Genetic and biochemical analyses of chromosome and plasmid gene homologues encoding ICL and ArCP domains in *Vibrio anguillarum* strain 775

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Abstract Anguibactin, the siderophore produced by *Vibrio anguillarum* 775 is synthesized from 2,3-dihydroxybenzoic acid (DHBA), cysteine and hydroxyhistamine via a nonribosomal peptide synthetase (NRPS) mechanism. Most of the genes encoding anguibactin biosynthetic proteins are harbored by the pJM1 plasmid. In this work we report the identification of a homologue of the plasmid-encoded *angB* on the chromosome of strain 775. The product of both genes harbor an isochorismate lyase (ICL) domain that converts isochorismic acid to 2,3-dihydro-2,3-dihydroxybenzoic acid, one of the steps of DHBA synthesis. We show in this work that both ICL domains are functional in the production of DHBA in *V. anguillarum* as well as in *E. coli*. Substitution by alanine of the aspartic acid residue in the active site of both ICL domains completely abolishes their isochorismate lyase activity in vivo. The two proteins also carry an aryl carrier protein (ArCP) domain. In contrast with the ICL domains only the plasmid encoded ArCP can participate in anguibactin production as determined by complementation analyses and site-directed mutagenesis in the active site of the plasmid encoded protein, S248A. The site-directed mutants, D37A in the ICL domain and S248A in the ArCP domain of the plasmid encoded AngB were also tested in vitro and clearly show the importance of each residue for the domain function and that each domain operates independently.

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Introduction

Vibrio anguillarum is a marine bacterium responsible for both marine and freshwater fish epizootics throughout the world (Actis et al. 1999). Many serotype O1 strains of *V. anguillarum* possess the 65 kb virulence plasmid pJM1 or pJM1-like plasmids encoding an iron-scavenging system, which is essential for virulence in this bacterium (Conchas et al. 1991; Crosa 1980; Di Lorenzo et al. 2003; Tolmasky et al. 1988a, b). This system includes a low-

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molecular-weight iron-binding compound, anguibactin, which once secreted, competes for bound iron within the host fish (Actis et al. 1988; Wolf and Crosa 1986). The iron-anguibactin complex is then internalized by a transport system that includes the FatA, -B, -C, and -D proteins and requires the TonB2 complex for energy-transduction to the outer membrane (Actis et al. 1988; Stork et al. 2004). Anguibactin possesses both a catechol and a hydroxamate group and it has been characterized as ω-N-hydroxy-ω-N ((2′-(2″,3″-dihydroxyphenyl)thiazolin-4′-yl)carboxy) histamine by crystal X-ray diffraction studies and chemical analysis (Jalal et al. 1989). Retrobiosynthesis and experimental data showed that anguibactin is synthesized from 2,3-dihydroxybenzoic acid (DHBA), cysteine, and hydroxyhistamine (Di Lorenzo et al. 2004a).

The genes encoding proteins involved in the biosynthesis and transport of anguibactin lie on a 25 kb contiguous region of the virulence plasmid (Tolmasky et al. 1988a). An additional gene cluster was identified in a non-contiguous region of pJM1 and the products encoded by the genes in this cluster are homologues of enzymes involved in the biosynthesis and activation of DHBA (Di Lorenzo et al. 2003). We have also reported the presence of this cluster in the pJM1-like plasmid pJHC-1 of strain 531A including the gene encoding the bi-functional AngB protein: its amino end conferring an isochorismate lyase activity required for the production of DHBA and its carboxyl end providing an aryl carrier protein domain, functioning in anguibactin assembly (Welch et al. 2000). Furthermore, the carboxy-terminal end of AngB can be synthesized from the 3′ region of angB, as an independent polypeptide which, like the carboxy terminal portion of AngB, also functions as an aryl carrier protein involved in anguibactin assembly (Welch et al. 2000). This pJHC1 plasmid-mediated angB gene is essential not only for anguibactin biosynthesis but also for the production of DHBA in the 531A strain since when this strain was cured of the pJM1-like plasmid pJHC1, resulting in strain S531A-1, it could no longer produce DHBA (Welch et al. 2000). Previous work in our laboratory had shown that the prototypic strain 775 when cured of the pJM1 plasmid, generating plasmidless strain H775-3, still produced DHBA (Chen et al. 1994). Recently a partial sequence of an open reading frame (orf) with

homology to the plasmid-encoded angB gene was identified in the chromosomal DNA of V. anguillarum 775 within a gene cluster encoding vanchrobactin biosynthesis and transport genes (Alice et al. 2005; Balado et al. 2006; Naka et al. 2008). We report in this work the complete sequence of this chromosomal gene, vabB, from strain 775 and the analyses of its domains as compared to the plasmid-encoded version. Our results also explain the phenotypic differences in DHBA production between the 531A and 775 strains of V. anguillarum.

Materials and methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are described in Table 1. V. anguillarum was cultured at 25°C in either trypticase soy broth or agar supplemented with 1% NaCl (TSBS and TSAS, respectively). For experiments to determine iron uptake characteristics, the strains were first grown on TSAS supplemented with the appropriate antibiotics and then in M9 minimal medium (Sambrook and Russell 2001) supplemented with 0.2% Casamino acid, 5% NaCl, the appropriate antibiotics and either ethylenediamine-di-(o-hydroxyphenyl acetic acid) (EDDA) for iron-limiting condition or ferric ammonium citrate (FAC) for iron-rich conditions. Antibiotic concentrations used for V. anguillarum were ampicillin (Ap) 500–800 µg/ml, rifampicin (Rif) 100 µg/ml, spectinomycin (Sp) 100 µg/ml, chloramphenicol (Cm) 10–15 µg/ml and kanamycin (Km) 100–200 µg/ml.

E. coli strains were grown in Luria–Bertani (LB) medium in the presence of the appropriate antibiotics. Antibiotic concentrations used for E. coli were Ap 100 µg/ml, Sp 100 µg/ml, Cm 30 µg/ml, Km 50 µg/ml and trimethoprim (Tp) 10 µg/ml.

General methods

Plasmid DNA preparations were performed using the alkaline lysis method (Sambrook and Russell 2001). Sequence quality plasmid DNA was generated using Wizard® Plus SV Minipreps (Promega). Restriction endonuclease digestion of DNA was performed under the conditions recommended by the supplier (Invitrogen, Roche, NEB). Transformations in the E. coli

Table 1 Strains and plasmids used in this study

Bacterial strains	Genotype and relevant characteristics	Source or reference
Vibrio anguillarum		
775(pJM1)	Wild type; Pacific ocean prototype	Crosa et al. (1980)
531A(pJHC1)	Wild type; Atlantic ocean prototype	Tolmasky et al. (1988b)
775(pJM1::Ω)	775 carrying pJM1 with Ω transcription-translation terminator at the *Bgl*II site in *angB*	This study
775(pJM1::K)	775 carrying pJM1 with a Klenow modification at the *Bgl*II site in *angB*	This study
775*vabB*::Cm	775 with the *vabB* gene replaced by the chloramphenicol resistance cassette gene	This study
775*vabB*::Cm(pJM1::Ω)	775 with the *vabB* gene replaced by the chloramphenicol resistance cassette gene, carrying pJM1 with Ω transcription-translation terminator at the *Bgl*II site in *angB*	This study
775*vabB*::Cm(pJM1::K)	775 with the *vabB* gene replaced by the chloramphenicol resistance cassette gene, carrying pJM1 with a Klenow modification at the *Bgl*II site in *angB*	This study
CC9-16(pJHC9-16)	Anguibactin-deficient, iron transport-proficient	Walter et al. (1983)
CC9-8(pJHC9-8)	Anguibactin-deficient, iron transport-deficient	Walter et al. (1983)
Escherichia coli		
XL1 blue	*recA1, endA1, gyrA46, thi, hsdR17, supE44, relA, lac*F′ [*proAB*+, *lacI*^{q}, *lacZ*ΔM15 Tn*10*(tet^r)]	Stratagene
HB101	*supE44 hsd20* (r^{-B} m^{-B}) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	Boyer and Roulland-Dussoix (1969)
BLR(DE3)	F⁻ *ompT hsdS*^{B} (r^{-B} m^{-B}) *gal dcm*Δ(*srl-recA)306*::Tn*10*(tet^B) (DE3)	Novagen
AN192	*proC14 leu-6 trpE38 thi-1 fhuA23 lacY1 mtl-1 xyl-5 rpsL109 azi-6 tsx-67 entB402*	Staab and Earhart (1990)
Salmonella typhimurium		
enb-1	LT2 derivative, enterobactin⁻, Sm^r	Pollack and Neilands (1970)
enb-7	LT2 derivative, enterobactin⁻, DHBA⁻, Sm^r	Pollack and Neilands (1970)
Plasmids		
pJM1	Indigenous plasmid in strain 775	Crosa et al. (1980)
pJHC1	Indigenous plasmid in strain 531A	Tolmasky et al. (1988b)
pJHC9-16	pJM1 derivative carrying TAF and transport genes,	Tolmasky et al. (1988a,b)
pJHC9-8	pJM1 derivative carrying only the TAF region	Tolmasky et al. (1988a,b)
pRK2073	Helper plasmid for conjugation, Tp^r, Tra$^+$	Figurski and Helinski (1979)
pCR®2.1-TOPO®	Cloning vector, Ap^r, Km^r	Invitrogen
SuperCos 1	Cosmid vector, Ap^r	Stratagene
pTW-MEV	Suicide vector, R6K ori, *sacB*, Ap^r	Welch et al. (2000)
pTW99	pBR322 with kanamycin resistance gene from pUC4K cloned in *Sal*I site	Welch et al. (2000)
pBR322	Cloning vector, Tc^r, Ap^r	Bolivar (1978)
pKD46	pSC101 oriTS, P$_{araB}$ *g b exo*, Ap^r	Datsenko and Wanner (2000)
pKD3	R6K ori, Cm^r, Ap^r	Datsenko and Wanner (2000)
pQE60	T5 phage promoter expression vector, Ap^r	Qiagen
pET29b	T7 phage promoter expression vector, Km^r	Novagen
Cos#2	SuperCos 1 harboring a 15 kb fragment from 775 genomic DNA	Lab collection

Table 1 continued

Bacterial strains	Genotype and relevant characteristics	Source or reference
pAngB	*angB* gene of plasmid pJM1 cloned by PCR in pTW99	This study
pCR-*vabB*	1.1 kb PCR fragment from 775 genomic DNA containing the *vabB* gene cloned in pCR®2.1-TOPO®	This study
pVabB	*Cla*I-*Bam*HI fragment from pCR-*vabB* cloned in pBR322	This study
pAngB-D37A	pAngB with mutation D37A in ICL domain	This study
pVabB-D71A	pVabB with mutation D71A in ICL domain	This study
pAngB-S248A	pAngB with mutation S248A in ArCP domain	This study
pQEAngB	*angB* cloned as a *Nco*I-*Bgl*II fragment in pQE60	This study
pQEAngB-D37A	pQEAngB with mutation D37A in ICL domain	This study
pQEAngB-S248A	pQEAngB with mutation S248A in ArCP domain	This study
pEntC	*entC* cloned in pET29b	This study
pVibE	*vibE* cloned in pET29b	Keating et al. (2000)
pSfp	*sfp* cloned in pET29b	C. T. Walsh

strains HB101, XL1 blue and BLR(DE3) and other cloning strategies were performed according to standard protocols (Sambrook and Russell 2001). DNA sequencing reactions were carried out by the OHSU-MMI Research Core Facility (http://www.ohsu.edu/core) using a model 377 Applied Biosystems Inc. automated fluorescence sequencer. Sequencing primers were designed using Oligo 6.8® primer analysis software and purchased from the OHSU-MMI Research Core Facility (http://www.ohsu.edu/core) and Invitrogen. DNA and protein sequence analysis were carried out at the NCBI using the BLAST network service (Altschul et al. 1990). The web server Tcoffee (http://igs-server.cnrs-mrs.fr/Tcoffee/tcoffee_cgi/index.cgi) was used for multiple sequence alignment (Notredame et al. 2000). Three-dimensional models of the ArCP domains of AngB and VabB were built using the web server 3D-JIGSAW (http://3djigsaw.com/).

Plasmids were transferred from *E. coli* to *V. anguillarum* by triparental conjugation as previously described (Tolmasky et al. 1988a).

Construction of the complementing clone

The fragment containing the *angB* gene from the pJM1 plasmid was amplified by PCR and cloned into the pBR322 derivative pTW99 as previously described for the *angB* gene of plasmid pJHC1 (Welch et al. 2000). The generated construct, pAngB, was sequenced to confirm that the *angB* gene sequence was not mutated during the PCR amplification or the cloning. A 1.1 kb fragment containing the chromosomal *vabB* gene of strain 775 was amplified by PCR using the VabB-*Cla*IF (5′-CAATCGATTTTTATGCGTAGAC-3′) and the VabB-*Bam*HIR (5′-AGTGGATCCGGTATACTCAAA-3′) primers and genomic DNA from 775 as a template. The forward and the reverse primers were modified to contain restriction endonuclease sites (underlined in the sequence) suitable for cloning downstream of the promoter of the tetracycline resistance gene of pBR322. Reactions consisted of 3 min at 95°C followed by 30 cycles of 45 s at 94°C, 1 min at 55°C, and 2 min at 72°C followed by a single cycle at 72°C for 10 min. The PCR product was cloned in the pCR®2.1-TOPO® vector using the TOPO TA PCR Cloning Kit (Invitrogen). The 1.1 kb *Cla*I-*Bam*HI fragment was subcloned into the *Cla*I-*Bam*HI sites of pBR322 to generate the pVabB plasmid carrying the *vabB* gene. After recloning in pBR322 the entire *vabB* gene was sequenced to verify that no mutation was generated in the gene during amplification or cloning.

Site-directed mutagenesis

Site-directed mutants were generated using the Quickchange™ site-directed mutagenesis kit (Stratagene). Plasmids pAngB and pVabB as template and the primer pairs D37A-U (5′-TCGGTTGTTCTAGTTCACGCTTTACAGGCTTATTTTCTCA-3′)/D37A-L (5′-TGAGAAAATAAGCCTGTAAAGCGTGAACTAGAACAACCGA-3′) and D71A-U (5′-GCGGTACTGCTCATTCACGCTATGCAAAAGTACTT

TATCA-3′)/D71A-L (5′-TGATAAAGTACTTTTGC ATAGCGTGAATGAGCAGTACCGC-3′) were used for the mutation in the ICL domain of the plasmid (pAngB-D37A) and chromosomal (pVabB-D71A) copy, respectively. For the mutant in the ArCP of the plasmid copy primers S248A-U (5′-TGATTTT CCTTGGACTTGATGCGATACGCATAATGACA CT-3′) and S248A-L (5′-AGTGTCATTATGCGTAT CGCATCAAGTCCAAGGAAAATCA-3′) were used with plasmid pAngB as template to generate pAngB-S248A. The procedure was performed according to the manufacturer recommendations, with 16 cycles consisting of: 30 s at 95°C followed by 1 min at 55°C and 12 min at 68°C. For the amplification, *Pfu* polymerase was used and after the PCR, the mixture was treated with *Dpn*I to cleave the parental DNA. Then 1 μl of the mixture was transformed in the XL1 blue chemically competent cells provided with the Quickchange™ kit. Site-specific mutations were confirmed by DNA sequencing with the appropriate primers and the entire mutant genes (*angB* or *vabB*) were sequenced to verify that no other region was affected during mutagenesis.

Construction of *V. anguillarum* mutant strains by allelic-exchange

Mutants 775(pJM1::Ω) and 775(pJM1::K) were generated as previously described by Welch et al. (2000), employing the suicide vector pTW-MEV harboring either the *angB*::Ω or the *angB*::K mutation.

To construct mutant 775*vabB*::Cm, the *vabB* gene was replaced with the chloramphenicol resistance cassette in the cosmid vector Supercos harboring a 15 kb insert containing the *vabB* gene (Cos#2) using the *λ*red system (15). The chloramphenicol acetyl transferase (*cat*) gene was amplified from plasmid pKD3 with primers *vabB*-P1 (5′-GGCTTTAAGG-CATCACGAACGAGAAAAGCCTCTTTTAAC AG TGTAGGCTGGAGCTGCTTC-3′) and *vabB*-P2 (5′-AACTGGTTGCTCTTCAGT AGAACGCGTGTCC GTTACTTGACATATGAATATCCTCCTTAG-3′) that have 40 bases of homology to the *vabB* sequence at their 5′-end and 20 bases of homology with pKD3 at the 3′-end. The PCR product was transferred by electroporation in HB101 cells harboring Cos#2 and pKD46. Cells were made electro-competent by washing the cells grown in the presence of arabinose (0.2%) four times with ice-cold sterile water at 0°C.

After electroporation, cells were plated on LB plates containing Cm at 37°C. At this temperature the pKD46 plasmid is lost and only cells containing Cos#2 where the *vabB* sequence is replaced with the *cat* gene are able to grow. Plasmid isolation and restriction digest was performed to assure no rearrangements occurred during the mutagenesis. The *Not*I fragment containing the entire insert from the cosmid was cloned in vector pTW-MEV. The *vabB* knock-out was obtained using the pTW-MEV vector containing *vabB*::Cm, as described for the plasmid mutants. The same procedure was repeated in 775(pJM1::Ω) and 775(pJM1::K) strains to obtain the double mutants.

Determination of DHBA and anguibactin production

DHBA was assayed by employing the Arnow reaction for cathecols (Arnow 1937). In addition, a growth assay with *Salmonella* strains that require DHBA, was used to confirm the presence of this catechol (Pollack and Neilands 1970).

The siderophore anguibactin was detected by three methods. Chrome azurol S (CAS) plates were used to test production of iron-binding compounds by each strain (Schwyn and Neilands 1987). The results obtained on CAS plates were confirmed by a bioassay using strains CC9-16 and CC9-8 that detect specifically anguibactin synthesis, as previously described (Tolmasky et al. 1988a; Walter et al. 1983). Furthermore, strains were tested for sensitivity to the iron-chelating compound EDDA via growth assay in liquid minimal medium.

AngB protein expression and purification

The complete *angB* gene was cloned in the expression vector pQE60 under the control of the T5 promoter (pQEAngB). Cloning into the *Bgl*II site of pQE60 adds a *C*-terminal hexahistidine tag and two extra amino acids to the overexpressed protein (GSHHHHHH). Site-directed mutations in the overexpression clone were generated as described above using primer pairs D37A-U/D37A-L (pQEAngB-D37A) and S248A-U/S248A-L (pQEAngB-S248A), the *angB* gene cloned in pQE60 as template. The AngB proteins were purified from *E. coli* XL1 blue containing the expression vector. Cultures were

grown in LB broth supplemented with Ap at 37°C to an OD_{600} of 0.3 and then cooled to room temperature. Gene expression was induced with 100 mM isopropyl-b-D-thiogalactopyranoside (IPTG) at 25°C for 4 h. The harvested cells were resuspended in 25 mM Tris, pH 8.0, 300 mM NaCl and 2 mM $MgCl_2$, lysed by sonication and the lysate clarified by centrifugation. The supernatant was incubated with Qiagen Ni–NTA Superflow resin for 16 h at 4°C. Proteins were eluted with a step gradient of five column volumes of lysis buffer containing 25–200 mM imidazole in 25 mM increments. Fractions containing purified AngB proteins (as determined by 12% SDS–PAGE analysis) were pooled and dialyzed against 20 mM Tris, pH 8.0, 50 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT and 10% glycerol. Samples were concentrated using a Centricon YM-3 (Amicon) and quantified from absorbance at 280 nm using the predicted molar extinction coefficient e = 48790 (http://au.expasy.org/tools/protparam.html).

Expression and purification of EntC, Sfp and VibE

The *entC* gene was amplified from *E. coli* HB101 genomic DNA using primers EntC-*Nde*I (5′-TGTG-GAGGATCATATGGATA-3′) and EntC-*Xho*I (5′-CTCGCTCGAGATGCAA TCCA-3′) and cloned in the corresponding sites of the expression vector pET29b. The resulting plasmid pEntC expressed the EntC protein as a translational fusion of a *C*-terminal hexahistidine tag. The construct was transferred to strain BLR(DE3) for expression. EntC was expressed and purified as described above for AngB with few modifications. Cells were grown in LB broth supplemented with kanamycin at 37°C to an OD_{600} of 0.5 prior inductions with 500 mM IPTG. Harvested cells were resuspended in 50 mM Tris, pH 8.0, 300 mM NaCl and 20 mM b-mercaptoethanol and then disrupted by sonication.

The construct overexpressing the Sfp and the VibE proteins as translational fusions to *C*-terminal hexahistidine tags were received from the laboratory of Professor Christopher T. Walsh (Harvard Medical School). VibE was expressed and purified as previously described (Keating et al. 2000). Sfp was expressed and purified following the same procedure of the AngB proteins with minor modification. Strain BLR(DE3) harboring pSfp was cultured in LB broth

supplemented with kanamycin at 37°C to an OD_{600} of 0.6 prior induction with 1 mM IPTG for 5 h at 25°C.

Assay for isochorismate lyase activity

Isochorismate lyase activity of the ICL domain of the AngB protein was determined by a coupled assay with L-lactate dehydrogenase, LDH (Rusnak et al. 1990). In this assay the concentration of pyruvate in the reaction mixture was calculated from the decrease of absorbance at 340 nm due to NADH oxidation. Pyruvate and 2,3-dihydro-2,3-dihydroxybenzoic acid are formed from isochorismic acid in equimolar amounts in the reaction catalyzed by isochorismate lyases. EntC catalyzes the conversion of chorismic acid to isochorismic acid, the substrate of the ICL domain. Reaction mixtures contained 100 mM Tris, pH 7.5, 6 mM $MgCl_2$, 1.3 mM chorismic acid, 76 nM EntC, 80 nM AngB (wild type and mutant proteins), 0.2 mM NADH and 7 units of LDH. The reaction mixtures were incubated at 25°C for 40 min and the OD_{340} measured every 5 min. After 40 min, virtually all the NADH was oxidized in the reaction mixtures containing an active ICL domain.

Analysis of covalent [14C] salicylation of the ArCP domain

Reaction mixtures contained 75 mM Tris, pH 7.5, 10 mM $MgCl_2$, 2 mM tris(carboxyethyl)phosphine (TCEP), 200 mM Coenzyme A, 1 mM Sfp (the phosphopantetheinyl transferase) and 10 mM AngB (wild type and mutant proteins). After 1 h incubation at 25°C to obtain holo-AngB, 10 mM ATP, 100 mM [14C] salicylate and 1 mM VibE (adenylation enzyme) were added to the mixtures and the samples were incubated at 25°C for additional 60 min before precipitation with 10% trichloroacetic acid (TCA) and 60 mg of BSA. Pellets were washed twice with 10% TCA, redissolved in 88% formic acid and submitted for liquid scintillation counting. The percentage of label protein was calculated from the cpm, the specific activity of [14C] salicylate and the moles of AngB protein present in the reaction (Marshall et al. 2002).

The accessory proteins Sfp and VibE used in this assay are not the proteins specific for the AngB protein and the anguibactin system. It was previously shown that Sfp and VibE work to high efficiency in

the phosphopantetheinylation of AngB and activation of the substrate to be loaded on AngB (Marshall et al. 2002). Salycilate is not the actual substrate for VibE or AngB but it was shown to work in this system and the natural substrate DHBA is not available in radioactive-labeled form (Marshall et al. 2002).

Nucleotide sequences accession number

The GenBank accession numbers for the sequences were as follows: for *angB*, AY312585 for strain 775 and AF311973 for strain 531A; for *entC*, M36700.

The sequences of the *vabB* genes of 775 and 531A strains were deposited with GenBank and assigned the accession numbers DQ066620 and DQ066621.

Results

### Identification of a chromosomal *angB* homologue in strain 775

Previous work in our laboratory had shown that strain 775 of *V. anguillarum* when cured of the pJM1 plasmid (H775-3) still produced 2,3-dihydroxybenzoic acid, DHBA (Chen et al. 1994). On the contrary the plasmid-less strain derivative of 531A (S531A-1) could no longer produce DHBA (Welch et al. 2000). Furthermore, it was also shown that in the 531A strain only one of the genes of the DHBA biosynthetic pathway, *angB*, encoded on the pJM1-like plasmid pJHC1 was required to complement strain S531A-1 (Welch et al. 2000). Recently a partial sequence of an open reading frame (*orf*) with homology to the plasmid-encoded *angB* gene was identified in the chromosomal DNA of *V. anguillarum* 775 within the vancrobactin gene cluster (Alice et al. 2005; Naka et al. 2008). Completion of the nucleotide sequence of the chromosomal gene revealed an *orf* of 1038 bp that encodes a predicted protein with 64% identity and 78% similarity to the plasmid-encoded AngB. The chromosomally encoded AngB-homologue (VabB) of 775 possesses the isochorismate lyase (ICL) and the aryl carrier protein (ArCP) domains that are also found in the plasmid-encoded AngB (Fig. 1a). ICL domains are involved in DHBA biosynthesis and catalyze the conversion of isochorismic acid to 2,3-dihydro 2,3-dihydroxybenzoic acid

(Rusnak et al. 1990). The ArCP domain is the domain required for tethering of activated DHBA and thus required for siderophore biosynthesis (Gehring et al. 1997; Welch et al. 2000).

Sequencing of the same chromosomal locus of *V. anguillarum* 531A showed that the same *orf* is present in this strain but a deletion of a base at the 5′-end of the gene resulted in premature termination of translation. As shown in Fig. 1b, in 531A the predicted product of the *vabB* gene is only 94 amino acids long. Welch et al. (2000) previously showed that in 531A the *angB* gene is required for DHBA and anguibactin production, thus the frame-shift generated by the base deletion in the chromosomal *vabB* gene of 531A resulted in the plasmid-encoded AngB becoming essential for DHBA production.

Mutational analysis of the *angB* gene in strain 775

To test the functionality of the *vabB* gene in strain 775 we generated a mutant, 775(pJM1::Ω), consisting of the insertion of the Ω transcriptional-translational terminator (Frey and Krisch 1985) at the *Bgl*II site in the 5′-end of the *angB* gene (Fig. 2). This mutant was tested for its ability to produce DHBA by the Arnow test, specific for catechols, and the results were confirmed by bioassay using *Salmonella* strains *enb-1* and *enb-7* that require DHBA and/or enterobactin to grow in iron limiting conditions. Anguibactin production was determined by CAS agar (data not shown) and growth in iron limiting conditions. To confirm that the iron-binding compound detected on CAS agar is actually anguibactin, bioassays employing strains CC9-8(pJHC9-8) and CC9-16(pJHC9-16) were performed as explained in the "Materials and methods" section. As shown in Fig. 2, strain 775(pJM1::Ω) did not produce anguibactin but it was still able to produce DHBA. Since it is known that in strain 531A, the ArCP domain of AngB can be produced as a polypeptide separate from the ICL domain, a strategy was designed to mutate only the ICL domain. Mutant 775(pJM1::K) is a 4 bp insertion at the *Bgl*II site in *angB*, generated by cleaving the *Bgl*II site and filling in the ends with the Klenow fragment of DNA polymerase I prior to re-ligation. This mutant could still produce anguibactin as determined by CAS and bioassay without any DHBA exogenously added to the media since it was able to
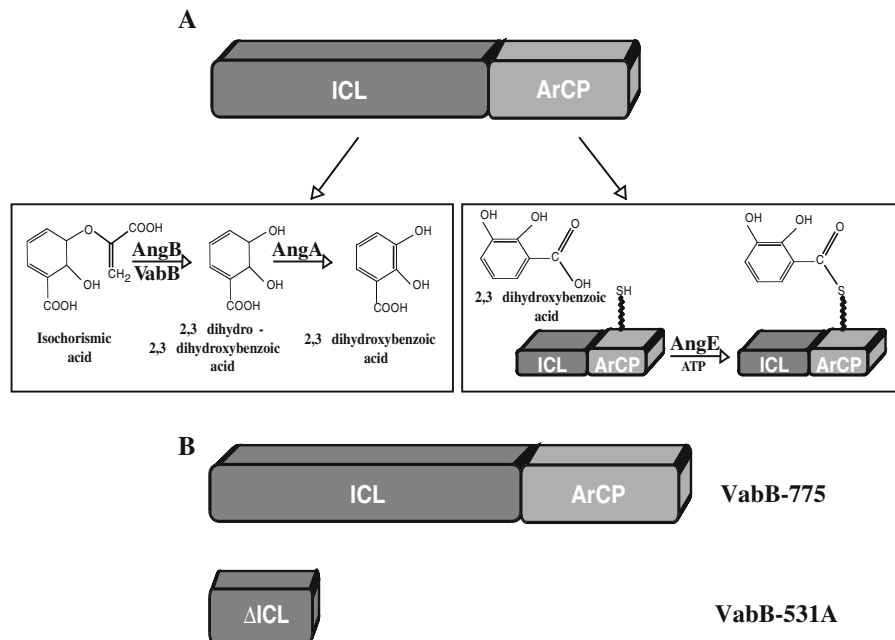
Fig. 1 Domain organization of plasmid-encoded AngB and chromosomally encoded VabB. **A** The ICL and ArCP domain organization of the AngB protein of *V. anguillarum* 775 and their putative function in DHBA and anguibactin production, respectively, are shown. **B** Schematic representation of the predicted *vabB* products from *V. anguillarum* 775 and 531A. The 531A VabB possesses only a truncated ICL domain
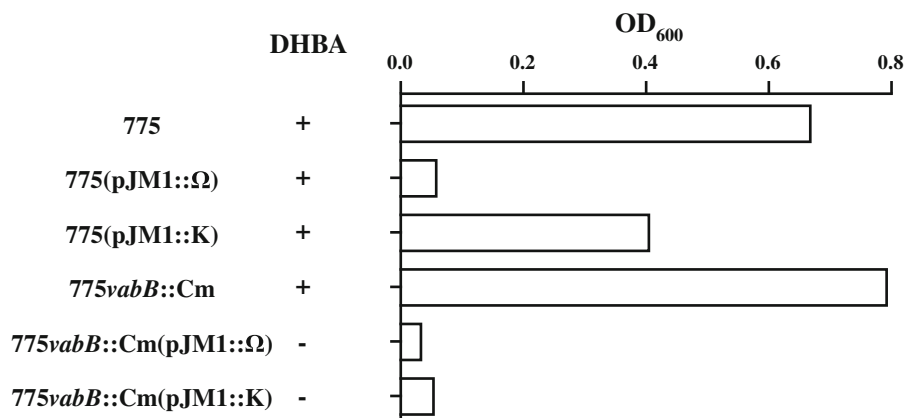
Fig. 2 DHBA and anguibactin production of mutants of the *V. anguillarum angB* and *vabB* genes. The *angB*, *vabB* and the double mutants with their phenotype in DHBA and anguibactin production are shown and compared to the wild type strain. Presence of DHBA in the supernatant of cultures grown in iron-limiting conditions was analyzed by using the Arnow reaction and confirmed by bioassays with *Salmonella* strain *enb-1* and *enb-7*. Anguibactin production by the different strains was detected by CAS agar, bioassays and growth in iron limiting conditions. The results obtained for the growth in iron limiting conditions are represented as the OD_{600} at 48 h of each strain grown in M9 supplemented with 2 μM EDDA. Shown is a representative growth experiment

produce DHBA endogenously (Fig. 2). Therefore, the ICL domain of VabB in 775 must be functional although not essential while the ArCP of AngB is essential for anguibactin biosynthesis. Furthermore,

the results obtained with mutant 775(pJM1::K) indicate that also in 775 the plasmid-encoded ArCP domain can be translated and function independently of the ICL domain.

Mutational analysis of the *vabB* gene and generation of double *angB/vabB* mutant in strain 775

To confirm that the *vabB* gene product is only functional in DHBA production and that the AngB protein possesses the only ArCP domain involved in anguibactin biosynthesis, we generated a mutation in the *vabB* gene by replacing almost the complete gene with a chloramphenicol resistance gene as explained in the "Materials and methods" section. The *vabB* gene was replaced by the *vabB*::Cm construct in 775, 775(pJM1::Ω) and 775(pJM1::K) at its chromosomal location in each strain and the resulting derivatives analyzed for DHBA and anguibactin production. Anguibactin was determined by CAS agar, bioassay and growth of the strains in minimal media supplemented with 2 μM ethylenediamine-di-(*o*-hydroxyphenyl acetic acid), EDDA. The presence of DHBA in the supernatant was determined as described above for the *angB* mutants. Strain 775(pJM1::Ω) although not able to synthesize anguibactin can still produce DHBA (Fig. 2). Strain 775*vabB*::Cm and strain 775(pJM1::K) are not affected in DHBA and in anguibactin production, while both double mutants, 775*vabB*::Cm(pJM1::Ω) and 775*vabB*::Cm(pJM1::K), are unable to produce either compound (Fig. 2). As expected, addition of DHBA to the growth media restored siderophore production in strain 775*vabB*::Cm(pJM1::K), data not shown. Clearly both ICL domains function in the synthesis of DHBA and they are the only two ICL domains present in strain 775 involved in the production of DHBA, an essential precursor of anguibactin. This product can then be incorporated in the siderophore only by the plasmid-encoded ArCP domain.
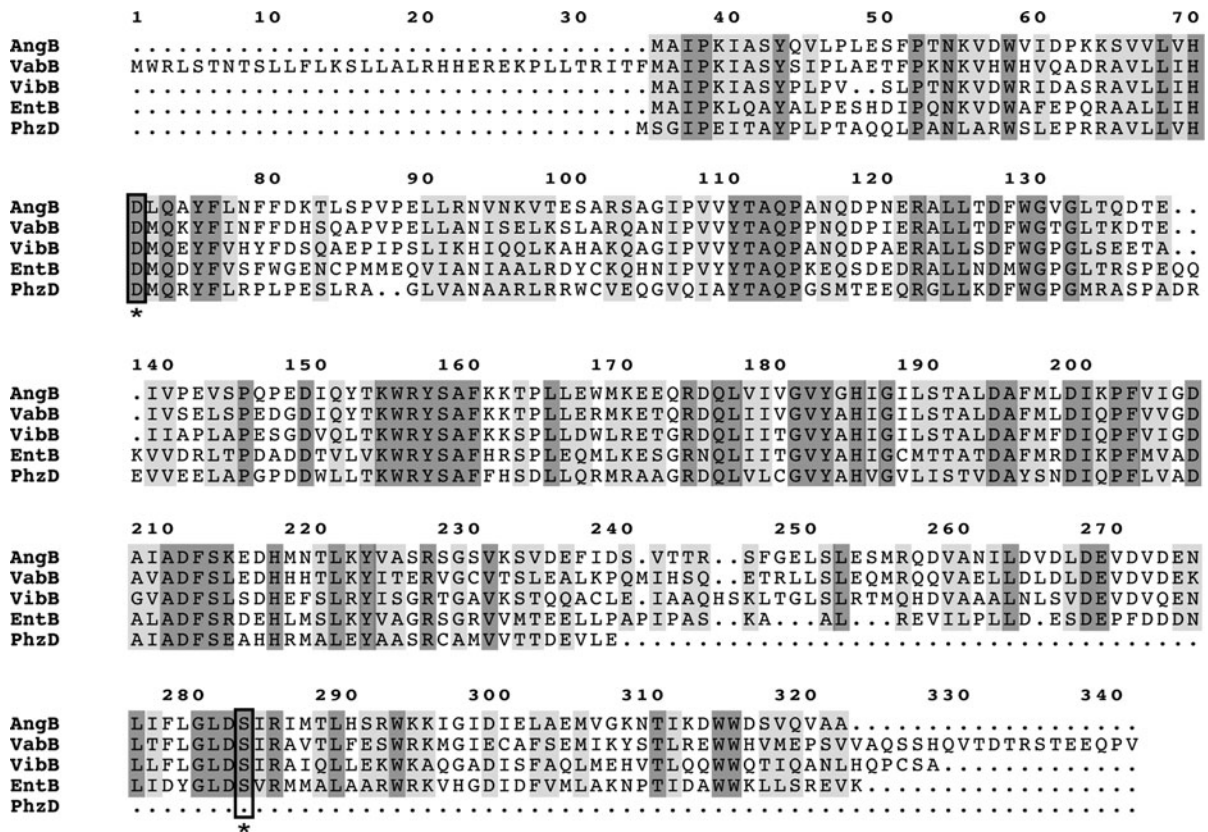
Characterization of the VabB and AngB functions in vivo

The *angB* and *vabB* genes from *V. anguillarum* were cloned in vector pBR322 or its derivative pTW99 downstream of the tetracycline resistance gene promoter generating pAngB and pVabB, respectively. To analyze the functionality of both domains, we mutated conserved residues in the ICL and ArCP domain by site-directed mutagenesis. In the ICL domain the residue to be mutated was chosen based

on an alignment of this domain with several other ICL domains. In the isochorismate lyase PhzD from *Pseudomonas aeruginosa*, an aspartic acid residue in the active site is essential for 2,3-dihydro-3-hydroxyanthranilic acid production from 2-amino-2-deoxyisochorismate (Parsons et al. 2003). The PhzD protein possesses only an ICL domain and it is involved in the biosynthesis of phenazine (Parsons et al. 2003). The same aspartic acid and several surrounding residues are conserved in AngB and VabB as well as in the ICL domain of several enzymes involved in DHBA production (Fig. 3). This conserved aspartic acid residue was mutated to an alanine in the construct harboring either the *angB* or the *vabB* gene generating the constructs pAngB-D37A and pVabB-D71A, respectively. In the ArCP domain of AngB, the residue mutated to an alanine was the conserved serine (S248) that has been shown for the AngB protein of strain 531A to be essential for the attachment of the phosphopantetheinyl arm (Welch et al. 2000). Since we have shown that the ArCP domain of VabB is not functional in anguibactin production, we did not mutate this domain, although it is worthy to note that a conserved serine (S283) in the chromosomally encoded VabB corresponds to the serine residue 248 of AngB and to the conserved serine residue of other ArCP domains (Fig. 3).

The wild type and mutant constructs were used to complement strain AN192 (an *E. coli entB* mutant) as well as the plasmid terminator mutant 775(pJM1::Ω) and the double *angB/vabB* mutant of *V. anguillarum*.

The *E. coli* strain AN192 can be complemented for DHBA production by both wild type *angB* and *vabB* genes of *V. anguillarum* (Fig. 4a). Strain AN192 complemented with pAngB or pVabB were demonstrated to produce enterobactin as determined by growth in iron-limiting conditions and by bioassay with the *Salmonella* strains (Fig. 4a). Enterobactin production in strain AN192 does not require the ArCP domain of AngB since the mutation in *entB* in this strain affects only the functionality of the ICL domain of EntB and not of its ArCP domain (Staab and Earhart 1990). The ICL D to A mutants in either of the pAngB or pVabB constructs were not able to complement strain AN192, clearly showing that the aspartic acid residue is indeed essential for the activity of the ICL domain of both proteins (Fig. 4a).

```
              1        10         20         30         40         50         60         70
AngB    ..............................................MAIPKIASYQVLPLESFPTNKVDWVIDPKKSVVLVH
VabB    MWRLSTNTSLLFLKSLLALRHHEREKPLLTRITFMAIPKIASYSIPLAETFPKNKVHWHVQADRAVLLIH
VibB    ..............................MAIPKIASYPLPV..SLPTNKVDWVRIDASRAVLLIH
EntB    ..........................MAIPKLQAYALPESHDIPQNKVDWAFEPQRAALLIH
PhzD    ..........................MSGIPEITAYPLPTAQQLPANLARWSLEPRRAVLLVH

                       80         90        100        110        120        130
AngB    DLQAYFLNFFDKTLSPVPELLRNVNKVTESARSAGIPVVYTAQPANQDPNERALLTDFWGVGLTQDTE..
VabB    DMQKYFINFFDHSQAPVPELLANISELKSLARQANIPVVYTAQPNQDPIERALLTDFWGTGLTKDTE..
VibB    DMQEYFVHYFDSQAEPIPSLIKHIQQLKAHAKQAGIPVVYTAQPANQDPAERALLSDFWGPGLSEETA..
EntB    DMQDYFVSFWGENCPMMEQVIANIAALRDYCKQHNIPVYYTAQPKEQSDEDRALLNDMWGPGLTRSPEQQ
PhzD    DMQRYFLRPLPESLRA..GLVANAARLRRWCVEQGVQIAYTAQPGSMTEEQRGLLKDFWGPGMRASPADR
        *

             140        150        160        170        180        190        200
AngB    .IVPEVSPQPEDIQYTKWRYSAFKKTPLLEWMKEEQRDQLVIVGVYGHIGILSTALDAFMLDIKPFVIGD
VabB    .IVSELSPEDGDIQYTKWRYSAFKKTPLLERMKETQRDQLIIVGVYAHIGILSTALDAFMLDIQPFVVGD
VibB    .IIAPLAPESGDVQLTKWRYSAFKKSPLLDWLRETGRDQLIITGVYAHIGILSTALDAFMFDIQPFVIGD
EntB    KVVDRLTPDADDTVLVKWRYSAFHRSPLEQMLKESGRNQLIITGVYAHIGCMTTATDAFMRDIKPFMVAD
PhzD    EVVEELAPGPDDWLLTKWRYSAFFHSDLLQRMRAAGRDQLVLCGVYAHVGVLISTVDAYSNDIQPFLVAD

             210        220        230        240        250        260        270
AngB    AIADFSKEDHMNTLKYVASRSGSVKSVDEFIDS.VTTR..SFGELSLESMRQDVANILDVDLDEVDVDEN
VabB    AVADFSLEDHHHTLKYITERVGCVTSLEALKPQMIHSQ..ETRLLSLEQMRQQVAELLDLDLDEVDVDEK
VibB    GVADFSLSDHEFSLRYISGRTGAVKSTQQACLE.IAAQHSKLTGLSLRTMQHDVAAALNLSVDEVDVQEN
EntB    ALADFSRDEHLMSLKYVAGRSGRVVMTEELLPAPIPAS..KA...AL...REVILPLLD.ESDEPFDDDN
PhzD    AIADFSEAHHRMALEYAASRCAMVVTTDEVLE.....................

             280        290        300        310        320        330        340
AngB    LIFLGLDSIRIMTLHSRWKKIGIDIELAEMVGKNTIKDWWDSVQVAA..................
VabB    LTFLGLDSIRAVTLFESWRKMGIECAFSEMIKYSTLREWWHVMEPSVVAQSSHQVTDTRSTEEQPV
VibB    LLFLGLDSIRAIQLLEKWKAQGADISFAQLMEHVTLQQWWQTIQANLHQPCSA............
EntB    LIDYGLDSVRMMALAARWRKVHGDIDFVMLAKNPTIDAWWKLLSREVK..................
PhzD    .......................
                *
```

Fig. 3 Multiple sequence alignment of isochorismate lyase enzymes. All the proteins in this alignment except PhzD participate in DHBA biosynthesis as well as in siderophore biosynthesis. PhzD possesses only the ICL domain and it is involved in the biosynthesis of phenazine. The identical residues are shown shaded in *darker grey* while similar amino acids are shaded in *lighter grey*. The two conserved residues in the ICL and ArCP domains that have been selected to be replaced by an alanine in the AngB or VabB proteins are *boxed* and indicated with an *asterisk*

Complementation of both *V. anguillarum* mutants with pAngB restores anguibactin production while strains containing pVabB still cannot produce the siderophore; nevertheless both constructs in the double mutant restored DHBA production (Fig. 4b). As it was the case in *E. coli*, the ICL mutant constructs could not complement the double *V. anguillarum* mutant for DHBA production (Fig. 4b). Furthermore, the ArCP mutant in AngB (pAngB-S248A) could not complement strain 775(pJM1::Ω) for anguibactin production although it was able to restore DHBA production in strain 775*vabB*::Cm(pJM1::Ω) (Fig. 4b and data not shown). These results further confirm that the ArCP domain of AngB is the only one that can tether DHBA to be utilized in anguibactin production while either of the plasmid or chromosomal ICL domain can intervene in DHBA production.

Activities of the ICL and ArCP domain of AngB in vitro

To assess the functionality in vitro of the AngB domains, we over-expressed the wild type and the mutant proteins as *C*-terminal His-tagged proteins in *E. coli* as described in the "Materials and methods" section.

To determine ICL activity, an L-lactate dehydrogenase-coupled assay was used in which pyruvate concentration in the reaction mixture was calculated from the amount of NADH oxidized, measured by the decrease of absorbance at 340 nm (Rusnak et al. 1990). In the reaction that converts isochorismate to 2,3-dihydro-2,3-dihydroxybenzoic acid catalyzed by ICL domains, pyruvate is produced in equimolar amounts with 2,3-dihydro-2,3-dihydroxybenzoic acid

Fig. 4 Complementation of the *entB* mutant of *E. coli* and the mutants of *V. anguillarum* 775. **A** Complementation of the *E. coli entB* mutant AN92 with the wild type and mutant constructs of *angB* and *vabB* of *V. anguillarum*. The ability to produce DHBA and enterobactin of each strain is measured as the ability to grow in iron-limiting conditions and by bioassay with the *Salmonella* strains *enb1* and *enb7*. Shown is a representative growth experiment. **B** The phenotypes in DHBA and anguibactin production of the complemented *angB* mutants are shown and compared to the wild type strain. DHBA production was measured by the Arnow test on supernatant of cultures grown in iron-limiting conditions. Growth in iron-limiting conditions (minimal media supplemented with 2 μM EDDA) is used to determine the ability of each strain to produce anguibactin. Shown is a representative growth experiment
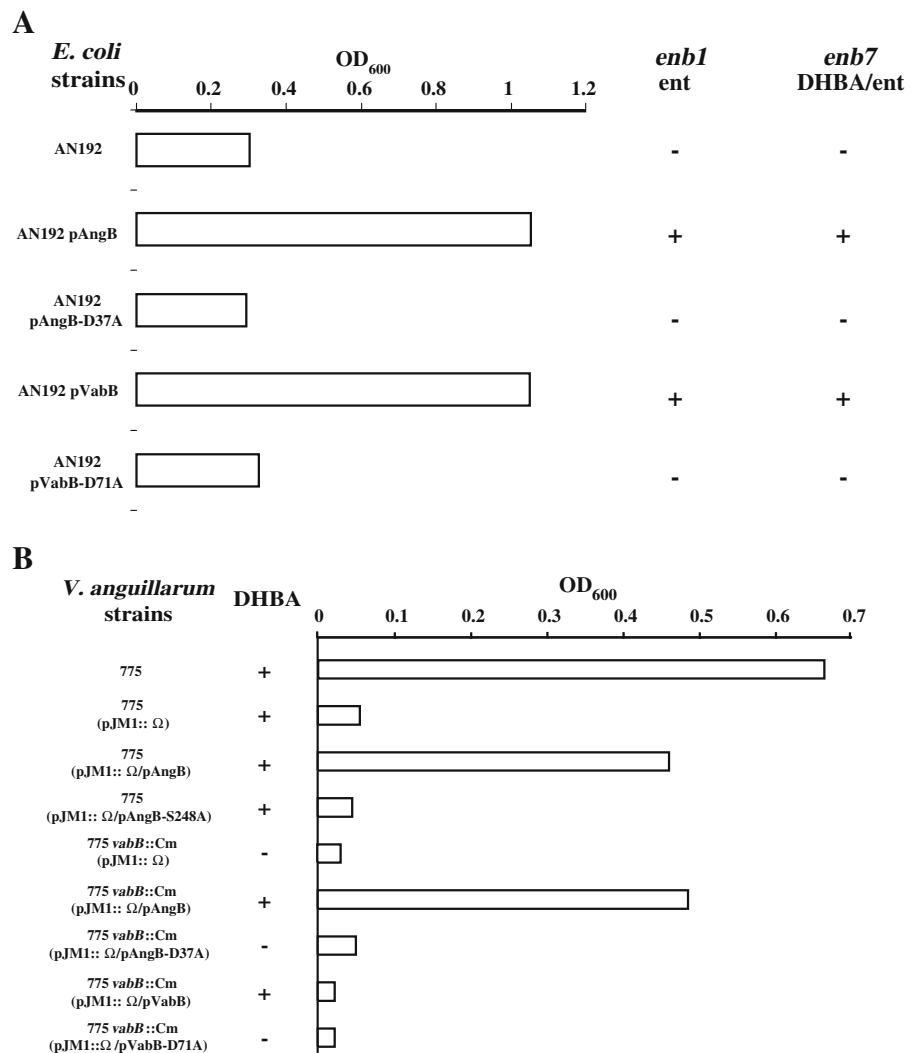
(Fig. 5a). As shown in Fig. 5b, NADH was completely oxidized to NAD^+ in the reaction containing wild type AngB or the ArCP mutant protein. On the contrary the ICL mutant protein behaved as the no-ICL sample in the oxidation of NADH confirming the results obtained in vivo for this mutant protein (Fig. 5b).

Functionality of the ArCP domain was determined by in vitro salicylation with ^{14}C labeled salicylate of the purified proteins (Marshall et al. 2002). Although the wild type AngB protein purified from *E. coli* is obtained partially in its holo form, i.e. with the phosphopantetheine arm already tethered to the ArCP domain (data not shown), holo-ArCP was formed in vitro prior to the salicylation reaction by incubation with the phosphopantetheinyl transferase of *Bacillus*

subtilis, Sfp (Quadri et al. 1998) and coenzymeA (CoA). Figure 6 shows that the wild type AngB protein as well as the ICL mutant can be salicylated in vitro to similar extent while the percentage of the ArCP S248A mutant labeled with [^{14}C] salicylate is similar to that of the sample in which no adenylation domain (VibE) was added. These results are in agreement with the results obtained for the AngB-S248A mutant in anguibactin production in vivo.

Discussion

The iron uptake system mediated by the plasmid pJM1 is composed by the 348 Da siderophore anguibactin that is synthesized from DHBA, cysteine

Fig. 5 In vitro ICL reaction with purified wild type and mutant AngB proteins. **A** Scheme of the in vitro ICL reaction. Isochorismic acid, the substrate for AngB is obtained from chorismic acid by addition of purified EntC protein. Production of pyruvate from isochorismic acid is measured as the oxidation of NADH by L-lactate dehydrogenase in the reaction mix. **B** Production of pyruvate expressed as nanomoles is plotted over time for each AngB protein. Each point is calculated as the mean of three independent experiments in which the same amount of proteins and substrate were used. The *error bars* indicate the standard error of the mean (SEM)
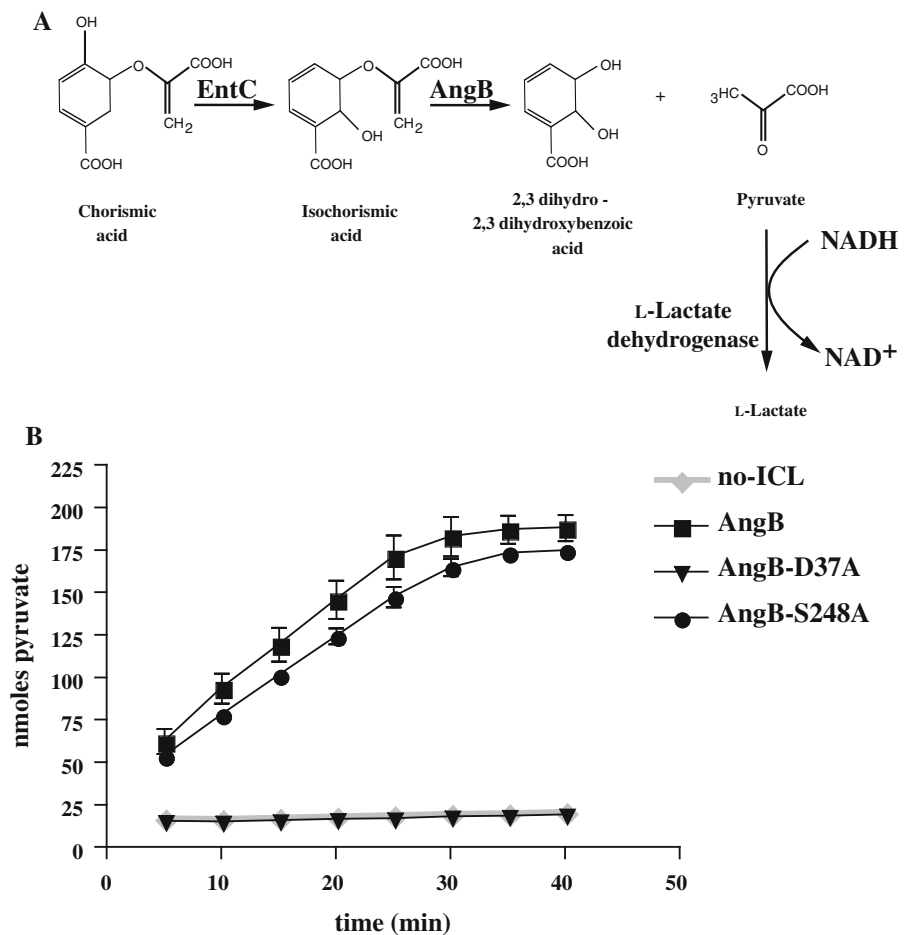
and hydroxyhistamine, and a membrane receptor complex specific for ferric-anguibactin. This system is an important virulence factor for the fish pathogen *V. anguillarum* (Wolf and Crosa 1986).

Sequencing of the pJM1 plasmid of strain 775 revealed a gene cluster (*orf39* to *43*) that encodes products that share homology with proteins involved in synthesis and activation of DHBA (Di Lorenzo et al. 2003). A similar cluster was identified on the pJM1-like plasmid pJHC1 of strain 531A and it was shown that the *angB* gene (*orf41*) in this cluster is essential for DHBA and anguibactin production in this strain (Welch et al. 2000). One interesting difference between these two *V. anguillarum* strains was revealed by the study of their plasmidless derivatives. H775-3, the 775 derivative, is proficient in DHBA production while S531A-1, the 531A derivative, is not. Therefore, we hypothesized that an additional copy of the *angB* gene might be present

on the chromosome of strain 775. We identified an *orf* with homology to the plasmid-encoded *angB* gene in the chromosomal DNA of *V. anguillarum* 775 within the vanchrobactin cluster. Sequencing of this *orf* revealed that it encoded a protein 78% similar and 64% identical to the plasmid-encoded AngB, although 57 amino acids longer. The predicted amino acid sequence of the chromosomal homologue includes the ICL and ArCP domains as it is the case for the AngB protein. Surprisingly, the same sequence was found on the 531A chromosomal DNA but deletion of a base at the 5′-end resulted in a non-functional truncated protein as the truncation occurs in the ICL domain before the conserved residues of the active site.

Furthermore, in this work we also characterized the two homologues of 775 and assessed their functions by a combination of knock-out and site-directed mutations. We clearly showed that the ArCP
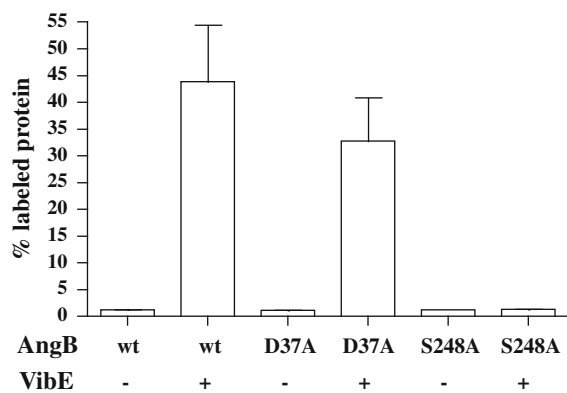
Fig. 6 In vitro salicylation reaction with purified wild type and mutant AngB proteins. The percentage of ArCP domain labeled with [^{14}C]-salicylate is shown with each *bar* calculated from the mean of three independent experiments in which the same amounts of proteins and substrate where used. As controls each AngB protein was incubated without the adenylating enzyme VibE. The means for the wild type and the D37A mutant AngB proteins are not significantly different when compared by the *t*-test at $P \leq 0.05$ ($P = 0.45$). The *error bars* indicate the standard error of the mean of the results from replicate samples. wt, wild type AngB; D37A, D37A mutant of AngB; S248A, S248A mutant of AngB

domain of the plasmid-encoded copy is essential for anguibactin biosynthesis, while the ICL domain of either the chromosomal or the plasmid homologue can be used in DHBA production. The two ICL domains can also complement an *E. coli entB* mutant for DHBA production.

Site-directed mutagenesis of the ICL domain of the two proteins of 775 confirmed that the active site of this domain corresponds to the active site of PhzD, an isochorismate lyase that is not part of the DHBA biosynthetic pathway and is not associated with an ArCP domain (Parsons et al. 2003). In vitro experiments with the purified plasmid-encoded AngB protein demonstrated that the site-directed mutant in the ICL domain was affected in the isochorismate lyase activity but its ArCP domain could still be loaded with [^{14}C]-salicylate as it is the case for the wild type protein. The same was true for the ArCP domain mutant (S248A), whose ability to convert isochorismate to 2,3-dihydro-dihydroxybenzoic acid was not altered while the loading of [^{14}C]-salicylate was completely abolished. Thus, the in vitro results confirmed those obtained in vivo in *V. anguillarum* and *E. coli*. Furthermore, the in vitro experiments with the site-directed mutants (D37A and S248A) clearly showed the importance of each residue for the domain function.

The identification and characterization of the chromosomally encoded homologue *vabB* add another chapter to the complex tale of the iron-uptake systems of *V. anguillarum* species. The chromosomally encoded gene lies within a cluster of other genes dedicated to DHBA biosynthesis and activation and these genes have been shown to be part of the vanchrobactin biosynthetic pathway (Alice et al. 2005; Balado et al. 2006; Naka et al. 2008). Naka et al. (2008) showed that the ArCP of VabB perfectly functions in vanchrobactin assembly line. The fact that this clearly functional ArCP domain is not able to participate in anguibactin production suggests a system specificity of the AngB-ArCP domain for the anguibactin system.

In the enterobactin system, three residues have been identified that, when mutated, result in a decreased rate of enterobactin production (Lai et al. 2006). Two of these residues map within helix 3 of EntB crystal structure and helix 3 is suggested to be the major recognition element on EntB-ArCP domain for EntF, the downstream elongation module (Drake et al. 2006; Lai et al. 2006). AngB-ArCP and VabB-ArCP homology models were generated using 3D JIGSAW web server (Bates et al. 2001). Interestingly, the two models differ in helix 3 where AngB-ArCP seems to lack in the model a helix 3 and to have a longer helix 2, whereas the VabB-ArCP domain shows a 4 helices model highly similar to the EntB structure. As a consequence, we propose that other recognition elements in the ArCP domain are required for the interaction of AngB with AngM, the downstream module (Di Lorenzo et al. 2004b). The proposed difference in the interaction-surface of the two ArCP domains could explain the inability of the VabB-ArCP domain to function in anguibactin production.

Furthermore, short *N*-terminal and *C*-terminal sequences have been shown to be involved in protein–protein interactions in the NRPS family (Hahn and Stachelhaus 2004). The VabB protein presents amino acid extensions at both ends when compared with the other homologues including *V. anguillarum* AngB and these extra sequences at the *N*- and *C*-terminus could be the specific signature of the vanchrobactin system that prevent VabB from interacting with the other proteins of the anguibactin system. The system specificity of the anguibactin pathway does not apply to DHBA biosynthesis since

the ICL domain activity seems to be independent of the other enzymes and strain background.

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