This is to certify that the

dissertation entitled

Genetic and physiological characterization of a selenite-resistance determinant from an F-like plasmid of Stenotrophomonas maltophilia ORO2

presented by

Jonathan James Caguiat

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology

Major professor
Julius H. Jackson

Date 9/22/95

GENETIC AND PHYSIOLOGICAL CHARACTERIZATION OF A SELENITE-RESISTANCE DETERMINANT FROM AN F-LIKE PLASMID OF STENOTROPHOMONAS MALTOPHILIA ORO2

By

Jonathan James Caguiat

A DISSERTATION

Submitted to
Michigan State Univesity
in partial fufillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology

1995

ABSTRACT

GENETIC AND PHYSIOLOGICAL CHARACTERIZATION OF A SELENITE-RESISTANCE DETERMINANT FROM AN F-LIKE PLASMID OF STENOTROPHOMONAS MALTOPHILIA ORO2

By

Jonathan James Caguiat

Stenotrophomonas maltophilia ORO2 displayed growth resistance to several heavy metal salts when it was first isolated from mercury contaminated soil. Transformation of *Escherichia coli* HB101 with genomic DNA from *S. maltophilia* ORO2 yielded transformants containing pOR1, a 100 kb plasmid that conferred resistance to Se(IV), Pb(II) and Hg(II). Cloning of *Hin*dIII fragments from pOR1 into pBR322 revealed that selenite-resistance was encoded by a 4 kb fragment in the recombinant plasmid, pLJ100. Southern hybridizations of cloned pOR1 fragments to blots of agarose electrophoretic gels containing digestions of pOR1 resolved 60% of the physical map of pOR1. Double restriction endonuclease analysis located the position of the uncloned fragments on the map. Transcription and translation in an *in vitro* expression system showed that the 4 kb *Hin*dIII fragment encoded a 35 kDa polypeptide. The nucleotide sequence of this fragment revealed that it contained a 2.2 kb segment identical to transposon, Tn*1000*; a 400 bp segment identical to *rep2A* from *repFIC*; and open reading frames for a 3.7 kDa hypothetical polypeptide and a 35 kDa polypeptide not associated with Tn*1000*. The sequence of 15 N-terminal amino acid residues from the purified 35 kDa polypeptide matched the amino acid sequence of the 35 kDa open reading frame. Amino acid sequence comparison of this new selenite dissimilatory reduction polypeptide, SedR, to other known sequences did not reveal a relationship that implied its function. Growth experiments demonstrated that the selenite-resistant strains reduced selenite to

elemental selenium. Earlier experiments suggested that glutathione, glutathione reductase, thioredoxin, and thioredoxin reductase may be involved in this pathway. Experiments with *E. coli* mutants for glutathione and thioredoxin biosynthesis and reduction showed that the wild type strain, which was already resistant to selenite, depended upon glutathione and thioredoxin reductase. The recombinant plasmid, pLJ100, increased the enhanced resistance of the wild type and the mutant strains to selenite. The 4 kb insert may influence selenite resistance by encoding a redoxin that is reduced by other reductases, producing a reductase that reduces other redoxins or encoding a regulator that induces some other selenite-resistance pathway. In this study, I located the position of selenite-resistance on the physical map of pOR1, sequenced a 4 kb fragment that conferred selenite-resistance and identified a 35 kDa polypeptide encoded by this fragment.

Dedications

To my wife, Tani Spielberg,
and to my parents,
Carlos and Julianna Caguiat.

Acknowledgments

I would like to thank all the people I worked with as a graduate student:

Dr. Julius H. Jackson, my mentor, for giving me support and guidance to achieve my degree,

Dr. Patricia A. Herring for her eagerness always to help me solve a problem or answer a question,

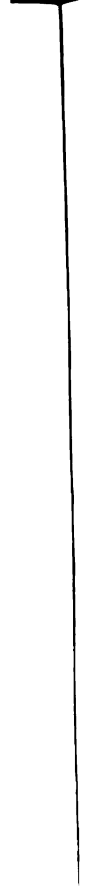
Dr. Michael A. Winrow for teaching me new molecular techniques,

Dr. Loren R. Snyder for providing me with space in his lab for a year while Julius was in Atlanta,

My other committee members, Dr. Michael Bagdasarian, Dr. Craig S. Criddle, and Dr. Richard C. Schwartz for their advice and

Mr. Tony B. Griffin for helping me adjust to Atlanta during my year visit to Clark Atlanta University.

TABLE OF CONTENTS



RES

Tab

1.

2.

3.

4.

5

6.

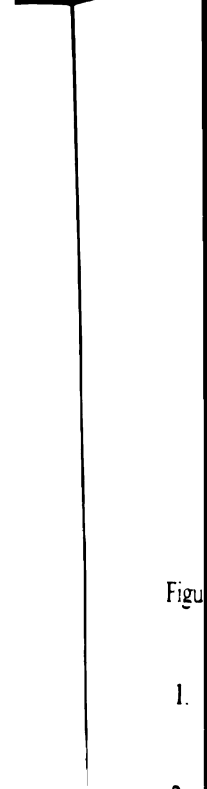
7.

8.

9

10.

LIST OF TABLES



Figu

1.

2.

3.

4

5.

6.

7.

8.

9.

10.

LIST OF FIGURES

INTRODUCTION

Selenium is a group VIA element similar to sulfur and tellurium. It exists in the +6, +4, 0 and -2 oxidation states as selenate (SeO_4^{2-}), selenite (SeO_3^{2-}), elemental selenium (Se^0) and selenide (HSe^-), respectively. The presence of each depends upon the redox potential and pH (Geering *et al.*, 1967; McNeal and Balistrier, 1989; Elrashidi *et al.*, 1989; Masscheleyn *et al.*, 1991). Selenate exists under highly oxidizing conditions, whereas selenite exists under mildly oxidizing conditions (Elrashidi *et al.*, 1987). Although both are soluble, selenate tends to be more mobile (Alemi *et al.*, 1991) and more available for biological absorption because selenite has a much higher affinity for metal oxides than selenate in soil (Christensen *et al.*, 1989; Balistrier and Chao, 1990; Zhang and Sparks, 1990). Elemental selenium and selenide exist under reducing conditions. Elemental selenium exists as a red or black crystal. Selenide is present as hydrogen selenide gas, methyl selenide gas (Chau *et al.*, 1976) metal selenide ores or organic selenide (Stadtman, 1990; Heider and Böck, 1993). Since elemental selenium and selenides are insoluble, they are not available for biological absorption.

Microbes play a major role in the selenium cycle (Shrift, 1964). There are three types of overlapping reactions in this cycle: oxidation and reduction, immobilization and mineralization, and methylation (Doran, 1982). First, several genera of bacteria are known to oxidize and reduce each inorganic species of selenium. Strains which reduce selenate and selenite to elemental selenium under aerobic conditions (Levine, 1925; McReady *et al.*, 1965; Weiss *et al.*, 1965; Burton *et al.*, 1987; Lortie *et al.*, 1992; Maiers *et al.*, 1988) and by anaerobic respiration (Oremland *et al.*, 1989; Rech and Macy, 1992; Steinberg *et al.*, 1992) are the most common. *Micrococcus lactilyticus* reduces selenite to selenide (Woolfolk and Whiteley, 1962), *Thiobacillus ferrooxidans* oxidizes copper selenide to elemental selenium (Torba and Habashi, 1972) and *Bacillus megaterium* oxidizes elemental selenium to selenite (Sarathchandra and Watkinson, 1981). Secondly,

bacteria immobilize inorganic selenium by incorporating it into organic compounds and mineralize organic selenium by converting it back to inorganic selenium. Thirdly, they methylate inorganic and organic forms of selenium to form dimethyl selenide and dimethyl diselenide which diffuse into the atmosphere (Doran and Alexander, 1977; Thompson-Eagle and Frankenberger, 1989; Chau *et al.*, 1976).

Selenium enters the environment through industrial processes (Haygarth, 1994). It is used in staining glass or masking the color of iron oxides in glass; in the pigmentation of plastics, paints and ceramics; as an antioxidant in inks, vegetable oils and lubricants; and in the treatment of fungal infections and dandruff (Newland, 1982). Selenium is released into the atmosphere by the combustion of coal and oil (Nriagu, 1988), and leeches into the environment from stored coal (Yang *et al.*, 1983) or during the mining and refining of phosphate, uranium, copper, lead and zinc (World Health Organization, 1987).

Agricultural activity in arid regions that contain high levels of naturally occurring selenium can cause serious problems. The selenosis of migratory birds in the Kesterson National Wildlife Refuge in the San Joaquin Valley in California is a well known example (Presser and Ohlendorf, 1987; Tanji *et al.*, 1986). Marine sedimentary rock originating from volcanic dust or eroded igneous rock that contained high concentrations of selenium was deposited in this area during the Cretaceous period (Trelease and Beath, 1949; Davidson and Powers, 1957; Presser and Ohlendorf, 1987; Presser, 1994). Because the region is dry and a layer of clay is located immediately below the soil, runoff from the irrigation of farm land collects underneath the fields and evaporates to concentrate selenium and other salts that leach from the soil. A subsurface drainage system was developed to remove this water and direct it to the San Francisco Bay *via* the San Luis Drain. However, politics and funding restrictions blocked the completion of this project (Marshall, 1985). Since it was built only as far as the Kesterson reservoir, the water from this drain was diverted into the wildlife refuge. Subsequently, several species of migratory birds were poisoned (Ohlendorf *et al.*, 1986; Ohlendorf, 1989).

Animal and humans suffering from selenium poisoning exhibit various symptoms. Livestock feeding on selenium accumulating plants contract "alkaline disease," which is characterized by emaciation, lameness and tail, hair and hoof loss (Trelease and Beath, 1949). Humans also lose their hair and nails (Yang *et al.*, 1983). Birds experience muscular atrophy, weight loss and embryo deformities (Ohlendorf *et al.*, 1986; Ohlendorf, 1989). Bacteria sensitive to selenite exhibit complete growth inhibition. Many resistant strains demonstrate a growth curve with an increased lag phase (Leifson, 1936; McReady *et al.*, 1965).

The mechanisms for selenium toxicity are not well understood. Elemental selenium and selenate are not toxic because they are not highly reactive. Selenate is toxic only once it has been converted to selenite (Martin, 1973). Selenite may interfere with protein function by oxidizing sulfhydryl groups to form disulfides (RSSR) and unstable selenosulfides (RS-Se-SR) (Ganther, 1971; Martin, 1973; Doran, 1982; Nakagawa, 1988). Figure 1 demonstrates how selenite may become toxic in the presence of reduced glutathione (GSH) (Whiting *et al.*, 1980; Shamberger, 1985). It reacts with reduced glutathione to form selenodiglutathione (GSSeSG) (2). This compound is converted to selenoperoxide ($GSSe^-$) by reacting with one of the following: another molecule of reduced glutathione (3); glutathione reductase and NADPH (4) (Ganther, 1968; Ganther, 1971); or thioredoxin, thioredoxin reductase and NADPH (Ren *et al.*, 1993; Björnstedt *et al.* 1992; Holmgren and Kumar, 1988). $GSSe^-$ reacts with reduced glutathione (7) or glutathione reductase and NADPH (6) to generate selenide (HSe^-). Seko *et al.* (1988) observed that HSe^- reacts with oxygen to form elemental selenium and a superoxide ion (O_2^-), a free radical, that may damage DNA (Shamberger, 1985) or lipids (Seko *et al.*, 1988). Cell growth may also be inhibited by depletion of NADPH and competitive inhibition of thioredoxin reductase (Björnstedt *et al.*, 1992; Kumar *et al.*, 1992). When *Escherichia coli* thioredoxin reductase and thioredoxin, or calf thymus thioredoxin are added to GSSeGS in the presence of oxygen, a high level of non-stochiometric NADPH

H

H

GS

GS

GS-

GS-

GS-

HSe

HSe

Figure
et al.,

$$HSeO_4^- + 2\ GSH \longrightarrow HSeO_3^- + GS\text{-}SG + H_2O \quad (1)$$

$$HSeO_3^- + 4\ GSH \longrightarrow GS\text{-}Se\text{-}SG + GS\text{-}SG + OH^- + 2\ H_2O \quad (2)$$

$$GS\text{-}Se\text{-}SG + GSH \rightleftharpoons GS\text{-}Se^- + GS\text{-}SG + H^+ \quad (3)$$

$$GS\text{-}Se\text{-}SG + NADPH \xrightarrow{\text{glutathione reductase}} GS\text{-}Se^- + GSH + NADP^+ \quad (4)$$

$$GS\text{-}Se^- + H_2O \rightleftharpoons Se^0 + GSH + OH^- \quad (5)$$

$$GS\text{-}Se^- + NADPH + H_2O \xrightarrow{\text{glutathione reductase}} HSe^- + GSH + NADP^+ + OH^- \quad (6)$$

$$GS\text{-}Se^- + GSH \rightleftharpoons HSe^- + GS\text{-}SG \quad (7)$$

$$HSe^- + [O] \longrightarrow Se^0 + OH^- \quad (8)$$

$$HSe^- + [O] + 2\ GSH \longrightarrow HSe^- + GS\text{-}SG + H_2O \quad (9)$$

Figure 1. Possible reactions of selenate, selenite and selenide with glutathione (Whiting *et al.*, 1980; Shamberger, 1985).

oxidation
oxidizes
to restor
may int
selenome
1957; Tu
likely cau

S

non-cova
(Wagner
1978; Di
selenocys
proteins
Stadtman
of eukary
deiodinas
the active
formate d
(Cone *et a*

TH

with four
SelC is a
al., 1988)
form amin
selenocyste
tRNAs (Le

oxidation occurs. Since the conversion of HSe^- to elemental selenium is slow (8), it oxidizes and starts the cycle again. Subsequently, large quantities of NADPH are required to restore thioredoxin and thioredoxin reductase to their reduced state. Finally, selenium may interfere with protein function by replacing sulfur (Shrift, 1954). Since selenomethione does not inhibit growth when it replaces methionine (Cowie and Cohen, 1957; Tuve and Williams, 1961; Frank *et al.*, 1985), selenocysteine appears to be the most likely cause of toxicity (Heider and Böck, 1994).

Selenium is also an important element in animal and bacterial metabolism. It exists non-covalently bound to the active centers of some bacterial xanthine dehydrogenases (Wagner and Andreesen, 1970) and nicotinic dehydrogenases (Imhoff and Andreeson, 1978; Dilworth, 1982). It is covalently bound to some tRNAs (Wittwer, 1983), selenocysteine and selenomethionine. Selenomethionine is randomly incorporated into proteins and does not play an important role in protein function (Sliwkowski and Stadtman, 1984). Selenocysteine, on the other hand, is a key residue in the active centers of eukaryotic glutathione peroxidase (Forstrom *et al.*, 1978) type I iodothyronine deiodinase (Behn *et al.*, 1990) and plasmid protein P (Burk, 1991). It is also present in the active centers of prokaryotic hydrogenase (Rieder *et al.*, 1984; Muth *et al.*, 1987), formate dehydrogenase (Jones *et al.*, 1979; Zinoni *et al.*, 1986), and glycine reductase (Cone *et al.*, 1976).

The *E. coli* pathway for selenium incorporation into selenocysteine is associated with four genes: *selA*, *selB*, *selC* and *selD* (Böck *et al.*, 1991; Heider and Böck, 1994). SelC is a specific tRNA which possesses the opal termination code (UCA) (Leinfelder *et al.*, 1988). This new tRNA is charged with a serine residue. SelA converts seryl-tRNA to form aminoacrylyl-tRNA (Forchhammer and Böck, 1991). SelD adds HSe^- to form selenocysteyl-tRNA and also supplies selenide for the synthesis of selenium containing tRNAs (Leinfelder *et al.* 1990). Finally, SelB is a translation factor that incorporates

selenocys

codon (Z

A

understoo

cell by th

sulfate t

system (

selenide

resistant

showed

(Gerrard

mimic s

different

was iso

persona

Xanthor

(Pallero

chemica

and Au

Ag(I) a

maltopi

as pOR

detecte

into *E.*

3) in th

After p

selenocysteine into a growing polypeptide chain (Forchhammer *et al.*, 1989) at a UGA codon (Zinoni *et al.*, 1986; Zinoni *et al.*, 1987).

Although the mechanism for the incorporation of selenium into cell protein is well understood, the pathways for its transport and reduction are not clear. Selenate enters the cell by the sulfate transport system (Brown and Shrift, 1980). Selenite may enter by the sulfate transport system (Lindblow-Kull *et al.*, 1985) or a selenite specific transport system (Brown and Shrift, 1982). Once inside the cell, both oxyanions are reduced to selenide or elemental selenium. McCready *et al.* (1965) and Levine (1925) observed that resistant strains accumulated elemental selenium inside the cells. Electron microscopy showed that it collects on the cell wall and membrane but not in the cytoplasm of *E. coli* (Gerrard *et al.*, 1974). The discrepancies in these results and the ability of selenium to mimic sulfur suggest that selenium metabolism is probably complex and involves several different pathways.

Pseudomonas maltophilia Oak Ridge Research Institute strain O2 (ATCC 53510) was isolated from a mercury contaminated site in Oak Ridge, TN in 1986 (N. Revis, personal communication). This designation was changed by Swings *et al.* (1983) to *Xanthomonas maltophilia.* Recently, it was renamed *Stenotrophomonas maltophilia* (Palleroni and Bradbury, 1993). *S. maltophilia* ORO2 displayed a capacity for the chemical transformation of several different heavy metal salts. It reduced Se(IV), Hg(II) and Au(III) to their elemental states and formed insoluble complexes with Pb(II), Cd(II), Ag(I) and Cr(III). Initial agarose electrophoretic gels of total genomic DNA from *S. maltophilia* ORO2 demonstrated that it contained a single 100 kb plasmid band designated as pOR1 (Figure 2, lane 1). The two smaller plasmid bands seen in this gel were not detected in earlier gels. Total genomic DNA from *S. maltophilia* ORO2 was transformed into *E. coli* strain HB101. Selenite-resistant transformants contained pOR1 (Fig. 2, lane 3) in the new strain MJ800, which also demonstrated resistance to Pb(II) and Hg(II). After purifying pOR1 from MJ800 using a modified alkaline lysis protocol (Kado and Liu,

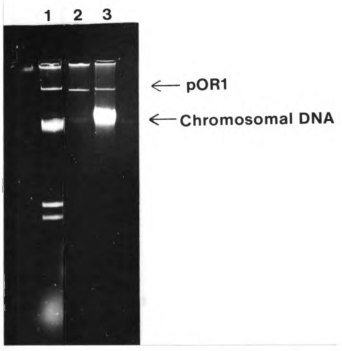


Figure 2. Association of a 100 kb plasmid, pOR1, conferring the transformation of Hg(II), Se(IV) and Pb(II). Lane 1: *Stenotrophomonas maltophilia* ORO2 total genomic DNA. Lane 2: Purified pOR1 from MJ800. Lane 3: Total genomic DNA from MJ800.

1981; Crosa *et al.*, 1994), it was digested with *Hin*dIII, ligated into pBR322 and transformed into HB101. Agarose gel electrophoresis of small scale plasmid preparations of selenite-resistant colonies showed that selenite-resistance was located on a 4 kb, *Hin*dIII fragment from pOR1 (Fig. 3). This recombinant plasmid was called pLJ100 in strain MJ801.

S. maltophilia ORO2 and *E. coli* may use glutathione and glutathione reductase to reduce selenite to elemental selenium (Blake *et al.* unpublished). In Table 1, *S. maltophilia* ORO2 grew in the two control cultures containing selenite or buthionine-sulfoximine, an inhibitor of the glutathione reductase synthesis pathway (Griffith and Meister, 1979) However, it failed to grow in the presence of both chemicals. Thus, *S. maltophilia* required glutathione for resistance to selenite. Stopped flow spectrophotometry was used to predict a possible pathway for the reduction selenite to elemental selenium. Glutathione and H_2SeO_3 reacted to form GS-Se-SG with the production of the intermediates shown in Figure 4 (Blake *et al.*, personal communication). When NADPH and glutathione reductase or cell extracts from *S. maltophilia* ORO2 grown in selenite were added, elemental selenium was released and glutathione was returned to its reduced state. Thus, glutathione reductase may be involved in the bacterial reduction of selenite.

In this study, I localized the position of selenite-resistance on the physical map of pOR1, sequenced the 4 kb *Hin*dIII fragment which conferred resistance to selenite in *E. coli* and identified a 35 kDa polypeptide, SedR, encoded by this fragment.

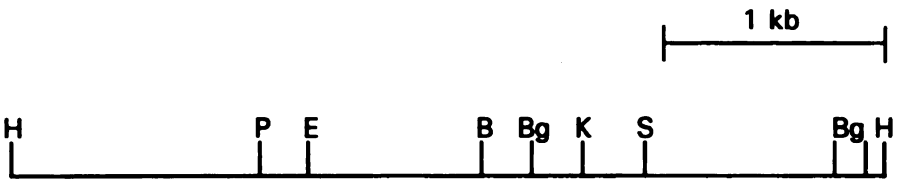


Figure 3. Physical map of the 4 kb, *Hin*dIII insert in pLJ100. This cloned fragment was ligated into pBR322 and confers resistance to Pb(II) and Se(IV). Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*R1; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; and S, *Sph*I.

Table 1. Dependence of *Stenotrophomonas maltophilia* ORO2 upon reduced glutathione for growth in LB broth containing 10 mM selenite.

Strain	Growth conditions		Growth
	Selenite	BSO_a	
S. maltophilia ORO2	+	-	+
S. maltophilia ORO2	-	+	+
S. maltophilia ORO2	+	+	-

[a]BSO, L-buthionine-[S,R]-sulfoximine, irreversibly inhibits γ-glutamylcysteine synthetase in the pathway for glutathione (GS) biosynthesis.

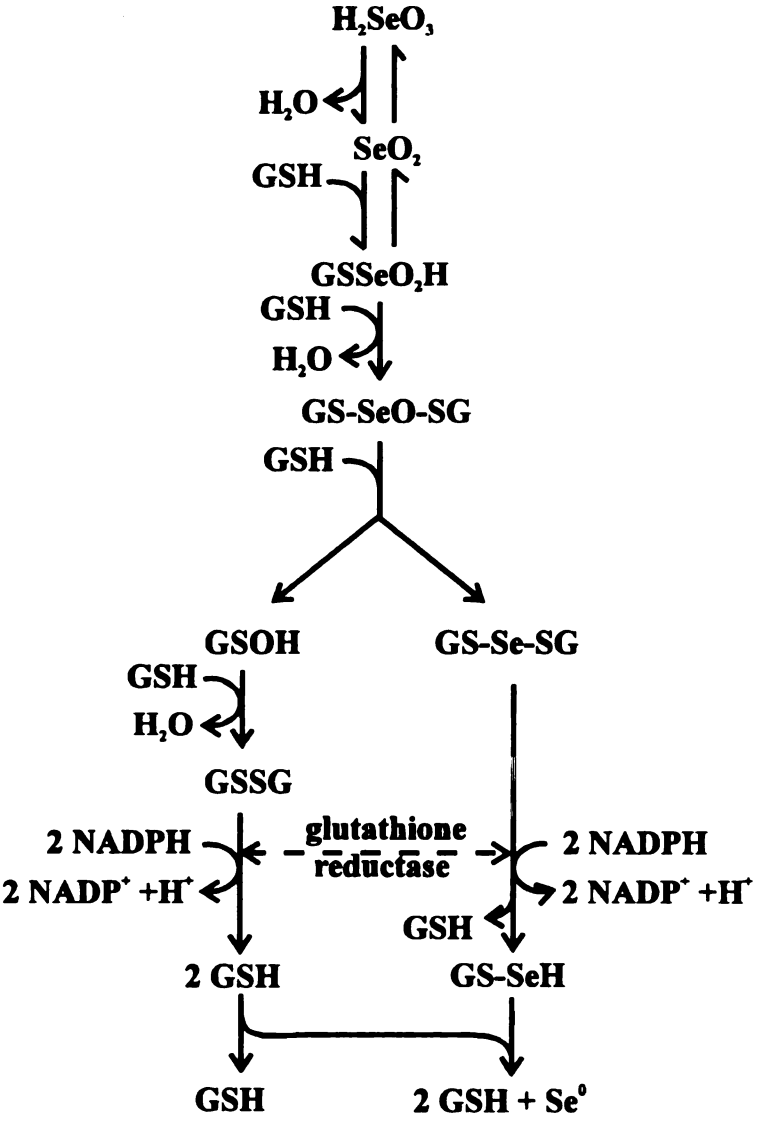


Figure 4. Proposed pathway for Se(IV) reduction in *Stenotrophomonas maltophilia* ORO2 and *E. coli.*

MATERIALS AND METHODS

Strains, plasmids, and media. The strains and plasmids used in this study are listed in Table 2. M-9 minimal medium (Ausubel *et al.*, 1992) for *E. coli* contained 0.6% (w/v) Na_2HPO_4, 0.3% (w/v) KH_2PO_4, 0.1% (w/v) NH_4Cl, 0.055% (w/v) NaCl, 1 mM $MgSO_4$ and 0.5% (w/v) D-glucose. Luria Bertani broth (LB broth) contained 1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 0.5% (w/v) NaCl and 1 mM NaOH in distilled water (Ausubel *et al.*, 1992). MacConkey agar was obtained from Difco Laboratories and prepared according to the manufacturer's instructions (Holt and Krieg, 1994). Terrific broth contained 1.2% (w/v) bacto-tryptone, 2.4% (w/v) bacto-yeast extract, 0.4% (v/v) glycerol, 17 mM KH_2PO_4 and 72 mM K_2HPO_4 dissolved in distilled water (Tartof and Hobbs, 1987). All agar plates contained 1.6% (w/v) agar. When required, media was supplemented with 0.5 mg % (w/v) thiamine, 0.4 mM L-amino acid(s), 100 μg/ml Ampicillin, 60 μg/ml kanamycin and 40 mM selenite. Cultures were grown at 37 °C with aeration in a baffled flask placed in a New Brunswick Scientific Co., Inc. gyrotory water bath shaker at 200 rpm.

Restriction endonucleases. Restriction endonucleases were obtained from Promega. *Acc*65I, *Bgl*II, *Hin*dIII, *Sac*I, *Eco*ICRI and *Sph*I were stored in 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.4, 50 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 0.5 mg/ml bovine serum albumin (BSA), and 50% (v/v) glycerol. *Bam*HI was stored in 10 mM Tris-HCl, pH 7.4, 300 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mg/ml BSA and 50% (v/v) glycerol. *Eco*RI and *Pst*I were stored in 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.15% DTT, 0.5 mg/ml BSA and 50% (v/v) glycerol. *Acc*65I and *Bgl*II required a 10 x digestion buffer containing 60 mM Tris-HCl, pH 7.9, 60 mM $MgCl_2$, 1.5 M NaCl and 10 mM DTT. *Bam*HI and *Hin*dIII required a 10 x digestion buffer containing 60 mM Tris-HCl, pH 7.5, 60 mM $MgCl_2$, 1 M NaCl and 10 mM DTT.

Table 2. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference
Strains		
Stenotrophomonas matophilia ORO2	Ap^r, Se(IV)^r, Pb(II)^r, Hg(II)^r, Au(III)^r, Ag(I)^r, Cd(II)^r, Cr(III)^r	ATCC 53510
Escherichia coli HB101	Δ(*gpt-proA*)*62*, *leuB6*, *thi-1*, *lacY1*, *recA*, *rpsL20*, (Str^r), *ara-14*, *galK2*, *xyl-5*, *mtl-1*, *supE44*, Δ(*mcrBC-hsdRMS-mrr*)	Boyer and Roulland-Dassiox, 1969
Escherichia coli DH5α	F′/*endA1*, *hsdR*($r_k^- m_k^+$), *supE44*, *thi-1*, *recA1*, *gyrA*, (Nal^r), *relA1*, Δ(*lacZYA-argF*)$_{U169}$ (*φ80lacZΔM15*)	Hanahan, 1983
Escherichia coli JM109	F′ *traD36*, *proA*$^+$, *proB*$^+$, *lacI*^{q}, *lacZΔM15*/*recA1*, *endA1*, *gyrA96*, (Nal^r), *thi*, *hsdR17*, *supE44*, *relA1*, Δ(*lac-proAB*), *mcrA*	Yanisch-Perron *et al.*, 1985
Escherichia coli XL1-Blue MRF′ Kan	F′ *proAB lacI*^{q}*ZΔM15* Tn*5* (Kan^r)/Δ*(mrcA)183*, Δ(*mrcCB-hsdSMR-mrr*)*173*, *endA1*, *supE44*, *thi-1*, *recA*, *gyrA96*, *relA1*, *lac*	Short and Sorge, 1992; obtained from Stratagene
Escherichia coli AB1157	F$^-$, *ara-14*, *leuB6*, *lacY1*, *supE44*, *rfbD1*, *thi-1*, *malT1*, *ara-14*, *galK2*, *xyl-5*, *mtl-1*, *his G4(oc)*, *argE3*	Apontweil and Berends, 1975
JF420	AB1157, *gor*$^-$	Oden *et al.*,1994; from Guangyong Ji, unpublished.

Table 2 (cont'd)

Strain or plasmid	

Table 2 (cont'd)

Strain or plasmid	Relevant characteritics	Reference
JF432	AB1157, *trxB*, *gal*$^+$	Oden *et al.*,1994; from Guangyong Ji, unpublished
JF1070	AB1157, Δ*proAB lac*	Oden *et al.*,1994; from Guangyong Ji, unpublished
JF1097	AB1157, *trxA*::*kan gshA*	Oden *et al.*,1994; from Guangyong Ji, unpublished
JF2014	AB1157, *trxA*::*kan gor*	Oden *et al.*,1994; from Guangyong Ji, unpublished
JF2062	AB1070, *trxA*::*kan*	Oden *et al.*,1994; from Guangyong Ji, unpublished
JF2200	AB1070, *gshA*::*kan*	Oden *et al.*,1994; from Guangyong Ji, unpublished
JF2201	AB1070, *gshB*::*kan*	Oden *et al.*,1994; from Guangyong Ji, unpublished

Table 2 (cont'd)

Table 2 (cont'd)

Strain or plasmid	Relevant characteristics	Reference
X2642	HB101 containing pBR322	Bolívar *et al.*, 1977, Sutcliffe, 1978, Boyer and Roulland-Dassiox, 1969
MJ800	HB101 containing pOR1	Figure 1 and Figure 7
MJ801	HB101 containing pLJ100	Figure 3
MJ810	DH5α containing pLJ145	Figure 7
MJ811	DH5α containing pLJ169	Figure 7
MJ812	DH5α containing pLJ171	Figure 7
MJ813	DH5α containing pLJ193	Figure 7
MJ814	DH5α containing pLJ195	Figure 7
MJ815	DH5α containing pLJ196	Figure 7
MJ816	DH5α containing pLJ197	Figure 7
MJ820	DH5α containing pSP73	Krieg and Melton, 1979

Table 2 (cont'd)

Table 2 (cont'd)

Strain or plasmid	Relevant characteristics	Reference
MJ821	DH5α containing pLJ200	Figure 13
MJ822	DH5α containing pLJ201	Figure 13
MJ823	DH5α containing pLJ202	Figure 13
MJ824	DH5α containing pLJ203	Figure 13
MJ825	DH5α containing pLJ204	Figure 13
MJ826	DH5α containing pLJ205	Figure 13
MJ830	HB101 containing pT7-4	Tabor and Richardson, unpublished
MJ831	HB101 containing pGP1-2	Tabor and Richardson, 1985
MJ832	HB101 containing pLJ270	Figure 17
MJ833	HB101 containing pLJ271	Figure 17
MJ834	MJ831 containing pT7-4	Tabor and Richardson, 1985

Table 2 (cont'd)

Table 2 (cont'd)

Strain or plasmid	Relevant characteristics	Reference
MJ835	MJ831 containing pLJ270	Figure 17
MJ836	MJ831 containing pLJ271	Figure 17
MJ840	HB101 containing MRF' Kan	Short and Sorge, 1992; from Stratagene
MJ841	MJ840 containing pOR1	Table 6 and Table 7
MJ844	MJ800 containing pLJ193	Table 6 and Table 7
MJ845	MJ800 containing pUC19	Table 6 and Table 7
MJ846	JM109 containing pLJ193	Table 5
MJ847	JM109 containing pUC19	Table 5
MJ848	HB101 containing pLJ280	Figure 20
MJ849	HB101 containing pLJ281	Figure 20
MJ850	HB101 containing pLJ291	Figure 20

Table 2 (cont'd)

Strain or plasmid	Relevant characteristics	Reference
MJ851	HB101 containing pLJ294	Figure 20
MJ852	HB101 containing pLJ307	Figure 20
MJ853	HB101 containing pUC19	Norrander *et al.*, 1983
Plasmids		
pBR322	Ap^r, Tc^r, ColE1	Bolívar *et al.*, 1977, Sutcliffe, 1978
pUC19	Ap^r, *lacZ'*	Norrander *et al.*, 1983
pOR1	100 kb F-like plasmid from *S. maltophilia* ORO2, $Pb(II)^r$, $Se(IV)^r$, $Hg(II)^r$	Latinwo *et al.*, 1990
pLJ100	pBR322 containing the 4 kb *Hin*dIII fragment from pOR1, $Pb(II)^r$ $Se(IV)^r$	Latinwo *et al.*, 1990
MRF' Kan	F' *proAB*, *lacI*^{q}*ZΔM15*, Tn*5* (Kan^r)	Short and Sorge, 1992, from Stratagene

Table 2 (cont'd)

Table 2 (cont'd)

Strain or plasmid	Relevant characteristics	Reference
pLJ145	pUC19 containing the 3.5 kb *Acc*65I fragment from pOR1	Figure 7
pLJ169	pUC19 containing the 7.2 kb *Acc*65I fragment from pOR1	Figure 7
pLJ171	pUC19 containing the 7.8 kb *Acc*65I fragment from pOR1	Figure 7
pLJ193	pUC19 containing the 13.4 kb *Bam*HI fragment from pOR1	Figure 7
pLJ195	pUC19 containing the 11.4 kb *Hin*dIII fragment from pOR1	Figure 7
pLJ196	pUC19 containing the 8.0 kb *Sac*I fragment from pOR1	Figure 7
pLJ197	pUC19 containing the 11.8 kb *Sac*I fragment from pOR1	Figure 7
pSP73	Ap^r, SP6 and T7 RNA polymerase promoters	Krieg and Melton, 1979
pLJ200	pSP73 containing the 4 kb *Hin*dIII fragment from pOR1	Figure 13
pLJ201	*Bam*HI deletion in pLJ200	Figure 13
pLJ202	*Eco*RI deletion in pLJ200	Figure 13

Table 2 (cont'd)

Table 2 (cont'd)

Strain or plasmid	Relevant characteristics	Reference
pLJ203	*Pst*I deletion in pLJ200	Figure 13
pLJ204	*Kpn*I deletion in pLJ200	Figure 13
pLJ205	*Sph*I deletion in pLJ200	Figure 13
pGP1-2	Kan^r, T7 RNA polymerase	Tabor and Richardson, 1985
pT7-4	Ap^r, T7 RNA polymerase promoter	Tabor and Richardson, unpublished
pLJ270	pT7-4 containing the 4 kb *Hin*dIII fragment from pOR1	Figure 17
pLJ271	pT7-4 containing the 4 kb *Hin*dIII fragment from pOR1	Figure 17
pLJ280	*Hin*dIII/*Eco*RI segment from the pLJ100 insert containing Tn*1000* and *rep2A* in pBR322	Figure 20
pLJ281	*Hin*dIII/*Eco*RI fragment from the pLJ100 insert containing SedR and the 3.7 kDa ORF in pBR322	Figure 20
pLJ291	*Hin*dIII/*Pst*I fragment from the pLJ100 insert containing SedR in pUC19	Figure 20

Table 2 (cont'd)

Table 2 (cont'd)

Strain or plasmid	Relevant characteristics	Reference
pLJ294	*Hin*dIII/*Eco*RI fragment containing SedR and the 3.7 kDa ORF in pUC19	Figure 20
pLJ307	*Hin*dIII/*Bam*HI segment containing part of Tn*1000* in pBR322	Figure 20

Se(IV)^r - selenite-resistance
Ag(I)^r - silver-resistance
Pb(II)^r - lead-resistance
Cd(II)^r - cadmium-resistance
Hg(II)^r - mercury-resistance
Cr(III)^r - chromate-resistance
Au(III)^r - gold resistance

*Eco*RI and *Pst*I required a 10 x digestion buffer containing 900 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$ and 500 mM NaCl. *Sac*I required a 10 x digestion buffer containing 100 mM Tris-HCl, pH 7.5, 70 mM $MgCl_2$, 500 mM KCl and 10 mM DTT. *Sph*I required a 10 x digestion buffer containing 100 mM Tris-HCl, pH 7.4, 100 mM $MgCl_2$ and 1.5 M KCl. *Eco*ICRI required a 10 x digestion buffer containing 60 mM Tris-HCl, pH 7.5, 60 mM $MgCl_2$, 500 M NaCl and 10 mM DTT.

Total genomic preparations. Total genomic DNA was prepared using a method described by Ausubel *et al.* (1992). Cells from 100 ml of *E. coli* and 50 ml of *S. maltophilia* were harvested at 14,500 x g and resuspended in 9.5 ml of TE solution which contained 10 mM Tris-HCl, pH 8.0 and 0.5 mM EDTA. The suspension was mixed with 0.5 ml of 10% (w/v) sodium dodecyl sulfate (SDS) and 50 µl of 20 mg/ml proteinase k. This mixture was incubated at 37 °C for 1 hour. The preparation was then mixed with 1.8 ml of 5 M NaCl and 1.5 ml of of a solution containing 10% (w/v) cetyltrimethylammonium bromide (CTAB) and 0.7 M NaCl. After incubating the preparation at 65 °C for 20 min, it was extracted once with 5 ml of 24:1 (v/v) chloroform:isoamyl alcohol and a second time with an equal volume of 1:1 (v/v) phenol and chloroform. The DNA was then precipitated with 0.6 volumes of isopropanol, centrifuged at 20,000 x g, gently mixed with 5 ml of 70% (v/v) ethanol, centrifuged at 20,000 x g, dried under a vacuum after discarding the ethanol and resuspended in 1.5 ml of TE containing 5 µg/ml of RNase.

Plasmid preparations. Purification of pOR1 was performed by a modified alkaline-lysis protocol (Kado and Liu, 1981 and Crosa *et al.*, 1984). After growing MJ800(pOR1) in LB broth containing 40 mM selenite for 1-2 days at room temperature in a shaking water bath, 0.3 g of harvested wet cells were resuspended in 2 ml of TE (pH 8.0), mixed with 5.5 ml of lysing solution (3.4% (w/v) SDS and 0.032 M NaOH dissolved in TE) and incubated at 65 °C for 45 min. The lysed cells were gently mixed with 0.412 ml of 2 M Tris-HCl, pH 7.2 and 2 ml of 5 M NaCl. After incubating the preparation on ice in the refrigerator overnight, it was centrifuged at 20,000 x g for 20 min, and the

supernatant was poured through cheese cloth into a new tube. Plasmid DNA was precipitated with 0.6 volumes of isopropanol for 20 min at room temperature, centrifuged at 20,000 x g for 15 min, gently mixed with 5 ml of 70% (v/v) ethanol, centrifuged at 20,000 x g for 10 min, dried under a vacuum after discarding the ethanol and resuspended in 1 ml of TE containing 5 μg/ml RNase. Small scale plasmid preparations were performed using an alkaline lysis procedure (Birnboim and Doly, 1979). The strain harboring the desired plasmid was grown overnight in 5 ml of LB broth containing the appropriate antibiotic(s). Cells from 1.5 ml of culture were harvested at 14,000 x g for 1 min, resuspended in 0.1 ml of TE, gently mixed with 0.2 ml of lysing solution (1% (w/v) SDS and 0.2 N NaOH dissolved in TE) and incubated on ice for 5 minutes. Cell debris and SDS were precipitated by adding 0.15 ml of 3 M potassium acetate (pH 5.2) and incubating the sample on ice an additional 5 min. After centrifuging the preparation at 14,000 x g for 15 min, the supernatant was poured into a new tube. Plasmid was precipitated with 1 ml of 95 % (v/v) ethanol, centrifuged at 14,000 x g for 15 minutes, mixed with 0.5 ml of 70 % (v/v) ethanol, centrifuged at 14,000 x g for 5 minutes, dried under a vacuum after discarding the ethanol and resusupended in 30 μl of TE containing 5 μg/ml of RNase. Restriction enzyme digests of the plasmid contained 5 μl of plasmid, 2 μl of 10 x digestion buffer, 0.5 μl of the appropriate enzyme (5 U) and 12.5 μl of distilled water. Digestions were mixed with 10 x loading buffer, incubated at 37 °C for 1-2 hr and separated on a 0.8% agarose gel. All gels were stained for 15 min at room temperature in water containing 0.5 μg/ml ethidium bromide. Large quantities of DNA were purified using Promega's Maxipreps (Sambrook *et al.*, 1989). A 250 ml culture harboring the desired plasmid was grown in LB-broth containing the appropriate antibiotic at 37 °C overnight in a shaking water bath. The cells were harvested at 14,000 x g for 15 min, resuspended in 15 ml of resuspension buffer (50 mM Tris-HCl, pH7.5, 10 mM EDTA and 100 mg/ml RNase) and mixed with 15 ml of lysing solution (0.2 M NaOH, and 1% SDS). When the solution cleared, it was neutralized with 15 ml of 2.55 M potassium acetate (pH

4.8), cen
tube. Th
isopropa
mixed wi
packed in
adding 12
EDTA a
through
followed
The vacu
was drie
resin by
centrifug
C
of pOR1
µl of 10
Magic C
into the
was push
an Epper
resin by
centrifug
et al., 19
(300 mM
pUC19 (
pH 7.4,
coli strai

4.8), centrifuged at 14,000 x g for 15 minutes and poured through cheese cloth into a new tube. The DNA was precipitated at room temperature for 20 minutes with 0.6 volumes of isopropanol, centrifuged at 14,000 x g for 15 minutes, resuspended in 2 ml of TE and mixed with 10 ml of maxiprep resin. Using a Promega vacuum manifold, the resin was packed into a maxiprep column. Resin remaining in the centrifuge tube was removed by adding 12 ml of column wash solution (200 mM NaCl, 20 mM Tris-HCl, pH7.5, 5 mM EDTA and 50% (v/v) ethanol) to the tube, decanting it into the column and pulling it through with the vacuum manifold. An additional 13 ml of column wash solution, followed by 5 ml of 80% (v/v) ethanol were pulled through the column to wash the resin. The vacuum was applied for 15 extra min to remove as much liquid as possible. The resin was dried at 1,300 x g for 5 min in a clinical centrifuge. The DNA was eluted from the resin by incubating it with 1.5 ml of distilled warm (65 °C) water for 1 min and centrifuging the column for 5 min in a clinical centrifuge at 1,300 x g.

Cloning of pOR1 fragments. *Acc*65I (*Kpn*I), *Hin*dIII, *Sac*I or *Bam*HI digestions of pOR1 containing 2 µl (20 U) restriction endonuclease; 88 µl (0.9 µg) of pOR1; and 10 µl of 10 x digestion buffer were incubated overnight at 37 °C and mixed with Promega's Magic Cleanup kit resin. The resin was packed into a Cleanup kit column by pushing it into the column with a 3 ml syringe; washed with 2 ml of 80% (v/v) isopropanol, which was pushed through the column with the syringe; and dried by centrifuging the column in an Eppendorf tube for 20 seconds at 14,000 x g. Plasmid fragments were eluted from the resin by incubating it with 30 µl of distilled warm (65 °C) water for 1 minute and centrifuging the column in a new tube at 14,000 x g for 20 seconds. Ligations (Maniatus *et al.*, 1989) consisted of 15 µl (0.4 µg) of digested pOR1, 2 µl of 10 x ligation buffer (300 mM Tris-HCl, pH 7.8, 100 mM $MgCl_2$, 100mM DTT and 5 mM ATP), 2 µl of pUC19 (50 ng) and 1 µl of T4 DNA ligase (3U), which was stored in 10 mM Tris-HCl, pH 7.4, 50 mM KCl, 0.1 mM DTT, 0.1 mM EDTA and 50% (v/v) glycerol. *Eschericia coli* strain DH5α was transformed with 15 µl (0.34 µg) of ligase reaction, and 100 µl of

the tr

ampic

ampic

appro

8.0, 1

al., 19

desire

-80 °C

(Hana

LB br

10 ml

transfo

0.01 M

gently

incuba

mixed

in vol

14,000

plates

LB br

*Bam*H

purifie

were i

bromp

gel to

the transformation reaction was plated on McConkey agar plates containing 100 μg/ml of ampicillin. White transformants were grown in 5 ml of LB broth containing 100 μg/ml of ampicillin. Small scale plasmid preparations from 1.5 ml of culture were digested with the appropriate enzyme, mixed with 10 x loading buffer (20% (w/v) ficoll, 0.1 M EDTA, pH 8.0, 1% (w/v) SDS, 0.25% (w/v) bromphenol blue and 0.25% xylene cyanol (Ausubel *et al.*, 1989)) and electrophoresed through a 0.8% (w/v) agarose gel. Cultures containing the desired recombinant plasmid were mixed 1:1 (v/v) with 50% (v/v) glycerol and stored at -80 °C.

Transformations. Competent cells were prepared using a modified $CaCl_2$ method (Hanahan, 1983). A 100 ml culture containing the desired strain of *E. coli* was grown in LB broth to an optical density of 0.4. Cells were centrifuged at 3,000 x g, resuspended in 10 ml of 0.15 M NaCl, centrifuged at 3,000 x g again, resuspended in 1 ml of transformation buffer (15% (v/v) glycerol, 0.1 M $CaCl_2$, 0.01 M Tris-HCl, pH 8.0 and 0.01 M $MgCl_2$), and frozen at -80 °C. The cells were thawed on ice, and 0.1 ml was gently mixed with the 0.03-0.5 μg of plasmid DNA. The transformation mixture was incubated on ice for 30 min, heat shocked for 2 min at 42 °C, incubated on ice for 15 min, mixed with 1 ml of LB-broth, and incubated at 37 °C for 45 min. Cells were either plated in volumes of 0.1 ml, or the entire transformation was plated by centrifuging them at 14,000 x g for 10 sec, resuspending them in residual supernatant and spreading them on plates with the appropriate selection. Single colonies were inoculated into 5 ml cultures of LB broth and grown overnight for small scale plasmid preparations.

Southern blot analysis. *Bgl*II, *Acc*65I (*Kpn*I), *Hin*dIII, *Eco*ICRI (*Sac*I), and *Bam*HI restriction endonuclease enzyme digestions consisting of 25 μl (0.25μg) of purified pOR1, 3 μl of 10 x digestion buffer, and 2 μl (approximately 20 U) of enzyme were incubated overnight at 37 °C and separated on a 0.8% agarose gel until the bromphenol blue dye migrated 11.5 cm. The fractionated DNA was transferred from the gel to a Biorad Zeta Probe membrane (Reed and Mann, 1985, Sambrook *et al.*, 1989)

using Bi

in 0.2 M

800 mM

min; and

and soa

filter pa

on the

1982); t

and the

the Tran

transfer

overnigh

removed

stratalink

Restricti

of DNA

incubate

Sea Plac

bands w

was mix

column

isopropa

in an Ep

incubatin

Eppendo

in the ope

using Biorad's Trans-Blot Cell apparatus. The gel was soaked in 0.25 M HCl for 10 min; in 0.2 M NaOH and 0.5 M NaCl for 30 min, twice; in 5 x TAE buffer (20x TAE contained 800 mM Tris-HCl, 400 mM acetate, 20 mM EDTA and glacial acetic acid, pH 7.4) for 10 min; and in 0.5 x TAE buffer for 10 min. The membrane was cut the same size as the gel and soaked in 0.5 x TAE for 10 min. Two pads from the apparatus and two sheets of filter paper the same size as the gel were briefly soaked in 0.5 x TAE. The gel was placed on the gel holder from the Trans-Blot Cell apparatus in the following order (Danner, 1982): the first pad, the first sheet of filter paper, the gel, the second sheet of filter paper and the second pad. After removing all air bubbles, the gel holder was closed and placed in the Trans-Blot tank so that the membrane faced the cathode. The tank contained 0.5 x transfer buffer, cooled to 4 °C. The DNA was tranferred to the membrane at 40 V overnight and was completed at 80 V for 1 hr the following day. The membrane was removed from the gel holder; washed with 1 x TAE buffer; treated in a Stratagene UV stratalinker 2400 to fix the DNA to the membrane; and air dried.

Fragments were isolated for nick translation using the following protocol. Restriction endonuclease digestions of the recombinant plasmids containing 43 µl (20 µg) of DNA, 5 µl of 10 x digestion buffer, and 2 µl (20 U) of restriction endonuclease were incubated at 37 °C for 2 hr and separated on a 0.8% agarose gel using FMC Bio Products' Sea Plaque agarose. The gel was stained with ethidium bromide and the desired fragment bands were excised from the gel. Up to 500 µl of melted agarose containing the fragment was mixed with 1 ml Promega's PCR Prep resin. The resin was packed onto a PCR Prep column by pushing it through the column with a 3 ml syringe, washed with 2 ml 80% (v/v) isopropanol which was pushed through the column, and dried by centrifuging the column in an Eppendorf tube for 20 sec at 14,000 x g. DNA was eluted from the resin by incubating it with 50 µl of double distilled water for 1 minute and centrifuging it in a new Eppendorf tube at 18,500 x g for 20 sec. The sample was incubated at 65 °C for 30 min in the opened Eppendorf tube to concentrate the DNA and remove excess isopropanol.

DNA fragments were labeled by using the following 50 µl reaction from Promega's nick translation system (Rigby *et al.*, 1977 and Sambrook *et al*, 1989): 3 µl of deoxyadenosine triphosphate (300 mM), 3 µl of deoxyguanosine triphosphate (300 mM), 3 µl of deoxythimidine triphosphate (300 mM); 5 µl of 10 x nick translation buffer (500 mM Tris-HCl, pH 7.2, 100 mM $MgSO_4$ and 1mM DTT); 0.5 to 1.0 µg of DNA dissolved in 23 µl of water; 5 µl of DNA polymerase/DNaseI mix (DNA polymeraseI [1 U/µl], 0.2 ng/µl DNaseI, 50% (v/v) glycerol, 50 mM Tris-HCl, pH 7.2, 10 mM $MgSO_4$, 0.1 mM DTT and 0.5 mg/ml nuclease free BSA); and 7 µl of [α-^{32}P] deoxycytidine (70 mCi at 400 Ci/mmol and 10 mCi/ml). After incubating the reaction for 1 hr at 15 °C, 5 µl of 0.25 M EDTA (pH 8.0) were added to stop the reaction. To measure the percent incorporation, 1 µl of the reaction was added to 99 µl of 0.2 M EDTA (pH 8.0), and 3 µl of this dilution was placed, in duplicate, on Angel 934 AH fiber filters. The filters were dried under a heat lamp. One was placed in a scintillation vial containing 10 ml of scintillation fluid. The other filter was washed twice for 5 min in 50 ml of 0.5 M sodium phosphate (pH 6.8), dried under a heat lamp and placed in another scintillation vial. Percent incorporation was calculated by dividing the counts per minute (cpm) of the washed filter by the cpm of the unwashed filter and multiplying this quotient by 100. Fragments with greater than 30% incorporation were used for hybridization. The labeling reaction was diluted with 0.45 ml of TE, concentrated for 5 min at 3000 x g in an Amicon Microcon, diluted with 0.45 ml of TE, boiled for 5 min and added to a hybridization bottle containing a blot and hybridization buffer.

Hybridizations were achieved using Bellco's microhybridization oven and a protocol (Church and Gilbert, 1984) modified for Biorad's Zeta Probe membrane. The membrane, rolled tightly in a Bellco nylon mesh, was placed in a hybridization bottle containing 0.2 x SSC buffer. The mesh and membrane were unraveled against the wall of the bottle so that all air bubbles were eliminated. After decanting the SSC buffer, 40 ml of hybridization buffer (1mM EDTA, 7% (w/v) SDS, and 0.5 M $NaHPO_4$ [0.5 M sodium],

pH 7.2

replace

The blo

EDTA

in 1% (

wrappe

develop

were n

consisti

U) of r

samples

samples

distilled

(w/v) S

was stai

slabs we

30 µl o

After inc

PCR Pre

T

transferr

Kan cells

°C. The

streptomy

selenite a

Kan) were

pH 7.2) were added, and the blot was incubated at 65 °C for 15 min. The buffer was replaced with 40 ml of fresh hybridization buffer, and the denatured probe was added. The blot was incubated overnight at 65 °C; washed in 150 ml of 5% (w/v) SDS, 1 mM EDTA and 40 mM $NaHPO_4$ (40 mM sodium), pH 7.2 at 65 °C for 1-2 hr, twice; washed in 1% (w/v) SDS, 1 mM EDTA, and 40 mM $NaHPO_4$, pH 7.2 at 65 °C for 1-2 hr, twice; wrapped in plastic; and placed on film in an autoradiogram cassette. The film was developed after exposing it overnight at -80 °C.

Plasmid mapping by digesting excised pOR1 fragments. Uncloned fragments were mapped using a modified technique described by Danna (1980). Digestions consisting of 897 µl (9 µg) of purified pOR1, 100 µl of 10 x digestion buffer and 3 µl (30 U) of restriction enzyme were incubated overnight at 37 °C, divided into two 500 µl samples and treated separately with Promega's Clean Up system. The DNA from both samples was eluted into the same tube with 100 µl (50 µl for each cleanup reaction) of distilled warm (65°C) water, mixed with 10 x loading buffer and fractionated on a 0.8% (w/v) Sea Plaque agarose gel until the bromphenol blue dye migrated 11.5 cm. The gel was stained with ethidium bromide and selected bands were excised from the gel. The gel slabs were melted at 65 °C and used in the following digestions: 100 µl of melted gel slab, 30 µl of 10 x digestion buffer, 168 µl of water and 2 µl (20 U) of restriction enzyme. After incubating the digestions overnight at 37 °C, the DNA was purified using Promega's PCR Preps system and separated on a 0.8% (w/v) agarose gel.

Transfer of MRF′ Kan to HB101 and MJ800. The F′ episome, MRF′ Kan, was transferred from XL1-Blue MRF′ Kan to HB101 and MJ800 by mating. XL1-Blue MRF′ Kan cells were mixed on an LB plate with HB101 or MJ800 and incubated for 2 hr at 37 °C. The cells were scraped off the plates and streaked onto an LB plate containing streptomycin and kanamycin for mating with HB101 and onto an LB plate containing selenite and kanamycin for the mating with MJ800. HB101(MRF′ Kan) and MJ800(MRF′ Kan) were named MJ840 and MJ841, respectively.

transf

M-9 r

M-9 r

supple

the in

strain

plasmi

10^{-7} a

50 wer

similar

by 10^{-6}

by 10^{-6}

then pl

subclon

(Krieg

restrict

pLJ100

was inc

sample

pSP73 a

DNA lig

pLJ200.

using Pro

EcoRI, *Ba*

Incompatibility analysis. The cloned pOR1 fragments inserted into pUC19 were transformed into JM109 using the method described above, except JM109 was grown in M-9 minimal medium. All transformation reactions were spread on two types of plates: M-9 minimal medium plates supplemented with ampicillin and M-9 minimal medium plates supplemented with ampicillin and 0.4 mM proline. Figure 12 illustrates the protocol for the incompatibility experiments with selection for neither plasmid (Berquist, 1987). A strain containing the competing plasmids was grown overnight under selection for both plasmids, diluted 10^{-6} in medium without selection, grown overnight, diluted by 10^{-6} or 10^{-7} and plated on medium without selection. To identify colonies which lost a plasmid, 50 were spotted onto a plate containing selection for each plasmid.

Incompatibility experiments with selection for one of the competing plasmids was similar to the protocol above. The strain containing the competing plasmids was diluted by 10^{-6} in medium containing selection for one of the plasmids, grown overnight, diluted by 10^{-6} or 10^{-7} and plated on medium with selection for same plasmid. Colonies were then plated onto a plate containing selection for the other plasmid.

DNA sequence determination. The 4 kb *Hin*dIII fragment from pOR1 was subcloned between the SP6 and T7 phage RNA polymerase promoters in plasmid, pSP73 (Krieg and Melton, 1987), to create plasmid pLJ200 for sequencing (Fig. 13). A restriction endonuclease digestion containing 1 µl (1 µg) of pSP73, 10 µl (1 µg) of pLJ100, 3 µl of 10 x digestion buffer, 14 µl of distilled water and 2 µl (20 U) of *Hin*dIII was incubated for 3 hr at 37 °C and treated with Promega's Clean Up system. The eluted sample was ligated at 4 °C for 12-15 hr in a reaction containing 10 µl (0.4 µg) of digested pSP73 and pLJ100; 2 µl of 10 x ligation buffer; 7 µl of distilled water; and 1 µl (3U) of T4 DNA ligase. Transformation of DH5α with 10 µl (0.2 µg) of the ligated DNA yielded pLJ200. A large scale preparation was performed on a 250 ml culture of MJ821 (pLJ200) using Promega's Wizard Maxiprep system. This purified DNA was used to create *Sph*I, *Eco*RI, *Bam*HI, *Pst*I, and *Acc*65I (*Kpn*I) deletions in the 4 kb insert of pLJ200 (Figure

13). R
digest
treate
contai
µl (6 U
reactio
plasmi
Terrific
they w
260 nm
primer
derivati
promote
the 4 kb
primers
Universit
synthesiz
base anal
dissolved
and 12 pm
Biochemis
Catalyst 8
products.
In
system (Zu
cloned pO
DNA in a

13). Restriction endonuclease digestions containing 10 µl (6.8 µg) of pLJ200, 2 µl of 10 x digestion buffer, 6 µl of distilled water and 2 µl (2 U) of restriction endonuclease were treated with Promega's Clean Up system. The products were ligated in reactions containing 5 µl (0.6 µg) of DNA, 5 µl of 10 x ligation buffer, 38 µl of distilled water and 2 µl (6 U) of T4 DNA ligase. Transformation of DH5α with 5 µl (0.06 µg) of each ligation reaction yielded strains with the desired deletions in the pLJ200 insert. To obtain these plasmids for sequencing, DH5α containing the desired plasmid was grown overnight in Terrific broth at 37 °C. After purifying plasmids with the small scale plasmid preparation, they were treated with Promega's Clean Up system. Preparations which demonstrated a 260 nm:280 nm optical density ratio between 1.8 and 2.0 were used for sequencing. Dyed primer reactions were used to determine partial sequences of the inserts in pLJ200 and its derivative deletion plasmids. This reaction used dyed primers from the SP6 or T7 promoter; nucleotide bases; and nucleotide base analogs (Sanger *et al.*, 1977) to amplify the 4 kb insert by the polymerase chain reaction (PCR). Dyed terminator reactions used primers synthesized by the Macromolecular Structural Facility at Michigan State University with a Perk and Elmer Applied Biosystems model 394 oligonucleotide synthesizer. These reactions contained the synthesized primers, nucleotide bases and dyed base analogs in PCR reactions. Dyed primer reactions contained 3 µg of plasmid dissolved in 15 µl of distilled water. Dyed terminator reactions contained 2 µg of plasmid and 12 pmol of primer dissolved in 20 µl of distilled water. At the MSU-DOE-PRL Plant Biochemistry Facility, automated flourescent sequencing was performed using the ABI Catalyst 800 for Taq cycle sequencing and the ABI 373A Sequencer for the analysis of products.

***In vitro* protein expression.** Promega's S30 coupled transcription and translation system (Zubay, 1973 and Zubay, 1980) was used to express the polypeptides encoded by cloned pOR1 fragments. The 50 µl reaction contained the following: 2 µg of plasmid DNA in a volume of 12 µl of water, 20 µl of premix minus methionine (1.25 mM all 20

amino

triphosp

glutama

(RNasi

[ethane

^{35}S me

the reac

adding

harvest

of load

blue. 20

10 µl w

fragmen

orientat

a small

of 10 x

(stored

Boehrin

Promeg

for 1 hr

mM Zn

Plasmid

(1 8 µg

*Hin*dIII

digested

pLJ100

amino acids, except methionine; 5 mM adenosine triphosphate; 1.25 mM cytosine triphosphate, guanosine triphosphate and uridine triphosphate; 525 mM potassium glutamate; and 50 mM phosphoenol pyruvate), 2 µl (80 Units) of RNase inhibitor (RNasin) from Promega (stored in 20 mM N-[2-hydroxyethyl]piperizine-N'-[ethanesulfonic acid], pH 7.6; 50 mM KCl; 8 mM DTT and 50% (v/v) glycerol), 1 µl of ^{35}S methionine (1200 Ci/Mmol at 10 mCi/ml) and 15 µl of S30 extract. After incubating the reaction for 2 hr at 37 °C, the protein from 10 µl of the reaction was precipitated by adding it to 40 µl of acetone and incubating it on ice for 15 min. The protein was harvested at 18,500 x g for 5 min, dried under a vacuum for 15 min, resuspended in 20 µl of loading buffer (100 mM Tris-HCl, pH 6.8; 4% (w/v) SDS, 0.2% (w/v) bromphenol blue, 20% (w/v) glycerol and 8% (w/v) β-mercaptoethanol) and boiled for 5 min. Then, 10 µl were fractionated on a 12% SDS polyacrylamide gel.

***In vivo* protein expression using T7 RNA polymerase.** The 4 kb *Hin*dIII fragment was subcloned from pLJ100 into pT7-4 (Tabor and Richardson) in both orientations to give plasmids, pLJ270 and pLJ271 (Fig. 17). Purified pT7-4 plasmid from a small scale preparation of MJ830 (pT7-4) was mixed with 88 µl of distilled water, 10 µl of 10 x digestion buffer, 1 µl (10 U) of *Hin*dIII and 1 µl (0.05 µg) of DNase-free RNase (stored in 10 mM Tris-HCl, pH 7.0, 50 mM $CaCl_2$ and 50% (v/v) glycerol) from Boehringer Mannheim. The plasmid was digested for 1 hr at 37 °C and purified with Promega's Clean Up system. After treating it with 4 units of shrimp alkaline phosphatase for 1 hr at 37 °C in a reaction containing 100 mM glycine-NaOH, pH 9.6, 1 mM $MgCl_2$, 1 mM $ZnCl_2$ and 1 mM p-nitrophenyl phosphate; it was incubated for 30 min at 65 °C. Plasmid pLJ100 was digested at 37 °C for 1 hr with *Hin*dIII in a reaction containing 10 µl (1.8 µg) of DNA, 34 µl of distilled water, 5 µl of 10 x digestion buffer, and 1 µl (1 U) of *Hin*dIII. After treating the digestion with Promega's Clean Up system, it was mixed with digested pT7-4 for 12-15 hr at 15 °C in a ligation reaction containing 8 µl (0.4 µg) of pLJ100, 5 µl (0.3 µg) of pT7-4, 2 µl of 10 x ligation buffer, 3 µl of distilled water and 2 µl

(6 U) c
reaction
cloned
MJ832
with the
µl of 1
with 5 µ
with pl
transfor
three str
of 600 r
Harveste
mM Tris
and 0.0
fractiona

S

phoresis
Protean
wiped wi
reagents
14.0 ml o
HCl/SDS
0.116 ml
between th
sec-butano
temperatur
dried. The

(6 U) of T4 DNA ligase. Transformation of DH5α with 10 μl (0.035 μg) of the ligation reaction yielded a transformant containing pLJ270 (Fig. 17). The 4 kb fragment was cloned in the opposite orientation by performing a small scale plasmid preparation on MJ832 (pLJ270), digesting the preparation with *Hin*dIII as described for pT7-4, treating it with the Clean Up system and religating it in a reaction containing 17 μl of DNA (2 μg), 2 μl of 10 x digestion buffer and 1 μl (3U) of T4 DNA ligase. Transformation of DH5α with 5 μl (0.5 μl) of ligation reaction yielded pLJ271 (Fig. 17). HB101 was transformed with plasmid, pGP1-2 (Tabor and Richardson, 1985) to give MJ831. MJ831 was transformed with pLJ270, pLJ271 and PT7-4 to give MJ834, MJ835 and MJ836. All three strains and MJ831 were grown at 30 °C to an optical density of 0.25 at a wavelength of 600 nm, incubated for 30 min at 42 °C, and grown at 37 °C for an additional 90 min. Harvested cells from 6 ml of culture were resuspended in 0.1 ml of cracking buffer (60 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 1% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and 0.01% (w/v) bromphenol blue). After boiling the samples 5 min, 30 μl were fractionated on a 12% SDS polyacrylamide electrophoretic gel.

SDS polyacrylamide gel electrophoresis. SDS polyacrylamide gel electrophoresis (Ausubel *et al.*, 1992 and Laemmli, 1970) was performed using a Bio-Rad Protean II Slab Cell apparatus. Two thoroughly cleaned glass plates and two spacers wiped with ethanol were assembled in the apparatus. The following filtered, resolving gel reagents for a 12% gel were mixed and degassed for 15 min: 12.25 ml of distilled water; 14.0 ml of 30% (w/v) acrylamide and 0.8% (w/v) bisacrylamide; and 8.75 ml of 4 x Tris-HCl/SDS (1.5 M Tris-HCl, pH 8.8 and 0.4% SDS). The solution was gently mixed with 0.116 ml of 10% (w/v) ammonium persulfate and 0.020 ml of TEMED and poured between the plates to a level of 4 cm from the top. After layering 1 ml of water saturated sec-butanol on the top of the running gel, it was allowed to polymerize for 1 hr at room temperature. The sec-butanol was decanted. The gel was rinsed with distilled water and dried. The following stacking gel reagents were mixed and degassed for 15 min: 9.15 ml

of wa

Tris-H

with C

top o

Cell a

25 mL

migrat

bromp

β-lacto

and my

at app

respect

in a so

dried in

at -80 °

acetic a

brilliant

water fo

88% (v/

photogra

Harveste

polypept

(w/v), 0.

mM β-m

polyacryla

thioglycol

of water; 0.95 ml of 30% acrylamide and 0.8% (w/v) bisacrylamide; and 3.75 ml of 4 x Tris-HCl/SDS (0.5 M Tris-HCl, pH 6.8 and 0.4% SDS). The solution was gently mixed with 0.15 ml of 10% (w/v) ammonium persulfate and 0.015 ml of TEMED, and poured on top of the running gel. After the stacking gel polymerized, the Bio-Rad Protean II Slab Cell apparatus was assembled with the upper and lower reservoir buffers both containing 25 mM Tris-HCl, pH 8.3, 200 mM glycine and 0.1% (w/v) SDS. Protein samples migrated through the stacking gel at 100 V and the running gel at 200 V until the bromphenyl blue dye reached the bottom of the running gel. Protein standards, lysozyme, β-lactoglobulin, carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase B and myosin (H-chain), were obtained from Bethesda Research Laboratories and migrated at apparent molecular weights of 15.4, 18.1, 28.3, 43.3, 69.8, 105.1 and 215.5 kDa, respectively. For autoradiography, the running gel was fixed for 1 hr with gentle shaking in a solution containing 50% (v/v) methanol, 3% (v/v) glycerol, 10%(v/v) acetic acid; dried in a Bio-Rad model 583 gel drier at 80 °C for 2 hr; and exposed to film, overnight, at -80 °C. For coomassie staining, gels were fixed in 50% (v/v) methanol, 10% (v/v) acetic acid, and 40% distilled water for 30 min and stained in 0.05% (w/v) coomassie brilliant blue (Bio-Rad), 50% (v/v) methanol, 10% (v/v) acetic acid and 40% distilled water for 4 hr. They were destained with 7% (v/v) acetic acid, 5% (v/v) methanol and 88% (v/v) distilled water for 2 hr. After rinsing them with distilled water, they were photographed.

N-terminal amino acid sequence determination of the SedR polypeptide. Harvested MJ836 (pGP1-2 and pLJ271) cells that were induced to synthesize the SedR polypeptide were resuspended in 100 µl of 2 x sample buffer (0.2 M sucrose, 6% SDS (w/v), 0.125 mM Tris-HCl, pH6.9, 4 mM EDTA, 0.5% (w/v) bromphenol blue and 286 mM β-mercaptoethanol), incubated for 15 min at 65 °C and separated on a 12% SDS polyacrylamide electrophoretic gel. The upper electrode buffer contained 0.1 mM sodium thioglycolate. After electrophoresis, polypeptides were transferred from the running gel to

a sheet of Biorad's polyvinylidene diflouride (PVDF) membrane (Speicher, 1989) using Biorad's Trans-Blot Cell apparatus. The apparatus was assembled as mentioned in the protocol for DNA transfer, except Towbin buffer (Towbin *et al.*, 1979), which contained 25 mM Tris, 192 mM glycine and 20% (v/v) methanol, was used as the transfer buffer. Polypeptides were transferred to the membrane at 30 V for 15-20 hr. The polypeptides on the membrane were then stained with 40% (v/v) methanol and 0.025% (w/v) coomassie blue R for 15 min and destained with 50% (v/v) methanol for 5 min. The SedR polypeptide was excised, and the first 15 N-terminal amino acid residues were sequenced by Edman degredation (Edman and Begg, 1967) using a Perk and Elmer Applied Biosystems model 494 protein/peptide sequencer (Matsudaira, 1987). This work was performed by the Macromolecular Structure Facility at Michigan State University.

Nucleotide and protein sequence analysis. The Macintosh program, Amplify (Engels, unpublished), was used to predict whether a primer selected from a known nucleotide sequence in a fragment would amplify an unknown segment of the fragment in the polymerase chain (PCR) reaction. Adjacent nucleotide sequences were identified using a nucleotide sequence comparison program from Intelligenetics Geneworks (Smith *et al.*, 1981; Smith and Waterman, 1981). This program located identical stretches between nucleotide sequences so that a complete sequence could be assembled from partial overlapping sequences. Nucleotide and polypeptide sequences were analyzed by a basic local alignment search tool (Blast) (Altschul *et al.*, 1990) at the National Center for Biotechnology Information (NCBI). This program compared nucleotide and polypeptide sequences to other known sequences and identified segments that were similar or identical to segments of the known sequences. Information on using Blast was obtained by sending electronic mail to blast@ncbi.nlm.nih.gov with the word HELP in the body of the message. Sequences were examined for open reading frames (Tzagoloff, 1982) using a program from Intelligenetics Gene Works for the Macintosh.

Growth curves. LB Broth was inoculated 1:100 with an overnight culture containing X2642 (pBR322), MJ800 (pOR1) or MJ801 (pLJ100). These new cultures were grown at 30 ^{o}C in a baffled flask at 200 rpm. Turbidity was measured every hour using a Klett Summerson Colorimeter with a no. 59 filter. After 2 hr, 1 M sodium selenite was added to give a final concentration of 40 mM selenite. For every hour from 3 hr to 12 hr, 25 ml of cells were harvested at 14,000 x g; resuspended in 1 ml of a buffer containing 10 mM potassium phosphate and 1 mM EDTA, pH 7.1; sonicated; and centrifuged at 20,000 x g. The supernatant was poured into new tubes, frozen at -20 °C, and total protein was determined at a later date using a Bradford assay (1976). Both the cells and the elemental selenium which became associated with the cells contributed to turbidity. Thus, the same growth experiment was performed for X2642 (pBR322) in the absence of selenite to establish a linear correlation between turbidity and total protein for cells not associated with elemental selenium (Fig. 21). This standard curve was used to determine the expected turbidity attributed to cells alone for strains grown in selenite (Fig. 22).

Total protein assay. Total protein from cell extracts were determined using a method described by Bradford (1976). Bradford reagent which, was obtained from BioRad, contained 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol and 8.5% (w/v) phosphoric acid. Standard assays contained 5 ml of Bradford reagent and 10 to 100 μg of BSA dissolved in 1 ml of 10 mM potassium phosphate and 1 mM EDTA, pH 7.1. Absorbance was measured at 595 nm. A standard curve demonstrating a linear correlation between absorbance and protein was used to determine the amount of protein in an unknown sample.

Bioremediation experiments. The dialysis tubing experiments (Komori *et al.*, 1990) were assembled as shown in Figure 20. The top of a no. 14 rubber stopper was removed with a saw so that it fit firmly inside the top of a 600 ml beaker. Five holes that could firmly hold 2 ml conical, screw cap tubes (Fisher) were drilled evenly around the circumference of the top. Another hole was drilled through the center for a bubble tube.

The bottoms of five 2 ml conical tubes were removed. Four Spectral/Por 4 dialysis tubes with a molecular weight cut off of 14,000 (Baxter) were sealed at one end and fit around the neck of four of the screw cap tubes that were inserted into one of the holes around the circumference of the stopper. The fifth hole also contained a screw cap tube, but it was used to withdraw samples from the medium. The bubble tube was inserted through the hole in the center of the stopper. The 600 ml beaker was filled with 350 ml of LB broth, and the assembled stopper was placed firmly in the beaker. Aluminum foil was placed around the top of the beaker to seal it, and the whole apparatus was sterilized in an autoclave. Three systems were assembled. To each system, sodium selenite was added to a concentration of 10 mM. Separate cultures of *S. maltophilia* ORO2, MJ800 (pOR1) and HB101 were grown overnight in 250 ml of LB broth at 25 °C, harvested at 3000 x g, and resuspended in 40 ml of LB broth. Samples of 10 ml were added to dialysis bags immersed in the medium and allowed to grow at 25 °C. Oxygen was introduced to each system by bubbling sterile air through the bubble tubes. Selenite concentrations were measured 12 and 24 hr later.

Selenite concentrations were measured by mixing 0.5 ml of 1 M sulfuric acid and 0.5 mM 2-mercaptobenzimidizole with 0.5 ml of LB broth containing selenite. After allowing the reaction to incubate at room temperature for 2 hr, the formation of a selenite/2-mercaptobenzimidizole complex was measured at 318 nm (Blake, personal communication). The amount of selenite in a sample was determined from a standard curve that established a linear correlation between the amount of selenite and absorbance at 318 nm.

Non-sterile batch culture experiments were performed using *S. maltophilia* ORO2. An overnight culture of *S. maltophilia* ORO2 grown in sterile M-9 minimal medium was diluted 1:100 in non-sterile M-9 minimal medium containing 100 mM selenite, 0.5% acetate and 0.4 mM cysteine. A similar culture that did not contain *S. maltophilia* ORO2 was also started. Both cultures were aerated at 25 °C in baffled flasks at 200 rpm.

RESULTS

Growth curves of *S. maltophilia* ORO2, HB101, MJ800, and MJ801. To establish a definition for selenite resistance, the ability of each strain to grow in LB broth containing 40 mM selenite was followed with a Klett Summerson colorimeter using a no. 59 filter. Each strain was introduced to selenite during early log phase and turbidity was measured every half hour. In addition to the cells, the elemental selenium, which precipitated during the growth of these strains, also contributed to the turbidity. Thus, the apparent growth rate, μ (doublings/hr), was calculated for each strain grown in the presence and absence of selenite of selenite (Table 3). Even with the contribution of elemental selenium to turbidity, HB101 failed to grow in selenite. It demonstrated an apparent growth rate of less than 0.15 doubling/hr. *S. maltophilia*, MJ800 (pOR1) and MJ801 (pLJ100) grew in selenite at apparent growth rates of 0.83, 0.75 and 0.67 doublings/hr, respectively. Thus, pOR1 and the 4 kb *Hin*dIII insert in pLJ100 conferred selenite-resistance in HB101.

Physical mapping and size determination of pOR1. The physical map of pOR1 was constructed using *Bgl*II, *Acc*65I (*Kpn*I), *Hin*dIII, *Sac*I (*Eco*ICRI) and *Bam*HI restriction enzymes. Digestions of pOR1 in Figure 5 were fractionated by agarose gel electrophoresis, and the length and number of each fragment produced was determined to calculate a size of 100 kb for pOR1 (Table 4). The gel was Southern blotted to a nylon filter and cloned *Acc*65I, *Hin*dIII, *Sac*I and *Bam*HI fragments were used as probes in hybridizations to identify adjacent pOR1 fragments (Fig. 6). In lane 2 of Figure 6, the 4 kb *Hin*dIII fragment hybridized to the 4.3 kb, 2.8 kb and 1.3 kb *Bgl*II fragments. Thus, these three fragments were adjacent, with the probe containing the complete 1.3 kb *Bgl*II fragment and a portion of the other two fragments. The 16.5 kb and 21.5 kb *Bgl*II fragments also displayed weak signals, but digestions of these fragments with *Hin*dIII and a digestion of the 4 kb *Hin*dIII fragment with *Bgl*II revealed by agarose gel

Table 3. Influence of pOR1 and pLJ100 on cell growth in the presence of selenite.

Organism	Plasmid	[a]Apparent growth rate μ (doublings/hr)	
		- Selenite	+ selenite[b]
S. maltophilia ORO2	pOR1	1.6	0.83
E. coli HB101		1.2	< 0.15
MJ800	pOR1	0.81	0.75
MJ801	pLJ100	0.71	0.67

[a]Selenite (40 mM) was added to early log phase cultures growing at 30 °C. The growth rate $\mu = 1/g$, where the generation time $g = \ln 2/k$ and k is the instantaneous growth rate constant.

[b]The contribution of selenite to turbidity was included in these calculations.

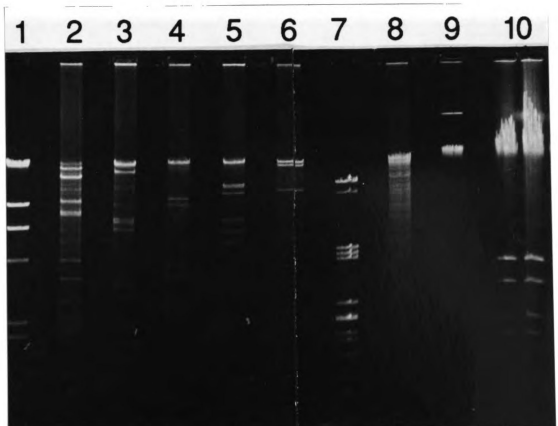


Figure 5. Agarose gel of pOR1 digestions. Lane 1: *Hin*dIII digested λ DNA. Lane 2: *Bgl*II digested pOR1. Lane 3: *Acc*65I (*Kpn*I) digested pOR1. Lane 4: *Hin*dIII digested pOR1. Lane 5: *Sac*I digested pOR1. Lane 6: *Bam*HI digested pOR1. Lane 7: *Pst*I digested λ DNA. Lane 8: *Hin*dIII digested *E. coli* HB101 total genomic DNA. Lane 9: Total genomic DNA from MJ800 (pOR1). Lane 10: Total genomic DNA from *Stenotrophomonas maltophilia* ORO2.

Table 4. Fragment sizes generated by restriction endonuclease digestions of pOR1.

Fragment sizes (kb)				
*Bgl*II	*Acc*65I (*Kpn*I)	*Hin*dIII	*Eco*ICRI (*Sac*I)	*Bam*HI
21.5	26.4	33	23.6	44
16.5	26.4	23.6	15.0	26.2
16.6	19.0	11.7	14.0	13.4
10.2	7.4	10.0	13.6	6.1
8.8	7.2	4.3	11.8	4.3
7.5	6.2	4.0	8.0	2.5
4.0	3.5	2.7	6.7	1.3
3.7	2.4	2.7	5.7	1.3
2.8	2.2	2.5	2.4	1
2.4		2.2		
2.3		1.9		
2.1		1.3		
1.4				
1.3				
101.1	100.7	99.9	100.8	100.1

The sum of the fragments sizes are noted at the bottom of the table.

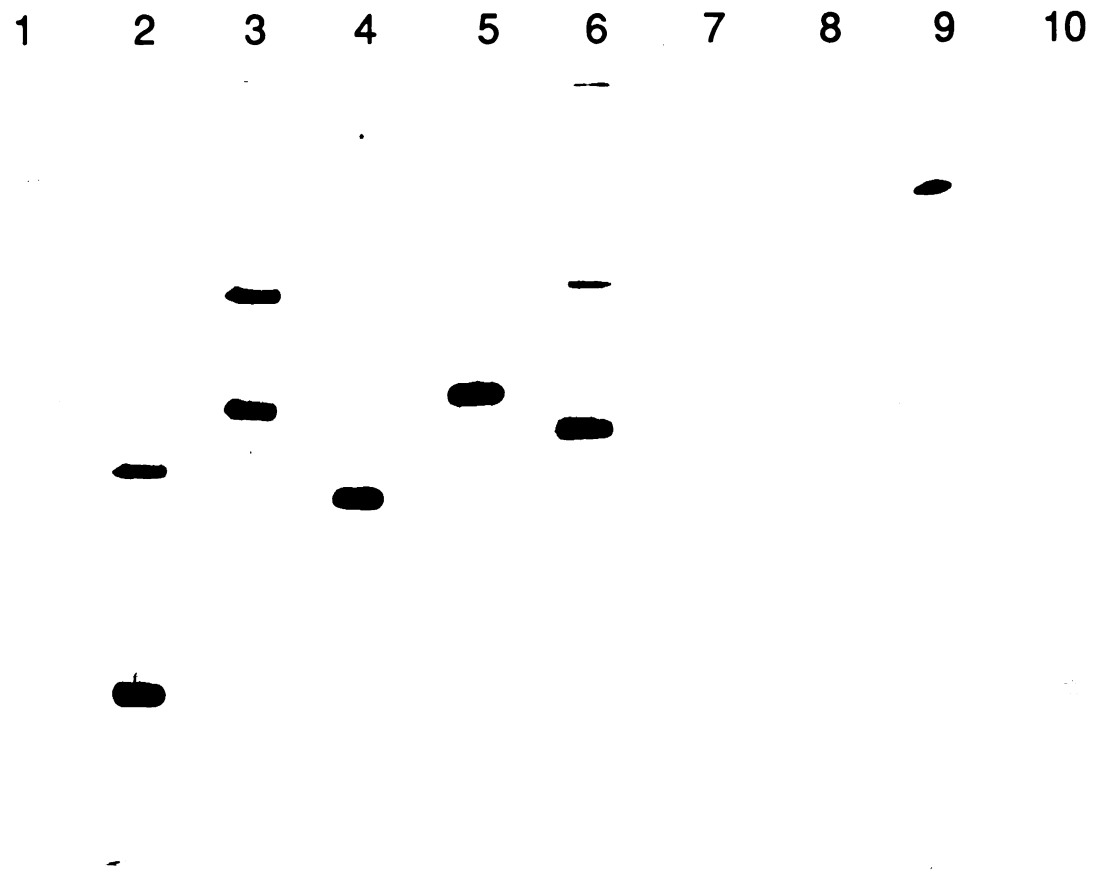


Figure 6. Southern analysis of pOR1 digestions using the 4 kb *Hin*dIII fragment from pOR1 as a probe. Lane 1: *Hin*dIII digested λ DNA. Lane 2: *Bgl*II digested pOR1. Lane 3: *Acc*65I (*Kpn*I) digested pOR1. Lane 4: *Hin*dIII digested pOR1. Lane 5: *Sac*I digested pOR1. Lane 6: *Bam*HI digested pOR1. Lane 7: *Pst*I digested λ DNA. Lane 8: *Hin*dIII digested *E. coli* HB101 total genomic DNA. Lane 9: Total genomic DNA from MJ800 (pOR1). Lane 10: Total genomic DNA from *Stenotrophomonas maltophilia* ORO2.

electrophoresis that none of the resulting fragments were similar in size. The 4 kb *Hin*dIII probe was not located near these two fragments. The location of other pOR1 fragments was determined by repeating this experiment with the cloned pOR1 fragments shown in Figure 7.

Approximately 60% of the map was constructed by using cloned pOR1 fragments as probes in hybridizations. The rest of the map was completed by using double restriction enzyme digestions. For example in Figure 8, lane 1 contained two 26.4 kb *Acc*65I (*Kpn*I) fragments digested with *Bgl*II, lane 4 contained one 21.5 kb *Bgl*II fragment digested with *Acc*65I and lane 6 contained two 16.5 kb *Bgl*II fragments digested with *Acc*65II. The restriction enzyme digestion products of 11.2 and 10.3 kb in lane 1 and 4 suggested that the 21.5 kb *Bgl*II fragment was cut in half by *Acc*65I and contained a segment of each 26.4 kb *Acc*65I fragment. The digestion products of 16.1 and 15.2 kb in lanes 1 and 6 suggested that the other half of each 26.4 kb *Acc*65I fragment contained a part of one of the 16.5 kb *Bgl*II fragments.

Stability of pOR1 in *S. maltophilia* ORO2. Under laboratory conditions, it appeared that pOR1 was converted to smaller plasmids in *S. maltophilia* ORO2. In Figure 5, lane 10, pOR1 was no longer detectable, and four smaller plasmid bands that were present were not in the original gel electrophoretic profiles of *S. maltophilia* ORO2. In addition, the 4 kb *Hin*dIII fragment hybridized to pOR1 from *E. coli* in lane 9 of Figure 6 and to two smaller plasmid bands from *S. maltophilia* ORO2 DNA (lane 10) but not to a 100 kb plasmid band from S. *maltophilia* ORO2. The four small S. *maltophilia* ORO2 plasmid bands were excised from an agarose gel and digested with *Hin*dIII (Fig. 9). Plasmid bands A and C both yielded fragments of 4.9 kb and 3.7 kb. Plasmid bands B and D both yielded fragments of 3.6 kb. Since the 4 kb *Hin*dIII fragment from pOR1 hybridized to plasmid bands A and C, these plasmids contained a homologous sequence. No relationship could be established between the plasmids in bands B and D.

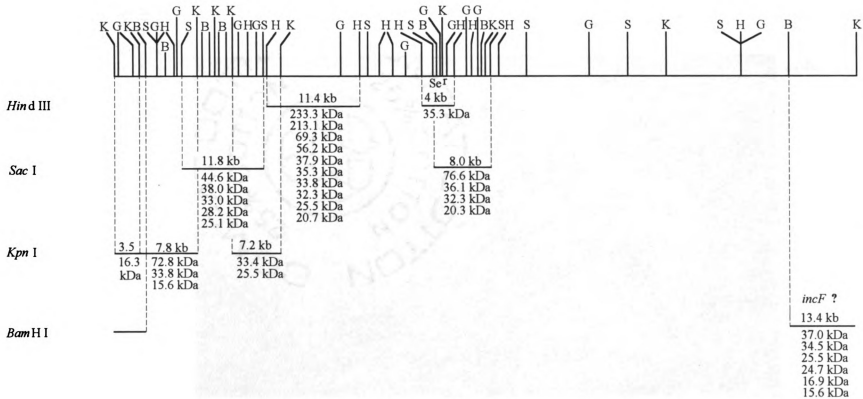


Figure 7. Physical map of pOR1. Sizes of polypeptides encoded by each cloned fragment are listed below the lines representing each pOR1 segment. Abbreviations: B, *Bam*HI; G, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; and S, *Sac*I.

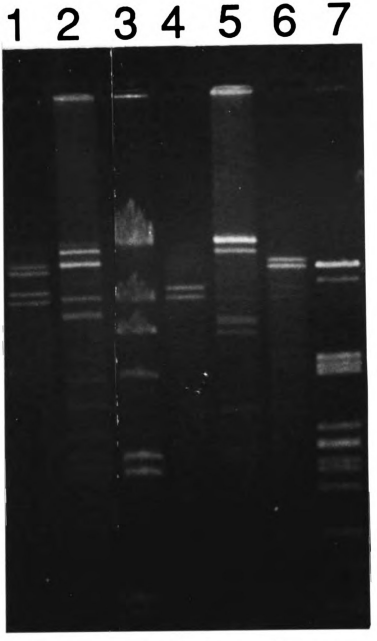


Figure 8. Agarose gel used to map two 26.4 kb *Acc*65I (*Kpn*I), a 21.5 kb *Bgl*II and two 16.5 kb *Bgl*II fragments. Lane 1: *Bgl*II digested 26.4 kb *Acc*65I fragments. Lane 2: *Bgl*II digested pOR1 plasmid. Lane 3: *Hind*III digested λ DNA. Lane 4: *Acc*65I digested 21.5 kb *Bgl*II fragment. Lane 5: *Acc*65I digested pOR1 plasmid. Lane 6: *Acc*65I digested 16.5 kb *Bgl*II fragments. Lane 7: *Pst*I digested λ DNA.

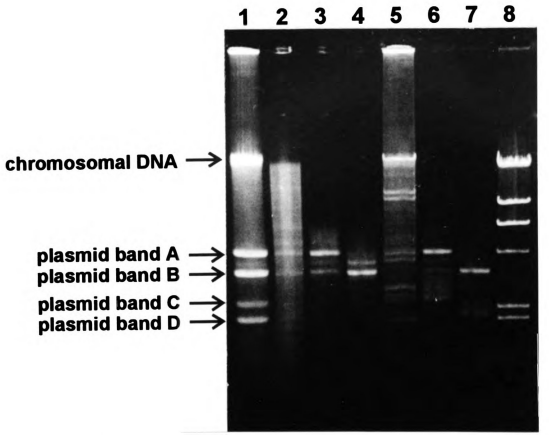


Figure 9. *Hin*dIII digestions of *Stenotrophomonas maltophilia* ORO2 plasmid bands. Lane 1: *S. maltophilia* ORO2 total genomic DNA. Lane 2: *S. maltophilia* ORO2 total genomic DNAdigested with *Hin*dIII. Lane 3: Plasmid band A digested with *Hin*dIII. Lane 4: Plasmid band B digested with *Hin*dIII. Lane 5: pOR1 digested with *Hin*dIII. Lane 6: Plasmid band C digested with *Hin*dIII. Lane 7: Plasmid band D digested with *Hin*dIII. Lane 8 λ DNA digested with *Hin*dIII.

***In vitro* expression of pOR1 fragments**. The size and number of detectable polypeptides encoded by each pOR1 fragment was determined by introducing each into an *in vitro* transcription and translation system (Zubay, 1973) and separating the products with a 12% SDS polyacrylamide gel. A fragment was considered to encode a polypeptide if its electrophoretic profile contained a band different in size from those in the profile of the pUC19 or pBR322 control vectors (Figure 10). The 11.8 kb *Sac*I fragment produced 44.6, 38.0, 33.0, 28.2 and 25.1 kDa polypeptides. The 7.8 kb *Acc*65I fragment encoded 72.8, 33.8 and 15.6 kDa polypeptides. The 3.5 kb *Acc*65I fragment generated a 16.3 kDa polypeptide. The 13.4 kb *Bam*HI fragment produced 37.0, 34.5, 25.5, 24.7, 16.9 and 15.6 kDa polypeptides. The 11.4 kb *Hin*dIII fragment generated 233.3, 213.1, 69.3, 56.2, 37.9, 35.3, 33.8, 32.8, 25.5 and 20.7 kDa polypeptides. The 7.2 kb *Acc*65I fragment encoded 33.4 and 25.5 kDa polypeptides. The 8.0 kb *Sac*I fragment produced 76.6, 36.1, 32.3 and 20.3 kDa polypeptides. The 4 kb *Hin*dIII fragment generated a 35.3 kDa polypeptide. This was the only consistent band observed in all other expression experiments with this fragment (Fig. 11). The other bands observed in Figure 10 for the 4 kb *Hin*dIII fragment were not detected previously. The calculated size of the polypeptide(s) produced by each fragment are presented below the line which represents each fragment in Figure 7. All polypeptides encoded by these fragments were probably not detected by this system. Two dimensional gel electrophoresis would distinguish between some of the pOR1 polypeptides which are similar in size to those of pUC19 and pBR322.

Incompatibility experiments. Preliminary nucleotide sequence data showed that the 4 kb *Hin*dIII fragment from pOR1 contained a segment from the transposon, Tn*1000*, which was originally discovered in the *E. coli* F-plasmid. To determine if pOR1 was related to the F-plasmid, incompatibility tests were conducted using pUC19 and JM109. The *proA* and *proB* genes were deleted in JM109 so that proline synthesis depends upon an F′ episome containing $proA^+$ and $proB^+$. JM109 was transformed with each

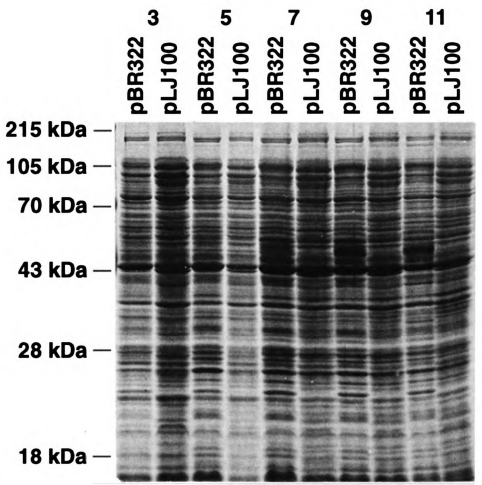


Figure 10. *In vitro* transcription and translation of cloned pOR1 fragments. Lane 1: 11.7 kb *Sac*I fragment. Lane 2: 7.4 kb *Acc*65I (*Kpn*I) fragment. Lane 3: 3.5 kb *Acc*65I (*Kpn*I) fragment. Lane 4: 13.4 kb *Bam*HI fragment. Lane 5: pUC19. Lane 6: 11.8 kb *Hin*dIII fragment. Lane 7: 7.2 kb *Acc*65I (*Kpn*I) fragment. Lane 8: 7.7 kb *Sac*I fragment. Lane 9: No DNA. Lane 10: 4 kb *Hin*dIII fragment. Lane 11: pBR322. The 4 kb *Hin*dIII fragment was the only one cloned in pBR322. All other fragments were cloned into pUC19.

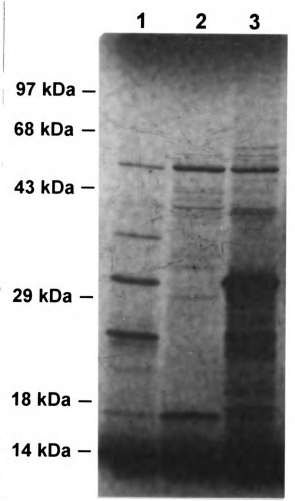


Figure 11. *In vitro* transcription and translation of the 4 kb, *Hin*dIII insert in pLJ100. Lane 1: pLJ100. 2: no plasmid 3: pBR322.

recombinant of pUC19 containing a pOR1 insert and spread on minimal ampicillin plates supplemented with and without proline. All transformants grew on both plates, except for the one carrying pLJ193 which contained a 13.4 kb *Bam*HI fragment from pOR1. It grew on the ampicillin plate supplemented with proline but failed to grow on the ampicillin plate lacking proline. However, it grew when a transformant from the ampicillin and proline plate was streaked onto an ampicillin plate without proline. To investigate this result more closely, the following incompatibility experiment was performed (Fig. 12). Overnight cultures of MJ846 (pLJ193) and MJ847 (pUC19) were diluted by 10^{-6} in minimal medium with proline (selection for neither plasmid), grown overnight and plated on minimal medium plate with proline. Fifty colonies from these plates were placed on minimal medium plates with ampicillin and proline (selection for pUC19 and pLJ193) and on minimal medium plates lacking ampicillin and proline (selection for the episome). All colonies of MJ847 (pUC19) grew on both plates. All colonies of MJ846 (pLJ193) grew on the ampicillin and proline plate, but none grew on the plate lacking ampicillin and proline (Table 5). The F′ episome was eliminated in the presence of pLJ193 but was retained in the presence of pUC19.

To determine if the 13 kb insert in pLJ193 contained an incompatibility determinant for pOR1, the same experiment was performed for MJ844 (pOR1 and pLJ193) and for MJ845 (pOR1 and pUC19). Both strains retained selenite-resistance, but none of the MJ844 colonies and 74% of the MJ845 colonies retained ampicillin-resistance (Table 6). When this experiment was repeated with selection for one of the plasmids during competition (ampicillin or selenite), all MJ844 and MJ845 colonies retained selenite-resistance and 74% of the colonies from both strains retained ampicillin-resistance (Table 7). It was not clear whether pOR1 out competed the CoelE1 incompatibility determinant from pUC19 or an incompatibility determinant from the 13 kb *Bam*HI insert in pLJ193.

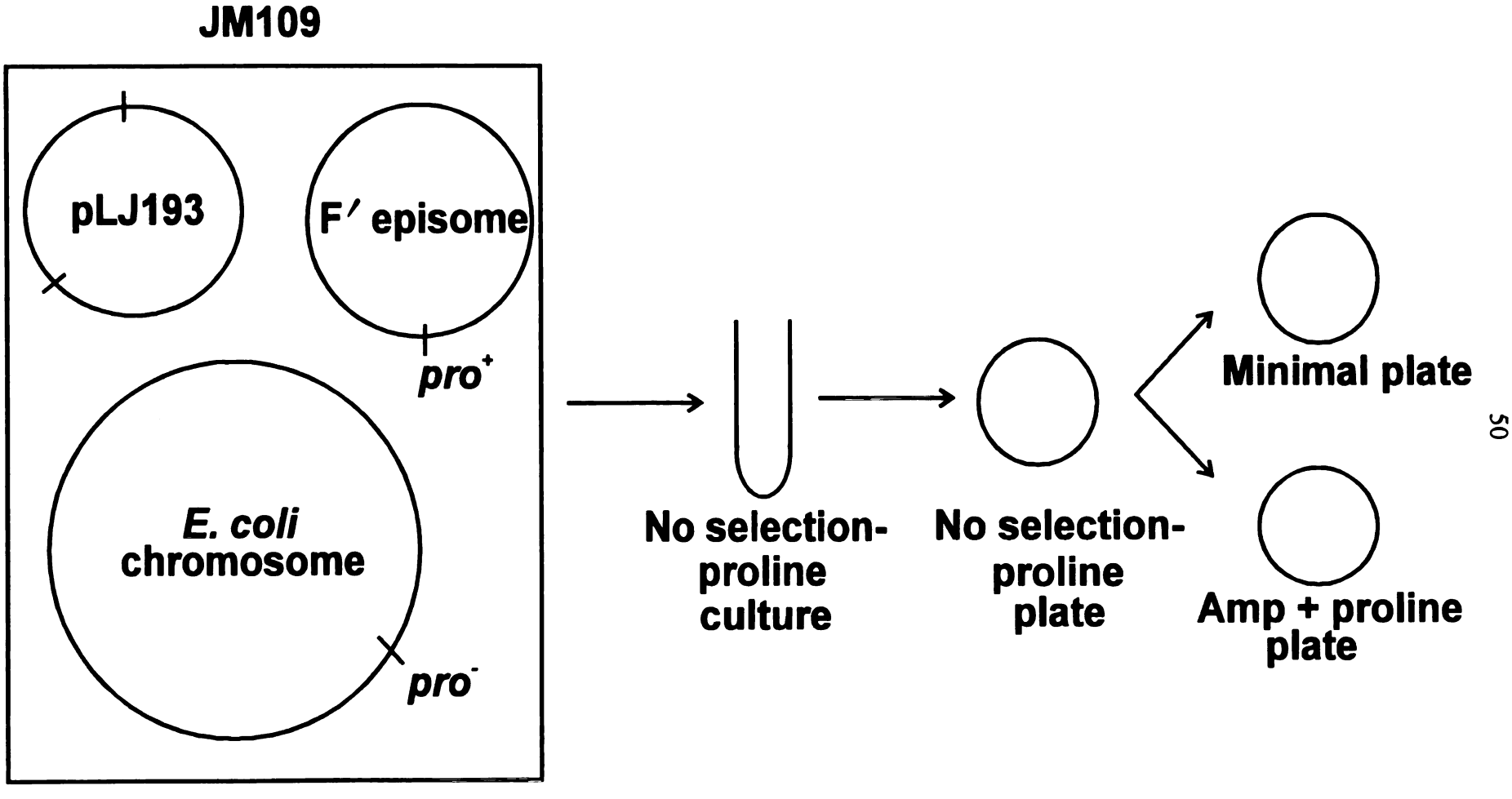


Figure 12. Incompatibility testing and analysis for pOR1 and F.

Table 5. Incompatibility experiments using the F′ episome from JM109 and pLJ193 that contains a 13 kb *Bam*HI fragment from pOR1.

	Percent colony growth	
M-9 medium	MJ846(F′+pLJ193)	MJ847(F′ episome+pUC19)
Minus proline	0	100
Proline + ampicillin	100	100

Table 6. Incompatibility experiments with selection for neither plasmid.

	Percent colony growth of HB101 strains						
LB broth	pUC19	pLJ193	pOR1	MRF′ Kan	pOR1 + pLJ193	pOR1 + pUC19	pOR1 + MRF′ Kan
Ampicillin	100	100	NA	NA	0	74	NA
Selenite	NA	NA	100	NA	100	100	100
Kanamycin	NA	NA	NA	100	NA	NA	100

NA - not applicable

Table 7. Incompatibility experiments with selection for one of the plasmids.

	Percent colony growth of HB101 strains		
LB broth	pOR1 + pLJ193	pOR1 + pUC19	pOR1 + MRF' Kan
Ampicillin	74	74	NA
Selenite	100	100	100
Kanamycin	NA	NA	100

NA - not applicable

Two plasmids are defined as incompatible only if elimination is reciprocal during competition. Since elimination was not reciprocal in the incompatibility experiments above, pOR1 was tested directly by mating it with a strain that contained an F'episome. An inconsistent *pro* marker in MJ800 (pOR1) made it difficult to transfer the F' episome from JM109 to MJ800. Thus, MJ800 was mated with XL1- Blue MRF' Kan, which possessed a kanamycin-resistance marker on the episome, MRF' Kan. This new strain, MJ841, retained selenite and kanamycin resistance in both types of incompatibility experiments, without selection for either plasmid and with selection for one of the plasmids (Table 6 and 7). These results suggested that pOR1 may not contain an *incF* determinant, may contain a CoelE1 replication origin, or carries and uses an additional replication origin unrelated to the one from the F-plasmid.

Nucleotide sequence determination of the 4 kb fragment. The nucleotide sequence of the 4 kb, *Hin*dIII fragment from pOR1 was determined using the strategy shown in Fig. 13. It was subcloned between the T7 and SP6 phage promoters in plasmid, pSP73, to create the recombinant plasmid, pLJ200. Dyed primers for the T7 and SP6 phage promoters were used to sequence the insert at both ends. Since each reaction was accurate through 300 bp only, deletions in pLJ200 were constructed using the restriction endonuclease sites shown in Figure 13, and the sequence was determined from these sites using the dyed SP6 primer. There were not enough restriction enzyme sites in the 4 kb fragment to obtain a complete sequence. The Macintosh program, Amplify (Engels, 1993), helped predict primers which had a strong potential to amplify new segments of the 4 kb insert in a polymerase chain reaction (PCR). These primers were synthesized and used with dyed base analogs to complete the sequence of the pLJ200 insert. The nucleotide sequence of the 4 kb fragment is shown in Figure 14.

Sequence analysis. The DNA sequence was analyzed by a basic local alignment search tool (Blast) at the National Center for Biotechnology Information (NCBI) (Altschul *et al.*, 1990) This program compared it to other known DNA sequences (Fig. 13). A 2.2

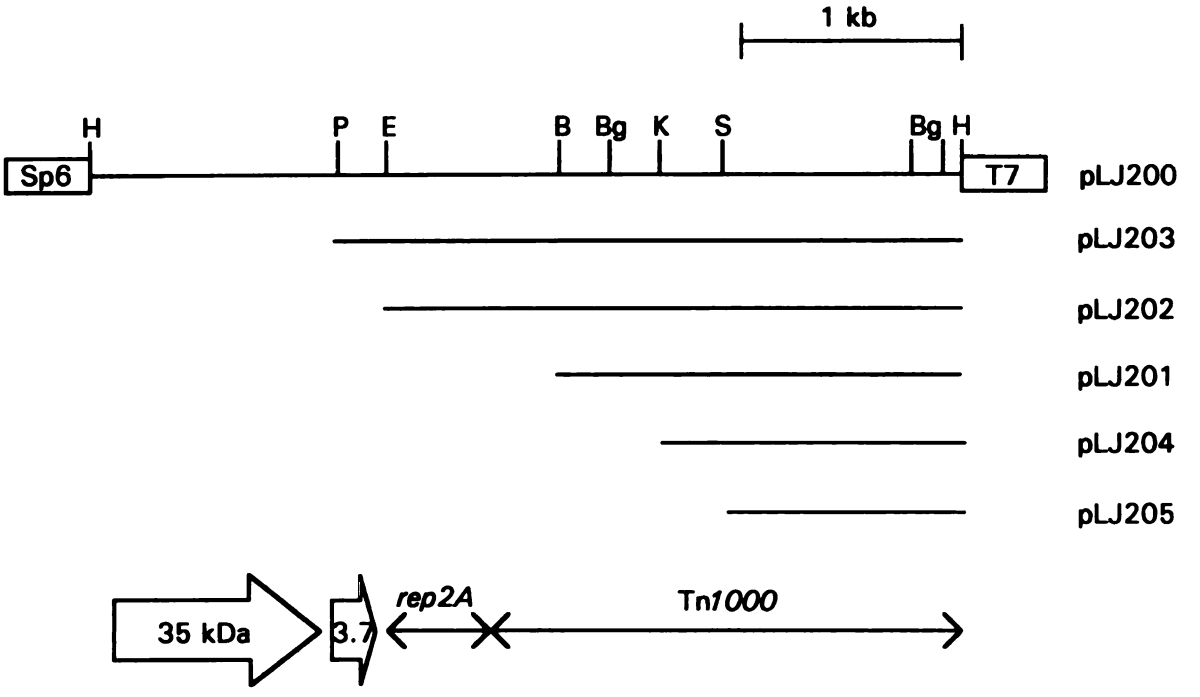


Figure 13. Sequence features of the 4 kb, *Hin*dIII fragment from pOR1. The location of a 35 kDa open reading frame, a 3.7 kDa hypothetical open reading frame, a portion of *rep2A* and a 2.2 kb segment of Tn*1000* are noted below deletions used to sequence this fragment. The open reading frames are not in the same frame. Abbreviations: *Bam*HI (B); *Bgl*II (Bg); *Eco*R1 (E); *Hin*dIII (H); *Kpn*I (K); *Pst*I (P); and *Sph*I (S).

Figure 14. Nucleotide sequence of the 4 kb, *Hin*dIII fragment from pOR1.

AAGCTTTCAG CACCTTAAAA ACAAATTCCA ATAATAGAAA AAACAAATGC CAAGGTAATG 60
CAATTCAGTT ATTGCAGAAA ATATACTTTA TCTATCATCC TCACATTAGA TGGGGATGAT 120
TAATATATTC CATGAGACTC TTTATGAATA CTATAAAAAT AAAGCTAAAC CTAATTGATT 180
ATGATTCAAT TGTAAATATT GAATTTCCCT GCCTCCTATC TGAAATAGAG ATAGAGTTAT 240
TATCTCAATT ACTGAAGGGG TATTCTGTAA ATGAGATTTC CAAGCGAAGA AACAGAAGTA 300
TCAAAACAGG CTCGTGCCAA AAAATGAAAT TATATAAAAA ACTTAACGTA AAAAGCGATC 360
TGACTTTATG GGGAGATGTT TTTTTAAGAT TTAAAGCCTA CCTACAACCT AAAAATATCA 420
TCTGTGATAA TTTTAATAGG TCTGTTTTAC CAGTTGTTTC ATCTAAAGGG GAGAGTATGG 480
CGCACTACAA CATATATTAC CAACCGATCT ATAACGCAAA AAATGGAAAT ATTGCTGGAT 540
GTGATGTTAC TATTGCGTTA AAAAATTCAG ATGGTAGTGC TTTGGCACTA GATTCTGACA 600
GAATTAATTA TAACCCCAAT GATAACAAAG TATCTTATTT GTGTGGACAT ATCAACAAAC 660
TATTCTCACC GATTAAAAAT AATCTTCCGC ATGGTTTTTT TATTACGATA AACATTAATC 720
CTGAAGATAT TCTTACCTGT GATATCGAAA GGGAGTGTTT ACATTTTATT AAAGTTTTCG 780
GAACGGAACG GATAAGACTT GTCTTGCAGT TTTCAACTAA AGAAGAACTT TACATAATAA 840
GAAGATACCA GTCTTCTTTA AGACGTATCA GAAATAATAA TGTTTATTTA TCGCTTAATG 900
ATTTCGGTAT GGGATATGCT GAGCTTTCAC ACTTGCAAAA CATACCTTTC TCCTATGTAA 960
ACCTACATAA AACAATGTTC CATGATATAG AAAGCAATAG TTTAACAGAT ATAATAGCAA 1020
CTACAATTAT TGATTTACCC AAACCCCCCT CATATAGATG TTATTGCTGA TGGTATAGAA 1080
ACGAAGAAAC AGGCAGGTTA TATGATTGAA AGAGGAGTTA AATATTTAAA AGGCATTGCA 1140
TTATCATCAC CGCTACCTGC AGATGCATTT GTACGAAAAT TGTTAGCTTC ATTAAAACAA 1200
GTCTAAAAAT CAGAACCACC CATATAGCGG GTGGTTCTGA TTTTTTTAAT ATCACCTGGT 1260
AAATATTATA CCATTTTCAT TTGATATGAA TTTCCTTAAA ATTGATATCA GCGAAGATTA 1320
TAATTTTCGA GTGCGCAAAA CCTGTCCGAT TTGTTAAACA ACAAAAAGAT TAAGGGTTTA 1380
TTGGTATGAA ATAAGAAAAA AACCTCCTTT GAATTCAAAG GAGGAGAAAA ATAAGGCTTA 1440
CTTCAAGTCA CCCCCACTAT CAATGGAAGT TTCTTATTTA TACCTGTTCT TAATGTTAAA 1500
TGTGATGTGT AAATTCCTAA ATTTAAATCT ATTGCCCATT TTGGGGTAAA TTTCTTTGCT 1560
CAAGGGTAAT TGTCATGGCG GTCACCAAAC AATAACGTAG CTGGCGAAGT TCCATTGCGA 1620
TTCTCCGGAG AAGGCTGGGA TATTTACATT AAATCAACAT GCTGTCAGAA GAAAGCACTG 1680
AGTAGAGAAT GGCGCGCATA ACAAAACAAC CGACAGGTTG ACTTTTCAGA ATAGGGGCAG 1740
TTTGGTGATC GTCACCAGTA TATGGGGTTT GAGGGCCAAT GGAACGAAAA CGTACGTTAA 1800
GGAGATAATT CGTTGTTTAT ATTTAAATTT AGAGCTCTCA GTTCCCCTTT TAAAATATCC 1860
TCTGGCAACG TGAATGTATA ATGGCCCAAC ATATTGATAT GCCCGTTGCA TCAGGGGGAG 1920
ATAAGCCGGA GCGATATCTT TCAATCTATA ATTTCCTTTT CCCATTACGG GCGCAATCCA 1980
AGCTCCCAAC GCTTCCTTCA TATAGAGCGT GTTCCACAAG AACCCACTGA TTAGTAACCA 2040
GGCCCAGCGC CCCCAGTTGA TCCTTCCTGC CCTTCACGAT AACGCTTTCT GATCTCTCCG 2100
CGTTGTCCGT AACAAATCGC ACGAGCCACA GCGTGGCGGC CTTCTCCTCG ATTAAGCTGC 2160
GTCAGGATCC GCCGACGATA ATCTTCATCA TCAATATAAT TGAGGAGATA TAGCGTTTTG 2220
TTTACACGCC CTACTTCCAT AATTGCCTGT GCCAGTCCTG ATGGGCGCGA GTTTTCAGTA 2280
AAGAGCGAAT GAGTTCTGAC GCATGAATTG TACCCAACTT CAGCGAACCA GCGGTTCGAT 2340
TCATCTCATC CCACTGACTC TCCGCTTTTG ACAGATCTGC ATATCCTCGG GCCAACTTAT 2400
CCAGTACTCC GTAGTTTGCC GATTTATTCA CCCGCCAGAA CACCGCCTCA CCTGCATCGG 2460
CAAGCCGGGG GGAAAACTGA TACCCCAGTA GCCAGAACAG ACCGAAAATA ATATCGCTGC 2520
TACCCGCAGT GTCTGTCATG ATTTCACCTG GATTCAGCCC TGTCTGCTGC TCAAGAAGTC 2580
CTTCCAGTAC AAAAATCGAA TCCCGTAATG TACCGGGTAC CACAATGCCA TGGAACCCAG 2640
AGTACTGATC AGATACGAAG TTATACCAGG TGATGCCTCG TCCAGAACCA AAATATTTTC 2700
TGTTAGATCC TGAGTTGATG GTCTTCACTG GTGTGACAAA GCGCATGCCG TCAGCTGATG 2760
CCACTTCTCC ACCTCCCCAA CGACCAGCAA GCTCCAGTGT GGACTGAAAA TCAACCAGGC 2820
GGGCATTGGC GCTGACCAGC GTTTCTGCAC GAAGGTAATT CTGTTTCACC CAACTGAGCC 2880
GATGGCGGGT CAGTGCTGGT ATATTGTGCT TTATCAGCGG TTCCAGTCCG ATATTACAGG 2940
CTTCAGCCAT CAATACCGCA CATAAACTGA TGTGCAAATC TTGCGCTCGA GCACCAGATT 3000
CACTGACATG CGCAAACTCA TGTGTAAATC CTGTCTGGGC ATCTATCTCA AGTAACAGTT 3060
CCGTTAAATC TACCGGTGGG AGTAGCTTGT TTGATCCGAT TATATAAGAC GGAACCAATG 3120
ATGGTGTCTC TTCCTGTTTC TCCAGACCAA CTGATAGTCA GGGATGGATA TTTACCTTCA 3180
TTACAGATAT GAGCTGCCGC ATTCTTTTCA AATCGTGATG CCACGGCTTT CCAGGTCTCA 3240
TCCAGCTGAA TAGCCAGATG TTGCACACCT TTACGTCCAT CGACAGGATG TCCCAGTGTC 3300
CGACAGACAG GAATACGCTG AGTCTGCCAC TCTTCACCTT GCAACAACTT CTCGCGAGGA 3360
TCTCCCCAGC GATCACTGTT TTCAAGCCAG ATGTTCCCGG CGGCGCAGTG CATCCTGAAG 3420
GCGTTCCACC AAACATAGTG AATAACCTGC ACGCTGTATC CGTCCCTCCG CATCGTATAC 3480
GAGGCGTTTC CAGGGACCGG TGATAATATG TTCAGGCGCA TCATCAAGGA TGCGCTTTTT 3540
CGAACCATTC AGTTCTGCCA GATAATGAAT CGCAGCCAGT ACATGTTCAC CTGCCGGTGC 3600

Figure 14 (cont'd)

```
CGCACGGAAA TGCAGGTCCC GCAACACCGC CGGAAGAAAA CGTTTAACCC GACCGTACTG  3660
CTCAACCATT TCGTCATGGA AATTATTGTT CTGTGGACGA GCAAGTTCAT TAACCTTGCT  3720
TACAGATTCT GCCAGTCTGT TTTTGGGTAC GCACTTGAAG ATAACCTGCC TGAGATCTGG  3780
GACATCTGTA TTATCATCCA GCAACAATGC ACATGTCCGC GCCAGTAACA ATGCGGCCTG  3840
ATCAAGATCT TTCAGTGTCC TGAGTCTTTT TTTTTGCCCG GTTTTCTTTT CTTCGCGGAT  3900
AATGTCCAGA ATTAGCATAT CAAGCACATC AACGGCATCG TCTAATGCCG TTATTTCCTG  3960
TGCTTTAACG AATGCAGTAA GTACAGCAAG CTT                               3993
```

kb *Sac*I/*Hin*dIII portion, bases 1764-3995, was identical to the complement of bases 3771-5981 of transposon, Tn*1000*. This segment of Tn*1000* contained the C-terminal end of the transposase, *tnpA* (Broom *et al.*, 1993). Bases 1411-1764 were identical to the *rep2A* gene from the *repFIC* replication origin. Previous research demonstrated that Tn*1000* interrupted a *repFIC* replication origin in the F-plasmid (Berquist *et al.*, 1986; Saadi *et al.*, 1987; Willetts and Skurray, 1987). Thus, Tn*1000* may also interrupt a *repFIC* replication origin in pOR1. Analysis of the 4 kb fragment using IntelliGenetics, Gene Works (Tzagoloff, 1982), predicted that the other 1.4 kb *Sac*I/*Hin*dIII segment contained open reading frames for a 35 kDa polypeptide (Fig. 15, row 2) from base pair 132 to 1067 and a 3.7 kDa polypeptide (Fig. 16) from base pair 1102 to 1203. There were no apparent -35 and -10 promoter sequences for these open reading frames.

Sequence determination of the N-terminal region of the 35 kDa polypeptide. A level of expression higher than the one observed for the *in vitro* transcription and translation system was required to obtain enough of the 35 kDa polypeptide for amino acid sequencing reactions. The 4 kb insert from pLJ100 was subcloned in both orientations into plasmid, pT7-4, to obtain expression of both strands from the T7 phage promoter located on this vector. The two new recombinant plasmids, pLJ270 and pLJ271 (Fig.17), were transformed into HB101 possessing pGP1-2 (Tabor and Richardson, 1985), a plasmid which encoded T7 RNA polymerase at 42 °C. After inducing expression of the 4 kb insert with T7 RNA polymerase, the samples were separated on a 12% SDS polyacrylamide gel (Fig. 18). Lane 3 revealed that pLJ271 in MJ836 encoded a 35 kDa polypeptide, which was not detected in Lanes 1, 2 and 4. These lanes contained extracts from MJ835 (pGP1-2 and pLJ270), MJ834 (pGP1-2 and pT7-4) and MJ831 (pGP1-2), respectively. MJ834 synthesized a 32 kDa polypeptide not encoded by the other plasmids.

The 12% SDS polyacrylamide gel of the polypeptides expressed *in vivo* was electroblotted to a PVDF membrane (Speicher, 1989). After staining the blot, the band containing the 35 kDa polypeptide was excised, and the sequence of 15 N-terminal amino

Figure 15. Putative nucleotide and amino acid sequences of SedR. The first row is the nucleotide sequence, the second row is the amino acid sequence predicted by Intelligenetics, Geneworks, and the third row is the partial N-terminal sequence determined from the purified polypeptide.

```
AAGCTTTCAGCACCTTAAAAACAAATTCCAATAATAGAAAAAACAAATGCCAAGGTAATG     60

CAATTCAGTTATTGCAGAAAATATACTTTATCTATCATCCTCACATTAGATGGGGATGAT    120

TAATATATTCCATGAGACTCTTTATGAATACTATAAAAATAAAGCTAAACCTAATTGATT    180
           M  R  L  F  M  N  T  I  K  I  K  L  N  L  I  D  Y     17
                      M  N  T  I  K  I  K  L  N  L  I  D  Y     13

ATGATTCAATTGTAAATATTGAATTTCCCTGCCTCCTATCTGAAATAGAGATAGAGTTAT    240
  D  S  I  V  N  I  E  F  P  C  L  L  S  E  I  E  I  E  L  L     37
  D  S                                                           15

TATCTCAATTACTGAAGGGGTATTCTGTAAATGAGATTTCCAAGCGAAGAAACAGAAGTA    300
  S  Q  L  L  K  G  Y  S  V  N  E  I  S  K  R  R  N  R  S  I     57

TCAAAACAGGCTCGTGCCAAAAAATGAAATTATATAAAAAACTTAACGTAAAAAGCGATC    360
  K  T  G  S  C  Q  K  M  K  L  Y  K  K  L  N  V  K  S  D  L     77

TGACTTTATGGGGAGATGTTTTTTTAAGATTTAAAGCCTACCTACAACCTAAAAATATCA    420
  T  L  W  G  D  V  F  L  R  F  K  A  Y  L  Q  P  K  N  I  I     97

TCTGTGATAATTTTAATAGGTCTGTTTTACCAGTTGTTTCATCTAAAGGGGAGAGTATGG    480
  C  D  N  F  N  R  S  V  L  P  V  V  S  S  K  G  E  S  M  A    117

CGCACTACAACATATATTACCAACCGATCTATAACGCAAAAAATGGAAATATTGCTGGAT    560
  H  Y  N  I  Y  Y  Q  P  I  Y  N  A  K  N  G  N  I  A  G  C    137

GTGATGTTACTATTGCGTTAAAAAATTCAGATGGTAGTGCTTTGGCACTAGATTCTGACA    620
  D  V  T  I  A  L  K  N  S  D  G  S  A  L  A  L  D  S  D  R    157

GAATTAATTATAACCCCAATGATAACAAAGTATCTTATTTGTGTGGACATATCAACAAAC    680
  I  N  Y  N  P  N  D  N  K  V  S  Y  L  C  G  H  I  N  K  L    177

TATTCTCACCGATTAAAAATAATCTTCCGCATGGTTTTTTTATTACGATAAACATTAATC    740
  F  S  P  I  K  N  N  L  P  H  G  F  F  I  T  I  N  I  N  P    197

CTGAAGATATTCTTACCTGTGATATCGAAAGGGAGTGTTTACATTTTATTAAAGTTTTCG    800
  E  D  I  L  T  C  D  I  E  R  E  C  L  H  F  I  K  V  F  G    217

GAACGGAACGGATAAGACTTGTCTTGCAGTTTTCAACTAAAGAAGAACTTTACATAATAA    860
  T  E  R  I  R  L  V  L  Q  F  S  T  K  E  E  L  Y  I  I  R    237

GAAGATACCAGTCTTCTTTAAGACGTATCAGAAATAATAATGTTTATTTATCGCTTAATG    920
  R  Y  Q  S  S  L  R  R  I  R  N  N  N  V  Y  L  S  L  N  D    257

ATTTCGGTATGGGATATGCTGAGCTTTCACACTTGCAAAACATACCTTTCTCCTATGTAA    980
  F  G  M  G  Y  A  E  L  S  H  L  Q  N  I  P  F  S  Y  V  N    277

ACCTACATAAAACAATGTTCCATGATATAGAAAGCAATAGTTTAACAGATATAATAGCAA   1040
  L  H  K  T  M  F  H  D  I  E  S  N  S  L  T  D  I  I  A  T    297

CTACAATTATTGATTTACCCAAACCCCCCTCATATAGATGTTATTGC                1067
  T  I  I  D  L  P  K  P  P  S  Y  R  C  Y  C                   312
```

```
ACCTACATAAAACAATGTTCCATGATATAGAAAGCAATAGTTTAACAGATATAATAGCAA        1020

CTACAATTATTGATTTACCCAAACCCCCCTCATATAGATGTTATTGCTGATGGTATAGAA        1080

ACGAAGAAACAGGCAGGTTATATGATTGAAAGAGGAGTTAAATATTTAAAAGGCATTGCA        1140
                        M  I  E  R  G  V  K  Y  L  K  G  I  A         13

TTATCATCACCGCTACCTGCAGATGCATTTGTACGAAAATTGTTAGCTTCATTAAAACAA        1200
  L  S  S  P  L  P  A  D  A  F  V  R  K  L  L  A  S  L  K  Q         33

GTCTAA                                                              1206
  V                                                                   34
```

Figure 16. Hypothetical 3.7 kDa polypeptide from the 4 kb *Hin*dIII insert in pLJ100.

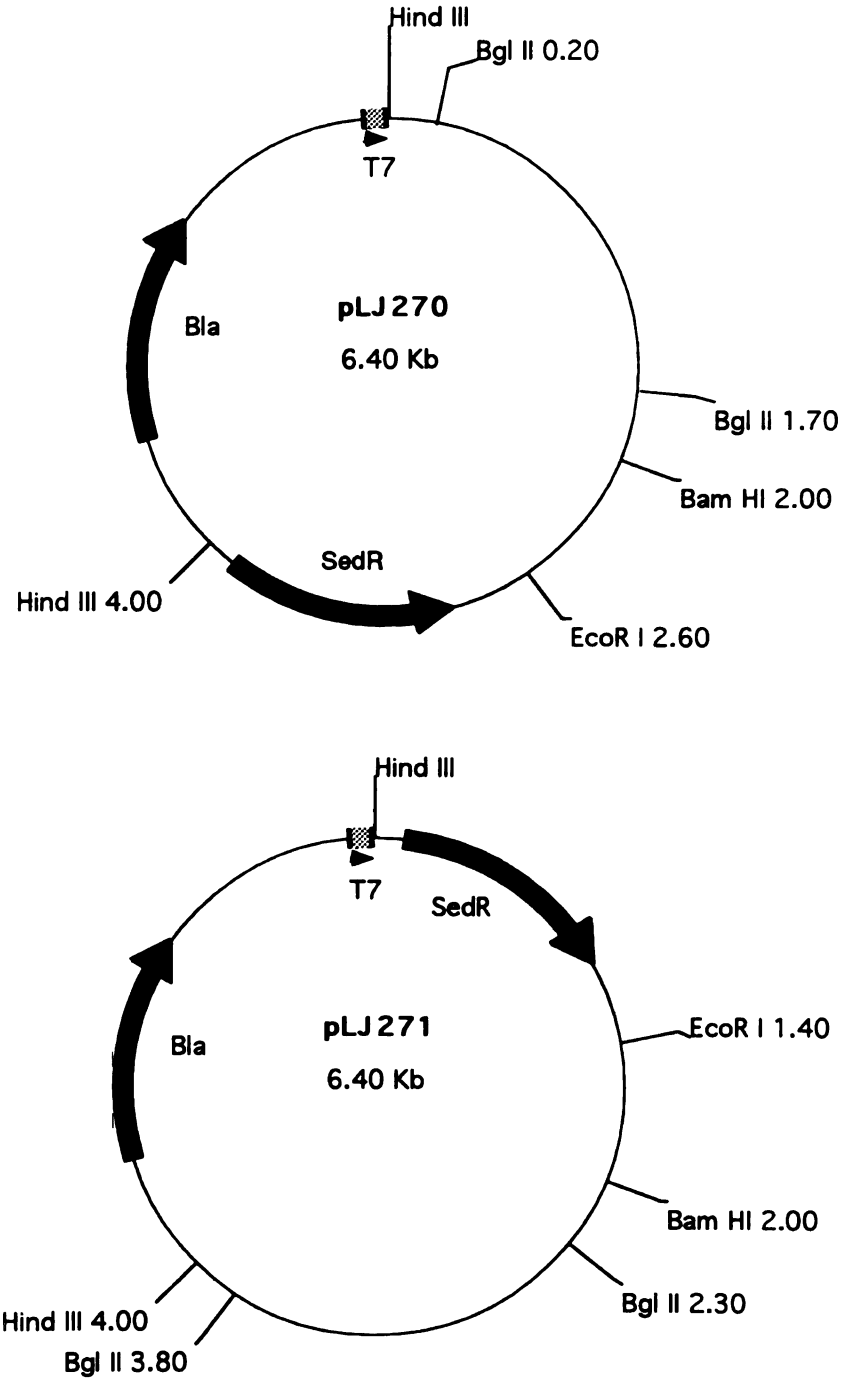


Figure 17. Recombinant plasmids used for *in vivo* expression of SedR.

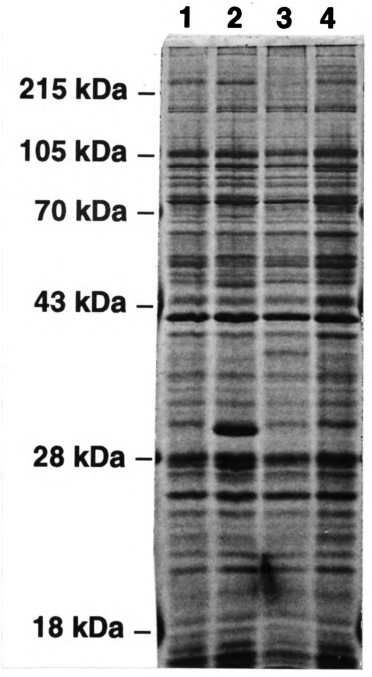


Figure 18. SDS polyacrylamide gel of the expressed SedR polypeptide. Lane 1: MJ835 (pGP1-2 and pLJ270). Lane 2: MJ834 (pGP1-2 and pT7-4). Lane 3: MJ836 (pGP1-2 and pLJ271). Lane 4: MJ831 (pGP1-2).

acid residues was determined. It matched the open reading frame predicted by Gene Works, except translation started four residues downstream of the predicted start site (Fig. 15, row 3). This new polypeptide was designated as the selenite dissimilatory reductase polypeptide or SedR.

Sequence analysis of SedR. Blast analysis (Altshul *et al.*, 1990) of the SedR amino acid sequence did not suggest a possible function for this polypeptide (Fig. 19). Residues 1-83 were 67% similar to residues 1-83 in the YAHA protein, a truncated, hypothetical polypeptide located near the *E. coli* genes encoding proteins for the synthesis of choline glycine betaine, which is involve in osmoregulation (Lamark et al., 1991). A segment from residue 25-75 also was 66% similar to residues 143-193 of the UvrC polypeptide which is involved in excision repair in *E. coli* (Sharma *et al.*, 1986). The same segment of SedR was 62% similar to residues 151-201 of the RcsB polypeptide which is involved in the regulation of colonic acid and capsule synthesis in *E. coli* (Stout and Gottesman, 1990). The regions of RcsB and UvrC similar to SedR contained helix turn helix motifs, which are involved in DNA binding, located from residues 42-64 in the SedR polypeptide.

Deletion analysis of the 4 kb, *Hin*dIII fragment from pOR1. To determine if *sedR*, the 3.7 kDa hypothetical polypeptide or some other segment of the 4 kb *Hin*dIII fragment from pOR1 was responsible for selenite-resistance, the deletions shown in Figure 20 were constructed, transformed into HB101 and tested for selenite-resistance (Table 8). Each strain was introduced to 40 mM selenite during early log phase. Turbidity was measured 22 to 30 hours later. The positive control, MJ801 (pLJ100), was the only strain that exhibited resistance to selenite. MJ848 (pLJ280), MJ849 (pLJ281) and MJ852 (pLJ307), did not grow much better than X2642 (pBR322), and MJ850 (pLJ291) and MJ851 (pLJ294) failed to grow as well as MJ853 (pUC19). Plasmids, pLJ270 and pLJ271 (Fig. 17), were ideal for making more deletions in the 4 kb fragment. Before introducing the deletions, they were tested for selenite resistance in strains MJ832

```
>sp|P21514|YAHA_ECOLI HYPOTHETICAL PROTEIN IN BETT 3'REGION (FRAGMENT).
          >pir|S10897|S10897 hypothetical protein (betT 5' region) -
          Escherichia coli (fragment) >pir|S15178|S15178 hypothetical protein
          - Escherichia coli >gp|X52905|ECBET_1 Escherichia coli betT, betI,
          betB and betA genes. [Escherichia coli]
          Length = 126

 Score = 170 (78.7 bits), Expect = 3.3e-17, P = 3.3e-17
 Identities = 35/83 (42%), Positives = 56/83 (67%)

Query:     1 MNTIKIKLNLIDYDSIVNIEFPCLLSEIEIELLSQLLKGYSVNEISKRRNRSIKTGSCQK 60
             MN+   ++ L ++ + V++  P  +SE E  LL  L++G SV EIS+ RNRS KT S QK
Sbjct:     1 MNSCDFRVFLQEFGTTVHLSLPGSVSEKERLLLKLLMQGMSVTEISQYRNRSAKTISHQK 60

Query:    61 MKLYKKLNVKSDLTLWGDVFLRF 83
              +L++KL ++SD+T W D+F ++
Sbjct:    61 KQLFEKLGIQSDITFWRDIFFQY 83


>gp|X03691|ECUVRC_2 E. coli uvrC gene for DNA repair. [Escherichia coli]
           Length = 211

 Score = 101 (46.8 bits), Expect = 1.4e-05, P = 1.4e-05
 Identities = 20/51 (39%), Positives = 34/51 (66%)

Query:    25 LSEIEIELLSQLLKGYSVNEISKRRNRSIKTGSCQKMKLYKKLNVKSDLTL 75
             LSE E++++  + KG  VNEIS++ N S KT +  + +++ KLN+  D+ L
Sbjct:   143 LSERELQIMLMITKGQKVNEISEQLNLSPKTVNSYRYRMFSKLNIHGDVEL 193
                           helix turn helix motif


>sp|P376>sp|P14374|RCSB_ECOLI REGULATOR OF CAPSULE SYNTHESIS B COMPONENT.
           >pir|JV0068|BVECCB rcsB protein - Escherichia coli
           >gp|M28242|ECORCSBC_2 capsule synthesis regulator component B
           [Escherichia coli] >gp|L11272|ECORCSC_2 rcsB gene product
           [Escherichia coli]
           Length = 216

 Score = 101 (46.8 bits), Expect = 1.4e-05, P = 1.4e-05
 Identities = 24/51 (47%), Positives = 32/51 (62%)

Query:    25 LSEIEIELLSQLLKGYSVNEISKRRNRSIKTGSCQKMKLYKKLNVKSDLTL 75
             LS  E E+L    +G+ V EI+K+ NRSIKT S QK     KL V++D+ L
Sbjct:   151 LSPKESEVLRLFAEGFLVTEIAKKLNRSIKTISSQKKSAMMKLGVENDIAL 201
                           helix turn helix motif
```

Figure 19. Blast analysis of the SedR polypeptide sequence. Helix turn helix motifs present in UvrC and RcsB are underlined.

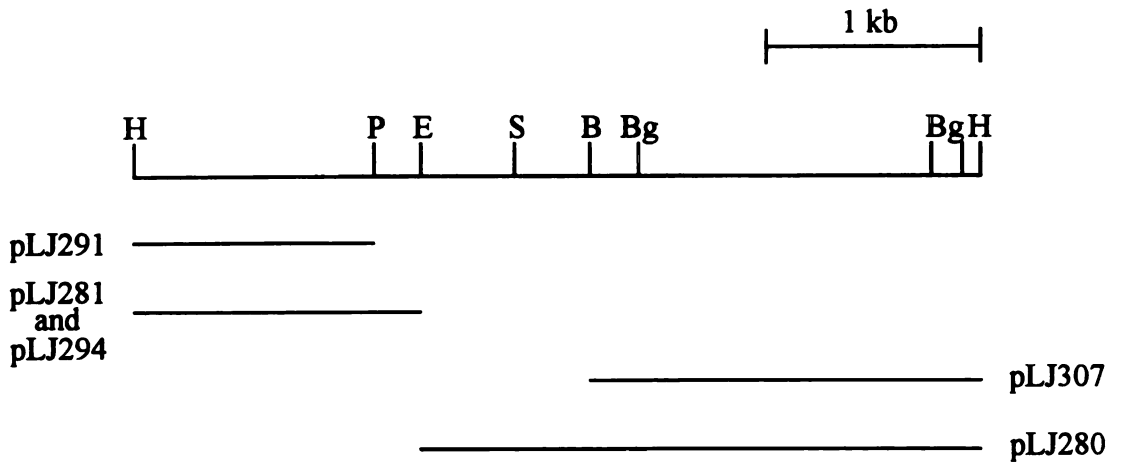


Figure 20 Deletions analysis of the 4 kb, *Hin*dIII fragment. The fragments from pLJ280, pLJ281 and pLJ307 were cloned into pBR322. Plasmid, pLJ281, contains the open reading frames for SedR and the hypothetical 3.4 kDa polypeptide. Plasmids, pLJ291 and pLJ294, were cloned under the control of the *lac* promoter in pUC19. Lines show regions remaining in the deletion.

Table 8. Influence of selenite on HB101 strains containing plasmids with different segments of the 4 kb *Hin*dIII fragment.

Strain	Plasmid	Turbidity (Klett Units)
MJ801	pLJ100	330
MJ830	pT7-4	78
MJ832	pLJ270	86
MJ833	pLJ271	89
X2642	pBR322	59
MJ848	pLJ280	82
MJ849	pLJ281	71
MJ852	pLJ307	3
MJ853	pUC19	64
MJ850	pLJ291	27
MJ851	pLJ294	2

(pLJ270) and MJ833 (pLJ271) (Table 8). Neither strain demonstrated resistance to selenite. Thus, it appeared that the whole 4 kb fragment and pBR322 are required for the expression of selenite resistance.

In pLJ291, *sedR* was cloned under the control of the *lac* promoter in pUC19, and in pLJ294, both *sedR* and the hypothetical 3.7 kDa polypeptide were cloned under the control of the *lac* promoter. MJ850 (pLJ291), MJ851 (pLJ294) and MJ853 (pUC19) were grown to a turbidity of 90 Klett Units and introduced to IPTG to induce expression from the *lac* promoter. After 1 hr of expression, selenite was added. MJ850 and MJ851 did not demonstrate an immediate ability to reduce selenite by producing a red color. These experiments suggested that SedR and the hypothetical 3.7 polypeptide may not play a direct role in selenite-resistance.

Growth experiments of X2642, MJ800 and MJ801 in selenite. In earlier experiments, the growth of X2642 (pBR322), MJ800 (pOR1) and MJ801 (pLJ100) in LB broth containing 40 mM selenite was followed by measurements of turbidity with a Klett Summerson colorimeter. Both the cells and the elemental selenium which became associated with the cells contributed to the turbidity. To determine the turbidity attributed to cells alone, turbidity and total protein at each time point were measured for X2642 grown without selenite (Fig. 21A) and for the three strains in the presence of selenite. A plot of total protein versus turbidity for X2642 grown without selenite was used to establish a linear correlation of turbidity to protein mass (Fig. 21B). The expected turbidity for each strain grown in selenite was then determined from the total cell protein measured at each time point. Figure 22 shows a plot of turbidity versus time and expected turbidity vs time. Normally, bacteria growing in logarithmic phase follow the equation, $\frac{dx}{dt} = kx$, where x is cell mass or turbidity, t is time and k is the instantaneous growth rate constant. However, the growth of cells exposed to selenite followed $\frac{dx}{dt} = C$, where C is constant. Not all the cells in the population survived to reproduce. By integrating this

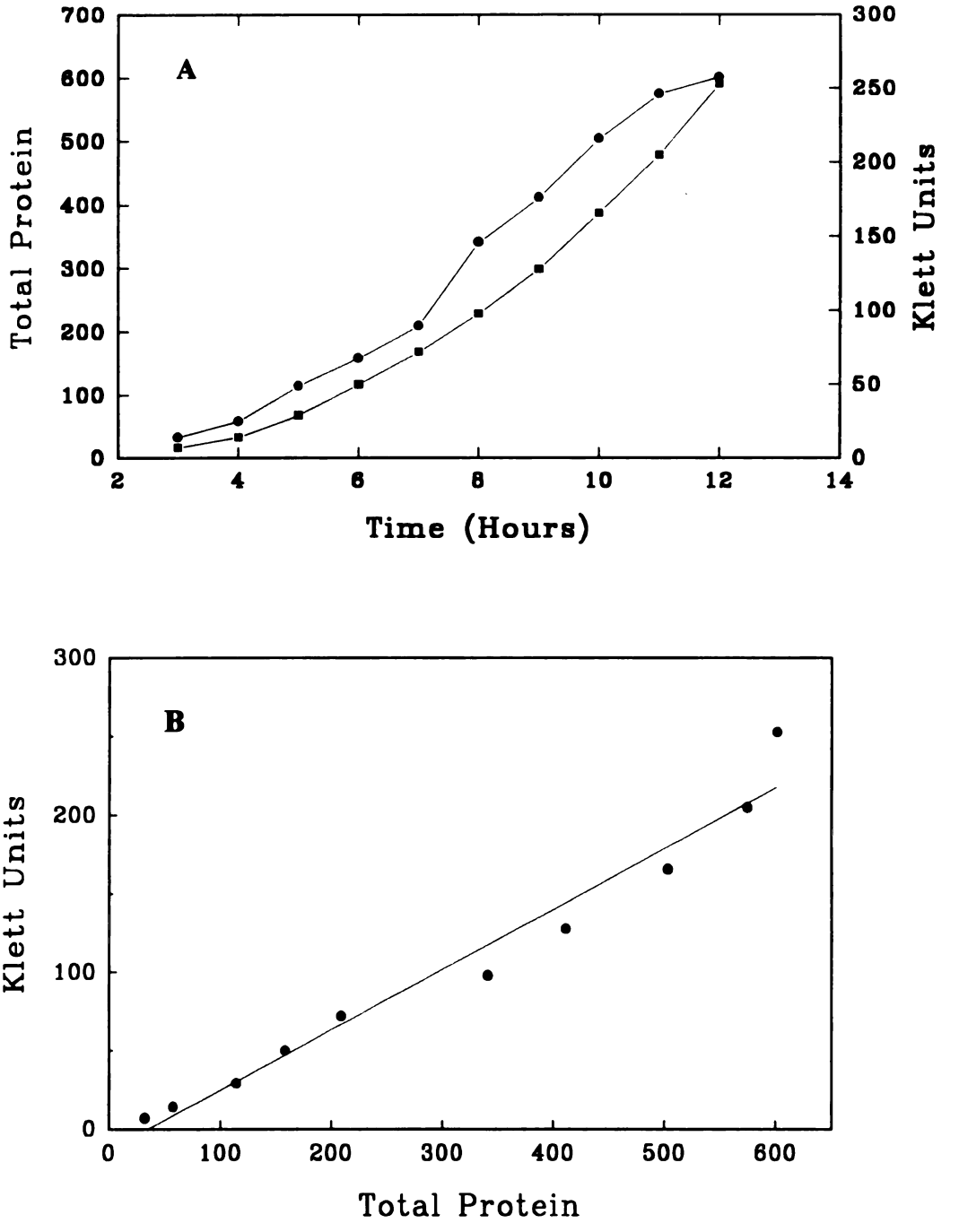


Figure 21. Correlation of turbidity to protein mass for X2642 grown in the absence of selenite. A: Growth of X2642 measured by ■ turbidity and ● protein mass. B: Linear correlation between turbidity and protein mass.

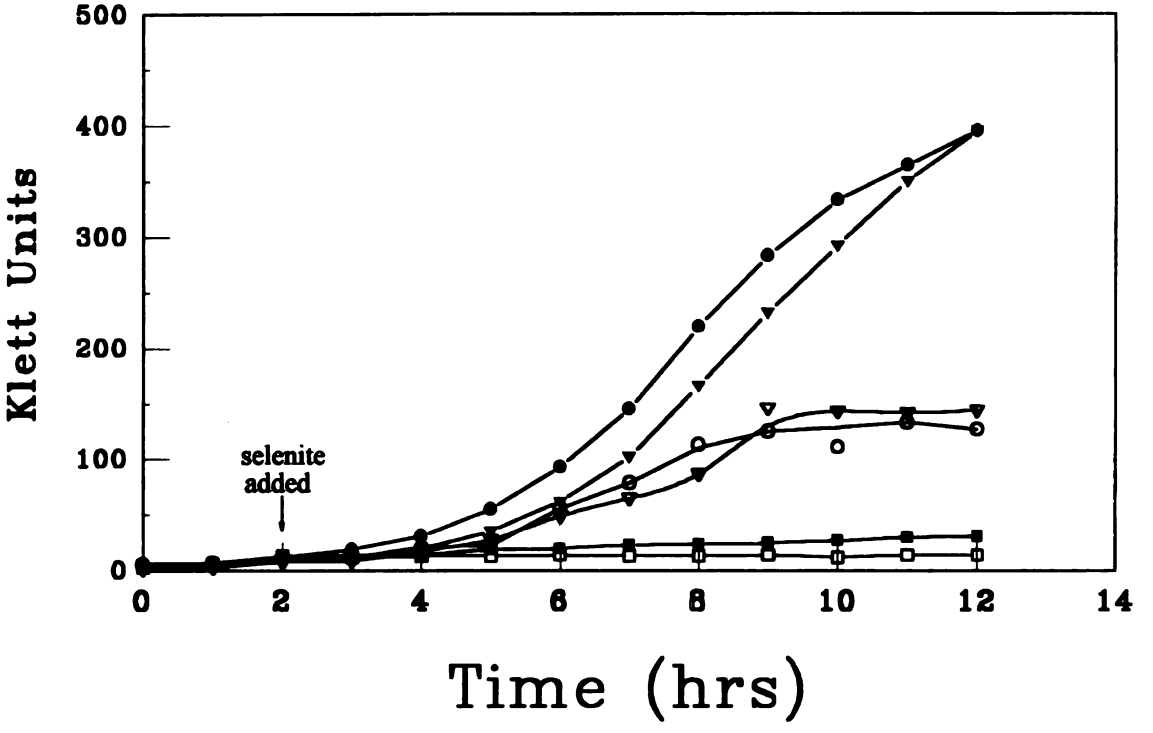


Figure 22. Influence of 40 mM selenite on the growth of X2642 (pBR322), MJ800 (pOR1) and MJ801 (pLJ100). Symbols: ■ X2642 - observed; □ X2642 - expected; ● MJ801 - observed; O MJ801 - expected; ▼ MJ800 - observed; ▽ MJ800 - expected.

equation and solving for C, $C = \frac{x_1 - x_2}{t_1 - t_2}$ which is the slope of a linear curve. Linear regression for the time points between 5 and 8 hours demonstrated that MJ800 (pOR1) grew at a C value of 19 Klett units/hr with an R^2 of 0.997 and MJ801 (pLJ100) grew at a C value of 29 Klett units/hr with an R^2 of 0.995. X2642 (pBR322) was completely inhibited and reduced little selenite. HB101 containing pLJ100 or pOR1 exhibited inhibited growth, but began reducing selenite within one hour after it was added and was most active during stationary phase.

Protein extracts from hours 3, 5, 7, 9 and 11 of the growth curves for X2642 grown without selenite and MJ801 grown in 40 mM selenite were elecotrophoresed through a 12% SDS polyacrylamide gel (Fig. 23). Throughout the growth curve, X2642 produced a 25 kDa polypeptide that was not produced by MJ801, and MJ801 synthesized a 42 kDa polypeptide that was not made by X2642. During stationary phase, X2642 also encoded a 51 kDa polypeptide which was not generated by MJ801. Other experiments demonstrated that when X2642 was grown in 40 mM selenite, it still contained the 25 kDa polypeptide but not the 42 and 51 kDa polypeptides. When MJ801 was grown in the absence of selenite, it encoded the 51 kDa polypeptide but did not contain the 25 and 42 kDa polypeptides during stationary phase (data not shown).

Cysteine requirement for selenite-resistance. *S. maltophilia* ORO2, HB101, MJ800 and MJ801 did not grow on M-9 minimal medium containing selenite. Since glutathione was shown to be necessary for selenite resistance, three amino acids, glutamate, cysteine and glycine, which constitute glutathione were used as supplements. Table 9 shows that each strain did not grow on minimal medium plates unless they were supplemented with 0.4 mM cysteine. When the other two amino acids were present and cysteine was absent, none of the strains grew on minimal plates containing 20 mM selenite.

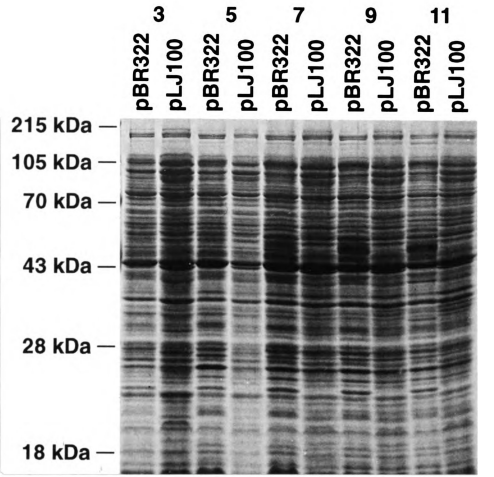


Figure 23. SDS polyacrylamide gel of extracts from X2642 (pBR322) grown in the absence of selenite and JM801 (pLJ100) grown in 40 mM selenite. Samples from 3, 5, 7, 9 and 11 hours during the growth of each strain were separated on a 12% polyacrylamide electrophoretic gel.

Table 9. Cysteine requirement for growth on minimal plates containing selenite.

	Growth			
Minimal Plates with	*S. maltophilia* ORO2	HB101	MJ800	MJ801
Cysteine	+	+	+	+
Selenite	-	-	-	-
Cysteine + Selenite	+	+	+	+

S. maltophilia, HB101, MJ800 (pOR1) and MJ801 (pLJ100) were streaked on M-9 minimal salts plates containing 0.5% glucose, 20 mM selenite and 0.4 mM cysteine. Growth was detected by the formation of colonies.

Genetic investigation of the role played by glutathione, glutathione reductase, thioredoxin and thioredoxin reductase in selenite-resistance. To determine the requirement of glutathione and glutathione reductase for selenite reduction, strains of *E. coli* with mutations in the genes for glutathione synthesis, *gshA* (γ-glutamycysteine synthetase) and *gshB* (glutathione synthetase), and glutathione reductase, *gor* (Oden *et al.*,1994), were transformed with pBR322 and pLJ100. *E. coli* strains with mutations in thioredoxin, *trxA*, and thioredoxin reductase, *trxB*, (Oden *et al.*, 1994), were also tested because they have been found to reduce selenite to elemental selenium (Holmgren and Kumar, 1988). Each strain was introduced to 40 mM selenite in early log phase and measured for growth after 24 hours with a Klett Summerson colorimeter. The wild type strain containing pBR322 already appeared to be resistant to selenite because it displayed a turbidity of 210 Klett units (Table 10). Both glutathione mutants (*gshA* and *gshB*) containing pBR322 and the thioredoxin reductase mutant (*trxB*) containing pBR322 did not grow well. They exhibited turbidities of 30, 26 and 30 Klett units, respectively. These results suggested that the wild type strain relied on glutathione and thioredoxin reductase, instead of glutathione reductase, to convert selenite to elemental selenium. The glutathione reductase (*gor*) and the thioredoxin (*trxA*) mutants containing pBR322 maintained some resistance to selenite with turbidities of 170 and 119 Klett units. All strains containing pLJ100 exhibited an enhanced ability to grow in the presence of selenite. Selenite-resistance conferred by pLJ100 did not appear to depend upon any of the genes tested above. Perhaps, the 4 kb insert from pLJ100 is involved in some other pathway used to reduce selenite to elemental selenium.

Bioremediation experiments using *S. maltophilia* ORO2 and MJ800. Dissolved sodium selenite is clear in solution. When cells convert it to elemental selenium, it formed a red precipitate which became associated with the cells. To determine if *S. maltophilia* ORO2, MJ800 (pOR1) and HB101 could remove selenite from LB broth under sterile conditions, each strain was grown overnight, harvested, resuspended and

Table 10. Growth of glutathione, glutathione reductase, thioredoxin and thioredoxin reductase mutants in 40 mM selenite.

		Turbidity		Relative Turbidity
Strain	Mutant	pBR322	pLJ100	pLJ100/pBR322
JF1070	w.t. - AB1157	210	308	1.5
JF420	*gor*	170	315	1.9
JF2200	*gshA*	30	196	6.5
JF2201	*gshB*	26	147	5.7
JF432	*trxB*	30	187	6.2
JF2062	*trxA*	119	189	1.6
JF1097	*trxA* and *gshA*	52	135	2.6
JF2014	*trxA* and *gor*	89	180	2.0

trxA - thioredoxin
trxB - thioredoxin reductase
gor - glutathione reductase
gshA - γ-glutamylcysteine synthetase
gshB - glutathione synthetase

placed in dialysis bags which were immersed in liquid medium containing 10 mM selenite. After 24 hours of growth under aerobic conditions, the dialysis bags contained red cells that accumulated elemental selenium (Fig. 24). The LB broth outside the bags was still yellow, but there was no measurable decrease in selenite concentration.

Since LB broth is expensive to use as a nutrition source, M-9 minimal medium containing cysteine was used in a non-sterile batch reactor to determine whether *S. maltophilia* could remove selenite from water containing 100 mM selenite. This level of selenite was supposed to prevent the reactor from becoming contaminated by inhibiting the growth of other bacteria. If this experiment was successful, *S. maltophilia* ORO2 could be used in a sequencing batch reactor to remove selenite from a continuous flow of water. A sequencing batch reactor consists of a series of batch reactors. While *S. maltophilia* ORO2 removes selenite in one reactor from contaminated water supplemented with M-9 salts and cysteine, other batch reactors are filling. When the reactor finishes removing the selenite and the cells settle, the water is drained off the top of the cells and the reactor is filled again. The initial batch culture experiment was not successful. Both the control (no added bacteria) and the experimental culture did not grow well. Due to a lack of time, this work was not pursued further.

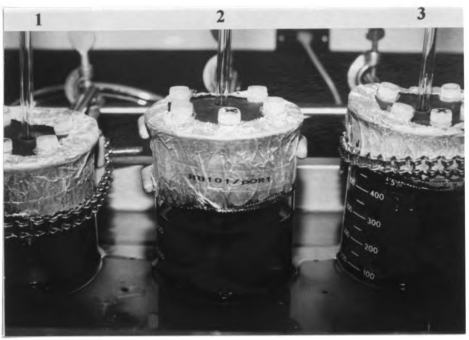


Figure 24. Removal of selenite from LB broth. Each strain, *S. maltophilia* ORO2 (1), MJ800 (2), and HB101 (3), was grown ovenight in 250 ml of LB broth, pelleted and resuspended in 40 ml of LB broth. Samples of 10 ml were added to dialysis bags immersed in LB broth containing 10 mM selenite.

DISCUSSION

The physical map of pOR1 from *S. maltophilia* ORO2 reveals a size of 100 kb. Experimental tests for F incompatibility are inconclusive. However, the *Bam*HI and *Hin*dIII maps of pOR1 and the F-plasmid (Skurray *et al.*, 1977; Childs *et al.*, 1977; Ohtsubo and Ohtsubo, 1977; Johnson and Willetts, 1980; Cheah and Skurray, 1986) are highly similar (Fig. 25). The fragments created by digestions of both plasmids with both enzymes are similar in size and present in the same order. The transposon, Tn*1000*, appears to be located in a similar position on each map. In addition the 13 kb *Bam*HI fragment from pOR1, suspected of containing an incompatibility determinant for the F-plasmid, is located in a position corresponding to the incompatibility determinant of the F-plasmid. Finally, Tn*1000* interrupts *repFIC* in the F-plasmid (Berquist *et al.*, 1986; Saadi *et al.*, 1987; Willetts and Skurray, 1987) and may interrupt *repFIC* in pOR1. Hybridizations using a probe for *repFIA* (Courtuier *et al.*, 1980) may verify the presence of an F-plasmid replication origin in pOR1. If pOR1 contains a replication origin for the F-plasmid, *Stenotrophomonas maltophilia* is an unusual host. The F-plasmid can move by conjugation to other genera of bacteria and replicate, but it is not well maintained in the absence of selection. It transfers to *Proteus* (Falkow *et al.*, 1964, Datta and Hedges, 1972), *Erwinia Chrysanthemi* (Chatterjee and Starr, 1972), *Pseudomonas flourescens* (Mergeay and Gertis, 1977) *Pseudomonas aeruginosa* (Guiney, 1982) and *Legionella pneumophila* (Wiater *et al.*, 1994). It also recombines with itself (Palchaudhuri and Maas, 1976), pBR322 (Guyer, 1978), and the chromosomes of *E. coli* (Davidson *et al.*, 1974) and *Pseudomonas syringae* (Leary *et al.*, 1984). The instability of pOR1 in *S. maltophilia* and of the F-plasmid in genera of bacteria other than *E. coli* may explain why the F-plasmid is not observed in natural bacterial isolates.

A partial polypeptide map of pOR1 is also presented in this study. SDS polyacrylamide gel electrophoresis of polypeptides expressed by cloned pOR1 fragments

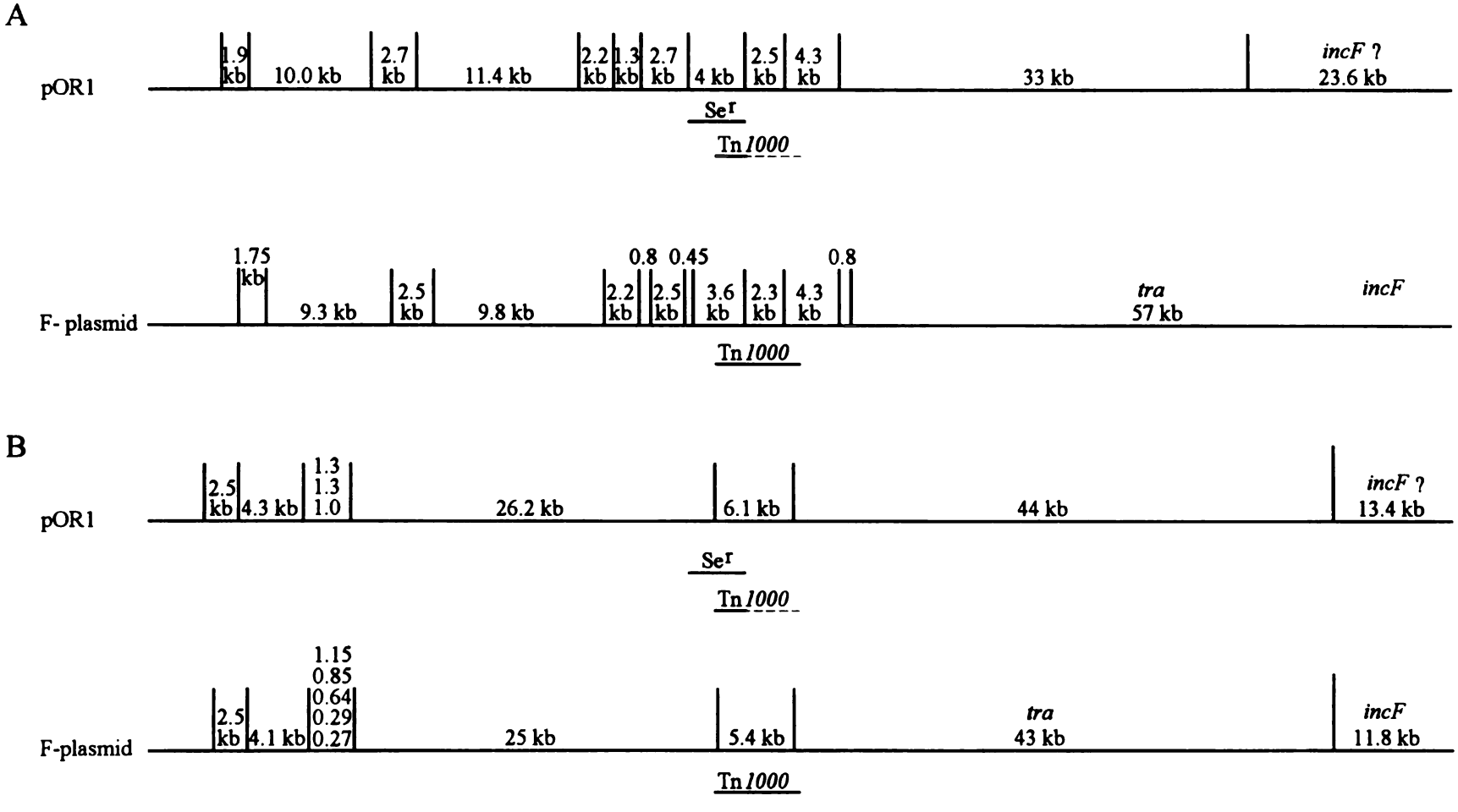


Figure 25. Comparison of the *Hin*dIII (A) and *Bam*HI (B) physical maps of pOR1 and the F-plasmid (Childs *et al.*, 1977 and Ohtsubo and Ohtsubo, 1977). Tn*1000* from pOR1 is extrapolated (dotted line) from the sequence of the 4 kb insert in pLJ100.

in an *in vitro* transcription and translation expression system (Zubay, 1973) demonstrates that pOR1 encodes at least 32 polypeptides ranging in size from 15.6 to 233 kDa.

A 4 kb, *Hin*dIII fragment from pOR1 confers resistance to selenite. Comparison of the nucleotide sequence of this fragment to other nucleotide sequences by blast analysis (Altschul *et al.*, 1990) shows that a 2.2 kb segment is identical to Tn*1000* (Broom *et al.*, 1993); a 400 base pair segment, adjacent to Tn*1000,* is identical to *rep2A* from the *repFIC* replication origin (Berquist *et al.*, 1986; Saadi *et al.*, 1987; Willets and Skurray, 1987); and a 1.4 kb segment, unrelated to Tn*1000* and *repFIC,* contains open reading frames for a 35 kDa polypeptide, SedR, and a 3.7 kDa hypothetical polypeptide. Comparison of the amino acid sequences to other known amino acid sequences does not reveal a possible function for either polypeptide. Polyacrylamide gel electrophoresis of protein encoded by this fragment *in vitro* using a transcription and translation system (Zubay, 1973) and *in vivo* using a T7 RNA polymerase expression system (Tabor and Richardson, 1985) confirms the existence of *sedR*. The sequence of the 15 N-terminal amino acid residues from this polypeptide verifies its origin on the 4 kb fragment. The percentage of acrylamide used to detect SedR is not high enough to resolve a 3.7 kDa polypeptide. Thus, it is unknown if the hypothetical 3.7 kDa polypeptide is encoded by the 4 kb fragment. The complete 4 kb insert in pLJ100 appears to be required for the expression of selenite-resistance. Deletion of Tn*1000* and of both polypeptides eliminates selenite-resistance. MJ832 and MJ833 that contain subclones of the 4 kb fragment in pT7-4 in both orientations, does not exhibit selenite-resistance. Expression of *sedR* and the 3.7 kDa hypothetical polypeptide using the *lac* promoter in pUC19 also fails to induce selenite-resistance. Hence, the vector from pLJ100, pBR322, appears to play a role in resistance to selenite. Even HB101 cells transformed with pLJ100, purified from selenite-resistant cultures of MJ801, fail to exhibit resistance to selenite, immediately. To obtain a selenite-resistant colony, a transformant must be grown overnight at 37 °C, incubated overnight at room temperature and plated without dilution onto an LB plate containing

selenite. Then, only a few selenite-resistant colonies appear. This inconsistent expression may be explained by a poor ability of the gene products from the 4 kb fragment to interact with *E. coli* proteins involved in selenite-resistance. Introduction of this fragment into a selenite-sensitive strain of *S. maltophilia* or *Pseudomonas* may give better expression.

The influence of the 4 kb fragment on selenite resistance is unknown. Experiments using glutathione, glutathione reductase, thioredoxin and thioredoxin reductase mutants suggest that *E. coli* strain AB1157 uses glutathione and thioredoxin reductase, instead of glutathione and glutathione reductase, for resistance to selenite. Since thioredoxin can reduce selenite to elemental selenium (Holmgren and Kumar, 1988), this result is not surprising. However, all mutants carrying the 4 kb fragment exhibit enhanced resistance to selenite. Thus, pLJ100 did not appear to require any of the above components to confer selenite-resistance. This plasmid may influence selenite-resistance by producing a new type of redoxin that is reduced by several different reductases, encoding a reductase that reduces several different redoxins or producing a regulator that induces some other pathway.

Selenite-resistant strains of bacteria may use three mechanism to relieve the toxicity of selenite. They may reduce it to elemental selenium (McReady *et al.*, 1965), prevent it from entering the cell (Weiss *et al.*, 1965) or incorporate it into selenomethionine (Scala and Williams, 1962), which does not interfere with protein function when it replaces methionine (Cowie and Cohen, 1957; Tuve and Williams, 1961; Frank *et al.*, 1985). The growth experiments on MJ800 (pOR1), MJ801 (pLJ100) and X2642 (pBR322) demonstrate that the 4 kb fragment confers selenite-resistance by reducing it to elemental selenium. X2642 (pBR322) inoculated into LB broth containing 40 mM selenite fails to grow and reduce selenite to elemental selenium. The selenite resistant strains, MJ800 (pOR1) and MJ801 (pLJ100) grow and reduce selenite in LB broth containing 40 mM selenite. Therefore, to grow in a rich medium containing selenite, HB101 must reduce it to elemental selenium. The selenite reducing activity was the

highest during stationary phase. This observation may be explained by the presence of high levels of glutathione in *E. coli* during this phase (Fahey et al., 1978). It is unknown if these resistant strains exclude selenite from cell or incorporate it into selenomethionine.

Selenite may inhibit cell growth by oxidizing sulfhydryl groups to form unstable selenosulfides (RSSeSR) (Ganther, 1971; Martin, 1973; Doran, 1982; Nakagawa, 1988); by reacting with glutathione, glutathione reductase, thioredoxin, thioredoxin reductase and NADPH to generate deleterious oxygen free radicals (Seko *et al.*, 1988); or by replacing sulfur in cysteine to form selenocysteine, which interferes with protein synthesis (Shrift, 1954; Heider and Böck, 1993). This last idea is confirmed by selenite-resistant, *cysK*, *E. coli* mutants (Fimmel and Loughlin, 1977) and previous research demonstrating that cysteine and other sulfur containing compounds relieve the growth inhibition of some strains grown in minimal medium containing selenate (Oremland, 1994) and selenite (Fels and Cheldelin, 1949). The cysteine requirement for *S. maltophilia* ORO2, MJ800 (pOR1) and MJ801 (pLJ100) to grow on M-9 minimal medium plates containing selenite is also in agreement with these studies. When cysteine and selenite are present in M-9 salts medium, the cells use the available cysteine, instead of inadvertently incorporating selenium from selenite into cysteine. The cysteine requirement may also be explained by the need to produce large quantities of glutathione to reduce selenite to elemental selenium. Since cysteine is a major component of glutathione, the cell may not be able to produce cysteine rapidly enough to synthesize the glutathione needed to detoxify selenite. Nevertheless, the pathway for selenite-resistance encoded by pOR1 and pLJ100 cannot compensate for the absence of cysteine in M-9 salts medium containing selenite.

Extracts of MJ801 (pLJ100) grown in 40 mM selenite contain an extra 43 kDa polypeptide not observed in X2642 (pBR322) extracts from cells grown in the absence of selenite and lack a 25 kDa and a 51 kDa polypeptide present in X2642 extracts from cells grown in the absence of selenite. The roles these polypeptides play in selenite-resistance is

unclear. Obtaining a short N-terminal amino acid sequence of these polypeptides may indicate a possible identity and mechanism for selenite-resistance.

In the bioremediation experiments, *S. maltophilia* ORO2, MJ800 (pOR1) and HB101 inside dialysis tubing immersed in LB broth containing selenite reduce the selenite which diffuses into the tubing and sequester it as elemental selenium. Although *S. maltophilia* ORO2 does not grow in non-sterile, M-9 minimal salts medium containing 0.5% acetate and 100 mM selenite, it may grow when glucose is present as a carbon source or when selenite is present at lower concentrations. Because selenate is the form of selenium that contaminates water under oxidized conditions (Masscheleyn, 1990), most studies have concentrated on using strains that reduce selenate to elemental selenium using aerobic (Lortie *et al.*, 1990) and anaerobic (Macy, 1994; Steinberg *et al.*, 1992; DeMoll-Decker and Macy, 1993) bacteria. For bioremediation using *S. maltophilia* ORO2, it probably must work in conjunction with another strain that reduces selenate to selenite to effectively remove selenium from contaminated water.

To obtain an efficient system for selenium bioremediation, a clear understanding for the genetic and biochemical processes are important. The mechanism of bacterial resistance to selenite appears to be complex and may involve several pathways. This work establishes a genetic background which begins to explore the mechanisms for selenite-resistance in bacteria.

In conclusion, the 100 kb F-like plasmid, pOR1, from *S. maltophilia* ORO2 confers resistance to Pb(II), Hg(II) and Se(IV) in *E. coli*. A 4 kb *Hin*dIII fragment from this plasmid confers resistance to lead and selenite. This fragment encodes a 35 kDa polypeptide designated as the selenite reduction polypeptide or SedR. The mechanism for selenite-resistance appears to involve the reduction of selenite to elemental selenium. However, the influence of the 4 kb fragment or *sedR* on selenite-resistance still must be resolved.

LIST OF REFERENCES

Alemi, M. H., D. A. Goldhamer, and D. R. Nielsen. 1991. Modeling of selenium transport in steady-state unsaturated soil columns. J. Environ. Qual. **20:**89-95.

Altshul, 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.

Ausubel, F. M. R., Brent, R. E. Kingston, D. D. Moore, J. G Seidman, J. A. Smith, and K. Struhl. 1989. Current protocols in molecular biology. John Wiley and Sons, Inc., New York.

Behne, D., A. Kyriakopoulos, H. Meinhold, and J. Kohrle. 1990. Identification of type I iodothyronine 5'-deiodinase as a selenoenzyme. Biochem. Biophys. Res. Commun. **173:**1143

Balistrier, L. S., and T. T. Chao. 1990. Adsorption of selenium by amorphous iron oxyhydroxide and manganese dioxide. Geochim. Cosmochim. ACTA **54:**739-751.

Berquist, P. L., S. Saadi, and W. K. Maas. 1986. Distribution of basic replicons having homology with RepFIA, RepFIB and RepFIC among IncF group plasmids. Plasmid **15:**19-34.

Berquist, P. L. 1987. Incompatibility. p. 37-78 *In* K. G. Hardy (ed.) Plasmids - a practical approach. IRC Press, Oxford.

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

Björnstedt, M., S. Kumar, and A. Holmgren. 1992. Selenodiglutathione is a highly efficient oxidant of reduced thioredoxin and a substrate for mammalian thioredoxin reductase. J. Biol. Chem. **267:**8030-8034.

Blake, R. II, S. Bardhan, N. Revis, L. M. Latinwo, J. J. Caguiat, and J. H. Jackson. A mechanism for microbial-dependent reduction of selenite to elemental selenium conferred by a 100 kb plasmid in Stenotrophomonas maltophilia. unpublished.

Böck, A., K. Forchhammer, J. Heider, W. Leinfelder, G. Sawers, B. Verprek and F. Zinoni. 1991. Selenocysteine: The 21st amino acid. Mol. Microbiol. **5:**515-520.

Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heynecker, and H. W. Boyer. 1977. Construction of useful cloning vectors. Gene **2:**95-113.

Boyer, H. W., and D. Roulland-Dussoix. 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli.* J. Mol. Biol. **41:**459-472.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. **72:**248-254.

Brown, T. A., and A. Shrift. 1980. Assimilation of selenate and selenite by Salmonella typhimurium. Can. J. Microbiol. **26:**671-675.

Brown, T. A., and A. Shrift. 1982. Selective assimilation of selenite by *Escherichia coli.* Can. J. Microbiol. **28:**307-310.

Burk, R. F. 1991. Molecular biology of selenium with implications for its metabolism. FASEB J. **5:**2274-2279.

Burton, G. A., Jr., T. H. Giddings, P. DeBrine, and R. Fall. 1987. High incidence of selenite-resistant bacteria from a site polluted with selenium. Appl. Environ. Microbiol. **53:**185-188.

Chatterjee, A. K., and P. M. Starr.1972. Genetic transfer of episomal elements among *Erwinia* species and other enterobacteria: F'*lac*$^+$. J. Bacteriol. **111:**169-176.

Chau, Y. K., P. T. S. Wong, B. A. Silverberg, P. L. Luxon, and G. A. Bengert. 1976. methylation of selenium in the aquatic environment. Science **192:**1130-1131.

Cheah, K.-C. and R. Skurray. 1986. The F plasmid carries an IS*3* insertion sequence within *FinO.* J. Gen. Microbiol. **132:**3269-3275.

Childs, G. J., H. Ohtsubo, E. Ohtsubo, F. Sonnenberg, and M. Freundlich.1977. Restriction endonuclease mapping of the *Escherichia coli* K-12 chromosome in the vicinity of the *ilv* genes. J. Mol. Biol. **117:**175-193.

Christensen, B. T., F. Bertelsen, and G. Gissel-Nielsen. 1989. Selenite fixation by soil particle-size separates. J. Soil Sci. **40:**641-647.

Church, G. M., and W. Gilbert. 1983. Genomic sequencing. Proc. Natl. Acad. Sci. USA. **81:**1991-1995.

Cone, J. E., R. M. Del Río, J. N. Davis, and T. C. Stadtman. 1976. Chemical characterization of the selenoprotein component of clostridial glycine reductase: identification of seleoncysteine as the organoselenium moiety. Proc. Natl. Acad. Sci. USA **73:**2659-2663.

Couturier, M., F. Bex, P. L. Berquist, and W. K. Maas. 1980. Identification and classification of bacterial plasmids. Microbiol. Rev. **52:**375-395.

Cowie, D. B., and G. N. Cohen. 1957. Biosynthesis by *Escherichia coli* of active altered proteins containing selenium instead of sulfur. Biochim. Biophys. Acta. **26:**252-261.

Crosa, J., M. Tolmasky, L. Actis, and S. Falkow. 1994. Plasmids, p. 365-386. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood and N. R. Krieg (ed.), Manual of Methods for General Bacteriology. American Society for Microbiology, Washington D. C.

Danna, A. J. 1980. Determination of fragment order through partial digests and multiple enzyme digests. Meth. Enzymol. **65:**449-467.

Danner, D. B. 1982. Recovery of DNA fragments from gels by transfer to DEAE-paper in an electrophoresis chamber. Anal. Biochem. **125:**139-142.

Datta, N., and Hedges, R. W. 1972. Host ranges of R factors. J. Gen. Microbiol. **70:** 453-460.

Davidson, D. F., and H. A. Powers. 1959. Selenium content of some volcanic rocks from western United States and Hawaiian islands. U.S. Geol. Surv. Bull. **1084C:**69-81.

Davidson, N., R. C. Deonier, S. Hu, and E. Ohtsubo. 1974. Electron microscope hetero-duplex studies of sequence relations among plasmids of *Escherichia coli*, p.56-65. *In* D. Schlesinger (ed.), Microbiology. American Society for Microbiology, Washington D. C.

Dilworth, G. L. 1982. Properties of the selenium-containing moiety of nicotinic acid hydroxylase from *Clostridium barkeri*. Arch. Biophys. Biochem. **219:**30-38.

Doran, J. W. 1982. Microorganisms and biological cycling of selenium. Adv. Microb. Ecol. **6:**17-32.

Doran, J. W., and M. Alexander. 1977 Microbial transformation of selenium. Appl. and Environ. Microbiol. **33:**31-37.

Edman, P., and G. Begg. 1967. A protein sequenator. Eur. J. Biochem. **1:**80-91.

Elrashidi, M. A., D. C. Adriano, S. M. Workman, and W. L. Lindsay. 1987. Chemical equilibria of selenium in soils: a theoretical development. Soil Sci. **144:**141-152.

Elrashidi, M. A., D. C. Adriano, and L. Lindsay. 1989. Solubility, speciation, and transformation of selenium in soils, p. 51-63. *In* L. W. Jacobs (ed), Selenium in agriculture and the environment, Soil Sci. Soc. Am. Spec. Publ. 23, Am Soc. Agronomy, Madison.

Fahey, R. C., W. C. Brown, W. B. Adams, and M. B. Worsham. 1978. Occurrence of glutathione in bacteria. J. Bacteriol. **133:**1126-1129.

Falkow, S., J. A. Wohlhieter, R. V. Citarella, and L. S. Baron. 1964. Transfer of episome elements to *Proteus*: I. Transfer of F-linked chromosomal determinants. J. Bacteriol. **87:**209-219.

Fels, I. G., and V. H. Cheldelin. 1949. Selenate inhibition studies III: the reversal of selenate inhibition in *Escherichia coli*. Arch Biochem. **22:**323-324.

Fels, I. G., and V. H. Cheldelin. 1948. Methionine in selenium poisoning. J. Biol. Chem. 176:819-828.

Fimmel, A. L. and R. E. Loughlin. 1977. Isolation and characterization of *cysK* mutants of *Escherichia coli* K12. J. Gen. Microbiol. **103:**37-43.

Forchhammer, K., W. Leinfelder, and A. Böck. 1989. Identification of a novel translation factor necessary for the incorporation of selenocysteine into protein. Nature (London) **342:**453- 456.

Forchhammer, K., and A. Böck. 1991. Selenocysteine synthase from *Escherichia coli*. J Biol. Chem. **266:**6324-6328.

Forstrom, J. W., J. J. Zakowski, and A. L. Tappel. 1978. Identification of the catalytic site of rat liver glutathione peroxidase as selenocysteine. Biochemistry **17:**2639-2644.

Frank, P., A. Licht, T. D. Tillius, K. O. Hodgson, and I. Pecht. 1985. A selenomethionine-containing azurin from an auxotroph of *Pseudomonas aeruginosa*. J. Biol. Chem. **260:**5518-5525.

Ganther, H. E. 1970. Reduction of the selenotrisulfide of glutathione to a persulfide analog by glutathione reductase. Biochemistry **10:**4089-4098.

Ganther, H. E. 1968. Selenotrisulfides. Formation by the reduction of thiols with selenious acid. Biochemistry **7:**2898-2905.

Geering, H. R., E. E. Cary, L. H. P. Jones, and W. H. Allaway. 1967. Solubility criteria for the possible forms of selenium in soils. Soil Sci. Soc. Amer. Proc. **32**:35-40.

Gerrard, T. L., J. N. Telford, and H. H. Williams. 1974. Detection of selenium deposits in *Escherichia coli* by electron microscopy. J. Bacteriol. **119:**1057-1060.

Guiney, D. G. 1982. Host range of conjugation and replication functions of the *Escherichia coli* sex plasmid F'*lac*: comparison with the broad host-range plasmid RK2. J. Mol. Biol. **162:**699-703.

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

Heider, J. and A. Böck. 1993. Selenium metabolism in micro-organisms. Adv. Microb. Physiol. **35:**71-109.

Högberg, J., P. Garber, and A. Stahl. 1988. Studies on selenite-induced DNA fragmentation and the role of poly(ADP-ribose)polymerase in selenite toxicity, p. 74-78. *In* A. Wendel (ed.), Selenium in biology and medicine. Springer-Verlag, Berlin.

Holmgren, A., and S. Kumar. 1988. Reactions of the thioredoxin system with selenium, p. 47-51. *In* A. Wendel (ed.), Selenium in biology and medicine. Springer-Verlag, Berlin.

Holt, J. G., and N. R. Krieg 1994. Enrichment and isolation, p.179-215 *In* P. Gerhardt, R. G. E. Murray, W. A. Wood and N. R. Krieg (ed.), Manual of Methods for General Bacteriology. American Society for Microbiology, Washington D. C.

Imhoff, D., and J. R. Andreeson. 1978. Nicotinic acid hydroxylase from *Clostridium barkeri*: selenium-dependent formation of active enzyme. FEMS Microbiol Lett. **5:**155-158.

Jackson, J. H., R. Blake, II, S. Bardhan, and N. Revis. 1988. Bacterial reduction of selenite to elemental selenium. FASEB J. **2:**A1347.

Jones, J. B., G. L. Dilworth, and T. C. Stadtman. 1979. Occurrence of selenocysteine in selenium-dependent formate dehydrogenase of *Methanococcus vanielii*. Arch. Biophys. Biochem. **195:**255-260.

Kado, C. L. and S.-T. Liu. 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1356-1373.

Komori, K., A. Rivas, K. Toda, and H. Ohtake. 1990. A method for the removal of toxic chromium using dialysis-sac cultures of a chromate-reducing strain of *Enterobacter cloacae* HO1. Appl. Microbiol. Biotechnol. **33:**117-119.

Kumar, S., M. Björnstedt, and A. Holmgren. 1992. Selenite is a substrate for calf thymus thioredoxin reductase and thioredoxin and elicits a large non-stoichiometric oxidation of NADPH in the presence of oxygen. Eur. J. Biochem. **207:**435-439.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680-685.

Latinwo, L., L. Overbye, J. Caguiat, R. Blake, N. Revis, and J. H. Jackson. 1990. Reduction of selenium, mercury and lead salts from a toxic waste site in Abstracts of the 90th Annual Meeting of the American Society for Microbiology **329:**Q244.

Leary, J. V., M. D. Thomas, and E. Allingham. 1984. Conjugal Transfer of *E. coli* F'*lac* from *Erwinia chrysanthemi* to *Pseudomonas synringae* pv. glycinea and the apparent stable incorporation of the plasmid into the pv. glycinea chromosome. Mol. Gen Genet. **198:**125-127.

Leifson, E. 1936. New selenite enrichment for the isolation of typhoid and paratyphoid (*Salmonella*) bacilli. Am. J. Hyg. **24:**423-432.

Leinfelder, W., Zehelein, E., Mandrand-Berthelot, M.-A. and A. Böck. 1988. Gene for a novel tRNA species that accepts L-serine and cotranslationally inserts selenocysteine. Nature **331:**723- 725.

Leinfelder, W., K. Forchhammer, B. Veprek, E. Zehelein, and A. Böck. 1990. *In vitro* synthesis of selenocysteinyl-$tRNA_{UCA}$ from seryl-$tRNA_{UCA}$: involvement and characterization of the *selD* gene product. Proc. Natl. Acad. Sci. USA **87:**543-547.

Levine, V. E. 1925. The reducing properties of microorganisms with special reference to selenium compounds. J. Bacteriol. **10:**217-263.

Linblow-Kull, C., Kull, F. J. and A. Shrift. 1985. Single transporter for sulfate, selenate and selenite in *Escherichia coli*. J. Bacteriol. **163:**1267-1269.

Lortie, L., W. D. Gould, S. Rajan, R. G. L. McCready, and K.-J. Cheng. 1992. Reduction of selenate and selenite to elemental selenium by a *Pseudomonas stutzeri* isolate. Appl. Environ. Microbiol. **58:**4042-4044.

Macy, J. M. 1994. Biochemistry of selenium metabolism by *Thauera selenatus* gem. nov. sp. nov. and use of the organism for bioremediation of selenium oxyanions in San Joaquin valley drainage water, p. 421-444. *In* W. T. Frankenberger, Jr. and S. Benson (ed.), Selenium in the Environment Marcel Dekker, Inc., New York.

Marshall, E. 1985. Selenium poisons refuge, California politics: Drainage for the San Joaquin Valley has dumped selenium into a wildlife refuge and pitted two federal agencies against each other. Science **229:**144-146.

Martin, J. L. 1973. Selenium assimilation in animals p. 663-669. *In* Organic Selenium Compounds: Their Chemistry and Biology. John Wiley and Sons, New York.

Masscheleyn, P. H., R. D. Delaune, and W. H. Patrick 1990. Transformation of selenium as effected by sediment oxidation-reduction potential and pH. Environ. Sci. Technol. **24:**91-96.

Matsudaira, P. 1987. Sequence from picomole quantities of proteins onto polyvinylidene diflouride membranes. J. Biol. Chem. **262:**10035-10038.

McCready, R. G. L., J. N. Campbell, and J. I. Payne. 1965. Selenite reduction by Salmonella heidelberg. Can. J. Microbiol. **12:**703-714.

McNeal, J. M., and L. S. Balistrieri. 1989. Geochemistry and occurrence of selenium: an overview, p.1-13. In L. W. Jacobs (ed), Selenium in agriculture and the environment, Soil Sci. Soc. Am. Spec Publ. 23, Am Soc. Agronomy, Madison.

Mergeay, M., and J. Gerits. 1978. F'-plasmid transfer from *Escherichia coli to Pseudomonas flourescens*. J. Bacteriol. **135:**18-28.

Muth, E., E. Morschel, and A. Klein. 1987. Purification and characterization of an 8-hydroxy-5-deazaflavin-reducing hydrogenase from archaebacterium *Methanococcus voltae*. Eur. J. Biochem. **169:**571-577.

Nakagawa, T., E. Aoyama, N. Kobayashi, H. Tanaka, M. Chikuma, H. Sakurai, and M. Nakayama. 1988. Thiol exchange reactions involving selenotrisulfides. Biophys. Res. Commun. **135:**183-188.

Newland, L. W. 1982. Arsenic, Beryllium, Selenium and Vanadium, p. 27-67. *In* O. Hutzinger (ed.), The handbook of environmental chemistry - anthropogenic compounds, vol. 3B. Springer Verlag, Berlin.

Norrander, J. T., T. Kemp, and J. Messing. 1983. Construction of improved M13 vectors using oligonucleotide-directed mutagenesis. Gene **26:**101-106.

Oden, K. L., T. B. Gladysheva and B. P. Rosen. 1994. Arsenate reduction mediated by the plasmid-encoded ArsC protein is coupled to glutathione. Mol. Microbiol. **12:**301-306.

Ohlendorf, H. M., D. J. Hoffman, M. K. Saiki, and T. W. Aldrich. 1986. Embryonic mortality and abnormalities of aquatic birds: apparent impacts of selenium from irrigation drainwater. Sci. Total Environ. **52:**49-63.

Ohlendorf, H. M. 1989. Bioaccumulation and effects of selenium in wildlife, p. 133-177. *In* L. W. Jacobs (ed), Selenium in agriculture and the environment, Soil Sci. Soc. Am. Spec. Publ. 23, Am Soc. Agronomy, Madison.

Ohtsubo, H., and E. Ohtsubo. 1977. Repeated DNA sequences in plasmids, phages and bacterial chromosomes, p. 49-64. *In* A. I. Bukhari, J. A. Shapiro, and S. L. Adhya (ed.), DNA insertion elements, plasmids and episomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Oremland, R. S. and J. P. Zehr. 1986. Formation of methane and carbon dioxide from dimethylselenide in anoxic sediments and by a methanogenic bacterium. Appl. Environ. Microbiol. **52:**1031-1036.

Oremland, R. S. 1994. Biogeochemical transformations of selenium in anoxic environments, p. 389-419. *In* W. T. Frankenberger, Jr. and S. Benson (ed), Selenium in the Environment. Marcel Dekker, Inc., New York.

Painter, E. P. 1941. The chemistry and toxicity of selenium compounds with special reference to the selenium problem. Chem. Rev. **28:**179-213.

Palchaudhuri, S., and W. K. Maas. 1976. Fusion of two F-prime factors in *Escherichia coli* studied by electron microscope heteroduplex analysis. Molec. Gen. Genet. **146:**215-231.

Palleroni, N. J., and J. F. Bradbury 1993. *Stenotrophomonas*, a new bacterial genus for *Xanthomonas maltophilia* (Hugh 1980) Swings *et al.* 1983. Int. J. Syst. Bacteriol. **43:**606-609.

Presser, T. S. 1994. Geologic origin and pathways of selenium from the California coast ranges to the west-central San Joaquin Valley, p. 139-155. *In* W. T. Frankenberger, Jr. and S. Benson (ed), Selenium in the Environment. Marcel Dekker, Inc., New York.

Presser, T. S., and H. M. Ohlendorf. 1987. Biogeochemical cycling of selenium in the San Joaquin Valley, California, USA. Environ. Manage. **11:**805-821.

Reed, K. C., and D. A. Mann. 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucl. Acids Res. **13:**7201-7221.

Ren, X., M. Bjornstedt, B. Shen, M. L. Ericson, and A. Holmgren. 1993. Mutagenesis of structural half-cystine residues in human thioredoxin and effects on the regulation of activity by selenodiglutathione. Biochemistry **32:**9701-9708.

Rieder, R., R. Camack, and D. O. Hall. 1984. Purification and properties of the soluble hydrogenase form *Desulfovibrio desulfuricans* (strain Norway 4). Eur. J. Biochem. **145:**637-643.

Rigby, P. W. J., Dieckmann, M., Rhodes, C., and Berg, P. 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol Biol. **113:**237-251.

Saadi, S., W. K. Maas, D. F., Hill and P. L. Berquist. 1987. Nucleotide sequence analysis of repFIC, a basic replicon present in incFI plasmids p307 and F, and its relation to the repA replicon of incFII plasmids. J. Bacteriol. **169:**1836-1846.

Sanger, F., Nicklen, S., and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. **74:**5463-5467.

Sarathchandra, S. U. and J. H. Watkinson 1981. Oxidation of elemental selenium to selenite by *Bacillus megaterium*. Science **211:**600-601.

Scala, J. and H. H. Williams. 1962. The enhancement of selenite toxicity by methionine in *Escherichia coli*. Arch. Biochem. Biophys. **99:**363-368.

Seko, Y., Y. Saito, J. Kitahara, and N. Imura. 1988. Active oxygen generation by the reaction of selenite with reduced glutathione *In vitro*, p. 70-73. *In* A. Wendel (ed.), Selenium in biology and medicine. Springer-Verlag, Berlin.

Shamberger, R. J. 1985. The genotoxicity of selenium. Mutation Res. **154:**29-48.

Short, J. M. and J. A. Sorge. 1992. *In vivo* excision properties of bacteriophage lambda zap expression vectors. Methods Enzymol. **216:**495-508.

Shrift, A. 1964. A selenium cycle in nature? Nature (London) **201:**1304-1305.

Shrift, A. 1954. Sulfur-selenium antagonism. I. antimetobolite action of selenate on the growth of *Chlorella vulgaris*. Am. J. Bot. **41:**223-230.

Skurray, R. A., H. Nagaishi and A. L. Clark. 1978. Construction and *Bam*HI analysis of chimeric plasmids containing *Eco*RI DNA fragments of the F sex factor. Plasmid **1:**174-186.

Sliwkowski, M. X. and T. C. Stadtman. 1984. Incorporation and distribution of selenium into thiolase from *Clostridium kluyveri* J. Biol. Chem. **260:**3140-3144.

Smith, T. F., M. S. Waterman, and W. M. Fitch. 1981. Comparative biosequence metrics. J. Mol. Evol. **18:**38-46.

Smith, T. F., and M. S. Waterman. 1981. Identification of common molecular subsequences. J. Mol. Biol. **147:**195-197.

Speicher, D. W. 1989. Microsequencing with PVDF membranes: efficient electroblotting, direct protein adsorption and sequencer modifications, p. 24-35. *In* T. Hugli (ed.), Techniques in Protein Chemistry. Academic Press, San Diego.

Stadtman, T. C. 1990. Selenium biochemistry. Annu. Rev. Biochem. **59:**111-127.

Steinberg, N. A., J. S. Blum, L. Hochstein, and R. S. Oremland. 1992. Nitrate is the preferred electron acceptor for growth of freshwater selenate-respiring bacteria. Appl. Environ. Microbiol. **58:**426-428.

Sutcliffe, J. G. 1978. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. **43:** 77-90.

Swings, J., P. De Vos, M. Van den Mooter, and J. De Ley. 1983. Tranfer of *Pseudomonas maltophilia* (Hugh 1981) to the genus *Xanthomonas* as *Xanthomonas maltophilia* (Hugh 1981) comb. nov. Int. J. Syst. Bacteriol. **33:**409-413.

Tabor, S., and C. C. Richardson. 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. **82:**1074-1078.

Tanji, K., L. Läuchli, and J. Meyer. 1986. Selenium in the San Joaquin Valley. Environment **28(6):**6-11, 34-39.

Tartof, K. D., and C. A. Hobbs. 1987. Improved media for growing plasmid and cosmid clones. Bethesda Res. Lab. Focus **9:**12.

Thompson-Eagle, E. T. and W. T. Frankenberger, Jr. 1990. Protein-mediated biomethylation in evaporation pond water. Env. Tox. Ch. **9:**1453-1462.

Torma, A. E. and F. Habashi. 1972. Oxidation of copper(II) selenide by *Thiobacillus ferrooxidans*. Can. J. Microbiol. **18:**1780-1781.

Towbin, H., T. Staehelin and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350-4354.

Trelease, S. F., and O. A. Beath. 1949. Selenium - Its geological occurrence, and its biological effects in relation to botany, chemistry, agriculture, nutrition, and medicine. The Champlain Printers, Burlington, VT.

Tzagoloff, A. 1982. Mitochondrial genetics, p. 308. *In* P. Seikevits (ed.), Mitochondria, Plenum Publishing Corp., New York.

Wagner, R., and J. R. Andreesen. 1970. Selenium requirement for active xanthine dehydrogenase from *Clostridium acidiurici* and *Clostridium cylindrosporum*. Arch. Microbiol. **121:**255-260.

Weiss, K. F., J. C. Ayres, and A. A Kraft. 1965. Inhibitory action of selenite on *Escherichia coli*, *Proteus vulgaris*, and *Salmonella thompson*. J. Bact. **90:**857-8621.

Wiater, L. A., A. Marra, and H. A. Shuman. 1994. *Escherichia coli* F-plasmid transfers to and replicates within *Legionella pneumophila*: an alternative to using an RP4-based system for gene delivery. Plasmid **32:**280-294.

Willetts, N., and R. Skurray. 1987. Structure and function of the F factor and mechanism for conjugation, p.1110-1133. *In* F. C. Neidhardt, J. C. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. F. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology Washington D. C.

Wittwer, A. J. 1983. Specific incorporation of selenium into lysine- and glutamate-accepting tRNA's from *Escherichia coli*. J. Biol. Chem. **258:**8637-8641.

Woolfolk, C. A. and H. R. Whiteley. 1962. Reduction of inorganic compounds with molecular hydrogen by *Micrococcus lactilyticus*. J. Bacterol. **84:**647-658.

World Health Organization. 1987. Sources, transport, and cycling of selenium in the environment, p. 36-43. In Environmental health criteria 58 - selenium. The International Programme on chemical safety, Geneva.

Yang, G, S. Wang, R. Zhou, and S. Sun. 1983. Endemic selenium intoxication of humans in China. Am. J. Clin. Nutr. **37:**872-881.

Zhang, P., and D. L. Sparks. 1990. Kinetics of selenate and selenite adsorption/desorption at the geothite/water interface. Environ. Sci. Technol. **24:**1848-1856.

Zinoni, F., A. Birkmann, W. Leinfelder, and A. Böck. 1987. Cotranslational insertion of selenocysteine into formate dehydrogenase from *Escherichia coli* directed by a UGA codon. Proc. Natl. Acad. Sci. **84:**3156-3160.

Zinoni, F, A. Birkmann, T. C. Stadtman, and A. Böck. 1986. Nucleotide sequence and expression of the selenocysteine-containing polypeptide of formate dehydrogenase (formate-hydrogenase-lyase-linked) for *Escherichia coli*. Proc. Natl. Acad. Sci. USA **83:**4650-4654.

Zubay, G. 1973. *In vitro* synthesis of protein in microbial systems. Annu. Rev. Gen. 7:267-287.