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Physiological control of amonabactin biosynthesis in *Aeromonas hydrophila*

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Summary. Amonabactin is a siderophore from *Aeromonas hydrophila* which is produced in two biologically active forms composed of the phenolate 2,3-dihydroxybenzoic acid (DHB), lysine, glycine, and either trytophan (amonabactin T) or phenylalanine (amonabactin P). Amonabactin biosynthetic mutants (generated by chemical mutagenesis) that either produced no amonabactin or overproduced the siderophore were isolated and identified on chrome azurol S siderophore detection agar. Amonabactin-negative mutants were of two categories. One type produced no phenolates and used exogenous DHB to synthesize amonabactin (both forms) while the other type excreted DHB but not amonabactin. This suggests an amonabactin biosynthetic pathway composed of two segments, one producing DHB and the other assembling amonabactin from DHB and the amino acids. Overproduction mutants used amonabactin poorly or not at all, indicating that they contained lesions in amonabactin utilization. Adding the analog D-tryptophan to wild-type *A. hydrophila* cultures reduced synthesis of both amonabactin T and amonabactin P and lengthened the lag phase in iron restricted medium. The tryptophan and phenylalanine forms of amonabactin may be synthesized by a single assembly pathway that contains a novel enzyme (sensitive to D-tryptophan) which inserts either tryptophan or phenylalanine into amonabactin.

Key words: Siderophores — Amonabactins — Amonabactin mutants — *Aeromonas hydrophila*

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Introduction

Amonabactin is a recently described phenolate siderophore produced by bacteria belonging to the Gram-negative genus *Aeromonas* (Byers 1987; Barghouthi et al. 1989). The various species of *Aeromonas* cause a fatal hemorrhagic septicemia in fish; in humans they cause wound and blood infections as well as acute gastroenteritis (Khardori and Fainstein 1988). The siderophore amonabactin is synthesized in two biologically active forms that are composed of 2,3-dihydroxybenzoic acid (DHB), lysine, glycine and either tryptophan (amonabactin T) or phenylalanine (amonabactin P). Amonabactin is the predominant siderophore in *A. hydrophila* and *A. caviae,* although some *A. sobria* strains produce this siderophore. Supplementing cultures of *A. hydrophila* with either L-phenylalanine or L-tryptophan altered siderophore synthesis to yield mainly amonabactin P or amonabactin T, respectively, suggesting that the type of amonabactin made may depend on the intracellular concentrations of L-tryptophan and L-phenylalanine. Separate purification of amonabactin T and amonabactin P was accomplished by a combination of production and polyamide chromatographic methods (Barghouthi et al. 1989). Amonabactin may be important in the virulence of *Aeromonas* species, representing one of the means for acquisition of iron from a host.

Present studies with amonabactin mutants revealed an iron-regulated amonabactin biosynthetic pathway composed of two arms, one synthesizing 2,3-dihydroxybenzoic acid and the second arm assembling amonabactin from DHB, lysine, glycine and either tryptophan or phenylalanine. Experiments with the analog D-tryptophan indicated a single amonabactin assembly pathway

that may synthesize both amonabactin T and amonabactin P.

Materials and methods

Microorganism and culture media. A. hydrophila 495A2 and the procedures for growth in the Chelex-100-treated glucose/mineral salts medium at various iron concentrations and in L-medium have been described (Barghouthi et al. 1989). Amonabactin mutant isolation is described below. Preparation of the minimum CAS siderophore detection agar (without amino acid or vitamin supplements) of Schwyn and Neilands has also been published (Barghouthi et al. 1989). All cultures were incubated at 30° C.

Thin-layer chromatography of amonabactin and DHB. Amonabactins T and P and DHB were separated and identified on thin-layer plates of polyamide (Schleicher & Schüll G1600) as described by Barghouthi et al. (1989) with *n*-butanol saturated with 1.7% ammonium acetate as the solvent. Standards of DHB (Aldrich Chemical Co.) and amonabactins T and P (separately purified by the methods of Barghouthi et al. 1989) were included. Photographic negatives of thin-layer chromatograms were prepared with ultraviolet illumination. The negatives were scanned and the position and heights of the density peaks of ultraviolet fluorescent compounds were plotted using a Zeineh soft laser scanning densitometer/plotter (LKB Instruments).

Assay for amonabactin and DHB synthesis. The assay described by Barghouthi et al. (1989), in which excreted phenolates first were partially purified on small columns of polyamide and then analyzed by thin-layer chromatography, was used. Total phenolate excretion was estimated by assay for dihydroxyphenolates (Evans 1947).

Chemical mutagenesis. Amonabactin mutants of *A. hydrophila* 495A2 were isolated by treating mid-log phase cells (suspended in 0.1 M citrate pH 5.5) with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) at a final concentration of 50 μg/ml for 50 min at 30° C. The mutagen was removed by centrifuging and resuspending the cells in fresh citrate buffer. Nutrient broth (Difco) cultures were prepared and incubated at 30° C overnight. Mutant clones with lesions in amonabactin production were identified by plating the culture on the minimum CAS agar medium. The colonies were scored for presence or absence of a siderophore-like halo and for relative size of the halo.

Minimum inhibitory concentration (MIC) of ethylenediamine-di-(o-hydroxyphenylacetic acid) (EDDA). Melted L-medium agar or glucose/mineral salts medium agar containing EDDA at various concentrations (Barghouthi et al. 1989) was cooled to 45° C and then seeded with 100 CFU/ml of the test *A. hydrophila* isolate. The organism was sensitive to the temperature (45–50° C) of liquid agar and brief exposure to agar at 50° C caused significant loss of viable cells. The seeded agar was poured into plates which were incubated for 24 h at 30° C. The MIC was then estimated by noting the presence or absence of colonies. The capacity of the test isolate to use amonabactin was determined by placing paper disks (6.3 mm diameter) containing 4–10 ng amonabactin/disk on the surface of seeded agar containing the appropriate MIC of EDDA. A zone of growth surrounding the disk indicated use of amonabactin to reverse EDDA inhibition.

Results and discussion

Phosphate concentration and iron regulation of amonabactin synthesis

Regulation of amonabactin production in *A. hydrophila* 495A2 was similar to siderophore regulation in other microorganisms in which synthesis is inversely proportional to the amount of iron added to the culture. However, the precise concentration of iron required to stop amonabactin excretion by *A. hydrophila* depended on the amount of potassium phosphate in the Chelex-100-treated glucose/mineral salts culture medium (Fig. 1). At a potassium phosphate level of 33 mM phosphate, amonabactin production was not detected above 0.89 μM iron. At 43 mM phosphate, an iron level higher than 17 μM was required to halt amonabactin synthesis. In the range of 0.18–1.0 μM iron, phenolate production was higher in the 43 mM phosphate medium. Analyses (by polyamide thin-layer chromatography) of the phenolates produced at all iron levels (except those preventing phenolate synthesis) showed that both amonabactins T and P were produced. There probably is no relationship between the severity of iron restriction and the form of amonabactin produced. Increased amonabactin production at an elevated phosphate level is unexplained, although it may have been caused by a decrease in the availability of iron due to formation of iron-phosphate complexes.

The mechanism by which iron regulates siderophore production in *A. hydrophila* presently is unknown. *Escherichia coli* has a number of iron-controlled operons, all of which appear to be regulated (at least partly) by the product of the *fur*

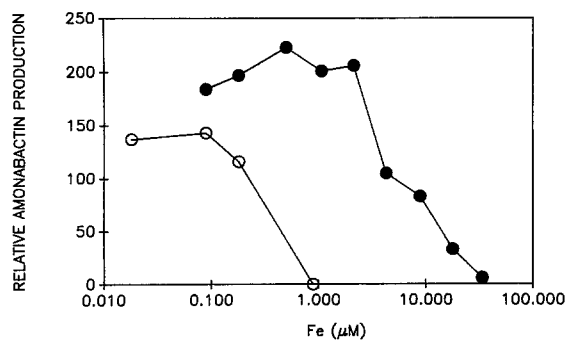

Fig. 1. Phosphate influence on iron control of amonabactin biosynthesis in glucose/mineral salts medium (Chelex-100-treated) containing (○) 33 mM and (●) 43 mM potassium phosphate. The relative amonabactin production = μM DHB excreted/A_{600} where A_{600} measures the culture turbidity

gene (Hantke 1982; de Lorenzo et al. 1987). This product (Fur protein) is a negative repressor of transcription that uses ferrous iron as a corepressor. Some of the operons of this iron regulon encode biosynthesis of siderophores, although others are involved in production of various substances such as toxins (e.g. Calderwood and Mekalanos 1987). A consensus sequence (Fig. 2) for a palindromic Fur-binding site has been deduced (de Lorenzo et al. 1987; DeGrandis et al. 1987; Calderwood and Mekalanos 1988). This control mechanism may be common in bacteria. The iron-regulated diphtheria toxin gene from corynephage β has a possible Fur-binding sequence (Tai and Holmes 1988). *A. hydrophila* also may have an iron regulon that uses a Fur-like negative repressor. Sequence analysis of the *A. hydrophila* gene for its hemolytic cytotoxin aerolysin (Howard and Buckley 1986) revealed an AT-rich palindromic sequence (upstream from the putative promoter sequence) that may represent the iron control site (Fig. 2). The aerolysin gene also has been cloned from another species, *Aeromonas sobria* (Husslein et al. 1988). Comparison of the sequences of the *A. hydrophila* and *A. sobria* regions coding for aerolysin revealed 77% similarity at both the nucleotide and amino acid levels (Husslein et al. 1988). Although the regulatory regions upstream from the aerolysin genes in both species have a high A+T content, they show only 46% similarity. The aerolysin genes appear highly conserved while their regulation is divergent. The *A. sobria* regulatory region is characterized by a sequence (AATAAAA) that is repeated 8 times in 300 bp but it is not clear how (or if) this sequence is involved in regulation or how iron participates in control. The molecular mechanism by which iron regulates production of amonabactin is also unknown; however, analysis (now in progress) of the promoter region of a recently cloned *A. hydrophila* gene that encodes a step in biosynthesis of amonabactin (Barghouthi, S., unpublished) may reveal the mechanism.

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Consensus        5' GATAATGATA------ATCATTATC

A. hydrophila    5' -TTAAT-ATATTGCGCAT-ATTAA-
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Fig. 2. Comparison of the Fur-binding consensus sequence (de Lorenzo et al. 1987; DeGrandis et al. 1987; Calderwood and Mekalanos 1987) with a possible similar regulatory site for the *A. hydrophila* aerolysin gene (Howard and Buckley 1986). Spaces (−) are inserted to give maximum alignment; areas of similarity are underlined

Characterization of amonabactin biosynthetic mutants

To evaluate the amonabactin biosynthetic and utilization systems and their genetic complexity, *A. hydrophila* 495A2 was treated with NTG and strains with mutations in the amonabactin system were identified on the minimum CAS siderophore detection agar. Colonies on this agar were scored for presence or absence of a siderophore-like halo and for the size of the halo. Two categories, nonproducers and overproducers, were isolated and characterized (Table 1). When grown in low-iron (0.18 μM) culture medium, the group of mutants that failed to produce amonabactin (showed no halo on CAS agar) were subdivided into two types, those that excreted DHB (identified by thin-layer chromatography) and those that excreted no detectable phenolates. Adding DHB to the cultures of the phenolate-negative isolates allowed them to synthesize amonabactin (both T

Table 1. Pertinent characteristics of representative types of *A. hydrophila* amonabactin mutants

Amonabactin mutant category	Strain	CAS halo	Excrete amonabactin	Iron regulation	Excrete DHB	Amonabactin produced with added DHB	Utilize amonabactin	EDDA MIC
Nonproducers	SB101	absent	no	—	no	yes	yes	40
	SB201	absent	no	—	yes	—	yes	40
Overproducers	SB301	oversize	yes	yes	—	—	yes	80
	SB303	oversize	yes	yes	—	—	no	20

Mutants were generated by chemical mutagenesis with NTG and were isolated on CAS siderophore detection agar medium (without amino acid or vitamin supplements); amonabactin production (with or without added 40 μM DHB and at 0.18 μM or 34 μM iron) as well as DHB excretion (at 0.18 μM iron) were demonstrated by thin-layer chromatography; amonabactin utilization was assessed by measuring reversal of EDDA inhibition; — = not determined. The MIC (minimum inhibitory concentration) of EDDA is reported as μg/ml of L-agar medium; the MIC of the wild-type amonabactin producer (*A. hydrophila* 495A2) was 100 μg/ml. Mutant strain SB303 grew as a small (minute) colony; all other strains demonstrated normal colony size and appearance

and P forms). Therefore, like enterobactin biosynthesis in *E. coli* (Earhart 1987; Ozenberger et al. 1989), the amonabactin biosynthetic system probably is composed of at least two arms: the DHB segment produces this precursor and a second group of enzymes assembles amonabactin from DHB (either an endogenous or exogenous DHB source) and the amino acids. Owing to their inability to produce a siderophore, these mutants were more sensitive than the wild-type parent to EDDA inhibition; they used amonabactin (either form) to reverse EDDA inhibition (Table 1).

A second category of amonabactin mutants was identified by the oversized siderophore halo surrounding the colonies. Theoretically, an oversized halo might result from a mutation in the amonabactin utilization process (depriving the cells of iron chelated by amonabactin) or from a lesion in iron regulation of siderophore synthesis (Schwyn and Neilands 1987). No regulatory mutants have yet been identified in *A. hydrophila*; amonabactin synthesis by all of the overproduction mutants examined to date was prevented by growth in high-iron (34 μM) medium. Despite their excessive (2–3 times that of the wild-type) production of amonabactin, all of the overproduction mutants showed a minimum inhibitory concentration (MIC) for inhibition by EDDA that was less than that of the wild type (Table 1). This suggests that the overproduction mutants had an impaired amonabactin utilization process that impeded iron acquisition from ferric amonabactin. One mutant strain (SB303) appeared to have a complete block in the use of amonabactin; this strain also was very sensitive to EDDA inhibition (Table 1). Future experiments characterizing the specific lesions in these mutants should help to identify various components (for example, a possible amonabactin receptor) required for efficient use of ferric amonabactin as an iron source.

Inhibition of amonabactin synthesis by D-*tryptophan*

It was shown previously (Barghouthi et al. 1989) that adding either L-tryptophan or L-phenylalanine to iron-deficient cultures of *A. hydrophila* 495A2 markedly shifted siderophore synthesis to favor amonabactin T or amonabactin P, respectively. Although adding L-phenylalanine caused predominant amonabactin P production, exclusive synthesis of amonabactin T was obtained with an L-tryptophan supplement. These effects are shown in Figs. 3 and 4. Adding the analog D-tryptophan to *A. hydrophila* 495A2 cultures inhibited synthesis of both amonabactin T and amonabactin P and caused apparent excretion of DHB (Figs. 3 and 4). A physiological consequence of growth in the presence of D-tryptophan was increased sensitivity to the iron restriction caused by EDDA. Adding D-tryptophan to *A. hydrophila* cultures in the glucose/mineral salts medium containing 15 μg EDDA/ml increased the apparent length of the lag phase by several hours (data not shown), suggesting that the analog impaired amonabactin production.

The mechanism by which D-tryptophan inhibited siderophore synthesis is unknown; however, the assembly segment of the amonabactin biosynthetic pathway may contain an enzyme able to bind and insert either tryptophan or phenylalanine into amonabactin. The enzyme may not distinguish between D-tryptophan and L-tryptophan. If D-tryptophan is an inactive analog and if this enzyme is involved in synthesis of both forms of amonabactin, then inhibition of the enzyme by binding of D-tryptophan would curtail synthesis of both amonabactins T and P. A single amonabactin assembly system that senses the intracellular availability of phenylalanine and tryptophan may represent a physiological control mechanism that affords rapid and automatic switching between production of the two forms of amonabactin. This might be necessary to assure an adequate

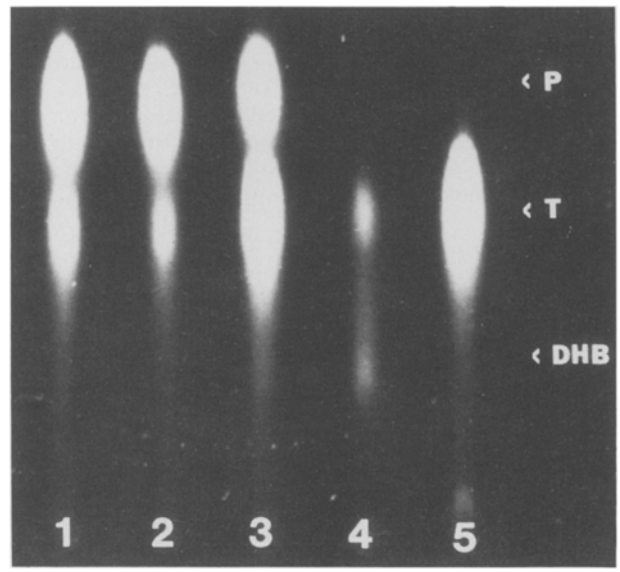

Fig. 3. Control of amonabactin synthesis by aromatic amino acid supplements. Thin-layer chromatogram showing fluorescent phenolates excreted by *A. hydrophila* 495A2 during growth in unsupplemented medium (lane 3) and in medium containing (0.3 mM) D-phenylalanine (lane 1), L-phenylalanine (lane 2), D-tryptophan (lane 4) and L-tryptophan (lane 5)

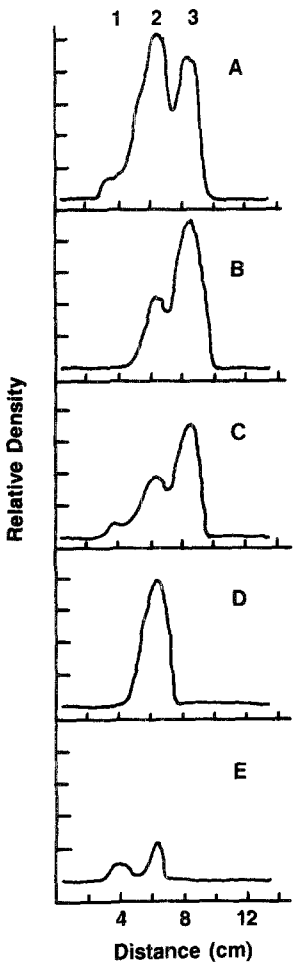

Fig. 4. Inhibition of amonabactin production by D-trytophan. Scans of photographic negatives of thin-layer chromatograms of excreted fluroescent compounds showing production of DHB (peak 1), amonabactin T (peak 2) and amonabactin P (peak 3) in cultures that were unsupplemented (A) or supplemented (0.3 mM) with L-phenylalanine (B), D-phenylalanine (C), L-tryptophan, (D), and D-tryptophan (E)

supply of aromatic compounds in the cell during the heavy demand placed on the aromatic pathway by amonabactin production.

Adding D-phenylalanine (0.3 mM) did not inhibit amonabactin production. In fact, D-phenylalanine supplements shifted predominant synthesis to amonabactin P, an effect similar to L-phenylalanine (Figs. 3 and 4). The reason for the lack of inhibition by D-phenylalanine is unknown; it might be due to the presence of a phenylalanine racemase in *A. hydrophila*.

The frequency of mutation (by NTG treatment) to the amonabactin-negative phenotype in the present studies was estimated to be fairly high (10^{-3}). This also might be taken as evidence for a single genetic system encoding enzymes for production of both forms of amonabactin. From the physiological and mutant characterization studies, genes (which are probably organized in several transcriptional units) in the amonabactin system may be grouped as follows.

1) Genes encoding enzymes for biosynthesis of DHB.

2) Genes encoding enzymes for assembly of amonabactin from DHB and the amino acids. This group may include a novel enzyme capable of inserting either tryptophan or phenylalanine into the siderophore.

3) Genes for utilization of amonabactin, probably including a specific receptor.

4) Genes involved in the iron regulation of amonabactin production. Although the genetic studies have not yet identified a regulatory gene(s), physiological studies indicate there must be one or more.

Several characteristics of amonabactin resemble other microbial siderophores but it has a unique aromatic amino acid composition and its biosynthetic pathway may use one or more novel enzymes. Amonabactin also may be important in the virulence of certain strains of the *Aeromonas* species. The genetics, physiology, biosynthesis, regulation, coordination chemistry and possible role in pathogenesis of amonabactin deserve detailed study. These studies should be assisted by the recent cloning of an amonabactin biosynthetic gene, as reported by Barghouthi et al. at the 1989 Annual Meeting of the American Society for Microbiology (abstract D214).

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This publication is dedicated to the memory of Charles E. Lankford, a pioneer in microbial iron research, who died August 10, 1989.

References

Barghouthi S, Young R, Olson MOJ, Arceneaux JEL, Clem LW, Byers BR (1989) Amonabactin, a novel tryptophan- or phenylalanine-containing phenolate siderophore in *Aeromonas hydrophila*. J Bacteriol 171:1811–1816

Byers BR (1987) Iron, siderophores, and virulence in *Aeromonas hydrophila*. In: Winkelmann G, van der Helm D, Neilands JB (eds) Iron transport in microbes, plants and animals, VCH Publishers, Weinheim, pp 111–116

Calderwood SB, Mekalanos JJ (1987) Iron regulation of Shiga-like toxin expression in *Escherichia coli* is mediated by the *fur* locus. J Bacteriol 169:4759–4764

DeGrandis S, Ginsberg J, Toone M, Climie S, Friesen J, Brunton J (1987) Nucleotide sequence and promoter mapping of the *Escherichia coli* Shiga-like toxin operon of bacteriophage H-19B. J Bacteriol 169:4313–4319

deLorenzo V, Wee S, Herrero M, Neilands JB (1987) Operator sequences of the aerobactin operon of plasmid ColV-K30 binding site of the ferric uptake regulation (*fur*) repressor. J Bacteriol 169:2624–2630

Earthart CF (1987) Ferrienterobactin transport in *Escherichia coli*. In: Winkelmann G, van der Helm D, Neilands JB (eds) Iron transport in microbes, plants and animals VCH Publishers, Weinheim, pp 67–84

Evans WC (1947) Oxidation of phenol and benzoic acid by some soil bacteria. Biochem J 41:373–382

Hantke K (1982) Negative control of iron uptake systems in *Escherichia coli*. FEMS Microbiol Lett 15:83–86

Howard SP, Buckley JT (1986) Molecular cloning and expression in *Escherichia coli* of the structural gene for the hemolytic toxin aerolysin from *Aeromonas hydrophila*. Mol Gen Genet 204:289–295

Husslein V, Huhle B, Jarchar T, Lurz R, Goebel W, Chakraborty T (1988) Nucleotide sequence and transcriptional analysis of the *aerCaerA* region of *Aeromonas sobria* encoding aerolysin and its regulatory region. Mol Microbiol 2:507–517

Khardori N, Fainstein V (1988) *Aeromonas* and *Plesiomonas* as etiological agents. Annu Rev Microbiol 42:395–419

Ozenberger BA, Brickman TJ, McIntosh MA (1989) Nucleotide sequence of *Escherichia coli* isochrosimate synthetase gene *entC* and evolutionary relationship of isochorismate synthetase and other chorismate-utilizing enzymes. J Bacteriol 171:775–783

Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160:47–65

Tai S-PS, Holmes RK (1988) Iron regulation of the cloned diphtheria toxin promoter in *Escherichia coli*. Infect Immun 56:2430–2436

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