The non-haem chloroperoxidase from *Pseudomonas fluorescens* and its relationship to pyrrolnitrin biosynthesis

Sabine Kirner,¹ Susanne Krauss,¹ Gisela Sury,¹ Stephen T. Lam,² Jim M. Ligon² and Karl-Heinz van Pée³

Author for correspondence: Karl-Heinz van Pée. Tel: +49 351 463 4494. Fax: +49 351 463 5506.
e-mail: chr@ctch02.chm.tu-dresden.de

¹ Institut für Mikrobiologie,
Universität Hohenheim,
Garbenstrasse 30, D-70593
Stuttgart, Germany

² Department of Molecular
Genetics, CIBA Agricultural
Biotechnology, Research
Triangle Park, North
Carolina, USA

³ Institut für Biochemie,
Technische Universität
Dresden, D-01062 Dresden,
Germany

The non-haem chloroperoxidase gene (*cpoF*) from the pyrrolnitrin producer *Pseudomonas fluorescens* BL914 was cloned using an oligonucleotide derived from part of the N-terminal amino acid sequence of chloroperoxidase (CPO-P) from *Pseudomonas pyrocinia* as a probe. Based on the overexpression of *cpoF* in *Escherichia coli* and the stability of CPO-F against higher temperatures and proteases, the enzyme was purified to homogeneity. Partial characterization of the enzyme showed that it belongs to the class of bacterial non-haem CPOs. To investigate the role of CPO-F in pyrrolnitrin biosynthesis, the *cpoF* gene was inactivated by insertion of a kanamycin cassette. Exchange of the chromosomal *cpoF* gene against the disrupted copy had no influence on pyrrolnitrin production demonstrating that CPO-F was not involved in pyrrolnitrin biosynthesis.

Keywords: *Pseudomonas fluorescens*, pyrrolnitrin, chloroperoxidase, gene disruption

INTRODUCTION

Haloperoxidases are a widely distributed family of enzymes catalysing the formation of carbon–halogen bonds in the presence of hydrogen peroxide, halide ions and a suitable organic substrate. According to molecular and catalytic properties, they can be divided into two classes: haem-type and non-haem-type haloperoxidases. The haem enzymes, such as chloroperoxidase (CPO) from *Caldariomyces fumago* (Hager *et al.*, 1966), usually contain protoporphyrin IX as prosthetic group and exhibit catalase and peroxidase activities (Neidleman & Geigert, 1986). The non-haem enzymes can be subdivided into two groups: eukaryotic haloperoxidases which contain vanadium (Vilter, 1984; Wever *et al.*, 1985) and bacterial non-haem haloperoxidases which require neither metal ions nor any other cofactors (Hecht *et al.*, 1994). According to the halide ions that they can oxidize, haloperoxidases are designated as chloroperoxidases, bromoperoxidases and iodoperoxidases.

Naturally occurring halogenated compounds have been isolated from many different organisms including pro-

karyotes, fungi, plants and mammals. It has been widely accepted that haloperoxidases are involved in the formation of organohalogenes (Neidleman & Geigert, 1986; van Pée, 1990). Among the most interesting and best studied organohalogenes are the chlorinated antibiotics like chloramphenicol (Ehrlich *et al.*, 1947), 7-chlorotetracycline (Duggar *et al.*, 1948), vancomycin (McCormick *et al.*, 1956) and pyrrolnitrin (Arima *et al.*, 1964) that are produced by bacteria.

Pyrrolnitrin, a chlorine-containing phenyl-pyrrole derivative with antifungal activity, was isolated from a number of *Pseudomonas* strains (Arima *et al.*, 1964; Elander *et al.*, 1968; Imanaka *et al.*, 1965). On the basis of metabolites isolated, a hypothetical pathway for pyrrolnitrin biosynthesis (Fig. 1) was postulated (van Pée *et al.*, 1980). From the pyrrolnitrin producer *Pseudomonas pyrocinia*, a CPO (CPO-P) was isolated that catalysed the chlorination of monodechloroaminopyrrolnitrin to aminopyrrolnitrin *in vitro* (Wiesner *et al.*, 1988) and the last step of pyrrolnitrin biosynthesis, the oxidation of the amino group of aminopyrrolnitrin to the nitro group of pyrrolnitrin (Kirner & van Pée, 1994). This strongly suggested the participation of this CPO in pyrrolnitrin biosynthesis.

To further investigate the role of non-haem CPOs in

Abbreviations: CPO, chloroperoxidase; *cpo*, gene encoding chloroperoxidase; DIG, digoxigenin

Fig. 1. Hypothetical biosynthetic pathway of pyrrolnitrin biosynthesis according to van Pée *et al.* (1980). All of the shown intermediates with the exception of 7-chlorotryptophan have been isolated.

pyrrolnitrin biosynthesis, a genetic approach was adopted. Here we describe the cloning of a CPO gene (*cpoF*) from *Pseudomonas fluorescens* BL914, the purification of the corresponding enzyme, its partial characterization, a gene replacement experiment and its effect on pyrrolnitrin biosynthesis.

METHODS

Bacteria, plasmids and culture conditions. *P. fluorescens* BL914 (Hill *et al.*, 1994), with or without plasmid pCIB137, containing a global regulatory gene for antifungal activity (Gaffney *et al.*, 1994) was grown at 30 °C, either on LB medium or on *Pseudomonas* minimal medium (PMM), consisting of 35 mM $K_2HPO_4 \cdot 3H_2O$, 22 mM KH_2PO_4 , 8 mM $(NH_4)_2SO_4$, 25 mM sodium succinate and 1.2 mM $MgSO_4$. *Escherichia coli* TG1 (Gibson, 1984) and *E. coli* S17-1 (Simon *et al.*, 1983) were routinely grown either in liquid LB medium or on LB agar plates at 37 °C. When required for the maintenance of plasmids, antibiotics were added at the following concentrations: ampicillin, 100 $\mu g\ ml^{-1}$; tetracycline, 20 $\mu g\ ml^{-1}$; kanamycin, 50–100 $\mu g\ ml^{-1}$. For routine cloning and subcloning in *E. coli*, pUC18 and pUC19 (Vieira & Messing, 1982) and pBR322 (Bolivar *et al.*, 1977) were used. *E. coli* transformants carrying pUC18 or pUC19 were selected on LB agar containing ampicillin (100 $\mu g\ ml^{-1}$), X-Gal (40 $\mu g\ ml^{-1}$) and 0.2 mM IPTG. For isolation of the kanamycin cassette, pUC4K obtained from Pharmacia was used.

Bacterial conjugation. Plasmid DNA was introduced from *E. coli* into *P. fluorescens* by a biparental procedure utilizing the donor host *E. coli* S17-1 with *tra* genes integrated into the donor strain chromosome (Simon *et al.*, 1983). Selection for

exconjugants was performed on PMM agar containing kanamycin and tetracycline allowing only recombinant *Pseudomonas* cells to grow.

DNA isolation and manipulations. Total DNA was prepared as described by Davis *et al.* (1980). Plasmid DNA was isolated by the alkaline lysis method (Sambrook *et al.*, 1989). Agarose gel electrophoresis, restriction endonuclease digestions, treatment with alkaline phosphatase and DNA ligations were performed according to standard procedures (Sambrook *et al.*, 1989). DNA fragments were isolated from agarose gels with the GeneClean II kit (Dianova) as recommended by the supplier. Oligonucleotides and DNA probes for use in Southern hybridization (Southern, 1975) were labelled with digoxigenin (DIG) as recommended by the manufacturer (Boehringer Mannheim) with the DIG Oligonucleotide 3'-End Labelling Kit and the DIG DNA Labelling Kit, respectively.

Transfers and hybridizations. DNA fragments separated by agarose gel electrophoresis were denatured (0.5 M NaOH, 1.5 M NaCl), equilibrated (0.25 M NaOH, 1.5 M NaCl) and transferred (Sambrook *et al.*, 1989) to a nylon membrane (Schleicher & Schuell). Colony screening (Sambrook *et al.*, 1989) was performed on Hybond-N gridded membranes (Amersham-Buchler) according to the manufacturer's instructions. Detection of hybridizing DNA fragments and colonies was performed with the DIG Luminescent Detection Kit (Boehringer Mannheim).

Hybridization experiments using a mixed 17-mer oligonucleotide [TTT(C)TAT(C)AAA(G)GAT(C)TGG GG] that corresponded to the amino acid residues 12–17 of the N-terminus of CPO-P from *P. pyrocinia* (Wolframm *et al.*, 1988) as the probe, were carried out overnight at 43 °C in $2 \times$ SSC (1 $\times$ SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). The membranes were stringently washed twice in $2 \times$ SSC containing 0.1% SDS at 43 °C for 15 min and then twice in 1 $\times$ SSC containing 0.1% SDS at 43 °C for 15 min.

Using the DIG-labelled 1.2 kb *KpnI* fragment as a probe for identification of the original, functional *cpoF* gene and the disrupted *cpoF* copy, hybridizations and washing steps were carried out at 80 °C, as described above. The last stringent wash was performed twice for 15 min in $0.1 \times$ SSC, 0.1% SDS.

Subcloning and construction of the replacement vector. For subcloning in *E. coli*, fragments were isolated from agarose gels and ligated with linearized pUC18. For inactivation of the *cpoF* gene, pUC4K was digested with *Bam*HI and the 1.3 kb fragment containing the kanamycin cassette was ligated into a 0.3 kb deletion in the *cpoF* gene, obtained by digestion of pSK380 with *Bgl*II, resulting in pSK480. After digestion of pSK480 with *Eco*RI and partial digestion with *Hind*III, the generated fragments were separated by agarose gel electrophoresis. The appropriate 4.8 kb *Eco*RI–*Hind*III fragment was isolated and ligated to *Eco*RI–*Hind*III-digested pBR322 resulting in pSK48D.

Enzyme assays, protein electrophoresis and protein measurements. Brominating activity was measured using the monochlorodimedone assay (Hager *et al.*, 1966) with 42 μM monochlorodimedone, 8.8 mM H_2O_2 , 0.1 M NaBr, 10 mM sodium azide in 1 M sodium acetate buffer, pH 5.5, at 25 °C, recording the decrease of monochlorodimedone absorbance at 290 nm due to the formation of monobromomonochlorodimedone. One unit of CPO catalysed the bromination of 1 μmol monochlorodimedone to monobromomonochlorodimedone in 1 min. Sodium azide was necessary for the inhibition of haem-containing catalases and peroxidases.

For electrophoresis of native enzymes, 7.5% (w/v) polyacrylamide gels (pH 7.5) were used (Maurer, 1964). Brominating activity was detected after native electrophoresis by the conversion of phenol red to bromophenol blue as described by Pfeifer *et al.* (1992). SDS-PAGE was performed according to Schagger & von Jagow (1987) using 4% stacking gels and 10% resolving gels. For estimation of subunit sizes, low molecular mass standards (Pharmacia) were used. Gels were stained for proteins with Coomassie Blue R250.

Protein concentrations were determined using the method of Whitaker & Granum (1980).

Purification of CPO-F from *E. coli* TG1 containing pSK230. For purification of CPO-F, *E. coli* TG1 containing *cpoF* on a 2.3 kb *XhoI* fragment in pUC18 (pSK230) was used. *E. coli* cells were grown in 6 × 11 LB medium for 24 h in the presence of ampicillin and IPTG, harvested by centrifugation, resuspended in 4 vols 10 mM ammonium acetate buffer, pH 7.0, and disrupted by sonication for 6 min (Branson Sonifier 450, 160 W, 50% duty cycle, 4 °C). The crude extract obtained by centrifugation (22 100 g, 4 °C, 30 min) of the sonicate was stored at -20 °C or used immediately for further purification.

Ammonium sulphate was added to the crude extract to 35% saturation at 0 °C. After centrifugation (22 100 g, 4 °C, 20 min), ammonium sulphate was added to the supernatant to 50% saturation. The pellet obtained by centrifugation was dissolved in the original volume of 10 mM ammonium acetate buffer, pH 7.0, and dialysed twice against this buffer for 2 h each time. The protein solution was slowly heated to 55 °C on a water bath with continuous stirring and then centrifuged immediately (22 100 g, 4 °C, 20 min). The supernatant of the heat treatment was diluted with buffer to a protein concentration of 5 mg ml⁻¹ and 0.01 vols trypsin solution (10 mg ml⁻¹ in 1 mM HCl) was added. After incubation at 37 °C for 16 h, the solution was concentrated and washed with 10 mM sodium acetate buffer, pH 5.5, by ultrafiltration (Amicon YM30 membrane). The resulting protein solution, concentrated to about one-fifth of its original volume, was loaded onto a DEAE-Sephacel column (1.5 × 6 cm), equilibrated with 10 mM sodium acetate buffer, pH 5.5. After washing the column with this buffer, proteins were eluted using a gradient (100 ml) of 0–0.6 M NaCl in buffer. Fractions (1.5 ml) with at least 20% of the brominating activity of the most active fraction were pooled and concentrated to about one-tenth of its original volume by ultrafiltration.

The concentrated solution was loaded onto a Sephacryl S 300 column (2.5 × 160 cm), equilibrated with 0.1 M ammonium acetate buffer, pH 6.8. Fractions (2 ml) with at least 10% of the brominating activity of the most active fraction were pooled, concentrated by ultrafiltration and stored at -20 °C.

Chlorination of indole to 3-chloroindole by CPO-F. Chlorination of indole was performed as described by Bantleon *et al.* (1994) with 0.1 mM indole, 100 mM NaCl, 29 mM H₂O₂ in a total volume of 10 ml of 1 M sodium acetate buffer, pH 4.5. The reaction was started by addition of 1 U of brominating activity of CPO-F and incubated at room temperature. The reaction was analysed by HPLC at 280 nm using a C₁₈ reverse phase column (125 × 4.6 mm) with methanol/water (60:40, v/v) as the eluent. Under these conditions 3-chloroindole was eluted at 9.5 min.

Measurement of pyrrolnitrin production. Cultures were incubated in LB medium for 48 h and extracted with 0.7 vols ethyl acetate. The extract, after evaporation, was taken up in a small volume of ethanol and analysed by thin-layer chromatography on silica gel with toluene as the eluent. Pyrrolnitrin was detected by spraying with van Urk's reagent (Stahl & Kaldewey, 1961).

The samples were additionally analysed by HPLC at 252 nm using a C₁₈ reverse phase column (125 × 4.6 mm) with methanol/water (70:30).

RESULTS

Cloning, subcloning and partial restriction mapping of *cpoF* from *P. fluorescens* BL914

The oligonucleotide derived from the N-terminal amino acid sequence of CPO-P from *P. pyrocinia* hybridized with a 9 kb *EcoRI* fragment of total DNA from *P. fluorescens* BL914 (Fig. 2). The enriched gene library was prepared by separating *EcoRI*-digested total DNA on an agarose gel and ligating the isolated DNA in the range of 9 kb into pUC18. Transformation of *E. coli* with the ligation mixture yielded about 400 transformants. Colony hybridization led to the identification of a single recombinant clone harbouring plasmid pSK900. Crude extracts of this clone showed brominating activity in the same range as *P. fluorescens* BL914.

For restriction mapping, pSK900 was digested with different restriction enzymes and the fragments were separated by agarose gel electrophoresis. Southern blotting and hybridization studies with the oligonucleotide probe led to the partial restriction map shown in Fig. 3. By ligating a 3.8 kb *EcoRI*–*BamHI* fragment into pUC18, a subclone (pSK380) was obtained that showed moderate overexpression of brominating activity and was suitable for the gene replacement experiment. Further subcloning using *XhoI* resulted in pSK230 containing *cpoF* on a 2.3 kb insert. *E. coli* clones harbouring pSK230 overexpressed brominating activity about 50 times, compared to *P. fluorescens* BL914 and *E. coli* clones harbouring pSK900, and were used for enzyme purification. The smallest insert conferring brominating activity was a 1.2 kb *KpnI* fragment (Fig. 3).

Disruption of *cpoF*

For construction of the gene replacement vector, the 3.8 kb insert of pSK380 was used. Digestion of pSK380 with *BglII* resulted in a 300 kb deletion (Figs 3 and 4). As a selectable marker, the 1.3 kb kanamycin cassette from pUC4K was inserted at the site of the deletion, yielding pSK480. *E. coli* clones harbouring pSK480 did not produce CPO-F or any other haloperoxidase (Fig. 5). For introduction of the disrupted *cpoF* gene into *P. fluorescens* by conjugation, the insert of pSK480 was religated into pBR322 resulting in the donor plasmid pSK48D that was transferred from *E. coli* S17-1 into *P. fluorescens* BL914 by biparental mating. As pSK48D cannot replicate in *Pseudomonas*, only such *P. fluorescens* clones could survive on PMM medium containing kanamycin that had the marker gene integrated into the chromosome. Single crossover mutants could be distinguished from double crossover mutants by screening the kanamycin-resistant clones for loss of tetracycline resistance. Approximately 90% of the kanamycin-resistant exconjugants were tetracycline-sensitive. Analysis of the total DNA of these

Fig. 2. (a) Agarose gel electrophoresis and (b) Southern hybridization of total DNA from *P. fluorescens* BL914, plasmid DNA and total DNA from single and double crossover mutants of *P. fluorescens* BL914 using the 1.2 kb insert of pSK120 as a probe. Lanes: 1, *Pst*I-digested λ -DNA; 2, *Eco*RI-digested total DNA from *P. fluorescens* BL914; 3, *Eco*RI-digested pSK900; 4, *Eco*RI-*Bam*HI-digested total DNA from *P. fluorescens* BL914; 5, *Eco*RI-*Bam*HI-digested pSK380; 6, *Eco*RI-*Bam*HI-digested pSK480; 7, *Eco*RI-*Bam*HI-digested total DNA from a single crossover mutant of *P. fluorescens* BL914; 8, *Eco*RI-*Bam*HI-digested total DNA from a double crossover mutant of *P. fluorescens* BL914.

Fig. 3. Partial restriction map of the 3.8 kb *Bam*HI-*Eco*RI insert of pSK380. The smallest subclone exhibiting brominating activity is marked with a bold line.

clones by Southern hybridization showed that they only contained a copy of the *cpoF* gene, inactivated by the incorporation of the kanamycin cassette (Fig. 2).

In *P. fluorescens* BL914 pyrrolnitrin production is regulated by a global regulatory gene for antifungal activity which had been cloned into pCIB137 (Gaffney *et al.*, 1994). Therefore, pCIB137 was introduced into the double crossover mutants of *P. fluorescens* BL914 by conjugation. Analysis of clones containing only an inactivated copy of the *cpoF* gene and pCIB137 for pyrrolnitrin production showed that they still produced pyrrolnitrin at the same level as *P. fluorescens* BL914(pCIB137) containing an active copy of the *cpoF* gene.

Purification of CPO-F from *E. coli* TG1 harbouring pSK230

CPO-F was overexpressed by *E. coli* clones harbouring pSK230. These clones produced about 50 times more of the enzyme than *P. fluorescens* BL914. The enzyme was purified by fractionated ammonium sulphate precipitation between 35–50% ammonium sulphate saturation, followed by heat treatment at 55 °C, digestion of contaminating proteins with trypsin, removal of digested, smaller proteins and peptides by ultrafiltration, ion

Fig. 4. Construction of the gene replacement vector pSK48D. The approximate location of *cpoF* is indicated by bold lines.

exchange chromatography on DEAE Sephacel and molecular sieve chromatography on Sephacryl S 300. From 6 g of cells, 22 mg of electrophoretically homogeneous CPO (Fig. 5) with a specific activity of 3.8 U

Fig. 5. Native PAGE of crude extracts and purified CPO-F (a) stained for protein and (b) stained for brominating activity. Lanes: 1, crude extract of *P. fluorescens* BL914 harbouring pCIB137; 2, crude extract of *E. coli* harbouring pSK380; 3, crude extract of *E. coli* harbouring pSK480; 4, CPO-F from *P. fluorescens* BL914 purified from *E. coli* harbouring pSK230.

Table 1. Purification of CPO-F from *E. coli* harbouring pSK230

Step	Total protein (mg)	Total activity (U)	Specific activity [U (mg protein) ⁻¹]	Yield (%)
Crude extract	1680	176	0.1	100
Ammonium sulphate precipitation	263	168	0.6	95
Heat treatment	166	130	0.8	74
Trypsin digestion	108	120	1.1	68
DEAE Sephacel	40	100	2.5	57
Sephacryl S 300	22	84	3.8	48

mg⁻¹ and a recovery of 48% were obtained. The results of a typical purification procedure are summarized in Table 1.

Partial characterization of CPO-F

Purified CPO-F was not inhibited by azide and also showed other properties characteristic for bacterial non-haem haloperoxidases. The M_r of the native enzyme was estimated to be 70000. However, with a subunit size of M_r 29000, as estimated by SDS-PAGE (not shown), it was not possible to decide whether the native enzyme comprises two or three identical subunits. Bromination of monochlorodimedone is strongly dependent on acetate with an optimal acetate concentration of 0.5 M. The enzyme is very stable against hydrogen peroxide. At a concentration of 150 mM it still has about 50% of the

activity shown at a concentration of 15 mM which is the optimal hydrogen peroxide concentration for bromination of monochlorodimedone by CPO-F. The pH optimum for this reaction is at pH 5.5. The chlorination of indole by CPO-F demonstrated that it is a CPO.

DISCUSSION

The fact that the non-haem CPO from *P. pyrocinia* catalyses *in vitro* the last two steps in pyrrolnitrin biosynthesis (Fig. 1), the chlorination of monodechloroaminopyrrolnitrin to aminopyrrolnitrin (Wiesner *et al.*, 1988) and the oxidation of the amino group of aminopyrrolnitrin to the nitro group of pyrrolnitrin (Kirner & van Pée, 1994), strongly suggested the participation of this enzyme in pyrrolnitrin biosynthesis.

However, the lack of substrate- and regiospecificity of bacterial non-haem CPOs (Itoh *et al.*, 1993; Bongs & van Pée, 1994; Burd *et al.*, 1995), the high K_m values for hydrogen peroxide (1.6–30 mM) and bromide (11–69 mM) (Burd *et al.*, 1995), as well as the presence of these enzymes in bacteria not known to produce halo-metabolites (Bantleon *et al.*, 1994; Burd *et al.*, 1995), casted some doubt on their role in the biosynthesis of halometabolites and nitrocompounds. To see whether bacterial non-haem haloperoxidases are really involved in the biosynthesis of halometabolites and nitrocompounds, we decided to replace the functional chromosomal *cpoF* in a pyrrolnitrin-producing strain by a disrupted copy and measure the effect of this gene replacement on pyrrolnitrin production. However, it was not possible to use *P. pyrocinia* for this experiment, as this strain is a poor producer of pyrrolnitrin and is resistant to many antibiotics used for selection in molecular genetics. Therefore, an oligonucleotide derived from part of the N-terminal sequence of *P. pyrocinia* CPO was used to clone a corresponding enzyme from the pyrrolnitrin producer *P. fluorescens* BL914. The gene was originally cloned on a 9 kb *EcoRI* fragment. Subcloning resulted in a 3.8 kb *EcoRI*–*BamHI* (pSK380) and a 2.3 *KpnI* fragment (pSK230) containing *cpoF*. The 3.8 kb fragment was used for the construction of a replacement vector and the 2.3 kb fragment for isolation of CPO from recombinant *E. coli* clones. The isolation of CPO-F was based on the overexpression in *E. coli* and on the temperature and protease stability of bacterial non-haem haloperoxidases (van Pée, 1988; Zeiner *et al.*, 1988). Determination of a few of its properties showed that the isolated enzyme belonged to the bacterial non-haem CPOs. The M_r of the subunits (29 000) was very similar to that of other bacterial non-haem haloperoxidases (Pelletier *et al.*, 1994; Bantleon *et al.*, 1994; Burd *et al.*, 1995).

Replacement of the chromosomal *cpoF* gene in *P. fluorescens* BL914 by an inactivated copy via homologous recombination yielded mutants of *P. fluorescens* BL914 that, when transformed with the global regulatory gene for antifungal activity on the plasmid pCIB137 (Gaffney *et al.*, 1994), produced pyrrolnitrin at the same level as the wild-type strain containing pCIB137. This clearly proved that CPO-F cannot be involved in either the chlorination of monodechloroaminopyrrolnitrin or in the oxidation of aminopyrrolnitrin.

The catalysis of unspecific halogenation (Bongs & van Pée, 1994) and unspecific oxidation of amino to nitro groups (Itoh *et al.*, 1993; Kirner & van Pée, 1994; Burd *et al.*, 1995) by bacterial non-haem haloperoxidases can be explained by the reaction mechanism of these enzymes, as suggested by Hecht *et al.* (1994). Bacterial non-haem haloperoxidases contain no cofactors or metal ions (Haag *et al.*, 1991; Hecht *et al.*, 1994); they only consist of amino acids. Comparison of the amino acid sequences and investigation of the three-dimensional structure of these enzymes revealed that they contain a catalytical triad, consisting of serine, histidine and aspartate residues (Hecht *et al.*, 1994). The serine residue of this triad reacts with acetate forming a serine/acetate ester. This ester is

not hydrolysed by water, but by hydrogen peroxide, resulting in the formation of peracetic acid (van Pée *et al.*, 1994) which, as a strong oxidizing agent, can oxidize halide ions leading to the formation of hypohalide ions, powerful halogenating agents, or can oxidize aromatic amino groups to nitro groups. This mechanism explains the lack of substrate specificity, observed for bacterial non-haem haloperoxidases and why these enzymes could falsely be thought to be involved in the biosynthesis of metabolites like pyrrolnitrin.

Dairi *et al.* (1995) showed that in 7-chlorotetracycline biosynthesis, the halogenating enzyme is also not a bacterial non-haem haloperoxidase. Comparison of the gene for the chlorination step in 7-chlorotetracycline biosynthesis showed that there is no similarity between this gene and bacterial non-haem haloperoxidases and haem-containing haloperoxidases (Facey *et al.*, 1996). The fact that non-haem haloperoxidases and haem-containing haloperoxidases (Facey *et al.*, 1996) are not involved in the specific formation of halometabolites by bacteria, raises the question what kind of halogenating enzymes are actually involved in the biosynthesis of pyrrolnitrin and other halometabolites?

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