A gene cluster involved in the biosynthesis of vanchrobactin, a chromosome-encoded siderophore produced by *Vibrio anguillarum*

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Vibrio anguillarum serotype O2 strains produce a catechol siderophore named vanchrobactin, which has been identified as *N*-[*N'*-(2,3-dihydroxybenzoyl)-arginyl]-serine. This work describes a chromosomal region that harbours the genetic determinants necessary for the biosynthesis of vanchrobactin. The authors have identified the genes involved in 2,3-dihydroxybenzoic acid (DHBA) biosynthesis (*vabA*, *vabB* and *vabC*) and activation (*vabE*), and a gene (*vabF*) encoding a non-ribosomal peptide synthetase, which is putatively involved in the assembly of the siderophore components. Also described are the identification and characterization of genes encoding a putative vanchrobactin exporter (*vabS*) and a siderophore esterase (*vabH*). In-frame deletion mutants in *vabA*, *vabB*, *vabC*, *vabE*, *vabF* and *vabH* were impaired for growth under conditions of iron limitation, and the analysis of culture supernatants by chrome azurol-S and cross-feeding assays showed almost no production of siderophores in any of the *vabABCEF* mutants. In addition, deletion mutations of *vabA*, *vabB* and *vabC* abolished production of DHBA, as assessed by chemical and biological analyses. Complementation of each mutant with the corresponding gene provided *in trans* confirmed the involvement of this gene cluster in the biosynthesis of DHBA and vanchrobactin in *V. anguillarum* strain RV22. Based on chemical and genetic data, and on published models for other catechol siderophores, a model for vanchrobactin biosynthesis is proposed.

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INTRODUCTION

Bacterial pathogens have developed a series of mechanisms to obtain iron from the host. One of the main strategies is the synthesis and secretion of siderophores that can remove iron from the host iron-binding proteins, and internalize it to the cell (for a recent review, see Wandersman & Delepelaire, 2004).

The fish pathogen *Vibrio anguillarum* is the causative agent of vibriosis, a highly fatal haemorrhagic septicaemia affecting marine and freshwater fish species throughout the world (Toranzo & Barja, 1990). Up to 23 serotypes, designated O1–O23, are recognized in the European system (Pedersen *et al.*, 1999), with O1 and O2 being the main ones implicated in disease (Toranzo *et al.*, 1997). Although the virulence mechanisms of *V. anguillarum* are not fully understood, it is known that the ability to scavenge iron

through the utilization of siderophores contributes significantly to virulence (Wolf & Crosa, 1986). Two clearly different siderophore-mediated systems have been described in *V. anguillarum* strains belonging to serotypes O1 and O2 (Conchas *et al.*, 1991). In most pathogenic strains of serotype O1, the system is mediated by the 65 kb plasmid pJM1, which harbours genes for the biosynthetic machinery, and utilization of the catecholate-type siderophore anguibactin (Stork *et al.*, 2002). Recently, it has been reported that the biosynthesis of anguibactin needs chromosomally encoded enzymes, in addition to those encoded by pJM1 genes (Alice *et al.*, 2005).

On the other hand, all serotype O2 strains tested so far, and some plasmidless serotype O1 strains, produce a siderophore that is encoded by hitherto uncharacterized chromosomal genes, and is not related to the pJM1 plasmid-mediated system (Stork *et al.*, 2002). The biological activity deduced from cross-feeding assays pointed initially to a functionality related to the *Escherichia coli* 2,3-dihydroxybenzoic acid (DHBA)-containing siderophore enterobactin (Lemos *et al.*, 1988; Conchas *et al.*, 1991). This chromosome-encoded system produces a siderophore called vanchrobactin, which has been recently isolated from the serotype O2 strain RV22, and chemically characterized as *N*-[*N'*-(2,3-dihydroxybenzoyl)-arginyl]-serine (Soengas *et al.*, 2006).

Abbreviations: A-domain, adenylation domain; ArCP, aryl carrier protein; CAS, chrome azurol-S; DBS, 2,3-dihydroxybenzoyl-serine; DHBA, 2,3-dihydroxybenzoic acid; EDDA, ethylenediamine-di-(*o*-hydroxyphenyl-acetic acid); MFS, major facilitator superfamily; NRPS, non-ribosomal peptide synthetase; PCP, peptidyl carrier protein; TE, thioesterase.

The GenBank/EMBL/DBJ accession number for the nucleotide sequence reported in this paper is AM168450.

The genes necessary for DHBA biosynthesis are also present and expressed in the chromosome of the anguibactin-producing *V. anguillarum* O1 strain 775 (Chen *et al.*, 1994); in addition, a chromosomal gene cluster involved in DHBA synthesis and activation has been identified in this strain (Alice *et al.*, 2005). Most of these genes have functional homologues in the pJM1 plasmid, except *angA_{ch}* (encoding a putative 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase), which is essential for DHBA and anguibactin synthesis, and for which only the chromosomal copy seems to be functional.

Since vanchrobactin is a catechol siderophore (Lemos *et al.*, 1988; Soengas *et al.*, 2006), we hypothesized that homologues of the genes described in the chromosome of serotype O1 strains, and some additional genes, could be responsible for biosynthesis of DHBA and the siderophore produced by plasmidless *V. anguillarum* strains. Thus, the aim of this study was the identification and characterization of the genes involved in the biosynthesis of vanchrobactin.

METHODS

Bacterial strains, plasmids and media. Strains and plasmids used, as well as those derived from this study, are listed in Table 1. *V. anguillarum* serotype O2 strain RV22 was routinely grown at 25 °C in tryptic soy agar and broth (Difco), supplemented with 1 %

NaCl (TSA-1 and TSB-1, respectively). *E. coli* strains were routinely grown at 37 °C in Luria-Bertani (LB) medium (Pronadisa) supplemented with the appropriate antibiotics. All strains were stored frozen at -80 °C in LB broth containing 20 % (v/v) glycerol. Ampicillin sodium salt and kanamycin (Sigma-Aldrich) stock solutions in ultrapure water (100 mg ml⁻¹ and 50 mg ml⁻¹, respectively) were filter-sterilized, and stored at -20 °C.

DNA manipulations. Standard DNA manipulations were carried out as described by Sambrook & Russell (2001). Total genomic DNA from *V. anguillarum* was purified with the Easy-DNA kit (Invitrogen). Plasmid DNA purification, and extraction of DNA from agarose gels, were carried out using kits from Qiagen. DNA-probe labelling and Southern blot analysis were performed with the ECL DNA-labelling and detection system (Amersham Biosciences). PCR reactions were carried out in a T-Gradient Thermal Cycler (Biometra), with *Taq* polymerase BioTaq (Bioline). For inverse PCR, chromosomal DNA was digested with a single restriction enzyme, self-ligated, and used as template in a PCR reaction with the Expand Long Template PCR System (Roche Diagnostics), using suitable primers.

DNA sequences were determined with the dideoxy-chain-termination method on either plasmid or PCR products using GenomeLab DTCS-Quick Start Kit with a CEQ 8000 DNA Sequencer (Beckman Coulter). Sequences were examined and assembled using BioEdit version 7.0.4.1 (Hall, 1999). The European Bioinformatics Institute and the NCBI services were used to consult the DNA and protein sequence databases with FASTA3 and BLAST algorithms. The protein families database of alignments and hidden Markov models (Pfam), of the Sanger Institute,

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
<i>V. anguillarum</i>		
RV22	Serotype O2 wild-type strain isolated from diseased turbot (Spain)	Lemos <i>et al.</i> (1988)
MB3	RV22 <i>vabE</i> -defective mutant	This study
MB6	RV22 <i>vabS</i> -defective mutant	This study
MB10	RV22 <i>vabC</i> -defective mutant	This study
MB11	RV22 <i>vabB</i> -defective mutant	This study
MB12	RV22 <i>vabA</i> -defective mutant	This study
MB14	RV22 <i>vabF</i> -defective mutant	This study
MB19	RV22 <i>vabH</i> -defective mutant	This study
<i>E. coli</i>		
DH5α	<i>supE4 ΔlacU169 (Φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Laboratory stock
S17-1-λpir	<i>tpr smr recA thi pro hsdR-M+RP4::2-Tc::Mu Km::Tn7 λpir</i>	Herrero <i>et al.</i> (1990)
<i>S. enterica</i> serovar <i>Typhimurium</i>		
<i>enb-1</i>	Enterobactin biosynthesis mutant; can use enterobactin as an iron source	Pollack <i>et al.</i> (1970)
<i>enb-7</i>	Enterobactin biosynthesis mutant; can use enterobactin and DHBA as iron sources	Pollack <i>et al.</i> (1970)
Plasmids		
pGEM-T Easy	PCR cloning vector, Ap ^R	Promega
pWKS30	Low-copy-number cloning vector, Ap ^R	Wang & Kushner (1991)
pNidKan	Suicide plasmid, pCVD442 derivative, <i>pir</i> , Km ^R	Mouriño <i>et al.</i> (2004)
pHRP309	Low-copy-number vector, Gm ^R	Parales & Harwood (1993)
pMB13	<i>vabA</i> gene and promoter cloned in pHRP309, Gm ^R	This study
pMB14	<i>vabC</i> gene and promoter cloned in pHRP309, Gm ^R	This study
pMB15	<i>vabF</i> gene and promoter cloned in pHRP309, Gm ^R	This study
pMB16	<i>vabH</i> gene and promoter cloned in pHRP309, Gm ^R	This study

was utilized to predict the protein domain organization (Bateman *et al.*, 2004). The prediction of transmembrane helices in proteins was carried out using program HMMTOP version 2.0 (Tusnady & Simon, 2001).

Cloning and sequencing of a gene cluster involved in vanchrobactin biosynthesis. In order to clone the genes responsible for the biosynthesis of vanchrobactin, in the chromosome of serotype O2 strain RV22, we used PCR to look for the presence of a homologue of the *angE* gene originally cloned from the chromosome of a pJM1-like plasmid-bearing *V. anguillarum* O1 strain 90-11-287 (Holmstrom & Gram, 2003). As a result, we found an ORF that showed 97% identity to the nucleotide sequence of this *angE* gene. An internal fragment of the *angE* homologue amplified in RV22 was labelled, and used as a probe in Southern hybridizations to identify suitable restriction sites surrounding this locus. This information was used to carry out an inverse PCR reaction, and a 7 kb fragment was obtained. Successive inverse PCRs and primer walking allowed us to extend sequencing in two directions to a fragment of approximately 18 kb.

Mutant construction. Gene deletions in *V. anguillarum* RV22 were constructed by using PCR amplifications of two fragments of each gene and flanking regions, which when ligated together would result in an in-frame (nonpolar) deletion (Fig. 1). The oligonucleotides used to amplify the upstream and downstream ends of each gene are listed in Table 2. Suitable restriction sites were added to the oligonucleotides. Construction of in-frame deletions of *vabA*, *vabC*, *vabE*, *vabB*, *vabS* and *vabF* was accomplished in several steps. The PCR-amplified 3'-end gene fragments were ligated into pWKS30, and resulting plasmids were cut with suitable enzymes, and ligated to the 5' ends of the PCR fragments of each gene. Each deleted allele cloned in pWKS30 was digested with *NotI* and *ApaI*, and ligated into the suicide vector pNidKan (Mouriño *et al.*, 2004). As a pCVD442 derivative, pNidKan contains R6K *ori*, requiring the *pir* gene product for replication, and the *sacB* gene, conferring sucrose sensitivity. The resulting plasmids were mated from *E. coli* S17-1- λ pir into *V. anguillarum* RV22, and transformants, with the plasmid integrated in the chromosome by homologous recombination, were selected on agar medium containing 50 μ g kanamycin ml⁻¹ (resistance conferred by pNidKan) and 50 μ g ampicillin ml⁻¹ (specific antibiotic to select *V. anguillarum* RV22). A second recombination event was obtained by selecting for sucrose (10%) resistance. This process led to the production of the following mutant strains of *V. anguillarum*: MB12 (Δ *vabA*), MB10 (Δ *vabC*), MB3 (Δ *vabE*), MB11 (Δ *vabB*), MB6 (Δ *vabS*), MB14 (Δ *vabF*) and MB19 (Δ *vabH*). Deletion of the parental gene was verified in all cases by Southern blot hybridization. DNA sequencing of the region involved in the deletion was carried out to ensure that all constructs were in-frame.

Complementation of *V. anguillarum* mutants. The vanchrobactin biosynthetic genes *vabA*, *vabC* and *vabF*, and also the *vabH* gene, along with their own promoter sequences, were PCR amplified from *V. anguillarum* RV22 chromosome using specific primers. Suitable restriction sites were added to the oligonucleotides (Table 2). The *vabF* gene was amplified from strain MB19 (Δ *vabH*) using proFesV_*XbaI* and VacF_*XbaI* primers (Table 2) to incorporate the promoter upstream of *vabH*. The amplified DNA fragments were cloned into the pHRP309 vector (Parales & Harwood, 1993) at the appropriate restriction site, and subsequently transformed into the *E. coli* S17-1- λ pir strain. The resulting plasmids (Table 1) were mated from *E. coli* S17-1- λ pir into the corresponding *V. anguillarum* mutant, and transformants were selected on agar medium containing 10 μ g gentamicin ml⁻¹ (resistance conferred by pHRP309) and 50 μ g ampicillin ml⁻¹ (to select for *V. anguillarum*). The pHRP309 recombinant plasmids were isolated from the *V. anguillarum* mutants, and digested with appropriate restriction enzymes to verify the presence of the inserts.

Growth under iron-limited conditions, and test for siderophore production. To test the ability of *V. anguillarum* defective mutants to grow under iron-limited conditions, overnight cultures in LB of the parental and mutant strains were adjusted to an OD₆₀₀ of 0.5, and diluted 1:15 in CM9 minimal medium (Lemos *et al.*, 1988) containing the iron chelator ethylenediamine-di-(*o*-hydroxyphenylacetic acid) (EDDA) at 5 μ M. Cultures were incubated at 25 °C, with shaking at 150 r.p.m., and growth (OD₆₀₀) and siderophore production were measured after 22 h incubation. Siderophore production was measured using the chrome azurol-S (CAS) liquid assay (Schwyn & Neilands, 1987). In addition, the Arnow test (Arnow, 1937) was used for spectrophotometric measurement of catechol in each sample. A non-inoculated CM9 sample, containing EDDA at an appropriate concentration, was used as negative control and spectrophotometric blank for the CAS liquid assay.

Cross-feeding assays. The biological activities of the supernatants produced by the mutant strains were determined by performing cross-feeding experiments. We tested the ability of culture supernatants from *V. anguillarum* and *Salmonella enterica* serovar Typhimurium (Table 1) siderophore-defective mutants to cross-feed different indicator strains that were defective in DHBA and/or siderophore synthesis. It is known that vanchrobactin can substitute the function of enterobactin to supply iron to enterobactin-deficient mutants (Lemos *et al.*, 1988; Conchas *et al.*, 1991). Two mutants of *S. enterica* serovar Typhimurium LT2 (Pollack *et al.*, 1970), which are deficient in two different biosynthetic steps of enterobactin production, were used to detect vanchrobactin and DHBA production: (i) strain *enb-7*, which can use exogenous enterobactin, vanchrobactin or

Fig. 1. Physical map of (a) the vanchrobactin biosynthesis genes of *V. anguillarum* serotype O2 strain RV22; and (b) their respective homologues, which have been described as part of the chromosomal anguibactin biosynthesis gene cluster of serotype O1 strain 775 (Alice *et al.*, 2005). The ruler at the top indicates nucleotide positions. ORFs are depicted as arrows, which indicate the direction of transcription, and the numbers displayed vertically show the start and end points of each gene. Deleted regions within each gene are shown as hatched boxes.

Table 2. Oligonucleotide primers used for mutant construction, and number of amino acids deleted

Oligonucleotide	Sequence (5'→3')*	Total no. of aa/deleted aa
<i>ΔvabE</i> mutant		541/419
<i>ΔVacE</i> 1	GCGGATCCGTGCAAAACCAACAGCGCTC	
<i>ΔVacE</i> 2	GCAGAGATAGAATTCAGCAC	
<i>ΔVacE</i> 3	GCGAATTCCACATCCATTTCGTGATGA	
<i>ΔVacE</i> 4	GCAAGCTTCAATATCCCCGTGGTGTACA	
<i>ΔvabB</i> mutant		286/219
<i>ΔVacB</i> 1	CGGGATCCGGTCTTAATCTACGCGACGC	
<i>ΔVacB</i> 2	GCGAATTCATCGTGAATGAGCAGTACCG	
<i>ΔVacB</i> 3	GCGAATTCGAATCATGGCGCAAGATGGG	
<i>ΔVacB</i> 4	GCAAGCTTTGTTACTACTCAAGGACGTC	
<i>ΔvabA</i> mutant		258/204
<i>ΔVacA</i> 1	CGTCTAGATGATCCACAACAGATTACGC	
<i>ΔVacA</i> 2	GCGGATCCTACCGCATAACCAATCCCCT	
<i>ΔVacA</i> 3	GCGGATCCGAAGACATTGCTGGGGTTGT	
<i>ΔVacA</i> 4	GCGAATTCAGGTGTACCAACAGCGACTG	
<i>ΔvabC</i> mutant		393/337
<i>ΔVacC</i> 1	GCTCTAGACAACCCAGCAATGTCTTCG	
<i>ΔVacC</i> 2	GCGGATCCCATGGTGTGTGAGGCGAAG	
<i>ΔVacC</i> 3	GCGGATCCACAGGGGCAAAATGAGCAC	
<i>ΔVacC</i> 4	GCAAGCTTCTCAATTAAACGAAAGCGG	
<i>ΔvabF</i> mutant		2835/2777
<i>ΔVacF</i> 1	CGTCTAGATGGTATTGATGCTCGATGGC	
<i>ΔVacF</i> 2	CGGGATCCCCAAATACCTTGCTGTGTGC	
<i>ΔVacF</i> 3	GCGGATCCCATGAAGATATCTTGGCCCC	
<i>ΔVacF</i> 4	GCGAATTCTAAGATGAGAGCAAACCACC	
<i>ΔvabS</i> mutant		437/313
<i>ΔExpV</i> 1	GCGGATCCCTTTCATCCACTCCAACAGC	
<i>ΔExpV</i> 2	GCGAATTCCTAGCACCAACGATGTCGAT	
<i>ΔExpV</i> 3	GCGAATTCATCCAATGCCATGATAAGGCC	
<i>ΔExpV</i> 4	GCAAGCTTCAACAGCAGGGT	
<i>ΔvabH</i> mutant		401/232
<i>ΔFesV</i> 1	GCTCTAGAAGAGGATGCCAATCAGTGTG	
<i>ΔFesV</i> 2	GCGGATCCTGAATCGAAACCGAGCCACT	
<i>ΔFesV</i> 3	GCGGATCCGGTCACGATCGACTCTGTTG	
<i>ΔFesV</i> 4	GCAAGCTTGACTGATGGCATTACGCTG	
Complementations		Position
proVacA_ <i>Sma</i> I	GCCCCGGGCATCCAATAGCTGATTAGCC	2010
VacA- <i>Sma</i> I	GCCCCGGGAGCAGCAATATTGTCAGCCC	910
proVacC_ <i>Kpn</i> I	GCGGTACCATACCGCATAACCAATCCCC	1720
VacC_ <i>Kpn</i> I	GCGGTACCGCTGAATTGCTCAGGCCAAG	3180
proFesV_ <i>Xba</i> I	GCTCTAGAATCCATGGGCTTCATGACCT	17800
FesV_ <i>Xba</i> I	CGTCTAGACAAGCATGAATGAACAGCGC	16050
VacF_ <i>Xba</i> I	GCTCTAGAGATACGCACTGGTTTCGTCG	7460

*Restriction sites used are underlined.

DHBA to overcome iron-limiting conditions; and (ii) *enb-1*, which can use only enterobactin or vanchrobactin for iron supply (Conchas *et al.*, 1991; Lemos *et al.*, 1988).

We designed two cross-feeding assays: (i) *V. anguillarum* MB10 *vabC*-defective mutant was used as an indicator strain to test MB3(*ΔvabE*), MB14(*ΔvabF*), and *S. enterica* *enb-7* and *enb-1*; (ii) *enb-7* and *enb-1* were used as indicators to test MB10(*ΔvabC*), MB3(*ΔvabE*) and MB14(*ΔvabF*) mutants. In each assay, strain RV22 was used as positive

control. Each indicator strain was inoculated into CM9 minimal medium containing a concentration of 2,2'-dipyridyl that was higher than the MIC for that strain. Strains to be tested were cultured on LB agar, and cells were harvested with a sterile loop, and placed on the surface of the indicator-strain plates. The results were scored as positive when tester cells promoted the growth of indicator strains.

Phylogenetic tree construction. A total of 410 adenylation-domain (A-domain) sequences were retrieved from UniProt/

TrEMBL/SWISS-PROT databases (Apweiler *et al.*, 2004; Boeckmann *et al.*, 2003). These sequences were aligned with the A-domain sequences predicted for *V. anguillarum* RV22 VabE (A-domain) and VabF (A1- and A2-domains) proteins, using CLUSTAL W software (Thompson *et al.*, 1994). The resulting alignments were visually inspected, and, when appropriate, manually adjusted. The phylogenetic tree was built using the amino acid sequence that contained the A-domain substrate-binding pocket (positions 190–331) (Stachelhaus *et al.*, 1999). Phylogenetic trees relied on the modules SEQBOOT, PROTDIST, NEIGHBOR and CONSENSE of the PHYLIP package (Felsenstein, 1996). Bootstrap analysis was used to assess statistical support for relationships via branch and bound analysis of 1000 replicated datasets. The tree obtained with the initial 413-sequence multiple alignment was further used to select 56 aa sequences that grouped together with our working sequences. These were employed to construct a second phylogenetic tree, with the same methodology described above.

RESULTS AND DISCUSSION

Our previous studies have indicated that plasmidless serotype O2 strains produce a catechol siderophore whose synthesis is mediated by chromosomal genes (Conchas *et al.*, 1991; Lemos *et al.*, 1988), in contrast with the well-known pJM1-mediated system described for *V. anguillarum* serotype O1 strain 775 (Crosa & Walsh, 2002; Stork *et al.*, 2002). Sequence analysis of an 18 kb chromosomal fragment from *V. anguillarum* strain RV22 (see Methods) revealed the presence of eight ORFs, named *dahP*, *vabA*, *vabC*, *vabE*, *vabB*, *vabS*, *vabF* and *vabH* (Fig. 1), whose deduced protein products showed significant similarity to proteins involved in siderophore-mediated iron utilization in other bacterial species (Table 3). While DahP and VabB showed 69 and 97 % identity, respectively, to partially sequenced DahP and AngB_{ch} serotype O1 chromosomally encoded homologues (Alice *et al.*, 2005), the VabA, VabC, VabE proteins showed an identity higher than 97 % to AngA_{ch}, AngC_{ch} and AngE_{ch}, respectively, which are encoded in the chromosome of *V. anguillarum* serotype O1 775 strain, and are involved in the synthesis of DHBA (Alice *et al.*, 2005) (Table 3). The same gene arrangement was observed for the RV22 and 775 homologues, and a putative Fur box predicted within the *vabA*–*vabC* intergenic region was found to be 100 % identical to that reported between *angA_{ch}* and *angC_{ch}* (Fig. 1). The three remaining genes of this cluster, *vabS*, *vabF* and *vabH*, are transcribed in the same DNA strand as *vabB* (with a putative Fur box identified upstream of *vabH*); homologues of these genes have not been described to date in *V. anguillarum* (Table 3), and they are analysed in detail below.

vabABC are involved in DHBA synthesis in *V. anguillarum* RV22

To test the role of these genes in DHBA and vanchrobactin production, individual in-frame deletions of *vabABC* were constructed. When the mutants were cultured in iron-sufficient conditions (CM9 plus 10 µM ferric sulfate), no significant differences in growth levels were observed with respect to the parental strain RV22. However, under iron-restricted conditions (CM9 plus 5 µM EDDA),

MB12(Δ*vabA*), MB11(Δ*vabB*) and MB10(Δ*vabC*) mutants were severely affected in their ability to grow, in comparison with the parental strain (Fig. 2a).

These cultures were also analysed by the CAS assay to detect the production of siderophores in supernatants. The supernatants from the three null mutants cultured under iron-restricted conditions showed that no significant siderophore levels were produced, with the absorbance in the CAS assay being between 5- and 10-fold lower than in the parental strain (Fig. 2b). In addition, analysis of the supernatants by the Arnov test revealed that DHBA production was completely abolished in MB12 and MB11 mutants, and reduced about sixfold in the MB10 mutant (Fig. 2b). The cross-feeding assays showed that *V. anguillarum* RV22 wild-type and *S. enterica* *enb-1*, but not *enb-7*, promoted the growth of the *vabC* mutant (Table 4). Similarly, supernatants of the *vabC* mutant failed to complement *enb-7*. Together, these results indicate that *vabA*, *vabB* and *vabC* are most probably involved in the synthesis of DHBA in *V. anguillarum* RV22, and that they appear to be necessary for siderophore production, and growth under iron-limited conditions.

Although the *vabC*-null mutant produced residual amounts of DHBA, it has been reported that MenF, which is involved in the biosynthesis of menaquinones, synthesizes isochorismate, resulting in the production of low amounts of enterobactin in an *E. coli* *entC* mutant (Buss *et al.*, 2001). An analogous situation was reported for *V. anguillarum* serotype O1, when an *angC* mutant was found to produce residual amounts of DHBA, but this production was abolished in an *angC*–*menF* double mutant (Alice *et al.*, 2005).

When MB10 and MB12 mutants were complemented with plasmids pMB13 (containing the *vabC* gene) and pMB14 (containing the *vabA* gene), respectively, the growth levels under iron-restricted conditions were restored to the wild-type level (Fig. 2a), indicating that *vabC* and *vabA* are essential for iron uptake. In addition, the complemented mutants showed levels of DHBA and siderophore production comparable with those observed in the parental strain (Fig. 2b), confirming that *vabC* and *vabA* are essential for DHBA and siderophore biosynthesis in strain RV22.

Characterization of *vabE* and *vabF*, and prediction of vanchrobactin components

In catechol-type siderophores, DHBA must be activated by a 2,3-dihydroxybenzoate-AMP ligase. The remaining siderophore components are further selected and activated by non-ribosomal peptide synthetases (NRPSs), and assembled with DHBA to form the final siderophore molecule (Earhart, 1996; Walsh *et al.*, 1990). The RV22 VabE sequence is nearly identical to AngE_{ch}, which is encoded by the chromosome of the *V. anguillarum* O1 strain 775. AngE_{ch} is a 2,3-dihydroxybenzoate-AMP ligase involved in the synthesis of the siderophore anguibactin, and it is responsible for the selection and activation of DHBA (Alice

Table 3. Proteins with homology to products of the *V. anguillarum* *vab* gene cluster

<i>V. anguillarum</i> protein (no. of aa, kDa)	Accession no.	Identity (%)	Similarity (%)
DAHP synthase (305, 33·07)			
DAHP synthase (<i>V. anguillarum</i>)	AAO92362	69	84
DAHP synthase (<i>Vibrio vulnificus</i>)	AAO07756	58	72
DAHP synthase (<i>E. coli</i>)	AAN79680	55	70
DAHP synthase (<i>Shigella boydii</i>)	ZP_00697486	57	69
VabA (258, 28·32)			
AngA _{ch} (<i>V. anguillarum</i>)	AAW66007	98	98
ViuA (<i>V. vulnificus</i>)	AAO07757	60	77
VibA (<i>Vibrio cholerae</i>)	AAF93939	54	72
EntA (<i>Bacillus cereus</i>)	AAP09266	45	61
VabC (397, 43·39)			
AngC _{ch} (<i>V. anguillarum</i>)	AAW66008	98	99
AngC (<i>V. anguillarum</i>)	AAO92360	70	84
ViuC (<i>V. vulnificus</i>)	AAO07758	66	79
VibC (<i>V. cholerae</i>)	AAC45925	62	80
VabE (541, 60·31)			
AngE _{ch} (<i>V. anguillarum</i>)	AAW66009	97	98
AngE (<i>V. anguillarum</i>)	AAO92359	62	76
VibE (<i>V. cholerae</i>)	ZP_00750963	62	77
ViuE (<i>V. vulnificus</i>)	NP_937357	60	72
VabB (286, 32·73)			
AngB _{ch} (<i>V. anguillarum</i>)	AAW66010	97	98
AngB (<i>V. anguillarum</i>)	AAO92358	64	78
VibB (<i>V. cholerae</i>)	AAG00563	59	76
ViuB (<i>V. vulnificus</i>)	NP_937360	53	71
DhbB (<i>A. baumannii</i>)	AAN28931	50	67
VabS (437, 46·27)			
Probable MSF protein (<i>C. violaceum</i>)	AAQ59909	59	74
P45 (<i>A. baumannii</i>)	AAN28937	48	69
P43 (<i>E. coli</i>)	CAA40706	48	70
MSF protein (<i>S. boydii</i>)	ZP_00697587	48	70
VabF (2835, 316·52)			
CbsF (<i>C. violaceum</i>)	AAQ59905	44	60
NRPS (<i>Yersinia frederiksenii</i>)	ZP_00829712	51*	68*
Peptide synthetase (<i>B. cereus</i>)	AAP09419	27	47
EntF (<i>E. coli</i>)	AAN79148	40*	55*
NRPS (<i>Thermobifida fusca</i>)	AAZ55900	32	48
DhbF-like (<i>A. baumannii</i>)	AAN28936	42*	60*
VabH (429, 45·55)			
Enterochelin esterase (<i>Y. frederiksenii</i>)	ZP_00829710	38	54
Enterochelin esterase (<i>Y. bercovieri</i>)	ZP_00823579	38	53
Enterochelin esterase (<i>C. violaceum</i>)	NP_901901	35	54
Enterochelin esterase (<i>Y. intermedia</i>)	ZP_00833719	39	53
Fes (<i>E. coli</i>)	AAN79146	37	53
Enterochelin esterase (<i>S. enterica</i>)	NP_806023	35	52

*Identity/similarity restricted to C-terminal module.

et al., 2005). On the other hand, VabF (with a predicted size of 2835 residues) is homologous to members of the NRPS family (Table 3) that are involved in the final assembly of siderophores, for instance *E. coli* EntF (Rusnak *et al.*, 1991), *Chromobacterium violaceum* CbsF, and *Acinetobacter*

baumannii Dhbf-like proteins (Dorsey *et al.*, 2003) (Table 3).

To ascertain the role of VabE and VabF in vanchrobactin synthesis in RV22, we constructed in-frame deletion mutants

Fig. 2. (a) Growth (OD₆₀₀) after 22 h incubation of *V. anguillarum* RV22, and MB3($\Delta vabE$), MB6($\Delta vabS$), MB10($\Delta vabC$), MB11($\Delta vabB$), MB12($\Delta vabA$), MB14($\Delta vabF$) and MB19($\Delta vabH$) mutants, in CM9 minimal medium supplemented with $Fe_2(SO_4)_3$ (10 μM ; hatched bars), or the iron chelator EDDA (5 μM ; grey bars). (b) DHBA production measured by the Arnow test (A_{510} ; hatched bars), and siderophore production measured by the CAS supernatant assay (A_{630} ; grey bars). In the CAS assay, lower values indicate higher siderophore production. Results are expressed as mean values ($\pm$ SD) from three independent experiments.

of *vabE* and *vabF*. As shown in Fig. 2a, both mutants were significantly impaired for growth under iron-limited conditions, and siderophore levels in supernatants were less than 20 % of those detected in the parental strain, indicating that these mutations result in a severe reduction of siderophore synthesis (Fig. 2b). These results are comparable with those obtained for *vabA*, *vabB* and *vabC* mutants. However, analysis of the supernatants using the Arnow test showed a significant production of DHBA (about 50 % of that produced by the wild-type) in *vabE*- and *vabF*-null mutants

(Fig. 2b). DHBA production in *vabE* and *vabF* mutants was verified in cross-feeding bioassays using the enterobactin-deficient *S. enterica enb-7* as the indicator strain. Results showed that both *vabE* and *vabF* mutants could promote the growth of *enb-7*, but not *enb-1*, indicating that *vabE* and *vabF* mutants produce DHBA, but are unable to synthesize vanchrobactin (Table 4). Complementation of *vabF* mutation with the parental *vabF* gene, provided *in trans* (plasmid pMB15), restored the wild-type phenotype in growth ability and siderophore production (Fig. 2a, b).

Table 4. Results of cross-feeding experiments with various indicator strains

Strain	Ability to cross-feed		
	MB10 (<i>ΔvabC</i>)	<i>enb-1</i>	<i>enb-7</i>
RV22	+	+	+
<i>enb-1</i>	+	NA	+
<i>enb-7</i>	–	–	NA
MB10 (<i>ΔvabC</i>)	NA	–	–
MB3 (<i>ΔvabE</i>)	+	–	+
MB14 (<i>ΔvabF</i>)	+	–	+

NA, Not applicable.

These results are in agreement with the hypothesis that VabE and VabF are two NRPSs involved in the final steps of vanchrobactin assembly. Since these steps are subsequent to the production of DHBA, this molecule would be produced normally in *vabE* and *vabF* mutants, but it could not be used for the synthesis of vanchrobactin.

Since VabE and VabF are homologous to well-described NRPSs involved in siderophore biosynthesis, and these enzymes exhibit a modular organization (Crosa & Walsh, 2002), we subjected the amino acid sequences of VabE and VabF to a domain-prediction analysis. Results indicated that VabE contains a unique A-domain, and that VabB consists of a single aryl-carrier-protein (ArCP) domain. Therefore, VabE and VabB could constitute an initiation module with the A–ArCP structure. VabF shows a seven-domain structure, organized into two modules: the N-terminal module (VabF module 1) follows the C–A–PCP (condensation–adenylation–peptidyl-carrier-protein) domain structure, whereas the C-terminal module (VabF module 2) contains a C–A–PCP–TE (condensation–adenylation–peptidyl-carrier-protein–thioesterase) domain structure. In addition, we were able to locate the conserved core motifs of the catalytic domains (Marahiel *et al.*, 1997) in each of the VabE, VabB and VabF predicted domains (data not shown).

In order to elucidate VabE and VabF substrate specificity, we applied the non-ribosomal code (Challis *et al.*, 2000; Stachelhaus *et al.*, 1999). A sequence alignment of the predicted adenylation domains of VabE and VabF with the well-characterized adenylation domain of the *Bacillus subtilis* gramicidin synthetase A (GrsA) was carried out to locate the 10 residue positions that are crucial for substrate binding. Results of this analysis suggest that either DHBA or salicylate, and serine, are activated by VabE and VabF, respectively. However, it seems clear that DHBA should be the actual substrate activated by the initiation module (VabE–VabB), not only because the closest VabE homologues are 2,3-dihydroxybenzoate-AMP ligases (Table 3), but also because vanchrobactin is a catechol-type siderophore (Lemos *et al.*, 1988; Soengas *et al.*, 2006).

Since the sequence alignments were not sufficient to establish VabF substrate-specificity prediction, we constructed a neighbour-joining tree from a multiple sequence alignment of A-domain substrate-binding pocket sequences for substrate determination (Di Vincenzo *et al.*, 2005). The results confirmed our previous specificity predictions for VabE and VabF. The VabE A1-domain was clustered with a bootstrap value of 66 % into a branch containing DHBA and salicylate-specific A-domains (Fig. 3). This cluster could be further divided in two subgroups: one including some well-characterized proteins with DHBA-AMP ligase activity, such as *E. coli* EntE or *Vibrio cholerae* VibE; and a second subgroup including proteins with salicyl-AMP ligase activity. The VabE A-domain was clustered with a bootstrap value of 68 % into the subgroup of 2,3-dihydroxybenzoate-AMP ligases. Interestingly, the VabF A1-domain clustered together with a bootstrap value of 95 % into the branch that grouped A-domains responsible for the selection and adenylation of amino acids with a positively charged side chain. The VabF A2-domain clustered, with a bootstrap value of 96 %, with serine-specific A-domains, such as those of the *E. coli* and *Shigella sonnei* EntF proteins, and the A-1 and A-2 domains of *Pseudomonas syringae* SyrE (Fig. 3).

According to these analyses, vanchrobactin should be composed of at least the following residues: (i) DHBA; (ii) serine; and (iii) an amino acid from the group ornithine/lysine/arginine, since these amino acids belong to the same specificity-pocket family (Rausch *et al.*, 2005). The recent elucidation of vanchrobactin chemical structure (Soengas *et al.*, 2006) showed that this siderophore is a N-[N'-(2,3-dihydroxybenzoyl)-arginy]-serine (Fig. 4c). Thus, the structural chemical data are in complete agreement with the component prediction based on the amino acid sequences of VabE and VabF. These data, together with the mutagenesis analysis described above, strongly suggest that VabE and VabF are the key enzymes involved in vanchrobactin biosynthesis.

VabS as a putative siderophore exporter

VabS is a predicted protein of 437 aa that shows homology to the 12-transmembrane-segment members of the major facilitator superfamily (MFS) class of proton-motive-force-dependent membrane efflux pumps (Paulsen *et al.*, 1996), such as the *E. coli* EntS (P43), a protein encoded within the *ent* operon that participates in the secretion of enterobactin (Furrer *et al.*, 2002). The VabS protein shares conserved domains with members of the MFS family, especially the amino acid motifs that define it (Pao *et al.*, 1998).

The prediction of transmembrane structure of *V. anguillarum* VabS using the TMHMM program showed the characteristic 12-transmembrane segments, equivalent to those described for the *E. coli* enterobactin siderophore exporter P43 (EntS) (Furrer *et al.*, 2002), with the exception of transmembrane domain 11. An analogous situation was reported for the *A. baumannii* P45 protein (Dorsey *et al.*, 2003).

Fig. 3. Neighbour-joining tree of 56 adenylation domains based on the alignment of the substrate-binding pockets. Proteins are named according to their SWISS-PROT entry names (<http://www.ebi.uniprot.org>). The relevant closest homologues to *V. anguillarum* VabF A1- and A2-domains, and VabE A-domain, are also denoted, with species name and protein nomenclature. Substrate specificity is shown in parentheses.

In order to gain an insight into the role of *vabS* in vanchrobactin-mediated iron acquisition, we constructed a deletion mutant. The *vabS* mutant showed a reduction in its growth ability under iron-limited conditions (Fig. 2a). Interestingly, when the Arnow assay was carried out with *vabS* mutant supernatants, not only did it yield values similar to the RV22 wild-type during the first hours of culture (data not shown), but these values showed an increase of 50 % after 22 h, with respect to the wild-type (Fig. 2b). However, the levels of siderophore production in the *vabS* mutant, as determined by the CAS assay, did not show significant differences with respect to the wild-type (Fig. 2b). In this regard, it has been reported that the *E. coli* P43 mutant is unable to secrete enterobactin efficiently, its growth ability being supported by 2,3-dihydroxybenzoyl-serine (DBS), an enterobactin breakdown product that retains iron-chelating activity, and freely diffuses out of the cells (Furrer *et al.*, 2002; Hantke, 1990; Rabsch *et al.*, 2003). Something similar could

occur in *V. anguillarum* RV22, and a precursor or breakdown product of vanchrobactin could also act as a siderophore. These products could be detected by Arnow and CAS assays, although their efficiency in supplying iron to the cell would be inferior to the natural siderophore, and would probably result in lower growth levels (Fig. 2a). If the cells have not taken up enough iron, this would produce an increase in DHBA and/or other vanchrobactin precursors (Fig. 2b). These results, together with the comparative analysis of the protein sequence, suggest that VabS could be involved in the export of vanchrobactin.

***vabH* encodes a putative siderophore-degrading enzyme**

In most bacteria, once a ferric-siderophore complex enters the cytoplasm, iron is removed by a mechanism requiring an esterase activity. To test the potential role of VabH in

Fig. 4. Proposed model for vanchrobactin biosynthesis pathway. (a) Intermediates and enzymes involved in the DHBA biosynthesis. (b) Proposed model for vanchrobactin assembly, based on enterobactin and anguibactin biosynthesis machinery (Crosa & Walsh, 2002), and according to the domain specificity/arrangement of the NRPS VabE–VabB and VabF proteins determined in this work, and vanchrobactin chemical structure (c).

siderophore utilization, we constructed an in-frame deletion mutant of the *vabH* gene. As shown in Fig. 2a, the MB19($\Delta vabH$) null mutant was impaired for growth under iron-limited conditions, although both the CAS assay and the Arnow test showed a significant production of siderophore and DHBA, respectively (Fig. 2b). This could be evidence of the inability of the *vabH* mutant to utilize intracellular ferric–vanchrobactin. When the MB19 mutant strain was transformed with the pMB16 plasmid containing the *vabH* gene, the growth ability under iron-limited conditions was restored to wild-type levels. In addition, DHBA production and siderophore production in the complemented mutant gave values that were similar to those obtained with the parental strain (Fig. 2a).

In *E. coli*, the internalized ferric–enterobactin complex is degraded to ferrous iron (Fe^{2+}) and the linear trimer, dimer and monomer of DBS by means of a ferric–enterobactin esterase (Fes) (Brickman & McIntosh, 1992; Furrer *et al.*, 2002). The predicted translation product of *vabH* is a protein of 401 aa, with 53 % similarity to Fes (Table 3),

suggesting that this gene could be the *E. coli* fes orthologue. However, other Fes homologues do not play a role in iron release from the ferric–siderophore, but they do play a role in the degradation of the siderophore. This is the case for CbsH, a Fes homologue with a peptidase activity involved in degradation of chrysobactin, which is a siderophore produced by *Erwinia chrysanthemi* that has a structure closely similar to vanchrobactin (Rauscher *et al.*, 2002). Thus, although more work is needed to unravel the precise role of this gene in vanchrobactin utilization, *V. anguillarum* VabH could play a potential role in degrading vanchrobactin after its internalization.

A biosynthetic model for vanchrobactin

From the genetic data reported here, as well as the chemical structure and previous reports for enterobactin and anguibactin synthesis (Crosa & Walsh, 2002), we propose the model for the biosynthesis of vanchrobactin shown in Fig. 4.

DAHP synthase is not one of the enzymes directly connected to siderophore synthesis, but catechol-type siderophore biosynthesis itself starts with the conversion of chorismate into the DHBA, and DAHP is an intermediate in chorismate synthesis (Kloosterman *et al.*, 2003). According to the role assigned to the *E. coli* homologues (Earhart, 1996; Walsh *et al.*, 1990), the enzymes involved in DHBA biosynthesis from chorismate in *V. anguillarum* RV22 should be an isochorismate synthase (VabC), an isochorismatase (VabB), and a 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase (VabA) (Fig. 4a).

Once DHBA is synthesized, 2,3-dihydroxybenzoate-AMP ligase activity is necessary for DHBA activation (Crosa & Walsh, 2002). The protein encoded by *vabE* is the candidate for activating DHBA to its acyl-adenylate derivative in an ATP-dependent manner, and for transferring this activated species to holo-VabB. According to enterobactin and anguibactin synthesis models (Ehmann *et al.*, 2000), VabB could be involved in DHBA biosynthesis through its isochorismatase activity, and in further binding of DHBA through its ACP domain.

The adenylation domains of NRPS are responsible for the selection and incorporation of either one amino acid or one carboxy acid into the final product (Crosa & Walsh, 2002). According to the sequence analysis and chemical data mentioned above, the VabF A1-domain should activate an arginine residue, and, through its PCP domain, the 2,3-dihydroxybenzoyl-arginine precursor should be synthesized. The VabF A2-domain should then activate a serine residue, since this is the substrate predicted by the non-ribosomal code, and on the basis of the high homology showed by the VabF A2-domain and *E. coli* EntF (Fig. 3), which is a well-characterized ATP-dependent serine-activating enzyme (Reichert *et al.*, 1992). The TE domain is the last domain to play a role in the biosynthesis of a non-ribosomal peptide, releasing the final product (Lautru & Challis, 2004). The TE domain predicted in the VabF module 2 would have this role, releasing vanchrobactin into solution.

In conclusion, from the results described here and those reported by others (Alice *et al.*, 2005), it seems reasonable to speculate that the vanchrobactin biosynthesis cluster, or at least part of it, is shared by serotypes O1 and O2 of *V. anguillarum*, and that vanchrobactin is probably the functional siderophore in strains lacking the pJM1-type plasmid. A study of the distribution of the genes described here is required for other strains in order to clarify the evolution of plasmid- and chromosomal-mediated siderophore biosynthesis in *V. anguillarum*.

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