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Cloning of a *Vibrio cholerae* Vibriobactin Gene Cluster: Identification of Genes Required for Early Steps in Siderophore Biosynthesis

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***Vibrio cholerae* secretes the catechol siderophore vibriobactin in response to iron limitation. Vibriobactin is structurally similar to enterobactin, the siderophore produced by *Escherichia coli*, and both organisms produce 2,3-dihydroxybenzoic acid (DHBA) as an intermediate in siderophore biosynthesis. To isolate and characterize *V. cholerae* genes involved in vibriobactin biosynthesis, we constructed a genomic cosmid bank of *V. cholerae* DNA and isolated clones that complemented mutations in *E. coli* enterobactin biosynthesis genes. *V. cholerae* homologs of *entA*, *entB*, *entC*, *entD*, and *entE* were identified on overlapping cosmid clones. Our data indicate that the vibriobactin genes are clustered, like the *E. coli* enterobactin genes, but the organization of the genes within these clusters is different. In this paper, we present the organization and sequences of genes involved in the synthesis and activation of DHBA. In addition, a *V. cholerae* strain with a chromosomal mutation in *vibA* was constructed by marker exchange. This strain was unable to produce vibriobactin or DHBA, confirming that in *V. cholerae* VibA catalyzes an early step in vibriobactin biosynthesis.**

Virtually all organisms require iron for growth, but in most aerobic environments, iron availability is limited. Many microorganisms have developed efficient means to obtain iron and under conditions of iron deprivation produce low-molecular-weight iron-chelating compounds called siderophores (23). These compounds are synthesized and secreted into the environment, where they bind ferric iron with high affinity. In gram-negative bacteria, outer membrane receptors, including FepA in *Escherichia coli* (30) and ViuA in *Vibrio cholerae* (10, 54), bind the ferrisiderophore complex. Transport of the ferrisiderophore into the cell is dependent upon TonB and specific periplasmic and inner membrane proteins (22, 38).

Vibriobactin, the only known siderophore produced by *V. cholerae*, is a catechol (18) (see Fig. 1). The prototype catechol, enterobactin, which is produced by *E. coli*, is a cyclic trimer of 2,3-dihydroxybenzoyl-L-serine (33, 37, 59). Vibriobactin also contains three 2,3-dihydroxybenzoyl residues, but they are linked to a backbone of norspermidine, a compound rarely encountered in bacterial species (18). Two of the 2,3-dihydroxybenzoyl moieties are linked to the backbone via L-threonine residues, while the third is directly linked to the norspermidine (Fig. 1).

The genetics and biosynthesis of enterobactin have been studied extensively (14, 57) (Fig. 1). The *ent* genes are clustered in three operons on a 22-kbp region of the *E. coli* chromosome and are coordinately regulated by the Fur repressor (11). Under conditions of iron starvation, chorismate is converted to 2,3-dihydroxybenzoic acid (DHBA) by the products of the *entABC* genes (64). The first step in this pathway is the conversion of chorismate to isochorismate, which is catalyzed by EntC, isochorismate synthase. The gene encoding this 43,000-molecular-weight monomeric protein has been cloned and sequenced (15, 27, 34), and EntC has homology with other enzymes which use chorismate as a substrate. Isochorismate is also a precursor in the synthesis of menaquinone, and a second isochorismate synthase gene, *menF*, has recently been identified in *E. coli* (12). EntB, isochorismatase, catalyzes the second step in the pathway. The gene for this 35,500-molecular-weight protein has been sequenced (26, 32), and recombinant EntB has been purified and characterized (42). EntB is believed to be a bifunctional protein; the C-terminal region of this protein has been shown to possess EntG activity, part of the enterobactin synthetase activity (52). EntA, 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase, catalyzes the third step in the dedicated pathway, which is the final step in the synthesis of DHBA. The gene for this 26-kDa protein has been cloned and sequenced (26, 32). The protein has been purified to homogeneity, and the catalytic mechanism of this NAD-dependent dehydrogenase has been investigated (26, 43).

In the final steps of enterobactin biosynthesis, DHBA and L-serine are converted to enterobactin by enterobactin synthetase, the product of the *entDEFG* genes (28, 60). The conversion of DHBA to enterobactin is not well understood but appears to be similar to the synthesis of peptide antibiotics (8). EntE, 2,3-dihydroxybenzoate-AMP ligase, is a homodimer with a subunit molecular weight of 59,000 (41, 53). This protein activates DHBA during the enterobactin synthetase reaction (41).

Less is known about the synthesis of vibriobactin, and prior to this work no information existed on the genetic organization of the genes for vibriobactin biosynthesis. Vibriobactin synthesis requires chorismate (18), and *V. cholerae* strains secrete DHBA, which is believed to serve as an intermediate in vibriobactin biosynthesis. These data suggested that at least the initial steps in vibriobactin biosynthesis would be similar to those observed in *E. coli* (Fig. 1). Thus, we reasoned that *V. cholerae* and *E. coli* might possess functionally similar genes involved in the early steps of catechol biosynthesis. We further speculated that if the genes for vibriobactin synthesis and utilization are clustered, as the enterobactin genes are in *E. coli*, we could isolate many or all of the vibriobactin genes by cloning a small number of genes and then characterizing the neighboring genes. We describe here the cloning and partial characterization of *V. cholerae* genes which complement specific

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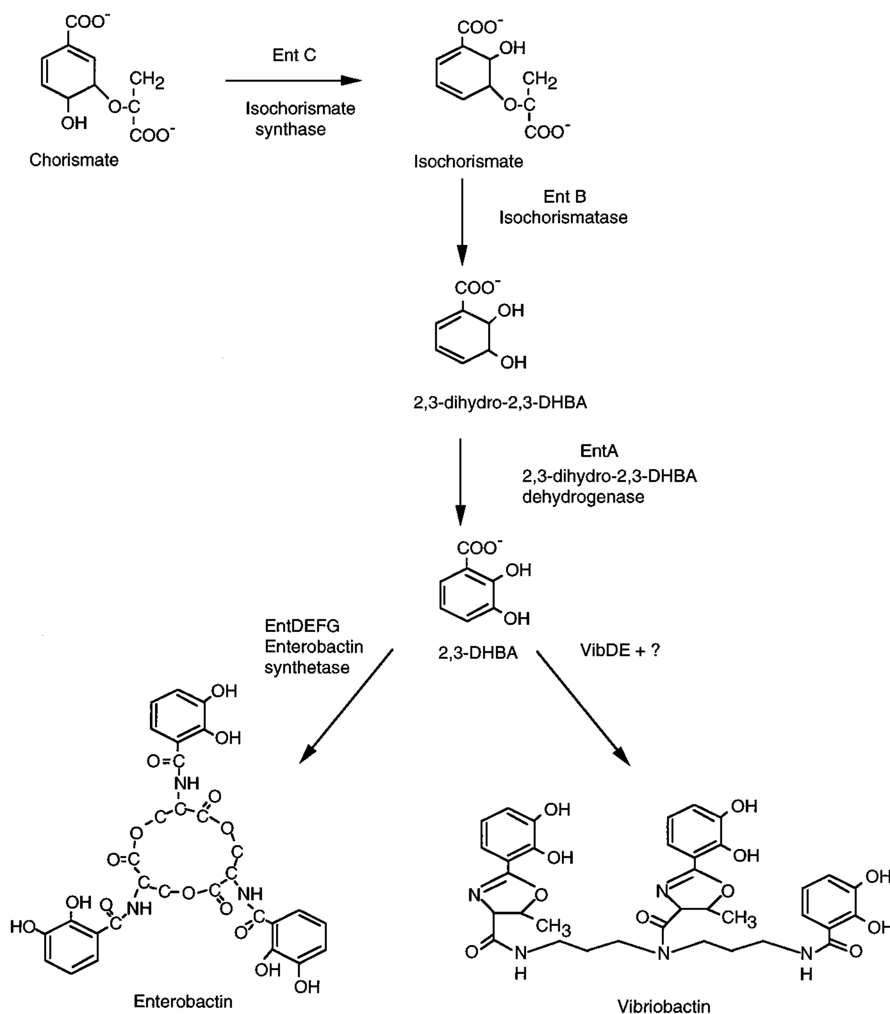

FIG. 1. Biosynthesis of enterobactin in *E. coli*, as adapted from references 28 and 64, and the proposed pathway for vibriobactin biosynthesis in *V. cholerae*.

enterobactin synthesis gene mutations in *E. coli*. As observed in *E. coli*, there is clustering of these genes, but the organization of the genes in *V. cholerae* is different. We also present the nucleotide sequences of the *vibA*, *vibB*, *vibC*, and *vibE* genes, the *V. cholerae* homologs of *entA*, *entB*, *entC*, and *entE*, respectively. A *V. cholerae* strain with a chromosomal *vibA* mutation was constructed and shown to be blocked in the synthesis of both DHBA and vibriobactin.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this work are listed in Table 1. All strains were maintained at −80°C in L broth plus 20% glycerol.

Media, reagents, and bacterial growth. Strains were grown on L agar or in L broth at 37°C. L medium plus the iron chelator ethylenediamine di(*ortho*-hydroxyphenylacetic acid) (EDDA), deferrated by the method of Rogers (39), was used for the iron-deficient medium. The concentrations of antibiotics, when added, were 250 μg/ml for carbenicillen, 12 μg/ml for tetracycline, 50 μg/ml for kanamycin, and 200 μg/ml for streptomycin.

Siderophore production and utilization bioassay. Strains were grown overnight at 37°C in L broth and washed once in saline. L agar containing EDDA (100 μg/ml) was inoculated with 10^4 bacteria per ml and poured into plates. Solutions of $FeSO_4$ (10 mM) or fully grown bacterial cultures were spotted (10-μl drops) onto the surface of the hardened agar and allowed to dry. Following overnight incubation, plates were observed for zones of growth around each compound or culture spot.

Detection of siderophores. Chrome Azurol S (CAS) agar, on which siderophore-secreting colonies produce an orange halo over the blue background, was used for the detection of siderophores in the complementation experiments (47). The Arnow test was used to detect secretion of catechols (4). Thin-layer chromatography was performed with 0.2-mm-thick silica gel plates (DC-Plastikfolien Kieselgel 60 F_{254}; E. Merck, Darmstadt, Germany) and a solvent system of *n*-butanol–acetic acid–water (12:3:5 by volume) to separate phenolate siderophores. The plates were examined under UV illumination and sprayed with 0.1 M $FeCl_3$ in 0.1 N HCl to detect characteristic iron-binding compounds. Standards were 2,3-DHBA and enterobactin (provided by C. F. Earhart, University of Texas at Austin). High-pressure liquid chromatography was performed with an Ultrasphere-ODS 25-cm-long C_{18} reverse-phase column. Samples were dissolved in methanol–1% phosphoric acid (1:1) prior to loading and were chromatographed at a flow rate of 1 ml per min. Peaks were detected by determining the absorbance at 220 nm.

Recombinant DNA techniques. Chromosomal DNA was isolated by the method of Marmur (29). Construction of cosmid banks, restriction enzyme analysis, agarose gel electrophoresis, and Southern hybridizations were carried out by standard molecular biology protocols (44). DNA sequencing was performed with a Sequenase DNA sequencing kit, version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio) or with an Applied Biosystems Prism 377 DNA sequencer (Perkin-Elmer Corp.).

Triparental matings were performed as follows: Donor, recipient, and mobilizing plasmid strains were grown in L broth with appropriate antibiotics to mid-log phase. Cultures were washed once in saline and then resuspended in the original volume of L broth. Fifty microliters of each was mixed and spread on an L agar plate, and the plates were incubated overnight at 37°C. The bacterial lawn was harvested with L broth, diluted, and plated on CAS agar containing the appropriate amino acid supplements and antibiotics.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source and/or reference(s)
Strains		
Lou15	*V. cholerae* El Tor	49
EWV100	Lou15 *vibA*::Tn*5*	This study
EWV101	Lou15 *vibD*::miniTn*10*	61
0395	*V. cholerae* classical	S. Calderwood
MBG14	O395 *viuA*::Tn*phoA*	10, 17
CA401	*V. cholerae* classical	49
2076-79	*V. cholerae* non-O1	J. Oliver; 51
324	*V. vulnificus*	3
BE2-542	*V. alginolyticus*	3
M474801	*V. parahaemolyticus*	3
UT1	*A. hydrophila*	University of Texas at Austin
PS9	*P. shigelloides*	13
SA101	*S. flexneri* 2a	24
HB101	*E. coli*	7
1017	HB101 *ent*::Tn*5*	13
P678-54	*E. coli* minicell strain	1
AN193	*E. coli entA*	C. Earhart
AN192	*E. coli entB*	C. Earhart
AN90	*E. coli entD*	C. Earhart
AN93	*E. coli entE*	C. Earhart
AN117	*E. coli entF*	C. Earhart
AB1515.43	*E. coli entG*	C. Earhart; 52
ID2	*E. coli fes*	C. Earhart; 36
Plasmids		
pRK340	Temperature-sensitive Tn*5* donor	R. Meyer
pRK2013	Mobilizing plasmid	44
pAT153	Cloning vector	44
pACYC184	Cloning vector	44
pLAFR1	Cosmid cloning vector	J. Leong
pUC18	Cloning vector	63
pWSK29	Cloning vector	58
pBluescript SK$^-$	Cloning vector	Strategene
pUCD800	*sacB* gene	16
pMPS11	*entBA* genes cloned in pACYC184	46
pVSA1	pLAFR1 cosmid clone carrying *vibAD*	This study
pVSB1	pLAFR1 cosmid clone carrying *vibABCD*	This study
pJSV78	pAT153 carrying the 3.8-kbp *Eco*RI-*Sal*I fragment of pVSA1 with *vibA* plus 4.0-kbp pLAFR1 sequences	This study
pUC22	pAT153 containing the 2.2-kbp *Eco*RI fragment of pVSA1 with *vibC* and part of *vibA* and *vibE*	This study
pJSV90	pACYC184 containing the 9.0-kbp *Eco*RI fragment of pVSA1 with *vibD*	This study
pJSV78::Tn*5*	pJSV78 containing *vibA*::Tn*5*	This study
pJSV76	pAT153 containing the *Eco*RI fragment from pJSV78::Tn*5*	This study
pJSV76S	pJSV76 with the 1.9-kbp *Eco*RV fragment containing the *sacB* gene inserted into the *Sca*I site	This study
pVIB127	pWSK29 containing an *Nhe*I partial digestion fragment of pVSB1 containing *vibBCE* and most of *vibA*	This study
pVIB128	Bluescript SK$^-$ containing the *Bam*HI fragment of pVIB127 with *vibCE* and most of *vibA* and *vibB*	This study
pVIB139	pWSK29 containing the *Nhe*I fragment of pVSB1 with *vibC*, *vibE*, *vibB*, and most of *vibA*	This study

Preparation of minicells and [^{35}S]methionine labeling of proteins were performed as previously described (31). Gel lanes were loaded with equivalent numbers of counts per minute of ^{35}S-labeled protein.

Transposon mutagenesis and construction of a *vibA* disruption in *V. cholerae*. Tn*5* mutagenesis of pJSV78, using the temperature-sensitive plasmid pRK340, was performed as previously described (24). Cells containing mutagenized plasmids were tested on CAS plates to screen for loss of siderophore production. A plasmid in which a Tn*5* mutation had abolished *vibA* function was named pJSV78::Tn*5*.

To obtain a null *vibA* mutation on the *V. cholerae* chromosome, the *Eco*RI fragment containing the disrupted *vibA* gene from the plasmid pJSV78::Tn*5* was subcloned into the *Eco*RI site of pAT153, yielding pJSV76. An *Eco*RV fragment containing the *sacB* (16) gene was then inserted into the *Sca*I site within the *amp* gene of pJSV76 to give pJSV76S. The plasmid pJSV76S was transferred to *V. cholerae* Lou15 by electroporation as previously described (20). A Tc^r, Km^r, sucrose-sensitive transformant which contained a plasmid that comigrated with pJSV76S was selected. This transformant was grown for four passages in L broth plus kanamycin, and serial dilutions were plated on L agar plates containing 5% sucrose and kanamycin. A sucrose-resistant, Tc^s, Km^r colony was isolated, and the presence of Tn*5* within the chromosomal *vibA* gene was confirmed by Southern hybridization (data not shown).

Nucleotide sequence accession number. The GenBank accession number for the *vibABCE* sequence is U52150.

RESULTS

Cloning of the vibriobactin biosynthesis genes of *V. cholerae*. To isolate the genes responsible for vibriobactin biosynthesis, we initially screened for DNA sequences that complemented

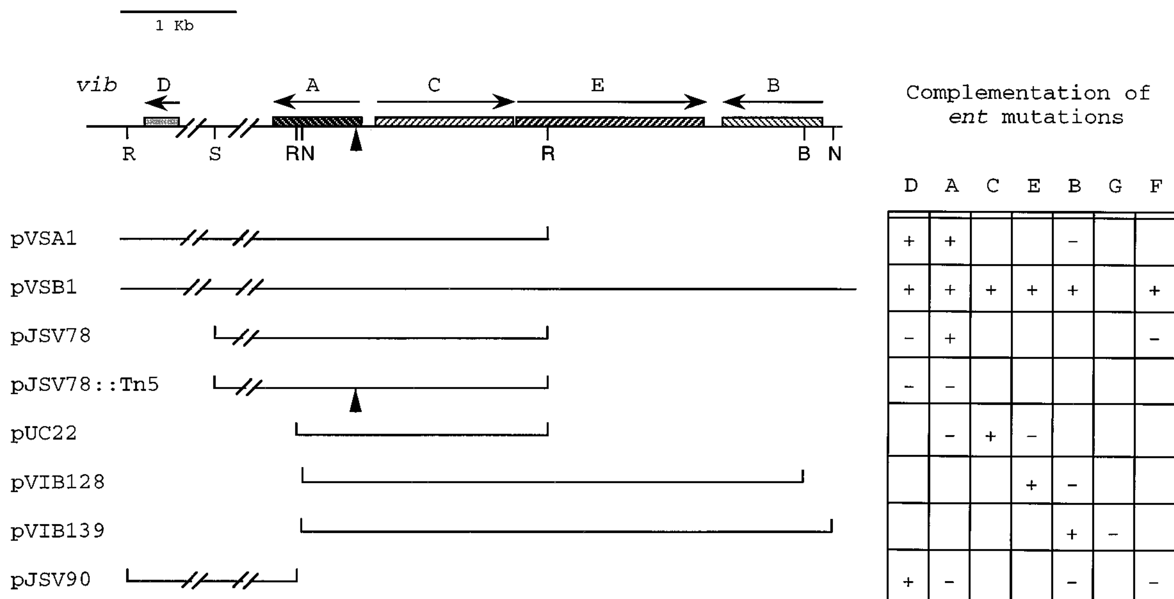

FIG. 2. Organization of the vibriobactin biosynthesis genes. The arrows indicate the direction of transcription of the *vib* genes. Selected restriction sites are indicated below the line. The enzyme abbreviations are as follows: R, *Eco*RI; S, *Sal*I; N, *Nhe*I; and B, *Bam*HI. The arrowheads in *vibA* and in pJSV78::Tn*5* indicate the site of the Tn*5* insertion. The abilities of the plasmids to complement enterobactin biosynthesis gene mutations are indicated at the right. +, complementation; −, no complementation. Empty boxes indicate that the complementation was not tested. Strains used for complementation were as follows: *entA*, AN193; *entB*, AN192; *entC*, SA101; *entD*, AN90; *entE*, AN93; *entF*, AN117; and *entG*, AB1515.43 (see Table 1).

E. coli mutations in either *entA* or *entB*. These genes were chosen because they encode proteins in the pathway for the synthesis of DHBA, which was expected to be a common intermediate in the synthesis of enterobactin and vibriobactin (Fig. 1). To identify these genes, a cosmid bank of partially *Eco*RI-digested *V. cholerae* El Tor strain Lou15 DNA was constructed in pLAFR1. The cosmid bank, containing approximately 1,200 independently cloned DNA fragments, was transferred via triparental mating to the *E. coli entA* mutant AN193 or the *entB* mutant AN192. AN193 and AN192 carrying the cosmid clones were screened on CAS agar for colonies with orange halos, indicative of siderophore production. Cosmid clones pVSA1, complementing the *entA* mutation in AN193, and pVSB1, complementing the *entB* mutation in AN192, were isolated by this method.

Several assays were used to confirm that the functional complementation of enterobactin mutants by the *V. cholerae* genes was due to the production of DHBA and enterobactin. AN193 carrying pVSA1 and AN192 carrying pVSB1 were both positive in the Arnow test for the synthesis of catechols. To further characterize products secreted by these strains, their culture supernatants were analyzed specifically for the production of DHBA and enterobactin by thin-layer chromatography and by reverse-phase high-pressure liquid chromatography. In each case, the mutant carrying the complementing plasmid produced compounds which comigrated with enterobactin and DHBA standards (data not shown). In bioassays, the production of functional enterobactin was confirmed by demonstrating that each strain carrying the cosmid could stimulate the growth of *E. coli* 1017, a siderophore mutant blocked in the pathway between DHBA and enterobactin, under conditions of iron limitation (data not shown).

To determine whether the *entA* and *entB* analogs are linked to each other and to other *V. cholerae* siderophore genes, the cosmids pVSA1 and pVSB1 were tested for complementation of other enterobactin biosynthetic mutants by the CAS agar assay. The cosmid pVSA1 complemented the *entA* and *entD* mutations, and the cosmid pVSB1 complemented mutations in the *entABCDEF* genes (Fig. 2). Restriction mapping and Southern hybridizations indicated that pVSA1 and pVSB1 overlap physically, and we concluded that the *entA* and *entD* homologs are located on the region of overlap between the two cosmids whereas the *entB* homolog is in a region unique to pVSB1 (Fig. 2). Because there was not an *E. coli entC* mutation that possessed a well-defined EntC phenotype, we tested complementation in a *Shigella flexneri* strain that had been previously shown to lack a functional *entC* gene (45). Introduction of the cosmid pVSB1 into *S. flexneri* SA101 yielded a strain with an Arnow-positive phenotype, directly demonstrating the presence of an EntC function on the cosmid.

Complementation of the *entF* mutation, as assayed on a CAS plate, was unanticipated, since EntF activates serine, which is not a precursor for vibriobactin synthesis. Chromatographic analysis of the *entF* mutant strain with and without pVSB1 confirmed that this cosmid promoted the synthesis of a siderophore in *E. coli*, suggesting that pVSB1 contained all the genes necessary for the production of a siderophore in *E. coli*. This compound, however, did not comigrate with either enterobactin or vibriobactin. This is not surprising, since *E. coli* does not synthesize the norspermidine found in vibriobactin (62) and VibF is unlikely to activate the serine to form authentic enterobactin. The structure of this novel siderophore has not been determined.

Linkage of the vibriobactin biosynthesis genes to *viuA*, the gene for the vibriobactin outer membrane receptor protein (10, 54), was investigated. The cosmids were tested for complementation of *V. cholerae* MBG14, which contains a *viuA* mutation. Neither of the cosmids complemented the *viuA* mutation. In addition, a probe from the *viuA* gene did not hybridize with either of the cosmids (data not shown). In *V. cholerae*, a

second vibriobactin utilization gene, *viuB*, maps adjacent to *viuA* (9). *viuB* complements an *E. coli fes* mutation, and it is believed that ViuB, like Fes, is responsible for intracellular removal of iron from the iron-siderophore complex. The cosmid pVSB1 did not complement the *fes* mutation in *E. coli* ID2, consistent with this cosmid lacking the *viuA-viuB* gene region. The cosmid pVSB1 was also tested for complementation of mutations in genes for the inner membrane enterobactin transport proteins. Data describing these vibriobactin transport genes will be reported elsewhere (61).

Localization of the vibriobactin gene cluster and sequence of the genes responsible for the synthesis and activation of DHBA. The vibriobactin biosynthetic gene region was initially localized on the cosmids by identification of *vibA*. To accomplish this, the cosmid pVSA1 was digested with *Sal*I, and each fragment was ligated with pAT153 and transformed into the *entA* mutant strain AN193. Only AN193 carrying a cloned 7.8-kbp fragment produced orange halos on CAS agar. Additionally, only this subclone, named pJSV78, rendered AN193 positive in the Arnow test. The gene for this *entA* homolog, *vibA*, was mapped to a 3.8-kbp *Eco*RI-*Sal*I DNA fragment, since the remaining 4.0-kbp *Eco*RI-*Sal*I fragment of the insert in pJSV78 was shown by hybridization and restriction mapping to be pLAFR1 vector sequences from pVSA1.

To determine the location of *vibA* on the plasmid, pJSV78 was mutagenized with Tn*5* and transformed into AN193. A transformant that failed to produce siderophore on CAS agar and in low-iron T medium was isolated. Restriction enzyme analysis showed that the Tn*5* had inserted into a 2.2-kbp *Eco*RI fragment (Fig. 2). Sequences outside this *Eco*RI fragment also were required for VibA function, since AN193 carrying pUC22, the *Eco*RI fragment subcloned into pAT153, failed to produce siderophore on CAS agar or in T medium (Fig. 2).

To determine if other genes for vibriobactin synthesis are located near *vibA*, plasmids containing various portions of this region were constructed (Table 1) and tested for complementation of mutations in *entBCDEF* (Fig. 2). These data showed that the other genes for synthesis and activation of DHBA map together immediately upstream of *vibA* (see below). An open reading frame (ORF) complementing an *entD* mutation was found to lie approximately 6 kbp downstream of *vibA*. This ORF will be described elsewhere.

The nucleotide sequence of the region required for complementation of *entABCE* mutations was determined on both strands. This sequence contained four ORFs, which correspond to the *vibABCE* genes (Fig. 2).

The *vibA* ORF was identified genetically in two ways. First, complementation of an *entA* mutation was observed with pJSV78, which contains the intact *vibA* ORF, but not with pUC22 or pJSV90, which contain the upstream and downstream ORFs, respectively, but not the intact *vibA* ORF (Fig. 2). Second, DNA sequencing indicated that the Tn*5* insertion that inactivated *vibA* function is located within this ORF (Fig. 2). This Tn*5* insertion should not be polar on downstream genes, since the gene downstream of *vibA* is encoded on the opposite strand (data not shown). The *vibA* gene has two potential initiating methionines, separated by six nucleotides. The presence of multiple potential initiating methionines is not uncommon in siderophore synthesis genes. Regulation of vibriobactin synthesis by iron is mediated by the iron-regulatory protein Fur (55). Consistent with this, a potential Fur box (ATAAATGCAAGCAATTTCC) was identified which overlaps the −35 consensus sequences.

vibA encodes a 262-amino-acid protein. VibA has a predicted molecular weight of 28,000 and a predicted pI of 5.31. This is consistent with the 28-kDa protein visualized in minicells (Fig. 3). This 28-kDa protein was not observed in minicells containing the plasmid pJSV78::Tn*5* (data not shown). A Blast search of the National Center for Biotechnology Information nonredundant database (2) was used to identify sequences with homology to VibA. The most similar proteins were EntA from *E. coli* and DhbA from *Bacillus subtilis* (see Table 2). The Blast search also identified a large number of oxidoreductases with significant homology to VibA. Nahlik et al. (32) previously noted homology between EntA and a family of short alcohol-polyol-sugar dehydrogenases. The N-terminal segment of VibA has homology to the consensus pyrimidine nucleotide binding pocket of these dehydrogenases, including the presence of the properly spaced conserved glycines. In addition, a search of the Prosite database showed that amino acids 145 to 173 of VibA are a perfect match to the signature pattern for short-chain-type alcohol dehydrogenases. These data suggest that VibA is also a member of this family.

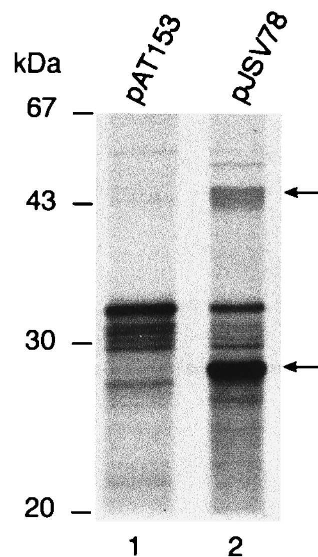

FIG. 3. Minicell analysis. The ^{35}S-labeled proteins produced by minicells of strain P678-54 containing the vector pAT153 (lane 1) or pJSV78 (lane 2) were visualized by autoradiography. The positions of VibA (molecular weight, 28,000) and VibC (molecular weight, 44,000) are indicated by arrows. The positions of molecular size markers are shown on the left.

Upstream of the *vibA* ORF, and transcribed in the opposite direction, is *vibC*. This ORF was shown to complement the *entC* defect in *S. flexneri* SA101. *vibC* is the only intact ORF present on the plasmid pUC22. When this plasmid was introduced into strain SA101 containing the plasmid pMPS11, which encodes the *entA* and *entB* genes, the cells secreted catechol as determined by the Arnow assay. Untransformed SA101, or SA101 containing either pMPS11 or pUC22 alone, was negative in this assay.

vibC also has two potential methionine initiation codons, separated by only three nucleotides. The predicted VibC protein contains 395 amino acids with a total molecular weight of 43,600, consistent with the size of the protein observed in minicells (Fig. 3). The predicted pI is 6.12. A Blast search with the VibC sequence identified homology to the *E. coli* EntC protein and to EntC homologs from other organisms (Table 2). The EntC sequence is similar to those of other proteins that utilize chorismate as a substrate (15), including TrpE and PabB, and VibC also exhibits significant homology to these proteins.

vibE is located downstream from *vibC*. *vibC* and *vibE* are

TABLE 2. Selected homologies of vibriobactin biosynthesis proteins to sequences from other organisms[a]

Vib protein and homologous proteins (reference)	Percent identity	Percent similarity[b]
VibA		
B. subtilis DhbA (40)	42	59
E. coli EntA (32)	40	63
VibC		
B. subtilis DhbC (40)	44	65
Pseudomonas fluorescens EntC homolog (EMBL Y09356)	41	50
E. coli EntC (15, 34)	40	56
A. hydrophila AmoA (5)	40	47
B. subtilis MenF (53)	36	46
VibE		
B. subtilis DhbE (40)	53	63
Pseudomonas aeruginosa EntE homolog (48)	52	61
E. coli EntE (41, 53)	50	61
VibB		
V. vulnificus VenB (25)	59	66
E. coli EntB (26, 32)	49	60
B. subtilis DhbB (40)	49	58

[a] Homologies were calculated by using the Bestfit program of the Genetics Computer Group.
[b] Includes conservative substitutions.

encoded on the same strand and are separated by only 10 nucleotides, suggesting that they may be cotranscribed. This ORF was identified as the functional homolog of *entE*, because it is the only intact ORF present on the plasmid pVIB128 (which complements the *entE* mutation) that is not also present on pUC22 (which does not complement that mutation) (Fig. 2). The predicted VibE protein has a molecular weight of 60,000 and a predicted pI of 6.10. In a Blast search, VibE was found to have homology to *E. coli* EntE and to several EntE homologs from other organisms (Table 2). In addition, it has a lower degree of homology to other adenylate-forming enzymes, including EntF.

vibB is downstream of *vibE* and is encoded on the opposite strand. An *entB* mutation is complemented by pVIB139 but not by pVIB128. These plasmids have similar inserts, except pVIB139 contains the entire *vibB* ORF while pVIB128 contains only part of this ORF (Fig. 2). This confirms the assignment of this ORF as *vibB*. *vibB* and *vibE* are separated by approximately 100 bp. This intergenic region contains a long inverted repeat sequence that may function as a transcription terminator.

vibB encodes a 293-amino-acid protein with a predicted molecular weight of 32,500 and a predicted pI of 5.27. VibB has sequence homology to EntB and other EntB homologs (Table 2). *E. coli* EntB is a bifunctional protein with both EntB and EntG activities (52). Interestingly, the plasmid pVIB139, which complemented the *entB* mutation, failed to complement an *entG* mutation. The interpretation of this result is unclear. VibB may also be bifunctional but may fail to substitute for EntG, possibly because it catalyzes the synthesis of vibriobactin and cannot function in enterobactin synthesis or because it fails to interact properly with other enterobactin synthetase proteins. Alternatively, VibB may have only one function, and an EntG-like activity either is not required for vibriobactin synthesis or is encoded in a separate gene. The role of EntG activity in enterobactin biosynthesis is unknown.

Inactivation of the *vibA* gene. To determine the role of these proteins in *V. cholerae*, the wild-type *vibA* gene on the *V. cholerae* chromosome was replaced with the Tn*5* insertion mutation by marker exchange, using the Tn*5* mutation that is present in pJSV78::Tn*5*. The presence of the disrupted *vibA* gene on the chromosome was confirmed by Southern blotting (data not shown). The resulting strain, EWV100, had increased sensitivity to the iron chelator EDDA relative to the parent strain. In L broth, the MIC of EDDA for EWV100 was 16 μg/ml while that for the parent strain Lou15 was greater than 500 μg/ml. EWV100 was negative in the Arnow assay, indicating that it secreted neither DHBA nor vibriobactin. When analyzed in bioassays (Table 3), EWV100 was unable to stimulate the growth of a *V. cholerae vibD* mutant, indicating that it did not secrete vibriobactin. The growth of EWV100 was stimulated by wild-type *V. cholerae* in a bioassay, confirming that vibriobactin utilization is unimpaired in the *vibA* mutant. Growth of EWV100 was also stimulated by the *vibD* mutant strain EWV101. This was most likely due to EWV100 taking up DHBA secreted by the *vibD* mutant and converting it to vibriobactin, consistent with EWV100 possessing a defect in an early step in vibriobactin biosynthesis. Introduction of pJSV78, which contains the wild-type *vibA* gene, restored both the synthesis of catechols, as detected in an Arnow assay, and the ability to stimulate the growth of the *vibD* mutant in a bioassay (Table 3), confirming that the phenotype observed in EWV100 is due to the *vibA* mutation.

Sequences closely related to the *vibA* and *vibC* genes were found only in *V. cholerae* strains. The *Eco*RI insert of plasmid pJSV22 encoding all of *vibC* and most of *vibA* was used to probe genomic DNA isolated from *V. cholerae*, several other *Vibrio*, *Aeromonas*, and *Plesiomonas* species, and *E. coli* to determine whether homologous sequences are present in these strains (data not shown). Hybridization to an 18-kbp *Sal*I fragment was observed with DNA from *V. cholerae* strains, including Lou15, the El Tor parent of the cosmid library, and the classical strains CA401 and O395. Hybridization to DNA from the *V. cholerae* non-O1 strain 2076-79 was also observed, but the size of the hybridizing fragment was smaller, indicating some divergence of sequence in this region between this strain

TABLE 3. Effect of a *vibA* mutation on vibriobactin biosynthesis and utilization[a]

Indicator strain	Zone of stimulation (mm) with:				
	$FeSO_4$	Lou15	EWV100 (*vibA*::Tn*5*)	EWV100/ pJSV78	EWV101 (*vibD*::Tn*10*)
EWV100	22	15	0	22	18
EWV100/pJSV78	24	27	0	18	20
EWV101	18	30	0	20	0

[a] Cultures of the indicator strains were seeded into L agar containing EDDA, and various *V. cholerae* strains were spotted onto the medium. $FeSO_4$ was spotted onto sterile disks placed on the medium. The zones of growth were measured 18 h after inoculation.

and the O1-serotype *V. cholerae* strains. The DNAs of the other *Vibrio* species tested, *Vibrio vulnificus*, *Vibrio alginolyticus*, and *Vibrio parahaemolyticus*, as well as those of *Aeromonas hydrophila*, *Plesiomonas shigelloides*, and *E. coli* showed no hybridization to the probe, even under conditions of reduced stringency.

In the reciprocal experiment, the cloned *E. coli ent* genes (*entCABE*) from the plasmid pCP410 (35) were hybridized to genomic DNA of various *V. cholerae* strains and other *Vibrio* species. No hybridization was seen between labeled *E. coli* DNA and the DNA of any of the strains tested, even that of the *V. cholerae* biotypes containing sequences which functionally complement the *E. coli entA* defect (Fig. 2). These data indicate that although *V. cholerae* and *E. coli* siderophore biosynthesis genes encode gene products which are analogous in function, the DNA sequences are sufficiently different to prevent hybridization even under reduced-stringency conditions.

DISCUSSION

In this paper, we have presented a preliminary characterization of genes responsible for vibriobactin biosynthesis in *V. cholerae*. We showed that homologs of *entABCDE* are located on two overlapping cosmids clones and that these cosmids functionally complement *E. coli* mutants defective in enterobactin biosynthesis. We further characterized and sequenced a region of the chromosome containing genes responsible for the synthesis and activation of the vibriobactin biosynthetic intermediate DHBA. A *vibA* mutant was constructed by marker exchange, and this strain produced no catechols and grew poorly under conditions of iron starvation. Taken together, our data strongly support the pathway for vibriobactin biosynthesis shown in Fig. 1. Specifically, they support the model that in *V. cholerae*, DHBA is produced from chorismate by proteins homologous to EntA, EntB, and EntC. There is less information about the conversion of DHBA to vibriobactin. Our data support the model that this reaction shares some features with the late steps in enterobactin biosynthesis, since *V. cholerae* contains proteins functionally homologous to EntD and EntE. Recently, *ent* homologs from the gram-positive organism *B. subtilis* have been cloned by functional complementation of enterobactin biosynthetic mutations (19, 40). This finding supports the model that catechol synthesis pathways are strongly conserved, even between gram-positive and gram-negative bacteria.

As in other bacteria, the *V. cholerae* siderophore biosynthesis genes are clustered on the chromosome; however, the gene organization in *V. cholerae* is different from that in *E. coli*. For example, in *E. coli*, the genes for DHBA synthesis and activation are cotranscribed in a single operon, whereas in *V. cholerae*, the genes are adjacent but are organized into at least three transcription units. In *E. coli*, the genes for enterobactin transport are intermingled with genes for enterobactin biosynthesis. The outer membrane protein responsible for vibriobactin transport, *viuA*, does not appear to be encoded on either of the cosmids, pVSA1 or pVSB1, and its position relative to the genes described here is not known.

Interestingly, sequences hybridizing with this region were found only in *V. cholerae* genomes. El Tor, classical, and non-O1 biotypes of *V. cholerae* contained sequences which hybridized to a vibriobactin region probe; however, other *Vibrio* species, *P. shigelloides*, *E. coli*, and *A. hydrophila*, an organism which also produces DHBA as an intermediate in siderophore biosynthesis (6), exhibited no significant homology to this region. Although the genera *Plesiomonas* and *Aeromonas* are also members of the *Vibrionacaeae* family, the fact that *V. cholerae* appears to possess a unique siderophore system is not surprising since Andrus et al. (3) have previously shown that siderophore production and utilization are quite specific even among different *Vibrio* species. Additionally, the *E. coli entCABE* genes encoded by the plasmid pCP410 failed to hybridize to the *V. cholerae* chromosome even under conditions of low stringency. These data reflect the fact that functional homology between gene products can exist even in the absence of strong DNA homology.

The role of vibriobactin synthesis and utilization in the life cycle of *V. cholerae* is unknown. It was shown previously that genes for the synthesis and utilization of vibriobactin are not absolutely required for growth of *V. cholerae* in animal models (21, 50). Many organisms possess multiple systems for iron transport, an observation that may reflect the overall importance of iron acquisition. Consistent with this, *V. cholerae* strains defective in the utilization of both vibriobactin and heme were more attenuated in animal models than were strains carrying a mutation in only one of these systems (21, 56). Like many pathogens, *V. cholerae* must survive and grow in a variety of environments. Nearly all bacteria that can live in the soil and water produce one or more siderophores. It may be that the production and utilization of vibriobactin are most significant during environmental growth of *V. cholerae* and thus it could play a major role in the transmission of *V. cholerae*.

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REFERENCES

1. **Adler, H. I., W. D. Fisher, A. Cohen, and A. A. Hardigree.** 1967. Miniature *Escherichia coli* cells deficient in DNA. Proc. Natl. Acad. Sci. USA **57:**321–326.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
3. **Andrus, C. R., M. Walter, J. H. Crosa, and S. Payne.** 1983. Synthesis of siderophores by pathogenic *Vibrio* species. Curr. Microbiol. **9:**209–214.
4. **Arnow, L. E.** 1937. Colorimetric determination of the components of 3,4-dihydroxyphenylalanine tyrosine mixtures. J. Biol. Chem. **118:**531–537.
5. **Barghouthi, S., S. M. Payne, J. E. L. Arceneaux, and B. R. Byers.** 1991. Cloning, mutagenesis, and nucleotide sequence of a siderophore biosynthetic gene (*amoA*) from *Aeromonas hydrophila*. J. Bacteriol. **173:**5121–5128.
6. **Barghouthi, S., R. Young, M. O. J. Olson, J. E. L. Arceneaux, L. W. Clem, and B. R. Byers.** 1989. Amonabactin, a novel tryptophan- or phenylalanine-containing phenolate siderophore in *Aeromonas hydrophila*. J. Bacteriol. **171:**1811–1816.
7. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459–472.
8. **Bryce, G. F., and N. Brot.** 1972. Studies on the enzymatic synthesis of the cyclic trimer of 2,3-dihydroxy-*N*-benzoyl-L-serine in *Escherichia coli*. Biochemistry **11:**1708–1715.
9. **Butterton, J. R., and S. B. Calderwood.** 1994. Identification, cloning, and sequencing of a gene required for ferric vibriobactin utilization by *Vibrio cholerae*. J. Bacteriol. **176:**5631–5638.
10. **Butterton, J. R., J. A. Stoebner, S. M. Payne, and S. B. Calderwood.** 1992. Cloning, sequencing, and transcriptional regulation of *viuA*, the gene encoding the ferric vibriobactin receptor of *Vibrio cholerae*. J. Bacteriol. **174:**3729–3738.
11. **Calderwood, S. B., and J. J. Mekalanos.** 1987. Iron regulation of Shiga-like toxin expression in *Escherichia coli* is mediated by the *fur* locus. J. Bacteriol. **169:**4759–4764.
12. **Daruwala, R., O. Kwon, R. Meganathan, and M. E. Hudspeth.** 1996. A new isochorismate synthase specifically involved in menaquinone (vitamin K_2) biosynthesis encoded by *menF* gene. FEMS Microbiol. Lett. **140:**159–163.
13. **Daskaleros, P. A., J. A. Stoebner, and S. M. Payne.** 1991. Iron uptake in

Plesiomonas shigelloides: cloning of the genes for the heme-iron uptake system. Infect. Immun. **59:**2706–2711.
14. **Earhart, C. F.** 1996. Uptake and metabolism of iron and molybdenum, p. 1075–1090. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol. 1. ASM Press, Washington, D.C.
15. **Elkins, M. F., and C. F. Earhart.** 1988. An *Escherichia coli* enterobactin cluster gene with sequence homology to *trpE* and *pabB*. FEMS Microbiol. Lett. **56:**35–40.
16. **Gay, P., D. Le Coq, M. Steinmetz, T. Berkelman, and C. I. Kado.** 1985. Positive selection procedure for entrapment of insertion sequence elements in gram-negative bacteria. J. Bacteriol. **164:**918–921.
17. **Goldberg, M. B., V. J. DiRita, and S. B. Calderwood.** 1990. Identification of an iron-regulated virulence determinant in *Vibrio cholerae*, using Tn*phoA* mutagenesis. Infect. Immun. **58:**55–60.
18. **Griffiths, G. L., S. P. Sigel, S. M. Payne, and J. B. Neilands.** 1984. Vibriobactin, a siderophore from *Vibrio cholerae*. J. Biol. Chem. **259:**383–385.
19. **Grossman, T. H., M. Tuckman, S. Ellestad, and M. S. Osburne.** 1993. Isolation and characterization of *Bacillus subtilis* genes involved in siderophore biosynthesis: relationship between *B. subtilis sfp*^{0} and *Escherichia coli entD* genes. J. Bacteriol. **175:**6203–6211.
20. **Henderson, D. P., and S. M. Payne.** 1993. Cloning and characterization of the *Vibrio cholerae* genes encoding the utilization of iron from haemin and haemoglobin. Mol. Microbiol. **7:**461–469.
21. **Henderson, D. P., and S. M. Payne.** 1994. *Vibrio cholerae* iron transport systems: role of heme and siderophore iron transport in virulence and identification of a gene associated with multiple iron transport systems. Infect. Immun. **62:**5120–5125.
22. **Klebba, P. E., J. M. Rutz, J. Liu, and C. K. Murphy.** 1993. Mechanisms of TonB-catalyzed iron transport through the enteric bacterial cell envelop. J. Bioenerg. Biomembr. **25:**603–611.
23. **Lankford, C. E.** 1973. Bacterial assimilation of iron. Crit. Rev. Microbiol. **2:**273–331.
24. **Lawlor, K. M., P. A. Daskaleros, R. E. Robinson, and S. M. Payne.** 1987. Virulence of iron transport mutants of *Shigella flexneri* and utilization of host iron compounds. Infect. Immun. **55:**594–599.
25. **Litwin, C. M., T. W. Rayback, and J. Skinner.** 1996. Role of catechol siderophore synthesis in *Vibrio vulnificus* virulence. Infect. Immun. **64:**2834–2838.
26. **Liu, J., K. Duncan, and C. T. Walsh.** 1989. Nucleotide sequence of a cluster of *Escherichia coli* enterobactin biosynthesis genes: identification of *entA* and purification of its product 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase. J. Bacteriol. **171:**791–798.
27. **Liu, J., N. Quinn, G. A. Berchtold, and C. T. Walsh.** 1990. Overexpression, purification, and characterization of isochorismate synthase (EntC), the first enzyme involved in the biosynthesis of enterobactin from chorismate. Biochemistry **29:**1417–1425.
28. **Luke, R. K. J., and F. Gibson.** 1971. Location of three genes concerned with the conversion of 2,3-dihydroxybenzoate into enterochelin in *Escherichia coli* K-12. J. Bacteriol. **107:**557–562.
29. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. **3:**208–218.
30. **McIntosh, M. A., C. L. Pickett, S. S. Chenault, and C. F. Earhart.** 1978. Suppression of iron uptake deficiency in *Escherichia coli* K-12 by loss of two major outer membrane proteins. Biochem. Biophys. Res. Commun. **81:**1106–1112.
31. **Mills, M., and S. M. Payne.** 1995. Genetics and regulation of heme iron transport in *Shigella dysenteriae* and detection of an analogous system in *Escherichia coli* O157:H7. J. Bacteriol. **177:**3004–3009.
32. **Nahlik, M. S., T. J. Brickman, B. A. Ozenburger, and M. A. McIntosh.** 1989. Nucleotide sequence and transcriptional organization of the *Escherichia coli* enterobactin biosynthesis cistrons *entB* and *entA*. J. Bacteriol. **171:**784–790.
33. **O'Brien, I. G., and F. Gibson.** 1970. The structure of enterochelin and related 2,3-dihydroxy-*N*-benzoylserine conjugates from *Escherichia coli*. Biochim. Biophys. Acta **215:**393–402.
34. **Ozenberger, B. A., T. J. Brickman, and M. A. McIntosh.** 1989. Nucleotide sequence of *Escherichia coli* isochorismate synthetase gene *entC* and evolutionary relationship of isochorismate synthetase and other chorismate-utilizing enzymes. J. Bacteriol. **171:**775–783.
35. **Pickett, C. L., L. Hayes, and C. F. Earhart.** 1984. Molecular cloning of the *Escherichia coli* K-12 *entACGBE* genes. FEMS Microbiol. Lett. **24:**77–80.
36. **Pierce, J. R., C. L. Pickett, and C. F. Earhart.** 1983. Two *fep* genes are required for ferrienterochelin uptake in *Escherichia coli* K-12. J. Bacteriol. **155:**330–336.
37. **Pollack, J. R., and J. B. Neilands.** 1970. Enterobactin, an iron transport compound from *Salmonella typhimurium*. Biochem. Biophys. Res. Commun. **38:**989–992.
38. **Postle, K.** 1993. TonB protein and energy transduction between membranes. J. Bioenerg. Biomembr. **25:**591–601.
39. **Rogers, H. J.** 1973. Iron-binding catechols and virulence in *Escherichia coli*. Infect. Immun. **7:**445–456.
40. **Rowland, B. M., T. H. Grossman, M. S. Osburne, and H. W. Tabor.** 1996. Sequence and genetic organization of a *Bacillus subtilis* operon encoding 2,3-dihydroxybenzoate biosynthetic enzymes. Gene **178:**119–123.
41. **Rusnak, F., W. S. Faraci, and C. T. Walsh.** 1989. Subcloning, expression, and purification of the enterobactin biosynthetic enzyme 2,3-dihydroxybenzoate-AMP ligase: demonstration of enzyme-bound (2,3-dihydroxybenzoyl)adenylate product. Biochemistry **28:**6827–6835.
42. **Rusnak, F., J. Liu, N. Quinn, G. A. Berchtold, and C. T. Walsh.** 1990. Subcloning of the enterobactin biosynthetic gene *entB*: expression, purification, characterization, and substrate specificity of isochorismatase. Biochemistry **29:**1425–1435.
43. **Sakaitani, M., F. Rusnak, N. R. Quinn, C. Tu, T. B. Frigo, G. A. Berchtold, and C. T. Walsh.** 1990. Mechanistic studies on *trans*-2,3-dihydroxybenzoate dehydrogenase (EntA) in the biosynthesis of the iron chelator enterobactin. Biochemistry **29:**6789–6798.
44. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
45. **Schmitt, M. P., and S. M. Payne.** 1991. Genetic analysis of the enterobactin gene cluster in *Shigella flexneri*. J. Bacteriol. **173:**816–825.
46. **Schmitt, M. P., and S. M. Payne.** 1988. Genetics and regulation of enterobactin genes in *Shigella flexneri*. J. Bacteriol. **170:**5579–5587.
47. **Schwyn, B., and J. B. Neilands.** 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160:**47–56.
48. **Serino, L., C. Reimmann, H. Baur, M. Beyeler, P. Visca, and D. Haas.** 1995. Structural genes for salicylate biosynthesis from chorismate in *Pseudomonas aeruginosa*. Mol. Gen. Genet. **249:**217–228.
49. **Sigel, S. P., and S. M. Payne.** 1982. Effect of iron limitation on growth, siderophore production, and expression of outer membrane proteins of *Vibrio cholerae*. J. Bacteriol. **150:**148–155.
50. **Sigel, S. P., J. A. Stoebner, and S. M. Payne.** 1985. Iron-vibriobactin transport system is not required for virulence of *Vibrio cholerae*. Infect. Immun. **47:**360–362.
51. **Simpson, L. M., M. A. Dry, and J. D. Oliver.** 1987. Experimental *Vibrio cholerae* wound infections. FEMS Microbiol. Lett. **40:**89–93.
52. **Staab, J. F., and C. F. Earhart.** 1990. EntG activity of *Escherichia coli* enterobactin synthetase. J. Bacteriol. **172:**6403–6410.
53. **Staab, J. F., M. F. Elkins, and C. F. Earhart.** 1989. Nucleotide sequence of the *Escherichia coli entE* gene. FEMS Microbiol. Lett. **59:**15–20.
54. **Stoebner, J. A., J. R. Butterton, S. B. Calderwood, and S. M. Payne.** 1992. Identification of the vibriobactin receptor of *Vibrio cholerae*. J. Bacteriol. **174:**3270–3274.
55. **Stoebner, J. A., and S. M. Payne.** 1988. Iron-regulated hemolysin production and utilization of heme and hemoglobin by *Vibrio cholerae*. Infect. Immun. **56:**2891–2895.
56. **Tashima, K. T., P. A. Carroll, M. B. Rogers, and S. B. Calderwood.** 1996. Relative importance of three iron-regulated outer membrane proteins for in vivo growth of *Vibrio cholerae*. Infect. Immun. **64:**1756–1761.
57. **Walsh, C. T., J. Liu, F. Rusnak, and M. Sakaitani.** 1990. Molecular studies on enzymes in chorismate metabolism and enterobactin biosynthetic pathway. Chem. Rev. **90:**1105–1129.
58. **Wang, R. F., and S. R. Kushner.** 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. Gene **100:**195–199.
59. **Wilkins, T. D., and C. E. Lankford.** 1970. Production by *Salmonella typhimurium* of 2,3-dihydroxybenzoylserine and its stimulation of growth in human serum. J. Infect. Dis. **121:**129–136.
60. **Woodrow, G. C., I. G. Young, and F. Gibson.** 1975. Mu-induced polarity in the *Escherichia coli* K-12 *ent* gene cluster: evidence for a gene (*entG*) involved in the biosynthesis of enterochelin. J. Bacteriol. **124:**1–6.
61. **Wyckoff, E. E., and S. M. Payne.** Unpublished data.
62. **Yamamoto, S., S. Shinoda, M. Kawaguchi, K. Wakamatsu, and M. Makita.** 1983. Polyamine distribution in *Vibrionaceae*: norspermidine as a general constituent of *Vibrio* species. Can. J. Microbiol. **29:**724–728.
63. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
64. **Young, I. G., L. Langman, R. K. J. Luke, and F. Gibson.** 1971. Biosynthesis of the iron-transport compound enterochelin: mutants of *Escherichia coli* unable to synthesize 2,3-dihydroxybenzoate. J. Bacteriol. **106:**51–57.