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Diversity of siderophore genes encoding biosynthesis of 2,3-dihydroxybenzoic acid in *Aeromonas* spp.

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Most species of the genus *Aeromonas* produce the siderophore amonabactin, although two species produce enterobactin, the siderophore of many enteric bacteria. Both siderophores contain 2,3-dihydroxybenzoic acid (2,3-DHB). Siderophore genes (designated *aebC*, *-E*, *-B* and *-A*, for *a*eromonad *e*nterobactin *b*iosynthesis) that complemented mutations in the enterobactin genes of the *Escherichia coli* 2,3-DHB operon, *entCEBA(P15)*, were cloned from an enterobactin-producing isolate of the *Aeromonas* spp. Mapping of the aeromonad genes suggested a gene order of *aebCEBA*, identical to that of the *E. coli* 2,3-DHB operon. Gene probes for the aeromonad *aebCE* genes and for *amoA* (the *entC*-equivalent gene previously cloned from an amonabactin-producing *Aeromonas* spp.) did not cross-hybridize. Gene probes for the *E. coli* 2,3-DHB genes *entCEBA* did not hybridize with *Aeromonas* spp. DNA. Therefore, in the genus *Aeromonas*, 2,3-DHB synthesis is encoded by two distinct gene groups; one (*amo*) is present in the amonabactin-producers, while the other (*aeb*) occurs in the enterobactin-producers. Each of these systems differs from (but is functionally related to) the *E. coli* 2,3-DHB operon. These genes may have diverged from an ancestral group of 2,3-DHB genes.

Keywords: 2,3-dihydroxybenzoic acid, *Aeromonas* spp., amonabactin, enterobactin, *Escherichia coli*, iron acquisition, siderophore genes, siderophores

Introduction

Production of the low molecular mass, ferric chelating molecules called siderophores is the usual iron gathering tactic of many bacterial and fungal microorganisms (Byers 1987). Although siderophores belong to several chemical categories, many of them contain catecholate (phenolate) groups that form all or part of the iron chelation center of the molecules (Matzanke 1991). Many siderophores are based on the iron binding capacity of the phenolate 2,3-dihydroxybenzoic acid (2,3-DHB) (Table 1). These siderophores range in structure from the free monomer 2,3-DHB and its single amino acid conjugates (e.g. 2,3-DHB–glycine) to more complex molecules like enterobactin (the cyclic triester of 2,3-DHB–serine), as well as those (e.g. parabactin) with a spermidine backbone and those (e.g. amonabactin) containing several different amino acids. The phenolate siderophores are produced by a diverse group of microbial physiological classes ranging from animal and plant pathogens to free-living and symbiotic nitrogen fixing microorganisms (Table 1).

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In *Escherichia coli*, the biosynthetic enzymes that catalyze the conversion of chorismic acid (the common intermediate in aromatic biosynthesis) to 2,3-DHB are encoded by an operon designated *entCEBA(P15)*. It is located within the enterobactin cluster of genes (listed in clockwise order on the chromosome), *entD, fepA, fes, entF, fepE, fepC, fepG, fepD, fepB, entC, entE, entB, entA, P15*, which spans about 22 kb near minute 13 on the *E. coli* chromosome (Pickett *et al.* 1984, Nahlik *et al.* 1987, 1989, Liu *et al.* 1989, 1990, Ozenberger *et al.* 1989, Rusnak *et al.* 1989, 1990, Brickman *et al.* 1990). These genes are coordinately expressed as four transcripts originating from two bidirectional, iron-regulated control regions. One of these control regions is located between the ferric enterobactin transport gene *fepB* and the first gene (*entC*) of the 2,3-DHB operon. The *fepB* transcript is initiated anticlockwise and upstream of the *entC* gene, but on the opposite DNA strand. The promoter sequences, as well as two iron-responsive (Fur protein binding) sites, for the divergent transcripts are located in the 103 bp that separate *fepB* and *entC*. The enzymatic activities of the 2,3-DHB operon, with the exception of *P15* (which is phenotypically undefined), are known. The products of *entC*, *entB* and *entA* (isochorismate synthetase, 2,3-dihydro-2,3-dihydroxybenzoate synthetase and 2,3-

Table 1. Siderophores containing 2,3-DHB

Trivial name[a]	Amino components[b]	Producing organisms	References[c]
Agrobactin	sperm, thr	*Agrobacterium tumefaciens*	Ong *et al.* 1979
Aminochelin	putrs	*Azotobacter vinelandii*	Page & von Tigerstrom 1988
Amonabactin	lys, gly and trp or phe	*Aeromonas* spp.	Barghouthi *et al.* 1989
Azotochelin	lys	*Azotobacter vinelandii*	Corbin & Bulen 1969, Knosp *et al.* 1984
Chrysobactin	lys, ser	*Erwinia chrysanthemi*	Persmark *et al.* 1989
2,3-DHB	none	actinomycetes *Acinetobacter calcoaceticus Brucella abortus*	Dyer *et al.* 1964, Ratledge & Chaudrey 1971, Smith *et al.* 1990, Lopez-Goni *et al.* 1992
Enterobactin (enterochelin)	ser	*Escherichia coli Salmonella typhimurium Shigella* spp. *Klebsiella* spp. *Enterobacter* spp. *Aeromonas* spp. *Serratia marcescens Erwinia carotovora*	O'Brien & Gibson, 1970, Pollack & Neilands 1970, Perry & San Clemente 1979, Payne *et al.* 1983, Andrus & Payne 1983, Barghouthi *et al.* 1989, Bull & Loper 1991, Angerer *et al.* 1992, Tarkkanen *et al.* 1992, Podschun *et al.* 1992
Fluvibactin	*nor*-sperm, thr	*Vibrio fluvialis*	Yamamoto *et al.* 1993
Itoic acid	gly	*Bacillus subtilis*	Ito & Neilands 1958
Myxochelin A	lysinol	*Angiococcus disciformis*	Kunze *et al.* 1989
None	thr	*Klebsiella oxytoca Rhizobium trifolii Rhizobium leguminosarum*	Korth 1970, Skorupska *et al.* 1988, Patel *et al.* 1988
None	ala, thr	*Bacillus circulans*	Kobaru *et al.* 1983
None	thr, gly	*Rhizobium* spp. RA-1	Modi *et al.* 1985
None	leu	*Azospirillum lipoferum*	Saxena *et al.* 1986
None	arg	*Pseudomonas stutzeri*	Chakraborty *et al.* 1990
None	ala, lys	*Rhizobium* spp.	Jadhav & Desai 1992
None	*nor*-sperm	*Vibrio fluvialis*	Yamamoto *et al.* 1993
Parabactin	sperm, thr	*Paracoccus denitrificans*	Tait 1975
Protochelin	putrs, lys	'Bacterium DMS5746'	Taraz *et al.* 1990
Spirilobactin	orn, ser	*Azospirillum brasilense*	Bachawat & Ghosh 1987
Vibriobactin	*nor*-sperm, thr	*Vibrio cholerae*	Griffiths *et al.* 1984

[a]Siderophores are listed alphabetically by trivial name; if none available, compounds are so designated. Not listed are anguibactin, a catecholate siderophore (produced by *Vibrio anguillarum*) that does not contain 2,3-DHB (Jalal *et al.* 1989) and a siderophore (produced by *Acinetobacter baumannii*) containing an unidentified phenolate (Echenique *et al.* 1992).

[b]Abbreviations of amino components: ala, alanine; arg, arginine; gly, glycine; leu, leucine; lys, lysine; orn, ornithine; phe, phenylalanine; putrs, putrescine; sperm, spermidine; ser, serine; thr, threonine; trp, tryptophan.

[c]References document the first description of the siderophore in one or more of the listed organisms.

dihydro-2,3-dihydroxybenzoate dehydrogenase, respectively) catalyze sequential conversion of chorismate to 2,3-DHB. The *entE* product (2,3-dihydroxybenzoate-AMP ligase) activates the carboxyl group of 2,3-DHB for final steps of siderophore synthesis. The amino acid sequence of the EntC protein has significant similarities with other chorismate-binding proteins (TrpE and PabB) which is taken as evidence for a family of chorismate utilizing enzymes (Ozenberger *et al.* 1989). The EntB protein may be bifunctional because an activity (previously assigned to an '*entG*' locus which was not revealed by sequence data) in the final steps of enterobactin synthesis from 2,3-DHB and serine is encoded by the *entB* 3′ terminus (Staab & Earhart 1990).

In the enterobactin producing enteric bacteria *E. coli, Salmonella* spp. and *Shigella* spp., the 2,3-DHB genes (as well as other genes involved in assembly and utilization of enterobactin) are highly conserved (Schmitt & Payne 1988, 1991, Faundez *et al.* 1990). Although the native siderophore of *Shigella flexneri* is the hydroxamate aerobactin, 10% of the *S. flexneri* isolates produce both aerobactin and the phenolate siderophore enterobactin (Payne *et al.* 1983). However, an essentially complete, but unexpressed, set of enterobactin genes is present on the chromosome of enterobactin-negative *S. flexneri* strains (Schmitt & Payne 1988). Genetic analyses of these strains revealed several differences (including an amber codon in the 5′ portion of the 2,3-DHB gene *entC*) from the enterobactin-producing strains which may account for lack of expression of the enterobactin genes (Schmitt & Payne 1991).

Do the bacteria that produce siderophores containing 2,3-DHB (Table 1) have similar 2,3-DHB biosynthetic pathways and are the genes that encode the enzymes in the pathways conserved or divergent? The 2,3-DHB genes have been identified and cloned from several organisms. *Vibrio cholerae* produces the phenolate siderophore vibriobactin and a 21.2 kb piece of DNA that complemented mutations in the *E. coli* 2,3-DHB genes *entC* and *entA* was cloned from *V. cholerae* (Stoebner & Payne 1988). Probes for the *entCEBA(P15)* operon failed to hybridize with *V. cholerae* DNA digests, suggesting little DNA sequence homology between the 2,3-DHB operons of each organism but the presence in *V. cholerae* of genes

that are functionally similar to the *E. coli entCEBA(P15)* operon (Stoebner & Payne 1988).

The operon (*fct cbsCEBA*) that includes the 2,3-DHB genes has been cloned from the plant pathogen *Erwinia chrysanthemi* (Franza *et al.* 1991, Franza & Expert 1991). Although the first gene (*fct*) of the operon is involved in transport of the *E. chrysanthemi* siderophore chrysobactin, the gene order of the 2,3-DHB biosynthetic genes (*cbsCEBA*) is identical to that of *E. coli*. Probes for *E. coli entEBA* genes hybridized at low stringency with the *E. chrysanthemi* 2,3-DHB genes.

Bacillus subtilis produces the simple phenolate siderophore 2,3-DHB–glycine. Cloned *B. subtilis* DNA complemented *E. coli* strains with mutations in the *entE, entB, entA* and *entC* genes (Grossman *et al.* 1993), suggesting that genes equivalent to those in the *E. coli entCEBA (P15)* operon occur in *B. subtilis*.

The Gram-negative genus *Aeromonas* can be divided by DNA–DNA reassociation kinetics into at least 14 hybridization groups (HG) and the type of siderophore made is characteristic of the HG (Zywno *et al.* 1992, Byers & Arceneaux 1993). Most of the aeromonad HG produce the phenolate siderophore amonabactin. From an amonabactin-producing isolate, a gene (*amoA*) that complemented an *E. coli entC* mutation was cloned and sequenced (Barghouthi *et al.* 1991). The nucleotide sequence of *amoA* is only 58% identical to *entC* but the deduced amino acid sequence of the *amoA* product has a carboxy-terminal similarity with EntC protein of 79%. Immediately downstream from *amoA* is a gene that complements an *E. coli entE* mutation, suggesting that the 2,3-DHB genes in amonabactin-producing *Aeromonas* spp. may be of the same order on the chromososme as *E. coli entCEBA(P15)*. The *Aeromonas* spp. HG 8/10 and HG 9 produce enterobactin, the siderophore of enteric bacteria. Present studies were done to determine if the genes encoding biosynthesis of 2,3-DHB in the enterobactin-producing *Aeromonas* spp. resembled either the *E. coli entCEBA(P15)* operon or the 2,3-DHB genes in the amonabactin-producing aeromonads, or if they might be yet another version of the 2,3-DHB genes. Analyses of the cloned 2,3-DHB genes from enterobactin-producing *Aeromonas* spp. suggest the presence of genes that are functionally equivalent to, but evolutionarily distinct from, the 2,3-DHB genes of both *E. coli* and the amonabactin-producing aeromonads.

Materials and methods

Bacteria, plasmids, routine cultivation and culture media

The *E. coli* and *Aeromonas* spp. strains and the plasmids (with their relevant characteristics and sources) used for the gene cloning and mapping studies are listed in Table 2. Additional wild-type strains (total of 188) of the *Aeromonas* spp. (obtained from S. Barghouthi, C. Lobb, J. Bertolini, S. Stuart, S. Zywno, L. Pickering and L. Ford) and clinical isolates (total of 16) of *E. coli* (provided by M. Lundrigan) were used in the hybridization studies. The type of siderophore produced by the *Aeromonas* spp. isolates was previously determined (Zywno *et al.* 1992). All cultures were preserved at −80 °C in nutrient broth (Difco Laboratories, Detroit, MI) containing 25% glycerol. Both the *E. coli* and *Aeromonas* spp. cultures usually were grown (at 37 and 30 °C, respectively) on nutrient broth or Luria–Bertani (LB) broth or agar (Miller 1972). The chrome azurol S (CAS) siderophore detection agar was prepared as described by Barghouthi *et al.* (1989); appropriate nutritional supplements were added to the CAS agar to support growth of the *E. coli* strains.

Preparation and screening of gene banks for entC*-equivalent genes: bioassay for enterobactin production*

Chromosomal DNA was prepared by the method of Marmur (1961). Plasmid DNA was isolated using Qiagen columns as instructed by the manufacturer (Qiagen, Chatsworth, CA). A genomic library of *Aeromonas* spp. SS279 was constructed by standard methods (Maniatis *et al.* 1982). Briefly, chromosomal DNA was partially digested with *Sau*3A and ligated to the *Bam*HI site of the cosmid vector pJB8. Restriction and nucleic acid modifying enzymes were obtained from Promega (Madison, WI). The ligated DNA then was packaged with a commercial system ('Pack-a-gene', obtained from Promega) as directed by the manufacturer. *E. coli* SAB11 was infected with the phage preparation and ampicillin-resistant transformants were isolated. The transformants were screened on CAS siderophore detection agar for complementation of the *entC* mutation in the *E. coli* SAB11 chromosome. Complementation of the *entC* mutation by an *entC*-equivalent gene product permits synthesis of 2,3-DHB and subsequent assembly of enterobactin, producing a colony with a yellow–orange siderophore halo on the CAS agar. Recombinant plasmids prepared in subcloning procedures also were analyzed with the CAS agar procedure for their ability to complement mutations of *ent* genes present in various strains of *E. coli*. The plasmids were introduced into *E. coli* strains by transformation (Davis *et al.* 1980). Siderophore production was also determined in a bioassay as previously described (Fleming *et al.* 1985) employing the *E. coli* indicator strains AN193 and AN90 to detect the production of 2,3-DHB and enterobactin, respectively.

Mapping and subcloning

The recombinant plasmids were mapped by single and multiple digestion with various restriction enzymes as described by Maniatis *et al.* (1982). For subcloning, the recombinant and recipient plasmids were similarly digested, and the DNA fragments mixed and then ligated with T4 ligase. When a specific restriction fragment was to be subcloned, it was purified from agarose gels by the 'Prep-a-Gene' system (Bio-Rad Laboratories, Richmond, CA) as described by the manufacturer.

Table 2. Bacterial strains and plasmids used for gene cloning

Strain or plasmid	Genotype or phenotype	Reference or source
E. coli strains		
HB101	*proA2 leu lacY1 ara14 galK2 xyl5 mtl1 recA12 hsdR hsdM supE44*	S. M. Payne
SAB11	Like HB101 but *entC*	Barghouthi *et al.* 1991
C600	F^-, *thi*-1, *thr-t leuB6 lacY1 tonA21 supE44*	Maniatis *et al.* 1982
AN90	*thi leuB proC trpE lacY mtl xyl rpsL azi fhuA tsx supA entD*	S. Stuart
AN93	Like AN90 but *entE* (instead of *entD*)	S. M. Payne
AN193	Like AN90 but *entA* (instead of *entD*)	S. Stuart
AN193-60	Like AN193 but *recA*	M. McIntosh
AN192-60	Like AN193-60 but *entB* (instead of *entA*)	M. McIntosh
DK214	*thi leuB proC trpE lacY mtl xyl rpsL azi fhuA tsx purE fepB*	C. Earhart
MM294	*endol B1* r_k	S. M. Payne
Aeromonas species		
SS279	human fecal isolate, produces enterobactin	S. Stuart
SS279R	spontaneous Rf^r SS279	this study
BAM1	Isogenic with SS279R but *aebC*::Km^r	this study
Plasmids		
pJB8	cosmid Ap^r	S. M. Payne
PSUP202	pBR325::*mob* Tc^r Cm^r Ap^r	Simon *et al.* 1983
pRK340	Tc^r Km^r Tn*5*	S. M. Payne
pRK2013	ColE1 *mob*$^+$ *tra*$^+$ (RK2) Km^r	S. M. Payne
pITS55	*entCEBA(P15)* Ap^r	M. McIntosh
pSB315	pSUP202::*amoA*	Barghouthi *et al.* 1991
pGM3	pJB8::*aebC, aebE, aebB*; complements *E. coli entC, -E, -B* mutations	this study
pGM4	pJB8::*aebC, aebE, aebB, aebA*; complements *E coli entC, -E, -B, -A* mutations	this study
pSS11	pSUP202::*aebC, aebE, aebB*; subclone of pGM3	this study
pSK11	pSS11::Kn^r	this study
pSS117	pSUP202::*aebC, aebE*; deletion derivative of pSS11, complements *E. coli entC, entE*	this study
pSS1156	pSUP202::*aebC*; deletion derivative of pSS11, complements *E. coli entC*	this study
pSS116	pSUP202::*aebB*; deletion derivative of pSS11, complements *E. coli entB*	this study
pSS115	pSUP202::*aebC, aebE*; deletion derivative of pSS11, complements *E. coli entC, entE*	this study
pSS113	pSUP202::*aebC*; deletion derivative of pSS11, complements *E. coli entC*	this study
pSK113	pSS113 *aebC*::Kn^r; does not complement *E. coli entC*	this study
pST1	pSS113::Tn*5*; complements *E. coli entC*	this study
pST2	pSS113 *aebC*::Tn*5*; does not complement *E. coli entC*	this study
pST3	pSS113::Tn*5*; complements *E. coli entC*	this study
pJELA3	pSUP202::*aebC, aebE, aebB, aebA*; subclone of pGM4; complements *E. coli entC, -E, -B, -A*	this study

Mutagenesis of aeromonad aeb *genes: marker exchange mutagenesis*

Mutagenesis with transposon Tn*5* was used to localize the cloned *aebC* gene on plasmid inserts. The desired plasmids were introduced (by transformation) into *E. coli* C600, a strain harboring the temperature sensitive plasmid pRK340 which carries Tn*5*. Transformants were selected on agar containing the appropriate antibiotics which was incubated at 41 °C. The resulting transformants were pooled, diluted and subcultured several times in LB broth at 41 °C. Plasmids (most of which should be the recombi-

nant plasmid with Tn*5*) were prepared from the LB broth culture and were transformed into *E. coli* SAB11. The resulting transformants were screened on CAS agar. Recombinant plasmids lacking *entC*-complementing activity were selected for mapping. The insertion point of the Tn*5* in the recombinant plasmid was determined by single digestion with *Hin*dIII, *Eco*RI, *Xho*I and *Bam*HI.

The insertion of a cassette (Km^r 'GenBlock', obtained from Pharmacia, Piscataway, NJ) encoding kanamycin resistance into the appropriate restriction enzyme sites of cloned DNA also was employed to map the gene loci. The cassette and the plasmid were digested similarly, and the mixed DNA fragments were ligated overnight with T4 ligase. The ligated DNA was transformed into *E. coli* SAB11. The transformants were selected on LB agar with kanamycin (50 μg/ml) and then they were screened on CAS agar for loss of *entC*-complementing activity. The resulting inactivated recombinant plasmids were mapped with various restriction enzymes to locate the insertion.

For marker exchange mutagenesis of *aebC*, plasmid pSK113 (pSS113 *aebC*::Km^r) was mobilized into *Aeromonas* spp. SS279R, a spontaneous rifamycin resistant (Rf^r) mutant derived from *Aeromonas* spp. SS279, by triparental mating with the method of Barghouthi *et al.* (1991) using *E. coli* MM294/pRK2013 (mobilizing strain) and *E. coli* SAB11/pSK113 (mutagenizing strain). The exconjugants were cultured in LB medium containing rifamycin (75 μg/ml) to select for *Aeromonas* spp. SS279R and kanamycin (50 μg/ml) to select for *aebC*::Km^r. The resulting cells were subcultured several times in LB medium without antibiotics to allow for allelic exchange. The cells then were plated on CAS agar containing kanamycin (50 μg/ml). Colonies without a siderophore halo growing on this agar (indicative of replacement of *aebC* with *aebC*::Km^r) were transferred to LB agar containing chloramphenicol (20 μg/ml) to determine sensitivity to this antibiotic, assuring that pSK113 had been lost. One such siderophore negative, kanamycin resistant, chloramphenicol sensitive strain designated *Aeromonas* spp. BAM1 was selected.

Colony and dot blot hybridization

Colony and dot blots were prepared as described by Maniatis *et al.* (1982). DNA probes were prepared as restricted fragments purified from agarose gels employing the 'Prep-a-Gene' system (Bio-Rad Laboratories). The DNA probes were non-radioactively labeled with the 'Genius system' (Boehringer Mannheim, Indianapolis, IN) as directed by the manufacturer. The membranes were hybridized with labeled probe DNA overnight at 65 °C. After hybridization, the membranes were washed twice for 5 min at room temperature with 2 × SSC containing 0.1% SDS. For high stringency conditions the blots were also washed twice for 15 minutes at 68 °C with 0.1 × SSC containing 0.1% SDS. Low stringency conditions consisted of two washes for 15 min at 42 °C with 2 × SSC containing 0.1% SDS. Membranes were then developed as directed by the manufacturer.

Results

Cloning 2,3-DHB genes from an enterobactin-producing Aeromonas *spp.*

By its complementation of the *entC* mutation in *E. coli* SAB11, an *entC*-equivalent gene (*amoA*) previously was identified in a gene library prepared from an amonabactin-producing strain of *Aeromonas* spp. (Barghouthi *et al.* 1991). The *E. coli* SAB11 strain is unable to produce enterobactin and grows as a colony without a siderophore halo on the CAS siderophore detection agar. Complementation of the *entC* mutation permits 2,3-DHB synthesis and subsequent assembly of enterobactin, producing a colony with a visible yellow–orange halo on the blue CAS agar. In present studies, we used the same strategy to clone 2,3-DHB genes from the enterobactin-producing *Aeromonas* spp. SS279. Chromosomal DNA that had been partially digested with *Sau*3A was used to prepare a gene bank (in the cosmid vector pJB8) that was hosted in *E. coli* SAB11. The library was screened on CAS agar for complementation of the *entC* mutation. One thousand individual colonies were examined; five demonstrated a siderophore halo. Plasmids were prepared from the five colonies and used to transform *E. coli* SAB11. All transformants from all five plasmid preparations were positive for siderophore synthesis of CAS agar, demonstrating that the phenotype was due to the presence of the recombinant plasmid. These plasmids were designated pGM1 through 5. Synthesis of enterobactin by *E. coli* SAB11 harboring these plasmids was confirmed by bioassay for growth stimulation of enterobactin-requiring *E. coli* strains. Electrophoretograms of *Eco*RI–*Sal*I digests of pGM3 and pGM4 demonstrated that the two plasmids contained different inserts of 35–40 kb of *Aeromonas* spp. DNA. The cloned gene from the enterobactin producing *Aeromonas* spp. which complemented the *entC* mutation of *E. coli* SAB11 was designated *aebC* (*a*eromonad *e*nterobactin *b*iosynthesis).

To subclone the *aebC* gene to a smaller DNA fragment and insert that fragment into a mobilizable plasmid, *Eco*RI fragments of pGM3 and pGM4 were subcloned in the high copy number plasmid pSUP202. The recombinant plasmids were transformed into *E. coli* SAB11 and were screened on CAS agar for siderophore production. A subclone (designated pSS11) of pGM3 was positive for siderophore production; it contained an 8.6 kb insert. A second positive subclone (termed pJELA3) of pGM4 contained a 14.5 kb insert. The two plasmids were mapped by restriction enzyme digestion (Figure 1). The 8.6 kb insert of pSS11 was identical to the left end of the 14.5 kb insert of pJELA3.

To determine if 2,3-DHB genes in addition to *aebC* also had been cloned, the recombinant plasmids were tested for complementation of known mutations in the *E. coli* enterobactin genes. The plasmid pGM3 and its subclone, pSS11, complemented mutations in the *entC*, *entE* and *entB* genes (*E. coli* strains SAB11, AN93 and AN192-60, respectively). Neither plasmid complemented the *entA*

mutation in *E. coli* AN193-60. However, the larger subclone pJELA3 (derived from pGM4) complemented mutations in all 2,3-DHB biosynthetic genes, *entC, -E, -B* and *-A*. The cloned complementing genes were designated *aebC, aebE, aebB* and *aebA*. The enterobactin transport gene *fepB* exists immediately upstream from the *E. coli entCEBA(P15)* operon (Brickman *et al.* 1990). The plasmids pGM3 and pSS11 failed to complement the *fepB* mutation contained in *E. coli* DK214, suggesting that a gene functionally equivalent to *fepB* had not been cloned with the *aeb* genes.

*Gene order of the 2,3-DHB genes (*aebC, -E, -B *and* -A*) of enterobactin-producing* Aeromonas *spp.*

To initially compare the 2,3-DHB biosynthetic genes from *Aeromonas* spp. to those of *E. coli*, the order of the genes cloned from the enterobactin-producing *Aeromonas* spp. was determined. Plasmid pJELA3, but not pSS11, complemented the mutation in *entA*, indicating that the aeromonad *entA* equivalent existed within the right end 5.9 kb of DNA of the insert in pJELA3. Deletion mapping of various fragments of the 8.6 *Eco*RI insert of pSS11 was used to determine the order and relative position of the various genes involved in the biosynthesis and activation of 2,3-DHB. The resulting plasmids were analyzed for their ability to complement various mutations (Figure 2). Deletion of the left end (2.4 kb *Eco*RI–*Bgl*II fragment) of the 8.6 *Eco*RI insert of pSS11 had no effect on the *entC* complementing activity while deletion of the 1.2 kb *Eco*RI–*Sal*I fragment from the right end resulted in the loss of *entB* activity. The loss of *entE* activity was caused by removal of the 3.0 kb *Eco*RI–*Bam*HI fragment from the right end of the insert and also by the deletion of the internal 2.0 kb *Xho*I fragment. The *Xho*I deletion in pSS116 also resulted in the destruction of *entC* activity. The 3.0 kb *Bgl*II–*Bam*HI fragment in plasmid pSS113 contained the *entC* activity (*aeb*C). The deletion analysis suggests that the gene order for the *aeb* genes is *aebCEBA*, identical to the *entCEBA(P15)* operon of *E. coli*.

Insertion mapping of the aebC *activity*

The effect (on complementing actvity for *E. coli ent* mutations) of insertion of either a kanamycin resistance (Km^r) cassette or Tn*5* into the cloned aeromonad DNA was used to define the position of the *aebC* gene in the insert. Neither *entC, -E* nor *-B* complementing activity was eliminated by the insertion of the Km^r cassette in the *Bgl*II site of pSS11; however, when the insertion was in the *Xho*I site of pSS113, the *entC*-like activity of *aebC* was inactivated (Figure 2). To localize *aebC* on the DNA insert in pSS113, the plasmid was mutagenized with Tn*5*. Of several Tn*5* insertions, *entC* complementing activity was destroyed by Tn*5* at 820 bp to the left of the *Xho*I site

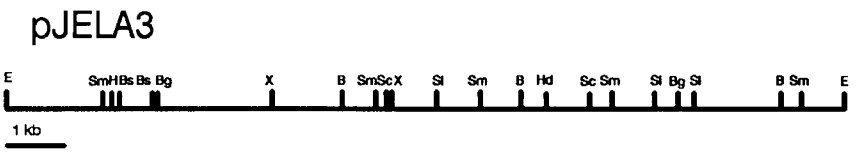
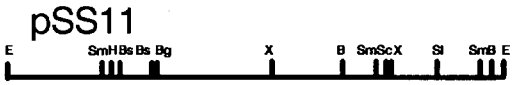


Figure 1. Restriction maps of pSS11 and pJELA3. Symbols: B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EII; E, *Eco*RI; H, *Hpa*I; Hd, *Hin*dIII; Sc, *Sac*I; Sl, *Sal*I; Sm, *Sma*I; X, *Xho*I.



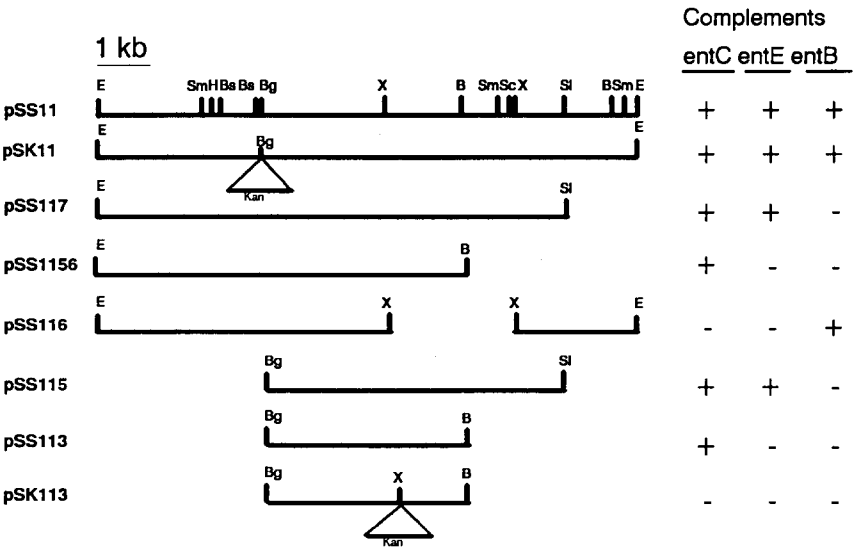


Figure 2. Derivatives of pSS11 and their ability to complement mutations in *entC, entE* or *entB* carried in *E. coli* strains SAB11, AN93 and AN192-60, respectively. Deletions and insertions are shown in relation to pSS11. Symbols: B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EII; E, *Eco*RI; H, *Hpa*I; Hd, *Hin*dIII; Sc, *Sac*I; Sl, *Sal*I; Sm, *Sma*I; X, *Xho*I; kan, Km^r GenBlock™; +, complementation; −, no complementation.

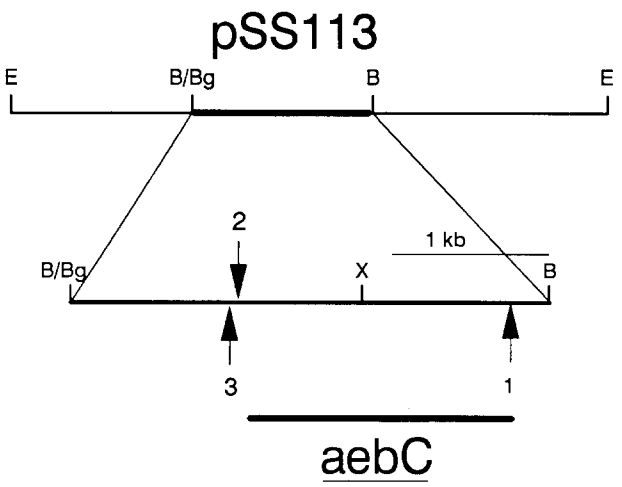


Figure 3. Tn*5* mutagenesis of the cloned *aebC* gene. Numbered arrows indicate sites of insertions. Insertion 2 (which mapped 820 bp to the left of the *Xho*I site) disrupted *aebC* activity; insertions 1 (250 bp to the left of the *Bam*HI) and 3 (860 bp to the left of the *Xho*I site) did not affect *aebC* activity. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; X, *Xho*I.

(Figure 3); however, another Tn*5* insertion that mapped only 40 bp away (at 860 bp to the left of the *Xho*I site) retained the *entC* complementing activity. Another insertion site of Tn*5*, 250 bp to the left of the *Bam*HI site, also did not affect activity. Therefore, *aebC* (the *entC*-equivalent of enterobactin-producing *Aeromonas* spp.) mapped to a 1.7 kb region of the 3.0 kb *Bgl*II–*Bam*HI DNA insert of pSS113. This region begins at 1.3 kb to the right of the *Bgl*II site and extends for 1.7 kb to a point 0.250 kb to the left of the *Bam*HI site (Figure 3).

Marker exchange mutagenesis

The complementation data suggest that the *aeb* genes cloned from the enterobactin-producer *Aeromonas* spp. SS279 encode enzymes for enterobactin biosynthesis. To confirm this point, the plasmid pSK113 (*aebC*::Km^r, Figure 2) was mobilized into *Aeromonas* spp. SS279R and the exconjugants were selected in medium containing rifamycin (to select for *Aeromonas* spp. SS279R) and kanamycin (to select for *aebC*::Km^r). The resulting cells then were subcultured several time in medium without antibiotic addition to allow for marker exchange. This population was screened on CAS agar containing kanamycin to identify enterobactin-negative, Km^r colonies (indicative of exchange of *aebC*::Km^r for *aebC* in the chromosome). The chloramphenicol sensitivity (assuring loss of pSK113) of one such isolate, *Aeromonas* spp. BAM1, was confirmed. The marker exchange experiment indicates that insertion-inactivation of the *entC*-complementing activity of the aeromonad gene *aebC* also yields a mutated gene that blocks enterobactin production in *Aeromonas* spp. when the inactive gene is present in the chromosome.

Hybridization of probes for the 2,3-DHB biosynthesis genes

Cloning of the siderophore genes from an enterobactin-producing aeromonad isolate made possible an assessment (by hybridization with DNA probes) of the relatedness of the genes encoding the 2,3-DHB biosynthetic enzymes in *E. coli*, in the amonabactin-producing *Aeromonas* spp. and in the enterobactin-producing *Aeromonas* spp. The gene probes for the *E. coli* 2,3-DHB genes were prepared from the plasmid pITS55 which was digested with *Eco*RI and *Ava*I to yield a 6.0 kb *Eco*RI fragment containing *entEBA* and a 1.1 kb *Eco*RI–*Ava*I fragment with *entC*. The probe for the *amoA* gene was isolated as a 1.4 kb *Bam*HI fragment from plasmid pSB315. The *aeb* gene probe was a 2.0 kb *Xho*I fragment from the plasmid pSS11 (Figure 3). The probes were hybridized (at both high and low stringencies) to colony blots of strains of *Aeromonas* spp. and *E. coli* (Table 3). When 76 isolates of *Aeromonas* spp. (54 enterobactin and 22 amonabactin producers) and 16 *E. coli* strains were tested with the *entEBA* probe, hybridization was evident only with the *E. coli* strains. Similarly, the *entC* probe hybridized only to the *E. coli* strains. Although fewer strains were tested, the *aebCE* gene probe hybridized only with the enterobactin-producing strains of *Aeromonas* spp. and with neither the amonabactin-producing isolates nor the *E. coli* strains, indicating significant differences in the nucleotide sequences of the 2,3-DHB genes of the three physiological types.

An interesting situation was noted with the gene probe for *amoA*, the *entC*-equivalent from amonabactin-producing aeromonads. All of the 22 amonabactin-producers hybridized with the *amoA* probe, whereas none of the 22 enterobactin-producing aeromonad strains did so. However, the *amoA* gene probe also hybridized with chromosomal DNA of the *E. coli* strains. The percent identity of the DNA sequence between *amoA* and *entC* is

Table 3. Hybridization of siderophore gene probes

Organism tested	Gene probes[a] (number of isolates positive/number tested)			
	entC	*entEBA*	*amoA*	*aebCE*
E. coli (enterobactin-producing)	16/16	16/16	16/16	0/4
Aeromonas spp. (enterobactin-producing)	0/54	0/54	0/22	9/9
Aeromonas spp. (amonabactin-producing)	0/22	0/22	22/22	0/10

[a]Gene probes for the *E. coli* 2,3-DHB genes were prepared from pITS55 (Nahlik *et al.* 1989) by digestion with *Eco*RI and *Ava*I to yield a 6.0 kb fragment containing *entEBA* and a 1.1 kb fragment with *entC*. The probe for *amoA* was a 1.4 kb *Bam*HI fragment from pSB315 (Barghouthi *et al.* 1991). The probe for *aebCE* was a 2.0 kb *Xho*I fragment of pSS11 (this study).

58% (Barghouthi *et al.* 1991), which suggests that the *amoA* probe had not hybridized to the *E. coli entC* gene. Moreover, the *E. coli entC* probe failed to hybridize to the aeromonad DNA, suggesting that *amoA* and *entC* do not cross-hybridize. To examine this paradox, dot blot analyses were performed with *entC*, *entEBA* and *amoA* as probes. Once again the *E. coli ent* probes hybridized only with the DNA from *E. coli* strains and not with *Aeromonas* spp. DNA or with pSB315, which carries the *amoA* gene. The *amoA* probe hybridized with chromosomal DNA from both *E. coli* and an amonabactin-producing *Aeromonas* spp. strain, but not to plasmid DNA of pITS55 which carries *E. coli entCEBA(P15)*. The inability of specific probes for *entC* and *amoA* to cross-hybridize suggests that the *amoA* probe hybridized to a gene sequence other than *entC* in the *E. coli* DNA. It is possible that the portion of the *amoA* probe outside of the *amoA* gene hybridized with *E. coli* DNA. On the other hand, chorismate utilizing enzymes (like the products of *amoA*, *entC*, *pabB*, *trpE* and possibly others) represent a family of evolutionarily related enzymes with significant amino acid sequence identities (Ozenberger *et al.* 1989, Barghouthi *et al.* 1991). It may be that the *amoA* gene probe hybridized with an *E. coli* locus (other than *entC*) derived from this family.

Discussion

The similarities in function and proximity on the chromosome of the genes encoding the 2,3-DHB biosynthetic enzymes in amonabactin-producing *Aeromonas* spp., in enterobactin-producing *Aeromonas* spp. and in other bacteria suggest more than coincidental relatedness among these systems. The genus *Aeromonas* has at least two distinct, but functionally and probably evolutionarily related, genetic systems for the biosynthesis and activation of 2,3-DHB. One of these is found in the amonabactin-producing species, the other in the enterobactin-producing types. Each of these systems differs from, but also is functionally related to, the 2,3-DHB operon found in *E. coli* and possibly other bacteria. In addition to *E. coli* and *Shigella* spp., the restriction maps of the 2,3-DHB genes of *E. chrysanthemi*, for the *entC* and *entE* counterparts (*amoAB*) of amonabactin-producing aeromonads, and for the *enCEBA* equivalents in the Gram-positive microorganism *B. subtilis* also have been reported (Figure 4). Despite the apparent functional compatibility of the gene products, comparison of these maps suggests divergence at the nucleotide sequence level, as well as rearrangements in the gene order of the *B. subtilis* system. Gene probes for the two aeromonad types and for the *E. coli* 2,3-DHB genes did not cross-hybridize; however, cross-hybridization of probes for the *E. coli* genes with the *E. chrysanthemi cbsCEBA* genes (Franza & Expert 1991) indicates a closer relationship between these two. The 2,3-DHB biosynthetic systems in most or all of the phenolate siderophore producing organisms may have evolved from a single ancestral group of 2,3-DHB genes. The monomer (2,3-DHB) may be the archetypical phenolate siderophore; it remains the siderophore of *Brucella*

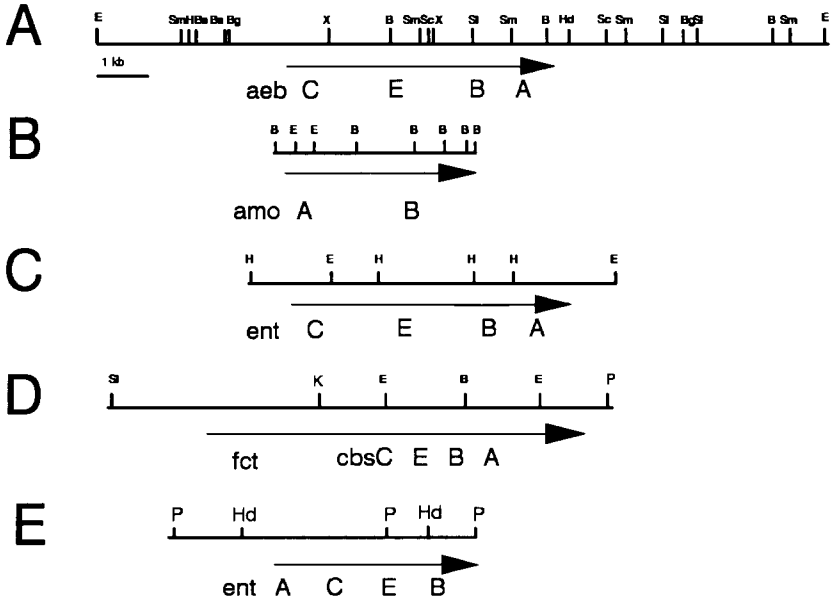


Figure 4. Restriction maps and approximate positions of the 2,3-DHB genes of five organisms (arrows indicate most probable direction of transcription): (**A**) *aebCEBA* from enterobactin-producing *Aeromonas* spp. (this study); (**B**) *amoAB* (*entCE* equivalents) from amonabactin-producing *Aeromonas* spp. (Barghouthi *et al.* 1991); (**C**) *entCEBA(P15)* from *E. coli* (Nahlik *et al.* 1989); (**D**) *cbsCEBA* from chrysobactin-producing *E. chrysanthemi*; the *fct* gene is the chrysobactin receptor (Franza & Expert 1991); (**E**) *entACEB* (equivalent to similarly designated *E. coli* genes) from *B. subtilis* (Grossman *et al.* 1993; T. Grossman & B. Rowlands, personal communication). Symbols: B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EII; E, *Eco*RI; H, *Hpa*I; Hd, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Sc, *Sac*I; Sl, *Sal*I; Sm, *Sma*I; X, *Xho*I.

abortus and perhaps other organisms (Table 1). The modern array of structurally diverse 2,3-DHB-containing siderophores may be the result of evolutionary radiation in which the chemical and ferric chelating properties of the siderophores were altered by linking one or more of the 2,3-DHB monomers to other compounds, fitting the siderophores to certain environmental niches.

The *E. coli fepB* gene (which encodes a protein that shuttles ferric enterobactin across the periplasmic space) is located immediately upstream from *entC* (Brickman *et al.* 1990). We did not find a *fepB*-complementing gene upstream of the closed *aebC* gene of enterobactin-producing *Aeromonas* spp. This does not rule out the existence of an enterobactin transport gene upstream of *aebC*, but if one is present, it is not the functional equivalent of the *E. coli fepB* gene. Enterobactin uptake genes have not yet been identified in the *Aeromonas* spp.

In *E. coli*, the final synthesis (and excretion) of enterobactin from serine and 2,3-DHB requires the products of the genes *entB, -D* and *-F*. The assembly of enterobactin in the aeromonads may resemble that in *E. coli* because we noted that an *Eco*RI fragment of plasmid pGM3 complemented mutations in both *E. coli* genes *entD* and *entF* (unpublished data). The relevant aeromonad genes (tentatively designated *aebD* and *aebF*) have not been subcloned. The gene products of *aebC, -E, -B, -A, -D* and *-F* may represent all enzymes necessary for the biosynthesis of enterobactin in *Aeromonas* spp.

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