Biometals (2014) 27:559–573
DOI 10.1007/s10534-014-9729-4

Functional analysis of pyochelin-/enantiopyochelin-related genes from a pathogenicity island of *Pseudomonas aeruginosa* strain PA14

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Received: 12 March 2014 / Accepted: 17 March 2014 / Published online: 30 March 2014

Abstract Genomic islands are foreign DNA blocks inserted in so-called regions of genomic plasticity (RGP). Depending on their gene content, they are classified as pathogenicity, symbiosis, metabolic, fitness or resistance islands, although a detailed functional analysis is often lacking. Here we focused on a 34-kb pathogenicity island of *Pseudomonas aeruginosa* PA14 (PA14GI-6), which is inserted at RGP5 and carries genes related to those for pyochelin/enantiopyochelin biosynthesis. These enantiomeric siderophores of *P. aeruginosa* and certain strains of *Pseudomonas protegens* are assembled by a thiotemplate mechanism from salicylate and two molecules of cysteine. The biochemical function of several proteins encoded by PA14GI-6 was investigated by a series of complementation analyses using mutants affected in potential homologs. We found that *PA14_54940* codes for a bifunctional salicylate synthase/salicyl-AMP ligase (for generation and activation of salicylate), that *PA14_54930* specifies a dihydroaeruginoic acid (Dha) synthetase (for coupling salicylate with a cysteine-derived thiazoline ring), that *PA14_54910* produces a type II thioesterase (for quality control), and that *PA14_54880* encodes a serine *O*-acetyltransferase (for increased cysteine availability). The structure of the PA14GI-6-specified metabolite was determined by mass spectrometry, thin-layer chromatography, and HPLC as (*R*)-Dha, an iron chelator with antibacterial, antifungal and antitumor activity. The conservation of this genomic island in many clinical and environmental *P. aeruginosa* isolates of different geographical origin suggests that the ability for Dha production may confer a selective advantage to its host.

Keywords Genomic island · Siderophore · *Pseudomonas* · Pyochelin · Dihydroaeruginoic acid

Electronic supplementary material The online version of this article (doi:10.1007/s10534-014-9729-4) contains supplementary material, which is available to authorized users.

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Introduction

The Gram-negative bacterium *Pseudomonas aeruginosa* is found ubiquitously in soil and aquatic environments and is virulent towards a large variety of organisms including plants, nematodes, amoeba, insects, and mammals (D'Argenio et al. 2001; Gallagher and Manoil 2001; Plotnikova et al. 2000; Pukatzki et al. 2002). Over the past century, it has

emerged as an important opportunistic human pathogen causing several nosocomial infections (Van Delden and Iglewski 1998). More notably, *P. aeruginosa* is recognised as the main cause for chronic lung infection in patients suffering from the genetic disease cystic fibrosis (Lyczak et al. 2002). The ability of *P. aeruginosa* to cause a wide range of acute and chronic infections is based not only on its large arsenal of secreted virulence factors and secondary metabolites but also on its metabolic versatility and high potential for adaptation. Not surprisingly therefore, the *P. aeruginosa* genome is larger than those of most sequenced bacteria and varies between 5.5 and 7 Mbp (Schmidt et al. 1996; Lee et al. 2006). The difference in genome size between strains results from the so-called accessory genome which consists of extrachromosomal elements (e.g. plasmids) and of chromosomal insertions of foreign DNA blocks acquired by horizontal gene transfer. These insertions tend to cluster at certain loci which have been termed "regions of genomic plasticity" (RGP; Mathee et al. 2008). The presence of these chromosomal inserts may confer a selective advantage to the strain and, according to their gene content, these inserts are tentatively assigned as pathogenicity, symbiosis, metabolic, fitness, or resistance islands (Juhas et al. 2008). In many cases however, a functional analysis of the relevant genes has not yet been carried out.

Here we focus on a DNA block inserted at RGP5 in the highly virulent strain PA14 (Mathee et al. 2008). This block, which carries 23 genes (*PA14_54850 to PA14_55090*), was originally termed genomic island 6 (PA14GI-6; Song and Zhang 2009) but later described as PA14GI-3 (Song and Zhang 2011); we will use the original designation PA14GI-6 here. Sequence analysis predicted that PA14GI-6 qualified as a pathogenicity island as it carries genes for the biosynthesis of a putative siderophore, for TonB-dependent receptors and ABC transport systems (Song and Zhang 2009). Indeed, some of the PA14GI-6 genes closely ressemble those specifying pyochelin (Pch) and enantiopyochelin (EPch) (Fig. 1), two enantiomeric siderophores produced by *P. aeruginosa* and *P. protegens*, respectively (Youard et al. 2011). Biosynthesis of Pch and EPch proceeds via a thiotemplate mechanism in which salicylate is coupled to two molecules of cysteine which are cyclized and modified during the assembly (Fig. 2; reviewed by Youard et al. 2011). Here we investigate the function of the Pch/EPch-related siderophore biosynthesis genes of PA14GI-6 and we determine the structure of the molecule they specify.

Materials and methods

Bacterial strains, plasmids and culture conditions

Table 1 lists the strains and plasmids used in this study. Bacteria were routinely cultivated at 37 °C (*Escherichia coli* and *Pseudomonas aeruginosa*) and 30 °C (*Pseudomonas protegens*) on nutrient agar and in nutrient yeast broth (Stanisich and Holloway

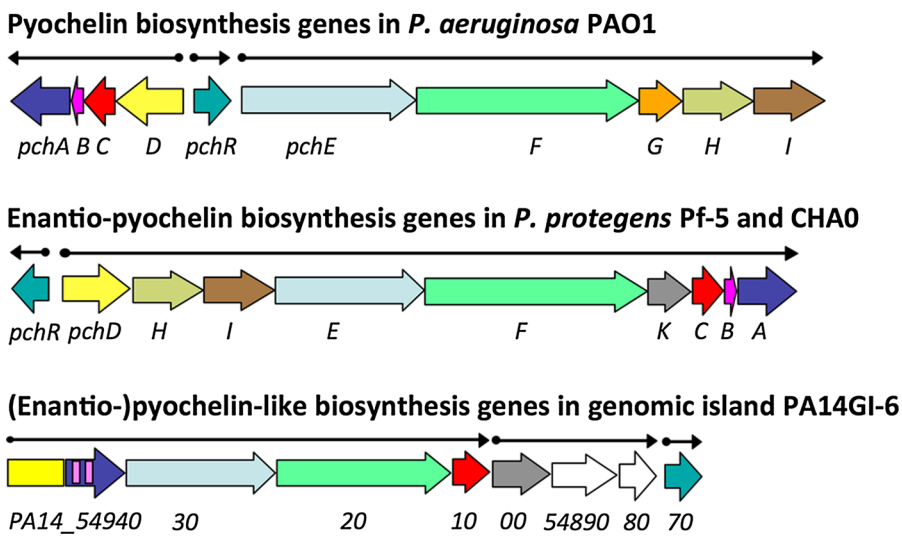

Fig. 1 Pch and EPch biosynthesis genes of *P. aeruginosa* and *P. protegens*, respectively, and their potential counterparts in genomic island PA14GI-6. Genes with significant homology are colored identically

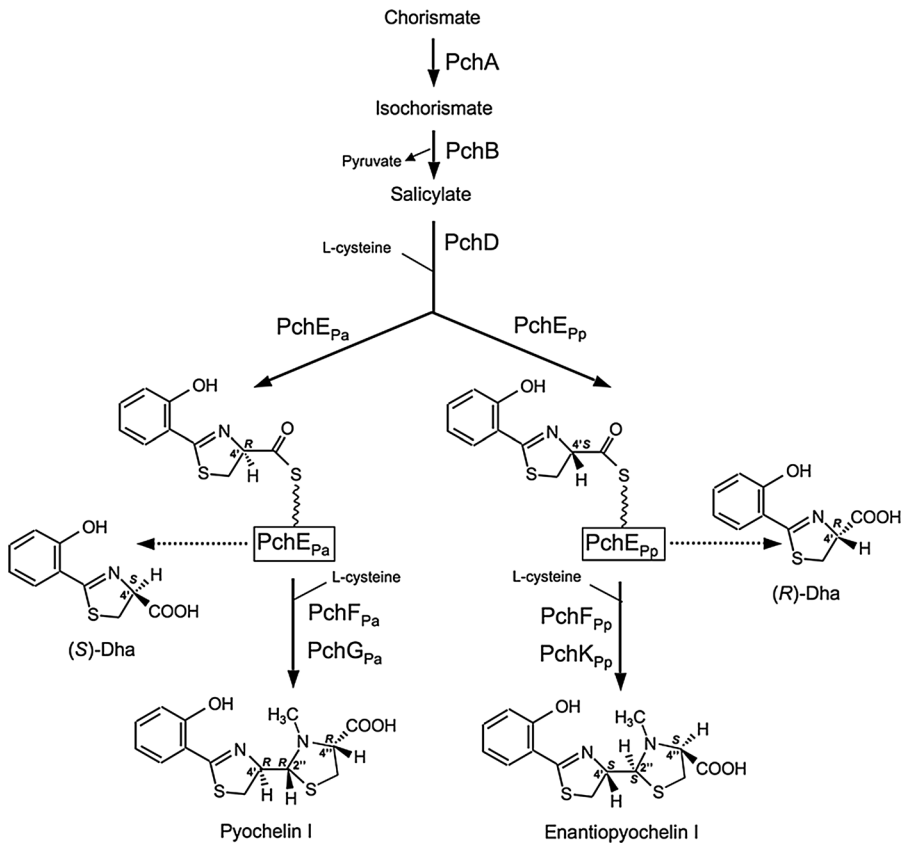

Fig. 2 Biosynthesis of Pch and EPch in *P. aeruginosa* and *P. protegens*, respectively. Both siderophores are produced by a thiotemplate mechanism from salicylate and two cysteine molecules, which are cyclized during the assembly and undergo a certain number of modifications (reviewed in Youard et al. 2011). For Pch, these are (1) epimerization of the first cysteine by PchE, (2) reduction of the second thiazoline ring by PchG and (3) subsequent *N*-methylation by PchF. EPch assembly is essentially identical except that it is the second cysteine which is epimerized by a so far uncharacterized mechanism and that reduction appears to be carried out by PchK which has no sequence homology with PchG. Note that premature hydrolysis of the thioester bond of the PchE-attached compound can generate the byproduct Dha (Serino et al. 1997; Patel et al. 2003; Quadri et al. 1999)

1972). To facilite uptake of heterologous DNA during conjugation and transformation, *P. aeruginosa* and *P. protegens* strains were grown at 43 and 35 °C, respectively. For metabolite extraction, bacteria were grown in GGP medium (Carmi et al. 1994) in which iron is present but not immediately accessible, thus inducing the expression of siderophore biosynthesis genes. Production of siderophores was assessed on solid Chrome Azurol S (CAS) agar (Schwyn and Neilands 1987), which turns orange in the presence of iron-chelating molecules. Amino acid complementation experiments with *E. coli* strain MG1655-C were carried out on solid M9 medium with 0.5 % glycerol as a carbon source (Sambrook and Russell 2001). When needed, antibiotics were added to the media as follows. For *E. coli* ampicillin (Ap) was used at 100 μg ml^{-1} while kanamycin (Km) and tetracycline (Tc) were used at 25 μg ml^{-1}. For selection of Ap-resistant plasmids in *P. aeruginosa*, carbenicillin (Cb) was used at 250 μg ml^{-1}. Tc was used at 125 μg ml^{-1} for both *P. aeruginosa* and *P. protegens*. Counterselection of *E. coli* donor cells during mutant construction occurred with chloramphenicol (Cm) at 10 μg ml^{-1}; mutant enrichment was performed with tetracycline at 20 μg ml^{-1} and carbenicillin at 2 mg ml^{-1} (for *P. aeruginosa*) or cycloserine at 50 mg ml^{-1} (for *P. protegens*). To activate transcription from *tac* promoters, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at 1 mM final concentration.

Table 1 Bacterial strains and plasmids

Name	Relevant characteristics	Reference/Source
E. coli strains		
DH5α	*recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1* Δ(*lacZYA-argF*) *U169* (ϕ 80d*lacZ*ΔM15)	Sambrook and Russell (2001)
MG1655	Wild type	Bachmann (1987)
MG1655-C	Δ*cysE*	Sturgill et al. (2004)
P. aeruginosa strains		
PALS128	*pvdB*	Visca et al. (1992)
PAO6334	*pvdB*, *pchG*::ΩSp/Sm	This study
PAO6342	*pvdB,* Δ*pchC*	Reimmann et al. (2004)
PAO6382	Δ*pvdF*	Hoegy et al. (2009)
PAO6383	Δ*pvdF,* Δ*pchBA*	Michel et al. (2007)
PAO6398	Δ*pvdF, pchD*::ΩSp/Sm	This study
PAO6399	Δ*pvdF,* Δ*pchE*	Youard et al. (2007)
PAO6402	Δ*pvdF, pchR*::ω	Youard and Reimmann (2010)
PA14	Wild type	Lee et al. (2006)
PA14-98	PA14 Δ*pvdF*	This study
PA14-99	PA14 Δ*pvdF* Δ*pchDCBA* Δ*pchR* Δ*pchEFGHI*	This study
PA14-106	PA14-99 with *PA14_54940* to *PA14_54910* under P_{tac} control	This study
PA14-109	PA14-99 with *PA14_54940* to *PA14_54910* under P_{tac} control and *PA14_54900* to *PA14_54880* under $lacI^{Q}$-P_{tac} control	This study
A30	Environmental isolate Pa4, Algeria	N. Benzidane
A31	Environmental isolate Pa5, Algeria	N. Benzidane
A34	Environmental isolate Pa20, Algeria	N. Benzidane
A35	Environmental isolate Pa22, Algeria	N. Benzidane
A44	Clinical isolate H3471, University hospital Lausanne, Switzerland (CHUV)	D. Blanc
A47	Clinical isolate H7, CHUV	D. Blanc
A90	Clinical isolate 7B5, University hospital Geneva, Switzerland (HUG)	C. Van Delden
A93	Clinical isolate 36D5, HUG	C. Van Delden
A97	Environmental isolate 10289, CHUV	D. Blanc
A107	Environmental isolate 12049, CHUV	D. Blanc
A112	Environmental isolate 13072, CHUV	D. Blanc
A116	Environmental isolate 7800, CHUV	D. Blanc
A117	Environmental isolate 7852, CHUV	D. Blanc
A118	Environmental isolate 7854, CHUV	D. Blanc
A120	Environmental isolate 7868, CHUV	D. Blanc
A122	Environmental isolate 8093, CHUV	D. Blanc
A123	Environmental isolate 8510, CHUV	D. Blanc
A124	Environmental isolate 8727, CHUV	D. Blanc
A134	Clinical isolate 822, CHUV	D. Blanc

Table 1 continued

Name	Relevant characteristics	Reference/Source
A135	Clinical isolate 1161, CHUV	D. Blanc
A137	Clinical isolate 2114, CHUV	D. Blanc
P. protegens strains		
CHA1222	Δ*pchR*, *pvd* ::Tn*1733*, Km^r	Youard and Reimmann (2010)
CHA1238	Δ*pvdF*	Reimmann (2012)
CHA1262	Δ*pvdF*, Δ*pchK*	This work
CHA1264	Δ*pvdF,* Δ*pchE*	This work
Plasmids		
pET21a-*cysE*	pET21a derivative carrying *cysE* of *E. coli*, Ap^r	Sturgill et al. (2004)
pME497	Mobilizing plasmid, Ap^r	Voisard et al. (1994)
pME3087	Suicide vector, ColE1 replicon, Tc^r	Voisard et al. (1988)
pME6000	pBBR1-based cloning vector, Tc^r	Maurhofer et al. (1998)
pME6032	$lacI^Q$-P_{tac} expression vector, Tc^r	Heeb et al. (2002)
pME6123	Identical with pME3087-*pchD*::ΩSp/Sm, Tc^r, Sp/Sm^r	Serino et al. (1997)
pME6176	Suicide plasmid to delete *pchDCBA*, *pchR*, and *pchEFGHI* in *P. aeruginosa*, Tc^r	Reimmann et al. (2001)
pME6180	Suicide plasmid to generate *pchG*::ΩSp/Sp in *P. aeruginosa*,Tc^r	Reimmann et al. (2001)
pME7152	Suicide plasmid to delete *pvdF* in *P. aeruginosa*,Tc^r	Michel et al. (2007)
pME9244	Suicide plasmid to delete *pchK* in *P. protegens*,Tc^r	This study
pME9751	Suicide plasmid to delete *pchE* in *P. protegens*,Tc^r	This study
pME10075	pME6032 with *PA14_54870* under P_{tac} control, Tc^r	This study
pME10201	pME6000 with *PA14_54930* under P_{lac} control, Tc^r	This study
pME10202	pUCPSK with *PA14_54940* under P_{lac} control, Ap^r	This study
pME10205	pME6000 with *PA14_54900* under P_{lac} control, Tc^r	This study
pME10208	pME3087 carrying a 1.2 kb BamHI-HindIII fragment containing the 3′ part of *PA14_54950* and the 5′ part of *PA14_54940* under P_{tac} control, Tc^r	This study
pME10209	pME6032 with *PA14_54880* to *PA14_54900* under P_{tac} control, Tc^r	This study
pME10219	pME3087 carrying a 2.8 kb EcoRI-XbaI fragment containing the 3′ part of *PA14_54910, lacI*^{Q}- P_{tac}, and the 5′ part of *PA14_54900*, Tc^r	This study
pME10225	pUCPSK with *PA14_54910* under P_{lac} control, Ap^r	This study
pUCPSK	ColE1-pRO1600 shuttle vector, Ap^r	Watson et al. (1996)

DNA manipulations and sequencing

DNA manipulations were carried out according to standard procedures (Sambrook and Russell 2001). Preparations of plasmid DNA were made using Jetstar (Genomed GmbH) and QIAprep Spin Miniprep (Qiagen, Inc.) kits. Chromosomal DNA of *P. aeruginosa* and *P. protegens* was purified as described by Gamper et al. (1992). DNA fragments were extracted from gels and purified with the Invisorb® fragment cleanUp kit from Invitek. For Southern blotting, chromosomal DNA of *P. aeruginosa* strains was digested overnight with BamHI, separated on a 0.8 % agarose gel, and transferred to a Hybond-N nylon membrane (Amersham Biosciences). DNA probe labeling, hybridization, and detection were done with the DIG DNA labeling and detection kit (Roche) according to the manufacturer's procedures. A DNA probe covering the entire genomic island was prepared from 7 PCR fragments generated from chromosomal DNA of PA14 using the primer pairs GI-1/GI-2bis, GI-5/GI-6, GI-7/GI-8, GI-9bis/GI-10bis, GI-13/GI-14, GI-17bis/GI-18bis, and GI-21bis/GI-22bis (Table S1). Transformations of bacterial strains were done by electroporation (Farinha and Kropinski 1990). Constructs based on PCR techniques (for oligonucleotides see Table S1) were sequenced commercially by GATC Biotech.

Construction of mutants and overexpression strains

Gene replacement mutants of *P. aeruginosa* and *P. protegens* (Table 1) were generated as described (Schnider et al. 1995; Ye et al. 1995; Laville et al. 1998). The suicide plasmids carrying the relevant mutations were constructed as follows. First, two PCR fragments were amplified using the template DNA and primer pairs listed in Table S2. Then, the two fragments were combined by overlap extension PCR, and cloned into pME3087, using the restriction sites introduced by the two flanking PCR primers (see Tables S1 and S2). For construction of the suicide plasmid pME10219, which was used to place the *PA14_54900* to *PA14_54880* operon under $lacI^{Q}$-P_{tac} control, overlap extension PCR was used to combine three PCR fragments as detailed in Tables S1 and S2.

The resulting suicide plasmids were mobilized from *E. coli* DH5α to the *P. aeruginosa* and *P. protegens* recipient strains using the helper plasmid pME497 and subsequently integrated into the chromosome with selection for tetracycline resistance. Excision of the vector via a second crossing-over was obtained by enrichment for tetracycline-sensitive cells. Mutants were identified by PCR and sequence analysis. Supplementary Table S2 lists the primer pairs used for generating the suicide plasmids, the *P. aeruginosa* and *P. protegens* recipient strains used for mutant construction, and the designation of the mutants generated in this way.

PCR screening to identify potential GI-6 carriers

Pseudomonas aeruginosa isolates were screened for the presence of GI-6 using the degenerate primers DpchE1 and DpchE5 (Table S1) which were designed based on sequence comparison of *pchE* from *P. aeruginosa*, *P. protegens*, *Burkholderia cenocepacia*, *Burkholderia pseudomallei* and *Burkholderia thailandensis*. The primers specify the amino acid sequences HWRSIPY and NGKV/I/LDR which flank the epimerase domain of PchE from *P. aeruginosa* strain PAO1. PCR amplification was performed with GoTaq polymerase (Promega) and whole cell templates in the presence of 5 % DMSO. After initial template denaturation at 95 °C for 2 min, 5 cycles of amplification were performed with denaturation at 95 °C for 1 min, annealing at 65 °C for 1 min, and elongation at 72 °C for 2 min. Then, another 25 cycles were carried out during which the annealing temperature was lowered to 58 °C. A final extension was done during 3 min. These conditions amplified a fragment of 1,409 bp, characteristic for a *pchE* gene encoding an epimerase domain (such as *pchE* of *P. aeruginosa* PAO1), and a fragment of 524 bp corresponding to a *pchE* homolog devoid of sequences specifying an epimerase domain (such as *PA14_54930* or *pchE* of *P. protegens*).

Construction of expression plasmids

To analyse the function of PA14GI-6-encoded genes in complementation experiments a series of expression plasmids were constructed. For constitutive expression of *PA14_54940* (plasmid pME10202) and *PA14_54910* (plasmid pME10225) from P_{lac}, PCR fragments of 3 kb (amplified from chromosomal DNA of PA14 using primers 54940fw and 54940bw2) and

Table 2 Predicted functions of PA14-GI6 specified proteins based on bioinformatics analyses

Protein of PA14GI-6	Protein size (amino acids)	Homologous protein[a]	Identical amino acids (%)	Amino acid stretch with homology	Predicted function of the PA14GI-6 protein
PA14_54940	994	PchD (Pa)	52	9–522	Bifunctional protein with salicylate synthase and salicyl-AMP ligase activities
		PchA (Pa)	30	693–975	
		Irp9 (Ye)	41	567–981	
PA14_54930	1,113	PchE (Pp)	48	1–1,062	Dha synthetase
PA14_54920	1,487	PchF (Pp)	45	19–933	Desmethyl-enantiopyochelin synthetase
		PchF (Pp)	38	955–1,055	
PA14_54910	271	PchC (Pp)	43	14–271	Type II thioesterase
PA14_54900	384	PchK (Pp)	31	12–333	Reductase
PA14_54890	634	No homology to Pch proteins of Pa and Pp			Motifs indicate saccharopine dehydrogenase, NAD binding and ATC hydrolase activities
PA14_54880	292	CysE (Ec)	50	51–287	Serine acetyl transferase
PA14_54870	302	PchR (Pa)	34	143–289	AraC-type regulator

[a] Note that if homology was detected to proteins of *P. aeruginosa* (Pa) and *P. protegens* (Pp), only the protein with stronger homology is listed

0.9 kb (primer pair AM-36/AM-37) were cloned via BamHI/SacI and BamHI/XhoI, respectively, into pUCPSK. For constitutive expression of *PA14_54930* (plasmid pME10201) and *PA14_54900* (plasmid pME10205), PCR fragments of 3.5 kb (obtained from PA14 with primer pair AM-1/AM-2) and 1.1 kb (primer pair AM-11/AM-12), were cloned into pME6000 between the restriction sites HindIII/EcoRI and EcoRI/BamHI, respectively. Inducible expression of the entire *PA14_54900-PA14_54890-PA14_54880* operon under P_{tac} control was obtained with pME10209 as follows. A 3.8-kb PCR fragment was PCR amplified from PA14 using the primer pair AM-11 and AM-13, trimmed with EcoRI and BglII and cloned into pME6032 between the same sites. The *PA14_54870* expression plasmid pME10075 was constructed in a similar way. The gene was PCR-amplified from PA14 DNA with the primer pair PA14-AraC-fw/PA14-AraC-bw, and cloned under P_{tac} control into pME6032 using the restriction sites EcoRI and XhoI of the two primers.

Metabolite extraction and analysis

Pseudomonas aeruginosa strains were grown in GGP medium for 2 days to stationary phase (OD_{600} ~ 7–11). For HPLC analysis, acidified culture supernatants were extracted with ethyl acetate, dried under reduced pressure, then dissolved in 60 % (vol/vol) methanol—10 mM H_3PO_4, and injected into HPLC systems as reported previously (Reimmann et al. 1998; Youard et al. 2007). Compounds were identified based on their retention times and UV spectra. Preparation of Dha and Pch standards has been described previously (Serino et al. 1997). Electrospray mass spectrometry experiments were done on a microTOF LC from Brucker Daltonics. Analytical Thin-layer chromatography (TLC) was carried out with Merck TLC silica gel $60F_{254}$ on aluminum sheets and *n*-butyl alcohol/water/acetic acid 4:1:1 (v/v/v) as the mobile phase. Compounds were detected by fluorescence at 365 nm or by spraying with a solution of $FeCl_3$ in MeOH.

Results

Bioinformatic analysis of *PA14_54940* to *PA14_54870*

As illustrated in Fig. 1, the genes of PA14GI-6, which are potentially involved in the biosynthesis and regulation of a Pch/EPch-related compound, appear to be organized in two operons and a separately transcribed gene. A detailed bioinformatic analysis (Table 2) showed that the N-terminal part of PA14_54940 has strong homology with the salicyl-AMP ligase PchD of *P. aeruginosa* while its C-terminal part weakly ressembles isochorismate synthase

PchA. Interestingly, a stronger homology of the C-terminal part was found with Irp9 of *Yersinia enterocolitica*, an enzyme which converts chorismate directly to salicylate. We thus predicted that PA14_54940 may have salicylate synthase and salicyl-AMP ligase activities.

The stop codon of *PA14_54940* overlaps with the start codon of *PA14_54930,* which codes for a protein ressembling the peptide synthetases PchE of *P. protegens* ($PchE_{Pp}$) (Table 2) and *P. aeruginosa* ($PchE_{Pa}$) (not shown). These proteins couple salicylate to the first L-cysteine moiety. During Pch biosynthesis (Fig. 2), L-cysteine is converted by a dedicated epimerase domain of $PchE_{Pa}$ to its D-enantiomer. Such a domain is absent from $PchE_{Pp}$ and PA14_54930, suggesting that PA14_54930 is a Dha synthetase, generating a molecule whose stereochemical configuration at the chiral center C4′ is identical with that of EPch (Fig. 2).

The third gene of this first operon is *PA14_54920*; its start codon overlaps with the stop codon of *PA14_54930*. PA14_54920 displays homology to the Pch and EPch synthetases PchF of *P. aeruginosa* ($PchF_{Pa}$) and *P. protegens* ($PchF_{Pp}$), respectively, with a closer relatedness to the latter (Table 2). However, unlike the two PchF proteins, PA14_54920 does not have a dedicated methyltransferase domain required for adding a methyl group to the thiazoline rings of Pch and EPch (Fig. 2). Moreover, the C-terminal part of PA14_54920 has no ressemblance to the thioesterase domains of $PchF_{Pa}$ and $PchF_{Pp}$. Instead, it presents a typical NAD binding motif, suggesting that product release may be carried out by a thioester reductase mechanism (Du and Lou 2010). *PA14_54910*, last gene of the *PA14_54940* to *PA14_54910* operon, encodes a putative type II thioesterase, which is most closely related to PchC of *P. protegens*. Its *P. aeruginosa* homolog was shown to be required for quality control of Pch biosynthesis by removing wrongly charged substrates from the peptide synthetases PchE and/or PchF (Reimmann et al. 2004).

The second operon potentially implicated in the biosynthesis of a Pch/EPch-related compound is comprised of three genes. The first one, *PA14_54900*, codes for a protein which is related to PchK of *P. protegens*. Although the function of PchK has not been demonstrated experimentally, the protein is believed to be a functional homolog of PchG, carrying out the reduction of the second thiazoline ring (Fig. 2). In addition to the motifs shared with PchK, bioinformatics identified a motif which is caracteristic of proteins carrying out an epimerase reaction, indicating that PA14_54900 could be a bifunctional protein.

The stop codon of *PA14_54900* overlaps with the start codon of *PA14_54890.* Its deduced amino acid sequence does not reveal homology to proteins implicated in the biosynthesis of Pch and EPch. Based on its NAD binding motif and a certain resemblance with saccharopine dehydrogenases, PA14_54890 may function as an oxidoreductase.

The last gene of the operon, *PA14_54880*, has no counterpart in the Pch/EPch gene clusters either. However, its deduced amino acid sequence revealed strong homology to CysE of *Escherichia coli*, an enzyme which converts serine to *O*-acetylserine during cysteine biosynthesis.

The operon *PA14_54900* to *PA14_54880* is followed by the separately transcribed gene *PA14_54870*, which codes for an AraC-type regulator ressembling PchR of *P. aeruginosa* ($PchR_{Pa}$). This protein, and its counterpart in *P. protegens* ($PchR_{Pp}$) bind their cognate siderophores Pch and EPch, respectively, and upregulate the transcription of the Pch/EPch biosynthesis and transport genes (Lin et al. 2013; Youard et al. 2011).

Taken together, bioinformatics revealed that the siderophore biosynthesis genes of PA14GI-6 appear to be more related to the EPch biosynthetic genes than to those specifying Pch, suggesting that the stereochemistry of the molecule specified by PA14GI-6 could be identical with that of EPch.

Functional analysis of PA14GI-6 genes

In the following, we tested the predicted functions of several PA14GI-6 genes by complementation analyses using *P. aeruginosa*, *P. protegens* and *E. coli* mutants affected in potential homologs. We first evaluated whether *PA14_54940* could restore Pch production in *P. aeruginosa* mutants deleted for *pchBA* (PAO6383) or carrying an Ω cassette inserted in *pchD* (PAO6398), respectively. Both mutants are pyoverdine (Pvd) negative due to a deletion in *pvdF*, allowing the production of Pch to be evaluated visually by the formation of an orange halo on CAS agar plates. As shown in Fig. 3a, constitutive expression of *PA14_54940* from the *lac* promoter of plasmid pME10202 fully restored Pch production in PAO6383 and also led to a minor production of Pch

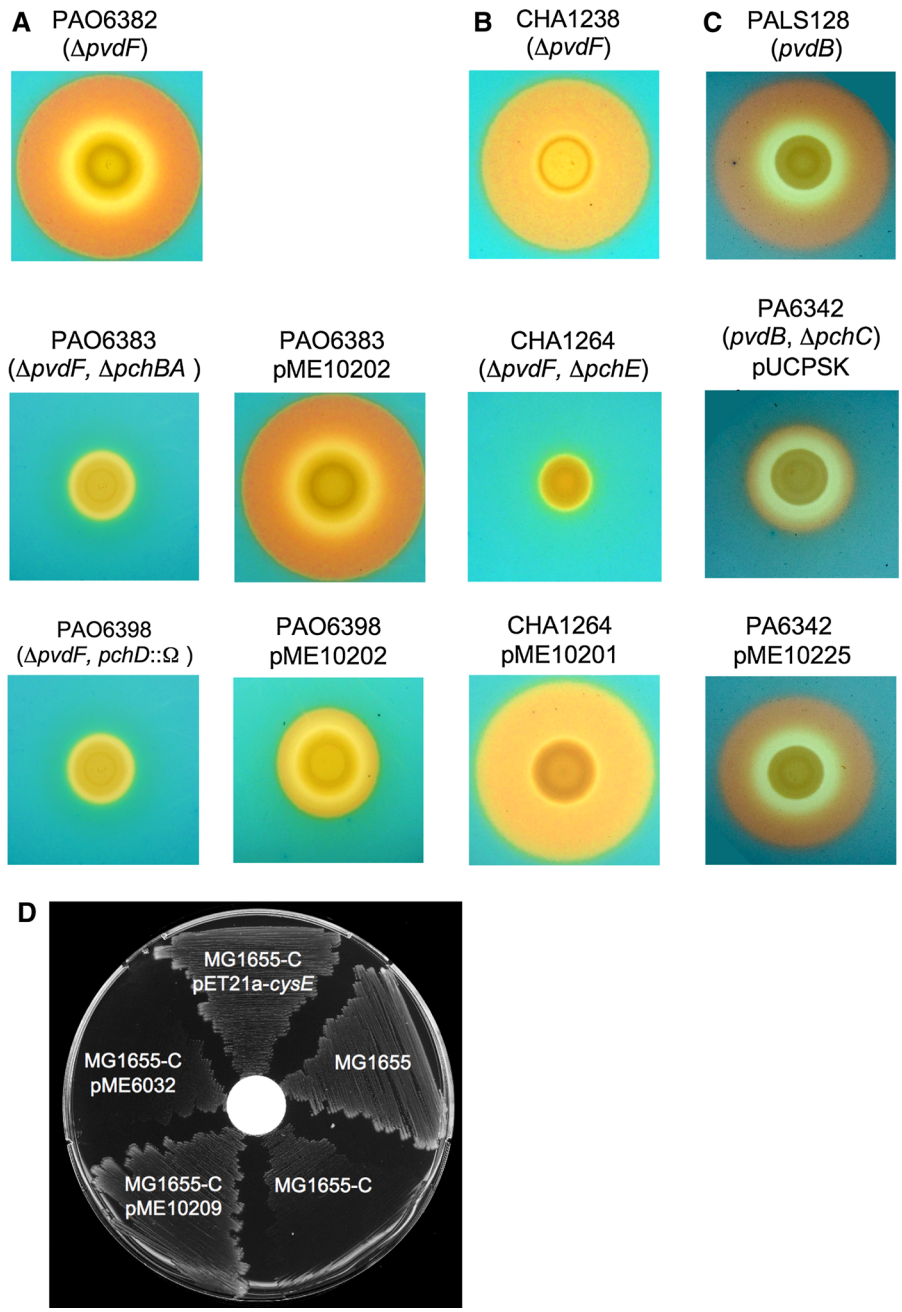

Fig. 3 Functional analysis of *PA14_54940* (**a**), *PA14_54930* (**b**), *PA14_54910* (**c**) and *PA14_54880* (**d**) by complementation analyses. Formation of orange halos around bacterial colonies of Pvd-negative strains grown on CAS agar plates is indicative of Pch (**a**, **c**) or EPch (**b**) production. Serine acetyl transferase activity was identified by cysteine-independent growth of the indicated *E. coli* strains on M9 minimal medium containing 1 mM IPTG. Note that growth of the *cysE* deletion mutant MG1655-C occurred only in close proximity of a filter paper soaked with DL-cysteine

in PAO6398. We thus conclude that *PA14_54940* encodes a bifunctional enzyme with salicylate synthase and salicyl-AMP ligase activities.

Next, we addressed the function of *PA14_54930*, which was expressed under P_{lac} control in plasmid pME10201. Introduction of this plasmid restored production of EPch in CHA1264, a Pvd-negative *P. protegens* mutant deleted for *pchE* (Fig. 3b). By contrast, *PA14_54930* was unable to restore Pch production in the analogous *P. aeruginosa* mutant PAO6399 (data not shown). These data show that *PA14_54930* specifies indeed a dihydroaeruginoic acid synthetase which is expected to generate the same stereochemical configuration at the chiral center C4′ than does PchE of *P. protegens* (Fig. 2).

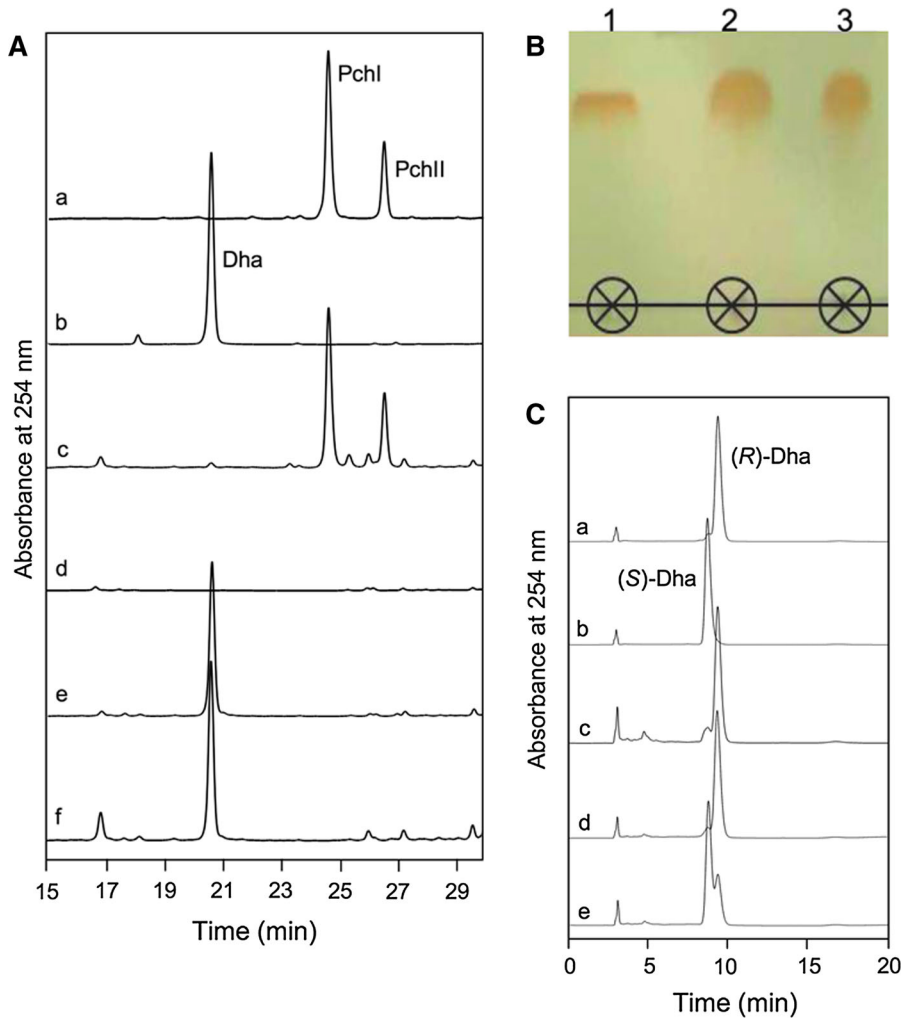

Fig. 4 Identification of the metabolite specified by PA14GI-6 as (*R*)-Dha. **a** HPLC analysis of culture supernatant extracts from PA14-98 (*trace c*), PA14-99 (*trace d*), PA14-106 (*trace e*) and PA14-109 (*trace f*) compared with standards of Pch (*trace a*) and Dha (*trace b*). Note that due to the spontaneous isomerisation at the chiral center C2″, Pch occurs as a mixture of two isomers. EPch was not used as a standard here as the retention times of its two isomers are identical with those of PchI and PchII on the reversed phase C-18 column used. **b** Migration of the PA14GI-6-specified metabolite was compared with a Dha standard by thin layer chromatography. *Lane 1* metabolite; *lane 2* co-spot; *lane 3* Dha standard. **c** Chiral HPLC of culture supernatant extract from PA14-109 (*trace c*) compared with standards of (*R*)-Dha (*trace a*) and (*S*)-Dha (*trace b*). *Trace d*, metabolite mixed with (*R*)-Dha; *trace e*, metabolite mixed with (*S*)-Dha

The function of *PA14_54920* was not tested because it seemed unlikely that a protein lacking a dedicated methyltransferase domain would be able to replace PchF in *P. aeruginosa* or *P. protegens*.

Instead, we showed that *PA14_54910* is a functional homolog of the type II thioesterase PchC. In agreement with previous observations (Reimmann et al. 2004), we found that pyochelin production was reduced, but not abolished, in the Pvd-negative *pchC* mutant PAO6342, compared with production in its PchC-positive parent strain PALS128 (Fig. 3c). Constitutive expression of *PA14_54910* on plasmid pME10225 partially restored Pch production, confirming the predicted role of this PchC homolog in quality control of siderophore assembly.

We then evaluated wether *PA14_54900* would be able to complement the Pvd-negative *pchK* mutant CHA1262. However, no siderophore production by CHA1262 was observed when *PA14_54900* was expressed from the *lac* promoter of pME10205 (data not shown).

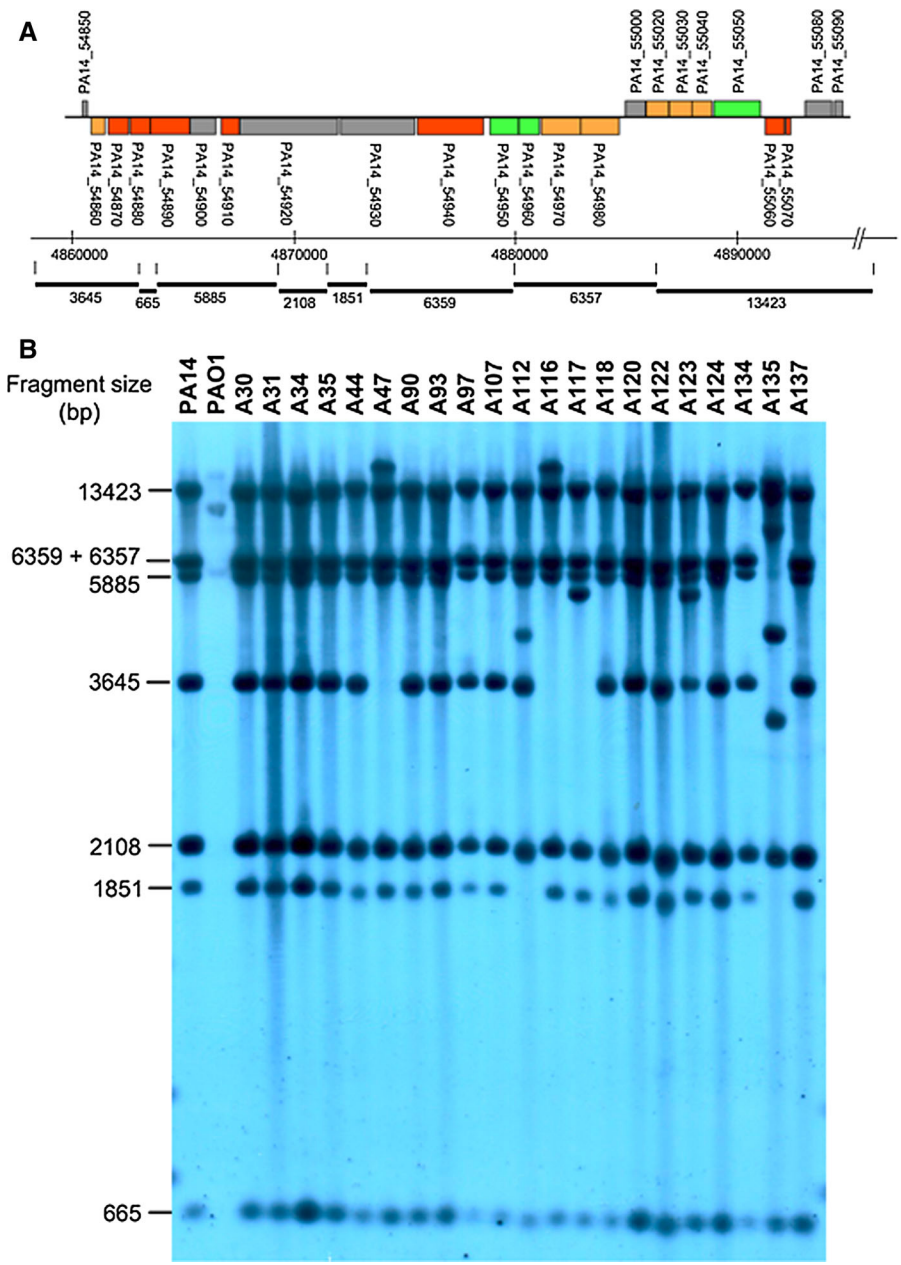

Fig. 5 Occurrence of GI-6 in environmental and clinical *P. aeruginosa* isolates. **a** Genes of GI-6 in the chromosome of strain PA14 and size of DNA fragments generated by in silico restriction with BamHI (in bp). **b** BamHI fragments of chromosomal DNA of PA14, PAO1 and of 21 GI-6 carriers were separated by agarose gel electrophoresis, transferred to a nylon membrane and hybridized against a PA14GI-6-specific digoxygenin-labeled DNA probe

To demonstrate the activity of the predicted serine *O*-acetyltransferase PA14_54880, we tested whether *PA14_54880* would complement a *cysE* mutation in the *E. coli* strain MG1655-C. As shown in Fig. 3d, cysteine-indepent growth occurred in the corresponding wildtype strain MG1655, but not in MG1655-C. Growth was restored in this mutant with *E. coli cysE* gene (carried by pET21-*cysE*) and with *PA14_54880* (carried by plasmid pME10209) confirming the predicted function of this gene.

Finally we tested if the AraC-type regulator PA14_54870 would be able to replace PchR in *P. aeruginosa* or *P. protegens*. However, expression of this gene under P_{tac} control did not allow the Pvd-negative *pchR* mutants PAO6402 and CHA1222 to produce Pch and EPch, respectively (data not shown).

Clearly, PA14_54870 is not able to function as a transcriptional activator with Pch or EPch as effectors.

Structural identification of the metabolite specified by PA14GI-6

Based on bioinformatics and complementation experiments we predicted that the molecule specified by PA14GI-6 would be essentially composed of two cysteine-derived rings joined to salicylate. To purify large amounts of this molecule for structural identification, we generated the two Pvd and Pch-negative overexpression strains PA14-106 and PA14-109, in which genes of the first operon (*PA14_54940* to *PA14_54910*) or those of both operons (*PA14_54940* to *PA14_54910* and *PA14_54900* to *PA14_54880*) are expressed under P_{tac} control. HPLC analysis (Fig. 4a) of culture supernatant extracts showed one major peak, irrespective of whether one (trace e) or both operons (trace f) had been overexpressed. This peak was different from those corresponding to Pch/EPch (traces a [Pch standard] and c [extract of the Pvd-negative strain PA14-98]) and was not produced with an extract of the Pvd- and Pch-negative strain PA14-99 in which the two operons had not been artificially overexpressed (trace d). Surprisingly, the retention time of this peak was identical with that of Dha. For further structural identification of the PA14GI-6-specified metabolite, the culture supernatant extract of PA14-109 was washed, purified on a metal-free silica gel column and subsequently analyzed by electrospray mass spetrometry. The ionic profile presented a peak at *m/z* 224 ($M + H^+$) corresponding to the molecular weight of Dha (MW = 223). TLC analysis confirmed these results in that the PA14GI-6-specified metabolite co-migrated perfectly with a synthetic Dha standard (Fig. 4b). Finally, we investigated the stereochemistry of the compound by chiral HPLC and found that it had the same retention time as (*R*)-Dha, the byproduct of EPch biosynthesis (Fig. 4c).

Identification of GI-6 in clinical and environmental *P. aeruginosa* isolates

To easily detect potential GI-6 carriers among natural isolates of *P. aeruginosa*, a PCR-based screening method was developed which is based on the size differences of *pchE* and *PA14_54930*. This method produces a 1,409 bp fragment caracteristic for $pchE_{Pa}$, and an additional fragment of 524 bp characteristic for a gene resembling *PA14_54930*. We screened 110 clinical (isolated from patients) and 42 environmental *P. aeruginosa* isolates from different geographical origins and identified a total of 21 potential GI-6 carriers (data not shown). To test whether the entire genomic island was conserved in these candidates, chromosomal DNA was isolated, cleaved with BamHI and hybridized against a PA14GI-6- specific probe. As illustrated in Fig. 5, Southern analysis revealed that most isolates had a banding pattern which was identical to that of PA14. In some cases, the pattern was slightly modified, probably because not all BamHI sites are conserved. Although we cannot exclude that certain isolates of a given collection might be directly related, the identification of GI-6 in clinical as well as environmental isolates from different geographical origin suggests that GI-6 confers (or had conferred at some stage) a selective advantage to its carrier.

Discussion

Comparing the accessory genome of the *P. aeruginosa* strains C3719, LESB58, PAO1, PA7, PA14, PA2192, PACS2 revealed a total of 89 RGP's, in which different inserts of foreign DNA are found (Klockgether et al. 2011). These DNA inserts, which apparently have been acquired by horizontal gene transfer, contribute to the ongoing evolution of the *P. aeruginosa* genome by conferring specific phenotypes that are advantageous under certain selective conditions. In this work we have focused on PA14GI-6, one of the inserts found at RGP5 of strain PA14. As illustrated in Fig. 1, this insert harbors a gene cluster which ressembles those for the biosynthesis of the enantiomeric siderophores Pch and EPch. While EPch has so far been found in certain strains of *P. protegens* only (Youard et al. 2011), Pch seems more widely distributed and has been isolated from several pseudomonads (*P. aeruginosa*, *P. fluorescens*, *P. akbaalia*), from members of the *Burkholderia cepacia* complex, and recently also from the plant pathogen *Streptomyces scabies* (Cox et al. 1981; Cuppels et al. 1987; Terano et al. 2002; Thomas 2007; Phoebe et al. 2001; Seipke et al. 2011). We thus hypothesized that an ancester of the genomic island PA14GI-6 may have

been involved at some stage in the dissemination of Pch/EPch biosynthetic genes between these different bacterial species and genera and wondered what compound this island would specify todate. A detailed bioinformatics analysis of its Pch/EPch-related gene cluster (*PA14_54940* to *PA14_54870*) predicted a compound consisting of salicylate linked to two cysteine-derived thiazoline rings which may have the same stereochemical configuration as those of EPch (Table 2). Having a large set of mutations in potential homologs of these genes at hand, we were able to test and confirm the predicted functions of PA14_54940 as a bifunctional salicylate synthase/salicyl-AMP ligase (for generation and activation of salicylate; Fig. 2), of PA14_54930 as a Dha synthetase (for coupling salicylate with a cysteine-derived thiazoline ring; Fig. 2), of PA14_54910 as a type II thioesterase (for quality control; Reimmann et al. 2004), and of PA14_54880 as a serine *O*-acetyltransferase (for increased cysteine biosynthesis; Kredich 1996).

Expression of the entire gene cluster and structural analysis of the specified metabolite revealed its identity with (*R*)-Dha (Fig. 4), an iron chelator with antibacterial, antifungal and antitumor activity (Carmi et al. 1994; Elliot et al. 1988). Despite these interesting properties, the identification of Dha came as a surprise, as the gene cluster clearly codes for a PchF homolog (PA14_54920), and for two potential tailoring proteins (PA14_54900 and PA14_54890). Obviously, biosynthesis of a larger metabolite containing two thiazoline rings is terminated prematurely, and a biosynthetic intermediate is released from the thiotemplate PA14_54930 giving rise to Dha. Indeed, Dha was described as a byproduct of Pch biosynthesis in *P. aeruginosa*, namely when the PchE-bound intermediate hydroxyphenyl-thiazoline is released by slow hydrolysis of the thioester bond (Serino et al. 1997; Patel et al. 2003; Quadri et al. 1999).

Why then would biosynthesis not proceed to the end? Given that Dha contains only a single thiazoline ring it is likely that during evolution, one or several mutations in PA14_54920 have rendered this PchF homolog non-functional. Analysis of the characteristic sequence motifs in the predicted adenylation-thiolation-condensation/cylization domains, and comparison with the motifs of PchF from *P. aeruginosa* and *P. protegens*, revealed a very good motif conservation except for an alternative key residue in the PA14_54920 thiolation domain (GGTSL instead of GGDSL). However, replacing the threonine codon against an aspartate codon did not alter metabolite production (data not shown), suggesting that additional mutations in *PA14_54920* may prevent biosynthesis to proceed.

In conclusion, we have shown here that PA14GI-6 does not (or no longer) code for a Pch/EPch-related siderophore but has evolved to confer the ability of Dha production to its host. The maintenance of GI-6 in a large number of clinical and environmental *P. aeruginosa* isolates (Fig. 5) indicates either that Dha production could confer a selective advantage under certain conditions or that the presence of this rather small GI does not have a fitness cost.

Acknowledgments We wish to thank Zeb A. Youard for help with degenerate primer design, Paul Majcherczyk for HPLC analyses, and Nadja Leimer and Luis Alejandro González Blázquez for carrying out initial experiments on this project. This work as supported by the Swiss National Foundation for scientific research (project 31003A-132998).

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