Identification and Characterization of Zinc-Responsive Genes in *Pseudomonas Fluorescens*

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IDENTIFICATION AND CHARACTERIZATION OF ZINC-RESPONSIVE GENES IN *PSEUDOMONAS FLUORESCENS*

by

Thomas L. Wilson

A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
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Thomas L. Wilson
IDENTIFICATION AND CHARACTERIZATION OF ZINC-RESPONSIVE GENES IN *PSEUDOMONAS FLUORESCENS*

Thomas L. Wilson

Western Michigan University, 2000

Genes responsive to elevated concentrations of zinc were identified via transposon mutagenesis in the common soil bacterium, *Pseudomonas fluorescens* ATCC13525. Some of these genes were essential to maintain metal homeostasis in the bacterial cell. DNA sequences of the transposon-tagged genes were determined by DNA sequencing of arbitrary PCR products. DNA sequence analysis indicated that one gene was similar to P-type ATPases responsible for transporting metal ions out of the cell. The corresponding mutant was sensitive to zinc, cadmium, and lead indicating that this gene may be responsible for defending *Pseudomonas fluorescens* against these metal ions. Four zinc-induced genes shared similarity with pyoverdine synthetase genes. The corresponding mutants were unable to produce the iron-scavenging siderophore pyoverdines. One other mutant was sensitive to zinc, cadmium, and copper. The gene targeted by the transposon in this mutant did not show any homology to known genes in databases. This may indicate the presence of a novel and heretofore unidentified cellular mechanism playing a role in metal tolerance.
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INTRODUCTION

Metal-rich environments occur naturally from the weathering of rocks or other geological events or from man-made efforts such as mining and smelting factories (Roane et al., 1996). Because of this, metal pollution has fast become a widespread problem. Traditional remediation methods include the physical removal of contaminated soils. Alternative methods are being sought due to the increasing cost of excavation and the dwindling available landfill space. This has lead to the possibility of utilizing microorganisms for the bioremediation of contaminated sites. Alternative microbiological methods of bioremediation are being used to restore contaminated soil and sediments, aquatic systems, and nuclear waste (White and Gadd, 1998; Crawford and Crawford, 1996). Further characterization of the mechanisms used by bacteria to cope with exposure to toxic levels of heavy metals could prove beneficial to the environment and human health.

Thus, the bioavailability of heavy metals, such as cadmium, mercury, zinc, and copper, has sharply increased in recent years (Mergeay, 1995). Some metals, including cadmium, mercury, and lead, have traditionally been thought to be toxic for cells at any concentration. This previous notion may be premature, as cadmium has recently been discovered to have a biological role in marine diatoms (Lane and Morel, 2000). This is the only organism that uses cadmium as a cofactor for enzymatic reactions.
Cadmium is known to be toxic for most other organisms because it binds to proteins with sulfur-containing amino acids rendering them nonfunctional. Other metals, such as zinc and copper, are known to be required for the metabolism of the cell. For example, zinc is required for many essential metalloenzymes (Prasad, 1993; Kendrick et al, 1992). The functions of zinc in these metalloenzymes can be catalytic or structural. An example of an enzyme in which zinc plays a catalytic role includes carbonic anhydrase. Zinc plays a structural role in enzymes such as aspartate transcarbamylase, which is involved in the synthesis of pyrimidines. The roles of zinc ions are utilized in the enzyme leucine aminopeptidase for the hydrolysis of N-terminal amino acid residues from proteins, peptides, and amino acid amides.

Zinc has also shown to be very important in the role of regulating gene expression. An example of this are the "zinc fingers", which are motifs in DNA binding proteins. The specific location and action of these structural motifs within regulatory proteins aide with the recognition of certain promoter regions and, therefore, regulate gene expression (Garret and Grisham, 1995; Prasad, 1993). Zinc is also a co-factor of the DNA polymerase and RNA polymerase that are essential for the polymerization of DNA and RNA, respectively (Kendrick, 1992).

In spite of the need for essential metals such as zinc and copper, at high intracellular concentrations, these metals may result in severe illnesses in humans and other animals. The elevated levels may also lead to growth deficiencies in plants and animals (Silver and Phung, 1996; Prasad, 1993). Therefore, zinc, copper, iron, and other metals are essential micronutrients for all cells, but at too high of concentrations they may become toxic. In fact, certain human genetic disorders may result in
improper balances of these essential metals within the body. Examples of this are Wilson’s and Menkes’ diseases. Analysis of the defective genes identified in patients with these diseases has revealed a gene encoding a copper translocating protein as the source of malfunction (Phung et al., 1994; Vulpe et al., 1993).

Metal ion pumps and resistance mechanisms are also common in many different genera of bacteria because these are thought to have evolved relatively early in the earth’s history as the microbes were constantly exposed to very harsh conditions, including heavy metal exposure. An example of an efflux pump utilized by most cells is the P-type ATPase. This pump uses an efflux mechanism that translocates metal ions across the cellular membrane.

The P-type ATPase forms a membrane bound efflux mechanism. This term refers to the phosphoenzyme intermediate that is formed by the phosphorylation of a conserved aspartate residue as part of the translocation cycle. Another conserved residue among P-type ATPases includes a proline residue forming a portion of the ion translocation pathway in the protein. Also conserved are domains for ATP binding and energy transduction (Rensing et al., 1999; Solioz and Vulpe, 1996; Lutsenko and Kaplan, 1995). Azide treatment of cells with active P-type ATPases does not result in sensitivity indicating that the mode of transport is not due to proton motive force (Beard et al., 1997). However, transport of metals is sensitive to vanadate (Solioz and Vulpe, 1996).

A common example of a P-type ATPase is the Na⁺-K⁺ pump found in the plasma membrane of virtually all animal cells. The Na⁺-K⁺ pump is an antiporter that actively transports Na⁺ out of the cell against its electrochemical gradient while
pumping $K^+$ into the cell. The resulting $Na^+$ gradient is vitally important for the cells as it contributes to transport of sugars and amino acids. This gradient is also exploited by nerve cells to produce nerve impulses (Alberts et al., 1994).

Resistance to Poisonous Metals - Cadmium

Many bacteria have acquired resistance systems that chelate, detoxify, or pump poisonous metals out of the cell. A well-documented example of a cadmium efflux pump used by the bacterium *Staphylococcus aureus* is the CadA P-type ATPase. This heavy metal efflux system confers resistance to cadmium by translocating the ions out of the cell. The *cadA* gene has also been shown to confer resistance to zinc when induced by cadmium, zinc, cobalt, lead, and bismuth. Resistance to bismuth and lead is also possible but uncertain (Silver and Walderhaug, 1992).

CadA consists of six transmembrane $\alpha$-helices and is 727 amino acids in length (Silver et al., 1989; Silver and Walderhaug, 1992). Cadmium recognition and binding of CadA is thought to occur primarily on the cytoplasmic side of the periplasmic membrane at the first two cysteines at positions 23 and 26. The next region of the protein is referred to as the transduction domain because it is involved in moving the cadmium substrate to the membrane channel. The membrane channel itself contains a proline residue at position 372 that is highly conserved and positioned between another pair of cysteine residues (Solioz and Vulpe, 1996). These four cysteines, the two at the binding site and the two in the membrane channel, are the only four in the entire amino acid sequence. Also highly conserved is an aspartate
at position 415 that is phosphorylated in the ATPase enzyme cycle. A lysine residue at position 489 is conserved and functions in ATP binding. This is followed by more highly conserved sequences starting at approximately position 600 and finally ending with a lysine at position 727 (Silver et al., 1989; Silver and Walderhaug, 1992). The diagram of CadA is illustrated in Figure 1.

A second open reading frame of the cad operon encodes cadB. The cadB-mediated response is not an efflux mechanism and results only in a low-level resistance (Smith and Novick, 1972). The mechanism of CadB has not been determined but has been hypothesized to bind free cadmium ions (Perry and Silver, 1982; Nucifora et al., 1989; Silver and Walderhaug, 1992).

The characterization of the cadA operon of S. aureus has also resulted in the identification of another cadmium resistance open reading frame. This open reading
frame encodes for the soluble protein CadC. CadC is a repressor protein of 122 amino acids that inhibits the transcription of the *cad* operon (Silver and Walderhaug, 1992; Tsai et al., 1997). CadC, the DNA-binding, negatively acting regulatory protein, binds the *cad* DNA and inhibits transcription of the operon. Furthermore, CadC has been shown to dissociate from the *cad* operon upon exposure to Cd(II), Bi(II), and Pb(II) (Endo and Silver, 1995). CadC has also been proposed, not conclusively, to sequester intracellular cadmium and possibly present it to the CadA efflux pump to be expelled to the outside of the cell (Ivey et al., 1992). It is proposed that cadmium is transported into the cell by a manganese transporter (Perry and Silver, 1982). This exposure to cadmium results in the CadC repressor protein dissociating from the promoter allowing for the transcription of the *cadA* gene (Nies and Silver, 1995).

**Homeostasis of Essential Metals - Zinc**

As opposed to toxic metals, bacteria must carefully regulate their exposure to essential metals, which are needed in low concentrations, but at too high of concentrations may become detrimental to the cell. An example of an enzyme playing a role in zinc homeostasis in *Escherichia coli* is the ZntA protein. This P-type ATPase was recently identified after sequencing the *E. coli* genome. Identified within the sequence was the P-type ATPase motif (Rensing et al., 1997). This gene and its protein product were analyzed in detail (Beard et al., 1997; Rensing et al., 1997).
In *E. coli*, *zntA* is responsible for resistance to zinc, cadmium, and lead. Production of everted vesicles, where accumulation inside these vesicles is considered to represent metal ion efflux out of an intact cell, revealed the accumulation of zinc, cadmium, and lead (Rensing *et al.*, 1997; Rensing *et al.*, 1998). The metal transport was not sensitive to azide treatment but to vanadate. This indicates the mode of transport was not due to proton motive force, but was due to P-type ATPase activity (Beard *et al.*, 1997; Rensing *et al.*, 1997). Also, ZntA has also been shown to be highly similar to CadA of *S. aureus*.

Mercury was shown to induce *zntA* activity, but it did not contribute to mercury resistance in *E. coli* (Sharma *et al.*, 2000). Interestingly, at pH values above 6.5, cadmium inhibits ZntA activity while there is no inhibition by zinc or lead. This could be due to the strong complexes cadmium may form with cysteines within ZntA at this pH. Since zinc and lead have lower affinities for the sulfur ligands as compared to cadmium, only cadmium shows this inhibition (Sharma *et al.*, 2000).

Transcriptional regulation of *zntA* occurs via ZntR (Brocklehurst *et al.*, 1999). This transcriptional regulator is induced by zinc, cadmium, and lead. Interestingly, the induction range of *zntA* begins at zinc concentrations much greater than that needed for sufficient growth. Thus, ZntR prevents expression of *zntA* until Zn(II) concentrations exceed those for optimal growth and approach toxic levels (Brocklehurst *et al.*, 1999).
Other Zinc Homeostasis Mechanisms

Zinc homeostasis in *E. coli* has also been shown with the high-affinity uptake system *znuABC* and its regulator Zur (Rensing *et al*., 1999; Patzer and Hantke, 1998). *ZnuABC* constitutes a periplasmic-binding protein-dependent uptake system for zinc ions in *E. coli*. The regulator, Zur, is a repressor protein that has similarities to Fur, a regulator protein of the iron uptake systems (Rensing *et al*., 1999). It is hypothesized that, when enough zinc is in the cell, Zur represses the expression of *znuABC*. As the concentrations of zinc ions decrease inside the cell, the metal activated Zur provides for the expression of *znuABC* promoting zinc uptake (Patzer and Hantke, 1998).

Thus, the P-type ATPase transporter proteins are efflux pumps used by bacteria to exclude the toxic metal ions from the cytoplasm. The mechanism is utilized to prevent exposure to the poisonous ions as well as to regulate levels of essential metals within the cell. This resistance mechanism is just one example of a variety of proteins utilized by bacteria to maintain metal homeostasis within the cells.

Siderophores and Iron Starvation

Many bacteria also utilize a variety of other mechanisms to cope with exposures to toxic and essential heavy metals. Responses to heavy metal ions occur at the level of transcription of the genes encoding proteins that may be responsible for the uptake of the metals (Brown *et al*., 1998). *P. fluorescens*, for example, shows regulated gene expression when exposed to varying concentrations of iron. Because iron, much like zinc, is an essential metal needed for normal cellular functions, *P.*
fluorescens produces iron-scavenging siderophores when iron concentrations are low. The siderophores chelate ferric iron with a high degree of affinity and specificity (Stintzi et al., 1996). Fluorescent pseudomonads produce the characteristic yellowish-green siderophore pyoverdine (Rombel et al., 1995). A diagram of pyoverdine is illustrated in figure 2. This peptide can be analyzed quantitatively by observing its peak absorbance at 403 nm (McMorran et al., 1996).

![Pyoverdine structure](image)

Figure 2. General Chemical Structure of Pyoverdine From Glick et al. (1999).

Pyoverdine synthetases are enzyme complexes that synthesize the siderophore molecules without the aid of ribosomes. These peptide synthetases show significant sequence and structural similarity to other enzymes that synthesize peptides without ribosomes. An example of this is the gramicidin synthetase of Brevibacillus brevis, responsible for the production of the peptide antibiotic gramicidin (Kleinkauf and von Doehren, 1995).

The regulation of iron uptake occurs via the Fur protein, which senses the elevated iron concentration inside the cell. The transcription of the iron-regulated genes is repressed as intracellular iron binds the cytoplasmic protein. When the iron
concentrations decrease, iron dissociates from the Fur protein. Fur will not remain bound to the DNA promoter region, called the Fur-box, and this results in the active transcription of several iron-responsive genes (Simon and Walderhaug, 1992; Glick et al., 1999). Therefore, if intracellular concentrations of iron are low, the iron chelating siderophores will be synthesized and secreted from the cells.

Once the siderophores chelate the free iron in the environment surrounding the bacterium, it must be taken back into the cell and made available for the cellular metabolism. Because siderophores are hydrophilic, they can not pass freely into the cytoplasm and must be transported via membrane-bound proteins. Once inside the cell, the iron is released from the siderophore complex by one of two specific mechanisms. One mechanism entails the enzymatic cleavage of the siderophore releasing the chelated iron. A second mechanism enzymatically reduces the ferric iron to the ferrous state. The ferrous iron has a much lower affinity for the siderophore, and, thus, results in the release from the siderophore complex (Glick et al., 1999).

Thus, bacterial cells utilize a variety of different mechanisms to regulate essential metal homeostasis. One mechanism includes monitoring intracellular metal concentrations and transporting excess ions out of the cell. Other mechanisms involve secreting molecules to sequester extracellular metal ions that are brought back into the cell. These systems are regulated tightly to keep intracellular concentrations of the essential metals at levels required for normal metabolism of the cell.
Transposon Mutagenesis and Reporter Genes to Study Differential Gene Expression

This study utilized transposons to identify genes used by soil bacteria to cope with exposure to heavy metals. Transposable elements have proven to be useful tools for identifying gene function (Cai et al., 1998; Chen et al., 1993). This is because the insertion of the transposon disrupts the function of the gene. Transcriptional and translational stop signals within the transposon also result in a strong polar effect on genes downstream within the same operon. The functions of these genes can then be inferred. Also, transposons often encode genes responsible for antibiotic resistance (deBruijn and Rossbach, 1994), allowing for the identification of strains containing the transposon by selecting for antibiotic resistant strains.

Another beneficial aspect of certain transposons is the transcriptional monitoring of the mutated gene because of the inclusion of promotorless reporter genes within the transposon. An example of this is the transposon Tn5-B20, encoding a promotorless lacZ gene (Simon et al., 1989; deBruijn and Lupski, 1984). The Tn5-B20 transposon encodes a promotorless lacZ reporter gene and the nptII gene allowing for kanamycin resistance. When Tn5-B20 inserts behind an active promoter, the resulting β-galactosidase activity can be measured allowing for the quantification of transcription (Casadaban et al., 1983; Miller, 1992). A diagram of the Tn5-B20 transposon is illustrated in Figure 3.
Relevance of This Study

At environmental sites contaminated with elevated concentrations of heavy metals, soil microorganisms will be some of the first organisms to encounter this stress. For this reason, our laboratory has been investigating the differential gene expression of the common soil bacterium *Pseudomonas fluorescens* ATCC 13525 when exposed to heavy metals. The identification of the processes used by this organism to respond and adapt to toxic heavy metal ions may lead to future applications for bioremediation as well as the understanding of the localization of these toxic metals within the food web.

Using the transposon Tn5-B20, several loci responding to cadmium have been identified via transposon mutagenesis and arbitrary PCR (Rossbach *et al.*, 2000). Since a specific set of genes was identified in *P. fluorescens* that was differentially expressed when exposed to the toxic metal cadmium, we investigated the hypothesis that a different set of genes would respond to the essential metal zinc. Moreover, a second hypothesis was that some of the genes may play a role in helping the bacterial cell to cope with essential and toxic metal stress.
MATERIALS AND METHODS

Bacterial Strains and Plasmids

The genotypes and sources of the bacterial strains, vectors, and recombinant plasmids used in this study are listed in Table 1.

Media

Complex Medium for *E. coli*

The complex medium used for culturing *E. coli* was Luria-Bertani (LB) broth and agar (Miller, 1992) containing tryptone (10 g/l), yeast extract (5 g/l), and NaCl (5 g/l). *E. coli* cultures grown transduced with λ phage were grown in LB supplemented with 10 mM MgSO₄. For solid medium, 15 g/l agar was added.

Media for *Pseudomonas*

Complex Medium

Tryptone-yeast (TY) medium (Beringer, 1974) was used to grow *P. fluorescens* cultures. This complex medium contains tryptone (5 g/l), yeast extract (10 g/l), and CaCl₂ (0.5 g/l). For solid medium, 15 g/l agar was added.
Table 1

Bacterial Strains and Plasmids Used in this Study

<table>
<thead>
<tr>
<th>Strains or Plasmids</th>
<th>Relevant Characteristics</th>
<th>Source or Reference</th>
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</thead>
<tbody>
<tr>
<td><strong>Bacterial strains</strong></td>
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<tr>
<td><em>Pseudomonas fluorescens</em></td>
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</tr>
<tr>
<td>ATCC13525</td>
<td>Wild type</td>
<td>ATCC</td>
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<tr>
<td>Z1 (14C2)</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;, zinc-induced mutant of ATCC13525</td>
<td>This study</td>
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<tr>
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<td>ATCC12633</td>
<td>Wild type</td>
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<td>Relevant Characteristics</td>
<td>Source or Reference</td>
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<td><strong>Escherichia coli</strong></td>
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<td>DH5-α</td>
<td><em>supE44 lacU169</em></td>
<td>Sambrook <em>et al.</em> (1989)</td>
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<td><em>(Φ80lacZΔM15)</em> <em>hsdR17</em></td>
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| LE392MP             | *F*, *e14* (McrA*'), Δ(mcrC-mrr)*  
|                     | *Tc**, *hsdR514*, *supE44*,  
|                     | *supF58*, *lacY1* or Δ(*lacIZY*)*6*,  
|                     | *galK2*, *galT22*, *metB1*,  
|                     | *trpR55*, λ*           | Epicentre Technologies Corp. |
| **Phage**           |                          |                      |
| λc1857 Sam7         |                          | Epicenter Technologies Corp. |
| **Plasmids**        |                          |                      |
| pBluescript (KS+)   | Cloning and sequencing  
|                     | plasmid; *Ap**, *lacZ α* | Stratagene           |
| pLAFR3              | Cosmid cloning vector; Mob*,  
|                     | *Tra*, *IncP*; *Tc*  | Staskawicz, *et al.*, 1987 |
| pTW1000             | pLAFR3 carrying ~32 kb  
|                     | fragment of mutant Z1; *Tc**,  
|                     | *Km*  | This study |
| Km*R pTW1001        | pLAFR3 carrying ~32 kb  
|                     | fragment of mutant Z1; *Tc**,  
|                     | *Km*  | This study |
| pTW1002             | pBluescript KS* carrying an  
|                     | 11 kb *EcoRI* fragment of  
|                     | pTW1000; *Ap**, *Km*  | This study |
| pTW1003             | pBluescript KS* carrying a  
|                     | 3 kb *EcoRI* fragment of  
|                     | pTW1000; *Ap*  | This study |
| pTW1004             | pBluescript KS+ carrying a  
|                     | 1.7 kb fragment of pTW1002;  
|                     | *Ap*  | This study |
| pTW1005             | pLAFR3 carrying ~32 kb  
|                     | *Sau3AI* fragment of  
|                     | ATCC13525; *Tc*  | This study |
| pTW1006             | pBluescript KS+ carrying a  
|                     | 2.1 kb *EcoRI* fragment of  
|                     | pTW1005; *Ap*  | This study |
| pTW1007             | pBluescript KS+ carrying a  
|                     | 9.8 kb *EcoRI* fragment of  
|                     | pTW1006 | This study |
**Defined Medium**

The minimal medium used for culturing *P. fluorescens* was low phosphate medium. This medium contains a salt solution with the following ingredients: NaCl (4.68 g/l), KCl (1.49 g/l), NH₄Cl (1.07 g/l), (NH₄)₂SO₄ (0.43 g/l), 0.01 M MgCl₂, 0.27 mg/l, Tris-base (14.33 g/l). The pH of the salt solution was adjusted to 7.0-7.4 with concentrated HCl and autoclaved. A 10X salt solution was made and diluted to a 1X concentration with autoclaved glycerol (5.0 ml/l), filter sterilized thiamine (0.4 ml/l), autoclaved peptone (1%), and autoclaved CaCl₂ (0.1 mM). This was added to autoclaved water agar (15 grams agar/ 1l of water).

**Growth Conditions**

*P. fluorescens* cultures were routinely grown at 28°C. *E. coli* was grown at 37°C. Broth cultures were incubated in a gyratory shaker at 200 rpm (New Brunswick Scientific, Edison, NJ).

**Antibiotics**

The following antibiotics were added to *P. fluorescens* growth media: kanamycin (Km; 50 µg/ml) and tetracycline (Tc; 20 µg/ml). The antibiotics supplemented into *E. coli* growth media were as follows: kanamycin (20 µg/ml) and ampicillin (Ap; 100 µg/ml). All values are final concentrations.
Minimal Inhibitory Concentration (MIC) for One Metal

The minimal inhibitory concentration (MIC) was determined by a spread plate technique as outlined by Mergeay (1995). The optical density (O.D.) of a 10 ml overnight culture of TY broth containing *P. fluorescens* was measured at 600 nm using a Beckman DU 640 spectrophotometer. Dilutions of the culture were made and spread onto TY agar plates and incubated at 28°C for 2 days. The dilution resulting in 30-300 colony forming units (CFU) was determined and correlated to the O.D.\textsubscript{600}. An appropriate dilution was plated onto TY plates supplemented with metal in increasing concentrations. The plating was performed in duplicate. The MIC was the metal concentration that resulted in no colony growth after 2 days of incubation at 28°C. The analysis of the MIC with lead was performed on low phosphate medium to prevent precipitation of the metal.

MIC for Several Metals

When comparing the MIC of different metals for several mutant strains at the same time, the method of Lim and Cooksey (1993) was utilized. Ten microliters of an overnight TY, Km\textsuperscript{50} culture were spotted onto TY plates with increasing concentrations of metal. Bacterial growth was observed after 24 and 48 hours. The metal concentration that resulted in no confluent bacterial growth was determined as the MIC of that metal.
Transposon Mutagenesis

Transposon mutagenesis was performed via conjugation of *P. fluorescens* with *E. coli* strain S17-1 that contained the suicide vector pSUP102::Tn5-B20 and was described by Rossbach *et al.* (2000). The mutant strains were stored in 27% glycerol (final concentration) in microtiter plates at -80°C.

Screening for Stress-Responsive Gene Fusions

The 5,000 mutant strains were retrieved from storage microtiter plates at -80°C and thawed and replica plated onto plates containing TY, Km$^{50}$ or TY, Km$^{50}$ with 1.0 mM zinc sulfate (final concentration). All plates also contained X-gal (5-bromo-4-chloro-3-indolyl galactosidase) at a final concentration of 40 mg/ml. The plates were incubated at 28°C for 2 days. Differential gene expression was observed with colonies of varying color intensities. Colonies that showed a darker shade of blue when exposed to the metal ion as compared to the colonies grown without the metal were putatively identified as having genes tagged by the transposon that were induced in the presence of the ion. Colonies displaying a lighter shade of blue or even white when exposed to the metal as compared to the colonies grown without the metal were putatively identified as having genes tagged by the transposon that were repressed in the presence of the metal ion. The putative strains were streaked onto plates with or without the metal to verify responsiveness to the stress. When screening for differential gene expression for other stresses, the TY plates were supplemented with CdCl$_2$ (150 µM), CuSO$_4$ (1.0 mM), ethanol (4%), NaCl (500mM),
HgCl₂ (8 µM), naladixic acid (10 µg/ml), cumene hydroperoxide (300 µM), and H₂O₂ (1.2 mM). All values are final concentrations. Mutant strains were also observed for heat stress by growth at 32°C.

Screen for Siderophore Production

Mutant and wild type strains of *P. fluorescens* were streaked on *Pseudomonas* F agar (Difco, Detroit, MI). Production of the siderophore pyoverdine was noted from the characteristic yellowish-green pigmentation secreted around the bacterial colonies (Difco Manual, 10th edition). Further analysis of siderophore production was investigated by observing a peak pyoverdine absorbance at 403 nm (McMorran *et al.*, 1996). Ten milliliter broth cultures of wild type and mutant strains were incubated overnight. A 1.0 ml aliquot was pelleted at 10,000 rpm for 5 min. The supernatant was scanned at all wavelengths from 370-540 nm using a Beckman DU 640 spectrophotometer.

β-Galactosidase Enzyme Assays

The differential expression of the genes tagged by the Tn5-B20 transposon were analyzed quantitatively by measuring the β-galactosidase activities with ortho-nitrophenyl β-D-galactopyranoside (ONPG) according to Miller (1992) with minor modifications. Overnight cultures of 10 mls TY, Km⁵⁰ were diluted 1:10 ensuring an O.D₆₀₀ of approximately 0.27. The refreshed cultures were incubated for an additional 2 hours. Control cultures did not contain additional metals. When identifying gene expression with a specific metal, that metal was supplemented in the
overnight and refreshed media. Often the metal slowed the growth of the cultures. However, the control cultures, after two hours of refreshed growth, grew to an O.D.₆₀₀ of approximately 0.7. The mutant strains that exhibited severe growth inhibition in the presence of the metals, Z₁ and Z₉, could not grow to sufficient overnight turbidity and were only exposed to the metals for 2 hours (Rossbach et al., 2000). The remaining steps of the β-galactosidase enzyme assay protocol were followed exactly as published by Miller (1992). Metal concentrations used for the β-galactosidase enzyme assay were 1.0 mM ZnSO₄, 100 µM CdCl₂, 1.0 mM Pb(C₂H₃O₂)₂, 1.0 mM CuSO₄, 2.5 µM HgCl₂, 1.0 mM NiCl₂, and 62.5 µM AgNO₃. For colored metal solutions, TY broth solutions containing the metals were used as blanks when reading absorbance values from the spectrophotometer.

Growth Curves

The O.D.₆₀₀ of 10 ml overnight cultures of the mutant or wild type strains grown in TY was measured to ensure that each broth contained approximately equal numbers of bacterial cells. These cultures were diluted 1:150 in 300 ml Erlenmeyer flasks with TY broth. The cultures were incubated for 60 hours. The absorbances in the flasks were measured using a Beckman DU 640 spectrophotometer at 600 nm every 2 hours for the first 24 hours, every 4-5 hours for the second day, and every 10-12 hours on the third day. Zinc sulfate (1.5 mM) or copper sulfate (1.5 mM) were added at an O.D.₆₀₀ of 1.0 (approximately 6.0 hours after refreshment). The values
indicate final metal concentrations. The growth curves were performed in duplicate and the values averaged.

Isolation, Preparation, and Analysis of DNA

Isolation of Bacterial Total Chromosomal DNA

The total chromosomal DNA was isolated from the bacterial cells by a modified protocol from Ausubel et al. (1995). A 1.5 ml aliquot from a 10 ml overnight culture was centrifuged for 3 minutes at room temperature at 10,000 rpm. After decantation of the supernatant, the pellet was washed in 1 ml TE buffer (pH 8.0), pelleted by centrifugation, and resuspended in 350 µl of TE buffer. Added to the resuspended cells was 50 µl of 10% SDS and 100 µl pronase (2.5 mg/ml; Boehringer Mannheim, Indianapolis, IN) and incubated at 37°C for 1-2 hours. Then, 100 µl of 5.0 M NaCl and 80 µl of CTAB/NaCl solution (10% cetyltrimethylammonium bromide in 0.7 M NaCl). were added and incubated at 65°C for 10 minutes. This was followed by adding an equal volume of chloroform/isoamyl alcohol (24:1), shaken well for one minute, and centrifuged at 12,000 rpm for 5 minutes at room temperature. The upper phase was added to an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). This was followed with another extraction with phenol/chloroform/isoamyl alcohol (25:24:1) as described above. The upper phase was removed and collected in a new microcentrifuge tube. The DNA was precipitated with the addition of 2 volumes of cold 100% ethanol, spooled with closed-end capillary tubes, and resuspended in 100 µl H₂O.
Isolation of Plasmid DNA

Small-Scale Plasmid Isolation (Miniprep)

Small-scale plasmid DNA isolation was performed by the alkaline lysis method (Sambrook et al., 1989; modified from Birnboim & Doly, 1979). Low copy number plasmids were isolated from a 3.0 ml overnight culture and resuspended in 50 µl H₂O. The isolation of high copy number plasmids was from a 1.5 ml overnight culture and dissolved in 100 µl H₂O.

Plasmid Isolation for Sequencing

Pure DNA used for sequencing was isolated using the Wizard Plus Miniprep DNA Purification System according to the manufacturer’s protocol (Promega, Madison, WI). DNA was eluted from the miniprep column with 50 µl H₂O.

Mid-Scale Plasmid Isolation

The Qiagen Midiprep Kit (Qiagen GmbH, Hilden, Germany) or the Wizard Plus Midiprep Purification System (Promega, Madison, WI) was used for the isolation of midi-scale plasmids.
DNA Digestion With Restriction Endonucleases

Restriction digests were performed with endonucleases and their appropriate buffers obtained from New England Biolabs (Beverly, MA), Gibco BRL (Gaithersberg, MD), and Boehringer Mannheim (Indianapolis, IN). Digestions were performed according to the protocols of the manufacturer. The reaction times and volumes varied according to the concentration of the DNA and the amount needed.

Agarose Gel Electrophoresis

Agarose Gel Preparation

Agarose gels used for general analysis were prepared with 0.7% agarose (w/v) agarose (Sea-Kem LE) in 1x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) buffer by heating to a boil. For the separation of DNA bands of small sizes, 2.0 % agarose gels were used.

Agarose Gel Loading

To aide the loading of DNA into the wells of the agarose gel, 10% loading dye (0.25% bromphenol blue, 0.25% xylene-cyanol FF, 15% Ficoll Type 400 in H_2O) was added to the DNA samples. At least one of two DNA size standards were also loaded into the gel. These included the λ digested with *Hind*III and the 1-Kilobase DNA Ladder (Gibco BRL, Gaithersberg, MD) that were diluted to a final concentration of 0.1 µg/µl with loading dye and used according to the manufacturer’s protocols.
Electrophoresis

Agarose gel electrophoresis was performed in 1 x TAE buffer (see Agarose Gel Preparation) with applied voltage that varied according to the size of the gel and the time desired to complete the electrophoresis.

DNA Visualization and Gel Photodocumentation

Following electrophoresis, agarose gels were soaked in 0.5 µl/ml ethidium bromide in 1 x TAE buffer for a minimum of 15 minutes. The DNA was visualized by placing the gel on a Fotodyne Foto/Prep Ultraviolet transilluminator (Hartland, WI) using the analytical mode. Photographs of the stained, illuminated gels were taken using a Fotodyne FCR-10 camera (settings: F-stop, 4.5; exposure time 1.0 sec) with Polaroid 667 black and white film.

Isolation of DNA From Agarose Gels

A phenol-freeze method was used to isolate a desired band of DNA from an agarose gel by first cutting agarose containing the DNA fragment into very fine pieces with a sterile scalpel and transferring it to a microcentrifuge tube. To the microcentrifuge tube, 0.3 ml of buffered phenol were added and mixed thoroughly by vortexing. The agarose-phenol mixture was placed at -80°C for 10 minutes, centrifuged at room temperature at 12,000 rpm for 10 minutes, and the resulting upper phase was collected in a new microcentrifuge tube. To the remaining agarose-phenol phase, 0.3 ml of 1 x TAE buffer was added, frozen at -80°C for 10 minutes,
centrifuged at room temperature at 12,000 rpm, and the upper phase was combined with the previously isolated aqueous layer. An equal amount of chloroform/isoamyl alcohol (24:1) was added to the tube, vortexed, and centrifuged at room temperature for 3 minutes at 12,000 rpm. The upper phase was again collected. NaCl at a final concentration of 100 mM was added to the DNA solution followed with the addition of 2 volumes cold ethanol. Precipitation occurred at -80°C for 30 minutes or at -20°C for 2 hours. The DNA was pelleted by centrifugation at 4°C for 30 minutes, washed with 200 µl of 70% cold ethanol, and centrifuged at 4°C for 15 minutes. The DNA pellet was dried by a SpeedVac Model DNA 120 (Savant, Holbrook, NY) for 10 minutes at room temperature and resuspended in 20 µl H₂O.

**Dephosphorylation of Linearized Vectors**

The 5’-phosphate group from linear plasmid DNA was removed with Calf Intestine Phosphatase (CIP; Boehringer Mannheim, Indianapolis, IN) according to Sambrook (1989). The deactivation of the enzyme was accomplished by heating to 70°C for 10 minutes. The DNA was purified by phenol/chloroform extraction and precipitated by adding one fifth the volume of 7.5 M NH₄OAc and twice the volume of cold ethanol. The DNA solution was placed at -80°C for 30 minutes followed by centrifugation at 4°C for 30 minutes. The resulting pellet was washed with cold 70% ethanol and centrifuged at 4°C for 15 minutes. The DNA pellet was dried under vacuum (see Isolation of DNA From Agarose Gels) and resuspended in H₂O.
**Ligations**

T4 DNA Ligase (Gibco BRL, Gaithersberg, MD) was used to perform ligations according to Sambrook *et al.* (1989) in a final volume of 15 µl. Ligation mixtures were incubated at 15°C overnight.

**Preparation of Competent Cells**

*E. coli* DH5-α competent cells were prepared according to the procedure of Sambrook *et al.* (1989). The cells were aliquoted into 350 µl samples that were mixed with 150 µl of sterile 87% glycerol and stored in cryogenic tubes at -80°C until needed.

**Transformation of Competent Cells**

Competent cells were transformed with the heat shock treatment according to the procedure of Sambrook *et al.* (1989) by using 100 µl of stock cells.

**Genomic Library Construction**

**Packaging of Total Chromosomal DNA Into Cosmids**

The total chromosomal DNA of *P. fluorescens* was partially digested with the *Sau3AI* restriction enzyme by incubating equal amounts of DNA with diluted concentrations of enzyme. The samples were digested for 1 hour at 37°C and heat inactivated by incubation at 65°C for 20 minutes. Samples of 5 µl of the digested
products were run on a gel as illustrated in Figure 4. Since DNA fragments of approximately 30 kb were desired, only DNA from reactions shown in lanes 8-11 were used. The partially digested total DNA was packaged into the cosmid pLAFR3 as described by Staskawicz et al. (1987) and ligated according to Ish-Horowicz and Burke (1981). The final digested cosmid product resulted in pLAFR3 “sticky arms” that were compatible for an insert digested with *Bam*HI. Since *Sau3AI* restriction sites are compatible with *Bam*HI, the partially digested total DNA was ligated with the digested cosmid.

![Figure 4. Partial Digest of Total DNA for Packaging. The total DNA of *P. fluorescens* was digested with diluted concentrations of *Sau3AI*. Lane 1 contains the most enzyme and the completely digested DNA. Lane 18 contains the lowest concentration of enzyme. Samples represented in lanes 8-11 were used for packaging. *λ* represents the marker *λ/HindIII*.](image_url)
The ligated vectors were packaged with the MaxPlax Lambda Packaging Kit according to the manufacturer’s protocol (Epicentre Technologies Corporation, Madison, WI). One hundred microliters of packaged cosmids were resuspended in 100 µl Phage Buffer. *E. coli* DH5-α cells were infected by adding 100 µl of bacteria with 100 µl the resuspended, packaged cosmids. This mixture was incubated at 37°C for 45 minutes. Then, 1 ml of LB broth supplemented with 10 mM MgSO₄ was added to the infected cells and incubated at 37°C for an additional 45 minutes with frequent agitation. The resulting colonies were screened for the pLAFR3 cosmid by plating on LB, Tc²⁰ agar plates supplemented with 10 mM MgSO₄. Approximately 60 CFU per plate were observed. This procedure was repeated until approximately 1,440 colonies were obtained. Plasmid DNA isolation of tetracycline resistant (Tcᴿ) strains verified cosmids with inserts of 32-45 kb. The genomic library strains were stored in glycerol and kept at -80°C.

**Packaging of Mutant Z1 Total Chromosomal DNA Into Cosmids**

The total chromosomal DNA of mutant strain Z1 was packaged into pLAFR3 as described above. The resulting cells were screened on Lb, Tc²⁰, Km²⁰ agar plates supplemented with 10 mM MgSO₄ to select for the cosmid which would carry the transposon Tn5-B20 and surrounding chromosomal DNA.
Packaging Efficiency Assay

The efficiency of the packaging extracts of the MaxPlax Lambda Packaging Kit (Epicentre Technologies Corporation, Madison, WI) was performed according to the manufacturer’s protocol. This included the infection of the control \textit{E. coli} LE392MP with the ligated \(\lambda\) control DNA \(\lambda c1857\). The packaging efficiency was identified as \(3.63 \times 10^9\) plaque forming units (PFU) per \(\mu\)g of DNA.

Southern Analysis

Southern Blotting

A modified method by Sambrook \textit{et al.} (1989) for Southern blotting was used for the transfer of DNA fragments by capillary action from a 0.7\% agarose gel to a positively charged nylone membrane (Boehringer Mannheim, Indianapolis, IN).

Preparation of Chemiluminescent Probes

DNA probes used for blotting were digested by restriction endonuclease activity and separated on an agarose gel by electrophoresis. The desired DNA probe was excised and purified from the gel (see Isolation of DNA from Agarose Gels) and labeled with digoxygenin 11-dUTP according to the manufacturer’s protocol (DIG DNA Labeling Kit; Boehringer Mannheim, Indianapolis, IN).
DNA-DNA Hybridization

Hybridization of the digoxygenin-labeled probe to the DNA on the nylon membrane was performed at 68°C. This and all nylon washing procedures were done according to the manufacturer’s protocol (DIG DNA Detection Kit; Boehringer Mannheim, Indianapolis, IN).

Chemiluminescent Detection

The hybridizing DNA fragments were detected with the chemiluminescence substrate CSPD according to the manufacturer’s protocol (DNA Detection Kit; Boehringer Mannheim, Indianapolis, IN). The nylon membrane was sealed in a plastic hybridization bag and exposed to Kodak X-OMAT X-ray film for a minimum of 20 minutes. Film developing occurred in Kodak Developer (D-19) for 5 minutes. The X-ray film was then washed in water for 2 minutes, fixed in Kodak Fixer for 5 minutes, washed again in water for 5 minutes, and allowed to air dry.

Primers

Oligonucleotide primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) or Gibco/BRL/ Life Technologies (Rockville, MD). The analysis of the quality of the oligonucleotide primers was done using the Primer Calculator program provided by Williamstone Enterprises (http://www.williamstone.com) or the NetPrimer program provided by Premier
Biosoft International (http://www.premierbiosoft.com/netprlaunch.html). All the oligonucleotide primers used in this study are listed in Table 2.

**Table 2**

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<th>Primer name</th>
<th>Sequence</th>
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</tbody>
</table>

* indicates a primer that was not 100% homologous to isolated DNA sequence
Polymerase Chain Reaction (PCR)

A 50µl PCR reaction was prepared with concentrations recommended by the manufacturer (Sigma, St. Louis, MO or Gibco BRL/ Life Technologies, Rockville, MD). The procedure used with REDTaq polymerase from Sigma (St. Louis, MO), included 1 x REDTaq PCR reaction buffer, 5 µl template DNA (1-10 nM), 0.2 mM dNTP's, primers at 0.5 µM each, and 2.5 U REDTaq DNA polymerase. If Taq DNA polymerase from Gibco BRL/Life Technologies was used, then 5 µl template DNA (1-10 nM) was mixed with 1 x PCR Buffer minus Mg, 1.5 mM MgCl₂, 0.5 µM of each primer, 0.2 µM dNTP’s, and 2.5 Units of Taq DNA Polymerase. The PCR reaction volumes were brought to 50 µl with sterile water. The reaction mixtures were covered with 40 µl of mineral oil. The PCR was performed in an automated thermocycler (DNA Thermo Cycler 480, PE Biosystems, Foster City, CA) with the following cycle profile: 30 cycles x [(1 minute at 95°C)(1 minute at 55°C)(2 minutes at 72°C)]. The results of the reaction were documented by loading 10 µl of PCR product into a 0.7% agarose gel, electrophoresed, and stained with ethidium bromide (see Agarose Gel Electrophoresis).

Arbitrary PCR

Arbitrary PCR was performed as described by Rossbach et al. (2000) and O'Toole (1998). This procedure allowed for the amplification of the genetic region
immediately downstream of the transposon as shown in Figure 5 (Caetano-Anolles, 1993). This procedure was performed on the regions flanking the Tn5-B20 insertions in the zinc-responsive mutants as well as for all cadmium-responsive mutants isolated from a previous study in our laboratory (Rossbach, 2000; Kukuk, 1997).

![Figure 5](image)

**Figure 5.** Mechanisms and Primers of Arbitrary PCR Used to Isolate the DNA Sequences Downstream of the Transposon.

The sequences of the isolated PCR products were analyzed by using the Tn5Ext primer and a mixture of the arbitrary primers ARB1 and ARB2. Five microliters of an overnight mutant strain culture grown in 10 mls TY, Km\(^{50}\) was used as the template DNA. The reaction conditions were as follows: 6 x [(30 seconds at 95°C)(30 seconds at 35°C)(2 minutes at 72°C)] followed with 30 x [((30 seconds at 95°C)(30 seconds at 45°C)(2 minutes at 72°C)]. A second round of PCR was performed using mixes as described above, except that 1 µl of the previous PCR was used as the template DNA and the primers used were Tn5Int and ARB2. The reaction conditions for the second round were as follows: 30 x [((30 seconds at 95°C)(30 seconds at 45°C)(2 minutes at 72°C)]. The final PCR product was checked
by loading 10 µl of PCR product into a 2.0% agarose gel and documented as previously described.

**DNA Sequence Determination**

The DNA sequences adjacent to the right end of the Tn5-B20 in all mutant strains was determined using the Tn5int primer that is complementary to the right border of the transposon (IS50). Sequencing of the *zacA* gene was performed using M13, M13 reverse, and costume made primers listed in Table 2.

Automated fluorescent DNA sequencing was performed at the MSU-DOE-PRL Plant Biochemistry facility with the PE Biosystems (Foster City, CA) Catalyst 800 for Taq cycle sequencing and the ABI 377 sequencer and also at the WMU DNA Sequencing Facility by the dideoxy-chain termination method with the ABI PRISM Dye Primer Sequencing Kit (PE Biosystems, Foster City, CA) by using the ABI PRISM 310 Genetic Analyzer (PE Biosystems, Foster City, CA). Here, 0.5 µg template DNA, 3 µl 5 x Buffer, and 2 µl Big Dye Terminator were used in a total sequencing reaction of 20 µl. The GeneAmp PCR System 9700 (PE Biosystems, Foster City, CA) was used to perform the sequencing reactions at the following conditions: 25 cycles x [(5 seconds at 96°C)(5 seconds at 45°C)(4 minutes at 68°C)]. The CENTRI.SEP columns with sphadex was used to remove excess Big Dye Terminator nucleotides according to the manufacturer’s recommendations.
DNA Sequence Analysis

The SeqEd program (Applied Biosystems, Foster City, CA) was used to assemble, translate and analyze the DNA sequences. The CodonUse 3.1 program (Conrad Halling, University of Chicago) with the usage table specific for *P. fluorescens* was used to identify open reading frames (ORF). Predicted DNA and amino acid sequences were compared to sequences in the GenBank database by using the BLAST 2.0 search programs provided by the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/BLAST/).
RESULTS

Determination of Minimal Inhibitory Concentration (MIC)

To identify working concentrations of the metals used in this study, the MIC of several different metals was determined. According to the method of Mergeay (1995), the MIC of ZnSO₄ was determined to be 2.0 mM, CdCl₂ was 200 µM, and CuSO₄ was 2.0 mM with TY medium. The MIC of Pb(C₂H₃O₂)₂ was 1.0 mM with low-phosphate medium (Figure 6). The metal concentrations used in this study were slightly less than the MIC as *P. fluorescens* was challenged but still allowed to grow.

Screening of Mutant Bank with Zinc

The entire 5,000 strains of a previously created Km⁺ *P. fluorescens* mutant bank were replica plated onto TY medium with Xgal and TY medium with Xgal containing 1.0 mM ZnSO₄. This resulted in the identification of 8 mutants that appeared to be induced and four that were repressed. Ten of the zinc-responsive mutants are shown in Figure 7.

Verification of Tn5-B20 Insertions in *P. fluorescens* Mutants

To verify that the Tn5-B20 transposon had generated single and random insertions into the genome of the mutants a hybridization experiment was performed. The total DNA of the zinc-induced mutants was isolated, digested with the restriction
Figure 6. The Minimal Inhibitory Concentrations (MIC) of ZnSO₄, CdCl₂, Pb(C₂H₃O₂)₂, and CuSO₄ for *P. fluorescens*. Experiments were performed on TY medium except the Pb(II) MIC was performed on low-phosphate medium. Y-axis: % of survival. X-axis: ZnSO₄ concentration (mM). Data points represent the average of duplicate experiments.

enzyme *EcoRI* and separated via gel electrophoresis. The probe used was the 1.9 kb *EcoRI-SacI* fragment of pMC1403 containing the *lacZ* gene. A single hybridizing fragment was visible in the *E. coli* positive control because of the *lacZ* gene that it harbors. No hybridizing fragment was observed in *P. fluorescens* indicating that the chromosomal DNA does not contain a *lacZ* gene. One hybridizing fragment was also seen in a second positive control, C11, from another mutant strain isolated previously (Rossbach, 2000). One hybridizing fragment was also evident in all mutant strains
Figure 7. Results of the Screen of the Mutant Bank for Differential Gene Expression in the Presence of 1.0 mM ZnSO$_4$. A. Colonies of mutants strains growing on TY medium without metal supplementation. B. Mutants growing on TY medium with 1.0 mM ZnSO$_4$. C. Designations of the mutants. 

except for mutants 19C9 and 28F1. In these mutants, two hybridizing fragments were visible indicating that the transposon had inserted twice into the genomes. Thus, these two mutants were not analyzed further. Figure 8 illustrates the results of the Southern blot for the zinc-induced mutants. Another blot was performed probing for the transposon insertion in the zinc-repressed mutants, Z7-Z10, also showing single hybridizing fragments of different sizes each (data not shown).

**β-Galactosidase Enzyme Assays of Zinc-Responsive Mutants**

To quantitatively measure the induction or repression of the gene tagged by Tn5-B20 β-galactosidase enzyme assays were performed. Figure 9 illustrates the results of the β-galactosidase enzyme assays for mutants Z2, Z3, Z5, Z6, Z7, Z9, and Z10. Mutants Z4 and Z8 are omitted as these strains were characterized in a previous study (Rossbach *et al.*, 2000). Mutants Z2, Z3, Z5, and Z6 displayed an
1. *E. coli*
2. *P. fluorescens*
3. C11
4. Z1
5. Z6
6. 19C9
7. 28F1
8. Z4
9. Z2
10. Z3
11. Z5
12. λ
13. pMC1403/
    *EcoRI-Sacl*

Figure 8. Southern Blot Showing the Hybridization of Total DNA From Zinc-Induced Mutants and Control Strains Probed With the *lacZ* Gene of Tn5-B20.

induction of approximately 2-fold when exposed to 1.0 mM ZnSO$_4$ and mutants Z7, Z9, and Z10, showed a repression of β-galactosidase activity that was not statistically significant. Mutant Z1 showed the strongest induction of all mutants (6-fold; 234 Miller Units without zinc, 1360 Miller Units with zinc).

Screening of Zinc-Responsive Mutants with Other Metals and Stresses

The insertion of the reporter gene transposon resulted in differential color variations of the mutants when grown on TY plates allowed for them to be screened under a variety of environmental stresses on with media containing Xgal. Mutants Z1 through Z10 were screened for their responsiveness to different metals and other stresses. These included CdCl$_2$, CuSO$_4$, HgCl$_2$, oxidative, solvent, osmotic, and heat
Figure 9. Quantitative β-Galactosidase Enzyme Assay of Zinc-Responsive Mutants. The induction or repression of the genes tagged by the transposon was measured from cultures exposed to 1.0 mM ZnSO₄. White bars represent enzyme activity in TY medium without metal supplementation. Black bars represent enzyme activity when exposed to zinc. Assays were performed in duplicate and the values averaged. Student t-test analysis revealed only mutants Z2-Z5 to be significantly different for the two treatments. Y-axis: β-galactosidase enzyme activity measured in Miller Units. X-axis: zinc-responsive mutant strains. Error bars indicate the standard error.

Shock stresses. The gene tagged by Tn5-B20 in mutant strain Z4 was induced when exposed to Zn(II), Cd(II), Cu(II), EtOH, cumene hydroperoxide, and heat shock. Mutant Z1 was induced only by Zn(II), Cd(II), and Cu(II) and repressed by heat shock. No other mutant showed induction by Cd(II). All other mutant strains showed repressed gene expression in the presence of by Cu(II) and cumene hydroperoxide. Mutant strain Z2 was repressed by EtOH. Mutants Z7, Z8, Z9, and Z10 showed repressed gene expression when exposed to every stress tested. The NaCl, HgCl₂, naladixic acid, and H₂O₂ stresses all showed no effect with mutants, Z1 through Z6 (Table 3).
<table>
<thead>
<tr>
<th>Mutant</th>
<th>ZnSO₄</th>
<th>CdCl₂</th>
<th>CuSO₄</th>
<th>EtOH</th>
<th>Cumene Hydroperoxide</th>
<th>Heat Shock</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Z2</td>
<td>+</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Z3</td>
<td>+</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Z4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Z5</td>
<td>+</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Z6</td>
<td>+</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Z7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Z8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Z9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Z10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ induction when grown on TY plates containing X-gal
- repression when grown on TY plates containing X-gal
0 no color change of colonies on TY plates with added stress
Genomic Analysis of the Z1 Gene Tagged by Tn5-B20

Arbitrary PCR was performed to amplify the genes that were differentially expressed when exposed to metal stress. This experiment resulted in the successful amplification of approximately 200-400 bases immediately downstream of the insertion site of the transposon in 9 out of 10 mutants. The arbitrary PCR DNA sequence of mutant Z1 showed 60% similarity, when translated, to ZntA of *E. coli* and 56% similarity to CadA of *S. aureus*. DNA sequence analysis of the arbitrary PCR product from mutant Z1 indicated that the transposon had inserted just downstream of a DNA region encoding the Cys-Pro-Cys (CPC) motif. This motif is a common characteristic of P-type ATPases. The sequence also revealed the presence of a conserved aspartate residue downstream of the transposon that is important for the phosphorylation cycle of the protein (Figure 10).

```
P.f.Zl   ... VISTPVTIVSGLAAAARKGILIKGAGVLYLEGGYKLDYLDLABDTITGTHGKVPCTDYLPE ...  
E.c.ZntA 392 VISTPAATTSGLAAAARRGLIKGGAALQOLGRTQVAFDKTGTLTVCNKPKRTAHPA 454  
S.a.CadA 371 VISTPISLVSAIGNAAKKGVLGKVVGYLEKLGAIKTVAFKDTGLTKGVEVVTDFEVT 433
```

Figure 10. Alignment of the Predicted Protein Sequence From the Translated Arbitrary PCR Product of Mutant Z1 With the P-type ATPases ZntA of *E. coli* (*E.c. ZntA*) and CadA of *S. aureus* (*S.a. CadA*). The asterisk highlights the aspartate residue that is predicted to be phosphorylated in P-type ATPases. Identical amino acids are shaded. Amino acid residue numbers are given on each line.
Isolation and Identification of the Gene Tagged in Mutant Z1

Packaging of Mutant Z1 Total Chromosomal DNA Into the Cosmid pLAFR3

Since the arbitrary PCR analysis resulted in only a partial sequence of the gene tagged in mutant Z1, the entire gene was isolated by a different method. The total chromosomal DNA of mutant Z1 was isolated, partially digested with Sau3AI, ligated to pLAFR3, and transduced into DH5-α cells as described in Materials and Methods. The resulting cells were selected on LB, Tc$^{20}$, Km$^{50}$ plates supplemented with 10 mM MgSO$_4$. A clone was identified that was Tc$^R$ and Km$^R$. This was named as pTW1000.

The region downstream of the transposon in clone TW1000 was mapped with restriction sites and subcloned by isolating an 11 kb EcoRI fragment that was ligated with pBluescript KS+ vector and was named pTW1002 (Figure 11). The 1.7 kb HpaI-HindIII fragment of pTW1002 that contained the immediate end of the transposon was subcloned into pBluescript KS+ and termed pTW1004 (Figure 11).

Isolation of the Wild Type Gene Tagged in Mutant Z1

To isolate the corresponding wild type gene tagged in mutant Z1 and identify its DNA sequence, a genomic library of 1,440 strains containing P. fluorescens DNA was created to isolate the corresponding wild type gene.

The 1.7 kb HpaI-HindIII fragment from pTW1004 was used as a probe to screen the genomic library for the wild type gene. The library was screened by
Figure 11. Restriction Map of Cosmid Clone pTW1000 and Derived Subclones. Abbreviations: E, EcoRI; H, HindIII; Hp, HpaI; and P, PstI.
pooling together minipreps from the microtiter plates of the genomic library. The Southern blot resulted in a hybridizing fragment from one of the pooled miniprep solutions. To identify exactly which clone contained the gene of interest, PCR was performed using primers TW2 and TW3 that are specific for the *zacA* sequence (Figure 12). The expected 66 bp fragment resulting from amplification with these primers was identified in one of the ten clones. This clone was named pTW1005 (Figure 12).

Sequence Determination the Gene Tagged in Mutant Z1

Subclones of pTW1005

An *EcoRI* digest of pTW1005 was subjected to Southern analysis to identify a fragment containing *zacA*. The 1.7 *HpaI-HindIII* fragment of pTW1004 was used as a probe. A 2.7 kb hybridizing fragment was identified. This fragment was excised from an agarose gel and ligated into the pBluescript KS+ vector resulting in pTW1006 (Figure 12). The wild type DNA was used for sequencing when possible. Therefore, pTW1006 was used for most of the sequencing. However, since pTW1005 randomly packaged the total DNA, the extreme right end of the gene tagged in mutant Z1 was not contained in pTW1005 (Figure 12). Thus, pTW1006 also did not contain the right end of the gene. Therefore, the sequence determination of the right end of the gene tagged in mutant Z1 was determined from subclones pTW1002 and pTW1004.
Figure 12. Genetic Map of pTW1005 and pTW1006. The large open arrows indicate open reading frames (ORF) of \textit{zacA} and \textit{rpoS}. The small closed arrow indicates the position of the transposon insertion in mutant Z1 with relation to \textit{zacA}. Small open arrows indicate the location and orientation of some primers used in this study. Abbreviations: E, \textit{EcoRI}; Pv, \textit{PvuII}; and X, \textit{XhoI}. 
Analysis of Gene Sequence Tagged in Mutant Z1

After further sequencing of the gene, it was determined that the gene shared highest similarity, when translated, to *cadA* of *S. aureus*. This is in contrast to the finding with the original arbitrary PCR product showing highest similarity to *zntA*. This is probably because the arbitrary PCR product was much shorter and the region originally sequenced happened to encode the membrane channel of ZntA. This is plausible because of *cadA* and *zntA* both being P-type ATPases are 35% similar to each other. With the sequence of the gene tagged in mutant Z1 determined, similarity with *cadA* was 45% and similarity with *zntA* was 41% (Figure 13).

Highlighted in the boxes of Figure 13 are motifs conserved among P-type ATPases. The CPC motif equivalent to amino acid position 371 of CadA is part of the transmembrane channel of the protein (Nucifora *et al.*, 1989). The aspartate residue and the highly conserved sequence following it at position 415 of CadA is hypothesized to be the phosphorylated in the ATPase cycle (Silver *et al.*, 1989). The same residue and conserved sequence is found in both *zntA* and the gene tagged in mutant Z1. Also, a conserved lysine residue is found at the equivalent amino acid position 567 of CadA (Figure 13). This residue could be a conserved ATP binding site.

Upon sequencing of the gene, the expected N-terminus of the deduced protein was not identified because the sequence started only at the amino acid position equivalent o position 122 of CadA (Figure 13). Sequence analysis also showed 93% similarity, when translated in the opposite direction, with the C- terminus of RpoS
Figure 13. Alignment of the Predicted Protein Sequence of the Gene Tagged in Mutant Z1 from *P. fluorescens* ATCC13525 CadA of *S. aureus* and ZntA of *E. coli*. The arrow indicates the position of the transposon insertion. Identical amino acids are shaded. The CPC and putative phosphorylation and ATP binding sites are boxed. Amino acid residue numbers are given on each line.
of *Pseudomonas tolaasi* (Figure 12).

A possible explanation for this unexpected result is that the *zacA* gene was scrambled during the ligating and packaging of the pTW1005 cosmid clone. To verify this, PCR was performed with wild type chromosomal DNA and two sets of primers, TW11 with TW15 and TW16 with TW15 (Figure 12). The expected size of the PCR product resulting with primers TW11 and TW15 is 1.9 kb. This was indeed observed. If the structure found to be present on pTW1005 was identical to the chromosomal DNA of *P. fluorescens*, then a PCR product of 2.3 kb would be observed using primers TW16 and TW15. This was not observed.

The Gene Tagged in Mutant Z1 Is Induced by Several Metals

Because the gene tagged in mutant Z1 was similar to *cadA* of *S. aureus* and *zntA* of *E. coli*, the gene was analysed to determine if it was responsive to a similar spectrum of metals. The β-galactosidase activity of mutant strain Z1 was measured to be significantly induced approximately 6-fold with exposure to Zn(II), 3.6-fold with Hg(II), 2.6-fold with Cd(II), 2.6-fold Pb(II), 2-fold with Ni(II), and 1.5-fold with Ag(I). Exposure to Cu(II) resulted in enzyme activity that was not significantly induced compared to that with no metal. The induction of the gene is illustrated in Figure 14.
Figure 14. β-Galactosidase Enzyme Assay of Mutant Z1 with Several Metals. The induction of the tagged gene was measured for several metal when xposed to 2 hours. Assays were performed from duplicate cultures and the values averaged. Student t-test analysis showed significant induction when exposed to all of the metals tested except for copper. Y-axis: β-galactosidase enzyme activity measured in Miller Units. X-axis: metal ions supplemented into the medium. Error bars indicate the standard error.

Sensitivity of Zinc-Responsive Mutants to Other Metals

To test our hypothesis, whether the genes displaying differential expression play a role in metal tolerance in *P. fluorescens*, the MIC for several different metals according to the method of Lim and Cooksey (1993) was determined for all mutants. The metals used were Zn(II), Cd(II), Cu(II), Ni(II), Co(II), Ag(I), and Hg(II). The results are summarized in Table 4. Because of metal solubility problems with the complex medium, the MIC for Pb(II) was determined using low phosphate medium according to the method of Mergeay (1995). All zinc-responsive mutants except for
Zl and Z9 showed MIC values identical to the wild type culture. Mutant Z1 showed a lower MIC of Zn(II) and Cd(II) compared to the wild type. For this reason, the gene tagged by the transposon in mutant strain Z1 was named *zacA* for zinc and cadmium tolerance. Mutant Z1 was also more sensitive to Pb(C$_2$H$_3$O$_2$)$_2$ compared to the wild type. Mutant Z9 not only showed a lower MIC compared to the wild type with Zn(II) and Cd(II), and but also with Cu(II).

**Mutants Z1 and Z9 Are Sensitive to ZnSO$_4$**

To further prove that the disruption of the tagged genes in strains Z1 and Z9 leads to metal sensitivity, the growth characteristics of wild type, Z1, and Z9 were observed after addition of zinc to broth cultures (Figure 15). The addition of zinc in log phase growth had a negligible effect on the wild type culture as the growth with the metal soon regained growth comparable to the culture without added metal (Figure 15A). Inhibition of growth was seen in mutant Z1 after the addition of zinc as compared to the culture without metal added (Figure 15B). Growth of mutant Z1 did recover, however, approximately 14 hours after the addition of zinc. Severe inhibition of growth was observed in mutant Z9 after the addition of zinc as compared to the culture without metal added. Growth of mutant Z9 was not able to recover up to approximately 50 hours after the addition of the metal (Figure 15C).
Table 4
The MIC of Different Metal Ions For *P. fluorescens* Wild Type and Mutant Strains

<table>
<thead>
<tr>
<th>Metal</th>
<th>Wild Type, Z2-Z8, and Z10</th>
<th>Z1</th>
<th>Z9</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnSO₄</td>
<td>8 mM</td>
<td>4 mM</td>
<td>4 mM</td>
</tr>
<tr>
<td>CdCl₂</td>
<td>2 mM</td>
<td>1 mM</td>
<td>1 mM</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>4 mM</td>
<td>4 mM</td>
<td>1 mM</td>
</tr>
<tr>
<td>NiCl₂</td>
<td>4 mM</td>
<td>4 mM</td>
<td>4 mM</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>2 mM</td>
<td>2 mM</td>
<td>2 mM</td>
</tr>
<tr>
<td>AgNO₃</td>
<td>0.25 mM</td>
<td>0.25 mM</td>
<td>0.25 mM</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>0.040 mM</td>
<td>0.040 mM</td>
<td>0.040 mM</td>
</tr>
</tbody>
</table>

Mutant Z9 Is Sensitive to CuSO₄

The growth characteristics of wild type and Z9 were observed after addition of copper to broth cultures (Figure 16). The addition of 2.0 mM copper in log phase growth had no effect of the wild type culture, as the growth was almost identical to that of the culture with metal added (Figure 16A). Inhibition of growth was seen in mutant Z9 after the addition of copper as compared to the culture without metal added. Growth did recover, however, approximately 25 hours after the addition of copper (Figure 16B).
Figure 15. Growth Characteristics of Wild Type, Z1, and Z9 Cultures With and Without Exposure to 1.5 mM ZnSO₄. A. Growth of *P. fluorescens* ATCC13525 over time. B. Growth of *P. fluorescens* mutant Z1 over time. C. Growth of *P. fluorescens* mutant Z9 over time. Open circles represent bacterial growth without the addition of zinc. Black squares represent growth with Zn(II). The arrows show the time at which the metal was added. Y-axis: optical density measured at 600 nm (O.D.₆₀₀). X-axis: time in hours. The data points are the average of two individual cultures.
Figure 16. Growth Characteristics of Wild Type and Z9 Cultures With and Without Exposure to 2.0 mM CuSO₄. A. Growth of *P. fluorescens* ATCC13525 over time. B. Growth of *P. fluorescens* mutant Z9 over time. Open circles represent bacterial growth without the addition of copper. Black squares represent growth with copper. The arrows show the time at which the metal was added. Y-axis: optical density measured at 600 nm (O.D.₆₀₀). X-axis: time in hours. The data points are the average to two individual cultures.
The sequence of the gene tagged in mutant Z9 was determined from sequencing the arbitrary PCR product. No similarity was shown to other known genes. Other mutants that showed no similarity to known genes were strains Z7 and Z8. Thus, this gives no indication for a function of these genes. The DNA sequence of the gene tagged in mutant Z10 could not be determined (Appendix A).

The gene tagged in mutant Z4, which was induced by most stresses analyzed, showed 30% sequence similarity, when translated, to the zinc metalloprotease NprE of *B. amyloliquefaciens* (Appendix A). The NprE protein is a catalytic enzyme that requires zinc for its activity (Vasantha *et al.*, 1984).

Genomic Analysis of the Genes Tagged in Mutants Z2, Z3, Z5, and Z6

The analysis of the arbitrary PCR products from the genes tagged by the Tn5-B20 transposon in mutants Z2, Z3, Z5, and Z6 resulted in the identification of several different open reading frames (ORF) that shared high similarity, when translated, with pyoverdine synthetase genes of *Pseudomonas aeruginosa* (Figure 17 and Appendix A). The ORF tagged in mutant Z2 shared highest similarity, when translated, with the pyoverdine synthetase D protein (PvdD) of *P. aeruginosa*. PvdD, a large protein of 2448 amino acids that is involved in the synthesis of pyoverdine, contains sequences that probably arose via gene duplication since various regions of this protein show high similarity to each other. The genomic region adjacent to the transposon insertion in mutant Z2 shared 55% and 53% identity with amino acids 176
to 310 and 1237 to 1371 of pyoverdine synthetase D of *P. aeruginosa* (Figure 17A). An independent transposon insertion in the same gene of mutant Z5 seems to have occurred since the DNA region adjacent to the transposon shared, when translated, 59% identity with the C-terminal region (amino acids 2168 to 2283) of the same enzyme (Figure 17B). The translated genomic DNA region adjacent to the transposon insertion in mutant Z3 displayed 80% identity to another enzyme also involved in the biosynthesis of pyoverdine, pyoverdine synthetase F of *P. aeruginosa* (Figure 17C). The translated DNA region of mutant Z6 shared 53% identical amino acids with gramicidin S synthetase of *Brevibacillus brevis* and 50% similarity to pyoverdine synthetase B of *P. fluorescens* ATCC17400 (Figure 17D). Gramicidin, compared to pyoverdine, is another cyclic peptide that is synthesized non-ribosomally.

**Screen for Siderophore Production**

Since the arbitrary PCR results indicate that the genes tagged in mutants Z2, Z3, Z5, and Z6 may be involved in the synthesis of pyoverdine, all of the zinc-responsive mutants were screened for their ability to synthesize siderophores. Since the insertion of the transposon into the DNA disrupts the function of the gene, those four mutants should not be able to produce pyoverdine. Pyoverdine production was detected by growing the mutant strains on *Pseudomonas* F medium (Figure 18). When produced by *P. fluorescens*, pyoverdine displays a characteristic yellowish-green color that becomes very evident on *Pseudomonas* F medium. All of the zinc-
Figure 17. Similarities of the Putative Amino Acid Sequences Deduced From the Tn5-B20 tagged ORFs in Zinc-Induced *P. fluorescens* Mutants Z2, Z5, Z3, and Z6. A. Alignment of the predicted protein sequence from the translated arbitrary PCR product of mutant Z2 (*P. f.* Z2) with two regions in pyoverdine synthetase D of *Pseudomonas aeruginosa* (*P. a.* PvdD). B. Alignment of the predicted protein sequence from the translated arbitrary PCR product of mutant Z5 (*P. f.* Z5) with a different region of pyoverdine synthetase D of *P. aeruginosa* (*P. a.* PvdD). B. Alignment of the predicted protein sequence from the translated arbitrary PCR product of mutant Z3 (*P. f.* Z3) with pyoverdine synthetase F of *P. aeruginosa* (*P. a.* PvdF). D. Alignment of the predicted protein sequence from the translated arbitrary PCR product of mutant Z6 (*P. f.* Z6) with gramicidin S synthetase of *Brevibacillus brevis* (*B. b.* GrsA) and with pyoverdine synthetase B (*P. f.* PvsB) of *P. fluorescens* strain ATCC17400. Identical amino acids are shaded. Amino acid residue numbers are given on each line.
Figure 18. Illustration of Siderophore Production. Strains were grown on *Pseudomonas* F medium to enhance the production of the yellowish-green siderophore pyoverdine.
responsive mutants, except for strains Z2, Z3, Z5, and Z6, produced a yellowish-green color.

Another characteristic of pyoverdine is that, when analyzed spectrophotometrically, it has a peak absorbance of 403 nm (McMorran et al., 1996). The supernatants from overnight cultures of the mutants were analyzed. Figure 19 shows an example of the wavelength scan for the pyoverdine peak with mutant Z6. Only mutants Z2, Z3, Z5, and Z6 did not show the characteristic peak at 403 nm. These findings further prove that the transposon had, indeed, inserted into genes responsible for the production of pyoverdine.

Figure 19. Spectrophotometric Analysis of Siderophore production. The supernatant of wild type and mutant cultures were scanned with a Beckman DU spectrophotometer for a peak absorbance of 403 nm. This graph illustrates the absorbance pattern of wild type (top purple line) and mutant Z6 (bottom green line). The blank used to eliminate background is shown as the blue line. Y-axis: absorbance. X-axis: wavelengths scanned in nm.
Induction of Pyoverdine Synthetase Genes During Zinc Excess or Iron Limiting Conditions

Since the tagged genes of mutants Z2, Z3, Z5, and Z6 share similarity with genes normally expressed during iron starvation, the induction of these genes was investigated quantitatively with β-galactosidase enzyme assays. To create conditions of iron starvation, the cells were grown overnight in the presence of the iron chelator 2,2'-dipyridyl. When grown in the presence of Zn(II), all four mutants were approximately 2-fold induced (Figure 10). During iron starvation, however, strains Z2, Z3, Z5, and Z6 were induced approximately 4.5-, 4.3-, 9.5-, and 1.5-fold, respectively (Figure 20.)
Figure 20. β-Galactosidase Enzyme Assay of Mutants Z2, Z3, Z5, and Z6. The level of induction of the genes was measured when the strains were exposed to 0.2 mM 2,2'-dipyridyl. White bars, TY medium only; black bars, TY medium with iron chelator. Y-axis: β-galactosidase enzyme activity measured in Miller Units. X-axis: mutant strains. Assays were performed in triplicate and the values averaged.
DISCUSSION

In this study, genes used by the soil bacterium *P. fluorescens* to cope with heavy metal exposure were identified and characterized. Transposon mutagenesis with the Tn5-B20 reporter transposon was used to create strains with mutated genes that were analyzed for induction or repression upon exposure to heavy metal stresses.

Of 10 zinc-responsive mutants, 6 had tagged genes that were induced and 4 had tagged genes that were repressed when exposed to 1.0 mM ZnSO$_4$. Genetic analysis of the DNA region downstream of the Tn5-B20 insertion was performed by elucidating the sequence of the arbitrary PCR product. A few of the resulting sequences from the zinc-responsive mutants shared similarity with sequences from unfinished microbial genomes. Others did not share sequences with any known gene. Thus, these genes are responsive to exposure to ZnSO$_4$, but there is no indication for their exact function.

Mutant Z1 had a gene tagged by the transposon that was shown to be induced when exposed to most metals tested including Zn(II), Hg(II), Cd(II), Pb(II), Ni(II), and Ag(I). This mutant was sensitive to Zn(II), Cd(II), and Pb(II). The gene was termed *zacA* because of its sensitivity for zinc and cadmium. When the DNA flanking the Tn5-B20 insertion was sequenced, it was determined to be similar 45% similar to *cadA* of *S. aureus* and 41% similar to *zntA* of *E. coli*. CadA and ZntA are P-type ATPases that translocate Zn(II) and Cd(II) out of the cell thereby allowing for the resistance of *S. aureus* and *E. coli* to the metals.
Many of the highly conserved amino acid residues that are present in CadA are also present in ZacA. The entire sequence of CadA contains four cysteine residues. The first two cysteines are located at positions 23 and 26 (Nucifora et al., 1989). This is where cadmium recognition and binding is thought to occur in *S. aureus*. The last pair of cysteines is located in the membrane channel at positions 371 and 373. Between the pair of cysteines in the channel is a proline residue (Nucifora et al., 1989). Together, the last pair of cysteines and the proline comprise the highly conserved CPC motif. In ZacA, with the protein sequence as determined to this date, similarity started with amino acid 122 of CadA. Therefore, the first two putative cadmium-binding cysteines are not present in ZacA. The highly conserved CPC motif, however, is present (Figure 20). Thus, the CPC motif could be an integral structure necessary for the translocation of the metal across the membrane since it is located within the transmembrane channel. A third cysteine residue is present, however, in ZacA comparable to position 511 of CadA. In CadA an aspartate at position 415 is followed by a highly conserved sequence of TGTL(or l)T (Nucifora et al., 1989). This motif is also present in ZacA (Figure 20). This aspartate is proposed to be phosphorylated in the ATPase cycle. Finally, a lysine residue at position 489 of CadA is proposed to bind ATP (Silver et al., 1989). This lysine was not present in ZacA or ZntA. A lysine residue is conserved, however, in all three proteins at position 567 of CadA.

The N-terminus of ZacA can not be described conclusively because the protein sequence only starts similarity with CadA at position 122 of CadA. It is possible that, if this were the structure of the protein, ZacA could be used as a pump
if the metals were presented to it. This concept might be plausible since a possible mechanism of the CadC protein, a separate cytoplasmic protein, is to sequester intracellular cadmium and present it to CadA (Ivey et al., 1992). It is possible that a similar protein in *P. fluorescens* binds intracellular Zn(II) and Cd(II) and presents the metal to ZacA to be expelled from the cell.

If the sequence of ZacA determined in this paper is complete as reported here, analysis of the DNA sequence should reveal a start codon at the position where the similarity starts. The first start codon was, however, observed 224 bases upstream from the site where the gene similarity starts. Moreover, the CodonUse profile starts to show high probability of an ORF at the same point where the similarity starts. This would speak against the sequence determined in this study to be the entire gene. Hence, the gene may have been scrambled during packaging of the genomic library clone. This could explain the other truncated gene, *rpoS*, that was located in the opposite orientation upstream of the *zacA* sequence. Further evidence of a scrambled *zacA* gene was provided by PCR that was not able to show that the structure found on the cosmid clone was equivalent to the genomic structure.

Also, an interesting feature of ZacA is that it was isolated and identified to be induced in the presence of Zn(II) and Cd(II) even though *P. fluorescens* was not isolated from a metal contaminated site. Since similar mechanisms of CadA and ZntA are used by *S. aureus* and *E. coli*, respectively, to be resistant to high levels of the metals, ZacA of *P. fluorescens* probably functions in homeostatic mechanisms. Based on amino acid similarity and metal sensitivity ZacA is probably responsible for
zinc and cadmium tolerance of \textit{P. fluorescens} by translocating these ions out of the cell by P-type ATPase activity.

Interestingly, a previous study in our laboratory has revealed another zinc and cadmium tolerance system, \textit{czy}, that is similar to the metal resistance mechanism used by \textit{Alcaligenes eutrophus} (Feng, 2000). Thus, it seems that these homeostatic genes of \textit{P. fluorescens} could be the ancestral genes used by highly metal resistance bacteria. Because the chromosomal genes already allowed for a lower level maintenance of the metal concentrations within the cell, the genes, during selection, probably evolved through duplications and mutations. Once duplicated, the genes were free to mutate to confer metal resistance with highly efficient mechanisms and often are found on plasmids.

The tagged gene of mutant Z9 showed no match to any known gene. This mutant also was shown to be repressed when exposed to 1.0 mM ZnSO$_4$. Interestingly, this mutant was also sensitive to Zn(II), Cd(II), and Cu(II). Thus, the gene tagged with the transposon is repressed when exposed to zinc and is involved with Zn(II), Cd(II), and Cu(II) tolerance. However, the exact function of this gene is, as of yet, unknown.

Another mutant, Z4, was also identified as a cadmium-induced mutant. The gene tagged in this mutant was induced by several stresses including elevated concentrations of Zn(II), Cd(II), Cu(II), EtOH, cumene hydroperoxide, and temperature. Therefore, this gene may represent a general stress response gene.

When the partial DNA sequence of this gene was translated, it showed similarity to NprE of \textit{B. amyloliquefaciens}. The NprE protein is a catalytic enzyme, bacillolysin,
that requires zinc for its proteolytic activity (Vasantha et al., 1984). It is interesting to note that NprE is a protease that would degrade other proteins. Thus, the exposure to a variety of stresses must require the cell to produce the protease possibly to prevent the accumulation of damaged proteins.

Zinc-induced mutants Z2, Z3, Z5, and Z6 were all induced approximately 2-fold when exposed to 1.0 mM ZnSO$_4$. However, in iron limited conditions, strains Z2, Z3, Z5, and Z6 were induced approximately 4.5-, 4.3-, 9.5- and 1.5-fold, respectively. Genetic analysis of the genes disrupted by the transposon in strains Z2, Z3, and Z5 showed DNA sequence similarities to pyoverdine synthetase genes. Mutant Z6 showed highest similarity to a gene encoding gramicidin S synthetase of B. brevis. However, a slightly lower sequence similarity was also seen with pyooverdine synthetase B of P. fluorescens ATCC17400. Since both gramicidin and pyoverdine are both relatively similar because they are peptides made by large enzyme complexes, it was concluded that the tagged gene in mutant Z6 encoded for a pyoverdine synthetase. Furthermore, mutants Z2, Z3, Z5, and Z6 were all unable to produce pyoverdine. Thus, it was concluded that all of the tagged genes were indeed involved in the synthesis of pyoverdine.

The induction of siderophore synthetase genes during exposure to elevated concentrations of Zn(II) has not been previously described. However, it has been shown that the amount of pyoverdine production was stimulated by zinc (Hoefte, 1994). A possible explanation for this phenomenon could be that, when the bacterial cells are flooded with excess Zn(II), the cells are overwhelmed with Zn(II) entering the cell. This would cause a decrease in the available Fe(II) that would enter the cell.
The excess Zn(II) may bind the siderophores with a higher affinity than Fe(II). If the Zn(II) binds the siderophore, Fe(II) will not be brought into the cell. This would create a greater need for Fe(II) further inducing the production of more siderophores including pyoverdine.

Another possible explanation for pyoverdine synthetase induction when *P. fluorescens* is exposed to elevated concentrations of Zn(II) could be that, when the metal is flooding the cell and the cytoplasm, the ions are also interacting with Fe(II) regulating genes. This could occur if Zn(II) bound a protein that was repressing transcription of pyoverdine synthetase genes. This could release the repressor protein from the DNA and allow for the eventual production of pyoverdine. An example of a regulator protein of Fe(II) transporting genes is Fur (ferric uptake regulator). When iron is present in the cytoplasm, Fe(II) binds to Fur which then binds to the promoter of genes that, when translated, result in the transport of iron into the cell. Under iron-limited conditions, Fe(II) is not available to bind Fur allowing for the dissociation from the promoter and resulting in the transcription the iron-regulated genes. It has been shown that metals such as Zn(II), Cd(II) and others also bind Fur (Bagg and Neilands, 1987). Thus, Zn(II) could also be used as a co-repressor of Fur. This, however, implies that excess Zn(II) would prevent the transcription of pyoverdine synthetase genes. In this study, however, Zn(II) was shown to induce the expression of siderophore genes. This implies that Zn(II) may also interact with proteins other than Fur to regulate these genes.

In conclusion, we were able to establish that a specific set of genes were identified in *P. fluorescens* that are expressed when the bacterium is exposed to
elevated concentrations of zinc. Most of the genes identified were different from cadmium-responsive genes reporter in an earlier report (Appendix B, Rossbach et al., 2000). Moreover, genes were also identified that are responsive to essential and toxic metal stress thereby modulating metal homeostasis within the cell.
Appendix A

Genetic Analysis of Arbitrary PCR Products From Genes Tagged by Tn5-B20 in Zinc-Responsive *P. fluorescens* Mutants
<table>
<thead>
<tr>
<th>Mutant</th>
<th>Sequence Similarity</th>
<th>% Identity (identical aa/total aa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z1</td>
<td>ZntA of <em>E. coli</em></td>
<td>60% (35/58)</td>
</tr>
<tr>
<td>Z2</td>
<td>Pyoverdine synthetase D of <em>P. aeruginosa</em> (N-terminus)</td>
<td>55% (74/135)</td>
</tr>
<tr>
<td></td>
<td>Pyoverdine synthetase D of <em>P. aeruginosa</em> (C-terminus)</td>
<td>54% (73/135)</td>
</tr>
<tr>
<td>Z3</td>
<td>Pyoverdine synthetase F of <em>P. aeruginosa</em></td>
<td>80% (95/119)</td>
</tr>
<tr>
<td>Z4</td>
<td>Zinc metalloprotease NprE of <em>B. amyloliquefaciens</em></td>
<td>30% (20/66)</td>
</tr>
<tr>
<td>Z5</td>
<td>Pyoverdine synthetase D of <em>P. aeruginosa</em></td>
<td>59% (69/116)</td>
</tr>
<tr>
<td>Z6</td>
<td>Gramicidin S synthetase I of <em>B. brevis</em></td>
<td>53% (19/36)</td>
</tr>
<tr>
<td></td>
<td>Pyoverdine synthetase B of <em>P. fluorescens ATCC17400</em></td>
<td>50% (18/36)</td>
</tr>
<tr>
<td>Z7</td>
<td>No match</td>
<td>-</td>
</tr>
<tr>
<td>Z8</td>
<td>No match</td>
<td>-</td>
</tr>
<tr>
<td>Z9</td>
<td>No match</td>
<td>-</td>
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<tr>
<td>Z10</td>
<td>ND</td>
<td>-</td>
</tr>
</tbody>
</table>

ND, Not determined due to unobtainable sequence from the sequencing reaction
No match, no significant similarity to any sequence in the database using BLASTX or BLASTN
Appendix B

Genetic Analysis of Arbitrary PCR Products From Genes Tagged by Tn5-B20 in Cadmium-Responsive *P. fluorescens* Mutants
<table>
<thead>
<tr>
<th>Mutant</th>
<th>Sequence Similarity</th>
<th>% Identity (identical aa/total aa)</th>
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</thead>
<tbody>
<tr>
<td>C1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td><em>Shewanella putrefaciens</em> 4317 unfinished genome</td>
<td>33% (28/83)</td>
</tr>
<tr>
<td>C4</td>
<td>Contig54 of <em>Pseudomonas aeruginosa</em> unfinished genome</td>
<td>77% (24/33)</td>
</tr>
<tr>
<td>C6</td>
<td>Iron-uptake factor PiuB of <em>aeruginosa</em> unfinished genome</td>
<td>31% (50/161)</td>
</tr>
<tr>
<td>C7</td>
<td>Ferric transport ATP-binding protein (AfuC) of <em>Actinobacillus pleuropneumoniae</em></td>
<td>45% (58/125)</td>
</tr>
<tr>
<td>C8</td>
<td>Zinc metalloprotease NprE of <em>Bacillus amyloliquefaciens</em></td>
<td>30% (20/66)</td>
</tr>
<tr>
<td>C9</td>
<td><em>Vibrio cholerae</em> 666 1741 unfinished genome</td>
<td>61% (36/59)</td>
</tr>
<tr>
<td>C10</td>
<td>Acyl CoA synthetase of <em>Vibrio cholerae</em></td>
<td>46% (19/41)</td>
</tr>
<tr>
<td>C11</td>
<td>CzrR of <em>Pseudomonas aeruginosa</em></td>
<td>80% (40/50)</td>
</tr>
<tr>
<td>C12</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>C13</td>
<td>Contig 2277.1 of <em>Bordetella bronchiseptica</em> unfinished genome</td>
<td>60% (42/69)</td>
</tr>
<tr>
<td>Mutant</td>
<td>Sequence Similarity</td>
<td>% Identity (identical aa/total aa)</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------------------------------------------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>C15</td>
<td>Contig 343 of <em>Salmonella typhi</em> unfinished genome</td>
<td>38% (52/135)</td>
</tr>
<tr>
<td>C16</td>
<td>No match</td>
<td>-</td>
</tr>
<tr>
<td>C17</td>
<td>No match</td>
<td>-</td>
</tr>
</tbody>
</table>

ND, Not determined because of poor sequencing reaction
No match, no significant similarity to any sequence in the database using BLASTX or BLASTN


