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Complete Sequence of Virulence Plasmid pJM1 from the Marine Fish Pathogen *Vibrio anguillarum* Strain 775

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The virulence plasmid pJM1 enables the fish pathogen *Vibrio anguillarum*, a gram-negative polarly flagellated comma-shaped rod bacterium, to cause a highly fatal hemorrhagic septicemic disease in salmonids and other fishes, leading to epizootics throughout the world. The pJM1 plasmid 65,009-nucleotide sequence, with an overall G+C content of 42.6%, revealed genes and open reading frames (ORFs) encoding iron transporters, nonribosomal peptide enzymes, and other proteins essential for the biosynthesis of the siderophore anguibactin. Of the 59 ORFs, approximately 32% were related to iron metabolic functions. The plasmid pJM1 confers on *V. anguillarum* the ability to take up ferric iron as a complex with anguibactin from a medium in which iron is chelated by transferrin, ethylenediamine-di(*o*-hydroxyphenyl-acetic acid), or other iron-chelating compounds. The *fatDCBA-angRT* operon as well as other downstream biosynthetic genes is bracketed by the homologous ISV-A1 and ISV-A2 insertion sequences. Other clusters on the plasmid also show an insertion element-flanked organization, including ORFs homologous to genes involved in the biosynthesis of 2,3-dihydroxybenzoic acid. Homologues of replication and partition genes are also identified on pJM1 adjacent to this region. ORFs with no known function represent approximately 30% of the pJM1 sequence. The insertion sequence elements in the composite transposon-like structures, corroborated by the G+C content of the pJM1 sequence, suggest a modular composition of plasmid pJM1, biased towards acquisition of modules containing genes related to iron metabolic functions. We also show that there is considerable microheterogeneity in pJM1-like plasmids from virulent strains of *V. anguillarum* isolated from different geographical sources.

The fish pathogen *Vibrio anguillarum* strain 775 is the causative agent of vibriosis, a highly fatal hemorrhagic septicemic disease (3). This bacterium disseminates in the vertebrate host by using the otherwise unavailable iron bound by high-affinity iron binding proteins, such as transferrin and lactoferrin. Furthermore, *V. anguillarum* 775 has the ability to grow in vitro in media in which iron is chelated by transferrin, ethylenediamine-di(*o*-hydroxyphenyl-acetic acid), and other iron chelators (15, 40). The metabolic pathway supporting the ability of this bacterium to grow under iron-limiting conditions is linked to the presence in the bacterial cells of the virulence plasmid pJM1 (15).

Iron metabolic plasmids are rare; in addition to the pJM1-like plasmids only the pColV-K30 family of plasmids, identified in human clinical strains of *Escherichia coli* and other enteric bacteria, have been associated with iron metabolism. However, the pJM1 and pColV-K30 plasmid-mediated iron uptake systems are unrelated (17, 49). pJM1-like-plasmids, usually around 65 to 67 kbp, have been reported by us and others in different virulent *V. anguillarum* strains isolated from many epizootics throughout the world (30, 32, 46). In this family of plasmids the best characterized is the 65-kb pJM1 plasmid that has been isolated from the *V. anguillarum* strain 775 and is the one that we chose to sequence. The diverse biochemical features of the pJM1 gene products and the possibility of horizontal evolution provided by the existence of insertion elements surrounding some of these genes make this fascinating plasmid also of interest for the study of evolutionary ecology.

Here we report the nucleotide sequence and annotation of the entire pJM1 virulence plasmid from *V. anguillarum* strain 775.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *V. anguillarum* strain 775 harboring the virulence plasmid pJM1 was grown in M9 minimal medium supplemented with mineral salts and Casamino Acids (Difco) as previously described (51). Plasmids carrying subclones from pJM1 were propagated in *E. coli* HB101 in Luria-Bertani medium in the presence of chloramphenicol (30 μg/ml). Plasmid DNA was extracted using the Qiagen Midi kit.

Determination of the pJM1 sequence. Sequencing primers were designed using Oligo 6.8 primer analysis software and purchased from the Oregon Health and Science University-Molecular Microbiology and Immunology (OHSU-MMI) Research Core Facility (http://www.ohsu.edu/core) and Invitrogen. The DNA template used for sequencing reactions was either the complete pJM1

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plasmid or fragments cloned using pBR325 as cloning vehicle. DNA sequencing reactions were carried out manually by the chain-termination method and by the OHSU-MMI Research Core Facility using a model 377 Applied Biosystems Inc. automated fluorescence sequencer. Base calling was performed on a Macintosh computer with the ABI base-calling program. Cycle sequencing was performed with AmpliTaq FS DNA polymerase with "Big-Dye" labeled terminators, both from Applied Biosystems Inc. The Sequencher software (Gene Code Corporation) was used for sequence assembly and editing. Codon usage tables were generated by the GCG Wisconsin Package. Coding regions were identified with BLAST (6). Coding sequences with associated ribosome binding sites were predicted with GENEMARK (9) and GLIMMER (http://www.tigr.org/software/glimmer/) and compared to the BLAST alignments to establish the coding sequence start. Regions lacking identified open reading frames (ORFs) and giving no BLAST results but revealing, on visual inspection, coding sequences with ribosomal binding sites were included in the annotation. G+C content analyses were performed with Oligo 6.8 primer analysis software.

Nucleotide sequences accession number. The complete circular nucleotide sequence of pJM1 from *V. anguillarum* strain 775 has been deposited in the GenBank sequence library and assigned the accession number AY312585.

RESULTS

Nucleotide composition. The circular plasmid is comprised of 65,009 bp, and the overall G+C content is 42.6%, which is slightly lower than the average G+C content of the two *V. anguillarum* 775 chromosomes (44%) (36). The G+C content is also reflected by the lack of restriction sites for *Asc*I (GGC GCGCC), *Not*I (GCGGCCGC), and *Srf*I (GCCCGGGC) and the presence of only one site for *Fse*I (GGCCGGCC). The bias towards A and T in the third codon position is also a consequence of this structural feature (data not shown).

With no demonstrated origin of replication (see below), position 1 of the sequence was arbitrarily assigned to 290 nucleotides upstream of a *Bam*HI site, which corresponds to the last base of the *angM* gene (ORF1). A total of 86.6% of the plasmid genome appears to have a coding function, and 41 of the 59 ORFs either correspond to biochemically characterized proteins or can be correlated with assigned or described functions by high degrees of similarity to sequences in the databases. Additionally, 18 ORFs predicted by GENEMARK, GLIMMER, or visual inspection but having no significant similarity to sequences in the databases may be functionally relevant (Table 1). Two additional genes, *rnaA* and *rnaB*, were found on the pJM1 plasmid and were shown to encode two antisense RNAs. The ORFs and the two antisense RNA genes are distributed between the strands in a ratio of 36 on one and 25 on the other strand.

ORF analysis. A graphical representation of the 59 known or predicted ORFs, and the two antisense RNA genes, appears in Fig. 1. Their relationships to representative homologues in databases are detailed in Table 1. Two ORFs, identified by using GLIMMER software, are not included in Table 1 because they are encoded within ORF54 in the opposite strand, they show no homology with any known proteins or ORFs in GenBank, and no obvious Shine-Dalgarno sequence was identified upstream of the AUG of these ORFs.

Based on gene similarity search results, functions associated with ORFs may be classified into the following categories: utilization of iron, transposition, and replication-partition (Table 1).

In Fig. 2 the G+C content of each ORF is shown, including the relative position of insertion sequence (IS) elements.

Utilization of iron. The predicted metabolic abilities linked to pJM1 center on iron uptake and utilization (Fig. 1). We have demonstrated that the genes *fatDCBA* (ORF3 to ORF6) confer on pJM1-carrying bacteria the ability to utilize ferric anguibactin and so are part of the pathway of iron utilization (2, 4, 5, 26). We also demonstrated that the genes *angM* (ORF1), *angR* (ORF7), *angT* (ORF8), *angN* (ORF10), and *angBG* (ORF41) encode nonribosomal peptide synthetases while *angU* (ORF9) and *angH* (ORF13) encode tailoring enzymes for the biosynthesis of anguibactin, since transposon insertions in each of these genes resulted in no siderophore production (17, 40–42). Other ORFs encoded on pJM1 (ORF39 to ORF43 and ORF59) show high similarity to siderophore biosynthetic genes found in other bacteria, and some of them could intervene in anguibactin biosynthesis. The homologies in Table 1 suggest that the predicted polypeptides of these ORFs could play a role in the early stages of anguibactin biosynthesis: ORF39 (AngD) is a phosphopantetheinyl transferase; ORF41 (AngBG) possesses the isochorismate lyase and aryl carrier protein (ArCP) domains; ORF42 (AngE) is the 2,3-dihydroxybenzoic acid (DHBA) AMP ligase; and ORF43 (AngC) is an isochorismate synthetase. ORF40 may have a function in the transport of ferric anguibactin into the cell cytosol. Upstream of *angM* (ORF1) there is another ORF (ORF59) with similarity to a stretch of 159 amino acids within the amino acid sequence of the 2,3-dihydro-DHBA dehydrogenase of *Vibrio cholerae*, the last enzyme in the DHBA biosynthetic pathway (52). Further upstream of ORF59 is a small (23-amino-acid) ORF that corresponds to the amino-terminal end of the 2,3-dihydro-DHBA dehydrogenase. It is thus possible that ORF59 could have been generated by a frameshift of the original full-length gene, resulting in a shorter ORF truncated at the amino-terminal end. The small ORF upstream of ORF59 has not been included in Table 1.

Retrobiosynthesis of anguibactin indicates that it is composed of one molecule of DHBA, one of cysteine, and one of *N*-hydroxyhistamine (1, 22). Cysteine is converted to a thiazoline ring, through cyclization, in the process of synthesis, and *N*-hydroxyhistamine is obtained from the modification of histidine.

In *V. anguillarum* strain 775, the anguibactin precursor DHBA is synthesized by chromosome-encoded proteins, as shown by the ability of the plasmidless strain to produce DHBA (11), although some virulent strains of *V. anguillarum*, such as 531A, rely on the pJHC1 (a pJM1-like plasmid)-encoded AngBG protein for the synthesis of this precursor (50). The amino terminus of the pJHC1-encoded AngBG possesses the isochorismate lyase activity, thereby explaining the need for this protein for the synthesis of DHBA in this strain (50). Analysis of mutations in the *angB* ORF of pJHC1 provided evidence that, in addition to *angB*, an overlapping gene, *angG*, exists at this locus and that it encodes polypeptides which are in frame to the carboxy-terminal end of the isochorismate lyase. The carboxy terminus of AngBG encodes an ArCP domain that is also present in the internal AngG polypeptides and is where phosphopantetheinylation occurs at a conserved serine residue, the phosphopantetheinylate moiety acting as an acceptor of an activated aryl or amino acid group (50). In strain 775 the *angBG* gene is also found on the pJM1 plasmid (ORF41); however, we do not know at present if the product

TABLE 1. Summary of ORFs identified by significant homology (BLAST search) or prediction or previously experimentally verified

ORF	Length (amino acids)	CDS (start codon–stop codon)[a]	Gene or function of closest relative (source)	Data bank reference	Identity[b] (%)	e value[c]
1	706	1–2118c	NRPS[d] VibF (*V. cholerae*)	gb|AAG00566.1|	209/535 (39)	e-103
2	302	2207–3115c	Transposase for ISV-A1 (*V. anguillarum*)	gb|AAA81774.1|	301/302 (99)	e-173
3	314	3578–4522	Iron transport protein FatD (*V. anguillarun*)	gb|AAA25641.1|	314/314 (100)	e-141
4	317	4519–5472	Iron transport protein FatC (*V. anguillarum*)	gb|AAA25642.1|	317/317 (100)	e-139
5	322	5526–6494	Iron transport protein FatB (*V. anguillarum*)	gb|AAA91580.1|	322/322 (100)	e-178
6	726	6598–8778	Iron transport protein FatA (*V. anguillarum*)	gb|AAA91581.1|	726/726 (100)	0.0
7	1,048	8863–12009	NRPS AngR (*V. anguillarum*)	gb|AAA79860.1|	1,048/1,048 (100)	0.0
8	252	12006–12764	NRPS AngT (*V. anguillarum*)	gb|AAA79861.1|	252/252 (100)	e-150
9	442	12862–14190c	Rhizobactin biosynthesis protein RhbE (*Sinorhizobium meliloti*)	gb|AAK65920.1|	237/435 (54)	e-143
10	956	14314–17184c	NRPS VibF (*V. cholerae*)	gb|AAG00566.1|	332/931 (35)	e-157
11	306	17396–18316	Transposase for ISV-A2 (*V. anguillarum*)	gb|AAA81776.1|	305/306 (99)	6-174
12	322	18437–19405	Transposase (*Vibrio vulnificus*)	gb|AAO10886.1|	301/322 (93)	e-152
13	386	19652–20854	Histidine decarboxylase AngH (*V. anguillarum*)	emb|CAA83945.1|	386/386 (100)	0.0
14	536	20970–22580	ABC transporter homologue (*E. coli*)	gb|AAN79727.1|	137/548 (25)	3e-27
15	560	22577–24259	Putative ABC transporter (*Streptomyces coelicolor*)	emb|CAC17507.1|	132/461 (28)	2e-40
16	105	24444–24761	Orf1 ISVme (*V. metschnikovii*)	gb|AAN33020.1|	24/96 (25)	1.4
17	117	24758–25111	Orf2 ISVme (*V. metschnikovii*)	gb|AAN33021.1|	41/103 (39)	e-15
18	513	25171–26712	Orf3 ISVme (*V. metschnikovii*)	gb|AAN33022.1|	217/526 (41)	2e-99
19	304	26817–27731c	RepA (*Aeromonas salmonicida*)	gb|AAK97757.1|	52/205 (25)	e-07
20	343	27728–28759c	Related to DNA mismatch repair protein (*Streptococcus mutans*)	gb|AAN59686.1|	28/93 (30)	0.67
21	362	29673–30761c	Transposase for IS*801* (*Pseudomonas syringae*)	emb|CAA40540.1||	171/341 (50)	8e-92
22	128	31031–31417	Unknown (*V. vulnificus*)	gb|AAO07608.1|	34/99 (34)	0.014
23	99	31555–31854	Conserved hypothetical protein (*Pseudomonas putida*)	emb|CAC86752.1|	54/92 (58)	e-22
24	96	31878–32168	Hypothetical protein (*P. putida*)	emb|CAC86751.1|	48/83 (57)	2e-21
25	409	32297–33526c	GLIMMER, GENEMARK prediction; no homology			
26	127	33513–33896c	GLIMMER prediction; no homology			
27	323	33902–34873c	ParB (*Xylella fastidiosa*)	gb|AAF85627.1|	64/218 (29)	5e-16
28	273	34870–35691c	ParA (*Leptospira interrogans*)	gb|AAN51545.1|	74/247 (29)	3e-22
29	514	35892–37436	Putative bacteriophage protein (*Salmonella enterica*)	gb|AAO69541.1|	101/307 (32)	5e-32
30	115	37505–37852	GLIMMER prediction; no homology			
31	103	37883–38194	GLIMMER prediction; no homology			
32	264	38616–39410	GLIMMER, GENEMARK prediction; no homology			
33	104	39738–40052	GLIMMER, GENEMARK prediction; no homology			
34	116	40152–40451	GLIMMER prediction; no homology			
35	93	40396–40677	GLIMMER prediction; no homology			
36	136	40727–41137	GLIMMER prediction; no homology			
37	176	41246–41776	GLIMMER prediction; no homology			
38	306	41945–42865c	Transposase for ISV-A2 (*V. anguillarum*)	gb|AAG33855.1|	306/306 (100)	e-174
39	239	43053–43772	NrgA (*Photorhabdus luminescens*) similar to EntD (*E. coli*)	gb|AAO17175.1|	91/227 (40)	3e-40
40	251	43873–44628	Iron(III) ABC transporter (*Vibrio parahaemolyticus*)	dbj|BAC62003.1|	183/250 (73)	e-101
41	287	44827–45690	AngB/G (*V. anguillarum*)	gb|AAG33854.1|	287/287 (100)	e-151
42	546	45741–47381c	DHBA-AMP ligase (*V. cholerae*)	gb|AAF93937.1|	307/536 (57)	0.0
43	393	47362–48543c	Isochorismate synthase (*V. vulnificus*)	gb|AAO07758.1|	252/392 (64)	e-149
44	306	48880–49800	Transposase for ISV-A2 (*V. anguillarum*)	gb|AAG33856.1|	306/306 (100)	e-174
45	277	49943–50776c	DHAP[e] synthase (*V. vulnificus*)	gb|AAO07756.1|	149/268 (55)	2e-82
46	513	50796–52337c	Orf3 ISVme (*V. metschnikovii*)	gb|AAN33022.1|	217/526 (41)	2e-99
47	117	52397–52750c	Orf2 ISVme (*V. metschnikovii*)	gb|AAN33021.1|	41/103 (39)	e-15
48	105	52747–53064c	Orf1 ISVme (*V. metschnikovii*)	gb|AAN33020.1|	24/96 (25)	1.4
49	115	53664–54011	GLIMMER prediction; no homology			
50	153	55181–55561c	Putative transposase (*Klebsiella pneumoniae*)	emb|CAA09339.1|	98/151 (64)	4e-55
51	112	55679–55951c	Putative arylsulfatase regulatory protein (*E. coli*)	gb|AAN83151.1|	24/97 (24)	0.73
52	140	55971–56393c	GLIMMER prediction; no homology			
53	389	56383–57552c	Hypothetical protein (*Pseudomonas putida*)	gb|AAN662271.1|	34/101 (33)	0.002
54	980	57830–60772	Transposase (*Mesorhizobium loti*)	dbj|BAB54451.1|	431/965 (44)	0.0
55	194	60891–61475	Putative resolvase (*Serratia marcescens*)	gb|AAN52496.1|	183/194 (94)	8e-88
56	464	61553–62947	SpnT (*S. marcescens*)	gb|AAN52497.1|	430/464 (92)	0.0
57	144	62944–63378	RecX (*E. coli*)	dbj|BAA16560.1|	54/146 (36)	e-18
58	299	63430–64329	Transposase for ISV-A2 (*V. anguillarum*) (partial)	gb|AAG33855.1|	293/293 (100)	e-166
59	175	64326–64853c	VibA (*V. cholerae*) (partial)	gb|AAF93939.1|	91/159 (57)	2e-42

[a] CDS, coding sequence; c, complementary strand.
[b] Shown as number of identical amino acids/total number of amino acids in the region of homology identified by BLAST.
[c] An e value of >0.4 indicates no homology.
[d] NRPS, nonribosomal peptide synthetase.
[e] DHAP, 2-dehydro-3-deoxyphosphoheptonate aldolase.

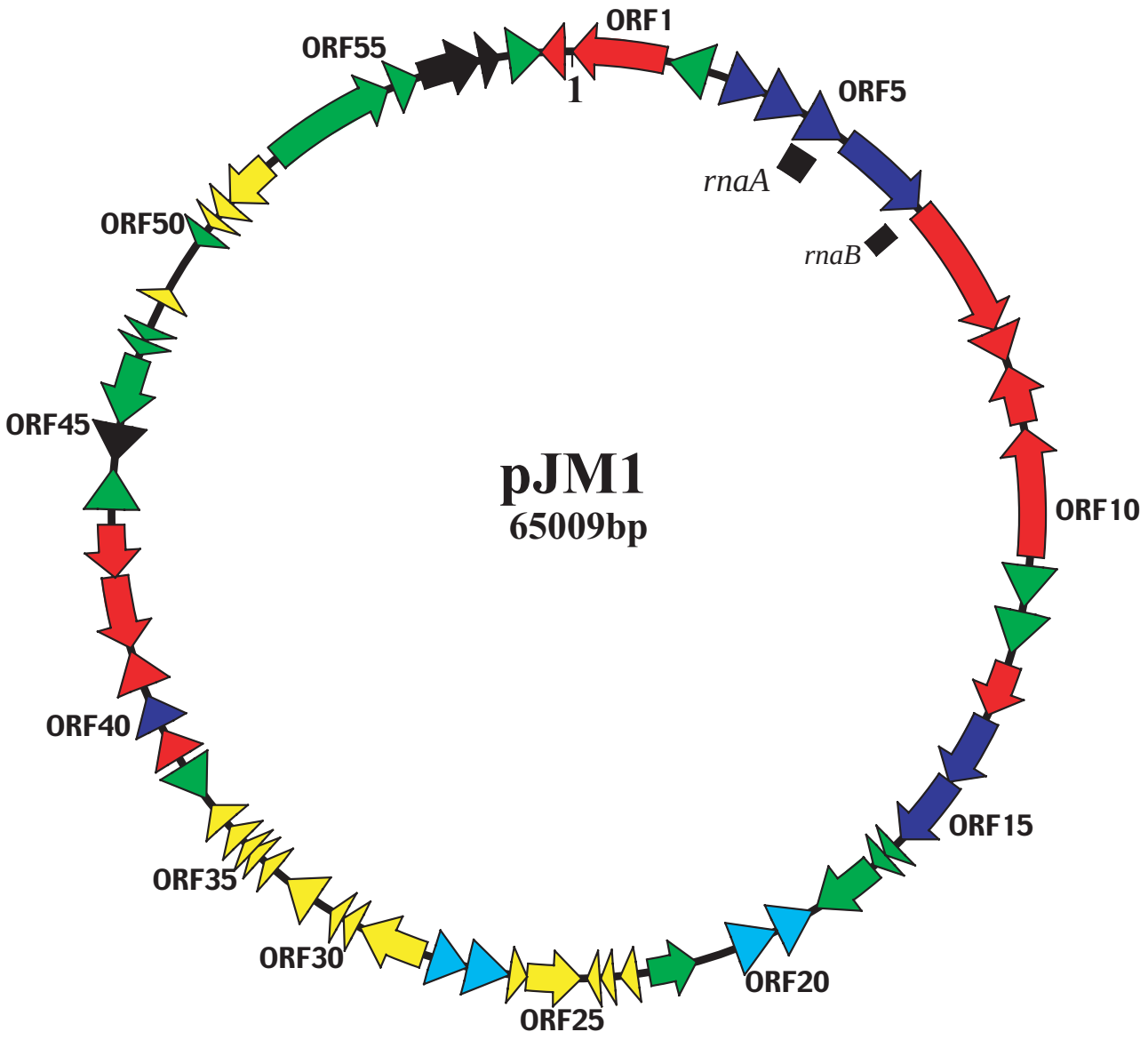

FIG. 1. Schematic representation of the ORFs on the two strands of pJM1 DNA. Red, ORFs related to biosynthesis of the siderophore; blue, ORFs related to transport of the siderophore; green, ORFs related to IS elements and composite transposon; cyan, ORFs related to replication and partitioning; yellow, conserved hypothetical ORFs and ORFs with no known functions; black, ORFs with functions that do not fall in any of the above categories. The black blocks represent genes encoding antisense RNAs: *rnaA*, antisense RNAα, and *rnaB*, antisense RNAβ.

of this gene is essential for anguibactin biosynthesis, as is the case for strain 531A.

The enzymology of anguibactin biosynthesis is still under investigation; however, predictions can be made based on our knowledge of the structure of this siderophore, genetic evidence, and functions of potential biosynthetic proteins inferred by homology studies (17, 39). The results of this combined genetic and in silico analysis are shown in the model in Fig. 3. In the biosynthesis of other phenolic siderophores, activation of DHBA, before loading on the specific ArCP domain, occurs by the action of a 2,3-dihydroxybenzoate-AMP ligase, VibE in vibriobactin biosynthesis in *V. cholerae*, and EntE in enterobactin biosynthesis in *E. coli*. ORF42 in pJM1 shows homology with VibE and EntE adenylation domains (Table 1), suggesting that it could act as the 2,3-dihydroxybenzoate-AMP ligase in anguibactin biosynthesis. The activated DHBA is then ligated to the phosphopantetheinyl moiety in AngBG. Phosphopantetheinylation of this protein might have occurred by the action of the pJM1-encoded AngD (ORF39), showing homology to phosphepantotheinyl transferases. The pJM1 plasmid-encoded proteins AngR (ORF7), AngM (ORF1), and AngN (ORF10) must play a role in subsequent biosynthetic steps, although AngR, in addition to its biosynthetic function, is also essential for regulation of iron transport gene expression (13, 16–18, 34, 39, 42, 51). Cysteine, one of the anguibactin precursors, is likely activated by the A domain of AngR. Activated cysteine is then loaded onto the peptidyl carrier protein (PCP) domain of AngM. Although the AngR amino acid sequence

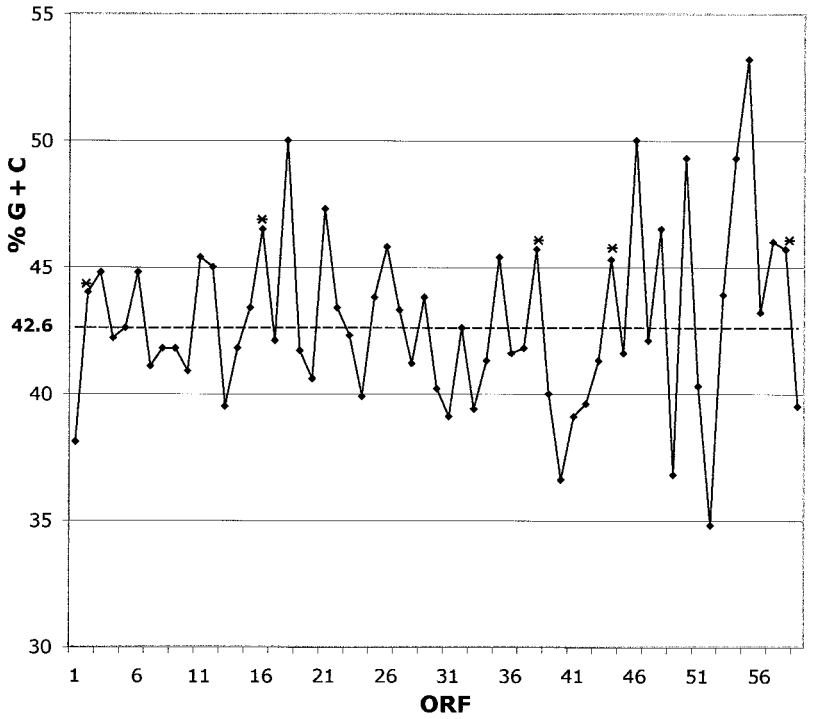

FIG. 2. G+C contents of the ORFs of pJM1. Dashed line, mean G+C content of pJM1 DNA (42.6%). Asterisks indicate the highly related ISV-A1 and ISV-A2.

also contains a PCP domain, this may not be functional because an essential serine is replaced by alanine in this domain. The condensation (C) domain of AngM then catalyzes the formation of a peptide bond between DHBA and cysteine, resulting in the DHBA-cysteine dipeptide bound to the PCP domain of AngM. Another plasmid-encoded protein, AngN, contains two tandem cyclization (Cy) domains that are involved in the cyclization of the cysteine moiety to form the thiazoline ring. Another Cy domain is found in the AngR protein; however, the essential first aspartic acid is replaced by asparagine in the highly conserved Cy motif, suggesting that also this domain of AngR is not functional. Anguibactin is

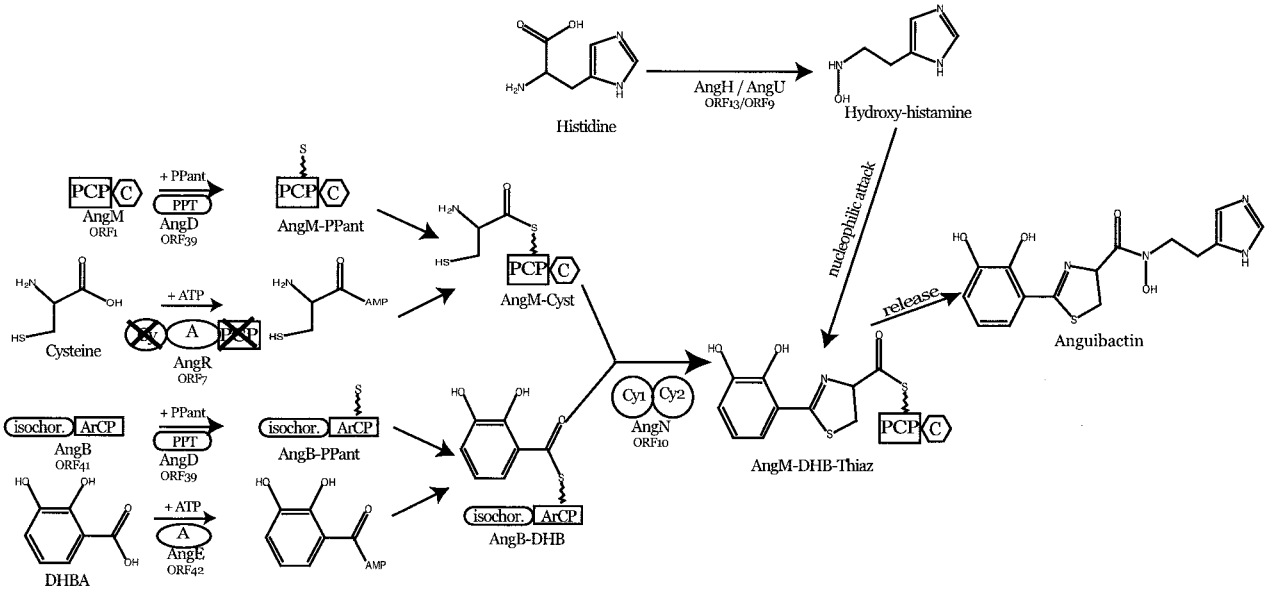

FIG. 3. Model of anguibactin biosynthesis. The predicted ORF numbers from Table 1 are given under the protein designations. Abbreviations: DHB, DHBA and radicals thereof such as a diphenolic ring; Cyst, cysteine; Thiaz, thiazoline ring; PPant, phosphopantetheinyl moiety; isochor., isochorismate.

released from the PCP domain of AngM by nucleophilic attack of *N*-hydroxyhistamine to the phosphopantetheinyl arm of the PCP domain of AngM. *N*-Hydroxyhistamine is produced by modification of histidine catalyzed by the histidine decarboxylase AngH (ORF13) (41) and a monooxygenase homologue, AngU (ORF9). AngT (ORF8), the thioesterase identified in this system, is not included in this model because it does not appear to be strictly necessary for anguibactin production, since an *angT* mutant results in only a 17-fold-lower yield of anguibactin (17, 51).

After anguibactin is synthesized, it is secreted to the extracellular space. Two ORFs (ORF14 and ORF15), downstream of *angH*, related to the ABC-type transporter, are perhaps involved in secretion as part of the complex that exports anguibactin to the extracellular space. After the siderophore is secreted and bound to iron, the ferric siderophore complex is transported to the cytosol via a highly specific transport system (2, 4, 5, 26, 40, 44, 45). In *V. anguillarum* 775 this system includes the outer membrane receptor FatA (ORF6), which binds ferric anguibactin and shuttles it to the periplasm (2, 5). The energy necessary for this transport is mediated by a chromosomally encoded TonB-ExbB-ExbD complex, which interacts with the FatA protein (M. Stork, M. L. Lemos, and J. H. Crosa, unpublished data).

The next step in internalizing the ferric siderophore complex involves the periplasmic binding protein FatB (ORF5). FatB is a lipoprotein (4) that is anchored to the inner membrane, unlike the *E. coli* homologues FhuD and FepB, which are free in the periplasm (25, 37).

We believe that the last step in internalization of ferric anguibactin involves the inner membrane proteins FatD (ORF3) and FatC (ORF4) and that these catalyze the transport of ferric anguibactin from the periplasm to the cytosol (5, 26). In other systems, such as the ferrichrome and enterobactin systems, the energy for this transport through the inner membrane is provided by an ATP-binding protein (7, 24, 31). ORF40 shows homology with many of these iron(III) ATP-binding proteins and could provide the missing ATP-binding domain of the permease complex, although the activity of chromosomally encoded ATP-binding proteins cannot be ruled out.

The genes *fatABCD* together with the genes *angR* and *angT* are located in the iron transport-biosynthesis (ITB) operon (Fig. 1) (51). Regulation of this operon is carried out at the transcriptional level by the positive regulator AngR and the chromosomally encoded negative regulator Fur and at the posttranscriptional level by two antisense RNAs, RNAα and RNAβ, encoded within this operon by the *rnaA* and *rnaB* genes, respectively (Fig. 1) (12, 35, 47, 48). These two genes have been shown to encode RNAs that are not translated. Another positive regulator of the ITB operon, the *trans*-acting factor (TAF), is encoded in a region noncontiguous to this operon encompassing ORF29 to ORF53 (40, 45, 50). The TAF determinants have not been identified as yet, and no ORF included in the TAF region shows homology with regulatory proteins, with the exception of ORF51, which shows very low homology (24% identity) to an *E. coli* arylsulfatase regulatory protein.

Insertion elements and transposons. The ITB operon and the biosynthetic genes *angU* (ORF9) and *angN* (ORF10) are located within a structure that resembles a transposon, flanked by the almost identical ISV-A1 and ISV-A2 elements (44). There is a second putative composite transposon on pJM1 that contains homologues of genes (ORF39 to ORF43) involved in the synthesis of DHBA, one of the anguibactin precursors (Table 1). These genes are also organized as a cluster that is flanked by identical ISV-A2 sequences. 2 shows very clearly that the ISV-A2-flanked DNA has an average G+C content of 39.3%, significantly lower than the average G+C content of the pJM1 plasmid, suggesting a horizontal acquisition of this composite gene region. A third set of ORFs, ORF1 (*angM*) and ORF59, is flanked by ISV-A1 and ISV-A2 sequences, although the ISV-A2 element is interrupted at the 5′ end of the putative transposase gene by an insertion of a sequence containing four ORFs (ORF54 to ORF57). It is of interest that one of these ORFs (ORF54) shows homology to a transposase gene, while another, ORF55, shows high similarity to a resolvase. ORF56 shows similarity to *spnT*, a *Serratia marcescens* gene encoding a protein with no homologues, although when overexpressed it affected both sliding motility and prodigiosin production in this bacterium (21). The other ORF in this cluster, ORF57, encodes a predicted protein with homology to RecX, a *recA* regulator (38). Furthermore this cluster is flanked by 5-bp direct repeats, suggesting that the interruption of the ISV-A2 transposase gene (ORF58) could have resulted from insertion of a transposon containing these four ORFs.

Besides ISV-A1 and ISV-A2, pJM1 carries several hypothetical IS elements (Table 1). Two of them show homology to a *Vibrio metschnikovii* ISVme insertion sequence, and they both contain all three ORFs found in this IS element. These two identical ISVme-like sequences flank a region of 24,085 bp containing 27 ORFs (from ORF19 to ORF45), almost half of the coding capacity of pJM1. Another IS element, RS1 carrying ORF21, which shows homology to a transposase, is found as a single copy in pJM1. Two other ORFs, ORF12 and ORF50, show similarity to transposases; however, no other components of ISs were identified.

Replication and partition. The pJM1 plasmid replicates at a copy number of 1 to 2 in *V. anguillarum* cells (10). ORF19 to ORF28, encoding hypothetical proteins that could be involved in replication and partition, are clustered adjacent to the TAF region. ORF19 shows homology with the RepA protein of *Aeromonas salmonicida* plasmid pRAS3 (28). The presence of four 21-bp direct repeats in the proximity of ORF19, spanning bp 29013 to 29136, and the relatively low G+C content of 33.7% are consistent with the existence of a possible *oriV* in this region. The predicted amino acid sequences of ORF27 and ORF28 show similarity to those of ParB and ParA, respectively. In other systems, the plasmid-encoded ParA and ParB proteins form one operon autoregulated by the Par proteins. The ParA protein is an ATPase that assembles with ParB subunits and forms a nucleoprotein complex that binds to a *cis*-acting centromere-like site, *parS* (27). As predicted for ParA proteins, ORF28 exhibits at its N terminus an ATP-GTP-binding consensus sequence (19). In other bacteria, homologues of *parA* and *parB* map adjacent to the chromosomal and plasmid origin regions of replication (29, 33), and this is the case for pJM1. Experiments are being carried out to characterize the functions of the pJM1 replication region.

The microheterogeneity of pJM1-like plasmids in strains of *V. anguillarum*. Different virulent *V. anguillarum* strains har-

Strain	Geographic source	*Bam*HI restriction pattern*
775	Washington	I
133-S	Washington	I
V15	Washington	V
PB1	Tukushima, Japan	I
PT77022	Tukushima, Japan	IV
PT77052	Tukushima, Japan	I
PT77082	Tukushima, Japan	IV
PT77097	Tukushima, Japan	I
PT223	Tukushima, Japan	I
LS-173	Oregon	I
LS-174	Oregon	I
507	Maine	III
521	Maine	II
521A	Maine	III
522A	Maine	II
522C	Maine	II
523B	Maine	II
527	Maine	III
528	Maine	III
531A	Maine	II
531B	Maine	II
532	Maine	III
533	Maine	II
535	Maine	II
540A	Maine	II
543A	Maine	III
547B	Maine	III
500	New Hampshire	III
509	New Hampshire	III
R61	Galicia, Spain	VI
R72	Galicia, Spain	VI

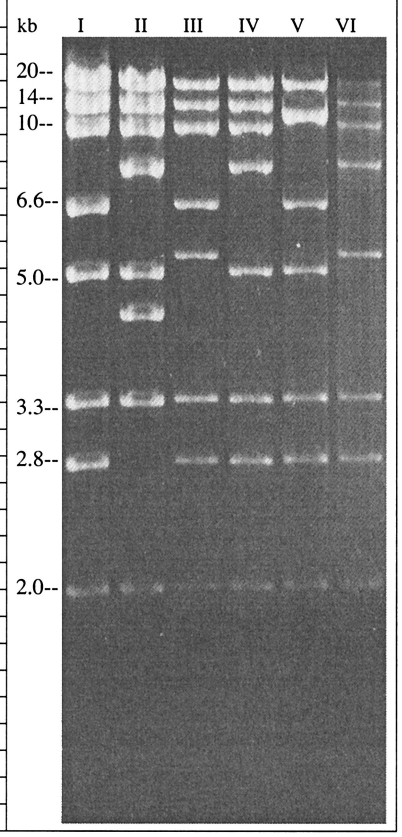

FIG. 4. Microheterogeneity of pJM1-like plasmids from *V. anguillarum* strains. *, "kb" indicates sizes in kilobase pairs of each of the pJM1 *Bam*HI restriction endonuclease fragments.

boring the pJM1-like-plasmids showed microheterogeneity in their restriction endonuclease patterns. A listing of the *Bam*HI restriction fragment length polymorphisms as well as the *Bam*HI profiles for several naturally occurring pJM1-like virulence plasmids that we have examined is given in Fig. 4.

DISCUSSION

The presence of genes of a complex biosynthetic pathway for a siderophore and the carriage of the iron transport operon together with its role in virulence of *V. anguillarum* 775 are unique features of plasmid pJM1 (15, 16, 17). The 65,009-nucleotide sequence, with an overall G+C content of 42.6%, revealed 59 genes and ORFs encoding functions associated with utilization of iron, transposition, and partition. Approximately a third of these ORFs and genes are related to iron metabolic functions, including ferric anguibactin transport proteins, nonribosomal peptide enzymes, and other proteins essential for the biosynthesis of the siderophore anguibactin. The majority of the ORFs encoding iron metabolic functions were experimentally demonstrated to be involved in anguibactin-mediated iron metabolism (ORF1 to ORF13). Other ORFs with homology to proteins involved in the biosynthesis and activation of the precursor of anguibactin DHBA, such as ORF39 to ORF43, were also identified. Our recent work has demonstrated that one of these, ORF42, named *angE*, encodes a 2,3-dihydroxybenzoate-AMP ligase that could act in activating DHBA. In a previous publication we reported that the *angE* homologue in plasmid pJHC1, a pJM1-like plasmid found in *V. anguillarum* strain 531A, was not essential for anguibactin production (50). A deletion that eliminated a region including the *angB* and *angE* genes was complemented by just the *angB* gene, demonstrating the existence of a chromosomal homologue of the *angE* gene in this strain (50). We have recently identified in the 775 strain chromosome homologues of *angE* and other genes involved in the production, activation, and incorporation of DHBA in the anguibactin biosynthetic pathway; therefore, the pJM1 plasmid-carried *angE* gene in the 775 strain might not be essential for anguibactin biosynthesis (unpublished data). It is of interest that the amino acid sequence of this chromosomally encoded AngE is identical to that reported previously (reference 20 and our unpublished results). It is also of interest that ORF39 to ORF43 are organized as a cluster that is flanked by ISV-A2 sequences and that the DNA in this cluster shows an average G+C content of 39.3%, significantly lower than the average G+C content of the pJM1 plasmid. It is tempting to speculate that this ISV-A2-flanked structure is a transposon that has been horizontally

acquired from other bacteria. Furthermore, the existence of chromosome homologues for some of these ORFs suggests the attractive possibility that there could also have been an integration event in one of the two *V. anguillarum* chromosomes. Curiously, the ITB operon, *fatDCBA-angRT* (ORF3 to ORF8, respectively), and other anguibactin biosynthetic genes, *angU* (ORF9) and *angN* (ORF10), located downstream of this operon, are also bracketed by the highly related ISV-A1 and ISV-A2 ISs. Nevertheless, the fact that ORFs and genes on pJM1 are flanked by IS elements does not necessarily imply that the composite structures are transposons, since the relative orientation of the flanking IS elements is not always as found with the IS elements flanking known composite transposons such as Tn*5*, Tn*7*, and Tn*10* (8, 14, 23). The positions of IS elements forming a composite transposon-like structure, corroborated by the G+C content of the pJM1 sequence, suggest a modular composition of the virulence plasmid pJM1 of *V. anguillarum* strain 775 biased towards acquisition of transposon modules containing genes related to iron metabolic functions.

An important final step is the secretion of the siderophore to the extracellular milieu, a still-unsolved question for siderophore-mediated iron transport systems. In this vein, although not yet proven, ORF14 and ORF15 could function in the export of anguibactin, since these ORFs show homology to ABC transporters involved in efflux. Elucidation of their function, if any, will have to await current genetic analysis.

Regions that have features consistent with replication and partitioning functions have been identified adjacent to the TAF region. It is possible that the product of ORF19 is a replication protein that interacts with the 21-bp repeats located in proximity. Whether these sequences are truly involved in these functions await further analysis, which is currently being carried out.

We also report in this work considerable microheterogeneity in pJM1 plasmids isolated from many parts of the world, even from both the east and west coasts of the United States. Previous work has also shown microheterogeneity in many other examples of pJM1-like plasmids (30, 32, 42, 43). For instance the pJM1-like plasmid pJHC1 from strain 531A shows two major changes, two extra insertions of the RS1 sequence and also an *angR* gene that has a single nucleotide change that results in an amino acid substitution in the AngR protein (H267N). This change was associated with an increased anguibactin production not only in the 531A strain but also in other strains in which the AngR protein shows this H-to-N alteration (42). In other cases the existing microheterogeneity has only been characterized by restriction endonuclease analysis without further genetic analysis (30, 32, 43).

Recently a partial sequence of a pJM1-like plasmid (pEIB1) was submitted to GenBank (accession number AY255699). This plasmid is another example of the microheterogeneity of the pJM1-like plasmids: pEIB1 differs from pJM1, in the sequence of at least two anguibactin biosynthesis genes. The *angR* sequence has the H267N substitution found in several pJM1-like plasmids that leads to a higher siderophore production phenotype (42), while the *angN* sequence shows an internal deletion compared with the *angN* gene present in pJM1. The deletion in this pJM1-like plasmid resulted in a frameshift splitting the *angN* gene (ORF10 in pJM1) into two ORFs, truncating the first Cy domain of AngN while leaving the second Cy domain intact. It would be of interest to know whether the strain harboring the pJM1-like plasmid with this *angN* deletion is still able to produce anguibactin. Since there is no information on the origin of the strain, we cannot identify whether this plasmid is one of the already described pJM1-like plasmids. Furthermore, this partial sequence submission erroneously assigns homology of the ORF JM15, which is 100% homologous to our ORF56, to the TcbA insecticidal toxin from *Photorhabdus luminescens* (GenBank accession number AAC38627). By our analysis ORF56, and thus JM15, shows only low homology (20% identity) to an 110-amino-acid stretch of the 2,504-amino-acid-long TcbA protein. Conversely, we found a strikingly high homology (92% identity [Table 1]) of the predicted 464-amino-acid product of ORF56, and thus JM15, to the 464-amino-acid-long SpnT (GenBank accession number AAN52497), an *S. marcescens* protein possibly involved in both sliding motility and prodigiosin production in this bacterium (21).

Our complete sequence analysis and annotation of pJM1 present for the first time the entire sequence of an iron metabolic regulon that is in its majority encoded on a plasmid and that is an essential factor of virulence in *V. anguillarum* infections of fish.

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