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# Hydroxamate Production by *Aquaspirillum magnetotacticum*

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**Spent culture fluids from *Aquaspirillum magnetotacticum* MS-1 grown at high (20 μM) but not low (5 μM) iron concentration contained material yielding a positive hydroxamate test. Cells possessed six major outer membrane proteins. Three outer membrane proteins ranging from 72,000 to 85,000 daltons were coordinately produced at iron concentrations conducive to hydroxamate production. A 55,000-dalton iron-repressible outer membrane protein was also present in strain MS-1 cultured at low but not high ferric quinate concentration. Culture fluids from strain MS-1 which were hydroxamate positive augmented growth of a *Salmonella typhimurium* siderophore-deficient (*enb-7*) mutant in low-iron medium, suggesting a role of hydroxamate in uptake of iron by the cell.**

Numerous bacterial proteins, including cytochromes, catalases, peroxidases, superoxide dismutases, ribotide reductases, and nitrogenases, contain iron (15). Because of its insolubility at neutral pH under aerobic conditions, iron is usually unavailable for direct uptake by cells (16). Under conditions of low iron concentration (less than 1 μM), many microorganisms produce iron chelators, termed siderophores (16). These are assimilated into gram-negative cells by means of specific receptor proteins located in the outer membrane (16, 18, 20).

Siderophores have been detected in spent culture fluids from aerobes and facultative anaerobes but are apparently not produced by strict anaerobes or the lactic acid bacteria (15, 16). No information exists concerning siderophore production by obligate microaerophiles.

*Aquaspirillum magnetotacticum* (13) is a gram-negative, obligately microaerophilic chemoheterotroph, 2.0% of which (dry weight) is iron. Although proteins and hemoproteins of this organism contain iron, most of this metal is compartmentalized within its magnetosomes, which are intracellular enveloped crystals of the iron oxide magnetite (2). Virtually nothing is known of the manner in which cells of this organism sequester iron. However, in both its natural habitat and its culture medium the total iron concentration is 20 μM. In nature, the iron may be complexed with humic substances or plant-derived organic acids. In the culture medium that we used, iron was chelated with quinic acid.

This study was initiated to determine whether *A. magnetotacticum* uses a high-affinity (siderophore) system similar to those used by other gram-negative organisms for iron acquisition.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Cells of *A. magnetotacticum* MS-1 (ATCC 31632) and of a nonmagnetic mutant *A. magnetotacticum* strain, NM-1A, were cultured microaerobically in chemically defined growth medium (MSGM) as previously described (3). The iron source, ferric quinate, was provided at concentrations of 0, 5, 10, 20, or 40 μM. $FeSO_4$ was omitted from the culture medium mineral solution, and for studies involving spectrophotometric analysis of supernatant fluids, resazurin was omitted. A ferric chloride-sodium citrate mixture with a citrate-to-iron molar ratio of 1:1 or 20:1 (an iron concentration of 20 μM) was used in lieu of ferric quinate. Without added iron, MSGM contained 0.35 μM iron, as determined by the ferrozine method (19). No attempts were made to completely remove iron from the culture medium.

*Salmonella typhimurium* LT-2 *enb-7*, an enterobactin-deficient mutant (a gift from J. B. Neilands, Department of Biochemistry, University of California at Berkeley), and *S. typhimurium* ATCC 14028 were maintained on nutrient agar slants and subcultured twice each month. To promote siderophore production, *S. typhimurium* ATCC 14028 was cultured for 48 h at 37°C on a rotary shaker in 0.25% (wt/vol) Casamino Acids (Difco Laboratories, Detroit, Mich.) solution containing 0.2 mM $MgCl_2$ and adjusted to pH 7.5 (18).

**Isolation of outer membrane proteins.** *A. magnetotacticum* MS-1 and NM-1A were grown to early stationary phase in 1-liter batch cultures. Cells were harvested by centrifugation (7,000 × *g* for 15 min at 4°C) and suspended in 10 ml of 50 mM potassium phosphate buffer (pH 6.8). Outer membrane proteins (OMPs) were isolated by the procedure of Schnaitman (17). Briefly, DNase and RNase (Sigma Chemical Co., St. Louis, Mo.) were each added to cell suspensions at a final concentration of 0.1 mg/ml. Cells were ruptured by two passes through a precooled French pressure cell (16,000 $lb/in^2$). Unbroken cells and cellular debris were removed by centrifugation at 7,000 × *g* for 15 min at 4°C. The resulting supernatant fluid was ultracentrifuged at 200,000 × *g* for 60 min at 4°C. The brown pellet, containing both inner and outer cell membranes, was suspended in 10 ml of 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid (HEPES, pH 7.4) containing 2% (vol/vol) Triton X-100 and 10 mM $MgCl_2$. The unsolubilized outer membrane fraction was collected by ultracentrifugation (200,000 × *g* for 60 min at 4°C) and washed once in 10 mM HEPES (pH 7.4) to removed residual Triton X-100. The solubilized cytoplasmic membrane proteins were precipitated with cold 95% ethanol overnight at −12°C and collected by centrifugation (7,000 × *g* for 30 min at 4°C). Protein determinations were made by the procedure of Lowry et al. (11) with bovine serum albumin as a standard. The activity of succinic dehydrogenase, a cytoplasmic membrane enzyme, was assayed in each cell fraction (5) to assess the purity of the outer membrane fraction.

**Electrophoresis and analysis of OMPs.** OMPs and molecular weight standards (Bio-Rad Laboratories, Richmond, Calif.) were solubilized and separated by the electrophoretic

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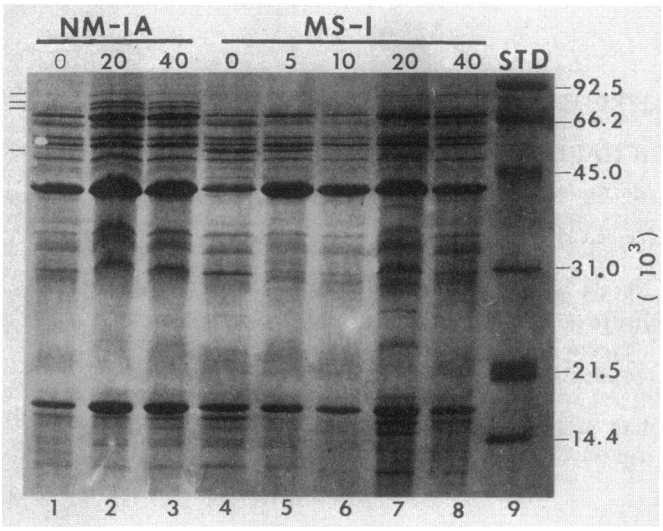


FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of OMP profiles from *A. magnetotacticum* NM-1A and MS-1 cultured at various iron concentrations (indicated above each lane, micromolar). Each lane contained 30 μg of purified (17) OMP. Dashes to the left of the gel indicate the positions (reading upward) of the 55,000-, 72,000-, 76,000-, and 85,000-dalton proteins. Lane 9, on the right, contained the molecular mass standards (in kilodaltons) indicated.

methods of Laemmli (9). OMPs were stacked in a 4% acrylamide gel at a constant current of 10 mA. The current was increased to 20 mA as OMPs entered a 12% acrylamide separating gel. OMPs were stained with Coomassie brillant blue and quantitated with a Helena Quick Scan R&D densitometer (Helena Laboratories, Beaumont, Tex.) at $A_{590}$.

**Detection of iron chelators.** Spirilla were grown in 500-ml batch cultures containing iron in the form of ferric quinate at 5, 20, and 40 μM or in the form of ferric citrate with a molar ratio of citrate to iron of either 20:1 or 1:1 (20 μM Fe). Spent culture fluids were freed from cells by centrifugation (7,000 × *g* for 15 min) and vacuum filtered through 0.45-μm Metricel GA-6 filters (Gelman Sciences, Inc., Ann Arbor, Mich.). The fluids were concentrated to 1/45 their original volume by flash evaporation at 35°C. Each sample was then carefully adjusted to pH 7.0 with 1 N HCl or NaOH, filter sterilized, and stored at 4°C until assayed. Uninoculated culture media at each iron concentration and spent culture fluids (concentrated 1/10 their original volume) from *S. typhimurium* ATCC 14028 cultures were prepared in an identical manner to those of controls.

The Arnow test (1) was used for the detection of phenolate-type iron chelators. The positive controls were 400 μM catechol (Sigma) and 1.5 mM 2,3-dihydroxybenzoic acid (Aldrich Chemical Co., Inc., Milwaukee, Wis.); the negative controls were 1.5 mM solutions of acetohydroxamic acid (Aldrich) and deferoxamine (a generous gift from CIBA-GEIGY Corp., Summit, N.J.). A modification of the Csaky reaction was used to detect secondary hydroxamic acids (7). The negative controls were catechol and 2,3-dihydroxybenzoic acid; the positive controls were acetohydroxamic acid and deferoxamine.

**Siderophore activity.** The effect of spirillum spent culture fluids on growth of the enterobactin-deficient *S. typhimurium* LT-2 *enb-7* mutant was examined. To each sidearm flask containing 100 ml of Davis minimal medium (4) with no added iron was added 1.0 ml of an overnight culture of the *S. typhimurium* LT-2 *enb-7* mutant grown in Davis minimal medium without iron or citrate. To each inoculated test flask was added 1 ml of either pre- or postgrowth fluids (prepared as described above) from strain MS-1 cultured in MSGM containing 5 or 20 μM ferric quinate. To each control flask was added 1.0 ml of either *S. typhimurium* ATCC 14028 spent culture supernatant fluid or uninoculated Davis minimal medium (prepared as described above). Growth of the *S. typhimurium* LT-2 *enb-7* mutant at 37°C (in a shaking water bath) was monitored at an optical density at 660 nm.

## RESULTS

**OMPs and iron.** Six major OMPs, with masses ranging from 16,400 to 64,500 daltons, were produced by strains MS-1 and NM-1A (Fig. 1). The OMP preparation appeared to be relatively free of cytoplasmic membrane proteins in that it contained only 7.0% of the total succinic dehydrogenase activity of the various cell fractions.

When cultured with low (0 or 5 μM), not high (10, 20, or 40 μM), concentrations of added ferric quinate, strain MS-1 produced a 55,000-dalton OMP (Fig. 1, lanes 4 to 8). This 55,000-dalton iron-repressible OMP (IROMP) comprised 13.0 and 4.0% of the total major OMP of cells cultured with 0 and 5 μM concentrations of added ferric quinate, respectively. This IROMP also comprised 1.0% of the total OMP of strain NM-1A cells grown with no added ferric quinate (Fig. 1, lane 1). Three minor OMPs (72,000, 76,000, and 85,000 daltons) not present at low iron concentrations were produced by cells of each strain cultured with 20 or 40 μM ferric quinate (Fig. 1, lanes 2, 3, 7, and 8).

Strain MS-1 cells cultured in medium containing 400 μM sodium citrate and 20 μM ferric chloride produced, in addition to the 55,000-dalton IROMP, another IROMP, this one of 58,000 daltons (Fig. 2, lane 1). Cells cultured with 20 μM sodium citrate and 20 μM ferric chloride produced neither the 58,000- nor the 55,000-dalton IROMP (Fig. 2, lane 4). Cells grown with either 5 μM ferric quinate or 20 μM ferric citrate with a citrate-to-iron molar ratio of 20:1 (i.e., conditions of low iron availability) produced the 55,000-dalton IROMP (Fig. 2, lanes 1 and 2). This protein was absent from cells cultured with ferric quinate concentrations greater than or equal to 10 μM (Fig. 1, lanes 6 to 8) or with ferric citrate concentrations of 20 μM with a citrate-to-iron molar ratio of 1:1 (Fig. 2, lanes 3 and 4).

**Siderophores.** Catechol-type iron chelators were not de-

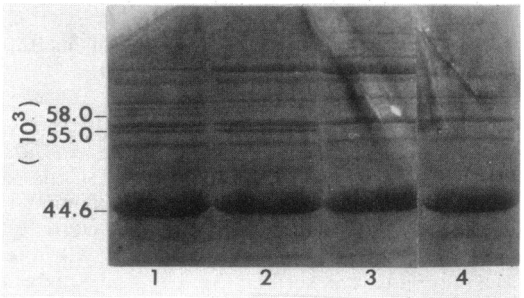


FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of OMP profiles from *A. magnetotacticum* MS-1 cultured with citrate-to-iron molar ratios of 20:1 or 1:1 (lanes 1 and 4, respectively) or with 5 or 20 μM ferric quinate (lanes 2 and 4, respectively). Each lane contained 30 μg of purified (17) OMP. The positions of the iron citrate-induced IROMP (58,000 daltons), the 55,000-dalton IROMP, and the major OMP (44,600 daltons) are indicated.

tected in spent culture fluids from strains MS-1 or NM-1A (by means of the Arnow reaction). Hydroxamate-type iron chelators were produced by cells of each strain (Table 1), as evidenced by positive hydroxamate tests. Hydroxamates were detected in culture media of cells grown at a 20 or 40 μM ferric quinate concentration. Surprisingly, spent medium from strain MS-1 cultured with less (5 μM) ferric quinate consistently failed to yield a positive hydroxamate reaction (Table 1). Culture fluids from cells of strain NM-1A grown at either a 5 or 20 μM ferric quinate concentration were consistently positive, however (Table 1).

Culture fluids obtained from strain MS-1 cultured with 20 μM ferric citrate at a citrate-to-iron molar ratio of 1:1 were positive in the hydroxamate test, whereas those obtained from cells grown at a 20:1 molar ratio were not (Table 1).

**Siderophore activity.** The enterobactin-deficient *S. typhimurium* LT-2 *enb-7* mutant did not grow in low-iron-concentration medium in the absence of exogenously supplied chelators (Fig. 3). Phenolate or hydroxamate (12, 15) siderophores have been shown to allow for the growth of this mutant in low-iron-concentration medium. Culture fluids from *S. typhimurium* ATCC 14028 (wild type) markedly stimulated the growth of the *S. typhimurium* LT-2 *enb-7* mutant, whereas uninoculated sterile Davis minimal medium (negative control) treated similarly had little effect (Fig. 3). Neither growth obtained with spent culture fluids from spirilla cultured with 5 μM ferric quinate nor growth obtained with uninoculated spirillum culture medium containing 20 μM ferric quinate exceeded that obtained with uninoculated, unsupplemented Davis minimal medium (Fig. 3). At 10 h, the *enb-7* mutant supplied with spent culture supernatant fluid from spirillum strain MS-1 cells cultured with 20 μM ferric quinate showed a 50% higher culture $A_{660}$ than when supplied with unsupplemented Davis minimal medium (negative control) or with uninoculated medium containing 20 μM ferric quinate (Fig. 3).

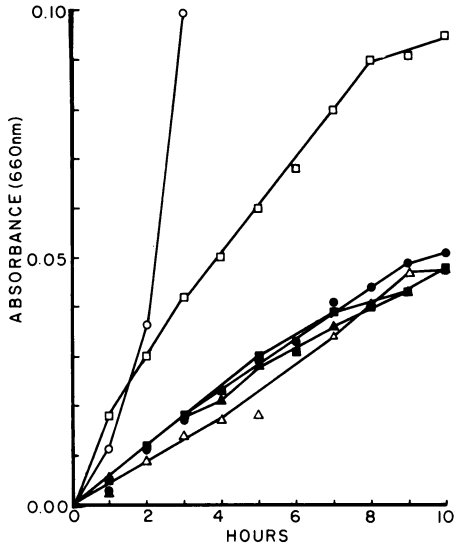


FIG. 3. Growth response of *S. typhimurium* LT-2 *enb-7* mutant cultured in Davis minimal medium supplemented with uninoculated culture medium containing 20 μM ferric quinate (■) or 5 μM ferric quinate (▲) or supplemented with spent culture fluids from strain MS-1 grown with 20 μM ferric quinate (□) or 5 μM ferric quinate (△). Controls contained Davis minimal medium (negative control) (●) or spent culture fluids from wild-type cultures of *S. typhimurium* ATCC 14028 (positive control) (○).

## DISCUSSION

Our results indicate that magnetic cells of *A. magnetotacticum* produced hydroxamate material when cultured with 20 or 40 μM added ferric quinate but not when grown with 5 μM ferric quinate. The nonmagnetic mutant, strain NM-1A, produced hydroxamates at both iron concentrations tested (5 and 20 μM ferric quinate). Because these results were unexpected in the light of iron concentration effects on siderophore production by enteric bacteria (10, 12, 15, 16), we repeated this study using an alternate source of iron, ferric citrate. At physiological pH and with a 20-fold molar excess of citrate, the ferric citrate complex can be expected to exist in a highly polymerized state (14, 16), rendering iron less available to cells. *A. magnetotacticum* MS-1 cells responded through their hydroxamate production to the available iron concentration in their culture medium. At a 20 μM concentration of iron, supplied in the form of ferric citrate, cells produced hydroxamates when the molar ratio of citrate to iron was 1:1 but not 20:1. Since the latter situation represents low available iron concentration for *A. magnetotacticum*, these results corroborated those obtained with ferric quinate; magnetic cells of this species produce secondary hydroxamates only when cultured under iron-sufficient conditions.

Although in enteric bacteria siderophore synthesis is derepressed by iron deficiency, hydroxamate synthesis by cells of *A. magnetotacticum* is apparently repressed by iron deficiency or is induced by available iron.

With low concentrations of available iron, many bacteria synthesize OMPs which function as receptors for siderophores (8, 10, 15, 16, 18). The 55,000-dalton IROMP in *A. magnetotacticum* may not function in iron transport via hydroxamates, as its synthesis was repressed at iron concentrations necessary for hydroxamate production. Thus, it may be a component of another iron uptake system not involving hydroxamates. Cells of strain NM-1A did not produce the IROMP but did produce hydroxamates, suggesting that a mutation(s) resulting in the loss of magnetite production may be associated with the gene(s) directing the synthesis of the IROMP.

In the enteric bacteria, iron storage proteins or those

TABLE 1. Hydroxamate and IROMP production by *A. magnetotacticum*

| Strain and supplement (μM) | Hydroxamate production (μM)[a] | Production of 55,000-dalton IROMP[b] |
|---|---|---|
| MS-1 | | |
| Ferric quinate | | |
| 5 | <5 | + |
| 20 | 49 | − |
| 40 | 53 | − |
| Citrate iron[c] | | |
| 20 (1:1) | 36 | − |
| 20 (20:1) | <5 | + |
| NM-1A | | |
| Ferric quinate | | |
| 5 | 47 | −[d] |
| 20 | 77 | − |

[a] Values are deferoxamine equivalents.
[b] +, Production; −, no production.
[c] Values in parentheses indicate the ratio of citrate to iron.
[d] Inferred result based upon absence of the IROMP from electrophoretic protein profiles of strain NM-1A cells cultured with 0, 20, and 40 μM concentrations of added ferric quinate.

involved with nonspecific iron transport are usually large (8, 16). The 72,000-, 76,000-, and 85,000-dalton OMPs detected in *A. magnetotacticum* cells cultured at a 20 or 40 μM concentration of ferric quinate may serve a role in iron metabolism comparable to those of OMPs of similar size and produced under similar conditions by the enteric bacteria (16). Alternatively, their coordinate production under conditions in which cells also produce hydroxamates suggests that these OMPs may be involved in hydroxamate secretion or binding or both.

The 58,000-dalton IROMP produced by *A. magnetotacticum* cells cultured with citrate may be a component of a citrate-mediated iron uptake system similar to that of *Mycobacterium smegmatis* (14) or *Escherichia coli* (16, 20).

The fact that only spent culture fluids which tested positively for hydroxamates stimulated growth of the *S. typhimurium* LT-2 *enb-7* mutant is consistent with a physiological role of this material in iron transport (e.g., as a siderophore) in *A. magnetotacticum*.

We do not know why hydroxamates are produced at high iron concentration and less so at low iron concentration by *A. magnetotacticum* MS-1. Two of the three catecholate siderophores of *Azotobacter vinelandii* are produced to some extent by cells cultured with 25 μM iron (6). Recently, we have detected hydroxamate production at high (20 μM) iron concentrations by *Aquaspirillum bengal*, *Aquaspirillum serpens*, and *Aquaspirillum polymorphum*, which are not magnetic (L. Paoletti and R. Blakemore, unpublished results). Thus, the magnetic spirillum appears not to be unique in this respect. This is an unusual pattern, and although not many published studies include results of hydroxamate analysis at both high and low iron concentrations, this appears to be the first report of bacterial hydroxamate production at high iron concentration. Our results also suggest that some available iron is necessary to induce synthesis of this material and of the 72,000- to 85,000-dalton OMPs detected.

Siderophore production at high iron concentration may be common among free-living organisms which accumulate this metal or require it for metabolism but live in environments in which it is normally or transiently abundant.

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