

DISSIMILATIVE FE(III) REDUCTION
BY ALTEROMONAS PUTREFACIENS STRAIN 200

Thesis by
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Dedicated to Coach and Mrs. Di

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ABSTRACT

Complementary genetic and biochemical analyses have been used to study the (dissimilative) iron reduction system of *Alteromonas putrefaciens* strain 200. Preliminary kinetic data suggested that *A. putrefaciens* 200 possessed a ferri-reductase that was physiologically distinct from either cytochrome oxidase or nitrate reductase. A suite of iron-reduction-deficient mutants was generated via chemical (EMS) and transposon (Tn5) mutagenesis procedures. A newly developed screening technique was subsequently used to identify mutants deficient in both high-rate and low-rate iron reduction activity. A conjugal gene transfer system was developed for mobilization of IncP1-based cloning vectors to *A. putrefaciens* 200. The broad host range (IncP1) cosmid cloning vector pVK100 was used to construct *A. putrefaciens* 200 gene clone banks in *E. coli* strains HB101 and S17-1 (mobilizing strain). Both three-way and two-way mating (conjugation) procedures were used to mobilize the gene clone banks into the suite of iron-reduction-deficient mutants during genetic (complementation) analysis. Two iron reduction clones (designated S4-E-2 and S18-F-4) were identified by their ability to restore iron reduction activity to several of the iron-reduction-deficient mutants. Preliminary biochemical characterization of selected mutant strains has indicated that cytochrome content may play an important role in the iron reduction process. Based on the results of the complementary genetic and biochemical studies, the iron reduction mutants have been placed into four classes: Class I (deficient in both high-rate and low-rate iron reduction activity, complemented by clones S4-E-2 and S18-F-4, possible

b- or *c*-type cytochrome mutants), Class II (deficient in both high-rate and low-rate iron reduction activity, complemented by clone S4-E-2 but not by S18-F-4), Class III (proficient in high-rate iron reduction activity, but deficient in low-rate iron reduction activity, not complemented by either clone S4-E-2 or S18-F-4), and Class IV (deficient in both high-rate and low-rate iron reduction activity, but not complemented by either clone S4-E-2 or S18-F-4, possible *d*-type cytochrome or anaerobic regulatory mutant).

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CHAPTER 1

INTRODUCTION AND RESEARCH OBJECTIVES

Microorganisms have long been implicated as important participants in the natural cycling of such critical elements as carbon, nitrogen, sulfur and phosphorus (for reviews see Brock, 1988; Schlegel, 1986). In several cases, the molecular mechanisms of the microbially-catalyzed process are understood at the biochemical and genetic level. Microorganisms implicated in the natural cycling of nitrogen, for example, have received considerable attention because of their ability to affect the nitrogen supply to plants in soil environments. The physiological basis and regulation of the microbial systems involved in both nitrification and denitrification (see below) are well understood. However, only recently have microorganisms begun to receive attention as major contributors to the biogeochemical cycling of heavy metals such as lead, mercury, manganese and iron. The apparent ability of microorganisms to mediate the transformation of iron coupled with the participation of iron as a reactive redox species in natural waters has prompted recent speculation that iron-transforming bacteria play an important role in global iron cycling (Ehrlich, 1981), in regulating the rate of lake acidification (Kelley et al., 1982), in biologically induced mineral formation (Herlihy et al., 1985) and in competition for available organic matter in natural waters and sediments (Lovley, 1987). Studies concerning the molecular details and functional role of microbial iron reduction systems, however, are generally lacking.

Most studies concerned with microbial iron reduction have focused on ecological aspects of the process (Nealson, 1983; Lovley and Phillips, 1986; Ghiorse, 1987). Several studies have addressed microbial iron reduction from a mechanistic viewpoint, although generally through the use of indirect methods.

The ability of *Escherichia coli* to reduce Fe(III) was first described by Starkey and Halvorson in 1927; however, only recently has the (dissimilative) iron reduction system of *E. coli* been investigated at a mechanistic level. Williams and Poole (1987) have investigated the Fe(III) reduction system of *E. coli* strain K12. Cell free

extracts harvested from cultures grown either aerobically, microaerophilically or anaerobically reduced Fe(III) when provided with reducing power in the form of NADH. When haem-deficient mutants were grown in the presence of glucose, the corresponding membrane fractions were still capable of reducing Fe(III) at rates identical to the rates of the parent strain. A similar result was found with the membrane fraction of a double quinone mutant (*ubiAmenA*) provided with Fe(III) and glucose (Williams and Poole, 1987). These results suggested that cytochromes and quinones were not involved in the Fe(III) reduction system in *E. coli* K12. Further cell fractionation experiments demonstrated that the Fe(III) reduction activity was soluble and not membrane bound. Thus, it appeared that the ferri-reductase activity was not respiratory chain-linked. Ferri-reductase activity associated with the soluble fraction is generally attributed to ferrisiderophore reductases involved in iron assimilation pathways (Neilands, 1982). At high redox potential and at neutral pH, iron is found predominantly in the particulate Fe(III) form (Stumm and Morgan, 1981). In order to meet nutritional iron requirements, bacteria that propagate under such conditions generally express a high-affinity iron uptake system. When grown under iron-limited conditions, these bacteria induce specific membrane receptor proteins capable of binding and transporting Fe(III) (complexed by siderophores) to the cell cytoplasm. Once in the cytoplasm, ferri-reductases are thought to release the metal from the Fe(III)/siderophore complex (Arceneaux and Byers, 1980). Expression of the iron assimilation system in *E. coli* is controlled by the level of Fe(II) in the cell (Neilands, 1982). However, in the studies by Williams and Poole (1987) with cell free extracts of *E. coli* K12, Fe(III) reduction activity was not effected by the concentration of iron used in the growth medium. In addition, the concentration of haem added to the medium had no effect on Fe(III) reduction activity. Relatively high haem concentrations were found to inhibit ferri-reductase activity in *Bacillus subtilis* (Lodge et al., 1982). The

mechanistic details and functional role of the ferri-reductase activity displayed by the cell free extracts of *E. coli* K12 remain unclear.

Although the ferri-reductase activity of *E. coli* K12 was found predominantly in the soluble fraction, such has not been the case in all bacteria. Lascelles and Burke (1978) studied the iron reduction process in membrane preparations from *Staphylococcus aureus*. In the absence of a suitable reductant, the membrane fractions were unable to reduce Fe(III). However, when provided with either L-lactate, glycerol-3-phosphate or NADH, the membrane fractions expressed Fe(III) reduction activity. This finding suggested that the Fe(III) reduction activity of *S. aureus* was respiratory chain-linked. In addition, each membrane fraction expressed L-lactate dehydrogenase, glycerol-3-phosphate dehydrogenase and nitrate reductase activities. To determine if nitrate reductase was capable of reducing Fe(III) in the membrane fractions, azide (N_3^-) was added to each preparation. Although nitrate reductase activity was completely inhibited, Fe(III) reduction activity was still detected. This finding suggested that a reductase other than nitrate reductase was capable of reducing Fe(III) in *S. aureus*.

In membrane preparations of *Spirillum itersonii*, Fe(III) was reduced in the presence of NADH or succinate (Dailey and Lascelles, 1977). In a series of respiratory inhibitor studies, rotenone but not antimycin A or 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO) inhibited Fe(III) reduction activity in the membrane preparations of *S. itersonii*. Furthermore, Fe(III) activity was completely inhibited by molecular oxygen, but was restored to full activity when electron transport to O_2 was blocked with antimycin A. These results suggested that the Fe(III) reduction process was respiratory chain-linked and that the site of electron transfer to Fe(III) was situated upstream from the *b*- and *c*-type cytochromes of the electron transport system (see below).

A series of studies by Ottow and coworkers (Ottow, 1970; Ottow and Glathe, 1971; Munch and Ottow, 1982; Munch and Ottow, 1983) investigated the physiological basis of Fe(III) reduction in various soil organisms including *Clostridium*, *Pseudomonas*, *Aerobacter* and *Bacillus* species. Munch and Ottow (1983) proposed that Fe(III) reduction activity was respiratory chain-linked, and that the terminal electron transfer step was catalyzed by two physiologically distinct enzymes, nitrate reductase and ferri-reductase. They based this hypothesis on studies with *Aerobacter aerogenes*, a soil microorganism that was capable of both nitrate and Fe(III) reduction. Their results demonstrated that nitrate-reductase-deficient mutants were also impaired in the ability to reduce Fe(III). In addition, when NO_3^- and Fe(III) were provided simultaneously to liquid cultures, the amount of Fe(II) produced by the nitrate-reductase-deficient mutant was significantly lower than the amount of Fe(II) produced by the parent strain. In subsequent studies with various glucose-fermenting species (e.g., *Clostridium butyricum*), Fe(III) reduction activity was detected even though the wild-type strain was unable to reduce NO_3^- . It was not conclusively demonstrated, however, that Fe(III) reduction activity was respiratory chain-linked, and not due to secondary chemical reduction of Fe(III) by various fermentation products.

The iron reduction system of the magnetotactic bacterium *Aquaspirillum magnetotacticum* was recently investigated by Paoletti and Blakemore (1986, 1988). Cell free extracts of *A. magnetotacticum* were shown to enzymatically reduce Fe(III) in the presence of NADH, and incorporated most of the Fe(II) intracellularly in the form of magnetite (Fe_3O_4), a mixed-valence iron oxide. Ferri-reductase activity was located primarily in the periplasmic space, was inhibited by O_2 , but unaffected by the respiratory inhibitors HQNO, antimycin A or rotenone. These results were different from those of previous studies which demonstrated that intact cells of *A. magnetotacticum* were capable of coupling proton translocation to Fe(III) reduction

(Short and Blakemore, 1986). In addition, a non-magnetic strain (unable to accumulate Fe(II) intracellularly) was capable of accumulating Fe(III) (as ferrihydrite), thereby suggesting that the iron reduction system was not used solely for iron assimilation. The molecular details and functional role of the iron reduction system in magnetotactic bacteria remain unclear.

Ferri-reductase activity (coupled to sulfur oxidation) was also reportedly found in whole cell fractions of the chemolithotrophic acidophile *Thiobacillus ferrooxidans* (Sugio et al., 1985). This conclusion was based on stoichiometric arguments, and did not address the possibility of an indirect (chemical) mechanism whereby metabolic intermediates resulting from aerobic respiration reduced the Fe(III) in solution. Earlier studies suggested that *T. ferrooxidans* was capable of utilizing either reduced sulfur or Fe(II) as an energy source during aerobic growth (Kinsel, 1960; Unz and Lundgren, 1961; Sugio et al., 1984). A sulfur:oxygen oxireductase was implicated in the transfer of electrons from sulfur to molecular oxygen (Kelly et al., 1982). However, recent work by Sugio and coworkers (1985) suggested that a sulfur:ferric iron oxido-reductase was also capable of coupling the oxidation of elemental sulfur to the reduction of Fe(III) under anaerobic conditions. The ferri-reductase activity was located predominantly in the periplasmic space, and was therefore thought to be involved in proton translocation via the extracytoplasmic mechanism proposed by Hooper and DiSpirito (1985). The enzyme was purified to an electrophoretically homogeneous state and exhibited maximum activity at near neutral pH. Earlier studies with *T. thiooxidans* (Kino and Usami, 1982) failed to detect any Fe(III) reduction activity under anaerobic conditions.

Arnold and coworkers (1986) have investigated the iron reduction system of the non-fermenting chemotroph *Alteromonas putrefaciens* 200. The Fe(III) reduction activity of *A. putrefaciens* 200 was compared (on a per cell basis) to the

Fe(III) reduction activity of several other high-rate iron reducers (e.g., *Bacillus pumilus*, *Bacillus circulans*, *Pseudomonas aeruginosa*) and found to be nearly an order of magnitude greater (Arnold, 1987). The physiological basis of the Fe(III) reduction system of *A. putrefaciens* 200 was studied in a series of respiratory inhibitor experiments. In cells grown at high oxygen tension, the respiratory inhibitors HQNO, DCCD (dicyclohexyl-carbodiimide), dicumarol and quinacrine inhibited ferri-reductase activity; however in cells grown at low oxygen tension, only quinacrine effectively inhibited ferri-reductase activity (see Figure 1.1).

Furthermore, the Fe(III) reduction rate for cells grown at low oxygen tension was nearly eight fold higher than the Fe(III) reduction rate for cells grown at high oxygen tension. The oxygen utilization rate doubled when cells were grown at low oxygen tension. These results suggested the Fe(III) reduction system was respiratory chain-linked and that a highly branched electron transport chain containing two ferri-reductases (one constitutive and one induced by growth at low oxygen tension) were involved in the iron reduction system of *A. putrefaciens* 200. Additional Fe(III) reduction rate studies with this microorganism included experiments in which liquid cultures were separated from goethite particles by a dialysis membrane (14 kD pore size). Fe(III) reduction activity was not detected until the membrane was physically ruptured, indicating that iron oxide/cell contact was required for reductive dissolution to occur, and that a soluble (reducing) metabolic intermediate was not involved in the iron reduction process. Preliminary iron reduction experiments with cell free extracts of *A. putrefaciens* 200 were not successful, but further work is necessary.

Based on the studies described above, it is apparent that the physiological basis and functional role of (dissimilative) Fe(III) reduction in bacteria are not well understood. In some species (e.g., *E. coli* K12, *Bacillus subtilis*), Fe(III) reduction activity was located in the cell cytoplasm and appeared to be involved in iron

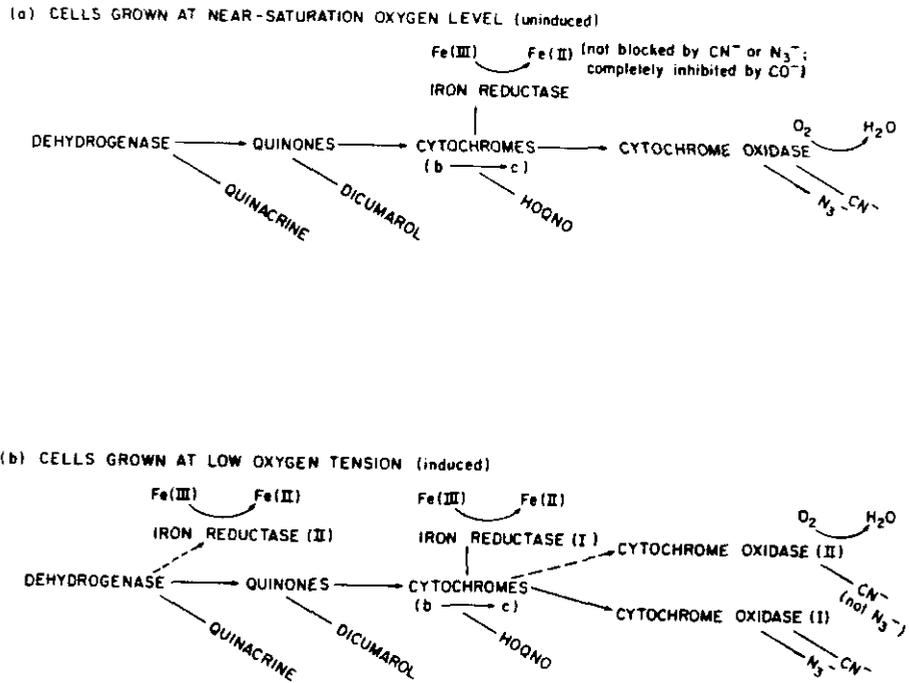


Figure 1.1 Summary of results from respiratory inhibitor study of *Alteromonas putrefaciens* liquid cultures grown at either high (a) or low (b) oxygen tension (from Arnold et al., 1986).

assimilation; in other species (e.g., *Staphylococcus aureus*, *Spirillum itersonii*, *Alteromonas putrefaciens*), Fe(III) reduction activity was located in the membrane fraction and appeared to be respiratory chain-linked; while in magnetotactic bacteria (e.g., *Aquaspirillum magnetotacticum*), the functional role of the Fe(III) reduction system was largely unknown.

The main objective of the present study was to determine the molecular mechanism of the iron reduction process of *Alteromonas putrefaciens* 200. *A. putrefaciens* 200 was selected for such a study based on several criteria including the apparent dissimilatory nature of the Fe(III) reduction process, the relatively high Fe(III) reduction activity displayed by cells grown under low oxygen tension, and the likelihood that the iron reduction process is respiratory chain-linked. Anaerobic respiration (proton translocation) coupled to Fe(III) reduction has not yet been unequivocally demonstrated in any microorganism.

Alteromonas putrefaciens 200 was isolated in 1980 from produced oil field fluids and was subsequently implicated as the causative agent of pipeline corrosion (Obuekwe, 1980; Obuekwe et al., 1981; Obuekwe and Westlake, 1982). Gram-negative facultative anaerobes similar if not identical to *Alteromonas putrefaciens* 200 were also isolated from natural waters and sediments, spoiling fish, dairy products and clinical specimens (Iizuka and Komagata, 1964; Lee et al., 1977; Semple and Westlake, 1987). Taxonomic identification and classification of these isolates generally involved gross morphological and biochemical characterization. The large number of characteristics unique to these isolates resulted in the establishment of a new genus designated *Alteromonas* in *Bergey's Manual of Systematic Bacteriology* (Baumann et al., 1984). The minimum characteristics required for classification as an *Alteromonas* spp. (see Figure 1.2) included a gram-negative stain reaction, the ability to use molecular oxygen as a terminal electron acceptor, the inability to ferment D-glucose, the presence of a polar flagellum for

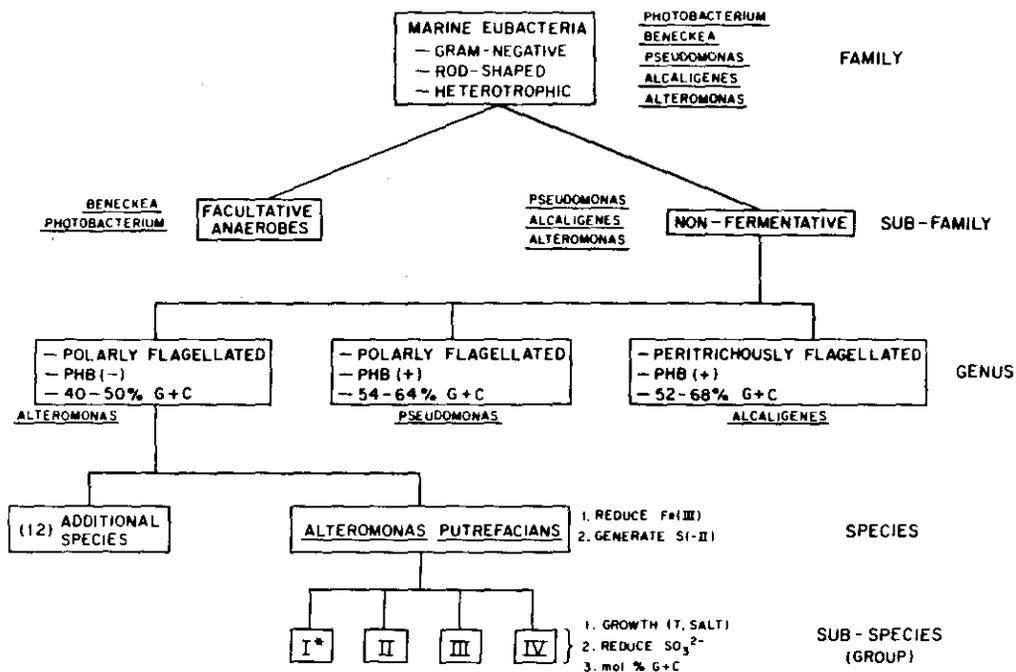


Figure 1.2. Proposed taxonomic guidelines for differentiation between marine eubacteria (e.g. *Photobacterium*, *Beneckea*, *Pseudomonas*, *Alcaligenes*, *Alteromonas*) with similar morphological and biochemical characteristics.

cell motility, the inability to accumulate polyhydroxybutyrate as an intracellular reserve product and a DNA base composition of between 38% and 49% G + C (T_m , Bd). This last characteristic differentiates the genus *Alteromonas* from genera with similar morphological and biochemical characteristics (e.g., *Pseudomonas* and *Alcaligenes*). *Alteromonas putrefaciens* 200 was originally classified as a *Pseudomonas* spp. (Obuekwe, 1980); however, its DNA base composition [42.3% G + C (T_m , Bd)] was clearly different from that found with other *Pseudomonas* spp. and resulted in its reclassification as an *Alteromonas* species (Semple and Westlake, 1987). *Alteromonas putrefaciens* isolates were differentiated from other alteromonads by their ability to reduce Fe(III) and generate hydrogen sulfide under anaerobic conditions (see Figure 1.2). Semple and Westlake (1987) recently proposed a classification system to differentiate between *Alteromonas putrefaciens* strains. *A. putrefaciens* 200 was classified as a Group I-type based on growth at 4°C in the presence of 4.5% (but not 7.5% NaCl), the reduction of both thiosulfate ($S_2O_3^{2-}$) and sulfite (SO_3^{2-}) to hydrogen sulfide, and its low DNA G + C composition. In addition to catalyzing the reduction of O_2 , $S_2O_3^{2-}$ and SO_3^{2-} , *A. putrefaciens* 200 was capable of reducing fumarate, nitrate, trimethylamine-N-oxide, manganese oxides, and ferric iron (Obuekwe, 1980). Furthermore, *A. putrefaciens* 200 was capable of oxidizing a wide variety of organic compounds including sugars, oxy- and hydroxy acids and several aromatics as sole carbon and energy source. Because of its metabolic versatility, *A. putrefaciens* 200 will provide a unique system for future study of bacterial respiration.

Based on the studies of Obuekwe (1980) and Arnold et al. (1986), the Fe(III) reduction system of *Alteromonas putrefaciens* 200 appeared to be respiratory chain-linked. It is now well-established that the major function of bacterial respiration is the electrogenic translocation of protons out of the cytoplasmic membrane, thereby generating a proton motive force across the membrane that consists of two additive

components, pH and electrical potential (Mitchell, 1961). The proton motive force, when of sufficient magnitude, reverses the direction of a proton translocating ATPase, resulting in the net synthesis of ATP. Under aerobic conditions, the electrons are transferred to molecular oxygen; under anaerobic conditions, the electrons can be transferred to alternative electron acceptors.

The physiological basis of bacterial respiration (oxidative phosphorylation) has been studied extensively and several comprehensive reviews exist (Haddock and Jones, 1977; Anraku, 1988). In most chemotrophic aerobes and facultative anaerobes the energy required for cell metabolism is derived from respiratory chain-linked oxidation-reduction reactions. Aerobic respiration has been shown to involve the transfer of reducing equivalents from an organic donor (via NADH) to molecular oxygen. In some bacteria, organic donors can transfer electrons directly to the respiratory chain. In others, NAD(P)H is used to carry reducing equivalents to the respiratory system. Aerobic respiratory chains generally contain a wide variety of electron-transporting components including iron-sulfur (Fe-S) proteins, flavoproteins, quinones and cytochromes. The Fe-S proteins and flavoproteins are generally found in the dehydrogenases which catalyze the initial oxidation of the organic donor, whereas cytochromes (iron-containing haemoproteins) are generally found at the terminal end of the electron transport chain and catalyze the reduction of O_2 to H_2O (see Figure 1.3). In the absence of O_2 , facultatively anaerobic and obligately anaerobic bacteria are capable of utilizing alternative electron acceptors during respiration. These alternative electron acceptors include both organic (e.g., fumarate, trimethylamine-N-oxide) and inorganic [e.g., CO_2 , NO_3^- , NO_2^- , SO_4^{2-} , $S_2O_3^{2-}$ and possibly Fe(III)] compounds. Although it appears thermodynamically feasible to couple dissimilatory Fe(III) reduction with respiratory chain-linked (oxidative) phosphorylation ($E_o' = 772$ mV), iron-driven respiration has not been unequivocally demonstrated in pure cultures. Anaerobic respiratory chains have

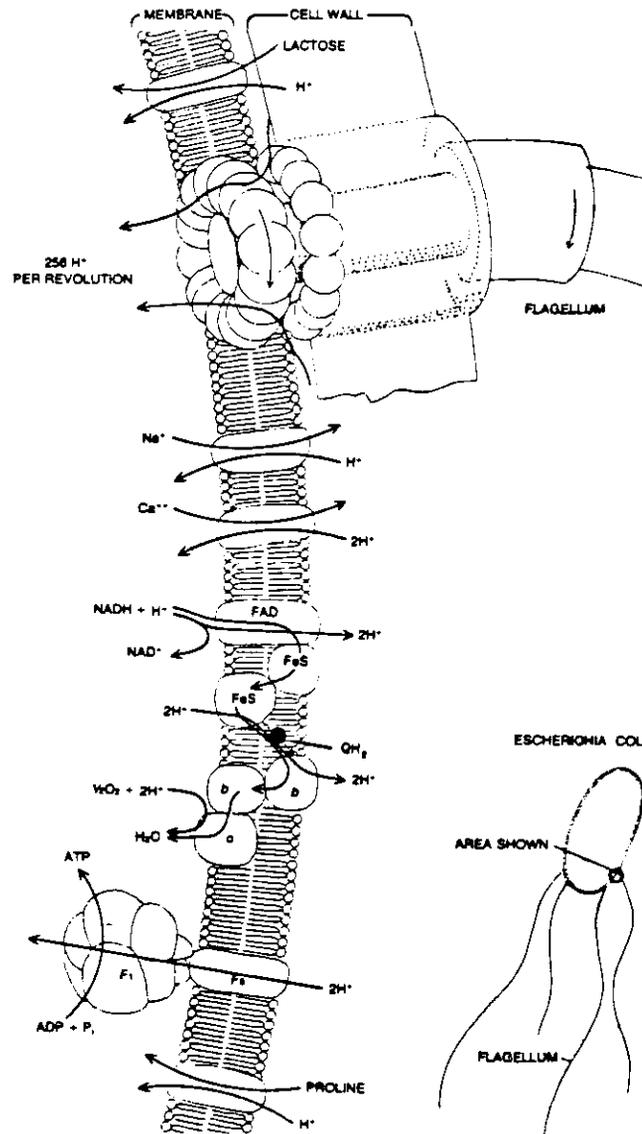


Figure 1.3. Schematic representation of the *E. coli* aerobic respiratory system showing relevant respiratory chain components including a proton-translocating ATPase (from Hinkle and McCarty, 1978).

been found to contain elements similar to those of their aerobic counterparts (Jones, 1983) with the addition of appropriate reductases to catalyze the terminal electron transfer step. The topological organization of the anaerobic respiratory chain and the biochemical properties of their components have been studied extensively and several comprehensive reviews exist (e.g., Haddock and Jones, 1977; Poole, 1983; Ingledew and Poole, 1984; von Jagow and Sebald, 1980; Gennis, 1987). When grown at high oxygen tension, in the presence of a non-fermentable substrate, the cytochrome complement of *E. coli* includes cytochromes *b*₅₅₆, *b*₅₆₂ and *o*. Cytochrome *o* is directly involved in proton translocation. Mutants in the *cyo* locus were still capable of growth at wild-type rates (Au et al., 1985). This was attributed to the ability of a cytochrome *d* complex to function in the place of cytochrome *o* when required. The cytochrome *d* complex is expressed when cells are grown at low oxygen tension or grown to high cell density (Miller and Gennis, 1983). When expressed under these conditions, the *d*-type haem imparts a darker pigmentation to cells and membrane preparations. Mutants in the *cyd* locus encoding cytochrome *d* were also capable of growth at wild-type rates, probably due to the ability of cytochrome *o* to function in its place. Mutants in both *cyo* and *cyd* were unable to grow on non-fermentable substrates. Purified cytochrome *d* was nearly 25-fold less sensitive to cyanide than cytochrome *o*; while the affinity of cytochrome *d* for O₂ was nearly 10-fold greater than the affinity of cytochrome *o* for O₂ (Gennis, 1987). The two oxidases are thought to provide the cells with two different pathways for electron transfer, thereby maximizing electron transfer capacity under a variety of growth conditions.

Of particular interest to the study of respiratory chain-linked reduction of Fe(III) is the anaerobic electron transport system to nitrate. Several previous studies (e.g., Munch and Ottow, 1983) have suggested that nitrate reductase was capable of reducing Fe(III) under anaerobic conditions. The nitrate reductase

system of *Escherichia coli* is the best characterized nitrate reductase system and has served as a model for several studies concerning respiratory chain physiology and regulation. When incubated under anaerobic conditions and in the presence of NO_3^- , *E. coli* was found to synthesize two membrane-bound proteins, cytochrome b_{556} and NO_3^- reductase. Under the same growth conditions and in the presence of selenium and molybdenum, formate dehydrogenase was synthesized and thereby allowed nitrate-respiring cells to grow on the corresponding non-fermentable substrates (Enoch and Lester, 1975). Electron transfer from formate to nitrate by cells grown under nitrate-respiring conditions was found to proceed via two major membrane-bound complexes, the formate dehydrogenase/Fe-S protein complex and the cytochrome b_{556} /nitrate reductase complex. Electron transfer between the two complexes involved an as yet uncharacterized quinone/menaquinone component. The nitrate reductase moiety spanned the cytoplasmic membrane with the electron-accepting site toward the periplasm and the nitrate-reducing "cleft" toward the cytoplasm (Ingledew and Poole, 1984). Because of the topology of the nitrate-reductase complex, NO_3^- transport across the inner membrane was required before reduction could occur. Since NO_3^- was also required to induce nitrate reductase synthesis, it was subsequently proposed that molecular oxygen inhibited both electron transfer to NO_3^- and synthesis of nitrate reductase by preventing nitrate transport into the cytoplasm via a nitrate/nitrite antiporter (Jones et al., 1980). Recent work has provided evidence that nitrate reductase synthesis is indeed repressed in the presence of O_2 by exclusion of NO_3^- from the cytoplasm; however, nitrate reductase activity was detected in the presence of O_2 after abolishing the NO_3^- permeability barrier (Noji and Taniguchi, 1987). A similar mechanism for oxygen control of nitrate uptake was recently reported to exist in *Pseudomonas aeruginosa* (Hernandez and Rowe, 1987). The molecular details of the oxygen-controlled nitrate transport system have not yet been determined.

The molecular mechanism of nitrate reductase expression has also been studied at the genetic level. Mutants defective in nitrate reductase activity were isolated and used to characterize the nitrate reduction system in *E. coli* (Guest, 1969; Glaser and DeMoss, 1972; MacGregor, 1975). Mutants defective in the various structural components, assembly functions, and co-factors required for a functional nitrate reductase were isolated. Further biochemical and genetic analysis of the nitrate-reductase-deficient mutants demonstrated the presence of a single regulatory element (*fnr* gene product) that was responsible for induction of the nitrate reductase genes (Fimmel and Haddock, 1979; Chippaux et al., 1981; Stewart, 1982). In addition, the strains deficient in *fnr* were also deficient in other anaerobic respiratory enzymes including formate dehydrogenase and fumarate reductase (Lambden and Guest, 1976; Newman and Cole, 1978). Gene expression studies with the promoter from a fumarate reductase structural gene demonstrated that the *fnr* gene product was required for induction of fumarate reductase activity (Jones and Gunsalus, 1987).

One of the major objectives of the present study is to determine the physiological basis of ferri-reductase activity in nitrate-respiring cultures of *Alteromonas putrefaciens* 200. If ferri-reductase activity is found to be physiologically distinct from nitrate reductase activity, then the physiological basis of the Fe(III) reduction system of *Alteromonas putrefaciens* 200 will be examined at the genetic and biochemical level using the nitrate reductase system of *Escherichia coli* as a model system. The overall approach to be taken to study the molecular mechanism of dissimilative iron reduction will involve isolation and characterization of a suite of iron-reduction-deficient mutants. Such an approach will necessitate the development of an efficient genetic system for use with this microorganism. Specific research objectives, therefore, will include the development of a screening technique and mutagenesis procedures for isolation of a suite of iron-reduction-

deficient mutants, the development of a gene transfer system and construction of an *A. putrefaciens* gene clone bank. With the facility to transfer cloned DNA into the suite of iron-reduction-deficient mutants, discrete DNA fragments containing loci required for iron reduction activity can be identified via genetic (complementation) analysis. Additional biochemical studies will provide information concerning the defects in each mutant. Results from this study will be used to postulate a molecular model of the dissimilative iron reduction system of *Alteromonas putrefaciens* 200. The following four chapters focus specifically on each of the research objectives described above.

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CHAPTER 2

**THE EFFECTS OF NITRATE, MOLECULAR OXYGEN AND CYANIDE
ON DISSIMILATIVE IRON REDUCTION ACTIVITY BY
ALTEROMONAS PUTREFACIENS 200**

I. INTRODUCTION

In previous studies, the physiological basis of microbial iron reduction was determined indirectly by measuring iron reduction activity in the presence of competing electron acceptors and/or respiratory inhibitors. From these kinetic data, the electron transport chain components involved in microbial iron reduction were identified. However, much remains uncertain concerning respiratory chain physiology and expression in the presence of Fe(III) and competing electron acceptors. Hammann and Ottow (1974) demonstrated that *Clostridium butyricum*, although unable to reduce NO_3^- , was capable of reducing Fe(III), suggesting that a ferri-reductase (physiologically distinct from nitrate reductase) was involved in the iron reduction process. The work by Ottow and co-workers (Ottow, 1970; Ottow and Glathe, 1971; Munch and Ottow, 1977) suggested that two physiologically distinct enzymes (nitrate reductase and ferri-reductase) were each capable of reducing Fe(III). They based their conclusions on studies with *Aerobacter aerogenes*, a facultative anaerobe capable of reducing either NO_3^- or Fe(III) under anaerobic conditions. In the presence of NO_3^- , however, iron reduction activity was inhibited. Furthermore, mutants deficient in nitrate reductase activity were also deficient in ferri-reductase activity (although some iron reduction activity was still detected). Based on these findings, Munch and Ottow (1983) proposed that nitrate reductase was capable of reducing Fe(III), but that NO_3^- was the preferred terminal electron acceptor (i.e., competitive inhibitor). However, these findings have been questioned by Obuekwe (1981) based on subsequent studies with *Alteromonas putrefaciens* 200 (then classified as *Pseudomonas* sp. 200). He suggested that the inhibition of iron reduction activity reported by Ottow was actually the result of a secondary chemical reaction, namely the oxidation of ferrous iron (produced via Fe(III) reduction) by nitrite (produced via nitrate reduction). Conclusions based on Obuekwe's results

must be approached with caution because of several oversights in the experimental approach (see below).

Further studies with *A. putrefaciens* have centered on the relationship of iron reduction and oxygen utilization. Respiratory inhibitor studies with *Alteromonas putrefaciens* 200 have suggested that a branched respiratory chain and two ferri-reductases (one constitutive and one induced via growth at low oxygen tension) are involved in Fe(III) reduction (see Figure 1.1, Arnold et al., 1986). The inducible system was capable of Fe(III) reduction at rates nearly ten fold higher than the constitutive system. In addition, both ferri-reductases were relatively insensitive to cyanide, whereas cytochrome oxidase activity (both constitutive and induced) was severely inhibited by cyanide. Fe(III) reduction activity was not observed in the presence of molecular oxygen; however, preliminary studies (Arnold, 1987) indicated that Fe(III) reduction activity may be restored by blocking electron transport to molecular oxygen with cyanide. However, these results were inconclusive due to the narrow range of cyanide concentrations employed in those experiments.

The goal of this portion of the project was to clarify uncertainties concerning the physiological role of iron reduction in relation to NO_3^- and O_2 utilization. First, the inhibitory effect of NO_3^- on Fe(III) reduction was studied using *Alteromonas putrefaciens* as a model organism. Second, the effect of O_2 on iron reduction activity was studied using cyanide as an inhibitor of electron transport to O_2 . Results from these studies should provide a better understanding of the physiological basis of dissimilative iron reduction by *Alteromonas putrefaciens* 200.

II. MATERIALS AND METHODS

A. Simultaneous Reduction of Fe(III) and NO_3^-

In each experiment, a 1.1 L liquid culture of wild-type *Alteromonas putrefaciens* 200 was grown in a Biostat M (B. Braun Instrument Co.) batch reactor.

Growth medium (pH 7.0) consisted of lactate medium as described by Obuekwe (1980) [per liter: 0.5 g K_2HPO_4 , 2.0 g Na_2SO_4 , 1.0 g NH_4Cl , 0.15 g $CaCl_2$, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.5 g yeast extract, 3 ml 60% sodium lactate syrup]. Low levels of iron (for satisfaction of microbial nutritional requirements) were added (to 4 mg/L as Fe) from a filter-sterilized $FeCl_3 \cdot 6H_2O$ stock solution. Microbial growth was initiated by adding 1.5 ml from a dense overnight culture of *A. putrefaciens* 200. Two sets of experiments were carried out: in the first set, oxygen tension was maintained at or near saturation (200 μM) throughout the growth period; in the second set, the oxygen tension was reduced to $\leq 1.0\%$ (2 μM) during the final 3 hours of the growth period. Temperature was held constant at 30°C. Microbial growth was monitored by periodic measurement of absorbance at a wavelength of 600 nm. When the target optical density was reached ($OD_{600} = 0.50$) chloramphenicol was added (to 0.25 mM) to halt cell growth. The culture oxygen utilization rate was then measured by monitoring the time-dependent oxygen profile for 5 minutes. Nitrilotriacetic acid (NTA) was then added to 1.86 mM . The pH was held constant (7.0 ± 0.1) via manual addition of 2 N NaOH. Residual oxygen was subsequently purged from the system with high-purity N_2 gas. To determine the effect of Fe(III) on the rate of nitrate reduction, $FeCl_3 \cdot 6H_2O$ and KNO_3 (Sigma) were added simultaneously to the liquid cultures. For each set of growth conditions, a series of three experiments were carried out: in the first experiment, $FeCl_3 \cdot 6H_2O$ was added to 1.86 mM (as Fe(III)) and KNO_3 to half that concentration (as NO_3^-); in the second experiment, Fe(III) was again added to 1.86 mM , while NO_3^- was added to twice that concentration. In the third (control) experiment, NO_3^- was added to 1.86 mM while Fe(III) was omitted. Immediately following these additions, and at 1 minute time intervals thereafter, 1 ml aliquots were withdrawn from the batch and used to determine the concentration of nitrite in solution via the procedures of Montgomery and Dymack (1961). Each 1 ml aliquot was quenched in

diozotized sulfanilic acid coupled to N-(1-naphthyl)ethylenediamine (NED) and the absorbance measured at 550 nm ($\epsilon_{550} = 1.94 \times 10^4 \text{ M}\cdot\text{cm}^{-1}$). To determine the effect of NO_3^- on the rate of Fe(III) reduction, a similar set of experiments was carried out. In this series, ferrozine (1,4,6-tripyridyl-1,3,5-triazine; Sigma) was used to determine the concentration of Fe(II) in solution (see below). Ferrozine (to 7.2 mM) was added to the batch before the simultaneous addition of $\text{FeCl}_3\cdot 6\text{H}_2\text{O}$ and KNO_3 . The production of ferrous iron was monitored for a period of 30 minutes. For each set of growth conditions, a series of three experiments were carried out: in the first experiment, Fe(III) was added to 1.86 mM and NO_3^- added to half that concentration; in the second experiment, Fe(III) was again added to 1.86 mM while NO_3^- was added to twice that concentration; in the third (control) experiment Fe(III) was again added to 1.86 mM while NO_3^- was omitted. Immediately following these additions, and at 1 minute intervals thereafter, 3 ml aliquots were withdrawn from the batch and immediately placed on ice. At the conclusion of the experiment (approximately 30 minutes) the aliquots were centrifuged (4000xg, 10 minutes; Sorvall RC3B centrifuge) and the concentration of Fe(II) determined colorimetrically by measuring the absorbance at 562 nm. Fe(II) forms a magenta-colored tris-complex with ferrozine ($\epsilon_{562} = 2.8 \times 10^4 \text{ M}^{-1}\cdot\text{cm}^{-1}$) in the neutral pH range (Stookey, 1970).

B. Fe(III) Reduction in the Presence of Molecular Oxygen and Cyanide.

A series of 10 experiments were carried out to determine the effect of cyanide on iron reduction activity in the presence of molecular oxygen. In each experiment, a 1.1 L liquid culture of *A. putrefaciens* 200 was grown under high oxygen tension ($> 200 \mu\text{M}$) in a Biostat M (B. Braun Instrument Co.) batch reactor. Growth medium (pH 7.0) consisted of lactate medium (Obuekwe, 1980) as described above. Iron for satisfaction of microbial nutritional requirements was added (to 4 mg/L as Fe) from a filter-sterilized $\text{FeCl}_3\cdot 6\text{H}_2\text{O}$ stock solution.

Microbial growth was initiated by adding 1.5 ml of a dense overnight culture to the 1.1 L batch, and growth monitored by periodic A_{600} measurements. Temperature was held constant at 30°C. At a target optical density [$A_{600} = 0.25 \text{ (cm}^{-1}\text{)}$] chloramphenicol was added (to 0.25 mM) to halt cell growth. Nitrilotriacetic acid (NTA) was then added to 1.86 mM, accompanied by the addition of 2 N NaOH to maintain a constant pH (7.0). Ferrozine (to 7.2 mM) was subsequently added to the batch followed by the addition of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (to 1.86 mM as Fe). The oxygen tension was maintained at near saturation levels [$1.8 \times 10^{-4} \text{ M}$ ($\pm 0.1 \text{ M}$)] throughout the time period required for each addition. The oxygen supply was immediately cut off after the addition of Fe(III) and the oxygen utilization rate was monitored for a period of 10 minutes. Two minutes after cutting off the oxygen supply, and at two minute intervals thereafter, 3 ml aliquots were withdrawn from the batch and immediately placed on ice. At the conclusion of the experiment, these aliquots were used to determine the concentration of Fe(II) in solution (as previously described). After this initial 10 minute period, the oxygen tension was again raised to $1.8 \times 10^{-4} \text{ M}$, followed by the addition of KCN (Baker) to prescribed levels. A series of 10 experiments was carried out in this manner, changing the final concentration of CN^- varied in each case. The $[\text{CN}^-]$ ranged from $0.3 \times 10^{-5} \text{ M}$ to $10 \times 10^{-5} \text{ M}$ (final concentration as CN^-). After CN^- addition (and cutoff of the oxygen supply), the concentration of O_2 and Fe(II) were determined at 1 minute intervals for a period of 20 minutes. At the end of this 20 minute period, O_2 was purged from the system with high-purity N_2 gas, and the production of Fe(II) was monitored (under anaerobic conditions) for approximately 10 minutes.

III. RESULTS AND DISCUSSION

A. Simultaneous Reduction of Fe(III) and NO_3^- by Alteromonas putrefaciens 200.

The experimental approach used to study the simultaneous reduction of NO_3^- and Fe(III) by *Alteromonas putrefaciens* 200 was similar to the approach used by Obuekwe (1981). Batch cultures of *Alteromonas putrefaciens* 200 were grown to a target optical density and subsequently provided with nitrate and ferric iron under anaerobic conditions. NO_3^- and Fe(III) reduction rates were determined by measuring the production of nitrite and ferrous iron in solution. In Obuekwe's experiments, batch cultures were allowed to incubate anaerobically in the presence of NO_3^- and Fe(III) for a period of 12 hours without taking precautions against the induction of alternative electron transport pathways. Since *Alteromonas putrefaciens* 200 possesses an inducible (high-rate) ferri-reductase (Arnold et al., 1986) and an inducible (high-rate) nitrate reductase (see below), the rate data presented by Obuekwe may reflect induction of either system during the time course of the experiment. Interpretation of those data must be approached with caution, especially with respect to the physiological basis of the iron reduction process. In the present study, chloramphenicol was added prior to the additions of NO_3^- and Fe(III), thus halting protein synthesis and prohibiting the induction of alternative electron transport pathways during the time course of the experiment. In addition, Obuekwe used the phenanthroline technique (Krishna-Murti et al., 1966) to determine the amount of Fe(II) in solution. This technique entails withdrawing an aliquot from the batch culture provided with NO_3^- and Fe(III) and quenching the aliquot in a phenanthroline/ammonium acetate buffer. Ferrous iron measurements taken via the phenanthroline technique will therefore reflect the secondary effects of Fe(II) oxidation by NO_2^- . In the present study, ferrozine was added directly to the liquid culture, thereby providing a method for determining the amount of Fe(II)

produced *in situ*. Since ferrozine complexes Fe(II) on a time scale much faster than that of the reoxidation of Fe(II) to Fe(III) (Arnold, 1987), this technique eliminates the secondary effect of Fe(II) oxidation by NO_2^- . The production of NO_2^- and Fe(II) was monitored under anaerobic conditions for 30 minutes. Results from both sets of experiments are given in Figure 2.1. The production of Fe(II) [i.e., dissimilative reduction of Fe(III)] by cultures grown at either high (constitutive) or low (induced) oxygen tension was unaffected by the presence of NO_3^- . The concentration of NO_3^- was not a factor in either case. Similarly, the production of NO_2^- [i.e., reduction of nitrate] by cultures grown at either high or low oxygen tension was unaffected by the presence of Fe(III). Since the concentration of nitrate did not affect the nitrate reduction rate in these experiments, it was assumed that NO_3^- was provided at saturating levels.

As indicated in Figure 2.1 and summarized in Table 2.1, prolonged growth at low oxygen tension ($< 2 \mu\text{M}$) induced high-rate nitrate reduction activity. The activity of the induced (high-rate) nitrate reduction system was more than two-fold greater than the activity of the constitutive (low-rate) system. It is not known whether induction of high-rate nitrate reduction activity was due to the expression of a second (high-rate) nitrate reductase, or simply due to enhancement of the constitutive system. Also evident from the data presented in Table 2.1, and as noted in previous studies with *A. putrefaciens* 200 (Arnold et al., 1986), the induced ferri-reductase system was capable of iron reduction at rates nearly 8-fold greater than its constitutive counterpart. Oxygen utilization rates doubled after prolonged growth under low oxygen tension.

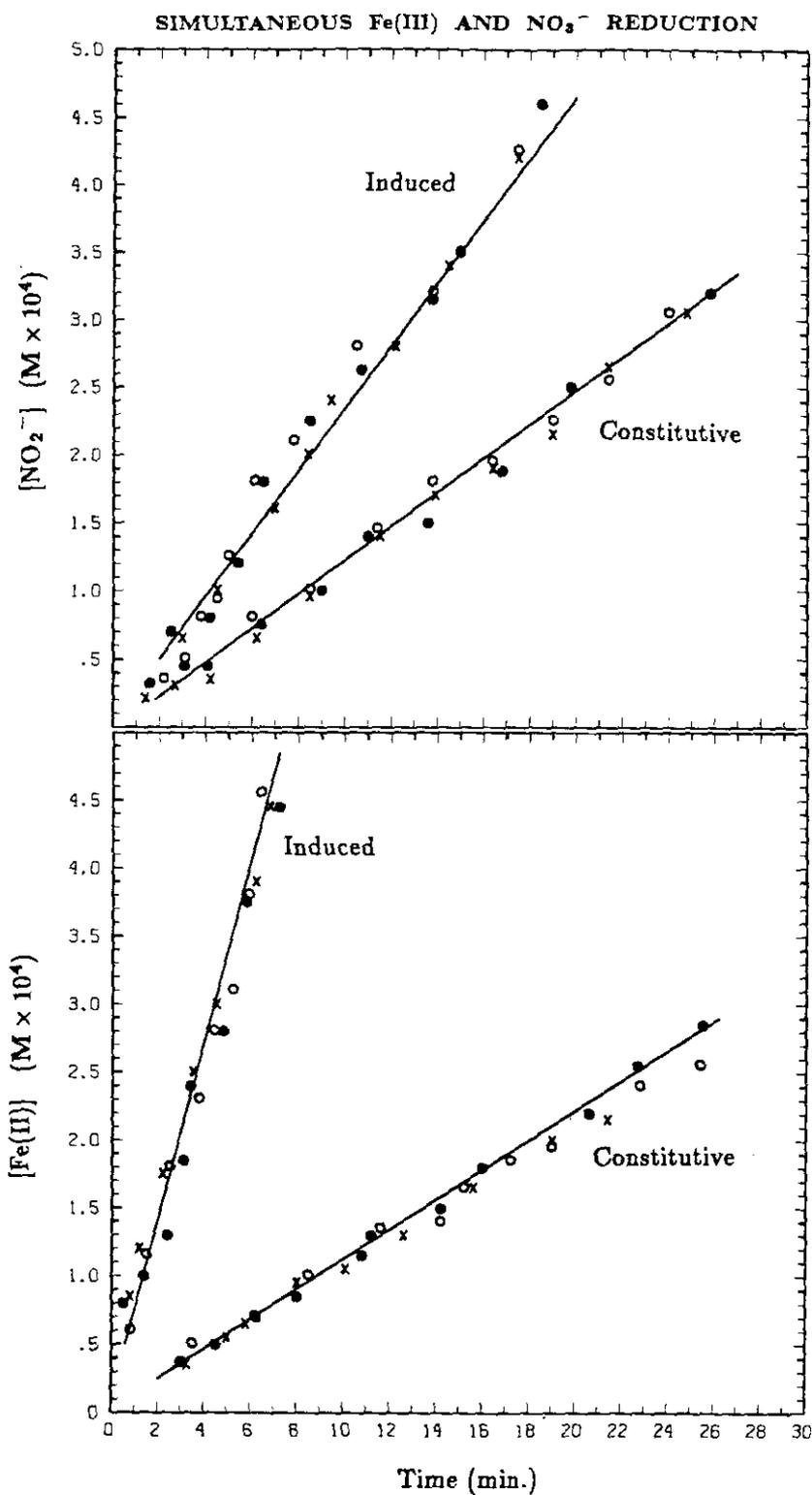


Figure 2.1. Production of NO₂⁻ (top) and Fe(II) (bottom) by batch cultures of *Alteromonas putrefaciens* 200 provided with NO₃⁻ and Fe(III) simultaneously. (●, [NO₃⁻] or [Fe(III)] = 0; x, [NO₃⁻] = 2 [Fe(III)]; ◐, [NO₃⁻] = 1/2 [Fe(III)]).

Table 2.1. Experimentally Determined Rate Data:
Simultaneous Fe(III) and NO₃⁻ Reduction and
Corresponding Oxygen Utilization Rates^a

Growth Conditions	V _{max,NO₃⁻}	V _{max,Fe(III)}	V _{max,O₂}
High oxygen tension ^b	1.3 (1.3) ^d	1.1 (1.1)	2.1 (8.4)
Low oxygen tension ^c	2.7 (2.7)	7.8 (7.8)	4.2 (16.8)

- Notes:
- All rates reported in $\underline{M}/\text{min} \times 10^5$
 - [O₂] > 200 μM)
 - [O₂] < 2 μM
 - Values in parentheses are normalized on a per mole electron basis.

Expression of alternative anaerobic respiratory systems in response to low oxygen tensions is not unprecedented in bacteria. A second (high-rate) cytochrome oxidase is expressed under low oxygen tension in *E. coli* (Castor and Chance, 1959; Green and Gennis, 1983), *Pseudomonas putida* (Sweet and Peterson, 1978), and *Klebsiella aerogenes* (Moss, 1956). Expression of constitutive nitrate reductase activity is generally not found in cells grown aerobically, and specific conditions are usually required (see below) to induce expression of nitrate reductase activity. In *A. putrefaciens* 200, however, nitrate reductase activity was expressed when cells were grown under highly aerobic conditions (Figure 2.1). This finding is different from that found with the nitrate reductase system of *Escherichia coli*, in which NO₃⁻ is reduced only when cells have been exposed to anaerobic conditions in the presence of NO₃⁻ for sufficient time to induce nitrate reductase (Stewart and MacGregor, 1982). Furthermore, in light of the recent study of Noji and Taniguchi (1987), which demonstrated that molecular oxygen inhibits expression of the *E. coli* nitrate reductase operon by excluding NO₃⁻ (required to induce the operon) from

the cytoplasm, *A. putrefaciens* 200 probably regulates expression of nitrate reductase activity in a manner different from that found in the enterics. The ability to express nitrate reductase activity immediately in response to low oxygen tension may afford *A. putrefaciens* 200 a selective advantage in environments involving aerobic/anaerobic interfaces (e.g., freshwater and marine sediments).

Results given in Table 2.1 and Figure 2.1 suggest that Fe(III) and NO_3^- are reduced by two physiologically distinct terminal reductases: the iron reduction rates (both constitutive and induced) measured in the presence of NO_3^- were identical to the Fe(III) reduction rate measured in the absence of NO_3^- . In addition, the nitrate reduction rates (after growth at either low or high oxygen tension) measured in the presence of Fe(III) were identical to the nitrate reduction rates measured in the absence of Fe(III). In previous studies with *A. putrefaciens* 200 (Obuekwe, 1980; Arnold et al., 1986), molecular oxygen was shown to inhibit both nitrate and iron reduction activity. It therefore appears that *A. putrefaciens* 200 possesses three physiologically distinct enzymes, each capable of transferring electrons to a specific terminal electron acceptor. Under aerobic conditions, electrons are preferentially transferred to O_2 ; under anaerobic conditions, and in the presence of NO_3^- and Fe(III), electrons are transferred to NO_3^- and Fe(III) simultaneously. This finding contradicts thermodynamic arguments that biological reduction of Fe(III) should not occur until NO_3^- disappears (Munch et al., 1978; Nealson, 1983). Reasons for this apparent discrepancy are not known, but may be the result of a novel biological control mechanism involved in expression of alternative anaerobic electron transport pathways. A comparison of the relative (apparent) rates of electron transport by the aerobic and anaerobic respiratory systems can provide preliminary insight into the biological control elements involved in anaerobic electron transport. If it is assumed that a 4-electron transfer is required to reduce molecular oxygen, and that a one electron transfer is required to reduce either Fe(III) or NO_3^- , then

an electron balance can be used to compare the relative rates of electron transfer by the aerobic and anaerobic respiratory systems on an equivalent basis. If the overall electron transfer rate of both systems is limited by the same element of the electron transport chain, then the (apparent) maximum rate of electron transfer to O_2 [i.e., $4V_{\max,O_2}$] should equal the combined (apparent) maximum rates of electron transfer to NO_3^- and Fe(III) [i.e., $V_{\max,NO_3^-} + V_{\max,Fe(III)}$] under anaerobic conditions. An electron balance based on the electron transfer rates given in Table 2.1 (values in parentheses) indicates that, after aerobic growth, the rate of electron transfer to Fe(III) and NO_3^- is nearly three fold slower than the rate of electron transfer to O_2 . After microaerophilic growth, the rate of electron transfer to Fe(III) and NO_3^- is two fold slower than the rate of electron transfer to O_2 . These results suggest that the overall electron transport rate to O_2 is controlled by a respiratory chain element different from that element controlling the overall electron transfer rate to NO_3^- and/or Fe(III). If anaerobic electron transfer was limited at the level of ferri-reductase and nitrate reductase, then the components of the anaerobic electron transport chain involved in the reduction of NO_3^- and Fe(III) should be in a reduced state during anaerobic respiration. It may therefore be possible to identify those components by comparing the difference spectra of membrane preparations from cells respiring aerobically vs. cells actively reducing NO_3^- and/or Fe(III). Such experiments will be the subject of future work.

B. Fe(III) Reduction Activity in the Presence of Molecular Oxygen and Cyanide

Previous respiratory inhibitor studies with *A. putrefaciens* 200 (Arnold et al., 1986) suggested the presence of a complex electron transport chain whose composition depended largely on the oxygen tensions maintained during growth (Figure 1.1). When cells were grown under high oxygen tension, a CN^- -sensitive cytochrome oxidase was detected along with a (low-rate) ferri-reductase whose activity was essentially unaffected by the presence of CN^- . When the cells were

grown microaerophilically, a second (high-rate) ferri-reductase was expressed whose activity was also unaffected by the presence of CN^- . Preliminary studies on Fe(III) reduction in the presence of O_2 (Arnold, 1987) demonstrated that Fe(III) reduction does not occur until residual oxygen has been purged from the system. However, when CN^- was added, Fe(III) reduction occurred even in the presence of molecular oxygen. Preliminary experiments by Arnold (1987) also suggested that the relative increase in Fe(III) reduction activity (in the presence of cyanide) might be proportional to the relative inhibition of the oxygen utilization rate. The following model was proposed: if the rates of electron transport to O_2 and Fe(III) were limited at the cytochrome oxidase and ferri-reductase levels, respectively, then the relative increase in the rate of Fe(III) reduction should equal the relative decrease in the rate of oxygen utilization, i.e.,

$$\frac{V_{\text{Fe(III)}}}{V_{\text{max,Fe(III)}}} = 1 - \frac{V_{\text{O}_2}}{V_{\text{max,O}_2}} \quad (1)$$

If the relative inhibition of oxygen utilization is proportional to the concentration of cyanide, then

$$V_{\text{O}_2} = \frac{V_{\text{max,O}_2}}{1 + K_i [\text{CN}^-]}, \quad (2)$$

where K_i is the apparent inhibition constant between CN^- and cytochrome oxidase activity. Substituting equation (2) into equation (1) yields an expression for the apparent iron reduction rate as a function of $[\text{CN}^-]$:

$$V_{\text{Fe(III)}} = V_{\text{max,Fe(III)}} \left[1 - \left\{ 1 + K_i [\text{CN}^-] \right\}^{-1} \right] \quad (3)$$

Unfortunately, this analysis is not straightforward, as the concentration of free cyanide in solution will be affected by complexation with various iron species. To investigate the speciation of cyanide in batch experiments in which Fe(III), O₂ and cyanide were supplied simultaneously to batch cultures of *A. putrefaciens*, the computer code MINEQL (Westall et al., 1976) was used to calculate the equilibrium speciation of cyanide and iron in the culture medium (lactate medium plus NTA, Fe(III), NaCN). Thermodynamic data corresponding to relevant equilibria were primarily from Martell and Smith (1970) and have been summarized previously (Arnold et al., 1986). Equilibria and thermodynamic data were expanded here to include both ferro- and ferri-cyanide complexes. Correction of the thermodynamic data to 30°C was accomplished by using the integrated van't Hoff equation (Stumm and Morgan, 1981). Iron and cyanide speciation were approached systematically by fixing the pH (7.0) and increasing the total cyanide concentration stepwise from 10⁻⁵ M to 10⁻² M. Fe(III) was permitted to precipitate as Fe(OH)₃ (s,amorph) in all simulations. More stable Fe(III)-bearing minerals were not considered.

As illustrated in Figure 2.2, when [CN⁻] < 10⁻³ M, the equilibrium concentration of CN⁻ is limited by the HCN acidity constant (pK_a, 30°C = 9.2). Above a total cyanide concentration of 10⁻³ M, [CN⁻] is greatly affected by the formation of ferri-cyanide complexes. When [CN⁻] > 3 x 10⁻³ M, the predominant cyanide species is Fe(CN)₆³⁻. In the concentration range employed in these experiments (i.e. [CN⁻] < 10⁻⁴ M), however, cyanide/iron complexation is negligible.

To test the cyanide inhibition model described above, a series of 10 experiments was carried out in which the final concentration of cyanide added to aerobically grown liquid cultures was systematically varied. When a target optical density had been reached, chloramphenicol was added (to halt cell growth),

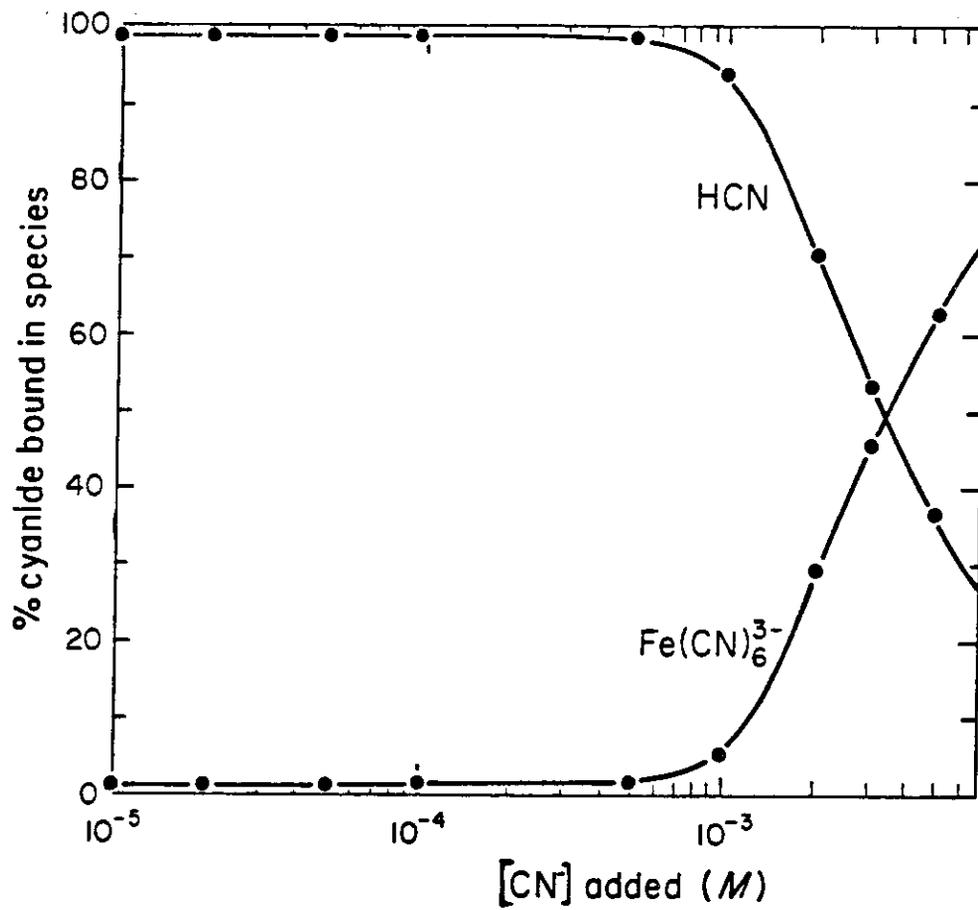


Figure 2.2. Equilibrium speciation of cyanide and iron in lactate medium as a function of [CN⁻] added to batch cultures. The computer code MINEQL was used to calculate equilibrium values in each simulation.

followed by the successive additions of NTA, ferrozine, and ferric iron. The uninhibited O₂ utilization rate was measured, followed by the addition of CN⁻ to preselected levels. The oxygen utilization and Fe(III) reduction rates were then measured simultaneously. The ferrozine technique, used in these experiments to measure Fe(II) production *in situ*, eliminates the effect of secondary reoxidation of Fe(II) to Fe(III) in the presence of O₂. Kinetic data from this study are summarized in Table 2.2.

Table 2.2. Results of Cyanide Inhibition Study

[CN ⁻] (Mx10 ⁵)	$\frac{V_{O_2}}{V_{max,O_2}}$	$\frac{V_{Fe(III)}}{V_{max,Fe(III)}}$	$V_{Fe(III)}(M/min \times 10^5)$	
			observed ^c	predicted ^d
10.0	0.02	0.52	0.16	0.31
5.0	0.05	0.57	0.18	0.29
3.0	0.07	0.52	0.16	0.29
2.0	0.09	0.32	0.10	0.28
1.5	0.14	0.29	0.09	0.27
1.0	0.20	0.23	0.07	0.26
0.8	0.27	0.26	0.08	0.25
0.6	0.40	0.16	0.05	0.23
0.3	0.64	0.09	0.03	0.19
0.0	1.00	0.05	0.02	0.00

- Notes:
- a. $V_{max,O_2} = 1.40 \times 10^{-5} M/min$
 - b. $V_{max,Fe(III)} = 0.35 \times 10^{-5} M/min$
 - c. experimental results.
 - d. model predictions.

The observed oxygen utilization rates were directly proportional to the concentration of CN^- added to the liquid culture ($K_i = 4.7 \times 10^5 \text{ M}^{-1}$, see Figure 2.3). However, the Fe(III) reduction activities observed experimentally were not in agreement with the Fe(III) reduction activities predicted from the model described above (see Table 2.2). The observed Fe(III) reduction rates were generally two- to five-fold lower than the predicted rates. Furthermore, when oxygen utilization was completely inhibited ($[\text{CN}^-] > 1 \times 10^{-4} \text{ M}$), iron reduction rates were only 50% of the maximum iron reduction rate measured under anaerobic conditions (see Table 2.2). The inability to completely restore Fe(III) reduction activity to cells whose electron transport system to O_2 had been completely blocked (i.e., $[\text{CN}^-] > 1 \times 10^{-4} \text{ M}$) could be due to kinetic limitations imposed by a respiratory chain component upstream from the ferri-reductase but downstream from the branchpoint to O_2 . If ferri-reductase activity had limited the overall iron reduction rate, then Fe(III) reduction activity should have been completely restored to maximal rates by the (complete) blockage of electron transport to O_2 . This finding is different from the results of previous inhibitor studies with *Spirillum itersonii* (Dailey and Lascelles, 1977) in which Fe(III) reduction activity was completely restored to membrane preparations when electron transport to O_2 was blocked. From that observation, it was concluded that ferri-reductase activity limited the overall iron reduction rate. The results of the present study suggest that ferri-reductase activity may not limit overall iron reduction activity by *A. putrefaciens* 200. The identity of the electron transport chain component which limits constitutive iron reduction activity by *A. putrefaciens* 200 has not yet been resolved.

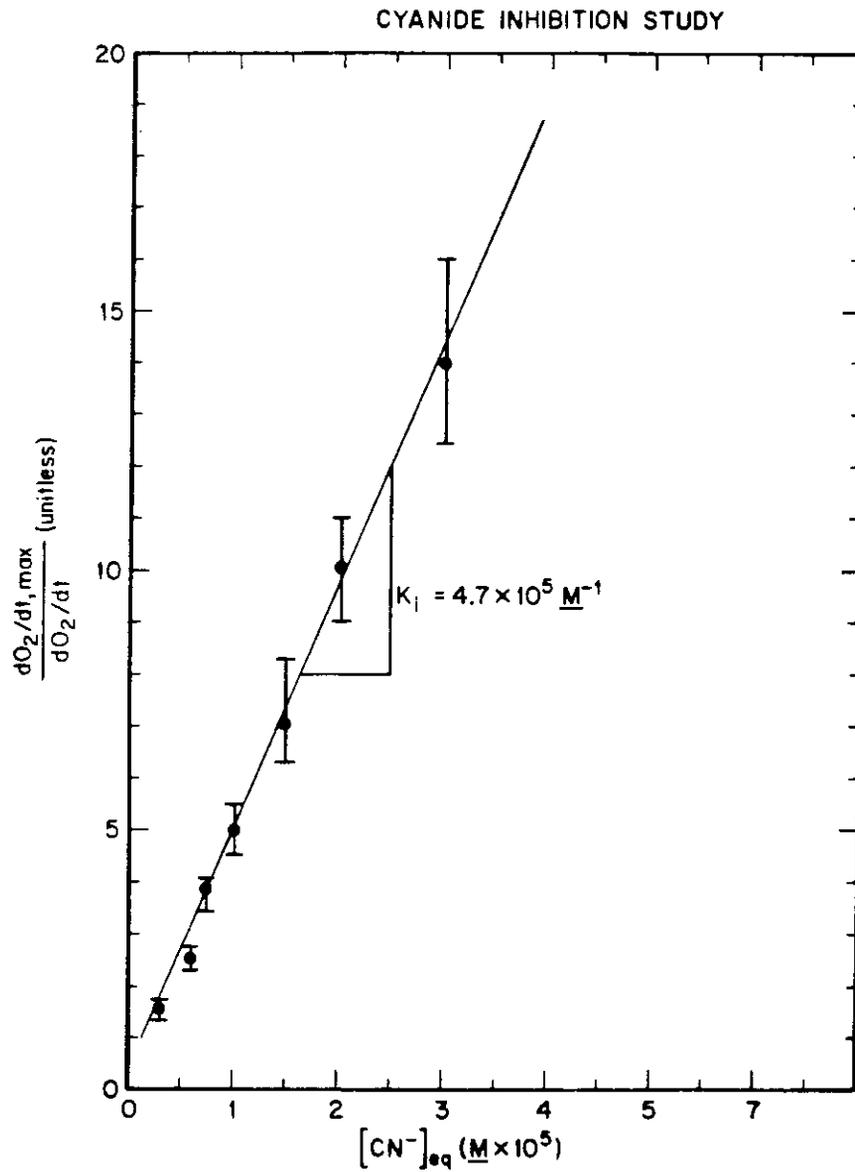


Figure 2.3. Relative inhibition of oxygen utilization by cyanide in batch cultures of *Alteromonas putrefaciens* 200.

IV. SUMMARY

The physiological basis of microbial iron reduction was investigated in a series of batch experiments using the facultative anaerobe *Alteromonas putrefaciens* 200 as a model organism. The combined effects of nitrate, molecular oxygen and cyanide on iron reduction activity were used to study mechanistic aspects of respiratory chain physiology and expression in the presence of alternative electron acceptors. Results indicated that *A. putrefaciens* 200 was capable of reducing NO_3^- and Fe(III) simultaneously under anaerobic conditions but was unable to reduce either electron acceptor in the presence of molecular oxygen. In addition, each system possessed a low-rate and a high-rate component. Expression of low-rate nitrate reductase activity occurred when cells were grown aerobically in the absence of nitrate. Molecular oxygen completely inhibited Fe(III) reduction activity; when electron transport to O_2 was blocked by the addition of cyanide, however, Fe(III) reduction activity was restored, although not at maximal rates. This finding suggests that ferri-reductase activity does not limit iron reduction activity by *A. putrefaciens* 200. Overall, these results suggest that *Alteromonas putrefaciens* 200 possesses a highly branched respiratory chain terminating with at least three physiologically distinct terminal reductases: a cytochrome oxidase, a nitrate reductase and a ferri-reductase.

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CHAPTER 3

**DEVELOPMENT OF A SCREENING TECHNIQUE
AND MUTAGENESIS PROCEDURES FOR
ISOLATION OF IRON-REDUCTION-DEFICIENT MUTANTS**

I. INTRODUCTION

Molecular studies aimed at elucidating the pathway and mechanism of microbial electron transport chains depend to a large extent on the ability to isolate respiratory-deficient mutants. The availability of iron-reduction-deficient mutants will facilitate both genetic and biochemical characterization (see Chapter 5) of the iron reduction system of *Alteromonas putrefaciens* 200. Mutant strains are generally isolated by combining mutagenesis procedures with a screening technique designed to detect specific phenotypic changes. The screening technique must be sensitive enough to allow for detection of mutants deficient in that activity, and in addition, facilitate rapid, reliable screening of tens of thousands of potential mutants. Assuming that a representative bacterial genome contains approximately 3,000 loci, of which 1,000 are structural genes (as in the case of *Escherichia coli*; Bachmann and Low, 1980), the number of potential mutants that must be screened in order to ensure a 99.0% chance of detecting a defect in a single structural gene is approximately 5,000 (assuming all genes are identical with respect to absolute mutation frequency). The probability relation that is frequently used to describe this phenomenon [i.e. $P = 1 - (1-f)^N$, where N is the number of attempts required to ensure a probability P (= 0.99) of detecting a random event that occurs with an absolute frequency f (= 1/1000)] has been used in previous genetic studies (Clark and Carbon, 1979) to calculate the gene bank number size required for cloning genes in nitrogen fixation. Intuitively, it is analogous to having a 99.0% chance of randomly selecting the one colored ball out of a box containing 999 uncolored others.

The detection of respiratory-deficient mutants is often complicated by the existence of secondary metabolic systems (e.g., multi-branched respiratory chains, fermentation pathways) that can support normal growth even in the absence of the respiratory component of interest. In this manner, mutants lacking a particular

electron transport chain component may not show a respiratory-deficient phenotype. This problem has been circumvented by employing screening strategies that are designed specifically for the respiratory system under study. For example, *E. coli* mutants lacking a cytochrome *d* terminal oxidase complex have been identified (Willison et al., 1981; Green and Gennis, 1983) by their failure to oxidize an artificial electron donor (N,N,N',N'-tetramethyl-p-phenylenediamine) that donates electrons directly to that branch of the electron transport chain. Dehydrogenase-deficient *E. coli* mutants have been identified (Creaghen and Guest, 1972) by their inability to grow aerobically on such non-fermentable substrates as lactate and succinate. A similar strategy (Au and Gennis, 1987) has been used to identify *E. coli* mutants lacking the cytochrome *o* terminal oxidase complex. Suicide substrates have also been employed in the isolation of respiratory-deficient mutants. Nitrate-reductase-deficient mutants have been identified by their resistance to normally lethal doses of chlorate (reduced to hypochlorous acid by nitrate reductase) during anaerobic growth (Piechaud et al., 1967). A second screening technique developed (Pichinoty, 1963) for the detection of nitrate-reductase-deficient mutants was based on the use of a colorimetric reagent [diazotized sulfanilic acid coupled with N-(1-naphthyl)-ethylenediamine (NED)] that forms a pink-colored tertiary complex with nitrite, the reduced form of the terminal electron acceptor. A screening technique specific for the detection of iron-reduction-deficient mutants has not been reported.

Several investigators (see below), however, have isolated both iron- and manganese-reducing microorganisms via traditional enrichment techniques that may be applicable to the development of a screening technique for the detection of iron-reduction-deficient mutants. At neutral pH and under oxidizing conditions iron and manganese are found predominately in the solid phase as Fe(III)-hydroxides and Mn(IV)-oxides (Stumm and Morgan, 1981). The chemical speciation of iron and

manganese at neutral pH has been exploited in the development of enrichment strategies used to isolate Fe- and Mn-reducing microorganisms from both terrestrial (Bromfield, 1954; Troshanov, 1968; Bautista and Alexander, 1972) and aquatic (DeCastro and Ehrlich, 1970; Ghiorse and Hirsch, 1982; Myers and Nealson, 1988) environments. The presence of Fe- and Mn-reducing microorganisms was indicated by clearing zones (i.e., visible reduction to soluble Fe(II) and Mn(II) forms) around colonies incubated anaerobically (or microaerophilically) on solid media supplemented with the appropriate oxide. The absence of clearing zones around such colonies might then indicate the presence of iron- or manganese-reduction-deficient mutants.

In addition, the availability of several colorimetric reagents for the detection of reduced iron makes it possible to assay for iron reduction activity on a quantitative basis. Both phenanthroline- (American Public Health Association, 1985) and ferrozine-(2,4,6-tripyridyl-1,3,5-triazine; Stookey, 1972) based methods have been employed in spectrophotometric determination of microbial Fe(III)-reduction activity (e.g., Jones et al., 1983; Arnold et al., 1986). Ferrozine forms a bidendate tris-complex with ferrous iron and is stable in the neutral pH range (pH 4-9). Phenanthroline-based methods require highly acidic conditions (pH < 3). In addition, ferrozine complexes ferrous iron on a time scale much faster than that of the reoxidation of the ferrous to ferric species (Carter, 1971). Thus, the ferrozine trap technique provides an attractive alternative for *in situ* measurement of iron reduction activity by microorganisms that propagate at neutral pH. Such measurement can be made without the loss of cell viability and in the presence of molecular oxygen.

The development of mutant isolation procedures is also essential to the genetic analysis of bacteria with no known genetic systems (Lidstrom, 1989). Stable mutant strains have been shown to arise spontaneously in bacteria (e.g., Darzins and

Chakrabarty, 1984), although the frequency of such mutation is generally quite low. The molecular mechanism of spontaneous mutation of nucleic acids is thought (Drinkwater et al., 1980; Duncan and Miller, 1980) to involve heat (free energy)-induced depurination and/or deamination of component nucleotides. Inaccurate base pairing during DNA replication will then result in a change (replacement, addition, or deletion) in the original nucleotide sequence. Frameshift mutations (i.e., addition or deletion of a base pair) are potentially the most harmful since each codon after the frameshift will be misread including stop-signal information. It is also possible to induce mutations in bacteria at frequencies several orders of magnitude higher than the corresponding background spontaneous rate, using agents called mutagens. Many times the specificity (i.e., site of action) of induced mutations will be different from those occurring spontaneously (Coulondre and Miller, 1977; Coulondre et al., 1978). In addition, certain "hotspots" in bacterial genomes are more susceptible to mutation than others (Benzer, 1961). For these reasons it is important to use a variety of mutagenesis techniques in the analysis of genes required for functional expression of bacterial systems.

Techniques developed to induce mutations in bacteria include radiation mutagenesis, various chemical treatments, and transposon mutagenesis. The molecular basis of action of the mutagenic agents associated with each technique has been studied extensively and several excellent reviews exist (e.g., Drake, 1970; Drake and Baltz, 1976; Miller, 1983; Bennett, 1985). Each technique has proven effective in a wide range of microorganisms and their mutagenic potential has been well documented.

Ultraviolet radiation causes dimerization of adjacent pyrimidines (primarily thymines) resulting in distortion of the DNA helix and subsequent interference in both replication and transcription. Several investigators (e.g., Miller, 1972) have been successful in isolating specific mutants by exposing liquid cultures to UV light.

Because of its highly non-specific nature (i.e., can affect all nucleotides), UV light provides an attractive alternative to other mutagenic agents. However, many bacteria contain repair systems that reverse UV-induced dimerization.

Chemical reagents commonly used to induce mutation in bacteria include intercalating dyes (e.g., acridine orange), base analogues (e.g., 5-bromouracil), alkylating agents (e.g., ethyl methane sulfonate) and reagents that cause base transition mutations (e.g., nitrous acid, hydroxylamine). Of these chemical mutagens, the alkylating agents ethyl methane sulfonate (EMS) and N'-methyl-N'-nitro-N-nitrosoguanidine (NTG) are considered two of the most powerful. The molecular basis of their action is well documented and has recently been reviewed (Lemontt and Generoso, 1982). Alkylation at the O⁶ position of the purine ring results in hydrolysis of the purine-deoxyribose bond. During subsequent DNA replication, random insertion of a thymine residue in place of the deleted guanine will result in a GC to TA transversion. Alkylating agents do in fact show a high frequency of GC to TA transversions. The effectiveness of alkylating agents as chemical mutagens resides in their ability to form the required DNA adducts independently of the *recA*-dependent repair systems generally used by bacteria to defend against UV-induced mutation. Drawbacks to their use include problems with mutational specificity and intercellular diffusion barriers.

Transposon mutagenesis has proven to be an effective method for insertional inactivation of DNA target sites. The properties of transposons have been reviewed extensively (Kleckner, 1981) and their application to genetic analyses is well established (Foster, 1984). After transfer of the donor plasmid to an appropriate host strain, transposons are thought to autoreplicate (via self-encoded replicases) and insert a second copy in the host genome. Insertion into a target gene generally results in disruption of that gene function.

Transposable elements have been grouped into three distinct classes (Kleckner, 1981) on the basis of their regulatory control mechanisms, genetic make-up, and DNA sequence homology: Class IA (simple insertion molecules), Class IB (insertion modules flanking a selectable determinant), Class II (contiguous sequences encoding at least two proteins involved in transposition and a selectable marker), and Class III (transposing bacteriophages). The transposing properties inherent to each class are indicative of their mutagenic potential. The absence of a selectable marker precludes use of Class IA transposons in most genetic studies. Class II transposons insert preferentially into plasmid genomes (Kretshmer and Cohen, 1979) thus limiting their use as chromosomal mutagens. Faulty excision in the genome of host strains other than *E. coli* precludes use of transposing phages (Class III). Thus, transposable elements of the Class IB type (Table 3.1) appear to be the best candidates for use as mutagenic agents in *Alteromonas putrefaciens* 200. The antibiotic resistance conferred by several transposons in this class (i.e., Tn₅, Tn₉, Tn₁₀) facilitates selection of recipient strains, and the relatively non-specific (i.e., quasi-random) distribution of insertion sites reported with these transposons further advocates their potential as mutagenic agents.

Table 3.1. Examples of Class IB transposable elements (Kleckner, 1981).

<u>Element</u>	<u>Module at ends</u>	<u>Selectable marker</u>	<u>Size (kb)</u>
Tn ₅	IS ₅₀	Km ^r	5.7
Tn ₉	IS ₁	Cm ^r	2.5
Tn ₁₀	IS ₁₀	Tc ^r	9.3

The advent of broad-host-range cloning vectors as transposon delivery vehicles (see Chapter 4) has expanded the list of bacteria susceptible to transposon mutagenesis. Transposon mutagenesis has been successfully applied to a diverse

group of gram-negative bacteria including *Bordatella* (Weiss and Falkow, 1983), *Rhizobia* (Beringer et al., 1978), *Erwinia* (Chatterjee et al., 1983), *Myococcus* (Kuner and Kaiser, 1981), *Thiobacillus* (Davidson et al., 1985), the aforementioned enteric bacteria, and various *Pseudomonas* spp. (Krishnapillai et al., 1981; Tsuda et al., 1984; Monticello et al., 1985).

Experimental procedures developed for transposon mutagenesis generally involve either transposition achieved by means of plasmid incompatibility or transposition from a "suicide" donor plasmid. The latter method holds the most promise for mutagenesis of genetically uncharacterized microorganisms since plasmid content is generally not known in these bacteria. Two types of broad-host-range suicide vectors have been used in transposon mutagenesis: (i) mobilizable plasmids containing a transposon and bacteriophage Mu (recipient-strain-specified restriction directed toward Mu DNA sequences eliminates the vector from transconjugants after transfer) and (ii) mobilizable plasmids containing transposons joined to narrow-host-range replicons that are unable to replicate in the new host strain. Since mutagenic frequency is the product of vector transfer frequency and transposon insertion frequency, the isolation of stable mutants is contingent upon efficient plasmid mobilization (see Chapter 4).

A variety of transposon delivery vehicles have been used successfully in gram-negative bacteria. For example, *Pseudomonas putida* has been successfully mutated (Monticello et al., 1985) by conjugative transfer of plasmid pJB4JI (pHIJI::Mu::Tn₅), a type (i) "suicide" vector. Suicide vector pJB4JI has also been used to mutate *Caulobacter crescentus* (Ely and Croft, 1982), *Rhizobium* spp. (Beringer et al., 1978), and *Thiobacillus versutus* (Davidson et al., 1985). An example of a type (ii) suicide transposon vector is the colicin plasmid pLG221 (colIb *drd-1 cib*::Tn₅). Suicide vector pLG221 was used to introduce Tn₅ insertions into

the chromosome and indigenous plasmids of *Pseudomonas* sp. B13 and *Pseudomonas putida* PaW340 (Weightman et al., 1984). The broad-host-range R plasmid RK2 has been used to construct additional families of "suicide" donor plasmids. Hybrid plasmid pSUP5011 ([pBR322-*mob*]::Tn5), constructed by linkage of the *mob* (*oriT*) sequence of RK2 to a ColE1 replicon (pBR322) harboring transposon Tn5, can be mobilized into the broad-host-range of RK2 but does not replicate outside *E. coli* (Simon et al., 1983). Because of these features, plasmid pSUP5011 (see Figure 4.1) was chosen as a potential transposon delivery vehicle for use with *Alteromonas putrefaciens* 200.

II. MATERIALS AND METHODS

A. Strains, media, and growth conditions

The strains and plasmids used in this study are listed in Table 3.2.

A rifamycin-resistant strain of *Alteromonas putrefaciens* 200 was isolated by plating approximately 10^9 cells on nutrient agar supplemented with rifamycin SV (Sigma; 100 g/L).

Alteromonas putrefaciens 200 strains were grown at 30°C on lactate medium (pH 7.0) as described by Obuekwe (1980) [per liter: 0.5 g of K_2HPO_4 , 2.0 g of Na_2SO_4 , 1.0 g of NH_4Cl , 0.15 g of $CaCl_2$, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.5 g of yeast extract, 3 ml of 60% sodium lactate syrup] except during mutagenesis experiments when L broth (Miller, 1972) was used for liquid cultures. Minimal salts solution (MSS) was essentially lactate medium (pH 7.0) minus yeast extract and lactate. *Salmonella typhimurium* LT2 and *Escherichia coli* strains were grown at 37°C in L broth (Miller, 1972). MSS-supplemented medium was prepared by adding filter-sterilized supplements to sterile medium. Antibiotics were added at the following final concentrations: rifamycin SV (100 mg/L), tetracycline (20 mg/L), kanamycin (40 mg/L). Media employed in test for total auxotrophy consisted of MSS (pH 7.0) supplemented with 0.2% (w/v) glucose (MSSG). Specific auxotrophs were screened

on MSSG medium supplemented with various combinations of amino acids, vitamins and purine/pyrimidine bases (Miller, 1972). Agar (Difco) was added to 1.5% (w/v) for plates. Nutrient agar was supplemented with filter-sterilized $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (to 0.15 mM as Fe) for use in screening experiments.

Table 3.2. Strains and plasmids used in this study.

Strain or Plasmid	Relevant Characteristics	Source or Reference
<i>Alteromonas putrefaciens</i> strain 200		
Wild Type	High-rate iron reducer	D.W.S. Westlake
R100	Rifamycin resistant (100 mg/L)	This study
<i>Salmonella</i>		
<i>typhimurium</i> LT2	Unable to reduce iron	M. Simon
<i>E. coli</i>		
HB101	<i>recA</i> , <i>hsdR</i> , <i>hsdM</i>	M. Lidstrom
CSR603	<i>recA</i> , <i>hsdR</i> , <i>hsdM</i>	D. Bartlett
S17-1	chromosomally-integrated RK2	A. Puhler
Plasmids		
pVK100	Cosmid cloning vector (Tc^r , Km^r , Mob^+)	Knauf & Nester
pRK2013	Helper plasmid (pBR322 replicon, Km^r , Tra^+)	D. Bartlett
pSUP5011	Transposon delivery vehicle ($\text{Tn}5$, pBR322 replicon, Km^r , Mob^+)	A. Puhler

B. Screening Technique.

Three methods were developed and tested for their ability to detect both high-rate and low-rate iron reduction activity by *Alteromonas putrefaciens* 200. The first method is a modification of a technique recently employed (Arnold et al., 1988)

for the *in situ* detection of ferrous iron production (dissimilative iron reduction) in the presence of molecular oxygen. Each well of a 96 well tissue culture dish (Microtest Plate III, Becton Dickinson and Co.) was half-filled (0.15 ml) with sterile lactate medium and immediately inoculated with an appropriate strain. The dish lid was sealed and the assembly incubated for 24 h. (30°C) with vigorous shaking. A solution (pH 7.0) containing ferrozine (to 37.2 mM) and equimolar FeCl₃·6H₂O (to 1.86 mM as Fe) and nitrilotriacetic acid (NTA) was then added to each well. The formation of magenta-colored liquid cultures was monitored visually for 30 minutes. Previous respiratory inhibitor studies (Arnold et al., 1986) indicated that HQNO (2-n-heptyl-4-hydroxyquinoline-N-oxide) was a potent inhibitor of low-rate (constitutive) iron reduction activity by *A. putrefaciens* 200 but did not affect high-rate (inducible) iron reduction activity. HQNO was added to several wells at a level (10⁻⁴ M) previously shown to be inhibitory to constitutive iron reduction activity. *E. coli* HB101 (low-rate iron reduction activity) and *Salmonella typhimurium* LT2 (unable to reduce iron) were used as control strains.

The second method was adapted from the technique developed by Piechaud et al. (1967) for detecting mutants defective in nitrate reductase activity. Colonies for screening were transferred from master plates to sterilized filter paper (Whatman No. 4) that had been previously soaked in a solution (pH 7.0) consisting of 1% sodium lactate (v/v) and equimolar FeCl₃·6H₂O (1.86 mM as Fe) and NTA. A fine mist of filter-sterilized ferrozine (37.2 mM) was immediately sprayed on the filter paper to wet the colonies. The formation of magenta-colored colonies was monitored visually for 30 minutes.

The third method tested as a potential screening technique was adapted from the enrichment procedures (see above) used to isolate iron- and manganese-reducing microorganisms from various natural water systems. Colonies were grown

both aerobically and anaerobically (BBL anaerobic cannisters; 0.1 vol % oxygen) on nutrient agar (Difco) supplemented with one of several iron oxides (to 1 mM as Fe). The oxides included lepidocrocite (γ -FeOOH), goethite (α -FeOOH), hematite (α -Fe₂O₃) and amorphous ferric hydroxide (Fe(OH)₃). Each oxide (except ferric hydroxide, added as FeCl₃·6H₂O) was synthetically prepared (Pfizer Corp.) and added to sterile molten nutrient agar cooled to 50°C to avoid problems associated with high-temperature conversion of the iron oxides to hematite (α -Fe₂O₃), the thermodynamically-favored oxide form. When the colonies were of sufficient size (i.e., 0.5 mm), each was checked for the presence of clearing zones (indicative of iron reduction activity). In addition, a fine mist of filter-sterilized ferrozine (37 mM) was sprayed over each plate and the colonies visually monitored for the presence of ferrous iron (i.e., magenta-coloration).

C. Mutagenesis Procedures

1. Chemical Mutagenesis (Ethyl Methane Sulfonate)

A fresh overnight culture (0.2 ml) of wild-type *Alteromonas putrefaciens* 200 was used to inoculate 15 ml LB in a 125 ml flask. Cells were grown at 30°C for 12 h. (to 2×10^{10} cells/ml) with vigorous shaking, harvested, washed and resuspended in an equal volume MSS (pre-warmed to 30°C). The conditions required for mutagenesis (90% kill) of the wild-type strain were determined by adding 10 μ L ethyl methane sulfonate (EMS) to 1 ml of the washed cell suspension and incubating (30°C) with gentle mixing over a range of treatment times. Immediately after treatment, cells were washed and resuspended in MSS; serial dilutions were plated onto nutrient agar for enumeration of viable cells and determination of survival curves.

To test the mutagenic potential of this technique, the EMS-treated cells were subsequently tested for total auxotrophy by replica-plating onto MSSG agar plates. Wild-type *A. putrefaciens* 200 is capable of growth on unsupplemented MSSG agar

plates. Specific auxotrophs were identified by replica-plating master plates onto MSSG plates supplemented with various combinations of amino acids, vitamins, and purine/pyrimidine bases. Specific auxotrophs were picked off the master plates and stored in LB:glycerol (85:15 vol %) at -80°C for future use.

Those experimental conditions used to test total auxotrophy were also used to generate *A. putrefaciens* 200 mutants deficient in iron-reduction activity. EMS-treated cells were washed and resuspended in MSS before plating serial dilutions onto nutrient agar supplemented with FeCl₃·6H₂O (to 0.15 mM as Fe). After 36 hours growth (30°C), the resulting colonies were screened for aberrant iron reduction activity via screening technique No. 3. Those colonies showing aberrant magenta-color formation were picked to nutrient agar plates and rescreened for subsequent verification as mutants deficient in Fe(III) reduction capability.

2. Transposon Mutagenesis

Transposon mutagenesis of wild-type *Alteromonas putrefaciens* 200 was accomplished by two-way (bi-parental) crosses with appropriate donor [*E. coli* S17-1 (pSUP5011)] and recipient [rifamycin resistant *Alteromonas putrefaciens* 200] strains. A spontaneous rifamycin resistant *Alteromonas putrefaciens* 200 strain was isolated by plating approximately 10⁹ cells on nutrient agar supplemented with 100 mg/L rifamycin SV (Sigma). Single isolates were restreaked onto identical medium and stored in LB:glycerol (85:15 vol %) at -80°C. To determine the conditions (i.e., donor:recipient ratio, mating time) that optimized the frequency of transposition, a series of mating experiments were carried out. Mid-log phase liquid cultures of donor and recipient cells were washed in LB, mixed in pre-determined ratios (1:1, 1:4, 1:10), spotted (0.10 ml total volume) onto L plates, and incubated for various times (1, 4, 12, 24 h.) at 30°C. After this incubation period, the mating mixture was washed from the mating plate with MSS buffer, serially diluted, and plated onto solid medium [i.e., nutrient agar

supplemented with Rf (100 mg/L) and Km (50 mg/L)] that selected for stable inheritance of Tn₅ in the *A. putrefaciens* 200 genome and against the donor *E. coli* strain. Since pSUP5011 is unable to replicate outside of *E. coli* (i.e., contains a narrow host-range ColE1 replicon), the transposon mutants should also be chloramphenicol sensitive. A random sample of 25 Rf^rKm^r single colony isolates was tested for chloramphenicol (50 mg/L) sensitivity. Those conditions that optimized the frequency of Tn₅ inheritance in the *A. putrefaciens* 200 genome were used in transposon mutagenesis experiments to generate iron-reduction-deficient mutants. The screening technique employed in detecting aberrant iron-reduction activity with the Tn₅ mutants was identical to that technique (No. 3) used previously in the EMS mutagenesis experiments. Appropriate dilutions of the mating mixtures were plated on selective medium [i.e., NFRK plates: nutrient agar supplemented with ferric iron (2 mM as Fe), Rf (100 mg/L) and Km (50 mg/L)] and incubated for 72 h. A fine mist of ferrozine (30 mM) was then sprayed on the resulting single colonies (i.e., Tn₅ recipients) and any light magenta-colored colonies were picked to NFRK plates. Single colonies were rescreened as a check for transposon stability and stored at -80°C. Stable Tn₅ mutants showing light-magenta coloration were checked for iron reduction activity in batch liquid culture as described above for the EMS-generated iron-reduction-deficient strains. Overnight cultures (5 ml), grown in lactate medium containing both Rf and Km were used to inoculate the reactor vessel (Biostat M, B. Braun) containing 1 L of lactate medium supplemented with Km (50 mg/L) only. Rifamycin was omitted because of its rapid decomposition in the presence of light. To ensure that Km did not affect iron reduction activity in liquid culture, control experiments were run with Tn₅ mutants that had previously shown a positive phenotype on NFRK plates.

D. Iron Reduction Activity of Potential Iron-Reductase-Deficient Mutants

To test the potential mutants for both high-rate and low-rate iron reduction activity, batch cultures of each were grown on Westlake medium (supplemented with kanamycin for Tn5 mutants) in a Biostat M (B. Braun Co., Inc.) fermenter either at near saturation (200 μM) or at low (2 μM) dissolved oxygen concentration. At a target optical density of $A_{600} = 0.25$, chloramphenicol was added (to 0.25 mM) to halt cell growth during the subsequent 30 minute iron reduction experiment. Chloramphenicol was previously shown (Arnold et al., 1986) not to inhibit the rates of oxygen utilization or iron reduction in batch cultures of wild-type *A. putrefaciens* 200. The oxygen supply was then cut off and the oxygen utilization rate monitored at 10 second time intervals for 5 minutes. High-purity nitrogen gas was then bubbled vigorously through the reactor vessel to purge the system of residual oxygen. Addition of equimolar $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (1.86 mM as Fe) and nitrilotriacetic acid (NTA) immediately followed. Ferrous iron measurements (American Public Health Assoc., 1985), initiated within 30 sec. of Fe(III) and NTA addition, were taken at 2 minute time intervals for approximately 30 min. The pH (7.0 ± 0.1) was held constant throughout the time duration of each experiment by manual addition of acid (2N H_2SO_4) or base (2N NaOH). Oxygen utilization and iron reduction rates were calculated from the time-dependent concentration profiles.

III. RESULTS AND DISCUSSION

A. Screening Technique

Preliminary work in this study centered on the development of a screening technique for detection of iron-reduction-deficient mutants of the facultative anaerobe *Alteromonas putrefaciens* 200. Each screening technique is based in part on the observations that ferrozine is active at pH 7 (Stookey, 1970), complexes ferrous iron on a time scale faster than that of the reoxidation of the ferrous to

ferrous iron on a time scale faster than that of the reoxidation of the ferrous to ferric species (Carter, 1971), and does not affect iron reduction activity by *A. putrefaciens* 200 (Arnold et al., 1988). Each of the three methods developed and tested for use in this study were able to detect iron reduction activity by this microorganism. The ability of the strain to induce a second high-rate iron reduction system required that the screening technique also be able to distinguish between strains deficient in either the high-rate (inducible) or low-rate (constitutive) iron reduction systems. The first method (Microtest Plate) showed a high degree of sensitivity since liquid cultures of *A. putrefaciens* 200, *Escherichia coli* HB101 and *Salmonella typhimurium* LT2 provided with ferric chloride and ferrozine were distinguishable (Figure 3.1) based on the intensity of the magenta-color formed. The addition of HQNO did not affect the rate of magenta-color formation. However, it appeared that the high-rate iron reduction system was fully induced during growth in the micro-test plate wells, since fully induced liquid cultures (grown under low oxygen tension in the Biostat M and transferred to the wells) appeared magenta-colored immediately after ferrozine addition whereas cultures grown for 24 hours in the micro-titre plate wells required nearly 30 minutes to reach the same color intensity.

The second method developed and tested for use as a screening technique yielded results similar to those of the first method, except the color change occurred more rapidly. Wild-type colonies, transferred to filter paper that had previously been soaked in an equimolar FeCl_3 /NTA and lactate solution (pH 7), turned magenta-colored within seconds of spraying with a ferrozine mist. In addition, concentric rings of varying magenta-color intensity were noted within each colony. The intensity was highest for the middle cells and decreased radially until the cells of the nascent outer ring appeared nearly colorless (see Figure 3.2).

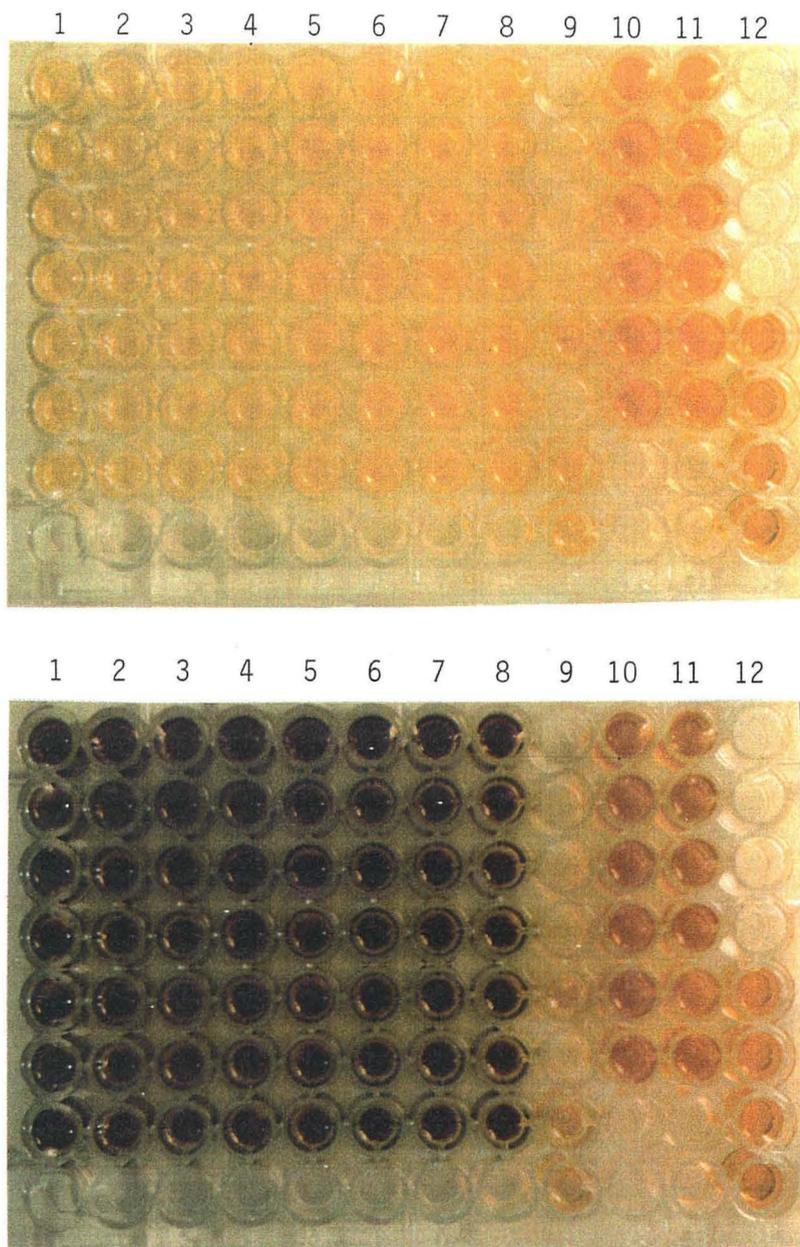


Figure 3.1. Screening Technique No. 1 (MicroTest Plate Technique) before (top) and after (bottom) the addition of ferrozine to each well (1-8: *A. putrefaciens* 200 WT; 9: open; 10-11: *E. coli* HB101; 12: *S. typhimurium* LT2).

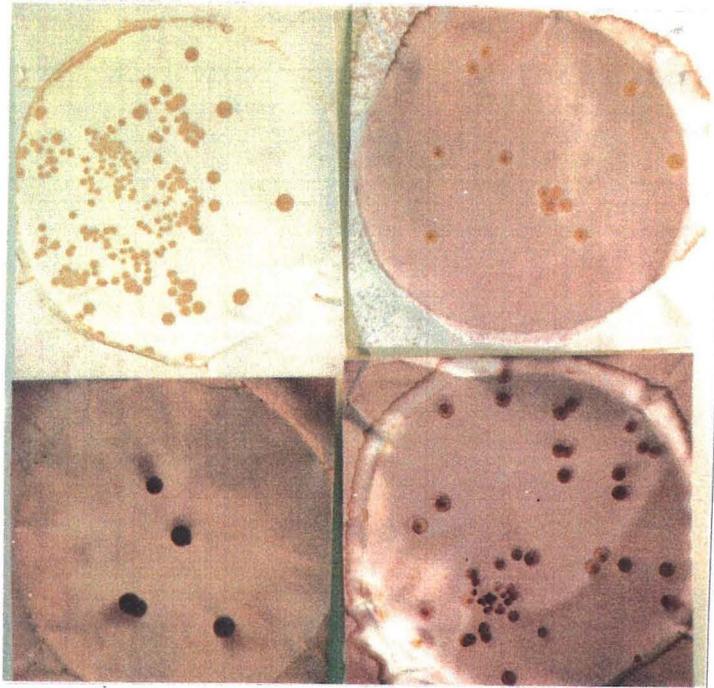


Figure 3.2. Screening Technique No. 2: Replica Plating Technique. Before replica plating *A. putrefaciens* WT, filter paper was soaked in a solution (pH 7) of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ /NTA, lactate at the following concentrations: upper left (0 mM, 0%); upper right (10^{-3} mM, 0%); bottom right (10^{-1} mM, 0%); bottom left (10 mM, 0.1%).

The third screening technique was adapted from the various enrichment protocols used previously to isolate Mn- and Fe-reducing microorganisms from terrestrial and aquatic environments. It was reasoned that iron reduction activity could be detected by the presence of clearing zones (i.e., reductive dissolution) around colonies incubated anaerobically on nutrient agar supplemented with one of several commercially available synthetic iron oxides (e.g., hematite, goethite, lepidocrocite). Ferric chloride (i.e., amorphous ferric hydroxide in solution at neutral pH) was also included in the series. Serial dilutions of wild-type *A. putrefaciens* 200 were spread on the iron oxide-supplemented nutrient agar plates and incubated in anaerobic jars (< 0.1 volume % oxygen) for two weeks at 30°C. Colonies failed to appear after the two week anaerobic incubation period, however colonies appeared on the same plates within 36 hours after exposure to aerobic conditions. Clearing zones were not evident around the periphery of the aerobically grown colonies, most likely due to the inhibitory effect of molecular oxygen on iron reduction activity by this microorganism (Arnold et al., 1986), or due to rapid reoxidation of any soluble (reduced) iron that may have been produced. After the 36 hours aerobic incubation period, the plates were again placed in an anaerobic jar and incubated overnight at 30°C. Iron reduction activity could then be detected since the same colonies appeared magenta-colored after spraying with a fine mist of ferrozine (10 mM). In addition, the degree of magenta-color intensity depended on the identity of the iron oxide added to the nutrient agar (results not shown). Several investigators (e.g., DeCastro and Ehrlich, 1970; Jones, 1983; Lovley and Phillips, 1986) had previously reported that iron reduction activity was inversely related to the degree of crystallinity of the oxide provided to the microorganism (i.e., amorphous or poorly crystallized oxides were more readily reduced than the more crystalline forms). This phenomenon was observed (data not shown) in the above experiments since the intensity of magenta-colored colonies followed

a similar pattern [i.e., hematite (α -Fe₂O₃) < goethite (α -FeOOH) = lepidocrocite (γ -FeOOH) < ferric hydroxide (Fe(OH)₃,amorph.)]. Furthermore, the high intensity of magenta-colored colonies grown aerobically on nutrient agar supplemented with ferric chloride (i.e., Fe(OH)₃,amorph.) suggested that this method holds potential for use as an effective screening technique (see Figure 3.3).

Immediately after being sprayed with ferrozine, wild-type colonies of *A. putrefaciens* 200, grown aerobically on solid medium produced concentric rings of varying magenta-color intensity (see Figure 3.3). As observed in screening technique No. 2, the intensity was highest for the middle cells and decreased radially until the cells of the nascent outer ring appeared nearly colorless. It was reasoned that the inner cells had been induced for high-rate iron reduction activity (possibly due to low oxygen tension at the colony center) while those cells of the outer ring were uninduced and expressed the constitutive iron reduction system. This reasoning was supported by the observation that colonies of *E. coli* HB101 (low-rate iron-reducer) produced a light magenta-coloration, and that *Samonella typhimurium* LT2 (unable to reduce iron) failed to produce magenta-colored colonies after being sprayed with ferrozine (see Figure 3.4). Screening Technique No. 3 was chosen for future use based on its high sensitivity and its potential for rapid screening of tens of thousands of potential iron-reduction-deficient mutants.

B. Mutagenesis Procedures

Mutagenesis experiments included optimization of the conditions required (90% kill) for chemical treatment of wild-type *A. putrefaciens* 200 with ethyl methane sulfonate (EMS). Survival curves (i.e., viable cell counts as a function of treatment time) are given in Figure 3.5. Those conditions included incubating 1 ml of washed cells (2×10^{10} per ml) with 10 μ L of EMS at 30°C with gentle mixing for 60 minutes. The ability of EMS to generate stable mutations in the *A. putrefaciens* 200 genome was tested by screening treated (90% kill) colonies for total auxotrophy.

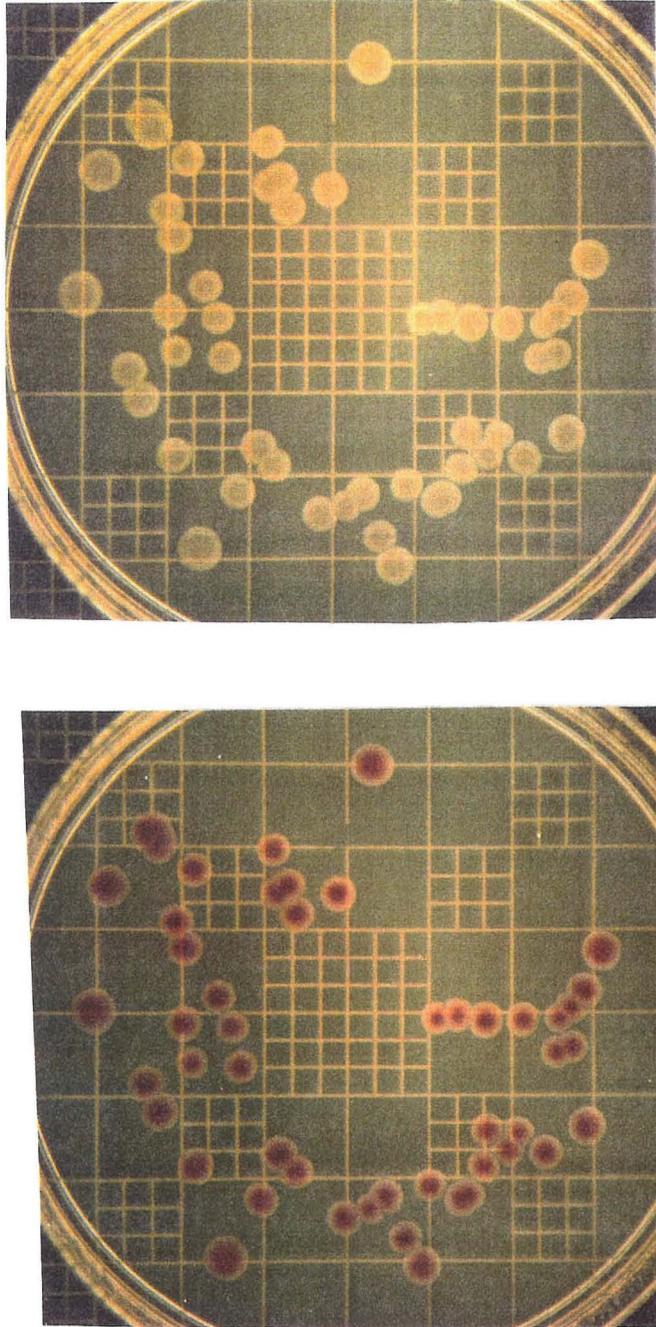


Figure 3.3. Screening Technique No. 3: *A. putrefaciens* 200 colonies before (top) and after (bottom) spraying with a fine mist of ferrozine (10 mM). Nutrient agar was supplemented with $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (to 15 mM) as Fe).

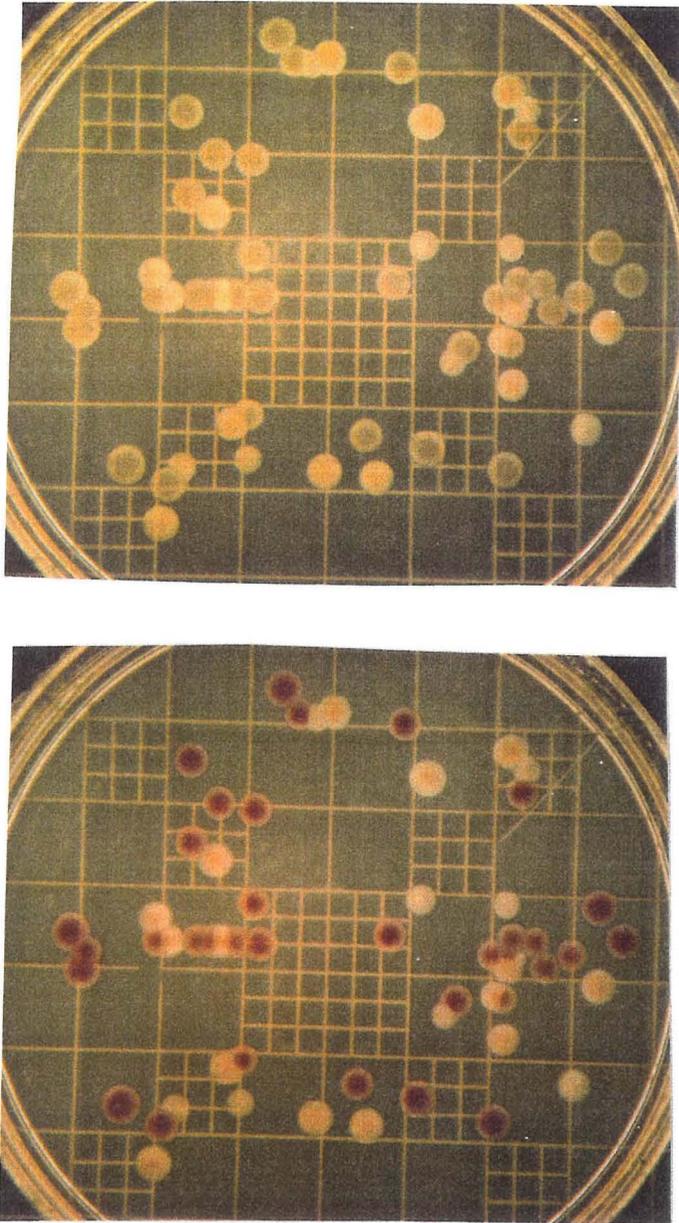


Figure 3.4. Screening Technique No. 3 applied simultaneously to a mixture of strains: *A. putrefaciens* 200 (magenta-colored), *E. coli* HB101 (light red), *S. typhimurium* LT2 (white) before (top) and after (bottom) ferrozine spray.

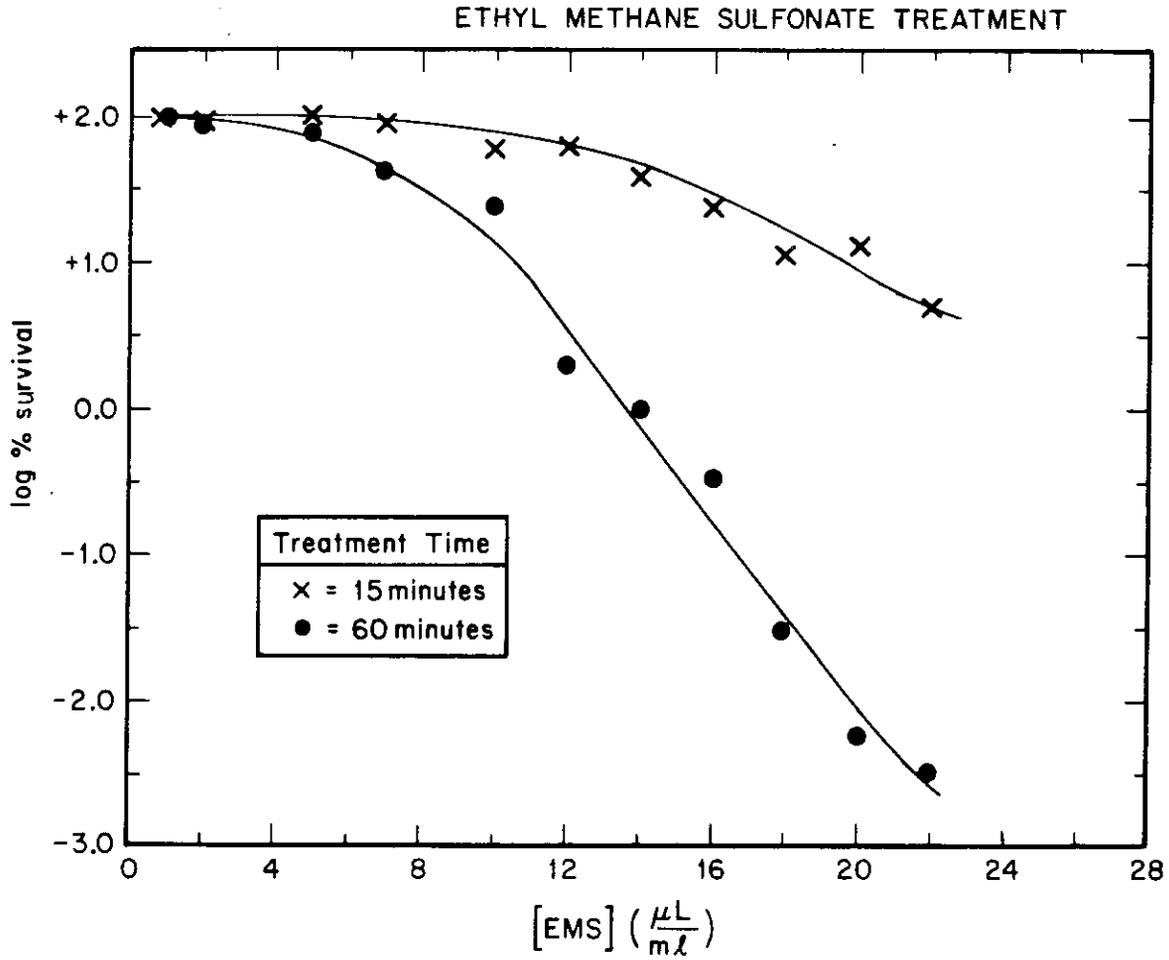


Figure 3.5. Survival curves of *Alteromonas putrefaciens* liquid cultures exposed to varying concentrations of ethyl methane sulfonate (EMS).

The frequency of total auxotrophy was calculated at 0.4% (12,000 colonies screened). Of the auxotrophs screened, 20% required either methionine or threonine for growth (data not shown). Since EMS appeared to generate stable mutations, those conditions found to be optimal for generating specific auxotrophs were again employed to generate strains of *A. putrefaciens* 200 deficient in iron reduction activity.

Screening technique No. 3 was used to screen EMS-treated cells for aberrant iron reduction activity. Of the first 12,000 colonies screened, eight showed extremely light magenta-coloration. The eight potential iron-reduction-deficient mutants were purified by a single-colony transfer to nutrient agar plates. Batch cultures of each potential mutant were tested for oxygen utilization and iron reduction activity. Four of the isolates (false negatives) were capable of iron reduction and oxygen utilization at rates nearly identical to those of the wild-type strain; however, the four other isolates (F18, F37, Wanda, Flamingo) showed extremely low iron reduction activity by the constitutive system. In addition, F37 was unable to reduce iron via the inducible (high-rate) iron reduction system. The inducible iron reduction systems of F18, Flamingo and Wanda were not affected. Mutants defective only in the inducible iron reduction system (i.e., with wild-type constitutive activity) were not detected. Both the constitutive and induced iron reduction systems of isolate F37 were capable of iron reduction at rates 4% those of the respective wild-type system. Figure 3.6 illustrates the iron-reduction-deficient phenotype of the four EMS-generated mutants. Table 3.3 lists the corresponding Fe(III) reduction rate data for each iron-reduction-deficient mutant. Speculation as to the defects in the four EMS-generated iron-reduction-deficient mutants will be discussed later after additional genetic and biochemical evidence (Chapter 5) has been presented.

Table 3.3. Oxygen Utilization and Fe(III) Reduction Rate Data for the EMS- and Tn5-generated Iron Reduction Mutants

Strain	<u>Oxygen Utilization Rate</u> ^a		<u>Fe(III) Reduction Rate</u>	
	Constitutive ^b	Induced ^c	Constitutive ^b	Induced ^c
Abiotic Control	-	-	0.01	-
WT	1.00	1.00	1.00	1.00
R ^r	1.05	0.98	1.00	0.95
EMS Mutants				
F37	0.87	0.58	0.04	0.04
Flamingo	0.89	0.95	0.05	0.82
F18	0.90	0.94	0.05	1.00
Wanda	0.89	0.95	0.14	0.85
Transposon Mutants				
T121	1.00	0.85	0.01	0.02
T176	1.00	0.92	0.01	0.01
T191	1.00	1.00	0.01	0.02
T243 (positive control)	1.05	0.92	0.90	0.95

- Notes:
- (a) All rates normalized to WT rates
 - (b) Constitutive rates: oxygen utilization = 1.40×10^{-5} M/min
iron reduction = 0.35×10^{-5} M/min
 - (c) Rate induction: oxygen utilization = 2X
iron reduction = 8X

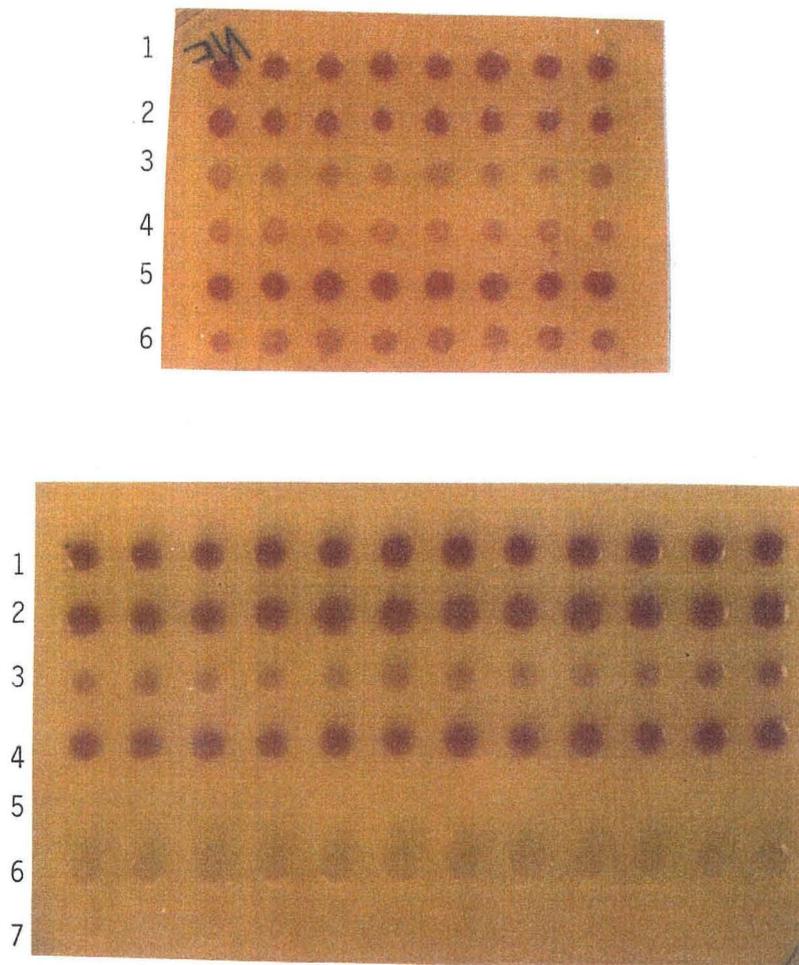


Figure 3.6. Screening Technique No. 3 applied to EMS-generated iron-reduction-deficient mutants: top (1-*A. putrefaciens* WT; 2-Rf^r; 3-F18; 4-Flamingo; 5-Banana; 6-Wanda) and bottom (1-*A. putrefaciens* WT; 2-Rf^r; 3-F37; 4-Rf^r; 5-empty; 6-*E. coli* HB101; 7-*S. typhimurium* LT2).

Experimental techniques developed for transposon mutagenesis concentrated on optimization of the frequency of transposition. Since transposition is dependent on efficient transfer of the broad-host-range plasmid (pSUP5011) harboring the transposon (Tn $\underline{5}$), preliminary experiments centered on development of an efficient conjugation system for use with the donor [*E. coli* S17-1 (pSUP5011)] and recipient [*Alteromonas putrefaciens* 200 R100] strains. Experimental procedures that optimized the conjugation frequency (see Chapter 4 for data) were as follows: Mid- to late-log phase donor and recipient cells were washed in LB, mixed in a 1:4 (donor:recipient) ratio, and immediately spotted (0.1 ml total volume) onto L plates. After an incubation period of 4 h. at 30⁰C, the spot mating was washed from the L plate with MSS and serially-diluted onto NFRK plates that selected for Tn $\underline{5}$ (Km^r) transposition and against the donor *E. coli* strain (Rf^S). Spontaneous background resistance of *A. putrefaciens* R100 to Km (50 mg/L) was $< 1 \times 10^{-10}$, and that of *E. coli* S17-1 (pSUP5011) to Rf (100 mg/L) was 2×10^{-10} . The optimum transposition frequency (i.e., frequency of Km^r *A. putrefaciens* R100 colonies) was approximately 1×10^{-4} under the conditions described above. Identical conditions (i.e., mating ratios, mating time) were used in two-way crosses (see Chapter 4) to determine the frequency of mobilization of the broad host range cloning vector pVK100 from *E. coli* S17-1 to *A. putrefaciens* R100. In this case the optimum conjugation frequency was approximately 1×10^{-2} . Since pVK100 and pSUP5011 harbor identical *mob* sites (and presumably are under identical regulatory control in *E. coli* S17-1), they should be mobilized into *A. putrefaciens* Rf100 recipient strains at identical frequencies. Thus, the frequency of transposition of Tn $\underline{5}$ into the genome of *A. putrefaciens* Rf100 (after successful mobilization of pSUP5011) is estimated to be approximately 1×10^{-2} . This value is at the upper end of the transposition frequencies reported with Tn $\underline{5}$ (Bennett, 1985). Since pSUP5011 harbors a narrow-host-range replicon (ColE1) it should be unable to replicate

outside of *E. coli*. However, there have been some reports (Y. Tsygankov, personal communication) of integration of the entire suicide plasmid into the recipient genome after transposition. To examine this problem, 25 Km^r Rf^r *A. putrefaciens* colonies were randomly picked from the NFRK selection plates and tested for chloramphenicol (50 mg/L) sensitivity. Of the 25 strains tested, two were resistant to chloramphenicol, and presumably had pSUP5011 integrated into their chromosomes. To verify the presence of pSUP5011 in the two chloramphenicol-resistant strains, a plasmid mini-prep (as described above) was done on each strain, and analyzed via agarose gel electrophoresis (see Figure 3.7). It appears as though approximately 10% of all Tn₅ mutants will inadvertently integrate the suicide plasmid pSUP5011 into the chromosome. For this reason each iron-reduction-deficient Tn₅ mutant was also checked for chloramphenicol sensitivity. Each iron reduction-deficient mutant was sensitive to chloramphenicol.

Those colonies that grew on the NFRK Tn₅-selection plates after 96 hours incubation at 30°C were sprayed with a fine mist of ferrozine (10 mM) and visually monitored for aberrant magenta-color formation. Any light magenta-colored colonies were restreaked for single colony isolation and rescreened on NFRK selection plates (see Figure 3.8). Those Tn₅ mutants showing a stable iron-reduction-deficient phenotype were saved in LB:glycerol (-80°C) for subsequent testing of both constitutive and induced iron reduction activities in batch liquid cultures. Of the 12,000 Tn₅ mutants screened in this manner, four showed extremely light magenta-color formation. Their corresponding constitutive and inducible iron reduction activities are listed in Table 3.3. Each of the four appears to have lost its ability to reduce iron via both the constitutive and inducible iron reduction systems. In addition, they appear to have an impaired ability to utilize oxygen and a significantly slower growth rate. To make certain that each of these four strains were derivatives of *A. putrefaciens* 200 and not the *E. coli* S17-1 donor

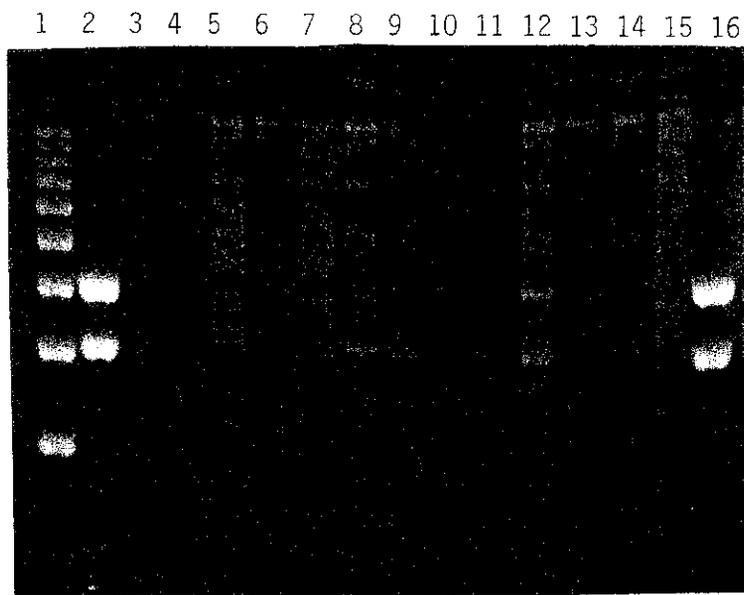


Figure 3.7. *Hind*III digested mini-preps from randomly selected Km^r (Tn5) strains of *A. putrefaciens* 200: 1-marker; 2-*E. coli* S17-1 (pSUP5011); 3-*A. putrefaciens* 200 WT; 4-open; 5-15- Km^r ; 16-*E. coli* S17-1 (pSUP5011). Lanes 8-11 are dilutions of identical prep. Note bands corresponding to pSUP5011 in lane (8-11) and lane 12.

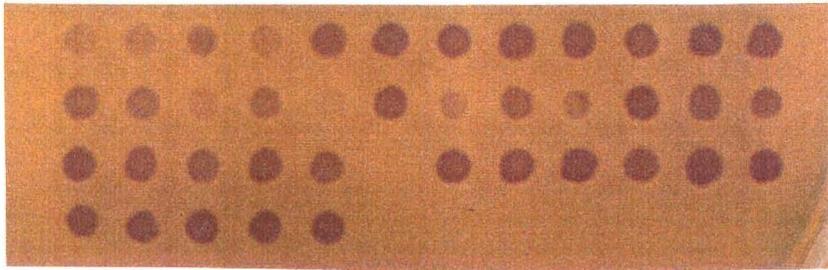


Figure 3.8. Screening Technique No. 3 applied to 40 potential iron-reduction-deficient transposon mutants (colonies are overnight cultures of Rf^r Km^r strains picked from a two-way mating between *E. coli* S17-1 (pSUP5011) and *A. putrefaciens* 200 Rf^r). T121 is located at row no. 2, column no. 5.

strain or a contaminant, each was compared to the wild-type strain for morphological differences at the colony (3X magnification) and individual cell (1000X magnification) levels. All four appeared identical to the wild-type strain. In addition, chromosomal DNA was prepared from each via the Micro-Marmur procedure (see Chapter 4), cut with the same restriction enzyme (*Hind*III), separated by agarose gel electrophoresis and compared to wild-type chromosomal DNA prepared in an identical manner. The genetic "fingerprints" of the four Tn₅ iron-reduction-deficient mutants (Figure 3.9) were identical to the wild-type *A. putrefaciens* strain, and easily distinguishable from that of *E. coli* S17-1.

IV. SUMMARY

The facultative anaerobe *Alteromonas putrefaciens* 200 was chosen as a model system for study of the process by which microorganisms mediate the dissimilative reduction of iron. *A. putrefaciens* 200 has proven to be an attractive system for such a study since it is able to reduce iron at rates an order of magnitude faster than those previously reported for other iron-reducing organisms. Earlier studies suggested that the iron reduction system of *A. putrefaciens* 200 was respiratory-chain linked and consisted of a constitutive (low-rate) and an inducible (high-rate) component. Results presented here summarize preliminary studies aimed at development of a screening technique and mutagenesis procedures for isolation of iron-reduction-deficient mutants. Chemical (ethyl methane sulfonate) and transposon (Tn₅) mutagenesis procedures were used to generate stable mutations in the *A. putrefaciens* 200 genome, as verified by testing for specific autotrophs. Mutants in both high-rate and low-rate iron reduction activity were identified via a newly developed screening technique for detection of iron-reduction-deficient mutants, using both EMS and Tn₅ mutagenesis procedures.

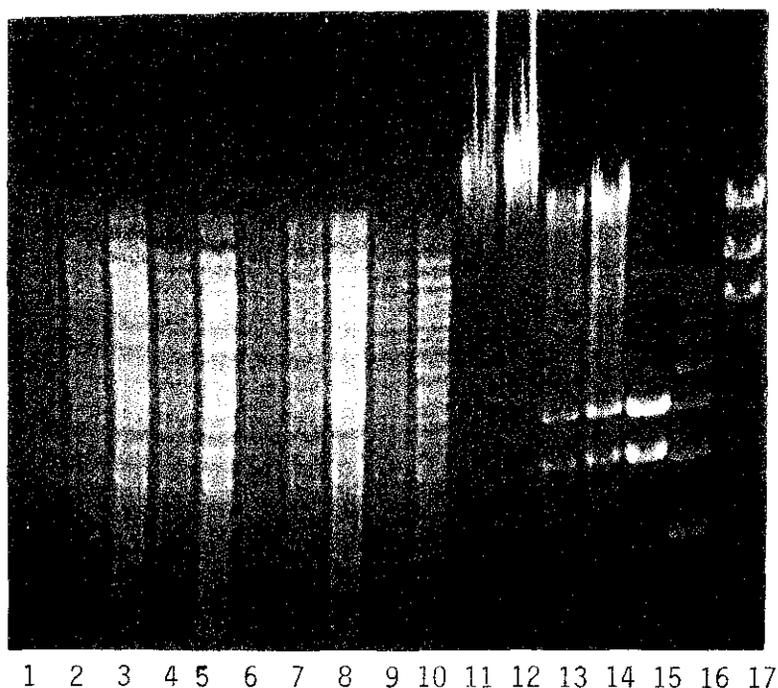


Figure 3.9. *Hind*III-digested chromosomal DNA preps (via Micro-Marmur procedure) of iron-reduction-deficient transposon mutants T121 (lanes 6-8), T176 (4-5) and T191 (1-3), *A. putrefaciens* 200 WT (9-10), *A. putrefaciens* WT uncut (11-12), *E. coli* S17-1 (pSUP5011) (13-14), and a plasmid mini-prep of *E. coli* S17-1 (pSUP5011) (15). Lanes 16 and 17 are markers.

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CHAPTER 4

**DEVELOPMENT OF A GENE TRANSFER SYSTEM AND CONSTRUCTION OF
AN ALTEROMONAS PUTREFACIENS 200 GENE CLONE BANK**

INTRODUCTION

The development of a screening technique and mutant isolation procedures was the first step in establishing a genetic system for functional analysis of the iron reduction system of *Alteromonas putrefaciens* 200. Of equal importance to the development of a working genetic system in this microorganism is the ability to transfer genes (i.e., cloned DNA fragments) into a host of choice for mutant complementation studies. Several recent advances in gene transfer technology have facilitated such genetic studies, and hence have provided a wealth of new information on gene organization and expression in bacteria other than the *Enterobacteriaceae*, many with exotic catalytic capabilities. Examples of the diverse range of bacterial functions that have been studied as a direct result of new gene transfer technology include nitrogen fixation (e.g., Ditta et al., 1980), xenobiotic biodegradation (e.g., Ramos et al., 1987), methanol oxidation (e.g., Nunn and Lidstrom, 1986), photosynthesis (e.g., Klug and Drews, 1984), and methanogenesis (e.g., Worrell et al., 1988).

In addition, genetic studies have played a key role in molecular analysis of anaerobic respiration in bacteria, although *Escherichia coli* has been used almost exclusively as the model system (e.g., Stewart and MacGregor, 1982; Green and Gennis, 1983; Rondeau et al., 1984; Jones and Gunsalus, 1987). Genetic analysis of nitrate reduction by various *Pseudomonas* spp. is one exception and has recently received attention because of its potential use in anaerobic biodegradation processes (J. M. Tiedje, personal communication). Each of these studies involved strategies similar to those described above, namely the isolation and characterization of mutants deficient in the respiratory activity of interest, followed by gene cloning experiments aimed at phenotypic restoration (complementation) of that activity. This type of complementation analysis requires that a wild-type allele be expressed (in *trans*) in a recipient mutant strain. The wild-type genes are

generally introduced as part of a hybrid cloning vector that is transferred to the recipient. The formation of partial diploid strains can result in functional expression of the cloned genes and phenotypic restoration of wild-type activity. In theory, the recircularized recombinant plasmid (i.e., vector plus insert) will complement the previously inactivated loci. Isolation and characterization of the cloned genes facilitate subsequent studies on gene organization and regulation in the original host.

The strategy used to develop gene transfer systems in gram-negative bacteria generally involves either construction of a new cloning vector derived from an indigenous plasmid, or application of a known gene transfer system to the strain under study. The latter approach is preferred for gram-negative bacteria due to the existence of broad-host-range cloning vectors. In most cases, this approach involves application of one of the three principal methods of bacterial gene transfer: generalized transduction (uptake of DNA packaged in a bacteriophage capsid), direct transformation (uptake of naked DNA from the surrounding medium), or conjugation (transfer of DNA involving cell-to-cell contact). Although other mechanisms for gene transfer do exist (e.g., capsduction in *Rhodobacter capsulatus*; Marrs, 1974; cell fusion in *Bacillus subtilis*; Schaeffer and Hotchkiss, 1978) these three methods are considered the principal routes by which bacteria exchange genetic material. Generalized transduction involves the accidental packaging of bacterial DNA (instead of phage DNA) inside a bacteriophage capsid during the lytic cycle of phage infection. Upon lysis, the hybrid phage is free to infect a new host, and does so by the same mechanism used to promote transfer of phage DNA (i.e., by first adsorbing to the host cell wall and then injecting its DNA across the cell envelope). Once the transduced bacterial DNA has entered the new host, it is either integrated into the recipient genome via homologous recombination, stably maintained as an autonomous replicon (as in the case of plasmid transduction),

totally degraded by host-specified nuclease action, or retained as a stable DNA fragment in the original transduced cell (abortive transductants; Masters, 1985). Generalized transduction is mechanistically distinct from specialized transduction, the process whereby chromosomal DNA adjacent to a specific prophage attachment site is incorporated into the DNA of a temperate phage during faulty excision from a lysogen. Because of its non-specific DNA packaging mechanism, generalized transduction has been employed as a genetic tool in a variety of bacteria including the enterics (Brooks-Low and Porter, 1978), *Rhizobium* spp. (Kondorosi and Johnston, 1981), *Staphylococci* (Lacey, 1975), and various pseudomonads (Holloway, 1979). Although the narrow host range of most *Pseudomonas* phages limits their utility during intra-species transfer (Holloway, 1979), there are reports of generalized transduction between *Pseudomonas aeruginosa* and *Pseudomonas putida* (Chakrabarty and Gunsalus, 1970). Since *Alteromonas putrefaciens* 200 was originally classified as a pseudomonad (Obuekwe, 1980), preliminary work on the development of a transduction system in this bacterium centered on identification of a *Pseudomonas* phage (either temperate or virulent) capable of infecting *Alteromonas putrefaciens* 200. Because they had been isolated from *Pseudomonas aeruginosa* and *Pseudomonas putida*, respectively, bacteriophages B3 (Holloway and Monk, 1959) and gh-1 (Lee and Boezi, 1966) were chosen for this study. Bacteriophage B3 is a temperate transducing phage of *Pseudomonas aeruginosa* and has an apparent DNA content (i.e., packageable DNA size) of 45 kb. Bacteriophage gh-1 is a virulent phage of *Pseudomonas putida* and has an apparent DNA content of 30 kb. Each phage was tested for its ability to infect wild-type *A. putrefaciens* 200. If infection was possible, a series of experiments were then conducted to determine if the phage was capable of transducing specific (auxotrophic) markers in *A. putrefaciens* 200.

Direct (or natural) transformation is generally defined as the process by which bacteria take up naked DNA from the surrounding environment and incorporate it into their genome. It is not surprising to find that bacteria have evolved a system for acquiring foreign DNA from the environment. A recent study (Paul et al., 1987) has shown that nucleic acids make up a large component of the total organic matter in aquatic environments. Although the presence of dissolved DNA is thought to benefit the indigenous microbial population primarily as a reservoir of nitrogen and phosphorous (made available by extracellular nuclease action), the ability to acquire extracellular DNA through genetic exchange with other (lysed) organisms may also aid in the process of microbial adaptation, especially in constantly changing environments such as estuarine and coastal marine ecosystems.

Although the molecular details of transformation vary between bacterial groups, the overall process is considered equivalent in all bacteria and consists of three steps: (i) binding (recognition) of the incoming DNA to the cell surface, (ii) processing of the DNA to render it competent for transfer across the cell envelope, and (iii) incorporation of the DNA into the recipient genome. The molecular basis of the transformation process, however, depends on the identity of the recipient strain and the nature of the incoming DNA. For example, classical transformation studies with the gram-positive bacterium *Bacillus subtilis* (Venema, 1979) have shown that the process is regulated by host gene functions, independent of the primary structure of the incoming DNA fragment. However, in the case of the gram-negative strain *Haemophilus influenzae* (Smith and Danner, 1981) the incoming DNA fragment must contain a rare 11 bp sequence (also found throughout the recipient genome) for recognition and uptake to occur. Due to the heightened interest in identification of suitable vectors for gene cloning, more recent work has concentrated on the development of methods for transformation

with plasmid DNA. Since plasmids can be found in a variety of topological forms (e.g., CCC, OC, linear) recipient strains have developed highly specific mechanisms for processing the plasmid before uptake and incorporation proceed. The numerous experimental protocols that have been used to transform various bacteria reflect the diversity of the bacterial transformation mechanisms. Saunders et al. (1984) give a thorough review of the various procedures used in past transformation studies, and have outlined a strategy for devising transformation protocols in bacteria with no known natural transformation systems. The strategy addresses the major factors that affect plasmid transformation frequency. Those factors include the nature of the plasmid DNA (e.g., size, concentration, required recognition sequences, topological state), the physiological state of the recipient cells (e.g., growth phase, nutritional status), the procedures used to induce artificial competency in the recipient cell (e.g., identity and concentration of divalent cations, proto(sphero)plasting, heat-pulsing), and problems with expression in the new host strain (e.g., replicon recognition, extra- and intra-cellular nuclease action, concentration of antibiotics used in selection). The major factors that were deemed important to the development of a transformation system in the gram-negative bacterium *Alteromonas putrefaciens* 200 included the nature of the plasmid (e.g., size, origin of replication), the physiological state of the recipient cells (e.g., growth phase), exposure time and temperature, selection media, and the presence of extracellular nucleases. Each of these factors was examined in an array of experiments (see Materials and Methods section) designed to promote uptake of previously selected plasmid cloning vectors (pVK100 and pSP329). The plasmids were chosen based on their size and ability to replicate in a broad range of gram-negative bacteria (see below). *Alteromonas putrefaciens* 200 is also known to produce copious quantities of an extracellular DNase (Obuekwe, 1980), and this may be an important factor in development of an efficient transformation system.

This problem may be overcome by isolating mutants deficient in extracellular nuclease production. Such a strategy may be applicable with *Alteromonas putrefaciens* 200.

The most recent advances in gene transfer technology have been made in the area of bacterial conjugation. Conjugation is the process whereby bacteria exchange genetic material via direct contact between donor and recipient cells. In most gram-negative bacteria, self-transmissible plasmids mediate the conjugation process and in some instances, also possess the ability to mobilize chromosomal DNA across species lines (Holloway, 1979; Barth, 1979), thereby providing the impetus for development of broad host range cloning systems. Development of such systems in gram-negative bacteria has focused on manipulation of the self-transmissible plasmids that possess the ability to replicate and be stably maintained in a wide range of bacteria. This ability is particularly attractive when developing a cloning system in bacteria with no known gene transfer capability (Lidstrom, 1989).

Broad host range plasmids have been placed into incompatibility groups based on their inherent replication and partitioning functions. Several recent reviews (e.g., Schmidhauser et al., 1987; Mermod et al., 1986; Lidstrom, 1989) have outlined the important features and uses of broad host range plasmids in gram-negative bacteria. Of the five classes of broad host range plasmids isolated from gram-negative bacteria (IncC, N, P, Q and W), three (IncP, Q and W) have been used in construction of broad host range cloning vehicles. The basic strategy employed in construction of a broad host range cloning vector involves separating the loci required for conjugal transfer (designated as *tra* functions) from those required for replication and maintenance. In this manner, a high level of biological containment is achieved; the cloning vector contains only replication and maintenance functions (i.e., is not self-transmissible) but may be mobilized into an appropriate recipient strain by providing it with a second "helper" plasmid that

appropriate recipient strain by providing it with a second "helper" plasmid that contains the complementary *tra* functions. The procedure normally involves a three-way mating between *E. coli* donor (harboring the cloning vector), *E. coli* helper (harboring the "helper" plasmid) and recipient strains. Such systems have been derived from the self-transmissible parent plasmid of each of the IncP, W and S incompatibility groups. Cloning vectors derived from the self-transmissible IncP plasmids appear particularly attractive, largely due to their ability to replicate and be stably maintained in most gram-negative bacteria.

The IncP plasmids have been subdivided into three main groups (IncP1, IncP2, and "others") based on heteroduplex analysis and by the antibiotic resistance conferred by each (Yakobson and Guiney, 1983). The IncP1 plasmids were all isolated from clinical specimens taken from the same hospital (Birmingham, UK) in 1969 (Holloway et al., 1979). It is interesting to note that IncP plasmids were not found in clinical isolates before the medical use of antibiotics became a widespread practice (Datta and Hughes, 1983). Except for only minor variations in their primary structure, the five members of the IncP1 subclass (RK2, RP4, RP1, R18, R68) are indistinguishable. They each confer resistance to kanamycin, tetracycline and ampicillin, are identical by both restriction map (Currier and Morgan, 1981) and hybridization analysis (Burkardt et al., 1979), and are approximately 60 kb in size. Analysis of the physical and genetic map of RK2 (see Figure 4.1) reveals that the genetic loci encoding functions required for either antibiotic resistance, conjugal transfer, replication, or maintenance and partitioning are scattered throughout the plasmid. Molecular analysis of each of these genetic regions has been the subject of several recent studies (e.g., Bechhofer and Figurski, 1983; Bechhofer et al., 1986; Cross et al., 1986; Pohlman and Figurski, 1983; Merryweather et al., 1986; Lanka and Barth, 1981; Schmidhauser and Helinski, 1985) and definitively reviewed by Thomas and Smith (1987). Conjugal transfer of RK2 requires essentially three

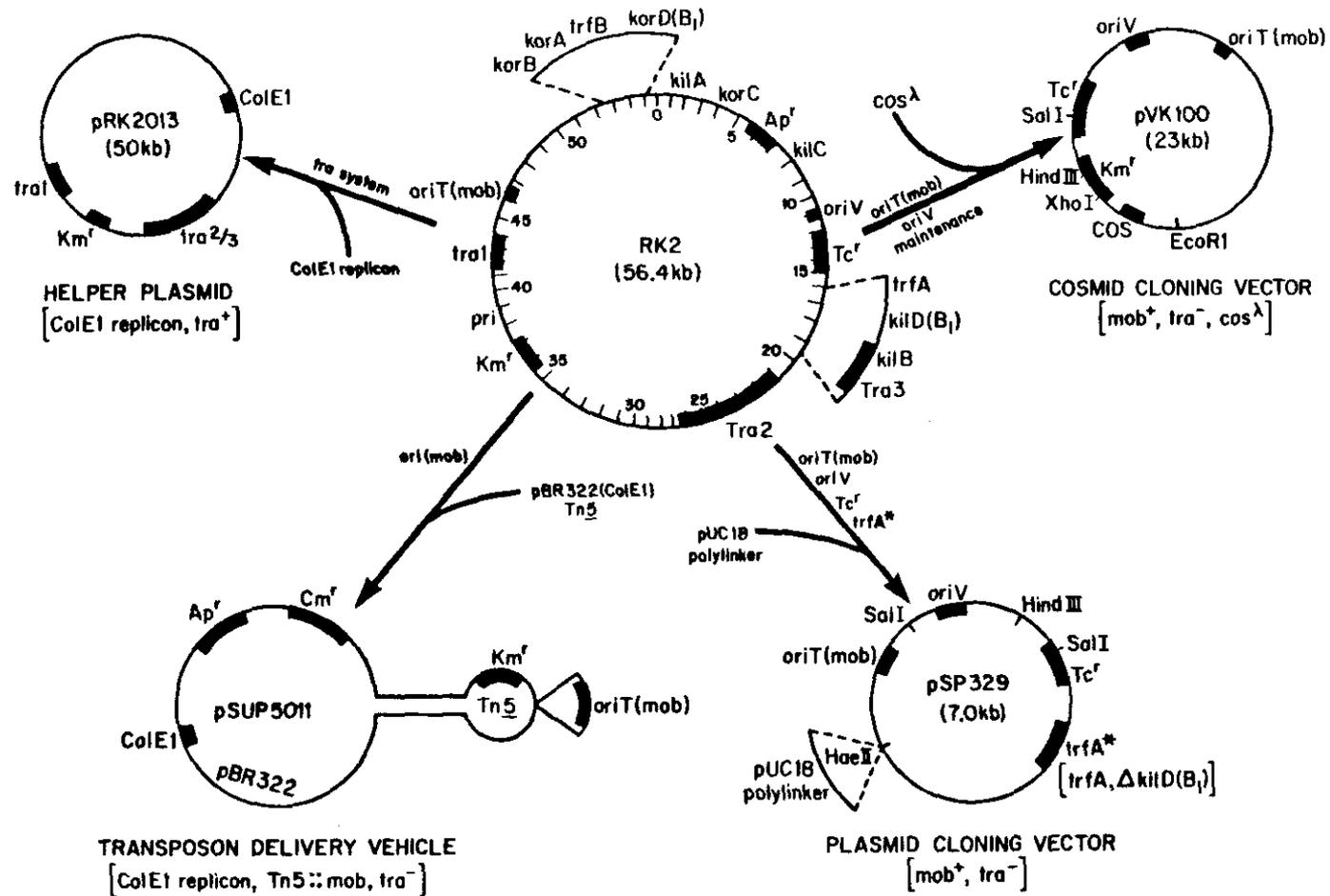


Figure 4.1. Structure, relevant genetic loci and nuclease cleavage sites of IncP1 plasmid RK2 and the RK2-based plasmids used in this study (relative positions on the RK2 map correspond to the distance (kb) from a unique *EcoRI* site at position 0; RK2 derivatives not drawn to scale). The RK2 derivatives included pVK100 (cosmid cloning vector), pSP329 (plasmid cloning vector), pRK2013 (helper plasmid) and pSUP5011 (*Tn5* delivery vehicle).

regions: a *cis*-acting origin of conjugal transfer (*oriT*, also designated *rlx*, *nic*, *mob*), transfer genes (*tra1*, *tra2/3*) encoding the structural components required for assembly of the sex pilus, and *pri* whose gene product acts as a DNA primase facilitating single-stranded DNA synthesis at *oriT* (mutations in the *pri* locus decrease the frequency of RK2 conjugal transfer). Only two loci are required for replication: the origin of vegetative replication (*oriV*), a 393 bp sequence (immediately upstream from a *dnaA* consensus binding site) that consists of five-17 bp tandem repeats capable of binding the gene product of the second locus required for replication, *trfA*. The *trfA* gene product (*trans*-acting replication function) is itself a DNA binding protein (consisting of two polypeptides in a α -helix-turn α -helix structural motif) that promotes DNA polymerase A binding at *oriV*. Expression of the *trfA* gene product is regulated (and hence the fundamental level of conjugative control) by the *trfB* operon, a set of genes that are also required for efficient maintenance and partitioning of RK2. The *trfB* operon consists of the *kor* (*kill over ride*) genes *A*, *B*, *C*, and *D* whose gene products control expression of their respective *kil* gene complements, (*A*, *B*, *C*, and *D*). The *kil* genes encode membrane proteins essential for efficient partitioning of RK2 to daughter cells during cell division. It is thought that overproduction of the *kil* gene products disrupts the integrity of the cytoplasmic membrane and is therefore lethal to the cell.

The basic strategy employed in construction of broad host range cloning vectors derived from RK2 has entailed separation and either deletion or mutation of the four genetic regions described above. In addition to harboring the necessary replication and maintenance functions, most cloning vectors also encode several selectable markers, one of which can be inactivated by insertion of a DNA fragment at a unique restriction site contained within that locus (i.e., allows for detection of hybrid vectors). Cloning vectors also generally possess a strong promoter(s) that

can actively transcribe cloned DNA. The first broad host range cloning vector derived from RK2 (designated pRK290) was originally constructed for use with *Rhizobium meliloti* in studies on bacteria-plant symbioses (Ditta et al., 1980). A *Rhizobium* gene clone bank was generated by directly transforming an appropriate *E. coli* strain with pRK290 containing random fragments of the *R. meliloti* genome. It is generally found, however, that the relatively large size (approximately 20 kb) of vectors such as pRK290 limits their allowed insert size during direct transformation experiments. Prohibitively low transformation frequencies (nearly identical to the frequency of background spontaneous marker expression) are generally the case for plasmids > 20 kb (Saunders et al., 1984).

Since it is advantageous to clone the largest DNA fragment possible (thereby minimizing the number of clones in the gene bank), gene cloning studies can be limited by the size of the cloning vector. Prohibitively low transformation frequencies have been overcome by constructing smaller cloning vectors (e.g., deletion derivatives of RK2) or by using broad host range cosmid vectors. Schmidhauser et al. (1983) identified the minimal RK2 replicon (pTJS175) required for conjugal transfer (*oriT*) and replication (*oriV*) in the broad host range of the parent plasmid. Stable maintenance of pTJS175 in the broad host range of RK2 also required the presence of the *trfA* region (coordinates 12.0 to 20.0 kb; see Figure 4.1) and the *korA-korB* fragment (coordinates 52.5 to 56.0) of RK2. A mini-RK2 replicon (pRK252) lacking the *korA-korB* region was unstable in nearly all the gram-negative strains tested (*Azotobacter* spp. being the sole exception). This finding was attributed to the absence of the *kor* functions required for regulation of *kil* gene expression from the *trfA* operon. Not surprisingly, when the *trfA* promoter and the *kilD* (B_1) regions were deleted (resulting in mini-RK2 plasmid pTJS75), the *korA* and *korB* loci were not required for stable maintenance. Mini-RK2 replicon pSP329 (see Figure 4.1) was constructed by ligation of a pUC18 polylinker

(containing additional cloning sites) to pTJS75. Based on its small size (7.0 kb) and ability to replicate and be stably maintained in a wide range of gram-negative bacteria, mini-RK2 replicon pSP329 was chosen as a potential cloning vector for development of a gene transfer system in *Alteromonas putrefaciens* 200.

The second strategy used to overcome prohibitively low transformation frequencies has involved construction of broad host range cosmid cloning vectors derived from the parent plasmid RK2. Cosmids, possessing the *cos* locus (cohesive ends) of phage lambda are packaged *in vitro* in lambda phage particles only if they have received an insert of appropriate size (Hohn, 1979). Phage lambda will package any DNA fragment containing the *cos* locus as long as the fragment is from 75 to 105% of its native length (i.e., 38 to 55 kb). Thus, a 23 kb plasmid that contains a *cos* locus will be packaged by phage lambda only after receiving an insert of approximately 20 kb in length. Broad host range cosmids have been constructed by inserting the lambda *cos* locus into broad host range plasmids. Knauf and Nester (1982) constructed the broad host range cosmid pVK100 by inserting the *cos* locus into a mini-RK2 replicon (see Figure 4.1). The resulting vector (23 kb in length) can be used to clone relatively large DNA fragments (between 20-30 kb). Transfer of the hybrid vector to an appropriate *E. coli* strain (via lambda-mediated transfection) allows for permanent storage of the DNA fragments in a gene clone bank. The clone bank can then be mobilized into the host strain of choice via the mating procedures described above. Based on its broad host range features and ability to carry large fragments of cloned DNA, cosmid pVK100 was selected as a potential cloning vector for development of a conjugation system in *Alteromonas putrefaciens* 200.

The broad host range cloning vectors derived from the IncP1 plasmid group are mobilized into the host range of RK2 by "helper" plasmids in which the RK2 transfer system has been joined to a narrow host range replicon. Figurski and

Helinski (1979) constructed the most widely used "helper" plasmid (pRK2013) by ligating the RK2 transfer system to the ColE1 replicon pBR322 (see Figure 4.1). "Helper" plasmid pRK2013 is thought to provide the conjugation machinery (in *trans*) required for efficient transfer of mobilization-proficient (mob^+), transfer-deficient (tra^-) cloning vectors such as pSP329 and pVK100. Because it harbors a ColE1 replicon, pRK2013 can not replicate in host strains other than *E. coli*. A similar strategy has been used in the development of broad host range transposon delivery vehicles: mobilization-proficient (mob^+), transfer-deficient (tra^-) plasmids containing an appropriate transposon have been joined to ColE1 replicons thereby providing a broad host range mobilization system for transposon mutagenesis in a wide range of gram-negative bacteria (see Chapter 3 for further discussion).

Simon and co-workers (1983) have also developed a cloning system that eliminates the need of a "helper" strain (harboring a "helper" plasmid such as pRK2013) during tri-parental matings. By integrating the entire RK2 plasmid into the chromosome of *E. coli* 294 (via P1vir transduction), they succeeded in constructing a broad host range mobilizing strain that carries the IncP1 transfer system integrated in its chromosome. RK2-encoded resistances to tetracycline and kanamycin (see Figure 4.1) were eliminated by Mu-phage and Tn7 insertion, respectively. The resulting strain (designated *E. coli* S17-1) is also deficient in host-specified restriction thereby adding to its attractiveness as an intermediate host for gene bank storage. Since it can mobilize IncP1-based (mob^+) cloning vectors without the aid of a "helper" strain, mobilization of a gene clone bank stored in S17-1 simply requires a two-way cross between S17-1 and the selected recipient. The broad host range mobilizing strain *E. coli* S17-1 was chosen as the host strain for permanent storage of an *Alteromonas putrefaciens* 200 gene clone bank.

A major portion of the work described in this chapter is devoted to the development of a gene cloning system in *Alteromonas putrefaciens*. With the facility

to transfer cloned DNA into mutant strains, complementation studies of the iron reduction system can proceed. The approach taken in development of a gene transfer system in *Alteromonas putrefaciens* has not been to construct new cloning vectors derived from indigenous plasmids, but instead has been aimed at capitalizing on the broad host range capability of plasmid cloning vectors derived from the IncP1 incompatibility group. Gene transfer systems based on the IncP1 plasmids have been shown to facilitate gene transfer in most gram-negative bacteria and, hence, hold considerable promise for development of a genetic system in *Alteromonas putrefaciens*.

II. MATERIALS AND METHODS

A. Strains, Media, and Growth Conditions

The strains, plasmids and bacteriophage used in this study are listed in Table 4.1. A rifamycin-resistant strain of *Alteromonas putrefaciens* 200 was isolated by plating approximately 10^9 cells on nutrient agar supplemented with rifamycin SV (Sigma; 100 mg/L). *Alteromonas putrefaciens* R^{rf} was purified by two single-colony transfers and saved in LB:glycerol (85:15 vol %) at -80°C.

Table 4.1. Strains, Plasmids and Bacteriophage Used in This Study

Strain	Relevant Characteristics	Source
<i>Alteromonas putrefaciens</i>		
Strain 200	Wild-type, high-rate iron reducer	D.W.S. Westlake
Strain 200, R ^{rf}	Rifamycin-resistant	This study
<i>Serratia marcescens</i>	Wild-type, produces extracellular DNase	P. Arps
<i>Pseudomonas aeruginosa</i> ATCC 15692	Transduced by bacteriophage B3	ATCC
<i>Pseudomonas putida</i> ATCC 17453	Transduced by bacteriophage gh-1	ATCC

Table 4.1 (continued)

Escherichia coli

HB101	<i>recA</i> , <i>hsdR</i> , <i>hsdM</i>	M. Lidstrom
HB101 (pVK100)	HB101 with pVK100	Knauf and Nester
HB101 (pSP329)	HB101 with pSP329	This study
CSR603 (pRK2013)	<i>recA</i> , with pRK2013	D. Bartlett
S17-1	<i>recA</i> , chromosomally-integrated IncP1 transfer system	R. Hanson
S17-1 (pVK100)	S17-1 with pVK100	This study
S17-1 (pSUP5011)	S17-1 with pSUP5011	A. Puhler
Plasmids	Relevant Characteristics	Source
pVK100	Cosmid cloning vector [Mob ⁺ , <i>cos</i> , Tc ^r , Km ^r]	Knauf and Nester
pRK2013	Helper plasmid [ColE1 replicon, Tra ⁺ , Km ^r]	D. Bartlett
pSUP5011	Transposon delivery vehicle [ColE1 replicon, Mob ⁺ , Tn5, Km ^r]	A. Puhler
pSP329	Mini-RK2 replicon [Mob ⁺ , Tc ^r]	T. Schmidhauser
Bacteriophage	Relevant Characteristics	Source
B3	Temperate transducing phage of <i>P. aeruginosa</i>	ATCC
gh-1	Virulent phage of <i>P. putida</i>	ATCC

A. putrefaciens strains were grown at 30°C on lactate medium (pH 7) as described by Obuekwe (1980) [per liter: 0.5 g K₂HPO₄, 2.0 g Na₂SO₄, 1.0 g NH₄Cl, 0.15 g MgSO₄·7H₂O, 0.5 g yeast extract, 3 ml 60% sodium lactate syrup] except during mating experiments when L broth (Miller, 1972) was used.

Escherichia coli strains were grown at 37°C in L broth. *Pseudomonas aeruginosa* (ATCC 15692) and *Pseudomonas putida* (ATCC 17453) strains were grown in liquid media as described by Holloway (1962) and Lee (1966), respectively. MSS (minimal salt solution) was essentially lactate medium minus yeast extract and lactate.

Antibiotics were added to both liquid and solid media at the following final concentrations (mg/L): rifamycin SV (100), tetracycline (10), kanamycin (50), chloramphenicol (50), streptomycin (50). When required for iron reduction screening procedures, filter-sterilized FeCl₃·6H₂O was added to nutrient agar to a final concentration of 0.15 mM (as Fe).

B. Gene Transfer Techniques

1. Generalized Transduction

The layer agar technique of Holloway and Fargie (1960) was used to prepare both the phage lysate and titers of B3 and gh-1. Phage stocks were stored at 4°C until needed. To test the ability of each phage to infect wild-type *A. putrefaciens* 200, two different techniques were employed. The first technique is based on the liquid culture method described by Miller (1972): a single plaque was picked with a Pasteur pipette from each of the B3 and gh-1 phage titers and resuspended in 0.3 ml saline buffer (Holloway and Fargie, 1960). The phage suspension was then mixed with 0.3 ml of an *A. putrefaciens* 200 overnight that was grown in lactate medium and washed and resuspended in equal volume saline buffer. The mixtures were allowed to incubate at 30°C for 10 minutes, then transferred to a 250 ml flask containing 30 ml of lactate medium, and incubated for 6 h. with vigorous shaking. OD₆₀₀ measurements were taken every 30 minutes to monitor cell growth and the

onset of cell lysis indicative of phage infection. As a control, each phage was tested on its host strain via identical procedures.

The second technique used to test the ability of phages B3 and gh-1 to infect *A. putrefaciens* 200 entailed cross-streaking the respective phages with an inoculum of wild-type cells. An aliquot (0.1 ml) of each phage lysate (approximately 10^{10} pfu/ml) was seeded in a straight line across a nutrient agar plate. A loopful of a fresh colony of *A. putrefaciens* 200 was then streaked across the phage and the plates incubated at 30°C. The cross-streak was examined periodically for three days to determine if the phage were capable of infecting *A. putrefaciens* 200. Control experiments were run by cross-streaking phages B3 and gh-1 on both *Pseudomonas aeruginosa* and *Pseudomonas putida*.

2. Direct Transformation

Figure 4.2 outlines the strategy used to determine if cloning vectors pSP329 and pVK100 were capable of directly transforming wild-type *A. putrefaciens* 200. Procedures followed for large scale preps of pSP329 (in *E. coli* HB101) and pVK100 (in *E. coli* HB101) were essentially those of Maniatis et al. (1982). Two rounds of CsCl-banding preceded the final phenol/chloroform extraction. Mid-log phase cells of *A. putrefaciens* (500 ml) were harvested, washed in equal volume MSS, resuspended in 500 ml cold 10 mM MgSO₄ and held on ice for 15 minutes. The cells were then dispensed into 12 separate aliquots of 30 ml each, harvested a second time, and resuspended in 15 ml of cold CaCl₂ buffer. The concentration of Ca²⁺ in aliquots 1-6 was 50 mM and in aliquots 6-12 was 100 mM. After 30 minutes on ice, the cells were again harvested and resuspended in 3 ml cold CaCl₂ buffer (50 mM or 100 mM). Competent cells (0.2 ml) from each aliquot were mixed with plasmid DNA (500 ng in 0.1 ml T₁₀E₁ buffer) and held on ice for 15 minutes. Mixtures 5, 6, 11 and 12 were then heat-pulsed for 10 minutes at 42°C. Mixtures 3, 4, 9 and 10 were heat pulsed for 2 minutes at the same temperature,

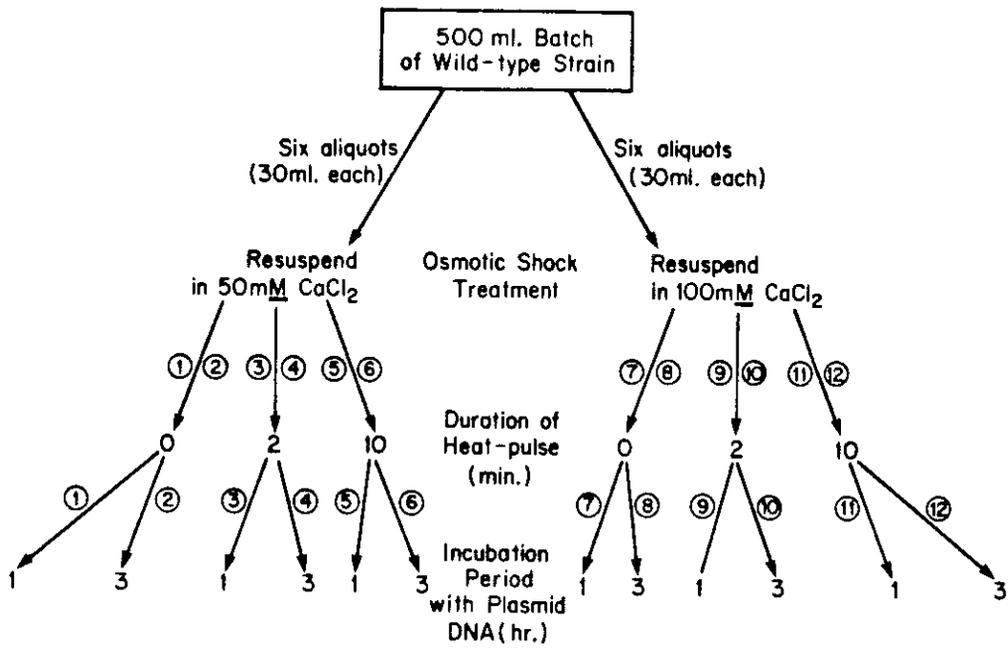


Figure 4.2. Overall strategy used in direct transformation experiments with *Alteromonas putrefaciens* 200.

while mixtures 1, 2, 7 and 8 received no heat treatment. After heat pulsing, the mixtures were allowed to cool at room temperature for 10 minutes before adding 3 ml of lactate medium to each. Mixtures 1, 3, 5, 7, 9 and 11 were then incubated with vigorous shaking for 90 minutes at 30°C, while mixtures 2, 4, 6, 8, 10 and 12 were incubated under the same conditions for 3 hours. Control experiments were run using cells only (i.e., no plasmid DNA). A second set of control experiments were run using *E. coli* strain HB101 as the recipient. Serial dilutions of each mixture were plated on nutrient agar supplemented with 5 mg/L tetracycline.

To circumvent the problem of extracellular nuclease production by *A. putrefaciens* 200 during transformation experiments, a series of mutagenesis experiments were run to generate an *A. putrefaciens* mutant deficient in extracellular DNase activity. Mutagenesis procedures employed in generation of DNase-deficient mutants were essentially identical to those used in previous experiments to generate iron-reduction-deficient mutants of *A. putrefaciens* 200 (see Chapter 3). Ethyl methane sulfonate (EMS) was again employed as the chemical mutagen. However, instead of plating EMS-treated liquid cultures on nutrient agar (supplemented with ferric iron), the mutagenized cells were plated on DNase Test Agar (Difco) supplemented with methyl green (0.01%). Approximately 12,000 EMS-treated colonies (i.e., those surviving 90% kill treatment) were screened on this medium. Those colonies that did not produce a clearing zone were picked directly from the plate and rescreened on identical medium to ensure stability of the DNase-deficient phenotype.

3. Conjugative Gene Transfer Systems

Mobilization of the broad host range cloning vectors pSP329 and pVK100 into *A. putrefaciens* 200 was attempted in three-way crosses between *E. coli* CSR603 (pRK2013) mobilizer, *E. coli* HB101 (pVK100 or pSP329) donor and *A. putrefaciens* 200 Rf^r recipient strains. Procedures used to transform *E. coli* strain HB101 with

pSP329 and pVK100 were identical to the direct transformation procedures described in the previous section. Mid-log phase liquid cultures of donor, mobilizer and recipient strains were washed in LB, mixed in various ratios (1:1:1, 1:1:4, 1:1:10) spotted (0.1 ml total volume) onto nutrient agar, and incubated at 30°C over a range of mating times (1 h. to 24 h.). After the mating period, the cells were washed from the mating plate with MSS and serially diluted onto selective medium. The selective medium consisted of nutrient agar supplemented with rifamycin SV (100 mg/L) and tetracycline (10 mg/L). Transconjugates were visible after 3 days growth at 30°C and were purified by two single-colony transfers on selective media.

Mobilization of cloning vectors pVK100 and pSP329 into *A. putrefaciens* was also attempted via two-way crosses between an *E. coli* mobilizing strain (S17-1 harboring either pVK100 or pSP329) and an *A. putrefaciens* 200 Rf^f recipient. Mid-log phase liquid cultures of the *E. coli* mobilizing donor and *A. putrefaciens* recipient strains were washed in LB, mixed in various ratios (1:1, 1:4, 1:10), spotted (0.1 ml total volume) onto nutrient agar, and incubated at 30°C over a range of mating times (1 h to 24 h). After the mating period, the cells were washed from the mating plate with MSS and serially diluted onto selective media (nutrient agar supplemented with rifamycin SV (100 mg/L) and tetracycline (10 mg/L). Colonies that appeared after 3 days were purified by two single-colony transfers to selective media. The frequencies of background (spontaneous) resistance of each *E. coli* strain and of *A. putrefaciens* 200 Rf^f to rifamycin SV and tetracycline were calculated by plating serial dilutions of each on nutrient agar supplemented with rifamycin SV (100 mg/L) and tetracycline (10 mg/L).

To ensure that each plasmid had been stably maintained in all transconjugates, a plasmid mini-prep was performed on a random sample of 15 colonies from each mating. Overnight cultures (1.5 ml) of each potential transconjugate were washed and resuspended in 0.15 ml SET buffer (20% sucrose,

50 mM Tris pH 7.6, 50 mM EDTA). RNaseA (20 μ l of a 10 mg/ml stock in 10 mM NaAc, pH 4.5) and lysozyme (50 μ l of a 5 mg/ml stock in SET buffer) solutions were added and the mixture was incubated at room temperature for 5 minutes. Approximately 0.35 ml of lytic mix (10% SDS, 10 N NaOH) was then added and the mixture placed on ice for 10 minutes. Addition of an equal volume of sodium acetate (3 M) was followed by a 60 minute incubation period (on ice) to precipitate the SDS and chromosomal DNA. After centrifuging (14,000xg for 10 min. at 4°C) an equal volume of isopropanol was added to the supernatant and the plasmid DNA was allowed to precipitate at room temperature for 5 minutes. After centrifugation, the pellet was washed with 1 ml of 70% ethanol and dissolved in 20 μ L T₁₀ (pH 7.5) buffer. The mini-prep was subsequently digested with *Hind*III and its presence verified via agarose gel (0.8%) electrophoresis.

C. Construction of an *Alteromonas putrefaciens* 200 Gene Clone Bank

Figure 4.3 outlines the overall strategy followed in construction of an *Alteromonas putrefaciens* 200 gene clone bank. A modified version (Ann Wopat, Univ. of Washington) of the Marmur (1961) procedure for DNA isolation was used to isolate total DNA from a 50 ml liquid culture of wild-type *A. putrefaciens* 200 grown to early stationary phase in lactate medium. The final pellet was dissolved in T₁₀E_{0.5} buffer and stored at 4°C until needed. DNA fragments in the 15-20 kb size range were obtained by size fractionation of a *Hind*III partial digest of the wild-type DNA preparation. Conditions required for optimum size fractionation in the 15-20 kb size range were determined by digesting (20 μ L total volume) 10 μ g of chromosomal DNA with 10 units of *Hind*III (New England Bio Labs) at 37°C over a range of incubation times (1 to 60 minutes). In each digestion, *Hind*III was added to pre-warmed (37°C) digestion mix (containing distilled H₂O, digestion buffer and *A. putrefaciens* DNA) and the tubes were immediately immersed in a 37°C water bath. Each reaction was stopped by adding 1 μ L of 0.2 M EDTA and the reaction

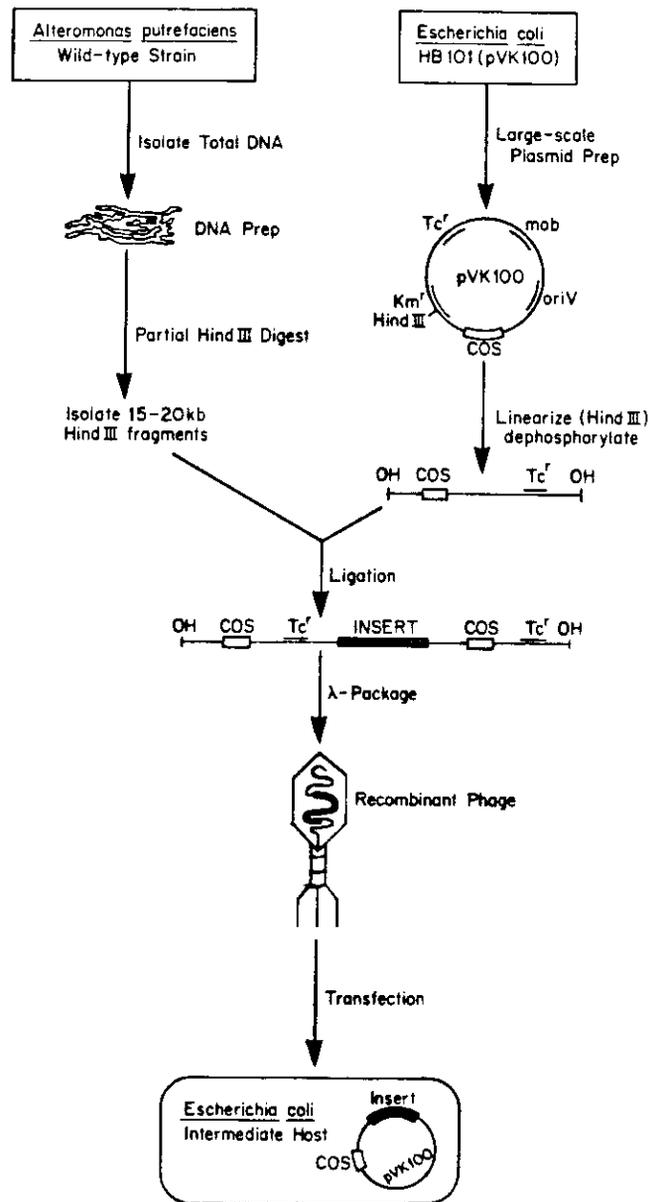


Figure 4.3. Schematic of overall strategy used in construction of an *Alteromonas putrefaciens* 200 gene clone bank. *E. coli* strains HB101 and S17-1 were used as intermediate hosts.

mix was then placed on ice. Aliquots from the time series were analyzed via agarose gel (0.8%) electrophoresis. To scale-up the total amount of DNA digested (to 0.10 mg) an identical set of experiments was run using 10X the amount of each digestion mix component. A small aliquot of each large scale digest was analyzed via agarose gel (0.8%) electrophoresis to ensure that the entire 15-20 kb size range was covered by the time series. The large-scale partial digests were then combined (approximately 0.9 mg total DNA) and size fractionated via agarose gel (0.8%) electrophoresis. The bands in the gel corresponding to the 15-20 kb size range were sliced out and placed in an electro-separation chamber (Elutrap; Schleicher and Schuell, Inc.). The 15-20 kb DNA fragments were then electroluted from the gel slice, extracted once with an equal volume of phenol/chloroform, once with an equal volume of chloroform, and were precipitated with 0.1 volume sodium acetate (3 M). The DNA was then washed in 3 volumes cold ethanol and resuspended in T₁₀ buffer. The dissolved DNA was immediately heated to 65°C for 15 minutes to inactivate any residual DNase activity. A small aliquot was run on an agarose gel to check for fragment size and yield.

The procedure of Birnboim and Doly (1979) as described by Maniatis et al. (1982) was used for large-scale preparation of the cosmid cloning vector pVK100 (in *E. coli* HB101). The plasmid prep was CsCl-banded two times before extracting the ethidium bromide with isopropanol (saturated with 20 X SSC) and dialyzing for 6 hours against two changes of T₁₀E₁ buffer. To ensure the identity of the purified plasmid, a diagnostic check was performed by double digestion with *SalI-XhoI* and *HindIII-EcoRI*. The appropriate bands were visualized via agarose gel electrophoresis. Approximately 100 µg of the purified cosmid was digested with *HindIII*, extracted once with phenol/chloroform and precipitated as described previously. Residual DNase activity was inactivated by heating the resuspended pellet (T₁₀ buffer) at 65°C for 15 minutes. The linearized cosmid was subsequently

dephosphorylated by treating 75 μg cosmid DNA with 1 unit calf intestinal phosphatase (Boehringer Mannheim) for 30 min. at 37°C. A single phenol/chloroform extraction and precipitation step again preceded heat treatment at 65°C for 15 minutes. After each treatment step (linearization and dephosphorylation), an aliquot (2 μg) of the cosmid prep was loaded on an agarose gel to check for plasmid size and yield. The linearized, dephosphorylated cosmid was resuspended in T₁₀ buffer and stored at 4°C until needed.

T4 DNA ligase (Boehringer Mannheim) was used to ligate the 15-20 kb fragments of *A. putrefaciens* 200 DNA to linearized, dephosphorylated pVK100. Insert and vector DNA were mixed in a 5:1 (insert:vector) ratio, heated at 65°C for 15 minutes and allowed to cool at room temperature for 30 minutes. Ligation buffer and T4 DNA ligase were then added (10 μL total reaction volume) and the mixture was incubated at 16°C overnight. An aliquot (1 μL) of the ligation mix was run on an agarose gel to check for concatamer size and yield.

A commercially available DNA packaging kit (Boehringer Mannheim) was used to package 0.5 μg of the ligation mix into lambda-phage particles. Wild-type lambda DNA was packaged as a positive control. The recombinant phage were stored at 4°C until needed. *E. coli* strains HB101 and S17-1 were chosen as the intermediate hosts for gene bank storage. Each strain was prepared for transfection by resuspending an early-stationary phase liquid culture (10 ml) in 0.4 vol MgSO₄ (10 mM) and incubating (with vigorous shaking) at 32°C for 45 minutes. The transfection-competent cells were stored at 4°C until needed. Two separate clone banks were generated by transfecting each host strain with the recombinant lambda phage. A mixture of 100 μL cells and 50 μL phage prep was incubated at 37°C for 20 minutes (no shaking), followed by the addition of 0.65 ml LB. The mixture was then incubated (with vigorous shaking) at 37°C for 90 minutes. The positive lambda control (i.e., the packaging of wild-type lambda DNA) was also carried through the

same procedure and checked via an appropriate lambda phage titer. After the 90 minute incubation period, appropriate serial dilutions of the transfected cells were plated on selective media [i.e., L agar supplemented with 20 mg/L tetracycline (HB101) or 50 mg/L streptomycin and 20 mg/L tetracycline (S17-1)] and incubated at 37°C for 36 hours. Approximately 4,000 recombinant colonies were picked from each of the two clone banks and permanently stored via two methods: the first method entailed picking approximately 4,000 colonies from the original selection plates of each clone bank and transferring them individually to a 96-well tissue culture dish (MicroTest Plate III, Becton Dickinson Co.) containing 0.2 ml of LB:glycerol (85:15 vol %) supplemented with tetracycline (10 mg/L) in each well. The inoculated dishes were then incubated at 37°C for 12 h, sealed with UV-irradiated pressure sensitive film (Becton Dickinson and Co.) and stored at -80°C. When transferring the individual colonies of the clone bank to the tissue culture dish, care was taken to pick only one-half of the cell mass. The remaining cell mass was washed from the masterplates with LB and pooled in 10 ml LB:glycerol (85:15 vol %) for permanent storage (*en masse*) at -80°C. A random sample of 25 members from each clone bank were checked for the presence of pVK100 and the size of the cloned insert. Each strain was grown overnight in LB supplemented with tetracycline (200 mg/L), and the recombinant cosmid isolated via the mini-prep procedures described above. The mini-prep was subsequently digested with *Hind*III and loaded on an agarose gel to check for insert size and yield.

III. RESULTS AND DISCUSSION

A. Development of a Gene Transfer System

The ability to transfer cloned genes into *Alteromonas putrefaciens* 200 is of primary importance to complementation studies of the previously generated suite of iron-reduction-deficient mutants. Once discrete DNA fragments containing loci

required for iron reduction activity are isolated, additional studies on gene organization and expression of the iron reduction system are possible. Since *A. putrefaciens* 200 does possess an indigenous plasmid (T. DiChristina, unpublished data), it may be possible to construct a new cloning system by joining the appropriate gene transfer functions (if any) of the indigenous plasmid to a narrow host range replicon (e.g., ColE1). However, since *A. putrefaciens* 200 is a gram-negative bacterium, and because gene transfer systems derived from such bacteria are known to mediate chromosomal DNA transfer amongst a wide range of microorganisms (Barth, 1979), preliminary work centered on identification of a broad host range gene transfer system applicable to *A. putrefaciens* 200. Gene transfer systems based on each of the three principal routes by which bacteria exchange genetic information (i.e., transduction, transformation, conjugation) were chosen for this study.

Aleromonas putrefaciens 200 was originally classified as a member of the genus *Pseudomonas* (Obuekwe, 1980). Since bacteriophage isolated from various members of this genus are capable of transducing chromosomal DNA across species lines (Holloway, 1979), an initial study was undertaken to identify a *Pseudomonas* transducing phage capable of infecting *A. putrefaciens* 200. Because they had been isolated from *Pseudomonas aeruginosa* and *Pseudomonas putida*, respectively, bacteriophages B3 (Holloway, 1960) and gh-1 (Lee and Boezi, 1966) were chosen for this study. B3 is a temperate phage, capable of transducing chromosomal DNA from *P. aeruginosa*. The prophage can not be induced to enter the lytic cycle by artificial means (e.g., UV light), therefore induction experiments were not conducted with this phage. Phage gh-1 is virulent with *P. putida* forming clear plaques on solid media. Two techniques, one in liquid medium and the other on solid medium, were used to determine if *A. putrefaciens* 200 was within the immunity range of each phage. The first technique, based on the liquid culture method

range of each phage. The first technique, based on the liquid culture method described by Miller (1972), consisted of inoculating a liquid culture of *A. putrefaciens* 200 with a fresh phage plaque and monitoring cell growth and subsequent lysis over a range of incubation times. The second technique consisted of cross-streaking a phage stock with a loopful of *A. putrefaciens* 200 cells and checking for cell lysis after colony growth. Identical results were obtained via both techniques: neither bacteriophage B3 (see Figure 4.4) nor bacteriophage gh-1 (data not shown) were capable of inducing the lytic cycle in *A. putrefaciens* 200. In addition, neither phage was capable of inducing a lytic response when incubated with any strain other than its original host (see Figure 4.4). These results suggest that the immunity range of *Pseudomonas* phages B3 and gh-1 is highly specific, and most likely limits the transducing capability of each to intra-strain crosses. It is possible, however, that the phages do enter the cell but are unable to induce the lytic cycle (under the experimental conditions employed in these experiments) in strains other than their original host. Problems with host specificity are not uncommon to inter-species transduction experiments (Holloway, 1979); thus, since recent morphological and biochemical evidence (Semple and Westlake, 1987) indicate that *A. putrefaciens* 200 is not a *Pseudomonas* spp., it is not surprising to find that this strain is immune to infection by phages B3 and gh-1.

The strategy employed in development of a cloning system for direct transformation of *A. putrefaciens* 200 centered on the choice of a suitable plasmid vector, and on optimizing the experimental conditions required to induce artificial competency for uptake of that plasmid. Figure 4.2 outlines the array of experiments used to examine the major factors that can affect plasmid transformation frequency. Those factors included osmotic shock treatment, heat-pulse treatment, the physiological state of the recipient cells, plasmid concentration and exposure time,

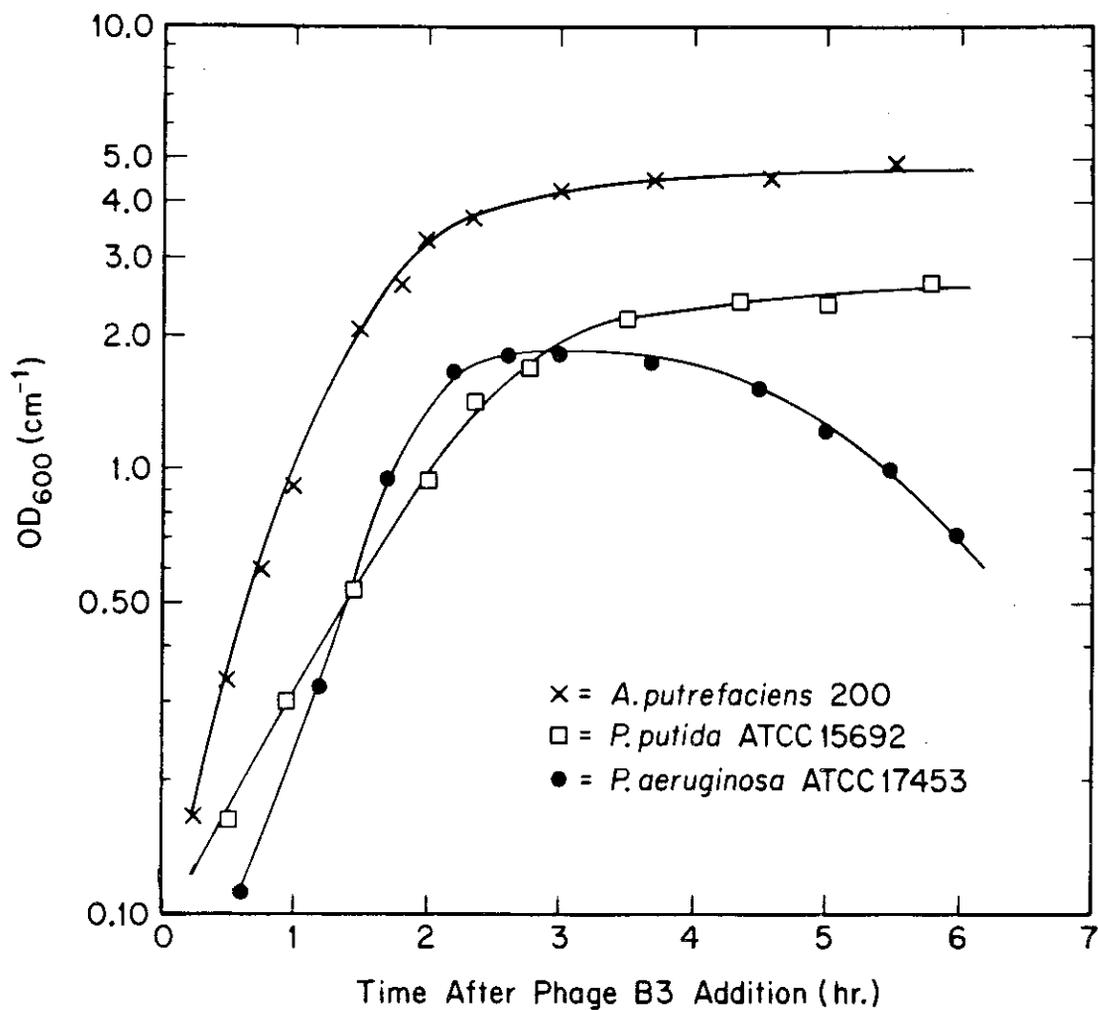


Figure 4.4. Results of the liquid culture technique used to determine the immunity range of *Pseudomonas aeruginosa* phage B3. Each strain was incubated in the presence of a fresh phage B3 plaque for a period of 14 h. Cell lysis was monitored via OD₆₀₀ measurements.

and the concentration of antibiotics in the selection media. It has generally been found that transformation frequencies are highest when cells are harvested at mid-log phase and immediately treated to induce artificial competency for plasmid uptake (Brown et al., 1979). In each transformation experiment described below, mid-log phase cells ($A_{600} = 0.8$) were harvested immediately prior to treatment. Initial treatment consisted of an osmotic shock. A myriad of osmotic shock treatments have been used previously to optimize plasmid transformation frequency in gram-negative bacteria (Saunders et al., 1984). Each transformation procedure generally involves incubating the harvested cells in the presence of a combination of divalent cations. Although the exact mechanism is not yet clear, several investigators (e.g., Weston et al., 1981) have suggested that divalent cations neutralize the negatively-charged outer membrane thus allowing DNA binding and subsequent uptake to proceed. Other investigators (e.g., Brown et al., 1979; van Alphen et al., 1978) have suggested that treatment with divalent cations exposes outer membrane proteins required for DNA transport into the cytoplasm. In either case, CaCl_2 treatment has proven the most effective in promoting plasmid uptake in *E. coli*, while Mg^{2+} was superior to Ca^{2+} in promoting transformation of *Pseudomonas* spp. (Mercer and Loutit, 1979). Both CaCl_2 and MgSO_4 were used in series to osmotically shock exponentially-growing liquid cultures of *A. putrefaciens* 200. Although both cations are present in the lactate growth medium (at 1.35 mM and 0.41 mM, respectively), the concentrations of CaCl_2 and MgSO_4 used in these experiments were nearly two orders of magnitude higher. Immediately after addition of plasmid DNA, the calcium-treated cell suspension was heat-pulsed at 42°C over a range of incubation times. Previous studies (Weston et al., 1981) have shown that a heat-pulse is essential for efficient transformation of *E. coli* cells. The mechanism by which a heat-pulse facilitates direct transformation has not yet been resolved, however, a secondary reaction may occur during heat treatment, namely

the inactivation of extracellular DNase activity, that may allow plasmid uptake to occur. Since *A. putrefaciens* 200 produces copious quantities of an extracellular DNase (see below), heat-pulsing was included in the transformation experiments. Problems with cell viability after prolonged heat-treatment of *A. putrefaciens* cells were encountered however. Less than 1 in 10^5 cells survived the 10 minute heat-pulse at 42°C (see Table 4.2).

An additional variable examined in the transformation experiments included the time of exposure to the plasmid DNA [approximately 500 ng of plasmid DNA was used in each transformation experiment]. The potential recipients were given sufficient time (3 hours) for plasmid uptake and subsequent marker expression before plating on selective media. Table 4.2 presents the results of the transformation experiments described above.

No evidence was obtained that either plasmid pSP329 or cosmid pVK100 was transformed into wild-type *A. putrefaciens* 200 under any of the conditions employed in the transformation experiments described above. These two cloning vectors differ in size and in their broad host range maintenance properties. The inability to transform *A. putrefaciens* 200 with the relatively small (7.0 kb) plasmid pSP329 suggests that size is not the limiting factor. Cosmid pVK100 possesses a full complement of the functions required for replication and stable maintenance in the host range of RK2. Mini-RK2 replicon pSP329, on the other hand, carries only the minimal functions required for replication and stable maintenance in a narrow range of bacteria (*E. coli*, *Azobacter* spp. and *P. putida*). The absence of a full complement of maintenance functions in pSP329 could therefore account for its apparent inability to transform *A. putrefaciens* 200. This is not the case in fact, since conjugation experiments (see below) show that both pVK100 and pSP329 can be stably maintained by *A. putrefaciens* 200.

Table 4.2. Results of Direct Transformation Experiments.

Sample No.	[CaCl ₂] (mM)	Heat pulse (42 ⁰ C) (min)	Plasmid Exposure Time (hr)	Tc ^r Frequency	
				pSP329	pVK100
1	50	0	1	<5x10 ⁻⁸	<6x10 ⁻⁹
2	50	0	3	<8x10 ⁻⁹	<1x10 ⁻⁹
3	50	2	1	<5x10 ⁻⁸	<6x10 ⁻⁹
4	50	2	3	<8x10 ⁻⁹	<1x10 ⁻⁹
5	50	10	1	nd	nd
6	50	10	3	nd	nd
7	100	0	1	<5x10 ⁻⁸	<6x10 ⁻⁹
8	100	0	3	<8x10 ⁻⁹	<1x10 ⁻⁹
9	100	2	1	<5x10 ⁻⁸	<6x10 ⁻⁹
10	100	2	3	<8x10 ⁻⁹	<1x10 ⁻⁹
11	100	10	1	nd	nd
12	100	10	3	nd	nd
Positive Controls					
<i>E. coli</i> HB101	50	2	1	2x10 ⁻⁴	nd
<i>E. coli</i> S17-1	50	2	1	nd	1x10 ⁻⁴

Note: nd = not determined

Of major concern to the transformation procedures with *A. putrefaciens* 200 was the production of extracellular DNase by this bacterium (see Figure 4.5). Procedures designed to inactivate DNase activity via prolonged heat-pulse treatment were ineffective due to problems with cell viability of temperatures above 40°C. A series of experiments were therefore undertaken to generate a DNase-deficient mutant of *A. putrefaciens* 200, which could be used in transformation experiments similar to those described above. Ethyl methane sulfonate (EMS)-treated liquid cultures (generated via mutagenesis procedures identical to those used in Chapter 3) were plated on DNase Test Agar (Difco) and screened for the inability to produce extracellular DNase. Of the 12,000 colonies screened via this technique, none exhibited a DNase-deficient phenotype. Transposon mutagenesis was not used in this study.

It is possible that experimental parameters other than those considered here are responsible for the apparent inability to induce transformation in *A. putrefaciens* 200. For example, several studies (Saunders et al., 1984) have shown that certain recipient strains will take up plasmid DNA only if it is provided in a specific topological form (e.g., CCC, OCC or linear). Such factors were not pursued in this study.

The inability to develop either a generalized transduction or a direct transformation system in *A. putrefaciens* 200 necessitated the development of alternative gene transfer procedures. The relatively recent discovery that IncP1-based cloning vectors (i.e., those vectors containing the *mob* site of the broad host range plasmid RK2) can be mobilized into a wide range of gram-negative bacteria (Barth, 1979) suggests that such cloning systems may be applicable to *A. putrefaciens* 200. Such an approach is now being widely used to analyze microbial functions in bacteria with no known natural gene transfer capability (Lidstrom, 1989). Both three-way and two-way mating procedures were used in experiments

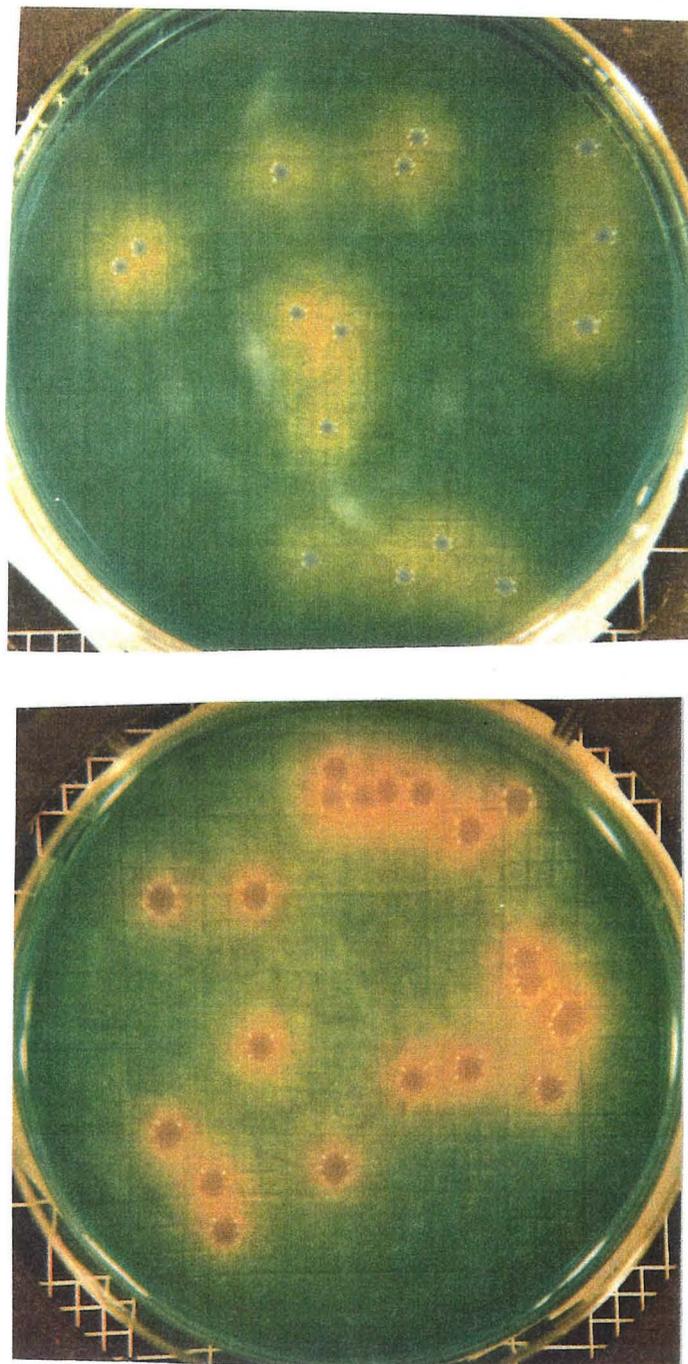


Figure 4.5. Wild-type strains of *Serratia marescans* (top) (positive control) and *Alteromonas putrefaciens* 200 (bottom) grown on DNase test agar. Clearing zone around periphery of each colony indicates extracellular DNase activity.

designed to optimize the frequency of conjugal transfer of cloning vectors pSP329 and pVK100. Both vectors contain the *mob (oriT)* locus of RK2 and therefore have the potential to be mobilized into the host range of RK2 when provided with the necessary IncP1 *tra* functions in *trans*. In three-way mating experiments, "helper" plasmid pRK2013 (containing the IncP1 *tra* system) was used to mobilize the cloning vectors from an *E. coli* HB101 donor strain to an *A. putrefaciens* 200 recipient. In the two-way mating experiments, *E. coli* mobilizing strain S17-1, containing the IncP1 transfer system integrated in its chromosome, was used to mobilize the cloning vector into an *A. putrefaciens* 200 recipient. Initial experiments indicated that both cloning vectors could be transferred to and stably maintained in *A. putrefaciens* 200. Optimization of the frequency of conjugal transfer of each vector to *A. putrefaciens* 200 was accomplished by systematically varying two experimental parameters: the ratio of donor, helper and recipient strains, and the mating time. Since transfer frequencies are generally much higher on solid surfaces than in liquid media (where pili may not be expressed), all matings were done on L agar. Results from the series of mating experiments are given in Figure 4.6 and Table 4.3.

To ensure that the recipient strains did indeed receive the appropriate vector, the plasmids were isolated from a random sample of transconjugates. It appears (Figure 4.7) that cloning vectors pSP329 and pVK100 are mobilizable into *A. putrefaciens* 200 at relatively high frequencies. Approximately 1% of all potential recipients receive cosmid pVK100, while pSP329 is mobilized into *A. putrefaciens* 200 at a frequency nearly two orders of magnitude lower. This difference might be attributed to the inherent maintenance properties of each plasmid. Cosmid pVK100 contains a full complement of the RK2 replication and maintenance system, whereas the mini-RK2 replicon pSP329 contains only the minimal number of functions (i.e., *oriT* and *trfA*^{*}) required for stable replication and maintenance in

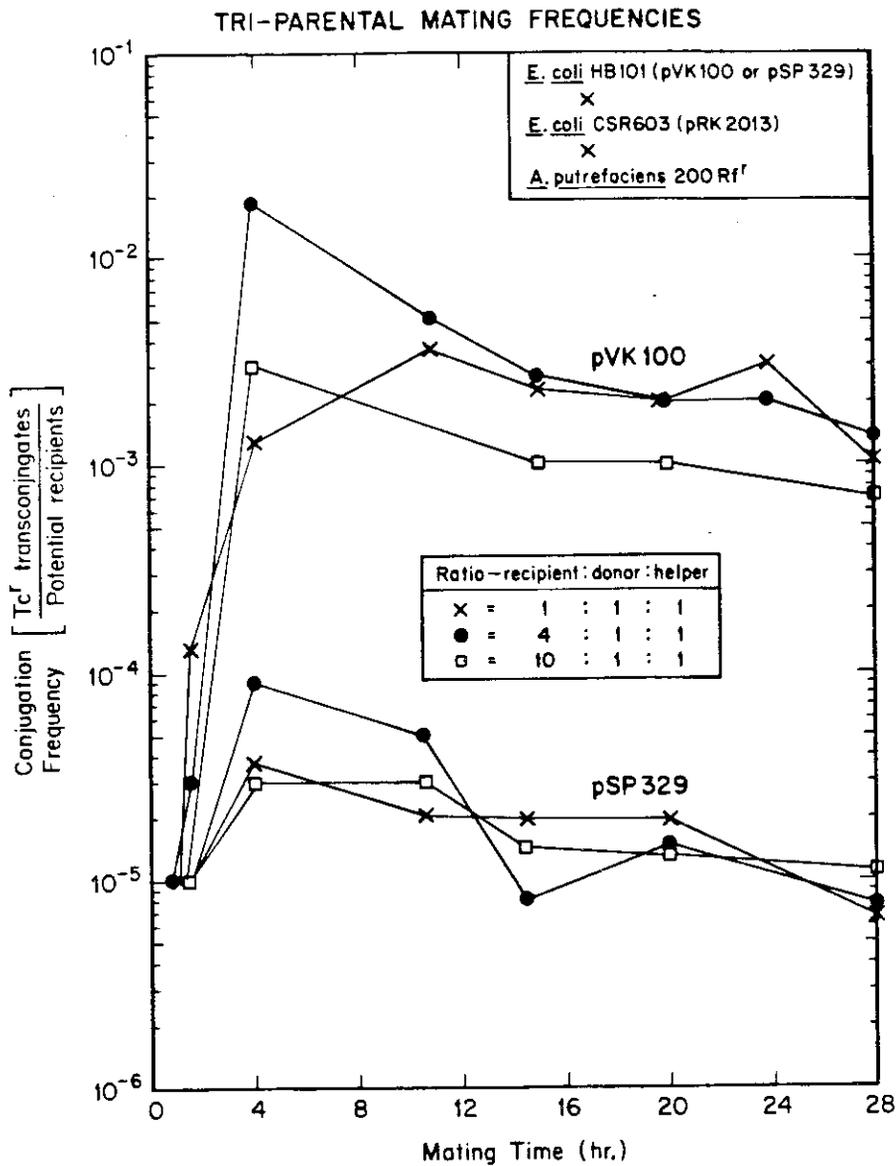


Figure 4.6. Mating frequencies from two series of three-way mating experiments between *Alteromonas putrefaciens* Rf^r, *E. coli* CSR603 (pRK2013) and *E. coli* HB101 (pVK100 or pSP329) strains. In each series of matings the mating time and ratio of the recipient, donor and helper strains were varied. Mating frequencies are based on the frequency of Rf^r, Tc^r colonies arising on selection media. The frequency of background resistance to Rf^r, Tc^r was $<1 \times 10^{-9}$.

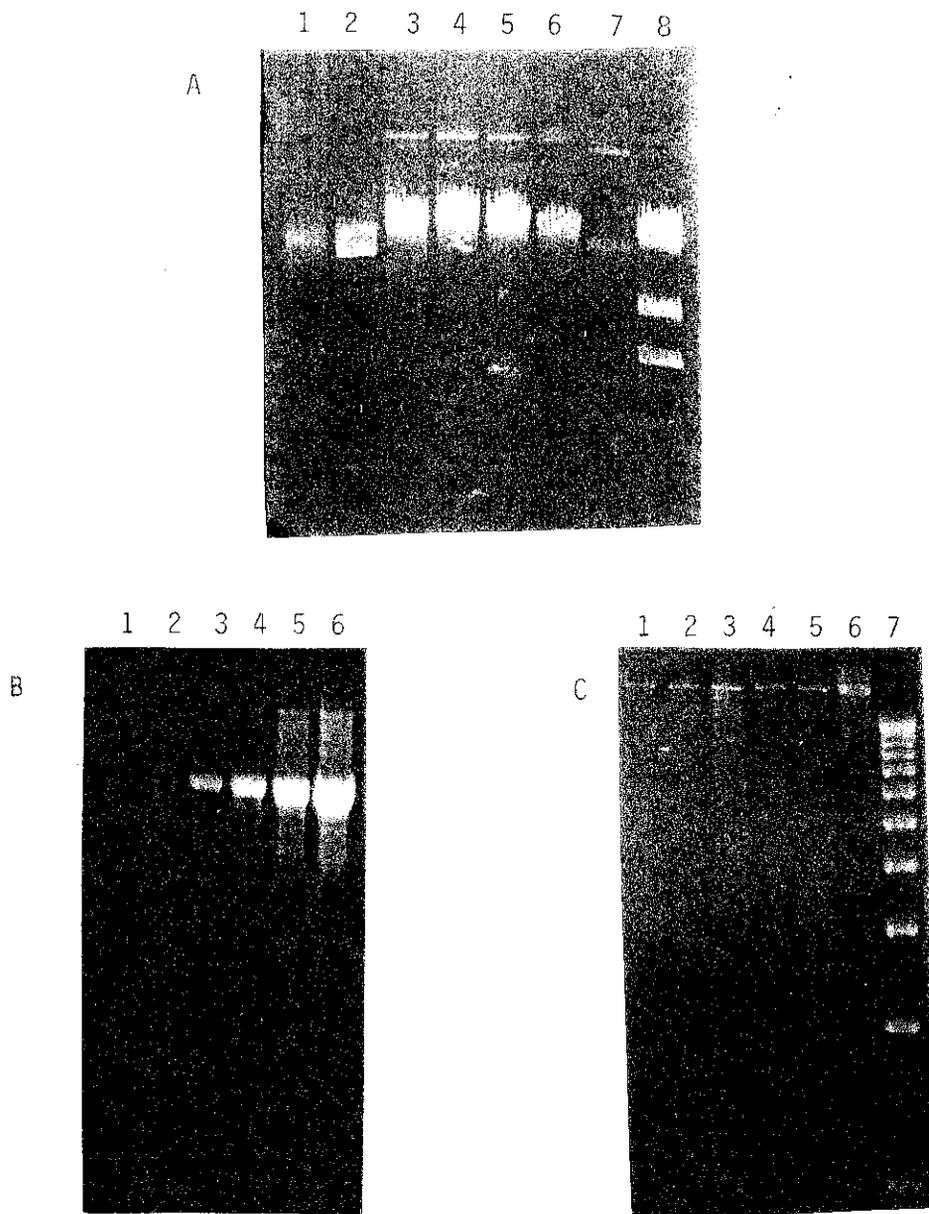


Figure 4.7. Plasmid mini-preps of *Alteromonas putrefaciens* transconjugates: Gel A [three-way cross with *E. coli* HB101 (pVK100) and *E. coli* CSR603 (pRK2013)] - Transconjugates (lanes 3-6), *E. coli* HB101 (pVK100, lane 7). Gel B [three-way cross with *E. coli* CSR603 (pRK2013) and *E. coli* HB101 (pSP329)] - *E. coli* HB101 (pSP329, *Hind*III cut, lane 2), transconjugate mini-prep *Hind*III cut (lanes 3-6). Gel C [two-way cross with *E. coli* mobilizing strain S17-1 (pVK100)] - transconjugate mini-prep *Hind*III cut (lanes 1-5), *E. coli* S17-1 (pVK100, *Hind*III cut, lane 6).

E. coli and *P. putida* (Schmidhauser and Helinski, 1985). Those maintenance functions encoded on pVK100 and not found in pSP329 may be responsible for the apparent decrease in conjugation frequency. It is interesting to note that the frequency of conjugal transfer of pVK100 is independent of the type of mating system used in vector mobilization (see Table 4.3). It was thought that regulation of pRK2013-encoded *tra* gene expression during three-way matings might be different than regulation of a chromosomally-integrated set of identical genes (i.e., during two-way mating) which could result in different mating frequencies. This was not noted for conjugal transfer of pVK100 to *A. putrefaciens* 200. Two-way matings with pSP329 (in *E. coli* S17-1) were not tested here, but might be of interest to future studies concerned with regulation of expression of plasmid maintenance functions.

Table 4.3. Maximum frequencies^a of conjugal transfer of cloning vectors SP329 and pVK100 to *A. putrefaciens* 200.

Mating System	Cloning Vector	
	pSP329	pVK100
Three-way Mating ^b	1x10 ⁻⁴	2x10 ⁻²
Two-way Mating ^c	----- ^d	1x10 ⁻²

- Notes:
- a. Maximum frequencies taken from Figure 4.6.
 - b. Three-way matings included *E. coli* HB101 donor, *E. coli* HB101 (pRK2013) helper and *A. putrefaciens* Rf^f recipient strains.
 - c. Two-way matings included *E. coli* S17-1 mobilizing and *A. putrefaciens* Rf^f recipient strains.
 - d. not determined.

B. Construction of an *Alteromonas putrefaciens* 200 Gene Clone Bank.

The ability to transfer cosmid cloning vector pVK100 into *A. putrefaciens* 200 opens up a myriad of possible studies concerning gene organization and regulation in this bacterium. When carrying a random distribution of cloned DNA fragments (i.e., as a gene clone bank), the recombinant cosmid can facilitate complementation

studies of mutant strains of choice. Cosmid pVK100 was used as the cloning vehicle in construction of an *A. putrefaciens* 200 gene clone bank. As outlined in Figure 4.3, the overall strategy involved insertion of a *Hind*III partial digest of wild-type *A. putrefaciens* 200 DNA into the corresponding site on pVK100 followed by transfer of the recombinant cosmid into an *E. coli* strain for permanent storage as a mobilizable gene clone bank. Checks were made after each step of the procedure to ensure that each reaction was proceeding efficiently. The *Hind*III partial digest was prepared for ligation by size fractionation of the 15-20 kb DNA fragments (see Figure 4.8). Cosmid pVK100 was prepared for ligation via *Hind*III digestion and subsequent dephosphorylation reactions (see Figure 4.9). The concatamers resulting from ligation of the wild-type DNA fragments to the linearized (dephosphorylated) vector (see Figure 4.9) were packaged into lambda phage particles, and subsequently used to transfect an *E. coli* host strain for permanent storage as a gene clone bank. Two separate clone banks were constructed: one in *E. coli* strain HB101, the other in *E. coli* mobilizing strain S17-1. Mobilization of the gene clone banks into selected *A. putrefaciens* (mutant) recipient strains can therefore be accomplished by either three-way or two-way mating procedures. A random sample of 25 members from each clone bank was checked for the presence of pVK100 and the size of the cloned insert (see Figure 4.10). Approximately 90% of all clone bank members received a recombinant vector (i.e., vector plus insert) and the remaining 10% received pVK100 without an insert. This may have been the result of inefficient dephosphorylation leading to self-concatamerization of the linearized vector. Of those clone bank members that did receive a cloned DNA fragment, the average insert size was approximately 22 kb. It should be noted that several clone bank members did harbor as many as four (cloned) *Hind*III fragments (see Figure 4.10). This fact may be important in subsequent subcloning experiments.

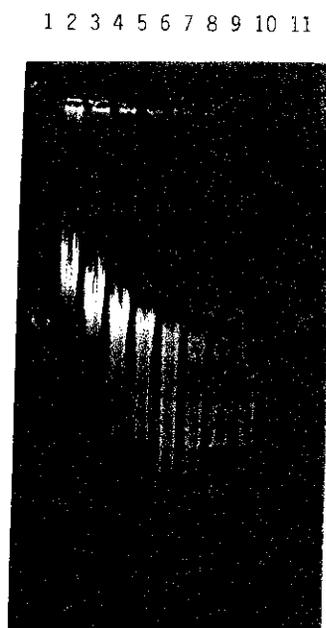


Figure 4.8. *Hind*III partial digest of *Alteromonas putrefaciens* chromosomal DNA prep: 15-20 kb size fractionation on 0.8% agarose gel. Lanes 1 and 11: markers (21, 15, 9 kb). Lanes 2-10: increasing time of *Hind*III digestion (1 min. to 60 min.).

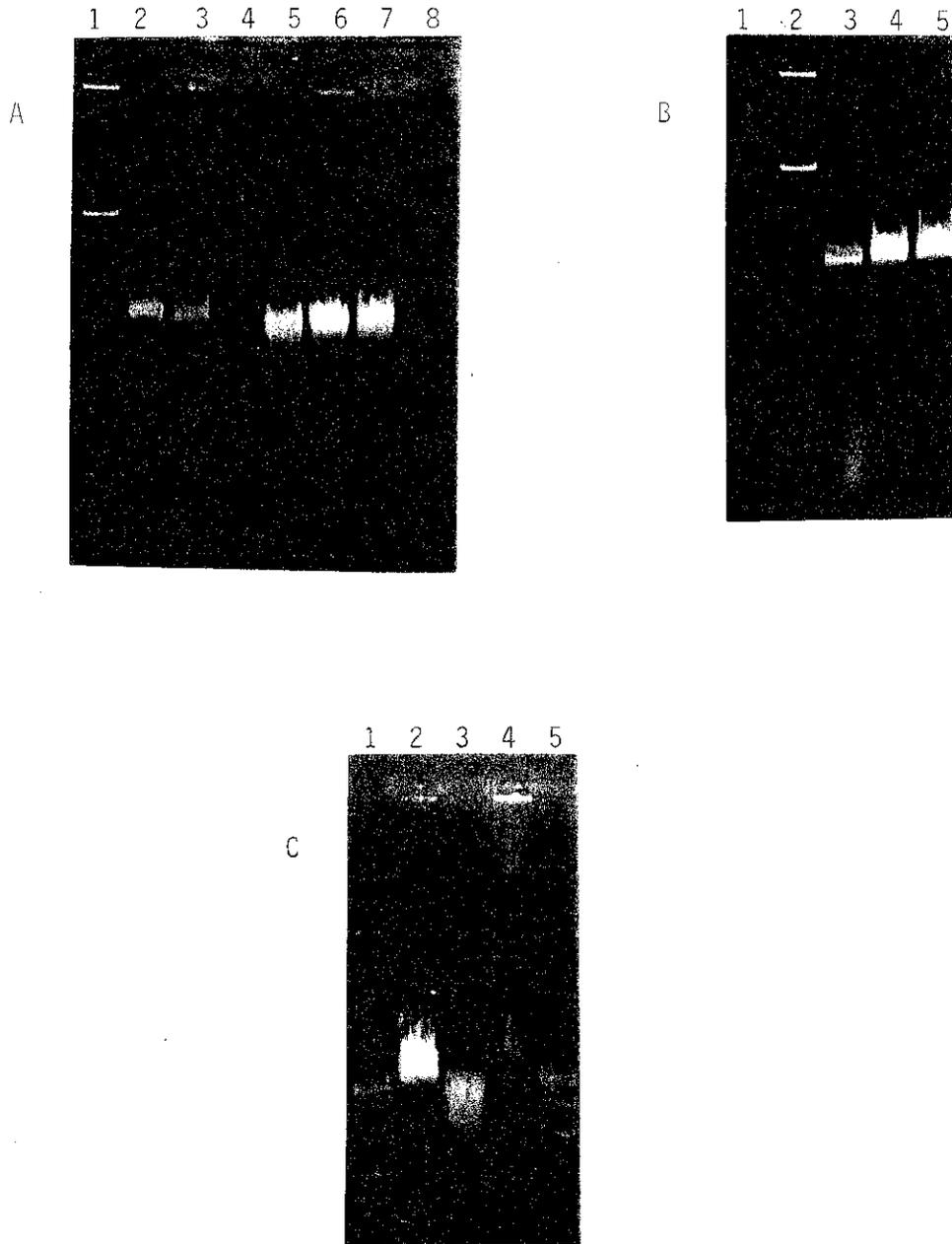


Figure 4.9. Gel A - large scale prep of cosmid pVK100 (lane 1 - uncut, Lanes 2 and 3 - *Hind*III digested) and 15-20 kb size fragments of *A. putrefaciens* chromosomal DNA (lanes 4-8). Gel B - pVK100 (lane 2 - uncut, 3 - after *Hind*III digestion, 4 - after dephosphorylation, 5 - after phenol/chloroform treatment). Gel C - ligation reaction (lane 2 - linearized and dephosphorylated pVK100, 3 - 15-20 kb *A. putrefaciens* DNA partial digest, 4 - long chain concatomers after ligation reaction).

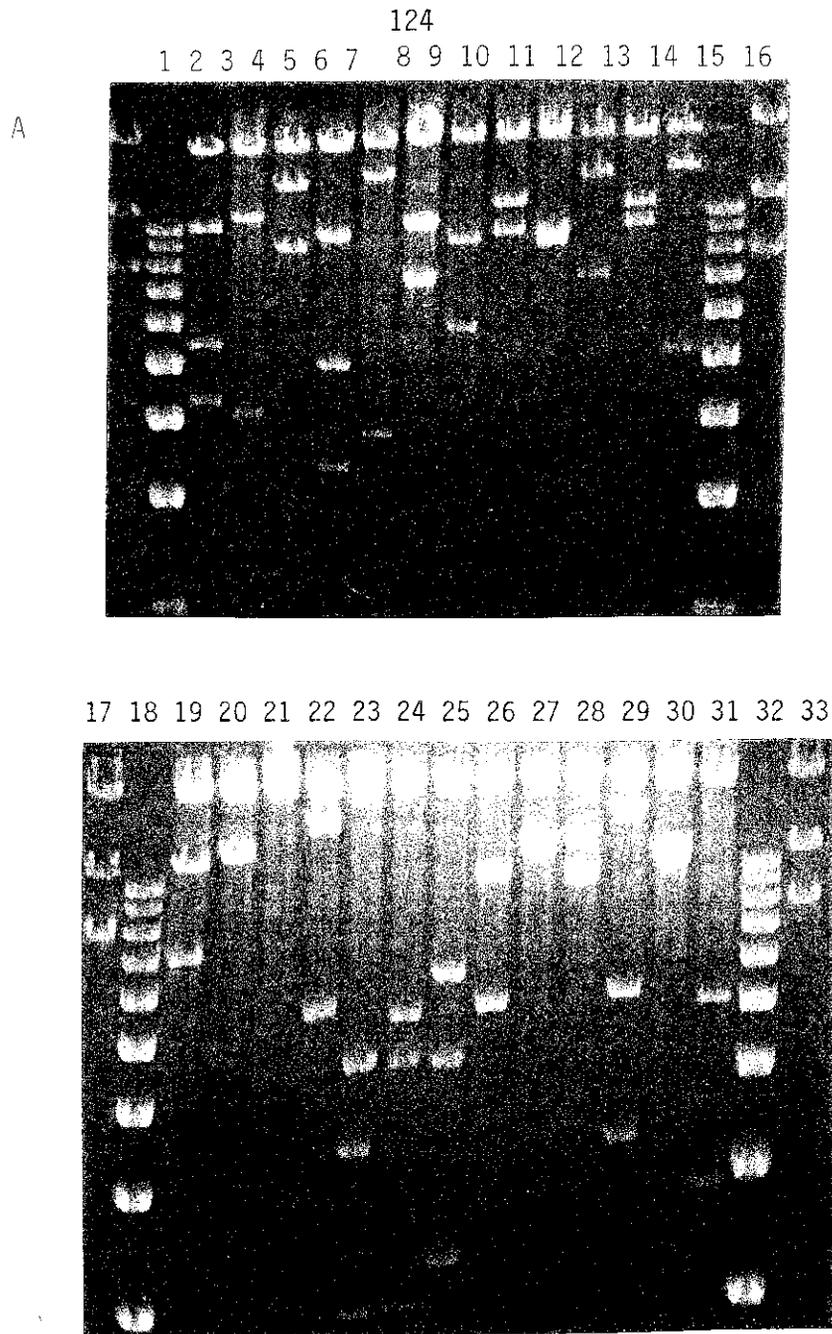


Figure 4.10. *Hind*III digested mini-preps of randomly selected members of the two *A. putrefaciens* 200 gene clone banks stored in *E. coli* HB101 (Gel A) and *E. coli* mobilizing strain S17-1 (Gel B). In each mini-prep, note the band corresponding to pVK100 and the multiple (cloned) *Hind*III fragments of *A. putrefaciens* 200 DNA (Markers - Lanes 1, 2, 15-18, 32, 33).

IV. SUMMARY

The three principal methods by which bacteria exchange genetic material (transduction, transformation, conjugation) were systematically examined in a series of experiments aimed at developing an efficient gene transfer system in *Alteromonas putrefaciens* 200. Initial attempts at the development of a suitable transduction or transformation system proved unsuccessful. The inability of several *Pseudomonas* transducing phages to infect *A. putrefaciens* 200 was attributed to their narrow host range. The production of an extracellular DNase by *A. putrefaciens* 200 most likely prevented efficient transformation of artificially-induced competent cells. Conjugal transfer of IncP1-based cloning vectors was possible, however, via three-way or two-way mating procedures. *E. coli* mobilizing strain S17-1 was capable of mobilizing broad host range cosmid pVK100 into *A. putrefaciens* at relatively high frequencies (1 transconjugate per every 100 potential recipients). An *A. putrefaciens* 200 gene clone bank was constructed utilizing pVK100 as the cloning vehicle and either *E. coli* strain HB101 or *E. coli* strain S17-1 as the clone bank host. With the facility to transfer cloned DNA into selected (mutant) strains, complementation analysis of the iron reduction system of *A. putrefaciens* 200 can proceed.

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CHAPTER 5

**GENETIC COMPLEMENTATION ANALYSIS AND
PRELIMINARY BIOCHEMICAL CHARACTERIZATION
OF ALTEROMONAS PUTREFACIENS
IRON-REDUCTION-DEFICIENT MUTANTS**

I. INTRODUCTION

The main objective of the present study was to determine the physiological basis of dissimilative iron reduction by *Alteromonas putrefaciens* 200. The overall approach taken to study the molecular mechanism of Fe(III) reduction involved isolation and characterization of a suite of iron-reduction-deficient mutants. Such an approach necessitated the development of an efficient genetic system for use with this microorganism; previous work included the development of a screening technique and mutagenesis procedures for isolation of a suite of iron-reduction-deficient mutants (Chapter 3), the construction of an *A. putrefaciens* 200 gene clone bank and the development of a gene transfer system (Chapter 4). With the facility to transfer cloned DNA into the suite of iron-reduction-deficient mutants, discrete DNA fragments containing loci required for iron reduction activity can be identified via genetic (complementation) analysis. Additional biochemical studies can provide information concerning the defects in each mutant. Results from the complementary genetic and biochemical analyses can then be used to postulate a molecular model of the dissimilative iron reduction system.

As illustrated in Figure 5.1, complementation analysis requires that a wild-type allele be expressed (*in trans*) in a recipient mutant strain. The wild-type genes are generally introduced as part of a hybrid cloning vector that is transferred to the recipient. The formation of partial diploid strains can result in functional expression of the cloned genes and phenotypic restoration (complementation) of wild-type activity. Complementation testing must be approached with caution, however, since phenotypic expression can be affected by unforeseen factors such as polar mutations and dominance of a mutant gene (i.e., an inactive mutant polypeptide forming an inactive oligomer with a normal polypeptide from

COMPLEMENTATION ANALYSIS

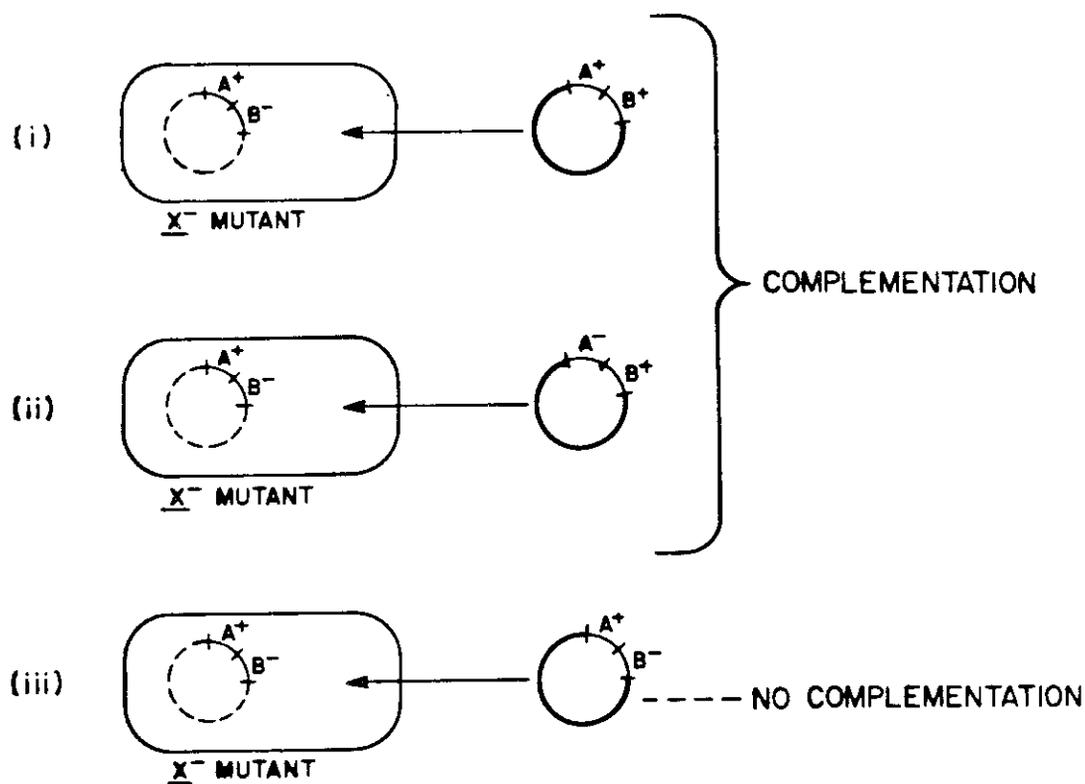


Figure 5.1. Complementation Analysis: In the hypothetical example illustrated above mutant X^- (phenotype X^- ; genotype: A^+B^-) served as the recipient strain for various recombinant cloning vectors. The hybrid cloning vectors contained DNA fragments isolated from strains with the following genotypes: (i) A^+B^+ , (ii) A^-B^+ and (iii) A^+B^- . In cases (i) and (ii), the formation of stable diploid strains has restored wild-type X^+ phenotype. However, in case (iii) complementation has not occurred since mutations are in the same locus (B gene).

a wild-type gene; Beckwith et al., 1985). In theory, the recircularized recombinant plasmid carrying a DNA fragment containing the wild-type gene will complement the previously inactivated loci.

Both chemical (ethyl methane sulfonate) and transposon (Tn5) mutagenesis procedures have been used to generate a suite of *A. putrefaciens* 200 mutants deficient in iron reduction activity. Mutants in both high-rate (induced) and low-rate (constitutive) iron reduction activity were identified via a newly developed technique for screening mutagenized *A. putrefaciens* 200 cultures. The broad host range cosmid cloning vector pVK100 (IncP1) was mobilized into *A. putrefaciens* at relatively high frequencies (one transconjugate per every 100 potential recipients) by either two-way or three-way mating (conjugation) procedures. An *A. putrefaciens* 200 gene clone bank was constructed in pVK100 and housed in *E. coli* mobilizing strain S17-1, thus facilitating complementation studies via two-way crosses between the gene clone bank and the suite of iron-reduction mutants. The present study details the results of such complementation analysis, and in addition, outlines preliminary biochemical characterization of the iron-reduction-deficient mutants.

II. MATERIALS AND METHODS

A. Complementation Experiments

Figure 5.2 outlines the overall strategy followed in complementation analysis of the previously generated suite of iron reduction-deficient mutants of *Alteromonas putrefaciens* 200. The *A. putrefaciens* 200 gene clone bank housed in *E. coli* mobilizing strain S17-1 (and stored permanently in the individual wells of the MicroTest plates) was used in two-way matings to transfer the clone bank to the suite of iron-reduction-deficient mutants. A 96-pronged "Clonemaster" stamp (Immusine Laboratories, Inc.) was used to transfer the 96 clone bank members contained in a single MicroTest plate to an identical plate containing 50 μL LB in each well. It was estimated that each prong transferred approximately 10 μL of cells

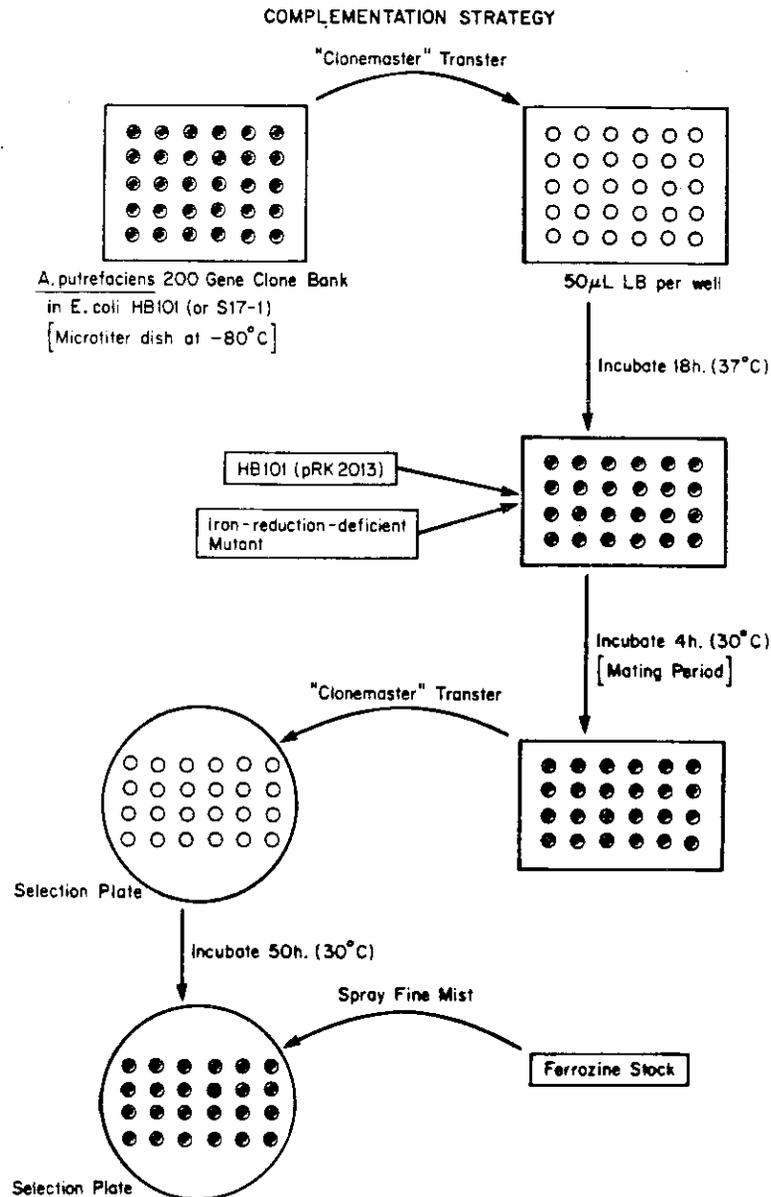


Figure 5.2. Overall strategy used in complementation of the iron-reduction-deficient mutants of *Alteromonas putrefaciens* 200.

during inoculation. The 96 clone bank members were subsequently incubated 18 hours at 37°C without shaking. After the 18 hour incubation period, a previously selected iron-reduction-deficient (Rf^f) mutant [grown to log-phase in LB supplemented with rifamycin (100 mg/L) and kanamycin (50 mg/L if required), washed and resuspended in equal volume LB] was added to each well of the MicroTest plate with an automated 8-pronged pipettor (EDP-M8; Rainin Instrument Co.). The mixture of recipient and donor cells (4:1 ratio; 0.1 ml total volume) was then incubated at 30°C for 4 hours. After the 4 hour mating period, the "Clonemaster" stamp was used to transfer the mating mixture from each of the 96 wells to selective media. The selective media consisted of nutrient agar supplemented with rifamycin SV (100 mg/L), kanamycin (50 mg/L if required), tetracycline (10 mg/L) and ferric chloride (to 0.15 mM as Fe). The selection plates were incubated 50 hours at 30°C and the resulting colonies sprayed with a fine mist of filter-sterilized ferrozine (0.15 mM; Sigma). The formation of magenta-colored colonies was monitored for 15 minutes and any positive phenotypes picked to identical selection media for single-colony isolation. After two single-colony isolations, each transconjugate was tested in liquid culture for iron reduction activity after growth at either high ($> 200 \mu\text{M}$) or low ($< 2 \mu\text{M}$) oxygen tension. A 5 ml liquid culture grown overnight in lactate medium supplemented with rifamycin SV (100 mg/L), kanamycin (50 mg/L if required) and tetracycline (5 mg/L) was used to inoculate a 1.5 L batch reactor (Biostat M, B. Braun Co.) containing 1 L of identical medium. At an optical density of $A_{600} = 0.25$ both oxygen utilization and iron reduction rates were measured as previously described (see Chapter 3). The iron reduction activities of the complemented transconjugate was compared to the activity of the original iron-reduction-deficient mutant, and to the activity of a randomly selected transconjugate that produced a negative iron reduction phenotype during complementation experiments.

In order to classify the mutants into complementation groups, an array of two-way matings were carried out. Each iron-reduction-deficient mutant was mated individually with each clone bank member that had previously restored the positive iron reduction phenotype to a mutant strain. Experimental procedures were identical to those used to mobilize pVK100 from *E. coli* mobilizing strain S17-1 into *A. putrefaciens* 200 (see Chapter 4). Negative controls consisted of individual two-way matings between each mutant and either *E. coli* S17-1 (pVK100) or a randomly selected clone bank member that had previously failed to restore a positive iron reduction phenotype to that particular mutant. To test the effect of the cosmid vector on iron reduction activity, pVK100 was mobilized into *A. putrefaciens* 200 (via the two-way mating procedures described above), and a resulting transconjugate screened for iron reduction activity on solid media via Screening Technique No. 3 (see Chapter 3) and tested for iron reduction activity in liquid culture.

The recombinant cosmids harbored in each clone were isolated via the mini-prep procedures described previously (see Chapter 4). DNA from each mini-prep was digested with *Hind*III and the cloned DNA fragments analyzed via agarose gel (0.8%) electrophoresis.

B. Biochemical Analyses

Selected mutant strains were analyzed biochemically via two procedures: reduced-minus-oxidized difference spectra and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Liquid cultures of each mutant strain were prepared as follows: 5 ml overnight cultures grown in lactate medium (see Chapter 3 for composition) supplemented with rifamycin SV (100 mg/L) and kanamycin (30 mg/L if required) were used to inoculate 1.5 L of identical medium in a Biostat M (B. Braun Instrument Co.) batch reactor. Cultures were grown at either high ($> 200 \mu\text{M}$) or low ($< 2 \mu\text{M}$) oxygen tension to a target optical density

of $A_{600} = 0.25 \text{ (cm}^{-1}\text{)}$. Chloramphenicol was added (to 0.25 mM) and the cells immediately harvested, washed and resuspended in MSS buffer (see Chapter 3 for composition). Aliquots for measurement of reduced-minus-oxidized difference spectra were concentrated 100X in MSS buffer and stored at -20°C . Aliquots for subsequent SDS-PAGE analyses were resuspended in original volume MSS and stored at 4°C .

Before spectroscopic analyses were carried out, the cells were lysed by passage through a French pressure cell at 20,000 psi. Unlysed cells and cell debris were removed by centrifugation at 14,000xg for one minute. The cell-free extract was stored on ice until ready for use. A dual-beam spectrophotometer (Shimadzu, MPS-2000) was used to measure the difference spectra: several grains of sodium dithionite (Sigma) were added to the cell free extract of the sample cuvette, and $1.5 \mu\text{L}$ of 10 mM ferricyanide (Sigma) was added to the reference cuvette. The difference spectrum was measured immediately after reagent addition.

The proteins expressed in each mutant during growth at either high ($> 200 \mu\text{M}$) or low ($< 2 \mu\text{M}$) oxygen tension were resolved by conventional SDS-PAGE procedures. A modified version of the Laemmli method (Laemmli, 1970) was used in each case. The separation and stacking gels consisted of 15.0% and 4.6% acrylamide, respectively. Each gel was stained with Coomassie blue before being photographed.

III. RESULTS AND DISCUSSION

Figure 5.2 outlines the overall strategy followed in complementation analysis of the previously generated suite of *Alteromonas putrefaciens* mutants deficient in iron reduction activity. The two *A. putrefaciens* gene clone banks, stored in *E. coli* HB101 and *E. coli* S17-1, respectively, were used in separate mating experiments. Each mating experiment was designed to identify specific clones that were capable

of restoring a positive iron reduction phenotype to selected mutant strains. Of the approximately 4,000 clones contained in the S17-1 clone bank, 192 were used in two-way matings with transposon mutant T121 (see Table 3.3, for Fe(III) reduction rate data). The 192 clones were contained in individual wells of two randomly selected Microtest Plates (S4 and S18). The clones in each Microtest Plate were transferred to a second mating plate (containing LB in each well) and allowed to grow to mid-log phase. After this initial growth period, an inoculum of transposon mutant T121 was added to each well, and the mixture of *E. coli* S17-1 donor and *A. putrefaciens* Rf^f recipient strains (at a 1:4 ratio) was incubated for 4 h. at 30°C. After the 4 h. mating period, portions of the 192 mating mixtures were transferred to antibiotic plates that selected for T121 (presumed) transconjugates (i.e., Rf^f, Km^r, Tc^r) and against the *E. coli* S17-1 donor strain (i.e., Rf^s, Km^s). Since the selective media had also been supplemented with ferric iron, Screening Technique No. 3 (see Chapter 3) was used to detect any T121 transconjugates in which the positive iron reduction phenotype had been restored. Of the 192 clones mated with T121 and screened for iron reduction activity via Screening Technique No. 3, two [designated S4-E-2 and S18-F-4 (plate-row-column)] appeared to have restored a positive iron reduction phenotype to mutant T121. Figure 5.3 is a photograph of selection plate S18 immediately after it had been sprayed with a fine mist of ferrozine. It appeared as though a positive iron reduction phenotype had been restored to T121 after mating with clone S18-F-4. All 91 other (presumed) transconjugates retained their negative iron reduction phenotype. A similar result was obtained with mating plate S4 (data not shown). A positive iron reduction phenotype was restored to T121 via mating with clone S4-E-2. All 91 other (presumed) transconjugates on mating plate S4 retained the negative iron reduction phenotype.

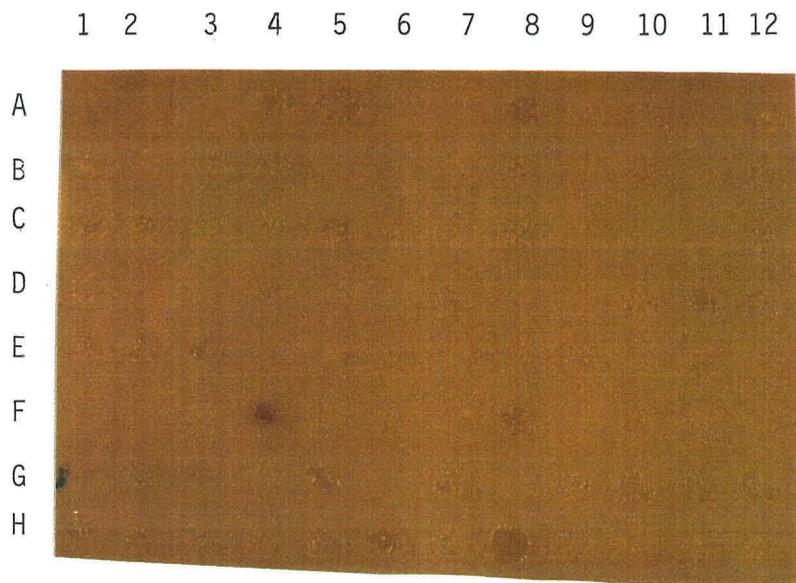
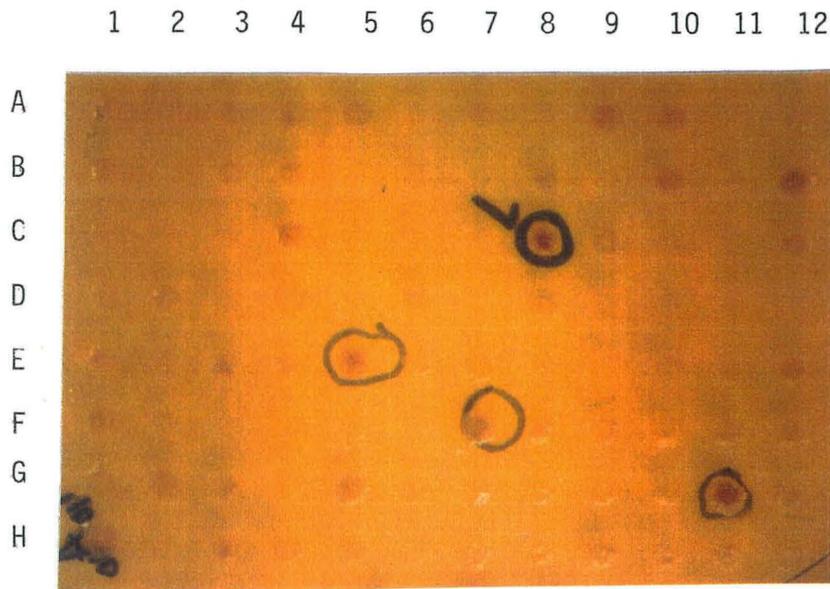


Figure 5.3. Selective plate S18 (after spraying with ferrozine) showing the 96 (presumed) transconjugates arising from individual two-way matings between *A. putrefaciens* mutant T121 and the 96 *E. coli* clones contained in the individual wells of Microtest dish S18. Note the appearance of a magenta-colored (presumed) transconjugate corresponding to clone position S18-F-4.

Complementation analysis using the *A. putrefaciens* gene clone bank harbored in *E. coli* HB101 consisted of an identical experimental procedure with one modification: *E. coli* strain CSR603 (pRK2013) was included in the mating mixture to provide the necessary IncP1 transfer (*tra*) functions for mobilization of the recombinant vector. Of the approximately 4,000 clones contained in the *E. coli* HB101 clone bank, 96 were used in three-way matings with ethyl methane sulfonate (EMS)-generated iron-reduction-deficient mutant F37 (see Table 3.3). The 96 clones were contained in a randomly selected Microtest Plate designated H4. After application of Screening Technique No. 3 to those colonies (presumed transconjugates) arising on appropriate selection media, it appeared that clone H4-C-8 was capable of restoring a positive iron reduction phenotype to mutant F37 (see Figure 5.4).

In order to classify the remaining EMS- and Tn5-generated iron reduction deficient mutants into complementation groups, an array of individual matings were carried out. Each of the iron-reduction-deficient mutants listed in Table 3.3 were mated individually with clones S4-E-2 and S18-F-4 via the spot mating procedure previously described (see Chapter 4). Clone H4-C-8 was not included in this study. Screening Technique No. 3 was again applied to those colonies (presumed transconjugates) arising on appropriate selection media. Negative controls consisted of individual two-way matings between each mutant and either *E. coli* S17-1 (pVK100) or two randomly selected clone bank members (S4-A-10 and S18-D-11) that previously failed to restore a positive iron reduction phenotype to T121 (see Figure 5.3). To test the effect of the cosmid vector on the positive iron reduction phenotype, pVK100 was mobilized into an *A. putrefaciens* R^F strain and screened on solid media via Screening Technique No. 3. It should be noted that transposon mutants T206, T208 and 219 (phenotypically deficient in iron reduction activity) were included in the array of mating experiments, however, their



H4

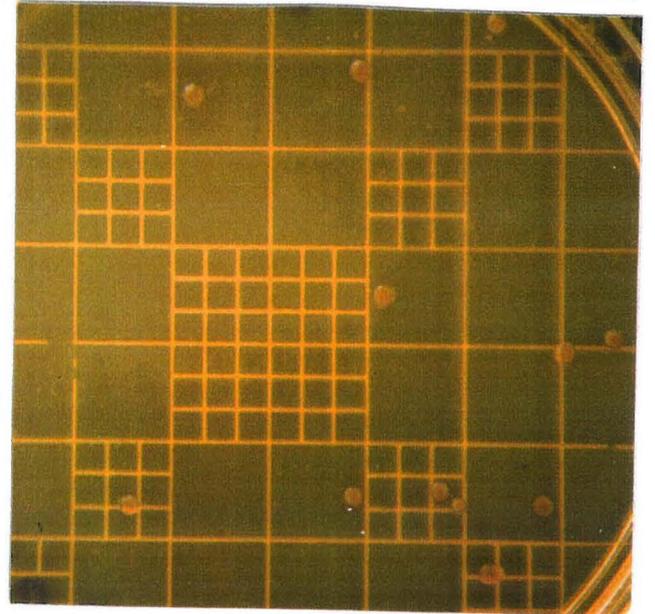
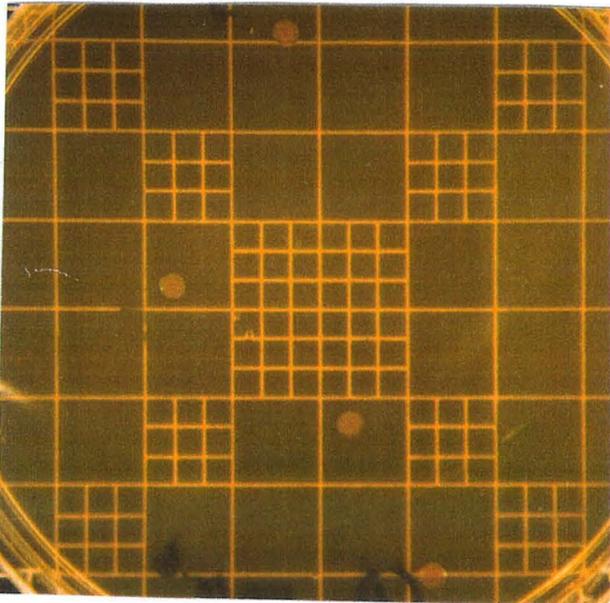
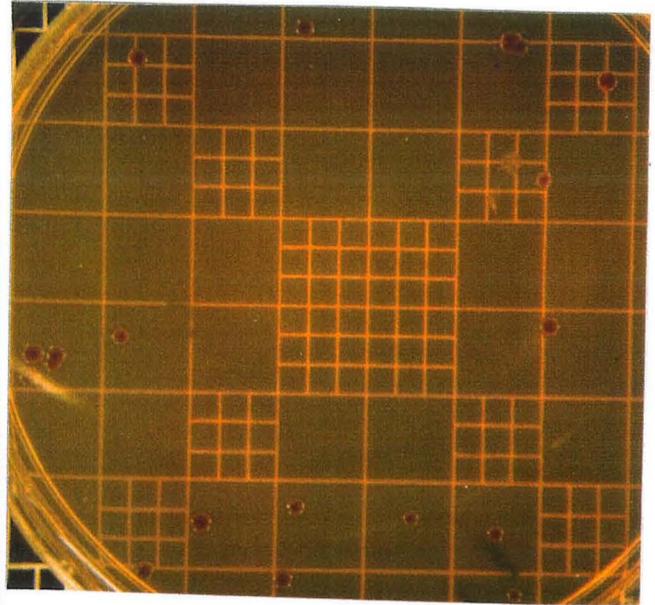
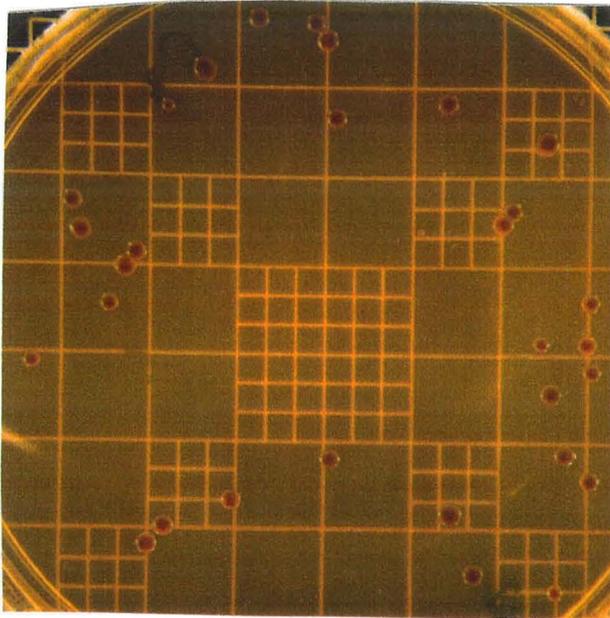
Figure 5.4. Selective plate H4 (after spraying with ferrozine) showing the 96 (presumed) transconjugates arising from individual two-way matings between *A. putrefaciens* mutant F37 and the 96 *E. coli* HB101 clones contained in the individual wells of Microtest dish H4. Note the appearance of a magenta-colored (presumed) transconjugate corresponding to clone position H4-C-8.

corresponding iron reduction and oxygen utilization rates have not yet been determined. Results from the array of complementation experiments described above are given in Figures 5.5, 5.6 and 5.7 (results shown only for matings with T121, T191 and Flamingo, respectively). Table 5.1 is a compilation of the results from complementation experiments with each of the mutants listed in Table 3.3.

From the preliminary data presented in Table 5.1, it appears that the iron-reduction-deficient mutants fall into at least 3 distinct complementation groups: Group I (T121, T206, T208) complemented by both S4-E-2 and S18-F-4 clones; Group II (T176, T191, T219) complemented only by clone S4-E-2; and Group III (F18, Wanda, Flamingo, F37) not complemented by either clone. All positive and negative control matings produced transconjugates with corresponding positive and negative iron reduction phenotypes. A transconjugate selected from the mating between mutant T121 and clone S18-F-4 (designated T121A) was tested for low-rate iron reduction activity in lactate medium supplemented with the appropriate antibiotics. The low-rate iron reduction activity of transconjugate T121A was nearly identical (data not shown) to the low-rate iron reduction activity of the wild-type strain grown under similar conditions (i.e., under high oxygen tension). The high-rate iron reduction system of T121A has not yet been tested in liquid medium. As a negative control, a transconjugate selected from the mating between mutant T121 and clone S18-D-11 (designated T121B) was tested for low-rate iron reduction activity in liquid medium in a manner identical to the T121A test described above. T121B, however, was unable to reduce Fe(III) after growth at high oxygen tension.

A

B



C

D

Figure 5.5. Results from individual two-way matings between *A. Heromanas putrefaciens* iron-reduction-deficient (transposon) mutant T121 and *E. coli* S17-1 clones S4-E-2 (A), S18-F-4 (B), S4-A-10 (C) and S17-1 (pVK100) (D).

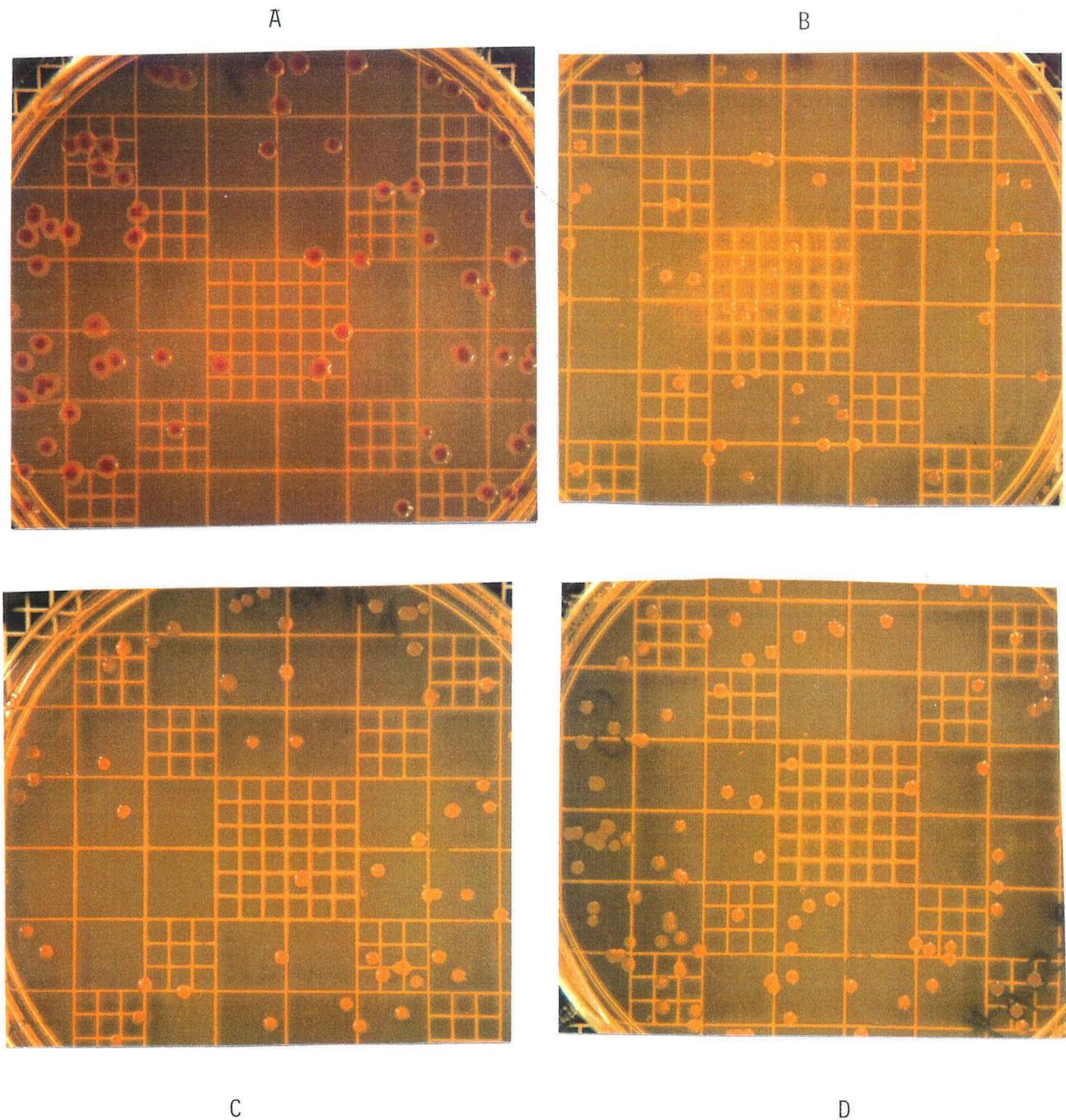


Figure 5.6. Results from individual two-way matings between *A. putrefaciens* iron-reduction-deficient (transposon) mutant T191 and *E. coli* S17-1 clones S4-E-2 (A), S18-F-4 (B), S4-A-10 (C) and S17-1 (pVK100) (D).

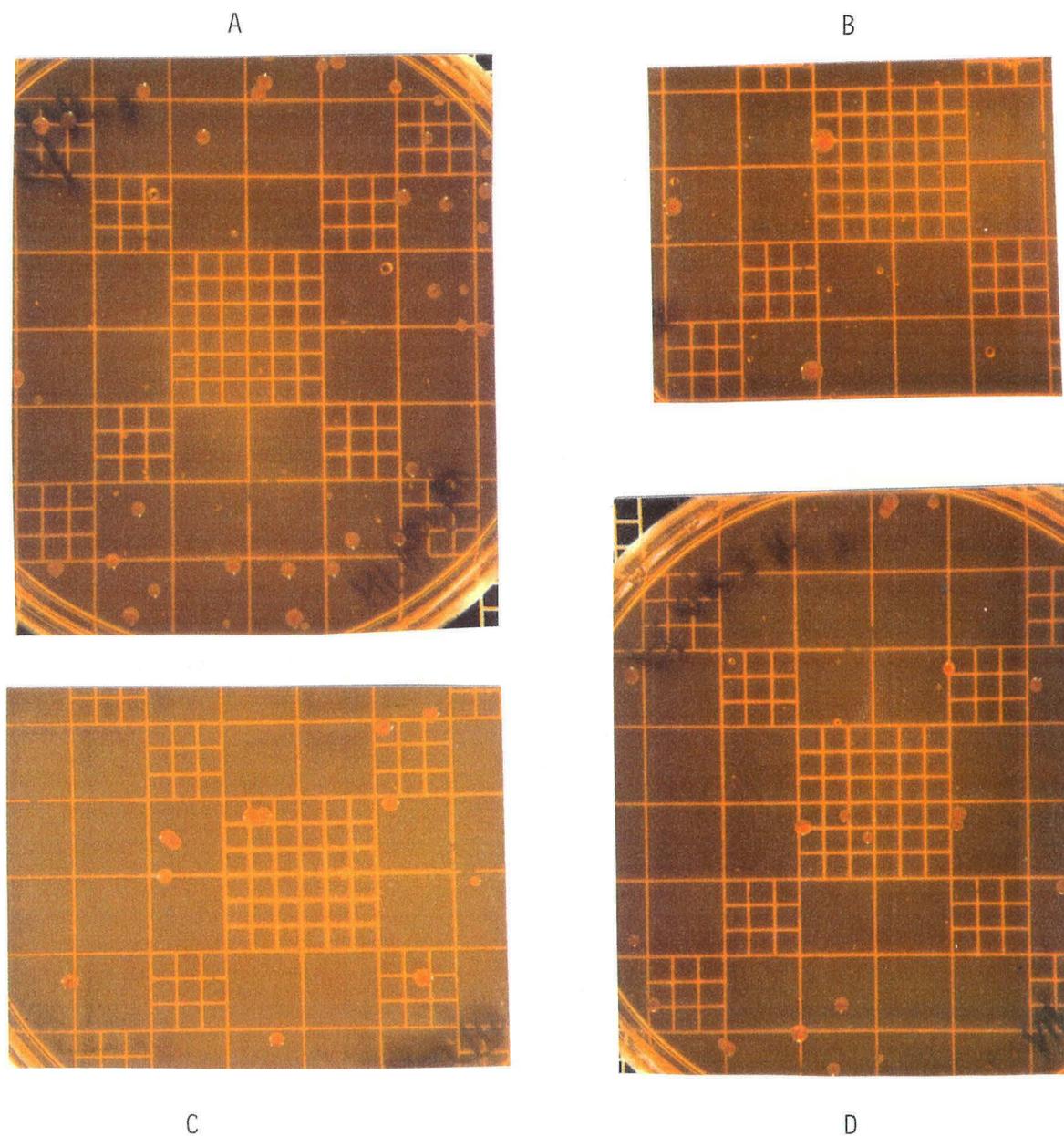


Figure 5.7. Results from individual two-way matings between *A. putrefaciens* iron-reduction-deficient (EMS) mutant Flamingo and *E. coli* S17-1 clones S4-3-2 (A), S18-F-4 (B), S4-A-10 (C) and S17-1 (pVK100) (D).

Table 5.1. Results from individual two-way matings between iron-reduction-deficient mutants and selected clones from the *A. putrefaciens* gene clone bank housed in *E. coli* S17-1.

Mutant Strain	<u>Two-way Mating Partner</u>				<i>E. coli</i> S17-1 (pVK100)
	S4-E-2	S18-F-4	S4-A-10	S18-D-11	
Tn5 Mutants					
T121	+	+	-	-	-
T176	+	-	-	-	-
T191	+	-	-	-	-
T206	+	+	nd	nd	nd
T208	+	+	nd	nd	nd
T219	+	-	nd	nd	nd
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EMS Mutants					
F18	-	-	-	nd	-
Wanda	-	-	-	nd	-
Flamingo	-	-	-	nd	-
Banana	+	+	+	+	+
F37	-	-	-	nd	-
<hr style="border-top: 1px dashed black;"/>					
Positive Control					
Rf ^f	+	+	+	+	+

Notes: + = positive iron reduction phenotype.
 - = negative iron reduction phenotype.
 nd = not determined.

The finding that Group I strains are complemented by both S4-E-2 and S18-F-4 suggests that the cloned inserts contain a common internal fragment. To determine if the cloned inserts contained overlapping *Hind*III fragments, plasmid mini-preps of both S4-E-2 and S18-F-4 were digested with *Hind*III and analyzed via agarose gel electrophoresis. Results are presented in Figure 5.8.

It appears that both inserts contain a *Hind*III fragment of approximately 9 kb in size. Further restriction analysis is needed to determine if these two 9 kb *Hind*III fragments are identical.

Preliminary biochemical characterization of the iron reduction mutants from each of the three complementation groups included SDS-PAGE and reduced-minus-oxidized spectral analyses. Transposon mutants T121, T176 and T191 and EMS-generated mutant F37 were selected for spectral analysis. Two cell free extracts of each mutant were prepared from liquid cultures grown at high and low oxygen tension. The reduced-minus-oxidized difference spectra of each extract was measured and compared to the difference spectra of cell free extracts of the wild-type strain grown at either high or low oxygen tension. When grown at high oxygen tension, the reduced-minus-oxidized difference spectra of wild-type *A. putrefaciens* 200 (Figure 5.9) contains absorption maxima at 420 nm (Soret band), 522 nm (corresponding to *b*- and *c*-type cytochromes) and 550 nm (corresponding to *c*-type cytochromes). When grown at low oxygen tension, absorption maxima are again found at 420 nm, 522 nm and 550 nm along with a fourth maxima at 630 nm (corresponding to a *d*-type cytochrome). It is not uncommon to find the induction of a second high-affinity *d*-type cytochrome after prolonged growth under low oxygen tension. Such a phenomenon has been noted in several other gram-negative bacteria including *E. coli* (Castor and Chance, 1959; Green and Gennis, 1983), *Pseudomonas putida* (Sweet and Peterson, 1978), *Proteus vulgaris* (Moyed and O'Kane, 1956) and *Klebsiella aerogenes* (Moss, 1956). It is thought that the induction

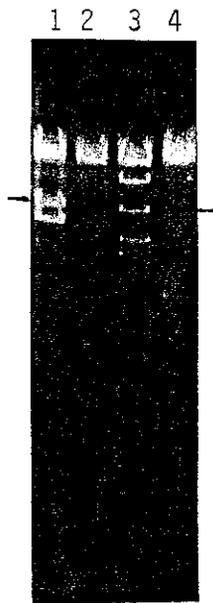


Figure 5.8. *Hind*III-digested mini-preps of *E. coli* S17-1 clones S18-F-4 (lane 1), S4-E-2 (lane 3) and S17-1 (pVK100) (lanes 2 and 4). Tick corresponds to 9 kb size fragments.

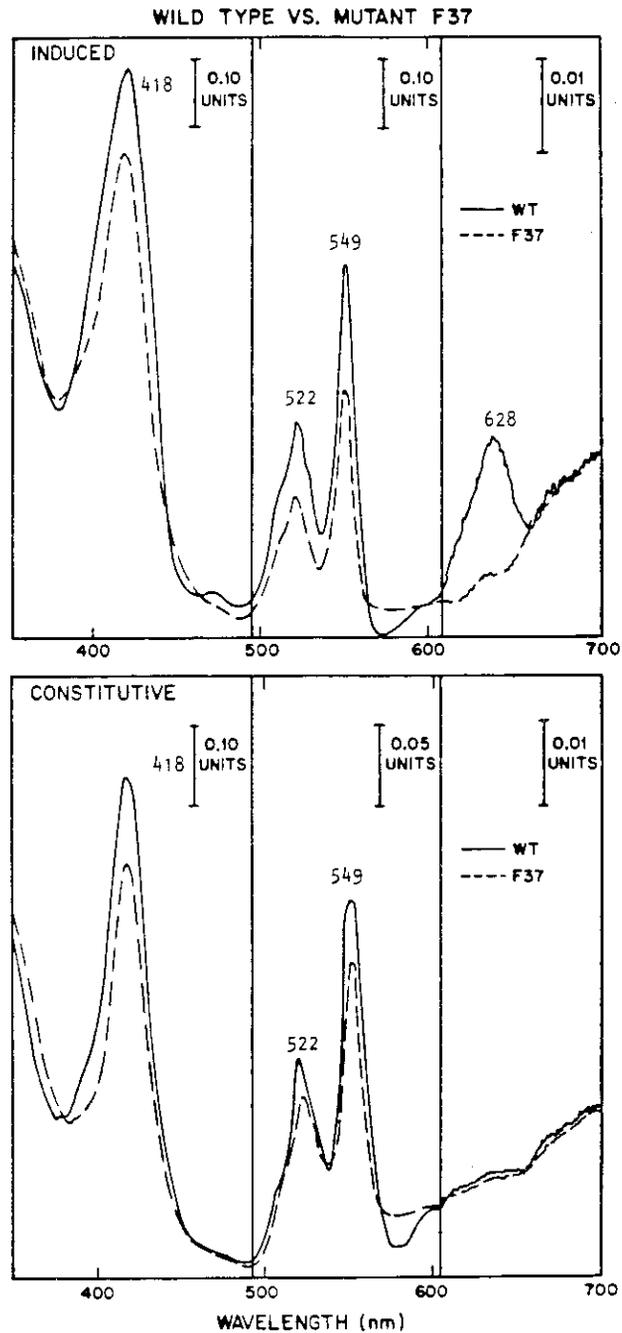


Figure 5.9. Reduced-minus-oxidized difference spectra of cell free extracts of *A. putrefaciens* 200 wild-type and iron-reduction-deficient (EMS) mutant F37 for cells grown at low (top) and high (bottom) oxygen tension.

of a high-affinity (i.e., low K_m) cytochrome oxidase affords the microorganism selective advantage in response to low O_2 conditions.

The reduced-minus-oxidized difference spectra of mutants F37 and T121 were significantly different from the corresponding spectra of the wild-type strain. The reduced-minus-oxidized difference spectrum of mutant F37 grown at high oxygen tension was nearly identical to the corresponding spectrum of the wild-type strain grown under the same conditions (see Figure 5.9). However, when grown at low oxygen tension, mutant F37 failed to express the *d*-type cytochrome that normally absorbs at 630 nm (see Figure 5.9). The inability of mutant F37 to express a *d*-type cytochrome coincides with its inability to reduce iron at high rates (see Table 3.3). This observation suggests that *d*-type cytochromes may play a role not only in aerobic respiration (i.e., to scavenge O_2 at micromolar concentrations) but also in the high-rate iron reduction system of *A. putrefaciens* 200. Although the precise phenotype of mutant F37 is unclear, several possibilities exist. Either F37 is a *d*-type cytochrome mutant and *d*-type cytochromes are required for iron reduction activity, or it is a mutant in a common regulatory element, or it is a mutant in a non-regulatory element required by both systems. It is not unprecedented for a microorganism to use the same electron transport chain component in two different systems. For example, *Aerobacter* spp. are thought to use the same terminal reductase to transfer electrons to either NO_3^- or Fe(III) under anaerobic conditions (Munch and Ottow, 1983). Although thermodynamic arguments preclude the use of Fe(III) as a terminal electron acceptor until O_2 has been nearly exhausted (Ghiorse, 1987), it would not be unreasonable for a facultative anaerobe to use the same electron transport chain component (induced via growth under low oxygen tension) to transfer electrons to either O_2 or Fe(III); the respiratory system would then be poised to use either O_2 or Fe(III) depending on their availability. On the other hand, the inability of mutant F37 to either reduce iron at a high-rate or express the

d-type cytochrome also suggests that a common regulatory element controls expression of both systems under low oxygen tension. In *Escherichia coli*, a single regulatory protein (Fnr) controls expression of several anaerobic respiratory enzymes including nitrate reductase (Stewart, 1982) and fumarate reductase (Jones and Gunsalus, 1987). *A. putrefaciens* 200 may possess a similar control mechanism for the expression of its anaerobic respiratory enzymes. The mechanism by which *A. putrefaciens* 200 controls expression of anaerobic respiratory enzymes in the presence of alternative electron acceptors is unknown, and will be the subject of future work.

The reduced-minus-oxidized spectra of cell free extracts of mutant T121 also differed significantly from the spectra of the wild-type strain. The absorption maxima (520 nm) corresponding to the complement of *b*- and *c*-type cytochromes was not present in spectra measured from T121 cultures grown at either high or low oxygen tension (see Figure 5.10). In addition, the absorption maxima corresponding to the *c*-type cytochrome (550 nm) had shifted to 556 nm and was significantly reduced in size (see Figure 5.10). The absorption maxima corresponding to the *d*-type cytochrome of mutant T121 grown under low oxygen tension was nearly identical to the corresponding maxima for the wild-type strain grown under the same conditions. This finding suggests either that *b*- and *c*-type cytochromes are involved in both the high- and low-rate iron reduction systems of *A. putrefaciens* 200 or that T121 is deficient in an element required by both iron reduction systems. Results from previous (respiratory inhibitor) studies (Arnold et al., 1986) suggested that *b*- and *c*-type cytochromes were involved only in the low-rate iron reduction of *A. putrefaciens* 200. Hydroxyquinoline-N-oxide (HQNO), thought to disrupt the quinone-cytochrome *b* cycle in aerobic electron transport systems, was shown to inhibit low-rate iron reduction activity, but did not affect the high-rate iron reduction system (see Figure 1.1). These results suggest that *b*- and *c*-type

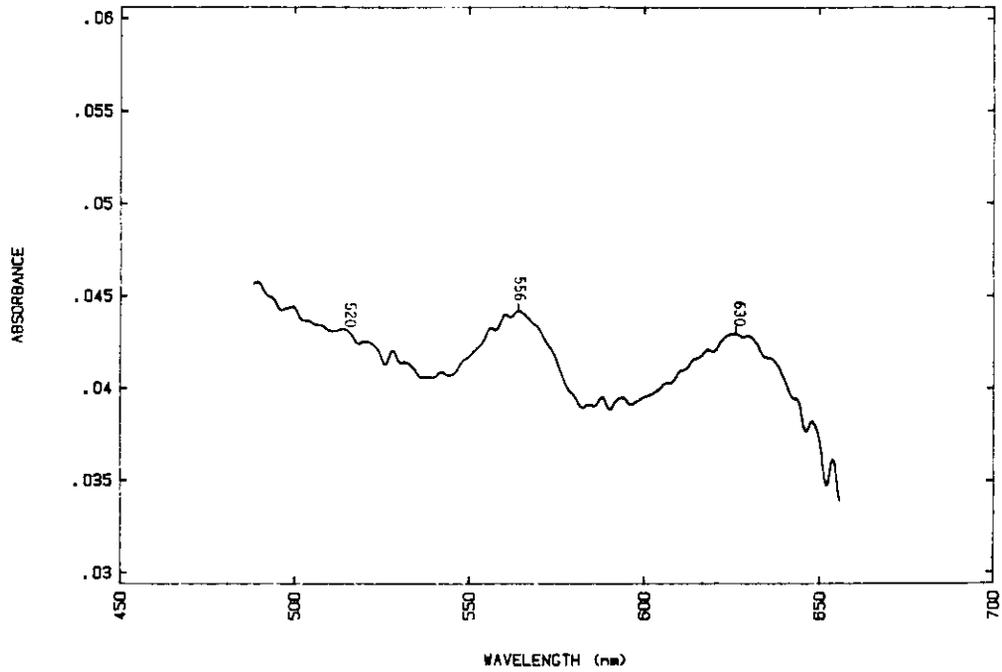


Figure 5.10. Reduced-minus-oxidized difference spectra of cell free extracts of iron-reduction-deficient (transposon) mutant T121 grown at low oxygen tension (data not shown for cells grown at high oxygen tension). Corresponding wild-type spectra given in Figure 5.9 (top).

reduction system (see Figure 1.1). These results suggest that *b*- and *c*-type cytochromes may be involved in the low-rate iron reduction system of *A. putrefaciens* 200; however, since T121 is also deficient in high-rate iron reduction activity, the molecular details of the high-rate system remain unclear.

Results from the SDS-PAGE analysis of the whole cell fraction of each iron reduction mutant (grown at both high and low oxygen tension) are presented in Figure 5.11. A fairly strong signal corresponding to a high MW (approximately 80 kD) protein was found for the whole cell fraction prepared from liquid cultures grown at high oxygen tension. This signal was significantly reduced, however, for cells grown under low oxygen tension. Due to the large number of protein bands in each lane, differences between the protein patterns of the wild-type and mutant strains were difficult to discern. SDS-PAGE analysis of separated cell fractions or two-dimensional gel analyses may be required to resolve the differences between the proteins expressed by each iron-reduction-deficient mutant and the wild-type strain.

Based on the combined results of the genetic (complementation) study (Table 5.1), the preliminary biochemical analysis (described above) and the corresponding mutant rate data (Table 3.3), the iron reduction mutants have been placed into four separate classes (see Table 5.2).

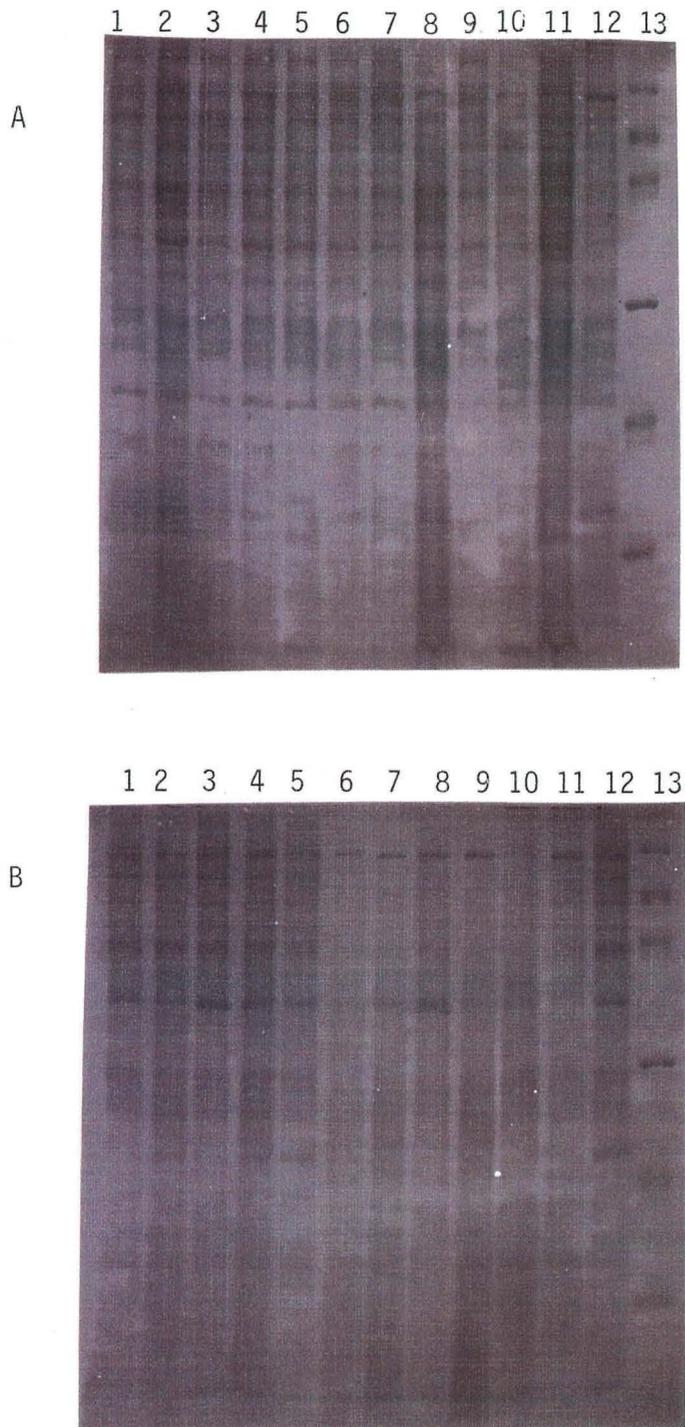


Figure 5.11. SDS-PAGE analysis of EMS-generated iron reduction mutants [Gel A: 1-Banada(L), 2-Flamingo(L), 3-Wanda(L), 4-F18(L), 5-F37(L), 6-WT(L), 7-Banana(H), 8-Flamingo(H), 9-Wanda(H), 10-F18(H), 11-F37(H), 12-WT(H), 13-markers] and transposon mutants [Gel B: 1-191(L), 2-176(L), 3-121(L), 4-243(L), 5-WT(L), 6-191(H), 7-176(H), 8-121(H), 9-243(H), 10-WT(H), 11-WT(H), 12-WT(L), 13-markers]. (H) = grown at high oxygen tension; (L) = grown at low oxygen tension.

Table 5.2. Proposed Classification System for the Iron-Reduction-Deficient Mutants

Class or Strain	Mutant Iron Reduction Activity		Complementing Clone		Detectable Phenotypic Defects
	low-rate	high-rate	S4-E-2	S18-F-4	
Class I					
T121	-	-	+	+	<i>b-</i> or <i>c-</i> type cytochrome
T206	-	-	+	+	
T208	-	-	+	+	Not known
Class II					
T176	-	-	+	-	None detected
T191	-	-	+	-	None detected
T219	-	-	+	-	None detected
Class III					
Flamingo	-	+	-	-	Not known
F18	-	+	-	-	Not known
Wanda	-	+	-	-	Not known
Class IV					
F37	-	-	-	-	<i>d</i> -type cytochrome

Note: + = positive iron reduction phenotype
 - = negative iron reduction phenotype

IV. PROJECT SUMMARY

The physiological basis of microbial (dissimilative) iron reduction was investigated using the facultative anaerobe *Alteromonas putrefaciens* strain 200 as a model organism. Preliminary kinetic data suggested that *A. putrefaciens* 200 possessed a ferri-reductase that was physiologically distinct from either cytochrome oxidase or nitrate reductase (Chapter 2). *A. putrefaciens* 200 was capable of reducing NO_3^- and Fe(III) simultaneously under anaerobic conditions but was unable to reduce either electron acceptor in the presence of O_2 . Previous studies demonstrated that *A. putrefaciens* 200 was also capable of utilizing Mn(IV) and S(IV) as alternative electron acceptors (see Chapter 1). In addition, *A. putrefaciens* 200 was capable of utilizing a wide range of organic compounds as sole carbon and energy source. The metabolic versatility displayed by this microorganism suggests that it is poised to couple the oxidation of a wide range of organic compounds to the reduction of a variety of alternative electron acceptors found in natural waters and sediments. *A. putrefaciens* spp. may therefore play an important role in the biogeochemical cycling of key elements such as carbon, nitrogen, manganese, sulfur and iron. The molecular details of the (dissimilative) iron reduction process have remained largely unstudied, however, and were the subject of further investigation.

Complementary biochemical and genetic analyses were used to study the molecular basis of the (dissimilative) iron reduction system of *A. putrefaciens* 200. A suite of iron-reduction-deficient mutants was generated via chemical (EMS) and transposon (Tn5) mutagenesis procedures. A newly developed screening technique was subsequently used to identify mutants deficient in both high-rate and low-rate iron reduction activity (Chapter 3). A conjugal gene transfer system was developed for mobilization of IncP1-based cloning vectors to *A. putrefaciens* 200. The broad host range (IncP1) cosmid cloning vector pVK100 was used to construct an

A. putrefaciens 200 gene clone bank in *E. coli* strains HB101 and S17-1 (mobilizing strain) (Chapter 4). Both three-way and two-way mating (conjugation) procedures were used to mobilize the gene clone banks into the suite of iron-reduction-deficient mutants during genetic (complementation) analysis. Two iron reduction clones (designated S4-E-2 and S18-F-4) were identified by their ability to restore iron reduction activity to several of the iron-reduction-deficient mutants. Preliminary biochemical characterization of selected mutant strains has indicated that cytochrome content may play an important role in the iron reduction process. Based on the results of the complementary genetic and biochemical studies, the iron reduction mutants have been placed into four classes: Class I (deficient in both high-rate and low-rate iron reduction activity, complemented by clones S4-E-2 and S18-F-4, possible *b*- or *c*-type cytochrome mutants), Class II (deficient in both high-rate and low-rate iron reduction activity, complemented by clone S4-E-2 but not by S18-F-4), Class III (proficient in high-rate iron reduction activity, but deficient in low-rate iron reduction activity, not complemented by either clone S4-E-2 or S18-F-4), and Class IV (deficient in both high-rate and low-rate iron reduction activity, not complemented by either clone S4-E-2 or S18-F-4, possible *d*-type cytochrome or anaerobic regulatory mutant). Future work will focus on further genetic and biochemical characterization of each class of iron reduction mutants.

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APPENDIX

**BIOEXTRACTION (REDUCTIVE DISSOLUTION) OF IRON
FROM LOW-GRADE IRON ORE USING PRIMARY EFFLUENT
WASTEWATER AS SOLE CARBON AND ENERGY SOURCE**

I. INTRODUCTION

The natural leaching of metals from insoluble minerals has been practiced for thousands of years (Brierly, 1982), however the central role that microorganisms play in mediating such processes has only recently been realized. A better understanding of the process by which bacteria catalyze the extraction of metals from naturally-occurring ores is due in a large part to the numerous studies on metal leaching by species of the genera *Thiobacillus* and *Sulfolobus*, both acidophilic chemolithotrophs. Initial leaching studies with these microorganisms (e.g., Ehrlich and Fox, 1967; Brock et al., 1972; Guay et al., 1977) concentrated on defining the optimum environmental conditions (e.g., temperature, pH, redox potential, particle size, substrate concentration) for the bioextraction process. More recent investigations have centered on the biochemical (Sugio et al., 1985) and genetic (Davidson et al., 1985) bases of the microbially-catalyzed reactions. Silverman (1967) first supported the idea that *Thiobacillus* and *Sulfolobus* species catalyze the oxidative dissolution of component metals (e.g., Fe, S, U, Sb, Mo) from metal-rich ores by both direct and indirect mechanisms. Direct oxidative dissolution is thought to involve direct attack by the bacteria on the ore surface and utilization of component minerals as suitable substrates for meeting bacterial energy requirements. For example, it is now generally accepted that members of the genera *Thiobacillus* and *Sulfolobus* are capable of oxidizing both the reduced iron and sulfur found in sulfidic minerals (e.g., pyrite). Reducing power derived from this oxidation is used to generate intracellular energy via classical (chemiosmotic) ATPase-coupled reaction pathways. Energy obtained in this manner is then used to fix carbon dioxide and thus support autotrophic growth. Under acidic conditions, the metabolically-generated by-products of the initial oxidation (e.g., Fe(III), S(VI)) are then released from the ore. In addition, these by-products may participate as strong chemical oxidants in subsequent reaction with uranium- and copper-

containing ores. The leaching process is then termed indirect. The indirect mechanism entails the Fe(II)-Fe(III) iron cycle whereby Fe(III) produced by bacterially-catalyzed Fe(II)-oxidation participates in secondary chemical oxidation and subsequent dissolution of otherwise insoluble nonferrous metals from various sulfides and oxides (e.g., FeS_2 , CuFeS_2 , UO_2). The mining industry has taken advantage of these microbially-driven processes to lower production costs and to aid in the development of more environmentally sound processes.

The coal and steel industries, in particular, have benefited from the recent advances in microbial leaching technology. When utilized in co-culture, *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* are able to accelerate the rate of oxidative dissolution of FeS_2 by several orders of magnitude (Brierly, 1982). Although this process adds an additional step in the overall reduction to elemental iron, it dramatically lowers transportation costs since the acid-soluble Fe(III) by-product is easily collected in the produced leachate. In addition, desulfurization (Hoffmann et al., 1981) of coal and low-grade iron ore by sulfur-oxidizing bacteria (before high temperature ore-beneficiation steps) can lessen the impact of SO_2 emissions to the atmosphere. If, however, the iron ore is of low sulfur content, it may be economically more attractive to implement a bacterially-catalyzed reductive dissolution step (at neutral pH, where Fe(II) is water soluble) preceding the high-temperature ore-beneficiation processes. Although several iron-reducing microorganisms have been isolated (Ottow, 1968; deCastro and Ehrlich, 1970; Brock and Gustafson, 1976; Obuekwe, 1980) the development of a bacterially catalyzed reductive dissolution process has been essentially overlooked. The purpose of this study was to test the ability of an iron-reducing microorganism to mediate the reductive dissolution of iron from a naturally-occurring semi-taconite iron ore, and to develop guidelines for scale-up to a commercially viable batch process. Figure A.1 shows a proposed scheme of the unit processes required for the

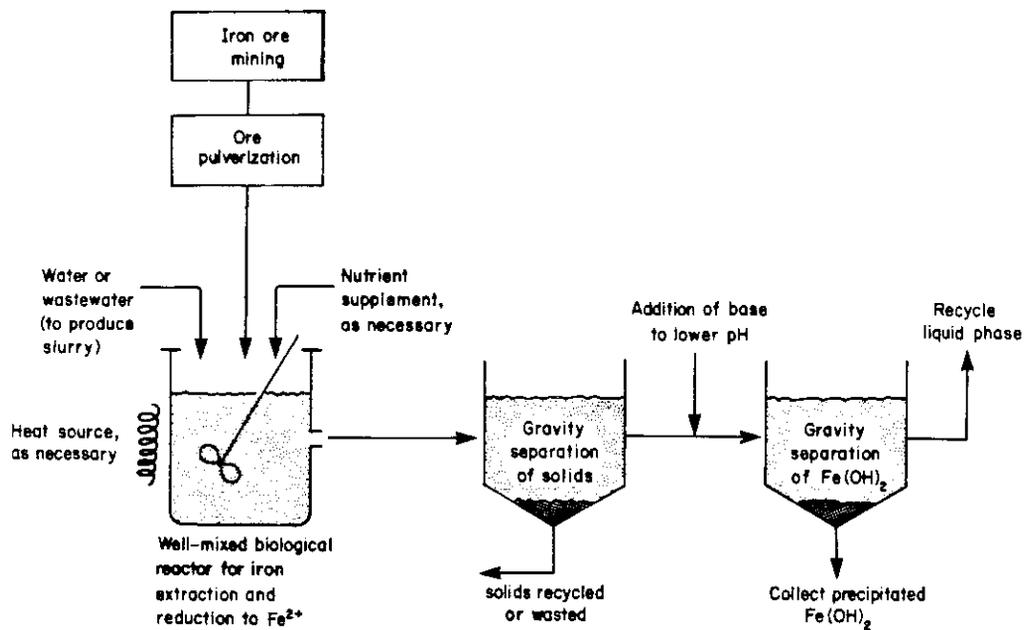


Figure A.1. Proposed scheme of the unit processes required for the reductive dissolution of iron from Fe(III)-bearing ores and subsequent recovery of Fe(II) (from Arnold, 1987).

reductive dissolution (bioleaching) of iron from Fe(III)-bearing ores with subsequent recovery of Fe(II) (Arnold, 1987). Commercial application of the proposed scheme depends on overall process kinetics, and therefore is most likely limited by the rate at which the iron-reducing microorganism (or consortia) catalyzes the reductive dissolution of iron from the representative iron ore. One such iron-reducing microorganism, *Alteromonas putrefaciens* strain 200, has been shown (Arnold et al., 1988) to mediate the reductive dissolution of several iron oxides (e.g., α -Fe₂O₃(s), FeOOH(s)) at rates high enough (mM·hr⁻¹ levels) to warrant further study with respect to its commercial applicability.

II. EXPERIMENTAL DESIGN

The ability of the marine eubacterium *Alteromonas putrefaciens* strain 200 (chemoheterotroph, facultative anaerobe) to catalyze the reductive dissolution of ferric iron from a naturally-occurring semi-taconite iron ore was tested in two series of batch reactor experiments. The two series of experiments differed solely in the identity of the substrate provided to the batch culture for microbial growth and reducing power. In the first set of experiments, a defined lactate medium was used; in the second set of experiments, wastewater obtained from the primary treatment effluent line at the San Jose Creek Wastewater Treatment Plant (Whittier, CA) was used. Lactate is the preferred carbon/energy source for iron reduction by *A. putrefaciens* 200 (Obuekwe, 1980). An abiotic control was also run for each set of experiments to determine the relative contributions of both chemical- and photo-induced reductive dissolution reactions. The experimental procedure outlined below was designed to facilitate pilot study scale-up to a commercially viable batch process.

A. Defined Lactate Medium as Substrate.

In each experiment, the batch reactor (Biostat M, B. Braun Instrument Co.) was filled to 1.5 L with lactate medium (see Chapter 2 for composition) and

autoclaved at 121°C for 30 minutes. Iron for satisfaction of microbial nutritional requirements was added from a filter sterilized FeCl₃ stock solution. Aerobic growth was initiated by adding 1.5 ml from a dense overnight culture of *A. putrefaciens* 200. Gas flow ($Q_{\text{air}} = 0.7$ L/min) and mechanical agitation provided mixing energy throughout the growth period. Temperature was held constant at 31°C. Microbial growth was monitored by periodic measurement of absorbance at $\lambda = 600$ nm. In order to induce high-rate iron reduction activity (Arnold et al., 1986b) air flow rates were reduced to maintain oxygen tensions $\leq 2 \mu\text{M}$ during the final 3 hours of aerobic growth.

At an A_{600} of 1.00 (2×10^9 cells/ml) the batch culture was purged of O₂ with high-purity, O₂-free nitrogen gas. Complexing agents (Sigma Chemical Co.) nitrilotriacetic acid (NTA) and/or ethylenediaminetetracetic acid (EDTA) were then added to appropriate levels, followed by addition of iron ore No. 1589. Solution pH was maintained at 7.0 ± 0.1 during chemical additions. Ferrous iron measurements (Arnold et al., 1986a) were initiated immediately following addition of iron ore No. 1589 and continued at regular intervals throughout the duration of the experiment (12 hours). The following system parameters were varied:

- (i) oxygen tension during aerobic growth of batch cultures of *A. putrefaciens* 200 (i.e., induction of high-rate iron reduction activity)
- (ii) complexing agent (either NTA and/or EDTA)
- (iii) concentration of complexing agent (1 mM, 10 mM)
- (iv) concentration of iron ore No. 1589 (1 mM, 10 mM as Fe(III))
- (v) iron ore particle size (2 μm , 10 μm).

The average particle size (10 μm) of naturally-occurring iron ore No. 1589 was lowered by repeated grinding (1 hour) on a Retsch-type mortar grinder. Microscopic visualization revealed an average particle size of approximately 2 μm after grinding.

B. Primary Effluent Wastewater as Substrate.

The experimental procedure used in the second set of batch experiments was essentially identical to the protocol followed in the experiments with lactate as the carbon/energy source, with the following exceptions:

- (i) The substrate supplied for both microbial growth and reducing power consisted of domestic wastewater (pH 7) (obtained from the primary effluent line at the San Jose Creek Wastewater Treatment Plant, Whittier, CA; see Table 3 for wastewater characteristics) supplemented with yeast extract (Difco; 0.25 g/L). Control experiments were run using yeast extract (pH 7) as the sole substrate for microbial growth and reducing power.
- (ii) Filter-sterilized (0.22 μm Nucleopore filter) wastewater medium (pH 7) was added to the empty batch reactor (autoclaved 30 min. at 121°C), followed by the initiation of temperature ($30^{\circ}\text{C} \pm 0.1$), pH (7.0 ± 0.1), and oxygen flow rate (0.7 L/min) control.
- (iii) Batch growth was initiated via the addition of a 1.5 ml culture grown for two days in wastewater medium supplemented with yeast extract (0.25 g/L).

The procedures for monitoring both microbial growth and reductive dissolution of iron ore No. 1589 were identical to those procedures used in the experiments with lactate as the sole carbon/energy source.

Table A.1. San Jose Creek WWTP (Whittier, CA)
Primary Effluent Quality
(L.A. County Sanitation District, 1985)

<u>Constituent</u>	<u>Concentration (mg/L)</u>
suspended solid	91.
Ammonia-N	19.0
Total COD	269.
Total BOD	135.
pH	7.14

III. RESULTS

Experimental data from the series of batch experiments is given in Table A.2 and Figure A.2.

Table A.2. Reductive Dissolution of Iron Ore No. 1589
by *A. putrefaciens* 200: Experimentally

Determined Rate Data						
Growth Medium	[Fe(III)] type ^a	{NTA} (mM)	{EDTA} (mM)	(I/U) ^b	Initial Rate ^c	Rate ^c after 1 hour
Lactate	10 mM Fe ₂ O ₃ ^d	10	--	U	2.0	2.0
Lactate	1 mM #1589	1	--	U	2.0	0.1
Lactate	10 mM #1589	10	--	U	2.0	0.3
Lactate	10 mM #1589	10	--	I	2.0	0.3
Lactate	10 mM #1589	10	10	U	3.0	0.5
Lactate	10 mM #1589 (2 μm particle)	10	10	U	3.0	1.0
Wastewater	10 mM #1589 (2 μm particle)	10	10	I	1.0	0.2

^a average iron ore particle size = 10. μm except where noted.
^b I = induced for high-rate ferri-reductase activity.
 U = uninduced for high-rate ferri-reductase activity.
^c M⁻¹hr⁻¹ × 10³
^d from Arnold et al., 1987.

- (i) Initial reductive dissolution rates are nearly identical to those rates obtained from previous experiments (Arnold et al., 1988) that used α-Fe₂O₃ (hematite) as the Fe(III) form during reductive dissolution experiments.
- (ii) A 10-fold increase in equimolar [NTA], [Fe(III)] yields a 3-fold increase in the rate of iron reduction.
- (iii) Induction of the high-rate iron reduction system in *A. putrefaciens* 200 does not appreciably enhance the rate of reductive dissolution of iron ore No. 1589.
- (iv) Addition of equimolar EDTA (plus NTA) yields a nearly two-fold increase in the reductive dissolution rate.
- (v) A decrease in iron ore particle size (from 10 μm to 2 μm) yields a 10-fold increase in initial iron reduction rate when equimolar EDTA and NTA are included.

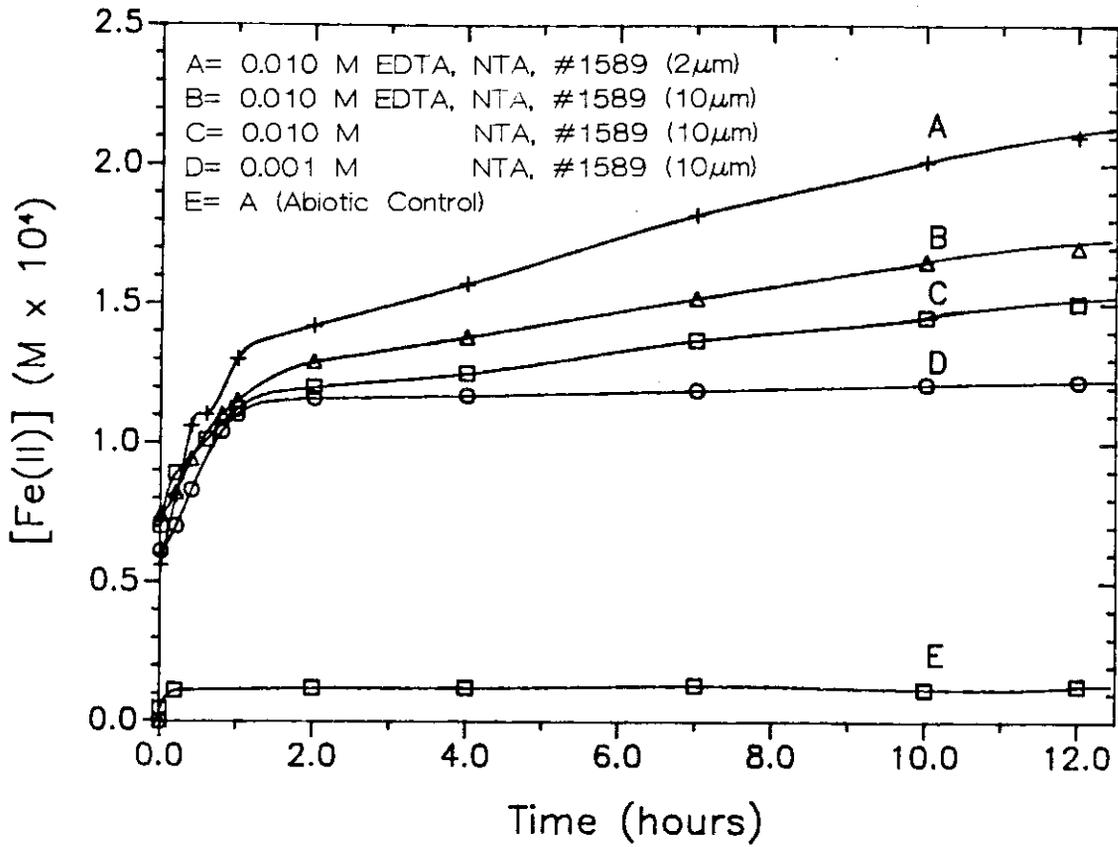


Figure A.2. Kinetic data from series of iron ore reductive dissolution experiments using filter-sterilized primary effluent wastewater as carbon and energy source.

- (vi) *A. putrefaciens* 200 was capable of growth on a medium consisting of primary effluent wastewater supplemented with yeast extract (0.25 g/L); the microorganism was unable to grow or reduce iron on yeast extract (pH 7) alone. The doubling time for batch cultures grown on wastewater medium was approximately 90 minutes (40 minutes on lactate medium).
- (vii) *A. putrefaciens* 200 was capable of catalyzing the reductive dissolution of iron from low-grade iron ore No. 1589 using primary effluent wastewater as its sole substrate for microbial growth and reducing power. Initial reductive dissolution rates ($1.0 \times 10^{-5} \text{ M} \cdot \text{hr}^{-1}$) were approximately 33% of the rate measured when batch cultures were grown on defined lactate media.
- (viii) Oxygen utilization rates ($3 \times 10^{-4} \text{ M} \cdot \text{hr}^{-1}$) of batch cultures grown on wastewater media were also 33% of the rate measured when batch cultures were grown on defined lactate media.

IV. SUMMARY

It appears that *Alteromonas putrefaciens* strain 200 is a suitable microorganism for use in a microbially-catalyzed process for the bio-extraction (reductive dissolution) of iron from low-grade iron ore. Scale-up of such a process for commercial application is made even more attractive by the ability of the microorganism to utilize a relatively inexpensive organic substrate (primary effluent wastewater) as the carbon and energy source. Yeast extract was added to the wastewater prior to the growth period as a nutrient supplement. Yeast extract is generally included in complex media to provide such essential growth factors as vitamins and trace metals for microbial cultivation.

Results from the batch experiments described above indicate that the rate of reductive dissolution of iron from low-grade ore is affected by several factors, including the concentration of iron ore (as Fe(III)) provided to the batch culture, the presence of complexing agents NTA and EDTA, and the identity and concentration of substrate provided for microbial growth and reducing power. At saturating solid Fe(III) concentration [10 mM iron ore as Fe(III)], the addition of equimolar EDTA and NTA resulted in a measurable increase in the initial and overall reductive dissolution rates. Whether the complexing agents increase total soluble Fe(III) or complex such inhibitory metals as Zn, Cu, Hg, and Pb contained within the iron ore is not known. A decrease in iron ore particle size enhances reductive dissolution rates, probably by increasing the surface area available for microbial attachment. Microorganism/Fe(III) particle contact is required for iron reduction by *A. putrefaciens* 200 (Arnold et al., 1988). Particle size effects, however, are not as pronounced as previously thought most likely because of the highly aggregated nature of Fe(III)-oxide particles at neutral pH (Stumm and Morgan, 1981).

Substitution of primary effluent wastewater for lactate as substrate during growth and reductive dissolution experiments resulted in a 67% reduction in reductive dissolution rates. In addition, oxygen utilization rates were nearly 30% of the rate measured when batch cultures were provided with lactate as reducing power. This is most likely a result of the electron flow limitations imposed on microbial metabolic processes by the less desirable substrate form. Log-phase growth rates for cultures grown on wastewater media were nearly two-fold slower than the values measured for batch cultures grown on lactate medium. A late log-phase cell density of approximately 2.0×10^9 cells/ml ($A_{600} = 1.0$) was obtained after 24 hours of growth on wastewater medium as opposed to 12 hours in the case of lactate medium. Nevertheless, the initial rates of iron ore reductive dissolution

($1.0 \times 10^{-5} \text{ M}\cdot\text{hr}^{-1}$) observed in the wastewater substrate/iron ore batch experiments are of sufficient magnitude to allow scale-up to a commercially viable batch process using the experimental details described here as the fundamental protocol. Since reductive dissolution rates after one hour are relatively slow, an additional step should include recycle of the ore remaining in the reactor (after the initial one hour treatment) into a series of batch reactors for similar bioleaching treatment with *Alteromonas putrefaciens* 200.

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