Interaction of *Escherichia coli* with catecholamine hormones, transferrin and lactoferrin

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by

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Abstract

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The correlation between stress and increased susceptibility to infectious disease has been widely established. A direct consequence of stress, whether physical or mental, is the release of catecholamine stress hormones (adrenaline and noradrenaline), which as well as reducing immune function, have recently been shown to directly enhance the growth of a variety of Gram positive and Gram negative bacteria. The mechanism of catecholamine-induced growth stimulation involved catecholamines facilitating iron removal from the high affinity mammalian iron binding proteins transferrin and lactoferrin. In the case of *Escherichia coli*, the direct binding of transferrin was also an important part of this process. However, the precise mechanism(s) by which catecholamines enabled bacterial access to transferrin was not clear, and neither was the identity of the *E. coli* protein(s) responsible for the binding of transferrin. Using the enteropathogenic *E. coli* strain E2348/69 as model organism, the objectives of this project were therefore to determine how catecholamines facilitated iron removal from transferrin and lactoferrin, identify and characterise the *E. coli* transferrin-binding protein, and to investigate globally the effects of catecholamine stress hormones on *E. coli* growth and virulence.

In this study, using a combination of electron paramagnetic resonance spectroscopy, chemical and polyacrylamide gel electrophoresis techniques, it was found that catecholamine stress hormones form a complex with the iron(III) associated with transferrin and lactoferrin, causing its reduction into iron(II), to which these proteins have much lower affinity. This enables the dissociation of iron from transferrin or lactoferrin, which then becomes available for bacterial uptake through ferric or ferrous iron uptake systems. It was also shown that although catecholamines enabled *E. coli* to acquire iron from transferrin, the amounts of iron made available by the stress hormones, while sufficient to enable growth, the levels of iron actually within the catecholamine-treated bacteria was not enough to switch off the expression of iron-regulated genes. The effects of catecholamines on expression of *E. coli* proteins was also investigated, and it was found that expression of intimin, important in formation of attaching and effacing lesion, was up-regulated in the presence of catecholamines.

Investigation of the role of quorum sensing in the mechanism of *E. coli* catecholamine responsiveness was also undertaken. Analysing catecholamine responsiveness of a series of *E. coli* E2346/89 *luxS* mutants showed that *luxS* is not required for the ability of *E. coli* to interact with catecholamine stress hormones.

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Con amore / With love

Sara

To my daughter Noemi

ABBREVIATIONS

6-OHDA	6-hydroxydopamine
A	absorbance
ACTH	corticotrophin
AI	autoinducer
Amp	ampicillin
APCs	antigen presenting cells
APS	ammonium persulphate
ATP	adenosine triphosphate
Bfp	bundle-forming pili
BSA	bovine serum albumin
CAs	catecholamines
Cm	chloramphenicol
CNS	Central Nervous System
CRH	corticotrophin-releasing hormones
Da	Daltons
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DO	dopamine
DOB	dobutamine
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EPEC	enteropathogenic *E. coli*
EPI	epinephrine
EPR	electron paramagnetic resonance
Fbp	periplasmic ferric iron binding protein
Fe	iron
Fur	ferric uptake regulation
Fz	ferrozine
G.A.S.	General Adaptation Syndrome
GC	glucocorticoids
GDP/GTP	guanosine diphospate / guanosine triphospate

GR	glucocorticoid receptor
g-value	shading coefficients measure the solar energy transmittance
HCl	hydrochloride
HPA	hypothalamic-pituitary-adrenal
HSP90	heat shock protein 90
HT	hypothalamus
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
IFN-γ	interferon γ
IL	interleukin
IPTG	isopropyl β-D-1-thiogalactopyranoside
ISO	isoprenaline
kb	kilobase
kDa	kilodalton
LbpA and B	two protein components lactoferrin receptor
LEE	locus of enterocyte effacement
Lf	lactoferrin
LfBp	lactoferrin binding protein
LOS	lipooligosaccharide
LPS	lipopolysaccharide
mT	millitesla
MW	molecular weight
NE	norepinephrine
NK	natural killer cells
OD	optical density
ORF	open reading frame
PAMPs	pathogen-associated molecular patterns
PMN	polymorphonucleocyte
PRRs	pattern recognition receptors
R LPS	rough LPS
SAM	sympathetic-adrenal medullary
SB	protein sample loading buffer
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis

SELDI	Surface-enhanced laser desorption/ionization
TbpA and B	two protein components transferrin receptor
TEMED	*N*,*N*,*N*′,*N*′-Tetramethylethylenediamine
Tet	tetracycline
Tf	transferrin
TfBp	transferrin binding protein
Tir	translocated intimin receptor
Tris	tris(hydroxymethyl)aminomethane
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
Δ mutant	deletion mutant
μCi	microcuries

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Chapter 1 – Introduction

The correlation between stress and disease has been well established over the last century. Stress in its many forms has been associated with the aetiology of a variety of medical conditions ranging from life threatening diseases such as cancer and heart failure to more trivial gum disease, acne, psoriasis, and also with an 'increased susceptibility to infectious disease'. The latter is a condition that refers to an 'effect' that stress has not only on the host immune system but also on the pathogenic agents themselves directly, and it is this aspect that will be the major focus of my thesis.

First it is important to give a brief introduction on the definition of stress, what are its effects on the host immunity and which stress-released compounds are directly responsible for the increased susceptibility to infectious diseases by both direct and indirect interaction with pathogenic agents.

1.1 Definition of stress

In our modern life the word '*stress*' is used and overused, although it is more like an abstract concept that everybody feels but which cannot be easily defined. A student during exam period, a person going to an interview, a mother worried about her child, somebody experiencing a car accident, an injury or a surgical operation, they will be all subjected to a stress event, although the stressors are of different nature. Stressors are agents that produce stress at anytime and they can be grouped in the following categories (Reiche *et al.*, 2004; Steptoe, 2000):

- **Physical stressors**: e.g. a car accident, surgical operation, burns, exposure to extreme environmental conditions;
- **Chemical stressors:** e.g. exposure to drugs, toxic agents, anaesthetics, bacterial toxins;
- **Psychological stressors:** e.g. student exams, writing a thesis, relationship difficulties.

The perception of stress varies drastically between individuals and only when a certain event is perceived by the brain as stressful may there be a physiological response (McEwen, 1998; Rabin, 2005).

The human and animal response to stress is generally associated with the 'fight or flight' response (Selye, 1976) in which the physiological response to a stressor results in the activation of the sympathetic nervous system, causing the release of catecholamines and glucocorticoids into the bloodstream. The 'fight or flight' response

has also been associated with the human and animal behavioural response to stress, which correlates with the observation that under threat or stress animals either fight if the conditions seem favourable for victory, or flight if the chances of defeat are greater. However, if this is true for male behaviour it is not entirely applicable to female, even though the fight or flight physiological response is similar in both sexes. From a behavioural point of view the response to stress in female is better described as 'tend and befriend' (Taylor *et al.,* 2000) since the first instinct of the female is to protect her offspring and avoid confrontation if possible. This difference in behaviour in response to stress between sexes may be associated to the fact that in most animal species, the female has a major role for the care and protection of the offspring which thus is correlated to the survival of the species.

In 1936 the physiologist Dr. Hans Selye, was the first to medically define *stress* as "the state manifested by a specific syndrome which consists of all the non specifically-induced changes within a biological system" (Selye, 1976). This syndrome, better known as stress syndrome or general adaptation syndrome (G.A.S.) consists of three phases: the alarm reaction, the stage of resistance and the stage of exhaustion. (Selye, 1946). In the first phase, the alarm response, the stressor causes the release of catecholamines by the adrenal medulla and production of glucocorticoids by the adrenal cortex. The function of these hormones, better known as stress hormones, is to help restore homeostasis. If continuous exposure to stressor is not so deleterious as to result in death, the phase of resistance occurs, in which the body manages to restore the balance of homeostasis and the level of stress hormones normalise. However, if the stressors persist, the phase of exhaustion occurs, which is characterised by a similar but stronger physiological response to the one obtained during the alarm reaction, and fatigue of the immune system can resolve in disease, or in extreme cases death. The G.A.S. theory is still used today as an outline of the stress response, but in light of more recent studies some adjustments have to be made, such as those suggested by McEwen (McEwen, 2005), who emphasised that the G.A.S. can no longer be used to represent a stereotypical response to all kinds of stress, since the hypothalamic-pituitary-adrenal axis and the noradrenergic and adrenergic nerves have different patterns of response according to the different type of stressor. McEwen also suggested that the final stage of the G.A.S. theory is the one in most need of refocusing, due to the fact the deleterious effects observed in the "exhaustion" phase are not due to a fatigue of the immune system but rather an excessive immune response (McEwen, 1998). Stress itself

is not wholly bad, for example, secretion of stress hormones in response to threat enhances awareness, increases heart rate and blood pressure, allowing for fast reaction to a dangerous situation. However, when the stress is protracted the state of 'stress' transforms into 'stressed out' and the outcome becomes negative causing disease or even death (McEwen, 2005).

What is then the correlation then between stress and disease? To answer this question it is necessary to observe in more detail how intertwined the central nervous, the endocrine and the immune systems are.

1.2 Interactions between the central nervous system, endocrine and immune system during stress

It seems obvious to correlate stress and its impact on health since the central and peripheral nervous system and the endocrine and immune systems share many common signal mediators and receptors (Fig.1.1). In fact, the physiological response to stressors results in a close interaction between the central nervous system (CNS), the endocrine and the immune system that is modulated by a complex bi-directional system of signalling pathways. The two major pathways that control the stressor response and have a direct impact on the immune function are the hypothalamic-pituitary-adrenal (HPA) and the sympathetic-adrenal medullary (SAM) axis (Chrousos, 1995; Elenkov *et al.*, 2002; Padgett *et al.*, 2003; Reiche *et al.*, 2004).

Response of the body to stress results in the activation of the CNS via blood-borne neurosensory and limbic signals which stimulate the hypothalamus (HT) to release corticotrophin-releasing hormones (CRH). CRHs play a fundamental role in coordinating the neuroendocrine response to stressors (Dunn *et al.*, 1990) by stimulating the synthesis of pro-opiomelanocortin by the anterior pituitary gland, which is subsequently then cleaved to corticotrophin (ACTH), β-endorphin and α-melanocyte-stimulating hormones. The ACTHs consequently stimulates the production of glucocorticoids (GC) from the adrenal gland. The CRHs stimulate the locus ceruleus to produce tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of norepinephrine (NE), which is then synthesised and secreted. CRH further activates the autonomic nervous system to release NE from peripheral sympathetic nerve termini and in addition NE and epinephrine (EPI) are secreted from the adrenal medulla.

High levels of circulating catecholamines (CAs) and glucocorticoids also termed ‘stress hormones’, will be present during stress and are responsible for ensuring that an adequate stress response is triggered. In addition they have bi-directional effect on a variety of immune cells, which will be discussed in detail later, which possess receptors for them (Chrousos, 1995; Elenkov *et al.*, 2002; Padgett *et al.*, 2003; Reiche *et al.*, 2004).

Catecholamines and glucocorticoids comprise the two major classes of stress hormones but during a stress response other compounds are involved, for example the level of opioids (responsible for reduction of pain during fight-flight response), arginine vasopressin (together with CRH induce pro-opiomelanocortin synthesis), somatosin (inhibitor of growth factor synthesis) and dopamine (inhibits release of prolactin from HT) is increased.

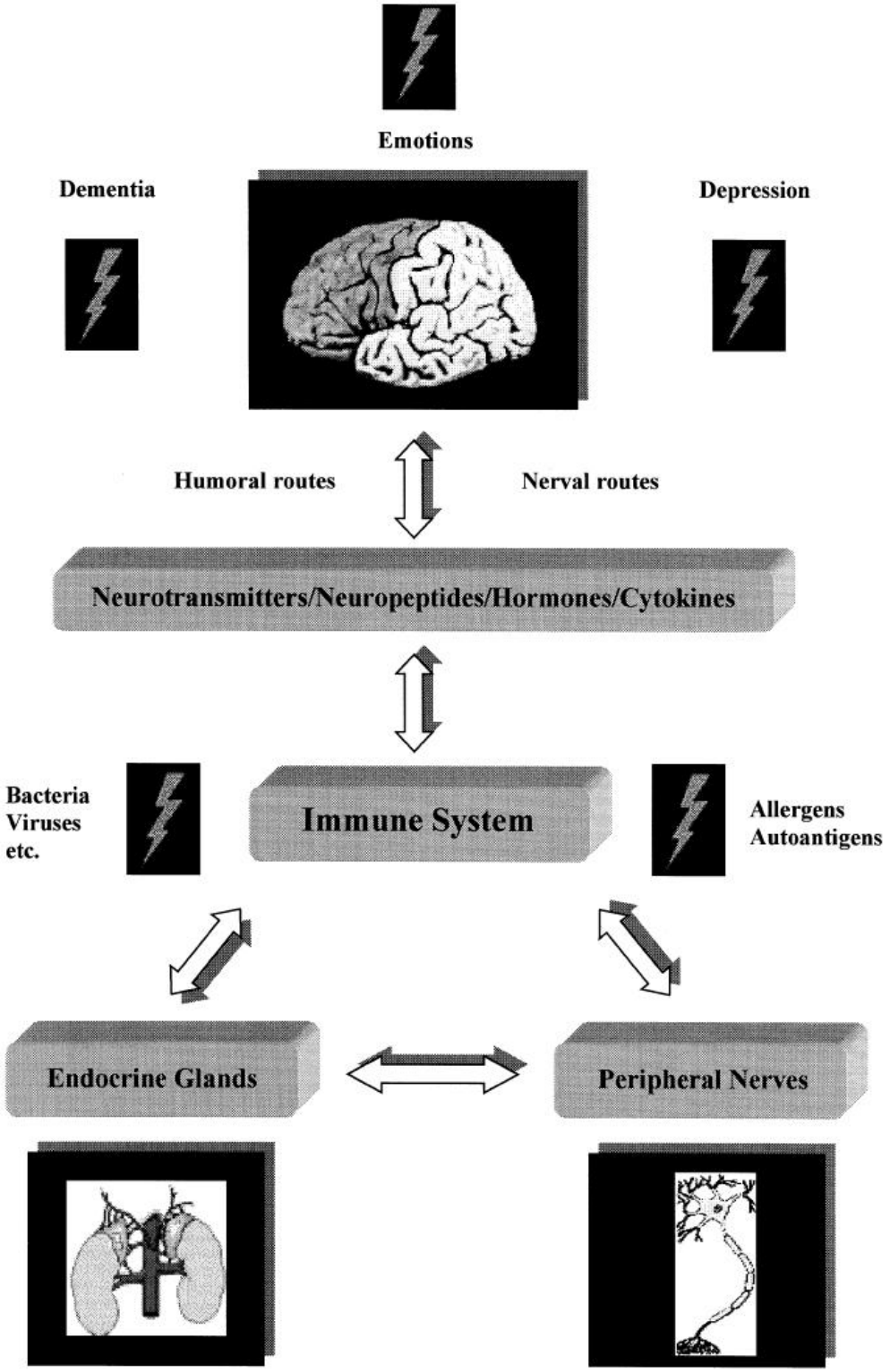


Fig. 1.1 Bi-directional network communication between the central and peripheral nervous system and the endocrine and immune systems (Haas *et al.*, 2001)**.**

1.3 Glucocorticoids

GC hormones are lipophilic molecules that belong to the steroid family of hormones. Their site of action on the immune system during stress, depends on the presence of GC receptors (GR) on the target cell (Padgett *et al.*, 2003), with GR being found on a variety of immune cells such as macrophages, monocytes, dendritic cells and T lymphocytes (Haas *et al.*, 2001; Morale *et al.*, 2001; Padgett *et al.*, 2003; Reiche *et al.*, 2004). GR's are comprised of three regions: an N-terminal domain involved in transactivation (Hoeck *et al.*, 1990), a DNA binding domain that is responsible to bind DNA through the two zinc fingers (La Baer *et al.*, 1994) and the C-terminal domain or ligand binding domain involved in transactivation, dimerization and heat shock protein 90 (HSP90) binding (Howard *et al.*, 1990; Kanelakis *et al.*, 2002). The inactive form of GR's exist as a part of an oligomeric complex bound to HSP90 in the cytoplasm that upon ligand binding dissociate and translocate into the nucleus where they complex with GC via the zinc finger of the DNA-binding domain. This complex then modulates the expression and transcription of a variety of effector molecules.

Cortisol is the one of the most important human and animal GC hormones and it is essential for a variety of cardiovascular, immunologic, metabolic and homeostatic functions. During stress the increased systemic levels of cortisol result in an increase of blood pressure and blood sugar levels and decrease of the immune response, focusing on the preparation for a fight-flight response. In fact, cortisol is an immunosuppressant and inhibits the mitogen-induced proliferative responses of T lymphocytes, the lytic activity of natural killer (NK) cells, the production of cytokines such as interleukin-1 (IL-1), IL-2, T-cell growth factor, serum immunoglobulin production, suppressor cells, monocyte function and cytotoxic responses (Black, 1994b; Chrousos, 1995; Marchetti *et al*., 2001; Padgett *et al.*, 2003; Reiche *et al.*, 2004). GCs also bind to receptors on the surface of antigen presenting cells (APCs), which suppresses the production of IL-12 causing reduction interferon-γ (IFN-γ) production by antigen-primed CD^{4+} T cells, the main inducer of Th1 response. This, results in a shift towards a Th2 type response associated with an increase of IL-4 release by T cells (Blotta, *et al.*, 1997; Elenkov, *et al.*, 1996; Elenkov *et al.*, 1999).

1.4 Catecholamines

The other main group of 'stress hormones' are the catecholamines, and it is this class of hormones that it is of particular interest to this thesis.

CAs are compounds synthesised from tyrosine (Fig.1.2) which contain a catechol group comprising of a benzene ring with two adjacent hydroxyl groups, and opposing primary or secondary amine side chains. The rate-limiting enzyme of catecholamine synthesis is tyrosine hydroxylase, whose activation and inhibition is regulated by a complex feedback system involving respectively a cAMP-dependent protein kinase and NE (Rang *et al.*, 1999; Ruffolo *et al.*, 1994).

At the sympathetic division of the autonomic nervous system, the catabolism of tyrosine begins with the first hydroxylation step, which occurs in the cytoplasm of postganglionic nerve termini and it is catalysed by the enzyme tyrosine hydroxylase. The next enzymatic step, catalysed by L-aromatic amino acid decarboxylase, again occurs in the cytoplasm of postganglionic sympathetic nerve termini, though the product of this reaction, dopamine, which is the first of the CAs to be synthesised, is transported and stored in storage vesicles in the sympathetic nerve terminal.

Dopamine can be further processed by the enzyme β-hydroxylase to produce norepinephrine, where, in association with adenosine triphosphate (ATP) it is retained and stored in the vesicle until an action potential triggers its release. This action potential, which is propagated through the length of the nerve's axon, is the result of electrical impulses originating at the central nervous system (CNS) level as a result of stress related stimuli, and it is caused by the depolarization and repolarisation of the neuronal membrane and consequent exchange of Na^+/K^+ ions (Fig.1.3). Upon the arrival of a sufficient action potential the release by a calcium dependent mechanism, exocytosis, occurs and the NE storage vesicle fuses with the neuronal plasma membrane with consequent discharge of NE that will then mediate the response of the effector organ by interacting with the adrenoceptor (Rang *et al.*, 1999; Ruffolo *et al.*, 1994).

The released NE can also undergo different fates:

- NE can be re-taken up through $uptake_1$ system into the sympathetic nerve terminal where it is either re-stored into storage vesicles or metabolised by monoamine oxidase (MAO) in the mitochondria.

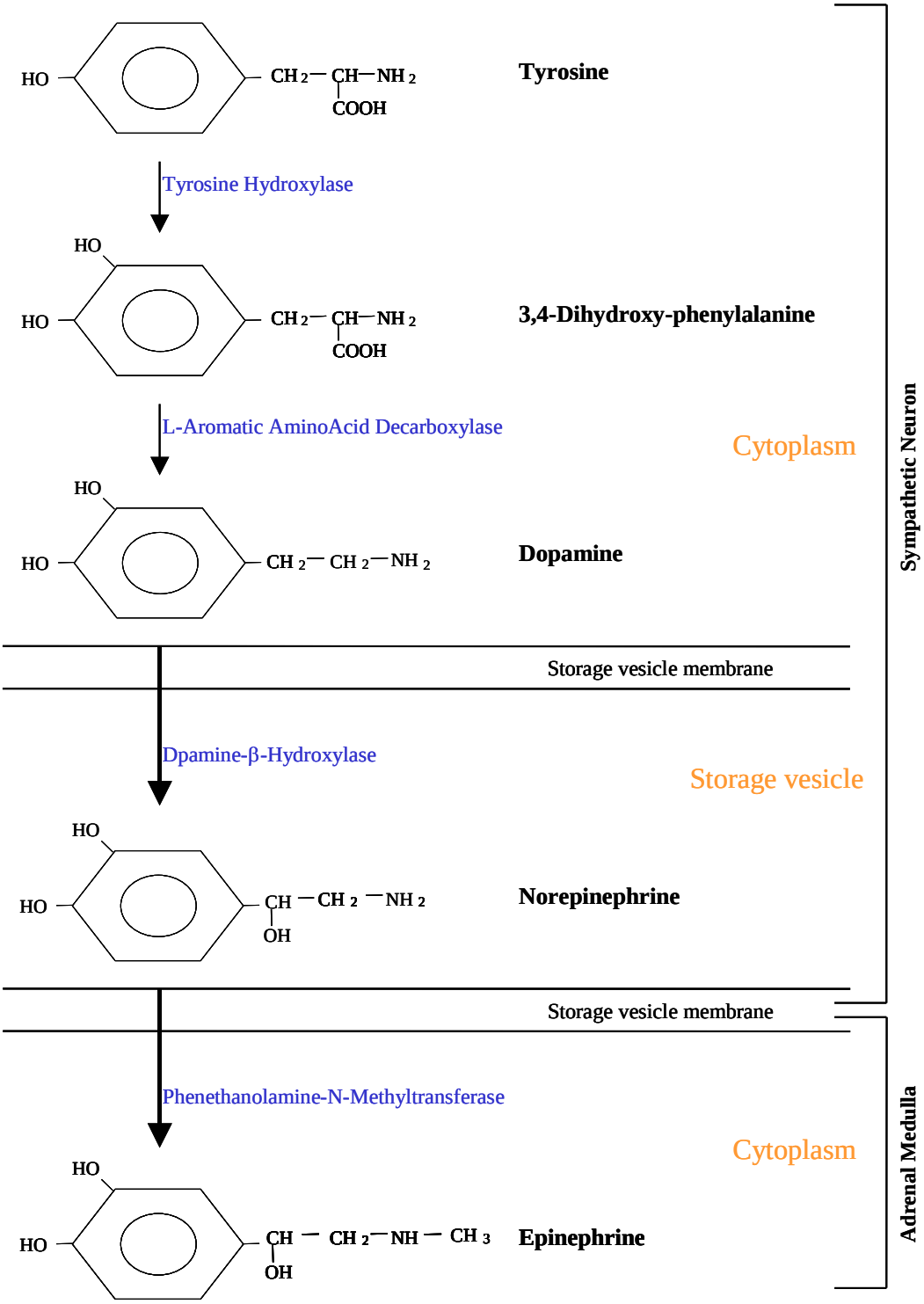


Fig.1.2 Catecholamines biosynthetic pathway. Illustrated are the enzymatic steps involved in the biosynthesis of the catecholamines dopamine (DO), norepinephrine (NE) and epinephrine (EPI). DO and NE synthesis occurs in postganglionic sympathetic nerve terminal, whereas the four enzymatic steps leading to the synthesis of EPI occurs in the adrenal medulla.

- NE diffuses away from the adrenoceptor and it is taken up by the uptake$_2$ system on the effector organ where it is metabolised either by catechol-O-methyl-transferase (COMT) or by MAO.
- Released NE interacts with the α_2-adrenoceptor at the nerve terminal to induce the auto-inhibitory feedback mechanism to control NE release by reducing intracellular cAMP (cAMP promotes Ca^{2+} influx and consequent depolarisation of the neuronal membrane, thus stimulating the release of NE).

The final product of tyrosine catabolism, EPI, is synthesised in the adrenal medulla by N-methylation of NE. This reaction is catalysed by phenethanolamine-N-methyl transferase, which is not present in the nerve terminal. EPI is then accumulated in storage granules in chromaffin cells and upon stimulation is released together with a small amount of NE directly into the circulation.

It has been mentioned already that CAs trigger a specific response in the effector organs by interacting with the adrenoreceptors that they express. Adrenoceptors are a class of G-protein coupled receptors, organised on the membrane of the effector cells in a 7- loop serpentine structure, and can be divided into two main subgroups: α and β adrenoceptors, as first suggested by Ahlquist (Ahlquist, 1948). According to his observation, different CAs showed different orders of potency of response in different adrenoceptor subtypes, measured as follows (Rang *et al.*, 1999):

Receptor	Order of agonist potency
α	NE > EPI > ISO (isoprenaline)
β	ISO > EPI > NE

The adrenoreceptors can be further subdivided into α1, α2 and β1, β2 and β3 subclasses (Ahlquist, 1948; Rang *et al.*, 1999; Ruffolo *et al.*, 1994).

The difference between α and β receptors stand also in the pathway by which they trigger the effector response (Fig.1.4). Both adrenoceptor groups function as intermediaries in transmembrane signalling pathways involving receptor, G-protein and effector. They all communicate with the cytoplasm of the effector cell by activation of a G-protein complex, which in its inactive form is present as a heterotrimeric complex comprised of three subunits named α, β and γ, in which the α-subunit is bound to guanosine diphosphate (GDP). Upon binding of the neurotransmitter to the receptor, the G-protein is then activated and GDP is instead replaced by GTP (guanosine triphospate), to which the two subunits β and γ, have less affinity and dissociate from

the complex. This dissociation is the trigger point for the activation of the secondary messengers, which then stimulate the different pathways by which the different adrenoceptor stimulate the effector response (Rang *et al.*, 1999):

- The α1 and α2 receptor are coupled to phospholipase C and their effect mainly depends on the release of intracellular Ca^{2+};
- The α2 also negatively couples to adenylate cyclase to reduce cAMP and inhibits Ca-channel (negative feedback of NE release from the terminal nerve);
- The β-adrenoceptors react by stimulating adenylate cyclase.

The most abundant adrenoceptors involved in the mediation of neurotransmitter response of immune cells apparently are the β2-adrenoceptors (Madden, 2003). As already mentioned, during stress the level of CAs is drastically increased in order to prepare the body for the "fight or flight" response by increasing the heart rate, blood pressure and circulating glucose level, which are essential to increase awareness and to provide energy to respond adequately to the imminent 'difficult' situation. However, the general effect of neuroendocrine catecholamine hormones (norepinephrine, epinephrine and dopamine) on the immune response is mainly inhibitory, since all the effort during 'dangerous' circumstances need to be spent to prepare the body to 'fight or flight'. These effects include the inhibition of T-cell proliferation induced by mitogens, NK cell activity, neutrophil phagocytosis, release of lysosomal enzymes from neutrophils and macrophage activity; whereas they stimulate B-cell antibody production and cytotoxic T cell function (Black, 1994a; Padgett *et al.*, 2003).

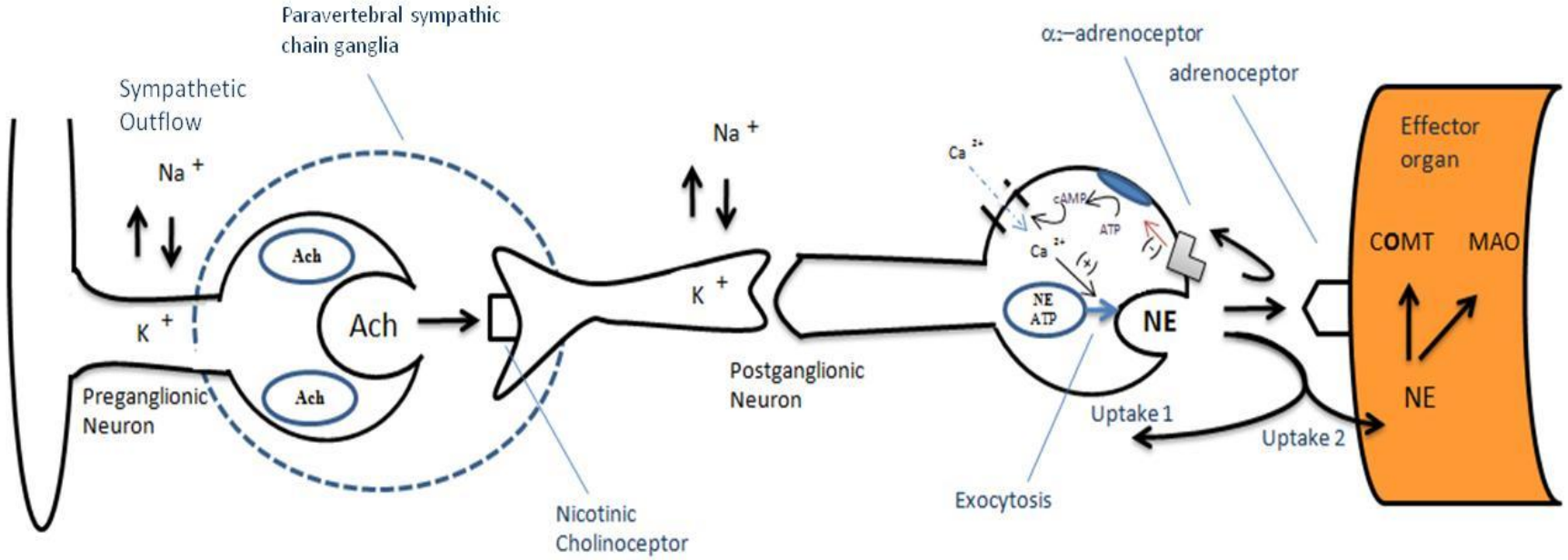


Fig.1.3 Schematic representation of the neurohumoral transmission in the sympathetic division of the autonomic nervous system. Short preganglionic neurons originate in the spinal cord to make synaptic connections in the peripheral autonomic ganglia. Upon arrival of an action potential to the preganglionic nerve terminal generated by the Na+/K+ ion exchange consequent the depolarization and repolarisation of the neuronal membrane, acetylcholine (Ach) is released by exocytosis, which diffuses across the synaptic cleft and interacts with the cholinoceptor on the cell body of the postganglionic neuron. This interaction allows the propagation of the action potential that elicits the release of the neurotransmitter (NE) at the postganglionic nerve terminal by exocytosis. The released NE can then undergoes several fates: 1) diffuses across the neuroeffector junction to stimulate the effector organ response by interacting with the adrenoceptor; 2) is reuptaken through uptake$_1$ system into the sympathetic nerve terminal where is either restored into storage vesicles or metabolised by monoamine oxidase (MAO) in the mitochondria; 3) diffuses away from the adrenoceptor and is uptaken by the uptake$_2$ system on the effector organ where is either metabolised by catechol-O-methyl-transferase (COMT) or MAO; 4) the NE interacts with the α_2-adrenoceptor at the terminal nerve level to induce the autoinhibitory feedback mechanism to control NE release by reducing intracellular cAMP (cAMP promotes Ca^{2+} influx consequent depolarisation of the neuronal membrane, thus stimulating release of NE).

1.5 Stress related infectious diseases depend on the role that stress hormones play in immunosuppression? Or there is more to it?

Initially the general consensus on the role played by catecholamines in stress-related increased susceptibility to infectious disease was negative, attributed to the direct effects on these compounds on host immune system, resulting in its impairment. It is perceivable that this in itself could explain why there might be increased risk of developing an infectious disease in stressed individuals, and so until recently the direct interaction between stress hormones and infectious agents was not taken into consideration (Freestone *et al.*, 2008; Lyte, 1993; Lyte, 2004).

An adult human carries approximately 10^{12} microbes associated with his epidermis, 10^{10} microbe inhabiting the oral cavity and 10^{14} microbes in his alimentary tract (Luckey, 1972). The latter, comprises the intestinal microflora which commonly consists of between 500-1000 bacterial species that live in symbiosis within our gastrointestinal tract (Sonnenburg *et al.*, 2004; Xu *et al.*, 2003), and play an important role in the development, structure, physiology, immunity, and maintenance of a healthy gut (Barbara *et al.*, 2005). One of the most important roles of the intestinal microflora is the development and promotion of the gut neuromotor function to enable appropriate digestion of food. The gastrointestinal tract is innervated by more than 100 million neurons (Costa *et al.*, 2000; Santos *et al.*, 2000), which play a fundamental role in restoring normal homeostasis during a stressful episode. Thus gut bacteria are routinely subjected to close contact with catecholamine stress hormones that are released by the enteric nervous system during a stress response, or that spill over from the circulation, causing significant local increases in CAs levels (Aneman *et al.*, 1996; Eisenhofer *et al.*, 1997). Moreover, outside the host, bacteria are exposed to catecholamines produced by protozoa (Janakidevi *et al.*, 1966) and plants (Apaydin *et al.*, 2000). Keeping these notions in mind, with the widespread nature of catecholamines it is not surprising that bacteria have evolved methods of interaction with these molecules. The evidence suggests that while commensal bacteria are normally living in symbiosis with their host, given favourable conditions they could then assume the role of an opportunistic pathogen (Tlaskalova-Hogenova *et al.*, 2004; Dowd, 2007a). But how can bacteria sense when the most suitable time for invading the host organism is?

The fact that bacteria are able to respond to environmental stimuli such as temperature, water availability, pH, osmolarity etc. is widely established, and in a similar fashion, bacteria have evolved mechanisms to understand when the host is under stress by interacting with our own stress hormones. In 1992 a new scientific field named microbial endocrinology was born, which was aimed at studying the intersection between endocrinology and microbiology in regard to stress-related host susceptibility to infectious disease, and to explain how infectious organisms utilise hormones present within the host as a means of 'sensing' their surroundings and as environmental cues to initiate growth and pathogenic processes (Lyte, 2004).

A direct interaction between stress hormones and bacteria had been observed as early as 1930 by Renaud and Miget (Renaud *et al.*, 1930) when they described an unusual fulminatory (within 6 hrs) case of gas gangrene caused by a syringe contaminated with clostridial spores in a patient whom was injected with adrenaline to treat urticaria. Although later it became clear that inadequately cleaned syringes could transmit diseases between individuals, the interesting fact was the observation that the dose of *Clostridium perfringens* needed to cause an infection was reduced by 4 logs in presence of a therapeutic concentration of epinephrine (Cooper, 1946; Evans *et al.*, 1948). In 1948 Evans *et al.* (Evans *et al.*, 1948) injected guinea pigs with a solution containing Gram positive and Gram negative bacteria supplemented with either epinephrine or a saline solution (control). Tissues analysis showed that in the bacteria supplemented with epinephrine the bacterial growth was enhanced when compared over the controls. Later studies (Lyte *et al.*, 1992) using a serum-based minimal medium (serum-SAPI) designed to mimic the *in vivo* conditions a bacterium would experience in its host, showed that addition of catecholamines such as norepinephrine, epinephrine and dopamine enhanced the growth of three strains of *Escherichia coli*, *Yersinia enterocolitica* and *Pseudomonas aeruginosa* when compared to the control samples, although the catecholamine metabolites normetanephrine and 4-hydroxy-3-methoxyphenylglycol did not affect the growth of the bacteria. These results indicate not only that NE enhanced the growth of these bacteria in a serum-based medium but also that the effect observed was not simply a consequence of a nutritional enrichment of the medium since incubation with normetanephrine, which contains one more methyl group than norepinephrine and hence would serve as a better energy source for growth, did not stimulate bacterial growth. Moreover, later studies (Freestone *et al.*,

1999) showed that catecholamine responsiveness was widespread amongst bacterial pathogens demonstrated that the effect of CAs was not only exclusive to a few species but it was a wide spread phenomenon. Freestone *et al.* tested 23 species of Gram negative and Gram positive pathogens showing that NE was able to enhance the growth of the majority of species tested. They also showed that the majority of the gram negative and not the gram positive bacteria exposed to NE were producing heat stable autoinducers (AI) (previously identified in *Escherichia coli* O157:H7*)* (Lyte *et al.*, 1996b; Lyte *et al.*, 1996a) able to induce growth within and cross species without further presence of NE. Chromatographic characterisation of autoinducers representative of Gram negative bacteria showed a high level of structural similarity between the species, although structural determination is still in progress (Freestone, unpublished data).

Other studies (Neal *et al.*, 2001) aimed to test the ability of catecholamine inotropes and other drugs used in intensive care units (ICUs) (28 compound tested) to stimulate the growth of a variety of bacteria. An investigation using coagulase-negative *Staphylococci* (associated with intravascular catheters which correlate with infections in ICUs patients) in a serum-based minimal medium demonstrated that inotrope compounds lacking the dihydroxybenzoyl moiety were not able to stimulate bacterial growth (Fig.1.5) indicating that the two adjacent hydroxyl groups on the benzene ring were an essential feature.

Animal model studies of traumatic stress (Alverdy *et al.*, 2000) had shown that the level of norepinephrine present in the gut lumen of mice that had undergone partial hepatectomy was significantly increased. Inoculation of *Pseudomonas aeruginosa*, opportunistic pathogens usually found during post-surgical complications, in hepatectomied mice, showed enhanced ability to adhere to the gut mucosa. Norepinephrine was also found to induce synthesis of *Pseudomonas aeruginosa* PA-1 adhesin both *in vitro* and *in vivo*. Other studies have reported that NE could enhance *E. coli* 0157:H7 adherence to gut tissues in a bovine ligated ileal loop model of infection (Vlisidou *et al.*, 2004). Norepinephrine has also been found to stimulate expression of the K99 pilus adhesin in enterotoxigenic *E. coli* (Lyte *et al.*, 1997b) and type 1 fimbriae in commensal *E. coli* (Hendrickson *et al.*, 1999). Other examples of virulence factors found to be enhanced by catecholamines were the shiga-like toxins (SLTs) of *E. coli* O157:H7 (Lyte *et al.*, 1996a).

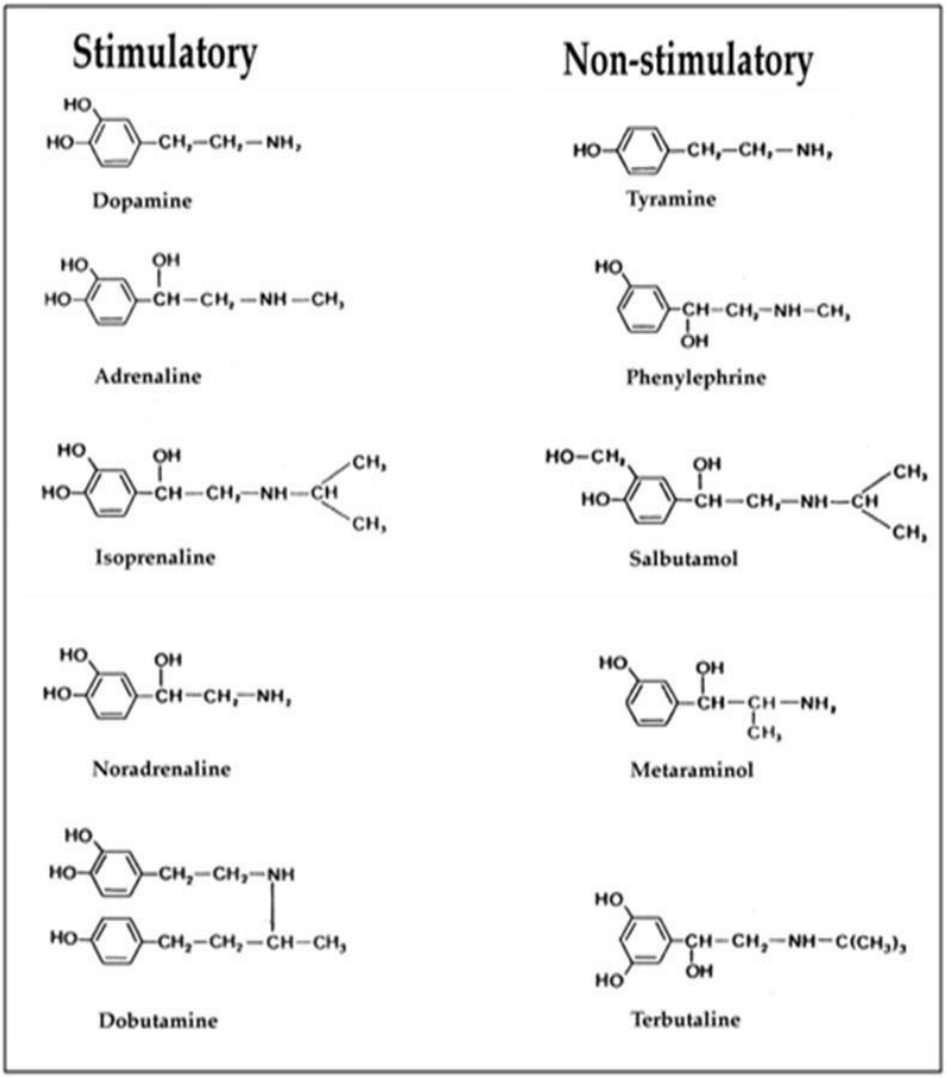


Fig.1.4 Structure of stimulatory and non-stimulatory compounds of bacterial growth. The structures of catecholamine and related drugs used in ICUs are illustrated above and as can be seen all stimulatory compounds are comprised of a benzene ring with two adjacent hydroxyl groups. (Taken from (Neal *et al.*, 2001))

Therefore not only can catecholamines enhance growth in a variety of bacterial species but they can also stimulate the production of a range of virulence factors, revealing how fundamental their role on the outcome of an infection is by closely and directly interacting with the both the host and the infectious agent.

1.6 Bacteria and their host's competition for iron

One of the most crucial elements for the growth and survival of many organisms (including bacteria) is iron. In nature, iron exists in its ferric (FeIII or Fe^{3+}) and ferrous (FeII or Fe^{2+}) cationic forms, whose redox potentials differ greatly (-300 mV

to +700 mV (Andrews *et al.*, 2003)) according to which molecule the iron is bound to, and it is this flexibility that makes the iron an essential redox mediator in biological systems. Therefore, iron is involved in a variety of enzymatic reactions essential to cellular processes such as amino acid and nucleotide synthesis, DNA synthesis, electron transport, activation of oxygen, peroxide reduction and photosynthesis (Wandersman *et al.*, 2004).

Notwithstanding the abundance of iron on earth, the availability of free usable iron in nature and in host organisms is restricted since the oxidised form of iron is insoluble, whereas the reduced form is toxic for the majority of macromolecules present in biological systems due to its ability to catalyse Fenton reactions and produce harmful free radicals (Fig.1.5).

- $Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot}$
- $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$

Fig.1.5 Fenton-type reactions. Iron reacts with oxygen with the release of free radicals, which are toxic to the host (Lewin, *et al.*, 2006); thus free iron in the body is usually sequestered by transport and storage protein systems.

Most cell-based living organisms have evolved several strategies to ensure uptake, transport and storage of iron. Therefore, the ability of invading organisms and hosts to compete for the acquisition of iron is one of the major factors that determine the outcome of a pathogenic infection.

1.6.1 Iron sequestering strategy of the innate immune defence

Fundamental to innate immunity is the recognition, through pattern recognition receptors (PRRs) of a variety of pathogen-associated molecular patterns (PAMPs) shared by large groups of microorganisms, which can then trigger an appropriate immune response by providing the signal needed for the induction of an adaptive immune response.

Another important ability of the innate system to resist bacterial invasion depends heavily on restricting access of the bacteria to free iron. Most microbial pathogens require a minimum iron concentration of 10^{-6} M however the body creates a virtually free-iron environment (10^{-18} M of iron in normal tissue fluids (Bullen *et al.*, 2005)) where almost all the free iron present is associated with transport and storage proteins

such as transferrin, lactoferrin, ferritin and hemoproteins (Ong *et al.*, 2006; Weinberg, 1978), of which the high affinity ferric iron-binding proteins transferrin (Tf) and lactoferrin (Lf) are most important, generally being 30-40% saturated with iron (Bullen, *et al.*, 2006). Tf and Lf are acute-phase proteins and their concentrations closely reflect conditions of stress and infection. Serum Tf and Lf belong to the family of transferrins, which are glycoproteins with a molecular weight of approximately 80 kDa. They consist of two homologous lobes, N- lobe and C-lobe connected by short peptide linkers, which are ordered and helical in Lf but unstructured in Tf. Each lobe can be further subdivided into two subdomains named respectively N1 and N2 and C1 and C2 which form a cleft, each of which contains a highly specific Fe^{3+} binding site (Caccavo *et al.*, 2002; Wally *et al.*, 2007). Human serum transferrin (Tf) is synthesised in the liver and released into the plasma where its main function is to transport ferric iron from the gut and to deliver it to requiring cells *via* specific surface transferrin receptors (TFR) by endocytosis (Rao *et al.*, 1983). Upon internalisation of the Tf-receptor complex, iron is released into the acidic endosome (pH 5.5) and transferred into the cytoplasm where it can be incorporated into metalloproteins, stored into ferritin (storage proteins) or chelated to smaller molecules (Ong *et al.*, 2006). In humans, Lf is found in milk, tear fluid, saliva and other bodily secretions such as pancreatic juice, small intestine secretions, bile etc. The affinity of Lf for ferric iron is approximately 300 times higher than in Tf, allowing it to maintain iron at lower pH, characteristic of the body secretions in which it is found (Caccavo *et al.*, 2002). Human Lfs are stored in specific granules of polymorphonucleocytes (PMN) from where they are released upon activation. Although Lf binds and transports ferric iron, its main function is not as an iron transport protein but as an antimicrobial and anti-inflammatory agent (Caccavo *et al.*, 2002). Lf binds with high affinity to lipid A, the toxic moiety of the lipopolysaccharide, or to endotoxin of Gram-negative bacteria and exerts its bactericidal activity by damaging the outer membrane of the bacteria. Moreover, Lf has been found to decrease the release of pro-inflammatory cytokines such as IL-1, IL-2 and TNF-α and to enhance the cytotoxicity of monocyte and natural killer cells (Caccavo *et al.*, 2002).

In addition to Tf and Lf, other molecules play an important role in iron-withholding strategy during infection, such as lipocalin proteins, hepcidin peptides, natural resistance-associated macrophage proteins 1 (Nramp1) and ferritins.

Lipocalin is a small extracellular protein that in addition to participating in several biological functions such as biosynthesis of prostaglandins, regulation of cellular homeostasis and immunity, plays an important role in the outcome of a bacterial infection by binding to catecholate-type siderophores and preventing them from uptaking iron for the bacteria (Flo *et al.*, 2004).

Hepcidin is an antimicrobial peptide, who's expression in hepatocytes is enhanced by IL-6 following inflammation (Nemeth *et al.*, 2004). Hepcidin peptides play an important role in lowering the absorption of intestinal iron and therefore in maintaining a low concentration of iron in the plasma during infection (Nemeth *et al*., 2004).

Nramp plays an important role in regulating the metabolism and release of iron acquired by phagocytosis from erythrocytes but is not Tf or Lf- associated. Induction of macrophages with LPS, bacterial toxin or cytokine such as TNF-α or Il-1 enhanced the expression of Nramp and soon after phagocytosis. Nramp is rapidly recruited onto the membrane of maturing phagosomes where it helps sequestering iron from the bacteria by extruding the iron (Ong *et al.*, 2006).

The major roles of ferritin include iron storage and detoxification. This protein is present in all mammalian cells and predominantly in macrophages and hepatocytes. The transcription and translation of ferritin is regulated by pro-inflammatory cytokines. A small amount of ferritin has also been found in the plasma (ng/l) and possible physiological functions for this portion of protein include: messenger with hormonal effect of the small intestine; regulation of transferrin synthesis by hepatocytes; iron scavenging toward leaked ferrous ions from damaged cells during infection (Ong *et al.*, 2006).

1.6.2 Bacterial strategies for host-iron acquisition

In order to overcome the lack of 'usable' iron encountered both in the host and in the environment, bacteria have evolved several means to obtain iron. These can be described via two main mechanisms: direct contact, which involves interaction between the bacterium and the exogenous iron/haem source or direct uptake of ferrous

iron through G protein-like transporters; or indirect, through use of extracellular 'iron scavenger' molecules, such as siderophores and hemophores, which are synthesised and released by the bacterium (Andrews *et al.*, 2003; Wandersman *et al.*, 2004).

Under non-oxidising anaerobic conditions, representative of the mammalian small intestine and stomach, iron can be predominantly found in its soluble ionic Fe^{2+} state. A variety of bacteria that colonise the intestinal tract (such as *E. coli, Salmonella enterica, Helicobacter pylori* etc.) can uptake iron through the ferrous iron transporter membrane protein FeoB. This ferrous iron transporter is expressed under anaerobic and low iron conditions by the *feoB* gene. FeoB is a G-coupled protein composed of two regions: a hydrophilic N-terminal domain that exhibits GTPase activity and contains a conserved guanine nucleotide Fe^{2+} binding site required for the uptake of the iron; and a hydrophobic C-terminal domain consisting of membrane spanning α-helices (7-12) (Hantke, 2003; Marlovits *et al.*, 2002).

Another method by which a variety of bacteria can uptake iron in the host is by directly binding to the host iron-binding proteins Tf and Lf. Several Gram negative and positive bacterial species have been found to bind Tf and Lf through specific surface receptors. For Gram negative species such as *Haemophilus influenza* (Khan *et al.*, 2007a; Khan *et al.*, 2007b), *Neisseria meningitis* (Perkins-Balding *et al.*, 2004) and *Neisseria gonorrhoeae* (Price *et al.*, 2004) that do not produces siderophores, acquisition of iron from Tf and Lf plays a major role in the outcome of an infection and occurs via iron regulated TonB dependent surface receptors, consisting of two protein components TbpA and TbpB or LbpA and LbpB, which bind to Tf and Lf respectively. Upon complex formation the Tf/Lf-iron is removed and transported across the bacterial outer membrane where it binds to periplasmic ferric iron-binding proteins, FbpA, that shuttle the iron across the periplasm to the ABC permease system that in turn it translocates it into the cytosol (Andrews *et al.*, 2003). Specific Tf (Modun *et al.*, 1998) receptors were also found to be expressed on the cell wall of Gram positive bacteria such as *Staphylococcus aureus* and *S. epidermidis* (Modun *et al.*, 1998).

Bacteria can also obtain iron in a similar receptor-mediated fashion from haem (Genco *et al.*, 2001).

Indirect iron acquisition by the bacteria is achieved by means of high-affinity extracellular iron chelators named siderophores. Siderophores are low molecular

weight compounds (<1000 Da) with high affinity towards ferric iron, that are synthesised and released by the bacteria under low iron conditions (Andrews *et al.*, 2003). Siderophores chelate ferric iron usually forming hexadentate octahedral complexes typically employing hydroxamates, α-hydroxycarboxylates and catechol ligands. Fe^{3+}-siderophore complexes are uptaken by the bacterial cell through TonB-dependent outer membrane receptors (i.e. FepA, FecA, FhuA). These receptors are expressed under iron starvation and are proteins comprising 22 β−stranded tubes that traverse the outer membrane acquiring a β-barrel like structure with a 'plugged' central channel. The function of a 'plug' or 'cork' is to limit access to the periplasm and upon binding of the ferric iron-siderophore complex undergoes conformational changes to enable its passage. Bacteria often possess more than one type of outer membrane receptor to allow cross recognition (Andrews *et al.*, 2003). Bacteria can also uptake iron from haemoglobin and haemopexin via haemophores (Genco *et al.*, 2001).

Once bacteria reach stationary growth phase or enter into an iron repleted environment, they store the iron excess within storage compounds such as Dps proteins, ferritins and bacterioferritins (Andrews *et al.*, 2003).

1.7 How do catecholamines affect bacterial growth?

As described in section 1.6, both the host and the bacteria are strongly competing for the acquisition of iron, although, under normal physiological conditions, a combination of iron depletion and a healthy immune system provides the host with the means to avoid infections and create an environment that permits the microflora to coexist in a balanced manner with the host. However, when the host is under stress a large amount of catecholamines are produced and the balance between the host and the bacteria is shifted to the detriment of the host. Freestone *et al.* (Freestone *et al.*, 2000) demonstrated by testing the ability of norepinephrine to stimulate the growth of clinical isolate *E. coli* with different fractions of bovine serum (previously separated by size exclusion chromatography) that the presence of Tf was essential for the growth induction process. Freestone *et al* showed that NE forms a complex with Tf and Lf, somehow weakening the normally high binding affinity of these proteins, such that the bacteria were able to bind and acquire iron from these proteins. Later work (Freestone *et al.*, 2002; Freestone *et al.*, 2003; Freestone *et al.*, 2007b) showed that in

addition to NE, inotropes such as EPI, DO, isoprenaline, dobutamine and certain catecholamine metabolites and dietary factors could also facilitate bacterial access to Tf and Lf-complexed iron. The mechanism by which the catecholamines and related compounds were able to facilitate bacterial access to the normally securely bound Tf/Lf-complexed was not fully understood, and is therefore an objective of this current project. Freestone and co-workers also provided evidence that *E. coli* can bind TF, though the identity of the *E. coli* TF-binding protein has yet to be determined; identification of the *E. coli* Tf/Lf binding protein is also a project objective.

1.8 Enteropathogenic *Escherichia coli E2348/69*

In view of the fact that the bacteria strain used as a model for the purpose of this thesis is the human clinical isolate enteropathogenic *Escherichia coli (*EPEC) E2348/69 (serotype O127:H6) a brief introduction to its pathogenesis and the systems adopted by this strain to acquire iron and cause infection will be given in this section.

EPEC are the major causative agents of infant diarrhoea worldwide, which constitutes a major problem in developing countries, where each year they claim the life of over 2 million victims, predominantly infants below the age of 5 (S. C. Clarke, *et al.*, 2003). The pathogenesis of EPEC is dependent on the ability of the bacteria to cause attaching and effacing (AE) lesions on the surface of host epithelial cells, which result in the reduction of the absorptive capacity of the intestinal mucosa and electrolyte unbalance causing diarrhoea. The mechanism of EPEC pathogenesis has been proposed to involve four stages (Donnenberg *et al.*, 1992; Knutton *et al*., 1998). The first stage involves the expression, under favourable conditions, of bundle-forming pili (Bfp), intimin and short surface filaments EspA. Through Bfp and EspA the bacteria are able to adhere to epithelial cells and via a type III secretion system, the translocated intimin receptors (Tir) and a number of undetermined effector molecules are injected directly into the host cells causing cell-signalling alterations and eventually depolymerisation of actin and loss of microvilli (stage two). At this stage (third stage) the EspA filaments are removed and the bacteria attach directly onto the host cells via adhesin intimin-modified Tir complex formation. In the fourth stage the translocated effector compounds interfere with the host cell processes leading to electrolyte loss and ultimately cell death.

As for other *E.coli* strains, the acquisition of iron plays a fundamental role in the outcome of an infection, and EPEC strains generally possess more than one system to acquire iron. *E. coli* it is known to comprise of at least seven iron-acquisition systems which genes (~35 iron-repressed genes) are strictly regulated by the transcriptional repressor ferric uptake regulation protein, Fur. The main system of *E. coli* for ferric iron acquisition is via siderophores. *E. coli* has been found to express under iron restricted conditions up to six siderophore receptors (Cir, FecA FepA, FhuA, FhuE, Fiu) although the only siderophores produced endogenously are enterobactins (McHugh *et al.*, 2003). Conversely, the expression of FeoB receptors on the outer membrane of *E. coli* is essential for the acquisition of ferrous iron in anaerobic environment such is the gut. Previous studies (Stojiljkovic *et al.*, 1993) observed that the ability of *feo* mutant *E. coli* strains to colonise mouse intestine was drastically impaired which also demonstrates the importance of a functional Feo system during infection.

1.9 How can bacteria sense when their host is under stress?

No genomic evidence for the existence of a classical mammalian adrenergic receptor exists in any bacterial species. The first study to determine the nature of possible bacterial receptors for the catecholamines was carried out by Lyte *et al*. (Lyte *et al.*, 1993) who used 3 α and β adrenergic antagonists, to investigate their ability to block CA induced growth responses in Gram negative bacteria. However, these antagonists had little effect in blocking growth, leading to the conclusion that the receptors through which bacteria sense stress hormones were neither the classical mammalian α or β adrenergic-type receptors. On the contrary, in later studies Sperandio *et al.* (Sperandio *et al.*, 2003) showed that α and β adrenergic antagonists, phentolamine and propanolol, could inhibit EPI and NE-induced motility and expression of genes in the locus of enterocyte effacement (LEE) in *E. coli* 0157:H7. They later identified as the putative CA receptors the two quorum sensing component regulators that control flagella and LEE expression, QseBC and QseEF (Sperandio *et al.*, 2002), which were also associated with specificity toward the *luxS*-dependent autoinducer-3 (Sperandio *et al.*, 2003) suggesting a potential cross communication between the bacterial quorum sensing and the EPI/NE host signalling system. They afterwards demonstrated that radio-labelled NE could bind to the sensory kinase QseC

protein in *E. coli* 0157:H7 (Clarke *et al.*, 2006), although they observe the α adrenergic antagonist phentolamine but not the β-adrenergic antagonist propanolol could interfere with the binding, which is in contradiction with previous results obtained from the same research group (Sperandio *et al.*, 2003). However, the effect of these or other antagonists was not observed on the most important stage of the bacterial-CA response mechanism, the initiation of growth.

Thus, disagreements between and within research groups on the nature of the CA receptors exist. In order to offer a solution to these controversies a more comprehensive study was performed by Freestone *et al.* (Freestone *et al.*, 2007a). These workers used a wide range of antagonists and CAs to demonstrate the existence of specificity bacterial catecholamine responsiveness, and the possible role of adrenergic and dopaminergic type receptors in three major enteric pathogens, *E. coli* 0157:H7, *Salmonella enteric and Yersinia enterocolitica*. From their results, Freestone *et al*. (Freestone *et al.*, 2007a) concluded that initial recognition by the bacteria of NE and EPI that results in stimulation of growth is mediated by receptors that show α adrenergic specificity. Surprisingly, none of the adrenergic antagonists were able to inhibit DO-induced bacterial growth, whereas dopaminergic antagonists such as chlorpromazine were able to block it. Investigations on the mechanisms by which the antagonists were blocking bacterial growth revealed that they did not effected the ability of CAs to mediate Fe acquisition from Tf or the ability of bacteria to accept Fe from CA-Tf or the induction of CA-AI. However the α adrenergic antagonists were causing reduction of cellular uptake of radio-labelled NE, supporting the hypothesis that they were blocking a specific signal transduction pathway. These observations were consistent between the three species analysed, suggesting that the response pathways through which CAs are sensed and initial growth is stimulated in the bacteria is unlikely to be dependent by the previously identified QseC or QseE receptors (Sperandio *et al.*, 2002), especially since *Y. enterocolitica* does not contain homologues to QseBC or QsecEF.

There are still many unclear aspects on the mechanism/s by which CAs recognised and enhance bacterial growth and virulence gene expression, although it is clear that bacteria can sense when the host is under stress and they can use stress hormones as a cues to prepare to invade the defences of their "compromised" host and so ensure their proliferation and survival.

1.10 Aims and Objectives

The hypothesis behind my PhD project is that the interaction between CA hormones and bacteria such as *E. coli* can play a fundamental role in the pathogenesis of stress related bacterial infection. The overall aim is to investigate the interaction between bacteria and catecholamine hormones to obtain a better understanding of the catecholamine response mechanisms that lead to the development of stress related bacterial infection.

The precise aims of the project are to determine the exact mechanism by which CAs can facilitate iron removal from Tf and Lf, to identified the *E. coli* transferrin and lactoferrin binding proteins (TfBp and LfBp), and to globally investigate how CAs enhance the ability of *Escherichia coli* to causes stress related infection, using a combination of proteomic, mutagenesis and chemical techniques.

Chapter 2 – Materials and Methods

2.1 MATERIALS

2.1.1 Bacterial strains

Details of the bacterial strains used in this thesis are summarised in the table below.

Strain	Relevant characteristics	Source or reference
E2348-69 *Strep^r*	O127:H6, *Strep*^{r}	(Haigh, 1999)
DH5α	F$^-$, Δ80*lacZ*ΔM15, *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*(r_K^- m_K^+), *relA1*, Δ(*lacIZYA-argF*), U169	(Hanahan, 1983)
CC118λpir	*araD139*, Δ(*ara leu*)7697, Δ*lacX74*, Δ*phoa20*, *galE*, *galK*, *thi*, *rpsE*, *rpoB*, *argE*(Am), *recA1*, Rif^r, *λpir*	(Manoil *et al.*, 1985)
SM10λpir	*thi*1, *thr*1, *leuB*6, *supE*44, *tonA*21, *lacY*1, *recA*::RP4-2-Tc::Mu *Km*^{r}, *λpir*	(Simon *et al.*, 1983)
E2348-69 deletion *luxS*	O127:H6, *Strep*^{r}, everything deleted except 4 codons and stop codon of *luxS* using pRDH126	(Haigh, unpublished)
E2348-69 Term (TAA) *luxS*	O127:H6, *Strep*^{r}, frameshift at codon 2 by introduction of TAA codon within *luxS* using pRDH127	(Haigh, unpublished)
E2348-69 *Tet luxS*	O127:H6, *Strep*^{r}, insertion of *Tet*^{r} within *luxS* using pVS72	(Haigh, unpublished)
E2348-69 *Cm luxS*	O127:H6, Strep^r, insertion of Cm^r within *luxS* using pVS98	(Haigh, unpublished)

Table 2.1 Bacterial strains used in this thesis

2.1.2 Bacterial culture media

The basic media used to grow bacterial strains during the experiments were the following:

Bovine serum: Adult bovine serum, B9433, SIGMA, UK

DMEM: Dulbecco's Modified Eagle Medium, D5671, SIGMA, UK

Luria Broth (LB): 1 % ($^{w}/_{v}$) tryptone (Oxoid), 0.5 % ($^{w}/_{v}$) yeast extract (Oxoid), and 0.5 % ($^{w}/_{v}$) NaCl, adjusted to pH 7.0 with 1 M NaOH

Luria Agar (LA): LB solidified with 1.5 % ($^{w}/_{v}$) agar

SAPI media: 2.7 mM glucose, 6.25 mM ammonium nitrate, 1.84 mM KH_2PO_4, 3.35 mM KCl, 1.01 mM $MgSO_4$, adjusted to pH 7.5 with 1 M KOH

Serum-DMEM: 30 % ($^{v}/_{v}$) Bovine serum, 70 % ($^{v}/_{v}$) SAPI medium

Serum-SAPI: 30 % ($^{v}/_{v}$) Bovine serum, 70 % ($^{v}/_{v}$) SAPI medium

SOB broth: 2 % ($^{w}/_{v}$) tryptone (Oxoid), 0.5 % ($^{w}/_{v}$) yeast extract (Oxoid), 0.05 % ($^{w}/_{v}$), NaCl, 0.0186 % ($^{v}/_{v}$) KCl, adjusted to pH 7.0 with 1 M NaOH

Sucrose agar: 1 % ($^{w}/_{v}$) tryptone (Oxoid), 0.5 % ($^{w}/_{v}$) yeast extract (Oxoid), adjusted to pH 7.0 with 1 M NaOH, 1.5 % ($^{w}/_{v}$) agar. Following autoclaving it was made up to 6 % ($^{v}/_{v}$) sucrose using sterile 40 % ($^{w}/_{v}$) sucrose stock solution.

Where appropriate, the liquid media components were sterilised by autoclaving at 125 ^{o}C for 15 minutes and stored at room temperature until required.

For plate culture of bacteria, culture agar was melted and cooled to 50 ^{o}C and antibiotic or additional chemicals were added, if required, before pouring approximately 25 ml/plate into sterile 90 mm plastic Petri dishes. The solidified agar plates were then stored at 4 ^{o}C.

Antibiotics were added to solid or liquid culture media, when required, at the following concentrations unless stated otherwise: 50 μg/ml streptomycin (Strep), 100 μg/ml ampicillin (Amp), 50 μg/ml kanamycin (Kan), 10 μg/ml chloramphenicol (Cm), 10 μg/ml tetracycline (Tet).

The *lacZ* inducer isopropyl β-D-1-thiogalactopyranoside (IPTG, 25 μg/ml) and substrate 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal, 25 μg/ml) were added to LA plates for blue/white colony selection.

Iron repleted and iron restricted media were prepared respectively, by addition of 100 μM $Fe(NO_3)_3$ or 300 μM 2,2'-dipyridyl.

2.1.3 Bacterial strain preservation

Bacterial strains were maintained by weekly re-plating in LA supplemented with appropriate antibiotics. When required, stocks plates were replaced with frozen stock cultures that were preserved at -80 ^{o}C in cryoprotectant medium (25 % ($^{v}/_{v}$) glycerol, 75 % ($^{v}/_{v}$) LB).

2.1.4 Proteins

Lactoferrin from human milk (L4894), human holo-transferrin (Siderophilin, iron-saturated) (T-4132), human transferrin (Siderophilin, partially saturated) (T-3309) and human apo-transferrin (T-4382) were purchased from SIGMA, UK. Peroxidase conjugated ChromPure human transferrin was purchased from Jackson ImmunoResearch laboratories, inc, UK, 009-030-050.

2.1.5 Antibodies and enzymes

For detection of transferrin and lactoferrin, the primary antibodies used were anti-human transferrin, developed in goat (T-6265) and anti-human lactoferrin, developed in rabbit (L-3262), which were obtained from SIGMA, UK. The cross recognition secondary antibodies for transferrin and lactoferrin were respectively anti-goat Immunoglobulins G (IgG) peroxidase conjugated developed in rabbit (A-5420) and anti-rabbit IgG peroxidase conjugate developed in goat (A0545) bought from SIGMA, UK.

All restriction enzymes were purchased from New England BioLabs, UK. The restriction enzymes used in the experiments in this thesis were: *Bam*HI, *Xba*I, *Sph*I, *Hinc*II, and *Pst*I. *Taq* DNA polymerases, T4 DNA ligase, dNTPs, t-RNA, RNase and all the reagents used in molecular genetics were purchased from New England BioLabs or Promega, UK.

2.1.6 Catecholamines

All the catecholamines used were purchased from SIGMA, UK: (−)-Epinephrine (+)-bitartrate salt (EPI), E4375; (±)-Epinephrine hydrochloride (EPIHCl), E4642; L-(−)-Norepinephrine (+)-bitartrate salt monohydrate (NE), A9512; dopamine hydrochloride (DO), H8502; Dobutamine hydrochloride D0676 (DOB); (−)-Isoproterenol (+)-bitartrate salt (isoprenaline) (ISO), I2760.

2.1.7 Plasmids

Details of the cloning vectors used in this thesis are summarised in the table below.

Plasmid	Relevant characteristics	Source or reference
pUC19	ColE1, *bla*, *lacI*,*lacZ*	(Yanisch-Perron, *et al.*, 1985)
pRDH10	*sacRB, mobRP4, ori* R6K, Cm^r, Tet^r	(R. D. Haigh, 1999)
pRT733	*oriR6K, mobRP4, bla,* Tn*phoA, Kan*^{R}	(Taylor, *et al.*, 1989)
pKD46	*oriR101*, *bla*, *araC*, ParaB, λ-red recombinase, *rep*A101ts, ORF *tL3*	(Datsenko *et al.*, 2000)

Table 2.2 Plasmids

2.1.8 DNA size estimation ladders

1 kb DNA ladder, New England BioLabs, UK, N3232S

100 bp DNA ladder, New England BioLabs, UK, N3231S

Prestained SDS-PAGE Standards, broad range, BIO-RAD, 161-0318

2.1.9 Chemicals

All chemicals were purchased from SIGMA, UK, Fisher Scientific, UK, BIO-RAD, UK or GE Healthcare, UK, unless stated otherwise.

2.1.10 Buffers and solutions

- **Polyacrylamide electrophoresis**

SDS PAGE: the following recipes are for 5ml gel volumes:

12 % Resolving gel: 1.6 ml dH_2O; 2 ml 30 % acrylamide mix; 1.3 ml 1.5 M Tris (pH 8.8); 0.05 ml 10 % SDS; 0.05 ml 10 % ammonium persulphate (APS) 0.002 ml TEMED

8 % Resolving gel: 2.3 ml dH_2O; 1.3 ml 30 % acrylamide mix; 1.3 ml 1.5 M Tris (pH8.8); 0.05 ml 10% SDS; 0.05 ml 10 % ammonium persulphate (APS) 0.003 ml TEMED

Stacking gel: 0.68 ml dH_2O; 0.17 ml 30 % acrylamide mix; 0.13 ml 1 M Tris (pH 6.8); 0.01 10 % SDS; 0.01 ml 10 % APS; 0.001 ml TEMED

Urea gel: 2 ml 10 x TBE; 7.2 g urea; 4.5 ml 30 % acrylamide mix; 0.2 ml APS; 0.02 ml TEMED; up to 20 ml with dH_2O

- **Electrophoresis buffers**

10x TBE buffer: 1 M Tris-HCl pH 8.8, 0.1 M H_3BO_3, 50 mM EDTA. Used at 1x

50x TAE buffer: 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M Na_2 EDTA pH 8, dH_2O up to 1 l. Used at 1x

5x SDS PAGE running buffer: 15 g Tris base, 72 g glycine, 5 g SDS, dH_2O up to 1 l. Used at 1x

Western blot Blocking buffer: 5 % **($^{w}/_{v}$)** BSA in Blot Wash buffer

Western blot Wash buffer: 150 mM NaCl, 10 mM Tris-HCl pH 7.5, 0.05 % ($^{v}/_{v}$) Tween-20

SDS protein sample loading buffer (SB): 4 ml distilled water, 1 ml 0.5 M Tris-HCl pH 6.8, 0.8 ml glycerol, 1.6 ml 10% ($^{v}/_{v}$) SDS, 0.4 ml 0.05% ($^{v}/_{v}$) DTT, 0.2 ml 0.05 % ($^{w}/_{v}$) bromophenol blue

Urea protein sample loading buffer: 100 mM Tris-HCl pH 7.5, 10 % glycerol

TBS: 150 mM NaCl, 10 mM Tris-HCl pH 7.5

TBS-T: 5 ml of Tween20 made up to 1 l ml with TBS.

Western Blot electrotransfer buffer: ice cold, 5.9 g Tris, 2.9 g glycine, 100 ml methanol, 3.4 ml 10 % SDS, dH_2O up to 1 l.

2-D Gel rehydration buffer: 0.850 g urea (Aristar, UK 452043), 0.304 g thiourea, 0.08 g Chaps, 10 µl ampholyts, 24 µl DeStreak™, 10 µl bromophenol blue (0.25 % solution); up to 2 ml with npH_2O.

2-D Gel electrophoresis Buffer I: 5.40 g urea, 3.75 ml 1.5M TRIS-HCl pH 8.8, 3 ml 10 % SDS, 3 ml glycerol, 0.3 g DTT, up to a final volume of 15 ml using npH_2O.

Buffer II: as buffer I but instead of DTT 0.375 g Iodoacetamide is added.

- **PAGE and Western blot Stain and de-stain solutions**

Drying solution: 20 % ($^{v}/_{v}$) ethanol, 10 % ($^{v}/_{v}$) glycerol.

Ponceau S stain: 0.2 % **($^{w}/_{v}$)** Ponceau S, 1 % **($^{v}/_{v}$)** acetic acid. De-stain with H_2O or TBS.

PVDF membrane de-stain: 50 % ($^{v}/_{v}$) methanol, 10 % ($^{v}/_{v}$) acetic acid.

PVDF membrane stain: 50 % ($^{v}/_{v}$) methanol, 0.1 % ($^{w}/_{v}$) Coomassie brilliant blue R250.

SDS-PAGE gel de-stain: 25 % ($^{v}/_{v}$) isopropanol, 10 % ($^{v}/_{v}$) glacial acetic acid.

SDS-PAGE gel stain: 0.1 % **($^{w}/_{v}$)** Coomassie brilliant blue R250, 40 % **($^{v}/_{v}$)** methanol, 10 % **($^{v}/_{v}$)** glacial acetic acid.

Urea gel de-stain: 10 % ($^{v}/_{v}$) methanol, 10 % ($^{v}/_{v}$) acetic acid.

Urea gel stain: 0.05 % **($^{w}/_{v}$)** Coomassie brilliant blue R250, 40 % **($^{v}/_{v}$)** methanol, 10 % **($^{v}/_{v}$)** glacial acetic acid.

Gel stain for sequencing: RAPIDstain Reagent, Calbiochem, UK, 553215.

- **Solution for Maxi Prep**

Solution I: 50 mM glucose, 10mM EDTA, 25 mM Tris-HCl pH8.0.

Solution II: 0.2 M NaOH, 1% SDS.

Solution III: 3 M potassium acetate, adjusted to pH 5.0 with glacial acetic acid.

2.2 METHODS

All experiments that needed reproducible repetitions were performed at least in triplicate unless stated otherwise. Results were statistically analysed as necessary by unpaired, two- tailed t-test.

2.2.1 PROTEOMIC ANALYSIS

2.2.1.1 Bacterial binding Assays of Tf and Lf

Inoculums of *Escherichia coli* strain E2348/69 were grown overnight at 37 ^{o}C in Luria Broth (LB). Approximately 500 µl of the overnight culture (OD_{600}~2.00) or adequate amounts of logarithmic growth culture (OD_{600}~0.6) equalised to a similar cell density of around 10^8 bacteria/ml, were centrifuged at room temperature at 10,000 rpm for 10 minutes. The bacterial pellets were washed twice in SAPI minimal medium supplemented with 50 mM of TRIS-HCl at pH 7.5 (SAPI-TRIS pH 7.5) in order to remove any LB media left within the pellets. The presence of TRIS-HCl pH 7.5 is necessary to maintain the pH at values higher than 5.5 to prevent bacterial acidification of the SAPI medium, since binding of iron (Fe), transferrin (Tf) or lactoferrin (Lf) is de-stabilised at low pH. The bacteria cells were then resuspended in 1 ml of SAPI-TRIS pH 7.5 and incubated statically at 37 ^{o}C for 30 minutes to allow the bacteria to recover and adapt to the new media. The amount of the cultures used in Tf or Lf binding assays were adjusted to obtain cultures of equal cell density (OD_{600} ~2.00) (i.e. approx. 10^8 cells/ml) using spectrophotometer ultrospec 2000 (Pharmacia Biotech, UK) set at a wavelength of 600 nm. An aliquot of 10 µg of either Tf or Lf were admixed to the normalised bacterial cultures and incubated at 37 ^{o}C for 1 hour to allow Tf or Lf to bind to the outer membrane proteins and/or receptors of the bacteria. Following centrifugation at 10,000 rpm for 10 minutes, the pellets were washed 3 times in PBS to remove un-bounded TF or LF. The pellets were resuspended in 50 µl of PBS and 50 µl of 2x SDS protein sample loading buffer.

2.2.1.2 Analysis of the environmental factor which regulate expression of the Tf / Lf binding protein

Iron availability: Inoculums of E2348/69 were grown overnight in LB (control), in LB supplemented with 100 μM of $Fe(NO_3)_3$ (iron repletion) and in LB with addition of 300 μM dipyridyl (iron depletion).

Growth phase: Inoculums of E2348/69 were grown in LB, iron repletion and iron depletion and the cells were collected at OD_{600} 0.6 (logarithmic phase) and after overnight incubation (stationary phase).

Osmolarity: Inoculums of E2348/69 were grown overnight in LB prepared with 0 %, 5 % or 10 % NaCl.

The bacteria were harvested by centrifugation at 10,000 rpm at room temperature and the binding assay was performed as described in section 2.2.1.1

2.2.1.3 Competition assay for Tf and Lf binding

For the Tf/Lf competition binding assay the protocol described in section 2.2.1.1 was followed. However, after the step in which the bacteria cells were resuspended in 1 ml of SAPI-TRIS pH 7.5 and incubated statically at 37 ^{o}C for 30 minutes, 5 μg or 50μg of either Tf or Lf were admixed to the samples and incubated at 37 ^{o}C for 1 hour. Following centrifugation at 10,000 rpm for 10 minutes, the pellets were washed twice in SAPI-TRIS pH 7.5. The bacteria cells were then resuspended in 1 ml of SAPI-TRIS pH 7.5 and 5 μg or 50 μg of either Tf or Lf were admixed to the samples and incubated at 37 ^{o}C for 1 hour. In the samples in which 50 μg of Tf or Lf was added first then 5 μg of the competitor protein was added after and vice versa. The bacterial Tf/Lf binding assays were then centrifuged at 10,000 rpm for 10 minutes and the pellets were washed 3 times in PBS. The pellets were resuspended in 50 μl of PBS, followed by 50 μl of 2x SDS protein sample loading buffer, and boiled for 10 minutes. The lysates were then centrifuged at 10,000 rpm for 10 minutes, and the supernatant separated on PAGE gels and Western blotted as described below (sections 2.2.1.7 and 2.2.1.8).

2.2.1.4 *E. coli* protein fractionation

Bacterial cultures were harvested by centrifugation at 8000 rpm for 10 minutes at 4 ^{o}C washed twice and then resuspended in envelope buffer (EB 10 mM TRIS pH 7.5). The samples were frozen overnight at –80 ^{o}C to increase the lysis of the bacterial cells by sonication. The sonicator (Soniprep 150, Sanyo) was set at 6-8 microns and the cells were lysed in 8 cycles of 15 second sonication followed by 45 second cooling. The lysed samples were centrifuged at 10,000 rpm for 10 minutes to remove the debris and unlysed cells. The supernatant contained the total bacterial protein fraction (membrane and cytoplasmic proteins). The supernatants containing total proteins extracts were centrifuged at 50,000 rpm for 10 minutes using a Beckman TL-100 ultracentrifuge to separate membrane proteins from cytoplasmic proteins. The supernatant contained the bacterial cytoplasmic protein fraction, while the pellets contained the bacterial total membrane protein fraction. When only outer membrane proteins were required, the pellets consisting of total membrane proteins were re-suspended in EB supplemented with 2 % Triton X-100 (EBT) (Schnaitman, 1971; Wooldridge, *et al.,* 1992) and extracted by incubation at room temperature with occasional mixing for 30 min. Triton insoluble pellets were collected by ultracentrifugation and re-extracted for further 30 min in EBT.

The concentration of the protein samples were determined using the Bradford assay (Bradford, 1976) and diluted or concentrated by freeze drying, as required. Standardised protein samples were mixed with adequate amounts of 2x SB prior to PAGE gel loading (1:1).

2.2.1.5 One dimensional PAGE protein preparation

Standardised OD_{600} of bacteria cells were harvested by centrifugation and resuspended in (1:1) PBS: 2x SB. The cells were lysed by boiling at 95 ^{o}C for 15 minutes, during which time the bacteria lysed and released proteins which were also denatured and solubilised. The protein lysates were then centrifuged at 10,000 rpm for 10 minutes to remove debris and unlysed cells, and loaded directly onto SDS-PAGE gels. Standardised protein samples were denatured in a heat block at 95 ^{o}C for 5 min and loaded onto SDS-PAGE (if just protein profile or Western blotting was required).

When the proteins were prepared for on blot binding assay (section 2.2.1.9), the samples were incubated at 4 ^{o}C or/and 37 ^{o}C for 30 min and then loaded onto the gel.

Different temperatures of preparation of the membrane extracts were adopted to test solubility (4 ^{o}C) and allow the refolding (37^{o}C) of the proteins (i.e. 95 ^{o}C irreversible denaturation of proteins).

2.2.1.6 Protein preparation for 2D electrophoresis

The protein concentrations of samples for 2-D analysis were quantified using 2-D Quant kit (Amersham, GE Healthcare, UK 80-6483-56) according to the manufacturer's protocol. The final concentration of the protein samples needed to be approximately 100 μg (in 7 cm strip) in a maximum of 50 μl total volume. The proteins were then solubilised and desalted using the 2D sample prep for insoluble proteins kit (Pierce perbio Bioscience, 89866, 89862) following the manufacturer's protocol. During the next step the samples were mixed with 75 μl of rehydration buffer in order to obtain a final volume of 125 μl, which was then loaded onto the rehydrating/focusing tray inserted onto Protean IEF cell (BIO-RAD, UK). The 7 cm ReadyStrip IPG Strip (BIO-RAD, UK, 163-2099) was gently applied gel side down onto the sample making sure the + and – markings were respectively positioned on the cathode and anode and that no bubbles were trapped under it. The samples were rehydrated passively for 1 hour. Each IPG strip was then overlaid with 1 ml of mineral oil and the samples were actively rehydrated for 17 hours. The next day 2 wicks were soaked in a 3.5 % solution of DTT and in npH_2O and were added respectively between the anode and cathode and each IPG strip, and the samples were focused using the preset protocol in the Protean IEF cell with the following isoelectric focusing conditions: Start Voltage 0 V; End Voltage 4,000 V; Volt-Hours 8-10,000 V-hr; Ramp rapid; Temperature 20 ^{o}C. The IPG strips containing the separated proteins were then positioned on a pre-casted 12 % Resolving gel (no stacking gel is required) and overlaid with Protean plus overlay agarose (BIO-RAD, UK, 163-2092). After the first dimension isoelectric focusing, the resolved proteins were then separated by second dimension SDS PAGE according to the methods in section 2.2.1.7.

2.2.1.7 One dimensional SDS PAGE separation of proteins

Proteins or IPG strips from first dimension isoelectric focusing were loaded onto appropriate percentage mini (10 cm x 8 cm) SDS-PAGE gels and separated using a

Mini-PROTEAN II system (BIO-RAD, UK) at a constant 50 mA (25 mA/gel) for approximately 1 hour (or as required). Proteins intended for sequencing were loaded onto large format (20 cm x 18 cm) SDS gels and were separated in the PROTEAN II XL system (BIO-RAD, UK) at 250 mA constant for approximately 6 hours. All SDS PAGE gels were separated in 1x running buffer. After electrophoresis gels were stained or Western blotted as appropriate. The different iron binding isoforms of Tf were separated using 6 % acrylamide gels containing 6 M urea, by electrophoresis at 4 °C and 70 V for 7 hrs in 1x TBE buffer. After electrophoresis gels were stained as described in section 2.2.1.10.

2.2.1.8 Western blotting

Protein samples separated by SDS PAGE were electroblotted at 250 mA for 1 hour using a Trans-Blot cell (BIO-RAD, UK) onto either polyvinylidene fluoride (PVDF, Immobilon-P, Millipore, UK IPVH00010) or nitrocellulose membranes (Trans-Blot transfer medium, BIO-RAD, UK, 162-0097). The blots were blocked overnight in blocking buffer at 4 ^{o}C and probed with antibodies as described below. All blots between treatments were washed three times in TBS-T and then incubated in anti-Tf or anti-Lf primary antibodies diluted 1:5000 in blocking buffer for 2 hours at RT on a rotator shaker, and re-washed three times in TBS-T. Cross recognition by primary antibodies was determined using 1:20000 dilutions in blocking buffer of the appropriate HPR-conjugated secondary antibodies (section 2.1.5). The secondary antibodies were incubated with the blots for 1 hr at RT on a rotatory shaker. The blots were finally washed twice in TBS-T and once in TBS and antibody cross reactivity determined using a using ECL+plus Western Blotting Detection System (Amersham GE Healthcare, UK, RPN2132). Immuno-reactive protein bands were visualised on x-ray films (Amersham Hyperfilm ECL, GE Healthcare 28906837) (Sambrook *et al.*, 1989).

2.2.1.9 On-blot Tf and Lf bacterial binding assay

Partially denatured *E. coli* total membrane proteins prepared as described in section 2.2.1.4 were separated on SDS-PAGE gels and blotted onto PVDF membranes. The blotting buffer was prepared with the omission of methanol to favour refolding of the proteins. The protein samples were arranged as duplicate mirror

images and divided into two, in order to enable direct correlation of protein band and Tf-binding. One half of the blot was stained with Coomassie and used for sequencing, whereas the other half of the blot was prepared for Tf-binding and was blocked overnight in Blocking buffer. To identify Tf or Lf binding proteins, next day the blot was washed three times in TBS-T and incubated for 1 hour with either 1 μg/ml of peroxidise-conjugated ChromPure human Tf in blocking buffer or 10 μg/ml of Human Lf also prepared in blocking buffer. The Tf-HPR blots were developed directly as described in section 2.2.1.8, whereas binding of Lf was visualised after incubating the samples with the adequate primary and secondary antibodies (section 2.2.1.8). Tf or Lf binding proteins were determined by lining up the x-ray film with the Tf-binding half of the blot with the Coomassie stained blot. Proteins which lined up were sequenced by PNACL (section 2.2.1.11) to determine their identity.

2.2.1.10 Gel staining

SDS PAGE and urea gels were washed with nanopure water and incubated with the appropriate staining solution (section 2.1.10) for as long as required. The excess stain was then removed and the de-stain solution was added until the protein bands were clearly visible. The gels were preserved either in nanopure water at 4 ^{o}C or dried in drying solution until a picture was taken or protein sequence performed.

2.2.1.11 Protein sequencing

Proteins sequencings were performed by the University of Leicester in house Protein Nucleic Acid Chemistry Laboratory (PNACL), using an Applied Biosystems 477 sequencer to provide the N-terminal protein sequence or peptide mass fingerprinting (http://www.le.ac.uk/mrctox/pnacl/).

2.2.1.12 Recording of protein bands on gels or X-ray films

Proteins bands on gels and X-ray films were scanned and analysed using a GS-710 Calibrated Imaging Densitometer connected to Quantity one software (BIO-RAD, UK).

2.2.2 MUTATION OF *E. coli E2348/69*

2.2.2.1 Oligonucleotide primers

The DNA sequences of ompA, ompC and Kan genes were retrieved from the National Centre for Biotechnology Information (NCBI) database and the oligonucleotide primers were designed using the SEQED, MAP program of the Wisconsin Molecular Biology Package, Version 9.1-UNIX. The primer melting temperatures (Tm) were established using the web program oligo calculator (http://www.pitt.edu/~rsup/OligoCalc.html) and were checked for potential primer dimer formation using the "FastPCR" program (Microsoft Visual Studio 6.0, Visual Basic) and aligned against the *E. coli* genome either using the SPECIALISED BLAST ALIGN program available at the NCBI database (http://www.ncbi.nlm.nih.gov/) or via the Gene Tool (Lite version 1.0) or BLAST tool available at Sanger Institute (http://www.sanger.ac.uk/).

All the oligonucleotide primers used during this experiment were synthesised by Eurofins MWG operon, Germany.

The oligonucleotide sequences used for the *omp* deletion mutagenesis were:

OMPA_F1 5′ AGCGGATCCCGCGATTCTCTTC 3′

OMPA_R1 5′ TGTTCTAGACATTTTTTGCGCCTCGTTATC 3′

OMPA_F2 5′ CCGTCTAGATAAGTTCTCGTCTGGTAG 3′

OMPA_R2 5′ AATGCATGCCACCAGCATACGCGC 3′

OMPC_F1 5′ TGTTGGATCCTTATTTCGCCATTCCGC 3′

OMPC_R1 5′ GACAGTACTTTTCTAGACATGTTATTAACCCTC 3′

OMPC_F2 5′ CTGGTTTACTCTAGATAATCTCGATTGATATCG 3′

OMPC_R2 5′ CATGGGCATGCCTTCAAGAACGG 3′

The oligonucleotide sequences used for the mutations based on λ-red recombinase system were:

Kan_F 5′ GACAGCAAGCGAACCGG 3′

Kan_R 5′ TTGGTCGGTCATTTCGAACC 3′

OMPC_R1λ

5'CCGGTTCGCTTGCTGTCAGTACTTTAACTTTCATGTTATTAACCCTC 3'

OMPC_F2λ

5' GGTTCGAAATGACCGACCAAGTTTACCAGTTCTAATCTCGATTGATATCG 3'

2.2.2.2 DNA extraction

Boiling techniques were used to extract DNA as follows. Inoculums of bacteria were grown overnight at 37 ^{o}C in shaking incubator in LB; 100 µl of the overnight culture (OD_{600} nm 5.0) was spun at 10,000 rpm for 10 min and the pellet was re-suspended in 100 µl of nanopure water. To extract DNA the cells were boiled at 95 ^{o}C for 10 min, centrifuged at 10,000 rpm for 10 minutes, and the supernatant containing DNA collected and preserved at –20 ^{o}C.

2.2.2.3 PCR reactions

Polymerase chain reaction amplification (PCR), which is technique based on the principle that a temperature resistant DNA polymerase (Taq) catalyses the extension of DNA fragments within two defined opposite primers forward (F) and reverse (R), can be used to amplify DNA fragments of interest. DNA samples (2.5 µl, ~10 pg/µl) were mixed with the following reagents (*Taq* PCR Kit, New England BioLabs, UK, E5000S):

2.5 µl of 10X PCR buffer

1.25 µl of 50mM $MgCl_2$

0.5 µl of 10mM dNTP mix

0.2 µl of Thermostable (Taq) DNA polymerase (5 units/ µl)

1 µl of 5 µM of F and R primers

16.05 µl of nanopure water

The PCR mixtures were mixed by centrifugation and amplified using PCT-0220 DNA Engine Dyad Peltier Thermal Cycler, manufactured by MJ Research, USA. The cycle of the amplification reaction was carried out according to the following steps:

Initial denaturation of the DNA at 95 ^{o}C for 5 minutes

95 ^{o}C for 30 seconds denaturation

55 ^{o}C for 30 seconds annealing

72 ^{o}C for 1 minute elongation

The PCR cycle was repeated 30 times.

Final elongation was performed at 72 ^{o}C for 4 minutes.

2.2.2.4 Colony PCR amplification

All transformed or conjugated colonies were checked by colony PCR as follows. Using sterile toothpicks, single colonies of interest were inoculated from the original agar plate containing selective markers into 100 μl of nanopure water; the same colony was also streaked out into a numbered square of selective media plate/s, which was previously divided into 56 equal squares. The colony PCR samples were boiled at 95 ^{o}C for 10 minutes and spun down for 10 minutes at 13,000 rpm. The supernatants were transferred into fresh Eppendorf tubes and the PCR performed as described in section 2.2.2.3. Any PCR samples of interest were checked by restriction digestion, and sequenced.

2.2.2.5 PCR product purification

PCR products were purified using a column PCR purification kit (cat no. 28106) QUIAGEN according to the following protocol: 5 volume of buffer PB were admixed to 1 volume of the PCR buffer. The mixture was added to the QIAquick column and centrifuged at 13,000 rpm for 1 minute to bind the DNA onto the column.

The flow-through was discarded and the column was washed with 0.75 ml of buffer PE and centrifuged for 1 minute at 13,000 rpm. The flow-through was discarded and the column re-spun 1 minute. The column was then transferred in a clean Eppendorf tube. 40 μl of nanopure water were added to the centre of the column, let stand for 1 minute and the column was centrifuged for 1 minute at 13,000 rpm to elute the DNA.

2.2.2.6 Purification of DNA by ethanol precipitation

To each DNA sample 1/10 volume of 3 M sodium acetate, 2 volumes of 100% ethanol and 1 μl of 10 mg/ml t-RNA were added, mixed and incubated on ice for 10 minutes. The mixtures were centrifuged for 10 minutes at 13,000 rpm, and the resultant pellet which contains the precipitated DNA washed in 70 % ethanol and dried in a vacuum drier for approximately 5 minutes. The DNA pellet was re-dissolved in 20 μl of sterile autoclaved nanopure water.

2.2.2.7 Digestion of PCR products and cloning vectors

The PCR products and cloning vectors were digested or double digested (as required) with the appropriate restriction enzymes: 20 μl of DNA was admixed with 1 μl of restriction enzymes A and B (for double digestions), 5 μl of digestion buffer and nanopure water were added to obtain a final volume of 50 μl. The reaction mix was incubated at 37 ^{o}C overnight.

2.2.2.8 DNA ligation

The PCR products were ligated into cloning vectors either by two way ligation (PCR product + cloning vector) or three way ligation (2 different PCR products + cloning vector) using the following reaction mix: X μl of DNA fragment/s (1:5 ratio vector/insert), 1 μl of T4 DNA ligase (New England BioLabs, UK, M0202L), 2 μl of 10x T4 DNA buffer, 2 μl of 10 mM dATPs and nanopure water to a total volume of 20 μl.

2.2.2.9 Gel extraction of DNA fragments

Puncturing and melting a small hole in a 0.5 ml Eppendorf tube to construct a sieve-like container and filling it with Polymer wool^{TM} (used in aquarium filter system, Interpet LTd, Dorking, Surrey) enabled the creation of a fast and cheap homemade gel extraction system that was quite effective; the glass wool extraction system was autoclaved prior use. The DNA band of the potential ompA and ompC mutation construct were cut out from the agarose gel with a scalpel and inserted in the sieve-like container, which was placed inside a 1.5 ml Eppendorf collection tube and spin twice at 13,000 rpm for 2 minutes. The fluid recovered contained the eluted DNA which was then purified using QIAGEN PCR purification kit as described in section 2.2.2.5.

2.2.2.10 Visualisation of DNA fragments

All DNA fragments were checked for size on 1.5 % agarose gel containing 500 ng/ml of ethidium bromide and electrophoresed at 100 V in TAE buffer at room temperature as required. The DNA fragments were visualised by UV transillumination (UVP Dual Intensity Transluminator) and recorded using a Olympus camera connected with Image Quant 100 capture software (GE Healthcare, UK)

2.2.2.11 DNA sequencing

All DNA samples of interest were sequenced by the Protein and Nucleic Acid Chemistry Laboratory (University of Leicester) using the 3730 automated sequencer. The DNA sequences were compared with the GeneBank databases using the BLAST program available at NCBI website and aligned using Gene Tool or Chromas (Technelysium Pty Ltd.)

2.2.2.12 Preparation of electrocompetent cells

All electrocompetent cells were prepared as follows: 1:100 dilution of an overnight bacterial culture was inoculated in 100 ml of pre-warmed Luria Broth and incubated at 37 ^{o}C in a shaking incubator set at 220 rpm until reaching an OD_{600} of 0.6. The culture was then transferred into ice-cold falcon tubes and kept at 4 ^{o}C throughout the protocol. The bacteria were centrifuged at 4000 rpm for 15 minutes. The supernatant was discarded and the bacterial pellets washed 4 times in ice-cold nanopure sterile water to remove any salts from the samples. The pellets were then re-suspended in 300 μl of nanopure sterile water, aliquoted in 40 μl volumes and transformed by electroporation.

2.2.2.13 Electroporation of electrocompetent cells

10 μl of the vector/insert samples were admixed with the 40 μl aliquots of electrocompetent cells and transferred to an ice-cold 0.2 cm cuvette (BIO-RAD, 1652086). The cuvettes were placed in a gene pulser apparatus (BIO-RAD) and electroporation was performed at 1.5 KV with a resistance of 1000 ohms and a capacitance of 25 μF. Samples with time constant readings between 21 and 23 were considered acceptable. 1 ml of SOB medium was added to the cuvette to recover the transformed samples and transferred into Eppendorf tubes. The bacteria were incubated for 60 minutes in a 37 ^{o}C water bath; 100 and 900 μl of the bacterial cultures were inoculated into LA plates supplemented with appropriate antibiotics and incubated at 37 ^{o}C overnight.

2.2.2.14 Preparation and transformation of $CaCl_2$ competent cells

The $CaCl_2$ competent cells were obtained following the methodology described by Dagbert *et al.* (Dagbert *et al.*, 1979). Inoculums of bacteria were grown overnight in LB at 37 ^oC in a shaking incubator. The next day a 1:50 dilution of the culture was transferred in 25 ml of LB and grown at 37 ^oC in a shaking incubator until it reached an OD_{600} of 0.6. The cells were cooled on ice and harvested by centrifugation at 4,000 rpm for 15 min at 4 ^oC. From this step onward the cells and reagents were kept at 4 ^oC. The cells were washed in 5 ml of 0.10 M $MgCl_2$, 0.1 M, centrifuged, and the pellets resuspended in 5 ml of 0.1 M $CaCl_2$ and incubated in ice for 30 minutes. The cells were re-harvested and resuspended in 1 ml of 0.1 M $CaCl_2$. Aliquots (100 μl) of $CaCl_2$ competent cells were admixed with 10 μl of ligated plasmid DNA, incubated on ice for 5 minutes and then heat shocked in a 42 ^oC water bath for 90 seconds. The transformed cells were recovered by admixing with 1 ml of LB and then incubated at 37 ^oC for 45 minutes to allow the expression of the resistance antibiotic present in the plasmid before plating the sample on LA supplemented with desired antibiotic and reagents (i.e. 25 μg/ml IPTG, 25 μg/ml X-GAL for blue/white selection).

2.2.2.15 Mini prep

1.5 ml of bacterial cultures were transferred into Eppendorf tubes and centrifuged for 10 minutes at 10,000 rpm; plasmid prep was then performed using QIAprep spin miniprep kit (250) purchased from QIAGEN, UK, 27106. The reaction pellets were resuspended in 250 μl of buffer P1 and 250 μl of buffer P2 were added and by inverting the tubes 6 times gently the samples were mixed. Buffer N3 (350 μl) was then added and the samples were gently mixed and centrifuged at 13,000 rpm for 10 minutes. The supernatants were transferred into QIAprep spin columns by pipetting and centrifuged at 13,000 rpm for 60 seconds. The flow-through was discarded and the columns washed with 0.75 ml of buffer PE and centrifuged at 13,000 rpm for 60 seconds. The flow-through was discarded and the columns were further centrifuged to remove any remaining buffer. The columns were placed into clean Eppendorf tubes and the plasmid DNA was eluted by adding 50 μl of npH_2O, which was left to stand for 60 seconds. The plasmid DNA was recovered by centrifugation at 13,000 rpm for 60 seconds.

2.2.2.16 Maxi prep

Large amounts of plasmid DNA (from bacteria carrying low copy number plasmid) were obtained by an alkaline lysis method based on that of Birnboim *et al.* (Birnboim *et al.*, 1979). Overnight cultures (50 ml) were harvested at 8,000 rpm (Beckman Coulter) for 10 minutes. The supernatant was discarded and the pellet resuspended into 5 ml of solution I supplemented with 5 µl of 10 mg/ml RNase, 10 ml of solution II and mixed gently by repeated inversion. 7.5 ml of solution III was added and the mixture was shaken vigorously and centrifuged at 4,000 rpm for 20 minutes. The supernatant was transferred into a clean Falcon tube and mixed with 20 ml of (24:1) Chloroform/isoamyl alcohol solution and centrifuged at 4,000 rpm for 20 minutes. The top layer of the mixture was transferred into a fresh falcon tube to which 30 ml of 95% ethanol was added. The samples were centrifuged at 4,000 rpm for 30 minutes, the supernatant discarded and the pellet was allowed to dry (for approximately 20 minutes in air). The pellet containing the DNA was resuspended in 100-200 µl of npH_2O. 5 volumes of buffer PB were added to 1 volume of the DNA sample and the mixture was applied to a QIAquick column and centrifuged at 13,000 rpm for 1 minute. Buffer PB (500 µl) was added to the column, and after centrifugation at 13,000 rpm for 1 min the DNA samples were purified according to section 2.2.2.5.

2.2.2.17 Conjugation: *ompA* deletion mutation

Inoculums of donor and recipient strains were grown overnight in LB supplemented with appropriate antibiotics at 37 ^{o}C in shaking incubator. The next day appropriate dilutions of the overnight cultures were transferred into fresh LB without addition of antibiotic and grown until an OD_{600} of 0.6. Once the specified OD was reached, 1 ml of each culture was transferred and centrifuged at 8,000 rpm for 2 min in the same Eppendorf tube. The pellet was resuspended in 100 µl of LB and spotted onto a sterile 25 mm nitrocellulose filter disk (0.45 µm pore size; Millipore). The conjugation mixture was incubated at 37 ^{o}C for 3 hours and the bacteria were washed free from the filter into 1 ml of LB. Dilutions of the conjugation mixture were spread on LB supplemented with appropriate antibiotics to select for the trans-conjugant colonies and incubated overnight at 37 ^{o}C. The next day any trans-conjugant colonies were streaked onto fresh media for purity.

2.2.2.18 Sucrose selection

Sucrose plate selection is used to select for clones that have undergone double homologous recombination events and excised the suicide vector from the chromosome. Single colonies of potential trans-conjugants were grown overnight at 37 ^{o}C in LB supplemented with appropriate antibiotic. Dilutions of the culture were spread onto 6 % sucrose agar plates and incubated overnight at 30 ^{o}C. Sucrose resistant colonies resistant to specified antibiotics were verified by PCR as the true deletion mutants.

2.2.2.19 Mutation with λ-red recombinase system: *ompC* deletion mutation

Inoculums of *E. coli E2348/69* carrying the pK46 λ-red plasmid were grown overnight in LB at 30 ^{o}C. 1:100 dilution of the culture was transferred into fresh media and grown at 30 ^{o}C until OD_{600} 0.5; 0.1 % arabinose was then added to the culture and further incubated at 30 ^{o}C until the OD_{600} once again reached 0.6. The bacteria were transferred to a water bath set at 42 ^{o}C incubated for 15 minutes and then cooled down in ice for 10 minutes. The cells were prepared for electroporation as described in section 2.2.2.12, however instead of npH_2O a more osmotically friendly solution was used which contained 1 mM MOPS and 20 % glycerol. Electrocompetent bacteria (40 μl) were mixed with 10 μl of PCR construct and electroporated as described in section 2.2.2.13. The bacteria were plated onto LB plate containing the appropriate antibiotics and the potential mutants were checked by PCR and sequencing.

2.2.3 IRON UPTAKE ANALYSIS

2.2.3.1 Intracellular ^{55}Fe[Tf] uptake in mutant and WT strains exposed to CAs

Inoculums of bacteria were grown in LB at 37 ^{o}C overnight. The next day 1:100 dilution of the bacteria were made in fresh LB and the cultures grown until late exponential (OD_{600} 1.6-1.8). The cells were harvested by centrifugation at 4,000 rpm for 15 minutes and washed twice and resuspended in SAPI-100 mM Tris pH 7.5 (SAPI-Tris) supplemented with 1 μg/ml of [^{55}Fe]-Tf (2x 10^5 CPM) and incubated 4 hours at RT in presence or absence of 100 μM of NE. The cells were harvested at 4,000 rpm for 15 minutes and washed twice in PBS to remove any residual [^{55}Fe]-Tf.

The bacterial pellets were then re-suspended in 100 μl of PBS and 2 ml of Optiphase Safe scintillation fluid (Canberra-Packard, UK) was added; radiolabel uptake was determined using the tritium channel of a Minaxi Tris-Carb 400 series scintillation counter.

2.2.3.2 ^{55}Fe labelling of Tf

[^{55}Fe]-Tf was prepared according to Freestone *et al*. (Freestone *et al.*, 2000). Human Apo-Tf was incubated for 5 hours at 37 ^{o}C in the presence of 25 μCi of $^{55}FeCl_3$ (1.5 μg of Fe/mg of protein), using 2 mM of sodium citrate as the iron donor. Unincorporated ^{55}Fe was removed by spin column chromatography (Micro Bio-spin 6 columns, BIO-RAD, UK, 732-6221).

2.2.3.3 Electron paramagnetic resonance (EPR)

Tf (6 mg/ml) in 400 μl of a 50 mM Tris-HCl, pH 7.5 only, or containing several concentrations of NE, ranging from 10 mM to 0.3 mM, were frozen in liquid nitrogen and analysed by JEOL-RE1X EPR Spectrometer. EPR analysis of 0.1 mM solutions of $Fe(NO_3)_3$ or $FeSO_4$ were similarly prepared with or without the addition of NE. The parameters of the machine were set in accordance to Borisenko et al. (Borisenko, *et al.*, 2000) as follow: Centre Field: 150 mT; Sweep Width: 80 mT; Field Modulation: 1.0 mT; Microwave Power: 10mW; Time Scan: 14 min; Temperature: 77 K. The samples were analysed in the Department of Chemistry, University of Leicester.

2.2.3.4 Ferrozine analysis

Human holo-Tf and Lf (2 mg/ml) were incubated with a range of CAs concentration (4 mM was the most suitable concentration) in 10 mM Tris pH 7.5 with or without the presence of 0.4 mM ferrozine at room temperature for 24 hours. Every 15 minutes readings at A 560 were automatically taken using the Varioskan Flash spectrophotometer connected to SkanIt software 2.4.1 (Thermo, UK). Various concentration of bovine serum were also analysed using this methodology.

2.2.3.5 Trace metal analysis

Inoculums of *Escherichia coli* strain E2348/69 were grown overnight in shaking incubator at 37 ^{o}C; 5 ml of the overnight culture was harvested by centrifugation at 10,000 rpm for 10 minutes, washed and re-suspended in 5 ml of serum-SAPI. 1:100 dilution of the culture was transferred into fresh serum-SAPI media to which 100 μM of the catecholamine (Epi or NE or DO) or 100 μM of Fe was added. A negative control consisting of serum-SAPI only was also prepared. The cultures were grown overnight statically in CO_2 incubator at 37 ^{o}C. The next day dilutions of the cultures were plated out onto LA for enumeration of growth levels (CFU/ml). For iron uptake analysis, the bacterial cells were harvested by centrifugation at 10,000 rpm for 10 minutes and washed 3 times in 10 mM Tris-HCl,pH 7.5. The bacteria were re-suspended in 500 μl of 10 mM TRIS pH 7.5 and sonicated. The sonicates were centrifuged twice for 10 minutes at 13,000 rpm in order to remove cellular debris and the supernatant frozen at -80 ^{o}C and lyophilised using a freeze dryer (ModulyoD, Thermo). The lyophilised samples were analysed at the Department of Geology, University of Leicester, with a JY Ultima 2 ICP-OES (Inductively-Coupled Plasma Optical Emission Spectrometry).

Chapter 3 – Identification of the *E. coli* Transferrin and Lactoferrin binding proteins

3.1 INTRODUCTION

Iron is an essential element for the survival of many organisms; therefore it is not surprising that its successful acquisition by bacteria and its retention by the host plays a fundamental role in the outcome of an infection. As previously mentioned (Chapter 1), a direct consequence of stress, whether physical or mental, is the release of stress hormones such as catecholamines (Padgett *et al.*, 2003), which not only affect the immune response of the host but also have a direct effect on the bacteria itself, using them as a cue to initiate an opportunistic infection. It has been previously demonstrated (Freestone, *et al.*, 1999; Freestone *et al.*, 2002; Lyte, *et al.*, 1996a; Lyte *et al.*, 1997a; Lyte *et al.* 1997b; Lyte *et al.*, 2003) that CAs hormones can enhance the rate of growth of a variety of both pathogenic and commensal Gram-negative and Gram-positive bacteria as well as stimulate the expression of bacterial virulence factors. Moreover, Freestone *et al.*, (Freestone *et al.*, 2000) demonstrated that CAs such as Epi and NE can form complexes with the host iron binding proteins transferrin (Tf) or lactoferrin (Lf), facilitating iron removal from these proteins by a not yet fully characterised mechanism. Although to date there is no genomic evidence for *Escherichia coli* Tf and Lf binding proteins (TfBp/LfBp), Freestone *et al.* (Freestone *et al.*, 2000; Freestone *et al.*, 2002; Freestone, *et al.*, 2003) have shown that the ability of Tf and Lf to physically interact with *E. coli* plays an important role in the mechanism of CA-mediated growth stimulation.

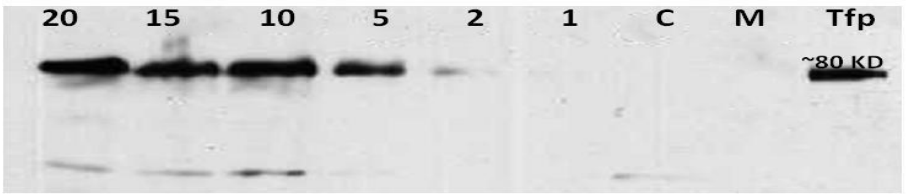
The aim of this chapter is to identify and characterise the Tf and Lf binding proteins in *Escherichia coli* E2348/69.

3.2 RESULTS

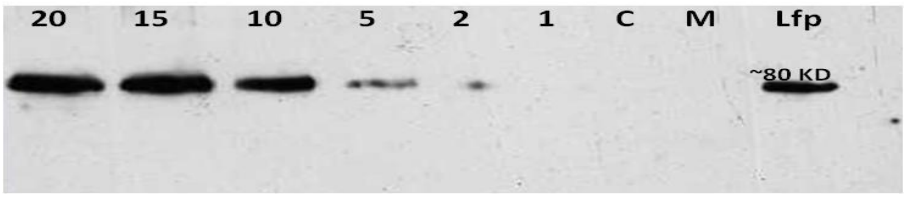
3.2.1 Characterisation of the binding of Tf and Lf on *Escherichia coli E2348/69*

In order to assess the binding of transferrin and lactoferrin proteins to *Escherichia coli* E2348/69, the bacteria were grown overnight in LB. Binding assays were then performed as described in section 2.2.1.1 in SAPI-TRIS minimal medium pH 7.5 to which 10 μg of either Tf or Lf were added. The total membrane protein extracts of the bacteria were prepared as described in section 2.2.1.5, separated by SDS-PAGE and the binding of Tf and Lf visualised by Western blot analysis and immunoblotting assay using respectively anti-human transferrin or anti-human lactoferrin primary antibodies. Cross recognition was determined using specific HPR-conjugated secondary antibodies (section 2.1.5). Figure 3.1 shows that both Tf (3.1a) and Lf (3.1b) bind to *E. coli* E2348/69.

The binding of Tf (or Lf) to the bacteria plays an important role in the bacterial growth response to stress hormones, although the *E. coli* TfBp have not yet been characterised (Freestone *et al.*, 2000). CAs facilitate the removal of iron associated with Tf (or Lf), which become available to the bacteria in an iron restricted environment such as can be found in body fluids, therefore it was important to investigate how the availability or absence of iron might affect the ability of the bacteria to bind to these proteins. *E. coli* was grown in LB (control) and LB supplemented with either 100 μM $Fe(NO_3)_3$ (iron repletion) or 300 μM of dipyridyl (iron depletion; dipyridyl is a high affinity iron chelator) and Tf and Lf binding assays were performed on bacterial cultures during their logarithmic and stationary growth phases. In Fig.3.2 it can be seen that Tf was binding to the bacteria independently of the presence or absence of iron, although when the bacteria were in the logarithmic growth phase there was increased binding to bacteria grown under conditions of iron deprivation. Similar results were seen for Lf (data not shown). The Tf/Lf blots were then scanned using a densitometer connected with Quantity one program that enabled determination of the volumes ($OD*mm^2$) of the bands corresponding to the Tf and Lf that was bound to the bacteria (Fig.3.3). Fig.3.3 shows that the availability of iron did not affect the ability of stationary growth phase bacteria to bind Tf and Lf ($p>0.05$).



3.1a



3.1b

Fig.3.1 Binding of Tf and Lf to *E. coli*. Inoculums of *E. coli* were grown o/n at 37 ^{o}C. 500 μl of the bacterial growth were harvest by centrifugation, washed twice, resuspended in SAPI-TRIS pH 7.5 and incubated at 37 ^{o}C for 1 hr with or without (control sample) the addition of 10 μg of human holo-Tf. The bacteria were then washed three times and resuspended in 2x SB. The bacteria were boiled at 95 ^{o}C for 10 minutes and the total protein extracts were separated on 12 % SDS-PAGE. The binding of Tf (3.1a) and Lf (3.1b) were visualised by immunoblotting using respectively anti-human transferrin or anti-human lactoferrin primary antibodies. Cross recognition was determined using appropriate secondary antibodies HPR conjugated (section 2.1.5). The gel loading order was as follow: a range between 20-1 μl of bacterial lysate from the Tf (3.1a) or Lf (3.1b) binding assay; bacterial lysate without Tf/Lf (C); prestained SDS-PAGE standards (M); 0.1μg of Tf or Lf.

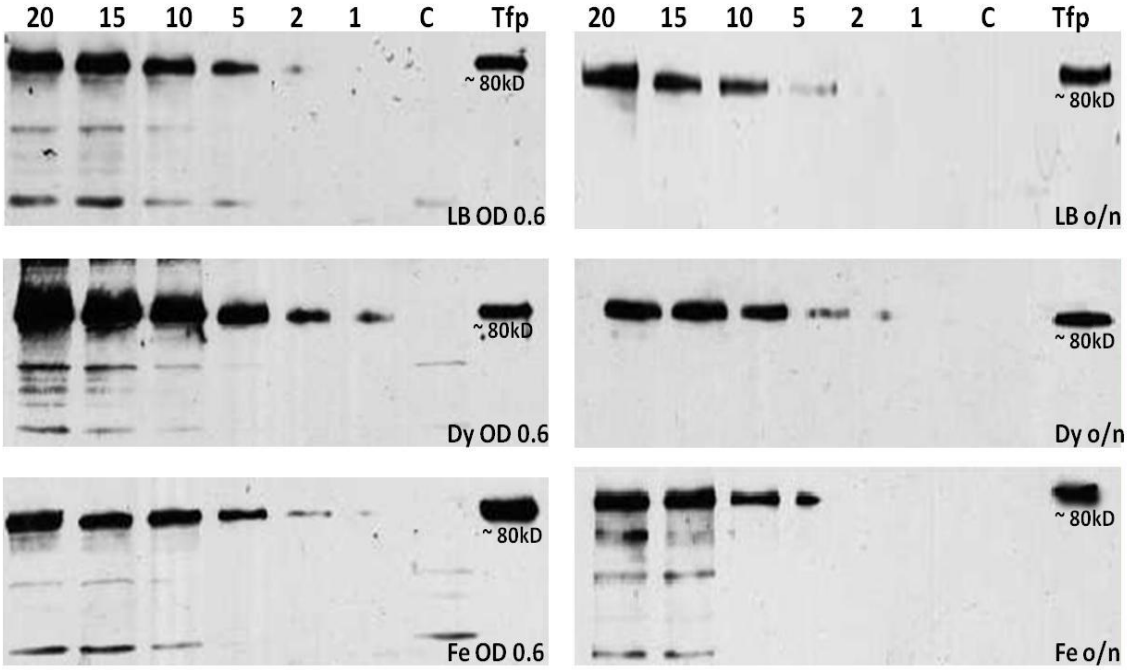
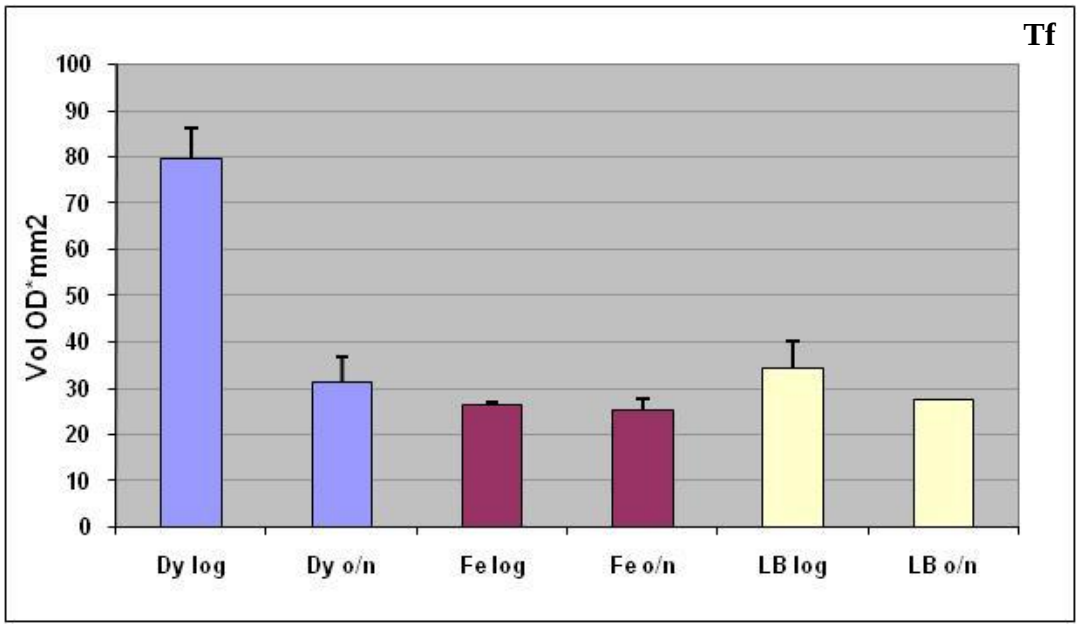


Fig.3.2 Effect that iron and growth phase has on the ability of *E. coli* E2348/69 to bind Tf. Inoculums of *E. coli* were grown until OD_{600} 0.6 (logarithmic phase, log) or overnight (stationary phase, o/n) in LB (control) or LB supplemented with either 300 μM dipyridyl (Dy, iron depletion) or 100 μM of $Fe(NO_3)_3$ (Fe, iron repletion). Binding assay was performed as described in section 2.2.1.1. 20-1 μl of the sample incubated with 10 μM of Tf; bacterial lysate without Tf (control, C); prestained SDS-PAGE standards (M); 0.1 μg Tf. Similar results to those shown in the Fig. were also obtained with Lf (data not shown).

3.3a



3.3b

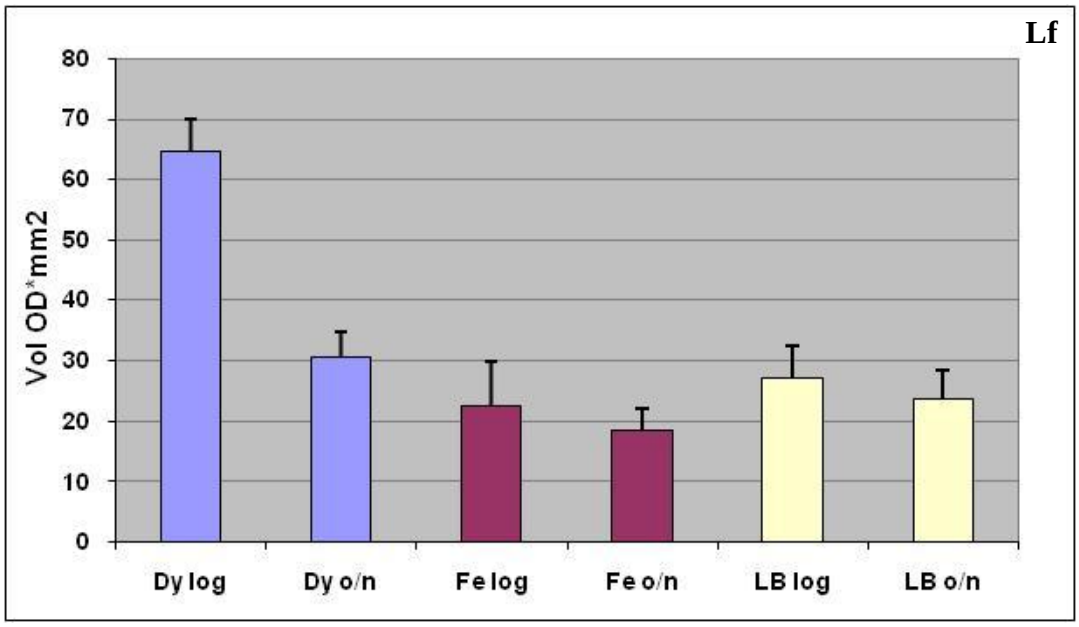


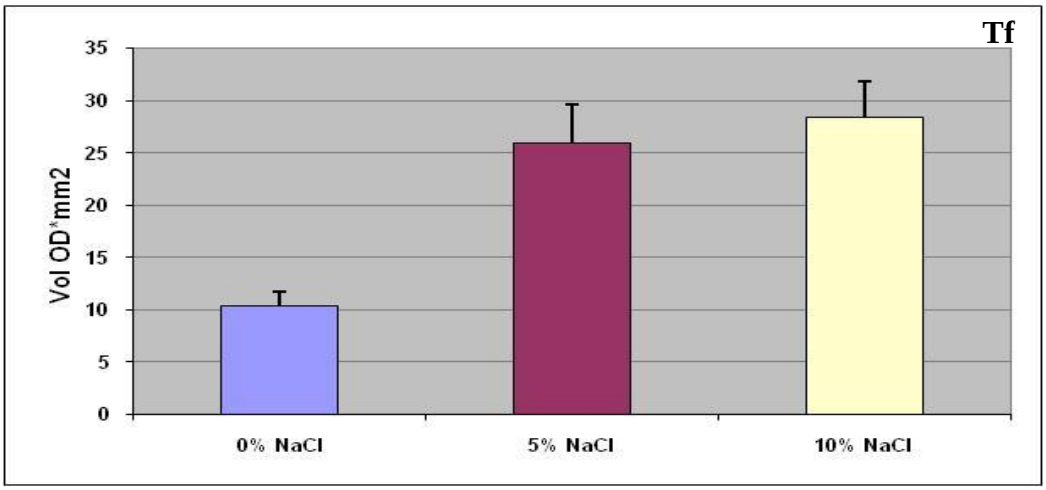
Fig. 3.3 Binding assay of Tf and Lf to *E.coli E2348/69* grown in different iron conditions. The bacteria were grown in different iron conditions and a Tf/Lf binding assay was performed as described in Fig.3.2. The histograms show the volume (OD*mm^2) of the bands representing Tf (3.3a) or Lf (3.3b) bound to the bacteria were determined using a Quantity one program connected to a densitometer. The data shows there was no significant difference ($p>0.05$) between the Tf and Lf that was bound by the bacteria when they were in their stationary phase. When the binding assay was performed using bacteria in their logarithmic growth phase, iron depletion had a significant ($p<0.05$) impact on the ability of the bacteria to bind to both Tf and Lf.

When the binding assay was performed on bacteria in their logarithmic growth phase, however, iron availability had a great impact on the amount of Tf and Lf bound

by the bacteria. The cultures of *E. coli* that were grown in an iron depleted environment bound significantly more ($p<0.05$) Tf and Lf when compared with the control.

The effect of osmolarity on the ability of *E coli* to bind Tf and Lf was also investigated. The bacteria were grown in 1 % ($^{w}/_{v}$) tryptone, 0.5 % ($^{w}/_{v}$) yeast extract supplemented with either 0 % ($^{w}/_{v}$), 5% ($^{w}/_{v}$) or 10 % of NaCl prior to the Tf/Lf binding assay. From Fig. 3.4 it can be seen that increasing osmolarity apparently enhances the binding of Tf and Lf to *E. coli* ($p<0.05$).

3.4a



3.4b

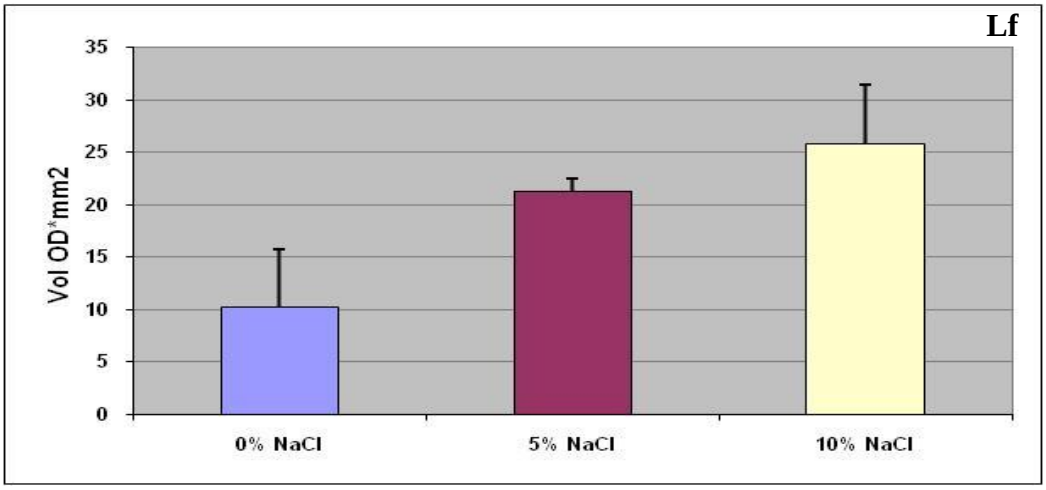


Fig. 3.4 Binding assay of Tf and Lf to *E.coli* E2348/69 grown at different osmolarities. *E.coli* was grown in 1 % ($^{w}/_{v}$) tryptone, 0.5 % ($^{w}/_{v}$) yeast extract supplemented with either 0 % ($^{w}/_{v}$), 5 % ($^{w}/_{v}$) or 10 % of NaCl prior to performing a Tf (3.4a) or Lf (3.4b) binding assay and LfBp The histograms show that Tf and Lf binding is enhanced by increasing osmolarity ($p<0.05$)

3.2.2 Identification of TfBp and LfBp

Once it was established that *Escherichia coli* E2348/69 bound both Tf and Lf, it was important to determine which of the bacterial membrane protein/s were actually the binding 'receptor/s'. Bacteria were grown overnight in LB supplemented with either 300 μM dipyridyl (Dy, iron depletion) or 100 μM of $Fe(NO_3)_3$. The next day outer membrane preparations were prepared as described in section 2.2.1.4. Prior to loading on 12 % SDS-PAGE, the membrane proteins were incubated in loading buffer for 30 minutes at either 4 ^oC or 37 ^oC, in order to determine the solubility of the binding proteins at different temperatures. Incubation at 37 ^oC in the presence of SDS allowed partial denaturation of the proteins, which were then expected to refold once separated from the denaturant. The membrane proteins were then separated on SDS PAGE gel, and blotted onto PVDF membrane in Tris-glycine buffer (no SDS or methanol denaturing agents were added in order to assist the in-blot refolding of the proteins). The blots containing the transferred membrane proteins were divided into two identical sections. On one half of the blot the following on-blot binding assay was performed: the blot was incubated with either with 1 μg/ml human HPR-conjugated TF (Fig.3.5a) or 10 μg/ml Lf (Fig.3.5b). Although the Tf-HRP conjugate allowed Tf binding to be visualised directly, Lf binding required incubation with primary anti-Lf antibodies and cross recognition secondary HPR conjugated antibodies, as described in section 2.2.1.8. Once it was determined which *E. coli* membrane proteins were binding to Tf and Lf, the corresponding proteins on the other half of the blot were sequenced by the University of Leicester PNACL (Fig.3.5b). The sequences of the proteins to which Tf and Lf were binding corresponded to OmpC and OmpA proteins (Fig.3.5c).

That the same *E. coli* proteins appeared to be binding both Tf and Lf was surprising, as bacteria tend to express individual Tf and Lf binding proteins. Therefore, OmpA and OmpC, competition assays were performed in order to establish whether these Omp proteins had preference for Tf over Lf, and *vice versa*, or if they were both binding Tf and Lf simultaneously (possibly at different sites). *E. coli* was grown at 37 ^oC o/n in LB and the next day were washed and resuspended in SAPI-TRIS pH 7.5. For the competition assay the protocol described in section 2.2.1.1 was followed, however the binding assay was performed twice, thus the bacteria were incubated first with 5 μg or 50 μg of either Tf or Lf (section 2.2.1.3). The bacteria were then harvested and washed twice in SAPI-TRIS pH 7.5 and the second binding assay performed by incubating the

samples in which 50 μg of Tf or Lf was added first with 5 μg of the 'competitor' respectively and *vice versa* (Fig.3.6). From Fig. 3.6 it can be seen that Tf and Lf both bind to the *E. coli* OmpA and OmpC proteins without competition. Moreover, the *E. coli* Tf and Lf Bp does not seem to favour any of the two iron transport proteins, and the bacteria bind to Tf and Lf, irrespective of which was added first.

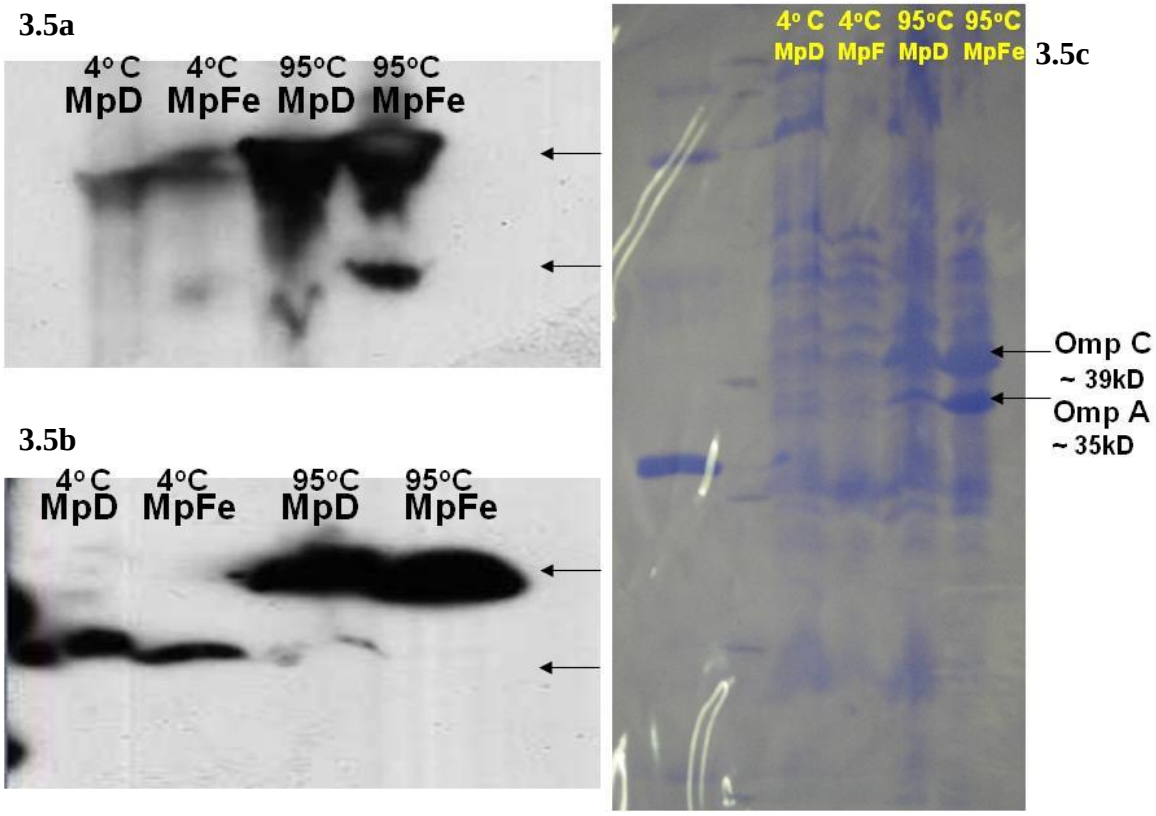


Fig.3.5 On blot binding of Tf and Lf to membrane protein extracts of *E. coli*. *E. coli* membrane proteins were blotted onto PVDF and a Tf/Lf-on blot binding assay performed directly on the blot by incubating with 1 μg/ml Tf-HPR (3.4a) or 10 μg/ml Lf (3.4b) as described in sections 2.2.1.8 and 2.2.1.9. Prior to the membrane protein extraction the bacteria were grown o/n in iron depletion and repletion conditions. The data shown suggests that the Tf and Lf *E.coli* binding proteins are not iron regulated since there is no significant ($p>0.05$) difference in Tf and Lf bound to proteins extracted from bacteria grown under iron replete and iron limited culture media. The *E. coli* proteins to which Tf and Lf bound were sequenced (N-terminal) and corresponded to the OmpA and OmpC porin proteins (3.4c) (Appendix2). The data also shows that that the OmpA protein is more soluble at 4 ^{o}C than OmpC protein.

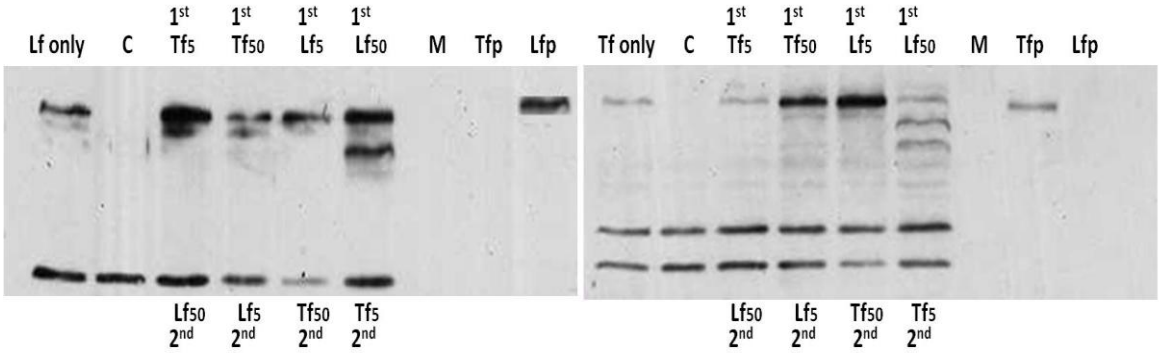


Fig.3.6 Competition assay for the binding of Tf and Lf by the *E. coli* Tf/Lf binding protein(s) and Lf. *E. coli* was grown o/n in LB and a Tf/Lf competitive binding assay was performed as described in section 2.2.1.3. The cells were first incubated with 5 μg or 50 μg of either Tf or Lf (as labelled). The bacteria were then washed twice in SAPI-TRIS pH 7.5 and the second binding assay performed by incubating the cultures in which 50 μg of Tf or Lf was added first with 5 μg of the 'competitor' (Lf or Tf), and vice versa. The data shows that Tf and Lf were bound by the bacteria independently of which protein was added first or second in the assay. The binding proteins did not seem to show any preference for either Tf or Lf, although it must be kept in mind that the detection of Tf and Lf was performed using different primary and secondary antibodies which may have different affinity and may not be the most suitable reagents for comparison. However, it is still possible to observe that both Tf and Lf can simultaneously bind to *E. coli.*

3.3 SUMMARY

1. Both Tf and Lf bind to viable *Escherichia coli* E2348/69
2. Tf and Lf bind to the outer membrane porin proteins OmpC and OmpA, although they seem to bind preferentially to OmpC
3. The bacteria can bind both Tf and Lf simultaneously

3.4 DISCUSSION

3.4.1 The OmpC and OmpA porins are the transferrin and lactoferrin binding proteins in *Escherichia coli* E2348/69

Successful competition for iron between the host and the invading organisms is one of the most important factors that determine the outcome of an infection. Both the host and bacteria have evolved several methods to uptake and store iron. One of the mechanisms by which catecholamines stimulate the growth of a variety of Gram positive and negative bacteria is by facilitating iron removal from Tf and Lf (Freestone *et al.*, 2000) by a not yet fully described mechanism. CAs have been found to form a trimeric complex with Tf or Lf and their associated iron, which is believed to destabilise the bond between the Tf or Lf and the iron, facilitating iron removal which then becomes available to the bacteria to be uptaken by several mechanisms. It was also observed that the NE-Fe complex could be directly internalised in the cytoplasmic fraction of the bacteria, whereas Tf remained bound to the outer membrane fraction. Although, specific transferrin and lactoferrin binding proteins have been found in Gram negative species such as *Haemophilus influenza* (Khan *et al.*, 2007a; Khan *et al.*, 2007b), *Neisseria meningitis* (Perkins-Balding, *et al.*, 2004) and *Neisseria gonorrhoeae* (Price, *et al.*, 2004) that do not produce siderophores, there is no genomic evidence for *Escherichia coli* Tf or Lf binding proteins (Freestone *et al.*, 2000; Freestone *et al.*, 2002; Freestone *et al.*, 2003).

In this chapter it was shown that both Tf and Lf were binding to the outer membrane of *E. coli*. The Tf and Lf proteins were allowed to directly bind to the outer membrane protein extracts which were previously separated on a SDS-page gel and blotted onto PVDF membrane and the identity of the Tf and Lf binding proteins in *E. coli* E2348/69, determined by protein sequencing, and corresponded to the outer membrane proteins OmpA and OmpC. These proteins, which will be investigated in more details in chapter 4, belong to the family of porins. OmpC belongs to the family of classical porins, which are non-specific channels that allow the passage of small hydrophilic solutes through the outer membrane of the bacteria (Nikaido *et al.*, 1980). OmpA is a multifunctional porin that can function as channel, but also has a variety of other roles such as maintaining the structural stability of the outer membrane of the bacteria, participating in conjugation by interacting with F-plasmid, functioning as phage receptor as well as

constituting an important target in the immune response (Smith *et al.*, 2007). OmpA exists in two conformational states that comprise an open and a closed pore (see Chapter 4), which could explain why Tf and Lf predominantly bind to OmpC when both OmpC and OmpA proteins are present (Fig.3.5). OmpA porins are more soluble than the classical porins and can be extracted by SDS at lower temperatures, and to be denatured they need to be boiled at 100^{o}C. In contrast, the tight trimeric structure of OmpC proteins strongly associates with the peptydoglican layer making them highly insoluble in SDS alone, higher temperatures are required to solubilise these proteins, which would otherwise be largely left behind in the insoluble fraction (Nikaido, 1996). Since OmpA proteins are more soluble it is possible that this could explain why in figure 3.5b Lf proteins bind solely to OmpA and not to OmpC (which may have remained in the insoluble fraction) when the samples were incubated at 4 ^{o}C prior gel loading. From Fig. 3.5b it appeared that the expression of the OmpA proteins seemed to be reduced when the bacteria were grown in absence of iron, although the expression of either OmpA or OmpC proteins has never been shown to be iron-dependent. However, the stability of the *ompA* transcript has been found to be dependent on the bacterial growth rate (Emory *et al.*, 1990), and when the cell growth rate is slower, the *ompA* mRNA becomes vulnerable to RNase cleavage, and its cellular level can be reduced by as much as a factor of 4. Therefore, this could explain why the amount of OmpA protein is reduced in the samples that were grown in low iron conditions, because, although all the bacterial cultures were harvested in the logarithmic phase of growth (OD_{600} 0.6) in order to extract the outer membrane protein fractions, the samples grown in iron depletion reached the desired OD_{600} between 3-5 hours later than the sample grown in the presence of iron.

From the results shown it was demonstrated that Tf and Lf bound to the bacteria whether or not they required iron (Fig.s 3.2 and 3.3). Notwithstanding the fact that expression of the *E. coli* Tf and Lf binding proteins does not appear to be iron regulated, the bacteria responded to their need for iron by binding more to Tf or Lf. In contrast, stationary phase bacteria bound Tf and Lf in a similar fashion irrespective of the iron conditions in which they were grown. There was no significant difference ($p>0.05$) between the Tf/Lf bound to the control bacteria and the bacterial sample grown in conditions of iron excess, although it must be appreciated that LB is not a fully iron deficient medium.

The Tf and Lf binding assay was also performed on bacteria which were grown in different osmotic conditions. From Fig. 3.4 can be seen that at higher osmotic pressure (5 % and 10 % NaCl) the binding of Tf and Lf to the bacteria is significantly higher ($p<0.05$) than when the samples were grown in low osmolarity media. Since OmpC porins are expressed at high temperature (37 ^{o}C and higher) and high osmolarity (0.15 M NaCl and higher (Nikaido, 1996) (See section 4.1.1, chapter 4), conditions that are generally found in the body, these results seem to indicated that at low osmolarity the expression of OmpC is reduced and so consequently is the binding of Tf and Lf. However, it is also plausible that since the expression of OmpF porins is favoured by low temperature and osmolarity, conditions usually found outside the body where the bacteria are not likely to encounter Tf and Lf, the bacteria grown in low osmolarity media express OmpF porins which could have less affinity for Tf and Lf; this would explain the reduction in Tf/Lf binding observed. In fact, expression of the *ompF* and *ompC* genes is differentially co-regulated by medium osmolarity (Matsuyama *et al.*, 1984). However, since *ompC* and *ompF* are also regulated by the temperature, and the growth of the bacteria was at 37 ^{o}C, it is also possible that there was just a reduction in the expression of OmpC (Nikaido, 1996). Since only OmpC proteins are expressed inside the human body by invading pathogens and these were the only proteins identified as TfBp and LfBp by sequencing, OmpF was not looked at further for the purpose of this thesis.

In this chapter it has been shown that both Tf and Lf seem to bind to the outer membrane proteins OmpC and to a lesser extent OmpA, therefore it was investigated whether the *E.coli* TfBp and LfBp were showing preference towards either Tf or Lf by performing a competition binding assay which consisted in incubating the bacterial samples with 50 μg of Tf or Lf first, followed after washes by the addition of 5 μg of the 'competitor' respectively and vice versa. Both Tf and Lf bound to the bacteria independently from whether they were added first or second in the assay or whether they were added in excess (50 μg) or in low concentration (5 μg which was approximately the threshold of the lower detection limit). Although human Tf and Lf are similar in structure and sequences (61.4 % identical) (Wally *et al.*, 2007) and play an important role in mammalian iron transport, they differ in their affinity for iron as well as their receptor binding properties. In fact, the human transferrin receptor/s bind only Tf and two distinct bacterial transport systems (TbpA/TbpB and LbpA/LbpB) are

used by the bacteria to acquire iron from Tf and Lf in bacterial species such as *Haemophilus influenza* (Khan *et al.*, 2007a; Khan *et al*., 2007b), *Neisseria meningitis* (Perkins-Balding *et al.*, 2004) and *Neisseria gonorrhoeae* (Price *et al.*, 2004). It is therefore intriguing that in *E. coli* E2348/69 both Tf and Lf seem to bind to both OmpC and OmpA, which do not possess sequence homology to other known Tf and Lf binding receptors such as TbpA/TbpB or LbpA/LbpB (Appendix 3). It is possible that either they bind to a separate, conserved 'binding site' or they bind to different sites on the porins, although further studies need to be carried out to resolve this puzzle. Previous studies aimed at investigating the antimicrobial activity of Lf, interestingly showed that the outer membrane proteins of other Gram negative bacteria of the *Enterobacteriaceae* family, which produce siderophores as a means to obtain ferric iron such as *Salmonella typhimurium* (Naidu *et al.*, 1993) and *Shigella flexneri* (Tigyi *et al.*, 1992) were binding Lf and it was suggested that the Lf-binding proteins might be porins. It is possible then that all the bacteria that belong to this family could bind to Tf and Lf through their porin channels. The antimicrobial properties of Lf are well established and by binding to the surface of bacteria Lf can deprive the bacterial cell of iron, as well as destabilising the surface and mediating bacterial uptake and killing during phagocytosis (Gado *et al.*, 1991; Valenti *et al.*, 2005). However, even though the binding of Lf to the bacteria is intended by the body as a natural defence against invading pathogens, during a stress episode the increased level of CAs could allow bacteria to take advantage and enable them to subtract the iron from Lf (and Tf), and by binding these host proteins, bacteria facilitate and enhance this host iron acquisition process.

3.4.2 Future work

In this chapter the *Escherichia coli* E2348/69 OmpC and OmpA outer membrane proteins were found to be the bacterial receptors for human Tf and Lf. However, to unequivocally confirm their identity and to understand the role that they play in the acquisition of host iron, the genes for *ompC* and *ompA* will need to be deleted. Chapter 4 shows how the *ΔompA/C* mutants were created and characterised in the context of the bacterial CAs response mechanism. In the future it would be interesting to study other bacteria belonging to the family *Enterobacteriaceae* in order to determine whether porins are the Tf and Lf binding proteins conserved unanimously among this family.

Chapter 4 – Mutation of the OmpA and OmpC outer membrane proteins in *Escherichia coli* E2348/69 and characterisation of their role in catecholamine responsiveness

4.1 INTRODUCTION

In chapter 3, transferrin/lactoferrin-binding and other proteomic assays suggested that OmpA and OmpC outer membrane porin proteins were capable of binding Tf and Lf (TfBp and LfBp). Considering the high copy number and conservation of OmpC and OmpA proteins within the *Enterobacteriaceae* family coupled with the fact there is no genomic evidence for an *Escherichia coli* TfBp, it is altogether more intriguing that Tf and Lf seem to bind to these proteins. In order to confirm whether this was true, the genes encoding OmpA and OmpC needed to be deleted. In this chapter the construction of *ompA* and *ompC* deletion mutants was undertaken, and their initial characterisation and role in the mechanism of *E. coli* responses to stress hormones investigated.

4.1.1 Classical porins: OmpC

The OmpC protein belongs to the family of classical porins along with OmpF and PhoE. These are trimeric proteins (Cowan *et al.*, 1992) that constitute relatively non-specific pores or channels that enable the passage of small hydrophilic molecules through the outer membrane (Nikaido *et al.*, 1980). In addition, OmpC and OmpF have been found to associate with the peptidoglycan layer and seem to play an important role in maintenance of the surface structure (Nogami *et al.*, 1983). The molecular weights of OmpF, OmpC and PhoE in *Eschericchia coli* K12 are 38,306, 37,083 and 36,782 Da respectively. The trimeric structure of the porin pores are due to hydrophobic inter-subunit regions, which appear to be important in the assembly and folding of the porin trimer in aqueous solution (Cowan *et al.*, 1992). Each subunit consists of a channel which is formed when the polypeptide chains of each subunit traverses the outer membrane 16 times as antiparallel β-strands, forming a β-barrel structure (Buchanan 1999; Cowan *et al.*, 1992; Nikaido, 1996). The membrane–traversing β-strands are connected by short internal (one to four residues) and long external loops. The sequences of external loops vary greatly between enteric bacterial species and consist mainly of a large number of charged (mostly acidic) amino acids, and it is believed they interact with LPS *via* cation bridges. Moreover, one external loop folds inwardly into the lumen of the β-barrel structure narrowing the pore size to produce channels with wide entry and exit points and a short central constriction (‘eyelet’), providing the solute-discriminating properties of the porins (Cowan *et al.*, 1992; Nikaido, 1996). This

allows only small molecules (M_r ~600) to diffuse through the outer membrane. The rigid structure of the porin eyelet depends on electrostatic forces created by the opposing positively and negatively charged side chains within the eyelet, which favour entry of small hydrophilic molecules (Nikaido *et al.*, 1981). Nikaido *et al.* (Nikaido *et al.*, 1983) determined the diameter of the pores for OmpF, OmpC and PhoE to be 1.16nm, 1.08nm and 0.84nm respectively. Although these porins are highly homologous (the sequence of OmpF and PhoE share 63 % similarity; whereas OmpC and OmpF are nearly identical, the major difference being the presence of a 15-residue insertion in the external loop that is not present in OmpF), they do show differences in the affinity toward charged ions. PhoE allows preferentially the passage of anions whereas OmpF and OmpC favour the passage of cations (Benz *et al.*, 1985). Site-directed mutagenesis showed that the specificity toward the different charged solutes was associated with the nature of the residues located in the eyelet. The most important residue for the anionic preference observed in PhoE is Lys-125, which in other classical porins is replaced by Gly-131 (Bauer *et al.*, 1989). Outer membrane porin proteins are very abundant in *E. coli* and constitute 2 % of the total protein mass. The OmpF, OmpC, and PhoE porins are each encoded by the *ompF, ompC and phoE* genes. PhoE porins are uniquely expressed under phosphate starvation condition, whereas OmpF porins are inhibited by high osmolarity and high temperature, indicating that these two channel proteins are mainly expressed outside of an animal host, where the bacteria will be subjected to lower temperatures and osmotic pressure and a more dilute environment (Jo *et al.*, 1986; Matsuyama *et al.*, 1984; Nikaido, 1996). Conversely, OmpC porins are expressed at high temperature (~37 ^{o}C) and osmolarity (0.15 M NaCl), conditions mimicking human bodily fluids, indicating that in the host and in general culture media the major porins expressed will be the OmpC proteins (Nikaido *et al.*, 1985). This may be due to the reduced size of the pore that enables exclusion of larger molecules (i.e. bile salts) and generally slows the rate of diffusion though the outer membrane, enhancing survival of the bacteria in the host.

4.1.2 Outer membrane proteins: OmpA

OmpA is a monomeric protein of molecular weight 35,152 Da (in *Escherichia coli K12)*. OmpA is expressed in the bacterial cell in high quantities (10^5 molecules/cell) and is responsible for a variety of functions such as participating in the structural

stability of the outer membrane of the bacteria, increasing the efficiency of conjugation by interacting with F-plasmid, functioning as phage receptor as well as constituting an important target in the immune response (Smith *et al.*, 2007). The feature of OmpA protein that is more interesting for our study is their behaviour as channels or pores. The structure of OmpA resembles the monomers of the classical porins (OmpC, OmpF and PhoE) and it was initially thought that the N-terminal domain, consisting of 171 amino acids (organised in anti-parallel amphipathic β-strand that traversed the membrane 8 times to confer a β-barrel structure) constituted the pore, whereas the C-terminal domain was associated with peptydoglican, conferring stability to the outer membrane (Bond *et al.*, 2002; Nikaido *et al*., 1980; Nikaido, 1996; Pautsch *et al.*, 2000; Smith *et al.*, 2007; Sugawara *et al.*, 1994; Vogel *et al.*, 1986). Sugawara *et al.* (Sugawara *et al.*, 1992) demonstrated that OmpA proteins acted as a porin-like diffusion channel, and although the size of the OmpA pores were estimated to be approximately 1 nm in diameter (similar to that of the classical porins), the flux of the solutes was 50 fold slower than observed in OmpF and OmpC. In addition ompA pores show a lack of continuous passage for water or solutes. Using the technique of transport-specific density gradient fractionation, Sugawara *et al.* (Sugawara *et al.*, 1994) demonstrated that OmpA existed in two stable alternative conformers, which constituted an open and a closed pore, and they observed that only 2-3 % of the population was in the open conformation. Although the temperature of their experiments did not emulate physiological conditions they proposed that the reason the solute flux was reduced in OmpA porins was due to the fact the majority of the channels were in a closed conformation. NMR spectroscopy studies (Arora *et al.*, 2001) and molecular dynamic studies (Bond *et al.*, 2002) revealed a degree of conformational flexibility in the structure of the OmpA proteins that confirmed the existence of both stereoisomers and suggested a gating mechanism based on switching ion pairs to open and close the channel. It was later suggested that the two OmpA conformations were temperature dependent (Zakharian *et al.*, 2003) and it was shown that OmpA formed two interconvertible conductance states which corresponded to a small channel (36-140 pS between 15-37 ^{o}C) and a large channel (115-373 pS between 21-39 ^{o}C) and that in physiological temperature the larger pores could predominate. It was also suggested that the larger pore would need to be comprised of both N and C terminal domains resulting in a 16 β-barrel structure, whereas the 8 β-barrel structure constituted the small pore

closed form (Smith *et al.*, 2007)). Although the structure of OmpA has been widely studied some issues remain to be resolved regarding the regulation and abundance of the two conformers in bacterial cells.

4.2 RESULTS

4.2.1 Construction of Δ*ompA* mutant in E2348/69

In order to confirm the binding of Tf and Lf to the outer membrane proteins OmpA and OmpC, deletion mutation of these genes was performed in *E. coli* E2348/69. The general cloning strategy adopted to create the deletion mutant is summarised in Fig. 4.1 and will be discussed in more detail in the following section.

The initial step was to recover sequences of the *ompA* and *ompC* genes (Appendix 4-5) from NCBI database and design primers to amplify flanking regions of approximately 250-300 bp between the upstream and the start codon (ATG) and between the stop codon (TAA) and the downstream region of the specified genes. The primers were designed to obtain specified PCR products (Fig.4.2.), which included the start and stop codon of the target genes, in order to create in frame non-polar deletion mutation. Moreover, the paired flanking regions were designed to be of similar size in order to keep the mutation central within the construct fragments, so as to improve the likelihood of a successful homologous recombination. The length of the PCR product obtained for the upstream and downstream region of the genes were respectively 251, 255 bp for *ompC* gene and 280, 294 bp for *ompA* as can be seen in Fig. 4.2. The PCR fragments and the cloning vector puC19 (Yanisch-Perron *et al.*, 1985) were double digested with appropriate restriction enzymes (Fig.4.3) and ligated to form the recombinant plasmid *via* a two or three way ligation. Since positive colonies were obtained with the three-way ligation strategy, the following results were based on the products obtained by this system.

Plasmid puC19 (Yanisch-Perron *et al.*, 1985) is a high copy number *E. coli* plasmid of 2686 bp length (Fig. 4.2), whose cloning site lies within the *lacZ* gene. Insertion of DNA causes disruption of the *lacZ* gene and enables recognition of the colonies containing the recombinant plasmid by plating them on an LA plate supplemented with IPTG, and X-gal. IPTG induces the expression of β-galactosidase, which cleaves the substrate X-gal producing blue coloured colonies. Disruption of the *lacZ* gene by

insertion of the mutant construct enables recognition of the colonies carrying the recombinant plasmid, which are not producing the enzyme and appear white. Moreover it contains an ampicillin (Amp) antibiotic cassette that enables further selection.

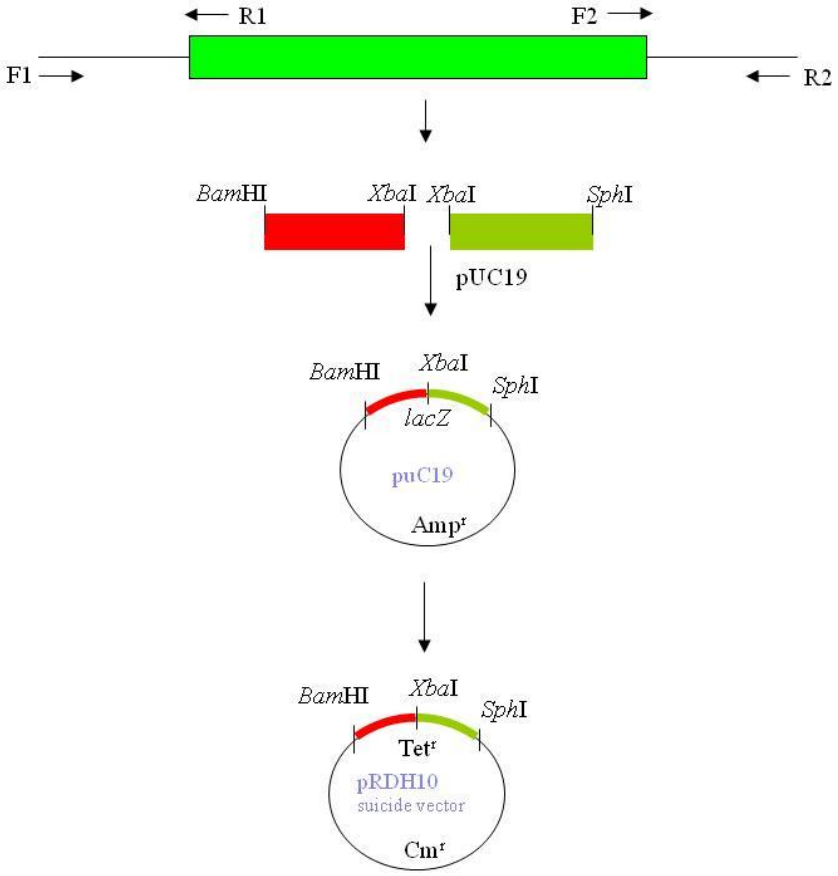


Fig 4.1 *ompA* and *ompC* cloning strategy. PCR fragments of ~250-300 bp length from the upstream and downstream regions of the target genes were double digested with *Bam*HI/*Xba*I or *Xba*I/*Sph*I and ligated in puC19 cloning vector (previously digested with *BamHI* and *SphI*), which was transformed into *E. coli* DH5α. Insertion of the construct results in the disruption of the *lacZ* gene. Using this (IPTG, X-gal) blue/white selection system the recombinant colonies were isolated, the plasmids recovered, and the construct inserted into the suicide vector pRDH10. pRDH10 contains a *ori*R6K origin of replication and will only replicate in strains which carry the π protein which is encoded on the lysogenic phage λpir, thus the recombinant plasmids were transformed into *E. coli* strain CC118λpir. pRDH10 also carries the mobRP4 which is used to mobilise the recombinant plasmid during conjugation, and so additional transformation in SM10λpir strain carrier of the integrated plasmid encoding the RP4-2 genes was necessary. Also, pRDH10 contains two antibiotic resistance genes for chloramphenicol (Cm) and tetracycline (Tet) (within the polylinker region) and a *SacRB* gene that encodes the enzyme levansucrase, which is inducible by growth in the presence of sucrose and which expression results in a lethal toxicity to the bacteria allowing selection of resolved merodiploids. The donor strain was conjugated with E2348/69 and merodiploids were selected for resistance of Cm and sensitivity to Tet. The E2348/69 merodiploids were resolved by sucrose selection and the mutant were checked by PCR and sequencing.

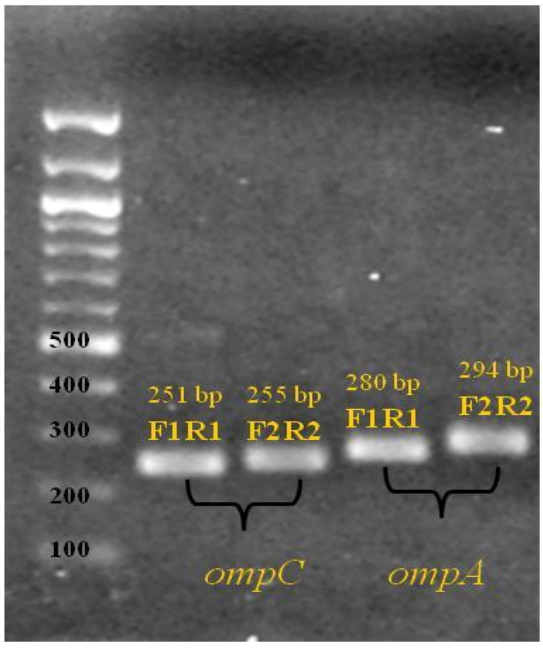


Fig.4.2 PCR amplification of the upstream and downstream region of the *ompC* and *ompA* genes. The PCR fragments were amplified using coupled F1R1 and F2R2 primers designed for the *ompC* and *ompA* genes. The PCR products obtained were in concordance with the expected sizes are labelled in the Fig.

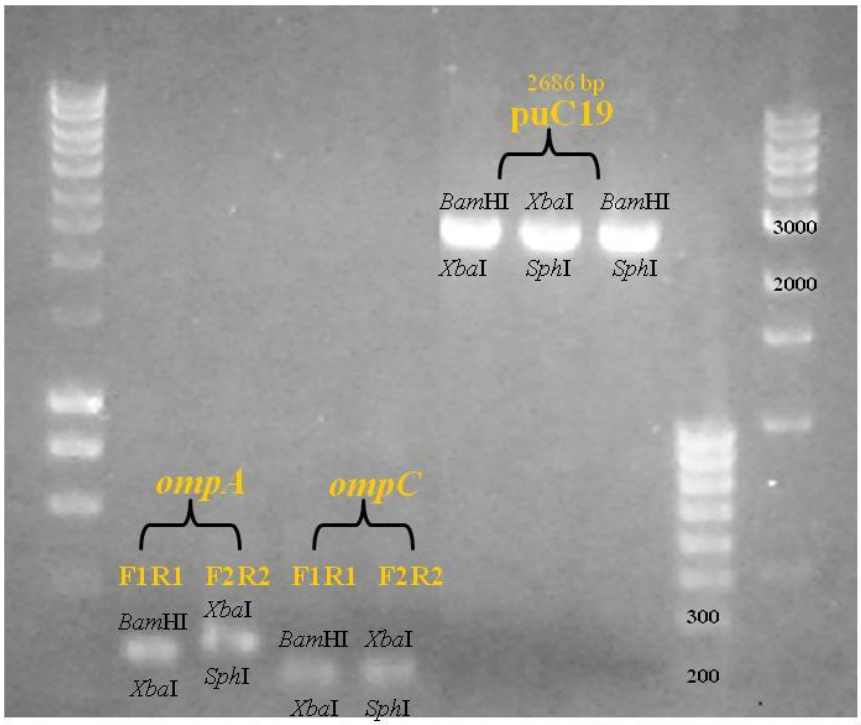


Fig.4.3 Double digestion of the PCR products of the cloning construct and puC19 cloning vector. The PCR products shown in Fig.4.2 and the puC19 vector were double digested with either *Bam*HI and *Xba*I or *Xba*I and *Sph*I to allow two way ligation of the upstream (F1R1) or downstream (F2R2) regions of the *ompC* and *ompA* genes into the vector. The puC19 vector was also digested directly with *Bam*HI and *Sph*I to enable three way ligation into the plasmid of both F1R1 and F2R2 PCR fragments. Since the three ligation system produced positive colonies the following results are based on the products obtained with this system.

The mixture containing the construct/vector ligations was transformed by electroporation at 1.5 KV with a resistance of 1000 ohms and a capacitance of 25 μF into electrocompetent *E. coli* strain DH5α (1st transformation). Cultures with time constant readings between 21-23 were considered as acceptable. The electroporated cultures were then inoculated onto LA plates supplemented with Amp (100 μg/ml). Since only the colonies with incorporated plasmid survived, they were recovered from the plate (with 2 ml of LB), inoculated into LB, grown overnight and plasmids extracted using the miniprep method described in section 2.2.2.15.

The recovered plasmids containing a mixture of recombinant *ompA* or *ompC* insert and re-ligated puC19 plasmid were each digested with *Hinc*II and *Pst*I restriction enzymes, whose restriction sites were present in the vector but which had been lost in the recombinant puC19 plasmid. This double digestion improved the efficiency of further transformations of the circular recombinant plasmid, since the digested vectors, now in linear form, could not be transformed into $CaCl_2$ competent *E. coli* DH-5α (2nd transformation).

The transformed cultures were plated onto an IPTG-X-gal-Amp LA plate and any single white colonies which grew up were further re-streaked on an IPTG-X-gal-Amp plate and analysed by colony PCR (data not shown). However, after screening many hundreds of colonies, the sizes of the *OmpA/C* PCR products were found to be either too short or longer than expected. Therefore, since these results were observed on several occasions (data not shown), a few colonies were chosen and recombinant plasmids were retrieved by mini-prep and digested with *Bam*HI and *Sph*I restriction enzymes. In Fig. 4.4 it is possible to see the digested *ompA* and *ompC* construct fragments and puC19 vector. The sizes of the *ompA* and *ompC* constructs should have been 574 and 506 base pairs, respectively. However, the *ompA* construct obtained was slightly shorter than the expected 500bp, whereas the *ompC* construct was longer than expected at approximately 800bp. In order to elucidate how this could have happened, PCR products using F1R2 primer sets were amplified from the ligation mixture used for the first transformation of the recombinant plasmid into *E. coli* DH-5α and from the plate prep mixtures obtained after the transformation. As can be seen in Fig. 4.5, from the ligation mixtures two PCR products were amplified, one of the expected length and the other shorter or longer than the expected length respectively for *ompA* and *ompC*

constructs. These anomalous DNA products were of similar length to the PCR products amplified from the plate prep mixture recovered after the 1st transformation (Fig.4.5).

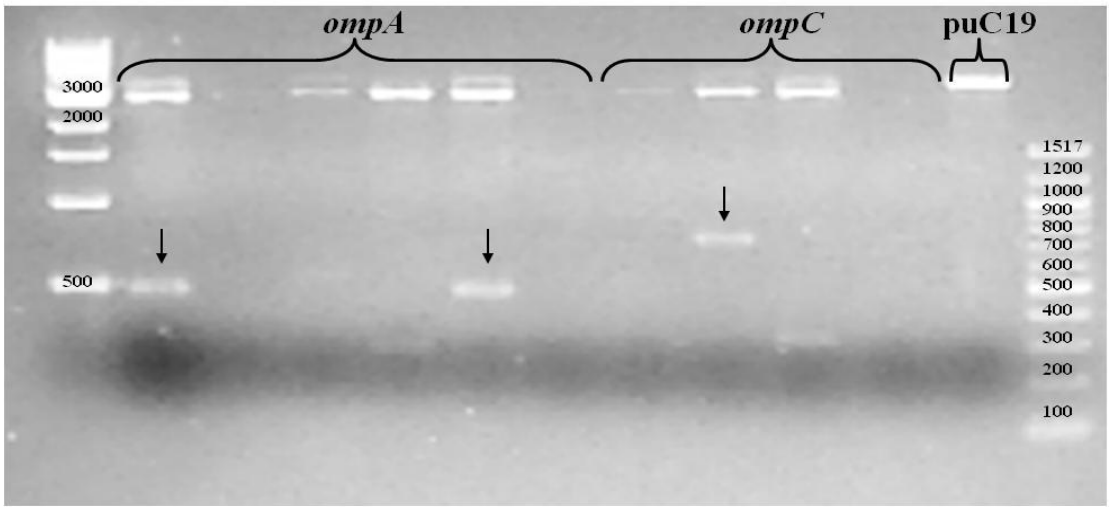


Fig.4.4 Digestion of the recombinant puC19 vectors. Recombinant puC19 vectors were retrieved by miniprep from overnight culture of white colonies containing the potential constructs and double digested with *Bam*HI and *Sph*I restriction enzymes. After screening many white colonies it became clear that the length of the inserts obtained after digestion for both *ompA* and *ompC* genes were not of the expected sizes. Therefore, recombinant plasmids of a few selected colonies (indicated by the arrows) were sent for DNA sequencing.

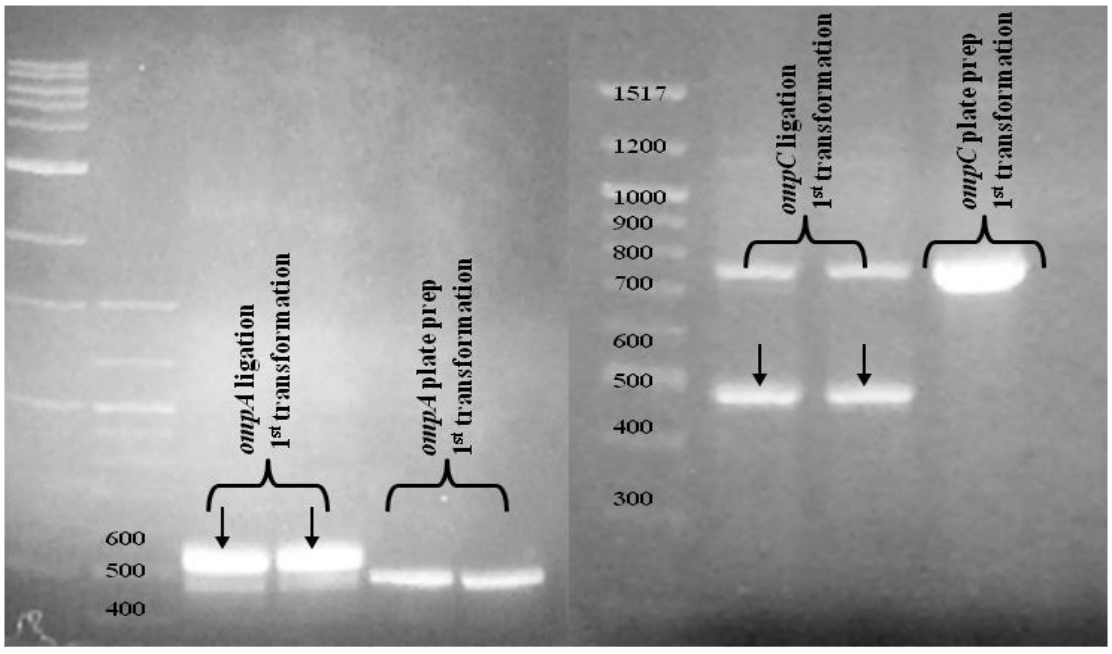


Fig.4.5 PCR products of the *ompA* and *ompC* constructs obtained from the ligation mixture and from the plate prep recovered after the 1st transformation. Using F1R2 primers to amplify *ompA* or *ompC* mutant constructs it was possible to demonstrate that two PCR products were obtainable from the ligation mixture used for the first transformation into *E. coli* DH-5α; whereas only one PCR product (of unexpected size) was amplified from the miniprep mixture of the bacteria recovered from the LA Amp plate after the 1st transformation.

Even thought the intensity of the bands and therefore the concentrations of the PCR products of the potential *ompA* and *ompC* constructs were higher in the ligation mixture, only the anomalous PCR products were successfully transformed, explaining why only these products were recovered following the 2nd transformation in *E. coli* DH-5α (Fig.4.4). In order to clarify why these incorrect sized constructs were favoured during transformation, the recombinant plasmids of Fig.4.4 (marked by arrows) were sent to the University of Leicester PNACL service for sequencing. The retrieved sequences were then aligned against the whole genome database entry of *E. coli*. Interestingly, only half of the sequence (originally amplified with the F2R2 primers) aligned with the appropriate downstream region of both *ompA* and *ompC* genes, whereas the upstream fragments, which corresponded to the promoter region of the genes, (originally amplified with F1R1 primers) aligned in a different region of the E2348/69 DNA (database comparisons gave identities of the translated proteins. These were, respectively, for the *ompA* and *ompC* F1R1 constructs: a putative fimbrial-like adhesin protein and an inner membrane protein). This was curious, since when the primers were initially checked there was no visible sign of more than one amplified PCR products for each pair of primers (Fig.4.2). This seemed to indicate that in high copy number the promoter region of the *omp* genes was deleterious for the cells and thus transformation of only the unexpected sized DNA fragment was favoured. To overcome this problem, the PCR products of these potential constructs were extracted from the agarose gel, purified, digested with *Bam*HI and *Sph*I (Fig.4.6) and ligated into suicide vector pRDH10 (Haigh, 1999).

Plasmid pRDH10 is a low copy number suicide vector 8190 bp long that contains an origin of replication, *ori*R6K that can only be replicated in strains that carry the π protein encoded in the lysogenic phage λpir. The plasmid also encodes two antibiotic genes conferring resistance to chloramphenicol (Cm) and tetracycline (Tet). The cloning site lies within a tetracycline antibiotic cassette causing selective inactivation of the gene upon successful recombination. The plasmids were electroporated into CC118λpir strain and the colonies containing the recombinant plasmid were selected for resistance to Cm (Cm^R) and sensitivity to Tet (Tet^S). Potential colonies containing the insert of the right size were further analysed by sequencing as described below. Fig. 4.7 shows the products obtained by amplifying using PCR the CC118λpir colonies that were resistant to Cm and sensitive to Tet. After screening many colonies two of them

were identified as containing the recombinant plasmid that carried the potential *ompA* insert of the construct mutation (Fig. 4.7).

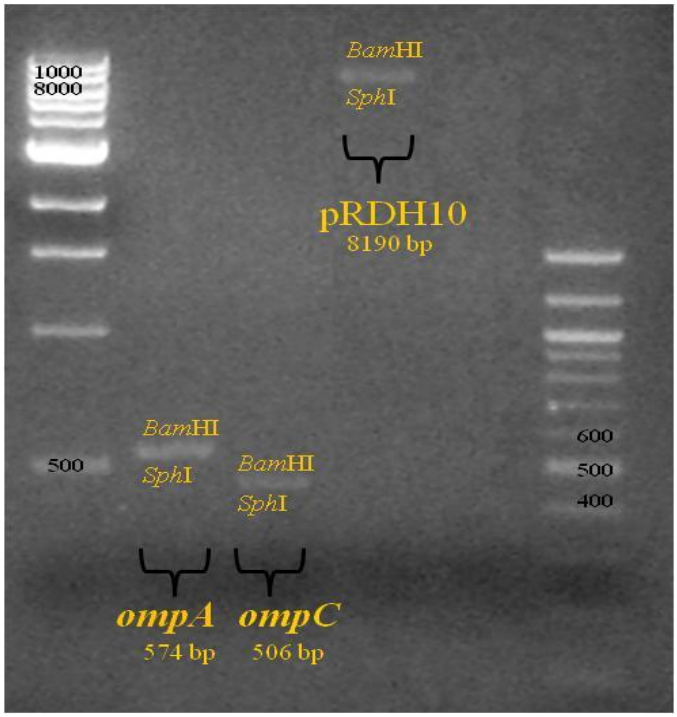


Fig.4.6 Double digestion of the *ompA* and *ompC* pRDH10 plasmid constructs. After excising the bands of interest from the agarose gel shown in Fig.4.5, the DNA was extracted and the fragments double digested with BamHI and SphI and the concentration was checked on a 1.5 % agarose gel prior to ligation (1: 5 vector/insert ratio) into pRDH10 plasmid.

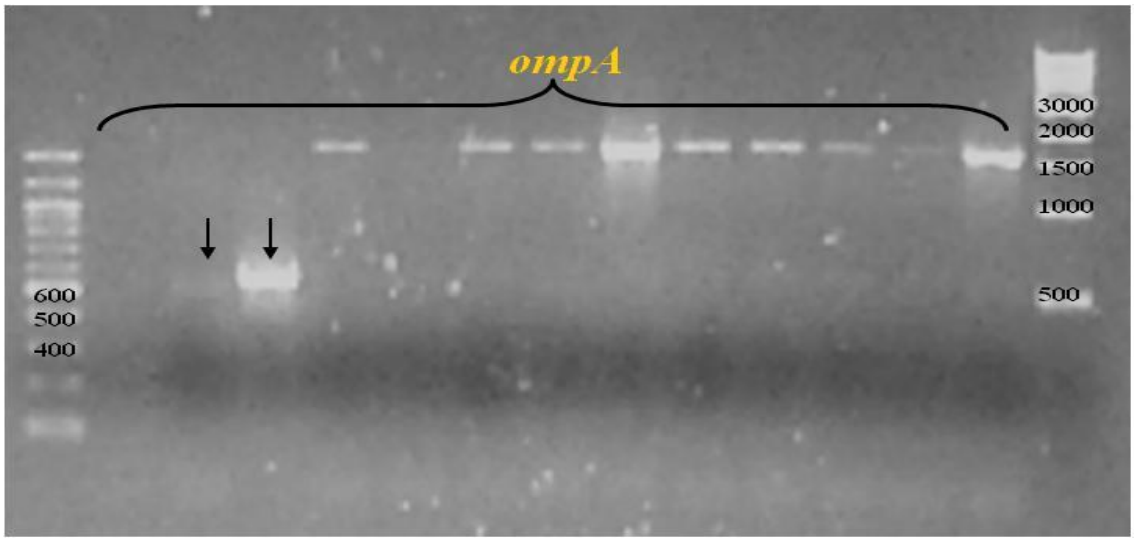


Fig.4.7 Colony PCR of the *ompA* construct after transformation into strain CC118λpir. Colony PCR was performed using F1R2 primers to screen Cm^R and Tet^S colonies carrying the potential recombinant plasmid containing the *ompA* mutation construct. PCR products obtaining from two colonies, marked by arrows, were of the expected sizes and were sent for sequencing.

Unfortunately even though repeating several times the electroporation of the pRDH10 plasmids carrying the *ompC* mutation construct into CC118λpir, it was never possible to obtain a positive colony after transformation. Therefore the *ompC* deletion mutants in E2348/69 was obtained using a different strategy, which will be described later on in this chapter.

Two colonies (Fig.4.7 marked by arrows) containing the potential *ompA* mutation construct were sent for sequencing to the University of Leicester PNACL service and the resultant sequences were then aligned using GeneTool to E2348/69 genomic DNA. Fortunately, the obtained sequences corresponded fully to the originally amplified upstream and downstream region of the *ompA* gene.

The plasmid vector pRDH10 also possesses the mobRP4 gene that is used to mobilise the mutant carrying plasmid during conjugation, however this requires an additional transfer genes which is encoded on the RP4-integrated plasmid in SM10λpir strain. Therefore the recombinant vector carrying the *ompA* mutation construct were recovered from the colonies of interest by maxiprep and transformed by electroporation into strain SM10λpir. The pRDH10 plasmid was first transformed into CC118λpir strain as the transformation efficiency and the quality of prepared plasmid DNA from strain SM10λpir was consistently very poor, even using preparative techniques such as CsCl gradient centrifugation (Haigh, 1999). The Cm^R and Tet^S SM10λpir colonies were screened by colony PCR for the recombinant plasmid carrying the *ompA* mutation construct, DNA fragments of the expected size were obtained, which can be seen in Fig. 4.9. From Fig. 4.9 it is possible to see that all the selected SM10λpir colonies were carrying recombinant pRDH10 plasmids containing the *ompA* mutation construct, therefore two colonies (marked in Fig.4.9 by the black arrows) were chosen for conjugation with the E2348/69 wild type strain. Both bacterial cultures were grown overnight and the following day transferred into fresh media without addition of any antibiotics until their growth was exponential, at an OD_{600} of about 0.6. One ml of each culture was then consecutively transferred into the same Eppendorf tube and spun at 8,000 rpm for 2 minutes. The pellet was re-suspended in LB and spotted onto a sterile 25 mm nitrocellulose filter disk on an LA plate and conjugated for 6 hrs at 37 ^oC. The trans-conjugant colonies were recovered from the filter and incubated overnight in LB supplemented with Cm.

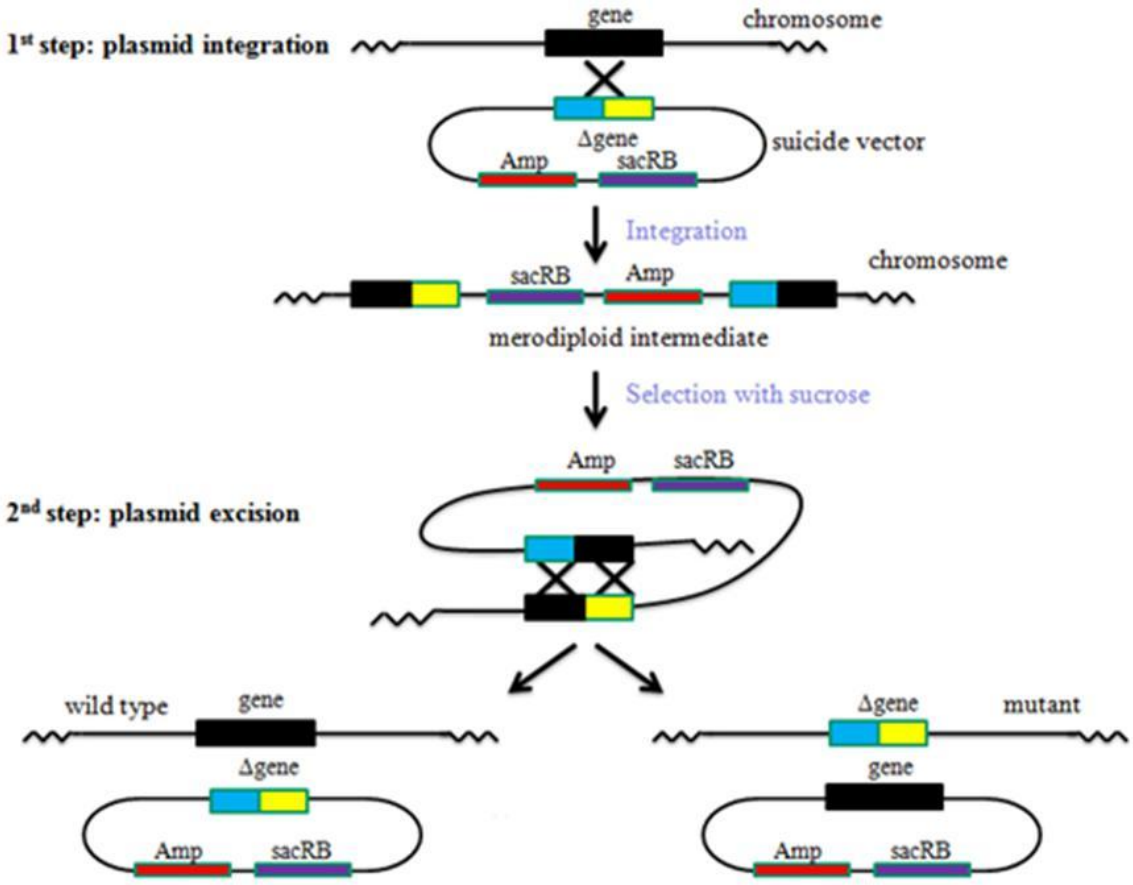


Fig.4.8 Homologous recombination. The allelic exchange of DNA material between the deletion construct and the wild type gene occurs in two main steps each involving homologous recombination. In the first strep the recombinant pRDH10 suicide vector carrying the mutant allele is introduced into strain E2348/69 strain by conjugation. This strategy allows selection of the Cm^R transconjugant colonies in which integration of the recombinant plasmid into the wild type allele by homologous recombination, results in formation of the merodiploid intermediate. Further selection of the E2348/69 strain is possible by plating the colonies on LA supplemented with streptomycin, due to its innate streptomycin resistance. The resultant $Strep^R$ Cm^R merodiploid are then resolved on 6 % sucrose grown, where the second homologous recombination event occurs and the plasmid is excised from the genome, and the merodiploid resolves to give either mutant or wild type colonies. Not all the merodipoid resolve thus, a further selection is possible due to the counter selectable marker encoded in the *sacRB* gene contained in the integrated pRDH10 that encodes the enzyme levansucrase, which is inducible by growth in the presence of sucrose and which expression results in a lethal toxicity to the bacteria.

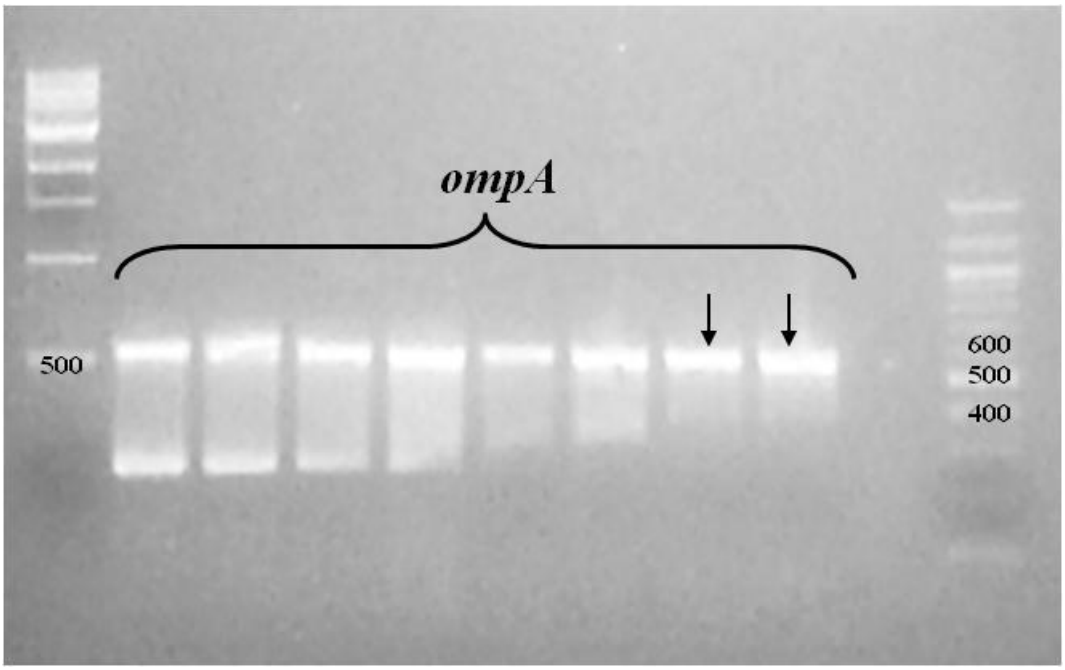


Fig.4.9 Colony PCR of *ompA* mutation fragments in SM10λpir. After transformation colony PCR was performed on Cm^R Tet^S colonies using ompA_F1 and ompA_R2 primers. All the screened colonies were carrying recombinant plasmid containing a *ompA* mutation construct fragment of the expected size; bands indicated by the arrows were further analysed.

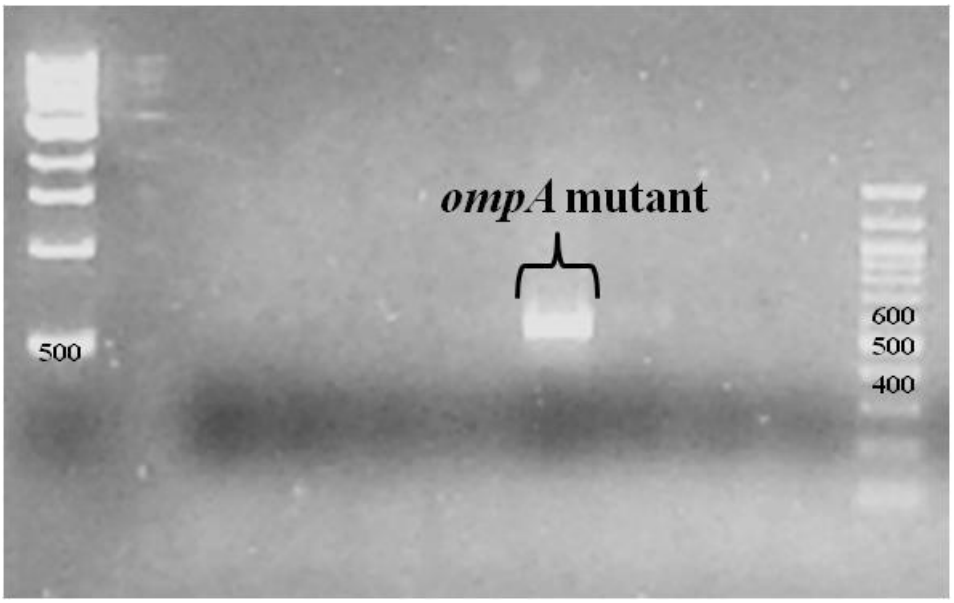


Fig. 4.10 PCR amplification of Δ*ompA* mutant in *E. coli* strain E2348/69. After conjugation, $Strep^R$ Cm^R transconjugant colonies were selected and the merodiploids were resolved by plating them on 6 % sucrose agar. E2348/69 $Strep^R$ Cm^S colonies were screened by colonies PCR using ompA_F1 and ompA_R2 primers. The PCR products were visualised on 1.5 % agarose gel electrophoresis and the size of the products was of the expected length (cells that did not give any PCR products were WT E2348/69, as determined by PCR in which the elongation step was protracted for 3 min, data not shown). The DNA of the potential *ompA* mutant was extracted by boiling the cells at 95 ^oC, purified using a QIAgen genome DNA purification kit and sent for sequencing to University of Leicester PNACL. The sequences obtained were blasted with the whole genome of E2348/69 and the fragment corresponded to the upstream and downstream regions of the *ompA* gene confirming that the *ompA* gene had been deleted and that the Δ*ompA* mutant had been obtained.

This allowed selection of the Cm^R trans-conjugant colonies in which integration of the recombinant plasmid in the wild type allele by homologous recombination resulted in the formation of merodiploid intermediate products (Fig.4.8).

Further selection of the E2348/69 strain containing the *ompA* mutation was made possible by plating the colonies onto LA supplemented with streptomycin, due to the characteristic innate antibiotic resistance of this particular strain. The $Strep^R$ Cm^R E2348/69 colonies carrying the merodiploid intermediate product in their genome were then resolved on 6 % sucrose agar incubated at 30 ^oC. During this step a second homologous recombination event occurred and the plasmid was excised from the bacterial genome. The merodiploid resolved to give either mutant (as shown in Fig.4.8) or wild type colonies.

However not all the merodiploids resolve, therefore it was the presence of the counter-selectable marker, *sacRB* gene (Blomfield *et al.*, 1991) in pRDH10 that enabled further selection. The *sacRB* sequence encodes the enzyme levansucrase, whose expression results in a lethal toxicity to the cell which is inducible by growth in the presence of sucrose. Thus growth of pRDH10-containing merodiploids on sucrose agar (Blomfield *et al.*, 1991) at 30 ^oC was used as a positive selection for bacteria within which secondary recombination events have occurred and resulted in plasmid excision. The resultant colonies are massively enriched for resolved merodiploids, and were screened by colony PCR and the potential mutant sequenced. Fig. 4.10 shows the PCR product amplified from a colony of E2348/69, which DNA sequence confirmed to be a *ΔompA* mutant.

4.2.2 Construction of *ΔompC* mutants in strain E2348/69

As previously mentioned despite several transformations of pRDH10 plasmid containing the *ompC* deletion construct DNA fragment into strain CC118λpir, it did not prove possible to obtain any positive colonies. Therefore, due mainly to time restriction, another approach was used to achieve colonies of an E2348/69 *ΔompC* mutant. The methods adopted involved the use of the phage λ-red recombinase system, where PCR products provided the homologous template for recombination with the target gene (Fig. 4.11)

The previously designed ompC_F1 and ompC_R2 primers were retained and used to construct the mutant. However ompC_R1 and ompC_R2 primers first needed to be

adapted to the new mutation strategy and new primers named ompC_R1λ and ompC_F2λ were synthesised that resembled the previous ones in position without the artificial restriction site and with the incorporation of 'a tail' DNA sequence complementary to a *kanamycin (kan)* gene cassette.

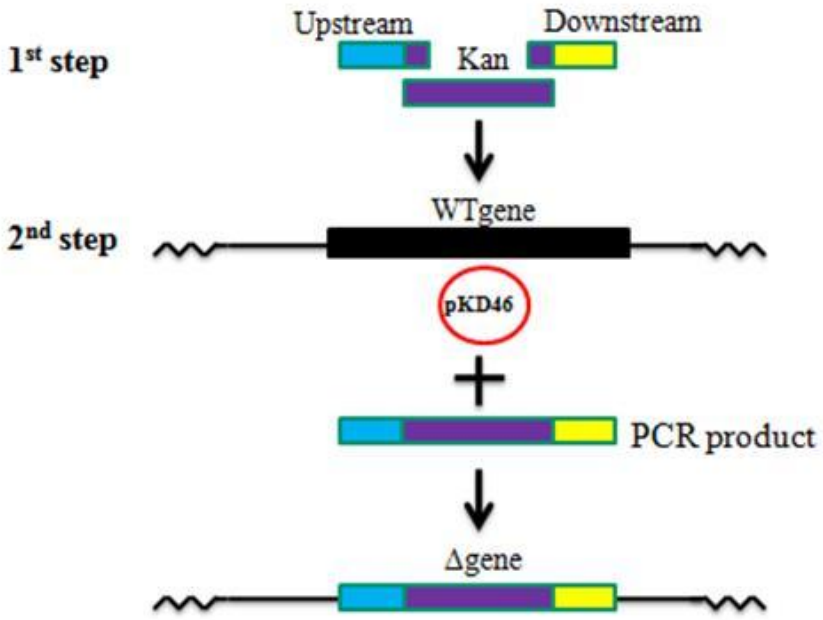


Fig. 4.11 Construction of the ΔompC mutant. The first step for constructing a Δ mutation based on the λ red recombinase system was to obtain a suitable template of the *ΔompC* gene that will be used for homologous recombination with the target gene. The PCR products of the upstream and downstream regions of the target gene and a *Kan* cassette selectable marker (1:1:1 ratio) constituted the template. The second step involved activation by arabinose of the λ red genes: *γ*, *β* and *exo* carried in pKD46 plasmid contained in the WT strain E2348/69, which encoded the Gam, Bet and Exo products. The function of these products was fundamental for the success of the mutation because Gam is responsible for the inhibition of the host RecBCD exonuclese V that enables the cells to degrade linear DNA, whereas Bet and Exo can access to DNA ends promoting recombination. The plasmid was then removed at 42 ^{o}C, to avoid unwanted recombinational events under no-inducing conditions, and the PCR template introduced by electroporation in electrocompetent E2348/69. Homologous recombination occurs and the Strep^{R}Kan^{R} colonies carrying the Δgene were screened by PCR and sequencing.

4.12a **4.12b**

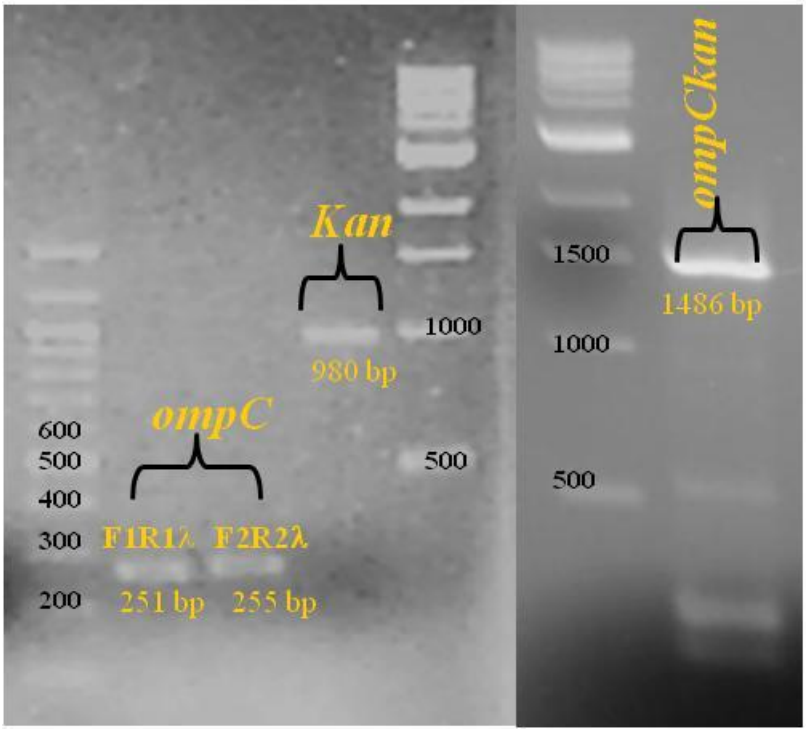


Fig.4.12 OmpC deletion construct PCR products. 4.12a: PCR product of the upstream and downstream regions of *ompC* gene and of the *kan* gene cassette. 4.12b: PCR product of the *ompC* deletion construct obtained by PCR amplification of the DNA fragments of gel a mixed in ratio 1:1:1.

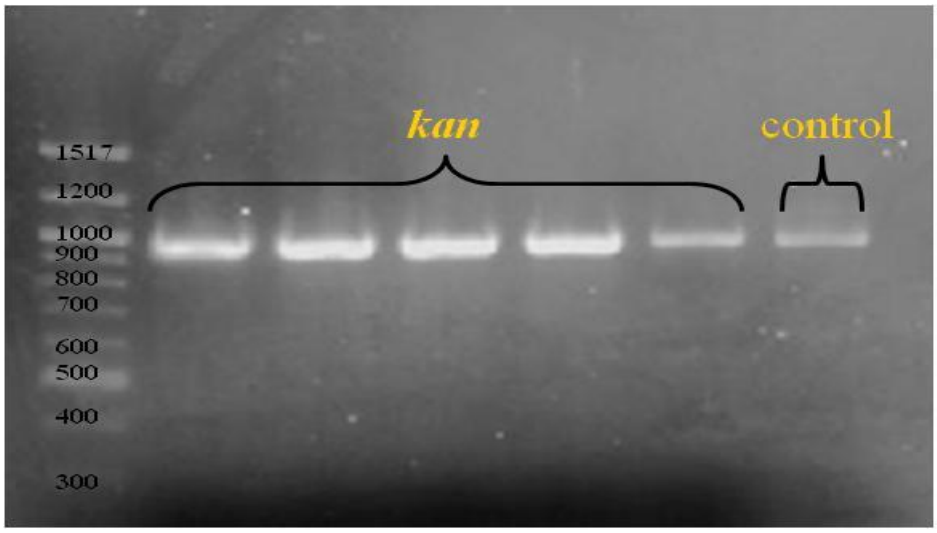


Fig.4.13 Colony PCR of Kan gene fragments in E2348/69. Kan_F and Kan_R primers were used to screen Strep^R Kan^R colonies that had undergone homologous recombination in order to select for potential *ΔompC* mutants. All the colonies checked gave PCR products of 980 bp, which corresponded to the expected size of the *kan* gene as compared with the control. The control used was the original PCR product obtained by amplification of the *TnPhoA Kan*^{R} gene contained in pRT733 vector.

These primers enabled the construction of a PCR product containing a *kan* cassette within the upstream and downstream regions of the target gene. This PCR product provided the template for homologous recombination and replacement of the *ompC* gene with the deleted form, plus insertion of the *kan* cassette to provide a suitable recognition marker for the mutant colonies (Fig.4.11). With this mutation strategy the start and stop codons of the gene were included in the construct to obtain a non-polar mutation. In Fig. 4.12a the PCR products of the upstream and downstream regions of the *ompC* gene and the PCR products of the *kan* cassette can be seen (251, 255, and 980 base pairs long, respectively). The *kan* gene was amplified using Kan_F and Kan_R primers that were design against the sequence of pRT733 plasmid which contained *TnPhoA* Kan^R gene (Taylor, *et al.*, 1989).

Once the three individual DNA fragments were obtained, the PCR products were mixed together in 1:1:1 ratio and a PCR amplification reaction was performed (section 2.2.2.3). The resulting PCR products, as seen in figure 4.12b included the *kan* cassette gene within the upstream and downstream region of *ompC* and constituted the template for homologous recombination (Fig.4.11) that was performed directly, by introducing the linear PCR products by electroporation into previously 'activated' electrocompetent WT E2348/69 cells prepared as described below.

The WT E2348/69 bacteria cells used for this type of mutation were carrying a pKD46 vector (Datsenko *et al.*, 2000). Plasmid pKD46 is a low copy number, temperature-sensitive replication plasmid that contains the λ red recombinase system of bacteriophage origin. This system includes three genes *γ, β* and *exo*, which encode respectively Gam, Bet and Exo products. The function of these products is fundamental for the success of the mutation because Gam is responsible for the inhibition of the host RecBCD exonuclese V that enables the cells to degrade linear DNA, whereas Bet and Exo can access DNA ends promoting recombination. These genes are strictly regulated by an arabinose inducible promoter P_{araB} to avoid recombination events under non-inducing conditions. Moreover pKD46 possess a small ORF, *tL3* that seems to greatly enhanced the number of recombinants, for unknown reasons (Datsenko *et al.*, 2000).

E2348/69 was grown overnight at 30 ^{o}C in LB. The next day 1:100 dilution of the overnight culture was transferred in fresh LB and the bacteria were re-grown at the same temperature until an OD_{600} of 0.6 was reached. Approximately 30 minutes

before reaching the target OD, 0.1 % arabinose was added to the bacteria in order to activate the λ red recombinase system. The bacteria were then incubated at 42 ^{o}C for 15 minutes, to enable the removal of the pKD46 vector. The activated electrocompetent E2348/69 cells were electroporated with the *ompC* PCR product templates, and homologous recombination occurred. The resultant colonies were plated on to LA supplemented with Kan and Strep antibiotics and incubated overnight at 37 ^{o}C. Single Strep^{R}Kan^{R} colonies were then checked by colony PCR using Kan_F and Kan_R primers to select for colonies carrying the *kan* gene and therefore potentially the *ΔompC* E2348/69 mutant (Fig.4.13). Using the Kan primers the first three colonies shown in Fig. 4.13 gave PCR products of the expected size of 980 bp, and were selected as the potential *ΔompC* mutant and re-streaked to check for purity on Strep/Kan LA. The selected colonies were finally checked by PCR using ompC_F1 and ompC_R2 primers (Fig. 4.14) and since the PCR products were of the expected size (1486bp), they were sent for confirmatory sequencing, and were found to be E2348/69 *ΔompC* mutant colonies.

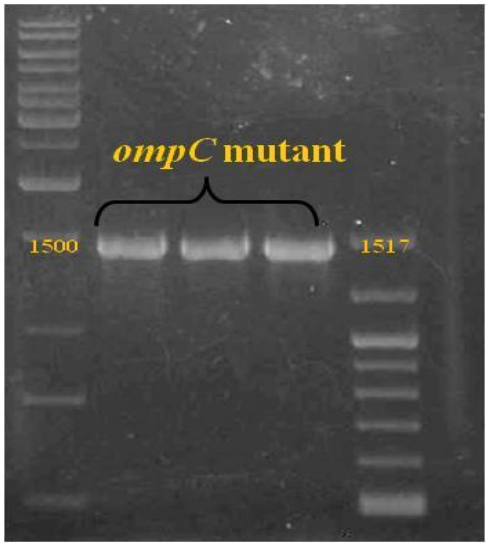


Fig. 4.14 PCR amplification of *ΔompC* mutant in *E. coli* strain E2348/69. The first three colonies that produced amplified PCR products of the expected size in Fig.4.13, were further checked by PCR using ompA_F1 and ompA_R2 primers to confirm that the amplified DNA fragments were of the expected size of 1486bp, which corresponded to the length of the replaced *ΔompC* gene. Since the PCR product size seemed to be of the right length the genomic DNA of the potential *ompC* mutant was extracted by boiling the cells at 95 ^{o}C, and purified using QIAgen DNA purification kit and sent for sequencing to PNACL. The obtained sequences were compared with the whole genome of E2348/69 and against the *kan* gene sequence. The results of the database comparison indicated that the sequence of the DNA fragment corresponded to the upstream and downstream regions of the *ompC* gene with within the *kan* gene, confirming that the *ompC* gene had been deleted and that the *ΔompC* mutant had been obtained.

4.2.3 Characterisation of *E. coli* E2348/69 Δ*ompA* and Δ*ompC* mutants

Once the *ompA* and *ompC* deletion mutants were obtained, tests were performed to show whether the actual loss of the OmpA and OmpC proteins, affected E2348/69 binding of Tf and Lf, uptake of iron, and responsiveness to catecholamines and growth generally. These tests were performed in order to elucidate the role these porin proteins play in the mechanism by which *E. coli* interacts with Tf and Lf and within the catecholamine response mechanism generally.

4.2.3.1 Outer membrane protein profile and on-blot Tf and Lf binding of *E. coli* E2348/69 *ΔompA* and *ΔompC* mutants compared with WT

Outer membrane protein preparations of the E2346/89 *omp* mutants and the WT strain were prepared by sonication and ultracentrifugation as described in section 2.2.1.4 and separated by SDS PAGE (Fig. 4.15). From the outer membrane protein profile shown in Fig. 4.15 it is possible to see the missing protein bands that corresponded each to the expected positions of OmpA and OmpC. Although the protein concentrations of the outer membrane protein extracts were equalised prior to loading, the expression of OmpC in the *ΔompA* mutant and OmpA in the *ΔompC* mutant, when compared with their expression in the WT, was significantly increased ($p<0.05$). Moreover, even though the total outer membrane protein profile is very similar when comparing the mutants with the WT strain, expression of several proteins become altered. In Fig. 4.15, it can be seen that some protein bands are either not present in the WT (bands 1 and 2), or are over-expressed in the mutants (band 3), or very much reduced/missing (bands 4 and 5). These proteins were sequenced and their identities obtained by comparing the sequences against the protein database (Appendix 7). Band 1: outer membrane protein C precursor; Band 2: outer membrane protein A precursor; Band 3: elongation factor Tu; Band 4: major outer membrane lipoprotein precursor; Band 5: 50s ribosomal protein. The reason why the bands corresponding to OmpA and OmpC precursors have lower MW than OmpA and OmpC porins is most likely due to the fact that they have been degraded by proteases present in the membrane fraction.

Once it was confirmed that the OmpA and OmpC proteins bands were missing in the outer membranes of the respective mutants, Tf and Lf binding assays were performed (directly on blotted outer membrane protein preps using the on-blot methodology described in section 2.2.1.9) using either 1 μg/ml of Tf-HPR or 10

μg/ml of Lf protein and the appropriate primary and secondary antibodies as described in section 2.2.1.8.

From Fig. 4.16 it can be seen that Tf (Lf data not shown but comparable to Tf) binds to OmpA and OmpC in the WT control, whereas the bands are missing where either OmpA or OmpC should had been in the respective outer membrane preparation of the mutants. This indicates that transferrin and lactoferrin are indeed binding to these porin proteins.

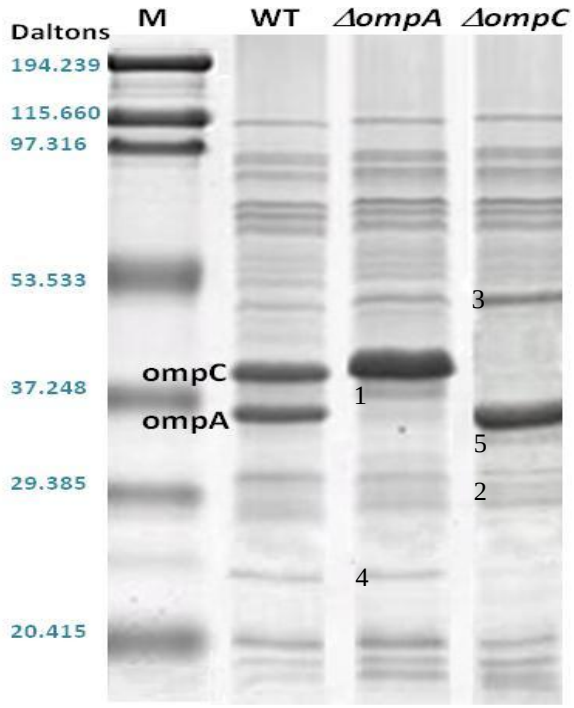


Fig.4.15 Wild type and mutants outer membrane proteins' profile. Outer membrane protein extracts of *E. coli* WT: wild type, *ΔompA*: deletion *ompA* mutant and *ΔompC*: deletion *ompC* mutant were obtained as described in section 2.2.1.4. The proteins were separated on a 12 % SDS PAGE at 60 mA and visualised using Coomassie stain. Protein bands that were expressed differentially between the WT and mutants were sequenced (peptide mass fingerprinting) and compared with protein database sequences (Appendix 7). Key: 1: outer membrane protein C precursor; 2: outer membrane protein A precursor; 3: elongation factor Tu; 4: major outer membrane lipoprotein precursor; 5: 50s ribosomal protein. The reason why the bands corresponding to OmpA and OmpC precursors have lower MW than OmpA and OmpC porins is most likely due to the fact that they have been degraded by outer membrane proteases present in the membrane fraction.

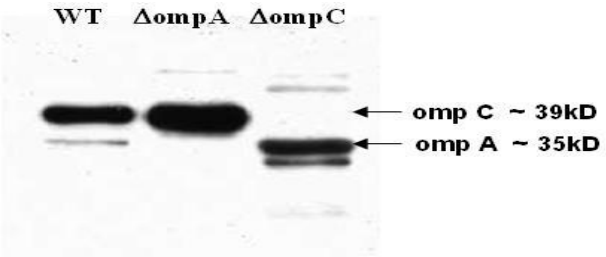


Fig.4.16 On blot Tf binding assay. The outer membrane proteins extracts shown in Fig.4.15 were electroblotted at 250 mA onto PVDF. The blots were then incubated with 1 μg/ml of Tf-HPR. In the WT samples Tf binds predominantly to OmpC and very weakly to OmpA. In the *ΔompA* mutant the Tf binds only to OmpC, whereas in the *ΔompC* mutant Tf binds only to OmpA. Tf seems to bind more selectively to OmpC, however in the absence of it binds to OmpA.

4.2.3.2 Tf and Lf binding assay

Transferrin and lactoferrin binding assays were performed in order to determine whether the absence of either OmpA or OmpC affected the *in vivo* binding of Tf and Lf to the *E. coli*. WT, *ΔompA* and *ΔompC* mutant strains were grown overnight at 37 ^{o}C in LB. The next day 1:100 dilutions of the bacterial cultures were transferred into fresh media and grown until exponential phase, around OD_{600} 0.6. The cultures were harvested by centrifugation, washed twice and re-suspended in SAPI-TRIS, pH 7.5 supplemented with 10 μg/ml Tf or Lf, or with no additions, in case of the control samples. All assays were then incubated for 1 hr at 37 ^{o}C, following which the cells were harvested by centrifugation and washed twice in PBS. The bacterial pellets were re-suspended in PBS and 2x SDS SB, boiled to lyse the bacterial and solubilise all the released proteins, and then run on a 12 % SDS PAGE gel. The gel was Western blotted, and immuno-probed as described in section 2.2.1.8, using anti-human transferrin, developed in goat or anti-human lactoferrin, developed in rabbit. The cross recognition secondary antibodies were respectively anti-goat IgG peroxiodase conjugated and anti-rabbit IgG peroxidase conjugated.

Fig. 4.17 shows the intensity of the binding of Tf to the WT and mutant *E. coli* strains (the Lf data is not shown but was similar to that shown for Tf). There was less binding of Tf to the *ΔompA* mutant than to the WT, and there was a further reduction in the *ΔompC* mutant. The western blots of the Tf and Lf binding assays were analysed using gel densitometry scanning. Fig. 4.18 shows the Volume OD* mm^2 of the bands on blots of the (a) Tf binding assay and (b) of the Lf binding assay. Using a Student T-test it was possible to determine that the difference in binding of Tf and Lf when comparing the WT strain and *ΔompA* mutant was not significant ($p>0.05$); whereas there was a

significant reduction in the binding of Tf and Lf to the *ΔompC* mutant ($p<0.05$) when compared to the WT, suggesting that Tf and Lf bind to OmpC to a greater extent than to OmpA.

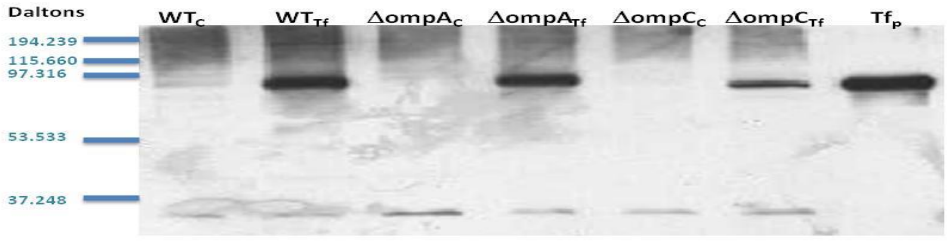
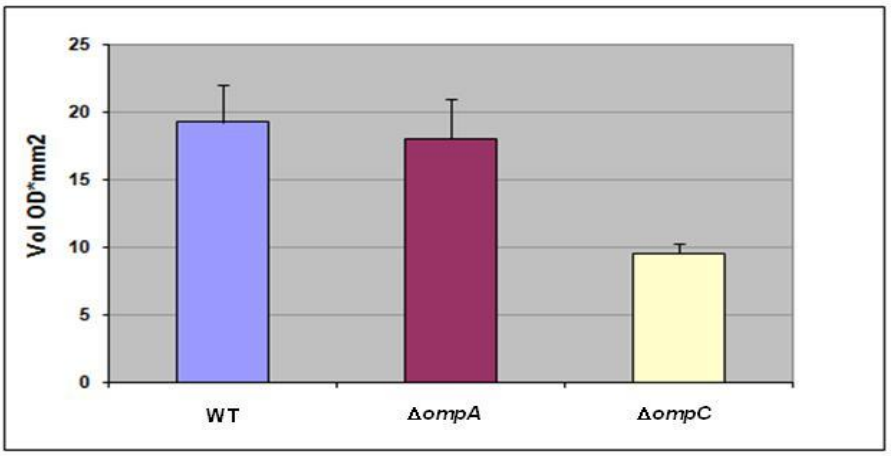
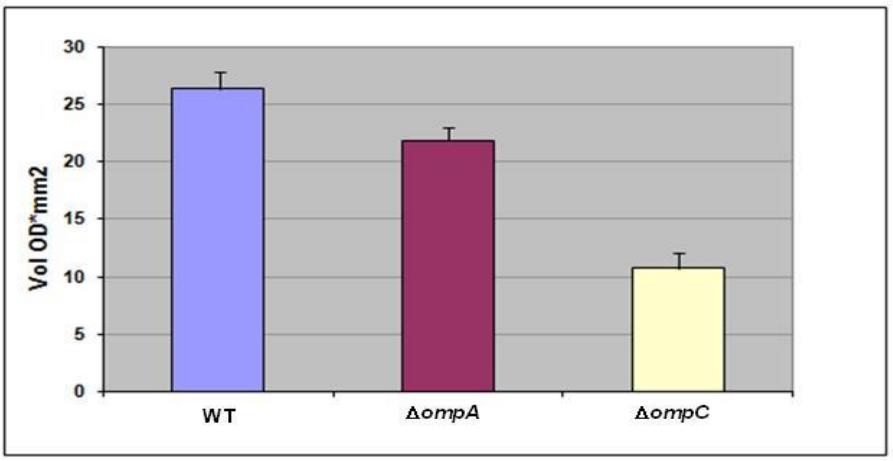


Fig.4.17 Tf binding assay. Analysis of the binding of Tf to *E. coli* was performed in WT and *ompA* and *ompC* mutants strains. The negative controls of the assay are the bacteria samples, both mutants and WT, in which the analysis conditions were maintained, however without the addition of Tf. Tf_p (holo human transferrin protein) was used as positive control (~80 kD). Western blot analysis was performed as described in section 2.2.1.8.



Tf 4.18a



Lf 4.18b

Fig. 4.18 Tf and Lf binding assay. The immunoblot in Fig. 4.17 was scanned using a GS-710 Densitometer connected to Quantity One 4.5.0 program that enabled determination of the area and intensity of the volume of the Tf-antibody recognised bands, allowing comparison between them. The diagrams show the Vol OD* mm^2 of the bands on blots of the (4.18a) Tf binding assay and (4.18b) of the Lf binding assay. The difference between the binding of Tf and Lf to the WT and the *ΔompA* mutant is not significant ($p>0.05$), whereas the binding of Tf and Lf to the *ΔompC* mutant is significantly lower ($p<0.05$).

4.2.3.3 Analysis of the OmpA and OmpC Mutants' growth characteristics in the presence or absence of catecholamines

After checking for the absence of OmpA and OmpC in the *E. coli* mutants and how this affected the binding of Tf and Lf to the bacteria, bacterial growth of the mutant was assessed in both the presence and absence of catecholamines.

The omp mutants and WT strains were grown overnight in LB or DMEM minimal media and next day diluted 1:1000 in fresh LB or DMEM and re-grown at 37 ^{o}C for 24 hrs. The OD was measured at A 600 nm and recorded every 15 minutes. Fig.4.19 shows the growth curves of the WT, *ompA* and *ompC* deletion mutant strains when cultured in LB. The pattern of the growth curves of the WT and *ΔompA* strains are similar when grown in either LB or DMEM (Fig.4.19 and Fig.4.20) though once the stationary phase was reached there is a slight decline in OD readings in the *ΔompA* mutant culture. In contrast, when grown in LB the *ΔompC* mutant showed an overall lower level of final growth, and reached stationary phase before the WT (Fig.4.19). As observed for the *ΔompA* mutant, once the stationary phase was reached the *ΔompC* mutant also appeared to undergo a decline in cell density faster than WT. When grown in DMEM minimal media the *ΔompC* mutant grew more slowly and reached a plateau at a lower OD_{600} than the WT or *ΔompA* strains, thought its final cell density was similar to WT (Fig.4.20).

A more accurate technique to assess the number of bacteria present within a culture is the viable (CFU/ml) count. Therefore, high (~10^4 CFU/ml) and low (~10^2 CFU/ml) inoculums of WT and *ΔompA* and *ΔompC* mutant bacteria were cultured in serum-SAPI media and grown statically for 16 hrs in 5 % CO_2 humidified incubator set at 37 ^{o}C. The next day viable counts of the cultures were carried out by making serial dilutions of the cultures and then plating onto LA plates. Fig. 4.21 shows that the final cell density of the cultures starting from a high inoculum of ~10^4 CFU/ml is not significantly different ($p>0.05$) for either the WT or mutant strains irrespective of whether they were grown in presence or absence of CAs or iron. In contrast, when starting from a lower inoculum of ~10^2 CFU/ml (Fig.4.22), a cell density more representative of an *in vivo* situation, the final cell density of both mutant cultures varied significantly from the WT when grown in presence of the various CAs. All cultures reached a similar final cell density when iron was used as growth inducer, indicating that the *ΔompA* and *ΔompC* mutants were able to withstand the osmotic

stress of growing in a high serum-containing medium. When comparing the WT negative control (no catecholamines) with the samples grown in presence of CAs, there was a significant increase in the final cell number ($p<0.001$) in the cultures grown in the presence of NE, Epi, Do and Iso. Comparison of the negative control of *ΔompA* mutant with the cultures grown in presence of catecholamines showed again NE and Do induced a significant increase ($p<0.001$) in final growth levels. In stark contrast to the WT, however there was a significant reduction in growth in the presence of isoprenaline ($p<0.05$). Epi also did not induce the growth the *ΔompA* mutant, and the final cell density of this culture did not differ significantly from the un-supplemented negative control ($p>0.05$). The growth responses of the *ΔompC* mutant to catecholamines generally followed the pattern of responsiveness shown by the *ΔompA* mutant. The *ΔompC* mutant grew significantly better in the presence of NE, and Do than the other catecholamines tested ($p<0.001$). In contrast with the *ΔompA* strain the *ΔompC* mutant did show growth induction by Epi, though this was consistently a log or more lower than the Epi-induced growth stimulation seen with the WT, although it must be noted that the final growth levels of the un-supplemented control cultures of the two *omp* mutant strains was also lower than the WT. The *omp* mutant's growth was particularly inhibited by Iso, again in marked contrast to the positive growth responsiveness to this catecholamine of WT E2346/89.

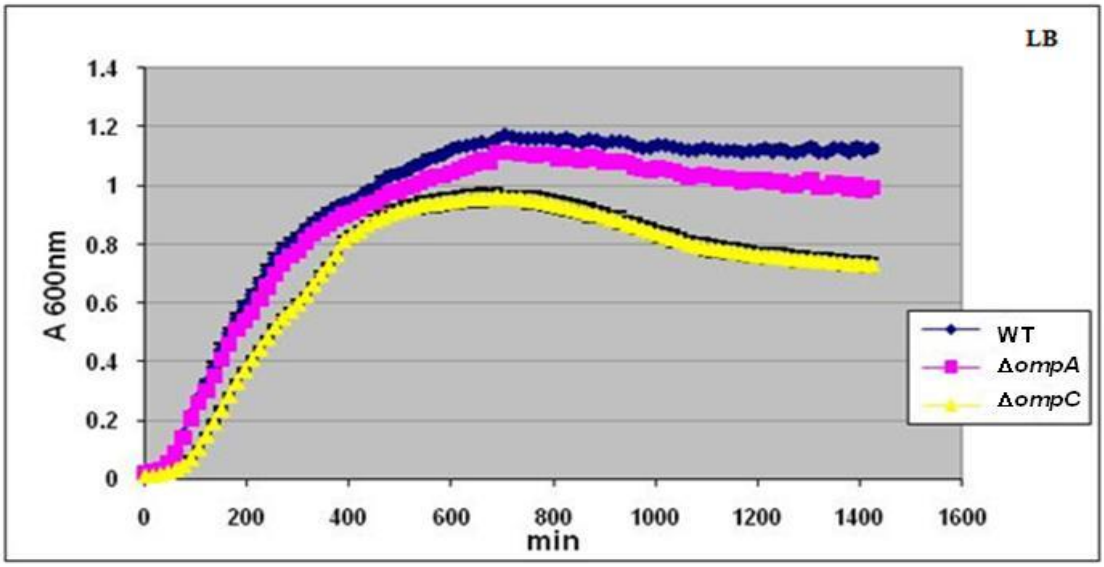


Fig.4.19 Growth characteristics in LB of *E. coli* WT and *ompA* and *ompC* mutants. Bacteria were grown overnight in LB and the next day diluted 1:1000 in fresh LB and incubated at 37 ^{o}C for 24 hrs. OD readings at absorbance of 600 nm were taken every 15 min. Blue: wild type; pink: *ompA* deletion mutant; yellow: *ompC* deletion mutant.

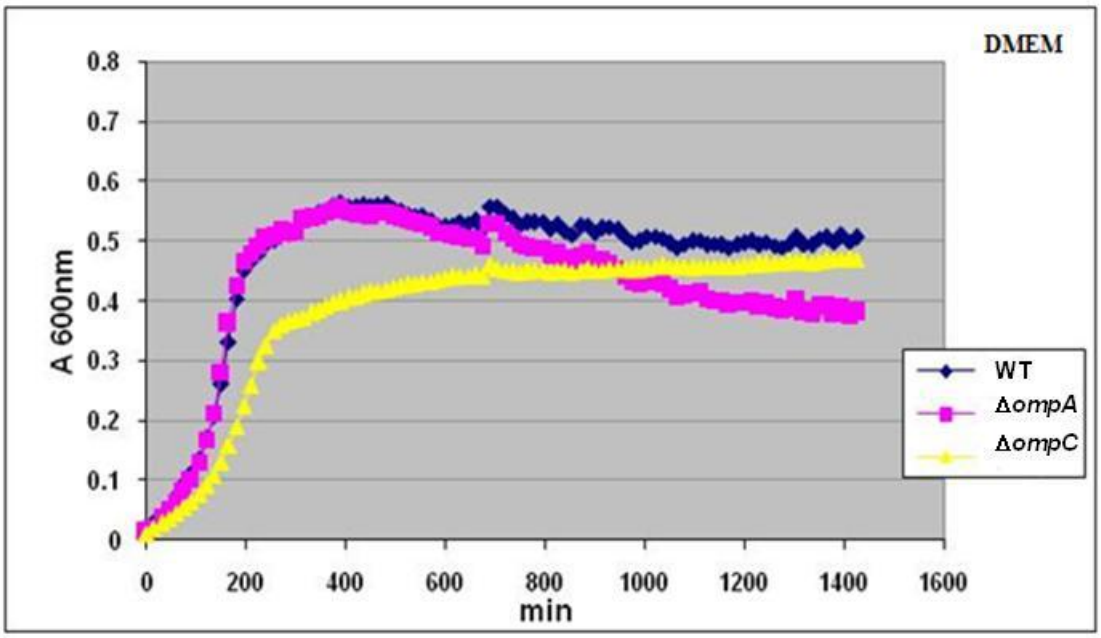


Fig.4.20 Growth characteristics in DMEM of WT *E. coli* and *ompA* and *ompC* mutants. Bacteria were grown overnight in DMEM, and the next day diluted 1:1000 in fresh DMEM and incubated in a Varioskan spectrophotometer at 37 ^{o}C for 24 hrs. OD readings at an absorbance of 600nm were taken every 15 min. Key: blue: wild type; pink: *ompA* deletion mutant; yellow: *ompC* deletion mutant.

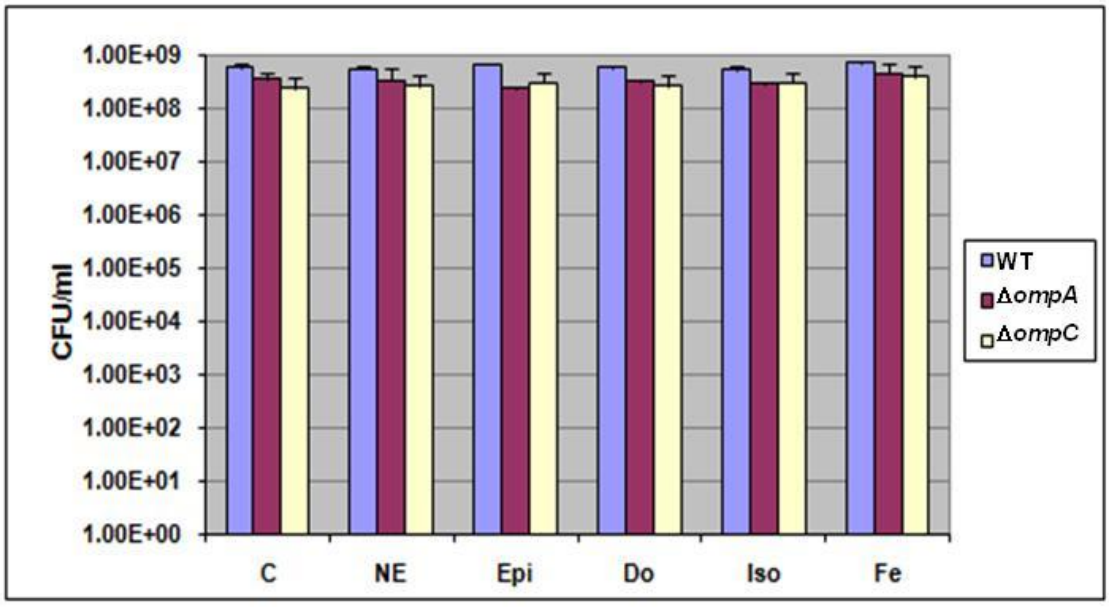


Fig.4.21 Growth responsiveness to catecholamines of high inoculums (~10^4 CFU/ml) of *E. coli* wt and *omp* mutants. Bacteria were grown for 16 hrs in serum-SAPI media at 37 ^{o}C in CO_2 incubator in presence or absence (C, negative control) of 100 μM CAs (NE, norepinephrine, Epi, epinephrine, Do, dopamine, Iso, isoprenaline or Fe ($Fe(NO_3)_3$ (a positive control). When starting with high cell density inoculum no significant ($p>0.05$) difference was observed between the WT and mutant cultures.

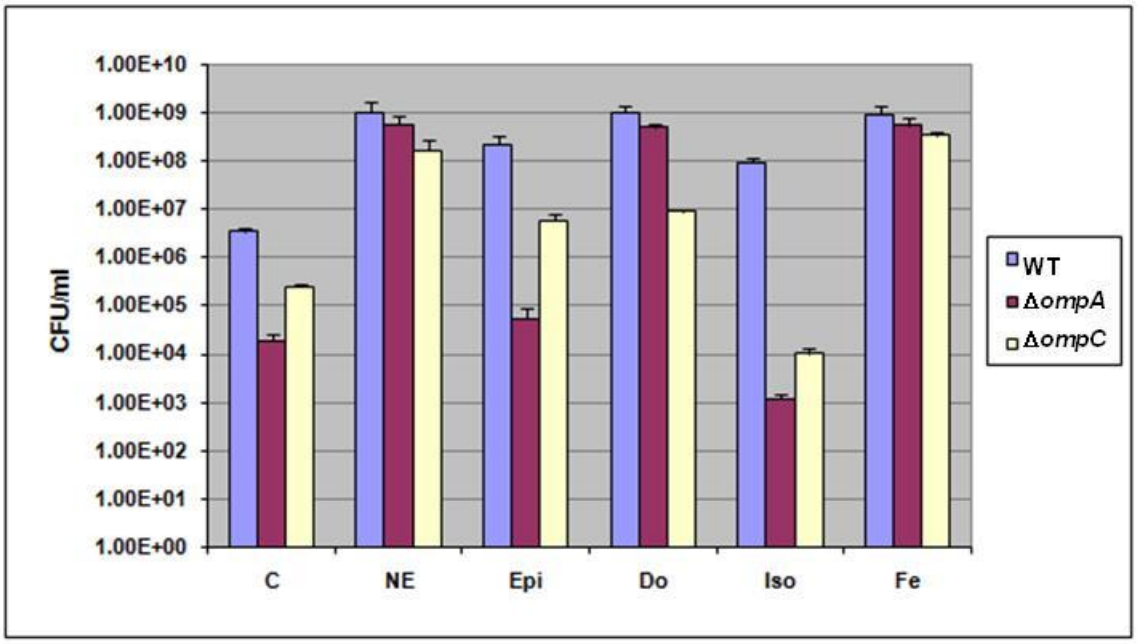


Fig.4.22 Growth responsiveness to catecholamines of low inoculum (~10^2 CFU/ml) of *E. coli* wt and *omp* mutants. Bacteria were grown for 16 hrs in serum-SAPI media at 37 ^{0}C in a CO_2 incubator in presence or absence (negative control) of 100 μM CAs or $Fe(NO_3)_3$ (positive control). There is significant ($p<0.001$) difference in the CFU/ml between the negative control and the samples grown in CAs in both WT and mutant strains for exception of the *ΔompA* specimen grown in Epi, which did not differ in final growth levels from the negative control ($p>0.05$).

4.2.3.4 ΔompA and ΔompC mutant uptake of Tf-complexed iron in presence and absence of catecholamines

Intracellular uptake of Tf-complexed iron by the *ΔompA* and *ΔompC* mutants was carried out using [^{55}Fe]Tf prepared using the methodology described by Freestone *et al*. (Freestone *et al.*, 2000). The bacteria were grown overnight in LB and the next day were diluted 1:100 and re-grown until they reached the stationary phase at OD_{600} of approximately 1.6-1.8. Such high inoculums (~10^8) were chosen in order to test the uptake of Tf-iron independently of any differences in growth rate. The WT and mutant cultures were washed in SAPI-Tris pH 7.5 and re-suspended in duplicate 2x 2 ml of fresh SAPI-Tris supplemented with 1 μg/ml of [^{55}Fe]Tf (2x 10^5 CPM) and incubated for 4 hrs in presence or absence of 100 μM NE. After the 4 hours the [^{55}Fe]Tf supplemented cultures were harvested by centrifugation and washed twice in PBS to remove any [^{55}Fe]Tf. The pellets were then re-suspended in 100 μl of PBS and 2 ml of Optiphase Safe scintillation fluid was added; radiolabel uptake was determined using the tritium channel of a scintillation counter. From Fig. 4.23 it can be seen that there was no significant difference between the WT and mutants samples

when incubated in absence of NE ($p>0.05$), whereas addition of NE resulted in increased uptake of [^{55}Fe] for all 3 strains ($p<0.05$), though the final magnitude of the incorporated [^{55}Fe] varied considerably between mutant and WT. The uptake of [^{55}Fe] in the *ompA* mutant in the presence of NE was significantly less than that of either the WT or *ompC* mutant. Uptake of [^{55}Fe] by the *ompC* mutant grown in the presence of NE was moderately reduced compared with WT. Collectively, this suggests that OmpA is more important than OmpC in mediating uptake of catecholamine-released iron from Tf.

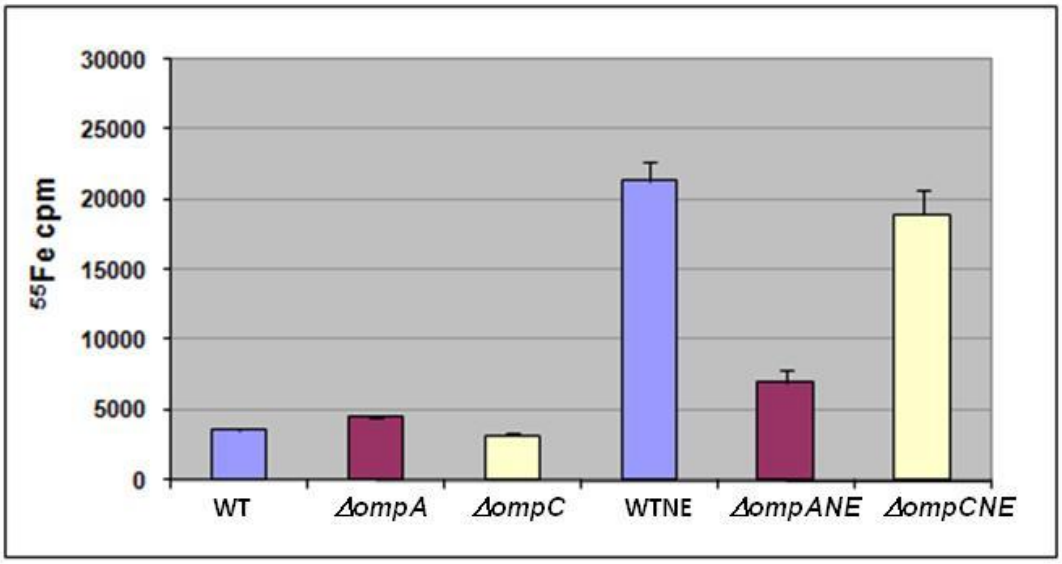


Fig.4.23 Acquisition of intracellular ^{55}Fe. High inoculums (~10^8 CFU/ml) of WT, ompA and ompC mutant strains were incubated for 4 hrs with [^{55}Fe] associated with Tf. The uptake of [^{55}Fe] by the WT and mutant strains was determined using scintillation counting as described in section 2.2.3.1. There was no significant difference in ^{55}Fe uptake between the WT and mutants samples when NE was absent ($p>0.05$), whereas addition of NE resulted in increased uptake of [^{55}Fe] for all 3 strains ($p<0.05$), though the amount of ^{55}Fe incorporation was less in both mutant strains, particularly *ΔompA*.

4.3 SUMMARY

1. *ΔompA* and *ΔompC Escherichia coli E2348/69* mutants were created and characterised
2. Both OmpA and OmpC are capable of binding to Tf and Lf, although both host iron binding proteins seem to bind predominantly to OmpC
3. The OmpA porin could be a candidate entry point for CAs into *E. coli*
4. Collectively OmpA seems to be more important than OmpC in mediating uptake of catecholamine-released iron from Tf

4.4.1 Deletion mutation troubleshooting: *ompA* gene

The mutation strategies adopted were aimed at obtaining non-polar deletion mutations and complete removal of the *ompA* and *ompC* genes. Non-polar mutations were used to minimise effects on the expression of surrounding genes. The Δ*ompA* mutant was a good example of a non-polar mutant since the gene was completely removed leaving only in-frame the start and stop codon within the 6 base pairs of the restriction site of *Xba*I (Fig.4.24).

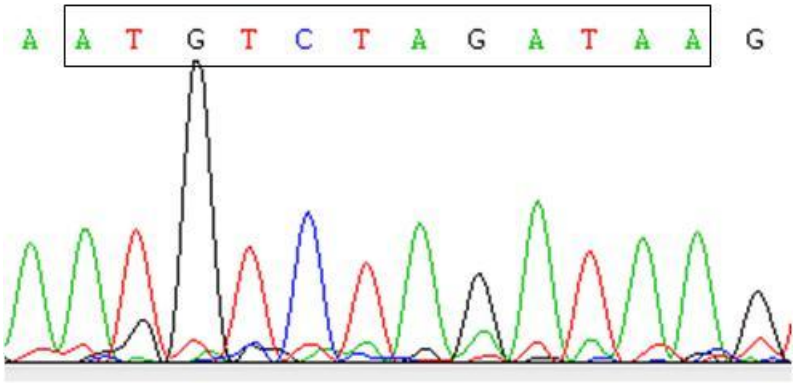


Fig.4.24 Chromatogram obtained from sequencing the *ΔompA* mutant using ompA_F1 primer. Highlight within the box, are the start (ATG) and stop (TAA) codon between the 6 base pair of the *Xba*I restriction site (TCTAGA). It is possible to observe that the mutation is in frame.

Although the deletion mutation of the *ompA* gene was eventually obtained, initially some difficulties were encountered due to the fact that the region upstream of the gene, which corresponded to the promoter region originally amplified by the ompA_F1 and ompA_R1 primer pairs, when expressed in a high copy number plasmid such is puC19, seemed to have deleterious effects on the bacteria. When the primers were initially checked by PCR, there was no sign that these primers would amplify other regions of the *E. coli* chromosome since there was only one PCR product visualised by agarose gel (Fig. 4.2). However, there was another PCR product that must have had low homology since its concentration must have been below the detection limit of ethidium bromide (< 1-5 ng). Despite this, both the expected and unexpected DNA inserts when ligated into puC19 vector should have

been successfully transformed. However, after transformation only plasmids carrying the unexpected sized insert were recovered by plate prepping the total transformed colonies (Fig.4.5). After sequencing the recombinant plasmids using puC19_F and puC19_R primers provided by PNACL, it was observed that the sequence of the downstream region initially amplified by ompA_F2 and ompA_R2 primer pair contained the correct fragment whereas the other half of the sequence was amplified from a completely different region of the chromosome (*yehE* gene). The detailed map of the *ompA* gene was retrieved from NCBI (Entrez gene → EcoGene) Fig.4.25, and shows that the reason that these mutants were not viable could be due to the presence in the promoter region of transcription factor binding sites (in brown), which in high copy number could interfere with the survival of the cell.

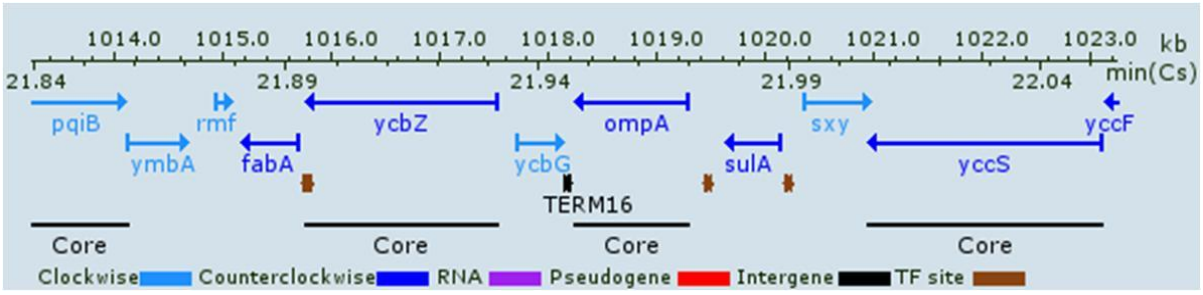


Fig.4.25 *ompA* gene's map in *Escherichia coli* (K12). The detailed gene map of *ompA* was retrieved from a link found in the NCBI webpage search engine, EcoGene (http://ecogene.org/geneInfo.php?eg_id=EG10669). The map shows the position and direction of transcription (dark blue) the *ompA* gene in the *E. coli* chromosome and the presence of a transcription factor binding site (in brown) in the promoter region of the gene. The gene map belongs to the *Escherichia coli* K12 species because the genomic DNA sequence of *E. coli* E2348/69 strain have just been assembled and become available at the Wellcome Trust Sanger Institute website, although it has not been released and it does not yet have any gene annotation (ongoing gene project). By database screening the DNA sequence, which included the1000 bp region upstream and downstream of the *E. coli* K12 *ompA* gene, against the E2348/69 genome, it was possible to see that the sequence of the *ompA* gene and the promoter region are highly conserved in *Escherichia coli* species (although the upstream and downstream genes may vary).

Cloning and sequencing analysis (Movva *et al.*, 1980b) of the regulatory region upstream of the *ompA* gene showed that this region comprised the promoter region, the 5'-untranslated region and the region corresponding to the signal peptide to this secretory protein. The promoter region had been shown to be highly homologous to the promoter region of the *lac* and *gal* genes, as the sequences of the 'Pribnow box'

and the RNA polymerase recognition site are almost identical. Since the puC19 plasmid is carrying the *lacZ* gene, the similarity of these promoters and their binding sites could have interfered with the survival of the bacteria carrying the recombinant plasmid by altering the normal expression pattern of the cell. In the region of about 140 bp from the start codon of *ompA*, there is an additional start codon of the elongated OmpA precursor protein, which constitutes the peptide extension at the NH_2- terminal end of OmpA which functions as signal peptide in protein secretion across the membrane (Movva *et al.*, 1980a). Two more start codon were identified, which lead to the synthesis of a pentapeptide and a heptapeptide and they all share a common ribosome-binding site (Movva *et al.*, 1980b). Over-expression of these molecules could have contributed to the detrimental effect on the bacteria that are carrying high copy numbers of the upstream region of the *ompA* gene within the puC19 plasmid. However, when the mutation construct was ligated directly into the low copy number plasmid pRDH10 and transformed, the *ΔompA* mutant bacteria were eventually obtained.

4.4.2 Deletion mutation troubleshooting: *ompC* gene

A similar problem to the one observed while trying to obtain the *ΔompA* mutants (section 4.4.1) was encountered when making the deletion mutation of the *ompC* gene, although the presence of an additional copy of the upstream region of this gene seemed to be deleterious even when expressed in a low copy number plasmid such as pRDH10.

Using the EcoGene database, it was possible to retrieve the detailed map of the promoter region of *Escherichia coli ompC* gene. From Fig.4.26 it is possible to observe that the upstream region of the *ompC* gene is much more complex from a regulation point of view than the *ompA* gene, and comprises several transcription factor binding sites which could together, even in a low copy number plasmid, compromise the survival of the bacteria carrying the recombinant plasmid. The expression of the *ompC* gene is affected by several environmental stimuli such as osmolarity, pH, nutrient availability, temperature and presence of toxins (Batchelor *et al.*, 2005). The regulation of this gene is very complex and involves the interaction of several regulatory compounds such as the two components systems EnvZ-OmpR (Maris *et al.*, 2005) and CpxA-CpxR (Batchelor *et al*., 2005), the small RNA micF

and the global regulator leucine-responsive regulatory protein (Lrp) (Ferrario *et al.*, 1995). As can be seen in Fig.4.27 several regulatory components bind the region upstream the *ompC* gene and included in the mutation construct of the upstream region of the gene are the binding sites of Lrp, OmpR and CpxR. Some of these proteins are not only involved in the regulation of *ompC* gene but are also involved in the regulation of other genes. For example, Lrp is known to participate in the control of expression of over 40 polypeptides (Calvo *et al.*, 1994)) involved in the regulation of the amino acid synthesis, pilin biosynthesis, maintaining the chromosomal structure and organisation and regulation of transport proteins such as OmpC and OmpF (Ferrario *et al.*, 1995). The OmpR and CpxR proteins are involved in the regulation of several genes in response to environmental osmolarity whose functions range from regulation of transport proteins (i.e. OmpC and OmpF) (Maris *et al.*, 2005) to regulation of virulence factors (i.e. curli fibers) (Jubelin *et al.*, 2005). The presence of these DNA binding sites for these global regulator proteins in the upstream fragment of the *ompC* deletion construct seem to give a plausible explanation for the lack of survival of the bacteria carrying the recombinant plasmid, probably due to a general unbalance of the bacteria's physiology.

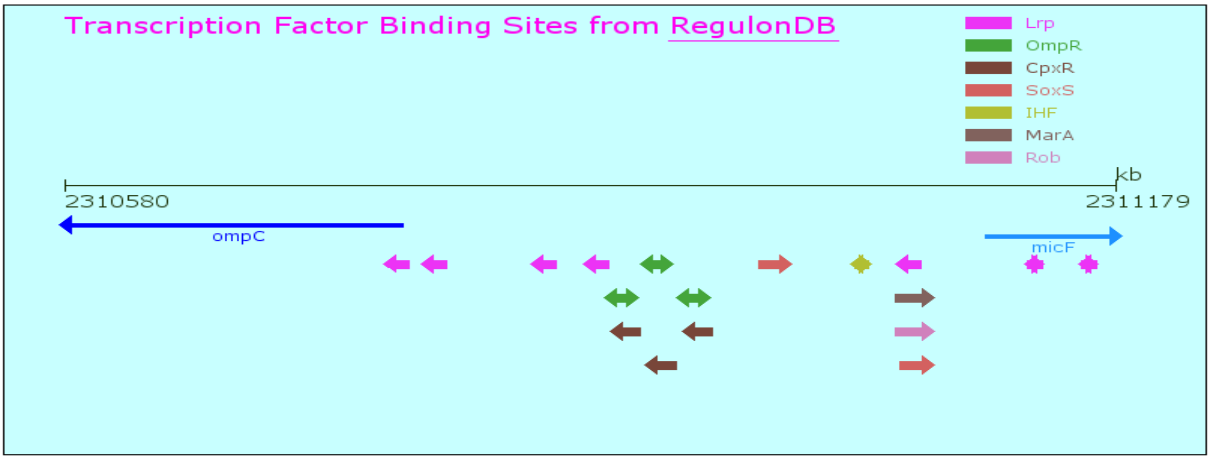


Fig.4.26 Promoter region map of *Escherichia coli* K12 *ompC* gene. The detailed promoter region map of *ompC* gene was retrieved from a link found in the NCBI webpage search engine, EcoGene (http://ecogene.org/regulon.php?eg_id=EG30063). The map shows the position and direction of transcription (dark blue) of the *ompC* gene in the *E. coli* K12 chromosome and the transcription factor binding sites in the promoter region of the gene. The gene map belongs to the *Escherichia coli* K12 species because the genomic DNA sequence of *E. coli* E2348/69 strain has just been assembled and made available at the Wellcome Trust Sanger Institute website, although it has not been released and it does not as yet have any gene annotation (ongoing gene project). By comparing the DNA sequence of the upstream region of the gene within the ompC_F1 and ompC_R1 primer pair of E2348/69 against the *E. coli* K12 genome it was possible to see that the DNA sequences were conserved between the species.

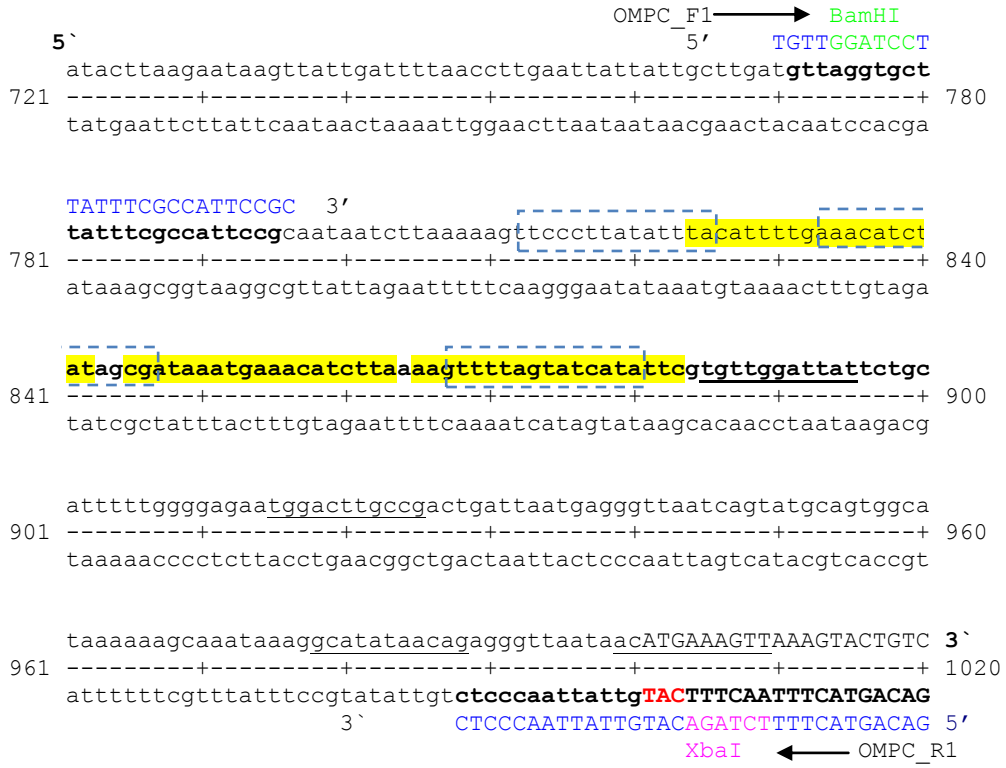


Fig.4.27 Upstream sequence of the *ompC* gene within the ompC_F1 and ompC_R1 primer pair. The DNA binding sites of the upstream region of the *ompC* gene within ompC_F1 and ompC_R1 are labelled as follows: underlined in black with single line (DNA binding sites of Lrp); highlighted in yellow (DNA binding sites of OmpR); contained within the dashed boxes (DNA binding sites of CpxR).

A new approach for obtaining the deletion of the *ompC* gene was therefore necessary and the methods adopted involved the use of the phage λ-red recombinase system, where linear PCR products provided the homologous template for recombination with the target gene, thus overcoming the problem of expressing the recombinant plasmid encounter with the above mentioned system. However, since bacterial genes are recurrently present in clusters or operons, the insertion of transposon or antibiotic resistance cassettes could affect transcription of the downstream genes resulting in the formation of polar mutants, with different phenotypes compared to the deletion non-polar mutants (Link *et al.*, 1997). To overcome this possible effect on downstream genes, a 'non-polar' antibiotic resistance gene cassette was used for the disruption of the *ompC* gene, which has no promoter or terminator sequences and lays between the start and stop codon of the *ompC* gene maintaining downstream transcriptions and translations. Thus, using this mutation technique, eventually the *ΔompC* mutant was obtained.

4.4.3 Membrane protein profiles of ΔompA and ΔompC mutants

Using SDS-PAGE analysis, it was possible to observe from the resulting membrane protein profiles that the OmpA and OmpC bands were absent in *ΔompA* and *ΔompC* bacterial mutant strains, respectively (Fig.4.15). Interestingly, in the absence of either *ompA* or *ompC* the mutant bacteria over-expressed either OmpC or OmpA respectively, presumably to supplement the requirement for transport and structural systems (Fig.4.15). This possibility was reinforced as examination of the membrane protein profile of the *ΔompA* mutant strain showed an extra protein, which was not present in the WT and the *ΔompC* mutant (Fig.4.15, band 1) that corresponded to an accumulation of the outer membrane protein C precursor, confirming that the cells were overproducing OmpC. Conversely, from the membrane protein profile of the *ΔompC* mutant there was also an extra band (Fig.4.15, band 2) that corresponded to the outer membrane protein A precursor, again confirming that the absence of the OmpC proteins in this mutant strain was overcome by the production of additional OmpA porins. In addition, the protein bands corresponding to elongation factor Tu and the 50s ribosomal protein (Fig.4.15 bands 3 and 5) were each found to be more highly-expressed or altogether present in only in the *ΔompC* strain, as can be seen by comparison of the outer membrane protein profile of this mutant with the one of the WT and *ΔompA* strain. Although these proteins are obviously a contamination from the cytoplasmic protein fraction, their presence could suggest that the cells were actively synthesising outer membrane protein A precursor in much higher amounts than in the WT strain to overcome the lack of OmpC, which instead of a single pore protein, is a trimeric porin and thus the requirement of more OmpA porins (possibly at a ratio to 3:1) to replace a single OmpC. Although this reasoning is very conjectural, the fact remains that these proteins, in order to have been recovered in the outer membrane protein fractionation, must had been present in the cytoplasmic fraction of the *ΔompC* mutant strains in greater concentration than in the WT and *ΔompA* mutant strain, since the membrane preparations were performed in parallel and followed identical procedures from similarly grown overnight cultures. This hypothesis could also be supported by the observation of a further difference in the outer membrane protein profile of *ΔompC* mutant, in which the protein band (Fig.4.15 band 4) corresponding to the major outer membrane lipoprotein precursor was absent. Both OmpA and the lipoprotein precursors are exported from the

cytoplasm to the periplasmic space and the outer membrane in a Sec machinery-dependent manner (Hayashi *et al.*, 1985; Matsuyama *et al.*, 1993; Yakushi *et al.*, 1997) . However, accumulation of pre-OmpA proteins precede that of pro-lipoproteins (Hayashi *et al.*, 1985), thus, if indeed the concentration of OmpA precursor proteins was high enough, this could have caused a 'traffic jam' in the system and interfered with the translocation of lipoprotein precursors. The idea of a traffic jam in the Sec system has been previously suggested by Matsuyama *et al.* (Matsuyama *et al.*, 1993) during their investigations into the role that SecD played in the translocation of proteins such as OmpA and maltose-binding proteins (MBP) across the cytoplasmic membrane in which they observed that the accumulation of precursor proteins was the result of a traffic jam caused by the stacking of the non-released mature proteins. In a similar fashion the over-expression of OmpA proteins could have caused a blockage in the *Sec* translocation system.

4.4.4 Phenotypic characterisation of OmpA and OmpC and their role in the bacterial catecholamines' response mechanism

After obtaining the *ΔompA* and *ΔompC E. coli E2348/69* mutants, several phenotypic characterisations were performed to understand the role that these proteins play in the bacterial response to stress hormones.

4.4.4.1 OmpA and OmpC bind to Tf and Lf

The Δ*omp* mutants allowed the confirmation that indeed both Tf and Lf were binding to OmpA and OmpC (See Chapter 3 for discussion), although in the presence of both porins, OmpC was significantly favoured as the TfBp and LfBp. The assay of Tf and Lf binding to the WT and *ΔompA* and *ΔompC* mutant strains (Fig.4.18) showed that the absence of OmpA did not affect significantly the binding of these proteins to the bacteria, whereas the absence of OmpC caused a sizeable reduction in the ability of the cells to bind both Tf and Lf. This difference in binding may be due to the fact that OmpC forms three structurally stable and rigid pores whose conformation seems to favour binding (although the nature of the bond is not clear) (Nikaido *et al.*, 1981; Nikaido, 1996) whereas OmpA is a multifunctional protein and although it can function as a channel, exists in two alternative conformers, which form open and closed pores (Sugawara *et al.*, 1994). Since a portion of the OmpA pores are present on the bacterial cells as closed channels maybe this conformation

does not allow the binding of Tf and Lf either due to the obstruction of the binding site, or simply to the stereotypic impediment that this closed conformation poses. However in the *ΔompC* mutants, which lack the OmpC transport channels and whose function seems to be replaced by OmpA pores, it is possible that there is an increase in the proportion of the open pore stereoisomers in the outer membrane of the bacteria that allows some binding of Tf and Lf. Collectively from these results (Fig. 4.16-4.18), it seems that although OmpA can bind both Tf and Lf, it does not seem to play a major role in the binding of these proteins when OmpC is expressed in the outer membrane of *Escherichia coli*. Another reason for the bacteria to favour OmpC over OmpA as Tf and Lf binding protein, can be given by examining previous studies (Caccavo *et al.*, 2002; Eda, *et al.*, 1996) on the ability of Lf to bind to human monocytic cell leukaemia cell line THP-1. Eda *et al.* (Eda *et al*., 1996) documented an increase in Lf binding to the cells as they mature into macrophages. Scatchard analysis of the [^{125}I]-Lf binding to THP-1 indicated the presence of several high and low affinity Lf binding receptors. Since it has previously been found that Lf can bind to host cells via multiple receptors possessing different affinities, by extension it is possible that *E.coli* might also express more than one Tf/Lf binding protein. This could also explain the Tf/Lf binding assay was performed in the control and mutant strains there was only a slight ($p>0.05$) decrease in the Tf/Lf binding ability of the mutant lacking OmpA (a low affinity receptor) compared with the control strain, whereas the absence of OmpC (a high affinity receptor) causes significant ($p<0.05$) reduction in the ability of the bacteria to bind to Tf and Lf.

4.4.4.2 OmpA: Possible entry point for the CAs?

The role that OmpA seems to play in the mechanism of direct bacterial response to stress hormones is as a possible entry point for the internalisation of catecholamines such as NE and Do (see section 1.5, Fig.1.4 for the CA structures) or as receptor specific recognition site for growth induction of these CAs. In fact, when low *E. coli* inoculums (to be reflective of the numbers of bacteria likely to be present at the start of an infection) of *ΔompA* mutants were grown in serum-SAPI in presence or absence (control) of CAs, it was possible to observe that the growth of the bacteria supplemented with NE and DO was significantly higher ($p<0.001$) than the control sample (and so similar to the response seen in the WT strain). In contrast, while Epi stimulated log-fold increases in growth of the WT strain, it had much less effect on

the growth of the *omp* mutants ($p>0.001$). This significant reduction in catecholamine growth induction was even more pronounced with the larger-structured catecholamine Iso. The OmpA and OmpC porins show some size selectivity, favouring hydrophilic molecules of less than 600 Da; it is therefore probable that due to the bulky chemical side chains of Epi and Iso that these were the site of entry into the bacteria.

The uptake of Tf-complexed iron was also reduced ($p<0.05$) in the *ΔompA* strain compared with the WT and *ΔompC* mutant strains. Previous studies (Freestone *et al.*, 2000) had shown that for *E. coli* grown in SAPI medium supplemented with either [^{55}Fe]Tf/Lf in presence or absence (control) (+/-) of NE or [^{55}Fe]Lf +/- NE, or serum-SAPI +/- [^{3}H]NE, that NE-stimulated growth was accompanied by simultaneous uptake of both [^{55}Fe] and [^{3}H] into the bacterial cell. Detection of radioactivity in cytoplasmic fraction of the bacteria showed that both [^{55}Fe] and [^{3}H]NE were internalised in the cytoplasm of these cells suggesting that one of the mechanism by which NE could provide iron for bacterial growth is by acting in a siderophore-like fashion. However, in the current study since only [^{55}Fe] uptake was assessed and not [^{3}H]CAs, it is not possible to confirm whether the CAs were internalised in the omp mutants without testing this hypothesis first. As already mentioned above (Freestone *et al.*, 2000) internalisation of NE into the bacterial cytoplasm as been previously detected, and later studies (Freestone *et al.*, 2007a) showed that α-adrenergic antagonists were able to block NE- and Epi- induced bacterial growth, whereas D_2-dopaminergic antagonists were able to block growth induction mediated by DO, although increase of CAs concentration reduced the effect of the antagonist. These observations suggest that CAs-induced growth inhibition is competitive and that there is evidence for the existence of either α-adrenergic and dopaminergic CAs uptake systems or receptors for initial recognition of CAs. Freestone *et al.* (Freestone *et al.*, 2007a) also observed that although the antagonists blocked CAs responsiveness, the internalisation into bacterial cells of ^{3}H-NE was never completely blocked, suggesting that there could be more than one entry point for CAs. Thus, OmpA could be one of the entry points for larger CAs, although more tests need to be carried out to confirm this.

NE, DO and Epi all enhanced the growth of the *ΔompC* mutant when compared with the WT strain, although the general growth responsiveness to the CAs in the *ΔompC* mutant seems to be weaker than in the WT strain. However, examination of

the growth curves for this mutant in various culture media showed that it grows less effectively than the WT strain (Fig.4.19-4.20). However, in serum-SAPI medium both the *ΔompA* and *ΔompC* strains reached a similar final cell density to their WT parent when iron was used as the growth inducer, indicating that the *ΔompA* and *ΔompC* mutants were able to withstand the osmotic stress of growing in a high serum-containing medium. This result also suggests that the overall differences in the magnitude of CA growth stimulation between the WT and the *ΔompC* could be explained by the reduction of Tf binding to the outer membrane of the mutant bacteria.

4.4.5 Future work

Deletion of the *ompA* and *ompC* genes was initially performed in order to determine whether or not their products were binding Tf and Lf. It has been shown that both OmpA and OmpC can bind to Tf and Lf. Moreover, preliminary analysis of the role that these proteins in the overall mechanism of CAs response to stress hormones have shown that OmpA and OmpC, may be playing a more complex an important role than only acting at Tf and Lf binding proteins. It would be interesting to investigate these mutants further, focusing on their responses to CA-induced growth, uptake and virulence factor production using a wider variety of CA inotropes, adrenergic and dopaminergic antagonists. It would be also very useful to create combinations of double mutants lacking both *ompA* and *ompC* (keeping in consideration that there is high possibility that the bacteria would not be able to survive a double porin deletion) or *ompA/C* and *entA* (gene responsible for the synthesis of enterobactin) or *OmpA/C* and *tonB* (gene responsible for active siderophore uptake) genes, which have been previously found essential for NE-stimulated growth in *E. coli* 0157:H7 (Freestone, *et al.*, 2003). This will provide a more definitive understanding of the specificity and the role that porins play in the recognition or uptake of CAs and Fe within the mechanism of the *E. coli* response to stress hormones. Finally, even though *omps* mutants were created using mutation strategies aimed to obtain non-polar mutants, and SDS-PAGE of membrane extracts clearly showed that the OmpC and OmpA proteins have been deleted, if time had allowed it would have been appropriate to confirm that indeed there were not polar or secondary effects by complementation of the WT gene.

Chapter 5 – Elucidation of the mechanism by which catecholamines remove iron from transferrin and lactoferrin

5.1 INTRODUCTION

Iron, has long been established as an essential nutrient for the growth of commensal and pathogenic bacteria alike. The human iron binding proteins Tf and Lf play an important role in the iron-withholding strategy of the innate immune defence (Ong *et al.*, 2006) by maintaining in the host an iron limited environment aimed at disfavouring the growth of invading microorganisms, whilst also preventing the toxic effects that free iron have on the host organism due to its ability to catalyse Fenton reactions to produce free radicals.

Previous studies (Freestone *et al.*, 1999; Freestone *et al.*, 2002; Freestone *et al.*, 2003; Lyte *et al.*, 1996a; Lyte *et al.*, 1997a; Lyte *et al.*, 1997b) have shown that CA hormones can enhance the growth rate of a variety of both pathogenic and commensal Gram-negative and Gram-positive bacteria by 10 million fold or more, as well as stimulate the expression of bacterial virulence factors such as *E. coli* 0157:H7 shiga toxin (Lyte *et al.*, 1996a).

Freestone *et al.*, (Freestone *et al.*, 2000) demonstrated that CAs such as EPI and NE can form complexes with the host iron binding proteins transferrin (Tf) and lactoferrin (Lf), facilitating iron removal from these proteins by a not yet fully characterised mechanism. The aim of this chapter is therefore to understand the mechanism/s that enable removal of iron from Tf and Lf by CAs. Techniques that have been used include EPR, ferrozine-ferrous iron binding studies and urea gel analysis.

5.2 RESULTS

5.2.1 EPR analysis of Tf and NE

In order to confirm the binding of catecholamines to Tf- associated Fe, EPR analysis of transferrin-catecholamine complex formation was performed in collaboration with Dr. Jonathan Woodward and Remya Muralikuttan, from the Department of Chemistry, University of Leicester.

EPR is a technique that enables the study of chemical species with one or more unpaired electrons. The rationale behind the use of EPR is that electrons are charged particles, which spin in a circular motion creating a magnetic field, thus every electron has a magnetic dipole moment and spin quantum number. In chemical species with paired electrons the total spin angular momentum is zero, whereas compounds with unpaired electron are susceptible to magnetic fields and thus generate specific signals that can be detected by EPR analysis. Since the Fe in Tf is paramagnetic it will produce a specific signal detectable by an EPR spectrometer.

Norepinephrine (NE) was chosen as the test catecholamine because of its widespread distribution in the human body and involvement in the mammalian stress response, and because reports describing the interaction of stress hormones with Tf have largely focussed on this catecholamine. Tf-NE samples for EPR analysis were prepared by mixing 6 mg/ml of holo-Tf with (or without in the case of the control sample), several concentrations of catecholamine ranging from 6 mM to 0.3 mM buffered in 50 mM Tris-HCl, pH 7.5, after which, samples were immediately frozen in liquid nitrogen and analysed using a JEOL-RE1X EPR Spectrometer. EPR analysis parameters were: centre field: 150 mT' sweep width: 80 mT, field modulation: 1.0 mT, microwave power: 10 mW, time scan: 14 min and the reaction was conducted at a temperature of 77 K

Fig. 5.1 shows the typical double peak EPR signal of di-ferric transferrin at g-value of 4.3 (150 mT field strength), which is universally accepted as the signature spectrum of iron-replete holo-Tf (Hoefkens *et al*., 1996) even though the reason for this double peak has not yet been fully explained. Fig. 5.2a and 5.2b show that after the addition of NE to di-ferric Tf the double peak EPR signal is lost and a new single peak EPR signal is produced.

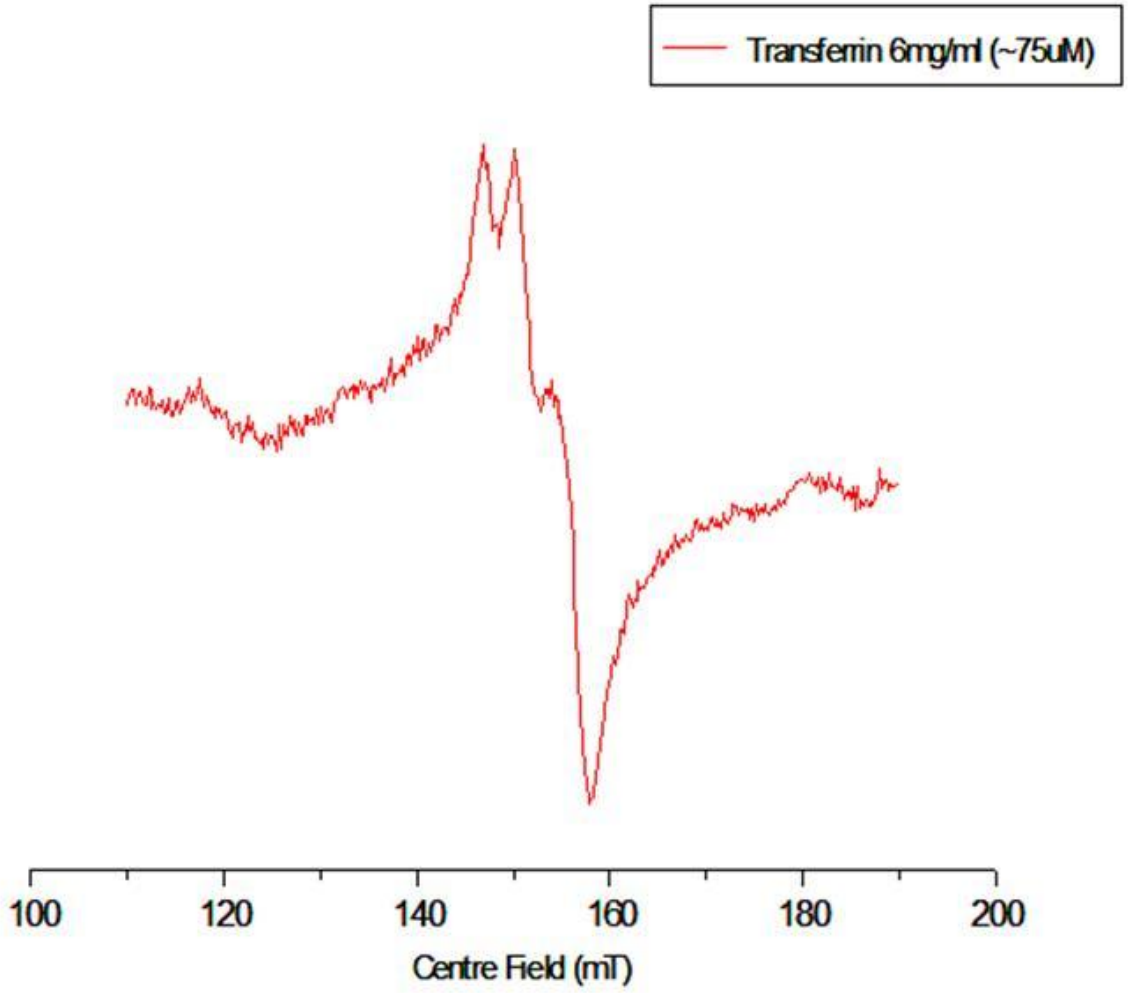
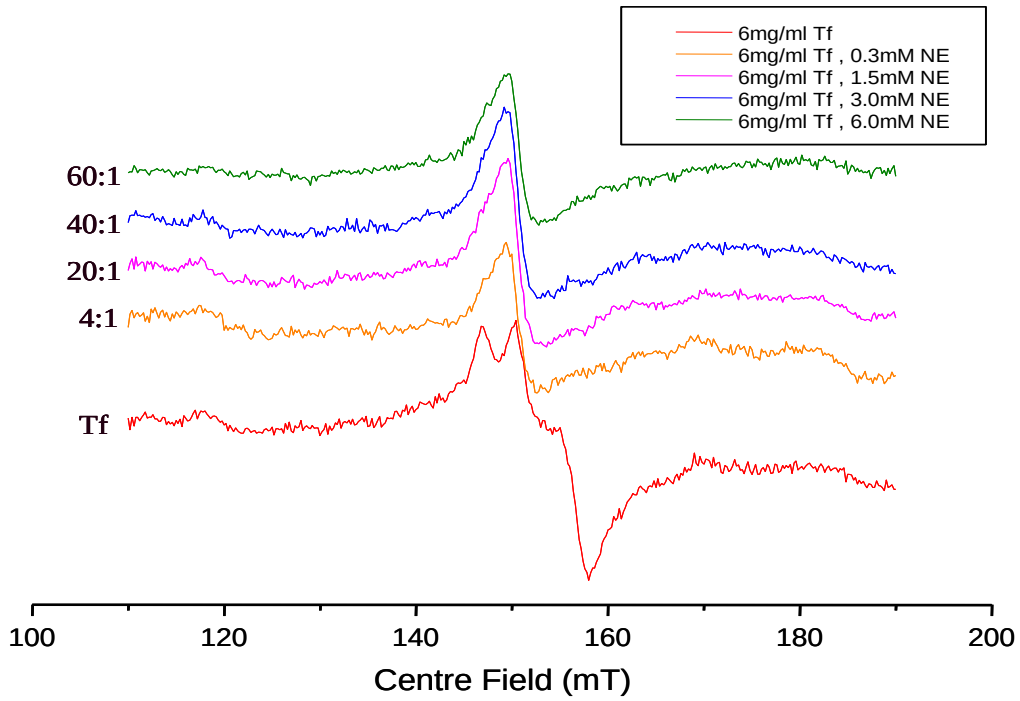


Fig. 5.1 EPR spectrum of Fe associated with human holo-Tf. The red trace shows the characteristic double peak EPR signal of holo-Tf; the Tf shown has a g-value of 4.3 (150 mT). (Borisenko *et al.*, 2000; Hoefkens *et al.*, 1996).

5.2a



5.2b

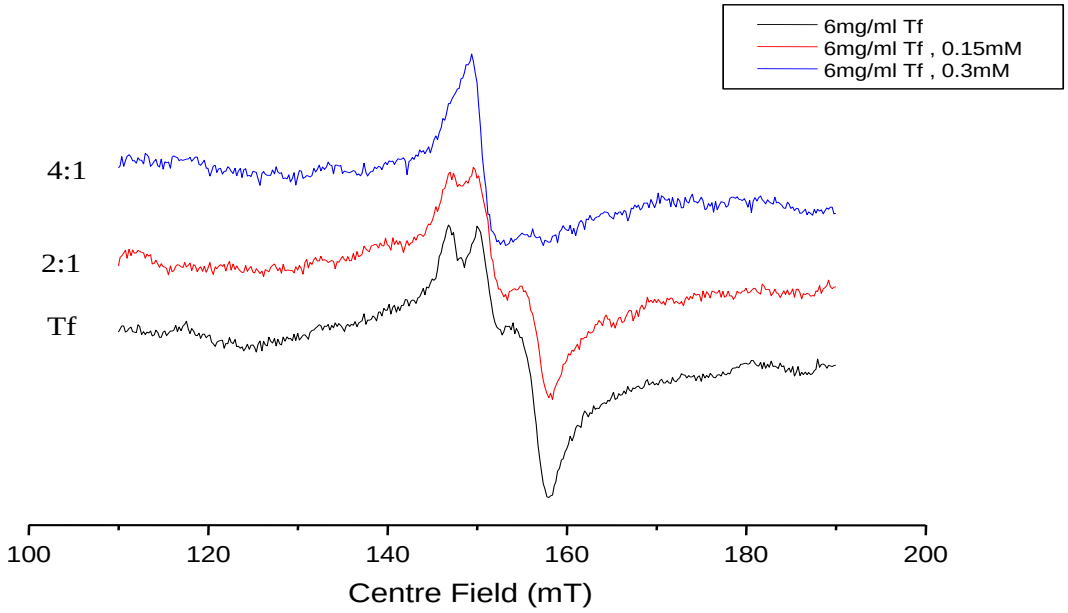


Fig. 5.2 EPR spectrum of Fe associated with human holo-Tf in presence and absence of NE. The traces in red (5.2a) and in black (5.2b) show the typical double peak EPR signature of Fe(III) associated with human di-ferric Tf. The addition of NE (5.2a, 5.2b) at the concentrations shown resulted in the loss of the double peak EPR signal and appearance of a single peak EPR signal. The minimal concentration ratios of NE:Tf necessary to switch to a single peak EPR signal is 4:1. However, it was possible to observe an intermediate peak with lower NE:Tf ratio of 2:1 (red spectrum, 5.2b).

To catalyse the switch from a double to single peak, a minimal molar ratio 4:1 of NE:Tf was found to be necessary. However, the addition of a lower concentration of NE, 0.15 mM, with NE:Tf ratio of 2:1, enabled the visualisation of what appeared to be an intermediate peak. It is interesting to note that the disappearance of the typical holo-Tf EPR signal and the appearance of the single peak on the Tf EPR spectrum was complete by the time the Tf-NE samples were prepared for analysis (approximately 5 minutes), which suggests that the effect of NE on the Fe associated with Tf was very rapid. This is in contrast with previous urea gel analysis that suggested catecholamine-mediated removal of iron from Tf required hours of incubation (Freestone *et al.*, 2000; Freestone *et al.*, 2002; Freestone *et al.*, 2003).

Since it had been indirectly shown that catecholamines can bind Tf-complex iron (Freestone *et al*., 2000; Freestone *et al.*, 2002; Freestone *et al.*, 2003) we decided to investigate the EPR signal of inorganic ferric and ferrous iron in the presence or absence of NE, in order to determine whether the single peak EPR signal was due to the binding of these two compounds.

The spectra in Fig.s 5.3 and 5.4 show that the addition of NE to both ferric and ferrous iron salts produced an EPR-detectable signal that peaked at 150 mT, a g value very similar to that obtained when the human holo-Tf was incubated with NE. This seems to indicate that Fe in both its ferric and ferrous states can form a complex with NE, and that the EPR signal formed when NE is added to Tf was due to the catecholamine directly binding the Tf-complexed Fe. An interesting difference between the interaction of NE with Tf-complexed iron and iron free in solution is the rate at which the NE-Fe complex was formed. When iron was complexed with Tf the formation of the NE-Fe complex was very rapid and effectively complete by the time the EPR analysis commenced. However, the rate of NE complex formation with free iron was much slower in comparison, and the signal strength of the resulting EPR peak also increased over time. This suggests that the NE-Fe complex formed when the iron is bound to Tf is probably facilitated by internal structural elements within the Tf iron-binding domain.

Although Fig.s 5.2-5.4 showed that NE can form a complex with both organic and inorganic iron, it is still not clear how NE can subtract the iron from Tf, since the affinity of Tf for Fe(III) is very high (Tf binds ferric iron with association constant of ~ 10^{22} M^{-1}). In contrast the affinity of Tf to Fe(II) is very low, and since

catecholamines can reduce inorganic iron, we used ferrozine (a ferrous iron binding dye) and urea-acrylamide gel electrophoresis analysis, to determine whether CAs were catalysing iron removal from Tf by reducing the high affinity Fe(III) to low affinity Fe(II).

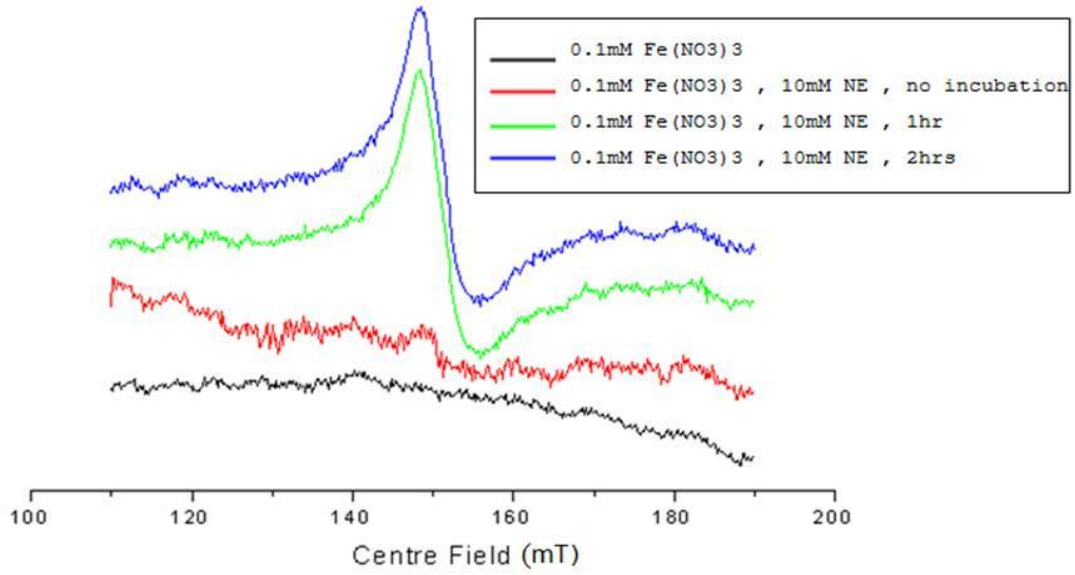


Fig. 5.3 EPR spectrum of inorganic ferric iron in presence and absence of NE. The traces in black show that there is no EPR signal of ferric iron without the presence of NE. The traces in red, green and blue show a single peak at 150 mT indicative of Fe presumably complexed with NE, after 0, 1 and 2 hrs incubation respectively at room temperature.

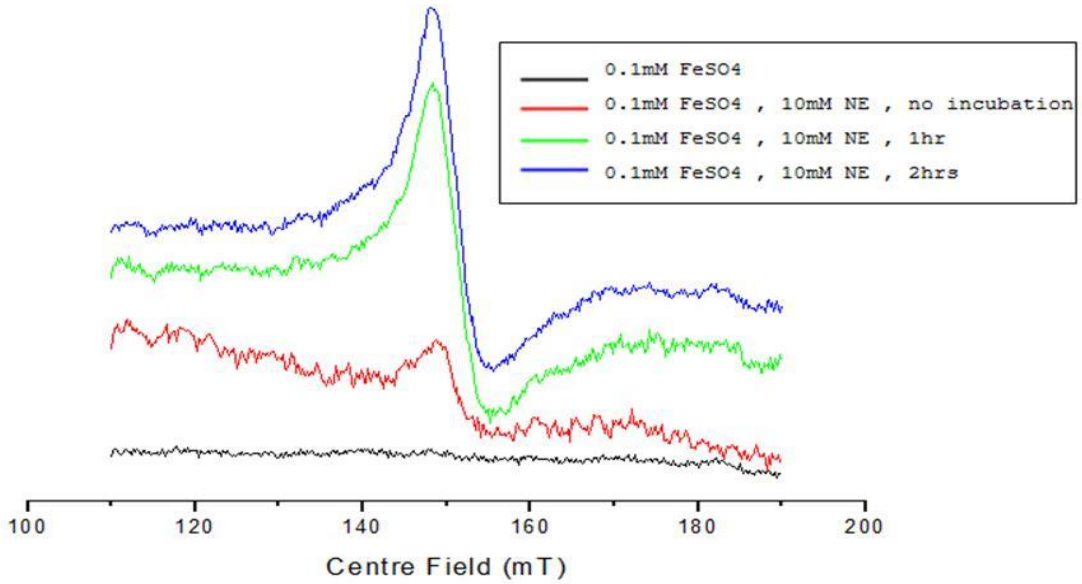


Fig. 5.4 EPR spectrum of inorganic ferrous iron in presence and absence of NE. In black no EPR signal of ferrous iron without the presence of NE. In red, green and blue single peak at 150 mT of Fe^{2+} in presence of 10 mM NE respectively after 0, 1 and 2 hrs incubation at room temperature.

5.2.2 Effect of CAs on Tf-Fe(III)

In order to understand how the Tf associated iron can be 'stolen' by NE and other catecholamines, ferrozine analysis was performed. Ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt) (fz) is an iron chelator that complexes specifically with Fe(II) aqueous solutions producing a magenta colour with maximum absorbance at 560 nm. The rationale for using ferrozine was that if CAs were able to reduce the Fe(III) associated to Tf into Fe(II), this would then explain how the Fe was lost from Tf due to its low affinity for ferrous iron.

Inorganic ferric and ferrous iron of concentrations ranging from 0 to 200 μM were mixed with 400 μM of fz buffered in 10 mM Tris-HCl, pH 7.5. The absorbance of these solutions at 560 nm was recorded over a period of 24 hrs; however the A560 nm values of the individual reagents did not change over time (data not shown), indicating that the fz was not capable of spontaneously reducing ferrous iron. The standard curves in Fig. 5.5 shows that a linear relationship occurs between Fe(III) concentration and A560 nm; ferrous salts did not form a complex with fz, confirming the specificity of fz for Fe(II).

Once it was established that fz specifically complexed Fe(II), fz was incubated for 24 hr with each of the individual reagents used in the Tf-catecholamine interaction experiments, in order to determine whether the readings obtained at A560 nm were indeed due to the magenta colour developed by the formation of a fz-Fe(II) complex and not artefacts due to, for example, catecholamine oxidation. Fig. 5.6 showed that all the reagents individually incubated with fz at room temperature did not complex with fz.

To determine the most suitable concentration of CAs to be used experiments a preliminary assay using concentrations of NE ranging from 0 to 4000 μM was performed. Holo-Tf (2 mg/ml, molar concentration 25 μM) of human origin was incubated at room temperature with the different concentrations of NE for 24 hrs in 10 mM Tris-HCl pH 7.5 buffer; fz-Fe(II) complex formation was monitored at A560 nm and data recordings were taken every 15 minutes. From Fig.5.7 it can be seen that that the concentration of NE giving the fastest rate of fzFe(II) complex formation was 4000 μM; this concentration was therefore used during subsequent experiments with NE and other catecholamines.

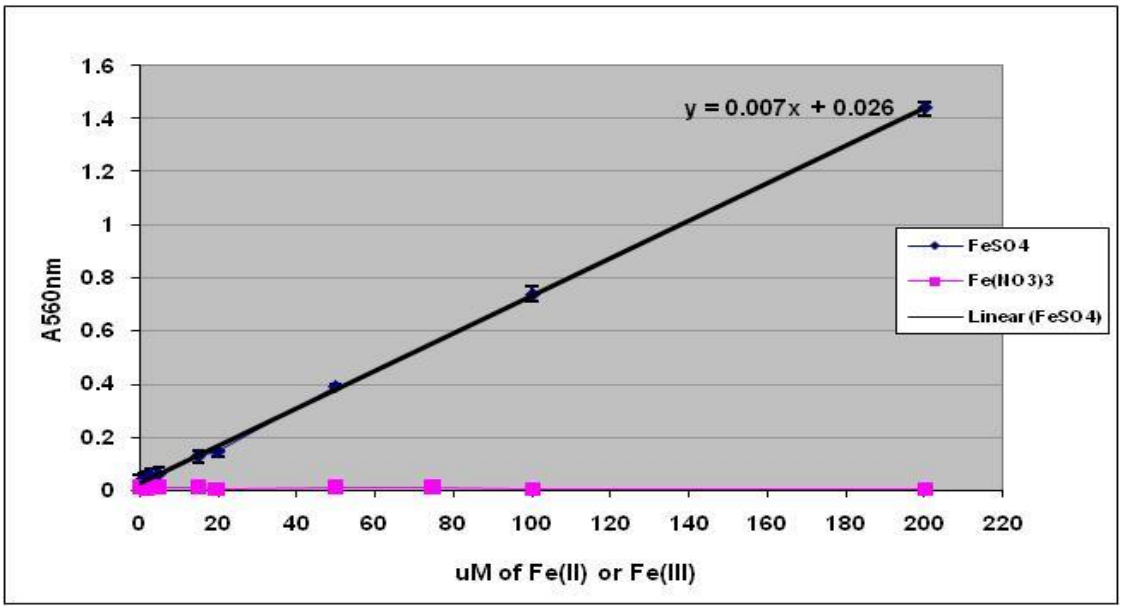


Fig. 5.5 Fe(II) and Fe(III) standard curve of the Fe-fz complex formation at absorbance of 560nm. Fe(II) formed a complex with fz resulting in formation of a magenta colour compound and producing a linear standard curve with respect to Fe(II) concentration. Fe(III) did not complex with fz.

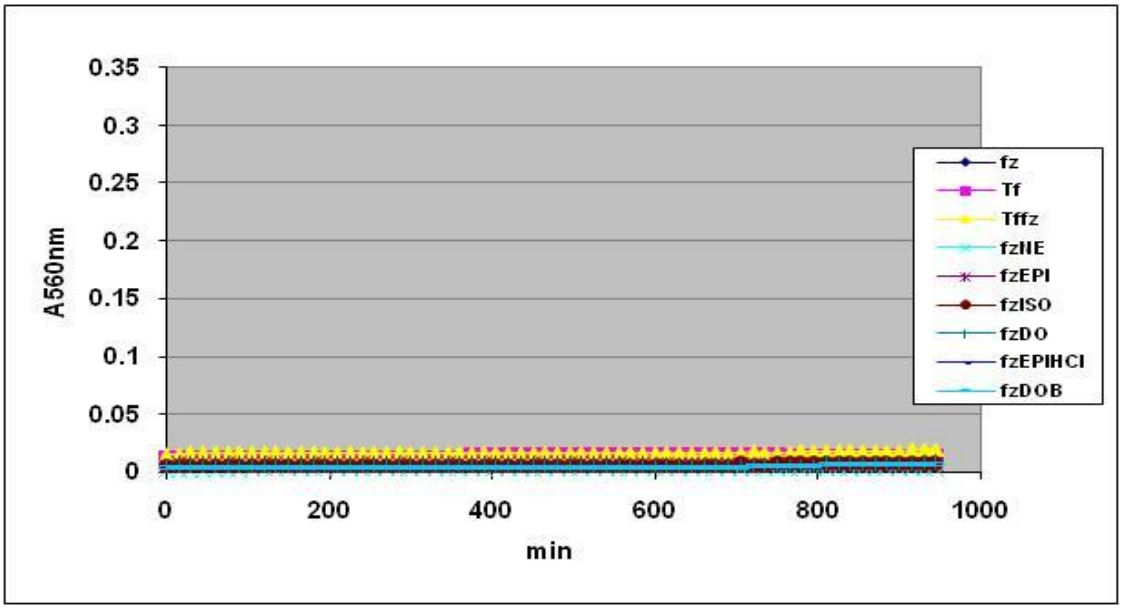


Fig. 5.6 Control samples. All the catecholamines at a concentration of 4 mM that were used during the experiment were incubated with 400 μM of fz buffered in 10 mM Tris-HCl, pH 7.5 at room temperature for 24 hrs. Only the period for the first 16 hrs is shown because the readings did not change between 24 and 16 hrs. Fz and Tf were also incubated separately and together at concentration of 400 μM and 2 mg/ml respectively.

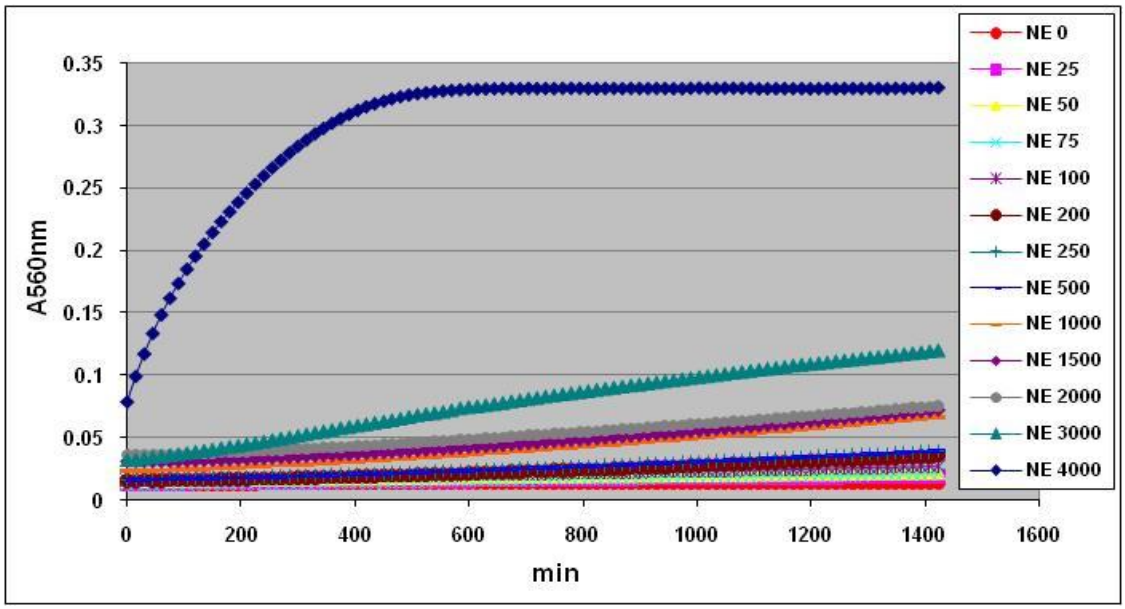


Fig. 5.7 NE dose-response effect on Tf(FeIII) reduction. Human holo-Tf, (2 mg/ml) which corresponds to 50 μM of total Fe, was buffered in 10 mM Tris-HCl pH 7.5 and incubated for 24 hrs at room temperature with concentrations of NE ranging from 0 μM to 4000 μM; fz was used at 400 μM. As the concentration of NE increases reduction of Tf(FeIII) to FeII can be observed and a fz(FeII) complex appears whose increase is proportional to the iron lost from Tf.

Urea-acrylamide gel electrophoresis analysis was also performed on the Tf-NE-fz mixtures shown in Fig. 5.7 in order to visualise the iron-binding status of the Tf (Heaphy, 1981) in these samples. The urea-acrylamide gel in Fig. 5.8 demonstrates not only that catecholamines were able to reduce the iron from Fe(III) to Fe(II) but also that the Fe was actually lost from the Tf.

Fig. 5.8 confirms that NE-mediated loss of iron from Tf is concentration-dependent; as the concentration of NE increased so did the loss of Fe from Tf, visualised in Fig. 5.8 as a shift from di-ferric iron-saturation to its mono-ferric and iron free forms. The Tf sample incubated with 4000 μM of NE for 24 hr lost almost all its iron, which the ferrozine analyses in Fig. 5.7 shows had been reduced to Fe(II). It is interesting to note that in the Tf sample incubated with 4000 μM NE without fz, iron loss was less than when fz was present. This shows the fz is clearly acting as sink for Fe(II), enabling the NE to reduce further Fe(III) to Fe(II) from the Tf, which otherwise would remain complexed with the Tf, by shifting the reaction equilibrium towards the production of Fe(II).

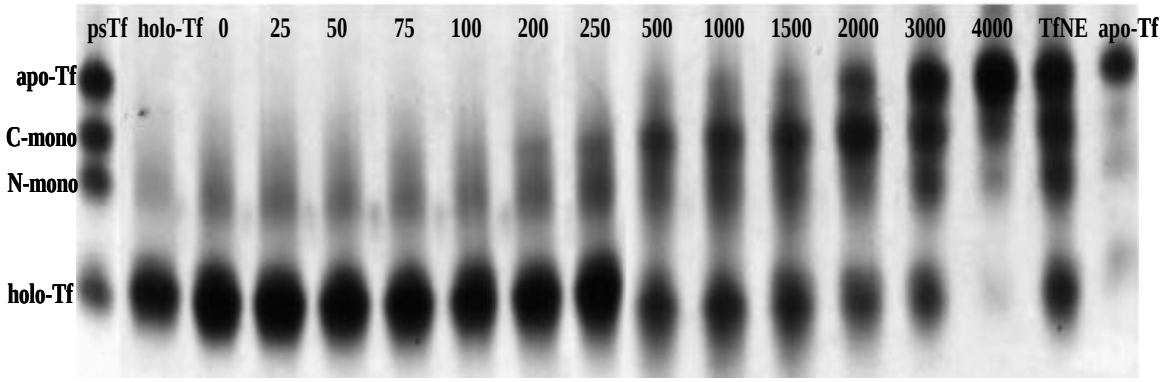


Fig. 5.8 Urea-acrylamide gel electrophoresis of the effects of increasing NE concentration on Tf iron binding. psTf: partially iron saturated solution of human-Tf (apo-Tf, iron-free Tf; C-mono, monoferric Tf-Fe C-terminal domain; N-mono, monoferic Tf-Fe, N terminal domain; holo-Tf, iron saturated Tf, Tf-Fe2. 0 to 4000 μM NE+Tf+fz; Tf+NE; apo-Tf. The samples (0-4000 μM) from Fig. 5.7 were run on a urea-acrylamide gel electrophoresis for 6 hrs at 70 V. ps-Tf was used to show the different degrees of Fe saturation of Tf, and holo-Tf and apo-Tf were used as controls. Tf-NE was the sample incubated with 4000 μM of NE without the presence of fz, which produced spectrophotometric data close to zero (the data recorded in Fig. 5.9) since without fz there could be no Fe(II) complex formation.

Fig. 5.9, shows that norepinephrine complexed the Tf associated Fe(III), reducing it to Fe(II), thereby producing in the presence of fz a strong magenta coloured compound indicative of fz-Fe(II) complex formation. Urea gel analysis of the Tf samples shown in Fig. 5.9 was also carried out (Fig. 5.10a,b). The gels were scanned using a densitometer connected with Quantity one program that enabled determination of the different ratios of the Tf iron binding isoforms at the different time points (Fig. 5.10c). From Fig. 5.10a and 5.10b it is apparent that the loss of iron in the presence of a Fe(II) sink (Fig.5.10a) resulted in the complete shift of Tf into its apo or iron free form within the first few hours of contact with NE. However, in the absence of a ferrous iron sink (5.10b) there was still loss of iron, though the rate and extent of iron removal was slower and seemed to plateau when ~50-60 % of the apo isoform was reached, suggesting that the NE was possibly still bound to Fe that remained complexed within the Tf.

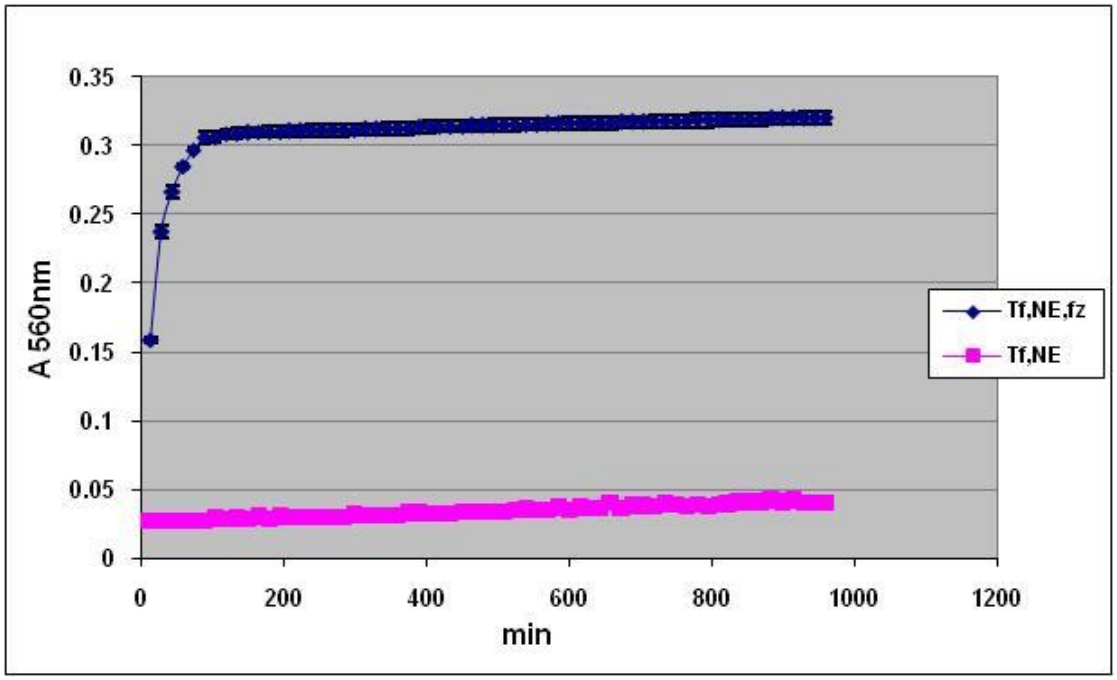
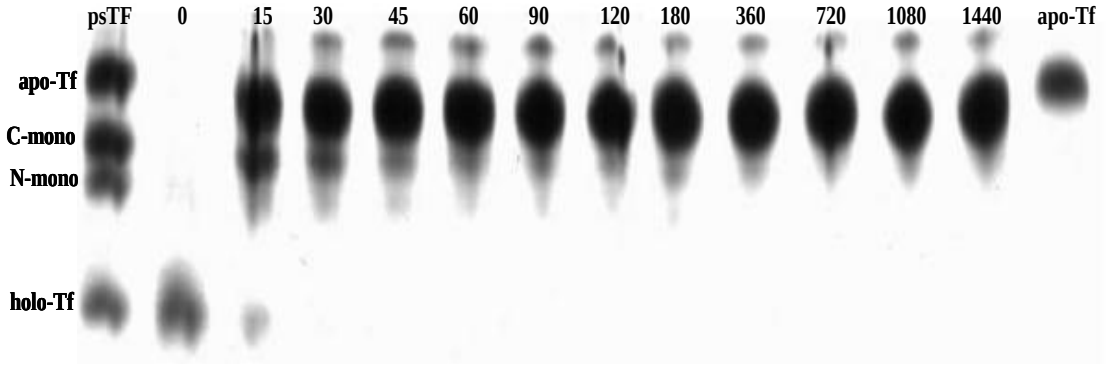
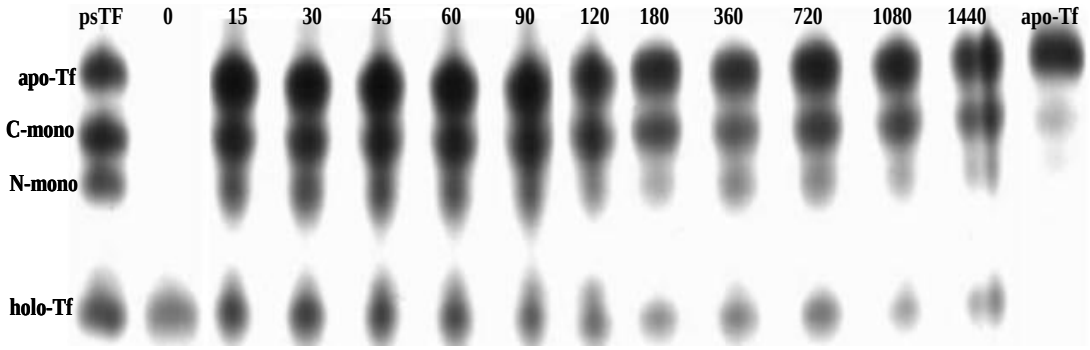


Fig. 5.9 The effect of NE effect on Tf(FeIII) binding. Human holo-Tf, (2 mg/ml) Tf was incubated at room temperature for 24 hr with either 4 mM NE and 400 μM fz (blue) or NE only (pink). Both Tf samples were buffered with 10 mM Tris-HCl pH 7.5. Measurements of fz-Fe complex formation were taken every 15 min using Varioskan flash densitometer set at A560nm.

5.10a



5.10b



5.10c

TfNEfz	min 0	15	30	45	60	90	120	180	360	720	1080	1440
apo-Tf	0%	67%	79%	88%	89%	91%	93%	95%	97%	98%	98%	98%
C-mono	0%	26%	18%	11%	11%	8%	7%	5%	3%	2%	2%	2%
N-mono	1%	5%	3%	1%	1%	1%	0%	0%	0%	0%	0%	0%
holo-Tf	99%	2%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%

TfNE	min 0	15	30	45	60	90	120	180	360	720	1080	1440
apo-Tf	0%	43%	46%	46%	47%	47%	48%	50%	50%	53%	54%	58%
C-mono	0%	29%	29%	35%	30%	30%	32%	29%	32%	34%	33%	32%
N-mono	0%	13%	12%	8%	11%	11%	10%	11%	9%	6%	6%	5%
holo-Tf	100%	15%	13%	11%	12%	12%	10%	10%	9%	7%	7%	5%

Fig. 5.10 Iron-binding by Tf in the presence of NE and ferrozine. The Tf-NE/fz samples used in Fig. 5.9 were frozen at the time points shown (0-1440 min) and analysed for changes in iron binding status using urea gel electrophoresis as described in Materials and Methods, chapter 2, section 2.2.1.7. The Tf iron standards consisted of psTf (partially saturated human Tf), apo-Tf and holo-Tf. In the samples (Fig. 5.10a) incubated in presence of fz, the loss of iron from Tf was rapid and within ~1 hr 30 min all the iron was lost from holo-Tf, which shifted to its mono/apo-iron binding isoform. Whereas in the Tf-NE samples incubated without fz (Fig. 5.10b), there was still loss of iron, though this was much slower and not all the iron was removed from the Tf. Fig. 5.10c shows the percentage difference in the distribution of the iron binding isomers of Tf shown in the urea gels in Figs 5.10a and 5.10b, calculated using Quantity one program densitometer analysis program.

The ability of other catecholamines to reduce and remove Tf-complexed iron was also investigated. Like NE, Epinephrine (EPI) was also able to reduce the Fe(III) associated to Tf to Fe(II) facilitating the loss of Fe which was then taken up by the fz (Fig. 5.11). In the presence of a ferrous iron sink (Fig. 5.12a) it was possible to observe a more rapid and complete loss of iron from Tf, and a shift to the apo isoform of Tf of ~94 %. However, in the absence of fz (Fig. 5.12b) there was still significant loss of iron from Tf but the process was slower and only ~67 % of the total iron was lost after 24 hrs.

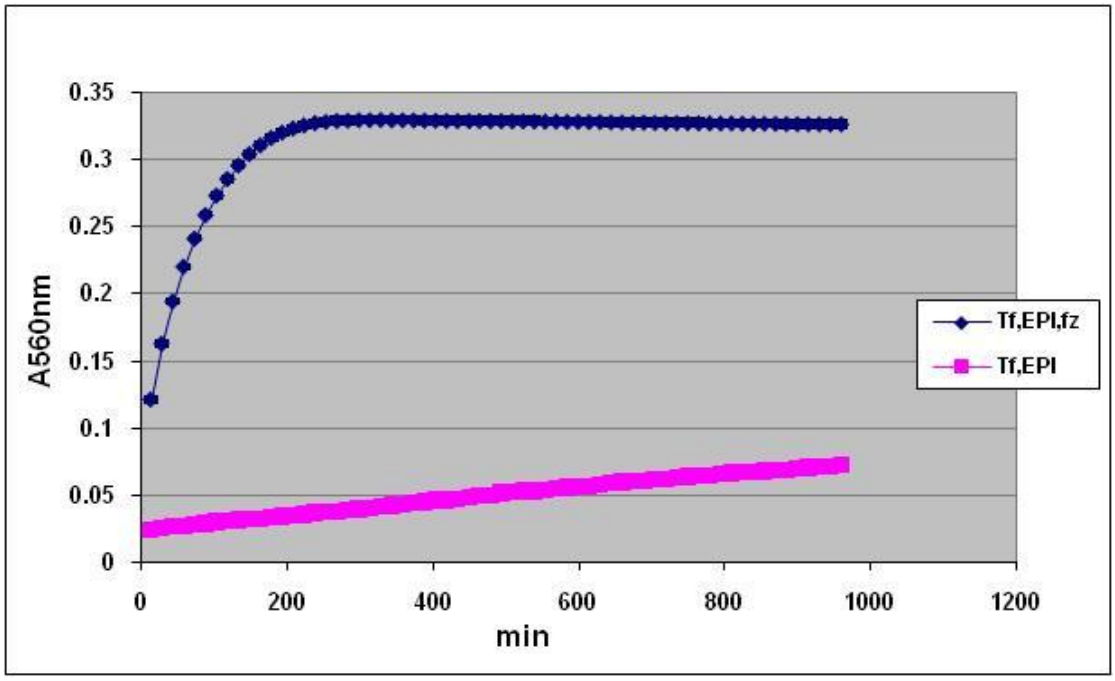
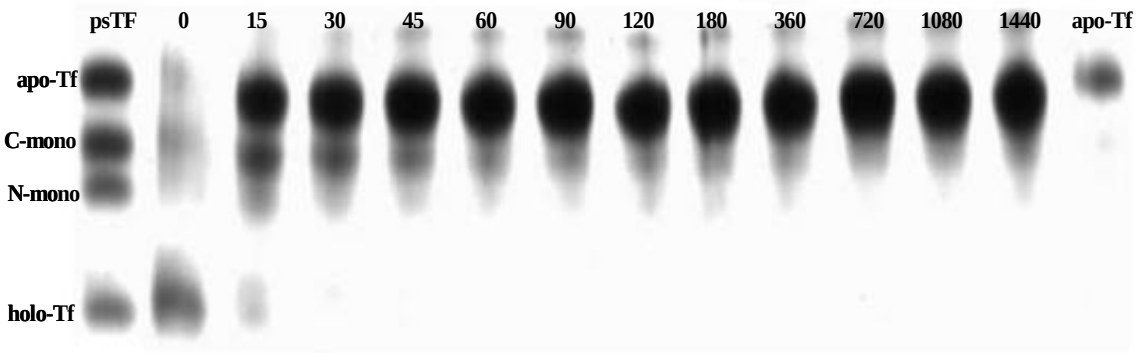
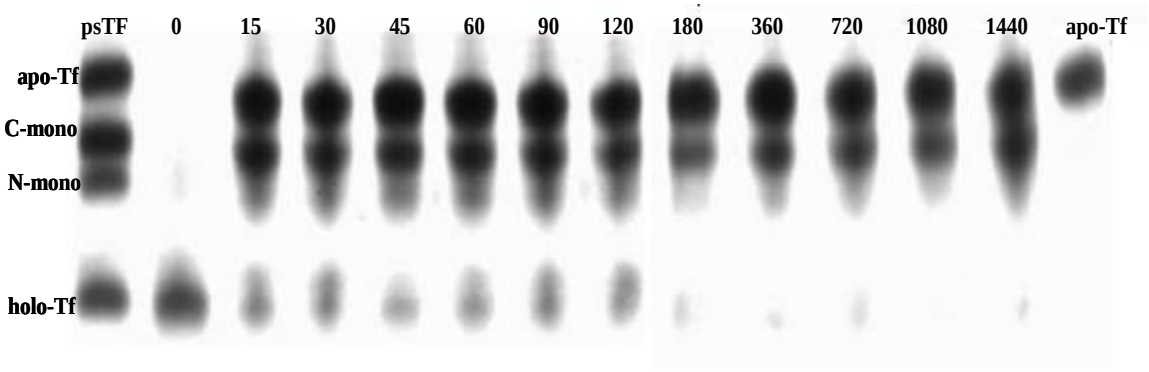


Fig. 5.11 The effect of EPI on Tf(FeIII) binding. The details for these sets of experiments are as described in the legend for Fig. 5.9. Holo-Tf was incubated with EPI and fz (blue) or holo-Tf and EPI only (pink).

5.12a



5.12b



5.12c

TfEPIfz	min 0	15	30	45	60	90	120	180	360	720	1080	1440
apo-Tf	12%	65%	75%	84%	87%	88%	89%	91%	92%	94%	94%	94%
C-mono	22%	26%	22%	15%	12%	11%	10%	8%	7%	6%	6%	6%
N-mono	9%	6%	3%	1%	1%	1%	1%	1%	1%	0%	0%	0%
holo-Tf	57%	3%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%

TfEPI	min 0	15	30	45	60	90	120	180	360	720	1080	1440
apo-Tf	0%	49%	50%	54%	54%	54%	56%	58%	60%	65%	66%	67%
C-mono	0%	35%	34%	32%	32%	39%	32%	31%	34%	32%	30%	29%
N-mono	1%	9%	9%	8%	8%	7%	7%	7%	5%	3%	4%	4%
holo-Tf	99%	7%	7%	6%	6%	0%	5%	4%	1%	0%	0%	0%

5.12 Iron-binding by Tf in the presence of EPI and ferrozine. The iron-binding isoforms of the Tf shown in the urea gel in Fig. 5.11 were subject to urea gel electrophoresis and gel densitometry scanning as described in the legend to Fig. 5.10. Fig. 5.12 a and b show the urea gel analysis of Tf incubated with EPI and fz and EPI only; Fig. 5.12c shows the percentage difference in the distribution of the iron binding isomers of Tf shown in the urea gels in Figs 5.12a and 5.12b.

Isoprenaline (ISO) was also able to reduce the Fe(III) from Tf into Fe(II), which then formed a complex with fz giving the characteristic magenta colour development indicative of the presence of ferrous iron (Fig. 5.13). The Tf samples of Fig. 5.13 were similarly analysed by urea gel electrophoresis and densitometry scanning (Fig. 5.14). Fig 5.14a shows that when Tf was incubated with ISO in the presence of fz, the catecholamine became a very potent remover of Tf-complexed iron, and almost all the Tf-bound Fe was lost within ~1 hr 30 min; whereas in absence of fz (Fig. 5.14b and 5.14c) around only 60-70 % of the iron was lost during the same time interval.

A similar pattern of Fe loss was observed in the Tf samples incubated with NE, EPI and ISO in presence of fz (Figs.10a, 12a and 14a). However, ISO alone seemed to enable faster loss of iron from TfFe(III) than the other catecholamines, indicating that its effect on TfFe(III) is stronger.

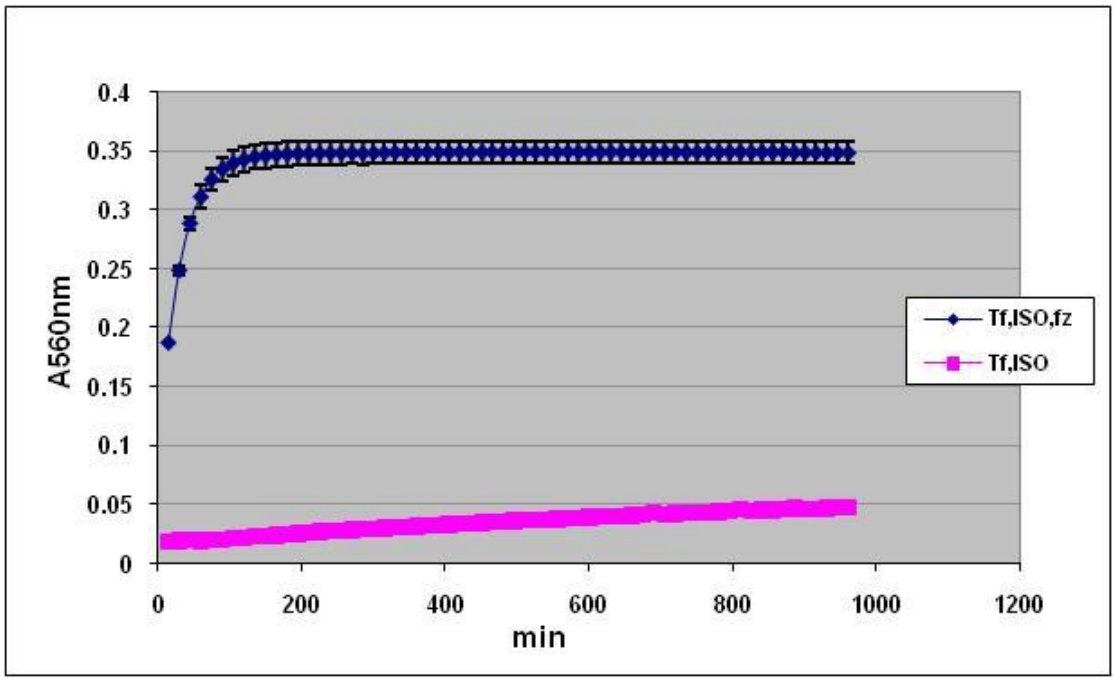
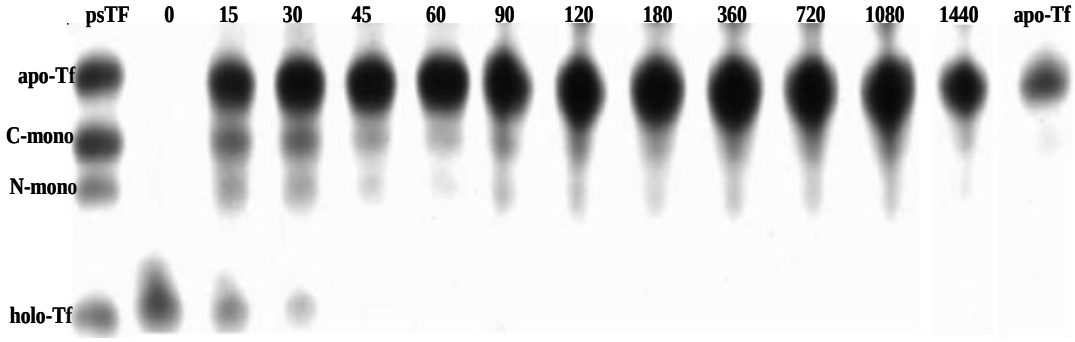
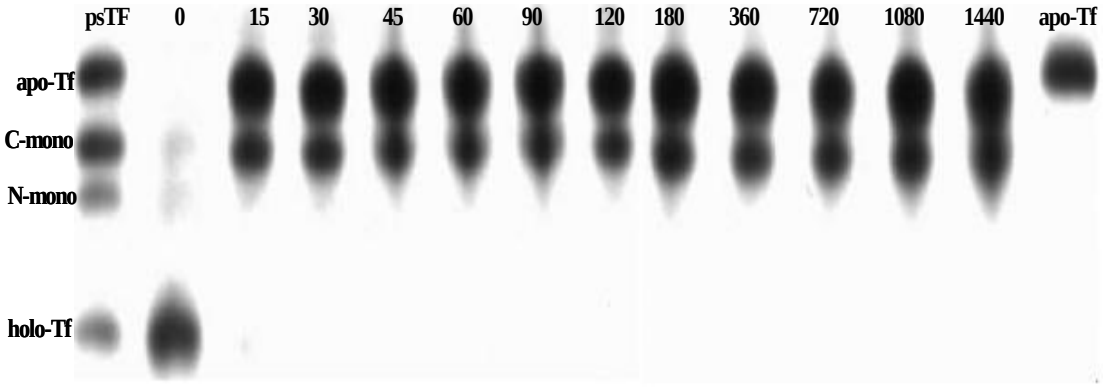


Fig. 5.13 The effect of ISO on Tf(FeIII) binding. The details for these sets of experiments are as described in the legend for Fig. 5.9. Blue, human holo-Tf was incubated with ISO and fz; pink, human holo-Tf and ISO only.

5.14a



5.14b



5.14c

TfISOfz	min 0	15	30	45	60	90	120	180	360	720	1080	1440
apo-Tf	0%	61%	74%	88%	90%	92%	92%	93%	94%	94%	94%	99%
C-mono	0%	23%	18%	10%	8%	7%	7%	6%	5%	5%	6%	1%
N-mono	0%	7%	5%	2%	2%	1%	1%	1%	1%	1%	0%	0%
holo-Tf	100%	9%	3%	0%	0%	0%	0%	0%	0%	0%	0%	0%

TfISO	min 0	15	30	45	60	90	120	180	360	720	1080	1440
apo-Tf	0%	64%	65%	66%	66%	67%	67%	67%	68%	69%	70%	70%
C-mono	5%	33%	34%	34%	34%	33%	33%	33%	32%	31%	30%	30%
N-mono	2%	3%	1%	0%	0%	0%	0%	0%	0%	0%	0%	0%
holo-Tf	93%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%

Fig. 5.14 Iron-binding by Tf in the presence of ISO and ferrozine. The iron-binding isoforms of the Tf shown in the urea gel in Fig. 5.11 were subjected to urea gel electrophoresis and gel densitometry scanning as described in the legend to Fig. 5.10. Fig. 5.14 a and b show the urea gel analysis of Tf incubated with ISO and fz and ISO only; Fig. 5.14c shows the percentage difference in the distribution of the iron binding isomers of Tf shown in the urea gels in Fig.s 5.14a and 5.14b.

The effect of dopamine (DO) on the TfFe(III), which can be seen in Fig. 5.15, was different from that observed with the catecholamines NE, EPI and ISO. Incubating DO with Tf and fz did not result in the expected Fe(II)-fz complex formation and the mixture did not take a magenta colouration, but was instead a dark brown colour developed which unfortunately still absorbed at 560 nm. However, urea gel electrophoresis showed that there was still loss of iron from Tf at the end of the 24 hr incubation with the DO, though it was still much lower (~30 %) than observed with the other catecholamines (Figs. 5.16a, b and c). What may be significant is that apart from differences in chemical structure, NE, EPI and ISO were purchased as bitartrate salts whereas DO was obtainable only as a hydrochloride. Thus, it was necessary to establish whether the difference in potency of the dopamine to remove Tf iron was due to the catecholamine structure, or the chemistry of the catecholamine anion. Fortunately, epinephrine is available as both a bitartrate and hydrochloride salt. Therefore, the EPI bitartrate experiment shown in Fig. 5.12 was repeated using a similar concentration of epinephrine hydrochloride (EPIHCl) (Fig. 5.17-5.18).

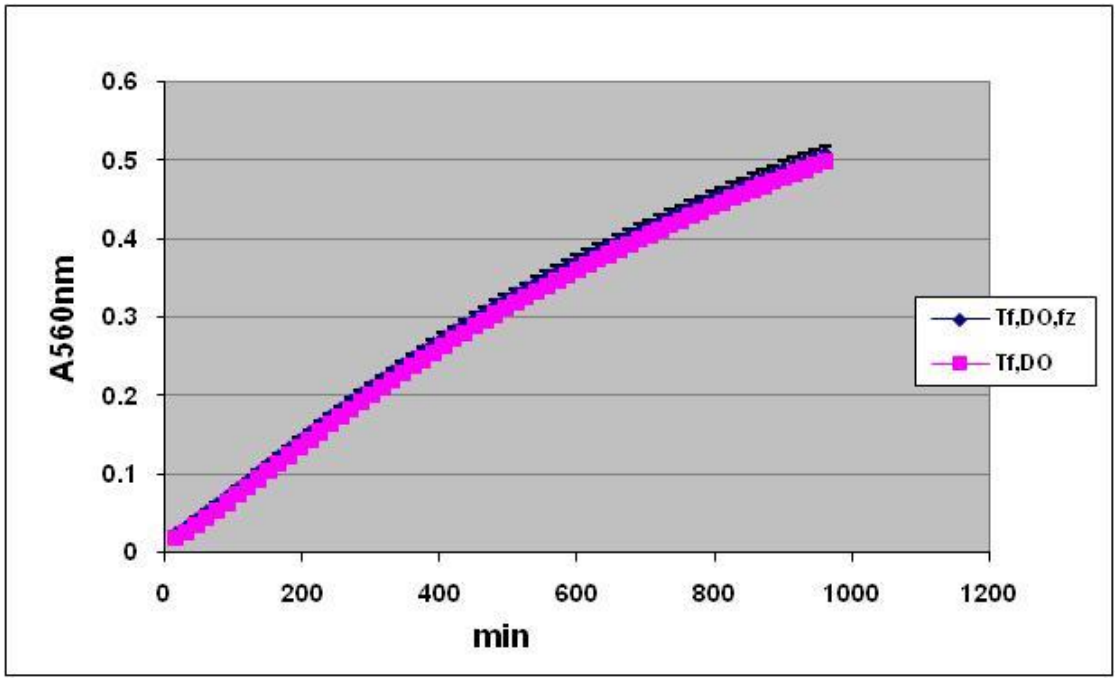
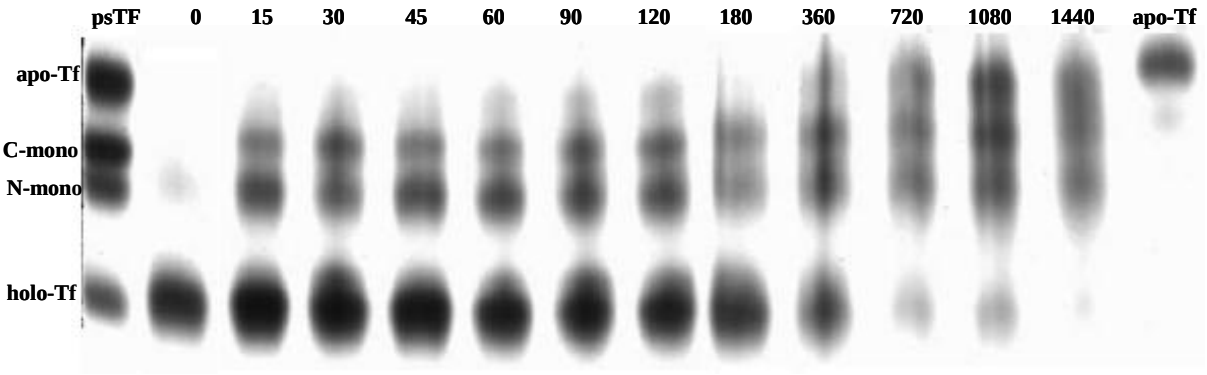
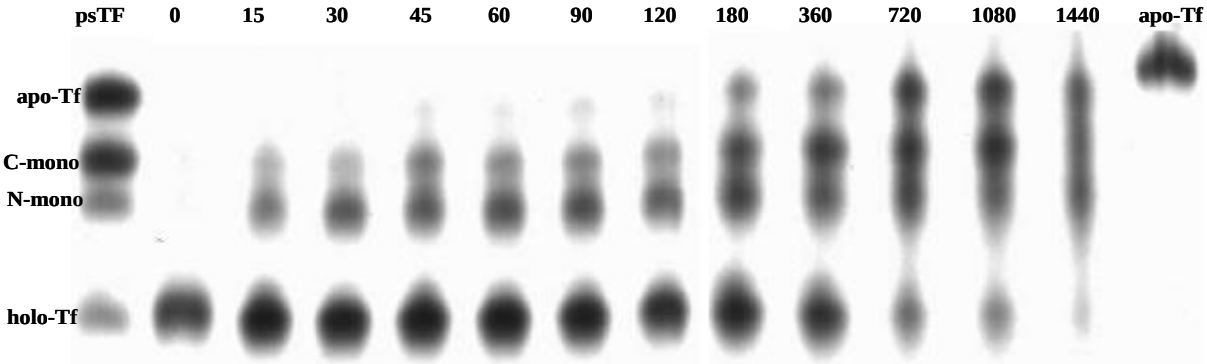


Fig. 5.15 The effect of DO on Tf(FeIII) binding. The details for these sets of experiments are as described in the legend for Fig. 5.9. Blue, human holo-Tf incubated with DO and fz; pink, human holo-Tf and ISO only.

5.16a



5.16b



5.16c

TfDOfz	min 0	15	30	45	60	90	120	180	360	720	1080	1440
apo-Tf	0%	1%	1%	2%	2%	2%	2%	3%	11%	27%	27%	29%
C-mono	0%	11%	12%	17%	17%	13%	16%	16%	26%	32%	34%	38%
N-mono	3%	23%	24%	18%	18%	26%	25%	25%	28%	33%	32%	32%
holo-Tf	97%	65%	63%	63%	63%	59%	57%	56%	35%	8%	7%	1%

TfDO	min 0	15	30	45	60	90	120	180	360	720	1080	1440
apo-Tf	0%	0%	0%	1%	1%	1%	1%	7%	11%	25%	27%	28%
C-mono	0%	7%	8%	14%	11%	12%	14%	23%	30%	33%	32%	37%
N-mono	0%	17%	26%	22%	26%	27%	27%	25%	21%	26%	34%	22%
holo-Tf	100%	76%	66%	63%	62%	60%	58%	45%	38%	16%	7%	13%

Fig. 5.16 Iron-binding by Tf in the presence of DO and ferrozine. The iron-binding isoforms of the Tf shown in the urea gel in Fig. 5.15 were subjected to urea gel electrophoresis and gel densitometry scanning as described in the legend to Fig. 5.10. Fig. 5.16a and 5.16b show the urea gel analysis of Tf incubated with ISO and fz and ISO only; Fig. 5.16c shows the percentage difference in the distribution of the iron binding isomers of Tf shown in the urea gels in Figs 5.14a and 5.14b.

As was observed for dopamine hydrochloride, the presence of fz in the reaction mixture of TfFe(III) and EPIHCl (Fig. 5.17) did not give a positive reading regarding the formation of the fzFe(II) complex (the same absorbance curve was obtained in absence of fz). This suggested that the presence of the hydrochloride anion in the Tf – catecholamine mixture could indeed affect the outcome of the catecholamine-catalysed iron reduction reaction. Moreover, the loss of Fe from Tf both in the presence (5.18a) or absence (5.18b) of fz was significantly reduced for the EPI hydrochloride salt; the reaction effectively halted when ~ 24 % and 17 % of the apo-Tf isoform was obtained (5.18c), when compared respectively with NE, EPI and ISO where the loss of iron was close to 100 % in the presence of fz and ~ 70 % in the absence of fz (Fig 5.10, 5.12 and 5.14).

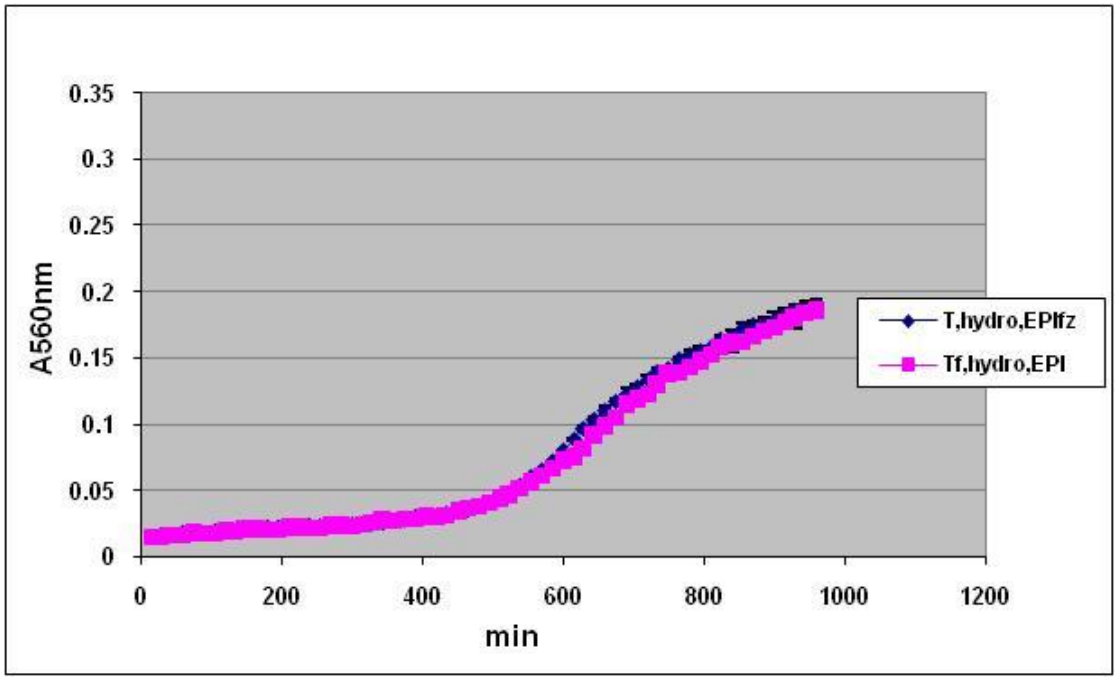
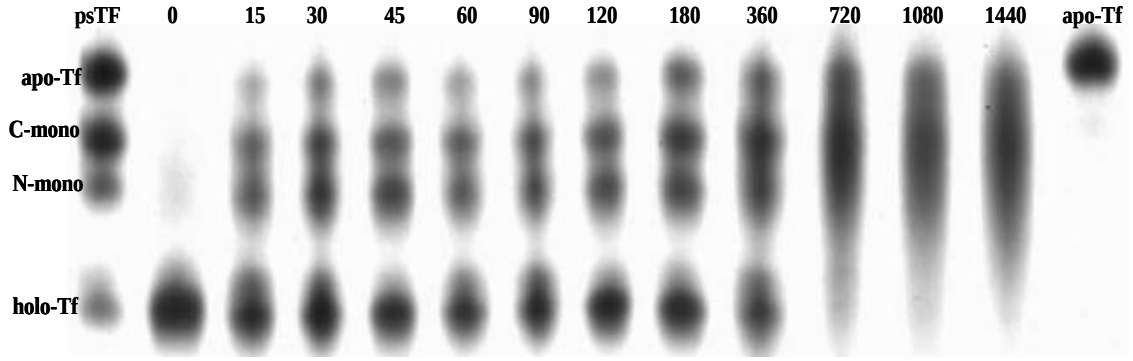
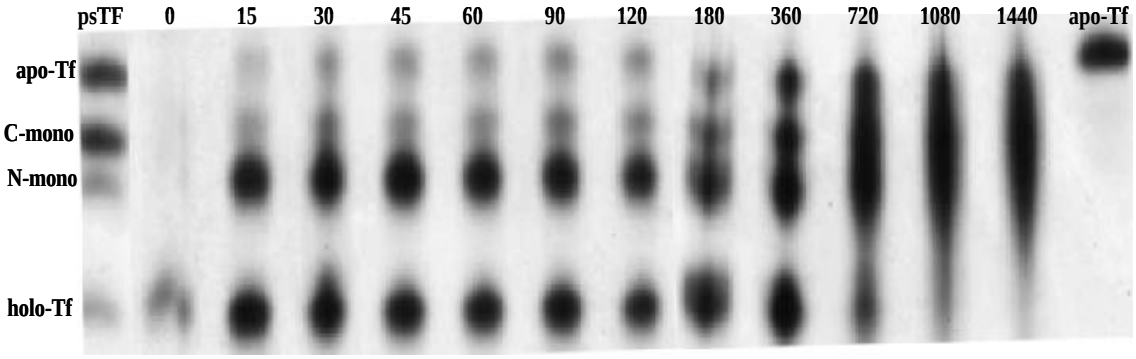


Fig. 5.17 The effect of EPI hydrochloride on Tf(FeIII) binding. The details for these sets of experiments are as described in the legend for Fig. 5.9. Blue, human holo-Tf was incubated with EPIHCl and fz; pink, human holo-Tf and EPIHCl only.

5.18a



5.18b



5.18c

TfEPIHClfz	min 0	15	30	45	60	90	120	180	360	720	1080	1440
apo-Tf	0%	5%	8%	9%	9%	10%	10%	14%	16%	23%	23%	24%
C-mono	1%	18%	22%	22%	21%	22%	21%	26%	30%	35%	37%	37%
N-mono	4%	24%	24%	23%	26%	24%	30%	24%	23%	30%	30%	30%
holo-Tf	95%	53%	46%	46%	44%	44%	39%	36%	31%	12%	10%	9%

TfEPIHCl	min 0	15	30	45	60	90	120	180	360	720	1080	1440
apo-Tf	0%	0%	2%	2%	3%	4%	4%	5%	12%	15%	17%	17%
C-mono	0%	5%	7%	4%	5%	8%	7%	16%	19%	29%	33%	37%
N-mono	0%	44%	45%	54%	52%	45%	47%	35%	33%	43%	44%	44%
holo-Tf	100%	51%	46%	40%	40%	43%	42%	44%	36%	13%	6%	2%

Fig. 5.18 Iron-binding by Tf in the presence of EPI hydrochloride and ferrozine. The iron-binding isoforms of the Tf shown in the urea gel in Fig. 5.17 were subject to urea gel electrophoresis and gel densitometry scanning as described in the legend to Fig. 5.10. Fig. 5.18a and 5.18b show the urea gel analysis of Tf incubated with ISO and fz and ISO only; Fig. 5.18c shows the percentage difference in the distribution of the iron binding isomers of Tf shown in the urea gels in Fig.s 5.18a and 5.18b.

Fig.5.19 shows that little Fe(II) was formed when dobutamine (DOB) was incubated with holo Tf in the presence of fz. Fig. 5.20 shows that incubation of Tf with DOB produced a similar pattern of iron loss from holo-TfFe(III) to that seen in the samples incubated with DO hydrochloride and EPI hydrochloride (Figs. 5.16 and 5.18). This suggests that the presence of the hydrochloride anion in the dobutamine preparation interfered with the ability of the catecholamine to reduce the Fe(III) associated with the holo Tf.

It is also interesting to note that independent of the order by which CAs were added to the holo-Tf mixture, and regardless of presence or absence of fz, iron loss from the Tf was invariably associated with the N- terminal iron binding domain, suggesting that possibly it is more readily accessible to the CAs.

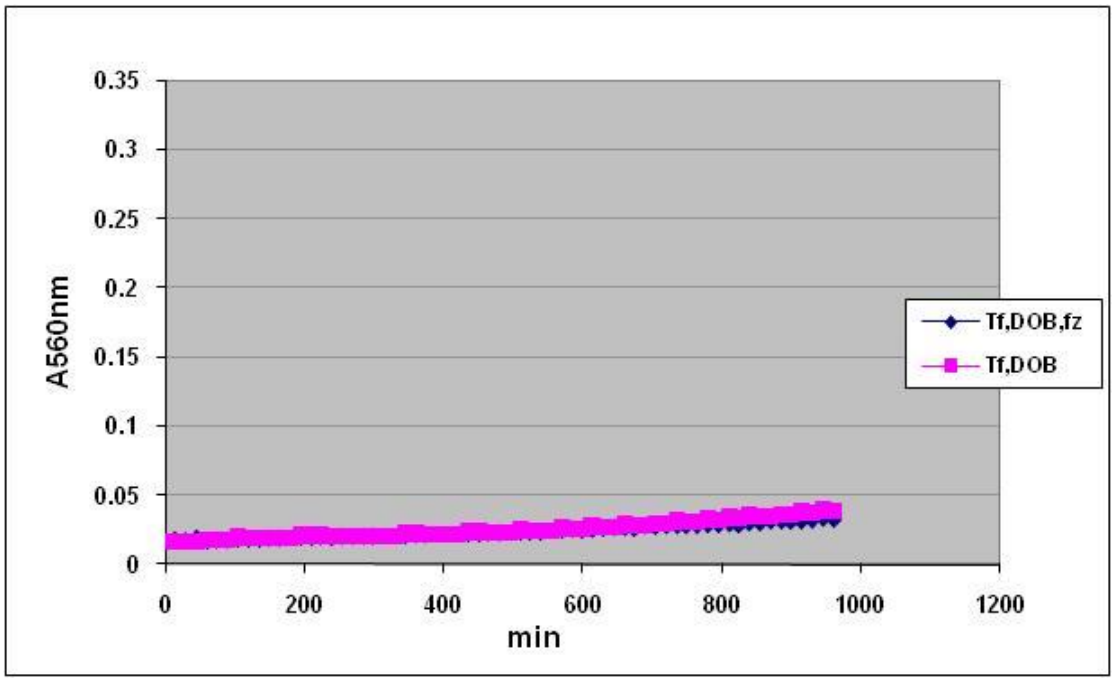
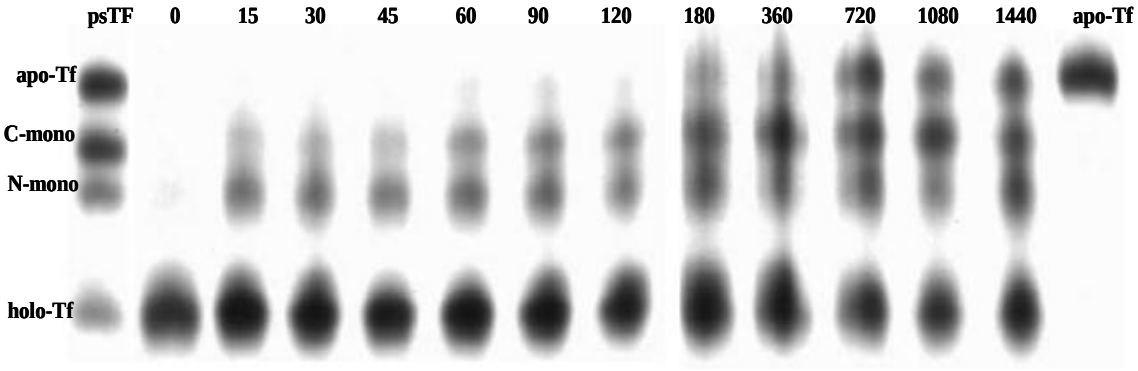
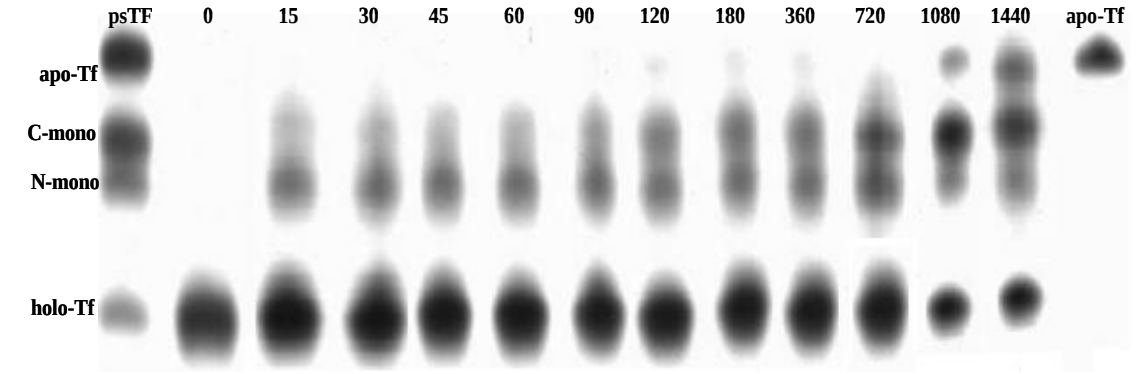


Fig. 5.19 The effect of Dob hydrochloride on Tf(FeIII) binding. The details for these sets of experiments are as described in the legend for Fig. 5.9. Blue, human holo-Tf was incubated with DOB and fz; pink, human holo-Tf and DOB only

5.20a



5.20b



5.20c

TfDOBfz	min 0	15	30	45	60	90	120	180	360	720	1080	1440
apo-Tf	0%	0%	0%	0%	1%	1%	1%	9%	10%	16%	17%	22%
C-mono	0%	7%	7%	8%	14%	11%	13%	24%	29%	24%	32%	28%
N-mono	1%	14%	17%	16%	13%	17%	17%	16%	12%	18%	10%	16%
holo-Tf	99%	79%	76%	76%	72%	71%	69%	51%	49%	42%	41%	34%

TfDOB	min 0	15	30	45	60	90	120	180	360	720	1080	1440
apo-Tf	0%	0%	0%	0%	0%	1%	1%	1%	1%	2%	7%	15%
C-mono	0%	5%	5%	7%	8%	11%	14%	15%	16%	30%	42%	47%
N-mono	0%	15%	16%	17%	17%	17%	18%	17%	17%	8%	14%	15%
holo-Tf	100%	80%	79%	76%	75%	71%	67%	67%	66%	60%	37%	23%

Fig.5.20 Iron-binding by Tf in the presence of EPI hydrochloride and ferrozine. The iron-binding isoforms of the Tf shown in the urea gel in Fig. 5.19 were subject to urea gel electrophoresis and gel densitometry scanning as described in the legend to Fig. 5.10. Fig. 5.20 a and b show the urea gel analysis of Tf incubated with ISO and fz and ISO only; Fig. 5.20c shows the percentage difference in the distribution of the iron binding isomers of Tf shown in the urea gels in Fig.s 5.20a and 5.20b

5.2.3 Analysis of the effects of catecholamines on lactoferrin iron binding

The effect of NE on the iron valency of human saturated LfFe(III) was also analysed using ferrozine (Fig. 5.21). Urea gel electrophoresis analysis could not be performed on Lf due to the poor resolution of Lf on urea-acrylamide gels (Freestone, *et al*., 2000) From the data shown in Fig. 5.21 it is possible to conclude that when iron saturated Lf (III) is incubated with NE and fz, the NE reduces the iron associated with Lf from ferric to ferrous oxidation state. The loss of iron from Lf was a constant and continuous process, and after 24 hrs incubation with NE and fz at room temperature nearly all of the Lf-complexed ferric iron was reduced to ferrous.

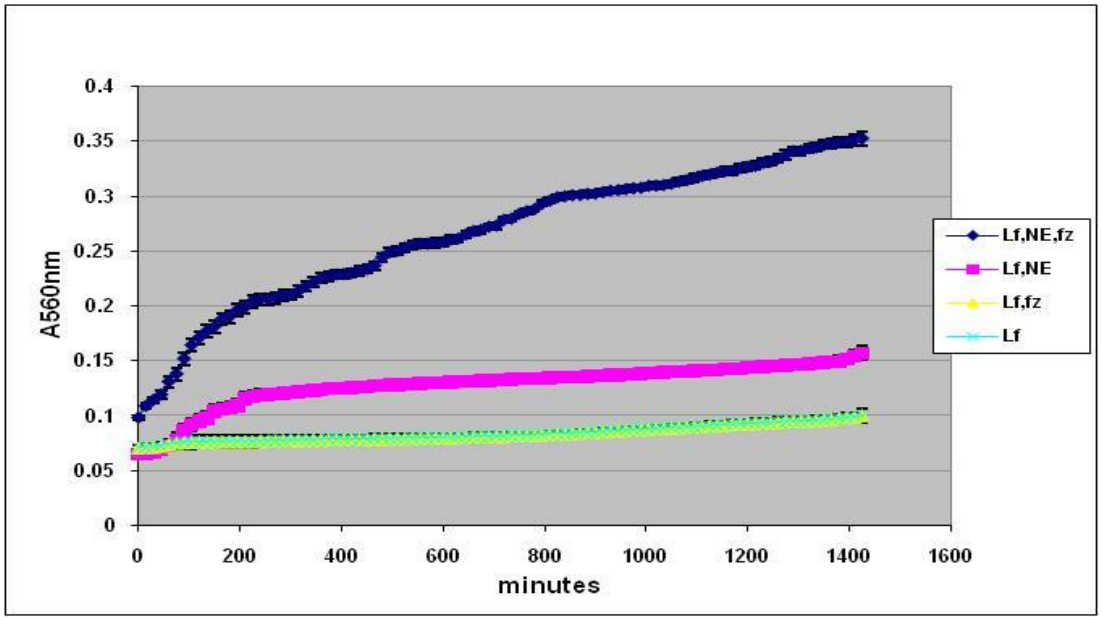


Fig.5.21 Effect of NE on Lf Fe(III) iron binding. In blue, 2 mg/ml of human iron saturated Lf, containing approximately 50 μMof Fe(III) were incubated at room temperature for 24 hr with 4 mM of NE and 400 μM of fz; in pink human saturated Lf and NE were incubated in absence of fz (control). In yellow and green Lf was incubated respectively with and without fz to determined whether the reading were an artefact or not. All samples were buffered in 10 mM Tris-HCl pH 7.5. Readings were taken every 15 min using Varioskan flash densitometer at 560nm. The reduction of Fe(III) associated with Lf to Fe(II) by NE was a constant but slower process when compared to the samples containing Tf instead of Lf (Fig. 5.9). However, there was still loss and reduction of iron from Lf and after 24 hr over 90% of the Lf Fe(III) had been converted to Fe(II).

5.2.4 Effect of NE on serum Tf Fe(III) reduction

It would be valuable to be able to visualise if catecholamine stress hormones or inotropes were affecting Tf iron binding status in whole blood or serum, as this could be a useful diagnostic tool to indicate if inotrope supplementation, such as occurs in

the treatment of acutely ill ICU patients, was compromising the bacteriostatic nature of the patient's blood. To investigate this possibility, NE was mixed with bovine serum in presence and absence of fz, in order to determine whether it was possible to observe the reduction of Fe(III) present in the serum Tf into Fe(II) and consequently Fe(II)-fz-complex formation. From Fig.5.22 it was possible to observe that in the samples containing 1 % BS, NE and fz Fe(II)-fz complex formation was detectable, indicating that the presence of the catecholamine in the serum reduced the Tf Fe(III) to Fe(II). However, when the percentage of BS was increased to 6 % (Fig.5.23) and above (Fig.5.23), it was no longer possible to obtain readings specific for the formation of Fe(II)-fz but instead a dark brown colour was observed (Fig.5.24). This suggests that following addition of NE, redox reactions may have occurred in the serum, which at low concentration of BS seemed not to interfere with the formation of the Fe(II)-fz complex, although they were problematic at high concentrations. This suggests that ferrozine analysis may not be the most suitable technique to determine the reduction of Fe(III) associated with Tf in a complex organic fluid, although it did reveal that the mechanism by which CAs remove iron from serum Tf is by ferric iron reduction.

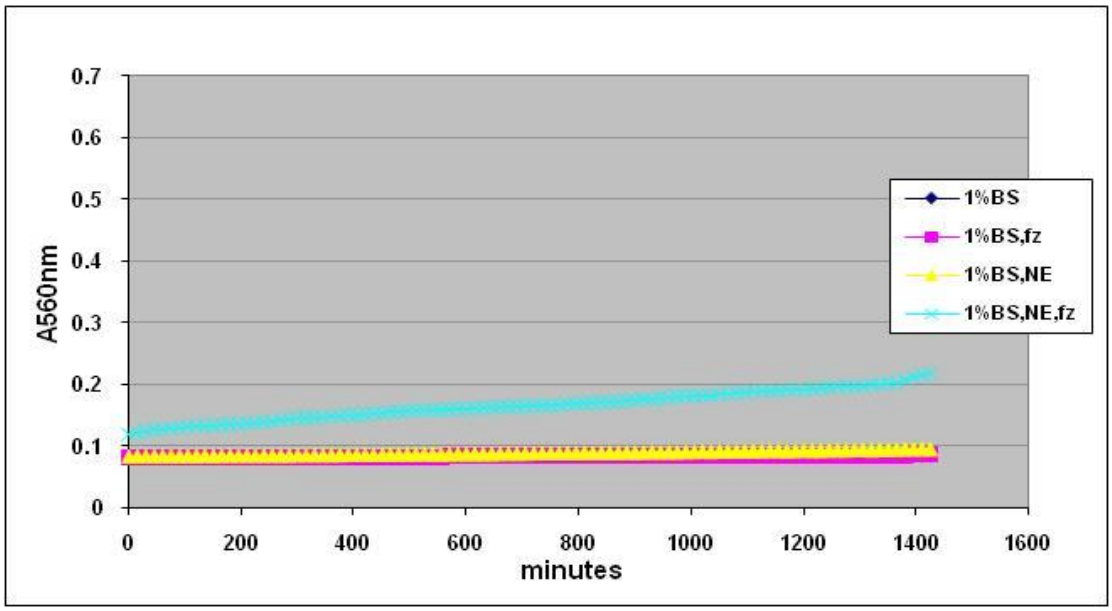


Fig.5.22 Effect of NE on Fe(III) binding in 1 % serum. A solution containing 1 % BS in 99 % PBS was incubated at room temperature for 24 hrs in the presence and absence of 4 mM of NE and fz, the sample was labelled as in the diagram. A560 nm readings were taken every 15 min using a Varioskan flash densitometer in order to determine if Fe(II)-fz complex formation was taking place. As can be seen from the above diagram, only for the sample containing BS, NE and fz (light blue line) was it possible to detect fz-Fe(II)-complex formation. The other assay components did not absorb at 560 nm.

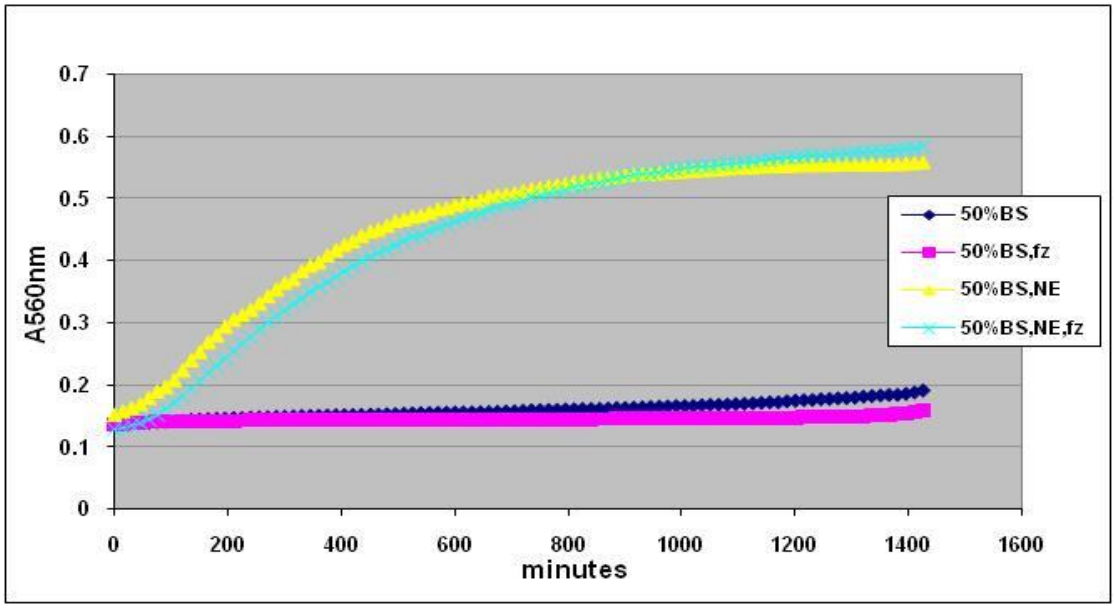


Fig.5.23 Effect of NE on Tf Fe(III) binding in 50 % serum. A solution containing 50 % BS in 50 %PBS was incubated at room temperature for 24 hrs with combinations of 4 mM NE and 400 μM fz as labelled in the histogram. Reading were taken every 15 min at 560 nm using a Varioskan flash densitometer in order to monitor Fe(II)-fz complex formation.

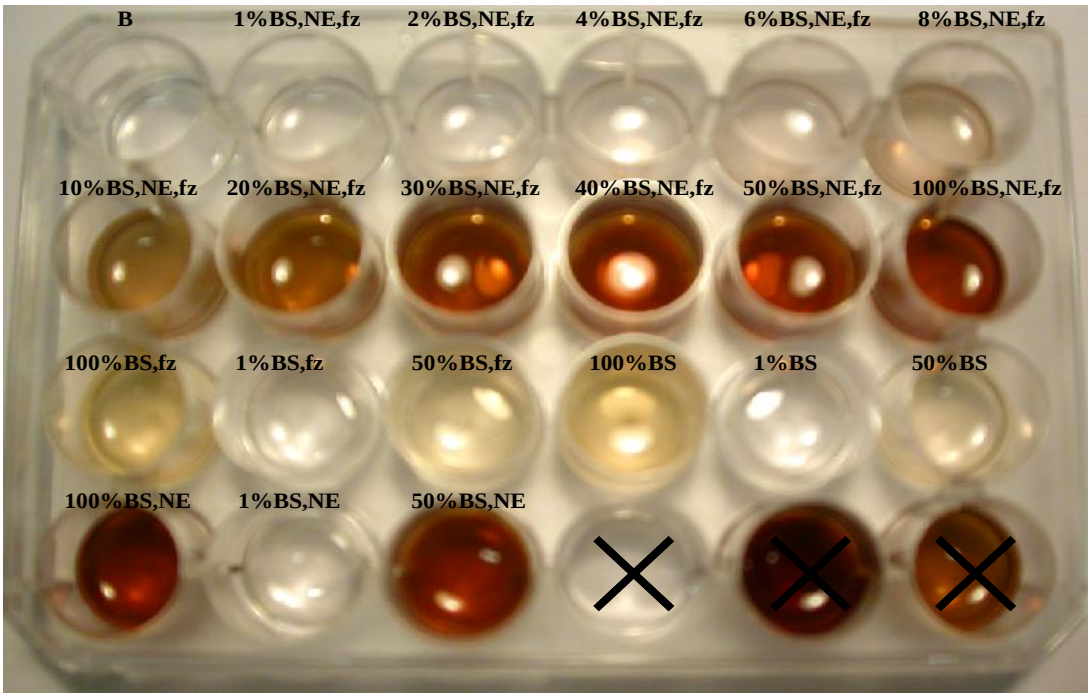


Fig.5.24 Pictures of the effect of NE on Tf Fe(III) binding at different serum concentrations. Aliquots of this 24 well plate were analysed using a Varioskan spectrophotometer at A560 nm. As shown in the Fig., different volumes of serum were mixed with PBS, NE and fz, which were incubated at room temperature for 24 hr. The addition of NE caused a redox reaction within the serum mixture that resulted on the development of a brown colouration and a high absorbance at 560 nm (Fig.5.23).

5.3 SUMMARY

1. It is possible to visualise catecholamine-mediated iron loss from the Fe(III)-iron binding protein transferrin using EPR spectroscopy
2. Use of the iron(II)-specific dye indicator ferrozine revealed that catecholamines induce iron loss from transferrin and lactoferrin via reduction of the transferring-complexed Fe(III) to Fe(II), an iron valency for which transferrin has much reduced binding affinity
3. Catecholamines also facilitate iron loss from lactoferrin via Fe(III) reduction

5.4 DISCUSSION

Iron is an essential nutrient for the growth of the majority of bacteria and in the case of pathogenic bacteria, the availability of host iron constitutes a determining factor for the outcome of an infection. Thus, one of the defence mechanisms of the host is to create an iron-limited environment where all of the iron present is inaccessible to the bacteria, by way of being associated with transport and storage proteins such as transferrin, lactoferrin, ferritin and the haemoproteins (Weinberg, 1978). However, it had previously been shown (Freestone *et al.*, 2000) that catecholamines can form a complex with Tf and Lf facilitating the removal of iron, which then becomes readily available for bacterial growth, even though the exact mechanism by which this process was occurring was not fully understood. The importance of this research became clear considering the implications that the ability of stress hormones to remove iron from Tf and Lf would have on a variety of clinical conditions ranging from sepsis (Neal *et al.*, 2001) to staphylococcal biofilm formation in intravenous lines (Lyte *et al.*, 2003) or stress related intra-abdominal sepsis of intestinal commensal gram negative bacteria (Freestone *et al.*, 2002).

Given the importance of the catecholamine-transferrin (lactoferrin) interaction, a precise elucidation of its mechanism of action needed to be carried out and this chapter was focused on discovering exactly how the CAs can lower the affinity of the iron bound to these high affinity Fe-binding proteins.

5.4.1 Complex formation of CAs with iron associated to Tf

The idea of CAs being able to subtract iron from Tf or Lf it seemed altogether extraordinary considering the affinity that these molecules have for ferric iron. For example, Tf has a very high affinity for Fe(III), which it binds with association constant of ~ 10^{22} M^{-1} (Lewin *et al.*, 2006). Notwithstanding, it is indisputable that catecholamines such as NE can interfere with the iron associated to Tf since the characteristic double peak EPR signal of Tf (Hoefkens *et al.*, 1996) was replaced by a single peak (Fig.5.2) even at NE:Tf ratio as low as 4:1. In addition there is strong evidence of a Tf-CAs complex resulting in iron removal in the literature (Freestone *et al.*, 1999; Freestone *et al*., 2000; Freestone *et al.*, 2003). Our results were similar to previous studies, (Borisenko *et al*., 2000) aimed at understanding the mechanism of toxicity of an analogue of dopamine, 6-OHDA, and its possible use as a chemotherapeutic drug for the treatment of patients with neuroblastoma. Here they showed that the interaction between 6-hydroxydopamine (6-OHDA) and the iron associated to transferrin resulted in the disappearance of the characteristic double peak EPR signal of holo-Tf and appearance of a single peak EPR signal at 150 mT. This suggested the mechanism involved iron release, which was toxic to the organism due to its ability to catalyse Fenton reactions and produce free radicals. Thus, there was strong evidence that the dopamine analogue 6-OHDA released the iron bound to transferrin by reduction. Modelling studies aimed at understanding the pathogenesis of Parkinson's disease showed that the interaction of 6-OHDA with the iron storage protein ferritin also resulted in the release of Fe(II) (Monteiro *et al.*, 1989). However, due to the nature and toxicity of the neurotoxin 6-OHDA towards catecholaminergic neurons and its strong redox power, it was possible that the effects observed were unique to this molecule, thus it was necessary to perform EPR analysis on naturally derived stress related CAs.

We have also shown that the 150 mT single peak EPR signal obtained by incubating inorganic ferric and ferrous iron with NE (Fig.5.3 and Fig.5.4) is similar to the one obtained by incubating Tf with NE, whereas there is no EPR signal obtained by ferric and ferrous iron alone. This seems to indicate that the single peak represents the EPR signal emitted by iron when bound to NE, although the interaction of NE with inorganic iron appear to be much slower than when the iron is associated with Tf, as can be seen comparing the signal at time 0 of Figs. 5.3 and 5.4 with the one of

Fig. 5.2. However, this does not indicate that the single peak EPR signal (150 mT) observed is solely characteristic of iron binding by NE, on the contrary it seems to be a signal common to other iron-catechol interaction since it had previously been observed with 6-OHDA (though only with inorganic $FeSO_4$), although EPR analysis should be carried out on other CAs and catechol compounds to confirm this. Moreover, we obtained similar single peak EPR signal when incubating both inorganic ferric and ferrous ion with NE, however there in no suggestion in the literature (El-Ayaan *et al.*, 1998; Martinez *et al.*, 1990) that NE could form a complex with Fe(II), on the contrary there seem to be no binding formation with the reduced form of iron. In fact, NE had also been found to bind with (FeIII) at the active site of phenylalaline hydroxylase (Martinez *et al.*, 1990), whereas the CAs was not able to bind the ferrous form of the enzyme. In addition Gerard *et al.* (Gerard *et al.*, 1999) showed that the equilibrium constant of Fe(III)-CAs complex is much higher (~ 2.0) than the one obtained from the Fe(II)-CAs complex (~ -1.5). This might indicate that the EPR signal obtained from the incubation of NE with ferrous iron (Fig.5.4) could actually be the signal of the complex Fe(III)-NE resulted from NE oxidizing the Fe(II) into Fe(III). El-Ayaan *et al*. (El-Ayaan *et al.*, 1998) showed that incubation of inorganic Fe(II) with NE resulted in Fe(III)NE complex formation which in anaerobic acid solution reacts to yield Fe(II) and the semiquinone form of NE as illustrated in Fig. 5.25. In aqueous solution of neutral pH (the assay conditions in the experiments in this chapter) the hydroxyl residues of NE are in their ionic form, thus there is movement of electrons between the molecular species. It is not unreasonable therefore to suggest that NE could form a complex with Fe(III) associated with Tf, causing its reduction into Fe(II) (which could be taken up by a ferrous iron sink if in proximity, as described in section 5.4.2), lowering the Fe affinity for Tf resulting in its loss. The liberated Fe(II) in the presence of atmospheric oxygen could then be re-oxidised back into Fe(III) and re-form a complex with NE. To support this theory, previous studies performed by Freestone *et al.* (Freestone *et al.*, 2003) showed that NE was able to remove iron from holo-Tf and shuttle the iron through a dialysis membrane in which was then taken up by apo-Tf. Since apo-Tf binds Fe(III) with higher affinity than NE, it seems that it was able to extract the iron(III) from it, alternatively, if the NE was bound with Fe(II) then most likely the apo-Tf would not had been able to extract the iron. Freestone *et al.* (Freestone *et al.*, 2003) have also shown using mutants for

enterobactin synthesis and uptake systems that the ferric binding siderophore enterobactin is essential in inducing norepinephrine-stimulated growth of *E. coli.* Again, this reinforces the idea that NE complex with Fe(III) and not Fe(II).

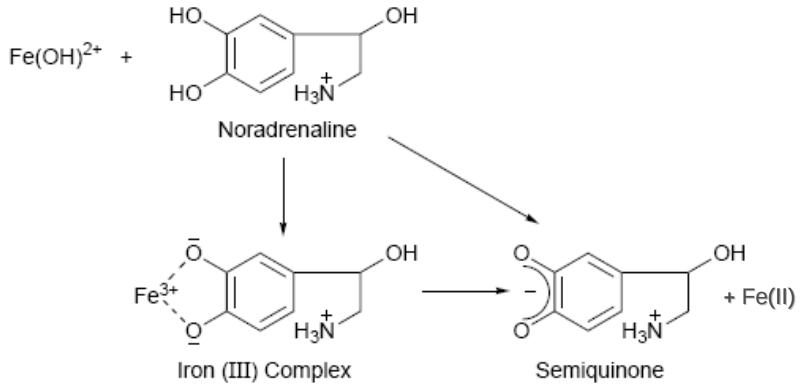


Fig. 5.25 Binding of inorganic Fe(III) with NE and reduction of Fe(III) ↔ Fe(II) by NE. (Fig. adapted from (El-Ayaan *et al.,* 1998)).

As already mentioned, the speed of the complex formation between the iron associated with NE and the inorganic iron, seems to vary greatly, suggesting that the structure of Tf somehow facilitated NE:Fe complex formation. More information on how the chemical structure of Tf may increase the speed of CAs-Fe complex formation can be gained from previous studies (Andersson *et al.,* 1988) on the elucidation of the feedback inhibition mechanism in mammalian catecholamine pathway of tyrosine hydroxylase by NE and Epi. Resonance Raman studies on tyrosine hydroxylase performed by Andersson *et al.* (Andersson *et al.,* 1988) demonstrated the presence of a blue-green bidentate tyrosine-Fe(III)-catecholamine complex, reinforced by the observation of the release-induced by denaturing condition of NE and EPI from tyrosine hydroxylase. Moreover, analysis of the energies of the catecholamine-iron(III) charge transfer transition indicated a mixture of histidine and carboxylate residues at the iron coordination site. The chemical composition at the iron coordination site is also conserved in other non-haem iron enzymes such as phenylalanine hydroxylase and lipoxygenase (Andersson *et al.*, 1988) that had been found to associate with CAs. Comparison between the iron-coordinating residues in the N- and C- lobe of Tf and Lf, which are highly conserved and consist of an aspartic acid, two tyrosine an histidine and a carboxyl group (Wally *et al.*, 2007) (Fig.5.26), with the iron coordination site of tyrosine hydroxylase and other iron associated

enzymes, reveals areas of homology. Therefore it appears that certain highly conserved active site iron binding residues play an important role in the interaction between NE and protein-complexed iron by providing a suitable acidic environment that promotes protein- iron-complex formation and iron reduction (El-Ayaan *et al.*, 1998).

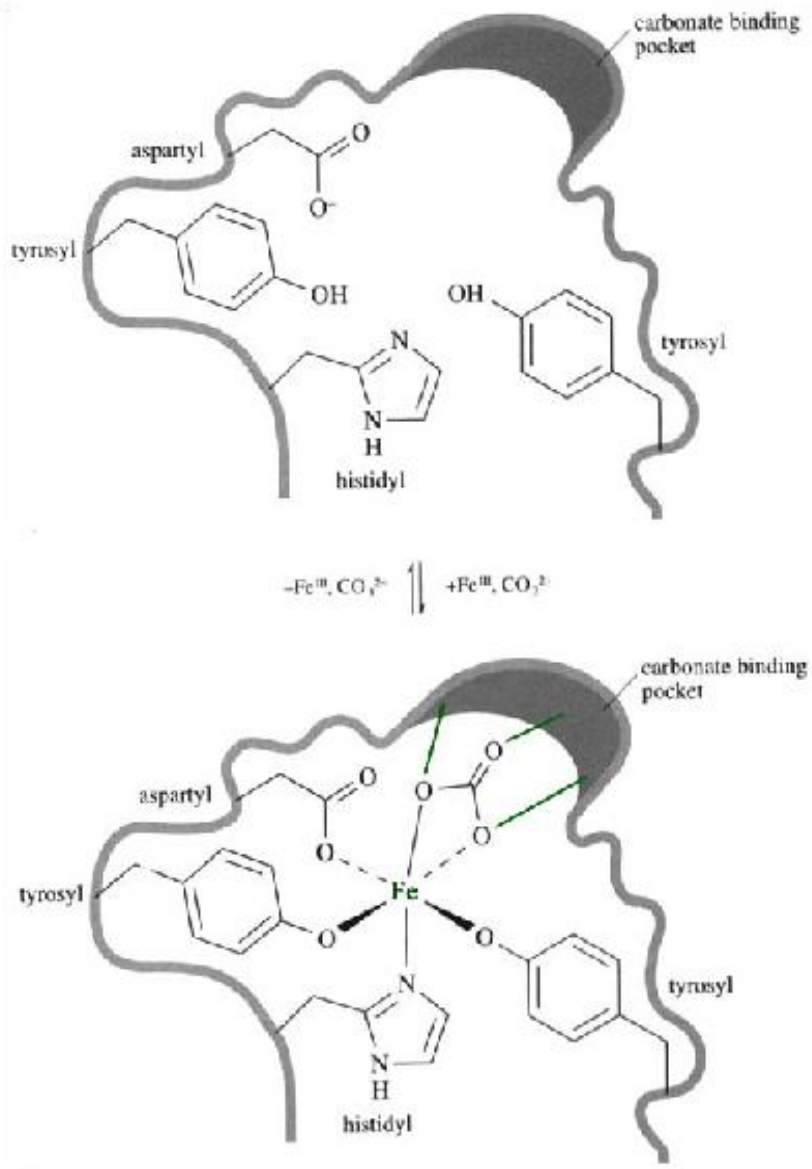


Fig.5.26 Iron binding active site of Tf. Represented above are the highly conserved iron-coordinating residues in the N- and C- lobe of Tf (and Lf), which consist of an aspartic acid, two tyrosine and a histidine group. Iron binding is mediated by a carboxyl group (http://openlearn.open.ac.uk/file.php/2986/S343_1_009i.jpg).

5.4.2 CAs reduce Tf associated Fe(III) to Fe(II)

Although EPR analysis showed that a complex between NE and iron was formed which possessed a characteristic EPR signal that differed from the EPR signal of the iron associated with Tf, it did not elucidate the exact mechanism by which the iron was removed from Tf. Knowing that Tf has very low affinity for Fe(II) (association constant of ~10^3 M^{-1} (Wandersman *et al.,* 2004)) and based on observations in previous studies (Borisenko *et al*., 2000; Monteiro *et al.*, 1989) it was proposed that a possible mechanism by which CAs could remove Fe(III) from Tf was to reduce it to Fe(II). Ferrozine and urea gel analysis have provided the tool that enabled us to determine that the mechanism of Tf iron removal of all the CAs tested (norepinephrine, epinephrine and isoprenaline) was the reduction of Fe(III) to iron Fe(II). The Tf-complexed iron is then lost as Fe(II) which can be taken up *in vitro* by a ferrozine chemical iron sink, whose function in an *in vivo* situation could be performed by a bacterial Fe(II) uptake system such as Feo in *E. coli* (Kammler *et al.*, 1993) which is then used for growth. This could be an additional mechanism by which bacteria can uptake catecholamine-released host-sequestered iron. As mentioned above (section 5.4.1) comparing the current results with the earlier work of Freestone *et al.* (Freestone *et al.,* 2003) it is possible to suggest that one of the mechanisms by which NE supplies iron to the bacteria is by forming a complex with Fe(III) from which the siderophore enterobactin then extracts the iron. Freestone *et al*. (Freestone *et al.*, 20003) also observed a significant difference in Tf-iron uptake when the bacteria were in direct contact with Tf compared with Tf separated by a dialysis membrane. When the bacteria were in direct-contact with Tf and NE their incorporation of Tf iron was ~3.7 fold higher than the non-catecholamine treated control culture. This could indicate that another mechanism by which bacteria uptake NE mediated iron access from Tf when in direct contact could be through the ferrous iron uptake system FeoB, enabling the bacteria to function as a ferrous iron sink, in a similar fashion to that observed *in vitro* with ferrozine. This theory seem the more reasonable when comparing our results in which Tf and CAs were incubated in presence and absence of ferrozine. In the case of Tf incubated with NE, Epi (bitartrate) and Iso in presence of ferrozine, almost all the Fe(III) associated with the Tf protein was reduced to Fe(II) and taken up by ferrozine. This iron loss was also visualised by urea gel electrophoresis analysis (Fig. 5.10, 5.12 and 5.14), which

showed that after approximately 2 hours the Tf was completely shifted to its iron free apo isoform. However, in the samples incubated without ferrozine, the loss of iron was markedly reduced (Fig. 5.10, 5.12 and 5.14), indicating that without a ferrous iron sink to uptake the release Tf iron and shift the reaction toward Fe(III) reduction, NE was either possibly still bound to the Fe within the protein, and thus unable to form other complexes, or NE was reducing, oxidising and forming a complex with Fe(III), which could then be reuptake by the fraction of apo-Tf present forming a redox cycle. This hypothesis is consistent with the findings of Freestone *et al*. (Freestone, *et al.*, 2000) who used ^{55}Fe-labelled Tf, ^{3}H-NE and size exclusion chromatography to show that NE could remove Tf iron under denaturing conditions (urea gel electrophoresis) but that under native conditions (size exclusion chromatography), the Tf, the radiolabelled iron and ^{3}H-NE all remained associated with each other.

It was not possible to determine whether this mechanism could be applied to all CAs since I could not observe iron reduction from Tf in the samples incubated with dopamine and dobutamine, although this was most likely due to different chemical forms in which they were sold (hydrochloride salts), whereas the other CAs were purchased as bitartrate salts. The presence of chloride anion in the CAs tested seemed to greatly reduce the DO or DOB reduction of Tf Fe(III) into Fe(II). Iron release from Tf was also reduced, as can be seen by comparing the result obtained with Epi bitartrate and Epi hydrochloride (comparing Fig. 5.11 and 5.12 with 5.17 and 5.18). Moreover El-Ayaan *et al.* (El-Ayaan *et al.*, 1998) reported the presence of chloride ions markedly interfering with NE- inorganic Fe(III) complex formation by participating in the reaction and binding the iron. Although, it is possible that both chloride and bitartrate anions could potentially be chelating Tf-associated iron, Freestone *et al*. (Freestone *et al.*, 2007c) showed that non-bitartrate containing catechol-compounds such as catechin and neutralised chlorogenic and tannic acids could directly remove Tf iron. In addition, non-catechol-containing bitartrate salts such as normetanephrine did not remove Tf iron. This indicates that the iron-removal associated with the noradrenaline and adrenaline bitartrate compounds are due to the complexation of iron by the catechol moiety, and not the compound anion, reinforcing the idea that indeed the presence of hydrochloride was interfering with the assay.

It was observed that CAs remove the iron firstly from the N- cleft of Tf, as observed in Fig. 5.10, 5.12, 5.14, 5.16, 5.18 and 5.20, in which the first isoform to appear and disappear is the N-mono isoform, suggesting a preference or more easy route of access to the iron in this domain of the Tf protein, which could be the entry point of CAs. This, however it is not entirely surprising since many studies show that although the N- and C- lobe share a 56 % similarity, the rate of iron release from the C-lobe is considerably slower the one of the C-lobe (Wally *et al*., 2006).

It was noted that dobutamine, which is the structurally the largest CA used in our experiments, was the least effective at removing iron from Tf, since the percentage of the Tf holo-form when comparing it with any other CAs was much higher (~30 %), suggesting that molecular size is an important factor for the ability of CAs to remove iron from Tf.

5.4.3 CAs reduce Fe(III) associated with Lf into Fe(II)

Previous studies (Freestone *et al.*, 2000) have shown that NE facilitated the removal of iron from Tf and Lf, and could provide it for bacterial growth in a similar fashion. Therefore, we tested whether the same mechanism observed for catecholamine-mediated removal of Tf-Fe, involving ferric iron reduction, could be applied to Lf as well.

According to our results, the mechanism by which NE was able to remove the iron from Lf also involved reduction of the Fe(III) associated with Lf into Fe(II), which was then taken up by the ferrous iron chelator ferrozine (Fig.5.21), as determined for Tf. Although compared with NE-removal of Tf iron there was a significant difference in the speed by which NE could reduce the iron in Lf. In fact, the NE-induced loss of iron from Lf was a constant and continuous process, taking 24 hours incubation with NE and fz to reduce the valency of the Lf Fe(III) to Fe(II) iron; in the case of Tf it took only 2 hours for the NE to reduce most of the Fe(III). This could be explained by the fact that although transferrin and lactoferrin are quite similar in sequence and structure, and coordinate iron in a similar fashion, they do differ in their affinity for iron (Wally *et al.*, 2007). Wally *et al.* (Wally *et al.*, 2007) showed that the differences between Lf and Tf that could affect the iron binding affinity involved the variation in the structure of their inter-lobe linker, which is helical in Lf, whereas unstructured in Tf. The rigid structure of the helical inter link is also stabilised by a disulfide bond

between the linker and the C-lobe of Lf, which is absent in Tf. Other differences include the fact that the C- lobe in Lf is rotated closer to its N- lobe, than in Tf, and the openness of the C- lobe is higher in Tf since the structure of the C lobe hinge is unstructured in Tf, whereas it is composed of a β-stand in Lf. Thus, the binding site of Lf is more structured than that in Tf, making the removal of Lf-complexed iron more difficult. Biologically, is not surprising that the Lf affinity for iron is higher since the only known function of the Lf protein is anti-bacterial and it is found in body fluids such as lymph, mucosal secretions and milk where it is more likely to come into contact with fluids with a lower pH than serum Tf, and a variety of bacterial species. This alone could explain the differences in the comparative ease with which catecholamines were able to liberate Tf iron compared with Lf observed in our assays.

5.4.4 Possible use of EPR as diagnostic tool?

In this chapter the mechanisms by which CAs can facilitate the removal of iron from the host proteins, transferrin and lactoferrin have been elucidated, and we have shown that when the CA:Fe complex is formed and it is in proximity to bacteria, they can uptake it and use it for their growth. These effects may be clinically important in the intensive care setting, where stress hormone levels coupled with inotrope supplementation could be affecting the Tf-iron binding status of acutely ill patients. Where this therapy is being used it must be allied to a greater monitoring of bacterial infection, highlighting the need for a fast response diagnostic technique to determine the state of CAs-iron interaction. We tested the hypothesis that either ferrozine assays of Tf-iron binding status or EPR analysis performed in body fluid such as serum (or blood) could have a potential role as diagnostic tool for the detection of the catecholamine-compromised iron binding state of Tf. Although we established that the use of ferrozine analysis may not be the most suitable technique to determine the reduction of Fe(III) associated with Tf in complex organic fluids such as serum, EPR analysis could be used as an alternative diagnostic tool. EPR analysis could be performed on blood samples of ICU patients to whom inotropes were administered to determine whether their Tf was compromised causing the blood to become a less inhibitory/more bacterial friendly environment, and thus more likely to be infected. EPR would be a simple analysis and the test would require only a small amount (~0.4 ml) of blood to be frozen and to determine its EPR spectrum. The feasibility of the

use of this technique is supported by the fact that Tf has a unique characteristic double peak EPR signal of 150 mT (Borisenko *et al.*, 2000; Hoefkens *et al.*, 1996) that becomes a single EPR peak of 150 mT when associated with CAs. Currently, one major limitation is that the current method used to produce the frozen Tf pellet needed for EPR analysis (Muralikuttan, 2006) is quite rudimentary and would be in contradiction to health and safety regulations for hospitals due to the use of open liquid nitrogen. This problem could be overcome with the use of an automated enclosed sample collection system connected to an adequate computer program.

5.4.5 Future work

More studies should be carried out to establish whether EPR analysis is a suitable diagnostic to determine whether the serum Tf in severely ill ICU patients is compromised, and therefore associated with increased risk of infection. Thus more EPR analysis should be performed on a wider variety of endogenous catecholamine stress hormones and inotropes to determine whether the single peak EPR signal is shared between all CA-Tf-Fe complexes. Although, the fact that Borisenko *et al.* (Borisenko *et al.*, 2000) obtained similar results with 6-OHDA to the present study, is encouraging. Moreover, if this technique is to be proposed as diagnostic tool more experiments using human body fluids need to be carried out, using ICU patient blood samples.

Chapter 6 – Additional investigations of the interaction of *Escherichia coli* E2348/69 with catecholamines stress hormones

6.1 INTRODUCTION

6.1.1 Protein profiles and iron storage analysis of *E. coli E2348/69* strains exposed to catecholamines

In the last century it has been widely demonstrated that stress is correlated to the aetiology of a variety of medical conditions including increased susceptibility to infection. In this context in the 1990s a new field of research evolved called microbial endocrinology (section 1.5) which has contributed greatly to the general acceptance that bacteria inhabiting a host can sense when that body is under stress, are capable of directly interacting with mammalian catecholamine stress hormones, using them to enhance their growth and up-regulate expression of virulence factors (Freestone *et al.*, 1999; Freestone *et al*., 2000; Freestone *et al*., 2002; Lyte *et al.*, 1992; Lyte, 1993; Lyte, *et al.*, 1996a; Lyte *et al.*, 1997a; Lyte *et al*., 1997b; Lyte 2004; Sperandio *et al.*, 2001; Sperandio *et al.*, 2003; Vlisidou *et al*., 2004).

In the previous chapters (chapter 3 and 4) it was shown that bacteria are able to directly bind to transferrin and lactoferrin through their outer membrane porins, although the binding of these proteins alone is not necessarily favourable to the bacteria, in terms of the antimicrobial activity of lactoferrin (Caccavo *et al.*, 2002; Ong, *et al.*, 2006; Valenti *et al.*, 2005). However, when there are catecholamines present at the same time as the lactoferrin, the situation can drastically change in favour of the bacteria. It was demonstrated (chapter 5) that catecholamines can form a complex with transferrin and lactoferrin which causes the reduction of their associated iron(III) into iron(II) to which the affinity of Tf and Lf is substantially reduced, enabling the bacteria to highjack and utilise the iron. However, the effects that catecholamines have on the bacteria are not only restricted to providing the iron for bacterial growth but it has also been observed that they stimulate the expression of bacterial virulence factors in a variety of bacterial species (Lyte *et al.*, 1996a; Lyte *et al.*, 1996b; Lyte et al., 1997a; Lyte *et al.*, Lyte *et al.*, 2003). In order to investigate how catecholamines alter the protein profile of the bacteria and the storage of iron within the cytoplasm, a preliminary study into the biochemistry of the bacteria under the influence of catecholamines, using SDS PAGE and trace metal analysis was undertaken.

6.1.2 Characterisation of *E. coli E2348/69 luxS* mutant

The final aspect of the CAs-response mechanism that will be investigated in this chapter, involves the characterisation of *luxS* mutants in *E. coli* E2348/69. The l*uxS* gene has been previously shown to have metabolic or/and cell signalling functions in a variety of species (Vendeville *et al.*, 2005). Sperandio *et al*. using microarray analysis linked *luxS* with the role of global regulator after showing that the expression of over 400 genes were altered in an *E. coli 0157:H7 luxS* mutant (Sperandio *et al*., 2001). Moreover, they suggested the involvement of *luxS* in regulation of type III secretion, motility and LEE expression in *E. coli* 0157:H7 (Sperandio *et al.*, 2001; Sperandio *et al.*, 2003) as well as participating in cross-communication between CAs (EPI and NE) and the *E. coli luxS*-dependent AI-3 response system (Clarke *et al.*, 2006; Kendall *et al.*, 2007; Sperandio *et al.*, 2003). Keeping these notions in consideration, it was unexpected to find that microarray analysis of NE-treated WT *E. coli* 0157:H7 here in Leicester, did not show any significant difference in the expression of genes involved in quorum sensing such as *luxS, LEE,* or *qseAB/EF* (Freestone *et al.* unpublished data). Therefore, after obtaining the plasmids used for the construction of the *E. coli* 0157:H7 *luxS* mutants, they were used here to make *luxS* mutant in several *E. coli* strains using the same mutation strategy adopted by Sperandio *et al.* (Sperandio *et al.*, 2003) which involved the insertion of a tetracycline (Tet) or a chloramphenicol (Cm) cassette within the *luxS* gene. In addition, the creation of a *luxS* in frame deletion mutant, a premature termination mutant (Term) and mutants with a Cm cassette insertion were also made for comparison purpose by Dr Haigh (Department of Genetics, University of Leicester). The mutants were also constructed in several *E. coli* strains including the 0157:H7 strain used by the Sperandio group, in this chapter I will present a preliminary characterisation of the *luxS* mutants in *E. coli* E2348/69, looking into their growth rate in presence and absence of CAs (Sperandio *et al*., 2003).

6.2.1 CAs effect on membrane protein expression

In order to determine how CAs were affecting proteins expression, inoculums of *E. coli* were grown for 16 hours in DMEM or DMEM-serum media with and without the addition of 100 μM of CAs (NE, EPI, DO) or 100 μM of $Fe(NO_3)_3$. DMEM was chosen in preference to the SAPI medium allow bacterial growth in the absence of serum and CAS (SAPI is too nutritionally poor to support bacterial growth when used alone). Cultures were also grown in presence of both CAs and $Fe(NO_3)_3$, to help distinguish CA-specific effects on bacteria from the iron-related effects of the CAs. The bacteria were harvested and the total membrane protein fractions were prepared as described in section 2.2.1.4. The amount of protein present in the membrane extracts was measured and normalised, and equal amounts of protein were separated by SDS-PAGE. Fig. 6.1 shows that the total membrane protein profiles of *E. coli* grown in the presence of catecholamines (in this particular gel only proteins from the NE and DO-treated cultures are shown, however the protein profile of the bacteria grown in EPI is similar to the other CAs (data not shown). The proteins extracted from the CA-treated bacteria were more similar in appearance to the control than to the protein profile of the bacteria grown in excess iron. For the bacteria grown with iron, the pattern of protein expression changed, affecting the expression of iron-regulated proteins. However, the protein profile of the bacterial sample grown in presence of both iron and CAs was identical to the one of the bacteria grown in presence of iron alone.

Although the total membrane protein preparation gave a general idea of the protein profile of *E. coli* grown in presence of CAs and iron, in order to identify proteins showing differences in expression, a less complex (protein species rich) outer membrane protein preparation was used. Fig. 6.2 show that the outer membrane protein profile of bacteria grown in presence of CAs again showed more similarity with the protein profile of the control bacteria, and the expression of iron regulated proteins did not seem to be affected by the catecholamine. In the table in Fig. 4.2 (4.2c) the proteins that are differentially expressed in bacteria grown in presence of CAs are shown. When compared with the control, up-regulation was observed of the expression of proteins involved in favouring the entry and attachment of the bacteria

to the host cells (outer membrane protein X), in the formation of pili (Outer membrane lipoprotein bfpB) or the attachment protein intimin. These proteins were repressed in the bacteria grown in iron repleted conditions. Furthermore, the outer membrane proteins OmpA and OmpC, which have been previously found (chapter 3 and 4) to be involved in the binding of Tf and Lf (OmpC) and in the mechanism of CAs dependent uptake of iron (OmpA), were apparently down-regulated in the bacteria grown in an excess of iron. However, proteins involved in cell growth and cell wall formation (Membrane-bound lytic murein transglycosylase E, Lipopolysaccharide core biosynthesis protein rfaZ and N-acetylmuramic acid 6-phosphate etherase) were expressed in bacteria grown in presence of iron but not in the control and CA-treated bacteria.

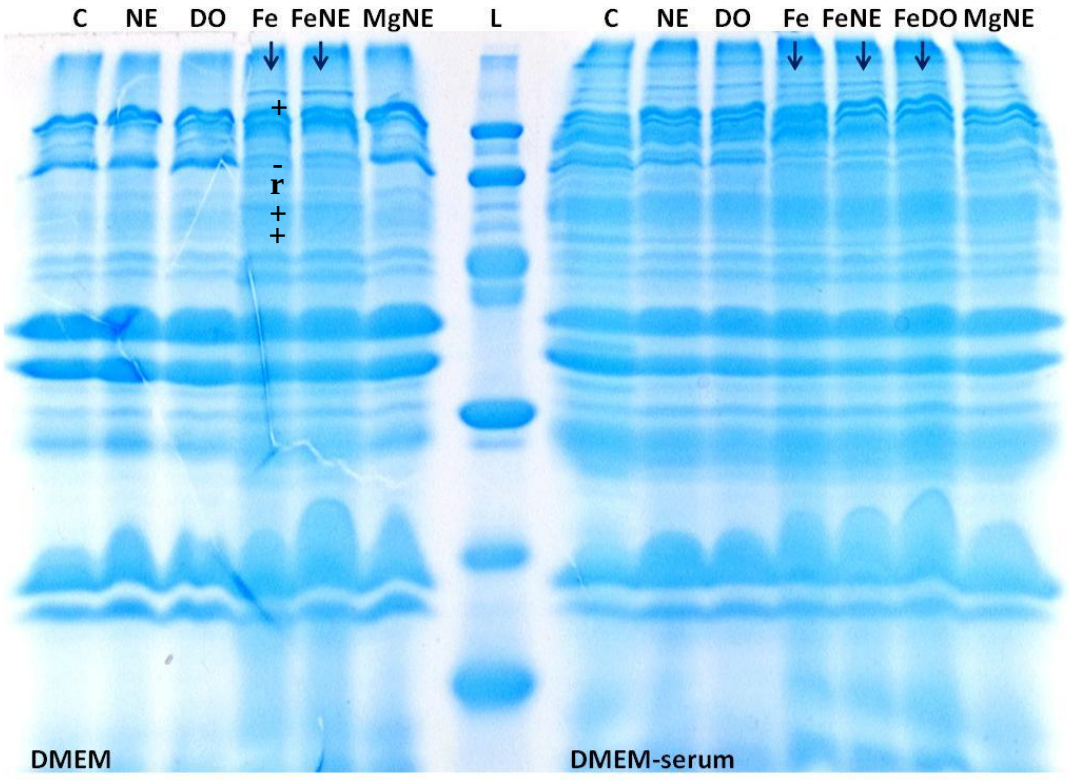
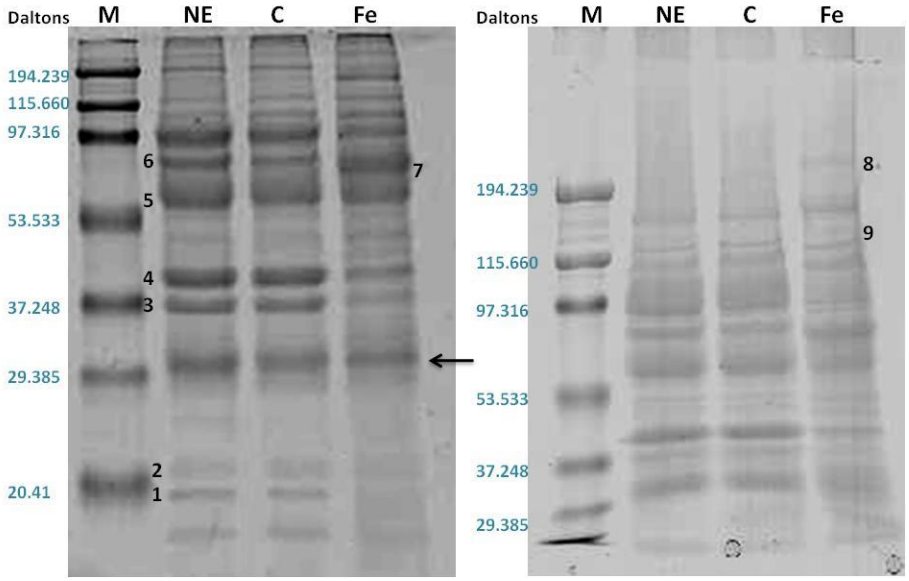


Fig.6.1 12% SDS-PAGE of the total membrane proteins profiles of CA and Fe-treated *E. coli*. *E. coli* was grown overnight (16 hrs) statically in CO_2 at 37 ^{o}C in DMEM medium or serum-DMEM medium (control, C) with additions of 100 μM of catecholamines (NE or DO); 100 μM of $Fe(NO_3)_3$ (Fe); both CAs and iron (FeNE, FeDO); NE and 100μM $MgSO_4$ (MgNE). The bacterial cultures grown in the presence of Mg and NE or DMEM or DMEM-serum ware considered as controls. Key: + present in iron conditions only; - absent in iron conditions; r repressed in iron conditions. The membrane protein profiles show that *E. coli* grown in presence of CAs is more similar to the controls than the iron-excess treated bacteria. Addition of Fe affected the profile of the NE-treated bacteria, while Mg did not repress Addition of iron to the catecholamines results in the expression of iron-regulated proteins not seen with the NE alone. The black arrows mark the samples in which Fe was present.

6.2a **6.2b**



6.2c

Bands	Proteins identity	M_r ~ kDa	Function	C	CA	Fe
1	**Outer membrane protein X precursor**	17	Promote bacterial adhesion and entry in mammalian cells	+	+	-
2	**Peptidoglycan-associated lipoprotein precursor**	22	Involved in maintaining the cell envelope integrity; associated with the Tol system	+	+	↓
3	**OmpA**	35	Porins involved in CAs dependent iron acquisition	+	+	↓
4	**OmpC**	37	Porin involved in Tf and Lf binding	+	+	↓
5	**Outer membrane lipoprotein BfpB**	58	Involved in the biogenesis of Bundle-Forming Pili	+	↑	+
6	**Intimin**	94	Attachment protein	+	↑	↓
7	**Lipopolysaccharide core biosynthesis protein RfaZ**	81	Involved in alternative pathway for the synthesis of rough LPS not substrate for O-antigen attachment	-	-	+
8	**N-acetylmuramic acid 6-phosphate etherase**	197	Required to utilise peptidoglycan fragments obtained from environment or endogenous cell wall	-	-	+
9	**Membrane-bound lytic murein transglycosylase E**	118	Involved in bacterial growth	-	-	+

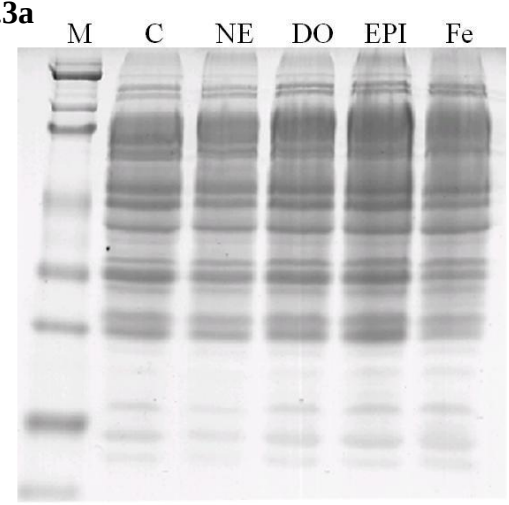
Fig.6.2 Outer membrane proteins profiles of CA and Fe-treated *E. coli*.: 12 % (Fig.6.2a) and 8 % (Fig.6.2b) SDS-PAGE gels of the outer membrane proteins of *E. coli* grown in presence or absence (C) of 100 μM of NE or 100 μM Fe in DMEM-serum for 16 hrs in a CO_2 incubator at 37 ^{o}C. Proteins that showed differences between the profiles were sequenced and identified by PNACL and are summarised in table 6.2c (+ : present; - : absent; ↑ : upregulated; ↓ : downregulated). The protein bands marked by the black arrows show similar intensity confirming that the difference between the samples was due to difference in protein expression and not to different loading concentrations.

6.2.2 Detection of internal bacterial iron and proteomic analysis

It has been shown that CAs such as Epi and NE can form complexes with the host iron binding proteins transferrin (TF) or lactoferrin (LF), facilitating iron removal from these proteins, which become available to the bacteria to utilise (Freestone *et al.*, 1999; Freestone *et al.*, 2002; Lyte *et al*., 1996b; Lyte *et al.,* 1997a; Lyte *et al.,* 1997b; Lyte *et al.,* 2003). Notwithstanding the fact that the presence of CAs in a serum-based media allowed the bacteria to grow by providing them with Tf (or Lf) derived iron, the membrane protein profiles in section 6.2.1 suggested that the CA-treated *E. coli* did not seem to be iron repleted, since the expression of several iron-regulated proteins was not affected by the presence of CAs. In order to examine whether the bacteria grown in the presence of CAs were iron repleted, the intracellular iron levels in the total protein extracts of *E. coli* grown in serum-SAPI in presence or absence of CAs and iron were analysed by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) at the Department of Geology (University of Leicester) using a JY Ultima 2 ICP-OES. ICP-OES is a technique that allows the detection of trace elements at concentrations as low as <10 ppm present in biological matter and naturally occurring material such as water, sediments, soils, rocks etc. This technique is based on a type of emission spectroscopy using inductively coupled argon plasma to produce excited atoms and ions that then emit electromagnetic radiation at wavelengths characteristic of a particular element whose emission intensity is indicative of the concentration of within the sample being analysed, in this case iron (http://en.wikipedia.org/wiki/ICP-AES). The protein concentrations of the extracts analysed by ICP-OES were normalise and similar in concentration (Fig. 6.3a). However, even though it has been shown that catecholamines deliver iron to bacteria, the data in Fig.6.3b shows clearly that that there was no significant difference ($p>0.05$) between the total levels of iron present in the bacteria grown in serum-SAPI alone or with the addition of CAs. The iron concentration of bacterial cells grown in excess iron was significantly ($p<0.05$) higher than both control and the CAs induced *E. coli*. These results indicate that the bacteria grown in presence of CAs are not iron repleted, which concurs with the protein profiles shown in Fig.6.1.and 6.2. The ICP-OES protein extracts were also separated into total membrane and cytoplasmic protein fractions, and analysed by 2-D PAGE. The cytoplasmic profile is shown in Fig.6.4a and the total membrane protein profile in Fig.6.4b. Overall, Fig.6.4 again shows that

the profile of *E. coli* grown in serum-SAPI in the presence of CAs (in this case NE) is more similar to the protein profile of control bacteria than to the bacteria grown in excess iron. This data reinforces the results described in section 6.2.1, and suggests that although bacteria grown in presence of CAs can acquire enough iron from Tf or Lf to grow, the amounts of iron they are internalising is not enough to affect the expression of certain iron regulated proteins. The protein profiles of bacteria grown in the presence of DO and EPI were also analysed by 2D SDS PAGE and the results were similar to the ones obtained for NE were observed (data not shown).

6.3a



6.3b

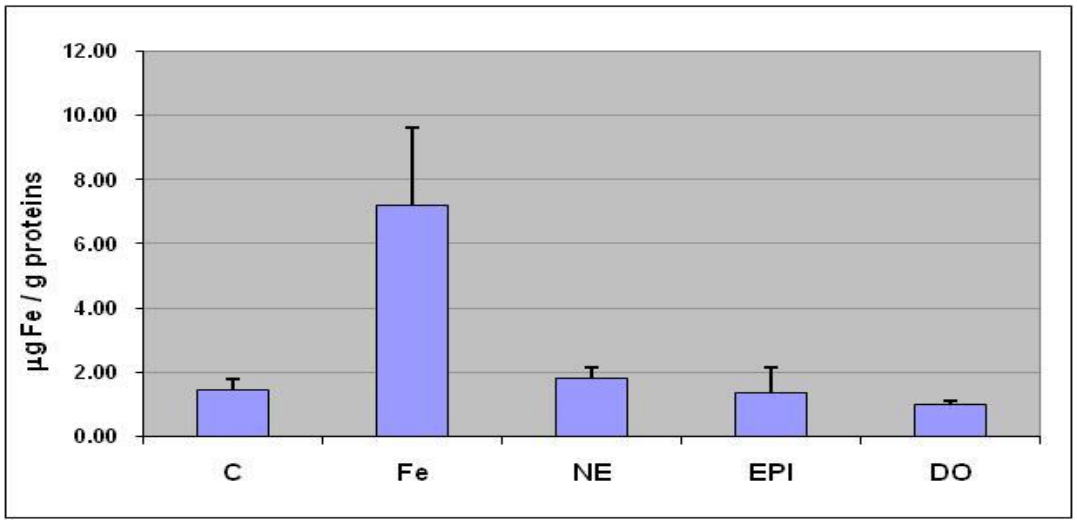


Fig.6.3 Trace metal analysis of cytoplasmic iron storage of CA and Fe-treated *E. coli*. Inoculums of *E. coli* were grown overnight in serum-SAPI in the presence or absence of 100µM NE, EPI, DO or $Fe(NO_3)_3$. Extracts were made of the bacteria, and total protein concentrations measured, and normalised; the similarity in protein levels was also checked by SDS PAGE (Fig.6.3a). The protein extracts were then analysed by ICP-OES to determine the level of iron present (Fig.6.3b). The data shows that the levels of intracellular iron were very similar between CA-treated and control bacteria ($p>0.05$).

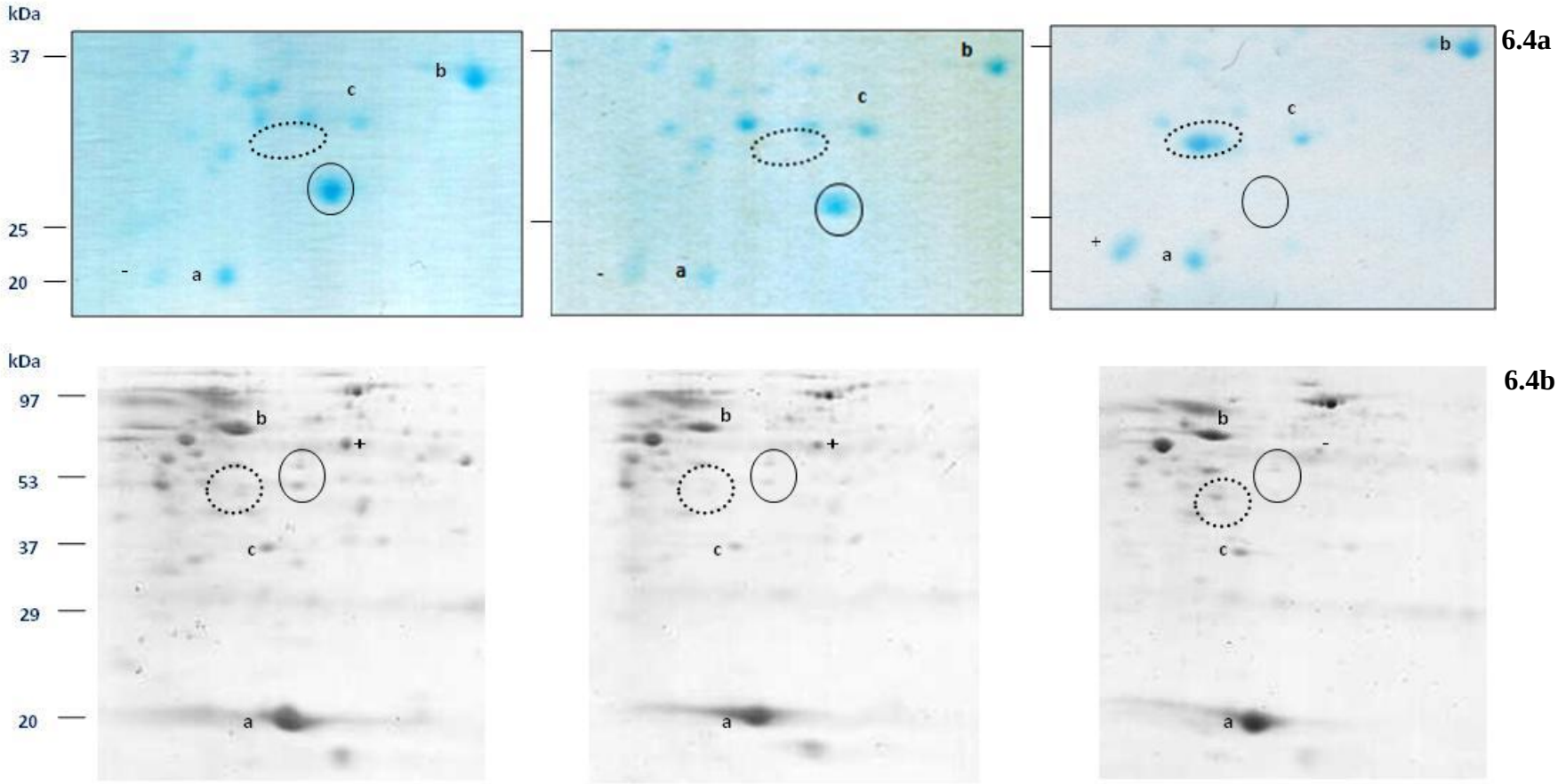


Fig.6.4 2-D PAGE total membrane and cytoplasmic protein profiles of NE and Fe-treated *E. coli*. Portions of the protein extracts analysed by ICP-OES were separated into cytoplasmic (Fig.6.4a) and total membrane proteins fractions (Fig.6.4b) and analysed by 2D SDS-PAGE. The bands labelled with a,b and c were used as references to compare loading between the gels. Protein bands encircled by the dotted line were expressed only in iron excess; whereas protein bands encircled by continuous line were expressed in the control sample and in the bacterial sample grown in CAs. Bands labelled with + or – were respectively upregulated or downregulated.

6.2.3 Intra- and Inter-species signalling in *E. coli* E2346/89

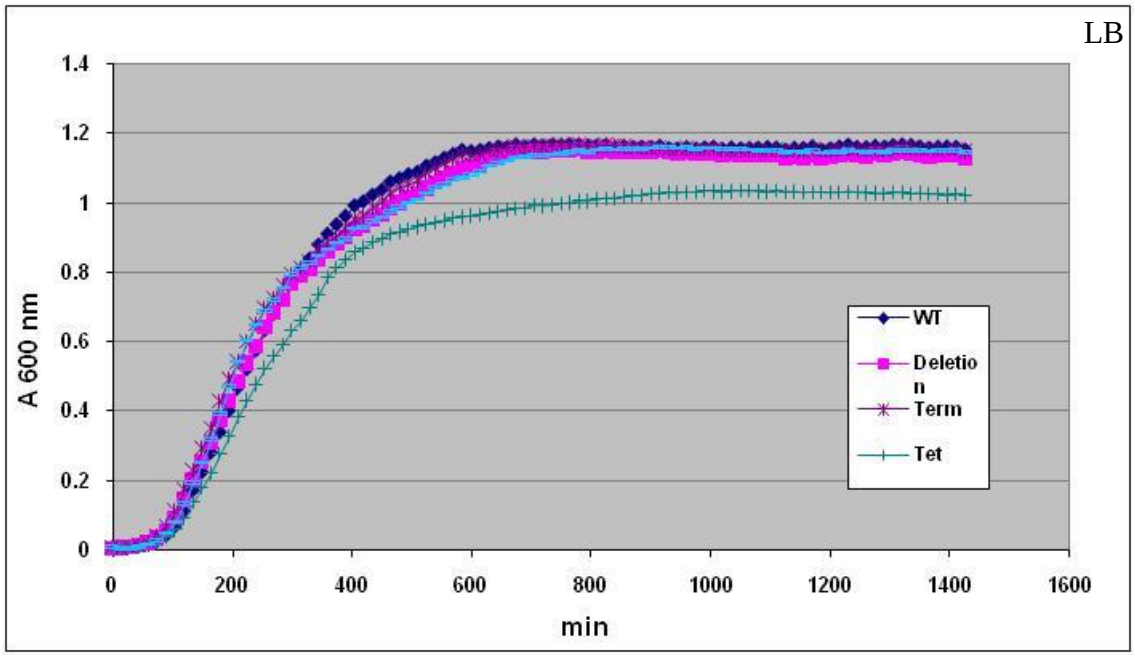
The four *E. coli E234/69 luxS* mutant strains analysed were created using the following strategies:

- Deletion: the *luxS* promoter is intact but the resultant transcript encodes only three amino acids;
- Term: the luxS gene is intact but a premature stop codon TAA has been created at codon 4;
- Tet: insertion mutation of the *luxS* gene with tetracycline cassette;
- Cm: insertion mutation of the *luxS* gene with chloramphenicol cassette.

All the mutants were validated using PCR and DNA sequencing.

Growth profiles of *E. coli* E2348/69 WT and mutant strains were obtained by growing the bacteria in LB or DMEM overnight at 37 ^{o}C. Next day 1:1000 dilutions of the cultures were transferred into fresh LB and DMEM and incubated at 37 ^{o}C for 24 hours; OD_{600} readings were taken every 15 minutes. From Fig. 6.5 it can be seen that the deletion, Term and Cm *luxS* mutant strains growth in LB (Fig.6.5a) and DMEM (Fig.6.5b) were similar to that of their WT parent. In contrast, the Tet mutants showed marked growth reduction in both LB and DMEM media when compared with the WT strain. The *luxS* mutants were then tested on their ability to grow in a serum-based media (serum-SAPI) in presence or absence of catecholamines (NE, EPI or DO) (Fig.6.6). The data in Fig.6.6 shows that the deletion, Term and Cm *luxS* mutant strains showed growth responses to the CAs that were comparable to the WT. Once again, the Tet mutant grew more poorly in serum-SAPI, which may explain why it had a generally reduced response to the CAs, particularly to EPI. The data also shows very clearly that the *luxS* gene is not required for catecholamine-mediated growth induction.

6.5a



6.5b

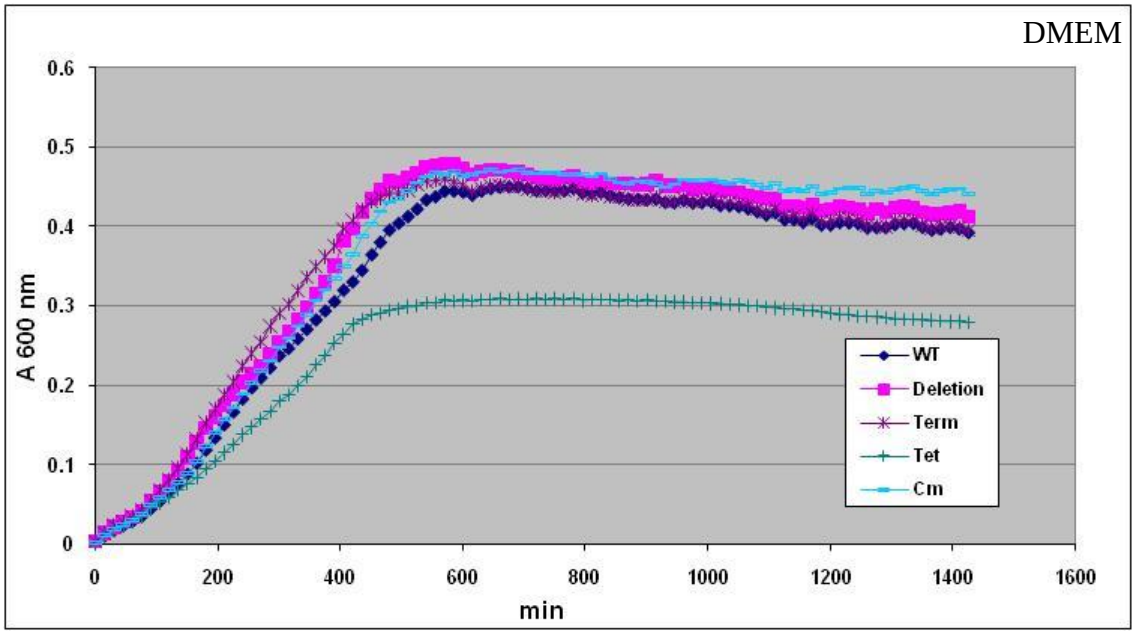


Fig.6.5 Growth curve of *luxS E. coli E2348/69* mutants and WT in LB and DMEM. Inoculums of *E. coli* and the *E. coli luxS* mutants shown were grown o/n in LB or DMEM. Next day 1:1000 dilutions of the overnight culture were transferred in fresh LB or DMEM and incubated at 37 ^{o}C for 24 hrs, with OD readings at absorbance of 600 nm taken every 15 min. The growth curve of nearly all the *luxS* mutants were similar to the one of the WT, apart from the *E. coli* mutant in which the *luxS* gene was inactivated by inserting a Tet cassette, in which the growth was consistently poorer than the WT in both LB and DMEM media.

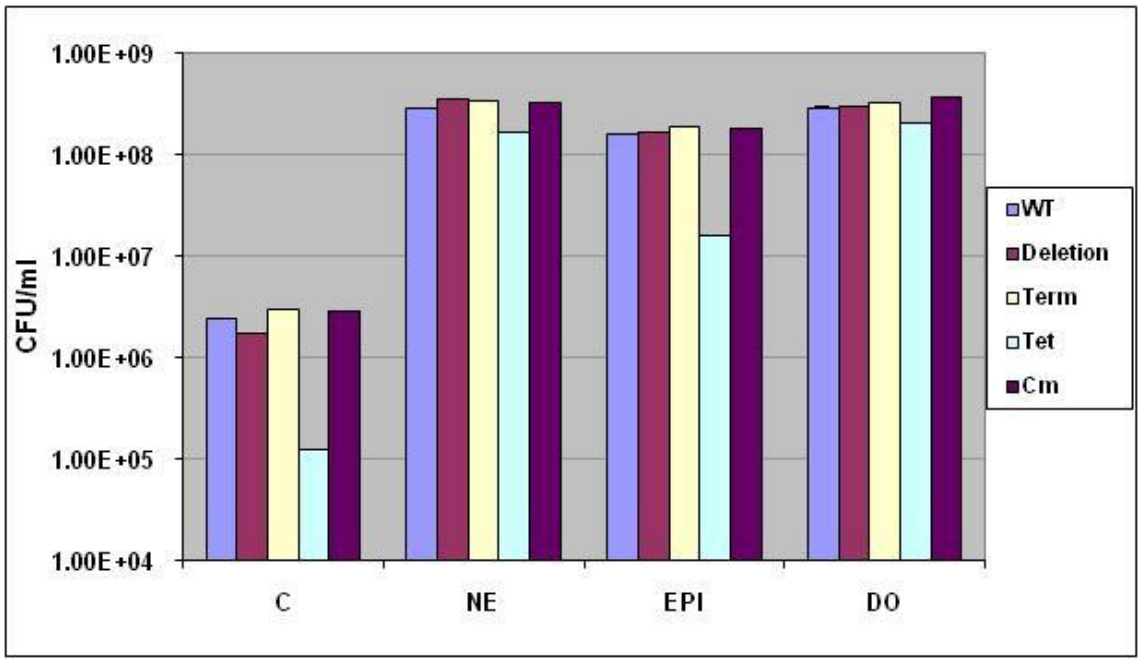


Fig.6.6 Effect of CAs on the growth of *E. coli* E2348/69 WT and *luxS* mutants. Starting inoculums of about 10^2 bacteria were grown in serum-SAPI in presence or absence 100 μM of CAs (NE, EPI, DO) at 37 ^{o}C in CO_2 incubator for 16 hours. The CA-induced bacterial growth in serum-based media of the mutant strains was similar to the one of the WT. Only the *luxS* Tet mutant showed reduced growth both in presence and absence of CAs, particularly to EPI.

6.3 SUMMARY

1. The protein profile of *E. coli* grown in presence of CAs are more similar to the protein profile of control bacteria than bacteria grown in excess iron
2. The expression of iron-regulated protein in *E. coli* is not affected by the presence of CAs in serum-based media.
3. Bacteria grown in presence of CAs in serum-based media are not iron repleted.
4. Proteins involved in entry and attachment of the bacteria to the host are upregulated in *E. coli* grown in the presence of CAs in serum-based media.
5. The *E. coli* E2348/69 *luxS* gene is not required for catecholamine-growth induction.

6.4.1 Effects of CAs on proteins expression and iron storage in *E. coli E2348/69*

The growth of CA exposed bacteria is enhanced dramatically when compared with control bacteria grown in a minimal serum-based media (chapter 4) and is mainly due to the increased iron bioavailability (Freestone *et al*., 2000). However, it addition to enabling the bacteria to highjack iron from Tf or Lf CAs have also been shown stimulate a variety of proteins important to *E. coli* virulence (Lyte *et al.*, 1992; Lyte *et al.*, 1996a; Lyte *et al.*, 1996b; Lyte *et al.*, 1997a; Lyte *et al.*, 1997b; Lyte *et al.*, 2003). Therefore, CAs affect bacteria more profoundly than just by providing iron for their growth. Although, the fact remains that iron removal from Tf or Lf is a major contribution toward the ability of the bacteria to replicate in such a number to become worrisome for their host. Thus, it is reasonable to consider that the CAs would allow bacteria to acquire enough iron to affect the expression of iron-regulated proteins. However, in this chapter it was shown that the level of total iron present in the bacteria induced to grow by the addition of CAs was not comparable to the level found in bacteria grown in the presence of excess iron. Thus, CA-stimulated bacteria are not iron repleted, and the iron acquired through CAs, although sufficient for growth, is not adequate to turn off expression of iron-regulated proteins, many of which will be virulence genes. In fact, the expression of proteins involved in attachment and entry into mammalian cells were not suppressed by the presence of CAs (Fig.6.2), although they were down regulated in the iron-fed bacteria. These proteins included the outer membrane protein X (OmpX), outer membrane lipoprotein BfpB and intimin. OmpX is a 17 kDa protein that plays a fundamental role in bacterial adhesion and entry into mammalian cells as well as participating in the resistance against the complement system (de Kort *et al.*, 1994; Vogt *et al.*, 1999). The outer membrane lipoprotein BfpB is required for the biogenesis of bundle-forming pili, which play a role in the pathogenesis of enteropathogenic *E. coli* by causing the formation of bacterial microcolonies that bind to the epithelial cells of the small intestine (Ramer *et al.*, 1996). Intimin was not only expressed in the bacteria grown in presence of CAs but it was actually upregulated when compared with the control sample. This latter protein is essential for the formation of the attaching and effacing lesions, through which *E. coli* EPEC mediates its pathogenicity (Kumar *et al.*, 2001; Phillips *et al.*, 2000). The fact that CA affected bacteria were upregulating

virulence factors it is not surprising since it has been demonstrated by others (Lyte *et al.*, 1997a; Lyte *et al.*, 1997b; Vlisidou *et al*., 2004). Moreover, several recent microarray investigations of global responsiveness to CAs indicate that NE can result in the increased expression of genes involved in host cells attachment (Bansal *et al.*, 2007; Dowd, 2007b), although direct comparability between these two studies is not possible due to the use of different culture media and growth conditions.

The OmpA and OmpC proteins (chapter 3 and 4) have been found to be down-regulated in the bacteria that were grown in excess of iron but when grown in presence of CAs, when comparing their outer membrane protein profiles. The fact that these proteins remained expressed at high levels by the bacteria grown with CAs, reinforced the importance of their role in the binding of Tf or Lf and uptake of iron.

It was also interesting to note (Fig.6.2) that the proteins that were found to be up-regulated in the bacterial sample grown in iron excess were all involved in bacterial growth, but not virulence factors. These proteins included the lipopolysaccharide core biosynthesis protein RfaZ, the N-acetylmuramic acid 6-phosphate etherase and the membrane-bound lytic murein transglycosylase E. Lipopolysaccharide core biosynthesis protein RfaZ is synthesised from the *rfaZ* gene which is part of the *rfa* cluster that contains genes involved in the synthesis of lipopolisaccharide (LPS) and the attachment of the O antigen to the core. However, RfaZ is involved in the synthesis and completion of the hexose region of the core which constitute the lipooligosaccharide (LOS) which is a form of rough LPS (R LPS) that arises from a different modification pathway than the LPS core to which O-antigen is attached (Klena *et al.*, 1992a; Klena, *et al.*, 1992b; Schnaitman *et al.*, 1993). N-acetylmuramic acid 6-phosphate etherases are enzymes required for the utilisation and recycling of peptidoglycan fragments obtained from the environment or endogenous cell wall (Jaeger *et al.*, 2008). Whereas the membrane-bound lytic murein transglycosylase E are enzymes involved in the enlargement and division of the sacculus during bacterial growth (Kraft *et al.*, 1998).

6.4.1.1 Future work

Although the use of 1D and 2D SDS-PAGE can give a general overview of the protein expression, to have a more complete idea on how CAs affect the bacteria it would be useful to perform more comprehensive studies involving modern techniques such as Surface-enhanced laser desorption/ionization (SELDI) and microarray analysis (these studies are ongoing in our laboratory).

6.4.2 Is *luxS* involved in catecholamines responsiveness in *E. coli* E2346/89?

Sperandio *et al.* (Sperandio *et al.*, 2001; Sperandio *et al*., 2003) were the first group to report evidence of a global regulatory role for *luxS* in *E. coli* 0157:H7. They also suggested an involvement of the *luxS* in the regulation of type III secretion, motility and LEE expression in the above-mentioned *E. coli* strain. Moreover, evidence has been presented for cross communication between CAs such as EPI and NE and a *luxS*-dependent AI-3 response system (Clarke *et al.*, 2006; Kendall *et al*., 2007; Sperandio *et al.*, 2003). The plasmids (pVS72, pVS98) used to created the *luxS* mutants were obtained from Dr Sperandio and used to recreate the pVS72, pVS98 transposon mutants in several *E. coli* backgrounds including *E. coli* 0157:H7 strains *Sakai, NCTC12900* and *86-24* and *E.coli* 0127:H7 strain E2348/69. The mutant used in the *luxS* microarray and other studies (Sperandio *et al*., 2001; Sperandio *et al.*, 2003) were insertion Tet *luxS* mutants of *E. coli* 0157:H7 strains *86-24* created using construct plasmid pVS72, the other mutant was also an insertion mutation constructed using pVS98 plasmid comprising of a Cm resistance gene instead. As already mentioned in section 6.1.2 for comparison purposes in frame deletion and Term mutants were also made. In this chapter (section 6.2.3) the *luxS* mutants in *E. coli* E2348/69 were tested for their ability to grow in LB and DMEM media and in serum-based media analysed for their ability to respond to the catecholamines EPI, NE or DO. The results in section 6.2.3 consistently showed a reduction in the ability of the *luxS* Tet mutant to grow in all the tested media, both laboratory and host-like, when compared with the WT; the other mutants all showed a similar phenotype to their WT parent. Moreover, the CA-induced growth response was similar in all the *luxS* mutants, although the response pattern of the luxS Tet mutants was reduced possibly as a consequence of its reduced ability to grow in serum-based media. The ability to grow in poor and rich and serum-based media was also tested for the *luxS* mutant in

other *E. coli* strains (Freestone *et al*. manuscript in preparation), although the pattern of response to the CAs was similar in all the mutant strains tested strains, some strain variation were observed in the growth responses to different culture media. For example, when the *E. coli* 0157:H7 (*Sakai*) *luxS* mutants and WT were tested for their ability to grow in succinate as carbon source media, it was observed that Tet and also the Cm *luxS* mutant showed marked growth reduction not seen with the deletion and Term mutants, however in richer media and serum-SAPI media only the Tet mutants showed a reduced growth phenotype. From Fig.6.7 it can be seen that the upstream genes adjacent to the *luxS* gene in the two above mentioned *E. coli* strains are not maintained and they are expressed in opposite directions.

Escherichia coli O127:H6 str. E2348/69

Escherichia coli O157:H7 str. Sakai

Fig.6.7 Genomic map of the *luxS* gene and the upstream and downstream coding regions. The genomic maps of *E. coli* 0127:H6 and 0157:H7 were obtained from NCBI web site (http://www.ncbi.nlm.nih.gov/).

Sequencing of the plasmids used to make the *luxS* mutations (data not shown, Freestone *et al.* unpublished) showed that the *Tet* gene was in reverse orientation to the *luxS* suggesting that these mutants may be polar and affect the expression of upstream and downstream genes. The *Cm* gene was inserted in the same orientation of the *luxS* gene, thus less subjected to polar effects. Although deletion and Term mutant gave more reproducible results between species and therefore represent better mutation strategies, which seemed to be less inclined to cause polar effects and

misleading phenotypes than transposon insertion mutations (at least in this particular case). Studies from our laboratory (Freestone *et al.* unpublished) also showed that none of the deletion and Term *luxS E. coli* mutant in any the strains tested showed the mobility defect that were observed for the Tet mutants.

These results are in concordance with previous studies (Wang, *et al.*, 2005) that analysed the effect of *luxS* deletion mutation on *E. coli K12*. Wang *et al.* showed using microarray analysis that the expression of fewer than 50 genes, the majority of which were involved in metabolism, were found to be different from the WT strain. In addition, no effect on growth and mobility was observed in this mutant suggesting that the differences from their study and the Sperandio study (Sperandio *et al*., 2001; Sperandio *et al*., 2003) were caused by strain variations. However, our study suggested that the difference in phenotype observed seem to be caused by a polar effect or secondary mutations. In *E. coli* 0157:H7 a regulatory RNA gene, *sraD*, is positioned which if disrupted by the tet transposon could explain the variations in the phenotype observed between the different types of *luxS* mutant, and different *E. coli* strain backgrounds.

6.4.2.1 Future work

Given the possibility that the *luxS* insertion mutants used in the Sperandio studies (Sperandio *et al*., 2001; Sperandio *et al.*, 2003) were polar mutants, it would be useful to perform microarray analysis on the deletion, Term *luxS* mutants and the two insertion mutants in *E. coli* 0157:H7 and on the other *E. coli* strains that were mutated, in order to determine whether the results obtained in the Sperandio studies were not an artefact and whether the phenotype observed was conserved between different strains or was indeed specific to *E. coli* 0157:H7 as Wang *et al.* (Wang *et al.*, 2005) suggested. Moreover it will be interesting to investigate role that *luxS* plays in inter-cellular signalling pathways, though the data presented in this chapter clearly shows that *luxS* is not required for *E. coli* inter-kingdom signalling.

Chapter 7 – Discussion

7.1 Stress hormones and increased susceptibility to infection

The consequences of increased CAs levels during a stressful episode (mental or physical) on bacterial growth and the expression of virulence factors, has been widely reported (Freestone *et al.*, 1999; Freestone *et al.*, 2002; Freestone *et al.*, 2003; Lyte *et al.*, 1996a; Lyte *et al.*, 1997a; Lyte *et al.*, 1997b), however the precise mechanism by which these processes occur are still very much under investigation.

There are real clinical implications for the ability of bacteria to recognise catecholamines. For instance, patients in an intensive care environment will not only have a high level of circulating catecholamine in their blood stream, but catecholamine inotropes such as dobutamine and isoprenaline and dopamine will also be administered in cases of septic and cardiogenic shock (Dellinger *et al*., 2004) in order to maintain a minimal arterial pressure to prevent vascular perfusion (Hollenberg *et al.*, 2004; LeDoux *et al.*, 2000). Sepsis caused by overgrowth and translocation of gut bacteria such as *E. coli* is a recurrent surgical complication (Nieuwenhuijzen *et al.*, 1999) and the mortality rate of severe sepsis of ICU patients hospitalised is unacceptably high (Dellinger, 2003; Friedman, *et al.*, 1998). Thus, even though patients are undergoing careful antibiotic prophylaxis, they could die from infections unrelated to their initial condition, originating from their own microflora. Keeping these notions in mind, it is clear that there exists a precarious balance between the positive and detrimental effects of the use of catecholamine inotropes in ICU scenarios, which has not been taken into consideration until now. The involvement of stress hormones in infection is not only restricted to the human medical situation. Stress-related colonisation by pathogenic bacteria such as *E. coli* 0157:H7 and *Salmonella enterica* is commonly observed on farmed animals (Freestone *et al.*, 2008) and could consequently present a potential hazard for humans through the dissemination into the food chain, and causing a major health and economic concern for the meat production industries. It has been previously shown that just a mild handling stressor, such as a single day weighing, was enough to unbalance the microflora of pigs, which resulted in increased number of *E. coli* and other coliform species in the faeces of test animals when compared to controls (Dowd *et al.*, 2007a). This demonstrates that a common management practise considered by humans to not be stressful, could be perceived very differently by the livestock undergoing the stress, in such a manner that could lead to changes of the gut

environment which might favour any pathogen subsequently acquired by the animals. In addition, it has been shown by Toscano *et al.* (Toscano *et al.*, 2007) that bacteria (*Salmonella thyphimurium*) pre-treated with NE before infecting young pigs with the pathogen, were present in greater numbers and more widely distributed in the analysed gut tissues. Given the widespread nature of the CAs and other dietary catecholamines present in human food chain, and their obvious effect on enteropathogenic bacteria (Freestone *et al.*, 2007), these observations should be noted and taken into consideration by farmers and food companies in order to provide better conditions during the husbandry, transport and handling of livestock, in conjunction with greater monitoring of the bacterial population, with the purpose of minimising the risk associated with the consumption of food.

The importance of understanding the effect that catecholamines have on the stimulation of bacterial growth and virulence factors and the role that they play in the outcome of an infection, and awareness on this topic is fundamental both from a medical and economical point of views. The experiments performed during the course of this thesis were aimed to answer some of the questions raised on the mechanism by which CAs can stimulate the bacteria and increase susceptibility to infectious diseases in *E. coli.*

The major difficulty encountered by bacteria during the invasion of a host is the almost complete absence of free iron in the body. This iron is commonly associated with transport and storage proteins such as transferrin, lactoferrin, ferritin and hemoproteins (Weinberg, 1978) and thus inaccessible to the bacteria. It has been previously showed that CAs could complex with Tf and Lf and facilitate iron removal from these proteins, which then becomes available for bacterial uptake, enabling their growth in normally bacteriostatic host fluids such as blood (Freestone *et al.*, 2000; Freestone *et al.*, 2002; Freestone *et al.*, 2007b; Freestone *et al.*, 2008). However it was not clear how CAs could remove iron(III) from Tf or LF, since these iron-binding protein have very high affinity for ferric Fe. In the current study it was demonstrated, using EPR and ferrozine analysis (chapter 5), that CAs can directly bind with the Fe(III) associated with Tf or Lf causing reduction of the Tf/Lf-coordinated Fe(III) into Fe(II), an iron valency for which these proteins have a much reduced binding affinity . Complex formation of CAs to Tf or Lf and reduction of the associated Fe(III) into Fe(II), provides an important vehicle by which pathogenic bacteria can acquire iron,

which can be a fundamental factor for the outcome of an infection and that is normally inaccessible and safely stored into host iron binding proteins. Urea gel electrophoresis of CAs-treated Tf showed that CAs were removing the iron associated with Tf initially from the N- iron binding domain of Tf, suggesting that this domain could be the initial entry point for the CAs. Moreover, other studies have shown that although the N- and C- lobe share a 56 % homology, the rate of iron release from the C-lobe is considerably slower the one of the N-lobe (Wally *et al.*, 2006), which is consistent with our observations. In this it was also demonstrated that CAs were able to remove iron from Lf, however the removal of iron from Lf was considerably a slower process (approximately 24 hours in the presence of an iron sink) when compared with removal of iron from Tf (within one hour in presence of an iron sink). Although Tf and Lf show considerable similarity in protein sequence and structure, and coordinate iron in a similar fashion, they nonetheless display significant differences in their affinity for ferric iron. Wally *et al* (Wally *et al.*, 2007) showed that these differences lay in the variation in the structure of their inter-lobe linker, which is helical in Lf, and unstructured in Tf, making the removal of iron more difficult from Lf, which may explain the comparative differences in the potency of catecholamine effects of Tf and Lf observed in our current study.

Once the iron has been removed from Tf or Lf, the bacteria are free to uptake it through several routes. Freestone *et al.* (Freestone *et al.*, 2000; Freestone *et al.*, 2003) have previously shown using entA and tonB *E. coli* mutants that functional synthesis and uptake enterobactin systems were required for CA stimulated bacterial growth. However, analysis on the ability of these mutants to uptake ^{55}[Fe] associated with Tf mediated by NE (Freestone *et al.* unpublished data), (Fig.7.1), although confirming that enterobactin is important for CAs-induced iron uptake, also showed that when the bacteria were incubated in proximity of [^{55}Fe]Tf there was a drastic ($p<0.05$) increase in the ability of *E. coli* to acquire iron both in WT and mutants strains, when compared to the level of iron uptake by the bacteria that were separated from the [^{55}Fe]Tf by a dialysis membrane. These observations in conjunction with the discovery that the mechanism by which NE can facilitate iron loss from Tf is by reduction, suggest that NE-induce iron uptake from Tf or Lf in *E. coli* involves both the bacterial ferric (enterobactin) and ferrous (Feo) iron uptake systems, when Tf or Lf are in close proximity to the bacteria. Therefore, the uptake of iron seems also to

be favored by the binding of Tf or Lf to the bacteria. Freestone *et al.* (Freestone *et al.*, 2000; Freestone *et al.*, 2002; Freestone *et al.*, 2003) have shown that the ability of Tf and Lf to physically interact with *E. coli* plays an important role in the mechanism of CA-mediated growth stimulation, though, there is no genomic evidence for *Escherichia coli* Tf and Lf binding proteins. In this study it was found that the identity of the Tf and Lf binding protein in *E. coli* was mainly the outer membrane proteins OmpC. However, a portion of the Tf and Lf was also able to bind to OmpA to which binding was more pronounced in the absence of OmpC, as demonstrated by on blot binding assay in the *E. coli* OmpC deletion mutant (chapter 4, Fig.4.16).

^{55}Fe-incoporation CPM/ml				
	Non-contact - NE	**Non-contact + NE**	**Contact - NE**	**Contact + NE**
Wild type	4929	30697	37179	128653
entA	706	198	10056	17199
tonB	1246	723	11094	28477

Fig.7.1 Acquisition of iron from [^{55}Fe]Tf by *E. coli* ferric uptake mutants. *E. coli entA* and *tonB* mutants and WT strain were incubated both in contact or separated by a dialysis membrane with [^{55}Fe]Tf (10^5 cpm) according to Freestone *et al.* (Freestone *et al.*, 2000) and analysed for [^{55}Fe] uptake.

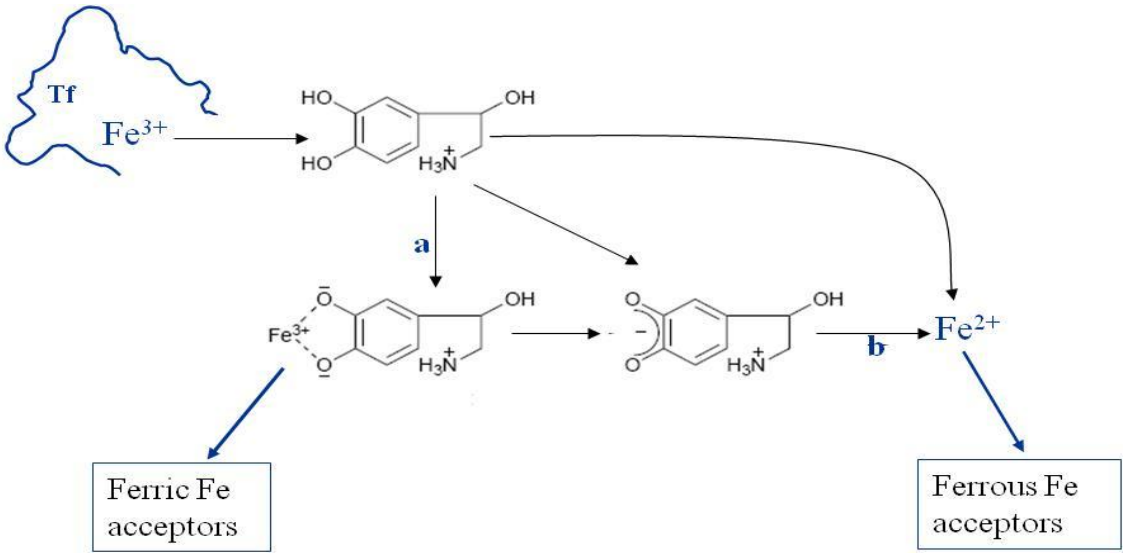


Fig.7.2 Mechanism of reduction of Fe(III) associated with Tf to Fe(II) by NE and routes of iron uptakes by the bacteria. This illustration represents the possible mechanism involved in the reduction of iron associated with Tf by NE and showed that steps from which the different *E. coli* uptake systems can intervene and uptake the iron. The first step (a) shows the complex formation between NE and Fe(III), probably causing conformational changes in Tf/Lf which allows removal of ferric iron as Fe(III)-NE complex enabling enterobactin to uptake Fe(III) from NE, to which it has a major affinity. The second step (b) shows that the NE can also reduce the Fe(III) into Fe(II) and the semiquinone form of NE at which point the free Fe(II) can be uptaken, when in proximity of the bacteria by the ferrous system uptake such as Feo.

From these results it seems that the main function of OmpA porins in the CAs-response mechanism appears to be a role in the internalisation of iron from CA-Fe complexes (chapter 4, Fig.4.23). Freestone *et al.* (Freestone *et al.*, 2000) demonstrated that bacteria can uptake iron from Tf and Lf by direct internalization of CAs-Fe complexes, in which the CAs act as a pseudo-siderophore. This observation is consistent with the current finding that internalization of CA-removed Tf-complexed iron is drastically reduced in the OmpA deletion mutant when compared with the WT and the OmpC strains, and that the OmpA growth response to larger CAs is drastically reduced. This suggests that OmpA could be one entry point for CAs-Fe complex, most likely mainly for larger CAs such as EPI and ISO, although further studies need to be carried out in using radiolabelled catecholamines of different molecular weights in order to confirm the precise role of this protein within the bacterial CAs-response mechanism.

It is well established now that the main mechanism in which CAs induce bacterial growth in *E. coli* (and a variety of other bacterial species) is by providing iron for bacterial growth from the host iron binding proteins Tf or Lf. Therefore it was

surprising when we analysed the intracellular iron levels of catecholamine-growth stimulated that the bacterial cells were not iron repleted (chapter 6, Fig.6.3). Although the *E. coli* cells were obtaining sufficient iron to grow, the amount of iron obtained was not enough to affect the expression of iron-regulated genes. Expression of proteins involved in attachment and entry into mammalian cells were not suppressed by the presence of CAs (chapter 6, Fig.6.2), although they were clearly down-regulated in the bacteria grown in iron excess. Moreover, intimin, which is essential for the formation of the attaching and effacing lesions, through which *E. coli* EPEC mediates its pathogenicity (Kumar *et al.*, 2001; Phillips *et al.*, 2000) was not only expressed in the bacteria grown in presence of CAs but it was actually found to be upregulated when compared with the control cultures (chapter 6, Fig.6.2). It has been previously observed in other species that CAs were able to induce the expression of virulence factors such as PA-1 adhesin in *Ps. aeruginosa* (Alverdy *et al.*, 2000), shiga-like toxins (SLTs) (Lyte *et al.*, 1996a) or production of autoinducers (Lyte *et al.*, 1996b; Freestone *et al*, 1999) in *Escherichia coli* strains. However, this is possibly the first time it was shown that one of the reasons why bacteria became more 'aggressive' in presence of CAs in a serum-based media is due to the fact that they obtain sufficient iron to grow although not enough to turn off virulence genes or at least this is what it has been observed in the pathogenic EPEC *E. coli* E2348/69.

It has been previously shown that bacterial CAs-response is not just simply a consequence of iron acquisition and that the bacteria can sense stress hormones through specific receptors. Freestone *et al.* (Freestone *et al.*, 2007a) using a wide range of antagonists and CAs and observed a specificity of bacterial catecholamine responsiveness regarding the possible role of adrenergic and dopaminergic -type receptors in three major enteric pathogens, *E. coli* 0157:H7, *Salmonella enterica and Yersinia enterocolitica*. From their results, Freestone *et al*. (Freestone *et al.* 2007a) concluded that initial recognition by the bacteria of NE and EPI that results in stimulation of growth is mediated by receptors that show α-adrenergic specificity. Moreover, none of the adrenergic antagonists were able to inhibit DO-induced bacterial growth, whereas dopaminergic antagonists were able to block it. Investigations into the mechanisms by which the antagonists were blocking bacterial growth revealed that they did not affect the ability of CAs to mediate Fe acquisition from Tf or the ability of bacteria to accept Fe from CA-Tf or the induction of CA-AI.

However the α adrenergic antagonists were causing reduction of cellular uptake of radio-labelled NE, supporting the hypothesis that they were blocking a specific signal transduction pathway. Clarke *et al*. (Clarke *et al.*, 2006) showed *in vitro* binding of NE to the two-component regulatory system QseC proposing this protein as the receptor for NE and EPI in *E. coli 0157:H7.* Moreover they suggested that QseC also recognised a novel *luxS*-dependent autoinducer AI-3 and evidence has been presented for cross communication between CAs such as EPI and NE and a *luxS*-dependent AI-3 response system (Clarke *et al.*, 2006; Kendall *et al.*, 2007; Sperandio *et al.*, 2003). However, most of the preliminary observations towards these suggestions were based on experiments performed using *E. coli* 0157:H7 *Tet* insertional *luxS*- mutants (Sperandio *et al.*, 2001). It was shown in this study that mutation of the *Tet* insertion *luxS* mutant in an *E. coli* E2348/69 strain obtained using the same plasmids adopted by the Sperandio group (Sperandio *et al.*, 2001) produced different phenotypes to *luxS* mutants obtained via an in frame deletion mutant, Term and Cm mutant (chapter 6, Fig.6.5, 6.6) suggesting that the construct used to obtained the Tet mutants was causing polar effects and therefore my well have an artefact phenotype. These observations were also confirmed in *luxS* mutant performed with the same strategies in *E. coli* 0157:H7, thus this study and other ongoing research in my laboratory has unearthed uncertainties on the role that *luxS* plays in the biology of *E. coli.* However, what is certain is that *luxS* is not required for the first initial contact with the CAs, and is not involved in the most important stage of bacterial pathogenesis, the ability of bacteria to initiate growth.

7.2 Future work

As well as providing answers to the questions set at the beginning of this project, the work in this thesis has also opened up many questions. Although the CAs-response mechanism in bacteria has been widely investigated, there are still some uncertainties and unanswered questions regarding which receptor/s on bacteria can sense and internalise CAs. This aspect is of most interest and needs to be elucidated in more detail. In particular, the identity of the CA receptors involved in catecholamine-growth induction is a priority. Further analysis if there is cross communication between intra-kingdom signalling (quorum sensing) and inter-kingdom signalling (microbial endocrinology) would be interesting (and is ongoing in my laboratory). In

the context of infection susceptibility and ICU patients, another important objective would be to translate the methodologies used in Chapter 5 to the clinical setting, that is use EPR/ferrozine analysis to determine whether inotropes and endogenous stress hormones are de-stabilising in blood Fe-bound to Tf. This research could provide additional and useful insight into the infection risk of ICU patients on inotrope support. Elucidation of the role of the *E. coli* TfBP and LfBP in survival of bacteria *in vivo* would also be interesting, as it would illustrate whether OmpA and OmpC play a similar role in other enteropathogenic bacteria.

Appendix 1

Publications completed during the course of this thesis:

Freestone, P. P., Sandrini, S. M., Haigh, R. D., & Lyte, M. (2008). Microbial endocrinology: How stress influences susceptibility to infection. *Trends in Microbiology, 16*(2), 55-64.

Appendix 2

N-terminal protein sequence results. Highlighted in yellow are the N-terminal aminoacids sequenced by PNACL that were blasted against the *E. coli* genome using the NCBI database and that allowed to determine the identity of the Tf and Lf binding proteins as the porins OmpC and OmpA.

>gi|215487434|ref|YP_002329865.1| outer membrane porin protein C [Escherichia coli O127:H6 str. E2348/69]
MKVKVLSLLVPALLVAGAANAAEIYNKDGNKLDLYGKVDGLHYFSDNDSKDGDKTYMRLGFKGETQVTDQ
LTGYGQWEYQIQGNEPESDNSSWTRVAFAGLKFQDVGSFDYGRNYGVVYDVTSWTDVLPEFGGDTYDSDN
FMQQRGNGFATYRNTDFFGLVDGLNFAVQYQGKNGSPSGEGMTGVTNNGRDVLEQNGDGVGGSITYDIGE
GFSFGGAISSSKRTAEQNNYGIGNGDRAETYTGGLKYDANNVYLAAQYTQTYNATLAGDLGWANKAQNFE
VVAQYQFDFGLRPSVAYLQSKGKNLNANIAGRTYDDEDLLKYVDVGATYYFNKNMSTYVDYKINLLDDNQ
FTRAAGINTDDIVALGLVYQF

>gi|215486075|ref|YP_002328506.1| outer membrane protein A [Escherichia coli O127:H6 str. E2348/69]
MKKTAIAIAVALAGFATVAQAAPKDNTWYTGAKLGWSQYHDTGFIPNNGPTHENQLGAGAFGGYQVNPYV
GFEMGYDWLGRMPYKGSVENGAYKAQGVQLTAKLGYPITDDLDVYTRLGGMVWRADTKSNVYGKNHDTGV
SPVFAGGVEYAITPEIATRLEYQWTNNIGDAHTIGTRPDNGMLSLGVSYRFGQGEVAPVVAPAPAPAPEV
QTKHFTLKSDVLFTFNKATLKPEGQAALDQLYSQLSNLDPKDGSVVVLGYTDRIGSDAYNQALSERRAQS
VVDYLISKGIPADKISARGMGESNPVTGNTCDNVKQRAALIDCLAPDRRVEIEVKGIKDVVTQPQA

Appendix 3

Sequence homology blast results of OmpA, OmpC of *E. coli* E2348/69 and TbpA, TbpB, LbpA, LbpB of *Neisseria meningitis*. Using the alignment blast tool of ClustalW2 program, (http://www.ebi.ac.uk/Tools/clustalw2/index.html) the protein sequences of OmpA and OmpC of *E. coli* E2348/69 were aligned for similarity against the protein sequences of LbpA/LbpB and TbpA/TbpB of *Neisseria meningitides*. From the blast results it is possible to observe that there is no homology between the protein sequences.

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score
1	TbpA_Neisseria	908	2	OmpA_Escherichia	346	14

Alignment

```
CLUSTAL 2.0.10 multiple sequence alignment

TbpA_Neisseria        MQQQHLFRLNILCLSLMTALPVYAENVQAEQAQEKQLDTIQVKAKKQKTRRDNEVTGLGK 60
OmpA_Escherichia      ------------------------------------------------------------

TbpA_Neisseria        LVKSSDTLSKEQVLNIRDLTRYDPGIAVVEQGRGASSGYSIRGMDKNRVSLTVDGVSQIQ 120
OmpA_Escherichia      ------------------------------------------------------------

TbpA_Neisseria        SYTAQAALGGTRTAGSSGAINEIEYENVKAVEISKGSNSSEYGNGALAGSVAFQTKTAAD 180
OmpA_Escherichia      ------------------------------------------------------------

TbpA_Neisseria        IIGEGKQWGIQSKTAYSGKDHALTQSLALAGRSGGAEALLIYTKRRGREIHAHKDAGKGV 240
OmpA_Escherichia      ------------------------------------------------------------

TbpA_Neisseria        QSFNRLVLDEDKKEGGSQYRYFIVEEECHNGYAACKNKLKEDASVKDERKTVSTQDYTGS 300
OmpA_Escherichia      ------------------------------------------------------------

TbpA_Neisseria        NRLLANPLEYGSQSWLFRPGWHLDNRHYVGAVLERTQQTFDTRDMTVPAYFTSEDYVPGS 360
OmpA_Escherichia      ----------------------------------MKKTAIAIAVALAGFATVAQAAP-- 23
                                                         *           *     *

TbpA_Neisseria        LKGLGKYSGDNKAERLFVQGEGSTLQGIGYGTGVFYDERHTKNRYGVEYVYHNADKDTWA 420
OmpA_Escherichia      ------------------------------------------------------KDNTWY 29
                                                                              **

TbpA_Neisseria        DYARLSYDRQGIDLDNRLQQTHCSHDGSDKNCRPDGNKPYSFYKSDRMIYEESRNLFQAV 480
OmpA_Escherichia      TGAKLG----------------------------------WSQYHDTGFIPNNGPTHENQL 56
                        * *                                   * *     *

TbpA_Neisseria        FKKAFDTAKIRHNLSINLGYDRFKSQLSHSDYYLQNAVQAYDLITPKKPPFPNGSKDNPY 540
OmpA_Escherichia      GAGAFGGYQVNPYVGFEMGYDWLG-RMPYKGSVENGAYKAQGVQLTAKLGYPITDDLDVY 115
                         **            ***              *  *        *   *       *

TbpA_Neisseria        RVSIGKTTVNTSPICRFGNNTYTDCTPRNIGGNGYYAAVQDNVRLGRWADVGAGIRYDYR 600
OmpA_Escherichia      TRLGGMVWRADTKSNVYGKNHDTGVSPVFAGG----------VEYAITPEIATRLEYQWT 165
                          *           * *  *   *   **          *            *

TbpA_Neisseria        STHSEDKSVSTGTHRNLSWNAGVVLKPFTWMDLTYRASTGFRLPSFAEMYGWRAGESLKT 660
OmpA_Escherichia      NNIGDAHTIGTRPDN-------------------------GMLSLGVSYRFGQGEVAPV 199
                             *                                 *    *    **
```

```
TbpA_Neisseria        LDLKPEKSFNREAGIVFKGDFGNLEASYFNNAYRDLIAFGYETRTQNGQTSASGDPGYRN 720
OmpA_Escherichia      VAPAPAPAP-------------EVQTKHFTLKSDVLFTFNKATLKPEGQ----------- 235
                        *                     *      *  *   *    **
TbpA_Neisseria        AQNARIAGINILGKIDWHGVWGGLPDGLYSTLAYNRIKVKDADIRADRTFVTSYLFDAVQ 780
OmpA_Escherichia      ----------------------AALDQLYSQLSN----------------------LDPKD 252
                                            * *** *                          *

TbpA_Neisseria        PSRYVLGLGYDHPDGIWGINTMFTYSKAKSVDELLGSQALLNGNANAKKAASRRTRPWYV 840
OmpA_Escherichia      GS--VVVLGYTDRIGSDAYNQALSERRAQSVVDYLISKGIPADKISARGMGESNP------ 305
                       *  *  ***    *    *        * **   * *         *

TbpA_Neisseria        TDVSGYYNIKKHLTLRAGVYNLLNYRYVTWENVRQTAGGAVNQHKNVGVYNRYAAPGRNY 900
OmpA_Escherichia      --VTG--NTCDNVKQRAALIDCLAPDRRVEIEVKGIKDVVTQPQA--------------- 346
                        * *  *       **     *         *

TbpA_Neisseria        TFSLEMKF 908
OmpA_Escherichia      -------- 
```

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score
1	TbpA_Neisseria	908	2	OmpC_Escherichia	371	16

Alignment

CLUSTAL 2.0.10 multiple sequence alignment

```
TbpA_Neisseria        MQQQHLFRLNILCLSLMTALPVYAENVQAEQAQEKQLDTIQVKAKKQKTRRDNEVTGLGK 60
OmpC_Escherichia      --------MKVKVLSLLVPALLVAGAANAAEIYNKDGNKLDLYG---------KVDGLHY 43
                              ***       *    *     *                      * **

TbpA_Neisseria        LVKSSDTLSKEQVLNIRDLTRYDPGIAVVEQGRGASSGYSIRGMDKNRVSLTVDGVSQIQ 120
OmpC_Escherichia      FSDND-----------------------SKDGDKTYMRLGFKGETQVTDQLTGYGQWEYQ 80
                                                  *         *      **  *    *

TbpA_Neisseria        SYTAQAALGGTRTAGSSGAINEIEYENVKAVEISKGSNSSEYGNGALAGSVAFQTKTAAD 180
OmpC_Escherichia      -------IQGNEPESDNSSWTRVAFAGLKFQDVGSFDYGRNYG-------VVYDVTSWTD 126
                             *                  *           **       *        *

TbpA_Neisseria        IIGE--GKQWGIQSKTAYSGKDHALTQSLALAGRSGGAEALLIYTKRRGREIHAHKDAGK 238
OmpC_Escherichia      VLPEFGGDTYDSDNFMQQRGNGFATYRNTDFFGLVDGLNFAVQYQGKNG---SPSGEGMT 183
                          *  *           *   *        *   *       *    *

TbpA_Neisseria        GVQSFNRLVLDEDKKEGGSQYRYFIVEEECHNGYAACKNKLKEDASVKDERKTVSTQDYT 298
OmpC_Escherichia      GVTNNGRDVLEQNGDGVGGSITYDIGEGFSFGG------------AISSSKRTAEQNNYG 231
                       **    * **       *    * * *     *                  *      *

TbpA_Neisseria        GSNRLLANPLEYGSQSWLFRPGWHLDNRHYVGAVLERTQQTFDTRDMTVPAYFTSEDYVP 358
OmpC_Escherichia      ------------------------------------------------------------

TbpA_Neisseria        GSLKGLGKYSGDNKAERLFVQGEGSTLQGIGYGTGVFYDERHTKNRYGVEYVYHNADKDT 418
OmpC_Escherichia       ---------IGNGDRAETYTGG---------------------------------------
244
                             *          *

TbpA_Neisseria        WADYARLSYDRQGIDLDNRLQQTHCSHDGSDKNCRPDGNKPYSFYKSDRMIYEESRNLFQ 478
OmpC_Escherichia      ------LKYDANNVYLAAQYTQTYNATLAGDLGWANKAQN------------------FE 280
                            *  **     *     **      *                          *

TbpA_Neisseria        AVFKKAFDTAKIRHNLSINLGYDRFKSQLSHSDYYLQNAVQAYDLITPKKPPFPNGSKDN 538
OmpC_Escherichia      VVAQYQFDFG-------------------LRPSVAYLQ----------------------- 299
                        *    **                    *  *  ***

TbpA_Neisseria        PYRVSIGKTTVNTSPICRFGNNTYTDCTPRNIGGNGYYAAVQDNVRLGRWADVGAGIRYD 598
OmpC_Escherichia      ----SKGKNLN------------------ANIAG-----RTYDDEDLLKYVDVGATYYFN 332
                           * **                    ** *        *   *    ****
```

```
TbpA_Neisseria          YRSTHSEDKSVSTGTHRNLSWNAGVVLKPFTWMDLTYRASTGFRLPSFAEMYGWRAGESL 65
OmpC_Escherichia        KNMSTYVDYKINLLDDNQFTRAAGINTDDIVALGLVYQF---------------------- 371
                                *              **          *  *

TbpA_Neisseria          KTLDLKPEKSFNREAGIVFKGDFGNLEASYFNNAYRDLIAFGYETRTQNGQTSASGDPGY 718
OmpC_Escherichia        ------------------------------------------------------------

TbpA_Neisseria          RNAQNARIAGINILGKIDWHGVWGGLPDGLYSTLAYNRIKVKDADIRADRTFVTSYLFDA 778
OmpC_Escherichia        ------------------------------------------------------------

TbpA_Neisseria          VQPSRYVLGLGYDHPDGIWGINTMFTYSKAKSVDELLGSQALLNGNANAKKAASRRTRPW 838
OmpC_Escherichia        ------------------------------------------------------------

TbpA_Neisseria          YVTDVSGYYNIKKHLTLRAGVYNLLNYRYVTWENVRQTAGGAVNQHKNVGVYNRYAAPGR 898
OmpC_Escherichia        ------------------------------------------------------------

TbpA_Neisseria          NYTFSLEMKF 908
OmpC_Escherichia        ----------
```

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score
1	TbpB_Neisseria	703	2	OmpA_Escherichia	346	3

Alignment

```
CLUSTAL 2.0.10 multiple sequence alignment

TbpB_Neisseria          MNNPLVNQAAMVLPVFLLSACLGGGGSFDLDSVDTEAPRPAPKYQDVPSKKPEARKDQGG 60
OmpA_Escherichia        ----------MKKTAIAIAVALAG-----FATVAQAAPKDNTWYTGAKLGWSQYHDTG-- 43
                                  *           * *         *   **     *

TbpB_Neisseria          YGFAMRFKRRNWYPPSNPKENEIRLSEGDWEQTGNGNIKNPSKQKNIIDALSGNGEAPLQ 120
OmpA_Escherichia        ------------FIPNNGPTHENQLGAG---------------------AFGGYQVNPYV 70
                                  * *    *  * *                        *  *    *

TbpB_Neisseria          DSSQQGEGISKVTDYHDFKYVWSGFFYKQIGNTIKKDDSSSKIIEARNGPDGYIFYKGTD 180
OmpA_Escherichia        G---------------FEMGYDWLG------------------------------------ 80
                        .                  * * *

TbpB_Neisseria          PSRKLPVSGSVEYKGTWDFLTDVQANQKFTDLGSAFTKSGDRYSAFSGELDYIVRKEEDK 240
OmpA_Escherichia        ---RMPYKGSVENG-----AYKAQGVQLTAKLGYPITDDLDVYTRLGG------------ 120
                             *  ****           *  *    **    *   * *    *

TbpB_Neisseria          KDGHVGLGLTTEITVNFEKKTLSGKLIKNNMVINNGDEPTTQYYSLEAQVTGNRFNGKAM 300
OmpA_Escherichia        --------------------------------MVWRADTKSNVYGKNHDTGVSPVFAGGVE 149
                                                         *      *          * *

TbpB_Neisseria          VTDKPENSKSKQHPFVSDSSSLSGGFFGPQGEELGFRFLSNDNKVAVVGSAKTKDETASS 360
OmpA_Escherichia        YAITPEIATRLEYQWTNN-----------------------------IGDAHTIGTRPDN 180
                            **                                        * * *

TbpB_Neisseria          GGTSGGASVSASNGATGTSSGNSNLTTVLDAVELTPDGKEIKDLDNFSNAAQLVVDGIMI 420
OmpA_Escherichia        GMLSLGVSYRFGQ-------------------------------------------GEVA 197
                        *  * * *                                                 *

TbpB_Neisseria          PLLSTESGNGQADKGKNGGTDFTYTTTYMPESDKKDTKAQTGAGGMQTASDAAGVNGGQA 480
OmpA_Escherichia        PVVAPAPAPAPEVQTKH----FTLKSDVLFTFNKATLKPEGQAALDQLYSQLSNLDPKDG 253
                        *             *     **          *   *    *   *  *
```

```
TbpB_Neisseria        GTKTYKVEACCSNLNYLKYGLLTRENSNSVMQTVRNSSQAAARTAQGAQSMFLQGERTDE 540
OmpA_Escherichia      --------------SVVVLGYTDRIGSDAYNQALSE------RRAQSVVDYLIS------ 287
                                    *   *  *     *          * **

TbpB_Neisseria        KEIPKEQKVVYLGTWYGHIAANGTSWTGNASDQQSGNRAKFDVNFKDKKITGTLTAANRQ 600
OmpA_Escherichia      KGIPADK-----------ISARG----MGESNPVTGN-----------------TCDNVK 315
                      * **              * * *       *    **                  *  *

TbpB_Neisseria        EATFTIDAMIDDNGFKGTAKTGNDGFAPDQNSSTGTYKVHIANAEVQGGFYGPNAEELGG 660
OmpA_Escherichia      QRAALIDCLAPDR-----------------------RVEIEVKGIKDVVTQPQA----- 346
                           **    *                        * *          * *

TbpB_Neisseria        WFAYPGNGQTKNAQENAQASSGNGNSAVSATVVFGAKRQQLVK 703
OmpA_Escherichia      ------------------------------------------
```

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score
1	TbpB_Neisseria	603	2	OmpC_Escherichia	371	11

Alignment

CLUSTAL 2.0.10 multiple sequence alignment

```
TbpB_Neisseria        MNNPLVNQAAMVLPVFLLSACLGGGGGSFDLDSVETKQHNPQSEAPKYQDEQTEKPAKPD 60
OmpC_Escherichia      ---MKVKVLSLLVPALLVAGAAN-AAEIYNKDGNKLDLYGKVDGLHYFSDNDSKDGDK-- 54
                            *       *  *             *                  *       *

TbpB_Neisseria        EAQAENDRPAYGFAVKIPRRFWYPRNKEDHKALSEADWEKLGAGAPNEFPQKNEISNMTE 120
OmpC_Escherichia      -------------------------------------TYMRLGFKGETQVT---------- 68
                                                           **

TbpB_Neisseria        GTLNESIQPGEDGKSRVEGYTKFEYVRSGYIYRNGLPMERIDGSVRNGPKGYLFYKGSNP 180
OmpC_Escherichia      --------------DQLTGYGQWEYQIQ----------------------------GNEP 86
                                  **   **                                  *  *

TbpB_Neisseria        SQALPTGKAIYKGTWDYVTDAKMGQKFSQLGGSQAGDRYGALSAEEADVLRNKNEAKESQ 240
OmpC_Escherichia      ESDNSS----------WTRVAFAGLKFQDVGSFDYGRNYG--------VVYDVTSWTDVL 128
                                     *  * **   *    *  **        *

TbpB_Neisseria        TDFGLTSEFEVDFAAKTMTGKLYRNNRITNNETENRDKQIKRYDIQADLHGNRFKGKALA 300
OmpC_Escherichia      PEFG---------------GDTYDSDNFMQ-----------------QRGNGFATYRNT 155
                        **               *  *                        ** *

TbpB_Neisseria        ADKGVTNGSHPFISDSDSLEGGFYGPKGEELAGKFLSKDNKVAAVFGAKQKDKKEGENAA 360
OmpC_Escherichia      DFFGLVDGLN-FAVQYQGKNG---SPSGEGMTG----------VTNNGRDVLEQNGDGVG 201
                         *   *   *        *     * **   *                    *

TbpB_Neisseria        GLATETVIDAYRITGGEFKKEQIDSFGDVKKLLVDGVELSLLPSEGNKAAFQHGIEQNGV 420
OmpC_Escherichia      GSITYDIGEGFSFGG----------------------AISSSKRTAEQNNYGIG-NGD 236
                      *  *          *                        *         **  **

TbpB_Neisseria        KATVCCSNLDYMSFGKLSKENKDDMFLQGVRTPVSDVAARTEANAKYRGTWYGYIANGTS 480
OmpC_Escherichia      RAETYTGGLKYDAN-------------------------NVYLAAQYTQTYNATLAGDLG 271
                       *       * *                          * *  *    *

TbpB_Neisseria        WSGEASNQEGGNRAEFDVDFSTKKISGTLTAKDRTSPTFIITAAIQDNGFEGTAKTGENG 540
OmpC_Escherichia      WANKAQN--FEVVAQYQFDFGLRPSVAYLQSKGKN-----LNANIAGRTYD-------DE 317
                      *   * *      *     **        *  *          * *

TbpB_Neisseria        FALDPKNTGNSHYAHIEATVSGGFYGKNAIEMGGSFSFPGNAPEGKQEKASVVFGAKRQQ 600
OmpC_Escherichia      DLLKYVDVGATYYFNKNMST----YVDYKINLLDDNQFTR--AAGINTDDIVALGLVYQF 371
                        *     *   *            *    *       *      *      *  *   *

TbpB_Neisseria        LVQ 603
OmpC_Escherichia      ---
```

```
SeqA Name               Len(aa)  SeqB Name               Len(aa)  Score
==========================================================================
1    LbpA_Neisseria     937      2    OmpA_Escherichia   346      13
==========================================================================
```

Alignment

```
CLUSTAL 2.0.10 multiple sequence alignment

LbpA_Neisseria        MNKKHGFQLTLTALAVAAAFPSYAANPETAAPDAAQTQSLKEVTVRAAKVGRRSKEATGL 60
OmpA_Escherichia      --------MKKTAIAIAVALAGFAT--------VAQAAPKDNTWYTGAKLGWSQYHDTGF 44
                              ** * * *    *          **           ** *      **

LbpA_Neisseria        GKIVKTSETLNKEQVLGIRDLTRYDPGVAVVEQGNGASGGYSIRGVDKNRVAVSVDGVAQ 120
OmpA_Escherichia      --IPNNGPTHEN-------------------QLGAGAFGGYQVN---------------- 67
                        *     *                     * ** ***

LbpA_Neisseria        IQAFTVQGSLSGYGGRGGSGAINEIEYENISTVEIDKGAGSSDHGSGALGGAVAFRTKEA 180
OmpA_Escherichia      ----PYVGFEMGYDWLG------RMPYK-----------GSVENG--------------- 91
                            *   **   *         *            **   *

LbpA_Neisseria        ADLISDGKSWGIQAKTAYGSKNRQFMKSLGAGFSKDGWEGLLIRTERQGRETHPHGDIAD 240
OmpA_Escherichia      ----------------AYKAQGVQLTAKLGYPITDD------------------------ 111
                                    **      *    **     *

LbpA_Neisseria        GVAYGINRLDAFRQTYGIKKPSEGGEYFLAEGESELKPVAKVAGNGNYLNNQLNRWVKER 300
OmpA_Escherichia      ----------------------------------LDVYTRLGG---------------- 120
                                                        *       *

LbpA_Neisseria        IEQNQPLSAEEEAMVREAQARHENLSAQAYTGGGRILPDPMDYRSGSWLAKLGYRFGGRH 360
OmpA_Escherichia      -------------MVWRADTKSNVYGKNHDTGVSPVFAGGVEYAITPEIATR-------- 159
                                ** * *           **          *      *

LbpA_Neisseria        YVGGVFEDTKQRYDIRDMTEKQYYGTDEAKKFRDKSGVYDGDDFRDGLYFVPNIEEWKGD 420
OmpA_Escherichia      ------------------------------------------------------------

LbpA_Neisseria        QKLIRGIGLKYSRTKFIDEHHRRRRMGLLYRYENEKYSDNWADKAVLSFDKQGVATDNNT 480
OmpA_Escherichia      --------LEYQWTNNIGDAHT-----------------------------IGTRPDNGM 182
                              * *  *  *  *                                *    **

LbpA_Neisseria        LKLNCAVYPAVDKSCRASADKPYSYDSSDRFHYREQHNVLNASFEKSLKNKWTKHHLTLG 540
OmpA_Escherichia      LSLGVSYRFGQGEVAPVVAPAPAPAPEVQTKHFTLKSDVLFTFNKATLK----------- 231
                      * *                 *  *         *      **       **

LbpA_Neisseria        FGYDASNAISRPEQLSHNAARISEYSDYTDKGDKYLLGKPEVVEGSVCGYIETLRSRKCV 600
OmpA_Escherichia      -----------PEGQAALDQLYSQLSNLDPK----------------------------- 251
                               **         *  *    *

LbpA_Neisseria        PRKINGSNIHISLNDRFSIGKYFDFSLGGRYDRKNFTTSEELVRSGRYVDRSWNSGIVFK 660
OmpA_Escherichia      ----DGSVVVLGYTDRIGSDAYNQALS----ERRAQSVVDYLISKGIPADKIS------- 296
                           **        **     *          *       *   *   *

LbpA_Neisseria        PNRHFSLSYRASSGFRTPSFQELFGIDIYHDYPKGWQRPALKSEKAANREIGLQWKGDFG 720
OmpA_Escherichia      -ARGMGESNPVTG----------------NTCDNVKQRAALIDCLAPDRRVEIEVKG--- 336
                        *     *                          ** **    *  *      **

LbpA_Neisseria        FLEISSFRNRYTDMIAVADHKTKLPNQAGQLTEIDIRDYYNAQNMSLQGVNILGKIDWNG 780
OmpA_Escherichia      ----------IKDVVTQPQA---------------------------------------- 346
                                *

LbpA_Neisseria        VYGKLPEGLYTTLAYNRIKPKSVSNRPGLSLRSYALDAVQPSRYVLGFGYDQPEGKWGAN 840
OmpA_Escherichia      ------------------------------------------------------------

LbpA_Neisseria        IMLTYSKGKNPDELAYLAGDQKRYSTKRASSSWSTADVSAYLNLKKRLTLRAAIYNIGNY 900
OmpA_Escherichia      ------------------------------------------------------------
```

```
LbpA_Neisseria        RYVTWESLRQTAESTANRHGGDSNYGRYAAPGRNFSL 937
OmpA_Escherichia      -------------------------------------
```

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score
1	LbpA_Neisseria	937	2	OmpC_Escherichia	371	7

Alignment

CLUSTAL 2.0.10 multiple sequence alignment

```
LbpA_Neisseria        MNKKHGFQLTLTALAVAAAFPSYAANPETAAPDAAQTQSLKEVTVRAAKVGRRSKEATGL 60
OmpC_Escherichia      ------MKVKVLSLLVPALLVAGAAN---------------------------------- 20
                            * * *    ***

LbpA_Neisseria        GKIVKTSETLNKEQVLGIRDLTRYDPGVAVVEQGNGASGGYSIRGVDKNRVAVSVDGVAQ 120
OmpC_Escherichia      -----AAEIYNKDGNK--LDLYGKVDGLHYFSDNDSKDG-------DKTYMRLGFKGETQ 66
                            *  **       **     *            *       **        *  *

LbpA_Neisseria        IQAFTVQGSLSGYGGRGGSGAINEIEYENISTVEIDKGAGSSDHGSGALGGAVAFRTKEA 180
OmpC_Escherichia      VT-----DQLTGYG---------QWEYQ-----------------------------IQG 83
                           * ***          **

LbpA_Neisseria        ADLISDGKSWGIQAKTAYGSKNRQFMKSLGAGFSKDGWEGLLIRTERQGRETHPHGDIAD 240
OmpC_Escherichia      NEPESDNSSW---TRVAFAGLKFQDVGSFDYGRN-------------------------Y 115
                          **  **      *      *   *    *

LbpA_Neisseria        GVAYGINRLDAFRQTYGIKKPSEGGEYFLAEGESELKPVAKVAGNGNYLNNQLNRWVKER 300
OmpC_Escherichia      GVVYDVTSWTDVLPEFGG-DTYDSDNFMQQRGN----------GFATYRNTDFFGLVDG- 163
                      ** *           *                *           *   * *      *

LbpA_Neisseria        IEQNQPLSAEEEAMVREAQARHENLSAQAYTGGGRILPDPMDYRSGSWLAKLGYRFGGRH 360
OmpC_Escherichia      -----------LNFAVQYQGKNGSPSGEGMTGVTNNGRDVLEQNGDGVGGSITYDIGEGF 212
                                     *       *    **      *                 *  *

LbpA_Neisseria        YVGGVFEDTKQRYDIRDMTEKQYYGTDEAKKFRDKSGVYDGDDFRDGLYFVPNIEEWKGD 420
OmpC_Escherichia      SFGGAISSSKR------TAEQNNYGIGNGDRAETYTG------------------------ 243
                        **      *         *   **           *

LbpA_Neisseria        QKLIRGIGLKYSRTKFIDEHHRRRRMGLLYRYENEKYSDNWADKAVLSFDKQGVATDNNT 480
OmpC_Escherichia      -------GLKYDAN---------------NVYLAAQYTQTYNATLAGDLG---------- 271
                             ****                   *    *

LbpA_Neisseria        LKLNCAVYPAVDKSCRASADKPYSYDSSDRFHYREQHNVLNASFEKSLKNKWTKHHLTLG 540
OmpC_Escherichia      -----------------WANKAQNFEVVAQYQF--------------------DFGLRPS 294
                                      * *                                  *

LbpA_Neisseria        FGYDASNAISRPEQLSHNAARISEYSDYTDKGDKYLLGKPEVVEGSVCGYIETLRSRKCV 600
OmpC_Escherichia      VAYLQSKGKNLNANIAGRTYDDEDLLKYVDVGATYYFNKN-------------------- 334
                        *  *                        * * *  *   *

LbpA_Neisseria        PRKINGSNIHISLNDRFSIGKYFDFSLGGRYDRKNFTTSEELVRSGRYVDRSWNSGIVFK 660
OmpC_Escherichia      ------------------MSTYVDYKIN-LLDDNQFTR-----AAGINTDDIVALGLVYQ 370
                                       * *       *   **        *   *     * *

LbpA_Neisseria        PNRHFSLSYRASSGFRTPSFQELFGIDIYHDYPKGWQRPALKSEKAANREIGLQWKGDFG 720
OmpC_Escherichia      F------------------------------------------------------------ 371

LbpA_Neisseria        FLEISSFRNRYTDMIAVADHKTKLPNQAGQLTEIDIRDYYNAQNMSLQGVNILGKIDWNG 780
OmpC_Escherichia      ------------------------------------------------------------

LbpA_Neisseria        VYGKLPEGLYTTLAYNRIKPKSVSNRPGLSLRSYALDAVQPSRYVLGFGYDQPEGKWGAN 840
OmpC_Escherichia      ------------------------------------------------------------

LbpA_Neisseria        IMLTYSKGKNPDELAYLAGDQKRYSTKRASSSWSTADVSAYLNLKKRLTLRAAIYNIGNY 900
OmpC_Escherichia      ------------------------------------------------------------
```

```
LbpA_Neisseria        RYVTWESLRQTAESTANRHGGDSNYGRYAAPGRNFSL 937
OmpC_Escherichia      -------------------------------------
```

SeqA Name	Len(aa)	SeqB Name	Len(aa)	Score
1 LbpB_Neisseria	725	2 OmpA_Escherichia	346	9

Alignment

CLUSTAL 2.0.10 multiple sequence alignment

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LbpB_Neisseria        MCKPNYGGIVLLPLLLASCIGGNFGVQPVVESTPTAYPVTFKSKDVPTPPPAKPSIEITP 60
OmpA_Escherichia      ------------------------------------------------------MKKTAIAIAV 10
                                                                               *   * *

LbpB_Neisseria        VNRPAVGAAMRLPRRNTAFHREDGTEIPNSKQAEEKLSFQEGDVLFLYGSKGNKLQQLKS 120
OmpA_Escherichia      ALAGFATVAQAAPKDNTWYTG-------------AKLGWS--------------------- 37
                             *   *  **                   **

LbpB_Neisseria        EIHKRDSDVEIRTSEKENKKYDYKFVDAGYVYVKGKDEIKWTSDYKQFSNRLGYDGFVYY 180
OmpA_Escherichia      ----------------------QYHDTGFIPNNGP----------THENQLGAGAFGGY 64
                                           *  *     *             * **   *  *

LbpB_Neisseria        SGERPSQSLPSAGTVEYSGNWQYMTDAKRHRAGKAVGIDNLGYYTFYGNDVGATSYAAKD 240
OmpA_Escherichia      QVN--------------------------PYVGFEMGYDWLGRMPYKG-SVENGAYKAQG 97
                                                  *   * * **     *  *    * *

LbpB_Neisseria        VDEREKHPAKYTVDFGNKTLTGELIKNQYVKPSEKQKPLTIYNITADLNGNRFTGSAKVN 300
OmpA_Escherichia      VQLTAKLG---------------------------------YPITDDLDVYTRLG----- 119
                      *    *                                  * ** **      *

LbpB_Neisseria        PDLAKSHANKEHLFFHADADQRLEGGFFGDKGEELAGRFISNDNSVFGVFAGKQNSPVPS 360
OmpA_Escherichia      -----------GMVWRADTKSNVYG--------------KNHDTGVSPVFAGGVEYAITP 154
                                 **      *                 *   *  ****

LbpB_Neisseria        GKHTKILDSLKISVDEASGENPRPFAISPMPDFGHPDKLLVEGHEIPLVSQEKTIELADG 420
OmpA_Escherichia      EIATRLEYQWTNNIGDAHTIGTR-------PDNG-------------MLSLGVSYRFGQG 194
                         *            *     *        ** *              *        *

LbpB_Neisseria        RKMTVSACCDFLTYVKLGRIKTERPAAKPKAQDEEDSDIDNGEESEDEIGDEEEGTEDAA 480
OmpA_Escherichia      ---------------EVAPVVAPAPAPAPEVQTKHFT----------------------- 216
                                     **  *  *

LbpB_Neisseria        AGDEGSEEDEATENEDGEEDEAEEPEEESSAEGNGSSNAILPVPEASKGRDIDLFLKGIR 540
OmpA_Escherichia      ----------------------------------LKSDVLFTFNKATLKPEGQAALDQLY 242
                                                        *         *         *

LbpB_Neisseria        TAETNIPQTGEARYTGTWEARIGKPIQWDNHADKEAAKAVFTVDFGKKSISGTLTEKNGV 600
OmpA_Escherichia      SQLSNLDPK----------------------DGSVVVLGYTDRIGSDAYNQALSERR-- 277
                          *                          *        *   *       * *

LbpB_Neisseria        EPAFRIENGVIEGNGFHATARTRDDGIDLSGQGSTKPQIFKANDLRVEGGFYGPKAEELG 660
OmpA_Escherichia      ------AQSVVD---YLISKGIPADKISARGMGESNP---------------------VT 307
                            *            * *   * *   *

LbpB_Neisseria        GIIFNNDGKSLGITEGTENKVEADVDVDVDVDVDADADVEQLKPEVKPQFGVVFGAKKDN 720
OmpA_Escherichia      GNTCDNVKQRAALIDCLAPDRRVEIEVKGIKDVVTQPQA--------------------- 346
                      *    *                      *    **

LbpB_Neisseria        KEVEK 725
OmpA_Escherichia      ----- 
```

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score
1	LbpB_Neisseria	725	2	OmpC_Escherichia	371	12

Alignment

```
CLUSTAL 2.0.10 multiple sequence alignment


LbpB_Neisseria        MCKPNYGGIVLLPLLLASCIGGNFGVQPVVESTPTAYPVTFKSKDVPTPPPAKPSIEITP 60
OmpC_Escherichia      ------------------------------------------------------------

LbpB_Neisseria        VNRPAVGAAMRLPRRNTAFHREDGTEIPNSKQAEEKLSFQEGDVLFLYGSKGNKLQQLKS 120
OmpC_Escherichia      -----------------------------------------------------MKVKVLSLLVP 11
                                                                           *   *  *

LbpB_Neisseria        EIHKRDSDVEIRTSEKENKKYDYKFVDAGYVYVKGKDEIKWTSDYKQFSNRLGYDGFVYY 180
OmpC_Escherichia      ALLVAGAANAAEIYNKDGNKLDLYGKVDGLHYFSDND----SKDGDKTYMRLGFKGETQV 67
                                 *   * *      *  *    *      *      ***  *

LbpB_Neisseria        SGERPSQSLPSAGTVEYSGNWQYMTDAKRHRAGKAVGIDNLGYYTFYGNDVGATSYAAKD 240
OmpC_Escherichia      TDQLTG-----------YGQWEYQIQGNEPESDNSS-WTRVAFAGLKFQDVGSFDYGRN- 114
                            * * *                          ***   *

LbpB_Neisseria        VDEREKHPAKYTVDFGNKTLTGELIKNQYVKPSEKQKPLTIYNITADLNGNRFTGSAKVN 300
OmpC_Escherichia      ----------YGVVYDVTSWT---------------------DVLPEFGGDTYD------ 137
                           * *      *                           *

LbpB_Neisseria        PDLAKSHANKEHLFFHADADQRLEGGFFGDKGEELAGRFISNDNSVFGVFAGKQNSPVPS 360
OmpC_Escherichia      ---------------SDNFMQQRGNGFATYRNTDFFG--LVDGLNFAVQYQGKNGSPSGE 180
                                     *    **         *             **  **

LbpB_Neisseria        GKHTKILDSLKISVDEASGENPRPFAISPMPDFGHPDKLLVEGHEIPLVSQEKTIELADG 420
OmpC_Escherichia      GMTGVTNNGRDVLEQNGDG----------------------------VGGSITYDIGEG 211
                       *                *                          *    *      *

LbpB_Neisseria        RKMTVSACCDFLTYVKLGRIKTERPAAKPKAQDEEDSDIDNGEESEDEIGDEEEGTEDAA 480
OmpC_Escherichia      -------------FSFGGAISSSK-----RTAEQNNYGIGNGDRAETYTG---------- 243
                                  * *               * **   *   *

LbpB_Neisseria        AGDEGSEEDEATENEDGEEDEAEEPEEESSAEGNGSSNAILPVPEASKGRDIDLFLKGIR 540
OmpC_Escherichia      ------------------------------------------------------------GLK 246
                                                                                   *

LbpB_Neisseria        TAETNIPQTGEARYTGTWEARIGKPIQWDNHADKEAAKAVFTVDFGKKSISGTLTEKNGV 600
OmpC_Escherichia      YDANNVYLA--AQYTQTYNATLAGDLGWANKAQNFEVVAQYQFDFGLRPSVAYLQSKG-- 302
                           *      * ** *  *       * * *      *    ***       *  *

LbpB_Neisseria        EPAFRIENGVIEGNGFHATARTRDDGIDLSGQGSTKPQIFKANDLRVEGGFYGPKAEELG 660
OmpC_Escherichia      ----------------------KNLNANIAGRTYDDEDLLKYVDVGATYYFN-------- 332
                                             *         *  *      *

LbpB_Neisseria        GIIFNNDGKSLGITEGTENKVEADVDVDVDVDVDADADVEQLKPEVKPQFGVVFGAKKDN 720
OmpC_Escherichia      --------------KNMSTYVDYKINLLDDNQFTRAAGIN---TDDIVALGLVYQF---- 371
                                    *        *      *            * *

LbpB_Neisseria        KEVEK 725
OmpC_Escherichia      ----- 
```

Appendix 4

DNA sequence of *ompA* gene plus 1000 bp upstream and downstream the gene of *Escherichia coli K12*. The primers were design to include the upstream region and the start codon of gene (ompA_F1 and ompA_R1) and the stop codon and the downstream region of the gene (ompA_F2 and ompA_R2) as labelled below. The primers were designed against *E. coli K12* because the *E. coli* E2348/69 genomic sequence was not yet completed and was not available.

```
          gcggcaagattaatttatgttttcccgtcaccaacgacaaaatttgcgaggctctttccg
        1 ---------+---------+---------+---------+---------+---------+ 60
          cgccgttctaattaaatacaaaagggcagtggttgctgttttaaacgctccgagaaaggc

b          R  Q  D  *  F  M  F  S  R  H  Q  R  Q  N  L  R  G  S  F  R  -

          aaaatagggttgatctttgttgtcactggatgtactgtacatccatacagtaactcacag
       61 ---------+---------+---------+---------+---------+---------+ 120
          ttttatcccaactagaaacaacagtgacctacatgacatgtaggtatgtcattgagtgtc

b          K  *  G  *  S  L  L  S  L  D  V  L  Y  I  H  T  V  T  H  R  -

          gggctggattgattatgtacacttcaggctatgcacatcgttcttcgtcgttctcatccg
      121 ---------+---------+---------+---------+---------+---------+ 180
          cccgacctaactaatacatgtgaagtccgatacgtgtagcaagaagcagcaagagtaggc

b          G  W  I  D  Y  V  H  F  R  L  C  T  S  F  F  V  V  L  I  R  -

          cagcaagtaaaattgcgcgtgtctctacggaaaacactacagccgggcttatcagtgaag
      181 ---------+---------+---------+---------+---------+---------+ 240
          gtcgttcattttaacgcgcacagagatgccttttgtgatgtcggcccgaatagtcacttc

b          S  K  *  N  C  A  C  L  Y  G  K  H  Y  S  R  A  Y  Q  *  S  -

          ttgtctatcgcgaagatcagcccatgatgacgcaacttctactgttgccattgttacagc
      241 ---------+---------+---------+---------+---------+---------+ 300
          aacagatagcgcttctagtcgggtactactgcgttgaagatgacaacggtaacaatgtcg

b          C  L  S  R  R  S  A  H  D  D  A  T  S  T  V  A  I  V  T  A  -

                                      HincII
                                        |
          aactcggtcagcaatcgcgctggcaactctggttaacaccgcaacaaaaactgagtcggg
      301 ---------+---------+---------+---------+---------+---------+ 360
          ttgagccagtcgttagcgcgaccgttgagaccaattgtggcgttgtttttgactcagccc

b          T  R  S  A  I  A  L  A  T  L  V  N  T  A  T  K  T  E  S  G  -
          aatgggttcaggcatctgggctacccttaacgaaagtaatgcagattagccagctctccc
      361 ---------+---------+---------+---------+---------+---------+ 420
          ttacccaagtccgtagacccgatgggaattgctttcattacgtctaatcggtcgagaggg

b          M  G  S  G  I  W  A  T  L  N  E  S  N  A  D  *  P  A  L  P  -

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      421 ---------+---------+---------+---------+---------+---------+ 480
          gaacggtgtgacacctcagttaccaagcgcgaaatgcgtgcccgttaatgtcacaccact

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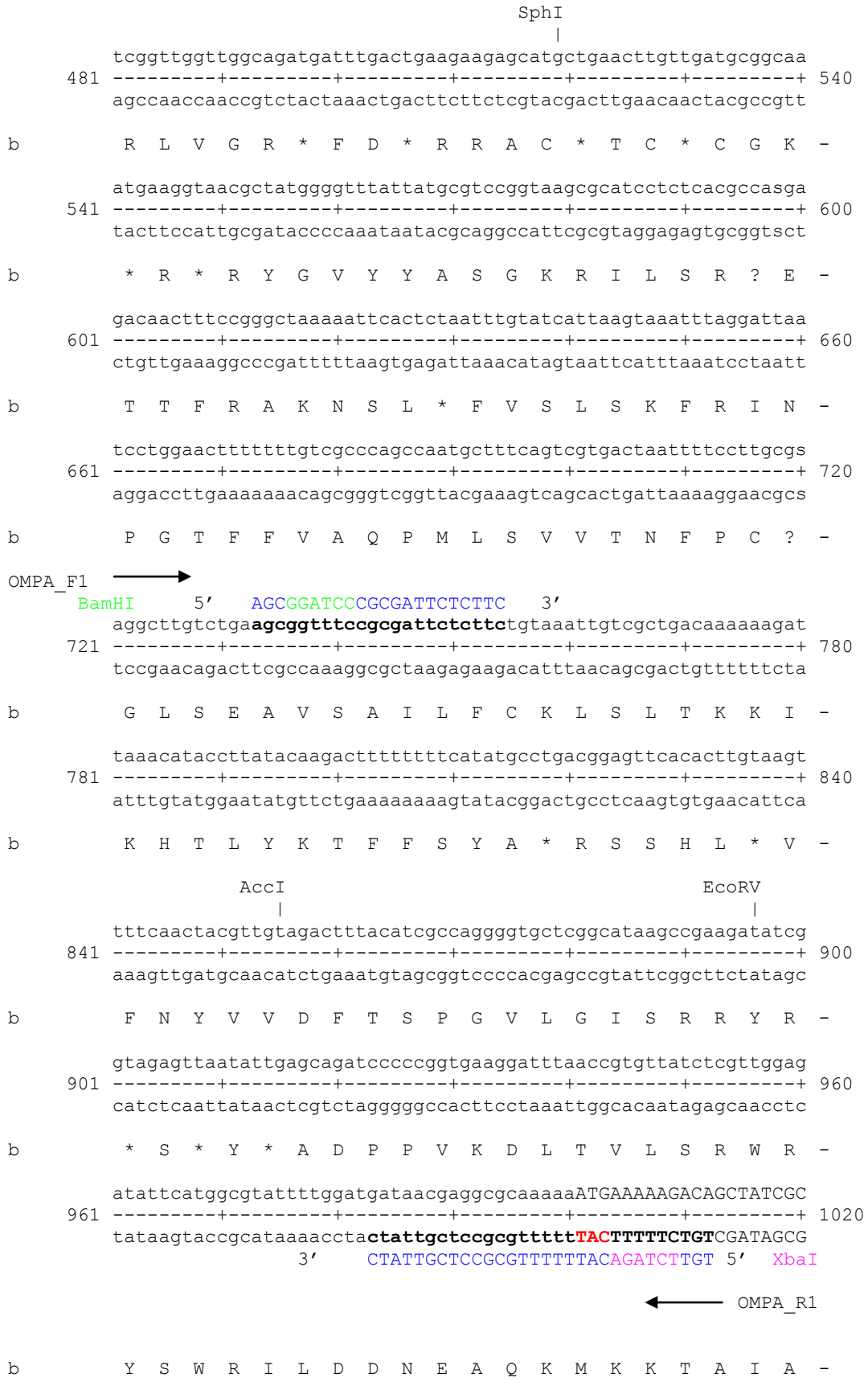
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b * R * R Y G V Y Y A S G K R I L S R ? E -
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b T T F R A K N S L * F V S L S K F R I N -
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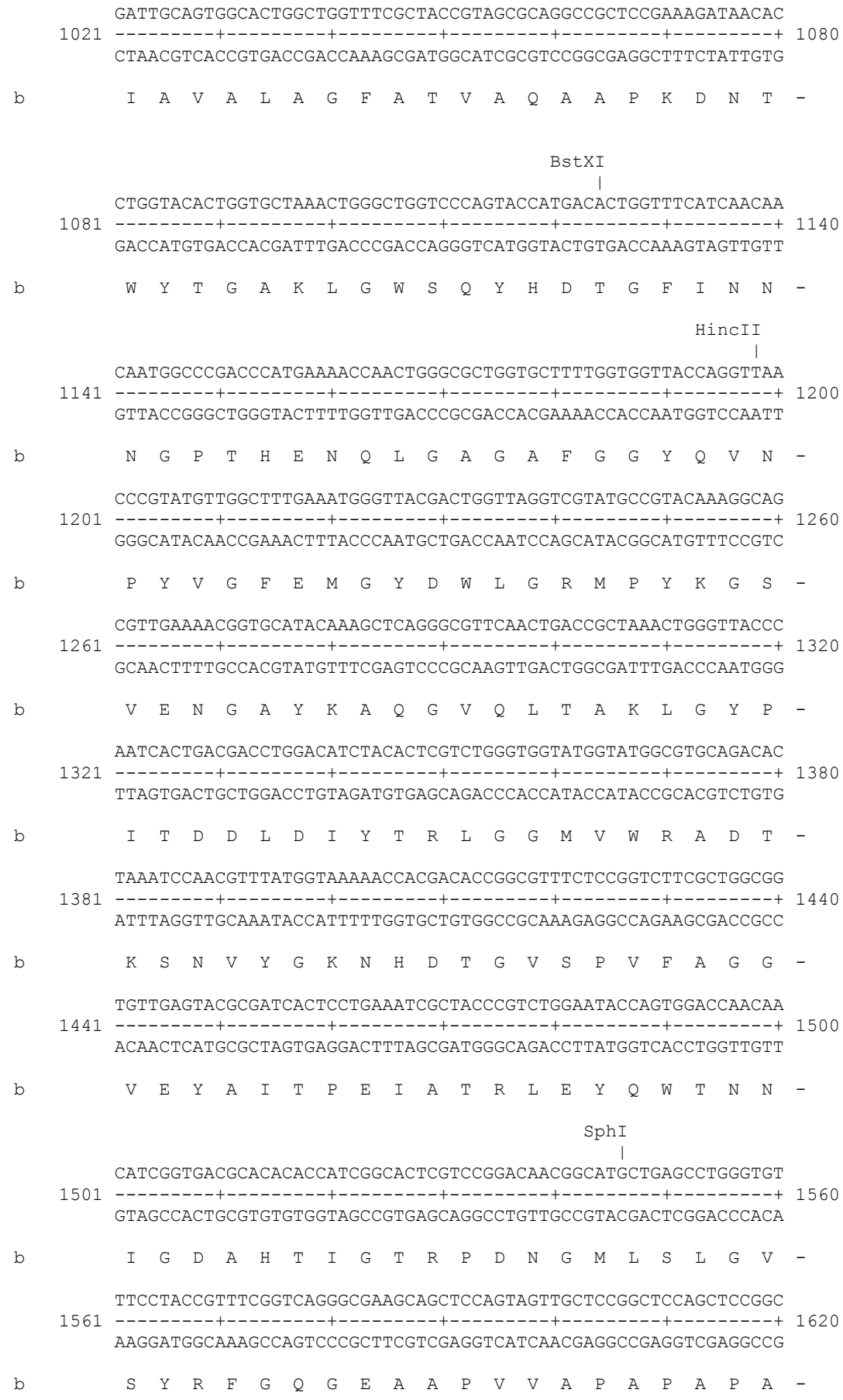
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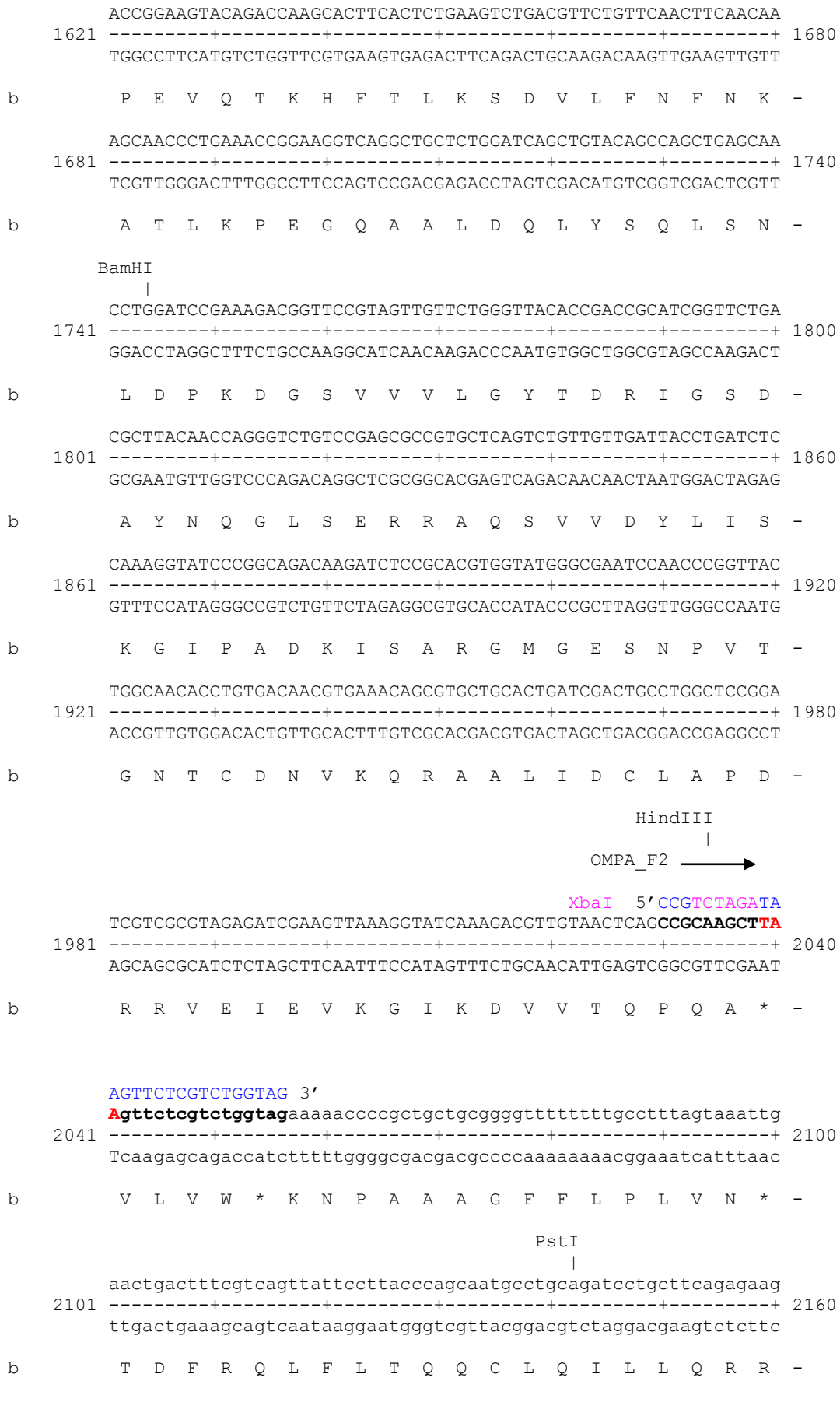
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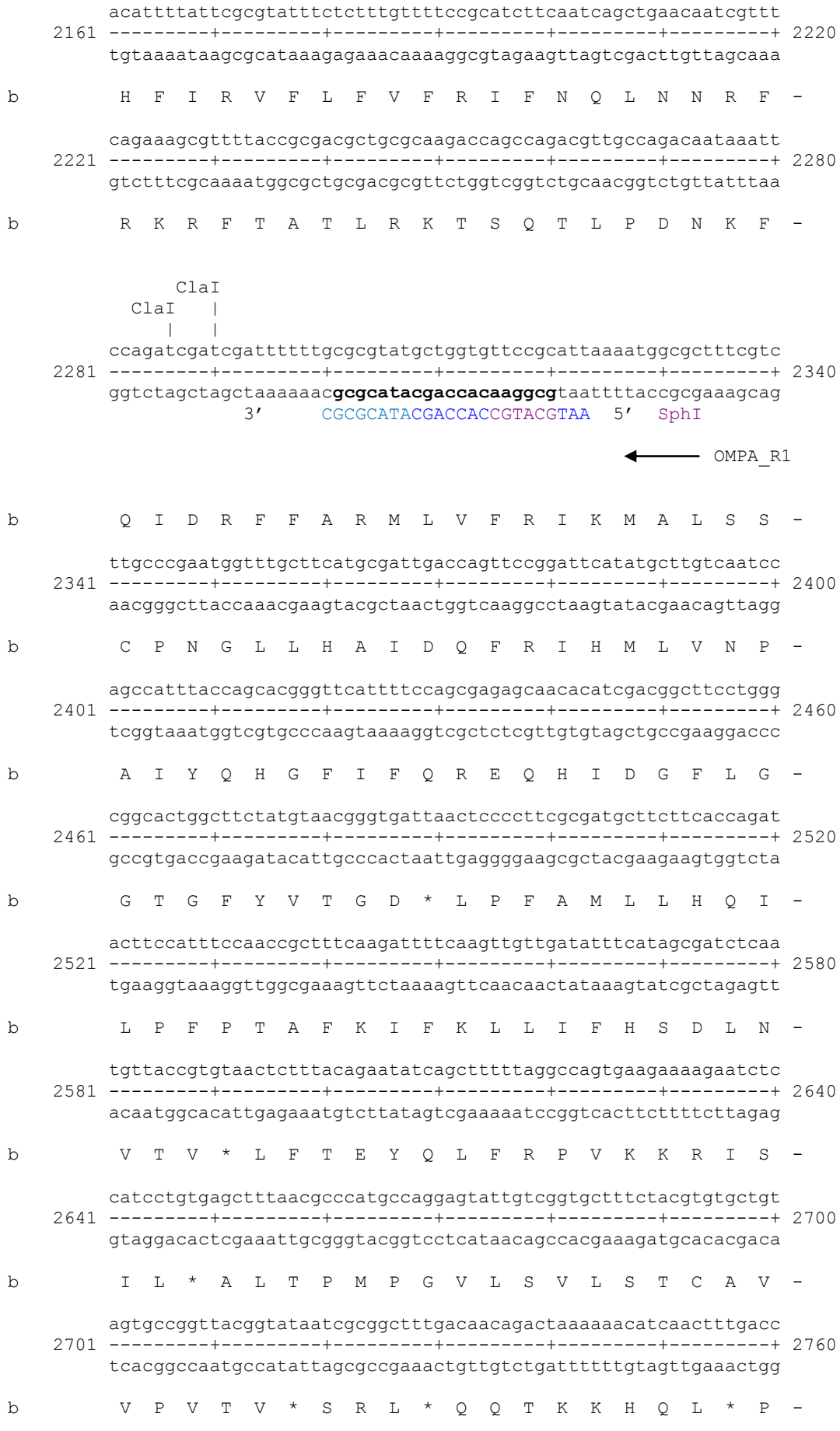
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        taatgctttgaacgtaccgcactggaccaaggactatggctatcgatagtcctttataaa

b        L  R  N  L  H  G  V  T  W  F  L  I  P  I  A  I  R  K  Y  L  -

                                                          SacII
                                                            |
        gctcagccacatttgattgacgaaaacgatcctttattcagtgatactcaaccgcggctg
    2821 ---------+---------+---------+---------+---------+---------+ 2880
        cgagtcggtgtaaactaactgcttttgctaggaaataagtcactatgagttggcgccgac

b        L  S  H  I  *  L  T  K  T  I  L  Y  S  V  I  L  N  R  G  C  -

                                                              DraII
                                                                |
        caatttgcgctggagcagttgctgcatacgcgagcatcctcctcttttatgctggcgaag
    2881 ---------+---------+---------+---------+---------+---------+ 2940
        gttaaacgcgacctcgtcaacgacgtatgcgctcgtaggaggagaaaatacgaccgcttc

b        N  L  R  W  S  S  C  C  I  R  E  H  P  P  L  L  C  W  R  R  -

        gccccggaagagtctgagtatctgaatcttattgccaatgccgcgcgtacgctacaaagc
    2941 ---------+---------+---------+---------+---------+---------+ 3000
        cggggccttctcagactcatagacttagaataacggttacggcgcgcatgcgatgtttcg

b        P  R  K  S  L  S  I  *  I  L  L  P  M  P  R  V  R  Y  K  A  -

        gatgcaggccaactggtgggcggtcactatgaggtttccgg
    3001 ---------+---------+---------+---------+- 3041
        ctacgtccggttgaccacccgccagtgatactccaaaggcc

b        M  Q  A  N  W  W  A  V  T  M  R  F  P   -
```

Enzymes that do cut:

AccI	BamHI	BstXI	ClaI	DraII	EcoRV	HincII	HindIII
PstI	SacII	SphI					

Enzymes that do not cut:

ApaI	BanII	EagI	EcoRI	KpnI	NotI	SacI	SalI
SmaI	SpeI	SstI	XbaI	XhoI	XmaI		

PRIMERS:

OMPA_F1 5′ AGCGGATCCCGCGATTCTCTTC 3′ Tm:59

OMPA_R1 5′ TGTTCTAGACATTTTTTGCGCCTCGTTATC 3′Tm:59

OMPA_F2 5′ CCGTCTAGATAAGTTCTCGTCTGGTAG 3′ Tm:60

OMPA_R2 5′ AATGCATGCCACCAGCATACGCGC 3′ Tm:61

Appendix 5

DNA sequence of *ompC* gene plus 1000 bp upstream and downstream the gene of *Escherichia coli E2348/69*. The primers were design to include the upstream region and the start codon of gene (ompC_F1 and ompC_R1) and the stop codon and the downstream region of the gene (ompC_F2 and ompC_R2) as labelled below.

```
          cgccggaggaggggaggtcgtcgcggcaatgttgtcgtagatctgccaggtcacgtaac
        1 ---------+---------+---------+---------+---------+---------+ 60
          gcggcctcctccccctccagcagcgccgttacaacagcatctagacggtccagtgcattg

b          A  G  G  G  G  G  R  R  G  N  V  V  V  D  L  P  G  H  V  T  -

                                          HincII
                                           AccI|
                                          SalI||
                                             |||
          gccaggtatcgacgcgcttttggattgcgtgggtaatgtcgacaatctggtaacttttat
       61 ---------+---------+---------+---------+---------+---------+ 120
          cggtccatagctgcgcgaaaacctaacgcacccattacagctgttagaccattgaaaata

b          P  G  I  D  A  L  L  D  C  V  G  N  V  D  N  L  V  T  F  I  -

                    HincII
                         |
          ctttcagccaggcgttaacggcactttgtatcattacccccatcgtcaccagtaacacaa
      121 ---------+---------+---------+---------+---------+---------+ 180
          gaaagtcggtccgcaattgccgtgaaacatagtaatgggggtagcagtggtcattgtgtt

b          F  Q  P  G  V  N  G  T  L  Y  H  Y  P  H  R  H  Q  *  H  N  -

          tgatcaacagtaaaaagaagcgggtaatgctccccggtaggagtgaaaagcgggtcgtgg
      181 ---------+---------+---------+---------+---------+---------+ 240
          actagttgtcatttttcttcgcccattacgaggggccatcctcacttttcgcccagcacc

b          D  Q  Q  *  K  E  A  G  N  A  P  R  *  E  *  K  A  G  R  G  -

          ccgttgtctctttctgacgcattcgtgtttatgacctgttaaaacttcgcgaagtgatag
      241 ---------+---------+---------+---------+---------+---------+ 300
          ggcaacagagaaagactgcgtaagcacaaatactggacaattttgaagcgcttcactatc

b          R  C  L  F  L  T  H  S  C  L  *  P  V  K  T  S  R  S  D  R  -

          ggcagtataaagggtacagtgtgaaattccagactcttacctgattgatgattttctatt
      301 ---------+---------+---------+---------+---------+---------+ 360
          ccgtcatatttcccatgtcacactttaaggtctgagaatggactaactactaaaagataa

b          A  V  *  R  V  Q  C  E  I  P  D  S  Y  L  I  D  D  F  L  F  -

          ctcccgtgatatttatcatcctgcattgggggaaatagatgtacaagcgccattttgctg
      361 ---------+---------+---------+---------+---------+---------+ 420
          gagggcactataaatagtaggacgtaacccccttttatctacatgttcgcggtaaaacgac

b          S  R  D  I  Y  H  P  A  L  G  E  I  D  V  Q  A  P  F  C  C  -

          tacaaataacctacaaaaagcccaatgaaattaattgcaaataaacgaagattgctggaa
      421 ---------+---------+---------+---------+---------+---------+ 480
          atgtttattggatgtttttcgggttactttaattaacgtttatttgcttctaacgacctt

b          T  N  N  L  Q  K  A  Q  *  N  *  L  Q  I  N  E  D  C  W  K  -
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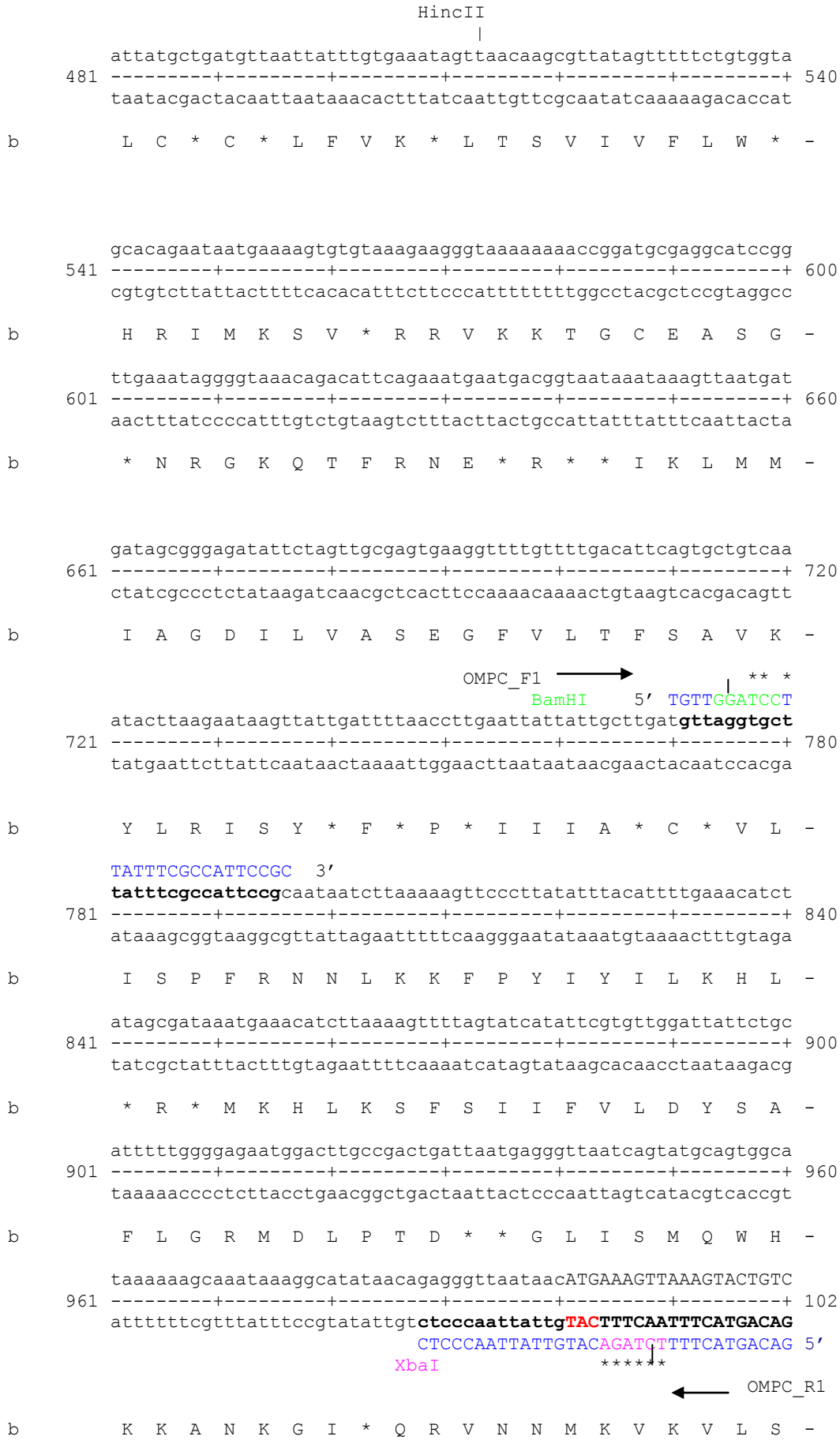
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b L C * C * L F V K * L T S V I V F L W * -
gcacagaataatgaaaagtgtgtaaagaagggtaaaaaaaaccggatgcgaggcatccgg
541 ---------+---------+---------+---------+---------+---------+ 600
cgtgtcttattacttttcacacatttcttcccatttttttttggcctacgctccgtaggcc
b H R I M K S V * R R V K K T G C E A S G -
ttgaaataggggtaaacagacattcagaaatgaatgacggtaataaataaagttaatgat
601 ---------+---------+---------+---------+---------+---------+ 660
aactttatccccatttgtctgtaagtctttacttactgccattatttatttcaattacta
b * N R G K Q T F R N E * R * * I K L M M -
gatagcgggagatattctagttgcgagtgaaggttttgttttgacattcagtgctgtcaa
661 ---------+---------+---------+---------+---------+---------+ 720
ctatcgccctctataagatcaacgctcacttccaaaacaaaactgtaagtcacgacagtt
b I A G D I L V A S E G F V L T F S A V K -
OMPC_F1 ** *
BamHI 5′ TGTTGGATCCT
atacttaagaataagttattgattttaaccttgaattattattgcttgatgttaggtgct
721 ---------+---------+---------+---------+---------+---------+ 780
tatgaattcttattcaataactaaaattggaacttaataataacgaactacaatccacga
b Y L R I S Y * F * P * I I I A * C * V L -
TATTTCGCCATTCCGC 3′
tatttcgccattccgcaataatcttaaaaagttcccttatatttacattttgaaacatct
781 ---------+---------+---------+---------+---------+---------+ 840
ataaagcggtaaggcgttattagaatttttcaagggaatataaatgtaaaactttgtaga
b I S P F R N N L K K F P Y I Y I L K H L -
atagcgataaatgaaacatcttaaaagttttagtatcatattcgtgttggattattctgc
841 ---------+---------+---------+---------+---------+---------+ 900
tatcgctatttactttgtagaattttcaaaatcatagtataagcacaacctaataagacg
b * R * M K H L K S F S I I F V L D Y S A -
atttttggggagaatggacttgccgactgattaatgagggttaatcagtatgcagtggca
901 ---------+---------+---------+---------+---------+---------+ 960
taaaaacccctcttacctgaacggctgactaattactcccaattagtcatacgtcaccgt
b F L G R M D L P T D * * G L I S M Q W H -
taaaaaagcaaataaaggcatataacagagggttaataacATGAAAGTTAAAGTACTGTC
961 ---------+---------+---------+---------+---------+---------+ 1020
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XbaI ******
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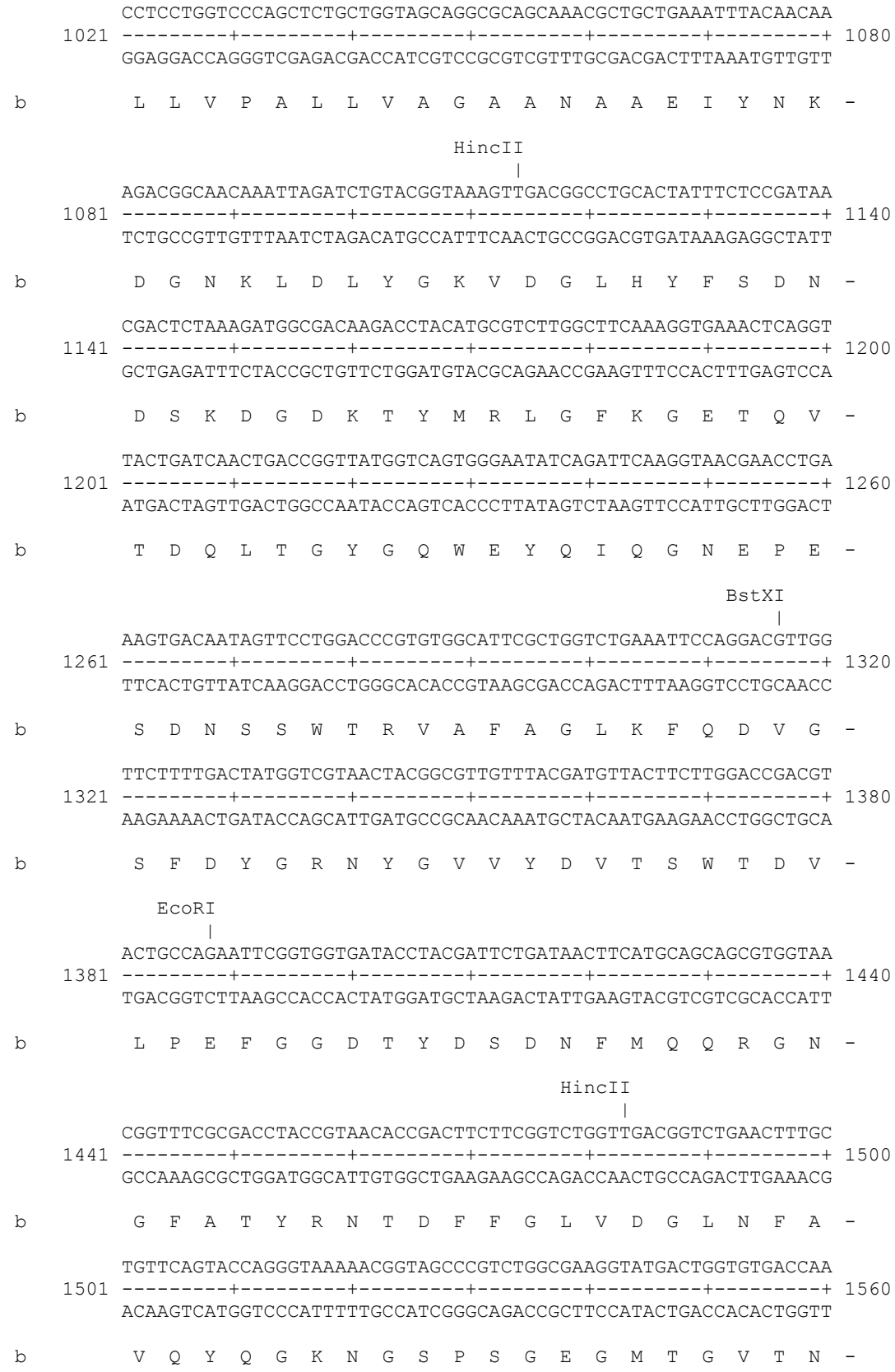
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1081 1140
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b D G N K L D L Y G K V D G L H Y F S D N -
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1141 1200
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b D S K D G D K T Y M R L G F K G E T Q V -
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1201 1260
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b T D Q L T G Y G Q W E Y Q I Q G N E P E -
BstXI
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1261 1320
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b S D N S S W T R V A F A G L K F Q D V G -
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1321 1380
AAGAAAACTGATACCAGCATTGATGCCGCAACAAATGCTACAATGAAGAACCTGGCTGCA
b S F D Y G R N Y G V V Y D V T S W T D V -
EcoRI
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1381 1440
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b L P E F G G D T Y D S D N F M Q Q R G N -
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CGGTTTCGCGACCTACCGTAACACCGACTTCTTCGGTCTGGTTGACGGTCTGAACTTTGC
1441 1500
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b G F A T Y R N T D F F G L V D G L N F A -
TGTTCAGTACCAGGGTAAAAACGGTAGCCCGTCTGGCGAAGGTATGACTGGTGTGACCAA
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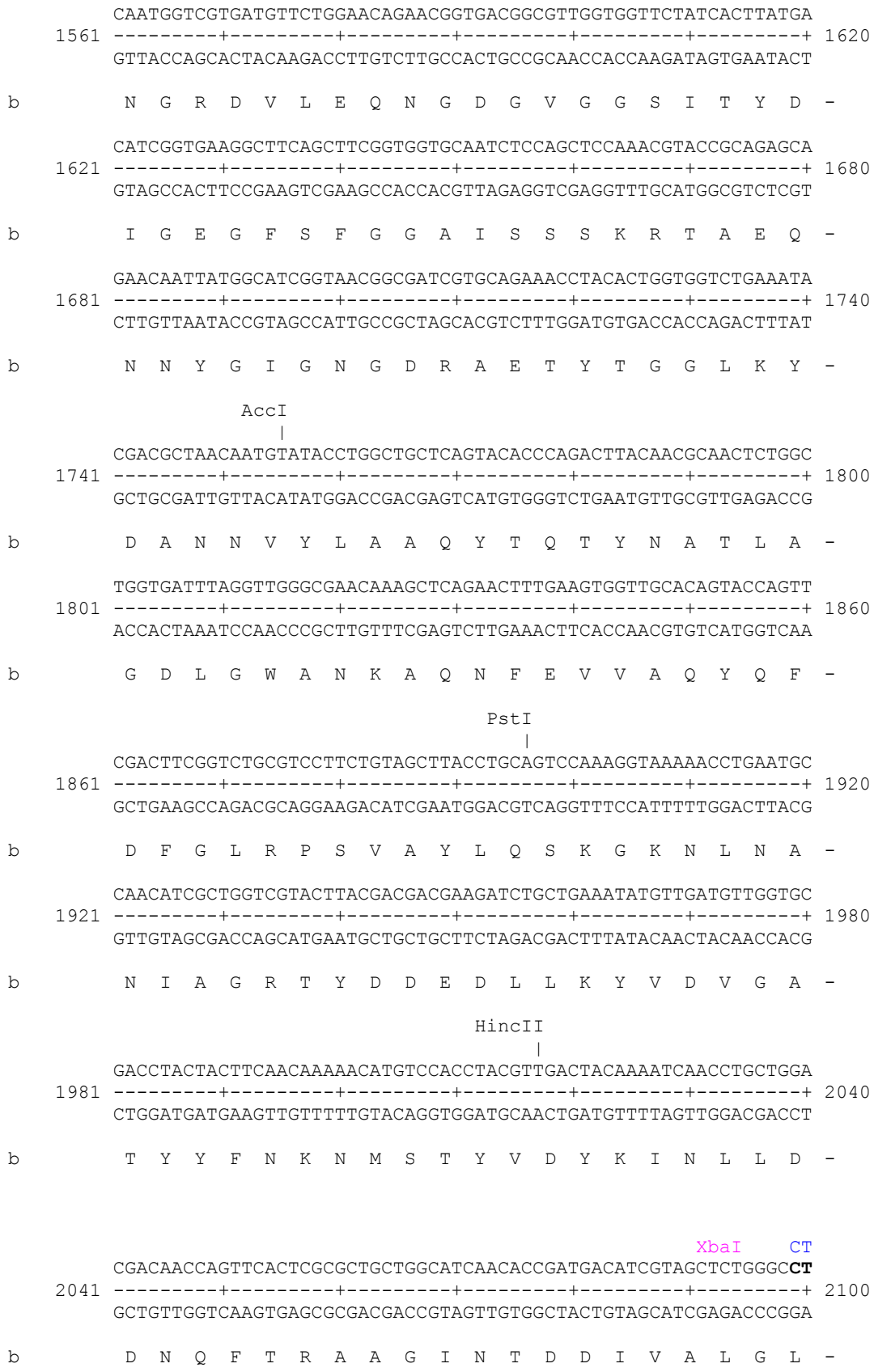
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b I G E G F S F G G A I S S S K R T A E Q -
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1681 ---------+---------+---------+---------+---------+---------+---------+ 1740
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b N N Y G I G N G D R A E T Y T G G L K Y -
AccI
|
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1741 ---------+---------+---------+---------+---------+---------+---------+ 1800
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b D A N N V Y L A A Q Y T Q T Y N A T L A -
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1801 ---------+---------+---------+---------+---------+---------+---------+ 1860
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b G D L G W A N K A Q N F E V V A Q Y Q F -
PstI
|
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1861 ---------+---------+---------+---------+---------+---------+---------+ 1920
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b D F G L R P S V A Y L Q S K G K N L N A -
CAACATCGCTGGTCGTACTTACGACGACGAAGATCTGCTGAAATATGTTGATGTTGGTGC
1921 ---------+---------+---------+---------+---------+---------+---------+ 1980
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b N I A G R T Y D D E D L L K Y V D V G A -
HincII
|
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1981 ---------+---------+---------+---------+---------+---------+---------+ 2040
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b T Y Y F N K N M S T Y V D Y K I N L L D -
XbaI CT
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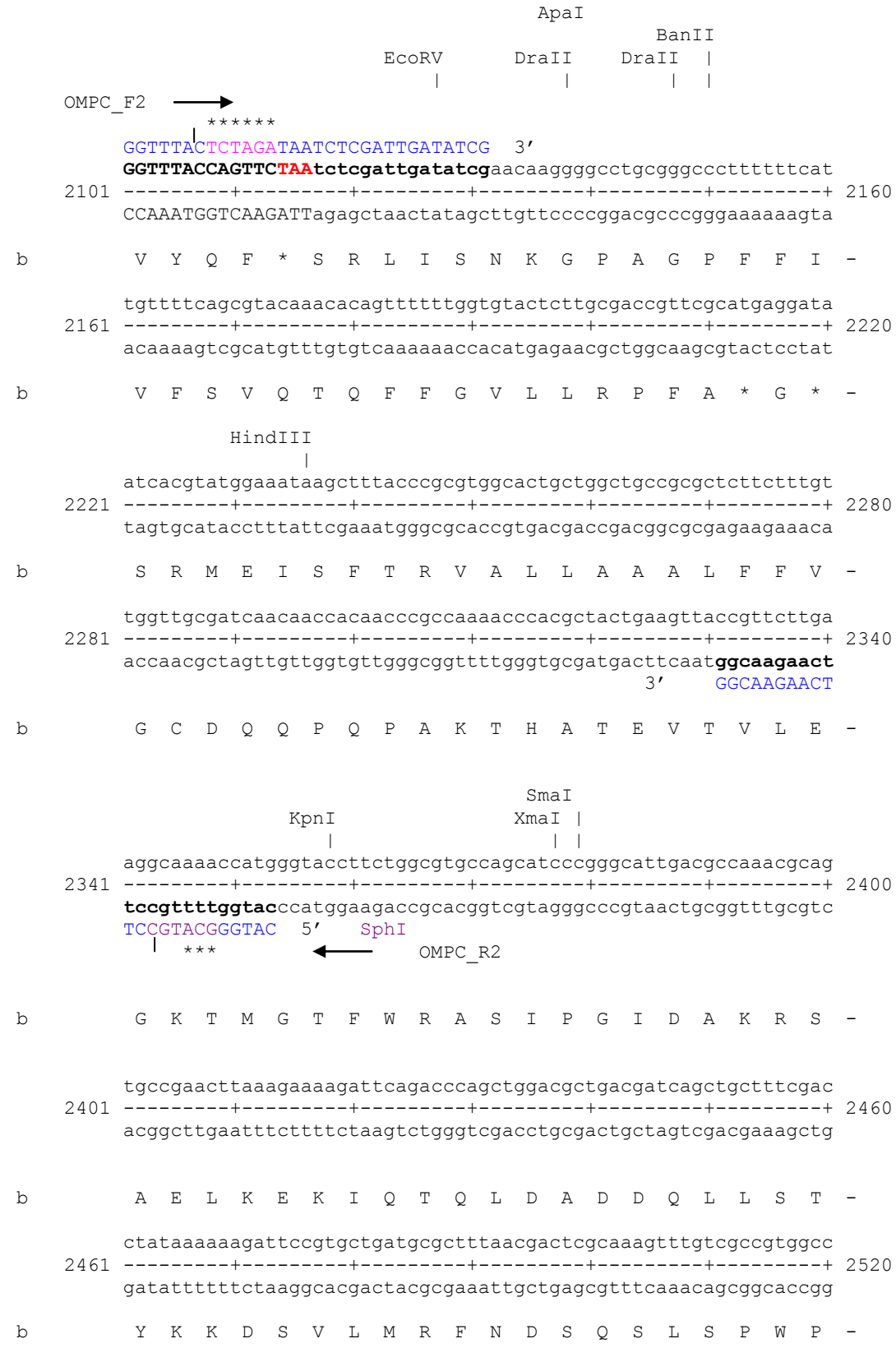
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KpnI
XmaI
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***
OMPC_R2
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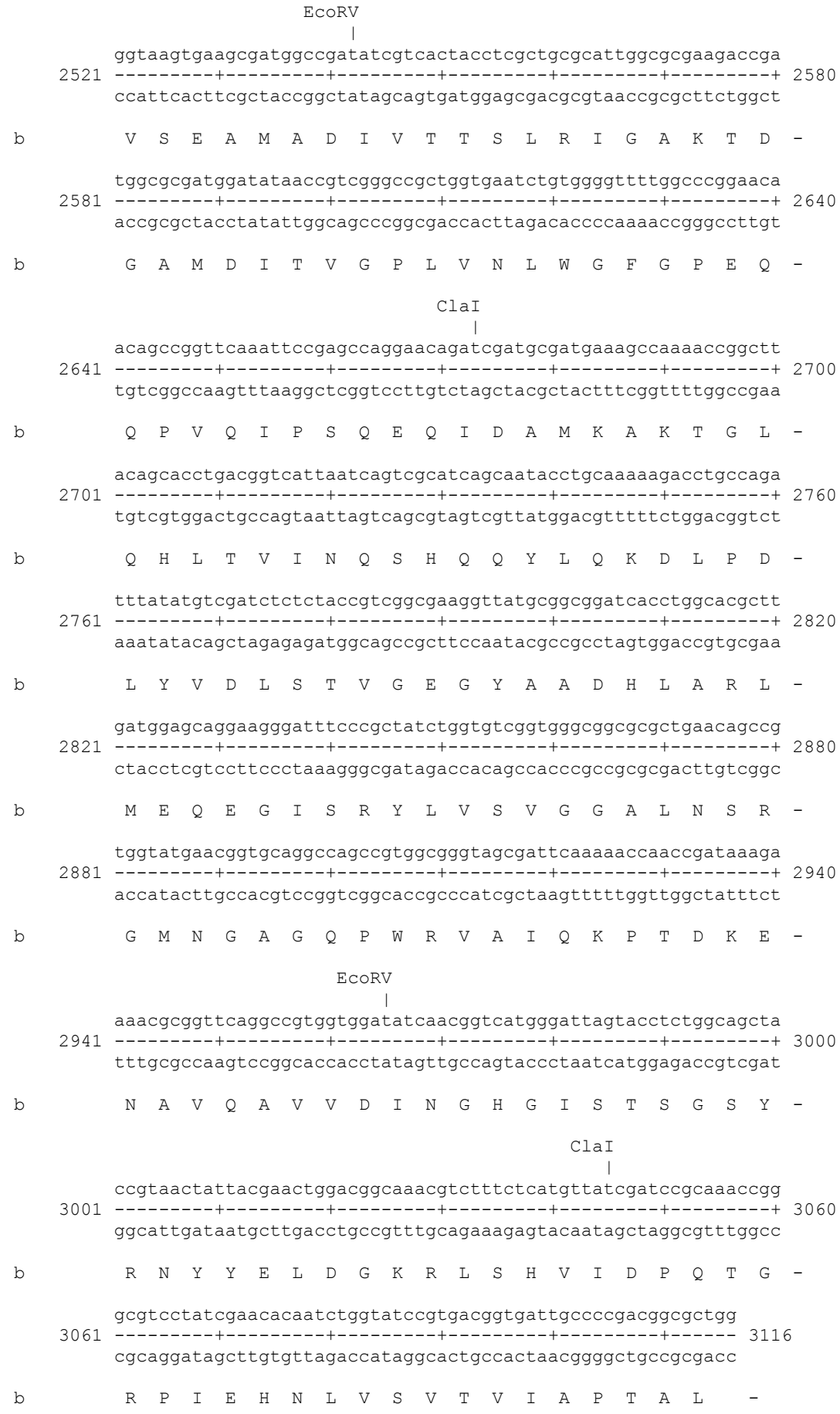
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3061 cgcaggatagcttgtgttagaccataggcactgccactaacggggctgccgcgacc 3116
b R P I E H N L V S V T V I A P T A L -

Enzymes that do cut:

AccI ApaI BanII BstXI ClaI DraII EcoRI EcoRV
HincII HindIII KpnI PstI SalI SmaI XmaI

Enzymes that do not cut:

BamHI EagI NotI SacI SacII SpeI SphI SstI
XbaI XhoI

PRIMERS:

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OMPC_R1 5′GACAGTACTTTTCTAGACATGTTATTAACCCTC 3′ Tm:59

OMPC_F2 5′CTGGTTTACTCTAGATAATCTCGATTGATATCG 3′ Tm:59

OMPC_R2 5′CATGGGCATGCCTTCAAGAACGG 3′ Tm:59

Kan_F 5′ GACAGCAAGCGAACCGG 3′ Tm:52

Kan_R 5′ TTGGTCGGTCATTTCGAACC 3′ Tm:52

OMPC_R1λ Tm:68

5'CCGGTTCGCTTGCTGTCAGTACTTTAACTTTCATGTTATTAACCCTC 3'

OMPC_F2λ Tm:69

5' GGTTCGAAATGACCGACCAAGTTTACCAGTTCTAATCTCGATTGATATCG 3'

Appendix 6

***ompA* sequence from Sanger.** The sequence of the ompA gene became available after a year from the starting of the project. In blue font are the upstream and downstream regions of the *ompA* gene that were amplified by PCR to construct the deletion mutant.

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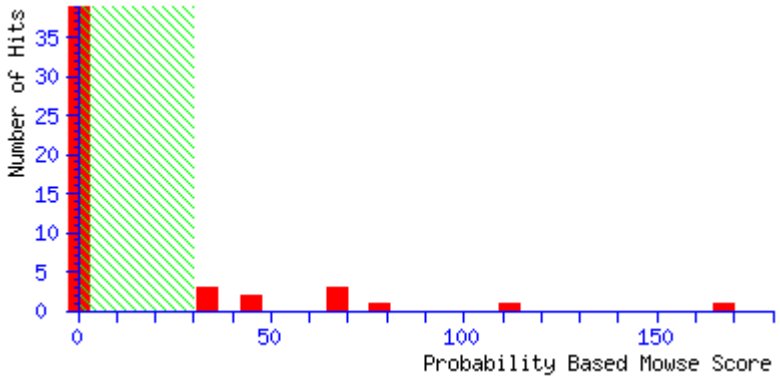
CCCGTATGTTGGCTTTGAAATGGGTTATGACTGGTTAGGTCGTATGCCATACAAAGGCAG
CGTTGAAAACGGTGCATATAAAGCTCAGGGCGTTCAGCTGACCGCTAAACTGGGTTACCC
AATCACTGACGATCTGGACGTATACACTCGTCTGGGTGGTATGGTATGGCGTGCAGACAC
TAAATCCAACGTTTATGGTAAAAACCACGACACCGGCGTTTCTCCGGTCTTCGCTGGCGG
TGTTGAGTATGCGATCACTCCTGAAATCGCTACCCGTCTGGAATACCAGTGGACCAACAA
CATCGGTGACGCACACACCATCGGCACTCGTCCGGACAACGGCATGCTGAGCCTGGGTGT
TTCCTACCGTTTCGGTCAGGGCGAAGTAGCTCCAGTAGTTGCTCCGGCTCCAGCTCCGGC
ACCGGAAGTACAGACCAAGCACTTCACTCTGAAGTCTGACGTTCTGTTCACCTTCAACAA
AGCAACCCTGAAACCGGAAGGTCAGGCTGCTCTGGATCAGCTGTACAGCCAGCTGAGCAA
CCTGGATCCGAAAGACGGTTCCGTAGTTGTTCTGGGTTACACTGACCGCATCGGTTCTGA
CGCTTATAACCAGGCTCTGTCCGAGCGTCGTGCTCAGTCCGTTGTTGATTACCTGATCTC
TAAAGGTATCCCGGCAGACAAAATCTCCGCACGTGGTATGGGCGAATCCAACCCGGTTAC
TGGCAACACCTGTGACAACGTGAAACAGCGTGCTGCACTGATCGATTGCCTGGCTCCGGA
TCGTCGCGTAGAGATCGAAGTTAAAGGCATCAAAGACGTTGTAACTCAGCCGCAGGCT**TA**
AGTTCTCGTCTGGTAGAAAAACCCCGCAGCTGCGGGGTTTTTTTTTCGCCTTTAGTAAAT
TGAACTGACTTTTGACAGTTATTCCTTACCCAGCAATGCCTGCAGATCCTGCTTCAAAGA
AGACATTTTATTCGCGTATTTCTCTTTGTTTTCCGCATCTTCAATCAGCTGAACAATCGT
TTCAGAAAGCGTTTTACCGCGACGCTGCGCAAGGCCAGCCAGACGTTGCCAGACGATAAA
TTCCAGATCGATCGATTTTTTGCGCGTATGCTGGTGTTCTGCATTAAAATGGCGCTTCCG
TCTGGCCCGAATGGTCTGCTTCATGCGATTGACCAGTTCCGGATTCATATGCTTGTCAAT
CCAACCATTTACCAGCACGGGTTCATTTTCCAGCGAGAGCAACTCATCGACGGCTTCCTG
GGCGGCACTGGCTTCTATGTAACGGGTGATTAACTCCCCTTCGCGGTGCTTCTTCACCAG
ATACTTCCATTTCCAACCGCTTTCAAGATTTTCAAGTTGTTGATATTTCATAGCGATCTC
AATGTTACCGTGTAACTCTTTACAGAATATCAGCTTTTTAGGCCAGTGAAGAAAAGAATC
TCCATCCTGTGAGCTTTAACGCCCATGCCAGGAGTATTGTCGTTGCTTTCTACGTGTGCT
GCAGTGCCGGTTACGGTATAATCGCGGCTTTGACAACAGACTAAAAAACATCAACTTTGA
CCATTACGAAACTTGCATGGCGTGACCTGGTTCCTGATACCGATAGCTATCAGGAAATAT
TTGCTCAGCCACATTTGATTGACGAAAACGATCCTTTATTCAGTGATACTCAACCGCGAC
TGCAATTTGCGCTGGAGCAGTTGCTGCATACGCGAGCATCCTCCTCTTTTATGCTGGCGA
AGGCCCCGGAAGAGTCTGAGTATCTGAATCTTATTGCTGATGCCGCGCGTACGCTACAAA
GCGATGCAGGCCAACTGGTGGGCGGTCACTATGAGGTTTCCGG

Appendix 7

Peptide mass fingerprinting sequencing blast results of proteins in Fig.4.15.
PROBABILITY BASED MOWSE SCORE

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 30 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

BAND 1

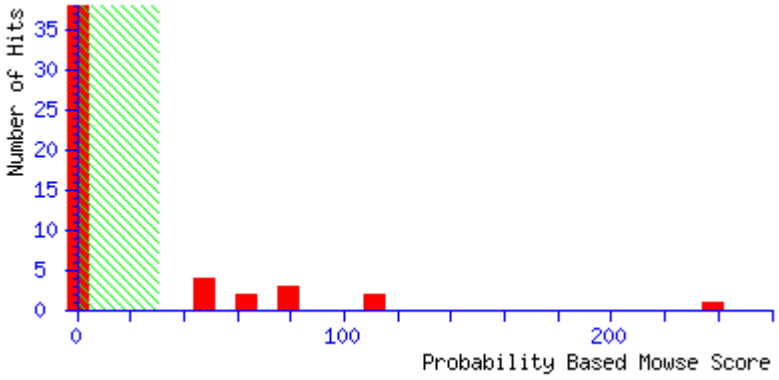


OMPC_ECOLI Mass: 40343 Score: 167 Queries matched: 9 emPAI: 0.71
Outer membrane protein C precursor - Escherichia coli (strain K12)

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
11	443.6433	885.2720	884.4141	0.8580	0	45	0.002	1	R.GNGFATYR.N
16	470.1631	938.3116	938.4709	-0.1593	0	(18)	0.79	1	R.AETYTGGLK.Y
17	470.1712	938.3279	938.4709	-0.1430	0	35	0.013	1	R.AETYTGGLK.Y
18	470.2093	938.4041	938.4709	-0.0668	0	(17)	0.91	1	R.AETYTGGLK.Y
41	568.7164	1135.4182	1135.4856	-0.0673	0	40	0.006	1	K.NMSTYVDYK.I + Oxidation (M)
42	568.7329	1135.4513	1135.4856	-0.0342	0	(31)	0.046	1	K.NMSTYVDYK.I + Oxidation (M)
63	645.7172	1289.4198	1289.5677	-0.1479	0	47	0.0014	1	K.FQDVGSFDYGR.N
70	674.7744	1347.5343	1347.6783	-0.1440	0	79	7.1e-007	1	K.INLLDDNQFTR.D
76	720.2701	1438.5256	1438.6769	-0.1513	0	49	0.00077	1	K.YVDVGATYYFNK.N

Proteins matching the same set of peptides:
OMPC_ECO57 Mass: 40483 Score: 167 Queries matched: 9
Outer membrane protein C precursor - Escherichia coli O157:H7

Band 2

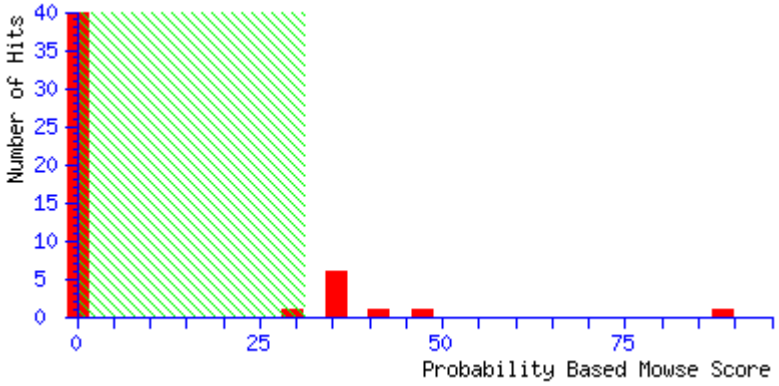


OMPA_ECOLI Mass: 37292 Score: 238 Queries matched: 6 emPAI: 0.78
Outer membrane protein A precursor - Escherichia coli (strain K12)

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
2	409.6357	817.2569	817.4269	-0.1700	0	58	0.00013	1	R.LGGMVWR.A
21	458.2124	914.4103	914.5185	-0.1082	0	56	0.00016	1	K.AQGVQLTAK.L
51	607.6966	1213.3787	1213.6125	-0.2338	0	80	6.1e-007	1	R.AALIDCLAPDR.R
52	611.7254	1221.4363	1221.6605	-0.2242	0	48	0.00085	1	R.AQSVVDYLISK.G
61	640.7419	1279.4693	1279.6409	-0.1715	0	67	1.2e-005	1	K.DGSVVVLGYTDR.I
147	867.7261	2600.1565	2600.2871	-0.1306	0	67	1.1e-005	1	K.NHDTGVSPVFAGGVEYAITPEIATR.L

Proteins matching the same set of peptides:
OMPA_ECO57 Mass: 37292 Score: 238 Queries matched: 6
Outer membrane protein A precursor - Escherichia coli O157:H7

Band 3

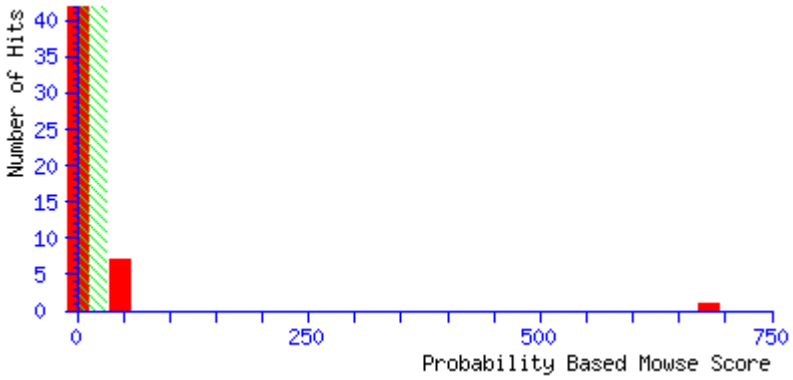


EFTU_ECOLI Mass: 43457 Score: 345 Queries matched: 12 emPAI: 1.10
Elongation factor Tu - Escherichia coli (strain K12)

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
71	514.2219	1026.4293	1026.5822	-0.1529	0	76	1.7e-006	1	R.AGENVGVLLR.G
74	535.2590	1068.5035	1069.5226	-1.0191	2	9	8.4	10	-.MSKEKFER.T + Oxidation (M)
79	586.2498	1170.4850	1170.6496	-0.1646	0	92	6e-008	1	K.VGEEVEIVGIK.E
83	607.7561	1213.4976	1213.6230	-0.1254	0	51	0.00061	1	K.FESEVYILSK.D
84	609.6866	1217.3587	1217.5564	-0.1977	0	76	2.1e-006	1	K.ALEGDAEWEAK.I
92	411.7916	1232.3529	1232.6091	-0.2562	0	19	0.94	1	K.GYRPQFYFR.T
100	652.3603	1302.7060	1302.7759	-0.0698	0	67	1.5e-005	1	K.TTLTAAITTVLAK.T
149	745.3631	1488.7116	1488.8704	-0.1588	0	37	0.016	1	R.QVGVPYIIVFLNK.C
188	594.2496	1779.7270	1779.9375	-0.2105	0	67	1.5e-005	1	K.MVVTLIHPIAMDDGLR.F
192	599.6292	1795.8658	1795.9325	-0.0666	0	(62)	5.6e-005	1	K.MVVTLIHPIAMDDGLR.F + Oxidation (M)
194	601.9135	1802.7186	1802.8799	-0.1613	0	25	0.24	1	R.GITINTSHVEYDTPTR.H
220	982.8716	1963.7287	1963.9527	-0.2241	0	66	1.9e-005	1	R.ELLSQYDFPGDDTPIVR.G

Proteins matching the same set of peptides:
EFTU_ECOL6 Mass: 43457 Score: 345 Queries matched: 12
Elongation factor Tu - Escherichia coli O6
EFTU_ECO57 Mass: 43457 Score: 345 Queries matched: 12
Elongation factor Tu - Escherichia coli O157:H7

Band 4

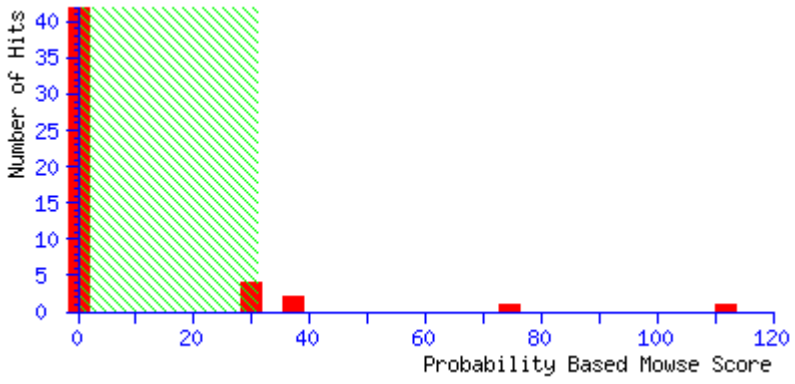


BFPB_ECO11 Mass: 58507 Score: 682 Queries matched: 23 emPAI: 2.04
Major outer membrane lipoprotein precursor - Escherichia coli (strain K12)

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
1	409.6286	817.2426	817.4181	-0.1755	0	21	0.82	2	K.DIENSIK.L
20	437.1806	872.3466	872.4603	-0.1137	0	55	0.00035	1	K.DNLGVIDK.N
26	441.2082	880.4018	881.4276	-1.0259	1	18	1.1	1	R.TSSANMKK.I + Oxidation (M)
29	445.1714	888.3282	888.4916	-0.1635	0	44	0.0035	1	K.SLAATVAEK.M
52	474.6795	947.3445	947.4924	-0.1478	0	36	0.02	1	K.DVISGGVSSK.S
74	509.7025	1017.3904	1017.5859	-0.1954	0	53	0.00046	1	R.FSISILPGGK.Y
78	553.7737	1105.5329	1105.6608	-0.1278	0	57	0.00016	1	K.LEGVHGIILR.S
80	558.2104	1114.4062	1114.6095	-0.2033	0	28	0.13	1	K.LLGGALNGSASR.T
86	579.6641	1157.3136	1157.5030	-0.1894	0	60	7.8e-005	1	K.FDSDVDFWK.D
93	612.7549	1223.4952	1223.6874	-0.1921	0	41	0.0062	1	K.NILHADTSLLK.S
104	639.7622	1277.5098	1277.6615	-0.1517	0	(66)	1.9e-005	1	R.NGNTLVLAGYEK.K
105	640.2459	1278.4772	1277.6615	0.8156	0	69	1e-005	1	R.NGNTLVLAGYEK.K
148	734.7617	1467.5088	1467.6842	-0.1754	0	104	3.1e-009	1	R.NESVDQGVGTTSFK.L
174	845.8169	1689.6192	1689.8685	-0.2494	0	106	2.1e-009	1	K.INEYINTLNAQLER.Q
195	606.9402	1817.7988	1817.9635	-0.1647	1	67	1.7e-005	1	K.KINEYINTLNAQLER.Q
223	653.2661	1956.7764	1956.9105	-0.1341	1	54	0.00035	1	K.FDSDVDFWKDIENSIK.L
233	994.4313	1986.8480	1987.0660	-0.2180	0	92	5.2e-008	1	K.VSVVTSASVTTMSGQPVPLK.V
234	663.6800	1988.0182	1987.0660	0.9522	0	(25)	0.25	1	K.VSVVTSASVTTMSGQPVPLK.V
242	1071.5228	2141.0311	2141.1216	-0.0905	0	124	2.8e-011	1	K.LILGSDGSYSISTSTSSVIVR.T
260	804.3586	2410.0539	2410.1441	-0.0902	0	34	0.031	1	K.TDSIYIGNSSFQTYHGEPLPGK.L
264	841.3219	2520.9440	2519.2829	1.6611	0	50	0.00069	1	R.SSTPLGFDEVLSMIQDSSGIPIVK.H
265	846.3029	2535.8869	2535.2778	0.6091	0	(35)	0.022	1	R.SSTPLGFDEVLSMIQDSSGIPIVK.H + Oxidation (M)
268	884.6247	2650.8522	2651.0914	-0.2392	0	102	4.6e-009	1	K.NSISSDSNSSSGSSGSSGSSSSDSGAELK.F

Proteins matching the same set of peptides:
BFPB_ECO27 Mass: 58452 Score: 682 Queries matched: 23
Major outer membrane lipoprotein precursor - Escherichia coli (strain K12)

Band 5



RL6_ECO57 Mass: 18949 Score: 112 Queries matched: 3 emPAI: 0.74
50S ribosomal protein L6 - Escherichia coli O157:H7

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
61	494.6999	987.3852	987.5237	-0.1385	0	60	9.3e-005	1	R.TLNDAVEVK.H
71	575.7858	1149.5571	1149.6758	-0.1187	0	48	0.0012	1	K.APVVVPAGVDVK.I
72	585.2672	1168.5198	1168.6564	-0.1366	0	67	1.4e-005	1	K.QVIGQVAADLR.A

Proteins matching the same set of peptides:
RL6_ECOL5 Mass: 18949 Score: 112 Queries matched: 3
50S ribosomal protein L6 - Escherichia coli O6:K15:H31 (strain 536 / UPEC)
RL6_ECOL6 Mass: 18949 Score: 112 Queries matched: 3
50S ribosomal protein L6 - Escherichia coli O6
RL6_ECOLI Mass: 18949 Score: 112 Queries matched: 3
50S ribosomal protein L6 - Escherichia coli (strain K12)
RL6_ECOUT Mass: 18949 Score: 112 Queries matched: 3
50S ribosomal protein L6 - Escherichia coli (strain UTI89 / UPEC)

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