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Cloning, Mutagenesis, and Nucleotide Sequence of a Siderophore Biosynthetic Gene (*amoA*) from *Aeromonas hydrophila*†

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Many isolates of the *Aeromonas* species produce amonabactin, a phenolate siderophore containing 2,3-dihydroxybenzoic acid (2,3-DHB). An amonabactin biosynthetic gene (*amoA*) was identified (in a *Sau*3A1 gene library of *Aeromonas hydrophila* 495A2 chromosomal DNA) by its complementation of the requirement of *Escherichia coli* SAB11 for exogenous 2,3-DHB to support siderophore (enterobactin) synthesis. The gene *amoA* was subcloned as a *Sal*I-*Hin*dIII 3.4-kb DNA fragment into pSUP202, and the complete nucleotide sequence of *amoA* was determined. A putative iron-regulatory sequence resembling the Fur repressor protein-binding site overlapped a possible promoter region. A translational reading frame, beginning with valine and encoding 396 amino acids, was open for 1,188 bp. The C-terminal portion of the deduced amino acid sequence showed 58% identity and 79% similarity with the *E. coli* EntC protein (isochorismate synthetase), the first enzyme in the *E. coli* 2,3-DHB biosynthetic pathway, suggesting that *amoA* probably encodes a step in 2,3-DHB biosynthesis and is the *A. hydrophila* equivalent of the *E. coli entC* gene. An isogenic amonabactin-negative mutant, *A. hydrophila* SB22, was isolated after marker exchange mutagenesis with Tn*5*-inactivated *amoA* (*amoA*::Tn*5*). The mutant excreted neither 2,3-DHB nor amonabactin, was more sensitive than the wild-type to growth inhibition by iron restriction, and used amonabactin to overcome iron starvation.

Most isolates of the mesophilic *Aeromonas* species produce either of the two iron-transporting siderophores, amonabactin and enterobactin (5). Amonabactin is the predominant siderophore in isolates phenotypically identified as *A. hydrophila* and *A. caviae*, while enterobactin is found in most *A. sobria* strains (5). Enterobactin (also called enterochelin) is the indigenous siderophore of *Escherichia coli* and certain other enteric bacteria (26, 28). Members of the genus *Aeromonas* are aquatic, gram-negative bacteria that can be pathogenic, causing diseases in hosts ranging from fish to humans (19). Acquisition of iron from a host is essential for growth of a pathogen during an infection (7, 38), and production of amonabactin (but not enterobactin) confers on an aeromonad isolate the capacity to obtain iron from serum Fe-transferrin (24), suggesting that amonabactin may be an aeromonad virulence factor.

Both amonabactin and enterobactin contain 2,3-dihydroxybenzoic acid (2,3-DHB) conjugated to amino acids. Amonabactin is synthesized in two biologically active forms, each composed of 2,3-DHB, lysine, glycine, and either tryptophan (amonabactin T) or phenylalanine (amonabactin P) (5). Enterobactin is a cyclic triester of 2,3-dihydroxybenzoylserine (26, 28). In *E. coli*, the cluster of enterobactin biosynthesis and utilization genes occurs in several transcriptional units located near min 13 on the chromosome (1). The biosynthesis of enterobactin involves several genes which have been designated *entA* to *entG* (6, 20, 21, 27, 29, 30, 36). The products of *entC*, *entB*, and *entA* catalyze the sequential conversion of chorismate, the branch point intermediate in aromatic biosynthesis, to 2,3-DHB; these genes occur in a transcriptional unit consisting of *entCEBA*(*P15*), the expression of which is controlled by a nucleotide sequence ("iron box") upstream from *entC* that responds to the Fur repressor, the ferrous binding regulatory protein of the iron regulon (3, 6, 21, 27, 36). The nucleotide sequence of *entC* suggests that it is a member of a family of genes with a common evolutionary origin that encode chorismate-utilizing enzymes (27). The *P15* locus is phenotypically undefined (6). The product of *entE* activates the carboxyl group of 2,3-DHB with ATP (29, 37) for the final stages of enterobactin synthesis, the coupling of 2,3-DHB and L-serine. These assembly reactions are catalyzed by the products of *entD*, *entE*, *entF*, and possibly *entG* which are believed to exist in a multienzyme complex. Sequence data reveal no separate *entG* gene; EntG activity is encoded by the *entB* 3′ terminus, suggesting that EntB is a bifunctional protein (36). Like enterobactin production, the amonabactin biosynthetic scheme also includes two arms, one synthesizing 2,3-DHB and the other assembling amonabactin from 2,3-DHB and the amino acids. Amonabactin-negative mutants are of at least two categories; one type excretes only 2,3-DHB, while the other uses exogenous 2,3-DHB to produce both forms of amonabactin (4).

To develop strains and methods to assess the importance of amonabactin in virulence of *A. hydrophila* and to initiate studies of the genetic system encoding amonabactin biosynthesis and of its relatedness to the enterobactin genes, we cloned an amonabactin biosynthetic gene (*amoA*). Its nucleotide sequence was determined, and it was mutagenized with Tn*5*. With the inactivated gene (*amoA*::Tn*5*), an isogenic amonabactin-negative mutant of *A. hydrophila* then was constructed, and some of its physiological properties were determined. This work is reported here.

MATERIALS AND METHODS

Bacteria, plasmids, media, restriction enzymes. The relevant characteristics and sources of the bacterial strains and the plasmids used in this study are given in Table 1. The

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† This paper is dedicated to the memory of Charles E. Lankford, a pioneer in microbial iron metabolism.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype	Reference or source
Aeromonas strains		
495A2	Wild-type *A. hydrophila*; produces amonabactin (Amo$^+$)	4
SB22	Isogenic with 495A2, but *amoA111* (*amoA*::Tn*5*)	This study
SB221	Amo$^+$ (marker rescue of SB22)	This study
E. coli strains		
HB101	*proA2 leu lacY1 ara-14 galK2 xyl-5 mtl-1 recA12 hsdR hsdM supE44* Sm^r; produces enterobactin (Ent$^+$)	H. Boyer
SAB11	As for HB101, except Ent$^-$; produces enterobactin from exogenous 2,3-DHB	This study
JM101	F′ *traD36 lacI^q* (*lacZ*)*M15 proAB*/*supE thi* (*lac-proAB*)	C. Jones
AN193	*thi leuB proC trpE lacY mtl xyl rpsL azi fhuA tsx supA entA403*	S. Stuart
AN192	As for AN193, but *entB402* instead of *entA*	S. Stuart
AN93	As for AN193, but *entE405* instead of *entA*	C. Earhart
C600	F$^-$ *thi-1 thrT leuB6 lacY1 tonA21 supE44*	22
MM294	*endoI B1* r_K	2
Plasmids		
pJRD215	IncQ *cos*$^+$ *mob*$^+$ Km^r Sm^r (derived from RSF1010)	11
pSUP202	pBR325::*mob* Tc^r Cm^r Ap^r	34
pRK340	Tn*5* Tc^r Km^r	R. Meyer
pRK2013	ColE1 *mob*$^+$ *tra*$^+$ (RK2) Km^r	R. Meyer
pITS55	Cloned *entCEBA* (from *E. coli*)	27
pMPS32	Cloned *fepB fepC entCE* (from *S. flexneri*)	32
pSB215	pJRD215::*amoA*; complements *E. coli* SAB11	This study
pSB315	pSUP202::*amoA*; complements *E. coli* SAB11	This study
pSB316	pSB315 *amoA*::Tn*5*; does not complement *E. coli* SAB11	This study
pSB317	Deletion derivative of pSB315; complements *E. coli* SAB11	This study
pSB319	Deletion derivative of pSB315; does not complement *E. coli* SAB11	This study
M13mp18	DNA sequencing vector	New England Biolabs
M13mp19	DNA sequencing vector	New England Biolabs

method for preparation of the Chelex 100-treated, glucose-mineral salts culture medium has been described elsewhere (5). This medium (containing various levels of added iron) was used for cultivation of the *A. hydrophila* strains; for growth of *E. coli* strains, the medium was supplemented with the required growth factors. The chrome azurol S (CAS) siderophore detection agar was prepared as previously described (4, 5). The L-medium broth and agar were prepared by published methods (9). To determine sensitivity, estimated as the MIC, of *A. hydrophila* to the iron restriction imposed by the chelating agent ethylenediamine-di-(*o*-hydroxyphenylacetic acid) (EDDA), the agent was incorporated at various concentrations in L medium (4). The nutrient agar and the components of NY medium (that contained, per liter, 8 g of nutrient broth and 2 g of yeast extract) were obtained from Difco Laboratories, Detroit, Mich. Restriction enzymes were purchased from Boehringer Mannheim, Indianapolis, Ind., or Promega Biotech, Madison, Wis.; they were used under conditions recommended by the suppliers.

Isolation of a 2,3-DHB-requiring *E. coli* host. To obtain a *recA* strain of *E. coli* which also required exogenous 2,3-DHB for assembly of enterobactin and which would be appropriate for complementation by cloned *A. hydrophila* amonabactin genes, chemical mutagenesis with *N*-methyl-*N*′-nitro-*n*-nitrosoguanidine (4) was used to produce enterobactin-negative mutants of *E. coli* HB101. Mutants were identified and isolated on CAS agar. To determine which of the mutants could synthesize enterobactin with added 2,3-DHB, each isolate was transferred to CAS agar as a streak and a paper disc containing 5 nmol of 2,3-DHB was placed on each streak. Those isolates able to use exogenous 2,3-DHB to assemble enterobactin were apparent from the siderophore halos around growth adjacent to the disc. The 2,3-DHB requirement of one isolate (designated *E. coli* SAB11) was confirmed; enterobactin was detected by thin-layer chromatography (TLC) only in supernatants of low-iron (0.18 μM) cultures that were supplemented with 2,3-DHB (10 μM). The mutant phenotype of *E. coli* SAB11 was complemented by the plasmid pITS55 which carries *entCEBA*, a transcriptional unit encoding 2,3-DHB synthesis in *E. coli* (27). Complementation also was achieved with pMPS32, a recombinant plasmid carrying genes of the enterobactin cluster (including *entC* and *entE* but not *entB* or *entA*) from *Shigella flexneri* (32). These data suggest that *E. coli* SAB11 is a 2,3-DHB-biosynthetic mutant (possibly *entC*) that retains the capacity to assemble enterobactin.

Amonabactin preparation, assay for phenolates, and TLC of siderophores. Amonabactins T and P were separately purified from supernatants of low-iron cultures (supplemented with either L-tryptophan or L-phenylalanine) of *A. hydrophila* 495A2 by previously published methods (5). Culture supernatant was assayed for excreted phenolates by the method of Evans (15). Amonabactin in culture supernatants was identified by polyamide TLC (5), and enterobactin was identified with a two-dimensional TLC system (25) using plates of cellulose (Eastman Kodak, Rochester, N.Y.).

Preparation of DNA. Chromosomal DNA was prepared from *A. hydrophila* 495A2 by the method of Marmur (23). The DNA was stored at −20°C. Plasmid DNA was prepared by the alkaline lysis method (22) and then was purified by two-step cesium chloride-ethidium bromide density gradient centrifugation (17). The plasmid DNA was stored at 4°C. Electrophoretic analysis of DNA in agarose gels has been described previously (22). Analyses for plasmids in *A. hydrophila* strains were performed as described by Maniatis et al. (22). Transformation of *E. coli* strains with plasmid DNA was done by published procedures (10).

Construction and screening of a gene library for an amonabactin gene. By standard methodology (22), *A. hydrophila* 495A2 chromosomal DNA was partially digested with

*Sau*3A1 and ligated to the *Bam*HI-digested cosmid vector pJRD215 (11). The ligated DNA then was packaged with a commercial system (Packagene; Promega Biotech) according to the manufacturer's instructions. The recombinant cosmids were transduced into the siderophore-negative *E. coli* SAB11 (which requires 2,3-DHB to synthesize enterobactin); 1,500 clones hosting recombinant cosmids then were isolated on NY agar containing kanamycin (20 μg/ml). These clones were screened on CAS agar for complementation (evident as a siderophore halo surrounding the clone) of the siderophore-negative phenotype of *E. coli* SAB11.

Hybridization methods. Previously described procedures were used to prepare, purify, and label DNA probes and for hybridization of the labeled probes to genomic or plasmid DNA (22). The DNA used for probe preparation was purified from the desired electrophoretic bands. The probes were labeled with biotin-11-dUTP (obtained from BRL Laboratories, Gaithersburg, Md.). Hybridization was done on nitrocellulose membranes (Trans-blot, obtained from Bio-Rad Laboratories, Richmond, Calif.). The biotinylated probes were assayed with the Blu Gene Nucleic Acid Detection System (BRL Laboratories).

Marker exchange mutagenesis of *A. hydrophila*. The cloned amonabactin gene *amoA* was inactivated by Tn*5* insertion which was accomplished by mating (for 6 h at 37°C on an L-agar plate) *E. coli* SAB11/pSB315 and *E. coli* C600/pRK340::Tn*5*. The cells, suspended in sodium chloride solution (0.85%), were used to inoculate 50 ml of NY broth containing chloramphenicol (35 μg/ml) and kanamycin (20 μg/ml). After overnight incubation at 41°C (which prevents replication of pRK340), this culture was diluted and spread on NY agar containing chloramphenicol and kanamycin (at the above concentrations) and ampicillin (100 μg/ml). After incubating at 41°C, colonies appearing on this agar were screened on CAS agar for the siderophore-negative phenotype. For marker exchange mutagenesis of wild-type *A. hydrophila* 495A2, plasmid pSB316 (pSB315::Tn*5*) was mobilized into *A. hydrophila* by triparental mating (14, 16). One milliliter of each of the overnight cultures (in L broth) of *A. hydrophila* 495A2 (recipient), *E. coli* MM294/pRK2013 (mobilizing strain), and *E. coli* SAB11/pSB316 (mutagenizing strain) was mixed with the others. The cells were concentrated by centrifugation and then were spread on NY agar which was incubated at 30°C for 6 to 8 h. Cells on this agar then were streaked on nutrient agar containing kanamycin (20 μg/ml) and ampicillin (100 μg/ml). Oxidase-positive colonies (35) with *A. hydrophila* morphology were transferred to nutrient agar containing chloramphenicol (35 μg/ml) and ampicillin (100 μg/ml) to select for *A. hydrophila* harboring pSB316. Resistant colonies then were transferred and cultured through three cycles in NY broth without antibiotic additions to allow for marker exchange. The final culture was grown with kanamycin (20 μg/ml) to select for Tn*5*. The kanamycin-resistant organisms were screened on CAS agar for a loss of amonabactin production, indicative of replacement of *amoA* with *amoA*::Tn*5*.

DNA sequence determination and analysis. To determine the nucleotide sequence of *amoA*, the dideoxy chain termination method (31) was used. DNA fragments and deletion subclones (prepared with the Erase-a-Base system from Promega Biotech) were cloned into M13mp18 and M13mp19 and then were transformed into *E. coli* JM101. Required oligonucleotide primers were purchased from Research Genetics, Huntsville, Ala. The Sequenase DNA-sequencing system was obtained from United States Biochemical Corp., Cleveland, Ohio, and α-^{35}S-dATP (1,300 to 1,500 Ci/mmol) was from New England Nuclear, Boston, Mass. Reaction mixtures were electrophoresed in 6% polyacrylamide gels with the Base Runner system (International Biotechnologies, Inc., New Haven, Conn.), and the gels were exposed on X-Omat XRP5 film (Eastman Kodak Co.). Autoradiographs were read with a gel reader, and sequence data were analyzed with Pustell software (both purchased from International Biotechnologies, Inc.).

Nucleotide sequence accession number. The *amoA* sequence has been submitted to GenBank (accession number M63339).

RESULTS

Cloning the amonabactin biosynthetic gene *amoA*. A cloning strategy for an amonabactin biosynthetic gene was developed from the fact that the two siderophores amonabactin and enterobactin, predominantly synthesized by *A. hydrophila* and *E. coli*, respectively, contain 2,3-DHB. Because certain mutants of both organisms that cannot produce 2,3-DHB are able to assemble their siderophores if supplied with 2,3-DHB, it was reasoned that a mutation in an *E. coli* gene encoding a step in 2,3-DHB biosynthesis (yielding an enterobactin-negative phenotype) might be complemented by a recombinant plasmid carrying all or the necessary part of the 2,3-DHB biosynthetic genes cloned from an amonabactin-producing *A. hydrophila* strain. Such a complemented mutant would be expected to produce enterobactin and would be evident as a colony with a siderophore halo on CAS agar. To implement this approach, chromosomal DNA from wild-type *A. hydrophila* 495A2 was used to prepare a gene bank (in the cosmid vector pJRD215) that was hosted in the enterobactin-negative strain *E. coli* SAB11. To produce enterobactin, this strain (which was isolated after chemical mutagenesis of *E. coli* HB101; see Materials and Methods) either requires a DHB supplement or must harbor a recombinant plasmid carrying a gene encoding the missing step in the biosynthesis of 2,3-DHB. Fifteen hundred members of this gene bank were screened, and one siderophore-producing clone was isolated. It harbored a recombinant plasmid, designated pSB215. Analysis by TLC of the phenolates produced by *E. coli* SAB11/pSB215 (when grown in low-iron medium) identified enterobactin; no phenolates were found in the supernatant of low-iron *E. coli* SAB11 cultures harboring only the vector pJRD215. To ascertain whether loss of pSB215 caused reversion to the siderophore-negative phenotype, the culture was cured of the plasmid by growth for three culture cycles in L broth containing 0.1% sodium dodecyl sulfate. When this culture was spread on CAS agar, siderophore-negative colonies were apparent at a frequency of 2×10^{-3}; such colonies were sensitive to kanamycin and did not harbor detectable plasmids. Moreover, when pSB215 (purified by cesium chloride density gradient centrifugation) was used to transform *E. coli* SAB11, all of 200 transformants (selected on the basis of kanamycin resistance) were positive for siderophore production on CAS agar. Analysis of pSB215 with several restriction enzymes gave a size estimate of 3.4 kb for the inserted DNA. This size is smaller than expected; however, analysis of digested *A. hydrophila* chromosomal DNA with a probe representing the insert showed the same-sized fragment in chromosomal DNA, suggesting that no rearrangement had occurred (data not shown). Complementation of siderophore synthesis in *E. coli* SAB11 appeared to reside with the recombinant plasmid pSB215, and the activity was assumed to be due to a cloned

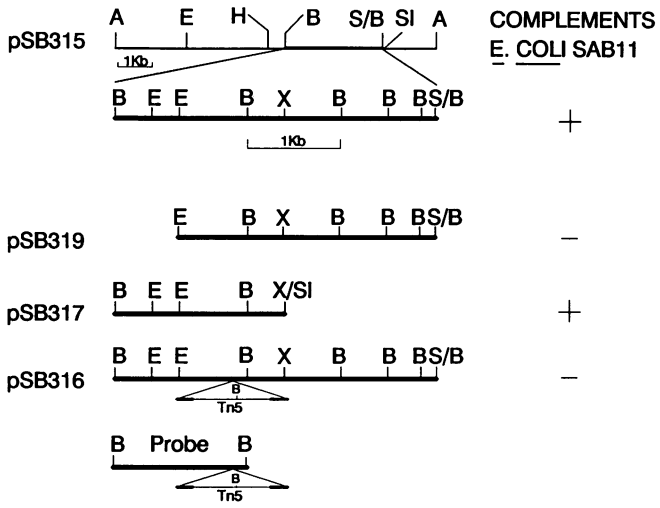

FIG. 1. Restriction maps of several *A. hydrophila* DNA inserts and their capacity to complement the *ent* mutation in *E. coli* SAB11. The vector (pSUP202) with inserted DNA (heavy line) is shown at the top of the figure, and the locations of Tn*5* in pSB316 and the *amoA*::Tn*5* probe are indicated. B, *Bam*HI; E, *Eco*RI; X, *Xho*I; Sl, *Sal*I; S/B, *Sau*3A1-*Bam*HI hybrid site; A, *Ava*I; H, *Hin*dIII; +, positive for complementation; −, negative for complementation.

A. hydrophila gene (designated *amoA*) possibly encoding a step in 2,3-DHB biosynthesis.

The DNA insert in pSB215 was directly subcloned in the tetracycline resistance gene of pSUP202 as a *Sal*I-*Hin*dIII fragment. The pSUP202 plasmid was selected because it usually is unstable in gram-negative bacteria unrelated to *E. coli* (34) and because, unlike the initial cloning vector (pJRD215), pSUP202 does not encode kanamycin resistance. This trait is useful in detection of transposition of Tn*5* which was to be used for insertion inactivation of the cloned *amoA*. The new plasmid, designated pSB315, also complemented *E. coli* SAB11, confirming that complementation activity resided in the cloned DNA.

Mapping and mutagenesis of *amoA*. The restriction map of pSB315 is shown in Fig. 1. To obtain a location for *amoA* on the cloned DNA and to determine which region would be useful for preparation of an *amoA* probe, several deletion derivatives (Fig. 1) were constructed and tested for complementing activity. A recombinant plasmid (pSB319) containing all of the DNA insert to the right of the *Xho*I site as well as the *Eco*RI site immediately to the left of the *Xho*I site was without activity in *E. coli* SAB11. However, pSB317, which retained all of the cloned DNA to the left of the *Xho*I site (including the two *Eco*RI sites), had *amoA* activity.

Mutagenesis of *amoA* with Tn*5* and mapping of the transposon insertion site gave additional information on the location of *amoA*. The kanamycin-resistant exconjugants of a mating between *E. coli* SAB11/pSB315 and *E. coli* C600/pRK340::Tn*5* were screened on CAS agar, and siderophore-negative colonies were isolated. The recombinant plasmid, designated pSB316, in one of these was analyzed by restriction mapping (Fig. 1). The size was 16.3 kb, a gain of 5.5 kb (the size of Tn*5*) over the 10.8-kb size of pSB315. The 1.4-kb *Bam*HI fragment in the DNA insert of pSB315 was not evident in the *Bam*HI digest of pSB316; instead, two new fragments of 3.2 and 3.7 kb in size were present (data not shown). The sum (6.9 kb) of the new fragments equaled the combined size of the 1.4-kb piece and Tn*5*. This transposon has a single *Bam*HI site that divides it into two pieces, accounting for the hybridization results. Mapping with additional restriction enzymes placed the Tn*5* insertion site at 1.25 kb from the left end of the DNA insert, about 0.5 kb to the left of the *Xho*I site (Fig. 1). Therefore, *amoA* occupied the left 1.7 kb of the insert and overlapped the right *Eco*RI site.

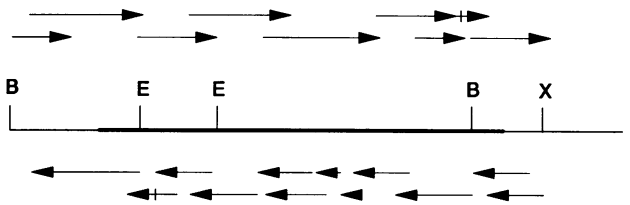

FIG. 2. Sequencing strategy of *amoA*. Priming sites for specific oligonucleotides are indicated by arrows with a vertical slash; the heavy line shows an open reading frame of 1,188 bp. Abbreviations are as in the legend to Fig. 1.

To tentatively characterize the extent of the genetic information present on the cloned *A. hydrophila* DNA, attempts were made to complement known enterobactin mutations with the recombinant plasmid. Neither *E. coli* AN193 (*entA*) nor *E. coli* AN192 (*entB*) were complemented by pSB315; however, the plasmid complemented *E. coli* AN93 (*entE*). This suggests that the 3.4-kb piece of cloned DNA may encode both the 2,3-DHB biosynthetic gene *amoA* and a gene functionally equivalent to the *E. coli* gene *entE*. The product of *entE* is the enzyme 2,3-DHB-AMP ligase which likely activates 2,3-DHB (29).

Nucleotide sequence of *amoA*. The sequencing strategy with subclones of pSB315 is shown in Fig. 2. The sequences of both strands of DNA encompassing *amoA* were determined (Fig. 3). A translational reading frame, beginning with a GTG codon for valine, was open for 1,188 bp. It was preceded by a polypurine sequence resembling a Shine-Dalgarno ribosome binding site (33). The −10 sequence (of the putative −35 and −10 sequences for transcription initiation) was overlapped by an iron box nucleotide sequence resembling the Fur repressor-binding site in other bacteria (3, 8, 12, 13). The Fur protein regulates expression of operons comprising an iron regulon, and production of amonabactin is controlled by iron (4, 5). The open reading frame would encode a possible AmoA protein of 396 amino acids (Fig. 3). Support for the conclusion that *amoA* encodes an enzyme in the 2,3-DHB biosynthetic pathway from chorismic acid (the branch point intermediate in aromatic synthesis) was obtained by comparing the possible AmoA amino acid sequence with that of the *E. coli* EntC protein (isochorismate synthase, the first enzyme in the 2,3-DHB pathway). Alignment of AmoA with EntC revealed an overall identity of 43%, with 73% similarity; however, the 103 C-terminal amino acids of AmoA showed 58% identity and 79% similarity with EntC (Fig. 4). These values are in good agreement with the identity of EntC with the TrpE and PabB proteins, which, like EntC, bind chorismic acid (27). These similarities have been taken as evidence for a family of chorismate-utilizing enzymes (27), to which *amoA* may belong. At the DNA level, *amoA* was 58% identical to *entC*.

Marker exchange mutagenesis with *amoA*::Tn*5*. One of the purposes of the present research was to construct isogenic mutants with lesions in amonabactin biosynthesis for subsequent study of their phenotypic properties. To this end, plasmid pSB316 (*amoA*::Tn*5*) was mobilized by triparental

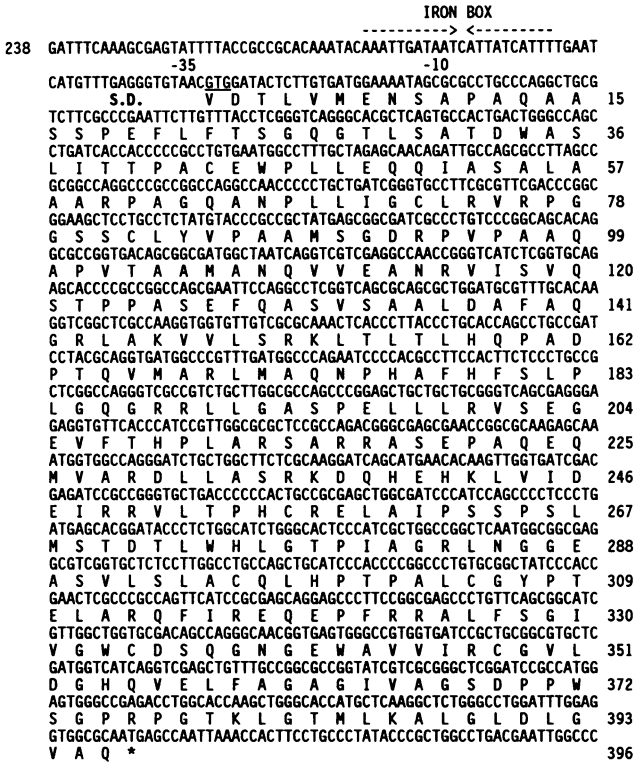

FIG. 3. Nucleotide sequence of the *A. hydrophila* locus *amoA* and its deduced amino acid sequence. Putative signals for a palindromic regulatory sequence (iron box), promoter region (−10, −35), ribosome binding site (S.D.), the translation start codon (single underline) for valine (V), and the stop codon (*) are indicated; amino acid numbers are shown on the right. The nucleotide sequence begins at nucleotide 238 from the left *Bam*HI site of the DNA insert shown in Fig. 1.

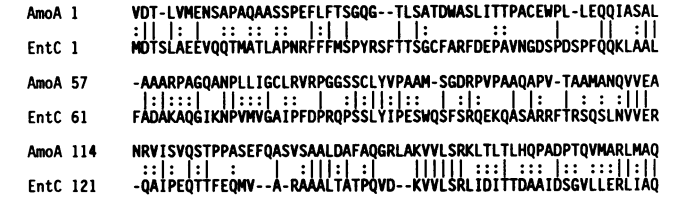

FIG. 4. Alignment of the deduced amino acid sequences encoded by the *A. hydrophila amoA* and the *E. coli entC* genes. Identical amino acids are indicated with a solid line; similar amino acids are indicated with a dotted line.

mating into wild-type *A. hydrophila* 495A2 and exconjugants were grown on medium containing ampicillin (to select for *A. hydrophila*) and kanamycin (to select for Tn*5*). Ampicillin- and kanamycin-resistant colonies which also were resistant to chloramphenicol (encoded on pSB316) then were grown for three culture cycles in NY broth without antibiotics to allow marker exchange and plasmid curing. From this population kanamycin-resistant cells were selected, and from these a siderophore-negative isolate, designated *A. hydrophila* SB22, was identified on CAS agar. Biochemical tests confirmed its identity as *A. hydrophila*; it contained no detectable plasmids.

To ascertain whether *A. hydrophila* SB22 contained the mutated *amoA*::Tn*5* in its genome, a probe was prepared from the left *Bam*HI fragments of the DNA insert in pSB316 (Fig. 1). In *Bam*HI-digested genomic DNA, this probe should detect *amoA* as a 1.4-kb fragment, *amoA*::Tn*5* as two pieces of 3.7 and 3.2 kb (because of the *Bam*HI site in Tn*5*), and Tn*5* at any locus. Hybridization signals were evident only at 3.7 and 3.2 kb in *Bam*HI-digested genomic DNA of the mutant *A. hydrophila* SB22 (data not shown). The probe detected only the expected 1.4-kb *Bam*HI fragment in wild-type *A. hydrophila* 495A2 DNA. Moreover, a probe prepared from the 2.5-kb *Bgl*II fragment of Tn*5* detected only the 3.7- and 3.2-kb *Bam*HI pieces in *A. hydrophila* SB22 (data not shown). These results provided evidence that *amoA*::Tn*5* had replaced *amoA* in the chromosome of *A. hydrophila* SB22 and that Tn*5* was not inserted at any other locus.

Characterization of *A. hydrophila* SB22. Like amonabactin-negative mutants of *A. hydrophila* produced by chemical mutagenesis (4), the marker exchange mutant *A. hydrophila* SB22 was more sensitive than the wild type to inhibition by the chelating agent EDDA. In L agar (seeded with 100 CFU/ml) containing EDDA, the MIC of EDDA for the wild type was 80 to 100 μg/ml; the MIC for *A. hydrophila* SB22 was 40 to 50 μg/ml. In L agar containing 50 μg of EDDA per ml, inhibition of the mutant strain was reversed by paper discs (placed on the seeded agar) containing 2 ng of either form of amonabactin. The increased sensitivity of *A. hydrophila* SB22 to EDDA probably was due to its inability to produce a siderophore; however, the mutant's amonabactin utilization system was intact.

Because amonabactin production was required for growth of *A. hydrophila* SB22 in L agar containing more than 50 μg of EDDA per ml, acquisition of the ability to grow at EDDA levels above 50 μg/ml was a measure of the reversion frequency of this mutant strain. At 70 μg of EDDA per ml, amonabactin-producing revertants formed colonies that were surrounded by satellite colonies (probably stimulated by amonabactin excreted by the revertant). The apparent frequency of reversion to amonabactin production was low, about 3×10^{-7}, indicating that *A. hydrophila* SB22 was a stable mutant.

The supernatant obtained from *A. hydrophila* SB22 cultures grown in low-iron (0.18 μM) glucose-mineral salts medium contained no detectable phenolates when assayed by chemical test and by polyamide TLC (Fig. 5). Of interest was the observation that when supplemented with 2,3-DHB (20 μM), *A. hydrophila* SB22 produced no amonabactin (Fig. 5). The inability of the mutant to produce amonabactin when supplied with 2,3-DHB may be caused by a polarity effect that decreases expression of amonabactin biosynthetic genes that are located downstream from the transposon insertion in *amoA*.

Marker rescue was accomplished by mobilizing a recombinant plasmid (pSB215) carrying *amoA* into *A. hydrophila* SB22. Exconjugants (screened on CAS agar) showed reversion to amonabactin production at a frequency of 10^{-3},

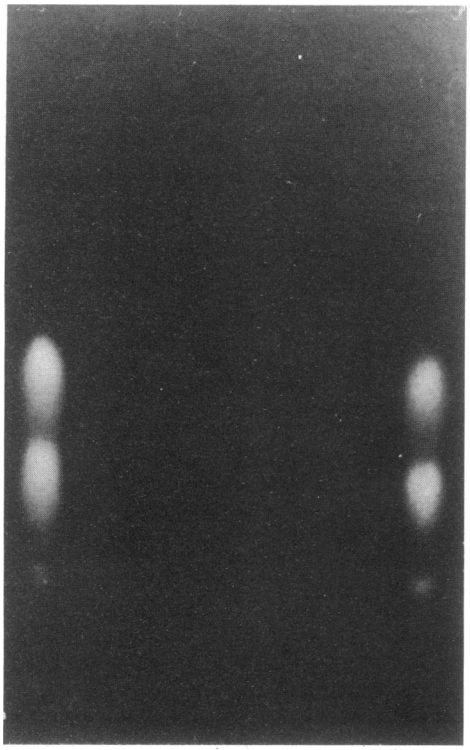

FIG. 5. Production of amonabactin by strains of *A. hydrophila*, demonstrated by polyamide TLC. Lanes: 1, strain 495A2 (wild type); 2, strain SB22 (isogenic amonabactin mutant); 3, strain SB22 grown with added 2,3-DHB (20 μM); 4, strain SB221 constructed by marker rescue of *A. hydrophila* SB22. Abbreviations: P, amonabactin P; T, amonabactin T; D, 2,3-DHB.

contrasting to the low spontaneous reversion frequency of 3×10^{-7}. *A. hydrophila* SB221, an amonabactin-producing isolate obtained by marker rescue, was sensitive to kanamycin (probably because of the loss of Tn*5*), and it produced both forms of amonabactin (Fig. 5).

DISCUSSION

By its complementation of the enterobactin-negative phenotype of *E. coli* SAB11 (a strain requiring exogenous 2,3-DHB for synthesis of enterobactin), the amonabactin biosynthetic gene *amoA* was identified in a gene bank of *A. hydrophila* genomic DNA. The nucleotide sequences of both strands of DNA encompassing *amoA* were determined. A translational reading frame, beginning with a GTG codon for valine, was open for 1,188 bp. Putative signals present in the sequence included a −10, −35 promoter region which was overlapped by a possible iron box sequence resembling the Fur repressor protein-binding site in *E. coli* and some other organisms (3, 8, 12, 13). This likely accounts for iron control of amonabactin synthesis (5) by regulating expression of *amoA* and genes in the same transcriptional unit. The deduced amino acid sequence encoded by *amoA* suggested an AmoA protein of 396 amino acids. Alignment of AmoA with the *E. coli* EntC protein (isochorismate synthetase, the first enzyme in the pathway from chorismic acid to 2,3-DHB), revealed 43% overall identity with 73% similarity; however, the sequence of the 103 C-terminal amino acids of AmoA showed 58% identity and 79% similarity with EntC. These values are like those obtained when EntC is compared with the chorismate-binding protein components TrpE and PabB of the enzymes anthranilate synthetase and *p*-aminobenzoate synthetase, respectively, both of which are initial enzymes in biosynthetic branches from chorismate (27). The regions of greatest resemblance also are found in the C-terminal halves of these proteins. It has been proposed that these proteins are members of a family of chorismate-binding proteins encoded by related genes (27). There was 58% identity of *amoA* and *entC* at the nucleotide level, and present data indicate that the *A. hydrophila* gene *amoA* probably encodes a protein that belongs in this family.

In *E. coli*, the importance of the EntC enzyme in metabolism is attested to by the fact that its reaction product (isochorismic acid) is the precursor of both 2,3-DHB and menaquinone (18). Production of 2,3-DHB from isochorismate is catalyzed by the products of two additional genes, *entB* and *entA*, which occur in *E. coli* in a transcriptional unit composed of *entCEBA(p15)* (6, 21, 27, 29, 30, 36). The close relationship of *amoA* and *entC* suggests that *A. hydrophila* has an operon resembling *entCEBA(p15)*. This speculation is supported by the finding that the cloned DNA containing *amoA* also complemented *E. coli* AN93, an *entE* mutant. The second gene (*entE*) of the *E. coli* operon encodes a 2,3-DHB-activating enzyme (2,3-DHB-AMP ligase) that probably is needed for incorporation of 2,3-DHB into enterobactin (29). The recombinant plasmid carrying *amoA* failed to complement either *entA* or *entB* mutations in *E. coli*, suggesting that if present in *A. hydrophila*, genes equivalent to *entA* and *entB* were not included in the piece of the cloned DNA or that they may be located elsewhere on the chromosome. These data also provide circumstantial evidence that *E. coli* SAB11, the strain isolated and used here for complementation by *A. hydrophila* DNA, is an *entC* mutant. The strain incorporated exogenous 2,3-DHB into enterobactin (indicating a functional *entE* gene), and the complementing *A. hydrophila* DNA lacked *entA* or *entB* activity.

The cloned *amoA* was inactivated by insertion of Tn*5*, and marker exchange mutagenesis of *A. hydrophila* with *amoA*::Tn*5* produced the isogenic amonabactin-negative strain *A. hydrophila* SB22. Hybridization analyses with appropriate probes indicated that *amoA*::Tn*5* had exchanged with the genomic *amoA* and that Tn*5* was present only at this chromosomal locus. While there is no direct evidence that loss of the *amoA* gene product is entirely responsible for the mutant phenotype, studies of the physiology of *A. hydrophila* SB22 gave expected results. Neither amonabactin nor 2,3-DHB was excreted, and the organism was more sensitive than its amonabactin-producing parent to growth inhibition by EDDA. Unlike the wild-type parent, *A. hydrophila* SB22 was unable to overcome the iron-restrictive condition imposed by heat-inactivated human serum, being unable to grow in such serum unless supplemented with iron or amonabactin (24). It used either form of amonabactin to reverse EDDA inhibition, indicating that its amonabactin utilization system was intact. The mutant did not produce amonabactin when supplied with 2,3-DHB. Mutant strains of *A. hydrophila* that are able to use exogenous 2,3-DHB for amonabactin synthesis have been isolated (4). It is possible that expression of one or more of the genes (which may be located downstream from *amoA* in the same transcriptional unit) involved in assembly of amonabactin was blocked by the Tn*5* insertion in *amoA*. Our preliminary evidence suggests the presence of a gene with *entE*-like activity adjacent to or near *amoA*. Mutation of *entC* by insertion inactivation

decreases expression of transcriptionally linked, downstream genes, preventing use of exogenous 2,3-DHB (27).

While amonabactin is the predominant siderophore in aeromonads phenotypically classified as *A. hydrophila* and *A. caviae*, about 80% of the *A. sobria* strains may be enterobactin producers (4, 39). Both of these siderophores utilize 2,3-DHB as a precursor. Comparison of the nucleotide sequences of *amoA* and *entC* revealed apparent divergence from a common ancestor, suggesting that the genes encoding 2,3-DHB production in *E. coli* are evolutionarily distant from their equivalents in amonabactin-producing aeromonads. The 2,3-DHB biosynthetic genes in enterobactin-producing aeromonads may resemble either the amonabactin (*amoA*-like) genes or the *E. coli* (*entC*-like) genes, or they may represent a third evolutionary branch. In fact, it is not yet known whether enterobactin biosynthesis and utilization in the aeromonads parallel the enterobactin system of *E. coli* and other enteric bacteria. This can, and should, be determined.

Cloning, sequence analysis, and mutagenesis of *amoA* offers a potential method for obtaining isogenic amonabactin-negative mutants in almost any amonabactin-producing isolate. This will facilitate determination of a possible role for amonabactin in virulence of *A. hydrophila*. It also opens the way for genetic analyses of the amonabactin system and for comparison of its organization and mechanism of iron regulation with the siderophore systems of other organisms.

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