JOURNAL OF BACTERIOLOGY, Mar. 1994, p. 1468–1474
0021-9193/94/$04.00+0

Vol. 176, No. 5

Isolation of Anaerobic Respiratory Mutants of *Shewanella putrefaciens* and Genetic Analysis of Mutants Deficient in Anaerobic Growth on Fe^{3+}

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Received 7 September 1993/Accepted 16 December 1993

A genetic approach was used to study (dissimilatory) ferric iron (Fe^{3+}) reduction in *Shewanella putrefaciens* 200. Chemical mutagenesis procedures and two rapid plate assays were developed to facilitate the screening of Fe^{3+} reduction-deficient mutants. Sixty-two putative Fe^{3+} reduction-deficient mutants were identified, and each was subsequently tested for its ability to grow anaerobically on various compounds as sole terminal electron acceptors, including Fe^{3+}, nitrate (NO_3^-), nitrite (NO_2^-), manganese oxide (Mn^{4+}), sulfite (SO_3^{2-}), thiosulfate ($S_2O_3^{2-}$), trimethylamine *N*-oxide, and fumarate. A broad spectrum of mutants deficient in anaerobic growth on one or more electron acceptors was identified. Nine of the 62 mutants (designated Fer mutants) were deficient only in anaerobic growth on Fe^{3+} and retained the ability to grow on all other electron acceptors. These results suggest that *S. putrefaciens* expresses at least one terminal Fe^{3+} reductase that is distinct from other terminal reductases coupled to anaerobic growth. The nine Fer mutants were conjugally mated with an *S. putrefaciens* genomic library harbored in *Escherichia coli* S17-1. Complemented *S. putrefaciens* transconjugants were identified by the acquired ability to grow anaerobically on Fe^{3+} as the sole terminal electron acceptor. All recombinant cosmids that conferred the Fer^+ phenotype appeared to carry a common internal region.

The ability of microorganisms to catalyze the dissimilatory reduction of ferric iron (Fe^{3+}) has been known for nearly a century (15). Only recently have such redox transformations been linked to microbial energy transduction and anaerobic growth. At least three Fe^{3+}-reducing bacteria (*Geobacter metallireducans* [16], *Desulfuromonas acetoxidans* [29], and *Shewanella putrefaciens* [1, 17, 25]) are now known to conserve energy for anaerobic growth by coupling the oxidation of organic compounds to the dissimilatory reduction of Fe^{3+}. Compared with the wealth of information that exists concerning the genetic and biochemical details of other anaerobic respiratory systems (e.g., nitrate [NO_3^-], fumarate, and trimethylamine *N*-oxide [TMAO] respiration) of members of the heterotrophic *Proteobacteria* (14, 35), relatively little is known about the molecular details of anaerobic Fe^{3+} respiration.

G. metallireducans and *D. acetoxidans*, strictly anaerobic members of the δ *Proteobacteria*, are capable of supporting anaerobic growth by coupling the complete oxidation of a variety of organic compounds to the reduction of Fe^{3+} (16). *G. metallireducans* expresses Fe^{3+} reduction activity when grown anaerobically on either NO_3^- or Fe^{3+} as the sole terminal electron acceptor (11). Conversely, *G. metallireducans* does not express NO_3^--reduction activity when grown anaerobically on Fe^{3+}, an indication that this bacterium expresses a distinct Fe^{3+} reductase linked to anaerobic growth. Direct contact between cells and Fe^{3+} oxide particles is required for Fe^{3+} reduction (17). Respiratory inhibitors of *b*- and *c*-type cytochrome functions and of cytochrome oxidase activity do not affect Fe^{3+} reduction activity in *G. metallireducans* (11). Results of cytochrome oxidation experiments, however, suggest that *c*-type cytochromes do participate in electron transport to Fe^{3+} (16). Similar cytochrome oxidation experiments with *D. acetoxidans* indicate that this S^0- and Fe^{3+}-respiring marine organism also utilizes *c*-type cytochromes during anaerobic respiration on Fe^{3+} (29).

The nonfermenting, facultative anaerobe *S. putrefaciens*, a member of the heterotrophic γ *Proteobacteria* (20, 31), displays remarkable respiratory versatility, as it is able to respire on a variety of compounds as sole terminal electron acceptors, including oxygen (O_2), NO_3^-, nitrite (NO_2^-), manganese oxide (Mn^{4+}), sulfite (SO_3^{2-}), thiosulfate ($S_2O_3^{2-}$), TMAO, fumarate, Fe^{3+}, and potentially several others (18, 24). As with members of the δ *Proteobacteria*, the physiology of anaerobic electron transport to Fe^{3+} in *S. putrefaciens* is based on several lines of indirect evidence. *S. putrefaciens* cells do not produce a soluble, extracellular component that is capable of reducing Fe^{3+}, and direct contact between cells and Fe^{3+} oxide particles is required before Fe^{3+} reduction activity can be detected (2). Electron transport to Fe^{3+} appears to be respiratory chain linked and to require dehydrogenase and *b*- and *c*-type cytochrome functions (1, 22, 27). Prolonged exposure to O_2 (a preferred terminal electron acceptor) does not irreversibly inhibit the Fe^{3+} reduction activity of *S. putrefaciens* (1, 3, 22). Furthermore, when cytochrome oxidase activity is blocked by cyanide, *S. putrefaciens* is capable of reducing Fe^{3+} in the presence of nearly saturating O_2 concentrations (3). However, aerobically grown *S. putrefaciens* cells do not translocate protons under anaerobic conditions with Fe^{3+} as the sole terminal electron acceptor, while anaerobically grown cells do exhibit this capability (25). The Fe^{3+} reduction activity of anaerobically grown *S. putrefaciens* cells has been reported to be formate dependent and localized to the outer membrane fraction (22). Because of its ability to grow anaerobically on Fe^{3+} as the sole terminal electron acceptor (17, 18, 24), *S.*

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putrefaciens is thought to contain at least one Fe^{3+} reductase coupled to energy transduction.

The main objectives of the present study were to generate an array of Fe^{3+} respiration-deficient mutants of *S. putrefaciens* and to isolate from the wild-type *S. putrefaciens* genome DNA fragments that restored anaerobic Fe^{3+} respiratory capability to the mutants. This report describes (i) the development of chemical mutagenesis procedures and plate assays to facilitate the screening of an array of Fe^{3+} reduction-deficient mutants, (ii) the characterization of the overall anaerobic respiratory capability of the putative Fe^{3+} reduction-deficient mutants, and (iii) the genetic analysis of mutants deficient only in the ability to grow anaerobically on Fe^{3+} as the sole terminal electron acceptor.

MATERIALS AND METHODS

Bacterial strain and antibiotic concentrations. The Fe^{3+}-reducing bacterial strain used in the present study (*S. putrefaciens* 200; NCIB 12577) was isolated from a crude oil pipeline in 1980 by Obuekwe (26). Strain 200 has been alternately referred to in the literature as *Pseudomonas* sp. strain 200 (1–3, 27, 28), *Pseudomonas ferrireductans* (1), and *Alteromonas putrefaciens* 200 (31, 32). On the basis of more recent DNA-DNA (31) and 16S rRNA (9) hybridization studies, strain 200 has been reclassified as a member of the newly formed genus *Shewanella* (20), a heterogeneous group of the γ *Proteobacteria*. When required in the present study, antibiotics were added at the following concentrations (except as noted otherwise): chloramphenicol, 25 μg ml^{-1}; rifamycin SV, 100 μg ml^{-1}; streptomycin, 100 μg ml^{-1}; and tetracycline, 20 μg ml^{-1}.

Development of plate assay-based screening techniques for the detection of Fe^{3+} reduction-deficient mutants of *S. putrefaciens*. To facilitate the screening and subsequent detection of *S. putrefaciens* mutants deficient in Fe^{3+} reduction activity, two separate plate assay-based screening techniques were developed. The application of both techniques consisted of spreading a cell suspension of *S. putrefaciens* on an appropriate solid medium and, after 5 to 7 days of aerobic growth (30°C), scoring the resulting colonies for a specific Fe^{3+} reduction-dependent phenotype. In the first plate assay, the growth medium consisted of Fe^{2+}- and $S_2O_3^{2-}$-containing triple sugar iron agar (TSI; Difco Laboratories, Detroit, Mich.). In the second plate assay, the growth medium consisted of nutrient agar (Difco) supplemented with 50 mM Fe^{3+} · citrate (AGR medium).

Chemical mutagenesis of *S. putrefaciens* and isolation of putative Fe^{3+} reduction-deficient mutants. Ethyl methanesulfonate (EMS; Sigma Chemical Co., St. Louis, Mo.) was used as a chemical mutagen to generate an array of mutant strains deficient in Fe^{3+} reduction activity. Liquid cultures of a spontaneous rifamycin-resistant *S. putrefaciens* derivative were grown (30°C) to late-log phase in LB medium (30) supplemented with rifamycin, harvested (4°C), and washed and resuspended in phosphate-buffered saline solution (PBS; 8.7 mM Na_2HPO_4, 1.5 mM $NaH_2PO_4 \cdot H_2O$, 0.15 M NaCl). EMS was added to a final concentration of 10 μg ml^{-1}, and the cell suspension was incubated (30°C) with gentle mixing for a predetermined time period (60 to 75 min) to achieve 90% kill. Surviving cells were spread on TSI and AGR medium and, after 5 to 7 days of aerobic growth (30°C), the resulting colonies were scored for a wild-type phenotype (designated TSI^+ and AGR^+, respectively; see below for a description). Approximately 10,000 EMS-treated colonies were screened in this manner. Colonies that displayed negative (or weak), TSI^- and AGR^-, phenotypes (putative Fe^{3+} reduction-deficient mutants) were subsequently tested for their overall anaerobic respiratory capability.

Testing of the putative Fe^{3+} reduction-deficient mutants for overall anaerobic respiratory capability. The spontaneous rifamycin-resistant *S. putrefaciens* derivative was tested for its ability to grow anaerobically on a defined minimal medium (SM medium) (23) supplemented with either lactate, succinate, or formate (15 mM final concentration) as the sole organic carbon substrate and either NO_3^- (15 mM), NO_2^- (3 mM), Mn^{4+} (30 mM as MnO_2 [5]), Fe^{3+} (50 mM as Fe^{3+} · citrate [17]), TMAO (25 mM), SO_3^{2-} (10 mM), $S_2O_3^{2-}$ (10 mM), or fumarate (5 mM) as the sole terminal electron acceptor. Solid SM medium (pH 7.9) was prepared by adding Bacto Agar (Difco) to a final concentration of 1.5% (wt/vol). Putative Fe^{3+} reduction-deficient mutants were tested for anaerobic growth on solid SM medium supplemented with lactate and all electron acceptors tested except for NO_3^-. For anaerobic growth on NO_3^-, *S. putrefaciens* required formate as an obligate organic carbon substrate (see below). The spontaneous rifamycin-resistant and putative Fe^{3+} reduction-deficient mutant strains were first grown aerobically on solid SM medium (supplemented with lactate and rifamycin) and then patched onto an array of SM plates containing rifamycin and the various combinations of organic carbon substrates and terminal electron acceptors listed above. The inoculated SM plates were then incubated under anaerobic conditions (GasPak Plus anaerobic system; Becton Dickinson and Co., Cockeysville, Md.) for 10 to 14 days before each patch was scored for visible growth. All incubations were carried out in the dark at 30°C. Prior to placement of the anaerobic canisters at 30°C, each was kept overnight at 4°C to ensure that anoxic conditions had developed before allowing strain growth. Anaerobic growth control experiments consisted of patching each mutant on two sets of SM control plates, one in which all electron acceptors were omitted and the other in which all primary organic carbon substrates were omitted. Specific mutant strains (designated Fer mutants) were selected for further study on the basis of their inability to grow anaerobically on Fe^{3+} as the sole terminal electron acceptor while retaining their ability to grow on all other electron acceptors tested. Only these Fer mutants were tested for anaerobic growth on Mn^{4+} and fumarate as sole terminal electron acceptors.

Testing of the Fer mutants for Fe^{3+} reduction activity. The Fer mutants were each tested for Fe^{3+} reduction activity in a liquid assay (1, 6). Batch cultures were grown under either highly aerobic (>200 μM dissolved O_2) or microaerobic (<2.0 μM dissolved O_2) conditions in a 1.5-liter bioreactor system (6) with strict monitoring of dissolved O_2 concentrations (Ingold O_2 probe; Ingold Electrodes, Wilmington, Mass.). The growth medium consisted of a semidefined lactate medium (27) supplemented with rifamycin. After a target optical density (A_{600} = 0.25) was reached, chloramphenicol was added to each batch culture and the reactor vessel was purged with N_2. Fe^{3+} · citrate was then added (15 mM final concentration), and the production of Fe^{2+} was monitored for 60 to 90 min with a ferrozine assay (34, 36). The assay of Lowry et al. (19) was used to determine protein content. Abiotic control experiments were previously carried out (6) to ensure that chemical Fe^{3+} reduction was negligible under the conditions used in these experiments.

Construction of an *S. putrefaciens* gene clone bank. *S. putrefaciens* genomic DNA was isolated via previously described procedures (21). The broad-host-range cosmid pVK100 (Tet^r Kan^r Tra^+ [13]) was used as a cloning vehicle for genetic manipulations of *S. putrefaciens*. A genomic library of *S. putrefaciens* DNA was constructed by ligating (T4 DNA

TABLE 1. Results of growth experiments with wild-type *S. putrefaciens* 200

Organic carbon substrate[a]	Growth[b] on the following terminal electron acceptor[c]:									
	None	O_2	NO_3^-	NO_2^-	Mn^{4+}	Fe^{3+}	SO_3^{2-}	$S_2O_3^{2-}$	TMAO	Fumarate
None	−	−	−	−	ND	−	−	−	−	−
Formate	−	−	+	−	ND	−	+	+	+	−
Acetate	−	+	−	−	ND	−	−	−	−	−
Succinate	−	+	−	+	ND	+	−	−	−	−
Lactate	−	+	−	+	+[d]	+	+	+	+	+

[a] Sole organic carbon substrate added to SM growth medium as described in the text.
[b] −, no growth; +, growth; ND, not determined.
[c] Sole terminal electron acceptor added to SM growth medium as described in the text.
[d] Based on the ability of the colony to form clearing zone on Mn^{4+}-supplemented solid medium.

ligase; Bethesda Research Laboratories, Inc., Gaithersburg, Md.) gel-purified, partially digested *Hin*dIII fragments (20- to 30-kb size fraction) of *S. putrefaciens* genomic DNA to *Hin*dIII-digested pVK100. The recombinant cosmids were packaged in lambda bacteriophage particles (Lambda packaging kit; Boehringer Mannheim Biochemicals, Indianapolis, Ind.), and the recombinant phage were used to transform *Escherichia coli* mobilizing strain S17-1 (Sm^r Mob^+ [33]). The *E. coli* S17-1 transformants were plated on LB agar (37°C) supplemented with streptomycin and tetracycline, and approximately 3,000 colonies were pooled in PBS and stored at −80°C. The recombinant cosmids were isolated from 25 randomly selected transformants via alkaline lysis procedures (30). Agarose gel electrophoresis of *Hin*dIII- and *Eco*RI-digested recombinant cosmids was carried out on 0.7% agarose in 0.5× TBE buffer containing 0.5 μg of ethidium bromide ml^{-1} (30). Restriction enzymes (Promega Corp., Madison, Wis.) were used according to manufacturer's instructions.

Genetic analysis of the Fer mutants. Genetic analysis of the Fer mutants was carried out via mating experiments with the *S. putrefaciens* wild-type gene clone bank. Recipient Fer mutants were grown to mid-log phase in LB medium (30°C) supplemented with rifamycin, and the *E. coli* S17-1 clone bank pool was grown for two doublings in LB medium (37°C) supplemented with streptomycin and tetracycline. The recipient and donor cells were harvested (4°C), washed in LB medium, mixed at the appropriate ratio (100-μl total volume), and incubated (30°C) on LB agar for a predetermined mating period. For optimal conjugation frequencies (10^{-3} to 10^{-4} transconjugants per potential recipient), the optimal mating period was 4 h and the optimal ratio of recipient to donor cells was 5:1 (data not shown).

Each mating mixture was serially diluted and plated on SM agar supplemented with lactate, rifamycin, and tetracycline. After aerobic incubation for 4 to 5 days at 30°C, the resulting transconjugants were patched onto SM agar supplemented with lactate, rifamycin, tetracycline (40 μg ml^{-1}), and Fe^{3+} · citrate (50 mM final concentration). The transconjugants were then incubated under anaerobic conditions (as described above) for 10 to 14 days at 30°C. Putative Fer^+ complements (i.e., transconjugants displaying the acquired ability to grow anaerobically on Fe^{3+} as the sole terminal electron acceptor) were purified once on SM agar supplemented with lactate, rifamycin, and tetracycline, and single colonies were tested a second time for anaerobic growth on Fe^{3+}. The purified Fer^+ complements were subsequently tested for Fe^{3+} reduction activity in liquid cultures with the ferrozine assay described above.

Background contamination of DNA in plasmid preparations from *S. putrefaciens* strains (data not shown) necessitated the use of alternative methods to identify the complementing DNA fragments in the Fer^+ transconjugants. To circumvent this problem, each Fer^+ transconjugant (donor) was mated with *E. coli* S17-1 (recipient), and recombinant cosmids were isolated from the resulting tetracycline-resistant *E. coli* S17-1 transconjugants as described above. The mating procedures used in these experiments were essentially identical to those described above, with the exception of the mating period (6 h). After the mating period, the mating mixtures were pooled in PBS and used to inoculate 100 ml of LB medium containing streptomycin and tetracycline. The inoculum was incubated aerobically (37°C) with vigorous shaking for 2 to 4 days. A loopful of the enrichment culture was then purified twice (37°C) on LB agar supplemented with streptomycin and tetracycline. Purified *E. coli* S17-1 transconjugants were examined for plasmid content as described above. *E. coli* S17-1 transconjugants that harbored complementing (Fer^+) recombinant cosmids were subsequently mated with the remaining Fer mutants, and the resulting *S. putrefaciens* transconjugants were tested for the ability to grow anaerobically on Fe^{3+}.

Testing of the *E. coli* S17-1 transformants for Fe^{3+} reduction activity. The *E. coli* S17-1 transformants that contained complementing (Fer^+) cosmids were tested for their ability to (i) express the TSI^+ and AGR^+ phenotypes in the two plate assay-based screening techniques, (ii) express Fe^{3+} reduction activity in liquid cultures, as detected with the liquid ferrozine assay, and (iii) grow anaerobically on Fe^{3+} as the sole terminal electron acceptor. The procedures used in these experiments were identical to those used to test the Fer mutant and transconjugant strains for Fe^{3+} reduction activity, except that streptomycin was substituted for rifamycin in all growth media. Each experiment was run in duplicate, once at 30°C and a second time at 37°C. A control strain (*E. coli* S17-1 harboring pVK100 without cloned DNA) was included in these experiments.

RESULTS

Coupling of the organic carbon substrate and the terminal electron acceptor during anaerobic growth. Specific coupling between the organic carbon substrate and the terminal electron acceptor was observed in growth experiments with *S. putrefaciens* (Table 1). *S. putrefaciens* was capable of growth on (i) formate and either NO_3^-, TMAO, SO_3^{2-}, or $S_2O_3^{2-}$ (but not O_2, NO_2^-, Fe^{3+}, or fumarate), (ii) acetate and only O_2, (iii) succinate and either O_2, NO_2^-, or Fe^{3+} (but not NO_3^-, TMAO, SO_3^{2-}, $S_2O_3^{2-}$, or fumarate), and (iv) lactate and all electron acceptors tested except for NO_3^-. Regardless of the organic carbon substrate used, *S. putrefaciens* was unable to form colonies when incubated anaerobically on solid SM

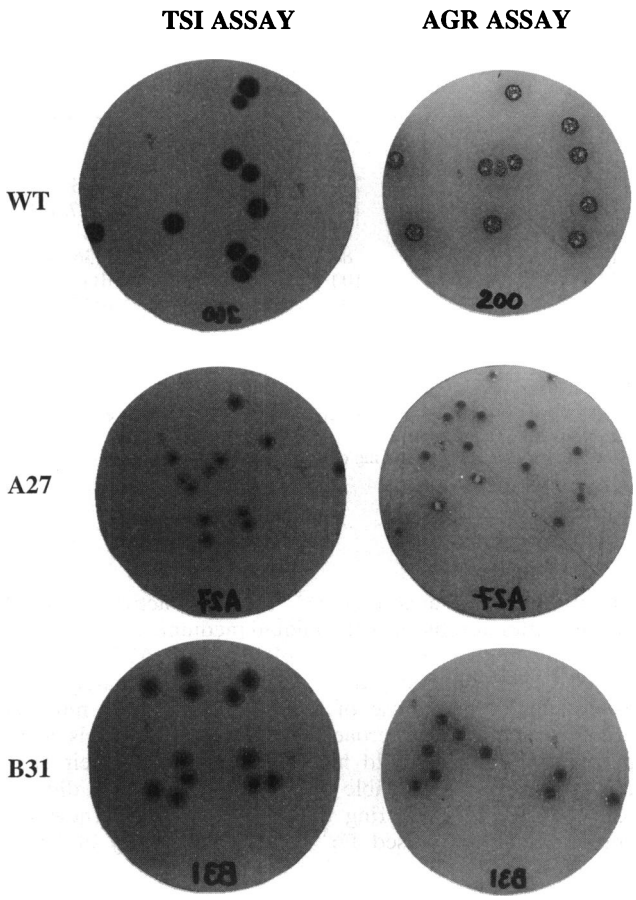

FIG. 1. TSI$^-$ and AGR$^-$ phenotypes of *S. putrefaciens* 200 class I (see text and Table 2) (Fer) mutants A27 and B31 (WT, wild-type strain). Serial dilutions of each strain were spread on the appropriate solid medium (as described in the text) and incubated aerobically for 5 (TSI assay) to 7 (AGR assay) days at 30°C. Note that the overlap of A27 colonies will result in the expression of an AGR$^+$ phenotype.

medium with Mn^{4+} as the sole terminal electron acceptor. However, clearing zones were evident around the vicinity of each patch after prolonged anaerobic incubation with lactate as the organic carbon substrate. This phenotype is thought to be indicative of respiratory chain-linked Mn^{4+} reduction by *S. putrefaciens* (see below) (23). Control experiments (Table 1) demonstrated that an organic carbon substrate and a terminal electron acceptor were required for anaerobic growth on solid SM medium. Although *S. putrefaciens* is capable of aerobic growth on citrate as an organic carbon source (26), it is incapable of coupling anaerobic citrate oxidation to Fe^{3+} reduction.

Development of plate assay-based screening techniques for the detection of Fe^{3+} reduction-deficient mutants of *S. putrefaciens*. Two plate assays were developed to facilitate the screening of large numbers of potential Fe^{3+} reduction-deficient mutants of *S. putrefaciens*. The first plate assay was based on the observation that wild-type *S. putrefaciens* formed a black iron sulfide precipitate on its colony surface when grown aerobically on TSI (TSI$^+$ phenotype; see Discussion; Fig. 1). The second plate assay was based on the observation that wild-type *S. putrefaciens* liquified the agar support directly below the colonies when grown aerobically on AGR medium

TABLE 2. Anaerobic respiratory capability of TSI$^-$ and AGR$^-$ mutants of *S. putrefaciens* 200

Mutant class	Growth deficiency[a]	No. of isolates
I	Fe^{3+} only	9
II	Fe^{3+} and NO_2^-	5
III	Fe^{3+} and SO_22-	2
IV	Fe^{3+} and $S_2O_3^{2-}$	15
V	SO_3^{2-} only	2
VI	$S_2O_3^{2-}$ only	8
VII	SO_3^{2-} and $S_2O_3^{2-}$	2
VIII	All electron acceptors except for O_2	16
IX	All electron acceptors except for NO_3^-	3
X	Fe^{3+} and NO_3^-	0[b]
XI	Fe^{3+} and TMAO	0[b]

[a] Defined as the inability to grow on solid SM medium with the listed compound as the sole terminal electron acceptor.
[b] Mutants with this phenotype were not isolated or detected.

(AGR$^+$ phenotype; see Discussion; Fig. 1). The AGR$^+$ phenotype was not detected when the wild-type strain was grown anaerobically on AGR medium. However, anaerobically grown colonies displayed the AGR$^+$ phenotype after exposure to O_2 for approximately 2 days. The AGR$^+$ phenotype was most evident with added 50 mM Fe^{3+} · citrate and undetectable at Fe^{3+} · citrate concentrations below 1 mM. After approximately 1 week of aerobic growth on AGR medium, wild-type colonies liquified enough agar to reach the bottom of the petri dish (Fig. 1), and the liquified agar in the colony "well" contained a high concentration of Fe^{2+} (data not shown).

Chemical mutagenesis of *S. putrefaciens* and isolation and phenotypic characterization of anaerobic respiratory mutants of *S. putrefaciens*. Approximately 10,000 EMS-treated colonies were screened via the TSI and AGR plate assays described above. Sixty-two EMS-treated colonies displayed a negative (or weak), TSI$^-$ or AGR$^-$, phenotype (see Fig. 1 for examples). The 62 putative Fe^{3+} reduction-deficient mutants were each tested for anaerobic growth on solid SM medium supplemented with lactate and each of the terminal electron acceptors listed above except for NO_3^-. (As a result of wild-type strain respiratory capability [Table 1], anaerobic growth on NO_3^- could only be tested with formate as an organic carbon substrate.) Results of the phenotypic characterization of the anaerobic respiratory capability of the 62 putative Fe^{3+} reduction-deficient mutants are presented in Table 2. On the basis of their anaerobic respiratory deficiencies, the mutants were placed into nine classes, each deficient in its ability to grow on one or more terminal electron acceptors: I (Fe^{3+} only; designated Fer mutants), II (Fe^{3+} and NO_2^-), III (Fe^{3+} and SO_3^{2-}), IV (Fe^{3+} and $S_2O_3^{2-}$), V (SO_3^{2-} only), VI ($S_2O_3^{2-}$ only), VII (SO_3^{2-} and $S_2O_3^{2-}$), VIII (all electron acceptors tested except for O_2), and IX (all electron acceptors tested except for NO_3^-). Mutants deficient in anaerobic growth on Fe^{3+} and NO_3^- (class X) and Fe^{3+} and TMAO (class XI) were not detected.

Fe^{3+} reduction activity of class I (Fer) mutants, deficient only in anaerobic growth on Fe^{3+} as the sole terminal electron acceptor. As observed in earlier studies (1), microaerobically grown *S. putrefaciens* expressed a high level of anaerobic Fe^{3+} reduction activity, approximately 10-fold higher than that of aerobically grown cells. All nine class I (Fer) mutants were deficient in anaerobic Fe^{3+} reduction activity after aerobic growth, while the seven strains isolated via the AGR plate assay (Fer mutants B25, B29, B31, B39, B41, B43, and B45) were also deficient in anaerobic Fe^{3+} reduction activity after

TABLE 3. Fe^{3+} reduction activity, O_2 utilization capacity, and complementing cosmids of *S. putrefaciens* 200 class I (Fer) mutants

Mutant	Isolation method[a]	Fe^{3+} rduction activity (%)[b]		O_2 utilization rate (%)[c]	Complementing cosmid(s)[d]
		Aerobic	Microaerobic		
200R[e]	NA	100	100	100	NA
B25	AGR	8.0	34	94	pB4
B29	AGR	7.0	16	100	pE43
B31	AGR	7.0	18	100	pG37, pB4
B39	AGR	1.0	10	82	pB4
B41	AGR	1.0	13	88	pF36, pI28
B43	AGR	5.0	12	100	pB4
B45	AGR	5.0	14	100	pP37
A5	TSI	16	99	99	pB4
A27	TSI	21	94	94	pB4

[a] NA, not applicable; AGR, AGR plate assay; TSI, TSI plate assay.
[b] For cells grown aerobically or microaerobically. Activity is expressed as a percentage of the wild-type activity (for 100% aerobically grown wild-type cells, 160 nmol mg of protein^{-1} min^{-1}; for 100% microaerobically grown wild-type cells, 1,500 nmol mg of protein^{-1} min^{-1}).
[c] The rate is expressed as a percentage of the wild-type rate (for 100% aerobically grown wild-type cells, 275 nmol mg of protein^{-1} min^{-1}).
[d] Recombinant cosmid(s) that complemented Fer mutants.
[e] Spontaneous rifamycin-resistant derivative of wild-type *S. putrefaciens* 200.

microaerobic growth (Table 3). The two Fer mutants isolated via the TSI plate assay (A5 and A27), although unable to grow anaerobically on Fe^{3+} as the sole terminal electron acceptor, retained the ability to express a high level of Fe^{3+} reduction activity (wild-type level) after microaerobic growth. In Fer mutants A5 and A27, the high-level Fe^{3+} reduction system does not appear to be coupled to anaerobic energy transduction. Each of the nine Fer mutants was capable of anaerobic fumarate respiration and also formed a clearing zone when tested for Mn^{4+} reduction. In addition, each of the nine Fer mutants was capable of utilizing O_2 at rates nearly identical to that of the wild-type strain (aerobically grown cells only; Table 3).

Genetic analysis of class I (Fer) mutants. Four randomly chosen *S. putrefaciens* class I (Fer) mutants (B29, B31, B41, and B45) were each mated with the *S. putrefaciens* gene clone bank pool harbored in *E. coli* S17-1. Approximately 1 in 500 *S. putrefaciens* transconjugants acquired the ability to grow anaerobically on Fe^{3+} as the sole terminal electron acceptor. Recombinant cosmids recovered from complemented transconjugants B29(pE43), B31(pB4), B31(pG37), B41(pF36), B41(pI28), and B45(pP37) contained *Hin*dIII inserts totalling 27.9, 23.0, 23.0, 23.3, 21.5, and 27.9 kb, respectively (Fig. 2). On the basis of the restriction patterns of *Hin*dIII-*Eco*RI double digests (Fig. 2), the cloned fragments in cosmids pE43 (i.e., complemented mutant B29) and pP37 (i.e., complemented mutant B45) appeared to be identical. Double digests of cosmids pB4 and pG37 (i.e., complemented mutant B31) indicated that the cloned fragments in these cosmids were also identical. All of the complementing cosmids (i.e., pE43, pB4, pG37, pF36, pI28, and pP37) contained a 13.5-kb *Hin*dIII cloned fragment with one internal *Eco*RI site (resulting in two *Hin*dIII-*Eco*RI fragments of 9.5 and 4.0 kb) (Fig. 2). Cosmids pF36 and pI28 complemented the same mutant strain, and the only common fragment was the 13.5-kb *Hin*dIII fragment noted above. Complementing cosmid pB4 was conjugally transferred into the remaining five Fer mutants (i.e., B25, B39, B43, A5, and A27), and the resulting transconjugants were tested for the ability to grow anaerobically on Fe^{3+}. *S. putrefaciens* transconjugants B25(pB4), B39(pB4), B43(pB4), A5(pB4), and A27(pB4) all displayed the ability to grow anaerobically on Fe^{3+} as the sole terminal electron acceptor (Table 3).

The B31, B45, and A27 *S. putrefaciens* transconjugant strains were also tested for anaerobic Fe^{3+} reduction activity in liquid cultures. After aerobic growth in liquid medium, *S. putrefaciens* transconjugants B31(pB4), B45(pP37), and A27(pB4) expressed Fe^{3+} reduction activity at levels approximately 3- to 15-fold higher than those of their respective (Fer mutant) parent strains; after microaerobic growth, the levels were approximately 4- to 5-fold higher than those of their (Fer mutant) parent strains (Table 4). Transconjugants that did not receive a cosmid conferring the Fer^+ phenotype (negative control strains) expressed Fe^{3+} reduction activity at levels

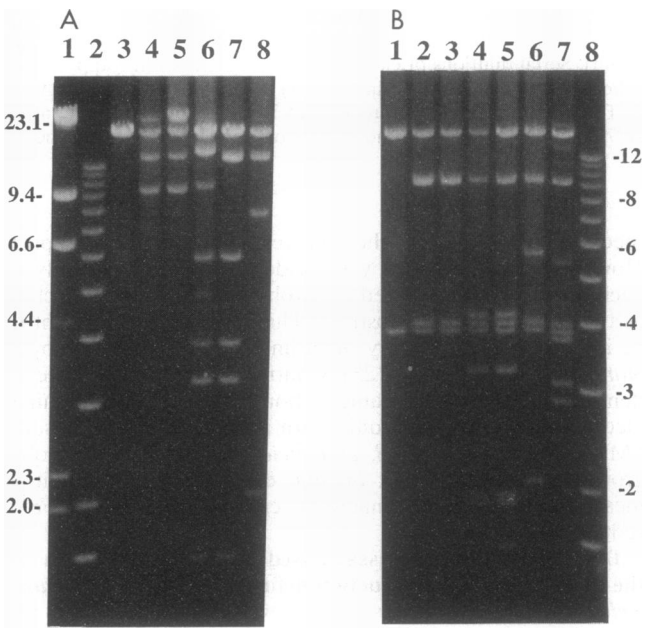

FIG. 2. (A) Agarose gel electrophoresis of *Hin*dIII-digested Fer^+ complementing cosmids. Lanes: 1, lambda markers; 2, 1-kb ladder markers; 3, pVK100 control; 4, pB4; 5, pG37; 6, pP37; 7, pE43; 8, pF36 (note that pP37 is only partially digested and that data are not shown for pI28). (B) Agarose gel electrophoresis of *Hin*dIII- and *Eco*RI-digested Fer^+ complementing cosmids. Lanes: 1, pVK100 control; 2, pB4; 3, pG37; 4, pP37; 5, pE43; 6, pF36; 7, pI28; 8, 1-kb ladder markers.

TABLE 4. Fe^{3+} reduction activity of complemented transconjugants of class I (Fer) mutants B31, B45, and A27

Strain	Anaerobic growth[a] on Fe^{3+}	Fe^{3+} reduction activity (%)[b]	
		Aerobic	Microaerobic
200R[c]	+	100	100
200R(pVK100)	+	100	100
B31	−	7.0	18
B31(pVK100)	−	11	12
B31(pB4)	+	109	67
B45	−	5.0	14
B45(pVK100)	−	7.0	10
B45(pP37)	+	44	54
A27	−	21	94
A27(pVK100)	−	16	ND
A27(pB4)	+	57	ND

[a] +, growth; −, no growth.

[b] For cells grown aerobically or microaerobically. Activity is expressed as a percentage of the wild-type activity (see Table 3, footnote *b*, for wild-type activity values). ND, not determined.

[c] Spontaneous rifamycin-resistant derivative of wild-type *S. putrefaciens* 200.

nearly identical to those of their respective (Fer mutant) parent strains. The *E. coli* S17-1 transformants that harbored complementing (Fer^+) cosmids pE43, pB4, pG37, pF36, pI28, and pP37 all tested negative for the TSI^+ and AGR^+ phenotypes, were unable to express anaerobic Fe^{3+} reduction activity in liquid cultures, and were unable to grow anaerobically on Fe^{3+} as the sole terminal electron acceptor (data not shown).

DISCUSSION

The molecular basis of microbial (dissimilatory) Fe^{3+} reduction is poorly understood. The major goal of the present study was to develop a genetic system for studying anaerobic Fe^{3+} respiration in *S. putrefaciens* 200. The genetic approach followed in the present study involved: (i) isolation of mutants deficient in anaerobic growth on Fe^{3+} as the sole terminal electron acceptor, (ii) identification of a group of the mutants (designated Fer mutants) that retained the ability to grow anaerobically on all anaerobic electron acceptors except for Fe^{3+}, and (iii) genetic complementation of the Fer mutants with an *S. putrefaciens* wild-type gene clone bank. Two separate plate assays were developed to facilitate the initial screening of putative Fer mutants. The plate assays were each based on a characteristic *S. putrefaciens* phenotype that correlated with Fe^{3+} reduction activity. In the first plate assay, wild-type *S. putrefaciens* formed a characteristic black iron sulfide precipitate on its colony surface after aerobic growth on TSI (TSI^+ phenotype). Since TSI contains both $S_2O_3^{2-}$ and Fe^{2+}, the production of an iron sulfide precipitate on TSI is generally thought to indicate S^{2-} production via either the reduction of $S_2O_3^{2-}$ or the degradation of cysteine (4). However, it has also been suggested (4) that, upon autoclaving, the Fe^{2+} component of TSI is oxidized to an amorphous Fe^{3+} oxide form. The formation of an iron sulfide precipitate on TSI may therefore indicate that a colony is capable of concomitantly reducing Fe^{3+} and producing S^{2-}. In the present study, a chemical analysis of iron speciation in (cooled) TSI indicated that the predominant iron form was Fe^{3+} and not ferrozine-extractable Fe^{2+}. Since *S. putrefaciens* 200 was capable of anaerobic growth on either Fe^{3+} or $S_2O_3^{2-}$ as the sole terminal electron acceptor, it was hypothesized that a (mutagenized) colony displaying a TSI^- phenotype might be deficient in either one or both anaerobic respiratory activities. This appears to be the case, as specific mutants deficient in the ability to grow anaerobically on Fe^{3+}, $S_2O_3^{2-}$, or both electron acceptors were detected by the TSI plate assay (see below). It also appears that these two anaerobically regulated respiratory systems are expressed during aerobic growth on TSI, possibly because of the development of anaerobic (or suboxic) conditions within the colony center.

The second plate assay was based on the observation that wild-type *S. putrefaciens* liquified the agar support directly below the colonies when grown aerobically on AGR medium (AGR^+ phenotype). For the expression of an AGR^+ phenotype, wild-type colonies required the presence of O_2 and a high concentration of Fe^{3+}. Previous studies (8) showed that under such conditions, wild-type *S. putrefaciens* colonies are capable of growing aerobically while also simultaneously reducing Fe^{3+}. It was hypothesized that (mutagenized) aerobically grown colonies that displayed an AGR^- phenotype might be deficient in Fe^{3+} reduction activity. This appears to be the case, as the AGR^- mutants detected by the AGR plate assay were deficient in anaerobic growth on Fe^{3+} (see below). The biological and chemical interactions that result in an AGR^+ phenotype are not known but may include a Fenton-type reaction (10) between the products of aerobic respiration and Fe^{3+} reduction (12, 37); the end products of this reaction then interact chemically with the agar medium.

Chemical mutagenesis of *S. putrefaciens* and application of the TSI and AGR plate assays resulted in the detection of a number of TSI^- and AGR^- mutants. The TSI^- and AGR^- mutants were classified according to their overall anaerobic respiratory capability and placed into nine mutant classes based on a deficiency in anaerobic growth on one or more electron acceptors. The isolation of a broad spectrum of mutants deficient in anaerobic growth on more than one electron acceptor suggests that the anaerobic electron transport systems in *S. putrefaciens* either may share components or may be under the control of a common regulatory element (e.g., FNR in *E. coli*). The isolation of class I (Fer) mutants provides evidence that *S. putrefaciens* expresses at least one terminal Fe^{3+} reductase that is distinct from other terminal reductases coupled to anaerobic growth, including NO_3^- reductase. Several investigators (as reviewed by Lovley [15]) had previously hypothesized that microbial (dissimilatory) Fe^{3+} reduction either was catalyzed directly by the enzyme NO_3^- reductase or was the result of a secondary chemical reaction (i.e., Fe^{3+} was reduced chemically by an organic compound or by bacterially produced NO_2^-, Mn^{2+}, or S^{2-}). Putative class I (Fer) mutants, isolated by a ferrozine spray screening technique and reported in an earlier study (8), have now all been reclassified as class VIII mutants (7). The ferrozine spray screening technique appears to detect only class VIII mutants (mutants deficient in growth on all electron acceptors except for O_2). Class I mutants may be undetectable with the ferrozine spray screening technique because the leaky Fer mutants (Table 3) produce a high ferrozine-Fe^{2+} background when grown aerobically on Fe^{3+}-supplemented nutrient agar.

A genetic analysis of four of the nine class I Fer mutants revealed that all recombinant cosmids that conferred the Fer^+ phenotype also carried a common internal region. This region consisted of two *Hin*dIII-*Eco*RI fragments of 9.0 and 4.5 kb. Each of the remaining five Fer mutants was also complemented by one of the original complementing clones (pB4) that contained this common region. This preliminary genetic analysis suggests that the genes required for anaerobic growth

on Fe^{3+} may be harbored on or near this region of the *S. putrefaciens* genome. Further analysis of the complementing fragments will provide information on the individual genes and gene products required for anaerobic respiration on Fe^{3+} as a sole terminal electron acceptor.

ACKNOWLEDGMENTS

Financial support for this study was provided in part by a Woods Hole Oceanographic Institution Postdoctoral Scholarship and a National Science Foundation Marine Biotechnology Fellowship (OCE-9023943 to T.J.D.) an Office of Naval Research Young Investigator Award (N00014-90-J-1917 to E.F.D.), and National Science Foundation Grant no. NSF-OCE-92-18532.

We thank Mark Barrett and Michael Torrente for laboratory assistance, Mary Lidstrom for technical advice, and Paul Dunlap and Dave Caron for providing laboratory space.

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