Molecular characterisation of PgaA, an antigen from periodontopathogen *Porphyromonas gingivalis*.

Thesis submitted for the degree of Doctor of Philosophy
at the University of Leicester

by

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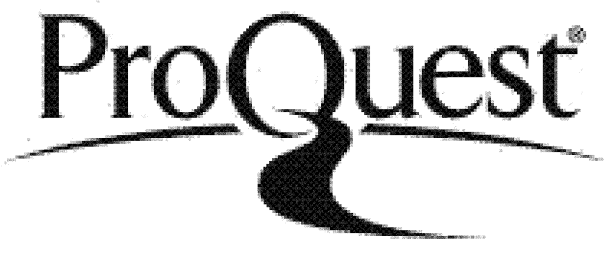

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Declaration.

The accompanying thesis submitted for the degree of Ph.D. entitled "Molecular characterisation of PgaA, an antigen from periodontopathogen *Porphyromonas gingivalis*" is based on work conducted by the author in the department of Microbiology and Immunology of the University of Leicester mainly during the period between January 1990 and September 1993.

All the work detailed in this manuscript 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: Gordon Riley Date: 22/3/96.

Abstract.

Molecular characterisation of PgaA, an antigen of periodontopathogen *Porphyromonas gingivalis.*

Gordon P. Rigg BSc (Manchester).

Molecular cloning techniques were used to investigate antigenic determinants of periodontopathogen *P.gingivalis* in an attempt to begin to explore the cell surface of this organism. A genomic library generated in *Escherichia coli* was probed with polyclonal antiserum specific for *P.gingivalis* whole cells. Clone pGPR1, which contained a 307 bp fragment of *P.gingivalis* DNA sequence, was found to react with both polyclonal antiserum and a monoclonal antibody (mAb) specific for the trypsin-like protease of this organism (Brick190). This fragment was used to probe a second *P.gingivalis* genomic library. Clone pGPR2 was shown to hybidise with the 307 bp probe. DNA sequence analysis revealed pGPR2 contains a 5,653 bp insert with seven open reading frames one of which shows significant DNA homology with the *rnh*B gene of *E.coli*. The 307 bp probe sequence was found to reside within an ORF predicted to encode a 455 amino acid (50 kDa) protein and this ORF was designated *pga*A (*P.gingivalis* antigen). The predicted amino acid sequence of PgaA contains a putative ABC signature for binding NTPs as well as a predicted transmembrane domain. Subclones of *pga*A in expression vector pTTQ18* were found to be reactive with a mAb specific for a 46 kDa species-specific antigen of the cell envelope of *P.gingivalis* (LDS28) as well as Brick190. One such subclone, pGPR7 was also shown to express a 46 kDa protein reactive in western blots with mAb LDS28. Minicell labelling of pGPR2-encoded proteins using pGPR2 subclones revealed that *pga*A directs expression of protein of multiple molecular weights (31-46 kDa) from its own promoter in *E.coli*, and that some of these forms may be caused by proteolysis of a 50 kDa precursor which itself shows a reduced apparent molecular weight (46 kDa) on SDS PAGE.

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To my parents, John and Dorothy I give my deepest thanks for their love, belief in me, patience and support over the years. This thesis is dedicated to you.

Finally, my special thanks to Sue for being with me, being patient, tolerant and being there for me. Thank you.

Contents.

List of Abbreviations.

ANUG	Acute necrotizing ulcerative gingivitis
AP	Adult periodontitis
BAMCA	Nα-benzoyl-L-arginine-7-amido-4-methyl-coumarin
BAPNA	benzoyl-L-arginine-*p*-nitroanilide
bp	base pairs
CIP	calf intestinal phosphatase
DIFP	diisoprpylfluorophosphate
DNA	deoxyribonucleic acid
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxcytosine 5'-triphosphate
dGTP	deoxyguanine 5'-triphosphate
dTTP	deoxythymidine 5'-triphosphate
dNTP	deoxynucleoside 5'-triphosphate
EDTA	ethylenediaminotetra-acetic acid
GJP	generalized juvenile periodontitis
IPTG	isopropylthiogalactoside
kb	kilobase
kDa	kilodaltons
KDO	2-keto-3-deoxymanno-octonic acid
LJP	localised juvenile periodontitis
LPS	lipopolysaccharide
mAb	monoclonal antibody
mins	minutes
NEB	New England Biolabs
ORF	open reading frame
PCMB	*p*-chloromethyl mercuribenzoate
PCR	polymerase chain reaction
PMN	polymorphonuclear leukocytes
PMSF	phenylmethonylsulfonyl fluoride
PRP	proline rich proteins
psi	pounds per square inch
RBC	red blood cells
RNA	ribonucleic acid
RP	refractory periodontitis
RPP	rapidly progressive periodontitis
SDS	sodium lauryl sulphate
SOD	superoxide dismutase
SSC	saline-sodium citrate
TEMED	N,N,N',N'-tetramethylethylenediamine
TLCK	tosyl-L-phenylalanine chloromethyl ketone
TLP	trypsin-like protease
U	unit
X-gal	5-bromo-4-chloro-3-indoyl-D-galactoside

Chapter 1

Introduction.

1.1 *Porphyromonas gingivalis*.

1.1.1 Taxonomy and serology.

The first researchers to report black-pigmented Gram-negative anaerobes in pure culture described "black colonies" isolated from clinical specimens taken from "throat, tonsils, infected surgical wound of the abdomen, from urine... and from the faeces of a case of chronic dyssentry..." (Oliver and Wherry, 1921). As they believed the pigment to be melanin they proposed the novel species *Bacterium melaninogenicum* to describe these bacteria. In recognition of Oliver and Wherry's report Bergeys' Manual of Determinative Bacteriology named this bacterium *Haemophilus melaninogenicum* (third edition, 1930) and later *Bacteroides melaninogenicus* (Roy and Kelley; Bergeys fifth edition, 1939). Other proposed name changes included *Ristella melaninogenicus* (Prévot, 1928) and *Fusiform nigrescens* (Schwabacher *et al.*, 1947) although the designation *Bacteroides melaninogenicus* persisted.

Despite proposed changes in the taxonomy of black-pigmented anaerobes, no further divisions occurred within the group *Bacteroides* until 1970 when it was realised that *B.melaninogenicus* could be regrouped into several subspecies by virtue of heterogeneity in the way that different strains are able to ferment carbohydrates (Holdeman and Moore 1970). The fermenting strains were split into two subgroups; *B.melaninogenicus* subspecies *melaninogenicus* which strongly ferment carbohydrate, and the weakly fermenting strains *B.melaninogenicus* subspecies *intermedius*. The completely asaccharolytic strains were subsequently redefined as *B.melaninogenicus* subspecies *asaccharolyticus*. Shortly after this Coykendall *et al.* (1980) proposed that the asaccharolytic strains of oral origin be known as *Bacteroides gingivalis* based on the observation that oral and non oral strains could be distinguished from each other by virtue of their differing G+C base pair content (Shah *et al.*, 1976, and van Steenberg *et al.*, 1979). Strains isolated from non oral sites retained the name *B.asaccharolyticus*. However, a later re-evaluation led to the generation of a further species namely *B.endodontalis* which, although asaccharolytic and isolated exclusively from oral sites, was considered

sufficiently different from *B.gingivalis* to be treated as a separate species (van Steenberg *et al.* 1982).

Today the genus *Bacteroides* is restricted only to *B. fragilis* and its close relatives (Shah *et al.*, 1989). The asaccharolytic black-pigmenting strains have been reclassified as *Porphyromonas* whereas the saccharolytic black-pigmenters are now known as *Prevotella* (the species names have remained the same). These rearrangements were made using a large number of criteria including DNA hybridisation and base pair composition, and rRNA sequence analysis (a review of which can be found in Collins and Shah, 1987). Thus *Bacteroides gingivalis* is now known as *Porphyromonas gingivalis*. Similarly *Bacteroides endodontalis* and *asaccharolyticus* have become *Porphyromonas endodontalis* and *asaccharolyticus* respectively.

The above changes in taxonomical groupings have subsequently been augmented by the application of serological analysis. Studies using antisera raised to *P.gingivalis* have shown little or no cross-reaction with outer membranes of *Prevotella* (formerly *Bacteroides*) species (Li and Bowden, 1990). In addition use of immunoelectrophoresis and immunoblotting (Reed *et al.*, 1980) has demonstrated that no common antigens are observed between saccharolytic and asaccharolytic black-pigmenting strains and that *Porphyromonas (Bacteroides) asaccharolyticus* isolated from non-oral sites is in all cases antigenically dissimilar to *P.gingivalis*. These data are in agreement with the allocation of a separate genus for the asaccharolytic strains and for several distinct species within the genus *Porphyromonas*. In addition, the distinct antigenic profile of *P. gingivalis* and the high prevalence of antibodies against *P.gingivalis* in the antisera of patients with periodontal disease (Hayakawa *et al.*, 1992, Ogawa *et al.*, 1991, Whitney *et al.*, 1992) could be of an advantage when immunological techniques are applied in the diagnosis and molecular analysis of bacteria and bacterial infections of the periodontum.

Within the classification *P.gingivalis* a number of serogroups have been observed using crossed-immunoelectrophoresis, and this has demonstrated shared and serospecific antigens between human and animal isolates of *P.gingivalis* (Parent *et al.*, 1986). Fisher *et al.* (1986) were able to show that there are two distinct serotypes within the human "biotype" which they named serogroups A and B, where Group A correlated with strains considered less virulent in animal models (van Steenberg *et al.*, 1987). These

data have been corroborated by Gmür *et al.* (1988) who have demonstrated that monoclonal antibodies (mAb) raised against formalinised *P.gingivalis* W83 cells (virulent and serogroup B) do not react in western blots against the less virulent strains. It is possible that antigenic components unique to and diagnostic for the B serotype might represent important virulence determinants.

A third serogroup has since been described, namely serogroup C (Fisher *et al.*. 1987) whose representative strain A7A1-28 is also virulent. It was shown that at least some of the *P.gingivalis* antigens characteristic of each serogroup are present on the bacterial cell surface (Fisher *et al.*, 1987). This latter observation is consistent with the data of Gmür *et al.* (1988) which suggests that some of the antigenic determinants recognised by their mAb were lipopolysaccharide (LPS) in nature.

Another approach for study of the heterogeneity amongst *P.gingivalis* strains has employed restriction fragment length polymorphism (RFLP) analysis of chromosomal DNA of *P.gingivalis* (Loos *et al.*, 1990). These workers analysed the RFLP "fingerprints" of 33 isolates, and found that nearly all of their agarose gel banding patterns were different. This genetically-based heterogeneity was irrespective of serotype hence demonstrating the poor discrimination of serotyping in the detection of inter-strain polymorphisms. The few strains sharing the same "fingerprint" were all commonly used laboratory strains and the authors have speculated that laboratory errors have resulted in duplication of the same strain (however one of these identical groups represented isolates taken from the same tribe of Indians two years apart and may explain their identical fingerprints). Notably strains W83 and W50 (serogroup B) had identical "fingerprints" yet these two strains differ significantly in their outer membrane polypeptide banding patterns in SDS PAGE (Kennel and Holt 1990). Ideally attempts to combine RFLP analysis, and serotyping data might be able to better define clonal groups of *P.gingivalis* and relate them to differing severities of periodontal disease.

1.1.2 Nutrition, physiology and cell components.

Transmission light microscopy of Gram-stained cells reveals *P.gingivalis* to be a small Gram-negative rod with a tendency to form short chains and

sometimes clumps. Electron microscopy (EM) of thin sample sections confirms this morphology and reveals inner and outer membranes separated by an electron-dense layer of peptidoglycan (Mansheim and Kasper, 1977). A layer of capsular material (about 30 nm) external to, and associated with the outer membrane is also visible (Mansheim *et al.*, 1978). Negative staining techniques have revealed numerous fimbriae of varying lengths (≤1.3 μm length) emanating from the cell surface (Handley and Tipler, 1986). In addition, immunogold-labelling using antibodies against the HA-Ag2 haemagglutinin has demonstrated the presence of a further cell surface appendage independent of fimbriae (Deslaurier and Mouton, 1992). This structure appeared as long amorphous strands emanating from the cell surface. This structure is invisible using other EM techniques and these authors postulate that the antibody conjugate binding stabilises this structure during processing for EM hence allowing its visualisation. Also visible under EM is the occurrence of numerous blebs or vesicles (10-500nm in diameter) which are the result of localised budding of the outer membrane. These outer membrane vesicles (OMV) are bag-like structures and are believed to contain periplasmic components.

Growth requirements of *P.gingivalis* include amino acids arginine, cysteine, histidine, serine, tryptophan, and small peptides as judged by substrate utilisation during chemostat studies (McKee *et al.*, 1986). It is believed that because of it's inability to utilise carbohydrate as an energy source *P.gingivalis* metabolises amino acids for energy. This anaerobic metabolism of amino acids results in short chain fatty acid by-products, namely acetate, propionate, butyrate, isovalerate and phenylacetate which are released into the culture media during growth. The requirement for small peptides and amino acids necessitates the existence of extensive proteolytic mechanisms by which nutritional requirements can be met. These criteria have been met by *P.gingivalis* which degrades proteins and peptides in the external environment for uptake into the cell and for subsequent metabolism. Another essential growth requirement of *P.gingivalis* is vitamin K. The reason for this is unknown but it has been speculated that it might function in some aspect of the electron transport chain (Gibbons and McDonald, 1960). Although the source of vitamin K *in vivo* is unknown, there is some limited evidence to suggest that some of the black-pigmented anaerobes are able to substitute steroid hormones for vitamin K (Kornman and Loesche, 1982).

The role of haemin in the physiology of *P.gingivalis* is pivotal to the understanding of growth and virulence. Haemin is the sole source of protoporphyrin for this bacterium without which growth can not occur. It is believed that *P.gingivalis* maybe unable to synthesise its own protoporphyrin and hence uses the tetrapyrrole group from haemin instead (Carmen *et al.*, 1990). Thus, the source of this crucial compound has to be environmental and *in vivo* is probably derived from the host's haemoglobin. This acquired protoporphyrin is then used to form part of the prosthetic group of cytochrome b, one of the major components of the electron transport chain (Shah and Williams, 1987). It is also probable that haemin is the main source of iron for the bacterium (Carmen *et al.*, 1990). This observation correlates with the notion that in many host-pathogen situations excess iron enhances bacterial infection and that virulence is associated with the ability of the bacterium to obtain iron from it's host (Payne, 1988). What is more, *P.gingivalis* has been shown to store haemin for later use in situations of haemin limitation and that this storage activity is mediated by the cell's outer membrane, thus giving *P.gingivalis* its characteristic black pigmentation (Grenier, 1991, and Rizza *et al.*, 1968).

1.2 Periodontal Disease and Bacterial Infection.

1.2.1 Introduction

Periodontal disease is a condition of the junction between teeth and periodontum. Its symptoms include localised inflammation, bleeding, and in severe cases destruction of bone and connective tissue; subsequent tooth loss at the affected site is common in the advanced stages of infection. It occurs with varying severity and symptoms and although affecting mostly healthy adults, it can target children and individuals with certain genetic and acquired diseases. It's causative agent is a mixed microbial infection of the subgingival crevice, certain species of which (*P.gingivalis* for example) are believed to play pivotal roles in the aetiology of periodontal disease. Preventative measures for the most part involve practice of good oral hygiene in order to discourage plaque-induced gingivitis, which otherwise might predispose some sites to more serious forms of periodontal disease (Marsh, 1990). Once the disease state is initiated it can be treated with antimicrobial agents and nominal dental surgery. However, this treatment is lengthy and

must be sustained for a long periods of time, and it is the inconvenience of this long-term therapy and it's widespread nature that justifies further research into this disease (Socransky *et al.*, 1981).

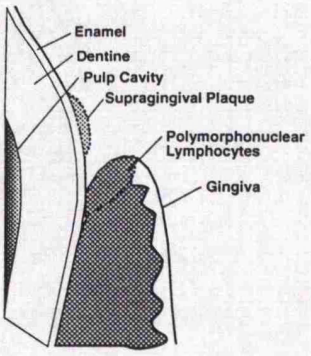
Although preventative measures are trivial it has been shown that the majority (70 to 90%) of adults over the age of 35 years show some degree of periodontitis (this is classically known as adult periodontitis). Whilst on a global scale information concerning the prevalence of periodontal disease is poor, some data suggest that it is the major cause of tooth loss in the over 30's, being more significant than dental caries in this age group (Barnes, 1980). As a world health problem, most populations studied have shown to be at risk of severe periodontitis regardless of established patterns of oral hygiene; this highlights the economic and global importance of gingival disease.

1.2.2 Disease progression and classification.

The stages of disease progression can be classified on the basis of immunopathological changes at the site of infection, and reflect differing levels of severity. The first of these stages as reviewed by Roit and Lehner (1980) is the initial lesion.

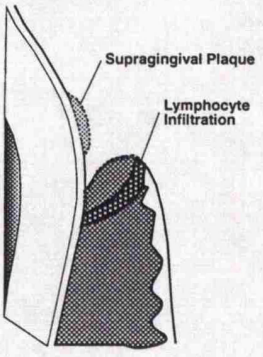
The **initial lesion** is characterised by an acute inflammatory response induced by components of the immune system in response to the presence of plaque in the gingival sulculus (figure 1.1). This response involves the migration of neutrophil granulocytes from the gingival blood vessels drawn by microbially-secreted chemotactic factors, through the junctional epithelium and oral sulcular epithelium, into the gingival sulculus. It is here that these neutrophils carry out anti-bacterial immune functions such as phagocytosis, whilst secreting further chemotactic factors (van Dyke *et al.*, 1980). There is also involvement of the humoral response in the form of complement activation and immunoglobulin generation, although on a small scale. This initial response itself is not serious in nature in that it represents a controlled immune response which restricts yet tolerates bacterial presence and multiplication in the gingival crevice (Schroeder and Attstrom, 1980). Immunological activity of this kind is common in healthy gingiva yet can progress further if plaque is allowed to accumulate resulting in an **early lesion.**

Figure 1.1 Diagrammatic representation of the stages in the developement of the advanced periodontal lesion.



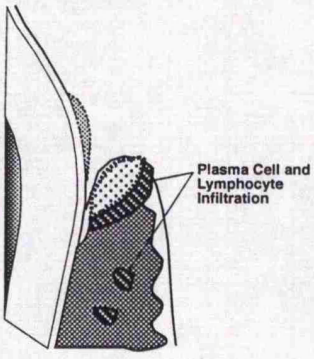
Initial Lesion

An acute localised inflammatory response characterised by dilation of the gingival blood vessels and exudation of fluid into the gingival crevice.



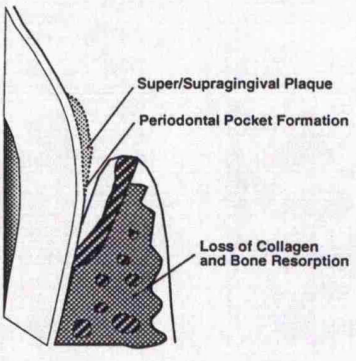
Early Lesion

Lymphocytes migrate to the site of infection and there is a increase in the exudation of serum immunoglobulins, complement, and fibrinogen. The early lesion is commonly found in normal gingiva when plaque control is not practiced efficiently.



Established Lesion

The lesion is still localised at the gingival margin but is dominated by plasma cells as oppose to lymphocytes. The gingival sulculus may deepen and the junctional epithelium may be converted to a pocket



Advanced Lesion

Pronounced, progressive pocket formation occurs with ulceration of the pocket epithelium, destruction of the collagenous periodontal ligament and irreversible bone resorption. The epithelial barrier between plaque and periodontal tissue is disrupted. This stage is classically termed periodontitis.

The **early lesion** develops within 4 to 10 days of plaque accumulation and is a condition in which large numbers of lymphocytes infiltrate the connective tissues and gingival crevice where they are believed to secrete further inflammatory mediators (figure 1.1). These lymphocytes are primarily of the T-cell type with small numbers of B-cells and macrophages (although the proportion of each cell type remains somewhat controversial; Ivanyi and Newman, 1986). In the vicinity of this lymphocyte infiltration there are degenerative changes in fibroblasts and also a localised loss of collagen. This stage is manifested by gingivitis (figure 1.1) which is seen as redness around the necks of the teeth and is frequently accompanied by bleeding of the gingiva either spontaneously or in response to minor trauma of the gums such as that caused by routine brushing.

The **established lesion,** also known as periodontitis, usually occurs within 2 to 3 weeks of initial lesion emergence and may remain stable for some time. The symptoms include gingival bleeding, the gums pulling away from their teeth and the formation of a periodontal pocket. This pocket formation is a critical point in disease progression and is caused by a deepening of the subgingival crevice (figure 1.1). This allows colonisation of the subgingival tooth surface and is characterised by apical migration of the junctional epithelium thus allowing bacterial secretions access to the connective tissues. The mechanism of pocket formation is unknown but it has been suggested that it arises as a direct consequence of the advance of plaque either mechanically or by some intrinsic ability of plaque to disrupt junctional epithelium (Shroeder and Attstrom, 1980). It is accompanied by a loss of collagen and infiltration of the surrounding connective tissue with complement and immunoglobins. and plasma cells, as oppose to lymphocytes which predominate in the early lesion (Schroeder *et al.*, 1976).

In the **advanced lesion,** where the immunological defence regimen is no longer effective chronic inflammatory damage occurs. The symptoms include a deepening of the periodontal pocket (figure 1.1), ulceration of the pocket epithelium, loss of collagen and soft connective tissue, and irreversible destruction of the periodontal ligament and alveolar bone. The sum product of this tissue destruction is the loosening and subsequent loss of dentition, which occurs irrespective of the health of the affected tooth. This corrosion can spread both laterally and apically. In addition, the gingival epithelium

can also degenerate further, allowing direct contact between plaque and periodontal tissue. This leads to direct contact between bacteria and components of the immune system (reviewed in Slots and Genco, 1984). This breakdown of the cementum-epithelial junction results in extensive leakage of blood cells and other blood components into the gingival crevice. Usually by this stage the damage done is irreversible in that even if successful therapy is administered the effected teeth remain loose and prone to mechanical injury-induced loss.

As mentioned there are certain high risk groups to be looked at in considering periodontal disease as well varying types of disease depending on the causative factors. These are shown in table 1.1. By far the most significant of these diseases are the "aggressive" forms namely localised juvenile periodontitis (LJP), generalised juvenile periodontitis (GJP), rapidly progressive periodontitis (RPP) and refractory periodontitis (RP).

Rapidly progressive periodontitis (RPP) is a rare form found in young adults who usually have had some previous history of juvenile periodontitis (reviewed in Nisengard *et al.*, 1988). Characteristic symptoms include bursts of destructive activity leading to severe alveolar bone loss, gingival bleeding and acute inflammation. The disease can lose its inflammatory character yet maintain its bone destroying properties. RPP and refractory periodontitis (RP) are symptomatically similar yet RP represents a form of the disease which is resistant to treatment (Nisengard *et al.*, 1988). Cases of RP are particularly rare and patients who suffer from it suffer continued periodontal breakdown despite treatment.

Juvenile periodontitis by definition affects individuals of the 12 to 20 age group. The two classic forms of the disease localised juvenile periodontitis (LJP) and generalised juvenile periodontitis (GJP) have similar symptoms in that in both cases there is little inflammation and little supragingival plaque (Nisengard *et al.*, 1988). However, the pattern of bone destruction differs. In LJP bone loss is extremely discrete and is usually localised to one tooth. In GJP bone loss is generalised in that it is able to spread laterally. In addition, susceptibility to LJP is familial and tends to affect young females. Prepubertal periodontitis also has a genetic component and is also more common in females. Occurring during the eruption of the primary teeth,

Table 1.1 Principal forms of periodontal disease.

I. Gingival diseases

A. Gingivitis

1. Nonspecific gingivitis.
2. Acute necrotizing ulcerative gingivitis (ANUG)

B. Manifestations of systemic disease and hormone disturbance -e.g.desquamative gingivitis and pregnancy-associated gingivitis.

C. Drug associated abberations -e.g.Dilantoin-induced gingival hyperplasia.

II. Muco-gingival conditions -e.g., gingival recession.

III. Periodontitis

A. Adult periodontitis

1. Slight
2. Moderate
3. Advanced
4. Refractory and rapidly progressive

B. Juvenile periodontitis

1. Prepubertal
2. Generalized juvenile periodontitis (GJP)
3. Localised juvenile periodontitis (LJP)

C. Periodontal abscess

IV. Pathology associated with occlusion

V. Other conditions (such as infection or trauma)

Adapted from Newman and Nisengard (1988).

prepubertal periodontitis is characterised by intensively red gums and subsequent rapid alveolar bone loss. This bone loss can be generalised or localised, the localised form tending to induce less inflammation (Nisengard *et al.*, 1988).

Other forms of gingival dysfunction can be associated with systemic disease and generalised systemic conditions. For example patients with insulin-dependent diabetes mellitus or juvenile diabetes frequently suffer severe periodontal disease exhibiting severe bone loss. Hormonal changes can also effect periodontal status in that there is a prevalence of periodontal disease in pregnant woman. This susceptibility has been linked to changes in plaque content and correlated with increased uptake of progesterone by dental plaque (Kornman and Loesche, 1982). There is also an increased susceptibility to periodontal disease in immunosuppressed individuals. Patients with human immunovirus infection (HIV) have been reported as having HIV-associated periodontitis and HIV-associated gingivitis (Murray *et al.*, 1989).

One final form of disease to consider is acute necrotizing ulcerative gingivitis (ANUG). Symptomatically ANUG manifests itself in painful ulcerative, necrotic gingival lesions which are most commonly associated with the gingival papillae (Nisengard *et al.*, 1988). ANUG patients are commonly under some form of physiological or psychological stress. It is for this reason that it is also known as "trench mouth" as it was seen to be common in battle-weary soldiers. Treatment focuses on effective plaque control and removal of infected tissue.

1.2.3 The importance of *P.gingivalis* in periodontal disease.

The involvement of different micro-organisms in periodontal disease presents a difficult and, in some instances unique problem for investigators. It has been estimated that between three to four hundred different species at some point inhabit the subgingival site (Socransky, 1981) and while no single micro-organism can be shown to be the cause periodontal disease, mixed infections are too complex to control and evaluate under experimental conditions. It is thus impossible to interpret Koch-Henle Postulates in relation to periodontal disease. We are however able to target our efforts by approach of circumstantial evidence. The presence of certain members of the

periodontal flora correlating with certain aspects of disease present such criteria.

There is compelling evidence pointing to *P.gingivalis* as having an important role in certain forms of periodontal disease. In a study by White and Mayrand (1981) it was shown that adult periodontitis patients with severe inflammation showed a greater proportion of Gram-negative anaerobic rods in subgingival samples when compared with healthy controls. Moreover, 31.8% of these isolates were shown to be oral *B.asaccharolyticus* (*P.gingivalis*). This evidence is in agreement with a plethora of previous data showing that black-pigmented anaerobic bacteria are recovered from the majority of advanced periodontitis and gingivitis sites (reviewed in Slots, 1981). There is also a positive correlation between the percentage of black-pigmented anaerobic bacteria present in infection and the severity of disease. Interestingly, it has been shown that *P.intermedia* tends to dominate the earlier, less severe forms of periodontal disease whereas *P.gingivalis* predominates in the more acute forms (Kiel *et al.*, 1983).

The application of animal models in assessing the virulence of *P.gingivalis* and other periodontopathogens has provided some tentative evidence to suggest the role of certain bacteria in periodontal disease. The strongest evidence for *P.gingivalis* in this context has been provided by Holt *et al.* (1988) and Klausen *et al.* (1991a; 1991b). Holt *et al.* (1988) demonstrated that monoinfection of rifampicin resistant *P.gingivalis* into the periodontal pockets of rifampicin-sterilised monkeys (*Macacae fascicularis*) resulted in rapid and significant alveolar bone loss as well as increases in levels of systemic antibodies against *P.gingivalis*. This was the first convincing animal data suggesting a role for *P.gingivalis* in periodontal disease in that previous experimental models either did not cater for monoinfection or infection of the gingival crevice. This data was later strengthened when Klausen *et al.* (1991a, 1991b) showed monoinfection of the periodontal pockets of gnotobiotic rats by *P.gingivalis* induced severe alveolar bone loss in these animal and that immunisation with *P.gingivalis* cells endowed protection against this bone loss. The problem with this animal model is that rats do not naturally suffer from periodontal disease (although *Macacae fascicularis* does) and as with most other animal models the circumstances surrounding infection are un-natural.

Further evidence for *P.gingivalis* having a significant role human periodontal disease can be seen when the humoral response to *P.gingivalis* is examined. Systemic anti-*P.gingivalis* antibodies are common at elevated titres in patients with adult periodontal disease (Curtis *et al.*, 1991; Nardin *et al.*, 1991; Ogawa *et al.*, 1991; Papaioannou *et al.*, 1991; Whitney *et al.*, 1992). These data do not directly implicate *P.gingivalis* in virulence, but do suggest some form of involvement in the immune response. Indeed the persistent presence of *P.gingivalis* whilst a humoral immune response is being mounted suggests that this micro-organism is some way evasive or tolerant of host defence mechanisms.

These lines of evidence pointing to *P.gingivalis* as a periodontopathogen have given researchers in the field of periodontology a focus. Subsequent experimentation has unearthed a rich variety of potential virulence factors which further suggest a role for *P.gingivalis* in periodontal disease.

1.2.4 Other organisms involved in periodontal disease.

Despite the large number of micro-organisms commonly isolated from periodontal pockets, it is believed that only a small number of species are involved directly in periodontal disease. Studies of adult periodontitis have shown that at least one of *P.gingivalis*, *P.intermedia* or *Actinobacillus actinomycetemcomitans* is almost always present (99.2% of sites) in progressive periodontal lesions (Slots *et al.*, 1985). *A.actinomycetemcomitans* is also closely associated with localised juvenile periodontitis as it has been isolated from upto 97% of LJP patients (Genco and Slots, 1984). *P.intermedia* along with oral spirochete *Treponema denticola* have been implicated in ANUG (Loesche *et al.*, 1982).

Other putative periodontopathogens include *Wolinella recta, Fusobacterium nucleatum, Eikenella corrodens* and some *Bacteroides* species, all of which are implicated in different forms of periodontal disease by virtue of frequency of isolation (Socranski *et al.* 1981). In addition, these bacteria have been shown to exhibit potential virulence factors and to be active in animal models (for a review of virulence of these organisms see Holt and Brammanti, 1991).

Current ideas concerning bacterial pathogens as part of mixed infections in oral disease tend to focus on an interdependence of individuals in such a mixed flora. There are clear examples of the importance of one bacterial type in the colonisation, emergence and survival of another in terms of attachment, nutrition and environmental factors. It is also possible that periodontopathic bacteria can assert virulence as a consortia. These factors will be considered in subsequent chapters.

1.2.3 The periodontum as an ecological niche.

The oral cavity hosts a rich variety of comensal micro-organisms which under normal circumstances show little or no pathogenic behaviour. However, as a mixed flora oral microbes show unique and interesting behaviour in that some members of this community have the ability to specifically bind among themselves and to other bacterial species. This specific interaction (coaggregation) along with the ability to bind hard and soft host tissues allows the formation of large multigeneric bacterial aggregates on host surfaces. This form of colonisation sports two aspects unique to the oral cavity. First, reports of bacteria-bacteria interactions are in themselves rare outside the oral cavity and are usually confined to genetic conjugation systems. Secondly, the formation of coaggregates in the oral ecosystem is unique in that bacterial interactions in other ecosystems are usually either nutritional or predatory relationships (Kolenbrander, 1988).

The establishment of surface-bound colonies is essential to oral bacteria in that it allows survival in a hostile environment. In the first instance colonisation and coaggregation render the participants resistance to being washed away by salivary flow. Secondly, in micro environments of mixed flora inter-bacterial nutritional relationships can be established. Thirdly it is possible that resistance to host defence factors might be enhanced by co-operation. Hence, it is the specificity of these adhesive and coaggregative activities and the formation of specific microbial environments that generates the tissue tropisms of these oral bacteria and determines their behaviour.

1.2.4 Colonisation and ecological changes during disease progression.

The initial colonisation of the subgingival crevice is mediated by plaque bacteria (table 1.2). The first stage in this chain of events is the attachment of the primary colonisers to the enamel surface of the dentition by *Streptococcus* and *Actinomyces* species. This is closely followed by attachment of *Haemophilus, Veillonella* and *Peptosteptococcus* species until all available sites on the enamel are occupied at which point coaggregative activity causes a build up plaque bacteria on the enamel surface (reviewed in Kolenbrander, 1988). It is this plaque build-up proximal to the gingival crevice that allows plaque bacteria to interact with the host immune system (Schroeder and Attstrom, 1980). These bacteria are able to metabolise carbohydrate derived from the host's diet, the result of which is the release of acidic metabolic by-products (Coulter and Russel, 1976). It is acidity which causes the cariogenic nature of plaque at the same creating an acidic, anaerobic core within the colony with the potential to sustain further anaerobic metabolism.

Many of these archetypal plaque bacteria have been shown to demonstrate coaggregative activities with *P.gingivalis* cells and vesicles (table 1.2) and several other Gram-negative periodontopathogens such as *Eikenella corrodens, Fusobacterium nucleatum,* and *Prevotella intermedia* (Kolenbrander, 1989). In addition to standard one on one interactions, these bacteria also have the potential to form coaggregative "bridges" (reviewed in Holt and Bramanti, 1991). Thus these coaggregative activities are probably important in the colonisation of these Gram-negative organisms which, once established in the gingival crevice eventually take over, resulting in a predominantly Gram-negative infection. This change in microflora is itself not well documented but probably contributes to the worsening disease situation where antagonism from the plaque bacteria encourages the formation of the periodontal pocket, pending the firm establishment of periodontopathogens.

Despite the potential for anaerobic growth within supra and sub-gingival plaque, it is unlikely that *P.gingivalis* is one of the earlier periodontopathogens to significantly colonise the periodontal niche. There are several reasons for this. First, the prevalence of *P.gingivalis* correlates positively with disease severity and also tends to be localised to sub gingival plaque (Kiel *et al.*, 1983). Secondly, it has been shown that the fermentation of carbohydrate in supra gingival plaque can cause a drop in pH which would be unsuitable for

Table 1.2 Examples of bacteria known to coaggregate with *P.gingivalis*.

Species name	Reference
Streptococcus sanguis	Stinson *et al.*, (1991)
Streptococcus gordonii	Stinson *et al.*, (1992)
Streptococcus salivarius	Kolenbrander (1989)
Streptococcus mitis	Nagata *et al.*, (1990)
Actinomyces viscosus	Ellen *et al.*, (1991), Bourgeau *et al.*, (1990) Rosenberg *et al.*, (1991)
Treponema denticola	Grenier, D. (1992)
Eikenella corrodens	Kolenbrander (1989)
Fusobacterium nucleatum	Kolenbrander (1989)

P.gingivalis growth (Takahashi *et al.*, 1990, and McDermid *et al.*, 1988). Thirdly, it has been shown that in continuous culture the growth of *P.gingivalis* is inhibited by the presence of carbohydrate (McKee *et al.*, 1988). It is much more likely that utilisation of available carbohydrate and a rise in pH is required before *P.gingivalis* can predominate and it is in this context that *Prevotella intermedia* has been implicated (Takahashi *et al.*, 1990).

P.intermedia is commonly isolated from both sub and supra-gingival plaque and has been found to be acid tolerant and cause increases in the pH of liquid cultures (Takahashi *et al.*, 1990). Thus *P.intermedia* is a potential candidate for modifying the early sub-gingival plaque in favour of *P.gingivalis* survival. In addition, *P.intermedia* is able to metabolise both carbohydrate and peptides as energy sources making it a good candidate for mediating progressive modification of plaque ecology. Takahashi *et al.* (1990) have speculated that leakage of dietary carbohydrate into the gingival crevice could potentiate growth of the acid-producing oral *Streptococci* and *Actinomyces* species. Subsequently growth of *P.intermedia* could raise the pH of the crevice hence favouring the growth of *P.gingivalis.*

Apart from progressive modifications in the physiological conditions of sub and supra gingival plaque, there is also the inter-dependency of different bacteria to be considered. These include the presence of the correct coaggregation partners to allow colonisation, and a nutritional interdependency with the ability to form micro environments mediated by coaggregative cages (Holt and Bramanti, 1991). For example, growth of *P.gingivalis* is believed to be stimulated by bacterially-derived naphthoquinone and succinate (Mayrand and McBride, 1980; Slots and Genco, 1984; Holt and Bramanti, 1991). In turn it has been shown that *T. denticola* is not isolated from sub gingival sites unless *P.gingivalis* is present (but not *vice versa*) and that *P.gingivalis* secretes a protein factor (> 50 kDa in mass) which stimulates the growth of this micro-organism (Simonson *et al.*, 1992). Interestingly coaggregation has also been demonstrated between *P.gingivalis* and *T.denticola* (Grenier, 1992).

There is evidence to suggest that there is bacterially-mediated inhibition of growth for certain members of the microflora. The release of bacteriocins by black pigmented bacteroides has been reviewed by Takazoe *et al.* (1984) and these bacteriocins have been shown to be active against oral *Streptococci* and

Actinomyces species as well as some Gram-negative species. Additionally it has been shown that haemin whilst promoting growth of *P.gingivalis* inhibits the growth of various Gram-positive plaque bacteria (Takazoe, 1984). In this context it is possible that in advanced forms of gingivitis, haemin derived from leakage of blood cells into the gingival crevice maybe in part responsible for diminution of the Gram-positive species in favour of the Gram-negative anaerobes.

These antagonistic and co-operative features of the sub and supra gingival microflora present a complex situation of infection. This complexity is so intense that the full picture may never be totally clear, yet it is evident that ecological aspects of the sub gingival microflora are of great importance in the understanding of periodontal disease.

1.3 Molecular and cellular aspects of periodontal disease.

1.3.1 Host defence mechanisms in periodontal disease.

The role of bacterial factors in disease pathogenesis can not be evaluated in context without first considering host defence mechanisms. Components of the immune system interact with bacterial secretions and cells, and it is the degree of success or severity of these immune responses which determine the outcome of bacterial challenge. Host responses to periodontal disease involve both humoral and cell-mediated immunity. The humoral response consists largely of antibody production and complement fixation, whereas the cell-mediated response is manifested by bacterial phagocytosis and subsequent killing. These activities are stimulated and co-ordinated by inflammatory mediators such as cytokines and products of tissue damage.

The frontline participants in the cell-mediated response are polymorphonuclear leukocytes (PMN) and macrophages. Of the different PMN cell types the neutrophil is most prominent in the periodontum and shows pronounced migrational activity from the peripheral blood into the site of microbial challenge (Repo *et al.*, 1990). This migration is in response to chemotactic factors such as activated complement components, products of the fibrinolytic and kinin-generating systems, specific factors from host cells

such as macrophages as well as bacterial secretory products (Hanazawa *et al.*, 1992). Neutrophils phagocytose bacterial cells and participate in the inflammatory response by releasing cytokines and collagenases (Gangbar *et al.*, 1990). Macrophages are also chemotactically drawn to the site of infection and, similarly engulf bacterial cells and release cytokines (Lindemann and Economou, 1988) as well as synthesising complement components and presenting antigens to T cells.

Prominent in the connective tissues of both healthy and diseased sites are T cells whose functions include control of the B and T cell immune response and killing of infected or transformed host cells. T cell-dependent functions are defined by different T cell subsets whose relative proportions are important in modulating the immune response to periodontopathogens (Manti *et al.*, 1984). Such control functions also influence the type of antibody response *via* release of lymphokines and cell-cell antigen presentation to B cells (Katz, 1992). A role for antibody in the periodontal lesion is suggested by the large number of activated B cells found at the site of infection (Gemell and Seymour, 1991; Ogawa *et al.*, 1991). Specific antibodies against periodontopathic bacteria are commonly found in the sera of patients and have the potential to neutralise bacterial attachment and toxins, to trigger complement, to block bacterial transport mechanisms and to aid opsonisation and phagocytosis (Cutler *et al.*, 1991a; 1991b). This production of immunoglobulin is enhanced by T cell-derived polyclonal B cell activators *in vitro* hence demonstrating the importance of T cells to the humoral response (Manti *et al.*, 1984; Ito *et al.*, 1986; Gemmel and Seymour,1992; Katz *et al.*, 1992).

The second mode of humoral defence in periodontal disease is by action of complement. Complement-mediated killing of bacteria can be directed by either antibody binding of the bacterial cell surface or by direct binding of C3b, leading to attack complex-formation and bacterial lysis. The triggering of the complement cascade leads to release of anaphylatoxins which have the effect of increasing vascular permeability and attracting neutrophils and macrophages. Complement fixation is also recognised by polymorphs and aids phagocytosis of periodontopathogens (Hurst and Wilton, 1992). A by-product of this reaction is the release of further inflammatory and tissue repair factors. The induction of clot formation in response to inflammatory and bacterially-derived damage can then immobilise invading cells.

1.3.2 Interaction of *P.gingivalis* with the host defence mechanisms.

In chronic periodontal disease a degree of persistence of infection and resistance against the immune system is evident. Understanding the reasons why requires knowledge of particular immune reactions. One such reaction involved in the combat of *P.gingivalis* infection is the ability to raise a specific antibody response capable of neutralising key bacterial components. The effectiveness of this specific response depends on the generation of the correct immunoglobin class and isotype so as to enable efficient complement fixation and recognition by phagocytes (van Steenberg *et al.*, 1985).

The antibody response to *P.gingivalis* in periodontitis patients has been noted by several workers to be of a high titre and primarily of the IgG subclass (Wilton *et al.* 1991; Whitney *et al.*,1992). In addition the primary subclass found in these studies (for both healthy and diseased individuals) was IgG2, an isotype which classically lacks strong complement fixation and is commonly raised in response to capsular polysaccharide. The role of antibody in opsonisation of *P.gingivalis* is clearly important. It has been shown that a significant enhancement of complement-dependent phagocytosis is seen in the presence of antibody (Cutler *et al.* 1991a; 1991b). This enhancement was obvious in immunised rabbits, whereas non-immunised rabbits produced serum which was non-opsonic for *P.gingivalis*. However, despite high levels of specific antibodies in periodontitis patients studied, very few of these individuals (3 of 17) had sera that could opsonise *P.gingivalis* (Cutler *et al.*, 1991a). This suggests that there maybe a strategy for evasion of opsonophagocytosis elicited by this organism.

Studies involving cells of the immune system from experimental infections have shown that interaction between cells from remote sites and those at the site of infection exert behaviour that may represent a significant host strategy for modulation of disease severity. Such data shows that whilst antibodies against *P.gingivalis* (and also *F.nucleatum*) are produced by both circulatory and diseased site-associated B cells, cultured B cells from diseased sites produce higher titres of specific anti-*P.gingivalis* antibodies than B cells of circulatory origin (Gemmel and Seymour, 1992). In athymic (essentially T cell

deficient) mice specific antibody to *P.gingivalis* is not produced in response to bacterial challenge unless splenic T cells from immunised animals are provided via transplant (adoptive transfer; Katz *et al.*, 1992). The subclass of resultant antibodies was also influenced by T cells, suggesting that T cell responses maybe of great importance in the host's defence strategy. *P.gingivalis* cells have been demonstrated to perturb the growth of T cell cultures (Manti *et al.*, 1984). It does this by inhibiting T cell division *in vitro* and altering CD4/CD8 subset ratios (T cells expressing CD4 are mostly of the T helper type whilst those expressing the CD8 surface marker are mostly of the cytotoxic type). This suggests a strategy for compromise of the immune system by altering cellular populations. The importance of T cell subset ratios in periodontal disease has been demonstrated by the effectiveness of certain immunomodulating drugs in altering T cell populations whilst simultaneously causing disease regression (Manti *et al.*, 1984).

Other significant activities of periodontopathogens in disease, apart from evasion of the immune response include the ability to enhance inflammatory damage and to induce bone resorption. These latter activities are thought to be partly mediated by bacterially-induced imbalances in the autocrine and immune systems of the host in the region of the gingiva and surrounding connective tissues. Of these systems the cytokine system is believed to be a major effector of inflamatory response and one particular cytokine interleukin-1 (IL-1) is believed to have important influence in this context. IL-1 enhances proliferation and generation of collagen, collagenase, protoeoglycan and prostaglandin in fibroblast cultures (Takada *et al.*,1991a). There are 2 classes of IL-1, namely IL-1a and IL-1b. Both forms have similar biological properties; IL-1b has been shown to be identical to osteoclast arming factor, an autocrine factor which can induce bone resorption Kabashima *et al.*, 1990). Both soluble IL-1b and cell-bound IL-1a are found in *P.gingivalis*-stimulated fibroblast cultures (Lerner *et al.*, 1990; Takada *et al.*, 1991a) and the soluble form found in *P.gingivalis*-stimulated gingival mononuclear cell cultures (Gemmel and Seymour, 1993). IL-1a can also be found in gingival crevicular fluid from periodontal disease patients (Kabashima *et al.*, 1990). In the light of these data it is possible that much of the bone resorptive and inflamatory properties of periodontitis may be attributed to the virulence factors which affect tissue factors such as IL-1. These will be considered along with other potential virulence determinants in the following section.

1.3.3 Virulence Factors and the Bacterial Cell Envelope.

The bacterial envelope consists of membranes and associated material and encompasses the cell cytosol. As a subcellular fraction it provides a discrete template for metabolic activities such as photosynthesis in *Rhodopseudomonas* species (Deisenhofer and Michael, 1989) and the electron transport chain (cytoplasmic face of the envelope), the organisation of topographic events such as chromosomal partitioning following cell division (Niki *et al.*, 1992) as well as providing an additional cellular compartment, the periplasm in the case of Gram-negative species (Nikaido and Nakae, 1979). The cell envelope is also the interface between the bacterial cell and its environment. In the Gram-negative envelope the inner membrane maintains active (usually energy dependent) control of macromolecule export and solute passage and is supported on its outside by a large heteropolymeric peptidoglycan molecule which protects the cell from osmotic lysis. External to this is the outer membrane which acts as a passive barrier to solvent molecules via protein pores or porins. These control the size and type of solvent interchange by virtue of pore size and of the charged environment within the pores (Cowan *et al.*, 1992; for a review see Benz, 1988).

In the case of commensal and pathogenic bacterium the cell's environment with which it must interact consists of the cells and connective tissue of its host. Thus the bacterial envelope and all surface structures encountered by the host and are all potential targets for the immunological response. This "dialogue" between host and pathogen during the course of evolution has resulted in structures in bacteria, often surface associated, whose function is to prolong infection by aggressive and evasive interaction with host defence, and to promote bacterial nutrition and attachment in a hostile environment. These virulence factors are now discussed starting with those of non-protein composition .

1.3.3.1 Bacterial Capsules.

The bacterial capsule is a high-molecular-weight polysaccharide material external to the bacterial cell wall. Whilst not found on all bacteria, capsules

are commonly associated with organisms associated with disease and isolated from diseased sites (Brook, 1987). Whilst functionally encapsulation may confer evasive protection against host defence mechanisms there is some evidence that capsular material may directly antagonise components of the immune response. It has been shown that implantation of purified capsular material from *Bacteroides fragilis* can cause abscess formation in rats and this demonstrates direct antagonism of host tissues by capsular polysaccharide (Onderdonk *et al.* 1977).

The composition of capsular polysaccharide varies between species and strains, though in general consists of linear or branched polysaccharide chains of one or more species of saccharide. This gives the cell surface a negatively charged, hydrophilic character which is believed to help the bacterial cell resist phagocytosis by reducing surface tension at the phagocyte cell surface (Moxon and Kroll, 1990). Also some capsular polysaccharides contain carbohydrate repeat structures identical to those found in host carbohydrates and it is believed that this mimicry may make it difficult for the immune system to recognise the bacterium (Vimr *et al.*, 1984). Capsular polysaccharide can also show intrinsically poor complement fixation resulting in reduced opsonophagocytosis and attack complex formation (Cross *et al.*, 1990).

Antibody production against capsular polysaccharides usually consists of a T cell-independent response. Such activation is generally characterised by weak primary and secondary responses with no maturation or immunoglobulin class switch being involved. Notably T-independent antigens show poor memory induction and this maybe important in chronic disease (Cross *et al.*, 1990).

To date there have been few studies of *P.gingivalis* capsule and it is not known whether many different polysaccharides exist amongst *P.gingivalis* strains. Whilst the polysaccharide structure for strains A7A1-28, 381 and W50 has not yet been defined, it has been shown to contain glucose, glucosamine, aminouronic acid, and galactosaminonouronic acid (Schifferle *et al.*, 1989). Capsules of *P.gingivalis* occur as electron dense, hydrophilic, heteropolymeric layers of about 15 nm thick which are closely associated with the outer membrane (Woo and Holt, 1979).

The presence and thickness of capsule varies between strains (Handley and Tipler, 1986) and this has provided scope for evaluation of the role of capsule in the disease process. Hydrophilic (encapsulated) strains of *P.gingivalis* have been shown to exhibit reduced phagocytosis by human leukocytes in culture when compared to hydrophobic strains (van Steenberg *et al.*, 1985). Encapsulated strains of *P.gingivalis* are also more virulent in the murine abscess model when compared to acapsular strains (Reynolds *et al.* 1989). The problem with such observations is that the presence/absence of capsule is often coincidental with aberrations in other putative virulence determinants such as protease activity. For example, the commonly studied W50/BE1 mutant, which has a lesion in its ability to metabolise haemin (Shah *et al.*,1988), exhibits a reduced capsular layer when compared its parent strain. This mutant is also avirulent in the mouse model (Marsh *et al.*, 1989) but shows reduced trypsin-like activity, and in this respect can not be used to ascertain the role of capsule in disease. Oddly, W50/BE1 was seen to be less hydrophobic than the parent strain and it has been suggested that the hydropathic character may be influenced by capsular composition as well as thickness (Haapasalo *et al.*, 1989).

The problems of determining defined roles for individual virulence factors should be overcome with the aid of newly developed techniques for generating isogenic mutants of *P.gingivalis*. Recently several groups have described the generation of isogenic mutants (Lepine *et al.*, 1993; Park and McBride, 1993). One such report describes the isolation of a Tn 4351 insertion-generated mutant which shows enhanced thickness of capsule when compared to it's parent strain (Genco *et al.*.1993). Mutants of this type should be of great use in assessing virulence and in cloning capsule genes.

1.3.3.2 Bacterial Endotoxin (LPS).

Classically bacterial endotoxin has been defined as the toxic fraction associated with the outer membrane which asserts its toxic properties through release of membranous material from dead cells or via outer membrane blebbing (Raetz, 1993). For the purpose of this introduction disease-active membrane components other than lipopolysaccharide (LPS) will be considered elsewhere as the classical biological activity of endotoxin is

now widely accepted as being caused by the LPS moeity of the outer membrane.

LPS occurs as a lipid/sugar chain(s), whose lipid portion is an integral membrane component of the Gram-negative outer membrane and whose carbohydrate portion is associated with the cells outer surface or glycocalyx. Functionally, LPS maintains the selective permeability and fluidity characteristics of the outer membrane being the dominant lipid moeity in the outer lipid bilayer (Benz, 1988; Raetz, 1993). The lipid component is referred to as lipid A and is considered to harbour the majority of the bio-active properties of the LPS molecule in that synthetic analogues of lipid A show extreme biological activities comparable to those of the whole LPS molecule (reviewed by Burrell, 1990). The generalised lipid A molecule consists of a ß (1,6)-linked glucosamine disaccharide to which is attached ester and amide fatty acids via phosphoester linkages. The contrast of the electronegative phosphoester groups against the uncharged hydrophobic fatty acid chains orientates the lipid A molecule in the membrane with the glucosamine groups proximal to the surface from which the carbohydrate species of the molecule extend (Raetz, 1993). Lipid A is itself insoluble in water and lacks biological activity. However the association of lipid A with some polysaccharide (e.g. 2-keto-3-deoxymanno-octonic acid, KDO) both increases solubility and toxicity.

The carbohydrate chain consists of two regions; the core polysaccharide and the O side chain. The core region consists of a number of KDO residues which are linked on one side to the lipid A glucosamine disaccharide and on the other to heptose, glucose, galactose and amino sugar residues. The latter core components are in turn attached to the O-specific chain which in the Enterobacteriaceae consists of polysaccharide repeats of various sugar groups including the rare 3,6-dideoxyhexoses, galactose, glucose, mannose and rhamnose. These polysaccharide chains can be up to 40 repeats in length and may be branched or linear, the length of which varies from molecule to molecule.

The LPS of *P.gingivalis* has been comprehensively studied and has been found on the surface of both cells and extracellular vesicles (Bramanti *et al.*, 1989 and Deslauriers *et al.*, 1990). Its carbohydrate component is typical of the *Enterobacteriaceae* and consists of hexose and hexosamine sugars, showing a typically "smooth" chemotype. The core/lipid A portion however consists of

phosphorylated KDO, is abundant in branched acyl groups in its fatty acid content, and lacks the 3-hydroxymyristate component usually associated with *Enterobacteriaceae* (Fujiwara *et al.*, 1989). Hence *P.gingivalis* LPS has some unique structural features. Attempts to serotype using *P.gingivalis* LPS as antigen have shown that there are at least two different antigenic groups present among LPS of different strains of *P.gingivalis* as well as one common species-specific antigen (Fujiwara *et al.*, 1989).

The biological properties of LPS include direct cytotoxicity, complement and Hageman factor activation, immunomodulation via cytokine induction, direct B-cell mitogenicity and T-cell independent antigenicity as well as in some cases functioning in haemagglutination and coaggregation (Holt and Bramanti, 1991). In addition to this *P.gingivalis* LPS (especially the lipid A region) has been observed to bind and store haemin, an activity essential for the growth and establishment of this organism in the periodontal pocket (Grenier, 1991).

The endotoxic effects of *P.gingivalis* LPS are extremely unusual. In comparison with *Escherichia coli* and *Salmonella typhimurium* and *S.minnesota*, *P.gingivalis* LPS shows little endotoxic activity as assayed in the Shwartzman reaction in rabbits (Sveen, 1977; Fujiwara *et al.*, 1990), the mouse LD^{50} model, the chicken embryo model, the *Limulus* amoebolysate assay (Mayrand and Holt, 1988). However, *in vitro* analysis of interactions of *P.gingivalis* LPS with cultured cells suggest a potential role for LPS in the perturbation of host defence by interfering with the expression of host-derived cytokines (Mayrand and Holt, 1988). These cytokines have in turn been implicated in the destruction of the supporting periodontal tissues. LPS from *P.gingivalis* has been shown to induce production of IL-1, PGE_2 and TNF from monocytes (as does LPS of *E.coli*; Garrison *et al.*, 1988, Isogai *et al.*, 1988, and Garrison *et al.*, 1989), IL-1, IL-6 and IL-8 from gingival fibroblasts and also to inhibit fibroblast growth in culture (Takada *et al.*, 1991a; Tamura *et al.*, 1992). Probably the most significant of these cytokines is IL-1 which can activate osteoclasts into bone resorption and induce fibroblasts into producing collagen extracellular matrix, collagenase and PGE_2 along with other cytokines such as IL-6, IL-8. TNF can also stimulate bone resorption and can augment the osteoclast activating activities of IL-1 as well as being able to stimulate IL-1 production itself (Takada *et al.*, 1991b). The effects of IL-1 are also apparent in the cells of the immune system where it activates B and T cell

proliferation and in the central nervous system where it induces symptoms associated with fever (Takada *et al.*, 1991b).

Extrapolation of the significance of *in vitro* observations to the *in vivo* situation is not yet possible, yet these results should be considered carefully in the light of the fact that bone resorption and tissue destruction represent significant histopathological changes in the disease state and that elevated levels of IL-1 are commonly found in the gingival crevicular fluid of patients with chronic inflammatory periodontal disease (Kabashima *et al.*, 1990). It has also been established that LPS molecules can be shed from the bacterial cell surface during infection (Raetz, 1993) and are capable of traversing the gingival crevicular epithelium hence penetrating the connective tissues (Schwartz *et al.*, 1972). Thus LPS may be a significant trigger in the genesis of tissue destruction seen in periodontal disease although as described next there are other factors elicited by *P.gingivalis* which have been postulated to fulfil a similar function.

1.3.3.3 Protein Components of the Outer Membrane.

The outer membranes (OM) of periodontopathogens have received much attention in recent years. In that until now little has been known about periodontopathogen virulence determinants a logical approach has been to analyse outer membrane components using simple techniques and then to follow on from this work by using more sensitive techniques.

The OM of *P.gingivalis* boasts a complex array of polypeptides in comparison to *E.coli*. Cell surface iodination of whole cells has revealed that there are between 7 and 14 major outer membrane proteins (MOMPS) with some degree of outer surface exposure (Kennel and Holt, 1990) of an estimated total of around 28 polypeptides (Deslauriers *et al.*, 1990) as oppose to *E.coli* which has around five MOMPS (Nikaido and Nakae, 1979). This polypeptide profile varies depending on the strain examined or the physiological conditions used for cell growth. Studies of the MOMPS of *P.gingivalis* are also complicated by the activity of membrane-associated proteolytic activities (Holt and Bramanti, 1991) which degrade outer membrane components creating extra bands.

Three outer membrane proteins of molecular masses 26, 46 and 19 kDa (Kennel and Holt, 1990, and Papaioannou *et al.*, 1991) only show expression in cells grown under haemin limitation. The 26 kDa protein has been shown to exposed on the surface and to bind to and to be rapidly internalised on exposure to haemin; it has been postulated that this protein may be important in cellular uptake of haemin (Kennel and Holt, 1990). Such proteins are of particular interest in that cells grown without haemin have been shown to be avirulent in the mouse model (McKee *et al.*, 1986). However, there is no direct evidence for these proteins having a role in haemin uptake and it is possible that haemin is simply an environmental factor governing the expression or surface localisation of these proteins (Robertson and Meyer, 1992).

Several studies have been made on the reactions between different antisera and the OM and intact cells of *P.gingivalis*. Laorisin *et al.* (1990) demonstrated that antisera from periodontitis patients contained high proportions of anti-*P.gingivalis* antibodies reactive with cell sonicates and that there were clear differences seen in the antibody binding of sera between adult periodontitis patients and rapidly progressive periodontitis patients. It was determined that antigens of high mass (82, 57 and 44 kDa) were recognised in adult periodontitis antisera and that antigens of low mass (44, 27, 25, and 18 kDa) were recognised in the sera from rapidly progressive periodontitis patients. Similar studies have shown that periodontitis patients have antisera more reactive to cell surface proteins of mass 55 and 47 kDa (Curtis *et al.*, 1991) and 73 and 46 kDa (Papaioannou *et al.*, 1991) when compared to sera from control individuals. Such results are important in three respects; first they demonstrate the presence of bacterial components probably expressed *in vivo* which react with the immune system and hence may reflect focal points of immune system evasion or alternatively susceptibility (useful for vaccine studies); secondly, a contrast between immune reactions from diseases of differing severity is apparent and thirdly they provide a rational focus for the investigation of specific bacterial cell surface components.

Much ground has been made in the analysis of cell surface and secretory enzymes of *P.gingivalis* and this is largely attributable to the ease with which (spontaneous) extracellular vesicles (ECV) can be prepared. These ECV consist of 66% protein, 21% sugars and 17.4% LPS with no detectable peptidoglycan (Grenier and Mayrand, 1989). When compared to the outer membrane such ECV show nearly identical polypeptide profiles (Williams

and Holt, 1985; Grenier and Mayrand, 1987). ECV also posses the same enzymatic properties (Smalley and Birss, 1987; Minhas and Greenman, 1989; Grenier and Mayrand, 1987) as well as similar haemagglutinating abilities as intact cells. In that the dimensions of ECV potentially allow them to penetrate anatomical barriers the study of these structures as virulence mediators in themselves is attractive; discussion of their virulence properties along with those of the cell surface follow (Kay *et al.*, 1990).

1.3.3.4 Hydrolytic Enzymes: Proteases

Proteolytic activity has been noted in subgingival plaque samples and is believed to predominantly correlate with the presence of oral spirochetes and *P.gingivalis* (Pederson and Lamberts, 1990) and is believed to be important in the process of tissue destruction seen in periodontal diseases. Proteolytic activity of a wide variety of oral microbes has been characterised and has shown *P.gingivalis* and oral treponemes *Treponema vincentii* and *T. denticola* to exhibit a rich variety of proteolytic activities (Suido *et al.*, 1986; Mäkinen *et al.*, 1988; Bretz *et al.*, 1990). Because of the broad spectrum of substrate specificity's various imaginative roles have been proposed for hydrolytic activities in the disease state such as their involvement in bacterial nutrition, substrate adhesion, evasion of the immune response and generation of tissue inflammation (reviewed by Mayrand and Holt, 1988).

P.gingivalis cells and vesicles have been shown to degrade a variety of protein substrates *in vitro* and whilst breakdown of substrates such as casein and BSA demonstrate proteolytic activity, degradation of interstitial tissue components and important proteins of the immune system has also been shown. Degradation of IgG, IgM, complement components C3 and C5, and unidentified components of whole serum by *P.gingivalis* cells has been demonstrated (Sundqvist *et al.*, 1985). It has been proposed that destruction of immunoglobulin and complement components could confer evasive protection on micro-organisms by compromising immune system function (Sundqvist *et al.*, 1985). In addition, degradation of C1-inhibitor, antithrombin, plasminogen, prekallikrein, prothrombinase complex, clotting factor X, a_2-antiplasmin and fibrinogen by *P.gingivalis* has been demonstrated (Nilsson *et al.*, 1985). C1-inhibitor is a serpin and a key modulator of both the classical complement pathway and the clotting pathway and its inactivation

could potentially inhibit clot-formation and improve vascular permeability (these events are seen in the angioedema caused by C1-inhibitor deficiency syndrome). Fibrinogen and Factor X are components of the clotting system and it conceivable that their depletion could result in compromised clot formation. The prothrombinase complex is also a precursor for the clotting system. Degradation of clotting system components may be important in that the fibrin matrix formed in blood clots is the support for tissue repair in inflammatory lesions and its disruption may prevent healing (Lantz *et al.*, 1991). In addition to degrading a_2-antiplasmin, *P.gingivalis* also degrades a_2-macroglobulin and a_1-antitrypsin (Carlsson *et al.*, 1984); these three are plasma proteinase inhibitors and are responsible for down-regulating the activity of tissue-degrading enzymes released by polymorphonuclear leukocytes and the control of steps in the proteinase cascade of human plasma. *P.gingivalis* has also been shown to degrade collagen which is a key component of the interstitial tissues (Mayrand and Grenier, 1984). However, ther is little data to support that *P.gingivalis* degrades these proteins *in vivo*.

Analysis of protease activities using protein purification techniques has shown that multiple proteases account for the proteolytic profile of *P.gingivalis* (table 1.3). These data are corroborated by substrate zymography studies which show protease activity of at least eight molecular weights (Grenier *et al.*, 1989) and by analysis of the mutant W50/BE1 which, though exhibiting reduced trypsin-like activity in its proteolytic profile, maintains normal collagenase-like activity (Marsh *et al.*, 1989). From these data it is evident that there are at least two classes of activity, the trypsin-like activity specific for amidolytic cleavage of arginine peptidyl links and collagenase activity specific for the sequence Gly-Pro which is repeated many times in the primary structure of mammalian collagens (table 1.3). These activities tend to be cell-associated (in the envelope fraction) and secretory (secretion localised to the ECV fraction).

Studies of proteases frequently attempt to classify the enzyme according to predetermined stereochemical classification of amino acid residues in the catalytic site, namely those of thiol-activated or serine proteases (although other groups do exist). Thiol-activated enzymes are activated by reducing agents such as ß-mercaptoethanol, and inhibited by sulfahydryl blocking group reagents iodoacetate, PCMB and N-ethylmaleimide; serine proteases are inhibited by compounds such as TLCK, DIFP and PMSF. The trypsin-like

Table 1.3 Proteases described for *P.gingivalis* and their properties.

Name and Strain	Starting Material	Molecular Weight (kDa)	Substrate	Activation (reduction)	Effect of Inhibitors (mM) EDTA	PMSF	TLCK	PCMB	Reference
glycylprolyl aminopeptidase 381	cell extract	75 (SDS PAGE/100°C)	Gly-Pro MCA	No	NI (1)	MI (0.5)	ND	WI(1)	Miyauchi *et al.*, 1989.
collagenase 381	cell envelope extract	90 (zymography)	gelatin, Collagen Type I,II,III	Yes	SI (5)	NI(1)	MI(1)	ND	Birkedall-Hanson *et al.*, 1988.
trypsin-like protease 381	cell extract	45 (gel filtration) 50 (SDS PAGE/100°C)	BAPNA, casein, gelatin, BSA	Yes	WI(10)	ND	MI (0.5)	NI (0.5)	Tsutsui *et al.*, 1987.
trypsin-like protease 33277	culture supernatant	300 (gel filtration) azoalbumen, azocoll	BAPNA, azocasein	ND	SI (1)	ND	SI (0.01)	SI (0.01)	Fujimura and Nakamura, 1987.
glycylprolyl protease 33277	outer membrane	29 (SDS PAGE/37°C) 19.5 (SDS PAGE/100°C)	Gly-Pro pNA, gelatin, lysozyme	No	NI (20)	MI (4)	SI(2)	MI(4)	Grenier and McBride, 1987.
trypsin-like protease 33277	cell extract	35 (gel filtration) collagen type IV	BAPNA, gelatin	Yes	SI (10)	SI (1)	ND	WI (1.5)	Sorsa *et al.*, 1987.
caseinolytic protease 381	culture supernatant	>100 (SDS PAGE/25°C) 35,32,18(100°C)	BAPNA, casein, lysozyme	Yes	NI (1)	ND	ND	MI(1)	Otsuka *et al.*, 1987.
thiol protease 381	cullture supernatant	49 (SDS PAGE/100°C)	BAPNA	Yes	NI (1)	WI (1)	SI(1)	MI(1)	Ono *et al.*, 1987.
glycylprolyl dipeptidylamino -peptidase / 381	culture supernatant	160 (gel filtration)	Gly-Pro MCA	ND	ND	MI (0.5)	ND	NI(1)	Abiko *et al.*, 1984.
trypsin-like protease 381	cell envelope extract	ND	BAPNA, BSA	Yes	SI (2)	ND	MI (0.1)	SI (0.1)	Yoshimura *et al.*, 1984.
trypsin-like protease W50	culture supernatant	58 (SDS PAGE/100°C)	BAPNA,fibronectin, collagen type I	Yes	ND	ND	ND	ND	Smalley *et al.*, 1988.
glycylprolyl-peptidase A7A1-28	cell extract	80 (SDS PAGE/100°C) 75 (gel filtration)	Gly-Pro pNA	ND	NI (5)	SI(5)	NI(1)	ND	Barua *et al.*, 1989.
gingipain H66	culture supernatant	50 (SDS PAGE/100°C) 44 (gel filtration)	BAPNA+other Arg compounds	Yes	SI (1)	NI	SI (1)	ND[g]	Chen *et al.*, 1992.
gingivain W83	culture supernatant	100 (SDS PAGE/100°C)	BAPNA	Yes	ND	ND	ND	ND	Roberts *et al.*, 1990 Shah *et al.*, 1990

Table 1.3 (continued) Proteases described for *P.gingivalis* and their properties.

Name and Strain	Starting Material	Molecular Weight (kDa)	Substrate	Activation (reduction)	Effect of Inhibitors (mM) EDTA	PMSF	TLCK	PCMB	Reference
gingipain H66	culture supernatant	50 (SDS PAGE/100°C) 44 (gel filtration)	BAPNA+other Arg compounds	Yes	SI (1)	NI	SI (1)	ND[g]	Chen *et al.*, 1990.
gingivain W83	culture supernatant	100 (SDS PAGE/100°C)	BAPNA	Yes	ND	ND	ND	ND	Roberts *et al.*, 1990, Shah *et al.*, 1990.
Pase-A 381	culture supernatant	105-110 smear (SDS PAGE/100°C)	casein	ND	MI (1)	SI (5)	MI (1)	NI (5)	Hinode *et al.*, 1991.
Pase-B 381	culture supernatant	72-80 smear (SDS PAGE/100°C)	casein, BAPNA + other Arg compounds	Yes	SI (1)	NI (5)	SI (1)	SI (5)	Hinode *et al.*, 1991.
Pase-C 381	culture supernatant	44 (SDS PAGE/100°C)	casein, BAPNA, BLPNA + other Arg compounds	Yes	SI (1)	NI (5)	SI (1)	MI (5)	Hinode *et al.*, 1991.
collagenase 1101	cell sonicate supernatant	94 (SDS PAGE/37°C) + degredation products	collagen,casein, + synthetic collagen substrate	No	WI(10)	NI (10)	MI (1)	ND	Lawson and Meyer, 1992.

Table 1.3 Proteases described for *P.gingivalis* and their properties. Given are substrate characteristic used in each study, apparent molecular weights and protease inhibition profiles. NI denotes no inhibition of protease activity for ta particular inhibitor; SI, strong inhibition; MI, moderate inhibition; WI, weak inhibition; ND, inhibition was not determined. Numbers in parenthesis next to the inhibition levels denote concentration of inhibitor used (mM).

protease(s) are invariably thiol activated exhibiting molecular weights of around 50 kDa, with higher mass forms (possibly multimers) >100 kDa and low molecular weight forms of between 18 and 35 kDa. These different molecular weights may represent different proteins or may reflect the difficulties in determining molecular weight of proteins (table 1.3). It is also possible that catalytically active forms may be generated by auto or heteroproteolysis or by subunit dissociation during preparation. Interestingly the recent duplicated independent cloning of a trypsin-like protease (TLP) gene showed the gene product to be 482 amino acid, 50kDa protein with active form molecular weights of 90kDa and 64kDa in SDS PAGE (table 1.3; Bourgeau *et al.*, 1992; Park and McBride, 1993).

TLP production in culture is enhanced by the presence of excess haemin and this correlates with enhanced virulence in the mouse model (Marsh *et al.*, 1988, and McKee *et al.*, 1986). TLP enzyme activity is stimulated by reducing conditions (such as those found in periodontal pockets) yet inhibited by salivary histatins (small peptides of unknown function; Nishikata *et al.*, 1990). Thus it may be that TLP function is modulated by environmental factors. There is also some evidence that TLP may have a role in intergeneric adhesion in that adhesion of *P.gingivalis* to *Actinomyces* species is inhibited by thiol proteinase inhibitors (Ellen *et al.*, 1991). Shah *et al.* (1989) have also shown TLP to have haemolytic activity and this may be important in liberating haem for nutritional purposes (although *P.gingivalis* has been shown to possess an additional haemolysin; Chu *et al.*, 1991). Thus TLP may be a multifunctional enzyme.

The importance of TLP has been shown in a number of studies involving substrate degradation. Purified TLP degrades fibronectin from human plasma and type I collagen (Smalley *et al.*, 1988; Uitto *et al.*, 1989); both of these are important cell matrix molecules, collagen being the scaffold for assembly of the extracellular matrix and cell motility and fibronectin being an important cell adhesion molecule active in maintaining contact inhibition of cell proliferation, controlling cell motility and cellular secretion. Fibronectin has also been shown to bind collagen, heparin sulphate and fibrin; clearly disturbances in such important matrix macromolecules could be disastrous for tissue integrity. In addition, disruption of basement membranes in ScaBER cell cultures has been observed (Shah *et al.*, 1992). Basement membranes are composed of collagen and laminin and provide an anatomical barrier

between epithelia and mesenchyme and are the structural support for epithelia. Interfering with this structure might disrupt tissue function by compromising epithelial-mesenchyme interactions (Grobstein, 1975).

Immunoglobulin degradation (IgG, IgM, and complement C3) by TLP has been shown and subsequent reductions in bactericidal activity of human serum have been demonstrated (Grenier, 1992b). As well as the ability of TLP to degrade fibrinogen (Lantz *et al.*, 1991) the TLP preparation designated Prase-C by Hinode *et al.*, (1992; see table 1.3) is also potentially capable of converting kininogen to kinin (kallikrein-like activity) and degrading a_2-macroglobulin (Hinode *et al.*, 1992). Evidence for further perturbation of host defence is seen with the direct interaction of TLP with neutrophils *in vitro* where exposure reduced phagocytic capacity and altered expression of immunoglobulin Fc and complement component receptors (Tai *et al.*, 1992). Also TLP-induced alterations in cell surface glycoproteins of human gingival fibroblasts have been reported by Uitto *et al.*, (1989) who described alterations in high mass cell surface glycoproteins and fibronectin, also noting induction of host collagenase and plasminogen activator production.

Degradation of collagen, apart from potentially worsening inflammation at the site of infection, could be of a nutritional advantage to *P.gingivalis* in that collagen is the most abundant protein in periodontal tissue. In that TLP degrades collagen, dissection of specific collagenase activities is difficult. Some collagenase activity is localised in the cellular fraction but the majority of activity is found in the ECV fraction (a small amount is found in the extracellular soluble fraction, Abiko *et al.*, 1984). With the aid of synthetic substrates a glycylprolyl dipeptidylaminopeptidase has been identified (table 1.3). Again reports of molecular weights vary. The dipeptidase described by Abiko *et al.* (1984) showed a 160 kDa mass (gel filtration) when purified from cells but a mass of 75kDa when isolated from cell-free extracts (table 1.3). Barua *et al.* (1989) showed a molecular mass of 75-80kDa depending on method of measurement and the recent cloning of a GlyPro dipeptidase of 75kDa (Nakamura *et al.*, 1992) supports the assignment of a molecular weight for this protein which consistently shows serine protease characteristics when challenged with inhibitors (table 1.3). At least other GlyPro-cleaving enzyme may exist as Grenier and McBride (1989) have reported one of mass 29kDa located on the cell surface. Lawson and Meyer (1992) have described a protein which can bind collagen and cleave basement membrane type IV collagen.

This protein does not bind GlyPro-containing substrates, is thiol activated, autoproteolytic and has a mass of 94kDa. This and the existence of one other collagenase report for an non GlyPro-cleaving, 35kDa protein which dimerises to 70kDa (Kato *et al.*, 1992) suggest that other collagenases or proteases able to degrade collagen exist (table 1.3). It is very probable that the ability of *P.gingivalis* to degrade collagen is of key importance to this bacterium.

Several genes encoding proteases have been cloned. Genes encoding proteins with trypsin-like protease activities have been cloned in *E.coli* (Arnott *et al.*, 1990; Bourgeau *et al.*, 1992; Otogoto and Kuramitsu, 1993; Park and McBride, 1993) and the differing sequences of these genes suggests that there are multiple trypsin-like proteases. One such TLP gene (designated *prt*T) was shown to express a protease with a molecular weight of 53.9 kDa and this is in agreement with much of the data gained from purification of trypsin-like proteases with molecular weights of 50 kDa (table 1.3). The *tpr* gene of Park and McBride (1993) also encodes a TLP of around 50 kDa molecular weight yet has a different nucleotide sequence from the *prt*T gene and this shows there must be at least two trypsin-like proteases of *P.gingivalis*. Genes encoding *P.gingivalis* collagenases have also been cloned in *E.coli*. The observation that the restriction maps for these clones differ and that the molecular weights of the proteins encoded therein also differ suggest that *P.gingivalis* also expresses multiple collagenases (Kato *et al.*, 1992; Nakamura *et al.*, 1992). Molecular weights of these proteins are 37.8 kDa and 75 kDa and the latter is roughly consistent with the observed molecular weight for purified collagenases (table 1.3).

1.3.3.5 Hydrolytic Enzymes: Lipases, Glycosidases and other Enzymic Activities.

Besides proteolytic activities *P.gingivalis* shows a variety of other enzymatic activities which could be important in virulence (see table 1.4). Again their potential roles fall into various categories; fulfilment of nutritional requirements, perturbation of host tissues and preparation of substrate for adhesion.

Direct evidence for bacterially-mediated breakdown of non-protein extracellular matrix (glycosaminoglycans) as yet do not exist and reports of

Table 1.4 Selected Enzymic activities of *P.gingivalis* .

I. Glycosidases.

ß-D-mannopyranosidase.[a]
N-acetyl-ß-D-glucosaminidase.[a]
N-acetyl-ß-D-galactosaminidase.[a]
Neuraminidase (sialidase).[b]

II. Sulphatases.

Glycosulphatase.[c]

III. Lipase.

Phospholipase A.[b]

IV. Phosphatases.

Acid phosphatase.[b]
Alkaline phosphatase.[b]

V. Free Radical-processing enzymes.

Catalase *(non-human biotypes only)*.[b]
Superoxide dismutase (SOD).[e]

VI. Miscellaneous activities.

DNAase.[b]
Keratinase.[b]
Chondroitin sulfatase.[b]
Hyaluronate Lyase.[f]

a Homer *et al.*, 1991.
b Holt and Bramanti, 1991.
c Slomiany *et al.*, 1993.
d Parent *et al.*, 1986.
e Amano *et al.*, 1990.
f Minhas and Greenman, 1989.

periodontopathogen glycosidases are rare. It has been shown that glycoproteins can be degraded by oral *Treponemes* and *P.gingivalis* (Homer *et al.*, 1990). In addition, sulphatase in gingival crevicular fluid has been found to be a reliable indicator of periodontal disease progression and correlates with the presence of *P.gingivalis* (Slomiany *et al.*, 1993). Current ideas concerning degradation of complex polysaccharides suggest an interdependent sequence of hydrolytic activities acting on terminal residues of glycosyl chains. Demonstrations that high levels of sulphate esterification of macromolecules can make them more resistant to degradation suggest that bacterial sulphatase activities such as those found in *Bacteroides melaninogenicus* and *Prevotella intermedia* (Roberton *et al.*, 1993) may be important in the degradation of host sulphated macromolecules such as mucin, and heparin and chondroitin sulphate. Similarly neuraminidase activity on terminal sialic acid residues (also inhibited by terminal sulphation; Roberton *et al.*, 1993) may render carbohydrate polymer susceptible to sequential degradation from its terminal end. Such degradation could perturb host defence by cleaving sugars from glycoproteins such as complement C1q and IgG hence altering their recognition. Alternatively active degradation of glycosaminoglycans (GAG's) such as keratin, and chondroitin and heparin sulphate polymer could disrupt tissue function as it has been shown that fibroblast behaviour is influenced by GAG 's (Schor *et al.*, 1987). It is entirely possible that the keratinase, chondroitin sulphatase and hyaluronidase activities of *P.gingivalis* (table 1.4) may be due to these concerted enzymic activities and not due to dedicated individual enzymes.

Glycosidase, sialidase, and glycosulphatase activities of *P.gingivalis* have been reported (table 1.4) but poorly characterised as yet. The cloning of a N-acetyl-ß-D-glucosaminidase gene (Lovatt and Roberts, 1994) and potential cloning of a glycosulphatase gene from *P.gingivalis* (this thesis) *via* generation of isogenic mutants should answer a variety of questions relating to the importance of these enzymes in virulence. One report of a 37 kDa glycosulphatase protein does exist. This enzyme cleaved sulphate from galactosyl and lactosylceramide sulphates (glycosphingolipids) derived from gingival epithelium as well as gingival proteoglycans rich in N-acetylgalactosamine-4-sulphate (Slomiany *et al.*, 1993). These results may be significant in that the integrity of glycosphingolipids on epithelial cells is crucial in cellular recognition and binding of ECM. Another enzyme from *P.gingivalis*, phopholipase A has been shown to release phosphatidyl choline and

phosphatidyl ethanolamine from hamster epithelial cells grown *in vitro* (Bulkacz *et al.*, 1985) and it is possible that concerted action of the sulphatase and phospholipase could represent a further virulence mechanism.

The superoxide dismutase (SOD) of *P.gingivalis* exists as three isozymes derived from a single 46 kDa apoprotein (Amano *et al.*, 1990) and catalyses the dismutation of superoxide (O_2^-) into O_2 and H_2O_2. It is a metalloprotein whose isoforms bind either Fe or Mn or both depending on the oxygen tension to which the cells producing the SOD are exposed. The role of this enzyme is to neutralise the oxygen burst (superoxide, O_2) elaborated by polymorphonuclear leukocytes which is intended to kill invading bacteria. Similar in function are catalase enzymes, although little is known about the catalase produced by animal-infecting forms of *P.gingivalis* .

1.3.3.6 Adhesins.

Adhesins fall into two broad classes, those responsible for host-tissue interaction and those involved in formation of microbial communities. As previously discussed oral intergeneric microbial communities serve to satisfy mutual nutritional and colonisation functions as well as to promote the interchange of genetic material between the members of the same and different species. Adhesins can be surface-bound or occur as appendages emanating from the cell surface, and those which bind carbohydrates are sometimes referred to as being "lectin-like". Receptors for adhesins are commonly polysaccharides such as those of the bacterial glycocalyx or the sugar residues found on glycoproteins (Holt and Bramanti, 1991)

Data relating to fimbrial adhesins and haemagglutinins for oral microbes is abundant. There have been, however several studies examining modulators of adhesive properties many of which are host derived and contents of saliva. The presence of small (100-150 aa) proline rich proteins (PRPs) in saliva has been shown to encourage of adhesion of various bacteria, including *P.gingivalis A.viscosus* and *S.mutans*, to hard dental tissues. This is only true in the case of adsorbed PRPs as soluble PRPs are not bound by the aforementioned species and suggests binding of PRPs to substrate induces some conformational change recognised by the bacterial receptors (Gibbons, 1989). Such conformational changes have been proposed to reflect the

presence of cryptitopes, binding sites which are hidden under certain circumstances. Other potential cryptitopes include the binding sites for fibrinogen, collagen and buccal epithelial cells for *P.gingivalis* (Gibbons , 1989; Lantz *et al.*, 1991). In the cases of collagen and fibrinogen binding of these substrates may be intrinsically linked with their degradation by *P.gingivalis* proteolytic enzymes. Binding to epithelia is enhanced by trypsin, neuraminidase and papain treatment of epithelial cells and it is conceivable that glycolytic enzymes may contribute to the exposure of cryptitopes and subsequent binding (Gibbons, 1989).

Cryptitopes and proteolytic activities have also been implicated in intergeneric coaggregation. Ellen *et al.* (1991) have shown *P.gingivalis* adhesion to *A.viscosus* shows an inhibition profile similar to that of TLP when coaggregation assays were performed in the presence of protease inhibitors. Thus it is possible that *P.gingivalis* proteases may be involved in expression of cryptitopes or may themselves function as adhesins. Enzyme-mediated processing of host components might also enhance the action of the two known *P.gingivalis* adhesins namely the fimbrial adhesin and the haemagglutinin.

The requirement of *P.gingivalis* for haem for growth is fulfilled by the targeting and subsequent lysis of red blood cells (RBC) thus releasing haemoglobin into the medium. This targeting is manifested by a specific adhesive event which through subsequent cross-linking causes the agglutination of RBCs (known as haemagglutination). *P.gingivalis* exhibits a number of haemagglutinins as well as at least one haemolysin. Molecular cloning experiments have led to reports of at least three haemagglutinins (Ikeda *et al.*, 1993; Lepine *et al.*, 1993; Madden *et al.*, 1993) and the persistence of haemagglutinating activity in isogenic mutants for at least two such cloned genes supports the argument for the existence of multiple haemagglutinins (Lepine *et al.*, 1993). The use of immunoelectron microscopy with antibodies raised against one such haemagglutinin, HA-Ag2 has shown this protein to be associated with long cell-bound strands of material distinct from fimbriae (Deslauriers *et al.*, 1992) which appear to represent a completely novel bacterial cell surface component.

Attempts to purify *P.gingivalis* haemagglutinins have reported the existence of molecular weights of 33, 38 kDa (Chandad and Mouton), 24, 37, and 42 kDa

(Inoshita *et al.*, 1986) and 40 kDa (Okuda *et al.*,1986). Haemagglutinating activity is exhibited by ECV, whole cells and extracellular protein (Kay *et al.*,1990; Deslauriers *et al.*, 1992) and has been shown to be inhibited by parotid saliva (Murakami *et al.*, 1990). Hence it is possible that saliva may control haemagglutination as an environmental factor. The haemagglutinins reported by Okuda *et al.* (1986) and Inoshita *et al.* (1986) both showed activities which were inhibited by L-arginine and it was suspected for some time that this haemagglutinin might well also have proteolytic activity as its activity was found to be inhibited by a variety of protease inhibitors (Nishikata *et al.*, 1989). However, a recent report has demonstrated independent molecular assignments for haemagglutinating and proteolytic activities using polyclonal antisera at the same time as confirming their similarities in inhibition and activation profiles (Shah *et al.*, 1992). It is possible that arginine-containing components of the RBC outer membrane may act as ligands for haemagglutination (hence competitive inhibition by L-arginine). One other possible ligand might be the lipid components of the erythrocyte membrane as haemagglutination has also been shown to be inhibited by phospholipase treatment of the RBC (Okuda *et al.*, 1986).

Fimbriae are common amongst both Gram-positive and Gram-negative bacteria consisting of repeating subunits of fimbrillin monomers and being involved in either cell attachment or conjugation. The fimbriae of *P.gingivalis* are thin, curly appendages of around 5 nm diameter and between 0.3 and 1.6 μm in length which emanate from the cell surface (Yoshimura *et al.*, 1984). The fimbrillin monomer exhibits both heterogeneity in molecular weight (41-49 kDa) and serospecificity between different *P.gingivalis* strains and isolates as well as some minor protein sequence anomalies in its N-terminal sequence (Lee *et al.*, 1991). Molecular cloning and sequencing of the fimbrillin gene has revealed it to exist as a single copy on the chromosome and to encode for a species specific protein possessing a putative N-terminal leader sequence with no known protein sequence homologues (Dickinson *et al.*, 1988).

Antibodies raised against *P.gingivalis* fimbriae are able to inhibit adhesion of *P.gingivalis* cells to buccal epithelial cells and it has been suggested that fimbriae may mediate attachment by *P.gingivalis* to buccal epithelia (Bramanti *et al.*, 1990). In addition purified fimbrillin and synthetic peptides of fimbrillin have been shown to inhibit *P.gingivalis* adhesion to saliva-coated hydroxyapatite beads and it is possible that these fimbriae may also be

involved in adhesion to saliva-coated surfaces, possibly being important in colonisation (Sharma *et al.*, 1993, Lee *et al.*, 1992). Other workers have shown that fimbrillin can induce IL-1 and neutrophil chemoattractant production in macrophages *in vitro* suggesting that not only may fimbriae be involved in the infiltration of periodontal tissues by neutrophils during disease but may also contribute to the inflammatory response by its effect on IL-1 production (Hanazawa *et al.*, 1992; 1993). It is these qualities of *P.gingivalis* fimbriae along with their ability to yield protective immunisation to infection in the gnotobiotic rat model that make fimbriae a potentially good candidate for vaccine manufacture (Ogawa *et al.*, 1992; Evans *et al.*, 1992b).

1.4 Aims.

At the time this project began (1989) little was known about the molecular pathogenicity of *P.gingivalis*. The now common molecular biology techniques had not been applied to the study of periodontopathogens. Preliminary cloning experiments had suggested that DNA instability may create problems in cloning *P.gingivalis* DNA in *E.coli* (Progulske-Fox *et al.*, 1989). It was necessary to adopt an approach which would circumvent such problems. This study attempts such an approach.

The first stage in this study was to be the molecular cloning of antigens from *P.gingivalis*. Cloning antigenic determinants was attractive for two reasons. First, several studies had shown that characteristic antigens are recognised in periodontal disease patients and must hence have a role in host-pathogen interactions (Curtis *et al.*, 1991; Papaioannou *et al.*, 1991). Secondly, that it would allow identification of small gene fragments using expression libraries and this has the advantage of avoiding expression of toxic proteins in the initial stages, so that subsequent isolation of a larger clones using the small gene fragment could occur in non-expression vectors. This second phase was by approach of colony hybridisation of a second non expression vector library. Determination of the DNA sequence of such clones would then allow designation of function for the antigen(s) using sequence similarities. Other data could be derived from the sequence relating to protein export and processing signals, about which little is known for *P.gingivalis*. In this way it was hoped we would identify potential virulence factors, porins and other cell surface structures so that the general biology of this organism might be better understood. Moreover, studying antigenic determinants prominent in disease might eventually aid vaccine development and hence constitute a positive step in the combat of periodontal disease.

Chapter 2

Materials and Methods.

2.1 Bacterial Strains and Plasmids.

Bacterial strains used in this study and their strain genotypes are given in table 2.1.1. Plasmids and their characteristics are given in table 2.1.2 .

Table 2.1 Bacterial Strains.

Organism/ Strain.	Source/ Genotype.	Reference.
E. coli LE392	F$^-$, *hsd*R514 (r^-_k, m^+_k), *Sup*E44, *Sup*F58,*l ac*Y1, *gal*K2, *gal*T22, *met*B1, *trp*R55, λ^-.	Murray *et al.*, 1977
E. coli JM101	*sup*E, *thi*D(*lac-pro*AB), F'[*tra*D36, *pr*AB, *lac*I_qZΔM15].	Yanisch-Perron *et al.*, 1985.
E. coli TG2	*sup*E, *hsd*5, *thi*D(*lac-pro*AB), D(*srl-recA*$^-$) 306::Tn10(*tet*^{R}), F'[*tra*D36, *pro*AB, *lac*I_q ZΔM15].	Maniatis *et al.*, 1991
E.coli DS410	*min*A, *min*B, *ara*, *xyl*, *mtl*, *azi*, *thi*.	Dougan and Sheratt, 1977
E.coli Sure™	e14$^-$(*mcr*A), D(*mcr*CB-*hsd*SMR-*mrr*)171, *sbc*C, *rec*B, *rec*J, *umu*C::Tn5(*kan* ^{R}), *uvr*C, *sup*E44, *lac*, *gyr*A96, *rel*A1, *thi*-1, *end*A1, F'[*tra*D36, *pro*AB, *lac*I_qZΔM15].	A. Greener, 1990
E.coli NM522	*sup*E, *thi*D(*lac-pro*AB), F'[*tra*D36, *pr*AB, *lac*I_qZΔM15], Δ(*hsd*MS*mcr*B)5(r^-_k, m^-_k *mcr*CB-)	Maniatis *et al.*, 1991
E. coli JM109(DE3)	e14$^-$(*mcr*A),*rec*A1, *end*A1, *gyr*A96, *hsd*R17 (r^-_k, m^+_k), *sup*E44, *thi*D(*lac-pro*AB), F`[*tra*D36,*pro*AB, *lac*I_qZΔM15], lc Its 857,*ind*1, Sa7,*nin*5, *lac*UV5-T7 gene 1)	Yanisch-Perron *et al.*, 1985.

Table 2.1 (continued) Bacterial Strains.

Organism/ Strain.	Source/Genotype.
P. gingivalis W83	Clinical isolate of unknown origin. MRC Dental Unit, London.
P. gingivalis 23A3A	American typed culture collection
P. gingivalis 11834B	Clinical isolate of unknown origin. MRC Dental Unit, London.
P. asaccharolyticus ATCC8503	American typed culture collection
P. endodontalis ATCC35406	American typed culture collection
P.intermedia NCTC9339	National Collection of typed cultures

Table 2.2 Plasmids used in this study.

Plasmid	Characteristics.	Reference
pTZ18R and pTZ19R	Ap^R. Phagemid cloning/expression vector.	Mead *et al.*, 1986.
pLG339	Km^R, Tc^R. Low copy number cloning vector.	Stoker *et al.*, 1982.
M13mp18 and mp19	Bacteriophage M13-derived vector for the production of single-stranded DNA for sequencing.	Yanisch-Perron *et al.*, 1985.
pTTQ18*	Ap^R Expression vector for tightly regulated expression of cloned genes.	Stark, 1987.
pGEM-5Zf(+)	Ap^R. Phagemid cloning/expression vector.	Promega Corps, 1991.

2.2 Bacterial and Bacteriophage Growth, storage, and manipulation.

2.2.1 Propagation of strains.

Routine culture of *P. gingivalis* and other *Porphyromonas* and *Prevotella* species was at 37°C on blood agar plates (4% blood agar base (Oxoid) supplemented with 5% defibrinated horse blood) in a humidified anaerobic cabinet (Don Whitley) with an atmosphere of 80% (v/v) N_2, 10% (v/v) H_2, and 10% (v/v) CO_2. For liquid culture freshly made BM nutrient broth at pH 7.4 (Shah *et al.*, 1976) was used (table 2.3). Cultures of *P. gingivalis* typically take 3 to 5 days to grow.

Table 2.3 Composition of Bacteroides Medium.

Component	Content
Trypticase Peptone (BBL)	1% (w/v)
Proteose Peptone (Difco)	1% (w/v)
Yeast Extract (Merck)	0.5% (w/v)
Glucose (BDH)	0.5% (w/v)
Sodium Chloride (BDH)	0.5% (w/v)
Cysteine Hydrochloride (BDH)	0.07% (w/v)
Sodium Carbonate ($NaHCO_3$)	0.1% (w/v)
40% NaOH Solution*1	adjust to pH 7.4
Hemin/Menadione Stock*2	1% (v/v)

*1 NaOH is used to adjust the pH to 7.4 after which the broth is autoclaved without addition of hemin/menadione.

*2 Stock solution contains 1ml menadione solution (Sigma; 100mg in 20ml 95% ethanol) and 1ml hemin solution (Sigma; 50mg in 1ml 40% NaOH) in a final volume of 100ml. This solution is then filter-sterilised (0.2 µm Acrodiscs, Gelman Sciences) and stored at -20°C.

E. coli was routinely subcultured at 37°C and with shaking at 200 rpm using Luria-Bertani medium (LB; 0.5% (w/v) NaCl, 0.5% (w/v) yeast extract, and 1% (w/v) trypticase peptone) with bacteriological agar (BBL) added to 1.5% final (w/v) as required. Other media used include B-agar (trypticase peptone 1% (w/v), NaCl 0.8% (w/v), and agar 1.5% (w/v) final) and TY medium (trypticase peptone 1.6% (w/v), yeast extract 1% (w/v), NaCl 0.5% (w/v). For soft top agar 0.6% agar was used. Where necessary media were supplemented antibiotics at the following concentrations: ampicillin 100 $\mu g\ ml^{-1}$, kanamycin 25 $\mu g\ ml^{-1}$, and tetracycline 25 $\mu g\ ml^{-1}$. All antibiotics were purchased from Sigma Chemical Company Ltd. For screening of recombinants using insertional inactivation of the *lacZ'* cassette, solid media was supplemented with 0.2 mg ml^{-1} X-gal and mM IPTG (both were purchased from Novabiochem).

Bacterial cells were collected by centrifugation at 3,300g for 5 min at 4°C in a Sorval centrifuge (Dupont). Small volumes of culture (≤1.5 ml) were centrifuged in Eppendorf microfuge (MSE) at 13,400 g for 1 min at room temperature.

M13KO7 helper phage stock preparation was carried out according to the recommendations of the suppliers (Pharmacia technical support). M13KO7 was streaked down a TY agar plate using a continuous side to side movement. 5ml of TY soft top agar at 42°C was then mixed with 0.5 ml of an NM522 culture (grown to an optical density >0.8) and poured over the plate starting at the dilute end of the streak and pouring towards its starting point by tipping the plate. After incubation overnight at 37°C single plaques were visible. An area of closely spaced plaques was scraped off and used to inoculate 200 ml of 2xTY broth supplemented with 70 $\mu g\ ml^{-1}$ kanamycin. After 10-16 h growth at 37°C with shaking at 300 rpm cells were collected by centrifugation. The supernatant was aspirated off and clarified of cells by two further centrifugation/aspiration steps. The supernatant was then filter sterilised and stored at 4°C.

2.2.2 Transformation of Bacterial cells with plasmid and M13mp18/mp19 DNA.

Bacteria were transformed using variations of either the CaCl method (Mandel and Higa, 1970) or the high efficiency electroporation method of Dower *et al* ., (1988). For the CaCl method 100 µl of an overnight culture was back-diluted into 10 ml of LB-broth and grown to mid exponential phase (an OD_{600} of around 0.5). Cells were harvested by centrifugation and washed once in 10 ml of ice-cold 10 mM NaCl solution. Cells were then made competent to take-up DNA by gently resuspending the cell pellet in 4 ml ice-cold CaCl (100mM) and incubating for 30 min on ice. The cells were then sedimented using a gentle centrifugation step (1,800g for 5 min at 4°C) and resuspended in 1 ml of ice-cold CaCl (100mM). Competent cells (100 µl aliquots) were then transformed by co-incubating with 5-20 µl plasmid DNA for 1 hour on ice, after which the cells were heat shocked (42°C for 3 min) and then immediately mixed with 1 ml ice-cold LB broth. The transformation was then left for 1 hour at 37°C, after which 100 µl portions were plated onto LB agar plates containing the appropriate antibiotics and incubated at 37°C overnight.

For electroporation 100 µl of an overnight culture was back-diluted into 10 ml of LB-broth and grown to mid-exponential phase (an OD_{600} of around 0.5). Cells were pelleted by centrifugation, and washed three times in 10 ml volumes of ice-cold water by alternate resuspension of cell pellets and re-centrifugation. Finally the cells were washed once in 10 ml ice-cold 10% glycerol and resuspended in 10% glycerol to a final volume of 80 µl. DNA (1-2 µl) was then mixed with the cell sample (40 µl aliquots) which was then pipetted deep into the electrode gap (2 mm) of an ice-cold Gene Pulser™ cuvette (Biorad). A high voltage pulse was the delivered through the sample using a Biorad Gene Pulser™ with pulse controller unit (pulse parameters were 25 µF capacitance, 200 Ω resistance, and 2.4 KV voltage to give a 0.48 kV cm^{-1} field strength). Time constants were typically in the 4.6-4.9 msec range depending on the purity (salt content) of the DNA sample. Immediately after the pulse the transformation was mixed with 1 ml of ice cold SOC recovery medium (table 2.4) and incubated for 1 hour at 37°C before plating out (100µl portions) onto LB agar with the appropriate antibiotics.

Transformation of M13mp18/mp19 single or double-stranded DNA was performed using the electroporation method except that the the 1h recovery period after transformation was shortened to 20 mins at room temperature. Cells were mixed with 3ml moltern (42°C) B-agar soft top supplemented with 10µl 100mM IPTG, 50µl 2% X-gal solubilised in dimethylformamide and 200µl of JM101 indicator cells and plated out onto B-agar (indicator cells were prepared by resuspending cells from a mid-log phase JM101 culture in a 1/100th volume of sterile L-broth). B-agar soft top has the same composition as B-agar (section 2.2) except that BBL agar is added to 1% (w/v). Plates were incubated overnight at 37 °C.

Table 2.4 Composition of SOC recovery medium.

Component.	Concentration.
Trypticase peptone	2% (w/v)/20 gl^{-1}
Yeast Extract	0.5% /5 gl^{-1}
NaCl	10 mM /0.57 gl^{-1}
KCl	25 mM /0.175 gl^{-1}
$MgCl_2$	10 mM /2.025 gl^{-1}
$MgSO_4$	10 mM /2.45 gl^{-1}
Glucose	20mM /3.6 gl^{-1}

2.3 Methods of Recombinant DNA Manipulation.

2.3.1 Extraction and Purification 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 a 400 ml overnight culture were split into two 200 ml portions and harvested by centrifugation. Each cell pellet was resuspended in 5 ml of ice-cold solution I (50mM glucose, 25 mM Tris-HCl pH8.0, 10 mM EDTA and 5 $mgml^{-1}$ lysozyme), transferred to a 30 ml Oakridge tube and left on ice for 30 mins. 10

ml of freshly made room temperature solution II (0.2M NaOH and 1% SDS) was added to each tube; the tubes were covered with Nesco film and mixed by inverting sharply five times. After a further 10 mins on ice, 7.5 ml of ice-cold solution III (5M potassium acetate buffer at pH4.8 which is 3 M wrt potassium and 5M wrt acetate content) was added, again with gentle mixing and a further 10 mins incubation on ice. The resultant precipitate was sedimented by centrifugation at 36,900g for 30 mins in a Sorval centrifuge using a Beckman SS-34 rotor cooled to 4°C. 18 ml of supernatant from each tube was mixed with 12 ml of isopropyl alcohol and allowed to stand at room temperature for 20 mins. The resultant DNA precipitate was pelleted by centrifugation in 30 ml Corex tubes for 30 mins at 3,500g and 20 °C; the supernatant was poured off, the tube inverted and the pellet allowed to air dry. Both pellets were resuspended in water and pooled together to give a final volume of exactly 17 ml. Exactly 17 g of ultrapure caesium chloride (weighed to the nearest 10 mg) was added, and allowed to dissolve after which the solution was placed in a Sorval 30 ml vertical rotor centrifuge tube. 1 ml of ethidium bromide (5 mg ml^{-1}) solution was added to the tube which was then filled with mineral oil, balanced and crimp-sealed. Chromosomal and plasmid DNA were then separated by centrifugation for 20 h at 40,000 rpm and 20 °C in a Sorval TV850 fixed angle rotor using a Sorvall OTD 60 Ultracentrifuge. DNA bands were visualised under UV light and the lower plasmid DNA band was collected by piercing the tube and dripping from the bottom. Ethidium bromide was extracted by mixing several times with caesium chloride-saturated isopropanol, keeping the lower aqueous phase. Finally, the DNA solution was cleared of remaining contaminants by dialysing against 10 l of sterile water overnight, changing the water once. The resultant DNA solution was aliquoted and stored a -20°C.

Small scale extraction of plasmid DNA was carried out using a variation of the alkaline lysis method described above. The cell pellet from 1.5 ml of overnight culture was resuspended in 100 µl ice-cold solution I by vortexing for 10 secs in a 1.5 ml eppendorf centrifuge tube. The cell suspension was then left on ice for 30 mins after which 200 µl of room temperature solution II was added and mixed in by inverting the tube sharply five times. After a further five minutes on ice 150µl of ice-cold solution III was added and a precipitate was allowed to form by standing on ice for a further five minutes. The supernatant was recovered after centrifugation in an Eppendorf centrifuge for 5 mins and mixed once with an equal volume (about 400 µl) of

phenol/chloroform (1:1), vortexed for 5 secs and centrifuged in an Eppendorf centrifuge for 1 min. The upper aqueous phase (about 400µl) was collected, carefully avoiding disturbance of the phenol/chloroform phase, and mixed with 1 ml of room temperature ethanol (this method of DNA precipitation works only with miniprep DNA and relies on contaminating salts and carrier RNA in the preparation as an aid to precipitation). After 5 mins the DNA precipitate was sedimented by centrifugation for 5 mins in an Eppendorf centrifuge. The ethanol was aspirated off and discarded, avoiding the DNA pellet which was then dried *in vacuo* for 5 mins using a vacuum desiccator. The pellet was resuspended in 60 µl of sterile nanopure water and stored at -20°C.

2.3.2 Extraction of M13mp18/mp19 and Phagemid Template DNA.

For template production in M13mp18/mp19 single plaques were isolated directly from fresh transformation plates, using laboratory strain JM101 for all M13 cloning and propagation.

5 ml of L-broth was co-inoculated with 100 µl of a JM101 overnight broth culture and a toothpick-isolated M13 plaque and incubated for 5 h at 37°C with vigorous aeration. The cells from a 1.5 ml aliquot of this culture were sedimented by centrifugation for 2 mins in an Eppendorf centrifuge and retained for plasmid miniprep analysis of replicative form (RF) DNA. The supernatant was aspirated off and completely clarified of cells by two further centrifugation/aspiration steps. 800µl of supernatant was mixed with 200 µl of a fresh solution of 20% (w/v) PEG 6000 and 2.5 M NaCl and allowed to stand at room temperature for 30 mins. The resultant phage precipitate was collected by centrifugation in an Eppendorf centrifuge for 5 mins. The supernatant was carefully removed using a drawn-out Pasteur pipette. To ensure removal of all the supernatant the Eppendorf was given a pulse centrifugation and any residual liquid removed from the pellet using a glass capillary tube. The pellet was resuspended in 200 µl 1.1 M sodium acetate buffer pH 7.0 and mixed with an equal volume of phenol/chloroform (1:1) by vortexing for 10 secs. The two phases were then re-established by centrifugation for 1 min after which the upper aqueous phase was aspirated off and mixed with an equal volume of chloroform. After a pulse centrifugation step the upper aqueous phase was removed avoiding

contamination with the lower organic phase, mixed with 1 ml of -20°C absolute ethanol and chilled to -20°C for 30 mins. The resultant DNA precipitate was collected by centrifugation in an Eppendorf centrifuge for 5 mins, the ethanol removed by aspiration and vacuum desiccation for 5 mins, and the pellet dissolved in 20 µl sterile nanopure water.

For single stranded DNA production using phagemid vectors (e.g. pTZ18/19) a freshly grown colony was used to co-inoculate 15 ml of 2xTY ampicillin broth along with 50µl of M13KO7 helper phage stock ($1x10^8$ pfu ml^{-1}). After 1 h at 37°C with shaking at 300 rpm (very slight growth should be visible) kanamycin was added to a concentration of 75 µg ml^{-1} and the culture allowed to grow overnight (18 h) at 37°C and 300 rpm. The cells were then harvested and the supernatant collected and thoroughly clarified of cells by two further centrifugation/aspiration steps. A 1/4 volume of 20% (w/v) PEG 6000, 3.5M NaCl solution was then added, and the solution mixed and allowed to stand on ice for 30 mins. The resultant phage precipitate was collected by centrifugation for 30 mins at approximately 11,000 g at 4°C (using an SS34 Sorval rotor at 11,000 rpm). The supernatant was removed and any remaining liquid was collected by giving the tube a quick re-centrifugation and using a capillary pipette to draw up supernatant from around the pellet. The pellet was resuspended in 400 µl of a solution of 20 mM Tris-HCl pH 7.5, 20 mM NaCl and mM EDTA, transferred to an Eppendorf and mixed with 400 µl of phenol/chloroform (1:1) by vortexing for 10 s. This was then centrifuged in an Eppendorf centrifuge for 2 min and the upper aqueous phase carefully collected and ethanol precipitated as described for M13 template DNA (above).

2.3.3 Extraction of Chromosomal DNA.

Extraction of bacterial chromosomal DNA was carried out using a variation of the phenol-extraction method of Saito and Miura (1963). Bacterial cells from a 10 ml of overnight broth culture were resuspended in 5 ml of solution I and left on ice for 30 mins. SDS and EDTA were then added to a final concentration of 1% and 50mM respectively and the preparation was then allowed to stand on the bench at room temperature until the turbidity cleared leaving the solution translucent (usually after about 20 mins). The cell lysate was then mixed with an equal volume of phenol/chloroform (1:1) by gentle

inversion for about 10 mins. After centrifugation at 4,000 g for 20 mins at 4°C 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. This aqueous phase was then re-mixed with phenol/chloroform, re-centrifuged and collected repeatedly until no longer turbid (usually three repetitions suffice). The cleared aqueous phase was then mixed with a one tenth volume of 3M 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 side of the tube thus causing an interface to form between two phases. Chromosomal DNA precipitated at this interface was collected by spooling around the end of a sterile glass Pasteur pipette and resuspended in sterile nanopure water.

2.3.4 Methods used routinely in Sub-cloning, Manipulation and quantitation of DNA.

Standard analysis of DNA was by estimating molecular weights in kb using agarose gel electrophoresis. DNA samples (usually around 20 µl) were mixed with 6x DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 15% (w/v) Ficoll) and pipetted into the wells of 0.7% Seakem agarose submarine slab gels made using TAE buffer (40 mM Tris-acetate pH 7.7, mM EDTA) with 0.5 µg ml^{-1} ethidium bromide. The gel was run in TAE buffer with ethidium bromide by applying 120 Volt potential difference accross the gel for about 2 h. The DNA was visualised by placing the gel on a longwave UV transilluminator and its molecular weight estimated by comparison with 1kb ladder DNA standards (Gibco BRL) run on the same gel.

DNA concentrations were measured by determining the A_{260} of the DNA in water using a Philips UV/VIS spectrophotometer and calculated under the assumption that an A_{260} of 1 represents a double-stranded DNA concentration of 50 µg ml^{-1}, a single-stranded concentration of around 40 µg ml^{-1} or an oligonucleotide concentration of about 20 µgml^{-1}. Alternatively DNA concentrations were estimated by comparing band intensities to those of known DNA standards after agarose gel electrophoresis.

Manipulation of DNA concentrations was by either diluting the DNA in water or by concentration using ethanol precipitation where samples were mixed with a one tenth volume of 3M sodium acetate buffer pH 5.2 and two

volumes of -20°C absolute ethanol and then incubated at -20°C for 30 mins. The DNA precipitate was then sedimented by centrfugation in an Eppendorf centrifuge and the ethanol supernatant removed. At this point if there was excessive salt in the sample the DNA was washed in chilled (-20°C) 70% ethanol and repelleted. The pellet was dried in a vacuum desiccator and resuspended in the desired volume of sterile nanopure water.

DNA sub-cloning was carried out by restriction endonuclease cleavage of purified plasmid DNA followed by isolation of subsequent restriction fragments and ligation into a plasmid vector for clonal propagation. Restriction enzyme digestion was performed using the guidelines provided by the manufacturers and typically involved cutting upto 1 µg of DNA for 1-2 h with 1 unit of enzyme in a volume of 20 µl using the reaction buffer supplied with the enzyme (Gibco BRL). If necessary the DNA was then diluted to 400 µl, mixed with phenol/chloroform (1:1) to denature the enzyme, centrifuged for 1 min and the upper aqueous phase removed and ethanol preciptated for digestion with a second restriction enzyme. Restriction digests were then run on 0.7% agarose gels as described above and the fragments for cloning excised directly from the gel.

DNA in agarose gel slices was purified using a Sephaglas™ bandprep kit (Pharmacia) following the manufacturers instructions. Resultant DNA was then co-precipitated with appropriately digested vector (typically 1µg vector) and resuspended in 7.5 µl sterile nanopure water. This was followed by addition of 2 µl 5X ligation buffer (Gibco BRL) and 0.5 µl T4 DNA ligase (Gibco BRL) and ligated for 2 to 4 h at 37°C. The molarity ratio of vector to insert depended on the DNA ends left by the restriction enzyme; for "sticky-ended" ligations vector/insert ratios were 1:1, for "blunt/sticky" ligations 1:1.5, and for "blunt-ended" ligations ratios of 1:2 were used.

2.3.5 Preparation of Genomic DNA for Library Construction.

Reaction kinetics for digestion of chromosomal DNA with restriction endonuclease *Sau 3a* were determined by running a test reaction from which aliquots were taken at 2.5 min intervals and mixed with 6x DNA loading buffer to stop the reaction. Subsequent agarose gel electrophoresis revealed the optimal time course to produce restriction with a class size bias in the

order of 5-10 kb. The resultant reaction consisted of 1 ml of W83 genomic DNA (approximately 100 µg), 110 µl of 10x reaction buffer 4 (Gibco BRL) and 6µl of *Sau3a* (42 units) and was incubated for 12 mins at 37°C after which the reaction was terminated by mixing for 10 secs with 250 µl of phenol/chloroform (1:1). The resultant emulsion was centrifuged in an Eppendorf centrifuge for 5 mins and the upper aqueous phase (1 ml) carefully aspirated off. This was then split into 3 portions each of which was ethanol precipitated (section 2.3.4), resuspended in 100 µl and pooled together.

Restricted DNA was sorted into fractions according to restriction fragment class size using sucrose density gradient centrifugation. 14.5 ml of an autoclaved (10 psi for 10 mins) solution of 20% (w/v) sucrose, 20 mM Tris-HCl pH 8.0, M NaCl, an 5 mM EDTA was placed in each of two ethanol-rinsed and dried 17 ml polyallomer tubes (dimensions 16x102 mm; Dupont). The tubes were covered with Nesco film and frozen at -20°C after which the tubes were allowed to thaw at 4°C for 13 hours. The 300µl of *Sau 3a* -restricted chromosomal DNA was gently layered onto the top of one of the gradients using 300µl of water to balance the second tube. Tubes were placed in an AH627 swing-out rotor being careful not to disturb the gradient. Tubes were centrifuged at 26,000 rpm at 4°C for 24h in an OTD Sorval Ultracentrifuge. Twenty seven five drop fractions were collected from the gradient by clamping the tube and plunging a narrow gauge hypodermic needle through the base of the tube. Fractions containing DNA were identified by ethanol-precipitating every third fraction into a volume of 50 µl and running 20 µl on a 0.7% agarose gel. DNA-containing fractions were dialysed overnight against TE buffer (100mM Tris HCl pH7.8, 10 mM EDTA), ethanol-precipitated and resuspended in a volume of 100µl.

2.3.6 Phosphatase Treatment of Vector DNA for High Efficiency Cloning.

High frequency recombination was achieved by removal of 5′ terminal phosphate groups from restricted vector hence preventing vector self-ligation. 100 µg of vector was cleaved with *BamH* I (50 U) for 4 h at 37°C in a volume of 50 µl. Complete digestion was confirmed when transformation of JM101 with cut vector yielded no transformants. 45 µl of this digest was mixed with 5µl of 10x calf intestinal phosphatase (CIP) buffer (10x CIP buffer consists of 0.5 M Tris-HCl pH 9.0, 10 mM $MgCl_2$, and mM $ZnCl_2$ and should be autoclaved)

followed by 1 µl of 5 $U\mu l^{-1}$ CIP (NEB). After 30 mins incubation at 37°C a further 0.5 µl of CIP was added and the reaction allowed to proceed for a further 30 mins at 37°C. The reaction was diluted to 200 µl and stopped by vortexing with an equal volume of phenol/chloroform (1:1). The mixture was centrifuged in an Eppendorf centrifuge for 2 mins and the upper aqueous phase carefully removed. This was then ethanol precipitated and the pellet washed with 70% ethanol, dried and resuspended in 50 µl sterile nanopure water. Phosphatase efficiency was assessed by determining transformation efficiencies for self-ligated phosphatased vector. 1 to 4 µl of this vector was then used in ligations.

2.3.7 Polymerase Chain Reaction.

Polymerase chain reaction (PCR) was performed using the method of Saiki *et al.*, 1988). Reactions (100µl) consisted of oligonucleotide primers (0.25 µM each), dNTPs (each at 200 µM final; Pharmacia), 10 ng template DNA, 1x reaction buffer (10 mM Tris-HCl pH 9, 50 mM KCl, 0.01% (w/v) gelatin, 1.5 mM $MgCl_2$ and 0.1% Triton X-100) and 2.5 units Taq DNA polymerase (NBS Biologicals). Concentration of $MgCl_2$ was initially determined empirically by running test reactions using 1.5 to 7 mM $MgCl_2$ and then choosing the $MgCl_2$ concentration optimal for specificity and amplification for all future reactions using that primer/template combination. Reactions mixtures were overlaid with 20 µl molecular biology grade mineral oil (United States Biologicals) and cycle-incubated (30 cycles) using a Perkin Elmer Cetus thermal cycler. Cycles consisted of 1 min at 94°C (denaturing step), 1 min at 55°C (annealing should be 5°C below the Tm of the primer with the lowest melting point) and 2 mins at 72°C (extension step) with a 5 min denaturing step (94°C) before cycling and a 5 min chase step (72°C) to complete partially polymerised chains. The aqueous phase was removed by pipetting from under the oil overlay and analysed (10 µl) on agarose gels.

2.4 DNA-DNA Hybridisation and Detection Techniques.

2.4.1 Transfer of DNA from Agarose Gels (Southern Blotting).

DNA was transferred to filters using a capillary blotting technique (Southern, 1975). DNA was separated by electrophoresis on 0.7% agarose gels. After running gels were photographed along side a ruler as a point of reference and then soaked in a solution of 0.25 M HCl for 7 mins to depurinate the entrapped DNA. The gel was rinsed in distilled water and then soaked in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 30 mins with gentle rocking. The gel was again rinsed in distilled water and then soaked with rocking for a further 30 mins in neutralising solution (0.5 M Tris-HCl pH7.5, 3M NaCl) and then rinsed in distilled water. The gel was then placed on six sheets of 20x SSC-soaked Whatman 3MM filter paper (20x SSC consists of 175.3 g l^{-1} NaCl and 88.2 g l^{-1} sodium citrate and is pH-adjusted to 7.0 with 5 M NaOH) and overlaid with a pre-soaked (10x SSC) square of Hybond N nylon membrane (Amersham). This in turn was overlaid one pre-soaked (10x SSC), four sheets of Whatman 3MM filter paper and a stack of dry paper towels, in that order (all filter paper and towels were cut to the same dimensions as the face area of the agarose gel and were layered carefully so as to avoid trapping air bubbles). The transfer sandwich was then evenly compressed by placing a weight (0.5 to 1.0 kg) on a glass plate on top of the paper towels. Transfer was overnight on the bench changing the towels once and ensuring the filter paper under the gel remained wet by providing an excess of 20x SSC. The transfer sandwich was then dismantled and the DNA fixed to the filter by wrapping the dried filter in Saran wrap and exposing the DNA side to UV light from a long-wave transilluminator for 5 mins.

2.4.2 Transfer of Bacterial Colonies for DNA-DNA Hybridisation.

Bacteria were grown on LB agar plates to a density of 100 to 200 cfu $plate^{-1}$ or alternatively individually toothpicked to give 100 small streaks $plate^{-1}$. Imprints of the bacterial growth were transferred onto marker pen-labelled 85 mm colony/plaque screen filters (NEN Biotechnology, Dupont) by dropping the filters onto the surface of the agar. Reference points were made in the agar by stabbing through the ready-made holes in the periphery of the filters. Filters were lifted off the plates in a single continuous motion from one side,

avoiding smearing the bacterial imprint. Filters were then placed bacteria-side up onto Whatman 3MM soaked in denaturing solution (section 2.4.1) for five mins and then on neutralising solution-soaked (section 2.4.1) Whatman filter (section 2.4.1) for 5 mins and then allowed to dry. Excess colonial material was gently scrubbed off using polyallomer wool soaked in 3x SSC (section 2.4.1) and again allowed to dry. Filters prepared this way can be used directly in DNA-DNA hybridisation experiments. The technique can also used to transfer bacteriophage plaques.

2.4.3 Radiolabelling of DNA *in vitro*.

DNA was labelled for detection in DNA-DNA hybridisation experiments using the random primer/extension method. Restriction digested-DNA was separated by electrophoresis on a 1% low melting point (LMP) agarose gel (BRL) and the restriction fragment(s) of interest excised from the gel using a sterile scalpel. The resultant agarose slice was added to sterile nanopure water (1.5 ml /g agarose) and placed in a boiling water bath for 5 mins. This molten slurry could then be used immediately for labelling or stored at -20°C and re-boiled for 3 mins immediately before use. The molten slurry was then kept at 37°C for 3 to 60 mins.

Table 2.5 Components of Oligonucleotide Labelling Buffer (OLB)

Solution A	100 µl Tris-HCL pH8.0,/$MgCl_2$ (1.25 and 0.125 M respectively) 18 µl ß-mercaptoethanol, 5 µl each of 100mM dATP, dTTP and dGTP (Pharmacia).
Solution B	2 M HEPES (pH adjusted to 6.6 using NaOH).
Solution C	Hexadeoxynucleotides (d_6(NTPs); Pharmacia); 50 OD units in 550 µl of TE Buffer pH7.0 (Tris-HCl 3mM, pH7.0 and 0.2 mM EDTA).

*Note: Solutions A, B and C should be stored at - 20 °C and should not be repeatedly freeze-thawed.

Labelling reactions were set up using a variation of the method of Feinberg and Vogelstein (1983). To 25 ng of DNA in molten agarose, 5 µl 5x OLB (5 x OLB consists of solutions A, B and C (table 2.5) in the ratio 10:25:15 respectively), nanopure water (to give a final volume of 25 µl), 0.5 µl Klenow large fragment DNA polymerase (4 units μl^{-1}; BRL) and 2.5 µl ^{32}P α-dCTP (10 $\mu Ci\ \mu l^{-1}$; Dupont) were added. Incubation was at room temperature for at least 5h.

2.4.4 Hybridisation and Detection of Radiolabelled Probe with DNA Immobilised on Filters.

Hybridisation and wash procedures were carried out in a rotary hybridisation oven (Hybaid) using cylindrical canisters. Southern blot or colony hybridisation filters were pre-treated in prehybridisation fluid (table 2.6) for 2h at 65 °C (25ml per Southern filter or canister containing six colony blot filters). Filters were then incubated overnight at 65 °C with hybridisation fluid containing the radiolabelled probe.

Table 2.6 Composition of Hybridisation Solutions.

Prehybridisation Fluid*3	3xSSC. 0.1% SDS. 5x Denhardt's solution*1. 6% PEG 6000. 200 µg ml^{-1} Denatured Salmon Sperm DNA*2.
Hybridisation Fluid*3	3xSSC. 0.1% SDS. 2x Denhardt's solution*1. 6% PEG 6000. 200 µg ml^{-1} Denatured Salmon Sperm DNA. Radiolabelled Probe*2.

*1 100x Denhardt's solution is 2% Ficoll, 2% BSA and 2% Polyvinolpyrollinidine.

*2 Salmon Sperm DNA is denatured by boiling and then forcing the solution through a narrow gauge syringe needle several times until no longer viscous.

*3 Solutions are stored at -20°C.

Signal specificity (95% or greater DNA homology) was defined by washing the filters in solutions for high stringency. This consisted of four 15 min

washes in a solution of 0.1% SDS and 0.1x SSC (1% SDS was used for colony/plaque screen filters) at 65 °C. Filters were 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 in the dark and the film exposed at -70 °C. Film was developed in an Agfa-Geveart automatic film processor.

2.5 DNA Sequencing.

2.5.1 DNA Sequencing Reactions.

DNA was sequenced using methods incorporating the dideoxynucleotide chain termination principle (Sanger *et al.*, 1977). The use of single-stranded DNA derived from M13 and Phagemid cloning vectors was used to produce high quality (sections 2.3.1 and 2.3.2) sequence data using either the Sequenase™ Version 2 or the Taquence™ sequencing kit (United States Biochemical Corporation). These kits facilitate template-dependant second strand DNA synthesis with random chain elongation termination due to dideoxynucleotide incorporation. Chain termination is restricted to only one of the four bases, with a different dideoxynucleotide in each of four separate termination reactions. Detection of second strand synthesis was by incorporation [α-^{35}S] ATP into DNA during polymerisation. Priming of extension was achieved using oligonucleotides with the universal (-40 bp) primer complementary to the ß-galactosidase cassette in M13/pTZ being used most frequently. Reactions were carried out according to manufacturer's instructions.

Double-stranded sequencing was carried out using either plasmid DNA or PCR product in conjunction with the ΔTaq™ cycle-sequencing kit (USB). Using this system as little as 50 ng DNA can yield DNA sequence. In addition use of bi-directional primers to vector sequence allows precise analysis of recombination events. The technique uses the same principle dideoxy chain termination and incorporation of [α-^{35}S] ATP steps, though relies upon multiple annealing/denaturation cycles to amplify second strand yield. Reactions were carried out according to manufacturer's instructions using the following cycle parameters:

Labelling	94 °C - 1 min	
	60 °C - 30 s	-75 Cycles.
Termination	95 °C - 30 s	
	72 °C- 1 min	-65 Cycles.

2.5.2 DNA Sequencing Polyacrylamide Gel Electrophoresis.

Chain termination products were resolved at the nucleotide level using Tris/Borate/EDTA (TBE) gradient gel electrophoresis (Biggins *et al.*, 1983; 10x TBE buffer consists of 121.1 g l^{-1} Tris-HCl, 55 g l^{-1} boric acid and 40 ml of 0.5M EDTA pH 8.0 per litre and is pH-adjusted to 8.3). Two glass plates (20cm x 50cm) were cleaned free of grease using Teepol and water. One plate surface was siliconised by applying a smear of dimethyldichlorosaline and allowing to dry. Plates were taped together with two 0.4 mm spacers separating their inner surfaces. The following solutions were prepared:

Solution A	7 ml 5x TBE acrylamide/urea mix
	45 µl 10% APS (ammonium persulphate)
	2.5 µl TEMED
Solution B	40 ml 0.5x TBE acrylamide/urea mix
	180 µl APS
	7.5 µl TEMED

The 0.5x TBE acrylamide urea mix consists of 430 gl^{-1} urea, 150 ml 30% Accugel acrylamide solution and 50 ml l^{-1} 10x TBE. The 5x mix is similar except that it contains 150 ml l^{-1} 10xTBE, with 50 gl^{-1} sucrose and 50 mg l^{-1} bromophenol blue. 14 ml solution B followed by 25 ml solution A was drawn up into a 25 ml pipette and mixed slightly by drawing up 3-4 air bubbles. This mixture was then run down between the glass plates avoiding introducing bubbles. A sharkstooth comb was inserted inverted at the top end of the gel into the acrylamide to form a large well. The gel was allowed to set at room temperature for 1h. Tape was then removed from the bottom of the gel and the gel clamped into a vertical gel electrophoresis stand with aluminium plates as heat sinks sandwiching the glass plates. The upper buffer reservoir was filled with 0.5x TBE and the lower reservoir with 1x TBE. The comb was removed from the top of the gel which was then pre-run for 30 mins at 40 W.

The upper surface of the gel was cleaned of urea by squirting it with 0.5x TBE buffer and the comb inserted with the points of the teeth biting into the acrylamide so as to form wells. Samples were heated to 70°C for 3 mins and 4 µl loaded into each well. Gels were run at a constant power of 40W for 3, 6, or 9h.

Once run the gel plates were prised apart with the non-siliconised plate down to retain the gel, which was then soaked in gel fix for 15 mins (gel fix is 10% methanol, 10% acetic acid) and rinsed in distilled water. The gel was transferred to pre-wet Whatman 3MM filter paper, overlaid with Saran wrap and dried in a vacuum drier for 1h at 80°C. Autoradiography was carried out by placing the film (Cronex, Dupont) in direct contact with the dried gel surface. Exposure was at room temperature.

2.5.3 Computer Analysis of DNA and Protein Sequence.

DNA sequence was read from sequencing gels by eye and assembled using programs of the Genetics Computer Group suite (University of Wisconsin; Devereaux *et al.*, 1984) running on a Silicon Graphics Crimson mainframe (SERC Daresbury Seqnet Facility, UK) under a UNIX platform. The program MAP was used to identify ORFs and restriction sites and works by creating an output file with these features shown underneath the nucleotide sequence. TRANSLATE was used for inferring protein sequences from DNA sequence and creates output files containing amino acid sequences in the GCG format. DNA and protein homologies were derived from database searches using either FASTA (Lipman and Pearson, 1985), or the BLAST series of search tools (Altschul *et al.*, 1990). Both programs find similarities between query sequence and the sequences of individual database entries and result in an output file containing database sequences with greatest similarity to that of the query sequence. The two programs differ in that FASTA finds and displays the longest single homologous stretch possible for query and database sequence, whereas BLAST finds and displays as many as possible alignments for each query and database sequence under given parameters. For creation of multiple alignments directly from query sequence and database the SOOTY and SWEEP programs were used. SOOTY interacts with the user to create a file containing data in a format recognised by SWEEP. SWEEP performs homology searches with a specified database and uses the best scoring entries

to create a multiple alignment from these entries and the query sequence. Databases used were the composite Genbank/European Molecular Biology Laboratory (Gen_EMBL) databases for DNA or the composite Swissprot/Protein Information Resource (OWL) database for proteins.

Protein analysis was carried out primarily by determining hydropathy profile using PEPPLOT (Kyte and Doolittle, 1982) and the programs SIGPEP and SIGSEQ (G. von Heine, 1986) to predict leader sequences and membrane-spanning domains. Attempts at structure prediction were carried out using PLOTSTRUCTURE and PEPTIDESTRUCTURE or the PREDICTPROTEIN Version 1.0 PHD neural network facility at EMBL Heidelberg (Sander and Schneider, 1991). Multiple alignments were performed using either CLUSTAL V or ALIEN using a PAM 250 matrix (Higgins *et al.*, 1994).

2.6 Techniques for Analysis of Proteins.

2.6.1 Expression of Cloned Genes in *E. coli.*

10 ml LB broth cultures supplemented with the appropriate antibiotics were inoculated with fresh bacterial colonies and incubated at 37°C with shaking at 200 rpm. For expression using either *lac* (M13 and pTZ vectors) or *tac* promoters (pTTQ18*) cells were allowed to grow to an OD_{600} of about 0.4 when IPTG was added to a concentration of 0.1 mM. Cells were allowed to grow to an OD_{600} of around 1.0 and were harvested by centrifugation. Cells were resuspended in a 1/10th volume of ice-cold 100mM Tris-HCl pH7.5. Optical densities of cell suspensions were adjusted to equivalence by dilution of the higher density cell suspensions. Cells were lysed by four 15 s sonication steps using a small sonication probe (Labsonic) at high power on ice with a 30 s cooling period (4°C) in between each step. Protein concentrations were determined using the coomassie blue method (Biorad). 100 µl protein lysate was added to 1 ml Coomassie blue reagent (diluted 1:4 in nanopure water) and the A_{525} was determined after 15-30 mins on the bench. Protein concentration was determined by comparison with a lysozyme standard curve in the 50 to 1000 µg ml^{-1} range.

2.6.2 Determination of Protein Molecular Weight Using PAGE.

SDS and CHAPS-PAGE were run using discontinuous buffer systems in the Mini-PROTEAN™ II system (Biorad). Gels were poured according to the manufacturer's instructions using 0.75 mm spacers and a 10 well 0.75 mm Teflon comb. For SDS-PAGE (Laemmli, 1970) the resolving gel was prepared first, overlaid with butan-1-ol and allowed to set. The butan-1-ol was removed and the top of the gel washed with distilled water. The resolving gel was overlaid with stacking gel into which the comb was set. The composition of the gels was as follows:

SDS-PAGE Resolving Gel	4.65 ml of 1.5M Tris-HCl pH8.8 / 0.2% SDS
(15 % Acrylamide)	4.95 ml of 30% Protogel Acrylamide
	348 µl 1% APS
	40 µl TEMED
SDS-PAGE Stacking Gel	3 ml of 0.5M Tris-HCl pH6.8 / 0.2% SDS
	1.04 ml of 30% Protogel Acrylamide
	2.12 ml distilled water
	150 µl 1% APS
	20 µl TEMED

The protein sample was mixed with 10x SDS PAGE loading buffer (12.5 ml Tris-HCl pH 6.8, 2 g SDS, 20 ml glycerol, 5 ml ß-mercaptoethanol, 100 mg bromophenol blue in a final volume of 50 ml) and boiled for 5 mins. Samples were loaded into the wells along side molecular weight standards (Sigma Chemicals Inc.) and the gel run in a Tris/glycine/SDS running buffer (3.02, 1 and 14.4 g l^{-1} respectively) at 150 V for 1-2h until the bromophenol blue dye front ran off. Gels were stained in coomassie blue solubilised in destain (10% acetic acid, 40% methanol) for 30 mins and then destained to achieve the desired contrast.

Non-denaturing CHAPS-PAGE electrophoresis (CHAPS is 3-[(3-chloramidopropyl) dimethylammonio]-1-propanesulphonate; Sigma) was carried out using a variation of the method of Cavinato (1986) scaled down for use in this thesis. Resolving and stacking gels were poured as for SDS-PAGE using the following recipes:

CHAPS-PAGE Resolving Gel	2.5 ml of 1.5 M Tris-HCl pH 8.8
(10 % Acrylamide)	100 µl 3.5mM CHAPS
	3.3 ml 30% Protogel Acrylamide
	3.8 ml distilled water
	250 µl 10% APS
	25 µl TEMED
CHAPS-PAGE Stacking Gel	2.5 ml of 0.5 M Tris-HCl pH 6.8
	100 µl 3.5 mM CHAPS
	1.73 ml 30% Protogel Acrylamide
	6.05 ml distilled water
	50 µl 10% APS
	5 µl TEMED

Samples were sonicated in a solution of 3.5mM CHAPS, 0.5 M Tris-HCl pH6.8 and mixed with 2x CHAPS-PAGE loading buffer (17% glycerol, 3.5 mM CHAPS, 0.02% bromophenol blue, 8% Ficoll and 100mM Tris-HCl pH 6.8). Samples were loaded without boiling next to reference proteins (Sigma) and the gel run at 150 V for 2-4 h in a Tris/glycine/CHAPS buffer system (3.02, 14.4 and 0.021 g l^{-1} respectively) until the dye front ran off. Gels were stained as for SDS-PAGE gels.

2.6.3 Radiolabelling of Proteins *in vivo* .

Plasmid-encoded proteins were labelled *in vivo* using *E.coli* DS410 minicells isolated by the method of Hallewell and Sheratt (1976). DS410 cultures harbouring the plasmid of interest were grown to stationary phase in Brain Heart Infusion broth (400 ml) containing the appropriate antibiotics. Cells were sedimented by centrifugation at 600 g for 5 mins. The supernatant was collected and centrifuged at 8,500g for 15 mins and the resultant pellet resuspended in 3 ml of ice-cold 1x M9 salts (1x M9 salts consist of 6 g l^{-1} Na_2HPO_4, 3 g l^{-1} KH_2PO_4, 0.5 g l^{-1} NaCl, and 1 g l^{-1} NH_4Cl and is adjusted to pH 7.4). Minicells were then cleared of vegetative cells by sedimenting the vegetative cells with centrifugation at 4,650 g for 20 mins at 4°C using 20 ml 5-20% (w/v) sucrose gradients in 1x M9 salts. Minicells were collected from the middle of the gradient and the density gradient centrifugation repeated once. The OD_{650} of the minicell suspension was determined and adjusted to an

OD_{650} of 2.0. Minicells were then pelleted by centrifugation in an Eppendorf centrifuge for 2 mins and resuspended in an equal volume of 30% glycerol 1x M9 salts for storage at -70°C.

For labelling 50 µl of minicells were pelleted in an Eppendorf centrifuge for 2 mins and resuspended in a solution of 1x M9, 0.4% glucose supplemented with 15 µl Methionine assay medium (MAA) per ml (MAA is available from Difco and is solubilised at a concentration of 525 mg in 5 ml). Remaining vegetative cells were killed by adding 10 µl of 200 µg ml^{-1} d-cycloserine. Minicells were incubated at 37°C for 90 mins, pelleted by centrifugation and resuspended in pre-warmed (37°C) M9/glucose/MAA. 12 µCi of ^{35}S-methionine was added and the minicells were incubated at 37°C for 45 mins. The minicells were then pelleted and resupended in 100µl of M9/glucose/MAA supplemented with 10 µl 2 mg ml^{-1} non-radiolabelled methionine and incubated for a further 15 mins at 37°C. Minicells were then washed twice by pelleting in an Eppendorf centrifuges for 2 mins and resuspending in 1x M9 salts. Finally minicells were resuspended in nanopure water, mixed with SDS-PAGE loading buffer, boiled and subjected to SDS-PAGE using ^{14}C-methylated molecular weight standards (Amersham). Resultant gels were fixed for 1 h in 10% acetic acid, 25 % isopropanol, dried *in vacuo* at 80°C and autoradiographed.

Labelling of minicells in the presence of protease inhibitors was performed by labelling in solution P whose composition follows: 0.5 ml of 10x M9 salts, 50 µl of 40% glucose, 80 µl of MAA solution (as above), 100 µl of each of 5 $mgml^{-1}$ PMSF and O.5M EDTA and 4.5 ml of mix A. Mix A consists of 10 µl of 1$mgml^{-1}$ stock solutions of each of the following protease inhibitors in 10 ml; pepstatin A, antipain, chymostatin and leupeptin.

2.6.4 Radiolabelling of Proteins using DNA-directed Translation *in vitro*.

Synthesis of radiolabelled protein *in vitro* was by the method of Zubay (1973) and involved addition of DNA template, amino acids and ^{35}S-methionine to cell-free (S-30) extracts of *E. coli* and subsequent synthesis of transcripts and translation products derived from the sequence of the DNA template. S-30 extracts of *E. coli* were purchased from Amersham and labelling reactions carried out according to the manufactrer's recommendations. DNA used was

either CsCl-purified in the case of plasmid DNA or purified using the Sephaglas™ bandprep method in the case of PCR products. Care was taken not to use DNA treated with RNAse (including templates for PCR reactions). 2-5 μg DNA were used per labelling reaction. All pipette tips and Eppendorfs used were RNAse free. Reactions were analysed using SDS-PAGE with fixing and autoradiography steps identical to those described for minicells.

2.7 Protein-Antibody Hybridisation and Detection Techniques

2.7.1 Preparation of Antibodies.

Rabbit polyclonal antibodies were prepared using *P.gingivalis* extracellular vesicles as a source of antigen as described by Harlow *et al.* (1988). Where necessary polyclonal antiserum was preadsorbed with *E.coli* by taking colony blots from plates confluent with *E.coli* using the method described below (section 2.7.4) and incubating them with antibody diluted 1:10 in PBS. Incubation was overnight with rocking at 4°C using three filters for 15 ml of diluted antiserum. Preadsorbtion was assessed using *E.coli* in the colony blot protocol described below and repeated until *E.coli* background was sufficiently reduced.

Purification of IgG from spinner culture supernatant (specifically monoclonal antibody Brick 190; see section 3)was by caprylic acid precipitation (Reik *et al.*, 1987). 4.7 ml culture supernatant was mixed with 2x volumes of 0.06 M acetate buffer pH 4.0 and the pH adjusted to 4.8 with HCl. 330 μl of caprylic was then added and the mixture stirred for 30 mins at room temperature. The resultant precipitate was sedimented by centrifugation in a Sorval SS34 rotor at 12,000 rpm for 30 mins at 4°C and the supernatant dialysed overnight (4°C) against 50x volumes of PBS with 200 mM EDTA. The supernatant was then mixed with an equal volume of saturated ammonium chloride solution with stirring for 30 mins at 4°C.The resultant precipitate was sedimented by centrifugation in a Sorval SS34 rotor at 12,000rpm for 20 mins and the pellet resuspended in 1.5 ml PBS. This was then dialysed twice against 50x volumes PBS (4°C). Any remaining insoluble material was removed by centrifugation in an Eppendorf centrifuge for 5 mins. Antibody concentration was determined by measuring the A_{280} and using the equation (P.T.O.)

$$[IgG]mg/ml = \frac{A_{280}}{E^{1\%}_{1cm}}.10$$

where $E^{1\%}_{1cm}$ is the molar extinction coefficient for IgG (13.5 at A_{280}).

2.7.2 Transfer of Protein from PAGE Gels (Western Blotting).

Proteins resolved using PAGE were transferred to either PVDF (Millipore Corporation) or Hybond C membrane using the electroblotting technique of Towbin *et al.* (1979). Either low range pre-stained (Biorad) or biotinylated molecular weight markers (Sigma) were used for electrophoresis to facilitate estimation of molecular weights on transfer filters. Gels were soaked for 5 mins in transfer buffer (14.4 gl^{-1} glycine, 3.02 gl^{-1} Tris-HCl and 10% methanol). The gel was then laid on top of two layers of pre-wet (transfer buffer) 3MM Whatman filter paper and overlaid with the pre-wet (transfer buffer) transfer filter (PVDF must first be wet with methanol). Two layers of pre-wet 3MM Whatman filter paper were laid over the filter and the whole assembly was sandwiched between the fibres and supports of the blotting apparatus which was then slid into retort grooves of the blotting tank. Electrotransfer was carried out in transfer buffer by applying a field with a potential difference of 120 V perpendicular to the plane of the gel and membrane so that the transfer filter was closest to the positive terminal. After 1 h transfer at 4°C the blotting sandwich was dismantled. PVDF filters can be stored dry and re-wet using methanol before use. Hybond C filters should not be allowed to dry out.

2.7.3 Detection of Protein Antigens on Western Blots.

Filters were first blocked by incubation with gentle rocking for 45 mins in 3% BSA solubilised in PBS. Filters were then washed by rinsing twice for five mins in wash solution (0.05% Tween 20 in PBS) before incubation for 2 h at room temperature with antibody diluted in 10-20 ml per filter coupling solution (1% BSA in PBS). Filters were then rinsed twice for five minutes in wash solution before incubation for 1 h at room temperature with second antibody diluted in coupling solution (10-20 ml per filter). Second antibody should be specific for first antibody and should be conjugated with horseradish peroxidase (HRP) for detection of the resultant immunoglobulin complex. If biotinylated markers were used streptavidin-HRP (Sigma) was

included at a dilution of 1/1000 for the detection of labelled proteins. Blots were then washed twice for five mins in wash solution and finally once in PBS. Visualisation of antibody antigen complexes was achieved by adding 2 ml of 30 mg ml^{-1} 4-chloro-1-naphthol (solubilised in ethanol) to 100 ml of PBS containing 60 µl 30% (v/v) H_2O_2 and incubating the filters for 5 to 15 mins in this solution. The reaction was stopped by washing the filters in distilled water. For extra sensitivity PVDF membrane blots can be observed under short wave UV light to visualise 4-chloro-1-naphthol development intermediates invisible in normal daylight.

2.7.4 Transfer of Bacterial Colonies for Protein-Antibody Hybridisation (Colony Immunoblotting).

Transfer of bacterial colonies to nitrocellulose filters for immunoblotting was done using the method described by Sambrook *et al.* (1990). Bacterial transformants were grown to a colony size of 0.1-0.2 mm at 37°C and then incubated at 4°C for 2 h. Nitrocellulose filters (9 cm diameter; Schneider and Schnell) were then placed on the agar plate surface and using a hypodermic needle dipped in Indian ink, the filters were stabbed through making a mark in the agar (this can be used as an orientation point after blotting). Filter were then peeled off carefully avoiding smearing of the colonies, and placed colony side up on agar plates supplemented with IPTG (this step can be omitted for expression independent of IPTG). After 2-4 h at 37°C the filters were placed on damp paper towels and exposed to chloroform vapours for 15 mins. Filters were then incubated in lysis buffer (100 mM Tris-HCl pH 7.8, 150 mM NaCl, 5 mM $MgCl_2$ 1.5% (w/v) BSA, with 1µg ml^{-1} pancreatic DNAase and 40 µg ml^{-1} lysozyme) with rocking at room temperature overnight. Filters were then transferred to a dish containing TNT buffer (100 mM Tris-HCl pH8, 150 mM NaCl and 0.05% Tween 20) and excess colonial material gently wiped off using paper tissues. Filters were ten washed twice in TNT for two 30 min periods. Filters were stored damp at 4°C until needed.

2.7.5 Detection of Antigen using Colony Immunoblots.

Filters prepared as above were incubated in blocking buffer (3% BSA in TNT) for 30 mins with rocking and then for 2 h with primary antibody diluted in

blocking buffer (7.5 ml per filter with antibody at 1/1000 for polyclonal antisera). Filters were then washed for 3x 10 mins in TNT with 0.1% BSA (TNTB), TNT with 0.1% BSA and 0.1% Nonidet P-40 (TNTBN), and once again in TNTB. Filters were then incubated with second antibody diluted in blocking buffer for 2 h at room temperature with agitation (usually diluted at 1/1000 this antibody should be horseradish peroxidase-conjugated and specific for the primary antibody). Filters were washed in TNTB, TNTBN and TNTB as described above and given a final rinse in TNT. 2 ml of 30 mg ml^{-1} 4-chloro-1-naphthol solubilised in ethanol was then added to 100 ml of developing buffer (Tris-HCl pH7.5, 150 mM NaCl, 0.01 % H_2O_2). Filters were incubated in developing buffer (10 ml per filter) for 5-15 mins with agitation. The reaction was stopped by immersing the filters in water and allowed to air dry.

2.7.6 Immunoprecipitation of Protein Antigen.

Immunoprecipitation was executed by conjugating antibody to tosylactivated M-280 Dynabeads (Dynal U.K. Ltd.) and using this conjugate to affinity-purify antigen radiolabelled using the Zubay system (section 2.6.4). Antibody (LDS28 IgM purified courtesy of Roger James, Leicester Department of Surgery) was bead-conjugated following the manufacturer's instructions using a Dynal magnetic particle concentrator (MPC, Eppendorf size). Purified antibody had been previously analysed for protein contaminants using SDS-PAGE analysis.

For immunoprecipitation 100 µl beads were washed by mixing 100 µl beads in 1 ml 0.1% BSA in PBS and pelleting beads using the MPC whilst aspirating the supernatant. Beads were then resuspended in 100 µl, 15 µl of which was made upto 100 µl using PBS/BSA and mixed with 30 µl of radiolabelled protein (section 2.6.4). The bead/protein mix was incubated with rocking for 2 h. Beads were then washed with once with 100 µl 0.1% Tween 20 in PBS (50 µl of which was saved for SDS-PAGE analysis), and 3x with 500 µl Tween/PBS). Beads were then mixed with 10x PAGE loading buffer and the antigen eluted from the beads by boiling for 3 mins. Beads were removed using the MPC and the supernatant was analysed using SDS-PAGE and autoradiography (section 2.6.3).

2.8 Fractionation of Subcellular Compartments.

2.8.1 Preparation of Bacterial Outer Membrane and Envelope Fractions.

Cells from a 200 ml bacterial culture (an OD_{600} of around 0.8 to 0.9) were harvested by centrifugation and resuspended in 20 ml 10 mM sodium phosphate buffer pH 7.5. The cell suspension was then centrifuged at 4°C and 10,000 rpm in a Sorval SS34 rotor for 10 mins after which the cell pellet was resuspended in 10 ml phosphate buffer and sonicated for 5x 30 sec bursts on ice with cooling in between bursts. Debris was removed by performing three consecutive centrifugation steps (6,000 rpm for 10 mins in a Sorval SS34 rotor at 4°C) keeping the supernatant each time. The supernatant was then made upto a volume of 25 ml using ice-cold phosphate buffer and centrifuged (see section 2.3.1) for 5 h at 40,000 rpm and 4 °C in a Sorval TV850 fixed angle rotor using a Sorvall OTD 60 Ultracentrifuge. The resultant brown pellet attached to the side of the tube represents the cell envelope fraction and was resuspended in 5 ml of phosphate buffer (4°C). Further purification was achieved by taking the envelope preparation, adjusting the volume to 25 ml with phosphate buffer and performing three or four consecutive ultracentrifugation steps at 40,000 rpm for 1.5 h (4°C). Envelope was finally resuspended in 0.5 ml of phosphate buffer and stored at -20°C.

Outer membrane fractions were prepared by taking 50-100 µl of envelope preparation and adding an equal volume of 30% sodium sarkosinate (Sarkosyl™, BDH). This solution was then mixed extensively by passing up and down a fine gauge hypodermic needle a few times followed by vortexing for 30 mins. The volume was adjusted to 1 ml with ice-cold phosphate buffer and centrifuged at 50.000 rpm for 10 mins at 4°C using a Sorval benchtop ultracentrifuge with a swing out rotor. The resultant pellet was resuspended in 1 ml phosphate buffer and re-centrifuged for 10 mins at 50,000 rpm for 10 min. Finally the pellet was resuspended in 30-60 µl and stored at -20°C.

2.8.2 Isolation of Extracellular Vesicles of *P.gingivalis*.

Vesicle preparation was by the method of Grenier and Mayrand (1987). Cells from 400 ml of *P.gingivalis* broth culture were removed by centrifugation at 15,000 g for 20 mins. Ammonium sulphate (192 g) was then added gradually

to the supernatant over 2 h so as to achieve 40% saturation. The resultant precipitate was collected by centrifugation at 20,000 g for 40 mins at room temperature and resuspended in 15 ml 50mM Tris-HCl pH 9.8, 0.5 mM DTT. This was then dialysed (room temperature) for 16 h against 3 l Tris/DTT buffer. Vesicles were then collected by centrifugation at 20,000 g for 40 mins and resuspended in 1-5 ml 50 mM Tris-HCl pH 7.2. Vesicles were stored at -20°C.

2.9 Assays for Enzymic Activity.

2.9.1 Proteolytic Activities.

For direct determination of proteolytic activities of bacterial colonies bacteria were grown aerobically or anaerobically on either LB (*E.coli*) or Bacteroides medium agar (*P.gingivalis*) supplemented with either 1% casein, or 1% skimmed milk. Proteolytic activity was determined by looking for zones of clearing around bacterial colonies or underneath when the colony material was washed off with PBS.

Specific trypsin-like activity was assayed for using the fluorimetric substrate of Kanaoka *et al.* (1977) using an assay developed for this thesis and described by Arnott *et al.* (1990). 500 µl of cell lysate (4°C) was mixed by vortexing with 2,473 µl of ice-cold assay buffer (50 mM Tris-HCl pH 7.2, 1% DMSO, and 20 mM $CaCl_2$) containing L-arginine-4-methylcoumaryl-7-amide (BAMCA; Sigma; 15µl of a 10 mM stock solution per 3 ml reaction) and 12 µl 2.5 M ß-mercaptoethanol. The assay mixture was then incubated at 37°C for 1 h after which any insoluble material was sedimented by centrifugation in an Eppendorf centrifuge for 5 mins. The supernatant was then assayed for fluorescence at 440 nm with excitation at 366 nm using a Perkin Elmer fluorimeter. Trypsin-like activity was compared to a two fold dilution series of 100 µg ml^{-1} bovine pancreatic trypsin diluted in assay buffer and assayed in relative fluoresence units mg^{-1} protein. This assay can detect as little as 10 ng of trypsin if the incubation time is extended to overnight.

For BAMCA substrate zymography PAGE gels were washed briefly in BAMCA assay buffer before being layed onto 3MM Whatman filter paper soaked in assay buffer containing BAMCA and incubated for 5 mins at 37°C.

Enzyme activity was visualised by placing the filter paper and gel directly onto a long wave UV transilluminator.

Glycyl-prolyl dipeptidase activity was assayed using a variation of the method of Abiko *et al* (1985). The assay was essentially the same as that for trypsin-like activity except that the substrate used was L-glycy-L-prolyl-4-methylcoumaryl-7-amide (GlyProMCA). Also a collagenase (*Clostridium histolyticum*) dilution series was used instead of trypsin as the reference enzyme and positive control.

2.9.2 Haemagglutination Tests.

Haemagglutination was assayed using sheep erythrocytes using the method of Deslaurier sand Mouton (1992). Erythrocytes were washed three times by centrifugation at 3,300g for 5 min at 4°C in a Sorval centrifuge (Dupont) resuspending the pellet each time in PBS. Erythrocytes were then resuspended in PBS at a concentration of 0.2% (v/v) and washed a further two times finally resuspending in PBS to give an OD_{600} of 0.5. 50 µl portions of erythrocytes were placed in the wells of a V-bottom microtiter plate. 50 µl of a two-fold dilution series of bacterial cells or cell extract was then added to the erythrocytes followed by incubation for 1 h at 37°C, then overnight at 4°C. Haemagglutination was identified by the formation of a diffuse distribution of erythrocytes as oppose to a red spot in the bottom of the V-shaped well. Either *P.gingivalis* whole cells or vesicles were used as a positive control.

Chapter 3

Molecular Cloning of a *P.gingivalis* Antigenic Determinant in *E.coli*.

3.1 Introduction

Identification of cloned antigenic determinants from pathogenic bacteria using antisera against whole bacterial cells should in principle provide data about cell surface components of the pathogen. Studies of the *P.gingivalis* cell surface have shown that there is a series of different sized proteins associated with the outer membrane (Kennel and Holt, 1990) and that of these, proteins with molecular weights of 55, 47 and 40kDa predominate in their reactions with sera from periodontitis patients (Curtis *et al.*, 1991). Given the prominence in their reaction with patient's sera it is likely that these proteins maybe important in disease and exposed to the cell surface. Molecular cloning of antigenic determinants by certain groups may have already identified some of these cell surface components. For example Joe *et al.* (1993) have reported the cloning of a 51 kDa cell surface antigen, whilst Abiko *et al.* (1990) have reported the cloning of 40 kDa antigen. The analysis of these cell surface components along with the antigens described in this thesis may eventually reveal much about the pathenogenicity of *P.gingivalis* and permit the development of vaccines based on cell surface antigens.

At the time when this project was launched, molecular cloning and expression of genes from *P.gingivalis* was in its infancy. It was thus necessary to develop a strategy for cloning with little or no background knowledge relating to the genetics of *P.gingivalis*. A previous attempt in our laboratory to clone proteases of *P.gingivalis* (Arnott *et al.*, 1990) had resulted in the cloning of a large DNA fragment encoding gene product(s) expressing weak trypsin-like protease (TLP) activity. This activity was not, however expressed stably making further experimentation difficult to reproduce. Similar stability problems had also been encountered by Progulske-Fox *et al.* (1989) in cloning a haemagglutinin gene and Dickinson *et al.* (1988) in cloning the fimbrial subunit gene from *P.gingivalis*. The reasons for such instability are unknown, but are likely to be caused by alterations in cloned DNA at the nucleotide level. The selectional pressures behind such alterations might be expression of toxic gene products or possibly features inherent in cloned DNA, for instance

inverted or homologous repeats which might encourage rearrangements. There is also no guarantee that *P.gingivalis* genes will direct protein expression in *E.coli*. It is possible that transcriptional and translational signals for *P.gingivalis* gene expression may not be recognised in *E.coli*. There may also be gene expression problems due to the presence of codons in *P.gingivalis* coding sequences which are rare in *E.coli* and that can not be accommodated by the *E.coli* translational machinery. The presence of such codons could severely restrict levels of expression of *P.gingivalis* genes in *E.coli* (Hartl *et al.*, 1994), or may cause premature translational termination at points where rare codons occur, thus resulting in the expression of truncated proteins. Other potential problems may arise in detecting the phenotype of *P.gingivalis* proteins expressed in *E.coli*. For example, incorrect post translational processing of expressed proteins could result in the loss of protein function due to improper folding. Likewise proteolytic degradation by *E.coli* proteases could result reduced levels of production of proteins expressed from cloned genes (Gottesman and Maurizi, 1992).

The chosen strategy was in the first instance to clone small *P.gingivalis* DNA fragments into a high copy number plasmid expression vector and to screen them for reactivity with polyclonal antisera raised against *P.gingivalis* whole cells. This method has the advantage of using small DNA fragments which are unlikely to encode whole functioning proteins, thus circumventing any hypothetical toxicity effects associated with protein function. Also the use of expression vectors with built in *E.coli* promoters ensures the transcription of cloned DNA (in the absence of cloned transcriptional terminators). The resultant protein truncations, being small would narrow down the scope for eventual epitope mapping. In addition, screening using polyclonal antiserum should support cloning of a wide variety of *P.gingivalis* antigens, hence widening the scope of the technique. Once an epitope-containing polypeptide has been identified the full length protein gene can then be captured using colony-probe hybridisation against a genomic library specially constructed for screening large, possibly unstable DNA fragments.

3.2 Results

3.2.1 Characterisation of anti-*P.gingivalis* polyclonal antibodies.

The rabbit polyclonal antisera was produced in our laboratory as detailed in the materials and methods using *P.gingivalis* whole cells as the source of antigen. It was found to show strong reactivity with *P.gingivalis* cell lysates and ECV in western blots. This reaction was characterised by a broad range molecular weight smear presumably because antibody reactive components were too numerous for resolution on SDS PAGE (data not shown).

3.2.2 Characterisation of mAb Brick 190.

Brick 190 was produced at Bristol Polytechnic by Dr. John Greenman and is a mouse monoclonal raised against a TLP purified from sonicated *P.gingivalis* ECV using gel permeation chromatography (J. Greenman, personal communication). Ascites of Brick 190 is able to neutralise the native TLP activity of *P.gingivalis* ECV and reacts with a variety of protein molecular weights in western blot experiments using *P.gingivalis* (J. Greenman, personal communication). The material used in this study was mouse monoclonal (IgG_1) hybidoma spinner culture supernatant and was a gift from John Greenman (Bristol Polytechnic).

Several attempts at SDS PAGE western blots using *P.gingivalis* cells and ECV probing with Brick 190 were made without success. However, when *P.gingivalis* cell lysates and ECV were spotted (5 µl) onto nitrocellulose with and without the presence of SDS (0.1%) and hybridised to Brick 190 using the same protocol as for western blot filters, a strong signal was detected in spots from non-SDS treated samples. The most likely explanation for this phenomenon is that mAb Brick 190 recognises a native or discontinuous epitope (Wilson, 1991) the structure of which is destroyed by the denaturing properties of SDS hence the failure of Brick 190 recognise antigen treated with SDS. Therefore western blots using native CHAPS gels were performed in the absence of SDS using *P.gingivalis* cell sonicates, ECV and both envelope and outer membrane preparations. Mobility and focusing of protein bands in the CHAPS PAGE system was first confirmed by running out individual proteins standards. Proteins resolved using the CHAPS system were seen to adopt both monomeric and multimeric forms the latter being consistent with a native conformation for these proteins (figure 3.1a). In addition these subunits also migrated distances roughly proportional to their molecular weights. Molecular weight determination of unknown elements was difficult however,

Figure 3.1a

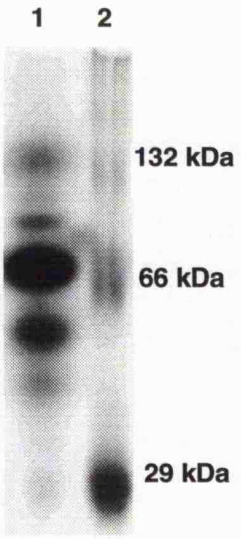


Figure 3.1b

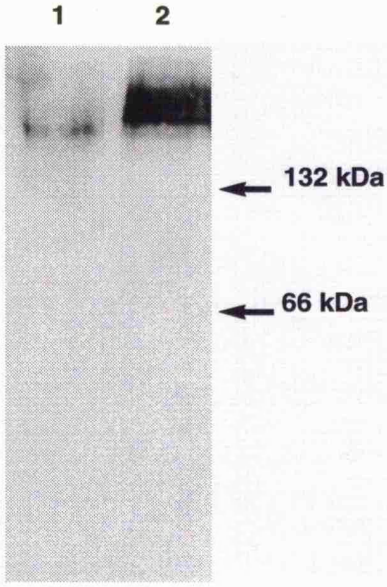


Figure 3.1c

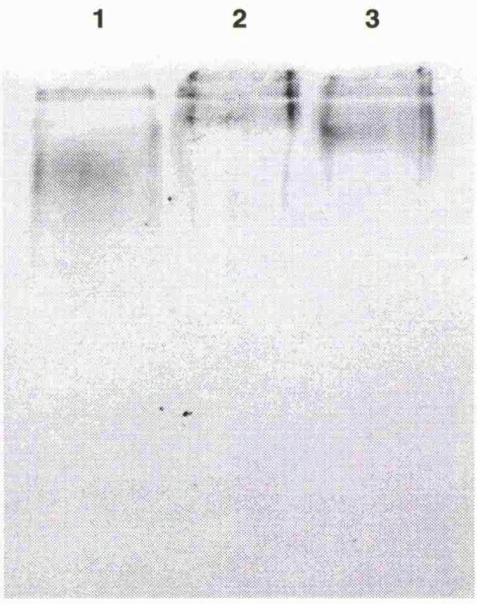


Figure 3.1d

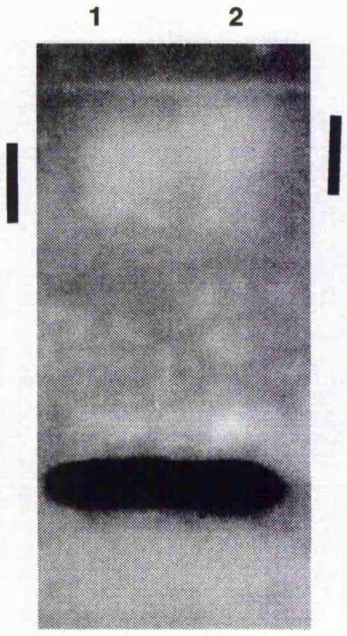


Figure 3.1 (a) Mobility of Proteins in the CHAPS PAGE system. Proteins were stained using coomassie brilliant blue. Shown is Albumen in lane 1 (monomer at 66 kDa molecular weight, dimer at 132 kDa) and carbonic anhydrase in lane 2 (29 kDa). **(b)** CHAPS PAGE western of P.gingivalis ECV (Lane 1) and cell sonicate (Lane 2) probed with mAb Brick 190. Shown are the approximate molecular weights of the albumen monomer and dimer. **(c)** CHAPS PAGE western of *P.gingivalis* whole cells, envelope and outer membrane fractions probed with mAb Brick 190 (Lanes 1, 2 and 3 respectively). **(d)** BAMCA substrate zymograph of *P.gingivalis* ECV and cell sonicates. The bar highlights the zone of fluorescence resulting from substrate cleavage. The intense dark band represents the bromophenol blue dye front.

in that standard protein molecular weight markers consist of several proteins which in the CHAPS system where multimerisation occurs, form confusing banding patterns. A crude method for molecular weight estimation in CHAPS western can be performed by taking a single protein which forms dimers (figure 3.1a) and to run it on the same gel as that used for western blotting. This track can then isolated, stained and compared along side with the western blot; the main problem is gel shrinkage during fixing which creates inaccuracy. Crude lysates of *E.coli* were also analysed on CHAPS PAGE in order to assess whether or not more complex samples could be resolved using this technique. *E.coli* cell sonicates also formed banding patterns in CHAPS PAGE confirming that the technique could be used to separate total *E.coli* protein (data not shown).

All *P.gingivalis* subcellular fractions tested including the outer membrane fraction reacted with Brick 190 in CHAPS PAGE western blots (figure 3.1b and c) showing a high molecular weight, low mobility response. This signal was characterised mostly by a smear, although band focusing could be seen in ECV (figure 3.1b).

The reaction of Brick 190 with the outer membrane fraction suggests that the Brick 190 antigen may be an outer membrane component or at least have some affinity for that subcellular compartment as well the others tested. The low mobility smearing in all samples suggests either an extremely high molecular weight for the Brick 190 antigen or a complexed composition which may contain non-protein components with aberrant electrophoretic properties in the CHAPS buffer system. A study of TLP activity using BAMCA substrate zymography of cell sonicates and ECV in CHAPS gels (figure 3.1c) revealed a fluorescent substrate utilisation pattern and electrophoretic mobility similar to that seen for the Brick 190 as described above (this activity verifies the non-denaturing qualities of the CHAPS gel system).

Given the similarity in high molecular weight banding patterns of both trypsin-like activity and Brick 190 antigen in CHAPS gels, and the association of both these characteristic with ECV and the cell surface (Roberts *et al.*, 1990), it is tempting to speculate that Brick 190 may indeed be specific for the TLP of *P.gingivalis* as suggested by Greenman. To further investigate this an attempt was made to confirm that Brick 190 is able to neutralise TLP activity. The

lower limit of detectable TLP activity from ECV and bovine pancreatic trypsin (Sigma) was determined using the BAMCA assay. The minimum detectable amount of trypsin per reaction was found to be 10 ng, whilst as little as 72 ng ECV could be detected in the presence of β–mercaptoethanol (β–mercaptoethanol was found to increase the TLP activity of ECV by a factor of 8 using this assay). Reactions were set up using the lowest amount of ECV and trypsin possible to give a repeatable BAMCA cleavage within the existing parameters. These reactions were set up in the absence of ß-mercaptoethanol to prevent damage to the mAb due to the breakage of sulfurhydryl bonds responsible for heavy and light chain bond integrity. In addition PBS which had performed well as a reaction buffer in a pilot study was used (data not shown). Dilutions of Brick 190 or a control antibody (anti-*Erwinia amylovora* LPS mAb) in PBS set to the same protein concentrations (25 mg ml^{-1} stock) were added to give a final volume of 1 ml and the reactions allowed to proceed. BAMCA cleavage by trypsin or ECV TLP was not inhibited by increasing titre of Brick 190, moreover proteolytic activity rose with increasing Brick 190 titre regardless of the presence of trypsin or ECV (data not shown). This increase in BAMCA-specific activity was not evident in the control antibody and could not be removed by capryllic acid precipitation of Brick 190. An explanation might be that this mAb contains a contaminant component that fluoresces at 440 nm hence giving false positive BAMCA breakdown.

3.2.3 Characterisation of mAb LDS28.

A thorough analysis of LDS28 was carried out in our laboratory by Andrew Wallace (Wallace *et al.*, 1992). LDS28 is specific for a *P.gingivalis* protein (43 kDa) present in cell lysates, and envelope, ECV and outer membrane fractions and reacts with gingivain, a preparation of TLP (Roberts *et al.*, 1990; Shah *et al.*, 1990). However the reaction of LDS28 against ECV was not against a single band but a complex ladder of molecular weights less than and exceeding the single 43 kDa band seen in other *P.gingivalis* preparations tested. This encouraged debate as to the presence of the 43 kDa band in a complexed format within the ECV. This antibody when immunogold-labelled also reacts with a cell surface exposed component of *P.gingivalis*. Attempts to use LDS28 to inhibit TLP or haemagglutinating activity of *P.gingivalis* ECV were largely unsuccessful (Wallace, A., unpublished results). Analysis of the

LDS28 reactive antigen for this thesis using different molecular weight marker preparations (Sigma biotinylated standards and Biorad prestained molecular weight markers) and linear regression has since suggested that the antigen's molecular weight is closer to 46 kDa than 43 kDa.

3.2.4 Generation of a *P.gingivalis* W83 Genomic Library in pTZ18R.

In order to ensure that the genomic DNA library would consist of a high proportion of recombinant molecules vector DNA was cleaved with *BamHI* and the 5` terminal phosphates removed using CIP. Vector prepared in this way is unable to self ligate and this ensures that the majority of transformants from ligations of vector and insert are the result of the generation of recombinant molecules. The cloning vector pTZ18R (figure 3.2) was chosen for several reasons. First, cloning into the *BamHI* allows insertional inactivation of the *lacZ'* cassette. This results in colonies unable to breakdown X-gal in *E.coli* strains harbouring the *lacZΔM15* mutation. Thus recombinant colonies are seen as white and non-recombinants as blue. Secondly, the ability to stimulate transcription from the *lacZ`* promoter by adding IPTG should also allow transcription of the cloned insert, a useful and controllable feature in the absence of a functioning cloned promoter. Thirdly, the pTZ series of vectors also carry a f1 origin of replication which allows production of single-stranded DNA templates *in vivo* in the presence of a helper phage. This template can be used for DNA sequencing insert DNA.

A preliminary experiment had shown that *E.coli* strain TG2(pTZ18R) when plated out onto X-gal produces slightly blue colonies in the absence of IPTG. This implies possible leaky expression of the *lacZ'* promoter. In order to eliminate this all media was supplemented with glucose (0.4% w/v). The result of this is that TG2(pTZ18R) grown on X-gal in the absence of IPTG no longer produces any blue colour in its colonies even after several days growth. This effect relies on the presence of a Crp (catabolite repression protein) binding site in the *lacZ`* promoter which is occupied by the Crp protein (Pastan and Adhya, 1976). In the presence of limiting glucose (or more accurately a subsequent excess of cAMP) enhanced Crp protein binding

Figure 3.2 Circular restriction map of pTZ18R

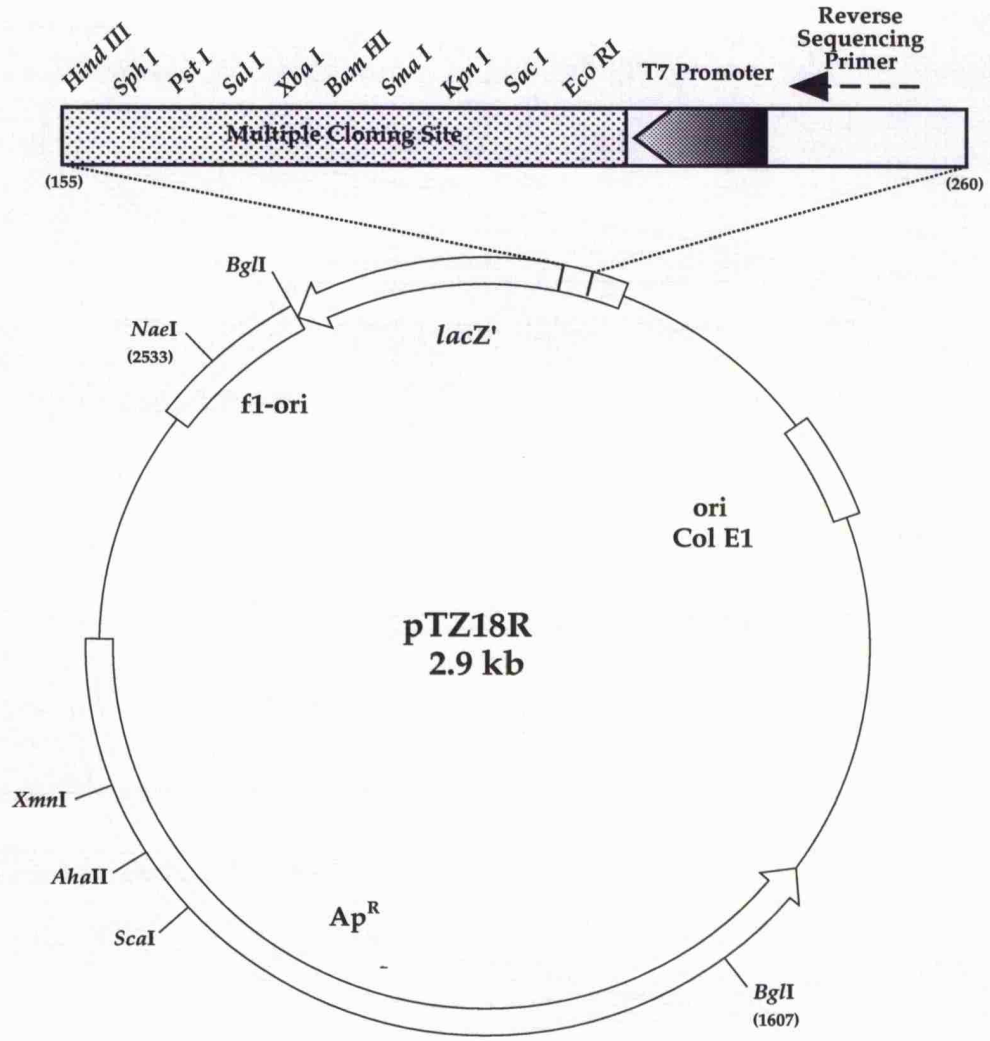


Figure 3.2 Circular restriction map of pTZ18R. Shown are restriction sites and antibiotic resistance genes. The multiple cloning site (MCS) is highlighted and expanded along with the position of the T7 promoter. Numbers in brackets denote relative coordinates of chosen restriction sites in bp.

stimulates transcription from the *lacZ*` promoter by interacting with RNA polymerase. The presence of excess glucose eliminates this effect by reducing cAMP availability, which in turn reduces Crp binding and enhancement of transcription. Hence the inclusion of glucose should tighten experimental regulation of protein expression and hopefully guard against leaky expression of any potentially toxic gene products.

Chromosomal DNA from *P.gingivalis* W83 was partially cleaved with the restriction endonuclease *Sau*3a to generate restriction fragments in the 0.1 to >12 kb range. This DNA was then fractionated according to size using sucrose density centrifugation and the resultant fractions analysed by agarose gel electrophoresis (figure 3.3a). Fraction 13 showed a DNA fragment class size of around 0.1 to 3 kb and was chosen for library construction. Test ligations were performed using 0.5, 1 and 2 μg of vector DNA (figure 3.3b, lanes 1-3 respectively) with approximately 0.5 μg of size selected chromosomal DNA each and analysed by agarose gel electrophoresis. Test ligations were then electrotransformed into *E.coli* TG2 and the efficiency of recombination calculated by determining the blue/white ratio of transformants plated out onto X-gal and IPTG. Ligation 3 showed an optimal blue/white ratio (a ratio of approximately 1:2) and transformation efficiency ($1.8x10^3$ cfu per μl ligation) and was chosen for screening. Ligations using 0.5 and 1 μg of vector DNA gave $1.8x10^2$ and $4.2x10^2$ cfu per μl respectively and blue/white ratios of 1:1.4 and 1:1.2. Twelve small scale DNA preparations were made from ligation 3 recombinants and cleaved with restriction endonuclease *Eco*RI in order to estimate the range of insert sizes produced by ligation 3. This was found to be between approximately 0.2 and 1.5 kb. The mean insert size was for ligation 3 was around 0.6 kb.

3.2.5 Screening the Library by Colony Immunoblotting.

Transformation plates were screened by colony immunoblotting with a rabbit polyclonal (M. Arnott and I.S. Roberts, unpublished data). Antisera was prepared by subcutaneous injection of rabbits with *P.gingivalis* (W83) whole cells in Freund's complete adjuvant with boosts in Freund's incomplete adjuvant. Screening was performed in the presence of IPTG without X-gal as described in section 2.7.5. Approximately 15,000 transformants from ligation 3 were screened. From these a total of five clones reactive with the anti-

Figure 3.3a Fractions of Size Selected Chromosomal DNA

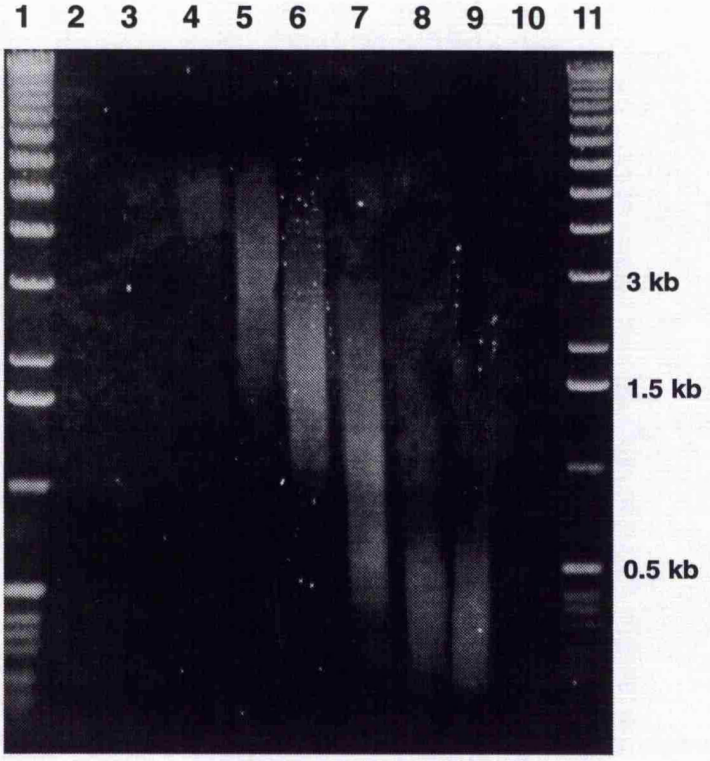


Figure 3.3b Ligation of Chromosomal DNA into pTZ18R.

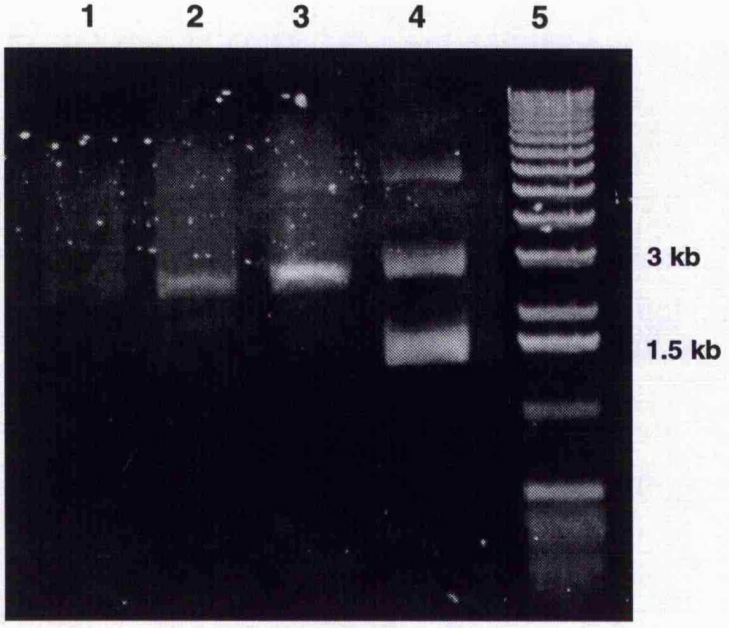


Figure 3.3 (a) Fractions of Size Selected Chromosomal DNA. *P.gingivalis* (W83) chromosomal DNA was partially restricted with *Sau*3a and size selected using sucrose density gradient centrifugation. Fractions were analysed using agarose gel electrophoresis. Lanes 1 and 11 contain DNA kb markers(BRL). Lane 4 contains fraction 10, lane five fraction 11 and so on. Numbering down the side denotes DNA molecular weight in kb. Fraction 13 (lane 7) was used in ligations with pTZ18R. (b) Ligation of Chromosomal DNA into pTZ18R. Numbering down the side denotes DNA molecular weight in kb (BRL). Lanes 1-3 containDNA ligations with different vector concentrations (see text for details). Lane 4 contains pTZ18R vector only.

P.gingivalis antisera were identified. Initially these were designated as clones A to E. Clones were then isolated by cross-referencing the colony immunoblot filters with the master plates lacking IPTG (the omission of IPTG from the master plates ensures restricted expression of cloned genes which might be deleterious to the host cell when over produced from the *lac*Z' promoter). Sub-cultures and cell suspensions in 20% glycerol for cryopreservation were made for each clone. Wherever possible subcultures were taken from the master plates so as to avoid propagation of possible mutant colonies.

3.2.6 Analysis of clones reactive with anti-*P.gingivalis* Polyclonal Antisera.

All five clones gave white colonies when subcultured on X-gal/IPTG and hence were likely to carry recombinant plasmids. Confirmation that all five clones harboured recombinant plasmid DNA was achieved by restriction enzyme digestion of small scale plasmid DNA extractions using *Eco*RI which cleaves pTZ18R once in the MCS (multiple cloning site; restricted DNA from clone D is shown in figure 3.4a). Insert sizes varied between around 0.2 to 1.5 kb. Clones were both toothpicked and streaked onto agar plates for single colonies and immunoblotted using rabbit anti-*P.gingivalis* polyclonal antisera. Clones A, B, C and E each gave some positive and some negative colonies. This suggested that there maybe a stability problem with A, B, C, and E or that they consisted of mixed populations of bacteria. To test this clones were restreaked for single colonies and reprobed with the antisera. Again clones A, B, C and E each gave some positive and some negative colonies suggesting that these clones were unstable. Only clone D gave consistently positive colonies, although this signal was fairly weak (figure 3.4b). This positive signal was not enhanced by the inclusion of IPTG in the immunoblot protocol and hence expression is probably due either to some promoter activity intrinsic for the cloned DNA or spurious promoter activity from the vector. Clone D was chosen for further analysis and was designated pGPR1.

3.2.7 Sequence analysis of pGPR1 insert DNA and Southern Hybridisation with *P.gingivalis* (W83) Chromosomal DNA.

Due to the small size of the pGPR1 insert (around 0.3 kb; see figure 3.4a) and the difficulties in analysing very small DNA fragments using agarose gel

Figure 3.4a Restriction Fragment of Clone D.

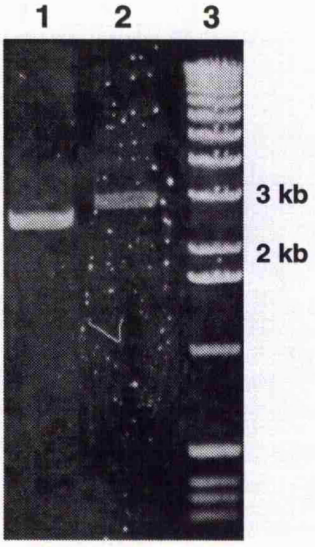


Figure 3.4b Clone D immunoblotted with Rabbit Polyclonal Antiserum.

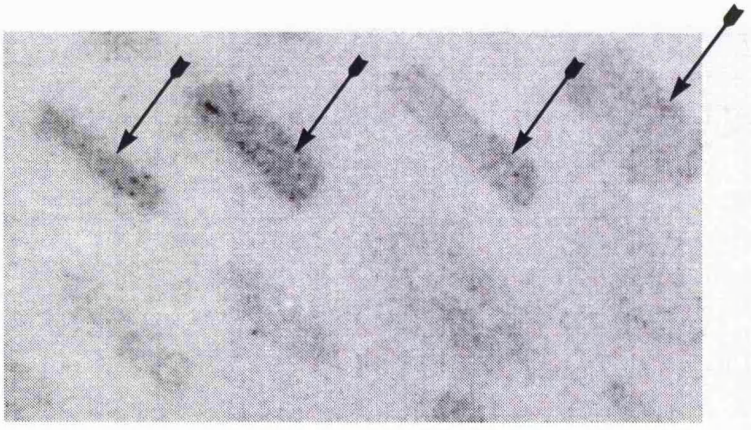


Figure 3.4 **(a)** Restriction Fragment of Clone D. Lanes 1 and 2 contain EcoRI-digestedDNA from pTZ18R and Clone D respectivley. Lane 3 contains DNA kb molecular weight markers. Molecular weights are shown down the side in kb. The shift in molecular weight seen in clone D in comparison to the vector pTZ18R is due to the presence of a roughly 0.3 kb insert. **(b)** Clone D immunoblotted with rabbit polyclonal antiserum. Colonies were toothpicked onto solid media and imprints taken onto nitrocellulose filters which were subsequently immunoblotted with anti-*P.gingivalis* polyclonal antiserum. Clone D is shown by arrows (top line). TG2(pTZ18R) are shown underneath for comparison.

electrophoresis the nucleotide sequence of pGPR1 was determined directly. The small size of the insert in pGPR1 meant that the entire nucleotide sequence could be determined on a single sequencing gel.

The DNA sequence of pGPR1 insert was found to be 307 bp in length. Its sequence and restriction map are given in figure 3.5a and the fragments orientation with respect to the vector is shown in figure 3.5b. No significant DNA homology was detected when the insert sequence was compared to DNA sequences within the composite Genbank/EMBL database. Reading frame analysis showed a number of small ORFs in all six phases and one large one whose co-ordinates exceed the range of the insert. This latter ORF translated in an orientation opposing the direction of *lac* Z′ expression. None of these ORFs shared any significant homologies with members of the composite OWL protein database. Due to the small size of this sequence lack of significant DNA or protein sequence homology was not unexpected in that weak sequence correlations often only become significant over greater distances (at least 100 amino acids; R.F. Doolittle, 1987). However the lack of identical or near identical DNA sequences in the database does suggest that this DNA has never before been sequenced and encodes a previously uncharacterised gene.

The restriction map of pGPR1 reveals that the entire insert can be excised from the vector on a single *Sal*I-*Eco*RI fragment with only very slight vector contamination (figure 3.5b). This fragment was radiolabelled and used to probe a Southern blot of *P.gingivalis* (W83) chromosomal DNA cleaved with different restriction enzymes (figure 3.6). In most cases the probe reacts with a different single restriction fragment of chromosomal DNA for each enzyme (the exception being *Hind*III which gives two fragments possibly as the result of a partial digestion). This suggests that the gene in question exists on the *P.gingivalis* chromosome as a single copy and that the insert is not the result of a ligation between two unrelated *Sau*3a restriction fragments *in vitro.* Interestingly, although the insert has a *Hinc* II site it only hybridises with a single *Hinc*II fragment on the chromosome. One possible explanation for this is that there is insufficient homology to hybidise due to the small size of the left hand *Hinc*II-*Sal*I.

Figure 3.5 (a)

```
     Sau3a  10                  30     HincII/Sau3a                 70                  90
  1  GATCATATGAGGAAAACGGTCAAAATCCAATCGAGTTAACCGATCAAATCCTGACCAAAAGGAATACACTGGGGAGAGCACTGACGACGACATTCCAAGA   100

         110                 130                 150                 170                 190
101  CCATCAGGACTATACCAAAGGGCTACTAGAGGGGTATGCAACAGATGAGGGGAGGTACAGGCCATTGCCCGTATTTGTATTCTATTCGGTAGAAGACATT   200

         210                 230                 250                 270                 290
201  CACACCAAACGCAAGGTGAGCCATTCACTATTCCAGAAATACCGACACATGGCCAGTTTCGGTTATTATCATGCGCTTACCGAGAATGCTTTCTTGGACT   300

       Sau3a
301  TTTGGATC  307
```

Figure 3.5 (b)

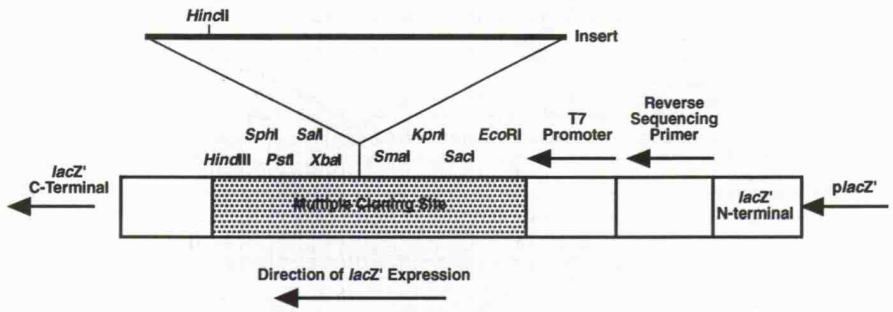


Figure 3.5 (a) Nucleotide sequence of pGPR1 insert showing *Sau*3a and *Hinc*II restriction endonuclease cleavage sites. Recognition sites are shown underlined. Terminal *Sau*3a sites shown are those spliced into the *Bam*HI site in the MCS of pTZ18R. **(b)** Orientation of pGPR1 insert with respect to *lacZ'*. Cloning of *Sau*3a ends into the MCS *Bam*HI resulted in the degeneration of this restriction site hence *Bam*HI is not shown. For a more detailed restriction map of pTZ18R see figure 3.1.

Figure 3.6a

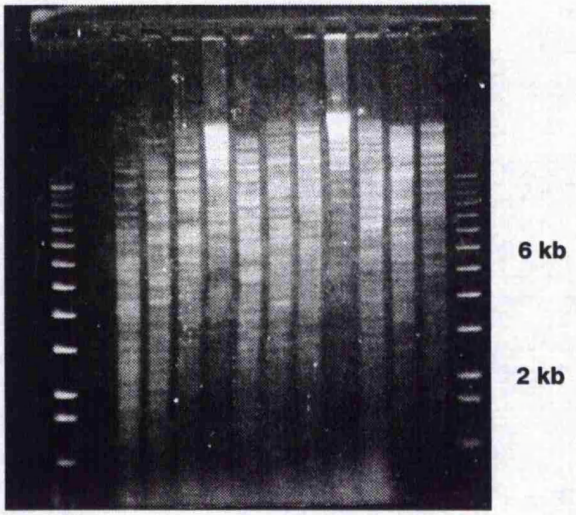


Figure 3.6b

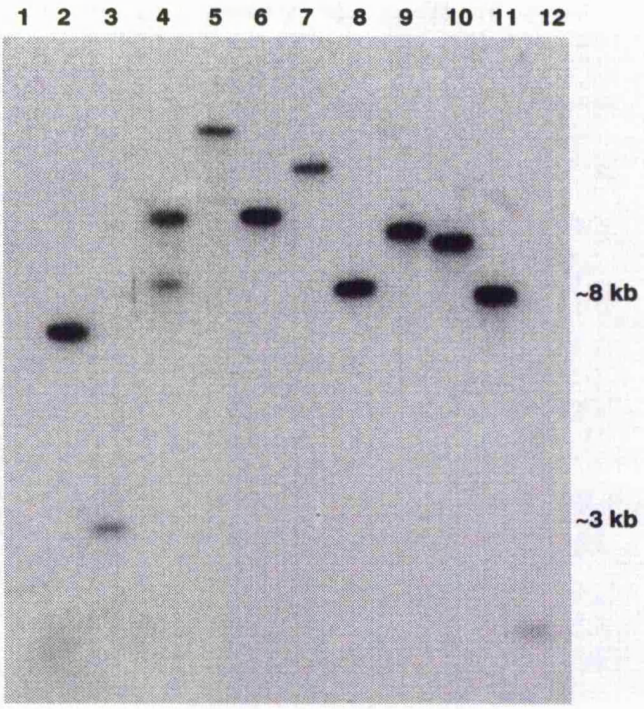


Figure 3.6 c. Southern blot analysis of *P.gingivalis* (W83) chromosomal DNA.

Lane	Restriction Enzyme	Fragment Size(s) Reacting with pGPR1 Probe.
1 (Empty)	-	-
2	*Cla* I	~6 kb
3	*Acc* I	~3 kb
4	*Hind* III	>12+~7.5 kb
5	*Xho* I	>12 kb
6	*Pst* I	~11 kb
7	*Bam* HI	>12 kb
8	*Eco* RI	~7.5 kb
9	*Sal* I	~11 kb
10	*Bgl* II	~11 kb
11	*Sma* I	~8 kb
12	*Hinc* II	~0.8 kb

Figure 3.6 (a) Agarose gel electrophoresis of restriction endonuclease-cleaved W83 chromosomal DNA. **(b)** The Southern blot of the same gel probed with radiolabelled pGPR1 insert. The probe was a 0.3 kb *Sal*I-*Eco*RI fragment of pGPR1 purified by excision from and LMP agarose gel and labelled with ^{32}P α-dCTP (see section 2.4.3). **(c)** Shows restriction enzymes used and the approximate molecular weight of hybridising fragments.

3.2.8 Characterisation of TG2(pGPR1) using monoclonal antibodies.

In order to further characterise the nature of the antigen expressed by TG2(pGPR1) antibodies described in the previous section were used as probes for immunoblotting. Subsequently toothpicked colonies of, and protein lysates of TG2(pGPR1) were subjected to immunoblot and western blot analysis.

Immunoblots of TG2(pGPR1) revealed that not only does pGPR1 react with the anti-*P.gingivalis* rabbit polyclonal antibody (figure 3.4b) but also strongly reacts with mAb Brick 190 (figure 3.7). Attempts at western blot analysis, however were unsuccessful in that no antibody-reactive bands could detected with either antibody using lysates of TG2(pGPR1). Lysates of TG2(pGPR1) also showed no signal with Brick 190 using CHAPS western blots. It is likely that the *P.gingivalis* antigen fragment expressed from this plasmid (estimated around 10 kDa assuming a maximum coding capacity for the 307 bp insert) maybe too small to be resolved using normal SDS PAGE and hence does not show using the standard western blot protocol.

3.2.9 Assays for pGPR1 Biological Activity.

Crude lysates of TG2(pGPR1) and TG2(pTZ18R) were prepared and assayed for Glycyl-Prolyl, TLP and haemagglutination activities. Using standard assay parameters pGPR1 tested negative for all of the above activities per mg protein when compared to the pTZ18R control (data not shown).

3.3 Discussion.

The use of antibodies for screening for antigens expressed in genomic libraries is a versatile technique whose success depends largely on the quality of antibodies used in screening. The utilisation of polyclonal antibodies in such a screening gives the advantage of looking for a large number of antigenic characteristics ranging from carbohydrate moieties such as LPS and capsular components, to proteins and peptides. Such antibodies maybe expected to react with proteins of all molecular weights depending on their immunogenicity. The use of whole *P.gingivalis* cells as antigen during the generation of polyclonal antisera should have generated predominantly cell

Figure 3.7 Immunoblot of TG2(pGPR1) with Brick 190.

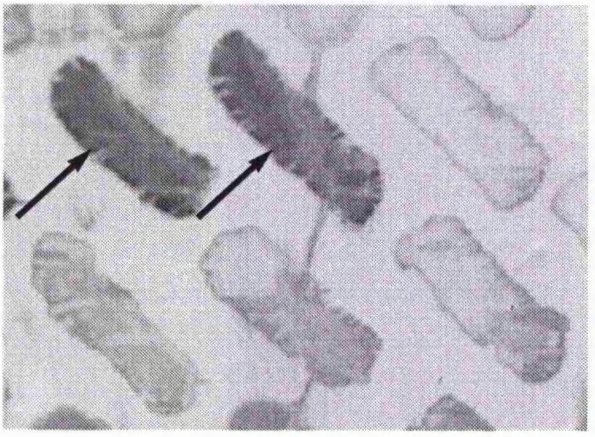


Figure 3.7 Immunoblot of TG2(pGPR1) with Brick 190. Colonies were toothpicked onto solid media and imprints talen onto nitrocellulose filters which were then immunoblotted with mAb Brick 190. TG2(pGPR1) is highlighted using arrows. TG2(pTZ18R) was also blotted for comparison and shows a faint reaction with Brick 190.

surface-specific antibodies, thus improving the chance of identifying virulence determinants and other components common to the cells surface. Previous studies have shown that anti-*P.gingivalis* antibodies specific for the cell surface also show a high degree of species specificity and hence genes cloned using such antisera maybe useful as diagnostic probes (Reed *et al.*, 1980; Chen *et al.*, 1986; Bowden and Nolette, 1990). Indeed cell surface components strongly immunogenic in the host have been demonstrated by analysis of patient and healthy subject antiserum and patterns of disease-related antigen expression established (Laorisin *et al.*, 1990; Papaioannou *et al.*, 1991; Curtis *et al.*, 1991).

Attempts at verifying the activity of Brick 190 to neutralise TLP activity were largely unsuccessful. The possible reason for this is that the original experiments were carried out using ascites which generally is of extremely high antibody titre, whereas the only available Brick 190 used in this thesis was from tissue culture supernatants. This material is of low titre and as mentioned possessed some intrinsic properties which made the TLP activity neutralisation experiment impossible. What is more this antibody was available only small quantities which, from batch to batch varied in their signal intensity. Despite reports of Brick 190 neutralising TLP, being raised against purified protease and reacting to a zone in CHAPS western blots similar to that seen in BAMCA substrate zymography, caution should exercised in assuming that this mAb is specific for the TLP of *P.gingivalis*. A second mAb, Brick 192 also raised against purified protease was found to react with a ladder in *E.coli*, a pattern characteristic of LPS (data not shown) and hence it is possible that the source antigen used to make mAb was not homogeneous. It is also possible that the TLP exists as a multicomponent complex and that other proteins of such a complex may co-purify with the TLP activity thus contaminating antigen preparations.

Initial library screening identified five clones only one of which, pGPR1 stably expressed an antigen reactive with *P.gingivalis* antiserum. This antigen was expressed from a 0.3 kb DNA fragment either under its own promoter activity, or from spurious promoter activity from the vector. One read-through ORF (approximately 10 kDa) predicted to be translated in the opposite orientation to that of *lacZ'* was detected, although this ORF showed no homology to any other known amino acid sequence. Presumably transcription of this ORF would be directed by the insert itself or by vector-

derived transcriptional activity firing against the direction of *lac*Z'. Vector-derived promoter activity of this kind is evident in some cloning vectors (C. Pazzani personal communication). The pGPR1 insert DNA occurs on the chromosome as a single gene copy of *P.gingivalis*, hence the use of colony blot-probe hybridisation of a *P.gingivalis* chromosomal DNA library in order to isolate the sequences surrounding the cloned insert should not be complicated by the risk of cloning multiple alleles.

Clone pGPR1 was shown react with the mAb Brick 190. This should prove to be an advantage in the characterisation of the pGPR1 host gene product in that mAb can be used as probes for protein expression, cellular localisation and for bacterial identification. Preliminary experiments using Brick 190 have demonstrated the antibody reacts with the *P.gingivalis* envelope, OMV and outer membrane fractions and that the epitope for this reaction encoded by pGPR1 is of the native or discontinuous type (structural conformation of this epitope is vital to its recognition by the mAb). This of course has the disadvantage that this antibody can not be used in SDS PAGE western blots, and hence the molecular weight of the Brick 190-specific protein could not be determined.

In order to answer these questions the next logical step was to clone a larger gene fragment encompassing the whole gene for the pGPR1 insert. DNA sequence analysis and other molecular genetic techniques could then be employed to assess the molecular weight of the whole pGPR1 gene, to speculate as to its function, expression and possible export to the bacterial cell surface.

Chapter 4

Isolation of DNA flanking the pGPR1 antigen-coding region from the Chromosome of *P.gingivalis* (W83).

4.1 Introduction.

The initial cloning and analysis of an epitope-coding gene fragment, may reveal something about the mature protein from *P.gingivalis*. However its main use is that of a probe can be of far more use by using that DNA fragment as a probe in the screening of a *P.gingivalis* (W83) genomic DNA library. First, this will result in cloning of the entire gene. Secondly it will allow cloning of neighbouring genes which may be part of some polycistronic operon. In numerous genetic system genes occur in functional clusters so that gene expression can be co-ordinated, and recombination of their heritable traits can occur *en bloc*. In bacteria polycistronic gene transcription is ubiquitous and facilitates the co-ordinated expression of several genes simultaneously. Where genes are clustered their respective proteins maybe involved interlinked processes in complex pathway such as those seen in metabolism and biosynthesis. This is also true of some virulence genes, for example the haemolysin gene of *E.coli* which occurs upstream of several genes responsible for the haemolysin protein export (Koronakis *et al.*, 1988. Thus cloning of DNA upstream and downstream of the *P.gingivalis* protein antigen gene may eventually give important insight into protein export and other virulence determinants that may reside within a cluster. Indeed four putative virulence genes, *prt*T, *prt*C, *sod*, and a haemagglutinin from *P.gingivalis* have all been cloned on the same DNA fragment (Madden *et al.*, 1993)

Once a suitable clone has been generated information about the number of genes present and their function can be deduced using restriction mapping and subsequent DNA sequence analysis. This information promotes accurate subcloning for subsequent protein over-expression experiments, generation of constructs for creation of isogenic mutants, promoter studies and protein engineering experiments. As mentioned the problem with generating such a clone maybe arise due to instability. For example cloning of *P.gingivalis*

protein antigens by Abiko *et al.*, (1990) and Joe *et al.*, (1993) resulted in production of massive amounts of cloned protein when expressed in *E.coli* from intrinsic *P.gingivalis* promoters, in far greater amounts than any protein observed in native *P.gingivalis* cells. Such over-expression would inevitably produce stress on *E.coli* cells producing these proteins and may encourage selectional pressures in favour of non-expressing mutants. In order to try to eliminate such problems a low copy number vector pLG339 was used to construct a genomic library. The advantage of a low copy number is a reduction of the gene dosage effect seen in high copy number plasmids and a subsequent reduction in expression levels of any potentially cytotoxic protein.

4.2 Results

4.2.1 Generation of a *P.gingivalis* W83 Genomic Library in pLG339.

The plasmid pLG339 (figure 4.1) although useful for its low copy number, does not have a MCS, a *lacZ'* used in screening for insertional inactivation using X-gal as a substrate, and does not give high yields of DNA from plasmid preparations. It contains a single *Bam*HI site suitable for cloning *Sau*3a fragments into the tetracycline resistance gene. By selecting for the plasmid using media supplemented with kanamycin, recombinants can be identified by screening for tetracycline sensitivity due to insertional inactivation of the tetracycline resistance gene. *E.coli* strain TG2 was chosen as a recipient for its *rec*A genotype which is likely to increase the stability of any recombinant molecules (Maniatis *et al.*, 1983). Unfortunately the *rec*A mutation in TG2 is a Tn10 insertion which renders TG2 resistant to tetracycline. As such, insertional inactivation of the tetracycline resistance gene in pLG339 could not be used as a screen for recombinant clones.

First pLG339 vector DNA was cleaved with *Bam*HI and phosphatased using CIP in order to prevent self ligation. This was then ligated to *Sau*3a-cleaved *P.gingivalis* (W83) chromosomal DNA fraction number 11 (see figure 3.3a). This chromosomal DNA fraction has a restriction fragment class size of around 1.5-7 kb and should be ideal for cloning large inserts. Ligations (20 µl) using approximately 2.5 µg of vector DNA and 0.5 µg of size selected chromosomal DNA were then transformed into TG2 and plated out onto kanamycin and tetracycline. Approximately 800 cfu per µl of ligation was

Figure 4.1 Circular map of vector pLG339.

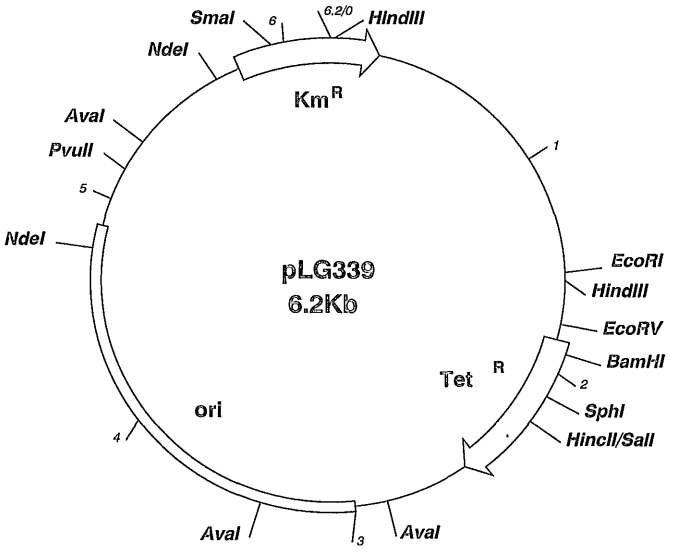


Figure 4.1 Circular map of vector pLG339. Antibiotic resistance genes are shown boxed with arrows denoting the direction of transcription. The origin of replication is simply shown boxed. Numbers shown in italics represent coordinates in kb. Restriction sites are as shown.

recovered. The frequency of recombination was determined by restriction enzyme analysis of small scale DNA preparations from 24 colonies. Around 60 % of transformants harboured recombinant molecules with inserts mostly exceeding 3 kb in length. This size of insert has coding capacity for a single protein of around 100 kDa and should be large enough to clone most prokaryotic genes.

4.2.2 Screening the Library using a Radiolabelled Probe.

Library transformants were individually toothpicked onto fresh agar plates (100 per plate), allowed to grow at 37°C overnight and then imprinted onto nitrocellulose filter as described in section 2.4.2. These filters were then hybridised with a radiolabelled *SalI-Eco*RI fragment of pGPR1. Of the 3000 recombinants screened one clone reacted strongly with the probe (figure 4.2a). This was designated pGPR2. Small scale DNA preparations of pGPR2 yielded single fragments of approximately 5 kb and 6 kb when cleaved with restriction enzyme *Eco*RI (figure 4.2b). Hence pGPR2 has an insert of approximately 5 kb. However when several minipreps were made from separate single colony inoculations, one of these minipreps showed variation in its restriction profile when cut with *Eco*RI (figure 4.2b). This variant was roughly vector size (6.2 kb). However when cut simultaneously with *NdeI* and *Eco*RI this variant gave a single 6 kb fragment (data not shown) and not predicted the 1.0 kb, 1.7 kb and 3.5 kb fragments expected from the pLG339 vector (figure 4.2b). One possibility is that this variant is a deletion-derivative of pGPR2 and hence caution should be exercised when using this clone. A large scale DNA preparation was made using inoculum from the original pGPR2 isolate and checked with restriction enzymes *Eco*RI, *NdeI*, *Eco*RV and *Bgl*II by cross referencing back to original miniprep DNA.

4.2.3 Restriction Mapping pGPR2 and its Hybridisation with Chromosomal DNA from Different Species.

In order to pinpoint the identity between the pGPR1 insert and the larger pGPR2 it was first necessary to create a detailed restriction map of pGPR2. This was done by performing double digests using a restriction enzyme that cuts pGPR2 once only in the pLG339 vector region (*Eco*RV) and an

Figure 4.2 a Isolation of Clone pGPR2 by Colony Hybridisation.

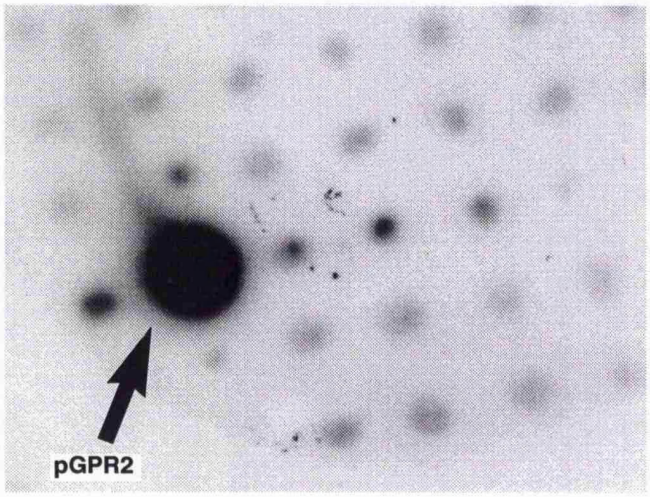


Figure 4.2 b Small scale DNA preparations of pGPR2.

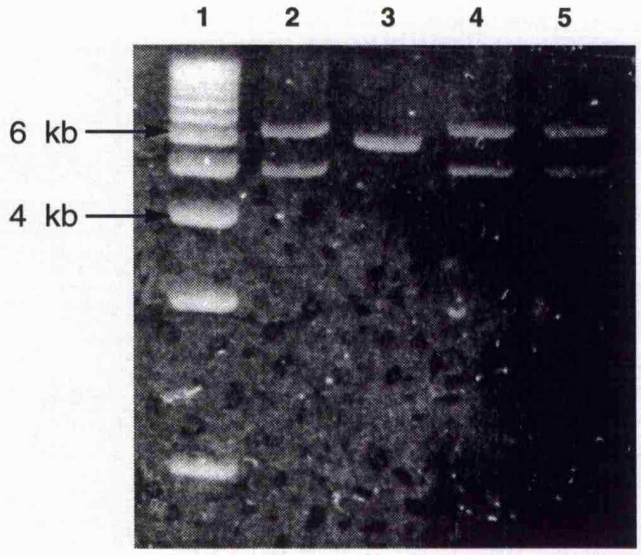


Figure 4.2 (a) Isolation of Clone pGPR2 by Colony Hybridisation. A *P.gingivalis* (W83) genomic DNA library generated in plasmid vector pLG339 was probed with a radiolabelled DNA fragment derived from the cloned insert of pGPR1. The clone pGPR2 was isolated and is shown hybridising with the probe (see arrow). (b) Small scale DNA preparations of pGPR2. DNA from colonies harbouring pGPR2 were cleaved with *Eco*RI and analysed by agarose gel electrophoresis (lanes 2 to 5). One of these minipreps showed an abberant restriction fragment profile (Lane 3). DNA molecular weight standards are shown in lane 1.

enzyme that cuts in the insert. Cleavage with the unique *Eco*RV site in the vector region reveals the relative position of the second enzyme site, thus allowing it to be mapped. The restriction map was subsequently confirmed using multiple restriction enzyme digests and is shown in figure 4.3. The pGPR1 insert contains a single *Hinc*II site and hence must map to one of the *Hinc*II sites in pGPR2.

Using the *Eco*RI-*Sal*I fragment of pGPR1 as a probe, Southern blots against single and multiple digests of pGPR2 were made (figure 4.4). The fragments reacting with the probe are highlighted in figure 4.5. The probe was seen to react with a 0.8 kb *Hinc*II fragment and this is consistent with the same probe reacting with a 0.8 kb fragment in Southern blots of *Hinc*II-cleaved chromosomal DNA of *P.gingivalis* (W83; see section 3.2.7). The reaction of the probe with the 1.2 kB *Xho*I-*Bgl*I fragment of pGPR2 and a 0.8 kb *Hinc*II fragment suggests that the pGPR1 insert maps to one of the three *Hinc*II sites between the *Xho*I and *Bgl*I sites. The reduction in size of the probe reactive fragment from 0.8 kb to 0.5 kb when *Hinc*II fragments are further digested with *Cla*I suggests that the pGPR1 insert reacts with the left hand of the two 0.8 kb *Hinc*II fragments (the *Cla*I restriction enzyme cuts several times to the left of the *Bgl*I site in the pGPR2 cloned fragment but due to the scarcity of sites in this region could not be mapped accurately and hence only the single *Cla*I site is shown on figure 4.5). Hence the mapping of the pGPR1 insert on pGPR2 is shown in figure 4.5.

The entire cloned fragment in pGPR2 was excised (using a *Hind*III-*Stu*I fragment) and used as a radiolabelled probe in Southern blots of *Bam*HI-cut chromosomal DNA from different *P.gingivalis* strains as well as *P.intermedia, P.endodontalis* and *B.assaccharolyticus*. This probe reacted with 12 kb and 1 kb fragments in *P.gingivalis* strains W83, 23A3A and 11834B using the wash procedure detailed in section 2.4.4 (data not shown) but did not react with *P.intermedia, P.endodontalis* or *B.assaccharolyticus*. This wash procedure is designed for the detection of DNA with strong similarity to the probe sequence and suggests that the *Hind*III-*Stu*I fragment of pGPR2 is unique to *P.gingivalis* and that identical sequence is not found in the other *Porphyromonas* species tested.

Figure 4.3 Restriction Map of pGPR2.

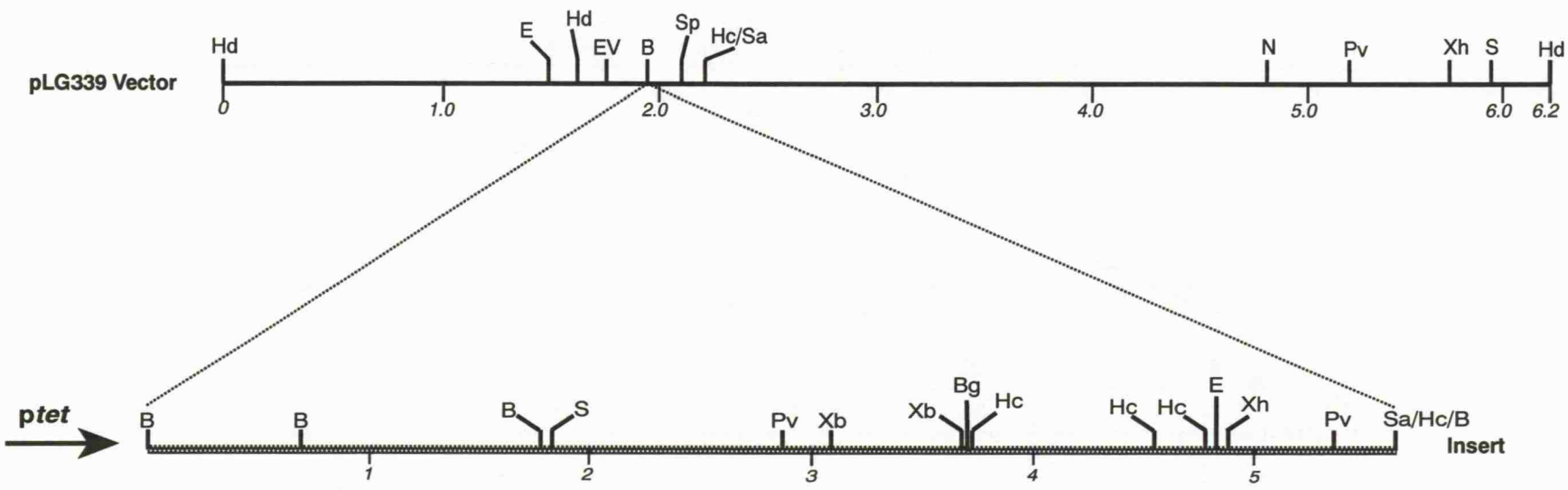


Figure 4.3 Restriction Map of pGPR2. Vector pLG339 is shown linearised at the *Hind*III site. Coordinates of vector and insert are shown in kb (italics). Restriction sites are as follows: B-*Bam*HI, Bg-*Bgl*I, E-*Eco*RI, EV-*Eco*RV, Hc-*Hinc*II, Hd-*Hind*III, N-*Nde*I, P-*Pst*I, Pv-*Pvu*II, Sa-*Sal*I, S-*Sma*I, Sp-*Sph*I, Xb-*Xba*I and Xh-*Xho*I. The direction of transcription into the cloned insert from the tetracycline resistance gene promoter is shown by an arrow.

Figure 4.4a Southern Blot Autoradiogram of pGPR2 DNA probed with a radiolabelled fragment of pGPR1.

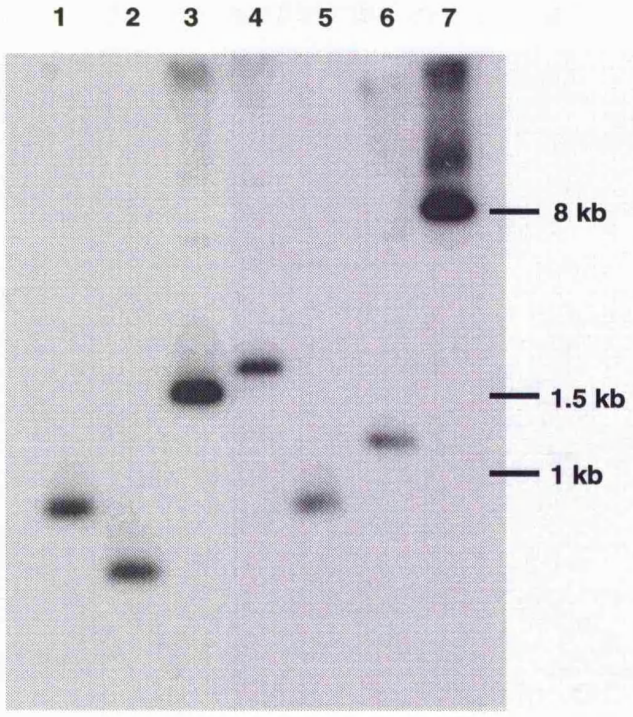


Figure 4.4 b Restriction endonuclease fragments of pGPR2 that hybridise with a probe derived from the pGPR1 cloned insert.

Lane Number/Enzyme	Restriction Fragment Size (kb)
1. *Hinc*II	**0.8**, ~10
2. *Hinc*II+*Cla*I	0.2, 0.4, **0.55**, 0.8, 3.5, 3.7
3. *Stu*I+ *Cla*I	**1.5**, 1.7, ~10
4. *Stu Bgl*II	**1.75**, ~9
5. *Hinc*II+*Bgl*II	**0.8**, ~10
6. *Xho*I+*Bgl*II	**1.2**, 4.2, ~5.9
7. *Eco*RV+*Bgl*II	4.2, **~8**

Figure 4.4 **(a)** Southern Blot Autoradiogram of pGPR2 DNA probed with a radiolabelled fragment of pGPR1. pGPR2 DNA was cleaved with different restriction endonucleases and Southern blotted in order to map the position of the pGPR1 cloned insert to pGPR2. Lanes 1-7 contain pGPR2 DNA cleaved with different restriction enzymes as detailed in figure 4.4b. **(b)** Restriction endonuclease fragment sizes of pGPR2 that hybridise with a pGPR1 cloned insert-derived probe. The table gives the fragment sizes of pGPR2 after cleavage with different restriction endonucleases as shown in the first column. Of these fragments those reactive with the pGPR1 probe in the Southern blot as shown in figure 4.4a are highlighted using bold text.

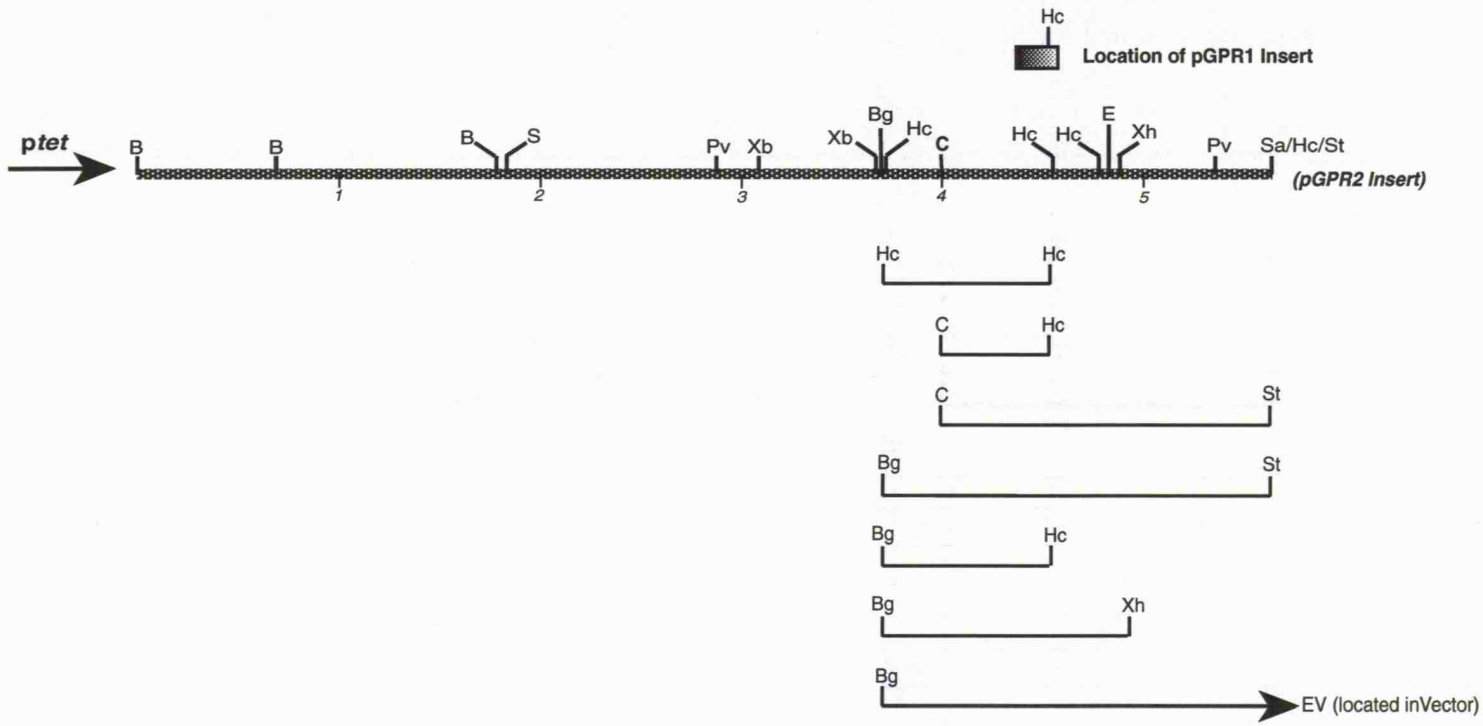


Figure 4.5 Localisation of the pGPR1 cloned insert with respect to pGPR2. Shown are the restriction fragments which hybridise to the pGPR1 probe (shown underneath the restriction map of pGPR2). The direction of transcription from the vector (pLG339) tetracycline resistance gene promoter, along with the putative localisation of the pGPR1 insert sequence is as shown. Restriction enzyme sites are abbreviated as in figure 4.3. Additional restriction sites not described in figure 4.3 are C-*Cla*I and St-*Stu*I. The arrow on the *Bg*lI/*Eco*RV restriction fragment highlights the fact that the *Eco*RV site is situated in the vector and not the cloned insert.

4.2.4 Assessment of Protease and Haemagglutination properties of pGPR2 and Immunoblot analysis using Polyclonal and Monoclonal Antibodies.

Both crude lysates and whole cells of TG2(pGPR2) and TG2(pLG339) were prepared and assayed for Glycyl-Prolyl dipeptidase activity, TLP activity and haemagglutination activity. In addition, cultures were grown anaerobically (see section 2.1.1) in LB broth and assayed for activity. The use of anaerobic conditions was intended to mimick the growth conditions under which *P.gingivalis* is cultured as it is possible that the enzymic activities to be tested for may be compromised by aerobic growth. Using the standard assay parameters pGPR2 tested negative for all of the above activities per mg protein when compared to the pLG339 control (data not shown). These experiments were repeated several times using cultures at different stages on their growth cycle in case expression was growth phase-dependent. Again results were consistently negative.

Clones were also grown aerobically and anaerobically on LB agar supplemented with 3% skimmed milk to test for general degradative activities such as lipase and non-specific proteolytic activity. This was felt necessary in that there is room for several genes to be encoded for on the pGPR2 insert and because of the wide variety of cell surface-associated enzymatic activities elicited on the cell surface of *P.gingivalis*. When grown on skimmed milk agar *P.gingivalis* produces a zone of clearing in the cream-coloured opaque appearance of the agar, underneath and around its colonies. Neither clone pGPR2 nor pLG339 produced any such clearing aerobically or anaerobically. Washing away the colony material failed to reveal any evidence of clearing. The lack of detectable activity however does not necessarily mean that there is not a protease gene present. For example the promoter for the antigen gene may have been excluded during cloning or simply might not function in *E.coli*. Alternatively the *P.gingivalis* translation initiation signals, if present may also not be recognised in *E.coli*. Hence pGPR2 may not necessarily express the cloned genes.

Colonies of TG2(pGPR2) and TG2(pLG339) were toothpicked onto LB-agar supplemented with kanamycin and tetracycine and immunoblotted using Brick 190, LDS28 and the *P.gingivalis* polyclonal antiserum. TG2(pGPR2) did not show an enhanced signal with either of the monoclonal antibody probes

when compared to TG2(pLG339), nor did it react with the *P.gingivalis* polyclonal antiserum. Lysates of TG2(pGPR2) were also western blotted using the available antibodies, but again did not show any signal when compared with the TG2(pLG339) negative control.

4.2.5 Subcloning of fragments of pGPR2 into pTTQ18* for Immunoblotting.

As it is possible that the pGPR2 cloned insert does not direct expression of antigen at levels sufficient for detection using imunoblotting, fragments of the pGPR2 insert were subcloned into an over expression vector. The plasmid cloning and expression vector pTTQ18* (figure 4.6a) was chosen. This is a high copy number vector with the *lacZ'* cassette and MCS downstream of the powerful *tac* promoter (De Boer, 1983). This promoter is a hybrid utilising the -35 bp promoter element of the *trp* operon and the -10 bp sequence and operator of the *lac* operon. Also a copy of the *lac*I^q allele is present which encodes the *lac* repressor protein. In the absence of IPTG this protein binds the *lac* operator and strongly prevents transcription of cloned genes, hence potentially restricting the leaky expression of toxic genes until expression is induced by addition of IPTG (Stark, 1987). The strain chosen for these experiments was *E.coli* Sure™. This strain has been shown to enhance stability of foreign cloned DNA and is recombination deficient (*rec*B and *rec*J). Sure™ is kanamycin (Tn5) and tetracycline (Tn10) resistant and should be grown on these antibiotics to ensure that these selectable markers are maintained (Greener, 1990).

Restriction fragments of pGPR2 were subcloned into the MCS of pTTQ18* and designated pGPR3, 4, 5 and 6 (figure 4.6b). These were screened for reactivity with *P.gingivalis* polyclonal antibody by colony blotting and using IPTG to induce expression from the *tac* promoter. Only one clone, pGPR3 reacted very faintly with this antibody. When single colonies of Sure(pGPR3) were streaked out for single colonies and immunoblotted the streak appeared blotchy and the different individual colonies varied in their signal intensity (figure 4.7; no such reaction was noted with Brick 190). In addition to this inspection of individual colonies revealed two distinct colony morphologies; the first form appeared as colonies characteristic of the *E.coli* strain Sure™ which bred true when individually streaked out; the second morphology

Figure 4.6a Circular Map of Vector pTTQ18* .

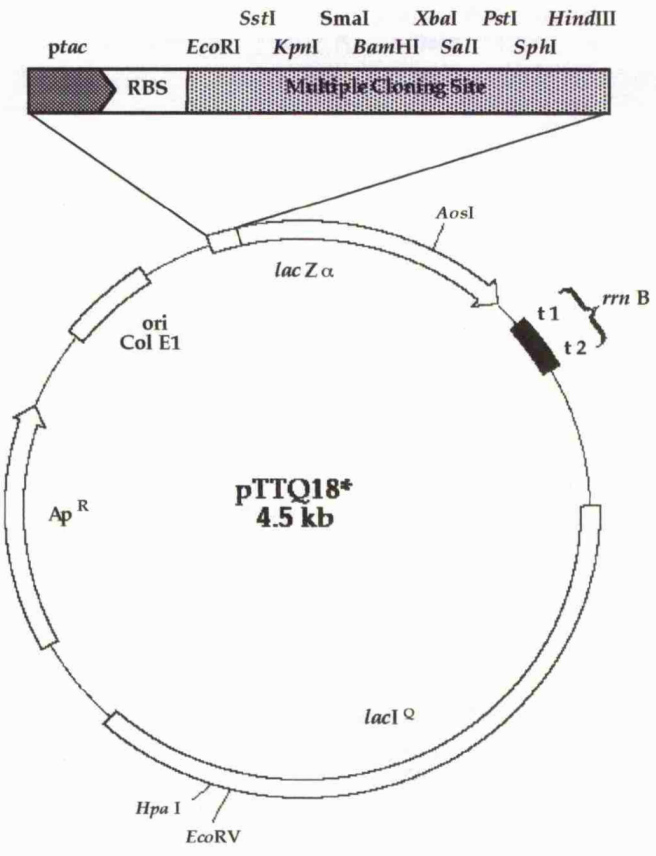


Figure 4.6b pGPR2 Subclones generated in pTTQ18*.

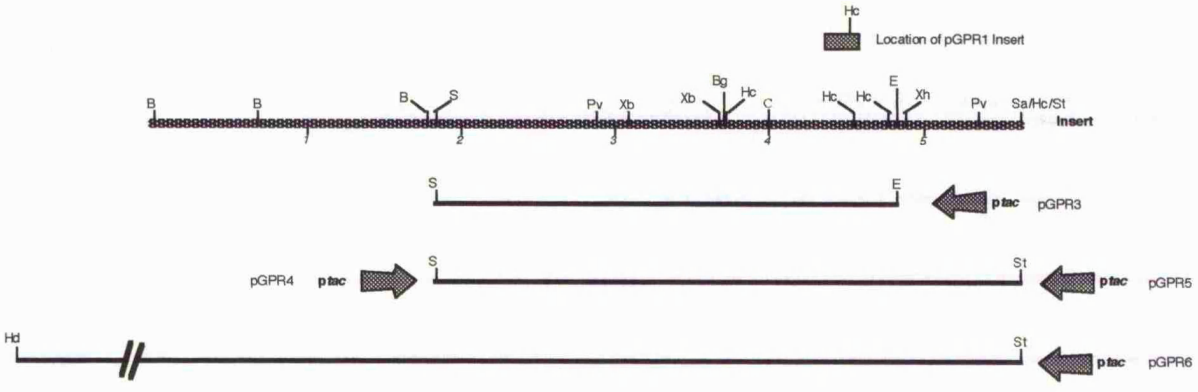


Figure 4.6 (a) Circular Map of Vector pTTQ18*. The MCS is shown expanded along with the position of unique restriction enzyme recognition sites. The transcriptional terminator from the *rrn*B gene is shown boxed in black. **(b)** pGPR2 Subclones generated in pTTQ18*. The orientation of each subclone with respect to p*tac* (the *tac* promoter) is shown using a shaded arrow. Note the *Sma*I-*Stu*I fragment was cloned in both orientations and hence represent pGPR4 and pGPR5. Also shown at the end of each construct are the enzymes used to generate the particular fragment used to clone into pTTQ18*. Restriction sites are abbreviated as in figure 4.3.

Figure 4.7.

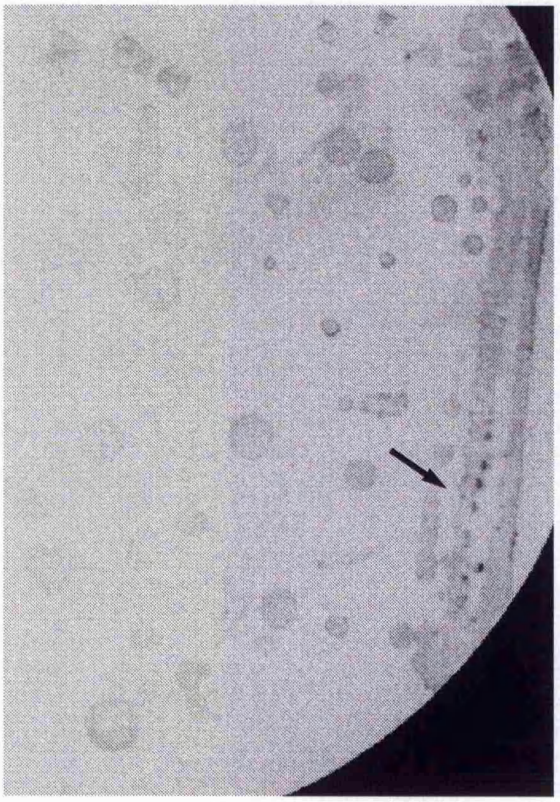


Figure 4.7 Unstable reaction of pGPR3 with anti-*P.gingivalis* polyclonal antibody. Clone pGPR3 was streaked out onto solid media and immunoblotted. Shown are polyclonal antisera -reactive colonies of varying signal intensity and blotchy appearance of the primary streak (arrow).

showed small, very flat translucent colonies which when individually streaked out gave rise to colonies of both morphologies. It is possible that these colonial variants and differing immunoblot profiles may be indicative of some form of instability.

In that simple subcloning had failed it was decided to attempt a more sophisticated method in which restriction fragments generated using *Sau*3a were cloned into the *Bam*HI site of pTTQ18*. The advantage with this method is that the high frequency of *Sau*3a cutting might generate a restriction fragment with improved stability and a significant reduction in size. These clones could then be screened using available antibodies.

Plasmid pTTQ18* was first cleaved with *Bam*HI and then treated with CIP. This DNA was then ligated with partially *Sau*3a-digested pGPR2 DNA. A series of test reactions had been performed to ensure that *Sau*3a digestion would not proceed to completion (yielding very small fragments) and had cut sufficiently so as to allow efficient cloning. Fortunately pGPR2 and pTTQ18* have different antibiotic resistance genes and this made it possible to select against transformants containing uncut or self ligated pGPR2. The ligation was transformed into Sure™ and plated onto kanamycin, tetracycline and ampicillin plates. Transformants were screened for recombinant molecules by looking for insertional inactivation of the *lacZ'* cassette which causes white colonies on media supplemented with X-gal. Individual recombinants (around 200) were toothpicked for screening with Brick 190 and LDS28. Of these only one reacted with Brick 190 and this was designated pGPR7 (figure 4.8a). Many of the recombinants, including pGPR7 reacted very strongly with LDS28 (figure 4.8b) with others showing weaker signal intensities. Four of these reactive clones were chosen for further analysis and designated pGPR8, 9 and 10 (figure 4.8b). The antibody LDS28 used here has been shown to react with Gingivain, the TLP preparation of H.N. Shah (Wallace *et al.*, 1992). Its reactivity with subclones of pGPR2 is entirely consistent with the reactivity of Brick 190 (which is also believed to be specific for TLP) with pGPR1 which maps to pGPR2. It is tempting to at this point to speculate that these two antibodies react with the same protein.

Figure 4.8a.

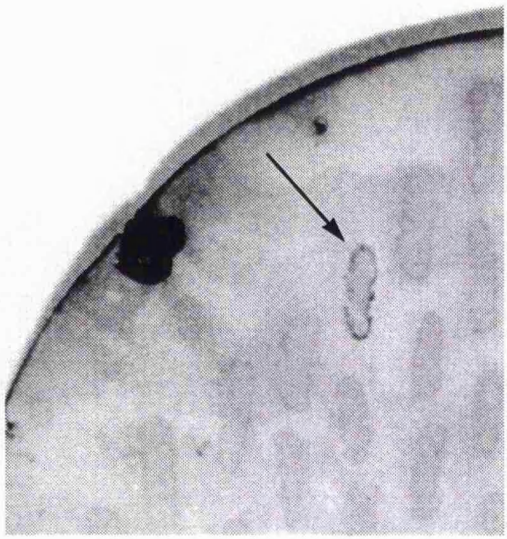


Figure 4.8b.

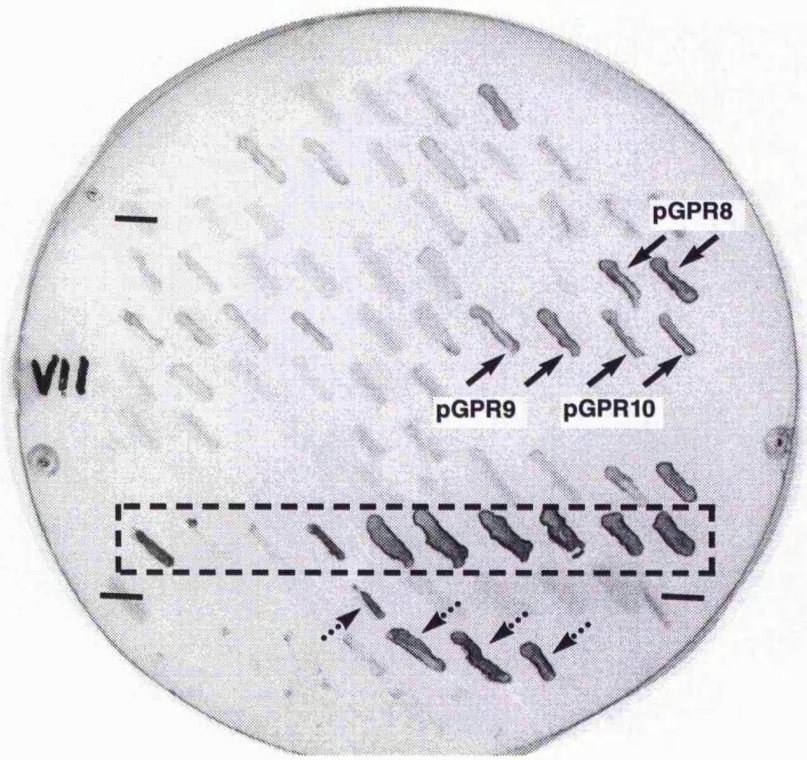


Figure 4.8 (a) Reaction of pGPR7 with mAb Brick 190. Clone pGPR7 is denoted by an arrow. **(b)** Reaction of *Sau*3a subclones of pGPR2 with LDS 28. Subclones reactive with LDS28 (pGPR7, 8, 9 and 10) were toothpicked onto solid media an immunoblotted. Subclones designated pGPR8, 9 and 10 are as shown and react with LDS28. DNA from subclone pGPR7 (pGPR7 streaks react strongly with LDS28 and are shown boxed) was used to transform Sure™. Transformants were toothpicked and immunoblotted with LDS28 (see below boxed area). Some transformants reacted with LDS28 and are highlighted with dotted arrows. Streaks of Sure™(pTTQ18*) were used as a negative control and did not react with LDS28 (shown using horizontal bars).

4.2.6 Analysis of Subclones Reactive with LDS28 and Brick 190.

In order to attempt to map the region responsible for the reactivity with LDS28 small scale DNA preparations were made of clones pGPR7 to 10. However, pGPR7 grew extremely slowly in broth culture and would not yield plasmid DNA. In addition when streaked for single colonies this clone yielded two colony types of similar morphology to those seen in pGPR3. Colony blots of these separate colony types with LDS28 revealed that the smaller colonies reacted strongly with LDS28, whereas the larger colony type elicited a weaker signal (figure 4.9). When large and small colonies were streaked individually out the large colony type persisted in producing only large colonies, whilst the small colony produced both colony types. Eventually DNA was prepared from pGPR7 by pooling a number of individual small scale DNA preparations and concentrating them 10 fold. This DNA however was present in very low amounts and would not cleave with restriction enzymes. It did however transform *E.coli* Sure™. Resultant transformants were individually toothpicked and screened with LDS28. Some of these transformants (4 out of the 18 screened) reacted strongly with LDS28 (figure 4.8b). As only a small proportion of transformants of pGPR7 react with LDS28, and when streaked for single colonies Sure™(pGPR7) sponateously develop eithers a positive or negative LDS28 reaction in immunoblots, it seems likely that pGPR7 is unstable.

Small scale DNA preparations of subclones pGPR8 to 10 were cleaved with restriction endonucleases *Hinc*II, *Sal*I and *Xba*I. *Hinc*II cleaves around the region encoding the antigen reactive with Brick 190 (pGPR1 insert) producing a distinctive 0.8 kb dimer and a 0.4 kb fragment. Likewise *Sal*I and *Xba*I cleave either side of the same region and can be used to give a crude restriction map of clones pGPR8 to 10. This is shown in figure 4.10. These clones all share the same region of the pGPR2 insert specifically the area surrounding the pGPR1 coding region. However after several passages on solid media clones pGPR8 to 10 exhibited diminished and eventually no reactivity with LDS28. What is more clones pGPR8 to 10 failed to show any reaction with Brick 190 in immunoblot experiments.

Subclones pGPR7 to 10 were all assayed for BAMCA-specific hydrolytic activity and haemagglutination activity. Assays were as standard except that pGPR7 lysates were prepared by resuspending bacteria grown on solid media

Figure 4.9 Clonal instability of pGPR7 .

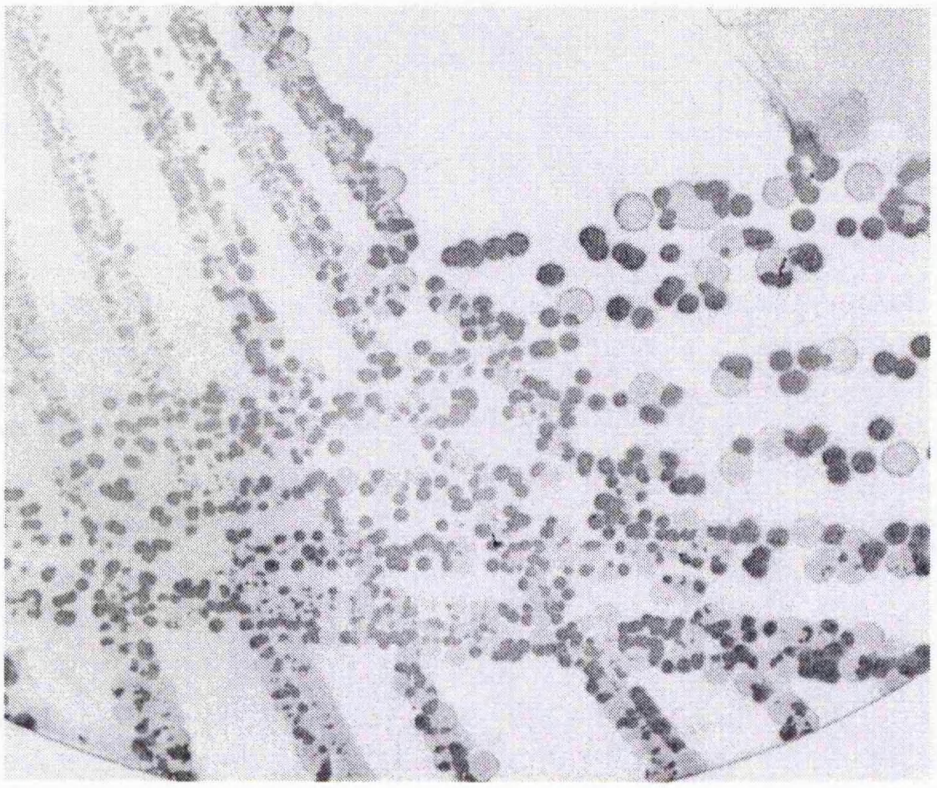


Figure 4.9 Clonal instability of pGPR7. Sure(pGPR7) was streaked for single colonies and immunoblotted using mAb LDS28. This blot revealed that individual colonies of pGPR7 react with different signal intensities when probed with LDS 28.

Figure 4.10 Approximate relative locations of cloned inserts in pGPR8, 9 and 10 with respect to pGPR2.

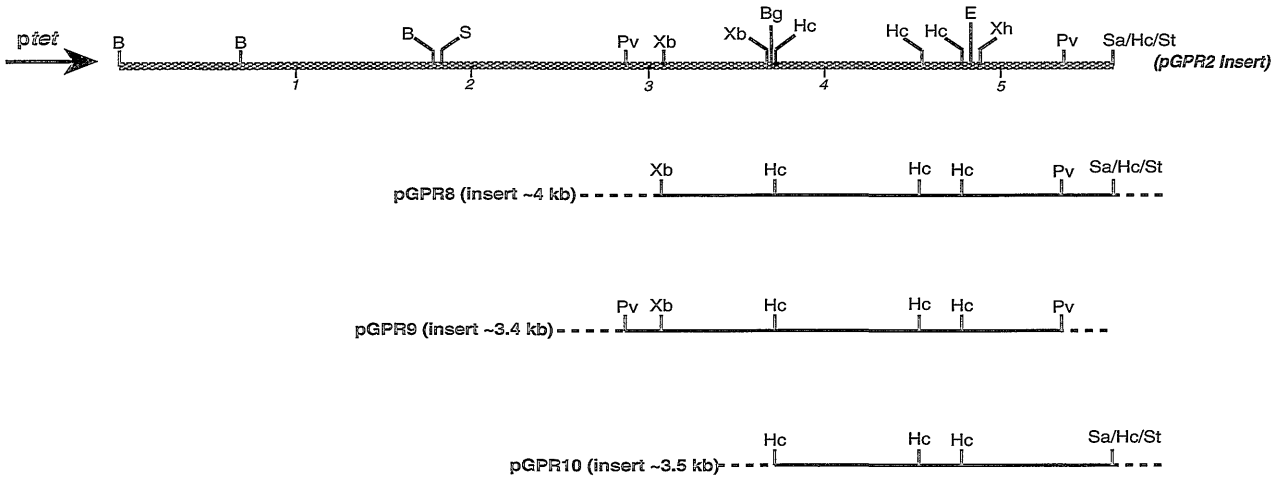


Figure 4.10 Approximate relative locations of cloned inserts in pGPR8, 9 and 10 with respect to pGPR2. Clones pGPR8, 9 and 10 were generated by ligating random *Sau*3a fragments of pGPR2 into plasmid pTTQ18*. The relationship between pGPR8, 9 and 10 cloned inserts and pGPR2 was investigated using restriction endonuclease analysis of small scale DNA preparations to detect characteristic *Hinc*II and *Xba*I/*Sal*I restriction fragments. Shown are known restriction sites for pGPR8-10, the total estimated size (kb) for each cloned insert and their position relative to the restriction site map of pGPR2. Restriction sites are as abbreviated in figure 4.3.

in a buffered solution (see 4.2.7). None of these subclones showed any siginificant BAMCA hydrolysis or haemagglutinating activities when compared to Sure(pTTQ18*) negative controls

4.2.7 Western Blot Analysis of Subclone pGPR7.

The purpose of western blot analysis of pGPR7 was to demonstrate antibody reactions with proteins of distinct molecular weight. Although the need to use non-denaturing CHAPS PAGE western blots for Brick 190 creates difficulties in determining molecular weights of reactive proteins, it should be possible to determine the molecular weight of the pGPR7-encoded antigen using LDS28 which will recognise SDS-denatured antigen.

Several attempts at using LDS28 against Sure(pGPR7) protein lysates of broth cultures in western blots were largely unsuccessful in that not only were Sure(pGPR7) broth cultures difficult to grow (section 4.2.6) lysates made from such cultures simply did not react with LDS28. What is more subcultures of such broths when grown on solid media and immunoblotted also did not react with LDS28 (data not shown). Eventually bacteria from LDS28-blotted positive colonies (see section 4.2.5) were resuspended in TE buffer supplemented with protease inhibitor PMSF (160µg ml^{-1}) and sonicated. These lysates were run out on both CHAPS PAGE and SDS PAGE gels and western blotted along side Sure(pTTQ18*) samples prepared in the same way. Sure(pGPR7) lysates prepared in this way reacted with both Brick 190 and LDS28 in CHAPS PAGE and SDS PAGE western blots respectively (figure 4.10a and b). The reaction with Brick 190 was not very pronounced (probably due to poor focusing) although the reaction with LDS28 was quite clear and was repeatable several times with the same and different batches of lysates. The latter shows the pGPR7 LDS28-reactive antigen to exhibit a protein molecular weight of slightly below 50 kDa and to be expressed regardless of the presence of IPTG during growth. The molecular weight of the Brick 190-reactive band using CHAPS PAGE was not determined.

Figure 4.11a.

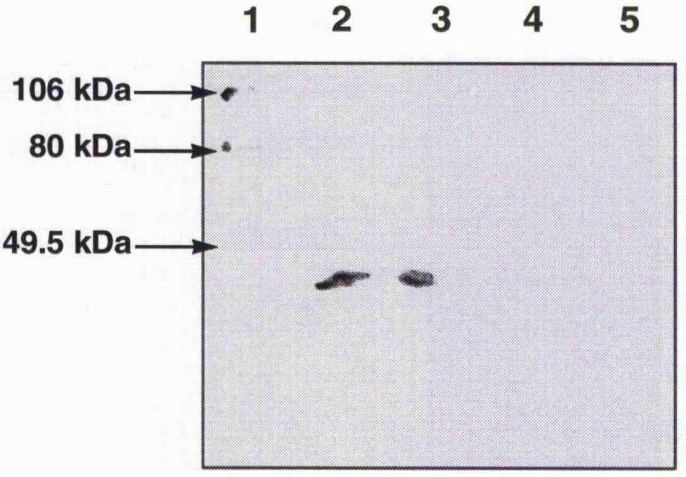


Figure 4.11b.

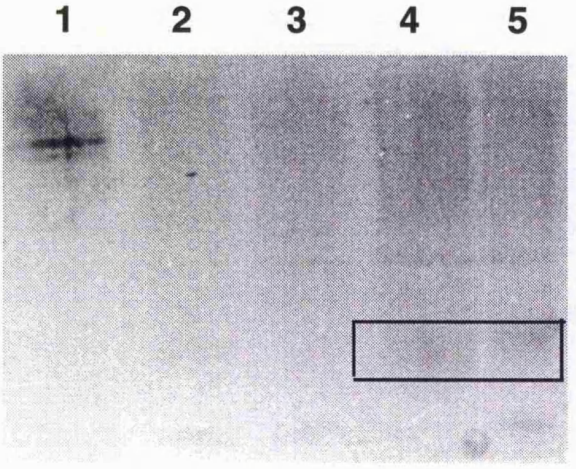


Figure 4.11 (a) Western blot of pGPR7 hybridised with antibody LDS28. Colonies of Sure(pGPR7) were resuspended in a solution of protease inhibitors, sonicated and western blotted with LDS28. Lanes 2 and 3 contain pGPR7 grown with and without IPTG respectively, and show a LDS28-reactive band of approximately 45 kDa molecular weight. Lanes 4 and 5 contain Sure(pTTQ18*) treated similarly with and without IPTG. Lane 1 contains prestained molecular weight markers (Biorad). Molecular weights (kDa) are shown down the left side. **(b)** CHAPS Western blot of clone pGPR7 hybridised with mAb Brick 190. Lysates were prepared as described above. Lane 1, *P.gingivalis* ECV, lanes 2 and 3 Sure(pTTQ18*) with and without IPTG, lanes 4 and 5 Sure(pGPR7) with and without IPTG respectively. Reactivity with Brick 190 using 4-chloro-1-naphthol and UV visualisation is shown boxed (lanes 5 and 6 only). Poor signal definition is due to the difficulties in photographing low intensity light from UV illumination using a UV filter.

4.3 Discussion.

Described is the cloning of a 5.5 kb *Sau*3a fragment (pGPR2) from *P.gingivalis* which carries gene(s) encoding a protein antigen. This antigen reacts with polyclonal antibodies raised against whole cells and mAb Brick 190 which was raised against a TLP purified from ECV. What is more either the same product or an additional product of this gene locus encodes the antigen specific for mAb LDS28. Southern blot analysis using a high stringency wash procedure suggests that the 5.5 kb fragment occurs once on the *P.gingivalis* chromosome. It follows that the genes cloned in pGPR2 should also occur as single copies on the *P.gingivalis* chromosome. Southern blot analysis revealed no hybridisation of the 5.5 kb fragment with chromosomal DNA from other *Porphyromonas* and *Bacteroides* strains and suggests that the 5.5 kb fragment may be unique to *P.gingivalis*.

From the initial stages of cloning pGPR2 problems had been encountered. A pGPR2 clone variant with restriction site polymorphisms most probably arising from a large DNA deletion was isolated despite attempts to maximise stability by using *rec*A strains and a low copy number vector to reduce gene dosage effects. There are at least two phenomena one must consider in trying to understand such rearrangements. First, a mechanism for deletion/rearrangement. These are likely to be caused either by the activity of mobile genetic elements (for example transposons and bacteriophage, neither of which require *rec* gene functions; bacteriophage λ is an exception to this), by damage to the DNA followed by misrepair (most likely to produce small point mutations), or by homologous recombination with for example components of the host cell chromosome (Ayala and Kiger, 1984). Certainly illegitimate recombination (transposon mediated) can account for DNA deletions, insertions, duplicative inversions and cointegrate replicon fusions. Natural transposons are a common feature on the chromosome of *E.coli* K-12, the strain from which most common laboratory strains are derived (Ronecker *et al..*, 1987) and their activity may account for much of the instability exprienced during cloning experiments. Even the F episome necessary for the production of M13 type bacteriophage and used in many cloning experiments encodes several mobile genetic elements, namely IS2, IS3 and gamma-delta (Hu *et al.*, 1988). Transpositional activities could also be intrinsic to cloned DNA. The second important factor in understanding clonal instability is the selectional pressure behind maintenance of a mutant phenotype. If the

protein expressed by a cloned gene is toxic or of some disadvantage to the cell, it will result in a loss of vigour and clones with such problems may grow slowly or not at all. Hence should a mutant containing a non-functioning protein arise it will outgrow the wild type. This may account for some of the instability problems seen here.

Similar problems were encountered in the stability of phenotype expression, although this may not reflect the same problem. For example clones pGPR7 and 3 both showed colonial variants, and variation in signal intensities for different colonies when probed with antibody. This phenomenon could be explained in either of two ways. First, it is possible that regulation of expression of the protein antigen is occurring. Hence some genetic switch may be involved. Secondly, DNA stability might be a problem. Also the elevated copy number of the pTTQ18* plasmid (up to 800 copies per cell) may cause gene dosage-dependent increases in gene expression and hence precipitate instability problems. Interestingly clone pGPR7 appeared to show a striking alteration in the yield and quality of plasmid DNA isolated from this clone and although unstable, reacted with both mAb used in this study. Also this expression was IPTG independent (section 4.2.7) suggesting the presence of cloned promoter activity. It is hence reasonable to assume that tight regulation of p*tac*-dependent expression using the *Lac* I^q gene and IPTG is of no advantage when trying to stabilise this clone.

Circumstantial evidence suggesting the presence of a region coding for a protease gene(s) on pGPR2 is amounting in that two antibodies allegedly specific for TLP recognise components expressed by pGPR2. However, caution should be exercised in making such assumptions. No reliable data exists dictating that Brick 190 was either raised against truly pure TLP or is specific for this enzyme. Secondly, although LDS28 reacts with gingivain in western blots it only reacts with one of several bands seen using coomassie blue staining (it is possible however that LDS28 reacts with a N or C terminal portion of TLP and the additional bands which don't react with LDS28 are simply breakdown products lacking the terminal portion for which LDS28 is specific). The mode of purification of gingivain involved affinity purification using a sepharose-conjugated thiol-binding agent 2,2'dipipyridyl disulphide (2,2'PDS) followed by elution using a pH gradient. The result of this is a pH-dependent purification of thiol containing proteins (Shah *et al.*, 1990). It is entirely possible that several proteins may co-purify at any particular elution

pH or that contaminant proteins may co-purify by virtue of affinity for the 2,2'PDS bound protein. This in conjunction with the fact that LDS28 was raised against ECV and not some purified preparation of TLP throws doubt on the specificity of LDS28 and the hypothesis that the pGPR2 antigen is the TLP. Notably none of the pGPR2 subclones tested so far exhibited any TLP activity.

The specificity of LDS28 and Brick 190 for a component of the cell surface or ECV suggests that the pGPR2 antigen(s) may have a cell surface localisation in *P.gingivalis*. What is more both antibodies produce a complex pattern in western blots. Brick 190 reacts with a high molecular weight smear in CHAPS PAGE whereas LDS28 reacts with a high molecular weight smear when run out on SDS PAGE without boiling (Wallace, A., unpublished results). In addition the complex banding pattern revealed when ECV are probed with LDS28 invites speculation about the presence of a covalently-linked complex resulting in an increase in apparent molecular weight of the 46 kDa protein (the lower molecular weight bands may be as a result of proteolytic breakdown). Given this "ladder"-like profile one wonders if this putative complex may involve covalent attachment to LPS (which also forms ladders whose bands correspond to the number of repeats in the O antigen chain). Such a complex would have to involve covalent linkages other than sulfurhydryl bonds in order to remain complexed during SDS PAGE in the presence of ß-mercaptoethanol which breaks -S=S- bonds. It is also not clear why this banding phenomenon occurs only in ECV. As ECV are very similar in composition to the *P.gingivalis* outer membrane (Grenier and Mayrand, 1987) it is likely that this complex pattern on SDS PAGE may be a consequence of the method of vesicle preparation.

The reaction of LDS28 with subclone pGPR7 in SDS PAGE western blots reveals a molecular weight for this antigen in the 40-50 kDa range and this is consistent with the observed mass of the *P.gingivalis* counterpart (46 kDa). However it is not clear as to whether pGPR7 encodes the whole antigen coding sequence as it was not possible to perform restriction digests on this DNA (section 4.3.6). Whilst pGPR7 reacts with both mAb used in this study it is not clear as to why pGPR8 to 10 did not react with Brick 190 especially as these clones contain the region of DNA previously reactive with brick 190 (the cloned insert of pGPR1). Subsequent sequence analysis may answer these questions.

Chapter 5

DNA Sequence analysis of Clone pGPR2.

5.1 Introduction

The first wide spread method of DNA sequencing was described by Maxam and Gilbert (1977) and involved chemical base-specific modification and cleavage of DNA in order to produce asymmetric DNA fragments for analysis on polyacrylamide gels. This method was superseded by the method of Sanger *et al.*, (1977) which relies on the incorporation of chain terminator inhibitors into an enzyme catalysed DNA polymerisation reaction. The ease and quality with which DNA is sequenced using the Sanger method has meant that dideoxy chain termination sequencing has substituted all other sequencing methods. The result is that over the past two decades the increase in the number of known DNA sequences has expanded exponentially. As a result of this today's more complete bacterial DNA databases contain approximately 22,187 sequences comprised of around 38,857,654 residues. Naturally, the number of predicted protein sequences has risen proportionally and the disciplines of molecular and structural biology have began to inter-link the primary and tertiary structure of proteins with protein function.

What are the experimental gains to be acquired from DNA sequence? First the primary DNA sequence may share or identity with other sequences and this may reveal the identity of the query sequence or suggest possible functions. The DNA base composition may also give clues as to the ancestral origins of the query sequence. Secondly identification of potential coding regions within a restriction map will facilitate subcloning and gene characterisation. The primary structure of proteins can then be accurately deduced and post-transitional modifications predicted from motifs within the amino acid sequence. Similarity with other proteins of known function or with known consensus activities may then suggest a function for a predicted protein. What is more any new sequence with relevant data will assume a position within future databases and hence contribute to future database searches. DNA sequence may also yield data as to how transcription occurs and subsequent translation. The key thing to realise is that sequence analysis is

primarily a predictive tool and should be used to direct and corroborate conventional forms of experimental data.

The determination of the nucleotide sequence of the 5.5 kb insert in pGPR2 probably represents the longest contiguous segment of *P.gingivalis* DNA to be sequenced to date. As little is known about *P.gingivalis* gene expression the gene characterisation described here in should provide valuable information for future projects.

5.2 Results

5.2.1 A Strategy for Determining the Nucleotide Sequence of pGPR2.

Several methods for dideoxy chain termination sequencing exist. Template DNA for sequencing reactions can be double or single stranded depending on the protocol used. In my experience the highest quality DNA sequence is derived from single stranded DNA. This method routinely and reproducibly gives a sequence yield of 400-450 (with a maximum of ~550) nucleotides per sequencing reaction and is limited within this range by the resolution of the polyacrylamide gel electrophoresis employed.

Single stranded DNA was produced by cloning of defined restriction fragments into chimaeric bacteriophages M13mp18 and M13mp19 (Yanish-Perron *et al.*, 1985). These vectors provide an extensive multiple cloning site in opposite orientations within the coding region for the *lac*Z′ cassette. This allows screening for insertional inactivation of the *lac*Z′ by cloned DNA. Production of single stranded DNA from either strand of a restriction fragment is also possible depending on the M13 vector used and subsequent fragment orientation within the vector. Hence a strategy using overlapping restriction fragments cloned in both orientations will give DNA sequence for both strands which sequences through the restriction sites used in cloning (figure 5.1). Where there is a scarcity of restriction sites double-stranded sequence can be acquired by bi-directional cloning of a larger fragment and then by directing sequencing reactions using oligonucleotide primers.

Figure 5.1 The Subcloning and Primer Strategy Used in Sequencing pGPR2

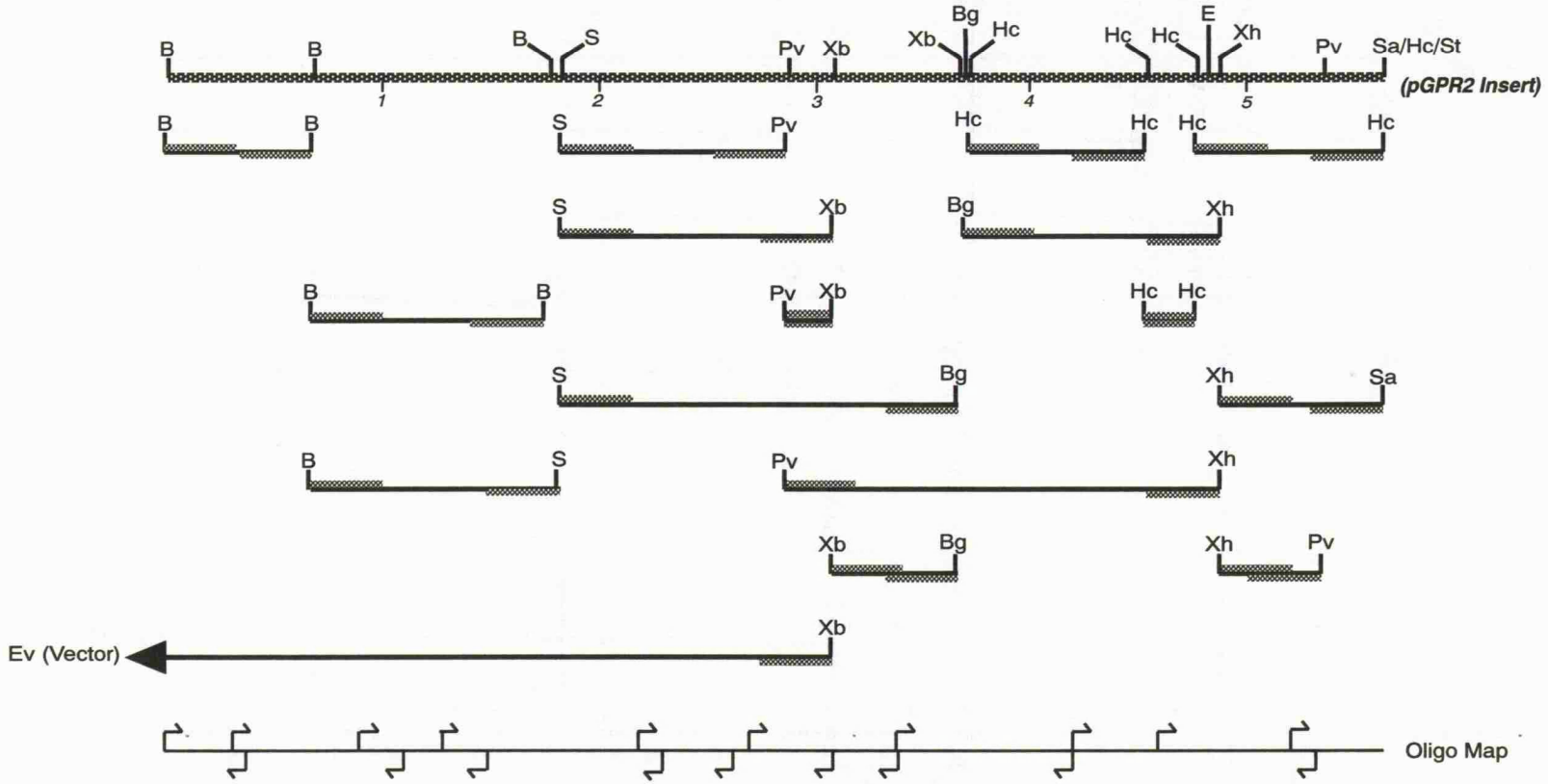


Figure 5.1 The Subcloning and Primer Strategy Used in Sequencing pGPR2. Fragments cloned bi-directionally into M13 are shown as solid lines bordered by restriction sites underneath the restriction map of the pGPR2 insert. The practical sequencing range using the M13 *lac* Z' C-terminal primer is shown using hatched boxes (▬▬) whose position above or below the restriction fragment line denotes which of the upper or lower strand sequence they represent. Also shown (below) is a map of the oligonucleotides used. The positions of the oligonucleotides used for sequencing relative to pGPR2 are shown using arrows; the arrows directions show the direction of priming during sequencing reactions and hence the direction of subsequent sequence data. Restriction sites are abbreviated as for figure 4.3.

5.2.2 Nucleotide Sequence of pGPR2 and Identification of Potential ORFs.

In all 11,306 residues of pGPR2 were sequenced (EMBL Accession No. x95938). This represents a 5.6 kb fragment sequenced on both strands and spans from the *Bam*HI site at the 5' end of the tetracycline resistance gene to a point close to (approximately 100 bp) the *Bam*HI at the 3' end of the tetracycline resistance gene. This DNA was shown to have a % GC content of around 48%, a value which is entirely consistent with reports of the % GC content of *P.gingivalis* being around 48-49% (Coykendal *et al.*, 1980). ORF analysis revealed the existence of seven major reading frames exceeding 120 residues in length (this cut off excludes proteins of around 11 kDa and below). These are shown in figure 5.2. When the 5.6 kb of sequence was compared to sequences in the GenEMBL composite DNA database using FASTA only one significant homology score was detected. This was over the first 410 residues in the region corresponding to ORF168 and constituted an uninterrupted 56.3% identity with the *rnh*B gene of *E.coli*. It is thus likely that the *P.gingivalis* homologue of *rnh*B maybe present on pGPR2. However sequences upstream and downstream of the *E.coli rnh*B allele did not show any significant homology with pGPR2. This suggests that pGPR2 contains an insert not previously sequenced and not contaminated with vector sequence.

The nucleotide sequence, relevant open reading frames and restriction sites of pGPR2 are shown in figures 5.3 and 5.4. The co-ordinates of the pGPR1 insert were found between base pairs 1046 and 1353 in figure 5.4 within the range of ORF455. The pGPR1 insert sequence was 100% identical with pGPR2 over this region. As pGPR1 is known to encode for a *P.gingivalis* antigen the ORF455 gene was redesignated *pga*A. As the translational start site for ORF199 overlaps the last few codons of *pga*A it is possible that translation of ORF199 and *pga*A may be coupled and that the two genes are transcribed on a single bicistronic mRNA.

Scrutinisation of the ORF pattern of pGPR2 reveals an apparent lack of ORFs greater than 10 kDa upstream of *pga*A for a stretch of 767 bp. A BLASTX search of this region (BLASTX translates DNA in all six frames and performs comparisons with protein databases; Altschul *et al.*, 1990) showed no small ORFs with sequence homology to any known protein suggesting the latter possibility is unlikely. In addition a large inverted repeat was

Figure 5.2a ORF Map of pGPR2.

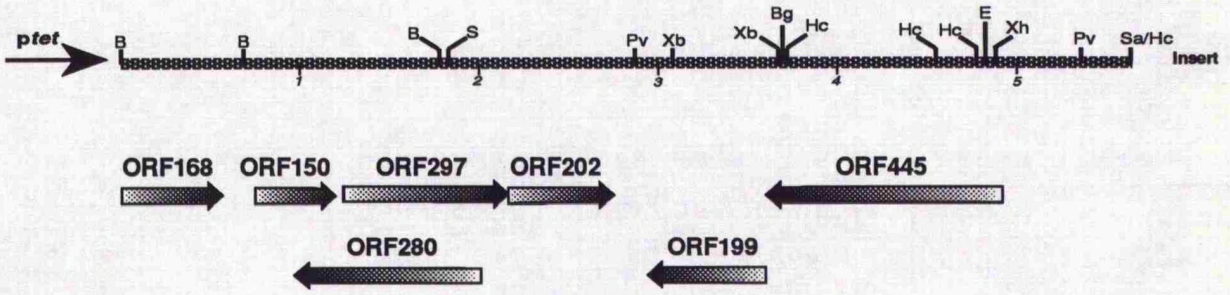
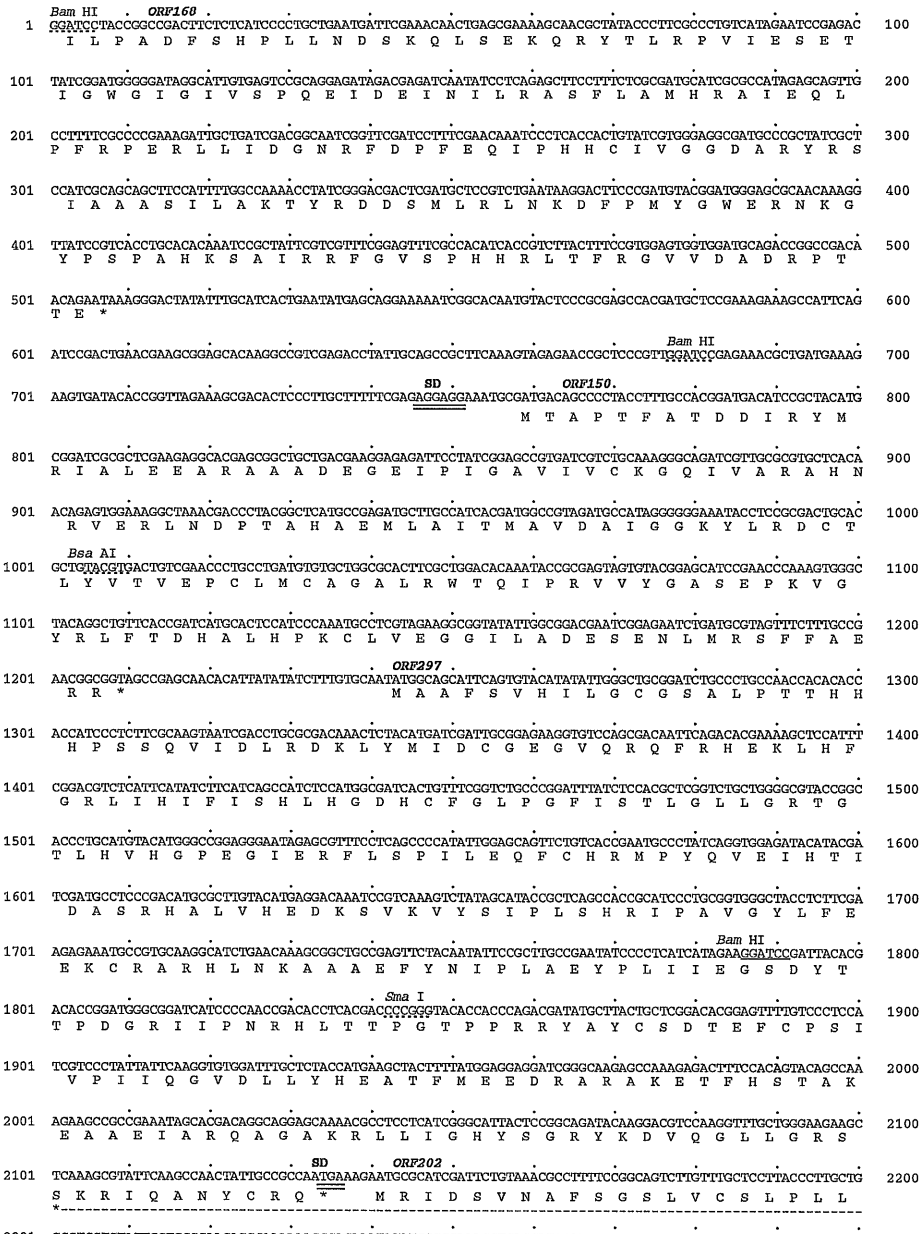


Figure 5.2b ORFs of pGPR2 and their molecular weights.

ORF	Predicted Molecular Weight
168 (start precedes sequence)	19.2 kDa
150	16.6 kDa
297	33.5 kDa
280	30 kDa
202	22.7 kDa
199	22.8 kDa
445	51.0 kDa

Figure 5.2 (a) ORF Map of pGPR2. Numbered arrows represent ORFs of pGPR2 and their transcriptional orientation. ORFs 297 and 280 occupy the same region of pGPR2 but are predicted to be translated from anti-parallel strands. ORF designations are derived from the number of codons predicted for each ORF. **(b)** ORFs of pGPR2 and their molecular weights. The predicted molecular weight of proteins encoded by each putative ORF is shown in kDa.

Figure 5.3 Nucleotide Sequence of the left hand *Bam*HI-*Pvu*II fragment of pGPR2.



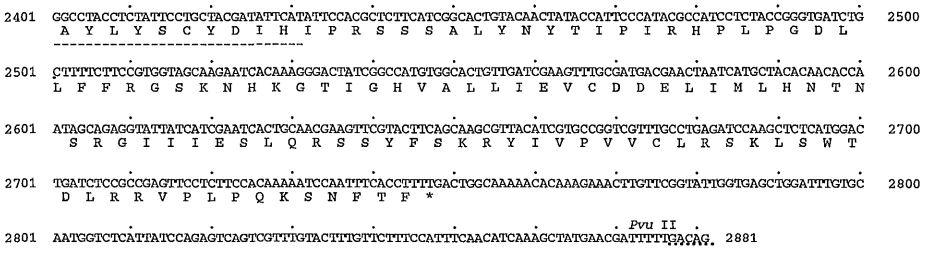
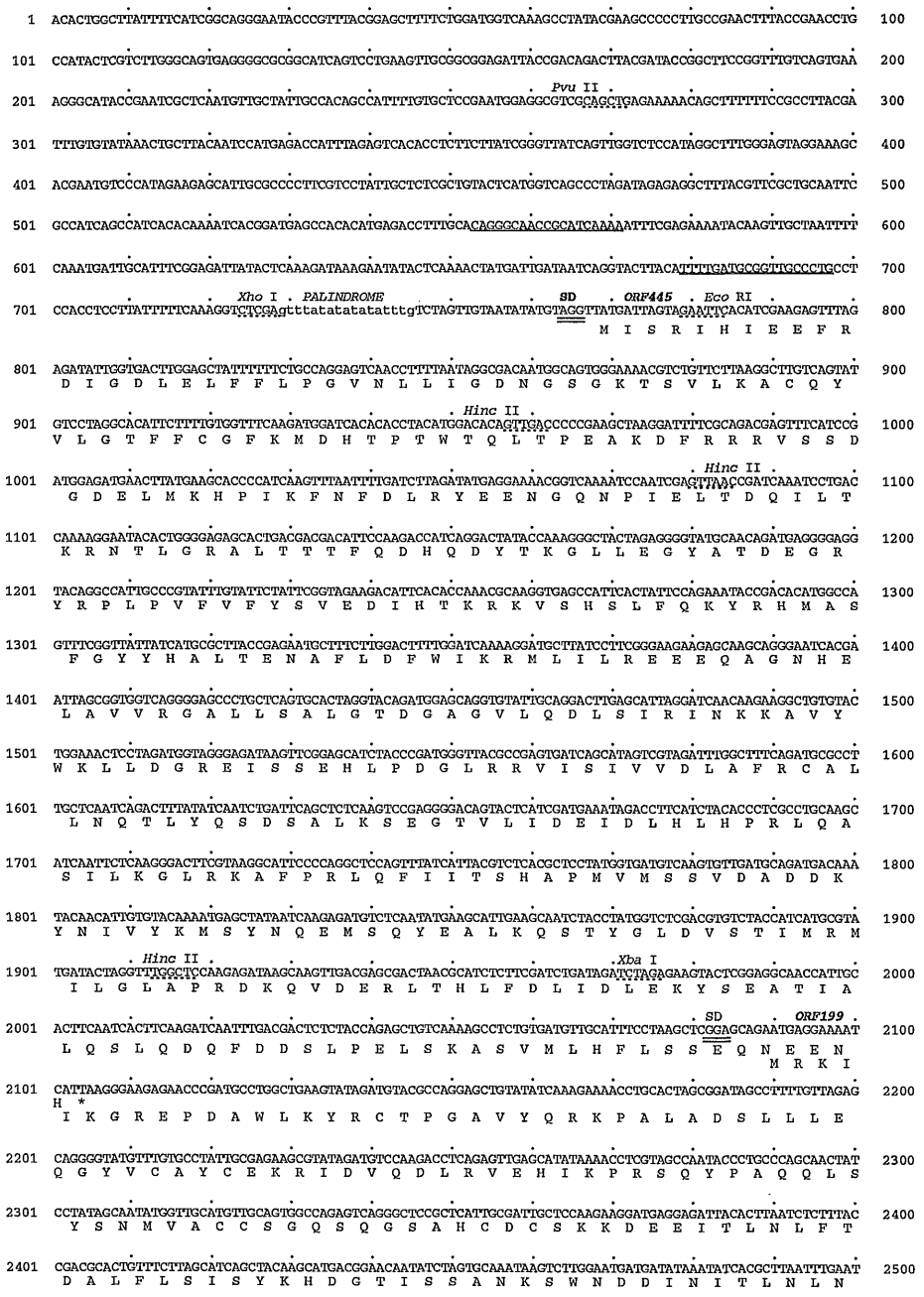


Figure 5.3 Nucleotide Sequence of the left hand *Bam*HI-*Pvu*II fragment of pGPR2 (figure 5.2). Nucleotide coordinates are given at the begining and end of each line with full stops every 10 bp above every tenth nucleotide. Coordinates begin at the *Bam*HI site at the pLG339 vector/insert junction and end at the central *Pvu*II site of the insert. Restriction enzyme site are shown in italics and their recognition sites underscored with a dotted line. Open reading frames are shown underneath their respective coding regions in single letter amino acid code with stop codons being represented by asterisks. Also shown are putative Shine Dalgarno translational initiation sites (double underlined and labelled in bold). The coding region for ORF280 is on the reverse complement of the strand shown and its coordinates represented by a dashed line.

Figure 5.4 Nucleotide Sequence of the right hand *Pvu*II-*Bam*HI fragment of pGPR2.



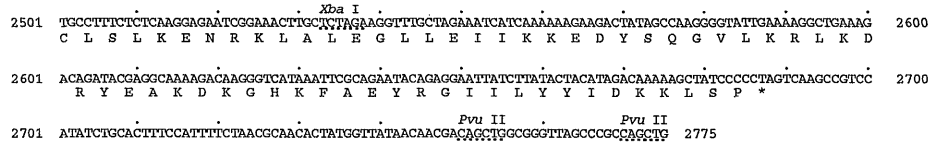


Figure 5.4 Nucleotide Sequence of the right hand *Pvu*II-*Bam*HI fragment of pGPR2 (figure 5.2). Shown is the coding strand which represents the reverse complement orientation of that shown on the ORF map in Figure 5.2a. Features are labelled as in Figure 5.3. Additional features include the presence of a putative stem loup structure whose stem is plain underlined and a palindromic stretch upstream of ORF 445 (designated *pga*A) which is shown in lowercase. The *Bam*HI site was not reached using DNA sequencing and hence is not shown, but is estimated to map approximately 0.1 kb upstream of the first coordinate as defined in this figure.

detected upstream of *pga*A (figure 5.4) using the IREP search tool which detects inverted repeats (Devereaux *et al.*, 1984). In addition to the 7 ORFs described so far three potential ORFs whose co-ordinates extend beyond the sequence and translate divergently with respect to *pga*A exist, although their predicted molecular weights are less than 10 kDa.

5.2.3 Sequence analysis of *Pga*A and its neighbour ORF199.

The putative *pga*A translate consists of 445 amino acids and has a theoretical molecular weight of 51.0 kDa. Immediately upstream of the start codon can be found a consensus Shine-Dalgarno sequence for directing ribosome binding of transcripts and translation initiation (figure 5.4). Also upstream of *pga*A and adjacent to the *Xho*I site there is a 17 bp AT-rich palindrome. Comparison of this sequence with the DNA database revealed no significant similarity with any other palindromic sequence. FASTA analysis revealed strong homology between the N-terminus of the PgaA protein sequence and the N-termini of RecF proteins from several different bacterial species (figure 5.5a). The RecF protein is believed influence RecA expression and *rec*F mutants show a compromised SOS response (Thomas and Lloyd, 1983). Multiple alignment of RecF sequences and PgaA (figure 5.5b) revealed highly conserved residues amongst all four RecF proteins and PgaA. However beyond the first 50 or so residues of PgaA CLUSTALV analysis (Higgins *et al.*, 1994) revealed very low homology with the RecF proteins which are all highly homologous throughout their lengths (data not shown). Hence it is unlikely that *pga*A encodes a RecF protein. A MOTIFS search (MOTIFS examines the PROSITE database of conserved amino acids motifs amongst groups of proteins) of the N-terminal of PgaA revealed that residues strongly conserved in the PgaA/RecF alignment follow closely the Walker type A motif of NTP-ases and kinases (Walker *et al.*, 1982; figure 5.5b). However a Walker type B consensus could not be detected in PgaA nor could motifs G-2, G-3 and G-4 of the GTPase superfamily (Bourne *et al.*, 1991). No other significant sequence similarities were noted for PgaA nor were N-terminal signal peptide consensus elements observed (von Heijne, 1986) although signal peptides are not an absolute requirement for protein secretion into the periplasmic space (Friedrich *et al.*, 1986).

Figure 5.5a Proteins sharing sequence homology with PgaA

PgaA Homologue	Identity	Reference
RecF *Proteus mirabilis*	27.7%/137aa	Skovgaard *et al.* (1990)
RecF *Salmonella typhimurium*	50.0%/46aa	Sandler *et al.* (1992)
Rec F *Haemophilus pleuropneumoniae*	45.4%/44aa	Loynds *et al.* (1992)
RecF *Escherichia coli*	52.2%/46aa	Adachi *et al.* (1984)

Figure 5.5b Multiple alignment of putative NTP binding folds of PgaA and RecF Proteins.

```
PgaA P.gingivalis          MI-SRIHIEEFRDIGDLELFFLPGVNLLIGDNGSGKTSVLKACQYVLGTFFCGFKMDHTPTW
RecF P.mirabilis           MILSRLLIRHFRNIEQADLPLADGFNFLVGPNGSGKTSILEAI-YTLGHGR-AFRSAQANRV
RecF S.typhimurium         S-LTRLLIKDFRNIENADLALSPGFNFLVGPNASGKTSVLEAI-YTLGHGR-AFRSLQPGRV
RecF H.pleuropneumoniae    MPLSRLIINNFRNLQSLDLELSPNFNFIVGHNGSGKTSLLEAI-FYLGHGR-SFKSHISNRI
RecF E.coli                S-LTRLLIRDFRNIETADLALSPGFNFLVGANGSGKTSVLEAI-YTLGHGR-AFRSLQIGRV
                                                        S     I
NTP Binding Consensus                           G    GKor    or
(Walker A-Type Motif)                                   T     V
```

Figure 5.5 (a) Proteins sharing sequence homology with PgaA. Homologies were determined using the FASTA search tool (Lipman and Pearson, 1985). Shown are % identities over lengths in amino acids. (b) Multiple alignment of putative NTP binding folds of PgaA and RecF proteins. Alignment were performed using CLUSTAL V (Higgins *et al.*, 1994). Totally conserverd residues are shown in bold typeface whilst conservative changes are underlined. The nucleotide binding motif is shown underneath the alignment (Walker et al, 1982).

Analysis of the composition of PgaA reveals a high prevalence of hydrophobic amino acid (35 % content by molarity). Hydropathy profile plots (Kyte and Doolittle, 1982) show several strongly hydrophobic patches within PgaA (figure 5.6). Of these only one is sufficiently hydrophobic (having a mean hybrophobicity greater than 1.5) over a stretch exceeding 15 amino acids (the approximate minimum thickness of a lipid bilayer Doolittle, 1986) so as to constitute the presence a potential membrane spanning region. Analysis of the antigenic index of PgaA (Jameson and Wolf, 1988) revealed multiple peaks of predicted high antigenicity (tending to an index value of 1.7) including peaks in the region of the pGPR1 insert coding stretch (figure 5.6).

The putative translational product of ORF199 encodes a protein of 22.8 kDa consisting of 199 residues. The translational start point of this protein overlaps the C-terminus of PgaA by approximately 16 bp and it is thus possible that these two cistrons are translationally coupled and transcribed as a single polycistronic message (McCarthy and Gualerzi, 1990). There is a potential consensus Shine-Dalgarno sequence 8 bp from the start codon for translational initiation. Analysis of the hydropathy profile of the ORF199 protein sequence revealed largely hydrophilic protein without hydrophobicity characteristics of transmembrane or membrane-associated proteins (Doolittle, 1986; data not shown) suggesting that the ORF199 may encode a soluble protein.

When the ORF199 protein sequence was compared to protein sequence databases using BLAST and FASTA search tools no significant homologies with other proteins were observed. Likewise a MOTIFS search of ORF199 search failed to reveal similarity to any known consensus sequences. However a search for motifs by eye revealed the presence of a small stretch of amino acids sharing key residue identities with the serine active site sequences from both serine proteases, lipases, and cutinases. This motif occurs at amino acid 80 of ORF199 and consists of residues CCSGQSQGS where serine and glycine residues (underlined) are highly conserved amongst lipases and cutinases (Brady *et al.*, 1990, Brzozowski *et al.*, 1991, Schrag *et al.*, 1991 and Martinez *et al.*, 1992) and where these and the first cysteine residue is conserved amongst serine proteases (Thornberry *et al.*, 1992 and Aitken, 1990a). These classes of enzymes are also known to have additional histidine

Figure 5.6 Secondary structure predictions for PgaA.

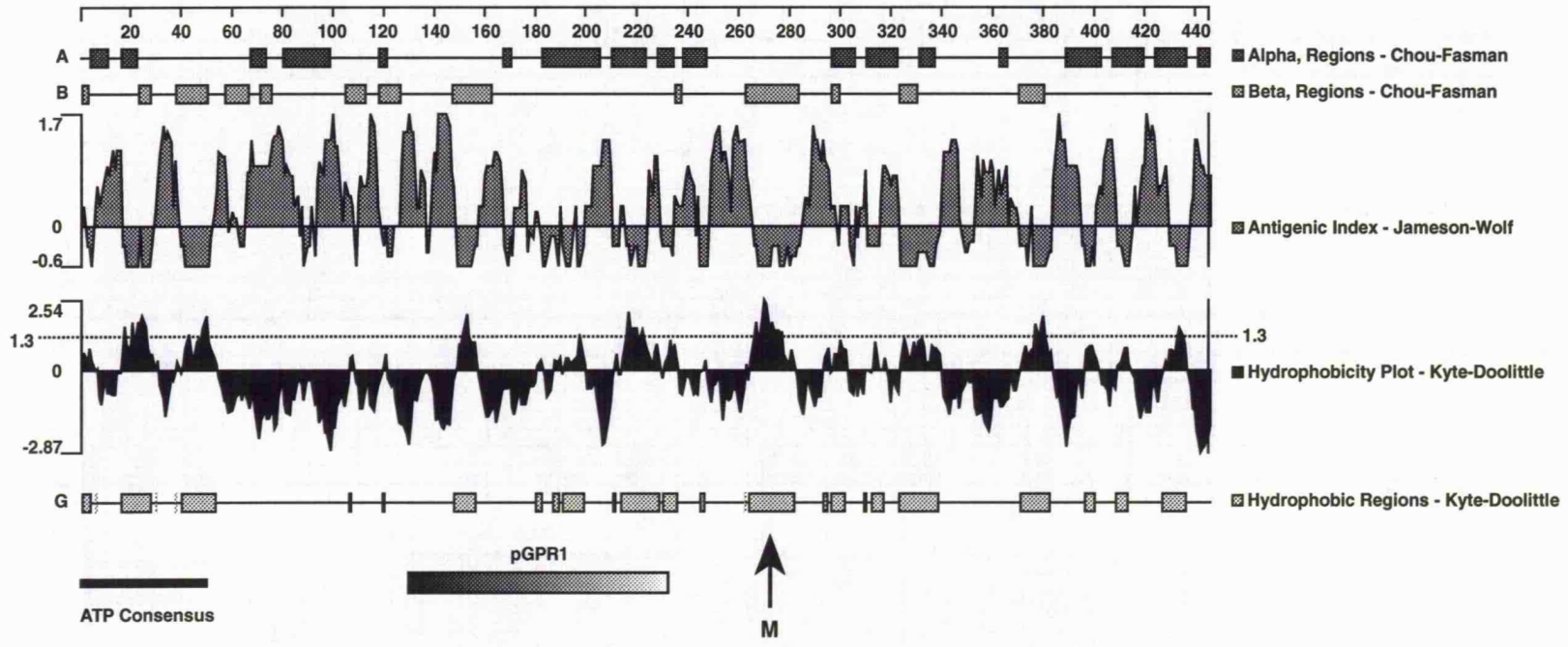


Figure 5.6 Secondary structure predictions for PgaA. Shown are Chou-Fasman α and β structure predictions (Chou and Fasman, 1978), the antigenic index (Jameson and Wolf, 1988) and hydropathy index (Kyte and Doolittle, 1982). The putative ATP-binding region is highlighted along with the position of the potential pGPR1 insert coding region. Also highlighted is the potential transmembrane domain (capital M).

and aspartic/glutamic acid residues in their active sites (Schrag *et al.*, 1991) and whilst the hypothetical ORF199 amino acid sequence contains such residues, it was not possible to identify additional active site consensus sequences in the ORF199 primary structure.

Since PgaA is predicted to react with mAb raised against the TLP of *P.gingivalis* yet shows no sequence similarities to known proteases and does not contain protease active site consensus sequences, whilst ORF199 contains the active site consensus for serine proteases, it is possible that ORF199 may have been generated due to aberrations in the DNA sequence and in fact be part of the PgaA coding sequence. In order to test this hypothesis a 0.9 kb fragment of DNA was amplified from the *P.gingivalis* (W83) chromosome using PCR with primers located upstream and downstream of the termination codon of PgaA and the start codon of ORF199. Double-stranded cycle sequencing of this PCR product using the aforementioned PCR primers to direct second-strand synthesis and labelling confirmed the translational stop/start organisation outlined in figure 5.4, detecting no inconsistencies in the DNA sequence of this region.

5.2.4 Sequence analysis of ORF 168, a putative *rnh* gene of *P.gingivalis*.

The start of ORF168 precedes the start of the cloned insert of pGPR2 and hence is lacking a translational start point and N-terminus. Analysis of the available predicted protein sequence reveals ORF168 to encode a protein of at least 19.2 kDa (168 residues). Comparison of this sequence with those of the protein databases shows ORF168 shares a 44.9% identity with the RNaseHII protein of *E.coli* over a 156 amino acid overlap (figure 5.7). This protein homology is consistent with the range of nucleotide homology shown by the corresponding region of pGPR2 with the *E.coli Rnh*B allele which codes for RNaseHII (section 5.2.2). Proteins of the RNaseH type degrade the ribonucleotide moiety on RNA-DNA hybrid molecules (Itaya *et al.*, 1990) such as the RNA primers for Okazaki fragment synthesis during chromosomal replication. There are two such RNases in *E.coli* which are unrelated in amino acid sequence. The *P.gingivalis* ORF168 only shows sequence homology to RNaseHII. Given the high level of similarity between RNaseHII and ORF 168, I suggest that ORF168 be referred to as *rnh*B_{LG} (*rnh* B-like gene) until the full extent of the coding sequence of *rnh*B_{LG} is determined and the biological activity characterised. Similar molecular weights of *rnh*B_{LG} (19.2 kDa) and

Figure 5.7 FASTA alignment of ORF168 with the Rnase HII protein of *E.coli*.

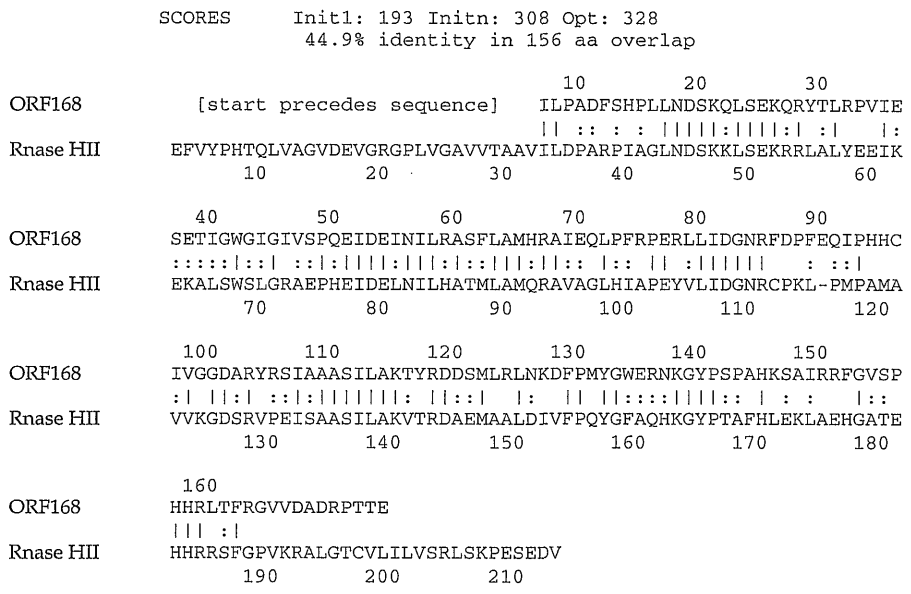


Figure 5.7 FASTA alignment of ORF168 with the Rnase HII protein of *E.coli*. Alignments were carried out using the method of Lipman and Pearson (1985). Positions of amino acid identities shared by ORF168 (*rnh*B_{LG}) and Rnase HII are denoted by vertical bars whilst conservative changes are denoted by colons. Amino acid co-ordinates are shown above or below the appropriate residues.

RNaseHII (22.5 kDa) suggest that only a small proportion of the *rnhB*$_{LG}$ cistron is absent in pGPR2.

5.2.5 Sequence analysis of ORFs 150, 297 and 202.

ORF150 occurs approximately 250 bp pairs downstream of *rnhB*$_{LG}$ (figure 5.3). The putative start codon is a methionine and is eight bp downstream of a strong Shine-Dalgarno consensus sequence for translational initiation (Shine and Dalgarno, 1974). A FASTA analysis of the predicted amino acid sequence ORF150 revealed that ORF150 shares significant sequence homology with several prokaryotic proteins (figure 5.8a) two of which, namely the Gef resistance protein (also known as ORF178) and the RibG protein are of known function. The gene *rib*G is a component the *rib* operon which encodes proteins for riboflavin synthesis in *B. subtilis*. RibG catalyses the conversion of 2,5-diamino-6-(ribosylamino)-4(3H)-pyrimidinone 5'-phosphate into amino-6-(ribosylamino)-2,4(1H,3H)-pyrimidinedione 5'-phosphate (Kil *et al.*, 1992). The product of this reaction is phosphatased and condensed with 3,4-dihydroxy-2-butanone 4-phosphate to create the final precursor required for riboflavin synthesis (Richter *et al.*, 1992).

Members of the *gef* gene family (a subset of the *hok* killer gene family which is defined using protein and DNA sequence similarities; Gerdes *et al.*, 1990) control plasmid maintenance in Gram-negative bacteria by killing plasmid-free segregants after cell division. The *gef* gene itself is located on the chromosome of *E.coli* and Gef is only toxic when overexpressed from multicopy plasmids. However, in that Gef is not plasmid encoded (unlike other members of the *gef* gene family) and therefore not involved in plasmid maintenance its function remains ambiguous (Gerdes *et al.*, 1990). ORF178 encodes protein of unknown function which is predicted to be membrane associated and can under certain circumstances confer resistance to Gef killing.

Multiple alignments of ORF150 and its homologues show ORF150 and 17 kDa ORF from *B.subtilis* contain a number of highly conserved residues. Also apparent on the multiple alignment is the presence of a conserved cluster of basic residues which would give the C-terminus of these proteins a strong net positive charge.

Figure 5.8 a Protein FASTA scores for ORF150.

ORF150 Homologue	FASTA Score (% identity/No residues)	Reference.
ORF178 *Escherichia coli*	40.8%/142 aa	Poulsen et al, 1992
17 kDa ORF (*scr* locus) *Bacillus subtilis*	43.2%/148 aa	Struck et al, 1990
ORF 2 (*trz* A locus) *Rhodococcus corallinus*	30.3%/109 aa	SeffensW., unpublished
RibG *Bacillus subtilis*	28.9%/142 aa	Sorokin et al, 1993.

Figure 5.8b Multiple alignment of ORF150 and its protein homologues.

```
ORF150 P.gingivalis     MTA---------PT----FATDDIRY-----MR-IALEEARAADEGEIPIGAVIVCKGQIV
ORF178 E.coli           MRR---------AFITGVFFLSEVEFSHEYWMR-HALTLAKRAWDEREVPVGAVLVHNNRVI
17 kDa ORF B.subtilis   MTQ-----------------------DELYMK-EAIKEAKKAEEKGEVPIGAVLVINGEII
ORF2 R.corallinus       LQRKRLEAQNSGSPVTAKHPMATQRYAAETAMLAVALEEAHIGLAEGASDWRSIFTLDGELV

                                    •
ORF150 P.gingivalis     ARAHNRVERLNDETAHAEMLAITMAVDAIGGKYLRDCTLYVTVEPCLMCAGALRWTQIPRVV
ORF178 E.coli           GEGWNRPIGRHDETAHAEIMALRQGGLVMQNYRLIDATLYVTLEPCVMCAGAMIHSRIGRVV
17 kDa ORF B.subtilis   ARAHNLRETEQRSIAHAEMLVIDEACKALGTWRLEGATLYVTLEPCPMCAGAVVLSRVEKVV
ORF2 R.corallinus       SRGHNQRIQDNDPSSHGETDAFRNAGR---HTTWRDKILVTTLAPCWYCTGMVRQFGFAKVV

                                    •
ORF159 P.gingivalis     YGASEPKVGYR-----LFTDHALHPKCLVEGGILADESENLMRSFFAERR------------
ORF178 E.coli           FGARDAKTGAAGSLMDVLHHPGMNHRVEITEGILADECAALLSDFFRMRRQGIKAQKKAQSS
17 kDa ORF B.subtilis   FGAFDPKGGCSGTLMNLLQEERFNHQAEVVSGVLEEECGGMLSAFFRELRKKKKAARKNLS-
ORF2 R.corallinus       MGETVNQPPPHG--------YDWLRELGVDIVDLGSPECIELLASYSA---REPDAWAEDGGQ
                                                          **  Cluster of  **
                                                          ** Basic Residues **

ORF150 P.gingivalis     --
ORF178 E.coli           TD
17 kDa ORF B.subtilis   -E
ORF2 R.corallinus       QW
```

Figure 5.8 (a) Protein FASTA scores for ORF150. Shown are the percentage of identical residues shared between ORF150 and the test sequence when aligned using the FASTA search tool (Lipman and Pearson, 1985). **(b)** Multiple alignment of ORF150 and its protein homologues. Amino acid sequences of proteins shown in figure 5.8a (excluding the RibG protein which is least similar to ORF150 and considerably longer than the other proteins of the alignment) were subject to multiple alignement to demonstrate residues common amongst all four proteins. Residues identical amongst all four proteins are shown in bold typeface while positions showing amino acid changes which are of a conservative nature are shown underlined. A conserved C-terminal cluster of basic residues is shown in italics (not found in ORF2 *R.corallinus*). The aspartate residue important in Gef resistance (ORF178) is shown using a bullet.

The 17 kDa ORF (figure 5.8a) is situated upstream of the gene encoding the small cytoplasmic RNA (*scr*) from *B. subtilis* (Struck *et al.*, 1990). Small cytoplasmic RNA is a stable, abundant RNA postulated to be involved in protein synthesis and targeting of secretory proteins to the membrane (Struck *et al.*, 1990). It is not known whether the 17 kDa ORF and small cytoplasmic RNA are functionally related. ORF2 (figure 5.8a) neighbours the s-triazine hydrolase gene (*trz*A locus; Steffens, unpublished results; accession L16534) from *Rhodococcus corallinus* which degrades compounds of the s-triazine herbicide group. These herbicides are important in weed control programs for major crops in the United States but are stable and accumulate in the environment, and hence bacterially mediated degradation of these compounds is of importance (Mulbry *et al.*, 1994). However the function of ORF2 from *R. corallinus* is not known.

Analysis of the hydropathy of ORF150 revealed the presence of three short (around 10 aa) strongly hydrophobic stretches (with a mean hydropathy > - 2; data not shown). Although these hydrophobic stretches are probably not long enough to be membrane-spanning, given their strong hybrophobicity it is possible that these may be involved with peripheral membrane interactions. A similar hydropathy profile has also been noted in the Gef resistance protein ORF178 (Poulsen *et al.*, 1992), and as the Gef protein itself is also predicted to be a membrane protein it has been speculated that Gef and the Gef resistance protein might interact with each other directly (Poulsen *et al.*, 1992).

Translation of ORF297 results in a predicted protein of 33.5 kDa molecular weight. FASTA analysis of the ORF297 predicted amino acid sequence revealed a 19.9 % identity over 286 aa shared with the arylsulfatase protein (Ats) of *Alteromonas carragenovora* (T. Barbeyron, unpublished results). Although the percentage identity of this score is a little low, the long range and the high content of conservative amino acid substitutions between the two proteins which show a 45.6% similarity when compared using the BESTFIT tool (figure 5.9) make it likely that this represents a significant FASTA homology. Hydrophobic cluster analysis (HCA)(Gaboriaud *et al.*, 1987) revealed a significant conservation in hydropathy for the two proteins as well as conserved clustering of histidine residues in the N-terminal half of each sequence (T. Barbeyron, personal communication). HCA analysis presents amino acid sequences as 2D columns of stacked, opened out helices

Figure 5.9 Bestfit comparison of ORF297 (*P.gingivalis*) and the Ats protein of *Alteromonas carragenovora.*

```
ORF297 P.gingivalis     1 MAAFSVHILGCGS--------------ALPTTHHHPSSQVIDLRDKLYMI 36
                          ::.:.. : |::|               ::|...| .....: :.:. |::
Ats A.carragenovora    14 LGCLAFTFNGSASETKNEWITLGTMAGPIPNAKHSQPANAMLVNGNTYVV 63

ORF297 P.gingivalis    37 DCGEGVQRQFRHEKLHFGRLIHIFISHLHGDHCFGLPGFISTLGLLGRTG 86
                          |.|:|.. |: .  |.: .:  :|:|||| ||. |||:::|     :  .
Ats A.carragenovora    64 DAGDGTAGQLAKVGLDIKNVDAVFLSHLHFDHTGGLPAILSLRWQTSARN 113

ORF297 P.gingivalis    87 TLHVHGPEGIERFLSPILEQFC------HRMPYQVEIHTIDASRHALVHE 130
                          .| |.||.|.:. :..|:| :.       . :| ||. .. .. : . |.:
Ats A.carragenovora   114 ELVVYGPPGTQQTVDGIFEYMTYGTLGHYGVPGQVPAPANTNIKVVEVED 163

ORF297 P.gingivalis   131 DKSVKVYSIPLSHRIPAVGYLFEEKCRARHLNKAAAEFYNIPLAEYPLII 180
                          :. :|: .:.: . |..  | :.. : .:  .| .|  :.:.|.:|.::.
Ats A.carragenovora   164 GTQLKLPDFTV.DVIRNSHYSWPKGSEEW--KKFQALSFKFSLQDYTVVY 210

ORF297 P.gingivalis   181 EGSDYTTPDGRIIPNRHLTTPGTPPRRYAYCSDTEFCPSIVPIIQGVDLL 230
                          .|.               |.|:.:. :.. . |  :...:.| :.|:::
Ats A.carragenovora   211 TGD--------------TGPSSAVEKLSSGVDL-LVSEMMDIDHTVNMI 244

ORF297 P.gingivalis   231 YHEATFMEEDR----ARAKETFHSTAKEAAEIARQAGAKRLLIGHYSGRY 276
                           .... |..::     :  .. | .:|:.:|:|:.|.. .|:|.| .. .
Ats A.carragenovora   245 KETNPQMPKGKFIGIHKHLSKHHLSPKQVGELAKAANVGSLVITHMAPGL 294

ORF297 P.gingivalis   277 KDVQGLLGRSSKRIQANY 294
                            |.|: ::  .|.:..:|
Ats A.carragenovora    295 -DTQAEIDFYTKQVASEY 311

 Percent Similarity: 45.620   Percent Identity: 21.168
```

Figure 5.9 Bestfit comparison of ORF 297 (*P.gingivalis*) and the Ats protein of *Alteromonas carragenovora.* Shown are pairwise comparisons for the two protein sequences with gaps introduced to optimise the alignment (dashed lines). Positions of residue identities are shown by vertical bars whilst colons denote conservative divergence between amino acids. Full stops denote positions of residue divergence which do not result in conflicting amino acid side chain charge. Protein coordinates are given at the begining and end of each line. Strongest homology between the two proteins occurs between ORF297 coordinates 37 and 107 an area with which ORF297 also shows strong homology to the Ats protein of *Mycobacterium leprae* (data not shown).

with hydrophobic residues boxed. Visual inspection of HCA plots allows cluster identification and infers structural conservation between proteins which do not show high levels of sequence identity (Saxena *et al.*, 1995). ORF297 also shares 24.7% identity over 77 amino acids with an arylsulfatase from *Mycobacterium leprae* which also shares cluster conservation compared with the Ats and ORF297 predicted amino acid sequences (the *Mycobacterium* genome sequencing project cosmid B1549). The *A.carragenovora ats* gene product has been shown to have a specificity for phenol sulphates and has a N-terminal leader sequence for directing transport into the periplasm (Barbeyron, unpublished results). Use of the SIGSEQ and SIGPEP search tools (von Heijne, 1986) reveals an apparent lack of conventional signal sequence in the predicted protein sequence of ORF297. However, analysis of the hydropathy profile reveals the presence of an N-terminal hydrophobic stretch which might function as a membrane anchor (figure 5.10).

ORF202 encodes predicted a 22.5 kDa protein with homology to several bacterial proteins (figure 5.11a) two of which have functions associated with the murein sacculus. Endopeptidase II (γ-D-glutamyl-L-diamino acid endopeptidase II) from *Bacillus sphaericus* is found only in the cytoplasm of sporulating cells and is thought to be important in the recycling of cell wall peptides during sporulation. It is a cysteine protease with a specificity for terminal L-ala-γ-D-glu-L-Xaa-Y units where L-Xaa represents L-lys, *meso*-diamino pimelate or ω-substituted *meso*-diamino pimelate; and Y is H D-ala or D-ala-D-ala (Hourdou *et al.*, 1992). Endopeptidase II has a strict requirement for terminal L-ala, and it is thought to be involved in hydrolysis of peptidoglycan crosslinks as part of an interdependent chain of peptidoglycan hydrolase activities.

The extracellular invasion associated protein (Iap) found in *Listeria* species, also known as p60 (figure 5.11a) has also been shown to have murein hydrolysing activity in cells expressing p60 and is by definition an autolysin (Wuenscher *et al.*, 1992). What is more, p60 is also capable of disrupting chains of *B.subtilis* cells and illiciting bacteriolytic activity. ORF202 is only homologous to the C-terminal ends of p60 and endopeptidase II (figure 5.11b) and may indicate the presence a common module within the larger p60 and endopeptidase II proteins. Notably, non of the aforementioned proteins exhibit consensus elements common amongst cysteine or serine proteases. However, site specific neutralisation of the single cysteine found in p60,

Figure 5.10 Hydrophobicity plot for ORF297.

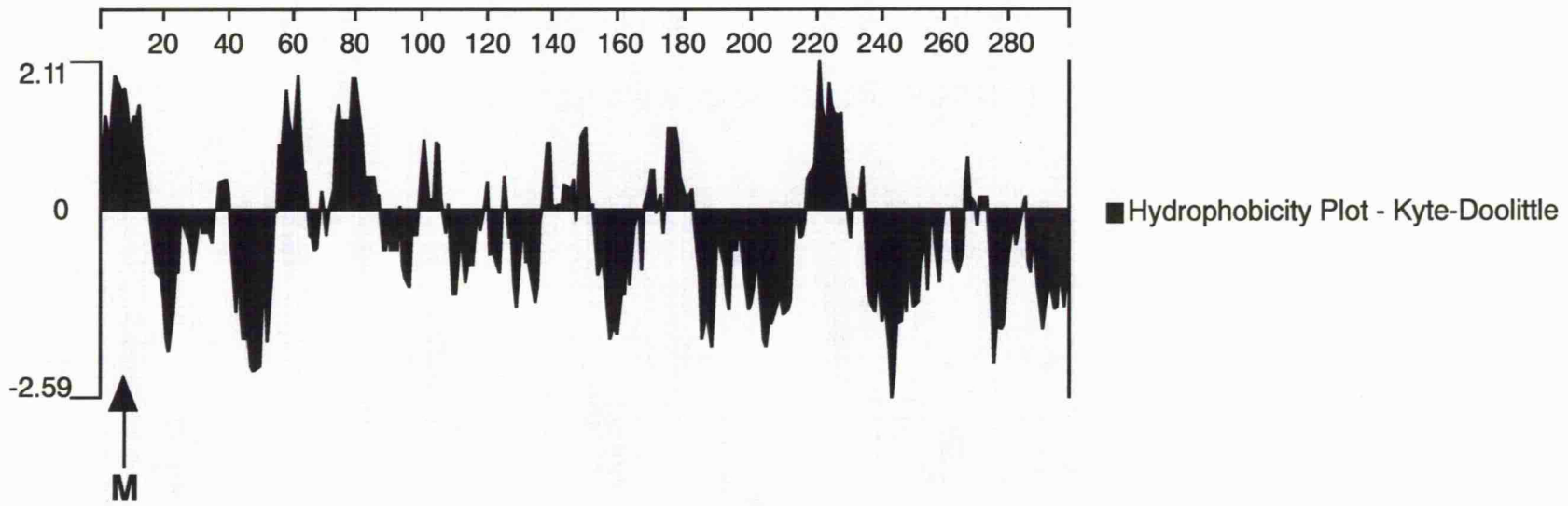


Figure 5.10 Hydrophobicity plot for ORF297. The hydropathy profile of ORF297 was constructed according to the method of Kyte and Doolittle (1982) using a window of 9 residues in order to accentuate the N-terminus. A possible N-terminal transmembrane domain is highlighted using a capital "M".

Figure 5.11a Table showing protein FASTA scores for ORF202.

ORF202 Homologue	FASTA Score (% identity/No residues)	Reference.
StarchDegrading Enzyme (SDE) *Clostridium acetobutylicum*	28.4%/88aa	Verhasselt, unpublished (Accession S31940)
p60 *Listeria species*	28.7/115aa	Kuhn *et al.*, 1988.
p54 *Enterococcus faecium*	27.2%/81aa	Fuerst *et al.*, 1989.
ORF17 *Escherichia coli*	26.5%/132aa	Friedrich *et al.*, 1986
endopeptidase II (EDP2) *Bacillus sphaericus*	27.3%/101aa	Hourdou *et al.*, 1992.

Figure 5.11b Multiple alignment of ORF202 and its protein homologues.

Figure 5.11 (a) Table showing protein FASTA scores for ORF202. Shown are the percentage of identical residues shared between ORF202 and the test sequence when aligned using the FASTA search tool (Lipman and Pearson, 1985). **(b)** Multiple alignment of ORF202 and its protein homologues.Amino acid sequences of proteins shown in figure 5.11a (excluding the SDE protein which strongly disrupts the alignment of conserved residues) were subjected to multiple alignment to demonstrate residues common to these protein homologues. Residues identical amongst all five proteins are shown in bold typeface while positions showing amino acid changes which are of a conservative nature are shown underlined. Protein coordinate start points are shown in parenthesis. The highly conserved cysteine residue crucial for p60 bacterial chain length disruption is shown using a bullet.

which is highly conserved amongst *Listeria* species variants, and which is conserved amongst all of the ORF202 homologues (figure 5.11b), results in ablation of the bacterial chain disruption properties of p60 (Wuenscher *et al.*, 1993). Given the conserved nature of this cysteine residue and its importance for enzyme activity it is tempting to speculate that this, and the surrounding highly conserved residues (figure 5.11b), may contribute to a hydrolase active site (Wuenscher *et al.*, 1993).

P.gingivalis ORF202 also shares homology with two proteins of unknown function namely p54 from *Enterococcus* (*Streptococcus*)*faecalium* and ORF17 from the *btu*CED operon of *Escherichia coli.* (figure 5.11b). p54 also contains a single conserved cysteine residue, and is homologous to ORF202 in its C-terminal. This protein is found in the bacterial cell envelope and its central region also shares tandem repeat homology with group I *Streptococcal* M proteins. ORF17 is found down stream of the *btu*CED operon whose protein products are involved in ATP-coupled periplasmic-protein-dependent import of vitamin B_{12}. However, ORF17 was shown not to be required for B_{12}-uptake and hence its function remains a mystery although sequence analysis has shown that it likely to posses a cleavable N-terminal signal sequence and lipoprotein processing site (see figure 5.12) and hence is likely to occur in the periplasm, outer membrane or as cell surface or extracellular protein (Friedrich *et al.*, 1986, and Pugsley *et al.*, 1990).

The region conserved amongst ORF202 homologues is also homologous to a starch degrading enzyme from *Clostridium acetobutylicum.* This enzyme shows good sequence identity with ORF202 although it does not align well in multiple alignments and hence is not shown in figure 5.11b. This may indicate that SDE represents a more distantly related member of the family of ORF202 sequence homologues although it does show conservation of the core residues DCSG and PGDL. SDE is an α-amylase (Verhasselt *et al.*, 1989) and contributes to the fermentation of starch by this organism by degrading its carbohydrate backbone.

Analysis of the hydropathy characteristics of ORF202 show the presence of an N-terminal hydrophobic stretch of sufficient length and hydropathy index so as to constitute a potential transmembrane domain (figure 5.12a; Doolittle, 1986). However a MOTIFS search revealed the presence of a consensus prolipoprotein processing site (figure 5.12a and b) at the N-terminus of

Figure 5.12 a Identification of a putative lipoprotein processing site in ORF202.

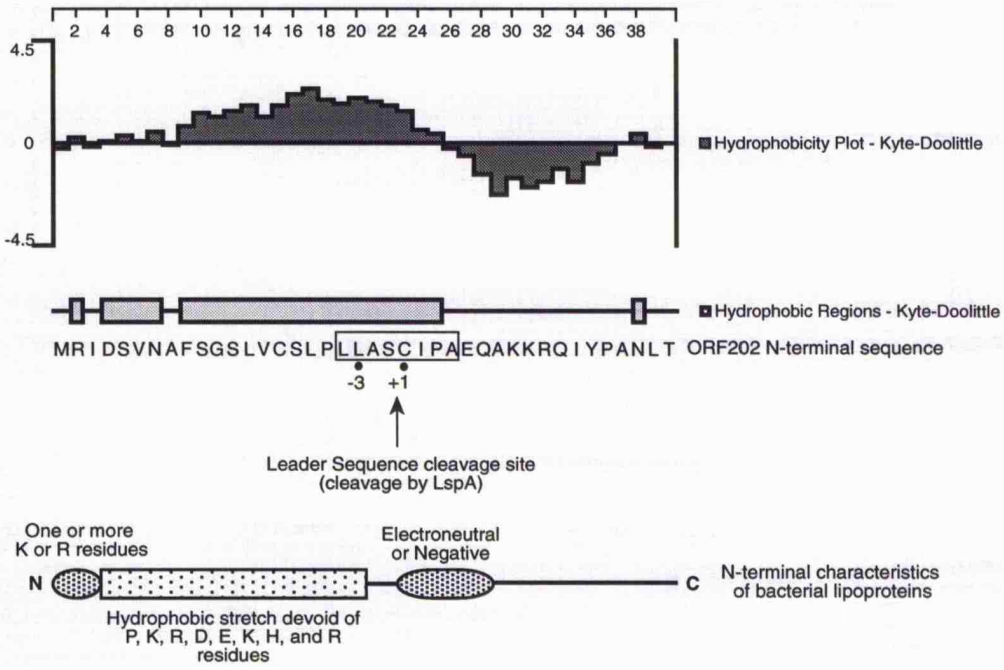


Figure 5.12b Hypothetical Processing of the ORF202 lipoprotein after cleavage.

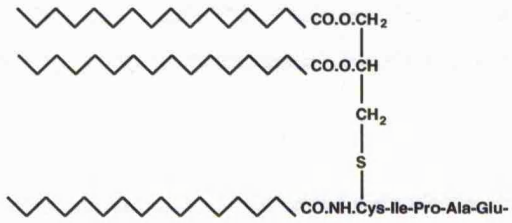


Figure 5.12 (a) Identification of a putative lipoprotein processing site in ORF202. The hydropathy characteristics of the ORF202 N-terminus are shown along with the corresponding amino acid sequence. A window of 1 was used for the hydropathy profile in order to accentuate terminal residues. Underneath is shown charge and hydropathy characteristics typical of bacterial lipoprotein precursers. The recognition site for cleavage is boxed and conforms to the consensus sequence LLAGCSSN where C at position 1 is an absolute requirement, and residues at positions -2, -1, +2, and +3 can be S, A, N or other neutral amino acids (Nielsen *et al.*, 1982; Aitken *et al.*, 1990)). **(b)** Hypothetical Processing of the ORF202 lipoprotein after cleavage. After proteolytic cleavage by LspA the resultant free C residue at the N-terminus of ORF202 is covalently linked via its free sulphur group to glycerol which is in turn acylated at two positions with, most frequently palmitic acid. In addition the free N-terminal amine is also fatty acylated. These lipid attachement intercalate directly with the membrane thus anchoring the lipoprotein at the surface (Aitken, 1990).

ORF202 suggesting that ORF202 encodes a lipoprotein. The role of the hydrophobic stretch and surrounding recognition elements in such proteins is to target the inner membrane and direct Sec-dependent transport of the bulk of the protein into the periplasmic space so that the protein is anchored to the inner membrane by the hydrophobic stretch (figure 5.12a). Additional features in the lipoprotein processing site then aid recognition by processing factors and participate in N-terminal cleavage by lipoprotein signal peptidase (LspA) and ligation to a fatty acid anchor (Pugsley *et al.*, 1993). It is not known, however whether or not *P.gingivalis* possesses mechanisms for lipoprotein processing as found in *E.coli* and other bacteria.

5.3 Discussion

Described above is the nucleotide sequence of a gene coding for an antigenic determinant designated PgaA. DNA sequence upstream and downstream of this gene was also determined in order to identify ORFs transcribed along with *pga*A. This was important in that functionally related genes in bacteria are often clustered and for two main reasons. First, polycistronic transcripts such as those found in the *Escherichia coli* haemolysin operon (Koronakis *et al.*, 1988) and the *Klebsiella oxytoca* pullulanase (Pugsley *et al.* 1990) allow co-ordinated expression of many proteins (upto 13 proteins for the long transcript in the pullulanase operon) with expression being regulated by altering the level of production of these large transcripts. Secondly in pathogenic bacteria it has been argued that certain loci related to virulence may be more prone to mutation so as to constitute the existence of a reservoir of adaptability to environmental changes (Moxon *et al.*, 1994). An example of this can be seen in the *kps* genes for group II capsule production in *E.coli* where two conserved gene regions flank a hypervariable region responsible for the generation of diversity of capsular antigens (Roberts *et al.*, 1988).

The presence of *pga*A as one gene in a locus of related genes was not confirmed by DNA sequence analysis as the function of genes identified in this study is not clear. Given the likelihood that *pga*A and ORF199 are translationally coupled and probably on the same transcript it is possible that their functions might also be related. It is also likely that ORFs 150, 297, and 202 are also functionally related for the same reasons. However there is no

link between ORFs 150, 297, and 202 and the *pga*A gene clusters and hence no reason to suppose that these are related.

Analysis of the DNA sequence 5' of *pga*A revealed the presence of a large (767 bp) non-coding region. Non coding regions of this size are rare in bacteria and have been termed "grey holes" (Daniels *et al.*, 1992). It has been suggested that they may represent gene remnants or alternatively encode for small proteins. However the DNA sequence upstream of *pga*A revealed the presence of two interesting features. The first is the presence of an inverted repeat possibly constituting the presence of a large stem loop structure predicted upstream of the *pga*A coding sequence. This stem loop would have a long stem (19 bp) and an extremely long loop of around 100 bp. Extragenic palindromic elements of this type are common in *E.coli* and have been postulated to be involved in maintaining chromosomal architecture, regulating gene expression, transcriptional termination and even DNA gyrase binding (Daniels *et al.*, 1992; Kil *et al.*, 1992). Alternatively transcriptional terminators in bacteria commonly feature such structures although the "stems" and "loops" are much shorter than the *pga*A palindrome and it difficult to see why a terminator should be present at the beginning of a transcript (Brendel and Trifnov, 1984). Stem-loops are also present at the 5' terminal of mRNA have been demonstrated to enhance transcript stability by blocking exoribonuclease activity (Bouvet and Belasco, 1992).

An element with a similar structure to the *Pga*A inverted repeat has been described upstream of the structural gene for type 1 fimbriae (*fim*A) in *E.coli*. This element consists of a 314 bp chromosomal DNA segment carrying the *fim*A promoter which is bordered by two 9 bp inverted repeats (Dorman and Higgins, 1987). Excision and reintegration by site-specific recombinases results in the *fim*A promoter firing into or away from the *fim*A gene and hence operating as a genetic switch controlling the expression of *fim*A. However the recombinase genes for this inversion are encoded upstream of the switching element (Dorman and Higgins, 1987) and no such ORFs are observed upstream of *Pga*A and hence *Pga*A does not closely resemble the *fim*A genetic switch.

The second DNA sequence feature upstream of *pga*A is a second smaller completely palindromic sequence (figure 5.2b) whose close dyad symmetry would not allow for the formation of a loop in the "stem-loop" type structure.

This type of palindrome is similar to operator sequences where DNA binding proteins recognise and bind DNA thus regulating transcription. Examples of this phenomenon can be found in the *lac* , *trp*, and *cro* operators (reviewed in Stryer, L., 1985). The position of this palindrome just upstream of *pga*A is consistent with this being an operator site for expression of *pga*A.

The molecular weight of the predicted PgaA (50kDa) protein is roughly consistent with the molecular weight of the LDS28-reactive protein expressed from clone pGPR7 (around 46 kDa; chapter 4 section 4.2.7) and the LDS28-reactive antigen seen in the outer membrane and gingivain preparations of *P.gingivalis* (again around 46 kDa; Wallace *et al.*, 1992). The small discrepancy in predicted and observed molecular weight could be due to the high hydrophobic amino acid content of PgaA. Certain hydrophobic amino acids bind increased amounts of SDS in SDS-PAGE resulting in an increase in protein electrophoretic mobility and reduction in predicted molecular weight (See and Jackowski, 1989).

Analysis of PgaA sequence did not yield data consistent with PgaA being a protease and this is consistent with the lack of BAMCA cleavage activity seen in subclones of pGPR2. However, all proteases do not conform to protein sequence consensus. Bacterial proteinases such as γ-D-glutamyl-L-diamino acid endopeptidase II from *B. spaericus* (described above; Hourdou *et al.*, 1992) a cloned trypsin-like protease and a cloned collagenase from *P.gingivalis* (Otogoto *et al.*, 1993; Kato *et al.*, 1992) have no known proteinase consensus sequence and little similarity to any other known proteases. However the downstream 22 kDa ORF199 does show a consensus for serine protease and cutinase active sites.

As the LDS28 antigen was shown to localise to the outer membrane in *P.gingivalis* (Wallace *et al.*, 1992) it follows that the PgaA protein sequence should exhibit signals for targeting the outer membrane. PgaA does not posses the classical N-terminal leader sequence for transport across the cytoplasmic membrane. This however is not an absolute requirement. Examples of Sec independent protein transport across the inner membrane include the porins (von Heijne, 1994), inner and outer membrane Tra proteins of the F sex factor of *E.coli* (Achtman *et al.*, 1979), Vitamin B_{12} transport protein BtuE (Friedrich *et al.*, 1986) and *Serratia marcescens* α-lytic protease and *E.coli* haemolysin (Pugsley *et al.*, 1990 and Pugsley, 1993). In the last two

examples a number of accessory proteins mediate secretion into the medium and it is possible that *P.gingivalis* may have similar uncharacterised secretory mechanisms. Other proposed secretory pathways include protein transport between inner and outer membrane *via* Bayer adhesion sites where porins (Smit and Nikaido, 1978) and penicillin binding protein 1b (Bayer *et al.*, 1990) have been shown to be translocated from inner to outer membrane. An example of evidence for an alternative secretory mechanism might be seen in the fimbrial subunit of *P.gingivalis* which has an extremely short N-terminal cleavage fragment (11 amino acids) which is not hydrophobic (Dickinson *et al.*, 1988) and does not resemble the bacteriocin-type leader sequence for Sec-independent ABC transporter-mediated secretion and processing (Havarstein *et al.*, 1995). Interestingly of the *P.gingivalis* proteases whose genes have been cloned and sequenced so far, none show features of the classical signal sequence (von Heijne, 1986) yet in *P.gingivalis* protease activity is found on the cell surface and in the surrounding medium (Kato *et al.*, 1992; Otogoto *et al.*, 1993; Park and McBride, 1993).

The presence of a putative transmembrane domain in PgaA is consistent with PgaA being a membrane protein. Secondary structure predictions (Chou and Fasman, 1978) suggest this stretch may be comprised of β-sheet forming residues. This is inconsistent with an inner membrane localisation where transmembrane spanning domains tend to be of the α-helices type conformation (Branden and Tooze, 1991). However β-type transmembrane domains are common in outer membrane proteins, especially porins (Cowan *et al.*, 1992) and this may suggest an outer membrane localisation for PgaA. It is unlikely that PgaA adopts a porin-like outer membrane conformation in that porins are composed of 16 anti parallel β-stretches. Another example of β-stretches in the outer membrane is seen in the Ton B protein (a periplasm-spanning protein involved in several solute uptake systems in Gram-negative bacteria; Klebba *et al.*, 1993). The outer membrane component of Ton B has been demonstrated to reside in a single C-terminal βαβ–rich transmembrane structure where hydrophobic residues of the β–strands are exposed to the membrane. It is possible that the β–rich hydrophobic stretch of Pga A may fulfil a similar role in the outer membrane. It must be stressed that care should be taken when examining structure predictions for transmembrane segments in that the measurements for predicting α/β forming characteristics of individual residues were taken from soluble proteins and are not

necessarily accurate for amino acids in the apolar phase (Chou and Fasman, 1978).

One puzzling feature in the sequence of PgaA is the presence of a kinase/ATP-binding consensus similar to that of the RecF DNA metabolism mediator. This enzyme is essential for the RecF DNA recombination repair pathway. Mutations in the RecF gene result in bacteria which that are conjugally recombination proficient but deficient in plasmid recombination and unable to efficiently induce an SOS response (Sandler *et al.*, 1991). RecF is believed to require ATP for its role in DNA metabolism. As mentioned it is unlikely that *pga*A represents a *rec*F gene of *P.gingivalis* although it does however posses a very similar ATP-binding consensus domain. Generally speaking ATP is used in energy dependent biological processes such as metabolism, cell signalling and inner membrane transport; ATP or ATP-binding proteins are not found in the periplasmic space. The role of the PgaA kinase/ATP-binding consensus sequence is unknown and whether it functions while the protein is in the cytoplasm remains a paradox. Equally puzzling is the function of PgaA. The sequence of PgaA has shed no light on the problem making further analysis difficult.

The *rnh*B gene of *E.coli* is present on the chromosome at 4.5 min on the genetic map between *lpx*B and *dna*E and encodes one of the two RNA-DNA hybrid specific RNases found in *E.coli.* Putative functions include creation of RNA primer for DNA replication of ColE1 type plasmids, suppression of initiation of DNA replication from sites other than *ori*C (the chromosomal origin of replication), degradation of RNA primers of Okazaki fragments, and involvement in DNA repair and SOS-induced mutagenesis (Itaya *et al.*, 1990). The *lpxBrnhBdnaE* type organisation is also conserved in *Salmonella typhimurium* and the fact that the $rnhB_{LG}$ allele is not neighboured downstream by *dna*E (DNA polymerase holoenzyme III, α-chain; Riley, 1993) suggests that *P.gingivalis* may exhibit a different transcriptional organisation with respect to genes responsible for bacterial cell division when compared to *E.coli* and *S.typhimurium* .

Analysis of the ORF150, ORF297, and ORF202 gene block suggested that these ORFs may be translationally coupled and on the same transcriptional unit and it is thus likely that the functions of the three are inter-related in some way. Their common feature is that they are all predicted to be

membrane-integral in some way. The existence of ORF280 which is predicated to translate from the antisense strand corresponding to the coordinates of ORF297 is puzzling. Given that the predicted amino acid sequence for ORF280 shares no sequence similarities with other known proteins I suggest that the likelihood is that ORF280 may be an artefact. It is however prudent to submit the ORF280 sequence to the database in case this does not turn out to be correct.

Analysis of the predicted amino acid sequence of ORF150 has given the fewest clues as to the function of ORF150. However, the presence of strongly conserved amino acids amongst all ORF150 homologues is intriguing and suggests a conserved structure/function for these proteins. The least related protein of these homologues, RibG contributes to the synthesis of 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione by performing a deaminase-type conversion (Richter *et al.*, 1992). It is possible that ORF150 and its homologues may function as deaminases or may have a substrate specificities for ribityl or pyrimidine containing compounds.

The Gef resistance protein encoded by ORF178 shows the strongest sequence similarity to the predicted amino acid sequence of ORF150 (figure 5.7a). Whilst the function of ORF178 is unknown, it is postulated to be an inner membrane protein as deduced from the proteins hydropathy profile (Poulsen *et al.*, 1992). The conserved cluster of basic amino acids in the C-terminus of the ORF150 homologues hence might constitute a "stop transfer" signal for these proteins, thus ensuring the correct topology in the inner membrane.

Gef killing is characterised by permeablisation and uncoupling of energy dependent processes of the inner membrane and it is possible that the function of ORF178 might also reside in the inner membrane (Gerdes *et al.*, 1990). The mechanism of Gef killing of plasmid-free segregants relies on tight control of *gef* expression by an antisense RNA. In plasmid or *gef*-free cells this antisense RNA is rapidly degraded and results in expression of the *gef* transcript which is long lived, and whose expression results in death of the plasmid-free cells. It is likely that this mechanism is also responsible for maintaining *gef* on the chromosome (Poulsen *et al.*, 1992).

ORF178 (the Gef resistance gene) was isolated due to its ability to protect from Gef-killing. However, sequence analysis of ORF178 from Gef resistant

and Gef susceptible strains show a polymorphism at position 64 in their predicted amino acid sequences, where only strains with an aspartate at position 64 confer Gef resistance. Knock out mutants of ORF178 do not show Gef resistance and hence it seems that the Asp 64 allele may have a modified function which confers Gef resistance. Interestingly, ORF150 and the 17 kDa ORF of *B.subtilis* both show Aspartate residues at the same relative position in their multiple alignments (figure 5.8b).

ORF297 is predicted to encode a protein of 33.5 kDa. Its primary structure and hydropathic clustering of amino acids bears similarity to the arylsulphatase (Ats) protein of marine bacterium *Alteromonas carragenovora* (Barbeyron *et al.*, in preparation) and hence might also be an arylsulphatase. Arylsulphatases or aryl-sulphate sulphohydrolases (E.C.3.1.6.1) are involved in metabolism of organic sulphur and sulphate and catalyse the hydrolysis of phenolic ester sulphates thus releasing sulphate as a sulphur source. Other arylsulphatase such as human lysosomal arylsulphatase B are specific for carbohydrate sulphates (N-acetylgalactosamine-4-sulphate; Peters *et al.*, 1990) Synthetic substrates include methylumbelliferyl-sulphate and p-nitrophenyl-sulphate. The *ats* gene of *A.carragenovora* encodes a 35.7kDa protein of outer membrane/periplasmic localisation which is thought to be involved in desulphation of carbohydrate seaweed compounds. It is not thought to be important in scavenging sulphate as sea water is abundant in sulphates and expression of the enzyme is sulphate-independent. It is however, believed to be involved in modifying carbohydrates by cleaving off sulphates, hence allowing secondary hydrolysis of carbohydrate for use as a carbon source (T. Barbeyron, personal communication).

Arylsulphatase activities of other prokaryotes have been described. The arylsulphatase gene of *Klebsiella aerogenes* (*ats*A) has been cloned in and the activity of the AtsA protein well defined (Murooka *et al.*, 1990). It hydrolyses arylsulfate esters to aryl compounds and inorganic sulfate and is also specific for methionine and taurine which have no organic ring structure (Azakami *et al.*, 1992). This enzyme however shows no protein sequence similarity to ORF297 or the arylsulphatase of *Alteromonas carragenovora* hence the latter seems to represent a distinct species of arylsulphatase, one to which ORF297 of *P.gingivalis* might belong. Downstream of *K.aerogenes ats*A is *ats*B which is a positive regulator of *ats*A. Expression of *ats*A in *K.aerogenes* is repressed by sulphate and cysteine and activated by monoamine compounds such as

tyramine, and it is believed that in this context arylsulphatase activity is important in scavenging sulphate from the environment for metabolism (Murooka *et al.*, 1990). *Escherichia coli* possesses *ats*AB homologues although arylsulphatase activity is not detectable. However antibodies reactive with *K.aerogenes* AtsA also react with an *E.coli* protein which is regulated in the same fashion as the *K.aerogenes* gene (Daniels *et al.*, 1992).

Sulphatase activities specific for sulphated carbohydrates have been described for several pathogenic Gram-negative anaerobes including *P.gingivalis* (Slomiany *et al.*, 1993). *Bacteroides melaninogenicus* has been shown to have a mucin-degrading sulphatase, whilst in *Prevotella* (formerly *Bacteroides*) *intermedius* a glycosulphatase desulphates glucose-6-sulphate and N-acetylglucosamine-6-sulphate (the latter is the major sulphated carbohydrate in mucin; Roberton *et al.*, 1993). It is speculated that the desulphation of sulpho-mucus glycoproteins and sulphated glycopeptides renders these polymers more susceptible to bacterial degradation (Roberton *et al.*, 1993). In addition enteric pathogen *Bacteroides thetaiotaomicron* has been shown to utilise chondroitin and heparin sulphate (these are component of host extracellular matrix). A genetic lesion in the interconnected chondroitin/heparin sulphate utilisation pathways of this organism resulted in an inability to compete with wild type *B.thetaiotaomicron* in the intestinal tracts of germ-free mice, thus demonstrating the importance of sulphated polysaccharides in bacterial colonisation (Cheng *et al.*,. 1992). Analysis of this transposon mutant revealed the presence of a regulatory gene for chondroitin/heparin utilisation (*chu*R), the encoded protein showing strong amino acid sequence homology with the AtsB gene of *K.aerogenes*. However, the utilisation of chondroitin/heparin sulphate was not due to classical arylsulphatase activity since the ability to catalyse the hydrolysis of phenolic ester sulphates (p-nitrophenol-sulphate) could not be detected. It seems that chondroitin/heparin sulphate degradation in *B.thetaiotaomicron* may be dependent on a sulphatase enzyme with a different catalytic specificity.

In periodontal disease sulphatase levels in the gingival crevicular fluid are recognised as a reliable indicator of periodontal disease progression and until recently it was believed that this sulphatase activity was entirely host-derived (Slomiany *et al.*, 1993). *P.gingivalis* has been demonstrated to produce an extracellular sulphatase capable of removing sulphate esters from glycosphingolipids (Slomiany *et al.*, 1993). These are the structural

components of gingival epithelial cell membranes and are crucial in cellular recognition processes, also participating in cell surface receptor interactions with proteins and proteoglycans of the extracellular matrix. Such perturbation could potentially result in damage to the cemento-epithelial junction, a symptom common in periodontal disease (Schroeder and Attstrom, 1980). The *P.gingivalis* sulphatase was also capable of desulphating proteoglycan rich in chondroitin-4-sulphate and dermatan sulphate with the sulphate ester group on C-4 of N-acetylgalactosamine. It was not, however active against salivary mucin (which has sulphate groups located at C-6 of galactose and C-6 of N-acetylglucosamine). This glycosulphatase has a molecular weight of 37kDa and, allowing for a certain amount of inaccuracy in SDS PAGE prediction of molecular weight and the sequence similarity of ORF150 with the sulphatase from *Alteromonas carragenovora* might be encoded by ORF150. Experiments to verify this speculation are currently under way in the laboratory of Ian Roberts.

The ORF202 shows several interesting protein sequence features, the first of which is that it possesses a putative lipoprotein signal sequence. In lipoproteins, lipoprotein signal peptidase (LspA) recognises key residues and subsequently cleaves off the N-terminal membrane anchor leaving a free cysteine at the protein N-terminus, which is then transacylated with lipid thus re-anchoring the protein to the membrane (Aitken, 1990b; Pugsley, 1993). Proteins of this kind localise to both the inner and outer membrane as well as being secretory. It is thus possible that the ORF202 protein may localise to the periplasmic face of the inner or outer membranes, the outer surface of the cell, or in the surrounding medium. Whilst the mechanism of lipoprotein export in Gram-negative bacteria is not well understood it has been shown that the presence of an aspartic acid residue at position 2 after processing can act as sorting code for specifying an inner membrane localisation (Pugsley, 1993) This is not true for *Klebsiella oxytoca* pullulanase where an aspartate residue at position 2 directs outer membrane localisation (Poquet *et al.*, 1993). ORF202 lacks such a sorting signal and hence by default may have an outer membrane localisation (figure 5.11a). Proteins of this sort are postulated to find their way to the outer membrane by a number of mechanisms. Transfer through points of inner/outer membrane contact by lipid flow have been suggested (Bayer *et al.*, 1990) along with mechanisms involving specific carrier proteins (Pugsley, 1993). Subsequently outer membrane lipoproteins could be released by proteolysis, through miscellation or by formation of ECV (Pugsley, 1993).

ORF202 is predicted to encode for a protein homologous with several proteins with degradative enzymic activities, namely Endopeptidase II from *B.spaericus* and the p60 protein found in *Listeria* species. Both of these enzymes are apparently hydrolases of peptidoglycan in Gram-positive bacteria. What is more Endopeptidase II has a strict requirement for terminal L-ala (Hourdou *et al.*, 1992) which is extremely rare in proteins. This implies that Endopeptidase II would also be incompatible with, for example, proteins containing D-ala residues; this really narrows the scope of this enzyme's specificity beyond that of peptidoglycan. The exact specificity of the p60 protein has not been determined, but it could also be specific for peptidoglycan sugar-amino crosslinks.

The p60 protein is large and seems to have a distinct tripartite domain structure with N and C-terminal domains separated by an extended, variable threonine-arginine repeat region (Kohler *et al.*, 1990). The N-terminus possesses a signal sequence and the C-terminus a domain conserved amongst *Listeria* species and *P.gingivalis* ORF202 homologues. It seems likely, given the conserved nature of the p60 C-terminus and its homology with Endopeptidase II, that the peptidoglycan hydrolase activity resides in this region. This belief is strengthened by the observation that sulfurhydryl blocking agents specific for the single cysteine in the conserved region (p60 contains only one cysteine) also block bacterial chain disruption activity (Wuenscher *et al.*, 1993).

The p60 protein has been speculated to be important in both cell division and bacterial virulence. Mutants expressing reduced levels of p60 (disruption of p60 itself is lethal) form long bacterial filaments as oppose to isolated cells in the wild type, presumably due to an inability to cleave peptidoglycan adhering mother and daughter cells after cell division. Such mutants are avirulent in animal models (Gutekunst *et al.*, 1992) yet are still invasive in experimental tissue culture monolayer experiments and bacteria remain both filamentous and competent to divide. In that p60 mutants can not however spread from cell to cell it is thought that the p60 may have some function other than peptidoglycan hydrolysis and may be involved in cell to cell spreading (Gutekunst *et al.*, 1992).

Given the aforementioned sequence data for ORF202 it seems likely that its encoded protein may be a peptidoglycan hydrolase. Such enzymes can be involved in housekeeping functions such as degrading peptidoglycan to allow cell growth or separation of mother and daughter cells following cell division. It is also possible that if the ORF202 translation product is involved in peptidoglycan breakdown, it might be involved an autolytic event responsible for the release of cytoplasmic virulence determinants. Cell breakage in group B *Streptococci* by an autolysin LytA (which shows an N-acetylmuramyl-L-alanine specificity) has been postulated to allow the release of pneumolysin and other toxic substance from the cytoplasm into the host *in vivo* (Berry *et al.*, 1989). Also *lyt*A mutants have been shown to exhibit severely attenuated virulence. A similar role has also been speculated for the p60 protein of *Listeria* (Wuenscher *et al.*, 1993). There is no evidence however that *P.gingivalis* undergoes autolysis *in vivo* or *in vitro*. Alternatively, release of an extracellular peptidoglycan hydrolase from a Gram-negative organism might induce lysis of Gram-positive species in a mixed microbial flora.

The amino acid sequence similarities between ORF202 and the α-amylase of *C.acetobutylicum* are curious. Starch is the main storage polysaccharide in plants and consists of α-1,4 linked glucose either unbranched (amylose) or branched with a low frequency substitution of α-1,6 linkages (amylopectin) with no amino acid component. Glucose is not a component of peptidoglycan (although it is the building block for N-acetylglucosamine which is a component of peptidoglycan) nor is it a component of its cross-linkages. Clearly the α-amylase substrate specificity is different than that of peptidoglycan-degrading enzymes. Nothing is known about the mechanism of catalysis of the starch degrading enzyme from *C.acetobutylicum* (P.Verhasselt, personal communication). However the enzyme is large and the homology with the ORF202 predicted amino acid sequence resides only in the very C-terminus of the α-amylase. It is thus possible that α-amylase activity is determined by a region outside the C-terminus, which may have thus far uncharacterised activity.

Analysis of the DNA sequence of the pGPR2 insert has identified several putative genes and allowed some speculation as to their possible functions. However it must be stressed that such predictions do not prove that these cistrons result in functional proteins *in vivo*. Direct evidence for expression is required and experimentation may be complicated by discrepancies in the

transcriptional and translational mechanisms of *E.coli* and *P.gingivalis*. The following section attempts to ascertain whether proteins are expressed from these ORFs, at least when expressed in *E.coli*.

Chapter 6

Expression Studies on *P.gingivalis* Antigen PgaA and Neighbouring ORFs in *Escherichia coli*.

6.1 Introduction

During the course of the cloning and characterisation of *pga*A difficulties in stable expression of an immuno-reactive clone arose. For example, expression vector constructs of pGPR2 whilst reacting strongly in immunoblots did not give signals in western blots unless cells from colonies were lysed in the presence of a proteinase inhibitors, and directly western blotted (pGPR7, section 4.2.7). Instability of pGPR2 could account for such observations should it encode for toxic proteins such as for example, an autolysin. However, it is also possible that the PgaA molecule is itself unstable. House keeping proteases of *E.coli* such as the Lon protease have been demonstrated to degrade abnormal proteins (Gottesman and Maurizi, 1992) and it is possible that proteolytic activity of this type could create difficulties when trying to detect an antigen that is degraded.

There is little data relating to expression of *P.gingivalis* genes and it is not known whether they are expressed normally in *E.coli*. As it is possible that genes cloned on pGPR2 are not constitutively expressed, or are expressed at very low levels or not at all, this could explain the difficulties encountered in detecting *P.gingivalis* antigen expression in *E.coli* harbouring pGPR2. In order to evaluate the extent to which gene expression is directed by pGPR2 in *E.coli*, it was first necessary radiolabel proteins and determine their molecular weights by SDS PAGE autoradiography.

6.2 Results

6.1.1 Combined Polymerase Chain Reaction and Zubay analysis of clone pGPR2 ORFs.

In order to study expression of pGPR2 genes, DNA was amplified from the chromosome of *P.gingivalis* by PCR and subjected to DNA-dependent, cell-free coupled *in vitro* transcription and translation as described originally by Zubay (1973). Cell-free, coupled *in vitro* transcription and translation systems

recognise transcriptional and translational start signals in DNA and RNA respectively, and mediate radiolabelled protein synthesis from exogenous DNA using standard biological mRNA, rRNA and tRNA intermediates. The advantage the Zubay system is that linear DNA fragments can be used as templates for transcription/translation. Thus there is no need for the use of plasmids for cloning which may contaminate expression studies by providing additional transcriptional activity (from vector promoters), or by expressing proteins with the same molecular weight as those of interest. What is more the use of PCR allows generation of desired DNA fragments in the absence of appropriate restriction sites and the amplification of potentially toxic genes without any of the deleterious effects associated with cloning of toxic genes (Resto *et al.*, 1992). Autoradiography of radiolabelled proteins resolved using SDS PAGE will reveal whether cloned *P.gingivalis* genes are recognised and expressed in *E.coli*, the molecular weights of those proteins expressed as well allowing expression of particular proteins to be assigned to each ORF.

Chromosomal DNA was used for the generation of two DNA fragments corresponding to the left and right half of the pGPR2 insert (fragments designated PCR L and PCR R respectively; figure 6.1a). PCR L consists of the very C-terminal of the *rnhB*$_{LG}$, all of ORFs 150, 297 and 280 along with the *rnhB*$_{LG}$ -ORF150 intergenic region and a C-terminally truncated ORF202. PCR R encompasses *pga*A and ORF199 and includes approximately 0.5 kb of DNA upstream of *pga*A. Primers used for these amplifications were those originally designed for DNA sequencing of the pGPR2 insert and were typically 17 nucleotides in length. Several pilot PCR amplifications revealed that good yields of DNA with the correct predicted molecular weight could be achieved using an annealing temperature of 47 °C with the highest product specificity at a $MgCl_2$ concentration of 0.15 mM final (figure 6.1b lanes 1 and 4). Verification that amplification was *P.gingivalis* (W83) chromosome-derived and not from contaminant pGPR2 or other DNA was achieved by carrying reactions out in the absence of chromosomal DNA template. Such reactions did not result in PCR products (data not shown).

The PCR reaction for PCR L showed multiple contamination of bands of low molecular weights. Possibly this may be a consequence of using relatively small primers for amplification. Verification of amplification of the correct target sequence was achieved by performing a Southern blot using the pGPR2 insert as a probe. PCR product run on an agarose gel and blotted in this way

Figure 6.1a Generation of Linear DNA fragments from the chromosome of *P.gingivalis* (W83) using Polymerase Chain Reaction.

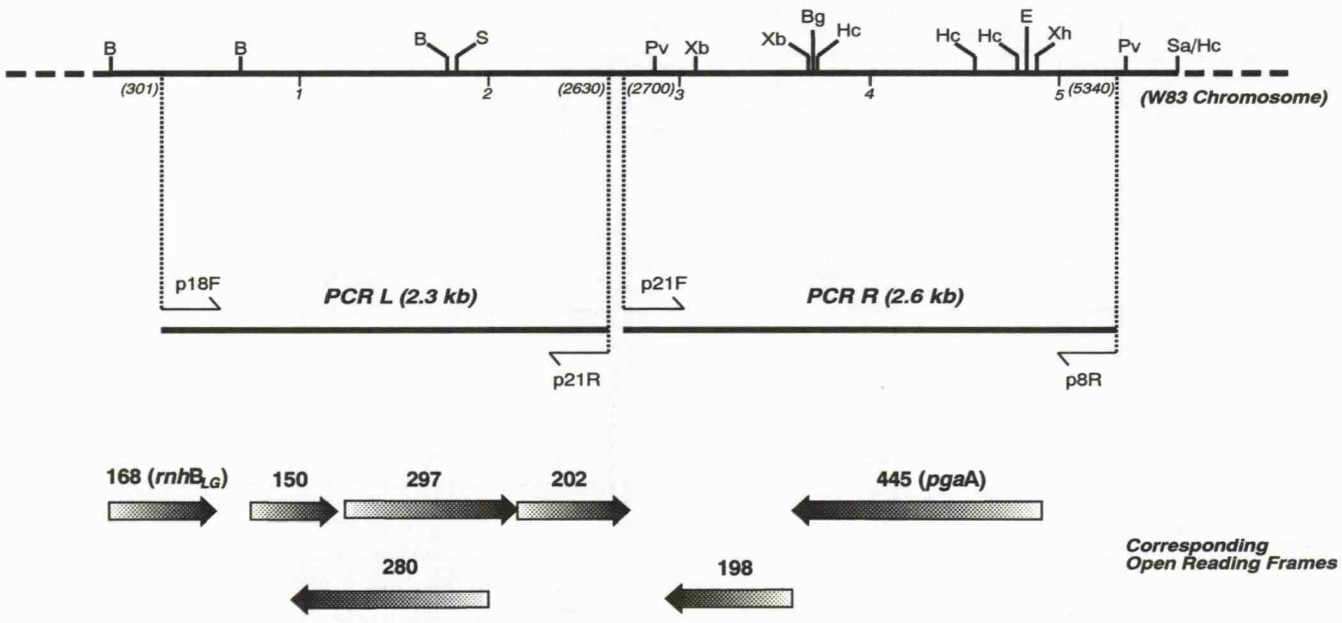


Figure 6.1 (a) Generation of linear DNA fragments from the chromosome of *P.gingivalis* (W83) using Polymerase Chain Reaction. Oligonucleotide primers p18F and p21R, and p21F and p8R were used to amplify PCR L and PCR R respectively. Numberepresent equivalent coordinates in the pGPR2 insert DNA in kb, whilst numbers in parentheses show the approximate position of the PCR primers in bp. Restriction sites are abbreviated as in figure 4.3. Also shown is the relative position of each open reading frame.

Figure 6.1b Generation of DNA fragments PCR L and PCR R.

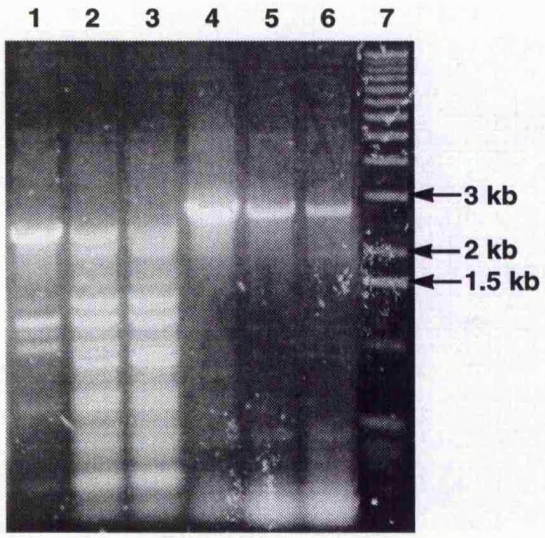


Figure 6.1c Southern hybridisation of fragments PCR L and PCR R with a pGPR2-derived radiolabelled DNA probe.

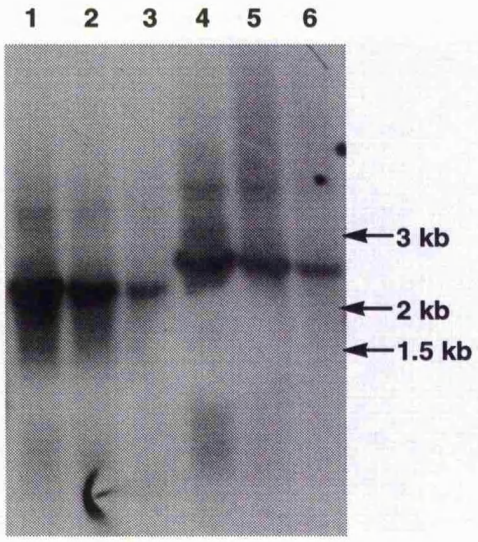


Figure 6.1 **(b)** Generation of DNA fragments PCR L and PCR R. PCR reactions were analysed using agarose gel electrophoresis. Lanes 1-3 and 4-6 show reactions generating PCR L and PCR R respectively. DNA in lanes 1 and 4 was amplified in the presence of 0.15 mM $MgCl_2$ whilst lanes 2 and 5, and 3 and 6 amplified in the presence of 2.5 and 3.5 mM $MgCl_2$ respectively. Lane 7 contains DNA molecular weight standards.**(c)** Southern hybridisation of fragments PCR L and PCR R with a pGPR2-derived radiolabelled DNA probe. A 5.5 kb *Hind*III/*Stu*I fragment of pGPR2 was used to probe a southern blot of PCR DNA as shown in figure 6.2c.The probe was seen to hybridise to fragments of molecular weights consistent with the predicted masses of amplifiifation products, and corresponding to the upper bands of DNA shown in figure 6.2b. Lane designations are as for figure 6.2b.

showed strong probe reactivity of molecular weight corresponding to the major high molecular weight bands (of around 2.3 and 2.6 kb; figure 6.1c) seen using agarose gel electrophoresis (figure 6.1b). This is consistent with these bands being products of the correct target sequences. Contaminant bands did not hybridise with the pGPR2 probe and hence are probably products of mis-annealing. DNA for use during *in vitro* transcription/translation was prepared by performing bandpreps of the more specific PCR reactions. Purity of band preparations was confirmed by analysis on agarose gels (data not shown).

DNA-dependant *in vitro* transcription/translation was performed according to the manufacturers instructions (Amersham) using ^{35}S-methionine to radiolabel synthesised protein. Reactions were analysed using SDS-PAGE as described in the materials and methods (figure 6.2). All reactions including the DNA-free reaction showed background labelling of bands. Reactions using the circular control vector showed strong labelling of a protein at around 30 kDa and this is probably the ampicillin resistance gene of pAT153 (figure 6.2, lane 1). However, no labelling of the tetracycline resistance gene could be visualised (a predicted molecular weight of about 40 kDa) despite reports of this gene having a strong, constitutively expressed promoter *in vitro* (Ehrlich *et al.*, 1985). The pGPR2 insert (excised using *Hin*dIII and *Stu*I and bandprepped) showed three proteins labelled at approximately 46 kDa, 33 kDa and 30 kDa whilst PCR R showed bands at 46 kDa and 30 kDa (although the 46 kDa band is faint and not conclusive; figure 6.2). No additional bands were seen in the PCR L reaction. Given the size and potential presence of the 46 kDa in both PCR R and the whole pGPR2 insert reactions it is likely that this represents the PgaA protein, and that *pga*A expression is derived from an upstream promoter element. Possibly the 30 kDa protein present in the whole insert reaction represents the product of ORF297. No labelled proteins of lower molecular weights corresponding to ORFs 150, 297 and 202 could be seen.

6.1.2 Minicell analysis of pGPR2 in *Escherichia coli* DS410.

Minicells are the product of aberrant partitioning during cell division in strains carrying the *min*A/*min*B genotype and exist as smaller than usual cells, devoid of chromosomal material (Dougan and Sheratt, 1977). Thus

Figure 6.2 SDS PAGE Fluorography of *in vitro* translation directed by DNA fragments PCR L and PCR R.

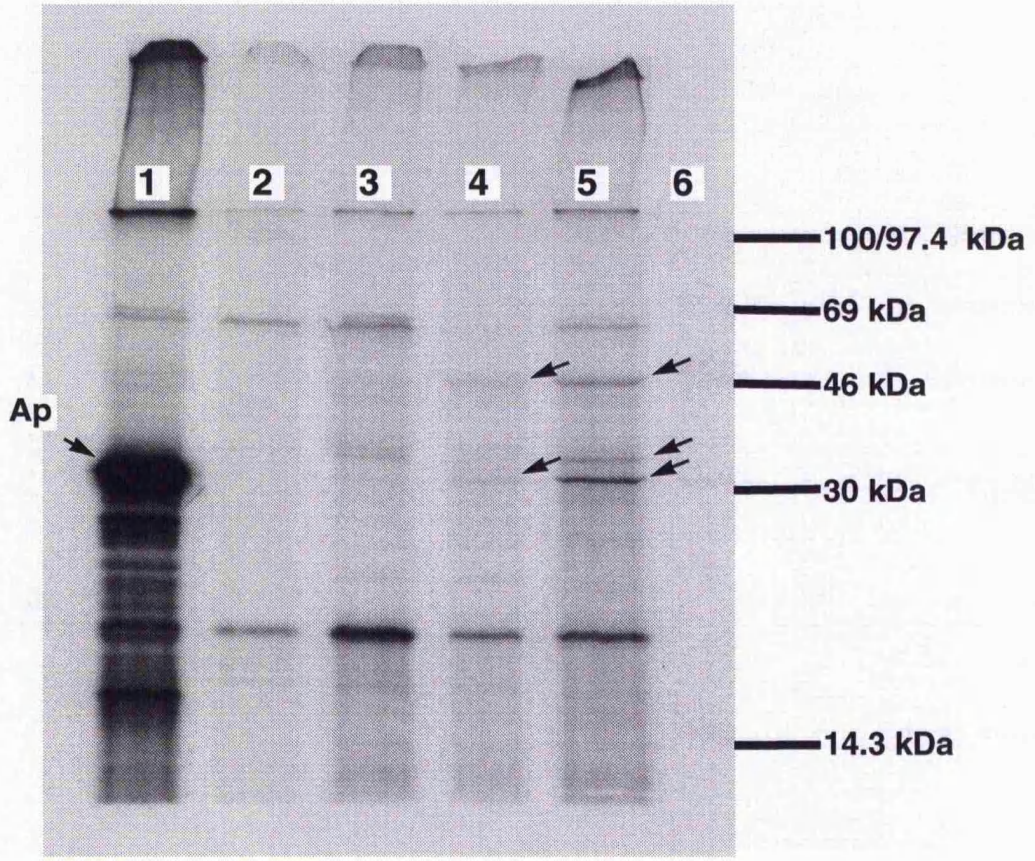


Figure 6.2 SDS PAGE Fluorography of *in vitro* translation directed by DNA fragments PCR L and PCR R.^{35}S-methionine-labelled Zubay reactions (20 ul) were run on a 12% SDS PAGE gel, fixed, treated with amplify and dried. Signal detection was with Kodak X-omat film for 24 hours. Lane 1 control plasmid pAT153 (circular), lane 2 reaction with no template, lanes 3 and 4 PCR L and PCR R-directed synthesis, lane 5 that of the pGPR2 insert (*Hind*III/*Stu*I fragment). Protein bands expressed that are not present in the template-free reaction (lane2) are highlighted by arrows. The ampicillin resistance gene of pAT153 is labelled Ap. No additional bands could be seen in the PCR L reaction when compared to the template-free reaction. Additional bands could be seen in the pGPR2 insert reaction and some faint bands of equilalent molecular weight in the PCR R reaction.

minicells are unable to direct protein synthesis except from long-lived mRNA species. By preincubating minicells at 37°C for an extended period, ant persisting mRNA wil be degraded, leaving the minicells unable to engage in *de novo* protein synthesis. When extra chromosomal replicons are present in minicell-producing strains such as DS410, some of this plasmid DNA can find its way into minicells and hence direct protein synthesis. As this is the only protein synthesis occurring in minicells it is possible to radiolabel these proteins using ^{35}S-methionine. The result is often very specific labelling of protein with very low backgrounds. Thus faintly expressed bands can be visualised, and this represents a distinct advantage over the Zubay technique, which shows very high backgrounds.

Plasmids pLG339 and pGPR2 were transformed into *E.coli* DS410 and minicells prepared from 400 ml cultures. To ensure no deletion or loss of plasmid had occurred miniprep DNA was also prepared from these cultures and analysed using restriction enzymes (data not shown). When minicells were labelled, pGPR2 strongly expressed non-vector proteins of 46 kDa 33 kDa and 31 kDa molecular weight in addition to several minor bands in the 35-46 kDa range and bands at around 25 kDa (figure 6.3a). Possibly, bands in the 25 kDa range might represent products of ORFs 202 and 199. (22.7 and 22.8 kDa respectively). The kanamycin resistance gene of pLG339 was also present in pGPR2 at around 30 kDa. Assuming that the 46 kDa band is encoded by *pga*A, the only ORF of sufficient size to code for a 46 kDa protein, and that the next largest predicted protein would be 33.5 kDa encoded by ORF297, it is possible that the minor bands may also be derived from *pga*A. To test this possibility and to investigate susceptibility of pGPR2-encoded proteins to *E.coli* proteases minicells were labelled in the presence of a cocktail of protease inhibitors. It is unknown as to whether such inhibitors can find their way inside minicells. It is, however possible that those derived from amino acids might gain access *via* the oligopeptide transport system. It is also possible that the destabilising effect of EDTA on the outer membrane might allow some entry of protease inhibitors into the periplasm.

Minicells labelled in the presence of protease inhibitors showed slightly reduced expression of all plasmid encoded proteins and this makes assessment of the effect of these inhibitors on faintly expressed bands such as those in the 25 kDa range difficult (figure 6.3b). No relative enhancement of any bands was seen. However, expression of the 33 kDa protein of pGPR2

Figure 6.3a SDS PAGE Autoradiogram of pGPR2 proteins labelled in DS410 minicells.

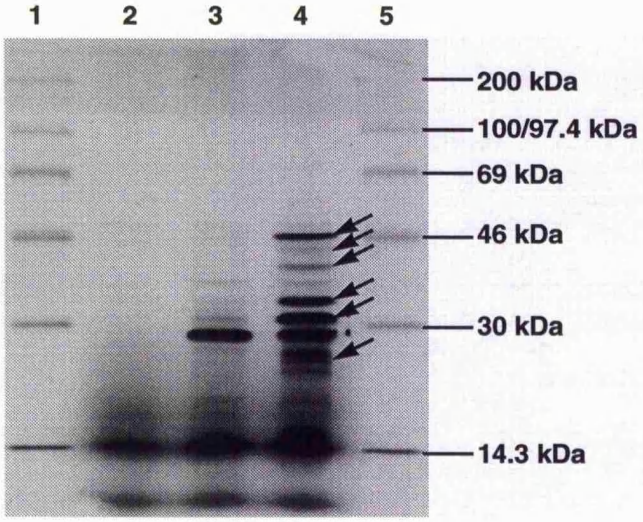


Figure 6.3b SDS PAGE Autoradiogram of pGPR2 proteins labelled in the presence of protease inhibitors.

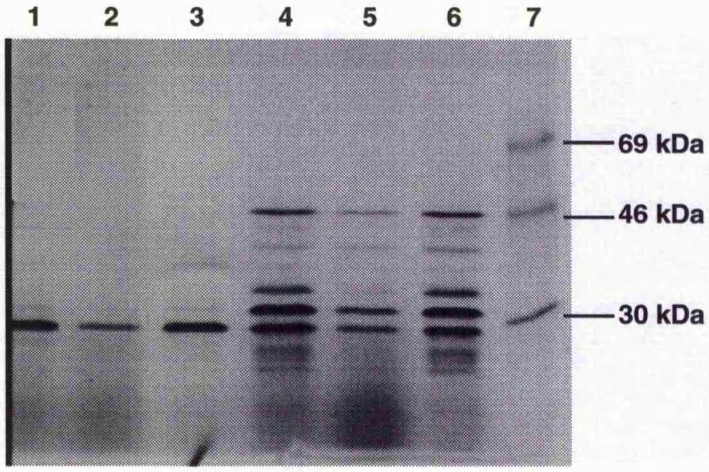


Figure 6.3 (a) SDS PAGE Autoradiogram of pGPR2 proteins labelled in DS410 minicells. Lane 2, plasmid-free minicells; lanes 3 and 4 minicells containing pLG339 cloning vector and pGPR2 respectively. Lanes 1 and 5 contain ^{14}C-methylated protein standards whose molecular weights are shown on the right. Several proteins expressed in pGPR2 not seen in vector pLG339 are highlighted using arrows and represent proteins encoded by the pGPR2 insert. **(b)** SDS PAGE Autoradiogram of pGPR2 proteins labelled in the presence of protease inhibitors. Lanes 1-3 pLG339 minicells; lanes 4-6 pGPR2 minicells. A cocktail of protease inhibitors was added to minicells either after (lanes 3 and 6) or before ^{35}S-labelling (lanes 2 and 5). Minicells not treated with protease inhibitors were run in lanes 1 and 4. Minicells labelled in the presence of protease inhibitors showed reduced labelling and loss of a 33 kDa pGPR2 insert encoded band.

(also seen in the Zubay reaction of pGPR2) was severely diminished in the presence of protease inhibitors suggesting that this band is derived from a degradation product of a higher molecular weight protein, probably the 46kDa *pga*A protein. This preliminary observation suggests that intrinsic proteolytic activity of *E.coli* should be considered when cloning foreign genes. The addition of protease inhibitors after labelling had no effect on the expression profile, suggesting that proteolytic degradation had occurred during labelling.

In order to clarify which pGPR2 ORFs were directing synthesis of which bands in minicells three deletions of pGPR2 were made (figure 6.4). The first consists of a deletion of the *Hinc*II fragments encoding *pga*A and ORF199 followed by self ligation to generate pGPR11. Plasmid pGPR12 was created by subcloning the 3.7 kb *Bam*HI fragment from pGPR2 into the *Bam*HI site of pLG339 and pGPR13 was created in the same way by subcloning the 1.7 kb *Bgl*I/*Bam*HI fragment of pGPR2 into pLG339. pGPR12 encodes *pga*A, ORF199, ORF202 and the C-terminal of ORF297 whilst pGPR13 only encodes *pga*A carrying a 100 bp truncation at the 5' end of the gene (figure 6.4). Minicell labelling with these constructs is shown in figure 6.5. Minicells of pGPR11 did not show any non vector-derived protein labelling (figure 6.5a) and suggests that despite strong promoter activity from the pLG339 teracycline resistance element, ORFs 150, 297 and 202 as well as *rhn*B_{LG} are not expressed within detectable limits from pGPR2 in *E.coli*. Radiolabelled protein was however expressed in pGPR12 and pGPR13 minicells. Minicells of pGPR12 (although showing a high background presumably due to contamination with vegetative cells) exhibit all of the bands seen in minicells and Zubay reactions of pGPR2. Given that pGPR12 only has coding capacity for three proteins these additional bands may be a consequence of proteolytic degradation of *pga*A and/or translation directed from internal start codons (or possibly internal transcriptional stop signals). Minicells of pGPR13 which encodes only *pga*A also shows protein of multiple molecular weight and suggests that all bands seen in minicells of pGPR2 are in fact derived solely from *pga*A (figure 6.5c). The truncation of *pga*A at the 3′ end (pGPR13) is predicted to reduce the molecular weight of PgaA by approximately 3 kDa. This deletion results in a very modest decrease in the apparent molecular weight of the 46 kDa band observed in minicells (figure 6.5c). However, a more pronounced decrease in the molecular weight of the 31 kDa and 33 kDa bands is clearly visible, and results in the 31 kDa protein running as a dimer

Figure 6.4 Generation of pGPR2 deletion subclones for expression in minicell producing strain DS410.

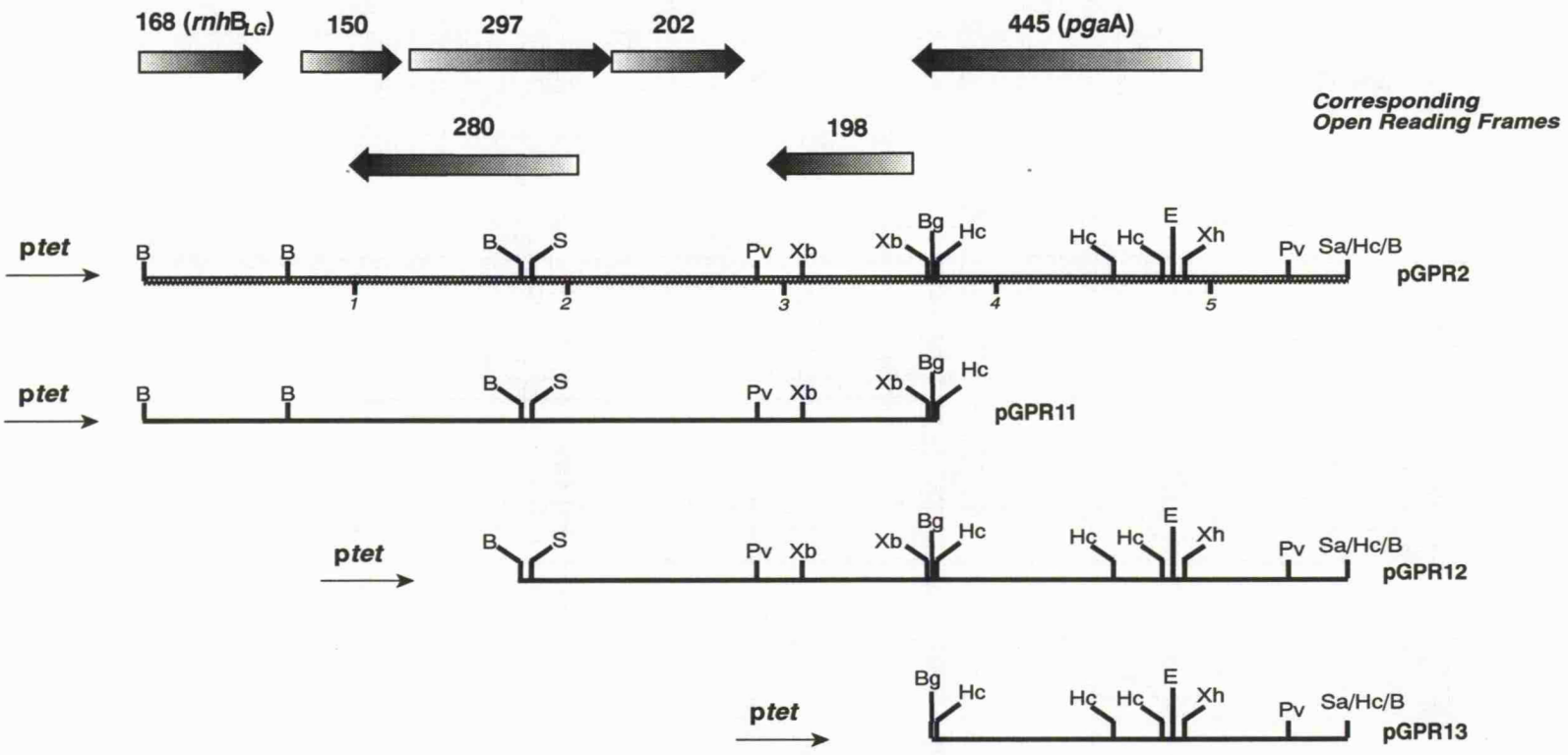


Figure 6.4 Generation of pGPR2 deletion subclones for expression in minicell producing strain DS410. pGPR11 was generated by deleting *Hinc*II from pGPR2 hence deleting ORF198 and *pga*A. Subclones pGPR12 and pGPR13 were created by excising the right hand *Bam*HI and *Bam*HI/*Bgl*II respectively and recloning them into pLG339. pGPR12 encodes intact ORF198 and *pga*A, whilst pGPR13 encodes only *pga*A with a small (approximately 100 bp) C-terminal truncation. ORFs are shown as hatched arrows above the restriction map.

Figure 6.5a Minicells of pGPR11

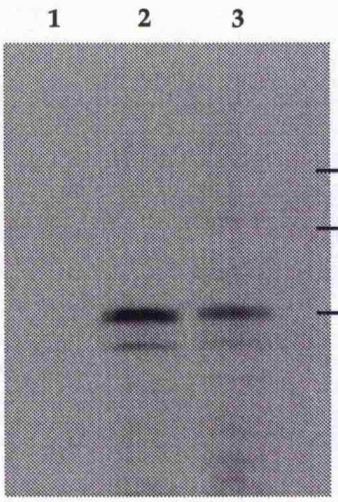


Figure 6.5b Minicells of pGPR12

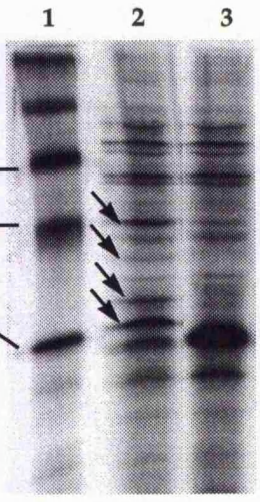


Figure 6.5c Minicells of pGPR13

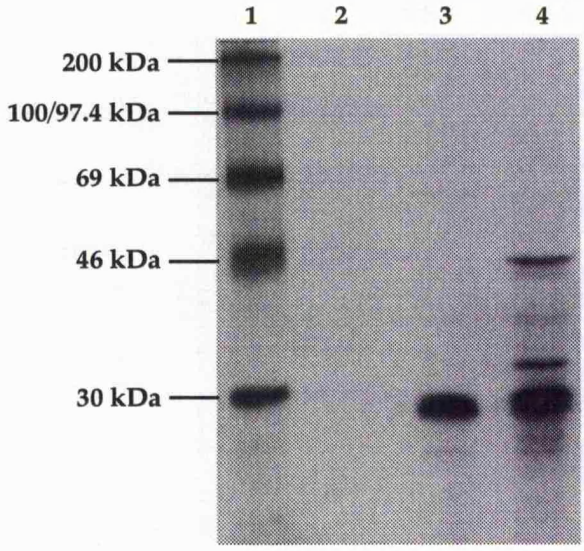


Figure 6.5 (a), (b) and (c) Minicells of pGPR11, pGPR12, and pGPR13 respectively. **(a)** Lane 1, plasmid-free minicells, lane 2 minicells of pLG339, Lane 3 minicells of pGPR11. No non-vector labelling could be seen in pGPR11 hence it seems that ORFs 168 (*rnh*B$_{LG}$), 150, 297, and 202 are not expressed. **(b)** Lane 1 molecular weight markers, lane 2, minicells of pGPR12, lane 3 minicells of pLG339. Arrows show non-vector bands in pGPR12 minicells. **(c)** Lane 1, molecular weight markers, lane 2 plasmid-free minicells, lane 3 minicells of pLG339, Lane 4 minicells of pGPR13.

with the kanamycin resistance gene protein. These data show that expression of *pga*A and other *P.gingivalis* genes in *E.coli* is complex and does not follow established, predictable patterns.

6.1.3 Immunoprecipitation of PgaA using LDS28-coupled Magnetic Particle Separation.

Immunoprecipitation of cell free extracts is a widely used technique for isolating a particular antigen in an antibody dependent fashion and implies DNA template directed expression of antigen for a specific antibody (Anderson and Blobel, 1983). The technique involves immobilisation of antibody to a high affinity matrix such as protein A sepharose or chemically activated (tosylated) magnetic particles and subsequent binding of radiolabelled antigen to that immobilised antibody. Alternatively antibody and antigen can be mixed directly and isolated by direct precipitation of the resulting immune complex or by mixing with *Staphylococcus aureus* cells which express protein A, a cell surface protein with a high binding affinity for most isotopes of IgG (Dicker *et al.*, 1991). Non specific binding to antibody can be eliminated by washing the matrix or precipitate, and antigen eluted from the matrix by denaturing the protein by boiling in SDS or by sharply dropping the pH to disrupt the antibody-antigen complex.

The mAb LDS28 is of the IgM isotype and will not allow protein A binding. Hence the method of using a chemically activated matrix to covalently bond LDS28 was chosen. M-450 tosylactivated Dynabeads (Dynal, U.K.) were activated by exposure to Borate pH9.5 and mixed with purified LDS28. Purified LDS28 was kindly supplied by Roger James (Leicester Department of Surgery) and its purity confirmed on SDS PAGE (data not shown; Brick 190 was not considered for use in immunoprecipitation in that this material was in short supply and there had been some previous difficulties in purifying this monoclonal; see section 3.2.5). The super-reactive tosyl groups of the beads react with the amino groups on the antibody creating a stable covalent attachment. Beads were then mixed with cell free translation reactions derived from pGPR2 and pLG339 DNA templates (*Sph*I-digested). Beads were then isolated using a magnetic particle concentrator and then washed four times in PBS. Protein eluted by boiling in SDS PAGE loading buffer was then analysed by SDS PAGE autoradiography (figure 6.6).

Figure 6.6a Zubay labelled proteins of pGPR2.

Figure 6.6b pGPR2-encoded proteins after Absorbtion to LDS28-coated Dynabeads.

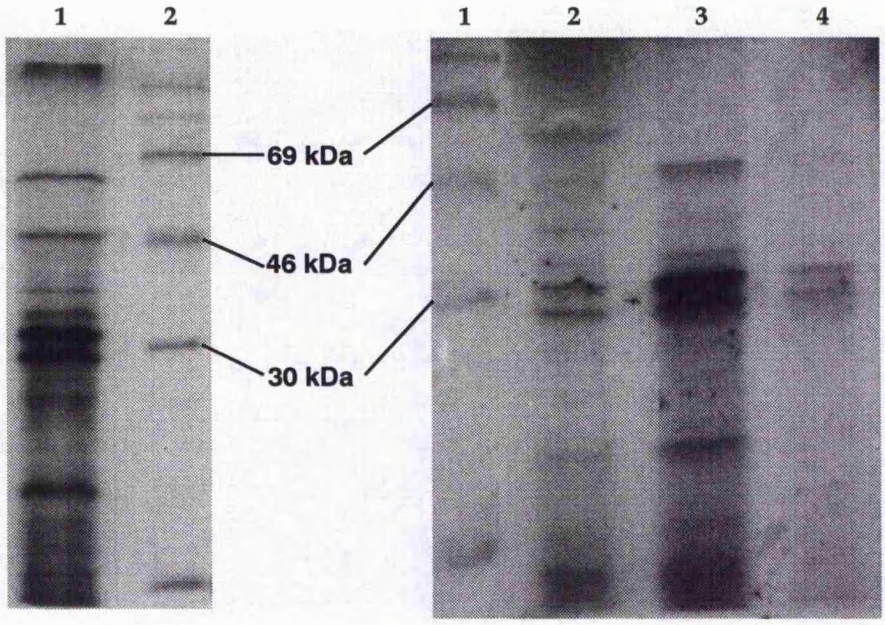


Figure 6.6c Control pLG339-encoded proteins after Absorbtion to LDS28-coated Dynabeads.

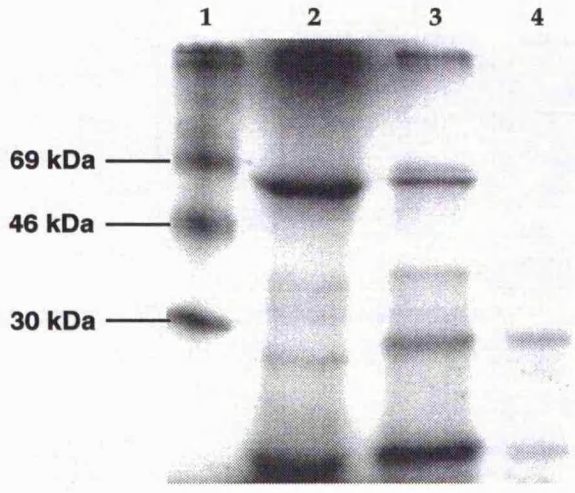


Figure 6.6 (a) Zubay labelled proteins of pGPR2. Labelling was performed using *Sph*I cleaved DNA termplate. ^{35}S labelling was checked for before immunoprecipitation. Lane 1, Zubay labelling of pGPR2 proteins, lane 2 molecular weight markers. **(b)** pGPR2 proteins after absorbtion to LDS28-coated Dynabeads. Elution was by boiling in SDS PAGE loading buffer for 5 mins. Lane 1, Molecular weight markers, lane 2 pGPR2 cell free translate supernatant after exposures to LDS28 coated beads, lane 3 supernatant collected from the beads during the first wash step, lane 4 material eluted from the beads after four washing steps. **(c)** Control pLG339 proteins after absorbtion to LDS28-coated Dynabeads. Lane 1, Molecular weight markers, lane 2 pLG339 cell free translate supernatant after exposures to LDS28-coated beads, lane 3 supernatant collected from the beads during the first wash step, lane 4 material eluted from the beads after four washing steps.

Initially beads were washed eight times with 1 ml of 0.1% Tween/PBS. However SDS PAGE fluorography of the material eluted from the beads by boiling in SDS PAGE sample buffer was unable to detect any radiolabelled protein in this fraction. Subsequently a less stringent wash procedure was adopted where beads were washed 4 times in 500 µl 0.1% Tween/PBS and this yielded bands in the pGPR2 eluent. A single band of around 30 kDa was very faintly visible in the eluent of the pLG339 and it is not clear as to whether the beads were non specifically adsorbing protein and this is what emerged in the bead eluents. Analysis of pGPR2 Zubay reactions immediately after adsorption to LDS28 Dynabeads did not suggest that any individual pGPR2 protein was strongly retained on the LDS28 matrix in that all bands were present relative intensity in this material (figure 6.6b, lane 3). However, proteins of molecular weights of around 30 and 32 kDa were eluted from the LDS28 coated beads in pGPR2 and it is possible that may be due to specific binding of radiolabelled antigen to the antibody-conjugated beads.

6.1.4 Subcloning of ORF297 using Polymerase Chain Reaction and its over -expression in *Escherichia coli*.

As no data exist to support the expression of proteins from ORFs 150, 297 and 202 the PCR generated fragment PCR L (figure 6.1a) was cloned into commercially available PCR cloning/over expression vector pGEM-T (Promega Corporation, 1991) following the manufacturers instructions. The principle of T-vector cloning relies on the intrinsic property of *Taq* polymerase to create 3'-terminal overhangs during PCR which most commonly consist of a single adenine residue. It is possible to create similar overhangs on blunt-cut DNA fragments by essentially setting up a PCR-like reaction in the presence of an excess of dTTP and in the absence of dATP, dGTP and dCTP so as to add a single T residue to the 3'-terminal. T-vector prepared in this way can not self ligate as its termini having T overhangs, are essentially self incompatible. However, these ends are compatible with standard "A-tailed" *Taq* PCR products and such DNA will ligate efficiently into T-vector. This results in the PCR fragment being cloned into the *lacZ'* cassette of pGEM-T and allows over expression of cloned genes from the convergent T7 and SP6 promoters which fire into *lacZ'* polylinker from both sides.

The cloning of genes against the direction of the *lacZ'* transcription allows strong expression from the T7 promoter in strains harbouring the T7 RNA polymerase gene (Davanloo *et al.*, 1984). Cloning in *E.coli* Sure™, which does not possess a copy of the T7 RNA polymerase gene, will not express genes cloned in the T7 orientation. This is important if there is any toxicity effect due to cloned genes which would be selected against in the event of leaky expression from, for example the *lacZ'* promoter. Once recombinants have been identified, plasmid DNA can be transformed into the T7 expression strain JM109(DE3). Strains carrying the DE3 genotype harbour the T7 RNA polymerase gene under the influence of the *lac*UV5 promoter which is IPTG-inducible, and results in production of the T7 RNA polymerase. The T7 RNA polymerase is then able to strongly direct expression of genes downstream of the T7 promoter.

Ligations were transformed into Sure™ and recombinants of PCR L/pGEM 5Zf(+) (this is the designation of the vector used in T-tailing; figure 6.7) were screened for by looking for white colonies on media supplemented with X-gal and IPTG. DNA from recombinants was simultaneously cleaved with restriction endonucleases *Sma*I and *Sal*I in order to determine the orientation of the insert with respect to the T7 promoter. A recombinant with the direction of transcription of ORFs 150, 297 and 202 against that of *lacZ'* was chosen and designated pGPR103.

DNA from pGPR103 was used to transform the strain JM109(DE3) for over expression of cloned genes. JM109(DE3/pGPR103) was grown in liquid culture and expression induced by the addition of IPTG. After cells were harvested and analysed on SDS PAGE (figure 6.8). Comparison of the JM109(DE3/pGPR103) polypeptide banding pattern with that of JM109(DE3/pGEM 5Zf(+)) revealed the presence of an additional band in JM109(DE3/pGPR103) lysate of around 31 kDa which presumably corresponds to expression of ORF297. Additional smaller bands corresponding to ORF150 and ORF202 (the latter of which has a C-terminal truncation) gene products could not be visualised.

Visual examination of cultures (10 ml) prepared under T7 expression as above revealed that JM109(DE3/pGPR103) forms aggregates when harvested by centrifugation and resuspended in a one tenth volume of PBS.

Figure 6.7 Circular Map of pGEM 5Zf(+).

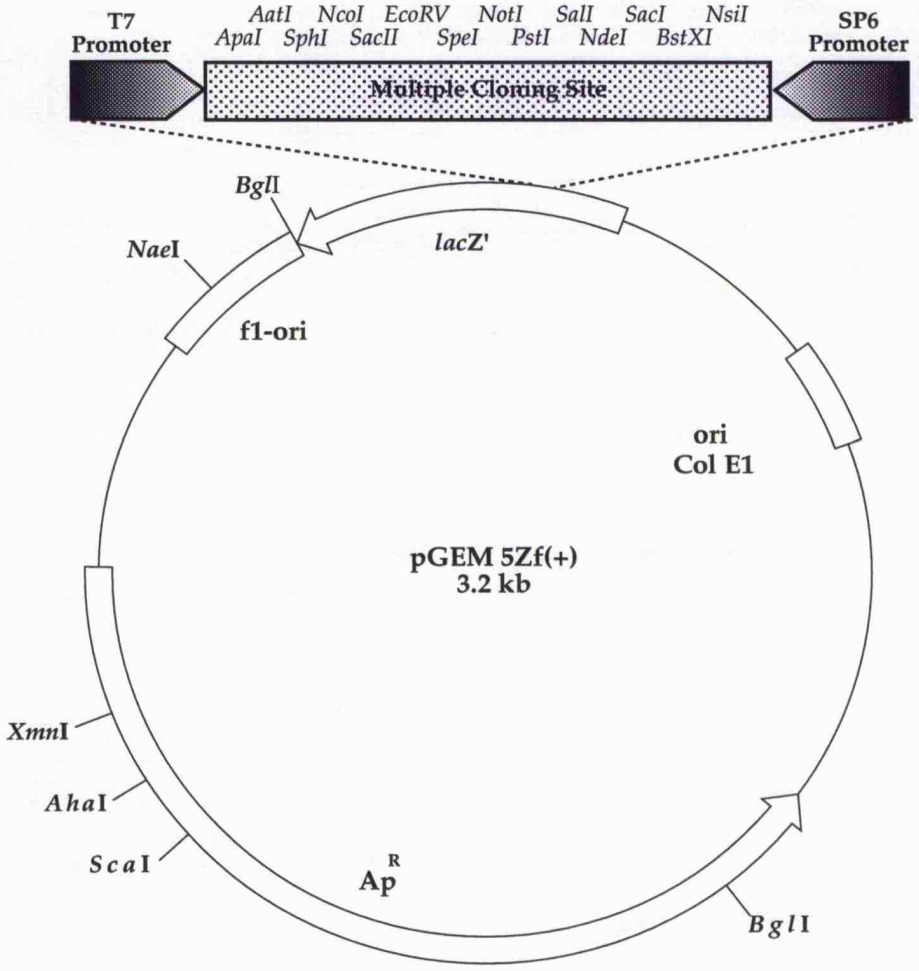


Figure 6.7 Circular Map of pGEM 5Zf(+). The polylinker is shown expanded along with restriction sites and orientations of T7 and SP6 promoters. The *Eco*RV site creates a single blunt-ended fragment and is used to clone PCR fragments in the T-vector cloning strategy. Areas specifying the *lacI*q and ampicillin resistance genes are shown as boxed arrows. The origin of replication is shown boxed.

Figure 6.8 SDS PAGE of *E.coli* expressing cloned genes from PCR L using the T7 promoter.

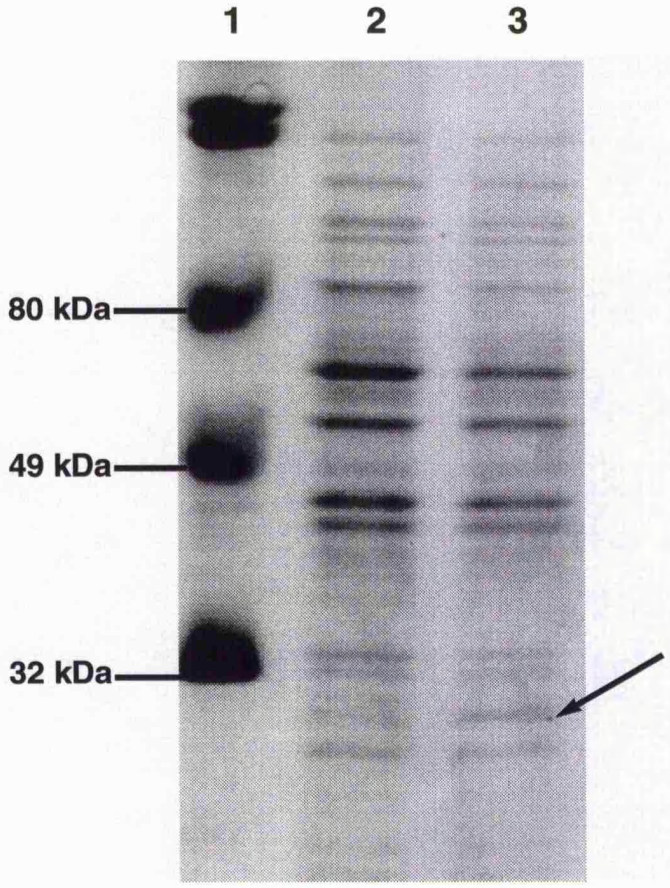


Figure 6.8 SDS PAGE of *E.coli* expressing cloned genes from PCR L using the T7 promoter. Lysates of JM109(DE3/pGPR103) and JM109(DE3/pGEM 5Zf(+)) were subjected to electrophoresis using SDS PAGE and then stained using Coomassie Blue. Lanes 2 and 3 contain lysates of JM109(DE3/pGEM 5Zf(+) and JM109(DE3/pGPR103)) respectively. Lane 1 contains SDS PAGE molecular weight markers whose molecular weights are labelled down the left hand side. A protein band over expressed in JM109(DE3/pGPR103) but not in JM109(DE3/pGEM 5Zf(+)) is visible at approximately 31 kDa (arrow), and presumably is encoded by the JM109(DE3/pGPR103) cloned insert.

JM109(DE3/pGPR103) cultures treated in this way and then disaggregated by a mixing using a bench top whirlymix, re-aggregated over about 10 mins both at room temperature and on ice. Aggregation was not seen in cultures of JM109(DE3/pGEM 5Zf(+)). Observation of cells under high power (x1000) magnification using phase contrast microscopy revealed no apparent differences in the size or shape of JM109(DE3/pGPR103) cells when compared to cells of JM109(DE3/pGEM 5Zf(+)). In addition no evidence of cell debris caused by cell lysis could be seen.

6.3 Discussion.

To date there have been few reported studies addressing the details of how and whether *P.gingivalis* genes are expressed in *E.coli* . Studies to date simply use over expression for cloning purposes and usually find that gene expression in *E.coli* is dependant on the vector promoter (Bourgeau *et al.*, 1992, Otogoto *et al.*, 1993, Lovatt and Roberts, 1994). Direct exceptions to this generalisation was noted by and McBride *et al.*, (1992) who cloned a *P.gingivalis* cell surface antigen which showed high levels of expression from its cloned promoter (later reported by Joe *et al.*, 1993). The notion of aberrant expression is in part supported by data described in this chapter.

Minicell and cell free system labelling of proteins encoded by pGPR2 and its derivatives has shown that expression of multiple protein products is directed solely by the *pga*A gene. The major bands expressed are of 31 kDa, 33 kDa and 46 kDa. The molecular weight of the 46 kDa protein is consistent with the observed molecular weight of the LDS28-reactive band seen in western blots of pGPR7 lysates and the observed molecular weight of the LDS28 antigen in the envelope and outer membrane fractions of *P.gingivalis*. However it is not clear as to why in *E.coli* LDS28 does not react with the multiple banding pattern. It may be that the LDS epitope is confined to a terminal part of the protein which is missing in truncated forms. The fact that the PgaA has a predicted molecular weight of 50 kDa yet expresses proteins with an observed molecular weight maximum of 46 kDa could be due several reasons.

One possible reason for the discrepancy in the predicted and observed molecular weights of PgaA might be that 50 kDa form is exquisitely sensitive to *E.coli* (and *P.gingivalis?*) proteases and is efficiently converted to the 46 kDa

form. The observation that minicell labelling of pGPR2 in the presence of protease inhibitors results in a strong reduction in relative signal strength of the 33 kDa band is consistent with the hypothesis that *E.coli* proteases are active against PgaA. It seems that the 33 kDa band may be a product of degradation of a higher molecular weight form. However minicell labelling in the presence of protease inhibitors did not result in the appearance of a 50 kDa band or drop in signal intensity of the 46 kDa band and suggests that if proteolytic processing of the predicted 50 kDa protein occurs, the protease(s) responsible is not affected by the cocktail of protease inhibitors when added exogenously to minicells. Proteolytic activity of this kind may reflect some of the difficulties encountered when trying to perform western blots of *pga*A expressing clones. Interestingly the 33 kDa band was also evident in cell free translates of pGPR2 (figure 6.6a). This may be significant in that cell free extracts lack the signal peptidase processing machinery for secretory proteins, whereas minicells maintain signal processing of membrane proteins (Achtman *et al.*, 1979). The lack of significant difference in the banding profiles using these two expression systems suggests that signal peptidases are not implicated in the observation of multiple forms of PgaA.

The presence of transcriptional and translational starts within the coding sequence of *pga*A is another possible reason for the generation of a multiple banding pattern from a single cistron. In that there is no promoter in the vector firing into *pga*A, and that there is a non coding 0.5 kb region upstream presumably translational and transcriptional start sites recognisable by *E.coli* must be present for directing expression of *pga*A. Within PgaA amino acid sequence there are several methionines which might also be responsible for internal translational start points. Protein of molecular weights of 45, 41.7, 30.6, 27.9 kDa and below are predicted to be produced from these hypothetical internal methionine starts and it is possible that the 45 kDa and 30.6 kDa predicted translates may be those that are observed in minicells and *in vitro* translates. Analysis for the presence of Shine-Dalgarno consensus elements proximal to these putative internal start codons shows that these methionines do not have this consensus sequence (data not shown). The Shine-Dalgarno consensus sequence is important in that it is complementary to the 16s ribosomal subunit and functions by targeting the ribosome and encouraging translational initiation. This interaction with the ribosome also considerably increases mRNA stability by protecting the transcript from processive exoribonuclease degradation (Wagner *et al.*, 1994). There is

however a good consensus Shine-Dalgarno sequence near the predicted start codon for the predicted 50 kDa (figure 5.3b) and it can not be ruled out that the 50 kDa protein may exhibit an aberrant molecular weight on SDS PAGE (See and Jackowski, 1989).

A further possibility is that premature translational stop points caused by the occurrence of codons which are rare in *E.coli* could give rise to truncated forms of PgaA. In such cases where a codon specifying an unavailable tRNA species during translation occurs, the ribosome can stall and yield a truncated peptide (Grosjean and Friers, 1982) or substitute a mispairing tRNA and proceed with peptide chain elongation (Faxen and Isaksson, 1994). Thus it is possible that premature translational termination could result in low molecular weight forms of PgaA and this phenomenon might be a problem when expressing *P.gingivalis* genes in *E.coli.*

A search for transcriptional start signals such as the σ^{70} -35 and -10 consensus sequences upstream of *pga*A was largely unfruitful. Searching for features of this type in DNA can be difficult in that very often even where the promoter has been experimentally identified these signals are degenerate (McClure, 1985). It is also possible that these consensus sequences are not relevant for *P.gingivalis* transcription in that it has been shown that antibodies to *E.coli* core RNA polymerase and σ^{70} subunit do not recognise their *P.gingivalis* counter parts and that the *P.gingivalis* core RNA polymerase shows a different sensitivity to transcriptional inhibitors *versus E.coli* RNA polymerase (Klimpel and Clark, 1990). The same study showed that cell extracts from *E.coli* and *P.gingivalis* do not efficiently transcribe chromosomal DNA in heterologous combination, but do efficiently transcribe their own chromosomal DNAs. However, *pga*A is expressed both *in vivo* and *in vitro* using the *E.coli* transcriptional machinery. It is not clear as to whether this expression is driven by a vector promoter, a genuine cloned promoter for *pga*A, or by a non-*P.gingivalis* promoter region of DNA which is spuriously recognised by the *E.coli* transcriptional machinery as a promoter. Really the only way to dispel doubts about how and from where *pga*A is transcribed would be to perform the appropriate transcript mapping experiments. Similar studies for other *P.gingivalis* genes may eventually clear up the problems of gene expression associated with *P.gingivalis* genes in *E.coli.*

No expression of ORFs 150, 297, and 202 was seen in either minicells or cell free translates. For ORFs 150 and 202 this is not entirely unexpected in that they are predicted to encode small proteins which may be difficult to visualise using standard SDS PAGE. However, expression of ORF297 should result in a protein of sufficient molecular weight for visualisation on SDS PAGE. It is possible though that the kanamycin resistance protein, having a similar molecular weight (30 kDa) could obscure the ORF297 protein on SDS PAGE (although no protein expression was seen in Zubay reactions of PCR L which encodes ORFs 150, 297, and 202 and does not include any vector sequence). Expression of ORFs 150, 297, and 202 was expected as the powerful tetracycline resistance gene promoter should have contributed to the expression of these ORFs. There may be a *P.gingivalis* transcriptional terminator in the *rnh*B_{LG}-ORF150 intergenic region, so that the influence of the tetracycline resistance gene promoter on the expression of ORFs 150, 297 and 202 is minimised.

Unlike ORFs 150 and 202, ORF297 does not posses a strong consensus Shine-Dalgarno. It is also possible that the mRNA for ORF297 contains codons that are rare in *E.coli* hence the level of expression inhibited by the scarcity of rare tRNA. A cursory comparison of the codon usage tables for *E.coli* and ORF297 reveals that ORF297 possesses a relatively high proportion of codons with an A residue at the third position (data not shown). The *E.coli* usage table was derived from 681 genes and should be representative of highly expressed genes for this organism as such genes show strong codon bias and will have greatest influence on codon distribution (the driving force of codon bias and the efficiency of translation are energetic constraints for codon-anticodon pairing; optimal codons are those with perfect Watson-Crick base pairing in the wobble position; Hartl *et al.*, 1994). In highly expressed genes of *E.coli*, A at the third base position is fairly rare (C and U are preferred; Grantham *et al.*, 1981) and it is thus possible that restricted availability of tRNA for such codons and poor processivity of translational machinery due to unfavourable base pairing might result in poor heterologous expression of *P.gingivalis* genes in *E.coli*. Unfortunately no codon frequency table has been compiled for *P.gingivalis* and it is hence impossible to predict expression levels of individual proteins in *P.gingivalis* (Hartl *et al.*, 1994). However, ORF297 does express at a modest level when introduced into a strong transcriptional over expression system as demonstrated above.

The attempts to immunoprecipitate radiolabelled PgaA using LDS28 revealed confusing results. Although the PgaA-derived 32 kDa band was retained by the LDS28-conjugated Dynabeads so was a 30 kDa band identical in mass to the vector-derived kanamycin resistance protein (figure 6.6b and c). If the kanamycin resistance protein is immunoprecipitated by LDS28-conjugated Dynabeads this would suggest that the whole immunoprecipitation procedure is non-specific. However, the 30 kDa band eluted from the beads may in fact be a PgaA-derived band masked in minicell experiments by the kanamycin resistance protein, which is of the same molecular weight. If this is the case *in vitro* expression of DNA fragment PCR R, which lacks the presence of the kanamycin resistance protein should show expression of a 30 kDa protein predicted to be derived from PgaA. Close examination of figure 6.2 (lane 4) shows that PCR R directs synthesis of a 30 kDa product *in vitro* and I suggest that this PgaA-derived product is that which is immunoprecipitated by LDS28. The 46 kDa form of PgaA was not precipitated by LDS28-conjugated Dynabeads. However, this form of PgaA is weakly expressed compared to the 30 and 32 kDa forms and it is possible that the 46 kDa form is present in the LDS28-Dynabead eluate at sub-detectable levels.

To close these data concur with the observation that expression of *P.gingivalis* genes in *E.coli* is unpredictable, and that when constructing gene libraries it is prudent to use an expression vector for screening (Klimpel and Clarke, 1992).

Chapter 7

Discussion.

The cell surface antigenic profiles of bacteria are of importance in host recognition of pathogens during infection, and this is demonstrated by the ability of many successful pathogens to vary their antigenic profiles, either by control of gene expression in response to environmental stimuli, or by random mechanisms caused by structural alterations at the genetic level (Robertson and Meyer, 1992). Periodontopathogen *P.gingivalis* exhibits a complex cell surface and expresses fimbriae, capsular material, haemagglutinin filaments, LPS, blebs which result in ECV as well as many as 17 surface exposed major outer membrane proteins (Holt and Bramanti, 1991). Many of these surface exposed components are antigenic in nature and those of which are unique amongst strains give the bacterium its characteristic serotype (Fisher *et al.*, 1987; Hanazawa *et al.*, 1991). Analysis of anti-*P.gingivalis* antibodies generated during immunisations, experimental infections and the immune systems of healthy and diseased individuals reveal that certain proteins of distinct molecular weights predominate in antibody recognition. These include molecular weights of around 46 kDa and 19 kDa for which antibodies are expressed in both healthy individuals and patients with periodontal disease and are expressed by the bacterium regardless of the levels of haemin in the growth medium (Kato *et al.*, 1987; Laorisin *et al.*, 1990; Papaioannou *et al.*, 1991). Other antigens are expressed at 115 kDa, 82 kDa, around 54 kDa, 40 kDa, 27 kDa, and 25 kDa (Laorisin *et al.*, 1990; Curtis *et al.*, 1991; Papaioannou *et al.*, 1991).

Interest in the significance of these antigens in disease and their possible use as diagnostic markers, vaccine development and targets for rational drug design has led to several groups, attempting to clone genes specifying these antigens. Antigens of molecular weights 21 kDa, 44 kDa and 52 kDa (McBride *et al.*, 1990), 40 kDa (Abiko *et al.*, 1990) and 200 kDa and 160 kDa (Hayakawa *et al.*, 1992) have been cloned but the functions of these proteins have yet to be elucidated. The reason for these proteins being of unknown function is ambiguous in that *P.gingivalis* should have porins, permeases and iron acquisition systems similar to those of other bacteria, and cloning and sequencing should facilitate identification of such genes.

Described is the stepwise molecular cloning of a gene which expresses a protein reactive with several antibodies raised against components of periodontopathogen *P.gingivalis*. This protein has a predicted molecular weight of 50 kDa but migrates with an maximum observed molecular weight of 46 kDa in SDS PAGE in *P.gingivalis* and *E.coli* and is designated *Pga*A. This protein encodes antigenic determinants for two monoclonal antibodies; Brick 190, the epitope for which is of the discontinuous type and disrupted by SDS used in PAGE analysis, and LDS28 the epitope for which is of the continuous type. These epitopes are probably at differing sites in the protein given the lack of cross reactivity of LDS28 with the Brick190-reactive 309 bp cloned internal fragment of *pga*A designated pGPR1. The pGPR1 *pga*A internal fragment also expressed an antigen reactive with an anti-*P.gingivalis* whole cell polyclonal antibody and the lack of success in using this polyclonal in western blots might also hint that the initial reaction against pGPR1 in colony blots of this antisera may have been due to conformational epitope(s).

The antigen for both Brick 190 and LDS28 was in both cases *P.gingivalis* ECV (Brick 190 antigen was in fact derived from a purified fraction of ECV with enhanced TLP activity) and given that ECV consist of outer membrane material and probably some periplasm (Grenier and Mayrand, 1979), it is likely that PgaA in *P.gingivalis* adopts either a periplasmic or outer membrane localisation or may even be secreted. LDS28-immunogold labelling of the outer membrane of intact *P.gingivalis* cells (Wallace *et al.*, 1992) also suggests that PgaA has an outer membrane localisation. Although it was not possible to detect bacterial signal sequences for secretion in the sequence of PgaA, there is a potential membrane spanning region and it is possible that export of this protein is by an unknown mechanism. Examples of proteins inserted through membranes without signal sequences include serine proteases of *Serratia marcesens*, the IgA protease of *Neisseria gonorrhoea*, the α-lytic protease of *Lysobacter enzymogenes* and the haemolysin of *E.coli* (reviewed by Pugsley, 1993). The observation that many of the examples of Gram-negative secretory pathway-independent secreted proteins given above are proteases may be circumstantial, though it is interesting to note that several of the *P.gingivalis* protease sequences reported so far also do not exhibit the classical signal sequence (Kato *et al.*, 1992; Otogoto *et al.*, 1993; Park and McBride, 1993).

Both LDS28 and Brick 190 have been implicated in having specificity for TLP and this enzyme is also expressed at the cell surface and in ECV (Roberts *et*

al., 1990). Brick 190 as well as being raised against a partially purified TLP also has been shown to neutralise its activity at high titre (John Greenman, personal communication); however attempts at repeating this experiment for this thesis were unsuccessful. Brick 190 was however shown to react with a high molecular weight smear in ECV envelopes and outer membrane preparations. LDS28 was seen to react with a 46 kDa protein the TLP "gingivain" preparation (kindly provided by H. Shah) but could not quench TLP or haemagglutination activity at high titre (Wallace *et al.*, 1992). The fact that gingivain can haemagglutinate erythrocytes and that subsequent experimentation has proved a separate molecular identity TLP and haemagglutinins (Shah *et al.*, 1992) suggests that the gingivain preparation is not homogenous and this statement is supported by the presence of multiple bands in this preparation (section 4.2.8).

Analysis of *P.gingivalis* cells and vesicles using the non denaturing CHAPS PAGE system revealed that both BAMCA cleavage (CHAPS PAGE substrate zymography) and LDS28 binding is localised to high molecular weight smear. A similar high molecular weight smear is also observed with LDS28 in SDS PAGE western blots of *P.gingivalis* when samples are not fully denatured by boiling (A.M. Wallace, personal communication). These data suggest that PgaA is in a conformation, or is associated with a structure which is resistant to detergent solubilisation, and when in this state the antigen runs with a far lower mobility than when solubilised. Thus PgaA may adopt a rigid conformation once folded that does not bind detergent proportionally, and hence runs with a lower apparent molecular weight. This is a characteristic also shown by some *E.coli* porins (Nikaido and Nakae, 1979). Alternatively, a high molecular weight complex (>2,000 kDa molecular weight) of the outer membrane has been described for *P.gingivalis* with which a 75 kDa antigenic determinant is associated (Yoshimura *et al.*, 1989); possibly PgaA may also be a component of this complex and this may explain the high molecular weight smear observed in CHAPS western blots.

E.coli clones of *pga*A did not exhibit TLP, haemagglutination or glycyl-prolyl dipeptidase activities, nor were protease consensus sequences noted. What is more no protease activity could be detected in any of the clones containing *pga*A constructed for this thesis. However, a cutinase/serine protease active site consensus sequence was found in the downstream open reading frame, ORF 199. As it is likely that ORF 199 and *pga*A are expressed from the same

transcript and are translationally coupled, it is possible that their functions might be inter-related. For example the two genes may encode separate subunits of the same protein and if this is the case one speculative hypothesis might be that *pga*A encodes a subunit of a PgaA/ORF199 oligomeric protease/cutinase whose activity requires both subunits; specificity of LDS28 and Brick 190 for the larger of the two subunits (PgaA) would be consistent with these mAb reacting with preparations of protease. This hypothesis could be tested further if antibodies raised against the protein encoded by ORF199 were also found to be specific for components of purified protease.

It is assumed that *pga*A was cloned along with its own promoter in that it is expressed in *E.coli* in the absence of any vector promoter. However the expression of PgaA in *E.coli* may be aberrant in that synthesis of products showing multiple molecular weights are directed by *pga*A alone. The reason for this is unknown but might be due to translation from within internal start codons within the gene, premature translational termination at the site of rare codons or from the degradative activity of *E.coli* proteases. What is more a truncation of this type may explain the discrepancy in the predicted (50 kDa) and observed (46 kDa) molecular weight for PgaA, although the 46 kDa form is also seen in western blots of *P.gingivalis* with LDS28 and *E.coli* Sure(pGPR7). The fact that ORF199 was not expressed, but is predicted to be on the *pga*A transcript is consistent with a breakdown in either trancription or translational coupling, and could be due to premature termination of either transcription or translation.

Expression of *pga*A from pGPR2 in *E.coli* minicells resulted in detectable expression of PgaA using radiolabelling. However, it was not possible to detect PgaA expression from pGPR2 using antibodies. An explanation for this might be that antibody techniques could not achieve the high levels of sensitivity required for detection. The cloning of *pga*A into over expression vector pTTQ18* did however result in detection of PgaA using antibodies, although it was found that expression from these constructs was unstable. It is also possible that some complex regulation of *pga*A expression may be involved. The presence of a palindromic sequence upstream of *pga*A may constitute an operator site and hence could be involved in modulation of *pga*A expression in *P.gingivalis* and *E.coli*. Also the presence of an 19 bp inverted repeat upstream of *pga*A is reminiscent of the *E.coli* fimbrial phase switch, where 9 bp inverted repeats are used to target a site specific inversion

of a DNA fragment encoding the promoter for *fim*A, the structural gene for type I fimbriae. Should such a site specific inversion of the promoter for *pga*A occur, this might be the explain difficulties encountered in detecting expression of PgaA.

Monoclonal antibodies exhibiting species specificity have the potential to be used as species-specific probes useful in the diagnosis of infection (Naito *et al.*, 1985; Chen *et al.*, 1986; Hayakawa *et al.*, 1992) and the identification of particular serotypes in bacterial infection (Gmür *et al.*, 1988). More recently the use of PCR in medicine has been implemented for use in diagnosis of infection (Spaepen *et al.*, 1992). PCR using specific primers has the advantage of being relatively quick and simple although difficult to use for quantitative analysis. Target sequences for amplification must be specific for the organism in question; possibly the use of serotype antigen genes would be a logical target for serotype specific PCR primer design. The *pga*A gene is located on the *P.gingivalis* chromosome apparently as a single copy. It shares no outstanding sequence homology with any other gene or protein apart from possessing a consensus phosphate binding domain and hence represents a novel protein of unknown function. In Southern blots the pGPR2 insert used as a probe was found to hybridise to all *P.gingivalis* strains tested but not other the *Porphyromonas* or *Bacteroides* species tested. This is consistent with the specificity of LDS28 which is also only reacts with *P.gingivalis* strains (Wallace *et al.*, 1992). It has been suggested that LDS28, having a high specificity for *P.gingivalis* might be useful as a diagnostic probe for this organism (Wallace *et al.*, 1992). Given that *pga*A has no DNA sequence homologues and encodes an antigen apparently unique to *P.gingivalis* it is possible that probes derived from *pga*A may be useful in identification of *P.gingivalis* and that possibly oligonucleotide primers derived from *Pga*A could be used for PCR-based diagnosis of *P.gingivalis* infections.

The cloning of cell surface and secretory components has implications for future vaccine development and manufacture of therapeutic agents. Immunisation with *Streptococcus mutans* as well as topical application of anti-streptococcal antibodies has been shown to provide protection against caries (reviewed by Roit and Lehner, 1980). Preliminary observations that anti-*P.gingivalis* antibodies augment immune reactions *in vitro* (Cutler *et al.*, 1991) make it tempting to speculate that the same might be true for periodontal disease. Cloning of genes encoding major antigenic determinants and

subsequent over-production in fusion protein purification systems may in the future allow generation of high affinity monoclonal antibodies using, for example phage display library technology (Hoogenboom *et al.*, 1991). The resulting chimeric antibodies can be enhanced for high avidity as well as engineered for antigen studies. Over expression of chimaeric antibody gene fusions in, for example yeast or bacterial systems could allow production of antibody on a process scale. Such antibodies might be useful at augmenting the immune response when added exogenously to infected gingiva and hence provide a useful therapeutic agent. Possibly PgaA might be one such target and the work laid down in this thesis instructive.

The cloning of *pga*A for over production of antigen may also be useful for vaccine development as it is found in all *P.gingivalis* strains tested so far and hence may provide cross-serotype protection. Generally speaking today's vaccines consist either of attenuated or dead organisms (Chatfield *et al.*, 1992) or of a mixture of antigens (Mitchell and Andrew, 1995). For PgaA to be useful in a vaccine one possible mode of delivery might be heterologous expression of *pga*A in attenuated *Salmonella* strains, and oral challenge. This method is attractive because it can be used orally and results in both secretory and humoral antibody responses (secretory antibodies may be of use in the gingival crevice) as well as cell mediated responses (Chatfield *et al.*, 1992). Alternatively, protein antigens covalently linked to capsular polysaccharide (T-independent) antigens have been shown to activate T-cells as well as B-cells and this results in a more efficient immune response against polysaccharide during immunisation, especially in infants (Siber, 1995). The latter is probably not a realistic method for *P.gingivalis* vaccine development in that as yet the capsular polysaccharide antigens of *P.gingivalis* remain poorly studied.

Along with *pga*A several other apparently unrelated genes were cloned and these include a cluster of three genes which are probably co-transcribed. All three have putative transmembrane domains and hence must be associated with inner or outer membrane. ORF202 possesses a lipoprotein cleavage and sorting signal and could localise in either membrane or be secreted. Sequence data for these proteins is abundant but isn't explicit enough to be able to allocate these proteins a probable function. One thing that is probable is that the functions of these proteins may be interconnected because of the

likelihood that they are co-translated. Two predictions stand which can be tested.

The first is for ORF297 which is homologous to an arylsulfatase protein from marine bacterium *Alteromonas carragenovora*. The amino acid sequence similarity of ORF297 and the arylsulfatase is not strong. Hydrophobic cluster analysis (HCA) of the two amino acid sequences revealed that they have two hydrophobic domain clusters in common, and this suggests a structural correlation between the two proteins (T. Barbeyron, unpublished). HCA analysis piles up the protein in question so that distant amino acids are clustered into a mosaic-like matrix from where highlighted hydrophobic clusters can be compared. The result is that distantly related proteins with poor search scores can be compared in terms of conserved structure where amino acids can be degenerate. This technique also revealed a strong cluster of histidines at the same relative position in the two proteins. It is thus possible that ORF297 might be an arylsufatase and might have some role in the break down of host sulphated molecules either extracellularly, or intracellularly for nutritional purposes. The over expression construct of ORF297, pGPR103 should facilitate assessment of arylsulfatase activity. What is more this clone also has an f1 origin or replication and hence can be used for preparing single stranded DNA for site-directed mutagenesis.

The second prediction of function is for ORF202 which encodes a potential lipoprotein. The predicted amino acid sequence of ORF202 is homologous with a domain from p60 found in *Listeria* species and endopeptidase II from *Bacillus sphaericus* and as these proteins hydrolyse peptidoglycan it seems likely that ORF202 is also a peptidoglycan hydrolase. To test this prediction, as it is possible that ORF202 may cause cell lysis when over expressed due to degradation of the murein sacculus, ORF202 could be cloned alone into pGEM-T (Promega Corporation) using PCR in the T7 orientation (figure 6.6). Genes cloned in this configuration should not be expressed unless they are cloned along with their own promoters, at least until they are introduced into a strain carrying the DE3 T7 RNA polymerase. ORF202 could then be over expressed (although probably being ultimately lethal to expressing cells) and its activity defined by looking for the ability to disaggregate chains of Gram-positive species such as *Listeria, Bacillus* and *Streptococci* species and to illicit bacteriolytic activity(Wuenscher *et al.*, 1993). In addition western blotting of such over expressing clones with anti-*P.gingivalis* antiserum might suggest a

cell surface localisation for the ORF202 translation product. Subsequent studies might then focus on studying the lipoprotein properties of ORF202, by for example using inhibitors of lipoprotein signal peptidase and determining the presence of unprocessed prolipoprotein using SDS PAGE.

Further experimentation to determine the role of PgaA in *P.gingivalis* virulence as an antigenic determinant or otherwise might centre around adopting a molecular form of Koch's postulates, namely that PgaA is always associated with pathogenic strains of *P.gingivalis*, that specific inactivation of *Pga*A should cause a measurable loss in virulence, and that the effect of the *pga*A mutation on virulence can be restored by complementation with a wild type *pga*A allele (Falkow, 1988). This approach could also be applied for the other ORFs described in this volume. Such an approach would first require a technique for making a *Pga*A *P.gingivalis* mutant. Allelic replacement has been achieved in *P.gingivalis* by cloning an insertionally inactivated allele of the gene in question into suicide vector pNJR5 and transferring that construct by mating from *E.coli* to *P.gingivalis* (Roberts, I.S., unpublished results). Upon suicide of the vector the inactivated allele inserts into the chromosomal allele *via* homologous recombination and if insertional inactivation is achieved using the chloramphenicol acetyl transferase gene, the mutant allele can be selected for by growing bacteria on chloramphenicol (Ostrowski and Roberts, unpublished). A *pga*A mutant of this type could be confirmed by looking for ablation of the antigen using western blotting, and its virulence assessed in for example gnotobiotic rat model (Klausen, 1991a; 1991b). Restoration of a *pga*A genotype could then be achieved by complementing the mutation *in trans* on a plasmid vector.

Other studies could then focus on expression of *Pga*A. Transcriptional activity can be assessed in *P.gingivalis* by measuring chloramphenicol acetyl transferase levels (Ostrowski and Roberts, unpublished) and environmental effectors of transcription investigated. Similarly primer extension (Maniatis *et al.*, 1992) of *P.gingivalis* RNA using *pga*A sequence oligonucleotides might reveal the transcriptional start of *pga*A. This data could then be used to characterise the promoter, and mutate the dyad and inverted repeat structures upstream of *pga*A and assess their role in the expression of *pga*A.

The work laid down in this thesis has proved challenging. Heterologous expression of *P.gingivalis* DNA in *E.coli* has allowed cloning and expression of

a novel antigenic determinant of *P.gingivalis* whose function is as yet ambiguous. These though are amongst the initial steps in attempting to understand the role of *P.gingivalis* in periodontal disease, and how an essentially commensal subgingival microbial flora can undergo a conversion so as to constitute an inflammatory infection. This thesis has attempted lay ground work for future studies and hopefully enlarge the debate relating to the molecular mechanisms involved in the pathogenesis of *P.gingivalis*.. In the six years since this study began improved molecular genetic techniques have developed for the study of this Gram-negative anaerobe and it is hoped they will solve some of the questions surrounding *pga*A and the other genes identified in this study.

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