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A Regioselective Tryptophan 5-Halogenase Is Involved in Pyrroindomycin Biosynthesis in *Streptomyces rugosporus* LL-42D005

Susanne Zehner,[1] Alexander Kotzsch,[1,4] Bojan Bister,[2] Roderich D. Süssmuth,[2] Carmen Méndez,[3] José A. Salas,[3] and Karl-Heinz van Pée[1,*]

[1]Institut für Biochemie
Technische Universität Dresden
01062 Dresden
Germany

[2]Institut für Chemie
Technische Universität Berlin
10623 Berlin
Germany

[3]Departamento de Biología Funcional e Instituto Universitario de Oncología del Principado de Asturias (I.U.O.P.A)
Universidad de Oviedo
33006 Oviedo
Spain

Summary

The antibiotic compound pyrroindomycin B contains an indole ring chlorinated in the 5 position. The indole ring is probably derived from tryptophan, and thus primers derived from conserved regions of tryptophan halogenases were used to amplify and clone a DNA fragment that was then used to isolate a tryptophan 5-halogenase gene (*pyrH*) from a cosmid library of the pyrroindomycin producer *Streptomyces rugosporus* LL-42D005. A gene disruption mutant in the tryptophan 5-halogenase gene no longer produced pyrroindomycin B, but still produced pyrroindomycin A, the nonhalogenated derivative. The halogenase gene could be overexpressed in *Pseudomonas fluorescens* BL915 ΔORF1 and was purified to homogeneity by immobilized metal chelate ion affinity chromatography. Chlorinating and brominating activities with tryptophan as a substrate were detected in cell-free extracts and for the purified enzyme.

Introduction

The pyrroindomycins A and B (Figure 1) were isolated by Ding et al. in 1994 [1]. Pyrroindomycin A and B exhibit good to excellent in vitro activity against gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* strains and poor activity against gram-negative bacteria, with pyrroindomycin A showing a higher antibacterial activity than the chlorinated derivative pyrroindomycin B [2]. The pyrroindomycins are composed of a pyrroloindole entity linked via an ester bond to an unbranched deoxytrisaccharide. A polyketide macro-ring system containing a tetramic acid functionality is connected to the other end of the trisaccharide through a glycosidic linkage. Whereas pyrroindomycin B contains a chlorine atom, pyrroindomycin A is the nonhalogenated derivative. Tryptophan is presumed to be the precursor for the pyrroloindole moiety [3]. The first step in the biosynthesis of the antifungal antibiotic pyrrolnitrin is the regioselective chlorination of tryptophan in the 7 position [4]. This is also assumed for the antitumor compound rebeccamycin [5]. Genes coding for enzymes catalyzing the regioselective chlorination of tryptophan in the 7 position have been isolated from several pyrrolnitrin-producing bacteria [6, 7]. Purification and characterization of the corresponding enzyme showed that it belongs to a novel type of halogenating enzymes. These halogenases are part of a two component system consisting of a flavin reductase and the actual halogenase. The flavin reductase is required for the formation of $FADH_2$ from FAD and NADH [8]. From the rebeccamycin producer *Lechevalieria aerocolonigenes*, a gene with high similarity to the tryptophan 7-halogenase gene from the pyrrolnitrin biosynthetic gene cluster was isolated [5], supporting the assumption that in rebeccamycin biosynthesis tryptophan is also halogenated in the 7 position. If the presumption that in the biosynthesis of pyrroindomycin B chlorination of tryptophan should take place was correct, it should be possible to detect the gene of this tryptophan 5-halogenase by PCR using primers derived from conserved regions of the tryptophan 7-halogenase genes isolated so far. The alignment of the amino acid sequences of the tryptophan 7-halogenases involved in pyrrolnitrin biosynthesis and the putative tryptophan 7-halogenase from the rebeccamycin producer showed the presence of a few conserved regions suitable for the constructions of PCR primers. Here we report the cloning and expression of a tryptophan 5-halogenase gene, the purification of the enzyme in active form, the identification of the enzymatic product, and the proof for the involvement of this tryptophan 5-halogenase in pyrroindomycin B biosynthesis.

Results and Discussion

Detection of the Tryptophan 5-Halogenase Gene

Comparison of published amino acid sequences of tryptophan 7-halogenases from pyrrolnitrin-producing bacteria [7] and the putative tryptophan 7-halogenase involved in rebeccamycin biosynthesis in *Lechevalieria aerocolonigenes* [5] allowed the identification of four conserved regions (Figure 2) suitable for the construction of four PCR primers. Using these primers in four combinations with total DNA from the pyrroindomycin producer *Streptomyces rugosporus* as the template DNA, a fragment of ~700 bp (*PCR-hal*) could be amplified with a combination of primers SZ003 and SZ005. Sequencing and analysis of the 700 bp PCR product shows an identity at the amino acid level to the tryptophan halogenases used for the sequence alignment be-

*Correspondence: karl-heinz.vanpee@chemie.tu-dresden.de
[4]Present address: Theodor-Boveri-Institut für Biowissenschaften (Biozentrum) der Universität Würzburg, Am Hubland, 97074 Würzburg, Germany.

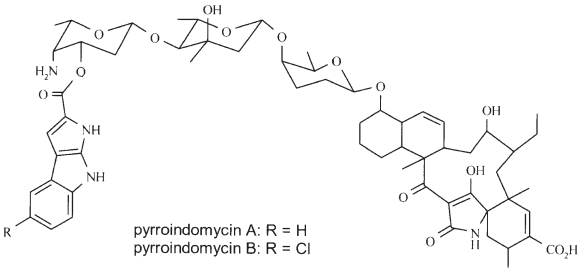

Figure 1. Chemical Structures of Pyrroindomycins A and B
See text for details.

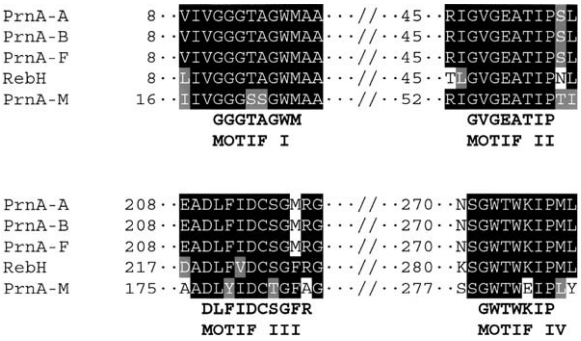

Figure 2. Comparison of Tryptophan Halogenase Sequence Regions Used for the Construction of PCR Primers for the Amplification of Tryptophan Halogenase Gene Fragments

PrnA-A, tryptophan 7-halogenase (trp 7-hal) from *Pseudomonas aureofaciens*; PrnA-B, trp 7-hal from *Burkholderia cepacia*; PrnA-F, trp 7-hal from *P. fluorescens*; RebH, trp 7-hal from *Lechevalieria aerocolonigenes*; PrnA-M, trp 7-hal from *Myxococcus fulvus*. Black boxes show conserved amino acid positions, gray boxes show similar amino acids, and conserved motifs are shown below the sequences.

tween 31% and 33%. When *PCR-hal* was used as the probe in colony screening of a cosmid library of *S. rugosporus*, five positive clones were detected. Subcloning of an ~4300 bp BamHI fragment from the positive cosmid clone 6C1 into pBluescript II SK (+) resulted in plasmid pB1G. Sequencing of a DNA region of 2564 bp around the PCR-*hal* hybridizing region revealed the presence of an *orf* of 1536 bp, named *pyrH* showing the typical high GC content of *Actinomyces* genes [9]. The deduced gene product revealed similarities to several known tryptophan halogenases. PyrH showed 56% similarity to the tryptophan 7-halogenase from *Pseudomonas fluorescens* whose in vitro activity has been demonstrated [10] and 52% sequence similarity to RebH from *Lechevalieria aerocolonigenes* [5]. The deduced amino acid sequence contains both crucial motifs of $FADH_2$-dependent halogenases [11]. The first motif (GxGxxG) is located near the N terminus of the protein and is known to be involved in the binding of nucleotide cofactors and is common to a number of FAD- and NAD(P)H-dependent enzymes [12]. The second motif (GWxWxIP), also conserved in all known $FADH_2$-dependent halogenases, is located in the middle of the deduced gene product. PyrH was assumed (later confirmed, see below) to be the tryptophan 5-halogenase of the pyrroindomycin biosynthetic gene cluster (the sequence was deposited in GenBank with accession number AY623051).

Heterologous Expression of the Tryptophan 5-Halogenase Gene *pyrH*, Purification of the Enzyme, and In Vitro Halogenation of Tryptophan

In order to assay the putative tryptophan 5-halogenase activity of PyrH, its coding gene was heterologously expressed in *Pseudomonas fluorescens* BL915 ΔORF1, as a N-terminal His-tagged protein, using plasmid pSZexM2. Overproduction of the protein was verified by SDS-PAGE of cell extracts (Figure 3). The protein was produced in soluble form. Halogenating activity in crude extracts from *P. fluorescens* BL915 ΔORF1 pSZexM2 was investigated by a specific enzyme assay. Although not absolutely necessary for halogenating activity in crude extracts, an overexpressed flavin reductase from *Escherichia coli* (Fre) [13] was added to the enzyme assay to ensure the formation of enough $FADH_2$ required by halogenases [8].

PyrH was purified to homogeneity by immobilized nickel chelate affinity chromatography (Figure 3). The purified enzyme did not contain flavin or any other cofactor with an absorbance in the visible range of the spectrum. Whereas the addition of FAD and of a flavin reductase was not necessary in the case of the crude cell-free extract, it was absolutely necessary for halogenating activity of the purified enzyme. Purified PyrH showed a turnover number of 8.13×10^{-3} s^{-1} and a K_m value of 0.15 mM with L-tryptophan as the substrate.

Using purified PyrH, in the presence of chloride and tryptophan, monochlorinated tryptophan ($[M+H]^+$ = 238.9 amu) was obtained, as detected by HPLC and HPLC-ESI-MS (Figure 4A). The position of chlorine in-

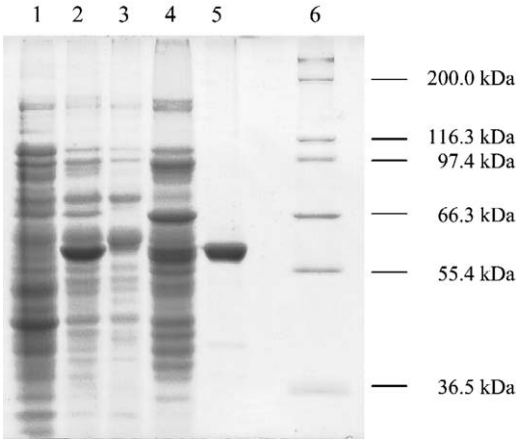

Figure 3. SDS-PAGE of Crude Extracts from *P. fluorescens* BL915 ΔORF1 pSZexM2 (*pyrH*) and of Purified PyrH

Lane 1, crude extract from *P. fluorescens* BL915 ΔORF1 pCIBhis (25 μg); lane 2, crude extract from *P. fluorescens* BL915 ΔORF1 pSZexM2 (*pyrH,* 20 μg); lane 3, run-through from the nickel chelate chromatography column (15 μg); lane 4, washing fraction (22 μg); lane 5, purified PyrH (7 μg); lane 6, molecular weight standard.

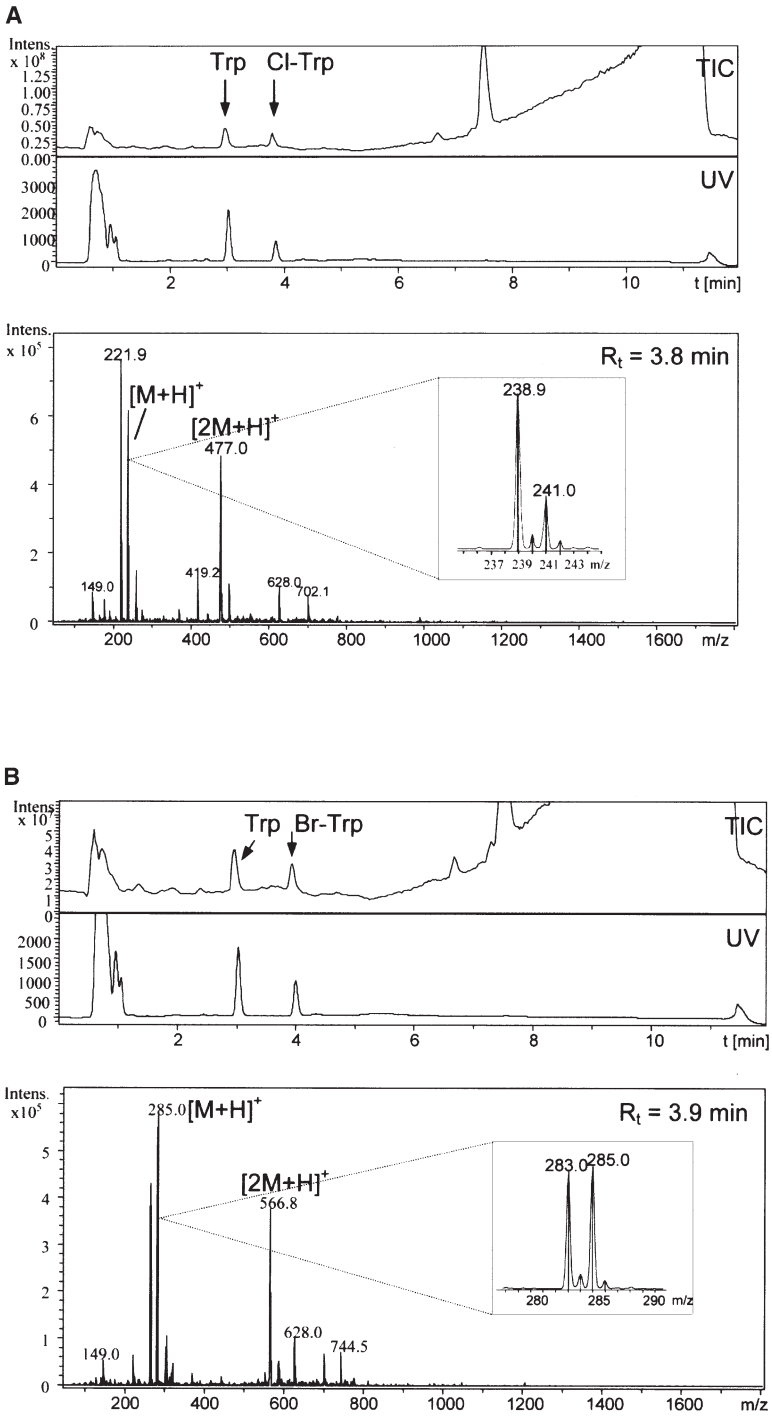

Figure 4. UV-Chromatograms and Total Ion Chromatograms of the Tryptophan 5-Halogenase Activity Assay

(A) UV- and TIC traces of the enzyme assay in the presence of chloride and corresponding mass spectra (R_t = 3.8 min) of 5-chlorotryptophan (Cl-Trp) ($[M+H]^+$ = 238.9 amu; $[2M+H]^+$ = 477.0 amu).

(B) UV- and TIC traces of the enzyme assay in the presence of bromide and corresponding mass spectra (R_t = 3.9 min) of 5-bromotryptophan (Br-Trp) ($[M+H]^+$ = 283.0 amu; $[2M+H]^+$ = 566.8 amu).

corporation was elucidated by ^{1}H-NMR (D_2O, 500.13 MHz). 2-H was observed at δ 7.35 (s), and 4-H at δ 7.62 (d) was found to be coupled with 6-H (dd) at δ 7.12 (J = 2.0 Hz). 6-H was also found to be coupled with 7-H at δ 7.35 (d, J = 8.7). These data for the chemical shift and coupling constants of the aromatic H of enzymatically produced chlorotryptophan were identical with the data obtained from authentic 5-chlorotryptophan. When chloride was substituted with bromide in the enzyme assay, monobrominated tryptophan was formed as identified by HPLC and HPLC-ESI-MS ($[M+H]^+$ = 283 amu) (Figure 4B). Chlorination is favored over the incorporation of bromide. Brominating activity is only about 75% of the chlorinating activity. At bromide concentrations <1 mM, 5-chlorotryptophan is also formed, although chloride was not added to the enzyme assay. This is due to the presence of chloride as an inevitable contamination in the enzyme assay. Only a single chlorinated or brominated product could be detected, respectively, showing that the enzyme was a halogenase catalyzing the regio-

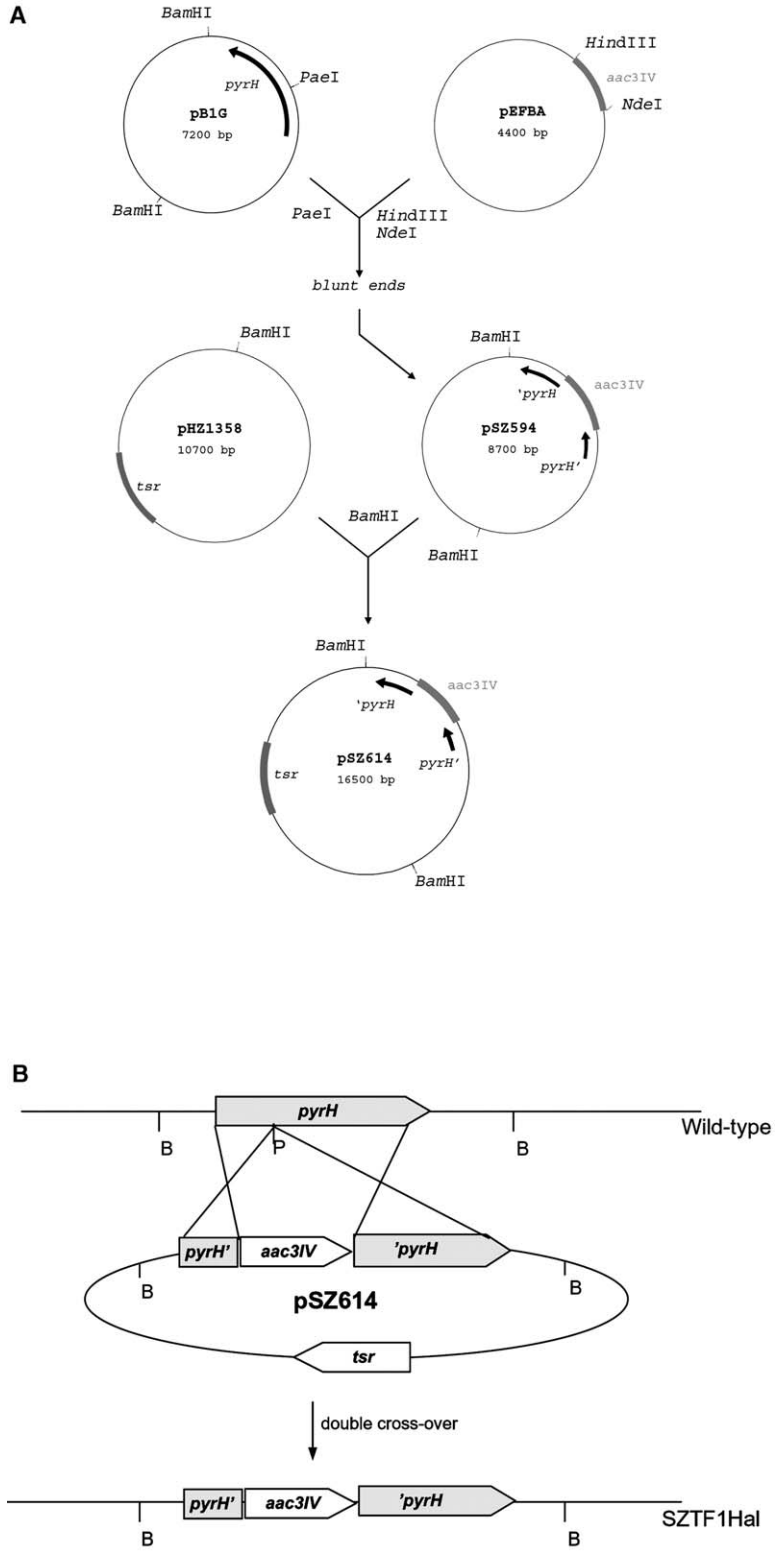

Figure 5. Generation of Mutant *Streptomyces rugosporus* SZTF1Hal

(A) Construction of plasmid pSZ614.

(B) Construction of the gene disruption mutant *Streptomyces rugosporus* SZTF1Hal. B, BamHI; P, PaeI.; *'pyrH* and *pyrH'* are each incomplete copies of *pyrH*; aac3IV is the apramycin resistance gene; *tsr*, thiostrepton resistance gene.

selective monohalogenation of tryptophan at the 5 position. Crude cell-free extract prepared from a control strain of *P. fluorescens* harbouring the vector plasmid without the tryptophan 5-halogenase gene, showed no halogenating activity.

Involvement of PyrH in Pyrroindomycin Biosynthesis

To find indications for the pyrroindomycin B biosynthetic gene cluster, flanking regions 3000 bp upstream and 1000 bp downstream of *pyrH*, respectively, were

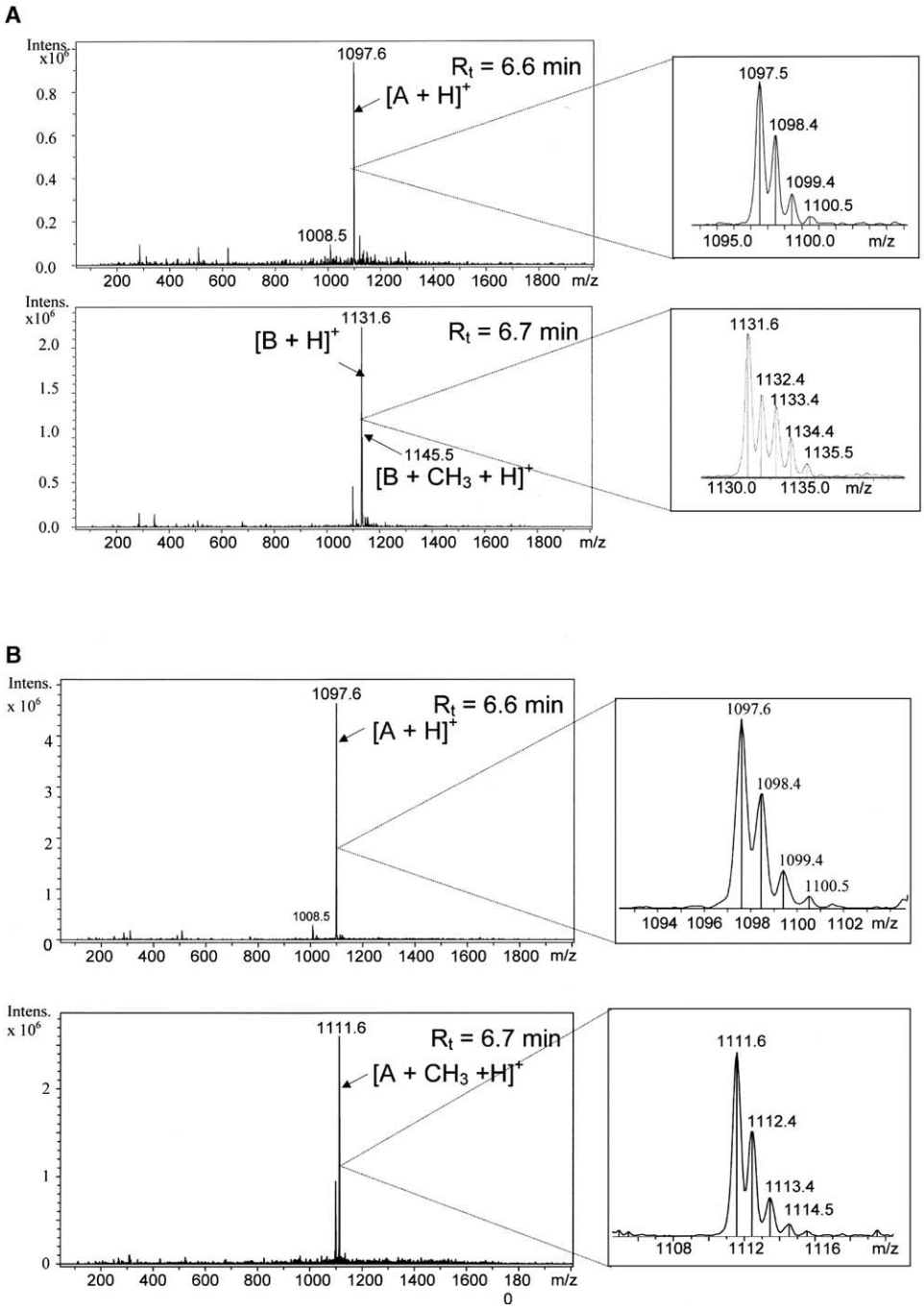

Figure 6. Data from the HPLC-ESI-MS Characterization of the Pyrroindomycin Metabolites from *Streptomyces rugosporus* SZTF1Hal and the Wild-Type Strain

(A) Mass spectra of pyrroindomycins produced by the wild-type strain. Pyrroindomycin A ($[M+H]^+$ = 1097.6 amu/R_t = 6.6 min); pyrroindomycin B ($[M+H]^+$ = 1131.6 amu/R_t = 6.7 min); methylated pyrroindomycin B ($[M+H]^+$ = 1145.5 amu/R_t = 6.7 min).

(B) Mass spectra of pyrroindomycins produced by *S. rugosporus* SZTF1Hal. Pyrroindomycin A ($[M+H]^+$ = 1097.6 amu/R_t = 6.6 min); methylated pyrroindomycin A ($[M+H]^+$ = 1111.6 amu/R_t = 6.7 min).

sequenced. Analysis of the DNA sequences for the presence of potential coding regions revealed three potential open reading frames, two upstream and one downstream of *pyrH*. All of them showed a high GC content, characteristic of *Streptomyces* genes and the bias in the third codon position characteristic of the genes of this genus. The deduced amino acid sequences showed no significant similarities with proteins involved in the biosynthesis of similar secondary metabolites (data not shown).

Therefore, in order to prove the involvement of PyrH in the biosynthesis of pyrroindomycin B, a gene disruption mutant was constructed. For this purpose, plasmid pSZ614 was generated. Plasmid pSZ614 containing the *pyrH* gene disrupted by an apramycin resistance gene (Figure 5A) is a bifunctional mobilizable plasmid that is unstable in *S. rugosporus*. This plasmid was introduced into the wild-type strain *S. rugosporus* by conjugation from *E. coli*. This experiment resulted in the apramycin-resistant and thiostrepton-sensitive mutant SZTF1Hal, where the mutated gene replaced the wild-type copy of the chromosome via homologous recombination (Figure 5B). The recombination event was confirmed by Southern analysis of chromosomal DNA from mutant SZTF1Hal and its comparison to the wild-type strain.

Mass Spectrometric Characterization of the Biosynthetic Products of the Mutant SZTF1Hal

To identify the pyrroindomycins produced by mutant SZTF1Hal, cell extracts of this strain were analyzed by HPLC-ESI-MS. The mass spectra derived from these investigations indicated the production of pyrroindomycin A and methylated pyrroindomycin A, but not that of pyrroindomycin B (Figure 6B). According to the characteristic isotopic pattern, the pyrroindomycin variants synthesized by mutant strain SZTF1Hal did not contain any chlorine atoms, as evident from the mass shifts of 34 atomic mass units to lower masses in comparison to the corresponding signals from the wild-type extracts (Figures 6A and 6B).

Significance

Regioselectively acting halogenases are enzymes that are responsible for the biosynthesis of a large variety of halogenated secondary metabolites. In many cases the biological activity of these metabolites is significantly influenced by the halogen substituents. Isolation and characterization of the genes of specific halogenases and understanding the biochemistry of these enzymes are prerequisites for genetic engineering aiming at the production of novel halogenated compounds by combinatorial biosynthesis. In the work presented here, a halogenase with novel regioselectivity was isolated. To our knowledge, this enzyme is the first tryptophan 5-halogenase and only the second halogenase purified in active form, and one of only three specific halogenases for which the enzymatic activity and the involvement in the biosynthesis of a halogenated metabolite could be shown. The availability of this specific halogenase is expected to open up novel possibilities for combinatorial biosynthesis and the enzymatic production of halogenated compounds.

Experimental Procedures

Bacterial Strains, Culture Conditions, and Vectors

Streptomyces rugosporus NRRL 21084 [1], *Escherichia coli* XL1 Blue MRA [Stratagene], *E. coli* JM109 (DE3) pEE1001 [14], and *Pseudomonas fluorescens* BL915 ΔORF1 [6] were used in this work. For sporulation, *S. rugosporus* was routinely grown at 30°C on agar plates containing MS medium for 7 days [15]. Cultures of *S. rugosporus* were grown on NZ Amine A medium [ATCC 172] at 30°C. *E. coli* was grown at 37°C in LB [16], supplemented with 100 μg ml^{-1} apramycin, 15 μg ml^{-1} tetracycline, or 100 μg ml^{-1} ampicillin. *P. fluorescens* strains were grown at 30°C on modified LB supplemented with 50 μg ml^{-1} kanamycin and 30 μg ml^{-1} tetracycline, if necessary, for 2 days. Vectors pOJ446 [17], pEFBA [18], and pHZ1358 [19] were described previously, and pBluescript II SK (+) was from a commercial source [Stratagene]. pCIBhis was constructed through cloning of a 270 bp PCR-fragment from the N-terminal expression region of pRSETB [Stratagene] including the polyhistidine tag and the MCS into the BamHI site of pPEH14 [4].

DNA Manipulation and Sequencing

Plasmid DNA preparations, restriction endonuclease digestions, alkaline phosphatase treatments, ligations, and other DNA manipulations were performed according to standard procedures for *E. coli* [16]. A genomic library of *S. rugosporus* was constructed in pOJ446 according to published procedures [16] and packaged with Gigapack III XL extract from Stratagene. Total DNA of *S. rugosporus* was isolated after treatment of the cells at 37°C for 1 hr with 10 mg ml^{-1} lysozyme in lysozyme buffer (10 mM Tris and 0.3 M saccharose [pH 8]). The cells were pelleted and resuspended in NAE buffer (0.15 M NaCl and 0.1 M EDTA [pH 8]). Proteinase K solution and SDS were added to a final concentration of 0.2 mg ml^{-1} and 2%, respectively. The cells were incubated at 37°C for a further 3 hr and at 60°C for an additional 30 min. DNA was purified by phenol/chloroform extraction and ethanol precipitation. Southern analysis, digoxigenin labeling of probes, hybridization, and detection were performed according to manufacturer's recommendations (Roche). Introduction of DNA into *S. rugosporus* was performed through *E. coli-Streptomyces* conjugation routinely used for *Streptomyces* [20]. DNA sequencing was performed by MWG biotech Ebersberg. Computer assisted database searches were carried out using the HUSAR program package (EMBL Heidelberg) and the BLAST program [21].

PCR Amplification

Degenerated oligonucleotides were designed to locate the halogenase gene in total DNA of *S. rugosporus*. They were derived from four conserved motifs present in the amino acid sequences of known tryptophan halogenase proteins (Figure 2). These oligonucleotides were used to attempt PCR amplification using total DNA from *S. rugosporus* as the template. The sequences of two sense oligonucleotides were SZ002 (motif II) 5′-TCGGYGTSGGCGARGC GACCRTCCC-3′ and SZ003 (motif I) 5′-TSGGCGGCGGCACYGC SGGMTGGATG-3′ and those of the two antisense oligonucleotides SZ004 (motif IV) 5′-AGCATSGGRATCTTCCAGGTCCABCC-3′ and SZ005 (motif III) 5′-GCCGGAGCAGTCGAYGAASAGGTC-3′. PCR mixtures contained 50 ng of template DNA, 50 pmol of each primer, 1 mM $MgCl_2$, 2.5 U Taq DNA Polymerase (MBI fermentas), 5% DMSO, and 0.2 mM dNTP-mix. Amplification was obtained by standard procedure with an annealing temperature of 63.5°C. The PCR product of the oligonucleotides SZ003 and SZ005 was named PCR-*hal* and subsequently used as the DNA probe for Southern hybridization.

Construction of Plasmids

From cosmid 6D1, a 4300 kb BamHI fragment was subcloned into the BamHI site of pBluescript II SK (+) yielding pB1G that contained the entire halogenase gene. For disruption of the halogenase gene, the apramycin resistance gene from pEFBA was cloned blunt-ended as a 1.5 kb HindIII-NdeI fragment into the blunt-ended PaeI site internal in the halogenase gene of pB1G leading to the plasmid pSZ594. The 5.8 kb BamHI fragment from pSZ594 was cloned into the same site of pHZ1358 yielding pSZ614 (Figure 5A). Plasmid pSZ614 was used for disruption of the chromosomal *pyrH* gene in *S. rugosporus* (Figure 5B).

Gene Expression

For the expression of *pyrH* in *P. fluorescens*, the gene was amplified by PCR. The following primers were used (restriction sites for BamHI and HindIII, respectively, are underlined and were included to facilitate ligation into pCIBhis): 5′-ATCG*GGATCC*CCATATGA TCCGATCTGTGGTGATCGTGGGTG-3′ for the 5′ end of the halogenase gene and 5′-ATGA*AAGCTT*TCATTGGATGCTGGCGAGGT ACTCGTAGCAGC-3′ for the 3′ end of the gene. PCR reaction con-

ditions were as follows: 100 ng of template DNA were mixed with 50 pmol of each primer, 1 mM $MgSO_4$, and 2 U *Pfu* DNA polymerase in a total volume of 50 μl containing 2 mM of each dNTP and 10% DMSO. The reaction was performed under standard conditions with an annealing temperature of 68.1°C. The PCR product was purified, digested with BamHI and HindIII, and ligated into the same sites of pCIBhis, generating pSZexM2. The amplified DNA sequence was verified by sequencing. The plasmid pSZexM2 was then introduced into *P. fluorescens* BL915 ΔORF1 by conjugation, as described earlier [22]. The halogenase gene was constitutively expressed in *P. fluorescens* under control of the tac promoter [4].

Preparation of Crude Extracts, Protein Electrophoresis, and Protein Measurements

Cells from a 2-day-old culture of *P. fluorescens* BL915 ΔORF1 pSZexM2 and control strain (containing pCIBhis) were harvested by centrifugation and washed twice with saline. The cells were resuspended in 100 mM potassium phosphate buffer (pH 7.2) and disrupted by sonication for 6 min (Branson sonifier 450, 160 W, 60% amplitude, 4°C). The crude extract was obtained by centrifugation (two times at 22,100 g, 4°C, 30 min) and immediately dialyzed against 10 mM potassium phosphate buffer (pH 7.2). Flavin reductase-containing extracts were prepared from *E. coli* JM109 (DE3) pEE1001 [14]. Cells from an overnight culture were harvested by centrifugation and washed once with 10 mM potassium phosphate buffer (pH 7.2). The cells were resuspended in 100 mM potassium phosphate buffer (pH 7.2) and disrupted by sonication for 2.5 min. The cell debris was removed by centrifugation and the crude extract containing Fre was used for enzyme assays after dialysis against 10 mM potassium phosphate buffer (pH 7.2) containing 10% glycerol. SDS-PAGE was performed according to Schägger and von Jagow [23] using 4.5% stacking gels and 10% resolving gels. For the estimation of protein size, Mark12 MW standard (Novex) was used. Gels were stained for proteins with Coomassie Brillant Blue R250. Protein concentrations were determined using the method of Whitaker and Granum [24].

Enzyme Assay

Halogenating activity was determined after incubating 100 μl of crude extract from *P. fluorescens* BL915 ΔORF1 pSZexM2 containing recombinant tryptophan 5-halogenase or 100 μl of purified PyrH, respectively, with 4.5 mU Fre [25], 10 μM FAD, 2.4 mM NADH, 25 mM NaCl or NaBr, respectively, 0.6 mM L-tryptophan, and 10 mM potassium phosphate buffer (pH 7.2) in a total volume of 200 μl at 30°C for 6 hr (crude extract) and for 90 min (purified PyrH), respectively. The reaction was stopped by boiling in a water bath for 5 min. After removal of precipitated protein by centrifugation, 20 μl of the assay mixture was analyzed by HPLC. HPLC was performed on a Nucleosil 100 RP-18 column (5μm, 125 mm × 4.6 mm, Bischoff) with MeOH/H_2O/trifluoracetic acid (40:60:0.1) as the eluent with a flow rate of 1 ml min^{-1} and UV detection at 220 nm The products were identified by comparison of their retention times with reference substances, HPLC-UV spectra, and HPLC-ESI-MS.

Purification of PyrH

All purification steps were performed at 4°C. For purification, the cell-free extract was dialyzed overnight against 10 mM potassium phosphate buffer (pH 7.2), and loaded onto a nickel-chelating sepharose fast flow column (44 ml column volume). The column was washed with buffer A (10 mM potassium phosphate buffer [pH 7.2] and 0.15 M NaCl) until the absorbance at 280 nm remained constant. After a further washing step with 180 ml of 40% buffer B (10 mM potassium phosphate buffer [pH 7.2], 150 mM NaCl, and 250 mM imidazole), PyrH was eluted with 100% buffer B. Fractions (2 ml) containing PyrH, as judged by SDS-PAGE, were pooled and dialyzed overnight against 10 mM potassium phosphate buffer (pH 7.2) containing 10% glycerol. The purified enzyme was stored at –20°C.

^{1}H NMR Analysis of 5-Chlorotryptophan

A large-scale reaction (6 × 3 ml) containing 10 mM potassium phosphate buffer [pH 7.2], 5 μM FAD, 25 mM NaCl, 0.6 mM L-tryptophan, 1,560 U catalase, 5 mM NADH, 169 mU flavin reductase (Fre), and 1.5 ml of cell-free crude extract was incubated at 30°C for 30 min with shaking (150 rpm). The assay mixtures were heated to 95°C for 5 min and protein was removed by centrifugation. The supernatant was fractionated by preparative HPLC using a Eurosphere-100 RP-18 column (5 μm, 250 mm × 20 mm, Knauer) with MeOH/H_2O/ammonium acetate (40:60:0.1) as the eluent at a flow rate of 5 ml min^{-1}. Fractions containing the chlorinated tryptophan derivative were pooled and methanol was evaporated in vacuo. The resulting aqueous solution was freeze dried. The remaining solid was dissolved in D_2O and analyzed by ^{1}H NMR. ^{1}H NMR spectroscopy was performed in a Bruker NMR spectrometer DRX-500 system (TMS d = 0). Authentic 5-chlorotryptophan was purchased from Apin Chemicals Ltd.

Isolation of Pyrroindomycins

Spores of strains *S. rugosporus* NRRL 21084 and *S. rugosporus* SZTF1Hal were inoculated in NZ Amine A medium and cultivated at 30°C and 170 rpm for 3 days. Each seed culture was used to inoculate (2.5%, v/v) four Erlenmeyer flasks containing 250 ml of SO7 medium [3]. After incubation for 8 days under the same conditions, cells were harvested by centrifugation. Since the pyrroindomycins were found to be predominantly associated with the cell pellet, the cells were resuspended in a small volume of water and were twice extracted by shaking for 2 hr with 1.5 volumes of ethyl acetate. The organic phase was carefully removed and evaporated in vacuo. The dried extracts from the pellets of each strain were redissolved in 100 μl methanol and applied to HPLC analysis.

HPLC and HPLC-ESI-MS Analysis

Detection of tryptophan derivatives and pyrroindomycins was performed by HPLC (Knauer, Berlin, Germany) on a reversed phase column (Lichrosphere 100 RP-18, 5.0 μm 250 × 4.6 mm, Bischoff), with acetonitrile and 0.1% trifluoroacetic acid (TFA) in water as eluent. A gradient from 10% to 100% acetonitrile over 20 min at a flow rate of 1 ml min^{-1} was used. From each strain 20 μl of the extracts were injected. Detection and spectral characterization of peaks were performed with a photodiode array detector. Pyrroindomycins were detected due to their characteristic UV/Vis spectra. The halogenation status of the compounds was monitored by HPLC-ESI-MS. For this purpose, cell-free extracts of the tryptophan halogenation assay or crude methanol extracts of pyrroindomycins from cell extracts of wild-type and mutant strains were analyzed by HPLC-ESI-MS. Separations were performed on a Merck Purospher column (RP-C18e, 125 × 4 mm, 4.0 μm) with a flow rate of 1.5 ml/min. The UV detector (λ = 214 nm) was connected in series with the mass spectrometer. Solvent A was 0.1% TFA and solvent B was acetonitrile/0.1%TFA (gradient 10%–100% over 10 min). Injection volumes were 20 μl. HPLC-ESI-MS analysis was performed on an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) coupled to a Bruker Esquire 3000plus (Bruker-Daltonics, Bremen, Germany) equipped with an ion-trap analyzer.

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Accession Codes

The sequence of PyrH was deposited in GenBank with accession number AY623051.