THE UNIVERSITY OF CALGARY

Effect of *fpvA* mutations on exotoxin A production in *Pseudomonas aeruginosa*

by

Julian Allister Oliver Wood

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOLOGICAL SCIENCES

CALGARY, ALBERTA

SEPTEMBER, 1997

Abstract

Pseudomonas aeruginosa is a common pathogen in immunocompromised patients, and is particularly important in lung infections of cystic fibrosis patients. Of the many virulence factors which are involved in *Pseudomonas* pathogenesis, exotoxin A is the most toxic. Exotoxin A (ETA) is subject to many levels of regulation, and its production is responsive to several environmental factors, including oxygen tension, temperature, pH, and iron concentration. This regulation seems to be coordinated by the *regAB* gene locus. RegAB is subject to the same regulation as ETA and is required for ETA production. Several factors have been shown to be involved in the regulation of the *regAB* locus, including *vfr*, *pvdS*, and *fur*. How these factors specifically affect the *regAB* locus in response to environmental conditions remains unclear. In an attempt to clarify the issue, we generated a tandemly duplicated *regA-lacZ* fusion in *Pseudomonas aeruginosa* strain PA103. This allowed us to monitor both transcription from the *regAB* promoters and ETA production, since a wild type copy of *regAB* remained after homologous recombination. The fusion strains were mutated using a Tn*5* derivative, and screened for altered beta-galactosidase production using a microtiter plate assay. Those mutants with altered beta-galactosidase production were assayed for ETA production and screened by Southern blot analysis to ensure insertion had not occurred in the *regAB* locus. One mutant was found to have the Tn*5* inserted into the gene for the ferripyoverdine receptor, *fpvA*. The mutation caused an increase in ADPRT activity in both low and high

iron growth conditions. An Ω interposon was inserted into the *fpvA* gene of wild type PA103, but this insertion had the exact opposite effect of the transposon insertion mutation. Pyoverdine levels in the mutants indicated no alteration of iron regulation. A mechanism explaining how these mutations affect FpvA function is postulated. Two other genes were also identified. One causes a reduction in ADPRT activity and the other is homologous to *fpvA*. Both are discussed based on their partial characterization.

Acknowledgements

First, I thank my supervisor, Dr. Doug Storey. He has always been helpful and encouraging.

I am grateful to the Alberta Heritage Foundation for Medical Research, the Canadian Cystic Fibrosis Foundation, and the University of Calgary for financial support throughout graduate school.

The people working in the Department of Biological Sciences have made graduate school a fun experience. I've made many good friends here.

I also thank my labmates and especially Dr. Isabelle Loubens for her continued insight and help on my project.

Finally, I extend my gratitude to my family, Rick, Joan, Jenn and Lexy for their continued support, encouragement and interest in my endeavours.

Table of Contents.

List of Figures.

List of Tables.

List of Abbreviations.

A	absorbance
α	alpha
ADP	adenosine diphosphate
ADPRT	ADP ribosyltransferase
AMP	adenosine monophosphate
Ap	ampicillin
ß	beta
BLAST	basic local alignment search tool
C-terminal	carboxy terminal
CAS	chrome azure S
Cb	carbenicillin
CF	cystic fibrosis
CFU	colony forming unit
CPM	counts per minute
CRP	cyclic AMP receptor protein
CTAB	cetyl trimethyl ammonium bromide
dCTP	deoxycytidine triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EF-2	elongation factor II
ELISA	enzyme-linked immunosorbent assay
ETA	exotoxin A

g	gram
Gm	gentamicin
GTE	glucose, Tris and EDTA buffer
h	hours
IVET	*in vivo* expression technology
L	liter
LB	Luria-Bertani broth
LD_{50}	lethal dose to kill 50% of animals
LPS	lipopolysaccharide
lys	lysine
M	molar
MCS	multiple cloning site
mg	milligram
mL	milliliter
mM	millimolar
mRNA	messenger RNA
MW	molecular weight
N	normal
N-terminal	amino terminal
NAD	nicotinamide adenine dinucleotide
NTA	nitrilotriacetic acid
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
OD	optical density
ONPG	o-nitrophenyl-ß-D-galactopyranoside
ORF	open reading frame
P1	promoter 1 of *regAB* operon

P2	promoter 2 of *regAB* operon
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFU	plaque forming units
PMSF	phenylmethylsulphonyl fluoride
R	resistant
RNA	ribonucleic acid
rpm	revolutions per minute
S	sensitive
s	second
σ	sigma
σ^{70}	RNA polymerase sigma factor 70
SDS	sodium dodecylsulphate
Sm	streptomycin
SMM	succinate minimal medium
sp	species
Spec	spectinomycin
SSC	standard sodium citrate
suc	sucrose
T1	transcript 1 of *regAB*
T2	transcript 2 of *regA*
TAE	tris-acetate-EDTA
TCA	trichloroacetic acid
TE	tris EDTA
Tet	tetracycline
Tris	tris (hydroxymethyl) aminomethane

TSBDC	trypticase soy broth - dialyzed and chelexed
UV	ultraviolet
VBMM	Vogel Bonner minimal medium
w / v	weight per volume
X-gal	5-chloro-4-bromo-3-indolyl-ß-D-galactopyranoside
°C	degrees Celsius
µg	microgram
µL	microliter
µM	micromolar
Ω	omega
bp	base pair
kb	kilobase

Chapter 1 Introduction

1.1 *Pseudomonas* Pathogenesis

Pseudomonas aeruginosa is an aerobic Gram-negative rod. These bacteria are ubiquitous in the environment, but have a preference for moist areas. They are also opportunistic pathogens that take advantage of compromised individuals, such as people suffering from diabetes, cancer and cystic fibrosis (CF). *P. aeruginosa* accounts for about 17% of all nosocomial infections, typically those incurred following invasive surgery or immunosuppression (Pollack, 1990). It is also responsible for 60% of respiratory tract infections in CF patients (Keramidas, 1987).

1.1.1 *P. aeruginosa* and Cystic Fibrosis

In CF patients, infection by *P. aeruginosa* is particularly important. Cystic fibrosis is an autosomal recessive disease characterized by the production of hyperviscous secretions in the respiratory tract that lead to pancreatic insufficiency, deficiencies in electrolyte transport and chronic pulmonary infections (McPherson and Dormer, 1987). Infections caused by *P. aeruginosa*, the primary pathogen isolated from CF patients (May *et al.*, 1991; Fitzsimmons, 1993), are notoriously refractory to antibiotic treatment (McPherson and Dormer, 1987; Collins, 1992).

1.1.2 Virulence Factors

1.1.2.1 Cell-associated Factors

P. aeruginosa produces a variety of virulence factors involved in adherence, proliferation and persistence. LPS seems to be involved in protection from phagocytosis (Pitt, 1989). Pili and flagella modulate binding to epithelial cells in the respiratory tract, while some non-pilus adhesins modulate binding to mucin (Simpson *et al.*, 1992). Alginate production appears to anchor the bacteria to epithelial cells after initial colonization, and its production in *P. aeruginosa* is almost unique to CF isolates (Govan and Harris, 1986). When CF isolates are grown on nutrient medium, the production of alginate causes colonies to take on a mucoid phenotype (Pollack, 1990).

1.1.2.2 Extracellular Factors

Local proliferation of *P. aeruginosa* infections is facilitated by the secretion of a variety of exoproducts. These include elastase, alkaline protease, phospholipase C, exoenzyme S and exotoxin A (ETA) (Pollack, 1990). Persistance of the bacterial infection, as seen in chronic infections of CF patients, involves a phenotypic conversion to mucoidy. In chronic infections, exoproducts are down-regulated while alginate and rough LPS (but not smooth LPS as seen in non-chronic isolates) production is increased (Hancock *et al.*, 1983). However, this phenomenon has only been noted in laboratory strains (Mohr *et al.*, 1990); population transcript assays on the sputa

from CF patients have shown that virulence factors are highly transcribed *in vivo* (Storey *et al.*, 1992; Raivio *et al.*, 1994).

1.1.2.2.1 Exotoxin A

Of *P. aeruginosa* virulence factors, ETA is the most toxic, with an LD_{50} of 0.2 μg on intraperitoneal injection in mice (Iglewski and Kabat, 1975). More than 90% of all clinical isolates produce exotoxin A (Bjorn *et al.*, 1977). ETA damages tissues through an ADP ribosyl transferase (ADPRT) activity. Incubation of ETA with elongation factor 2 (EF-2) results in the transfer of the ADP-ribosyl moiety of NAD^+ to EF-2. This covalent modification of EF-2 prevents peptide elongation in mammalian cells and ultimately results in their death. In the absence of EF-2, ETA still possesses the ability to hydrolyze NAD^+ (Iglewski *et al.*, 1977; Wick *et al.*, 1990a).

1.1.2.2.2 Siderophores

Almost all bacteria, with the exception of some lactobacilli, rely on iron for their metabolic processes (Braun, 1997). Iron is required as a cofactor for many enzymes, including both membrane-bound electron-transport chain enzymes and cytoplasmic enzymes. Acquisition of iron by bacteria is difficult in most environments, primarily because of its low solubility. To overcome these problems, bacteria have evolved several iron transport systems, without which they could not survive. The most potent are small molecules known as siderophores. These siderophores are so avid that they can remove iron bound to human transferrin or lactoferrin (Sriyosachati and Cox,

1986; Meyer *et al.*, 1996; Xiao and Kisaalita, 1997). *P. aeruginosa* synthesizes at least two siderophores, pyoverdine and pyochelin (Cox, 1980).

1.1.2.2.2.1 Pyoverdine

Pyoverdine is a small, peptide-containing quinoline chromophore capable of binding iron (III) very tightly. It has been demonstrated to be required for growth in human serum (Meyer *et al.*, 1996). Pyoverdine is on the order of 25 magnitudes stronger than pyochelin in its binding affinity for iron (III) (Visca *et al.*, 1992; Sriyosachati and Cox, 1986), with a stability constant of 10^{32} (Meyer *et al.*, 1990) It has also been shown to be produced during colonization of CF patients (Haas *et al.*, 1991). Presumably, pyoverdine is necessary for scavenging iron from host proteins in this iron depleted environment. Indeed, it is able to release iron from transferrin and lactoferrin (Xiao and Kisaalita, 1997), without the aid of prior proteolytic cleavage by elastase (Wolz *et al.*, 1994) as previously thought (Britigan *et al.*, 1993; Doring *et al.*, 1988).

There are at least 3 types of pyoverdines (Meyer *et al.*, 1997). Pyoverdine is synthesized by a variety of enzymes, some of which are produced from the *pvd* regulon. The *pvd* regulon consists of two open reading frames (ORFs) of unknown function, three pyoverdine synthetases (*pvdD*, *pvdE*, and *pvdF*), and a ferripyoverdine receptor, *fpvA* (Poole *et al.*, 1993). The receptor binds only iron-pyoverdine, or ferripyoverdine, complexes. PvdA, encoded by the *pvdA* gene at a separate locus from the *pvd* locus, is an L-ornithine N5-oxygenase.

(Visca *et al.*, 1994). Genes from the *envCD* locus are involved in the secretion of pyoverdine (Poole *et al.*, 1993).

Expression of pyoverdine is seen only under conditions of iron-limitation in wild type bacteria. The *envCD* locus, the *pvd* regulon and the *pvdA* gene are all under strict iron regulation mediated by the Fur repressor protein (Cunliffe *et al.*, 1995; Leoni *et al.*, 1996). *PvdA* is under the transcriptional control of *pvdS* (Leoni *et al.*, 1996), which is in turn controlled by Fur (Miyazaki *et al.*, 1995), but it is not clear how PvdS or Fur interacts with the *envCD*. Several genes in the *pvd* regulon contain consensus *pvdS* binding sites, suggesting indirect regulation by Fur.

1.1.2.2.2.2 Pyochelin

Pyochelin is another small iron-binding molecule, but is quite different from other siderophores in that it is a phenolate siderochrome. In contrast to pyoverdine, it contains neither hydroxamate nor catecholate chelating groups (Ankenbauer and Quan, 1994). There is some debate as to the role pyochelin plays in virulence. It seems to be required for optimal virulence and growth (Cox, 1982) and may assist in the production of hydroxyl radicals, causing local damage at the site of infection (Coffman *et al.*, 1990). Studies in a chronic pulmonary infection model in rats have shown that in *P. cepacia*, pyochelin may aid dissemination of infection (Sokol and Woods, 1988). In *P. aeruginosa*, however, pyochelin is neither capable of supporting growth in human serum, nor can it release iron from human transferrin (Ankenbauer *et al.*, 1985).

1.2 Regulation of Exotoxin A Production

Much of the work describing regulatory events in *P. aeruginosa* has been performed using growth curves in the laboratory. *P. aeruginosa* grows very quickly in an artificial aerobic environment, but exactly how this situation can be related to infectious growth in patients is somewhat ambiguous. Many of the same virulence factors, including ETA, are transcribed and expressed both in laboratory (Bjorn *et al.*, 1979) and clinical (Raivio *et al.*, 1994) situations, but their temporal and density-dependent expression is probably somewhat different. Nevertheless, growth in the laboratory environment provides an ideal system for varying environmental cues and monitoring the consequences. We therefore use growth curves in the laboratory to represent the expression of virulence factors in infection, given the previous correlations of regulation in the laboratory environment with regulation in infectious situations.

1.2.1 Environmental Regulation of ETA

ETA production is not constitutive (Liu, 1973). The level of expression varies in response to growth temperature, aeration and iron concentration (Liu, 1973; Wick *et al.*, 1990a). In particular, high iron concentrations depress ETA production, while low iron concentrations favour higher ETA production. This environment is similar to the situation in the CF lung, where iron is stored (in high affinity binding proteins) and difficult for the bacteria to obtain. Cell damage by exotoxin A can release the intracellular iron stores to the bacteria for scavenging. Strain differences can also account

for different levels of ETA production. For example, strain PAO1, the prototypical toxigenic strain, produces 10-fold less ETA than the hypertoxigenic strain PA103 (Wick *et al.*, 1990b). These observations suggest that ETA production is regulated in response to several environmental cues. The methods in which these cues affect regulation of virulence factors is the subject of intense study.

1.2.2 Transcriptional Regulation of ETA

To determine how ETA production was regulated, Lory (1986) analyzed *toxA* transcript levels using northern blot analysis. The gene *toxA* codes for exotoxin A (Gray *et al.*, 1984). It was found that in low iron conditions, there was a single *toxA* specific band that increased in intensity throughout the growth cycle of *P. aeruginosa*, but in high iron, there were no *toxA* specific bands found in total mRNA preparations. This suggested that regulation of ETA production occurred at the transcriptional level.

1.2.2.1 Regulation by RegA

1.2.2.1.1 Initial Evidence for RegA

The *regA* gene was discovered when several mutants of strain PA103 were created through N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis that were deficient in ETA production (Ohman *et al.*, 1980). NTG is an alkylating agent that produces G to A transitions upon DNA replication. One mutant, PA103-29, had normal production of all exoproducts except for elastase, which isn't produced by wild type PA103 either, and exotoxin A. This strain was

complemented with a PA103 gene bank and the gene which restored ETA production was isolated (Hedstrom *et al.*, 1986). They termed this gene *regA*. It is also known as *toxR*, as named by Wozniak *et al.*, (1987), who discovered it concurrently.

1.2.2.1.2 Requirement of *regA* transcription for ETA Expression

Analysis of ETA production in parallel with *regA* transcription showed that ETA is produced in two phases corresponding to two transcripts from the *regAB* locus(Frank and Iglewski, 1988). An early peak of *regAB* transcripts is seen in both low and high iron, although in high iron the peak is somewhat depressed, while a late peak of *regAB* is seen only in low iron conditions. Iron regulation of the *regAB* transcripts paralleled iron regulation of *toxA* transcripts, suggesting that not only is iron regulation controlled through the *regA* gene, but also reaffirming that ETA production requires *regA* transcription. ETA production corresponded with both *regAB* and *toxA* transcript accumulation. Analysis of the mutant PA103-29 showed that no *regAB* or *toxA* transcripts were present, consistent with the idea that *regA* was required for ETA production. Further studies by Frank and Iglewski (1988) showed that only the late peak of ETA was associated with an increase in ADPRT activity in the supernatant, suggesting that early ETA production remained intracellular, while later ETA was secreted.

1.2.2.1.3 Transcription of *regA*

Using one upstream probe and one internal probe to *regA*, Frank and Iglewski (1988) demonstrated that there were two transcripts, T1 and T2. T1 is a longer transcript produced in both low and high iron conditions early in the growth cycle, while T2 is a shorter transcript produced only under low iron conditions late in the growth cycle. Characterization of the promoter region was done in an effort to distinguish the T1 from the T2 transcript (Storey *et al.*, 1990). The 5' region upstream of the *regA* start codon was divided and subcloned into *lacZ* and *cat* translational and transcriptional fusion vectors. Two promoter regions were identified in this experiment, the P1 and P2 promoters. The P1 promoter constructs showed early reporter activity in both high and low iron, but no late activity, while the P2 constructs showed reporter activity late in the growth curve, and only under low iron conditions. It was concluded that these two promoters, P1 and P2, controlled production of the two observed transcripts, T1 and T2, since their activities and transcript accumulation patterns paralleled each other closely both temporally and in response to iron concentrations. This explained the biphasic transcript accumulation seen earlier for both *regA* and *toxA* transcripts.

Multiple copies of the *regA* locus have a profound effect when introduced into PA103 (Frank *et al.*, 1989). When *regA* is in a single copy, the P1 promoter is active and the T1 transcript is produced in both low and high iron conditions; however, in high iron conditions there is a significant decrease in promoter activity and hence

transcription (Frank and Iglewski, 1988). When multiple copies of *regA* are present on plasmids, T1 transcript accumulation is completely iron deregulated, with similar amounts of T1 transcripts in both low and high iron conditions (Frank *et al.*, 1989). In addition, T1 transcript accumulation in the multicopy situation is higher than in the single copy situation. This implies that there may be a repressor acting at P1 which is diluted out by multiple copies of the P1 promoter, allowing higher activities from P1. An activator, on the other hand, would presumably lead to lower levels of T1 transcript accumulation in the multicopy situation. This repressor would presumably require high iron conditions to be active, or an accessory factor which is activated by high iron. Expression of *toxA* closely paralleled *regA* transcription in both single and multicopy situations, reaffirming the relationship between these two genes.

1.2.2.1.4 Structure and Function of RegA

The exact mechanism by which RegA activates *toxA* is unclear, although some work has been done to elucidate their roles. The *regA* gene encodes a protein of about 28 kilodaltons. The most likely roles for the *regA* gene product are either a transcriptional activator or a sigma factor. Gel-shift assays failed to show any binding of RegA protein to upstream *toxA* sequences (Hamood and Iglewski, 1990); DNA footprinting analysis has not been reported. These observations argue against RegA as a transcriptional factor; however, good evidence in favour of this role has recently been presented by Walker *et al.* (1995). They demonstrated *in vitro* that in the absence of

RegA, *P. aeruginosa* RNA polymerase transcribes *toxA* mRNA poorly. With its addition, transcription increases dramatically (Walker *et al.*, 1994). With *E. coli* RNA polymerase holoenzyme, RegA is also required for efficient transcription of *toxA*, and antibodies against σ^{70} inhibited its transcription, suggesting that RegA is not a sigma factor. They also showed that RegA is required for formation of open-complexes at the *toxA* promoter. Deletion analyses of the upstream *toxA* sequences failed to determine a binding site for RegA, since the only deletion which did not support transcription also deleted the *toxA* promoter (Walker *et al.*, 1995).

Further studies by Raivio *et al.* (1996) defined three structure-function domains of RegA. Mutations were made throughout *regA* by inserting 6 bp linkers at several *Taq*I sites. The ETA activities of these mutants were analyzed over a growth curve in a strain containing a deletion of the *regAB* locus (PA103 Δreg), and the results suggested three domains: one for transcriptional activation, a second for efficiency of activation, and a third for iron regulation. These experiments support a scenario in which RegA functions as an alternative sigma factor.

1.2.2.2 Regulation by *regB*

Additional regulatory information was gained through the comparison of transcript accumulation patterns among different strains of *P. aeruginosa*. Strain PA103 has a biphasic *toxA* and *regA* accumulation pattern, but strain PAO1 only has the late peak of *toxA* and *regA* transcripts. When the *regA* gene was sequenced, it was

noted that there was an ORF of 228 bp just 6 bp downstream from the termination codon for *regA*. In PAO1, however, the ATG start codon for *regB* is mutated to an ACG (Wick *et al.*, 1990b). In a series of mix and match experiments, it was shown that by replacing the *regB* region of the PA103 *regAB* locus with the same region from PAO1, there was a five-fold drop in ETA activity compared to the PA103 *regAB* locus. Replacing the *regA* region had no effect, and replacing the upstream promoter regions was not attempted, even though there are three mutations in this area (Wick *et al.*, 1990b). While a product of the *regB* gene has never been identified experimentally, it does appear that this region is important in modulating ETA levels.

The role of the *regB* sequence was studied by Storey *et al.* (1991) in PA103-29. Upon sequencing of the *regAB* region from this mutant, they found two mutations, one upstream of the promoter region and one that introduced a premature stop codon into *regA*. This explained why there was no ETA activity in this strain, but didn't explain why there were no *regA* transcripts. It also implied that translation of *regA* transcripts was required for *regB* to be translated. This suggested that the ribosome may translate *regB* through a re-initiation event possibly requiring an anti-terminator. Consistent with this theory is the absence of any ribosome binding site upstream of the *regB* start codon.

When a P1-P2-*lacZ* translational fusion was introduced into PA103-29, Storey *et al.* (1991) found normal P2 activity but no P1 activity. In addition, they found that when this strain was complemented with the PA103 *regAB* locus, both the T1 and T2

transcripts bound a *regA*-specific probe but only the T1 transcript bound the *regB*-specific probe. This suggested that the *regB* ORF was required for P1 activity (but not P2 activity), and would be consistent with the idea that a repressor was functioning at the P1 regulatory site in the absence of RegB to inactivate the P1 promoter. It also explained the size differences between the T1 and T2 transcripts seen earlier. It didn't explain why PA103-29 did not have any P2 activity, since *regB* is not required for activity as in PAO1.

Attempts to express RegB using a variety of expression systems and promoters have given mixed results, nonetheless, it is clear that the *regB* ORF has some role in activating transcription at the P1 promoter. Some preliminary results using an expression vector where *regB* is under the control of the Tac promoter have shown increases in ETA production in both PA103 and PAO1, although these results require confirmation. Translational fusions to ß-galactosidase (Raivio, 1995) and glutathione S transferase have also suggested the synthesis of a small, antigenic protein derived from the *regB* ORF.

In a mutant of PAO1, designated Fe18, P1 promoter activity has been restored. In an effort to explain this, Ali (1993) sequenced the *regAB* region of Fe18 and found it to be identical to that in PAO1, where the *regB* ORF is missing, thus this mutant has P1 activity without a functional RegB or *regB* ORF. To explain, this, it was suggested that RegB may inactivate a repressor which acts at the P1 promoter. In PAO1, since RegB is absent, the repressor is free to inactivate the P1 promoter, hence no T1 transcript is observed. In Fe18, this repressor could be mutated to allow restored P1

transcription. Preliminary results with the above RegB overexpression vector have shown only a small increase in ETA activity in strain Fe18, as would be expected if the repressor were already inactive.

1.2.2.3 Regulation by Vfr

Some recent work has shown that there are additional regulatory factors acting at the P1 promoter. One gene, *vfr*, was cloned by its ability to complement ETA and protease deficient NTG mutants of strain PA103. These mutants were specifically deficient at the P1 promoter, and thus Vfr would appear to be a transcriptional activator at this site. The Vfr protein is 91% similar to the *E. coli* cyclic-AMP receptor (CRP) protein, and complements *E. coli crp* mutants. This suggests that Vfr may be a global regulator of virulence factors in *P. aeruginosa* (West *et al.*, 1994). Indeed, Vfr has been shown to be involved in regulating quorum sensing through modulation of *lasR* (Albus *et al.*, 1997). In *P. aeruginosa*, however, Vfr does not function in catabolite repression, and *E. coli* CRP cannot complement a *P. aeruginosa* Vfr mutation.

1.2.2.4 Regulation by Fur

The ferric uptake regulator (Fur) protein has also been implicated in regulation at the *regAB* locus. In an iron-sufficient environment, *E. coli* Fur normally binds free iron (Fe^{2+}) and a corepressor. This complex then binds to the promoter of iron sensitive genes and represses them (Prince *et al.*, 1991). In iron deficient conditions, Fur releases bound iron and derepresses these

genes. The production of high affinity iron binding siderophores is an example of a Fur-regulated system, both in *E. coli* (De Lorenzo *et al.*, 1988) and *P. aeruginosa* (Cunliffe *et al.*, 1995). In fact, Fur mutants in *P. aeruginosa* constitutively produce both pyoverdin and pyochelin (Hassett *et al.*, 1996). Many additional Fur regulated genes have been identified in *P. aeruginosa* using an *in vitro* cycle-selection strategy employing PCR and purified Fur protein to bind genomic DNA fragments containing Fur recognition sites (Ochsner and Vasil, 1996).

In contrast to the behaviour of Fur seen in *E. coli*, an over-expression vector of *E. coli* Fur in *P. aeruginosa* causes depression of ETA activities in PA103 and PAO1 under both high and low iron conditions. Further studies showed that no *toxA* transcript was produced in the presence of *E. coli fur*. Looking at the *regAB* operon, they found that at an early growth point, no T1 transcript was produced, but that a T2 transcript was still produced. This finding suggests that Fur may play a role at both the P1 and *toxA* promoters (Prince *et al.*, 1991). The *fur* gene has recently been cloned from *P. aeruginosa* by its ability to complement a *fur* mutation in *E. coli* (Prince *et al.*, 1993), and its activity in *P. aeruginosa* must be studied before making further interpretations about the *E. coli* Fur studies. Strains containing point mutations in Fur, however, do confirm its role in affecting the iron regulation seen at the *toxA* locus (Barton *et al.*, 1996).

Some studies using the *E. coli* Fur over-expression vector in strain Fe18 have also been performed. Compared to a vector control, *E. coli* Fur causes about a three-fold increase in early ETA activity

under low iron conditions, and a smaller but noticeable increase in ETA activity under high iron conditions (Ali, 1993). The repression seen by Prince *et al.* (1991) was reproduced in PAO1, thus the mutation in Fe18 also seems to have an effect with the interaction of Fur at the *regAB* P1 promoter.

Fur does not bind directly to either the *regAB* or *toxA* promoter regions (Ochsner *et al.*, 1995); consequently, its affect on these promoters must be through another factor. It is likely that this factor is *pvdS*.

1.2.2.5 Regulation by PvdS

PvdS is another global regulatory factor that was recently cloned and identified (Cunliffe *et al.*, 1995; Miyazaki *et al.*, 1995). PvdS appears to be an alternative sigma factor that plays a role both in the regulation of ETA production (Ochsner *et al.*, 1996) and in the regulation of pyoverdin production (Miyazaki *et al.*, 1995). The *pvdS* locus is strictly regulated by Fur (Ochsner *et al.*, 1995) in the traditional manner. The *pvdS* gene product is essential for transcription of the *regAB* (Ochsner *et al.*, 1996) and *pvdA* loci, and is probably involved in the regulation of other pyoverdin synthesis genes (Leoni *et al.*, 1996). A consensus binding site for PvdS has been defined as (G/C)CTAAATCCC by Rombel and colleagues (1995). This sequence was identified by point mutations in the *toxA* promoter and through comparison of the promoter regions between several iron regulated genes. The consensus sequence is also present in the promoters of *pvdA*, *pvdD*, and *pvdE* (Leoni *et al.*, 1996). There

is, however, additional complexity in the regulation of the *regAB* locus. It appears that *pvdS* is most important under microaerobic conditions, but under aerobic conditions another activator must be playing a role (Ochsner *et al.*, 1996).

1.2.2.6 Regulation by PtxR

A recent report has described the effect of yet another factor involved with regulation of ETA production (Hamood *et al.*, 1996). This factor, *ptxR*, was cloned through its ability to increase secretion of ETA in a secretion-deficient mutant of PAO1. The gene product of *ptxR* also increases levels of RegA, which presumably extends the effect to *toxA*, and works at the level of transcription. The predicted amino acid sequence of *ptxR* shows homology to the LysR family of transcriptional activators.

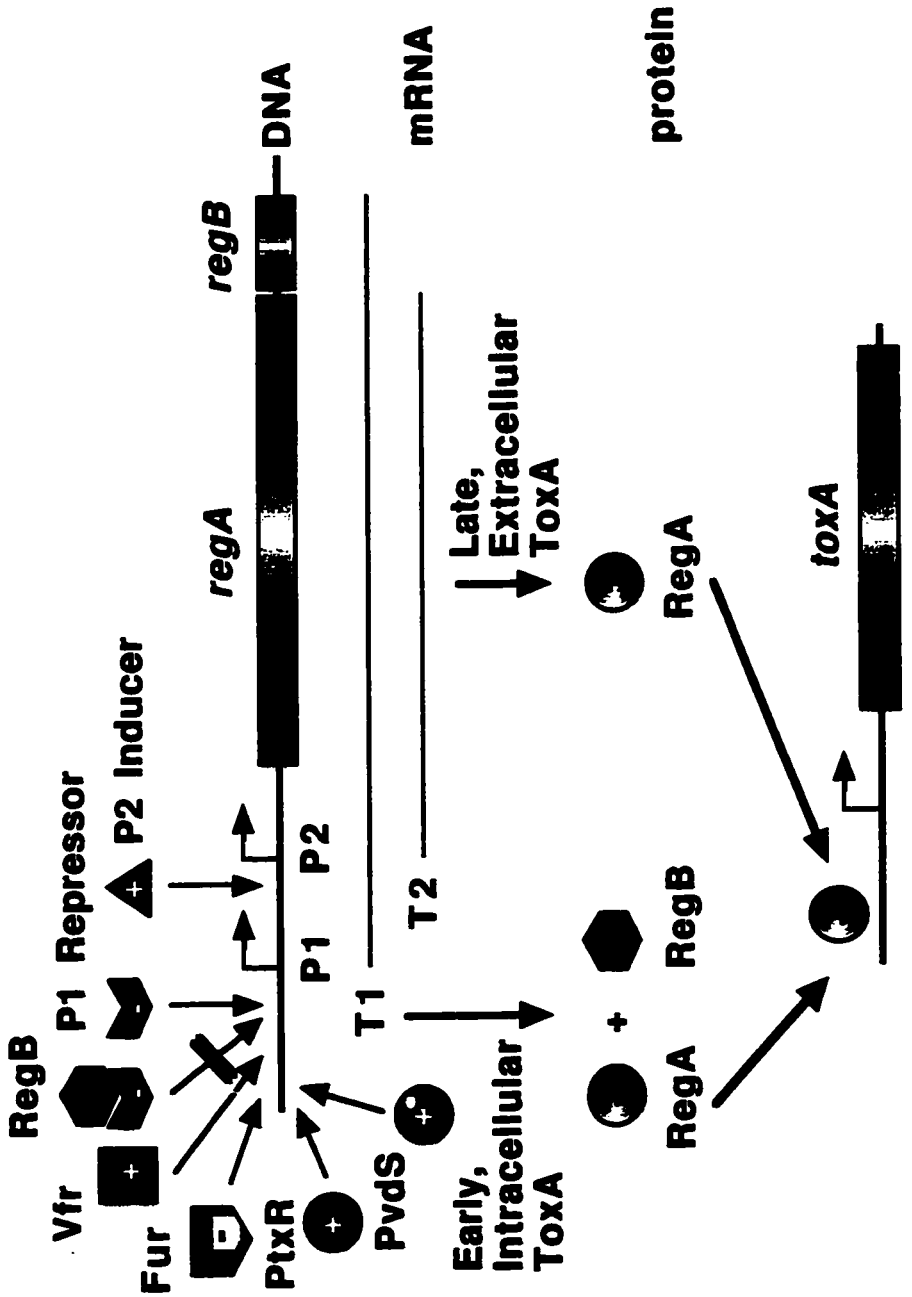
1.2.2.7 Model of transcriptional regulation at *regAB* locus

In the labarotory situation, regulation at the *regAB* locus in strain PA103 can be summarized in the following model (Figure 1). Early in the growth curve, the P1 promoter is active. A T1 transcript is produced that includes both *regA* and *regB* ORF's. Only intracellular ETA activity can be detected from ETA produced as a result of T1 transcription. The *regB* ORF somehow activates P1, probably by inactivating a P1 repressor. An anti-terminator may be responsible for allowing *regB* to be translated. Vfr is another regulatory protein which activates P1, while Fur appears to repress

it. Both PvdS and PtxR also play roles in activation at the P1 promoter. Later in growth, P2 promoter activity begins and P1 promoter activity drops. It is possible that transcription from the P2 promoter interferes with transcription from the P1 promoter, and the subsequent drop in *regB* allows increased repression at the P1 promoter. An activator for P2 promoter activity seems likely, and it too may interfere with the P1 promoter, causing repression. A T2 transcript containing only *regA* sequences is seen at this stage. ETA produced as a result of T2 transcripts is detected extracellularly. Both the T1 and T2 transcripts encode RegA which somehow activates *toxA*.

Figure 1. Diagrammatic representation of transcriptional events at the *regAB* locus in PA103.

In *P. aeruginosa* PA103, there are many factors controlling the transcription of the *regAB* locus, which in turn controls transcription of the *toxA* locus. The following figure summarizes the regulatory interplay at the *regAB* locus. Refer to the text for an explanation of events.


RegB
P1
Repressor
P2
Inducer
Vfr
Fur
PtxR
PvdS
regA
regB
DNA
P1
P2
T1
T2
mRNA
protein
Late,
Extracellular
ToxA
Early,
Intracellular
ToxA
RegA
RegB
RegA
toxA

1.3 Iron Transport across the Inner and Outer Membranes

As alluded to previously, iron is central in the regulation of many virulence factors involved in *Pseudomonas* pathogenesis. While pyoverdin and pyochelin are essential for scavenging iron, the question remains as to how the iron is translocated across the membranes, and how this in turn activates or represses genes involved in virulence.

1.3.1 Iron Chelators

Pseudomonas is capable of internalizing several very small iron-binding molecules. These small iron chelators include citrate (Cox, 1980), salicylic acid (Meyer, 1992), nitrilotriacetic acid (NTA) (Meyer and Hohnadel, 1992), and inositol polyphosphate (Smith *et al.*, 1994). While *Pseudomonas* cannot produce NTA, it can use iron-loaded NTA. The other molecules are probably produced in abundance only in conditions of iron abundance when the synthesis of more avid and energy-costly siderophores is repressed. These molecules, with the possible exception of NTA, are actively transported into the cell via specialized receptor mechanisms (Meyer *et al.*, 1996).

One well studied sytem that requires mention is the ferric citrate transport system in *E. coli*, reviewed by Braun (1997). Many of the proteins involved in transporting iron from ferric citrate into the cytoplasm, along with the accompanying proteins responsible for activation of the ferric citrate transport genes have been worked out. The transport proteins are all encoded from the *fecIRABCDE*

locus, and are regulated by the FecIR proteins also encoded from this locus. The system is novel in that it is induced by ferric citrate, but ferric citrate is never transported into the cytoplasm (Zimmermann *et al.*, 1984). Ferric citrate is first bound and transported into the periplasm by FecA, where it is then bound by the periplasmic binding protein, FecB. FecB delivers iron across the cytoplasmic membrane in an ATP-driven event in concert with membrane-bound FecD, FecC and FecE proteins. The translocation of ferric citrate across the outer membrane is driven by the electrochemical potential of the cytoplasmic membrane and the TonB-ExbB-ExbD complex (Braun, 1995). Curiously, in *fecB* mutants, the *fec* operon is still induced (Zimmermann *et al.*, 1984). This proved that ferric citrate transport into the cytoplasm is not required for induction. When ferric citrate binds to the FecA receptor, a signal is transmitted, likely via a conformational change, to the inner membrane-bound FecR protein. FecR interacts with cytoplasmic FecI, a member of the σ^{70}-family (Angerer *et al.*, 1995), to activate transcription of the *fec* operon. This system is similar to the pseudobactin siderophore transport system described in *P. putida* (Koster *et al.*, 1994), and may turn out to be the basis for other siderophore uptake systems as well.

1.3.2 Foreign Siderophores

Pseudomonads have also devised methods of using iron-loaded siderophores of foreign origin. Siderophores from other bacteria known to satisfy *Pseudomonas* iron requirements include pyoverdines from *P. fluorescens* and *P. chlororaphis*, cepabactin from *Burkholderia cepacia*, and enterobactin from *E. coli*. These

siderophores enter the cell through specialized receptors and porins (Meyer *et al.*, 1996).

1.3.3 Native Siderophores

Two siderophores have been described in *P. aeruginosa*, pyochelin and pyoverdine. The mechanism of their transport into the cells remains largely unknown, but current work is pointing towards a system similar to the *fec* mechanism in *E. coli*. There are several homologous systems that may all function through similar mechanisms, including the ferripyoverdine receptor (FpvA) from *P. aeruginosa* (Poole *et al.*, 1993), PbuA, the *Pseudomonas sp.* ferric pseudobactin M114 receptor protein (Morris *et al.*, 1994), PupA, the *P. putida* pseudobactin uptake protein (Bitter *et al.*, 1994), PupB, the *P. putida* ferric-pseudobactin receptor (Koster *et al.*, 1993), FptA, the *P. aeruginosa* PAO Fe(III)-pyochelin receptor (Ankenbauer and Quan, 1994) and FhuE, the *E. coli* outer-membrane receptor precursor protein for Fe(III)-coprogen and Fe(III)-rhodotorulic acid (Sauer *et al.*, 1990).

1.3.3.1 Ferripyoverdine

The complex of pyoverdine and iron is termed ferripyoverdine. Ferripyoverdine is bound by FpvA, the 86 kDa mature ferripyoverdine receptor. This receptor is encoded by *fpvA*, which lies in the middle of the *pvd* operon described earlier. It is hypothesized that translocation of ferripyoverdine is driven by a TonB system, as FpvA bares homology to other TonB-dependent receptors (Poole *et al.*, 1993). Exactly how this directs inactivation of iron-regulated genes

remains to be elucidated. The FpvA protein is translated initially as a precursor of 813 amino acids, and is later processed to a mature form. This assumes that translation starts from the first methionine after the first of two putative Shine-Dalgarno sequences, and that a 43 amino acid signal sequence is cleaved upon secretion, similar to that observed with the ferric pseudobactin receptor, PupB (Koster *et al.*, 1993).

1.3.3.2 Ferripyochelin

Similar to pyoverdin, iron-bound pyochelin (ferripyochelin) binds to a 76 kDa specific receptor encoded by *fptA* (Ankenbauer and Quan, 1994). This gene lies in a putative operon quite different from the *pvd* operon, and the function of the two adjactent ORFs is unknown (Ankenbauer and Quan, 1994). Pyochelin has also been reported to bind to a smaller, 14 kDa receptor (Sokol and Woods, 1983). In direct contrast to *fpvA*, *fptA* possesses a Fur box in its promoter region, but doesn't contain a TonB box in its N-terminal protein sequence (Ankenbauer and Quan, 1994). Recently, Wang and colleagues (1996) determined through *in vivo* expression technology (IVET) that FptA is selectively induced in a neutropenic mouse infection model. Another IVET experiment using an *in vitro* CF mucus system also identified *fptA* as a putative virulence factor (Wang *et al.*, 1996a). FptA is regulated in response to iron, but it seems that a unique AraC-like protein, designated PchR, is responsible for both activation and repression of *fptA* (Heinrichs and Poole, 1996).

1.4 Project Objectives

The objective of the current study was to evaluate what factors might specifically be affecting the *regAB* operon, particularly with respect to its iron regulation.

To determine which factors specifically act at the *regAB* promoters, two transcriptional fusions between the *regAB* promoters and *lacZ* were recombined onto the *P. aeruginosa* PA103 chromosome. This allowed easy measurement of the *regAB* P1 and P2 promoters. These reporter strains were mutagenized with a Tn*5* based transposon, and their ß-galactosidase activities measured in low iron conditions. Strains which displayed markedly different activities from parental controls were further analyzed, and DNA flanking the Tn*5* was eventually cloned.

Chapter 2 Materials and Methods

2.1 Media and Additives

Chemicals used to make media were purchased from BDH Inc., Gibco BRL (Life Technologies, Gaithersburg, MD), VWR Scientific of Canada Ltd., or Sigma Chemical Co., unless otherwise specified. All antibiotics were purchased from Sigma Chemical Co.

2.1.1 Liquid Media

Escherichia coli strains were grown in either Luria-Bertani (LB) broth (Sambrook *et al.*, 1989), SOB broth, or NZY broth. SOB broth is prepared by dissolving 20 g tryptone, 5 g yeast extract, 0.5 g NaCl, and 2.5 mL of 1M KCl in 1 L of distilled water. After adjusting the pH to 7.0 and autoclaving, 5 mL of autoclaved 2 M $MgCl_2$ is added. NZY broth is prepared by dissolving 10 g casein hydrolysate acid hydrolyzed vitamin-free, 5 g NaCl, 5 g yeast extract, and 2 g $MgSO_4 \cdot 7H_2O$ in 1 L distilled water and adjusting the pH to 7.5 with 10 M NaOH. All media were autoclaved for 15 minutes at 121°C before use.

Pseudomonas aeruginosa strains were grown in LB broth, Vogel Bonner minimal medium (VBMM), succinate minimal medium (SMM), or trypticase soy broth dialysate - chelexed (TSBDC). VBMM (Vogel and Bonner, 1956) is prepared by dissolving 3.0 g citric acid trisodium salt, 2.0 g citric acid, 10.0 g K_2HPO_4 (BDH Inc.), 3.5 g $Na(NH_4)HPO_4 \cdot 4H_2O$ and 0.2 g $MgSO_4 \cdot 7H_2O$ in 1 L of distilled water. The pH is brought to 7.0 with the addition of 10 M NaOH. Succinate minimal medium is prepared by dissolving 6.0 g of K_2HPO_4, 3.0 g of

KH_2PO_4, 1.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 4.0 g of succinic acid in 1 L of distilled water. The pH is brought to 7.0 with the addition of 10 M NaOH. TSBDC (Liu, 1973) is prepared by mixing 30 g of tryptic soy broth (Difco Laboratories) and 5 g of chelex resin (Bio-Rad Laboratories) in 90 mL of milliQ water for 4 hours. The concentrate was dialyzed against milliQ water overnight with stirring using 40 mm Spectra/Por dialysis tubing with a molecular weight cut-off of 6-8000 (Spectrum Medical Industries, Inc., Laguna Hills, CA) The liquid outside the dialysis tubing was autoclaved, and before use, 20 mL of similarly chelexed glycerol and 2.5 M monosodium glutamate were added.

2.1.2 Solid Media

To make solid media, liquid media was prepared as above and supplemented with agar to a final 1.5% concentration before being autoclaved and poured into sterile petri dishes.

Chrome azurol S (CAS) plates were prepared as described in Schwyn and Neilands (1987).

Top agar was prepared by adding agar to a 0.75% concentration in a suitable liquid medium. Before use, the top agar was melted in a steam bath and maintained at 48°C. Bacteria and phage were added to melted top agar and then poured onto solid agar plates pre-warmed to 37°C.

2.1.3 Antibiotics

For growth of *E. coli* containing plasmids, agar plates or liquid media contained either 50 μg/mL kanamycin, 100 μg/mL ampicillin,

10 μg/mL gentamicin, 10 μg/mL tetracycline, or 20 μg/mL streptomycin.

For growth of *P. aeruginosa* containing plasmids or chromosomal fusions, agar plates or liquid media contained either 300 μg/mL carbenicillin, 50 μg/mL gentamicin, 100 μg/mL tetracycline, 500 μg/mL spectinomycin or 500 μg/mL streptomycin.

2.1.4 Additives

To achieve high iron conditions in liquid media, 10 mg/mL $FeCl_3$ (Sigma) was added to a final 10 μg/mL concentration. Low iron conditions were maintained by washing glassware with 20% HCl (BDH Inc.) for 2 hours, rinsing with milliQ deionized water (Millipore), and autoclaving prior to growth in low iron media.

For *sacB* counterselection in *E. coli* and *P. aeruginosa*, sucrose (BDH Inc.) was added to a 5% concentration.

For blue/white selection of bacteria, 40 μL of 50 mg/mL ß-galactosidase chromogenic substrate X-gal (Gibco BRL) in N,N-dimethyl formamide (Sigma Chemical Co.) and 10 μL of 20 mM IPTG (Sigma Chemical Co.) was spread on agar plates before spreading bacteria.

2.2 Growth Curve Methodology

Primary overnight cultures of *P. aeruginosa* were grown under low iron conditions in 10 mL of succinate minimal medium or TSBDC medium with aeration (250 rpm) at 32°C in a Lab-Line incubator (Lab-Line Instruments, Inc.). Secondary cultures in low and high iron conditions were inoculated from the primary culture to give a final

OD_{540} of 0.02 (measured on a Beckman DU50 spectrophotometer (Beckman Instruments, Inc.) and grown under similar conditions.

Aliqots were removed at indicated time intervals and spun at 19 000xg in a Biofuge microcentrifuge (Baxter, Canlab). The supernatant was frozen at -70°C for exotoxin A assays and the cell pellet was resuspended in 1 ml of 1x A buffer (10x A buffer is 10.5 g anhydrous potassium phosphate dibasic, 4.5 g anhydrous potassium phosphate monobasic, 1 g ammonium sulphate, and 0.5 g sodium citrate in 100 mL distilled water). The cell suspension was also frozen at -70°C for ß-galactosidase assays.

All growth experiments were repeated at least once.

2.3 Strains and Plasmids

All strains, plasmids and phages used are listed in Table 1. *E coli* strains used to host plasmid manipulations included DH5α, HB101, JM105, XLOLR and XL1 Blue MRF'. Strains were stored in 15% glycerol/broth at -70°C. Phage and plasmids were stored at -20°C.

Table 1. Strains, phages and plasmids.

Strain, Phage or Plasmid	Description	Reference
E. coli		
DH5α	ϕ80d*lacZ*ΔM15Δ(*lacZYA-argF*)U169*deoR recA*1 *endA*1 *hsdR*17(r_k^-,m_k^+) *supE*44 *thr* d-*gyrA*96 [F' *proA*$^+$*B*$^+$*lacI*^{q}*Z*ΔM15]	Liss, 1987
JM109	e14-(McrA-) *recA1 endA1 gyrA96 thi-1 hsdR*17(r_k^-,m_k^+) *supE44 relA1* (Δ*lac-proAB*) [F' *traD36 proAB lacI*^{q}*Z*ΔM15]	Yanish-Perron *et al.*, 1985
HB101	*supE44 ara14 galK2 lacY1* Δ(*gpt-proA*)*62 rpsL20* (Str^r) *xyl-5 mtl-1 recA13* Δ(*mcrC-mrr*) *HsdS*$^-$(r^-m^-)	Sambrook *et al.*, 1989
XL1-Blue MRF'	Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *proABlacI*^{q}*Z*ΔM15 Tn10 (Tet^r)]	Stratagene Cloning Systems, La Jolla, CA
XLOLR	Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 thi-1 recA1 gyrA96 relA1 lac* [F' *proABlacI*^{q}*Z*ΔM15 Tn10 (Tet^R)] Su$^-$ λ^R	Stratagene Cloning Systems, La Jolla, CA
P. aeruginosa		
PAO1	*regA*$^+$*regB*$^-$ prototypical toxigenic strain, serotype O5	Holloway, 1969
PA103	*regA*$^+$*regB*$^+$ hypertoxigenic strain	Liu, 1966
PA103 N3	PA103 with *regA*::*lacZ* chromosomal fusion; Cb^R	this study
PA103 P3	PA103 with P1-*regA*::*lacZ* chromosomal fusion; Cb^R	this study
Pa103 N3 A156	PA103 N3::Tn*5*-B61; Cb^R, Gm^R	this study
PA103 N3 B17	PA103 N3::Tn*5*-B61; Cb^R, Gm^R	this study

Table 1. Strains, phages and plasmids, continued.

Strain, Phage or Plasmid	Description	Reference
P. aeruginosa		
PA103 P3 C44	PA103 P3 *regA*:: Tn*5*-B61; Cb^R, Gm^R	this study
PA103 P3 C86	PA103 P3 *fpvA*:: Tn*5*-B61; Cb^R, Gm^R	this study
PA103 N3 D81	PA103 N3::Tn*5*-B61; Cb^R, Gm^R	this study
PA103 P3 D168	PA103 P3::Tn*5*-B61; Cb^R, Gm^R	this study
PA103 *fpvA::Ω*	PA103 *fpvA::Ω*; Sm^R/$Spec^R$	this study
Lambda		
Zap Express™	λ vector with the pBK CMV MCS and excisable plasmid site	Stratagene Cloning Systems,La Jolla, CA
f1 filamentous		
ExAssist™ interference-resistant helper	excises pBK CMV from λ Zap Express	Stratagene Cloning Systems, La Jolla, CA
Plasmids		
pBK CMV	multi-purpose phagemid cloning vector; Km^R	Stratagene Cloning Systems, La Jolla, CA
pNOT19	pUC19 carrying *oriT*, *sacB*, and a unique NotI site; Cm^R, Ap^R	Schweizer, 1992
pZ1918	contains promoterless *lacZ* flanked symmetrically by the pUC19 MCS; Ap^R	Schweizer, 1993
pDF18-202	pUC18 containing a 1.9 kb PstI/XhoI*regAB* fragment at the PstI and SalI sites; Ap^R	Frank *et al.*, 1989
pDFC *lacZ*	pDF18-202 with promoterless *lacZ* inserted at *Cla*I in *regA*; Ap^R	this study

Table 1. Strains, phages and plasmids, continued.

Strain, Phage or Plasmid	Description	Reference
Plasmids		
pNRL	pNOT19 carrying *regA-lacZ* fusion; Ap^R	this study
pNRLP1	pNOT19 carrying P1-*regA-lacZ* fusion; Ap^R	this study
pSUP102::Tn*5*-B61	mobilizable pACYC184 suicide derivative carrying a Tn*5*-derived Tn*5*-B61 transposon; Tc^R, Gm^R	Simon *et al.*, 1989
pBK C86	pBK CMV carrying Tn*5*-B61 flanked by 1.3 kb of *fpvA* (cloned from genomic library of PA103 P3 C86); Km^R, Gm^R	this study
pBK C44	pBK CMV carrying Tn*5*-B61 flanked by *regA* (cloned from genomic library of PA103 P3 C86); Km^R, Gm^R	this study
pBK B17	pBK CMV carrying Tn*5*-B61 flanked by unknown sequence (cloned from genomic library of PA103 P3 C86); Km^R, Gm^R	this study
pMH1701	pJQ17 derivative that carries *sacRB* on a 2.6 kb *Pst*I fragment; Ap^R, Km^R, Suc^S	Hynes *et al.*, 1989
pHP45Ω	carries a 1.9 kb Sm^R/$Spec^R$ interposon cassete; Ap^R	Prentki and Krisch, 1984
pBK CS	pBK C86 with Tn*5*-B61 removed and *sacRB* cassette inserted; Km^R, Suc^S	this study
pBK CSΩ	pBK CS containing Ω interposon at *Xmn*I site in *fpvA*; Km^R, Suc^S, Sm^R/$Spec^R$	this study

Table 1. Strains, phages and plasmids, continued.

Strain, Phage or Plasmid	Description	Reference
Plasmids		
pBK Pup	pBK CMV containing *pup* homologous sequence; Km^R	this study
pBK Pup 2.6	pBK Pup subclone; Km^R	this study
pRK415	BHR Gram-negative vector based on pRK290; Tet^R	Ditta *et al.*, 1985
pRK *fpvA*	PAO1 *fpvA* cloned into *Hin*dIII/SacI sites of pRK415; Tet^R	this study
pUCP22 *fpvA*	PAO1 *fpvA* cloned into *Hin*dIII/SacI sites of pUCP22; Ap^R, Gm^R	Lamont, unpublished data.

2.4 DNA Methods

2.4.1 Purification of DNA

2.4.1.1 Purification of Chromosomal DNA

Bacteria were grown overnight in the appropriate medium under appropriate conditions. Genomic DNA was purified from these cultures as described in Ausubel *et al.* (1991).

2.4.1.2 Purification of Plasmid DNA by Mini-prep

Mini-preparations of plasmid DNA were performed based on the alkaline-lysis procedure first developed by Birnboim and Doly (1979). Bacteria were grown overnight in LB or SOB medium, pelleted in a microfuge, and resuspended in 200 µL GTE buffer (50 mM glucose, 25 mM Tris pH 8.0 and 10 mM EDTA pH 8.0). Cells were lysed with 300 µL of 0.2 N NaOH/ 1% SDS, iced for 5 minutes, and precipitated with 300 µL of 3 M potassium acetate, pH 4.8. After a further 5 minutes on ice, the mixture was centrifuged in a microfuge for 10 minutes and the supernatant precipitated with an equal volume of isopropanol. The precipitate was centrifuged in a microfuge for 10 minutes, washed with 70% ethanol, dried, and resuspended in 50 µL of deionized water. Before use, 1 µL of 50 mg/mL DNase-free RNase was added.

2.4.1.3 Purification of Plasmid DNA by CsCl Density Gradient Ultracentrifugation

Large scale plasmid preparations were obtained using a modification of the alkaline-lysis procedure and the CsCl density

gradient purification described by Garger *et al.* (1983). Bacteria were grown overnight in 250 mL of broth, spun for 10 minutes at 4000xg, and resuspended in 6 mL of glucose buffer (50 mM glucose, 25 mM Tris pH 8.0 and 10 mM EDTA pH 8.0). To digest cell walls, 1 mL of 20 mg/mL lysozyme in glucose buffer was added and the mixture incubated for 10 minutes at room temperature. The cells were then lysed with 14 mL of a 1% SDS, 0.2 M NaOH solution. After 5 minutes on ice, proteins and chromosomal DNA were precipitated with 7 mL of 3 M sodium acetate pH 4.8 and incubated for a further 15 minutes on ice. The precipitate was spun out at 8000xg and the supernatant extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was precipitated with 0.6 volumes of isopropanol and spun at 8000xg for 10 minutes. The pellet was dried at room temperature for 1 hour and then resuspended in 1 mL of deionized water.

One gram of CsCl was added to the DNA suspension and the volume measured. For every milliliter of the mixture, 80 µL of 10 mg/mL ethidium bromide and 10 µL of 1% (v/v) Triton X-100 was added. The mixture was pipetted into 1.2 mL Quick-Seal® tubes (Beckman Instruments, Inc.) and spun at 120,000 rpm for 90 minutes in a TLN 120 rotor using an Optima™ TLX Ultracentrifuge as per manufacturers instructions. After centrifugation, the tube was removed and the bright reddish band visualized under a hand-held UV lamp (Fisher Scientific) was removed with an 18 guage needle.

Ethidium bromide was removed from the sample by repeated extractions with TES-saturated n-butanol (TES buffer is 6.35 g Tris-HCl, 1.18 g Tris base, 3.36 g EDTA, and 2.92 g NaCl per liter

deionized water). The bottom (plasmid-containing) layer was then dialyzed against 1 L of TES buffer overnight at 4°C to remove salt. The resultant dialysate was used directly in further manipulations.

2.4.1.4 Purification of DNA for Sequencing Reactions

Plasmid DNA for automated ABI sequencing was prepared as outlined by the manufacturer (Applied Biosystems, Inc., Foster City, CA). Bacteria were grown overnight in SOB medium, pelleted in a microfuge, and resuspended in 200 µL GTE buffer (50 mM glucose, 25 mM Tris pH 8.0 and 10 mM EDTA pH 8.0). Cells were lysed with 300 µL of 0.2 N NaOH/ 1% SDS, iced for 5 minutes, and precipitated with 300 µL of 3 M potassium acetate, pH 4.8. After a further 5 minutes on ice, the mixture was centrifuged in a microfuge for 10 minutes and 1 µL of 50 mg/mL DNase-free RNase was added. Contaminating RNA was digested at 37°C for 20 minutes and the solution was extracted twice with equal volumes of chloroform. The supernatant was precipitated with an equal volume of isopropanol. The precipitate was then centrifuged in a microfuge for 10 minutes, washed with 70% ethanol, dried, and resuspended in 32 µL of deionized water. The DNA was then re-precipitated by addition of 8.0 µL of 4 M NaCl and 40 µL of 13% polyethylene glycol 8000. After incubation on ice for 20 minutes, the DNA was recovered by centrifugation for 20 minutes in a microfuge at 4°C, washed, dried, and resuspended as before.

2.4.1.5 Purification of DNA from Agarose Gels

After agarose gel electrophoresis, the ethidium bromide-stained DNA band was cut from the gel using a razor blade and placed in a microcentrifuge tube. The DNA was extracted from the agarose using a Geneclean II kit (Bio 101 Inc., La Jolla, CA) according to the manufacturers instructions.

Alternatively, DNA cut from an agarose gel was placed in Spectra/Por dialysis tubing (6-8000 molecular weight cut-off) containing 1 mL of TAE buffer. The tubing was oriented such that the agarose was nearest to the cathode. The DNA was then electroeluted overnight at 10 V in a standard agarose gel apparatus. To reduce adherence of the DNA to the dialysis tubing, the polarity on the electrophoresis apparatus was reversed and the voltage increased to 80 V for 1 minute. The TAE was then recovered and the DNA was precipitated for 20 minutes at -70°C with 0.1 volumes 3 M sodium acetate pH 4.8 and 2 volumes of 100% ethanol. The DNA was microcentrifuged for 20 minutes at 4°C, washed with 70% ethanol, dried, and resuspended in 10 μL of deionized water.

2.5 Enzymatic Manipulation

2.5.1 Digestion of DNA by Restriction Enzymes

Typical small scale restriction digestions were performed using 2 μL of a plasmid mini-preparation (100-300 ng), 1 μL of the appropriate 10x restriction buffer and 1 to 10 units of restriction enzyme in a total 10 μL volume. Restriction digests were incubated

at 37°C for 1 hour before further manipulation, except where otherwise noted. All enzymes and restriction buffers were purchased from either Gibco BRL, Pharmacia BioTech Ltd. (Upsalla, Sweden) or New England BioLabs, Inc. (Boston, MA).

Large scale digestions were typically performed in a total volume of 50 μL with an overnight incubation. Between 5 and 10 μg of DNA were digested with 10 to 30 units of restriction enzyme.

2.5.2 Ligation of Restricted DNA Fragments

DNA fragments were ligated using T4 DNA ligase. A typical reaction consisted of 100 ng vector DNA, 0.5 - 2.0 μg of insert DNA, 1.5 μL of 10X ligation buffer, 1 μL of 100 mM ATP (Pharmacia BioTech Ltd.), and 1 unit of ligase in a total volume of 15 μL. Ligase and buffer were purchased from either Gibco BRL or Pharmacia BioTech Ltd.

2.5.3 Blunt-End Ligation of Restricted DNA Fragments

The Takara DNA Blunting Kit (Clontech) was used to create blunt ends on restriction fragments. This kit employs T4 DNA polymerase to fill in 5' overhangs and to cut back 3' overhangs. T4 DNA ligase is used for the ligation.

2.6 Agarose Gel Electrophoresis

Agarose gels were routinely prepared using a 0.8% to 1.4% concentration of agarose in 50 mL of TAE buffer (50X TAE buffer is 242 g Tris base, 57.1 mL glacial acetic acid, and 100 mL 0.5 M EDTA

pH 8.0 in 1 L deionized water). Samples were electrophoresed at 100 V (with a Bio-Rad Model 200/2.0 power supply) and stained in TAE buffer containing 0.5 μg/mL ethidium bromide. The DNA bands were visualized and recorded using a medium wavelength UV trans-illuminator (UVP ImageStore 7500 Gel Documentation System, UVP Inc., Upland, CA).

2.7 Southern Blot Analysis

The Southern blot analysis was performed as outlined by Ausubel *et al.*, (1991).

2.7.1 Transfer of DNA to Nylon Membranes

DNA fragments separated on the agarose gel were depurinated in 500 mL 0.2 N HCl for 10 minutes, rinsed in deionized water, and placed on a piece of Whatman 3M chromatography paper soaked in 0.4 M NaOH. The chromatography paper rested on a glass plate and extended into a container of 0.4 M NaOH. A piece of nylon filter (Amersham Life Science Inc., Arlington Heights, IL) slightly smaller in both dimensions than the agarose gel was placed on top of the gel, followed by several pieces of chromatography paper and a stack of paper towels. To weigh down the apparatus, a flask containing 200 mL of water was placed on a second glass plate. The squash blot was allowed to proceed overnight before being baked at 80°C for 2 hours. The resultant blot could be stored at -70°C in saran wrap and tin foil.

2.7.2 Radio-labelling of DNA Probes

DNA for hybridization was digested and fragments purified as described above. Purified DNA fragments were labelled using the Oligolabelling Kit (Pharmacia) and ^{32}P-dCTP (Dupont NEN Products, Boston, MA) as per the manufacturers instructions. Unincorporated nucleotides were removed using NENsorb 20 nucleic acid purification cartridges (Dupont NEN Products) as per the manufacturers instructions.

2.7.3 Hybridization

The blot was prehybridized for at least 4 hours in 10 mL prehybridization solution at 42°C in a heat-sealable plastic bag (Gibco BRL). Prehybridization solution contains 0.5 mL 1 M KPO_4 pH 7.4, 5 mL of 20x SSC, 2 mL of 50x Denhardts solution, 100 µL 10 mg/mL salmon sperm DNA (Sigma), 10 mL 100% formamide, 2 mL 10% SDS and 0.4 mL deionized water. 20x SSC is 175.3 g NaCl and 88.2 g tri-sodium citrate in 1 L deionized water. 50x Denhardt's solution is 5 g Ficoll Type 400 (Pharmacia), 5 g polyvinylpyrrolidone (Sigma) and 5 g bovine serum albumin Fraction V (Sigma) in 500 mL deionized water.

After prehybridization, the solution was drained and 10 mL hybridization solution was added to the blot. Hybridization solution was prepared by dissolving 1.0 g dextran sulphate (ICN) in 10 mL prehybridization buffer. The labelled probe was added to the bag, sealed, and incubated overnight at 42°C.

The filter was then washed twice at room temperature with 200 mL of 2x SSC and 0.1% SDS for 15 minutes, 200 mL of 0.5x SSC

and 0.1% SDS for 15 minutes, and 200 mL of 0.1x SSC and 0.1% SSC for 15 minutes before a final 30 minute wash at 42°C with 0.1x SSC and 1.0% SDS.

If the filter was to be reprobed, it was quickly blotted with Whatmann paper upon removal from the final wash solution and wrapped in saran wrap while still wet; otherwise, the filter was air dried before autoradiography.

2.7.4 Visualization of Hybridization Signal

2.7.4.1 Autoradiography of Blots

Kodak XAR-5 Scientific Imaging Film (Eastman Kodak Company) was placed on top of the filter and enclosed in an X-ray cassette with image intensifiers. The film was exposed for 10 to 48 hours before development. The film was developed according to the manufacturers instructions.

2.7.4.2 Phosphoimaging

An erasable Type BAS-IIIS imaging plate (Fuji) was placed on a saran-wrapped radioactive membrane and exposed for between 20 minutes and 4 hours. The imaging plate was then transferred to a FUJIX BAS 1000 Bio-imaging analyzer and scanned into a computer. Hybridization intensities and DNA migrations were calculated using MacBas v2.2 software.

2.7.5 Probe Removal from Hybridized Blots

To remove probe from a wet filter, 200 mL of boiling 0.1% SDS is poured on the filter and allowed to cool to room temperature.

2.8 DNA Sequencing

2.8.1 Automated Sequencing

DNA for sequencing was prepared as described above. Five micrograms of double-stranded plasmid DNA, along with an appropriate primer, was submitted to the University of Calgary DNA Sequencing lab (Calgary, AB) for automated sequencing. Sequencing reactions were performed using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (ABI) and sequencing was performed on an ABI Model 370 automated sequencer. Sequencing corrections were made using EditView 1.0.1 analysis software.

2.8.2 Oligonucleotides used in Sequencing

Oligonucleotides used for sequencing were designed with the aid of Oligo 4.0 software to reduce the chance of primer-dimer formation and hairpin loops. Oligonucleotides were synthesized at the University of Calgary Core DNA Services lab (Calgary, AB). A summary of oligonucleotides used is presented in Table 2.

Table 2. Oligonucleotides.

Oligo	Description	Sequence
T7	sequencing	GTAATACGACTCACTATAG GGC
T3	sequencing	AATTAACCCTCACTAAAGG G
Tn*5* IS50R	sequence out from IS50R of a Tn*5* insertion	TAGGAGGTCACATGGAAGT CAGAT
pup1	sequence *fpvA* homologue	TCCCTGCAAAGCAGCGTTC
pup2	sequence *fpvA* homologue	TCCCGTCCGCTTGAAACAG
Bgl II linker	introduces a *Bgl* II site at a blunt site	5'-P-CAGATCTG

2.9 Transformation of Plasmid DNA

Both *E. coli* and *P. aeruginosa* were transformed using both chemical and electrochemical methods. Electroporation was used for all suicide-vector deliveries, while chemical transformation was used for routine plasmid deliveries.

2.9.1 Chemical Transformation

For chemical transformation, cells were prepared according to methods first described by Hanahan, 1983 and outlined in Sambrook *et al.* (1989). An overnight bacterial culture, inoculated from frozen stock, was subcultured into 100 mL SOB medium containing 20 mM $MgSO_4$ and grown aerobically until the OD_{600} reached between 0.4 and 0.6. The cells were chilled on ice for 10 minutes and pelleted at 2000xg at 4°C. The pellet was resuspended in 40 mL of FSB (pH 6.4) and placed on ice for a further 10 minutes. FSB contains 10 mM potassium acetate pH 7.5, 45 mM $MnCl_2 \cdot H_2O$, 10 mM $CaCl_2 \cdot H_2O$, 100 mM KCl, 3 mM hexamminecobalt chloride, and 10% glycerol. The cell suspension was then pelleted and resuspended in 8 mL of FSB before adding 280 µL of dimethyl sulphoxide (DMSO). After 15 minutes on ice, a further 280 µL of DMSO was added and the mixture was aliquoted, snap-frozen in liquid nitrogen and stored at -70°C until use.

To transform the cells, they were first thawed on ice and incubated with the DNA of interest for 30 minutes. The cells were heat-shocked at 42°C for 90 seconds (*E. coli*) or 37°C for 3 minutes (*P. aeruginosa*), followed by 2 minutes on ice, before being allowed to recover at 37°C with shaking in 1 mL of SOB broth. The recovering

cells were subsequently plated on appropriate antibiotic-containing agar plates.

2.9.2 Electrochemical Transformation

Electroporation of *P. aeruginosa* was carried out as described by Farinha and Kropinski (1990). A 50 mL cell culture was grown and harvested in a similar manner to that used in chemical transformation. The cell pellet was washed with 50 mL of 0.3 M sucrose, 25 mL of 0.3 M sucrose, and finally resuspended in 0.25 mL of 0.3 M sucrose. Only freshly-prepared cells were transformed. Salt-free DNA was added to 40 μL of cells and transferred to a 0.2 μM electrocuvette immediately before electroporation in a Bio-Rad Gene Pulser II apparatus with a Bio-Rad Pulse Controller II. The cells were electroporated at 2000 V with a capacitance of 25 μF and a resistance of 200 Ω. This typically generated a 5 ms electrical pulse. Following electroporation, the cells were allowed to recover in 2 mL of SOB for 90 minutes at 37°C before being spread on antibiotic agar plates.

E. coli cells were electroporated as described in Ausubel *et al.* (1991), with some modifications. A 100 mL culture was grown and harvested as above, but washed twice in 100 mL of ice-cold deionized and sterilized water before being washed in 8 mL of 10% glycerol, resuspended in 0.25 mL 10% glycerol, aliquoted and frozen at -70°C. DNA was added to the cells in the same manner as described above, and pulsed at 2500 V, 25 μF, and 200 Ω. Cells were recovered in 1 mL of SOB medium and plated after 1 hour at 37°C in a shaking incubator.

2.10 Transposon Mutagenesis

Plasmid pSUP102::Tn*5*-B61 (Simon *et al.*, 1989) was electroporated into *P. aeruginosa* strains N3 and P3. Recombinants were selected on LB agar containing 50 μg/mL gentamicin. Recombinants were checked on LB agar containing 300 μg/mL carbenicillin to confirm the presence of the *lacZ* fusion.

Recombinants were screened for alteration of promoter activity by measuring ß-galactosidase activity.

2.11 Screening of Transposon mutants

Mutants were inoculated into 2 mL of LB containing gentamicin and carbenicillin and grown for 24 hours in 5 mL Sarstedt snap-cap tubes at 37°C with shaking. 200 μL aliquots of the mutant cultures were transferred to a Falcon 3911 microtiter plate and assayed for ß-galactosidase activity as described below.

To obtain meaningful ß-galactosidase activities, the following calculation was applied to each well:

$$\text{activity} = (OD_{405} - \text{blank})/(OD_{620} - \text{blank}) * \text{time}$$

Wells with an OD_{620} below 1.0 or above 1.6 were ignored. All other wells were grouped so that a bell curve could be graphed. Mutants falling into the upper and lower limits of the bell curve were taken for further analysis. In total, 1000 mutants were screened.

While agar plates containing X-gal were used initially for screening, variability in the colour intensity of the colonies prevented reliable identification of mutants.

2.12 Homologous Recombination

DNA for homologous recombination was cloned into suicide vectors and electroporated into *P. aeruginosa*. Recombinants were selected on LB agar containing an appropriate antibiotic.

2.13 Construction of lambda Zap Express gene banks

Genomic DNA preparations were digested overnight with the appropriate restriction enzyme and electrophoresed on a 1.0% agarose gel. DNA within 2 kb of the size of the desired band was excised and purified by electroelution or by using the Gene Clean II kit.

Lambda Zap Express genomic libraries were constructed in accordance with the manufacturers instructions (Stratagene, La Jolla, CA). In brief, purified lambda vector DNA was digested for 2 hours with the appropriate restriction enzyme, phenol/chloroform extracted, ethanol precipitated, and resuspended in deionized water. 1 μg of digested vector arms was ligated to 0.1 μg of size-selected genomic DNA overnight at 16°C. The ligation mixes were then ethanol precipitated.

The precipitated ligation mix was packaged into lambda phage particles using the Gigapack Gold II kit (Stratagene, La Jolla, CA), in accordance with the manufacturers instructions. The resultant packaging mix was stored at 4°C in 500 μL SM buffer (5.8 g NaCL, 2.0 g $MgSO_4 \cdot 7H_2O$ and 50.0 mL 1M Tris-HCL (pH 7.5) in 1 L deionized water) containing 20 μL of chloroform until further use.

To titer the gene banks, 1 μL of the packaging mix was used to infect 200 μL of XL1 Blue MRF' host cells. Host cells were inoculated

from LB agar plates containing tetracycline into 10 mL of LB supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$ and grown overnight at 32°C with aeration. 100 µL of the overnight culture was subcultured into a similar medium and grown to an OD_{600} of 1.0. The cells were pelleted in a microcentrifuge and resuspended to an OD_{600} of 0.5 in 10 mM $MgSO_4$. 1 µL of packaged phage was added to 200 µL of the host cell suspension and incubated at 37°C for 15 minutes. Three mL of LB top agar, pre-equilibrated to 48°C, was added to the phage-bacteria mixture, supplemented with 15 µL 0.5 M IPTG and 150 µL 40 mg/mL Xgal, mixed and spread onto NZY agar plates. After overnight growth at 37°C, clear and blue plaques were counted to estimate the number of plaque forming units (PFU) in the library.

2.14 Plaque Blots

Five thousand PFU were plated on large 15 cm, two day old, NZY agar plates, in the same way as the phage libraries were titered. No blue/white selection was performed, and 8 mL of top agar was required to cover the plate.

The plates were overlayed after an overnight incubation with circular Hybond-N+ nylon membranes (Amersham Life Sciences) for 2 minutes, then removed and placed into 200 mL of a 1.5 M NaCl, 0.5 M NaOH solution. After 2 minutes, the membrane was neutralized for 5 minutes in a 1.5 M NaCl, 0.5 M Tris pH 8.0 solution, and rinsed for 30 seconds in a 0.2 M Tris pH 7.5 (containing 20 mL of 20x SSC) solution. The membrane was baked for 2 hours at 80°C, and then hybridized as outlined for Southern blot analysis.

Positively hybridizing plaques were removed from the plates with an Pasteur pipette and placed in 500 µL SM buffer (5.8 g NaCl, 2.0 g of $MgSO_4 \cdot 7H_2O$ and 50 mL of 1 M Tris pH 7.5 in 1 L of deionized water) containing 2 drops of chloroform for at least 2 hours. This stock could be stored at 4°C for at least 6 months with minimal drops in titer.

These phage stocks were either subjected to a second round of hybridization to purify the phage clone, or subjected to plasmid excision.

2.15 Plasmid Excision

To excise the plasmid from positive phage clones, *E. coli* strains XL1-Blue MRF' and XLOLR were grown to an OD of 1.0 as above but resuspended to an OD_{600} of 1.0 in 10 mM $MgSO_4$. 200 µL of XL1-Blue MRF' was combined with 200 µL of phage stock and 1 µL of ExAssist phage stock (titer of 10^{10}-10^{11} PFU). This was incubated at 37°C for 15 minutes in a Falcon 2059 snap cap tube. Three milliliters of NZY medium was added and the mixture was incubated overnight at 37°C with aeration. Bacteria were killed by a 20 minute 65°C incubation and removed by centrifugation at 1000xg: 50 µL of this filamentous phage stock was used to infect 200 µL of XLOLR for 15 minutes at 37°C. The bacteria were allowed to propagate for 45 minutes in 300 µL of NZY broth with shaking at 37°C before plating on LB agar containing kanamycin and gentamicin. This selected for clones containing the Tn*5*-B61 transposon and any adjacent sequences in the pBK CMV plasmid.

2.16 Assays

2.16.1 ADP-Ribosyltransferase Activity

EF-2 was prepared as described by Iglewski and Kabat (1975). To prepare EF-2, 45 g dry wheat germ (Rogers Foods Ltd., Armstrong, BC) was soaked on ice for 5 minutes in 360 mL of buffer I (50 mM Tris pH 8.0, 5 mM magnesium acetate, 50 mM KCl, 4 mM $CaCl_2$, 350 µL ß-mercaptoethanol, and 1 mL of 1 mg/mL PMSF in isopropanol with the latter two reagents added just before use) in a cold room. This mixture was then blended in a waring blender at top speed in 5 bursts of 10 seconds each with intervening 30 second pauses. Large solids were removed from the mixture by gravity filtration through cheese cloth, and the filtrate was centrifuged at 8000xg for 15 minutes to remove smaller debris. The supernatant was then further clarified by ultracentrifugation for 2 hours at 115,000xg. Contaminating lipids were removed by filtration over cheesecloth. Contaminating protein was precipitated with 30% ammonium sulphate, pelleted at 8000xg and discarded. The supernatant was then raised to 50% saturation with ammonium sulphate, and again pelleted. The pellet was retained and resuspended in 10 mL of buffer 2 (50 mM Tris pH 7.5, 1 mM EDTA, 350 µL/L ß-mercaptoethanol, and 1 ml of 1 mg/mL PMSF in isopropanol) and dialyzed overnight at 4°C against buffer 2. DTT and glycerol were added to the dialysate to 2 mM and 5% concentrations respectively prior to aliquoting and freezing.

ADP ribosyltransferase assays were performed as previously described by Frank and Iglewski (1988). Frozen aliquots of culture

supernatants collected as described above were thawed, and 10 µL was combined with 10 µL of urea-DTT solution (0.24 g urea, 65 µL 1 M DTT and 250 µL deionized water), mixed and incubated at room temperature for 15 minutes. To this mixture, 25 µL of T-II-C buffer, 25 µL of wheat germ extract, and 5 µL of ^{14}C-labelled NAD were added. T-II-C buffer is prepared with 125 µL 1 M Tris pH 7.0, 100 µL DTT, and 775 µL deionized water. The reaction was incubated for 45 minutes at room temperature and stopped by precipitation of the proteins with 10% TCA. The precipitated proteins were then collected by vacuum filtration over 0.45 µM nitrocellulose filters. The filters were placed in scintillation vials containing 5 mL of scintillation fluid and counted in an LKB Wallac 1215 Rack Beta liquid scintillation counter.

2.16.2 ß-Galactosidase Activity

ß-galactosidase assays were based on experiments described by Miller (1972), and modified to work in microtiter plates as done by Kolmar *et al.* (1994). Cells from growth curves or overnight cultures, resuspended in 1X A-Buffer, were transferred to a Falcon 3911 microtiter plate and spun at 3500 rpm for 10 minutes in an IEC Centra GP8R centrifuge (International Equipment Corporation, Needham Heights, MA). The supernatant was aspirated and the cells were resuspended in 200 µL of 1X A buffer (see Growth Curve Methodology), of which 10 µL was transferred to an ELISA plate. To the cells, 150 µL of lysis buffer (1.4 ml ß-mercaptoethanol, 3.0 mL of 10x Z-buffer, 5 µL of 10% SDS and 10.6 mL deionized water) and 10 µL of chloroform was added using a multichannel pipettor. 10x Z-

buffer is 16.1 g $Na_2HPO_4 \cdot H_2O$, 5.5 g $NaH_2PO_4 \cdot H_2O$, 0.75 g KCl, and 0.24 g $MgSO_4 \cdot 7H_2O$ in 100 mL of deionized water. After a 5 minute incubation, 100 µL of 4 mg/mL ONPG was added as substrate for the ß-galactosidase. The time elapsed between substrate addition and OD_{405} measurement was recorded. The OD_{405} was measured in an ELISA reader, as was the OD_{620} of the cell suspensions. Measurements on each sample were performed in duplicate. The ß-galactosidase activity was calculated using the following formula:

$$\text{activity} = (OD_{405} - \text{blank})/(OD_{620} - \text{blank}) * \text{time}$$

2.16.3 Analysis of Pyoverdine Production

Pyoverdine production was measured in both low and high iron conditions using both solid indicator media and using culture supernatants.

2.16.3.1 Growth on Chrome Azurol S Plates

Bacteria were spotted on chrome azurol S low and high iron plates and grown overnight at 37°C. The clear zone indicative of siderophore production was measured from its outer edge to the edge of the colony. Siderophores known to be responsible for this zone of clearing include both pyochelin and pyoverdine.

2.16.3.2 Spectrophotometric Analysis of Supernatant

Bacteria were grown in succinate minimal medium with and without iron for 24 hours at 32°C with aeration. Bacteria were pelleted at 2000xg and the supernatant was retained. The OD of the supernatant was measured from 350 nm to 450 nm. Pyoverdine typically peaked between 403 nm and 406 nm. The absorbance at this maximum was used as an indication of the pyoverdine production (Schwyn and Neilands, 1987).

2.17 Computer Analyses

ß-galactosidase activities were calculated using Microsoft® Excel (Microsoft, Redmond, WA). Oligonucleotides were designed using Oligo 4.06 (National Biosciences Inc., Plymouth, MN) primer analysis software. DNA sequences were first edited with EditView 1.0.1 software (ABI), then restricted and analyzed using DNAid+ 1.4 (École Polytechnique, France) and DNAsis 2.0 (Hitachi Software Engineering Co. Ltd.) software. Sequences were compared to the Genbank database using BLASTX (Gish and States, 1993) and BLASTN analysis (Altschul *et al.*, 1990), aligned using the Clustal W algorythm (Thompson *et al.*, 1994), and visualized using SeqVu 1.1 software (The Garavan Institute of Medical Research, Sydney, Australia). Plasmid maps were drawn using MacPlasmap (University of Utah, Salt Lake City, ID). Southerns were analyzed using MacBas 2.2 software (Fuji Photo Film Co., Ltd.)

Chapter 3 Results

3.1 Construction of strain PA103 N3 and PA103 P3

The construction of pNRL, used to create strain PA103 N3, and pNRLP1, used to create strain PA103 P3, is outlined in Figures 2 and 3 respectively. Detailed plasmid maps of pNRL and pNRLP1 are depicted in Figure 4.

These plasmids were electroporated into strain PA103 and recombinants were selected on LB containing 300 μg/mL carbenicillin. Integrants resulting from electroporation of PA103 with pNRL were termed N1 through N4, while those obtained using pNRL P1 were termed P1 through P4. Only P3 and N3 were fully analyzed.

To allow confirmation of *regAB* promoter activities with ADPRT activities, the *regAB* operon was maintained intact in these strains. Therefore, these recombinants were made using a single cross-over homologous recombination strategy. In fact, as can be seen from Figures 5 and 6, double integrations of both pNRL and pNRLP1 likely occurred, possibly because of large homologies of chromosomal and plasmid copies of *lacZ*. Nevertheless, ß-galactosidase (Figure 8) and ADPRT (Figure 9) activities of the constructs were predictable and comparable to wild type PA103.

Lanes 2 and 5 of Figure 5 show a Southern blot analysis of PA103 P3 genomic DNA probed with either a 2.3 kb *Eag*I fragment from pDF18-202 encompassing all of *regAB* or an internal 1.8 kb *Hin*cII *lacZ* fragment from pZ1918. The banding patterns and intensities of PA103 P3 probed with the *regAB* fragment can be

explained using the diagram of the *regAB* locus depicted in Figure 6c. The largest, 6.85 kb fragment is of a lesser intensity because *regA* is interrupted by *lacZ*, and thus less of *regA* is available for hybridization. In contrast, the 6.5 kb fragment contains an entire copy of *regAB*, and therefore hybridizes strongly. The 3.95 kb fragment is interrupted by a second insertion, and is therefore less intense, while the smallest 3.8 kb band is present in two copies, making it more intense.

Analysis of the same membrane with the *lacZ* probe (Figure 5 lane 5) highlights the 6.85 kb band and the 3.95 kb band, confirming that these bands contain the *lacZ* reporter juxtaposed to the *regAB* P1 promoter as predicted by Figure 6c.

The Southern blot analysis of PA103 N3 is shown in lanes 3 and 6 of Figure 5. It shows, like the analysis of PA103 P3, that two recombinations of the *lacZ* reporter construct occurred in the chromosome. Figure 6b depicts the genetic arrangement at the *regAB* locus. It is also useful to compare the Southerns of PA103 N3 with that of PA103 P3, when hybridized with the *regAB* fragment.

The band pattern of PA103 N3 is similar to that observed for PA103 P3. The 4.2 kb and 7.1 kb bands in PA103 N3 are of an increased size relative to the 3.95 kb and 6.85 kb bands seen in PA103 P3. The difference corresponds to the 247 bp deletion of the P2 promoter in PA103 P3. These same bands hybridize with the *lacZ* probe, indicating the same general arrangement in both strains. For the most part, the hybridization intensities seen for PA103 N3 are identical to those observed for PA103 P3, with one exception, the 4.2 kb band is more intense than its 3.95 kb counterpart. This could

possibly be explained by the presence of the P2 promoter which provides a greater amount of DNA for hybridization, but a similar increase in intensity for the 7.1 kb band over its 6.85 kb counterpart was not observed. One can see an increase in the intensity of the same band probed with *lacZ*, so perhaps this incongruency is related not to the structure, but to experimental error.

The integrations appeared very stable, since even after repeated passaging without any antibiotic pressure, both strains always retained their carbenicillin resistance (data not shown). Since the Southern blots were done long after passaging of the strains, it is assumed that the double recombination is stable too.

Figure 2. Construction of pNRL used to create strain PA103 N3.

To construct pNRL, used to create strain PA103 N3, plasmid pDF18-202 was digested with *Cla*I and blunt-end ligated to a 3.2 kb *Bam*HI-digested pZ1918 promoterless *lacZ* fragment. The *Bam*HI sites, being of the 5' variety, were preserved during cloning. The 5.1 kb *Kpn*I-*Pst*I *regA-lacZ* fragment from pDFC *lacZ* was ligated into similarly digested pNOT19 to create pNRL.

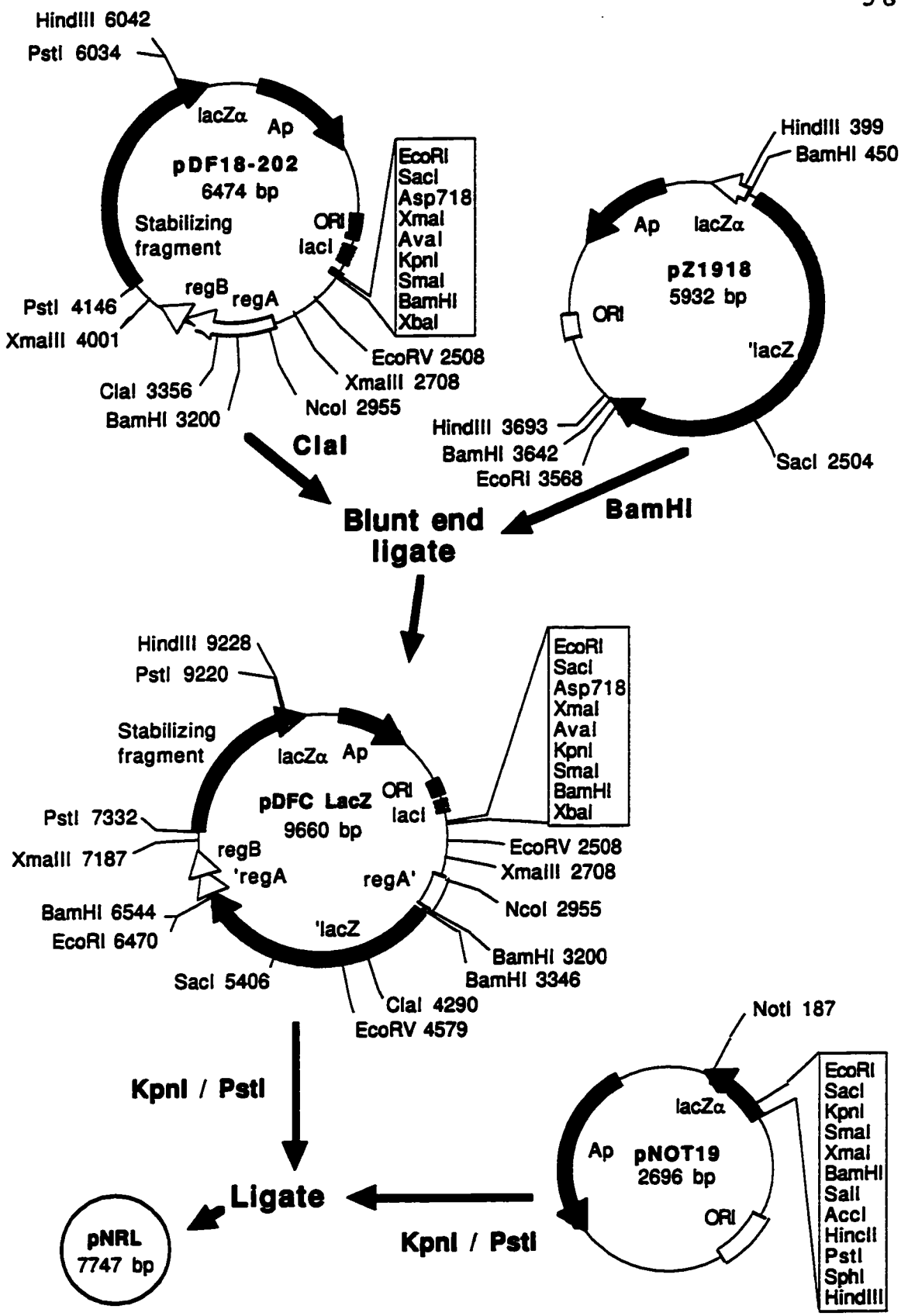

HindIII 6042
PstI 6034
lacZα
Ap
pDF18-202
6474 bp
Stabilizing
fragment
ORI
lacI
EcoRI
SacI
Asp718
XmaI
AvaI
KpnI
SmaI
BamHI
XbaI
regB
regA
PstI 4146
XmaIII 4001
ClaI 3356
BamHI 3200
EcoRV 2508
XmaIII 2708
NcoI 2955
HindIII 399
BamHI 450
Ap
lacZα
pZ1918
5932 bp
ORI
'lacZ
HindIII 3693
BamHI 3642
EcoRI 3568
SacI 2504
ClaI
BamHI
Blunt end
ligate
HindIII 9228
PstI 9220
Stabilizing
fragment
lacZα
Ap
pDFC LacZ
9660 bp
ORI
lacI
EcoRI
SacI
Asp718
XmaI
AvaI
KpnI
SmaI
BamHI
XbaI
PstI 7332
XmaIII 7187
regB
'regA
regA'
BamHI 6544
EcoRI 6470
'lacZ
SacI 5406
ClaI 4290
EcoRV 4579
EcoRV 2508
XmaIII 2708
NcoI 2955
BamHI 3200
BamHI 3346
KpnI / PstI
NotI 187
lacZα
Ap
pNOT19
2696 bp
ORI
EcoRI
SacI
KpnI
SmaI
XmaI
BamHI
SalI
AccI
HincII
PstI
SphI
HindIII
Ligate
KpnI / PstI
pNRL
7747 bp

Figure 3. Construction of pNRLP1 used to create strain PA103 P3.

To construct pNRLP1, used to create strain PA103 P3, a 7 kb *Kpn*I-*Nco*I pNRL fragment (missing both the *regA* P1 and P2 promoters) was blunt-end ligated to a *Kpn*I/*Eag*I 0.5 kb fragment from pDF18-202 (containing only the *regA* P1 promoter). In effect, pNRL P1 is pNRL with a 247 bp *Eag*I-*Nco*I *regA* P2 promoter deletion.

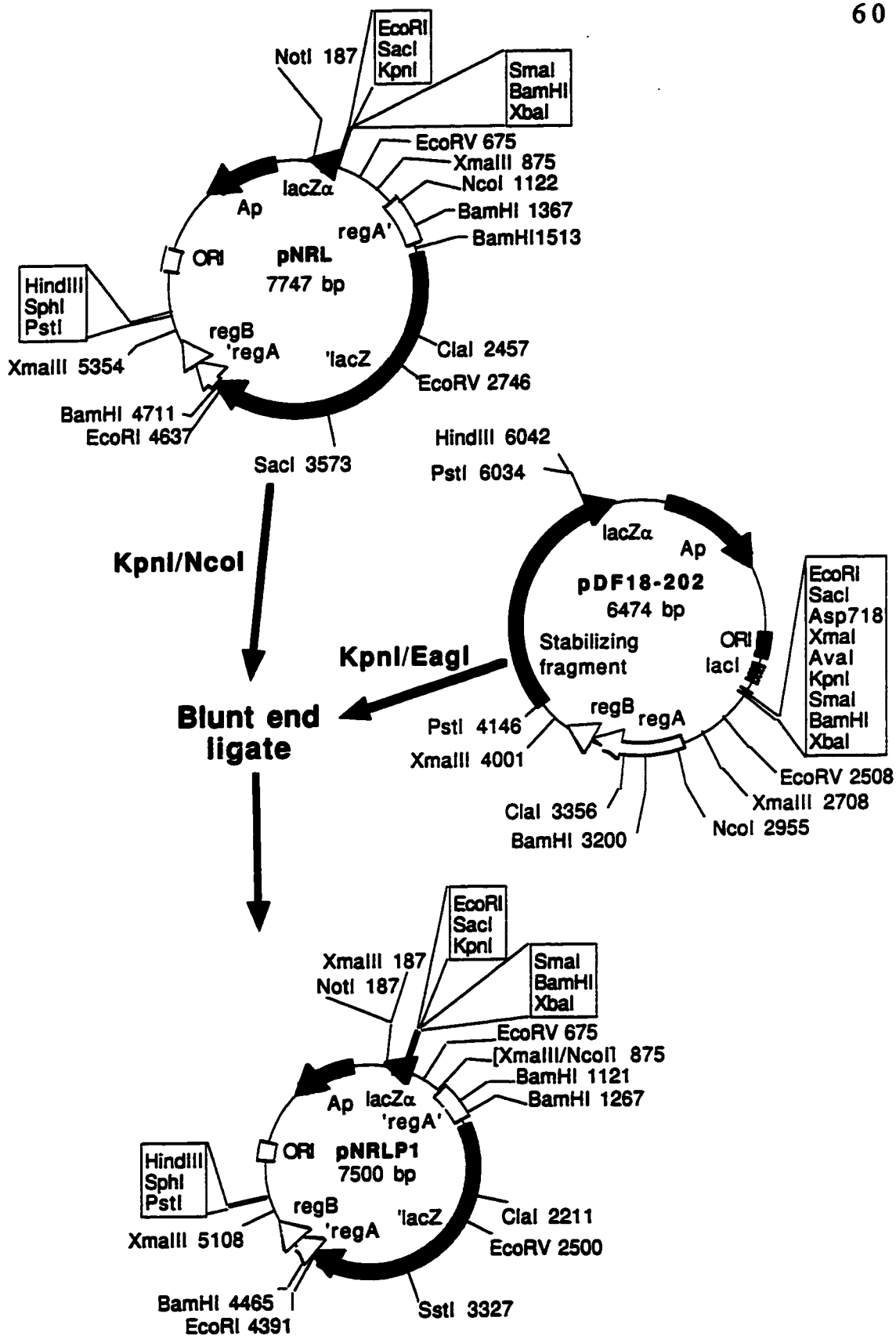
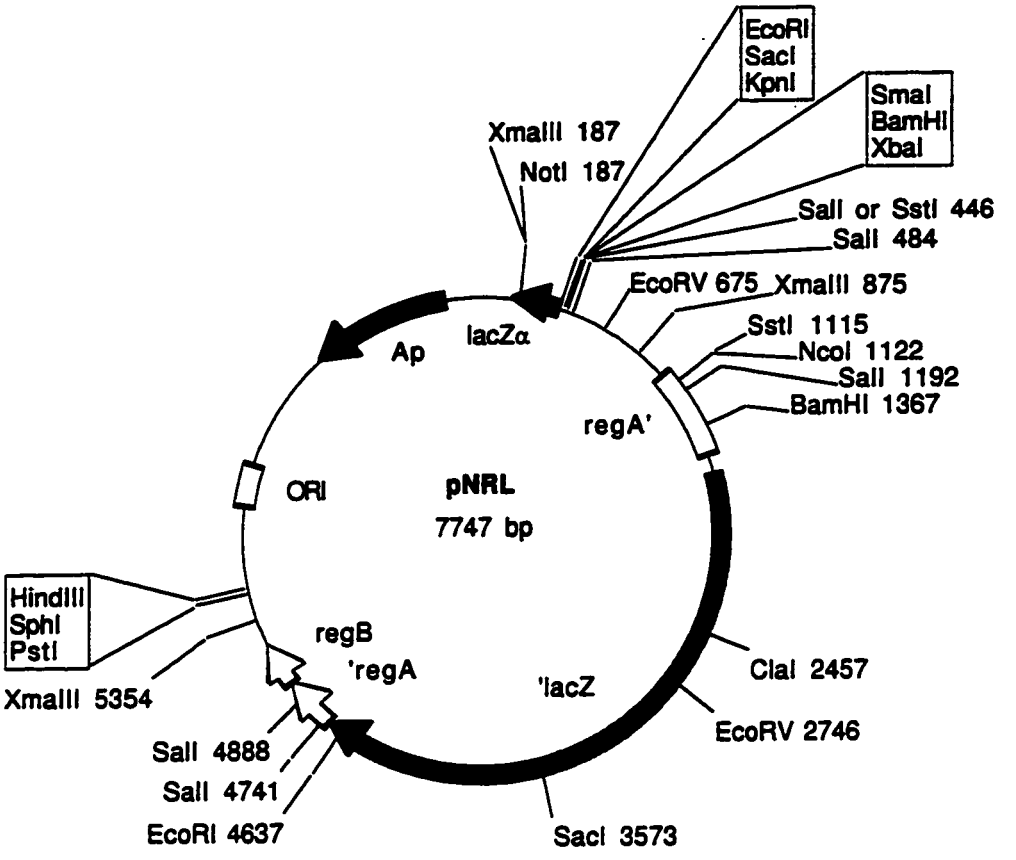

EcoRI
SacI
KpnI
NotI 187
SmaI
BamHI
XbaI
EcoRV 675
XmaIII 875
NcoI 1122
BamHI 1367
BamHI1513
lacZα
Ap
regA'
ORI
pNRL
7747 bp
HindIII
SphI
PstI
regB
'regA
'lacZ
ClaI 2457
EcoRV 2746
XmaIII 5354
BamHI 4711
EcoRI 4637
SacI 3573
KpnI/NcoI
HindIII 6042
PstI 6034
lacZα
Ap
pDF18-202
6474 bp
Stabilizing
fragment
ORI
lacI
EcoRI
SacI
Asp718
XmaI
AvaI
KpnI
SmaI
BamHI
XbaI
KpnI/EagI
Blunt end
ligate
regB
regA
PstI 4146
XmaIII 4001
ClaI 3356
BamHI 3200
EcoRV 2508
XmaIII 2708
NcoI 2955
EcoRI
SacI
KpnI
XmaIII 187
NotI 187
SmaI
BamHI
XbaI
EcoRV 675
[XmaIII/NcoI] 875
BamHI 1121
BamHI 1267
Ap
lacZα
'regA'
ORI
pNRLP1
7500 bp
HindIII
SphI
PstI
regB
'regA
'lacZ
ClaI 2211
EcoRV 2500
XmaIII 5108
BamHI 4465
EcoRI 4391
SstI 3327

Figure 4. Plasmids pNRL and pNRLP1

Shown are detailed plasmid maps of pNRL and pNRLP1 used in constructing strains PA103 N3 and PA103 P3, respectively. Apostrophes prefacing gene names indicate that 5' sequences of the gene are missing, while apostrophes following gene names are indicative of missing 3' sequences.


EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
XmaIII 187
NotI 187
SalI or SstI 446
SalI 484
EcoRV 675
XmaIII 875
SstI 1115
NcoI 1122
SalI 1192
BamHI 1367
lacZα
Ap
regA'
ORI
pNRL
7747 bp
HindIII
SphI
PstI
regB
'regA
'lacZ
ClaI 2457
XmaIII 5354
EcoRV 2746
SalI 4888
SalI 4741
EcoRI 4637
SacI 3573

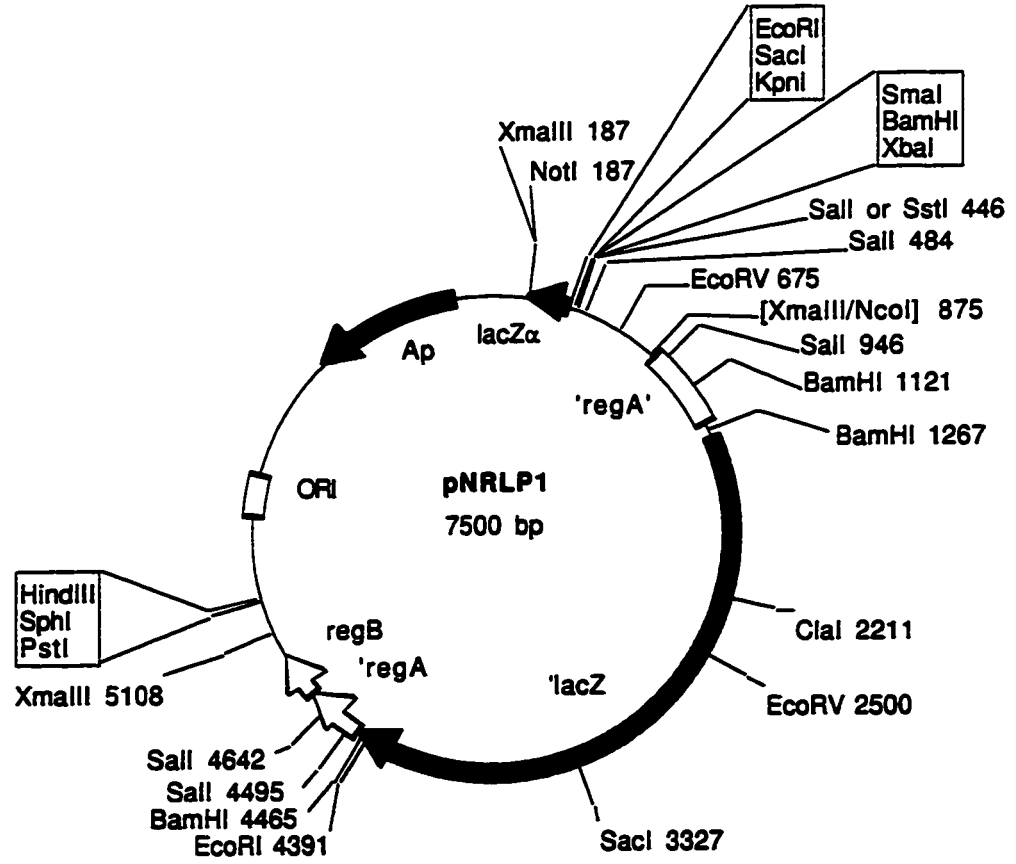

EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
XmaIII 187
NotI 187
SalI or SstI 446
SalI 484
EcoRV 675
[XmaIII/NcoI] 875
SalI 946
BamHI 1121
BamHI 1267
lacZα
Ap
'regA'
ORI
pNRLP1
7500 bp
HindIII
SphI
PstI
regB
'regA
'lacZ
ClaI 2211
XmaIII 5108
EcoRV 2500
SalI 4642
SalI 4495
BamHI 4465
EcoRI 4391
SacI 3327

Figure 5. Southern blot analysis of PA103 N3 and PA103 P3. *Eco*RI-digested genomic DNAs were probed with an *Eag*I (isoschizomer of *Xma*III) *regAB* fragment (Lanes 1-3) or a *Hin*cII internal *lacZ* fragment (Lanes 4-6). Lanes 1 and 4 contain PA103 genomic DNA, lanes 2 and 5 contain PA103 P3 genomic DNA, and lanes 3 and 6 contain PA103 N3 genomic DNA.

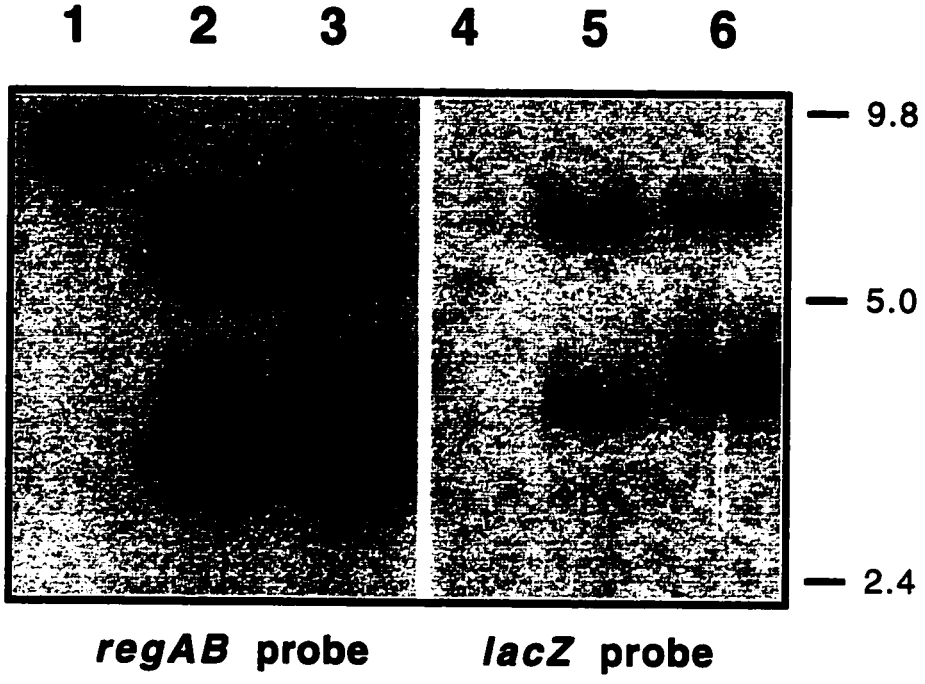
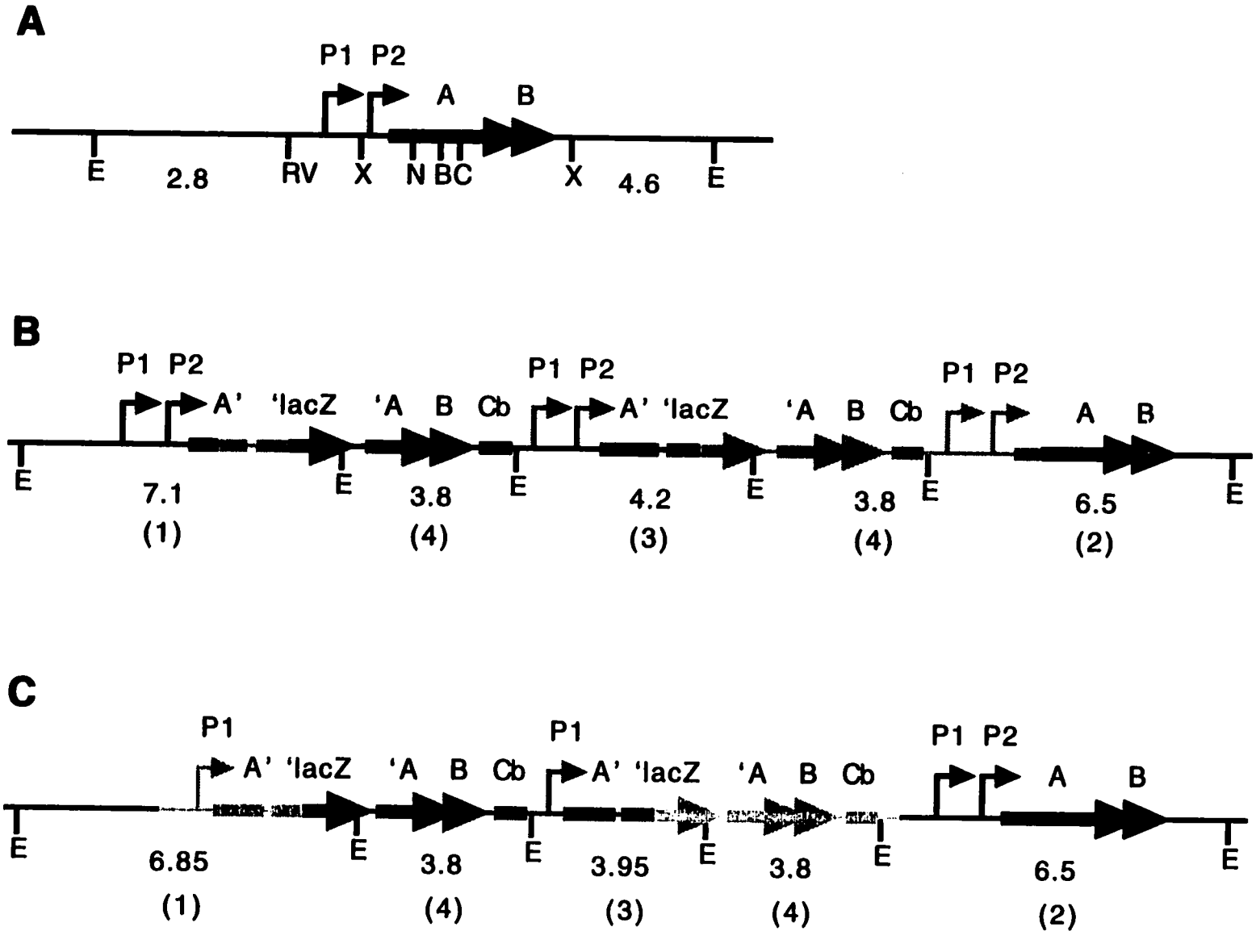

1 2 3 4 5 6
9.8
5.0
2.4
regAB probe
lacZ probe

Figure 6. Diagrammatic representation of the *regAB* locus in (A) PA103, (B) PA103 N3 and (C) PA103 P3.

A. The wild type PA103 *regAB* locus consists of two tandemly oriented genes, *regA* and *regB*, controlled by two tandemly oriented promoters, P1 and P2. **B.** The PA103 N3 *regAB* locus contains two copies of a P1-P2-*lacZ* fusion, as well as a P1-P2-*regAB* wild type operon. Plasmid pNRL integrated first into *regA*, and then a second copy of pNRL integrated into *lacZ*. **C.** The PA103 P3 *regAB* locus contains 2 copies of a P1-*lacZ* fusion, as well as a P1-P2-*regAB* wild type operon. Plasmid pNRLP1 integrated first upstream of the P1 promoter, and a second copy integrated into *lacZ*. Numbers below each operon represent *Eco*RI fragment sizes in kilobase pairs. Numbers in brackets represent bands probed with *regAB* from Figure 2. These numbered fragments correspond to bands from top to bottom in each lane of Figure 2. The diagram is not to scale. A - *regA*, B - *regB*; E - *Eco*RI, RV, *Eco*RV, X - XmaIII (*Eag*I), N - *Nco*I, C - *Cla*I.


A
P1 P2
A
B
E
2.8
RV
X
N BC
X
4.6
E
B
P1 P2
A' 'lacZ
'A B Cb
P1 P2
A' 'lacZ
'A B Cb
P1 P2
A B
E
7.1
(1)
E
3.8
(4)
E
4.2
(3)
E
3.8
(4)
E
6.5
(2)
E
C
P1
A' 'lacZ
'A B Cb
P1
A' 'lacZ
'A B Cb
P1 P2
A B
E
6.85
(1)
E
3.8
(4)
E
3.95
(3)
E
3.8
(4)
E
6.5
(2)
E

3.2 ß-galactosidase and ADPRT activities of PA103 N3 and PA103 P3

To confirm that the recombinant strains were reporting accurately on the activity of the *regAB* promoters, ß-galactosidase and ADPRT activity was measured throughout a growth curve (Figure 7).

Figure 8a illustrates ß-galactosidase activity of PA103 N3 throughout growth. P1 promoter activity is seen early in the growth curve in both low and high iron conditions, but P2 activity late in the growth curve is clearly inhibited by the presence of iron.

Figure 8b illustrates ß-galactosidase activity of PA103 P3 throughout a growth curve. P1 promoter activity can be seen early in the growth curve. There is little difference between growth in low and high iron conditions. Since the *lacZ* fusion in PA103 P3 does not contain the P2 promoter, no activity is seen late in the growth curve under low or high iron conditions.

Figure 9a illustrates ADPRT activity of PA103 N3 throughout a growth curve. Activities are somewhat less than those observed in wild type PA103, but the trends are consistent, with iron-regulated toxin accumulation late in the growth curve, and little iron-regulated ADPRT activity early in the growth curve. Iron regulation late in growth is somewhat derepressed. As seen in subsequent repetitions of this assay (Figure 20a), this late activity is probably erroneous.

Figure 9b illustrates ADPRT activity of PA103 P3 throughout a growth curve. Again, the levels observed are somewhat less than

seen in wild type PA103 (not shown), but similar to those seen in PA103 N3.

Figure 7. Growth curves of *P. aeruginosa* PA103 recombinants.

Primary cultures were grown overnight in TSBDC containing 300 μg/mL carbenicillin. Secondary cultures were inoculated into TSBDC to an OD_{540} of 0.02. Secondary cultures did not contain antibiotic. $FeCl_3$ (+Fe) was supplemented to 10 μg/mL or not added at all (-Fe). Aliquots taken from these growth curves were used for ß-galactosidase assays (Figure 8) and ADPRT assays (Figure 9).

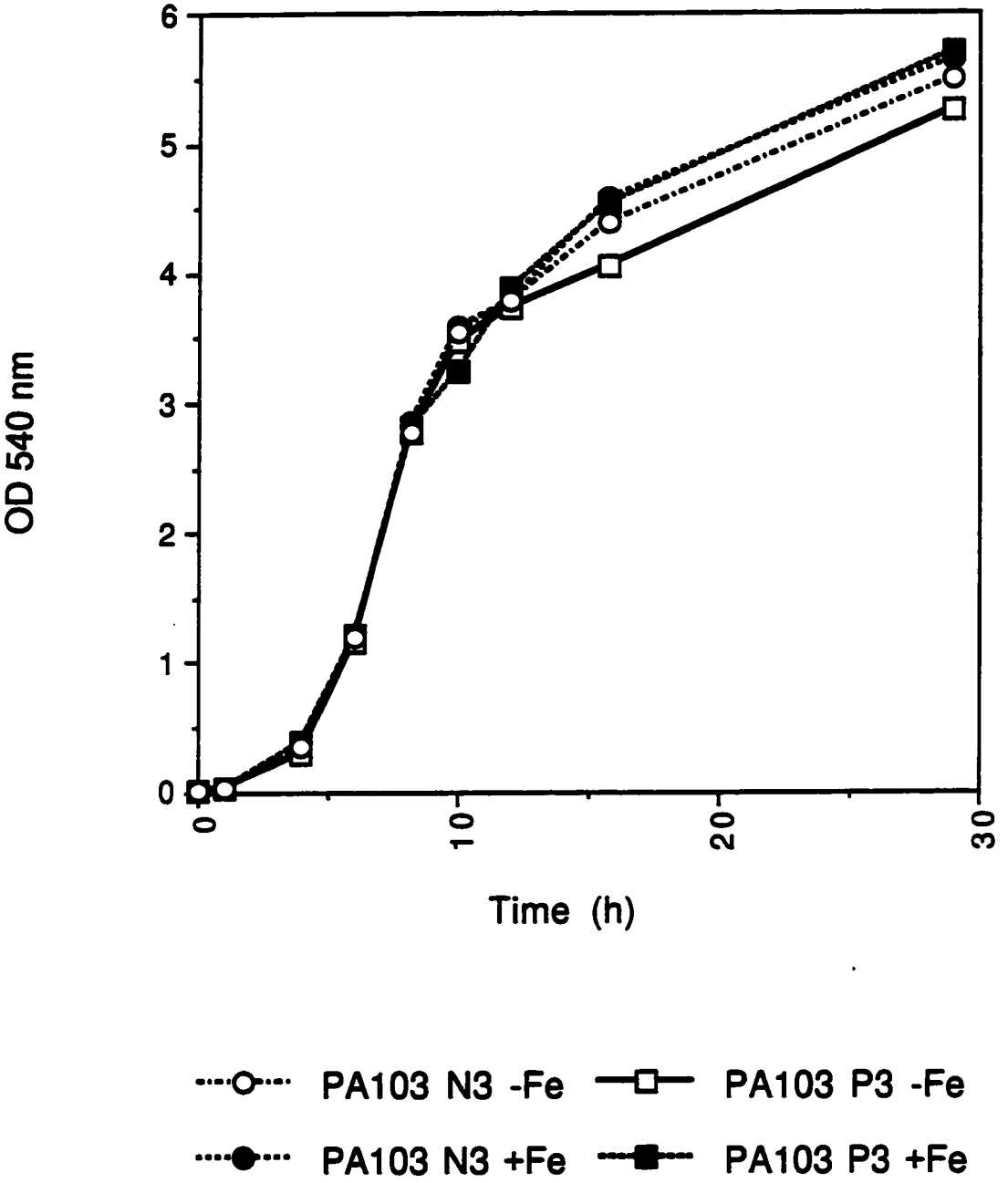

OD 540 nm
0
1
2
3
4
5
6
0
10
20
30
Time (h)
PA103 N3 -Fe
PA103 P3 -Fe
PA103 N3 +Fe
PA103 P3 +Fe

Figure 8. ß-galactosidase activities in *P. aeruginosa* PA103 recombinants.

Primary cultures were grown overnight in TSBDC containing 300 µg/mL carbenicillin. Secondary cultures were inoculated into TSBDC to an OD_{540} of 0.02. Secondary cultures did not contain antibiotic. $FeCl_3$ was supplemented to 10 µg/mL (+Fe) or not added at all (-Fe). Aliqots were removed at indicated time intervals, cells were resuspended in A-buffer, frozen, and assayed for ß-galactosidase activity. These trends were reproduced using standard Miller tube assays (Miller, 1972). **A.** PA103 N3 and **B.** PA103 P3.

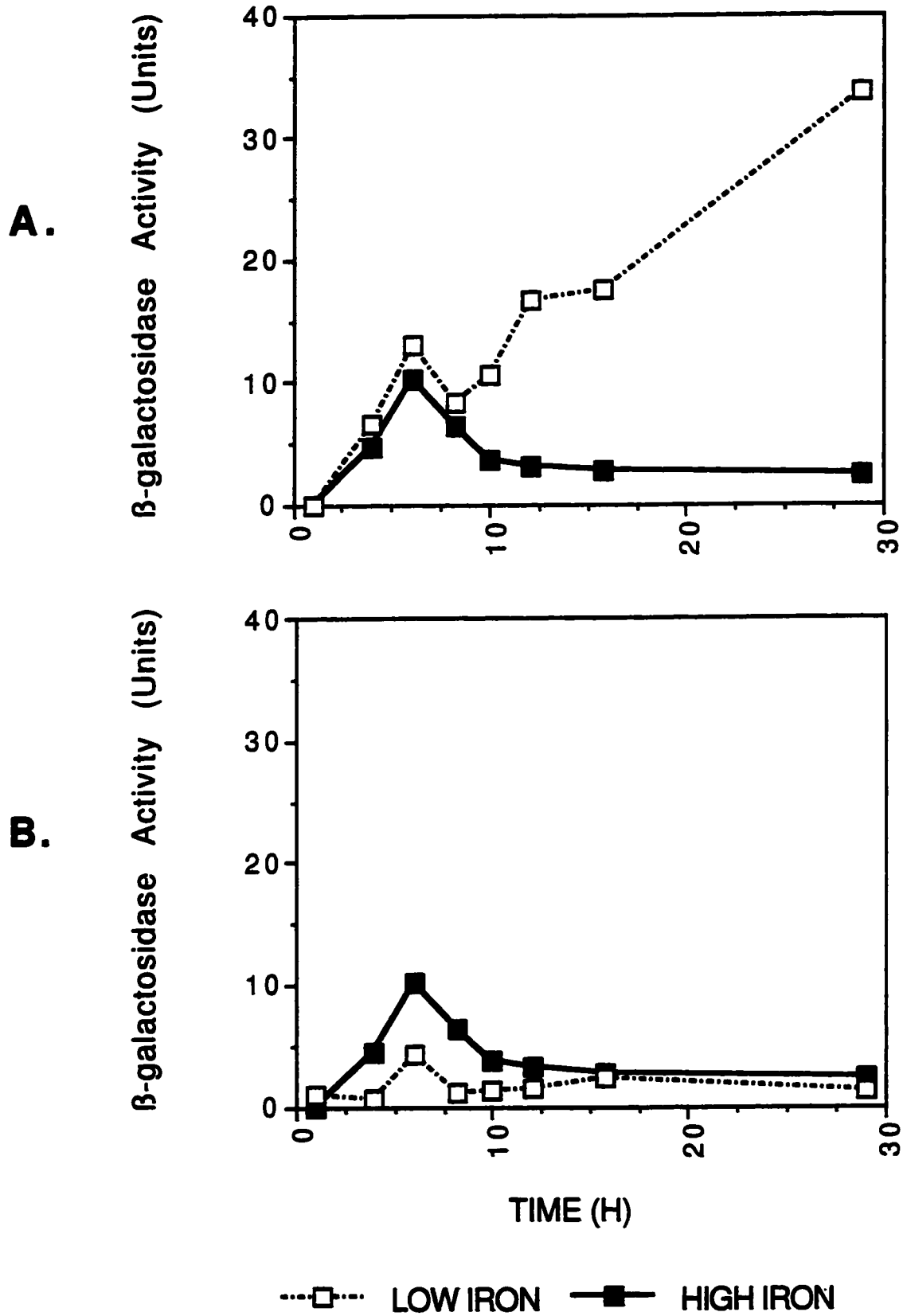
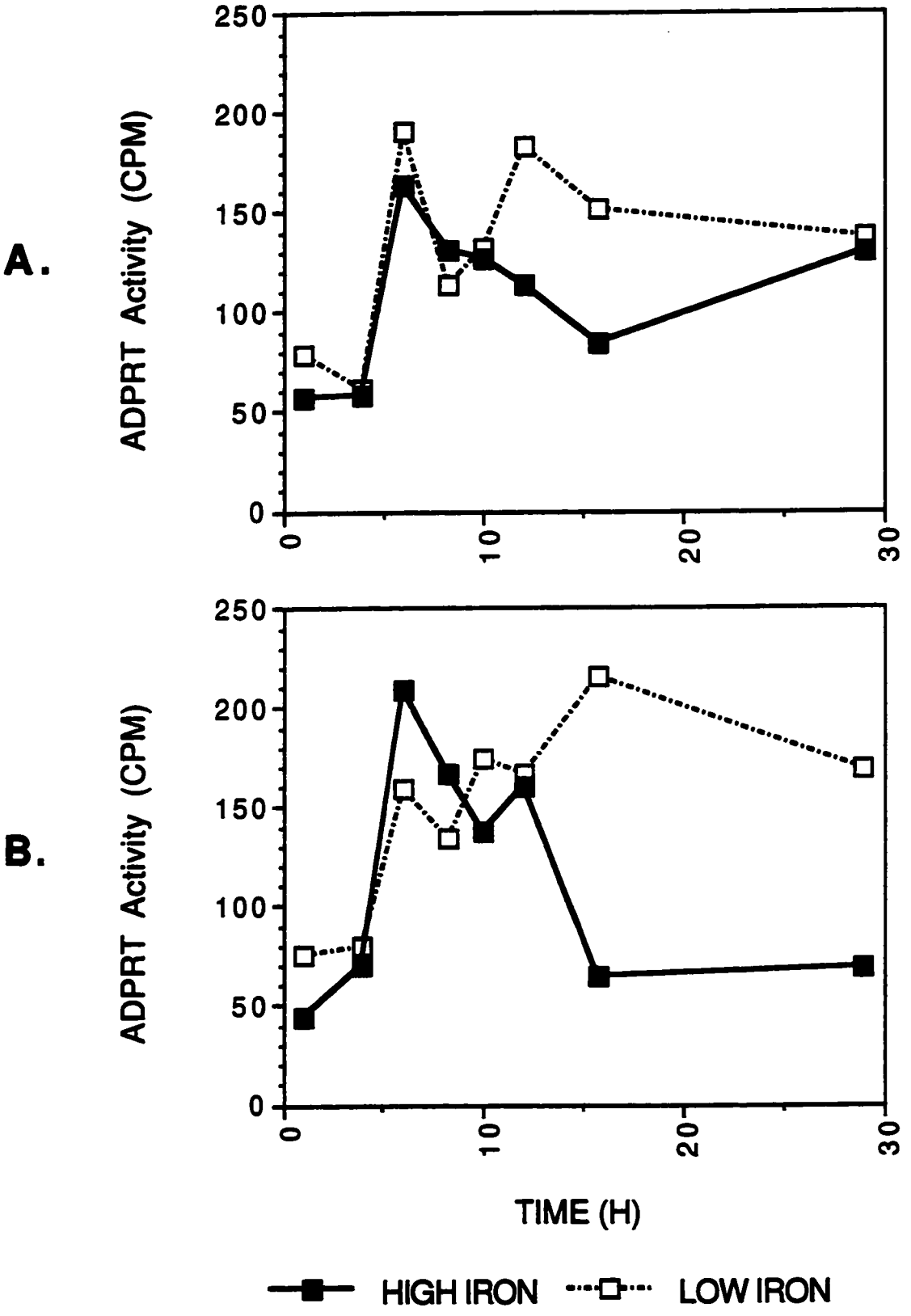

A.
B.
ß-galactosidase Activity (Units)
ß-galactosidase Activity (Units)
40
30
20
10
0
0
10
20
30
TIME (H)
LOW IRON
HIGH IRON

Figure 9. ADPRT activities of *P. aeruginosa* PA103 recombinants.

Primary cultures were grown overnight in TSBDC containing 300 μg/mL carbenicillin. Secondary cultures were inoculated into TSBDC to an OD_{540} of 0.02. Secondary cultures did not contain antibiotic. $FeCl_3$ was supplemented to 10 μg/mL (+Fe) or not added at all (-Fe). Aliqots were removed at indicated time intervals, and supernatants were frozen for storage before assaying for ADPRT activity. These trends were reproduced in subsequent assays when used as controls for further experiments. **A.** PA103 N3 and **B.** PA103 P3.


A.
B.
ADPRT Activity (CPM)
250
200
150
100
50
0
0
10
20
30
TIME (H)
HIGH IRON
LOW IRON

3.3 Transposition with Tn*5*-B61

To generate mutants altered in their expression of *lacZ*, and therefore potentially in the regulation of the *regAB* P1 and P2 promoters, both PA103 N3 and PA103 P3 were electroporated with pSUP102::Tn*5*-B61, a suicide plasmid carrying a Tn*5* derivative with gentamicin resistance (see Figure 17a for a diagram of the Tn*5* derivative, Tn*5*-B61). Recombinants, selected through carbenicillin and gentamicin resistance, were assayed for their ß-galactosidase activities (as reported by the P1 or P1-P2 promoters) in low iron medium. Twelve putative mutants were collected and grouped into 6 categories. Representatives from each category were further analyzed.

First, ß-galactosidase assays (Figure 11) and ADPRT assays (Figure 12) were performed over a growth curve (Figure 10) in low iron conditions.

Figure 11a depicts the ß-galactosidase activities of mutants derived from PA103 N3. These three mutants, PA103 N3 A156, PA103 N3 B17 and PA103 N3 D81, were representative of three classes of mutants: those with high activity early in growth (PA103 N3 A156), those with high activity later in growth (PA103 N3 D81), and those with lower activity throughout growth (PA103 N3 B17). ADPRT assays (Figure 12a) performed on the same samples confirmed that the mutations in both PA103 N3 B17 and PA103 N3 A156 affected ADPRT activity, while PA103 N3 D81 appeared unchanged.

Figure 11b illustrates the ß-galactosidase activities of PA103 P3-derived mutants. The corresponding ADPRT activities are shown in Figure 12b. Again, three groups of mutants are represented by the ß-galactosidase curves: those with high activity early (PA103 P3 C86), those with high activity late (PA103 P3 D168), and those with very high activity throughout growth (PA103 P3 C44). The ADPRT activity of PA103 P3 C86 was similarly high, but PA103 P3 C44 and PA103 P3 D168 activities were similar to the parental strain.

Genomic DNA from these mutants was prepared, digested with *Eco*RI, and probed with an internal 1.1 kb *Bam*HI-*Bgl*II Tn*5*-B61 gentamicin probe (Figure 13). All transpositions had occurred singularly and at different locations. The 3.8 kb band seen in all lanes of Figure 8 is due to a band from the *regAB-lacZ* fusion which has some identity to sequences in Tn*5* (Hindahl *et al.*, 1987). In fact, a 212 bp sequence homologous to Tn*5* present at the end of the 1.9 kb *regAB* clone was perpetuated throughout all cloning procedures. Less DNA was loaded into the control lanes, as judged by ethidium bromide staining of the gel prior to blotting, resulting in a fainter 3.8 kb band. Some lanes appear to have a second, less intense band. This is probably due to a cross-hybridization, an incomplete restriction digest, or insufficient washing. Comparison of the number of bands between genomic DNAs cut with different restriction enzymes reveals only one band in at least one of the preparations.

To ensure that transposition had not occurred into the chromosomal *lacZ* reporter gene, thereby artificially raising ß-galactosidase levels, genomic DNA from the transposon mutants was

digested with *Bam*HI, which cuts only on either side of *lacZ*, and hybridized with a *lacZ*-specific probe (Figure 14). None of the mutants appeared to have an insertion into *lacZ*, as suggested by their consistent size.

Figure 10. Growth curves of *P. aeruginosa* PA103 transposon mutants.

Primary cultures were grown overnight in TSBDC containing 300 μg/mL carbenicillin. Strains carrying a transposon were also grown with 50 μg/mL gentamicin. Secondary cultures were inoculated into TSBDC to an OD_{540} of 0.02. These cultures contained neither antibiotic nor $FeCl_3$. Aliquots taken from these growth curves were used for ß-galactosidase assays (Figure 11) and ADPRT assays (Figure 12).

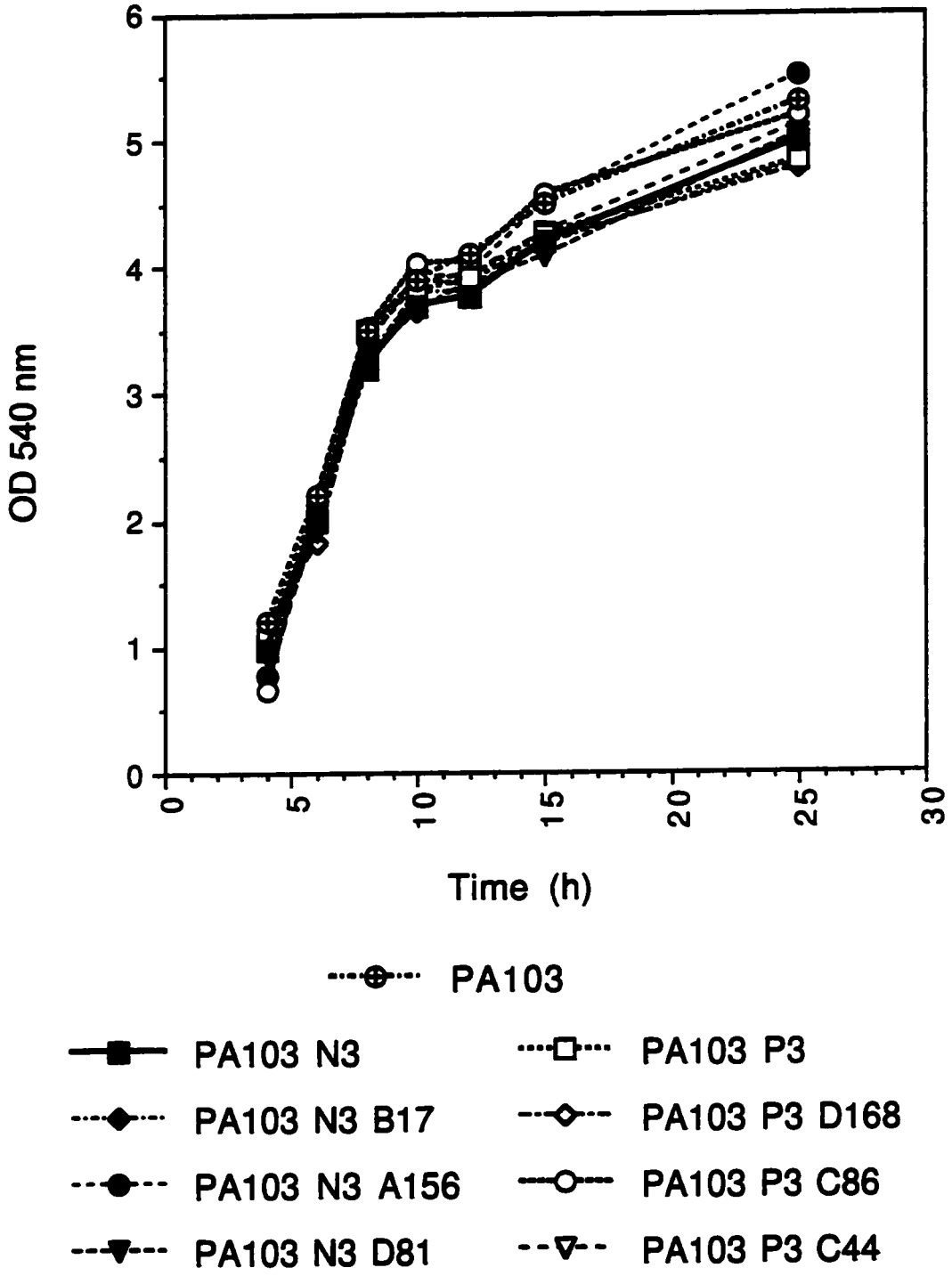
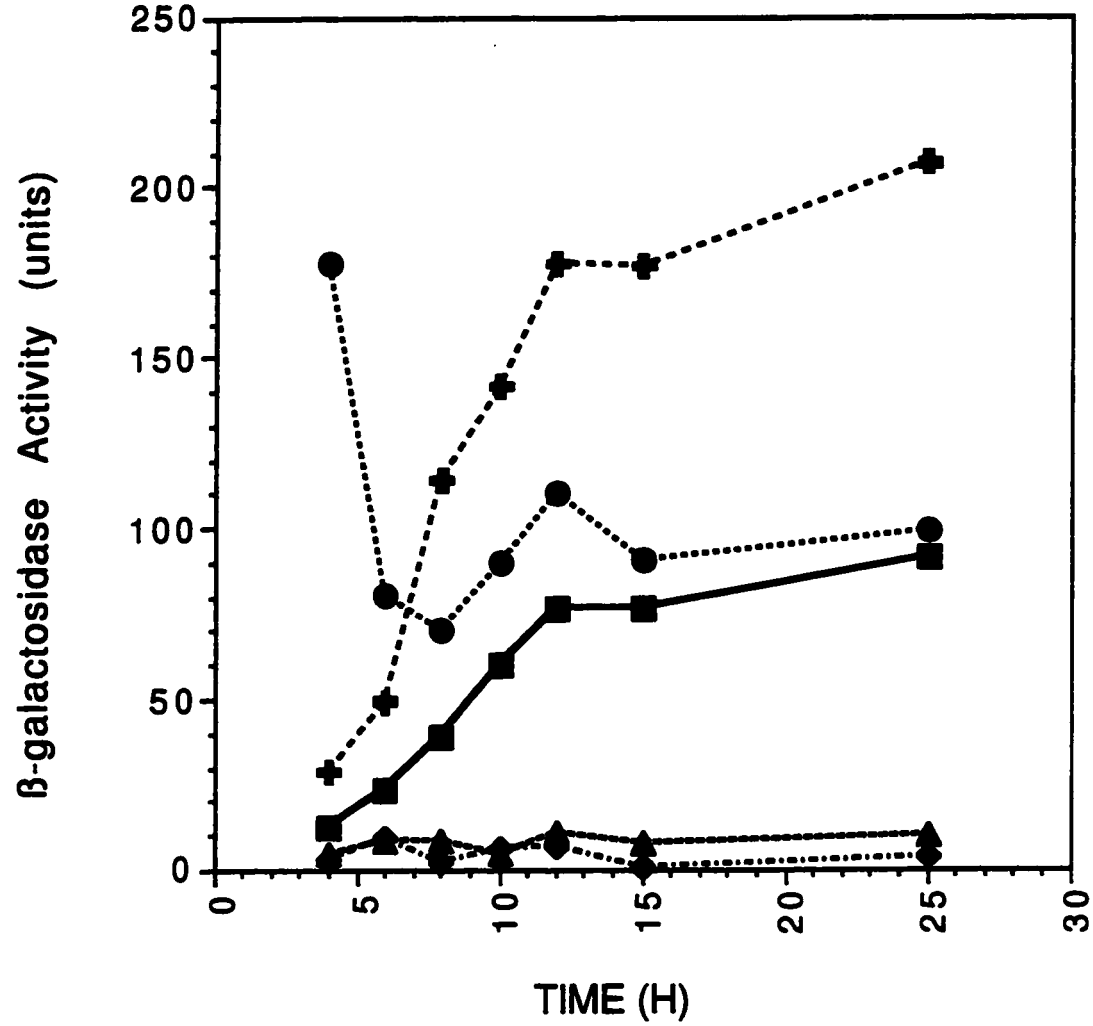
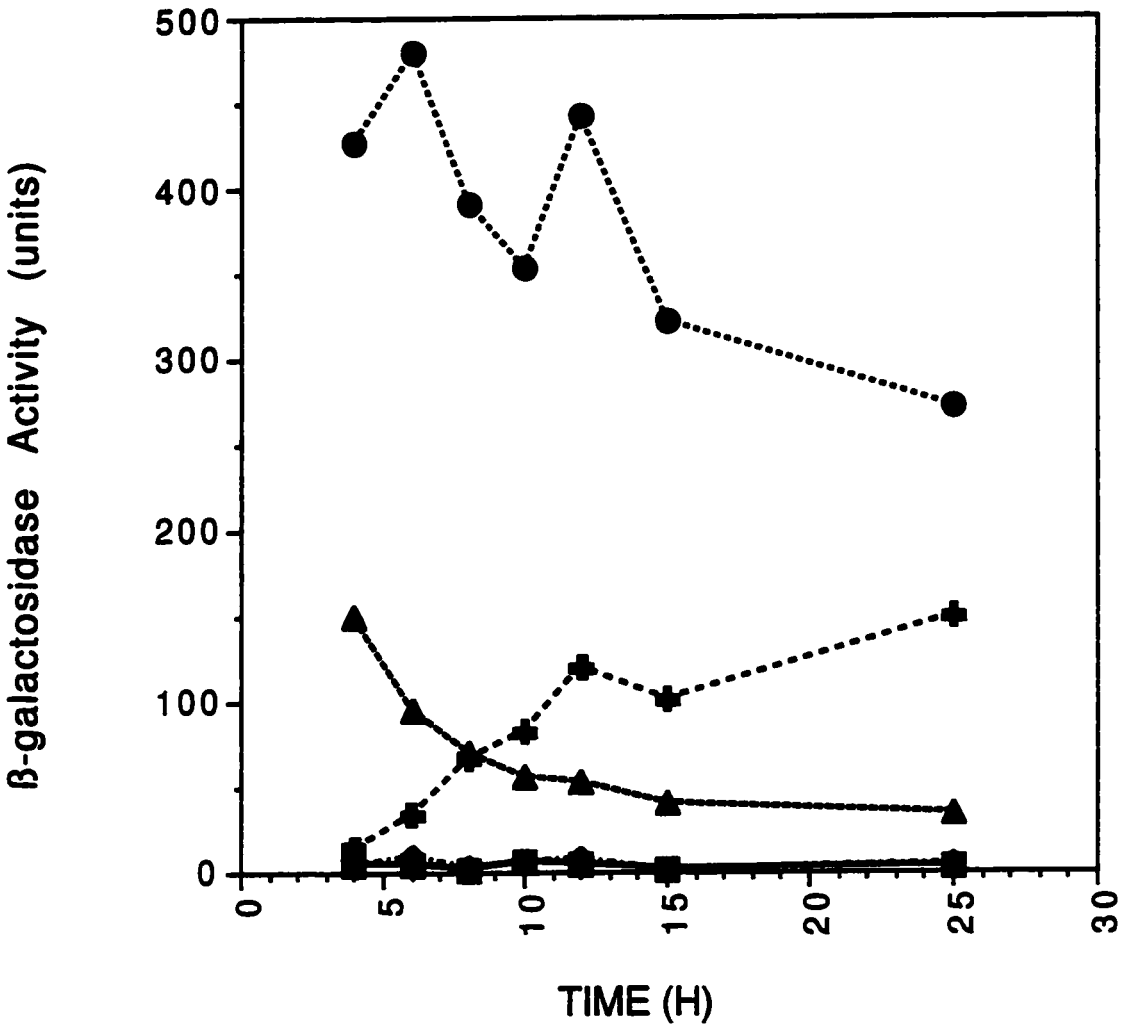

OD 540 nm
0
1
2
3
4
5
6
0
5
10
15
20
25
30
Time (h)
PA103
PA103 N3
PA103 P3
PA103 N3 B17
PA103 P3 D168
PA103 N3 A156
PA103 P3 C86
PA103 N3 D81
PA103 P3 C44

Figure 11. ß-galactosidase activities of PA103 transposon mutants.

Primary cultures were grown overnight in TSBDC containing 300 μg/mL carbenicillin. Strains carrying a transposon were also grown with 50 μg/mL gentamicin. Secondary cultures were inoculated into TSBDC to an OD_{540} of 0.02. These cultures contained neither antibiotic nor $FeCl_3$. Aliqots were removed at indicated time intervals, cells were resuspended in A-buffer, frozen, and assayed for ß-galactosidase activity. **A.** PA103 N3 transposon mutants and **B.** PA103 P3 transposon mutants.


250
200
150
100
50
0
β-galactosidase Activity (units)
0
5
10
15
20
25
30
TIME (H)
PA103
PA103 N3
PA103 N3 A156
PA103 N3 B17
PA103 N3 D81

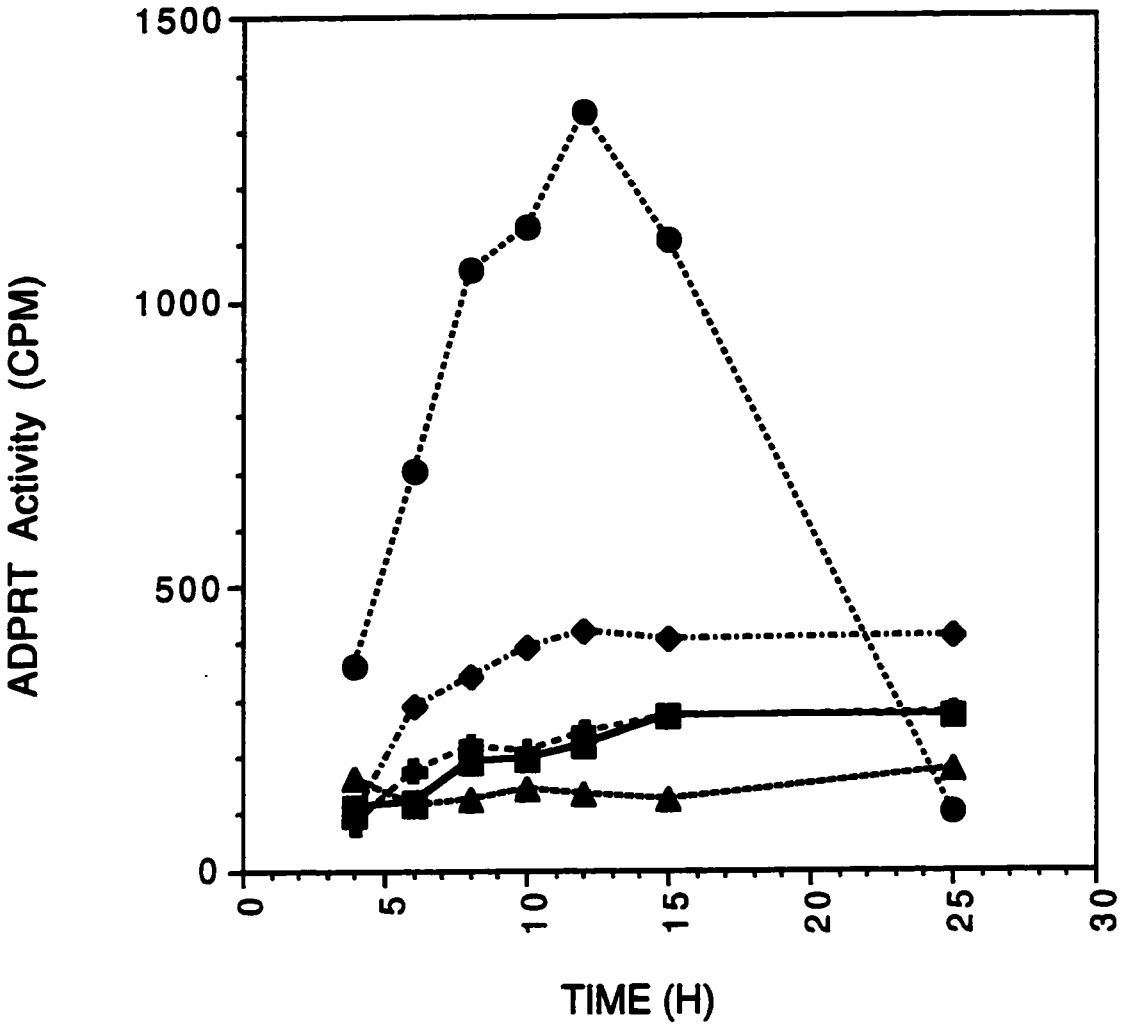
A.


500
400
300
200
100
0
ß-galactosidase Activity (units)
0
5
10
15
20
25
30
TIME (H)
PA103
PA103 P3
PA103 P3 C44
PA103 P3 C86
PA103 P3 D168

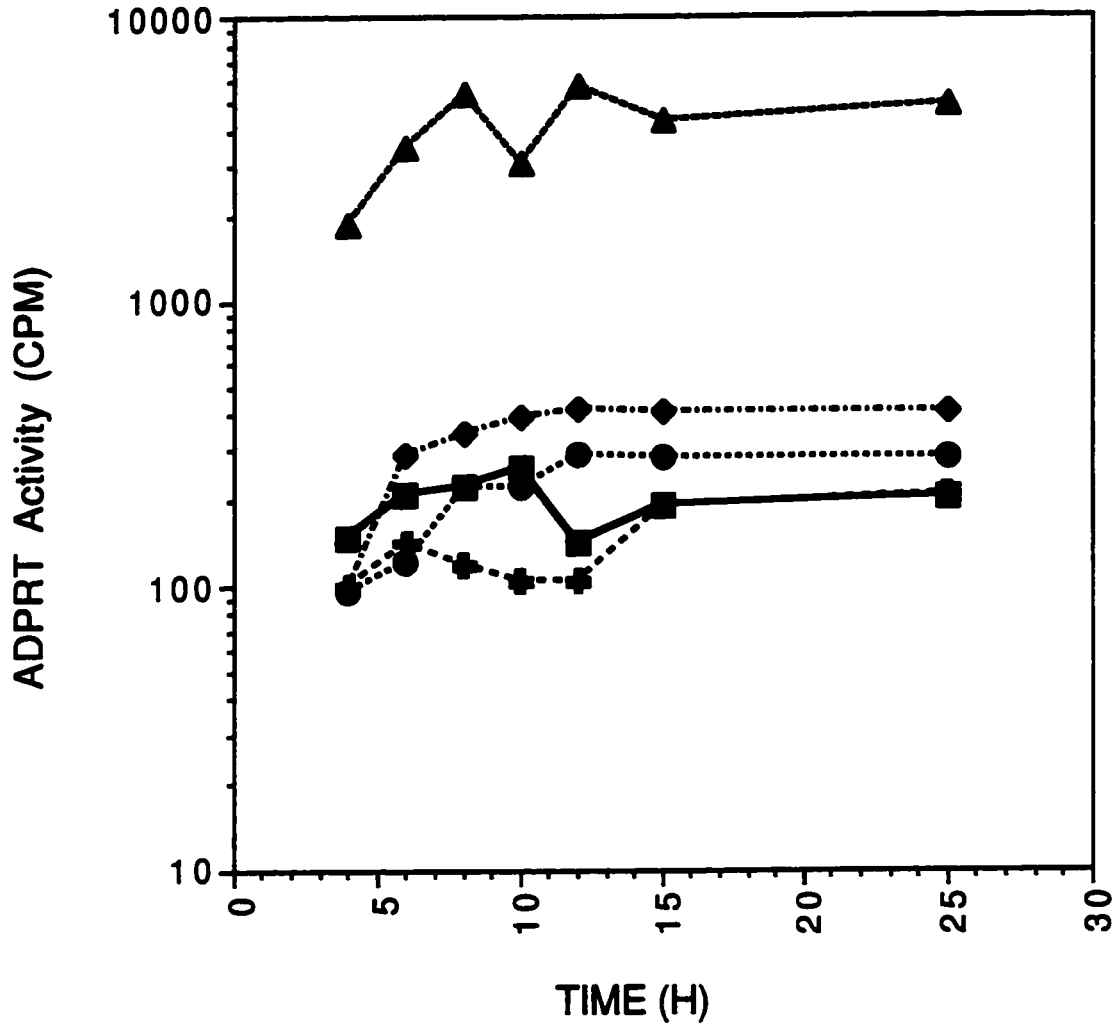
B.

Figure 12. ADPRT activities of PA103 transposon mutants.

Primary cultures were grown overnight in TSBDC containing 300 μg/mL carbenicillin. Strains carrying a transposon were also grown with 50 μg/mL gentamicin. Secondary cultures were inoculated into TSBDC to an OD_{540} of 0.02. These cultures contained neither antibiotic nor $FeCl_3$. Aliqots were removed at indicated time intervals, and supernatants were frozen for storage before assaying for ADPRT activity. The trends for PA103 N3 B17 and PA103 P3 C86 were reproduced in multiple subsequent experiments. **A.** PA103 N3 transposon mutants and **B.** PA103 P3 transposon mutants. Note that due to the high activity of PA103 P3 C86, the graph in Part B uses a logarithmic ordinate, whereas the graph in Part A uses a linear ordinate.


1500
1000
500
0
ADPRT Activity (CPM)
0
5
10
15
20
25
30
TIME (H)
PA103
PA103 N3
PA103 N3 A156
PA103 N3 B17
PA103 N3 D81

A.


10000
1000
100
10
ADPRT Activity (CPM)
0
5
10
15
20
25
30
TIME (H)
PA103
PA103 P3
PA103 P3 C44
PA103 P3 C86
PA103 P3 D168

B.

Figure 13. Southern blot analysis of PA103 N3 and PA103 P3 transposon mutants using a transposon-specific probe.

Genomic DNA from each of the transposon mutants was prepared, digested with either *Sal*I (lanes 1-7, 16) or *Eco*RI (lanes 8-14, 15) and electrophoresed on a 1.0% agarose gel. The DNA was transferred to a nylon membrane and hybridized with a *Bam*HI/*Bgl*II 1.1 kb transposon-specific fragment from pSUP102::Tn*5*-B61. Lanes 1 and 8: PA103 N3; 2 and 9: PA103 P3; 3 and 10: P3 C44; 4 and 11: N3 D81; 5 and 12: P3 C86; 6 and 13: P3 D168; 7 and 14: N3 B17, 15 and 16: N3 A156.

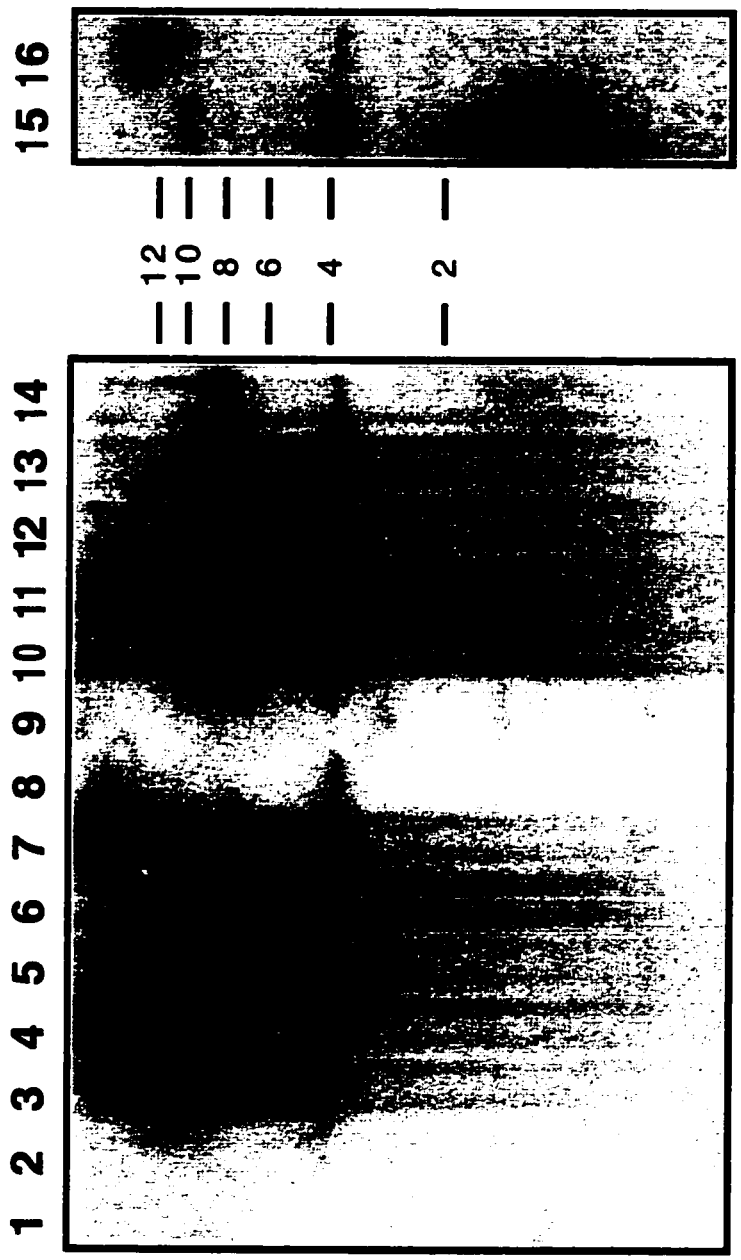
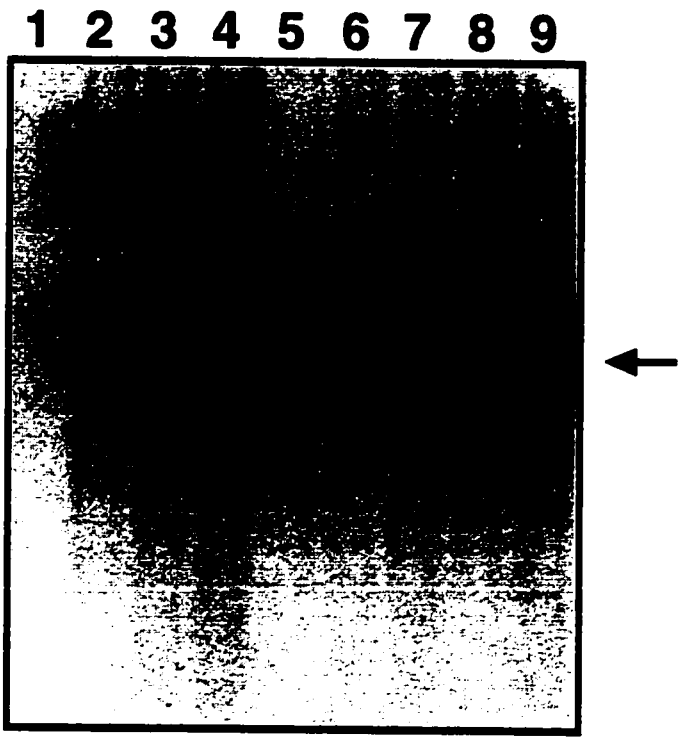

15 16
12
10
8
6
4
2
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 14. Southern blot analysis of PA103 N3 and PA103 P3 transposon mutants using a *lacZ*-specific probe.

Genomic DNA from each of the transposon mutants was prepared, digested with *Bam*HI and electrophoresed on a 1.0% gel. The DNA was transferred to a nylon membrane and hybridized with an internal *lacZ*-specific *Hin*cII 1.8 kb fragment from pZ1918. *Bam*HI cuts once in the *regAB* region, and on either side of *lacZ*. Lane 1: PA103, 2: PA103 N3, 3: PA103 P3, 4: P3 D168, 5: P3 C86, 6: N3 A156, 7: N3 D81, 8: N3 B17, 9: P3 C44.


1 2 3 4 5 6 7 8 9

3.4 Cloning of transposons and flanking DNA from PA103 P3 C44, PA103 N3 B17 and PA103 P3 C86

Two genomic Lambda Zap Express libraries of size-selected *Eco*RI genomic digests of all the mutant strains were prepared. Strains PA103 P3 C44 and PA103 N3 D81 genomic DNAs were pooled, as were genomic DNAs from strains PA103 P3 C86, PA103 P3 D168 and PA103 N3 B17. The pooled DNAs all displayed hybridizing bands of sufficiently different size as to allow selection of positive clones by subsequent Southern blot analysis (Figure 13 and 15).

The libraries were screened using the 1.1 kb Tn*5*-B61 probe and positive plaques were excised into plasmid form in strain XLOLR. Southern blot analysis on *Eco*RI-digested plasmid preps from gentamicin resistant colonies indicated that 3 of the 5 transposons along with the flanking DNA into which they inserted had been cloned (Figure 15). The flanking DNA, adjacent to the IS50R sequence, was sequenced using an IS50R specific primer.

Mutant C44 contains a transposon insertion disrupting amino acid 117 of the 249 amino acid *regA*. The transposon was oriented such that the *tac* promoter transcribed in the same direction as the P1 and P2 promoters of *regAB*. The insertion is just upstream of the *Cla*I site, into which *lacZ* was inserted, such that the *tac* promoter is probably driving *lacZ* transcription. While this was not immediately useful, it did indicate the methodology was working properly.

The sequence flanking the transposon insertion in mutant PA103 N3 B17 shows no significant homology to any sequence in the

non-redundant DNA databank using BLAST analysis. The sequence is shown in Figure 16.

Mutant PA103 P3 C86 contains an insertion at leucine 715 of the immature FpvA (ferripyoverdine receptor) protein (leucine 672 of the mature protein). Figure 17a depicts the *fpvA* genetic locus and the location of the transposon insertion. The transposon contains translational stops in all three frames within 33 amino acids of the junction between the 3' end of *fpvA* and the 5' end of the IS50R sequence. Thus, this mutation truncates the mature wild type FpvA protein from 770 amino acids to at most 705 amino acids.

Figure 15. Southern blot analysis of pBK CMV plasmids containing PA103 N3 and PA103 P3 transposon mutant inserts using a transposon-specific probe.

Plasmid DNA excised from positively hybridizing lambda plaques was prepared from strain XLOLR and electrophoresed on a 1.0% gel. The DNA was transferred to a nylon membrane and hybridized with a transposon-specific *Bam*HI/*Bgl*II 1.1 kb fragment from pSUP102::Tn*5*-B61. Lane 1: pBK CMV; lanes 2 and 3: pBK B17; lanes 4 - 7, 12, 13 and 16: pBK C44; lanes 8-11, 14 and 15: pBK C86.

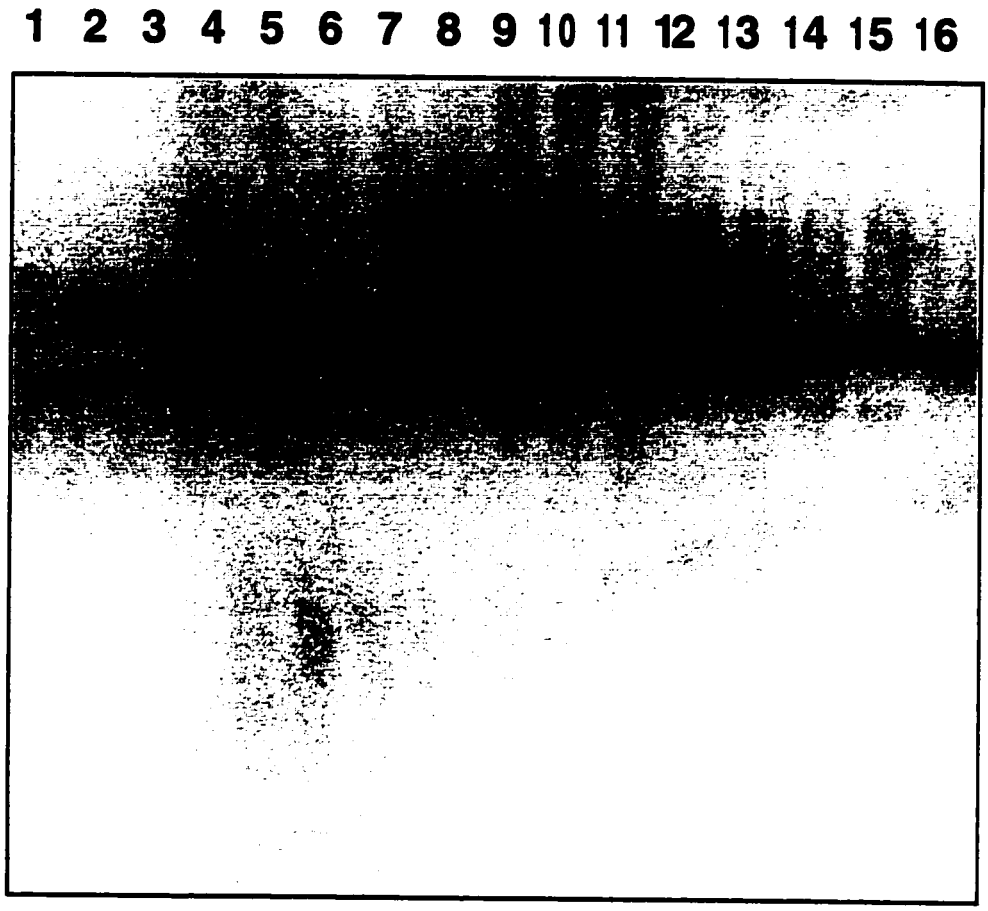

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

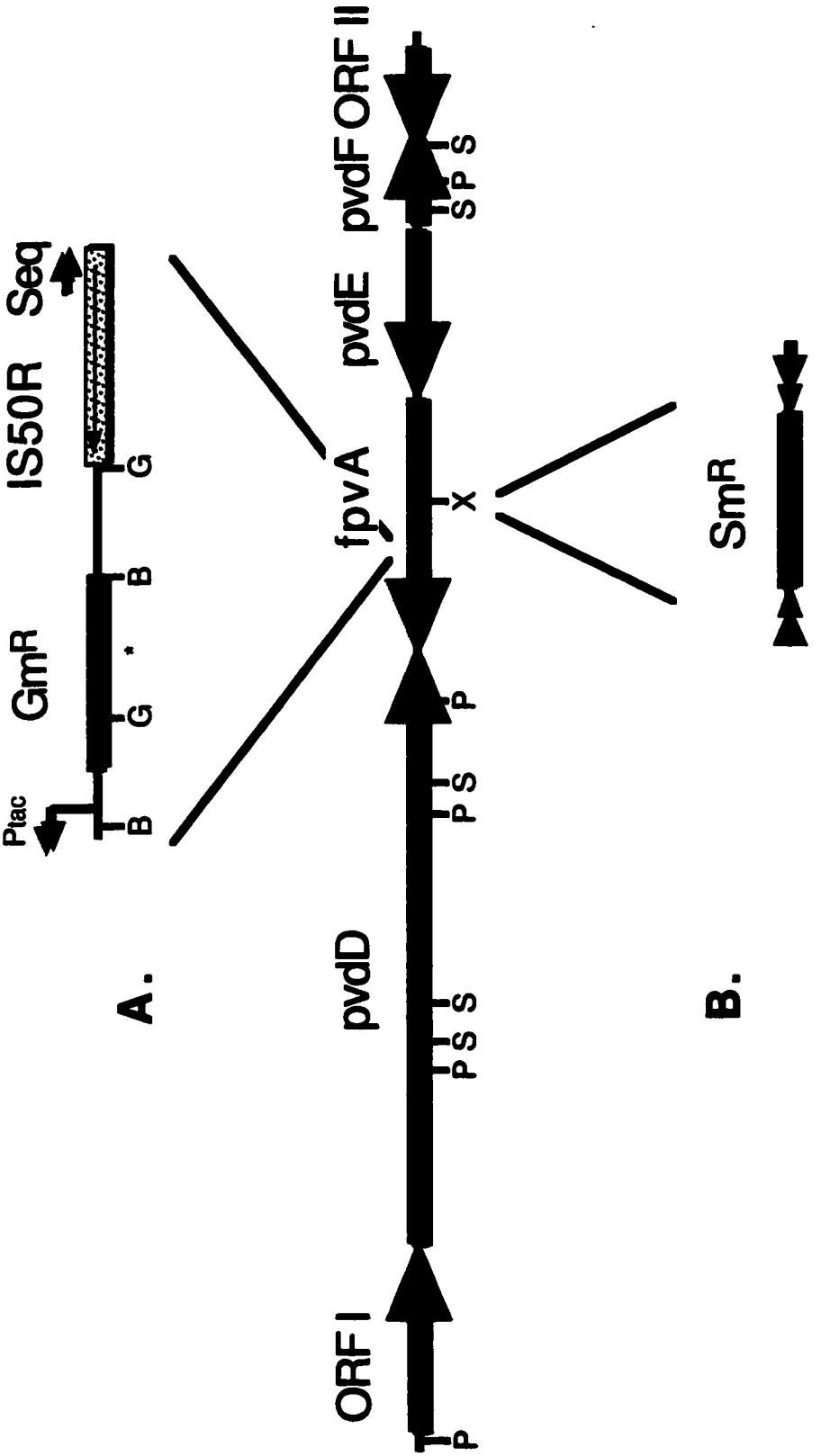
Figure 16. Sequence analysis of pBK B17.

DNA flanking the transposon insertion in PA103 N3 B17 was subcloned and partially sequenced using an IS50R-specific primer. The sequence is displayed in the 5' to 3' direction, and lies immediately adjacent to the Tn*5*-B61 IS50R sequence. Transposon specific sequence has been removed. The sequence has no homologies to sequences in Genbank as determined by either BLASTX or BLASTN.

```
        10         20         30         40         50         60
GCTTGGGGAG TGATACCTGT CTTCCGGGCA GCATCGGTAG GCTTGAGACC ATTTACCAAC

        70         80         90        100        110        120
ACCAGTCGGG CAGCATCCTC GCCAGGACCT CCGCGCAGGC GCATCAACTC AGCAAGGGCT

       130        140        150        160        170        180
GAAAACTGCT CTTCCGTCAT GCGCGACTTG GCCCCGGGAG TAAGCCGGCA ATGCAGGTCA

       190        200        210        220        230        240
ACAGTCGCTG GGCGCCGGCC TGGCTTGATA TATCGTTGCT GACCATATCT CCGCCAGCCG

       250        260        270        280        290        300
CTGCGGCCAG GCGACGCATG AATCCCATGG CAGCGCGATC AATCTCGGGA CCAACGTAGA

       310        320        330        340        350        360
GCACATCAAT CACGCCACGA AACTGGGCGG CGACTTCGAG AGCCGATTTC TCGTTGTCAG

       370        380        390        400        410        420
GTTGGCCATC ACTGAATGAA CAAAAGTCCC ACCGGGAATT GAATTGCAAG GCTGCTCCCC

       430        440        450        460        470        480
AGGGCCGCGG TCATGTTGGT ACTGCTCTCC CGCTCAGGAT GTGCTGGAAT TCCCGTGGTG

       490        500        510        520        530        540
GTCCCTGAAG GCAATCATCT CCCCGGTTGG GTTCCAGGGT GCTTTT.... ..........
```

Figure 17. Diagram of the *pvd* genetic locus in PA103 *fpvA* mutants.

Depicted in the diagram are the insertion points of the transposon Tn*5*-B61 and the Ω fragment into the *fpvA* gene of a wild type *pvd* regulon. **A.** PA103 P3 C86 contains a gentamicin-resistant Tn*5* derivative (Tn B61) inserted at leucine 672 of the mature FpvA protein. Shown are a *tac*-promoter, P_{tac}, and the binding site for the IS50R sequencing primer, Seq. **B.** PA103 *fpvA::Ω* contains a streptomycin/spectinomycin resistant Ω fragment with translational and transcriptional stops (shaded triangles) inserted at leucine 502 of the mature FpvA protein. The diagram is not drawn to scale. The asterisk represents the fragment used to probe for transposon-specific sequences. S - *Sma*I, P - *Pst*I, B - *Bam*HI, G - *Bgl*II.


A.
Ptac
B
GmR
G
*
G
B
IS50R
Seq
fpvA
pvdD
ORF I
P
P S
P S
P
pvdE
X
pvdF
ORF II
S P S
B.
SmR

3.5 Effect of iron on ADPRT activity in PA103 N3 B17 and PA103 P3 C86

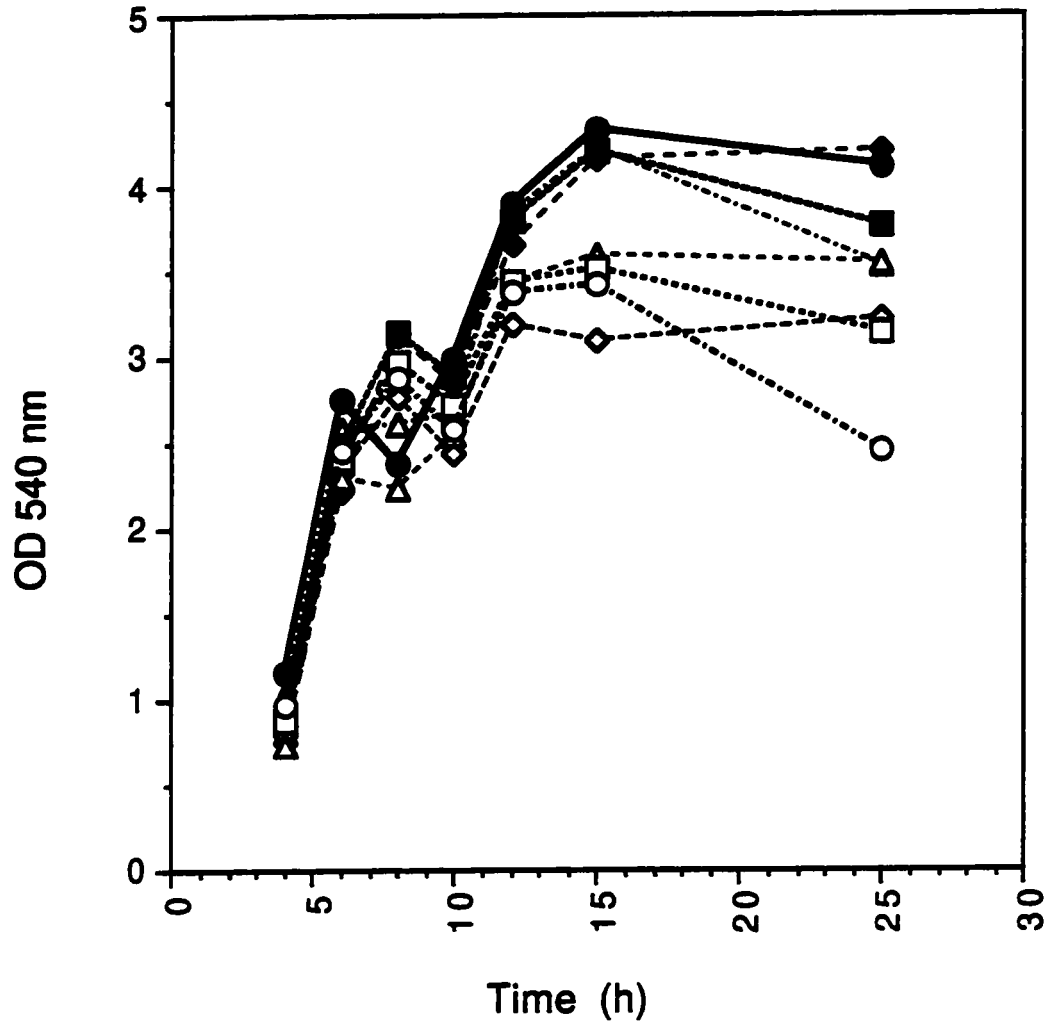
To assess the effects of iron on each of the cloned mutants, growth curves were performed both in low and high iron conditions (Figure 18). ADPRT and ß-galactosidase assays of PA103 P3 C86 and PA103 N3 B17 are shown in Figures 19 and 20 respectively.

In Figure 19, one can see that both the ADPRT and ß-galactosidase activity of PA103 P3 C86 are much higher in both low and high iron conditions than the parental strain. Much of the iron regulation seen late in growth appears to be alleviated (Figure 19a), although there is a significant decrease in ADPRT activity.

Figure 20a shows that the ADPRT activity of PA103 N3 B17 is about half that seen in PA103 N3 throughout growth. Iron regulation seems normal. The P1 and P2 promoter activities (Figure 20b) are similarly low, though still iron regulated.

Figure 18. Growth curves of *P. aeruginosa* PA103 P3 C86 and PA103 N3 B17 in low and high iron conditions.

Primary cultures were grown overnight in TSBDC containing 300 μg/mL carbenicillin. Strains carrying a transposon were also grown with 50 μg/mL gentamicin. Secondary cultures were inoculated into TSBDC to an OD_{540} of 0.02. Secondary cultures did not contain antibiotic. $FeCl_3$ was supplemented to 10 μg/mL (+Fe) or not added at all (-Fe). Aliquots taken from these growth curves were used for ß-galactosidase and ADPRT assays of PA103 P3 C86 (Figure 19) and PA103 N3 B17 (Figure 20).



PA103 P3 -Fe
PA103 P3 +Fe
PA103 N3 -Fe
PA103 N3 +Fe
PA103 N3 B17 -Fe
PA103 N3 B17 +Fe
PA103 P3 C86 -Fe
PA103 P3 C86 +Fe

Figure 19. Effect of iron on ADPRT and ß-galactosidase activity of PA103 P3 C86.

Primary cultures were grown overnight in TSBDC containing 300 µg/mL carbenicillin. Strains carrying a transposon were also grown with 50 µg/mL gentamicin. Secondary cultures were inoculated into TSBDC to an OD_{540} of 0.02. Secondary cultures did not contain antibiotic. $FeCl_3$ was supplemented to 10 µg/mL (+Fe) or not added at all (-Fe). Aliqots were removed at indicated time intervals, cells were resuspended in A-buffer, frozen, and assayed for ß-galactosidase activity. Supernatants were frozen for storage before assaying for ADPRT activity. ß-galactosidase and ADPRT activity trends were reproduced in at least two different experiments. **A.** ADPRT activities and **B.** ß-galactosidase activities of PA103 P3 C86.

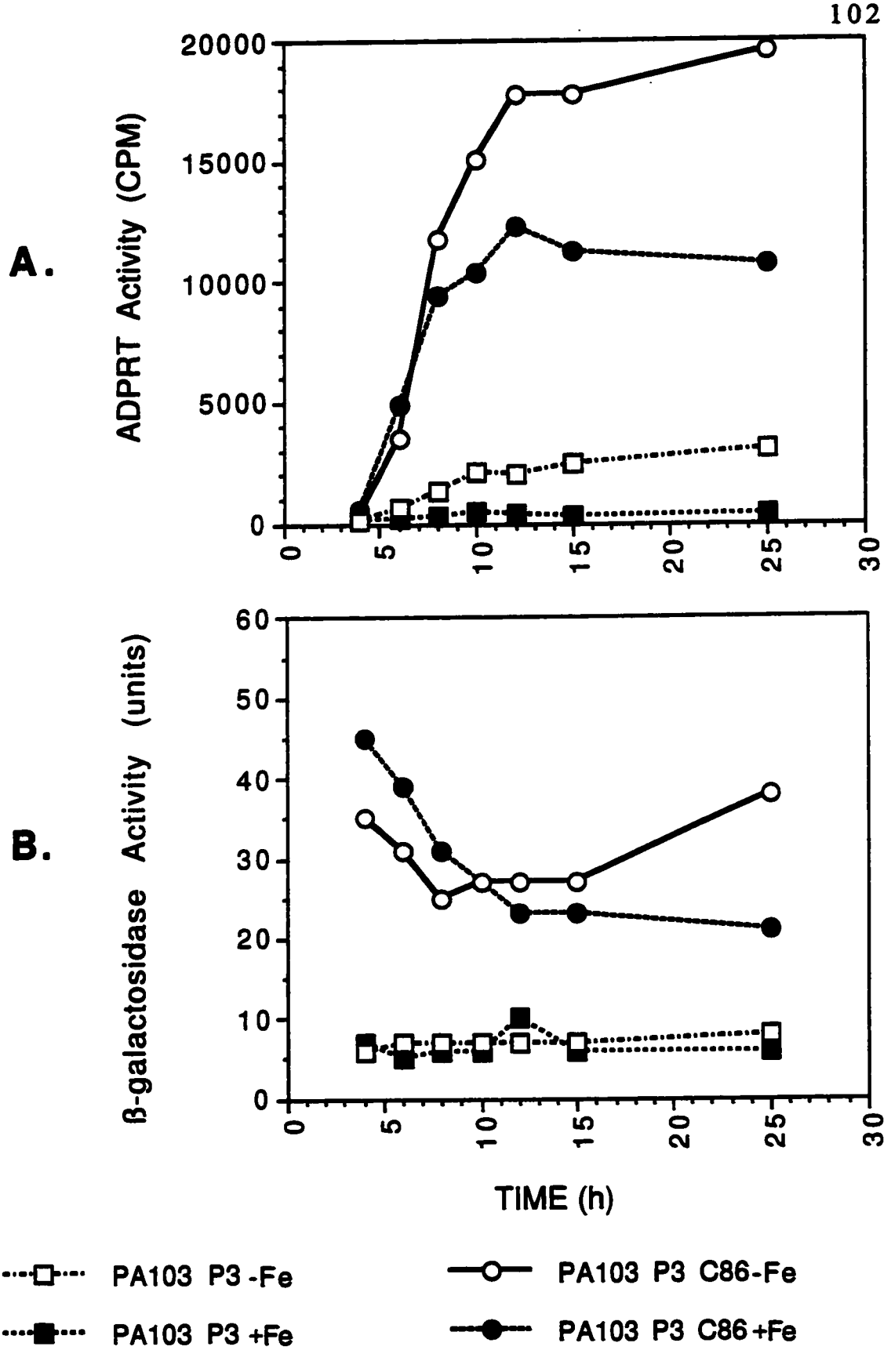
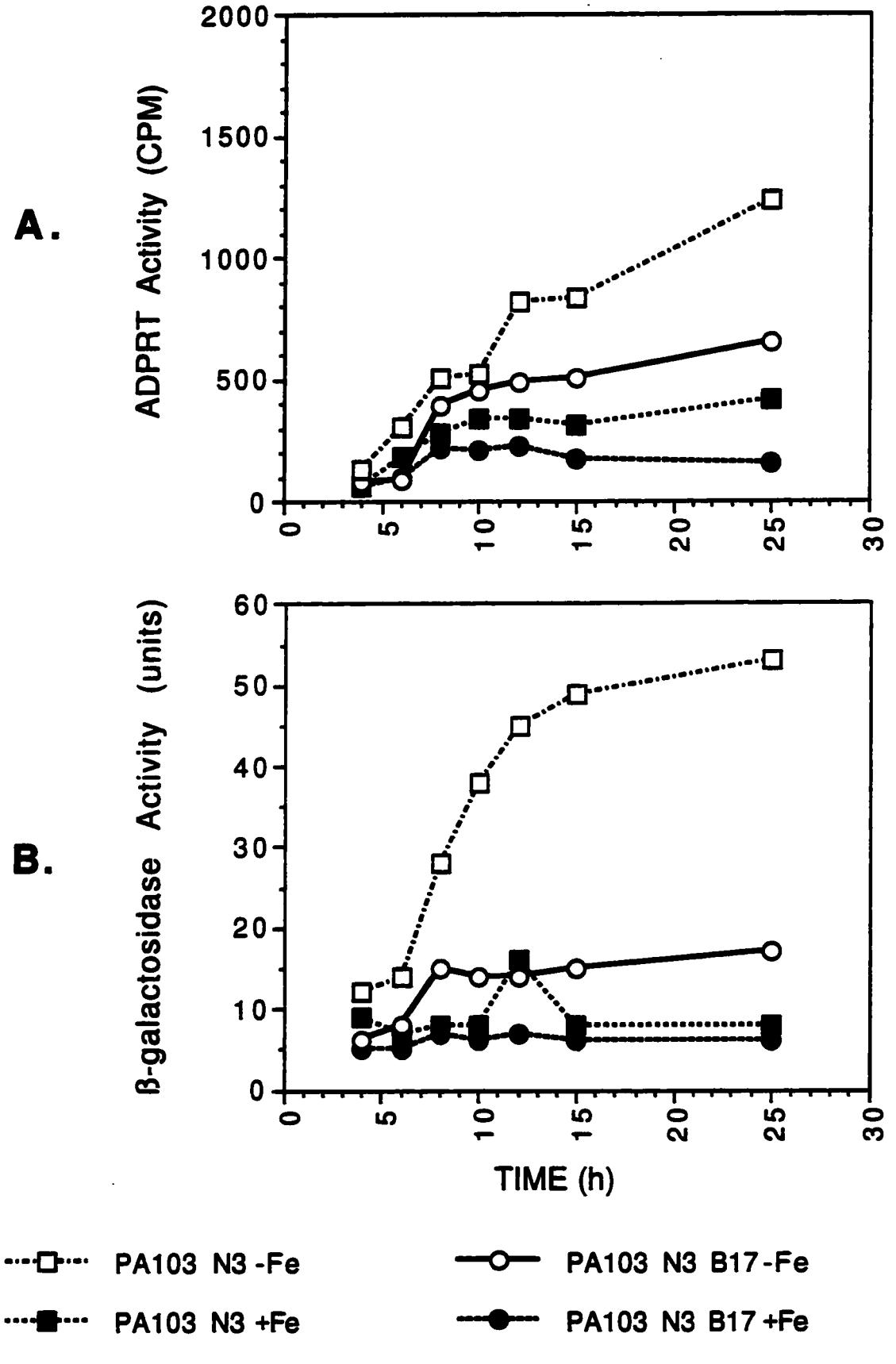

A.
ADPRT Activity (CPM)
20000
15000
10000
5000
0
0
5
10
15
20
25
30
B.
ß-galactosidase Activity (units)
60
50
40
30
20
10
0
TIME (h)
PA103 P3 -Fe
PA103 P3 +Fe
PA103 P3 C86-Fe
PA103 P3 C86+Fe

Figure 20. Effect of iron on ADPRT and ß-galactosidase activity of PA103 N3 B17.

Primary cultures were grown overnight in TSBDC containing 300 µg/mL carbenicillin. Strains carrying a transposon were also grown with 50 µg/mL gentamicin. Secondary cultures were inoculated into TSBDC to an OD_{540} of 0.02. Secondary cultures did not contain antibiotic. $FeCl_3$ was supplemented to 10 µg/mL (+Fe) or not added at all (-Fe). Aliqots were removed at indicated time intervals, cells were resuspended in A-buffer, frozen, and assayed for ß-galactosidase activity. Supernatants were frozen for storage before assaying for ADPRT activity. ADPRT and ß-galactosidase activity trends were reproduced in at least two different experiments. **A.** ADPRT activities and **B.** ß-galactosidase activities of PA103 N3 B17.


A.
ADPRT Activity (CPM)
2000
1500
1000
500
0
0
5
10
15
20
25
30
B.
β-galactosidase Activity (units)
60
50
40
30
20
10
0
TIME (h)
PA103 N3 -Fe
PA103 N3 +Fe
PA103 N3 B17-Fe
PA103 N3 B17 +Fe

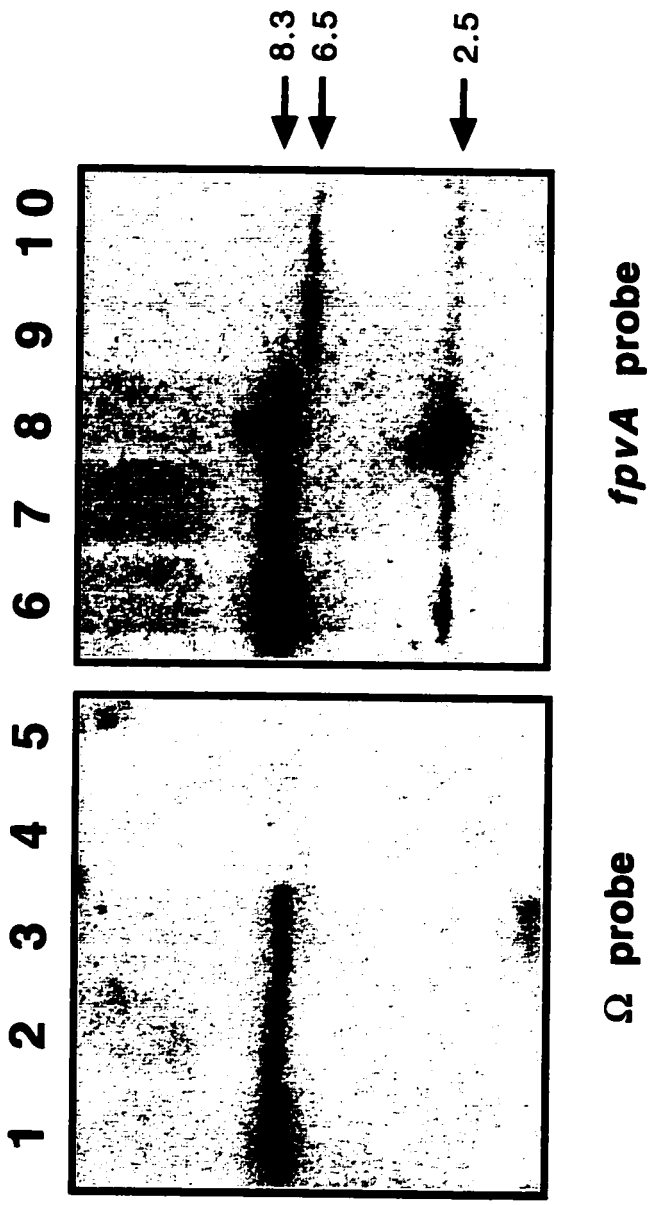
3.6 Construction of strain PA103 *fpvA::Ω*

A knockout of *fpvA* in wild type PA103 was constructed (see Figure 21 caption) to confirm the effect of an *fpvA* mutation on toxin production. To this end, plasmid pBK CSΩ, containing a 1.3 kb fragment of *fpvA* with an Ω fragment (containing transcriptional and translational stop signals), was electroporated into strain PA103 and recombinants were selected on LB agar containing 500 μg/mL spectinomycin.

Southern blot analysis of *Sma*I-digested genomic DNA from three recombinant strains with an Ω-specific and an *fpvA*-specific probe confirmed that the *fpvA::Ω* fragment had recombined with the chromosomal *fpvA* gene (Figure 21). *Sma*I does not cut within the Ω fragment. In all three recombinants, the same size bands hybridized with both probes, indicating that the *fpvA* genes in these strains also contained the Ω fragment. As expected, neither PA103 nor PAO1 contained a band hybridizing to the Ω probe. All of the bands hybridizing to the *fpvA* probe had increased by the size of the Ω fragment relative to the hybridizing bands in PA103 and PAO1. This confirmed that the resultant strain PA103 *fpvA::Ω* had an interruption of the *fpvA* gene, presumably at leucine 502 of the mature FpvA protein as predicted by the construction pBK CSΩ. The structure of the Ω mutant is diagrammed in Figure 17b.

Figure 21. Southern blot analysis of PA103 *fpvA::Ω* with Ω and *fpvA*-specific probes.

To generate an *fpvA* knockout mutant, plasmid pBK CSΩ was first constructed. To this end, plasmid pBK C86 was digested with *Pst*I and the resultant 7.5 kb fragment was ligated to a 2.6 kb *sacB Pst*I fragment from pMH1701, giving plasmid pBK CS. This essentially replaced the Tn*5*-B61 moiety of pBK C86 with the *sacB* gene from pMH1701, leaving the *fpvA* portion of pBK C86 intact. pBK CS was digested with *Xmn*I and ligated to a 1.9 kb *Bam*HI Ω fragment from pHP45Ω, in the presence of *Bgl* II linkers, to generate plasmid pBK CSΩ. This suicide plasmid was electroporated into *P. aeruginosa* PA103 and homologous recombinants were selected on spectinomycin agar plates. This strain was termed PA103 *fpvA::Ω*. Genomic DNA was prepared from *P. aeruginosa* strains PA103, PA103 *fpvA::Ω* and PAO1. After digestion with *Sma*I, DNA was electrophoresed on a 1.0% agarose gel and transferred to a nylon membrane. The membrane was hybridized with either a 1.9 kb Ω-specific *Bam*HI fragment from pHP45Ω (lanes 1-5) or a 750 bp *fpvA*-specific*Eco*RI/*Kpn*I probe derived from pBK C86 (lanes 6-10). Numbers indicate the sizes in kilobase pairs of the hybridizing DNA bands. Lanes 1-3 and 6-8: triplicate PA103 *fpvA::Ω* strains; lanes 4 and 9: PA103; lanes 5 and 10: PAO1.

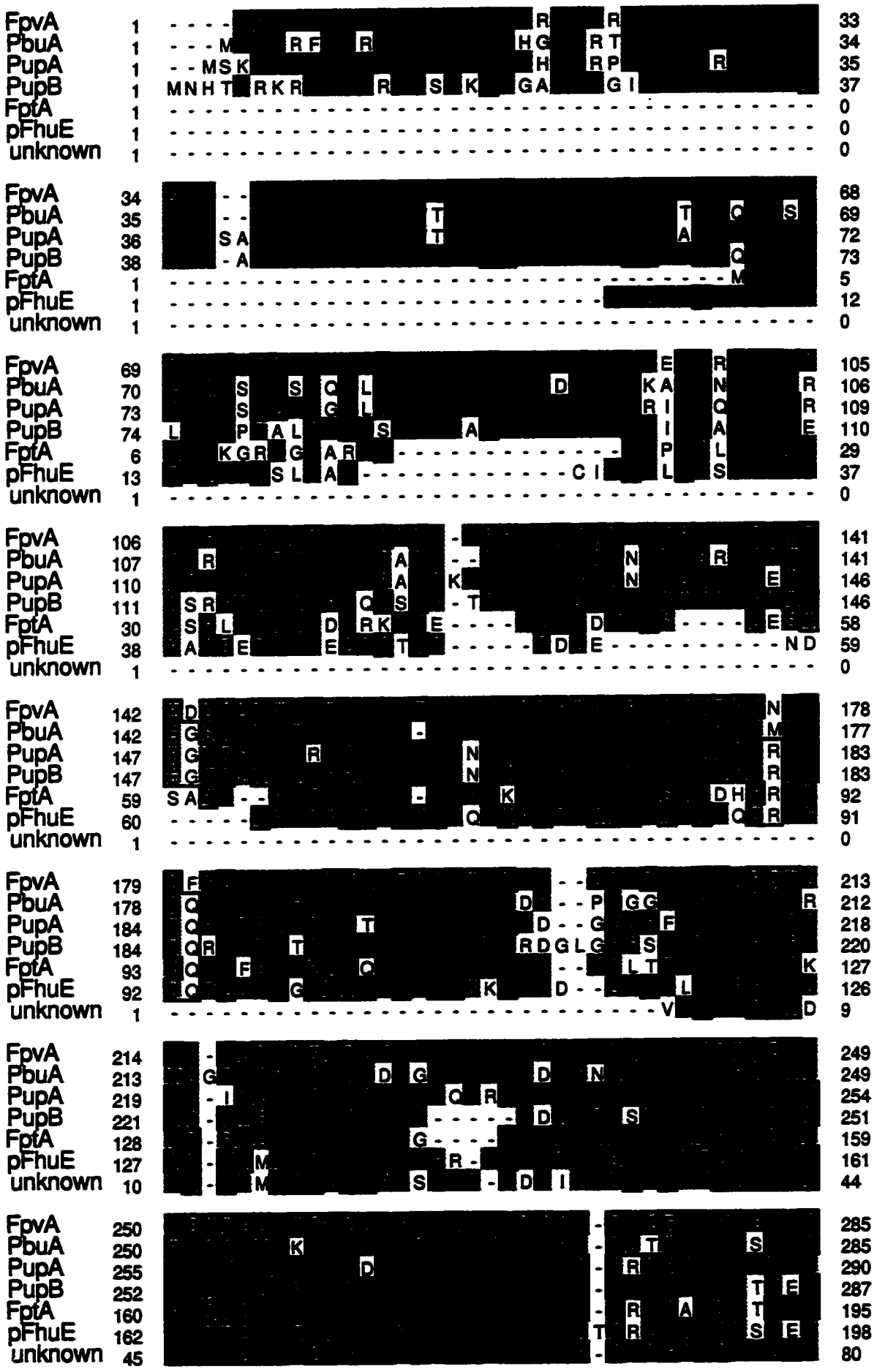

8.3
6.5
2.5
1 2 3 4 5
6 7 8 9 10
Ω probe
fpvA probe

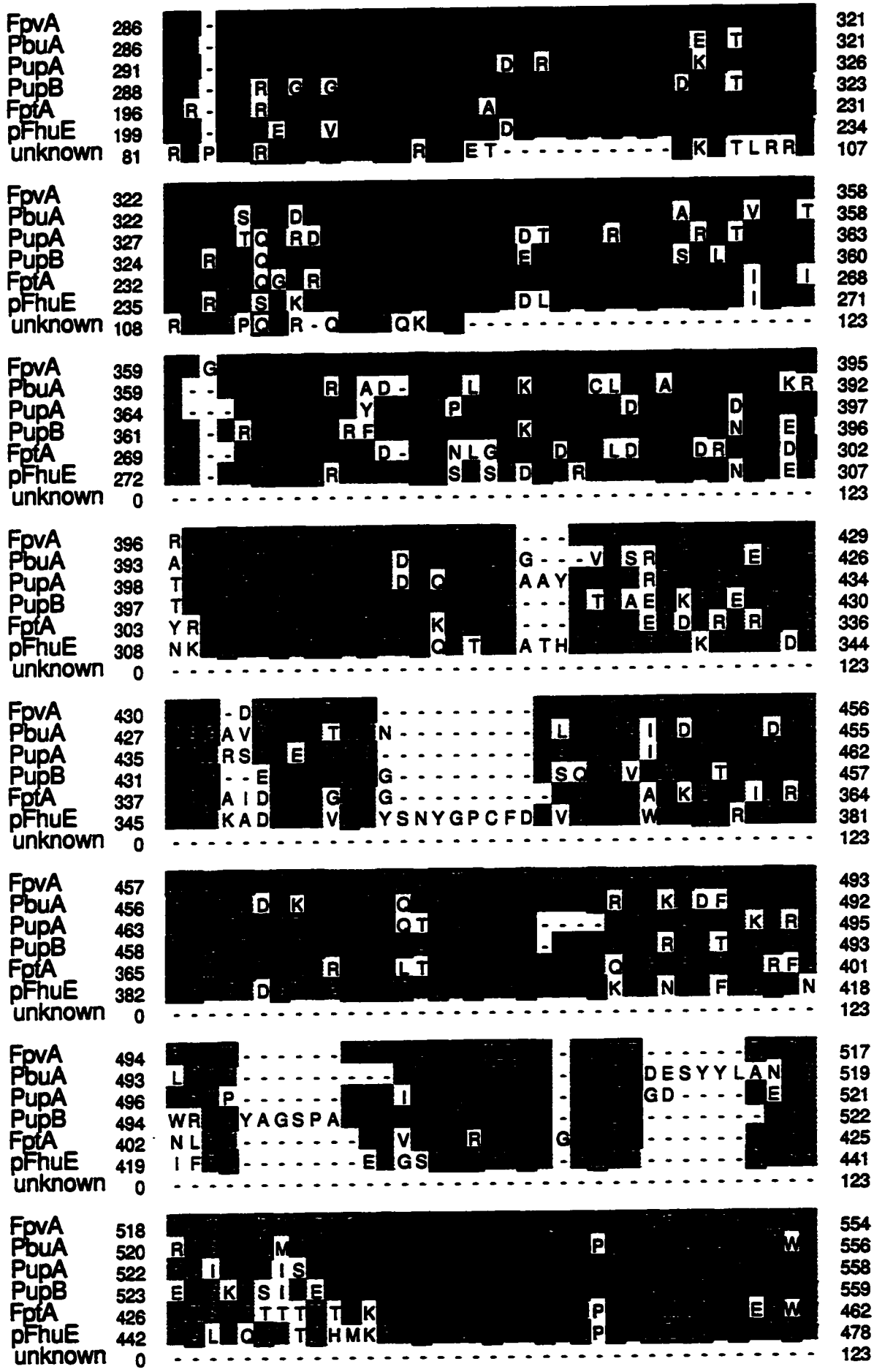
3.7 Cloning of an *fpvA* homologue

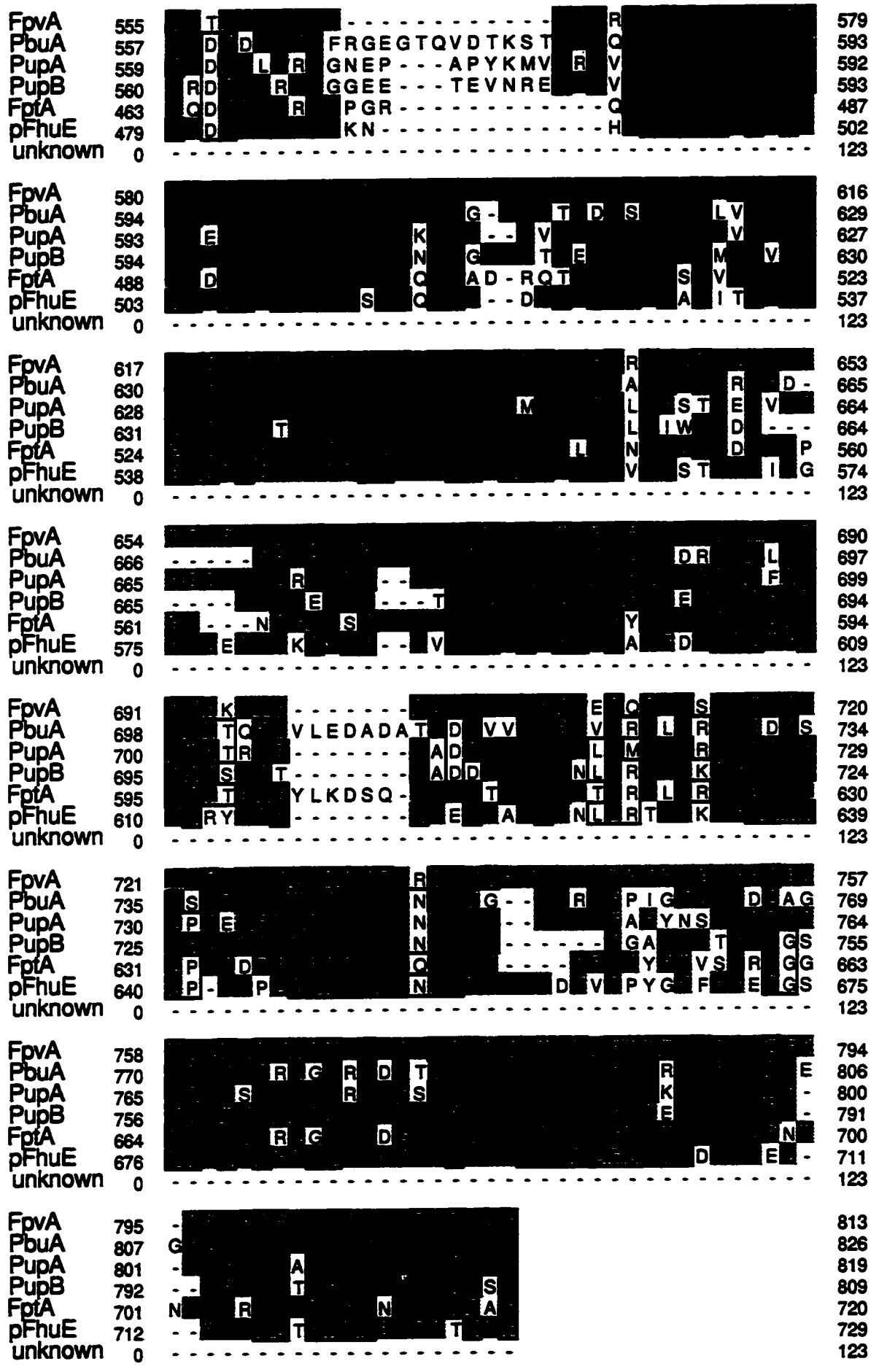
The pBK C86 clone contained a 1.3 kb insert of *fpvA* DNA, in addition to the transposon sequence. From this insert, a 750 bp *Eco*RI-*Kpn*I probe was prepared, and wild type PA103 genomic DNA was subjected to Southern blot analysis. Two bands hybridized - the expected 6.5 kb *Sma*I *fpvA* band as well as a smaller, 2.6 kb band (Figure 21). Interestingly, this band was also present in strain PAO1. A lambda Zap express library was constructed from genomic wild type PA103 DNA and screened with the *fpvA* probe in an attempt to clone out the wild type PA103 *fpvA* gene; however, only a clone of the 2.6 kb fragment could be obtained. This clone (pbK Pup) was partially sequenced, and BLAST analysis revealed that it was homologous to *fpvA*, but not identical. A protein alignment of this partial sequence with FpvA and other siderophore receptors is illustrated in Figure 22.

Figure 22. Protein alignment of several siderophore receptors from *Pseudomonas* and *E. coli* with partial sequence from pBK Pup.

pBK Pup was cloned from a *Sma*I genebank in Lambda Zap Express, where the inserted fragment hybridizes with an *fpvA*-specific 750 bp *Eco*RI/*Kpn*I probe. Partial sequencing and BLAST analysis of the sequence revealed it was probably a siderophore receptor. A hypothetical translation of the partial sequence aligns (using the Clustal W algorithm, Thompson *et al.*, 1994) with a high degree of homology to other siderophore receptors. Homology, indicated by the shaded areas, was identified through the GES algorythm, explained by Riek *et al.* (1995). Boxed amino acids share identity. All sequences were retrieved from the Genbank database. FpvA - ferripyoverdine receptor from *P. aeruginosa* PAO1 (Poole *et al.*, 1993); PbuA - *Pseudomonas sp.* ferric pseudobactin M114 receptor protein (Morris *et al.*, 1994); PupA - *P. putida* pseudobactin uptake protein (Bitter *et al.*, 1994); PupB - *P. putida* ferric-pseudobactin receptor (Koster *et al.*, 1993); FptA - *P. aeruginosa* PAO Fe(III)-pyochelin receptor (Ankenbauer and Quan, 1994); FhuE - *E. coli* outer-membrane receptor precursor protein for Fe(III)-coprogen and Fe(III)-rhodotorulic acid (Sauer *et al.*, 1990); unknown - translation of partial sequence from pBK Pup.


FpvA 1 33
PbuA 1 34
PupA 1 35
PupB 1 37
FptA 1 0
pFhuE 1 0
unknown 1 0
FpvA 34 68
PbuA 35 69
PupA 36 72
PupB 38 73
FptA 1 5
pFhuE 1 12
unknown 1 0
FpvA 69 105
PbuA 70 106
PupA 73 109
PupB 74 110
FptA 6 29
pFhuE 13 37
unknown 1 0
FpvA 106 141
PbuA 107 141
PupA 110 146
PupB 111 146
FptA 30 58
pFhuE 38 59
unknown 1 0
FpvA 142 178
PbuA 142 177
PupA 147 183
PupB 147 183
FptA 59 92
pFhuE 60 91
unknown 1 0
FpvA 179 213
PbuA 178 212
PupA 184 218
PupB 184 220
FptA 93 127
pFhuE 92 126
unknown 1 9
FpvA 214 249
PbuA 213 249
PupA 219 254
PupB 221 251
FptA 128 159
pFhuE 127 161
unknown 10 44
FpvA 250 285
PbuA 250 285
PupA 255 290
PupB 252 287
FptA 160 195
pFhuE 162 198
unknown 45 80


FpvA 286 321
PbuA 286 321
PupA 291 326
PupB 288 323
FptA 196 231
pFhuE 199 234
unknown 81 107
FpvA 322 358
PbuA 322 358
PupA 327 363
PupB 324 360
FptA 232 268
pFhuE 235 271
unknown 108 123
FpvA 359 395
PbuA 359 392
PupA 364 397
PupB 361 396
FptA 269 302
pFhuE 272 307
unknown 0 123
FpvA 396 429
PbuA 393 426
PupA 398 434
PupB 397 430
FptA 303 336
pFhuE 308 344
unknown 0 123
FpvA 430 456
PbuA 427 455
PupA 435 462
PupB 431 457
FptA 337 364
pFhuE 345 381
unknown 0 123
FpvA 457 493
PbuA 456 492
PupA 463 495
PupB 458 493
FptA 365 401
pFhuE 382 418
unknown 0 123
FpvA 494 517
PbuA 493 519
PupA 496 521
PupB 494 522
FptA 402 425
pFhuE 419 441
unknown 0 123
FpvA 518 554
PbuA 520 556
PupA 522 558
PupB 523 559
FptA 426 462
pFhuE 442 478
unknown 0 123


FpvA 555 579
PbuA 557 593
PupA 559 592
PupB 560 593
FptA 463 487
pFhuE 479 502
unknown 0 123
FpvA 580 616
PbuA 594 629
PupA 593 627
PupB 594 630
FptA 488 523
pFhuE 503 537
unknown 0 123
FpvA 617 653
PbuA 630 665
PupA 628 664
PupB 631 664
FptA 524 560
pFhuE 538 574
unknown 0 123
FpvA 654 690
PbuA 666 697
PupA 665 699
PupB 665 694
FptA 561 594
pFhuE 575 609
unknown 0 123
FpvA 691 720
PbuA 698 734
PupA 700 729
PupB 695 724
FptA 595 630
pFhuE 610 639
unknown 0 123
FpvA 721 757
PbuA 735 769
PupA 730 764
PupB 725 755
FptA 631 663
pFhuE 640 675
unknown 0 123
FpvA 758 794
PbuA 770 806
PupA 765 800
PupB 756 791
FptA 664 700
pFhuE 676 711
unknown 0 123
FpvA 795 813
PbuA 807 826
PupA 801 819
PupB 792 809
FptA 701 720
pFhuE 712 729
unknown 0 123

3.8 ADPRT activity of PA103 *fpvA::Ω*

The ADPRT activity of the *fpvA* omega mutant was compared to the wild type PA103 (Figure 24). PA103 P3 C86, containing a transposon insertion at L672 of the mature FpvA protein, exhibited a higher than normal toxin activity both in low and high iron (as shown in Figure 19). Growth curves of PA103 *fpvA::Ω* are shown in Figure 23. In contrast to PA103 P3 C86, PA103 *fpvA::Ω* exhibited much lower than normal ADPRT activity, both in low and high iron conditions (Figure 24). Specifically, in high iron conditions, PA103 *fpvA::Ω* exhibited almost no toxin activity both early and late in the growth curve.

Figure 23. Growth curves of *P. aeruginosa* PA103 and PA103 *fpvA::Ω* in low and high iron conditions.

Primary cultures were grown overnight in TSBDC. Secondary cultures were inoculated into TSBDC to an OD_{540} of 0.02. Neither primary nor secondary cultures contained any antibiotic. $FeCl_3$ was supplemented to 10 μg/mL (+Fe) or not added at all (-Fe). Aliquots taken from these growth curves were used for ADPRT assays of PA103 and PA103 *fpvA::Ω* (Figure 24).

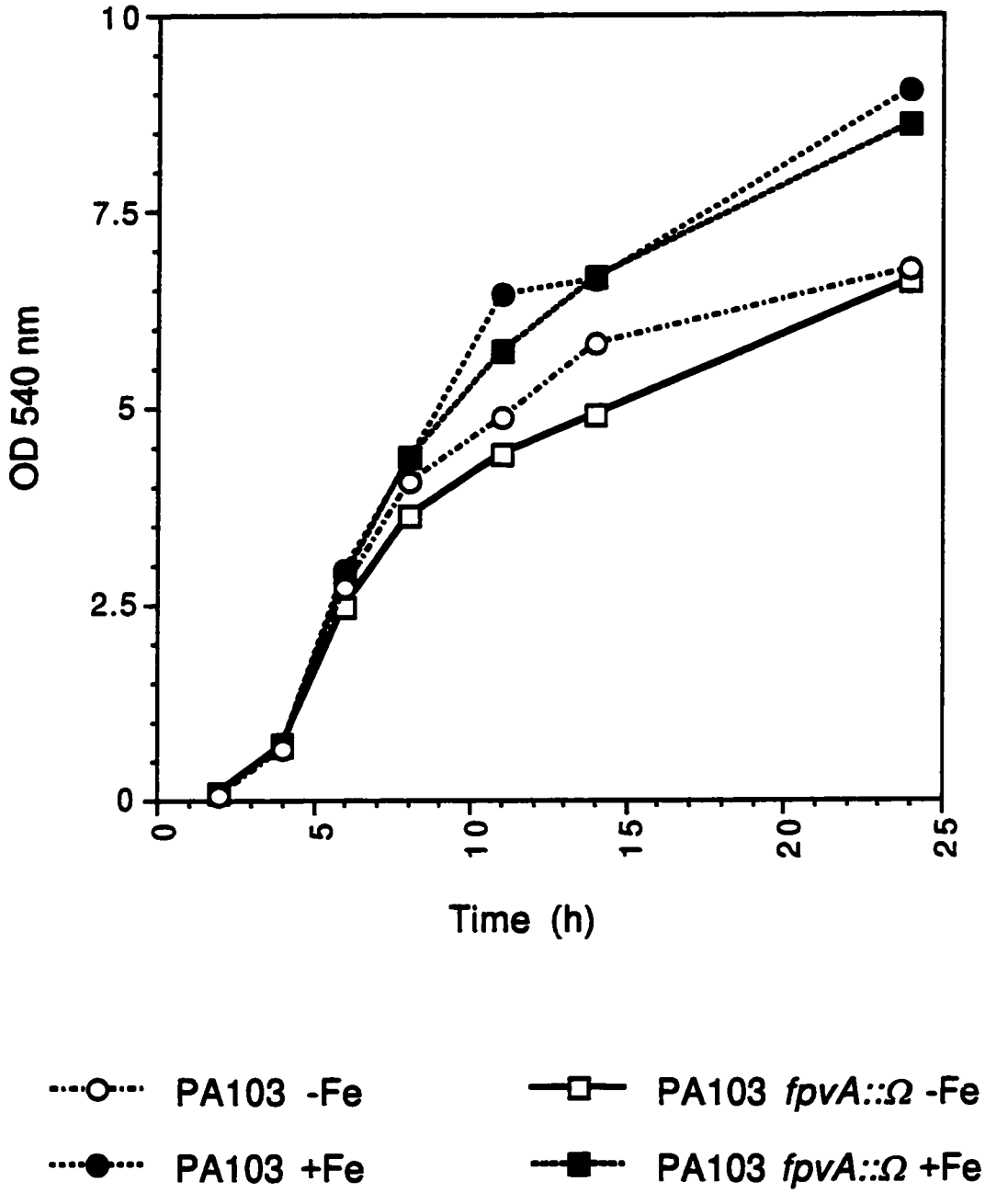
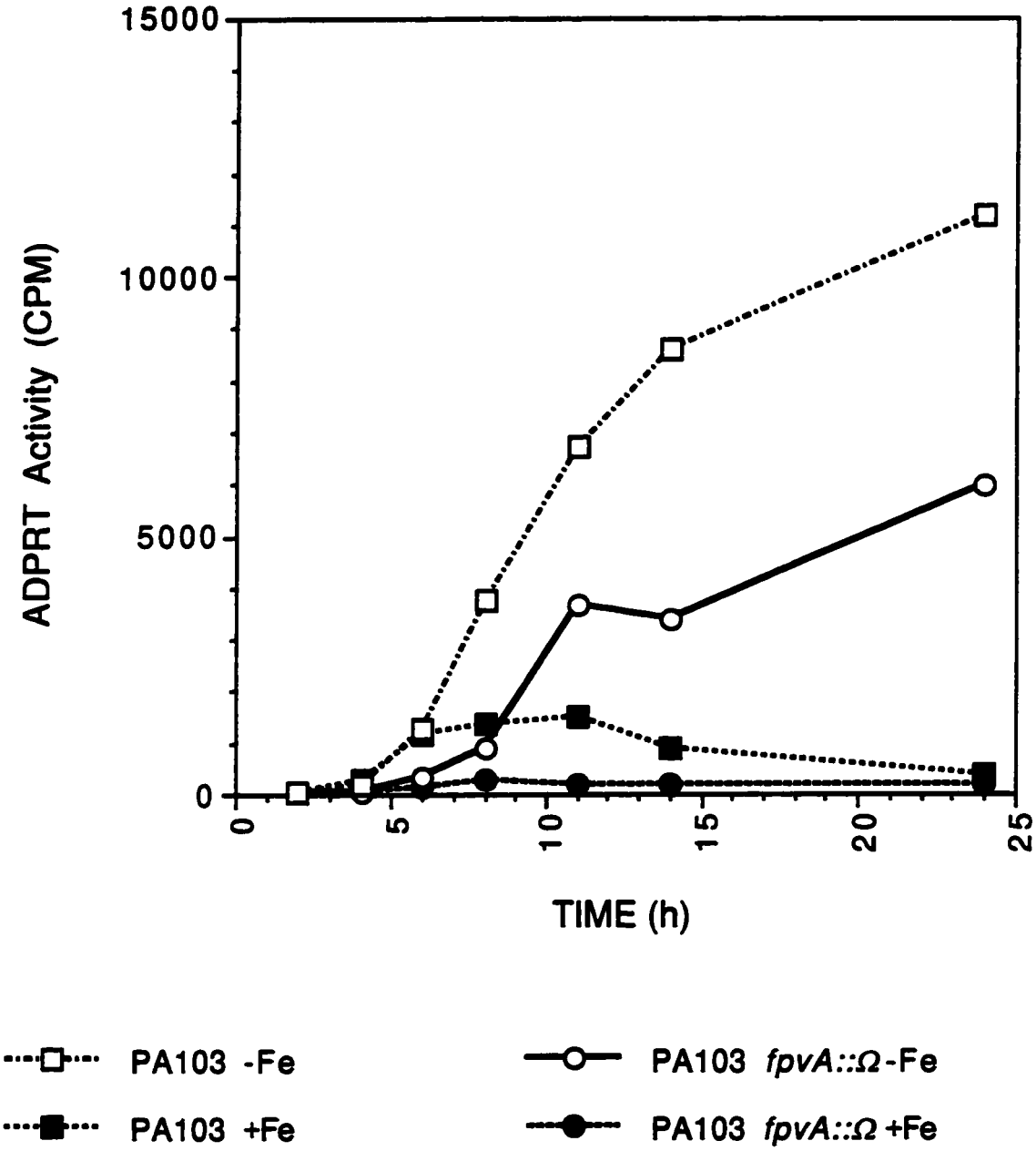

10
7.5
5
2.5
0
OD 540 nm
0
5
10
15
20
25
Time (h)
PA103 -Fe
PA103 +Fe
PA103 fpvA::Ω -Fe
PA103 fpvA::Ω +Fe

Figure 24. Effect of iron on ADPRT activity of PA103 *fpvA::Ω*.

Primary cultures were grown overnight in TSBDC containing 500 μg/mL spectinomycin. Secondary cultures were inoculated into TSBDC to an OD_{540} of 0.02. Secondary cultures did not contain antibiotic. $FeCl_3$ was supplemented to 10 μg/mL (+Fe) or not added at all (-Fe). Aliqots were removed at indicated time intervals and supernatants were frozen for storage before assaying for ADPRT activity. These trends were reproduced at least twice in subsequent experiments.


15000
10000
5000
0
ADPRT Activity (CPM)
0
5
10
15
20
25
TIME (h)
PA103 -Fe
PA103 +Fe
PA103 fpvA::Ω-Fe
PA103 fpvA::Ω +Fe

3.9 Pyoverdine production of PA103, PA103 *fpvA::Ω* and PA103 P3 C86

To see if iron regulation of pyoverdine had been affected by mutations in *fpvA*, bacteria were spotted on CAS plates. This also allowed us to see the effect (if any) of the P_{taq} promoter on the pyoverdine regulon. CAS plates indicate siderophore activity through different coloured halos surrounding the bacterial colony. Pyoverdine produces a yellow halo. The CAS plates showed that PA103 and PA103 *fpvA::Ω* both produce similar levels of pyoverdine, although part of the halo is undoubtedly attributable to pyochelin production, and that in both strains, iron regulation is unaffected (data not shown).

Pyoverdine production was measured quantitatively in supernatants of the two *fpvA* mutants as well as in wild type PA103 using a spectrophotometric assay (Table 3). The concentration of pyoverdine is proportional to the absorbance maximum observed between 403 and 409 nm (Schwyn and Neilands, 1987). The table shows that pyoverdine levels were similar between wild type PA103 and the transposon mutant, but that the *fpvA* knockout produced much less pyoverdine. Iron regulation of the pyoverdine regulon seemed unaffected.

Table 3. Effect of iron on pyoverdine activity of PA103, PA103 P3 C86 and PA103 *fpvA::Ω*.

Strain [a]	Iron Conc. [b]	λ_{max} (nm) [c]	Activity [d]
PA103	low	408	1.12±0.13
	high	-	0.00
PA103 P3 C86	low	409	1.15 ± 0.27
	high	-	0.00
PA103 *fpvA::Ω*	low	406	0.49 ± 0.17
	high	-	0.00

[a] Primary cultures were grown overnight in SMM, SMM containing 300 μg/mL carbenicillin and 50 μg/mL gentamicin, or SMM containing 500 μg/mL spectinomycin. Secondary cultures were inoculated into SMM to an OD_{540} of 0.02. Secondary cultures did not contain antibiotic. An aliquot was removed after 24 hours of growth and the supernatant was frozen for storage before being assayed for pyoverdine.

[b] In secondary cultures, $FeCl_3$ was supplemented to 10 μg/mL (high) or not added at all (low).

[c] The λ_{max} refers to the wavelength at which there is maximal absorbance between 350 and 450 nm.

[d] The activity is calculated by dividing the maximal absorbance with the OD_{540} of the culture. The error represents the standard deviation of the activity between duplicate experiments.

3.10 Complementation of PA103 *fpvA::Ω*

To confirm that the reduction of ADPRT activity seen in PA103 *fpvA::Ω* was due to the insertion mutation in *fpvA*, this strain was electroporated with a wild type copy of PAO1 *fpvA*, contained on either pUCP22 (a high copy number plasmid) or pRK415 (a low copy number plasmid). Growth curves for all complementations are depicted in Figure 25.

First, ADPRT assays of PA103 *fpvA::Ω* containing either a pRK415 plasmid control or pRK *fpvA* were compared over a growth curve in low and high iron (Figure 26). The expression of a functional FpvA protein increased ADPRT activity throughout growth, both in low and high iron conditions.

Second, the complemented PA103 *fpvA::Ω* ADPRT activities were compared to those obtained from PA103 containing the same pRK *fpvA* plasmid (Figure 27). The curves are very similar, with PA103 somewhat higher late in growth under low iron conditions. This indicates that PAO1 *fpvA* complements the mutant *fpvA* quite well.

Finally, the ADPRT activities from PA103 *fpvA::Ω* containing pRK *fpvA* were compared to those from the same strain containing a high copy number plasmid, pUCP *fpvA* (Figure 28). Here, one can see that in low iron conditions, overexpression of FpvA has caused a large increase in the amount of ADPRT activity.

Note that experimental data obtained from two separate experiments is presented on the same graph (Figures 26 and 28). While this is not normally practical, identical batches of all

reagents were used in both growth curves, making these comparisons feasible.

Figure 25. Growth curves of *P. aeruginosa* PA103 and PA103 *fpvA::Ω* complemented with pRK415, pRK*fpvA*, or pUCP*fpvA* in low and high iron conditions.

Primary cultures were grown overnight in TSBDC. Secondary cultures were inoculated into TSBDC to an OD_{540} of 0.02. Secondary cultures did not contain antibiotic. $FeCl_3$ was supplemented to 10 μg/mL (+Fe) or not added at all (-Fe). Aliquots taken from these growth curves were used in subsequent ADPRT assays (Figures 26, 27 and 28).

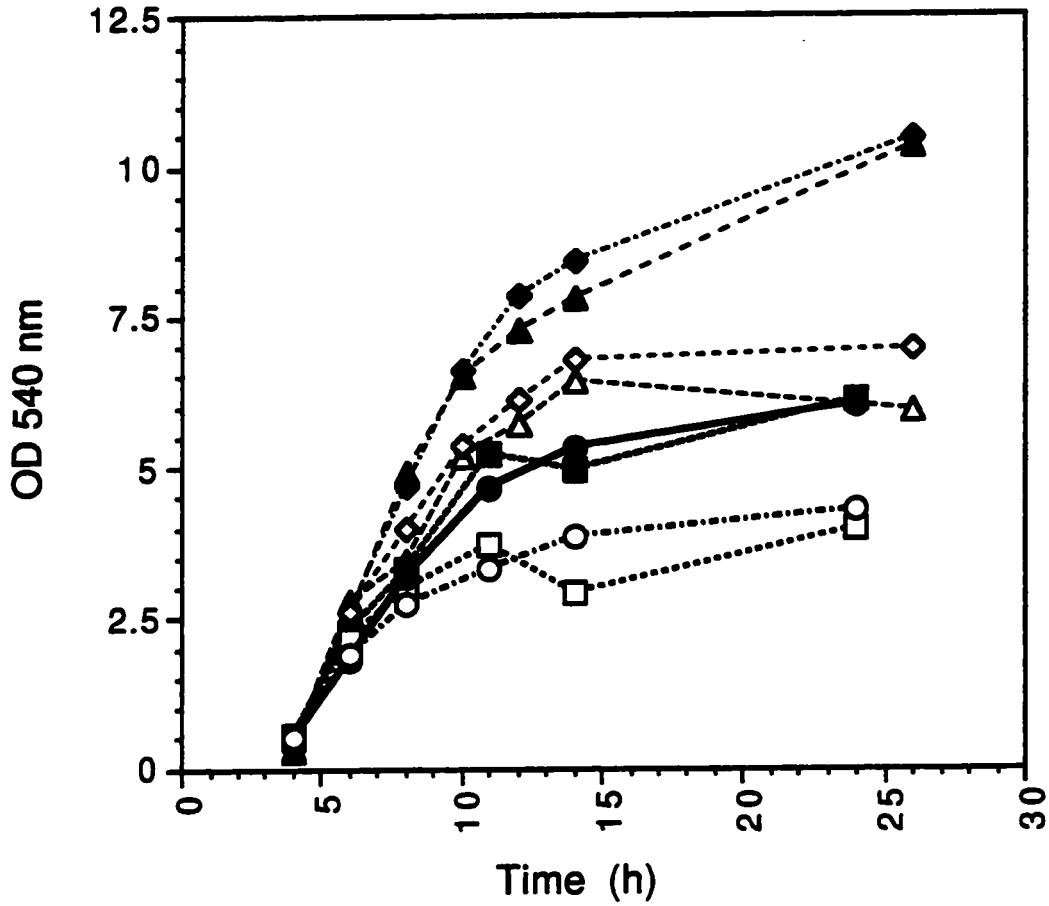

12.5
10
7.5
5
2.5
0
OD 540 nm
0
5
10
15
20
25
30
Time (h)
PA103 (pRKfpvA) -Fe
PA103 (pRKfpvA) +Fe
PA103 fpvA::Ω (pRKfpvA) -Fe
PA103 fpvA::Ω (pRKfpvA) +Fe
PA103 fpvA::Ω (pUCPfpvA) -Fe
PA103 fpvA::Ω (pUCPfpvA) +Fe
PA103 fpvA::Ω (pRK415) -Fe
PA103 fpvA::Ω (pRK415) +Fe

Figure 26. Effect of iron on ADPRT activity of PA103 *fpvA::Ω* complemented with either pRK415 or pRK *fpvA*

Primary cultures were grown overnight in TSBDC containing 100 μg/mL tetracycline (pRK *fpvA*). Secondary cultures were inoculated into TSBDC to an OD_{540} of 0.02. Secondary cultures did not contain antibiotic. $FeCl_3$ was supplemented to 10 μg/mL or not added at all. Aliqots were removed at indicated time intervals and supernatants were frozen for storage before assaying for ADPRT activity.

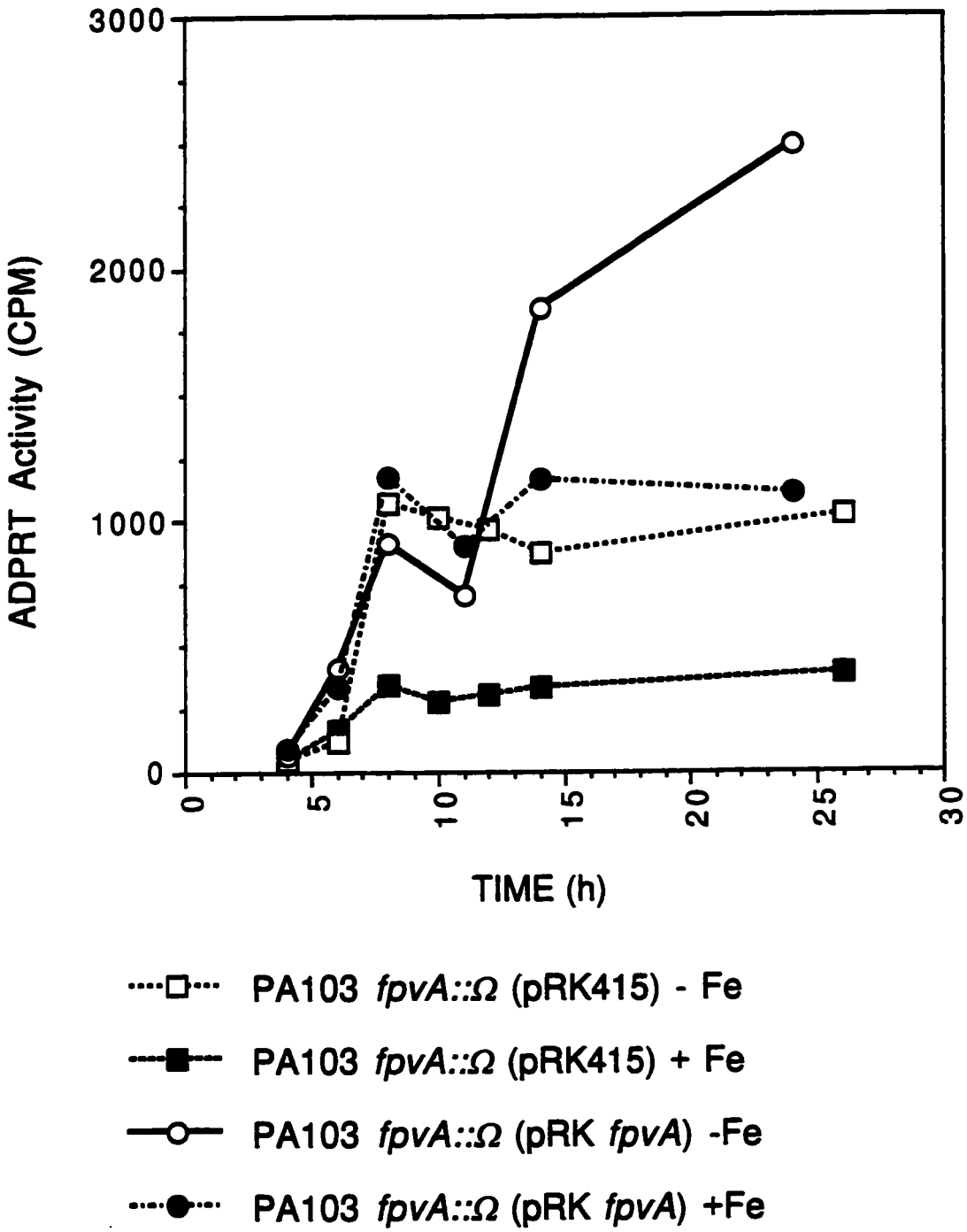

3000
2000
1000
0
ADPRT Activity (CPM)
0
5
10
15
20
25
30
TIME (h)
PA103 fpvA::Ω (pRK415) - Fe
PA103 fpvA::Ω (pRK415) + Fe
PA103 fpvA::Ω (pRK fpvA) -Fe
PA103 fpvA::Ω (pRK fpvA) +Fe

Figure 27. Effect of iron on ADPRT activity of PA103 and PA103 *fpvA::Ω* complemented with pRK *fpvA*.

Primary cultures were grown overnight in TSBDC containing 100 μg/mL tetracycline (pRK *fpvA*). Secondary cultures were inoculated into TSBDC to an OD_{540} of 0.02. Secondary cultures did not contain antibiotic. $FeCl_3$ was supplemented to 10 μg/mL or not added at all. Aliqots were removed at indicated time intervals and supernatants were frozen for storage before assaying for ADPRT activity.

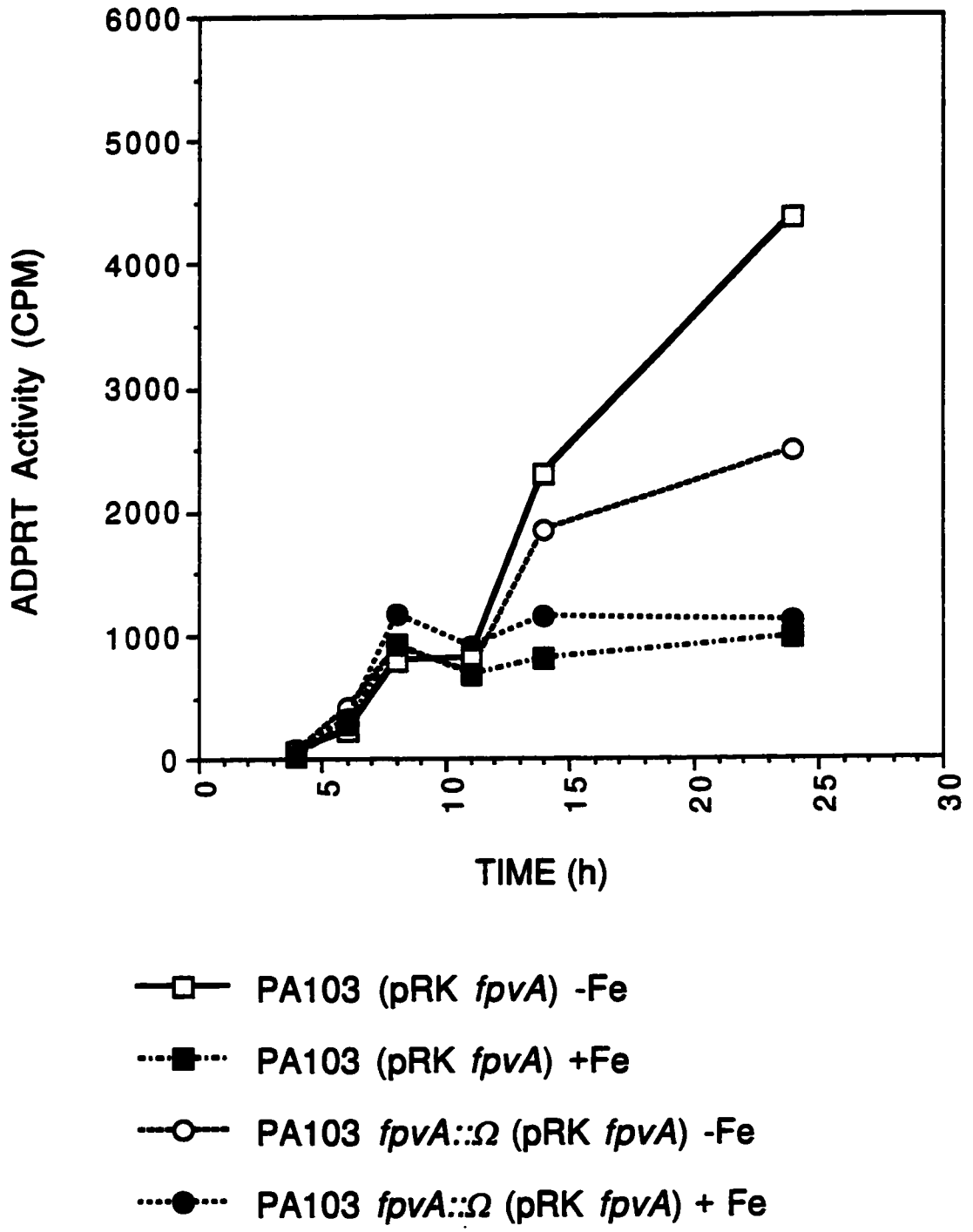
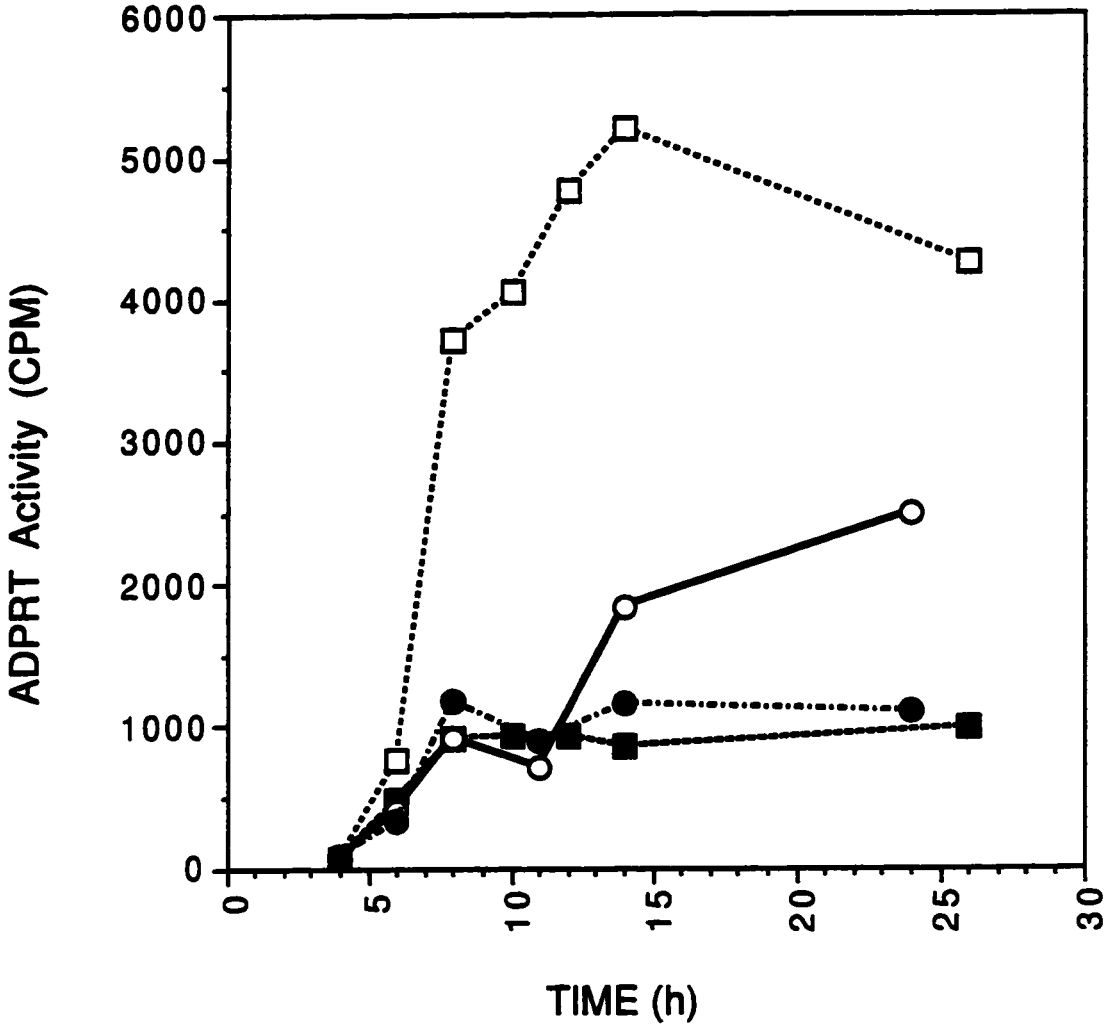

6000
5000
4000
3000
2000
1000
0
ADPRT Activity (CPM)
0
5
10
15
20
25
30
TIME (h)
PA103 (pRK fpvA) -Fe
PA103 (pRK fpvA) +Fe
PA103 fpvA::Ω (pRK fpvA) -Fe
PA103 fpvA::Ω (pRK fpvA) + Fe

Figure 28. Effect of iron on ADPRT activity of PA103 *fpvA::Ω* complemented with either pRK *fpvA* or pUCP *fpvA*.

Primary cultures were grown overnight in TSBDC containing 100 μg/mL tetracycline (pRK *fpvA*) or 300 μg/mL carbenicillin (pUCP *fpvA*). Secondary cultures were inoculated into TSBDC to an OD_{540} of 0.02. Secondary cultures did not contain antibiotic. $FeCl_3$ was supplemented to 10 μg/mL or not added at all. Aliqots were removed at indicated time intervals and supernatants were frozen for storage before assaying for ADPRT activity.


6000
5000
4000
3000
2000
1000
0
ADPRT Activity (CPM)
0
5
10
15
20
25
30
TIME (h)
PA103 fpvA::Ω (pUCP fpvA) -Fe
PA103 fpvA::Ω (pUCP fpvA) +Fe
PA103 fpvA::Ω (pRK fpvA) -Fe
PA103 fpvA::Ω (pRK fpvA) +Fe

Chapter 4 Discussion

4.1 Construction of *regAB* reporter strains in *P. aeruginosa* PA103

Two strains were constructed from *P. aeruginosa* PA103 to facilitate measurement of *regAB* promoter activity. Southern blot analysis (Figure 5) indicated that the *regAB* locus in both recombinant strains appeared to contain two *regA-lacZ* fusions (Figure 6). This could have arisen from two cross-over events, as depicted in Figures 29 and 30. This is plausible given the high efficiency of electroporation, which leads to a high number of plasmids per cell. Both strains seemed to be stable in this conformation, even in the absence of any antibiotic pressure. Note also that the Southern blots were done long after the strains had been in routine use. Why resolution to a single fusion did not occur is unknown, but there seemed to be some sort of pressure to maintain these particular gene configurations.

In both recombinant strains, expression from the fusions closely paralleled that from the wild type *regAB* locus. One strain, PA103 P3, reports solely on activity from the P1 promoter, while the other strain, PA103 N3, reports on the combined activity of the P1 and P2 promoters. This is evident from the ß-galactosidase activities of these strains shown in Figure 8. PA103 P3 shows slightly iron regulated activity very early in growth, as would be expected from studies by Storey *et al.* (1990) which showed that P1 activity is limited to early parts of the growth curve. In Figure 11b and Figure 19b, where ß-galactosidase activities of PA103 P3 are

also shown, there is very little P1 activity above the background level. Since the P1 activity is quite low in this region, more data points might give a better signal-to-noise ratio and more reproducible trends. Importantly though, we could easily see activation of this promoter through mutations elsewhere on the genome.

The P1 activity is probably also affected (lowered) by the deletion of all sequences immediately downstream from its transcriptional start site (T1). This includes the P2 promoter and any sites where factors may be binding to control P2 promoter activity, which likely have a simultaneous effect on P1 promoter activity. Since P1 promoter activity is lower than expected in the absence of the P2 promoter, this suggests that there is some factor which, while inhibiting P2 promoter activity, concurrently activates the P1 promoter. Removal of the factor could alleviate repression at the P2 promoter, while transcription from the P2 promoter itself would inhibit P1 transcription. This hypothesis agrees with the observed mutual exclusion of P1 and P2 promoter activity (Storey *et al.*, 1990).

Such a factor would bind between the P1 and P2 promoters. This places it in a position relative to the P2 promoter known among *E. coli* promoters to be a common binding site for activators (Gralla, 1991). At the same time, relative to the P1 promoter, this position is common for repressors. This contradicts the finding that removal of this putative P1 repression site causes a decrease in P1 promoter activity, when one would expect an increase. Perhaps repressor

locations can be different in *P. aeruginosa* compared to those in *E coli.*

When ADPRT activities are compared between the recombinant and the parental strains in the context of multiple *regAB* promoters, additional hints about their regulation are discerned. In Figure 9, parts A and B, both recombinant strains show between 50% and 65% of the activity seen by the parental PA103 strain (not shown) throughout a growth curve. This can also be seen in Figure 12. While the trends between the recombinant and parental strains were similar, the consistent decrease in ADPRT activity offers insight into *regAB* regulation. In the PA103 N3 construct (Figure 6b), there are 3 copies of the P1 and P2 promoters, in contrast to the normal single-copy situation. The decrease in activity could be explained by a saturation effect on the pool of activator molecules. The extra copies of the promoters could utilize a portion of the activator pool normally used to drive the *regAB* promoters, thereby decreasing P1 and P2 promoter activity and consequently ADPRT activity.

Likewise, in the PA103 P3 construct (Figure 6c), there are 3 copies of the P1 promoter, but only one copy of the P2 promoter. A similar decrease in activity is observed in this strain, implying that the activator is working on sequences upstream of the T1 transcription initiation site, and not sequences attributed to regulation of the P2 promoter. But if an activator binds upstream of the T1 initiation site, why does deletion of downstream sequences decrease P1 promoter activity? It would seem that the coordinate regulation of the P1 and P2 promoters is tightly coupled, such that segregation alters their regulation.

One might also notice that there are significant differences in the ADPRT activities between experiments. This is due to the many variables inherent to the ADPRT assay. These are accommodated through controls, but comparisons across experiments can be difficult, and no normalization is performed. Factors affecting the results obtained by the ADPRT assay are the medium, which after dialysis contains significantly different nutrient concentrations among batches, the EF-2 preparation, the activity of the isotope, and the growth rate of the strains. In all experiments, however, the trend is reproducible.

One particular discrepancy that bears mention is the sudden jump in ADPRT activity seen late in the growth of PA103 N3 under high iron conditions (Figure 9a), where one would not expect high activity. This may simply be due to experimental error, as activities shown in Figure 19a and seen in other experiments did not have a late, high ADPRT activity in high iron conditions.

Figure 29. Possible sequence of recombinatory events producing the PA103 N3 *regAB* locus.

Initially, a single cross-over event between pNRL and the *regAB* P1 and P2 promoters produced a single chromosomal fusion (1). As a result of the high efficiency of electroporation into the cells, a second cross-over event, probably in the *lacZ* gene, produced a double fusion (2). A - *regA*, B - *regB*, E - *Eco*RI, ORI - origin of replication.

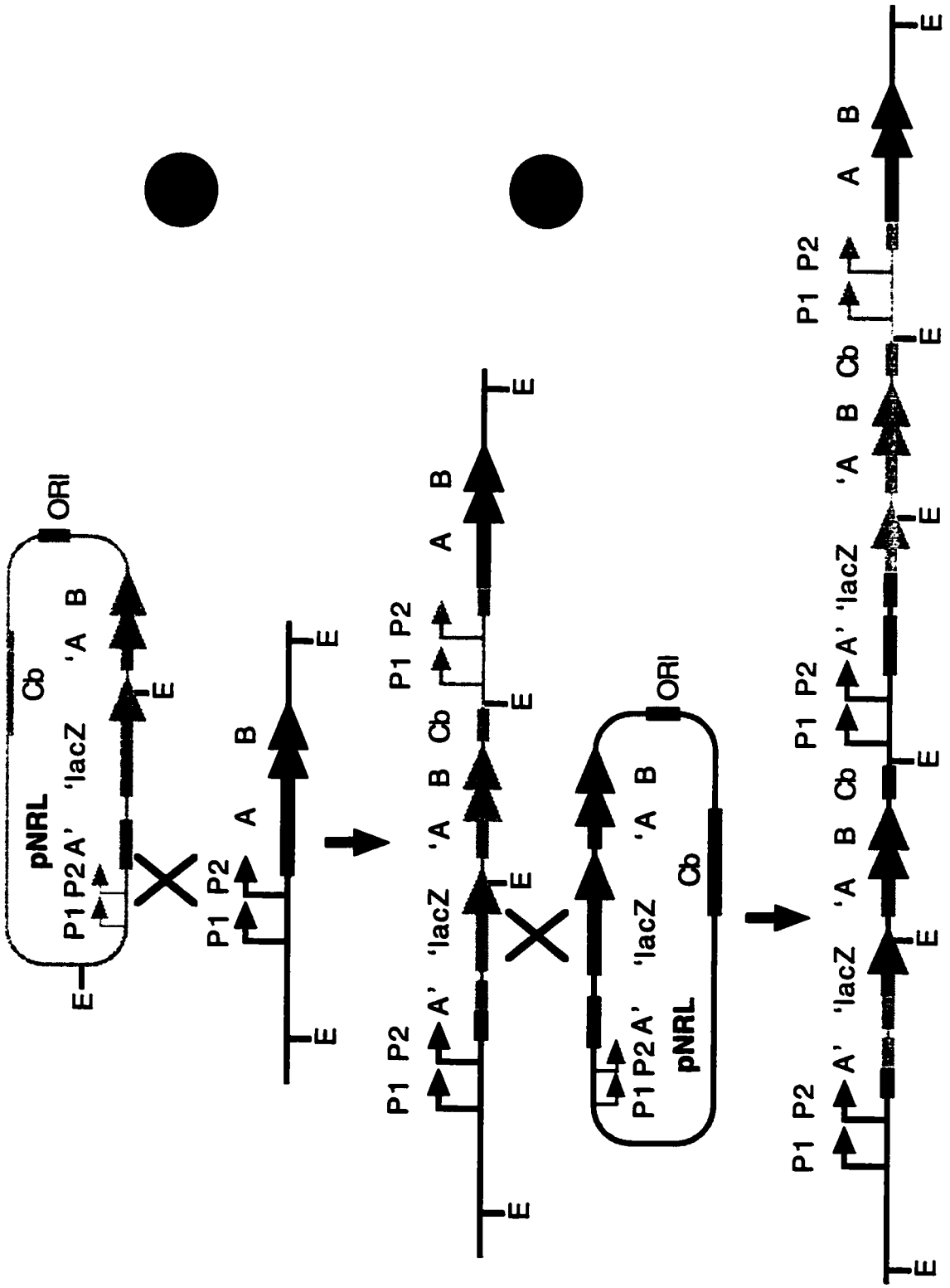
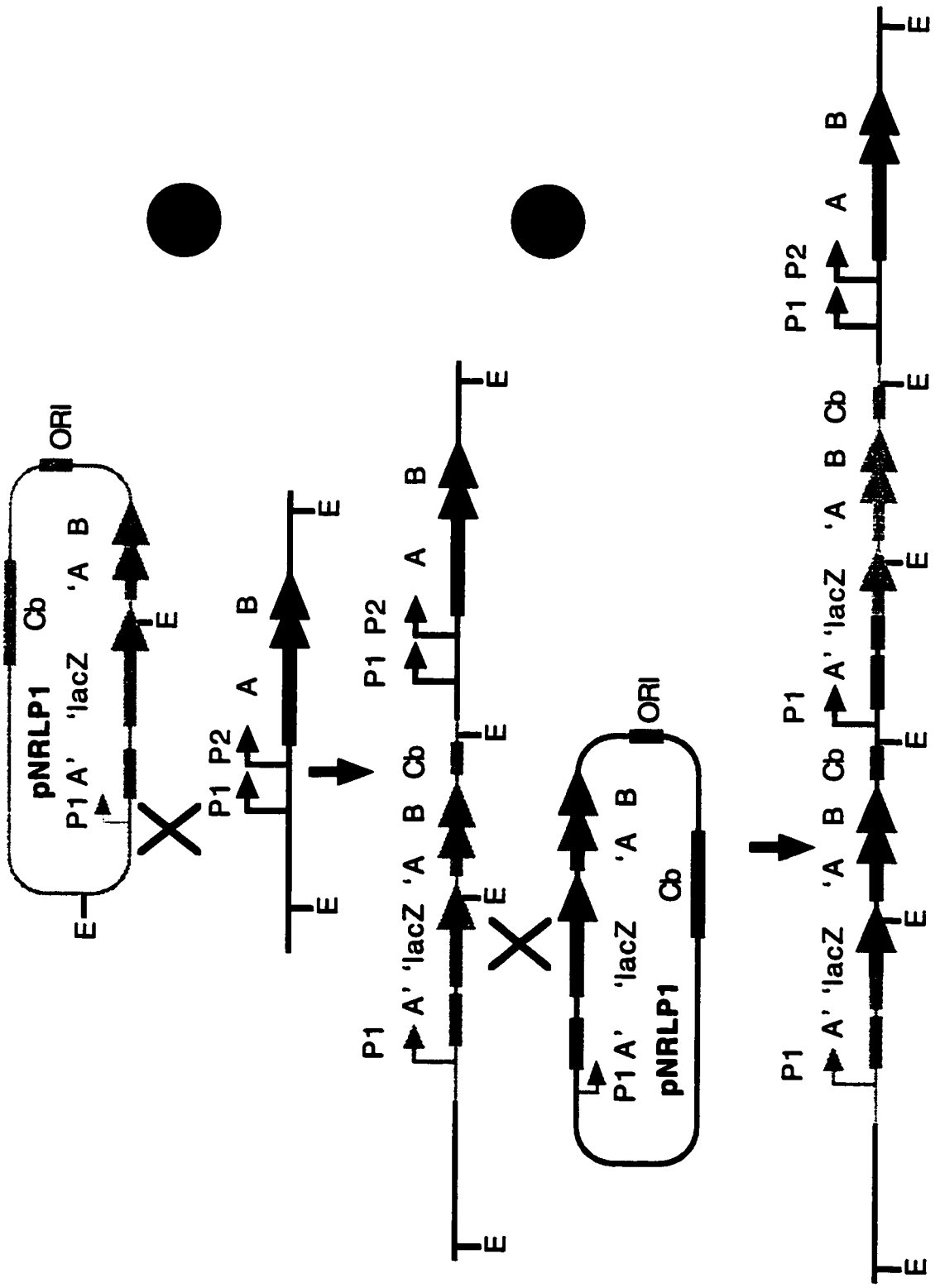

ORI
Cb
pNRL
'lacZ
'A
B
A
P1 P2
A'
E

Figure 30. Possible sequence of recombinatory events producing the PA103 P3 *regAB* locus.

Initially, a single cross-over event between pNRLP1 and the *regAB* P1 promoter produced a single chromosomal fusion (1). As a result of the high efficiency of electroporation into the cells, a second cross-over event, probably in the *lacZ* gene, produced a double fusion (2). A - *regA*, B - *regB*, E - *Eco*RI, ORI - origin of replication.


pNRLP1
Cb
P1 A'
'lacZ
'A
B
E
ORI
P1 P2
A
P1
A' 'lacZ
'A B Cb
P1 A' 'lacZ 'A B Cb

4.2 Generation of transposon insertion mutants affecting *regAB* transcription

Transposition into the recombinant *regAB* reporter strains was employed to alter regulation at the *regAB* locus. Transposon mutants altered in ß-galactosidase activity were relatively frequent, occurring more than once in every 100 strains screened. The high number of putative mutations may have been due to the difficulty in controlling strain growth, such that strains were assayed at varying stages of growth, or it may be because of the myriad factors involved in the regulation of *regAB*, many of which are interconnected by several networks of regulation. Other strains which appeared to have altered ß-galactosidase were discarded due to differences in growth. Only if a strain had achieved a certain OD_{600} range were its ß-galactosidase activities considered reliable. Mutants which were at least two standard deviations distant from the norm were chosen for further study. Initially, this consisted of 12 strains. These strains were classified into 6 groups based on their ß-galactosidase activity, with 3 from each parental strain. ADPRT assays were used to characterize their phenotypes further.

4.2.1 PA103 N3 transposon insertion mutants

Mutants derived from PA103 N3 displayed three phenotypes: high late ß-galactosidase activity, high early ß-galactosidase activity, or low ß-galactosidase activity (Figure 11a). ADPRT activities (Figure 12a) corroborated the latter two phenotypes, but the high late ß-galactosidase activity of strain PA103 P3 D81

contradicted the normal ADPRT activity. It is likely that this strain contained a transposon insertion close to one of the *regA-lacZ* fusions, such that the *tac* promoter upregulated *lacZ* transcription without altering *regAB* transcription.

The genetic defect in mutant PA103 N3 A156, with the high early ß-galactosidase and ADPRT activity, remains uncloned. This phenotype may also be a result of a proximal *tac* promoter that is not only upregulating *lacZ* transcription but also *regAB* transcription. This scenario is somehat unlikely, as Southern blot analysis of the region with a *lacZ* probe demonstrated no insertions into *lacZ* sequences (Figure 14). In addition, this scenario would necessitate a transcript covering the entire *regA-lacZ* fusion, the intervening sequences, and the wild type copy of *regAB* (about 10,000 nt). Thus, it is highly probable that this phenotype represents an insertion in either a repressor of the *regAB* operon, or an insertion such that the transcription of an activator of the *regAB* operon has been elevated. Since the effect is early, it is most likely a P1 promoter effect. We cannot, however, rule out the possibility that the P2 promoter has been activated prematurely. In the absence of a clone, it is difficult to discern between the two possibilities. Reconstructing this mutation in strain PA103 P3 might illuminate the situation.

The other PA103 N3 mutant, PA103 N3 B17, is interesting in that both promoters seem to be down-regulated. Since the effect is seen at both of the *regA-lacZ* fusions and through the *regAB* locus, it is extremely unlikely that an insertion into this locus has knocked out all three sets of promoters. There is the remote possibility that

the *tac* promoter is producing antisense mRNA through this entire locus, and inhibiting both ADPRT and ß-galactosidase activity by either the act of transcription or through hybridization to the sense strands, but this would necessitate an even longer transcript than mentioned above.

A more feasible theory involves regulators. It has already been shown that transcription of the P1 and P2 promoters is mutually exclusive, suggesting their tightly linked and coordinated regulation. It is not surprising that an effect on one would also affect the other. Since we still see iron regulation in PA103 N3 B17 (Figure 20), it seems most likely that the effect is on the P2 promoter. There is very little, if any, P1 promoter activity. The P2 promoter activity, however, is much lower than in the parental situation. Perhaps an activator has been sufficiently altered to allow minimal transcription to proceed from the P2 promoter, but has completely inhibited P1 promoter activity.

Cloning and partial sequencing of the DNA into which the transposon inserted in strain PA103 N3 B17 revealed no clues to its origin (Figure 16). While only a short stretch of DNA was sequenced, it was more than sufficient for a significant BLAST search. No DNA of similar sequence has been reported to date. Much more work is required with this clone to understand its profound effect on the *regAB* promoters.

4.2.2 PA103 P3 transposon insertion mutants

With mutants derived from PA103 P3, three phenotypes were again observed. Mutants demonstrated either high early ß-galactosidase activity, high late ß-galactosidase activity, or consistently high ß-galactosidase activity (Figure 10b). Only PA103 P3 C86 had a corresponding ADPRT phenotype (Figure 11b). The other two strains had parental ADPRT levels. Strain PA103 P3 C44, when cloned and sequenced, was shown to have inserted into *regA*. This was not indicated by Southern blot analysis (Figure 14), which could have also been performed using a *regA* probe to identify mutants which had inserted into *regA*. The insertion probably occurred in one of the two *regA-lacZ* fusions, which correspondingly upregulated transcription of the *lacZ* gene via the *tac* promoter without affecting the wild type copy of *regAB*. This implies that transcription, at least in this case, through both the fusion and the wild type *regAB* promoters, cannot occur, as previously postulated. Similarly, strain PA103 P3 D168, which was not cloned, probably contained an insertion which only affected transcription of *lacZ* and not *regAB*, since ADPRT activity was similar to the parental strain.

Mutant PA103 P3 C86, as mentioned, had drastically increased ADPRT and ß-galactosidase activity throughout a growth curve. When DNA adjacent to the transposon insertion was cloned and sequenced, it was found to have inserted into the *fpvA* gene. FpvA is the ferripyoverdine receptor, responsible for at least the initial binding of iron-loaded pyoverdine.

ß-galactosidase activity, controlled only by the P1 promoter in PA103 P3 C86, is fairly similar in both low and high iron medium (Figure 19). This is consistent with previous data suggesting that very little iron regulation occurs at the P1 promoter (Storey *et al.*, 1990). However, one can see that even in high iron, ADPRT activity is very high. There is still iron regulation of the P2 promoter, as the ADPRT activity does drop significantly late in growth. The effect is likely because the P1 promoter is remaining active late into growth. This would imply that both the P1 and the P2 promoter are active simultaneously in a low iron situation, which has not been seen before. By association, this would indicate that transcription from one promoter does not impede transcription from the other promoter, as put forth earlier. Northern analysis of the *regAB* transcripts throughout growth of this mutant would show whether both promoters were active simultaneously.

4.3 Effect of insertion mutations in *fpvA*

To confirm that the phenotype observed in PA103 P3 C86 was due to the mutation in the *fpvA* gene, an omega fragment with transcriptional and translational termination signals was inserted into the cloned portion of *fpvA* and homologously recombined into the PA103 chromosome using a suicide vector. This resulted in strain PA103 *fpvA::Ω*, diagrammed in Figure 17b. Southern blot analysis confirmed the insertion of the Ω fragment into the *fpvA* gene (Figure 21).

It would also have been useful to recreate the transposon insertion into *fpvA* in strain PA103 and verify its effect on ADPRT

activity. It is possible that the IS50R element transposed separately into a different gene which was responsible for the effect; however, since the IS50R element usually transposes in a conservative fashion, if it had transposed elsewhere on the chromosome, it would not have been found adjacent to the rest of Tn*5*-B61. IS50R was found adjacent to Tn*5*-B61, since an IS50R-specific primer was able to sequence clones possessing gentamicin resistance encoded by the transposon.

It may still be useful to prove that the effect is due to the transposon insertion, especially in light of the opposite effect seen in the *fpvA::Ω* mutation. There is some evidence to support that Tn*5* does not always transpose in a conservative fashion. In addition, IS50R can sometimes transpose several times independently of Tn*5*, especially if multiple suicide vectors are present in a cell, as is likely the case in high efficiency electroporation procedures. An improper excision of the Tn*5* or IS50R can also cause point and deletion mutations. These mutations can all occur while leaving the original Tn*5* intact, despite the influence of the transposase inhibitor coded by the resident Tn*5*, and are known to take place in several different genera (Hynes, 1997). A probe for IS50R could verify that insertion hadn't occurred in other parts of the chromosome. Alternatively, generalized transduction could be used to recreate the mutation in WT PA103. If cotransduction of the ADPRT phenotype and gentamicin resistance occurred, this would prove that the effect was due to the transposon insertion. Several phages can be used for generalized transduction in *P. aeruginosa*, including D3112 (Darzins and Casadaban, 1989), UT1 (Ripp *et al.*,

1994), F116 (Kidambi *et al.*, 1994), and B86 (Kilbane and Miller, 1988).

Curiously, ADPRT assays of PA103 *fpvA::Ω* had an opposite effect to that seen in PA103 P3 C86 (Figure 24). While PA103 P3 C86 demonstrated abnormally high ADPRT activities in both low and high iron conditions, PA103 *fpvA::Ω* exhibited unusually low activity. It is possible that this difference is attributable to the locations of the insertion mutaions. While PA103 P3 C86 contained an insertion at leucine 672, probably resulting in truncation shortly thereafter, PA103 *fpvA::Ω* was truncated at leucine 502. Polar effects could also have a role in this discrepancy, since a difference in pyoverdine production likely caused by polar effects is evident in the pyoverdine levels of these two strains (Table 3).

To understand how these mutations might affect the FpvA protein, and to explain how they could lead to opposite effects on ADPRT activity, we need to look at the structure of FpvA. The structure of FpvA has been proposed to be similar to that of *E. coli* FhuA (James and Lamont, 1997). FhuA is the ferrichrome-iron receptor in *E. coli*. It is also responsible for the uptake of the structural analogue albomycin, an antibiotic, and colicin M, a bacterial toxin. It also serves as the receptor for phage attachment by bacteriophages T5, T1 and ϕ80 (Killman *et al.*, 1996). The ferrichrome uptake system is believed to parallel the *fec* system in many respects. Both systems are dependent on energy provided through the TonB-ExbB-ExbD (Ton) system. The FhuD and FhuBC proteins are involved in transport of iron to the cytoplasm,

analogous to the FecB and FecCDE proteins, respectively (Braun and Hentke, 1991).

The mechanism by which FhuA transports iron complexes has been studied extensively using phage and antibiotic sensitivity as probes of function. FhuA is predicted to be composed of 50% ß-sheets, similar to the porins. This suggests that FhuA may form a channel where the ß-sheets are arranged in a ß-barrel. This proposition is supported by the innate resistance of FhuA to proteases, implying that a large amount of the protein is confined to the membrane (Koebnik and Braun, 1993). The surface-exposed regions of FhuA have been mapped using monoclonal antibodies (Moeck *et al.*, 1995). Mutations have shown that FhuA, while normally in a closed state, can be held in an open state, allowing free diffusion of molecules through its channel independently of the Ton system (Killmann *et al.*, 1993). This evidence has led to the gated-loop theory. It is proposed that FhuA can exist in two conformational states, one in which a large central loop blocks the central channel of FhuA, and a Ton-dependent energized conformation where the loop does not impede the channel. Deletion of this loop causes FhuA to assume an open configuration. Deletion of other loops, and different deletions of the main loop cause changes in permeability and specificity to FhuA ligands. (Killmann *et al.*, 1993).

A preliminary model of FpvA, originally proposed by Poole (1996), is similar to the model of FhuA (Figure 31). It is also predicted that ferripyoverdine uptake will ultimately employ a mechanism similar to that seen in the *fec* and *fhu* systems. In the diagram, one can see that both leucines into which the insertions

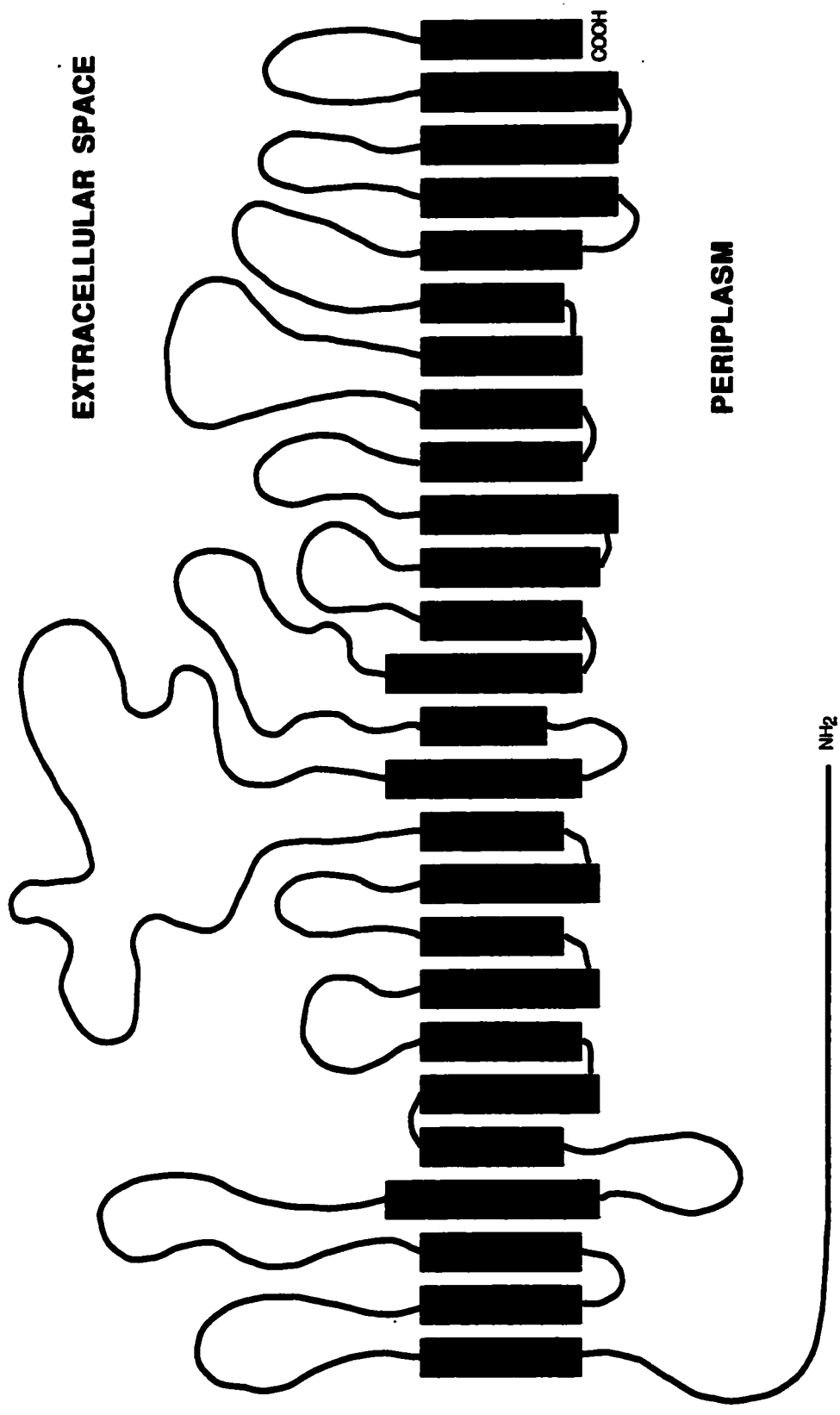
were made reside on predicted transmembrane regions. In both insertion mutations, the protein should truncate prematurely, shortly after the leucine residues.

So how can the difference between the transposon and interposon insertions be explained? It is not known whether or not FpvA is even inserted properly into the membrane. In FhuE, the outer-membrane receptor precursor protein for Fe(III)-coprogen and Fe(III)-rhodotorulic acid, the extreme end of the C-terminus is required for anchoring and insertion of the receptor into the outer membrane (Sauer *et al.*, 1990). In fact, insertion mutations throughout *fhuE* prevented insertions of the protein into the membrane, indicating that a particular conformation dictated by the primary structure is required either for signal cleavage or membrane insertion (Sauer *et al.*, 1987). The FpvA truncated proteins may well be localized in the periplasm.

As previously mentioned, the amino terminus of FecA is required for signaling of FecR. Perhaps the amino terminus of the transposon-mediated FpvA mutant protein is sufficiently intact to transmit this signal to a hypothetical 'FpvR' and cause constant activation of the transcriptional apparatus which ultimately controls the P1 promoter of *regA*. The interposon-mediated FpvA mutant, being somewhat shorter, may not be able to activate 'FpvR' at all.

Figure 31. Model depicting the proposed secondary structure of *P. aeruginosa* FpvA.

The FpvA model, originally proposed by Keith Poole and colleagues and communicated by James and Lamont (1997), consists of 26 transmembrane segments (shaded boxes) traversing the outer membrane of *P. aeruginosa*. The location of the two leucines marking the 5' ends of the insertion mutations in PA103 *fpvA::Ω* and PA103 P3 C86 are designated with an 'L'. The more N-terminal leucine represents the insertion point of the Ω interposon, while the more C-terminal leucine denotes the insertion site of the transposon in PA103 P3 C86. Both mutations should result in premature termination of the FpvA receptor. The largest loop is the gated loop.


EXTRACELLULAR SPACE
PERIPLASM
COOH
NH2

4.4 Transcriptional effects from *fpvA* insertion mutations

Pyoverdine levels in the mutants were measured for two reasons. First, there was a concern that the *tac* promoter might be interfering with transcription of other genes. Since the *tac* promoter transcribes into the *pvd* regulon, measurement of pyoverdine production gives an indication of the effect of the *tac* promoter. In the transposon mutant, PA103 P3 C86, pyoverdine levels were unaffected (Table 3), suggesting a negligible effect from the *tac* promoter.

PA103 *fpvA::Ω*, however, had significantly lower pyoverdine levels than the wild type strain. Strain PAO1 also showed lower pyoverdine levels when an interposon was placed into *fpvA* (James and Lamont, 1997). It would indicate that the transcriptional termination signals, present in the Ω interposon but absent in the transposon, are interfering with transcription of the *pvd* locus. Exactly how the *pvd* regulon is transcribed is unclear, but these data might argue that at least one other gene involved in pyoverdine synthesis uses a transcript coded by *fpvA*. Whether this involves the sense or anti-sense transcript from *fpvA* is unknown. Since *pvdD*, which is adjacent to *fpvA* (Figure 12), is transcribed in the opposite direction to *fpvA*, and *pvdF* is downstream from *pvdD*, *pvdF* could potentially be affected by a transcriptional stop in *fpvA*. While both *pvdD* (Merriman *et al.*, 1995) and *pvdE* (McMorran *et al.*, 1996) contain iron-starvation boxes (PvdS binding sites) (Oschner *et al.*, 1996), the absence of an iron-starvation box upstream of the *pvdF* coding sequence suggests that it could be co-transcribed with *pvdD*.

Alternatively, an unidentified ORF downstream from *fpvA* could be affected. A third possibility is that the drop in pyoverdine production is caused by protein-level interactions. If the FpvA protein is involved in regulation of the pyoverdine synthesis genes, as FecA is involved in the regulation of the *fec* operon, then a truncated FpvA protein as produced by PA103 *fpvA::Ω* may not function properly. The longer truncated protein produced by PA103 P3 C86 may have retained that function. Western blots will need to be done on cell fractions to resolve this issue.

Pyoverdine production is also regulated by iron concentrations in the environment. One question raised by mutating *fpvA* concerns effects on global iron regulation. Since both mutants maintained strict iron regulation of pyoverdine, it is clear that these two mutations in *fpvA* did not affect their iron regulatory apparatus. In contrast to *fpvA*, both *pvdD* and *pvdE* have well defined iron-starvation boxes, and therefore are probably regulated by the Fur repressor. While iron regulation of exotoxin A production seems disrupted at first glance, it may in fact be due to the increase in P1 activity, as suggested earlier. The P2 promoter is still regulated normally with respect to iron. It appears that the mutations in PA103 P3 C86 and PA103 *fpvA::Ω* have not affected global iron regulation, but have affected toxin activity through the P1 promoter of *regAB*.

4.5 Complementation of PA103 *fpvA::Ω*

Providing PA103 *fpvA::Ω* with a wild type copy of PAO1 *fpvA* restored ADPRT activities to wild type levels (Figure 27). The PAO1

fpvA gene, over the 400 nucleotides sequenced from pBK C86, is 97% identical to PA103 *fpvA* at the nucleotide level. It is probably even closer at the protein level. Thus it is not surprising that PAO1 FpvA can complement the mutation seen in PA103 *fpvA::Ω*. What is important to note is that alteration of ADPRT activities by mutation in *fpvA* is not a polar effect. Rather, it is an effect that manifests itself in the FpvA protein. This lends credence to the idea that it is FpvA, like FecA, which communicates to the transcriptional apparatus eventually responsible for controlling *regA* and *toxA*. The identity of any intermediate factors involved in the process is unclear at this time.

If FpvA is overexpressed in PA103 *fpvA::Ω* using a high copy number plasmid, the amount of ADPRT activity increases significantly in low iron conditions (Figure 28). In high iron conditions, no change is seen. This is consistent with the way in which FpvA is proposed to function. At high iron concentrations, the receptor is gated in a closed position and FpvA concentrations are low. There is no signaling to the inner membrane and ultimately no activation of transcription at the *regAB* locus. Thus, we see low levels of ADPRT activity. When iron is limiting, pyoverdine and FpvA are both synthesized (Cox and Adams, 1985; Gensberg *et al.*, 1992). Presumably, transport of ferripyoverdine through FpvA activates transcription. Concomitantly, higher production of the FpvA receptor allows more molecules of ferripyoverdine to bind, and consequently produces higher levels of transcriptional activation, which we see as a higher amount of ADPRT activity.

Iron regulation in both complementations is normal, supporting the notion that the P2 promoter is functioning normally, while the P1 promoter is over-activated.

Complementation of PA103 P3 C86 was complicated by the presence of the transposon and plasmid sequences on its chromosome, which provide resistance to several antibiotics. While complementation with pRK415-based plasmids should be possible, the strains had difficulty growing and the ADPRT activity of PA103 P3 C86 containing the plasmid control was drastically lower than the parent strain (data not shown). Passaging was performed in an attempt to select out tetracycline-resistant plasmid-containing strains with reasonable growth characteristics, which may have affected the membrane such that the secretion of ETA was altered, resulting in low ADPRT activities. Plasmid pRK*fpvA*, however, was maintained normally, perhaps due to the presence of the *P. aeruginosa* DNA insert. ADPRT assays from this strain containing pRK*fpvA* appeared similar to the parent strain, but in the absence of the proper control, conclusions cannot be inferred reliably, though some discussion of a potential complementation of PA103 P3 C86 is useful. If the mutant FpvA protein in PA103 P3 C86 is indeed causing a constant activation of FpvR and subsequently *toxA* transcription, would a WT FpvA protein be able to decrease that activation? It's possible some competition may occur, and thus we might see a decrease, but it would likely be small or even negligible.

4.6 A model for FpvA function

The similarity between FpvA and the Pup system was noted by Vrionis and Poole (1997). In particular, they noted that FpvA is pyoverdine-inducible, and that expression of the *pvdS* gene, the *pvd* operon and *fpvA* requires FpvA. This is consistent with what is known about the *E. coli* Fec and Fhu systems. It also resembles the Pup (ferric pseudobactin) uptake system in *P. putida*. In this system, PupI and PupR function analogously to FecI and FecR (Venturi *et al.*, 1995). Ochsner and Vasil (1996) have identified two Fur regulated proteins with some homology to PupI and PupR. These are candidates for the hypothetical proteins, FpvI and FpvR. The proposed model for ferripyoverdine transport thus parallels that seen in the Fec, Fhu and Pup systems (Figure 32).

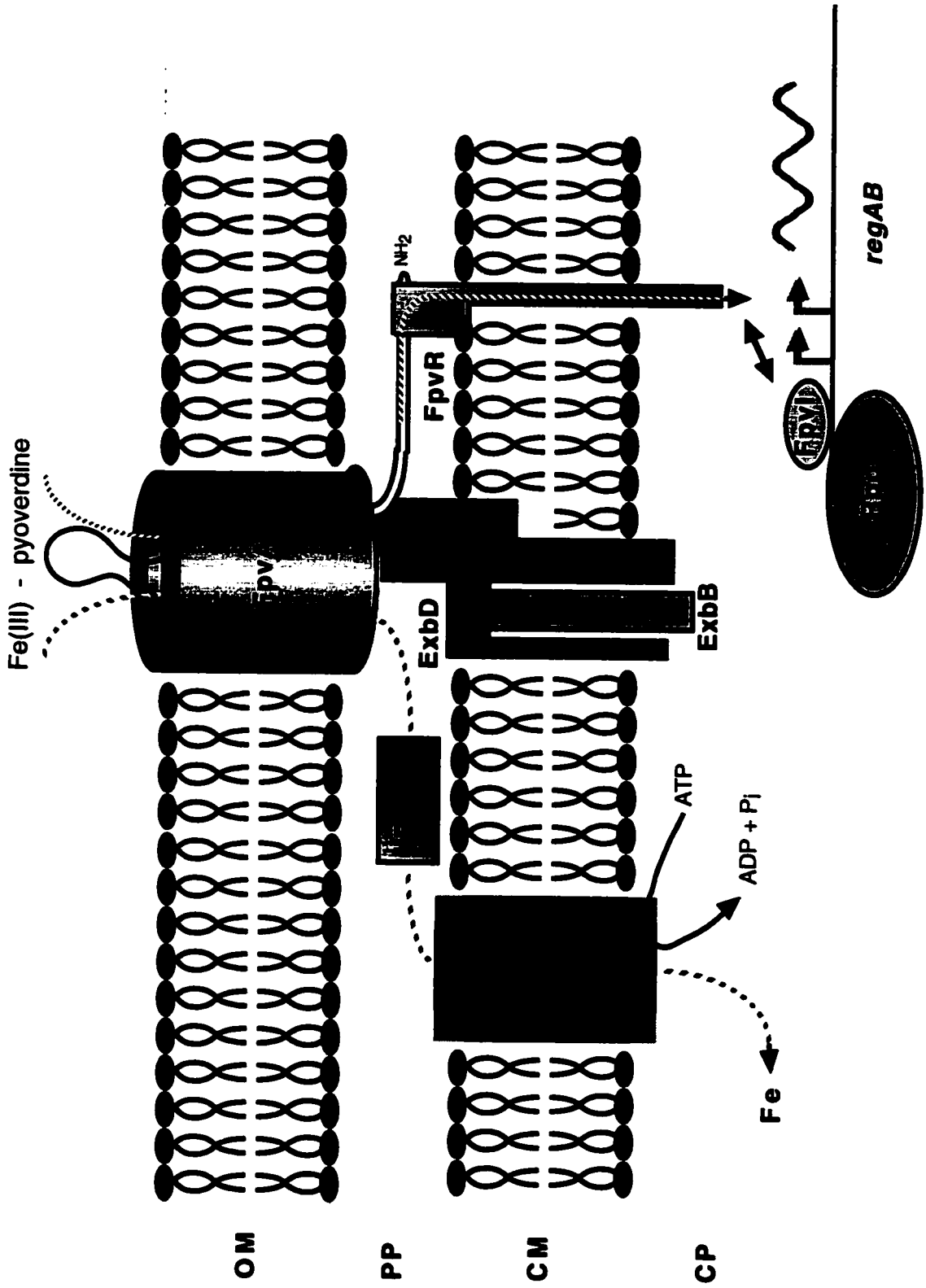
This leaves several questions unanswered. All of the Fpv proteins depicted in the model, with the exception of FpvA, remain to be positively identified. And what is the target gene? Our data would suggest that it is the P1 promoter of *regAB*. Or is it possible that RegA and RegB are analogous to the FecIR proteins? Both RegA and RegB are potentially membrane-bound proteins (Raivio, 1995). RegA is required for open-complex formation at the *tox* promoters. RegB can activate the P1 promoter. It would be a twist on the Fhu, Fec and Pup systems, but it is possible that RegA, in concert with RegB, is the alternative sigma factor activated by ferripyoverdine binding which activates *tox* transcription.

It is also unknown whether the FpvA transcriptional activation system is specific to the exotoxin A pathway. Is the transcription of

any other genes upregulated in the PA103 P3 C86 mutant? Are any other genes downregulated in PA103 *fpvA::Ω*? These questions will need to be answered using Northern analysis or transcriptional fusion reporter constructs. Possible candidates for an effect include *fur* and *pvdS*. It would also be informative to assay other iron regulated proteins to see if the potentially impaired transport of iron has affected their production. A particularly convenient system would be the fumarase and superoxide dismutase proteins (Hassett *et al.*, 1997). Finally, it is tempting to speculate that the mutation in PA103 N3 B17 has occurred in the putative FpvI or FpvR proteins, causing a decrease in the observed *regAB* promoter activity.

Figure 32. Model depicting the action of *P. aeruginosa* FpvA.

This model of the mechanism by which FpvA transports iron from ferripyoverdine and concurrently transmits a signal to activate transcription is based upon that proposed by Vrionis and Poole (1997), and Braun (1997). First, ferripyoverdine binds FpvA. FpvA undergoes a conformational shift and in concert with the Ton system transports ferripyoverdine to the periplasmic space. At this point, the ferripyoverdine is likely bound by 'FpvB'. The 'FpvB-ferripyoverdine' complex then releases its iron across the inner membrane through the action of as yet unknown proteins. Iron is reduced at this stage. Whether the pyoverdine is recycled to the extracellular space is unclear. The amino terminus of FpvA concomitantly signals the membrane-bound 'FpvR', which in turn causes the cytoplasmic alternative sigma factor 'FpvI' to initiate transcription of the target genes. OM - outer membrane; PP - periplasm; CM - cytoplasmic membrane; CP - cytoplasm; Rpo - RNA polymerase.


Fe(III) - pyoverdine
pv
FpvR
NH2
ExbD
ExbB
ATP
ADP + Pi
Fe
regAB
OM
PP
CM
CP

4.7 Another siderophore receptor from *P. aeruginosa*

In an attempt to clone the wild type PA103 *fpvA* gene, a second homologous gene was cloned. Using a probe derived from PA103 P3 C86, Southern blot analysis of PA103 genomic DNA identified a second 2.5 kb band in addition to the expected 6.5 kb band (Figure 21). This fragment was easily cloned from a PA103 genomic DNA lambda library, while cloning of the larger fragment has been elusive. Sequencing of this fragment and BLAST analysis revealed that it was probably a siderophore receptor similar to *fpvA*.

An alignment of the predicted protein sequence from this clone with other siderophore receptors (Figure 22) shows that while they all share homology, it is probably not a previously identified receptor. Interestingly, it most resembles *E. coli* FhuE. Over the first 74 translated amino acids from the unknown clone, it is 53% identical and 76% homologous to FhuE. This region is highly conserved across siderophore receptors, however, and the entire ORF must be cloned and sequenced before making strong conclusions about this sequence. It would also be interesting to see if a knockout in this gene had a similar effect to an *fpvA* knockout on ADPRT.

4.8 Future Studies

The discovery that FpvA is linked to *toxA* and *regAB* transcription has prompted many questions, several of which have already been posed. Much more work is needed to fully understand this system.

The localization of the mutant FpvA proteins needs to be determined. This is most easily achieved through analysis of intact cells with conjugate antibodies against FpvA. Information can also be discerned by the uptake of [^{55}Fe]-ferripyoverdine.

James and Lamont (1997) have a panel of linker insertion mutations into FpvA. It would be interesting to see if these mutants have any affect on ADPRT activity.

In a recent paper, a photoactivatable cross-linking siderophore which is taken up by FpvA was described (Ocaktan *et al.*, 1996). A set of pulse-chase experiments could identify factors involved further down the FpvA pathway.

Both the PA103 N3 A156 and PA103 N3 B17 strains look to be interesting mutants. More characterization of these mutants needs to be done.

The second siderophore cloned should be studied too. A knockout of this gene in WT PA103 might be interesting. One could label different siderophores and iron-binding molecules and see if such a mutant is rendered defective in their uptake.

4.9 Conclusions

This study has shown, by way of both transposon mutagenesis and insertion mutagenesis, that expression of the *fpvA* gene is somehow tied to transcription of the *regAB* promoters, and hence ETA production. The down-mutation caused by the Ω insertion can be complemented with PAO1 *fpvA* contained on a plasmid. Mutations at different locations in *fpvA* cause opposite effects on ADPRT activity. One possibility that explains these effects maintains that

the mutant FpvA proteins are located in the periplasm where their respective confirmations cause opposite effects on an inner membrane-bound signaling protein which activates transcription at the *regAB* locus. This is similar to the mechanisms described in the *E. coli* Fec and Fhu systems, and the *P. putida* Pup systems. Pyoverdine regulation is unaffected by mutations in *fpvA*.

In addition, it is likely that an activator functions upstream of the P1 promoter, given that some regulation still occurs despite deletion of sequences downstream from the T1 transcriptional start site.

Chapter 5 References

Albus, A.M., E.C. Pesci, L.J. Runyen-Janecky, S.E. West, and B.H. Iglewski. 1997. Vfr controls quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol.* **179**:3928-3935.

Ali, J.Y. 1993. Characterization of exotoxin A regulation in Fe18, a mutant of *Pseudomonas aeruginosa* strain PAO1. *Master's Thesis*. The University of Calgary, Calgary, AB.

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

Angerer, A., S. Enz, M. Ochs, and V. Braun. 1995. Transcriptional regulation of ferric citrate transport in *Escherichia coli* K-12. FecI belongs to a new subfamily of sigma 70-type factors that respond to extracytoplasmic stimuli. *Mol Microbiol.* **18**:163-174.

Ankenbauer, R., S. Sriyosachati and C.D. Cox. 1985. Effects of siderophores on the growth of *Pseudomonas aeruginosa* in human serum and transferrin. *Infect Immun.* **49**:132-140.

Ankenbauer, R.G. and H.N. Quan. 1994. FptA, the Fe(III)-pyochelin receptor of *Pseudomonas aeruginosa*: a phenolate siderophore receptor homologous to hydroxamate siderophore receptors. *J Bacteriol.* **176**:307-319.

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl (ed.). 1991. In *Current Protocols in Molecular Biology*. John Wiley & Sons, New York, N.Y.

Barton, H.A., Z. Johnson, C.D. Cox, A.I. Vasil, and M.L. Vasil. 1996. Ferric uptake regulator mutants of *Pseudomonas aeruginosa* with distinct alterations in the iron-dependent repression of exotoxin A and siderophores in aerobic and microaerobic environments. *Mol Microbiol.* **21**:1001-1017.

Birnboim, H.C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research.* **7**:1513-1523.

Bitter, W., I.S. van Leeuwen, J. de Boer, H.W. Zomer, M.C. Koster, P.J. Weisbeek, and J. Tommassen. 1994. Localization of functional domains in the *Escherichia coli* coprogen receptor FhuE and the *Pseudomonas putida* ferric-pseudobactin 358 receptor PupA. *Mol Gen Genet.* **245**:694-703.

Bjorn, M.J., B.H. Iglewski, S.K. Ives, J.C. Sadoff, and M.L. Vasil. 1978. Effect of iron on yields of exotoxin A in cultures of *Pseudomonas aeruginosa* PA-103. *Infect Immun.* **19**:785-791.

Bjorn, M.J., M.L. Vasil, J.C. Sadoff, and B.H. Iglewski. 1977. Incidence of exotoxin production by *Pseudomonas* species. *Infect Immun.* **16**:362-366.

Bjorn, M.J., P.A. Sokol and B.H. Iglewski. 1979. Influence of iron on yields of extracellular products in *Pseudomonas aeruginosa* cultures. *J Bacteriol.* **138**:193-200.

Braun, V. 1995. Energy-coupled transport and signal transduction through the Gram-negative outer membrane via TonB-ExbB-ExbD-dependent receptor proteins. *FEMS Microbiol Rev.* **16**:295-307.

Braun, V. 1997. Surface signaling: novel transcription initiation mechanism starting from the cell surface. *Arch Microbiol.* **167**:325-331.

Britigan, B. E., M. B. Hayek, B. N. Doebbeling & R. B. Fick. 1993. Transferrin and lactoferrin undergo proteolytic cleavage in the *Pseudomonas aeruginosa*-infected lungs of patients with cystic fibrosis. *Infect Immun.* **61**:5049-5055.

Coffman, T. J., C. D. Cox, B. L. Edeker & B. E. Britigan. 1990. Possible role of bacterial siderophores in inflammation. Iron bound to the *Pseudomonas* siderophore pyochelin can function as a hydroxyl radical catalyst. *J Clin Invest.* **86**:1030-1037.

Collins, F.S. 1992. Cystic fibrosis: molecular biology and therapeutic implications. *Science*. **256**:774-779.

Cox, C.D. 1980. Iron uptake with ferripyochelin and ferric citrate by *Pseudomonas aeruginosa*. *J Bacteriol*. **142**:581-587.

Cox, C.D. 1982. Effect of pyochelin on the virulence of *Pseudomonas aeruginosa*. *Infect Immun*. **36**:17-23.

Cox, C.D. and P. Adams. 1985. Siderophore activity of pyoverdin for *Pseudomonas aeruginosa*. *Infect Immun*. **48**:130-138.

Cunliffe, H. E., T. R. Merriman & I. L. Lamont. 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**:2744-2750.

Darzins, A. & M. J. Casadaban. 1989. In vivo cloning of Pseudomonas aeruginosa genes with mini-D3112 transposable bacteriophage. *J Bacteriol*. **171**:3917-3925.

De Lorenzo, V., M. Herrero, F. Giovannini, and J.B. Neilands. 1988. Fur (ferric uptake regulation) protein and CAP (catabolite-activator protein) modulate transcription of *fur* gene in *Escherichia coli*. *Eur J Biochem*. **173**:537-546.

Ditta, G., T. Schmidhauser, E. Yakobson, P. Lu, X.W. Liang, D.R. Finlay, D. Guiney, and D.R. Helinski. 1985. Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. *Plasmid.* **13**:149-153.

Doring, G., M. Pfestorf, K. Botzenhart & M. A. Abdallah. 1988. Impact of proteases on iron uptake of *Pseudomonas aeruginosa* pyoverdin from transferrin and lactoferrin. *Infect Immun.* **56**:291-293.

FitzSimmons, S.C. 1993. The changing epidemiology of cystic fibrosis. *J Pediatr.* **122**:1-9.

Frank, D. W., D. G. Storey, M. S. Hindahl & B. H. Iglewski. 1989. Differential regulation by iron of *regA* and *toxA* transcript accumulation in *Pseudomonas aeruginosa. J Bacteriol.* **171**:5304-5313.

Frank, D.W. and B.H. Iglewski. 1988. Kinetics of *toxA* and *regA* mRNA accumulation in *Pseudomonas aeruginosa. J Bacteriol.* **170**:4477-4483.

Garger, S.J., O.M. Griffith, and L.K. Grill. 1983. Rapid purification of plasmid DNA by a single centrifugation in a two- step cesium chloride-ethidium bromide gradient. *Biochem Biophys Res Commun.* **117**:835-842.

Gensberg, K., K. Hughes & A. W. Smith. 1992. Siderophore-specific induction of iron uptake in *Pseudomonas aeruginosa*. *J Gen Microbiol.* **138**:2381-2387.

Gish, W. and D.J. States. 1993. Identification of protein coding regions by database similarity search. *Nat Genet.* **3**:266-272.

Govan, J.R. and G.S. Harris. 1986. *Pseudomonas aeruginosa* and cystic fibrosis: unusual bacterial adaptation and pathogenesis. *Microbiol Sci.* **3**:302-308.

Gralla, J.D. 1991. Transcriptional control - lessons from an *E. coli* promoter data base. *Cell.* **66**:415-418.

Gray, G. L., D. H. Smith, J. S. Baldridge, R. N. Harkins, M. L. Vasil, E. Y. Chen & H. L. Heyneker. 1984. Cloning, nucleotide sequence, and expression in *Escherichia coli* of the exotoxin A structural gene of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA.* **81**:2645-2649.

Haas, B., J. Kraut, J. Marks, S.C. Zanker, and D. Castignetti. 1991. Siderophore presence in sputa of cystic fibrosis patients [published erratum appears in Infect Immun 1992 Mar;60(3):1261]. *Infect Immun.* **59**:3997-4000.

Hamood, A. N. and B. H. Iglewski. 1990. Expression of the *Pseudomonas aeruginosa toxA* positive regulatory gene (*regA*) in *Escherichia coli*. *J Bacteriol.* **172**:589-594.

Hamood, A.N., J.A. Colmer, U.A. Ochsner, and M.L. Vasil. 1996. Isolation and characterization of a *Pseudomonas aeruginosa* gene, *ptxR*, which positively regulates exotoxin A production. *Mol Microbiol.* **21**:97-110.

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

Hancock, R.E., L.M. Mutharia, L. Chan, R.P. Darveau, D.P. Speert, and G.B. Pier. 1983. *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis: a class of serum-sensitive, nontypable strains deficient in lipopolysaccharide O side chains. *Infect Immun.* **42**:170-177.

Hantke, K. 1983. Identification of an iron uptake system specific for coprogen and rhodotorulic acid in *Escherichia coli* K12. *Mol Gen Genet.* **191**:301-306.

Hassett, D.J., M.L. Howell, P.A. Sokol, M.L. Vasil, and G.E. Dean. 1997. Fumarase C activity is elevated in response to iron deprivation and in mucoid, alginate-producing *Pseudomonas aeruginosa*: cloning and characterization of *fumC* and purification of native *fumC*. *J Bacteriol.* **179**:1442-1451.

Hassett, D.J., P.A. Sokol, M.L. Howell, J.F. Ma, H.T. Schweizer, U. Ochsner, and M.L. Vasil. 1996. Ferric uptake regulator (Fur) mutants of *Pseudomonas aeruginosa* demonstrate defective siderophore-mediated iron uptake, altered aerobic growth, and decreased superoxide dismutase and catalase activities. *J Bacteriol.* **178**:3996-4003.

Hedstrom, R.C., C.R. Funk, J.B. Kaper, O.R. Pavlovskis, and D.R. Galloway. 1986. Cloning of a gene involved in regulation of exotoxin A expression in *Pseudomonas aeruginosa. Infect Immun.* **51**:37-42.

Heinrichs, D. E. & K. Poole. 1996. PchR, a regulator of ferripyochelin receptor gene (*fptA*) expression in *Pseudomonas aeruginosa*, functions both as an activator and as a repressor. *J Bacteriol.* **178**:2586-2592.

Holloway, B.W. 1969. Genetics of Pseudomonas. *Bacteriol Rev.* **33**:419-443.

Hynes, M.F. 1997. Personal communication.

Hynes, M.F., J. Quandt, M.P. O'Connell, and A. Puhler. 1989. Direct selection for curing and deletion of *Rhizobium* plasmids using transposons carrying the *Bacillus subtilis sacB* gene. *Gene.* **78**:111-120.

Iglewski, B.H. and D. Kabat. 1975. NAD-dependent inhibition of protein synthesis by *Pseudomonas aeruginosa* toxin. *Proc Natl Acad Sci U S A.* **72**:2284-2288.

Iglewski, B.H., P.V. Liu, and D. Kabat. 1977. Mechanism of action of *Pseudomonas aeruginosa* exotoxin A - adenosine diphosphate-ribosylation of mammalian elongation factor 2 *in vitro* and *in vivo*. *Infect Immun.* **15**:138-144.

James, E.E. and I.L. Lamont. 1997. Structure function relationships of the ferripyoverdine receptor FpvA of *Pseudomonas aeruginosa*. *Abstracts of the 97th General Meeting of the American Society for Microbiology.* B-233.

Keramidas, S. 1987. 1986 cystic fibrosis patient registry analyses . Cystic Fibrosis Foundation, Bethesda, Md.

Kidambi, S.P., S. Ripp, and R.V. Miller. 1994. Evidence for phage-mediated gene transfer among *Pseudomonas aeruginosa* strains on the phylloplane. *Appl Environ Microbiol.* **60**:496-500.

Kilbane, J.J. and R.V. Miller. 1988. Molecular characterization of *Pseudomonas aeruginosa* bacteriophages: identification and characterization of the novel virus B86. *Virology.* **164**:193-200.

Killmann, H., R. Benz and V. Braun. 1996. Properties of the FhuA channel in the *Escherichia coli* outer membrane after deletion of FhuA portions within and outside the predicted gating loop. *J Bacteriol.* **178**:6913-6920.

Kim, I., A. Stiefel, S. Plantor, A. Angerer, and V. Braun. 1997. Transcription induction of the ferric citrate transport genes via the N-terminus of the FecA outer membrane protein, the Ton system and the electrochemical potential of the cytoplasmic membrane. *Mol Microbiol.* **23**:333-344.

Kolmar, H., C. Frisch, G. Kleemann, K. Gotze, F.J. Stevens, and H.J. Fritz. 1994. Dimerization of Bence Jones proteins: linking the rate of transcription from an *Escherichia coli* promoter to the association constant of REIV. *Biol Chem Hoppe-Seyler.* **375**:61-70.

Koster, M., J. van de Vossenburg, J. Leong, and P.J. Weisbeek. 1993. Identification and characterization of the *pupB* gene encoding an inducible ferric-pseudobactin receptor of *Pseudomonas putida* WCS358. *Mol Microbiol.* **8**:591-601.

Koster, M., W. van Klompenburg, W. Bitter, J. Leong, and P. Weisbeek. 1994. Role for the outer membrane ferric siderophore receptor PupB in signal transduction across the bacterial cell envelope. *EMBO J.* **13**:2805-2813.

Leoni, L., A. Ciervo, N. Orsi & P. Visca. 1996. Iron-regulated transcription of the *pvdA* gene in *Pseudomonas aeruginosa*: effect of Fur and PvdS on promoter activity. *J Bacteriol.* **178**:2299-2313.

Liss, L. 1987. New M13 host: DH5αF' cells. In *Focus*. Bethesda, MD: Bethesda Research Laboratories. **9**:13.

Liu, P.V. 1966. The roles of various fractions of *Pseudomonas aeruginosa* in its pathogenesis. III. Identity of the lethal toxins produced *in vitro* and *in vivo*. *J Infect Dis.* **116**:481-489.

Liu, P.V. 1973. Exotoxins of *P. aeruginosa*. I. Factors that influence the production of exotoxin A. *J Infect Dis.* **128**: 506-513.

Lory, S. 1986. Effect of iron on accumulation of exotoxin A-specific mRNA in *Pseudomonas aeruginosa*. *J Bacteriol.* **168**:1451-1456.

May, T. B., D. Shinabarger, R. Maharaj, J. Kato, L. Chu, J. D. DeVault, S. Roychoudhury, N. A. Zielinski, A. Berry, and R. K. Rothmel. 1991. Alginate synthesis by *Pseudomonas aeruginosa*: a key pathogenic factor in chronic pulmonary infections of cystic fibrosis patients. *Clin Microbiol Rev.* **4**:191-206.

McMorran, B.J., M.E. Merriman, I.T. Rombel, and I.L. Lamont. 1996. Characterisation of the *pvdE* gene which is required for pyoverdine synthesis in *Pseudomonas aeruginosa*. *Gene*. **176**:55-59.

McPherson, M.A. and R.L. Dormer. 1987. The molecular and biochemical basis of cystic fibrosis. *Biosci Rep.* **7**:167-185.

Merriman, T.R., M.E. Merriman, and I.L. Lamont. 1995. Nucleotide sequence of *pvdD*, a pyoverdine biosynthetic gene from *Pseudomonas aeruginosa*: PvdD has similarity to peptide synthetases. *J Bacteriol.* **177**:252-258.

Meyer, J. M. 1992. Exogenous siderophore-mediated iron uptake in *Pseudomonas aeruginosa*: possible involvement of porin OprF in iron translocation. *J Gen Microbiol.* **138**:951-958.

Meyer, J. M., A. Neely, A. Stintzi, C. Georges & I. A. Holder. 1996. Pyoverdin is essential for virulence of *Pseudomonas aeruginosa*. *Infect Immun.* **64**:518-523.

Meyer, J. M., D. Hohnadel, A. Khan & P. Cornelis. 1990. Pyoverdin-facilitated iron uptake in *Pseudomonas aeruginosa*: immunological characterization of the ferripyoverdin receptor. *Mol Microbiol.* **4**:1401-1405.

Meyer, J.M. and D. Hohnadel. 1992. Use of nitrilotriacetic acid (NTA) bu *Pseudomonas* species through iron metabolism. *Appl Microbiol Biotechnol.* **37**:114-118.

Meyer, J.M., A. Stintzi, D. De Vos, P. Cornelis, R. Tappe, K. Taraz, and H. Budzikiewicz. 1997. Use of siderophores to type pseudomonads: the three *Pseudomonas aeruginosa* pyoverdine systems. *Microbiology.* **143**:35-43.

Miller, J.H. 1972. In *Experiments in Molecular Genetics.* Cold Spring Harbor, NY. Cold Spring Harbor Laboratory Press. pp. 352-355.

Miyazaki, H., H. Kato, T. Nakazawa & M. Tsuda. 1995. A positive regulatory gene, *pvdS*, for expression of pyoverdin biosynthetic genes in *Pseudomonas aeruginosa* PAO. *Mol Gen Genet.* **248**:17-24.

Moeck, G.S., M.J. Ratcliffe and J.W. Coulton. 1995. Topological analysis of the *Escherichia coli* ferrichrome-iron receptor by using monoclonal antibodies. *J Bacteriol.* **177**:6118-6125.

Mohr, C. D., L. Rust, A. M. Albus, B. H. Iglewski & V. Deretic. 1990. Expression patterns of genes encoding elastase and controlling mucoidy: co-ordinate regulation of two virulence factors in *Pseudomonas aeruginosa* isolates from cystic fibrosis. *Mol Microbiol.* **4**:2103-2110.

Morris, J., D. F. Donnelly, E. O'Neill, F. McConnell & F. O'Gara. 1994. Nucleotide sequence analysis and potential environmental distribution of a ferric pseudobactin receptor gene of *Pseudomonas sp.* strain M114. *Mol Gen Genet.* **242**:9-16.

Ocaktan, A., I. Schalk, C. Hennard, C. Linget-Morice, P. Kyslik, A.W. Smith, P.A. Lambert, and M.A. Abdallah. 1996. Specific photoaffinity labelling of a ferripyoverdin outer membrane receptor of *Pseudomonas aeruginosa. FEBS Letters.* **396**:243-247.

Ochsner, U. A. & M. L. Vasil. 1996. Gene repression by the ferric uptake regulator in *Pseudomonas aeruginosa*: cycle selection of iron-regulated genes. *Proc. Natl. Acad. Sci. U.S.A.* **93**:4409-4414.

Ochsner, U. A., A. I. Vasil & M. L. Vasil. 1995. Role of the ferric uptake regulator of *Pseudomonas aeruginosa* in the regulation of siderophores and exotoxin A expression: purification and activity on iron-regulated promoters. *J Bacteriol.* **177**:7194-7201.

Ochsner, U.A., Z. Johnson, I.L. Lamont, H.E. Cunliffe, and M.L. Vasil. 1996. Exotoxin A production in *Pseudomonas aeruginosa* requires the iron-regulated *pvdS* gene encoding an alternative sigma factor. *Mol Microbiol.* **21**:1019-1028.

Ohman, D.E., J.C. Sadoff and B.H. Iglewski. 1980. Toxin A-deficient mutants of *Pseudomonas aeruginosa* PA103: isolation and characterization. *Infect Immun.* **28**:899-908.

Olsen, R.H., G. DeBusscher, and W.R. McCombie. 1982. Development of broad-host-range vectors and gene banks: self-cloning of the *Pseudomonas aeruginosa* PAO chromosome. *J Bacteriol.* **150**:60-69.

Pitt, T.L. 1989. Lipopolysaccharide and virulence of *Pseudomonas aeruginosa. Antibiot Chemother.* **42**:1-7.

Poole, K., D. E. Heinrichs & S. Neshat. 1993. Cloning and sequence analysis of an EnvCD homologue in *Pseudomonas aeruginosa*: regulation by iron and possible involvement in the secretion of the siderophore pyoverdine. *Mol Microbiol.* **10**:529-544.

Poole, K., Neshat, S., Krebes, K., and D.E. Heinrichs. 1993. Cloning and nucleotide sequence analysis of the ferripyoverdine receptor gene *fpvA* of *Pseudomonas aeruginosa. J Bacteriol.* **175**:4597-604.

Prentki, P. and H.M. Krisch. 1984. *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene.* **29**:303-313.

Prince, R. W., C. D. Cox & M. L. Vasil. 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., D. G. Storey, A. I. Vasil & M. L. Vasil. 1991. Regulation of *toxA* and *regA* by the *Escherichia coli fur* gene and identification of a Fur homologue in *Pseudomonas aeruginosa* PA103 and PAO1. *Mol Microbiol.* **5**:2823-2831.

Raivio, T. L., E. E. Ujack, H. R. Rabin & D. G. Storey. 1994. Association between transcript levels of the *Pseudomonas aeruginosa regA*, *regB*, and *toxA* genes in sputa of cystic fibrosis patients. *Infect Immun.* **62**:3506-3514.

Raivio, T.L. 1995. Analysis of the role of the *regAB* operon in regulation of Exotoxin A Production in *Pseudomonas aeruginosa*. PhD Dissertation. The University of Calgary, Calgary, AB.

Raivio, T.L., D. Hoffer, R.W. Prince, M.L. Vasil, and D.G. Storey. 1996. Linker insertion scanning of *regA*, an activator of exotoxin A production in *Pseudomonas aeruginosa*. *Mol Microbiol.* **22**:239-254.

Riek, R.P., M.D. Handschumacher, S.S. Sung, M. Tan, M.J. Glynias, M.D. Schluchter, J. Novotny, and R.M. Graham. 1995. Evolutionary conservation of both the hydrophilic and hydrophobic nature of transmembrane residues. *J Theor Biol.* **172**:245-258.

Ripp, S., O.A. Ogunseitan, and R.V. Miller. 1994. Transduction of a freshwater microbial community by a new *Pseudomonas aeruginosa* generalized transducing phage, UT1. *Mol Ecol.* **3**:121-126.

Rombel, I. T., B. J. McMorran & I. L. Lamont. 1995. Identification of a DNA sequence motif required for expression of iron-regulated genes in pseudomonads. *Mol Gen Genet.* **246**:519-528.

Sambrook, J., E.F. Fritsch, and T. Maniatis.1989. *In Molecular cloning: a laboratory manual.* 2nd Ed. Cold Spring Harbor, NY. Cold Spring Harbor Laboratory Press.

Sauer, M., K. Hantke, and V. Braun. 1987. Ferric-coprogen receptor FhuE of *Escherichia coli*: processing and sequence common to all TonB-dependent outer membrane receptor proteins. *J Bacteriol.* **169**:2044-2049.

Sauer, M., K. Hantke, and V. Braun. 1990. Sequence of the *fhuE* outer-membrane receptor gene of *Escherichia coli* K12 and properties of mutants. *Mol Microbiol.* **4**:427-437.

Schweizer, H.P. 1992. Allelic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable *oriT* and the counter-selectable Bacillus subtilis *sacB* marker. *Mol Microbiol.* **6**:1195-1204.

Schweizer, H.P. 1993. Two plasmids, X1918 and Z1918, for easy recovery of the *xylE* and *lacZ* reporter genes. *Gene.* **134**:89-91.

Schwyn, B. & J.B. Neilands. 1987. Universal chemical assay for the detection and determination of siderophores. *Anal Biochem.* **160**:47-56.

Simon, R., J. Quandt and W. Klipp. 1989. New derivatives of transposon Tn*5* suitable for mobilization of replicons, generation of operon fusions and induction of genes in gram-negative bacteria. *Gene.* **80**:161-169.

Simpson, D.A., R. Ramphal and S. Lory. 1992. Genetic analysis of *Pseudomonas aeruginosa* adherence: distinct genetic loci control attachment to epithelial cells and mucins. *Infect Immun.* **60**:3771-3779.

Smith, A.W., D.R. Poyner, H.K. Hughes, and P.A. Lambert. 1994. Siderophore activity of *myo*-inositol hexakisphosphate in *Pseudomonas aeruginosa. J Bacteriol.* **176**:3455-3459.

Sokol, P.A. and D.E. Woods. 1983. Demonstration of an iron-siderophore-binding protein in the outer membrane of *Pseudomonas aeruginosa*. *Infect Immun.* **40**:665-669.

Sokol, P.A. and D.E. Woods. 1988. Effect of pyochelin on *Pseudomonas cepacia* respiratory infections. *Microb Pathog.* **5**:197-205.

Sriyosachati, S. and C. D. Cox. 1986. Siderophore-mediated iron acquisition from transferrin by *Pseudomonas aeruginosa*. *Infect Immun.* **52**:885-891.

Storey, D. G., D. W. Frank, M. A. Farinha, A. M. Kropinski & B. H. Iglewski. 1990. Multiple promoters control the regulation of the *Pseudomonas aeruginosa regA* gene. *Mol Microbiol.* **4**:499-503.

Storey, D. G., E. E. Ujack and H. R. Rabin. 1992. Population transcript accumulation of *Pseudomonas aeruginosa* exotoxin A and elastase in sputa from patients with cystic fibrosis. *Infect Immun.* **60**:4687-94.

Storey, D. G., T. L. Raivio, D. W. Frank, M. J. Wick, S. Kaye & B. H. Iglewski. 1991. Effect of *regB* on expression from the P1 and P2 promoters of the *Pseudomonas aeruginosa regAB* operon. *J Bacteriol.* **173**:6088-6094.

Thompson, J.D., D.G. Higgins, and T.J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**:4673-4680.

Venturi, V., P. Weisbeek, and M. Koster. 1995. Gene regulation of siderophore-mediated iron acquisition in *Pseudomonas*: not only the Fur repressor.*Mol Microbiol.* **17**:603-610.

Visca, P., A. Ciervo & N. Orsi. 1994. Cloning and nucleotide sequence of the *pvdA* gene encoding the pyoverdin biosynthetic enzyme L-ornithine N5-oxygenase in *Pseudomonas aeruginosa. J Bacteriol.* **176**:1128-1140.

Vogel, H.J. and D.M. Bonner. 1956. Acetylornithase of *Escherichia coli*: partial purification and some properties. *J Biol Chem.* **218**:97-106.

Vrionis, H. and K. Poole. 1997. Regulation of expression of the ferric pyoverdine receptor of *Pseudomonas aeruginosa. Abstracts of the 97th General Meeting of the American Society for Microbiology.* B-232.

Walker, S. L., L. S. Hiremath, D. J. Wozniak & D. R. Galloway. 1994. ToxR (RegA)-mediated *in vitro* transcription of *Pseudomonas aeruginosa toxA*. *Gene*. **150**:87-92.

Walker, S.L., L.S. Hiremath and D.R. Galloway. 1995. ToxR (RegA) activates *Escherichia coli* RNA polymerase to initiate transcription of *Pseudomonas aeruginosa toxA*. *Gene*. **154**:15-21.

Wang, J., A. Mushegian, S. Lory, and S. Jin. 1996a. Large-scale isolation of candidate virulence genes of *Pseudomonas aeruginosa* by *in vivo* selection. *Proc Natl Acad Sci USA*. **93**:10434-10439.

Wang, J., S. Lory, R. Ramphal, and S. Jin. 1996b. Isolation and characterization of *Pseudomonas aeruginosa* genes inducible by respiratory mucus derived from cystic fibrosis patients. *Mol Microbiol*. **22**:1005-1012.

West, S.E., A.K. Sample and L.J. Runyen-Janecky. 1994. The *vfr* gene product, required for *Pseudomonas aeruginosa* exotoxin A and protease production, belongs to the cyclic AMP receptor protein family. *J Bacteriol*. **176**:7532-7542.

Wick, M. J., D. W. Frank, D. G. Storey & B. H. Iglewski. 1990a. Structure, function, and regulation of *Pseudomonas aeruginosa* exotoxin A. *Annu Rev Microbiol*. **44**:335-363.

Wick, M. J., D. W. Frank, D. G. Storey & B. H. Iglewski. 1990b. Identification of *regB*, a gene required for optimal exotoxin A yields in *Pseudomonas aeruginosa*. *Mol Microbiol.* **4**:489-497.

Wick, M.J. and B.H. Iglewski. 1990. *Pseudomonas aeruginosa* exotoxin A. In: *Moss, J. Vaughan, eds. ADP-ribosylating toxins and G proteins. Insights into signal transduction.* pp. 31-44.

Wolz, C., K. Hohloch, A. Ocaktan, K. Poole, R. W. Evans, N. Rochel, A. M. Albrecht-Gary, M. A. Abdallah & G. Doring. 1994. Iron release from transferrin by pyoverdin and elastase from *Pseudomonas aeruginosa*. *Infect. Immun.* **62**:4021-4027.

Wozniak, D. J., D. C. Cram, C. J. Daniels & D. R. Galloway. 1987. Nucleotide sequence and characterization of *toxR:* a gene involved in exotoxin A regulation in *Pseudomonas aeruginosa*. *Nucleic Acids Res.* **15**:2123-2135.

Xiao, R. and W.S. Kisaalita. 1997. Iron acquisition from transferrin and lactoferrin by *Pseudomonas aeruginosa* pyoverdin. *Microbiology.* **143**:2509-2515.

Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene.* **33**:103-119.

Zimmermann, L., K. Hantke, and V. Braun. 1984. Exogenous induction of the iron dicitrate transport system of *Escherichia coli* K-12. *J Bacteriol.* **159**:271-277.

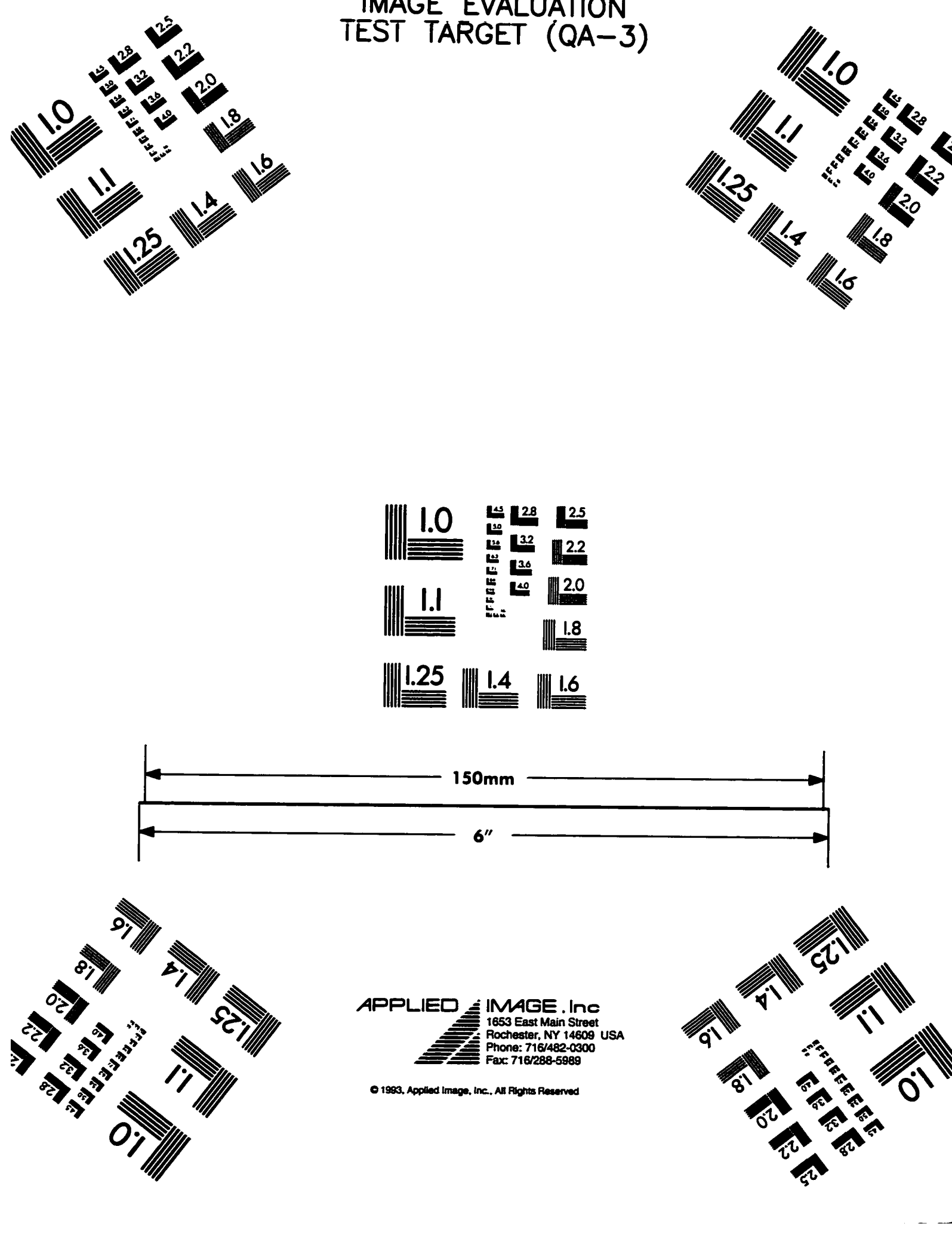


IMAGE EVALUATION
TEST TARGET (QA-3)
1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
150mm
6"
APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989
© 1993, Applied Image, Inc., All Rights Reserved