BIOASSAY OF A NOVEL SIDEROPHORE PRODUCED BY *PSEUDOMONAS FRAGI* AND OF AEROBACTIN PRODUCTION BY *ESCHERICHIA COLI*

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ABBREVIATIONS

Abbreviations of units

°C	degrees Celsius
Da	dalton(s)
g	grams(s)
h	hours(s)
kb	kilobase(s)
kDa	kilodalton(s)
L	litres(s)
M	molar
µM	micromolar
mM	millimolar
min	minute(s)
µL	microlitre(s)
mL	millilitre(s)
ng	nanogram(s)
nL	nanolitre(s)
nm	nanometre(s)
rpm	revolutions per minute
sec	second(s)
V	volts

Other abbreviations

<	less than
CAS	chrome azurol S
DHB	2,3-dihydroxybenzoate

EDDA	ethylenediamine-di(*o*-hydroxyphenyl acetic acid)
IROMP	iron repressible outer membrane protein
MBC	minimal bactericidal concentration
MIC	minimal inhibitory concentration
NA	Oxoid Nutrient Agar (code CM0003)
NB	Oxoid Nutrient Broth No. 2 (code CM0067)
p	probability (statistical significance)
RT	room temperature (approximately 22°C-24°C)
UV	ultraviolet

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SUMMARY

Food spoilage results in major economic losses in the domestic and export markets as well as in the home. In addition, the possibility of gastrointestinal concerns arise, if the food contains an extreme number of organisms. Refrigerated, aerobically stored meat is spoiled predominantly by psychrotrophic *Pseudomonas* species. These include *P. fragi*, *P. fluorescens* and *P. putida*. Previous work in this laboratory identified psychrotrophic pseudomonads as the major spoilage organisms of pork, stored aerobically at chill temperatures. Furthermore, siderophores were identified in this study as a major component of the ability of these organisms to grow under conditions of iron-deficiency and partial characterisation of a potentially novel siderophore-mediated iron acquisition system was carried out. This present work provided further insights into the biological nature of this system, through the use of bioassays. Bioassays for detection of the *E. coli* siderophores aerobactin and enterochelin were used as a base on which to develop a new bioasay detection system for this potentially novel pseudomoand siderophore. Responses were investigated to various iron sources as well as supernatants containing siderophore, of varying levels of purification. In order to completely characterise the biological and genetic basis of this system, new mutants were sought by transposon mutagensis which were defective in siderophore biosynthesis. These were to be used for determination of the biosynthetic pathway, characterisation of the genes involved, a new diagnostic bioassay and ultimately, a vaccine. A separate section of work studied a number of hydroxamate producing *E. coli* strains, of diverse sources. These strains were part of a previous investigation in this laboratory. The aerobactin bioassay, refined during the course of this project, was utilised in screening of these strains. Previously, these strains (of diverse origin) were identified as

producers of a hydroxamate of unknown type. All isolates that were hyroxamate positive, were aerobactin positive.

STATEMENT OF AUTHORSHIP

Except where reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis by which I have qualified for or been awarded another degree or diploma.

No other person’s work has been used without due acknowledgment in the main text of the thesis.

This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

CHAPTER 1

INTRODUCTION

1.1 Genus overview : *Pseudomonas*

The genus *Pseudomonas* is a remarkable group of species. Many members of the genus are widely distributed (Palleroni, 1984) and as a genus, *Pseudomonas* spp. inhabit an impressive diversity of ecological niches. At present, 79 species are officially recognised (Euzeby, no date) but, “the taxonomy of pseudomonads remains unclear” (Hayes, 2001). *P. aeruginosa*, a serious human pathogen, is amongst the most widely studied. Very few of the other “more environmental” species have been implicated in human disease but are common as food-spoilage organisms.

1.2 Genus overview : *Escherichia*

For many years, *E. coli* remained the only species in the genus, but now three species are recognised. One, *E. coli*, could be considered the most studied of all prokaryotes. It is commonly encountered as a laboratory organism, as a commensal of humans and other mammals and sometimes, as a serious animal or human pathogen. Five groups of pathogenic *E. coli* are recognised, based on virulence factors. The groups are enteroaggregative *E. coli* (EaggEC), enterohaemorrhagic (EHEC), enteroinvasive (EIEC) enteropathogenic (EPEC) and enterotoxigenic (ETEC). *E. coli* is an important agent of food and water-borne disease. For example, EHEC serotypes such as O157:H7 and O111 types cause the serious diseases haemolytic uraemic syndrome and haemorrhagic colitis. *E. coli* are not considered food spoilage organisms.

1.3 Food spoilage : An introduction

A food product, no longer acceptable for consumption by the consumer, is considered spoiled. Diverse intrinsic and extrinsic factors are responsible for food spoilage and include the type of animal, initial microflora, standard of hygiene and temperature control (Ebune & Prentice, 1995). Most spoilage results from microbial activity, although other factors, such as insects, may be involved (Adams & Moss, 1995). Foods spoiled by microbial activity will normally have "no public health significance" (Christian *et. al.*, 1997) even though they may be classified as spoiled, on the basis of odour and/or visual appearance. The major economic losses associated with food spoilage, might be reduced, if the complex interaction between microbial and biochemical factors in food spoilage was better understood (Huis in't Veld, 1996).

1.4 *Pseudomonas* spp. and pork spoilage

The most common spoilage organisms, across all foods, are *Pseudomonas* spp. (Adams & Moss, 1995). Spoilage of chilled meat is associated with a limited number of genera (Ayers, 1960) with pseudomonads predominating under aerobic storage conditions (Jay & Shelef, 1976). Specific spoilage changes that take place include production of ethyl ester during the early stages of spoilage, followed by formation of dimethyl sulphide, which results in "putrid and sulphury odours" (Borsch *et. al.*, 1996). The pseudomonad species isolated from raw pork include *P. aeruginosa* (Borah *et. al.*, 1992), *P. fluorescens* (Cheng *et. al.*, 1995) and *Pseudomonas* of unknown species (Blickstad and Molin, 1983; Blixt & Borch, 2002; Coates *et. al.*, 1995; Gill & Bryant, 1992). Pig carcass flora varies between sampling location (Miller *et. al.,* 1997) and during processing, *Micrococcus* spp. are the dominant flora, with pseudomonads comprising a smaller, but still substantial proportion (Gill & Bryant, 1992).

Psychrotrophic pseudomonads originate from many sources, and their utilisable substrates are many and varied (Huis in't Veld, 1996). This contributes to their advantage over competitors such as micrococci, lactobacilli and enterobacteria. Overall, *P. fragi* would seem to be the most important pork spoilage species. After chilled aerobic storage, *P. fragi* has the potential to dominate raw pork (comprising up to 93% of all organisms) with other species (including other pseudomonads), only present in low numbers (Blickstad & Molin, 1983).

1.5 Iron is essential for most, but not all, bacteria

For the majority of microbes, a restriction in iron availability results in sub-optimal growth. While the work described in this thesis centred on this concept, an investigation was also undertaken into an organism reported to have no requirement for iron, *Streptococcus pyogenes.*

Virtually all life has been observed to have a dependence on iron. This includes most bacteria. However, it has been discovered that a group of bacteria, mostly lactic acid bacteria, have no requirement for iron. This milestone in understanding of bacterial iron metabolism came when Archibald (1983) confirmed the suspicion (Neilands, 1981) that the nutritional requirements of *Lactobacillus plantarum* do not include iron. For many years prior to this, streptococci and lactic acid bacteria were recognised to require only limited nutrients (Whittenbury, 1978). This followed an investigation by Macleod and Snell (1947) who observed that maximum growth of some members of the genera *Enterococcus, Lactobacillus* and *Leuconostoc* was possible in chemically defined media without addition of iron. In addition, Marcelis *et. al.* (1978) demonstrated no inhibition of growth of *Enterococcus faecalis* under conditions of severe iron restriction (deferrated minimal media containing 15mM EDDA). Since Archibald's discovery, many species have been found to be capable of normal

growth without iron. As a substitute for iron, these organisms utilise cobalt and/or manganese (Clarke *et. al.*, 1997). Selected species from the genera *Borrelia, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Streptococcus* have been found to have no requirement for iron (Bruyneel *et. al.*, 1989; Memon, 1995; Pandey *et. al.*, 1994; Posney & Gherardini, 2000). In particular, Pandey and co-workers demonstrated virtually no accumulation of iron in 23 species of lactic acid bacteria. Strangely, two papers (Bezkorovainy *et. al.*, 1987; Kot *et. al.*, 1995) have reported conflicting results. These researchers observed accumulation/uptake of iron in species of *Bifidobacterium, Streptococcus* and *Lactobacillus*, including *L. plantarum* and have suggested it is surface bound. This is strange for organisms that have been reported in a number of studies to have no requirement for this metal and that therefore would not be expected to have the capability to bind it. It has been suggested that this may act as a detoxification mechanism (R.K.J. Luke, pers. comm.). However, the work of Bezkorovainy *et. al.* (1987) and Kot *et. al.* (1995) may be valid, because no other papers have reported investigations using bifidobacteria, nor *L. acidophilus*. Therefore, these species may be unusual among the lactic acid bacteria, in their ability to bind and maybe even utilise iron.

1.6 Bacterial iron acquisition and host iron sequestration

Iron is probably necessary for all pathogenic bacteria, and it isn't surprising that diverse mechanisms to obtain this precious metal have developed among the many species. Uptake of iron(III) is widespread. Much less common is uptake of iron(II), described for *E. coli* (Hantke, 1987; Kammler *et. al.*, 1993) and *P. aeruginosa* (Xiao & Kisaalita, 1998). The active transport of iron into the cell, against its concentration gradient, requires energy generated by the proton motive force (Letain, 1997). Employment of multiple means of iron acquisition

(such as siderophore-mediated uptake and acquisition directly from haem) is important both *in vivo* in a single host (Takase *et. al.*, 2000a) and when a pathogen's life cycle involves different hosts or a period in the external environment (Weinberg, 1999). Withholding and sequestering of iron by the host can be highly effective. The most common mechanism involves tight binding of iron to host proteins, such as haemoglobin or transferrin. Importance of host iron binding proteins in reducing virulence of a pathogen is exemplified by the work of Deak *et. al.* (1999) who produced transgenic plants expressing ferritin which resulted in increased resistance of the plants to multiple pathogens. The suggestion is that the cells of those plants had an elevated iron sequestering ability, as a result of the presence of ferritin (Deak *et. al.*, 1999) which can store up to 4500 iron atoms per molecule (Jurado, 1997) thereby reducing the amount available to a pathogen. A further mechanism of host defence through limitation of iron availability, has been described by Britigan *et. al.* (2000) - phagocyte removal of iron from siderophores.

The methods by which bacteria acquire iron have been divided into four groups (Weinberg, 1995) : 1) direct acquisition from haem, 2) direct acquisition from host lactoferrin and/or transferrin, 3) siderophore production and 4) acquisition from intracellular iron pools of the host. Lowering the pH can increase the availability of iron as can surface reduction of iron(III) to iron(II) (Neilands, 1992; Neilands, 1993; Neilands, 1995). Vartivarian and Cowart (1999) have described another iron acquisition system, constitutively expressed, based on extracellular reductases. It is independent of siderophore-mediated iron acquisition. This system was found by Vartivarian and Cowart (1999) to be more effective than siderophore-mediated mobilisation of iron from host iron-binding proteins. Many bacteria, including *Gardnerella vaginalis* (Jarosik *et. al.*, 1998)

and *Citrobacter* spp. (Khashe & Janda, 1996), have been found to employ more than one of the mechanisms just described, in order to acquire iron.

While all methods for obtaining iron, as detailed below, are phenotypically distinct, they can be genetically linked. This is the situation with haem utilisation and pyoverdine production in *P. fluorescens* where the absence of HemH affects both iron acquisition systems (Baysse *et. al.*, 2001).

A discussion of some of the methods of iron acquisition mentioned above follows.

1.6.1 Haem-iron acquisition

Acquisition of iron from haem sources is important for bacteria pathogenic for humans and animals. As limited free haem is present in the host, virulence of those bacteria that can utilise haem as a primary/sole iron source, would appear to be enhanced if they are haemolytic. However, haemolysin production is dependent on iron availability (Ulett *et. al.*, 2001). Haem uptake systems generally fall into two broad categories. In one type of system, a specific outer membrane receptor binds the haem-containing molecule (for example, haemoglobin) directly. Haem outer membrane receptor proteins have a very similar primary structure to siderophore receptors, suggesting a common function (Wandersman & Stojilijkovic, 2000). The haem uptake mechanism of *E. coli* O157 strain EDL933 appears to involve the direct binding of haem (Torres & Payne, 1997), as does *Bradyrhizobium japonicum* (Nienaber *et. al.*, 2001) and *Gardnerella vaginalis* (Jarosik & Land, 2001). Another mechanism of haem uptake is through the use of haemophores, which are secreted by the cell and bind haem, before attaching to an outer membrane receptor. Haem is then released from the haemophore and transported into the cell. Species to use this

haem-acquisition mechanism include *Serratia marcescens* (Letoffe *et. al.*, 1994) and *P. aeruginosa* (Letoffe *et. al.*, 1998; Ochsner *et. al.*, 2000).

1.6.2 Lactoferrin and transferrin as iron sources

A limited number of bacteria produce specific protein receptors which bind lactoferrin and/or transferrin directly, thereby enabling iron from that particular host protein to be utilised by the pathogen. Species most recognised for this form of iron acquisition are *Haemophilus influenzae* (Schryvers, 1989; Morton & Williams, 1990; Smoot *et. al.*, 1998) and the pathogenic neisseriae, *N. gonorrhoeae* (McKenna *et. al.*, 1988) and *N. meningitidis* (Archibald & DeVoe, 1979).

1.6.3 Siderophore-mediated iron acquisition

In a human host, invading bacteria may acquire the iron they need from their host, by means of siderophores. These compounds have been defined as "relatively low molecular weight, ferric iron specific chelating agents elaborated by bacteria and fungi growing under low iron stress" (Neilands, 1995). Siderophores, which fall into two main structural groups, catechols and hydroxamates, complex with Fe^{3+} and the complexes are recognised by specific receptors in the outer membrane. Iron is then released from siderophores by enzymes such as esterase (Greenwood and Luke, 1978) and reductases (Fischer *et. al.*, 1990; Vartivarian & Cowart, 1999). Another mechanism for iron release from siderophores has been suggested and demonstrated in *E. coli* and *Aeromonas hydrophila* by Stintzi *et. al.* (2000). This is a process termed ligand exchange and involves the ferric-siderophore complex exchanging its iron molecule with an iron-free siderophore at the cell surface. It has been shown that some aspects of siderophore-mediated iron acquisition can be species specific :

for example siderophores produced by a particular bacterium are able to remove iron only from the transferrin of its usual host (Morton and Williams, 1989). While a siderophore complex with iron(III) is the most common, it has been suggested (Xiao & Kisaalita, 1998) that pyoverdine is able to bind iron(II). Particular species tend to produce particular types of siderophore and this may be used in classification (Fuchs *et. al.*, 1997; Meyer *et. al.*, 1997).

Along with iron concentration, the Fur protein plays a major role in regulation of iron uptake. For example, in *P. aeruginosa* (Hassett *et. al.*, 1996) Fur controls expression of between 24 (Ochsner & Vasil, 1996) and 30 (Vasil & Ochsner, 1999) iron acquisition genes, while in *E. coli*, Fur regulates over 60 iron acquisition genes (Hantke, 2001). A variety of environmental conditions can also influence siderophore production and iron uptake. In *Pseudomonas fluorescens*, metals other than iron (such as cobalt and molybdenum), and compounds such as glycerol and glucose (Duffy and Defago, 1999). Vanadium alters the efficiency of siderophore complex formation by forming complexes with pyochelin and pyoverdine (Baysse *et. al.*, 2000) while in *Bacillus subtilis*, NaCl derepresses expression of genes encoding proteins involved in iron uptake (Hoffmann *et. al.*, 2002). Therefore, iron is not the sole regulator of the expression of siderophore synthesis genes and iron regulation genes, such as *fur*.

1.7 Siderophores of *Pseudomonas* and *Burkholderia* species

Pseudomonas and *Burkholderia* species have been to shown to produce a variety of siderophores. For *Burkholderia* spp., these include cepabactin (Meyer *et. al.*, 1989) and salicylic acid (Sokol *et. al.*, 1992). The siderophores produced by *Pseudomonas* spp. include aerobactin (Buyer *et. al.*, 1991), nocardamine (Meyer & Abdallah, 1980), quinolobactin (Mossialos *et. al.*, 2000), salicylic acid (Visca *et. al.*, 1993), pseudan IX (Royt *et. al.*, 2001), pseudobactin (Yang &

Leong, 1984), pyochelin (Cox *et. al.*, 1981), various structurally different pyoverdines (Ruangviniyachi *et. al.*, 2001; Sultana *et. al.*, 2001) and an unnamed siderophore by a *P. putida* strain (Boopathi and Rao, 1999). While a wide range of siderophores are produced by the Pseudomonaceae, their distribution is not random. As an example, salicylic acid is much more common than any other siderophore, among clinical isolates of *B. cepacia* (Darling *et. al.*, 1998). Although salicylic acid has been described as a siderophore of *Pseudomonas* and *Burkholderia*, its status as a siderophore in general, is questionable, following a clear rejection of the compound as a siderophore by Chipperfield and Ratledge (2000).

1.7.1 Pyochelin

Liu and Shokrani (1978) proposed the name pyochelin when first describing isolation of this siderophore. Pyochelin displays characteristics not completely compatible with the definition of hydroxamates or catecholates (Cox and Graham, 1979) and is regarded as a phenolate (Liu & Shokrani, 1978). It is small (325Da) (Cox *et. al.*, 1981), has a binding coefficient of approximately 10^5 (Cox and Graham. 1979) and its properties as a siderophore have been determined by Cox *et. al.* (1981). The first step of the biosynthetic pathway leading to the formation of pyochelin is similar to that of enterochelin. Isochorismic acid is formed from chorismic acid (Serino *et. al.*, 1995) with salicylic acid being the next compound in the pathway (Ankenbauer & Cox, 1988). One of the most important genes in ferric-pyochelin uptake is *pchR* as it controls synthesis of both pyochelin and FptA, the outer membrane receptor protein for ferric-pyochelin (Heinrichs & Poole, 1996).

1.7.2 Pyoverdine

The siderophore properties of pyoverdine have been demonstrated by Meyer and Abdallah (1978) and by Meyer and Hornsperger (1978). Approximately 50 pyoverdines have been described (Budzikiewicz, 1997). While all pyoverdines are very similar in overall structure, there are minor variations at the amino acid level (reviewed by Hohnadel and Meyer, 1988) which are important enough to determine specificity of that pyoverdine for its particular outer membrane receptor (Atkinson *et. al.*, 1998). Some strains produce receptors for pyoverdine(s) they do not produce, and are thereby able to utilise heterologous pyoverdine(s) (Hohnadel and Meyer, 1988; Ongena *et. al.*, 2002). Pyoverdines bind iron very tightly, with a binding coefficient in the order of 10^{32} (Meyer & Abdallah, 1978) and they can be recycled by the cell (Schalk *et. al.*, 2002). Early suggestions for the pyoverdine biosynthetic pathway included hydroxylation of L-ornithine to form L-N^5-hydroxyornithine, followed by acylation of this resulting compound and synthesis of chromophore (Visca *et. al.*, 1992b). All of the genes required for pyoverdine synthesis are located between 65min and 70min on the chromosome (Ankenbauer *et. al.*, 1986) as is the gene encoding FpvA, the ferric-pyoverdine receptor (Wade, 2001). More recently Meyer (2000) has proposed a hypothetical pyoverdine biosynthetic pathway, detailed as follows. A peptide synthetase is attached to N^5-hydroxyornithine followed by addition of ten amino acids and various steps of cyclisation, racemisation, formylation, sulphonation, desulphonation and dehydrogenation. The gene controlling this process is *pvdF* (McMorran *et. al.*, 2001).

RpoS is a protein widely known for its regulation of stress responses in Gram-negative bacteria. In *P. aeruginosa*, RpoS would appear to be a repressor of pyoverdine synthesis, as mutants deficient in RpoS expression, produce 1.5 fold more pyoverdine (Suh *et. al.*, 1999).

1.8 Siderophores of *Escherichia* species

1.8.1 Aerobactin

Aerobactin, a hydroxamate siderophore, was first isolated by Gibson and Macgrath (1969) from *Klebsiella mobilis* 62-1 (formerly called *Enterobacter aerogenes, Klebsiella pneumoniae* and *Aerobacter aerogenes*). In *E. coli* the aerobactin synthesis genes are commonly found on ColV plasmids, such as pColV-K30 (Carbonetti & Williams, 1984; Valvano *et. al.*, 1986), but they can also be located on the chromosome (Valvano & Crosa, 1988; Vokes *et. al.*, 1999). A total of five genes comprise the aerobactin-mediated iron acquisition system; the synthesis genes *iucA, iucB, iucC, iucD* and the outer membrane receptor gene, *iutA* (Bindereif *et. al.*, 1983). Among some genera of enterobacteria, such as *Shigella*, aerobactin production is almost universal (Reissbrodt & Rabsch, 1988). However, among *E. coli*, aerobactin production is less common and it was not until the early 1980s that a hydroxamate siderophore was reported in *E. coli* (Stuart *et. al.*, 1980), and subsequently identified as aerobactin (Braun, 1981). A detailed chemical characterisation (including structure) of aerobactin is available (Harris *et. al.*, 1979a). The structure of aerobactin is a centrally located citrate molecule joined to a modified lysine on either side via amide bonds. Biosynthesis involves acetylation (first suggested by Ford *et. al.*, 1986) and hydroxylation of the lysine molecules, followed by their attachment to the citrate (Earhart, 1996). Transport of this siderophore-iron complex across the cell membrane is related to transport of ferric-ferrichrome. Both require the inner membrane proteins FhuB, FhuC and FhuD with the outer membrane receptor for aerobactin being IutA (Braun *et. al.*, 1983), first isolated by Grewal *et. al.* (1982). Although ability to produce aerobactin is common among some pathogenic enterobacteria, it has been shown (Harris *et. al.*, 1979a; Harris *et. al.*, 1979b) that aerobactin has a lower affinity for iron than does enterochelin, the siderophore most commonly

produced throughout the family Enterobacteriaceae. Despite this lower affinity, aerobactin is more efficient in iron acquisition than is enterochelin (der Vartanian, 1988) and is recognised as a *E. coli* virulence factor (unlike enterochelin). A possible reason for this is that aerobactin can be reused by the cell (Braun *et. al.*, 1984). Aerobactin production has been demonstrated, or aerobactin genes have been detected, in many virulent strains isolated from humans (Jacobson *et. al.*, 1988) and animals, including ostriches (Knobl *et. al.*, 2001), dogs (Feria *et. al.*, 2001) and calves (Fecteau *et. al.*, 2001). Although its properties as a virulence factor are relatively well established, some studies (Nowrouzian *et. al.*, 2001a; Nowrouzian *et. al.*, 2001b) have shown that a large proportion of human commensal *E. coli* strains produce aerobactin (and fimbriae). These authors concluded that secretion of aerobactin (and presence of fimbriae) assists in colonisation and persistence of commensal strains.

1.8.2 Enterochelin

Commonly produced by a large number of strains proportion of Enterobacteriaceae (Reissbrodt & Rabsch, 1988), production of the catechol siderophore enterochelin, was first demonstrated in *E. coli, K. mobilis* (O' Brien & Gibson, 1970) and *Salmonella enterica* serovar Typhimurium (Pollack & Neilands, 1970). This followed research during the mid to late 1960s, which determined the intermediates in the biosynthetic pathway leading to enterochelin. Seven genes are known to be involved in the entire biosynthetic pathway from chorismic acid to enterochelin; *entA, entB, entC* (Young *et. al.*, 1971), *entD, entE, entF* (Luke & Gibson, 1971) and *entG* (Woodrow *et. al.*, 1975). Starting with chorismic acid (Gibson & Gibson, 1962; Gibson & Gibson, 1964), biosynthesis proceeds via isochorismic acid (Young *et. al.*, 1968), 2,3-dihydro-2,3-didroxybenzoic acid (Young *et. al.*, 1967a) and 2,3-dihydroxbenzoic acid (DHB)

(Young *et. al.*, 1967b) to enterochelin. Expression of genes involved in the formation of DHB are repressed under conditions of iron sufficiency (Young & Gibson, 1969) as is the conversion of DHB to enterochelin (Greenwood and Luke, 1981). Uptake of ferric-enterochelin occurs through FepA in the outer membrane and other Fep proteins (Meyer *et. al.*, 1990) in the inner membrane with dependence on TonB (Larsen *et. al.*, 1997). Although enterochelin has been clearly demonstrated to confer virulence on an otherwise avirulent strain of *E. coli* O111, (Rogers, 1973), unlike aerobactin it is not usually regarded as a full virulence factor in human infections but Furman *et. al.* (1994) found enterochelin to be necessary for *E. coli* virulence in mice. In another, enterochelin deficient mutants grew less in human monocytes (Gorbacheva *et. al.*, 2001). The lack of clear association between enterochelin and virulence may be because enterochelin-mediated iron acquisition is relatively inefficient and requires a high-energy expenditure. The occurrence of enterochelin production among enterobacteria is extensive, being found in environmental (Williams *et. al.*, 1989) and clinical (Podschun *et. al.*, 1993) isolates. Overall, the effect of enterochelin on virulence is not clear (de Lorenzo and Martinez, 1988).

1.9 Siderophore associated membrane proteins

An important step in siderophore-mediated iron acquisition is the binding of ferric-siderophore to receptor molecules on the outer membrane of the cell. This leads directly to entry of the siderophore-iron complex into the cell.

Sokol and Woods (1983) have demonstrated the iron-repressible nature of outer membrane receptor proteins. They found that in low-iron medium, outer membranes bound more than three times (15.6% compared to 4.1%) more pyochelin than occurred in high iron medium. This would suggest that an

elevated rate of synthesis of these proteins occurs under conditions of iron restriction.

Different iron-regulated outer membrane proteins (IROMPs) appear to have slightly different functions. Heinrichs *et. al.* (1991) isolated a 75kDa IROMP from *Pseudomonas aeruginosa* and found that it functioned (as a pyochelin receptor) in periods of severe iron limitation whereas the 14kDa IROMP known at that time, was utilised by the cell when the iron stress was less severe. However, Gensberg *et. al.* (1992) observed mutants deficient in either of these IROMPs that could still transport pyochelin. It would seem, therefore, that both outer membrane receptors can operate independently of the other, and that this may be important in pathogenesis. Some receptor proteins can be quite large, up to 90kDa in the case of *P. putida* WCS358 (Marugg *et. al.*, 1989), and in general the genes for the IROMPs don't appear to be plasmid borne (de Weger *et. al.*, 1986).

The structures of outer membrane receptor proteins with different functions can be very similar. This is true of *E. coli* FepA and FhuA (Clarke *et. al.*, 1997), the receptors for ferric-enterochelin and ferric-ferrichrome, respectively. The basic structure of FhuA is described as a C-terminal β-barrel of 22 antiparallel β-strands and an N-terminal cork domain with four-stranded β-sheets and short α-helices inside the barrel (Ferguson *et. al.* 1998; Ferguson *et. al.* 2000). Differences between the structures of these proteins relate to their surfaces (Clarke *et. al.*, 1997), where interactions occur with the siderophore-iron complexes. The similarities in such structures were evident in the work of Zhou *et. al.* (1995) who observed binding of ferric-enterochelin by both FepA and FecA, but different binding affinities. Moreover, patterns of inhibition by different monoclonal antibodies directed against either FepA of FecA indicate that different regions of the ferric-enterochelin complex are bound by the different receptors.

The various membrane proteins do not always exist independently. Rather, they can be cross-linked, in the manner that TonB is linked with FepA (Skare *et. al.*, 1993).

Of related interest is the evidence for various Ent proteins being located in the cell membrane and the suggestion that they have a role in enterochelin excretion (Greenwood, 1979; Hantash & Earhart, 2000).

A further means of iron transport through the membrane is by utilising ABC transporters. These are widely conserved among all bacteria and even Archaea (Koster, 2001), and are responsible for total passage of the iron-siderophore complex into the cytoplasm.

1.9.1 Fec

Frost and Rosenberg (1973) first described the presence of an iron uptake system utilising citrate. While not always classified as a true siderophore, citrate often serves the same purpose and can satisfy the iron requirements of those cells containing Fec proteins. Uptake of iron(III) citrate requires FecA (Wagegg and Braun, 1981) except when it is present at a concentration sufficient to allow diffusion without the need for FecA (Crosa, 1997). FecA is so widely expressed in coliform bacteria and the genes so extensively conserved, that it has been considered as a target for a potential vaccine (Lin *et. al.*, 1999). The overall topology of FecA is similar to both FepA and FhuA (Ferguson *et. al.*, 2002). In addition to FecA, four other Fec proteins (FecB, FecC, FecD, FecE) are associated with transport (Stadenmaier *et. al.*, 1989). Complex regulatory interactions occur between the Fec transport proteins and other Fec proteins, such as FecI and FecR (Ochs *et. al.*, 1996; Stiefel *et. al.*, 2001), while the role of Fur in iron(III) citrate uptake is in regulation of FecA only (Angerer & Braun, 1998).

1.9.2 Fep

Multiple *fep* genes are required for transport of ferric-enterochelin, and the first of these was characterised by McIntosh and Earhart (1977) who identified the enterochelin outer membrane receptor (FepA). The nature of the protein was unknown at that stage, but they noted a number of outer membrane proteins whose synthesis was derepressed during growth in iron deficient conditions, including an 81kDa protein, now known to be FepA. Discovery of further Fep proteins, including FepB (Pierce *et. al.* 1983) and FepC (Pierce and Earhart 1986), led to a more detailed picture of ferric-enterochelin transport. Pierce and Earhart (1986) describe FepB as having a periplasmic location and FepC probably being present in the cytoplasmic membrane. These authors have suggested that another protein, 29.5kDa in size, may be involved in ferric-enterochelin transport.

The mechanism of action of FepA (and its structure) in iron(III) enterochelin transport has been described by Buchanan *et. al.* (1999) as involving two hatches. The first is formed by the extracellular loop and once the ferric-enterochelin complex is recognised, the receptor closes at the extracellular surface. The N-terminal domain creates the second hatch, and movement of the iron-siderophore complex from FepA, is TonB dependent. It has been found (Sprencel *et. al.*, 2000) that additional Fep proteins other than FepA, such as FepB, do not alter the rate of ferric-enterochelin uptake. This is because all ferric-enterochelin complexes must pass through FepA first, and therefore this protein is the rate-limiting factor in uptake.

In addition to functioning as a ferric-enterochelin receptor, FepA is also the receptor for two colicins. Preliminary research by Guterman and Luria (1969), Guterman (1971a) and Guterman (1971b) suggested a common receptor for colicin B with further work by Hollifield Jr. & Neilands (1978) identifying FepA as

the colicin B receptor. Colicin D also gains entry to the cell through the FepA receptor (Pugsley & Reeves, 1976). There is however, a difference in binding affinity, with FepA having higher affinity for ferric-enterochelin than for colicin B or colicin D (Payne *et. al.*, 1997). Furthermore, different locations on FepA appear to be important for binding of these various compounds (Armstrong *et. al.*, 1990; Armstrong & McIntosh, 1995). In particular, the arginine molecules at positions 286 and 316 have been found to be essential for binding of ferric-enterochelin, colicin B and colicin D (Newton *et. al.*, 1997). It has been noted (Armstrong *et. al.*, 1990; Armstrong and McIntosh 1995) that mutations in various parts of the amino acid sequence can alter binding efficiency dramatically. Futhermore, these studies found that different parts of the sequence were important for binding of the different compounds. FepA appears to recognise and bind only compounds that are structurally related (Thulasiraman *et. al.*, 1998).

1.9.3 Fhu

Fhu proteins are required for transport into the cell, of the fungal siderophore complex, ferric-ferrichrome. Five proteins are involved. The most recently described of these is FhuE, the ferric-coprogen receptor of *E. coli* (Sauer *et. al.*, 1990). FhuA is the 85kDa outer membrane (Braun *et. al.*, 1973; Braun & Wolff, 1973) receptor (Hantke & Braun, 1975). The work of Koster and Braun (1989) has shown that FhuB is located in the cytoplasmic membrane and is 82 181Da in size (Koster & Braun, 1986). FhuC is between 28 433Da (Coulton *et. al.*, 1987) and 29 255Da in size (Burkharat & Braun, 1987) and is located in the inner membrane (Fecker & Braun, 1983) while FhuD has a periplasmic location (Koster & Braun, 1989) with a size of 33 206Da (Burkharat & Braun, 1987). The inner membrane Fhu proteins, such as FhuD, are able to bind hydroxamate

siderophores of different structures, and it has been shown that FhuD actually binds aerobactin more strongly than it does ferrichrome (Koster & Braun, 1990).

1.9.4 Fpt

The receptor for ferric-pyochelin is the 75kDa outer membrane receptor protein, FptA (Heinrichs *et. al.*, 1991) which has an exact size of 75 993Da (Ankenbauer & Quan, 1994). As mentioned previously (Section 1.9), other outer membrane receptor proteins, such as the 14kDa IROMP observed by (Sokol & Woods, 1983) have been suggested to play a role in ferric-pyochelin uptake. However, involvement of the 14kDa IROMP, or any other IROMP other than FptA now appears doubtful. The work of Gensberg *et. al.* (1992) provides evidence for this as they observed induction of synthesis of only Fpt in response to addition of pyochelin to *P. aeruginosa* grown under iron restriction. Ankenbauer (1992) has suggested a function for the 14kDa protein, which may be to facilitate functioning of, or to stabilise FptA. FptA shows homology to a number of outer membrane receptor proteins. Ankenbauer and Quan (1994) describe “significant homology” between FptA and FpvA, PupA, PupB and FhuE and “lower homology” of FptA with FhuA and FoxA. This is unusual, because pyochelin is a phenolate siderophore (Section 1.7.1), whereas the outer membrane proteins with which it shares most homology, for example FpvA and PupA/PupB, are receptors for hydroxamate ferric-siderophore complexes. From the investigations of Ankenbauer *et. al.* (1991) it appears that FptA recognises only a narrow range of pyochelin-like structures. These researchers created pyochelin analogues, with different properties and observed that some had a reduced efficiency of uptake.

1.9.5 Fpv

The pseudomonad outer membrane protein Fpv is the receptor for ferric-pyoverdine. The initial isolation was by Poole *et. al.* (1991) who calculated a molecular weight of 90kDa. However, mature FpvA is now known to have a size of 86 245Da and a high β-sheet content (Poole *et. al.*, 1993). Fpv does not appear to be the only ferripyoverdine receptor, as Gensberg *et. al.* (1992) observed expression of other possible receptor proteins when pyoverdine was added into an iron-deficient culture. Indeed, the utilisation of heterologous pyoverdines (Hohnadel and Meyer, 1988) would appear to confirm this. Interestingly, Fpv is able to bind iron-free pyoverdine, albeit with a 17-fold less affinity (Folschweiller *et. al.*, 2000) but this is not transported into the cell (Schalk *et. al.*, 2001). Like many of the enterobacterial outer membrane receptors, Fpv serves a dual purpose. It is also the receptor for the bacteriocin, pyocin S3 (Baysse *et. al.*, 1999). It has been shown that FpvA has a high degree of sequence homology with PupA and PupB and, based on sequence similarity with other TonB dependent outer membrane receptor proteins (Poole *et. al.*, 1993), it is also probably a TonB dependent receptor,

1.9.6 Iut

The *E. coli* IutA protein functions as the receptor for ferric-aerobactin and has a size of 77 345Da (Krone *et. al.*, 1983). The proteins FhuB, FhuC and FhuD are also required for transport of aerobactin through the cytoplasmic membrane (Braun *et. al.*, 1983). Even though the sequence of IutA is widely conserved among enterobacterial species, there is clear size variation. For example, the IutA proteins of *E. coli* and *K. mobilis* are 74kDa, while that for *Enterobacter cloacae* is 85kDa (Bouchet *et. al.*, 1994). Similarity is demonstrated by immunological cross-reactivity between IutA from *E. coli* and Iut from *E. cloacae*

(Crosa *et. al.*, 1988), *Erwinia carotovora* (Ishimaru & Loper, 1992), *K. mobilis* (Waters & Crosa, 1988) and *Shigella flexneri* (Marolda *et. al.*, 1987). It should also be noted that some species have multiple ferric-aerobactin receptors. Two receptors have been reported in *E. coli*, with sizes of 74kDa (McIntosh and Earhart, 1977) and 76kDa (Griffiths *et. al.*, 1985); in *E. clocae* (85kDa, 86kDa) (Bouchet *et. al.*, 1994); in *Klebsiella pneumoniae* (74kDa, 76kDa) (Williams *et. al.*, 1989) and in *S. flexneri* (74kDa) (Payne *et. al.*, 1983) and 77kDa (Bouchet *et. al.*, 1994).

IutA has a dual function. In addition to being the receptor for ferric-aerobactin, it is the receptor for cloacin DF13 produced by *Enterobacter cloacae* (Bindereif *et. al.*, 1982; van Tiel-Menkveld *et. al.*, 1981; van Tiel-Menkveld *et. al.*, 1982). In *E. coli*, the different receptors (74kDa and 76kDa) afford different levels of DF13 susceptibility, with cells containing the 76kDa receptor having a higher level of resistance to the cloacin (Marolda, 1991).

1.9.7 Pup

The Pup proteins are pseudomonad receptor proteins, required for pseudobactin uptake in *P. putida*. Comparatively little work has been undertaken on these receptors. Initial work on Pup, showed that PupA had a minor role in pseudobactin uptake and suggested the involvement of another Pup protein (Bitter *et. al.*, 1991). This protein was subsequently designated PupB, and shown to have 39.3% homology with PupA (Koster *et. al.*, 1993). PupA is 86 010Da in size (Bitter *et. al.*, 1991) while PupB has a size of 88 369Da (Koster *et. al.*, 1993).

1.9.8 Ton

Another membrane protein, TonB, has a key role in siderophore-mediated iron acquisition systems of many Gram-negative bacteria. It has also been

shown to be necessary for haem iron acquisition by *Rhizobium leguminosarum* (Wexler *et. al.*, 2001). TonB is relatively small, with the *P. putida* TonB having a size of 25 995Da (Poole *et. al.*, 1993) and that of *E. coli* TonB, a size of 26.1kDa - 26.6kDa (Postle & Good, 1983; Mann *et. al.*,1986). Energy is required for TonB-dependent transport following binding of the iron-siderophore complex to the outer membrane receptor (Kadner & McElhaney, 1978). Recognition of this as an energy-dependent process has been noted for ferric-enterochelin uptake (Pugsley & Reeves, 1977) with the energy being provided by the proton motive force (Bradbeer, 1993). This is an energy system not specific to iron-siderophore uptake, or even TonB-dependent transport. For example, Alberts *et. al.* (1994) describes the presence of the proton motive force in mitochondria. Two further proteins, ExbB and ExbD, are necessary for complete functioning of TonB (Braun *et. al.*, 1996; Karlsson *et. al.*, 1993a) for which they act as stabilisers (Karlsson *et. al.*, 1993b). Takase *et. al.* (2000b) constructed *tonB* mutants and observed no iron uptake when the cultures of these organisms were supplied with siderophore. However, when TonB function was restored by means of a plasmid encoding this protein, iron uptake returned to a level essentially the same as that in the wild type. Interestingly, in *P. aeruginosa*, a second TonB protein (designated TonB2) has been identified, but this protein appears to play a less important role in iron acquisition than does TonB1 (Zhao & Poole, 2000). The TonB dependent nature of siderophore-mediated iron acquisition is quite clear. However, in haemophore-mediated iron acquisition, this protein does not appear as important, because at least the first step in transport (haemophore binding to the outer membrane receptor) is TonB-independent (Letoffe *et. al.*, 1999). Theories on the mechanism of functioning of TonB and its location have been discussed at length by Klebba *et. al.* (1993). In summary, these are I) direct interaction in the periplasm between TonB and the outer membrane receptor; II) zones of adhesion

between the inner membrane and outer membrane, through which part of TonB is situated; and III) a mobile messenger to deliver signals, with TonB located in the cytoplasmic membrane.

1.10 *Pseudomonas* utilisation of siderophores can cross domain boundaries

All life can be classified initially on the basis of fundamental cellular properties, into the domains Prokarya and Eukarya. Among pseudomonads, there exists an ability to use a wide range of siderophores. This wide range is not restricted to compounds produced by species of the same genus, but crosses family and even domain boundaries. *Pseudomonas fluorescens* has been shown to possess receptors able to utilise structurally diverse pyoverdines produced by other pseudomonads (Mirleau *et. al.*, 2000). Furthermore, aerobactin and enterochelin can be used by some *Pseudomonas* species (Champomier-Verges *et. al.*, 1996; Loper & Henkels, 1999) as can the eukaryotic siderophores, coprogen (Meyer, 1992) and ferrichrome (Jurkevitch *et. al.*, 1992). In *E. coli*, it is not uncommon for strains to possess outer membrane receptors for siderophores they do not produce (Andrade *et. al.*, 2000), and thereby to be able to utilise a wider variety of siderophores. This increases the chances of survival and, with pathogens, of successful disease progression.

1.11 Siderophores as virulence factors

The role of siderophores in pathogenesis is variable, but overall, they do play a major, sometimes essential, role. Importance is dependent on the specific siderophore. Pyochelin has been shown to increase lethality of virulent *P. aeruginosa* but not to alter the virulence of avirulent bacteria (Cox, 1982). The suggestion was also made in that paper that pyochelin was likely to stimulate

expression of other virulence factors, thereby increasing virulence overall. *In vivo* studies using animals have demonstrated that mutants defective in siderophore production are much less virulent (Lehoux *et. al.*, 2000). In an *in vivo* investigation by Meyer *et. al.* (1996), pyoverdine appeared important in virulence of *P. aeruginosa* as growth of a mutant deficient in pyoverdine synthesis was fully inhibited in mice. It was therefore concluded that pyoverdine was essential for virulence in the murine system. At physiological pH, pyoverdine is unable to remove iron from transferrin or lactoferrin *in vivo* (Abdallah *et. al.*, 1989) but it can do so *in vitro* (Xiao & Kisaalita, 1997). Pyochelin does appear to be the most clinically important siderophore. From a clinical perspective, a correlation is apparent between pathogenicity of pseudomonads and ability to produce siderophore. In a study of uropathogenic *P. aeruginosa*, Visca *et. al.* (1992a) found 98% of strains produced pyochelin while 93% produced pyoverdine. They noted that all strains produced at least one siderophore, indicating the importance of siderophore-mediated iron acquisition in pathogenesis of this species. During an examination of *Burkholderia cepacia*, Sokol (1986) observed that pyochelin-producing clinical isolates were responsible for a severe infection in 86% of patients (61% of whom died) and a mild or moderate infection in 14% of patients. In contrast, pyochelin negative isolates resulted in a mild or moderate infection in 62% of patients. Work by Sokol (1987) has provided further evidence of the importance of siderophore-mediated iron acquisition in virulence by the finding that the ferric-pyochelin receptor was required for *P. aeruginosa* pathogenesis in mice. A number of species produce more than one siderophore. When this occurs, it would appear that one can satisfy the iron needs of the producing organism, and elimination of both is required before virulence is eliminated (Takase *et. al.*, 2000a).

Rogers (1973) examined an isolate of *E. coli* O111 and found that addition of a catechol siderophore enabled the organism to kill all mice within 18h, whereas those without the added siderophore did not exhibit any lethality. The importance of aerobactin as a virulence factor, independent of other virulence factors encoded by pColV-K30, has been demonstrated by Roberts *et. al.* (1989). They cured *E. coli* O78 of pColV-K30, introduced genes for aerobactin synthesis into the cells, and noted a complete restoration of virulence. Pathogenic strains are much more likely to produce aerobactin or contain the *iuc* gene (Prats *et. al.*, 2000). They found 94% of pathogenic *E. coli* O15:H1 produced aerobactin or contained the aerobactin genes (compared with 53% of non-pathogenic serotypes).

An interesting additional mechanism mediated by siderophores is described by Britigan *et. al.* (1992). Unrelated to iron, this paper describes endothelial cell injury brought about by pyocyanin and pyochelin, produced by *P. aeruginosa*.

In general, if a chelator is not available for use by an "iron-requiring" microorganism, then limited iron will be available to it, and virulence should then be decreased. Addition of iron has been shown to stimulate growth *in vivo* (Bullen *et. al.*, 1974) which may lead to increased virulence. This suggests an important role for iron in pathogenesis (Forsberg & Bullen, 1972).

1.12 Commercial applications of siderophores in plant pathology and human medicine

While siderophores are often used for iron acquisition by bacteria during pathogenesis and food spoilage, some soil bacteria can promote plant growth by chelating iron for their own use, and in doing so, making it unavailable for plant pathogens (Crosa, 1989). For example, Scher and Baker (1982) have shown

that the presence of *P. putida* can reduce the incidence of *Fusarium* wilt disease in cucumber from 84% to 40%. In other study, there was limited suppression of disease by a pyoverdine-deficient *P. fluorescens* mutant (Hamdan *et. al.*, 1991). To have an effect in all soil types (including those with a higher level of iron), strains could be genetically modified so that they produce siderophores over a broader range of soil iron levels (Dowling & O'Gara, 1994).

Siderophores have also been used clinically to reduce iron levels in human patients with iron overload. Preliminary work has been carried out on the suitability of enterochelin for this purpose, with some success (Guterman *et. al.*, 1978). Such use is not widespread with desferrioxamine a notable exception (Zanninelli *et. al.*, 1997), in use since the early 1960s (Bannerman *et. al.*, 1962; Smith, 1962).

1.13 Significance of research and aims of the project

Meat is a highly perishable product and provides a nutritious substrate for the growth of bacteria, therefore providing potential for foodborne disease and/or food spoilage under suitable conditions. The focus of the work which preceded that to be described in this thesis, was spoilage of aerobically stored pork, specifically that prepared for export to Singapore. That particular work (Coates, 1994; Coates *et. al.*, 1995) partly investigated pseudomonad attachment to meat but also discovered a potentially novel siderophore. Coates (1994) matched the properties of the (siderophore-like) compound of interest with key requirements for a siderophore and found little doubt that this compound was in fact a siderophore. Furthermore, the putative novel siderophore was found to be of key importance for growth of the organisms on pork, because mutants unable to utilise the siderophore had a generation time considerably longer than the wild type.

Singapore is a major importer of Australian pork and therefore pork spoilage is important in economic terms. Conditions encountered prior to arrival in the marketplace can be sub-optimal, leading to spoilage of the product and a shortening of the shelf life. Shelf life is more crucial for an exported meat product than it is for the domestic market because of the increased transit time between processing and retail display.

Prior to and concurrent with the work described in this thesis, colleagues in the laboratory had achieved partial chemical characterisation of this siderophore iron acquisition system and the main objective of this project was to achieve further biological and genetic characterisation. An important part of the prior chemical characterisation was the observation that the putative siderophore was negative when tested in the modified Csaky assay (Gillam *et. al.*, 1981) (for hydroxamate compounds) and the Arnow (1937) and Rioux (1983) assays (for catechol compounds). Most siderophores are either of a catechol or hydroxamate structure, and the result that the putative siderophore was neither, enhanced the possibility of it being novel. Siderophores have been identified which appear not to have a classic catechol or hydroxamate structure, as determined by a positive result in the above-mentioned chemical assays. These include legiobactin (from *Legionella pneumophila*) (Liles *et. al.*, 2000) and salicylic acid (from *B. cepacia*) (Sokol *et. al.*, 1992).

In part as a separate exercise, and in part as a model for the pseudomonad system, a collection of *E. coli* identified previously as hydroxamate producers, were to be tested for aerobactin production. Procedures developed and refined for the bioassay of aerobactin, were to be used in the development of a bioassay for the (putative) siderophore produced by the spoilage pseudomonad.

The aims of the two central parts of the project were :

1) to use an established bioassay method (for aerobactin), to screen a collection of hydroxamate-producing *Escherichia coli*, for ability to produce aerobactin.

2) to develop a bioassay for the (putative) siderophore produced by a *P. fragi* isolate implicated in the spoilage of pork.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Chemicals and their sources are listed in Table 2.1.

Table 2.1 : Chemicals

CHEMICAL, AND GRADE IF APPLICABLE	SUPPLIER
Acetic acid (analytical reagent)	Ajax
4-Aminobenzenesulphonamide	Ajax
Ammonium chloride	Ajax
Ampicillin sodium salt	Sigma
Boric acid	BDH
Bromothymol blue	BDH
Calcium chloride, anhydrous (analytical reagent)	Sigma
Chloramphenicol	Sigma
Chlorotetracycline hydrochloride	Sigma
Chrome azurol S	Sigma
Copper(II) sulphate, pentahydrate (analytical reagent)	BDH
2,2'-dipyridyl	Sigma
Ethylenediamine-di(o-hydroxyphenyl acetic acid)	Sigma
D-glucose, anhydrous (analytical reagent)	BDH
Glycerol, anhydrous (analytical reagent)	Ajax
Haemoglobin, bovine	Sigma
Haemin	Sigma
Hexadecyltrimethylammoniumbromide	Sigma
L-histidine	Merck
Hydrochloric acid (analytical reagent)	BDH
Iodine	May & Baker
Iron(III) chloride	Merck
Kanamycin sulphate	Sigma
L-leucine	Sigma
Magnesium chloride, hexahydrate (analytical reagent)	BDH
Magnesium sulphate, heptahydrate (analytical reagent)	BDH
Manganese sulphate, monohydrate (analytical reagent)	BDH
D-mannitol (general purpose reagent)	BDH
Methanol (HiPervSol for HPLC)	BDH
Methyl cyanide (general purpose reagent)	BDH
3-Morpholinopropanesulphonic acid	Sigma
Nalidixic acid	Sigma

N-(1-Naphthyl)ethylenediamine monohydrochloride	Sigma
Nitric acid (analytical reagent)	BDH
L-phenylalanine	Sigma
Piperazine-1,4-bis(2-ethanesulphonic acid)	Sigma
Polymyxin B sulphate	Sigma
Potassium phosphate monobasic (analytical reagent)	BDH
Potassium hydroxide	Sigma
Potassium iodide (analytical reagent)	Ajax
L-proline	Sigma
Sodium acetate, anhydrous (analytical reagent)	BDH
Sodium chloride (analytical reagent)	BDH
Sodium citrate tribasic, dihydrate (analytical reagent)	Ajax
Sodium dioxoarsenate (general purpose reagent)	Sigma
Sodium hydroxide (analytical reagent)	BDH
Sodium molybdate, dihydrate	Ajax
Sodium phosphate monobasic, anhydrous (analytical reagent)	BDH
Sodium succinate dibasic, hexahydrate	Sigma
Streptomycin sulphate	Sigma
D-sucrose (analytical reagent)	BDH
Sulphuric acid (technical grade)	Ajax
Thiamine hydrochloride	Sigma
L-tryptophan	Sigma
L-tyrosine	Sigma
Vancomycin hydrochloride	Sigma
Zinc sulphate, heptahydrate (analytical reagent)	BDH

2.1.2 Water

Throughout this project, water used was prepared by reverse osmosis and deionisation. Throughout this thesis, all such water will be referred to as "distilled water".

2.1.3 Cultures

Most cultures used were obtained from the culture collection of the Enteric Pathogenicity Laboratory in the Department of Agricultural Sciences, La Trobe University. Details of strains regularly used throughout this project appear in Table 2.2. Coates (1994), who originally described the *Pseudomonas* strain VIAS1, offered two suggested identifications. One (*P. putida*) was that provided

by the Microbiological Diagnostic Unit at The University of Melbourne; the other (*P. fragi*) was that based on results obtained with a Biolog kit. With the assistance of Oxoid Australia, an updated identification was carried out in 2001 by means of a Biolog GN test system (Oxoid; Melbourne, VIC). The use of this test essentially confirmed the identity as *P. fragi* (98% probability).

Table 2.2 : Strains of organisms and their known characteristics

STRAIN	CHARACTERISTICS	SOURCE OR REFERENCE
Pseudomonas fragi		
VIAS1	Pork spoilage isolate	Coates (1994)
mutant B	37kDa outer membrane receptor mutant of VIAS 1	Coates (1994)
mutant F	37kDa outer membrane receptor mutant of VIAS 1	Coates (1994)
mutant G	37kDa outer membrane receptor mutant of VIAS 1	Coates (1994)
Escherichia coli		
AN53	*entA fhuA thi*	Stuart *et. al.* (1980)
AN53N	*nal*^{R} derivative of AN53	EPL collection
AN90N	*entD fhuA leu proC thi trp nal*^{R} derived from AN90	EPL collection Cox *et. al.* (1970)
AN102	*fepC fhuA leu proC thi trp*	Cox *et. al.* (1970)
AN193	*entA fhuA leu proC thi trp*	I.G. Young
AN194	*fhuA leu proC thi trp*	Langman *et. al.* (1972)
AN194N	*nal*^{R} derivative of AN194	EPL collection
AN273	*fes fhuA proC leu thi trp*	Langman *et. al.* (1972)
DH1 (ATCC 33849)	*endA hsdR supE thi recA gyrA relA*	Hanahan (1983)
K-12 711(pColV)	*str*^{R} *lac pro his phe trp* and carries pColV	Smith (1974)
LG1522	*ara fepA lac leu mtl proC rpsL supE thi fhuA trpE xyl* carries pColV-K30iuc	Carbonetti and Williams (1984)
MW (serotype O18:H7)	Prototrophic meningitis isolate carrying pColV-K30	Smith and Huggins (1980)
RJ79	*fhuA leu proC thi trp* carries pRJ1000	Stuart *et. al.* (1980)
RJ80	*entA fhuA leu proC thi trp* carries pRJ1000	Stuart *et. al.* (1980)
SM10λ*pir*(pRT733)	*thi thr leu fhuA lacY supE recA*::RP4-2Tc::Mu:: carries pRT733 & λ transducing phage with *pir*	Taylor *et. al.* (1989)
Klebsiella mobilis		
62-1 (ATCC 25304)	*pheA trpC tyrA*	Gibson and Gibson (1962)

EPL : Enteric Pathogenicity Laboratory, Department of Agricultural Sciences, La Trobe University

Table 2.3 : *P. fragi* VIAS1 transconjugant strains isolated during this project

STRAIN DESIGNATION	KNOWN PHENOTYPE	METHOD OF ISOLATION
BMM5	Auxotrophic, small orange halo on CAS agar	Broth mating
BMM6	Auxotrophic, small orange halo on CAS agar	Broth mating
NM1	Prototrophic	Agar surface mating
NM2	Prototrophic	Agar surface mating
NM3	Prototrophic	Agar surface mating
NM4	Prototrophic	Agar surface mating
NM5	Prototrophic	Agar surface mating
NM6	Prototrophic	Agar surface mating

2.1.4 Media

Commercial media/supplements used, and prepared according to the manufacturer's instructions, are listed in Table in 2.4.

Table 2.4 : Commercial media/supplements

MEDIUM	MANUFACTURER	CODE
Bacteriological Peptone	Oxoid	L37
Bacto-Agar	Difco	0140-01
Casamino acids	Difco	0230-17
MacConkey Agar	Oxoid	CM0007
Nutrient Agar	Oxoid	CM0003
Nutrient Broth No. 2	Oxoid	CM0067
Pseudomonas Agar Base	Oxoid	CM0559
Purified Agar	Oxoid	L28
Simmon's Citrate	Oxoid	CM0155
Urea Agar Base	Oxoid	CM0053
XLD Medium	Oxoid	CM0469

CAS agar - Schywn and Neilands (1987)

CAS agar (1L) was prepared as follows :

Solution A 50mL of 2mM chrome azurol S

10mL of 1mM $FeCl_3$ in 10mM HCl

40mL of 5mM HDTMA

Solution B1 30.24g PIPES or 20.93g MOPS in 750mL H_2O

15g Purified Agar (Oxoid L28)

NaOH to adjust pH to either 6.8 (if using PIPES) or 7.2 (if using MOPS)

Solution B2 100mL of 10X deferrated MM9 (refer below for composition)

Supplements to Solution B (added after autoclaving)

$MgCl_2$ - 2mL

$CaCl_2$ - 100mL

Carbon source - 10mL

Solution C Appropriate growth factors depending on strain

MM9 stock solution per litre (10X concentration) - Schywn and Neilands (1987)

60g Na_2HPO_4

3g KH_2PO_4

10g NH_4Cl

5g NaCl

CAS agar - Alexander and Zuberer (1991)

CAS agar (1L) was prepared as follows :

Solution A 50mL of 2mM chrome azurol S

10mL of 1mM $FeCl_3$ in 10mM HCl

40mL of 5mM HDTMA

Solution B1 600mL H_2O

15g Purified Agar (Oxoid L28)

Solution B2 200mL H_2O

75mL deferrated MM9

20.93g MOPS

Solution C 4g glucose

4g mannitol

1.4mL of solution D

H_2O to a final volume of 70mL

Solution D prepared as a 50X stock solution

100mL H_2O

493g $MgSO_4$

11mg $CaCl_2$

1.17mg $MnSO_4$

1.4mg H_3BO_3

40µg $CuSO_4$

1.2mg $ZnSO_4$

1mg Na_2MoO_4

The four solutions were autoclaved separately and then growth factors or casamino acids added if required. The order of addition of the solutions was B2 to B1 first. Solution C was added next, followed by casamino acids or other growth factors if required. Finally, the dye mixture (solution A) was slowly added and thorough, but gentle, mixing effected.

Minimal media

For all minimal media, the base medium composition is provided. To this, supplements were added as appropriate for the strain(s) under investigation, mostly as sterile solutions after autoclaving. These supplements were a carbon source, magnesium sulphate, calcium chloride and if required, amino acids. When iron-deficient conditions were required, 2,2'-dipyridyl or EDDA were also included. When an iron-sufficient medium was required, iron(III) chloride was added as the source of iron. When necessary, M9 agar plates containing 2,2'-dipyridyl were used soon after preparation. However, plates, which contained

EDDA as iron chelator, were stored at least overnight at 4ºC before use. This was to allow time for slow binding of Fe to the EDDA (Miles and Khimji, 1975).

M9 agar - Anderson (1946) as modified by Miller (1972)

M9 agar (1L) was prepared as follows :

Solution A 800mL H_2O

15g purified agar (Oxoid L28)

Solution B 100mL 10X M9 stock solution (refer below for composition)

H_2O - volume dependent on volume of added supplements

Addition of supplements is detailed below.

M9 liquid medium - Anderson (1946) with modification by Miller (1972)

M9 liquid medium (100mL) was prepared as follows :

10mL 10X M9 stock solution (refer below for composition)

H_2O - volume dependent on volume of supplements

Addition of supplements as detailed in 2.1.3.4.

M9 stock solution composition per litre (10X concentration)

60g Na_2HPO_4

30g KH_2PO_4

10g NH_4Cl

5g NaCl

Minimal media supplements

After autoclaving, the basal M9 was cooled to approximately 55°C and sterile supplements added as required and as follows : 2mL of 1M $MgSO_4.7H_2O$ (Anderson, 1946) and 100µL of 500mM $CaCl_2$ (Miller, 1972) per litre. For iron-

sufficient conditions, iron(III) chloride was added to a final concentration of 200μM (Young & Gibson, 1979). Details of the concentrations of supplements used is shown in Table 2.5.

Table 2.5 : Concentrations of chemicals

CHEMICAL	STOCK SOLUTION CONCENTRATION	FINAL CONCENTRATION
Calcium chloride	500mM	50μM
2,2'-dipyridyl	10mM	100μM - 650μM
EDDA	139mM	9μM – 69μM
Iron(III) chloride	1mM	200μM
Glucose	1M	10mM
Haemin	10mM	10mM
Haemoglobin	0.1%	0.1%
Histidine	10mM	100μM
Leucine	3mM	30μM
Magnesium sulphate	1M	2mM
Phenylalanine	10mM	100μM
Proline	100mM	1mM
Sodium citrate	100mM	10mM
Sodium succinate	1M	10mM
Tryptophan	2mM	20μM
Tyrosine	100mM	100μM

Other media

Oxidation-Fermentation medium - Hugh and Leifson (1953)

O-F medium (1L) was prepared as follows :

Bacteriological Peptone (Oxoid L37)	2g
NaCl	5g
K_2HPO_4	0.3g
Agar	3g
Glucose	10g
Bromothymol blue	3mL
H_2O	to a final volume of 1L

Antibiotics

Solutions of antibiotics were prepared and used as indicated in Table 2.6.

Table 2.6 : Antibiotics

ANTIBIOTIC	STOCK SOLUTION CONCENTRATION	FINAL CONCENTRATION
Ampicillin	1mM, 27mM	10µM – 2.3mM
Chloramphenicol	1mM	10µM – 100µM
Kanamycin	1mM, 17mM	8µM – 343µM
Nalidixic acid	1mM, 43mM	10µM – 1.1mM
Polymyxin	1mM	10µM – 100µM
Streptomycin	1mM	10µM – 100µM
Tetracycline	1mM	10µM – 100µM
Vancomycin	1mM	10µM – 100µM

2.1.5 Buffers

The composition of the phosphate buffer was as given below.

200mM phosphate buffer - 2X stock solution (Gomori, 1955)

27.8g NaH_2PO_4

53.7g Na_2HPO_4

H_2O to 1L

For preparation of 200mL of 100mM phosphate buffer at pH 8.0;

5.3mL of 200mM NaH_2PO_4

94.7mL of 200mM Na_2HPO_4

H_2O to 200mL

The restriction endonuclease buffers (Promega; Madison, WI, United States) were colour-coded to match the appropriate restriction endonuclease. Details are shown in Table 2.7.

Table 2.7 : Details of restriction endonuclease buffers

RESTRICTION ENDONUCLEASE	BUFFER	COLOUR-CODE OF ENZYME AND BUFFER
*Bam*HI	E	Red
*Eco*RI	H	Pink
*Hin*dIII	E	Red
*Sac*I	J	Green
*Sal*I	D	Yellow
*Xho*I	D	Yellow

All other buffers were commercially available from QIAGEN (Melbourne, VIC). The QIAGEN codes for these buffers and names are listed below :

EB	Elution buffer	P1	Resuspension buffer
QBT	Equilibration buffer	P2	Lysis buffer
QC	Wash buffer	P3	Neutralisation buffer
QF	Elution buffer	PE	Wash buffer
QG	Solubilisation and binding buffer		

2.1.6 Solutions for preparation of competent cells

Competent Cell Solution 1 (Liu *et. al.*, 1996) (100mL) was prepared as follows :

10mL of 500mM $MgCl_2$

50mL of 20mM PIPES

40mL of H_2O

Competent Cell Solution 2 (Liu *et. al.*, 1996) (10mL) was prepared as follows :

1mL of 500mM $CaCl_2$

5mL of 20mM PIPES

1.5mL of glycerol

2.5mL of H_2O

2.2 Methods

2.2.1 Antibiotic preparation

Most antibiotics were dissolved in distilled water. Nalidixic acid was dissolved in NaOH then distilled water added to the desired volume (Gavan *et. al.*, 1971). Chloramphenicol was dissolved in methanol (one volume) and then distilled water (three volumes) according to Anhalt & Washington (1991). A buffer was used, initially for preparing the stock solutions of ampicillin. However, most

stock solutions of ampicillin were prepared in H_2O (Washington University GSC, no date). Alternatively, ampicillin was dissolved in a 100mM phosphate buffer, prepared as described by Gomori (1955). The two stock solutions of this buffer consisted of the following per litre :

27.80g (200mM) of NaH_2PO_4

53.65g (200mM) of $Na_2HPO_4.7H_2O$

2.2.2 Deferration

Minimal media (M9 and MM9), casamino acids and Nutrient Broth No. 2 were deferrated by treament with Chelex 100 (Bio-Rad; Hercules, CA, United States). The Chelex was added at the rate of 25% (w/v) according to the manufacturer's instructions. Deferration continued overnight (for a minimum of 18h) at 4ºC (Mickelsen & Sparling, 1981).

2.2.3 Ethylenediamine-di(*o*-hydroxyphenyl acetic acid) preparation

Preparation of (EDDA) was based on the method of Manninen & Mattila-Sandholm (1994). With constant stirring, the EDDA was dissolved in 5M KOH. Distilled H_2O was added next, to almost the final volume, and with continued stirring. Finally, pH was adjusted to pH 7.8 with 1M HCl. The resulting solution was passed through Whatman 4 filter paper (Whatman; Maidstone, Kent, England) into the final storage vessel.

2.2.4 Csaky assay for detection of hydroxamate compounds

The Csaky assay was carried out as described by Gillam *et. al.* (1981). The culture to be tested (1mL volume), was centrifuged in a microcentrifuge for 60sec at 13 000rpm to clear the cells from the supernatant liquid. The supernatant liquid was transferred to a new (previously unused) McCartney bottle

and 1mL 3M H_2SO_4 added. The sample was then autoclaved for 4h at 120°C and cooled, prior to addition of 3.5mL 2M sodium acetate. To this mixture, 1mL of sulphanilamide solution and 1mL of iodine solution were added before swirling and 5min incubation at RT. Next 1mL sodium arsenite solution was added, and finally 1mL *N*-(1-naphthyl)ethylenediamine. Thirty minutes was allowed for complete colour development after which time the sample was diluted to a total volume of 25mL. The absorbance of the resulting sample was then read in a Bio-rad Benchmark microplate reader (Bio-rad; Hercules, CA, United States) at 550nm.

2.2.5 Identification techniques

For the rapid presumptive identification of organisms as either *E. coli* or *P.fragi*, Oxidase Identification Sticks (code BR64A) (Oxoid; Melbourne, VIC) were most often used. Sometimes as an alternative, the two-tube oxidation/fermentation test (Hugh and Leifson, 1953), the test for the presence of urease or utilisation of citrate as a sole carbon source was carried out. The medium for the urease test was Urea Agar Base (code CM0053) (Oxoid; Melbourne, VIC) plus urea while Simmons Citrate Agar (code CM0155) (Oxoid; Melbourne, VIC) was used for the citrate test.

2.2.6 Bioassay for siderophores and iron sources

Different styles of bioassay were used, depending on the strain and sample involved. The indicator organisms were either poured into or spread on the surface of agar medium. Test strains or liquid test samples were applied in different ways, according to the type of strain or sample. Pour-plate bioassays were prepared as described by Miles and Khimji (1975).

For pour-plate bioassays, the M9 medium was prepared as described in Section 2.1.4, with the appropriate supplements. It was cooled to approximately 50°C in a waterbath and indicator organism added (1 mL per plate). The indicator strain was prepared in a similar manner, irrespective of the strain or siderophore under investigation. A NB culture was grown to log or early stationary-phase and centrifuged. The supernatant liquid was discarded and the pellet resuspended in 15mM NaCl. Slight variations were needed on occasions, in order to reduce cell density. This was achieved by increasing/decreasing the initial volume centrifuged or the volume of 15mM NaCl added. M9 bioassay plates (section 2.1.4) to be inoculated by the spread-plate method, were prepared in advance. The volume of resuspended cells spread on each plate was 100μL.

As appropriate, liquid samples were tested by application to sterile blank antimicrobial susceptibility disks (Oxoid; Melbourne, VIC: catalogue number HPO63A), added to wells (6mm in diameter) cut in the agar or spotted as drops, directly onto the surface. Cultures were tested by stabbing from an agar culture, into the agar of the bioassay plate (up to three times) with a straight wire. In most instances, four strains or samples were tested per plate. This number varied on rare occasions between two and six.

2.2.7 General inoculum preparation for use in iron-deficient media

Inocula were prepared in a standard way when liquid media or pour plates were to be inoculated, or when organisms were to be streaked onto iron-deficient agar, such as when tests were to be conducted for iron-related cross-feeding. An overnight NB culture (1mL) was centrifuged in a microcentrifuge tube at 13 000 rpm for 60sec. The supernatant liquid was decanted and the pellet vortexed and resuspended in 1mL 15mM NaCl. When *E. coli* LG1522 was to be used as the

indicator organism the pellet from 250μL of culture was resuspended in 1mL 15mM NaCl.

2.2.8 Antibiotic sensitivity testing

Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were determined by the broth dilution method, performed in a 24-well tissue culture tray (Costar; Cambridge, MA, United States). Five concentrations of each antibiotic were tested in NB (together with a control containing no antibiotic). Each well was prepared in the following manner. First, a volume of NB was added to each well, with allowance for the volume of antibiotic solution to be added subsequently. The final addition was 1mL log-phase NB culture of the test strain, resulting in a 2mL total volume. An enumeration was carried out to determine the initial cell density. MIC results were noted after 24h, at which time those wells without obvious growth were sampled for determination of the MBC. This was carried out by spreading 100μL onto NA. The lowest concentration of antibiotic, found not to contain viable and culturable cells, was recorded as the MBC.

2.2.9 Inducible acid tolerance

The procedure followed for inducible acid tolerance was that used previously (Button, 1999). Both adaptation and challenge were carried out in NB No. 2. From an overnight culture, a 10^{-1} dilution was made by the transfer of 500μL of culture into 4.5mL adaptation medium, pH 5.0 (acidified with 1M HCl). Adaptation was for 2h at 25°C. NB No. 2 without added HCl served as the control. Following the adaptation period, sub-lethal pH challenge was conducted in NB No. 2, acidified to pH 4.0 with 1M HCl. The length of challenge was 4h.

For enumeration purposes, 10^{-4} and 10^{-6} dilutions of samples taken before adaptation, after adaptation/before challenge and after challenge, were spread onto NA. Assessment of the affect on iron acquisition ability was on four different iron-deficient M9 and MM9 agars, by streak plating. The four media were : I) deferrated MM9 with glucose as carbon source; II) non-deferrated MM9 with glucose as carbon source; III) non-deferrated M9 with sodium succinate as carbon source and IV) non-deferrated MM9 with sodium succinate as carbon source.

2.2.10 Filter matings

Filter matings were performed as described by Coates (1994). Donor and recipient cells were mixed in a 1:10 ratio, and filtered through a 450nM nitrocellulose filter which was placed on an NA plate. This was incubated for 2h at 25°C. Following incubation, the filter was transferred to 10mL NB and vortexed to remove cells from the filter. This mating mixture was spread onto selective NA. This selective medium contained 1.075mM nalidixic acid (to select against the donor) and 77μM kanamycin (to select for transposon-carrying transconjugants), as used by Coates (1994).

2.2.11 Agar surface matings

Matings were based on the mating protocol of Zhao *et. al.* (1998), and this was used routinely to produce transconjugants of *P. fragi* VIAS1. The donor [*E. coli* SM10λ*pir*(pRT733)] and recipient (*P. fragi* VIAS1) strains were grown separately to approximate mid-log phase in NB at 25°C for the recipient and 37°C for the donor. At this point, 750μL aliquots of each culture were centrifuged together in a microcentrifuge. The supernatant liquid was decanted, and the

pellet resuspended in NB (approximately 15-20μL volume). This entire volume was transferred with a pipette to an NA plate (spotted onto the surface) and incubated for 18-24h at 25°C. By means of a sterile wire loop, the growth was removed from the NA plate and suspended in 1mL NB. Samples of this suspension (200μL) were then spread on NA containing nalidixic acid (43μM) and kanamycin (17μM) for selection of transconjugants.

2.2.12 Production of crude siderophore preparations

Different methods were used, depending on the genotype of the strain. For strains defective in siderophore uptake but still able to produce siderophore, such as *E. coli* AN102, preparations of cell-free culture supernatant containing siderophore was based on the method of Young (1976). The culture was grown in M9 liquid medium (Section 2.1.4) containing added iron (200μM). Conversely, for strains with functional siderophore receptors, deferrated M9 containing 2,2'-dipyridyl or EDDA as iron chelator was used. After the culture had reached stationary phase, it was centrifuged at 13 000 rpm in a microcentrifuge for 60sec and the supernatant liquid passed through a 220nM Millex syringe driven filter unit (Millipore; Bedford, MA, United States) into an acid-washed, sterile, glass Bijou bottle or into a sterile microcentrifuge tube.

2.2.13 Extraction of chromosomal DNA

Chromosomal DNA of *P. fragi* VIAS1 was extracted by means of a QIAGEN 500/G Genomic tip (QIAGEN; Melbourne, VIC). The tip was equilibrated with 10mL of QIAGEN Equilibration Buffer. Approximately 15mL of an overnight NB culture was vortexed and applied to the tip. Once the flow had stopped, the tip was washed twice with 15mL of QIAGEN Wash Buffer. Elution

was then carried out with QIAGEN Elution Buffer (15mL), prewarmed to 50°C. Precipitation of the DNA was by addition of 10.5mL of isopropanol, at RT. Centrifugation was selected as the method of recovery. This was performed for 10min at 4410 rpm, and at 4°C. The supernatant liquid was removed and the pellet washed in 4mL cold (4°C) 70% ethanol. The washed pellet was removed and added to a microcentrifuge tube containing 1.5mL cold (4°C) 70% ethanol and the contents of the tube centrifuged in a microcentrifuge at 13 000rpm for 10min. The ethanol was then removed and the pellet dried in air for approximately 10min before being resuspended in 500μL buffer EB (10mM Tris.Cl, pH 8.5). After the contents had redissolved, the tube was placed for approximately 45min in a waterbath set at 55°C. The redissolved DNA was checked for purity by determining the ratio of absorbance at 260nm to that at 280nm

2.2.14 Restriction enzyme digestion

Six restriction endonucleases were selected for digestion of the extracted chromosomal DNA. These were *Bam*HI, *Eco*RI, *Hin*dIII, *Sac*I, *Sal*I and *Xho*I. The digestion mixture initially contained 36μL of H_2O, 8μL of the DNA sample, 5μL of 10X buffer (see table 2.7 for details of the restriction endonuclease buffers) and 1μL of the enzyme. For subsequent restriction enzyme digests, the composition of the mixture was modified as necessary in line with results obtained. Digestion then proceeded for 19-21h at 37°C.

2.2.15 Extraction and purification of phagemid DNA

A QIAGEN Plasmid Mini Kit (QIAGEN; Melbourne, VIC) was used for extraction and purification of phagemid DNA. The phagemid vector used was

pBluescript (Short *et. al.*, 1988). From an overnight LB broth culture of *E. coli* containing pBluescript (cultured by M. Emmerling), 3mL was centrifuged for 10min at 4400 rpm. The pellet was resuspended in 300μL of buffer P1. Following addition of 300μL of buffer P2, the suspension was mixed gently and incubated for 5min at RT. To this, 300μL of chilled buffer P3 was added and gently mixed, prior to 5min incubation on ice. The sample was then centrifuged at 13 000 rpm in a microcentrifuge, for 10min. Prompt removal of the supernatant liquid followed and this was applied to a QIAGEN-tip 20, which had previously been equilibrated with 1mL of buffer QBT. Four washes followed (1mL buffer QC per wash). Elution took place with 800μL of buffer QF. Isopropanol (560μL volume at RT) was added to precipitate the phagemid DNA. The mixture was centrifuged for 30min at 10 000 rpm in a microcentrifuge, resuspended in 1mL 70% ethanol then re-centrifuged for 30min at 10 000 rpm. The ethanol supernatant was then removed and the pellet air dried and resuspended in buffer EB.

2.2.16 DNA cloning (gel extraction and ligation)

Digested chromosomal DNA, between 1.5kb and 6.0kb in size, was cut from an agarose gel with a razor blade, under UV light. Extraction of the DNA from the gel was by means of a QIAGEN QIAquick Gel Extraction Kit (QIAGEN; Melbourne, VIC). The gel piece was dissolved in buffer QG by incubation at 50°C coupled with regular vortexing. Once dissolved, one gel volume of isopropanol was added, and the sample mixed. The sample was next transferred to the QIAquick column and washed with 750μL of buffer PE. Remaining buffer PE was removed by centrifugation of the sample for 60 sec at 13 000 rpm. Elution was

with 50μL of buffer EB, carefully added to the centre of the QIAquick column membrane and then centrifuged at 13 000 rpm for 60sec.

An overnight ligation was used to ligate DNA fragments extracted from the agarose gel, into the pBluescript. Prior to this, the insert and the vector were precipitated overnight at -20°C in a total volume of 139.2μL. This comprised 29μL of insert/vector mixture, 5.8μL of 10M ammonium acetate and 104.4μL of 70% ethanol. Following this, the ligation was performed overnight at 14°C. The volume of the ligation mixture was 132.2μL: 30μL vector/insert mixture; 13.2μL 10X buffer; 200nL ligase; 88.8μL H_2O.

2.2.17 Agarose gel electrophoresis of DNA

Results of restriction enzyme digestions were visualised following horizontal agarose gel electrophoresis. When the agarose (0.4% in TAE buffer) gel had solidified, running buffer and ethidium bromide were added. TAE buffer, running buffer, sample buffer and the ethidium bromide solution were used as prepared by M. Emmerling. Sample buffer was added to each sample, prior to loading into the wells. This was at the rate of either 5% or 10%. Molecular weight markers were used to estimate fragment sizes and an undigested sample of DNA run to enable comparison of digested and undigested DNA. Running time was approximately 100min at 80V. The gel was then observed under UV light in a Gel Doc 2000 transilluminator (Bio-rad; Hercules, CA, United States). If the bands were faint, further ethidium bromide staining was carried out (30min), followed by two 30min washes in distilled water.

2.2.18 Preparation of competent *P. fragi* cells

Induction of competency was based on the chemical method of Liu *et. al.* (1996). From a 16h NB culture, a 1mL aliquot was transferred into 50mL of NB in a side-arm flask. This was incubated until an absorbance at 660nm of 0.6 was reached. Centrifugation (13 000 rpm) of the entire volume at 4°C for 10min followed. The supernatant liquid was decanted and the pellet resuspended in 10mL Competent Cell Solution 1 (Section 2.1.6). The resulting suspension was incubated on ice for 30min then centrifuged at 13 000 rpm, at 4°C for 10min. The supernatant liquid was removed and the pellet resuspended In 500μL of Competent Cell Solution 2 (Section 2.1.6). Following a further 30min incubation on ice, the cells were competent and ready to be transformed.

2.2.19 Transformation of *P. fragi*

The transformation procedure used, was that of Liu *et. al.* (1996). To 100μL of competent cells, 100ng of purified phagemid DNA was added and mixed well. A 30min incubation on ice followed. The mixture was heat shocked for 2min at 42°C and then immediately chilled on ice and incubated for 60min, on ice. NB (1mL) prewarmed to 25°C was added and the suspension incubated at this temperature for 60min, with shaking (180 rpm). The cells are then ready to be spread on media appropriate for plasmid selection.

2.2.20 Sterilisation and cleaning

Except where indicated, sterilisation was by autoclaving at 121°C for 15min. Exceptions were as follows. The O-F medium was autoclaved for 15min at 110°C. Many solutions were filter sterilised, by means of a 220nm Millex syringe driven filter unit (Millipore; Bedford, MA, United States). General cleaning

of glassware was in a Lab 905 dishwasher (Gallay; Melbourne, VIC) using Clean N detergent (Gallay; Melbourne, VIC) and rinse aid (Gallay; Melbourne, VIC). To reduce iron levels, glassware was soaked in 3M HNO_3 for a minimum of 16h, then rinsed three times in tap water and twice in distilled water.

2.2.21 Statistical analysis

The nature of this project required only a limited amount of statistical analysis. Where appropriate, statistical significance was assessed by the methods detailed in Gravetter and Wallnau (1992). On some occasions, Microsoft Excel 97 (Microsoft; Melbourne, VIC) was employed for statistical calculations.

CHAPTER 3

BIOASSAY DEVELOPMENT

3.1 Introduction

Methods available to determine production of siderophores include chemical, molecular genetic, immunological and microbiological procedures. The bioassay, a microbiological method, is relatively simple in principle and design and has high sensitivity. It can be highly specific for a particular siderophore and more reproducible than chemical methods such as the CAS agar assay, developed by Schwyn and Neilands (1987). The high reproducibility attainable with the bioassay was shown in a comparison of detection methods carried out by Le Roy *et. al.* (1993). These investigators observed inconsistent results in 30% of chemical (Csaky) assays but in the bioassay, only 6.7% of results were inconsistent. Justification for the choice of a bioassay over other methods has been well expressed by Neilands (1984) : “bioassays for siderophores are many orders of magnitude more sensitive than the best chemical assays”. In this review, Neilands discusses different types of bioassay. They can be specific for particular siderophores such as those for enterochelin or aerobactin, or more general like the *Arthrobacter* bioassay, used for detection of hydroxamate siderophores generally. A number of variations in the style of bioassay are possible and several of these were investigated and trialed in the preliminary stages of the work described in this chapter. All bioassays performed in this project were based on the work of Miles and Khimji (1975) who have discussed a number of problems which can be encountered with bioassays. Some of these problems were encountered during this project and were addressed as the methodology was refined. Development of a bioassay for siderophore(s)

produced by *P. fragi* was based on existing *E. coli* siderophore bioassays which were first refined until consistent, reproducible detection was achieved.

3.2 Check on reference strains : agar surface cross-feeding streak tests for detection of aerobactin and enterochelin

Prior to the commencement of work with bioassays, cross-feeding streak tests on the surface of agar were carried out to ensure correct responses by the reference strains. The technique, described by Gibson and Jones (1954) is very effective, but rarely used today - the bioassay method described below is used more commonly.

The first cross-feeding tests attempted were for detection of enterochelin and/or other DHB-containing compounds - hereafter referred to as "enterochelin/DHB compounds". *E. coli* strain AN194 (Ent^+) was used as producer and strain AN193 (Ent^-, Fep^+) as responder. Being a mutant defective in conversion of DHB to enterochelin, AN193 was unable to grow under the conditions of iron depletion used (minimal medium containing 100μM 2,2'-dipyridyl) but was able to grow under these conditions when cross-fed from actively growing AN194 or when provided with DHB. Colleagues in the laboratory carried out the DHB tests.

For preliminary aerobactin cross-feeding tests, *K. mobilis* 62-1 (Iuc^+) (Gibson & Gibson, 1962) was selected as the producing strain with *E. coli* LG1522 (Iuc^-, $IutA^+$) as responder (Carbonetti & Williams, 1984). The first isolation of aerobactin was made from *K. mobilis* 62-1 (Gibson and Macgrath, 1969). Clear cross-feeding was seen when either 2,2'-dipyridyl (125μM) or EDDA (278μM) was included as iron chelator in a deferrated M9 medium. Figure

3.1 illustrates a positive cross-feeding test and Figure 3.2 the absence of cross-feeding when AN194 (Ent^+, Iuc^-) was used as the "producing" strain.

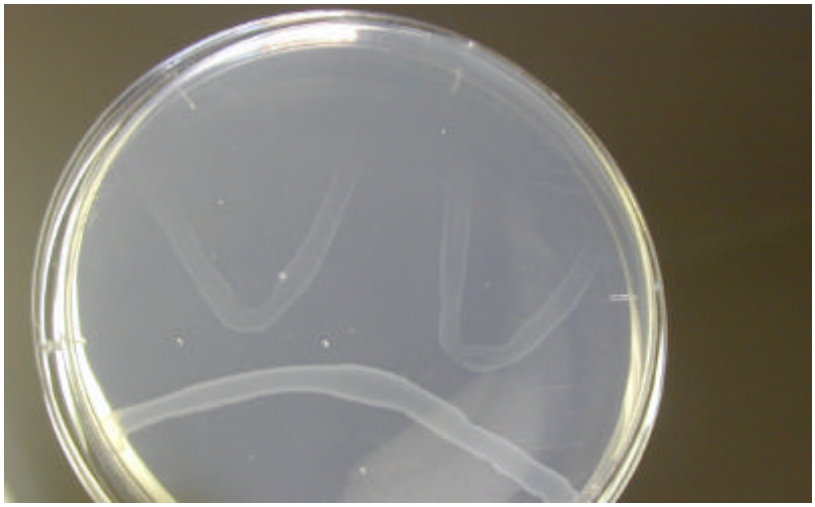


Figure 3.1 : Cross-feeding from *K. mobilis* 62-1 to *E. coli* LG1522

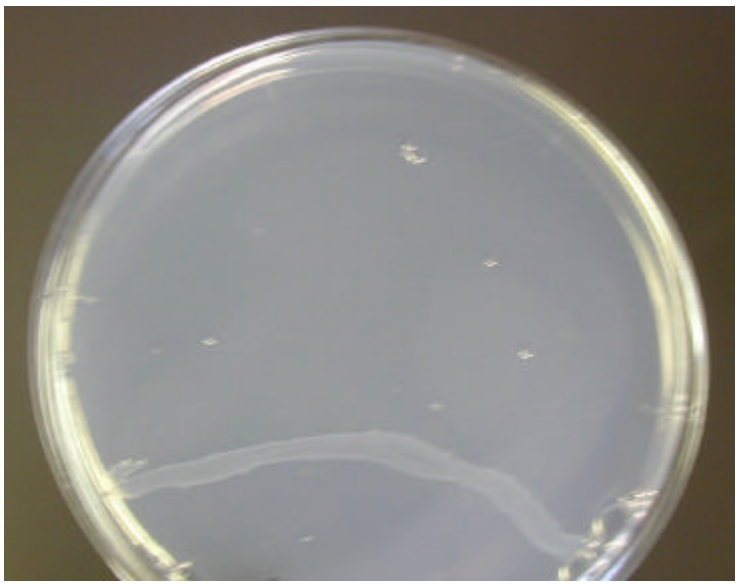


Figure 3.2 : Absence of cross-feeding from the *iuc* negative strain, AN194 to LG1522

3.3 Bioassay for enterochelin production

The bioassay method of Miles and Khimji (1975) was used to detect enterochelin production. The indicator strain was *E. coli* AN193. The chelator 2,2'-dipyridyl was included in the medium, initially at a final concentration of 100μM and later at 200μM. Noticeably different effects were observed with the

two concentrations, with growth of AN193 restricted to a smaller area around the drop of AN194 culture, when the higher level of 2,2'-dipyridyl was used. The results were clear after 18h incubation at 37°C, and there was little change from 1d to 2d of incubation. Incubation of 24h or less, was found to be optimal.

Different volumes of indicator culture (100μL, 200μL and 500μL) were included in the pour plates for the first series of enterochelin bioassays. The number of cells present in each aliquot was not determined. The size of indicator inoculum was not critical as inocula of varying visual turbidity resulted in clear responses. The source of enterochelin/DHB compounds, was a small drop of an *E. coli* AN194 culture, applied to the surface of the plate.

3.4 Bioassay for aerobactin production

Knowledge and experience gained in conducting bioassays for enterochelin/DHB compounds were applied to aerobactin bioassay procedures. These are more widely used because ability to produce aerobactin is sometimes determined during surveys of isolates (Podschun *et. al.*, 1992; Reissbrodt & Rabsch, 1988). For all aerobactin bioassays, the indicator strain was *E. coli* LG1522. Initial tests were performed with the aerobactin positive strains *E. coli* RJ80 and *K. mobilis* 62-1, with AN194 serving as a negative control. Concentrations of 2,2'-dipyridyl between 75μM and 300μM, were trialed in a deferrated M9 agar (section 2.1.4) before 125μM was found to be most suitable for the applications described here. Inoculum sizes ranging from 25μL to 100μL were tested. The success of the aerobactin bioassay was found to be somewhat dependent on the concentration of indicator cells. A consistent distribution of indicator cells during inoculation of a pour plate was too difficult to achieve with a volume as small as 25μL. An enumeration was carried out to determine the ideal

cell concentration, which was found to be approximately 10^7cfu/mL. Following determination of these conditions for achieving reproducible results, two further aerobactin-positive strains were tested : *E. coli* K-12 711(pColV) and *E. coli* O18:H7 strain MW. Both strains gave clear positive results under these conditions when inoculated as stabs directly from an NA culture.

The bioassay was also used for demonstrating the presence of aerobactin in the cell-free culture fluid of aerobactin positive strains. Cell-free culture supernatant was prepared as described in section 2.2.12 using 125μM 2,2'-dipyridyl as chelator. Culture supernatant of *E. coli* strains AN194, RJ79, K-12 711(pColV) and MW and *K. mobilis* 62-1, were tested. Clear growth of the indicator strain was observed around the wells containing cell-free culture supernatant from the aerobactin positive strains RJ79, K-12 711(pColV), MW and 62-1, but not around wells containing AN194 supernatant.

This aerobactin bioassay system was used to investigate the effect and suitability of different methods for applying cell-free culture supernatant and cultures themselves, to indicator plates. Cell free culture supernatant was applied directly on the surface, in wells or onto paper disks. Volumes, ranging from 1μL on disks to 50μL into wells, were investigated. Overall, it was found that 6mm diameter wells, holding 30μL liquid sample, were most suitable. Application of the liquid sample directly onto the agar surface (Figure 3.3) was not ideal because of the spreading which tended to occur before the sample had soaked into the agar. This problem increased if the agar plates were not completely dry when the sample was applied, as the sample tended to mix with surface moisture and spread widely across the surface.

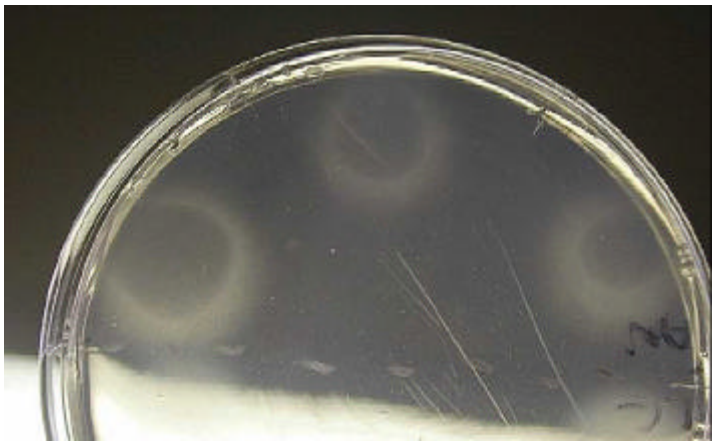


Figure 3.3 : Aerobactin bioassay with a liquid sample of cell-free culture supernatant applied directly onto the agar surface

Paper disks, such as Oxoid blank antimicrobial susceptibility disks are widely used for bioassays. An advantage of this procedure is that the location of the sample is clearly evident. This is particularly useful when the bioassay plate is to be photographed. Disk selection can be a critical factor. Disks were used as part of some bioassays trailed in this chapter. However, some of the disks appeared to influence the results, despite being sterilised by autoclaving prior to use. This effect was not clear-cut and although the basis (possibly chemical) was not determined, use of disks was discontinued. Generally, an overnight incubation period (15h-20h) for an aerobactin bioassay gave a strong, clear response. In some situations, such as when using a large volume (50µL) of cell-free culture supernatant, a positive result was observed in just 6h-8h (Figure 3.4). In this test, cell-free culture supernatant of strains K-12 711(pColV) and MW was used. It is clear that, under some conditions and with some samples of cell-free culture supernatant, a same-day result is achievable.

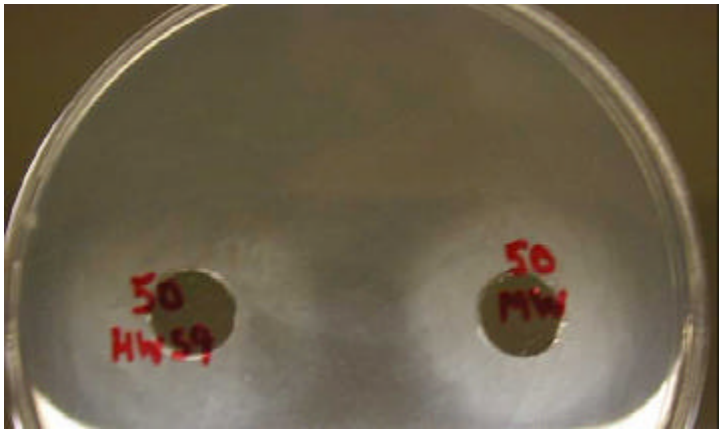


Figure 3.4 : Aerobactin bioassay using cell-free culture supernatants (50µL volume) of *E. coli* strains MW and K-12 711(pColV)

With most test strains or samples, incubation for longer than 40-50h was found to result in inconsistent or inconclusive results, due to background growth of the indicator strain. The number of strains or liquid samples tested on a single plate was limited to four, with regular and frequent observation of the plates necessary for conclusive results. Growth of the background strain distant from the samples after a relatively short period of incubation, may reflect rapid diffusion of aerobactin through the medium or high-level production of the siderophore. Alternatively, if the indicator strain is growing equally over the entire plate, then the level of iron restriction may not be sufficient to inhibit growth of that particular strain.

The final phase of the development of the aerobactin bioassay was to test actively growing cultures, rather than cell-free culture supernatant samples. From the commencement of the trials of this method, the test strains were stabbed into the agar from an NA culture (Figure 3.5). Some other researchers (Enard *et. al.*, 1988; Manninen & Mattila-Sandholm, 1994; Meyer *et. al.*, 1998) have used an aliquot of an overnight NB/LB broth grown culture, but here, a stab with a straight wire, from an NA plate/slope was found to deliver good results. The stab method

was used when strains were tested directly for the ability to produce aerobactin (Figure 3.5). Initially, up to 20 were tested per plate, but interference between test strains meant that the results were unclear. Routinely, to obtain clear results, a maximum of four cultures were tested per plate.

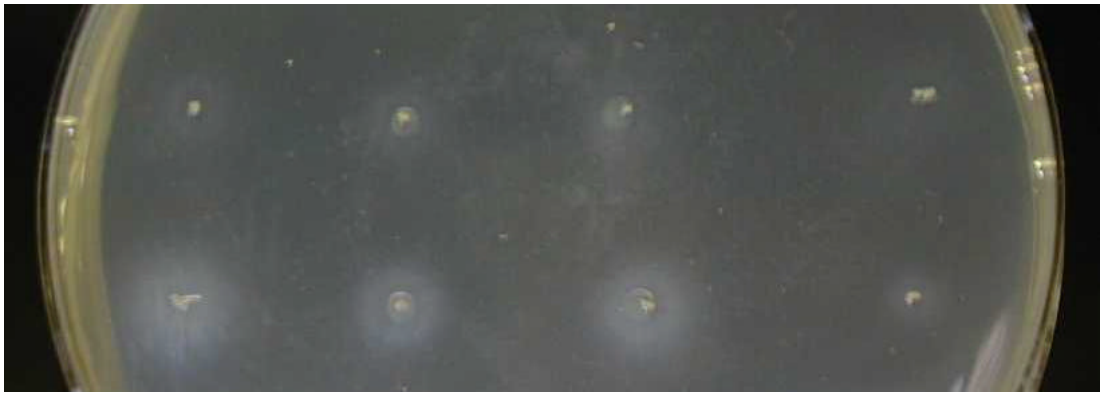


Figure 3.5 : Aerobactin bioassay using an actively growing culture (of *E. coli* O18:H7) stabbed into the agar

3.5 Pseudomonad cross-feeding tests and bioassays

In initial work, the mutants isolated previously by Coates (1994) were used. Although these were presumed to be defective primarily in uptake of the iron-sideropohore complex, it was felt that one or more may also be defective in siderophore biosynthesis. This thought reflected knowledge that genes for many siderophore-mediated, iron-acquisition systems, are clustered in operons. This is the case with genes for enterochelin, citrate, aerobactin and ferrichrome-mediated iron acquisition in *E. coli* (Earhart, 1996). Moreover, as with *pchR* of *P. aeruginosa*, one gene can control both synthesis of siderophore and expression of the respective outer membrane protein receptor (Heinrichs & Poole, 1993).

The strains used were the wild type (VIAS1) and three mutants derived from it, designated mutants B, F and G (Coates, 1994). Agar-surface cross-feeding tests were performed first. These were carried out on a variety of M9 and MM9 agar (Section 2.1.4). Factors which varied included the amount of iron

limitation (through deferration and acid washed glassware), carbon source (glucose, sodium succinate or sucrose) and level of phosphate which was classified as normal (as with M9) or low (as with MM9). These variables were tested in a number of combinations.

None of the minimal media tested, was able to support growth, on its own, of any of the VIAS1 IROMP mutants. This suggested that all the mutants were auxotrophic. This was supported by the (separate) observation that none of these mutants was able to grow in Schwyn and Neilands' (1987) CAS agar, but were able to do so when deferrated casamino acids was added. The mutants were also able to grow on some of the minimal media, when cross-fed by VIAS1. These media were M9 and MM9, to which no iron or chelator was added, and in which glucose was the carbon source. An interesting, additional observation was that once cross-fed, those mutants could be transferred to the same medium, on which they would grow without being supplied with any exogenous growth factors from the parent strain. Prior to being cross-fed, they would show no growth on such media. The suggestion that the IROMP mutants might be auxotrophic is not in agreement with the previous work of S. Ibro who observed growth in unsupplemented chemically defined medium. One explanation for this observation might be the inoculum type. S. Ibro inoculated minimal media mostly with an NaCl surface washing of growth from an NA slope. This could transfer sufficient growth factors (and possibly iron) from the complex medium into the minimal medium, enabling growth.

Further surface cross-feeding tests sought to investigate the growth response of the IROMP mutants to crude preparations of cell-free culture supernatant from VIAS1. These supernatant preparations were prepared by S. Ibro. One of these was prepared from a culture grown in an iron deficient minimal medium while the other was prepared from a culture grown in an iron sufficient

minimal medium. Testing was performed using M9 medium to which no iron or iron chelator was added. The result was a positive growth response of the IROMP mutants to both preparations, which was clearer when mutant F was the test organism. A repeat experiment gave essentially the same results.

When it was observed that mutants B, F and G were unable to grow unaided on minimal media, it was decided to attempt cross-feeding experiments on deferrated NA (deferrated Oxoid NB No. 2, code CM3, combined with Oxoid Purified Agar, code L28) containing 2,2'-dipyridyl. Firstly, the MIC of 2,2'-dipyridyl in deferrated NA was determined to be approximately 370μM. A cross-feeding experiment was then carried out under these conditions, but no cross-feeding was observed. This provided further evidence that the substance being cross-fed (see previous paragraph) was a growth factor not present in minimal medium but present in complex media, such as nutrient agar. Therefore, this would appear to be further evidence that mutants B, F and G are auxotrophic, for a yet to be determined growth factor.

With some siderophore bioassays, such as those used to detect aerobactin production, the test strains could be transferred directly from an iron-sufficient complex medium, such as nutrient agar with the aerobactin positive strains showing strong evidence of aerobactin production. However, the *P. fragi* indicator strains needed to be passaged in iron-deficient M9 first, so that their growth could be inhibited at a reasonable level of EDDA, and clear assessment of their response to liquid samples of siderophore and iron preparations made. Initially, two passages through deferrated M9 containing 100μM 2,2'-dipyridyl were tested. This was carried out by inoculating a second, identical deferrated M9 medium containing 100μM 2,2'-dipyridyl with actively growing culture from the first (approximately 1% inoculum). However, results in the subsequent bioassay

were not noticeably better than when a single passage was used. The passaged indicator culture was spread as a 10^{-2} dilution prepared in 15mM NaCl.

Initially, different levels of 2,2'-dipyridyl were used, with varying success. As the success varied, it was decided to investigate the use of EDDA, which, in general, gave better results. Concentrations of EDDA ranging from 9μM to 69μM were tested with VIAS1, before 25μM was selected. This concentration was close to the MIC of EDDA for VIAS1, and worked well only when the cells were passaged through an iron deficient medium first.

Results in this Section provide evidence that the compound being cross-fed from VIAS1 to the IROMP mutants, is unrelated to iron, as production appears to take place when sufficient iron is present in the medium. While VIAS1 is able to grow normally on a standard M9 medium, the IROMP mutants do not. In addition to auxotrophy, discussed earlier in this Section, other reasons are also possible to explain this observation. The M9 used, a chemically defined minimal medium without additional iron, may have an iron concentration below what is required for optimal growth. Under such conditions, VIAS1 growth is unrestricted as it is able to secrete siderophore. It is able to utilise the iron from this iron-siderophore complex, because the complex is able to gain entry into the cell via the intact IROMP. The IROMP mutants are however, defective in attachment and initial transport of the iron-siderophore complex and therefore they cannot obtain sufficient iron, unlike VIAS1. This would inhibit their ability to utilise the siderophore produced by VIAS1, and ultimately, restrict their growth. However, VIAS1 may constitutively synthesise another compound, capable of functioning as a siderophore, that is unable to be synthesised by the IROMP mutants. Inability to synthesise this hypothetical siderophore or siderophore-like compound may have occurred through the process of mutating the IROMP

genes, as the genes for this other compound may have been inactivated through the initial transposon mutagenesis.

The identity of this unknown compound may be citrate. Champomier-Verges *et. al.* (1996) reported *P. fragi* as a constitutive citrate producer. It is well known that citrate is an iron chelator. In some species, it exhibits full siderophore properties. These species include *Bradyrhizobium japonicum* (Guerinot *et. al.* 1990) and *P. aeruginosa* (Harding & Royt, 1990). Additionally, it may be that growth is made possible by the substance(s) cross-fed from the wild type, which results in expression of the gene(s) for another genetically related compound. Alternatively, the unknown substance may act as a signal for derepression of one or more genes, which are essential for growth in this medium. A further possibility is that cross-fed material is required in a very small (micromolar) concentration, and that growth-supporting amounts can be carried over in subcultured cells. If this were the scenario, further subculturing should eventually result in no growth. This was not tested. Whatever the nature and function of the unknown cross-fed substance, it did not appear to be iron-related because the cross-feeding did not take place in iron deficient medium. Consequently, although potentially interesting, this cross-feeding in iron-sufficient media was not investigated further.

Unlike the *E. coli* aerobactin and enterochelin bioassays, the bioassays that used pseudomonads as the indicator strain were prepared as spread plates rather than pour plates. This was because these *P. fragi* strains have an optimal growth temperature of 25°C. It was thought they may be more heat sensitive than *E. coli* and be more likely to be injured or killed in an agar pour plate at 45°C-50°C. Initially, pseudomonad inocula for pour plates were prepared in the same way as for *E. coli* : an overnight NB culture, centrifuged and resuspended in 15mM NaCl. However, this procedure resulted in growth, even in the presence of

relatively high amounts of iron chelator such as 2,2'-dipyridyl or more commonly, EDDA. It was presumed that iron levels in the inocula were adequate to allow growth. Carbonetti and Williams (1985) have suggested that several passages in iron-deficient media may be necessary before test strains of *E. coli* produce observable amounts of aerobactin. In the *P. fragi* work described here, it was found to be necessary to deplete the indicator strain (such as VIAS1) of iron, but not necessary to deplete the test strains, as suggested by Carbonetti and Williams (1985).

3.6 Summary

The initial objective of the work described in this Chapter was the acquisition of skills, knowledge and experience, from development of bioassays for established *E. coli* siderophores. The presence of biologically active aerobactin and enterochelin/DHB-containing compounds, in cell-free culture supernatant was demonstrated, as was production by actively growing cultures. Procedures for preparing indicator-strain inoculum and for applying test samples/strains were developed, as were procedures for achieving appropriate levels of iron deficiency in the bioassay medium. The pseudomonad work described in this chapter was a transition to work described later in this thesis. Progression was from established *E. coli* bioassays, to a bioassay for detection of the unknown siderophore(s). In addition, use of the aerobactin bioassay technique was carried out in a survey of a number of *E. coli* isolates, described in the next chapter.

Both the wild type strain and existing mutants were used to gain familiarity with this species of *Pseudomonas* and suitable bioassay conditions.

CHAPTER 4

INVESTIGATION OF THE HYDROXAMATE SIDEROPHORE PRODUCED BY *E. COLI* ISOLATES FROM THE ENTERIC PATHOGENICITY LABORATORY COLLECTION

4.1 Introduction

The catechol siderophore enterochelin was the first siderophore discovered in *Escherichia coli* (O'Brien and Gibson, 1970) and ability to produce it is very common throughout the Enterobacteriaceae (Reissbrodt & Rabsch, 1988). Although *E. coli* typically has receptors for a number of siderophores, aerobactin is the only hydroxamate siderophore it is known to produce. However, some strains are known to produce other hydroxamate-type siderophores (Payne, 1988). Ability to produce aerobactin is considered a virulence factor (Williams, 1979). Strains that produce it are more likely to be isolated from clinical material than from environmental sources (Podschun *et. al.*, 1992) and for example, are more often found in diarrhoeal stools than in non-diarrhoeal stools (de Fatima Martins *et. al.*, 2000). However, there are studies that have suggested aerobactin production is important for maintaining commensal *E. coli* strains (Nowrouzian *et. al.*, 2001a; Nowrouzian *et. al.*, 2001b).

This chapter describes a survey of isolates, collected during prior work in this laboratory (Stuart *et. al.*, 1982; Stuart, 1986). By a chemical method (Csaky assay), these isolates had been shown previously to produce hydroxamate when grown under iron-deficient conditions. Through use of a bioassay procedure, the ability of these isolates to secrete aaerobactin was investigated, with the aim of identifying a non-aerobactin producing isolate. Due to their high specificity, aerobactin bioassays will clearly detect aerobactin-producing isolates while not

detecting the production of other siderophores. Any isolate found not to produce aerobactin, but which remained hydroxamate positive, would be novel and of much interest

4.2 Check on properties of the indicator strain, *E. coli* LG1522

The indicator strain selected, *E. coli* LG1522, is probably the most widely used indicator strain in aerobactin bioassays. It was created by Carbonetti and Williams (1984) by addition of pColV-K30 to strain AN263 (Laird & Young, 1980). LG1522 cells are able to produce and secrete enterochelin but cannot utilise this siderophore, as they contain a mutation (*fepA*) which prevents effective binding of the ferric-enterochelin complex. Presence of the plasmid pColV-K30 provides aerobactin transport genes (*iut*) but not a functional set of the *iuc* genes required for aerobactin biosynthesis. In an iron-deficient medium, growth of LG1522 is stimulated by aerobactin, but should not be stimulated by enterochelin.

Strains of *E. coli*, like LG1522, which are able to synthesise enterochelin but possess a defective enterochelin binding/transport system have been shown (Young, 1976) to produce material of an intense red colour when grown in a chemically defined medium containing excess iron (III) (Figure 4.1). This colour intensified from pink to the intense red, as incubation continued. Young (1976) has discussed the mechanism behind this red colouration. In response to iron deprivation, cells secrete enterochelin which forms a complex with iron in the medium. This ferric-enterochelin complex cannot be transported into the cells, (because of the *fepA* mutation) and the state of iron-deficiency continues. This leads to further production and secretion of enterochelin resulting in a further increase in the concentration of ferric-enterochelin complexes and greater intensity of red colouration. This phenomenon was used during checking of the Enteric Pathogenicity Laboratory culture of LG1522.

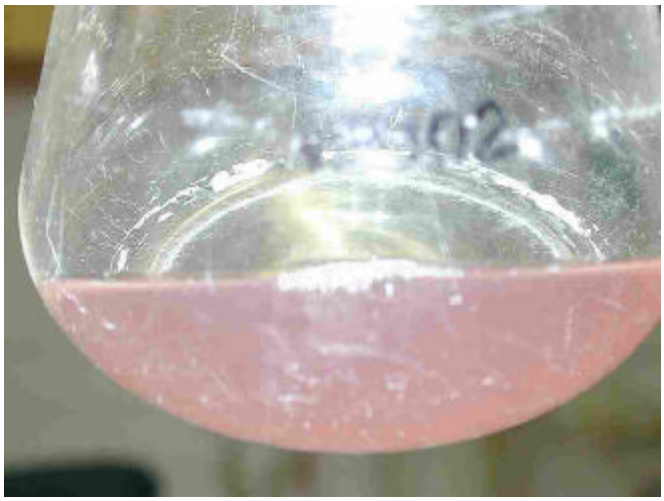


Figure 4.1 : Pink coloured *E. coli* AN102 culture

Upon initial checking of the LG1522 culture in the manner just described, no red colouration of the medium was observed. At first, it was thought that the *fepA* mutation may have reverted. This is known to occur at a relatively high frequency (Young, 1976). However, cultures derived from isolated single colonies on nutrient agar plates containing citrate, also failed to turn red. Further testing confirmed the expected auxotrophic requirements for leucine, proline and tryptophan (on M9 with and without these amino acids) and inability to produce acid from fermentation of xylose (on XLD medium) or lactose (on MacConkey agar). Other members of the laboratory were also unable to isolate cells with the appropriate LG1522 phenotype and a fresh culture of LG1522 was eventually obtained from Dr Richard Haigh at the University of Leicester. When tested, this culture immediately gave all responses characteristic of LG1522, most importantly, the red coloured culture supernatant. Characterisation of the first culture of LG1522 was not pursued further except to demonstrate its unsuitability for detection of aerobactin production (Table 4.1).

Table 4.1 : Mean diameter of growth (mm) of LG1522 and the possible FepA revertant of LG1522 in the presence of different functional siderophores

Presumed functional siderophore genes	LG1522	Possible FepA reverent of LG1522
iuc	4.8mm	6.4mm
iuc and *ent*	0.0mm	5.4mm

The original description (Young, 1976) of enterochelin overproduction resulting in a red colour of the ferric-enterochelin complex, was carried out using strain AN311. Preparation of enterochelin using this method has been described for other strains such as the *fepC* mutant AN102 (Payne, 1994). However, it appears not to have been described for either the *fepA* mutant LG1522 or the *fes* mutant AN273, which is able to transport ferric-enterochelin into the cells but cannot remove iron from the complex. Each of these strains was observed to yield a red coloured culture supernatant under the conditions described by Young (1976). This is expected, because while each of these strains has a different genotype, all are able to produce and excrete enterochelin but are unable to utilise ferric-enterochelin.

4.3 The hydroxamate-positive strains from the EPL collection

Reports of previous work (Stuart *et. al.*, 1982; Stuart, 1986) contain a summary of the human, environmental, bovine, porcine and avian origins of these isolates. In the investigation, 48 of the total 132 hydroxamate positive isolates were tested for their ability to produce aerobactin. In addition, a further eight non-hydroxamate producing strains from this collection were tested, as extra, negative control strains. In this chapter, they are referred to, according to the laboratory-assigned “ND” strain numbers.

4.4 First-stage aerobactin testing

For clarity of results, the first-stage bioassays were conducted in deferrated iron-deficient M9 containing 200μM 2,2'-dipyridyl. These conditions suppress background growth of the indicator strain, for long enough to enable a strong, clear zone of growth stimulation to be observed. Of the 48 strains, two (4.2%) were negative for aerobactin production after first-stage testing. These were ND412 and ND667.

Two interesting observations were made during these tests. First, some strains exhibited antibacterial activity against LG1522. The size of the inhibition zone varied between 0.5mm and 2.5mm. In some instances, further zones (up to four) were observed in addition to a one inhibitory and/or one stimulatory zone. These were sometimes more pronounced at lower levels of 2,2'-dipyridyl, such as 150μM. As many aerobactin synthesis and transport genes are located on ColV-K30 plasmids, it was thought likely that the inhibition reflected the production of a colicin. An illustrated example of a strain exhibiting a four-zone reaction is presented (Figure 4.2).

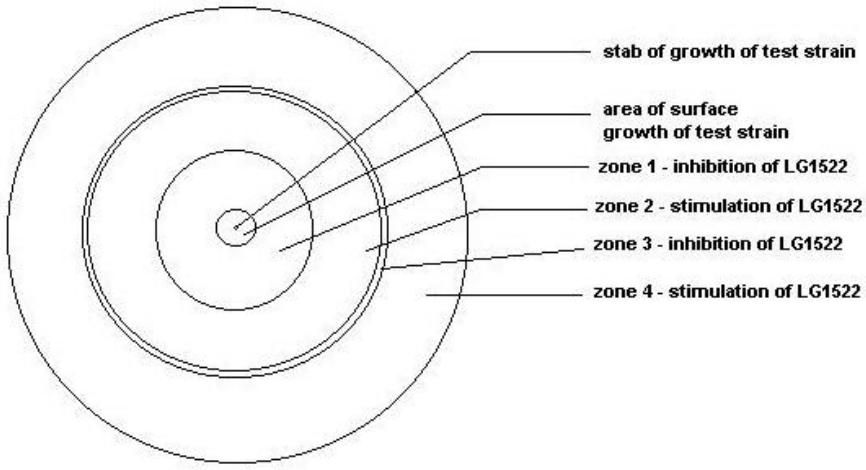


Figure 4.2 : Aerobactin bioassay appearance of ND437 after 184h incubation

The second interesting observation was that the degree of growth stimulation of LG1522 was not consistent. Some strains (such as ND533) were very intense stimulators while others (for example, ND818) exhibited weak stimulation. This variation may be due to differing rates of production and/or release of aerobactin.

For some isolates, copper resistance or sensitivity was known, from previous work in the laboratory. It was observed that the growth zone of LG1522 (after overnight incubation) around copper sensitive isolates (3.9mm) was significantly ($p < 0.00006$) larger than around copper resistant isolates (2.3mm).

None of the eight negative control strains from the collection gave indication of aerobactin production. The laboratory record indicated that when these isolates were first investigated, they produced a red coloured culture supernatant. These records did not, however, include details of the culture conditions when this observation was made. Some preliminary investigation of the conditions required to produce this red colour was therefore carried out. Of six strains tested, the red coloured culture supernatant was reproducible with only one, ND588, under iron-deficient conditions. This was quite unexpected, because previously, red coloured culture supernatant had only been observed under conditions of excess iron, as a result of the colour of the iron-siderophore complex. The reason behind this observation is unclear.

4.5 Follow up aerobactin testing of negative isolates

Those strains that were negative in the first stage aerobactin bioassays performed with 200μM 2,2'-dipyridyl were subsequently retested. This involved streaking on media containing either 150μM 2,2'-dipyridyl, and for those remaining negative, further streaking on media containing 125μM 2,2'-dipyridyl.

The reason for this multi-stage testing was that 2,2'-dipyridyl levels of 150μM and 200μM may be inhibitory for some strains, despite their ability to produce aerobactin. Such inhibition might reflect low-level production of aerobactin under conditions of mild iron deprivation. The lowest level of 2,2'-dipyridyl used, 125μM, was the minimum concentration that would inhibit LG1522 for a period of time long enough, for a reasonable aerobactin bioassay to be performed. "Reasonable" refers to the development of a clearly observable positive reaction, from aerobactin-producing strains within 24h. After this time (clearer after 48h), some slight stimulation of growth of LG1522 would often be observed in the vicinity of the negative control, AN194. However, this is not unexpected. Some strains harbouring a *fep* mutation are known to be only partially defective in binding/utilisation of ferric-enterochelin (S.M. Payne, pers. comm.).

4.6 Csaky assay for hydroxamates and CAS agar assay of aerobactin negative isolates

The two isolates (ND412 and ND667) which consistently yielded negative results during in aerobactin bioassays were of much interest and were tested for ability to produce siderophore as indicated by the CAS agar method of Milagres *et. al.* (1999). The growth medium used was M9 containing 125μM 2,2'-dipyridyl. Once siderophore production was confirmed, it was clearly necessary to confirm the production of hydroxamate in iron-deficient medium (deferrated M9 containing 125μM 2,2'-dipyridyl), by modified a Csaky assay. Growth was observed in this iron-deficient minimal medium (without supplements), although the growth under iron limitation was much reduced. Neither isolate was positive in this chemical assay for hydroxamte (Figure 4.3). The negative result from the modified Csaky assay may indicate loss of the aerobactin synthesis genes during storage.

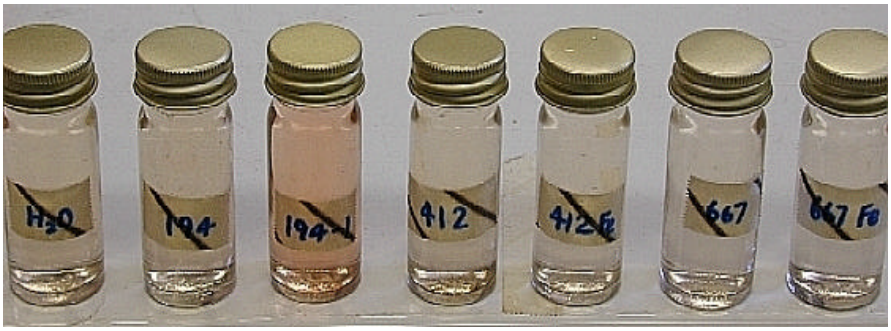


Figure 4.3 : Csaky assay results of tests on aerobactin negative strains
From L to R. Sample 1 (water control), Distilled water; Sample 2 (negative control), AN194; Sample 3 (positive control), RJ79 (AN194-1); Sample 4, ND412 low Fe culture supernatant; Sample 5, ND412 high Fe culture supernatant; Sample 6, ND667 low Fe culture supernatant; Sample 7, ND667 high Fe

4.7 Enterochelin/DHB compound production by selected strains

While ability to produce enterochelin is virtually universally present in the Enterobacteriaceae, it cannot be assumed that a given isolate will produce this siderophore. Some of the isolates described in this chapter were tested for ability to produce enterochelin/DHB compounds as described in Section 2.2.6, on an iron-deficient M9 medium containing 100μM 2,2'-dipyridyl with AN193 as the indicator strain. Most of the isolates tested did produce enterochelin/DHB compounds as expected (Appendix 4.2) but isolates ND541 and ND542 appeared not to. These isolates appear to produce neither enterochelin/DHB compounds, nor aerobactin. This suggests that they are non-siderophore producing strains, a rarity amongst *E. coli*. While the results obtained indicate that they do not produce siderophores, this raises the question of how they would be able to grow in the iron-deficient medium. It may be that these strains produce a non-aerobactin, non-enterochelin/DHB siderophore, undetectable by the procedures followed in the work described in this chapter.

4.8 Summary

Unlike ability to produce enterochelin, ability to produce aerobactin is not widespread among genera of Enterobactiaceae (Reissbrodt & Rabsch, 1988). Even within particular genera, *Klebsiella* in particular, this property is not widespread (Podschun *et. al.*, 1992). In the hydroxamate survey of *E. coli* conducted by Stuart *et. al.* (1982) previously in this laboratory, only 27.7% (132/476) of isolates were found to be hydroxamate positive. From this collection of 132 strains, 48 were selected and tested for production of aerobactin, the only hydroxamate siderophore known to be produced *E. coli*. One of the objectives was to search for indication of the presence of hydroxamate siderophore other aerobactin

The results obtained in this small survey illustrated that methodology and technique were appropriate. Of the 48 isolates, two were negative for aerobactin production in three bioassays of decreasing iron limitation. This suggested that a new hydroxamate siderophore may be present. However, an assay for the confirmation of the presence of hydroxamates was negative. It was assumed that these two strains have lost their capacity for aerobactin production during storage. While it seems likely that aerobactin is the only hydroxamate siderophore produced by *E. coli*,, a more extensive survey would be required to confirm this.

CHAPTER 5

ATTEMPTED ISOLATION OF A SIDEROPHORE PRODUCTION MUTANT OF *PSEUDOMONAS FRAGI* THROUGH TRANSPOSON MUTAGENESIS

5.1 Introduction

Previous work in association with this laboratory (Coates, 1994) produced mutants severely impaired in their ability to grow under conditions of iron deficiency. Three of these mutants were lacking an outer membrane protein, thought to be a receptor for the putative new siderophore (IROMP mutants). Genes controlling synthesis of the siderophore were not located. The work described in this chapter was designed to identify and characterise these genes, through the isolation of transposon induced mutants. A preliminary attempt was also made to clone the siderophore receptor genes, with selection based on their transfer from wild type cells to IROMP mutant cells. Had it been successful, such transfer should have enabled the mutants to utilise siderophore in a manner identical to the wild type. A similar approach was used by Baumler *et al.* (1998) when they transferred the gene for an outer membrane receptor of catechol siderophores from *Salmonella enterica* into an *E. coli fepA* mutant. This resulted in the *E. coli* being able to utilise enterochelin. It was also thought possible that biosynthesis genes might map close to receptor genes, and be cloned together with them.

5.2 Initial matings between *E. coli* SM10λ*pir*(pRT733) and *P. fragi* VIAS1

The procedure used for filter matings initially was that used by Coates (1994) during the isolation of the VIAS1 IROMP mutants referred to above. For

these transfers, the strains used were *E. coli* SM10λ*pir*(pRT733) (donor) and *P. fragi* VIAS1 (recipient): the donor strain harboured the same transposon (Tn*phoA*) as did the donor used by Coates. The only difference was that the plasmid used by Coates (1994) was pRT291. A similar transfer has been described by Rahme *et. al.* (1997) who used Tn*phoA* in pRT733, carried in *E. coli* SM10λ*pir*, to mutagenise a *P. aeruginosa* strain. The donor strain was created by Simon *et. al.* (1983). Further modifications to the strain were addition of the transducing phage from Kolter *et. al.* (1978) and inclusion of pRT733 by Taylor *et. al.* (1989).

Initial attempts to transfer the plasmid followed the method used by Coates (1994), described in Section 2.2.10. Many attempts were made with this filter mating method, but on no occasion, were any transconjugants isolated. Alterations were made, and investigations carried out, to determine what the problem might be. When the MICs for both the donor and recipient strains were checked, it was found that growth of VIAS1 was slightly impaired at 108μM nalidixic acid, with complete inhibition of growth at a concentration of 215μM. Growth of SM10λ*pir*(pRT733) was inhibited by 43μM nalidixic acid. Growth of VIAS1 was somewhat inhibited by 9μM kanamycin and completely inhibited by 17μM. Based on these results, the antibiotic concentrations chosen for routine selection were 17μM for kanamycin and 43μM for nalidixic acid. Despite this change in antibiotic concentration, other minor alterations (such as changes to the volume and the actual filter mating procedure) and a change from filter to broth matings, no transconjugants were obtained.

As the recipient strain SM10λ*pir*(pRT733) carries genes for ampicillin resistance and tetracycline resistance, in addition to chromosome and plasmid (transposon) located kanamycin resistance genes, the MICs for these two

antibiotics were determined as well (Table 5.1). The MBC profile of VIAS1 was also determined (Table 5.2).

Table 5.1 : Antibiotic MIC profile of *P. fragi* VIAS1 and *E. coli* SM10λ*pir*(pRT733)

ANTIBIOTIC	VIAS1	SM10λ*pir*(pRT733)
Ampicillin	25μM	> 100μM
Chloramphenicol	75μM	Not tested
Kanamycin	10μM	> 100μM
Nalidixic acid	215μM	< 43μM
Polymyxin	< 10μM	Not tested
Streptomycin	10μM	Not tested
Tetracycline	25μM	25μM
Vancomycin	> 100μM	> 100μM

Table 5.2 : Antibiotic MBC profile of *P. fragi* VIAS1

ANTIBIOTIC	MBC
Ampicillin	Not tested
Chloramphenicol	Not tested
Kanamycin	< 10μM
Nalidixic acid	< 25μM
Polymyxin	< 50μM
Streptomycin	< 25μM
Tetracycline	Not tested
Vancomycin	Not tested

5.3 Transfer of Tn*phoA* from *E. coli* SM10λ*pir*(pRT733) to *E. coli* AN194N.

Following repeated failure to transfer pRT733 from *E. coli* to *P. fragi*, transfer to another *E. coli* strain was undertaken in an attempt to demonstrate that :

a) the plasmid still contained the genes required for self-transmission, that is, was still a conjugative plasmid;
b) 37°C was a suitable temperature for this conjugation; and
c) conjugation to a member of the same species was possible under the conditions being used.

While all these were basic processes, it was hoped that a mating between two *E. coli* strains would reveal where the problem with the *Pseudomonas* mating lay.

A broth mating was carried out at 37°C between the *E. coli* strains SM10λ*pir*(pRT733) (donor) and AN194N (recipient). Strain AN194N was able to grow well even at a concentration of 215μM nalidixic acid. Prior to mating, a culture of each strain was grown to early log-phase (approximately 3.5h). The mating duration was approximately 4.5h and the mating mix was spread (200μL per plate) on NA selective medium containing 43μM nalidixic acid and 26μM kanamycin. This resulted in 7 colonies per 1mL of mating mix. A similar result was obtained following a repeat mating. The colonies were presumed to be AN194N derivatives as they all grew on M9 supplemented with leucine, proline and tryptophan but did not grow on unsupplemented M9. The transfer between *E. coli* strains was successful, albeit at a very low rate. This result indicated where problems with the method might be. A major problem might have been temperature, where 25°C may be too low (compared to 37°C). Furthermore, the filter mating may be unsuitable for transfer of this plasmid. Following this partial success, the mating was repeated at 25°C, the temperature necessary for mating involving VIAS1. No colonies were observed on the selective NA, even after 7d. Such results suggest that for a broth mating, 25°C is an unsuitable temperature when pRT733 is the plasmid to be transferred, even when transfer is between two *E. coli* cells.

5.4 Investigation of different mating methods between *E. coli* SM10λ*pir*(pRT733) and *P. fragi* VIAS1

Following a search of the literature for other mating methods, techniques described by Hirayama *et. al.* (1998), Kostal *et. al.* (1998) and Zhao *et. al.* (1998) were trialed. The standard broth mating, used previously, was carried out in parallel with these matings.

The mating method described by Hirayama *et. al.* (1998) was performed in the following manner. Donor cells were grown overnight on NA while recipient cells were grown to log phase in NB. In 400μL of recipient culture, two to three colonies of donor cells were suspended. The entire volume was spread onto a cellulose filter, which was placed on an NA plate and incubated for 6h at 25°C. The resulting growth was then suspended in NB and spread onto selective NA.

For the plasmid transfer method of Kostal *et. al.* (1998), donor and recipient cultures were grown to log phase in NB. From each culture, a 10μL aliquot was taken and mixed on a membrane filter. The filter was placed on an NA plate and incubated overnight. The resulting growth was suspended in 1mL 15mM NaCl and the mixture spread onto selective NA plates.

The mating method described by Zhao *et. al.* (1998) was performed as follows. Donor and recipient cultures were grown to log-phase in NB. A mixture of donor and recipient cultures was prepared (between 100μL and 750μL of each, depending on turbidity). The mixed culture was centrifuged, supernatant liquid discarded and the pellet resuspended in 20μL NB. This 20μL volume was applied to the centre of an NA plate and incubated overnight at 25°C. A suspension of this overnight growth was then prepared in 1mL NB and vortexed to aid even suspension. It was then spread onto selective NA plates.

Colonies were observed on the selective medium, only following the procedure of Zhao *et. al.* (1998). This method was therefore selected for further use.

5.5 Routine transfer of Tn*phoA* from *E. coli* SM10λ*pir*(pRT733) to *P. fragi* VIAS1

The method of Zhao *et. al.* (1998) was used for the production of higher numbers of VIAS1 transconjugants. The aim of this was to isolate at least one mutant defective in siderophore production.

One key refinement to the technique was to commence the mating when the donor strain's growth was in mid-log phase. This was found to occur after approximately 7h (Figure 5.1).

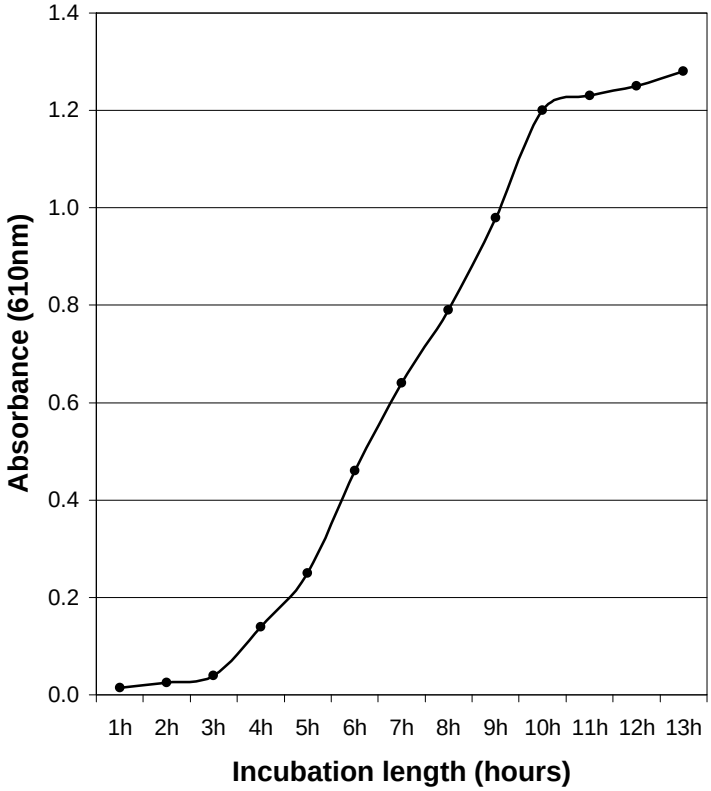


Figure 5.1 : Growth curve of *E. coli* SM10λ*pir*(pRT733)

This led to a substantial increase in number of transconjugants generated, compared to all previous matings (Figure 5.2), where the donor had been in early-log phase.

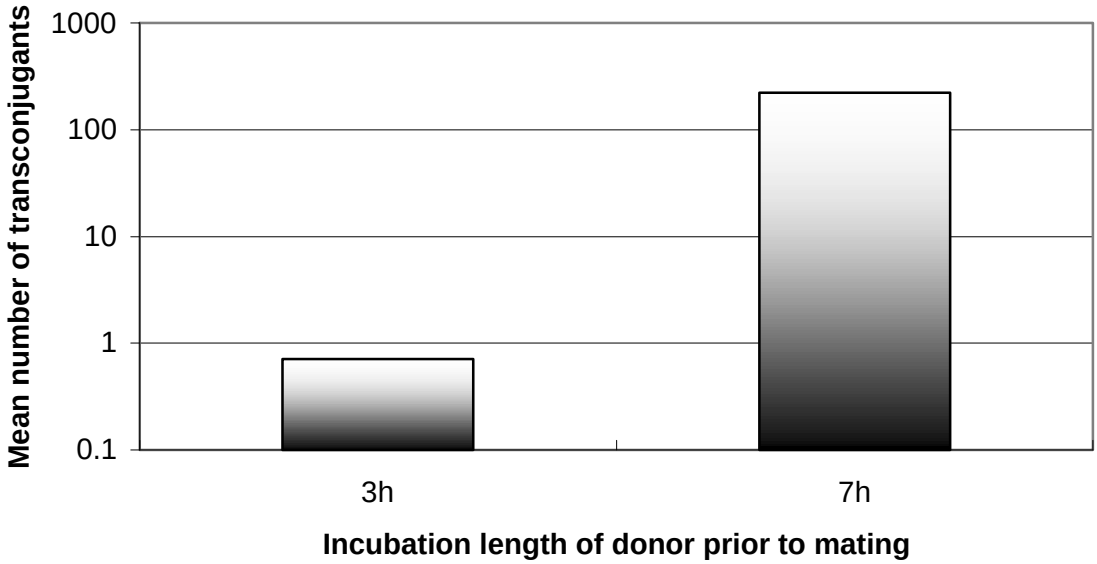


Figure 5.2 : Comparison of the numbers of transconjugants isolated following use of donor cells in early-log or mid-log phase at the commencement of mating

The number of transconjugants was such that dilution of the 200μL sample of mating mixture prior to spreading onto selective NA was not required. For each mating, two control plates were prepared. The first control medium was NA. Onto a third of the plate each, the mating mix, donor and recipient were spread to check that the cultures were viable and culturable. The second control involved spreading of 50μL of donor and recipient, each onto a half of the same selective NA as was used for the mating mix, to ensure their inability to grow on the selective medium. The selection medium used for all routine matings was NA containing 17μM kanamycin (for selection against the recipient) and 43μM nalidixic acid (for selection against the donor).

For most matings, the number of transconjugants isolated was low enough for each colony to be selected for further investigation. However, during the final mating, a large number of transconjugants were isolated with only 120-140 colonies being able to be used from each plate. The others were too close to each other, leading to uncertainly as to whether one or two colonies were present. In total, approximately 1900 transconjugants were obtained, most (over

99%) through the method of Zhao *et. al.* (1998). The remainder were generated by broth matings. Results of a direct comparison of these two methods through simultaneous matings are shown in Figure 5.3, from which it can be seen that the broth mating was much less successful under the conditions used.

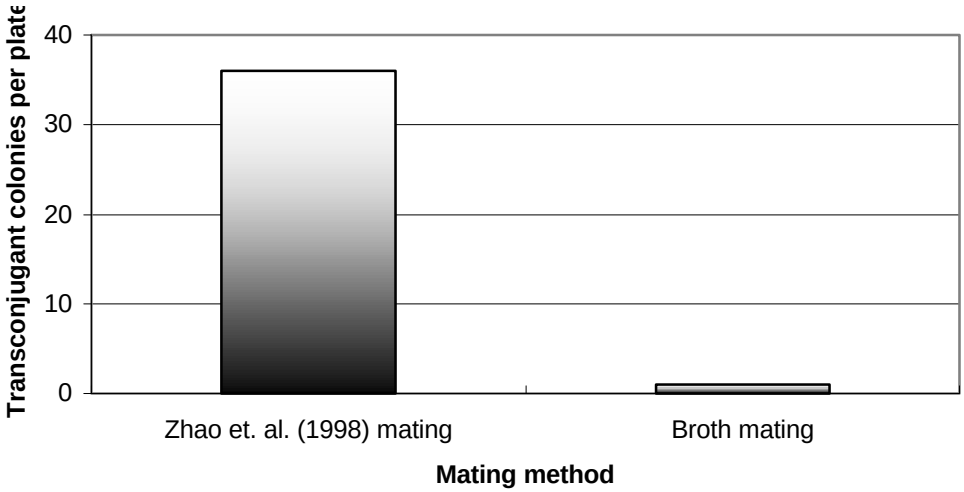


Figure 5.3 : Comparison of the mating method of Zhao *et. al.* (1998) with the standard broth mating

5.6 Screening for siderophore-deficient mutants

Several screening methods were investigated before selection of the one deemed most suitable. At first it was thought that screening would occur on CAS agar. However, initially there were a number of preparation problems associated with this agar (discussed in Chapter 8), and other methods were looked into while the problems with the CAS agar were rectified.

As the desired mutants were to have impaired ability to produce siderophore, much initial selection was based on their inability, or reduced capacity to grow in/on iron-deficient minimal media, relative to that of the wild type VIAS1 from which they were derived. These tests were generally conducted on M9 agar containing 25μM EDDA or in an M9 liquid medium containing 100μM 2,2'-dipyridyl.

The replica plating method was used to screen approximately 100 transconjugants. Three sets of media were used, in the order : M9 agar containing 25μM EDDA (to test ability to grow on an iron-deficient medium); M9 (to determine prototrophy or auxotrophy); and NA (to ensure viability/culturability and effective transfer via the velvet). As no impairment of growth, relative to that of the wild type, was noted on the iron-deficient medium, all transconjugants were assumed to have intact siderophore synthesis gene(s).

During some early matings, a small number of transconjugants were isolated. Growth of these was compared with growth of the wild type in liquid M9, with and without 2,2'-dipyridyl. (Figure 5.4).

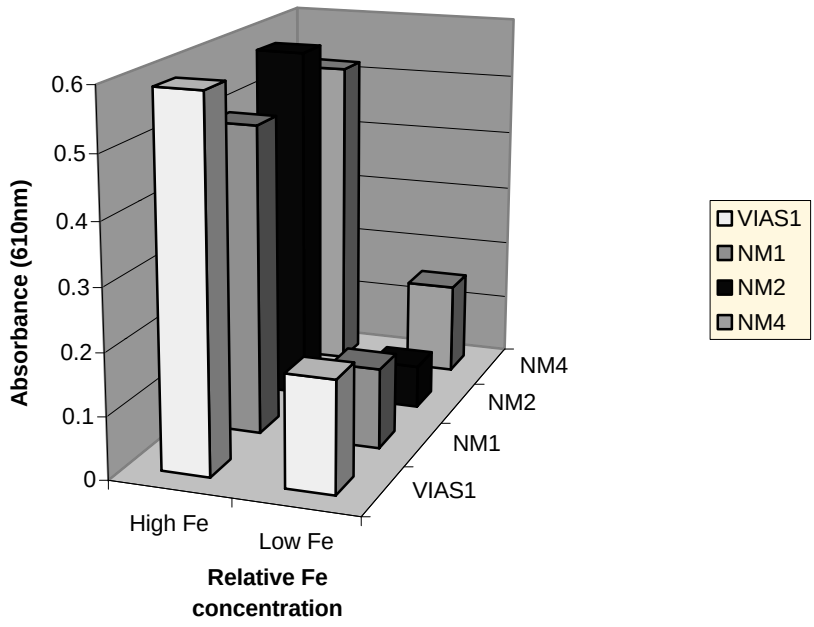


Figure 5.4 : Absorbance of transconjugant cultures compared to the wildtype in iron-deficient and iron-sufficient liquid M9 following 19h incubation

This was time consuming, but very suitable for clear identification of differences in growth related to impaired ability to acquire iron.

When problems with the preparation of CAS agar were settled, this method was employed for screening purposes, in place of the iron-deficient minimal

media. All factors considered, CAS agar was the best method for general screening for siderophore-defective mutants. This was because preparation of the medium was reasonably rapid, the distinction between negative and positive strains was clear (exemplified by growth comparisons of *E. coli* wildtype with siderophore deficient mutants), the testing process was not too time consuming. Furthermore, once the problems of preparation had been addressed, the procedure was relatively straight-forward. At first, screening was carried out on CAS agar in standard 90mm petri dishes. Only small numbers of colonies could be screened on a plate, because the rapid enlargement of the orange zone around siderophore-positive colonies complicated clear interpretation of the siderophore production status of particular colonies. The solution to this was to conduct the same screening in 96-well microtitre trays with each colony being screened in a separate well (Figure 5.5).

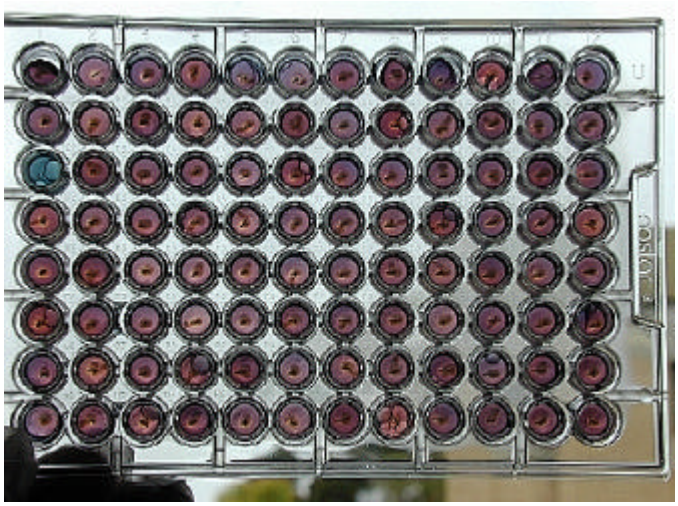


Figure 5.5 : A CAS agar 96-well microtitre tray following incubation with test strains

Use of microtitre trays also enabled more effective use of medium. The amount of medium required to fill all wells of a microtitre tray (approximately 24mL) was little more than that used in a petri dish, on which a maximum of 20 tests would be feasible because of zone spreading.

The suitability of CAS agar for differentiation between siderophore producers and non-producers was confirmed and monitored with two *E. coli* strains, one an enterochelin producer (DH1), the other (AN53N), an *entA* mutant unable to synthesise 2,3'-dihydroxybenzoate, an intermediate in the biosynthesis of enterochelin. At 25°C, the temperature at which growth of the pseudomonad colonies was tested, DH1 grew and produced a large orange zone around the colony. In contrast, growth of AN53N was limited with no orange zone evident. Similar results were observed when this experiment was repeated at 37°C. For each batch of CAS agar, the VIAS1 parent strain was included with the test strains, as a comparison. Test strains showing no orange zone or an orange zone smaller than that of the wild type, were selected for further investigation.

In most situations, complete investigation involved transfer to M9, to assess viability and prototrophy, followed by transfer to M9 agar containing 25μM EDDA, on which VIAS1 was grown concurrently for comparison. When the CAS agar used did not contain casamino acids, the isolates of interest could be transferred directly to M9 agar containing 25μM EDDA, as they should already have shown prototrophy, by their capacity to grow on that medium. In total, 15 colonies from the CAS agar in the 96-well microtitre trays were selected as potential siderophore-deficient mutants. Each of these was prototrophic and each grew just as well as VIAS1, on the iron-deficient M9 agar, suggesting they retained the siderophore producing ability of the parent strain from which they were derived.

The tranconjugants isolated in the broth mating comparison (Figure 5.3) were found to produce an extremely small orange zone on CAS agar, when screened initially by stabbing into this medium with a straight wire. However, when further tested, their growth was comparable with that of the wild type on

iron deficient M9. It was then decided to test these transconjugants (and only these) in a bioassay for haem utilisation on the same iron deficient M9. On this medium, growth of VIAS1 was strongly stimulated in the presence of haem, but no growth of the transconjugants was observed. At this stage, it was suspected that these may be haem utilisation mutants. Subsequent testing on iron sufficient M9 agar suggested that these mutants were auxotrophic.

While the failure to isolate a siderophore-defective mutant was disappointing, it should be remembered that no enrichment or specific selection for particular mutation(s) was included in the procedure. This was because none was known as the gene(s) and their product(s) have not been identified. Transposon mutagenesis supposedly creates mutants randomly. However, it is not really known how a given transposon inserts, with regard, for example, to particular affinity for a region on the chromosome, or even if such an affinity exists at all. An effect similar to that seen in this project, was experienced by Tsuda *et. al.* (1995), who were not able to isolate any mutants of interest in their tranposon mutagensis experiments. These researchers proposed that non-random insertion of the transposon was the “most likely reason” for their inability to isolate the mutant they desired. This would seem a reasonable explanation and may have been a factor in the in the experimental work described in this chapter.

All of these screening methods involved transfer of transconjugant colonies from the primary selection plate, which contained kanamycin for selection against wild type VIAS1. A potential problem with this, and the use of kanamycin for selection, is that kanamycin is bacteriostatic for *Pseudomonas* spp. This was demonstrated in the antibiotic sensitivity testing, described in the previous Section. For other organisms, kanamycin can also exert a bactericidal effect, due to accumulation in the cytoplasm of dissociated ribosomal 30S

subunits (Todar, 1996). The problem with using an antibiotic which is bacteriostatic, is that the sensitive cells are not eliminated from the population, but remain viable and culturable, and therefore are able to grow if transferred to a suitable growth medium. This could have occurred during the screening carried out in this section of work. The parent cells, remaining viable and culturable (but static) could have been transferred with transconjugant cells. In the absence of appropriate antibiotic selection, such parent cells could outgrow and mask the presence of transconjugant cells. Such a scenario would add immense confusion during attempts to purify the transconjugants. Overall, the results obtained (including auxotrophic transconjugants) did indicate that the selection used was suitable, and therefore, carryover of parent cells did not conceal the presence of transconjugants. With future work, consideration might be given to use of a bactericidal antibiotic for selection against VIAS1. Alternatively, purification of the transconjugants by streaking for single colonies on a non-selective medium prior to selection for the desired mutant phenotype could be carried out.

5.7 Attempted cloning of IROMP gene(s) by plasmid-mediated transfer from the wild type back to the IROMP mutant

A preliminary attempt was made to characterise the gene encoding the 37kDa IROMP. Full characterisation would have involved (DNA and amino acid) sequencing, and size analysis.

The first step in the attempted cloning of the gene(s) controlling the 37kDa IROMP was extraction of the genomic DNA from the wildtype strain. A UV scanning absorbance measurement of the extracted DNA was carried out from 220nm to 320nm. Absorbance measurements recorded at 260nm and 280nm were 0.2518 and 0.1587 respectively, giving a ratio of 1.59.

Following extraction, the genomic DNA of VIAS1 was digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Hin*dIII, *Sac*I, *Sal*I and *Xho*I as described in Section 2.2.14. These enzymes all have a high ligation efficiency (90%) and all recognise the phagemid vector used (pBluescript) in one location (MBI Fermentas, 2000). Use of this vector was recommended by M. Emmerling. Samples of digested genomic DNA were applied to a horizontal 0.4% agarose gel and electrophoresed, as described in Section 2.2.17. Results of the first set of digestions (Figure 5.5) indicated very few *Bam*HI recognition sites, as the bands were large, most being in the range of 20kb-30kb.

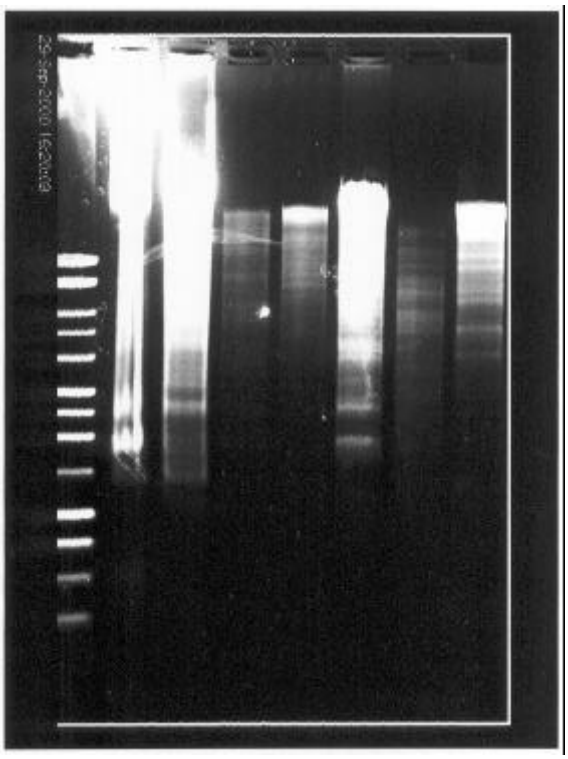


Figure 5.6 : Restriction endonuclease digestion of VIAS1 genomic DNA with six enzymes

Lane 1 : Molecular weight markers, Lane 2 : Undigested DNA, Lane 3 : Digestion with *Bam*HI, Lane 4 : Digestion with *Eco*RI, Lane 5 : Digestion with *Hin*dIII, Lane 6 : Digestion with *Sac*I, Lane 7 : Digestion with *Sal*I, Lane 8 : Digestion with *Xho*I

As this size range was unsuitable for subsequent cloning, *Bam*HI was not used further. Following further digestion of VIAS1 genomic DNA with the remaining five enzymes and visualisation of the electrophoresed fragments (Figure 5.6),

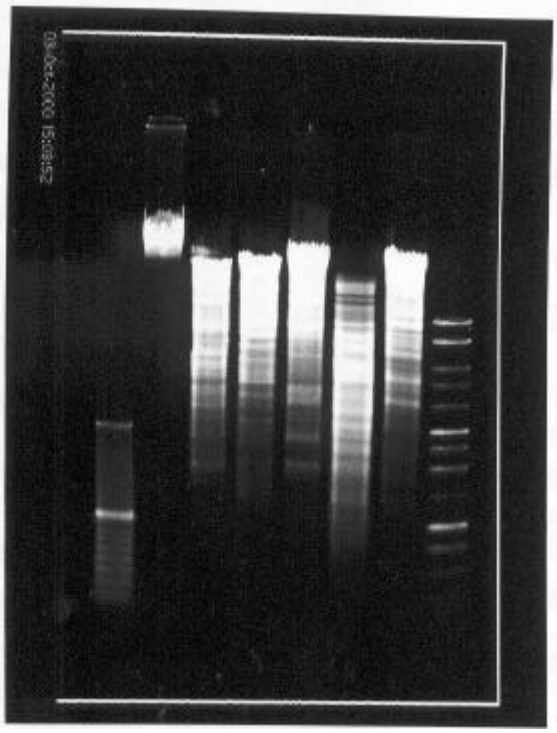


Figure 5.7 : Digestion of VIAS1 genomic DNA with five restriction endonucleases, except *Bam*HI

Lane 1 : Low molecular weight markers, Lane 2 : Undigested DNA, Lane 3 : Digestion with *Eco*RI, Lane 4 : Digestion with *Hin*dIII, Lane 5 : Digestion with *Sac*I, Lane 6 : Digestion with *Sal*I, Lane 7 : Digestion with *Xho*I, Lane 8 : High molecular weight markers

*Sal*I appeared the ideal restriction endonuclease as there were many recognition sites, but still fragments of an acceptable size. In addition, a larger range of fragment sizes were evident. A further advantage was that the largest fragments resulting from digestion with *Sal*I were smaller, than the largest fragments produced by the other enzymes.

With each of the remaining four enzymes, some DNA remained undigested but there was no such indication following digestion with *Sal*I. This can be seen in Figure 5.6 where each of the lanes (3, 4, 5, 7) containing DNA digested with *Eco*RI, *Hin*dIII, *Sac*I and *Xho*I respectively contain bands of the same size as the undigested DNA, in lane 2. This digestion was repeated, with similar results (Figure 5.7).

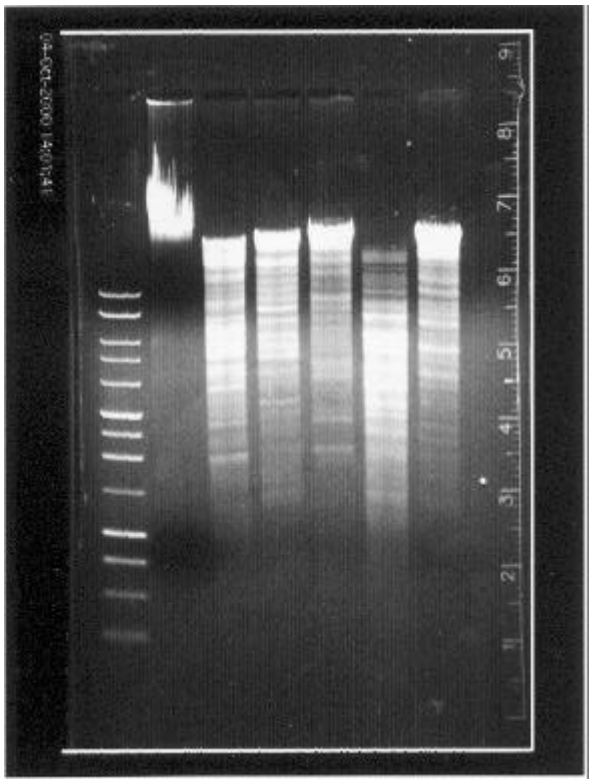


Figure 5.8 : Redigestion of VIAS1 genomic DNA with five restriction endonucleases, except *Bam*HI

Lane 1 : Molecular weight markers, Lane 2 : Undigested DNA, Lane 3 : Digestion with *Eco*RI, Lane 4 : Digestion with *Hin*dIII, Lane 5 : Digestion with *Sac*I, Lane 6 : Digestion with *Sal*I, Lane 7 : Digestion with *Xho*I

The next stage involved ligation of the fragments considered suitable, into the vector, as described in Section 2.2.16. This began with removal of parts of the gel containing bands between 1.5kb and 6.0kb in size. After discussion with M. Emmerling, this size range was thought to be an appropriate size to contain a gene encoding for a siderophore or its receptor. Once extracted from the gel, the bands of DNA were ligated into the vector, which itself, had been digested with the same five restriction endonucleases. Following ligation, any newly created phagemids (containing various fragments of genomic DNA from VIAS1) were ready to be transformed into the existing IROMP mutants.

The strain selected for transformation was Mutant B. This decision was based on earlier work (Section 3.5) which revealed that this mutant, appeared to

be “more defective” in iron acquisition than the other mutants. Prior to transformation, competency was induced in Mutant B by a chemical method (Section 2.2.18) that involving use of both $CaCl_2$ and $MgCl_2$. The efficiency of this method with *P. putida* is reported to be similar to that achieved by electroporation (Liu *et. al.*, 1996). Competent cells of Mutant B were then mixed with each of the five plasmid preparations (potentially) containing the different sized fragments generated by the five restriction endonuclease digestions. Selection for transformants was on an iron-deficient minimal agar medium (to select for the siderophore utilisation phenotype) containing 539μM ampicillin (to select for the phagemid). Two attempts were made at transforming Mutant B with VIAS1 DNA, but no transformant colonies were obtained.

These results are not totally surprising because, as discussed in Section 3.5, these 37kDa IROMP mutants appear also to be auxotrophic. Therefore, they would have had to acquire the gene for which they are auxotrophic as well, in order to grow on the selective medium. A solution to this, may have been to add (deferrated) casamino acids. However, the auxotrophic nature of these mutants was not determined in the earlier investigations and it was not known if casamino acids would have enabled growth. Overall, it was not possible to determine which part of the procedure followed was not successful. The gene of interest may have been isolated and inserted into the vector, but maybe the cells were not competent or were not transformed effectively. The reverse possibility is also possible, where the cells may have been competent but the gene not present in the vector. An additional explanation was use of an unsuitable vector. The pBluescript phagemid is a high copy number vector (Stratagene, 2002). West (1994) explicitly mentions that a low copy, or ideally a single copy vector is required when cloning iron-acquisition system genes in *E. coli*. The reason given by West (1994) is that iron-acquisition genes often contain membrane protein

genes, which, when present in a high number, may have a lethal effect on the cell. Such a situation might be expected where a high copy number vector is used. While these effects are described for *E. coli*, they could also occur in other Gram-negative bacteria, including *Pseudomonas* species. More suitable as a cloning vector therefore, would have been a low or single copy number plasmid. Examples of suitable vectors might include the following; the low copy number (less than ten copies per cell) plasmid pGA22, or the single copy number plasmid pOU71 (Pouwels *et. al.*, 1985). A much more detailed investigation would be necessary to cover all possibilities and to determine, beyond reasonable doubt, where the problem lay. Had there been time and resources, many further experiments could have been performed. In particular, transformation of the intact plasmid could have been checked. This would have demonstrated whether the cells were competent and the transformation efficiency could be calculated.

5.8 Summary

An attempt was made was to isolate mutants defective in synthesis of the putative new siderophore. Characterisation of the genes responsible was also attempted. Both attempts were unsuccessful in that the ultimate outcomes were not achieved, but were, successful in terms of progress made in this area. Much was learnt about CAS agar and a reliable method devised for its preparation and effective use. Furthermore, a reproducible method for Tn*phoA* transposon mutagenesis of *P. fragi* VIAS1 using *E. coli* SM10λ*pir*(pRT733) as the donor was developed. With methods for plasmid transfer and screening working well, it should only have been a matter of time before the mutant of interest was isolated. A full genetic and maybe protein characterisation of the siderophore synthesis and utilisation systems could then have been attempted.

CHAPTER 6

GROWTH RESPONSES OF *P. FRAGI* TO VARIOUS SIDEROPHORE PREPARATIONS, HAEM SOURCES AND OTHER POSSIBLE REGULATORS OF THE IRON STRESS RESPONSE

6.1 Introduction

Pseudomonads are able to utilise a wide range of siderophores and other means to satisfy their requirement for iron (see Section 1.10).

Earlier work in the laboratory by S. Ibro (unpublished data) produced preparations of putative VIAS1 siderophore (CAS-reactive material) purified to different extents. In the work described in this chapter, the ability of these preparations to stimulate growth of VIAS1 under iron-deficient conditions was tested. These bioassay procedures were also used to test the ability of VIAS1 and of mutants derived from it (Chapter 5), for their abilities to utilise a diverse range of potential iron sources.

6.2 Crude cell-free *P. fragi* culture supernatant

Cell-free culture supernatant (Section 2.2.12) from VIAS1 cultures grown in iron-deficient M9 containing 17μM EDDA and from cultures of VIAS1 and mutants NM1, NM2, NM3 and NM4, grown in iron-deficient M9 containing 100μM 2,2'-dipyridyl, were used. The strains used for testing of these supernatant preparations were VIAS1, NM1, NM2, NM3, NM4, NM5 and NM6. The bioassay procedure used was that described in Section 2.2.6. After incubation for 22h, zones of growth around the samples culture supernatant were small, and of low intensity. Citrate was seen to be the best stimulator of growth under these conditions of iron deficiency. (Table 6.1).

Table 6.1 : Growth zones of VIAS1 in response to crude cell-free culture supernatant and citrate

Test sample	Zone of Stimulation	Intensity of growth
100mM sodium citrate	32mm	Strong
VIAS1 supernatant from M9 + 100μM 2,2'-dipyridyl culture	18mm	Weak
VIAS1 supernatant from M9 + 17μM EDDA culture	20mm	Weak
NM1 supernatant	15mm	Weak

There was no observable stimulation of growth when a sample of 500nL rather than 30μL was inoculated directly onto the surface of the agar. No growth was observed around similar drops of culture supernatants from VIAS1, NM1, NM2 or NM3. This may have been because the volume size was too small.

6.3 C18+ semi-purified culture supernatant

The preparations of partially purified siderophore, supplied by S. Ibro, were prepared using reverse phase chromatography with a column containing C18. The column was pre-washed with 100% acetonitrile and 0.1% trichlorofluoroacetic acid (TFA). Following this, the cell-free culture supernatant was acidified to pH 2 with TFA and passed through the column. The column was washed with 0.1% TFA and the pooled washings designated C18-. Bound material was then eluted with 0.1% TFA in 100% acetonitrile and designated C18+. This C18+ material was dried by rotary evaporation and dissolved in 4mL H_2O.

In total, 33 preparations (thirty-two C18+ and one C18-) were tested for their ability to stimulate growth of VIAS1 and the IROMP mutants B, F and G. These preparations were mostly from cultures of VIAS1, but some were from cultures of mutants B, F and G. Full details of the samples are shown in Table 6.2.

Table 6.2 : Chemical and biological assay results of the C18+ and C18- preparations tested

PREPARATION NUMBER	SOURCE STRAIN	CAS TITRE (FROM S. IBRO)	SIZE OF GROWTH ZONE (MM)	
			13H INCUBATION	38H INCUBATION
1	VIAS1	1:32	8	9
2	VIAS1	1:32	2	3
3	VIAS1	No reaction	3	10
4	VIAS1	1:16	2	5
5	VIAS1	1:8	13	22
6	VIAS1	1:32	5	12
7	VIAS1	1:32	6	8
8	VIAS1	1:4	7	10
9	VIAS1	1:8	6	16
10	VIAS1	1:32	5	8
11	VIAS1	1:32	9	10
12	VIAS1	1:32	9	15
13	mutant F	1:2	2	9
14	VIAS1	1:32	6	10
15	mutant B	1:4	2	10
16	VIAS1	1:32	3	7
17	VIAS1	1:8	3	11
18	VIAS1	1:2	0	0
19	VIAS1	Not tested	0	8
20	VIAS1	1:32	5	11
21	VIAS1	1:4	11	14
22	VIAS1	1:4	8	9
23	VIAS1	Not tested	2	7
24	VIAS1	Not tested	2	7
25	VIAS1	Not tested	4	13
26	VIAS1	Not tested	0	0
27	VIAS1	1:32	8	10
28	VIAS1	Not tested	3	9
29	VIAS1	1:32	4	6
30	mutant F	Not tested	4	9
31	mutant B	1:8	4	9
32	mutant G	1:8	2	11
33	mutant B	1:4	4	10

The low-iron passaged inoculum (10^{-1} dilution) of VIAS1, mutant B or mutant F was spread onto an iron-deficient M9 agar plate containing 25μM EDDA. Samples (30μL) were tested in a well style bioassay as detailed in Section 2.2.6.

Of the 33 samples tested, only two (C18+ samples 18 and 26) failed to stimulate growth of VIAS1. Of particular interest was sample number 5. The growth zone around the well containing this sample was the most extensive and there was a small (1mm) zone of inhibition immediately adjacent to the well. The composition of the medium in which VIAS1 was grown for preparation of this

sample was unique in that glucose had been mixed with casein prior to addition to the medium with sodium succinate in the usual manner. The growth response to sample 4 was slightly less than that to sample 5 and again a slight zone of inhibition was evident. Growth around the well containing sample number 9, was intense, similar to that around sample 5, but with a smaller zone compared to that around the well containing sample 5.

Although earlier work indicated that the IROMP mutants may be auxotrophic, it was thought worthwhile to determine if they could respond to C18 semi-purified material. Mutants B and F were selected rather than mutant G, as this IROMP mutant appeared less sensitive to low iron stress. This theory was based on the results of preliminary cross feeding experiments. In these, mutant G was strongly cross fed by VIAS1. Under the same circumstances, mutants B and F did not grow. In fact, mutant G was observed to cross feed mutant F, further supporting the idea that mutant G had a higher capacity for growth under iron deprivation. The observation that mutant G responded to a compound produced by VIAS1 and, in addition, cross fed mutant F led to the suggestion that the genes for production of this growth-stimulatory compound may be associated with the genes encoding the 37kDa IROMP. This association could be geographic (for example co-location of the genes within an operon) or a common regulatory mechanism. Total results of these bioassays are shown in Appendix 3. Of the 33 samples tested, ten stimulated growth of mutant B (30%) and 11 stimulated growth of mutant F at (33%). Of the five preparations which clearly stimulated both mutants B and F, four (80%) were derived from cultures of IROMP mutants. Sample 5, which inhibited growth of VIAS1 closest to the well, exhibited the same inhibitory effect towards the growth of both mutants B and F. The finding that growth of the IROMP mutants was most clearly stimulated by their own partially purified supernatant, may indicate that the stimulatory

compound produced by the IROMP mutants being a different compound to the putative siderophore, produced by VIAS1. This is because uptake of this putative siderophore is presumed to be defective in the IROMP mutants. It is possible that the stimulatory compound is citrate. This explanation matches with all results. In low-iron medium, the IROMP mutants are stimulated by a substance which they cannot acquire through the 37kDa IROMP. Citrate is constitutively produced by *P. fragi* (Champomier-Verges *et. al.*, 1996). This may also be true for the IROMP mutants, derived from *P. fragi* VIAS1, and there is no reason to suggest that the IROMP mutants are defective in uptake and transport of citrate, into the cell.

It should be borne in mind that the procedure used by S. Ibro should have led both to purification and to concentration of components. The single sample (number 5) displaying antibacterial properties is potentially of great interest.

6.4 HPLC-purified supernatant

In order to further purify the CAS-reactive, siderophore-like material present in C18+ preparations, S. Ibro subjected it to reverse phase HPLC. Both C8 and C18 columns were used and bound material was eluted with a gradient of acetonitrile (in water). The elution profile is shown in Appendix 3, and should be consulted together with the results presented here. Also in Appendix 3 are full results of the bioassays described in this Section.

Prior to assessment of the growth-promoting activity of eluted fractions, it had to be determined whether the VIAS1 strain was capable of growing in the concentrations of acetonitrile used during elution from the HPLC column. A bioassay for acetonitrile was performed using the standard disk method on M9 agar (Section 2.2.6). At the concentrations tested, up to 100%, no inhibition of growth was evident.

The final set of fractions to be tested were from a C8 column and had been stored at 4ºC for seven months since running of the column. No information about the CAS activity of particular fractions was provided until after the bioassays had been read. The procedure used was that described in Section 2.2.6 and the results are shown in Table 6.3 for the sample volume of 30µL. Results of further bioassays, which tested the growth response of the same HPLC fractions but with variations to the test conditions, are included as Appendix

Table 6.3 : Effect of eluted HPLC fractions (sample size of 30µL) on growth of VIAS1 with 2d incubation on iron-deficient M9.

No growth stimulation			**Possible growth stimulation**	**Clear growth stimulation**			
				Weak	**Moderate**	**Strong**	
Volume eluted (mL)							
1.5	18.0	72.0	67.5	19.5	34.5	4.5	45.0
3.0	22.5	73.5		21.0	37.5	6.0	46.5
7.5	24.0	75.0		25.5	42.0	27.0	52.5
9.0	60.0	78.0		36.0	43.5	28.5	64.5
10.5	61.5	79.5		49.5	48.0	30.0	
12.0	63.0	81.0		54.0	51.0	31.5	
13.5	66.0	84.0		55.5	57.0	33.0	
15.0	69.0	87.0		58.5	76.5	39.0	
16.5	70.5	90.0		82.5	88.5	40.5	

The two areas of CAS activity (eluted volume of 30.0mL-33.0mL and 46.5mL and 48.0mL) were matched by growth-promoting activity. Some additional areas of growth promoting activity were also seen.

A second set of fractions was also tested. These were from a C18 column, to which CAS-reactive material from a C8 HPLC column, had been (re)applied. The CAS-reactive material from the fraction collected after 27mL had been subjected to electrospray mass spectrometry and found to contain a (single) species with a molecular weight of 434Da. The CAS activity of particular fractions was revealed until after the bioassays had been run and it would appear highly significant that CAS activity and growth promoting activity were both found

in the fractions collected at approximately 27mL-33mL and 47mL. These results are consistent with the view that the VIAS1-based bioassay described in this Chapter, is able to detect the putative siderophore produced by VIAS1, but also yields positive responses to other compounds. Further examination of the latter is described below.

6.5 *E. coli* siderophores and citrate

The ability of pseudomonads to utilise siderophores produced by members of other families, and other siderophore-like compounds, is of interest. Detection of receptors for utilisation of such compounds could be a novel finding. Such information could also provide insight into the niche occupied by a particular organism. For example, an organism capable of aerobactin utilisation might be expected to inhabit a location populated by pathogens, as aerobactin is a recognised virulence factor.

VIAS1 was tested by means of the well bioassay method described in Section 2.2.6. The samples tested were cell-free culture supernatants of VIAS1, NM1, NM2, NM3 and NM4, prepared from cultures grown under iron-deficient and iron-sufficient conditions. Other samples investigated were iron(III) chloride, sodium citrate, DHB (supplied by M. Wilkins) and *E. coli* crude cell-free culture supernatants likely to contain enterochelin (from AN194) or aerobactin (from RJ80). Growth was poor around the well containing 30μL DHB with growth only visible after approximately 40h. For other samples, when small sample volumes were used, growth was noted around the well containing the iron(III) compound and around the cell-free culture supernatants presumed to contain enterochelin and/or aerobactin. When this experiment was repeated, with a much larger volume of sample, identical samples stimulated growth. The growth stimulation was present after 16h and very clear after 25h, with zones of approximately

12mm for each sample, extending from the edge of the well. Utilisation of the iron(III) compound was expected, because iron is most commonly acquired by this organism as Fe^{3+}. Utilisation of enterochelin has been described for *P. fragi* (Champomier-Verges *et. al.*, 1996), but of most interest was the observation of possible aerobactin utilisation. Such utilisation by *P. fragi* has been mentioned in the literature (Labadie, 1999), but there no experimental evidence is available. Stimulation of growth by citrate only occurred when the larger sample volume was used. Taken together, these results suggest that VIAS1 expresses outer membrane receptors for iron(III) complexes with citrate, aerobactin and enterochelin. This ability to utilise structurally diverse siderophores is however not altogether uncommon, among *Pseudomons* spp. (Ongena *et. al.*, 2002). Both cell-free culture supernatants presumed to contain aerobactin or enterochelin were strong growth stimulators under iron-deficient conditions. It is not really possible to compare the effectiveness of exogenous (aerobactin, enterochelin) and endogenous siderophores because no attempt was made to quantify the concentration of siderophore in each sample.

Such utilisation of exogenous siderophore is worthy of comment. Of particular interest was utilisation of DHB by VIAS1. Based on similar experiments with *E. coli*, this would suggest that VIAS1 is able to convert DHB into a utilisable siderophore, such as enterochelin. Alternatively, DHB may function as a siderophore in its own right. A general explanation for exogenous siderophore utilisation may be that VIAS1 produces more receptors for uptake of iron complexes with exogenous siderophores as an energy-saving device and/or to gain access to iron which would normally be unavailable. Such siderophores may be present in the growth medium at a higher concentration (compared to endogenous siderophores) that they are utilised preferentially. Such preferential use may occur early, when few cells are growing. When the initial cells have

acquired enough iron by means of aerobactin/enterochelin, they may then switch to their own siderophore uptake system, and continue strong growth. Evidence of such a switch from exogenous to endogenous siderophore would probably not be detected in the growth curve. Although, if enough of the population were to undergo such a hypothetical switch of siderophore utilisation, a flattening of the growth curve, analogous to the well-known growth response for detection of a switch of carbon source utilisation by a culture, may be seen. Another explanation for ongoing utilisation of aerobactin/enterochelin is that those cells that are able to utilise these siderophores might commence growth early. Those cells that can efficiently utilise exogenous siderophores, compared to those that cannot, may have a selective advantage. Such a selective advantage could lead to development of a population of cells that have a high efficiency of aerobactin/enterochelin utilisation. A diversity of iron acquisition systems is not uncommon for meat spoilage pseudomonads (Labadie, 1999).

Also investigated, was the possibility that exogenous siderophore and/or citrate might stimulate growth of mutants NM1, NM2, NM3, NM4, NM5 and NM6. In the first instance, citrate and cell-free culture supernatants presumed to contain aerobactin or enterochelin, were tested. This experiment was conducted three times, and on all occasions, the cell-free culture supernatants presumed to contain aerobactin and enterochelin were better stimulators of growth than was citrate. Cell-free culture supernatants presumed to contain aerobactin consistently stimulated all strains. Cell-free culture supernatants presumed to contain enterochelin gave somewhat mixed results, but overall, were more stimulatory under these particular conditions of iron deficiency than was citrate, which was very limited in its ability to stimulate the growth of these strains. Negative controls (consisting of uninoculated medium) showed no growth. Results are summarised in Table 6.2. Such a finding that exogenous

siderophores and citrate can satisfy the iron acquisition requirements of *P. fragi* VIAS1 are in agreement with previous work (Champomier-Verges *et. al.*,1996).

Table 6.4 : Growth stimulation of VIAS1 and its mutants by various sideorphore preparations after overnight incubation

	RJ80 culture supernatant	AN194 culture supernatant	100mM sodium citrate	Homologous supernatant
VIAS1	4	4	4	1
NM1	1	2	0	0
NM2	3	3	1	0
NM3	1	1	0	0

Measurement is on a 1-5 scale with 1 representing light growth and 5 representing heavy growth

6.6 Haem iron acquisition

The previous section details the ability of VIAS1 to utilise a range of exogenous siderophore preparations and other compounds as sources of iron. Champomier-Verges *et. al.* (1996) have observed that the list of compounds that can serve as iron sources for *P. fragi* includes haemoglobin. In addition, Labadie (1999) has noted that haemoglobin strongly stimulates growth. Previous work in this laboratory (Wilkins, 2001) found that VIAS1 and a range of other pork spoilage isolates were able to utilise haem iron. Given these reports, the ability of VIAS1 to respond to haemoglobin and haem was tested in a well style bioassay (Section 2.2.6). The medium used for the haem-iron utilisation bioassays was as in previous bioassays (Section 2.2.6). The bioassay was used with samples of haemoglobin (0.1%) and haemin (10mM).

Clear utilisation by VIAS1 of both haemolglobin and haemin, confirmed the previous reports of haem-iron acquisition. Furthermore, each of the NM strains displayed a haem acquisition ability that was the same as their parent, as judged from the extent and intensity of growth surrounding wells containing sources of haem. This would suggest that the haem-mediated iron acquisition mechanisms

of these mutants were fully functional, or at least, each of the mutants displayed a fully functional phenotype. The ability of the IROMP mutants (B and F) to utilise haem-iron as a sole source of iron was also tested. No utilisation could be detected by either of these mutants. However, the medium used was an M9 (without deferration and without acid-washed glassware), without supplements, such as casamino acids. Therefore, based on previous observations (Section 3.5), absence of growth was probably due to auxotrophy rather than inability to acquire sufficient iron for growth.

6.7 Inducible acid tolerance : Relationship to regulation of iron uptake

Inducible acid tolerance refers to the ability of an organism to induce an acid tolerant state through adaptation at a sub-lethal pH, which subsequently enables survival or growth at a lethal pH. The Fur protein, a major factor in overall regulation of iron uptake, has been found to be influential in the development of an enhanced acid tolerant state in enteric bacteria. This aspect of inducible acid tolerance has been reviewed by Foster (1995).

In view of such findings, it was postulated here that inducible acid tolerance may have an effect on siderophore production by VIAS1. Therefore, a limited number of experiments to test this hypothesis were carried out as described in Section 2.2.9. In addition, an assessment was made of the role of inducible acid tolerance on the ability to acquire iron. This was determined on an iron-deficient M9 agar. Overall, no induction of enhanced acid tolerance was observed and therefore, the ability of those same cells to acquire iron was unchanged (Figure 6.1).

The results of this section were not totally unexpected, since inducible acid tolerance has not been described in any *Pseudomonas* species. As a result, the effect of inducible acid tolerance on iron acquisition could not be determined.

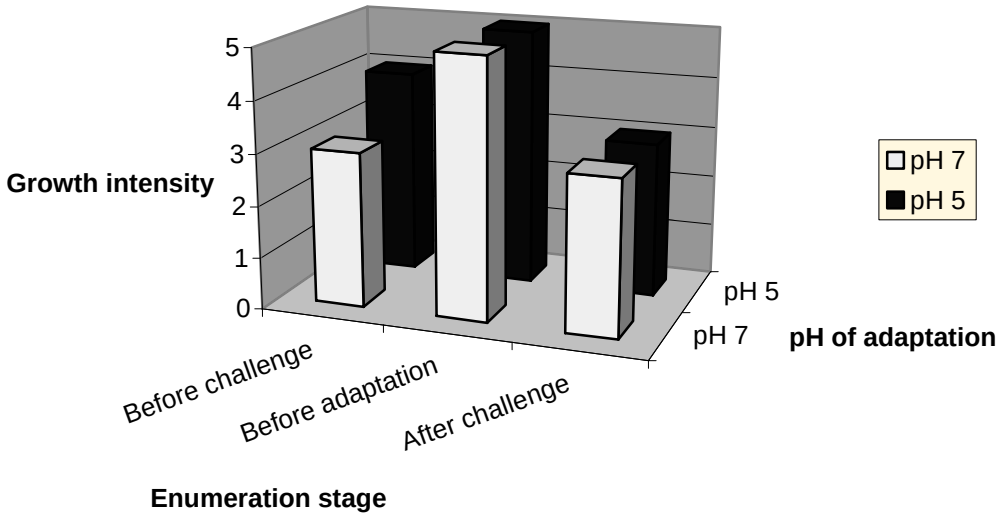


Figure 6.1 : Response of VIAS1 to low pH with and without prior adaptation at an intermediate pH

6.8 Summary

An objective of this project was the development of a bioassay for the apparently novel siderophore produced by the pork spoilage isolate, *P. fragi* VIAS1. Preliminary purification and chemical characterisation had been carried out previously, by S. Ibro, and the work presented in this chapter, was designed to determine whether this purified CAS-reactive material was able to stimulate growth of VIAS1. It was found to do so. Not all material which stimulated growth of VIAS1, was able to stimulate growth of mutants B and F. Cell-free culture supernatant from the NM strains did not stimulate the homologous NM strain. Strong, and relatively consistent growth of both VIAS1 and the NM strains, was noted in response to cell-free culture supernatant of *E. coli*, presumed to contain aerobactin. Cell-free culture supernatant, presumed to contain enterochelin was less stimulatory than was cell-free culture supernatant presumed to contain aerobactin, but was still more stimulatory than citrate. At the concentration used, stimulation by citrate was weak and inconsistent between strains.

VIAS1 and all NM strains tested were able to utilise haem compounds as a sole source of iron.

The results in this chapter demonstrate the diverse nature of iron acquisition in these pseudomonads. Also, further evidence is presented for the existence of a novel siderophore, the biological properties of which, include stimulation of growth of the wild type, under iron-deficient conditions, indicating that the compound, is capable of functioning as a siderophore.

CHAPTER 7

SIDEROPHORE-MEDIATED IRON ACQUISITION BY OTHER BACTERIA AND A FUNGUS

7.1 Introduction

Siderophore production is common in Gram-negative and Gram-positive bacteria, and in fungi. Bacteria and fungi produce many different siderophores and as discussed earlier (Section 1.10), siderophore from one organism can be utilised by another organism, even when they are of different domains. Furthermore, there are a number of methods by which microbial iron acquisition is possible (Section 1.6). Some of these may appear surprising, such as the presence of a system to acquire haem iron in *Bradyrhizobium japonicum*, a soil bacterium (Nienaber *et. al.*, 2001). The work described in this chapter was focussed on selected siderophore-mediated iron acquisition mechanisms in a diversity of Gram-positive and Gram-negative bacteria, as well as a fungus, *Botrytis cinerea.* This fungus is a major field and post-harvest plant pathogen (Hardy, 2001) and has been isolated from a wide range of economically important plant species (Leone, 1990). It has been shown to acquire iron by means of siderophores (Konetschny-Rapp *et. al.*, 1988). The section of work involving *B. cinerea* was undertaken in collaboration with C. Di Biase.

7.2 Cultures

Work described in this chapter involved strains detailed earlier (Section 2.2) and other strains described in Table 7.1.

Table 7.1 : Organisms used solely for work described in this chapter

Species	Strain (if known/applicable)	Source/reference
Bacillus cereus	Unknown	Teaching collection, Department of Microbiology, Monash University
Bacillus subtilis	BG1	Stasinopoulos *et. al.* (1998)
Botrytis cinerea	Unknown	T.V. Price, Department of Agricultural Sciences, La Trobe University
Klebsiella mobilis	MPS1	Isolated in this laboratory from a commercial pH 0-14 indicator solution
Micrococcus luteus	Unknown	Teaching collection, Department of Microbiology, Monash University
Pseudomonas aeruginosa	9305	V. Stanisich, Department of Microbiology, La Trobe University
Pseudomonas fluorescens	B52	Richardson (1981), Richardson and Te Whaiti (1978)
Shigella flexneri	Dv770/5	R. Haigh, Department of Genetics, University of Leicester
Streptococcus pyogenes	Unknown	Teaching collection, Department of Microbiology, Monash University

7.3 Bioassay for enterochelin production

For detection of enterochelin and/or DHB-related compounds, the method described in Section 2.2.6 was used. As some of the strains tested, were unable to grow at 37°C, the temperature of incubation was 25°C. Four strains produced results consistent with the production of enterochelin and/or DHB-related compounds. As expected, these included the *E. coli* strains DH1 and SM10λ*pir*(pRT733) as well as *K. mobilis* MPS1. *B. subtilis* BG1 also stimulated the growth of the indicator strain. While this could reflect production of enterochelin, a likely possibility is DHB because enterochelin has not been isolated from *B. subtilis*. An intermediate in *E. coli* enterochelin biosynthesis, DHB, has been identified as a siderophore of *B. subtilis* (Grossman *et. al.*, 1993). Production of the related compound, 2,3-dihydroxybenzoylglycine (itoic acid), by *B. subtilis* has also been reported to function as a siderophore (Ito & Neilands, 1958). The stimulation of AN193, observed around the growing culture of BG1, would be consistent with production of DHB and/or related compounds. AN193 is able to utilise exogenous DHB (Section 3.2) to synthesise enterochelin and

therefore, would be expected to grow in the presence of an organism secreting DHB.

7.4 Bioassay for aerobactin production

Ability to produce aerobactin was assessed with LG1522 as the indicator strain. Initial trials were conducted at 25°C because some of the strains tested grow optimally at this temperature. However, this incubation temperature was not suitable because growth of the indicator strain was poor and results with the control strains were not as expected. It was then decided to incubate the plates at 37°C, and to assay the cell-free culture supernatant, rather than the actively growing cultures from strains that were unable to grow at 37°C. Clear results were obtained. The only cultures, for which aerobactin production status was unknown, which yielded results consistent with aerobactin production, were *E. coli* SM10λ*pir*(prt733), *B. subtilis* BG1 and *K. mobilis* MPS1. Aerobactin production of each of these strains is of interest. *E. coli* SM10λ*pir*(pRT733) is a laboratory strain and would not be expected to produce aerobactin. Overall, very few *Klebsiella* spp. produce aerobactin (Reissbrodt & Rabsch, 1988). However, this result is not of special interest because while few species produce aerobactin, environmental species are just as likely to produce aerobactin as are other isolates. Aerobactin production by *B. subtilis* has not been reported. The work in this chapter did indicate, by bioassay, that *B. subtilis* BG1 is an aerobactin producer. However, further tests would be required before production of aerobactin could be confirmed. Hydroxamate siderophores have been reported in *B. subtilis* (Byers and Lankford, 1968) and *B. megaterium* (Byers *et. al.*, 1967), as well as the catechol compounds. Following the indication that BG1 may be an aerobactin producer, an attempt was made to grow BG1 in an iron-

deficient liquid M9. The purpose of this attempt was to collect the culture supernatant and determine by chemical assay (Csaky assay) the presence of hydroxamate compounds, and then to test specifically for aerobactin, by means of a bioassay. The attempt was unsuccessful as the organism did not exhibit any obvious growth in the medium.

A number of liquid samples were also tested in parallel. Those that were shown to stimulate LG1522 were sodium citrate, an iron(III) solution and supernatant from *E. coli* RJ80, the positive control.

7.5 Siderophore production by *Botrytis cinerea*

Confirmation of siderophore production by *B. cinerea* was carried out on CAS agar, inoculated by C. Di Biase. On this medium, the fungus grew slowly, with a small orange zone the result. A zone, orange in colour on CAS agar, is an indication of trihydroxamate production (Milagres *et. al.*, 1999). This demonstration of trihydroxamate production is in agreement with the findings of Konetschny-Rapp *et. al.*, (1988).

7.6 *Streptococcus pyogenes* and siderophore production

Results obtained from the tests described above, are inconclusive with regard to the siderophore production status of *S. pyogenes*. One reason is that growth was difficult to detect visually, on the bioassay medium. It may be possible that *S. pyogenes* and *E. coli* (and many Gram-negative organisms) differ in their requirements of a chemically defined medium. Previous investigations have failed to demonstrate production of siderophores by *Streptococcus pyogenes* or indeed any *Streptococcus* species (Eichenbaum *et. al.*, 1996; Evans *et. al.*, 1986; Memon and Kazi, 1994) or *Enterococcus faecalis* (Marcelis *et. al.*, 1978), previously classified as *S. faecalis*. Presumably they acquire iron by other

means, if they require iron at all. Most *S. pyogenes* are haemolytic (Baron *et. al.*, 1994) with high levels of haemolysin production occurring under iron-restricted conditions (Griffiths & McClain, 1988). It may be, therefore, that haem-iron acquisition is important in this species. Evans *et. al.* (1986) demonstrated that *S.* mutans acquires the metal as iron(II) and it is possible that acquisition of iron(II) rather than acquisition of iron(III) is usual in this genus.

7.7 Summary

A range of Gram-negative and Gram-positive bacteria and a fungus, were analysed for the presence of siderophore-mediated iron acquisition mechanisms. Results obtained with *B. subtilis, E. coli* SM10λ*pir*(pRT733) and *K. mobilis* MPS1 were consistent with production of both aerobactin and enterochelin or other DHB-related compounds. *E. coli* DH1 was found to produce enterochelin but not aerobactin.

CHAPTER 8

GENERAL DISCUSSION

8.1 Methodology

8.1.1 Reduction of contaminating iron levels in media

A reduction in the level of available iron in the media and glassware is absolutely necessary for any studies on siderophore production. In some situations, and with some species, the iron concentration needs to be kept extremely low for siderophore production. For example, repression of azotobactin synthesis is reported to occur when the concentration of iron in the medium rises to just 3μM (Tindale *et. al.*, 2000). In contrast, production of pseudomonad siderophores appears optimal when the iron concentration is 1μM-3μM (Prince *et. al.*, 1993). Minimal, chemically defined medium, generally has an iron content in the range 0.5μM to 3μM, mainly as a contaminant from the components of the medium (Weinberg, 1974).

Researchers initially investigating in the area of microbial iron acquisition employed 8-hydroxyquinoline extracted with chloroform to deplete media of available iron (Garibaldi & Neilands, 1956; Waring & Werkman, 1942). Many other methods are presently available to reduce iron levels in media. During preparation of media used during the work described in this thesis, four methods were used to reduce availability of iron to a level compatible with strong siderophore production. These were; I) acid washing of glassware, II) deferration of media, (III) use of high quality water and IV) use of an iron chelator (2,2'-dipyridyl or EDDA). Utilisation of the first three methods allowed the level of chelator to be kept lower than it would otherwise have been. This has many advantages including reduced cost, reduced danger of toxicity for the organisms

under study, and less hazard for the laboratory worker. This is particularly important for 2,2'-dipyridyl, which is reported to be mutagenic and possibly carcinogenic (Kuo & Lin, 1993; Lin *et. al.*, 1989). In non acid washed glassware, iron continually leaches out, particularly from the deeper layers. Soaking the items in concentrated acid for a period of time reduces this. Many acids can be used for the washing of glassware. Nitric was the acid of choice, and was used throughout this project because it "poses the least interference with subsequent fluorometric analyses for aromatic compounds" (Cox, 1994) and nitrates tend to be soluble (R.K.J. Luke, pers. comm.)

One of the most effective means of iron reduction is treatment with Chelex-100. For example, Davey *et. al.* (1970) reported a 92% reduction of iron in culture media through its use. A potential disadvantage is that metals other than iron are also chelated by Chelex-100. The work of Davey *et. al.* (1970) demonstrated that Chelex-100 was just as effective in reducing nickel concentrations, and was even more efficient at reducing some other metals; 95% of lead was removed and 99% of zinc, cadmium, manganese and copper. Literature provided with the product (Bio-Rad Laboratories, 1972) shows that there is a much higher preference for heavy metals. Fortunately, the metals other than iron which are chelated most strongly (such as copper and lead) are usually only required for bacterial growth in minute concentrations or not at all. Another aspect to consider when Chelex-100 is used, is how alteration of metal levels in the medium might affect siderophore production. An investigation by Visca *et. al.* (1992c) showed that pyoverdine production was affected by concentrations of other metals. For example, pyoverdine production was substantially reduced when cobalt(II) was present, even in small amounts, such as 5μM. Conversely, manganese(II) at a relatively high level (100μM) had virtually no effect.

Iron chelators are very effective in reducing iron availability. Their efficiency is such that they may be used as the sole method of reducing iron availability. This is true not only in minimal media, but even in complex media such as nutrient and Luria-Bertani agars. In fact, many researchers use these chelators in complex media, with no other means of reducing iron availability. As discussed above, a consequence of this approach is the need to use a large amount of chelator. Concentrations of EDDA required in minimal medium might be as large as 2.8mM (A.G. Brown, pers. comm). In contrast, in this project, EDDA concentrations between 15μM and 25μM were found to be effective, when used in combination with other methods for reducing iron levels. Overall, it is recommended that all available means for reduction of iron be used, so as to reduce the reliance on potentially hazardous and expensive iron chelators.

8.1.2 Effect of carbon sources on growth and iron availability

In terms of iron starvation, choice of carbon source can be very important, with some carbon sources “invoking a higher demand for iron” than others (Neilands, 1982). Glucose was often used as the carbon source in the CAS agar and minimal media. However in general, glucose generally decreases the cell’s requirement for iron, thereby decreasing production of siderophore (Payne, 1994): in particular glucose does not increase pyochelin production (Ankenbauer *et. al.*, 1986). During the work described in this thesis, carbon sources other than glucose (sodium succinate and sucrose) were incorporated into the various media, but because spoilage pseudomonads prefer glucose as a carbon source (Jackson *et. al.*, 1997), this was the carbon source used most commonly.

8.1.3 Citrate as a carbon source and siderophore

Citrate is often used as a carbon source in minimal media but its effects were not investigated here. This is because citrate can act as a siderophore, transporting iron to the cell (Frost & Rosenberg, 1973) and then actively across the cell membrane (Williams & Warner, 1981) therefore reducing or even eliminating the need for siderophore production. Although common as a mediator of iron acquisition, it is absent from some organisms, such as *Salmonella enterica* serovar Typhimurium or *Shigella flexneri* (Payne, 1988).

8.1.4 CAS agar

CAS agar was developed by Schwyn and Neilands (1987). The uniqueness and ease of interpretation behind the test system lies in the striking colours. When the plates are prepared, CAS agar should be blue. This is due to the chrome azurol S dye. During preparation of the medium this dye forms a complex with an added iron(III) solution. Addition of HDTMA to this mixture enables a more stable complex formation. The deep blue colour, changes to an orange when the iron(III) is removed from the complex, such as can occur in the presence of a siderophore or other chelating agent like EDDA.

Schwyn and Neilands (1987) described this as a “universal” assay for siderophore production. However, the number and diversity of papers describing modifications to the CAS agar for adaptation to the requirements of different organisms (Abdallah, 1998; Alexander & Zuberer, 1991; Buyer *et. al.*, 1989; Gram, 1996; Manninen & Mattila-Sandholm, 1994; Milagres *et. al.*, 1999; Shin *et. al.*, 2001) suggest that, in its original form, it has limited applications. The major modifications to the original method are to allow for the testing of Gram-positive bacteria and many fungi. This is because HDTMA is toxic to a large number of these organisms (Smith, 1998).

The main concern with initial preparation of CAS agar was its colour. In addition to various shades of blue, different shades of green and even brown were observed in this, and other (Alexander & Zuberer, 1991; A.G. Brown, pers. comm.) studies. During the work described in this thesis, it was imperative that the concentrations of the CAS agar components be kept constant. Very small variations were sufficient to result in a different colour of the agar. Consistency was not easily achieved when following the original Schwyn and Neilands (1987) method. The method of Alexander and Zuberer (1991) was found to produce more consistent results and the resulting CAS agar was found to be suitable for all applications and organisms for which it was used. Most of the organisms tested were Gram-negative bacteria, but the plant pathogenic fungus *Botrytis cinerea* was also found to grow and produce a coloured zone consistent with siderophore production.

Virtually all papers describing a CAS agar reaction indicate the colour change is from blue to orange. One study (Milagres *et. al.*, 1999) has found a distinction between the colour observed in the presence of hydroxamate (orange or reddish-orange) and catechol (reddish-purple) siderophores. In liquid medium, ferric-catechol complexes can be a red (Young, 1976) or purple (Payne, 1994) colour. Despite the number of studies which describe a universal orange colour in response to siderophore production, results obtained in this project, would seem to support the “two-colour detection system”. Enterochelin-positive strains clearly produced an orange zone while *B. cinerea*, which produces only hydroxamate siderophores (Konetschny-Rapp *et. al.*, 1988) produced a reddish-purple halo. If the results of (Milagres *et. al.*, 1999) are confirmed, and CAS agar can be used for discrimination between catechols and hydroxamates, the spectrum of applications for CAS agar should increase.

Although not used extensively for testing purposes, the CAS agar technique of Milagres *et. al.* (1999) was found to be excellent. Their method is truly universal. This is because they use a “half and half” technique which places the CAS agar in one half of the plate, and a medium preferred by the organism being tested on the other half. They used complex media such as malt extract agar (MEA) or NA as the preferred medium. However, such a medium would seem inappropriate for siderophore production. Therefore, a modification was made which altered the complex medium to an iron-deficient M9, containing 2,2’-dipyridyl. This medium was used as it is known to induce good siderophore production. The result was a clear indication of siderophore production (Figure 8.1).

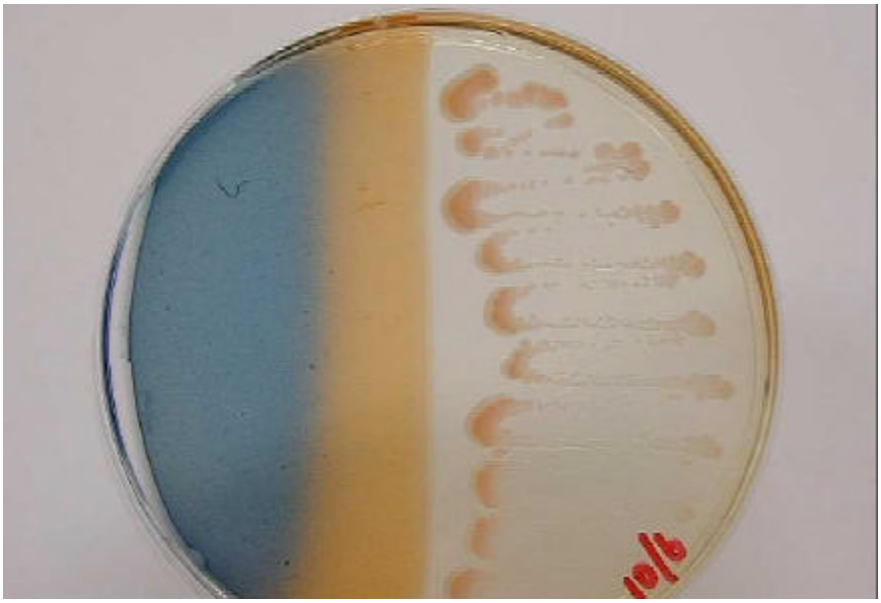


Figure 8.1 : An indication of siderophore production (orange zone on blue CAS agar) by *E. coli* O18:H7 strain MW when grown on iron-deficient M9.

8.2 *E. coli* AN102 is a *fepC* mutant and comparisons with AN273

Initially (Cox *et. al.*, 1970), when strain AN102 was isolated, only one *fep* mutation was known. Later, other *fep* genes and Fep proteins were discovered (Pierce *et. al.*, 1983; Pierce & Earhart, 1986), and a model of ferric-enterochelin

transport into the cell developed. The *fep* mutation of strain AN102 has been reported to be *fepA* (Wookey & Rosenberg, 1978), *fepB* (Pierce *et. al.*, 1983), *fepC* (Chenault & Earhart, 1991; Payne, 1994; Payne, pers. comm.; Pierce and Earhart, 1986) or *fepD* (Ozenberger, 1987).

Pierce *et. al.* (1983) have stated explicitly that AN102 is not a *fepA* mutant. They based their conclusion on complementation studies, ferric-enterochelin binding data, and the observation that AN102 is sensitive to colicin B, and suggested the strain to be a *fepB* mutant. However, Young (1976) has stated that ferric-enterochelin is a deep red colour. If AN102 were to be a *fepB* mutant, then ferric-enterochelin should accumulate in the periplasm and it could be reasonably expected that such an accumulation might result in at least some red colouration of the cells. However, this was not observed. Perhaps only low levels of ferric-enterochelin are able to accumulate. Such an explanation is consistent with an aspect of the growth of AN273, a *fes* mutant, under the same conditions. In iron-supplemented minimal medium, this strain has the same phenotype as *fepC* mutants but for a different reason. AN273 is able to transport ferric-enterochelin through the cell membrane and into the cytoplasm. Here, it accumulates because the absence of Fes results in an inability to remove the iron from the ferric-enterochelin complex. The observation of the cell pellet of this strain was the same as for AN102, which was no red colour. For both AN102 and the AN273 used as part of this work, intracellular accumulation might be at a low concentration, or at least a concentration that is too low to observe. Although AN102 is a ferric-enterochelin transport mutant, it could still accumulate ferric-enterochelin intracellularly. This is because AN102 has been noted by S.M. Payne (pers. comm.) to contain a "leaky" ferric-enterochelin transport system. Unlike with AN102, an absence of a red coloured cell pellet of AN273 under these conditions was unexpected. Greenwood (1979) clearly observed a cell pellet, red

in colour, once the culture had entered stationary phase, but in this work, the pellet was clearly white with a red coloured supernatant. Such a phenotype would appear to be a combination of strains AN273 (red cell pellet and red supernatant) and AN273-1 (white cell pellet and colourless supernatant), a *fer* mutant derivative of AN273 isolated by Greenwood (1979).

Complementation studies with plasmids containing various *fep* genes have been conducted by Ozenberger *et. al.* (1987). From the activation/inactivation of particular genes and combinations of genes and the ability/inability of the organism to grow under iron deficient conditions, it seemed relatively clear that AN102 is a *fepD* mutant. The most convincing case however, is for a mutation in *fepC*. This was demosntrated by Pierce and Earhart (1986) and confirmed by Chenault and Earhart (1991). Their work involved introduction of plasmids containing either functional *fepB* or *fepC* genes into AN102. The earlier study (Pierce & Earhart, 1986) described transformation of AN102 with a plasmid with a functional *fepB*, but containing a transposon insertion into *fepC*. This resulted in no growth under conditions of iron deprivation. Later (Chenault & Earhart, 1991), a fully functional *fepC* gene was introduced into AN102. This resulted in the return of the wild type growth phenotype under iron deficiency.

8.3 Meat spoilage control of pseudomonads

There are many methods available for food preservation. These include bacteriocins, natural antimicrobials from plants/animals, irradiation, microwaves, hydrostatic pressure, heat, ultrasound, electrical resistance heating, high voltage pulse techniques, surface treatment with organic acid, modified atmosphere packaging and aseptic processing (Gould, 1995). For meat, drying and low temperature are the more traditional methods with others of emerging interest being vacuum packaging, modified atmosphere and irradiation (Egan and Shay,

1988). Some are more effective than others and each can affect food in a characteristic manner. One of the most effective, safest and least damaging to food at effective concentrations is irradiation. Irradiation at low doses is particularly effective in reducing the number of pseudomonads, with 1kGy completely inhibiting growth (Dogbevi *et. al.*, 1999; Dogbevi *et. al.*, 2000). Although pork is the second most sensitive meat to irradiation treatment (Sudarmadji & Urbain, 1972) the low doses of irradiation required for inhibition of pseudomonads makes it a good choice for elimination of spoilage pseudomonads. This is particularly so as little change has been found in the sensory quality of pork, even when irradiated at a high level (Hannan & Thornley, 1957). For lengthening the shelf life of pork, radiation is especially effective. A dose of 0.5kGy, resulted in an increase in the shelf life of aerobically stored pork from 11d to 27d (Venugopal & Dickson, 1999). Provided guidelines, regulations and other requirements are adhered to, food treated with irradiation is safe, nutritious and does not resulted in the production of toxic compounds (Shea *et. al.*, 2000). Consumer and industry acceptance of irradiation as a means to treat food to reduce microbial load, is generally poor (Noy, 2001), despite the many advantages.

Due to the strictly aerobic metabolism of *Pseudomonas* species, modified atmosphere packaging (MAP) can be an effective control method. An atmosphere where CO_2 predominates has been found to effect much inhibition of pseudomonad spoilage organisms (Fang & Lin, 1994).

A potentially promising method for controlling pathogens in food has been suggested by Cheng *et. al.* (1995). These authors see a possible use for siderophores from *P. fluorescens* as a control method. Inhibitory effects (based on siderophore production) by other *Pseudomonas* species (*P. chlororapis*) have been demonstrated against important foodborne bacteria (*Salmonella enterica*

serovar Typhimurium) and fungi (*Aspergillus niger*) (Laine *et. al.*, 1996). This indicates that siderophores may be able to play a role in the control of foodborne microorganisms. Cheng *et. al.* (1995) also propose a use for the *P. fluorescens* organism itself in biocontrol. Being a key spoilage organism, such an idea would seem inappropriate, as spoilage bacteria are a major problem in food, as are pathogenic bacteria.

A practical application for the extension of the work described in this thesis could be the generation of monoclonal antibodies. These might be specific for the putative siderophore or for its IROMP receptor. Inactivation of either of these key components of the siderophore-mediated iron acquisition system might be expected to result in a substantial reduction in growth. This principle has been exploited by le Roy *et. al.* (1992) who substantially reduced the growth of an aerobactin producing *E. coli* in calf serum with a monoclonal antibody (mAb) specifically targeting aerobactin. Smith *et. al.* (1991) prepared mAb directed against a *P. aeruginosa* IROMP, and this approach could have been used in this work. Although an excellent idea in principle, they observed only low-level binding, which they suggest, is due to masking of binding sites on the IROMP by lipopolysachharide. A substantial increase in shelf life might be achieved, with mAb specific for the siderophore of interest or IROMP, provided high-level binding was possible. In practice, it is difficult to see how mAb would increase shelf life. This is because pork spoilage organisms mainly originate from the processing environment (Blixt & Borch, 2002) and therefore the mAb would be of limited use. An alternative approach may be the production of antibody by the animal following injection with IROMP as antigen. As *Pseudomonas* spp. can utilise various exogenous siderophores, there would be the need to use antigen representing many siderophores.

Such novel means to control spoilage organisms are required, as methods currently in use do not solve all problems. While current procedures tend to be adequate for meat marketed domestically, they are less satisfactory for most international markets. If shelf life could be increased and predicted more consistently, this may further international competitiveness of the Australian pork industry.

8.4 Virulence of recognised spoilage *Pseudomonas* spp.

Of the many species of *Pseudomonas*, only one, *P. aeruginosa*, is frequently associated with human disease. However, many other *Pseudomonas* species have been isolated from clinical material and in fact, most of the species, widely known and recognised as chief meat spoilers have been clearly implicated in sporadic cases of various diseases. These include pneumonia (Fujita *et. al.*, 1998) and septicemia (Macfarlane *et. al.*, 1991). The seriousness of such situations may increase dramatically, should the implicated pseudomonad possess multiple antibiotic resistance genes. This was found to be true in the two cases mentioned above and in fact, the wild type strain (VIAS1) used in this work was observed to exhibit multiple antibiotic resistance. Such a result is not of special interest. This is because among pseudomonads, at least *P. aeruginosa* is resistant to many antimicrobial compounds (Arakawa *et. al.*, 2000) so other *Pseudomonas* spp. may contain multiple antibiotic resistance genes. However, one of those antibiotics (vancomycin) to which VIAS1 was resistant, is of current interest because of the continuing clinical importance of vancomycin resistant bacteria (mainly *Enterococcus* spp.), particularly in nosocomial infectious (Fluit *et. al.*, 2001). Although an unlikely scenario, the possibility exists for pseudomonads from spoiled food to result in a nosocomial infection. The problem would be amplified if the isolate possessed unusual and/or high-level antibiotic resistance.

It is unknown whether the VIAS1 strain is a human pathogen, but if it is, its antibiotic resistance profile would prove a major problem in its control, as exemplified by the case described by Macfarlane *et. al.* (1991). They treated a patient with the two antibiotics found to be effective *in vitro*, but the disease did not regress and the organism persisted. Upon death of the patient, *P. putida*, an important spoilage pseudomonad, was recovered from its original *in vivo* location, indicating the ineffectiveness of the antibiotic treatment.

CHAPTER 9

CONCLUSION AND RECOMMENDATIONS

9.1 Conclusion

Prior investigations in conjunction with this laboratory identified a putative new siderophore from a pork spoilage *P. fragi* and provided some degree of characterisation, mainly of a chemical nature. During this project, biological and genetic investigations were carried out with a view to further characterising the putative siderophore. The main approach used was a bioassay, developed initially for aerobactin and enterochelin production, then applied to investigation of the putative new siderophore. Some preparations presumed to contain siderophore were found to stimulate growth of both wildtype and IROMP mutants, under iron-restricted conditions. These results are consistent with the chemical evidence for the presence of a siderophore. Furthermore, VIAS1, and various mutants derived from it, utilised exogenous siderophores and other iron sources, such as haemoglobin, as shown in a bioassay test. The possible growth response of VIAS1 to cell-free culture supernatant presumed to contain aerobactin would be a novel finding, if confirmed with pure aerobactin.

The aerobactin bioassays, developed during the early part of the project, were used for a study of hydroxamate-producing strains from the laboratory collection (Stuart *et. al.*, 1982; Stuart, 1986). All of these isolates which still produced hydroxamate, as detected by modified Csaky assay, were found to produce aerobactin. This result does not preclude the possibility that some *E. coli* produce another hydroxamate siderophore.

Bioassay procedures were applied in further iron utilisation investigations of a range of bacteria and a fungus. A possible novel finding was an indication of aerobactin production by a *B. subtilis* strain.

9.2 Recommendations

While only limited advances were made in some areas of this study, techniques developed or refined are available for use in future investigations. In particular, the mating method by Zhao *et. al.* (1998), combined with the CAS agar test system in a 96-well microtitre tray, should be suitable for isolation of further mutants. Availability of a suitable mutant would most probably facilitate complete chemical characterisation of the new siderophore. In addition, such a mutant would be the starting point for a comprehensive analysis of the siderophore's synthesis and function. Development of a reliable and effective bioassay test for the putative siderophore should also allow testing of both further food isolates and clinical isolates. This work could lead to development of further diagnostics, including ELISA and PCR based tests. If deemed to be important, for example clinically, then vaccine development may be an option. Antibodies could be directed against epitopes on either the IROMP or the putative new siderophore. Some success has been obtained in other studies (Lee *et. al.*, 2000; Mansouri *et. al.*, 1999; Staczek *et. al.*, 2000) where pseudomonad outer membrane proteins have been used as antigen.

APPENDICES

Appendix 1 : Taxonomy and classification of *Klebsiella mobilis*

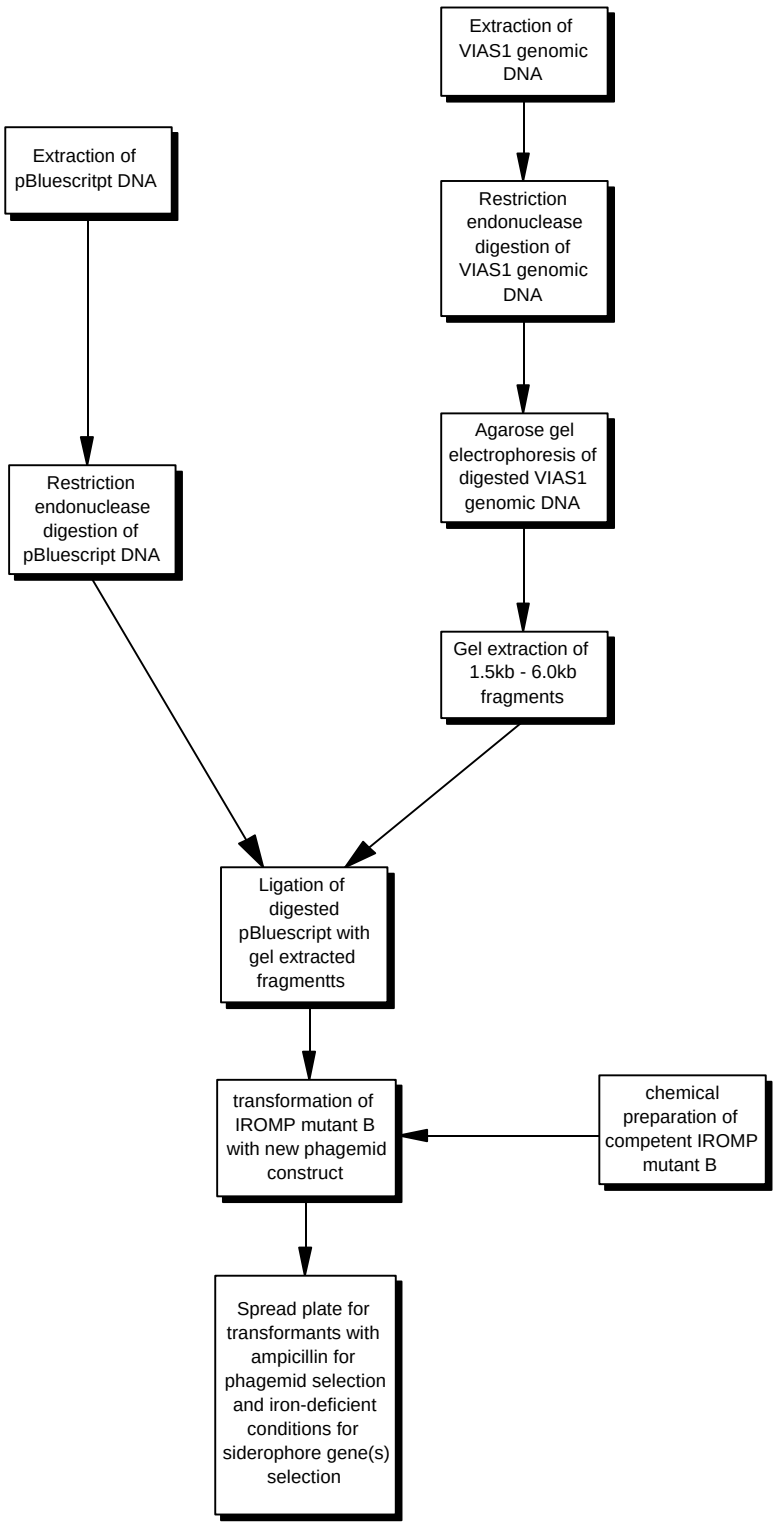
There is much confusion and debate surrounding the identity and taxonomic position of this organism. However, it does appear clear from all current evidence, that this organism should be classified *Klebsiella mobilis*, as proposed by Bascomb *et. al.* (1971).

Klebsiella mobilis is most often referred to as *Enterobacter aerogenes* with both names valid in bacteriological nomenclature, as listed in the List of Bacterial Names with Standing in Nomenclature (Euzeby, no date). Their names are therefore officially interchangeable. It has been found (Izard *et. al.*, 1980) that classification as *E. aerogenes* is not valid as the organism has more DNA relatedness to the type species of the genus *Klebsiella* (*K. pneumoniae*) than to the type species of the genus *Enterobacter* (*E. cloacae*). In addition, this investigation found that *E. cloacae* has similar DNA relatedness to *K. pneumoniae* (39%), *K. oxytoca* (40%) and *K. mobilis* (39%). In contrast, Izard *et. al.* (1980) observed much higher DNA relatedness between *K. pneumoniae* and *K. mobilis* (53%) or *K. oxytoca* (55%). Brenner *et. al.* (1972) has argued that *K. mobilis* not be included in the genus *Klebsiella*. They compared DNA relatedness between *K. pneumoniae* and a number of *Klebsiella* spp., including *K. mobilis*. Between *K. pneumoniae* and selected *Klebsiella* spp. they observed high DNA relatedness (80%+) and state that in their opinion, closely related bacteria demonstrate 75% DNA relatedness, which excludes *K mobilis*. These authors, however, did not include *K. oxytoca*, which has comparatively lower DNA relatedness to *K. pneumoniae*. *K. mobilis* is sometimes also described as *K. pneumoniae*. This appears clearly an invalid classification, as there are a number of fundamental biochemical (such as presence of ornithine decarboxylase and urease) and other differences (such as motility) between most strains of these

two organisms, including their DNA unrelatedness, as noted above. As well, it has been noted that there are further differences based on ability/inability to utilise certain compounds as carbon sources (Ferragut *et. al.*, 1983). In addition to its DNA relatedness, *K. mobilis* is clearly a member of the genus *Klebsiella*, from the work of Sivolodsky (1988). This study found a single unique feature of all *Klebsiella* spp., that was absent from 40 non-*Klebsiella* species in 22 genera from seven families. The feature is the capacity to give a "colour reaction with 5-aminosalicyclic acid".

To summarise, *K. mobilis* should not be classified as *E. aerogenes* because of this DNA unrelatedness to the type species of the *Enterobacter* genus. This puts it in the genus *Klebsiella*, based on DNA relatedness and its ability to form colour with 5-aminosalicyclic acid. Furthermore, classification as *K. pneumoniae* is not valid, based on fundamental differences between the two species. Therefore, the only valid name for this organism is *K. mobilis*, as proposed by Bascomb *et. al.* (1971).

Appendix 2 : Flowchart for DNA cloning procedure



Appendix 3 : *P. fragi* growth responses to siderophore preparations

A3.1 : C18+ and C18- semi-purified culture supernatant samples

A3.1.1 : Samples (by number) stimulating growth of IROMP mutants

Mutant B		Mutant F	
Possible stimulation	**Clear stimulation**	**Possible stimulation**	**Clear stimulation**
11	5 (with inhibition zone)	3	5 (with inhibition zone)
15	13	11	13
28	30	15	30
29	31	28	31
33	32	29	32
		33	

A3.2 : VIAS1 bioassay tests of purified HPLC fraction samples

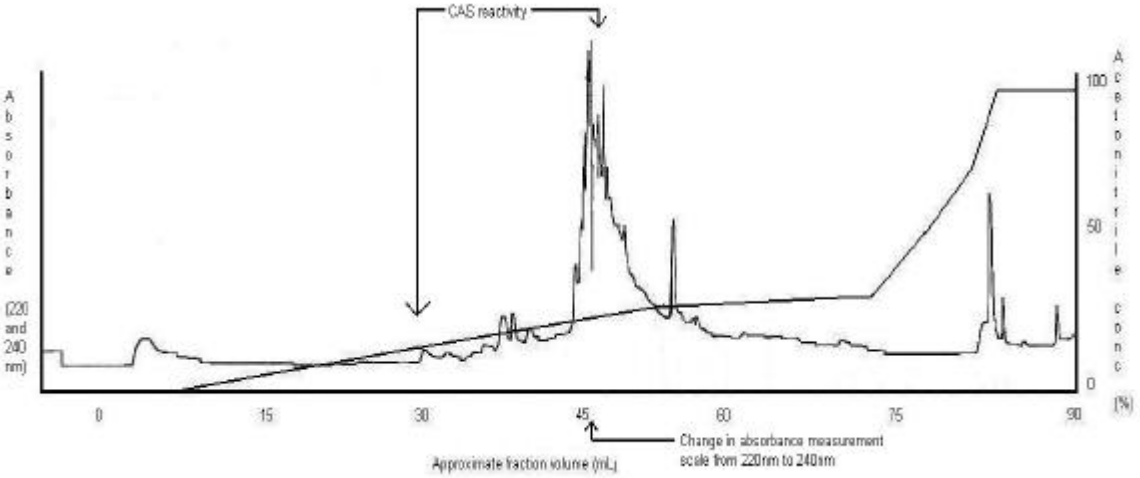
A3.2.1 : Large sample volume (30µL) with 1d incubation

No growth stimulation			Possible growth stimulation	Clear growth stimulation			
				Weak		Moderate	Strong
Volume eluted (mL)							
7.5	58.5	76.5	1.5	25.5	57.0	27.0	4.5
9.0	60.0	78.0	3.0	34.5	67.5	28.5	6.0
10.5	61.5	79.5	22.5	36.0	88.5	30.0	
12.0	63.0	81.0	24.0	40.5		31.5	
13.5	66.0	84.0	49.5	42.0		33.0	
15.0	69.0	87.0	54.0	43.5		37.5	
16.5	70.5			48.0		39.0	
18.0	72.0			51.0		45.0	
19.5	73.5			52.5		46.5	
21.0	75.0			55.5		64.5	

A3.2.2 : Small sample volume (500nL) with 2d incubation

No growth stimulation	Possible growth stimulation		Clear growth stimulation	
Volume eluted (mL)				
7.5	13.5	55.5	1.5	33.0
25.5	19.5	57.0	3.0	45.0
36.0	22.5	58.5	4.5	48.0
40.5	28.5	60.0	6.0	63.0
43.5	31.5	67.5	9.0	66.0
46.5	34.5	73.5	12.0	72.0
57.0	37.5	75.0	15.0	
61.5	39.0	76.5	16.5	
64.5	42.0	78.0	18.0	
69.0	45.0	79.5	21.0	
70.5	49.5	81.0	24.0	
87.0	51.0	82.5	27.0	
88.5	52.5	84.0	30.0	
90.0	54.0			

A3.3 : HPLC chromatogram of the work of S. Ibro



Appendix 4 : Results of the hydroxamate positive *E. coli* bioassays

A4.1 : Aerobactin bioassay results

A4.1.1 : Severe iron restriction (200μM 2,2'-dipyridyl)

Strain (ND No.)	Stimulation zone	Strain (ND No.)	Stimulation zone
		667	0.0
412	0.0	668	2.0
415	0.0	670	2.0
417	0.0	674	2.0
424	4.5	693	2.0
426	N/A	694	2.0
428	0.0	696	2.0
431	4.5	697	1.0
435	0.0	698	2.0
436	0.0	702	2.0
437	0.0	724	2.0
438	0.0	725	2.0
439	0.0	726	2.0
440	0.0	727	2.0
441	0.0	770	2.0
442	0.0	785	2.0
533	15.0	812	3.0
534	10.5	814	1.0
535	12.0	815	1.0
649	5.0	816	3.0
651	5.0	817	2.0
652	6.0	818	1.0
654	4.0	819	2.0
655	9.5	820	2.0
657	7.0	821	3.0

A4.1.2 : Moderate iron restriction (150μM 2,2'-dipyridyl)

Strain (ND No.)	Stimulation zone	Strain (ND No.)	Stimulation zone
		437	4.0
412	0.0	438	4.0
415	3.0	439	3.0
417	3.0	440	3.0
428	5.0	441	4.0
435	4.0	442	4.0
436	4.0	667	0.0

A4.1.3 : Low iron restriction (125μM 2,2'-dipyridyl)

Strain (ND No.)	Stimulation zone	Strain (ND No.)	Stimulation zone
412	0.0	667	0.0

A4.2 : Enterochelin bioassay results

A4.2.1 : Low iron restriction (100μM 2,2'-dipyridyl)

Strain (ND No.)	Stimulation zone	Strain (ND No.)	Stimulation zone
		435	2.0
412	5.0	534	5.0

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