Biosynthetic and regulatory elements involved in the production of the siderophore vanchrobactin in *Vibrio anguillarum*

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Some *Vibrio anguillarum* strains produce a catechol-type siderophore named vanchrobactin, whose biosynthetic pathway has not been completely elucidated. In addition to the previously described genes *vabA*, *vabC*, *vabB*, *vabE*, *vabF*, *vabS* and *vabH*, in the present study we have identified the genes encoding a DAHP (3-deoxy-D-arabino-heptulosonate-7-phosphate) synthetase (*vabG*), a phosphopantetheinyl transferase (*vabD*), a LysR-family transcriptional regulator (*vabR*) and a putative siderophore receptor (*fvfA*). A deletion affecting *vabG* or *vabD* greatly reduced growth under iron-limiting conditions, whereas deletion of *vabR* did not have significant effects. Vanchrobactin production was abolished in the *vabD* mutant, whereas the *vabG* mutant retained a residual vanchrobactin production ability. Reverse transcriptase-mediated PCR indicated that this 11-gene cluster is organized into six iron-regulated transcriptional units. Transcriptional *lacZ* fusions demonstrated that the ferric uptake regulator (Fur) protein is the main iron-responsive regulator of these genes. Interestingly, the *vabG* gene was strongly iron-repressed, but Fur was not essential for this repression. In addition, the maximal expression from the *vabG* promoter was achieved only in the presence of an intact copy of *vabR*. Analysis of the β -galactosidase activities of a *fvfA::lacZ* fusion in a *vabB* mutant and in the presence of added vanchrobactin suggested that a ferric-vanchrobactin-dependent activator plays a positive regulatory role in transcription of the *fvfA-vabD* operon. This possibility is reinforced by the presence of a predicted AraC box upstream of *fvfA*. We propose that vanchrobactin biosynthesis is subjected to a complex regulatory circuitry aimed at adjusting vanchrobactin production for the maintenance of iron homeostasis in *V. anguillarum*.

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INTRODUCTION

Iron is an essential nutrient for most bacteria, acting as a cofactor of many enzymes with redox activity. Most of the iron in biological systems is chelated by high-affinity iron-binding proteins, and thus bacterial pathogens have developed efficient mechanisms to obtain iron from host tissues (Ratledge & Dover, 2000). One of the main strategies is the synthesis of siderophores, high-affinity iron chelators that are released into the extracellular environment, where they complex iron and deliver it to the bacterial cell (Crosa, 1989; Wandersman & Delepelaire,

2004). Many siderophores are small peptides synthesized by non-ribosomal peptide synthetases (NRPSs), which are multimodular enzymes that produce a peptide without an RNA template (Crosa & Walsh, 2002; Schwarzer *et al.*, 2003). Other bacterial siderophores of non-polypeptidic nature are synthesized via NRPS-independent pathways (Challis, 2005).

Vibrio anguillarum is the causative agent of vibriosis, a generally fatal haemorrhagic septicaemic disease in a variety of marine animals (Toranzo & Barja, 1990). It is known that siderophore-mediated iron-acquisition systems play an important role in the pathogenicity of *V. anguillarum* for fish (Di Lorenzo *et al.*, 2003; Wolf & Crosa, 1986). Currently, two clearly different siderophore-mediated systems are known in *V. anguillarum*. Strains that harbour the 65 kb pJM1 or pJM1-like plasmids produce the siderophore anguibactin (Stork *et al.*, 2002), although its synthesis also requires chromosomal genes (Alice *et al.*, 2005; Chen *et al.*, 1994). Strains that lack pJM1-like plasmids produce a different catecholate siderophore (Conchas *et al.*, 1991; Lemos *et al.*, 1988), named vanchro-

Abbreviations: DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; DHBA, 2,3-dihydroxybenzoic acid; EDDA, ethylenediamine-di-(o-hydroxyphenyl acetic acid); Fur, ferric uptake regulator; FURTA, Fur titration assay; LTTR, LysR-type transcriptional regulator; NRPS, non-ribosomal peptide synthetase; PPTase, phosphopantetheinyl transferase.

The GenBank/EMBL/DBJ accession number for the sequence of *V. anguillarum* is AM168450.

A supplementary table showing the oligonucleotides used in this study is available with the online version of this paper.

bactin. Vanchrobactin is assembled from the precursors 2,3-dihydroxybenzoic acid (DHBA), serine and arginine (Soengas *et al.*, 2006), following an NRPS-based mechanism encoded by chromosomal genes (Fig. 1). VabE, VabB and VabF are three NRPSs that have been demonstrated to be essential for vanchrobactin biosynthesis (Balado *et al.*, 2006). Phosphopantetheinyl transferases (PPTases) are required for the NRPS to be functional, since they catalyse the essential post-translational activation of carrier proteins (Crosa & Walsh, 2002). To date, no candidate PPTase genes have been described in vanchrobactin-producing *V. anguillarum* strains.

DHBA, one of the vanchrobactin components, is synthesized from chorismate (an aromatic amino acid intermediate). One of the steps in the biochemical pathway that leads to chorismate is catalysed by the DAHP synthase, responsible for the production of 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP). This enzyme represents a key regulatory point in aromatic amino acid biosynthesis (Kloosterman *et al.*, 2003), and in a few species homologues of DAHP synthase genes have been

found linked to siderophore biosynthesis gene clusters (Di Lorenzo *et al.*, 2003; Osorio *et al.*, 2006), although no functional studies have been conducted.

The expression of genes involved in siderophore biosynthesis and uptake must be strictly regulated (Touati, 2000). The ferric uptake regulator (Fur) protein has been described as a major transcriptional regulator that represses the expression of many of the genes involved in iron acquisition (Braun *et al.*, 1998; Braun & Killmann, 1999; Escolar *et al.*, 1999). However, in some organisms, Fur can act indirectly as a positive regulator in controlling gene expression, for instance through the action of the small RNA RyhB (Masse & Gottesman, 2002). Genes involved in the biosynthesis of siderophores can also be subjected to transcriptional activation. There is increasing evidence that AraC-family activators are necessary for maximal expression of the respective siderophore biosynthetic and transport genes, and that these regulators require the cognate siderophore as a cofactor (Anderson & Armstrong, 2004; Brickman *et al.*, 2001; Fetherston *et al.*, 1996; Michel

Fig. 1. A proposed scheme for vanchrobactin biosynthesis and regulation. A circled plus symbol denotes activation and a circled minus symbol indicates repression. The Fur repressor protein, under conditions of high iron, represses transcription of the genes encoding VabCBAEDFHS and FvtA proteins (dashed lines ending in empty arrowheads). A dashed line ending in a filled arrowhead denotes negative regulation mediated by aromatic amino acids of genes encoding AroGFH proteins. The empty arrow arising from VabD shows the activation of the VabF peptide carrier protein (PCP) domains and VabB aryl carrier protein (ArCP) domain by VabD.

et al., 2005; Pelludat *et al.*, 1998). Similarly, LysR-type transcriptional regulators (LTTRs) activate the expression of genes involved in a wide range of cellular processes, such as amino acid biosynthesis (Panina *et al.*, 2001), virulence (Watnick *et al.*, 1998) and iron uptake (Vasil *et al.*, 1998). Most LTTRs are encoded by genes that are transcribed divergently from their target genes, and operate together with a small ligand that acts as a coinducer (Schell, 1993).

In a previous work, we characterized a cluster of seven genes involved in vanchrobactin biosynthesis and utilization, and proposed a preliminary biosynthetic model for vanchrobactin (Balado *et al.*, 2006). These genes included those responsible for the synthesis of DHBA from chorismate (*vabABC*), as well as the NRPSs involved in activation of DHBA (*vabE*), and the final assembly of DHBA, serine and arginine (*vabF*) to make vanchrobactin. Other genes putatively involved in siderophore export (*vabS*) and utilization (*vabH*) were also described as part of the cluster (Balado *et al.*, 2006). In the present study, we identified and characterized additional genetic determinants involved in the vanchrobactin biosynthetic pathway, and showed that vanchrobactin biosynthesis is subjected to a complex regulatory circuitry.

METHODS

Bacterial strains, plasmids and media. The strains and plasmids used, as well as those derived from this study, are listed in Table 1. *V. anguillarum* strains were routinely grown at 25 °C in Tryptic Soy Agar and Broth (Difco) supplemented with 1 % NaCl (TSA-1 and TSB-1, respectively). *Escherichia 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 with 20 % (v/v) glycerol. Ampicillin sodium salt and kanamycin (Sigma-Aldrich) stock solutions in ultrapure water (100 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 analyses were performed with the ECL DNA Labelling and Detection System (Amersham Biosciences). PCR reactions were carried out in a TGradient Thermal Cycler (Biometa), 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). The most relevant oligonucleotides used in this study are listed in Supplementary Table S1.

Table 1. Bacterial strains and plasmids used in this study

Resistance phenotypes: Amp^R, ampicillin; Gen^R, gentamycin; Neo^R, neomycin; Sm^R, streptomycin; Tp^R, trimethoprim.

Plasmid or strain	Relevant characteristic(s)	Reference or source
Plasmids		
pGEMT-Easy	PCR cloning vector, Amp ^R	Promega
pWKS30	Low-copy cloning vector, Amp ^R	Wang & Kushner (1991)
pT7-7	Cloning vector, Amp ^R	Tabor & Richardson (1985)
pHRP309	Low-copy-number reporter plasmid, Gen ^R	Parales & Harwood (1993)
pMB11	pHRP309 <i>fmtA::lacZ</i> fusion	This study
pMB12	pHRP309 <i>vabH::lacZ</i> fusion	This study
pMB19	pHRP309 <i>vabA::lacZ</i> fusion	This study
pMB20	pHRP309 <i>vabC::lacZ</i> fusion	This study
pMB33	pHRP309 <i>vabG::lacZ</i> fusion	This study
pMB34	pHRP309 <i>vabR::lacZ</i> fusion	This study
SuperCos1	Cosmid vector, Amp ^R , Neo ^R	Stratagene
<i>V. anguillarum</i> strains		
RV22	Wild-type serotype O2 strain isolated from diseased turbot (Spain)	Lemos <i>et al.</i> (1988)
775met11	Serotype O1 strain defective in Fur protein	Tolmasky <i>et al.</i> (1994)
MB11	RV22 <i>vabB</i> defective mutant	Balado <i>et al.</i> (2006)
MB14	RV22 <i>vabF</i> defective mutant	Balado <i>et al.</i> (2006)
MB53	RV22 <i>vabR</i> defective mutant	This study
MB54	RV22 <i>vabG</i> defective mutant	This study
MB67	RV22 <i>vabD</i> defective mutant	This study
<i>E. coli</i> strains		
DH5α	SupE4 Δ <i>lacU169</i> (Φ80 <i>lacZ</i> Δ <i>M15</i>) <i>hsd</i> R17 <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Laboratory stock
S17-1 λpir	Tp ^R Sm ^R <i>recA</i> , <i>thi</i> , <i>pro</i> , <i>hsdR</i> -M + RP4::2-Tc::Mu-Km::Tn7 λpir	Herrero <i>et al.</i> (1990)
XL1-Blue MR	Δ(<i>mcrA</i>)183Δ(<i>mcrCB</i> - <i>hsdSmr</i> - <i>mrr</i>)173 <i>endA1</i> <i>supE44</i> <i>thi-1</i> <i>recA1</i> <i>gyrA96</i> <i>relA1</i> <i>lac</i>	Stratagene
H1717	<i>araD139</i> <i>rpsL150</i> Δ(<i>argF</i> - <i>lac</i>) <i>relA1</i> <i>U169</i> <i>flbB5301</i> <i>deoC1</i> <i>ptsF25</i> <i>rbsR</i> <i>aroB</i> <i>fhuF</i> ::λplacMu	Hantke (1987)

DNA sequencing and bioinformatics tools. DNA sequences were determined with the dideoxy chain-termination method on either plasmid or PCR products using a 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 (EBI) and the NCBI services were used to consult the DNA and protein sequence databases with the FASTA3 and BLAST algorithms. The protein families database of alignments and HMMs (Pfam) of the Sanger Institute was utilized to predict the protein domain organization (Bateman *et al.*, 2004). The Virtual Footprint Promoter Matches from the online database PRODORIC Release 8.1 (<http://prodoric.tu-bs.de/>) was used to identify putative regulatory operators. MFOLD (3.1.2) (<http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html>) was used for RNA secondary structure prediction.

Construction of a *V. anguillarum* RV22 cosmid library. For construction of a cosmid library, genomic DNA from *V. anguillarum* RV22 was partially digested with the restriction enzyme *Sau3AI* and ligated into the *Bam*HI site of the SuperCos1 cosmid vector (Stratagene). Recombinant cosmids were packaged *in vitro* and transduced into *E. coli* XL1-blue MR (Stratagene). The cosmid library was screened by performing colony PCR on pools of recombinant clones using primers targeted to genes located at the 5' and 3' ends of the previously described partial vanchrobactin gene cluster (Balado *et al.*, 2006), and two cosmids, cosMB167 and cosMB69, were isolated. Cosmid DNA was purified using the QIAfilter Plasmid Midi Kit (Qiagen) and used for DNA sequencing.

Construction of *vabG*, *vabD* and *vabR* deletions by allelic exchange. Gene deletions in *V. anguillarum* RV22 were constructed by allelic exchange as previously described (Mouriño *et al.*, 2004). This process led to the obtention of *V. anguillarum* MB53 ($\Delta vabR$), MB67 ($\Delta vabD$) and MB54 ($\Delta vabG$) mutant strains. 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.

Growth under iron-limited conditions and test of 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 OD₆₀₀ 0.5 and diluted 1:15 in CM9 minimal medium (Lemos *et al.*, 1988) containing the iron chelator ethylenediamine-di-(*o*-hydroxyphenyl) acetic 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), which detects the presence of iron-chelating siderophore molecules. In addition, the Arnow test (Arnow, 1937), which specifically detects the presence of 2,3-dihydroxybenzoic acid (DHBA), was used for the spectrophotometric catechol measurement of each sample. A non-inoculated CM9 sample containing EDDA at appropriate concentrations was used as a negative control and as a spectrophotometric blank for the CAS liquid assay.

RNA purification and RT-PCR. *V. anguillarum* RV22 10 ml cultures were grown until exponential phase in high- and low-iron CM9 medium containing EDDA at 5 μ M or Fe₂(SO₄)₃ at 10 μ M, and total RNA was isolated with the RNA isolation reagent RNAwiz (Ambion) following the manufacturer's recommendations. RT-PCR reactions were performed with 1 μ g RNA pre-treated with RQ1 RNase-Free DNase (Promega) by using the M-MLV reverse transcriptase (Invitrogen). A negative control reaction for PCR was performed with total RNA without M-MLV reverse transcriptase to confirm the lack of genomic DNA contamination in each reaction mixture. All the

primers used to amplify the cDNA from putative *vabR*, *vabG*, *vabA*, *vabCE*, *vabHFSB* and *fvfAvabD* transcripts are listed in Supplementary Table S1. The operonic nature of the genes described in this study was tested by designing appropriate primers for reverse transcription located at the 3' end of the last gene for each predicted operon, and were named RT-1 to RT-6 (Fig. 2b).

Construction of *lacZ* transcriptional fusions, and β -galactosidase assays. DNA fragments corresponding to *V. anguillarum* presumptive *vabR*, *vabG*, *vabA*, *vabC*, *vabH* and *fvfA* promoter regions were obtained by PCR. The PCR-amplified putative promoter regions extended from about 300–700 bp upstream of the ATG start codon to about 50–100 bp downstream of the start codon for each tested gene. Thus, DNA fragments containing the putative promoter regions as well as additional nucleotides of the coding sequence were fused to a promoterless *lacZ* gene in the low-copy-number reporter plasmid pHRP309 (Parales & Harwood, 1993). The resulting transcriptional fusion constructs, *vabR::lacZ* (pMB34), *vabG::lacZ* (pMB33), *vabA::lacZ* (pMB19), *vabC::lacZ* (pMB20), *vabH::lacZ* (pMB12) and *fvfA::lacZ* (pMB11), were mobilized from *E. coli* S17-1 λ pir to *V. anguillarum* strains RV22 and 775met11 *fur* mutant by conjugation. The *V. anguillarum* strains carrying the promoter-*lacZ* fusion vector or control plasmid pHRP309 were grown in minimal medium CM9 under different iron-availability conditions. The β -galactosidase activities were measured by the method of Miller (1992). The data presented correspond to the mean of three independent experiments. When needed, purified vanchrobactin was obtained as previously described (Soengas *et al.*, 2006) and used at 15 ng ml⁻¹.

Fur titration assay (FURTA). The same DNA fragments used in *lacZ* transcriptional fusions, corresponding to presumptive promoter regions, were cloned in plasmid pT7-7 and transformed into the *E. coli* H1717 reporter strain (Hantke, 1987). The empty vector was used as a negative control. The colonies obtained were streaked onto MacConkey-lactose plates supplemented with 40 μ M ferric ammonium sulfate, as described elsewhere (Stojiljkovic *et al.*, 1994). The phenotype of colonies was checked after 24–48 h of incubation at 37 °C. Red colonies (Lac+) denote a FURTA-positive phenotype and indicate binding of the Fur protein to the promoter region transformed in the indicator strain (Stojiljkovic *et al.*, 1994).

Cross-feeding assays. The biological activities of the supernatants produced by parental and mutant strains were determined by cross-feeding experiments. We tested the ability of cell cultures of *V. anguillarum* siderophore-defective mutants (Table 1) to cross-feed different indicator strains that were defective in DHBA and/or siderophore synthesis. Two RV22 mutant strains that are deficient in vanchrobactin biosynthesis were used to detect vanchrobactin and DHBA production. Strain MB11 is a VabB-defective mutant, unable to produce DHBA (a step between chorismate and DHBA is blocked), and consequently it can use vanchrobactin and the intermediate DHBA to overcome iron limitation. The MB14 strain is blocked in the vanchrobactin assembly process (VabF-defective mutant), and thus it can only use vanchrobactin for iron supply, and not DHBA (Balado *et al.*, 2006).

Each indicator strain was inoculated into CM9 minimal medium containing 2,2'-dipyridyl at 80 μ M, a concentration higher than the MIC for these strains. Strains to be tested were cultured on LB agar plates supplemented with 100 μ M 2,2'-dipyridyl to induce activation of siderophore biosynthesis genes. The cells were harvested with a sterile loop and placed on top of the indicator strain plates. The results were scored as positive when tester cells promoted the growth of indicator strains. The RV22 strain was used as a positive control.

Fig. 2. (a) Physical map of the *vanchrobactin* biosynthesis genes of *V. anguillarum* RV22. ORFs are depicted as arrows, which indicate the direction of transcription, and the vertical numbers show the start and end points of each gene. Stem-loop structures denote putative transcriptional terminators. Dashed arrows with empty arrowheads show the regions and orientation used in transcriptional fusions. Predicted Fur boxes and a putative AraC box are shown above the ORFs. The predicted function for each gene product is shown under the ORFs. (b) Results of RT-PCR for operon mapping and for determination of iron regulation. RT denotes the binding site for primers used in reverse transcription (RT-1 to RT-6). Pairs of arrows facing each other denote the gene-specific primer pairs used in the subsequent PCR reactions. RNA for RT-PCR was purified from cells cultured under low-iron (1) and high-iron (2) conditions. Positive (+) controls are PCR reactions using chromosomal DNA as a template; negative controls (–) are PCR reactions without reverse transcriptase.

RESULTS AND DISCUSSION

Cloning of new ORFs linked to vanchrobactin biosynthesis genes

In a previous work, we described seven clustered genes involved in the biosynthesis and utilization of the siderophore vanchrobactin in *V. anguillarum* RV22 and proposed a preliminary model for vanchrobactin biosynthesis (Balado *et al.*, 2006). However, some of the functions necessary to complete the vanchrobactin biosynthesis pathway remained undescribed. In the present study, we have identified additional pieces in the biosynthetic pathway, and a new vanchrobactin biosynthesis and uptake model that now includes regulatory factors is presented in Fig. 1. We constructed a cosmid library from *V. anguillarum* RV22 and isolated three overlapping cosmids that hybridized with the vanchrobactin synthesis genes previously described (Balado *et al.*, 2006). Sequencing of the novel DNA contained in these cosmids resulted in the addition to the previously reported vanchrobactin gene cluster of four novel genes: *vabR*, *vabG*, *fvtA* and *vabD* (Fig. 2a). Downstream of the previously described *vabA* gene, we found genes *vabG* and *vabR*, which are transcribed from a divergently oriented promoter. The predicted VabG protein shows high similarity to members of the *E. coli*-type DAHP synthase family (Table 2). The deduced amino acid sequence of VabR shows similarity to several members of the LysR family of transcriptional activators (Schell, 1993). At the opposite side of this cluster, we found two genes downstream of *vabH* that are transcribed from the same DNA strand. The gene *fvtA* encodes a protein that shows similarity to a series of TonB-dependent outer membrane siderophore receptors, and its location linked to the biosynthesis genes suggests that this protein might be the receptor for the ferri-vanchrobactin complex. FvtA shows a 31 % identity to ViuA, the vibriobactin receptor of *Vibrio cholerae* (Butterton *et al.*, 1992), and to VuuA, the

vulnibactin receptor of *Vibrio vulnificus* (Webster & Litwin, 2000). The *vabD* gene encodes a predicted PPTase (Table 2), and shows a 29 % identity to *angD*, a plasmid-harboured gene described in anguibactin-producing *V. anguillarum* strains, and which proves to encode a functional PPTase (Liu *et al.*, 2005). We further sequenced the DNA regions in the vicinity of the *vab* cluster. Downstream of *vabR* we found two ORFs encoding a putative TonB-dependent outer membrane receptor, whose closest homologues have not been functionally characterized, and a putative lipase/esterase (data not shown). Downstream of *vabD* we found an ORF encoding a predicted protein which showed similarity to the E subunit of Na⁺-translocating NADH-quinone reductases.

VabD and VabG are new pieces in the vanchrobactin biosynthesis pathway

VabD is a putative PPTase (Table 2). We constructed by allelic exchange a *vabD*-defective mutant (strain MB67) and tested its ability to produce siderophores and to grow under iron limitation. Under iron-sufficient conditions, no significant differences in growth levels were observed with respect to the parental strain RV22. However, MB67 (Δ *vabD*) was impaired for growth under iron-restricted conditions (CM9 plus 5 μ M EDDA) (Fig. 3a). We predict that a mutation in this gene would not prevent DHBA production, while siderophore assembly should in turn be abolished. As expected, the CAS test showed no detectable levels of siderophore production in the *vabD* mutant (Fig. 3b), whereas significant levels of DHBA production were observed (Fig. 3c). These observations were further supported by the results obtained with the cross-feeding assays (Table 3). MB67 (*vabD* mutant) promoted growth of MB11 (*vabB* mutant); this may be explained by the accumulation of DHBA by MB67, which can be used by MB11 to complete vanchrobactin production. However,

Table 2. Proteins with homology to products of *V. anguillarum* genes *vabD*, *vabG* and *vabR*

<i>V. anguillarum</i> protein (no. of amino acids, molecular mass in kDa)	Accession number	Identity (%)	Similarity (%)
VabD (235, 26.96)			
EntD (<i>Marinomonas</i> sp.)	ZP_01076777	34	61
EntD (<i>Photobacterium profundum</i>)	YP_133485	34	55
VibD (<i>V. cholerae</i>)	EDN13617	34	55
EntD (<i>Pseudomonas fluorescens</i>)	YP_349971	35	55
VabG (351, 38.28)			
DAHP synthase (<i>Photobacterium damsela</i> subsp. <i>piscicida</i>)	CAJ76915	57	73
AroG (<i>V. vulnificus</i>)	NP_762766	55	70
DAHP synthase (<i>V. anguillarum</i>)	NP_943591	69	84
AroG (<i>Vibrio angustum</i>)	ZP_01235496	52	66
VabR (286, 32.85)			
LysR (<i>Vibrio alginolyticus</i>)	ZP_01260503	63	79
LysR (<i>Vibrio harveyi</i>)	EDL67341	55	74
LysR (<i>Vibrio splendidus</i>)	ZP_00988694	55	74
LysR (<i>Vibrio shilonii</i>)	ZP_01865776	48	69

Fig. 3. (a) Growth (OD₆₀₀) of *V. anguillarum* RV22, MB53 ($\Delta vabR$), MB54 ($\Delta vabG$) and MB67 ($\Delta vabD$) strains in CM9 minimal medium under iron-sufficient (Fe+; 10 μ M ferric sulfate) and iron-deficient (Fe-; 5 μ M EDDA) conditions. (b) Siderophore production measured by the CAS supernatant assay (A₆₃₀). (c) DHBA production measured by the Arnow test (A₅₁₀). In the CAS assay, lower values indicate higher siderophore production. Results are expressed as mean $\pm$ SD from three independent experiments.

vanchrobactin and for the growth of *V. anguillarum* RV22 under iron limitation.

A *vabD* homologue (*angD*) has recently been described in *V. anguillarum* strains that synthesize the siderophore anguibactin, and this gene proves to encode a functional PPTase (Liu *et al.*, 2005). AngD encodes a protein that shows 29% identity to the RV22 VabD protein. Based on protein similarity data and on our studies with the *vabD* mutant, VabD is likely the PPTase involved in vanchrobactin biosynthesis, playing a role in the post-translational phosphopantetheinylation of the aryl carrier protein (ArCP) domain of VabB and the two peptide carrier protein (PCP) domains of VabF (Balado *et al.*, 2006).

Gene *vabG* encodes a putative DAHP synthase (Table 2). DAHP synthases are key enzymes in the shikimate pathway, which leads to the biosynthesis of aromatic amino acids and of numerous secondary metabolites derived from them (Hodgson, 2000), and are strictly regulated via a series of complex mechanisms (Panina *et al.*, 2001). Vanchrobactin, as a catecholate siderophore, contains DHBA in its structure (Soengas *et al.*, 2006), and this molecule is itself synthesized from chorismate via the shikimate pathway. To ascertain the role of *vabG* in vanchrobactin biosynthesis, a non-polar defective mutant was constructed by allelic exchange. When this mutant (strain MB54) was cultured in iron-sufficient conditions (CM9 plus 10 μ M ferric sulfate), no significant differences in growth levels were observed with respect to the parental strain RV22 (Fig. 3a). However, the MB54 ($\Delta vabG$) mutant was severely affected in its ability to grow under iron-restricted conditions (CM9 plus 5 μ M EDDA), and chemical tests showed that siderophore and DHBA production were significantly abolished in this mutant (Fig. 3b, c). These results demonstrate that *vabG* is essential for growth of *V. anguillarum* RV22 under iron-limiting conditions, and suggest that VabG is part of the vanchrobactin biosynthesis pathway, acting as a putative DAHP synthase. Although other DAHP synthase genes have been described linked to siderophore biosynthesis gene clusters (Osorio *et al.*, 2006), we believe that our results represent the first experimental evidence of a DAHP synthase gene whose function is necessary for optimal siderophore production.

To test whether vanchrobactin biosynthesis is either partially or completely abolished in the *vabG* mutant, we tested the ability of strain MB54 to cross-feed a series of

MB67 could not cross-feed MB14 (*vabF* mutant), which can only use vanchrobactin. These results clearly demonstrate that *vabD* is essential for the biosynthesis of

Table 3. Results of cross-feeding experiments

The tester strains are listed in the column at the left, and their ability to cross-feed the four indicator mutant strains MB54, MB67, MB11 and MB14 is scored as positive, negative, or weak positive. NA, Not applicable; (+), weak positive.

Strain	Ability to cross-feed			
	MB54 ($\Delta vabG$)	MB67 ($\Delta vabD$)	MB11 ($\Delta vabB$)	MB14 ($\Delta vabF$)
MB54 ($\Delta vabG$)	NA	(+)	(+)	(+)
MB67 ($\Delta vabD$)	+	NA	+	–
MB11 ($\Delta vabB$)	+	–	NA	–
MB14 ($\Delta vabF$)	+	–	+	NA
RV22	+	+	+	+

vanchrobactin-deficient indicator strains (Table 3). Surprisingly, we found that the MB54 mutant was able to induce low levels of growth of MB14, a *vabF* mutant that can only use vanchrobactin to overcome the iron limitation. Similarly, MB54 also cross-fed MB11 ($\Delta vabB$), which can use both vanchrobactin and DHBA (Table 3). These results indicate that, although vanchrobactin biosynthesis is significantly reduced in the *vabG* mutant, it is not completely abolished. This observation would suggest that another gene or genes in *V. anguillarum* encodes a DAHP synthase activity that can partially, but not completely, substitute for the VabG function. This being the case, one gene could encode an isoenzyme mainly involved in the production of extra chorismate molecules for siderophore biosynthesis, while keeping a paralogue involved in the production of aromatic amino acids. In *E. coli* and other Gram-negative bacteria such as *V. cholerae*, up to three paralogous DAHP synthase genes (*aroF*, *aroG* and *aroH*), involved exclusively in amino acid biosynthesis, have been described coexisting in the same genome (Panina *et al.*, 2001, 2003). The necessity of having a specific isoenzyme involved in siderophore production is motivated by the fact that the paralogues involved in aromatic amino acid production are strictly regulated at the transcriptional (Pittard *et al.*, 2005), translational (Yanofsky, 2000), and enzymic activity levels (Pittard *et al.*, 2005). The interconnection between the biosynthetic routes leading to siderophore biosynthesis and to amino acids (two routes that share chorismate as an intermediate) has been recently evidenced in a *Bacillus subtilis* transcriptome study (Miethke *et al.*, 2006). These authors found that 11 amino acid biosynthesis genes were more than 40 % upregulated during iron starvation, and all of them encoded enzymes that are essential for the synthesis of the precursors threonine, glycine and DHBA of the siderophore bacillibactin.

The vanchrobactin biosynthesis gene cluster is organized into six iron-regulated transcriptional units

The three intergenic regions *vabR*–*vabG*, *vabA*–*vabC* and *vabH*–*fvtA* show a structure of divergent promoters.

Moreover, two putative Rho-independent transcriptional terminators were located by *in silico* analysis between the *vabG*–*vabA* and *vabE*–*vabB* stop codons (Fig. 2a). All these observations suggest that the *V. anguillarum* vanchrobactin biosynthesis gene cluster is organized in six transcriptional units: *vabR*, *vabG*, *vabA*, *vabCE*, *vabHFSB* and *fvtAvabD*. To confirm the transcriptional association of the three putative polycistronic messages, we carried out reverse-transcription reactions using primers located at the 3' end of the last gene for each predicted operon (primers RT-1 to RT-6 in Fig. 2b), and subsequent gene-specific PCR reactions were conducted. These RT-PCR experiments were carried out using RNA purified under conditions of both iron deficiency and iron sufficiency. The results obtained in this assay (Fig. 2b) confirmed the existence of three polycistronic mRNAs, one comprising the *vabH*, *vabF*, *vabS* and *vabB* genes, the second comprising *vabC* and *vabE*, and the last *fvtA* and *vabD*. However, these results do not rule out the possibility that additional promoters exist within this cluster, and may drive independent transcription of some of the genes. In addition, the transcripts of the six predicted transcriptional units were shown to be weakly expressed under iron-rich conditions (10 μ M ferrous sulfate) (Fig. 2b), whereas the intensity of the bands was significantly increased when using RNA samples from cultures supplemented with the iron chelator EDDA (5 μ M). Although RT-PCR carried out under these conditions is not the most accurate quantitative assay for RNA levels, it provides evidence that the expression of the genes of this cluster is indeed subject to regulation by iron.

The putative promoter regions contain consensus sequences for the binding of negative and positive regulators

Examination of the intergenic sequences of this cluster revealed three conserved Fur binding sites (Fig. 2a). The intergenic region *vabR*–*vabG* harbours a putative Fur box (GATAATAATTATCATTACG) with an identity of 15 out of 19 positions to the *E. coli* consensus iron box (de Lorenzo *et al.*, 1987) (the positions identical to the consensus are underlined). Two similar sequences,

GTAAATGATAATTGTTATT and GTTATTGATAAT-TATTATC, were found in the intergenic regions *vabH-fvtA* and *vabA-vabC* (Fig. 2a). The presence of these Fur binding sites would suggest that the Fur repressor exerts iron-dependent regulation of vanchrobactin synthesis genes. In order to gain further evidence that these promoters contain sequences for the binding of Fur, FURTA was carried out as described elsewhere (Stojiljkovic *et al.*, 1994) by cloning the presumptive promoter sequences containing potential Fur boxes into the FURTA indicator strain H1717 (Hantke, 1987). As a result, all the cloned promoters showed a typical FURTA-positive phenotype (evidenced as Lac⁺ colonies on MacConkey agar plates supplemented with excess iron), which suggests that the cloned sequences harbour sites for binding of the *E. coli* Fur protein.

In addition to the Fur-mediated repression, transcriptional regulators with a positive action have been described as playing roles in gene regulation (Chen & Crosa, 1996; Gallegos *et al.*, 1997). We therefore subjected the promoter sequences of this cluster to the PRODORIC database using the Virtual Footprint promoter analysis software, and as a result we predicted a putative AraC-type regulator binding site (TATTCATATCCATGGGCTTCATGACCTAA) in the negative strand of the *fvtA* gene, which includes the *fvtA* start codon (Fig. 2a).

Transcriptional *lacZ* fusions demonstrate that expression of vanchrobactin synthesis genes is iron regulated

To define the promoter activities and test whether the iron-mediated regulation of the vanchrobactin biosynthesis gene cluster occurred at the transcriptional level, as suggested by

the RT-PCR results (Fig. 2b), we constructed transcriptional fusions and examined their expression under high- and low-iron conditions. As a control, *V. anguillarum* RV22 carrying the pHRP309 plasmid without any promoter was analysed in parallel, exhibiting almost undetectable levels of β -galactosidase activity under all the growth conditions tested (data not shown). When the constructions were assayed in *V. anguillarum* RV22, high β -galactosidase activities were observed for *vabR::lacZ*, *vabG::lacZ*, *vabH::lacZ*, *fvtA::lacZ*, *vabC::lacZ* and *vabA::lacZ* fusions in low-iron medium (CM9 plus EDDA at 5 μ M), whereas the addition of 10 μ M Fe₂(SO₄)₃ to the culture medium resulted in the lowest β -galactosidase activity, indicating repression of promoter activity (Fig. 4). All the fusions with the exception of *vabR::lacZ* showed a reduction of ~90 % in their expression patterns under high-iron conditions with respect to levels shown under low-iron conditions (Fig. 4). In the case of the *vabR::lacZ* construction, this reduction was about 50 %. The transcriptional repression observed under iron-replete conditions is consistent with a predicted role for Fur in regulation. To demonstrate that the observed repression is indeed Fur-mediated, the fusions were conjugated into the *V. anguillarum fur* mutant strain 775met11 (Tolmasky *et al.*, 1994). The same iron conditions utilized for RV22 were now assayed with 775met11, and results are summarized in Fig. 4. Iron-dependent repression of promoters was not detected when the *vabH::lacZ*, *fvtA::lacZ*, *vabC::lacZ* and *vabA::lacZ* fusions were assayed in the *fur* mutant, and the β -galactosidase activities were high under both high- and low-iron conditions, indicating that these promoters are constitutive in the absence of Fur.

Surprisingly, the *vabG::lacZ* and *vabR::lacZ* fusions showed an unexpected response in the *fur* mutant. On

Fig. 4. Transcriptional analysis of the vanchrobactin biosynthesis genes carried out with *lacZ* fusions. Bars indicate β -galactosidase activities of cultures grown under iron-sufficient or iron-deficient conditions (CM9 plus 10 μ M ferric sulfate or 5 μ M EDDA). β -Galactosidase activities were assayed in the parental strain RV22 and in the 775met11 *fur* mutant.

the one hand, the *vabG::lacZ* fusion maintained a drastic iron-dependent repression in the *fur* mutant (Fig. 4). This result indicates that *vabG* transcriptional regulation is iron-dependent, but that Fur is not essential for this repression. Thus, there must be another regulator that controls the response of *vabG* to iron. The iron-dependent regulation of *vabG* is consistent with the above suggested role of VabG as a DAHP synthase mainly involved in siderophore biosynthesis.

Furthermore, the *vabR::lacZ* fusion maintained the reduction in β -galactosidase levels under high-iron conditions at $55 \pm 12\%$ of the levels shown under low-iron conditions. This fusion showed a similar β -galactosidase activity reduction in the Fur-deficient strain ($64 \pm 16\%$), which indicates that some levels of iron-mediated repression are still present in the absence of Fur (Fig. 4). This therefore suggests that Fur is not the main iron-responsive regulator of *vabR*. Notably, the maximal expression of the *vabR::lacZ* fusion achieved in strain 775met11 was reduced by more than 40% when compared to expression levels in the RV22 strain. This reduction could be explained in part by the fact that the 775met11 strain is not isogenic with RV22 and does not produce vanchrobactin.

The results obtained with *vabG::lacZ* and *vabR::lacZ* fusions suggest the necessary existence of additional regulation mechanisms different from Fur. Our results clearly indicate that: (1) *vabG* and *vabR* are iron regulated; (2) there is evidence that the *vabG-vabR* intergenic region harbours a Fur box, since it yields a strong FURTA-positive phenotype when transformed into the indicator strain H1717; (3) these results do not rule out the possibility that Fur plays a significant role in the regulation of either *vabG* or *vabR*, or both. In the absence of Fur, we found evidence of a strong iron-mediated repression. It could be that the repression of *vabG/vabR* is carried out by Fur as well as by a yet uncharacterized repressor, and that in the absence of Fur, this second repressor by itself accounts for nearly the same level of repression. Additional studies would be necessary to understand the role of the Fur-mediated repression in the expression of these two genes. It is also recognized that Fur may exert its effect indirectly by altering the expression of activators required for the expression of iron-regulated genes. Studies of the regulation of anguibactin biosynthesis genes, encoded in the pJM1 plasmid, have demonstrated that, in addition to the general mechanism mediated by Fur repression (Tolmasky & Crosa, 1991), other plasmid-encoded factors play a role in regulation, including the AngR protein (Tolmasky *et al.*, 1993; Wertheimer *et al.*, 1999), the trans-acting factor(s) (TAF) (Welch *et al.*, 2000), and an antisense RNA, RNA α (Chen & Crosa, 1996; Stork *et al.*, 2007).

Thus, transcriptional *lacZ* fusions evaluated under low- and high-iron conditions demonstrate that all these genes are strongly iron regulated, as suggested by the RT-PCR experiments (see above). However, the data we obtained using the two approaches do not show an exact correlation.

As an example, the expression of the *vabG* gene promoter showed a strong repression under high-iron conditions when measured by β -galactosidase assays, whereas the respective RT-PCR assay still showed a detectable amount of PCR product (Fig. 2b). We consider that the studies of *lacZ* fusion data are more suitable for quantitative comparisons than the RT-PCR assays, since the PCR-based exponential amplification of the RNA transcripts originally present in the sample could lead to an overestimation of the actual gene expression levels.

***vabR* encodes a putative LysR-family activator, and its deletion affects *vabG* expression**

VabR is similar to members of the LTTR family, which is the largest family of prokaryotic DNA-binding transcriptional factors (Schell, 1993). The domain analysis of VabR (a total of 286 residues) shows a LysR substrate domain from residues 61 to 278, which includes the helix–turn–helix DNA-binding motif at its N terminus. Most LTTRs are encoded by genes that are transcribed divergently from their target genes, and operate as transcriptional regulators activating expression together with a small molecule ligand that acts as a coinducer (Gallegos *et al.*, 1997; Schell, 1993). Notably, *vabR* and *vabG* are divergently transcribed (Fig. 2a), which suggests that *vabR* could exert a regulatory effect on *vabG*. To test this hypothesis, we designed an in-frame deletion mutant (MB53). This mutant did not show any significant alteration in vanchrobactin production or in the ability of *V. anguillarum* to grow under iron-limiting conditions (Fig. 3).

We then introduced the transcriptional fusion *vabG::lacZ* into the Δ *vabR* mutant, and compared the expression pattern from the *vabG* promoter with that obtained in the RV22 parental strain. As expected, this fusion was strongly repressed under conditions of iron excess, showing expression levels similar to those of the RV22 background. However, under iron-limiting conditions, the *vabR* mutant (MB53) strain showed a significant reduction of β -galactosidase activity of about 35–40% with respect to the wild-type RV22 (Fig. 5). These results clearly indicate that *vabR* encodes a regulatory factor necessary for maximal *vabG* expression. Other reported examples of LTTRs that cooperate with Fur in the regulation of gene expression include amino acid biosynthesis (Panina *et al.*, 2001), virulence (Watnick *et al.*, 1998) and iron uptake (Litwin & Quackenbush, 2001; Vasil *et al.*, 1998).

We have demonstrated that *vabR* exerts a regulatory role on the divergently transcribed *vabG* gene. However, it remained to be tested whether VabR also influences the expression of other vanchrobactin biosynthesis and transport genes, as well as regulating its own gene expression. To ascertain these possibilities, we introduced all the remaining gene fusions (*vabH::lacZ*, *fvtA::lacZ*, *vabC::lacZ*, *vabA::lacZ* and *vabR::lacZ*) into the *vabR* mutant. The results demonstrated that mutation of *vabR* did not have any detectable effect on the β -galactosidase activities of

Fig. 5. Transcriptional analysis of the *vabG* gene promoter carried out with *lacZ* fusions. Bars indicate β -galactosidase activities of cultures grown under iron-sufficient or iron-deficient conditions (CM9 plus 10 μ M ferric sulfate or 1.25 μ M EDDA) and in presence of 15 ng vanchrobactin ml^{-1} (Van 15). The β -galactosidase activity of transcriptional fusions was assayed in the parental strain RV22, in MB11 (*vabB* mutant) and in MB53 (*vabR* mutant).

these promoters (data not shown). Thus, it was demonstrated that *vabR* transcription is not self-regulated, and that the only direct target of VabR in the cluster described in this study is *vabG*.

We also tested whether VabR is involved in the regulation of *vabG* in response to vanchrobactin. To assess this possibility we measured *vabG::lacZ* expression in the presence and absence of exogenously added vanchrobactin, in strains MB11 ($\Delta vabB$) and MB53 ($\Delta vabR$), as well as in the parental strain. As shown in Fig. 5, under iron-sufficient conditions, no significant differences were observed in the mutants with respect to the parental strain. However, under iron-deficient conditions (1.25 μ M EDDA), addition of 15 ng vanchrobactin ml^{-1} caused a decrease of between 33 and 36% in the β -galactosidase values in the three assayed strains. This can be explained, as the addition of exogenous vanchrobactin is expected to produce a relaxation in the conditions of iron deficiency, leading to a lighter repression of the *vabG* promoter. Taking all the results into consideration, vanchrobactin is likely not involved in the VabR-mediated activation of the *vabG* promoter.

Transcriptional analysis of *fvbA::lacZ* in a vanchrobactin-deficient *vabB* mutant: dependence on a positive transcriptional regulator

The promoter region of *fvbA* contains a putative AraC box. This suggests that the *fvbA* gene is subjected to some kind of positive regulation. To ascertain this possibility, we introduced the *fvbA::lacZ* fusion into the parental strain as well as into the *vabB* mutant (MB11), and measured the β -galactosidase activities. We used less restrictive iron-limiting conditions (1.25 μ M EDDA) in order to allow growth of the *vabB* mutant and allow the comparison of the *vabB* data with those obtained with the parental strain. Under these conditions, no significant differences were observed in the β -galactosidase activity values between the parental and mutant strain (data not shown). Interestingly,

under iron-rich conditions, we found that the β -galactosidase activity of the *fvbA::lacZ* fusion into the *vabB* mutant was only $1.18 \pm 0.74\%$ of the activity observed under conditions of iron limitation. However, this same fusion into the parental strain RV22 yielded an appreciable basal expression level ($7.3 \pm 2.1\%$ of the expression levels under iron limitation) (Fig. 6). These results indicate that this promoter is downregulated in the absence of vanchrobactin (a sixfold decrease), and suggest that the Fe-siderophore complex plays a positive regulatory role. To test this possibility, we added purified vanchrobactin (at 15 and 30 ng ml^{-1}) to cultures of the *vabB* mutant, and analysed the variation in the β -galactosidase activities of the *fvbA::lacZ* fusion. The results showed an increase higher than threefold in the expression levels of the *fvbA* promoter (Fig. 6). This effect was vanchrobactin-dependent in a linear fashion, since by doubling the amount of vanchrobactin the β -galactosidase activity was similarly doubled. Remarkably, the addition of vanchrobactin to the

Fig. 6. Transcriptional analysis of the *fvbA* gene promoter carried out with *lacZ* fusions. The β -galactosidase activities are shown of cultures growing under iron-sufficient conditions (CM9 plus 10 μ M ferric sulfate). The β -galactosidase activity of transcriptional fusions was assayed in the parental strain RV22 and in the MB11 ($\Delta vabB$) vanchrobactin synthesis-defective mutant. Van 15, addition of vanchrobactin at 15 ng ml^{-1} ; Van 30, addition of vanchrobactin at 30 ng ml^{-1} .

parental strain harbouring the *fvfA::lacZ* fusion also resulted in a similar increase (Fig. 6). We also tested whether vanchrobactin is involved in the regulation of the transcription of other genes of the cluster. The analysis of the *vabH::lacZ*, *vabC::lacZ*, *vabA::lacZ* and *vabR::lacZ* fusions in strain MB11 ($\Delta vabB$) showed no significant differences in gene expression with respect to the parental strain (data not shown).

It has been reported that micro-organisms are able to sense siderophores in the extracellular environment, resulting in an upregulation of the siderophore uptake genes. One of these mechanisms is based on the activation of target genes by means of an AraC-type protein that requires the presence of the cognate siderophore to bind to specific promoter sequences (Anderson & Armstrong, 2004; Brickman *et al.*, 2001; Fetherston *et al.*, 1996; Pelludat *et al.*, 1998). It is tempting to speculate that the siderophore vanchrobactin is the effector molecule, activating a yet-uncharacterized AraC-type regulator that binds at the conserved AraC box in the *fvfA* promoter (Fig. 2). Our observation that the *fvfA* promoter is downregulated in the absence of vanchrobactin reinforces this hypothesis.

Conclusion

The results reported here indicate that the biosynthesis of the siderophore vanchrobactin in *V. anguillarum* proceeds through an NRPS-mediated pathway that is subjected to a complex regulatory network. Positive and negative regulatory mechanisms are involved, likely aimed at adjusting vanchrobactin production for the maintenance of iron homeostasis in *V. anguillarum*. A proposed model for the regulation of vanchrobactin synthesis and uptake is schematically summarized in Fig. 1. Future work will be directed to fill some of the gaps in our current knowledge of the regulatory network.

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