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Amonabactin, a Novel Tryptophan- or Phenylalanine-Containing Phenolate Siderophore in *Aeromonas hydrophila*

S. BARGHOUTHI,[1] R. YOUNG,[1] M. O. J. OLSON,[2] J. E. L. ARCENEAUX,[1] L. W. CLEM,[1] AND B. R. BYERS[1]*

Departments of Microbiology[1] and Biochemistry,[2] University of Mississippi Medical Center, Jackson, Mississippi 39216-4505

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***Aeromonas hydrophila* 495A2 excreted two forms of amonabactin, a new phenolate siderophore composed of 2,3-dihydroxybenzoic acid, lysine, glycine, and either tryptophan (amonabactin T) or phenylalanine (amonabactin P). Supplementing cultures with L-tryptophan (0.3 mM) caused exclusive synthesis of amonabactin T, whereas supplements of L-phenylalanine (0.3 to 30 mM) gave predominant production of amonabactin P. The two forms of amonabactin were separately purified by a combination of production and polyamide column chromatographic methods. Both forms were biologically active, stimulating growth in iron-deficient medium of an amonabactin-negative mutant. Of 43 additional siderophore-producing isolates of the *Aeromonas* species that were tested, 76% (19 of 25) of the *A. hydrophila* isolates were amonabactin positive, whereas only 19% (3 of 16) of the *A. sobria* isolates and all (3 of 3) of the *A. caviae* isolates produced amonabactin, suggesting a predominant synthesis of amonabactin in certain *Aeromonas* species.**

Aeromonas hydrophila and related aeromonads are gram-negative, freshwater pathogens of fish and humans. In fish they cause a fatal hemorrhagic septicemia, and in humans they cause wound, soft tissue, and blood infections as well as acute gastroenteritis (2, 5–7, 14). The first report of catecholate (phenolate) siderophore production by a strain of *A. hydrophila* was made by C. Andrus and S. M. Payne in 1982 (Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, CC I, II 11, p. 89). They later identified enterobactin as the siderophore produced by this strain (C. Andrus, and S. M. Payne, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, D13, p. 61). Enterobactin synthesis also was noted in another *A. hydrophila* isolate; however, catecholate siderophore synthesis by certain other strains of this organism could not be credited to enterobactin production (B. R. Byers, P. E. Byers, and J. E. L. Arceneaux, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, K184, p. 207). The present study describes purification and partial chemical and biological characterization of amonabactin, a new siderophore that contained 2,3-dihydroxybenzoic acid (DHB) and aromatic amino acids. Amonabactin was produced by many isolates of the genus *Aeromonas*.

MATERIALS AND METHODS

Organisms. *A. hydrophila* 495A2, which was isolated from a diseased aquatic animal, was obtained from C. Lobb of this Department. A mutant strain (*A. hydrophila* SB101) unable to produce amonabactin was isolated in this laboratory by treating *A. hydrophila* 495A2 with nitrosoguanidine and identifying amonabactin-negative colonies on chrome azurol S (CAS) (12) siderophore detection agar (manuscript in preparation). A total of 43 additional isolates of *Aeromonas* species (which were used only to determine which produced amonabactin) were obtained from the following: R. Brenden, Calgon Vestal Laboratories, St. Louis, Mo.; L. Pickering, University of Texas Health Science Center, Houston; T. Trust, University of Victoria, Victoria, British Columbia, Canada; T. Chakraborty, University of Würzburg, Würzburg, Federal Republic of Germany; F. W. Hickman-Brenner, Centers for Disease Control, Atlanta, Ga.; D. R. Nelson, University of Rhode Island, Kingston; the American Type Culture Collection, Rockville, Md. For routine maintenance, the cultures were grown on slants of brain heart infusion agar or nutrient agar (Difco Laboratories, Detroit, Mich.). All cultures were incubated at 30°C. The isolates also were kept in 25% glycerol in nutrient broth (Difco) at −70°C.

Purification of amonabactin. Amonabactin T and amonabactin P were separately purified from supernatants of *A. hydrophila* 495A2 after its growth in a low-iron minimal medium composed of the following (per liter): glucose, 5 g; $(NH_4)_2HPO_4$, 1 g; K_2HPO_4, 4 g; KH_2PO_4, 2.7 g. To lower metal contamination, the medium was treated with Chelex-100 (Bio-Rad Laboratories, Richmond, Calif.) by previously reported methods (1). After the Chelex-treated medium was filter sterilized, it was supplemented with filter sterilized solutions of high purity sulfate salts (Johnson-Matthey, Inc., Seabrook, N.H.) of magnesium (830 μM), manganese (40 μM), and iron (0.18 μM). Cultures (12-liter volume) were incubated at 30°C with aeration at 8 liters per min in a modified model 43-100 fermentor (The Virtis Co., Gardiner, N.Y.) in which exposed stainless steel components were coated with Teflon. For preparation of amonabactin T (the tryptophan-containing form of amonabactin), the medium was supplemented with 0.3 mM L-tryptophan, which caused exclusive production of amonabactin T. At maximum growth (usually after 12 to 18 h), the catecholate siderophore in the supernatant was readily detected by assay for dihydroxy phenolates (4) and by mixing 1 ml of supernatant with 0.005 ml of 1% ferric chloride, which resulted in a blue-purple color. The cells were removed by centrifuging the culture, and the phenolate(s) was adsorbed to polyamide (11) by passing the supernatant through a 5.5- by 13-cm column of polyamide (Woelm, Universal Adsorbents, Atlanta, Ga.) that had been previously washed with methanol, acetone, and sufficient water to remove the organic solvents. The column then was washed with water (1.5 liters) and then washed with 500 ml of 100% acetone. The amonabactin T then was eluted with methanol (usually 500 ml, or until elution of the iron-reactive siderophore was no longer evi-

* Corresponding author.

dent). The eluate was collected in Teflon vessels to retard formation of the siderophore-iron chelate. Where possible, Teflon was used to protect the siderophore from metals leached from glass vessels. The methanol solution was concentrated to 5 to 10 ml by vacuum evaporation, and the solution was transferred to a 500-ml Teflon beaker. The addition of 200 ml of ethyl acetate precipitated the amonabactin T and removed any DHB that might be present. After standing for 3 to 4 h at room temperature, the precipitate was collected by vacuum filtration on disks of Whatman no. 50 filter paper, and while on the filter it was washed with 200 ml of ethyl acetate. It was dried over desiccant and weighed. Allowing the filtrate to stand at room temperature overnight produced a second precipitation of amonabactin T, which also was collected, washed, and weighed. Usual yields of amonabactin T from 12 liters were about 250 mg from the first precipitation and about 40 mg from the second precipitation.

Amonabactin P (the phenylalanine-containing form) was prepared from 12-liter cultures grown as described above, except that the medium was supplemented with 30 mM L-phenylalanine instead of L-tryptophan. This caused predominant synthesis of amonabactin P. After the cells were removed by centrifugation, the amonabactin in the supernatant was adsorbed to a 5.5- by 13-cm column of polyamide. The column was washed with 1.5 liters of water, and the amonabactin P was eluted with 10% (vol/vol) acetone in water; the eluate was collected in 2-liter Teflon bottles. Usually 5 to 8 liters of 10% acetone was required to lower the concentration of amonabactin P in the eluate below the level detectable by the ferric chloride test. To retard oxidative decomposition of the siderophore, the eluate was sparged with nitrogen and kept in the dark. The acetone was removed from the eluate by vacuum evaporation, and the amonabactin P in the resulting aqueous solution was adsorbed to a fresh column (5.5 by 13 cm) of polyamide. After this column was washed with 500 ml of 100% acetone, the amonabactin P was eluted from the column with methanol and precipitated with ethyl acetate as described above for preparation of amonabactin T. Usual yields of amonabactin P were about 100 mg from the first precipitation and 10 mg from the second precipitation. The purified amonabactin T and amonabactin P were stored in a nitrogen-containing desiccator. For experimental use, the amonabactin preparations usually were dissolved (3 to 30 mg per ml) in 75% (vol/vol) ethanol in water.

Thin-layer chromatography of amonabactin. Amonabactin T, amonabactin P, and DHB were separated by thin-layer chromatography on plates of polyamide (G1600 from Schleicher & Schuell Co., Keene, N.H.; or Macherey-Nagel Polyamid-6 from Alltech Associates, Inc., Deerfield, Ill.). Some lots of the polyamide plates were contaminated with sufficient iron to cause formation of the colored siderophore-iron chelates during chromatography. Before use, iron was removed from these sheets by soaking them in 1 N HCl for 3 min and then rinsing them with water and drying them. The solvent system for polyamide chromatography was butanol saturated with aqueous 1.7% ammonium acetate. It was prepared by shaking butanol with excess 1.7% ammonium acetate in a separatory funnel and using the *n*-butanol layer. The amonabactins and DHB were visualized by fluorescence under UV light and with a spray of 1% ferric chloride. The tryptophan-containing amonabactin T was stained by spraying the chromatogram with Ehrlich reagent for tryptophan (13) and heating at 80°C until the color developed. For thin-layer chromatography to identify DHB, a two-dimensional solvent system (9) and cellulose plates (Chromagram 13255; Eastman Kodak Co., Rochester, N.Y.) were used.

Assay for amonabactin synthesis. To determine whether various *Aeromonas* isolates produced amonabactins T and P, the test organism was grown in the low-iron (0.18 μM) glucose-mineral salts medium. *A. sobria* AB3 and AB3-25 (obtained from T. Chakraborty) required the addition of pantothenic acid (10 μM) for growth. A 10-ml amount of culture supernatant was passed through a small column (0.5 by 1 cm) of polyamide. The column was washed with 2 ml of water, and adsorbed phenolates (including amonabactin T, amonabactin P, and DHB) then were eluted with 1 ml of methanol. Portions (usually 50 μl) of the methanol eluate were chromatographed by using the polyamide thin-layer system. If the siderophore concentration in the eluate was low, the eluate could be concentrated with a stream of nitrogen. Amonabactins T and P were visualized by UV fluorescence and 1% ferric chloride spray; amonabactin T was detected with Ehrlich reagent for tryptophan. If present, DHB also could be discriminated from amonabactin by its R_f and the blue-purple color of its fluorescence.

CAS siderophore detection agar medium. To determine presumptive siderophore production by isolates of *Aeromonas* species, a modification of the CAS agar medium of Schwyn and Neilands (12) was used. Siderophore production was apparent as a yellow-orange halo around the colonies; absence of a halo indicated the inability to produce a siderophore. To prepare the CAS agar medium, 5.3 g of NaOH and 30.24 g of piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) were dissolved in 750 ml of water. To this solution then were added 15 g of agar and 100 ml of a stock salt solution containing the following (per liter): KH_2PO_4, 3 g; NaCl, 5 g; NH_4Cl, 10 g. The agar was dissolved by heating; the solution then was sterilized by autoclaving and was cooled to 50°C in a water bath. Filter-sterilized solutions of the following components then were added: Casamino Acids (10%), 30 ml; glucose (20%), 10 ml; thiamine (200 μg/ml), 10 ml; nicotinic acid (200 μg/ml), 10 ml; $MgCl_2$ (1 M), 1 ml; $CaCl_2$ (0.1 M), 1 ml. Then 100 ml of a sterile solution containing a complex of chrome azurol S, iron, and hexadecyltrimethylammonium bromide was added; its preparation is described below. The medium then was mixed gently and plates were poured. CAS agar medium used to grow *A. sobria* strains AB3 and AB3-25 also contained 10 μM pantothenic acid to satisfy the growth requirement of these strains. The CAS-iron-hexadecyltrimethylammonium bromide solution was prepared by dissolving 60.5 mg of CAS (Fluka Chemical Corp., Ronkonkoma, N.Y.) in 50 ml of water and adding 10 ml of 1 mM $FeCl_3 \cdot 6H_2O$ (in 10 mM HCl). This solution then was added slowly to a solution containing 72.9 mg of hexadecyltrimethylammonium bromide (Fluka) in 40 ml water. The resulting solution was sterilized by autoclaving.

Biolabeling of amonabactin with [^{3}H]lysine. A modification of the previously described method (1) was used to biolabel amonabactin T and amonabactin P by growing *A. hydrophila* 495A2 with a labeled component, [^{3}H]lysine, of both forms. The organism was grown to maximum turbidity in 10 ml (contained in a 50-ml Nephalo flask) of iron-deficient (0.18 μM iron), Chelex-treated glucose-mineral salts medium that was supplemented with nonradioactive lysine (0.3 mM) and 1 to 2 mCi of [4,5-^{3}H]lysine (46 to 55 Ci/mmol; ICN Pharmaceuticals, Inc., Irvine, Calif.). For some experiments, the radioactive medium also contained various levels of either L-tryptophan or L-phenylalanine. After the culture was centrifuged to remove cells, the supernatant was passed through a polyamide column (0.5 by 2 cm). The column then was

washed with 2 ml of water, and the adsorbed siderophore was eluted with 10 ml of methanol. The eluate was reduced to about 1 ml with a stream of nitrogen. By using the polyamide thin-layer chromatographic system, portions of the eluate were chromatographed both separately and after mixing with purified amonabactin T and amonabactin P (and with the radioactive lysine) to confirm identities of the radioactive amonabactins. The chromatographic lanes were scanned and the radioactive peaks were plotted (and their amounts of radioactivity were determined) with an Imaging Scanner (Bioscan Instruments, Washington, D.C.) that was interfaced with a microcomputer-plotter (Apple Computer Corp.).

Growth inhibition by ethylenediamine-di-(*o*-hydroxyphenylacetic acid) (EDDA): utilization of siderophores to reverse EDDA inhibition. The capacity of the amonabactin-negative mutant *A. hydrophila* SB101 to utilize various siderophores to overcome EDDA inhibition of growth was determined by an agar-disk diffusion method. Melted L agar containing EDDA at the desired concentration was cooled to 50°C and then seeded with about 10^2 CFU of *A. hydrophila* SB101 per ml. The agar was poured into plates; after it solidified, sterile paper disks (6.3-mm diameter; Difco) containing the desired amount of the test siderophore or 10 μg of iron (as an iron salt) were placed on the surface of the agar. Before use, the disks were washed with 25 mM EDTA and rinsed with high-purity water. Zones of growth surrounding the disks indicated reversal of EDDA inhibition.

Other analyses and assays. Amino acid analyses were done by previously described methods (8) with 50-μg samples of amonabactin that were hydrolyzed in 3 M mercaptoethanesulfonic acid for 24 h at 110°C. A qualitative assay for the presence of DHB in amonabactin was done by hydrolyzing (under vacuum) 1-mg samples in 6 N HCl for 24 h at 110°C. The HCl was evaporated, and the residue was dissolved in methanol. DHB was identified by cochromatography with the authentic compound in a two-dimensional thin-layer chromatographic system (9). The presence of dihydroxy phenolates in the culture supernatant was determined as previously described (4). The possible presence of hydroxamates in culture supernatants was assayed by the Csaky method (3). UV absorption spectra of amonabactins T and P were determined with a model DU 70 UV/Vis spectrophotometer (Beckman Instruments). Water used for preparation of media and reagents was purified to approximately 18 MΩ resistivity by treatment with a reverse osmosis-charcoal demineralizer system (Millipore Corp., Bedford, Mass.).

RESULTS

Iron-regulated synthesis of amonabactin. Earlier studies (cited above) showed enterobactin synthesis by some strains of *A. hydrophila*. Although certain other *A. hydrophila* isolates produced a catechol during iron-deficient growth as evidenced by a positive chemical test (4) for the presence of a dihydroxy phenol in culture supernatants, extracting the supernatants with ethyl acetate (under conditions that yielded enterobactin) failed to recover the presumed phenolate in the extracts. This finding suggested that the putative siderophore (designated amonabactin) had not been previously identified. In *A. hydrophila* 495A2 (like other siderophore producing organisms), iron restriction activated or increased amonabactin synthesis, whereas adding the appropriate amount of iron prevented amonabactin production. In the glucose-mineral salts medium (containing 43 mM phosphate), an iron level higher than 17 μM stopped amonabactin synthesis. For production and purification of amonabactin, the organism was grown at 0.18 μM iron. Possible excretion of a hydroxamate siderophore was investigated by testing supernatants of low-iron cultures with the Csaky assay (3); readily detectable amounts of hydroxamic acids were not found.

Partial purification of mixed amonabactin T and amonabactin P. For analysis by thin-layer chromatography on polyamide sheets, phenolates present in low-iron (0.18 μM) culture supernatants first were partially purified on small polyamide columns (see Materials and Methods). Two possible phenolates at R_fs of 0.20 and 0.34 were noted on the chromatograms. Both compounds fluoresced (yellow-green) under UV light, and both reacted with iron (1% ferric chloride spray) to form a purple complex. In this thin-layer chromatographic system, a DHB standard appeared as an iron-reactive spot with blue UV fluorescence at R_f 0.10. Qualitative amino acid analysis of the partially purified siderophore hydrolyzed with mercaptoethanesulfonic acid indicated that lysine, glycine, phenylalanine, and tryptophan were present (although not as the free amino acids), possibly as components of one or both of the phenolates. Spraying the chromatograms with Ehrlich reagent for tryptophan (13) revealed that tryptophan was located only in the slower-moving phenolate at R_f 0.20. Accordingly, the tryptophan-containing phenolate was designated amonabactin T, and the faster-moving compound at R_f 0.34 was designated amonabactin P. To determine whether one of the compounds might be synthesized during only a portion of the culture cycle, samples collected at early-, middle-, and late-log phases were analyzed. Both amonabactin T and amonabactin P were present in all samples. Similar analyses were done with supernatants of cultures grown at iron concentrations from 0.08 to 17 μM; both amonabactins T and P were produced at all iron levels.

Preferential production of either amonabactin T or amonabactin P and biolabeling of both with [^{3}H]lysine. Adding L-tryptophan or L-phenylalanine to iron-deficient cultures markedly shifted siderophore synthesis to favor amonabactin T or amonabactin P, respectively. To visualize this effect, the siderophore was labeled by growing the organism in medium (supplemented with either L-tryptophan, L-phenylalanine, or neither) that contained [^{3}H]lysine. In cultures without the aromatic amino acid supplement, radioactive lysine biolabeled both amonabactins T and P (Fig. 1A). Adding L-tryptophan (0.3 mM) to the culture caused exclusive production of amonabactin T (Fig. 1B), and supplementing with L-phenylalanine (0.3 mM) caused predominant (but not exclusive) production of amonabactin P (Fig. 1C). A higher concentration (10 mM) of L-phenylalanine decreased apparent synthesis of amonabactin T, but some of the tryptophan-containing form was still made (Fig. 1D). Adding the phenylalanine precursor phenylpyruvic acid at concentrations from 0.3 to 1.2 mM also generated predominant amonabactin P synthesis in a concentration-dependent fashion, similar to that observed with L-phenylalanine addition (data not shown). Supplementing the cultures with L-tyrosine (10 mM) or shikimate (1.0 mM) had no effect on synthesis of amonabactin T or P (data not shown). Moreover, there was no apparent change in amonabactin production in cultures containing 0.3 mM each of lysine, glycine, or DHB (added separately and in various combinations).

Separate purification of amonabactin T and amonabactin P. The exclusive production of amonabactin T in L-tryptophan-supplemented cultures afforded a means for separate purification of this form (details are given in Materials and

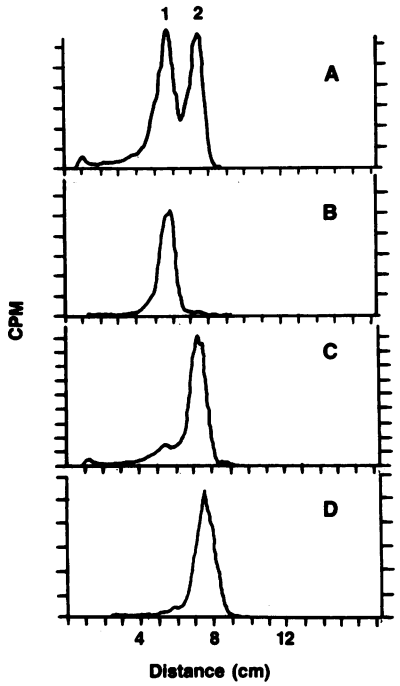

FIG. 1. Preferential production of amonabactin T or P by supplementing cultures of *A. hydrophila* 495A2 with L-tryptophan or L-phenylalanine, respectively. Radioactivity scans of thin-layer chromatograms of amonabactin labeled with [^{3}H]lysine showing production of amonabactin T (peak 1) and amonabactin P (peak 2) in cultures that were unsupplemented (A) or supplemented with 0.3 mM L-tryptophan (B), 0.3 mM L-phenylalanine (C), or 10 mM L-phenylalanine (D).

Methods). The usual yield was about 21 mg of amonabactin T per liter of supernatant. A procedure (detailed in Materials and Methods) for purification of amonabactin P was developed when it was discovered that this form of the siderophore was slowly but selectively eluted from polyamide columns by 10 to 12% (vol/vol) acetone in water (higher concentrations of acetone eluted both forms of amonabactin, although 100% acetone eluted neither). To insure that the level of amonabactin T was minimal in culture supernatants used to prepare amonabactin P, cultures were grown in low-iron (0.18 μM) medium containing 30 mM L-phenylalanine. Usual recoveries of amonabactin P were about 8 mg per liter of supernatant.

Polyamide thin-layer chromatography of the separately purified amonabactin T and amonabactin P did not reveal contamination of the preparations with the alternate form of the siderophore. Amino acid analyses of the preparations confirmed that amonabactin T was the tryptophan-containing species, whereas amonabactin P was the phenylalanine form; both also appeared to contain lysine and glycine. Similar but distinctly different UV absorption spectra were recorded for amonabactin T and P (Fig. 2). The amonabactin P spectrum, with absorption peaks at 314, 250, and 212 nm, resembled the DHB spectrum. The amonabactin T spectrum also had a broad absorption peak at 314 nm, suggestive of DHB; the additional peaks at 291 and 248 nm in this spectrum probably were due to the tryptophan content of amonabactin T. Both forms of amonabactin could be dissolved in 75% (vol/vol) ethanol in water. The preparations were only slightly soluble in water. Heating the aqueous mixtures in a boiling water bath dissolved the amonabactins

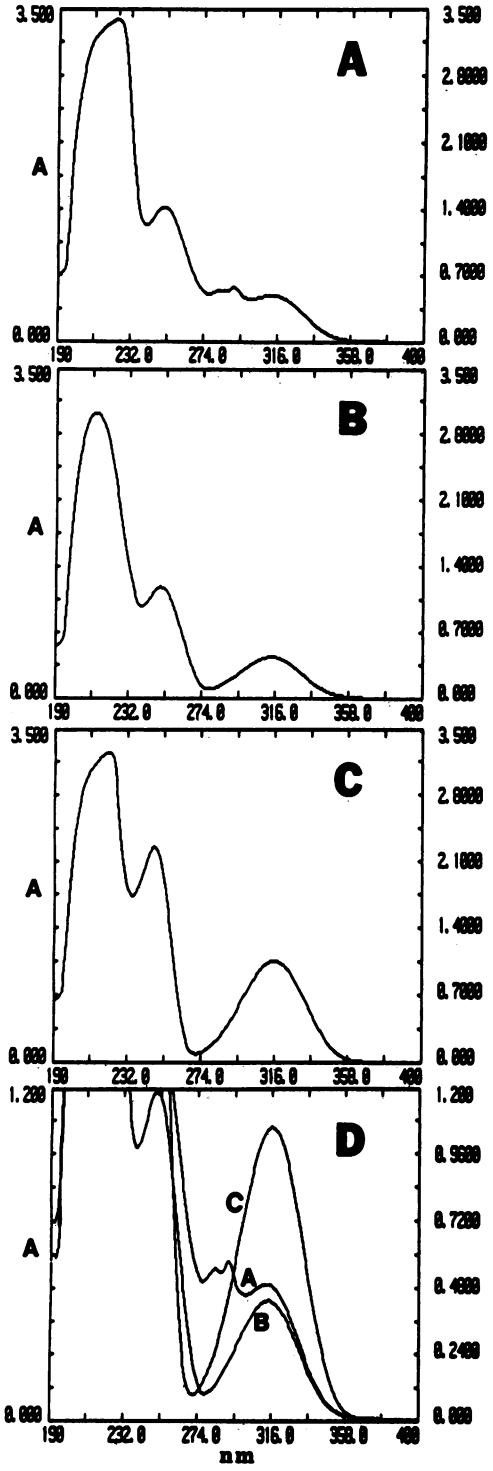

FIG. 2. UV adsorption spectra (in 75% ethanol in water at 50 μg/ml) of amonabactin T (A), amonabactin P (B); or DHB (C) and an expanded overlay of the three spectra (D).

in water; however, cooling to room temperature caused them to precipitate. Both amonabactin T and amonabactin P gave a positive chemical test (4) for dihydroxy phenols. Hydrolysis of the amonabactins with 6 N HCl released a product that was chromatographically identical to DHB in the two-dimensional thin-layer system (9), indicating that conjugated DHB was a component of both forms.

TABLE 1. Siderophore production by the *A. hydrophila* group[a]

Species	Total no. of isolates	No. (%) of isolates producing:	
		Amonabactin	Other phenolate(s)
A. hydrophila	25	19 (76)	6 (24)
A. sobria	16	3 (19)	13 (81)
A. caviae	3	3	0

[a] All isolates tested here were positive for siderophore production on the CAS agar of Schwyn and Neilands (12). Amonabactin was identified by thin-layer chromatography on polyamide (with butanol saturated with aqueous 1.7% ammonium acetate as the solvent).

Biological activity of amonabactins T and P; specificity of siderophore utilization. Mutant strains that are unable to produce amonabactin (either form) have been isolated by treating *A. hydrophila* 495A2 with nitrosoguanidine and subsequently identifying amonabactin-negative colonies on CAS siderophore detection agar (manuscript in preparation). One of these strains (SB101) was used in present studies to assess the biological activity of amonabactins T and P. Growth inhibition of strain SB101 by the chelating agent EDDA was reversed by either amonabactin T or amonabactin P; reversal of inhibition was apparent as zones of growth around paper disks containing amonabactin that were placed on the inoculated agar. For example, at an EDDA concentration of 40 μg/ml of L agar, growth inhibition was reversed by 1 ng of amonabactin P per disk and 4 ng of amonabactin T per disk. When tested at a concentration of 30 μg per disk, the siderophore ferrioxamine B (Desferal) also reversed EDDA inhibition, but at the same concentration the siderophores enterobactin, agrobactin, aerobactin, and schizokinen did not overcome inhibition. In an *A. hydrophila* strain (ATCC 11163) that produced enterobactin, growth inhibition by EDDA was reversed by disks containing enterobactin but not by either form of amonabactin.

Prevalence of amonabactin synthesis. To obtain an initial estimate of the prevalence of amonabactin synthesis in *A. hydrophila*, *A. sobria*, and *A. caviae* (members of the *A. hydrophila* group), 43 additional isolates (obtained from various sources, mentioned in Materials and Methods) were examined (Table 1). When plated on the CAS siderophore detection agar, all strains produced a siderophorelike halo. To ascertain which isolates produced amonabactin and whether both forms of amonabactin were made, siderophores in culture supernatants were partially purified on small polyamide columns and then analyzed by polyamide thin-layer chromatography. Amonabactins T and P were identified by UV fluorescence and by spraying with iron; Ehrlich reagent for tryptophan also was used to detect amonabactin T. The majority (76%) of the *A. hydrophila* isolates produced amonabactin; conversely, only 19% of the *A. sobria* isolates produced amonabactin. In all cases of amonabactin production, the synthesis of amonabactin T and amonabactin P was apparent. No isolate that synthesized only one form of amonabactin was identified in this survey. The remaining *A. hydrophila* and *A. sobria* isolates tested here produced a phenolate siderophore as evidenced by a positive test (4) for dihydroxy phenols; some (or all of them) may have produced enterobactin. Only three strains of *A. caviae* were examined; all three produced both forms of amonabactin. Three strains of *A. veronii* (5) also were analyzed. They did not produce amonabactin, although two *A. veronii* strains (1169-83 and 935-84) produced a siderophorelike halo on CAS agar medium.

DISCUSSION

Amonabactin, the new siderophore in *Aeromonas* species, was a catecholate based on DHB. It was synthesized in two forms, one containing tryptophan (amonabactin T) and the other containing phenylalanine (amonabactin P); both also contained lysine and glycine. Separate purification of each form was accomplished by a combination of methods involving preferential production, polyamide chromatography, and precipitation from ethyl acetate. Sole synthesis of amonabactin T by L-tryptophan-supplemented cultures permitted separate purification of amonabactin T from such cultures. The probable slight differences in the solubilities of amonabactin T and amonabactin P in certain organic-aqueous solvent systems afforded a means for separate purification of amonabactin P (by eluting it from polyamide with dilute aqueous acetone) and for distinguishing the two forms by polyamide thin-layer chromatography. Chemical structures are being determined and will be published separately.

Feeding the amonabactin-producing strain either of the siderophore precursors L-tryptophan or L-phenylalanine (or the phenylalanine precursor, phenylpyruvic acid) altered siderophore synthesis to yield mainly amonabactin T or amonabactin P, respectively. In fact, exclusive production of amonabactin T was achieved by supplementing with a low concentration (0.3 mM) of L-tryptophan. The addition of L-phenylalanine caused predominant amonabactin P production; however, even at a high level (30 mM) of L-phenylalanine some amonabactin T was synthesized. The difference in response of the organism to the two amino acids might be due to a preference for tryptophan in amonabactin synthesis, although it might also be explained by a deficiency in phenylalanine transport or by competing enzymes that may lower the effective concentration of phenylalanine. Simultaneous production of both amonabactin T and amonabactin P was evident at various points from early to late in the growth cycle and at different levels of iron restriction, suggesting that the organism may alternate between synthesis of the two forms. Although the process by which this is accomplished is unknown, it may involve a control system (either genetic or physiological) that senses the intracellular levels of tryptophan and phenylalanine. Both forms appeared biologically active. Unless there is an unanticipated functional difference between amonabactin T and P, synthesis of the two may be related to the fact that two components (DHB and an amino acid) of the siderophore are products of the aromatic pathway.

Although amonabactin synthesis may be more prevalent in strains that can be categorized biochemically as *A. hydrophila*, amonabactin production may be widespread in *Aeromonas* species. Of 25 *A. hydrophila* isolates (obtained from several laboratories), more than 70% produced amonabactin. Conversely, only about 20% of 16 *A. sobria* isolates produced amonabactin. The remaining isolates of both species studied here produced phenolates, and some may be enterobactin producers. The three representatives of *A. caviae* produced amonabactin, but more isolates of all the mesophilic aeromonads should be tested. Studies of siderophore utilization by various isolates suggested that the amonabactin-producing strains were not able to use enterobactin and vice versa. This indicates that use of amonabactin (like use of enterobactin and other siderophores) requires a specific system that likely includes an amonabactin outer membrane receptor. The existence of different siderophores in various isolates of *A. hydrophila* and *A. sobria* may be a useful phenotypic marker. Each of the *Aeromonas* species

contains more than one DNA hybridization group, but the hybridization groups within species cannot be separated biochemically (5, 10). The type of siderophore produced might be used to subdivide the motile aeromands. Also, it is not yet known whether amonabactin is important in the virulence of *Aeromonas* species, representing the mechanism (or one of several alternatives) for acquisition of iron from a host. Therefore, the function, genetics, and biosynthesis of this novel aromatic amino acid containing phenolate siderophore deserve more study.

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