Generation of a Novel Transposon Promoter Probe to Study Gene Regulation in *Porphyromonas gingivalis*

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by

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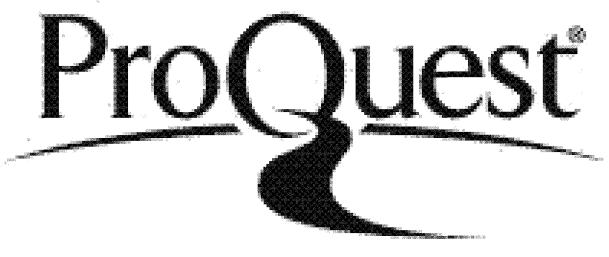

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Declaration

The accompanying thesis submitted for the degree of Doctor of Philosophy entitled "Generation of a Novel Transposon Promoter Probe to Study Gene Regulation in *Porphyromonas gingivalis*" is based on work conducted by the author in the department of Microbiology and Immunology at the University of Leicester during the period between October 1992 and October 1995.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other University.

Signed..Ana Ostrowski..... Date..19.8.97.....

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Contents

List of Abbreviations

Ap	Ampicillin
APS	Ammonium persulphate
bp	Base pair
CFU	Colony forming unit
CIP	Calf intestinal phosphatase
Cl	Clindamycin
Cm	Chloramphenicol
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminotetra-acetic acid
Gm	Gentamicin
IPTG	Isopropylthiogalactoside
kb	Kilo base
kDa	Kilodalton
Km	Kanamycin
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PMN	Polymorphonuclear leukocyte
SDS	Sodium lauryl sulphate
SOD	Superoxide dismutase
Sp	Streptomycin
SSC	Saline sodium citrate
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
Tp	Trimethoprim
Tt	Tetracycline
U	Units
X-gal	5-bromo-4-chloro-3-indoyl-D-galactoside

Abstract

Generation of a Novel Transposon Promoter Probe to Study Gene Regulation In *Porphyromonas gingivalis*

Anna Ostrowski

The transposon Tn*4351*, originally isolated from *Bacteroides fragilis*, has previously been used generate many isogenic mutants in the oral pathogen *Porphyromonas gingivalis*. Although such transposon mutants can determine the function of putative virulence genes they can not be used to study the regulation of these genes. To correct this deficiency Tn*4351* was manipulated to include a reporter gene to generate a type I promoter probe with which to study the regulation of putative virulence genes in *P. gingivalis* under different environmental conditions.

The manipulation of Tn*4351* was achieved by using PCR to amplify IS*4351*$_R$, one of the two directly repeated insertion elements that flank Tn*4351*. The PCR primers were designed to introduce a unique restriction enzyme site at the 5' end of IS*4351*$_R$ into which the reporter gene, a type III chloramphenicol acetyltransferase (*catIII*), could be cloned. Recreation of the entire transposon was achieved by cloning a 3.8 kb *Ava*I fragment from Tn*4351* into a unique *Ava*I restriction site within IS*4351*$_R$::*catIII* creating the transposon promoter probe Tn*4351*::*catIII*.

Tn*4351*::*catIII* was cloned into the *Bacteroides* suicide vector pJRD215 and transferred from *E. coli* into *P. gingivalis* by an optimised filter mating procedure, which resulted in the generation of transposon inserted mutants. Southern blot analysis of these mutants confirmed that transposition of Tn*4351*::*catIII* into the *P. gingivalis* genome occurred randomly. Screening of the transconjugants on media supplemented with chloramphenicol identified a number of mutants containing a transcriptional fusion with the *catIII* gene. A quantitative assay was also used to demonstrate CAT activity in these mutants. It is hoped that Tn*4351*::*catIII* will prove to be a useful genetic tool for studying the regulation of putative virulence determinants in *P. gingivalis*.

CHAPTER 1

INTRODUCTION

The importance of specific Gram-negative bacteria in the aetiology and pathogenesis of human periodontal disease has been increasingly appreciated in recent years. The black-pigmented Gram-negative oral anaerobe *Porphyromonas gingivalis* is generally regarded as a causative agent of adult periodontitis and a variety of other oral diseases in humans, as well as causing oral diseases in animals. Previously classed as "black-pigmented Bacteroides", a more appropriate term "black-pigmented oral anaerobes" is now generally used to describe and distinguish members of the genus *Porphyromonas* from other black-pigmented bacteria. Recent advances in the molecular genetic study of *P. gingivalis* has highlighted the role this organism may play in periodontitis and has allowed the relevance of putative virulence factors to be assessed.

1.1 Historical Overview

Black-pigmented Gram negative anaerobes were originally described by Oliver and Wherry in 1921 when they isolated such organisms from a variety of sites including the oral cavity, urine, human faeces and respiratory tract. Since then there have been many changes in the classification and nomenclature of these bacteria. Because of the black or brown pigmented colonies produced on blood agar plates by these organisms, which was assumed to be melanin, they were first described as *Bacterium melaninogenicum* (Oliver and Wherry, 1921). A few years later they were described in the third edition of Bergey's Manual of Determinative Bacteriology (Bergey, 1930) as *Haemophilus melaninogenicus*, due to their requirement for X and V growth factors which was characteristic of members of the genus *Haemophilus*. However, in the fifth edition of Bergey's manual produced in 1939 the generic name *Bacteroides* had been adopted and *Haemophilus melaninogenicus* was reclassified as *Bacteroides melaninogenicus* (Roy and Kelly, 1939). Despite subsequent attempts to regroup *B. melaninogenicus* into new

genera and species, for example *Ristella melaninogenicus* (Prévot, 1938), and *Fusiformis nigrescens* (Wilson and Miles, 1945), the nomenclature *B. melaninogenicus* took precedence as the proper description of the organism for several years.

The use of biological and immunological studies showed heterogeneity among strains of *B. melaninogenicus* (Courant and Gibbons, 1967; Sawyer *et al.*, 1962) and it soon became apparent that they could be taxonomially divided into several subspecies as a result of differing fermentation activities. The saccharolytic strains were regrouped as *B. melaninogenicus* subspecies *melaninogenicus*, moderately saccharolytic strains were regrouped as *B. melaninogenicus* subspecies *intermedius* and the asaccharolytic strains were regrouped as *B. melaninogenicus* subspecies *asaccharolyticus*.

However, the creation of a species that included both saccharolytic and asaccharolytic strains was not generally accepted and as new biochemical and chemical analyses were applied to the pigmented strains, heterogeneity among the groups became more apparent. Heterogeneity within *B. melaninogenicus* subspecies *asaccharolyticus* was demonstrated with regard to the electrophoretic mobility pattern of malate dehydrogenase (MDH) and differences in DNA base composition (Shah *et al.*, 1976). Strains with a slower MDH mobility and lower G + C content (46 - 48%) were found to be of oral origin, whereas strains with a faster MDH mobility and higher G + C content (52 - 54%) were isolated from non-oral sites. Further studies which looked at polypeptide patterns by SDS gel electrophoresis also revealed two distinct profiles (Swindlehurst *et al.*, 1977). The profile with major bands in the lower molecular weight region corresponded to the slow MDH mobility and low G + C content strains, whereas the profile that possessed a more evenly dispersed polypeptide pattern corresponded to the fast MDH mobility and high G + C content strains. The subspecies was initially elevated to species level, *Bacteroides asaccharolyticus* (Finegold and Barnes, 1977) and then due to confirmation of heterogeneity within this species (Shah and Hardie, 1979) further classification was achieved. The oral strains were assigned to the species *Bacteroides gingivalis*, and the species *B. asaccharolyticus* was retained for non-fermentative strains recovered from non-oral sites (Coykendall *et al.*, 1980). A third

group of asaccharolytic black pigmented bacteria was isolated from infected dental root canals and these could be differentiated genetically, physiologically and serologically from *B. gingivalis* and *B. asaccharolyticus* and were placed in a separate species *Bacteroides endodontalis* (van Steenbergen *et al.*, 1984).

Heterogeneity within the other subspecies of *B. melaninogenicus* led to revised nomenclature for these organisms. *B. melaninogenicus* subspecies *melaninogenicus* was elevated to three species, *Bacteroides denticola* (Shah and Collins, 1981), *Bacteroides melaninogenicus* and *Bacteroides loeschii* (Holdeman and Johnson, 1982). Strains of *B. melaninogenicus* subspecies *intermedius* were reclassified as *Bacteroides intermedius* or *Bacteroides corpris* (Johnson and Holdeman, 1983).

Further biochemical and chemical studies on *B. gingivalis, B. asaccharolyticus* and *B. endodontalis* demonstrated that these species formed a relatively homogeneous group quite unrelated to the *Bacteroides* type species *Bacteroides fragilis*. Ribosomal RNA homology (Johnson and Harich, 1986) and 16S rRNA cataloguing data (Paster and Dewhirst, 1988) confirmed the distinctiveness of these asaccharolytic taxa and on the basis of this and other data (for a review see Shah and Gharbia, 1993a) the genus *Porphyromonas* was proposed (Shah and Collins, 1988), reclassifying the asaccharolytic pigmented anaerobes as *Porphyromonas asaccharolyticus*, *Porphyromonas gingivalis* and *Porphyromonas endodontalis*.

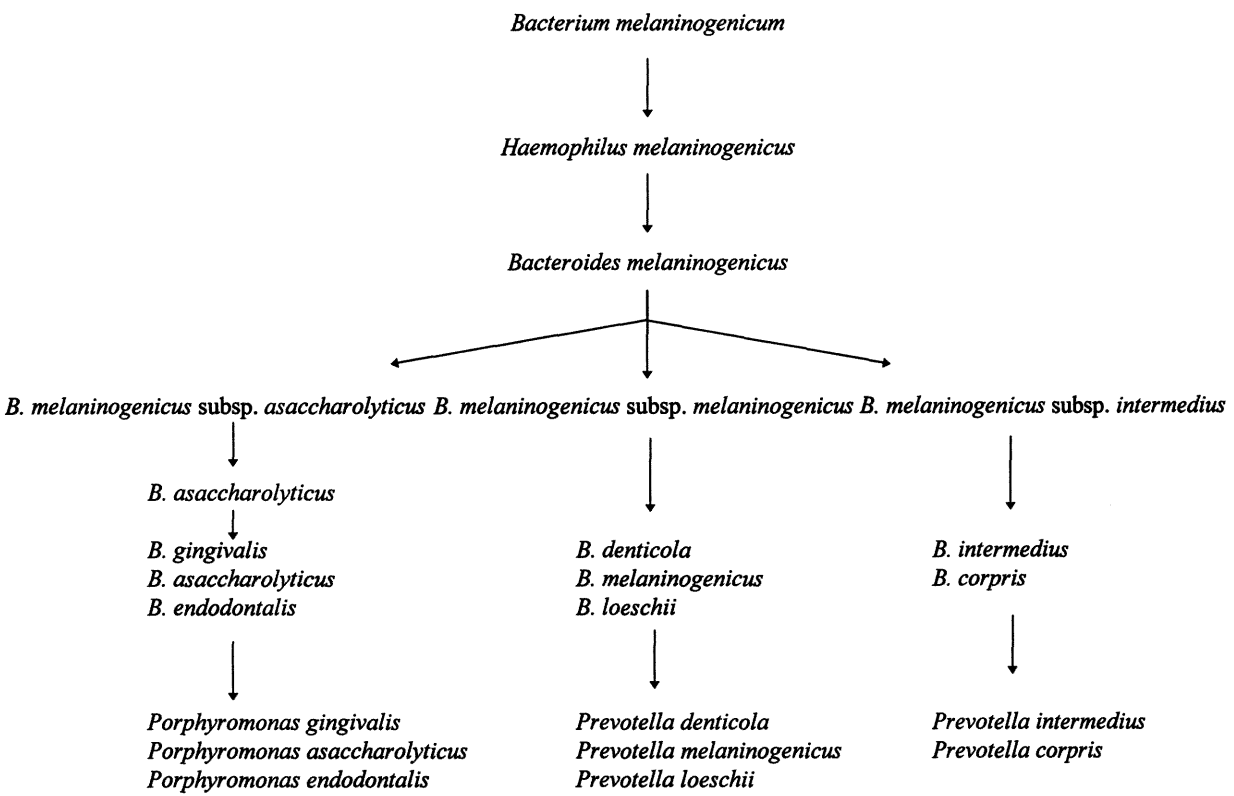
The saccharolytic *Bacteroides* species were also demonstrated to form a phenotypically and phylogenetically distinct group from *B. fragilis* and so it was proposed that they should be placed in the genus *Prevotella*, reclassifying these bacteria as *Prevotella melaninogenicus*, *Prevotella loescheii*, *Prevotella intermedius, Prevotella corpris* and *Prevotella denticola* (Shah and Collins, 1990).

At present seventeen distinct black-pigmented anaerobic species are recognised (Jousimies-Somer, 1995). Within the genus *Porphyromonas* two species, *Porphyromonas salivosa* and *Porphyromonas circumdentaria,* have been isolated from

gingival margins of cats (Love *et al.*, 1992) and five canine species have also been described. *Porphyromonas canoris* (Love *et al.*, 1994*), Porphyromonas cangingivalis* and *Porphyromonas cansulci* (Collins *et al.*, 1994) have been isolated from subgingival sites of dogs with naturally occurring periodontitis (Karjalainen *et al.*, 1993) and *Porphyromonas gingivicans* and *Porphyromonas crevioricanis* have been isolated from gingival crevicular fluid of beagles (Hirasawa and Takada, 1994). *P. gingivalis*-like Gram-negative rods have also been isolated from sheep (McCourtie and Poxton, 1990) as well as monkeys, cats, racoons and a jaguar (Laliberté and Mayrand, 1983). Most of the black-pigmented, Gram-negative species recognised belong to the genera *Porphyromonas* and *Prevotella* although some species, mainly of animal origin, still remain in the genus *Bacteroides,* for example *Bacteroides macacae* which is isolated from monkeys and *Bacteroides levii* which is isolated from the rumen of cattle. However, recent phylogenetic studies based on 16S rRNA sequence comparative analysis (Paster *et al.*, 1994) showed that these two species closely resemble species within the genus *Porphyromonas*, in fact *B. macacae* exhibits 99.5% sequence similarity with *P. salivosa* even though these two species show different phenotypic traits and are isolated from different hosts. It seems likely that *B. macacae* and *B. levii* will be moved to the genus *Porphyromonas* in the near future (Jousimies-Somer, 1995).

The taxonomy of anaerobic bacteria is still in a state of flux, the "pigmented anaerobic Gram-negative rods" have recently undergone significant taxonomic change. This change has been due the considerable heterogeneity in biochemical and chemical properties that have became apparent through detailed systemic studies on these microorganisms. It seems likely that with more sensitive nucleic acid analyses being developed all the time, that this trend will continue into the near future. A summary of the changes in nomenclature for the genus *Porphyromonas* is shown in figure 1.1.

Figure 1.1 Changes in nomenclature of black-pigmented anaerobes.



1.2 Characteristics of the Genus *Porphyromonas*

The genus *Porphyromonas gingivalis* belongs to the family Bacteroidaceae, a large family of Gram-negative, obligate anaerobes, nonsporing, non-motile rods. *Porphyromonas,* meaning porphyrin cell, aptly describes this genus which is characterised by the production of porphyrin pigment. The pigments, which are produced on blood agar plates from 3-10 days after subculture, are of two types, the dark brown/black pigment, originally thought to be melanin, has been identified as protohaem and the light brown, UV-fluorescent compound has been identified as protoporphyrin (Shah *et al.*, 1979). In contrast to the saccharolytic, black-pigmented, Gram-negative, anaerobic bacteria which produce rough, dry colonies, *Porphyromonas* species form smooth, slightly mucoid, wet, shiny, convex colonies which are 1 - 3 mm in diameter. The colonies darken progressively from the edge of the colony towards the centre after 6 - 10 days incubation until the entire colony is black due to the protoheam production. Optimum temperature for growth is 37°C and growth is not significantly affected by the addition of carbohydrates, although protein hydrolyates such as proteose peptone, trypticase or yeast extract do enhance growth (Shah and Williams, 1987). The major metabolic end products produced are acetic and butyric acids, together with lower levels of propionic, isobutyric and isovaleric acids.

Porphyromonas gingivalis.

The species *P. gingivalis* can be readily distinguished from the other species within the genus *Porphyromonas* by virtue of a variety of physiological and biochemical characteristics. Cells in liquid media are typically coccobacillus or rods of 0.5 by 1.0 to 2.0 μm and the colonies of this species take between 4 - 8 days to darken and are 1 - 2 mm in diameter. The principal respiratory quinones of this genus are unsaturated menaqinones and the number of polyprenyl side chains (isoprenes) of the menaquinones can be characteristic for different species. *P. gingivalis* is differentiated from *P. asaccharolyticus* in having nine isoprene units compared to ten isoprene units (Shah and Gharbia, 1993b). The species *P. gingivalis* can be set apart from the other members of

the genus in its ability to agglutinate sheep erythrocytes (Shah and Collins, 1988). DNA base composition is also a useful method of distinguishing between the different species within the genus *Porphyromonas*, the G + C molarity percent (G + C mol %) content of some of the species are listed in Table 1.1

Dietary carbohydrates do not reach subgingival sites and it is believed that *P. gingivalis* metabolises nitrogenous substrates as the major source of energy and these are probably derived from crevicular fluid and host-derived glycoproteins for example collagen, elastin and fibrinogen have been shown to be degraded by this bacterium (Lantz *et al.*, 1991). Proteases of *P. gingivalis*, such as the trypsin-like protease gingivain, degrade host derived proteins to generate a variety of peptides and amino acids. It has been shown that enzymatically digested protein fractions enhance the cell yield of *P. gingivalis* when compared with controlled nonenzymatic digested fractions (Shah and Gharbia, 1993c). Free amino acids are also fermented by this organism, for example aspartate, glutamate, histidine, serine, tryptophan and lysine (McKee *et al*, 1986), however the energy yield from these compared with the equivalent concentration of nitrogen-containing peptides is significantly lower (Shah and Gharbia, 1993c). The production of phenylacetic acid as an end product differentiates *P. gingivalis* from *P. asaccharolyticus* and *P. endodontalis* (van Steenbergen *et al.*, 1984). The production of this acid has been found to be directly proportional to the trypticase content of the media and L-phenylalanine, as well as peptides containing L-phenylalanine are also demonstrated to stimulate phenylacetic acid production (Bourgeau and Mayrand, 1983).

Other growth requirements of *P. gingivalis* include vitamin K and haemin. Although the role of vitamin K in the growth of asaccharolytic black-pigmented bacteria is not completely understood, it has been proposed that it is used as a precursor of menaquinone biosynthesis (Shah and Collins, 1980; Shah and Williams, 1987), which is involved in the electron transport system of these organisms. Haemin has also been shown to be important in the growth of *P. gingivalis*, which it breaks down into protohaem and protoporphyrin. It is thought that the characteristic black-pigment produced by this bacterium is due to the accumulation of haemin on the cell surface and

SPECIES	G + C mol %
P. gingivalis	45 - 48[1]
P. asaccharolyticus	50 - 52[1]
P. endodontalis	48 - 50[1]
P. salivosa	42 - 44[2]
P. canoris	49 - 51[3]
P. crevioricanis	44 - 45[4]
P. gingivicanis	41 - 42[4]

Table 1.1 The G + C content of some of the strains found within the genus *Porphyromonas*

1 Shah and Collins, 1988

2 Love *et al.*, 1992

3 Love *et al.*, 1994

4 Hirasawa and Takada, 1994

this may be a mechanism for haemin storage. The function of protoporphyrin is uncertain but it is thought that protohaem serves as the iron prosthetic group of cytochrome *b* found in the electron transport system of these bacteria (Shah and Gharbia, 1993b). Body fluids only contain low concentrations of free iron and this property acts as an important defence against invading microorganisms which require iron for growth. It is thought that *P. gingivalis* has a selective advantage when colonising the oral environment, by virtue of its ability to use haemin as an essential iron source (Marsh *et al.*, 1988). It is likely that *in vivo* the haemin derivatives, protohaem and protoporphyrin, are derived from the proteolysis of haemoglobin which has been found to be utilised more efficiently than other iron-containing compounds by *P. gingivalis* (Shizukuishi *et al.*, 1995). Haemolysin activity, which appears to be regulated by environmental haemin, has been described for this bacterium (Chu *et al.*, 1991) and two distinct haemolysin genes have been cloned from *P. gingivalis* into *E. coli* whose products are thought to be capable of lysing erythrocytes resulting in the liberation of haemoglobin (Karunakaran and Holt, 1993). Haptoglobin and hemopexin are compounds that rapidly bind free haemoglobin and haemin making it unavailable for use as an iron source, *P. gingivalis* is able to degrade both these iron-binding proteins as well as other iron-binding proteins such as plasma proteins, albumin and transferrin and is therefore capable of obtaining iron from them (Mayrand and Holt, 1988).

As well as being important physiologically, the availability of haemin has been shown to modulate the virulence of *P. gingivalis*. Cells grown in the chemostat under conditions of haemin-excess are virulent in mice, whilst those cultured under conditions of haemin-limitation show reduced pathogenicity (Mckee *et al.*, 1986). In addition it has been shown that trypsin activity of *P. gingivalis* is increased when bacteria are grown in haemin excess conditions, compared to haemin depleted conditions (Marsh *et al.*, 1988; Marsh *et al.*, 1994).

Because of the low levels of iron in the body fluids, competition and successful acquisition of available iron are essential for maintenance of pathological state. While haemolysin functions to raise the level of extracellular iron, the bacteria also require

iron-binding components to sequester the iron for their own use. The transport of haem and haem derived compounds is an energy-dependant process and an outer membrane receptor that is specific for the protoporphyrin IX ring appears to be involved in the initial binding of haem to the cell surface (Genco *et al.*, 1994). Originally it was supposed that the lipid A region of lipopolysaccharide was the active component for haemin binding (Grenier, 1991), however, it was later established that a 26 Kilodalton outer membrane protein (Omp26) may be important in haemin acquisition (Bramanti and Holt, 1992a). It was found that this Omp was actually regulated by haemin, being exported to the outer membrane during haemin-deprived conditions and then lost from the surface after a shift back to haemin-rich conditions. Seven strains of *P. gingivalis* examined were shown to express omp26 under haemin-starved conditions (Bramanti and Holt, 1992b). Recently however, it has been found that the binding and accumulation of haemin was induced by growth of cultures in the presence of haemin, rather than in haemin depleted conditions (Genco *et al.*, 1994). To explain these discrepancies a possible explanation has been put forward which suggests that the haemin-repressible protein found by Bramanti and Holt may be a low-affinity binding receptor and as such may be a component of a haemin-uptake system that is induced only under low environmental levels of haemin whereas the haemin-inducible proteins may be involved in high-affinity binding of haemin (Cutler *et al.*, 1995).

Strains of *P. gingivalis* isolated from humans can be readily distinguished from strains of *P. gingivalis* isolated from animals. Two biotypes of this species can be separated by nature of catalase activity, with catalase-negative and catalase-positive types corresponding to strains isolated from humans or animals respectively (Laliberté and Mayrand, 1983). Further studies have shown that the strains isolated from animals are also positive for glutamyl-glutamic acid arylamidase (GGA) and that the strains isolated from humans are negative for this enzyme activity (Fournier and Mouton, 1993). The *P. gingivalis* biotypes isolated from humans have been further separated into three or four serogroups based on immunodiffusion and immunoelectrophoresis tests (Fisher *et al.*, 1987; Nagata *et al.*, 1991).

In the last five years intra-species studies, using genetic techniques rather than the traditional biotyping and serotyping techniques, have highlighted extensive intragenic heterogeneity of *P. gingivalis*. Restriction endonuclease analysis (REA) has been used to give a more accurate indication of differences within the species (Loos *et al.*, 1990). Thirty three isolates were investigated and twenty nine distinct DNA fingerprints were identified. Of the sets of isolates that exhibited identical DNA fingerprints one set contained two separate isolates sampled from different patients two years apart in an isolated community; this result indicates that homogeneity can exists among strains within such isolated social groups. The other two sets of identical isolates contained strains W50 and W83, and strains 381-R, 2561 and 33277, which are clinical isolates of unknown origin and could represent identical isolates taken from the same patient. Further heterogeneity was highlighted by a study which used restriction fragment length polymorphism (RFLP) analysis on the fimbrillin locus of thirty nine isolates taken from human and animal sources, in which twenty five unique fingerprints were identified (Loos and Dyer, 1992). Studies using arbitrary primer PCR (AP-PCR) techniques confirmed these results with nine distinct banding patterns exhibited among nine strains of *P. gingivalis* analysed (Ménard *et al.*, 1992). These studies have recently been extended to include a total of ninety seven strains of *P. gingivalis*, which include laboratory strains and clinical isolates of human origin with diverse clinical and geographical origins, as well as thirty two strains isolated from the oral cavities of nine different animal species. Four nonameric oligonucleotides were used as single primers and the banding patterns of the different strains were compared (Ménard and Mouton, 1995). The conclusions of this study were that the strains could be clustered into three main groups, group I included all ninety seven strains isolated from humans and six strains isolated from monkeys. The strains in group II and group III were strongly differentiated from those in group I and included only strains of animal origin and it was suggested that these represented two cryptic species within the present *P. gingivalis* species. This data confirms earlier studies in which multi-locus enzyme electrophoresis (MLEE) on a hundred different isolates taken from the oral cavity of human and animals species identified three major divisions (Loos *et al.*, 1993). Division I contained all the strains isolated from the human oral cavity as well as four strains isolated from

monkeys, and divisions II and III contained only strains isolated from animals. It has been suggested that *P. gingivalis* strains isolated from non-human primates (NHP) consist of two distinct phyla, with strains isolated from NHP from the New World (America) demonstrating an animal biotype in group II, and strains isolated from NHP from the Old World (Africa) demonstrating a human biotype in group I (Ménard and Mouton, 1995). This suggests that the most recent strains of *P. gingivalis* isolated from humans are derived from strains isolated from Old World monkeys. It was also concluded from these experiments that no relationship can be established between specific clusters of *P. gingivalis* isolates and disease state, with strains isolated from healthy sites being distributed throughout the clusters. This suggests that all strains could be equally effective at colonising the host share a common virulence potential (Ménard and Mouton, 1995).

1.3 Plaque Development and Periodontal Disease

Periodontal disease is the general description for a variety of conditions which are characterised by inflammation and degradation of the periodontium, the supporting tissue of the teeth. The inflammatory response is produced as a result of complex, well-organised microbial communities which are known as dental plaque. The periodontal diseases can be divided into two general clinical groupings, namely gingivitis, where inflammation is confined to the gingival tissues, and periodontitis, where loss of alveolar support to the teeth occurs. The plaque in periodontal infections comprises of more than 200 bacterial species, although it is probable that only a minor number of these are capable of maintaining and sustaining pathological reactions in the periodontium (Moore *et al*, 1983). Preventative measures involve good oral hygiene and this is sufficient to control gingivitis and early periodontitis, however, active, severe periodontitis may often require extensive and costly treatment including application of antimicrobial agents, or surgery to remove damaged or necrotic tissue.

Plaque begins to develop as soon as teeth erupt, the initial phase of colonisation appears to involve deposition of microorganisms derived from saliva, onto the tooth surface

(Socransky *et al.*, 1977). Many of the early colonisers recognise components of the acquired pellicle, a thin coating that covers the surface of the tooth, which consists primarily of glycoproteins, mucins and enzymes found in saliva (Kolenbrander, 1988). The composition of plaque in periodontally healthy individuals is characterised by facultative bacteria, for example *Streptococcus, Haemophilus, Veillonellae* and *Actinomyces* spp. (Kolenbrander and London, 1993; Slots, 1977a; Socransky *et al.*, 1977). Once attached to the tooth surface the bacteria accumulate by proliferation and by the adherence of new bacteria species. An integral part of plaque formation is coaggregation, or cell to cell recognition of genetically distinct partner types, which results in the formation of adhesive interactions. Coaggregation is not generally seen in other ecosystems, but is essential in the oral environment for the colonisation of bacteria that can not adhere to molecules of the acquired pellicle (Kolenbrander, 1988; Kolenbrander and London, 1993). The plaque, which is known as the supragingival plaque, increases in thickness and becomes increasingly complex. The build-up of bacterial aggregates not only strengthens the plaque but can also lead to growth-enhancing or inhibitory influences of the bacteria upon one another (van Houte, 1982).

The gingival epithelium meets the enamel surface in a groove which is called the gingival crevice (figure 1.2a). In a periodontally healthy individual the gingival crevice is 1 - 2 mm deep and has a low bacterial density, the bacteria that are present are continuously eliminated by the gingival exudate and through desquamation of the junctional epithelium at the bottom of the crevice (Dahlén, 1993). If, due to poor oral hygiene, the supragingival plaque formed on the teeth is not removed, the accumulated bacteria can initiate an inflammatory reaction in the gingiva. This can occur within 1 - 4 days and a subgingival plaque begins to develop within the gingival crevice. The crevice provides loosely adherent or motile organisms with some protection from the cleansing action of the tongue and cheeks and once the subgingival plaque has accumulated, bacterial fermentation causes a drop in the oxidation-reduction potential, a change which favours the establishment and growth of facultative and eventually strict anaerobic bacteria (Coulter and Russell, 1976; Finegold, 1993; Moore *et al.*, 1983; Slots, 1977a; Slots, 1977b).

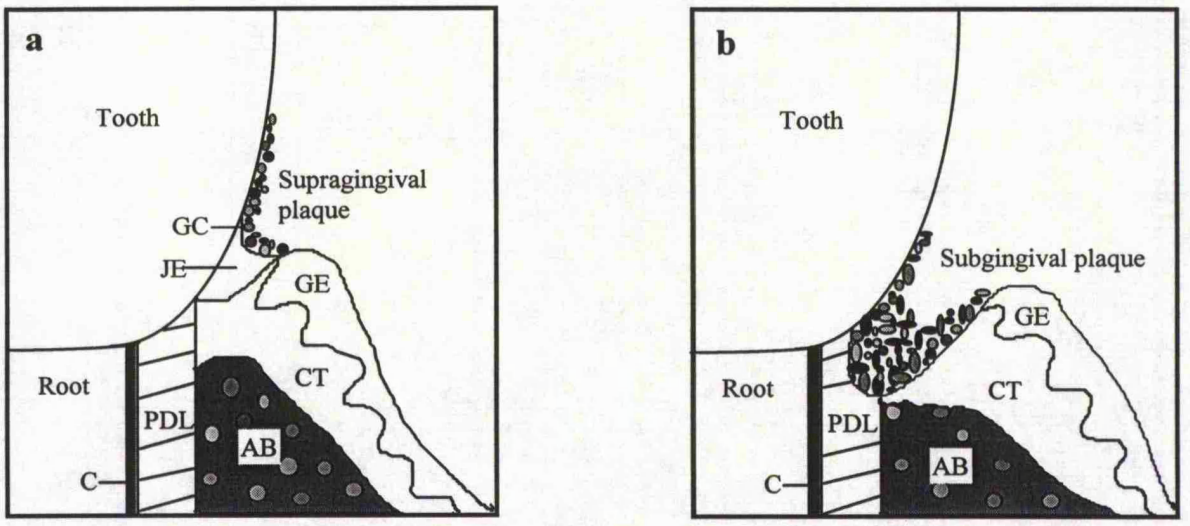


Fig. 1.2. Histopathology of the human gingival sulcus and surrounding tissues in health (**a**) and periodontitis (**b**). Figure **a** shows a diagrammatic representation of the build up of supragingival plaque on the surface of a tooth. The bacteria found in supragingival plaque are mainly Gram-positive facultative microorganisms. As the plaque develops subgingival plaque can develop in the gingival crevice and if this is not removed an inflammatory response is initiated by the host. Bacterial fermentation causes a drop in the oxidation-reduction potential which favours the growth of anaerobic microorganisms. Figure **b** demonstrates the result of prolonged inflammation in response to the subgingival plaque. The depth of the gingival crevice increases as the subgingival plaque develops. Cells of the immune system recruited to the area in response to the subgingival plaque bacteria stimulate host cells to release collagenases and other enzymes that can cause tissue damage. Eventually this results in destruction of the underlying connective tissue and periodontal ligament. As the inflammatory response persists increased osteoclast activity results in bone resorption. Abbreviations: JE, junctional epithelium; GE, gingival epithelium; CT, connective tissue; AB, alveolar bone; C, cementum; PDL, periodontal ligament; GC, gingival crevice. (Constructed from information present in Cutler *et al.*, 1995; Dahlén *et al.*, 1993; Holt and Bramanti, 1991)

Within 1 - 2 weeks the deposition of subgingival plaque leads to gingivitis where the inflammatory reaction persists in response to plaque. In chronic gingivitis, the most common form of this disease, the gingivae become swollen, red and bleed easily when probed, there is also a measurable exudate from the pocket but there is no evidence for loss of attachment to the teeth or alveolar bone destruction (Dahlén, 1993; Hirsch and Clarke, 1989). Bacteria that are routinely isolated from patients suffering with gingivitis include *Bacteroides intermedius,* which shows a recovery rate of > 70% in adults with gingivitis, *Actinomyces* species, *Streptococcus* species, *Fusobacterium* species and other types of black-pigmented anaerobic bacteria (Slots, 1977b; Dahlén, 1993). Gingivitis was previously thought to be a stage in the process leading inevitably to periodontal attachment loss, however, although periodontal destruction is rare in the absence of gingival inflammation, it is now recognised that gingivitis can be transient and reversible and that it can be present over long term without progressing to periodontal disease (Tanner and Stillman, 1993).

Periodontitis is defined as loss of alveolar support to the tooth, it is an inflammatory process that extends beyond the gingivae, leading to destruction of alveolar bone and connective tissues. The junctional epithelium, at the site of the gingival crevice, migrates downwards resulting in a relatively deep pocket between the tooth and the gingivae (figure 1.2b). The pocket is pathologically deepened by periodontal disease, resulting from the spread of the inflammation initiated by the formation and maturation of subgingival plaque. Continued inflammation around the periodontal ligament causes osteoclastic activity, resulting in irreversible damage to alveolar bone and periodontal ligament, which can lead to a loosening of teeth. The disease is highly prevalent in human populations world-wide, and is a major cause of tooth loss in adults over 35 years of age. The diseases appear to be active or inactive at any one time with disease progression and the destruction of periodontal tissues occurring in "bursts" rather than occurring continuously. The control of these "bursts" may be mediated by the hosts immune response or by interaction of periopathogenic bacteria with other microbes (Hirsch and Clarke, 1989). Bacteria can cause disease by direct invasion of the oral tissues or indirectly through various bacterial products such as enzymes which degrade

host tissues as well as destroying the functional activity of humoral antibodies directed against bacterial antigens (Listgarten, 1987; Slots, 1981; van Steenbergen *et al.*, 1986). Host defence mechanisms against subgingival plaque, although primarily protective, also have a place in the destruction process, in particular polymorphonuclear leukocytes (PMN) are believed to have the potential to cause both lytic and desquamative injury to oral epithelial cells (Altman *et al.*, 1992; Listgarten, 1987).

There are several clinically and microbiologically distinct periodontitis diseases, although their classification is incomplete, which are generally distinguished by the patients age at onset and the extent of the damage to oral tissues. These include adult periodontitis, localised juvenile periodontitis (LJP), rapidly progressive periodontitis (RPP) and refractory periodontitis (RP) (Tanner and Stillman, 1993). Adult periodontitis affects adults over the age of 35 years and is associated with mostly Gram-negative anaerobic asaccharolytic bacteria such as black-pigmented anaerobes, in particular *P. gingivalis* and *P. intermedius* (van Winkelhoff *et al.*, 1988a), *Treponema denticola*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *Wollinella recta* and *Eubacterium* species (Slots, 1977b; Tanner and Stillman, 1993).

Localised juvenile periodontitis is a rare form of angular alveolar bone destruction found in 12 - 26 year olds and generally only affects selected teeth, in particular the first molars and incisors. Unlike most forms of periodontal disease, LJP is characterised by minimal lesion-associated plaque and inflammation. The plaque that is developed with LJP contains few bacteria, but *A. actinomycetemcomitans* is often associated with the disease and is considered to be a primary causative agent (Genco *et al*, 1986; Zambon *et al.*, 1988). Several reports indicate a genetic component may be involved in LJP and could include a defect in PMN chemotaxis (Altman *et al.*, 1985; Genco *et al.*, 1986).

Rapidly progressive periodontitis (RPP) as its name suggests shows a relatively rapid rate of progression of disease. Acute inflammation, alveolar bone loss and bleeding are associated with this disease which is found in young adults who have usually had some previous history of juvenile periodontitis. Refractory periodontitis (RP) is

symptomatically similar to RPP however this type of disease shows a lack of response to therapy such as treatment with antimicrobial mouthrinses or treatment with systemic or local antibiotics. Bacteria species associated with disease progressing after therapy include *B. forsythus*, *P. intermedia*, *W. rectus*, *S. intermedius* and *P. gingivalis* (reviewed in Tanner and Stillman, 1993).

Periodontitis is also associated with patients with systemic diseases, for example individuals with insulin-dependant diabetes mellitus have a higher incidence and more severe periodontitis than age-matched controls (Dahlén, 1993; Mandell *et al*, 1992). There is also an increased susceptibility to periodontal disease in immunosuppressed individuals. Clinically distinct forms of gingivitis and periodontitis have been described in patients infected with the human immunodeficiency virus (HIV) in which the inflammation manifests itself as a fiery red line along attached gingivae, a feature not seen with other periodontal patients, and the periodontal lesions can progress very rapidly. Investigations on the microbiota associated with periodontitis in HIV related subjects reveals *Fusobacteria*, Spirochetes and *P. intermedia* as well as additional oral species that are not usually associated with sites of progressive periodontitis (Dahlén, 1993; Tanner and Stillman, 1993).

1.4 The Role of *P. gingivalis* in Periodontal Disease

Of the many bacteria that colonise the subgingival plaque, the black-pigmented anaerobe *P. gingivalis* has been implicated as a causative agent of adult periodontitis and rapidly progressive periodontitis as well as a variety of other periodontal diseases (Okuda and Takazoe, 1988; Slots, 1977b; Slots and Listgarten, 1988). Although mainly colonising subgingival sites of the oral cavity, *P. gingivalis* has also been recovered from the tongue, tonsils, saliva and rarely from the supragingival dental plaque (van Winkelhoff *et al.*, 1988a). The proportion of *P. gingivalis* in individuals with gingivitis has been shown to be less than 5% of the cultivable microflora, but its numbers can increase dramatically in advanced periodontitis and the high incidence of bacteria

isolated from active periodontal pockets gives an indication of the role it plays in periodontitis (Slots and Genco, 1984; Zambon *et al.*, 1981).

Initially it was thought that *P. gingivalis* could only be detected in diseased sites and never from healthy sites (Slots, 1977a; Slots, 1977b). However, recent studies which have used DNA probes to detect the presence of particular bacteria in periodontal pockets have shown that *P. gingivalis* can be detected in healthy individuals but at significantly lower numbers than in periodontal patients (Kojima *et al.*, 1993; Zambon *et al.*, 1981). Studies have also shown that there is an increasing prevalence of this organism within older age groups, with low numbers of bacteria isolated from 10 - 19 year olds and a maximum number of bacteria isolated from subjects aged 30 or more (Savitt and Kent, 1991). These observations probably reflect the fact that conditions are not suitable for the growth of *P. gingivalis* in younger individuals, but as the plaque matures the econiche of the subgingival plaque and periodontal pockets favour the growth of this bacterium (Takazoe *et al.*, 1984).

1.4.1 Conditions That Favour the Proliferation of *P. gingivalis*

The subgingival plaque is an extension of the supragingival plaque and as such the subgingival bacteria must be constantly affected by the supragingival organisms. Many factors could potentially regulate the levels of *P. gingivalis* and these include interaction with other bacterial flora, nutrient conditions, the pH, the oxidation-reduction potential and inhibitory factors in the gingival fluid.

Although *P. gingivalis* has been shown to adhere to buccal epithelial cells, erythrocytes and salivary components (Okuda *et al.*, 1981; Lamont *et al.*, 1992a), it is likely that initially it adheres to other organisms in the dental plaque. Coaggregation of *P. gingivalis* to some of the colonisers of dental plaque has been described (Table 1.2) and these interactions are thought to be important in the colonisation, proliferation and subsequent migration of this bacterium into the gingival crevice. Attachment of *P. gingivalis* to *Actinomyces* species has been suggested to be particularly significant for

Species name	Reference
Actinomyces viscosus	Ellen *et al.* (1988) Kolenbrander (1989) Rosenberg *et al.* (1991)
Eikenella corrodens	Kolenbrander (1989)
Fusobacterium nucleatum	Kolenbrander (1989)
Streptococcus gordonii	Lamont *et al.* (1994)
Streptococcus mitis	Lamont *et al.* (1992b)
Streptococcus salivarius	Kolenbrander (1989)
Streptococcus sanguis	Lamont *et al.* (1992b) Stinson *et al.* (1991)
Treponema denticola	Grenier (1992b)

Table 1.2 Microorganisms that form coaggregates with *P. gingivalis in vitro*.

subgingival colonisation by *P. gingivalis*. Specifically it has been shown that proliferation of *P. gingivalis* is favoured during the conditions that prevail during gingivitis, a disease in which *Actinomyces* species have been implicated as causative agents (Takazoe *et al.*, 1984).

The nutritional inter-dependency between *P. gingivalis* and other members of the microbial community is likely to be important for the establishment and proliferation of this bacterium prior to the initiation and progression of periodontal disease. Proliferation of *P. gingivalis* has been shown to be dependant on organisms that can produce succinate, which can replace the haemin requirement of this bacterium (Mayrand and Mcbride, 1980), as well as on organisms that can produce naphthoquinone, a vitamin K related compound (Sundqvist, 1993). A mutual, symbiotic, nutritional relationship has been described between *P. gingivalis* and *T. denticola*, a suspected periodontopathogen, where isobutyric acid and succinic acid are produced respectively by the organisms and are used as growth factors by each other (Grenier, 1992a).

The pH of the oral environment can also affect the proliferation of *P. gingivalis*. During the progression from health to disease the pH of the subgingival crevice increases from below neutrality, during health, to above pH 8, found during inflammation in periodontal pockets. Chemostat experiments have shown maximum growth of *P. gingivalis* occurs between pH 7 and pH 8, which corresponds to the conditions found in inflamed periodontal pockets. Interestingly, tissue degrading enzymes such as collagenase are produced optimally at neutral pH, whereas enzymes which help the bacteria evade the host response, such as trypsin-like activity, are produced optimally at pH 8 (McDermid *et al.*, 1988). It would appear that *P. gingivalis* is uniquely adapted to the environmental conditions found in the gingival crevice in that it is able to produce tissue degrading enzymes at the initial stage of pocket development, and then as the pH increases and a host response is initiated, enzymes that can help inactivate the hosts defences are produced.

1.4.2 Evidence for the Association of *P. gingivalis* with Periodontal Disease

Some evidence for the role of *P. gingivalis* in periodontal disease comes from the study of bacteria at different periodontal pocket depths. Positive correlations have been noted for the proportion of *P. gingivalis* in subgingival areas and periodontal pocket depth, with a higher proportion of this organism isolated from deeper pockets (Kojima *et al.*, 1993; Slots and Listgarten, 1988; Socransky *et al*, 1988; Zambon *et al*, 1981). Other studies, which focused on active lesions of periodontitis, demonstrated that there were elevated levels of *P. gingivalis* in active sites compared to inactive sites (Dzink *et al.*, 1988). It has also been suggested that after periodontal treatment levels of *P. gingivalis* decrease in successfully treated sites, whereas non-responding sites have levels of bacteria equal to or greater than the pre-treatment levels (Simonson *et al.*, 1992).

Serological studies have also been used to associate *P. gingivalis* with periodontal disease. It has been demonstrated that lower antibody levels against *P. gingivalis* are seen in early childhood, with progressively increasing levels of antibodies being seen among school age, puberty and adult subjects (Mouton *et al.*, 1981). It has also been shown that periodontal infections are associated with elevated antibody levels to *P. gingivalis* compared to healthy controls (Nakagawa *et al.*, 1994). Positive correlations have been made between antibody levels against surface antigens of *P. gingivalis* and alveolar bone loss (Naito *et al.*, 1987; Watanabe, 1989). Specifically it has been demonstrated that periodontitis patients have up to five times the level of IgG reactive with *P. gingivalis* compared to levels found in healthy controls (Nakagawa *et al.*, 1994).

Attachment to and penetration of epithelial cells is an early step in establishing infection in a variety of microorganisms such as *Salmonella* spp. or *Neisseria gonorrhoeae* (Finlay *et al.*, 1989; Shaw and Falkow, 1988). The demonstration that *P. gingivalis* can invade a human oral epithelial cell-line (KB) provides tentative evidence that this bacterium plays a role in the pathogenesis and progression of periodontal disease (Duncan *et al.*, 1993). Recently the study of the interaction of *P. gingivalis* with epithelial cells has been extended and it has been demonstrated that *P. gingivalis* can

invade cultured human pocket epithelial tissue, obtained during periodontal surgery of patients with advanced periodontitis (Sandros *et al.*, 1994). If invasion of oral epithelial cells can occur *in vivo* it would render the bacteria inaccessible to antibodies, phagocytes and other host defence mechanisms, as well as allowing multiplication of the bacteria in a nutritionally rich environment.

Further evidence that has been used to suggest a role for *P. gingivalis* in periodontal disease comes from animal studies. Initial experiments demonstrated that subcutaneous injection of *P. gingivalis* into animal models could induce skin lesions mostly resulting in phlegmonous abscesses (Baumgartner *et al.*, 1992; Kastelein *et al.*, 1981; van Steenbergen *et al.*, 1982). A subcutaneous chamber model has been described in mice which allows the virulence of *P. gingivalis* to be studied with respect to the interaction of this bacterium with the host response (Genco *et al*, 1991). These studies show that a systemic IgG response can be induced by *P. gingivalis* in serum and chamber fluid samples. Although this does not directly indicate a pathological role for *P. gingivalis*, further studies have shown that immunisation against *P. gingivalis* in these animal models leads to protection against secondary lesion formation and death (Dahlén and Slots, 1989; Genco *et al*, 1992; Katahira *et al.*, 1985; Okuda *et al.*, 1988). However, although these models allow the pathogenicity of the bacteria to be studied, these systems do not mimic the human pathological periodontal pocket. Some of the most convincing animal model data comes from experiments on non-human primates. The implantation of a rifampin-resistant strain of *P. gingivalis* into the periodontal microbiota of rifampin-sterilised monkeys (*Macaca fascicularis*) resulted in an increase in the systemic levels of antibody to the microorganism and rapid and significant bone loss. These results suggested that *P. gingivalis* was capable of inducing progression of periodontitis and could act as a pathogen in periodontal disease (Holt *et al.*, 1988). Immunisation of *M. fascicularis* with killed *P. gingivalis* was later shown to inhibit progression of periodontal tissue destruction (Persson *et al*, 1994). Further studies used gnotobiotic rat models which, unlike the subcutaneous chamber model in mice, allow oral colonisation, gingival tissue changes and the destruction of alveolar bone to be studied. It was demonstrated that monoinfection of the periodontal pockets of

gnotobiotic rats by *P. gingivalis* induced severe alveolar bone loss in these animals. In addition to this it was shown that immunisation of the gnotobiotic rats with *P. gingivalis* cells prior to infection by *P. gingivalis* resulted in a reduction of periodontal tissue loss (Klausen *et al.*, 1991).

The serological and animal model data as well as the studies on the invasion of epithelial cells all implicate *P. gingivalis* as an opportunistic periodontal pathogen that can survive and multiply within the periodontal pocket. It has been well documented that these bacteria have numerous potential virulence factors that are likely to enhance its colonisation as well as allowing it to evade the host immune response which is induced in response to the bacteria.

1.5 Host Response in Periodontal Disease

The host response in periodontal disease is comprised of a complex series of reactions to the microorganisms in the subgingival plaque. Periodontal disease can be described as a chronic inflammatory response in which the inflammation has several critical functions. Inflammation serves to destroy the injurious agent, if possible, and to remove it and its by-products from the body. Inflammation also has a role in repair or replacement of damaged tissues. However, prolonged inflammation due to dysregulation of the host immune system can lead to host-mediated destruction of gingivae and alveolar bone (figure 1.3). During the initial stages of inflammation vasodilation causes an increase in the permeability of blood vessels resulting in an increase in blood flow which is responsible for the redness and heat associated with inflammation (Listgarten, 1987). The vasodilation response is caused by the release of chemicals, such as histamine from mast cells and kinin derived from the activated complement cascade. Prostaglandins and leukotrienes are responsible for the long-term maintenance of the inflammation and are derived from enzymatic cleavage of arachidonic acid found in damaged cells, these factors can also be released in response to stimulation by certain components of complement (Listgarten, 1987).

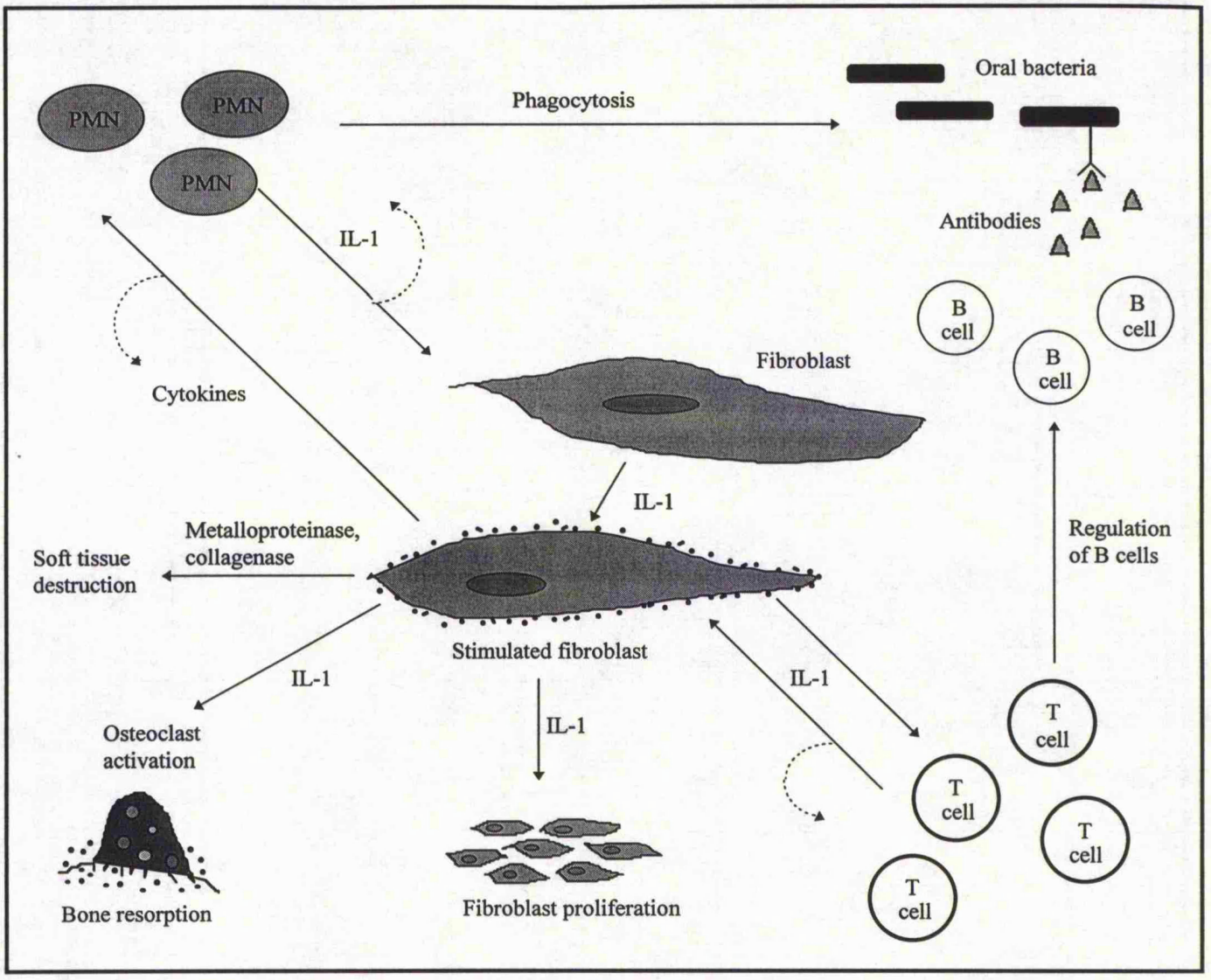


Fig. 1.3. Diagrammatic representation of some of the immunological processes involved in periodontal disease. In response to plaque bacteria an inflammatory response is induced. Phagocytic cells, such as PMN, are brought to the site by chemotaxis. The PMN produce cytokines, such as IL-1, which then acts on fibroblasts, T cells and other PMN. Fibroblasts are stimulated by IL-1 causing further production of IL-1, fibroblast proliferation and the release of enzymes that cause soft tissue destruction. IL-1 can also activate osteoclasts resulting in alveolar bone resorption. Cytokines produced by PMN or fibroblasts cause T cell proliferation. The cytokines produced by a particular cell can act on other cells or on the cell that produced it (as indicated by hash-lined arrows). Antigen presentation to B cells by activated T helper cells and the activation of T suppressor cells affect the homeostatic control of B cells which produce antibodies against the plaque bacteria (the diagram has been simplified such that antigen-presenting cells and the presentation of antigens to B cells on class II MHC molecules on T cells are not indicated). Diagram constructed from information present in Takada *et al.*, 1991a.

The early inflammation response is also associated with the migration of phagocytes, such as PMN's and monocytes, to the damaged area by chemotaxis and these components of the immune system can destroy invading organisms by phagocytosis (Cross and Kelly, 1990). The chemotactic factors are produced by microorganisms, other PMN's, kinins and components of the complement system. Several studies highlight the essential role of PMN in periodontal disease in particular patients with LJP are shown to have depressed PMN chemotaxis (Lavine *et al.*, 1979). PMN have the ability to destroy bacteria by the production of a variety of reactive metabolites which occurs as a result of a respiratory burst, this is termed oxygen-dependent killing. PMN can also kill phagocytosed organisms by oxygen-independent mechanisms by virtue of an array of enzymes, such as myeloperoxidase, lysosyme and elastase, packaged within primary and secondary granules which can be delivered *via* phagocytosis, secretion or cytolysis (Miyasaki, 1991; Thomas *et al*, 1988). However, PMN have a potential role in tissue destruction in periodontitis as their number at active periodontal sites increases as the disease progresses (Lamster and Novak, 1992). Studies have shown that activated PMN *in vitro* can cause desquamation of gingival epithelial cells by the action of extracellularly released granule proteases, such as collagenase, in a process termed "frustrated phagocytosis". The oxygen-dependant response of PMN can cause direct toxicity to host cells as well as lysis of these cells mediated by PMN oxidants (Altman *et al.*, 1992; Lamster and Novak, 1992).

Complement plays a major role in phagocytosis, chemotaxis, alteration of vascular permeability, killing of cells, lymphokine production, antibody synthesis, lysosomal enzyme release and bone resorption (Schenkein, 1982). The classical complement pathway involves the formation of insoluble antigen-antibody complexes which can then activate the complement cascade. The alternative pathway can be activated by lipoteichoic acid from Gram-positive bacteria and lipopolysaccharide of the Gram-negative cell wall, and results in an influx of phagocytic cells via chemotaxis, with resulting phagocytosis of microorganisms and release of lysosomal enzymes as well as stimulation of mast cells to release histamine hence contributing to inflammation.

The humoral immune response causes the synthesis of antibodies from activated B-cells, which predominate in later stages of inflammation (Ogawa *et al.*, 1989a). Secretary immunoglobulin A (IgA) has a role in prevention of attachment of microorganisms (Listgarten, 1987; Kilian, 1981). However, many pathogenic bacteria produce an IgA protease which is able to cleave IgA1 into Fab and Fc fragments, this property could induce a local paralysis of immune defence mechanisms allowing colonisation of the bacteria (Kilian, 1981; Sundqvist *et al.*, 1985). IgG has been shown to be the predominant class of immunoglobulin found in periodontitis and this immunoglobulin can readily cross blood vessel walls and enter tissue fluids where it can contribute to antibody dependant cellular cytotoxicity, the enhancement of opsonisation, the release of chemotactic factors and cause fixation of complement (Kono *et al.*, 1991). Increasing levels of IgG and other immunoglobulins during disease progression can, however, lead to continuation of the inflammatory response resulting in perpetuation of the disease state (Kono *et al.*, 1991).

The key components of the cell-mediated immunity are T cells of which there are several types. Helper T cells (T_H cells) are involved in the stimulation of other cells such as cytotoxic T cells or other T_H cells, they can also activate B-cells. Cytotoxic T cells (T_C cells) destroy target cells on contact, and suppressor T cells (T_S cells) regulate the immune responses, down-regulating antibody synthesis when it is no longer required. The role of T cells in periodontal diseases is highlighted by the observation that cells obtained from periodontal lesions show decreased mean $CD4^+/CD8^+$ subset cell ratios compared to healthy tissues (T cells expressing CD4 markers are mostly T helper cells, whereas T cells expressing CD8 markers are mostly cytotoxic or suppressor T cells). The result of the altered ratio of these important cells is to cause an imbalance in the regulation of the homeostatic gingival immune processes which can lead to over expression of proinflammatory components (Taubman *et al.*, 1988).

T cells, macrophages, PMN and fibroblast can produce cytokines which are multifunctional messengers that are believed to be important effectors of inflammation and the immune response. One of the most important cytokines produced in respónse to

periodontal disease is interleukin-1 (IL-1), of which there are two subclasses, IL-1 α and IL-1 β. These two subclasses have been demonstrated to be able to stimulate T_H cell synthesis, induce increased binding of PMN and monocytes to endothelial cells, increase production of prostaglandin by fibroblasts, induce lytic enzymes from lysosome-containing cells and stimulate bone resorption in tissue culture (Dinarello, 1990). IL-2 is involved in the proliferation of antigen stimulated T_H cells and the proliferation and differentiation of B-cells. TNF γ increases the activity of macrophages, whilst TNF β is a potent chemotactic which can enhance phagocytic cells.

Although cytokines have an important role as multifunctional inflammatory mediators of the immune response, during periodontal disease the cytokines can be inappropriately stimulated by bacterial components. This can lead to over stimulation or suppression of the cells of the immune system, resulting in soft tissue destruction and alveolar bone loss. Fibroblasts are the predominant cells involved in tissue repair mechanisms of chronic inflammatory lesions, including cellular proliferation and production of various matrices and enzymes (Genco and Slots, 1984; Listgarten, 1987). Under certain stimulation fibroblasts can produce IL-1 which in turn stimulates the fibroblasts to proliferate and generate matrix degrading enzymes such as collagenase and metalloproteinases, as well as prostaglandins and other cytokines (Takada *et al.*, 1991a). Macrophages are also shown to be stimulated by IL-1 to produce enzymes such as collagenase or prostaglandin. The collagenase would be expected to degrade the native collagen in the connective tissue, while the prostaglandin has several effects, including increased local vasodilation and permeability as well as promotion of bone resorption (Kabashima *et al.*, 1990).

Cytokines have been shown to be involved in bone resorption, in particular IL-1 (α and β) and TNF (α and β) are two of the most potent cytokine stimulators of bone resorption (reviewed in Hopps and Sisney-Durrant, 1991). IL-1β is thought to be the main bone resorption stimulator in active periodontal sites and it can also mediate soft tissue destruction through the stimulation of prostaglandin production and the induction of collagenase and other proteases. Confirmation of the role of IL-1β in periodontitis

comes from the observation that the levels of this cytokine are elevated in active periodontal sites compared to healthy sites (Hönig *et al.*, 1989; Stashenko *et al.*, 1991). Studies also indicate higher levels of IL-1α in the gingival crevicular fluid from diseased sites in periodontal patients compared to healthy sites (Kabashima *et al.*, 1990).

In summary it can be seen the chronic inflammatory response indicative of periodontitis is mediated by factors, such as IL-1 and other cytokines, that can regulate the immune response. Signals emitted by microorganisms, either directly or indirectly by numerous virulence factors produced by these bacteria, cause migration of macrophages, T cells and PMN to the underlying tissue. Release of proinflammatory cytokines from macrophages stimulates T cells, macrophages and B cells and results in further release of regulatory components. As the inflammatory response is prolonged fibroblasts are activated to release metalloproteinases and collagenases causing destruction of soft tissue. Meanwhile, osteoclasts attracted in response to the same signals resorb the adjacent alveolar bone. The overall result of these prolonged responses is the severe destruction of supporting periodontal tissue and alveolar bone which eventually leads to tooth loss.

1.6 Virulence Factors

Virulence factors can be considered to be a bacterial attribute or product that provides the organisms with a selective advantage for establishing infection and surviving within the host. Virulence factors may act either alone or in combination at various stages of infection. The periodontal pocket is unique in that it contains four types of surfaces, these are mineralised tissues such as cementum, keratinised epithelium and nonkeritinised epithelium (oral sulcular epithelium and junctional epithelium respectively), as well as the surfaces of other bacteria. Periodontal pathogens must be able to successfully colonise these different surfaces in order to survive in the environment. Known or putative virulence determinants of periodontal pathogens include capsules, fimbriae, outer membrane proteins, lipopolysaccharides, proteases and other enzymes, metabolites and endotoxin (Holt and Bramanti, 1991; Hofstad, 1992).

The role of the host immune response in disease progression and tissue destruction has been discussed (Section 1.5), in this section the putative virulence factors of *P. gingivalis* and the role these factors play in the onset and progression of periodontal disease are described.

1.6.1 Adhesins

The term adhesin is used to describe the components that play a role in host-parasite interactions. Adherence of bacteria to surfaces is the first step in colonisation and infection and it is generally accepted that bacterial cell surface components mediating attachment to specific host receptors are important pathological determinants which play a central role in virulence. It has been demonstrated that *P. gingivalis* can adhere to a variety of oral surfaces, including epithelial cells and erythrocytes (Okuda *et al.*, 1981), fibronectin-collagen complexes (Naito and Gibbons, 1988), salivary components (Gibbons and Hay, 1988) and other bacteria (Goldbourne and Ellen, 1991; Stinson *et al.*, 1991). In particular fimbriae and haemagglutinins are thought to have an important role in the adherence of *P. gingivalis*.

Fimbriae

Fimbriae are proteinaceous, filamentous, non-flagella appendages which project out from bacterial cell surfaces. Colonisation of the subgingival area by *P. gingivalis* is thought to be aided by fimbriae which have been shown to have an affinity for human crevicular epithelial cells (Hanazawa *et al.*, 1988), human buccal epithelial cells (Isogai *et al.*, 1988) and oral Gram-positive bacteria (Goldbourne and Ellen, 1991).

The fimbriae of *P. gingivalis* are described as curly, single-stranded filaments with a diameter of 5 nm, which are composed of helical polymers of repeated fimbrillin monomer subunits with a molecular mass of approximately 43 kDa (Yoshimura *et al.*, 1984a). Molecular cloning and sequencing of the fimbrillin gene has revealed that the fimbrillin sequence does not exhibit homology to sequences in other black-pigmented

oral anaerobes and shows no similarity to other known fimbrial protein sequences (Dickinson *et al.*, 1988). It has been shown that the majority of *P. gingivalis* isolates are fimbriated and possess the *fimA* gene (Loos and Dyer, 1992), although heterogeneity in the protein subunit has been described for different strains of *P. gingivalis* with respect to its size, N-terminal sequence and serospecificity and these differences may be important in the function and immune reactivities of the fimbriae (Lee *et al.*, 1991).

Although *P. gingivalis* fimbriae have long been implicated as playing a major role in adherence to gingival tissue surfaces, no conclusive genetic evidence for this had been obtained until recently. Studies demonstrated that adhesion of *P. gingivalis* to saliva-coated hydroxyapatite beads could be inhibited by the addition of purified fimbrillin or synthetic fibrillin peptides (Lee *et al.*, 1992). Further evidence was obtained by the inactivation of the *fimA* gene, resulting in a mutant that was incapable of expressing the 43 kDa FimA protein, and which had an adherence capacity that was reduced to one-third of the wild-type level (Hamada *et al.*, 1994). The mutant strain was shown not to cause the same extent of periodontal bone loss in a gnotobiotic rat model of periodontal disease relative to wild-type *P. gingivalis* (Malek *et al.*, 1994). Fimbriae were originally thought to be involved in haemagglutination (Okuda *et al.*, 1981), however the *fimA* mutant still retained a level of haemagglutination comparable to that of the wild-type strain, indicating that *fimA* function does not directly effect the expression of the haemagglutination activity in *P. gingivalis* (Hamada *et al.*, 1994).

Hydrophobicity of oral bacteria is thought to play a role in their adherence to host tissues, and it was thought that the number of fimbriae may contribute to the overall hydrophobicity of the cell (Watanabe *et al.*, 1992). Invasive strains of *P. gingivalis,* shown to cause spreading infections that often result in mortality in animal models, have been demonstrated to exhibit fimbriae with low hydrophobicity compared to non-invasive strains, that cause localised infections in animal models, which exhibit strong hydrophobicity properties (Naito *et al.*, 1993). However, the surface hydrophobicity of the *fimA* mutant was not significantly different from that of the wild-type strain (Hamada *et al.*, 1994). This indicates that the observed hydrophobicity of *P. gingivalis*

does not depend on the presence or absence of fimbriae but depends on some other surface component(s), that has not been examined as yet.

Electron microscopy was used to examine the interaction of *P. gingivalis* to human gingival fibroblasts and it was shown that during the adherence of wild-type bacteria long microvilli surrounding bacterial clumps appeared, however, these changes were not seen during the attachment of the *fimA* mutant bacterium. From this it was hypothesised that upon contact with epithelial cells wild-type bacteria can trigger a series of events that may lead to a reorganisation of cytoskeletal components, giving rise to microvilli which facilitate the contact between fimbriae and human oral tissue (Hamada *et al.*, 1994).

Recent findings suggest that a 72 kDa major cell-surface protein derived from *P. gingivalis* OMZ 409 could be another fimbria, as indicated by immunogold electron microscopy. These putative fimbriae differ from the fimbriae composed of the 43 kDa fimbrillin in terms of amino acid sequence, immunoreactivity and electron microscopic observation (Ogawa *et al.*, 1995). All *P. gingivalis* strains tested possessed this 72 kDa protein (Ogawa *et al.*, 1994a) although the function of these fimbriae and their functional and genetic relationship to the fimbrillin type fimbriae are as yet unknown.

Fimbriae from *P. gingivalis* have been shown to be important stimulators of the cellular and humoral immune response (Ogawa *et al.*, 1994b) and it has been found that plasma cells which form antibodies specific for *P. gingivalis* fimbriae increase significantly in the gingivae of patients with advanced periodontitis (Ogawa *et al.*, 1989b). Fimbriae or synthetic fimbrillin peptides have been shown to be able to stimulate monocytes and macrophages to release TNF α, IL-1 α, IL-1 β, IL-6 and IL-8, as well inducing macrophages to the express a neutrophil chemotactic factor (Hanazawa *et al.*, 1991; Hanazawa *et al.*, 1992; Ogawa *et al.*, 1991a; Ogawa *et al.*, 1994c). Fimbriae-stimulated bone resorption in *P. gingivalis* has been demonstrated *in vitro* and is thought to be regulated by cytokines (Kawata *et al.*, 1994), and the production of such cytokines can

also have profound effects on the development of inflammation, as described previously (section 1.5).

Haemagglutinin

The ability to attach to red blood cells is an important event in the interaction of many of the bacterial pathogens associated with periodontal disease. A large number of erythrocytes bathe the periodontal pocket during the progression of periodontal disease and a requirement for haem-containing compounds necessitates an interaction between periodontal bacteria and host cells rich in these compounds (Holt and Bramanti, 1991). Haemagglutination is a process that that can be defined as a sequence of two major events, firstly the bacterial adhesin attaches to red blood cells and secondly cross-linking of the erythrocytes occurs. Haemagglutinating activity has been demonstrated for *P. gingivalis* (Okuda and Takazoe, 1974), and although it was originally thought that fimbriae possessed haemagglutinating activity this has now been demonstrated not to be the case (Hamada *et al.*, 1994; Mouton *et al.*, 1989). Several groups have attempted to purify the haemagglutinin from *P. gingivalis* and results indicate that there is more than one haemagglutinin present in this species. Exohaemagglutinin preparations from culture media of *P. gingivalis* revealed three major proteins of 24, 37 and 44 kDa (Inoshita *et al.*, 1986), whereas Okuda *et al.* found a haemagglutinating protein of 40 kDa (Okuda *et al.*, 1986).

A surface antigen of *P. gingivalis* (HA-Ag2) that functioned as a haemagglutinating adhesin was identified (Mouton *et al.*, 1989). It was demonstrated that this antigen comprised of a protein complex composed of at least two major proteins of 33 and 38 kDa which were antigenically similar and contained at least one common epitope (Mouton *et al.*, 1989). However, it is not known whether HA-Ag2 is identical to the previously described haemagglutinins. Two distinct haemagglutinin-encoding genes have been identified in *P. gingivalis* strain 381 (Progulske-Fox *et al.*, 1989a). These genes, termed *hagA* and *hagB,* encode polypeptides of 125 and 50 kDa and have been expressed in *E. coli* (Progulske-fox *et al.*, 1989a). The *hagB* gene has also been cloned

and expressed in a live attenuated vaccine strain of *S. typhimurium*. The introduction of this strain into the gut of mice resulted in the stimulation of a mucosal and systemic immune response to the expressed *P. gingivalis* haemagglutinin (Dusek *et al.*, 1993).

The haemagglutinating activity of *P. gingivalis*, unlike lectins, is not inhibited by sugars, but is inhibited by arginine and lysine-containing peptides (Inoshita *et al.*, 1986; Okuda *et al.*, 1986). Generally arginine and lysine and their derivatives, serve as competitive inhibitors of trypsin-like proteases and so it has been suggested that the haemagglutinin of *P. gingivalis* has trypsin-like protease activity (Hoover *et al.*, 1992a; Nishikata *et al.*, 1989). Studies have shown that a band 3 protein on human erythrocytes is involved as a receptor protein for haemagglutinin and that arginyl residues may be important in the interaction between this protein and *P. gingivalis* (Hayashi *et al.*, 1992). Recent data suggests that haemagglutinin and trypsin-like protease are encoded as separate domains as part of the same protein. This is then post-translationally processed to produce separate molecules that then associate closely in the outer membrane of *P. gingivalis* (Cutler *et al.*, 1995; Kirszbaum *et al.*, 1995; Pike *et al.*, 1994).

1.6.2 Lipopolysaccharide

Lipopolysaccharide (LPS) is a macromolecule associated with the outer membrane of Gram-negative bacteria and is generally thought of as being responsible for the biological activity of endotoxin. The LPS of *Enterobacteriaceae*, specifically *E. coli* and *S. typhimurium,* possess structures considered to typify "classical" LPS. These LPS are composed of three covalently linked regions, the outer portion, called the O-polysaccharide, the core polysaccharide and a lipid complex which is termed lipid A. The outer-most region of LPS, the O-polysaccharide, consists of repeating and often branching carbohydrate units such as common hexoses i.e. glucose, galactose, mannose and rhamnose, as well as unique sugars called dideoxyhexoses, such as abequose, colitose and tyvelose (van Denmark and Batzing, 1987). The structure and arrangement of different carbohydrates of the O-polysaccharide are characteristic for different species of Gram-negative bacteria. The core polysaccharide lies between the O-polysaccharide

and the lipid A fraction and the structure of this component is less heterogeneous than the O-polysaccharide, with closely related strains of Gram-negative bacteria having similar core structures. Chemically the core consists of a unique eight-carbon ketosugar called 3-keto-2-deoxyoctonate (KDO), heptoses, glucose, galactose and acetylglucosamine, it is the KDO which forms a linkage between core polysaccharides and the lipid A moiety. The innermost component of LPS, the lipid A component, is embedded in the hydrophobic region of the outer membrane and consists of β(1,6)-linked glucosamine disaccharide with attached ester-, amide- and diester fatty acids (Raetz, 1993). Functionally the polysaccharide portion of the LPS defines the immunological specificity of the bacterium, while the lipid A is responsible for biological or endotoxic activity of the molecule (Holt and Bramanti, 1991; van Denmark and Batzing, 1987).

The carbohydrate LPS component of *P. gingivalis* is typical of *Enterobacteriaceae* and contains hexose and hexosamine sugars and shows a "smooth" chemotype (Bramanti *et al.*, 1989). However, many of the chemical and biological properties of the LPS from *P. gingivalis* do differ from the classical enterobacterial LPS (Hamada *et al.*, 1990). KDO, heptose and β-hydroxy myristic acid are unique to enterobacterial LPS, but these components were thought to be absent, at least in detectable amounts, in the LPS of *P. gingivalis* (Koga *et al.*, 1985; Mansheim *et al*, 1978). Recently a phosphorylated form of KDO has been shown to be present in *P. gingivalis* LPS and it is probable that the phosphorylation masked the presence of the components in previous studies (Bramanti *et al.*, 1989; Johne *et al.*, 1988; Kumada *et al.*, 1993). Other studies show that *P. gingivalis* LPS shows weak endotoxic activity compared to typical enterobacterial LPS (i.e. *E. coli* and *S. typhimurium* LPS) as tested by the Shwartzman reaction in rabbits (Mansheim *et al.*, 1978; Sveen, 1977). However, LPS from *P. gingivalis* can stimulate lymphocytes and macrophages from a mouse strain, C3H/HeJ, that is known as a classic LPS nonresponder as well as from the mouse strain C3H/HeN, a classic LPS responder strain suggesting that the Shwartzman reaction is not the best measure of toxicity of *P. gingivalis* LPS (Fujiwara *et al.*, 1990). Phosphate groups at the 1' and 4' positions of the glucosamine disaccharide backbone of lipid A are thought to be essential for high

toxicity and *P. gingivalis* lipid A structure lacks an ester-linked phosphate group at the 4' position which could account for the low toxicity reactions seen in the Shwartzman reaction for this bacterium (Johne *et al.*, 1988; Ogawa *et al.*, 1994d). In addition to this, differences in the fatty acid composition between *P. gingivalis* and *E. coli* LPS could reflect the differences seen in the activation of a host response (Fujiwara *et al.*, 1990).

LPS from *P. gingivalis* exhibits several biological activities. In particular the LPS can stimulate bone resorption at low levels (Iino and Hopps, 1984). The LPS can also cause polyclonal B-stimulation and induction of cytokine production from macrophages at a level that is comparable to that of enterobacterial LPS (Hofstad, 1992). It has been shown that *P. gingivalis* LPS can induce the production of IL-1 β, IL-6, IL-8, IFN γ and TNF α from human monocytes, furthermore the level of expression of IL-6 and IL-8 is higher than that induced by *E. coli* LPS (Agarwal *et al.*, 1995; Ogawa *et al.*, 1994d). As well as possessing bone resorption potential these cytokines can interact with fibroblasts and osteoblasts, mediating the production of metalloproteinases which are then capable of connective tissue degradation (Bramanti *et al.*, 1989). LPS from *P. gingivalis* has also been demonstrated to stimulate production of cytokines from human gingival fibroblasts, for example IL-6 which is important in the differentiation of activated B lymphocytes into antibody secreting cells (Takada *et al.*, 1991b; Yamaji *et al.*, 1995). Induction of IL-1 and PGE_2 from human fibroblasts by *P. gingivalis* LPS has also been noted (Sisney-Durrant and Hopps, 1991; Takada *et al*, 1991b). These are both potent stimulators of bone resorption and their release by LPS-stimulated fibroblasts would add to the pool of factors capable of bringing about alveolar bone loss, additionally PGE_2 causes continuation of the inflammatory response. Unlike LPS from other *Enterobacteriaceae,* the LPS from *P. gingivalis* is capable of stimulating the production of IL-8 from fibroblasts (Tamura *et al.*, 1992; Yamaji *et al.*, 1995). IL-8 is chemotactic and stimulatory for neutrophils and this results in accumulation of neutrophils in periodontal tissues whilst inducing neutrophil degradation and respiratory burst which would result in further tissue damage. It has also been demonstrated that main subclasses of IgG produced in response to *P. gingivalis* LPS are IgG2 followed by IgG4, with much lower levels of IgG1 and IgG3 being expressed. This is significant as IgG2

and IgG4 have been shown to be less efficient at binding complement components and less important in opsonisation and phagocytosis than either IgG1 or IgG3 (reviewed in Ogawa *et al.*, 1991b).

From these results it can be seen that the LPS from *P. gingivalis* is atypical. It contains phosphorylated KDO and lacks 3-hydroxymyristic acid which is a characteristic constituent of *Enterobacteriaceae* LPS. The endotoxic activity of *P. gingivalis* LPS, in terms of the Shwartzman reaction, is low although it has been found to be a potent stimulator of cytokines and PGE_2 which have important roles in the inflammation, tissue destruction and bone resorption associated with periodontal disease.

1.6.3 Capsules

The presence of an extracellular polysaccharide capsule, as defined by the electron microscopic visualisation of a ruthenium red, or India ink stained structure external to the cell wall, has been documented for *Porphyromonas* species (Okuda *et al.*, 1987; van Steenbergen *et al.*, 1987). These capsules appear as electron dense, hydrophilic, heteropolymeric layers of about 15 nm thick which are closely associated with the outer membrane (Mansheim and Coleman, 1980; Mayrand and Holt, 1988). Several studies have demonstrated the pathogenicity of encapsulated anaerobes, for example the formation of intra-abdominal abscesses in mice and rats by *B. fragilis* has been correlated with the presence of capsular material on these bacteria. In these experiments encapsulated *B. fragilis* strains, or purified capsular polysaccharide alone, induced abscesses, whereas non-encapsulated strains seldom caused abscesses (Onderdonk *et al.*, 1977). The capsules of *P. gingivalis* strains A7A1-28, W83 and 381 have been shown to contain predominantly amino sugars such as glucosamine, galactosamine and galactosaminuronic acid, with glucose being the only neutral sugar detected (Schifferle *et al.*, 1989). Differences in the sugars present in the capsular extracts from different strains of *P. gingivalis* have been noted, for example strain A7A1-28 does not contain galactose, a major component from extracts of strains 381 and W83 (Okuda *et al.*, 1987; Schifferle *et al.*, 1989).

Immunochemical studies on the capsular polysaccharide of *P. gingivalis* demonstrate that strains of this bacterium can be characterised according to the presence and immunoreactivity of a capsular antigen (van Winkelhoff *et al.*, 1993). The heat-stable antigen, which demonstrated the characteristics of a K-antigen and most likely represents a thermostable carbohydrate capsule, was found only in invasive strains of *P.gingivalis* (that is strains that induce abscesses or phlegmonous types of infections when injected into an animal model) and not on non-invasive strains. Of four invasive strains analysed three distinct serotypes of K-antigens could be recognised (van Winkelhoff *et al.*, 1993).

Bacterial hydrophobicity plays an important role in phagocytic ingestion and opsonisation (Absolom, 1988) and differences in hydrophobicity are seen between different strains of *P. gingivalis*. Strains of *P. gingivalis* that have a thick capsule, as indicated by India ink staining, demonstrate low hydrophobicity, appear to be more resistant to phagocytosis and require opsonisation with specific antibodies for complement-mediated killing. The presence of capsular material in these bacteria also correlates well with serum resistance and low chemiluminescence produced by PMN when exposed to *P. gingivalis* (van Steenbergen *et al.*, 1987; Sundqvist *et al.*, 1982; Sundqvist *et al.*, 1991; van Winkelhoff *et al.*, 1993). Encapsulated strains of *P. gingivalis* have also been demonstrated to be more virulent in the murine abscess model compared to acapsular strains (Reynolds *et al.*, 1989).

A Tn*4351* transposon induced capsular mutant (MSM-1) of *P. gingivalis* has been described that produces a capsule that is approximately twice as thick as the capsule found in the wild-type strain (Cutler *et al.*, 1994). MSM-1 exhibits greater resistance to phagocytosis when compared to the wild-type strain and incubation of the wild-type strain with PMN granule extracts results in a loss of viability, whereas, in contrast, MSM-1 is not killed by PMN granules under similar conditions. Antisera against the wild-type and MSM-1 confirmed antigenic differences in polysaccharide isolated from these strains and from this it can be hypothesised that the evasion of PMN phagocytosis

and killing appears to be associated with alterations in the capsular polysaccharide of strain MSM-1 (Cutler *et al.*, 1994).

An important component of the host immune response to periodontal pathogens during the progression of periodontal disease is an influx of phagocytes, in particular PMN, into the periodontal pocket. PMN can ingest bacteria by phagocytosis or they can kill the bacteria by oxidative or nonoxidative methods (Miyasaki, 1991). The serum resistance demonstrated by encapsulated bacteria and their ability to evade the action of PMN places these bacteria at an advantage when colonising and multiplying in the periodontal pocket. Mutants such as MSM-1 should prove extremely useful in identifying the exact role of capsules in periodontal infections.

1.6.4 Vesicles

The production of vesicles or "blebs" by *P. gingivalis* has been described by several groups (Handley and Tipler, 1986; Okuda *et al.*, 1981; Williams and Holt, 1985). Chemostat grown cultures of this bacterium display surface associated and extracellular vesicles (McKee *et al.*, 1986) and it is thought that they arise due to "blebbing" or extrusion from the outer cell membrane of intact cells during normal growth. The blebs are small, between 20 - 150 nm in diameter, and they have a polypeptide pattern that is similar, but not identical, to that of the outer membrane (Grenier and Mayrand, 1987; Williams and Holt, 1985). The structures are not empty, the contents of the periplasmic space becomes entrapped within the blebs as they are formed and this can be demonstrated as a moderately electron-dense staining material under electron microscopy (Deslauriers *et al.*, 1990).

The vesicles exhibit many of the biological activities of intact cells, for example they are capable of agglutinating erythrocytes (Grenier and Mayrand, 1987), they retain haemolytic activity (Chu *et al.*, 1991) and show collagenolytic and proteolytic activities (Grenier and Mayrand, 1987; Smalley and Birss, 1987). Vesicles have been shown to be associated with 80% of the extracellular activity of trypsin-like enzyme in culture

filtrates. The presence of the trypsin-like enzyme could be as a result of its envacuolation from the periplasmic space during bleb formation, or the enzyme could be integrated into the vesicle membrane (Smalley and Birss, 1987). It is possible that the release of proteolytic enzymes from the vesicles could be used to obtain peptides and haem compounds from host tissues, thus satisfying the nutritional requirements of the bacterium. In haemin limited environments large numbers of vesicles can be seen around *P. gingivalis* cells as well as free in the environment, whereas in haemin excess conditions fewer vesicles are seen on and around the cells (McKee *et al.*, 1986).

The role of vesicles in pathogenesis is unclear, however, growing evidence has indicated that vesicles may function as a virulence factor (Mayrand and Holt, 1988). Because of their small size it is thought that the vesicles could readily cross the epithelial cell barriers and as such act as a vehicle for delivering toxins and various proteolytic enzymes to the underlying connective tissue. Once the vesicles have penetrated deeply into the host tissue the action of host proteolytic enzymes could release the vesicles degradive enzymes into the tissue resulting in severe destruction (Holt and Bramanti, 1991). It has been observed that vesicles are capable of degrading type I collagen and fibronectin which are components of the connective tissue matrix in the periodontal ligament (Grenier and Mayrand, 1987; Smalley and Birss, 1987). LPS has also been demonstrated to be a major component of the *P. gingivalis* vesicle which could transport the LPS directly into the gingival connective tissue where it could exert its many biological functions resulting in stimulation of the inflammatory response, tissue destruction and bone resorption (Bramanti *et al.*, 1989).

Because vesicles have a similar polypeptide pattern as whole cells it has been suggested that they may be able to protect the infecting bacteria by competing for antibodies directed against whole cells so as to impede the hosts humoral defence mechanism (Deslauriers *et al.*, 1990). However it is also feasible that due to this property the vesicles can stimulate the hosts immune response which can then be directed against *P. gingivalis* whole cells. The vesicles from *P. gingivalis* have also been demonstrated to be resistant to the bactericidal activity of human serum possibly through a proteolytic

activity against host immunoglobulins and complement factors. It has been suggested that extracellular vesicles could indirectly protect other periodontal pathogens from complement action, thus allowing the overall progression of periodontal disease (Grenier and Bélanger, 1991). Vesicles may also be phagocytosed by PMN thereby depleting the phagocytic potential of these cells, complimentary to this they could also initiate the oxidative and non-oxidative killing mechanisms of PMN which could lead to host-mediated tissue destruction (Holt and Bramanti, 1991).

Vesicles have also been shown to have a role in coaggregation, an important step in the initial colonisation of many periodontal pathogens and have been demonstrated to mediate binding between homologous cells as well as noncoaggregating species. This suggests that the vesicles could act as coaggregation bridges, allowing species that can not normally adhere to each other, to aggregate via vesicles and thus contribute to the developing plaque and the progression of periodontal disease (Grenier and Mayrand, 1987). Vesicles can also act as a bridge between *Streptococcus mutans*, a pathogen of dental caries, and the tooth surface, and it has been suggested that the vesicles could promote the adherence of many other organisms to the pellicle, dentin and cementum of tooth surfaces (Kamaguchi *et al.*, 1995).

Although it is not known for certain whether the vesicles produced by *P. gingivalis* have a role as a virulence factor, it is clear that they have a potential role in disarming the host response. Putatively, the vesicles could aid the colonisation of periodontopathogens, as well as perpetuating periodontal disease by the release of LPS and proteolytic and other hydrolytic enzymes deep in the gingival tissues. However, due to the fact that the vesicles released by *P. gingivalis* share the same antigenic properties as whole cells it is feasible that the vesicles have the effect of stimulating the hosts immune response

1.6.5 Hydrolytic enzymes

A multitude of enzymatic activities are exhibited by *P. gingivalis* including protease, neuraminidase, β-N-acetyl-hexosaminidase, phospholipase A, alkaline phosphatase,

acid phosphatase, chondroitin sulphatase and keratinase activities (Holt and Bramanti, 1991). These hydrolytic enzymes are capable of degrading host macromolecules with the result of obtaining peptides required for the growth of the pathogens and it has been speculated that any contribution that these enzymes make towards the virulence of this bacterium may simply be coincidental with this process (Gharbia and Shah, 1993). Previously, it has been difficult to detect enzymatic activities due to the low specificity of some enzymes or the broad specificity of some of the substrates used. However, the development of fluorometric and chromogenic substrates for assaying these enzymes has improved the detection and subsequent analysis of particular activities (Gharbia and Shah, 1993).

Proteases

It has been demonstrated that *P. gingivalis* is highly proteolytic and is capable of degrading a number of different substrates (Hinode *et al.*, 1991; Holt and Bramanti, 1991). It has been observed that this organism can degrade host iron transport proteins such as albumin, haemopexin and haptoglobin (Mayrand and Holt, 1988) from which it is probable that this bacterium obtains haemin. Proteolytic degradation of IgA, IgG and IgM as well as complement factors C3, C4, C5 and factor B by *P. gingivalis* has been demonstrated and such activity may enable the bacteria to evade the host humoral defence mechanisms (Kilian, 1981; Schenkein, 1988; Sundqvist *et al.*, 1985). Alpha-1-antitrypsin and alpha-2- macroglobulin are the two main protease inhibitors found in serum, however proteases from *P. gingivalis* have been shown to degrade these proteins and it has been suggested that deficiency in serum protease inhibiting capacity could be correlated with periodontal disease (Bedi and Williams, 1994; Sandholm, 1986). While the contribution of bacteria-derived proteases on the degradation of host tissues is unknown, it has been shown that proteases from *P. gingivalis* are able to degrade fibrinogen and fibronectin (Bedi and Williams, 1994; Fujimura *et al.*, 1992; Lantz *et al.*, 1991) as well as collagen type I, III, IV and V. Since type I collagen is a major component of the connective tissue in the gingivae, periodontal ligament and alveolar bone and type IV collagen is a major component of the basement membrane, it is

probable that these bacteria-derived proteins have an important role in the tissue destruction seen during periodontal disease (Bedi and Williams, 1994; Birkedal-Hansen *et al.*, 1988; Kadowaki *et al.*, 1994).

Although many studies have been carried out on the proteases of *P. gingivalis*, the actual number and diversity of these enzymes is unknown. Early attempts to separate and characterise the enzymes has indicated a myriad of related but nonidentical proteases. Ambiguities have arisen since, despite common features, the purified proteins differed widely in characteristics such as molecular weight or substrate specificity (Table 1.3). Some of the differences described could be due to the fact that the enzymes have been isolated from different sources such as vesicles, from the supernatant of spent culture media or from the bacterial cells themselves. It is also possible that some of the proteolytic activities reported could be due to autolytic cleavage products of relatively few proteolytic enzymes. One study which used SDS-PAGE analysis suggested that there were eight proteases ranging in molecular weight from 29 kDa to 110 kDa, seven of which were found in vesicle and membrane preparations, three of these seven were also found in the supernatant and one band of activity was exclusively extracellular (Grenier *et al.*, 1989). It is only in recent years that structural data has enabled comparative analyses to be made between different protease genes. Sequence data from such studies should help to clarify the number and specificity of these enzymes (Bedi and Williams, 1994; Fletcher *et al.*, 1994; Madden *et al.*, 1995).

Studies on the proteases have attempted to classify the enzymes according to substrate specificity. Collagenolytic activity has been demonstrated in *P. gingivalis* (Mayrand and Grenier, 1985; Sundqvist *et al.*, 1987) and the enzyme(s) has been classified as a true collagenase due to its ability to cleave the helical domain of native triple helical collagen molecules (Birkedal-Hansen *et al.*, 1988). A collagenase gene *prtC* has been cloned and sequenced from *P. gingivalis* which encodes a 35 kDa molecular weight protein (Kato *et al.*, 1992; Takahashi *et al.*, 1991), and some of the trypsin-like

Table 1.3. Proteases described for *P. gingivalis*.

Name and Strain	Starting Material	Molecular Weight (kDa)	Substrate	Reference
Trypsin-like protease 381	cell envelope extract	ND	BAPNA, BANA, BSA	Yoshimura *et al.*, 1984b
Trypsin-like protease 33277	culture supernatant	300	azocasein, azocoll, BAPNA	Fujimura and Nakamura, 1987
glycylprolyl protease 33277	outer membrane	29	azocasein, azocoll, Gly-Pro*p*NA	Grenier and McBride, 1987
caseinolytic protease 33277	culture supernatant	ND	BANA, lysozyme	Otsuka *et al.*, 1987
collagenase 381	cell envelope extract	90	gelatin, collagen type I, II and III	Birkedal-Hansen *et al.*, 1988
glycylprolyl protease A7A1-28	cell envelope extract	80	Gly-Pro*p*NA	Barua *et al.*, 1989
gingivain W83	culture supernatant	100	BAPNA	Roberts *et al.*, 1990 Shah *et al.*, 1990
Pase-A 381	culture supernatant	105 - 110	casein, BAPNA	Hinode *et al.*, 1991
Pase-B 381	culture supernatant	105 - 110	casein, BAPNA	Hinode *et al.*, 1991
Pase-C 381	culture supernatant	44	casein, BAPNA	Hinode *et al.*, 1991
collagenase *prtC* ATCC 53977	cloned into *E. coli*	38	soluble or fibrillar collagen type I	Takahashi *et al.*, 1991
prtT 33277	cloned into *E. coli*	96 - 99	collagen, fibrinogen, fibronectin, BAPNA	Madden *et al.*, 1992
tpr W83	cloned into *E. coli*	50	BSA, azocoll, fibrinogen	Bourgeau *et al.*, 1992
prtH W83	cloned into *E. coli*	97	human C3 complement protein	Fletcher *et al.*, 1994
trypsin-like protease 381	culture extract	55	collagen type I, III, IV and V, fibrinogen fibronectin	Bedi and Williams, 1994

Table 1.3 Proteases described for *P. gingivalis* and their substrate specificities. Abbreviations, ND not done; BAPNA, benzoyl-L-arginine-*p*-nitoranalide; BANA, benzoyl-L-arginine-β-naphthylamide; BSA, bovine serum albumin; Gly-Pro*p*NA, glycl-L-proline-nitroanilide.

proteases isolated from *P. gingivalis* have also been shown to have collagenase activity (Bedi and Williams, 1994; Birkedal-Hansen *et al.*, 1988; Kadowaki *et al.*, 1994).

The production of trypsin-like enzymes, so called because these enzymes are able to degrade many of the synthetic substrates that are degraded by trypsin (Hinode *et al.*, 1991), distinguish *P. gingivalis* from the other black-pigmented anaerobic species, such as *P. asaccharolyticus* (Laughton *et al.*, 1982). Several such trypsin-like proteases have been isolated from *P. gingivalis* and these have generally been found to be thiol-activated enzymes that hydrolyse lysine and arginine-containing substrates (Curtis *et al.*, 1993; Fujimura *et al.*, 1993; Hinode *et al.*, 1991; Park and McBride, 1992). Trypsin-like protease activity has been shown to be increased with increasing concentrations of haemin (Marsh *et al.*, 1988) and cells grown in haemin excess conditions are found to be more virulent in a mouse killing model (McKee *et al.*, 1986). Following on from these studies it was found that a virulent strain of *P. gingivalis,* W50, exhibited a three fold greater trypsin-like activity than an avirulent strain (W50/BE1), indicating that this enzyme activity is important in the virulence of this bacterium (Marsh *et al.*, 1989; Smalley *et al.*, 1989). Trypsin-like proteases are also thought to be important in adherence of the bacteria to human epithelial cells (Gibbons, 1989). Treatment of these cells with trypsin-like enzyme enhances the attachment of *P. gingivalis* to cryptic receptors which are exposed after hydrolysis of arginine residues present on the epithelial cells (Gibbons, 1989; Grenier, 1992c). Trypsin-like proteases are also thought to be involved in the adhesion of *P. gingivalis* to *Actinomyces* species (Li *et al.*, 1991).

The characterisation of a trypsin-like protease, termed gingipain, that exhibits limited substrate specificity has been reported by several laboratories (Kadowaki *et al.*, 1994; Pavloff *et al.*, 1995; Pike *et al.*, 1994; Potempa *et al.*, 1995). Gingipain was shown to exist in two forms in all *P. gingivalis* strains tested. The form Arg-gingipain (RGP), was found to cleave peptides specifically on the carboxyl-terminal side of arginine residues, whereas the other, Lys-gingipain (KGP), cleaved specifically at the carboxly-terminal side of lysine residues. It was suggested that these two enzymes, working in concert, are responsible for most, if not all, of the trypsin-like activity of *P. gingivalis*

and that previously isolated trypsin-like enzymes that showed arginine and lysine specificity were in fact a mixture of the two different gingipain proteases (Potempa *et al.*, 1995). RGP was isolated from membranes, vesicles, or from the culture supernatant and was found to exist in several forms of different molecular weight. Trypsin-like proteases with a molecular weight of around 50 kDa have been frequently isolated from *P. gingivalis* (Curtis *et al.*, 1993; Fujimura and Nakamura, 1990), and in the studies on RGP it was found that a 50 kDa protease was either released in a free form into the culture media or was associated with haemagglutinins to form 110 or 95 kDa protein complexes (Pike *et al.*, 1994; Potempa *et al.*, 1995). The 110 and 95 kDa enzymes are closely related but the higher molecular weight enzyme form contains an extra 15 kDa polypeptide which is presumably involved in anchoring the enzyme to the membrane. Proteins in the range of 70 - 90 kDa were also isolated from vesicles and membranes and these proteins were found to consist of single polypeptide chains (Potempa *et al.*, 1995). The variety of 70 - 90 kDa molecular weights demonstrated are thought to be due to C-terminal processing of an initial polypeptide signal. KGP was also found to exist in a free form or as an enzyme-haemagglutinin complex (Potempa *et al.*, 1995). In summary, these authors suggested that the enzymes purified in other studies were different forms of RGP or KGP and that the difficulty in interpreting the data was due to the fact that either enzyme could occur as a single chain, free proteases or part of a protease-haemagglutinin complex with or without putative membrane anchorage polypeptides. Remaining diversity can be explained by the occurrence of enzymatic degradation products that could still retain proteolytic activity.

The cloning and expression in *E. coli* of trypsin-like enzymes encoded for by the genes *tpr* (Bourgeau *et al.*, 1992; Park and McBride, 1992), *prtT* (Otogoto and Kuramitsu, 1993), and *prtH* (Fletcher *et al.*, 1994) that do not share sequence homology with RGP, or each other, suggests that there is more than one protease with a specificity for arginine. The gene *tpr* encoding a distinct 64 kDa thiol protease has been cloned and sequenced from strain W83 (Bourgeau *et al.*, 1992; Park and McBride, 1992). A Tpr-deficient mutant of *P. gingivalis* has been constructed with the view to carry out *in vivo* pathogenicity studies (Park and McBride, 1993). Although the results of these studies

have not been reported as yet, such studies will determine whether the protease is associated with virulence or not. Another protease, *prtT,* isolated from strain 33277 has also been characterised (Madden *et al.*, 1992; Otogoto and Kuramitsu, 1993). In contrast to the *tpr* gene product PrtT hydrolyses the synthetic substrate BAPNA (benzoyl-L-arginine-*p*-nitroanalide). The gene was originally identified downstream of collagenase (*prtC*) and superoxide dimutase (*sod*) genes on a 5.9 kb *P. gingivalis* DNA fragment. Due to the close proximity of *prtT* and *prtC* it was proposed that the protease may have a role in further cleavage of degradation products of type I collagen produced by the action of collagenases (Otogoto and Kuramitsu, 1993). Haemagglutination activity was also found at the 3' end of the 5.9 kb fragment and it was originally believed to be encoded by a separate gene downstream of *prtT*. However, revised sequence analysis established that the *prtT* gene was larger than originally reported and that it actually encompassed the region encoding haemagglutination (Madden *et al.*, 1995). Finally, a 97 kDa protease, designated PrtH, that is normally found in the membrane vesicles has been cloned and expressed in *E. coli* (Fletcher *et al.*, 1994). This protease has been demonstrated to degrade the human C3 complement protein under defined conditions. Analysis of PrtH reveals that the predicted nascent product contains a protease domain followed by a haemagglutinin domain which are post-translationally processed by proteolytic events to produce a 43 - 54 kDa arginine-specific, thiol protease and a 43 - 53 kDa haemagglutinin (Kirszbaum *et al.*, 1995). The use of allelic exchange has enabled a *P. gingivalis* W83 strain with an inactive *prtH* gene to be made (Fletcher *et al.*, 1995). This mutant strain showed reduced proteolytic activity. In addition to this it was demonstrated that this mutant was dramatically less virulent than the wild-type strain in a mouse model of bacterial invasiveness. Some strains that did not contain sequences that hybridised with the prtH gene probe were also found to be less virulent than naturally occurring isolates which did carry the *prtH* gene sequence (Fletcher *et al.*, 1995). These results represent the first study elucidating the role of complement-degrading proteases in the pathogenesis of *P. gingivalis*.

Further molecular characterisation of this group of enzymes will clarify the relationship and relatedness between different proteases. Although the exact role that proteases

expressed by *P. gingivalis* may play in periodontal disease has not been determined, it has been demonstrated that they are potentially important not only in attachment, but also in the destruction of host defence mechanisms and destruction of the host tissues. The cloning and sequencing of these enzymes will allow their function in periodontal disease to be elucidated and comparison of cloned proteases will help to unravel the confusing data that surrounds the exact number and substrate specificity of these enzymes. The construction of specific isogenic protease mutants will allow the role that these enzymes play in virulence to be determined.

Exoglycosidases

Exoglycosidases catalyse hydrolytic cleavage of the glycosidic linkages beginning at the outer non-reducing end of oligosaccharide chains. Unlike the proteases, the glycosidases, neuraminidase and β-N-acetyl-hexosaminidase (β-Nahase) both of which are shown to be secreted by *P. gingivalis* (Minhas and Greenman, 1989; Moncla *et al.*, 1990; Tipler and Embery, 1985), have not been studied in any great detail. Recently the gene encoding for the β-Nahase of *P. gingivalis* W83 was cloned into *E. coli* and sequenced. It was found that this gene is present as a single copy on the chromosome and that it is absent from the chromosome of other members of the genus (Lovatt and Roberts, 1994). The action of β-Nahase is to remove terminal N-acetyl-glucosamine (GlcNAc) and N-acetyl-galactosamine (GalNAc) residues from oligosaccharides and glycoproteins (Cabezas, 1989). Proteoglycans are major constituents of gingival connective tissue and are composed of proteins covalently linked to glycosaminoglycan (GAG) chains and oligosaccharides. The proteoglycans have a role in regulating cell adhesion and growth, matrix formation, collagen fibril formation and binding of growth factors (Rouslahti, 1989; Uitto, 1991). The effect of β-Nahase on the proteoglycan function by enzymatic attack on GAGs could have a pronounced effect on the stability of the gingival tissue. It is also speculated that the β-Nahase could remove glycosidic residues from glycoproteins such as IgG which would aid the bacteria in evading host defence mechanisms (Koide *et al.*, 1977). The role of this enzyme in tissue destruction is currently unknown although it has been demonstrated that there are increased levels

of exoglycosidase activity in the gingival fluid of periodontal patients (Beighton *et al.*, 1992). A more precise role for this enzyme should be established by assaying the virulence potential of β-Nahase mutants generated by site-directed gene replacement (Lovatt, 1994).

Sialic acids are a family of 9-carbon carboxylated sugars usually found as terminal monosaccharides of animal oligosaccharides. The most common sialic acid is N-acetyl-neuraminic acid which is believed to be the biosynthetic precursor for all other members of the family (Varki, 1992). Neuraminidase cleaves terminal sialic acid residues from glycoproteins, glycolipids and oligosaccharides (Gharbia and Shah, 1993; Taylor *et al.*, 1992) and has been demonstrated to be able to modify host responses to bacterial infections. Desialidation of IgG decreases its ability to bind complement, whilst the action of neuraminidase on erythrocytes and leukocytes causes these cells to lose their glycoprotein receptors which leads to their removal from circulation. Furthermore the function of collagen synthesis, IL-2 and macrophage inhibitory factors are also reduced in the presence of sialidases (Aalto *et al.*, 1974).

There is evidence for two families of neuraminidases, with different requirements for a divalent metal ion to produce optimum activity (Roggentin *et al.*, 1993). Those enzymes which do not require metal have molecular weight values of around 42 kDa and represent the "small" bacterial neuraminidase family. The neuraminidase genes cloned from *Clostridium perfringens* and *Salmonella typhimurium* (Hoyer *et al.*, 1991; Taylor *et al.*, 1992) represent members of the small neuraminidase family. Neuraminidase genes that do require a metal ion for activity and have a molecular weight of greater than 60 kDa belong to the "large" family, for example the *Vibrio cholerae* neuraminidase is a member of this family and has a molecular weight of 90 kDa (Galen *et al.*, 1992; Taylor *et al.*, 1992).

Neuraminidase activity has been demonstrated in the dental plaque (Rogers *et al.*, 1979), in salivary secretions (Perlitsh and Glickman, 1966) and in crevicular fluid (Kitawaki *et al.*, 1983). Oral bacteria that exhibit neuraminidase activity include *P. gingivalis*, but

not *P. asaccharolyticus* or *P. endodontalis* (Moncla *et al.*, 1990), *Prevotella loecheii* and *Actinomyces viscosus*, and the genes encoding neuraminidase have been cloned and sequenced for the latter two bacteria species (Henningsen *et al.*, 1991; Takeshita *et al.*, 1991).

Although the role of neuraminidase in the virulence of oral bacteria is unknown, what is clear is that neuraminidase activity can affect the colonisation of oral surfaces. Treatment of epithelial cells with neuraminidase greatly reduces the number of the early colonising bacteria *S. sanguis* and *S. mitis* that attach, presumably because these bacteria possess adhesins which bind to sialic acid-containing receptors, which are destroyed upon treatment with neuraminidase (Gibbons, 1989). The elimination of these bacteria from the oral epithelial cells would facilitate the attachment of neuraminidase producing bacteria, such as *P. gingivalis*.

Proteoglycans interact with collagen and other fibrous proteins to form a sieve-like three-dimensional network that contributes to the overall integrity of the gingival connective tissue. It is likely that the glycosidases act in concert to break down gingival tissues, rendering the host tissue susceptible to subsequent secondary attack by bacterial proteases. The detection of β-Nahase activity in outer-membrane vesicles of *P. gingivalis* would suggest that the glycosidases could be transported deep into gingival tissues where they could cause preliminary tissue damage to the GAGs and associated glycoproteins which would effect the functional integrity of the host tissue (Minhas and Greenman, 1989; Holt and Bramanti, 1991). The action of glycosidases on host proteins such as IgG and complement factors can disrupt the hosts ability to respond to bacterial invasion which suggests that this group of enzymes could have a significant virulence potential in periodontal disease and associated tissue destruction.

Superoxide dismutase

The superoxide dismutases (SODs) are a family of metalloproteins that bind iron (Fe-SOD), maganese (Mn-SOD) or copper (CuZn-SOD) in their active site. The SOD gene

from *P. gingivalis* has been characterised and it has been demonstrated to exist as three isozymes derived from a single apoprotein. In anaerobic conditions three Fe-SOD isozymes are found, whereas in aerobic conditions a major Mn-SOD with two minor Fe-SOD isozymes have been demonstrated (Amano *et al.*, 1990). The presence of three isozymes from one apoprotein can be explained by different regulation of the type of metallo-SOD formed at the post-transcriptional level, for example by acetylation or phosphorylation. Such regulation could result in the two minor isozymes only accepting Fe, but the major isozyme being able to accept Fe or Mn (Amano *et al.*, 1990).

Phagocytosis of bacteria by PMN is accompanied by oxidative metabolism resulting in superoxide hydrogen peroxide, singlet oxygen and hydroxy radicals which contribute to the bactericidal activity of the PMN (Miyasaki, 1991). Generally aerobic and many anaerobic bacteria express enzymes such as superoxide dismutase, catalase and peroxidase which neutralise these toxic oxygen metabolites. Although strains of *P. gingivalis* isolated from animals have catalase activity, strains of this bacterium isolated from the human oral cavity exhibit only superoxide dismutase activity which catalyses dismutation of superoxide (O_2^-) into O_2 and H_2O_2 (Amano *et al.*, 1988). It has been demonstrated that out of the black-pigmented anaerobes *P. gingivalis* is the most aerotolerant in the presence of oxygen and shows the highest SOD activity, with a twofold increase in SOD specific activity occurring on exposure of this bacterium to air (Amano *et al.*, 1988; Amano *et al.*, 1990).

Importantly, the SOD from *P. gingivalis* has been demonstrated to protect the bacteria from killing by PMN and it appears that the biological functions of Fe-SOD and Mn-SOD may be the same in terms of being able to inhibit bacterial killing by PMN (Amano *et al.*, 1992). The importance of SOD activity to the survival of *P. gingivalis* is highlighted by the use of site-directed mutagenesis to disrupt the *sod* gene. The resulting mutant was demonstrated to show no SOD activity and as a consequence exhibited a rapid viability loss immediately after exposure to air, whereas the wild-type parent showed no decrease in viability for at least 5 hours under aerobic conditions (Nakayama, 1994). It has also been demonstrated that SOD activity, as well as being

induced by increased oxygen tension, undergoes a twofold increase under the condition of elevated temperature (Amano *et al.*, 1994). The use of recently developed temperature sensitive probes to detect the temperature in periodontal pockets, have revealed a higher temperature in pockets with periodontal disease compared to anatomically equivalent healthy sulci (Fedi and Kilroy, 1992). The temperature in diseased sites was found to be up to two degrees higher than at healthy sites and the induction of SOD activity at these elevated temperatures suggests that temperature stress might influence the pathogencity of *P. gingivalis*. It has been observed that heat shock treatment of murine macrophages results in an enhanced capacity to release superoxide ion (Reddy and Gangadharam, 1992). This observation would indicate the necessity of increased SOD activity to allow the invading bacteria to survive in the increased superoxide levels that could occur due to the raised temperature and subsequent increase in superoxide ion tension in periodontal pockets. Overall it can be speculated that aerotolerance would be an important attribute for periodontopathogens which are often exposed to air as well as oxidative stress due to the host response to invading pathogens.

1.7 Genetic Approaches to the Study of Virulence Factors.

Until relatively recently the molecular genetic study of putative virulence determinants from *P. gingivalis* was limited to the cloning and expression of the genes encoding these determinants into *E. coli* (Proglaske-fox *et al.*, 1989a; Roberts *et al.*, 1990). Such studies are useful for determining the structure of the gene, but are of little use for assessing the contribution that these factors may make to the aetiology of periodontal disease. The genetic approaches used to obtain isogenic mutant derivatives in other Gram-negative pathogens such as *E. coli*, by transposon mutagenesis or by the reintroduction of cloned DNA on plasmid suicide vectors for example, were not applicable for the study of *P. gingivalis* due to a lack of a suitable system for introducing DNA into this bacterium. The development of genetic systems in *P. gingivalis* has been severely hindered due to an apparent lack of naturally occurring plasmids, bacteriophages or transposons in this organism (Höhne *et al.*, 1993; Sako *et al.*, 1988; Sandmeier *et al.*, 1993). There have been few reports of plasmids in black-

pigmented Gram-negative anaerobes. Plasmids have been demonstrated in *P. melaninogenicus*, *P. intermedia, P. asaccharolyticus* and *B. levii* but no vectors have been constructed based on these plasmids for use in this group of bacteria (Höhne *et al.*, 1993; Sako *et al.*, 1987; Sako *et al.*, 1988; Yoshimoto and Umemoto, 1990). A species-specific insertion element, IS*1126*, that belongs to the IS*5* group within the IS*4* super-family of insertion elements, has been described for *P. gingivalis* (Maley *et al.*, 1992; Maley and Roberts, 1994). Insertion elements represent the simplest form of transposable elements, they are between 0.7 to 1.6 kb and encode only the proteins required for the transposition process (Galas and Chandler, 1989), however, Southern blot analysis of several different strains of *P. gingivalis* have revealed that multiple copies of this element are present on the chromosome and so it is unlikely that IS*1126* could be used for random insertional mutagenesis in *P. gingivalis* (Maley and Roberts, 1994). Transposons are larger and more complex than insertion elements, and these usually contain genes for auxiliary traits, such as antibiotic or heavy metal resistance as well as the genes for transposition. Many transposons are composite elements in which the auxiliary genes are bracketed by IS elements (Berg *et al.*, 1989), as yet no studies have been carried out to determine whether IS1126 exists as part of a composite transposon in *P. gingivalis* or not.

During the last ten years a genetic system of plasmid shuttle vectors and transposon suicide vectors has been developed for use in colonic *Bacteroides* species (Shoemaker *et al.*, 1986; 1989; 1991). Because of the relatively close phylogenetic relationship between the *Bacteroides* species and *P. gingivalis*, compared to other Gram-negative bacteria such as *E. coli* (Woese, 1987) it seemed appropriate to try and exploit the systems used in *Bacteroides* species as a means of genetic manipulation in *P. gingivalis.* Two distinct mechanisms of transferable antibiotic resistance have been described for intestinal *Bacteroides* and these determinants are either plasmid-encoded (Privitera *et al.*, 1979; Tally *et al.*, 1979; Welch *et al.*, 1979) or associated with the chromosome (Malamy and Tally, 1981; Marsh *et al.*, 1983; Tally *et al.*, 1981).

Transfer of clindamycin and tetracycline resistance markers in plasmid-free cells has been found to be due to conjugative transposons. These are self-transmissible elements that are normally integrated into the chromosome but can excise themselves and transfer by conjugation to the recipient (for a review on *Bacteroides* conjugative transposons see Salyers *et al.*, 1995). These elements are also capable of transferring plasmids either in *trans*, by providing the proteins required to form the mating pore, or in *cis*, where the conjugative transposon integrates into the plasmid and as well as creating the mating pore may also provide the origin of replication and proteins required for initiation of transfer (Salyers *et al.*, 1995).

The second system of transfer of antibiotic resistance determinants is by the conjugation of plasmids that encode such determinants. Three separate *Bacteroides* R plasmids were found to carry a gene (*erm*F) encoding resistance to the lincosamide antibiotics clindaymicin/erythromycin (Privitera *et al.*, 1979; Tally *et al.*, 1979; Welch *et al.*, 1979). Two of the plasmids, a 41 kb plasmid pBF4 (Welch *et al.*, 1979) and a 14.6 kb plasmid pBFTM10 (Tally *et al.*, 1982), were isolated from different strains of *B. fragilis,* the third plasmid pBI136 (82 kb) was isolated from *B. ovatus* (Smith and Macrina, 1984). The plasmids were demonstrated to be unrelated except for in the region of the highly conserved *erm*F determinant (Guiney *et al.*, 1984a; Shimell *et al.*, 1982), which was found in all three plasmids to be flanked by homologous direct repeats of approximately 1.2 kb (Guiney *et al.*, 1984b; Smith, 1985; Smith and Gonda, 1985; Welch and Macrina, 1981). These were subsequently shown to be transposons Tn*4351* and Tn*4400* on pBF4 and pBFTM10 respectively, which have over 90% sequence homology to each other (Robillard *et al.*, 1985; Salyers *et al.*, 1987; Shoemaker *et al.*, 1985), and Tn*4551* on pBI136 (Smith and Speigel, 1987). Although these plasmids can be transferred between *Bacteroides* species, differences in the control of plasmid replication between *E. coli* and *Bacteroides* species acts as a substantial barrier to plasmid transfer between these bacteria (Guiney *et al.*, 1984c)

A large number of shuttle-vectors have now been constructed based on these transposon-carrying plasmids that can be transferred to *Bacteroides* recipients by

conjugal transfer from *E. coli*. These chimeric vectors are capable of replication in *E. coli* and *Bacteroides* due to the presence of two origins of replication, one of which is usually taken from one of the *Bacteroides* cryptic plasmids while the other is taken from *E. coli* (Matthews and Guiney, 1986; Pheulpin *et al.*, 1988; Shoemaker *et al.*, 1986; Shoemaker *et al.*, 1989). Antibiotic resistance markers are provided by the *erm*F determinant which allows selection of the vectors in *Bacteroides*, Tn*4351* and Tn*4400* also encode a tetracycline resistance determinant that is only expressed in aerobically grown *E. coli* and not in *Bacteroides* or anaerobically grown *E. coli*. Many of the first shuttle vectors to be developed included *E. coli* mobilisation regions because it was assumed that such regions would be required if the vectors were to be mobilised by IncP plasmids. However subsequent results revealed that the *Bacteroides* plasmids that form the basis of the chimeric vectors already contained mobilisation regions that were recognised by broad host range IncP plasmids such as R751 (Salyers *et al.*, 1987).

These chimeric vectors were found to be suitable for use in *P. gingivalis* and the first successful report of the introduction of foreign DNA into *P. gingivalis* was by Progulske-fox *et al.* (1989b). The shuttle vector used in this study was pE5-2 (fig. 1.4) which was constructed from three plasmids pB8-51, a cryptic *Bacteroides* plasmid, Tn*4351* from pBF4 which was used to provide the antibiotic-resistance markers and RSF1010 an IncQ *E. coli* plasmid which contains a mobilisation factor that is recognised by R751 (Shoemaker *et al.*, 1985). This shuttle vector was transferred into two strains of *P. gingivalis* as well as into *P. intermedia*. Further studies using a vector pVAL-1 demonstrated the ability of such plasmids to be conjugally transferred into several strains of *P. gingivalis* (Dyer *et al.*, 1992). Recently suicide vectors, chimeric vectors that do not contain a *Bacteroides* origin of replication, have been used to generate isogenic mutants of putative virulence determinants in *P. gingivalis*, these include mutations of the superoxide dismutase gene (Nakayama, 1994), *trp* protease gene (Park and McBride, 1993), *fimA* (Malek *et al.*, 1994) and *gdh*, a gene that encodes for glutamate dehydrogenase (Joe *et al.*, 1994). It has now been demonstrated to be possible to electroporate DNA into *P. gingivalis* (Yoshimoto *et al.*, 1993), and this method has been used to introduce a disrupted copy of the *prtH* protease gene into *P.*

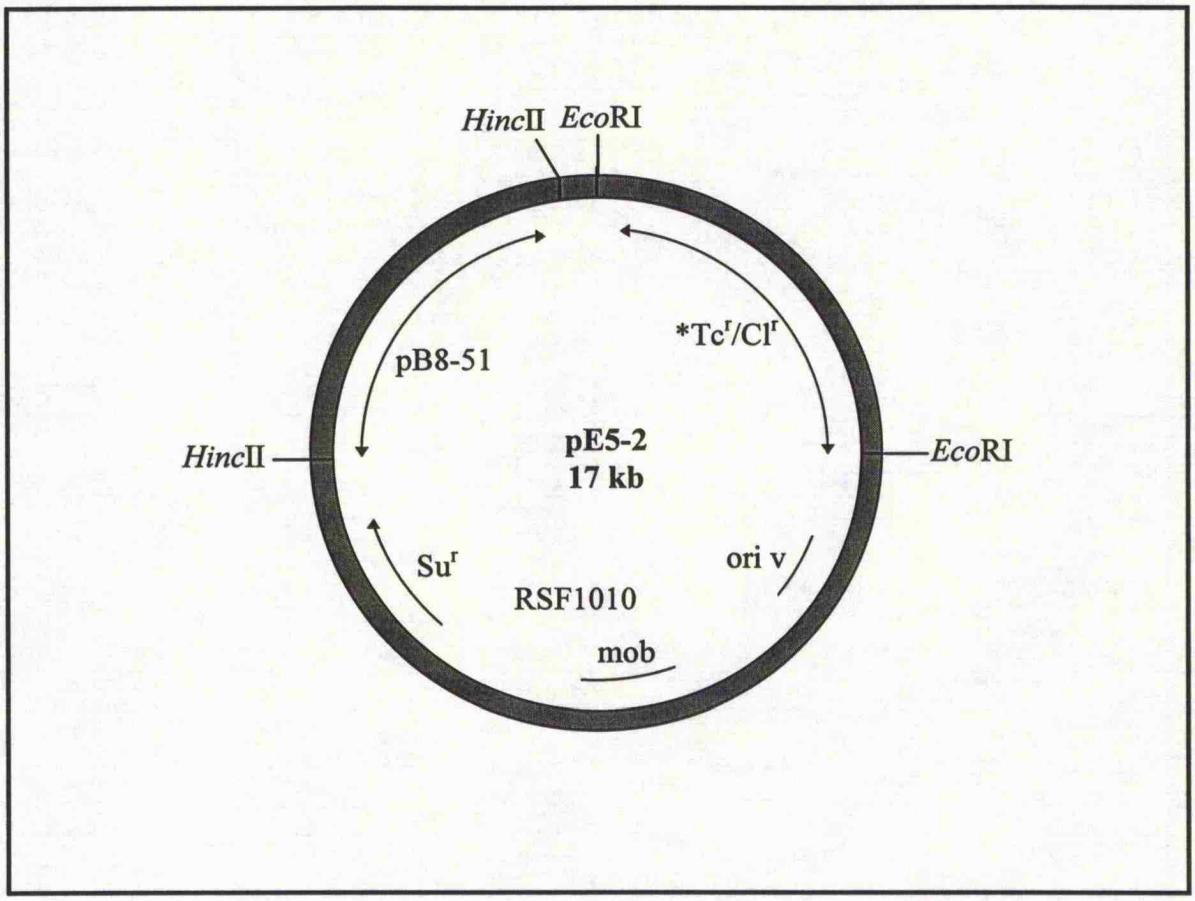


Fig. 1.4. Map of plasmid pE5-2. The plasmid pE5-2 was constructed by cloning a 3.8 kb *Eco*RI fragment from pBF4 into the *Eco*RI site of RSF1010 and by cloning the cryptic *Bacteroides* plasmid pB8-51 into the *Hinc*II site of RSF1010. The resulting 17 kb plasmid expresses the Su^r (sulphanilamide) and $*Tc^r$ (tetracycline) determinants in *E. coli* and the Cl^r (clindamycin) determinant in *Bacteroides* spp. The diagram demonstrates the position of the origin of replication (ori v) and the mobilisation region (mob).

gingivalis, resulting in a *P. gingivalis* W83 mutant that was defective in the *prtH* gene (Fletcher *et al.*, 1995).

Tn*4351* and Transposon Mutagenesis

Tn*4351*, originally isolated as part of the *B. fragilis* plasmid pBF4, has been used successfully to create many well-defined, single mutations in *P. gingivalis*. Shoemaker *et al.* first identified that the *ermF* determinant on pBF4 was part of a transposon during conjugation experiments in *E. coli* in which they mobilised the *Bacteroides* shuttle vector pE5-2 between *E. coli* strains (Shoemaker *et al.*, 1985). In these studies it was found that a 3.8 kb *Eco*RI fragment of pBF4 that contained the *ermF* determinant was capable of inserting randomly into the *E. coli* chromosome as well as into R751, the IncP plasmid used to mobilisable pE5-2 between strains. Furthermore it was found that the insertion events were RecA independent, occurring in *recA*$^-$ as well as *recA*$^+$ cells, and so it was determined that the clindamycin/erythromycin element was part of a transposon that was subsequently designated Tn*4351*. The transposon was later introduced into *B. uniformis* on R751 which can not be maintained in *Bacteroides* and so acts as a suicide vector. It was found that not only could Tn*4351* insert into the *B. uniformis* chromosome but it also mediated the cointegration of R751 into the chromosome in about half of the transconjugants (Shoemaker *et al.*, 1986). Approximately 13% of the transconjugants were found to be auxotrophic mutants which gave the first indication that Tn*4351* could be used for transposon mutagenesis in *Bacteroides* species. Shoemaker *et al.* suggested that from the evidence that had accumulated Tn*4351* was a composite, class 1 transposon similar to Tn*9* and that the direct repeats that flanked the *ermF* determinant were insertion sequences that were responsible for the transposition and cointegration events demonstrated in *B. uniformis* (Shoemaker *et al.*, 1986).

Sequence analysis of one of the direct repeats demonstrated homology between the structural organisation of this element and other IS elements. It was also demonstrated that this element could promote transposition in *recA E. coli* and so consequently the

direct repeats were identified insertion elements and given the designation IS*4351*. The element was found to be 1.155 kb and to contain partially matched (20 out of 25) terminal-inverted repeats. Such repeats in other IS elements are thought to be provide recognition sites for the element's transposase protein (Grindley and Reed, 1985). The element also contained three anti-parallel open-reading frames that were suggested to encode a transposase, a repressor, or an inhibitor such as is found in other IS elements (Grindley and Reed, 1985). Nine promoter-like sequences were also found within IS*4351* (Rasmussen *et al.*, 1987). The element was demonstrated to cause a 3-bp duplication upon transposition to a new site, such duplications are generally characteristic for a particular IS element and are thought to be the result of staggered nicks on the target DNA that are generated as part of the transposition process (Kleckner, 1981). Some composite transposons have been found to have only one functional flanking IS element, for example Tn*5* has IS elements that differ by 1 bp, the effect of this is to create a nonsense codon that affects the IS*50*$_L$ gene product involved in transposition resulting in the loss of the ability of this IS element to act independently (Iida *et al.*, 1983). Studies on IS*4351*$_L$ and IS*4351*$_R$ (donating the insertion sequences at either end of the transposon) demonstrated that both copies of the insertion element were capable of mediating cointegrate formation of a non-mobilisable plasmid that contained a chloramphenicol (Cm^r) marker, indicating that both elements were independently active, at least in *E. coli* (Hwa *et al.*, 1988). Cointegrates were detected by studying the frequency of transfer of Cm^r in the presence of a conjugative plasmid and it was demonstrated that the highest frequency of transfer occurred when Tn*4351* was used, and IS*4351*$_R$ (the element sequenced by Rasmussen *et al.*, 1987) was consistently less active than IS*4351*$_L$ (Hwa *et al.*, 1988).

The antibiotic resistance markers of Tn*4351* have also been studied in some detail. The transposon has a clindamycin/erythromycin resistance gene *ermF* that confers resistance to these antibiotics in *Bacteroides* species, and a novel tetracycline resistance gene *tetX* that confers tetracycline resistance on aerobically grown *E. coli* but not on anaerobically grown *E. coli* or *Bacteroides* and it is designated *Tc^r in the literature to distinguish it from other *Bacteroides* tetracycline resistance genes. Evidence suggests that *tetX*

encodes a NADP-requiring oxidoreductase. The origin of this gene is unknown but the low G + C content (32 mol %) compared to that of *B. fragilis* (42 mol %) and the fact that this gene does not function in *B. fragilis* suggests that it is not of *Bacteroides* origin (Speer *et al.*, 1991). The macrolide, lincosamide, streptogramin B resistance (MLS^r) determinant *ermF* from Tn*4351* was demonstrated to show striking sequence homology with *erm* genes from Gram-positive bacteria which suggested that like these genes *ermF* encodes for an rRNA methylase (Rasmussen *et al.*, 1986). The transcriptional control of *ermF* was found to be dependant on signals contained in the IS*4351* element situated immediately upstream of the *ermF* structural gene. The G + C content of *ermF* is 32 mol % which suggests that like *tetX* this gene is not of *Bacteroides* origin (Rasmussen *et al.*, 1986). However, IS*4351* has a similar G + C content to *B. fragilis* and IS*4351*-like sequences have been identified in the chromosome of this bacterium which suggests that this element is of *Bacteroides* origin (Rasmussen *et al.*, 1986). A putative evolutionary history of the generation of Tn*4351* can be summarised as follows; an invading *erm* sequence was transferred to *Bacteroides* species from a low G + C content bacterium on a plasmid or transposon. The *erm* was transferred onto the *Bacteroides* chromosome with its upstream regulation sequences, and this event was followed by movement of a copy of IS*4351* close to the start codon of *erm*. The IS*4351* then took over the upstream regulation of the gene and this was followed by a duplication event such that copies of this element now flanked the gene, resulting in the generation of a composite transposon (Rasmussen *et al.*, 1986).

Transposition of Tn*4351* has been demonstrated to occur randomly in *P. gingivalis* often as single insertions although cointegrate insertions can also occur (Dyer *et al.*, 1992; Hoover *et al.*, 1992b). Tn*4351* transposon mutagenesis has been used to generate mutants in *P. gingivalis* with considerable success and such mutagenesis has advantages over the allelic exchange mutagenesis used to transfer genes cloned and disrupted in *E. coli* back into *P. gingivalis*. Transposon mutagenesis is a one step process, Tn*4351* can be transferred into *P. gingivalis* by conjugation where it can transpose into the genome. Mutants of choice can be identified and the effects of the mutation can be studied. Furthermore Tn*4351* can be used as a marker for isolating the mutated gene which can

then be used to find the equivalent wild-type gene. Several studies have isolated Tn*4351*-generated mutants. A non-haemagglutinating Tn*4351*-induced mutant has been isolated that shows altered coaggregation activity (Takahashi *et al.*, 1995) and regulatory genes involved in fimbrial expression have also been mutated by this system (Terazawa *et al.*, 1995). Several studies have isolated pigment-deficient mutants, one of which also exhibits reduced trypsin-like protease activity as well as reduced haemagglutinin activity (Hoover and Yoshimuro, 1994). Two studies isolated Tn*4351*-generated mutants based on pigment deficiency, one of these mutants MSM-1 was found to have alterations in polysaccharide containing antigens that resulted in increased evasion of PMN phagocytosis (Genco *et al.*, 1995a). The second mutant MSM-3 was found to grow poorly in the presence of haemin, showed overproduction of vesicles compared to the wild-type strain and was more invasive than the wild-type strain in a mouse model. It was concluded that the mutation resulted in a decrease in the transport of haemin which resulted in an increase in the expression of several virulence factors which appear to be coordinately regulated by haemin (Genco *et al.*, 1995b).

The high frequency of transposition, together with the observed stability of the insertions indicates that Tn*4351* mutagenesis is a valuable tool for examining a variety of mutations in *P. gingivalis*. These mutations can give us important information on putative virulence genes and the role(s) that they may play in periodontal disease. Once virulence determinants have been identified it would be preferable to study the regulation of the genes and study the environmental factors present in the periodontal pocket that might effect the expression of these genes. It has already been determined that several factors such as haemin availability, temperature and pH can regulate the expression of several virulence genes although the precise mechanisms are unknown (Amano *et al.*, 1994; Marsh *et al.*, 1994; McDermid *et al.*, 1988; McKee *et al.*, 1986). Although at present it is possible to clone the promoter sequences from *P. gingivalis* into reporter vectors in *E. coli*, this is not an ideal situation for studying the regulation of these sequences. It has been determined that no antibiotic resistance genes that function in *Bacteroides* can function in *E. coli* (and *vice versa*), there are several possible reasons for this including problems related to codon usage and the fact that the majority of

codons found in *Bacteroides* genes may be recognised by minor tRNA of *E. coli* resulting in inefficient translation or a truncated protein, or both (Odelson *et al.*, 1987; Rasmussen *et al.*, 1986). It is likely that the study of the regulation of *P. gingivalis* virulence determinants in *E. coli* would be hindered by differences in the expression systems between the two species.

A useful tool that has been developed and used for the study of gene regulation in other Gram-negative bacteria is the manipulation of transposons to include reporter genes (Berg *et al.*, 1989). Reporter genes allow the regulation of genes to be assayed even when the bacterial gene product is difficult to assay on its own. For example the class 3 transposon Mu has been manipulated to include a promoterless *lac* reporter gene close to one end of Mu, which could then be used to study the activities and regulation of specific promoters by the easily scored Lac phenotype (Casadaban and Cohen, 1979). This type of transposon/reporter gene fusion is known as a "type I" fusion, it only monitors transcription because the reporter gene lacks a promoter but contains its own translation sequences, the transposon fusion need only insert into the target gene in the correct orientation for reporter gene expression. "Type II" fusions monitor translation as well as transcription, and the transposon fusion must insert into the target gene in the correct orientation and in the correct reading frame in order for the reporter gene to be expressed (Berg *et al.*, 1989). The *lacZ* reporter gene has also been incorporated as type I transcription probes into Tn*3* (Stachel *et al.*, 1985), Tn*5* (Kroos and Kaiser, 1984) and Tn*10* (Way *et al.*, 1984). Many of the reporter genes used are antibiotic resistance genes because the expression of these genes is easy to assess, for example a kanamycin resistance gene has been used to make a reporter gene fusion of Tn*5* that has been used in the study of gene expression in *Caulobacter crescentus* (Bellofatto *et al.*, 1984). The *phoA* gene product, alkaline phosphatase, has been used to make type II gene fusions. The gene product is only active in the periplasm or when excreted but is not active when sequestered in the cytoplasm. Tn*phoA* (Tn*5phoA*) has been designed to use this property, it detects genes that encode exported or membrane proteins, identifies structural features required for protein localisation and identifies protein domains that are outside the cell membrane (Manoil and Beckwith, 1985; 1986). Such

transposon/reporter gene fusions have proved instrumental in the discovery of genes that respond to particular environmental signals, if such a powerful genetic tool could be engineered for use in *P. gingivalis* the regulation of putative virulence genes, and the environmental stimuli that control these genes could help us have a better understanding of the factors involved in the progression of periodontal disease.

1.8 Aims of Investigation

In recent years the application of the molecular genetic system originally developed for use in *Bacteroides* species has allowed the genetic analysis of virulence determinants in *P. gingivalis*. In particular transposon mutagenesis, using the *B. fragilis* transposon Tn*4351*, has allowed the generation of *P. gingivalis* isogenic mutants. Such isogenic mutants allow the identification of putative virulence genes and give an indication of the role that these determinants may play in the pathogenicity of *P. gingivalis*. However, this system does not allow the regulation of the putative virulence genes to be analysed or the environmental factors that may affect the regulation of these genes to be determined. Transcriptional promoter probes, which fuse a gene of interest to a promoterless reporter gene whose product can be easily monitored, have be used with considerable success in many Gram-negative bacteria to study gene regulation (Berg *et al.*, 1989). The initial aim of this study was to manipulate the *B. fragilis* transposon Tn*4351* to include a type III chloramphenicol acetyl transferase (*catIII*) gene such that the manipulated transposon (Tn*4351*::*catIII*) could act as a type I transcriptional probe. Once the transposon had been manipulated the second aim of the project was to transfer Tn*4351*::*catIII* from *E. coli* into *P. gingivalis* in order to generate *P. gingivalis* transposon-inserted mutants. The final aim of the project was to demonstrate CAT activity from randomly selected transposon-inserted mutants and to determine whether differential CAT activity could be demonstrated for particular transposon-inserted mutants under different environmental stimuli.

CHAPTER 2

Materials and Methods

2.1 Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Table 2.1 and Table 2.2 respectively.

2.1.1 Growth Conditions and Media

Routine culture of *P. gingivalis* was at 37°C in a humidified anaerobic cabinet (Don Whitley) with an atmosphere of 80% (v/v) N_2, 10% (v/v) H_2, 10% (v/v) CO_2. Cultures were grown in *Bacteroides* medium (BM) at pH 7.4 (1% (w/v) trypticase peptone; 1% (w/v) proteose peptone; 0.5% (w/v) yeast extract; 0.5% (w/v) glucose; 0.5% (w/v) NaCl; 0.07% (w/v) cysteine HCl; 0.1% (w/v) $NaHCO_3$) (Shah *et al.*, 1976) to which haemin (50 μg/ml) and menadione (5 μg/ml) were added and with the addition of 1.5% (w/v) agar (BBL) as required or on blood agar base number 2 (BAB) (Oxoid) supplemented with 7% (v/v) defibrinated horse blood. *E. coli* cultures were routinely grown in Luria broth (L-broth) (1% (w/v) trypticase peptone; 0.5% (w/v) NaCl; 0.5% (w/v) yeast extract) at 37°C and with shaking at 200 rpm in an aerobic environment, with the addition of 1.5% agar as required. B-agar (0.1% (w/v) trypticase peptone; 0.8% (w/v) NaCl; 1.5% (w/v) agar), soft top agar (0.1% (w/v) trypticase peptone; 0.8% (w/v) NaCl; 0.6% (w/v) agar) and minimal medium (1.67% (w/v) agar; 10% M9 salts adjusted to pH 7.4 [6% (w/v) $NaHPO_4$; 3% (w/v) KH_2PO_4; 0.5% (w/v) NaCl; 1% (w/v) NH_4Cl]; 0.4% glucose; 0.1 mM $CaCl_2$; 2 mM $MgSO_4$) were used where stated. Antibiotics were added to the growth media where necessary at the following concentrations: ampicillin 100 μg/ml; clindamycin 5 μg/ml; chloramphenicol 25 μg/ml; gentamicin 100 μg/ml; kanamycin 50 μg/ml; streptomycin 25 μg/ml; tetracycline 10 μg/ml; trimethoprim 100 μg/ml. All antibiotics were purchased from Sigma Chemical Company Ltd. For screening of recombinants using insertional inactivation of the *lacZ* cassette solid media

Table 2.1 Bacterial Strains

Bacterial Strain	Relevant Characteristic	Reference or Source
E. coli		
LE392	F$^-$, *hsdR514* ($r_k^-m_k^+$), *supE44*, *sup*F58, *lacY1*, *galK2*, *galT22*, *metB1*, *trpR55*	Sambrook *et al.*, 1989
JM101	*supE*, *thi* Δ(*lac-proAB*), F'[*traD36*, *proAB*, *lacI^q*, *lacZΔM15*]	Yanisch-Perron *et al.*, 1985
TG2	*supE*, *hsd5*, *thiD*(*lac-proAB*), Δ(*srl-recA*$^-$) 306::Tn*10*(*tet^r*), F'[*traD36*, *proAB*, *lacI^q*, *lacZΔM15*]	Sambrook *et al.*, 1989
S17-1	*RecA*$^-$, *Tp^r*, *Sm^r* (ΩRP4-Tc::Mu-Kn::Tn*7*)[a]	Simon *et al.*, 1983
S17-1*	Δ(*srl-recA*$^-$) 306::Tn*10*(*tet^r*), *Tp^r*, *Sp^r* (ΩRP4-Tt::Mu-Km::Tn*7*)[b]	This study
J08 (BW103)	*deoB*, *rpsL*, *cir*, *recA1*, *leu*	Merryweather *et al.*, 1986
W3110	Thy$^-$, F$^-$, λ$^-$, *deoC2*	Bachmann, 1987
P. gingivalis		
33277	Laboratory Strain (Gm^r)	C. I. Hoover
381	Laboratory Strain (Gm^r)	C. I. Hoover
P. asaccharolyticus		
ATCC 8503	Type strain	American Type Culture Collection

[a] IncP plasmid RP4 inserted into the S17 chromosome via bacteriophage Mu. Tn7 was inserted in the remaining Km^r and provides the Tp^r, Sm^r phenotype.

[b] Δ(*srl-recA*$^-$) 306::Tn*10* (*tet^r*) transferred from TG2 to S17-1 by P1 phage transdudction to cause a deletion of the recA gene

Table 2.2 Plasmids

Plasmid	Characteristics	Reference
M13mp19	Bacteriophage cloning/sequencing vector	Yanisch-Perron *et al.*, 1985
pAO5	Ap^r (Cl^r) pBR328 vector containing Tn*4351**	This Study
pAO6	Ap^r (Cl^r) pBR328 vector containing Tn*4351*::*catIII*	This Study
pBR328	Ap^r, Cm^r, Tt^r non-mobilisable cloning vector	Soberon *et al.*, 1980
pGEM-T	Ap^r commercial T-tailed cloning vector	Promega Corporation Ltd.
pJRD215	*E. coli-Bacteroides* suicide vector Km^r, Rep^+, Mob^+	Davison *et al.*, 1987
pNJR12	*E. coli-Bacteroides* shuttle vector Km^r, Rep^+, Mob^+ (Tc^r, Rep^+, Mob^-)	Stevens *et al.*, 1992
pSA1	Ap^r, Km^r (Cl^r) pAO6/pJRD215 plasmid fusion (orientation 1) which acts as an *E. coli-Bacteroides* suicide vector	This study
pSA2	Ap^r, Km^r (Cl^r) pAO6/pJRD215 plasmid fusion (orientation 2) which acts as an *E. coli-Bacteroides* suicide vector	This study
pUC18	Ap^r cloning vector	Yanisch-Perron *et al.*, 1985
pUC18::IMC3	pUC18 containing a *catIII* gene	Murray *et al.*, 1988
pVOH1	Ap^r Cm^r (Cl^r) pBR328 vector containing Tn*4351*	Hwa *et al.*, 1988
R388	Conjugal, IncW, Tm^r, 33 kb plasmid	Avila and de la Cruz, 1988

Mob^+ and Mob^- can and can not be mobilised respectively; Rep^+ and Rep^- can and can not replicate respectively; Plasmid phenotypes within parentheses are expressed in *Bacteroides* species, and those outside paretheses are expressed in *E. coli*.

were supplemented with 40 μg/ml X-gal and 0.1 mM IPTG (both purchased from Novabiochem). Bacterial cells were routinely harvested by centrifugation at 3,300g for 5 minutes at 4°C in a Sorval centrifuge. Small volumes of culture (< 1.5 ml) were centrifuged in a bench top microfuge (MSE) at 13,400g for 1 minute at room temperature.

2.2 Transformation of Bacterial Cells with Plasmid and M13mp19 DNA

2.2.1 $CaCl_2$ Method

For transformation by the $CaCl_2$ method (Mandel and Higa, 1970) 100 μl of an overnight culture was back-diluted into 10 ml of L-broth which was then grown to mid-exponential phase (OD_{600} ~ 0.5). Cells were harvested by centrifugation (3,300g at 4°C for 10 minutes), washed once in 10 ml of ice-cold 10 mM NaCl solution, recentrifuged and then resuspended in 4 ml of ice-cold $CaCl_2$ (100 mM). The cells were then placed on ice for 30 minutes before being collected by gentle centrifugation (1,800g for 5 minutes at 4°C). The cell pellet was then resuspended in 1 ml ice-cold 100 mM $CaCl_2$ and separated into 100 μl aliquots. Between 5-20 μl of DNA (suspended in nanopure water) was mixed with the 100 μl competent cell aliquots and these were then placed on ice for 1 hour. After this time the cells were heat shocked at 42°C for 3 minutes. Immediately after heat-shocking 1 ml of L-broth was added to the cells which were then incubated for 1 hour at 37°C. The transformed cells were plated in 100 μl portions onto L-agar plates supplemented with the appropriate antibiotics. The plates were then incubated overnight at 37°C.

2.2.2 Electroporation Method

For transformation by electroporation 100 μl of an overnight culture was back-diluted into 10 ml of L-broth which was then grown to mid-exponential phase (OD_{600} ~ 0.5). Cells were chilled on ice for 15 minutes before being harvested (3,300g at 4°C for 10

minutes). The cells were then washed three times in 10 ml volumes of ice-cold nanopure water by alternate resuspension of cell pellets and recentrifugation. Finally cells were washed once in 10% (v/v) glycerol and resuspended in 80 µl of 10% (v/v) glycerol. Aliquots of 40 µl of competent cells were mixed with plasmid DNA (1-2 µl) that had been ethanol precipitated. The samples were then pipetted deep into the electrode gap (2 mm) of an ice-cold Gene Pulsor™ cuvette (Biorad). A high voltage pulse was delivered through the sample using a Biorad Gene Pulsor™ with pulse controller unit (pulse parameters were 25 µF capacitance, 200 Ω resistance and 2.4 KV voltage). Immediately after the pulse the transformation sample was mixed with 1 ml ice-cold SOC recovery medium (2% (w/v) trypticase peptone; 0.5% (w/v) yeast extract; 10 mM NaCl; 25 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$; 20 mM glucose) and incubated at 37 °C for 1 hour before being plated onto L-agar supplemented with the appropriate antibiotics. Plates were then incubated overnight at 37°C.

2.2.3 Transformation with Bacteriophage DNA

For transformation with bacteriophage M13mp19 single-stranded DNA competent cells were prepared essentially using the CaCl method. Competent cells (100 µl) of the *E. coli* strain JM101 were mixed with the construct DNA and heat-shocked at 42°C for 3 minutes. The transformed cells were then immediately mixed with 3 ml molten (42°C) soft top agar supplemented with 20 µl IPTG (100 mM), 50 µl 2% (w/v) X-gal solubilised in dimethylformamide and 200 µl JM101 indicator cells (JM101 cells at mid-log phase). The suspension was mixed and poured onto a B-agar plate and rocked to disperse. Plates were incubated at 37°C overnight.

2.3 Procedures for DNA Extraction

DNA extraction protocols used the following solutions:

Solution I (ice-cold):	50 mM glucose 25 mM Tris-HCl pH 8.0 10 mM EDTA 5 mg/ml lysozyme

Solution II:	0.2 M NaOH 35 mM SDS
Solution III (ice-cold):	5 M acetic acid 3 M potassium acetate

2.3.1 Small Scale Extraction of Plasmid DNA

Small scale plasmid extractions were carried out on stationary phase bacterial cultures. The cell pellet from 1.5 ml of overnight culture was resuspended in 100 µl of freshly made solution I and was placed on ice for 30 minutes. Solution II (200 µl) was then added and the tube was gently mixed by inversion before being replaced on ice. After 5 minutes 150 µl of solution III was added and the tube was returned to the ice for another 5 minutes. The supernatant was recovered after centrifugation in a bench top centrifuge (13,400g for 5 minutes). Protein was removed by phenol extraction (section 2.3.6) and then two volumes of ethanol were added and the DNA was precipitated at -20°C for a minimum of 30 minutes. The DNA pellet was obtained by centrifugation at 13,400g for 5 minutes after which the ethanol was aspirated off and the pellet was dried for 5 minutes using a vacuum desiccator. The DNA pellet was resuspended in 30 µl of nanopure water and stored at -20°C.

2.3.2 Small Scale Extraction of Low Copy Number Plasmid DNA

The extraction of low copy number plasmid DNA was carried out as above (section 2.3.1) with the following modifications. A 10 ml overnight culture was split into 6 x 1.5 ml eppendorfs. The small scale extraction was then carried out separately on each sample as described above. After the ethanol precipitation step the DNA pellet from the first sample was resuspended in 30 µl nH_2O. This suspension was then used to resuspend the DNA in sample 2 and so on until all the samples had been resuspended into a final volume of 30 µl nH_2O.

2.3.3 Large Scale Extraction of Plasmid DNA

Large scale preparation of plasmid DNA was carried out using the alkaline lysis method outlined by Birnboim and Doly (1979) followed by caesium chloride-ethidium bromide density gradient centrifugation. Cells from an overnight 400 ml culture were harvested by centrifugation (3,300g at 4°C for 10 minutes) in 2 x 200 ml aliquots. Both cell pellets were then resuspended in 5 ml of freshly made solution I and transferred to 30 ml Oakride tubes and left on ice for 10 minutes. 10 ml of solution II was then added to each tube which were gently mixed before being replaced on ice for a further 10 minutes. Solution III (7.5 ml) was then added and the tubes were gently mixed and replaced on ice for 5 minutes. Cell debris was removed from the plasmid by centrifugation at 4°C for 30 minutes at 36,900g in a Sorval centrifuge using a Beckman SS-34 rotor. The DNA was precipitated at room temperature for 20 minutes by the addition of 0.6 volumes of isopropyl alcohol to each tube. The resultant DNA precipitate was collected by centrifugation in 30 ml corex tubes at 3,500g for 20 minutes at 20°C. The DNA pellets were air dried before being resuspended in nanopure water and pooled together to form a final volume of 17 ml to which 17 g of caesium chloride was added. The solution was transferred to a Sorval 30 ml vertical rotor centrifuge tube and ethidium bromide was added to a final concentration of 50 μg/ml. Chromosomal and plasmid DNA were separated by centrifugation at 40,000 rpm for 20 hours at 20°C in a Sorval TV850 fixed angle rotor using a Sorval OTD 60 Ultracentrifuge. DNA was visualised under UV light and the lower plasmid DNA band was collected. Ethidium bromide was removed by mixing several times with an equal volume of caesium chloride saturated isopropanol, keeping the lower aqueous phase each time. Finally the caesium chloride was removed by exhaustive dialysis against distilled water at room temperature. The resultant DNA solution was aliquoted and stored at -20°C.

2.3.4 Extraction of Chromosomal DNA

The method used to extract chromosomal DNA was based on a method described by Saito and Muira, 1963. Bacterial cells from a 5 ml stationary phase culture were washed in 10 mM NaCl before being resuspended in 5 ml solution I and placed on ice for 30 minutes. After this time SDS and EDTA were added to a final concentration of 1% and 50 mM respectively and the preparation was left at room temperature until the turbidity cleared leaving the solution translucent (up to 20 minutes). Sodium perchlorate at a final concentration of 1 mM and 2 volumes of chloroform/isomyl alcohol (24:1 v/v) were added to the preparation which was mixed gently for 15 minutes. Centrifugation at 4,000g for 20 minutes at 20°C separated the preparation into two phases. The upper aqueous phase was carefully removed and an equal volume of phenol/chloroform (1:1 v/v) was added and mixed thoroughly by gentle inversion. The preparation was again separated into two phases by centrifugation at 4,000g for 20 minutes at 20°C and the upper aqueous phase was collected carefully using a truncated plastic pipette with a wide-bore so as to avoid mechanical shearing of the DNA. The phenol/chloroform extraction was repeated until there was no protein remaining at the interface (typically 2-3 phenol/chloroform extractions). The cleared aqueous phase was then mixed with a one tenth volume of 3 M sodium acetate solution pH 5.2 after which 3 volumes of cold absolute ethanol (chilled to -20°C) was slowly poured down the side of the tube causing an interface of precipitated DNA to form. The DNA was collected at the interface by spooling the DNA around the end of a rounded Pasteur pipette and resuspended in sterile nanopure water.

2.3.5 Extraction of M13mp19 Template DNA

For all M13mp19 cloning and propagation the *E. coli* strain JM101, which was sub-cultured from minimal medium plates supplemented with thiamine, was used. M13mp19 recombinant clones were transformed into JM101 as described (section 2.2.3) and white plaques were picked into 5 ml L-broth containing 100 µl of an

overnight culture of JM101 and incubated for 5 hours at 37°C with vigorous aeration. Two 1.5 ml aliquots of the cell culture were pelleted by centrifugation in a bench top centrifuge. The supernatant was retained for isolation of single-stranded template DNA and the replicative form (RF) was isolated by the small scale plasmid extraction method (section 2.3.1) so that the identity of recombinant M13mp19 clones could be confirmed. The supernatant (0.8 ml) was mixed with 200 µl of a freshly prepared solution of 2.5 M NaCl, 20% (w/v) PEG 6000 and incubated at room temperature for 30 minutes. The resultant phage precipitant was collected by centrifugation in a bench top centrifuge for 5 minutes. The supernatant was carefully removed using a drawn-out Pasteur pipette and discarded. The pellet was resuspended in 100 µl of 1.1 M Sodium acetate pH 7.0 and mixed with an equal volume of phenol/chloroform (1:1). Centrifugation for 1 minute in a bench top centrifuge re-established the two phases and the upper aqueous phase was removed and mixed with an equal volume of Chloroform. After a pulse centrifugation step the upper aqueous phase was removed and precipitated in 1 ml -20°C ethanol for 30 minutes. The DNA was collected by centrifugation in a bench top centrifuge for 5 minutes and resuspended in 17 µl nanopure water.

2.3.6 Phenol/Chloroform Extraction and Ethanol Precipitation

Phenol/chloroform extraction's were based on a method by Sambrook *et al.*, 1989. One volume of phenol/chloroform (1:1 v/v) equilibrated in Tris-HCl pH 7.5 was added to the DNA sample and mixed carefully. After centrifugation in a bench top centrifuge at 13,400g for 5 minutes the upper aqueous phase was removed avoiding the inter-phase. One volume of chloroform was added to the sample and mixed by inversion. The aqueous phase was again separated by centrifugation in a bench top centrifuge at 13,400g for 1 minute. Ethanol precipitation was performed by the addition of one tenth volume 3 M Sodium acetate and 2 volumes of absolute ethanol (-20°C) to the sample which was left at -20°C for a minimum of 30 minutes. The DNA was collected by centrifugation in a bench top centrifuge for 5 minutes at 13,400g the ethanol was aspirated off and discarded and the DNA pellet was then dried for 5 minutes in a vacuum desiccator before being resuspended in nanopure water.

2.4 Methods Routinely used in the Manipulation of DNA

2.4.1 Restriction Enzyme Analysis

Analysis of recombinant plasmids was achieved by estimating molecular weights in kb of restriction enzyme digested DNA using agarose gel electrophoresis. DNA samples were mixed with 6x DNA loading buffer (0.25% (w/v) bromophenol blue; 0.25% (w/v) xylene cyanol, 15% (w/v) ficoll) and pipetted into the wells of 0.7% (w/v) Seakem agarose slab gels made using TAE buffer (40 mM Tris-acetate pH 7.7; 1 mM EDTA). The electrophoresis was conducted in TAE buffer containing 0.5 μg/ml ethidium bromide with 1 - 5 V/cm between the electrodes. The DNA was visualised using a long wave UV transilluminator and photographed using Polaroid 667 film. The molecular weight of DNA was established by comparison with 1 kb ladder DNA standard (GIBCO/BRL) which was run along side the DNA sample.

Restriction enzymes and DNA modifying enzymes were purchased from GIBCO/BRL or Pharmacia Biochemicals Inc. and were used according to the manufacturer's instructions. Typically restriction enzyme analysis of small scale plasmid extractions involved digesting up to 1 μg DNA at 37°C for 1 hour using one unit of enzyme in a 20 μl volume. Cleavage of large scale DNA plasmid extractions for isolation of restriction fragments for subsequent sub-cloning into a plasmid vector were typically carried out using 10 μg DNA in a 300 μl volume at 37°C overnight using 2-3 units of enzyme. If a double digest was to be carried out the DNA was digested overnight as described above using the first restriction enzyme which was then inactivated by heating at 65°C for 20 minutes. The DNA was then ethanol precipitated (section 2.3.6) and the DNA pellet was resuspended in 100 μl nanopure water and digested with the second restriction enzyme according to the manufacturers instructions. Restriction digests were run on 0.7% (w/v) agarose gels as described above and the fragments required for cloning were cut out of the gel using a sterile scalpel blade.

2.4.2 Ligation of Purified DNA Fragments

Fragments excised from agarose gels were purified using a Sephaglas™ BandPrep kit (Pharmacia) according to the manufacturers instructions. Ligation of restriction fragments into plasmid vectors typically used 0.5-1 μg vector DNA and purified fragment, 0.5 μl T4 DNA ligase and 4 μl 5x ligation buffer (GIBCO/BRL) in a final volume of 20 μl. Ligation reactions were left at 14°C overnight.

2.4.3 Phosphatase Treatment of Vector DNA for High Efficiency Cloning

High frequency recombination was achieved by removal of 5' terminal phosphate groups from restricted vector thus preventing self-ligation. 10 μg of vector DNA was digested with the appropriate restriction enzyme and then purified using a Sephaglas™ BandPrep kit. The phosphatase reaction was carried out in a volume of 40 μl containing 20 μl of purified vector DNA, 16 μl of nH_2O and 4 μl of 10x calf intestinal phosphatase buffer (GIBCO BRL) and 1 U of calf intestinal phosphatase (CIP) (GIBCO BRL). The reaction was left at 37°C for 30 minutes after which time a further 1 U of CIP was added to the reaction. This was left at 37°C for a further 30 minutes before two phenol/chloroform extractions and a single chloroform extraction were carried out on the DNA. An ethanol precipitation was then carried out on the DNA which was finally resuspended in 20 μl nH_2O. Phosphatase efficiency was assessed by determining the transformation efficiencies for self-ligated phosphatased vector. 1-4 μl of this DNA was then used in ligations.

2.5 End Repair of Restriction Enzyme Sites

A large scale extraction of plasmid DNA was digested overnight with the appropriate restriction enzyme as described above. Two phenol/chloroform extractions followed by a single chloroform extraction were carried out to remove any contaminating restriction enzyme and the resulting aqueous phase containing the plasmid DNA was ethanol precipitated. After the DNA pellet was dried it was resuspended in 100 μl 1x Klenow

end repair buffer (50 mM Tris-HCl pH 7.5; 10 mM $MgCl_2$) containing 0.2 mM of the appropriate dNTP's (Pharmacia) and 1 unit of the large fragment of DNA polymerase I (Klenow fragment) (GIBCO/BRL). The end repair reaction was left at room temperature for 20 minutes after which time two phenol/chloroform extractions followed by a single chloroform extraction were carried out to remove any contaminating dNTP's or Klenow and the DNA was then ethanol precipitated. After the DNA pellet had been dried it was resuspended in 1x ligation buffer (GIBCO/BRL) and 0.5 μl T4 DNA ligase was added. The ligation reaction was left overnight at 14°C.

2.6 Conjugal Transfer of Plasmid DNA

2.6.1 Conjugal Transfer of Plasmid DNA Between Two Strains of *E. coli*

Donor and recipient were chosen on the basis that they exhibited different auxotrophic requirements to each other. Donor cells, containing the plasmid(s) to be transferred, and recipient cells were grown to an OD_{600} of between 0.3-0.5. 1 ml of recipient cells were pelleted for 1 minute in a bench top centrifuge (13,400g) after which the supernatant was removed and discarded. 1 ml of donor cells were then pelleted on top of the recipient cell pellet in a bench top centrifuge (13,400g) and the supernatant was again removed and discarded. The cell pellet was then resuspended in 50 μl L-broth and was spotted onto a sterile 0.45 μm nitrocellulose filter (Millipore) that had been placed onto the surface of a L-agar plate. 1 ml of donor cells only and 1 ml of recipient cells only were also centrifuged in a bench top centrifuge for 1 minute, resuspended in 50 μl L-broth and spotted onto sterile 0.45 μm nitrocellulose filters. The filters were then incubated at 37°C for 4 hours. After this time the filters were washed in 500 μl 1x M9 salts (0.6% (w/v) Na_2HPO_4; 0.3% (w/v) KH_2PO_4; 0.05% (w/v) NaCl; 0.1% (w/v) NH_4Cl; adjusted to pH 7.4) and 10-fold serial dilutions were plated onto selective plates (100 μl per plate). Donor only cells were plated onto L-agar plates supplemented with the appropriate antibiotics and recipient cells only were plated onto minimal medium plates supplemented with the appropriate amino acids. Donor/recipient transconjugants were plated onto minimal medium supplemented with the appropriate amino acids to

allow recipient cells to grow and with the appropriate antibiotics to select for transfer of the plasmid(s) and as a negative control donor only and recipient only cells were also plated onto these minimal medium plates. Plates were incubated at 37°C overnight (for L-agar plates) or for 2-3 days (for minimal medium plates).

2.6.2 Conjugal Transfer of Plasmid DNA From *E. coli* to *P. gingivalis*

Matings carried out between *E. coli* and *P. gingivalis* were based on the methods described by Shoemaker *et al.*, 1986, Maley *et al.*, 1992 and Hoover *et al.*, 1992b. Donor cells (*E. coli* strain S17-1* or HB101 carrying the plasmid to be transferred) and recipient cells (*P. gingivalis* strain 381 or 33277) were grown to an OD_{600} of 0.4 and between 0.6-0.9 respectively. 1 ml each of donor and recipient cells were mixed and then harvested by centrifugation at 13,400g for 1 minute, after which time the supernatant was removed and discarded. The cell pellet was then resuspended in 100 µl BM broth and spotted onto a sterile 0.45 µm nitrocellulose filter (Millipore) that had been placed onto the surface of a blood agar base (BAB) plate. 1 ml Donor cells only and 1 ml recipient cells only were also harvested by centrifugation, resuspended in 100 µl BM broth and spotted onto sterile filters on BAB plates. The filters were incubated aerobically at 37°C for 15 hours before being incubated anaerobically for a further 48 hours (for *P. gingivalis* strain 33277) or 72 hours (for *P. gingivalis* strain 381). Each cultured bacterial spot was then removed from the nitrocellulose filter with a sterile cotton swab and resuspended into 700 µl of BM broth. Ten-fold serial dilutions of donor cells only were plated onto L-agar plates supplemented with the appropriate antibiotics (Tm, Ap, Km, Sp and Tt at 100, 100, 50, 25 and 25 µg/ml respectively for S17-1* donor cells or Tm and Tt at 100 and 25 µg/ml respectively for HB101 donor cells). Ten-fold serial dilutions of recipient cells only were plated onto BAB plates (100 µl per plate). Transconjugants were selected on BAB plates supplemented with gentamycin (100 µg/ml) and clindamycin (5 µg/ml). As a negative control donor only and recipient only cells were also plated onto these selective plates. Donor only plates were incubated aerobically at 37°C overnight and recipient only and transconjugant cells

were incubated anaerobically at 37°C for 10-21 days depending on the recipient strain used.

2.7 Bacteriophage P1 Transduction

The P1 bacteriophage transduction procedure was based on a method by Silhavy *et al.*, 1984. A donor bacterial culture expressing a selective marker was grown to stationary phase and 5 µl of this culture was then used to inoculate a 5 ml L-broth containing 0.2% (w/v) glucose and 5 mM $CaCl_2$. The 5 ml culture was incubated for 30 minutes at 37°C with vigorous shaking after which time it was inoculated with 100 µl of a P1 *vir* lysate (5×10^8 plaque forming units per ml). The culture was incubated at 37°C with vigorous shaking for a further 2-3 hours until the cells had lysed. 100 µl chloroform was added to the lysate and mixed by vortexing briefly. Lysates were transferred to 1.5 ml eppendorf tubes and pelleted by centrifugation at 4,500g for 10 minutes. The supernatant was carefully removed to a sterile eppendorf tube and chloroform (20 µl per ml of lysate) was added and mixed by vortexing briefly. Lysates were stored at 4°C until required.

Recipient cells were grown in L-broth containing 2.5 mM $CaCl_2$ to an OD_{600} of 0.8. Three separate plastic conical phage tubes were inoculated with 1 ml of the recipient culture and to the first tube 100 µl of the P1 *vir* lysate prepared above was added and to the second tube 10 µl of the P1 *vir* lysate prepared above was added. No P1 *vir* lysate was added to the third tube. The cultures were incubated for 30 minutes at 30°C with vigorous shaking after which time 4 ml of L-broth/5 mM sodium citrate were added and the cultures were incubated for 1 hour at 30°C with vigorous shaking. The cells were collected by centrifugation at 3,300g at 4°C for 10 minutes. The pellet was resuspended in 100 µl L-broth containing 5 mM sodium citrate and plated onto L-agar containing 5 mM sodium citrate and supplemented with the appropriate antibiotics.

2.8 Polymerase Chain Reaction Procedures

2.8.1 PCR Using *Taq* DNA Polymerase

Polymerase chain reaction (PCR) amplification was carried in a total volume of 100 µl containing oligonucleotide primers (0.25 µM final concentration; oligonucleotides were obtained from the University of Leicester's oligonucleotide generating facility), dNTP's (each at 200 µM final concentration; Pharmacia), 10 ng template DNA, 1x PCR buffer (10 mM Tris-HCl pH 9.0; 50 mM KCl, 0.01% (w/v) gelatin; 1.5 mM $MgCl_2$; 0.1% (w/v) Triton X-100) and 2.5 units of *Taq* DNA polymerase (NSB Biologicals). Reaction mixtures were UV irradiated on a transilluminator for 15 minutes prior to the addition of template DNA and *Taq* DNA polymerase. Before amplification samples were overlain with 20 µl molecular biology grade mineral oil (United States Biologicals). Amplification was performed in a Perkin-Elmer Cetus thermal cycler for 30 cycles consisting of 1 minute at 95°C (denaturing step), 1 minute at 45°C or 55°C (annealing temperature depended on the template to be amplified. Annealing should be at 5°C lower than the Tm of the primer with the lowest melting point) and 2 minutes at 72°C (extension). A 5 minute denaturing step (95°C) was included before cycling to denature the DNA and a 5 minute extension step (72°C) was included after the cycling to complete partially polymerised chains. The PCR product was analysed by agarose gel electrophoresis and the DNA band was excised and purified using a Sephaglas™ BandPrep kit (Pharmacia).

2.8.2 PCR using *UlTma*™ DNA polymerase

Amplification by PCR using *UlTma*™ DNA polymerase was carried out in a volume of 100 µl containing oligonucleotide primers (0.25 µM final concentration), dNTP's (each at 40 µM final concentration; Pharmacia), 10 ng template DNA, 1x PCR buffer (10 mM Tris-HCl pH 8.0; 10 mM KCl; 0.002% Tween 20® (v/v)), 1.5 mM $MgCl_2$ and 3 units of *UlTma*™ DNA polymerase (Perkin-Elmer). Reaction mixtures were UV irradiated on a

transilluminator for 15 minutes prior to the addition of template DNA and *UlTma*™ DNA polymerase. Before amplification samples were overlain with 20 µl molecular biology grade mineral oil (United States Biologicals). Amplification reactions were then carried out as above (section 2.8.1) for 30 cycles under the same denaturing, annealing and extension criteria using a Perkin-Elmer Cetus thermal cycler.

2.9 DNA Sequencing

Nucleotide sequence was determined by the chain termination method described by Sanger *et al.* (1977) in which the random incorporation of dideoxynucleotide triphosphates instead of deoxynucleotides during template-dependant second strand DNA synthesis results in termination of DNA synthesis. The cloning vector M13mp19 containing the insert to be sequenced was used to generate single-stranded template DNA (section 2.3.5). Sequence reactions were performed using the Sequenase® Version 2.0 kit (United States Biochemical Corporation, USA) according to the manufacturers recommendations. Detection of second strand synthesis was by the incorporation of [α-^{35}S]dATP into DNA during the extension reactions. Priming of the extensions was achieved using oligonucleotides (oligonucleotides were obtained from the University of Leicester's oligonucleotide generating facility) or the universal -40 sequencing primer (United States Biochemical Corporation, USA).

Radiolabelled DNA fragments were separated by gradient gel electrophoresis (Biggin *et al.*, 1983). The solutions that were used in the preparation of the gel are shown below:

Gel Solution 1:	7 ml 5x TBE acrylamide/urea mix 45 µl 10% (v/v) ammonium persulphate (Biorad) 2.5 µl TEMED (Sigma Chemical company Ltd)
Gel Solution 2:	40 ml 0.5x TBE acrylamide/urea mix 180 µl 10% (v/v) ammonium persulphate (Biorad) 7.5 µl TEMED (Sigma Chemical company Ltd)
5x TBE acrylamide/urea mix (per litre):	430 g urea 150 ml 10x TBE*1 150 ml 40% (v/v) acrylamide (BDH) 50 g sucrose 50 mg bromophenol blue

0.5x TBE acrylamide/urea mix (per litre):	430 g urea 50 ml 10x TBE[*1] 150 ml 40% (v/v) acrylamide (BDH)

[*1] 10x TBE consists of 0.089M Tris-Hcl, 0.089M boric acid and 0.002M EDTA pH 8.0; the solution is then adjusted to pH 8.3

To prepare the gel two glass plates (50 cm x 20 cm) were cleaned free of grease using Teepol and water. One plate surface (inside face) was siliconised by applying a small amount of dimethyldichlorosaline which was allowed to dry. The two plates were separated by 0.4 mm spacers and taped together. 10 ml solution 1 followed by 12 ml solution 2 were drawn up into a 25 ml pipette and mixed slightly by drawing 3-4 air bubbles into the pipette. The mixture was run down between the glass plates and then the rest of solution 2 was used to fill the remaining cavity, being careful not to introduce any bubbles. A comb was positioned at the mouth of the gel and the sides of the glass plates were clamped. Gels were routinely left to set overnight.

The gel was run in a vertical electrophoresis system. The tape was removed from the plates and the gel was clamped into the vertical electrophoresis tank with aluminium plates to allow even distribution of heat. The top tank of the electrophoresis system was filled with 0.5x TBE buffer and the lower tank was filled with 1x TBE buffer. Prior to running the wells in the gel were rinsed with running buffer and the gel was pre-run for 30 minutes at 40 W. After this time the samples were heated to 70°C for 3 minutes and then 4 µl was loaded into each well. Gels were run at a constant power of 40 W for 3, 6 or 9 hours.

After electrophoresis the gel plates were prised apart and the gel was soaked in gel fix (10% (v/v) acetic acid; 10% (v/v) methanol) for 15 minutes after which time the gel was rinsed in distilled water. The gel was transferred to a pre-wet Whatman 3MM filter paper, wrapped in Saran wrap and dried in a vacuum dryer at 80°C for 1 hour. Autoradiography was carried out by placing the Cronex (Dupont) autorad film in direct contact with the dried gel which was then left at room temperature.

DNA homologies were derived from database searches using FASTA (Lipman and Pearson, 1985), a program that finds similarities between the query sequence and the sequences of individual database entries.

2.10 Southern Blot procedures

2.10.1 Transfer of DNA to Nylon Filters

DNA was transferred to filters as described by Southern, 1975. DNA was separated by agarose gel electrophoresis and the gel was then photographed along side a linear ruler as a point of reference. The DNA was de-purinated by soaking the agarose gel in 0.25 M HCl for 7 minutes. After briefly rinsing in distilled water the gel was placed in denaturing solution (0.5 M NaOH; 1.5 M NaCl) for 30 minutes with occasional shaking. The gel was again rinsed briefly in distilled water before being placed into neutralising solution (0.5 M Tris-HCl pH 7.5; 3 M NaCl) for a further 30 minutes. After this time the gel was again rinsed in distilled water and then placed onto six sheets of 3MM Whatman paper that had been pre-soaked in 20x SSC (20x SSC: 3 M Sodium chloride; 0.3 M trisodium citrate pH 7.0) being careful not to trap any air bubbles. A square of Genescreen hybridisation membrane (Biotechnology systems) was pre-soaked in 3x SSC and placed on top of the gel again being careful not to entrap any air bubbles. A 3x SSC soaked piece of 3MM Whatman paper was placed on top of the hybridisation membrane followed by 5 pieces of dry 3MM Whatman paper and a stack of dry paper towels. Finally a 500 g weight was placed on top the construction which was left for 5 hours so that DNA from the gel could transfer to the hybridisation membrane. During this time the lower sheets of 3MM Whatman paper were regularly soaked with 20x SSC and the upper paper towels were regularly replaced as they became saturated. After 5 hours the apparatus was dismantled and the hybridisation membrane was rinsed in 3x SSC, air dried, wrapped in Saran wrap and then exposed to UV light from a long wave transilluminator for 5 minutes to fix the DNA to the filter.

2.10.2 Production of a Radiolabelled Probe

DNA to be used as a radiolabelled probe was labelled using the random primer/extension method. Plasmid DNA was cleaved with the appropriate restriction enzyme and the fragments were separated by agarose gel electrophoresis on a 0.7% (w/v) agarose gel. The required DNA fragment was excised from the gel using a sterile scalpel blade and purified using a Sephaglas™ BandPrep kit. The DNA could then be stored at -20°C. Before use the DNA was reboiled for 5 minutes and then placed at 37°C for 10-20 minutes.

Labelling reactions were set up based on a method described by Feinberg and Vogelstein (1983). To 10 ng DNA in a 10 µl volume, 3 µl 5x OLB (5x OLB consists of solutions A, B and C (see below) in the ratio 10:25:15 respectively), 1 unit Klenow (GIBCO/BRL) and 1 µl ^{32}P α-dCTP (10 Ci/µl, Dupont) were added. The labelling reaction was then left for a minimum of 5 hours. Immediately before use the probe was placed in boiling water for 5 minutes.

Solution A:	1 ml 1.25 M Tris-HCl pH 8.0/0.125 M $MgCl_2$ 18µl β-mercaptoethanol 5 µl each of 100 mM dATP, dTTP and dGTP (Pharmacia)
Solution B:	2 M HEPES (pH adjusted to 6.6 using NaOH)
Solution C:	Hexadeoxynucleotides (d6(NTP's); Pharmacia); 50 OD units in 550 µl of TE buffer pH 7.0 (3 mM Tris-HCl pH 7.0; 0.2 mM ETDA)

2.10.3 Hybridisation of DNA Immobilised onto Filters using Radiolabelled Probes

Hybridisation and wash procedures were carried out in a rotary hybridisation oven (Hybrid) using cylindrical canisters. Southern blot filters were pre-treated in 20 ml prehybridisation fluid (see below) for 2 hours at 65°C. The prehybridisation fluid was then replaced with 20 ml hybridisation fluid (see below) containing the radiolabelled probe and the filter was incubated at 65°C overnight.

Prehybridisation Solution:	3x SSC
	0.1% (w/v) SDS
	5x Denharts*1
	6% (w/v) PEG 6000
	200 µg/ml denatured salmon sperm DNA*2
Hybridisation Solution:	3x SSC
	0.1% (w/v) SDS
	2x Denharts*1
	6% (w/v) PEG 6000
	200 µg/ml denatured salmon sperm DNA*2

*1 100x Denharts consists of 2% (w/v) Ficoll, 2% (w/v) BSA and 2% (w/v) Polyvinolpyrollinidine.

*2 Salmon sperm DNA was sheared by forcing it through a narrow gauge syringe needle several times and denatured by boiling prior to use.

After hybridising overnight the filters were washed 4 times, for 15 minutes at a time, at 65°C in 20 ml of a high stringency wash solution that consisted of 0.1% (w/v) SDS and 0.1x SSC (which allows for 95% DNA homology). The filter was then air dried, wrapped in Saran wrap and placed in an intensifying screen autoradiography cassette with the DNA side up. Filters were overlaid with Kodak X-Omat AR film which was exposed at -70°C. Films were then developed in an Agfa-Geveart automatic processing machine.

2.10.4 Removal of Bound Probe from a Filter

The probe was stripped from some filters following autoradiography so that the filter could be re-hybridised to a different probe. To remove the probe, filters were washed at 65°C for 1-2 hours in a solution of 0.005 M Tris-HCl pH 8.0, 0.002 M EDTA and 0.1x Denharts. Successful removal of the probe was confirmed by exposure of the filter to autoradiography film.

2.11 Chloramphenicol Acetyltransferase (CAT) Assay

The assay used was based on a method by Shaw, 1975. The strains to be assayed were grown to mid-log phase (OD_{600} 0.5) and then 10 ml of bacterial culture was harvested by centrifugation (3,300g at 4°C for 10 minutes). Cell pellets were washed in ice-cold 0.85% (w/v) NaCl and were again pelleted for 1 minute before being resuspended in 1 ml of ice-cold 0.85% (w/v) NaCl. The bacterial cell samples were then sonicated with a Braun Labsonic 200 sonicator using a small probe. The samples were sonicated for 15 seconds, with 30 second cooling intervals between each sonicating burst, until clearing had occurred. Assays were then carried out on the sonicated samples at 37°C using a Philips UV/VIS spectrophotometer. A reaction mix was set up containing 100 µl sonicated bacterial extract, 900 µl reaction solution (0.4 mg/ml 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) made up in 1 M Tris-HCl pH 7.8 and 0.1 mM Acetyl coenzyme A (Sigma Chemical Company Ltd.)). The reaction mix was left for 10 minutes at 37°C to equilibrate before the assay reaction was started by adding 22 µl of 5 mM chloramphenicol to the reaction mix. The absorbance of the reaction was measured at 412 nm immediately before the addition of Cm and then at 1 minute intervals for up to 10 minutes after the addition of Cm. The rate of increase in absorbance at 412 nm prior to the addition of Cm was subtracted from the observed rate after the start of the reaction and the net change in extinction per minute was divided by 13.6 to give the result in micromoles of Cm acetylated per minute (1 unit of CAT = 1 micromole of chloramphenicol acetylated per minute at 37°C). The amount of protein in the assay reaction was estimated using a BIORAD protein assay kit with lysozyme as a standard. Enzyme activity was finally expressed as units per milligram of protein. Each sample was assayed in triplicate and a mean value was calculated. The standard deviation for each sample was also calculated.

2.12 Detection of Neuraminidase and β-N-acetyl-hexosaminidase Negative Mutants

Transconjugant *P. gingivalis* clones were screened for negative neuraminidase and β-N-acetyl-hexosaminidase mutants using an assay based on a method devised by Camara *et al.*, 1991. Transconjugants were streaked onto BM agar plates (50 transconjugants per plate) and anaerobically incubated for 3 days at 37°C. Negative clones were then screened by using a 4 ml overlay of 0.75% (w/v) agarose dissolved in phosphate buffered saline (Sigma) supplemented with the fluorogenic neuraminidase substrate 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUAN; 100 μg/ml) (Sigma) or the fluorogenic β-N-acetyl-hexosaminidase substrate 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (MUAG; 100μg/ml) (Sigma). The plates were incubated aerobically at 37°C for 1 hour after which time the plates were observed under UV light using a hand-held longwave UV lamp. Positive clones produced a fluorescence under the UV light and negative clones were expected to not fluoresce under the UV light. As a control the neuraminidase and β-N-acetyl-hexosaminidase negative bacterium *P. assacharolyticus* was used to demonstrate the non-emitance of fluorescence whereas the wild-type neuraminidase and β-N-acetyl-hexosaminidase positive bacterium *P. gingivalis* 33277 was used to demonstrate the emitance of fluorescence.

CHAPTER 3

The Manipulation of Tn*4351* to Create a Type I Transcriptional Promoter Probe, Tn*4351*::*catIII*.

3.1 Introduction

Until quite recently the study of putative virulence genes isolated from *P. gingivalis* has relied on the cloning and subsequent expression of the genes in *E. coli* (Progulske-Fox *et al.*, 1989a; Roberts *et al.*, 1990). Such studies, although providing useful information on the structure of the proteins encoded by these cloned genes, does not allow an assessment to be made of the possible role that individual virulence factors may play in disease progression. The generation of *P. gingivalis* strains carrying mutations in particular virulence genes would enable the generation of isogenic strains and permit the role of specific virulence factors to be determined. However, the lack of naturally occurring plasmids or bacteriophages in *P. gingivalis* has, until recently, resulted in the inability to transfer genetically manipulated DNA into this species.

The application of genetic systems, originally developed for use in *Bacteroides* species, that can assist in the genetic manipulation of putative *P. gingivalis* virulence genes has been discussed extensively in section 1.7. In summary, a series of chimeric shuttle and suicide vectors have been developed that can be transferred from *E. coli* into *Bacteroides* spp. or *Porphyromonas* spp. (Dyer *et al.*, 1992; Shoemaker *et al.*, 1986; 1989). These vectors permit the generation of single mutations within putative virulence genes either by allelic exchange or through transposon mutagenesis (Fletcher *et al.*, 1995; Hoover and Yoshimura, 1994) and this has proved to be an efficient method for studying the virulence determinants from *P. gingivalis* thought to be important in the initiation and continuation of periodontal disease.

It has already been established that the oral environment alters dramatically during the progression of periodontal disease. The effect of environmental stimuli on the production of virulence determinants has been discussed previously (sections 1.2 and 1.4.1) and it has been demonstrated that several environmental stimuli such as pH (McDermid *et al.*, 1988), temperature (Amano *et al.*, 1994) and haemin levels (Marsh *et al.*, 1988; 1994) can have an effect on the expression of putative virulence factors. Once putative virulence factors have been identified by mutagenesis it would useful to have a means of assaying the effect that environmental stimuli have on the expression of these genes. Transcriptional promoter probes have been developed to study gene regulation in many bacteria species (Bellofatto *et al.*, 1984; Kroos and Kaiser, 1984; Way, 1984). These systems use reporter gene/transposon fusion's that can be readily transferred into the bacteria of choice resulting in random transposition of the reporter gene fusion into the chromosome. The fusion need only insert downstream of a promoter region in the correct orientation and does not have to be inframe for reporter gene expression to occur (Berg *et al.*, 1989). Recently transposon mutagenesis has been undertaken in *P. gingivalis* using the transposon Tn*4351* (Dyer *et al.*, 1992; Hoover *et al.*, 1992b), a transposon originally isolated from the *B. fragilis* plasmid pBF4. Because it has already been demonstrated that this transposon can be successfully transferred into *P. gingivalis* and that it can then transpose randomly into the genome to generate single mutations it seemed appropriate to use Tn*4351* as a starting point from which to develop a type I transcriptional probe with which to monitor promoter activity in *P. gingivalis*.

Several reporter genes are routinely used to detect bacterial promoter activity, the major consideration being an easy assay for the reporter gene. These genes include the *lacZ* gene of *E. coli* whose gene product, β-galactosidase, catalyses the hydrolysis of lactose to glucose and galactose. This gene is often employed as the reporter gene since β-galactosidase is easily and quantitatively assayed, is active in a variety of organisms and remains active as a fusion protein (Bassford *et al.*, 1978). The gene *phoA* has been used as a reporter gene to generate both type I and type II promoter probes. The PhoA protein, alkaline phosphatase, is only active extra-cytoplasmically in the periplasmic space (Berg *et al.*, 1989). As a consequence *phoA* is useful in that it can be used to

detect genes that encode exported or membrane proteins, and can be exploited as a tool to study the topology of proteins within the membrane (Manoil and Beckwith, 1985; 1986). The use of a sensitive indicator medium (containing the chromogenic substrate 5-Bromo-4-Chloro-3-Indoyl Phosphate) and a quantitative enzyme assay available for alkaline phosphatase provides a simple way to monitor the expression and localisation of particular proteins. Bacterial bioluminescence has proved to be a sensitive, non-invasive reporter for monitoring gene expression in a number of bacterial species particularly at the transcriptional level (Stewart and Williams, 1992). Production of light is catalysed by luciferase, a heterodimeric enzyme encoded by the *luxA* and *luxB* genes as shown:

$$FMNH_2 + RCHO^a + O_2 \xrightarrow{\text{luciferase}} FMN + RCOOH + H_2O + \text{light}$$

[a] Long-chain aliphatic aldehyde

However, there has only been one example of the use of luciferase in obligate anaerobes and this is because the bioluminescence reaction requires oxygen which is toxic to obligate anaerobes such as *P. gingivalis*.

3.2 Cloning Strategy

The structure of Tn*4351* (fig. 3.1) with flanking homologous direct repeats of IS*4351* lends itself to manipulation. In order to generate the reporter gene/transposon fusion ideally it is necessary to have a unique restriction enzyme site close to one end of the transposon into which the reporter gene can be cloned. Analysis of the complete nucleotide sequence of IS*4351*$_R$ (fig. 3.2) reveals two *Eco*RI restriction enzyme sites, only 19 bp apart, close to one end of this insertion element. One of these sites could be used as the cloning site for the reporter gene. Because IS*4351*$_R$ and IS*4351*$_L$ are homologous direct repeats of each other it would be feasible to use only one of the insertion elements during the initial cloning of the reporter gene. Subsequently the entire transposon of antibiotic resistance markers flanked by two homologous insertion elements could be recreated at a later cloning stage. The strategy of recreating the entire

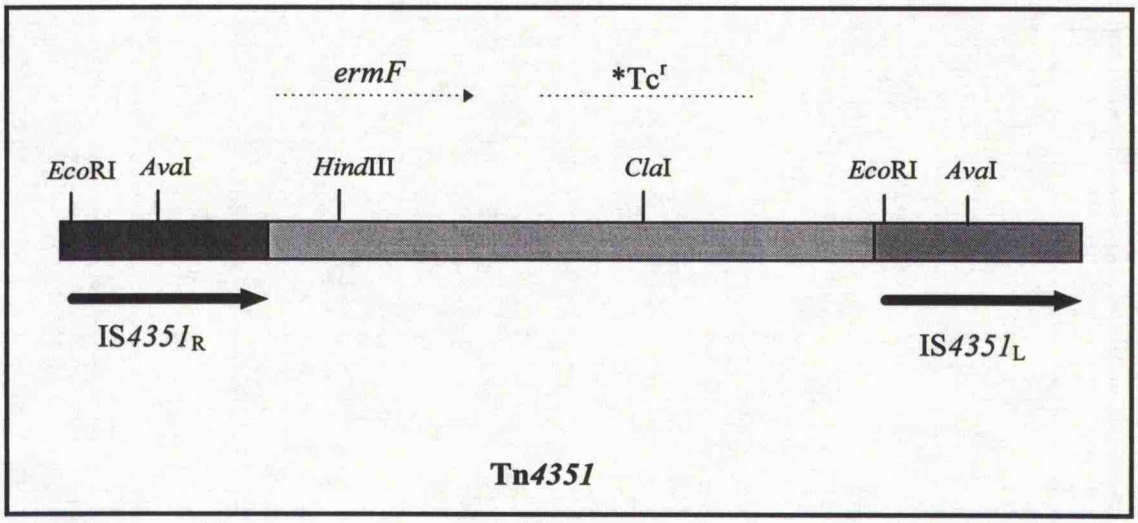


Fig. 3.1 Restriction map of Tn*4351* a composite transposon originally isolated from *B. fragilis*. *Eco*RI restriction enzyme sites depicted within both insertion elements represent two restriction enzyme sites situated close together. Positions of the antibiotic resistance determinants *ermF* and *Tc^r are indicated above the map, *ermF* confers resistance to clindamycin/erythromycin and *Tc^r confers resistance to tetracycline. The following diagrammatic representations are used.

IS*4351*$_L$ sequences

IS*4351*$_R$ sequences

Direction of the homologous direct repeats

*Eco*RI *Eco*RI

insR

1	GCTGAATTCAACTTGCAAATGCAACAGAATTCTGATTAATAATTTGTTTA AATTTTTCGT	60
61	TTGGCGTGAGGTATCCAAGTCTTTTACGAGGTCGATTATTGAGTTTA TTTTCAATCCACT	120
121	TAATCTGTTTGTTGGTTACTTCACTAAAGTCCTTACCCTTTGGGATAT ACTGCCTGATAA	180
181	GCCCGTTGGTGTTTTCATTGGCACCACGTTCCCATGAGTGGTATGGTTTGCAAAAATAGA	240
241	ATTTTATTTCCAATTTTT GCGCAATTT CCTCGTGCTTTGCAAACTCCTTT CCATTGTCAG	300
301	CCGTAATTGTGTGTATTAAGTTTTTCACTTTCCGCAGT GCCCATACTGCAATCTTAG CTA	360
361	CCGGGATGGCTTCTTTTCCCGACAACTTGCGTATCCAG ACCCTGCTTGTTGCTCT GTCGT	420
421	TAATGGTAAGAATGGCACCTTTGTGGTTCTTACCAATAATTGTATCTATCTCTA AATCAC	480
481	CAAATCTCTCCTTCAGTTCCACTATCTCGGGACGCTCATCAATATCCACCCTG CCTGGGA	540
541	TAAATCCTCGCCCTGCATTTTTAGAACCACGTTTGGCATACCTGCGACCTTG TCTGCGAA	600
601	GATATTTGTGCAGTTTGCCACCCCGCCGCTTATCCTCCCAAATCCAGCGATATA TCGTTT	660
661	CGTGAGATACCATCGCAATTCCCTCCAAGCGGCTCCTGCCGACAATCTGCTCCGGGCTGA	720
721	ATCCTTTCTTCA ACAGCTTTATT ATCCGTTTTCT CATTGCCGGTGTAAGCACTTCCTTGC	780
781	GATGTTTTTGCTGCTTGCGCCTGTCTG CTTTTCGCTGGGCAAGCTCC ATGCTATAGCTAC	840
841	CACTTCGGGCGTCGCAATTGCGCTTTATCTCCC TGTAAACAGTGCTTTTATCTAC TCCGA	900
901	TAGCTTCCGCTATTGCTTTTTTGCTCA TCGGTATTTGCAACATC ATAGAAATTGCATACC	960
961	TTTGTTCCTCGGTT ATATGTTTGCTCAT CTGCAAC TTTTTTTTCTTTGGACGGACAATTA	1020
1021	AAGCAAAGATAGCAAACTTTATCCATTCAGAGTGAGAGAAAGGGGGACATTGTCTCTCTT	1080
1081	TCCTCTCTGAAAAATAAATGTTTTT ATTGC TTATTATCCGCACCCAAAAAGTTGCATTTA	1140
1141	TAAGTTGAACTCAAG	

insL

AvaI

Fig. 3.2. Complete nucleotide sequence of the 1,155 bp insertion element IS*4351*$_R$ (Rasmussen *et al.*, 1987). Sequence shown 5' → 3'. The *Eco*RI and *Ava*I restriction enzyme sites are indicated on the sequence surrounded by a hashed lined box. Terminal inverted repeats (*insL* and *insR*) are indicated above the sequence. Three open reading frames are found within IS*4351*. ORF I extends from nucleotide 988 to nucleotide 11. ORF II extends from nucleotide 366 to nucleotide 622. ORF III extends from nucleotide 782 to nucleotide 1018.

transposon using a 3.8 kb *Ava*I fragment from Tn*4351* that encodes the antibiotic resistance markers, *ermF* and *Tc^r and which also incorporates the first 500 bases of IS*4351*$_L$ and the last 600 bases of IS*4351*$_R$ is demonstrated in figure 3.3. By cloning this 3.8 kb *Ava*I fragment into the unique *Ava*I restriction enzyme site within IS*4351*$_R$ the entire transposon is recreated. A cloning strategy was devised in order to generate the reporter gene/transposon fusion and is summarised below. Stages 2 - 4 are represented diagrammatically in figures 3.4 A, B and C.

1. In order that the reporter gene could be easily cloned into the unique *Eco*RI restriction enzyme site in IS*4351*$_R$, and so that the entire transposon could be recreated using the 3.8 kb *Ava*I fragment, a cloning vector had to be chosen that did not contain either of these restriction enzyme sites.
2. IS*4351*$_R$ was amplified by PCR using primers designed to eliminate one of the two *Eco*RI restriction enzyme sites. The resulting PCR product, IS*4351*$_R$*, was then cloned into the chosen vector (figure 3.4 A).
3. The reporter gene was amplified by PCR using primers designed to introduce *Eco*RI restriction enzyme sites into the 5' and 3' ends of the gene. The resulting PCR product was then cloned into the unique *Eco*RI restriction enzyme site within IS*4351*$_R$* (figure 3.4 B).
4. The entire transposon could then be recreated by cloning the 3.8 kb *Ava*I fragment from Tn*4351* into the unique *Ava*I restriction enzyme site within IS*4351*$_R$* (figure 3.4 C).
5. A biological assay was then devised to determine whether the manipulated transposon could still undergo transposition.
6. The manipulated transposon was finally cloned into a suicide vector that could then be transferred from *E. coli* into *P. gingivalis*.

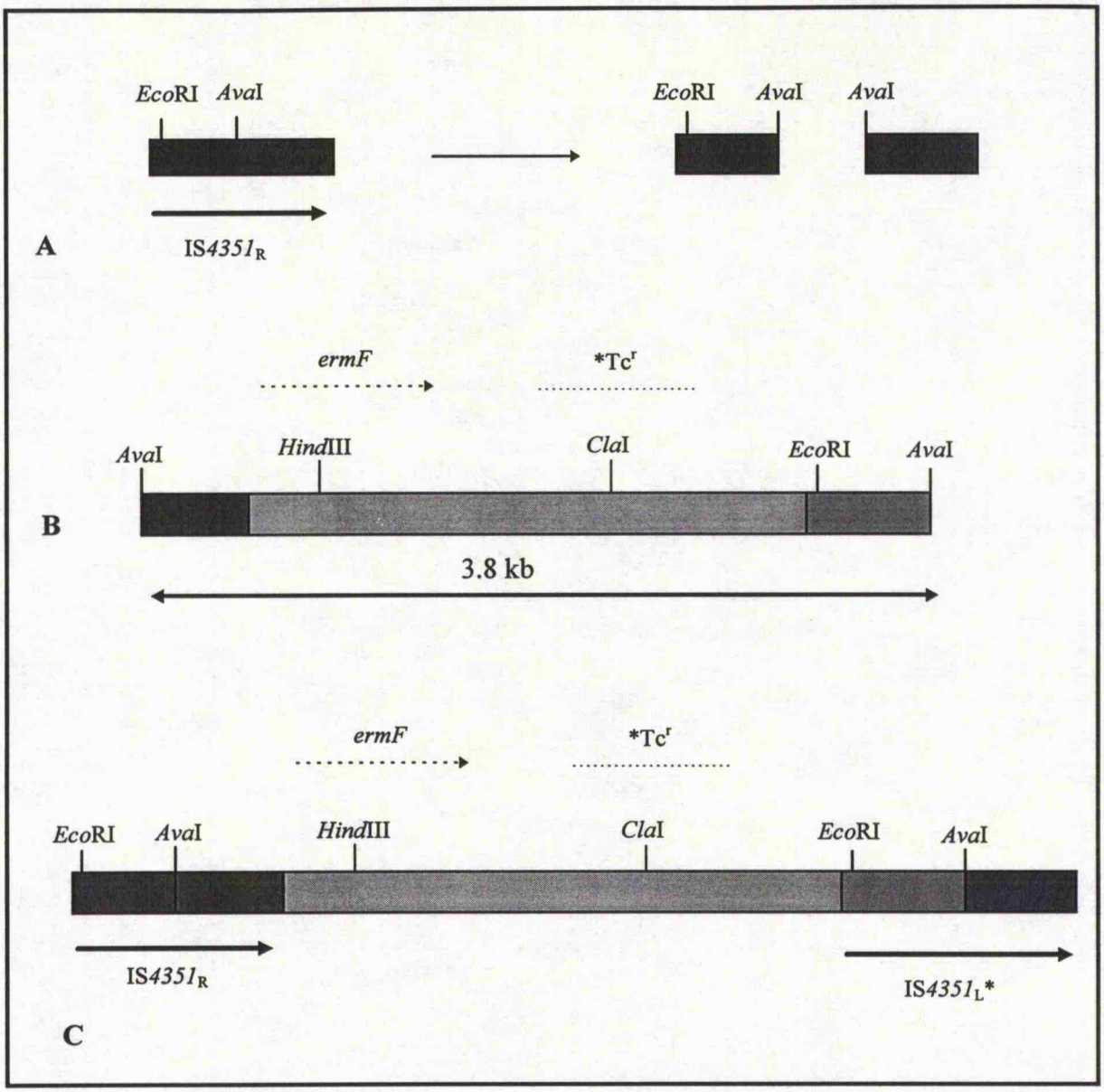


Fig. 3.3 Recreation of the transposon.

A. Cleavage of IS*4351*$_R$ with *Ava*I restriction enzyme.

B. Digestion of Tn*4351* with *Ava*I restriction enzyme to generate a 3.8 kb fragment that encodes the antibiotic resistance markers *ermF* and Tc^r. This fragment also contains the first 500 bp of IS*4351*$_L$ and the last 600 bp of IS*4351*$_R$.

C. Cloning the 3.8 kb *Ava*I fragment into the unique *Ava*I restriction enzyme site within IS*4351*$_R$ recreates the composite transposon designated Tn*4351** that is flanked by two insertion elements, one of which is an intact copy of IS*4351*$_R$ and the other of which, IS*4351*$_L$*, is a hybrid of IS*4351*$_R$ and IS*4351*$_L$.

IS*4351*$_L$ sequences IS*4351*$_R$ sequences

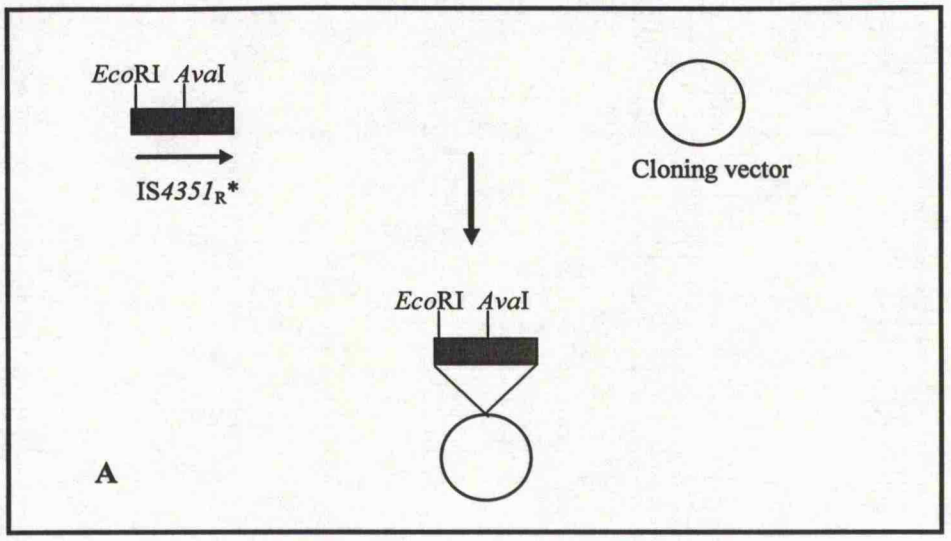


Fig. 3.4A

IS*4351*$_R$ is amplified by PCR using primers designed to remove one of the two *Eco*RI restriction enzyme sites. The PCR product IS*4351*$_R$* is cloned into a suitable vector.

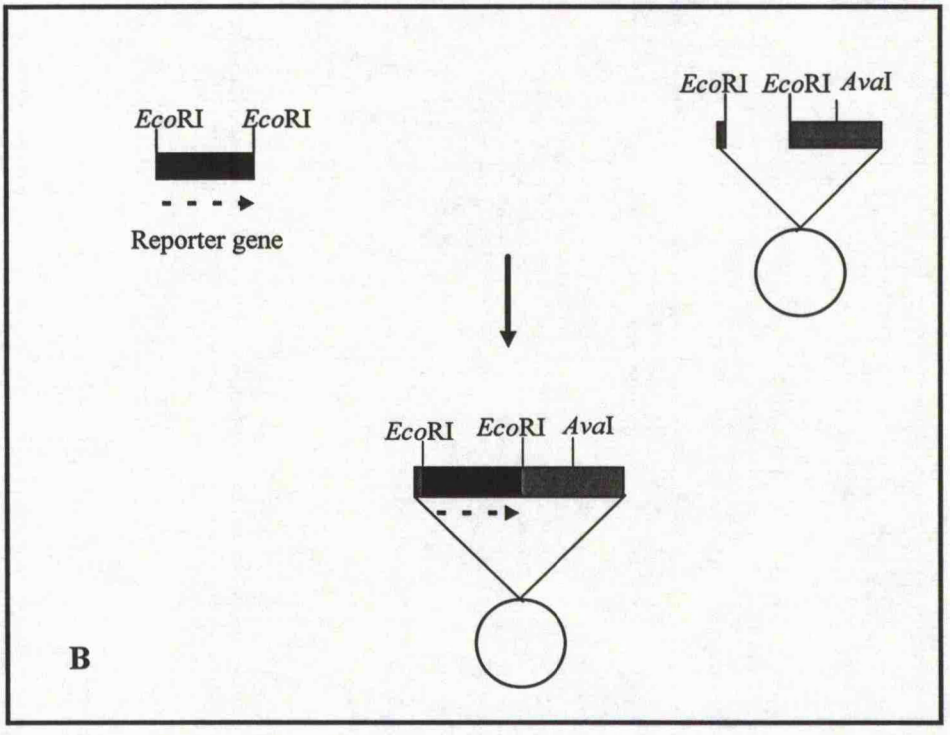


Fig. 3.4B

The reporter gene is amplified by PCR using primers designed to introduce *Eco*RI restriction enzyme sites to both the 5' and 3' ends of the gene. The reporter gene is then cloned into the unique *Eco*RI restriction enzyme site within IS*4351*$_R$*.

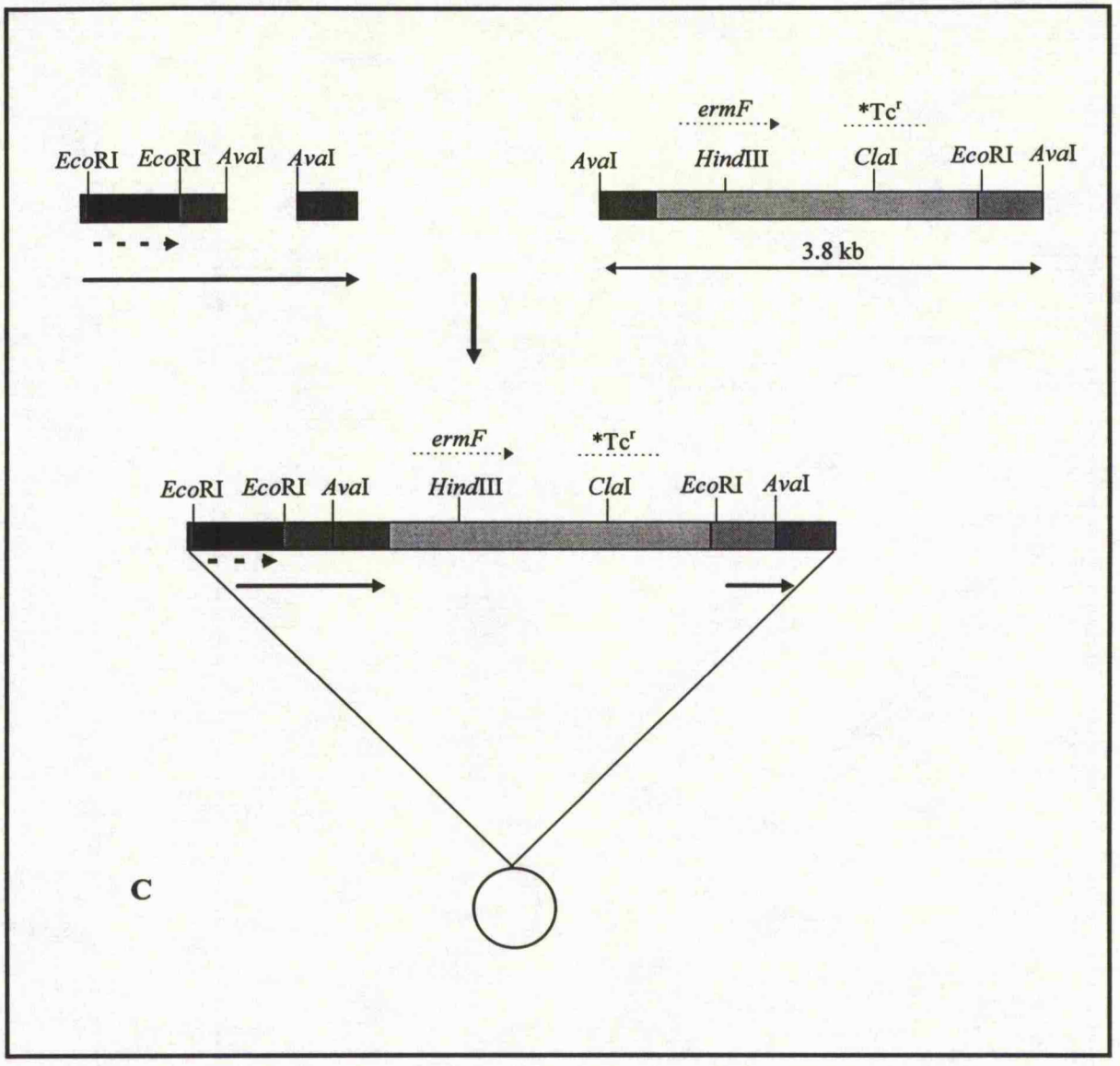


Fig. 3.4C

The 3.8 kb AvaI fragment from Tn*4351* is cloned into the unique *Ava*I restriction enzyme site within IS*4351*$_R$* recreating the entire transposon of antibiotic resistance markers flanked by two homologous direct repeats and including a reporter gene that is inserted in such a way that it can be used to detect promoter activity.

For Figures 3.4 A, B and C the following diagramatic representations are used:-

- - - ▶ Represents the direction of reporter gene expression

▬ IS*4351*$_R$ sequences

▬ IS*4351*$_L$ sequences

⟶ A solid arrow positioned underneath the insertion sequences represents the direction of the homologous direct repeats

3.3 Methods and Results

3.3.1 Stage 1: Creating a Suitable Cloning Vector

In order that the reporter gene can be cloned into the *Eco*RI restriction enzyme site found within IS*4351*$_R$, the cloning vector chosen must not contain any *Eco*RI restriction enzyme sites. Similarly if the unique *Ava*I restriction enzyme site located within IS*4351*$_R$ is to be used to recreate the entire transposon we also require that the cloning vector does not contain any *Ava*I restriction enzyme sites. The vector of choice is pBR328 (fig. 3.5) a non-mobilisable plasmid that was originally derived from pBR325 and pBR322 (Soberon *et al.*, 1980) and that is 4.9 kb in size and confers resistance to chloramphenicol, ampicillin and tetracycline.

Although pBR328 contains both *Eco*RI and *Ava*I restriction enzyme sites, it was chosen since it had previously been exploited in a biological assay in *E. coli* to study IS*4351* transposition (Hwa *et al.*, 1988). The assay was based on the ability of the two IS*4351* insertion elements to form cointegrates (an intermediary step of replicative transposition). In the study Tn*4351*, IS*4351*$_L$ and IS*4351*$_R$ were cloned into pBR328 and transfer of the chloramphenicol marker on pBR328 by cointegrate formation of the pBR328 based plasmids with a conjugative plasmid was then assayed (Hwa *et al.*, 1988). The use of this assay provides a rapid tool to determine what effect the reporter gene will have on the ability of the manipulated Tn*4351* to transpose. The cloning of the modified Tn*4351*, containing a reporter gene, into pBR328 would allow the frequency of transfer of Ap^r, via cointegrate formation, to be measured. This would demonstrate what, if any, effects the disruption of IS*4351*$_R$ has on the transposition of Tn*4351*.

The *Eco*RI and *Ava*I restriction enzyme recognition sites within pBR328 can be removed by end-repair, a "fill in" reaction that is catalysed by the large fragment of DNA polymerase I (Klenow fragment). The *Eco*RI restriction enzyme site was removed in the first instance and then the resulting plasmid was used in the reaction to remove

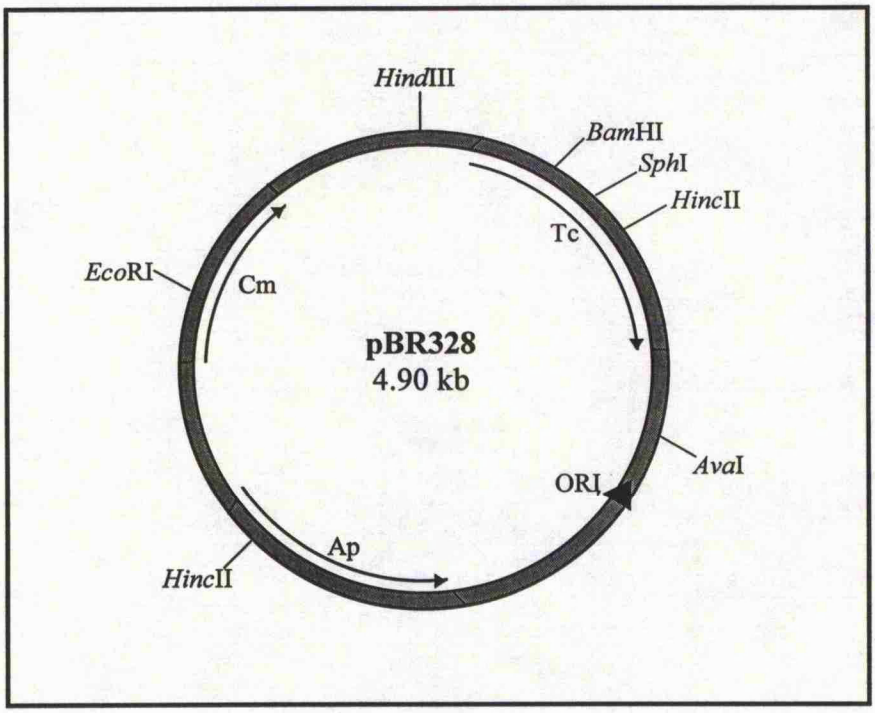


Fig. 3.5. Map of the 4.9 kb vector pBR328. The map indicates the positions of the antibiotic resistance genes ampicillin (Ap), chloramphenicol (Cm) and tetracycline (Tc). The position of the origin of replication (ORI) is also indicated on the map. Restriction enzyme sites used in the cloning procedures and in restriction enzyme analysis are indicated on the map.

the *Ava*I restriction enzyme site. Both *Eco*RI and *Ava*I restriction enzymes recognise a sequence of 6 bp which is cleaved one base in from the 5' end of the recognition sequence on both strands resulting in 5' overhangs (fig. 3.6). In the fill in reaction the plasmid was initially digested with the restriction enzyme according to the manufacturers recommendations and then Klenow was used to add complementary dNTP's to the 5' sticky ends of the plasmid. Contaminating Klenow and dNTP's were removed by phenol/chloroform extraction before a blunt end ligation reaction was set up using approximately 0.5 μg plasmid DNA to religate the plasmid back into a covalently-closed circle. The result of successful end-repairing of the 5' overhangs is to add 4 extra bp to each strand so that the recognition site now contains 8 additional bp (fig. 3.6) thus destroying the sequence recognised by the restriction enzyme.

The *Eco*RI restriction enzyme site in pBR328 lies within the region encoding the chloramphenicol resistance gene (fig. 3.5). The result of end repairing this site is to cause a frame shift mutation in the chloramphenicol resistance gene so that the RNA is no longer translated to generate a functional protein. Plasmids that had undergone end-repair of the *Eco*RI restriction enzyme and subsequent religation were transformed into the *E. coli* strain LE392. Transformants were then plated initially onto LA medium supplemented with Ap and Tt and were subsequently screened on LA supplemented with Tt, Ap and Cm. Transformants that could grow on both media still contained an intact *Eco*RI restriction enzyme site. However, transformants that failed to grow on the LA supplemented with Cm are likely to have lost the *Eco*RI restriction enzyme site by end-repair. Of 23 colonies that were plated in duplicate onto the two media, 5 colonies were found not to grow on medium supplemented with Cm. Confirmation of the loss of the *Eco*RI restriction site was achieved by restriction enzyme analysis of small scale extractions of chloramphenicol sensitive plasmid DNA (fig. 3.7). The end-repaired vector was identical to pBR328 except that the *Eco*RI restriction enzyme was no longer able to cleave within plasmid, the resulting vector was termed pAO1.

The *Ava*I restriction enzyme site in pBR328 does not lie within any of the antibiotic resistance markers and therefore a slightly different approach was taken to analyse *Ava*I

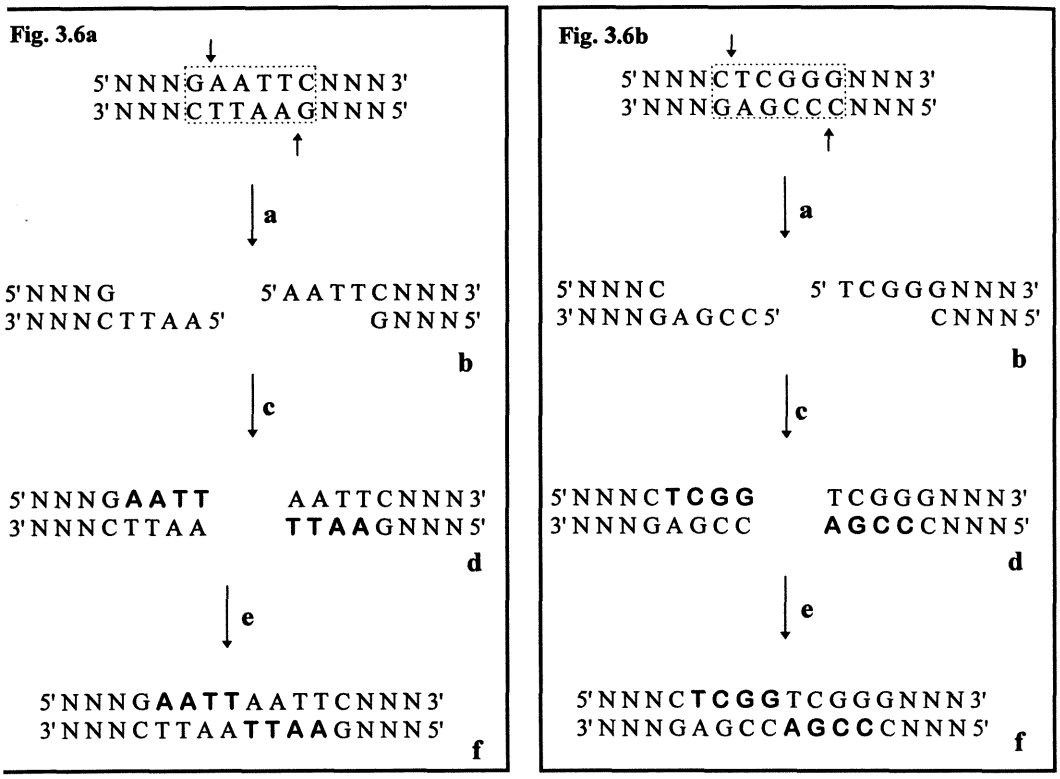


Fig. 3.6. End repair of *Eco*RI (fig. 3.6a) and *Aval* (fig. 3.6b) restriction enzymes sites in pBR328. 'lasmid DNA is digested with the appropriate restriction enzyme (**a**) which cleaves at the recognition site eaving 5' sticky ends (**b**). An end repair reaction is then carried out (**c**) during which Klenow catalyses the ddition of nucleotides to the sticky ends (**d**). A blunt end ligation (**e**) reforms the plasmid as a covalently-losed circle (**f**) which no longer has the restriction enzyme recognition sequences. Bold type letters e.g. **A,T,C** and **G** indicate bases added by Klenow during the end repair reaction, ⋯ indicates the restriction nzyme recognition site for *Eco*RI (fig 3.6a) and *Aval* (fig 3.6b) and ↑ ↓ indicate the cleavage sites of the estriction enzymes.

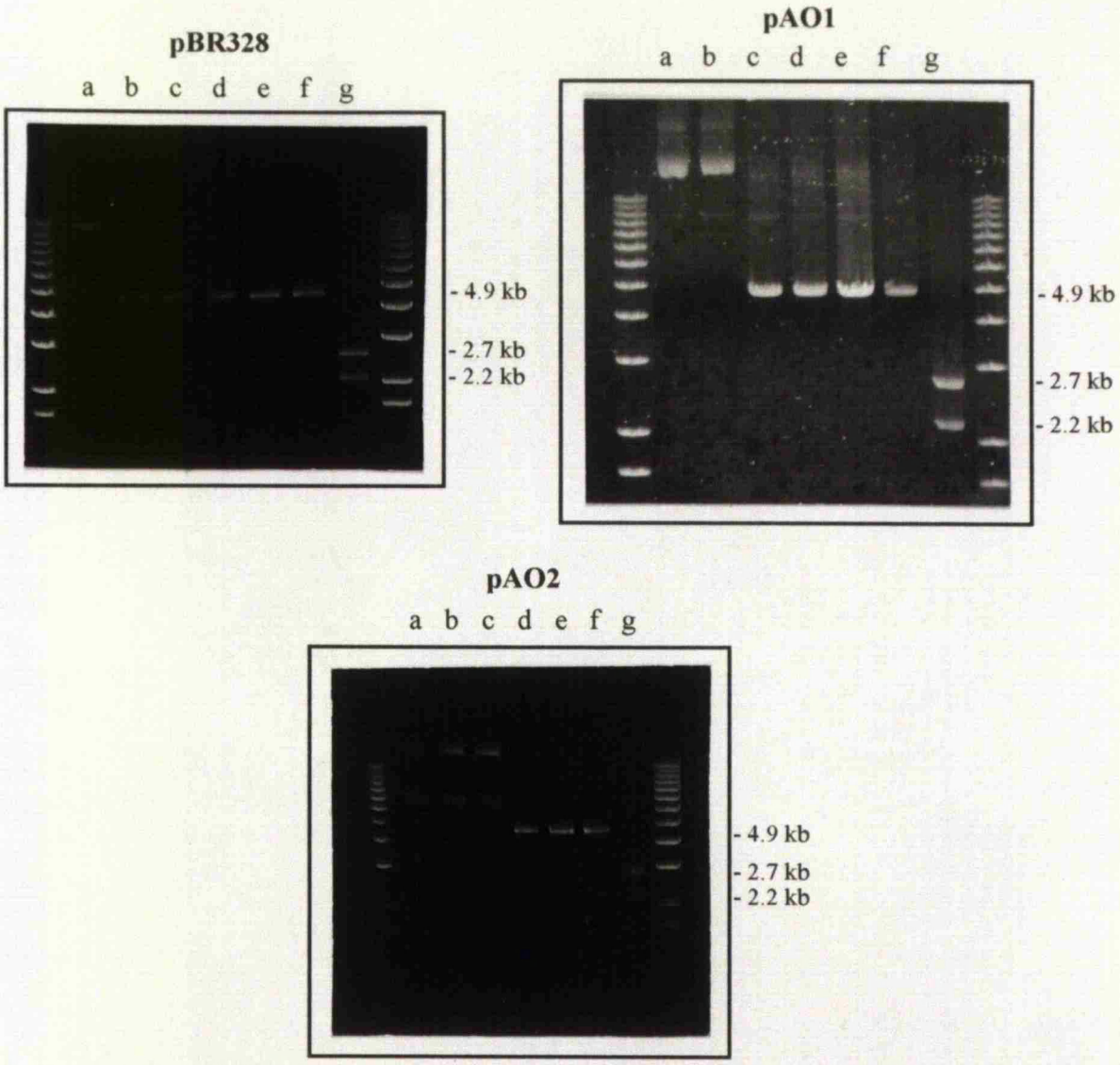


Fig. 3.7. Restriction enzyme digestion analysis of plasmids pBR328, pAO1 and pAO2. Plasmid DNA is run along side Kilo base (kb) ladder. The letters a - g represent DNA digested with the following restriction enzymes a = uncut DNA; b = *Eco*RI; c = *Ava*I; d = *Eco*RV; e = *Hin*dIII; f = *Bam*HI; g = *Hin*cII. In pBR328 all the restriction enzymes cut in the plasmid once to create a 4.9 kb fragment except *Hin*cII which cuts twice in the plasmid to create two bands of 2.2 and 2.7 kb. It can be seen that as a result of end repair of the recognition site for *Eco*RI in pBR328 the resulting plasmid pAO1 is no longer cut with *Eco*RI restriction enzyme. It can also be seen that as a result of end repair of the recognition site for *Ava*I in pAO1 the resulting plasmid pAO2 is no longer cut with *Ava*I restriction enzyme.

end-repaired vector. The loss of the *Ava*I restriction enzyme site within pAO1 and religation of plasmid DNA to form a covalently-closed circle was carried out as for the end-repair of the *Eco*RI restriction enzyme site within pBR328. However, before the *Ava*I end-repaired pAO1 vector was transformed into LE392 the ligated end-repaired pAO1 DNA was digested with *Ava*I restriction enzyme according to the manufacturer's instructions. The enzyme was subsequently inactivated by heating at 65°C for 20 minutes. The *Ava*I digested DNA was then transformed into LE392 by electroporation. An aliquot of the ligated DNA that had not been digested with *Ava*I restriction enzyme was also transformed into LE392 and acted as a control. It was expected that vector pAO1 that had not had the *Ava*I restriction enzyme site end-repaired would be linearised by the *Ava*I cleavage reaction and would therefore not be able to replicate in LE392. This was reflected in the number of transformants obtained for *Ava*I digested DNA compared to uncut DNA. A typical transformation of the uncut end-repaired vector DNA yielded 5.38 x 10^3 CFU/ml compared to transformation of the *Ava*I digested end-repaired DNA which yielded 84 CFU/ml (where transformations contained 0.5 - 1.0 μg/ml of DNA). Confirmation of *Ava*I end-repaired plasmid pAO1 was achieved by restriction enzyme digestion of small scale plasmid DNA extractions. The resulting vector, termed pAO2, could be distinguished from pAO1 by the inability of this vector to be cleaved by *Ava*I restriction enzyme (fig. 3.7).

3.3.2 Stage 2: Amplification of IS*4351*$_R$

The strategy undertaken for the amplification and subsequent sub-cloning of IS*4351*$_R$ is outlined in figure 3.8. Amplification of IS*4351*$_R$ was carried out by PCR using primers SAO1 and SAO2 (fig. 3.9). The sequence of primer SAO1 corresponds to the first 32 bases of the 5' → 3' strand of IS*4351*$_R$ as shown in figure 3.9. Primer SAO2 has a sequence identical to the last 32 bases of the complementary (3' → 5') strand of IS*4351*$_R$. The sequence of SAO1 is identical to that of IS*4351*$_R$ except for in the region of the *Eco*RI restriction enzyme site at nucleotides 4 to 9. The 2 bp alteration in the sequence from AATT to ATAT destroys the *Eco*RI restriction enzyme site but replaces

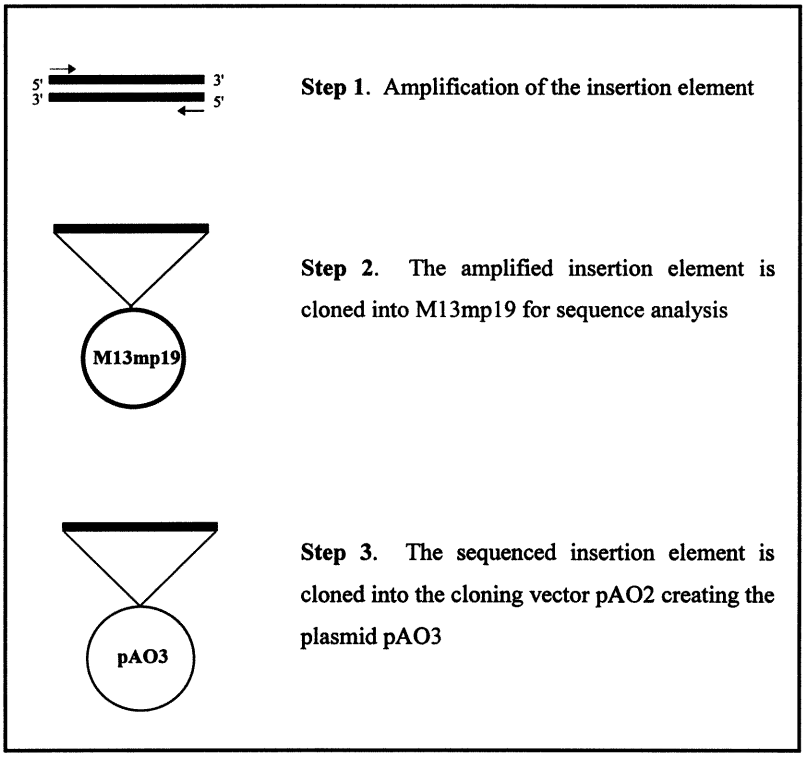


Fig. 3.8. Summary of the strategy undertaken for the amplification and sub-cloning of IS*4351*$_R$. IS*4351*$_R$ is amplified by PCR using primers designed to remove one of the two *Eco*RI restriction enzyme sites (Step 1). The amplified PCR product, IS*4351*$_R$*, is then cloned into the M13 based bacteriophage, M13mp19, so that single stranded template can be obtained for sequence analysis of the manipulated insertion element (Step 2). The sequenced IS*4351*$_R$* element can then be cloned into pAO2 creating plasmid pAO3 (Step 3).

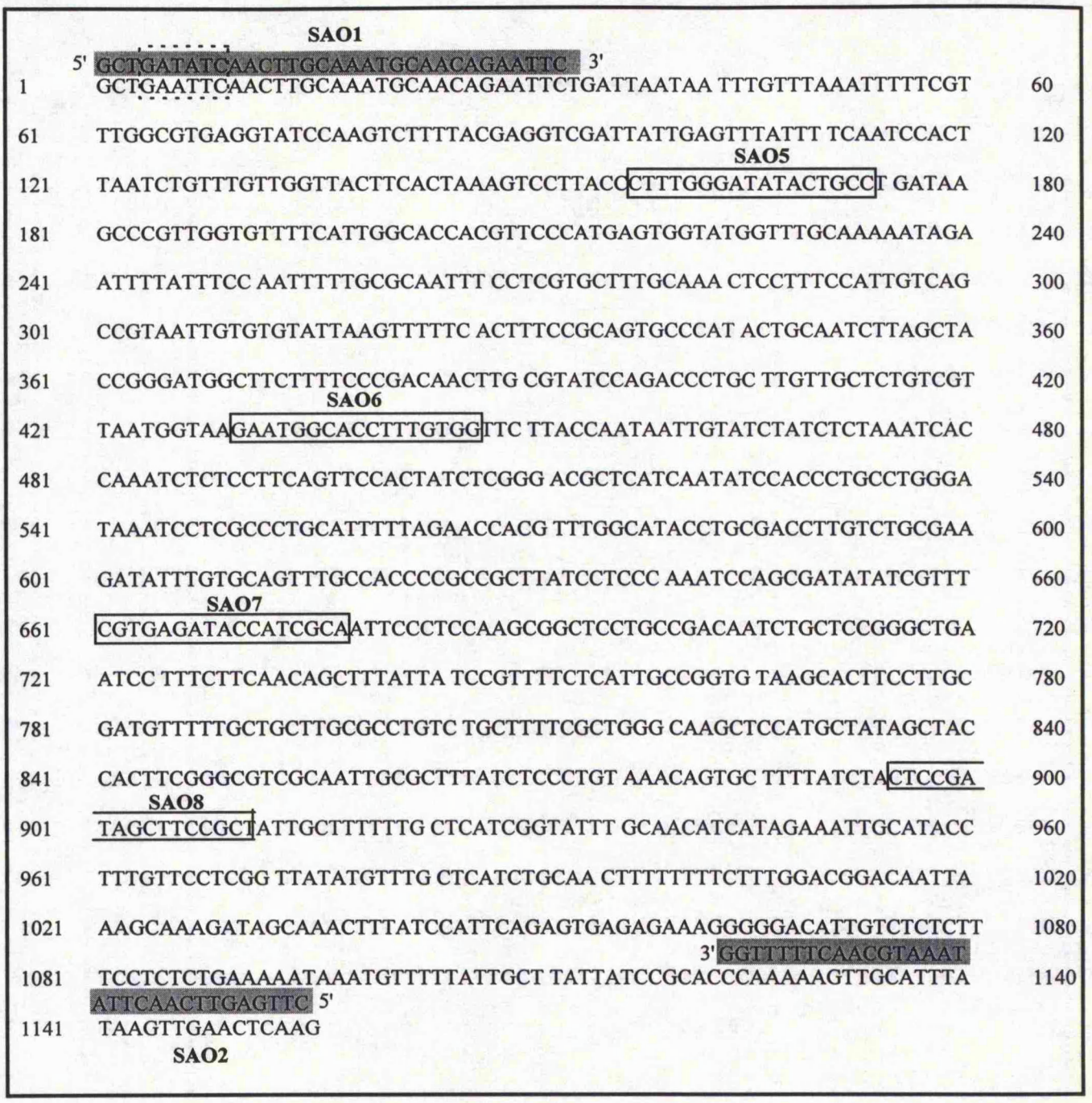


Fig. 3.9. Complete nucleotide sequence of IS*4351*$_R$ showing the primers used during PCR and sequencing reactions. The PCR primers, SAO1 and SAO2 are represented above the IS*4351*$_R$ sequence in a shaded box. The sequence for SAO2 was taken from the complementary (3' → 5') strand of IS*4351*$_R$ whereas the sequence for SAO1 was taken from the 5' → 3' strand of IS*4351*$_R$ shown above. SAO1 has an altered sequence to that of IS*4351*$_R$ the sequence GAATTC (identified by a hashed line box) was changed to GATATC which destroys the *Eco*RI restriction enzyme replacing it with an *Eco*RV restriction enzyme site. The sequencing primers, SAO5, SAO6, SAO7 and SAO8, are represented as a black box around the IS*4351*$_R$ sequence. Primers complementary to SAO5, SAO6, SAO7 and SAO8 were also used depending on the orientation of clones in M13mp19.

it with an *Eco*RV restriction enzyme site that can be used in subsequent cloning stages to analyse the orientation of inserts in the cloning vector.

The initial PCR reactions were carried out at 95°C for 1 minute denaturing, 45°C for 1 minute annealing and 72°C for 1 minute extension for 30 cycles using *Taq* DNA polymerase (section 2.8.1). A characteristic of *Taq* DNA polymerase terminal transferase activity is to add a single nucleotide to the 3' end of the PCR product and this nucleotide is almost exclusively an adenosine due to the strong preference of the polymerase for dATP (Clark, 1988; Marchuk *et al.*, 1991). This characteristic was exploited to clone the PCR product, IS*4351*$_R$*, into a commercially available PCR cloning vector pGEM®-T (Promega Corporation, 1991) which has been developed to include a single thymidine residue at the 3'-terminal of *Eco*RV blunt-cut vector DNA (fig. 3.10). The pGEM®-T vector is unable to self-ligate as the thymidine termini are incompatible, however, the *Taq* polymerase PCR products containing adenine termini are compatible with the pGEM®-T vector termini and will ligate efficiently into the vector. The pGEM®-T vector contains T7 RNA and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Insertion inactivation of the α-peptide by ligation of a *Taq* polymerase PCR product into the T-vector allows recombinant clones to be directly identified by colour screening on indicator plates supplemented with X-gal. Ligation of IS*4351*$_R$* into pGEM®-T vector was carried out according to the manufacturer's instructions and white colonies on LA plates supplemented with X-gal and IPTG were analysed by restriction mapping for the presence of the insert. Plasmid pGEM®-T found to contain an IS*4351*$_R$* insert was cleaved with *Sph*I and *Sal*I restriction enzymes to remove the IS*4351*$_R$* insert on a 1, 206 bp fragment which was subsequently cloned into *SphI*/*Sal*I digested M13mp19 from which template DNA could be made for sequence analysis. M13mp19 (fig. 3.11) is a filamentous *E. coli* bacteriophage containing 7.25 kb which exists as a single-stranded circle, although double-stranded forms arise as intermediates during DNA replication. The polylinker of M13mp19 is situated within the *lacZ* gene which allows selection of recombinant vectors by blue/white screening of M13mp19 plaques on the appropriate indicator plates. Confirmation of the presence of the

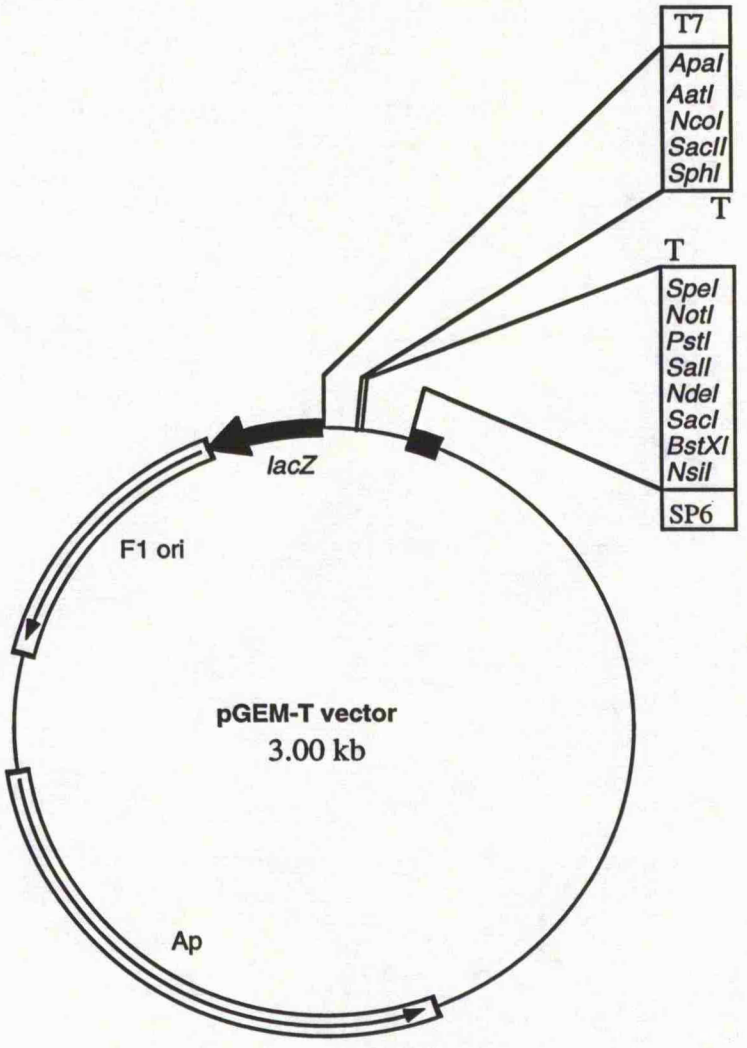


Fig. 3.10. Map of pGEM®-T vector. The vector is commercially prepared by cutting pGEM-5Zf DNA with *Eco*RV restriction enzyme and adding a 3' terminal thymidine to both ends. The polylinker is flanked by T7 RNA polymerase and SP6 RNA polymerase promoters. The vector contains a phage F1 region and an ampicillin resistance gene (Ap).

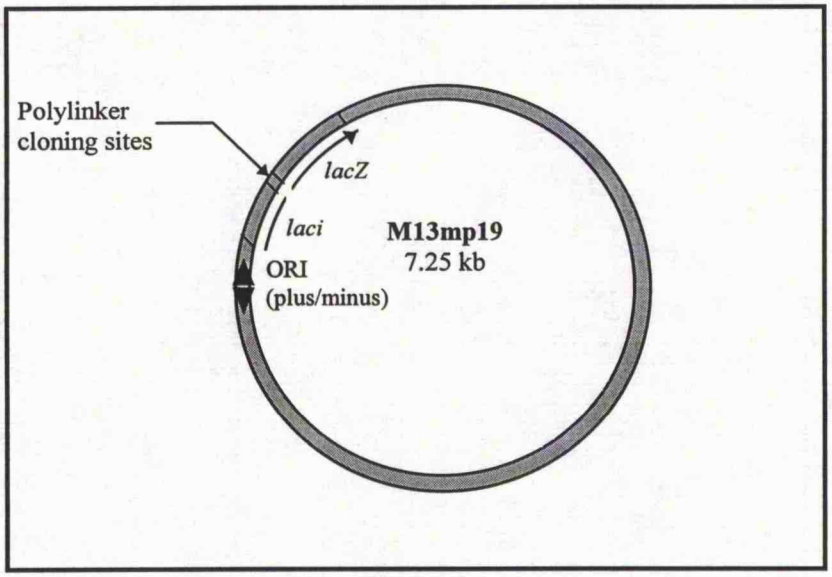


Fig. 3.11. Map of the M13-derived cloning and sequencing vector, M13mp19. The vector contains a polylinker site which lies within the lacZ gene allowing selection of inserts by blue/white screening on the appropriate indicator plates. The map also indicates the position of the origins of plus and minus strand replication.

IS*4351*$_R$* insert in M13mp19 was achieved by restriction enzyme analysis on double stranded (replicative form) DNA prepared from 'white' plaques. Once the insert had been confirmed single-stranded DNA was prepared for use as a template to sequence the insertion element.

The sequencing primers SAO5, SAO6, SAO7 and SAO8 are shown in figure 3.9. and the complementary primers SAO5R, SAO6R, SAO7R and SAO8R were also made so that depending on which orientation the IS*4351*$_R$* insert was cloned into M13mp19 the entire nucleotide sequence could be determined. In addition to these primers the M13 universal -40 sequencing primer was used. The wild-type IS*4351*$_R$ element was also cloned into M13mp19 and its nucleotide sequence was determined so that a direct comparison between the sequences of both elements could be made. The wild-type element was cloned from the plasmid pVOH1 (fig. 3.12) on a *Hind*III/*Sal*I fragment and cloned into the corresponding sites in M13mp19.

Sequence analysis of IS*4351*$_R$* identified three mutations in the amplified sequence compared to that of the wild-type (fig. 3.13). Two of the mutations were base changes and these were a T $\rightarrow$ C base change at nucleotide 919 resulting in a lysine to glutamic acid amino acid change in ORF1 and an A $\rightarrow$ C base change at nucleotide 482 which results in a phenylalanine to leucine amino acid change in ORF1. The third mutation occurred between nucleotides 254-258. The wild-type sequence has 5 thymine bases at this position however, the IS*4351*$_R$* sequence was found to have only 4 thymine bases at this position and resequencing of this part of the insertion element confirmed this finding. The result of a base deletion in this region is to cause a frame shift in ORF1 which truncates the transcript at nucleotide 243 instead of nucleotide 11. It is likely, as found in other insertion elements, that ORF1 which spans almost the entire length of the insertion element encodes for the transposase gene (Grindley and Reed, 1985) therefore the result of the truncation would be to cause the insertion element to become inactive.

Because of the number of mutations found in IS*4351*$_R$* it was decided to amplify the insertion element using *UlTma*™ DNA polymerase (Perkin Elmer) (section 2.8.2) which

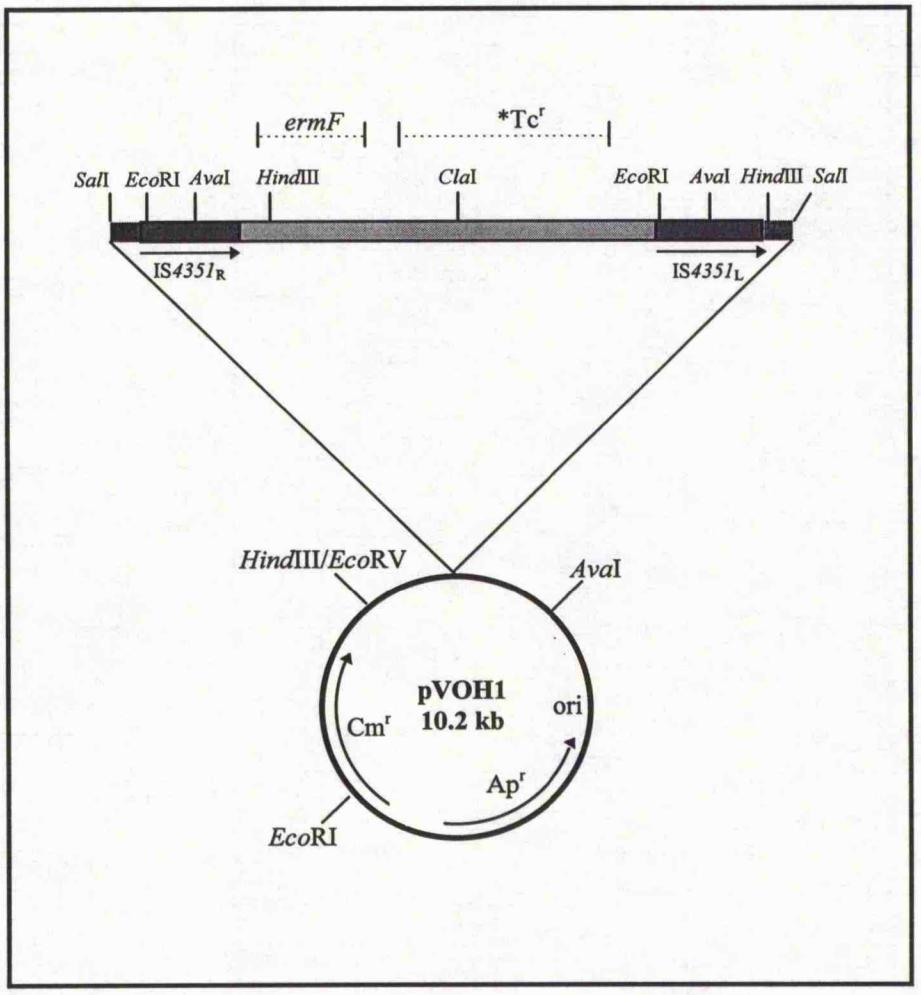


Fig. 3.12. Plasmid pVOH1. The plasmid was constructed by cloning a 6.2 kb *Sal*I fragment, containing Tn*4351*, from R751::Tn*4351* into a Tc^s derivative of pBR328 (created by deletion of the *Bam*HI restriction enzyme site of pBR328). The resulting plasmid was designated pVOH1. The pBR328 origin of replication (ori) and the genes conferring resistance to chloramphenicol (Cm^r) and ampicillin (Ap^r) are indicated on the diagram. The Tn*4351 ermF* determinant and $*Tc^r$ are also indicated on the diagram. ▬ represents R751 sequences, ▭ represents Tn*4351* sequences, ▬ represents IS*4351* sequences and —— represents pBR328 sequences (taken from Hwa *et al.*, 1988).

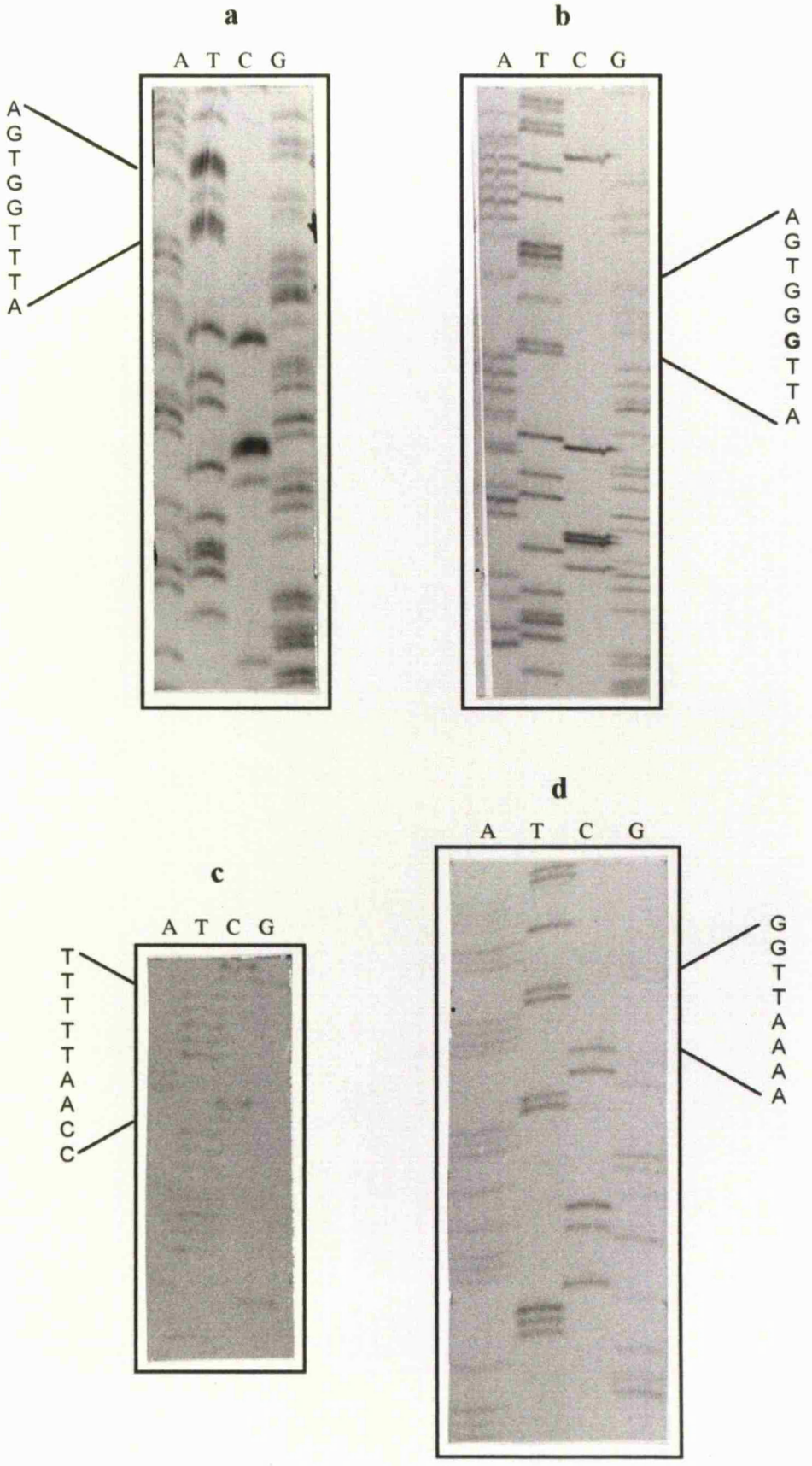


Fig. 3.13. Sequence analysis of *Taq* polymerase amplified IS*4351*$_R$

Fig. 3.13. Sequence analysis of *Taq* polymerase amplified IS*4351*$_R$. Sequence **a** represents the wild-type sequence whereas sequence **b** represents the *Taq* amplified sequence. The T to G base substitution at nucleotide 482 in sequence **b** is shown in bold type to the right of the sequence and can be compared to the wild-type sequence shown to the left of sequence **a**. Sequence **c** represents the wild-type sequence and is shown in the 5' - 3' direction as indicated in fig. 3.2. Sequence **d** represents the *Taq* amplified sequence and is shown in the complementary direction to sequence **c**. Sequence **d** demonstrates the deleted thymine base (shown as an adenine base on the complementary strand) between nucleotides 254 - 258 as indicated to the right of the sequence. This can be compared to the 5 thymine bases found in the wild-type sequence at this position shown to the left of sequence **c**.

has a 3' to 5' exonuclease proof-reading activity with no associated 5' to 3' nuclease activity. Theoretically the effect of the proof-reading activity may be to lower the misincorporation rate to 1 x 10^{-6} compared to 1 x 10^{-4} exhibited by *Taq* DNA polymerase under optimal conditions (where the misincorporation rate is measured as the number of base pair changes per total number of base pairs studied per cycle). The resulting PCR product was visualised by gel electrophoresis on a 0.7% agarose gel (figure 3.14). Control reactions using the PCR reaction mix minus the primers or the PCR reaction mix minus the template DNA were also carried out to demonstrate that the amplified product was not due to contaminated primers or template DNA. As expected no amplified PCR product could be seen for these control reactions (figure 3.14). The *UlTma*™ DNA polymerase does not add a nucleotide to the 3' end of the PCR product therefore, the amplified IS*4351*$_R$* PCR product could be blunt-end ligated directly into a *Hinc*II restriction enzyme site in M13mp19.

Sequence analysis of *UlTma*™ DNA polymerase PCR products revealed two base change mutations (fig 3.15). One of the mutations was a T $\rightarrow$ C base change at nucleotide 57. However, this mutation was silent and still retained a codon for glutamic acid in ORF1. The second mutation at nucleotide 675 was a G $\rightarrow$ A base change which causes an alanine to valine amino acid change in ORF1. Although the effect of such an amino acid change can not be predicted it was decided to continue using this amplified PCR product for two reasons. First, both alanine and valine are hydrophobic amino acids with similar side chains and therefore the effect of substituting one amino acid with the other may not be too detrimental to the protein structure. Secondly, before the manipulated transposon was to be transferred into *P. gingivalis* a biological assay was to be carried out in *E. coli* and this would enable the activity of the transposon to be assessed. As such, the effect of the mutation in the insertion element could be determined before any transposon mutagenesis was carried out. Sequence analysis of IS*4351*$_R$* also identified the altered *Eco*RI recognition site at nucleotides 4 - 9 and demonstrated the presence of a unique *Eco*RI restriction enzyme site at nucleotides 27 - 32.

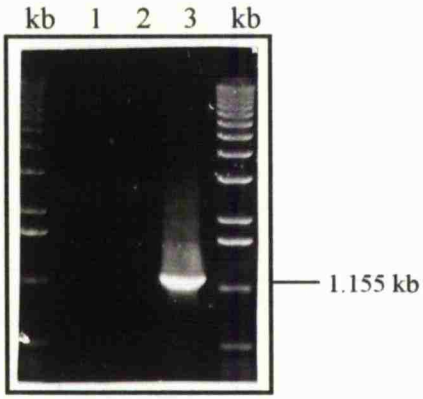


Fig. 3.14. Amplified IS*4351*$_{R}$. The plasmid pVOH1 was used as a template for a PCR reaction, using primers SAO1 and SAO2, to amplify the insertion element IS*4351*$_{R}$. The resulting 1.155 kb PCR product was visualised on a 0.7 % agarose gel (lane 3). Two negative controls were also carried out. The first control (lane 1) was carried out using the PCR reaction mix minus the primers. The second control (lane 2) was carried out using the PCR reaction mix minus template DNA. Kilo base (kb) ladder is run either side of the PCR products.

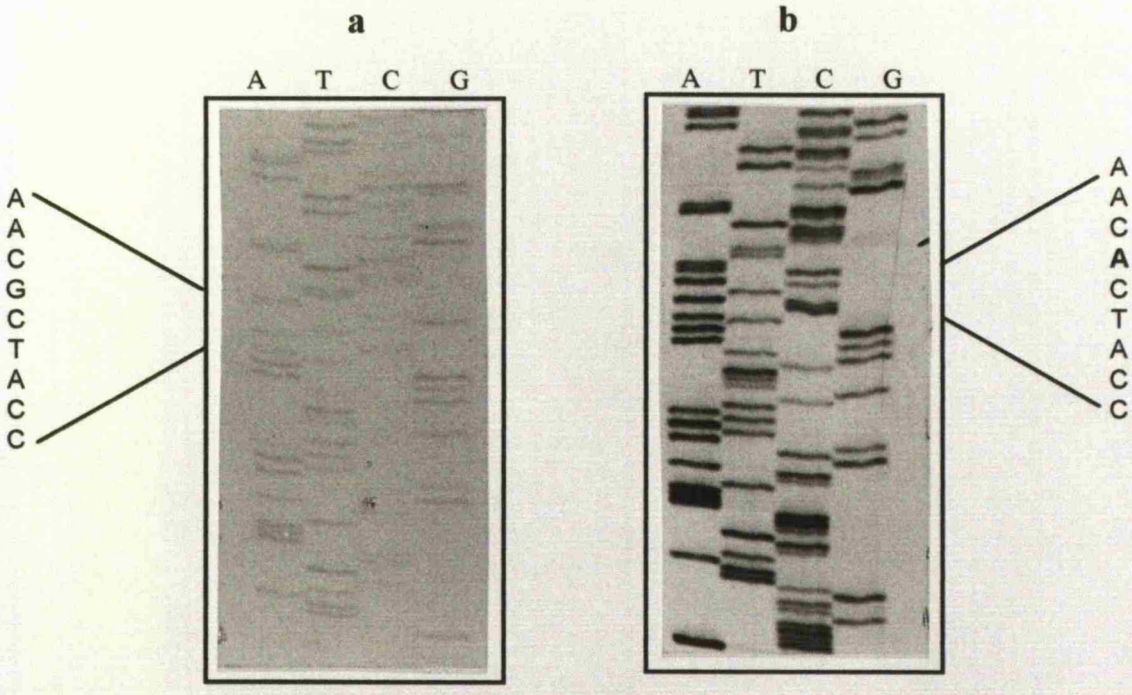


Fig. 3.15A. Sequence analysis of IS*4351*$_R$ amplified with *ULTma* polymerase. Sequence **a** represents the wild-type IS*4351*$_R$ sequence. Sequence **b** represents the IS*4351*$_R$ element amplified with *ULTma* polymerase. The G to A base substitution at nucleotide 675 in sequence **b** is indicated in bold-type to the right of the sequence and can be compared to the wild-type sequence indicated to the left of sequence **a**.

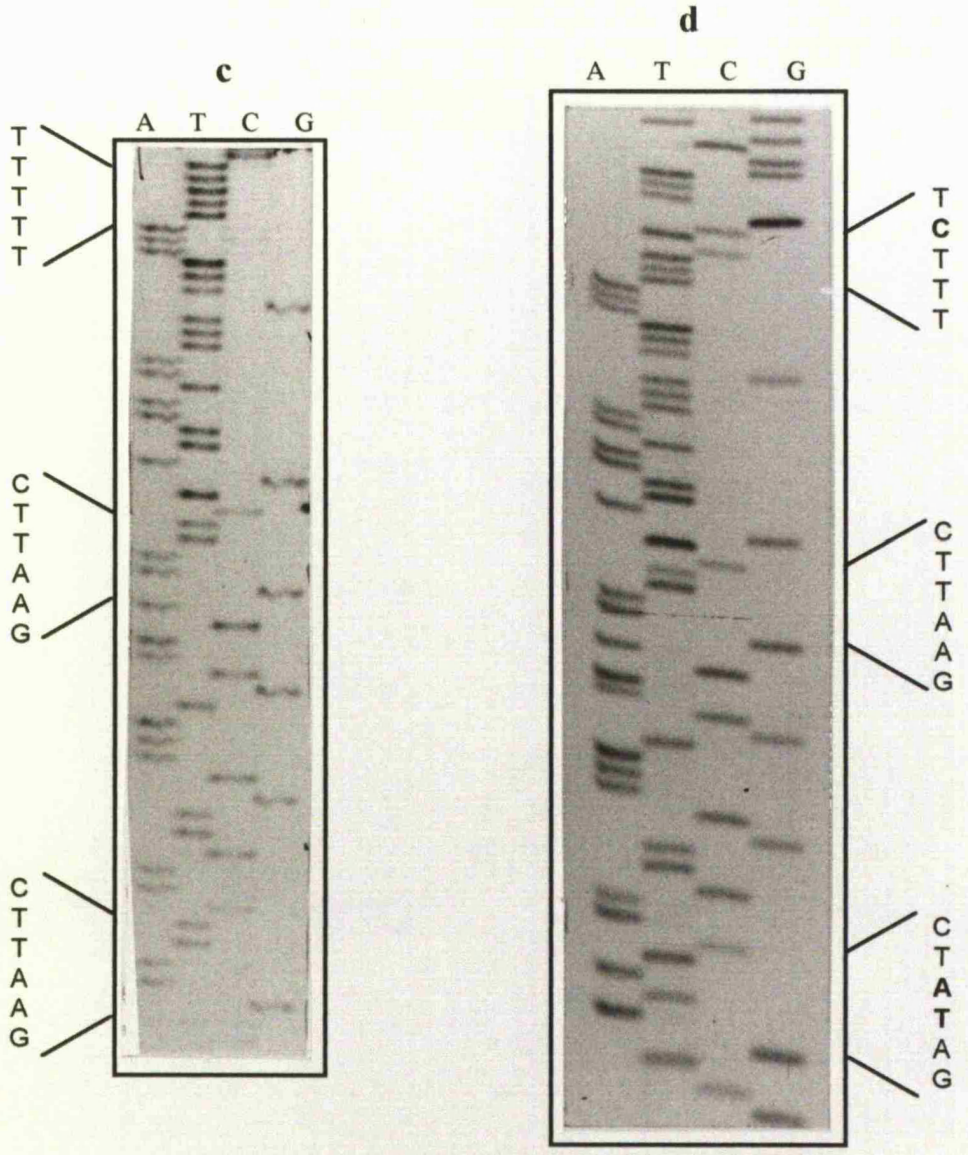


Fig. 3.15B. Sequence analysis of IS*4351*$_R$ amplified with *ULTma* polymerase. Sequence **c** represents the wild-type IS*4351*$_R$ sequence. Sequence **d** represents the IS*4351*$_R$ element amplified with *ULTma* polymerase. The T to C base substitution at nucleotide 56 in sequence **d** is indicated in bold-type to the right of the sequence. The AATT to ATAT base substitutions in sequence **d** resulting in destruction of the first *Eco*RI restriction enzyme site are shown to the right of the sequence. The remaining *Eco*RI restriction enzyme site (GAATTC) is also indicated to the right of the sequence. The base substitutions in the amplified sequence can be compared to the wild-type sequence shown to the left of sequence **c**.

A large scale plasmid extraction of the cloning vector pAO2 was digested with *Bam*HI and *Sph*I restriction enzymes which removed a 190 bp fragment of DNA from the vector. The sequenced insertion element, IS*4351*$_R$*, was then removed from M13mp19 on a *Bam*HI/*Sph*I fragment and sub-cloned into pAO2. The *Bam*HI and *Sph*I restriction enzyme sites in pAO2 lie within the tetracycline resistance gene therefore, clones harbouring pAO2/IS*4351*$_R$* recombinants were tetracycline sensitive due to disruption of the resistance gene. The ligation products were transformed into the *E. coli* strain LE392 and transformants were selected for on LA supplemented with Ap. These transformants were then plated in duplicate onto LA supplemented with Ap and Tt and onto medium supplemented with Ap only. Colonies that could not grow on medium supplemented with tetracycline were isolated from the LA Ap plates and restriction enzyme analysis of small scale plasmid DNA extractions from these were used to confirm the presence of an IS*4351*$_R$* insert within pAO2. The resulting vector was termed pAO3 (fig. 3.16) and restriction enzyme analysis of pAO3 confirmed the presence of unique restriction enzyme sites for *Eco*RI , *Ava*I, *Hin*dIII and *Bam*HI. Cleavage of the plasmid DNA with these restriction enzymes results in a single fragment of 5.89 kb (fig 3.17). The restriction enzymes *Hinc*II and *Eco*RV cleave twice in pAO3 resulting in fragments of 2.2 kb and 3.69 kb and 5.68 kb and 210 bp respectively (fig. 3.17).

3.3.3 Stage 3: Amplification of the Reporter Gene, *catIII*

When considering which reporter gene could be used as a *Porphyromonas* promoter probe two criteria had to be fulfilled. The first was that the reporter gene must be a gene that can be expressed after transfer into *P. gingivalis* and which is not normally expressed by *P. gingivalis*. The second criterion when selecting the reporter gene was that the gene sequence must not contain any *Ava*I restriction enzyme sites as this restriction enzyme site is required to be unique in IS*4351*$_R$* in order that the entire transposon can be recreated. Similarly, the reporter gene sequence was to be cloned into

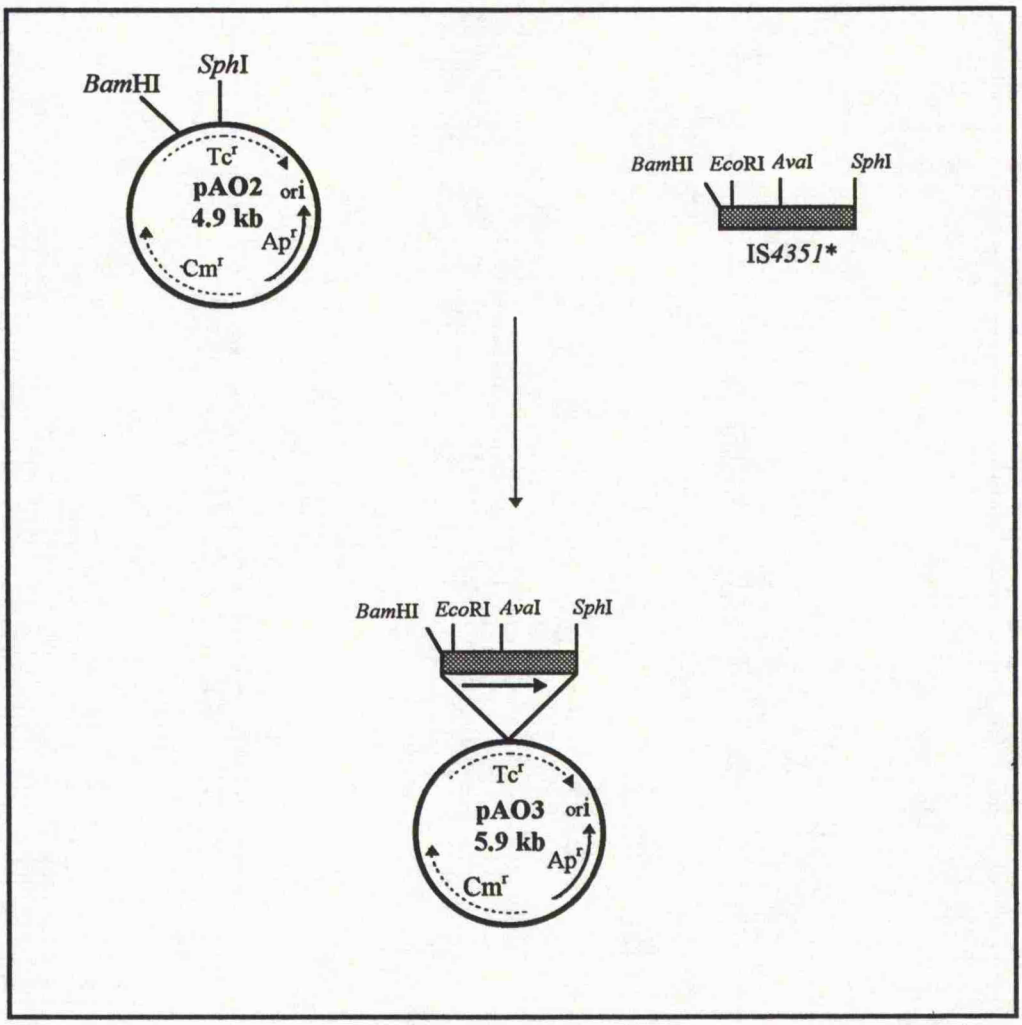


Fig. 3.16. Construction of plasmid pAO3. The amplified insertion element, IS*4351*$_R$* was removed from M13mp19 on a *Bam*HI/*Sph*I fragment. The cloning vector pAO2 was also digested with *Bam*HI and *Sph*I restriction enzymes. IS*4351*$_R$* was cloned into pAO2 creating the 5.9 kb plasmid pAO3. The pBR328 ampicillin resistance determinant (Ap^r) and the origin of replication (ori) are indicated on the diagram. The hashed line arrows represent the disrupted (non-functional) tetracycline (Tc^r) and chloramphenicol (Cm^r) resistance determinants. The solid arrow represents the direction of IS*4351*$_R$ * in pAO3.

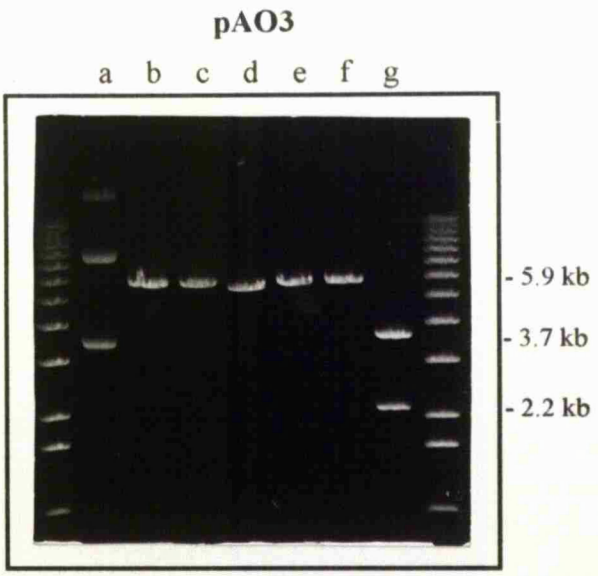


Fig. 3.17. Restriction enzyme digestion analysis of plasmid pAO3. The DNA was cleaved with restriction enzymes and separated by gel electrophoresis on a 0.7 % agarose gel. Kilo base ladder is run either side of the plasmid DNA. The letters a - g represent pAO3 plasmid DNA digested with the following restriction enzymes a = uncut DNA; b = *Eco*RI; c = *Ava*I; d = *Eco*RV; e = *Hin*dIII; f = *Bam*HI; g = *Hin*cII. The restriction enzymes *Eco*RI, *Ava*I, *Hind*III and *Bam*HI each cleave pAO3 once resulting in a single fragment of 5.89 kb. The restriction enzymes *Eco*RV and *Hinc*II each cleave pAO3 twice resulting in two fragments of 5.68 kb and 0.21 kb (this fragment can not be detected on the 0.7 % agarose gel), and 3.69 kb and 2.2 kb respectively

an *Eco*RI restriction enzyme site within pAO3 therefore, the reporter gene must not contain any internal *Eco*RI restriction enzyme sites.

The reporter gene of choice was a type III chloramphenicol acetyltransferase (*catIII*) contained on the pUC18 based plasmid pUC18:IM3 (Murray *et al.*, 1988). The *catIII* reporter gene fulfilled both criteria stated. In the first instance *P. gingivalis* strains W83, 33277 and 381 were found not to exhibit resistance to chloramphenicol. Bacteria were streaked in duplicate onto blood agar plates supplemented with 25, 10, 5, 2 or 0 μg/ml chloramphenicol and the plates were left anaerobically for up to 10 days. All three *P. gingivalis* strains tested only grew on medium that had not been supplemented with chloramphenicol. The *E. coli* strain LE392 that had been transformed with pBR328 which encodes a gene that confers resistance to chloramphenicol was used as a control. LE392 was streaked in duplicate onto LA supplemented with 25, 10, 5, 2 or 0 μg/ml chloramphenicol and the plates were left anaerobically overnight. Bacteria were found to be able to grow at all concentrations of chloramphenicol tested. Secondly the *catIII* gene sequence does not contain any *Ava*I or *Eco*RI restriction sites. This means that the *catIII* gene can be cloned into the unique *Eco*RI restriction enzyme site within IS*4351*$_R$* and then the entire transposon can subsequently be recreated by cloning the 3.8 kb *Ava*I fragment from Tn*4351* into the unique *Ava*I restriction enzyme site within IS*4351*$_R$*.

A diagrammatic representation of the amplification and cloning procedures undertaken at this stage is depicted in figure 3.18. The reporter gene was to be amplified by PCR using primers designed to introduce *Eco*RI restriction enzyme sites into either end of the gene. The reporter gene can then be cloned into the unique *Eco*RI restriction enzyme site within IS*4351*$_R$*. The entire nucleotide sequence and the predicted open reading frame of *catIII* is shown in figure 3.19 and the PCR primers, SAO3 and SAO4, used to amplify the reporter gene are also indicated on the diagram. The addition of arbitrary bases before the restriction enzyme site ensures efficient cleavage of the PCR product with *Eco*RI restriction enzyme. The open reading frame of *catIII* was amplified from pUC18:IM3 by PCR under the following conditions, 95°C for 1 minute denaturing,

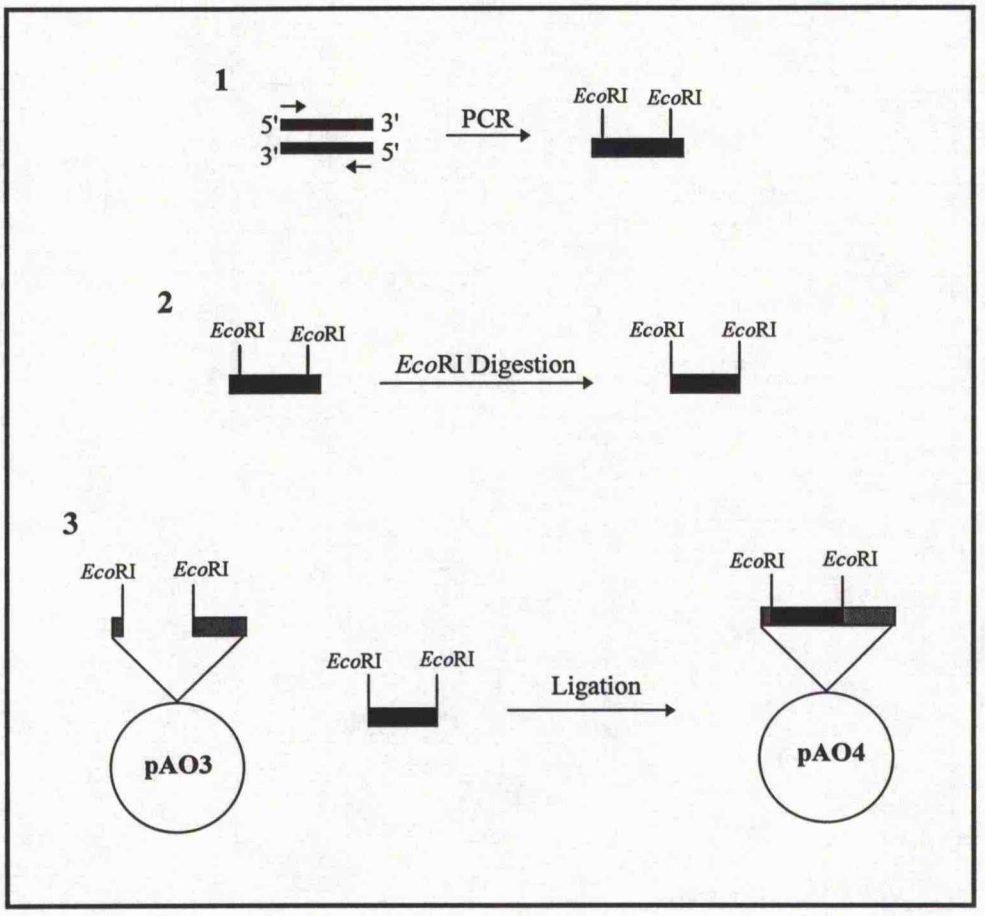


Fig. 3.18. Summary of the strategy involved in the amplification and subsequent subcloning of the reporter gene. The *catIII* reporter gene is amplified by PCR using primers designed to introduce *Eco*RI restriction enzyme sites into the ends of the reporter gene sequence (Step 1). The *catIII* PCR product can then be digested with *Eco*RI restriction enzyme resulting in *Eco*RI sticky-ends (Step 2). Finally the *catIII* sequence can be cloned into *Eco*RI digested pAO3 creating the plasmid pAO4 (Step 3).

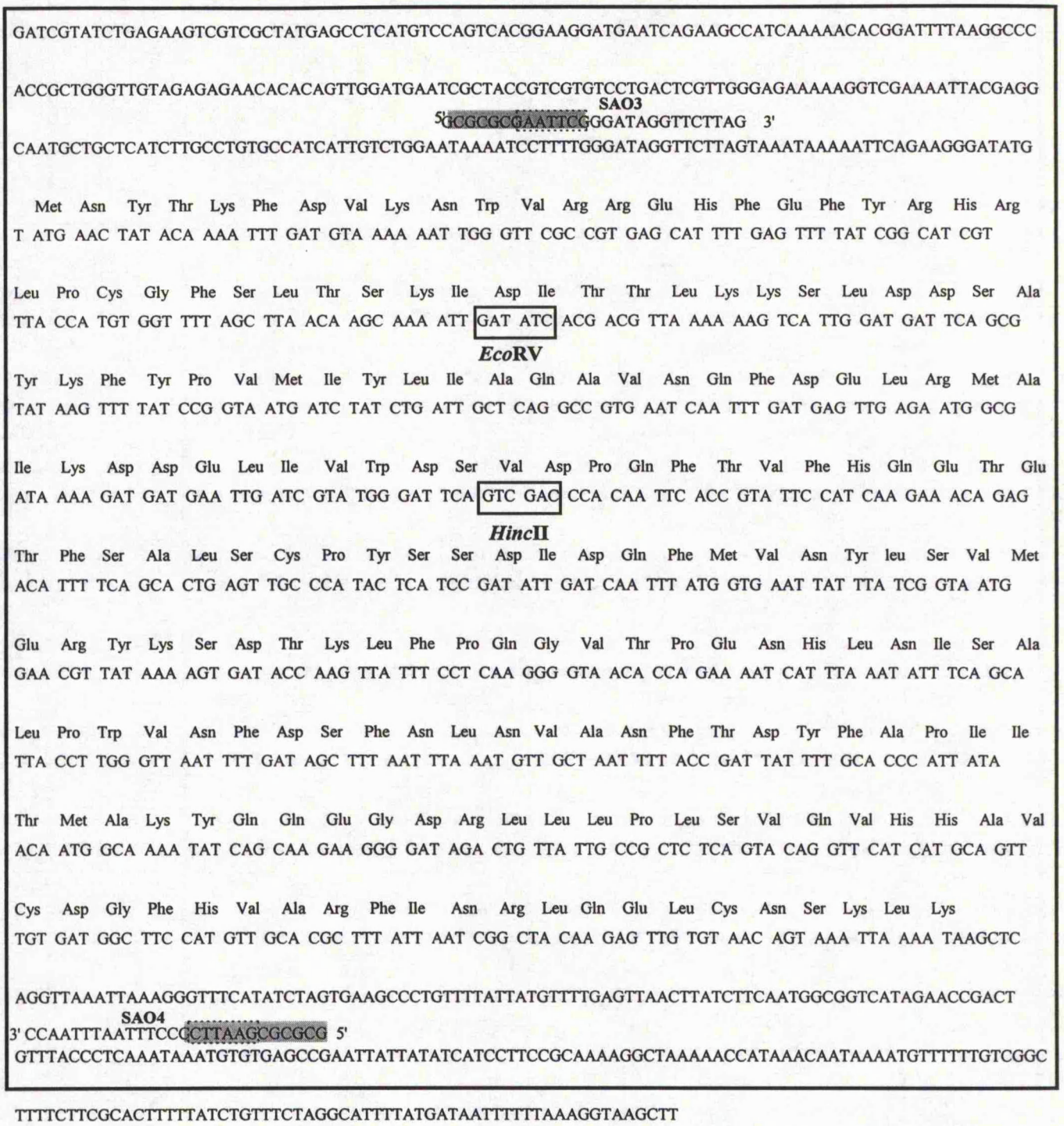


Fig. 3.19. Nucleotide sequence and predicted open reading frame of *catIII* (Murray *et al.*, 1988).

Sequence shown 5' → 3'. SAO3 and SAO4 represent the primers used in the PCR reaction to amplify the reporter gene. Shaded sequences within the primer represent non *catIII* sequences. Both primers have *Eco*RI restriction enzyme sites incorporated into the sequence which can be identified within the hashed line box and a GCGCGC sequence upstream of the *Eco*RI restriction enzyme site to allow efficient cleavage of the PCR product with the restriction enzyme. Unique restriction enzyme sites within the *catIII* sequence are indicated below the sequence.

56°C for 1 minute annealing and 72°C for 1 minute extension, for 30 cycles using *Taq* DNA polymerase. The resulting PCR product was visualised by gel electrophoresis on a 0.7% agarose gel (figure 3.20). Control PCR reactions were carried out using the PCR reaction mix minus the primers and the PCR reaction mix minus the template DNA. As expected no PCR amplification was seen for these reactions (figure 3.20). The amplified PCR product was purified by phenol/chloroform extraction to remove surplus *Taq* DNA polymerase or dNTP's and a reaction was carried out to cleave the *Eco*RI restriction enzymes sites at the 5' and 3' ends of the PCR *catIII* product. The plasmid pUC18 was also digested with *Eco*RI restriction enzyme which cleaves once in the polylinker of this cloning vector. A sticky-end ligation was carried out between approximately 0.5 μg *Eco*RI digested pUC18 and 0.5μg *catIII* PCR product and ligation products were transformed into LE392. The polylinker of pUC18 lies within the *lacZ* gene and so *catIII* inserts within the *Eco*RI restriction enzyme site of pUC18 can be screened for as white colonies on LA plates supplemented with Ap, X-gal and IPTG. Fourteen white colonies out of a total of 126 transformants were selected and plated onto LA supplemented with Ap, Cm (25 μg/ml) and 0.1 mM IPTG. If the PCR product is cloned into the polylinker site in the correct orientation relative to the promoter region of the *lacZ* gene then the *catIII* gene should be expressed resulting in clones that will be resistant to chloramphenicol. Four of the fourteen colonies were able to grow on LA supplemented with Cm, Ap and IPTG.

Confirmation of the *catIII* insert in pUC18 was achieved by restriction enzyme analysis of small scale extracted plasmid DNA and the resulting chloramphenicol resistant plasmid was termed pECAT (figure 3.21). The plasmid pUC18 has unique restriction enzyme sites for *Eco*RI, *Ava*I, *Hin*dIII, *Bam*HI and *Hin*cII which cleave the plasmid DNA once generating a single linear fragment of 2.686 kb (fig. 3.22). Plasmid pECAT which contains the 710 bp *catIII* insert has unique restriction enzyme sites for *Ava*I, *Hin*dIII and *Bam*HI restriction enzymes and these cleave the DNA once generating a single fragment of 3.396 kb whilst the restriction enzyme *Hin*cII cleaves pECAT twice, once in pUC18 sequences and once within the *catIII* insert, generating two fragments of 2.951 kb and 445 bp. Digestion of pECAT with *Eco*RI restriction enzyme demonstrates

Fig. 3.20. Amplified *catIII*. The plasmid pUC18:IM3 was used as a template for a PCR reaction, using primers SAO3 and SAO4, to amplify the reporter gene *catIII*. The resulting 0.71 kb PCR product was visualised on a 0.7 % agarose gel (lanes 1, 2 and 3). Two negative controls were also carried out. The first control (lane 4) was carried out using the PCR reaction mix minus the primers. The second control (lane 5) was carried out using the PCR reaction mix minus template DNA. Kilo base (kb) ladder is run either side of the PCR products.

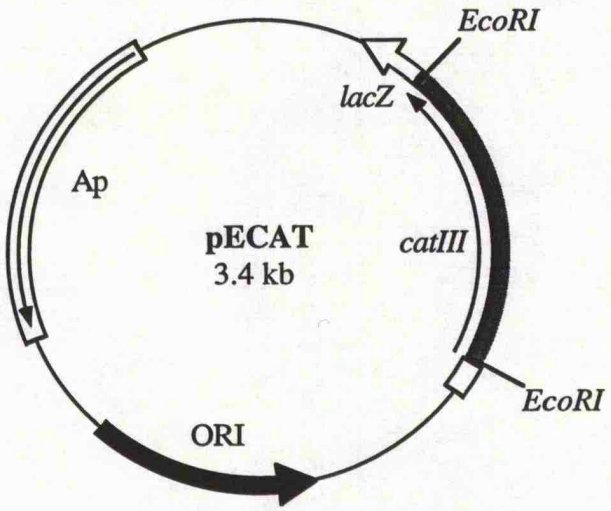


Fig. 3.21. Map of plasmid pECAT. The plasmid was constructed by inserting a 710 bp *Eco*RI fragment containing the amplified reporter gene, *catIII,* into the unique *Eco*RI restriction enzyme site within the polylinker of vector pUC18. The positions of the ampicillin resistance gene (Ap), the origin of replication (ORI) and the *lacZ* gene are indicated on the map.

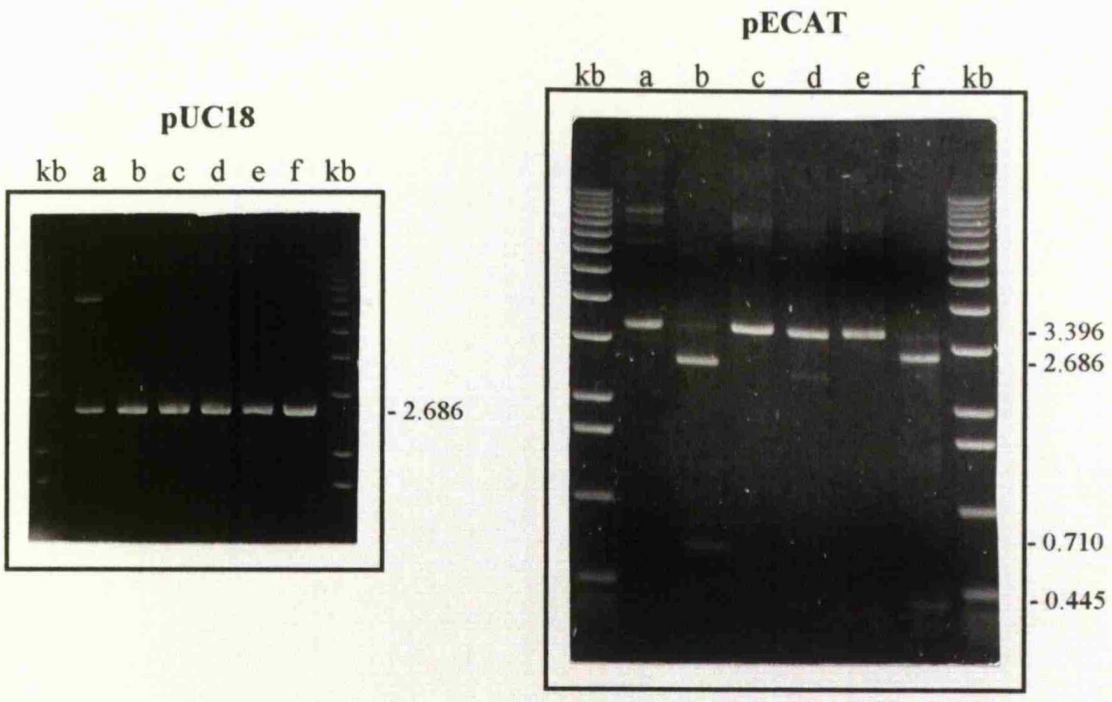


Fig. 3.22. The restriction profiles of plasmids pUC18 and pECAT. The DNA was cleaved with restriction enzymes and separated by gel electrophoresis on 0.7 % agarose gels. Kilo base ladder is run either side of the DNA digests. The letters a - f represent pUC18 and pECAT plasmid DNA digested with the following restriction enzymes a = uncut DNA; b = *Eco*RI; c = *Ava*I; d = *Hind*III; e = *Bam*HI; f = *Hinc*II. Size markers are indicated to the right hand side of the photographs. Plasmid pUC18 is cleaved once by each of the restriction enzymes tested resulting in a single fragment of 2.686 kb. Restriction enzymes *Ava*I, *Hind*III and *Bam*HI each cleave pECAT once resulting in a single fragment of 3.396 kb. The restriction enzymes *Eco*RI and *Hinc*II each cleave pECAT twice resulting in fragments of 2.686 kb and 0.71 kb and 2.951 kb and 0.445 kb respectively.

the 710 bp fragment corresponding to the *catIII* insert and the 2.686 fragment corresponding to pUC18 (fig. 3.22).

The *catIII* sequence was then cloned into IS*4351*$_R$* within pAO3. A large scale plasmid extraction of pECAT DNA was digested with *Eco*RI restriction enzyme and the 710 bp fragment encoding the *catIII* gene was purified from a preparative agarose gel as described in Materials and Methods. A large scale plasmid extraction of pAO3 was also digested with *Eco*RI restriction enzyme which cuts once within the IS4351$_R$* insert. The DNA was then purified from an agarose gel and treated with calf alkaline phosphatase to remove the 5' phosphate groups from the vector to prevent vector self-ligation. The 710 bp fragment from pECAT was then ligated with calf alkaline phosphatase treated pAO3 and this was transformed into the *E. coli* strain LE392. LE392 transformed with self-ligated calf alkaline phosphatase treated pAO3 generated approximately 20 transformants per plate. LE392 transformed with calf alkaline phosphatased treated pAO3 ligated with the 710 bp *catIII* fragment generated between 60-70 transformants per plate. Cleavage of DNA extracted from the transformants with *Eco*RI restriction enzyme analysis confirmed the presence of the 710 bp *catIII* insert in pAO3. Restriction enzyme analysis using *Eco*RV digestion of the plasmid DNA was used to determine the orientation of the *catIII* insert and plasmid pAO3 containing a *catIII* insert in the correct orientation was termed pAO4 (fig. 3.23). The *catIII* insert had to be orientated such that the 5' end of the gene was nearest to the *insR* sequence of IS*4351*$_R$ so that transposition of the promoter probe downstream of *P. gingivalis* promoter sequences would result in transcription of the reporter gene. When primer SAO1 was designed a two base pair substitution of the sequence was used to destroy the *Eco*RI restriction enzyme site (fig. 3.9). The substitution, however, generated an *Eco*RV restriction enzyme site which was essential for the determination of the orientation of the reporter gene within the clone. Insertion of *catIII* in the correct orientation in pAO3 results in fragments of 6.217 kb, 210 bp and 173 bp after cleavage of the plasmid DNA with *Eco*RV restriction enzyme. If *catIII* inserts in the opposite (incorrect) orientation in pAO3, then cleavage of the plasmid DNA with *Eco*RV restriction enzyme results in fragments of 5.811 kb, 579 bp and 210 bp. Of 15

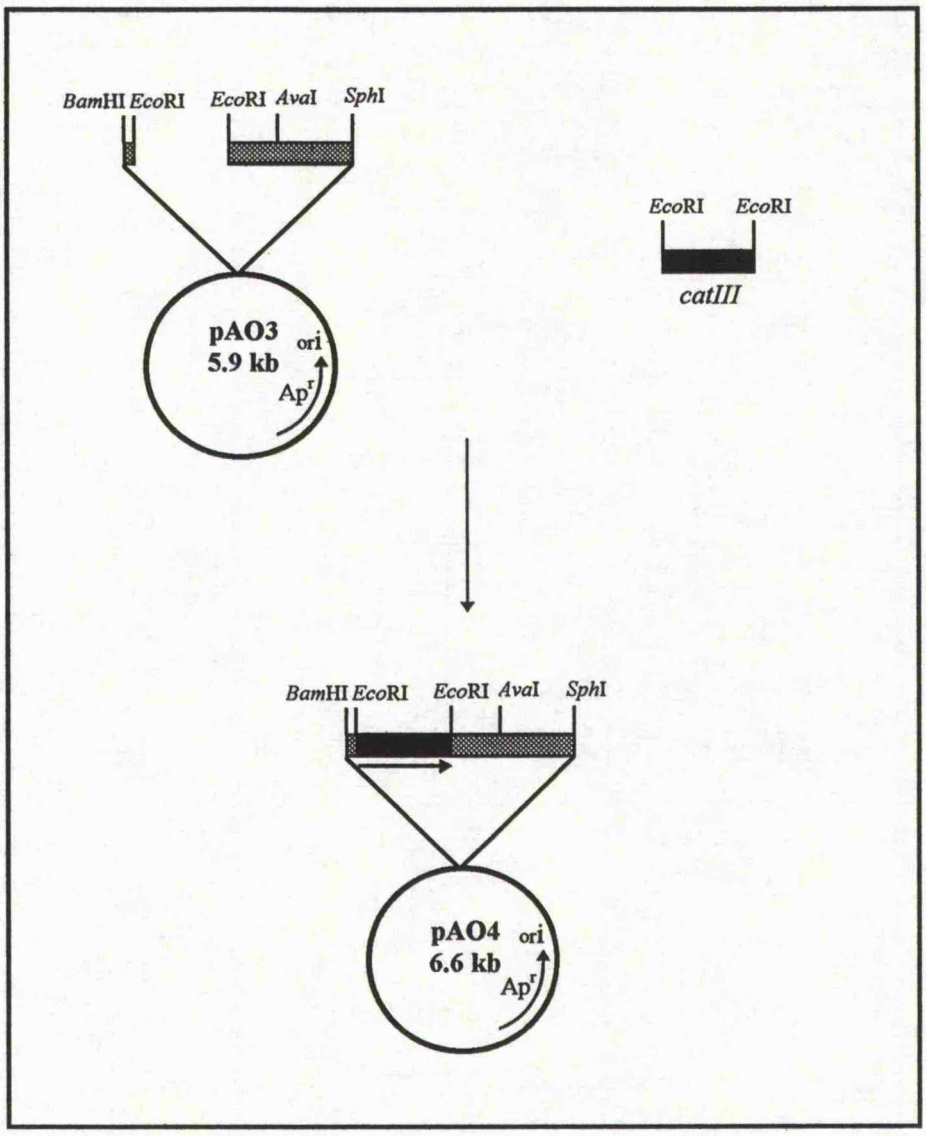


Fig. 3.23. Construction of plasmid pAO4. The *catIII* reporter gene was amplified by PCR using primers designed to introduce *Eco*RI restriction enzyme sites in to either end of the gene. After the amplified *catIII* was cleaved with *Eco*RI it could be cloned into the unique *Eco*RI restriction enzyme site of pAO3 resulting in the 6.6 kb plasmid pAO4. The solid arrow underneath *catIII* donates the direction of the reporter gene sequence

transformants analysed by restriction enzyme analysis, 5 transformants contained an incorrect insertion of *catIII* and 10 transformants contained *catIII* in the correct orientation and were designated pAO4. The restriction enzyme analysis of pAO4 is shown in figure 3.24. The restriction enzymes *Ava*I, *Hin*dIII and *Bam*HI cleave pAO4 once generating a single fragment of 6.60 kb. The restriction enzyme *Eco*RI cleaves pAO4 twice generating two fragments of 710 bp which corresponds to the *catIII* insert and 5.89 kb which corresponds to pAO3. Three recognition sites for *Hin*cII restriction enzyme are present in pAO4, two in pAO3 sequences and one in *catIII* sequences, cleavage of pAO4 with this restriction enzyme generates three fragments of 1.634 kb, 2.2 kb and 2.766 kb (data not shown). Three *Eco*RV restriction enzyme sites are also present in pAO4 one is found in the *catIII* sequence, one is found in IS*4351*$_R$* and the third is present in the pBR328 sequences. Cleavage of pAO4 with *Eco*RV restriction enzyme yields three fragments of 6.217 kb, 210 bp and 173 bp (fig. 3.24). Plasmid pAO4 demonstrated resistance to 25 μg/ml Cm and this was thought to be due to the orientation of the *catIII* gene relative to the tetracycline resistance gene promoter.

3.3.4 Stage 4: Recreation of the Entire Transposon

To recreate the entire transposon a 3.8 kb *Ava*I fragment from Tn*4351* was to be cloned into the unique *Ava*I restriction enzyme site in pAO4 to generate the putative transposon transcription probe Tn*4351*::*catIII*. The 3.8 kb *Ava*I fragment can also be cloned into the unique restriction enzyme site in pAO3 generating Tn*4351** which has an altered *Eco*RI restriction enzyme site and a mutation at nucleotide 675 in IS*4351*$_L$*.

Plasmid pVOH1, which contains a copy of Tn*4351*, was digested with *Ava*I restriction enzyme and the 3.8 kb fragment was purified from a preparative gel as described in the Materials and Methods. Vectors pAO3 and pAO4 were also digested with *Ava*I restriction enzyme which cleaves the DNA once within IS*4351*$_R$* and the linearised single fragment was purified from an agarose gel. Calf alkaline phosphatase was used to remove the 5' phosphate groups from the vectors to prevent vector self-ligation. A sticky-end ligation was carried out between the *Ava*I digested plasmids pAO3 and pAO4

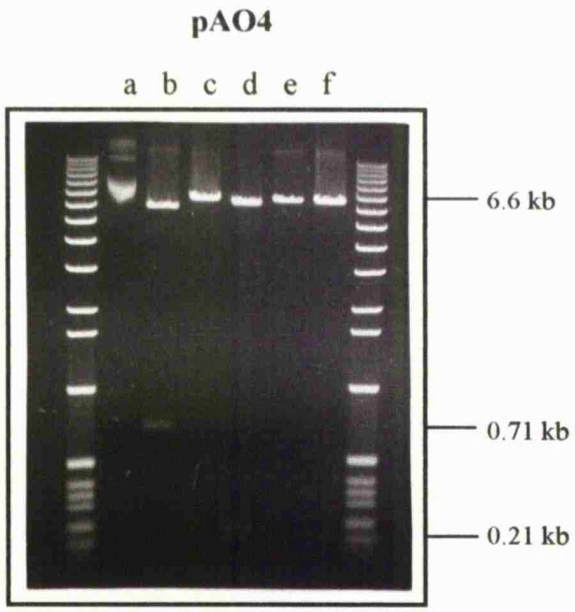


Fig. 3.24. Restriction enzyme digestion analysis of plasmid pAO4. The DNA was cleaved with restriction enzymes and separated by gel electrophoresis on a 0.7 % agarose gel. Kilo base (kb) ladder is run either side of the plasmid DNA. The letters a - f represent pAO4 plasmid DNA digested with the following restriction enzymes a = uncut DNA; b = *Eco*RI; c = *Ava*I; d = *Eco*RV; e = *Hin*dIII; f = *Bam*HI. The restriction enzymes *Ava*I, *Hind*III and *Bam*HI each cleave pAO4 once resulting in a single fragment of 6.6 kb. The restriction enzyme *Eco*RI cleaves pAO4 twice resulting in two fragments of 5.89 kb and 0.71 kb and *Eco*RV cleaves pAO4 three times resulting in fragments of 6.22 kb, 0.21 kb and 0.173 kb (the 0.21 kb fragment can be seen on the photograph above however, the 0.173 kb fragment can not be visualised). Size markers in kb are indicated to the right hand side of the photograph.

and the 3.8 kb *Ava*I fragment. Ligation products were then transformed by electroporation into LE392 and plated onto LA supplemented with Ap and Tt. Only clones that had the 3.8 kb *Ava*I insert which contains *Tc^r, the tetracycline resistance determinant of Tn*4351* which confers resistance in aerobically grown bacteria and not in anaerobically grown bacteria, were able to grow on medium supplemented with Tc. Restriction mapping of small scale plasmid extractions was used to determine the orientation of the *Ava*I fragment within these clones (fig. 3.25 and Table 3.1). Vector pAO3 with an *Ava*I insert in the correct orientation to create the transposon Tn*4351** was termed vector pAO5 (figure 3.26) and plasmid pAO4 with an *Ava*I insert in the correct orientation to create the plasmid Tn*4351*::*catIII* was termed plasmid pAO6 (figure 3.27).

The plasmid containing the cloned Tn*4351* was unstable and frequently underwent deletions causing loss of the transposon. This had previously been observed by Hwa *et al.*, 1988. To try to reduce the frequency of recombination the plasmids pAO5 and pAO6 were transformed into a *recA*$^-$ *E. coli* strain so that homologous recombination between the direct repeats would be abolished. Strain S17-1 was chosen for two reasons. First it is *recA*$^-$ by virtue of a point mutation within the *RecA* gene. Secondly S17-1 contains an insertion of an IncP plasmid in its chromosome which expresses the transfer genes required to allow mobilisation of *P. gingivalis* suicide vectors from *E. coli* into *P. gingivalis.* However, because S17-1 is *recA*$^-$ due to a single point mutation it was possible that it could revert back to the *RecA*$^+$. To overcome this possibility a P1 phage transduction was carried out. The *E. coli* strain TG2, which has a deletion of the *recA* region, was infected with P1 lysate. The *recA*$^-$ region of TG2 is linked to a tetracycline resistance gene which can be used as a selective marker. S17-1 was infected with the TG2 lysate and plated onto LA supplemented with tetracycline. Any S17-1 colonies that could grow on LA plates supplemented with tetracycline were checked for their *recA* phenotype. To confirm this the *E. coli* strains LE392, TG2, S17-1 and S17-1* were streaked onto an LA plate and exposed to UV light for 0, 10, 20, 40 and 60 seconds. Only the *RecA*$^+$ strain LE392 was able to grow after exposure to UV light for 60 seconds the other strains TG2, S17-1 and S17-1* did not grow after 10

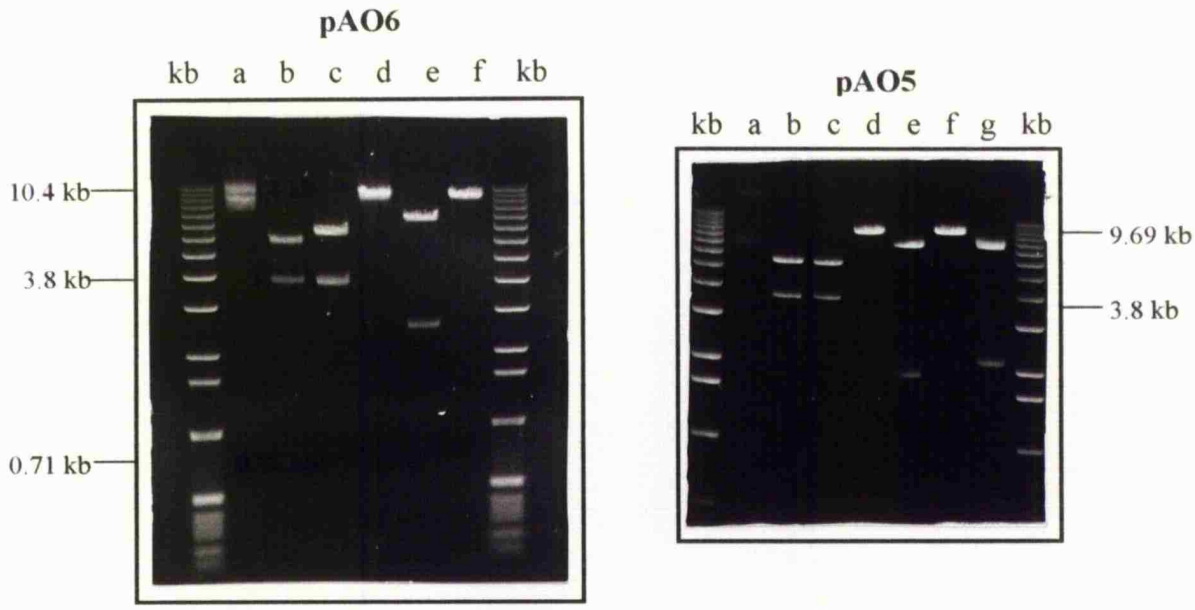


Fig. 3.25. Restriction enzyme analysis of plasmids pAO6 and pAO5. The DNA was cleaved with restriction enzymes and separated by gel electrophoresis on 0.7 % agarose gels. Kilo base (kb) ladder is run either side of the plasmid DNA. The letters a - f represent pAO6 and pAO5 plasmid DNA digested with the following restriction enzymes a = uncut DNA; b = *Eco*RI; c = *Ava*I; d = *Eco*RV; e = *Hin*dIII; f = *Bam*HI. Plasmid pAO5 was also digested with *Hinc*II (g). Size markers are indicated to either side of the two photographs.

	Size of fragments (kb) generated following cleavage of plasmid DNA with restriction endonucleases	
Restriction endonuclease	**pAO5**	**pAO6**
*Eco*RI	5.87, 3.8, 0.019	5.87, 3.8, 0.71, 0.019
*Ava*I	5.89, 3.8	6.6, 3.8
*Eco*RV	9.48, 0.21	10.02, 0.21, 0.173
*Hind*III	7.9, 1.79	7.9, 2.5
*Bam*HI	9.69	10.4
*Hinc*II	7.49, 2.2	—

Table 3.1. DNA fragments generated after cleavage of plasmids pAO5 or pAO6 with different restriction endonucleases.

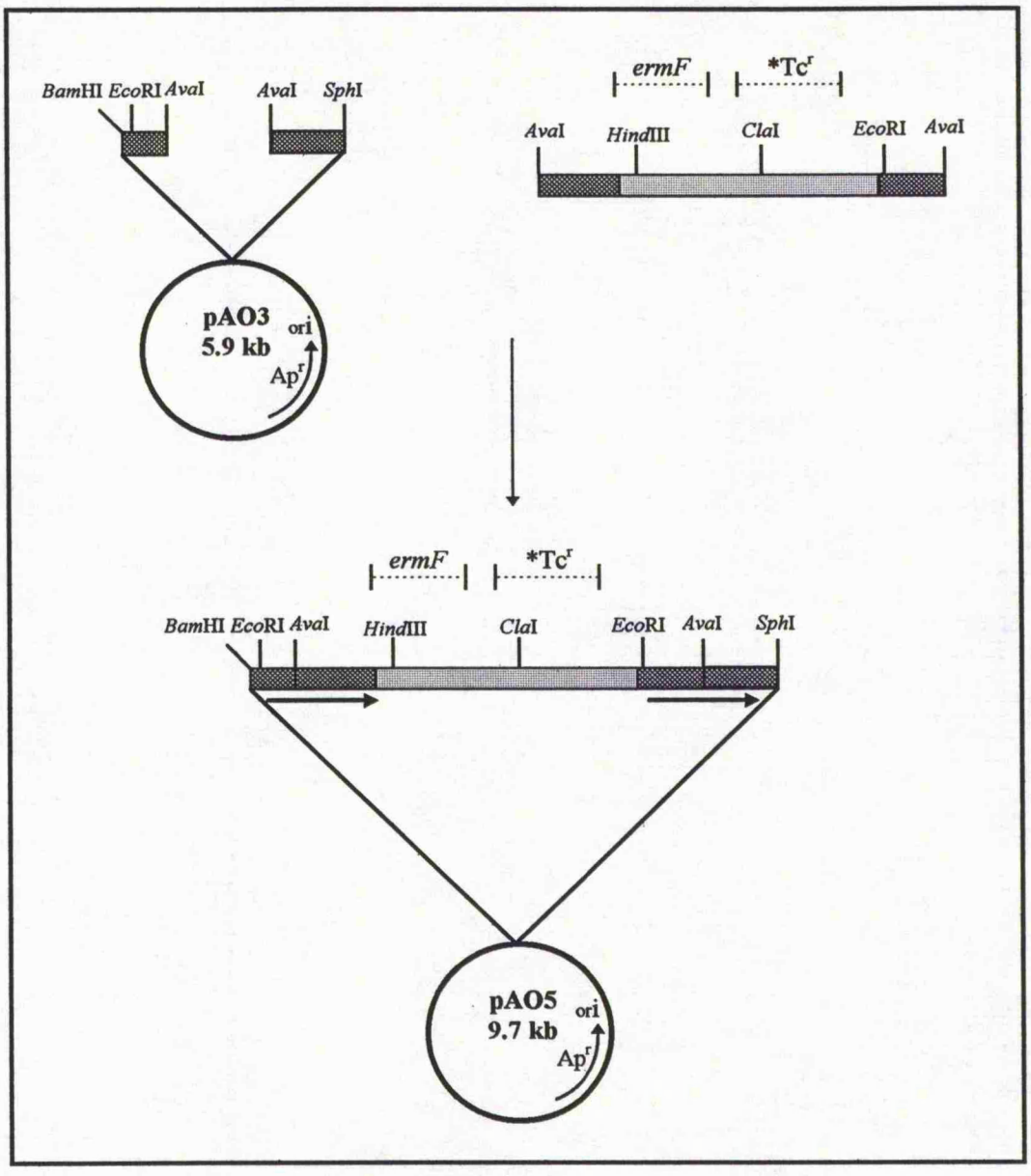


Fig. 3.26. Construction of plasmid pAO5. A 3.8 kb *Ava*I fragment from pVOH1 containing the Tn*4351 ermF* and *Tc^r resistance determinants as well as the last 600 bp of IS*4351*$_R$ and the first 500 bp of IS*4351*$_L$ was cloned into the unique *Ava*I restriction enzyme site of plasmid pAO3. The resulting plasmid was designated pAO5 and contains a complete copy of Tn*4351** The solid arrows underneath the transposon represents the direction of the insertion elements. The ampicillin resistance determinant (Ap^r) and the origin of replication (ori) are indicated on the diagram.

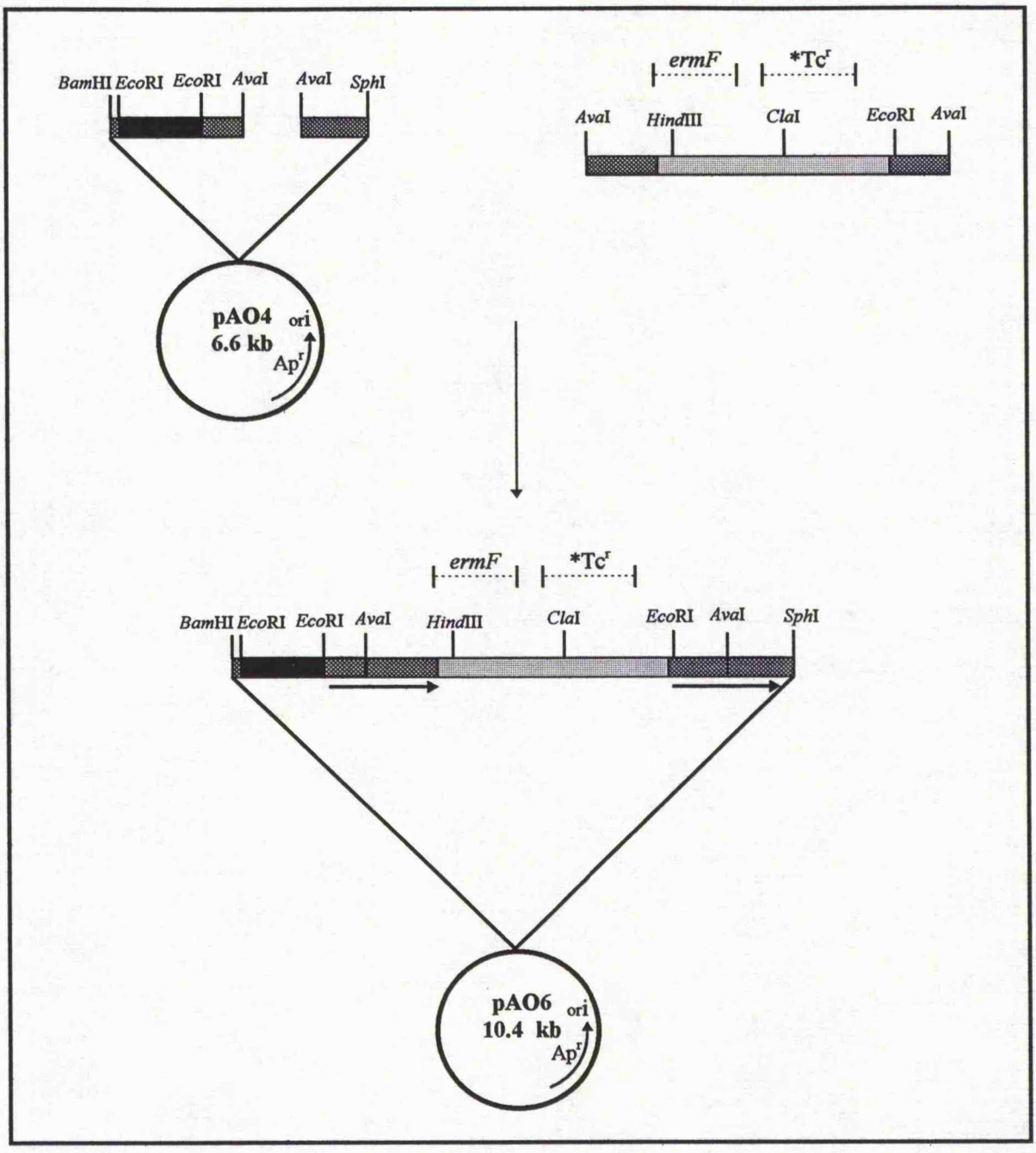


Fig. 3.27. Construction of plasmid pAO6. A 3.8 kb *Ava*I fragment from pVOH1 containing the Tn*4351 ermF* and *Tc^r resistance determinants as well as the last 600 bp of IS*4351*$_R$ and the first 500 bp of IS*4351*$_L$ was cloned into the unique *Ava*I restriction enzyme site of plasmid pAO4. The resulting plasmid was designated pAO6 and contains a complete copy of Tn*4351*::*catIII*. The solid arrows underneath the transposon represents the direction of the insertion elements. The ampicillin resistance determinant (Ap^r) and the origin of replication (ori) are indicated on the diagram.

seconds exposure to UV light and so confirmed the *recA*⁻ phenotype. The tetracycline resistant S17-1 colonies that could no longer revert to the wild-type RecA genotype were termed S17-1*. However, even after the insertion mutation in the *recA* gene in S17-1 to generate the *E. coli* strain S17-1*, pAO5 and pAO6 plasmid rearrangements were still observed.

3.3.5 Stage 5: Biological Assay

To determine whether the mutation at position 675, the altered *Eco*RI restriction enzyme site in Tn*4351* or the *catIII* insert had any effect on the activity of the manipulated transposon in terms of its ability to transpose, Tn*4351*::*catIII* and wild-type transposon, Tn*4351*, were assayed for cointegrate formation. The assay was carried out in *E. coli* and was based on an assay originally devised by Hwa *et al.*, 1988. The steps involved in the biological assay are shown in figure 3.28. An intermediary step of replicative transposition is the formation of cointegrates between the transposon and the recipient DNA. The plasmid pBR328 is a non-mobilisable plasmid and as such can not be transferred from one *E. coli* strain to another by conjugation. However, formation of a cointegrate between a conjugative plasmid and a pBR328 based plasmid carrying the transposon will be transferred between two strains. Cointegrate transconjugants can then be assayed by selecting for the antibiotic resistance markers found on the non-mobilisable plasmid pBR328 and the conjugative plasmid. In the first instance the plasmids pAO5, pAO6 or pVOH1, which all encode resistance to ampicillin, were transformed into a donor strain J08 which has a requirement for leucine when grown on minimal medium. A conjugative plasmid R388, which contains 33 kb and encodes resistance to trimethoprim, was then transformed into the same strain with either pAO5, pAO6 or pVOH1. R388 was used because it transfers between *E. coli* at high frequencies and contains no known IS elements (Avila and de la Cruz, 1988). The recipient strain LE392 was chosen as it has a requirement for tryptophan, methionine and thiamine when grown on minimal medium. Recipient and donor cells were mixed and transferred to nitrocellulose filters that had been placed onto the surface of LA plates. After incubation for 4 hours at 37°C the filters were washed in 1x M9 salts and 10-fold serial dilutions of the filter mix were carried out. Donor only cells were plated

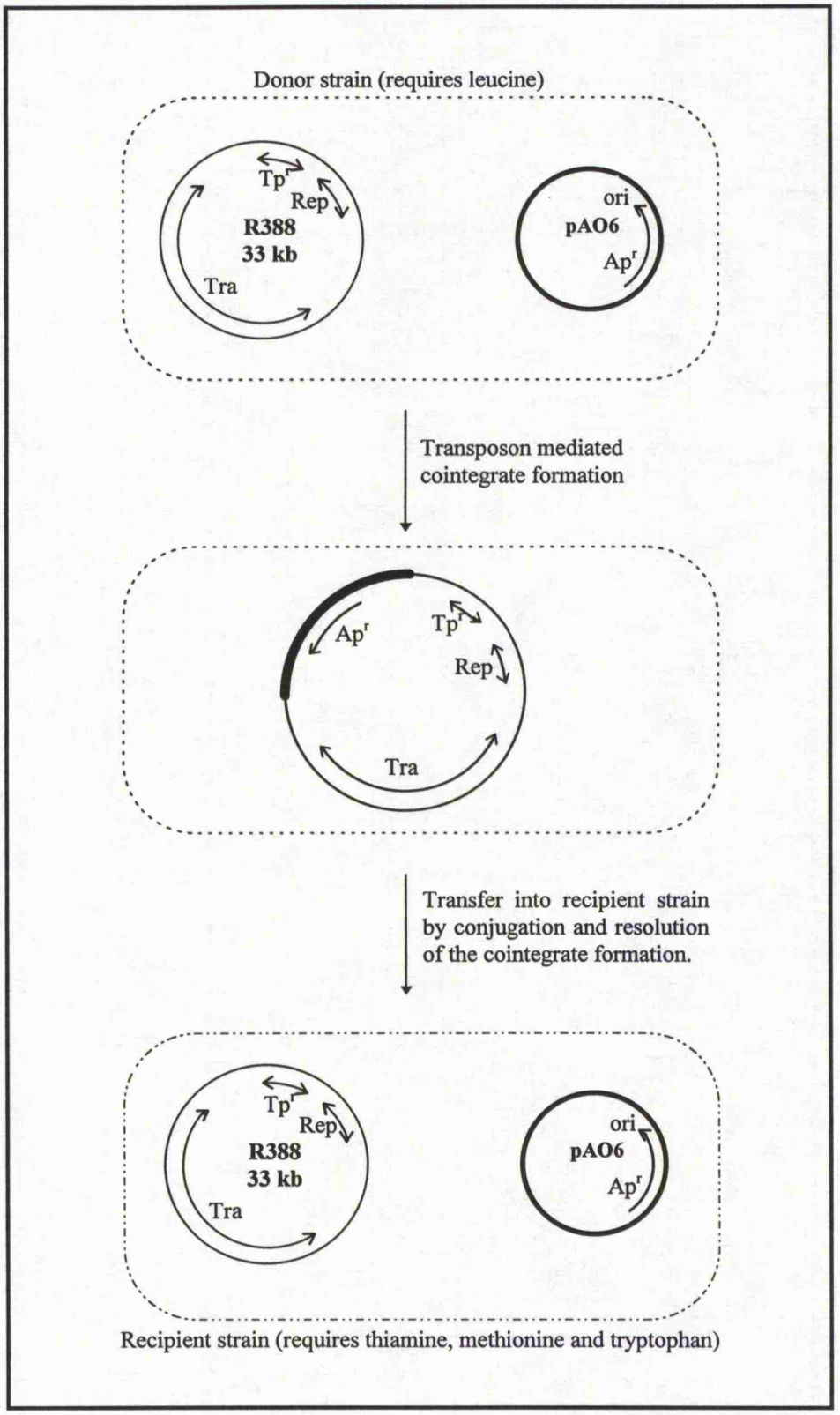


Fig. 3.28. Biological assay

Fig. 3.28. Biological assay for determining the activity of Tn*4351*::*catIII*. A filter mating is carried out between a donor strain (containing the non-mobilisable, pBR328 based plasmid, pAO6 which carries Tn*4351*::*catIII*, as well as a conjugative plasmid, such as R388) and a recipient strain that has different autotrophic requirements to the donor strain. Transposon mediated cointegrate formation between the two plasmids allows conjugation of both plasmids in to the recipient strain. Transfer of pAO6 can be detected by plating the recipient bacteria on to minimal medium containing thiamine, methionine and tryptophan and supplemented with ampicillin and trimethoprim. Ap^r and Tp^r represent the pBR328 ampicillin resistance determinant and the R388 trimethoprim resistance determinant respectively. The R388 and pAO6 origins of replication are indicated as Rep and ori respectively. Tra represents the R388 conjugal transfer region

in 10-fold serial dilutions onto LA plates supplemented with Ap and Tp whilst recipient cells were plated in 10-fold serial dilutions onto minimal medium plates supplemented with the amino acids tryptophan, methionine and thiamine. To select for transconjugants cells were plated onto minimal medium plates supplemented with the amino acids tryptophan, methionine and thiamine, which allows growth of LE392 recipient cells but does not allow the growth of J08 donor cells, and the antibiotics Ap and Tp, which selects for recipient cells that have received a cointegrate of R388 and the pBR328 based plasmids. Table 3.2 shows the frequency of transfer of the three plasmids. Plasmid pVOH1 contains a wild-type copy of Tn*4351* and so acts a control, pAO5 contains Tn*4351** and allows the efficiency of cointegrate formation (and thus transposition) of the manipulated Tn*4351* to be examined and pAO6 contains Tn*4351** with a *catIII* insert (Tn*4351*::*catIII*) and so allows the efficiency of cointegrate formation of the disrupted copy of Tn*4351* to be determined. The frequencies of transfer of all three plasmids, pVOH1, pAO5 and pAO6 (where the transfer frequency represents the number of CFU/ml of transconjugants per the number of CFU/ml of recipient cells), ranged from 2.8×10^{-4} to 7.65×10^{-6} and demonstrated that the efficiency of transfer for the plasmids containing a manipulated copy of Tn*4351* were comparable to that exhibited by pVOH1 containing the wild-type copy of Tn*4351*.

3.3.6 Stage 6: Development of a Suicide Vector

The shuttle vector pNJR12 (fig. 3.29) has been used in the genetic studies of *Bacteroides* species and *P. gingivalis* (Maley *et al.*, 1992; Stevens *et al.*, 1992). The vector is a chimeric vector composed of several components including a tetracycline resistance gene from the chromosomal element of *B. thetaiomicron* DOT which allows tetracycline selection in *Bacteroides* species and the *Bacteroides* plasmid pB8-51 which allows replication of the vector in *Bacteroides* species. The remainder of the shuttle vector contains the *Bacteroides* suicide vector pJRD215 (Davison *et al.*, 1987). This plasmid contains sequences, including a kanamycin resistance gene, from RSF1010 an IncQ plasmid that is able to replicate in most species of Gram-negative bacteria,

FREQUENCY OF TRANSFER

	pAO5/R388 (Ap^r/Tp^r)	pVOH1/R388 (Ap^r/Tp^r)
MATING 1	6.90×10^{-5}	2.10×10^{-4}
MATING 2	3.85×10^{-4}	ND
MATING 3	8.58×10^{-5}	4.30×10^{-4}
MATING 4	4.59×10^{-4}	6.50×10^{-5}
MATING 5	5.20×10^{-5}	9.49×10^{-5}

Table 3.2a

FREQUENCY OF TRANSFER

	pAO6/R388 (Ap^r/Tp^r)	pVOH1/R388 (Ap^r/Tp^r)
MATING 1	4.99×10^{-6}	7.65×10^{-6}
MATING 2	1.37×10^{-5}	4.58×10^{-5}
MATING 3	1.44×10^{-5}	3.70×10^{-5}
MATING 4	1.84×10^{-5}	3.94×10^{-5}
MATING 5	1.59×10^{-5}	4.30×10^{-4}
MATING 6	2.82×10^{-4}	6.50×10^{-5}

Table 3.2b

Table 3.2. Mobilisation frequencies of cointegrates formed between the non-mobilisable plasmids pVOH1, pAO5 and pAO6 with the conjugative plasmid R388. Frequency of transfer is expressed as number of transconjugants (cointegrates) per recipient. Table 3.2a represents the ability of plasmids containing Tn*4351** (pAO5) to form cointegrates compared to plasmids containing wild-type Tn*4351* (pVOH1). Table 3.2b represents the ability of plasmids containing Tn*4351*::*catIII* (pAO6) to form cointegrates compared to plasmids containing wild-type Tn*4351* (pVOH1). ND = not done.

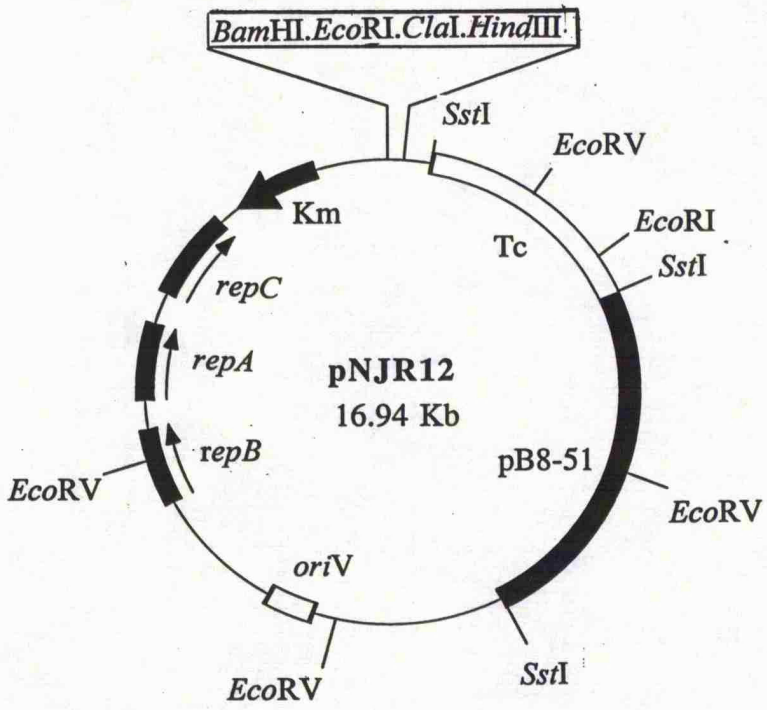


Fig. 3.29. Map of the chimeric *E. coli-Bacteroides* shuttle vector pNJR12. The vector contains the tetracycline DOT element (Tc) from *B. fragilis*. and the *Bacteroides* plasmid pB8-51 which allows selection and replication of pNJR12 within *Bacteroides* spp. The vector also contains a kanamycin resistance gene (Km) and the origin of replication (oriV) and *repA*, *repB* and *repC* genes from RSF1010 which allow for selection and replication of the vector within *E. coli*.

excluding *P. gingivalis* (Davison *et al.*, 1987), as well as a 432 bp polylinker cloning region that includes a multiple cloning site (MCS).

In order to generate a suicide vector the *Bacteroides* replicon region had to be removed from pNJR12 so that the vector could no longer replicate in *P. gingivalis*. A large scale plasmid extraction of the pNJR12 vector was digested with *Sst*I restriction enzyme generating three fragments. The 4.4 kb fragment contained the entire pB8-51 sequences, the 2.6 kb fragment contained the tetracycline resistance gene and the 10.2 kb fragment contained the wide-host range replicon and mobilisation functions of RSF1010 together with the kanamycin resistance gene and the MCS. The digested plasmid was then self-ligated to regenerate the 10.2 kb *Bacteroides* suicide vector pJRD215 (fig 3.30). Ligation products were transformed into LE392 and kanamycin resistant transformants selected. Small scale plasmid extractions were carried out on transformants so that restriction enzyme analysis could be used to detect clones that had lost the two smaller *Sst*I fragments. Because of the low copy number of pJRD215 (approximately 10 copies per cell) the small scale plasmid extraction technique was altered slightly to obtain maximum DNA yield for restriction analysis as described in section 2.3.2. Figure 3.31 shows the restriction analysis of pJRD215 digested with the restriction enzymes *Eco*RI, *Ava*I, *Hin*dIII, *Bam*HI and *Sst*I which each cleave the vector once resulting in a single fragment of 10.2 kb and with *Eco*RV which cleaves the vector twice resulting in two fragments of 7.46 kb and 2.74 kb. The removal of pB8-51 sequences results in a vector that is able to replicate and be selected for in *E. coli* but that is unable to replicate in *P. gingivalis* thus acting as a suicide vector.

In an attempt to increase the copy number of the suicide vector carrying Tn*4351*::*catIII*, the entire plasmid pAO6 was sub-cloned into pJRD215. This was achieved by digesting pAO6 and pJRD215 with *Bam*HI restriction enzyme. A sticky-end ligation was carried out between pAO6 and pJRD215 *Bam*HI digested plasmids and ligation products were subsequently transformed by electroporation into the *E. coli* strain S17-1*. Recombinants were selected for on LA supplemented with Sp, Tp and Tt, antibiotics that S17-1* is naturally resistant to, and Km and Ap to select for pAO6/pJRD215

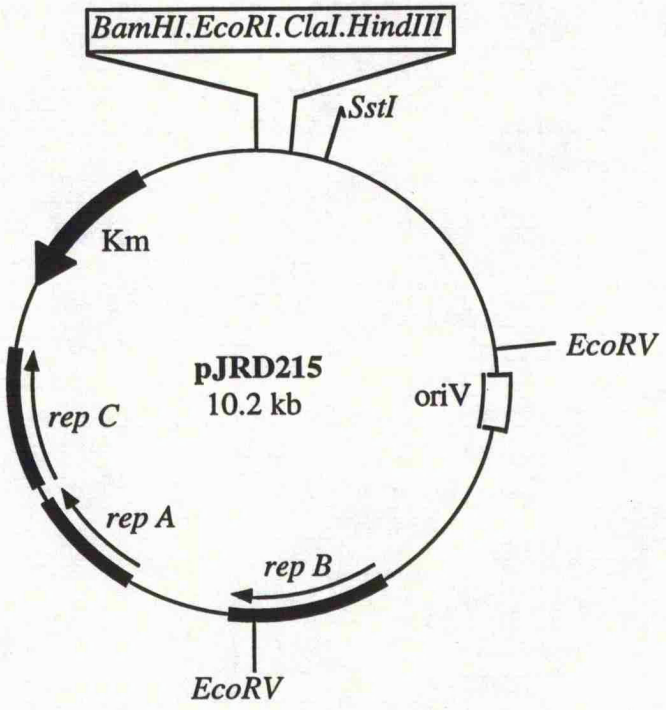


Fig. 3.30. Map of the *E. coli-Bacteroides* suicide vector pJRD215. The vector contains oriV and the genes *repA*, *repB* and *repC* from plasmid RSF1010 which allow pJRD215 to replicate in *E. coli*. The vector does not contain the appropriate genes to allow replication in *Bacteroides* spp. and as such acts as a suicide vector in this group of bacteria. The kanamycin resistance detrminant (Km) is recognised in *E. coli* but not in *Bacteroides* spp.

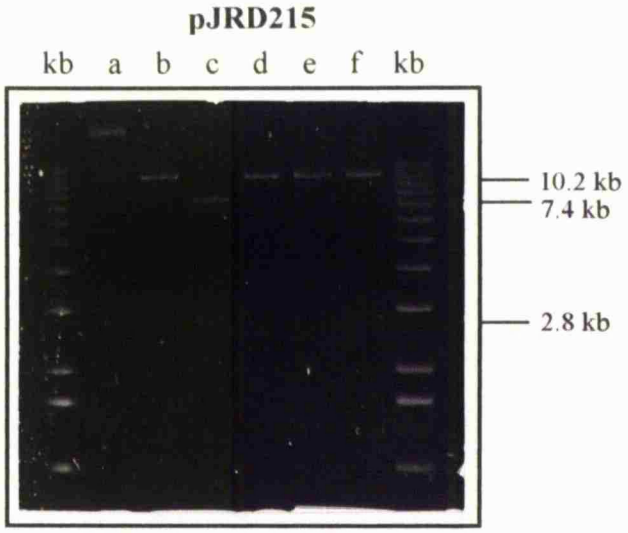


Fig. 3.31. Restriction enzyme analysis of suicide vector pJRD215. The DNA was cleaved with restriction enzymes and separated by gel electrophoresis on a 0.7 % agarose gel. Kilo base (kb) ladder is run either side of the digested plasmid DNA. The letters a - f correspond to plasmid DNA cleaved with the following restriction enzymes a = uncut DNA; b = *Eco*RI; c = *Eco*RV; d = *Hind*III; e = *Bam*HI; f = *Sst*I. The restriction enzymes each cleave the plasmid DNA once resulting in a single fragment of 10.2 kb, except for *Eco*RV which cleaves the plasmid twice resulting in two fragments of 7.4 kb and 2.8 kb. Size markers are indicated to the right hand side of the photograph

recombinants. Restriction mapping analysis was then used to confirm the presence of a pAO6/pJRD215 *Bam*HI fusion and to determine the orientation of the pAO6 insert. The resulting plasmids were termed pSA1 (fig. 3.32) and pSA2 (fig. 3.33) depending on the orientation of pAO6 in pJRD215. The restriction patterns of pSA1 and pSA2 and the size of fragments produced upon cleavage of the two vectors with different restriction enzymes are shown in figure 3.34 and Table 3.3.

Because the result of cloning pAO6 into pJRD215 is to increase the size of the suicide vector to twice its original size filter mating experiments were carried out in *E. coli* before the suicide vectors were transferred into *P. gingivalis* to confirm that the larger suicide vector could still be transferred. The *E. coli* strain S17-1*, which requires minimal medium supplemented with thiamine, tryptophan and methionine for growth, was used as the donor strain. Donor cells were mixed with the recipient strain W3110, which is prototrophic and requires minimal medium supplemented with thymine for growth, and were transferred onto nitrocellulose filters placed onto the surface of LA plates. After incubation for 4 hours at 37°C the cells were removed from the filter by washing with 1x M9 salts. Serial dilution's of donor cells only, recipient cells only and transconjugant cells were made and the appropriate dilution's were plated onto selective medium. Donor cells could be selected for on LA supplemented with Sp, Tp, Tt, Km and Ap, recipient cells could be selected for on minimal medium supplemented with thymine and transconjugants could be selected for on minimal medium supplemented with thymine and the antibiotics Km and Ap. Table 3.4 shows the frequency of transfer of pSA1 and pSA2, expressed as the number of transconjugants per recipient, between the two *E. coli* strains. The results demonstrate that the suicide vectors pSA1 and pSA2, despite their large size, are still able to be transferred between two *E. coli* strains.

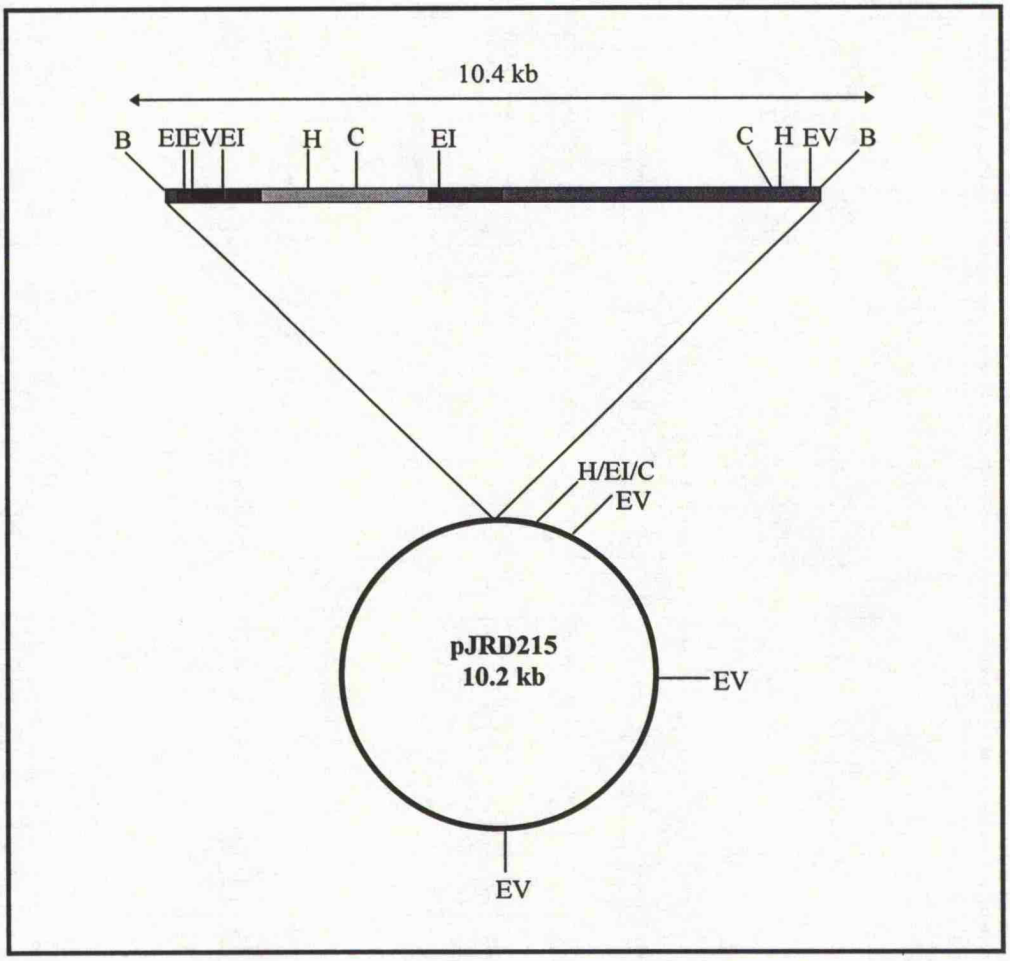


Fig. 3.32. Schematic representation of the suicide vector pSA1. The 10.4 kb plasmid pAO6 (containing Tn*4351*::*catIII*) was cloned, in the orientation demonstrated, into the *Bam*HI restriction enzyme site of the 10.2 kb suicide vector pJRD215 resulting in the suicide vector pSA1. ▬ Represents pAO6 sequences, ▬ represents Tn*4351** sequences, ▬ represents IS*4351* sequences and ▬ represents *catIII* sequences. Some restriction enzyme sites are indicated on the diagram where EI = *Eco*RI, EV = *Eco*RV, H = *Hind*III, C = *Cla*I, B = *Bam*HI

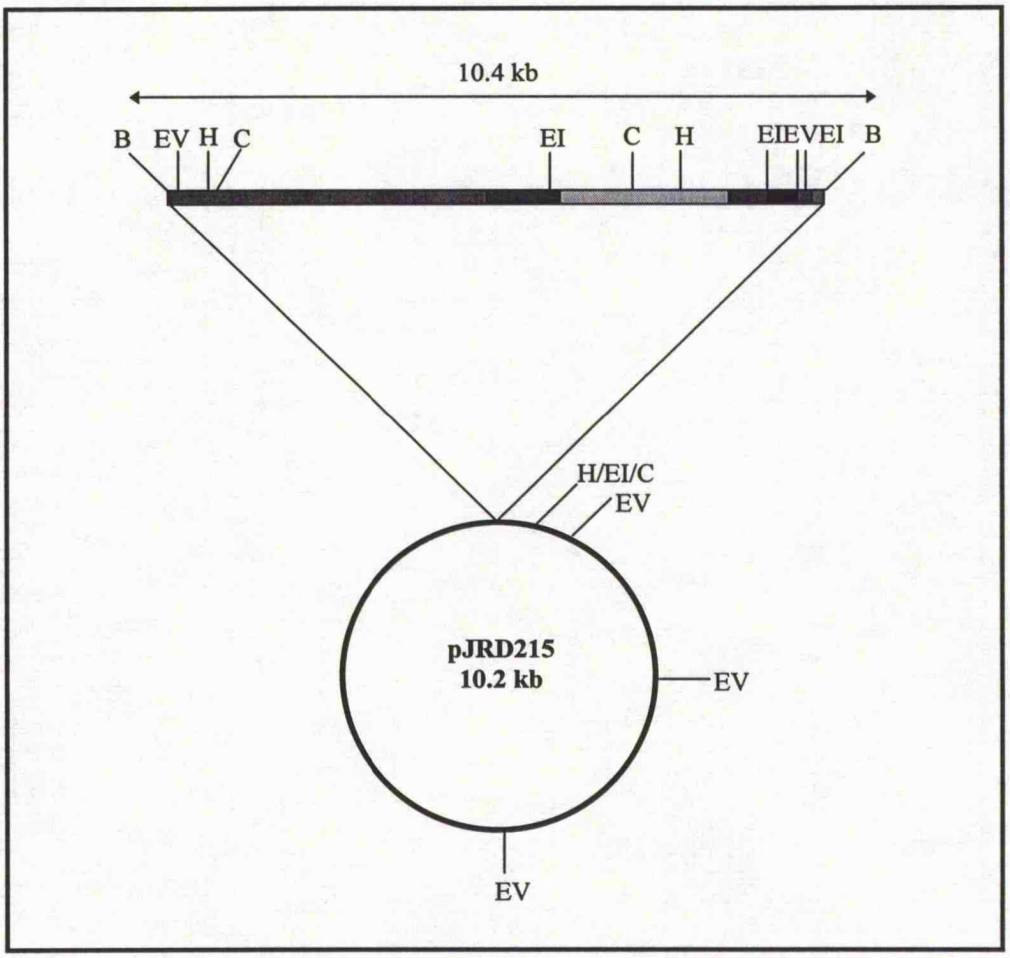


Fig. 3.33. Schematic representation of the suicide vector pSA2. The 10.4 kb plasmid pAO6 (containing Tn*4351*::*catIII*) was cloned, in the orientation demonstrated, into the *Bam*HI restriction enzyme site of the 10.2 kb suicide vector pJRD215 resulting in the suicide vector pSA2. ▬ Represents pAO6 sequences, ▭ represents Tn*4351** sequences, ▬ represents IS*4351* sequences and ▬ represents *catIII* sequences. Some restriction enzyme sites are indicated on the diagram where EI = *Eco*RI, EV = *Eco*RV, H = *Hind*III, C = *Cla*I, B = *Bam*HI

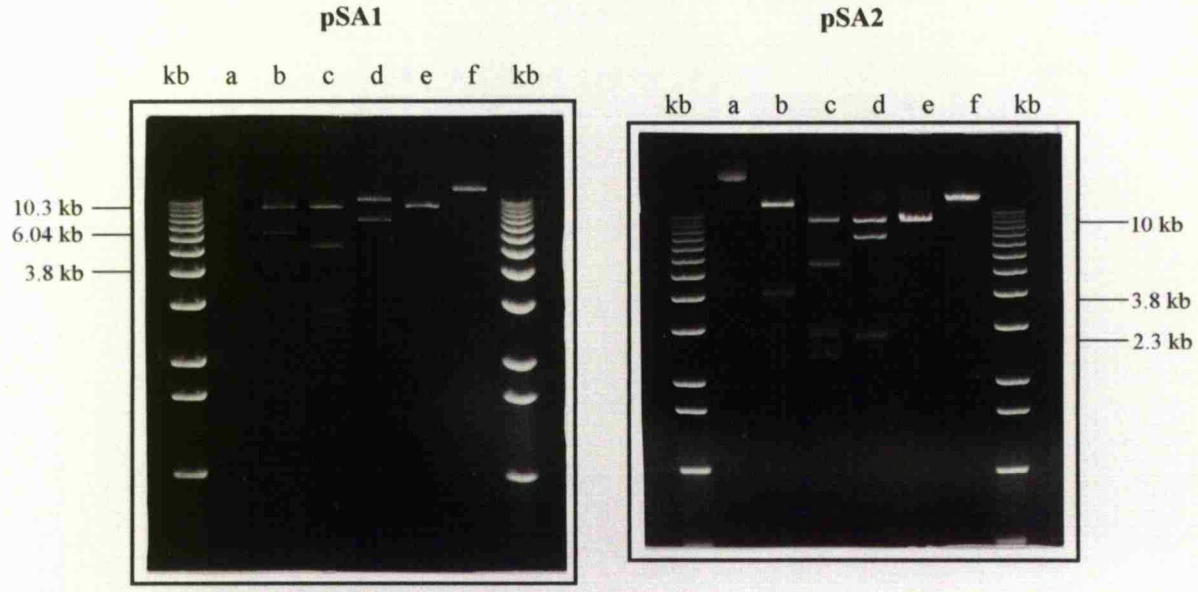


Fig. 3.34. Restriction enzyme analysis of plasmids pSA1 and pSA2. The DNA was cleaved with restriction enzymes and separated by gel electrophoresis on 0.7 % agarose gels. Kilo base (kb) ladder is run either side of the plasmid DNA. The letters a - f represent pAO6 and pAO5 plasmid DNA digested with the following restriction enzymes a = uncut DNA; b = *Eco*RI; c = *Eco*RV; d = *Hin*dIII; e = *Bam*HI; f = *Sst*I. Size markers are indicated to either side of the two photographs.

	Size of fragments (kb) generated following cleavage of plasmid DNA with restriction endonucleases	
Restriction endonuclease	**pSA1**	**pSA2**
*Eco*RI	10.03, 6.04, 3.8, 0.71, 0.019	15.8, 3.8, 0.71, 0.28, 0.019
*Eco*RV	9.98, 5.11, 2.78, 2.55, 0.173	10.04, 5.28, 2.78, 2.33, 0.173
*Hind*III	12.13, 7.9, 0.587	10.3, 7.9, 2.4
*Bam*HI	10.4, 10.2	10.4, 10.2
*Sst*I	20.6	20.6

Table 3.3. DNA fragments generated after cleavage of plasmids pSA1 or pSA2 with different restriction endonucleases.

FREQUENCY OF TRANSFER

	pSA1	pSA2
MATING 1	9.90×10^{-3}	3.57×10^{-2}
MATING 2	6.00×10^{-3}	3.90×10^{-3}
MATING 3	1.33×10^{-3}	1.35×10^{-3}
MATING 4	2.09×10^{-3}	3.97×10^{-2}
MATING 5	5.13×10^{-3}	4.19×10^{-2}
MATING 6	3.39×10^{-3}	2.94×10^{-2}

Table 3.4. Transfer frequency of suicide vectors pSA1 and pSA2 from *E. coli* strain S17-1* into *E. coli* strain W3110. Transfer frequencies are given as number of transconjugants per recipient.

3.4 Discussion

3.4.1 Generation of Transposon Promoter Probes

Manipulation of class II transposons, such as Tn*3*, to include promoterless reporter genes has been relatively straight forward due to the structure of these mobile elements (Berg *et al.*, 1989; Stachel *et al.*, 1985; Ubben and Schmitt, 1987) Class II transposons do not rely on insertion elements for transposition but instead comprise of independent units carrying the genes for transposition as well as a separate antibiotic resistance marker and these are flanked by closely related inverted repeats of approximately 38 bp. Deletions within the repeats or mutations within the transposase gene prevent transposition of the element and so promoter probes developed in class II transposons have taken advantage of the region encoding the antibiotic resistance marker. Manipulation of this section to include a promoterless reporter gene that is close to one end of the transposon does not affect the transposition function and several class II promoter probes have been developed (Berg *et al.*, 1989; Stachel *et al.*, 1985; Ubben and Schmitt, 1987). Manipulation of class I composite transposons has proved more difficult than manipulation of class II transposons and this is due to the symmetrical structure of composite transposons. These transposons carry a marker (such as drug resistance) flanked by two copies of an insertion element that are either inverse or direct repeats of each other. The insertion elements contain a long open reading frame encoding the transposase gene which spans almost the entire length of the element (Kleckner, 1981). Each element also contains perfect or nearly perfect terminal inverted repeats of 9-40 bp and these sequences are thought to serve as recognition sequences for the transposases enzymes in their role of fusing the ends of the insertion element to the target DNA (Iida *et al.*, 1983). Clearly, the majority of nucleotide sequences within the insertion elements are important for transposition to occur but despite this many transposon promoter probes have been generated using composite transposons. Tn*10* has been shown to have only one active insertion element, IS*10*$_R$, the other insertion element, IS*10*$_L$, is defective although the inverted repeats are still recognised by transposase (Kleckner, 1989). Because IS*10*$_L$ is non-functional this region can be easily

manipulated to include a reporter gene and as long as the inverted repeats are structurally intact such manipulations do not affect the ability of the entire transposon to undergo transposition (Way *et al.*, 1984). Similarly IS*50*$_L$, one of the flanking insertion elements of Tn*5*, has been found to be non-functional due to a single base pair substitution and only the most leftward 23 bases of this insertion element are required for transposition to occur. Therefore it has been feasible to insert promoterless reporter genes, including *lacZ* (Kroos and Kaiser, 1984), a kanamycin resistance determinant (Bellafatto *et al.*, 1984) and *phoA* (Manoil and Beckwith, 1985; 1986), near to the left end of Tn*5* without affecting the transposition activity of the transposon.

3.4.2 Manipulation of IS*4351*$_R$

Initial studies on the insertion elements that flank Tn*4351* indicated that at least one of the insertion elements was active and this was designated IS*4351*$_R$ (Rasmussen *et al.*, 1987). Further studies which focused on the ability of the individual elements to form cointegrates demonstrated that IS*4351*$_L$ showed a much higher frequency of cointegrate formation than did IS*4351*$_R$ (Hwa *et al.*, 1988). These results demonstrated that both insertion sequences were independently active, at least in *E. coli*, and therefore both elements encoded for a functional transposase. To date no studies have been undertaken to determine the effect on transposition of deleting or manipulating one of the insertion elements, however, in order to develop the promoter probe a reporter gene had to be inserted into one end of the transposon. It was decided to clone the reporter gene into IS*4351*$_R$ because not only did this element have two *Eco*RI restriction enzyme sites close to the end of the transposon, one of which could be used as the cloning site, but this element exhibited the lowest activity in terms of cointegrate formation. From this it can be speculated that IS*4351*$_R$ is less important for transposition (in terms of functional activity) than IS*4351*$_L$ and therefore would be more amenable to manipulation.

In order to create a unique restriction enzyme site into which the reporter gene could be cloned the PCR primer was designed to remove the *Eco*RI restriction enzyme site closest to the end of the transposon. This site lies within the region of the terminal

inverted repeat insR, therefore, insertion of a reporter gene into this site would have resulted in destruction of the transposase recognition site severely hindering transposition. Because the ends of IS*4351*$_R$ are inverted repeats (with 20 out of 25 bp homology) it was important to make the primers quite long (32-mers) so that each primer recognised and hybridised to the correct position of the sequence. The primer SAO1 was deliberately designed such that the 2 bp change used to destroy the *Eco*R1 restriction enzyme site resulted in the creation of an *Eco*RV restriction enzyme site. This was essential for the restriction enzyme analysis of pAO4 in order to determine the orientation of the *catIII* insert.

The long open reading frame spanning nucleotides 11 to 988 is thought to encode for the transposase. Cloning a reporter gene into the unique *Eco*RI restriction enzyme site disrupts the transposase transcript 15 bp from the 3' end of the coding sequence (fig. 3.25). It has been suggested that the asymmetric location of the long coding region relative to the terminal inverted repeats reflects the fact that lengthy transcription signals occur at the beginning of the gene while no special termination signals besides the stop codon are required at the end (Kleckner, 1981). This would suggest that it is feasible that transcriptional stop sequences within the reporter gene could cause termination of the transposase transcript resulting in a product that is only slightly truncated and which may still be active. However, a functional transposase will still be encoded by IS*4351*$_L$. The use of a biological assay in *E. coli* to study the activity of the transposon allows the effect of an insert within IS*4351*$_R$ to be rapidly determined prior to transposon mutagenesis experiments within *P. gingivalis*.

3.4.3 Amplification of IS*4351*$_R$ by PCR

Studies have shown *Taq* DNA polymerase to have a error rate of 1 in 10 000 bp per cycle which corresponds to one error in every 400 bp in the final product after 25 cycles (Saiki *et al.*, 1988). This corresponded to the mutation rate found when amplifying IS*4351*$_R$ with *Taq* DNA polymerase where one error was found in every 385 bp in the final product after 30 cycles. Sequence analysis of IS*4351*$_R$ identified three mutations

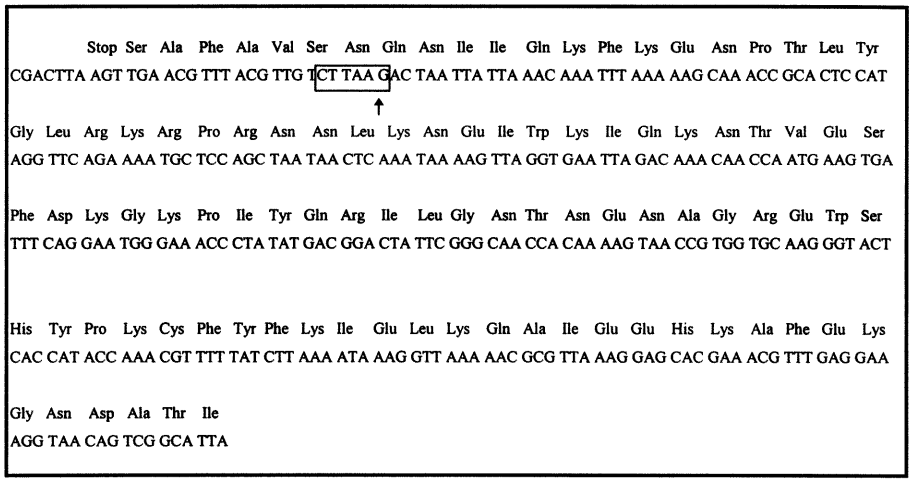


Fig. 3.35. Demonstration of the effect on the translation of the transposase protein by inserting a reporter gene into the *Eco*RI restriction enzyme site of IS*4351*$_R$*. Insertion into this site (indicated by a box and an arrow at the point of cleavage) results in the loss of the last 6 codons of the transposase protein resulting in a truncated product. The sequence is shown in the 3' - 5' direction. Only the last 101 codons out of 326 codons encoding the transposase protein are demonstrated

within the sequence, two of which were base substitutions and one of which was a deletion of a single nucleotide and these types of mutations have been shown to be the most common errors produced by polymerases during DNA synthesis (Clark, 1988). It was decided to re-amplify the insertion element using *UlTma* DNA polymerase for two reasons. First the T → C base change at nucleotide 919 results in an amino acid change of lysine to glutamic acid. During the recreation of the transposon the last 600 bp of IS*4351*$_R$ become the last 600 bp of the intact insertion element and so the substitution of lysine, which is a basic amino acid, to glutamic acid, which is an acidic amino acid, is likely to have severe consequences to the structure of the transposase protein. Secondly the deletion of a thymine nucleotide between nucleotides 254-258 causes a stop codon to occur in the nucleotide sequence resulting in a truncated translation of the transcript. As suggested above it is possible that the transposase gene could terminate within the reporter gene resulting in a product that could still exhibit some activity. The result of the bp deletion would cause the transposase transcript to terminate at nucleotide 243 resulting in a severe truncation of the transposase.

UlTma DNA polymerase is a highly thermostable, recombinant DNA polymerase, with an inherent 3' to 5' exonuclease proof-reading activity and no associated 5' to 3' nuclease activity. The 3' to 5' exonuclease proof-reading activity of *UlTma* DNA polymerase can improve fidelity by removing misinserted bases before extension occurs. The concentration of dNTP's are reduced during the *UlTma* DNA polymerase PCR reaction compared to the levels during the *Taq* DNA polymerase reaction and this is because high dNTP concentrations increase the error rate by driving the reaction in the direction of DNA synthesis and by decreasing the error discrimination at the extension step. Decreasing the level of dNTP's in the reaction causes the reaction to slow down, allowing the proof-reading activity of *UlTma* DNA polymerase time to correct misinserted bases (Eckert and Krunkel, 1992).

Despite the precaution of lowered dNTP concentrations, mutations were still identified in the *UlTma* DNA polymerase amplified IS*4351*$_R$ sequence. It is possible that by reducing the extension time this error rate could be reduced further by allowing less

time for a misinserted base to be misextended. Two mutations were found in the sequence, the first was a T → C base change at nucleotide 57 which did not effect the glutamic acid amino acid at the position of the mutation and so was not of concern. The second mutation a C → T base substitution at nucleotide 675 in ORF1 resulted in an alanine to valine amino acid change. Although a mutation in this part of the coding region would result in an amino acid change in the intact IS*4351*$_R$/IS*4351*$_L$ hybrid transposase gene, the change of alanine to valine is unlikely to affect the activity of the transposon. The amino acids alanine and valine are both neutral (nonpolar) and hydrophobic with very similar side chains that tend to cluster on the insides of proteins. Substitution of one of these amino acids with the other can be likened to a conservative amino acid substitution that is unlikely to affect the activity of the transposase.

3.4.4 Reporter Genes Routinely used to Detect Bacterial Promoter Activity

The reporter genes that are routinely used to detect bacterial promoter activity, such as *lacZ*, *phoA* and *luxA* and *luxB*, have already been described (section 3.1). However, many of these genes have proved to be inappropriate for use as reporter genes in *P. gingivalis*. For example, *lacZ* and *phoA* can not be used in *P. gingivalis* due to the fact that this bacterium naturally exhibits β-galactosidase and alkaline phosphatase activity (Minhas and Greenman, 1989; van Winkelhoff *et al.*, 1988b). Although in principle a Tn*4351* promoter probe containing a *lacZ* reporter gene could be used in any bacterium that does not encode for β-galactosidase and into which Tn*4351* can transpose (for example *P. assacharolyticus*) it has be observed that when the anaerobic *Bacteroides* spp. are grown on indicator plates containing the chromogenic substrate 5-Bromo-4-Chloro-3-Indolyl-β-D-Galactoside (X-gal) no colour change occurs (N. J. Shoemaker pers. commun.). Although the reason for this is unknown the result of the observation is that β-galactosidase activity can not be readily detected in these bacteria and as such *lacZ* would not be useful as a reporter gene in these bacteria. Similar observations were found when attempts were made to use an *E. coli* β-Glucuronidase (GUS) gene as a reporter gene in the *Bacteroides* species *B. thetaiotaomicron*, *B. vulgatus*, *B. ovatus* and *B. fragilis*. Gus activity could be readily assayed using cell extracts from these species

however, when the bacteria were grown on indicator plates containing the chromogenic substrate 5-Bromo-4-Chloro-3-Indoyl-β-glucuronide (X-glc) no blue colour was detectable (Feldhaus *et al.*, 1991). If the plates were left aerobically for 8 to 12 hours a faint blue colour did sometimes appear, although this result was not found to be consistent. It was suspected that the high concentration of the reducing agent cysteine in the *Bacteroides* medium may have been interfering with the development of the blue colour and so the level of this reagent in the medium was lowered five-fold but this did not result in an intensified colour reaction. The authors concluded that although GUS expression would be useful for localising and characterising *Bacteroides* promoters in cases where cell extracts could be used, screening for GUS expression was not feasible (Feldhaus *et al.*, 1991). It has been found that the *luxA* and *luxB* genes can be expressed in the anaerobic bacterium *Clostridium perfringens* and that bioluminescence gives a quantitative measure of the levels of active luciferase produced in the cells of this organism (Phillips-Jones, 1993). However, the bioluminescence reaction has a requirement for oxygen and as such luciferase would not be an appropriate reporter gene for use in the strict anaerobes such as *P. gingivalis*. The gene was able to be used in *C. perfringens* because many clostridial species are aerotolerant and remain viable for many hours following continual exposure to oxygen. In addition to this the clostridia possess high levels of soluble $FMNH_2$-requiring flavoproteins indicative of sufficiently high levels of cellular flavoproteins required for bioluminescence.

Genes that encode resistance to antibiotics have also been used in many transposon promoter probes (Berg *et al.*, 1989). Such genes can be readily detected on media plates supplemented with the appropriate antibiotic and many sensitive assay systems exist for quantifying the level of gene expression. A promoterless erythromycin resistance (Em^r) gene has already been used to generate transcriptional fusions to *Bacteroides* promoters (Smith, 1987). However, the assay for rRNA methylase (encoded for by Em^r) can not be done conveniently with crude bacterial extracts and therefore the promoter fusions could not be readily assayed (Smith, 1987).

The reporter gene chosen for use in the Tn*4351* promoter probe was a type III chloramphenicol acetyltransferase that was originally associated with the transmissible plasmid R387 from *Shigella flexneri*. Chloramphenicol acts as an inhibitor of prokaryotic peptidyltransferase activity and enzymatic acetylation catalysed by chloramphenicol acetyltransferase (CAT) is the commonest mechanism of bacterial resistance to the antibiotic (Shaw, 1967). Transfer of the acetyl group of acetyl-CoA to the primary hydroxy group of the antibiotic as a result of the CAT activity yields 3-acetylchloramphenicol, which fails to bind to bacterial ribosomes and so fails to inhibit polypeptide elongation. Inactivation studies of type III CAT have identified an essential histidine residue which is believed to function as a general base during catalysis. The critical imidazole group of the catalytic histidine base is thought to abstract a proton from the primary hydroxy group of chloramphenicol, thus promoting nucleophilic attack at the acetyl-CoA thioester bond (reviewed in Murray *et al.*, 1988). CAT has considerable value as a reporter gene in that it is a gene product which can be assayed with specificity and great sensitivity (Shaw, 1975). Initial detection of promoter/*catIII* fusions can be obtained on medium supplemented with chloramphenicol antibiotic. Enzyme activity can then be quantified by either measuring the chloramphenicol-dependant disappearance of acetyl-*S*-CoA, by the appearance of 3-*O*-acetoxy derivative of chloramphenicol or by the formation of reduced CoA.

3.4.5 Generation of a *catIII* Transcriptional Promoter Probe

In order that a transcriptional promoter probe can be generated the promoter sequences of the reporter gene must be removed, leaving the translation initiation sequences. To do this *catIII* was amplified from pUC18:IM3 using the primers indicated previously (fig. 3.17). The primers were placed such that the proposed transcriptional initiation and termination sequences were not amplified, but so that the putative ribosome-binding (Shine-Dalgarno) sequences were included in the sequences that were amplified. The resulting reporter probe need only insert in the correct orientation downstream from promoter sequences in order to be transcribed. Translation of the reporter gene is initiated from the *catIII* Shine-Dalgarno sequences and this results in a gene product that

is constant in length and amino acid sequence. Type II promoter probes, on the other hand, do not have their own transcription or translation initiation sites, both these processes are under the control of the promoter to which the reporter gene has fused. Fusions must insert into the target gene in the correct orientation and in the correct reading frame for expression to occur and the resulting hybrid proteins formed in type II fusions have an amino terminus of variable length encoded by the target gene.

The primers were designed to include *Eco*RI restriction sites so that the PCR product could be easily cloned into the unique *Eco*RI restriction enzyme site within the cloning vector pAO3. The use of restriction enzyme sites incorporated into the ends of primers in order to facilitate cloning was originally developed by Scharf *et al.*, 1986 who used this system to facilitate the cloning of human genomic fragments. It has been found that many restriction enzymes can not cleave DNA efficiently at a site that lies within a few bases of the end of a DNA fragment. For example, an 8 bp sequence containing a *Hin*dIII restriction endonuclease site shows no cleavage even after 20 hours digestion with *Hin*dIII endonuclease whereas a 12 bp sequence containing a *Hin*dIII restriction endonuclease site shows 75% cleavage with *Hin*dIII endonuclease after 20 hours digestion (New England Biolabs, 1993). Although experiments have found that *Eco*RI restriction enzyme can cleave sites which lie within a few bases of the end of a DNA fragment (greater than 90% cleavage of an 8 bp fragment containing an *Eco*RI recognition site occurs after 2 hours digestion with *Eco*RI endonuclease; New England Biolabs, 1993) arbitrary bases were included 5' to the restriction enzyme sites within the primers to be certain that cleavage would occur with maximum efficiency.

Rather than cloning the *Eco*RI digested PCR product directly into pAO3, the *catIII* sequence was first cloned into pUC18. The reason for this was two-fold; in the first instance the insertion of foreign DNA into the polylinker of pUC18 disrupts the *lacZ* gene and these clones can be identified as white colonies on the appropriate indicator plates. By using this kind of indicator system PCR products that have been efficiently digested with *Eco*RI endonuclease can be readily detected and such inserts can then be easily removed from pUC18, purified and then sub-cloned into the cloning vector.

Secondly, although at this stage the orientation of *catIII* in the vector was not important for subsequent cloning stages, the detection of chloramphenicol resistant clones enabled us to determine whether the amplified *catIII* gene was still functional. Selection of functional *catIII* expression in pUC18 demonstrated that there had been no detrimental mutations in the gene sequence as a result of the PCR amplification and so removed the need to sequence the *catIII* PCR product.

3.4.6 Frequency of Transfer of Tn*4351** and Tn*4351*::*catIII*

The biological assay carried out to determine whether the manipulated transposon, Tn*4351*::*catIII*, was still functionally active was based on an assay developed by Hwa *et al.* In the original experiments cointegrate formation between pVOH1 (the non-mobilisable plasmid pBR328 carrying a Tn*4351* insert) and the conjugative plasmid R388 was examined by looking at the frequency of transfer of the Cm^r marker from pBR328 or the $*Tc^r$ marker from Tn*4351*. Mobilisation frequencies for the two markers were found to be between 0.5×10^{-5} and 2.5×10^{-5} for Cm^r and between 0.2×10^{-6} and 2.8×10^{-6} for $*Tc^r$ (Hwa *et al.*, 1988). The differences in the transfer frequencies for the two markers can be explained by the fact that other transposon mediated processes apart from cointegrate formation can occur. In the first instance direct transposition of Tn*4351* into R388 would result in transconjugants with the phenotype $*Tc^r$, Cm^s, whereas inverse transposition involving the ends of Tn*4351* would result in transconjugants with the phenotype $*Tc^s$, Cm^r. It is also possible that cointegrate formation followed by an IS-mediated deletion of DNA inside Tn*4351* could also result in transconjugants with the phenotype $*Tc^s$, Cm^r (Hwa *et al.*, 1988).

To study the activity of Tn*4351*::*catIII* and Tn*4351** transfer of the Ap^r marker on pAO6 and pAO5 was used to examine cointegrate formation between these plasmids and R388. The Cm^r marker had been disrupted as a result of end-repairing the *Eco*RI restriction enzyme site in pBR328 and so could not be used asses the frequency of transfer. Frequency of transfer of Ap^r for pAO6 was found to be comparable to that found for pVOH1 with frequencies ranging from 2.82×10^{-4} to 4.99×10^{-6} for pAO6

and from 4.30 x 10^{-4} to 7.65 x 10^{-6} for pVOH1 (where the transfer frequency represents the number of CFU/ml of transconjugants per number of CFU/ml recipient cells). These results indicated that the presence of the reporter gene within IS*4351*$_R$ had not affected its ability to form cointegrates and that a single functional transposase gene was sufficient for transposon activity. The results also indicated that the 2 bp alteration in *insR* did not affect the recognition of this site by the transposase. The frequency of transfer for Ap^r demonstrated in these experiments was generally similar to the transfer frequency of Cm^r demonstrated previously (Hwa *et al.*, 1988). The frequency of transfer of Ap^r in mating 1, however, showed a ten fold lower frequency compared to the previously described results. Conversely transfer of Ap^r on pVOH1 in mating 5 and on pAO6 in mating 6 demonstrated a ten fold higher frequency than the previously described results. These discrepancies are most likely due to factors not examined in these experiments such as donor/recipient ratios which could affect the frequency of cointegrate formation. If conditions favoured direct transposition then the frequency of Ap^r transfer would be reduced. If conditions favoured cointegrate formation or inverse transposition then the frequency of transfer of Ap^r could be increased. The frequencies of transfer of Ap^r on pAO5 were marginally higher than those seen for pAO6 with frequencies ranging from 4.59 x 10^{-4} to 5.20 x 10^{-5}. Plasmid pAO5 contains Tn*4351** which has a mutation at nucleotide position 675 as well as an altered *Eco*RI restriction enzyme site. Plasmid pAO6 contains Tn*4351** with the *catIII* insert. It is possible that the presence of the *catIII* insert causes a reduction in the transposase activity in pAO6 compared to that seen in pAO5. However the activities for both plasmids are comparable to that exhibited by the control plasmid pVOH1 and so the increased frequency of transfer seen with pAO5 most likely represents differences in the mating conditions.

3.4.7 Frequency of Transfer of the Suicide Vectors pSA1 and pSA2

Plasmid pAO6 was cloned into the suicide vector pJRD215 on a *Bam*HI fragment in two orientations resulting in the suicide vectors pSA1 and pSA2. The plasmid pAO6 has a copy number of between 15 - 20 copies per cell (Soberon *et al.*, 1980) whereas the

suicide vector pJRD215 has a copy number of approximately 10 copies per cell (Davison *et al.*, 1987). For this reason it was decided to clone the entire pAO6 plasmid into the suicide vector rather than just the sequences containing the manipulated transposon because it was hoped that the resulting suicide vector would have a higher copy number than pJRD215. This proved to be the case and meant that analysis of kanamycin/ampicillin resistant transformants by small scale plasmid extraction and subsequent restriction enzyme analysis was less time consuming than if the suicide vector had remained at the same low copy number as pJRD215. It was not known whether the presence of such a large insert into pJRD215 would cause transfer of the plasmid to become less efficient and so to determine this filter mating experiments were carried out. The experiments measured the frequency of transfer of Km^r and Ap^r from a donor strain to a recipient strain of *E. coli*. The frequencies of transfer were found to be between 9.90×10^{-3} to 2.09×10^{-3} for pSA1 and between 3.97×10^{-2} to 2.94×10^{-3} for pSA2 (where the transfer frequency represents the number of CFU/ml of transconjugants per number of CFU/ml recipient cells). It was concluded from these results that the suicide vectors could be transferred efficiently, between *E. coli* strains at least. It appeared that a lower frequency of transfer was occurring for pSA1 compared to pSA2. Although this could be due to factors not studied in these experiments, such as donor/recipient ratios, it is also possible that the orientation of pAO6 in pJRD215 can affect the genes involved in transfer. Comparison of the frequency of transfer of pSA1 and pSA2 with the frequency of transfer of pJRD215 would have confirmed whether the differences seen were due to experimental conditions or due to the orientation of pAO6. However, because the differences in transfer frequencies for the two plasmids was, on average, less than ten fold it was decided to use both plasmids for transfer into *P. gingivalis*. The transposition frequencies for the two suicide vectors could then be compared with that of a control plasmid to determine whether there was any difference in the ability of the manipulated transposon to under go transposition depending on the orientation of the transposon in the suicide vector.

CHAPTER 4

Transfer of Tn*4351*::*catIII* into *P. gingivalis* and the Demonstration of Differential *catIII* Expression

4.1 Introduction

Several chimeric shuttle and suicide vectors have been developed for the transfer of DNA from *E. coli* into *Bacteroides* spp. One of the first shuttle vectors to be constructed was pE5-2 which contains an *E. coli* IncQ plasmid (RSF1010), a cryptic *Bacteroides* plasmid (pB8-51) and a 3.8 kb *Eco*RI fragment from the *B. fragilis* plasmid pBF4 which carries the *ermF* determinant of Tn*4351* (Shoemaker *et al.*, 1985). The plasmid pE5-2 was mobilised from *E. coli* to *E. coli* or from *E. coli* to *Bacteroides* spp. using a 52 kb conjugative IncP plasmid, R751 (Meyer and Shapiro, 1980), that confers resistance to trimethoprim (Shoemaker *et al.*, 1985). IncP plasmids, such as R751, which have a large host range among Gram-negative bacteria have been found to mobilise RSF1010 at very high frequencies (Bagdasarian *et al.*, 1981). The frequency of transfer of pE5-2 between two *E. coli* strains was found to be between 2.0×10^{-2} to 5.0×10^{-2} transconjugants per recipient. However, the frequency of transfer of this shuttle vector from *E. coli* to *B. uniformis* or *B. thetaiotaomicron* was found to be much lower at 10^{-6} transconjugants per recipient (Shoemaker *et al.*, 1985).

Further studies using R751 containing a Tn*4351* insertion demonstrated that the transposon could be transferred from *E. coli* into *Bacteroides* spp. where it could insert into chromosome of the recipient strain resulting in auxotrophic mutants (Shoemaker *et al.*, 1986). The matings were carried out aerobically on nitrocellulose filters which were incubated aerobically for 10 to 12 hours before the mating mixture was plated onto selective media and placed anaerobically for 48 hours (Shoemaker *et al.*, 1986). Using these conditions transconjugants were detected at a frequency of 10^{-5} to 10^{-6} transconjugants per recipient. Because plasmid R751 was found not to replicate in

Bacteroides spp. (Shoemaker *et al.*, 1985) these frequencies represented the frequency of transposition of Tn*4351* into *Bacteroides* spp. Using this system transposon mutagenesis has been carried out within the intestinal *Bacteroides* spp. to isolate several transposon mutants (Anderson and Salyers, 1989; Guthrie and Salyers, 1986).

Subsequently to the use of Tn*4351* in intestinal *Bacteroides* spp. several studies have described the introduction of Tn*4351* into *P. gingivalis* (Dyer *et al.*, 1992; Hoover *et al.*, 1992b; Progulske-Fox *et al.*, 1989b). These studies each used different strains of *P. gingivalis* and different mating conditions which resulted in transconjugants being detected at frequencies of between 10^{-7} to 10^{-10} transconjugants per recipient (Dyer *et al.*, 1992; Hoover *et al.*, 1992b; Progluske-Fox *et al.*, 1989b). The plasmid pE5-2 was one of the first vectors to be transferred into *P. gingivalis* (Progulske-Fox *et al.*, 1989b). In these experiments donor and recipient cells (*P. gingivalis* strains 381 and I 372) were grown to early log phase (1×10^8 to 2×10^8 CFU/ml). Cells were mixed, harvested by centrifugation and then spotted onto the surface blood agar plates. After anaerobic incubation for 24 hours bacterial cells were recovered from the plates by swabbing with a sterile cotton swab which was transferred to sterile media. The cell suspension was then streaked onto the appropriate selection plates which were left anaerobically for 96 hours (Progulske-Fox *et al.*, 1989b). Because pE5-2 does not contain a complete copy of Tn*4351* no transposition could occur. However, the frequency of transfer of pE5-2 from *E. coli* into *P. gingivalis* could be measured and was found to be 2.0×10^{-7} and 1.9×10^{-7} for strains 381 and I 372 respectively, which was ten-fold less frequent than the transfer of pE5-2 into intestinal *Bacteroides* spp. (Progulske-Fox *et al.*, 1989b).

Plasmid R751 containing a partial tandem duplication of Tn*4351* (R751::*Ω4) was found to exhibit a higher transposition frequency in *Bacteroides* spp. than R751 containing a single copy of Tn*4351* (Shoemaker *et al.*, 1986). Plasmid R751::*Ω4 (fig. 4.1) is unable to replicate in *P. gingivalis* or *Bacteroides* spp. and so acts as a suicide vector for delivery of Tn*4351* into these bacteria. R751::*Ω4 was transferred into *P. gingivalis* strain 33277 (Hoover *et al.*, 1992b). Donor and recipient cells were grown in liquid culture to an $O.D_{660}$ of 0.8 to 1.2. Mating mixtures were spotted onto the surface

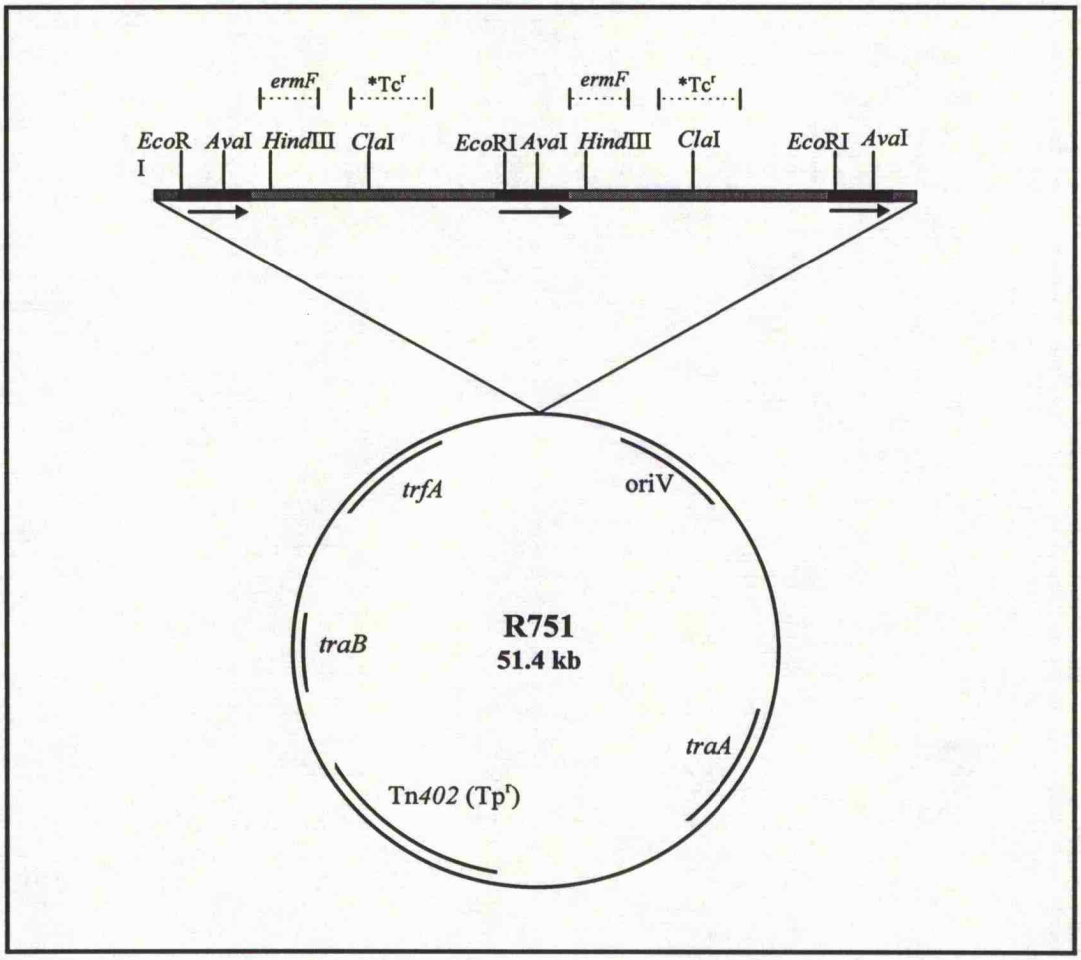


Fig. 4.1. R751::*Ω4. Partial tandem duplication of Tn*4351* inserted into the IncP, conjugative plasmid R751 results in plasmid R751::*Ω4. Tp^r refers to trimethoprim resistance. *Tc^r and *ermF* refer to the tetracycline resistance and clindamycin/erythromycin resistance determinants of Tn*4351*. The origin of replication of R751 is indicated by oriV and *traA* and *traB* represent the transfer genes. An additional gene required for replication of R751, *trfA*, is also highlighted. Arrows represent the direction of the insertion elements (▬) and shaded areas (▬) represent Tn*4351* sequences. The exact insertion point of *Ω4 in R751 is unknown, as indicated by striped areas (▬). The exact duplication point of *Ω4 is also not known. (Based of information present in Meyer and Shapiro, 1980; Shoemaker *et al.*, 1986).

of *Brucella* agar base plates and incubated aerobically for 2 to 4 hours before being transferred to anaerobic conditions for a further 36 to 48 hours (Hoover *et al.*, 1992b). After this time cells were harvested with sterile cotton swabs and plated onto the appropriate selection plates. The frequency of transposition was found to average 1.6 x 10^{-7} transconjugants per recipient for 10 independent matings (frequencies varied between 8.2 x 10^{-7} to 9.8 x 10^{-9}) which was approximately ten-fold lower than the reported frequency of transposition of Tn*4351* from R751::*Ω4 in *Bacteroides* spp. (Hoover *et al.*, 1992b; Shoemaker *et al.*, 1986).

Transposition of Tn*4351* from R751::*Ω4 was demonstrated in several strains of *P. gingivalis* (strains 381, W50, RB22D-1, 9-14K-1 and A7A1-28) using different mating conditions to those described above (Dyer *et al.*, 1992). A similar mass of donor and recipient cells were scraped from LA and blood agar plates respectively and mixed on the surface of a fresh blood agar plate. The mating mixture was then incubated aerobically or anaerobically for 15 to 21 hours. After this time the cells were removed from the plate, resuspended and plated onto selective media (Dyer *et al.*, 1992). A ten-fold lower frequency of transposition was observed when the mating mixture was incubated anaerobically compared to mating mixtures incubated aerobically. Transposition frequencies ranged from between 2.7 x 10^{-7} to 2.0 x 10^{-9} transconjugants per recipient for aerobically incubated mating mixtures, whilst anaerobically incubated mating mixtures demonstrated transposition frequencies of between 1.3 x 10^{-8} to 1.3 x 10^{-10} transconjugants per recipient depending on the recipient used (Dyer *et al.*, 1992).

Mating experiments also demonstrated that *Bacteroides* shuttle vectors, such as pNJR12, could be transferred into *P. gingivalis* (Maley *et al.*, 1992). The conditions used in these mating experiments were carried out using a broad adaptation of the procedure used for *Bacteroides* spp. (Shoemaker *et al.*, 1985) which was modified for use in *P. gingivalis*. Donor and recipient (*P. gingivalis* strain W83) cells were grown to early logarithmic phase between 1.0 x 10^{4} to 1.5 x 10^{8} CFU/ml. Donor and recipient cells were mixed in suspension and then harvested by centrifugation. The cell pellet was then resuspended and spotted onto nitrocellulose filters placed on blood agar plates.

The mating mixture was incubated anaerobically for 72 hours before the bacterial cells were removed from the filter with a sterile cotton swab and plated onto selective media (Maley *et al.*, 1992). The frequency of transfer of pNJR12 was found to be between 2.2 x 10^{-4} to 6.9 x 10^{-8} transconjugants per recipient depending on the donor/recipient ratio used (Maley *et al.*, 1992).

Using these systems, or variations of these systems, several transposon induced mutations have recently been described for *P. gingivalis*. The purpose of this study was to determine whether the manipulated transposon Tn*4351*::*catIII* was capable of being transferred into *P. gingivalis* and subsequently transposing into the *P. gingivalis* genome. If transposition did occur the next stage was to determine whether differential *catIII* expression could be achieved and whether this could be used to study gene regulation in *P. gingivalis*.

4.2 Results

4.2.1 Transfer of pSA1 and pSA2 from *E. coli* into *P. gingivalis* 381

The frequency of transfer of plasmids containing Tn*4351* was found to be more efficient into *P. gingivalis* 381 compared to transfer into four other strains tested (Dyer *et al.*, 1992), and as such this strain that was chosen as the recipient for transfer of the suicide vectors pSA1 and pSA2. Because the suicide vector generated for use in this study was derived from the shuttle vector pNJR12 it was decided to adapt the mating system used by Maley *et al.* which had been demonstrated to be an efficient means of transferring pNJR12 into *P. gingivalis* (Maley *et al.*, 1992). However, some modifications were made to this protocol. Donor and recipient cells were grown to early logarithmic phase (10^8 to 10^9 CFU/ml) as preliminary mating experiments using recipient starting cultures of less than 1 x 10^8 CFU/ml had failed to generate transconjugants (data not shown). In addition to this, an aerobic incubation step was included in the mating procedure since previous reports had indicated that aerobic incubation of the mating mixture resulted in an increased transfer/transposition frequency (Dyer *et al.*, 1992; Hoover *et al.*, 1992b).

The mating mixtures were incubated aerobically on nitrocellulose filters for 15 hours as this had been demonstrated to be the shortest aerobic incubation period required to achieve efficient transposition of Tn*4351* in *P. gingivalis* strain 381 (Dyer *et al.*, 1992). The plasmid R751::*Ω4 was also transferred into *P. gingivalis* 381 under the same mating conditions to provide a comparison for transposition frequencies between this plasmid and pSA1 and pSA2. R751::*Ω4 was transferred from the *E. coli* strain HB101, the strain that was used in the previous mating experiments (Dyer *et al.*, 1992; Hoover *et al.*, 1992b; Shoemaker *et al.*, 1986).

The suicide vectors pSA1 and pSA2 were both found to be unstable in *E. coli*. Typically the plasmids were found to have rearrangements resulting in a 4 - 5 kb deletion of the plasmid DNA. This deletion always resulted in the loss of the 3.6 kb *Eco*RI fragment from pSA1 and pSA2 that contained the Tn*4351 ermF* and *Tc resistance determinants. Filter mating experiments carried out using these deleted plasmids produced no transconjugants. Although these deleted plasmids were not analysed it was presumed that transposition of Tn*4351*::*catIII* from pSA1 or pSA2 into the donor strain genome had occurred. In order to avoid carrying out mating experiments with these deleted plasmids, overnight donor strain cultures, containing the unrearranged plasmid, were set up using bacteria taken from a glycerol stock prior to each mating experiment (glycerol stocks contained stationary phase donor strain bacteria in 15% sterile glycerol stored at -70°C). Restriction enzyme analysis of small scale plasmid extraction's of pSA1 and pSA2 were carried out prior to the mating experiment in order to detect and discard any donor cultures carrying rearranged suicide vectors.

Table 4.1 shows the frequency of transposition of Tn*4351*::*catIII* demonstrated when pSA1 and pSA2 were transferred into *P. gingivalis* 381 and clindamycin resistant transposon mutants were selected. These frequencies of transposition were compared to the frequency of transposition demonstrated when R751::*Ω4 was transferred into *P. gingivalis* 381. The average frequencies of transposition of Tn*4351* from pSA1, pSA2 and R751::*Ω4 were 5.27×10^{-7}, 2.3×10^{-7} and 8.97×10^{-7} respectively where transposition frequency represents the number of CFU/ml transconjugants (Gm^r/Cl^r) per

Table 4.1

Transposition Frequency[†]		
pSA1	**pSA2**	**R751::*Ω4**
1.70×10^{-7}	2.81×10^{-7}	2.36×10^{-6}
2.15×10^{-7}	3.36×10^{-7}	2.84×10^{-7}
1.20×10^{-7}	2.56×10^{-7}	4.54×10^{-7}
3.57×10^{-7}	1.27×10^{-7}	4.89×10^{-7}
7.79×10^{-7}	2.74×10^{-7}	—
1.24×10^{-6}	1.58×10^{-7}	—
4.66×10^{-7}	3.18×10^{-7}	—
8.68×10^{-7}	1.07×10^{-7}	—
Average: 5.27×10^{-7}	Average: 2.30×10^{-7}	Average: 8.97×10^{-7}

[†] Transposition frequencies are expressed as the number of CFU of transconjugants per CFU of recipient per millilitre

Table 4.1. Frequency of transposition of Tn*4351*::*catIII* or Tn*4351* from the suicide vectors pSA1, pSA2 and R751::*Ω4 into *P. gingivalis* 381. Vectors were transferred from *E. coli* S17-1* (pSA1 or pSA2) or HB101 (R751::*Ω4) into *P. gingivalis* 381 by filter mating. S17-1* (pSA1) were used at 1.2×10^8 CFU/ml, S17-1* (pSA2) were used at 1.41×10^8 CFU/ml and HB101 (R751::* Ω4) were used at 2.1×10^8 CFU/ml. Recipient cells (*P. gingivalis* 381) were used at 1.90×10^9 CFU/ml. The ratio of donor to recipient cells used were as follows; 1:16 for S17-1* (pSA1) to *P. gingivalis* 381, 1:14 for S17-1* (pSA2) to *P. gingivalis* 381 and 1:9 for HB101 (R751::* Ω4) to *P. gingivalis* 381.

CFU/ml recipient. These frequencies represent results from several individual matings from a single experiment using the same variables for each separate mating. The starting culture for *P. gingivalis* 381 was used at 1.90 x 10^9 CFU/ml and the starting cultures for S17-1* (pSA1), S17-1* (pSA2) and HB101 (R751::*Ω4) were used at 1.2 x 10^8, 1.41 x 10^8 and 2.1 x 10^8 CFU/ml respectively. The donor to recipient ratios were 1:16 for S17-1* (pSA1) to *P. gingivalis* 381, 1:14 for S17-1* (pSA2) to *P. gingivalis* 381 and 1:9 for HB101 (R751::*Ω4) to *P. gingivalis* 381. Tables 4.2a and 4.2b demonstrate the frequencies of transposition seen when pSA1 (Table 4.2a) and pSA2 (Table 4.2b) were transferred into *P. gingivalis* using different donor:recipient ratios. The frequencies of transposition demonstrated represent average frequencies of transposition for several separate mating experiments, each using different variables (i.e. donor:recipient ratios and starting CFU/ml). The frequencies range from between 1.05 x 10^{-7} to 5.23 x 10^{-9} for pSA1 and between 2.06 x 10^{-6} to 7.90 x 10^{-9} for pSA2. This represents an overall average frequency of transposition of 2.27 x 10^{-7} for pSA1 and 5.72 x 10^{-7} for pSA2.

4.2.2 Analysis of Clindamycin Resistant Transconjugants from *P. gingivalis* 381

To determine whether single insertions of Tn*4351* had occurred in *P. gingivalis* 381, Southern blot analysis was carried on randomly selected Cl^r transconjugants. Chromosomal DNA from the transconjugants was digested with *Hind*III restriction enzyme, separated by agarose gel electrophoresis and transferred by Southern blot to hybridisation membrane. The Southern blots were then probed with a 3.6 kb *Eco*RI radiolabelled fragment from pVOH1 which includes most of IS*4351*$_R$ and the *ermF* and *Tc^r regions of Tn*4351*. Figure 4.2 demonstrates the position of the 3.6 kb *Eco*RI fragment in Tn*4351* and the position of the *Hind*III restriction enzyme site. If a single Tn*4351* insertion occurs then hybridisation of the radiolabelled probe to *Hind*III digested chromosomal DNA should result in two fragments of > 2.1 kb and > 3.6 kb (figure 4.2).

Table 4.2a

Starting CFU/ml		Donor:Recipient ratio	Transposition Frequency[†]
Donor	**Recipient**		
3.00×10^8	1.90×10^9	1:6	1.05×10^{-7}
3.69×10^9	1.48×10^8	25:1	5.74×10^{-7}
4.50×10^8	1.91×10^9	1:4	5.23×10^{-9}
5.70×10^8	2.3×10^9	1:4	1.59×10^{-8}
6.40×10^8	1.4×10^8	4.6:1	2.14×10^{-7}
6.4×10^8	5.7×10^8	1:1	1.45×10^{-7}
1.2×10^8	1.91×10^9	1:16	5.27×10^{-7}

[†] Transposition frequencies are expressed as the number of CFU of transconjugants per CFU of recipient per millilitre

Table 4.2a. Transposition frequency of Tn*4351*::*catIII* from the suicide vector pSA1 into *P. gingivalis* 381. Results are from seven separate experiments and are representative of an average frequency for each experiment undertaken.

Table 4.2b

Starting CFU/ml		Donor:Recipient ratio	Transposition Frequency†
Donor	**Recipient**		
2.00×10^8	1.9×10^8	1:1	2.46×10^{-7}
1.78×10^8	1.65×10^8	1:1	2.06×10^{-6}
1.29×10^9	1.48×10^8	9:1	7.86×10^{-7}
6.3×10^8	1.9×10^9	1:3	7.90×10^{-9}
2.39×10^8	5.7×10^8	1:2.4	1.05×10^{-7}
1.41×10^8	1.91×10^9	1:14	2.30×10^{-7}

† Transposition frequencies are expressed as the number of CFU of transconjugants per CFU of recipient per millilitre

Table 4.2b. Transposition frequency of Tn*4351*::*catIII* from the suicide vector pSA2 into *P. gingivalis* 381. Results are from six separate experiments and are representative of an average frequency for each experiment undertaken.

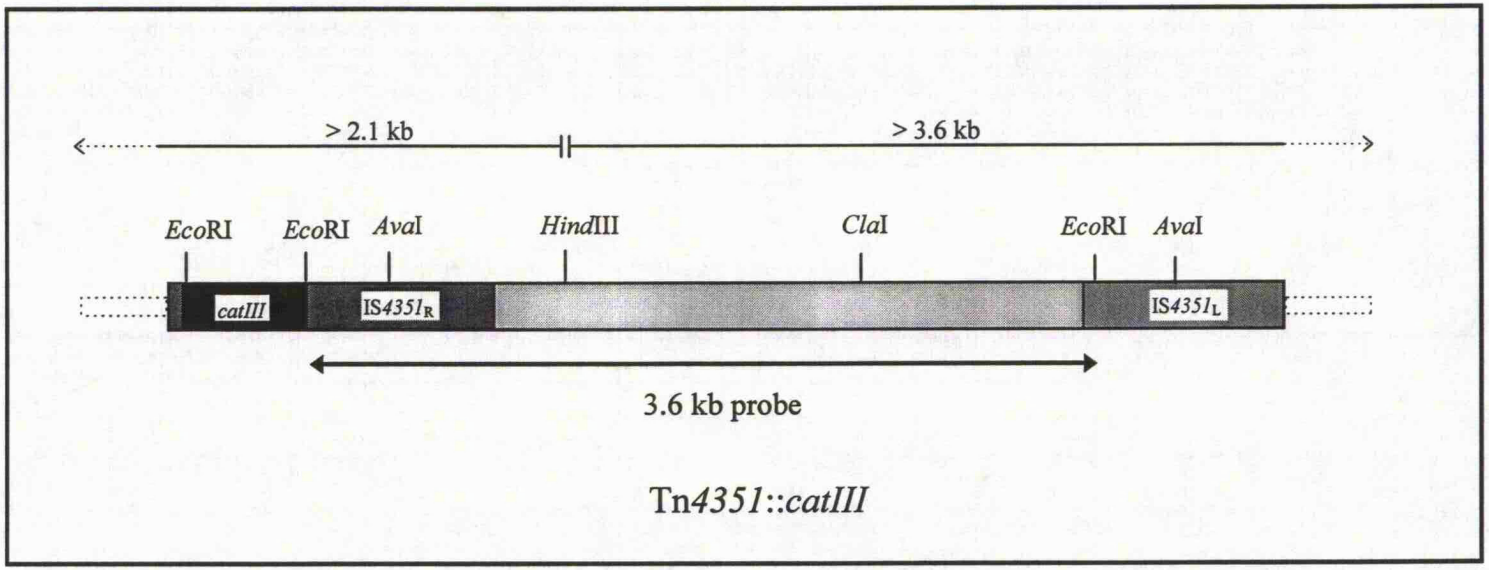


Fig. 4.2. Schematic representation of Tn*4351*::*catIII* indicating the 3.6 kb *Eco*RI fragment used as a probe in Southern blot analyses. The diagram also indicates the size of the fragments that would result from *Hind*III digestion of *P. gingivalis* chromosomal DNA that carried a single Tn*4351*::*catIII* insertion. Probing this digested DNA with the 3.6 kb radio-labelled fragment would result in two hybridised bands of > 2.1 kb and > 3.6 kb, as indicated by the hashed line arrow. Hashed line boxes either side of Tn*4351*::*catIII* represent chromosomal DNA.

Figures 4.3A and 4.3B show the autoradiographs of *Hind*III digested chromosomal DNA preparations, from transconjugants from matings involving pSA2, subjected to Southern blot analysis using a radiolabelled 3.6 kb *Eco*RI fragment from pVOH1 as a probe. Table 4.3 gives the sizes of the fragments that hybridised to the probe. The variable banding patterns seen for each transconjugant demonstrated that transposition into *P. gingivalis* 381 was random. However, the transconjugants were found not to contain single insertions of Tn*4351*::*catIII* as indicated by the fact that the 3.6 kb *Eco*R1 probe hybridised to three or more fragments. Hybridisation of *P. gingivalis* 381 chromosomal DNA with the 3.6 kb *Eco*RI probe was not observed indicating that the extra fragments were not due to the probe hybridising to homologous sequences in the *P. gingivalis* chromosome. Redigestion of transconjugant number 3 with *Hind*III restriction enzyme and subsequent Southern blot analysis using the 3.6 kb probe revealed that only four fragments were hybridised by the probe (as indicated in Table 4.3) and that the other bands seen in the autoradiograph were in fact partially digested fragments. The Southern blot analysis revealed two conserved fragments of 2.4 kb and 7.9 kb of which one, or both, appeared in all but one of the transconjugants. Digestion of pSA2 plasmid DNA with *Hind*III restriction enzyme results in three fragments of 10.3, 7.9 and 2.4 kb of which the 2.4 kb and 7.9 kb fragments contain Tn*4351* sequences. It was speculated that the 3.6 kb *Eco*RI probe was hybridising to the 2.4 kb and 7.9 kb fragments due to insertion of the plasmid into the chromosome. To confirm that pSA2 had inserted into the chromosome, Southern blot analysis was carried out on *Eco*RV digested transconjugant chromosomal DNA using a radiolabelled pJRD215 probe. The analysis revealed hybridisation of the probe to three fragments of 5.28 kb, 2.78 kb and 2.33 kb (data not shown) in transconjugants 3, 6 - 10 indicating that the plasmid had inserted into the chromosome and that there was a Tn*4351*::*catIII*/pJRD215 junction (corresponding to the 2.33 kb *Eco*RV fragment). For transconjugant number 2 the probe hybridised to three fragments of > 12, 5.28 and 2.78 kb, for transconjugant 4 the probe hybridised to three fragments of 5.28, 2.78 and 2.5 kb and for transconjugant number 5 the probe hybridised to four fragments of 7.0, 5.28, 2.78 and 2.6 kb. This indicated that these transconjugants contained complete plasmid sequences but did not contain a Tn*4351*::*catIII*/pJRD215 junction. Finally for transconjugant number 1 the

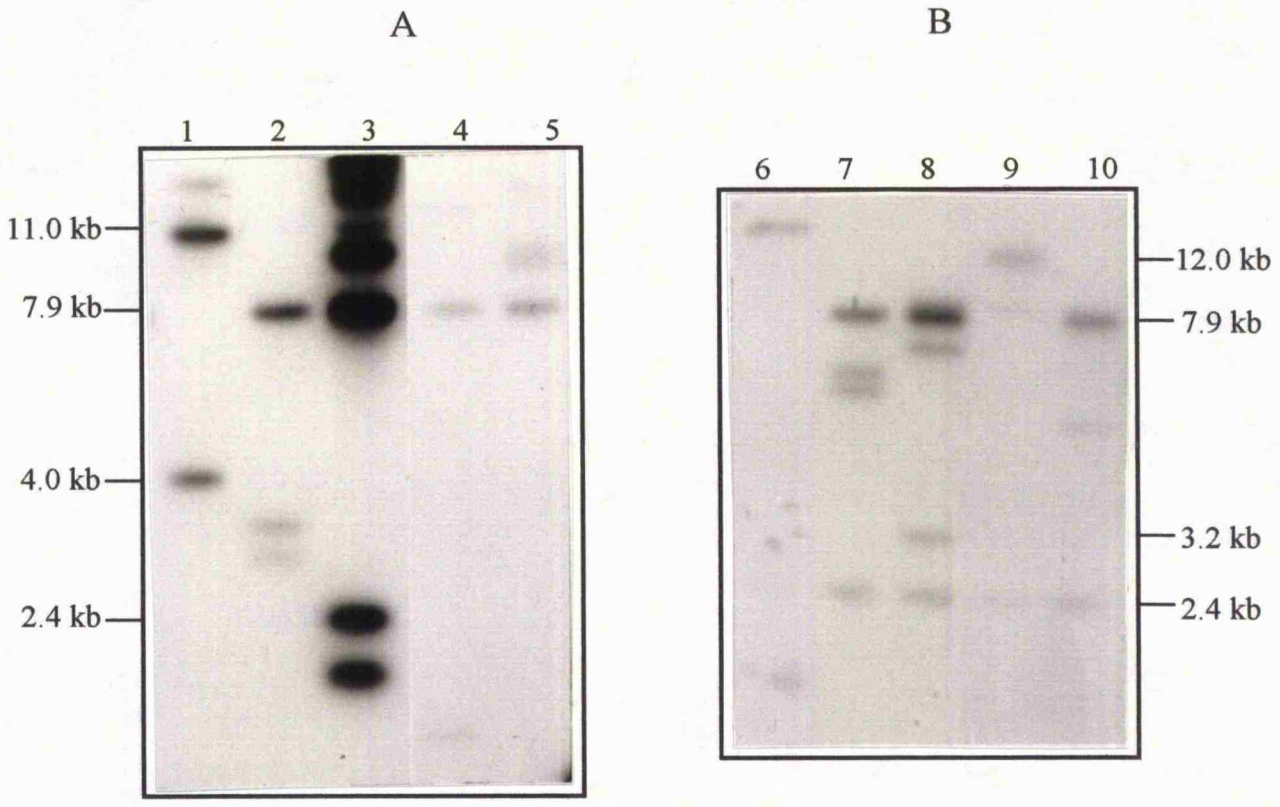


Fig. 4.3. Southern blot hybridisation analysis of *P. gingivalis* transconjugants. Chromosomal DNA from ten independent Cl^r/Gm^r transconjugants of *P. gingivalis* 381 transferred with the suicide vector pSA2 (lanes 1 - 10) were digested with *Hind*III restriction enzyme, separated by gel electrophoresis and transferred by Southern blot to hybridisation membrane. The membranes were hybridised with a [^{32}P] labelled 3.6 kb *Eco*RI fragment from pVOHI. Size markers (in kilo bases) are indicated at the sides. Hybridisation of *P. gingivalis* 381 DNA to the 3.6 kb probe was not observed (data not shown).

	Transconjugants (Gm^r/Cl^r)									
	1	2	3	4	5	6	7	8	9	10
Fragment Sizes (kb)	>12*	7.9	10.0	>12*	>12	>12	7.9	7.9	12.0	7.9
	11.0	3.5*	7.9	7.9	11.0*	2.9*	6.0	6.5	8.1*	4.6*
	4.0	3.0*	2.4*	2.0	10.0*	2.4*	5.5*	3.2*	2.4*	2.4*
			2.1*		7.9		2.4*	2.4*		

Table 4.3. Fragment sizes seen by Southern blot analysis of ten transconjugants from a S17-1* (pSA2)/*P. gingivalis* 381 filter mating. *Hind*III digested chromosomal DNA from the transconjugants was probed with a 3.6 kb *Eco*RI probe. The probe contains most of the IS*4351*$_R$ sequences and the regions encoding *ermF* and *Tc^r determinants of Tn*4351*. The probe would be expected to hybridise to two fragments of > 2.1 kb and > 3.6 kb if a single Tn*4351::catIII* insertion had occurred. The 3.6 kb probe hybridised to more than two fragments in all transconjugants. Conserved fragments of 2.4 kb and 7.9 kb were observed. The asterisk (*) represents the fragments that were also hybridised to by a *catIII* probe.

probe hybridised to a single fragment of > 12 kb. Chromosomal DNA from the transconjugants was also digested with *Hind*III restriction enzyme and subjected to Southern blot analysis using a radiolabelled *catIII* probe (data not shown). This probe hybridised to one or two fragments, indicated by an asterisk in Table 4.3, suggesting that in some cases there was more than one copy of IS*4351*::*catIII* in the chromosome. It was noted that the *catIII* probe hybridised to the conserved 2.4 kb *Hind*III fragment as would be expected if this fragment corresponded to the 2.4 kb *Hind*III fragment from pSA2.

Possible models for the insertion of Tn*4351*::*catIII* into the *P. gingivalis* chromosome are represented diagrammatically in figure 4.4. Transconjugants 3, 7 and 8 contained two copies of *catIII* as well as both the conserved 2.4 kb and the 7.9 kb fragments. This suggested that these transconjugants contained a cointegrate formation of two copies of Tn*4351*::*catIII* which flanked plasmid (pSA2) sequences (figure 4.4A). It was proposed that transconjugants 6 and 9 contained one copy of Tn*4351*::*catIII* with plasmid sequences attached to the transposon at a Tn*4351*::*catIII*/pJRD215 junction, as well as a separate IS*4351*::*catIII* insertion elsewhere in the chromosome (Figure 4.4B). Transconjugants 2 and 4 were proposed to contain a single copy of Tn*4351*::*catIII*, with plasmid sequences attached to the transposon at a Tn*4351*::*catIII*/pAO6 junction, as well as a separate IS*4351*::*catIII* insertion elsewhere in the chromosome (transconjugant number 2) or a separate IS*4351*$_L$ insertion elsewhere in the chromosome (transconjugant number 4) as demonstrated in figure 4.4C. Transconjugant number 10 was thought to contain a partially deleted cointegrate insertion as shown in figure 4.4D. Transconjugant number 5 was proposed to contain a transposon insertion similar to those described for model C as well as a separate partial Tn*4351*::*catIII* insertion, containing some attached pJRD215 plasmid sequences, elsewhere in the chromosome (figure 4.4E). Transconjugant number 1 did not contain either of the conserved fragments. Southern blot analysis of transconjugant number 1 demonstrated the hybridisation of the *catIII* probe to the > 12 kb fragment and not to the 11.0 kb or 4.0 kb fragments. In addition to this the pJRD215 probe hybridised to a fragment of > 12 kb but not to fragments of 5.28, 2.78 and 2.33 kb. It appeared from this that transconjugant

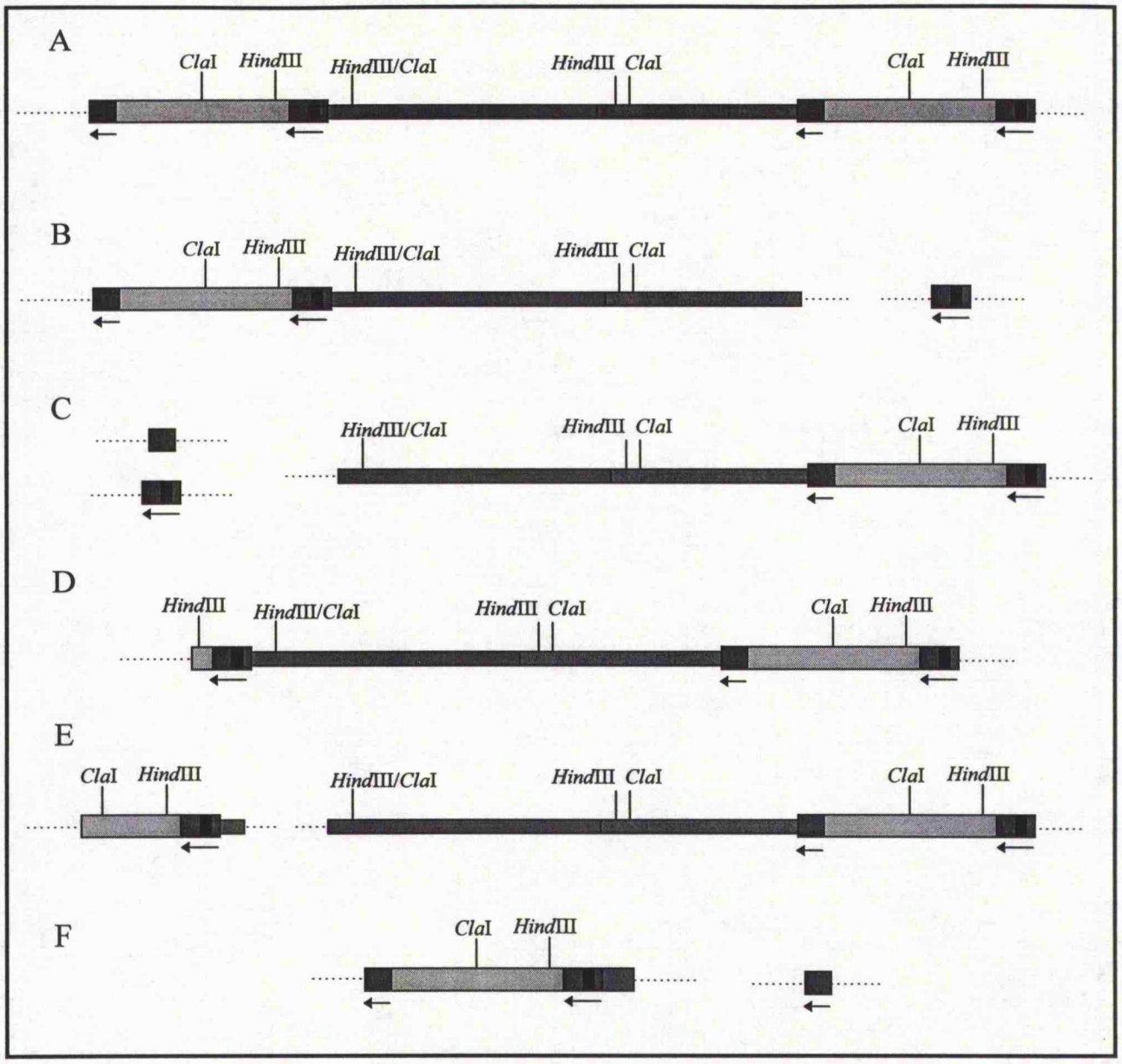


Fig. 4.4. Proposed models for the insertion of Tn*4351*::*catIII* into the *P. gingivalis* 381 chromosome from the plasmid pSA2. Model A applies to transconjugants 3, 7 and 8, model B applies to transconjugants 6 and 9, model C applies to transconjugants 2 and 4, model D applies to transconjugant 10, model E applies to transconjugant number 5 and model F applies to transconjugant number 1. Hashed lines represent chromosomal sequences. Arrows represent the direction of the insertion elements. ▬ represents pJRD215 sequences and ▬ represents pAO6 sequences. ▬ represents IS*4351* sequences, ▭ represents Tn*4351* sequences and ▬ represents the position of *catIII*.

number 1 contained a single Tn*4351*::*catIII* insertion that had some of the pJRD215 plasmid sequences attached to it as well as a separate IS*4351*$_L$ insertion elsewhere in the chromosome (figure 4.4F).

To test these proposals chromosomal DNA from transconjugants 3, 6, 7, 8 and 9 was digested with *Cla*I restriction enzyme and subjected to Southern blot analysis using a radiolabelled 2.4 kb *Hind*III fragment from pSA2 as a probe (figure 4.5). Chromosomal DNA from 1, 2, 4, 5 and 10 was also digested with *Cla*I restriction enzyme and then subjected to Southern blot analysis using a radiolabelled 7.9 kb *Hind*III fragment from pSA2 as a probe (figure 4.5). The expected fragments for the six models are shown in figures 4.6a and 4.6b. Due to the homologous nature of the insertion elements both probes can hybridise to fragments containing either of the insertion elements. Figure 4.7 demonstrates the Southern blot hybridisation analysis for transconjugants 1 - 10 using the radiolabelled 7.9 *Hind*III fragment as a probe (fig. 4.7A) or the radiolabelled 2.4 kb *Hind*III fragment as a probe (fig. 4.7B) and Table 4.4 lists the fragment sizes hybridised by the probe. The Southern blot analysis confirmed the insertion of a cointegrate formation in transconjugants 3, 7 and 8 and confirmed the proposed model for the Tn*4351*::*catIII* insertion in transconjugant number 6. However, Southern blot analysis of transconjugant number 9 did not confirm the proposed model for the Tn*4351*::*catIII* insertion. This model predicted that the 2.4 kb *Hind*III probe would hybridise to three fragments of > 1.9 kb, > 2.0 kb and 3.92 kb, instead the probe only hybridised to two fragments of 3.92 kb and 9.0 kb. It is possible that the 9 kb fragment represented a doublet or alternatively that the transposon had a deletion close to the *Cla*I restriction enzyme site such that the 2.4 kb *Hind*III probe only hybridised to two fragments. For the transconjugants that were analysed by Southern blot hybridisation using the 7.9 kb *Hind*III probe, only transconjugant number 1 demonstrated the expected banding pattern as predicted by the models in figure 4.6b. In transconjugant number 2 the 7.9 kb probe hybridised to four fragments rather than the expected three fragments and although all the bands appear with the same intensity, it is possible that one of these bands represents a partially digested fragment. For transconjugants numbers 4 and 5 the Southern blot analysis revealed that the 7.9 kb probe hybridised to the predicted number of fragments.

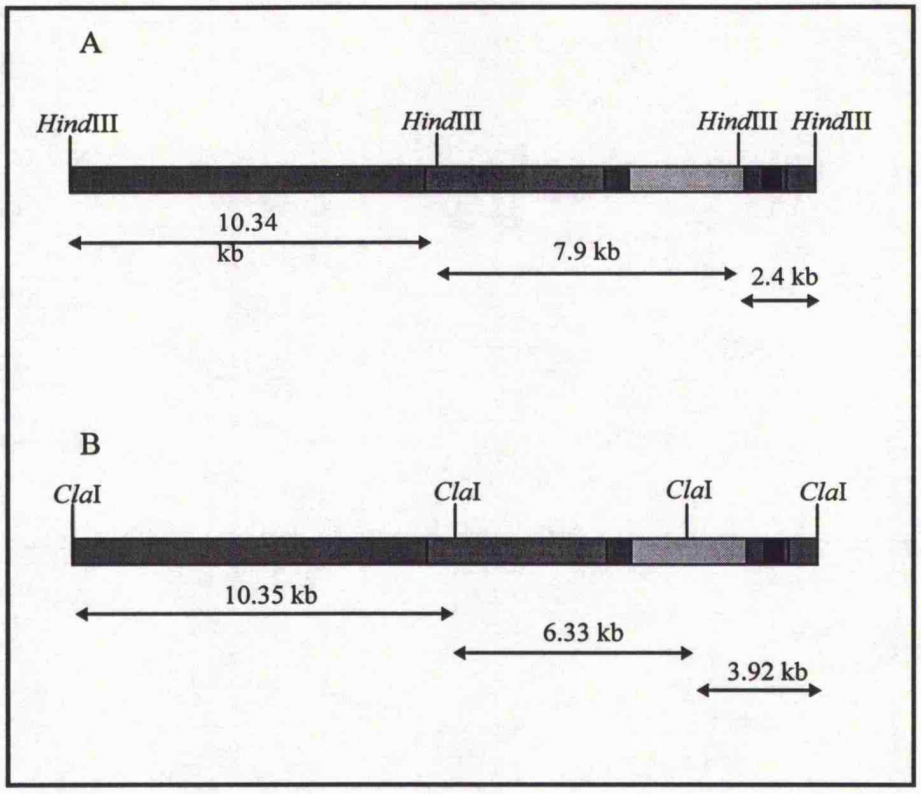


Fig. 4.5. Schematic drawing of the *Hind*III (A) and *Cla*I (B) restriction maps of pSA2. The 2.4 kb and 7.9 kb *Hind*III fragments were used as probes on *Cla*I digested transconjugants to determine the insertion patterns of Tn*4351*::*catIII* in *P. gingivalis* 381.

represents pJRD215 sequences, represents pAO6 sequences, represents IS*4351* sequences represents Tn*4351* sequences and represents *catIII*

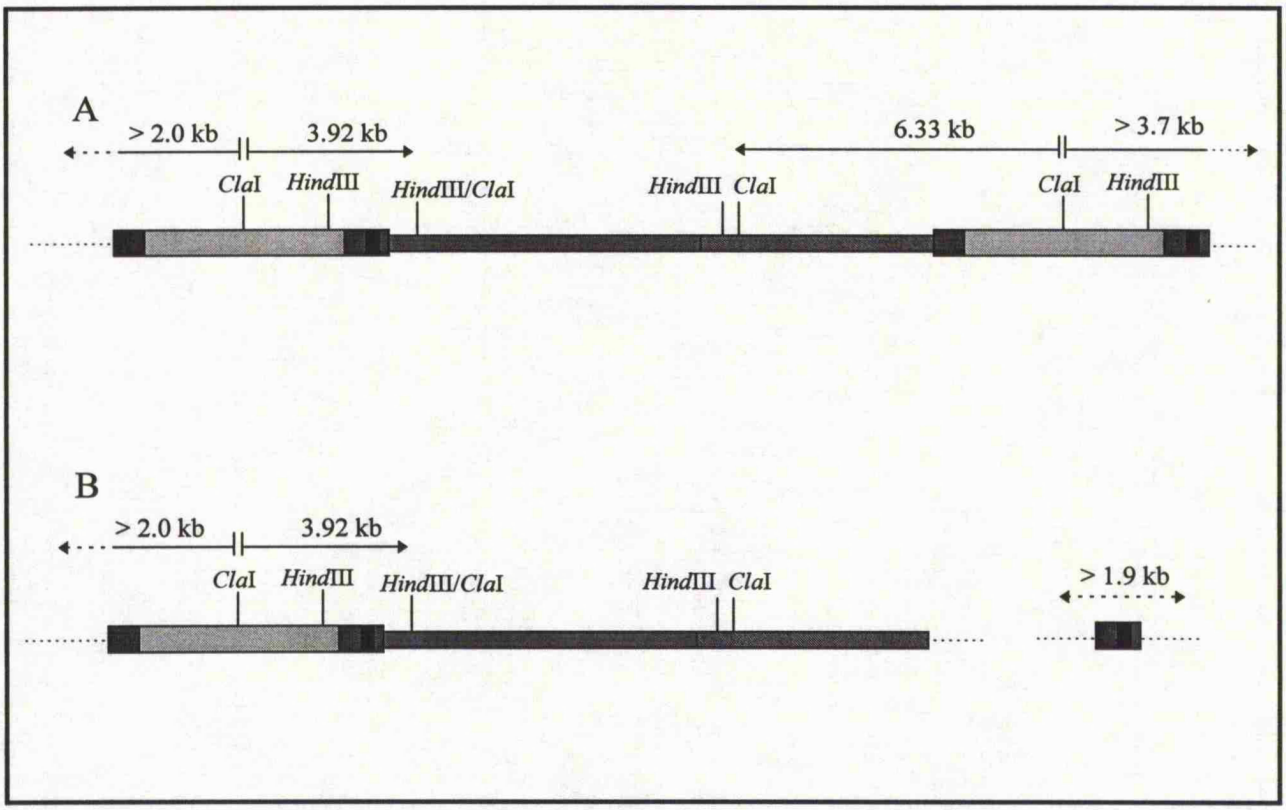


Fig. 4.6a. Fragment sizes predicted for transconjugants numbers 3, 7 and 8 (model A) and 6 and 9 (model B) from an S17-1* (pSA2)/*P. gingivalis* 381 filter mating digested with *Cla*I restriction enzyme and hybridised to a 2.4 kb *Hind*III probe from pSA2. Hashed line arrows represent fragments of unknown size while solid arrows represent fragments of defined size.

represents pJRD215 sequences, represents pAO6 sequences, represents Tn*4351* sequences, represents IS*4351* sequences and represents *catIII*.

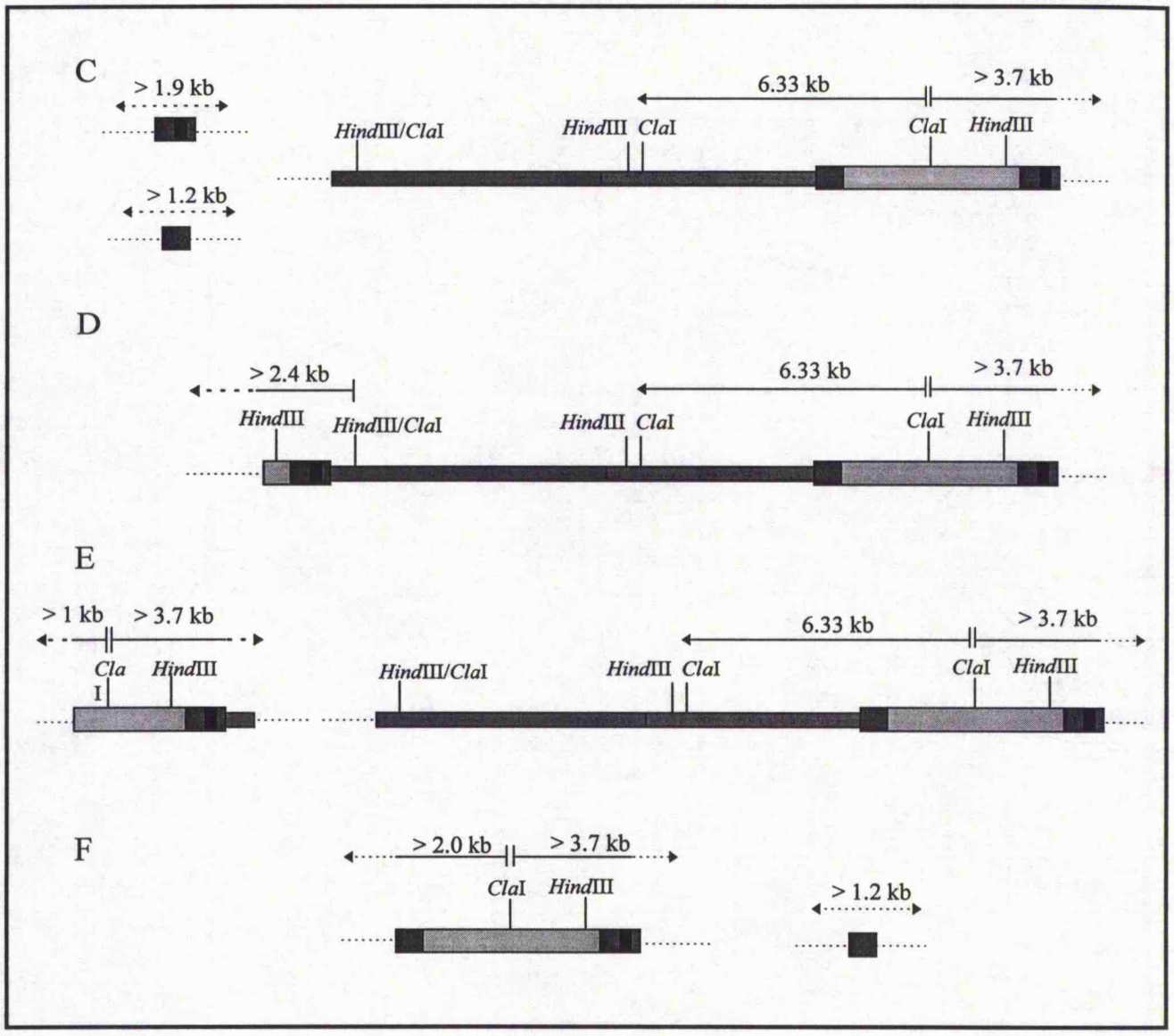


Fig. 4.6b. Fragment sizes predicted for transconjugants numbers 1 (model F), 2 and 4 (model C), 5 (model E) and 10 (model D) from an S17-1* (pSA2)/*P. gingivalis* 381 filter mating experiment digested with *Cla*I restriction enzyme and hybridised to a 7.9 kb *Hind*III probe from pSA2

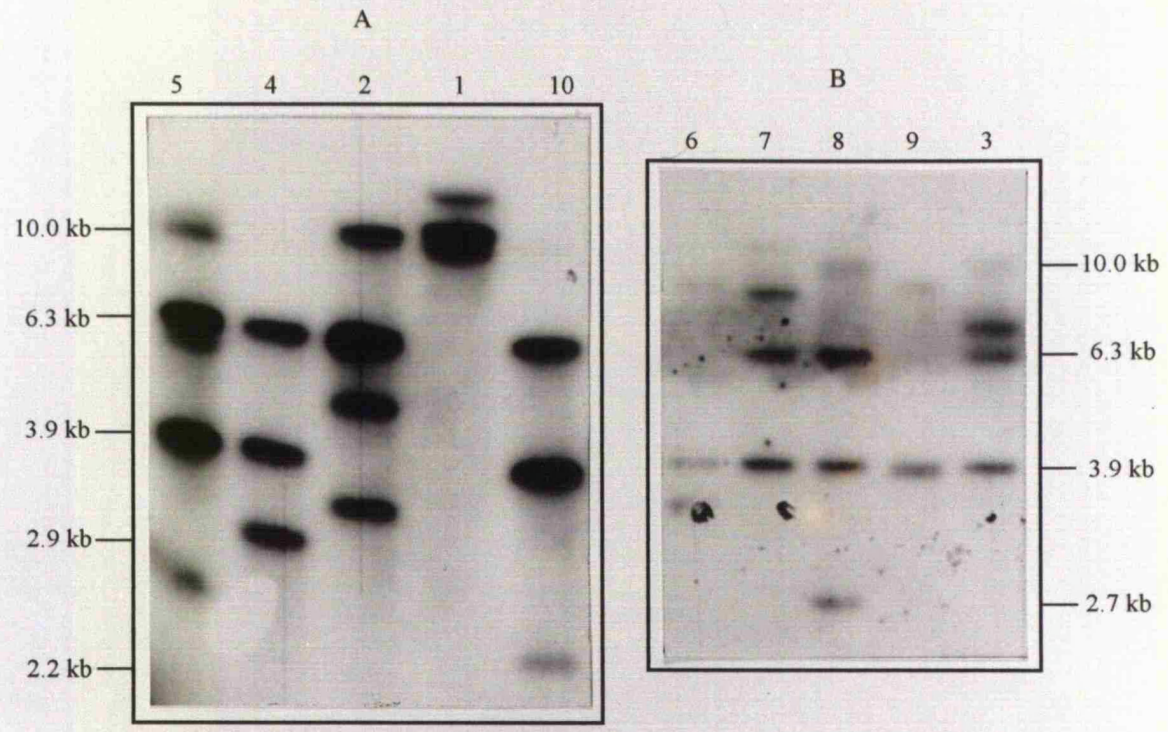


Fig. 4.7. Southern blot hybridisation analysis of *P. gingivalis* transconjugants. Chromosomal DNA from ten independent Cl^r/Gm^r transconjugants of *P. gingivalis* 381 transferred with the suicide vector pSA2 (lanes 1 - 10) were digested with *Cla*I restriction enzyme, separated by gel electrophoresis and transferred by Southern blot to hybridisation membrane. The membranes were hybridised with a [^{32}P] labelled 7.9 kb *Hind*III fragment from pSA2 (fig. 4.7A) or with a [^{32}P] labelled 2.4 kb *Hind*III fragment from pSA2 (fig. 4.7B). Size markers (in kilo bases) are indicated at the sides. Hybridisation of *P. gingivalis* 381 DNA to the 7.9 kb or 2.4 kb probes was not observed (data not shown).

Table 4.4a

	Transconjugants (Gm^r/Cl^r)				
	1	**2**	**4**	**5**	**10**
Fragment Sizes (kb)	> 13 11.0 9.0	10.0 6.3 4.9 3.2	6.3 3.9 2.9	10.0 6.3 5.9 3.9	6.3 3.9 2.2

Table 4.4b

	Transconjugants (Gm^r/Cl^r)				
	3	**6**	**7**	**8**	**9**
Fragment Sizes (kb)	10.0 7.0 6.3 3.9	9.0 3.9 3.4	> 13 8.5 6.3 3.9	10.0 6.3 3.9 2.7	9.0 3.9

Table 4.4. Fragment sizes seen after hybridisation of a 7.9 kb *Hind*III radiolabelled fragment (Table 4.4a) or a 2.4 kb *Hind*III radiolabelled fragment (Table 4.4b) from pSA2 to *Cla*I digested transconjugants from an S17-1* (pSA2)/*P. gingivalis* filter mating.

However, as well as the expected 6.33 kb fragment these transconjugants also appeared to contain the conserved 3.92 kb fragment. Since the 2.4 kb *Hind*III fragment is contained within the 3.92 kb *Cla*I fragment (fig. 4.5), the 3.92 *Cla*I fragment can not be present without the 2.4 kb *Hind*III fragment also being present unless transposon-induced rearrangements have occurred. Further Southern blot analysis could have allowed the true arrangement of these insertions to be characterised, however, since these transconjugants clearly did not contain the type of insertions that we were interested in no further analysis was carried out.

Figure 4.8 shows the Southern blot analysis of randomly selected transconjugants from S17-1* (pSA1)/*P. gingivalis* 381 filter mating experiments. Chromosomal DNA from the transconjugants was digested with *Hind*III restriction enzyme and subjected to Southern blot analysis using the 3.6 *Eco*RI probe. Table 4.5 demonstrates the fragment sizes hybridised by the radiolabelled 3.6 kb *Eco*RI probe. Chromosomal DNA from the transconjugants was also digested with *Hind*III and subjected to Southern blot analysis using a radiolabelled *catIII* probe (data not shown) and the fragments hybridised by this probe are indicated by an asterisks in Table 4.5. The Southern blot analysis of transconjugants 7, 8, 9 and 10 showed two fragments of > 2.1 kb and > 3.6 kb for each transconjugant that hybridised to the 3.6 kb *Eco*RI probe indicating single insertion of Tn*4351*::*catIII* into the chromosome. This represents a frequency of single insertions of Tn*4351*::*catIII* in *P. gingivalis* 381 of 30% when pSA1 is used as the suicide vector. Chromosomal DNA from the transconjugants was also digested with *Eco*RV restriction enzyme and analysed by Southern blot analysis using a radiolabelled pJRD215 probe to determine whether the plasmid had also inserted into the chromosome (figure 4.9). No hybridisation of transconjugant DNA with the pJRD215 probe was observed for transconjugants numbers 7 and 8 indicating that these transconjugants did not contain any plasmid sequences. Fragments of 8.5 kb and 4.5 kb were hybridised by the radiolabelled pJRD215 probe for transconjugants 9 and 10 respectively (The additional faint band seen for transconjugant 10 as well as additional bands seen for transconjugants 1 - 4 represent hybridised fragments from a previous Southern blot analysis that had not been efficiently removed from the hybridisation membrane). This

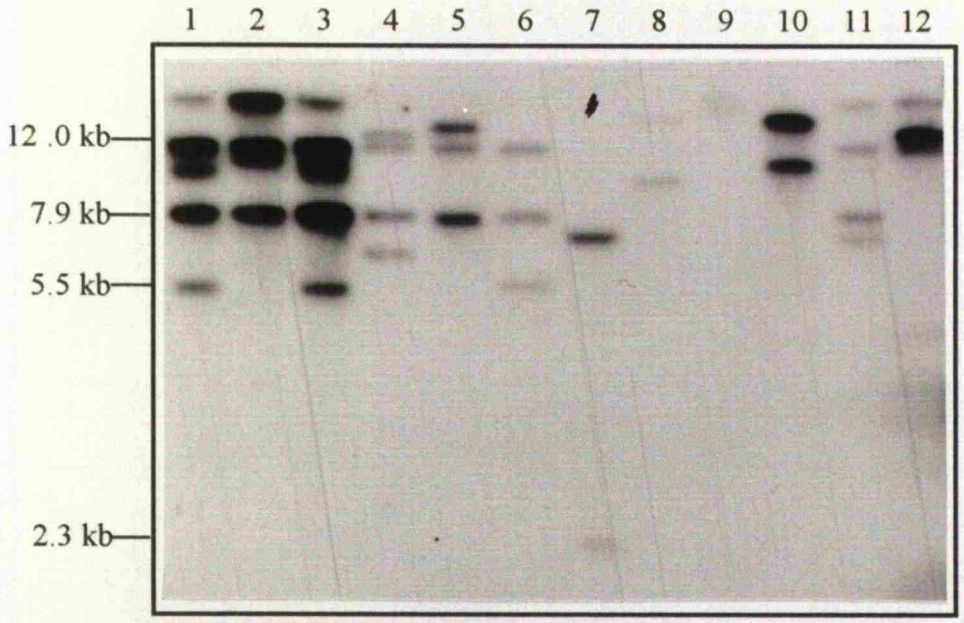


Fig. 4.8. Southern blot hybridisation analysis of *P. gingivalis* transconjugants. Chromosomal DNA from twelve independent Cl^r/Gm^r transconjugants of *P. gingivalis* 381 transferred with the suicide vector pSA1 (lanes 1 - 12) were digested with *Hind*III restriction enzyme, separated by gel electrophoresis and transferred by Southern blot to hybridisation membrane. The membrane was hybridised with a [^{32}P] labelled 3.6 *Eco*RI fragment from pVOH1. Size markers (in kilo bases) are indicated to the left. Hybridisation of the probe to *P. gingivalis* 381 DNA was not observed

	Transconjugants (Gm^r/Cl^r)											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Fragment sizes	12.0*	> 12*	12.0*	> 12*	> 12*	> 12*	7.3	> 12*	> 12	> 12	> 12*	> 12*
	10.0*	12.0*	10.0*	12.0*	12.0*	12.0*	2.3*	10.0	> 12*	11.5*	12.0*	> 12
	7.9	11.0	7.9	7.9	7.9	7.9					7.9	12.0*
	5.5	8.0	5.5	6.2		5.9					7.0	

Table 4.5. Fragment sizes seen by Southern blot analysis of *Hind*III digested transconjugant DNA from an S17-1* (pSA1)/*P. gingivalis* 381 filter mating experiment hybridised to a 3.6 kb *Eco*RI probe. The asterisk represents fragments that were also hybridised by a *catIII* probe. A conserved fragments of 12.0 kb can be seen for transconjugants 1 - 6 and 11 - 12. A conserved fragment of 8.0 kb can seen for transconjugants 1 - 6 and 11.

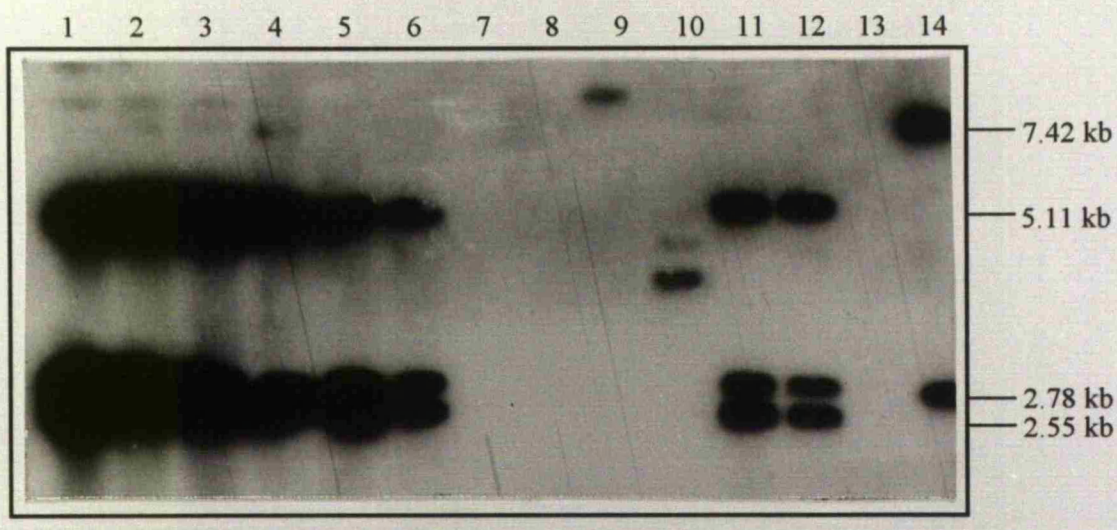


Fig. 4.9. Southern blot analysis of *P. gingivalis* transconjugants. Chromosomal DNA from twelve independent Cl^r/Gm^r transconjugants (lanes 1 - 12) of *P. gingivalis* 381 transferred with the suicide vector pSA1 were digested with *Eco*RV restriction enzyme, separated by gel electrophoresis and transferred by Southern blot to hybridisation membrane. The membrane was hybridised to a [^{32}P] labelled pJRD215 probe. Lane 13 represents *Eco*RV digested *P. gingivalis* 381 chromosomal DNA hybridised to the probe and lane 14 represents *Eco*RV digested pJRD215 hybridised to the probe. Size markers in kilo bases are indicated to the right of the autoradiograph.

indicated that these transconjugants did contain some plasmid sequences up to, but not including, the first *Eco*RV restriction enzyme site of pJRD215. For all other transconjugants, Southern blot analysis of transconjugant DNA demonstrated hybridisation of the pJRD215 probe to fragments of 5.11 kb, 2.78 kb and 2.55 kb, the fragment sizes expected if the entire plasmid had inserted into the chromosome. The Southern blot analysis of transconjugants 1 - 4 and 11, using the radiolabelled 3.6 kb *Eco*RI probe demonstrated hybridisation of the probe to four fragments in each transconjugant, two of which were the conserved 12.0 kb and 7.9 kb fragments which correspond to two of the *Hind*III fragments from pSA1, as well as to two other fragments of variable size. This suggested that these transconjugants contained cointegrate formation insertions (figure 4.10a). Transconjugants 5 and 6 were also proposed to be cointegrate formations. The > 12 kb fragment seen for transconjugant 5 was proposed to be a doublet and this theory was supported by the intensity of this band on the autoradiograph. A band of > 12 kb could also be seen for transconjugant 6 upon prolonged exposure of the autoradiograph. However, it is also possible that these transconjugants contained partially deleted cointegrate insertions such that the > 3.6 kb *Hind*III fragment was missing from transconjugant number 5 and the > 2.1 kb *Hind*III fragment was missing from transconjugant number 6 (figure 4.10b). Transconjugant 12 contained the conserved 12.0 kb fragment but not the conserved 7.9 kb fragment. It was proposed that this transconjugant contained a single copy of Tn*4351*::*catIII* with attached plasmid sequences (at a pJRD215/Tn*4351*::*catIII* junction) as well as a separate IS*4351*::*catIII* insertion else where in the chromosome (figure 4.10a (B)). No further analysis was carried out on these transconjugants.

4.2.3 Transfer of pSA1 and pSA2 from *E. coli* into *P. gingivalis* 33277

The appearance of Cl^r transconjugants on selective media plates from a *P. gingivalis* 381 mating take up to 21 days (Dyer *et al.*, 1992) therefore, it was decided to transfer the suicide plasmids pSA1 and pSA2 into the *P. gingivalis* strain 33277. Data suggested that transconjugants in this strain could appear on the selective plates after 7 - 10 days anaerobic incubation (Genco *et al.*, 1995a; Hoover *et al.*, 1992b). This was

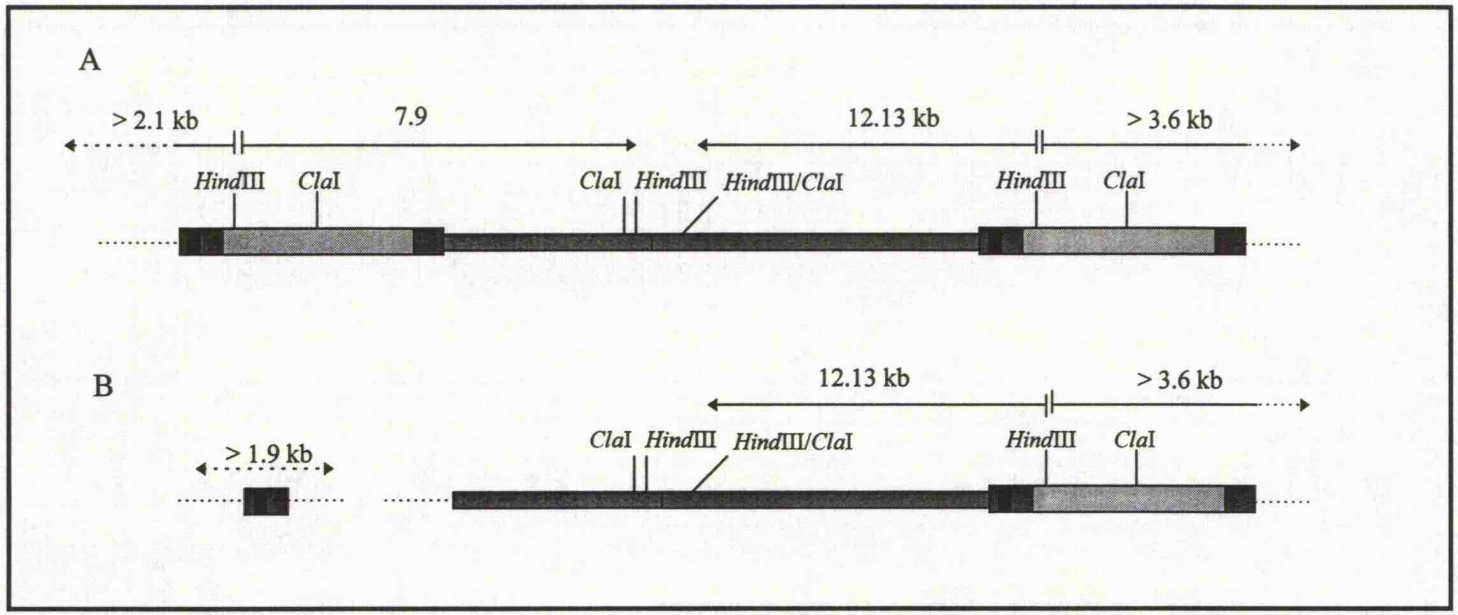


Fig. 4.10a. Expected sizes of *Hind*III fragments from transconjugants from a S17-1* (pSA1)/*P. gingivalis* 381 filter mating experiment which have a cointegrate formation inserted into the chromosome (fig. 4.10A) or a single insertion of Tn*4351*::*catIII* with attached plasmid sequences and a separate IS*4351*::*catIII* insertion elsewhere in the chromosome (fig. 4.10B). ▬ represents pJRD215 sequences, ▬ represents pAO6 sequences, ▭ represents Tn*4351* sequences, ▬ represents IS*4351* sequences and ▬ represents *catIII* sequences. Hashed lines represents chromosomal sequences. Solid arrows represent defined fragments whilst hashed line arrows represents fragments of variable size

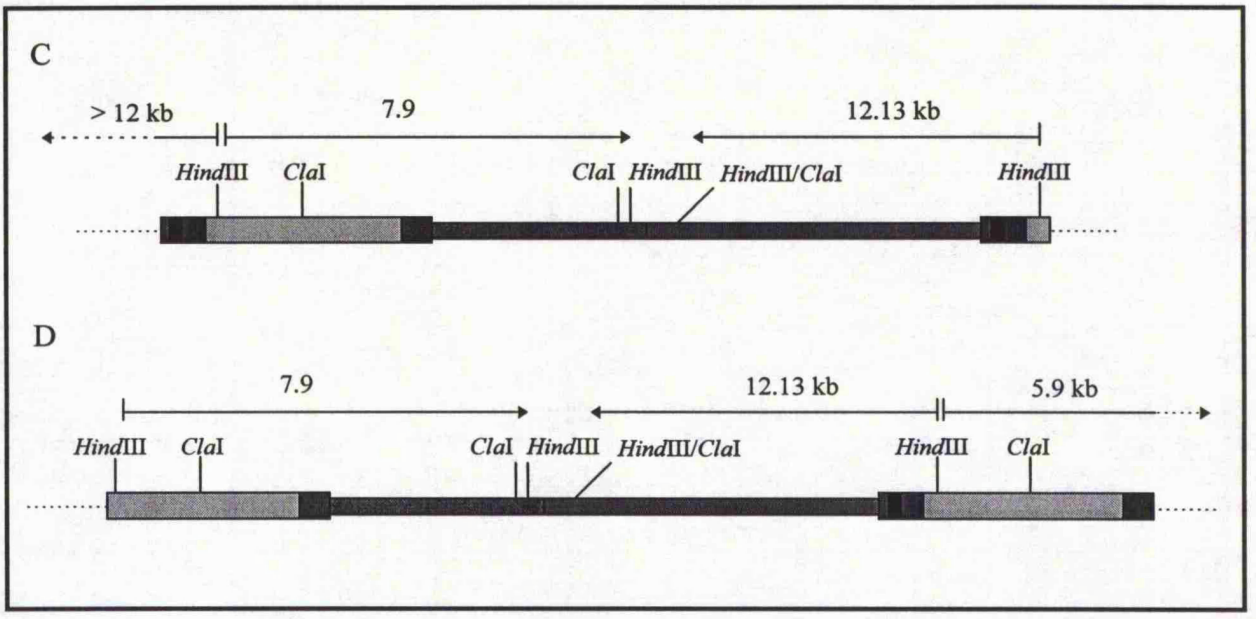


Fig. 4.10b. Two alternative models to describe the Tn*4351*::*catIII* insertion in transconjugnt number 5 (model C) and transconjugant number 6 (model D). ▬ represents pJRD215 sequences, ▬ represents pAO6 sequences, ▬ represents Tn*4351* sequences, ▬ represents IS*4351* sequences and ▬ represents *catIII* sequences. Hashed lines represent chromosomal sequences.

found to be the case and thus reduced the mating time considerably. Because strain 33277 has a shorter generation time than strain 381 mating mixtures were left on filters for 48 - 72 hours (including 15 hours aerobic incubation) rather than the 96 hours incubation used for strain 381. All other mating conditions remained as before (section 4.2.1). To determine whether the incubation time of the mating mixtures affected the frequency of transposition of Tn*4351*::*catIII* into strain 33277, filter mating experiments were carried out using two different incubation times. Table 4.6 demonstrates the frequency of transposition of Tn*4351*::*catIII* into *P. gingivalis* 33277 using either S17-1* (pSA1) or HB101 (R751::*Ω4) as the donor strain. The filter mating experiments were carried out using different donor/recipient ratios and the mating mixtures were incubated on filters for 48 or 72 hours before being plated onto selective media. The frequency of transposition was compared for each plasmid at the two incubation times. In general the frequency of transposition was higher for matings incubated for 48 hours although this was not true in all cases. It was concluded that factors such as donor/recipient ratios or starting CFU/ml for donor or recipient cells could effect the frequency of transposition regardless of whether the mating mixtures were incubated for 48 or 72 hours. Mating mixtures in future experiments were incubated for between 48 - 72 hours.

In order that the effect that the *catIII* insertion in IS*4351*$_R$ had on transposition frequency could be established, a control vector pSA3 was constructed. The vector was constructed from pAO5 (containing Tn*4351**) and pJRD215 in a cloning procedure similar to that used to construct pSA1 and pSA2 (section 3.3.6). Plasmids pAO5 and pJRD215 were cleaved with *Bam*HI restriction enzyme, ligated together and transformed into the *E. coli* strain S17-1*. Recombinants containing pSA3 were then selected on LA supplemented with Km and Ap. Restriction enzyme analysis of small scale plasmid extraction's of Km^r/Ap^r resistance colonies confirmed that pAO5 and pJRD215 had ligated together and allowed the orientation of pAO5 in pJRD215 to be determined. Plasmid pAO5 was found to have ligated into pJRD215 in the same orientation that pAO6 had ligated into pJRD215 to create plasmid pSA1 as illustrated in figure 4.11.

Table 4.6a

Starting CFU/ml		Donor/recipient ratio	Transposition Frequency†	
Donor	**Recipient**		**48 hours incubation**	**72 hours incubation**
2.5×10^8	1.93×10^8	1.3:1	8.47×10^{-7}	1.47×10^{-7}
2.5×10^8	7.9×10^8	1:3	8.46×10^{-8}	4.19×10^{-8}
2.7×10^8	1.29×10^8	2:1	9.17×10^{-7}	5.6×10^{-6}

Table 4.6b

Starting CFU/ml		Donor/recipient ratio	Transposition Frequency†	
Donor	**Recipient**		**48 hours incubation**	**72 hours incubation**
6.8×10^8	1.93×10^8	3.5:1	3.11×10^{-7}	1.26×10^{-6}
6.8×10^8	7.96×10^8	1:1.2	1.42×10^{-6}	2.62×10^{-7}
3.2×10^8	1.29×10^8	2.5:1	5.04×10^{-6}	2.17×10^{-7}

† Transposition frequencies are expressed as the number of CFU of transconjugants per CFU of recipient per millilitre

Table 4.6. Transposition frequency of Tn*4351*::*catIII* or Tn*4351* into *P. gingivalis* 33277 as a result of filter mating experiments using S17-1* (pSA1) as a donor (Table 4.6a) or HB101 (R751::*Ω4) as a donor (Table 4.6b). Mating mixtures were left on filters for 48 or 72 hours before being plated onto selective media.

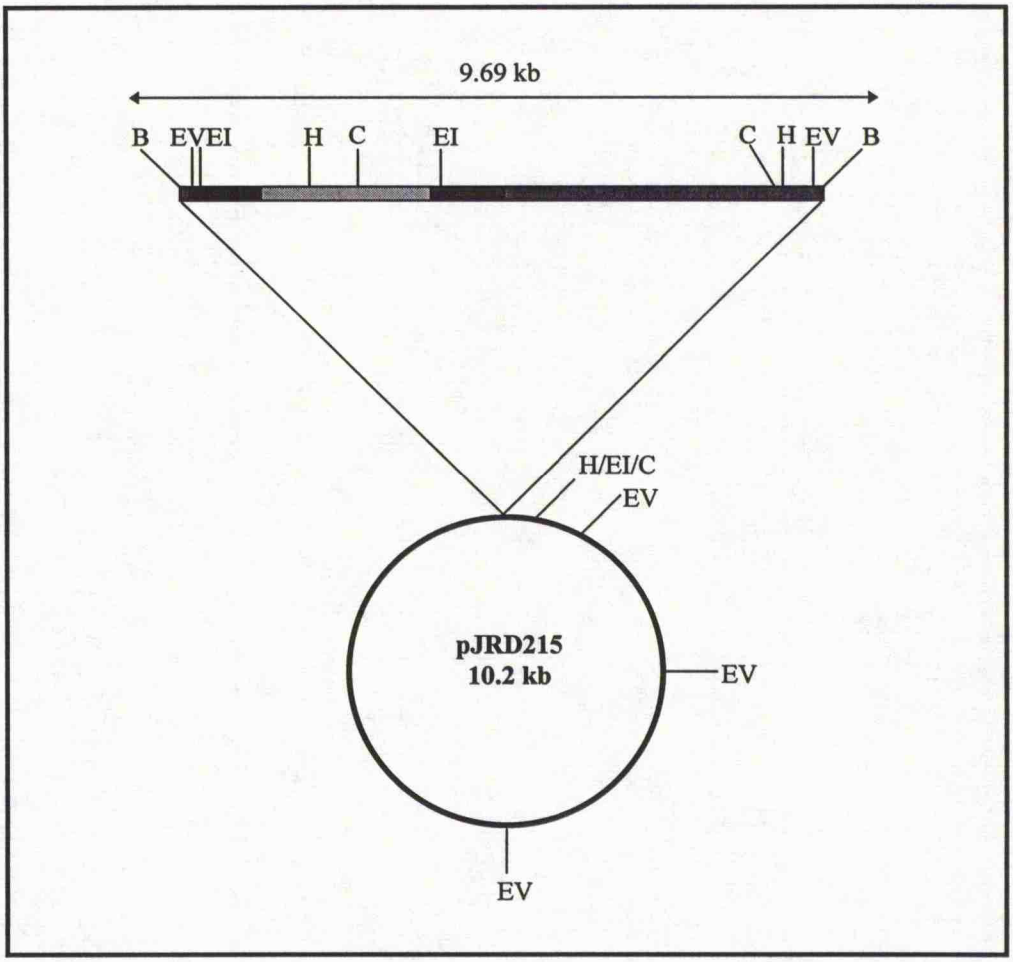


Fig. 4.11. Schematic representation of the suicide vector pSA3. The 9.69 kb plasmid pAO5 (containing Tn4351*) was cloned into the *Bam*HI restriction enzyme site of the 10.2 kb suicide vector pJRD215 resulting in the suicide vector pSA3. ▬ Represents pAO5 sequences ▭ represents Tn*4351** sequences and ▬ represents IS*4351* sequences. Some restriction enzyme sites are indicated on the diagram where EI = *Eco*RI, EV = *Eco*RV, H = *Hind*III, C = *Cla*I, B = *Bam*HI

The frequencies of transposition of Tn*4351*::*catIII* into *P. gingivalis* 33277 from plasmids pSA1 and pSA2 are shown in Tables 4.7a and 4.7b. These frequencies of transposition were compared to the transposition frequency of Tn*4351** into *P. gingivalis* 33277 from plasmid pSA3 (Table 4.7c) and to the transposition frequency of Tn*4351* into *P. gingivalis* 33277 from plasmid R751::*Ω4 (Table 4.7d) where the frequency of transposition represents the number of CFU/ml transconjugants (Gm^r/Cl^r) per CFU/ml recipient. The frequencies of transposition demonstrated represent average frequencies of transposition for several separate mating experiments, each using different variables (i.e. donor:recipient ratios and starting CFU/ml). The frequencies range from between 3.72×10^{-6} to 6.33×10^{-8} for pSA1, between 3.27×10^{-6} to 4.50×10^{-8} for pSA2, between 4.21×10^{-6} to 6.01×10^{-9} for pSA3 and between 9.55×10^{-6} to 1.49×10^{-7} for R751::*Ω4. This represents an overall average frequency of transposition of 7.02×10^{-6} for pSA1, 9.24×10^{-7} for pSA2, 9.91×10^{-7} for pSA3 and 2.13×10^{-6} for R751::*Ω4. From this it appears that the presence of the *catIII* insert within Tn*4351** does not reduce the frequency of transposition.

4.2.4 Analysis of Clindamycin Resistant Transconjugants from *P. gingivalis* 33277

Southern blot analysis was carried out on eleven randomly selected transconjugants from an S17-1* (pSA2)/*P. gingivalis* 33277 filter mating experiment. The chromosomal DNA from the transconjugants was digested with *Hind*III, separated by agarose gel electrophoresis and transferred by Southern blot to hybridisation membrane. The Southern blots were then probed with the 3.6 kb *Eco*RI radiolabelled fragment from pVOH1. Figure 4.12A demonstrates the Southern blot analysis of the transconjugants using the 3.6 kb radiolabelled fragment and Table 4.8 demonstrates the actual size of the fragments to which the probe hybridised. Chromosomal DNA from the transconjugants was also digested *HindIII* restriction enzyme and subjected to Southern blot analysis using a radiolabelled *catIII* probe (figure 4.12B). The size of the fragments that the *catIII* probe hybridised to are indicated by an asterisk in Table 4.8. Southern blot analysis of *Hind*III digested chromosomal DNA from transconjugants 1 - 11 using the radiolabelled 3.6 kb *Eco*RI probe demonstrated that no single Tn*4351*::*catIII* insertions

Table 4.7a

Starting CFU/ml		Donor:Recipient ratio	Transposition Frequency[†]
Donor	**Recipient**		
2.50×10^8	1.90×10^8	1.3:1	4.97×10^{-7}
2.5×10^8	7.96×10^8	1:3	6.33×10^{-8}
2.7×10^8	1.29×10^8	2:1	3.26×10^{-6}
7.4×10^8	1.39×10^9	1:2	3.72×10^{-5}
2.19×10^9	2.86×10^9	1:1.3	7.13×10^{-8}
1.46×10^9	2.86×10^9	1:2	1.40×10^{-7}
ND	1.29×10^8	ND	2.44×10^{-5}
ND	1.25×10^8	ND	4.22×10^{-6}
6.80×10^8	7.03×10^8	1:1	1.09×10^{-7}
1.40×10^8	6.90×10^8	1:5	2.80×10^{-7}

[†] Transposition frequencies are expressed as the number of CFU of transconjugants per CFU of recipient per millilitre

Table 4.7a. Transposition frequency of Tn*4351*::*catIII* from the suicide vector pSA1 into *P. gingivalis* 33277. Results are from separate experiments and are representative of an average frequency for each experiment undertaken. ND = not done.

Table 4.7b

Starting CFU/ml		Donor:Recipient ratio	Transposition Frequency[†]
Donor	**Recipient**		
8.30×10^8	1.90×10^8	4.3:1	1.20×10^{-6}
3.20×10^8	1.29×10^8	2.5:1	3.27×10^{-6}
6.10×10^8	1.39×10^9	1:2.3	2.11×10^{-7}
7.00×10^8	2.86×10^9	1:4	8.50×10^{-8}
ND	6.90×10^8	ND	1.03×10^{-6}
ND	5.34×10^9	ND	2.84×10^{-7}
ND	1.25×10^9	ND	1.27×10^{-6}
1.05×10^9	7.03×10^8	1.5:1	4.50×10^{-8}

[†] Transposition frequencies are expressed as the number of CFU of transconjugants per CFU of recipient per millilitre

Table 4.7b. Transposition frequency of Tn*4351*::*catIII* from the suicide vector pSA2 into *P. gingivalis* 33277. Results are from separate experiments and are representative of an average frequency for each experiment undertaken. ND = not done.

Table 4.7c

Starting CFU/ml		Donor:Recipient ratio	Transposition Frequency[†]
Donor	**Recipient**		
6.60×10^{8}	1.39×10^{9}	1:2	6.01×10^{-9}
1.46×10^{9}	2.86×10^{9}	1:2	2.04×10^{-8}
5.20×10^{8}	6.90×10^{8}	1:1.3	5.32×10^{-7}
ND	1.29×10^{8}	ND	4.21×10^{-6}
1.00×10^{6}	2.30×10^{7}	1:23	1.16×10^{-6}
4.00×10^{6}	4.00×10^{8}	1:100	1.68×10^{-8}

[†] Transposition frequencies are expressed as the number of CFU of transconjugants per CFU of recipient per millilitre

Table 4.7c. Transposition frequency of Tn*4351* from the suicide vector pSA3 into *P. gingivalis* 33277. Results are from separate experiments and are representative of an average frequency for each experiment undertaken. ND = not done.

Table 4.7d

Starting CFU/ml		Donor:Recipient ratio	Transposition Frequency[†]
Donor	**Recipient**		
1.29×10^9	2.86×10^9	1:2.2	1.49×10^{-7}
ND	1.39×10^9	ND	2.95×10^{-7}
ND	1.25×10^9	ND	3.02×10^{-6}
4.20×10^8	7.03×10^8	1:1.7	9.55×10^{-6}
6.50×10^7	2.04×10^9	1:31	2.73×10^{-7}
6.40×10^7	1.90×10^9	1:30	1.46×10^{-6}
6.50×10^7	4.00×10^8	1:6	1.5×10^{-7}

[†] Transposition frequencies are expressed as the number of CFU of transconjugants per CFU of recipient per millilitre

Table 4.7d. Transposition frequency of Tn*4351* from the suicide vector R751::*Ω4 into *P. gingivalis* 33277. Results are from separate experiments and are representative of an average frequency for each experiment undertaken. ND = not done.

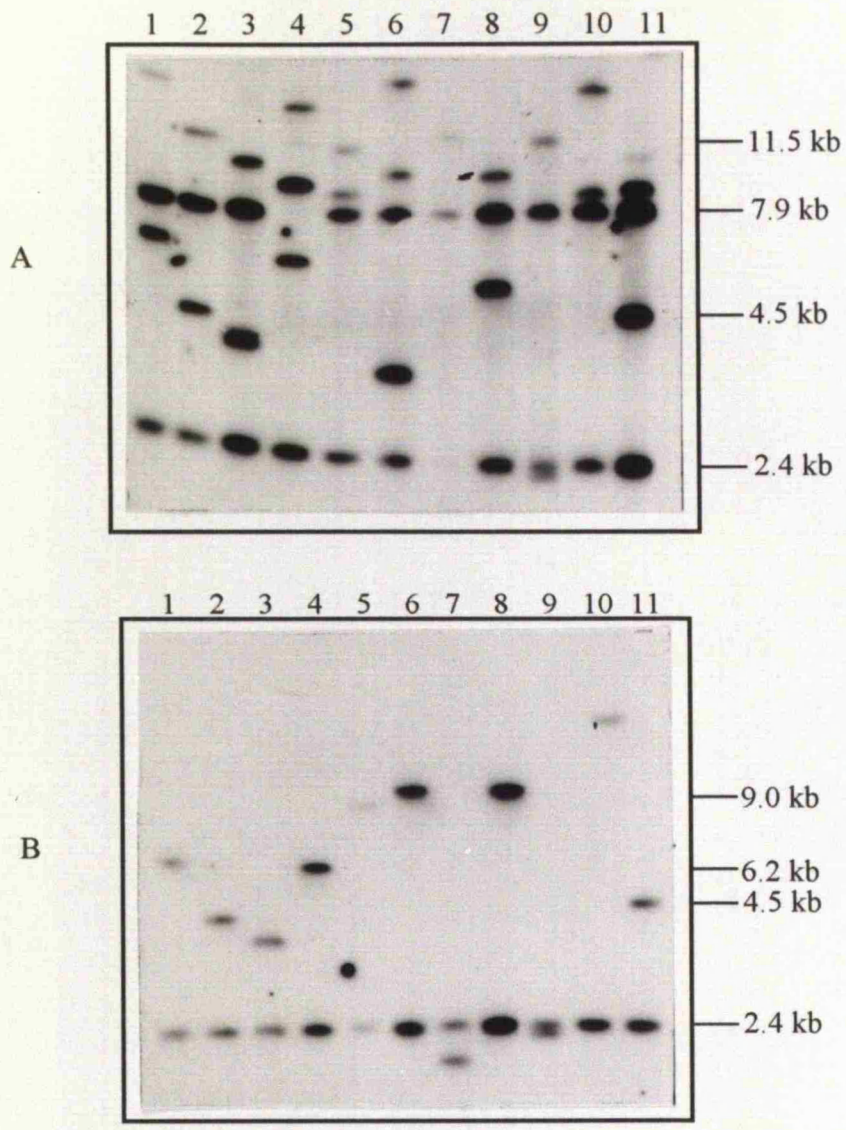


Fig. 4.12. Southern blot hybridisation analysis of *P. gingivalis* transconjugants. Chromosomal DNA from eleven independent Cl^r/Gm^r transconjugants of *P. gingivalis* 33277 transferred with the suicide vector pSA2 (lanes 1 - 11) were digested with *Hind*III restriction enzyme, separated by gel electrophoresis and transferred by Southern blot to hybridisation membrane. The membrane was hybridised with a [^{32}P] labelled 3.6 kb *Eco*RI fragment from pVOH1 (fig. 4.13A) or with a [^{32}P] labelled *catIII* fragment (fig. 4.13B). Size markers (in kilo bases) are indicated to the right. Hybridisation of *P. gingivalis* 33277 DNA to the 3.6 kb probe or to the *catIII* probe was not observed (data not shown).

	Transconjugants (Gm^r/Cl^r)										
	1	2	3	4	5	6	7	8	9	10	11
Fragment sizes (kb)	> 12	12.0	10.2	>12	11.5	9.0*	12.0	9.0*	11.5	>12*	8.3
	7.9	7.9	7.9	8.8	8.5*	7.9	7.9	7.9	7.9	8.2	7.9
	6.7*	4.6*	4.3*	6.2*	7.9	3.8	2.4*	5.2	2.4*	7.9	4.5*
	2.4*	2.4*	2.4*	2.4*	2.4*	2.4*	2.1*	2.4*	2.3*	2.4*	2.4*

Table 4.8. Fragment sizes seen by Southern blot analysis of eleven transconjugants from a S17-1* (pSA2)/*P. gingivalis* 33277 filter mating. *Hind*III digested chromosomal DNA from the transconjugants was probed with a 3.6 kb *Eco*RI probe. The probe contains most of the IS*4351*$_R$ sequences and the regions encoding *ermF* and *Tc^r determinants of Tn*4351*. The probe would be expected to hybridise to two fragments of > 2.1 kb and > 3.6 kb if a single Tn*4351::catIII* insertion had occurred. The 3.6 kb probe hybridised to more than two fragments in all transconjugants. Conserved fragments of 2.4 kb and 7.9 kb were observed. The asterisk (*) represents the fragments that were also hybridised by a *catIII* probe.

had occurred as indicated by the fact that the probe hybridised to four separate fragments for each transconjugant examined. The radiolabelled 3.6 kb *Eco*RI probe hybridised to two conserved fragments of 7.9 kb and 2.4 kb as well as two other fragments of variable size in transconjugants 1 - 3 and 5 - 11 and to the conserved 2.4 kb fragment and three other fragments of variable size in transconjugant number 4. Southern blot analysis of *Hind*III digested chromosomal DNA from transconjugants 1 - 11 using the radiolabelled *catIII* probe demonstrated that the 2.4 kb fragment contained a *catIII* insert. The fact that fragments of variable size were seen on the autoradiographs indicated that random insertion into *P. gingivalis* 33277 had occurred. It was speculated that transconjugants 1 - 3 and 5 - 11 contained cointegrate formation insertion as described previously (figure 4.4 model A) where the 7.9 kb and 2.4 kb fragments represent two of the *Hind*III fragments of pSA2. Transconjugant number 4 demonstrated the conserved 2.4 kb fragment and three other fragments, but did not have the conserved 7.9 kb fragment indicating that this did not contain a cointegrate insertion. It was proposed that in transconjugant number 4 two single insertions had occurred, one of which included insertion of pJRD215 plasmid sequences resulting in the conserved 2.4 kb fragment. To confirm the insertion patterns chromosomal DNA from transconjugants 1 - 11 was digested with *ClaI* restriction enzyme, separated by gel electrophoresis and analysed by Southern blot analysis using the radiolabelled 2.4 kb *Hind*III fragment from pSA2 (figure 4.13). Table 4.9 lists the sizes of the fragments hybridised to the 2.4 kb *Hind*III probe. Four fragments were hybridised by the 2.4 kb *Hind*III probe in each transconjugant (a partially digested fragment of > 12 kb was also hybridised by the 2.4 kb *Hind*III probe in transconjugant number 6). Conserved fragments of 6.3 kb and 3.2 kb corresponding to the 6.33 kb and 3.9 kb *Cla*I fragments of pSA2 were seen for transconjugants 1 - 3 and 5 - 11 confirming that these transconjugants did contain an insertion of a cointegrate formation. Transconjugant number 4, however, did not contain the expected 3.2 kb conserved fragment but did contain the 6.3 kb conserved fragment. This result suggested that transconjugant number 4 had originally contained a cointegrate insertion but subsequent transposon-induced rearrangements had deleted of some of the plasmid and transposon sequences. Figure 4.14 demonstrates two possible models to explain the banding patterns seen after

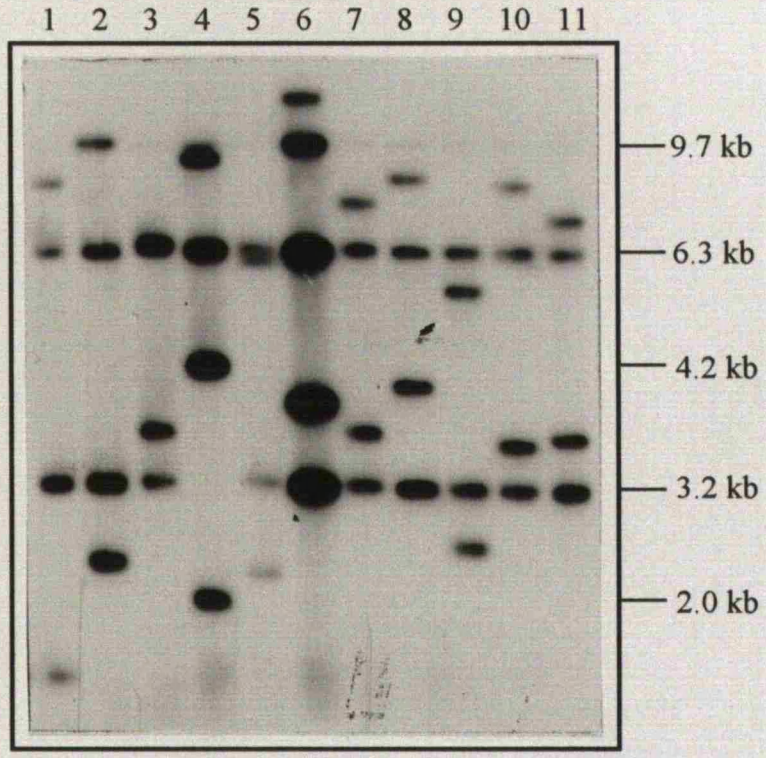


Fig. 4.13. Southern blot hybridisation analysis of *P. gingivalis* transconjugants. Chromosomal DNA from eleven independent Cl^r/Gm^r transconjugants of *P. gingivalis* 33277 transferred with the suicide vector pSA2 (lanes 1 - 11) were digested with *Cla*I restriction enzyme, separated by gel electrophoresis and transferred by Southern blot to hybridisation membrane. The membrane was hybridised with a [^{32}P] labelled 2.4 kb *Hind*III fragment from pSA2. Size markers (in kilo bases) are indicated to the right. Hybridisation of *P. gingivalis* 33277 DNA to the 2.4 kb probe was not observed (data not shown).

	Transconjugants (Gm^r/Cl^r)										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Fragment sizes (kb)	8.2	9.5	9.5	8.4	6.3	9.7	7.1	8.0	6.3	8.1	7.0
	6.3	6.3	6.3	6.3	5.9	6.3	6.3	6.3	5.5	6.3	6.3
	3.2	3.2	3.3	4.2	3.2	3.6	3.5	4.0	3.2	3.4	3.8
	1.8	2.3	3.2	2.0	2.2	3.2	3.2	3.2	2.4	3.2	3.2

Table 4.9. Fragment sizes seen after hybridisation of a 2.4 kb *Hind*III radiolabelled fragment from pSA2 to *Cla*I digested transconjugants from an S17-1* (pSA2)/*P. gingivalis* 33277 filter mating.

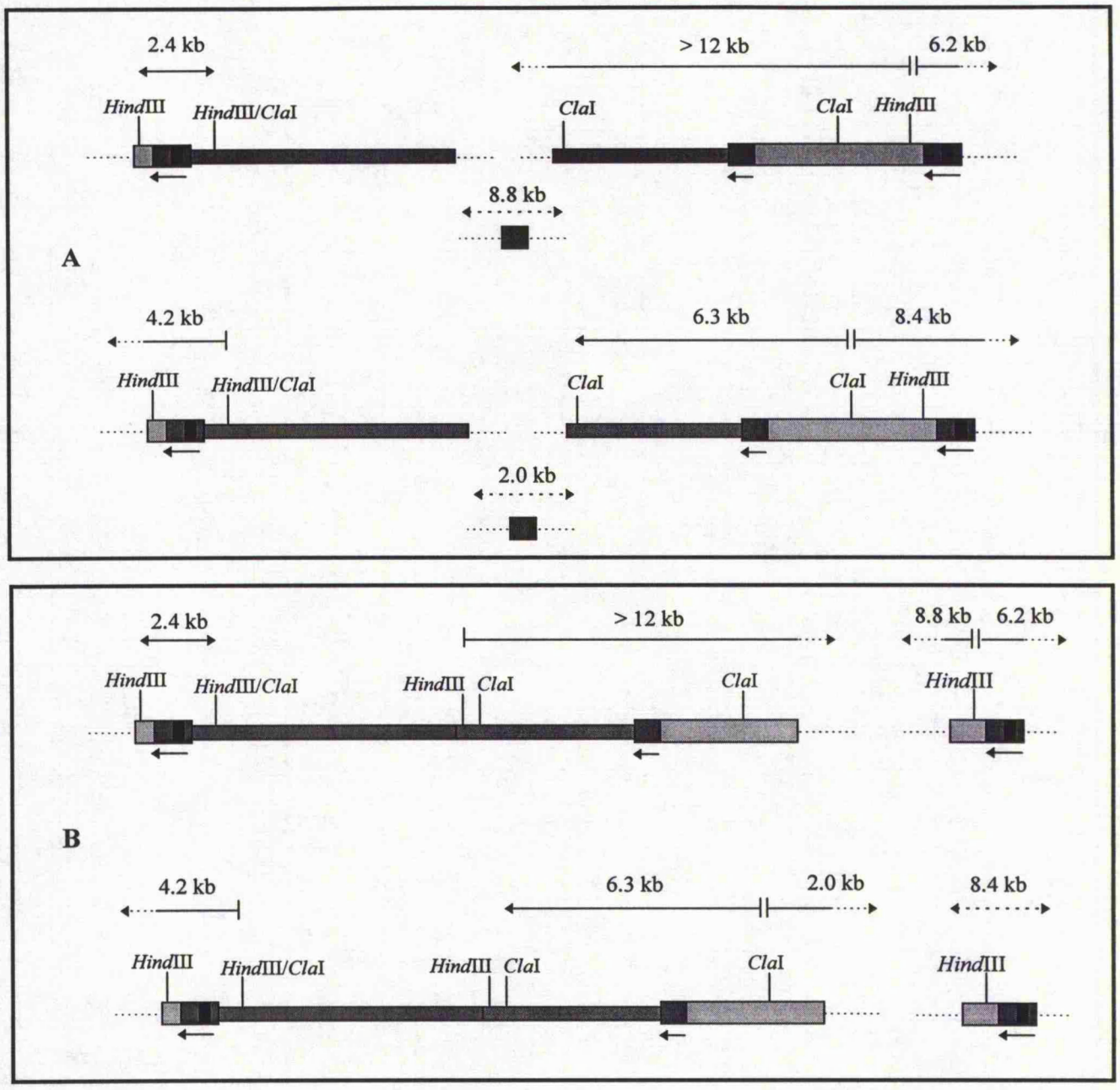


Fig. 4.14. Two possible models (models A and B) to explain the banding patterns seen when chromosomal DNA from transconjugant number 4, from an S17-1* (pSA2)/*P. gingivalis* 33277 filter mating experiment, was digested with *Hind*III and hybridised to the 3.6 kb *Eco*RI probe, or digested with *Cla*I and hybridised to the 2.4 kb *Hind*III probe. Hashed lines represent chromosomal sequences, arrows underneath insertion elements represent the direction of the insertion element. Solid arrows represent defined fragments whereas hashed line arrows represent fragments of unknown size. ▬ represents pJRD215 sequences, ▬ represents pAO6 sequences, ▬ represents IS*4351* sequences, ▬ represents Tn*4351* sequences and ▬ represents the position of *catIII*.

Southern blot analysis of *Hind*III digested chromosomal DNA from transconjugant 4 using the radiolabelled 3.6 kb *Eco*RI probe and the banding patterns seen after Southern blot analysis of *Cla*I digested chromosomal DNA from transconjugant 4 using the radiolabelled 2.4 *Hind*III probe. Both the models predicted that the transposon insertion in transconjugant number 4 contained two separate insertion events and therefore, no further analysis of this transconjugant was undertaken as the insertion was not useful for the study of the regulation of a single gene.

Figure 4.15 shows the Southern blot analysis of randomly selected transconjugants from S17-1* (pSA1)/*P. gingivalis* 33277 filter mating experiments. The autoradiograph shows transconjugants digested with *Hind*III restriction enzyme and subsequently analysed by Southern blot using the radiolabelled 3.6 *Eco*RI probe. Table 4.10 shows the size of the *Hind*III fragments hybridised by the 3.6 *Eco*RI probe. The *Hind*III digested chromosomal DNA from the transconjugants was also subjected to Southern blot analysis using the *catIII* probe (data not shown) and the fragment sizes that this probe hybridised to are indicated by an asterisk in Table 4.10. The Southern blot hybridisation analysis of transconjugants numbers 6, 9, 11 and 12 using the 3.6 kb *Eco*RI probe demonstrated that these transconjugants contained single insertions of Tn*4351*::*catIII* as indicated by the fact that the probe hybridised to two fragments of > 2.1 kb and > 3.6 kb. In accordance with this, when *Eco*RV digested chromosomal DNA from these transconjugants was subjected to Southern blot analysis using the radiolabelled pJRD215 probe, no hybridisation of the pJRD215 probe to the transconjugant DNA was observed indicating that no pJRD215 plasmid sequences had inserted into the chromosome (fig. 4.16). This represents a frequency of single Tn*4351*::*catIII* insertions into the *P. gingivalis* 33277 chromosome of 30% when pSA1 is used as the suicide vector. The Southern blot analysis of transconjugants 6 and 12 using the radiolabelled 3.6 kb *Eco*RI probe revealed that these two transconjugnts demonstrated identical banding patterns to each other suggesting that these two transconjugants may be siblings. Southern blot analysis of *Hind*III digested chromosomal DNA from transconjugants numbers 1 - 5, 7, 8 and 10 using the 3.6 kb *Eco*RI probe demonstrated the hybridisation of the probe to three fragments

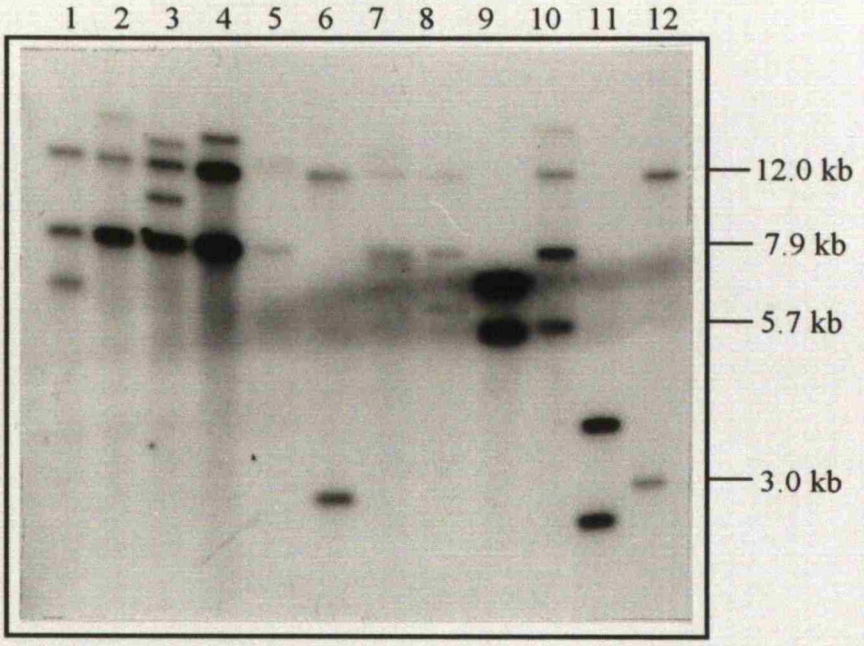


Fig. 4.15. Southern blot hybridisation analysis of *P. gingivalis* transconjugants. Chromosomal DNA from twelve independent Cl^r/Gm^r transconjugants of *P. gingivalis* 33277 transferred with the suicide vector pSA1 (lanes 1 - 12) were digested with *Hind*III restriction enzyme, separated by gel electrophoresis and transferred by Southern blot to hybridisation membrane. The membrane was hybridised with a [^{32}P] labelled 3.6 kb *Eco*RI fragment from pVOH1. Size markers (in kilo bases) are indicated to the right. Hybridisation of *P. gingivalis* 33277 DNA to the 3.6 kb probe was not observed (data not shown).

	Transconjugants (Gm^r/Cl^r)											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Fragment sizes	12.0*	>12*	>12*	>12*	>12*	11.5	>12*	12.0*	6.5*	>12*	4.0	11.5
	7.9	12.0*	12.0*	12.0*	12.0*	3.0*	12.0*	7.9	5.5	12.0*	2.8*	3.0*
	6.1	7.9	9.5	7.9	7.9		8.1	6.1		7.9		
			7.9				7.9			5.7		

Table 4.10. Fragment sizes seen by Southern blot analysis of *Hind*III digested transconjugant DNA from an S17-1* (pSA1)/*P. gingivalis* 33277 filter mating experiment hybridised to a 3.6 kb *Eco*RI probe. Conserved fragments of 12.0 kb and 7.9 kb were seen for transconjugants 1, 2, 3, 4, 5, 7, 8 and 10. Transconjugants 6, 9, 11 and 12 represent single insertions.

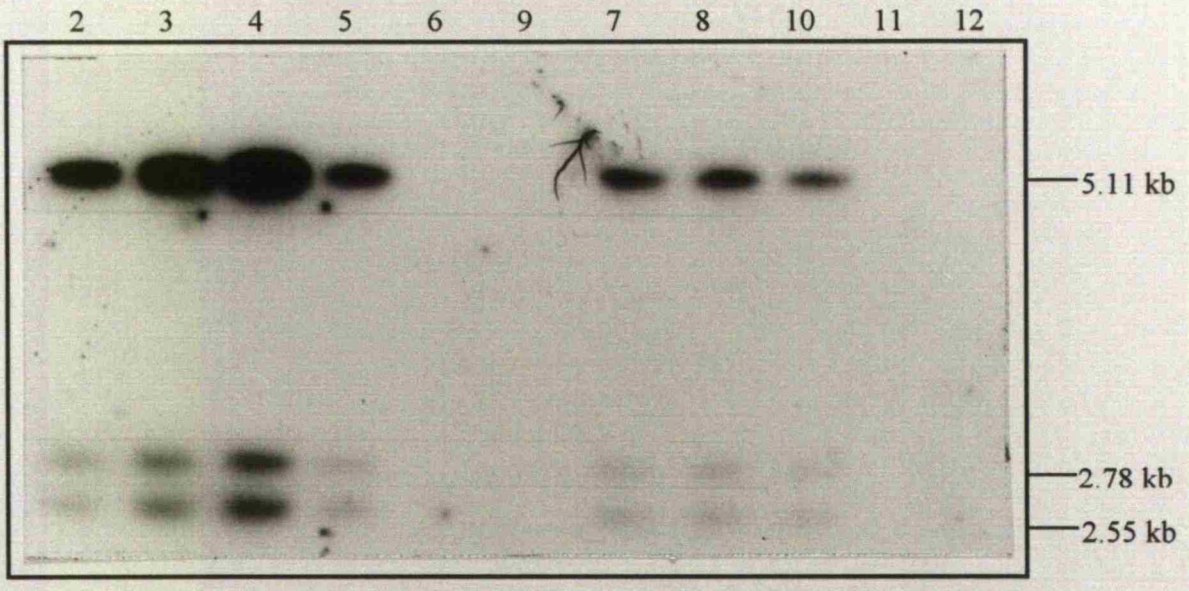


Fig. 4.16. Southern blot analysis of *P. gingivalis* transconjugants. Chromosomal DNA from eleven independent Cl^r/Gm^r transconjugants of *P. gingivalis* 33277 transferred with the suicide vector pSA1 were digested with *Eco*RV restriction enzyme, separated by gel electrophoresis and transferred by Southern blot to hybridisation membrane. The membrane was hybridised with a [^{32}P] labelled pJRD215 probe. Size markers in kilo bases are indicated to the right of the autoradiograph. Hybridisation of *P. gingivalis* 33277 chromosomal DNA to the probe was not observed (data not shown).

(transconjugants 1, 2, 4, 5 and 8) or four fragments (transconjugants 3, 7 and 10). In all cases two conserved bands of 12.0 kb and 7.9 kb could be identified and these were thought to correspond to the 12.13 kb and 7.9 kb *Hind*III fragments of pSA1. The chromosomal DNA from transconjugants 1 - 5, 7, 8 and 10 was digested with *Eco*RV restriction enzyme and then subjected to Southern blot analysis using the radiolabelled pJRD215 probe. The pJRD215 probe was found to hybridise to three conserved fragments of 5.11 kb, 2.78 kb and 2.55 kb in each transconjugant indicating that they did contain plasmid sequences (fig. 4.16). Transconjugant number 1 is not shown in figure 4.16, however, a separate Southern blot analysis did demonstrate hybridisation of the pJRD215 probe to three fragments of 5.11 kb, 2.78 kb and 2.55 kb for this transconjugant (data not shown). The analysis of transconjugants 1 and 8 using the 3.6 kb *Eco*RI probe demonstrated that these two transconjugants exhibited similar banding patterns to each other suggesting that they may be siblings. The Southern blot analysis of *Hind*III digested chromosomal DNA from transconjugants numbers 3, 7 and 10 using the radiolabelled 3.6 kb *Eco*RI probe demonstrated that these transconjugants contained cointegrate formation insertions as indicated by the fact that the probe hybridised to four fragments, two of which were the conserved bands of 12.0 kb and 7.9 kb. In the case of each of the transconjugants examined, the 12.0 kb fragment and one of the variable sized fragments were also hybridised by the radiolabelled *catIII* probe (data not shown). The Southern blot analysis of *Hind*III digested chromosomal DNA from transconjugants numbers 1 (and 8), 2, 4 and 5, using the radiolabelled 3.6 kb *Eco*RI probe showed conserved fragments of 12.0 kb and 7.9 kb and one other fragment of variable size in each transconjugants. The Southern blot analysis of *Hind*III digested chromosomal DNA from these transconjugants using the radiolabelled *catIII* probe demonstrated that the 12.0 kb fragment and the variable sized fragment were hybridised by the *catIII* probe for transconjugants 2, 4 and 5 and the 12.0 kb fragment only was hybridised by the *catIII* probe for transconjugant 1 (and 8). This suggested that transconjugants 1, (and 8), 2, 4 and 5 had originally contained a cointegrate insertion but then partial deletion of one of the transposons had occurred by transposon-induced rearrangements as indicated diagrammatically in figure 4.17. Model A applies to transconjugants numbers 2, 4 and 5 and model B applies to transconjugant number 1 (and 8).

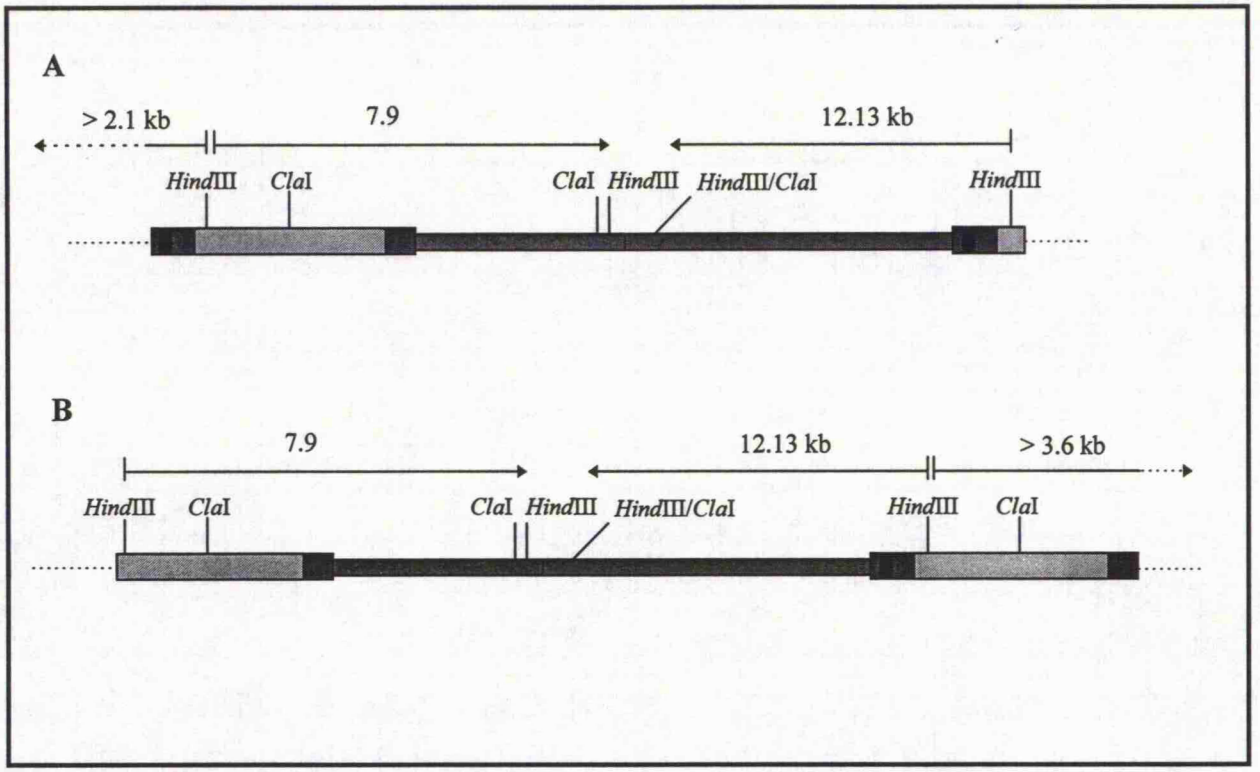


Fig. 4.17. Suggested models to account for the banding patterns seen when the 3.6 kb *Eco*RI probe was hybridised to transconjugants from an S17-1* (pSA1)/*P. gingivalis* 33277 filter mating. Model A applies to transconjugants 2, 4 and 5 whilst model B applies to transconjugants 1 and 8. ▬ represents pJRD215 sequences, ▬ represents pAO6 sequences, ▬ represents Tn*4351* sequences, ▬ represents IS*4351* sequences and ▬ represents *catIII* sequences. Hashed lines represent chromosomal sequences. Solid arrows represent defined fragments whilst hashed line arrows represents fragments of variable size.

4.2.5 Chloramphenicol Acetyltransferase (CAT) Activity of *P. gingivalis* 33277 Transconjugants

In order to determine the levels of CAT activity demonstrated by Cl^r transconjugants from matings between S17-1* (pSA1) and *P. gingivalis* 33277, the minimum inhibitory concentrations (MIC) for individual transconjugants were determined. It had already been established that *P. gingivalis* 33277 had an MIC of 2 μg/ml (Section 3.3.3) when plated onto BM agar supplemented with Cm. It was decided to determine the MIC for Cl^r transconjugants from matings between S17-1* (pSA1) and *P. gingivalis* 33277 using BM agar plates supplemented with 0, 2, 5 and 10 μg/ml Cm. The transconjugants were initially streaked onto BAB agar plates supplemented with gentamicin and clindamycin. These plates were then incubated anaerobically for 4 - 7 days or until the colonies had turned black. The transconjugants were then streaked onto the BM agar plates supplemented with 0, 2, 5 or 10 μg/ml Cm by placing a 50 square grid underneath the agar plate and using a sterile toothpick, inoculated from one of the transconjugants on the BAB plate, to make a diagonal streak across one of the squares on the surface of the agar plate until 50 separate transconjugants had been streaked per plate. A single toothpick was used to streak out a single transconjugant starting on the BM agar plate supplemented with 10 μg/ml Cm and then the same toothpick was used to streak out the transconjugant on the successive Cm dilution BM agar plates supplemented with 5 μg/ml, 2 μg/ml and 0 μg/ml Cm. The wild-type 33277 was streaked onto each plate as a negative control. In each case, without exception, growth of 33277 was only noted on BM agar plates that had not been supplemented with Cm. Table 4.11 indicates the number of transconjugants tested able to grow at the different Cm concentrations. It was found that 95% of the total number of transconjugants tested were able to grow on BM agar plates supplemented with 2 μg/ml Cm, 55% were able to grow on BM agar plates supplemented with 5 μg/ml Cm and almost 10% of the total number of transconjugants tested were able to grow at 10 μg/ml Cm. To determine whether it was possible to quantify the CAT activity of these transconjugants a CAT assay was undertaken on 15 randomly selected transconjugants. As a positive control the assay

	CHLORAMPHENICOL CONCENTRATION			
	0 μg/ml	**2 μg/ml**	**5 μg/ml**	**10 μg/ml**
Number of colonies able to grow at each concentration	643	608	356	57
Percent of total number scored able to grow at each concentration	100%	94.6%	55.4%	8.9%

Table 4.11. Percentage of Cl^r transconjugants able to grow on BM agar plates supplemented with different concentrations of chloramphenicol as indicated.

was also performed on the *E. coli* strain LE392 carrying the plasmid pECAT which contains the *catIII* gene inserted in the correct orientation upstream of the *lacZ* promoter. This strain had already been demonstrated to grow in media supplemented with 25 μg/ml Cm. The wild-type *P. gingivalis* strain 33277 was used as a negative control. Of the 15 transconjugants tested three were found to grow on BM supplemented with 5 μg/ml Cm, two were found to grow on BM agar supplemented with 2 μg/ml Cm and the remaining ten transconjugants were unable to grow on BM agar supplemented with Cm. Table 4.12 demonstrates the number of units of chloramphenicol acetylated by each transconjugant at 37°C (where 1 unit represents 1 nanomole of chloramphenicol acetylated per mg of protein per minute at 37°C). The positive control was found to acetylate 40.46 ± 3.79 units of chloramphenicol. Transconjugants 1, 3 and 6, all of which grew on BM agar supplemented with 5 μg/ml Cm, acetylated 6.80 ± 0.69, 7.08 ± 1.39 and 6.32 ± 1.82 units of chloramphenicol respectively, whilst transconjugants 2 and 5 which were able to grow on BM agar supplemented with 2 μg/ml Cm acetylated 3.10 ± 1.67 and 2.92 ±b 1.19 units of chloramphenicol respectively. The remaining transconjugants tested, transconjugants 4 and 7 - 15, were unable to acetylate chloramphenicol and this was in agreement with the MIC data which demonstrated that these bacteria were unable to grow on BM agar plates supplemented with 2 μg/ml Cm. The negative control, wild-type *P. gingivalis* 33277, was unable to acetylate chloramphenicol.

Southern blot analysis was carried out on transconjugants 1 - 15 to determine whether they contained single insertions of Tn*4351*::*catIII*. Chromosomal DNA from the transconjugants 1 - 15 was digested with *Hind*III, separated by agarose gel electrophoresis and transferred by Southern blot to hybridisation membrane. As controls, chromosomal DNA from wild-type *P. gingivalis* 33277 and plasmid DNA from the suicide vector pSA1 was also digested with *Hind*III restriction enzyme. These fragments were then subjected to Southern blot analysis using the 3.6 kb *Eco*RI radiolabelled probe from pVOH1 (fig. 4.18a). The fragment sizes displayed by this Southern blot are given in Table 4.13. The banding pattern seen for transconjugant number 13 was difficult to see on the autoradiograph, even after prolonged exposure of

Sample	Maximum Concentration of Cm allowing growth	Units of Chloramphenicol acetylated*
pECAT (positive control)	>25 μg/ml	40.46 ± 3.79†
Transconjugant 3	5 μg/ml	7.08 ± 1.39
Transconjugant 1	5 μg/ml	6.80 ± 0.69
Transconjugant 6	5 μg/ml	6.32 ± 1.82
Transconjugant 2	2 μg/ml	3.10 ± 1.67
Transconjugant 5	2 μg/ml	2.92 ± 1.19
Transconjugants 4, 7 - 15	0 μg/ml	0
33277 (negative control)	0 μg/ml	0

* Number of units of chloramphenicol acetylated per mg protein per minute at 37°C (where 1 unit represents 1 nano mole of chloramphenicol acetylated per minute at 37°C).

† Results are given as the mean ± the standard deviation.

Table 4.12. Number of units of chloramphenicol (Cm) acetylated by randomly selected transconjugants able to grow on BM agar plates supplemented with 0, 2, or 5 μg/ml Cm. The plasmid pECAT carried in the *E. coli* strain LE392 was used as a positive control and wild-type *P. gingivalis* 33277 was used as a negative control.

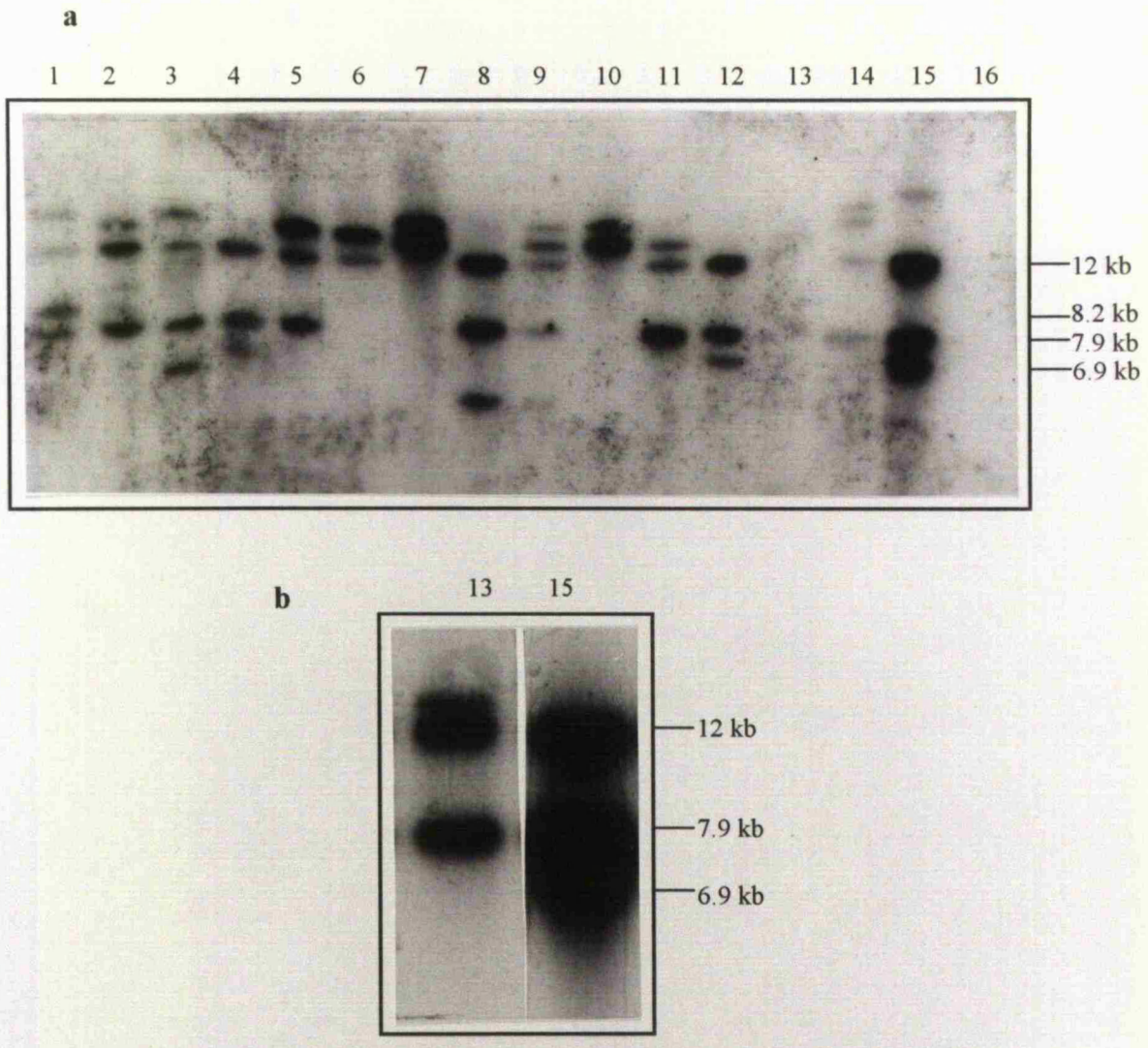


Fig. 4.18. Southern blot hybridisation analysis of *P. gingivalis* transconjugants. Chromosomal DNA from 15 independent Cl^r/Gm^r transconjugants of *P. gingivalis* 33277 transferred with the suicide vector pSA1 (lanes 1 - 15 fig. 4.18a) were digested with *Hind*III, separated by gel electrophoresis and transferred by Southern blot to hybridisation membrane. The membrane was hybridised with a [^{32}P] labelled 3.6 kb *Eco*RI fragment from pVOH1. Hybridisation of *P. gingivalis* 33277 DNA to the 3.6 kb *Eco*RI probe was not observed (lane 16 fig. 4.18a). Chromosomal DNA from transconjugants 13 and 15 was redigested with *Hind*III and subjected to further Southern blot analysis using the 3.6 kb *Eco*RI probe (fig. 4.18b). Size markers in kilo bases (kb) are indicated to the right.

	Transconjugants (Gm^r/Cl^r)														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Fragmen t sizes	>12*	>12*	>12*	12*	>12*	>12*	>12*	12*	>12*	>12*	>12*	12*	>12	>12	12*
	12*	12*	12*	7.9	12*	12*	>12	7.9	>12	>12	12*	7.9	12*	>12*	7.9
	8.2	9	7.9	7.5	7.9			5.9	12*		7.9	7.0	7.9	12*	6.9
	7.9	7.9	6.5						7.9					7.9	

Table 4.13. Fragment sizes seen by Southern blot analysis of *Hind*III digested Cm^r and Cm^s transconjugant DNA (transconjugants 1 - 15) from an S17-1* (pSA1)/*P. gingivalis* 33277 filter mating experiment hybridised to a 3.6 kb *Eco*RI probe. The asterisk represents fragments that were also hybridised by a *catIII* probe. A conserved fragments of 12.0 kb can be seen for all transconjugants. A conserved fragment of 7.9 kb can seen for transconjugants 1 - 5, 8 - 9 and 11 - 15.

the filter and so further Southern blot analysis using the radiolabelled 3.6 *Eco*RI probe was carried out on this transconjugant as indicated in figure 4.18b. Chromosomal DNA from transconjugant number 15 was reanalysed in order to determine whether the fainter fragments demonstrated in figure 4.18a were partially digested fragments. The second hybridisation analysis is shown in figure 4.18b and it can be clearly seen that only 3 fragments have been hybridised by the 3.6 *Eco*RI probe indicating that the additional fragments seen in the original autoradiograph were indeed partially digested fragments. The uneven migration of the different chromosomal samples was due to a warping effect on the gel plate used, however, conserved bands of 7.9 and 12 kb could be determined. Chromosomal DNA from the transconjugants was also digested with *Hind*III restriction enzyme and subjected to Southern blot analysis using the radiolabelled *catIII* probe (fig. 4.19). The fragment sizes that this probe hybridised with are indicated by an asterisk in Table 4.13 (a partially digested fragment of > 12 kb was also demonstrated by the *catIII* probe for transconjugant number 15). For transconjugants 1 - 5, 8 - 9 and 11 - 15 two conserved fragments of 12 kb and 7.9 kb were shown in the Southern blots using the radiolabelled 3.6 *Eco*RI probe. Southern blot analysis of *Hind*III digested chromosomal DNA from transconjugants 1 - 5, 8 - 9 and 11 - 15 using the radiolabelled *catIII* probe demonstrated hybridisation to the conserved 12 kb fragment but not to the conserved 7.9 kb fragment. Transconjugants 7 and 10 appeared to be siblings, each demonstrating a similar banding indicative of a single Tn*4351*::*catIII* insertion. Transconjugants numbers 1 - 3, 9 and 14 all appeared to contain cointegrate insertions as indicated by the fact that the 3.6 *Eco*RI probe hybridised to the two conserved fragments and to two other fragments of variable size. Two copies of *catIII* were also found by hybridisation analysis to be present in each of these transconjugants. Transconjugants numbers 4 - 5, 8, 11 - 13 and 15 were all proposed to contain cointegrate formations that had undergone transposon-induced rearrangements either prior to, or after insertion into the chromosome. The result of these rearrangements appeared to be the partial loss of one of the two Tn*4351*::*catIII* transposons as indicated previously in figure 4.17 (where transconjugants 5 and 11 fit into model A and transconjugants 4, 8, 12, 13 and 15 fit into model B). Results from Southern blot analysis using the radiolabelled 3.6 *Eco*RI probe indicated that transconjugant number 6 contained a cointegrate formation that had

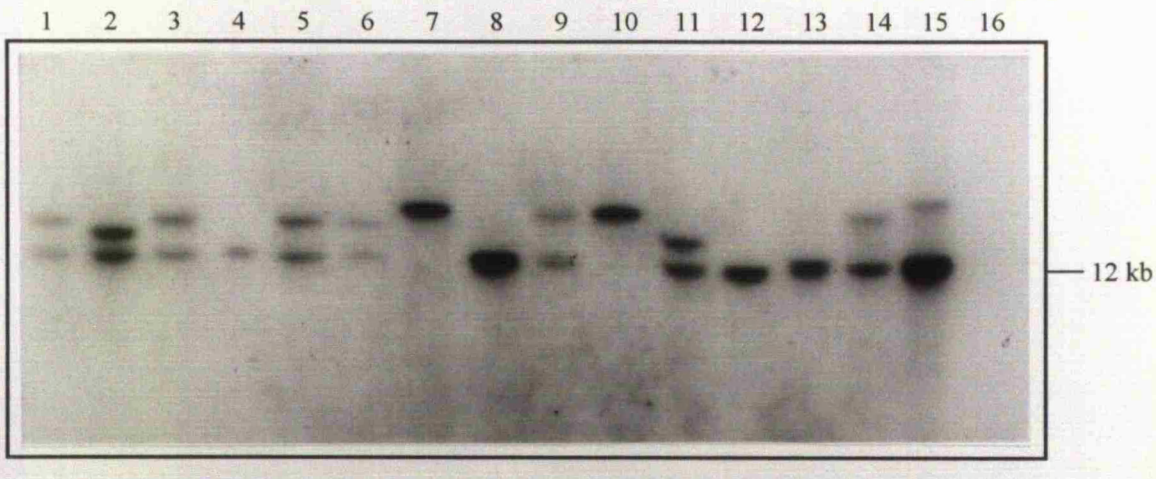


Fig. 4.19. Southern blot hybridisation analysis of *P. gingivalis* transconjugants. Chromosomal DNA from 15 independent Cl^r/Gm^r transconjugants of *P. gingivalis* 33277 transferred with the suicide vector pSA1 (lanes 1 - 15) were digested with *Hind*III, separated by gel electrophoresis and transferred by Southern blot to hybridisation membrane. The membrane was hybridised with a [^{32}P] labelled 0.7 kb *catIII* fragment. Hybridisation of *P. gingivalis* 33277 DNA to the 0.7 kb *catIII* probe was not observed (lane 16). Size markers in kilo bases (kb) are indicated to the right.

undergone transposon-induced rearrangements resulting in the loss of one of the two Tn*4351*::*catIII* transposons and partial loss of the second transposon as demonstrated in figure 4.20. It was speculated that this transconjugant also contained a separate IS*4351*$_R$::*catIII* insertion elsewhere in the chromosome which resulted in two bands in the Southern blot analysis when chromosomal DNA from transconjugant number 6 was digested with *Hind*III and probed with the radiolabelled *catIII* fragment.

4.2.6 Screening *P. gingivalis* 33277 Transconjugants for Neuraminidase and β-N-acetylhexosaminidase Negative Mutants

In order to determine whether Tn*4351*::*catIII* insertions could be used to study the regulation of potential virulence determinants an attempt was made to isolate a Cl^r transconjugant negative for neuraminidase or β-N-acetylhexosaminidase (β-Nahase) activity. These enzymes were chosen because not only are they thought to have a potential role as virulence determinants by virtue of their ability to break down host matrices (Gharbia and Shah, 1993; Koide *et al.*, 1977), but fluorometric assays allow a quick method of measuring the activity of these enzymes.

The assay used to detect neuraminidase or β-Nahase activity was based on a method developed previously to study neuraminidase activity in *Streptococcus pneumoniae* (Camera *et al.*, 1991). The *P. gingivalis* transconjugants were streaked along side the neuraminidase and β-Nahase positive wild-type *P. gingivalis* 33277 which acts as a positive control and the neuraminidase and β-Nahase negative bacteria *P. asaccharolyticus* which acts as a negative control. The substrates 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid (MUAN) or 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (MUAG) were used to detect for neuraminidase and β-Nahase activity respectively. Figure 4.21 demonstrates the mechanism of hydrolysis of MUAN by the action of neuraminidase resulting in release of the fluorogenic end product. Free 4-methylumbelliferone cleaved from the substrate by the action of the enzymes exhibits excitation at 365 nm and fluorescence emission at 450 nm which can be detected using a hand-help UV lamp. Each plate was examined for the absence of enzyme activity as

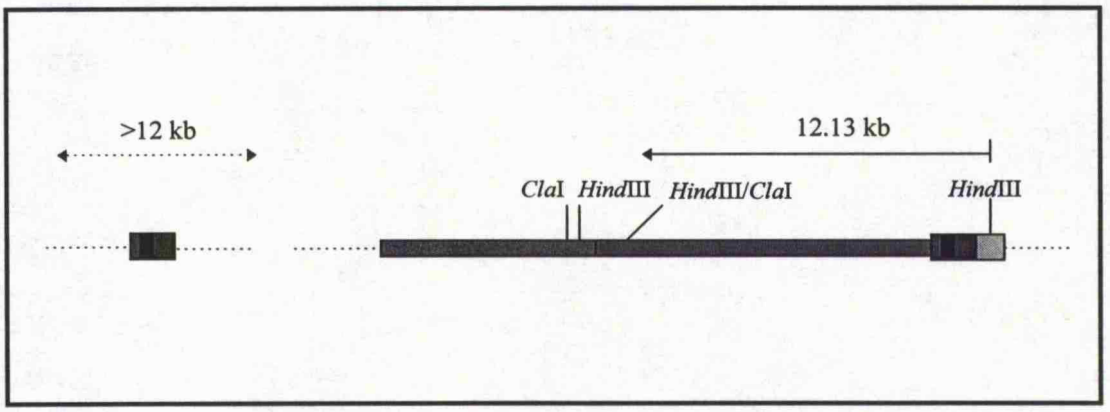


Fig. 4.20. Suggested model to account for the banding pattern seen after hybridisation of the 3.6 kb *Eco*RI probe and *catIII* probe to *Hind*III digested chromosomal DNA from transconjugant number 6.

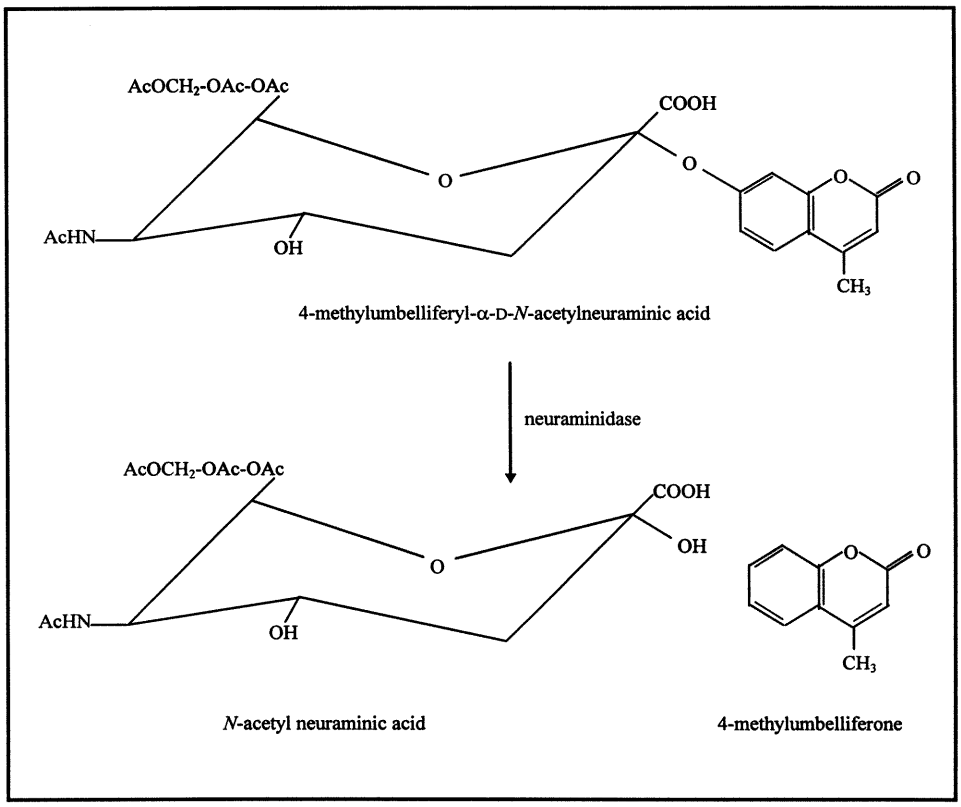


Figure 4.21. The mechanism of hydrolysis of the fluorogenic substrate 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid. The fluorogenic compound 4-methylumbelliferone is released from 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid as a result of cleavage of the substrate by neuraminidase (taken from Gharbia and Shah, 1993).

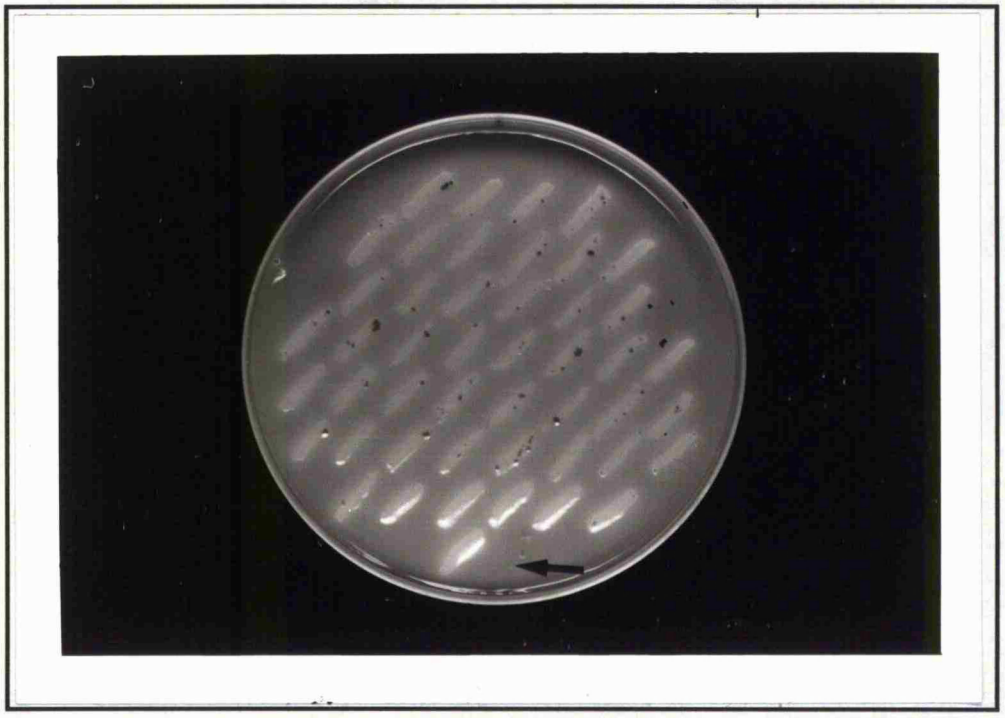
indicated by the non-fluorescence of a particular clone. Figure 4.22 demonstrates the fluorescence emission seen for the transconjugants when the plates were screened with the agarose overlay containing MUAG (fig. 4.22a) or MUAN (fig. 4.22b). Each plate also has a wild-type *P. gingivalis* 33277 (bottom left) positive control and a *P. assacharolyticus* (bottom right) negative control. In total 7000 transconjugants were screened for neuraminidase activity and 6750 transconjugants were screened for β-Nahase activity. However, none of the transconjugants were found to be negative for either of these enzyme activities.

4.3. Discussion

4.3.1 Frequency of Transposition

The aim of this part of the study was to determine whether Tn*4351*::*catIII* could be transferred into *P. gingivalis* for use as a promoter probe. Although it had already been determined that pSA1 and pSA2 could be transferred between two *E. coli* strains, it was not known whether they could be transferred from *E. coli* into *P. gingivalis*. The first objective was to devise a mating strategy which would allow efficient transfer of Tn*4351* into *P. gingivalis*. At the onset of this study the transfer of *Bacteroides* shuttle vectors, or *Bacteroides* suicide vectors containing Tn*4351*, into *P. gingivalis* had only recently been achieved and the literature varied in the mating strategies used to encourage conjugal transfer of these plasmids. The limited number of studies so far undertaken have each used different *P. gingivalis* strains as well as different mating protocols. Some studies suggested that long anaerobic incubation of mating mixtures was important (Maley *et al.*, 1992; Progulske-Fox *et al.*, 1989b), whereas other studies suggested that aerobic incubation for 4 - 24 hours prior to anaerobic incubation could improve the frequency of transfer (transposition) (Dyer *et al.*, 1992; Hoover *et al.*, 1992b). One study favoured 2 - 4 hours aerobic incubation prior to anaerobic incubation although an increased frequency of transposition was noted if the aerobic incubation time was increased to up to 24 hours (Hoover *et al.*, 1992b). This was confirmed by another study which suggested that incubation of the mating mixtures for < 15 hours or

a



b

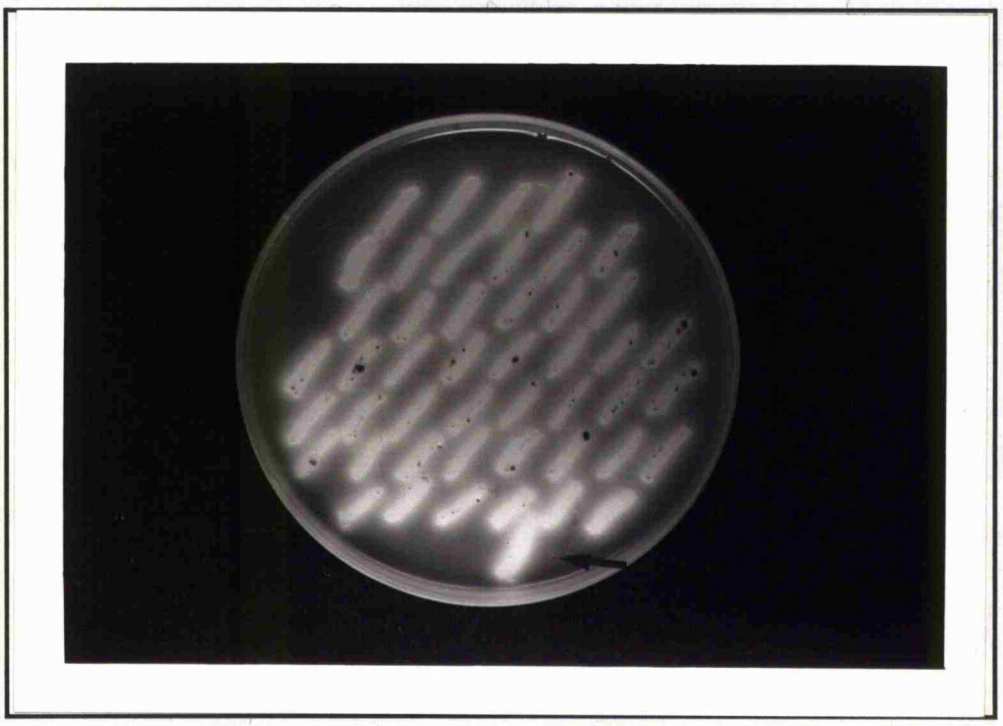


Figure 4.22. Plate assay for detection of the presence (or absence) of β-Nahase activity (fig. 4.22a) or neuraminidase activity (fig. 4.22b) in *P. gingivalis* 33277 Cl^r transconjugants. On each photograph the arrow demonstrates the position of the negative control, *P. asaccharolyticus,* (bottom right) and to the left of this the positive control, wild-type *P.gingivalis* 33277 (bottom left).

> 24 hours resulted in lower transfer frequencies (Dyer *et al.*, 1992). The discrepancy about whether or not to include an aerobic incubation period can be explained, in part, by the fact that each study used different strains of *P. gingivalis* which may exhibit different levels of aerotolerence. Therefore, the inclusion of an aerobic incubation period for one strain of *P. gingivalis* may result in an increased transposition frequency, whereas in another strain of *P. gingivalis* the inclusion of an aerobic incubation period may severely reduce the viability of the bacteria. Indeed it has been suggested that aerobic incubation of *P. gingivalis* strain W83 results in complete loss of viability of this bacterium (Maley, 1994).

Discrepancies were also found between different studies as to which donor to recipient cell ratio resulted in the most efficient frequency of transfer (transposition). One study found that increasing the donor to recipient ratio from 0.25 - 0.5 to 1.0 adversely affected the viability of recipient cells resulting in a reduction in the number of transconjugants (Hoover *et al.*, 1992b). Other studies, however, routinely used a donor to recipient ratio of 1.0 (Dyer *et al.*, 1992) whilst transfer of vectors into *P. gingivalis* W83 was found to be most efficient using excess donor to recipient cells (Maley, 1994). In contradiction to this, a recent study found that donor to recipient ratios of 1:30 to 1:500 resulted in the highest frequencies of transposition (Genco *et al.*, 1995a). Confusion also existed about whether transfer (transposition) frequency was growth phase dependant or not. One study found that growth phase did not affect transfer frequency (Dyer *et al.*, 1992) whereas other studies favoured the use of early log phase cells (10^8 - 10^9 CFU/ml) (Hoover *et al.*, 1992b; Progulske-Fox *et al.*, 1989b). However, conjugal transfer of plasmids into *P. gingivalis* strain W83 was only found to occur efficiently if starting cultures of less than 1 x 10^8 CFU/ml were used (Maley, 1994). The underlying conclusions from these studies were that strain variation can dramatically affect the efficiency of transfer (transposition) such that mating conditions must be optimised for the particular recipient strain used. The preliminary studies indicated that several factors, including starting CFU/ml, donor to recipient ratio and the use of aerobic incubation of the mating mixture, can all affect the outcome of *E. coli* to *P. gingivalis* conjugal plasmid transfer.

No definitive conclusions could be drawn as to the combination of variables that resulted in the most efficient transposition frequencies in *P. gingivalis* 381 and 33277 when R751::*Ω4, pSA1 or pSA2 were used as the suicide vectors. In general it was observed that for matings involving R751::*Ω4, pSA1 or pSA2 in *P. gingivalis* 381 and R751::*Ω4 or pSA2 in *P. gingivalis* 33277, a low donor to recipient ratio (i.e. 1:3 donor to recipient ratio) using donor and recipient cells in the magnitude of 10^8 and 10^9 CFU/ml respectively resulted in a lower frequency of transposition than if a higher donor to recipient ratio had been used (i.e. 1:20 donor to recipient ratio). However, a low donor to recipient ratio did result in a high frequency of transposition if the starting CFU/ml of both donor and recipient cells were in the magnitude of 10^8 CFU/ml (Tables 4.2 and 4.7). The use of starting CFU/ml of donor cells in excess to the starting CFU/ml of recipient cells also appeared to result in a higher frequency of transposition regardless of whether the starting CFU/ml of donor and recipient cells were in the magnitude of 10^8 or 10^9. Despite these conclusions it was clear that other factors must affect the efficiency of transposition. This was highlighted by a mating experiment which transferred pSA2 into *P. gingivalis* 381. A transposition frequency of 2.46×10^{-7} was achieved when a donor to recipient ratio of 1:1 was used. This was 10 fold lower than the highest frequency of transposition seen for mating experiments in strain 381 using pSA2 despite the starting CFU/ml of donor and recipient cells for both mating experiments being almost identical (2.00×10^8 and 1.9×10^8 compared to 1.78×10^8 and 1.65×10^8). In contrast to the above generalisations matings involving the transfer of pSA1 into *P. gingivalis* 33277 showed the highest frequency of transposition (3.72×10^{-5} transconjugants per recipient) when a donor to recipient ratio of 1:2 was used. The lowest frequency of transposition from pSA1 in *P. gingivalis* 33277 occurred when a similar donor to recipient ratio was used but the donor and recipient starting cultures were in the magnitude of 10^8 CFU/ml. Hence many factors, including the donor to recipient ratio, the starting CFU/ml of donor and recipient cultures, the suicide vector used and the recipient strain used are capable of affecting transposition frequencies.

The vector R751::*Ω4 (in the *E. coli* strain HB101) was used as a positive control. This plasmid was originally identified during mating experiments in which plasmid R751 was used to mobilise the shuttle vector pE5-2 between E. *coli* strains (Shoemaker *et al.*, 1986). One result of these matings was that in some transconjugants, Tn*4351* had inserted into R751. In the case of R751::*Ω4 a partial tandem duplication of Tn*4351* had inserted into R751 (Shoemaker *et al.*, 1986). When R751::*Ω4 was transferred from *E. coli* in to *Bacteroides* spp. the frequency of transposition was found to be 2 - 4 fold higher than from R751 carrying a single copy of Tn*4351* (Shoemaker *et al.*, 1986). This meant that transposition from R751::*Ω4 was occurring at a frequency that was easily detectable.

Mating experiments between HB101 (R751::*Ω4) and *P. gingivalis* 381 or 33277 had previously been demonstrated to result in a mean frequency of transposition of 2.7×10^{-7} transconjugants per recipient for strain 381 (Dyer *et al.*, 1992) and 1.6×10^{-7} transconjugants per recipient for strain 33277 (Hoover *et al.*, 1992b). Transfer of R751::*Ω4 into *P. gingivalis* strains 381 or 33277 in this study resulted in transposition frequencies 3 and 13 fold higher than the previously reported transposition frequencies (Dyer *et al.*, 1992; Hoover *et al.*, 1992b). These results show that the mating procedure developed in this study to transfer R751::*Ω4 into *P. gingivalis* 381 or 33277 produced a higher, more efficient frequency of transposition than previously reported. The significant increase in transposition frequency is likely to have been affected, in part, by the use of nitrocellulose filters as suggested by a recent study (Genco *et al.*, 1995a). In addition to this it is possible that factors such as differences in the donor to recipient ratios and starting CFU/ml of donor and recipient cells could have resulted in the higher transposition frequency demonstrated in this study.

For matings involving pSA2 and pSA1 in *P. gingivalis* 381, transposition occurred at a frequency 2 to 4 fold lower than the transposition frequency demonstrated from R751::*Ω4 in this strain. These results agreed with the previous findings that transposition of Tn*4351* from a plasmid containing a tandem duplication of Tn*4351* occurred at a higher frequency than from a plasmid containing a single copy of the

transposon (Shoemaker *et al.*, 1986). The generation of Cl^r transconjugants from matings involving pSA1 and pSA2 demonstrated that the suicide vectors pSA1 and pSA2 were capable of being mobilised from *E. coli* into *P. gingivalis* and that Tn*4351*::*catIII* could transpose into the *P. gingivalis* chromosome. The frequency of transposition of Tn*4351*::*catIII* resulting from transfer of pSA2 into *P. gingivalis* 33277 was also found to occur at a frequency 2 fold lower than the frequency of transposition of Tn*4351* found when R751::*Ω4 was transferred into this strain. However, the frequency of transposition of Tn*4351*::*catIII* found when pSA1 was transferred into *P. gingivalis* 33277 occurred at a frequency 3 fold higher than the frequency of transposition of Tn*4351* when R751::*Ω4 was transferred into this strain. The reason that transposition from pSA1 did not occur at a 2 - 4 fold lower frequency than from R751::*Ω4 in strain 33277, as expected (Shoemaker *et al.*, 1986), was unknown. It is possible that the use of donor starting cultures in the magnitude of 10^7 CFU/ml resulted in a decrease in the overall mean value for transposition frequency from HB101 (R751::* Ω4). It is also possible that taking the mean frequency of transposition of seven mating experiments involving R751::* Ω4, rather than the mean frequency of transposition of ten mating experiments as was the case for matings involving pSA1, resulted in a lower average frequency of transposition for matings involving R751::* Ω4 compared to matings involving pSA1.

A comparison of the frequencies of transposition of Tn*4351*::*catIII* for matings transferring pSA1 and pSA2 into *P. gingivalis* 33277 and 381 demonstrated that both the structure of the suicide vector and the recipient strain used have an affect on transposition frequency. The higher transposition frequencies of Tn*4351* and Tn*4351*::*catIII* demonstrated in *P. gingivalis* strain 33277 compared to strain 381 most probably reflects differences between the two strains. For example, it is possible that strain 381 is less aerotolerant than strain 33277 and that by reducing the aerobic incubation time of matings carried out in this strain an increase in the frequency of transposition would occur. A recent study has also demonstrated that transposition of Tn*4351* into strain 381 occurs at a lower frequency than into *P. gingivalis* strain A7436

and the authors suggested that this could perhaps be due in part to different restriction systems (Genco *et al.*, 1995a).

Transposition of Tn*4351*::*catIII* from pSA2 occurred at a higher frequency than transposition of the same transposon from pSA1 in *P. gingivalis* 381, whereas transposition of Tn*4351*::*catIII* occurred at a considerably higher frequency from pSA1 than transposition of the same transposon from pSA2 in *P. gingivalis* 33277. These observations may reflect a more efficient transfer frequency of pSA1 into strain 33277 than the transfer frequency of pSA2 or R751::*Ω4 into this strain. Such strain variation has been noted before in *P. gingivalis*. It has been demonstrated that strain W50 is not able to maintain the *Bacteroides* shuttle vector pE5-2 (Yoshimoto *et al.*, 1993). However, another *Bacteroides* shuttle vector, pVAL-1, that contains the same *Bacteroides* antibiotic resistance gene and cryptic plasmid as pE5-2 is maintained by strain W50 (Dyer *et al.*, 1992). It is also feasible that the actual transposition event is occurring at a higher frequency from pSA1 than from the other plasmids or that a higher transfer frequency coupled with a higher transposition frequency is resulting in the high number of transconjugants seen from matings involving pSA1. It is also conceivable that events such as inverse transposition, which would result in loss of the region of Tn*4351* that encodes for the Cl^r determinant, are favoured in strain 33277 from plasmids pSA2 and R751::*Ω4 resulting in a lower frequency of transposition (as measured by the number of Cl^r transconjugants per recipient) than from matings involving pSA1. The different frequencies of transposition demonstrated depending on the recipient strain and the suicide vector used, probably reflect the structural differences between the suicide vectors. It is possible that the orientation of pAO6 in pJRD215 has an affect on the mobilisation activity of the plasmids and/or on the transposon activity of Tn*4351*::*catIII* due to the orientation of the transposon with regard to the pJRD215 (RSF1010) origin of replication or mobilisation region or due to promoters in pJRD215 that transcribe through pAO6 into Tn*4351*::*catIII*.

It was decided that in addition to the use of R751::*Ω4 as a positive control plasmid, another plasmid, containing Tn*4351**, would be constructed. The use of such a control

plasmid enabled a direct comparison to be made between the transposition frequency of Tn*4351** compared to Tn*4351** containing a *catIII* insert (Tn*4351*::*catIII*). The suicide vector constructed was termed pSA3 and was identical to pSA1 in all respects except that it lacked the *catIII* insert. The *E. coli* strain S17-1* was used as the host for pSA3 which allowed a more direct comparison of transposition frequencies to be determined than when HB101 (R751::*Ω4) was used as the control strain. The frequencies of transposition of Tn*4351*::*catIII* from pSA2 and of Tn*4351** from pSA3 in *P. gingivalis* 33277 were similar, indicating that the presence of the *catIII* insert does not decrease the activity of the transposon Tn*4351**. However, the frequency of transposition of Tn*4351*::*catIII* from pSA1 in *P. gingivalis* 33277 was higher than the transposition frequencies of Tn*4351*::*catIII* and Tn*4351** demonstrated from pSA2 and pSA3 respectively. Since pSA1 and pSA3 are identical except for the presence of the *catIII* insert in pSA1 it would be expected that the transposition frequencies from pSA1 and pSA3 should be similar. It is possible that in fact the presence of the *catIII* insert in pSA1 is resulting in increased transposition activity in *P. gingivalis* strain 33277 compared to strain 381. The transposition frequencies of Tn*4351*::*catIII* seen when pSA1, pSA2 and pSA3 were transferred into *P. gingivalis* and the transposition frequencies of Tn*4351** seen when pSA3 was transferred into *P. gingivalis,* demonstrated that the presence of the *catIII* insert, the altered *Eco*RI restriction enzyme site in IS*4351*$_R$* and the mutation at nucleotide 675 in IS*4351*$_L$* had not detrimentally affected the transposition activity of the transposon.

In summary, it appears that the mating procedure developed for use in this study results in a higher frequency of transposition of Tn*4351* into *P. gingivalis* strains 381 and 33277 than previously described. For matings involving strain 381 transposition from R751::*Ω4 occurred at a frequency 2 and 4 fold higher than from pSA2 and pSA1 respectively. For matings involving strain 33277 transposition from all plasmids occurred at a higher frequency than demonstrated in strain 381. Transposition from pSA2 and R751::*Ω4 occurred at a 2 fold higher frequency in strain 33277 than in strain 381 and transposition from pSA1 occurred at a 30 fold higher frequency in strain 33277 than in strain 381. The frequency of transposition from pSA1 occurred at a considerably

greater frequency in strain 33277 than transposition from pSA2. These results most likely reflect structural differences between pSA1 and pSA2 due to the orientation of the pAO6 insertion in pJRD215. These differences could affect the transfer frequency and/or the transposition frequency of Tn*4351* from these plasmids into different *P. gingivalis* strains. Importantly, these results demonstrate that the suicide vectors pSA1 and pSA2 are capable of being efficiently transferred from *E. coli* into *P. gingivalis* and that transposition of Tn*4351*::*catIII* into the *P. gingivalis* chromosome can occur at a detectable frequency. It also appears that the presence of the *catIII* insert within Tn*4351** does not affect the transposition activity of this transposon in *P. gingivalis* and that in fact the presence of the *catIII* may even increase transposition frequencies when pSA1 is transferred into *P. gingivalis* 33277. The use of the filter mating procedure developed for use in this study will not only allow efficient transfer of pSA1 and pSA2 into *P. gingivalis* for analysis of promoter function, but the higher transposition frequencies will also allow Tn*4351* to be used more efficiently as a mutagenesis tool in this bacterium than previously demonstrated.

4.3.2 Analysis of Tn*4351*::*catIII* Chromosomal Insertions

One of the first conclusions drawn from the Southern blot analysis of *P. gingivalis* 381 and 33277 transconjugants was the difference between Tn*4351*::*catIII* transposon insertions from matings involving the suicide vector pSA1 and those involving the suicide vector pSA2. No single insertions of Tn*4351*::*catIII* were demonstrated in transconjugants isolated from matings involving pSA2 in either *P. gingivalis* strains tested (strains 381 and 33277). However, single insertions were demonstrated at a frequency of 30% for matings involving transfer of pSA1 into *P. gingivalis* 381 and 33277. Two theories exist as to the formation single insertions versus the formation of cointegrates. The first theory suggests that single insertions or cointegrates arise as alternative products of the resolution of an intermediate, while the second theory suggests that the cointegrate formation is the intermediate and that the resolution of the cointegrate results in single insertions (reviewed in Kleckner, 1981). Either way it can be postulated that the structure of pSA2 compared to the structure of pSA1 favours

either the resolution of the intermediate to generate cointegrates or does not favour the resolution of cointegrates to form single insertions. This could be due to the orientation of Tn*4351*::*catIII* in pSA2, compared to pSA1, and the close proximity of the transposon to pJRD215 promoter sequences. For example, Tn*4351*::*catIII* in pSA1 is positioned upstream of the pJRD215 *repC* gene such that the *repC* promoter could be acting upon ORFII and ORFIII in IS*4351*$_R$*. Although the function of ORFII and ORFIII are unknown, it can be postulated that the potential activation of these open reading frames by the *repC* promoter could alter the activity of Tn*4351*::*catIII* such that single and cointegrate insertions of the transposon occur. In pSA2 the pAO6 plasmid sequences lie between the *repC* promoter and the transposon. It can be speculated that the non-activation of ORFII and ORFIII by the *repC* promoter in pSA2 could result in the demonstrated exclusive insertion of cointegrates. A bias towards cointegrate insertion or single insertion has been noted for other transposons. For example, single insertions are five fold more frequent than cointegrate insertions for Tn*9* and 50 - 200 fold more frequent for Tn*5* and Tn*10*. However, single insertions and cointegrate insertions occur with equal frequencies in Tn*903* whereas Tn*1671* appears to give exclusively cointegrate insertions (Kleckner, 1981).

The original Tn*4351* transposon mutagenesis studies in *Bacteroides* spp. also demonstrated that insertion of cointegrate formations could occur (Shoemaker *et al.*, 1986). It was found that when Tn*4351* was transferred into *Bacteroides* spp. on the plasmid R751 then R751::Tn*4351* cointegrates inserted into the chromosome at a frequency of 50% (Shoemaker *et al.*, 1986). For *P. gingivalis* strains 33277 and W83 approximately 50% of transconjugants also contained both Tn*4351* and R751 sequences inserted into the chromosome (Hoover *et al.*, 1992b; Maley, 1994), whereas for *P. gingivalis* strain 381 70% of transconjugants were found to contain R751 sequences as well as Tn*4351* sequences inserted into the chromosome (Dyer *et al.*, 1992). Analysis of 8 randomly selected transconjugants from Tn*4351* mutagenesis experiments using the *P. gingivalis* strain A7436 indicated that only single insertions of Tn*4351* had occurred and that no R751 sequences had inserted into the chromosome (Genco *et al.*, 1995a). These results suggest that strain variation can result in different frequencies of single

Tn*4351* insertions into the *P. gingivalis* chromosome. This could possibly reflect a need for host proteins to assist in the resolution cointegrate formed between the plasmid carrying the transposon and the recipient *P. gingivalis* strain (or to resolve the intermediate into cointegrates or single insertions). Whilst strain variation did not appear to have affected the number of single insertions when pSA1 and pSA2 were transferred into strain 381 or 33277, strain variation could account for the fact that the insertions into *P. gingivalis* strain 33277 from matings involving pSA2 resulted, with one exception, in complete cointegrates whereas in strain 381 resulted in several types of insertions. In strain 381, matings involving pSA2 resulted in some complete cointegrate insertions, some deleted cointegrate insertions with separate IS insertions elsewhere in the chromosome and some insertions that could not be characterised. These latter insertions were thought to have resulted from transposon mediated deletion/inversion events which could have been mediated by any one of the eight terminal inverted repeats present in the cointegrate formation. Transposon mediated events could include inversions, deletions, adjacent insertions, duplications or inverse transposition, each of which could occur between the ends of the same IS element (intra-molecular transposition events) or between any one of the other six inverted repeats of the other three IS elements of the cointegrate (inter-molecular transposition events). Different Tn*4351*::*catIII* insertions were demonstrated by Southern blot analysis from matings involving the transfer of pSA1 into *P. gingivalis* 381 or 33277. The majority of non-single Tn*4351*::*catIII* insertions demonstrated from matings involving the transfer of pSA1 into strain 381 were found to be complete cointegrate insertions. Three of the non-single Tn*4351*::*catIII* insertions demonstrated from matings involving the transfer of pSA1 into strain 33277, were found to be complete cointegrate insertions, and five of the insertions were found to be partially deleted cointegrate insertions. Previous reports had not fully characterised the cointegrate insertions that resulted from the transfer of R751::*Ω4 into *P. gingivalis* (Dyer *et al.*, 1992; Hoover *et al.*, 1992b) although some indications were given that rearrangements had occurred. In strain W83 it was found that in mating experiments using R751::*Ω4 a small fragment of R751 sequence inserted into the *P. gingivalis* chromosome. Although these insertions were not characterised it was supposed that a region of R751 immediately adjacent to one of the

copies of IS*4351* had been carried into the chromosome by transposition of Tn*4351* (Maley, 1994). The same experiments also demonstrated that in W83 multiple, tandem, chromosomal insertions of Tn*4351* had occurred and that in addition to this random, single insertions of IS*4351* could transpose into the chromosome (Maley, 1994). Transposon mutagenesis studies in *P. gingivalis* 381 demonstrated that some transconjugants analysed by Southern blot analysis contained a single copy of Tn*4351* as well as a separate insertion event that was suggested to be an insertion of one of the IS*4351* elements (Dyer *et al.*, 1992). Transconjugants that contained R751 sequences were analysed by Southern blot analysis of *Pst*I digested chromosomal DNA using an R751::*Ω4 probe. The blot detected four conserved fragments as well as two other fragments in transconjugants that contained cointegrate formation insertions. In some cases one of the conserved *Pst*I fragments was missing suggesting that in these transconjugants a deletion or rearrangement event had occurred. Analysis of other transconjugants suggested that multiple transposon insertions had occurred or that cointegrates had sustained rearrangements during or after transposition (Dyer *et al.*, 1992). However, these rearrangements had not been characterised further and so no comparisons could be made between the rearrangements seen in the previous report (Dyer *et al.*, 1992) and those involving pSA1 and pSA2 in this study.

The frequency of cointegrate insertions (where a cointegrate is taken here to mean a complete or partially deleted cointegrate insertion) was found to be approximately 70% for strains 381 and 33277 for matings involving pSA1 and 100% for matings involving pSA2. Previous reports suggested that the frequency of cointegrate insertions was 50% in strain 33277 (Hoover *et al.*, 1992b) and 70% in strain 381 (Dyer *et al.*, 1992) for matings involving R751::*Ω4. The differences seen in the frequency of plasmid insertions demonstrated in this study and the previously reported frequencies of plasmid insertions (Dyer *et al.*, 1992; Hoover *et al.*, 1992b) probably reflects differences in the suicide vectors used. For all the previous studies the plasmid R751::*Ω4 was used to transfer the transposon into *P. gingivalis* whereas in this study a pBR328 and pJRD215 based plasmid was used as the carrying vehicle. The differences in the frequency of plasmid insertions could also reflect differences in the mating procedures used in this

study and the previously reported studies (Dyer *et al.*, 1992; Hoover *et al.*, 1992b). Analysis of transconjugants from matings involving the transfer of the control plasmid R751::*Ω4 into strain 381 and 33277 would have enabled us to determine whether the frequency of R751::*Ω4 cointegrate insertions occurred at a frequency of 50% for strain 33277 and at a frequency 70% for strain 381 as previously reported (Dyer *et al.*, 1992; Hoover *et al.*, 1992b) or at a frequency of 70% or 100% as indicated in this study. It is possible that the presence of the *catIII* insert within IS*4351*$_R$* could affect the resolution of pSA1 and pSA2 cointegrate formations due to the close proximity of the *catIII* insert to one of the inverted repeats in this insertion element. Alternatively the frequency of cointegrate insertions demonstrated in this study could reflect the alteration made to the terminal inverted repeat *insR*. In order to destroy one of the two *Eco*RI restriction enzyme sites found within IS*4351*$_R$, a 2 bp substitution was made to the sequence and this resulted in the imperfect inverted repeats having a match of 19 out of 25 bases instead of 20 out of 25 bases. The ends of the IS elements are thought to be important for the precise symmetrical joining of the transposon to the target DNA molecule (Grindley and Reed, 1985). Alteration of *insR* such that the number of mis-matched base pairs increases from 5 out of 25 to 6 out 25 could reduce the integrity of the ends such that the resolution of the cointegrates is inefficient. Such a loss of integrity of the terminal inverted repeat could be corrected in further studies by altering one or two base pairs of the *Eco*RI restriction enzyme site, of which one bp represents a mis-matched bp in the wild-type IS*4351* IS element, such that the number of mis-matched bases remains at 5 out of 25 or is even reduced to 4 out 25 mis-matched bases. This could potentially increase the frequency of transposition and increase the number of single insertions. The problem with substituting the bases in this way is that the *Eco*RI restriction enzyme site will no longer be converted into an *Eco*RV restriction enzyme site. Without the *Eco*RV restriction enzyme site the orientation of *catIII* in pAO4 can not be determined easily by restriction enzyme analysis.

In summary, the Southern blot hybridisation analysis of Cl^r transconjugants produced by matings which transferred pSA1 or pSA2 into *P. gingivalis* 381 or 33277, demonstrated that the position of Tn*4351*::*catIII* in pJRD215 can dramatically affect the percentage of

single insertions. The reason for this is unclear although it is presumably to do with the proximity of the transposon to important genes in the suicide vector such as the transfer genes, the origin of replication or more likely to plasmid promoter sequences. It is possible that by removing the transposon from pAO6 on different sized fragments and ligating these into pJRD215 that a higher frequency of single insertions could be obtained. Alternatively by cotransferring a donor strain with pSA1 and R751 and carrying out mating experiments between this strain and another *E. coli* strain, it will be possible to generate R751 plasmids containing a Tn*4351*::*catIII* insert. It would be hoped that as with R751::*Ω4, matings involving R751::Tn*4351*::*catIII* would demonstrate a higher frequency of single insertions. Insertion of Tn*4351*::*catIII* into R751 would also allow a direct comparison to be made between the transposition frequency of Tn*4351*::*catIII* compared to Tn*4351* especially if a partial tandem duplication of Tn*4351*::*catIII* in R751 could be generated.

4.3.3 Analysis of CAT Activity Data

The MIC data on transconjugants from matings involving S17-1* (pSA1) and *P. gingivalis* 33277 revealed that Tn*4351*::*catIII* could insert into the chromosome of the recipient bacteria in such a way as to give CAT activity. Indeed it was noted that almost 95% of the transconjugants tested were able to grow on BM agar plates supplemented with 2 μg/ml Cm, a concentration that the wild-type strain, 33277, was unable to grow at. It was found that 9% of the transconjugants tested were able to grow at Cm concentrations at least 10 μg/ml and although these transconjugants were not tested at higher concentrations of Cm it is possible that a proportion of these bacteria would have been able to grow at elevated Cm concentrations. The results of the MIC data demonstrated that CAT was capable of functioning in *P. gingivalis* and as such *catIII* was suitable for use as a reporter gene in this species. The results also indicated that the initial identification of transconjugants exhibiting CAT activity could be achieved with ease on BM agar plates supplemented with different levels of Cm. Because of the incubation time required to detect *P. gingivalis* growth the utilisation of a large scale detection system for analysis of reporter gene activity is an important consideration

when choosing which gene activity to study. The use of BM agar plates supplemented with Cm allowed the simultaneous detection of many transconjugants at one time and also allowed a direct comparison to be made between the growth of the wild-type bacterium 33277 and the transconjugants at each concentration of Cm.

In order to determine whether CAT activity could be tested quantitatively in *P. gingivalis* a spectrophotometric assay system was used to determine the CAT activity of 15 randomly selected transconjugants. The wild-type strain 33277 was used as a negative control and the *E. coli* strain LE392 carrying the plasmid pECAT was used as a positive control. The assay takes advantage of the generation of a free CoA sulphydryl group coincident with transfer of the acetyl group to Cm. The reaction of this CoA sulphydryl group with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) yields the mixed disulphide of CoA and thionitrobenzoic acid and a molar equivalent of free 5-thio-2-nitrobenzoate, which has a molar extinction coefficient of 13,600 at 412 nm (Shaw, 1975). The assay is performed at 37°C and the rate of increase in absorption of the sample at 412 nm prior to the addition of Cm is subtracted from the observed rate after the start of the reaction. The net change in extinction per minute is divided by 13.6 to give the result in micromoles per minute of Cm dependent DTNB reacted. This is equal to the rate of acetylation of Cm and since 1 unit of CAT is equal to 1 nanomole of Cm acetylated per minute at 37°C, the calculation also yields the number of units of enzyme in the cuvette. All values were adjusted such that the activity was given per mg of protein. The positive control, LE392 (pECAT), was found to have an activity of 40.46 ± 3.79 units of chloramphenicol acetylated per minute per mg of protein at 37°C (where 1 unit represented 1 nanomole Cm acetylated per minute). It was known that the plasmid pECAT, which contains *catIII* inserted upstream of the *lacZ* promoter, was capable of conferring resistance to at least 25 μg/ml chloramphenicol. The result from the assay demonstrated that high levels of CAT activity could be determined by this method. As expected no CAT activity could be demonstrated in the CAT assay for the wild-type strain 33277 as indicated by a zero rate of change in absorbance per minute at 412 nm. Of the fifteen transconjugants assayed 10 transconjugants did not exhibit CAT activity and this was confirmed by the MIC data on BM agar plates. Of the remaining

transconjugants tested, two were able to grow on at least 2 μg/ml Cm and three were able to grow on at least 5 μg/ml Cm. The CAT assay data from these transconjugants confirmed the MIC data. The transconjugants that were able to grow on at least 5 μg/ml Cm demonstrated a CAT activity that was approximately 6 fold lower than that demonstrated by LE392 (pECAT). The transconjugants that grew on Cm concentrations of at least 2 μg/ml Cm demonstrated a CAT activity that was 13 - 14 fold lower than that of LE392 (pECAT) and 2 - 3 fold lower than that demonstrated by the transconjugants that could survive on 5 μg/ml Cm.

The percent of Cm^s transconjugants identified for the CAT activity data was not in agreement with the overall MIC data which suggested that 95% of transconjugants were able to grow on BM agar supplemented with 2 μg/ml Cm. It is unfortunate that the random selection resulted in so many Cm^s transconjugants being selected for the CAT activity data. If time had allowed the transconjugants chosen for the CAT activity would have been selected from BM agar plates supplemented with the different concentrations of Cm. This would also have allowed the CAT activity of transconjugants able to grow on at least 10 μg/ml Cm to be assayed and the activity of these transconjugants could have then been compared to that of the positive control. However, the data obtained from the five transconjugants that did express CAT activity demonstrated that CAT activity could be assayed quantitatively in *P. gingivalis* and that the assay could distinguish between different levels of activity with great sensitivity. This is essential if the assay is to be used in the future to determine the effects of different environmental stimuli on a particular promoter (as measured by CAT activity).

Southern blot analysis of the transconjugants used in the CAT assay revealed that only two of the transconjugants, which were likely to be siblings of each other, contained single Tn*4351*::*catIII* insertions, but neither exhibited CAT activity. Of the five transconjugants that did demonstrate CAT activity, transconjugants numbers 1, 2 and 3 appeared to contain cointegrate insertions, transconjugant number 5 contained a partially deleted cointegrate insertion in which the *P. gingivalis* chromosome to cointegrate junction occurred at an intact copy of IS*4351*::*catIII* and transconjugant number 6

contained a partial copy of Tn*4351*::*catIII* with attached plasmid sequences as well as a separate IS*4351*::*catIII* insertion elsewhere in the chromosome. None of the transconjugants demonstrating CAT activity were found to contain a partial cointegrate insertion as indicated in model B of figure 4.17. This was expected as the proposed deletion of the cointegrate in this model results in loss of the IS*4351*::*catIII* at the *P. gingivalis* chromosome/cointegrate junction. For transconjugants numbers 1 - 3 and 5 two copies of *catIII* were present in the chromosome. However, since only one of the two *catIII* genes inserts in the correct orientation at the *P. gingivalis*/cointegrate junction only one of the copies of *catIII* is active in these transconjugants. In the case of transconjugant number 6 it would seem unlikely that the copy of *catIII*, inserted as part of the rearranged cointegrate, results in the CAT activity demonstrated. Since the *catIII* gene in this cointegrate is at least 12 kb downstream from the *P. gingivalis* chromosome it would be unlikely that an upstream promoter could activate this *catIII* gene, instead it is more likely that the *catIII* gene found in the separate IS*4351*::*catIII* insertion is activated which results in the CAT activity demonstrated.

Southern blot analysis of transconjugants from matings involving pSA1 had determined that single insertions occurred at a frequency of 30%. From this we would predict that 5 out of the 15 transconjugants analysed for CAT activity should have contained single insertions. In fact only 2 of the transconjugants analysed were found to contain single insertions although the reason for this is unknown. It is possible that the transconjugants selected were all products of the same filter mating experiment rather than from several separate mating experiments and that the conditions of the particular mating experiment did not favour the resolution of cointegrates to generate single insertions. In the future it would be interesting to determine whether different mating conditions can affect the percentage of single insertions. For example, if the mating mixture was only incubated on the filters for 48 hours, a reduced resolution of cointegrates compared to matings incubated on the filters for up to 72 hours might result. If this is the case then it would be important to determine the minimum time mating mixtures should be incubated for in order to achieve maximum resolution of cointegrates. The effect of the incubation time of mating mixtures could be determined

by Southern blot analysis of transconjugants from matings using the same donor and recipient ratios and starting CFU/ml but which have been incubated for different lengths of time.

Initially it was important to determine whether Tn*4351*::*catIII* could insert as a single copy into the *P. gingivalis* chromosome and whether the resulting transconjugants could demonstrate differential CAT activity. The Southern blot analysis demonstrated that no single insertions occurred when pSA2 was used as the suicide vector. The presence of two copies of *catIII* in different parts of the chromosome both of which could be demonstrating CAT activity would make analysis of the regulation of the promoters involved difficult. It was for this reason that CAT activity was not determined for transconjugants that resulted from matings involving pSA2. When pSA1 was used as the suicide vector, 30% of the transconjugants in both of the *P. gingivalis* strains tested contained single Tn*4351*::*catIII* insertions. At first the percent of single insertions seemed disappointing since it was assumed that two out of every three transconjugants would not contain useful promoter probe insertions. However, on examination of the Southern blot hybridisation data from transconjugants generated by the transfer of pSA1 into *P. gingivalis* 33277 or 381, it was apparent that the majority of insertions were cointegrates or partially deleted cointegrates. Since the structure of these cointegrates meant that only one copy of *catIII* could be at the chromosome/transposon junction in the correct orientation for activation from an upstream promoter there seemed no reason why these insertions could not function equally as well as a type I promoter probe as a single Tn*4351*::*catIII* insertion. The insertion of a cointegrate (or partial cointegrate) could in fact have an advantage over a single insertion. If a gene, disrupted by a single Tn*4351*::*catIII* insertion, was found to be regulated by environmental stimuli (as indicated by a change in CAT activity) the next step would be to generate a gene library from the transconjugant containing this particular *catIII* insertion. The gene library would then be probed with *catIII* to determine the location of the promoter of interest. Finally this clone would be sequenced in order to identify the gene lying downstream of this promoter. However, because a cointegrate insertion contains an *E. coli* origin of replication as well as selective antibiotic resistance markers it should be feasible to

digest the DNA from a transconjugant disrupted with a cointegrate insertion with a restriction enzyme that does not cleave within pSA1. The resulting fragments could be religated together and transformed into *E. coli*. Any ligation product containing the cointegrate formation will be capable of replicating in *E. coli* and can be selected for using the appropriate antibiotics. This would considerably reduce the amount of time spent cloning the gene located downstream of the promoter of interest. Despite the fact that the majority of Tn*4351*::*catIII* insertions into *P. gingivalis* 33277 from plasmid pSA1 would be suitable for studying the regulation of important promoters, a minority of the insertions, that do not contain a chromosome/*catIII* junction, would not demonstrate CAT activity.

4.3.4 Detection of Neuraminidase or β-Nahase Mutants

Although approximately 7000 transconjugants were screened in attempting to find a neuraminidase negative or a β-Nahase negative mutant all the transconjugants demonstrated these enzyme activities. It has been demonstrated that some bacteria, for example *Streptococcus pneumoniae*, contain at least two distinct enzymes with neuraminidase activity (Camara *et al.*, 1991). It is possible that *P. gingivalis* also contains more than one copy of the neuraminidase gene. If this is the case then both copies of the gene would have to be knocked out by transposon mutagenesis in order for the transconjugant to be selected as a negative mutant. Southern blot analysis of the *P. gingivalis* chromosome from several different strains revealed that this bacterium contains a single copy of the β-Nahase gene (Lovett, 1994), therefore a single insertion event should have generated a β-Nahase negative mutant. It seems likely that too few transconjugants had been screened to detect a negative mutant. However, it must be remembered that even if a negative mutant is generated by insertion of Tn*4351*::*catIII* into the gene of choice that there is still a one in two chance that the insertion will not be in the correct orientation to allow CAT activity to be assayed. It should also be considered that some of the insertions will not contain a chromosome/*catIII* junction and these insertions will not demonstrate CAT activity. This means that potentially two or three separate neuraminidase or β-Nahase negative mutants would have to be

generated before a negative mutant demonstrating CAT activity could be found. Once such a mutant had been found the next step would be to see how this gene was regulated. This could be achieved by carrying out CAT assays on negative mutants that had been grown under different environmental conditions. This would allow the exact conditions that up regulate the gene to be determined and the use of the sensitive CAT assay would enable even small changes in activity to be seen.

A non-specific approach may also be taken to determine the regulation of genes of interest. The CAT activity of several hundred transconjugants can be determined at one time by replica plating the transconjugants onto BM agar plates supplemented with different levels of Cm. The BM agar plates could be subjected to different environmental stimuli, such as increased or decreased haemin levels, increased incubation temperatures, alteration in the pH of the agar plates or increase in oxygen tension. Any transconjugant that demonstrated an altered CAT activity under the conditions of the environmental stimuli, as detected by growth of a particular transconjugant on a higher (or lower) concentration of Cm than noted under normal conditions, potentially has an insertion of Tn*4351*::*catIII* which is under the influence of a virulence gene promoter. Once an important transconjugant has been isolated the CAT activity of the gene under different stimuli can then be examined in more detail using the sensitive CAT assay. Environmental stimuli have been shown to regulate putative virulence genes and this is to be expected since during the progression from health to disease in the periodontal pockets bacteria have to be able to adapt to the change of environment in which both the temperature and pH levels increase and the oxygen tension decreases. Studies have already demonstrated that increasing the concentration of haemin in the *P. gingivalis* culture media results in an increase in trypsin-like protease activity (Marsh *et al.*, 1988; 1994). Conversely, decreasing the level of haemin results in an increase in collagenolytic activity (Marsh *et al.*, 1988; 1994). The pH of the media has also been found to alter enzymatic activities with a higher trypsin-like activity being noted at higher pH levels and higher hyaluronidase and collagenase activities being noted at lower pH levels (McDermid *et al.*, 1988). An increase in incubation temperature by a couple of degrees has been demonstrated to

result in increased levels of SOD activity and a decrease in the number of fimbriae on the bacterium cell surface (Amano *et al.*, 1994). These examples demonstrate the potential use Tn*4351*::*catIII* will have as a type I promoter probe for analysing the regulation of important virulence genes whose activity is determined by the changing environmental conditions that prevail during the progression from health to disease in the oral cavity.

CHAPTER 5

Discussion

Recent advances in the molecular genetic study of the relatively uncharacterised bacterium *P. gingivalis* has allowed the generation of isogenic mutants in this bacterium either by transposon mutagenesis, using Tn*4351*, or through allelic exchange using one of a number shuttle or suicide vectors capable of being transferred into *P. gingivalis*. The use of such systems has allowed the mutation, isolation and identification of genes thought to be important for the proliferation of *P. gingivalis* and for the continuation and progression of the diseased state in the oral cavity. However, these systems do not allow the expression and regulation of these genes to be analysed, or the environmental factors involved in the expression and regulation of these genes to be determined. Previously the analysis of factors that affect the regulation of such genes in *P. gingivalis* has been restricted to using Northern blot analysis to detect changes in the levels of mRNA of a particular gene under different environmental conditions (Amano *et al.*, 1994). The regulation of virulence genes in other bacteria has been studied with considerable success using type I transcriptional promoter probes which fuse a gene of interest to a promoterless gene whose product can be easily monitored (Bellofatto *et al.*, 1984; Manoil and Beckwith, 1985; 1986; Ubben and Schmitt, 1987; Way *et al.*, 1984). Such promoter probes are particularly useful for studying the regulation of genes whose functions are unknown or difficult to assay or for generating random insertions capable of forming transcriptional fusions at the site of insertion. The aim of this study was to manipulate the *Bacteroides* transposon, Tn*4351*, to include a reporter gene, generating a type I promoter probe with which to study the regulation of putative virulence genes in *P. gingivalis*.

At the onset of this study it was not known what the consequence of manipulating one of the two IS elements that flank Tn*4351* would have on the transposition activity of the transposon. Studies had indicated that both IS*4351*$_L$ and IS*4351*$_R$ were capable of independent transposition, although IS*4351*$_L$ demonstrated a higher level of

transposition than IS*4351*$_R$ (Hwa *et al.*, 1988). However, it was not known whether transposition of the entire transposon required functional transposase activity from both IS elements or whether the functional transposase from just one of the insertion elements would allow transposition to occur. The application of the biological assay for use in *E. coli* to assess transposon mediated cointegrate formation, and hence transposase activity, allowed the activity of Tn*4351*::*catIII* to be determined rapidly without the need to transfer the transposon into *P. gingivalis*. The biological assay determined that the manipulated transposon could mediate cointegrate formation and from this it was concluded that the insertion of *catIII* into IS*4351*$_R$, the 2 bp substitution to destroy the first *Eco*RI restriction enzyme site of IS*4351*$_R$::*catIII* and the mutation at nucleotide 675 in IS*4351*$_L$* had not resulted in loss of transposition activity. This was either because a truncated transposase protein was produced by IS*4351*$_R$ that retained some activity, or because a single functional transposase protein from IS*4351*$_L$* was all that was required for transposition to occur. A requirement for only one functional transposase protein has been demonstrated for other transposons. For example, several variants of Tn*10* and Tn*5* have been generated by taking advantage of the fact that most of the sequences within IS*10*$_L$ and IS*50*$_L$ are not required for transposition (Bellofatto *et al.*, 1984; Way *et al.*, 1984). Future experiments on Tn*4351* could delete sections of IS*4351*$_R$, keeping the essential terminal inverted repeat *insR* intact. The use of the biological assay in *E. coli* would then allow the rapid determination of the ability of these derivatives of Tn*4351* to form cointegrates and hence transpose. If the majority of IS*4351*$_R$ sequences are found to be nonessential for transposition then future manipulations of Tn*4351* could take advantage of this fact and use a deleted version of IS*4351*$_R$ as a cloning site for other reporter genes, antibiotic markers or promoters for further analysis of the regulation of *P. gingivalis* genes.

The mating protocol developed in this study proved to be a very efficient method for the transfer of suicide vectors from *E. coli* to *P. gingivalis*. The frequencies of transposition obtained by this study were found to be between 2 - 45 fold higher than the transposition frequencies previously reported for *P. gingivalis* (Dyer *et al.*, 1992; Hoover *et al.*, 1992b). Transposition frequency appeared to be affected by both the suicide vector used

to transfer the transposon into *P. gingivalis* and the recipient strain used. A higher frequency of transposition was seen from R751::*Ω4 than from pSA1 or pSA2 in *P. gingivalis* strain 381 and conversely a higher frequency of transposition was seen from pSA1 than from pSA2 or R751::*Ω4 in *P. gingivalis* 33277. The increased transposition frequencies of manipulated transposons above that of the wild-type precursors have been demonstrated previously, for example, derivatives of Tn*10* have levels of transposition 100-1000 fold higher than the transposition frequencies demonstrated for the wild-type Tn*10* (Way *et al.*, 1984). The initial transposon mutagenesis studies using Tn*4351* had already demonstrated that it was possible to transfer Tn*4351* into *P. gingivalis* (Dyer *et al.*, 1992; Hoover *et al.*, 1992b; Progulske-Fox *et al.*, 1989b). However, this study has demonstrated that is possible to achieve efficient and detectable levels of transposition in *P. gingivalis*, therefore the use of the mating procedure devised in this study will allow Tn*4351* to be used as an efficient mutagenesis tool for *P. gingivalis*.

The aim of this study was to manipulate Tn*4351* to include a reporter gene to generate a type I promoter probe for studying the regulation of gene expression in *P. gingivalis*. The manipulated transposon, Tn*4351*::*catIII*, was successfully transferred into *P. gingivalis* where it was found to insert randomly into the chromosome and this could result in differential CAT expression. The CAT activity generated by transposition of Tn*4351*::*catIII* into transcriptionally active areas of the *P. gingivalis* genome was detected with ease on BA agar plates supplemented with Cm and could also be assayed quantitatively. As such *catIII* is potentially an ideal reporter gene for use in a *P. gingivalis* promoter probe. The high percentage of Cm^r transconjugants produced in this study suggests that although random insertion of Tn*4351*::*catIII* is occurring, that the promoter probe may have a bias towards transposition into transcriptionally active regions. This type of bias towards transposition into transcriptionally active areas has been demonstrated for other transposons (Bellofatto *et al.*, 1984). If this is the case for Tn*4351*::*catIII* then this will be an advantage in using Tn*4351*::*catIII* as a promoter probe since in the majority of cases the transposon will insert within a gene. The study was unable to determine whether Tn*4351*::*catIII* could be used to study the regulation of

putative virulence genes under different environment stimuli. In order to establish whether Tn*4351*::*catIII* is a useful genetic tool, future work should concentrate on the isolation of a neuraminidase or β-Nahase negative mutant expressing CAT activity as well as on the isolation of transconjugants that demonstrate altered CAT activity under different environmental conditions. In the short term, the usefulness of Tn*4351*::*catIII* to study the environmental regulation of a putative virulence gene could be achieved by the isolation of Cl^r, beige pigmented mutants which were detected in this study at a frequency of 1 in 3000 Cl^r transconjugants. Other studies have demonstrated that beige mutants can exhibit a deficiency in trypsin-like protease activity and in haemagglutinin activity (Hoover *et al.*, 1994). Therefore, since trypsin-like protease activity has been found to be regulated by the concentration of haemin in the culture medium, a beige Cm^r mutant may well be able to demonstrate increased CAT activity in haemin excess conditions and decreased CAT activity when haemin levels are limiting (Marsh *et al.*, 1994).

Although this study indicates Tn*4351*::*catIII* has potential as a useful genetic tool, some problems still exist. The frequency of single insertions of Tn*4351*::*catIII* in *P. gingivalis* 381 and 33277, for matings involving the transfer of pSA1, was found to be 33.3%. The non-single insertions were found to be either complete or partially deleted cointegrate insertions. Because the complete cointegrate insertions and the majority of the partially deleted cointegrate insertions have one chromosome/*catIII* junction they are still useful as type I promoter probes. However, a minority of the partially deleted cointegrate insertions did not contain a chromosome/*catIII* junction and as such these insertions are not capable of forming transcriptional fusions. Although these partially deleted cointegrate insertions will automatically be selected against as having no CAT activity, the presence of such insertions will mean that more transconjugants will have to analysed than if all insertions were simple insertions, cointegrate insertions or partially deleted cointegrates with a chromosome/*catIII* junction. Since no other study has fully characterised Tn*4351* cointegrate insertions it is not known whether these partially deleted cointegrate insertions occur routinely as a result of transposon mediated deletions or whether they are an abnormality of Tn*4351*::*catIII* transposition.

It would be useful to determine what factors affect the frequency of cointegrate insertions so that the frequency of partially deleted cointegrate insertions not containing a chromosome/*catIII* junction can be reduced. Previous reports (Dyer *et al.*, 1992; Hoover *et al.*, 1992b) had suggested that R751::*Ω4 cointegrate insertions occurred at a frequency of 50% in strain 33277 (Hoover *et al.*, 1992b) and 70% in strain 381 (Dyer *et al.*, 1992). It seems probable that the differences in the frequency of single insertions seen in this study and the previous reports (Dyer *et al.*, 1992; Hoover *et al.*, 1992b) are a result of the different structures of the suicide vectors used. Future experiments should determine what, if any, affect the 2 bp substitution in *insR* has on the frequency of single insertions. It could also be established whether the position of Tn*4351*::*catIII* in pJRD215, with regard to the proximity of the transposon to pJRD215 promoter sequences, affects the frequency of single insertions. Alternatively plasmid R751 carrying a tandem, partial duplication of Tn*4351*::*catIII* could be generated in the hope that transposition of Tn*4351*::*catIII* from R751 would result in a higher frequency of single insertions and a lower frequency of partially deleted cointegrate insertions.

The versatility of the promoter probe might be increased by cloning other reporter genes into IS*4351*$_R$*. As described earlier (section 3.1) many reporter genes that have proved useful for studying gene regulation in other Gram-negative bacteria are not applicable for use in *P. gingivalis.* This is either because the reporter gene requires oxygen (luciferase), because *P. gingivalis* already encodes a gene with the same activity as commonly used reporter genes (alkaline phosphatase and β-galactosidase) or because the initial chromogenic detection system used to determine whether the reporter gene activity is present or not does not appear to work in *P. gingivalis* (β-galactosidase, β-glucuronidase, alkaline phosphatase). These problems limit the reporter genes that can be used effectively in *P. gingivalis*. The use of *catIII* demonstrates that antibiotic resistance determinants from other Gram-negative bacteria can be used as reporter genes in *P. gingivalis* and perhaps several Tn*4351* derivatives could be constructed each containing a different antibiotic resistance reporter gene. This would be advantageous because the manipulated transposons could then be used with a wide range of recipient

bacteria and suicide vectors that exhibit resistance to different antibiotics. Another gene that it would be useful to clone into Tn*4351* as an additional reporter gene is *xylE*, a gene which has been used as a reporter gene in several bacterial species and has become an invaluable tool for analysis of gene regulation in *Pseudomonas* (Curcic *et al.*, 1994; Konyecsni and Deretic, 1988). The gene *xylE* encodes for catechol 2,3 dioxygenase (CDO) which can convert catechol into 2-hydroxymuconic semialdehyde, a substance with a bright yellow colour (Nozaki, 1970). This provides the basis for scoring bacteria colonies carrying *xylE*, which turn yellow upon being sprayed with a solution of catechol. A promoterless *xylE* gene has been generated on a 1.4 kb cassette that can be removed on an *Eco*RI fragment (Curcic *et al.*, 1994). In the future it is hoped to obtain this cassette and clone it into the unique *Eco*RI site in pAO3 after which the entire transposon (Tn*4351*::*xylE*) could be regenerated using the 3.8 kb *Ava*I fragment as described previously.

The overall conclusions from this study are that it is possible to manipulate Tn*4351* to include a reporter gene and that the manipulated transposon can be transferred into the two *P. gingivalis* strains tested. Transposition of Tn*4351*::*catIII* into *P. gingivalis* was demonstrated to result in the random insertion of the transposon in the chromosome and the generation of transcriptional fusions at the site of insertion. The reporter gene, *catIII*, was found to be functional in *P. gingivalis* and CAT activity could be detected with ease allowing the rapid detection of transcriptional fusions. In addition to this it was demonstrated that CAT activity could be assayed quantitatively and with sensitivity in *P. gingivalis*. Differential CAT activity was demonstrated in different transconjugants and although the regulation of putative virulence genes under different environmental stimuli was not demonstrated, it can be envisaged that Tn*4351*::*catIII* will prove to be a powerful genetic tool as a type I promoter probe for use in *P. gingivalis* and *Bacteroides* spp. In addition to this, the mating procedure developed for use in this study has proved to be one of the most efficient systems developed for transfer of suicide vectors from *E. coli* into *P. gingivalis*. The use of this system will not only allow the efficient transfer and subsequent transposition of Tn*4351*::*catIII* into

P. gingivalis, but will also allow more efficient transposon mutagenesis to be carried out using Tn*4351* resulting in the generation of isogenic mutants at detectable levels.

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