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Reactivation of the vanchrobactin siderophore system of *Vibrio anguillarum* by removal of a chromosomal insertion sequence originated in plasmid pJM1 encoding the anguibactin siderophore system

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Summary

A chromosomal gene cluster encoding vanchrobactin biosynthesis and transport genes was identified in the *Vibrio anguillarum* serotype O1 strain, 775(pJM1), harbouring the anguibactin biosynthetic genes in the pJM1 plasmid. In this strain only anguibactin is produced as the vanchrobactin chromosome cluster has a RS1 transposition insertion into *vabF*, one of the vanchrobactin biosynthesis genes. Removal of this RS1 generating 775(pJM1) Δ *tnp*, still resulted in the detection of only anguibactin in specific bioassays. Surprisingly, when the pJM1 plasmid was not present as in the plasmidless strain H775-3, removal of the RS1 resulted in the detection of only vanchrobactin. These results thus can be interpreted as if presence of the pJM1 plasmid or of anguibactin itself is associated with the lack of detection of the vanchrobactin siderophore in bioassays. As high-performance liquid chromatography (HPLC) and mass spectrometry analysis demonstrated that both vanchrobactin and anguibactin were indeed produced in 775(pJM1) Δ *tnp*, it is clear that the pJM1-encoded anguibactin siderophore has higher affinity for iron than the vanchrobactin system in strains in which both systems are expressed at the same time. Our results underscore the importance of the anguibactin system in the survival of *V. anguillarum* 775 under conditions of iron limitation.

Introduction

In the environment as well as in the vertebrate host, iron is not readily available thus microorganisms evolved systems to utilize this metal that is essential for their metabolism. One of the systems is mediated by siderophores, small molecules capable of chelating iron with high affinity, which can be taken up via the bacteria-specific receptors as a ferric–siderophore complex (Crosa, 1989; Winkelmann, 2004). *Vibrio anguillarum* has been described as being part of the normal microflora of the aquatic environment (West *et al.*, 1983; Hidetoshi and Rivera, 2006), and is also known as an important pathogen that causes haemorrhagic septicaemia in salmonid fish with very high mortality if the disease is left untreated (Actis *et al.*, 1999). Twenty-three serotypes (O1–O23) have been described in this bacterium (Sorensen and Larsen, 1986; Grisez and Ollevier, 1995; Pedersen *et al.*, 1999); however, most vibriosis outbreaks in cultured salmonid fish and feral marine fish have been caused by strains belonging to serotypes O1 and O2, respectively (Toranzo and Barja, 1990;

Larsen *et al.*, 1994), whereas strains of serotypes O3 to O23 have been mainly isolated from marine environmental samples, including water, sediment, phyto- and zooplankton.

Many strains of *V. anguillarum* harbour the 65 kb plasmid pJM1 or pJM1-like plasmids encoding the siderophore anguibactin biosynthesis genes (Crosa, 1980; Tolmasky *et al.*, 1988; Welch *et al.*, 2000; Crosa and Walsh, 2002; Di Lorenzo *et al.*, 2003; Di Lorenzo *et al.*, 2004; Alice *et al.*, 2005) as well as the transport proteins for ferric anguibactin (Fig. 1A) (Actis *et al.*, 1985; 1986; 1988; 1995; Jalal *et al.*, 1989; Koster *et al.*, 1991), a major contributor to the virulence of this bacterium and to the bacterium's ability to obtain iron from the complex hydroxy-iron compounds found in the environment. However, it is puzzling that all these pJM1-containing *V. anguillarum* strains belong exclusively to the serotype O1, independently of the geographical region from where they were isolated (Toranzo *et al.*, 1983; Lemos *et al.*, 1988; Wiik *et al.*, 1989; Conchas *et al.*, 1991). Some of the other serotype O1 and all serotype O2 strains do not produce anguibactin; however, they are able to produce the DHBA-based chromosome-mediated siderophore vanchrobactin, whose structure was recently solved and its biosynthetic genes in the O2 strain RV22 were characterized (Lemos *et al.*, 1988; Balado *et al.*, 2006; Soengas *et al.*, 2006) (Fig. 1B). Recently our laboratory provided a possible explanation for the exclusive residence of pJM1 in O1 type strains. We demonstrated that the O1 lipopolysaccharide (LPS) side-chain is required to stabilize the pJM1 encoded ferric anguibactin specific receptor FatA in the outer membrane of *V. anguillarum* (Welch and Crosa, 2005). Furthermore, the intact O1 side chain is essential to withstand the bactericidal activity of fish serum during growth under iron limitation (Welch and Crosa, 2005).

It is of interest that some of the anguibactin biosynthesis genes in the O1 *V. anguillarum* 775(pJM1) strain exist as redundant functional copies in both the pJM1 plasmid and the chromosome, or are only harboured by the chromosome (Alice *et al.*, 2005). Thus, it is possible that the chromosomal functional duplicates in *V. anguillarum* 775(pJM1) are the remains of a cluster involved in the biosynthesis of an ancestral DHBA-based siderophore. Recently, molecular and physiological analysis of these genes in the *V. anguillarum* O2 type strain RV22 by Lemos group showed that they are involved in vanchrobactin biosynthesis (Balado *et al.*, 2006). In this work we demonstrate that the vanchrobactin cluster is also present in its entirety in the 775 chromosome and postulate a possible mechanism that resulted in the selection of the pJM1 plasmid-mediated anguibactin iron-scavenging system over that mediated by vanchrobactin in the pJM1-harboring O1 type strain of *V. anguillarum*.

Results

Identification of a chromosomal cluster of genes with homologies to siderophore biosynthesis and uptake genes in the *V. anguillarum* 775(pJM1)

Analysis and annotation of the complete sequence of the pJM1 plasmid resulted in the identification of an open reading frame, ORF39, a homologue of the *Escherichia coli* *entD* gene named *angD* [34% identity at the protein level and Accession No. CAB57861 (Di Lorenzo *et al.*, 2003)]. Purified AngD showed a phosphopantetheinyl transferase (PPT) activity that is necessary for the activation of nonribosomal synthetase carrier proteins (Liu *et al.*, 2005). Phosphopantetheinyl transferase operates by transfer of the 4'-phosphopantetheine (P-pant) moiety of coenzyme-A to a conserved serine residue of the peptidyl carrier protein (PCP) and aryl carrier protein (ArCP) domains of the carrier proteins involved in siderophore biosynthesis (Crosa and Walsh, 2002). To assess whether *angD* is necessary for anguibactin biosynthesis, we interrupted this gene in pJM1 with a kanamycin cassette as described in *Experimental procedures*. The chrome azurol S (CAS) assay analysis of this mutant showed that it could still produce anguibactin (Fig. 2A, panel 3;

panels 1 and 2 of Fig. 2A are controls). However, as PPT is essential for the incorporation of a P-pant group in PCP and ArCP domains of nonribosomal peptide synthases, it was expected that this gene would have been essential for siderophore biosynthesis (Walsh *et al.*, 1997). As we have previously demonstrated a functional redundancy for the pJM1-encoded anguibactin biosynthesis genes *angC* and *angE*, in the chromosome of *V. anguillarum* (Alice *et al.*, 2005), it was possible that a redundant copy of *angD* could also exist in the chromosomal DNA. To identify the putative chromosomal *angD* gene we further analysed the chromosome cluster harbouring *angC,E*, by screening of cosmid clones, inverse polymerase chain reaction (PCR) and nucleotide sequencing. We were finally able to identify a gene showing 29% identity at the protein level with the plasmid *angD* gene. A deletion mutant of this chromosomal *angD* gene still showed a halo on CAS, because the plasmid *angD* gene was functional (Fig. 2A, panel 4). For reasons that will become clear in the following paragraphs the chromosomal *angD* gene has now been renamed *vabD*. As expected, the double *angD-vabD* mutant did not produce anguibactin (Fig. 2A, panel 5). Furthermore, when the double mutant was complemented with either *angD* or *vabD*, the strains recovered the ability to produce anguibactin (Fig. 2A, panels 6 and 7). These results clearly show that both *angD* and *vabD* genes are functional, even if redundant, in the biosynthesis of anguibactin. In the process of sequencing the regions adjacent to *vabD* we came upon five additional ORFs (Fig. 3A). Four of them, *vabH*, *vabF*, *mbtH* and *vabS*, showed over 95% identity with those genes recently identified in the vanchrobactin biosynthesis locus of the O2 serotype RV22 strain (Balado *et al.*, 2006). We have also found another gene in this cluster, named *fvtA*, that showed 30% identity at the protein level with the *Bordetella pertussis* enterobactin receptor gene, *bfeA* (Accession No. AAA98536) (Beall and Sanden, 1995). We have recently identified that the product of this gene, FvtA, is the receptor for ferric vanchrobactin (to be published elsewhere). As the plasmidless RV22 strain belongs to the O2 serotype and the 775(pJM1) strain is of an O1 serotype we compared the vanchrobactin cluster in the latter strain with that of another plasmidless strain, 96F, that belongs to the O1 serotype. Comparison of the nucleotide sequences of the entire chromosomal locus between these two strains showed 98–100% identity in every one of the genes, except for the existence of the insertion of the RS1 element in the *vabF* gene from 775(pJM1) strain. Figure 3 shows a comparison of the 775(pJM1) with only the 96F chromosomal cluster as the RV22 cluster is highly related (more than 95% at the nucleotide level) to that of 96F. Figure 3A and B shows that the *vabF* gene in the chromosomal DNA of *V. anguillarum* 775(pJM1) is interrupted by the insertion sequence (IS) RS1, inserted at a CTTGAT sequence of the *vabF* gene. RS1 contains a transposase gene that show 100% identity to the RS1 originally described as present in the pJM1 plasmid (Di Lorenzo *et al.*, 2003), suggesting that the whole sequence of this IS element could have transposed from the plasmid to the chromosome. We have previously suggested that the chromosomal functional homologues of the anguibactin biosynthetic genes present on pJM1 could be remnants of a complete biosynthesis and uptake cluster for a chromosomal-mediated catechol siderophore, possibly vanchrobactin, in an ancestor of the 775(pJM1) strain that at some point during evolution had lost the ability to biosynthesize this siderophore (Alice *et al.*, 2005).

To corroborate the importance of *vabD* for the production of vanchrobactin, we generated an in-frame deletion of the *vabD* gene in the *V. anguillarum* 96F strain. This mutation results in a lack of vanchrobactin synthesis (Fig. 2B, panel 2). Although this mutant cannot produce vanchrobactin it can nevertheless be complemented with wild-type copies of either *angD* or *vabD* from *V. anguillarum* 775(pJM1) (Fig. 2B, panels 3 and 4). These results show not only that the two functional homologues from 775(pJM1) can operate for both anguibactin and vanchrobactin synthesis in 775(pJM1) but also can complement a mutation in the corresponding gene from the 96F strain.

Specific deletion of the RS1 transposon from the RS1-interrupted *vabF* gene in *V. anguillarum* 775(pJM1) results in vanchrobactin biosynthesis and competition for iron between anguibactin and vanchrobactin

In order to assess whether we could recover the functionality of the *vabF* gene in the *V. anguillarum* strains 775(pJM1) and its cured derivative H775-3, we decided to specifically remove the RS1 insertion element from this gene by using the splicing by overlap extension (SOE) PCR strategy as described in *Experimental procedures*. The H775-3 Δ *tnp* strain in which RS1 was deleted could now produce vanchrobactin (Table 1 and Fig. 2C, panel 1), demonstrating that the interruption of the *vabF* gene by the RS1 resulted in the cessation of the siderophore biosynthesis.

When we eliminated the RS1 sequence from the 775(pJM1) strain we obtained a positive bioassay for anguibactin but negative for vanchrobactin (Table 1) and a normal halo on CAS plates (Fig. 2C, panel 2). Therefore, it is possible that in *V. anguillarum*, anguibactin is more efficient in iron uptake than vanchrobactin or that the latter is actually not being produced. To discern between these two possibilities, we purified siderophore from supernatants of 775(pJM1) Δ *tnp* and RV22 as described by Soengas and colleagues (2006). After the first purification steps we identified fractions that were positive for vanchrobactin in specific bioassays. One of these fractions was further analysed by high-performance liquid chromatography (HPLC). The retention time of the peak of this fraction (R_f 9.01) was similar to vanchrobactin from the RV22 strain (R_f 9.04) (Fig. 4A). Figure 5 shows an HPLC with the profiles of anguibactin and vanchrobactin when they are run together. The six major peaks detected in this analysis were isolated and their biological activities tested in specific bioassays. As shown in Table 2, peak I and peak V were, respectively, identified as vanchrobactin and anguibactin by using specific bioassays for these siderophores. The remaining peaks II, III, IV and VI were negative in the bioassays (Table 2) while addition of ferric ammonium citrate (FAC) was used as a control for bacterial growth.

By using mass spectrometry (MS), we determined the nominal masses (m/z) of the purified compounds from RV22 and 775(pJM1) Δ *tnp* showing siderophore activities in bioassays and retention times of 9.04 and 9.01 respectively. In both cases the full electrospray ionization-mass spectra showed a major peak at m/z 398 [$M + H$]⁺ (data not shown), which corresponds to the molecular weight of vanchrobactin. To confirm the identity of these two purified compounds a MS spectrum with 30% collision energy was also run. Figure 4B show the presence in both samples of the parental peak at m/z 398 and the different fragments originated. These results clearly indicate that the putative vanchrobactin siderophore purified from the 775(pJM1) Δ *tnp* strain is identical to the vanchrobactin produced by RV22 as in both cases identical fragmentation products were obtained. From these results it is likely that the presence of anguibactin inhibits the activity of vanchrobactin without affecting its production. It is thus possible that anguibactin is a stronger iron chelator than vanchrobactin. To test this hypothesis we determined the iron chelating activity of purified anguibactin and vanchrobactin by the liquid CAS assay using EDTA as a standard (Fig. 6). The results indicate that the siderophore units of vanchrobactin are lower than EDTA while the siderophore units of anguibactin are higher than those of EDTA at concentrations below 50 μ M. Thus, in the case of the strain *V. anguillarum* 775(pJM1) Δ *tnp* which produces both siderophores, anguibactin must be the only active siderophore because it can scavenge iron more efficiently than vanchrobactin under conditions of iron limitation.

Conjugation of the pJM1 plasmid into the plasmidless strains 96F of serotype O1 and RV22 of serotype O2 reveals same behaviour as in strain 775(pJM1) Δ tnp in the competition for iron between anguibactin and vanchrobactin

In the previous section we showed that the 775(pJM1) Δ tnp strain produces both anguibactin and vanchrobactin and that the latter cannot operate in the presence of anguibactin. To determine whether the 96F and RV22 strains could also produce these two different siderophores, we designed an approach to mobilize for the first time the 65 kb pJM1 plasmid from *V. anguillarum* 775(pJM1) to each of these two strains as indicated in *Experimental procedures*. To determine whether the pJM1-mediated anguibactin outer membrane receptor FatA was produced, we performed Western blotting using a polyclonal FatA antiserum. As shown in Fig. 7, the FatA protein was detected in outer membrane preparations from all the strains that harboured the pJM1 plasmid, indicating that this protein is indeed produced only when the pJM1 plasmid is present. Interestingly, FatA is stable in the outer membrane in not only O1 but also the O2 strain. Bioassay results (Table 1) show that only anguibactin but not vanchrobactin can be detected in the strains harbouring pJM1, either the 96F(pJM1) or RV22(pJM1). For one of them, 96F(pJM1), we also performed high-performance thin-layer chromatography (HPTLC) and bioassays to assess whether this strain can produce both siderophores. Crude extracts obtained after the XAD-7 resin purification step from supernatants of cultures of the *V. anguillarum* serotype O1 strains 775(pJM1), 775(pJM1) Δ tnp, 96F and 96F(pJM1), as well as from the serotype O2 strain RV22 were fractionated by HPTLC (data not shown). The spots showing positive reaction after the FeCl₃ spray were extracted from the plate and their biological activity was tested by bioassays (Table 3). We detected two positive bands from 96F(pJM1) after FeCl₃ spray, while as expected, the 96F strain without pJM1 only showed one positive band. The two bands from 96F(pJM1) showed retardations factors of R_f of 0.31 and 0.12, respectively, concomitant with the presence of anguibactin [supernatant from 775(pJM1) showed R_f of 0.34] and vanchrobactin (supernatant from RV22 showed R_f of 0.15) whereas 96F without pJM1 showed a band with an R_f of 0.11. Those two bands were also found in supernatants of 775(pJM1) Δ tnp, that also showed two bands one with an R_f 0.32 that corresponds to anguibactin and another with an R_f 0.12 that is vanchrobactin. The bands showing higher R_f from 96F(pJM1), 775(pJM1) and 775(pJM1) Δ tnp showed positive anguibactin bioassays using the indicator strain CC9-16 Δ fvt (this strain has a deletion of the vanchrobactin receptor in the chromosome but still can take up anguibactin due to the presence of the plasmid-mediated anguibactin receptor FatA) whereas the bands showing lower R_f from 96F(pJM1), 96F and RV22 showed positive vanchrobactin results in the specific bio-assay using the indicator strain CC9-8. These results together with the fact that the chromosome cluster of 775(pJM1) Δ tnp and 96F are over 99% homology at the nucleotide level show that the siderophore produced by 96F is likely vanchrobactin. The behaviour of vanchrobactin in the presence of anguibactin is similar to the situation found with the 775(pJM1) Δ tnp: the two siderophores can be detected in the HPTLC but only anguibactin is identified in bioassays.

Is the RS1 element found inserted in the vabF vanchrobactin biosynthesis gene in the 775(pJM1) strain also present in the chromosome and/or plasmid of other *V. anguillarum* strains?

We wished to know whether the RS1 insertion element was a common feature in *V. anguillarum* strains. Thus, we determined the approximate number of copies of the RS1 sequence in several *V. anguillarum* strains by Southern blot hybridization. As shown in Fig. 8A, 775(pJM1) harbouring the pJM1 plasmid has at least six copies of RS1 in the total DNA (lane 1). Of those six bands, the band with the lowest molecular weight denoted with the open arrow is in the *vabF* gene as this band disappeared in 775(pJM1) Δ tnp in which the transposon was removed (lane 2). Another band, third largest band (denoted with the solid

arrow) in 775(pJM1), must be originated in the pJM1 plasmid because it is not present in the cured strain H775-3 (lane 3). Therefore, the four bands detected in H775-3 Δ tnp (lane 4) show that *V. anguillarum* 775(pJM1) must have at least four copies of RS1 in the chromosomal DNA in addition to that in *vabF*. Both plasmidless strains, 96F (lane 5) and RV22 (lane 7), do not harbour the RS1 transposon while 96F(pJM1) (lane 6) and RV22(pJM1) (lane 8) show a single band corresponding to the pJM1 plasmid DNA. All strains harbouring pJM1 from various geographical sources that we examined showed at least three bands indicating the presence of RS1 integrated in the chromosome of these strains (lanes 9–11). Furthermore, we performed PCR to determine the integrity of the *vabF* gene using primers complementary to the flanking regions of the transposon located within the 775(pJM1) *vabF* gene position. As shown in Fig. 8B the PCR fragment from 775(pJM1) (lane 1) is larger as compared with that from 775(pJM1) Δ tnp (lane 2) as the IS element was deleted in the latter strain. Polymerase chain reaction fragments from strains H775-3 (lane 3) and all strains harbouring pJM1-like plasmids (lanes 9–11) showed similar fragment sizes as those from 775(pJM1), whereas the PCR fragments from strains H775-3 Δ tnp, from the plasmidless strains 96F (lane 5) and RV22 (lane 7), and from plasmidless strains harbouring pJM1, 96F(pJM1) (lane 6) and RV22(pJM1) (lane 8) are similar to those from 775(pJM1) Δ tnp.

Discussion

At least two different siderophore-mediated iron uptake systems have been described in the bacterium *V. anguillarum*. One encoded on the pJM1 plasmid, mediated by the siderophore anguibactin, has only been identified in certain serotype O1 strains (Crosa, 1980). The other, of a chromosomal nature and mediated by the siderophore vanchrobactin, can be found and was characterized in the serotype O2 strain of *V. anguillarum* (Toranzo *et al.*, 1983; Lemos *et al.*, 1988; Wiik *et al.*, 1989; Conchas *et al.*, 1991; Soengas *et al.*, 2006). Notably, in natural isolates there has not been any report on the coexistence of these two siderophore systems. Recent work in the strain 775(pJM1) in our laboratory (Alice *et al.*, 2005) demonstrated the existence of functionally redundant chromosomal homo-logues of the pJM1-encoded anguibactin biosynthesis genes, especially those involved in DHBA biosynthesis. Therefore, one of our goals was to understand the relevance of these coexisting chromosome and plasmid functionally redundant genes.

We demonstrated in this work that the redundant functional chromosomal genes are part of a larger gene cluster encoding determinants for biosynthesis and uptake of vanchrobactin in the *V. anguillarum* 775(pJM1).

The chromosomal vanchrobactin biosynthesis cluster in the anguibactin-producing 775(pJM1) strain shows 99% identity at the nucleotide level to that found in another O1 strain, the plasmidless 96F, except that a transposition insertion of an IS element identical to the RS1 sequence encoded on the pJM1 plasmid occurred into the *vabF* chromosomal gene essential for vanchrobactin biosynthesis. We have also identified this insertion in all other O1 strains examined that carry a pJM1-like plasmid. Bioassays carried out with the RS1-deleted 775(pJM1) strain identified only anguibactin; however, HPLC fractionation and MS analysis revealed that in addition to anguibactin, vanchrobactin was also being synthesized. It is possible that these apparently contradictory results are due to anguibactin being a stronger iron chelator than vanchrobactin, and thus in the presence of anguibactin iron is sequestered from vanchrobactin, and cannot compete for the iron in the medium. This is likely due to the fact that anguibactin possesses the stronger chelating groups, hydroxamate and thiazolin in addition to catechol, whereas vanchrobactin only possesses the weaker chelating catechol moiety. From the results presented in this work as well as the preliminary results shown in our earlier publication (Alice *et al.*, 2005), we hypothesize that an ancestral

iron uptake system in *V. anguillarum* was mediated by vanchrobactin and that hegemony of the anguibactin-mediated system could have occurred by first acquisition of a pJM1-type plasmid by an O1 serotype strain harbouring the vanchrobactin cluster. As anguibactin is more efficient in binding iron than vanchrobactin, synthesis of the latter siderophore was no longer essential as it could not be used in the presence of anguibactin. Suppression of its production by insertional inactivation of *vabF* by an RS1 element, normally part of the pJM1 plasmid, resulted in the classical 775(pJM1) type strain.

Experimental procedures

Bacterial strains and media

Strains and vectors used in the present study are listed in Table 4. *Vibrio anguillarum* was incubated at 25°C into Trypticase soy broth or agar supplemented with 1% NaCl. *E. coli* strains were incubated at 37°C into LB broth or onto LB plates. *Vibrio anguillarum* was incubated in CM9 minimal medium containing 0.2% Casamino Acids, and 5% NaCl. Ethylenediamine-di-(*o*-hydroxyphenyl acetic acid (EDDA) (10–20 µM) was added to obtain iron limiting conditions. *Escherichia coli* strains were grown in CM9 with 0.01% thiamine and 0.004% tryptophan. Antibiotics were used at concentrations of 100 µg rifampicin ml⁻¹, 10 µg chloramphenicol ml⁻¹ and 50 µg kanamycin ml⁻¹ for *V. anguillarum*, and 50 µg ampicillin ml⁻¹, 30 µg chloramphenicol ml⁻¹, 50 µg kanamycin ml⁻¹ for *E. coli*.

Primers

Primers used in this study are listed on Table 5.

General methods

Plasmid and chromosomal DNA extraction, DNA ligations, transformations, colony hybridization, PCR, inverse PCR, cosmid cloning and agarose gel electrophoresis were carried out using standard methods (Sambrook and Russell, 2001).

DNA sequencing

DNA sequencing was conducted by the OHSU Molecular Microbiology and Immunology Research Core Facility, using an automated fluorescence sequencer model 377 (Applied Biosystems). Sequences were compared with reported sequences or sequences obtained using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST>) of the National Center for Biotechnology Information (Altschul *et al.*, 1997).

Detection of siderophores

The siderophores anguibactin and vanchrobactin were detected by the CAS assay (Schwyn and Neilands, 1987) or bioassays as described previously (Tolmasky *et al.*, 1988) using indicator strains as shown in Table 4.

Construction of the plasmid angD insertion mutant

A kanamycin cassette insertion mutant was constructed in the pJM1 plasmid-encoded *angD* gene. The kanamycin cassette was obtained by PCR using primers Km ClaI F and R, and the plasmid pKD4 (Datsenko and Wanner, 2000) as a template. The amplified fragment was cloned into pCR2.1, and the insert DNA was purified after digestion with ClaI. To amplify the *angD* gene, the primers *angD* knockout insert F and R were used. Polymerase chain reaction was performed as follows: denaturing for 5 min at 95°C followed by 30 cycles of amplification (30 s at 95°C, 30 s at 55°C, 2 min at 72°C) and extension of 7 min at 72°C using pJM1 DNA as a template and Taq DNA polymerase (Invitrogen) for the PCR reaction.

These conditions were used throughout this study. The amplified fragment was cloned into pCR2.1, and the insert DNA was purified after double digestion with XhoI and SpeI. The purified fragment was then cloned into pBluescript II digested with the same restriction endonucleases. The resulting plasmid was digested with ClaI and the kanamycin cassette was ligated into this site. This plasmid was double digested with XhoI and SpeI, and the purified fragment cloned into the suicide vector pDM4 resulting in plasmid pDMangD::Km which was then conjugated into *V. anguillarum* 775(pJM1). Plasmid cointegrates were selected on TSAS plates containing chloramphenicol and kanamycin. To counter-select for *E. coli* S17-1 λ pir overnight cultures of the exconjugant strains in TSBS plus kanamycin and rifampicin were streaked on TSAS plates with kanamycin, rifampicin and 15% sucrose. Insertion mutants of the *angD* gene were further confirmed by assessing chloramphenicol sensitivity, kanamycin resistance and the results of colony PCR, using the *angD* knockout insert F and R primers.

Construction of in-frame deletion mutants

Deletion mutants were constructed as described previously (Milton *et al.*, 1996) using the SOE PCR (Senanayake and Brian, 1995) with primers *vabD* mut-F, -R, -1 and -2 for the *vabD* mutant; *vabF* -F, -R, -1 and -2 for the *vabF* mutant, and *vabF* recov mut-F, -R, -1 and -2 for the RS1 deletion mutant. Polymerase chain reaction products were cloned into pCR2.1 and then subcloned into pDM4. Conjugation and selection of recombinants were conducted as described for the *angD* insertion mutant but without using kanamycin.

Complementations

Wild-type clones were constructed amplifying the corresponding wild-type genes by PCR. The reactions were conducted using primers *angD* comp SphI F and EcoRI R for *angD* complementation, primers *vabD* comp BamHI F and EcoRI R for *vabD* complementation. Amplified DNA fragments were cloned into the pCR2.1 cloning vector according to the manufacturer's protocol (Invitrogen), and nucleotide sequences were checked by DNA sequencing. Plasmid DNA was isolated and digested with restriction enzymes, ligated into pMMB208 (Morales *et al.*, 1991), transformed into the *E. coli* S17-1 λ pir strain, containing mobilization functions and conjugated into *V. anguillarum* 775(pJM1) as described above.

Conjugation of pJM1 into 96F or RV22

Polymerase chain reaction amplified fragments using primers *angD* integ F and R were cloned into pCR2.1. Plasmid DNA was then double digested with XhoI and SacI, and ligated at the same restriction sites of pDM4. After ligation, the ligated plasmid was transformed into *E. coli* S17-1 λ pir. This plasmid was then conjugated into *V. anguillarum* 775(pJM1), and colonies harbouring pJM1 integrated with pDM4 into the *angD* gene were selected on TSAS plate supplemented with chloramphenicol and rifampicin. The resulting plasmid was then purified and transformed in *E. coli* S17-1 λ pir which was then conjugated into either of the *V. anguillarum* 96F or RV22 strains. Chloramphenicol-resistant colonies were selected, and after an overnight culture, those cells that had lost pDM4 were screened by plating on TSBA plate with 15% sucrose. The carriage of pJM1 in the *V. anguillarum* strains was assessed by PCR amplification and subsequent restriction enzyme digestion of the plasmid DNA.

Siderophore isolation and analysis

All siderophore purification procedures were carried out using acid-washed glassware (with 0.1 N HCl) and the temperature during the purification procedure never exceeded 25°C. For the purification and characterization of vanchrombactin we used supernatants of the following *V. anguillarum* O1 strains 775(pJM1), 775(pJM1) Δ *tnp*, H775-3 Δ *tnp*, 96F, 96F(pJM1) and

the *V. anguillarum* O2 strain RV22 grown for 48 h in CM9 minimal medium. The cell-free supernatants were incubated with XAD7 resin (Sigma) in batch overnight at 25°C with shaking (200 r.p.m.). The resin was then washed twice with 1 volume of water and the crude siderophore extract eluted with 1 volume of methanol 100% and 1 volume of methanol 50% v/v. After this elution, samples were concentrated under vacuum. Crude extracts were then suspended in 1/10 of the volume in methanol 100% and the insoluble matter was separated by centrifugation. Siderophore activity was followed by separating the fractions onto HPTLC plates (Kieselgel 60 F_{254} 0.1 mm, Merck 1.11764) using, as a mobile phase, butanol : acetic acid : water (4:1:5 v/v/v, upper phase) and then developed using 1% (w/v) FeCl_3 in 0.5 N HCl. After spraying with FeCl_3 the positive spots were scrapped from the HPTLC and the samples were extracted twice with methanol 50% v/v to further testing by bioassays as described above. Pure anguibactin isolated from the *V. anguillarum* 775(pJM1) strain was used as control (Actis *et al.*, 1986). The presence of DHBA in the extracts analysed by HPTLC was detected by UV fluorescence and by using pure DHBA (Sigma) as standard. The retardation factor (R_f) for each component is depicted in Table 3. The identification of the catechol moiety in the samples was performed by the Arnow reaction using DHBA as an authentic standard (Arnow, 1937).

For the characterization of the 775(pJM1) Δtnp and the RV22 chromosomally-encoded siderophore vanchrobactin, cell-free supernatants were treated according with Soengas and colleagues (2006). The last purification step was performed using HPLC (Beckman Gold HPLC System) monitoring at 254 nm using a C18 reverse-phase HPLC column (YMC, AQ-312, 150 $\times$ 6 mm internal diameter). The composition of the mobile phases was: phase A, water 0.05% (v/v) trifluoroacetic acid (TFA) and phase B, acetonitrile 0.05% (v/v) TFA. Samples were eluted at a flow rate of 1 ml min⁻¹ using a linear gradient of mobile phase B from 0 to 50% over 10 min. Nominal masses of the ion species (m/z) detected during vanchrobactin purification from both strains were determined by MS, carried out at the BioAnalytical Shared Resource Core Facility at OHSU using a Thermo Electron LCQ Advantage ion trap mass spectrometer, equipped with an electrospray ionization source. The full electrospray ionization-mass spectra and those with collision energy (CE) 30% (MS2) were both acquired in a positive mode using as mobile phase 1:1 methanol : water for the full scan and 0.1% formic acid for MS2.

CAS iron-chelation assay

The CAS assay solutions were prepared according to Schwyn and Neilands (1987). The samples and reference absorbance measured at 630 nm, A_s and A_r , respectively, were determined after incubation at room temperature. EDTA pH 7.0 at different concentrations was used as positive control for chelating activity. Percentage of siderophore units were calculated as $[A_r - A_s/A_r] \times 100$.

Western blotting

All cultures were grown until exponential phase (OD_{600} 0.5–0.8) in CM9 media. After centrifugation, the supernatants were discarded and cell pellets were resuspended into 10 mM Tris buffer (pH 7.6) including proteinase inhibitor cocktail tablets (complete, EDTA-free) (Roche). Following centrifugation and after sonication, supernatants were transferred into new tubes and outer membrane fractions were isolated as described previously using 1.5% N-Lauryl sarcosine (Sigma) (Cobessi *et al.*, 2004). The concentration of outer membrane proteins was calculated by using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Bovine serum albumin was used as a standard. Five micrograms of outer membrane protein was separated for SDS-PAGE using 12% SDS-PAGE (Criterion™-XT Precast gel, Bio-Rad Laboratories) using XT-MOPS buffer (Bio-Rad Laboratories), and transferred for 4 h at 400 mA into Immobilon™-P membranes 0.45

µm (Millipore Corporation) using a Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad Laboratories). After blocking with 10% BSA in TBST (Tris 20 mM pH 7.6, NaCl 0.3 M and 0.05% Tween-20) overnight at room temperature, the membranes were washed with TBST for 10 min. Anti-FatA polyclonal antibody was diluted 1/15000 into TBST with 5% BSA. The membranes were incubated with this antibody for 1 h at room temperature and then washed three times with TBST (each time 10 min), and incubated for 1 h with goat anti-rabbit IgG [H + L, horseradish peroxidase conjugated (HRP) (Pierce Biotechnology)] as secondary antibody diluted 1/20 000 in TBST 5% BSA. The membrane was washed with TBST three times (each 10 min), and incubated for 5 min with the Immobilon™ Western Chemiluminiscent HRP substrate (Millipore Corporation) and developed using Kodak BioMax MR film (Kodak).

Southern hybridization

Primers, *orf21* F and R were constructed, and PCR was performed by using chromosomal DNA from H775-3 as a template. Amplified fragments were ligated into pCR2.1 and nucleotide sequences of inserted DNA were checked by sequencing. Plasmid DNA was digested with EcoRI and the insert was purified and used as a specific probe. Three micrograms of total DNAs from various *V. anguillarum* strains were digested with ClaI, electrophoresized in 1.5% agarose gels and transferred for 16 h at 300 mA into Hybond N⁺ membranes (GE Healthcare) using a Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad Laboratories) using as transfer buffer TBE (90 mM Tris-Borate, 2 mM EDTA). Labelling of the probe and hybridizations were conducted by using the AlkPhos Direct Labelling and Detection System with CDP-Star according to the manufacture's instruction (GE Healthcare).

Identification of the RS1 transposon in the *vabF* chromosomal gene by PCR

Primers *vabF tnp* check F and R complementary to the flanking regions of the transposon located within the 775(pJM1) *vabF* gene were used for the PCR reaction with various *V. anguillarum* chromosomal DNAs as templates.

Nucleotide sequence accession numbers

The nucleotide sequences described above were deposited in GenBank under the Accession No. AY738106 and DQ779149.

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Fig. 1. Structure of two types of siderophore produced by *V. anguillarum*

A. Anguibactin from O1 strain 775(pJM1) (Jalal *et al.*, 1989).

B. Vanchrobactin from O2 strain RV22 (Soengas *et al.*, 2006).

Fig. 2. Two functional homologues of the phosphopantetheinyl transferase genes are active in the biosynthesis of both anguibactin and vanchrobactin. CAS assays: (A) anguibactin; (B) vanchrobactin; (C) anguibactin and vanchrobactin

A. Lanes 1, 775(pJM1)-pMMB; 2, H775-3-pMMB; 3, 775(pJM1) Δ *angD*-pMMB; 4, 775(pJM1) Δ *vabD*-pMMB; 5, 775(pJM1) Δ *angD* Δ *vabD*-pMMB; 6, 775(pJM1) Δ *angD* Δ *vabD*-pMMB*angD*; 7, 775(pJM1) Δ *angD* Δ *vabD*-pMMB*vabD*.

B. Lane 1, 96F-pMMB; 2, 96F Δ *vabD*-pMMB; 3, 96F Δ *vabD*-pMMB*angD*; 4, 96F Δ *vabD*-pMMB*vabD*.

C. Lane 1, H775-3 Δ *tnp*; 2, 775(pJM1) Δ *tnp*. Three microlitres of overnight culture of each strains was spotted on CAS plate with the addition of 1 mM IPTG. Plates were incubated for 1 day at 25°C and for 3 days at 16°C.

Fig. 3. Comparison of the chromosomal loci involved in siderophore biosynthesis and transport in *V. anguillarum* strains 775(pJM1) and 96F

A. The slanted line box represents the transposon RS1 inserted in the *vabF* gene. Black thick vertical lines correspond to the sequence of CTTGATG, which are the duplicated sequences at site of insertion.

B. Detail of the transposon in *vabF*. The black arrow symbolizes the ORF21 corresponding to the transposase.

Fig. 4. Confirmation of vanchrobactin biosynthesis in the 775(pJM1) *tnp* strain

A. High-performance liquid chromatography (HPLC) chromatograms of vanchrobactin purified from 775(pJM1) *tnp* (left) and RV22 (right) strains.

B. Positive mode electrospray ionization-mass spectra of the purified siderophores shown in (A) using 30% collision energy. The nominal masses (m/z) of the parental ion species and different fragmentation products are indicated in the spectra.

Fig. 5. High-performance liquid chromatography (HPLC) profile of vanchrobactin and anguibactin. The fractions tested in the bioassays shown in Table 2 are indicated in roman numerals.

Fig. 6.

Relative affinities for Fe(III) of purified anguibactin and vanchrobactin. Chelating capability of purified anguibactin and vanchrobactin at various concentrations (10, 20 and 30 μ M) measured as percentage of siderophore units as indicated in *Experimental procedures*. Bars: white, vanchrobactin; black, EDTA; and grey, anguibactin. Values represent mean $\pm$ SD.

Fig. 7. Detection of the FatA receptor protein in the outer membrane fraction of several strains of *V. anguillarum* by Western blot using an anti-FatA serum. Outer membrane proteins were prepared from 775(pJM1), 96F, 96F(pJM1), RV22 and RV22(pJM1).

Fig. 8. Identification of RS1 in chromosome and/or pJM1-like plasmid DNA

A. Southern blot hybridization of *Cla*I-digested DNA to assess the relative copy number of RS1 in several *V. anguillarum* strains. Only the transposase gene *orf21* from RS1 was used as a probe to avoid problems with the bordering repeated sequences. Lane 1, 775(pJM1); 2, 775(pJM1) Δ *tnp*; 3, H775-3; 4, H775-3 Δ *tnp*; 5, 96F; 6, 96F(pJM1); 7, RV22; 8, RV22(pJM1); 9, 531A; 10, PT77022; 11, R61. The solid arrowhead points to the location of *orf21* in pJM1 of 775(pJM1), while the open arrowhead indicates the position of *orf21* in *vabF* of 775(pJM1).

B. Polymerase chain reaction to assess the site of interruption of *vabF* by RS1. Lane M, molecular marker; 1, 775(pJM1); 2, 775(pJM1) Δ *tnp*; 3, H775-3; 4, H775-3 Δ *tnp*; 5, 96F; 6, 96F(pJM1); 7, RV22; 8, RV22(pJM1); 9, 531A; 10, PT77022; 11, R61.

Table 1

Detection of siderophores by bioassays.

Strain tested	Indicator strains (siderophore utilization)	
	CC9-8 (ang ⁻ , van ⁺)	CC9-16Δ <i>fvxA</i> (ang ⁺ , van ⁻)
775(pJM1)	–	+
775(pJM1)Δ <i>tmp</i>	–	+
H775-3Δ <i>tmp</i>	+	–
96F	+	–
96F(pJM1)	–	+
RV22	+	–
RV22(pJM1)	–	+
FAC	+	+

EDDA (20 μM) was added into CM9 agar media with the *V. anguillarum* indicator strains. The tested strains were streaked on the plates. After 24 h incubation, the existence of halos around bacteria was observed. ang, anguibactin utilization; van, vanchrobactin utilization; +, growth; –, no growth; FAC, ferric ammonium citrate.

Table 2

Bioassays of the HPLC fractions shown in Fig. 5.

Fraction number	Retention time	Indicator strain (siderophore utilization)	
		CC9-8 (ang ⁻ , van ⁺)	CC9-16Δ <i>fvtA</i> (ang ⁺ , van ⁻)
I	8.90	+	-
II	9.42	-	-
III	9.75	-	-
IV	10.36	-	-
V	11.14	-	+
VI	11.35	-	-
FAC	NA	+	+

ang, anguibactin utilization; van, vanchrobactin utilization; +, growth; -, no growth; NA, not applicable; FAC, ferric ammonium citrate.

Table 3

Bioassays and Arnow test of positive spots extracted from the HPTLC.

Strain used for HPTLC	Retardation factor	Indicator strain (siderophore utilization)		Arnow
		CC9-8 (ang ⁻ , van ⁺)	CC9-16Δ <i>fvtA</i> (ang ⁺ , van ⁻)	
96F(pJM1)	R _f 0.31/R _f 0.12	-/+	+/-	+/+
96F	R _f 0.11	+	-	+
775(pJM1)	R _f 0.34 (anguibactin)	-	+	
RV22	R _f 0.15 (vanchrobactin)	+	-	+
775(pJM1)Δ <i>tnp</i>	R _f 0.32/R _f 0.12	-/+	+/-	+/+
Silica	Negative control	-	-	-
DHBA	ND	ND	ND	+

ang, anguibactin utilization; van, vanchrobactin utilization; +, growth, -, no growth; ND, not determined.

Table 4

Bacterial strains and plasmid used in this study.

Strain or plasmid	Phenotype	Reference
<i>Vibrio anguillarum</i> strains		
775(pJM1)	Wild type, Washington (serotype O1, pJM1)	Laboratory stock
775(pJM1)-pMMB	775(pJM1) harbouring pMMB208	This study
96F	Wild type, Maryland (serotype O1, plasmidless)	Lemos <i>et al.</i> (1988)
96F-pMMB	96F harbouring pMMB208	This study
RV22	Wild type, Spain (serotype O2, plasmidless)	Lemos <i>et al.</i> (1988)
531A	Wild type, Maine (serotype O1, pJM1-like)	Laboratory stock
PT77022	Wild type, Japan (serotype O1, pJM1-like)	Laboratory stock
R61	Wild type, Spain (serotype O1, pJM1-like)	Laboratory stock
H775-3	Plasmidless derivative of 775(pJM1)	Laboratory stock
H775-3-pMMB	H775-3 harbouring pMMB208	This study
H775-3 Δ tnp	H775-3 deleted transposon from <i>vabF</i>	This study
775(pJM1) Δ tnp	775(pJM1) deleted transposon from <i>vabF</i>	This study
775(pJM1) <i>angD</i> ::Km	775(pJM1) <i>angD</i> mutant	This study
775(pJM1) <i>angD</i> ::Km-pMMB	775(pJM1) <i>angD</i> ::Km harbouring pMMB208	This study
775(pJM1) Δ vabD	775(pJM1) <i>vabD</i> mutant	This study
775(pJM1) Δ vabD-pMMB	775(pJM1) Δ vabD harbouring pMMB208	This study
775(pJM1) <i>angD</i> ::Km Δ vabD	775(pJM1) <i>angD</i> and <i>vabD</i> double mutant	This study
775(pJM1) <i>angD</i> ::Km Δ vabD-pMMB	775(pJM1) <i>angD</i> ::Km Δ vabD harbouring pMMB208	This study
775(pJM1) <i>angD</i> ::Km Δ vabD-pMMBangD	775(pJM1) <i>angD</i> ::Km Δ vabD complemented with the <i>angD</i> gene	This study
775(pJM1) <i>angD</i> ::Km Δ vabD-pMMBvabD	775(pJM1) <i>angD</i> ::Km Δ vabD complemented with the <i>vabD</i> gene	This study
96F Δ vabD	96F <i>vabD</i> mutant	This study
96F Δ vabD-pMMB	96F Δ vabD harbouring pMMB208	This study
96F Δ vabD-pMMBvabD	96F Δ vabD complemented with the <i>vabD</i>	This study
96F(pJM1)	96F conjugated with pJM1	This study
RV22(pJM1)	RV22 conjugated with pJM1	This study
CC9-8	Anguibactin deficient, anguibactin transport system deficient	Walter <i>et al.</i> (1983)
CC9-16	Anguibactin deficient, anguibactin transport system proficient	Walter <i>et al.</i> (1983)
CC9-16 Δ fvzA	Anguibactin deficient, anguibactin transport system proficient, vanchrobactin transport system deficient	This study
<i>Escherichia coli</i> strains		
TOP10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>ara</i> Δ I39 Δ (<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^r) <i>endA1</i> <i>nupG</i>	Invitrogen
S17-1 λ pir	<i>thi pro hsdR hsdM</i> ⁺ <i>recA</i> RP4-2-Tc::Mu-Km::Tn7 λ pir	Simon <i>et al.</i> (1983)
Plasmids		
pCR2.1	T-vector, Km ^r Amp ^r	Invitrogen
pBluescript II	Cloning vector, Amp ^r	Stratagene
pDM4	Suicide plasmid <i>sacB</i> gene, R6K origin, Cm ^r	Milton <i>et al.</i> (1996)
pMMB208	<i>incQ</i> <i>lacIq</i> Ω <i>cat</i> Ω <i>Ptac</i> Cm ^r	Morales <i>et al.</i> (1991)
pDMangD::Km	pDM4 harbouring <i>angD</i> ::Km	This study

Strain or plasmid	Phenotype	Reference
pDM Δ <i>vabD</i>	pDM4 harbouring Δ <i>vabD</i>	This study
pDM <i>vabF</i> Δ <i>tnp</i>	pDM4 harbouring <i>vabF</i> Δ <i>tnp</i>	This study
pMMB <i>angD</i>	pMMB208 harbouring <i>angD</i>	This study
pMMB <i>vabD</i>	pMMB208 harbouring <i>vabD</i>	This study
pDM4 <i>angD</i>	Part of the <i>angD</i> gene cloned in pDM4	This study

Table 5

Primers used in this study.

Primer name	Sequence 5'-3'
Km ClaI F	<u>ATCGATGTGTAGGCTGGAGCTGCTTCGAAG</u> (underline, ClaI site)
Km ClaI R	<u>ATCGATCATATGAATATCCTCCTTAGTTCC</u> (underline, ClaI site)
<i>angD</i> knockout insert F	GCATGCATGTTTGTATCTCTCTCTTTCTAAGCAG
<i>angD</i> knockout insert R	GTTGTATGTTTTTCATGATTTGCAAAGAG
<i>vabD</i> mut-F	TCTTCTTAAAACGGACATCAGCG
<i>vabD</i> mut-R	AACCCATGAAGGCGAGTGAC
<i>vabD</i> mut-1	GCTTGACTTAAATAACACCAAGTCGTTTGAATGATGAGCATGC
<i>vabD</i> mut-2	GCATGCTCATCATTCCAAACGACTTGGTGTTATTTAAGTCAAGC
<i>vabF</i> recover mut-F	CATGGCTTATTCATCGATAAGC
<i>vabF</i> recover mut-R	TGACATTCTACGTCCTGAC
<i>vabF</i> recover mut-1	CAATGACAGGCGATGCATCTGCATCAAGCGAAGCGCTGATC
<i>vabF</i> recover mut-2	GATCAGCGCTTCGCTTGATGCAGATGCATCGCCTGTCATTG
<i>angD</i> comp SphI F	<u>GCATGCGTCTGATTTCCTCTTGTTATTCG</u> (underline, SphI site)
<i>angD</i> comp EcoRI R	<u>GAATTCCTAGCTCGATCTAATAAACTGGTTG</u> (underline, EcoRI site)
<i>vabD</i> comp BamHI F	<u>GGATCCACTCGATAGGTAATAAGGCATG</u> (underline, BamHI site)
<i>vabD</i> comp EcoRI R	<u>GAATTCCTATTTTATGTGTCGAAAAATGGG</u> (underline, EcoRI site)
<i>vabF</i> <i>tmp</i> check F	CAAATGCTCGATAGTGATTAATG
<i>vabF</i> <i>tmp</i> check R	CCAAATCACCAGTGCGGTACAT
<i>orf21</i> F	ATGCTCGCGTGCGGCACCAA
<i>orf21</i> R	CTACGCAGCTACGCTTAATC
<i>angD</i> integ F	ATTGAGGTGGGGTATGTTTCG
<i>angD</i> integ R	GAACAATGGTGAGGCTGTTG