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ORIGINAL PAPER

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# Reduction of diverse electron acceptors by *Aeromonas hydrophila*

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**Abstract** *Aeromonas hydrophila* ATCC 7966 grew anaerobically on glycerol with nitrate, fumarate, Fe(III), Co(III), or Se(VI) as the sole terminal electron acceptor, but did not ferment glycerol. Final cell yields were directly proportional to the amount of terminal electron acceptor provided. Twenty-four estuarine mesophilic aeromonads were isolated; all reduced nitrate, Fe(III), or Co(III), and five strains reduced Se(VI). Dissimilatory Fe(III) reduction by *A. hydrophila* may involve cytochromes. Difference spectra obtained with whole cells showed absorption maxima at wavelengths characteristic of *c*-type cytochromes (419, 522, and 553 nm). Hydrogen-reduced cytochromes within intact cells were oxidized by the addition of Fe(III) or nitrate. Studies with respiratory inhibitors yielded results consistent with a respiratory chain involving succinate (flavin-containing) dehydrogenase, quinones and cytochromes, and a single Fe(III) reductase. Neither anaerobic respiration nor dissimilatory metal reduction by members of the genus *Aeromonas* have been reported previously.

**Key words** Nitrate reduction · Fe(III) reduction · Co(III) reduction · Se(VI) reduction · Mesophilic aeromonads · Cytochromes · Estuarine bacteria

## Introduction

The role of mesophilic aeromonads as opportunistic pathogens of both cold- and warm-blooded animals, including humans, is well-documented (Khardori and Fainstein 1988; Altwegg and Geiss 1989). Numerous studies have focused upon the incidence of aeromonads in the environment with regard to their role in public health (Hazen et al. 1978; Rippey and Cabelli 1989). Less is known concerning the overall physiology and ecology of the group. Aeromonads grow aerobically, or anaerobically by fermentation (Farmer et al. 1992). Members of the genus reduce nitrate. However, nitrate reduction has not been reported to be coupled to growth. This study was undertaken to evaluate the role of nitrate as an electron acceptor for anaerobic growth and to determine whether mesophilic aeromonads, being facultative anaerobes, were, in fact, also capable of anaerobic respiration with nitrate, metals, or metaloids.

The Fe(III) concentration in freshwater sediments often exceeds that of other potential electron acceptors such as $O_2$, $NO_3^-$, and $SO_4^{2-}$, providing the potential for significant nutrient release through anaerobic organic-matter mineralization and phosphorous mobilization coupled to dissimilatory Fe(III) reduction (Myers and Nealson 1990; Caccavo et al. 1992; Lovley et al. 1993; Roden and Lovley 1993; Nealson and Saffarini 1994). Iron in sediments often exists as poorly crystalline Fe(III) oxides (Lovley 1993; Nealson and Saffarini 1994). The extent of diversity among dissimilatory metal reducers is currently being revealed. Dissimilatory Fe(III) reduction has been reviewed recently (Lovley 1993; Nealson and Saffarini 1994).

In contrast to Fe(III), selenium is one of the least plentiful (0.001 ppm) but most toxic elements in the Earth's crust (Doran 1982). Selenium is biologically interesting because small differences exist between concentrations that are generally deemed essential (0.05–0.1 ppm) and those that are generally toxic (4 ppm). Diverse soil organisms reduce Se(VI) to Se(IV) or to elemental selenium aerobically as a detoxification strategy, but to date only two are known to be capable of anaerobic dissimilatory Se(VI) reduction to elemental selenium: *Thauera selenatis* (Macy et al. 1993) and an isolate SES-3 (Oremland et al. 1994).

Cr(VI) contamination of soil can be extensive as a result of industrial activities such as leather tanning. Reduc-

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tion of highly toxic soluble Cr(VI) to insoluble and less toxic Cr(IV) could be important in Cr(VI) remediation (Lovley 1993; Cooke et al. 1995; Shen et al. 1996). Cr(VI) reduction can be coupled to growth by a novel *Desulfomaculum* isolate (Obraztsova and Tebo 1997). Cr(VI) reduction (detoxification) has previously been documented for a novel mesophilic aeromonad, *Aeromonas dechromitica*. However, anaerobic growth yields of this isolate on Cr(VI) have not been determined (Kvasnikov et al. 1985).

Our interest in the physiology of *Aeromonas* was stimulated by a previous finding that one member of this genus, an *Aeromonas veronii* strain isolated from Great Bay Estuary (N.H., USA), although not an Fe(III) reducer, indirectly supports iron cycling through syntrophic associations with other organisms (Knight et al. 1996). We isolated numerous aeromonads from Great Bay Estuary and its tributaries (Knight and Blakemore, unpublished work) and examined 24 isolates for their ability to grow anaerobically with Fe(III): all of them actively reduced this metal. We also examined their growth anaerobically with nitrate, with other metals, or with the metaloid selenium as sole electron acceptors. Our results indicate that *Aeromonas hydrophila* ATCC 7966 is capable of anaerobic respiration and that an electron transport chain comprised, in part, of *c*-type cytochromes is involved in dissimilatory reduction of diverse electron acceptors by this organism.

## Materials and methods

### Bacterial strains

*A. hydrophila* ATCC 7966 (hereafter *A. hydrophila*) was obtained from the American Type Culture Collection (ATCC; Rockville, Md., USA). Mesophilic aeromonad isolates were obtained from water samples and shellfish from Great Bay Estuary (N.H., USA) or from water or frogs from its freshwater tributaries (V. Knight and R. Blakemore, unpublished work). Aeromonad isolates were cloned and identified to the genus level by using standard phenotypic biochemical tests (Popoff and Vernon 1976; Carnahan et al. 1991), and to the species level by fatty acid analysis (MIDI, Newark, Del., USA).

### Media and cultivation

Mesophilic aeromonads were isolated on Rimler-Shotts *Aeromonas* medium (Shotts and Rimler 1973) by using membrane filtration (American Public Health Works 1992). All incubations were at 37° C unless otherwise noted. This temperature, which provided selection at initial stages of isolation, was optimal for growth of all isolates and for the ATCC strain. For aerobic growth, cells were grown with shaking in trypticase soy broth (TSB; Difco, Detroit, Mich., USA).

For anaerobic growth, cells were grown in basal salts medium (BSM) that contained the following ($gl^{-1}$): $NaHCO_3$ (2.5), $NH_4Cl$ (1.5), $NaH_2PO_4$ (0.6), KCl (0.1), yeast extract (Difco: 0.1), vitamin solution (Wolin et al. 1963; 10 ml), and mineral solution (Wolin et al. 1963; 10 ml). This medium was supplemented with 30 mM glycerol and 20 mM Fe(III) as Fe(III) citrate for *A. hydrophila*, or Fe(III) oxyhydroxide chelated with equimolar nitrilotriacetic acid for the aeromonad isolates. Growth with alternate electron acceptors was in BSM supplemented with 30 mM glycerol and one of the following as the sole electron acceptor (mM): $NaSO_4$ (20), $Na_2SO_3$ (20), $MnO_2$ (20), thiosulfate (20), trimethylamine *N*-oxide (TMAO; 20), $NaNO_3$ (20), fumarate (20), $Na_2SeO_4$ (10), Co(III)-EDTA (1), or $CrCl_3 \cdot 6\ H_20$ (1) unless otherwise noted. Concentrations of alternative electron acceptors were chosen based upon the toxicity of the respective compounds. Amorphous Fe(III) oxyhydroxide was synthesized from $FeCl_2 \cdot 6\ H_20$ as previously described (Lovley and Phillips 1988). Co(III)-EDTA was synthesized from $CoCl_2 \cdot 6\ H_20$ by peroxide oxidation as previously described (Girvin et al. 1993). Standard anaerobic technique was used throughout (Miller and Wolin 1974). Culture medium was boiled and cooled while being sparged with $N_2$ $CO_2$ (80:20, v/v) from which traces of oxygen had been removed by passage through hot copper filings. Triplicate cultures were initiated at an approximate concentration of $2 \times 10^6$ cells $ml^{-1}$ with bacteria from aerobically grown cultures.

For electron balance experiments, *A. hydrophila* was grown at 37° C in BSM supplemented with 3 mM glycerol and 20 mM nitrate, 20 mM ferric citrate, 10 mM Se(VI), or 1 mM Co(III)-EDTA for 48, 60, 120, or 24 h, respectively. For reductant studies, cells were inoculated at an initial concentration of $2 \times 10^7$ cells $ml^{-1}$ into BSM with substrate but lacking terminal electron acceptor, and were incubated for 24 h to allow acclimation to substrate prior to addition of sterile 20 mM ferric citrate. Substrates were added from sterile stock solutions as follows (final concentration in mM): acetate (15), caproate (5), ethanol (15), formate (15), glycerol (15), lactate (15), malate (5), methanol (5), phenol (0.5), pyruvate (15), succinate (15), toluene (0.5), or hydrogen (5) or JP8 (jet fuel); $6.25 \times 10^{-4}$ ml $ml^{-1}$, v/v). Concentrations of the substrates used were based upon their expected toxicity. After growth for 72 h, triplicate samples were analyzed for cell number and for the concentration of Fe(II). Fe(II) concentrations were normalized to cell number to facilitate comparison between substrates.

For fatty acid analysis, isolates were grown aerobically on trypticase soy agar (BBL, Cockeysville, Md., USA) for 24 h at 28° C according to the microbial identification protocol for aerobes (MIDI).

### Cytochrome spectra

*A. hydrophila* was grown for 16 h in BSM anaerobically with 20 mM Fe(III) citrate. Bacteria were collected by centrifugation at $10{,}000 \times g$ for 10 min, washed twice, and resuspended in 10 mM Hepes (pH 7) to a final cell density of $5.4 \times 10^{11}$ cells $ml^{-1}$ (1.25 mg protein $ml^{-1}$). Dithionite-reduced *minus* air-oxidized spectra were collected as previously described (Collins and Niederman 1976). To determine if reduced cytochromes could be oxidized by alternate terminal electron acceptors [Fe(III) or nitrate], cell suspensions were reduced by sparging with hydrogen prior to the addition of Fe(III) or nitrate. Cell suspensions were transferred to a Thunburg cuvette with 500 µl 5 mM Fe(III) citrate or 5 mM nitrate in the side arm and were placed under a steady stream of $H_2$ for 5 min. The contents of the side arm were then mixed with the cell suspensions and immediately placed in a DU-640 spectrophotometer (Beckman, Fullerton, Calif., USA). The control contained 500 µl 10 mM Hepes in the side arm. Azide was added from concentrated stock to a final concentration of 1 mM.

### Fe(III) reductase activity

*A. hydrophila* was grown for 16 h at 37° C anaerobically in BSM supplemented with 30 mM glycerol and either 20 mM ferric citrate or 20 mM sodium nitrate and was also cultured aerobically in BSM supplemented with 30 mM glycerol and 20 mM ferric citrate. Bacteria were collected by centrifugation at $10{,}000 \times g$ for 10 min, washed twice, and resuspended in 10 mM Hepes (pH 7.0). Bacteria were broken by three passages through a French pressure cell at 15,000 psi (140 MPa). For the separation of the membrane and cytosol fraction, cell suspensions were centrifuged for 10 min at $10{,}000 \times g$ to remove unbroken cells. Cell-free extracts were then centrifuged at $200{,}000 \times g$ for 1 h to collect the membrane fraction. Fe(III) reductase activity was measured as the accumulation of Fe(II) by a modification of the assay of Dailey and Lascelles

(1977) as previously described (Myers and Myers 1993). The assay was performed under anaerobic conditions at room temperature in glass cuvettes (TCS Medical Products, Huntington Valley, Pa., USA). The assay mix was sparged with nitrogen prior to addition of cell-free extract. The assay mix (total volume, 2 ml) consisted of the following: glycerol, 10 mM; Fe(III) citrate, 0.12 mM; ferrozine, 0.4 mM; $KHPO_4$ buffer (pH 7.5; mixture of $K_2HPO_4$ and $KH_2PO_4$), 28 mM; and approximately 0.5 mg cell-free extract protein. Accumulation of Fe(II) was measured by following the absorbance at 562 nm by using a Beckman DU-640 spectrophotometer. A molar extinction coefficient of 28,000 was used (Dailey and Lascelles 1977).

### Inhibitor studies

*A. hydrophila* was grown anaerobically in BSM supplemented with 30 mM glycerol and 20 mM Fe(III) citrate for 16 h at 37° C. Bacteria were collected by centrifugation at 10,000 × *g* for 10 min, washed twice and resuspended in 10 mM Hepes (pH 7.0). Cells for the inoculum were sparged with $N_2$ $CO_2$ for 10 min and inoculated in triplicate into 30 mM bicarbonate buffer (pH 7.0; final concentration, $1.35 \times 10^{10}$ cells $ml^{-1}$; approximately 1.25 mg protein $ml^{-1}$). These were supplemented with 5 mM glycerol and respiratory inhibitors as shown in Table 3. Inhibitor-free controls were used to determine the effect of each solvent. Cell suspensions were incubated for 10 min with appropriate respiratory inhibitor prior to the addition of 5 mM Fe(III) citrate to start the reaction. After incubation for 12 h at 37° C, the concentration of Fe(II) was determined. Percent inhibition over that of controls without respiratory inhibitor was determined after correcting for the blank and any inhibitory effect of the appropriate solvent.

### Analytical techniques

Fe(II) production was assessed by using ferrozine after treatment of the sample with 0.5 N HCl (Lovley and Phillips 1988). Ferrous ethylenediamine-HCl was used as a standard. Growth was determined by using acridine orange direct counts after samples had been treated with oxalate to solubilize Fe(III) (Lovley and Phillips 1988). Growth data presented in Fig. 1 were determined by optical density measurements at 600 nm. Protein concentration was measured by using the BCA protein assay (Pierce, Rockford, Ill., USA) using bovine serum albumin (Sigma, St Louis, Mo., USA) as a standard. Citrate and glycerol were measured by HPLC using a Beckman System Gold HPLC equipped with a Phenomenex (Torrance, Calif., USA) Relex organic acid column (300 × 7.8 mm; particle size, 0.5 μm) and a Beckman Model 156 refractive index detector. The mobile phase was 0.005 M $H_2SO_4$ at a flow rate of 0.5 ml $min^{-1}$. Nitrate and nitrite were measured by using ion chromatography (Dionex model DX 100) with an AS9-SC (Dionex, Sunnyvale, Calif., USA) column using an eluent of 1.8 mM $Na_2CO_3$:1.7 mM $NaHCO_3$ with a flow rate of 2.0 ml $min^{-1}$ as previously described (Oremland and Culbertson 1992). Selenium oxyanions were measured by using ion chromatography (Dionex model LCM) with an AS4A (Dionex) column using an eluent of 1.8 mM $Na_2CO_3$:1.7 mM $NaHCO_3$ with a flow rate of 2.0 ml $min^{-1}$ as previously described (Oremland and Culbertson 1992). Co(III) was quantitated by measuring the absorbance at 535 nm as previously described (Caccavo et al. 1996). Chromium was measured with a colorimetric assay by using *s*-diphenylcarbazide (Urone 1955).

Fatty acid methyl esters (FAME) were extracted according to the microbial identification protocol (MIDI, Newark, Del., USA). FAME were separated on an HP 5890 gas chromatograph equipped with an FID detector (Hewlett-Packard, Wilmington, Del., USA) equipped with a 25-m × 0.2-mm phenylmethyl silicone fused silica capillary column. Isolates were identified by the TBSA library version 3.9 (MIDI).

## Results

### Growth with diverse electron acceptors

*A. hydrophila* grew on glycerol anaerobically with nitrate or fumarate (Fig. 1) as terminal electron acceptor. Glycerol

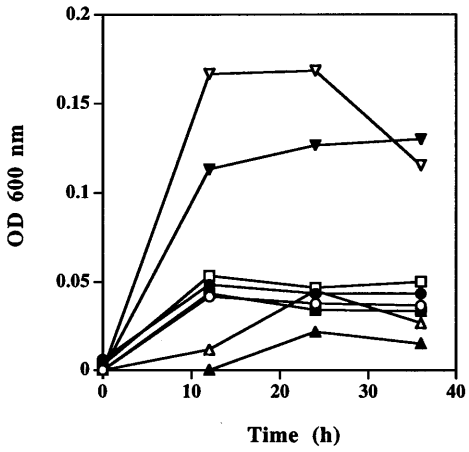


**Fig. 1** Anaerobic growth of *Aeromonas hydrophila* in basal salts medium supplemented with 30 mM glycerol and one of the following electron acceptors (20 mM): (▼) fumarate, (▽) nitrate, (■) thiosulfate, (□) Mn(IV), (▲) sulfite, (△) trimethylamine *N*-oxide, (●) sulfate, and (○) no added electron acceptor

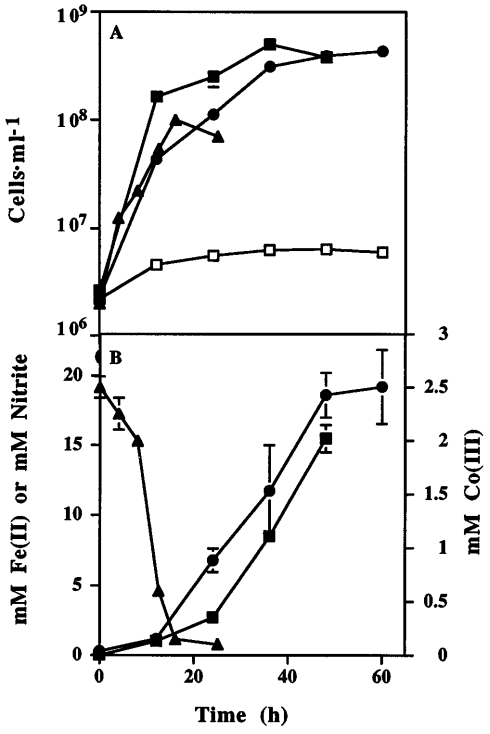


**Fig. 2** **A** Anaerobic growth, and **B** metal reduction by *Aeromonas hydrophila* in basal salts medium supplemented with 30 mM glycerol plus 20 mM ferric citrate, 20 mM sodium nitrate, or 1 mM Co(III)-EDTA. **A** Growth (cells $ml^{-1}$) with Fe(III) (●), $NO_3^-$ (■), Co(III) (▲), or no added electron acceptor (□). **B** Reduced electron acceptor formed (mM): Fe(II) (●) and $NO_2^-$ (■); or reduction (disappearance) or electron acceptor (mM): Co(III) (▲)

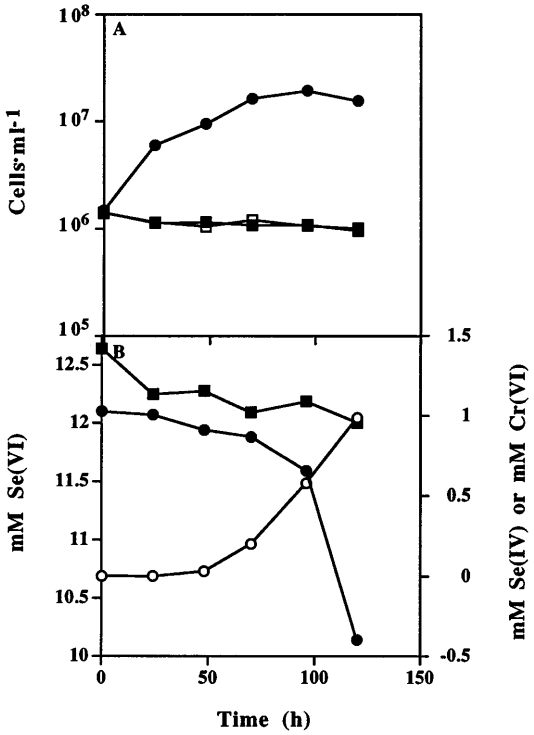


**Fig. 3** **A** Anaerobic growth, and **B** metal reduction by *Aeromonas hydrophila* in basal salts medium supplemented with 30 mM glycerol and 10 mM sodium selenate, or 1 mM Cr (VI). **A** (cells $ml^{-1}$) with selenate (●), Cr(VI) (■), or with no added acceptor (□). **B** Reduced electron acceptor formed (mM): Se(IV) (○); or reduction (disappearance) of electron acceptor (mM): Se(VI) (●) and Cr(VI) (■)

was not fermented, and no growth above that of the control lacking added electron acceptor occurred with sulfate, sulfite, thiosulfate, Mn(IV), or TMAO (Figs. 1). No growth occurred anaerobically without an added electron acceptor (Figs. 2A, 3A).

## Growth and reduction of nitrate, Fe(III), Co(III), Se(VI), and Cr(VI)

*A. hydrophila* grew anaerobically to a final cell density of $6.2 \times 10^8$ cells $ml^{-1}$ within 36 h in BSM supplemented with 30 mM glycerol and 20 mM nitrate (Fig. 2A). Reduction of nitrate occurred concomitantly with growth (Fig. 2B). *A. hydrophila* reduced 15 mM nitrate (all of the nitrate was reduced to nitrite) when grown anaerobically in BSM supplemented with 30 mM glycerol and 20 mM nitrate (Fig. 2B). Final cell yields and the amount of nitrate reduced after 48 h were directly proportional to the amount of nitrate provided over the range 1.25–10 mM (Fig. 4A).

*A. hydrophila* reached a final cell density of $4 \times 10^8$ cells $ml^{-1}$ (Fig. 2A) within 60 h when grown anaerobically in BSM supplemented with 30 mM glycerol and 20 mM ferric citrate. Reduction of Fe(III) occurred concomitantly with growth (Fig. 2). *A. hydrophila* reduced 20 mM Fe(III) when grown anaerobically in BSM supplemented with 30 mM glycerol and 20 mM Fe(III) citrate (Fig. 2B).

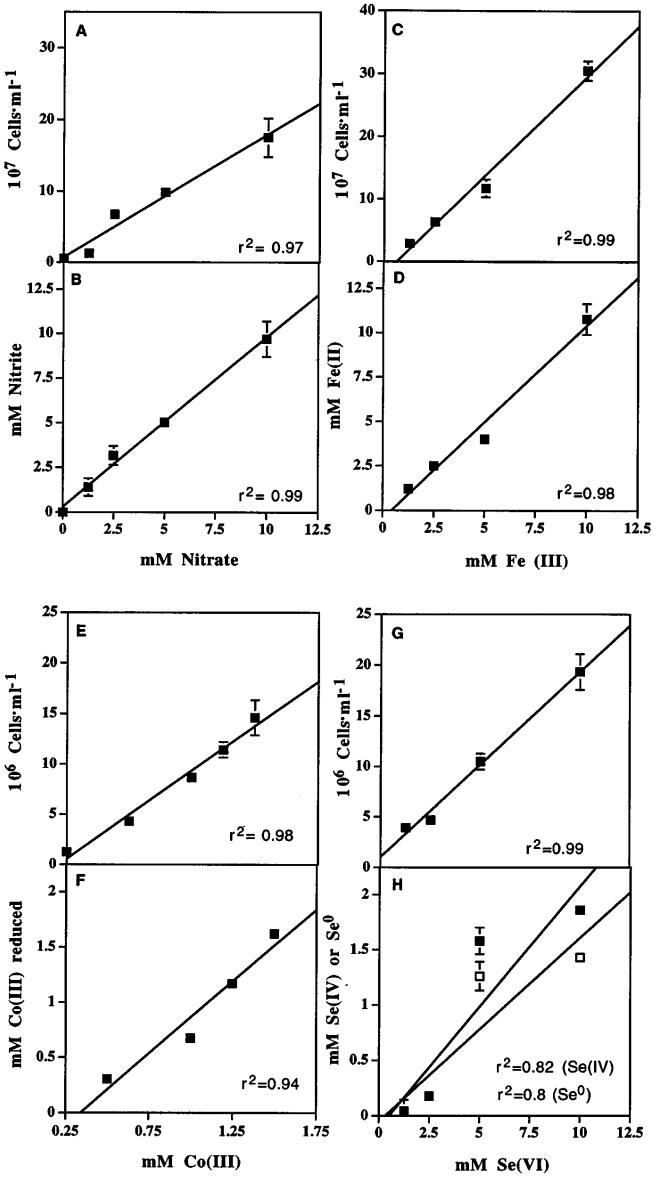


**Fig. 4A–H** Relationship of final cell yield (cell $ml^{-1}$) to concentration (mM) of electron acceptor provided, and of concentration (mM) electron acceptor reduced (or concentration of reduced electron acceptor) to concentration (mM) electron acceptor provided to *Aeromonas hydrophila* grown anaerobically in basal salts medium supplemented with 30 mM glycerol. **A** Cell yields vs. $NO_3^-$, **B** $NO_2^-$ produced vs. $NO_3^-$ provided, **C** cell yields vs. Fe(III) provided, **D** Fe(II) produced, vs. Fe(III) provided, **E** cell yields vs. Co(III) provided, **F** Co(III) reduced vs. Co(III) provided, **G** cell yield vs. Se(VI) provided, and **H** Se(IV) or $Se^0$ produced vs. Se(VI) provided

Final cell yields and the amount of Fe(III) reduced after 60 h were directly proportional to the amount of Fe(III) citrate provided over the range 1.25–10 mM (Fig. 4C). Growth in BSM supplemented with 30 mM glycerol did not occur when 20 mM unchelated Fe(III) oxyhydroxide was separated from the cells by dialysis tubing (data not shown). Growth of the control (unchelated ferric oxyhydroxide mixed with the cells) reached a final cell density of $1.2 \times 10^8$ cells $ml^{-1}$ after 48 h (data not shown).

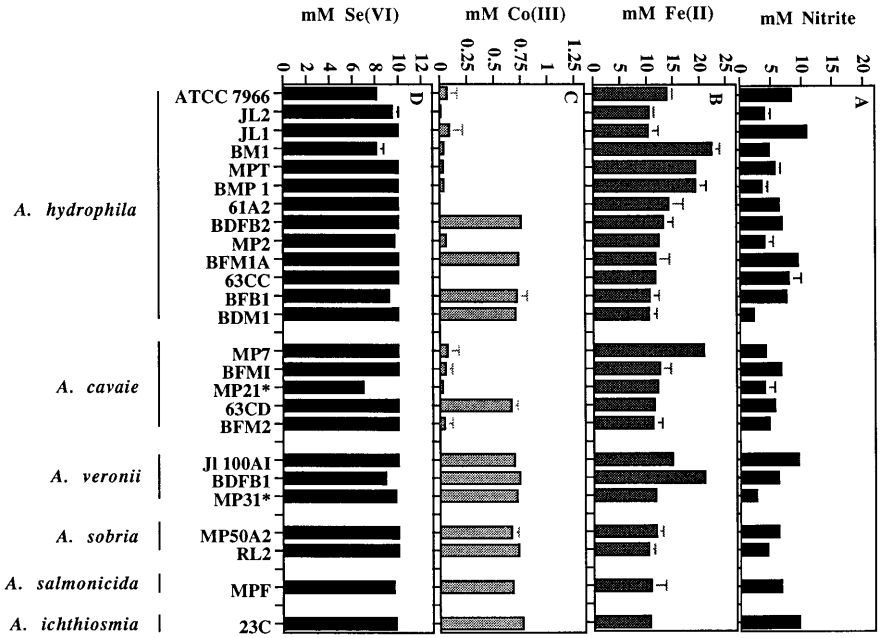


**Fig. 5** Metal and metaloid reduction by aeromonad isolates grown anaerobically in basal salts medium supplemented with 30 mM glycerol and one of the following electron acceptors: **A** 20 mM $NO_3^-$, **B** 20 mM Fe(III), **C** 1 mM Co(III), or **D** 10 mM Se(VI). Shown is the extent of reduction after incubation times with: $NO_3^-$, 48 h; Fe(III), 60 h; Co(III), 24 h; and Se(VI), 120 h

**Table 1** Balance of electrons and electron acceptors during growth of *Aeromonas hydrophila* in basal salts medium with different terminal electron acceptors (*NA* not applicable)

| Sample | Substrate used[a] (mmol) | % Carbon conversion to cells[a] | Electron acceptor reduced[b] (mmol) | Electrons produced[c] (mmol) | Electrons consumed[d] (mmol ) | Electron balance (%)[e] |
|---|---|---|---|---|---|---|
| No acceptor | 0 | NA | NA | NA | NA | NA |
| Nitrate | 2.8 | 22 | 14.2 | $3.1 \times 10^{-4}$ | $2.9 \times 10^{-4}$ | 93 |
| FE(III) | 1.3 | 33 | 11.9 | $1.3 \times 10^{-4}$ | $1.1 \times 10^{-4}$ | 89 |
| Se(VI) | 0.5 | 12 | 1.9 [Se(IV)] + 0.2 ($Se^0$) | $5.9 \times 10^{-5}$ | $4.6 \times 10^{-5}$ | 67 |
| Co(III) | 0.2 | 30 | 0.98 | $1.5 \times 10^{-5}$ | $9.8 \times 10^{-6}$ | 78 |

[a] Values are means of triplicate cultures corrected for background without substrate

[b] The increase in cell carbon was estimated to be approximately equal to the increase in protein concentration (Luria 1960)

[c] Calculated on the basis of the stoichiometric equations $C_3H_8O_3 + 3\ H_2O \rightarrow 3\ CO_2 + 14\ H^+ + 14\ e^-$ and corrected for carbon conversion to cell mass

[d] Calculated on the basis of the electron acceptor reduction as follows: $NO_3^- + H_2O + 2e^- \rightarrow NO_2^{2-} + 2\ OH$; $Fe(III) + e^- \rightarrow Fe(II)$; $SeO_4^{2-} + H_2O + 2\ e^- \rightarrow SeO_3^{2-} + 2\ OH^-$, and $SeO_4^{2-} + 4\ H_2O + 6e^- \rightarrow Se^0 + 8\ OH^-$; $Co(III) + e^- \rightarrow Co\ (II)$

[e] Electron balance calculated as (electrons consumed/electrons produced) × 100

*A. hydrophila* reached a final cell density of $1 \times 10^8$ cells $ml^{-1}$ (Fig. 2A) within 24 h when grown anaerobically in BSM supplemented with 30 mM glycerol and 2 mM Co(III)-EDTA. Reduction of Co(III) by *A. hydrophila* occurred concomitantly with growth (Fig. 2). *A. hydrophila* 2.5 mM Co(III) when grown anaerobically in BSM supplemented with 30 mM glycerol and 2.5 mM Co(III)-EDTA (Fig. 2B). The growth yield and the amount of Co(III) reduced after 24 h were directly proportional to the amount of Co(III)-EDTA provided over the range 0.5–1.5 mM (Fig. 4E).

*A. hydrophila* reached a final cell density of $2 \times 10^7$ cells $ml^{-1}$ (Fig. 3A) within 96 h when grown in BSM supplemented with 30 mM glycerol and 10 mM Se(VI). Reduction of Se(VI) by *A. hydrophila* occurred concomitantly with growth (Fig. 3). *A. hydrophila* reduced 2 mM Se(VI), and produced 1 mM Se(IV) within 120 h (Fig. 3B) when grown anaerobically in BSM supplemented with 30 mM glycerol and 10 mM Se(VI). The difference between Se(VI) reduced and Se(IV) formed is presumably due to the formation of elemental selenium as evidenced by the presence of red amorphous elemental selenium prior to filtration for analysis. Final cell yields and the amount of Se(VI) reduced after growth for 120 h were directly proportional to the amount of Se(VI) provided over the range 1.25–10 mM (Fig. 4G).

No growth above that of the control lacking a terminal electron acceptor ($1 \times 10^7$ cells $ml^{-1}$, Fig. 3A) occurred within 120 h with 1 mM Cr(VI) (Fig. 3A). *A. hydrophila* reduced 0.5 mM Cr(VI) within 120 h (Fig. 3B) when cultured anaerobically in BSM supplemented with 30 mM glycerol and 1 mM Cr(VI).

All 24 *Aeromonas* strains from Great Bay Estuary and the surrounding tributaries were capable of nitrate, Fe(III), or Co(III) reduction, and five strains reduced Se(VI) (Fig. 5).

## Electron balance

No glycerol dissimilation occurred in the absence of a terminal electron acceptor: *A. hydrophila* did not ferment glycerol. Stoichiometric conversion of glycerol occurred when the organism was grown anaerobically with nitrate, Fe(III), Se(VI), or Co(III) (Table 1). Corrections were made for the electrons consumed in biomass production and for the electrons produced in the "minus substrate" controls. In preliminary electron balance experiments, only 3 mM of 30 mM glycerol added was dissimilated (data not shown). The same amount of electron acceptor was reduced in cultures whether 3 or 30 mM glycerol was added (data not shown).

## Electron donors for Fe(III) reduction

*A. hydrophila* did not dissimilate citrate anaerobically as evidenced by HPLC analysis of culture fluids (data not shown). *A. hydrophila* reduced Fe(III) with glycerol, succinate, and lactate, producing 11.1, 3.1, and 1.0 mM Fe(II), respectively, after 72 h. A small amount of reduction (0.5 mM) occurred with $H_2$. Reduction occurred with pyruvate (3.41 mM). However, this is presumably due to acid production by fermentation since significant growth occurred in BSM without the addition of ferric iron and since the pH of spent culture medium was 4.8 (data not shown). No reduction by *A. hydrophila* occurred with acetate, caproate, ethanol, formate, JP-8 (jet fuel), malate, methanol, phenol, or toluene.

## Fe(III) reductase specific activity

Cell-free extracts of *A. hydrophila* grown anaerobically with Fe(III) reduced Fe(III) at a rate of 116 nmol $min^{-1}$ (mg protein)$^{-1}$ (Table 2). Extracts of cells grown aerobically ($O_2$ as the terminal electron acceptor) with Fe(III) reduced Fe(III) at 98% of this rate [114 nmol $min^{-1}$ (mg protein)$^{-1}$; Table 2]. Extracts of cells grown anaerobically with nitrate reduced Fe(III) at 35% of the rate [48 nmol $min^{-1}$ (mg protein)$^{-1}$] of cells grown anaerobically with Fe(III). Heat-inactivated (10 min at 100° C) extracts of cells cultured under any culture history did not reduce Fe(III) (the difference in the standard deviation is due to the sensitivity of the assay). Cell-free extracts of *A. hydrophila* grown aerobically with Fe(III), anaerobically with Fe(III), or anaerobically with nitrate (with NADH as the electron donor) reduced Fe(III) at 83%, 10%, or 41%, respectively, of the rate of those to which glycerol was added as the electron donor (Table 2).

To localize the reductase activity, extracts of cells grown anaerobically with Fe(III) were fractionated into membrane and cytosol fractions by ultracentrifugation as described by Myers and Myers (1992). Fe(III) reductase rates were 93% [108 nmol $min^{-1}$ (mg protein)$^{-1}$] and 14% [24 nmol $min^{-1}$ (mg protein)$^{-1}$] of the whole-cell rate for the membrane and cytosol fractions respectively (Table 2). No reduction occurred either in the absence of substrate or with heat-inactivated cell-free extracts.

**Table 2** Fe(III) reductase activity in cell extracts of *Aeromonas hydrophila* grown in basal salt medium supplemented with various electron acceptors. Results shown are averages of triplicate cultures ± standard deviations (*ND* not done)

| Culture history | Specific activity[a] | | |
|---|---|---|---|
| | 10 mM glycerol | 10 mM glycerol, heat killed | No glycerol, 0.5 mM NADH |
| Aerobic, Fe(III) | 114 ± 19 | 0 ± 11 | 95 ± 0 |
| Anaerobic, $NO^{3-}$ | 48 ± 25 | 10 ± 0 | 5 ± 6 |
| Anaerobic, Fe(III) | 116 ± 28 | 11 ± 19 | 48 ± 0 |
| Cytosol | 24 ± 22 | 0 ± 8 | ND |
| Membrane | 108 ± 23 | 0 ± 23 | ND |

[a] nmol $min^{-1}$ (mg protein)$^{-1}$

## Cytochromes

Dithionite-reduced *minus* air-oxidized absorption spectra obtained with intact cells of *A. hydrophila* grown anaero-

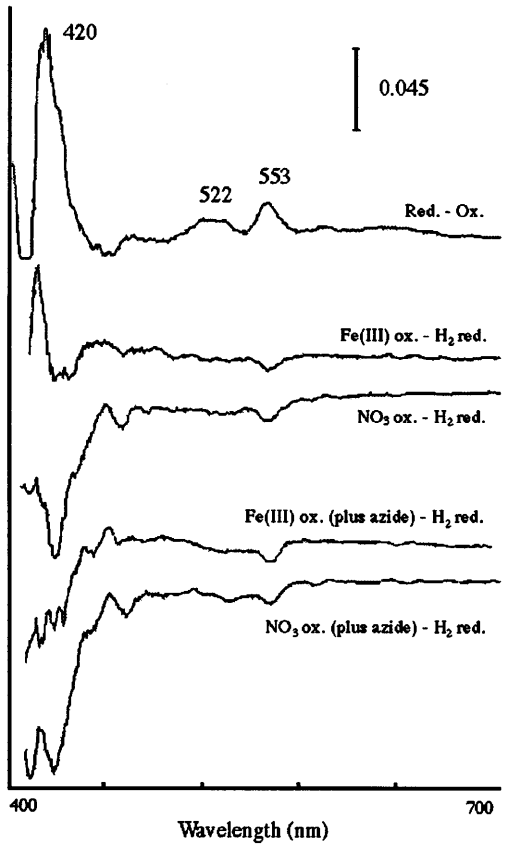


**Fig. 6** Room temperature difference spectra of cytochromes in intact, washed cells of *Aeromonas hydrophila* grown anaerobically in basalt salts medium supplemented with 30 mM glycerol and 20 mM Fe(III) citrate (*Red* reduced, *Ox.* oxidized)

**Table 3** Concentration and effect of respiratory inhibitors on Fe(III) reduction by cell suspensions of *Aeromonas hydrophila*. Inhibitors were dissolved in minimal solvent. Inhibition of Fe(III) reduction due to solvent-only control was subtracted from sample values (*HOQNO* 2-heptyl-4-hydroxyquinoline *N*-oxide, *CCCP* carbonly-cyanide-*m*-chlorophenylhydrazine, *NA* not applicable)

| Inhibitor | Inhibition site | Solvent | Concentration (M) | Specific activity[a] | Percent activity[b] |
|---|---|---|---|---|---|
| No inhibitor | NA | NA | NA | 1.69 ± 0.13 | 100 ± 7 |
| Quinacrine | (Flavins) dehydrogenase | $H_2O$ | $1 \times 10^{-4}$ | 0.19 ± 0.01 | 11 ± 7 |
| Rotenone | NADH dehydrogenase | Acetone | $1 \times 10^{-4}$ | 1.5 ± 0.25 | 109 ± 14 |
| Dicumarol | Quinones | 0.05 N NaOH | $1 \times 10^{-5}$ | 0.26 ± 0.03 | 28 ± 15 |
| HOQNO | Cytochrome *b* | Ethanol | $1 \times 10^{-5}$ | 0.35 ± 0.04 | 24 ± 1.5 |
| Azide | Terminal oxidase | $H_2O$ | $1 \times 10^{-3}$ | 1.1 ± 0.33 | 64 ± 20 |
| CCCP | Protonophore | Ethanol | $1 \times 10^{-5}$ | 0.07 ± 0.02 | 18 ± 6 |
| Heat-killed cells | NA | NA | NA | 0 ± 0.01 | 0 ± 0.1 |

[a] nmol $h^{-1}$ (mg protein)$^{-1}$
[b] Percent activity of cell suspensions without addition of inhibitor

bically with Fe(III) (Fig. 6) contained peaks characteristic of *c*-type cytochromes (absorption maxima at 420, 522, and 553 nm). Hydrogen-reduced cytochromes were oxidized by the addition of Fe(III) or nitrate. Oxidation of hydrogen-reduced cytochromes reduced by either of these alternate terminal electron acceptors was unaffected by the addition of azide (Fig. 6).

### Inhibitor studies

Fe(III) reduction by suspensions of washed cells grown anaerobically with Fe(III) was inhibited (Table 3) by the addition of quinacrine (89%), dicumarol (72%), 2-heptyl-4-hydroxyquinone *N*-oxide (HOQNO; 76%), carboyl-cyanide-*m*-chloropheylhydrazine (CCCP; 82%), to a lesser extent by azide (36%), but not by rotenone. Heat-killed cells (100° C for 10 min) failed to reduce Fe(III) (100% inhibition of the control rate).

## Discussion

Mesophilic aeromonads are facultative anaerobes that are ubiquitous in aquatic habitats. They have been reported by others to grow aerobically, or anaerobically by fermentation (Farmer et al. 1992). We report here that *A. hydrophila* carries out anaerobic respiration with nitrate, Fe(III), Co(III), Se(VI), or fumarate. We isolated 24 mesophilic aeromonads from Great Bay Estuary and its tributaries. These isolates were collected by using established (aerobic) selective and differential plating techniques for mesophilic aeromonads (Shotts and Rimler 1973), not by enrichment for metal reducers. We observed Fe(III) and $NO_3^-$ reduction by all *Aeromonas* isolates. Amorphous Fe(III) oxyhydroxide gave poor growth and reduction. However, by chelating the Fe(III) with citrate, EDTA, or nitrilotriacetic acid, increases were obtained in both final cell yields and the extent of reduction (data not shown). Although Fe(III) in sediments often exists as poorly crystalline Fe(III) oxides (Lovley 1993; Nealson and Saffarini 1994), use of this form of iron by metal reducers often occurs at a faster rate with chelated iron (Arnold et al. 1988; Dobbin et al. 1995).

Our data also show that five of our 24 aeromonad isolates can grow by means of dissimilatory selenate reduction. In one previous report, aeromonads had been isolated as part of a large group of organisms from a selenite-contaminated site (Burton et al. 1987). However, in that study only the resistance of these organisms to selenite and not the extent of growth had been examined (Burton et al. 1987). *A. hydrophila* is also capable of dissimilatory growth with Co(III). All of our aeromonad isolates were capable of Co(III) reduction. However, the extent varied among isolates. Only a limited number of bacteria are known to share the ability to grow in a dissimilatory manner using Co(III) as the terminal acceptor [Caccavo et al. 1994; Caccavo et al. 1996; Y. Gorby, (Batelle Pacific Northwest Laboratories Richland, Wash., USA), personal communication]. Cr(VI) reduction has been reported previously for a novel Cr(VI)-reducing aeromonad, *Aeromonas dechromatica* (Kvasnikov et al. 1985). However, the relatively small amounts of Cr(VI) reduction and low growth rates observed are suggestive of reductive detoxification of this metal and not of a role in anaerobic respiration. We obtained similar results with *A. hydrophila*.

We obtained representatives of five species of aeromonads within the 24 isolates collected. All of these isolates were capable of nitrate and Fe(III) reduction. The ability to reduce Se(VI) and the extent of Co(III) reduction varied among the isolates. In addition, we isolated three strains of *Aeromonas veronii* that were able to reduce Fe(III). However, we have previously reported that a different *A. veronii* strain isolated from Great Bay Estuary does not reduce Fe(III) (Knight et al. 1996). Therefore, this appears to be a variable trait within this species.

To avoid the possibility of terminal electron acceptor reduction due to pH or $E_0$ changes, we used the non fermentable substrate glycerol. Our electron balance results confirmed that glycerol was not fermented. Reduction of nitrate, Fe(III), Se(VI), and Co(III) are stoichiometrically coupled to dissimilation of glycerol. This supports anaerobic respiration by *A. hydrophila* coupled to each of these electron acceptors. The low electron balance for Se(VI)

and Co(III) could be due to low substrate usage or to the formation of undetected end products. In preliminary electron balance experiments, only 3 mM glycerol was dissimilated (data not shown) even though 30 mM glycerol was routinely added to the cultures of *A. hydrophila*. Furthermore, the same amount of electron acceptor was reduced when 3 or 30 mM glycerol was added to the culture medium.

Although *A. hydrophila* used several terminal oxidants, few electron donors were used to support growth. Of the potential substrates surveyed as electron donors, Fe(III) reduction occurred with glycerol, succinate, and – to a lesser extent – lactate. The reduction observed with pyruvate was due to fermentation of this substrate as evidenced by significant growth in controls with pyruvate but lacking in Fe(III).

Fe(III) reduction by *A. hydrophila* is dissimilatory in nature: growth and Fe(III) reduction occurred concomitantly, and no growth occurred in the absence of added Fe(III). The extent of Fe(III) reduction and the final cell yields were directly proportional to the quantity of Fe(III) supplied. Fe(III) reduction was enzymatic as evident from several observations: (1) the quantity of Fe(III) reduced increased with temperature (with a sharp decline at 45° C) (2) no reduction occurred when unchelated ferric oxyhydroxide was separated from the cells by dialysis tubing, and the (3) pH throughout growth remained circumneutral at 6.7.

The mechanism of Fe(III) reduction by *A. hydrophila* appears to be by a membrane-associated Fe(III) reductase. All of the Fe(III) reductase activity is located in the membrane fraction. It is interesting to note that cell extracts of bacteria previously grown on nitrate reduced Fe(III) at 41% of the rate of those grown anaerobically or aerobically on Fe(III). The results were similar to those obtained by others with the dissimilatory Fe(III) reducer *Geobacter metallireducens* strain GS-15 (Gorby and Lovley 1991). Nitrate-grown cells of GS-15 reduced Fe(III) at 50% of the rate of Fe(III)-grown cells (Gorby and Lovley 1991). Fe(III) reduction in washed cell extracts of *A. hydrophila* grown aerobically with Fe(III) occurred at the same rate [114 nmol $min^{-1}$ (mg protein)$^{-1}$] as that of cells grown anaerobically with Fe(III). This suggests that ferric reductase activity is constitutive and is not induced by anoxia. *Shewanella putrefaciens* sp. 200 produces two distinct reductases. A constitutive reductase that is produced under oxic and anoxic conditions is involved in an abbreviated electron transport chain under Fe(III)-reducing conditions (Arnold et al. 1986). The second reductase is induced during microaerobic growth and acts predominantly as an electron sink (Arnold et al. 1986).

*C*-Type cytochromes have been detected in all dissimilatory Fe(III) reducers (Arnold et al. 1986; Myers and Myers 1993; Nealson and Saffarini 1994; Caccavo et al. 1996) with one exception (Lovley et al. 1995). Dithionite-reduced *minus* air-oxidized spectra confirmed the presence of *c*-type cytochromes in *A. hydrophila.* Hydrogen-reduced cytochromes from *A. hydrophila* cells grown anaerobically with Fe(III) were oxidized by the addition of Fe(III) and nitrate, which suggests that they may be involved in the transfer of electrons to each of these electron acceptors. Reoxidation of hydrogen-reduced cytochromes was not blocked by the addition of azide. This inhibitor blocks the terminal oxidase of aerobically grown cells. Therefore, our results are suggestive of a distinct terminal reductase being utilized during anaerobic growth. The addition of HOQNO inhibited Fe(III) reduction. This inhibitor blocks transfer of electrons to cytochromes and is consistent with a role of cytochromes in electron transport to Fe(III). Pleiotropic mutants of *S. putrefaciens* sp. 200 that lack cytochromes are not capable of Fe(III) reduction (Saffarini et al. 1994). Mutants of *E. coli* K-12 that lack quinones and cytochromes reduced Fe(III) at rates similar to those of the parent strain. However, this reductase, unlike that of dissimilatory Fe(III) reducers, was a soluble enzyme not involved in dissimilatory metal reduction (Williams and Poole 1987).

Fe(III) reduction by washed suspensions of cells grown anaerobically with Fe(III) was not affected by the addition of rotenone. This suggests that NADH dehydrogenase of *A. hydrophila*, unlike that of many Fe(III) reducers (Arnold et al. 1986; Gorby and Lovley 1991), is repressed or inactive under Fe(III)-reducing conditions. In addition, extracts of cells grown anaerobically with Fe(III) reduced Fe(III) at 10% of the control rate (glycerol as donor) when NADH was added as an electron donor. This suggests that NADH is not an electron donor under these conditions for *A. hydrophila*. Quinacrine inhibited Fe(III) reduction to 11% of the control rate (no inhibitor) in suspensions of washed cells grown under Fe(III)-reducing conditions. This inhibitor blocks the flavin-containing enzymes such as succinate dehydrogenase. This suggests that succinate dehydrogenase, which is a means by which reducing equivalents enter the respiratory chain (via $FAD/FADH_2$ couple), is involved in the respiratory chain of *Aeromonas hydrophila* under Fe(III)-reducing conditions. Dicumarol inhibited Fe(III) reduction in cells grown anaerobically with Fe(III). This inhibitor blocks electron transfer to quinones and suggests that they may play a vital role in Fe(III) reduction for *A. hydrophila*. Addition of menaquinone to pleiotropic mutants of *S. putrefaciens* sp. 200 that lack quinones restored their ability to reduce Fe(III) (Myers and Myers 1994). These results suggest that dissimilatory Fe(III) reduction by *A. hydrophila* may involve a respiratory chain. Reducing equivalents that enter the chain via succinate dehydrogenase (EC 1.3.5.1; via $FAD/FADH_2$) are subsequently passed from quinones via cytochromes to an Fe(III) reductase. Additional work will be conducted to confirm the role of each of these components in anaerobic respiration.

Microbial Fe(III) reduction has been previously documented in Great Bay sediments and appears to play a significant role in biogeochemical cycling of this metal (Hines et al. 1984; Tugel et al. 1986). Dissimilatory metal reduction by mesophilic aeromonads has not been reported previously. In fact, little is known concerning their ecological niche. We have found that mesophilic aeromonads can be isolated throughout the year from Great Bay

Estuary, with highest cell numbers occurring in the late summer months (V. Knight and R. Blakemore, unpublished results), when Fe(III) reduction rates are the highest. Our results are consistent with anaerobic respiration as a means for the enhanced survival of this facultative pathogen of fish, amphibians, and humans in aquatic habitats and implicate this aquatic bacterium in biogeochemical dynamics of nitrate, metals, and metaloids.

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