Regulatory elements controlling lipase and metalloprotease production in *Pseudomonas fluorescens* B52

Conor Neil McCarthy BSc (Hons)

School of Health Science
Faculty of Health Sciences
Griffith University

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Health Sciences.

April 2003

Abstract

Psychrotrophic bacteria, such as *Pseudomonas fluorescens* B52, are a major cause of milk spoilage at refrigeration temperature due to the production of lipolytic and proteolytic enzymes. Regulatory mechanisms controlling the production of lipase and protease by the B52 *lipA* and *aprX* genes were investigated. Transposon mutagenesis identified the possible involvement of a poly-A polymerase enzyme which destabilises mRNA by 3' polyadenylation. A homologue of the *E. coli* EnvZ/OmpR two-component sensor/regulator system was identified by transposon mutagenesis and shown to repress lipase and protease production. This system responds to Na^+ and K^+ concentration in *E. coli* and these ions were also shown to repress lipase and protease expression in B52, however the EnvZ/OmpR system is not solely responsible for this. Assays of translational *lacZ* fusions with *aprX* and *lipA* were used to speculate on the mechanism by which Na^+ and EnvZ/OmpR repress the *aprX-lipA* operon. A membrane-bound sensor, MspA, which regulates protease production in *P. fluorescens* LS107d_2, was shown to exist in B52 but mutagenesis of the B52 *mspA* gene had no effect on lipase and protease expression. A homologue of the *P. fluorescens* CHA0 *rsmA* gene, encoding an RNA-binding translation repressor, was found in B52. Although *aprX* and possibly *lipA* contain consensus sequences for RsmA, mutagenesis of *rsmA* had no significant effect on lipase and protease expression. Repression of lipase and protease expression by Na^+ was increased by expression of the *P. fluorescens* M114 *pbrA* sigma-factor gene in B52.

Acknowledgements

I would like to thank Professor Ifor Beacham for providing me with the opportunity to undertake this PhD project, and for his help and guidance throughout the project. Thanks also to Rick Woods, Michelle Burger, Carie-Anne Beven, Nat Brown, Justin Boddey and Ian Peak for their experimental and technical suggestions which contributed to this work. Special mention goes to Rick Woods for his doctoral work which made an important contribution to the conclusions drawn from this study. Thanks to all those mentioned above and everyone else in the lab for their companionship during my time in the lab.

Finally, I thank my father Neil and brother Robin for their support, and my mother Sheena, who passed away before the submission of this thesis, for being there when I needed her, always. I dedicate this thesis to her.

Statement of Originality

This thesis contains original material that to the best of my knowledge has not been previously written or published by another person, except where due acknowledgement is given. This material has not been previously submitted for a degree or diploma in any University.

Conor Neil McCarthy BSc (Hons)

Contents

List of Abbreviations

A_n	absorbance at n nanometres
ATP	adenosine triphosphate
bp	base pairs
BLAST	basic local alignment search tool
BSA	bovine serum albumin
dATP	deoxyadenine triphosphate
dCTP	deoxycytosine triphosphate
dH_2O	sterile distilled water
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
ECF	extra-cytoplasmic functions
EDTA	ethylenediaminetetraacetic acid
g	grams
g	9.81 ms^{-2}
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	thousand base pairs
km	kanamycin
km^R	kanamycin resistant
LB	Luria-Bertani broth
lip^{++}	lipase over-producing
lip^-	lipase negative
mg	10^{-3} grams
min	minute(s)
ml	10^{-3} litres
mM	10^{-3} molar
mRNA	messenger RNA
ng	10^{-9} grams

nm	10^{-9} metres
nM	10^{-9} molar
OD	optical density
OmpR-P	phosphorylated OmpR protein
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFU	plaque-forming units
pmol	10^{-12} moles
pSK	pBLUESCRIPT SK
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature, approximately 23°C
SDS	sodium dodecyl sulfate
sec	seconds
TAE	40mM Tris acetate, 1mM EDTA, pH 8.0
TBE	45mM Tris-borate, 1mM EDTA
Tc^r	tetracycline resistance
TE	10mM Tris hydrochloride, 1mM EDTA, pH 8.0
Tris	Tris(hydroxymethyl)aminomethane
tRNA	transfer-RNA
μg	10^{-6} grams
μl	10^{-6} litres
μM	10^{-6} molar
V	Volts
v/v	volume per unit volume
w/v	weight per unit volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

List of Tables

List of Figures

Chapter 1

Introduction

1.1 Milk Spoilage by Psychrotrophic Bacteria

Psychrotrophic bacteria are a major cause of milk spoilage at refrigeration temperature. Psychrotrophy is defined as the ability to grow at or below 7°C (Suhren, 1989). In contrast, species such as *E. coli* are unable to growth below 9°C, possibly due to a block in translation (Morita, 1975; Herendeen *et al*, 1979; Broeze *et al*, 1978). Consequently, psychrotrophs can grow in milk stored at refrigeration temperatures, either before pasteurisation or in re-contaminated pasteurised milk. These bacteria produce extracellular lipases (glycerol-ester-hydrolase, E.C. 3.1.1.3) and proteases, which spoil milk and milk products by causing flavour defects.

The most common flavour defects are fruity and rancid flavours but putrid, potato, cheesy, bitter, unclean, soapy, yeasty and fishy defects have also been recorded (Cousin, 1982). Taste tests were performed on milk inoculated with lipolytic psychrotrophs and incubated at 4°C (Overcast and Skean, 1959). Testers reported rancid flavours sometimes accompanied and/or followed by bitterness. One sample was judged to taste sour and unclean. Taste tests on milk with added free fatty-acids showed that the most prominent causes of flavour defects are capric and lauric acids, which cause rancid, unclean, bitter and soapy flavours (Al-Shabibi *et al*, 1964). It has also been reported that even-numbered fatty acids from butyric to lauric contribute to rancid flavour (Scanlan *et al*, 1965), C_4 to C_{12} free fatty-acids cause rancid flavours, $C_4 - C_8$ cause butyric rancid flavours and C_{10}-C_{12} cause bitter and soapy flavours in butter (Cousin, 1989; Deeth and Fitzgerald, 1983; Woo and Lindsay, 1983).

Triglycerides comprise 95% of the lipid content of milk (Cousin, 1989). Lipases act on triglycerides primarily at the oil-water interface of lipid globules, liberating fatty acids by hydrolysis. Milk lipid globules contain surface proteins and protease activity is thought to enhance degradation by lipases (Fitzgerald and Deeth, 1983; AlKanhal *et al*, 1985).

Psychrotroph contamination usually originates from poorly sanitised dairy farm and processing equipment (Anderson *et al*, 1979). Contamination after pasteurisation can result in spoilage of products packaged for consumption, but growth of lipolytic psychrotrophs before pasteurisation can also cause spoilage due to the heat stability of these lipases. Psychrotroph lipases typically have high heat stability, many retaining significant activity after high-temperature short-time pasteurisation or even ultra-high-temperature treatment (Fitzgerald and Deeth, 1983). A lipase from a strain of *P. fluorescens* was found to be very stable at 100 to 150ºC, more so than at 60 to 80ºC, at which its stability was improved by Ca^{2+} (Fox and Stepaniak, 1983).

Strain B52 of the psychrotroph *Pseudomonas fluorescens* was chosen for this study because it has been studied previously (Richardson, 1981; McKellar *et al*, 1987), it has a highly lipolytic and proteolytic phenotype and the fluorimetric lipase assay performed well on cultures of this strain. The lipolytic and proteolytic abilities of B52 are encoded by the *lipA* (lipase) and *aprX* (alkaline metalloprotease) genes, which are cotranscribed (Woods, 2000; see section 1.3).

1.2 Factors affecting lipase and protease production

1.2.1 Temperature

Many psychrotrophs, including *P. fluorescens*, produce maximal extracellular enzyme levels at a temperature below the optimal growth temperature (McKellar, 1989). For example, lipase production by *P. fluorescens* W1 peaks at 8ºC while the minimum generation time occurs at 20ºC (Andersson, 1980), and production of extracellular lipase, proteases and periplasmic phosphatases by *P. fluorescens* MF0 peaks at 17.5ºC compared to an optimal growth temperature of 30ºC (Gügi *et al*, 1991; Merieau *et al*, 1993). The strain used in this study, B52, exhibits similar regulation (Woods, 2000). While generation time is shortest at 27ºC, lipase production by B52 peaks at 17ºC. Protease production is similar at 22ºC and 27ºC, even though growth is much slower at 22ºC. Regulation of lipase expression by temperature is unlikely to be at the level of transcription in B52 (Woods, 2000).

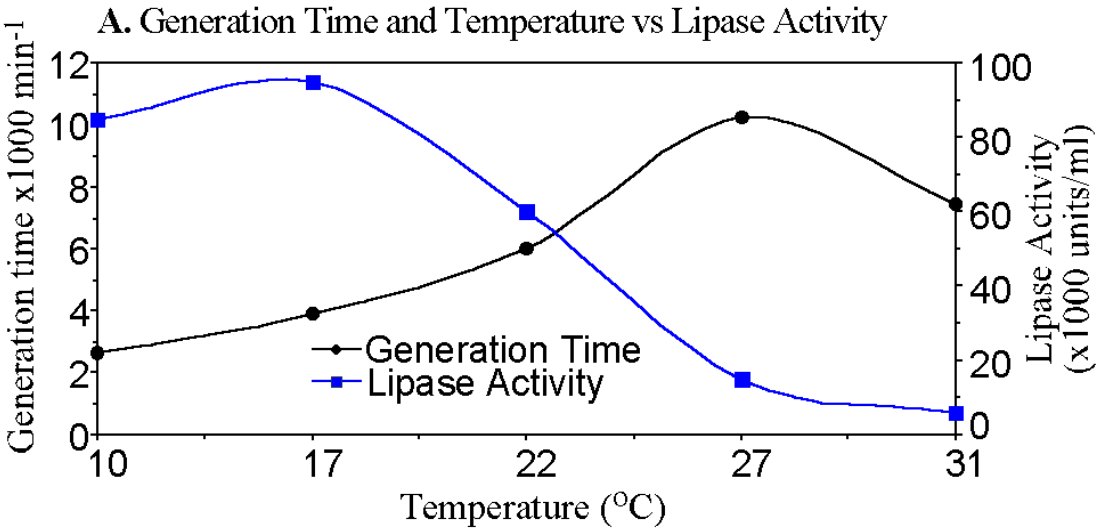


Figure 1.1. Lipase production versus generation time, showing that lipase production peaks at a lower temperature than is optimal for growth (Woods, 2000).

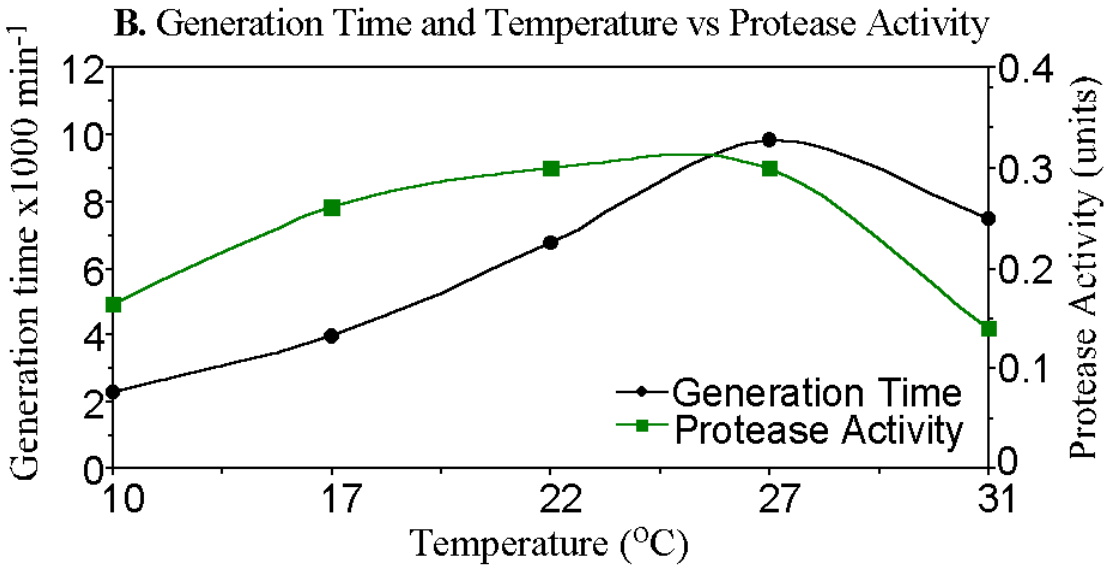


Figure 1.2. Protease production versus generation time, showing the absence of temperature-dependent regulation (Woods, 2000).

1.2.2 Growth Phase

Most reports indicate maximal production of lipase and protease by psychrotrophs occurs in late log-phase to early stationary-phase (McKellar, 1989) and the phenotype of B52 is consistent with these (Woods, 2000). Production of extracellular enzymes may be of no advantage at low cell densities due to diffusion of the enzymes and the nutrients liberated by their action. Because stationary-phase can occur at low cell density, quorum sensing could provide a more efficient signal for activating extracellular enzyme production than the onset of stationary-phase. Quorum-sensing organisms secrete autoinducers such as N-acylhomoserine lactone into the growth medium (reviewed by Swift *et al*, 2001). A high concentration of the compound is detected by a membrane-bound sensor to signal a high cell density.

1.2.3 pH

Optimal lipase and protease production by *P. fluorescens* and *P. fragi* typically occurs from pH 6 to 8 (Birkeland *et al*, 1985; McKellar, 1989). It has been reported that acidity affects the OmpR transcription regulator and the response to osmolarity (see section 1.2.8) (Heyde and Portalier, 1987; Bang *et al*, 2000; Sato *et al*, 2000).

1.2.4 Carbon Source

Carbon source has a complex effect on synthesis. Glucose usually represses extracellular enzyme synthesis in *Pseudomonas* sp. but stimulates on peptone medium (McKay, 1994; McKellar, 1989).

1.2.5 Iron

Iron (III) represses extracellular enzyme production by many psychrotrophs (McKellar, 1989). Lipase and protease production by *P. fluorescens* B52 is strongly repressed by 20 μM Fe^{3+}, while growth was fastest at 2 μM (McKellar *et al*, 1987). Up to 400 μM Fe^{3+} had only a slight effect on the lipase enzyme itself, and no effect on protease. B52 was also found to produce a siderophore, pyoverdine, which facilitates iron uptake in low-iron growth conditions. Addition of this compound to a B52 culture stimulated lipase production.

Woods (2000) measured the effect of Fe^{3+} on B52 in HEPES minimal medium by sampling cultures well into stationary phase. Maximum lipase production occurred in

2 μM Fe^{3+}, while production was reduced by more than 70% at 1 μM and was almost undetectable at 20 μM. Protease production was only slightly affected by zero added iron, peaked at 0.5 μM Fe^{3+} and maximum repression of 90% occurred at 12 μM (Figure 1.3). Measurement of *aprX* mRNA levels indicated that repression by iron occurs at the transcriptional level.

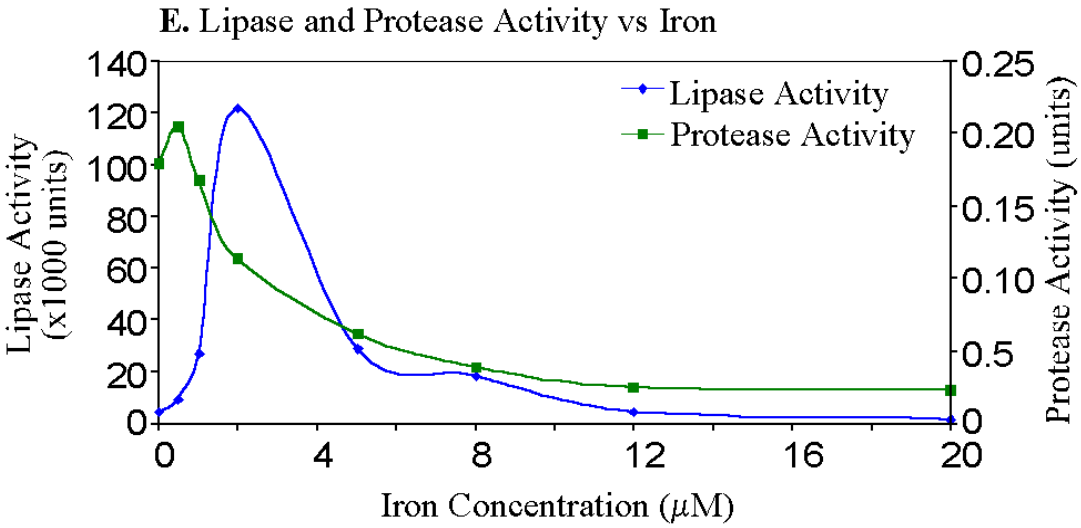


Figure 1.3. Lipase and protease production versus Fe^{3+} concentration (Woods, 2000).

A conserved iron-binding transcriptional regulator, Fur, was first identified in *Salmonella typhimurium* (for review see Litwin and Calderwood, 1993). A complex of Fur and Fe^{3+} binds to a consensus sequence in iron-regulated promoters and represses transcription. Fur homologues have been identified in *Pseudomonas putida* (Venturi *et al*, 1995), *N. gonorrhoeae* (Thomas and Sparling, 1994), *Vibrio cholerae* (Litwin *et al*, 1992), *Shigella flexneri* (Prince *et al*, 1991) and *Pseudomonas aeruginosa* (Prince *et al*, 1993). Structural homology of Fur between species is sufficient for *E. coli* Fur to regulate promoters from *P. fluorescens* M114 (O'Sullivan *et al*, 1994) and *P. aeruginosa* (Cunliffe *et al*, 1995), and Fur from *P. aeruginosa* and *N. gonorrhoeae* have been shown to regulate *E. coli* iron-regulated promoters (Prince *et al*, 1993; Thomas and Sparling, 1994).

Instead of being directly controlled by the Fur repressor, some iron-regulated genes in Pseudomonads are controlled via Fur-mediated repression of alternative sigma factors belonging to the extra-cytoplasmic functions (ECF) family. ECF sigma factors are a widely conserved protein family which replace σ^{70} in RNA polymerase and regulate production of extracytoplasmic proteins (Lonetto *et al*, 1994). Fur-regulated ECF sigma factors include PvdS in *P. aeruginosa* (Cunliffe *et al*, 1995), PfrI in *P. putida* WCS358 (Venturi *et al*, 1995), PsbS in *Pseudomonas* B10 (Leoni *et al*, 2002), PbrA in *P. fluorescens* M114 (Sexton *et al*, 1995; 1996) and FecI in *E. coli* (Van Hove *et al*, 1990; Ochs *et al*, 1995). Most genes regulated by Fur via ECF sigma factors are involved in iron uptake, but exotoxin A and protease from *P. aeruginosa* and a casein protease from *P. fluorescens* M114 are also controlled by this system.

1.2.6 The *prtIR* System

The *prtIR* operon in *P. fluorescens* LS107d_2 encodes an ECF sigma-factor, PrtI, and a transmembrane protein, PrtR, which is thought to be an activator of PrtI (Burger *et al*, 2000). Mutation of *prtI* or *prtR* results in the loss of protease production at 29°C, but not at 23°C. PrtI activates *aprX* either by binding to its promoter, or by activating another factor which results in the activation of *aprX*. A different mechanism can activate *aprX* at 23°C, but does not function at 29°C. It is not known if this system exists in B52.

1.2.7 Osmolarity and Salt Concentration

This study revealed that lipase and protease production by strain B52 is regulated by the concentration of Na^+ and K^+ ions, and this involves a homologue of the EnvZ-OmpR

regulatory system originally identified in *E. coli* and *Salmonella typhimurium*. The ratio of two *E. coli* outer membrane porin proteins, OmpF and OmpC, is determined by medium osmolarity (van Alphen and Lugtenberg, 1977) via the EnvZ-OmpR system (reviewed by Pratt *et al*, 1996). The pore of OmpC is smaller and has a lower flow rate than the OmpF pore (Nikaido and Rosenberg, 1983), so OmpC production is increased at high external solute concentration, while OmpF is repressed. Other genes and systems are also regulated by OmpR, including curli (thin fibrillar pili) biosynthesis in *E. coli*, the FadL outer-membrane protein involved in long-chain fatty-acid uptake in *E. coli*, the stationary-phase acid-tolerance response and the 40 kb *Salmonella* pathogenicity island I in *Salmonella enterica* serovar Typhimurium, and virulence in *Shigella flexneri* and *S. typhimurium* (Vidal *et al*, 1998; Higashitani *et al*, 1993; Bang *et al*, 2000; Lucas *et al*, 2000; Mills *et al*, 1998; Sleator and Hill, 2001). Curli production is particularly interesting, as it occurs only at temperatures below 30°C, in low osmolarity media and stationary phase (Prigent-Combaret *et al*, 2001). Curli production is controlled by a transcription activator, which is regulated by OmpR.

EnvZ proteins of *E. coli* and *S. typhimurium* span the cytoplasmic membrane with the C-terminal domain located in the cytoplasm (Liljeström 1986; Forst *et al*, 1987). EnvZ mediates expression of the porin genes by phosphorylating a DNA-binding protein, OmpR (Mizuno and Mizushima, 1990; Russo and Silhavy, 1991; Waukau and Forst, 1992). The EnvZ cytoplasmic domain is transautophosphylated on histidine-243 in a dimeric state before transferring the phosphate group to OmpR (Yang and Inouye, 1991; Roberts *et al*, 1994; Park *et al*, 1998). The rate of phosphotransfer is greatly increased if OmpR is bound to DNA, which causes a conformational change (Ames *et al*, 1999; Qin *et al*, 2001). The cytoplasmic domain also contains phosphatase activity (Aiba *et al*, 1989; Igo *et al*, 1989),

therefore the level of OmpR phosphorylation depends on the balance between the opposing activities, and increases with the osmolarity of the medium (Forst *et al*, 1990). The relative levels of OmpF and OmpC are determined by the concentration of phosphorylated OmpR (OmpR-P) (Lan and Igo, 1998).

Although porin expression is affected by high osmolarity generated by sucrose (Forst *et al*, 1988), experiments on EnvZ located in proteoliposomes and right-side-out membrane vesicles showed that EnvZ is not affected by sucrose, trehalose or glycine betaine (Jung *et al*, 2001). K^+ had the strongest effect on EnvZ, with Na^+ and other cations having a lesser effect. EnvZ was stimulated only when its cytoplasmic domain was exposed to these ions, not the periplasmic domain. This is consistent with observations that mutants with portions of the periplasmic domain deleted, or with the entire periplasmic domain replaced with an unrelated one from another sensory histidine kinase, are unchanged in their ability to regulate *ompF* and *ompC* in *E. coli* (Leonardo and Forst, 1996). However, a P159S mutation in the periplasmic domain of EnvZ prevents expression of the porin genes *in vivo*, and presumably prevents OmpR phosphorylation, but exhibits normal kinase activity when solubilised and assayed *in vitro*, indicating that the structure of the periplasmic domain can influence the activity of the cytoplasmic kinase (Kenney, 1997).

The effect of osmolarity or salt on the three catalytic activities of EnvZ is also unclear. Dutta *et al* (2000) identified mutations of Thr247 as abolishing phosphatase activity while retaining some autokinase and phosphotransferase activity, and used a fusion of the receptor domain of an aspartate chemoreceptor with the EnvZ cytoplasmic domain (Taz1-1) to measure the importance of phosphatase. Taz1-1 phosphorylates OmpR in response to aspartate instead of osmolarity, but this response was abolished by Thr247 mutations. It

was therefore concluded that the effect of the external signal is to reduce phosphatase activity. However, Mattison and Kenney (2002) measured the binding affinity of EnvZ for OmpR-P and concluded that it is likely to be too low for the osmolarity signal to regulate OmpR-P levels by altering EnvZ phosphatase activity, unless OmpR-P can undergo a conformational change which increases its affinity for EnvZ. In experiments on proteoliposomes and membrane vesicles, only the autophosphorylation activity of EnvZ was stimulated by cations (Jung *et al*, 2001). The phosphotransferase and phosphatase activities were unaffected.

OmpR is a member of the 'winged helix-turn-helix' family of DNA-binding transcriptional regulators (Martinez-Hackert and Stock, 1997). The N-terminal region contains the aspartate-55 phosphorylation site and interacts with RNA polymerase, while the DNA-binding domain is at the C-terminal end (Kato *et al*, 1989; Tsung *et al*, 1989; Delgado *et al*, 1993). Mutations in *rpoA* showed that OmpR-P directly contacts the alpha-subunit of RNA polymerase for both negative and positive regulation of the porin genes (Slauch *et al*, 1991).

Three phosphorylated OmpR (OmpR-P) dimers bind to three tandem repeated sequence elements upstream of *ompF* (Harlocker *et al*, 1995) and *ompC* (Maeda and Mizuno, 1990) (Figure 1.4). The two downstream sites in both *ompF* and *ompC* (F2, F3, C2 and C3) have a very low affinity for OmpR-P in isolation, and filling of these sites requires interaction with another OmpR-P bound to an adjacent site (Huang and Igo, 1996; Head *et al*, 1998). A fourth, low affinity OmpR-P site exists further upstream in *ompF* at -380 to –361, designated F4. Binding to this site requires protein-protein interaction, and may involve looped DNA (Huang *et al*, 1994; Rampersaud *et al*, 1994; Slauch and Silhavy, 1991).

Binding of OmpR-P to the *ompC* promoter activates transcription, and a single OmpR-P dimer bound at the C3 location is sufficient (Maeda and Mizuno, 1990). The *ompF* promoter is more complex, being activated by low concentrations of OmpR-P but repressed at higher concentrations (Slauch and Silhavy, 1989). The distant F4 site is required for repression of *ompF* at high osmolarity (Huang *et al*, 1994), but binding of OmpR-P to the four sites in *ompF* is not sufficient for repression, as shown by a C-terminal OmpR mutant which binds to *ompF* normally but fails to repress (Tran *et al*, 2000).

The mechanism by which *ompF* is activated at low osmolarity but repressed at high osmolarity is uncertain. It was thought that differences in affinity for OmpR-P may explain the regulation (Russo and Silhavy, 1991). In that model, binding of OmpR-P to the high-affinity site in *ompF* activates the gene at low osmolarity, while at high osmolarity, the low-affinity sites in both *ompF* and *ompC* would be filled, repressing *ompF* and activating *ompC*. However, detailed studies of binding affinities in the *ompF* and *ompC* promoters revealed that the difference in binding affinities between the two promoters is insufficient to explain the lack of *ompC* expression at low osmolarity (Head *et al*, 1998). Isolation of a T83I OmpR mutant, which activates *ompF* at low concentration but cannot repress *ompF* or activate *ompC* at high concentration, indicates that an OmpR-P conformational change may be involved (Mattison *et al*, 2002). A model was proposed in which OmpR-P is bound to the C1 site and all four *ompF* sites at low osmolarity, but the repressive DNA loop cannot form (Figure 1.4). At high osmolarity, OmpR-P binds in a different conformation, allowing the repressive *ompF* loop to form. The altered conformation activates *ompC* when bound to the three sites in *ompC*.

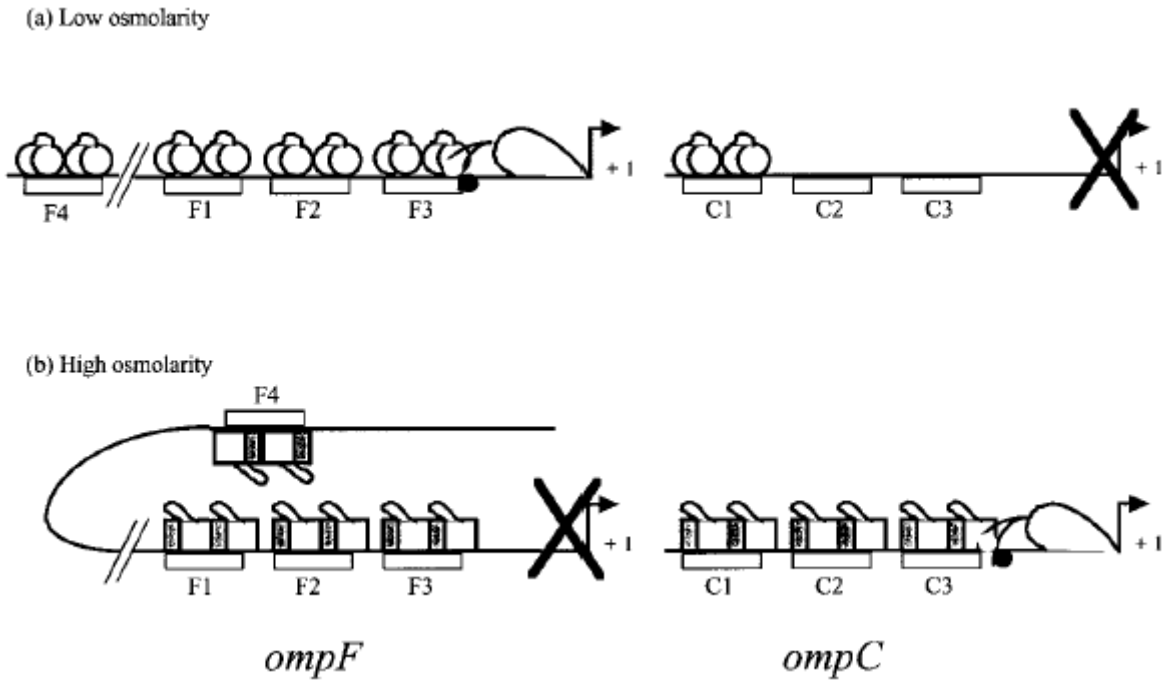


Figure 1.4. Model for the regulation of *ompF* and *ompC* by OmpR-P, showing the putative conformational change at high osmolarity. From Mattison *et al*, 2002.

The *E. coli ompF* gene is also regulated post-transcriptionally by an antisense RNA, MicF (Mizuno *et al*, 1984). MicF RNA binds to the 5' end of *ompF* mRNA and, in the presence of an unidentified protein, destabilises the message (Andersen and Delihas, 1990). MicF is required for *ompF* osmoregulation at low to medium osmolarity (Ramani *et al*, 1994), and is strongly activated by OmpR at 24°C, but not 37°C (Coyer *et al*, 1990). Levels of MicF in *E. coli* increase 10-fold at 37°C and above, and it is a major factor in thermal regulation of *ompF* (Andersen *et al*, 1989). The discovery that OmpR-EnvZ represses lipase production in B52 suggests that a temperature-regulated MicF-like antisense RNA may be involved in repression of lipase at the optimal growth temperature of B52. A homologue of *micF* was identified in *P. aeruginosa* by Southern blot (Esterling and Delihas, 1994), but it has apparently not been cloned or located in genomic sequence.

1.3 The *aprX-inh-aprDEF-prtAB-lipA* Operon

The lipase (*lipA*) and alkaline metalloprotease (*aprX*) genes of *P. fluorescens* B52 are located within an operon which also contains a protease inhibitor (*inh)*, a Type I (signal peptide independent) secretion system (*aprDEF*) and two autosecreted serine-protease homologues (*prtAB)* (Woods, 2000). A single transcription start site upstream from *aprX* is detectable. The promoter for this operon has high homology to the *aprX* promoter of *P. fluorescens* LS107d$_2$, which may be activated by the ECF sigma-factor PrtI (Burger *et al*, 2000).

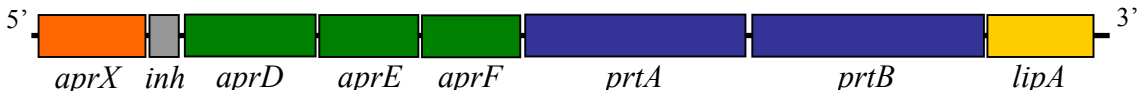


Figure 1.5. Structure of the *aprX-inh-aprDEF-prtAB-lipA* operon.

The *aprX-inh-aprDEF* genes are related to the *aprAIDEF* system of *P. aeruginosa*. The three-dimensional structure of AprA has been solved, and includes a calcium ion (Baumann *et al*, 1993). *P. aeruginosa* AprA is secreted by the AprDEF Type I secretion system (Guzzo *et al*, 1991; Duong *et al*, 1992). This system can also secrete *P. fluorescens* lipase, which contains a typical Type I secretion signal motif near the C-terminal end (Duong *et al*, 1994). B52 AprX is known to be secreted by AprDEF and LipA is probably secreted by the same system (Woods *et al*, 2001).

1.4 Aims of this Project

This project aimed to isolate and identify genes involved in regulation of lipase and protease production in *Pseudomonas fluorescens* B52, and to test for the involvement of homologues of genes identified in other strains or closely related species.

Chapter 2

Materials and Methods

2.1 Bacterial Strains and Plasmids

Table 2.1. Bacterial strains.

Strain	Genotype or Phenotype	**Use**	Source or Reference
E. coli			
TG1	*supE*, *thi*, *hsdD*5/F'*traD*36, Δ(*lac*,*pro*), *proAB*, *lac*^{q}*Z*, ΔM_{15}	General cloning	Amersham
S17.1λpir	RP4-2-Tc::Mu Km::Tn7 Tp Sm	Conjugation donor	Simon *et al*, 1983
XLOLR	Δ*(mcrA)183* Δ*(mcrCB-hsdSMR-mrr)173 endA1 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tc^{r})] Su^{-} λ^{r}	λ-ZAP excision	Stratagene
XL1-Blue MRF'	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB lacI*^{q}*Z.M15* Tn*10* (Tc^{r})]	λ-ZAP propagation	Stratagene
P. fluorescens			
B52	Rif^{r}, Ap^{r}, lipolytic and proteolytic.	Organism under study. Control organism in phenotype tests.	**Richardson, 1981**
B52B	Rif^{r}, Ap^{r}, *lipA*'-'*lacZ* fusion, Km^{r}, lipase negative.	*lipA*'-'*lacZ* fusion for regulatory studies	**Woods, 2000**
lip^{++}1,2,3	Rif^{r}, Ap^{r}, Km^{r}, overproduces lipase and protease	Transposon mutants which identified *envZ*	**This study**
lip^{-}10	Rif^{r}, Ap^{r}, Km^{r}, lipase and protease deficient	Transposon mutant which identified *pcnB*	**This study**

B52 *mspA*::pJP5608	Rif^r, Ap^r, Tc^r, *mspA*::pJP5608, lipolytic and proteolytic.	Mutant for testing the role of *mspA*	**This study**
B52 *rsmA*::Tc^r	Rif^r, Ap^r, Tc^r, *rsmA*::Tc^r, lipolytic and proteolytic.	Mutant for testing the role of *rsmA*	**This study**

Table 2.2. Plasmids.

Plasmid	Genotype	Description	Source or Reference
pBK-CMV	*neo*, *lacZ*α	Phagemid vector which contains inserts excised from λ-ZAP	Stratagene
pBLUESCRIPT SK+ (pSK)	*bla*, *lacZ*ΔM15	General cloning vector	Stratagene
pMMB207	*cat*	Broad host range vector for expression in *Pseudomonas*	Morales *et al*, 1991
pBBR1MCS	*cat*, *lacZ*α	Broad host range vector for expression in *Pseudomonas*	Kovach *et al*, 1994
pDM4	*cat*, *sacB*	Suicide vector with positive selection for double-crossover mutants using sucrose	Milton *et al*, 1996
pCUP408	*cat*, *pbrA*	*pbrA* clone from *P. fluorescens* M114	Sexton *et al*, 1996
pHP45Ω-Tc	Tc^r	Source of the Tc^r cassette	Fellay *et al*, 1987
pUTmini-Tn5 Km	*oriT, tnp**, *bla*, Km^r	Suicide vector for delivery of Tn5-derived Km^r transposon.	de Lorenzo *et al*, 1990
pBKpcnB	*neo*, *pcnB*	B52 *pcnB* gene in pBK-CMV	This study
pMMBpcnB	*cat*, *pcnB*	B52 *pcnB* gene in pMMB207	This study
pBKenvZ1.2	*neo*, *envZ*	B52 *ompR-envZ* operon lacking the 5' region of *ompR*	This study
pBKenvZ2.2	*neo*, *ompR-envZ*	Complete B52 *ompR-envZ* operon in pBK-CMV	This study
pBBR1ompRenvZ	*cat*, *ompR-envZ*	Complete B52 *ompR-envZ* operon in pBBR1MCS	This study
pBKmspA2	*neo*, *mspA*	B52 *mspA* gene in pBK-CMV	This study

pBKmspA4	*neo*	Religated *Bss*HII digest of pBKmspA2	This study
pJPmspAKO	Tc^r	Internal *mspA* fragment in pJP5608	This study
pBKrsmA1	*neo*	B52 *rsmA* clone lacking 3' end	This study
pSKrsmAKO	*bla*	Upstream and downstream *rsmA* fragments ligated together in pSK	This study
pSKrsmAKOTc	*bla*, Tc^r	pSKrsmAKO with a Tc^r cassette in the *Eco*RI site	This study
pDM4rsmAKOTc	*cat*, Tc^r	Insert from pSKrsmAKOTc in pDM4	This study

2.2 Media

2.2.1 Luria-Bertani (LB) broth or agar

Distilled water containing 1% tryptone, 0.5% yeast extract, 1% NaCl, and 1.5% agar if necessary, was autoclaved at 121°C for 15 minutes. After cooling to 40-50°C, any necessary antibiotics were added.

2.2.2 Luria-Bertani + 0.25% olive oil agar

0.25% sterile olive oil was added to autoclaved LB agar. The mixture was shaken vigorously before pouring into Petri dishes, and the foam was removed by flaming with a Bunsen burner.

2.2.3 Luria-Bertani + 1% tributyrin agar

1% sterile tributyrin was added to autoclaved LB agar. The mixture was shaken vigorously before pouring into Petri dishes, and the foam was removed by flaming with a Bunsen burner.

2.2.4 Luria Bertani + 2.5% skim milk agar

Double-strength LB agar and 5% w/v skim milk solution were autoclaved separately and mixed together before pouring into Petri dishes.

2.2.5 SOB and SOC broth

SOB broth was made by mixing a solution of salts with a solution of tryptone and yeast extract. The solutions were autoclaved at 121°C for 15 minutes before mixing to give a final concentration of 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 2% tryptone and 0.5% yeast extract. SOC broth was identical except glucose was added to the solution of tryptone and yeast extract for a concentration of 20 mM in the complete broth.

2.2.6 NZY broth or agar

Distilled water containing 0.5% yeast extract, 0.5% NaCl, 0.2% $MgSO_4 \cdot 7H_2O$, 1% NZ amine (casein hydrolysate), and 1.5% agar if necessary, was autoclaved at 121°C for 15 minutes. After cooling to 40-50°C, any necessary antibiotics were added.

2.2.7 Antibiotic concentrations

Table 2.3. **Antibiotic Concentrations**

Antibiotic	Dissolved in	For *E. coli* (μg ml^{-1})	For *P. fluorescens* (μg ml^{-1})
Ampicillin	70% Ethanol	100 (pSK) or 50 (others)	50
Kanamycin	dH_2O	50 (pBK-CMV) or 20 (others)	60
Chloramphenicol	Ethanol	30	200
Tetracycline	70% Ethanol	15	25
Rifampicin	Methanol	NA	50

2.3 DNA Preparations

2.3.1 Preparation of Plasmid DNA (Minipreps)

Small-scale purification of plasmid DNA was performed using the methods of Birnboim and Doly (1979) and Kraft *et al* (1988). Recombinant bacteria were grown overnight at 37°C in 2 ml LB. Most of the culture was transferred to a 1.5 ml Eppendorf tube. The bacteria were pelleted by spinning at 16000 *g* for 30 seconds in an Eppendorf centrifuge. The supernatant was discarded and the pellet was resuspended in 100 μl solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA) and left to stand for 5 minutes at room temperature. 200 μl of freshly made solution II (0.2 M NaOH, 1% SDS) was added and mixed in thoroughly by inversion. After standing on ice for 5 minutes, 150 μl solution III (3 M potassium acetate, pH 4.8) was mixed in by inversion. The solution was kept on ice for 5 minutes and spun at 16000 *g* for 5 minutes to pellet the cell debris. The supernatant was removed to a clean tube. An equal volume of TE-saturated 1:1

phenol/chloroform was added and the solution was vortexed for 5 minutes. The two phases were separated by centrifugation and the top layer was removed to a clean tube. The DNA was precipitated by adding 2 volumes 100% ethanol and spinning for 10 minutes at 16000 *g* in a refrigerated centrifuge. The supernatant was removed and the pellet washed with 1 ml 80% ethanol. The DNA was precipitated by spinning for 5 minutes at 16000 *g*. The pellet was dried under high vacuum for 1 - 2 minutes, resuspended in 90 µl dH_2O with 10 µl 10X RNase stock and incubated for 30 - 60 minutes at 37°C.

If the DNA was to be used for sequencing, it was further purified by extraction with an equal volume of TE-saturated phenol, followed by extraction with an equal volume of ether. The ether was removed, and 55 µl 20% PEG/2.5 M NaCl was added. The mixture was incubated on ice for one hour or overnight, and centrifuged for 15 minutes at 16000 *g*. The pellet was washed with 80% ethanol, dried under vacuum and resuspended in sterile dH_2O.

2.3.2 Preparation of Bacterial Genomic DNA

Genomic DNA was prepared by the method of Ausubel *et al* (1987). Either a colony was scraped off a plate, or 1.5 ml of liquid culture was pelleted in a 1.5 ml Eppendorf tube at 16000 *g* for 30 seconds. The bacteria were resuspended in 600 µl Tris/EDTA solution containing 0.5% SDS and 85 µg/ml proteinase K. This mixture was incubated at 37°C for one hour. 100 µl 5 M NaCl was mixed in, followed by 80 µl 10% hexadecyltrimethyl ammonium bromide (CTAB) solution in 0.7 M NaCl. The solution was incubated for 10 minutes at 65°C and extracted with an equal volume of 24:1 chloroform/isoamyl alcohol

followed by an equal volume of 1:1 phenol/chloroform. The DNA was precipitated from the aqueous phase with 0.6 volume isopropanol, the pellet was removed with a pipette tip, air dried and dissolved in 100 µl sterile dH_2O.

2.3.3 Oligonucleotide Synthesis

Single-stranded oligonucleotides were synthesised by commercial suppliers with purification by reverse-phase cartridge or HPLC.

2.4 Agarose Gel Electrophoresis of DNA

0.7% - 1.2% agarose minigels were made by adding 0.28 g – 0.48 g agarose to 40 ml 1X TAE buffer and boiling. The solution was cooled to 40-50°C before adding 2 µl ethidium bromide for a final concentration of 0.5 µg/ml. The solution was poured into a gel tray and wells were formed with an 8-tooth comb. Samples were mixed with 1/5 volume of dye solution containing 37% sucrose, 0.05% bromophenol blue and 50 mM EDTA before loading, and run at 100 V in a tank containing TAE buffer. A long-wave ultraviolet transilluminator was used to visualise the bands, which were recorded with a UVP GDS8000 gel documentation system.

2.5 Extraction of DNA from Agarose Gels

The desired region was excised from the gel with a scalpel and extracted using a QIAEX or QIAEX II kit according to the manufacturer's instructions. The gel sample volume was estimated by weight and 3 volumes of buffer QX1 plus 10 µl suspended QIAEX beads

were added. The mixture was incubated at 50°C for 10 minutes with vortexing every 2 minutes, then centrifuged for 30 seconds at 16000 *g*. The pellet was washed once in 500 µl buffer QX1 by vortexing, pelleted again at 16000 *g* for 30 seconds, then washed twice in buffer QX2 by vortexing and pelleting. The pellet was air-dried in a laminar-flow cabinet and the DNA eluted by resuspending in 20 µl dH_2O. The QIAEX beads were removed by centrifuging at 16000 *g* for 30 seconds and transferring the supernatant to a clean tube.

2.6 Measurement of DNA Concentration

A UV spectrophotometer was zeroed at 260 nm with distilled water in a 50 µl quartz cuvette. 50 µl of an appropriate dilution of DNA was placed in the cuvette and its absorbance was measured. A more dilute solution was used if the reading was greater than 1.0. The DNA concentration was calculated using the formula:

$$[\text{DNA}]\ (\mu g/ml) = A_{260} \times \text{dilution factor} \times 50\ (\text{dsDNA}) \text{ or } 20\ (\text{ssDNA oligos})$$

2.7 Restriction Enzyme Digests of DNA

Digests were performed in a 20 µl volume using 5 to 20 units of enzyme, a buffer recommended by the manufacturer, and 0.5 – 2 µg DNA. Digestion times were 1 – 2 hours. For ligations, the enzyme was inactivated by incubation at 65°C for 20 minutes. If required, the DNA was precipitated by adding a 1/10 volume of sodium acetate pH 5.2 and 2 volumes 100% ethanol, followed by centrifugation and washing as for plasmid minipreps.

If required, 3' recessed termini were filled in by adding 5 units Klenow polymerase and dNTPs to a concentration of 1 mM, and incubating for 30 minutes at 37°C.

2.8 DNA Ligation

Ligations were performed in a volume of 10µl or 20µl with 1 unit T4 DNA ligase and the appropriate volume of 10X ligase buffer (400 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP, 0.5 mM BSA). Vectors were used at a concentration of 20 to 60 ng/µl with a 3 to 5-fold molar excess of insert (Sambrook *et al*, 1989). If the vector was cut with two enzymes, it was either purified by gel extraction or the small fragment from the polylinker was cut with a third enzyme to prevent re-ligation. Ligation reactions were incubated at 16°C overnight.

2.9 Ethanol Precipitation of DNA

DNA solutions were precipitated by the addition of 0.1 volume of 3 M sodium acetate and 2 volumes of absolute ethanol at –20°C. The mixture was centrifuged at 16000 *g* for 10 minutes and the pellet was washed with 200 – 500 µl of 70% ethanol. It was centrifuged at 16000 *g* for 5 min if the wash could not be removed while leaving the pellet behind. The pellet was dried under vacuum for 1 minute and resuspended in the desired volume of dH_2O or 10 mM Tris-HCl.

2.10 Preparation of Competent Cells

2.10.1 $CaCl_2$ Method

One colony of *E. coli* strain TG1 was inoculated into 8 ml LB and grown to mid exponential phase ($A_{450} \approx 0.4$) at 37°C with shaking. The cells were pelleted by centrifugation and resuspended in 5 ml ice-cold 0.1 M $CaCl_2$. After 20 minutes on ice, the cells were pelleted again and all but approximately 100 µl of the supernatant was discarded. The cells were resuspended in the remaining liquid and incubated on ice for 1 to 24 hours before transformation.

2.10.2 Method of Hanahan (1983)

E. coli TG1 or DH5α was grown on a Psi (5 g/l yeast extract, 20 g/l tryptone, 5 g/l $MgSO_4$) plate overnight at 37°C. One colony was grown in 5 ml Psi medium at 37°C to an OD_{550} of 0.48. The culture was chilled on ice for 5 minutes and centrifuged for 5 minutes at 1480 *g*. The pellet was resuspended in 40 ml ice-cold TFB1 (100 mM RbCl, 50 mM $MnCl_2$, 30 mM Potassium acetate, 10 mM $CaCl_2$, 15% glycerol, pH 5.8), left on ice for 5 min. and centrifuged at 1480 *g* for 5 min. The pellet was resuspended in 4 ml ice-cold TFB2 (10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$, 15% glycerol, pH 6.8) and left on ice for 15 min. Aliquots were stored at –70°C.

2.11 Electroporation

Electroporation was performed in an E-C Apparatus Corporation EC100 electroporator according to the manufacturer's instructions. 500 ml of SOB broth was inoculated with a 5 ml overnight culture of the recipient *E. coli* strain, and grown to an OD_{550} of 0.8. The culture was chilled on ice and kept ice-cold for the rest of the protocol. It was centrifuged at 2500 *g* for 15 minutes; the pellet was washed by resuspending in ice-cold sterile dH2O and centrifuged again at 2500 *g* for 15 minutes. This washing step was repeated, and then the pellet was washed in ice-cold sterile 10% glycerol. The pellet was resuspended in a small quantity of ice-cold 10% glycerol at an OD_{550} of 100 – 200. For each transformation, a 40 µl aliquot of cells containing up to 2.5 µl of DNA was pulsed in an electroporation cuvette at 1800 V (1 mm cuvette) or 2800 V (2 mm cuvette). The cells were washed out of the cuvette with 200 µl SOC, added to another 800 µl SOC and incubated for 1 hour at 37°C before plating out.

2.12 Transformation

5 µl of the ligation product was added to a tube of competent cells and gently mixed. The tube was placed on ice for 5 minutes, and then heat shocked for 5 minutes in a 37°C water bath. 1 ml LB was added and the cells were incubated for 1 hour at 37°C to allow expression of the antibiotic-resistance genes. 100 µl was spread on LB agar plates containing the appropriate antibiotic(s) and these were incubated overnight at 37°C.

2.13 Conjugation

Conjugation was performed by the method of Franklin (1985). The *E. coli* donor and the *P. fluorescens* recipient were grown overnight in 2 ml LB containing appropriate antibiotics. The cultures were centrifuged and resuspended in 1 ml LB without antibiotics. Three pieces of Whatman filter were placed on an LB agar plate. 10 µl of the *E. coli* culture was placed on one filter, 10 µl of the *P. fluorescens* culture on another filter, and 10 µl of each on the third. After 8 hours incubation at 30°C, each filter was vortexed in 1 ml 0.9% NaCl. For plasmid transfers, a loopful of each suspension was streaked onto a plate containing the appropriate antibiotic plus rifampicin to select against the *E. coli* donor. For transposon mutagenesis or site-specific knockouts, 100 µl of each suspension was plated out instead of streaking with a loop. The controls containing only *E. coli* or *P. fluorescens* were checked for absence of growth after 48 hours at 27°C – 30°C.

2.14 Bacteriophage Lambda Packaging

A ligation with the λ-ZAP vector system (Stratagene) was packaged into λ phage particles with the Packagene system (Promega). 50 µl of Packagene extract was thawed on ice, 5 µl of the ligation product was added, and the mixture was incubated at room temperature for 3 hours. 445 µl of phage buffer (20 mM Tris-HCl pH 7.4, 100 mM NaCl, 10 mM $MgSO_4$) and 25 µl of chloroform were mixed in by inversion and the chloroform was allowed to settle to the bottom. 0.1% w/v gelatine and 7% v/v DMSO were added and the packaged library was stored at –70°C.

2.15 λ-ZAP Library Plating

1 – 2 µl of packaged ligation, amplified library or excised plaque suspension was added to 200 µl of XL1-Blue MRF' cells grown to an OD_{600} of 0.5 in LB supplemented with 10 mM $MgSO_4$ and 0.2% w/v maltose. The mixture was incubated at 37°C for 15 minutes, and then 3 ml of NZY top agar at 45 – 48°C was added. For checking the library by blue/white selection, 15 µl of 0.5 M IPTG and 50 µl of 250 mg/ml X-gal were also added. The mixture was poured onto NZY agar plates pre-heated to 37°C, and incubated overnight at 37°C.

2.16 λ-ZAP Library Amplification

A volume of packaged library containing ~50000 PFU was added to 600 µl of XL1-Blue MRF' cells grown to an OD_{600} of 1.0 in LB supplemented with 10 mM $MgSO_4$ and 0.2% w/v maltose. The mixture was plated out on a 150 mM NZY agar plate using 6.5 ml of NZY top agar, and incubated for 6 – 8 hours at 37°C. The plate was overlayed with 8 – 10 ml SM buffer and stored at 4°C overnight. The bacteriophage suspension was recovered with a 10 ml pipette into a sterile centrifuge tube, and incubated at room temperature for 15 minutes with 5% v/v chloroform. Cell debris was removed by centrifugation for 10 minutes at 500 *g*. The supernatant was removed to a sterile polypropylene tube, 0.3% v/v chloroform was added and aliquots were stored in 7% v/v dimethylsulfoxide at –80°C.

2.17 λ-ZAP Clone Excision

Inserts in the Stratagene λ-ZAP vector can be excised in a high copy-number phagemid vector, pBK-CMV. Excisions were performed according to the Stratagene protocol. Phage suspension (250 μl) from a positive plaque and 1 μl 'ExAssist' helper phage were added to 200μl of XL1-Blue MRF' cells resuspended in 10 mM $MgSO_4$ at an OD_{600} of 1.0. After 15 minutes incubation at 37°C, 3 ml of NZY broth was added. The culture was incubated with shaking at 37°C for 2 ½ hours or overnight; the *E. coli* was killed by heating at 65 – 70°C for 20 minutes and debris removed by centrifuging at 1000 *g* for 15 minutes. The resulting phagemids were plated by adding 10 – 100 μl phagemid suspension to 200 μl XLOLR cells resuspended in 10 mM $MgSO_4$ at an OD_{600} of 1.0. The mixture was incubated at 37°C for 15 minutes, 300 μl of NZY broth was added, the transfected cells were incubated at 37°C for 45 minutes, then 200 μl was plated onto LB/kanamycin (50 μg/ml).

2.18 Unidirectional Deletion of DNA

To facilitate sequencing, unidirectional deletion of inserts cloned in pSK were performed using the Erase-a-Base system (Promega Corporation) according to the manufacturer's instructions. Briefly, plasmid DNA was digested with a pair of restriction enzymes which left a 5' overhanging terminus on the vector and a 3' recessed terminus at the end of the insert. Aliquots of the digest were digested with exonuclease III for different lengths of time, and the remaining single-stranded DNA was removed with S1 nuclease. The

recessed terminus was filled using Klenow DNA polymerase fragment, and the blunt ends were ligated by T4 DNA ligase.

2.19 DNA sequencing

Sequencing was accomplished using an Applied Biosystems 377 automated sequencer. Labelled fragments were generated by cycle sequencing with a double-stranded DNA template and dye-labelled terminators according to the manufacturer's instructions. Unused terminators were removed by ethanol precipitation. The DNA sequence and chromatogram readouts were analysed using SeqEd or Chromas software.

2.20 Analysis of DNA and Protein Sequences

Open reading frame analysis, translations, restriction mapping, and isoelectric point prediction were performed using MacVector (Oxford Molecular). DNA and amino-acid homology between the sequences found in *P. fluorescens* and those in GenBank, SWISS-PROT, Brookhaven Protein Data Bank, PIR and the Transcription Factor Database was found using the BLAST Network Service provided by the National Centre for Biotechnology Information, USA (Altschul *et al*, 1990, 1997).

2.21 Polymerase Chain Reaction

Selected segments of DNA were amplified by the polymerase chain reaction (PCR) using a reaction mixture containing 5 µl *Taq* 10X buffer (Promega), 100 µM of each dNTP, 1.5 mM $MgCl_2$, 50 pmol of each primer, and 2.5 units of *Taq* polymerase. The volume was

made up to 50 µl with sterile dH_2O. Reactions were performed in a Hybaid PCR Sprint thermal cycler with the following program:

Cycle 1:	Remaining cycles:
2 min @ 95°C	15 sec @ 95°C
15 sec @ annealing temperature	15 sec @ annealing temperature
90 sec @ 74°C	90 sec @ 74°C

2.22 Synthesis of Radiolabelled DNA Probes

2.22.1 Hexamer Priming

The DNA fragment was radiolabelled by hexamer priming using the following reaction mixture:

5X labelling buffer	10 µl (Buffer + random hexamers)
0.5 mM dNTPs (T,C,G)	2 µl
Denatured template	3 µl (Incubated at 100°C for 5 min.)
10mg/ml BSA	2 µl
^{32}P-dATP	2 µl
Klenow fragment	1 µl (5 units)
dH_2O	30 µl

The reaction mix was left on the bench for 2 hours, followed by denaturation of the probe at 100°C for 5 min before use.

2.22.2 Nick Translation

A Nick Translation kit (Promega) was used to radiolabel DNA fragments with the following reaction mixture:

100 µM dNTP mix	10 µl
10X buffer	5 µl
DNA fragment	1 to 10 µl
[α-^{32}P]dCTP	7 µl
Enzyme mix	5 µl
dH_2O	to 50 µl

After incubation at 15°C for 60 minutes, 5 µl stop solution was added.

2.22.3 Primer Extension

If a suitable primer was available, labelled probes were made by primer extension with a reaction mixture containing 1X Taq buffer, 1.5 mM $MgCl_2$, 20 µM dNTP mix (A, G, T), 7µl [α-^{32}P]dCTP, 50 pmol primer, 2.5 units Taq DNA polymerase and 100 – 200 ng DNA template. The mixture was incubated at 95°C for 2 minutes followed by 25 cycles of the following program:

15 sec @ annealing temperature.
30 sec @ 74°C.
15 sec @ 95°C.

2.23 Colony Hybridisation

Solutions Used:

Denaturation solution 1.5 M NaCl, 0.5 M NaOH

Neutralising solution	1.5 M NaCl, 0.5 M Tris-HCl pH 7.5, 1 mM EDTA
20X SSC	3 M NaCl, 0.3 M Na_3-citrate
20X SSPE	3.6 M NaCl, 0.2 M Na_3PO_4 pH 7.7, 20 mM EDTA
100X Denhardt's solution	2% BSA, 2% Ficoll, 2% PVP
Pre-hybridisation solution	5X SSPE, 5X Denhardt's solution, 0.5% SDS

Colonies were transferred onto a circular nylon membrane by placing the membrane on the agar for 30 seconds. The membrane was left for 7 minutes on filter paper soaked in denaturation solution to lyse the bacteria and denature the DNA. The membrane was neutralised for 3 minutes on filter paper soaked in neutralising solution, followed by another 3 minutes on fresh neutralising paper. The membrane was washed in 2X SSC and dried on filter paper for 1 hour at room temperature or 15 minutes at 65°C. The DNA was covalently crosslinked to the membrane by exposure to UV from a transilluminator for 5 minutes. Membranes were placed in a Hybaid hybridisation bottle, separated by nylon mesh. To this was added pre-hybridisation solution and tRNA to a concentration of 20 μg/ml. The bottle was rotated in a hybridisation oven for 1 hour at 65°C for blocking of the membranes to occur. The probe was then added and allowed to hybridise to the membranes for 16 hours at 65°C. Membranes were washed twice in 2X SSPE, 0.1% SDS at room temperature for 10 minutes, and once in 1X SSPE, 0.1% SDS at 65°C for 15 minutes. Positive colonies were visualised by autoradiography (Section 2.26).

2.24 Plaque Screening

A circular positively-charged nylon membrane (Hybond-N+, Amersham-Pharmacia) was placed on the agar for 2 minutes. Three small holes were made in the membrane for later orientation. Denaturation, neutralisation and hybridisation were performed as for colony screening above, except a different hybridisation buffer (7% SDS, 10 mM EDTA, 0.5 M phosphate buffer, pH 7.2) was used, and UV-crosslinking was unnecessary with a positively-charged membrane. Positive plaques were visualised by autoradiography, or later by phosphoro-imaging. 0.2 μl of positive-control DNA was pipetted onto the orientation holes in the membrane to render these visible in the phosphoro-imager display.

Positive plaques were excised with a scalpel on a piece of agar 3 – 4 mm square. This was suspended in 500 μl SM buffer (100 mM NaCl, 0.2% $MgSO_4$, 50 mM Tris-HCl pH 7.5, 0.01% gelatine) with 20 μl chloroform, and stored at 4°C.

2.25 Southern Blotting

DNA samples were run on a TBE (45 mM Tris-borate pH 8.0, 1 mM EDTA) gel with λ/*Hind*III standards and a suitable positive control where possible. The gel was placed in a Bio-Rad Trans-Blot SD semi-dry transfer cell between two pieces of thick filter paper, with a piece of Hybond-N+ membrane on the bottom of the gel. The cell was run at 15 V for 30 minutes and the gel was checked on a UV trans-illuminator to ensure most of the DNA had been transferred. The DNA was denatured and hybridised with the probe by the same protocol used for plaque screening.

2.26 Autoradiography

Membranes were exposed to Fuji medical X-ray film in a Kodak cassette with an intensifying screen. The cassette was stored at -70°C for 1 to 3 days, and the film was developed by soaking in developer, water and fixer for 3, 1, and 5 minutes respectively.

2.27 Phosphoro-Imaging

Radiolabelled membranes were placed on a Kodak Storage Phosphor Screen SO-230 for 30 min – 24 hours. The screen was then scanned with a Bio-Rad Molecular Imager FX and the resulting image was analysed with Bio-Rad QuantityOne software.

2.28 Detection of Lipase Phenotype

The strain to be tested, together with a control, was patched onto an LB/0.25% olive oil plate or an LB/1% tributyrin plate and incubated 2 - 4 days at room temperature (≈23°C) for best expression of lipase. The size of the halo around the colony was compared to that of the control.

2.29 Detection of Protease Phenotype

The strain to be tested, together with a control, was patched onto an LB/2.5% skim milk plate and incubated as for olive-oil plates. The extent of clearing around the colony was compared to that of the control.

2.30 Fluorimetric Lipase Assay

Extracellular lipase activity was measured using the substrate 4-methylumbelliferyl-oleate (Roy, 1980; Stead, 1984; McKay *et al*, 1995), which is cleaved by lipase to liberate fluorescent 4-methylumbelliferone. The 1 ml reaction mixture contained 100 nM 4-methylumbelliferyl-oleate, 1.5 mM $CaCl_2$, 100 mM Tris-HCl pH 8.0, and 1 – 100 µl culture supernatant. Fluorescence was measured using a Hitachi F-4500 Fluorescence Spectrophotometer with excitation at 339 nm and emission at 444 nm. The lipase activity in pmol/min/ml supernatant (units) was calculated from the slope of the fluorescence graph and a standard curve of fluorescence from known quantities of 4-methylumbelliferone.

2.31 Protease Assay

Extracellular protease activity was measured using the method of Wassif *et al* (1995). The reaction mixture contained 0.5% w/v azocasein (Sigma), 2 mM $CaCl_2$, 50 mM Tris-HCl pH 8.0 and 50 µl culture supernatant in a volume of 500 µl. The mixture was incubated at 37°C for 30 minutes. The reaction was stopped and undigested protein precipitated by the addition of 125 µl 15% w/v trichloroacetic acid. The mixture was centrifuged at 16000 *g* for 5 minutes and the supernatant, containing azo-labelled free amino-acids, was removed and its A_{440} measured against a control reaction containing water instead of culture supernatant. Protease activity was defined as A_{440} units per 30 minutes for a 10% solution of culture supernatant.

2.32 β-galactosidase Assay

β-galactosidase activity from the *lacZ* gene in fusion constructs was assayed using the method described by Miller (1992). A 2 ml culture sample was centrifuged at 16000 *g* for 30 seconds and the pellet lysed by resuspending vigorously in 200 µl of B-PER (bacterial protein extraction reagent; Pierce Biotechnology, Inc., PO box 117, Rockford IL 61105, USA). The lysate was centrifuged at 16000 *g* for 10 minutes and the supernatant was used in the assay. The reaction mixture contained 0.1 – 100 µl lysate, Z buffer (60 mM $Na_2HPO_4.7H_20$, 40 mM $NaH_2PO_4.H_20$, 10 mM KCl, 1 mM $MgSO_4.7H_20$) and 40 mM β-mercaptoethanol in a volume of 1 ml. The reaction was started by adding 200 µl of 4 mg/ml ONPG, and incubated for 30 minutes. 500 µl of 1 M Na_2CO_3 was added to stop the reaction, and the A_{420} was measured.

The concentration of protein in the lysate was measured by the bicinchoninic acid method (Smith *et al*, 1985). Lysate (4 µl) or 10 µl of BSA standards (200 to 1200 µg/ml) was added to 200 µl of BCA working reagent (50 parts bicinchoninic acid to 1 part 4% $CuSO_4.5H_2O$) and incubated at 37°C for 30 minutes. The A_{562} was measured against a blank of Z buffer in BCA reagent. The amount of protein in the lysate was estimated from a standard curve of BSA samples. β-galactosidase specific activity was expressed in enzyme units/mg protein.

Chapter 3

Transposon Mutants

3.1 Mutagenesis by Non-Specific Transposition

Non-site-specific mutagenesis was used to locate genes involved in regulation of lipase and protease production in B52. The mini-Tn5-km transposon, containing a kanamycin resistance cassette, was transferred by conjugation into B52 in the suicide vector pUT. Exconjugants were screened by patching arrays of colonies onto LB-agar plates containing olive-oil or tributyrin to search for mutants with altered lipase expression. Several lipase-deficient mutants were isolated, together with three mutants which grew slightly more slowly but produced a significantly larger halo than those with wild-type expression. These lipase-overproducing mutants were designated $lip^{++}1$, $lip^{++}2$ and $lip^{++}3$.

To clone the region of transposon insertion in mutant strains, genomic DNA was digested with *Cla*I, for which there are no sites in the transposon. The genomic *Cla*I fragments were cloned into pSK and clones containing the transposon were selected by addition of kanamycin to the transformation mix after the one hour incubation in LB broth. These cultures were incubated at 37°C for several hours before being plated out.

The I1-out (5' CGCCGGTCTAGACTAGTT 3') and O1-out (5' TTCGTCGACAAGCTTGG 3') sequencing primers were designed to match sequences at the ends of the mini-Tn5 transposon. O1-out did not generate useable sequence but I1-out generated sequence from the region flanking the 'I' end of the transposon in the mutants studied. The sequences were translated in six frames and submitted to a BLAST search of all protein databases for putative identification of the disrupted gene.

3.2 EnvZ Osmolarity Sensor Mutants

All three mutants which overproduced lipase were shown by I1-out sequencing to have mini-Tn5-km disrupting a gene encoding a putative transmembrane sensor protein having a high degree of homology to the EnvZ sensor of *E. coli* and *Salmonella typhimurium*. The location of the transposon within the gene is different in all three mutants, showing that the mutants are independent and demonstrating the significance of EnvZ in lipase regulation. EnvZ and its cognate response regulator OmpR are the subject of Chapter 4.

3.3 PcnB Poly-A Polymerase Mutant

The transposon mutagenesis also produced a mutant, lip$^-$10, which appeared to be lipase-deficient, although it also grew more slowly than most other mutants. Sequencing from the I-end of the transposon in this mutant yielded sequence with high homology to the plasmid copy number B (*pcnB*) gene of *E. coli*, which encodes a poly-A polymerase (Cao and Sarkar, 1992). This gene was originally identified by mutations which reduced the copy number of ColE1-type plasmids (Lopilato *et al*, 1986; Liu and Parkinson, 1989). The poly-A polymerase enzyme typically adds a 15 to 40 nucleotide poly-A tail to mRNAs, which enhances the rate of degradation by enzymes such as polynucleotide phosphorylase, RNase II and RNase E (O'Hara *et al*, 1995).

3.3.1 Downstream Sequence from *pcnB*

No sequence downstream from *pcnB* was obtained. The *pcnB* gene of *P. syringae* pv. syringae has very high homology to B52 *pcnB*, and in this species *pcnB* is immediately

followed by *folK*, a gene required for folate biosynthesis, and *panBC*, two pantothenate synthesis genes. It seems unlikely that these genes would be involved in the regulation of lipase and protease production.

3.3.2 Cloning of Wild-Type *pcnB* and Mutant Complementation

A genomic library of *P. fluorescens* B52 was constructed in the λ-ZAP vector (Stratagene), a derivative of bacteriophage λ which contains a phagemid vector, pBK-CMV, in which the insert can be excised and handled as an ordinary plasmid-based clone. B52 genomic DNA was partially digested with *Sau*3A, fragments in the range of 5 kb to 10 kb were gel-purified by QIAEX, ligated with λ-ZAP vector arms and packaged in λ particles. The resulting library was titrated and found to contain few non-recombinants and a sufficiently high concentration of recombinant particles. Aliquots of this library, obtained by amplification (Section 2.16), were used for plaque screening experiments.

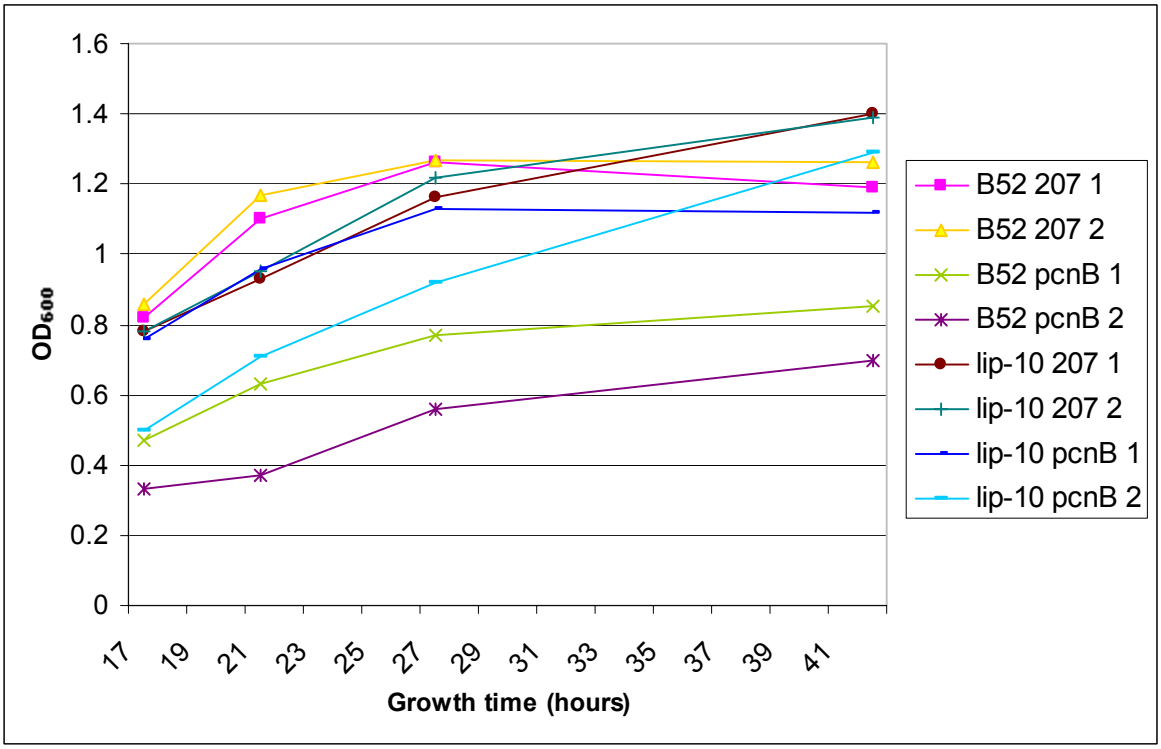
A subclone was made containing a *Sfi*I/*Not*I fragment which flanks the 'I' end of the transposon in the *Cla*I clone of the transposon insertion region. The gel-purified insert from this clone was radiolabelled by random hexamer priming and used to screen a 170 mm plate of the B52 λ-ZAP amplified library. A region containing a positive plaque was cored from the plate and the positive clone was isolated by secondary plaque screening. The insert was excised into the pBK-CMV phagemid vector and sequenced with the T3 and T7 vector primers to confirm that *pcnB* was not truncated at one end of the insert. The presence of *pcnB* was confirmed by sequencing the clone with a primer, *pcnB*-u1, designed to sequence upstream from within *pcnB*.

Restriction-enzyme mapping revealed that *Eco*RI and *Sac*I do not cut the wild-type *pcnB* clone insert, and these enzymes were used to clone the insert into the pMMB207 broad-host-range vector in *E. coli* S17.1. This pMMBpcnB plasmid was transferred to B52 wild-type and lip$^-$10 by conjugation. Lipase and protease activities were measured in liquid cultures of these strains grown in HEPES minimal medium at 22°C, with and without 150 mM NaCl. B52 and lip$^-$10 containing only pMMB207 were also assayed as controls (Figures 3.1-3.3).

It is clear from the complex assay results that the *pcnB* gene does not complement lip$^-$10. Lipase production appeared to be repressed by *pcnB* at low salt concentration, while being activated in 150 mM NaCl by *pcnB* in the mutant but not the wild-type. Protease production is repressed at low salt concentration by *pcnB* in the wild-type, and the lip$^-$10 mutant is protease-negative under these conditions with *pcnB* having no detectable effect. Similar results were obtained in 150 mM NaCl, except wild-type protease production was much lower due to repression by NaCl, and *pcnB* seemed to increase protease production by lip$^-$10 to roughly half wild-type levels. Complementation with *pcnB* stimulates both lipase and protease production by lip$^-$10 in 150 mM NaCl. The growth rates presented in Figure 3.1 show that the effect of *pcnB* knockout and overexpression cannot be explained by differences in growth rate, except that slow growth of B52/pMMBpcnB in low-salt media may be at least partly responsible for its lack of lipase and protease production.

Lip$^-$10 was originally isolated as being lipase deficient on 170mM NaCl, and duplicate number 2 of lip$^-$10/pMMB207 in Figure 3.2B produced approximately one third as much lipase as the wild-type in 150mM NaCl. The other duplicate produced far more lipase than the wild-type; however it also exhibited a very abnormal growth rate.

A



B

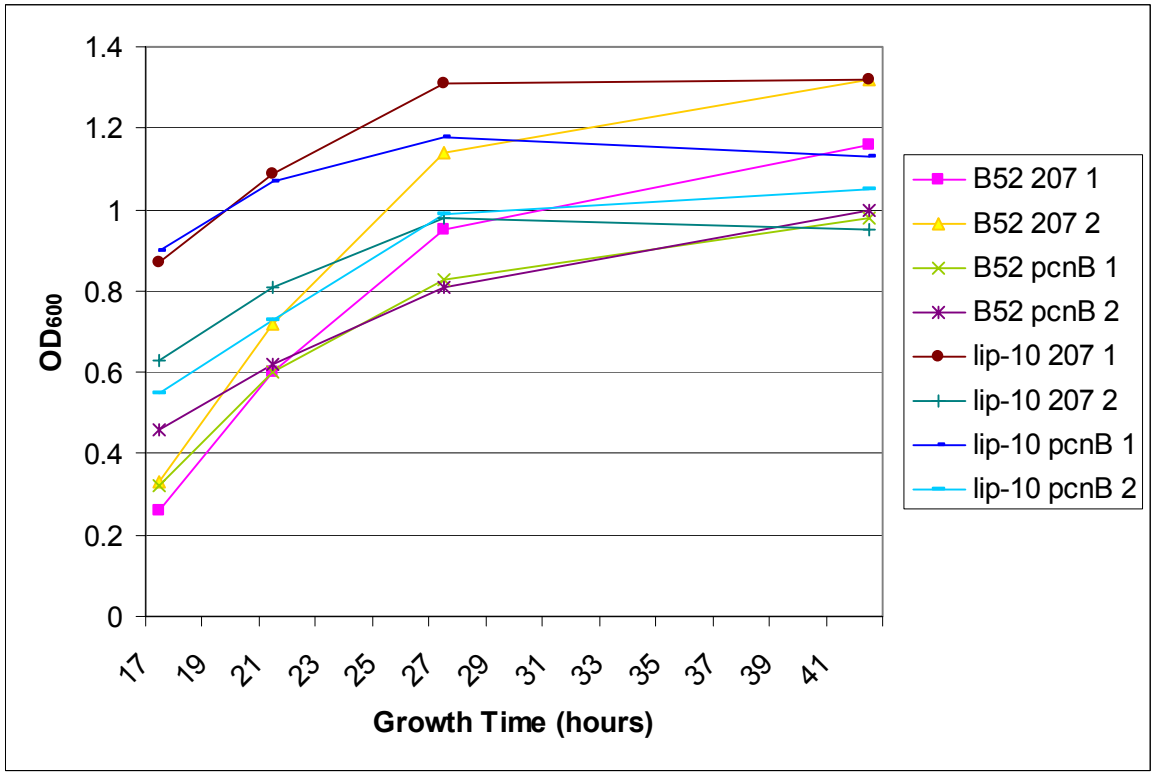
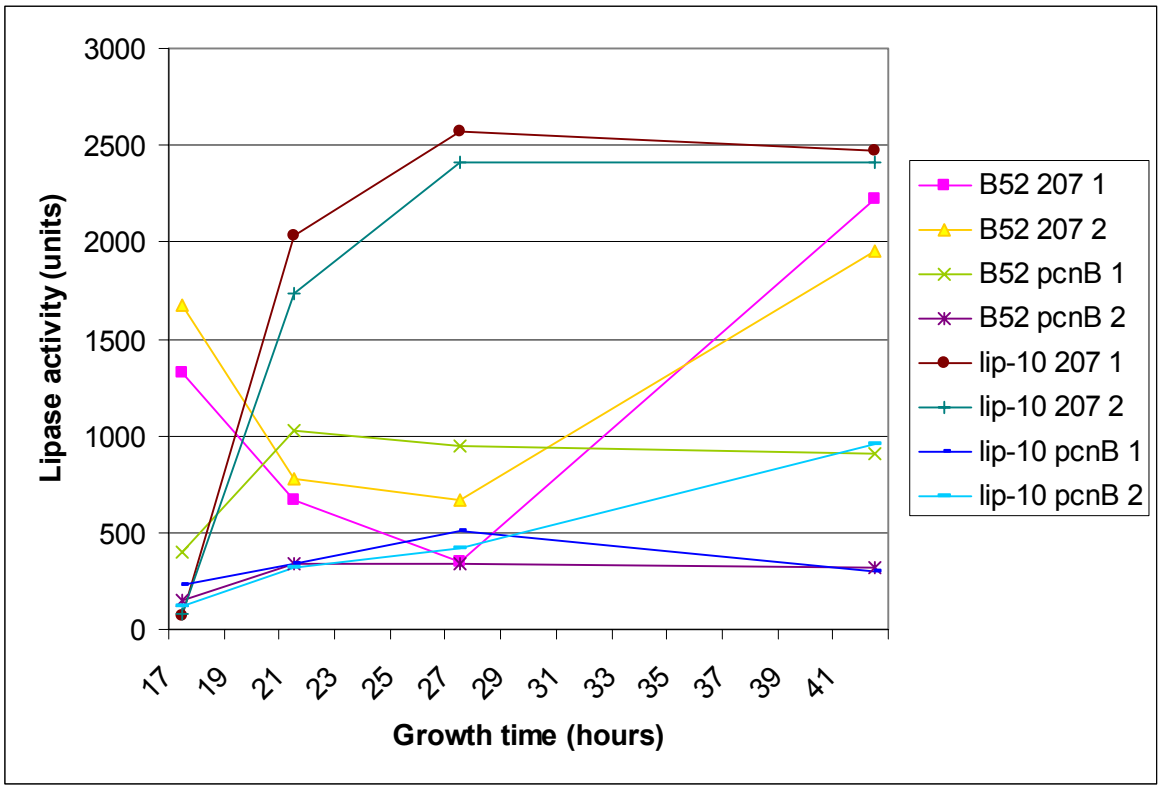


Figure 3.1. Growth of B52 and lip⁻10 cultures containing either pMMB207 vector or the complete *pcnB* gene in pMMBpcnB. Duplicate cultures of each strain were assayed (numbered 1 and 2). A, no NaCl; B, 150 mM NaCl.

A



B

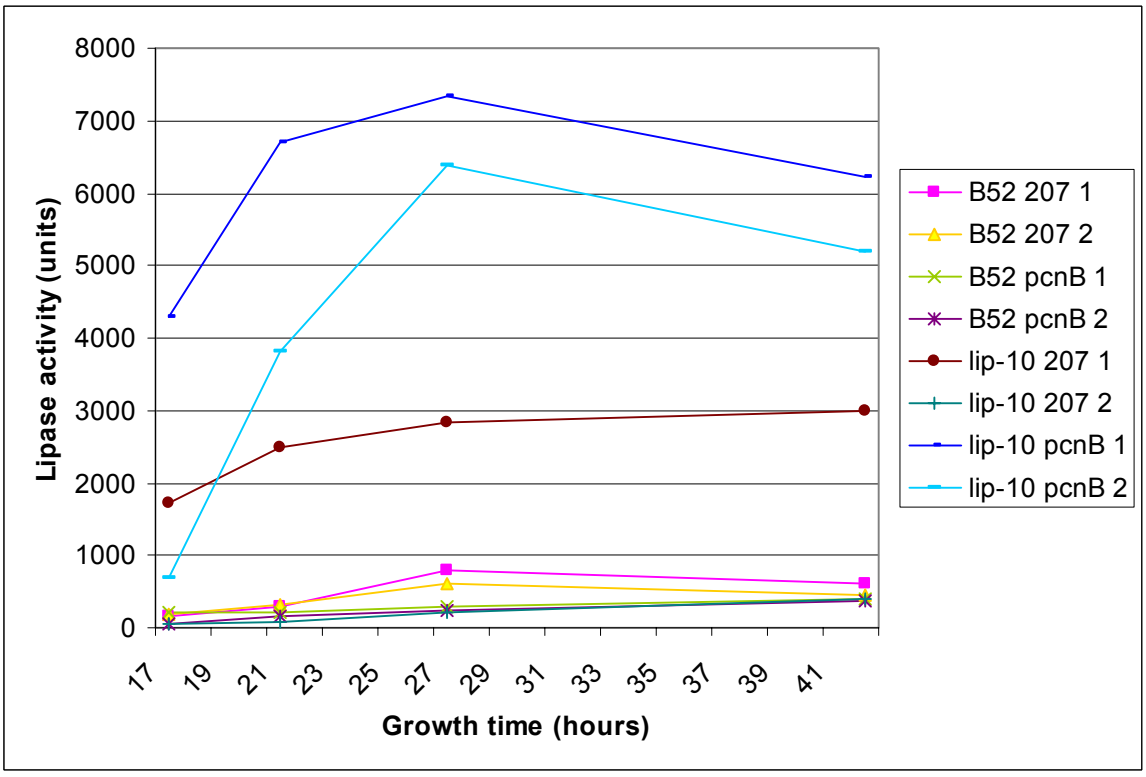
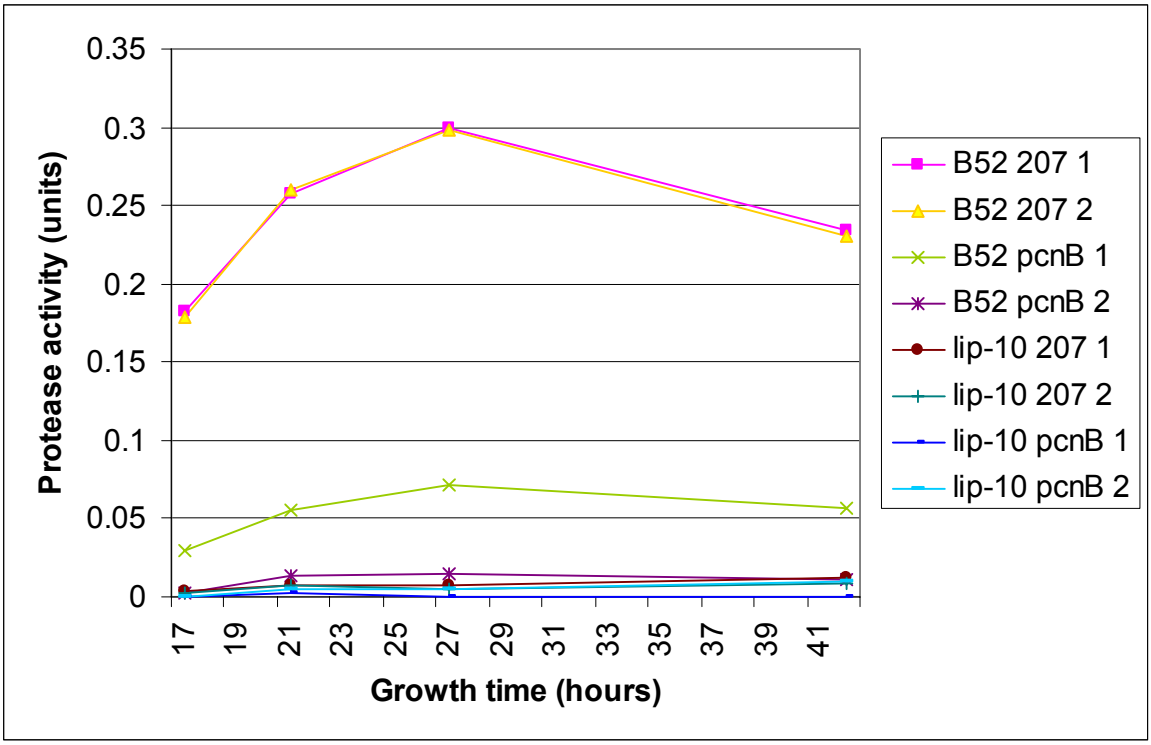


Figure 3.2. Lipase production by B52 and lip⁻10 cultures containing either pMMB207 vector or the complete *pcnB* gene in pMMBpcnB. Duplicate cultures of each strain were assayed (numbered 1 and 2). A, no NaCl; B, 150 mM NaCl.

A



B

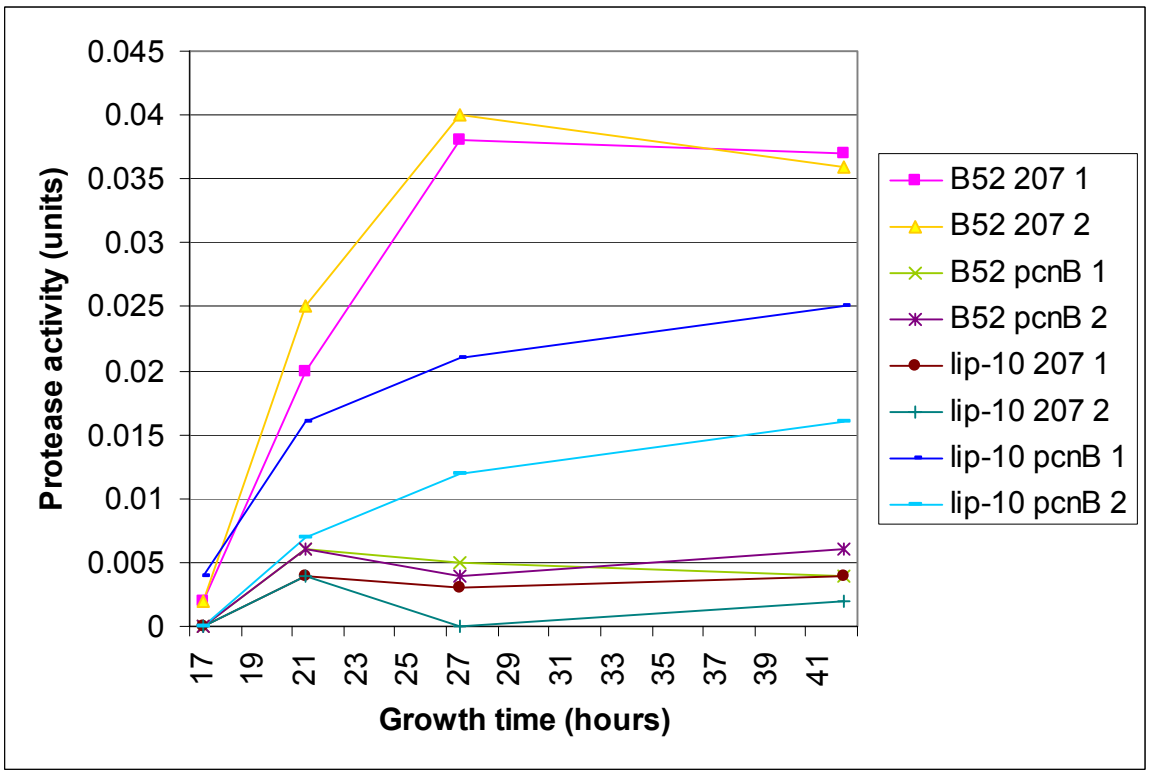


Figure 3.3. Protease production by B52 and lip⁻10 cultures containing either pMMB207 vector or the complete *pcnB* gene in pMMBpcnB. Duplicate cultures of each strain were assayed (numbered 1 and 2). A, no NaCl; B, 150 mM NaCl.

3.4 Discussion

Transposon mutagenesis of *P. fluorescens* was successful in identifying at least one gene, *envZ*, involved in the regulation of lipase and protease production. However, the lipase-deficient mutant, lip$^-$10, did not clearly identify the putative poly-A polymerase gene as being involved in regulation. Expression of *pcnB* in wild-type B52 and lip$^-$10 indicated that the genotypic difference between lip$^-$10 and wild-type B52 may be more than just a *pcnB*::mini-Tn5-km mutation. The transposition system used to generate lip$^-$10 is designed to allow multiple knockouts using different selectable markers (de Lorenzo *et al*, 1990), so it is unlikely that the transposon was remobilised after initially interrupting a different gene and leaving it damaged. It is possible, but unlikely, for a B52 cell to receive two copies of the transposon vector which subsequently targeted two locations on the genome. This could be tested by Southern blot with a mini-Tn5-km probe. The genotype of lip$^-$10 was not pursued further in this study.

Expression of multiple copies of *pcnB* in B52 wild-type seemed to cause significant inhibition of lipase and protease production. This effect is unclear in low-salt medium due to a reduction in growth rate, but is definite at high NaCl concentration. Although increased polyadenylation is likely to decrease the stability of many mRNAs, as it does in *E. coli* (Mohanty and Kushner, 1999), the effect on lipase and protease is well out of proportion with the effect on growth rate. Polyadenylation may have a particular effect on the stability of the mRNA containing *aprX* and *lipA*, or it may be an important factor in the regulatory system controlling lipase and protease production. In this regard, RNA processing and differential mRNA stability have been suggested as mechanisms of regulation of the *aprX-lipA* operon (Woods *et al*, 2001).

It is not easy to speculate on the apparent activation of lipase and protease expression in lip$^-$10 by *pcnB* overexpression in 150 mM NaCl media, given that *pcnB* was found to repress lipase and protease in wild-type B52. A non-polar *pcnB* knockout mutant is needed to determine more rigorously the effect of a *pcnB*-negative phenotype.

Several other lipase-negative mutants were isolated, but the region of transposon insertion in these mutants could not be cloned into pSK from digests with *Cla*I or several other enzymes which do not cut mini-Tn5-km. Partial digests with *Sau*3A might be successfully used for cloning these disrupted genes for further study.

Chapter 4

The EnvZ/OmpR System

4.1 Introduction

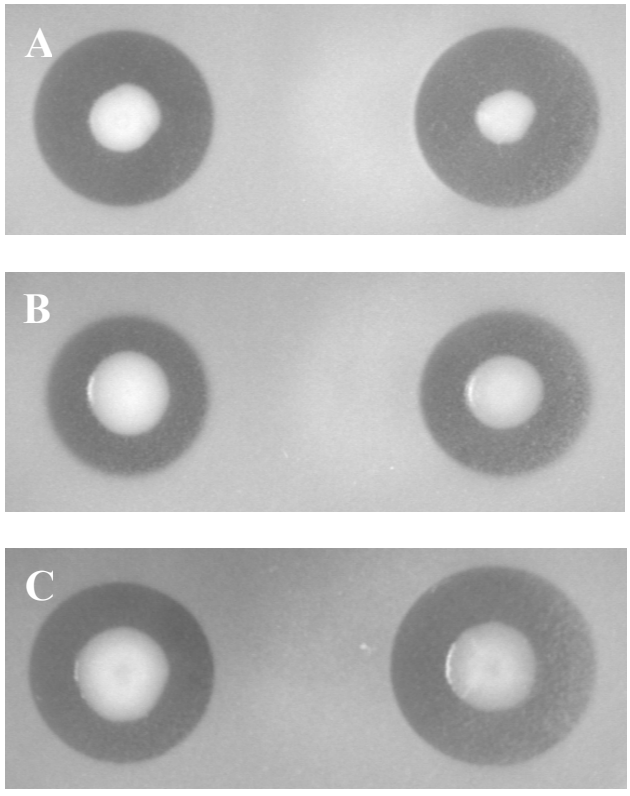
Three independent transposon mutations of the putative *envZ* osmolarity sensor gene were identified by their overexpression of lipase. EnvZ in *E. coli* is a transmembrane sensor which was thought to respond to osmolarity, but may be a sensor of solute concentration (Sleator and Hill, 2001; Jung *et al*, 2001; see Section 1.2.7). EnvZ activates the OmpR transcriptional regulator by phosphorylation (Waukau and Forst, 1992). The *ompR* gene is located upstream of *envZ* and the two genes constitute an operon (Comeau *et al*, 1985). Isolation of three independent *envZ* mutants demonstrated a clear involvement in regulation of lipase and protease production in B52.

There were no reports of regulation of extracellular enzymes of psychrotrophs by osmolarity in the literature, so B52 had always been grown on Luria-Bertani agar, a high osmolarity medium. In this Chapter we sought to confirm repression of lipase and protease by osmolarity on tributyrin and skim-milk agar and quantitate it in liquid culture. The complete sequence of *envZ* was required for an alignment of the entire putative EnvZ protein with its *E. coli* homologue, and sequencing upstream was required to locate a homologue of the *ompR* gene and confirm identity of the *ompR-envZ* operon. Final confirmation of the involvement of *ompR-envZ* in lipase and protease regulation would be provided by cloning the wild-type *ompR-envZ* from B52 and expressing it in the *envZ*::mini-Tn5-km mutant.

4.2 Regulation by Salt Concentration

4.2.1 Repression of Lipase and Protease Production by NaCl

B52 wild-type and the *envZ*::mini-Tn5-km mutants were patched onto Luria-Bertani agar plates containing skim-milk or tributyrin, for detecting lipase and protease respectively, and either 300 mM of added NaCl or no added NaCl. After incubation at room temperature for two days it was clear that lipase and protease expression is repressed by 300 mM NaCl, with protease production being almost abolished (data not shown). Later experiments showed that 150 mM added NaCl is sufficient to cause strong repression of lipase and protease production on agar, except for lipase at room temperature (Figure 4.1). Production of both enzymes is repressed by 150 mM NaCl at room temperature in liquid culture (Figure 4.4).



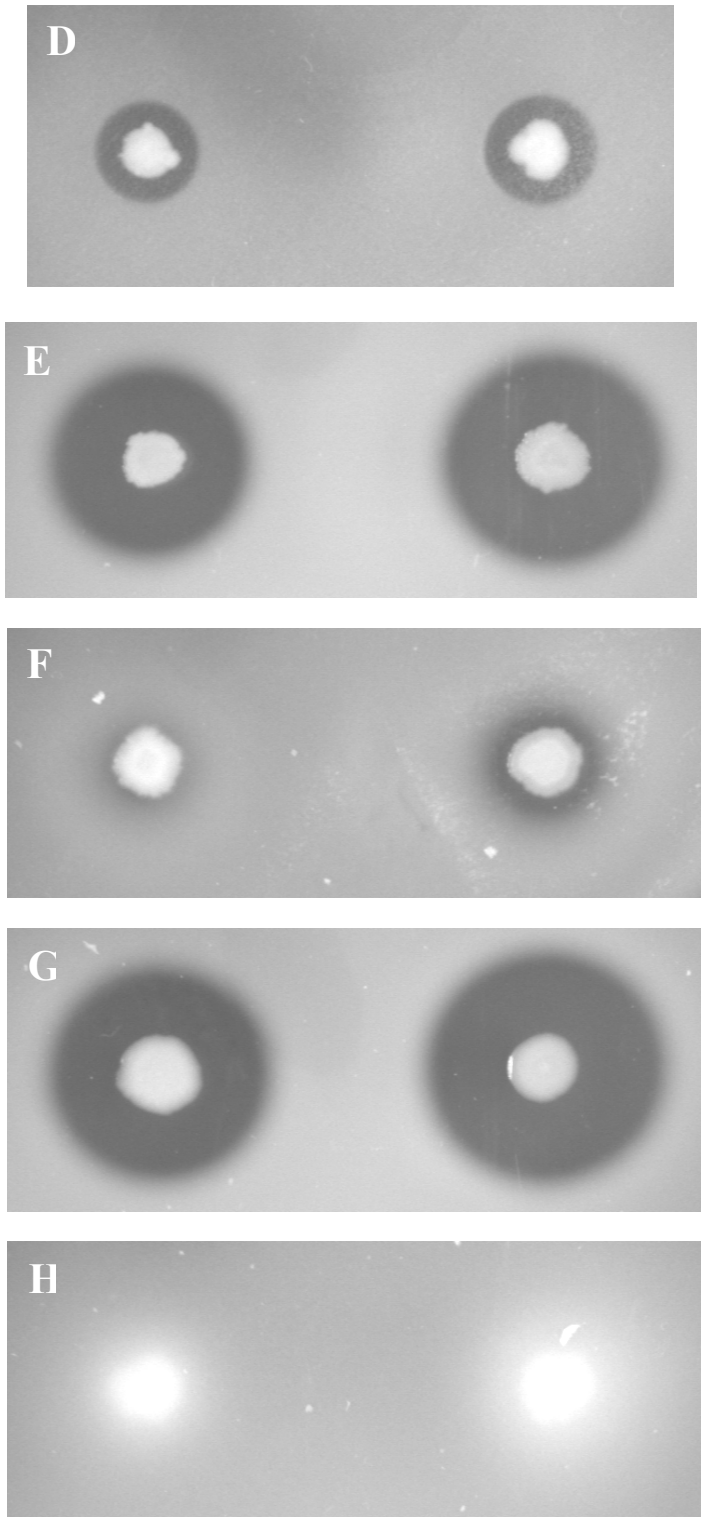


Figure 4.1. B52 wild-type (left) and the lip^{++}3 *envZ::*mini-Tn5-km isolate (right) patched onto LB tributyrin (TB) and skim-milk (SM) agar. **A**, TB, no added NaCl, 23°C. **B**, TB, 150 mM added NaCl, 23°C. **C**, TB, no added NaCl, 30°C. **D**, TB, 150 mM added NaCl, 30°C. **E**, SM, no added NaCl, 23°C. **F**, SM, 150 mM added NaCl, 23°C. **G**, SM, no added NaCl, 30°C. **H**, SM, 150 mM added NaCl, 30°C.

4.2.2 Lipase and Protease Production is Not Repressed by Osmolarity

LB-agar plates containing 200 mM sucrose or sorbitol and no added NaCl caused little or no lipase repression at 30°C. 300 mM arabinose and 300 mM maltose did not repress lipase or protease production on LB-agar at 17°C or 30°C (data not shown). These results are consistent with published observations that EnvZ does not respond to various sugars, glycine betaine, proline or Tris/2-morpholinoethanesulfonic acid and it is in fact a salt sensor (Jung *et al*, 2001).

4.2.3 Repression by Na^+ and K^+ is Identical in B52

Jung *et al* (2001) also reported that K^+ stimulated *E. coli* EnvZ at twice the level of Na^+. However, assays of liquid B52 cultures grown in HEPES minimal media found no significant difference between repression of protease production by 100 mM NaCl and 100 mM KCl (data not shown).

4.3 The *ompR-envZ* Operon of *P. fluorescens* B52

Sequencing of the B52 *ompR-envZ* operon was accomplished by a combination of sequencing from the I1-out primer from the transposon in the three mutants, sequencing from vector primers on two sets of unidirectional deletions of the I-end flanking sequence, sequencing from three primers designed from existing sequence, and sequencing from a vector primer on the wild-type *ompR-envZ* clone pBKenvZ1.2, which lacks the 5' end of *ompR*.

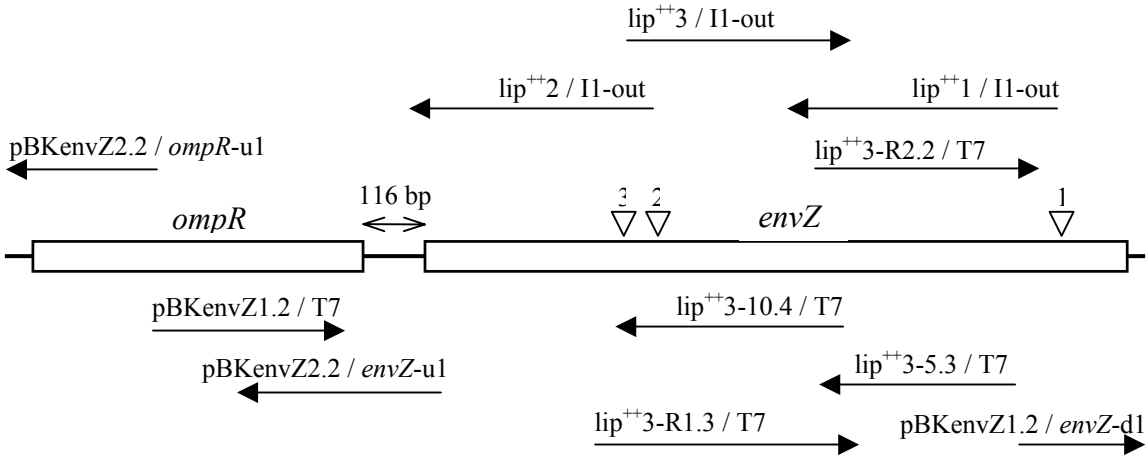


Figure 4.2. Structure of B52 *ompR-envZ* and sequencing strategy. The I1-out primer was used on *Cla*I clones from the three lip^{++} mutants, and reveals the location of the transposon in the *envZ* gene. The unidirectional deletion plasmids are lip^{++}3-5.3, –10.4, –R1.3 and –R2.2, and were sequenced with the T7 vector primer. The transposon insertion points are shown by inverted triangles.

A construct for unidirectional deletions was made by subcloning the transposon and I-end flanking sequence from the lip^{++}3 *Cla*I clone with *Not*I, then deleting the transposon with *Sfi*I, cloning the religated plasmid and using the *Cla*I and *Kpn*I sites in the vector for performing the deletions. Deletions in the opposite direction were accomplished by cutting out the insert with *Not*I, religating and choosing a clone with a reversed insert.

The operon sequence (Appendix 1) showed that *ompR* and *envZ* are present in the same order as found in *E. coli* (Mizuno *et al*, 1982; Comeau *et al*, 1985), but unlike *E. coli* the *envZ* coding sequence does not overlap that of *ompR*. The alignment in Figure 4.3 shows that the *P. fluorescens* B52 EnvZ protein contains the conserved sequence motifs present in type IA histidine kinases such as EnvZ (Kim and Forst, 2001). Sequence identity to *E. coli* EnvZ is particularly high surrounding the H-box motif, which includes the histidine site of phosphorylation. Overall similarity of B52 EnvZ is 92% with *P. fluorescens* Pf0-1,

90% with *P. syringae* pv. syringae, 87% with *Azotobacter vinelandii*, 85% with *P. aeruginosa* PA01 and 53% with *E. coli*.

Figure 4.3. Alignment of the *P. fluorescens* B52 EnvZ and *E. coli* EnvZ amino-acid sequences showing the conserved H-box, N, G1, F, G2 and G3 motifs, and the conserved positive amino-acid residue (+) at the end of helix 2 (Kim and Forst, 2001).

Overall similarity of B52 OmpR is 96% with *P. syringae* pv. syringae, 89% with *Azotobacter vinelandii* and *P. aeruginosa* PA01, and 82% with *E. coli*. B52 OmpR contains the conserved aspartate phosphorylation site found in *E. coli* OmpR and other homologues.

4.4 Complementation of the *envZ*::mini-Tn5-km Mutant

4.4.1 Cloning the wild-type *ompR-envZ* operon

A radiolabelled oligonucleotide probe was made using the *envZ*-u1 sequencing primer, which binds near the 5' end of *envZ* in the upstream direction. The primer was made by the same PCR program used for dye-labelled sequencing, except the extension temperature was 74°C. The template for the reaction was 0.2 μg of a *Cla*I clone of the *ompR-envZ* region from lip^{++}2.

The B52 λ-ZAP genomic library was screened with the *ompR-envZ* probe, two positive plaques were cored from the primary plate, and individual positive plaques were isolated by secondary plaque screening. The inserts from the two clones were excised into the pBK-CMV phagemid vector and DNA was purified from the resulting phagemid clones by miniprep. The inserts were sequenced from both ends with the T3 and T7 vector primers to check that the entire *ompR-envZ* operon was present. The insert in the pBKenvZ2.2 clone had no homology to *ompR* or *envZ* at either end; however, pBKenvZ1.2 contains *ompR* at the T3 end with 82 N-terminal amino-acids missing. This sequence was useful in completing the *ompR* sequencing. The presence of *ompR-envZ* in pBKenvZ2.2 was confirmed by sequencing from the *envZ*-u1 and *ompR*-u1 primers.

4.4.2 Expression of the *ompR-envZ* Operon in the *envZ*::mini-Tn5-km Mutant

Restriction mapping of the complete *ompR-envZ* clone showed that the entire insert could be removed with *Hind*III and *Spe*I. This fragment was cloned into the broad-host-range

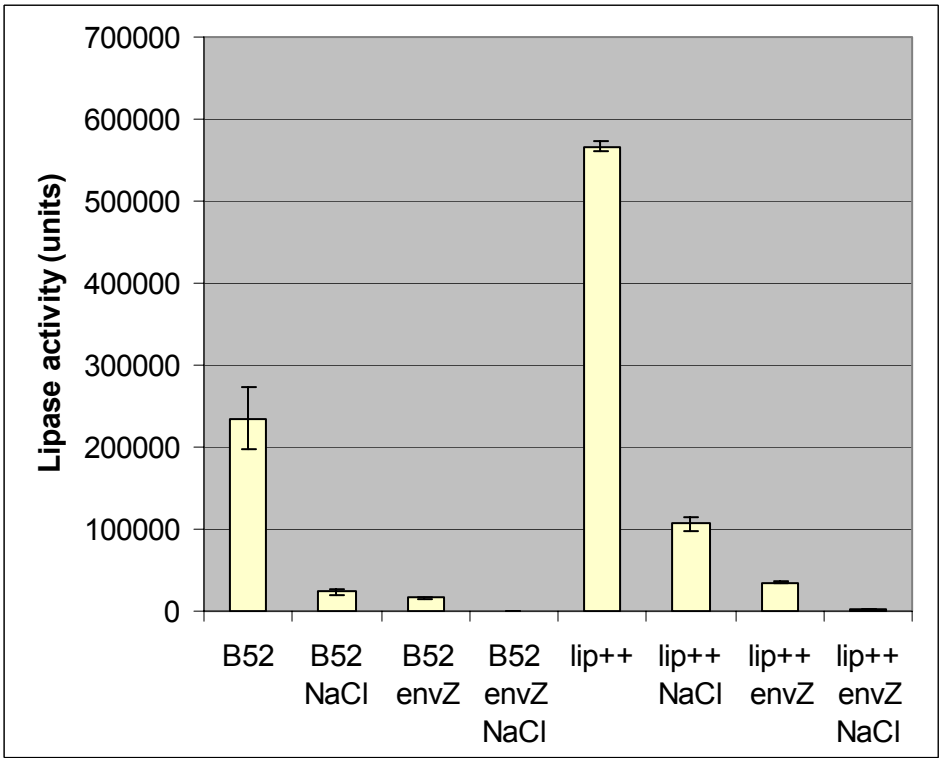
vector pBBR1MCS cut with *Hind*III and *Xba*I and grown in *E. coli* S17.1λ-pir (although λ-pir is not required for this vector). The clone was transferred into B52 wild-type and lip^{++}3 by conjugation. Assays on tributyrin and skim-milk plates showed that the presence of multiple copies of *ompR-envZ* strongly represses lipase and protease production even at low NaCl concentration (data not shown).

4.5 Liquid-Culture Assays of the Effect of *envZ* Mutation and *envZ-ompR* Overexpression

4.5.1 Lipase and Protease Assays

Lipase and protease assays were performed on B52 wild-type and lip^{++}3 containing either pBBR1MCS vector control or pBBR1ompRenvZ, in HEPES minimal media containing no NaCl or 150 mM NaCl, at 24°C. The assays showed that lipase production is substantially higher in the absence of functional EnvZ, with the difference being greater at high NaCl concentration. Protease production was increased little in low NaCl media by the lack of EnvZ, with the difference being more significant at high NaCl concentration. However, both lipase and protease production was still repressed by NaCl in the absence of functional EnvZ. Multiple copies of the *ompR-envZ* operon repressed lipase and protease production, with lipase being almost completely abolished at high NaCl concentration. The effect of NaCl was not due to an effect on the enzymes themselves during lipase and protease assays, because a relatively small quantity of culture supernatant was added to the assay mixture (Sections 2.30 and 2.31).

A



B

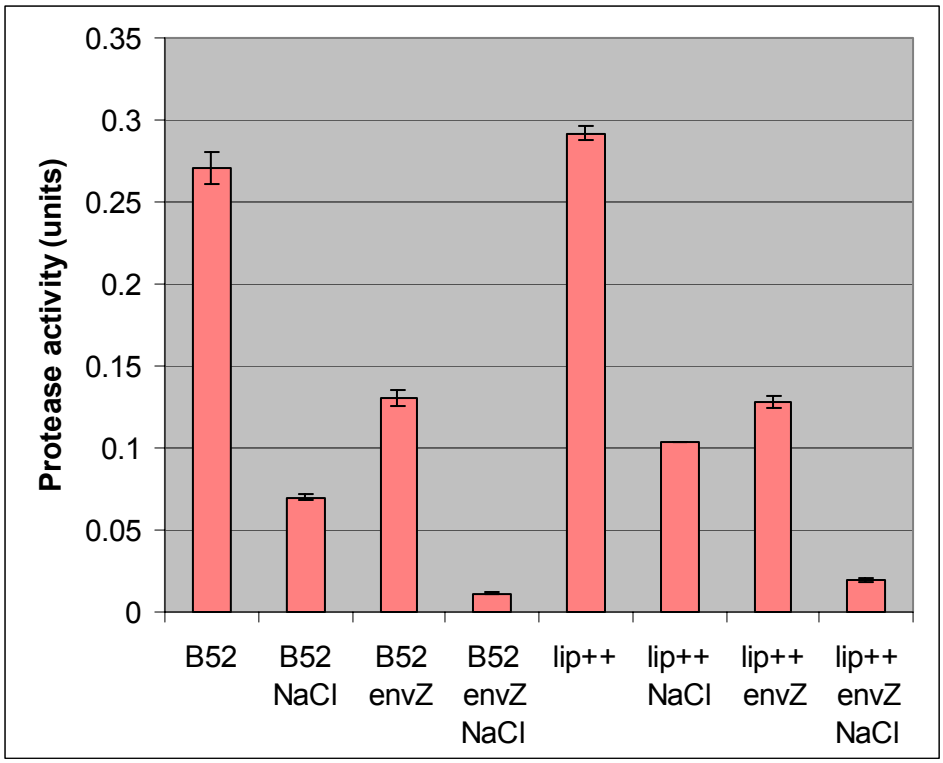


Figure 4.4. Liquid-culture assays of the effect of *ompR-envZ* overexpression on lipase and protease production. Error bars show the variation between duplicates. A, lipase assays; B, protease assays. Samples marked 'NaCl' contain 150 mM NaCl. Samples marked 'envZ' contain pBBR1ompRenvZ.

4.5.2 β-galactosidase Assays of *lacZ* Fusion Strains

Chromosomal translational *lacZ* fusions of *aprX* and *lipA* were constructed to monitor transcriptional and/or translational regulation of these genes (Woods, 2000). The B52 *aprX*'-'*lacZ* strain contains the N-terminal 35 codons of *aprX* fused to *lac*Z, and the B52B strain contains 17 N-terminal *lipA* codons fused to *lac*Z. Plasmids pBBR1MCS and pBBR1ompRenvZ were transferred into these fusion strains by conjugation, and the effects of *ompR-envZ* overexpression and NaCl were measured by β-galactosidase assays of liquid cultures. Repression of the *lipA* fusion by EnvZ/OmpR was much more substantial than repression of the *aprX* fusion (Figures 4.5 and 4.6). Addition of 150 mM NaCl did not repress either fusion significantly.

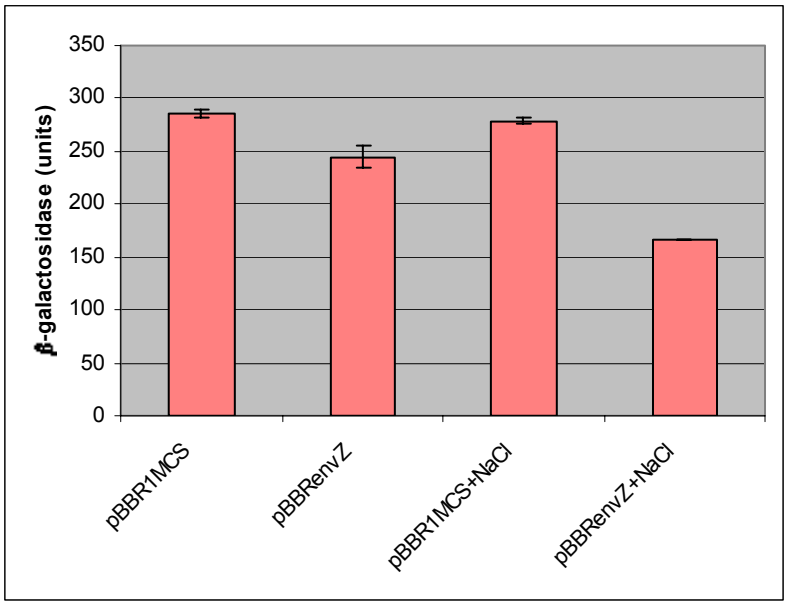


Figure 4.5. β-galactosidase assays of cultures of B52 *aprX'-'lacZ* containing pBBR1MCS (control) or pBBR1ompRenvZ. HEPES-minimal media containing no NaCl or 150mM NaCl was used. Error bars show the variation between duplicate cultures.

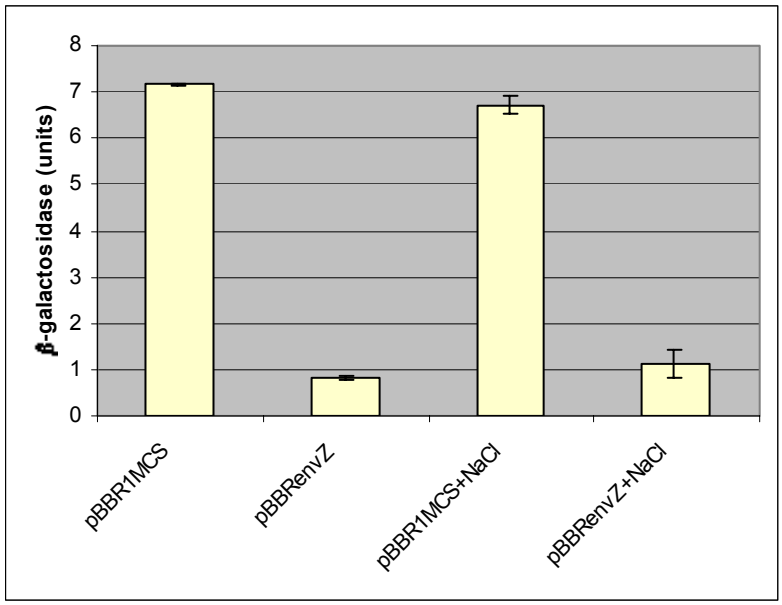


Figure 4.6. β-galactosidase assays of cultures of B52B (*lipA'-'lacZ*) containing pBBR1MCS (control) or pBBR1ompRenvZ. HEPES-minimal media containing no NaCl or 150mM NaCl was used. Error bars show the variation between duplicate cultures.

4.6 Attempted Knockout of the B52 *ompR* Gene

It is known that *E. coli* OmpR can be phosphorylated by histidine-kinases other than EnvZ (Forst *et al*, 1988, 1990; Nagasawa *et al*, 1993). This mechanism may be at least partly responsible for the repression of lipase and protease production by B52 at high NaCl concentration. If this is the case, a mutant lacking functional OmpR would exhibit reduced regulation by NaCl concentration.

The *ompR* gene is large enough to inactivate it by a single DNA crossover with an internal fragment of *ompR*. Two oligonucleotide primers were synthesised to produce the required fragment by PCR:

```
ompR-K5.2   5′ GTGAATTCAAGGGCCTTGAGCTG 3′

ompR-K3     5′ GTGGATCCATCACCAGGGCCTTG 3′
```

A single crossover mutant made with the product of these primers would have two copies of *ompR*, one with the first 292 nucleotides missing, and another with the last 198 nucleotides missing.

The PCR reaction was run with the wild-type *ompR-envZ* phagemid clone as template and annealing temperatures of 40°C for 4 cycles and 55°C for 21 cycles. The low initial annealing temperature was used because the restriction-site component of the primers does not bind to the template. The PCR product was cut with *Eco*RI and *Bam*HI, purified with QIAEX and cloned into the suicide vector pJP5608. The clone was checked by sequencing with the pJP5608E vector sequencing primer, which confirmed the presence of the desired 261 bp *ompR* fragment.

Despite several attempts, including a large conjugation on an agar slope in a 10 ml tube, and plating of the exconjugants on media containing no added NaCl, no *ompR* mutant was isolated. Knockout mutants of *ompR* have been constructed in *E. coli* (Comeau *et al*, 1985) and *Salmonella typhimurium* (Mills *et al*, 1998), but it is possible that OmpR is required for growth of *P. fluorescens* B52, even on low-salt medium.

4.7 Discussion

The isolation of three independent mutants of a homologue of the *E. coli* EnvZ osmolarity sensor which overproduces lipase and protease indicated that EnvZ and possibly osmolarity are involved in the regulation of lipase and protease production. Subsequent experiments showed that this is indeed the case, with overexpression of EnvZ and its response-regulator OmpR causing a high level of repression of lipase and protease

production, with these enzymes also being repressed by high NaCl concentration. Repression of lipase was substantially greater than repression of protease by NaCl or EnvZ/OmpR in liquid culture. The effect of high NaCl concentration is enhanced by the presence of multiple copies of *ompR-envZ*. The protease assays show this clearly, with protease production by wild-type B52 being reduced approximately four-fold by 150 mM NaCl, while the overproduction of EnvZ/OmpR increases protease repression by NaCl to more than ten-fold (Figure 4.4B). This is consistent with the notion that the EnvZ/OmpR system in B52 represses lipase and protease expression in response to NaCl concentration. Repression by EnvZ/OmpR in the absence of NaCl shows that some phosphorylation of OmpR could occur without stimulus by NaCl. This also occurs in *E. coli*, where *ompF* activation requires a low level of OmpR-P (Slauch and Silhavy, 1989). High osmolarity generated by non-ionic solutes such as sucrose, sorbitol, arabinose and maltose did not cause significant repression, a result consistent with a recent report on the properties of *E. coli* EnvZ (Jung *et al*, 2001).

The *envZ*::mini-Tn5-km mutation in B52 reduced but did not abolish repression of lipase and protease production by NaCl (Figure 4.4), so OmpR may be phosphorylated by another histidine-kinase enzyme, and either that enzyme or OmpR-P itself may respond to NaCl concentration. A broad range of species, such as *E. coli* and *P. aeruginosa*, contain large numbers of related histidine kinases (Kim and Forst, 2001), and 'crosstalk' between two-component systems, in which the histidine-kinase of one system phosphorylates the response-regulator of another, has been observed in *E. coli* (Forst *et al*, 1988, 1990; Nagasawa *et al*, 1993). However, available evidence suggests that NaCl can repress lipase production by an OmpR-independent mechanism. NaCl strongly repressed lipase production, but did not repress the *lipA*'-'*lacZ* fusions. In contrast, *ompR-envZ*

overexpression strongly repressed both lipase production and the *lipA*'-'*lacZ* fusion. Repression by NaCl in the *envZ*::mini-Tn5-km mutant may be due entirely to an OmpR-independent mechanism; however it seems equally likely that OmpR is phosphorylated independently of EnvZ due to crosstalk. An *ompR* mutant could not be isolated so this hypothesis could not be tested.

Genetic evidence strongly indicates that the *aprX* and *lipA* genes are cotranscribed onto a single mRNA (Woods, 2000; and see Figure 1.5). The relative lack of repression of the *aprX'-'lacZ* translational fusion by *ompR-envZ* in multicopy is therefore apparently inconsistent with the substantial repression of the *lipA'-'lacZ* translational fusion. The *aprX'-'lacZ* fusion was not regulated by NaCl, however measurements of *aprX* mRNA levels did show repression by NaCl (Woods, 2000), so the *aprX'-'lacZ* fusion may not be regulated as expected. One possible explanation for this is that repression of *aprX* by NaCl is due to reduction of mRNA stability or termination of transcription past the point where *lacZ* was inserted. The low levels of β-galactosidase from *lipA'-'lacZ*, and of *lipA* mRNA (Woods, 2000; and see Figures 4.5, 4.6), compared to *aprX'-'lacZ* and *aprX* mRNA, show that one or both of these mechanisms affect the 3' end of the mRNA where *lipA* is located. Regulation of mRNA decay downstream from the *lipA*'-'*lacZ* fusion point would explain the repression of lipase production by NaCl which is not detected by the fusion, and hence is apparently OmpR-independent (cf. Figures 4.4A and 4.6).

Chapter 5

Other Potential Regulators

5.1 Introduction

5.1.1 The MspA Sensor

A trans-membrane histidine-kinase sensor gene, *mspA*, was identified in *P. fluorescens* LS107d$_2$ by transposon mutagenesis which produced an *mspA* mutant (prot$^-$8) deficient in lipase and protease production (Burger, 2000). It was speculated that if *mspA* is present in *P. fluorescens* B52, it may be involved in regulation of lipase and protease in that strain also. Therefore it was necessary to clone the B52 *mspA* gene and determine the effects of both site-directed mutagenesis and overexpression from multiple plasmid copies.

5.1.2 The RsmA Translation Regulator

RsmA is an RNA-binding translation regulator which controls production of extracellular enzymes, including protease, by the soft-rot bacterium *Erwinia carotovora* subsp. *carotovora* (Cui *et al*, 1995; Chatterjee *et al*, 1995). RsmA binds to the ribosome-binding site of enzyme genes, repressing translation. Homologous regulatory systems are found in *E. coli* (Liu and Romeo, 1997) and *P. fluorescens* strains F113 (Aarons *et al*, 2000) and CHA0 (Blumer *et al*, 1999). RsmA is itself negatively regulated by binding to an RNA molecule, RsmB, which sequesters RsmA (Liu *et al*, 1998).

Comparison of sequences in the ribosome-binding region of *aprX* and *lipA* in *P. fluorescens* B52 with binding sites for the RsmA family revealed apparently significant similarity, especially in *aprX*. Cloning of the putative B52 *rsmA* gene and generation of a

site-directed *rsmA* mutant were required for investigation of the possibility that an RsmA homologue is involved in regulation of lipase and protease production in B52.

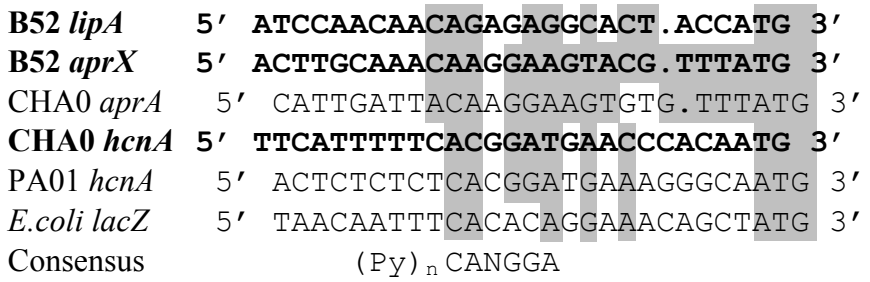


Figure 5.1. Alignment of B52 *lipA* and *aprX* ribosome-binding regions with RsmA-regulated sequences from *P. fluorescens* CHA0 alkaline protease (*aprA*) and hydrogen cyanide biosynthesis (*hcnA*) genes, and *P. aeruginosa* PA01 *hcnA* gene, with *E. coli lacZ* as an example of an unregulated gene. The consensus was identified by mutagenesis experiments, except for the first 'G' which was not tested (Blumer *et al*, 1999).

5.1.3 The PbrA Sigma-Factor

PbrA is an ECF sigma-factor which activates iron-regulated promoters in *P. fluorescens* M114 (Sexton *et al*, 1996). As lipase and protease production by B52 is also iron-regulated, it was speculated that a PrbA homologue may be involved. The *pbrA* gene of M114 is available on a broad-host-range vector, enabling its regulatory effect in B52 to be determined.

5.2 Cloning, Overexpression and Mutagenesis of B52 *mspA*

5.2.1 Cloning the Wild-Type *mspA* Gene

A radiolabelled DNA probe was made by extension of the MB02 sequencing primer which binds downstream of mini-Tn5-km in the LS107d$_2$ *mspA* mutant (Burger, 2000). A clone of the transposon insertion region from this mutant, pCLA8, was used as the template. The probe was used to screen the B52 λ-ZAP library on a 170 mm plate. Three positive plaques were selected for secondary screening, and one positive from each secondary plate was excised into pBK-CMV. The presence of *mspA* in these clones was confirmed by Southern blot of *Bss*HII digests of the three clones and pCLA8, using the same probe as described above (data not shown).

5.2.2 Sequencing of *mspRA*

A subclone of one of the pBK-CMV clones (pBKmspA2) was made by deleting approximately 2 kb from the T3 end of the insert using *Bss*HII and religating the plasmid so that it contained a shortened insert. Sequencing of this subclone (pBKmspA4) from the T3 vector primer revealed that in addition to the remaining portion of the insert, a small *Bss*HII fragment had been cloned as well, resulting in two inserts being present. A BLAST search found homology to DNA-binding response regulators, potentially the *mspR* gene. Downstream sequencing by primer walking located the *mspA* gene (Figure 5.2).

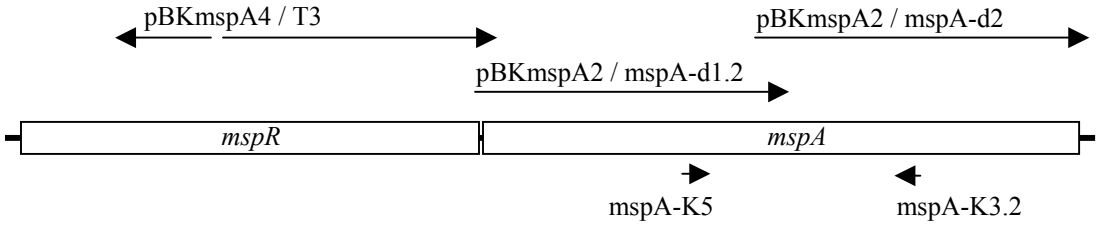


Figure 5.2. Sequencing strategy and PCR primers for making the *mspA* knockout construct. Sequencing template and primer names are shown. The pBKmspA4 plasmid contains a short *mspR* fragment in opposite orientation to the larger fragment.

5.2.3 Construction of an *mspA*::Tc^r Single-Crossover Mutant

An internal 413 bp fragment of *mspA* was made by PCR using the following primers:

```
mspA-K5     5′ TCGGATCCTCGCGAGGCGCGGC 3′
mspA-K3.2   5′ GCGAATTCGATCAGGTTGTTG  3′
```

Annealing temperatures of 50°C for 2 cycles and 55°C for 23 cycles were used for the PCR reaction. The PCR product and the suicide vector pJP5608 were cut with *Bam*HI and *Eco*RI, purified with QIAEX, ligated together and transferred into competent S17.1λ-pir. A putative recombinant containing the correct *Bam*HI/*Eco*RI fragment was checked by sequencing with the *mspA*-K5 primer. This pJPmspAKO plasmid was transferred into B52 by conjugation and a tetracycline-resistant mutant was isolated. The mutant was tested by PCR reaction on mutant genomic DNA and the pJPmspAKO construct using *mspA*-K5 and the pJP5608E vector primer (5' GGGCGATCGGTGCGGGC 3'). The PCR reactions yielded equal-sized fragments from the mutant and pJPmspAKO, of ~500 bp. To confirm the knockout of *mspA*, genomic DNA from B52 wild-type and *mspA*::Tc^r, and pBKmspA plasmid DNA, was cut with *Eco*RI and *Bam*HI, Southern blotted and then probed with a

radiolabelled DNA probe made by extension of the *mspA*-K5 primer on a pBKmspA template. The Southern blot showed that wild-type *mspA* is located on a single fragment with a *Bam*HI or *Eco*RI site at each end (Figure 5.4). An *mspA* single-crossover mutant would contain two copies of *mspA*, one with the 3' end truncated and the other with the 5' end truncated (Figure 5.3). A *Bam*HI-*Eco*RI digest of mutant DNA would therefore contain two fragments with *mspA* sequences, which was confirmed by the Southern blot (Figure 5.4).

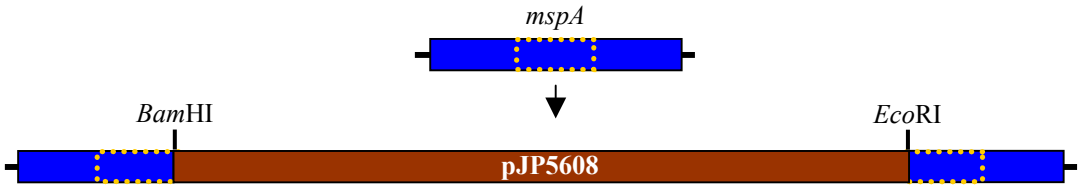


Figure 5.3. The *mspA* gene (blue) was disrupted by single crossover with an internal *mspA* fragment (yellow dots) cloned into pJP5608 (red).

5.2.4 Phenotype of the B52 *mspA*::pJP5608 Mutant

The mutant was patched onto Luria-Bertani agar plates containing skim-milk and either no added NaCl or 150 mM added NaCl. Unlike LS107d$_2$ prot$^-$8, which was clearly protease negative on skim-milk agar at low and high NaCl concentrations, protease production by the B52 *mspA* mutant was not significantly different to that of the wild-type (data not shown).

5.2.5 Effect of Multiple Copies of *mspRA*

The insert from pBKmspA was removed by digestion with *Pst*I and *Xba*I and cloned into the *Pst*I and *Xba*I sites of the pBBR1MCS broad host-range vector. This plasmid was

transferred into B52 by conjugation and several exconjugants were patched together with wild-type B52 onto skim-milk Luria-Bertani agar plates with no added NaCl and 150 mM added NaCl. No significant difference in protease production due to *mspRA* overexpression was observed.

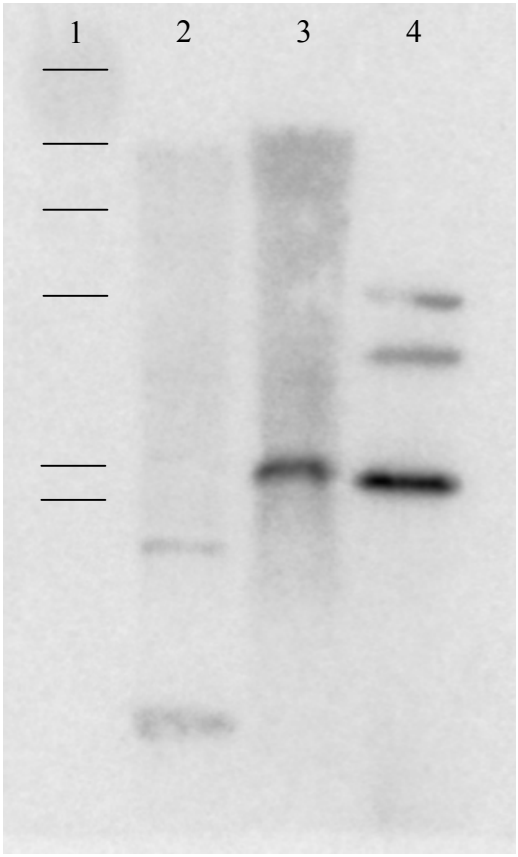


Figure 5.4. Southern blot of *Bam*HI/*Eco*RI digests showing disruption of *mspA*. Lane 1, λ-*Hind*III standards; 2, B52 *mspA* mutant genomic DNA; 3, B52 wild-type genomic DNA; 4, pBKmspA.

5.3 Mutagenesis of the B52 *rsmA* Gene

5.3.1 Isolation of a Fragment of B52 *rsmA* by PCR

An internal fragment of *rsmA* was amplified by PCR using two primers, *rsmA*-K5 and *rsmA*-K3, based on an alignment between *rsmA* sequences from *Erwinia carotovora*, *P. fluorescens* and *Serratia marcescens*. The product was cut with *Eco*RI and *Bam*HI and cloned into pSK. The insert from this clone was transferred into the *Eco*RI and *Bam*HI sites of pJP5608 for an unsuccessful attempt at generating a single-crossover mutant.

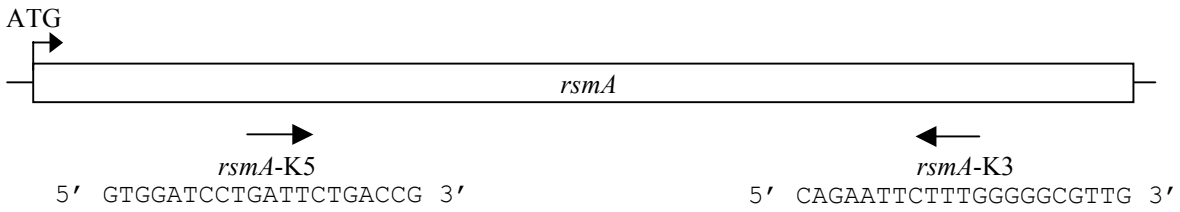


Figure 5.5. Primers used to amplify a fragment of the B52 *rsmA* gene.

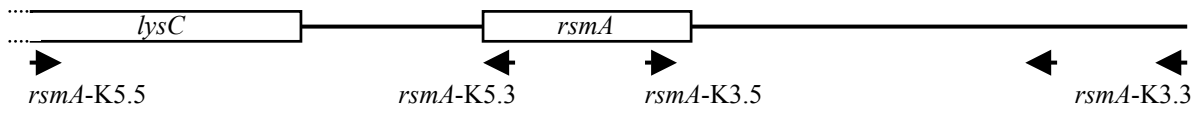
5.3.2 Cloning of B52 *rsmA* from the λ-ZAP library

A radiolabelled DNA probe was made by cutting the insert from the *rsmA* PCR clone with *Eco*RI and *Bam*HI and running a PCR reaction with the *rsmA*-K5 and *rsmA*-K3 primers and radiolabelled dCTP. The λ-ZAP library was screened with this probe; a positive plaque was cored and isolated by secondary screening. Excision and sequencing of this clone, pBKrsmA1, with T3 and T7 vector primers revealed that *rsmA* is at one end of the insert with thirty nucleotides missing from the 3' end, which truncates nine amino-acids from the C-terminal end of RsmA and substitutes a histidine for a lysine at the end of the truncated protein. Two more primary plaques were cored and isolated by secondary

screening, however one could not be excised and sequencing of the other excised clone showed that it contains *rsmA* truncated in the same location as in the first primary isolate. These results suggest that in *E. coli*, B52 *rsmA* is lethal if cloned into a vector with very high copy-number, such as pBK-CMV.

5.3.3 PCR Primer Design for the *rsmA* Double-Crossover Construct

Sequencing of pBKrsmA1 yielded sequence upstream from *rsmA*, enabling the design of a primer pair which amplifies a 480 bp product containing part of the aspartokinase gene *lysC* and 54 bp of the *rsmA* 5' end. The upstream primer for the other fragment, *rsmA*-K3.5, was based on the 3' end of the *rsmA* sequence present in the incomplete clone. Sequence was not available downstream from *rsmA*, however a sequence comparison between different species revealed a direct repeat which is 100% conserved in *P. aeruginosa*, *P. fluorescens*, *P. putida*, *E. coli*, *H. influenzae* and other species. The *rsmA*-K3.3 primer was designed on this sequence and when paired with *rsmA*-K3.5 was predicted to yield fragments of 437 and 587 nucleotides (the longer fragment was used).



rsmA-K5.5	5' AGTCTAGAAGTAGTCGGCGATAC 3'
rsmA-K5.3	5' GTGAATTCGCCATCACCGAT 3'
rsmA-K3.5	5' CTGAATTCACCGTGACCGTGCTC 3'
rsmA-K3.3	5' CTCTCGAGCCGAGTACTCTATCCA 3'

Figure 5.6. Map of the primer binding sites and primer sequences for the *rsmA* double-crossover knockout construct. The *rsmA*-K3.3 primer bound to a direct sequence repeat.

5.3.4 Assembly of the *rsmA* Double-Crossover Construct and Mutagenesis of *rsmA*

The *rsmA*-K5 primers were annealed at 45°C for 3 cycles and 52°C for 22 cycles in a PCR reaction. The *rsmA*-K3 primers were annealed at 45°C for 3 cycles and 58°C for 22 cycles. Both reactions yielded the expected products. The products were ethanol-precipitated, digested with *Eco*RI and ligated into a single fragment. The ligation products were ethanol-precipitated, purified by agarose gel electrophoresis and QIAEX extraction, digested with *Xba*I and *Xho*I and cloned into the *Xba*I and *Xho*I sites of pSK. This clone was cut with *Eco*RI (the vector *Eco*RI site was removed by *Xba*I and *Xho*I) and the tetracycline cassette from pHP45Ω-Tc was cloned into it on an *Eco*RI fragment. The construct was checked by sequencing with the T3 and T7 vector primers, which showed that the PCR products were correct and the tetracycline cassette was present between them. The complete *rsmA*::Tc^r construct was removed and cloned into the *Xba*I and *Xho*I sites of the *sacB* selection vector pDM4.

The *rsmA*::Tc^r construct in pDM4 was transferred from *E.coli* S17.1λ-pir to *P. fluorescens* B52 by conjugation. Several colonies from the conjugation grew on an LB agar plate containing 10 μg/ml tetracycline, 200 μg/ml chloramphenicol and 50 μg/ml ampicillin. Three of these colonies were grown in 1 ml LB broth containing the same antibiotic concentrations and plated onto LB agar containing 10 μg/ml tetracycline and 10% sucrose. Five out of the 200-300 colonies on each plate were patched onto 200 μg/ml chloramphenicol plates to check for vector suicide. Four colonies, all from plate 3, were chloramphenicol-resistant. One colony each from plates 1 and 2 was used for Southern blot and assay experiments.

5.3.5 Southern Blot Analysis of the *rsmA*::Tc^r Mutant

A radiolabelled nucleotide probe was made using the *rsmA*-K5.5 primer and the *rsmA*::Tc^r construct in pSK as a template. The template was first digested with *Eco*RV, which cuts within the Tc^r cassette. Labelled fragments were made by cycling 15 times at 55°C for 30 seconds, 74°C for 3 minutes and 95°C for 15 seconds. This primer binds to a single wild-type *Eco*RI fragment containing the entire region between the rsmA-5.5 and rsmA-3.3 primers, and to a shorter *Eco*RI fragment and the Tc^r cassette found in the *rsmA* mutant. The probe was used to Southern-blot *Eco*RI digests of wild-type B52 and the two mutant isolates. The probe bound to a single *Eco*RI fragment in the wild-type (Figure 5.8). A double crossover would result in disruption of *rsmA* by insertion of the Tc^r cassette, which contains an *Eco*RI site at each end (Figure 5.7). Therefore the probe would bind to a much shorter genomic fragment and the excised Tc^r fragment. The 2 kb Tc^r cassette fragment was faintly visible in the Southern blot of only one of the two *rsmA*::Tc^r mutants (Figure 5.8, lane 2). Two larger fragments were obtained from both mutants. A possible explanation for these fragments is a partial digest at the upstream *Eco*RI site in the Tc^r cassette, creating an additional fragment 2 kb larger than the upstream genomic fragment. Labelling of the Tc^r fragment would have been weak if most probe fragments did not extend into the Tc^r cassette during synthesis. However, it is clear from the Southern blot that *rsmA* was disrupted in both mutants.

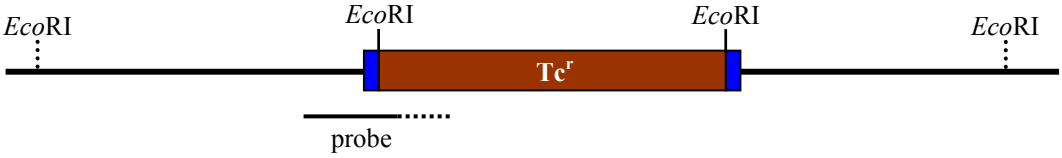


Figure 5.7. Structure of an *rsmA*::Tc^r double-crossover mutant. Most of *rsmA* (blue) was removed and replaced with the Tc^r cassette (red), which contains an *Eco*RI site at each end. The exact locations of the upstream and downstream *Eco*RI sites are unknown.

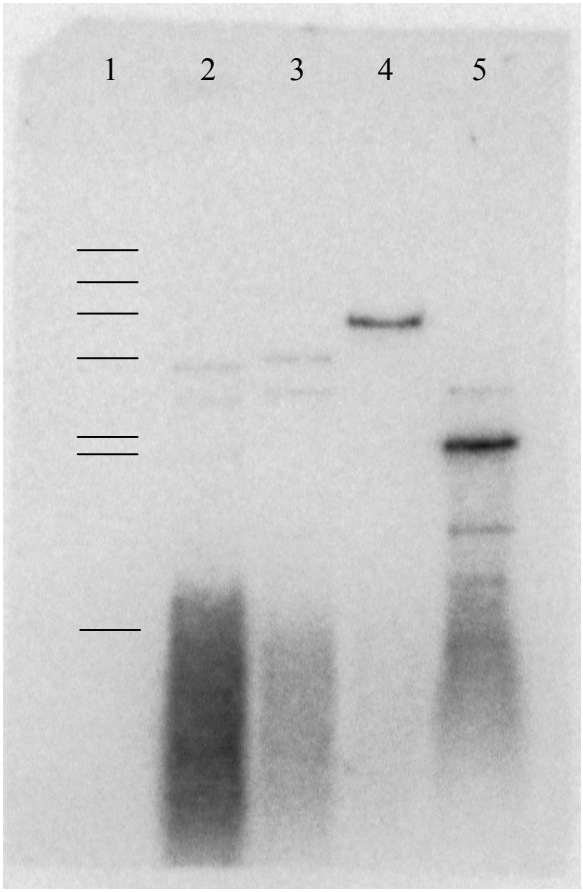


Figure 5.8. Southern blot of two *rsmA*::Tc^r mutants. Lane 1, λ-*Hin*dIII standards; 2 and 3, *RsmA*::Tc^r mutant genomic DNA cut with *Eco*RI; 4, B52 wild-type cut with *Eco*RI; 5, *RsmA* knockout contruct in pSK.

5.3.6 Lipase and Protease Phenotype of the *rsmA*::Tc^r Mutant

B52 wild-type and two *rsmA*::Tc^r isolates were patched onto skim-milk and tributyrin LB-agar plates with no added NaCl and 150 mM NaCl. Production of lipase and protease by the *rsmA*::Tc^r mutants on these plates was very similar to that of the wild-type. Assays of cultures grown in HEPES minimal medium with no NaCl showed no significant difference in protease production and only a small increase in lipase production by an *rsmA*::Tc^r mutant compared to the wild-type (Figure 5.9).

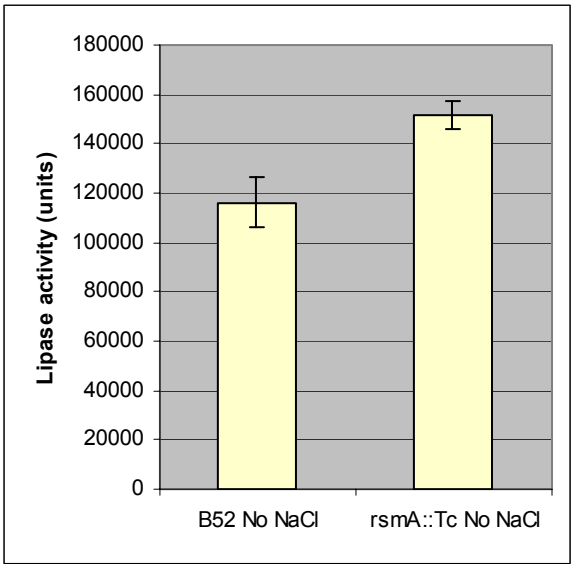


Figure 5.9. Lipase production by B52 wild-type and *rsmA*::Tc^r mutant. Error bars show the variation between duplicate cultures.

5.3.7 Multiple Copies of B52 *rsmA* are Lethal in *E. coli*

The complete wild-type *rsmA* gene was amplified from B52 by PCR using the *rsmA*-K5.5 and *rsmA*-K3.3 primers. The product was digested with *Bam*HI and *Eco*RI and cloning into the *Bam*HI and *Eco*RI sites of pSK, pBBR1MCS and pMMB207 was attempted. The pSK recombinant was a 2.2 kb plasmid, compared to the 2.96 kb of the vector, so a rearrangement presumably occurred. Cloning into pBBR1MCS and pMMB207 failed. The B52 *rsmA* gene appears to be lethal even in pMMB207, which miniprep yields suggest has a relatively low copy number compared to pSK. Due to this apparent lethality, *rsmA* could not be over-expressed in B52.

5.4 Effect of *pbrA* Over-Expression on Lipase and Protease Production

The ECF sigma-factor gene *pbrA* from *P. fluorescens* M114 was obtained in a broad-host-range vector in the plasmid pCUP408 (Sexton *et al*, 1996). The plasmid was transferred

into B52 by conjugation from *E. coli* S17.1. Liquid cultures of B52 containing either pCUP408 or pMMB207 were gown in HEPES minimal medium with no NaCl or 150 mM NaCl. Lipase production at high NaCl concentration was repressed approximately 10-fold by pCUP408 compared to the pMMB207 control, but was not affected at low NaCl concentration. Protease was also repressed, but the difference was less than two-fold with no NaCl. Repression of protease was higher with 150 mM NaCl, although the relatively low levels of protease at this salt concentration reduce the accuracy of the results.

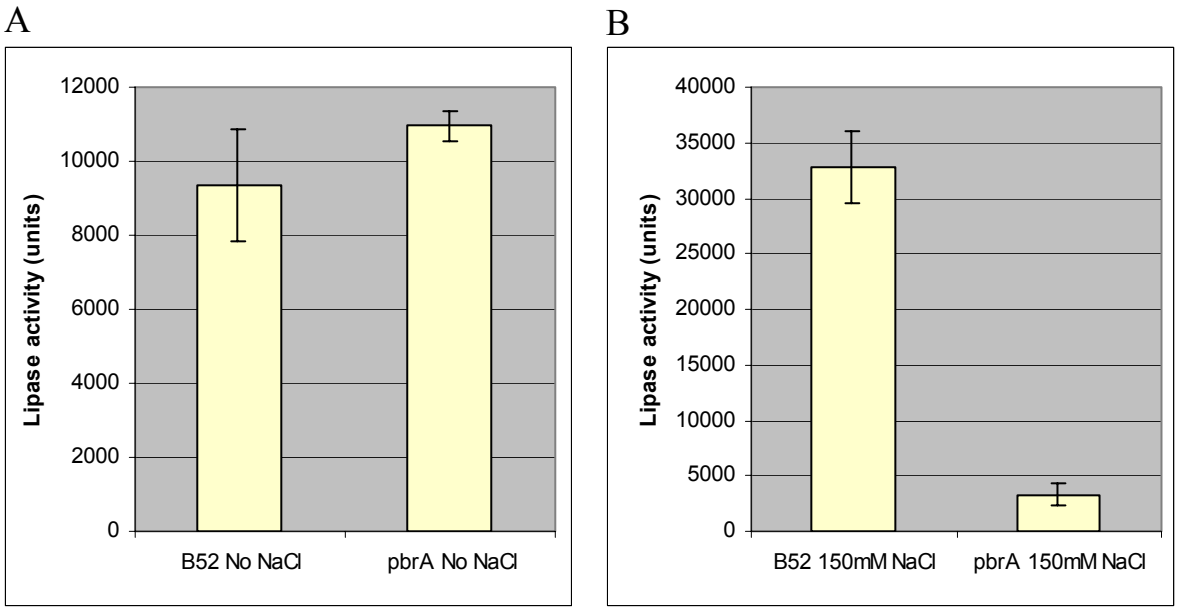


Figure 5.10. Lipase production by B52 containing pMMB207 or pCUP408 (*pbrA* clone) in media containing: A. no NaCl and B. 150 mM NaCl. Although lipase production was consistent between cultures within each assay experiment, it was variable across separate experiments inoculated from different starter cultures, hence the higher lipase production in B.

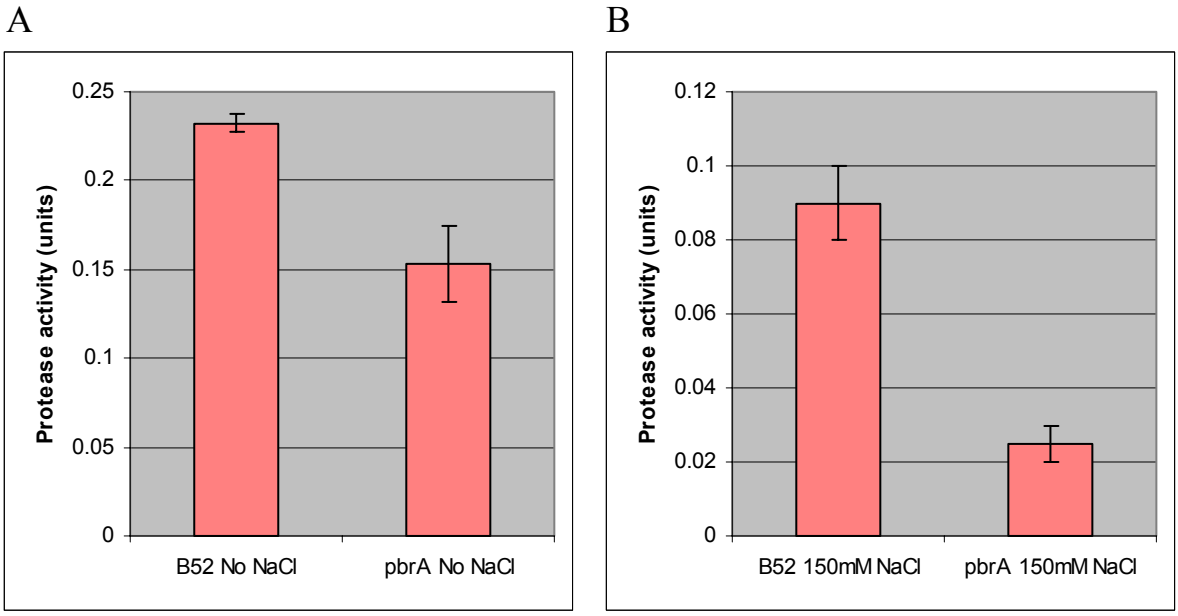


Figure 5.11. Protease production by B52 containing pMMB207 or pCUP408 (*pbrA* clone) in media containing A. no NaCl and B. 150mM NaCl.

5.5 Discussion

A two-component regulatory system, MspR/MspA, which is required for normal production of lipase and protease in *P. fluorescens* LS107d$_2$, was shown by disruption of *mspA* to have no effect on production of these enzymes in strain B52.

Potential recognition sequences for an RNA-binding protein, RsmA, which represses translation of extracellular enzyme genes in *Erwinia carotovora*, were found in the *aprX* and *lipA* genes of *P. fluorescens* B52. A gene for an RsmA homologue was found in B52, but the entire gene could not be cloned due to lethality in *E. coli*. Disruption of this gene by double-crossover had little or no effect on lipase and protease production in B52.

The ECF sigma-factor gene *pbrA* from *P. fluorescens* M114 strongly represses lipase production in B52 in 150 mM NaCl when expressed on a broad-host-range vector. The gene also represses protease production in 150 mM NaCl, but less strongly. These results are opposite to those observed in M114, in which PbrA activates expression of several iron-regulated genes, including a casein protease (Sexton *et al*, 1996). M114 PbrA appears to repress lipase and protease expression in B52 in response to NaCl. If this effect is at the transcriptional level, a PbrA homologue in B52 cannot perform a similar function because the *aprX*'-'*lacZ* fusion showed that the effect of NaCl is not at the transcriptional level. Alternatively, PbrA might activate a gene involved in post-transcriptional repression of lipase and protease expression in response to NaCl and/or OmpR-P. It may activate the *ompR-envZ* operon itself, and sequence upstream from *ompR* contains the '-10 box' identified for ECF sigma-factors such as PbrA (Sexton *et al*, 1996; Wosten, 1998), but no '-35 box' could be identified (Appendix 1). It is also conceivable that M114 PbrA binds to the promoter for a gene which opposes the effect of NaCl and/or OmpR-P on *aprX* and *lipA* expression in B52, but this binding does not result in transcription initiation, either because its position on the DNA is incorrect or its interaction with RNA polymerase is defective. In this case, if native B52 PbrA functions correctly as a sigma-factor it would activate extracellular enzyme production in B52.

Chapter 6

Conclusions and Future Research

6.1 The *pcnB* Gene

If regulation of lipase and protease production involves an antisense RNA functionally similar to MicF, and/or control of the stability of the *aprX-lipA* mRNA, polyadenylation by *pcnB* could play a significant role as it is known to affect RNA stability (O'Hara *et al*, 1995). Liquid culture assays of the *pcnB*::mini-Tn5-km mutant and the effect of *pcnB* overexpression did not show a clear complementation of the *pcnB* mutant or a well-defined effect of *pcnB* on lipase and protease production. If the genotype of the *pcnB* mutant is more than just disruption of *pcnB* by a transposon, a *pcnB* knockout would be required to provide a mutant with a known genotype to confirm involvement of *pcnB*. Downstream sequence from *pcnB* is also required to confirm that the phenotype is not due to a polar effect on a cotranscribed gene. A non-polar *pcnB* mutation would be required if the potential for a polar effect exists.

6.2 The *ompR-envZ* Operon

It is clear that lipase and protease production is repressed by NaCl concentration, probably via the OmpR/EnvZ system and also by an independent mechanism. According to the lack of repression of the *aprX*'-'*lacZ* fusion strain, this repression by NaCl does not occur at the level of *aprX* transcription. Repression of the *aprX*'-'*lacZ* fusion by iron showed that it does detect repression of transcription (Woods, 2000). The most likely mechanism for repression by OmpR-P and NaCl is regulation of mRNA degradation past the point where *lacZ* was fused with *aprX*. The *lipA* gene could be similarly regulated, but upstream from the *lacZ* fusion. An antisense RNA, similar to MicF of *E. coli*, may be responsible for regulation of mRNA degradation, as MicF is known to destabilise mRNA (Andersen and

Delihas, 1990). Isolation of a short antisense-RNA gene by transposon mutagenesis is unlikely, but it might be accomplished by screening a genomic library in a broad-host-range vector in B52, if sufficient transformation efficiency could be obtained, for example by electroporation. Clones with altered lipase or protease expression would contain potential regulatory genes.

OmpR-P may activate the antisense-RNA gene itself, or another unidentified regulatory factor. If such an OmpR-regulated gene could not be isolated by transposon mutagenesis or screening a library in B52, it may be possible to purify OmpR-regulated DNA fragments by using their affinity for OmpR-P. Substitution of glutamate for the phosphorylated aspartate residue may create an OmpR protein which is active without phosphorylation, as it does in *E. coli* (Lan and Igo, 1998). This would avoid the need to obtain phosphorylated OmpR.

It is clear from the phenotype of the *envZ*::mini-Tn5-km mutants that lipase and protease expression is still repressed in response to salt in the absense of EnvZ, though to a lesser extent. This may be partly due to phosphorylation of OmpR by another histidine-kinase, which are abundant in most bacteria (Kim and Forst, 2001). The response to salt may then be due to sensing of salt concentration by OmpR-P (Mattison *et al*, 2002). Transposon mutagenesis of one of the *envZ*::mini-Tn5-km mutants using a different selectable marker might succeed in locating another histidine-kinase which phosphorylates OmpR.

6.3 *mspA* and *rsmA*

Although homologues of *mspA* and *rsmA* were cloned from B52, disruption of these genes showed that they do not play a significant role in the regulation of lipase and protease production in B52. The Southern-blot result indicates that *rsmA* is disrupted in the B52 double-crossover mutant. The B52 RsmA protein is 100% identical to that of *P. fluorescens* CHA0, which contains an RsmA-regulated protease gene, *aprA*. The B52 *aprX* ribosome-binding site has high homology to that of CHA0 *aprA* (Figure 5.1), and matches the RsmA consensus sequence identified by Blumer *et al* (1999). Possible explanations for the lack of regulation by RsmA in B52 are a duplication of *rsmA*, lack of *rsmA* expression, or strong expression of the RsmA-sequestering RNA, RsmB.

6.4 The PbrA Sigma-Factor

The effect of expressing *pbrA* of *P. fluorescens* M114 in B52 provides evidence that an ECF sigma-factor is involved in regulation of lipase and protease expression in B52. Greater repression of *lipA* than *aprX*, and the enhancement of repression in 150 mM NaCl, suggests PbrA does not bind to the *aprX* promoter, but instead regulates a promoter involved in the response to NaCl, possibly even the *ompR-envZ* promoter. If this is the case, overexpression of *pbrA* in the B52 *aprX'-'lacZ* fusion strain will not regulate the *aprX* fusion.

If a close homologue of *pbrA* is present in B52, it could be isolated from the B52 λ-ZAP library using M114 *pbrA* as a probe. It is possible that M114 PbrA binds to the promoter for a gene which opposes the effect of NaCl and/or OmpR-P on *aprX* and *lipA* expression,

but fails to activate transcription of this gene, thereby repressing it instead. In that case, M114 *pbrA* may be more distantly related to its nearest homologue in B52. Screening with a *pbrA* probe might not succeed, and the B52 *pbrA* homologue could be isolated by transposon mutagenesis or possibly by screening a library in a broad-host-range vector.

6.5 Other Genes of Interest

The PrtI ECF sigma-factor and its activator, PrtR, found in *P. fluorescens* LS107d$_2$ (Burger, 2000) could be involved in regulation of *aprX* and possibly *lipA* expression in strain B52. If a homologous operon exists in B52, a probe made from LS107d$_2$ *prtIR* would locate it in the B52 λ-ZAP library. Over-expression of this operon on a broad-host-range vector in B52 would provide evidence for its involvement in regulation of *aprX* and *lipA*.

Regulation of lipase and protease expression by iron occurs at the transcriptional level (Woods, 2000). The response to iron concentration is likely to be mediated by the Fur repressor, which regulates PbrA in *P. fluorescens* M114 (Sexton *et al*, 1995). Overexpression of Fur from a related strain or species is likely to confirm its regulatory effect on lipase and protease in B52.

6.6 Conclusions

With the available evidence it is possible to construct a putative general model of the regulation of lipase and protease production in B52 (Figure 6.1). Iron acts at the level of transcription (Woods, 2000), and this is proposed to be via an ECF σ-factor. It is assumed

that M114 PbrA represses *ompR-envZ* in B52, but it may regulate another factor. Phosphorylated OmpR-P represses lipase and protease production indirectly by blocking ribosome binding and/or accelerating degradation of the mRNA. There must be a target site for degradation between the *lipA* ribosome binding site and the *lipA-lacZ* fusion point in order for the fusion to be regulated. Regulation of the *aprX*'-'*lacZ* fusion by OmpR-EnvZ was much weaker than regulation of protease production, so repression of *aprX* expression is due either to mRNA degradation continuing upstream from the target point for *lipA*, or other targets upstream from *lipA* but downstream from the *aprX*'-'*lacZ* fusion point (dotted line). It is possible that AprX secretion is restricted by degradation of the secretion genes on the mRNA, but this would be inefficient and accumulation of protease in the cytoplasm may be detrimental despite the effect of the inhibitor. In addition, S1 nuclease protection assays of *aprX* mRNA levels showed that these are regulated by NaCl at a similar level as protease production itself (Woods, 2000), and therefore mRNA degradation occurs up to the probe binding site near the 5' end of *aprX*. The unknown factor(s) which OmpR-P acts through, to effect mRNA degradation, is most likely an antisense RNA or RNA-binding protein. It is also conceivable that OmpR-P represses a factor which increases RNA stability.

The OmpR-independent effect of NaCl, and the effect of temperature, occurs via degradation of mRNA downstream from the *lipA*'-'*lacZ* fusion point. This explains the lack of regulation of the fusion. The simplest mechanism is that NaCl and temperature both work via the same intermediate which affects mRNA stability. Temperature may also decrease the stability of a stem-and-loop structure downstream from *lipA*, which could allow degradation to proceed upstream. NaCl cannot work by this mechanism because it increases the stability of nucleic acid duplexes.

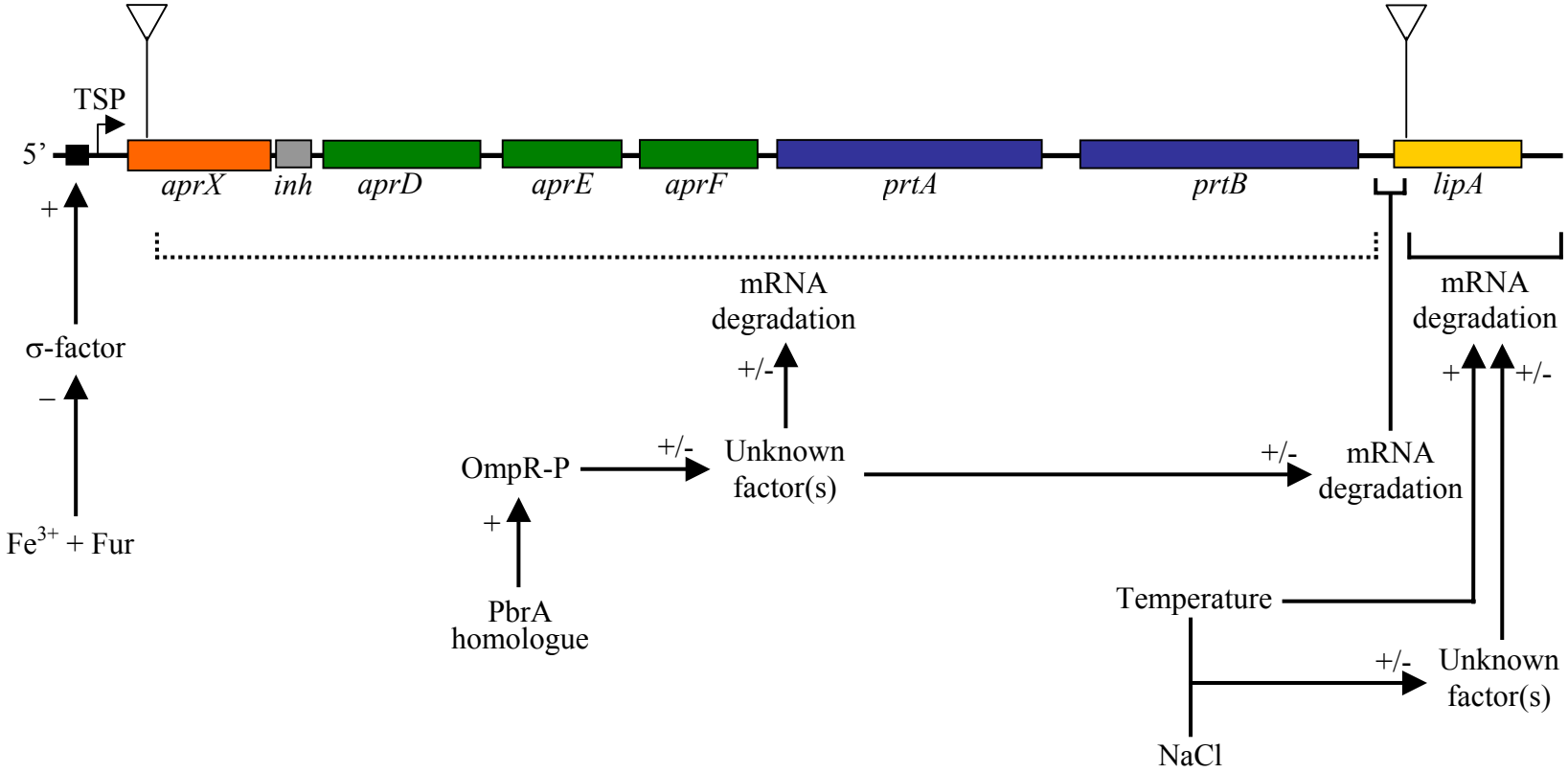


Figure 6.1. Putative regulatory model for lipase and protease production. The small black rectangle indicates the promoter, followed by the transcription start point (TSP). The inverted triangles mark the fusion points for the *aprX*'-'*lacZ* and *lipA*'-'*lacZ* translational fusions. The dotted line indicates a region where degradation of *aprX* mRNA may be initiated. See section 6.6 for a detailed description.

Appendix 1

```
CGGCCCGACTGGCGCCGCTACTTGGGTCAAAAGGCAGCCTGTTGACCCTCGAAATCGGAA   60
AATTGCTGCCCATCGCGGTAAAAAATAGCGAGGCAGCAAGCAGTAAAGTCTGACATTGCT  120
CAGCGCAAAGGCGGCGCATTATAACCAGCGTTCTGCAGCTGGGGGGGATTGCGCCGAAGG  180
TTGCAGGGGGCGGGTTCGCTGGCGGTAGAGGAAAAATCTGCTAACAATGCACACGGTGCG  240

TATAACGGCAGCTACGCCATAATGCGCGCCGAGATAAGAGGAGCATCTAATGAGCAGCAC  300
TGCACAAACTGCTGAAGGCGAAAAAATTCTCATCGTTGATGACGACCCGGGGCTGAGCAG  360
CCTGCTGGAGCGTTTTTTCAACTCCAAGGGCTACCGTGCCCGCGCGGTGCCAAACACCGA  420
GCAGATGGACCGCCTGTTGGGGCGCGAAGTGTTCAATCTGGTCGTACTCGACCTGATGCT  480
GCCCGGCGAAGACGGCCTCACCGCCTGCAAACGCCTGCGCGGTGCGAACAACCAGATCCC  540
                              ompR-K5.2 GTGAATTCAAGGGCCTTGAGCTG
CATCATCATGCTTACCGCCAAGGGTGATGAGTTGAGCCGCATCAAGGGCCTTGAGCTGGG  600
CGCTGATGATTACCTGGCCAAGCCGTTCAACCCGGACGAGTTGATGGCCCGGGTCAAAGC  660
CGTGTTGCGCCGTCAGGCAGCCCCGGTACCCGGCGCGCCAGGCAGCGAAGACGAAAGCGT  720
CACCTTTGGCGACTATGAACTGTCGCTGGCCACCCGTGAGCTCAAGCGTGGCGAAGAAGT  780
GCACATGCTGACCACCGGTGAGTTTGCCGTGCTCAAGGCCCTGGTGATGAACGCTCGCCA  840
                                  GTTCCGGGACCACTACCTAGGTG ompR-K3

GCCGCTGACGCGCGACAAACTGATGAACCTGGCCCGTGGCCGGGAGTGGGATGCATTGGA  900
GCGCTCCATCGACGTGCAGATTTCCCGTCTGCGCCGCATGATCGAGCCGGACCCGTCCAA  960
GCCGCGCTATATCCAGACGGTGTGGGGTGTGGGTTATGTGTTCGTGCCGGATGGCACCGC 1020
AACCAAGTGACCGATGATTTGCAGGCGCGGGCATCCGGCGTTTGACTCCATGAGAGTCAA 1080
CGGGATGCCCGGCGTCTGCAATTCTGCGAGCGCCGCTCGTTTCTGCAAGGTGTCCAGCCG 1140

TTTCCTATGAAAACCCCGTTGTGGTTCCCGCAAATTTCTTTTCAACGCACCCTTTGGCTG 1200
GTGCTGATCGTCGTTCTGTTTTCCAAGGCACTGACGCTGGTTTATCTGTTGATGAACGAA 1260
GACGTGCTGGTGGATCGGCAGTACAGCCACGGCGTCGCCCTGACGCTTCGTGCCTATTGG 1320
GCCGTCGATCCCGAAAACCGTGAAAAGGTCGCCAAGGCATCGACGCTGATTCGAGTCGAC 1380
GGCGCCGGTGTACCTGAAGGCGAGCAGCATTGGCCTTACAGCGAGATTTATCAGCGCCAG 1440
ATGCAGGCTGAGCTGGGGGACGACACCGAGGTGCGATTGCGCATGCATGTTTCGCCTGCG 1500
CTGTGGGTGAGGGCGCCGAGCCTGGGCGACGCGTGGATCAAAGTGCCGCTGTACCCGCAC 1560
CCGTTGCGGGGACAGAAGATCTGGAACGTGCTGGGCTGGTTCCTGGCAATTGGCTTGCTT 1620
TCAACGGCGTCCGCCTGGATTTTCGTGCGCCAGCTCAACCAGCCACTCAAACGCCTGGTC 1680
TTTGCCGCGCGCCAACTGGGGCAGGGGCGCAGTGTGCGCCTGCCGGTCAGCGATACGCCC 1740
AGCGAGATGACCGAAGTGTACGGCGCCTTCAACCAGATGGCGGAAGACGTTGAACAGGCC 1800
GGCCGCGAACGCGAGCTGATGCTGGCGGGCGTTTCCCACGACCTGCGCACACCGTTGACA 1860
CGGTTGCGGTTGTCCCTGGAGTTGATGGGCAACCACAACGACCTCACGGATGACATGGTG 1920
CGTGACATCGAGGACATGGACGCGATTCTCGACCAGTTCCTGGCGTTTATCCGCGATGGC 1980
```

```
CGCGACGAAGTGGTCGAGGAAGTCGACCTGACGGACCTGGTGCGTGAGGTGGTGGCGCCC 2040
TACAACCAGAACGGCGAACAGGTACGCATGCGCCTGGAGCCTATTCAGCCGTTTGCGTTG 2100
CGTCGGGTGTCGATGAAACGCCTGCTCAACAACCTCATTGGCAACGCCTTGCACCATGCC 2160
GGTTCCGATGTGGAAGTGGCGGCGTATGTGTCCGGCGACAGCACTGCGCCTTATGTGGTG 2220
TTGAGTGTGATGGACCGCGGCACGGGGATAGACCCGGCAGAGCTGGAAGGCATCTTCAAC 2280
CCGTTCACCCGTGGTGACCGTGCCCGGGGCGGCAAGGGCACGGGCTTGGGGTTGGCGATT 2340
GTGCGGCGGATTGCCTCGATGCATGGCGGCAATGTCGAGCTGCGTAACCGGGAGGAGGGT 2400
GGTCTGGAAGCGCGAGTGCGGTTGCCGCTTGGCCTGATGTTGCCTAGAGACGCGGTCTGA 2460
CACACAACAGAAATCAAATGTGGGAGCGG                                  2489
```

Appendix 1. Nucleotide sequence of the *ompR-envZ* operon of *P. fluorescens* B52. Arrows mark the Methionine start codons for OmpR and EnvZ, and stop codons are underlined. A possible -10 box for ECF sigma factors (consensus TCTRA) is marked in bold, but no -35 box is identifiable (Sexton *et al*, 1996; Wosten, 1998). The primers used for generating the *ompR* knockout construct are shown.

Appendix 2

In 1996, no software for the Windows platform was available at low or zero cost for the purpose of viewing and editing the output from Applied Biosystems automated DNA sequencers. To meet this need, “Chromas” was developed, based on a published description of the Applied Biosystems file format (Tibbetts, 1995). Available free of charge, Chromas displays the sequencing chromatogram and interpreted sequence contained within Applied Biosystems and Staden Chromatogram Format (SCF) files. The sequence can be edited for correction of errors and removal of vector sequence and the final result can be copied to the Windows “clipboard” for pasting into the NCBI BLAST search form. The sequence can also be exported to a file, with or without formatting, for publication or loading into other software. The free version of Chromas is still available, in addition to the later low-cost commercial version. Chromas is believed to be the most widely used software for viewing and editing Applied Biosystems and SCF files for small-scale DNA sequencing projects.

References

Aarons, S., Abbas, A., Adams, C., Fenton, A. and O'Gara, F. (2000). A Regulatory RNA (PrrB RNA) Modulates Expression of Secondary Metabolite Genes in *Pseudomonas fluorescens* F113. *J. Bacteriol.* **182**(14): 3913-3919.

Aiba, H., Nakasai, F., Mizushima, S. and Mizuno, T. (1989). Evidence for the physiological importance of the phosphotransfer between the two regulatory components, EnvZ and OmpR, in osmoregulation in *Escherichia coli*. *J. Biol. Chem.* **264**(24): 14090–14094.

AlKanhal, H.A., Frank, J.F. and Christen, G.L. (1985). Microbial protease and phospholipase C stimulates lipolysis of washed cream. *J. Dairy Sci. Tech.* **38**: 97-103.

Al-Shabibi, M.M.A., Langner, E.H., Tobias, J. and Tuckey, S.L. (1964). Effect of added fatty acids on the flavor of milk. *J. Dairy. Sci.* **47**:295-296.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990). Basic local alignment search tool. *J. Mol. Biol.* **215**: 403-410.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**: 3389-3402.

Ames, S.K., Frankema, N. and Kenney, L.J. (1999). C-terminal DNA binding stimulates N-terminal phosphorylation of the outer membrane protein regulator OmpR from *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **96**(21): 11792-11797.

Andersen, J., Forst, S.A., Zhao, K., Inouye, M. and Delihas, N. (1989). The Function of *micF* RNA: *micF* RNA is a major factor in the thermal regulation of OmpF protein in *Escherichia coli*. *J. Biol. Chem.* **264**(30): 17961-17970.

Andersen, J. and Delihas, N. (1990). *micF* RNA Binds to the 5' End of *ompF* mRNA and to a Protein from *Escherichia coli*. *Biochemistry* **29**: 9249-9256.

Anderson, R.E., Hedlund, C.B. and Jonsson, U. (1979). Thermal inactivation of a heat-resistant lipase produced by the psychrotrophic bacterium *Pseudomonas fluorescens*. *J. Dairy Sci. Tech.* **29**: 74-78.

Andersson, R.E. (1980). Microbial Lipolysis at Low Temperatures. *Appl. Env. Microbiol.* **39**: 36-40.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G. and Struhl, K. (1987). *Current protocols in molecular biology*. Wiley Interscience, New York.

Bang, I.S., Kim, B.H., Foster, J.W. and Park, Y.K. (2000). OmpR Regulates the Stationary-Phase Acid Tolerance Response of *Salmonella enterica* Serovar Typhimurium. *J. Bacteriol.* **182**(8): 2245-2252.

Baumann, U., Wu, S., Flaherty, K.M. and McKay, D.B. (1993). Three-dimensional structure of the alkaline protease of *Pseudomonas aeruginosa*: a two-domain protein with a calcium binding parallel beta roll motif. *EMBO J.* **12**(9): 3357-3364.

Birkeland, S.E., Stepaniak, L. and Sorhaug, T. (1985). Quantitative studies of heat-stable proteinase from *P. fluorescens* P1 by the enzyme-linked immunosorbent assay. *Appl. Environ. Microbiol.* **49**(2): 382-387.

Birnboim, H.C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucl. Acids. Res.* **7**(6): 1513-1523.

Blumer, C., Heeb, S., Pessi, G. and Haas, D. (1999). Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. *Proc. Natl. Acad. Sci. USA* **96**(24): 14073-14078.

Broeze, R.J., Solomon, C.J. and Pope, D.H. (1978). Effects of low temperature on *in vivo* and *in vitro* protein synthesis in *Escherichia coli* and *Pseudomonas fluorescens*. *J. Bacteriol.* **134**(3): 861-874.

Burger, M., Woods, R., McCarthy, C. and Beacham, I.R. (2000). Temperature regulation of protease in *Pseudomonas fluorescens* LS107d2 by an ECF sigma factor and a transmembrane activator. *Microbiology* **146**: 3149-3155.

Cao, G.-J. and Sarkar, N. (1992). Identification of the gene for an *Escherichia coli* poly(A) polymerase. *Proc. Natl. Acad. Sci. USA* **89**: 10380-10384.

Chatterjee, A., Cui, Y., Liu, Y., Dumenyo, C.K. and Chatterjee, A.K. (1995). Inactivation of *rsmA* Leads to Overproduction of Extracellular Pectinases, Cellulases, and Proteases in *Erwinia carotovora* subsp. *carotovora* in the Absence of the Starvation/Cell Density-Sensing Signal, N-(3-Oxohexanoyl)-L-Homoserine Lactone. *Appl. Env. Microbiol.* **61**(5): 1959–1967.

Comeau, D.E., Ikenaka, K., Tsung, K. and Inouye, M. (1985). Primary Characterization of the Protein Products of the *Escherichia coli ompB* Locus: Structure and Regulation of Synthesis of the OmpR and EnvZ Proteins. *J. Bacteriol.* **164**(2): 578-584.

Cousin, M.A. (1982). Presence and Activity of Psychrotrophic Microorganisms in Milk and Dairy Products: A Review. *J. Food Protection* **45**(2): 172-207.

Cousin, M.A. (1989). Physical and biochemical effects on milk components. In *Enzymes of Psychrotrophs in Raw Food*, pp 205-225. Edited by R.C. McKellar. CRC Press, Boca Raton, Florida.

Coyer, J., Andersen, J., Forst, S.A., Inouye, M. and Delihas, N. (1990). *micF* RNA in *ompB* Mutants of *Escherichia coli*: Different Pathways Regulate *micF* RNA Levels in Response to Osmolarity and Temperature Change. *J. Bacteriol.* **172**(8): 4143-4150.

Cui, Y., Chatterjee, A., Liu, Y., Dumenyo, C.K. and Chatterjee, A.K. (1995). Identification of a Global Repressor Gene, rsmA, of *Erwinia carotovora* subsp.

carotovora That Controls Extracellular Enzymes, N-(3-Oxohexanoyl)-L-Homoserine Lactone, and Pathogenicity in Soft-Rotting *Erwinia* spp. *J. Bacteriol.* **177**(17): 5108-5115.

Cunliffe, H.E., Merriman, T.R. and Lamont, I.L. (1995). Cloning and Characterization of *pvdS*, a Gene Required for Pyoverdine Synthesis in *Pseudomonas aeruginosa*: PvdS Is Probably an Alternative Sigma Factor. *J. Bacteriol.* **177**(10): 2744-2750.

Deeth, H.C. and Fitzgerald, C.H. (1983). Lipolytic enzymes and hydrolytic rancidity in milk and milk products. In *Developments in Dairy Chemistry – 2. Lipids*, pp 195-239. Edited by P.F. Fox. Elsevier Applied Science, Barking.

Delgado, J., Forst, S., Harlocker, S. and Inouye, M. (1993). Identification of a phosphorylation site and functional analysis of conserved aspartic acid residues of OmpR, a transcriptional activator for *ompF* and *ompC* in *Escherichia coli*. *Mol. Microbiol*. **10**(5): 1037-1047.

de Lorenzo, V., Herrero, M., Jakubzik, U. and Timmis, K.N. (1990). Mini-Tn5 Transposon Derivatives for Insertion Mutagenesis, Promoter Probing, and Chromosomal Insertion of Cloned DNA in Gram-Negative Eubacteria. *J. Bacteriol.* **172**(11): 6568-6572.

Duong, F., Lazdunski, A., Cami, B. and Murgier, M. (1992). Sequence of a cluster of genes controlling synthesis and secretion of alkaline protease in *Pseudomonas aeruginosa*: relationships to other secretory pathways. *Gene* **121**: 47-54.

Duong, F., Soscia, C., Lazdunski, A. and Murgier, M. (1994). The *Pseudomonas fluorescens* lipase has a C-terminal secretion signal and is secreted by a three-component bacterial ABC-exporter system. *Mol. Microbiol.* **11**(6): 1117-1126.

Dutta, R., Yoshida, T. and Inouye, M. (2000). The Critical Role of the Conserved Thr^{247} Residue in the Functioning of the Osmosensor EnvZ, a Histidine Kinase/Phosphatase, in *Escherichia coli*. *J. Biol. Chem.* **275**(49): 38645-38653.

Esterling, L. and Delihas, N. (1994). The regulatory RNA gene *micF* is present in several species of Gram-negative bacteria and is phylogenetically conserved. *Mol. Microbiol.* **12**(4): 639-646.

Fellay, R., Frey, J. and Krisch, H. (1987). Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for in vitro insertional mutagenesis of Gram-negative bacteria. *Gene* **52**: 147-154.

Fitzgerald, C.H. and Deeth, H.C. (1983). Factors influencing lipolysis by skim milk cultures of some psychrotrophic microorganisms. *Aust. J. Dairy Sci. Tech.* **38**: 97-103.

Forst, S., Comeau, D., Norioka, S. and Inouye, M. (1987). Localization and membrane topology of EnvZ, a protein involved in osmoregulation of OmpF and OmpC in *Escherichia coli*. *J. Biol. Chem.* **262**(34): 16433-16438.

Forst, S., Delgado, J., Ramakrishnan, G. and Inouye, M. (1988). Regulation of *ompC* and *ompF* Expression in *Escherichia coli* in the Absence of *envZ*. *J. Bacteriol.* **170**(11): 5080-5085.

Forst, S., Delgado, J., Rampersaud, A. and Inouye, M. (1990). In Vivo Phosphorylation of OmpR, the Transcription Activator of the *ompF* and *ompC* Genes in *Escherichia coli*. *J. Bacteriol.* **172**(6): 3473-3477.

Fox, P.F. and Stepaniak, L. (1983). Isolation and some properties of extracellular heat-stable lipases from *Pseudomonas fluorescens* strain AFT 36. *J. Dairy Res.* **50**: 77-89.

Franklin, F.C.H. (1985). Broad host range cloning vectors for Gram-negative bacteria. In *DNA cloning, A practical approach*, vol 1. Edited by D.M. Glover. IRL Press, Washington DC.

Gügi, B., Orange, N., Hellio, F., Burini, J.F., Guillou, C., Leriche, F. and Guespin-Michel, J.F. (1991). Effect of Growth Temperature on Several Exported Enzyme Activities in the Psychrotrophic Bacterium *P. fluorescens*. *J. Bacteriol.* **173**(12): 3814-3820.

Guzzo, J., Pages, J.-M., Duong, F., Lazdunski, A. and Murgier, M. (1991). *Pseudomonas aeruginosa* Alkaline Protease: Evidence for Secretion Genes and Study of Secretion Mechanism. *J. Bacteriol.* **173**: 5290-5297.

Hanahan, D. (1983). Studies on transformation of Escherichia coli with plasmids. *J. Mol. Biol.* **166**: 557-580.

Harlocker, S.L., Bergstrom, L. and Inouye, M. (1995). Tandem Binding of Six OmpR Proteins to the *ompF* Upstream Regulatory Sequence of *Escherichia coli*. *J. Biol. Chem.* **270**(45): 26849-26856.

Head, C. G., Tardy, A. and Kenney, L.J. (1998). Relative Binding Affinities of OmpR and OmpR-phosphate at the *ompF* and *ompC* Regulatory Sites. *J. Mol. Biol*. **281**: 857-870.

Herendeen, S.L., VanBogelen, R.A. and Neidhardt, F.C. (1979). Levels of Major Proteins of *Escherichia coli* During Growth at Different Temperatures. *J. Bacteriol.* **139**(1): 185-194.

Heyde, M. and Portalier, R. (1987). Regulation of major outer membrane porin proteins of Escherichia coli K 12 by pH. *Mol. Gen. Genet.* **208**(3): 511-517.

Higashitani, A., Nishimura, Y., Hara, H., Aiba, H., Mizuno, T. and Horiuchi, K. (1993). Osmoregulation of the fatty acid receptor gene *fadL* in *Escherichia coli*. *Mol. Gen. Genet.* **240**: 339-347.

Huang, K.J. and Igo, M.M. (1996). Identification of the bases in the *ompF* regulatory region, which interact with the transcription factor OmpR. *J. Mol. Biol.* **262**(5): 615-628.

Huang, K.J., Schieberl, J.L. and Igo, MM. (1994). A distant upstream site involved in the negative regulation of the *Escherichia coli ompF* gene. *J. Bacteriol.* **176**(5): 1309-1315.

Igo, M.M., Ninfa, A.J., Stock, J.B. and Silhavy, T.J. (1989). Phosphorylation and dephosphorylation of a bacterial transcriptional activator by a transmembrane receptor. *Genes Dev.* **3**(11): 1725-1734.

Jung, K., Hamann, K. and Revermann, A. (2001). K^+ Stimulates Specifically the Autokinase Activity of Purified and Reconstituted EnvZ of *Escherichia coli*. *J. Biol. Chem.* **276**(44): 40896-40902.

Kato, M., Aiba, H., Tate, S., Nishimura, Y. and Mizuno, T. (1989). Location of phosphorylation site and DNA-binding site of a positive regulator, OmpR, involved in activation of the osmoregulatory genes of *Escherichia coli*. *FEBS Lett.* **249**(2): 168-172.

Kenney, L.J. (1997). Kinase Activity of EnvZ, an Osmoregulatory Signal Transducing Protein of *Escherichia coli*. *Arch. Biochem. Biophys.* **346**(2): 303-311.

Kim, D.-j. and Forst, S. (2001). Genomic analysis of the histidine kinase family in bacteria and archaea. *Microbiology* **147**: 1197-1212.

Kovach, M.E., Phillips, R.W., Elzer, P.H., Roop II, R.M. and Peterson, K.M. (1994). pBBR1MCS: a broad-host-range cloning vector. *Biotechniques* **16**(5): 800-802.

Kraft, R., Tardiff, J., Krauter, K.S. and Leinwand, L.A. (1988). Using mini-prep plasmid DNA for sequencing double stranded templates with Sequenase. *Biotechniques* **6**(6): 544-546, 549.

Lan, C.-Y. and Igo, M.M. (1998). Differential Expression of the OmpF and OmpC Porin Proteins in *Escherichia coli* K-12 Depends upon the Level of Active OmpR. *J. Bacteriol.* **180**(1): 171-174.

Leonardo, M.R. and Forst, S. (1996). Re-examination of the role of the periplasmic domain of EnvZ in sensing of osmolarity signals in *Escherichia coli*. *Mol. Microbiol.* **22**(3): 405-413.

Leoni. L., Ambrosi, C., Petrucca, A. and Visca, P. (2002). Transcriptional regulation of pseudobactin synthesis in the plant growth-promoting Pseudomonas B10. *FEMS Microbiol. Lett.* **208**(2): 219-225.

Liljeström, P. (1986). The EnvZ protein of *Salmonella typhimurium* LT-2 and *Escherichia coli* K-12 is located in the cytoplasmic membrane. *FEMS Microbiol. Lett*. **36**: 145-150.

Litwin, C.M and Calderwood, S.B. (1993). Role of Iron in Regulation of Virulence Genes. *Clin. Microbiol. Rev.* **6**(2): 137-149.

Litwin, C.M., Boyko, S.A. and Calderwood, S.B. (1992). Cloning, sequencing, and transcriptional regulation of the Vibrio cholerae *fur* gene. *J. Bacteriol.* **174**(6): 1897-1903.

Liu, J. and Parkinson, J.S. (1989). Genetics and Sequence Analysis of the *pcnB* Locus, an *Escherichia coli* Gene Involved in Plasmid Copy Number Control. *J. Bacteriol.* **171**(1): 1254-1261.

Liu, M.Y. and Romeo, T. (1997). The Global Regulator CsrA of *Escherichia coli* Is a Specific mRNA-Binding Protein. *J. Bacteriol.* **179**(14): 4639-4642.

Liu, Y., Cui, Y., Mukherjee, A. and Chatterjee, A.K. (1998). Characterization of a novel RNA regulator of *Erwinia carotovora* ssp. *carotovora* that controls production of extracellular enzymes and secondary metabolites. *Mol. Microbiol.* **29**(1): 219-234.

Lonetto, M.A., Brown, K.L., Rudd, K.E. and Buttner, M.J. (1994). Analysis of the *Streptomyces coelicolor sigE* gene reveals the existence of a subfamily of eubacterial RNA polymerase σ factors involved in the regulation of extracytoplasmic functions. *Proc. Natl. Acad. Sci. USA* **91**:7573-7577.

Lopilato, J., Bortner, S. and Beckwith, J. (1986). Mutations in a new chromosomal gene of *Escherichia coli* K-12, *pcnB*, reduce plasmid copy number of pBR322 and its derivatives. *Mol. Gen. Genet.* **205**: 285-290.

Lucas, R.L., Lostroh, P., DiRusso, C.C., Spector, M.P., Wanner, B.L. and Lee, C.A. (2000). Multiple Factors Independently Regulate *hilA* and Invasion Gene Expression in *Salmonella enterica* Serovar Typhimurium. *J. Bacteriol.* **182**(7): 1872-1882.

Maeda, S. and Mizuno, T. (1990). Evidence for multiple OmpR-binding sites in the upstream activation sequence of the *ompC* promoter in *Escherichia coli*: a single OmpR-binding site is capable of activating the promoter. *J Bacteriol* **172**(1): 501-503.

Martinez-Hackert, E. and Stock, A.M. (1997). The DNA-binding domain of OmpR: crystal structures of a winged helix transcription factor. *Structure* **5**(1): 109-124.

Mattison, K. and Kenney, L.J. (2002). Phosphorylation Alters the Interaction of the Response Regulator OmpR with Its Sensor Kinase EnvZ. *J. Biol. Chem.* **277**(13): 11143-11148.

Mattison, K., Oropeza, R., Byers, N. and Kenney, L.J. (2002). A Phosphorylation Site Mutant of OmpR Reveals Different Binding Conformations at *ompF* and *ompC*. *J. Mol. Biol.* **315**: 497-511.

McKay, D.B. (1994). Molecular genetic and biochemical analysis of a lipolytic psychrotrophic pseudomonad. PhD thesis, Griffith University, Nathan, Queensland.

McKay, D.B., Dieckelmann, M. and Beacham, I.R. (1995). Degradation of triglycerides by a pseudomonad isolated from milk: the roles of lipase and esterase studied using recombinant strains over-producing, or specifically deficient in these enzymes. *J. Appl. Bacteriol.* **78**(3): 216-233.

McKellar, R.C. (1989). Regulation and control of synthesis. In *Enzymes of Psychrotrophs in Raw Food*, pp 153-171. Edited by R.C. McKellar. CRC Press, Boca Raton, Florida.

McKellar, R.C., Shamsuzzaman, K., San Jose, C. and Cholette, H. (1987). Influence of iron(III) and pyoverdine on extracellular proteinase and lipase production by *Pseudomonas fluorescens* B52. *Arch. Microbiol.* **147**: 225-230.

Merieau, A., Gugi, B., Guespin-Michel, J.F. and Orange, N. (1993). Temperature regulation of lipase secretion by *Pseudomonas fluorescens* MF0. *Appl. Microbiol. Biotech.* **39**: 104-109.

Miller, J.H. (1992). *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Mills, S.D., Ruschkowski, S.R., Stein, M.A. and Finlay, B.B. (1998). Trafficking of Porin-Deficient *Salmonella typhimurium* Mutants inside HeLa Cells: *ompR* and *envZ* Mutants Are Defective for the Formation of *Salmonell*a-Induced Filaments. *Infect. Immun.* **66**(4): 1806-1811.

Milton, D.L., O'Toole, R., Horstedt, P., and Wolf-Watz, H. (1996). Flagellin A is essential for the virulence of *Vibrio anguillarum*. *J. Bacteriol.* **178**(5): 1310-1319.

Mizuno, T. and Mizushima, S. (1990). Signal transduction and gene regulation through the phosphorylation of two regulatory components: the molecular basis for the osmotic regulation of the porin genes. *Mol. Microbiol.* **4**(7): 1077-1082.

Mizuno, T., Wurtzel, E.T. and Inouye, M. (1982). Osmoregulation of Gene Expression II: DNA sequence of the *envZ* gene of the *ompB* operon of *Escherichia coli* and characterization of its gene product. *J. Biol. Chem.* **257**(22): 13692-13698.

Mizuno, T., Chou, M.Y. and Inouye, M. (1984). A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (micRNA). *Proc. Natl. Acad. Sci. USA* **81**(7): 1966-1970.

Mohanty, B.K. and Kushner, S.R. (1999). Analysis of the function of Escherichia coli poly(A) polymerase I in RNA metabolism. *Mol. Microbiol.* **34**(5): 1094-1108.

Morales, V.M., Bäckman, A. and Bagdasarian, M. (1991). A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. *Gene* **97**: 39-47.

Morita, RY. (1975). Psychrophilic bacteria. *Bacteriol. Rev.* **39**(2): 144-167.

Nagasawa, S., Ishige, K., Mizuno, T. (1993). Novel Members of the Two-Component Signal Transduction Genes in *Escherichia coli*. *J. Biochem*. **114**: 35-357.

Nikaido, H. and Rosenberg, E.Y. (1983). Porin channels in *Escherichia coli*: studies with liposomes reconstituted from purified proteins. *J. Bacteriol.* **153**(1): 241-252.

Ochs, M., Veitinger, S., Kim, I., Welz, D., Angerer, A. and Braun, V. (1995). Regulation of citrate-dependent iron transport of *Escherichia coli*: FecR is required for transcription activation by FecI. *Mol. Microbiol.* **15**(1): 119-132.

O'Hara, E.B., Chekanova, J.A., Ingle, C.A., Kushner, Z.R., Peters, E. and Kushner, S.R. (1995). Polyadenylation helps regulate mRNA decay in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **92**: 1807-1811.

O'Sullivan, D.J., Dowling, D.N., deLorenzo, V. and O'Gara, F. (1994). *Escherichia coli* ferric uptake regulator (Fur) can mediate regulation of a pseudomonad iron-regulated promoter. *FEMS Micro. Lett.* **117**: 327-332.

Overcast, W.W. and Skean, J.D. (1959). Growth of certain lipolytic microorganisms at 4°C. and their influence on free fat acidity and flavor of pasteurized milk. *J. Dairy. Sci.* **42**: 1479-1485.

Park, H., Saha, S.K. and Inouye, M. (1998). Two-domain reconstitution of a functional protein histidine kinase. *Proc. Natl. Acad. Sci. USA* **95**: 6728–6732.

Pratt, L.A., Hsing, W., Gibson, K.E. and Silhavy, T.J. (1996). From acids to *osmZ*: multiple factors influence synthesis of the OmpF and OmpC porins in *Escherichia coli. Mol. Microbiol.* **20**(5): 911-917.

Prigent-Combaret, C., Brombacher, E., Vidal, O., Ambert, A., Lejeune, P., Landini, P. and Dorel, C. (2001). Complex Regulatory Network Controls Initial Adhesion and Biofilm Formation in *Escherichia coli* via Regulation of the *csgD* Gene. *J. Bacteriol.* **183**(24): 7213-7223.

Prince, R.W., Cox, C.D. and Vasil, M.L. (1993). Coordinate Regulation of Siderophore and Exotoxin A Production: Molecular Cloning and Sequencing of the *Pseudomonas aeruginosa fur* Gene. *J. Bacteriol.* **175**: 2589-2598.

Prince, R.W., Storey, D.G., Vasil, A.I. and Vasil, M.L. (1991). Regulation of *toxA* and *regA* by the Escherichia coli *fur* gene and identification of a Fur homologue in *Pseudomonas aeruginosa* PA103 and PA01. *Mol. Microbiol*. **5**(11): 2823-2831.

Qin, L., Yoshida, T. and Inouye, M. (2001). The critical role of DNA in the equilibrium between OmpR and phosphorylated OmpR mediated by EnvZ in *Escherichia coli*. *Proc Natl Acad Sci USA* **98**(3): 908-913.

Ramani, N., Hedeshian, M. and Freundlich, M. (1994). *micF* Antisense RNA Has a Major Role in Osmoregulation of OmpF in *Escherichia coli. J. Bacteriol.* **176**(16): 5005-5010.

Rampersaud, A., Harlocker, S.L. and Inouye, M. (1994). The OmpR protein of *Escherichia coli* binds to sites in the *ompF* promoter region in a hierarchical manner determined by its degree of phosphorylation. *J. Biol. Chem.* **269**(17): 12559-12566.

Richardson, B.C. (1981). The purification and characterisation of a heat-stable protease from *Pseudomonas fluorescens* B52. *N.Z. J. Dairy Sci. Tech.* **16**:172-176.

Roberts, D. L., Bennet, D. W. and Forst, S. A. (1994). Identification of the site of phosphorylation on the osmosensor, EnvZ, of *Escherichia coli*. *J. Biol. Chem.* **269**(12): 8728–8733.

Roy, R.N. (1980). Fluorimetric Assay of the Activity of Extracellular Lipases of *Pseudomonas fluorescens* and *Serratia marcescens*. *J. Appl. Bacteriol.* **49**: 265-271.

Russo, F.D. and Silhavy, T.J. (1991). EnvZ controls the concentration of phosphorylated OmpR to mediate osmoregulation of the porin genes. *J. Mol. Biol.* **222**(3): 567-580.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular Cloning: A laboratory manual. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York.

Sato, M., Machida, K., Arikado, E., Saito, H., Kakegawa, T. and Kobayashi, H. (2000). Expression of Outer Membrane Proteins in *Escherichia coli* Growing at Acid pH. *Appl. Env. Microbiol.* **66**(3): 943-947.

Scanlan, R.A., Sather, L.A. and Day, E.A. (1965). Contribution of free fatty acids to the flavor of rancid milk. *J. Dairy Sci.* **58**: 1582-1584.

Sexton, R., Gill, P.R. Jr, Callanan, M.J., O'Sullivan, D.J., Dowling, D.N. and O'Gara, F. (1995). Iron-responsive gene expression in *Pseudomonas fluorescens* M114: cloning and characterization of a transcription-activating factor, PbrA. *Mol. Microbiol.* **15**(2): 297-306.

Sexton, R., Gill, P.R. Jr, Dowling, D.N. and O'Gara, F. (1996). Transcriptional regulation of the iron-responsive sigma factor gene *pbrA*. *Mol. Gen. Genet*. **250**(1): 50-58.

Simon, R., Priefer, U. and Pühler, A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: Transposon mutagenesis in Gram negative bacteria. *Biotechnol.* **1**: 784-791.

Sleator, R.D. and Hill, C. (2001). Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence. *FEMS Microbiol. Rev.* **26**: 49-71.

Slauch, J.M., Russo, F.D. and Silhavy, T.J. (1991). Suppressor mutations in *rpoA* suggest that OmpR controls transcription by direct interaction with the alpha subunit of RNA polymerase. *J. Bacteriol.* **173**(23): 7501-7510.

Slauch, J.M. and Silhavy, T.J. (1989). Genetic analysis of the switch that controls porin gene expression in *Escherichia coli* K-12. *J. Mol. Biol.* **210**(2): 281-292.

Slauch, J.M. and Silhavy, T.J. (1991). *cis*-acting *ompF* mutations that result in OmpR-dependent constitutive expression. *J. Bacteriol.* **173**(13): 4039-4048.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**(1): 76-85.

Stead, D. (1984). Evaluation of a fluorimetric assay on the lipases from strains of milk psychrotrophic bacteria. *J. Dairy Res.* **51**: 123-130.

Suhren, G. (1989). Producer microorganisms. In *Enzymes of Psychrotrophs in Raw Food*, pp 3-35. Edited by R.C. McKellar. CRC Press, Boca Raton, Florida.

Swift, S., Downie, J.A., Whitehead, N.A., Barnard, A.M., Salmond, G.P. and Williams, P. (2001). Quorum sensing as a population-density-dependent determinant of bacterial physiology. *Adv. Microb. Physiol*. **45**: 199-270.

Tibbetts, C. (1995). Raw Data File Formats, and the Digital and Analog Raw Data Streams of the ABI PRISM™377 DNA Sequencer: A preliminary technical examination. Unpublished.

Thomas, C.E. and Sparling, P.F. (1994). Identification and cloning of a *fur* homologue from *Neisseria meningitidis*. *Mol. Microbiol.* **11**(4): 725-737.

Tran, V.K., Oropeza, R. and Kenney, L.J. (2000). A Single Amino Acid Substitution in the C terminus of OmpR Alters DNA Recognition and Phosphorylation. *J. Mol. Biol.* **299**: 1257-1270.

Tsung, K., Brissette, R.E. and Inouye, M. (1989). Identification of the DNA-binding domain of the OmpR protein required for transcriptional activation of the *ompF* and *ompC* genes of *Escherichia coli* by *in vivo* DNA footprinting. *J. Biol. Chem.* **264**(17): 10104-10109.

van Alphen, W. and Lugtenberg, B. (1977). Influence of osmolarity of the growth medium on the outer membrane protein pattern of Escherichia coli. *J. Bacteriol.* **131**(2): 623-630.

Van Hove, B., Staudenmaier, H. and Braun, V. (1990). Novel two-component transmembrane transcription control: regulation of iron dicitrate transport in *Escherichia coli* K-12. *J. Bacteriol.* **172** (12): 6749-6758.

Venturi, V., Ottevanger, C., Bracke, M. and Weisbeek, P. (1995). Iron regulation of siderophore biosynthesis and transport in *Pseudomonas putida* WCS358: involvement of a transcriptional activator and of the Fur protein. *Mol. Microbiol.* **15**(6): 1081-1093.

Vidal, O., Longin, R., Prigent-Combaret, C., Dorel, C., Hooreman, M. and Lejeune, P. (1998). Isolation of an *Escherichia coli* K-12 Mutant Strain Able To Form Biofilms on Inert Surfaces: Involvement of a New *ompR* Allele That Increases Curli Expression. *J. Bacteriol.* **180**(9): 2442-2449.

Wassif, C., Cheek, D. and Belas, R. (1995). Molecular Analysis of a Metalloprotease from *Proteus mirabilis*. *J. Bacteriol.* **177**(20): 5790-5798.

Waukau, J. and Forst, S. (1992). Molecular Analysis of the Signaling Pathway between EnvZ and OmpR in *Escherichia coli*. *J. Bacteriol.* **174**(5): 1522-1527.

Woo, A.H. and Lindsay, R.C. (1983). Statistical correlation of quantitative flavor intensity assessments and individual free fatty acid measurements for routine detection and prediction of hydrolytic rancidity off-flavors in butter. *J. Food Sci.* **48**: 1761.

Woods, R.G. (2000). The regulation of lipase and metalloprotease production in *Pseudomonas fluorescens* B52: A molecular analysis. PhD thesis, Griffith University, Southport, Queensland.

Woods, R.G., Burger, M., Beven, C.-A. and Beacham, I.R. (2001). The *aprX-lipA* operon of *Pseudomonas fluorescens* B52: a molecular analysis of metalloprotease and lipase production. *Microbiology* **147**: 345-354.

Wosten, M.M. (1998). Eubacterial sigma-factors. *FEMS Microbiol. Rev.* **22**(3): 127-150.

Yang, Y. and Inouye, M. (1991). Intermolecular complementation between two defective mutant signal-transducing receptors of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **88**(24): 11057-11061.