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A novel halogenase gene from the pentachloropseudilin producer *Actinoplanes* sp. ATCC 33002 and detection of in vitro halogenase activity

Ina Wynands, Karl-Heinz van Pée *

Institut für Biochemie, Technische Universität Dresden, D-01062 Dresden, Germany

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Abstract

A novel halogenase gene (*halB*) was isolated from a cosmid library of the pentachloropseudilin producer *Actinoplanes* sp. ATCC 33002. The halogenase has high identity (55%) to the flavin-dependent monodechloroaminopyrrolnitrin-3 halogenase from pyrrolnitrin biosynthesis and to the halogenases PltM and PltA (35% and 28%, respectively) involved in pyoluteorin biosynthesis. The enzyme has no sequence similarity to the flavin-dependent tryptophan halogenases. The gene could be heterologously expressed in *Pseudomonas aureofaciens* ACN as soluble protein. Chlorinating activity of HalB was shown with two synthetic substrates with structural similarity to pentachloropseudilin. HalB is the first halogenase from an actinomycete and only the third halogenase for which halogenating activity could be demonstrated in vitro.

Keywords: *Actinoplanes* sp.; Chlorinated antibiotic; Chlorination; Halogenase; Pentachloropseudilin; Invitro activity

1. Introduction

Halogenated metabolites can be found very frequently in nature [1]. While brominated compounds are predominantly produced by marine organisms, a large number of chlorinated metabolites can be found in organisms of terrestrial origin. Many of these halogenated metabolites possess antibiotic activity.

Until 1995, haloperoxidases were thought to be the type of halogenating enzyme involved in the biosyntheses of halogenated metabolites. However, this view changed when during the cloning of the chlorotetracycline biosynthetic gene cluster [2] and the elucidation of the biosynthetic pathway of the antifungal antibiotic pyrrolnitrin a novel type of halogenating enzymes could be detected [3,4]. The two halogenating enzymes involved in pyrrolnitrin biosynthesis, tryptophan 7-halogenase (PrnA) and monodechloroaminopyrrolnitrin 3-halogenase (PrnC), require $FADH_2$, chloride ions, and molecular oxygen for halogenating activity [5]. Genes for such $FADH_2$-dependent halogenases have in the meantime been detected in many bacterial biosynthetic gene clusters for chlorinated metabolites [6]. However, only in the case of the two halogenases involved in pyrrolnitrin biosynthesis, PrnA and PrnC, halogenating activity has been demonstrated in vitro [4,5].

The strain *Actinoplanes* sp. ATCC 33002 produces the phenylpyrrole derivative pentachloropseudilin (Fig. 1) which was detected by Cavalleri et al. in 1978 [7]. Until now, only very few data on the biosynthetic pathway of pentachloropseudilin are available [8].

* Corresponding author. Tel.: +49-351-463-4494; fax: +49-351-463-5506.

E-mail address: karl-heinz.vanpee@chemie.tu-dresden.de (K.-H. van Pée).

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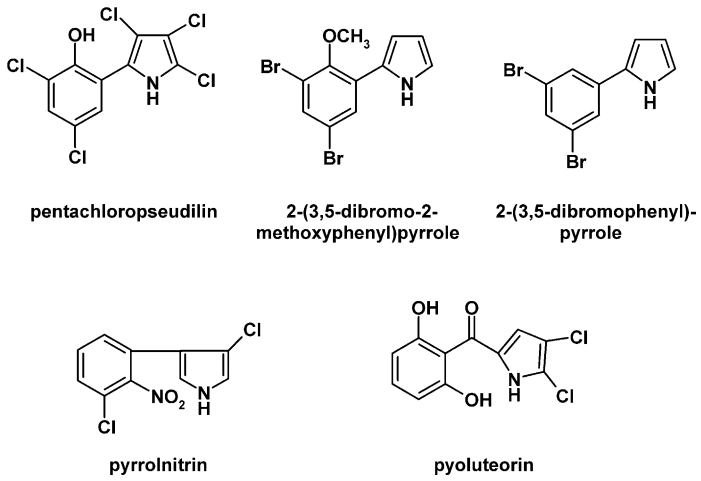

Fig. 1. Chemical structures of pentachloropseudilin, the two synthetic compounds used as substrates, pyrrolnitrin, and pyoluteorin.

Pentachloropseudilin contains chlorine atoms in the phenol ring as well as in the pyrrole ring and it is not known whether the incorporation of the five chlorine atoms is catalyzed by a single halogenase or by several enzymes. Because of its structural similarity to pyrrolnitrin (Fig. 1), we used the halogenase genes *prnA* and *prnC* [3,9] from the pyrrolnitrin biosynthetic gene cluster as probes to detect halogenase genes in the pentachloropseudilin producer *Actinoplanes* sp.

2. Material and methods

2.1. Bacterial strains and culture conditions

Actinoplanes sp. ATCC 33002 was cultured in soybean flour-glucose medium [10] at 28 °C for 3 days. *E. coli* was cultured in Luria–Broth (LB medium) [11] or HNB medium (3 g meat extract, 5 g yeast extract, 5 g peptone from meat, 5 g NaCl per litre) at 37 °C for 6–12 h. *Pseudomonas aureofaciens* and *P. fluorescens* were cultured at 30 °C in HNB medium for one day in genetic experiments and in MMM medium (25 g molasses, 2.1 g corn meal, 10 g malt extract, 30 g sucrose, 10 g yeast extract, 2 g K_2HPO_4 per litre, pH 7.0) for 3 days in expression experiments. In conjugation experiments PMM agar plates [12] were used. Antibiotics were added as follows: ampicillin 100 μg ml^{-1}, apramycin 100 μg ml^{-1}, chloramphenicol 34 μg ml^{-1}, tetracycline 15 μg ml^{-1} for *E. coli* and 30 μg ml^{-1} for *Pseudomonas*.

2.2. DNA manipulation, Southern blot analysis, cloning and sequencing of the halogenase gene

Total DNA was isolated using "2× Kirby mix" as described by Hopwood et al. [13]. Plasmids were routinely isolated from *E. coli* and *Pseudomonas* strains by an alkaline lysis method [11]. Conjugation from *E. coli* S17-1 into *Pseudomonas* was performed according to the protocol described by Hill et al. [14]. The DNA probes used were the *prnC* gene from *P. fluorescens* BL915 [3] and a 296-bp polymerase chain reaction (PCR) product which was amplified using the two PCR primers CHLST1+ (5′-GCGGCTGCAGSTGGDWSATYCCGYT-3′) and CHLST1-(5′-CCSSTGGATCCSCGGGTCSABGAAG-C-3′) derived from consensus sequences of known halogenases [6] and synthesized by MWG Biotech GmbH, Ebersberg, Germany. DNA probes were labelled with digoxygenin (Roche Diagnostic, Mannheim, Germany).

A cosmid library was constructed using the cosmid vector pOJ446 [15] and the Gigapack III Gold Packaging Extract (Stratagene, La Jolla, USA). The library was screened using the 296-bp PCR-product.

For sequence analysis, overlapping fragments were subcloned from the cosmid pIW3000Bcos10 into the vector pBluescript SK+ and sequenced with standard primers (MWG Biotech GmbH, Ebersberg, Germany). The nucleotide sequence data for *halB* have been deposited in the GenBank nucleotide sequence database under the Accession No. AF450451.

2.3. Heterologous expression of halB and enzyme assay

For construction of the *E. coli* expression plasmid pRSEThalB, *halB* was amplified by PCR (forward primer 5′-TATAAGATCTATGAGTTCAGCGCC-3′, reverse primer 5′-TATAAAGCTTTCAGAAGATCC-TCC-3′) and ligated into the *Bgl*II/*Hin*dIII sites of pRSETB. For construction of the expression plasmid pCIBhalB, *halB* was amplified by PCR (forward primer 5′-CAACTCTAGAAGGAGGCAGTCATGA-3′, reverse primer 5′-TATAAAGCTTTCAGAAGATCC-TCC-3′) and ligated into the *Xba*I/*Hin*dIII sites of pPEH14 [9]. Amplification of a DNA fragment containing the His_6 tag, Xpress antibody epitope, enterokinase recognition site and *halB* from the plasmid pRSEThalB (forward

primer 5′-TAACTCTAGAAAGGAGATATACATATGCGG-3′, reverse primer 5′-TATAAAGCTTTCAGAAGATCCTCC-3′) and ligation into the *Xba*I/*Hin*dIII sites of pPEH14 resulted in the expression plasmid pCIBhalBhis.

For expression of *halB* in *E. coli*, the strain BL21 (DE3) pLysS was used. The recombinant strain *E. coli* pLys pRSEThalB was grown in LB medium at 37 °C to an $OD_{600\,nm}$ between 0.4 and 0.6, induced with IPTG at a final concentration of 2 mM and further incubated at 30 °C. After harvesting the cells by centrifugation and resuspension in 100 mM potassium phosphate buffer (pH 7.2), cells were disrupted by grinding with glass beads using a mixer-mill. For expression of *halB* and *halB*his in Pseudomonads, the strains *P. aureofaciens* ACN [16] and *P. fluorescens* BL915 [14] were used. Bacteria were harvested by centrifugation after 3 days of growth, resuspended in 100 mM potassium phosphate buffer (pH 7.2), and disrupted by sonication. Crude cell extracts and the pellets were analyzed by SDS–PAGE [17] and Western blot analysis using the Anti-Xpress-HPR antibody (Invitrogen, Groningen, The Netherlands) to detect the Xpress antibody epitope contained in HalBhis.

The enzyme assay mixture contained 2.5 mM NADH, 12.5 μM FAD, 6.25 mM $MgCl_2$, 0.2 mM 2-(3,5-dibromo-2-methoxyphenyl)pyrrole or 2-(3,5-dibromophenyl)pyrrole as the organic substrates, and 200 μl of crude cell extract in a total volume of 800 μl. The assays were incubated at 30 °C for 16 h with shaking. After extraction for three times with 400 μl methyl-*tert*-butyl ether and concentration in vacuo, the extracts were analyzed by GC–MS with a GC Hewlett–Packard 5890, MSD 5971 and HP G1034C MS ChemSation software. The column used was an HP-1 column with a length of 11.5 m, an inner diameter of 0.21 mm and a film thickness of 0.33 μm. The injector temperature was 260 °C and the detector temperature was 290 °C. The oven program started at 80 °C for 1 min, heated to 310 °C with a rate of 20 °C min^{-1} and the final temperature was held for 3 min. As carrier gas helium was used with a flow rate of 1 ml min^{-1}.

3. Results and discussion

3.1. Hybridisation experiments, screening of the cosmid library and analysis of the positive clone

In Southern hybridisation experiment, using *prnC* as the probe hybridizing fragments could be detected (Fig. 2). However, no hybridisation signals could be found when *prnA* was used as the probe. This is consistent with the finding that $FADH_2$-dependent halogenases can be divided into two groups according to their substrate specificity, with one group acting on tryptophan or indole

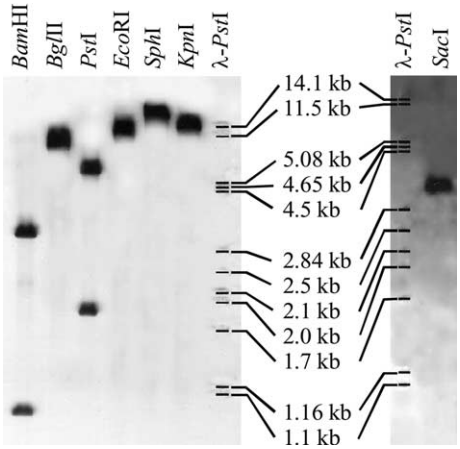

Fig. 2. Hybridisation of total DNA from *Actinoplanes* sp. after digestion with different restriction enzymes with the monodechloroaminopyrrolnitrin 3-halogenase gene (*prn*C) as the probe.

derivatives as substrates and the other group catalysing the halogenation of pyrrole or phenol derivatives [6].

Screening of the cosmid library by colony hybridisation with the 296-bp PCR product as the probe resulted in the detection of four positive cosmid clones. The clone containing the cosmid pIWcos10 was further analyzed. Subcloning of a 3.7-kb *Sac*I fragment into pUC18 and sequencing of the insert showed that this fragment contained a potential halogenase gene which was named *halB*.

3.2. Deduced protein sequence of HalB

Translation of the nucleotide sequences of *halB* into the corresponding amino acid sequence and comparison with the halogenating enzymes PrnC [3] and Cts4 [2] showed a high degree of identity of 44% and 51%, respectively. Whereas regions of high similarity can be found in the central part, the similarity at the end of the enzyme is rather low. A highly conserved motif GxGxxG which is known to be involved in the binding of nucleotide cofactors [18] is located near the N-terminus. In the central part of the enzyme, a second absolutely conserved motif containing two tryptophan residues (WxWxI) could be identified. Interestingly, both motifs are incomplete in PltD which has a sequence identity of 33% to PrnC and which is involved in pyoluteorin biosynthesis [19]. However, the function of PltD in pyoluteorin biosynthesis is not that of a halogenase, since the two halogen atoms present in pyoluteorin (Fig. 1) are incorporated by PltA and PltM. This shows that the overall sequence similarity is not a sufficient criterion for the annotation of halogenases, but that the two motifs (GxGxxG and WxWxI) are also essential criteria for the identification of $FADH_2$-dependent halogenases. The overall identity between the new halogenase HalB and other known halogenases from

the biosynthetic gene clusters of halogenated metabolites range between 28% for PltA from pyoluteorin biosynthesis [19] and 51% for Cts4 from chlorotetracycline biosynthesis [2] after correction of a sequencing error in the *cts4* gene [3,19].

3.3. Heterologous expression of halB in E. coli and in Pseudomonas strains and enzyme assay

Expression experiments of *halB* in *E. coli* resulted in the formation of insoluble protein. *HalB* was overexpressed as soluble protein in *P. aureofaciens* ACN from the expression plasmid pCIBhalB (Fig. 3(a)). Using the expression plasmid pCIBhalBhis which additionally contains the 6×His tag and the Xpress antibody epitope from the vector pRSETB overexpression of *halB* in *P. fluorescens* (Fig. 3(a)) could be proved by immunological detection of the Xpress antibody epitope (Fig. 3(b)).

The enzyme assays were carried out with crude cell extracts from *P. aureofaciens* ACN pCIBhalB and *P. fluorescens* BL915 pCIBhalBhis. Since flavin reductases are present in crude extracts [5] flavin reductase was not added. As controls, crude cell extracts of the host strains *P. aureofaciens* ACN and*P. fluorescens* BL915 were analysed for chlorinating activity. These controls did not show any halogenating activity with the substrates used. Halogenating activity was detected with the crude cell extract of *P. aureofaciens* ACN pCIBhalB with 2-(3,5-dibromo-2-methoxyphenyl)pyrrole (Fig. 1) as the substrate. The mass of the parent peak of the substrate (*m/z* 329/331/333, relative intensity 1:2:1) was shifted by *m/z* 34 in the parent peak of the product (*m/z* 363/365/367/369; relative intensity 3:7:5:1). This mass shift and the pattern of the parent peak obtained by GC–MS clearly showed that the obtained product contained an additional chlorine atom. However, the chlorinating activity of HalB with this substrate was very low. A much higher chlorinating activity for HalB was found when 2-(3,5-dibromophenyl)pyrrole (Fig. 1; *m/z* 299/301/303, relative intensity 1:2:1) was used as the substrate (Fig. 4). Again an additional chlorine atom was incorporated into the substrate with production of a compound with a mass shift of *m/z* 34 (*m/z* 333/335/337/339, relative intensity 3:7:5:1). No halogenating activity was detected

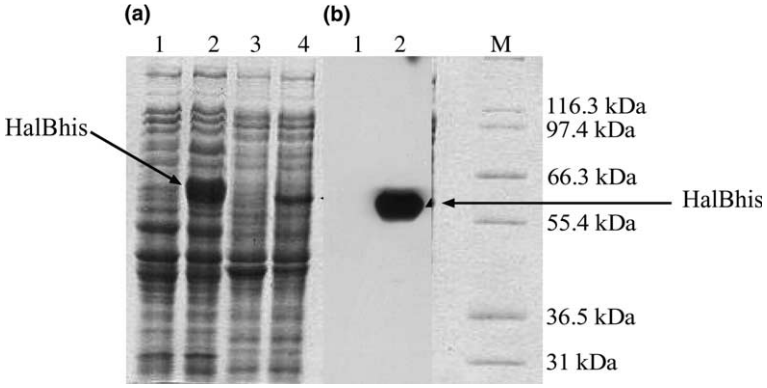

Fig. 3. SDS–PAGE (a) and Western blot (b) analysis of the crude cell extracts from *P. fluorescens* BL915 (lanes a1 and b1), *P. fluorescens* pCIBhalBhis (lanes a2 and b2), *P. aureofaciens* ACN (lane a3), and *P. aureofaciens* pCIBhalB (lane a4), molecular wheight marker (lane M).

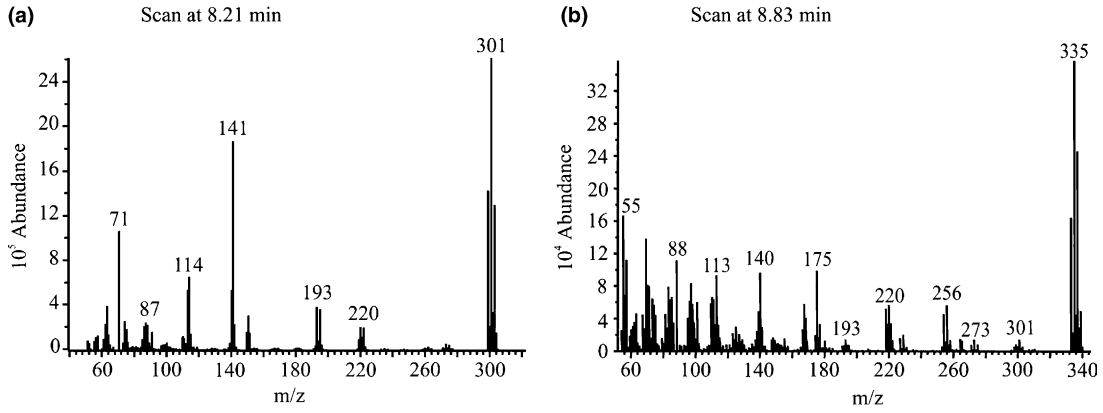

Fig. 4. Mass spectra of (a) 2-(3,5-dibromophenyl)pyrrole (*m/z* 299/301/303, relative intensity 1:2:1) used as the substrate in the enzyme assay with crude cell extract of *P. aureofaciens* pCIBhalB and of the product (b) identified as chloro-2-(3,5-dibromophenyl)pyrrole (*m/z* 333/335/337/339, relative intensity 3:7:5:1).

in the crude cell extract of *P. fluorescens* BL915 pCIB-halBhis with both substrates, indicating that the His tag might lead to not correctly folded HalB. The low activity of HalB is most likely due to the fact that both substrates are not the natural substrates for HalB. However, whereas for none of the many halogenase genes detected so far expression of the gene could be demonstrated, with the exception of *prnA* and *prnC*, chlorinating activity for HalB could be clearly shown. The very slow progress in the field of enzymatic halogenation is certainly mostly due to the fact that the recently detected $FADH_2$-dependent halogenase have high substrate specificity and that their natural substrates are not known or are not available. This is also due for HalB. However, the chlorinating activity of HalB with the substrates used in this study is much too low to allow their use in the purification and characterisation of the enzyme. This will have to await the identification of the natural substrate.

However, the fact that the two substrates which have quite some structural similarity to pentachloropseudilin (Fig. 1) are chlorinated by HalB, indicates that HalB could be involved in pentachloropseudilin biosynthesis and that at least one of the chlorination steps might occur quite late in the biosynthetic pathway. However, to prove this, identification of the complete pentachloropseudilin biosynthetic gene cluster is necessary.

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