Cloning of a second non-haem bromoperoxidase gene from *Streptomyces aureofaciens* ATCC 10762: sequence analysis, expression in *Streptomyces lividans* and enzyme purification

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The gene for BPO-A1, one of two non-haem bromoperoxidases in the tetracycline and 7-chlorotetracycline producer *Streptomyces aureofaciens* ATCC 10762, was cloned in the positive selection vector pIJ699 and expressed in *Streptomyces lividans* TK64. The cloned bromoperoxidase was over-produced up to 2800-fold by the *S. lividans* TK64 transformant. By taking advantage of the over-production of BPO-A1 and the heat stability of the enzyme, a new and simple purification procedure was developed. Subcloning into the vector pIJ487 and screening of recombinants by a newly developed histochemical assay located the *bpoA1* gene on a 2.1 kb *Bam*HI–*Hind*III fragment. The nucleotide sequence of the 2.1 kb fragment was determined; the *bpoA1* gene was identified within the sequence on the basis of the biased codon usage of *Streptomyces* genes and the presence of a nucleotide sequence encoding the N-terminal amino acid sequence obtained from the purified BPO-A1. Comparison of the deduced primary structure of BPO-A1 with those deduced for the non-haem chloroperoxidase CPO-P from *Pseudomonas pyrrrocinia* and the bromoperoxidase BPO-A2 from *S. aureofaciens* ATCC 10762 gave amino acid sequence identities of 49% and 40%, respectively.

Keywords: *Streptomyces aureofaciens*, bromoperoxidase BPO-A1/A2, non-haem haloperoxidase

INTRODUCTION

Haloperoxidases are widely distributed enzymes that catalyse the formation of carbon–halogen bonds in the presence of peroxides (e.g. hydrogen peroxide). The majority are haem-type oxidases with protoporphyrin IX as the prosthetic group (Dawson & Sono, 1987). A second group of haloperoxidases contains vanadium instead of haem (De Boer & Wever, 1988) and a more recently discovered third group, the bacterial non-haem haloperoxidases, does not require metal ions or any other cofactor (van Pée *et al.*, 1987; Wiesner *et al.*, 1988).

The first bacterial non-haem haloperoxidase, a chloroperoxidase (CPO-P) from *Pseudomonas pyrrrocinia*, was

detected by Wiesner *et al.* (1986). Another non-haem haloperoxidase was isolated from the 7-chlorotetracycline and tetracycline producer *Streptomyces aureofaciens* Tü24 (van Pée *et al.*, 1987). The enzyme isolated as a bromoperoxidase using the monochlorodimedone assay was later shown to be a chloroperoxidase (CPO-T; Burd *et al.*, 1992). Surprisingly *S. aureofaciens* ATCC 10762, another 7-chlorotetracycline and tetracycline producer, possessed two non-haem bromoperoxidases, BPO-A1 and BPO-A2 (Weng *et al.*, 1991). In immunological studies with antiserum raised against CPO-T, BPO-A2 cross-reacted with CPO-T but not with BPO-A1.

Up to now only very little is known about the reaction mechanism of bacterial non-haem haloperoxidases. Since the bacterial enzymes are able to catalyse the oxidation of halide ions without a prosthetic group or metal ions, one has to assume that the redox reaction takes place at certain

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amino acids of the peptide chain of the protein. Investigations by Haag *et al.* (1991) using the chloroperoxidases CPO-T from *S. aureofaciens* Tü24 and CPO-P from *P. pyrrocinia* gave the first evidence that a methionine residue is involved in the catalytic cycle of these enzymes.

The genes for BPO-A2 (Pfeifer *et al.*, 1992) and CPO-P (Wolfframm *et al.*, 1993) have been cloned and sequenced. The predicted amino acid sequence of BPO-A2 has only one methionine, which is consequently the only candidate for the postulated 'reactive methionine'. However, CPO-P possesses five methionines, none of them in a position similar to the methionine of BPO-A2 when the sequences are aligned (Wolfframm *et al.*, 1993); thus it was not possible to assign a putative reactive methionine for CPO-P.

BPO-A1 is in many respects different from BPO-A2 and CPO-P. Therefore the detection of amino acids conserved in all three proteins might give some information about the active site of bacterial non-haem haloperoxidases. In the following report we describe the cloning and sequencing of *bpoA1* and compare the deduced amino acid sequence with those of CPO-P and BPO-A2.

METHODS

Materials. Hydrogen peroxide (30%) was purchased from Merck, and monochlorodimedone from Sigma. Streptavidin-alkaline phosphatase complex, restriction endonucleases and bacteriophage T4 DNA ligase were obtained from Gibco/BRL and calf intestinal alkaline phosphatase (CIAP) was from Boehringer Mannheim. DEAE-Sephacel, Q Sepharose Fast Flow, Chelating Sepharose 6B and molecular mass standards for SDS-PAGE were obtained from Pharmacia. Amicon concentrators and ultrafiltration membranes (Diaflo, YM-10) were obtained from Amicon.

Bacterial strains, vectors and growth of cells. The gene library of *S. aureofaciens* ATCC 10762 was constructed in *S. lividans* TK64 (Hopwood *et al.*, 1983) using the plasmid pIJ699 (Kieser & Melton, 1988). Subcloning was done in the vector pIJ487 (Ward *et al.*, 1986). For DNA sequencing, DNA fragments were inserted into M13mp18 and M13mp19 (Yanisch-Perron *et al.*, 1985) and the phages were propagated in *Escherichia coli* TG1 (Maniatis *et al.*, 1982). Cultures of *S. lividans* TK64 harbouring recombinant plasmids were grown in soybean flour-mannitol (SM) medium (2%, w/v, soybean flour, 2%, w/v, mannitol) at 30 °C. Liquid media contained 5 µg thiostrepton ml⁻¹ and agar media 50 µg thiostrepton ml⁻¹ (kindly donated by E. R. Squibb and Sons Inc.). *E. coli* cells were grown in LB medium (Maniatis *et al.*, 1982) at 37 °C. Transformants carrying pUC18 and derivative plasmids were selected on agar media containing 100 µg ampicillin ml⁻¹, 40 µg X-gal ml⁻¹ and 0.2 mM IPTG. Recombinant M13 phages were identified, after infection of *E. coli* TG1, as white plaques in LB soft agar containing X-gal and IPTG.

Molecular biological methods. DNA from *S. aureofaciens* ATCC 10762 was isolated using '2 × Kirby mix' as described by Hopwood *et al.* (1985). Plasmids were isolated by an alkaline

lysis method (Kieser, 1984). *S. lividans* TK64 protoplasts were prepared and transformed as described by Hopwood *et al.* (1985). Protoplasts were regenerated on R2YE agar plates for 16 h and transformants were selected by overlaying the plates with 1 ml of a thiostrepton suspension in water (200 µg ml⁻¹). Phage DNA of M13 derivatives was extracted as described by Maniatis *et al.* (1982). DNA digestion with restriction enzymes, dephosphorylation with calf intestinal alkaline phosphatase and ligation were performed as described by Maniatis *et al.* (1982).

Hybridization studies. Total DNA was digested and separated by agarose gel electrophoresis. Southern blotting, prehybridization and hybridization were performed as described previously (van Pée, 1988) using a Nytran 13N nylon filter (Schleicher & Schüll). The hybridization conditions were 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium acetate, pH 7.0) containing 0.7% skimmed milk and 0.05% SDS at 68 °C; stringent wash conditions were 0.35 × SSC, 1% SDS at 60 °C.

The 2.1 kb *Bam*HI–*Hind*III fragment from pOP1974, containing the *bpoA1* gene used as a hybridization probe, was biotinylated with the nick-translation kit from Gibco/BRL according to the manufacturer's instructions.

Nucleotide sequence analysis. The nucleotide sequence of both strands of the 2.1 kb *Bam*HI–*Hind*III fragment containing the *bpoA1* gene was determined by the chain termination method (Sanger *et al.*, 1977) in an automated DNA sequencing system (ALF, Pharmacia) with fluorescence-labelled primers as described previously (Pfeifer *et al.*, 1992). The 2.1 kb *Bam*HI–*Hind*III fragment and subfragments generated with the restriction enzymes *Sac*I, *Sal*I, *Kpn*I and *Bgl*II were cloned in M13mp18 and M13mp19, and sequenced with the fluorescence-labelled universal primer. Gaps in the DNA sequence were closed by using additional fluorescence-labelled primers synthesized on a Cyclone Plus DNA synthesizer (Milligen). The DNA sequence was analysed for open reading frames (ORFs) and for the biased codon usage characteristic of *Streptomyces* genes by using the programs FRAMES and CODON PREFERENCES of the University of Wisconsin Genetics Computer Group (Devereux *et al.*, 1984). For the codon preference program, a codon usage table was generated from eight *Streptomyces* gene sequences as described by Sedlmeier & Altenbuchner (1992).

Construction of a genomic library of *S. aureofaciens* ATCC 10762. Total DNA of *S. aureofaciens* ATCC 10762 was partially digested with *Sau*3A and fractionated by electrophoresis in an agarose gel. Fragments in the 7–15 kb range were extracted from the gel (Tautz & Renz, 1983) and dephosphorylated. DNA of pIJ699 was digested with *Bgl*II and *Dra*I, and the fragments were separated on an agarose gel. The 5.0 kb vector fragment was extracted and ligated with the size-fractionated and dephosphorylated *S. aureofaciens* DNA fragments. The ligation mixture was then used to transform *S. lividans* TK64 protoplasts.

Screening haloperoxidase activities. For 'activity-screening' the thiostrepton-resistant colonies were transferred to SM agar containing thiostrepton and incubated for 7 d at 30 °C. The colonies from pairs of plates (88 colonies) were washed off with liquid SM medium and pooled in individual 1 litre flasks containing 200 ml liquid SM medium plus thiostrepton. The pool cultures were incubated at 30 °C for 5 d with shaking. The mycelium was harvested by centrifugation and the cells, suspended in twice their volume of 0.2 M Tris/SO₄ buffer (pH 8.3), were disrupted with a Branson sonifier J 17-A (twenty 30 s periods, 70 W, 4 °C). Cell debris was removed by centrifugation for 30 min at 22100 g. The supernatant solution was dialysed against 0.1 M sodium acetate buffer (pH 5.5) for 15 h. After centrifugation the pellet was discarded and the supernatant

solution was dialysed against 0.03 M Tris/SO₄ buffer (pH 8.3) for 15 h. The dialysed sample was applied to a DEAE-Sephacel column equilibrated with 0.03 M Tris/SO₄ buffer (pH 8.3). The column was washed with 0.2 M NaCl in 0.03 M Tris/SO₄ buffer (pH 8.3) and proteins were eluted with 0.7 M NaCl in the same buffer. Fractions containing bromoperoxidase activity were combined and then incubated for 15 min at 75 °C. Because the bromoperoxidase from *S. lividans* TK64 is inactivated by the heat treatment, only the bromoperoxidases from *S. aureofaciens* ATCC 10762 remained in solution. To distinguish between BPO-A1 and BPO-A2, samples showing bromoperoxidase activity after the heat treatment were examined by electrophoresis on a non-denaturing polyacrylamide gel. Positive pools were subdivided by further assays into smaller pools and eventually individual colonies were tested.

Subcloning in *S. lividans* TK64. For subcloning in *S. lividans* TK64, fragments were isolated from agarose gels and ligated with linearized and phosphatase-treated DNA of pIJ487 and transformed into *S. lividans* TK64 protoplasts. Colonies containing the bromoperoxidase gene were identified using a newly developed colony-screening procedure.

Colony-screening procedure. For colony-screening, the thiostrepton-resistant colonies were picked onto SM agar plates containing thiostrepton and incubated for 7 d at 30 °C. The plates were then flooded with lysozyme solution (2 mg ml⁻¹) in 0.3 M sucrose, 25 mM Tris/HCl (pH 8.0), 25 mM EDTA (pH 8.0), 5% (w/v) Triton X-100 and incubated for 1 h at 37 °C. The lysozyme solution was discarded, the plates washed once with water and afterwards flooded with 1 M sodium acetate buffer (pH 5.5), 0.002% phenol red, 1 M sodium bromide, 10 mM sodium azide, and 17.6 mM H₂O₂. Positive clones were identified by a blue halo around the colony, or as blue colonies after longer incubation.

Purification of BPO-A1 from recombinant *S. lividans* TK64 clones. All steps of the purification were performed at 4 °C unless otherwise stated. *S. lividans* TK64 harbouring plasmids encoding BPO-A1 was grown for 3 d; the cells were harvested (40 g wet wt), resuspended in 80 ml of 0.1 M ammonium acetate buffer (pH 6.8) and disrupted with a Branson sonifier J 17-A (twenty 30 s periods, 70 W, 4 °C). Cell debris was removed by centrifugation for 30 min at 22100 g. The supernatant solution was incubated at 75 °C for 15 min and precipitated proteins were removed by centrifugation. The heat-treated extract was applied at room temperature to a column (2.6 × 7.5 cm) of Q Sepharose Fast Flow, previously equilibrated with 20 mM ammonium acetate buffer (pH 6.8). After the column had been washed with 2 column volumes of the same buffer, the BPO-A1 activity was eluted with a linear gradient of 300 ml 0.1 M NaCl in the washing buffer. Fractions (3 ml) with activities more than 20% of the maximum were pooled and concentrated to 2–3 ml using an Amicon concentrator with a YM-10 membrane. The buffer was then changed by washing the concentrated solution twice with 20 mM potassium phosphate buffer (pH 7.0) in the Amicon apparatus. The concentrated solution was applied at room temperature to a Chelating Sepharose 6B column (2.6 × 10 cm) loaded with CuSO₄ (3 mg ml⁻¹ in H₂O) to about $\frac{2}{3}$ of its capacity and equilibrated with 20 mM potassium phosphate buffer (pH 7.0). Since BPO-A1 did not bind to the gel, it was eluted with the same buffer. Fractions (3 ml) with activities more than 20% of the maximum were combined and concentrated by ultrafiltration over a YM-10 membrane.

Enzyme assays. Brominating activity was measured spectro-

photometrically as described previously (Weng *et al.*, 1991). Protein concentration was determined by the Lowry method using bovine serum albumin as a standard.

Electrophoresis. For electrophoresis of native enzymes, 7.5% (w/v) polyacrylamide gels (pH 7.5) were used (Maurer, 1964). PAGE in the presence of 0.1% SDS was done according to Schägger & von Jagow (1987). Proteins of the low molecular mass calibration kit (Pharmacia) were used as standards. Gels were stained for proteins with Serva Blue R. Brominating activity was detected on native PAGE by the conversion of phenol red to bromophenol blue (Weng *et al.*, 1991).

RESULTS

Cloning and expression of the *bpoA1* gene from *S. aureofaciens* ATCC 10762

The gene encoding BPO-A1 from *S. aureofaciens* ATCC 10762 was isolated from a genomic library constructed in the plasmid pIJ699. Screening of 4400 thiostrepton-resistant colonies using the activity selection technique described in Methods gave two positive clones containing an insert fragment of 7.4 kb (pOP1974) or 7.9 kb (pOP1979). No clone containing *bpoA2* was isolated.

Restriction mapping and subcloning

Plasmids pOP1974 and pOP1979 exhibited nearly identical restriction patterns. Therefore only pOP1974 was used for further investigations. A partial restriction map is shown in Fig. 1. The insert contained no restriction sites for the endonucleases *Xba*I and *Xho*I. For subcloning, pOP1974 was digested with *Bam*HI and *Bam*HI–*Hind*III. Only the 3.8 kb *Bam*HI fragment (pOP1738) and the 2.1 kb *Bam*HI–*Hind*III fragment (pOP1721) gave bromoperoxidase activity when subcloned in pIJ487.

Southern blotting of total DNA digests

To confirm the origin of the cloned bromoperoxidase gene, Southern hybridization of the 2.1 kb *Bam*HI–*Hind*III fragment of pOP1974 was performed with *Bam*HI–*Hind*III-digested DNA from *S. aureofaciens* ATCC 10762 and *S. lividans* TK64. The biotinylated 2.1 kb

Fig. 1. Partial restriction map of the 7.4 kb fragment of pOP1974, containing the *bpoA1* gene from *S. aureofaciens* ATCC 10762. The abbreviations Ba, Bg, E, H, K, N, S and X indicate *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Kpn*I, *Nco*I, *Sst*I and *Xho*I, respectively. The 2.1 kb *Bam*HI–*Hind*III fragment which has been sequenced is marked with a bold line. The arrow indicates the location of the *bpoA1* gene, determined from the sequence data.

Fig. 2. Nucleotide sequence of the 2.1 kb *Bam*HI–*Hind*III fragment containing the *bpoA1* gene and the deduced amino acid sequence of BPO-A1. The sequence of amino acids underlined was determined by Edman degradation. The putative Shine–Dalgarno sequence of *bpoA1* and the potential promoter region are underlined. A palindromic sequence that overlaps the promoter regions and might play a role in the regulation of *bpoA1* expression is also indicated. An imperfect inverted repeat in the 3' flanking region of *bpoA1* presumably encodes a transcriptional terminator. A truncated ORF called *orf1* is upstream of *bpoA1*; the 118 amino acids of the C-terminal part of the protein ORF1 are given below the nucleotide sequence. In the intergenic region, a 17 bp palindromic sequence is indicated.

fragment of pOP1974 hybridized with a single 2.1 kb fragment of total DNA from *S. aureofaciens* ATCC 10762 but not with *S. lividans* TK64 DNA. These results

demonstrated that the cloned bromoperoxidase gene originated from the DNA of *S. aureofaciens* ATCC 10762 and not from *S. lividans* and that *bpoA1* is not similar in its

Fig. 3. SDS-PAGE of the bromoperoxidase produced by *S. lividans* TK64 harbouring pOP1738, BPO-A1 and BPO-A2 from *S. aureofaciens* ATCC 10762. Lanes: 1, 3.2 µg BPO-A2; 2, 3.2 µg BPO-A1 produced by *S. lividans* TK64(pOP1738); 3, 3.2 µg BPO-A1; 4, SDS marker proteins. The gel was stained with Serva Blue R.

DNA sequence to *bpoA2* despite its presence in the same strain (Pfeifer *et al.*, 1992). Furthermore no hybridization signals were found with the DNA of *S. aureofaciens* Tü24 and *P. pyrrocinia*. These species produce the chloroperoxidases CPO-T and CPO-P, respectively, which belong to the same group of non-haem haloperoxidases as BPO-A1.

Nucleotide sequence analysis

The nucleotide sequence of both strands of the 2.1 kb *Bam*HI–*Hind*III fragment was determined after subfragments with a size of about 500 bp had been cloned in M13mp18 and M13mp19. Only one complete ORF matching the very biased codon usage of *Streptomyces* was present (Fig. 2). This 825 bp ORF extended from position 1053 to 1878 downstream of the *Hind*III site and encoded a predicted protein of 275 amino acids. The calculated M_r of 30475 for the predicted polypeptide is in good agreement with the size of the BPO-A1 subunits (M_r 31000) as determined by SDS-PAGE (Fig. 3). Furthermore, the N-terminal amino acids of the deduced polypeptide are identical with the N-terminal amino acid sequence of BPO-A1 determined by Edman degradation of BPO-A1 purified from *S. aureofaciens* (Weng *et al.*, 1991). This clearly identifies the product of the cloned gene as BPO-A1. Upstream of the ORF a potential ribosome-binding site as well as a promoter (–35 and –10 regions) of the *E. coli* Eσ⁷⁰ type were identified by

DNA sequence comparison. An imperfect inverted repeat overlaps the putative promoter sequence and may be involved in the regulation of *bpoA1*. Another inverted repeat was found at the end of the gene, but its potential to form a stem-loop structure might be too weak for it to act as a transcriptional terminator. When the amino acid sequence of BPO-A1 was aligned with those of BPO-A2 and CPO-P (Fig. 4), pairwise comparison gave 40% amino acid identity for the BPO-A1/BPO-A2 pair, 49% for the BPO-A1/CPO-P pair, and 38% identity between BPO-A2 and CPO-P. The quite similar size of the three haloperoxidases and the high percentage of identical amino acids in their primary sequence indicate that they derive from a common ancestor. Furthermore, BPO-A1 seems to be more related to CPO-P than to BPO-A2.

A second ORF, truncated at its 5'-end and displaying the typical *Streptomyces* codon usage, was identified on the 2.1 kb fragment. This incomplete ORF ends 700 nucleotides upstream of the *bpoA1* gene at position 357 (Fig. 2), and encodes the last 118 amino acids from the C-terminal part of a putative protein called ORF1. It displays an overall amino acid identity of 34% with the C-terminal end of CwlA, an autolytic amidase from *Bacillus subtilis* (Foster, 1991). As shown in Fig. 5, many of the last 50 amino acids of ORF1 and CwlA are conserved in two other autolytic lysozymes, LysC from *Clostridium acetobutylicum* (Croux & Garcia, 1991) and Cbpm, the muramoyl-pentapeptide carboxypeptidase from *Streptomyces albus* (Joris *et al.*, 1983). From the sequence similarity of these regions in LysC and Cbpm, Croux & Garcia (1991) have postulated that the conserved domain is involved in recognition of the peptidoglycan substrate of these enzymes. The distance between the putative autolysin gene *orf1* and *bpoA1* makes it unlikely that they are part of an operon. Furthermore, an inverted repeat with a calculated ΔG of $-45 \text{ kcal mol}^{-1}$ at position 622–662 between the genes may terminate transcription of the autolysin gene.

Purification of BPO-A1 from *S. lividans* TK64 (pOP1738)

S. lividans TK64 containing pOP1738 produces the bromoperoxidase at high levels. When the purification method previously described for BPO-A2 (Pfeifer *et al.*, 1992) was used, no homogeneous protein was obtained. Therefore the purification procedure summarized in Table 1 was developed. The specific activity (46 U mg^{-1}) of the final product indicated a 51-fold purification. The bromoperoxidase isolated from *S. lividans* TK64(pOP1738) was identical to BPO-A1 from *S. aureofaciens* ATCC 10762 in its electrophoretic mobility on denaturing (Fig. 3) and non-denaturing polyacrylamide gels; it differed from BPO-A2 on native polyacrylamide gels (Fig. 6). When the gels were stained for brominating activity (Fig. 6a), several active bands could be seen. This is due to the formation of aggregates in buffers of low salt concentration. However, these bands could be detected only using activity staining, which is more sensitive than protein staining with Coomassie blue.

	1	**	+	+	+++++	+	*	*	+	++++	+	*	*	+
BPO-A1		MP ICTTRDG.	...	VEIFYKD	WGQ..	GRP	VV	FIHGW	PLNGD	AWQDQL	KAVV			
BPO-A2		MP FITVGQEN	STSIDLYED	HGT..	GQPVV	LIHGF	PLSGH	SWERQ	SAALL					
CPO-P		MP YVTTKDN.	...	VEIFYKD	WGP	KDAQPIV	FHHGW	PLSGD	DWDAQ	MLFFV				
	50		***	++++	*****	*	+	++	*	*	*	*	***	+
BPO-A1		DAGYRGIAHD	RRGHGHSTPV	WDGYDFDTFA	DDLNDLLTDL	DLRDVTLVAH								
BPO-A2		DAGYRVITYD	RRGFGQSSQP	TTGYDYDTFA	ADLNTVLETL	DLQDAVLVGF								
CPO-P		QKGYRVIAHD	RRGHGRSAQV	SDGHDMDHYA	ADAFAVVEAL	DLRNAVHIGH								
	100		*	++++	***	*	+	++	++	++	+	*	*	***
BPO-A1		SM GGGELARY	VGRHG..	TGR	LRS	AVLLSAI	PPVMIKSDKN	PDGV.	PDEVF					
BPO-A2		SM GTGEVARY	VSSYG..	TAR	IAK	VAFPLASL	EPFLLKTTDN	PDGAAPQEFF						
CPO-P		STGGGEVARY	VANDGQPAGR	VAKAVLVSAV	PPLMLKTESN	PEGL.	PIEVF							
	150		*		*	++	+	+	*	++	*	+	+	
BPO-A1		DALKNGVLTE	RSQFWKDTAE	G.FFSANRP.	GNKVTQGNKD	AFWYMAAQT								
BPO-A2		DGIVAAVKAD	RYAFYTGFEN	D.FYNLDENL	GTRISEEAVR	NSWNTAASGG								
CPO-P		DGFRKALADN	RAQFFLDVPT	GPFYGFNRA.	GATVHQGVIR	NWWRQMEGS								
	200		+	+	**	++++	+	****	**	+++	+++		++	
BPO-A1		IEGGVRCVDA	FGYTDFTEDL	KKFDIPTLVV	HGDDQVVP	DATGRKSAQI								
BPO-A2		FFAAAAAPT	W.YTDFRADI	PRIDVPALIL	HGTGDRTLPI	ENTARVFHKA								
CPO-P		AKAHYDGIKA	FSETDQTEDL	KSITVPTLV	HGEDDQIVPI	ADAALKSIKL								
	250		+	++	+	*	+++	+	*	++	*	*		
BPO-A1		IPNAELKVYE	GSSHGIAMVP	GDKEKFNRL	LEFLNK°									
BPO-A2		LPSAEYVEVE	GAPHG..	LLW	THAEVNTAL	LAFLAK°								
CPO-P		LQNGTLKTYP	GYSHG..	MLT	VNADVLNADL	LAFVQA°								

Fig. 4. Alignment of the deduced amino acid sequence of BPO-A1 with the sequences of BPO-A2 from *S. aureofaciens* ATCC 10762 and CPO-P from *P. pyrrocinia*. Amino acids identical in the three enzymes at corresponding positions are marked with an asterisk (*) above the alignment, and those conserved in two sequences with a +. BPO-A2 and CPO-P have 278 amino acids, whereas BPO-A1 contains 275. The methionines are in bold letters.

		**	**	*	+	++	***	**	***	**	*	+	+	+
ORF1	68	PDVDGYFGPK	TLAAVQKFQ	CTGLKPDGIV	GPLTWAKLDY	QSSQGT.TPV	WC°	118						
CwIA	230	NGIDGVYGP	KTADAIRRFQ	S	MYGLTQDGI	Y	GPKTKAKLEA	LLK°	272					
LysC	217	LEV	DGIEGPE	TEAAIKDFQ	S	IMGLTVDGI	W	GTNTSGAA.Q	QIFSRPLDGV	AY	267			
Cbpm	36	LAIDGQFGPA	TKAAVQRFQ	S	AYGLAADGIA	GP.TFNKI.Y	QLQDDCTPV	NF	85					

Fig. 5. Alignment of the C-terminal regions of ORF1, the *N*-acetylmuramoyl-L-alanine amidase CwIA of *Bacillus subtilis* and the autolytic lysozyme LysC of *Clostridium acetobutylicum* with the N-terminal region of the carboxypeptidase Cbpm from *Streptomyces albus*. CwIA has an overall length of 272 amino acids, LysC contains 324 and Cbpm contains 212. The circles (°) indicate the end of ORF1 and of CwIA. The numbers to the left and right of the sequences show the extremities of the CwIA, LysC and Cbpm domains aligned with ORF1. Amino acids conserved in all four sequences are indicated by an asterisk (*) above ORF1; those conserved in three sequences are marked with a +.

Table 1. Purification of bromoperoxidase BPO-A1 from *S. lividans* TK64(pOP1738)

Step	Total protein (mg)	Total activity (U)	Sp. act. [U (mg protein) ⁻¹]	Yield (%)
Crude extract*	480.0	440	0.9	100
Heat treatment	328.0	352	1.1	80
Q Sepharose Fast Flow	38.8	310	8.0	70
Chelating Sepharose 6B	5.7	260	46.0	60

* Prepared from 40 g of cells (wet wt).

DISCUSSION

The *bpoA1* gene of *S. aureofaciens* ATCC 10762 was cloned by constructing a genomic library of *S. aureofaciens* ATCC 10762 in *S. lividans* TK64 using the plasmid pIJ699. Screening of 4400 thiostrepton-resistant colonies using an activity selection technique yielded two positive clones, one containing an insert fragment of 7.4 kb (in pOP1974), and the other of 7.9 kb (in pOP1979). These contained very similar regions of DNA. Comparisons with the two bromoperoxidases of *S. aureofaciens* ATCC 10762 showed that the product of the cloned gene was identical to BPO-A1.

The coding sequence for BPO-A1 was located by

Fig. 6. Comparison by native PAGE of the bromoperoxidase produced by *S. lividans* TK64 harbouring pOP1738 with BPO-A1 and BPO-A2 from *S. aureofaciens* ATCC 10762. (a) Activity staining with phenol red for brominating activity. The bands showing brominating activity are marked with arrows. (b) Protein staining with Serva Blue R. Lanes: 1, 3.2 µg BPO-A1 from *S. lividans* TK64(pOP1738); 2, 2 µg BPO-A1; 3, 2 µg BPO-A2.

subcloning of the 7.4 kb fragment from pOP1974 in the plasmid pIJ487. The 2.1 kb *Bam*HI–*Hind*III fragment (pOP1721) contained all the information needed for BPO-A1 expression in *S. lividans* TK64.

DNA sequence analysis established that the regions flanking *bpoA1* and *bpoA2* are quite different. Pfeifer *et al.* (1992) reported an ORF with the opposite orientation immediately upstream of *bpoA2*; until now no function for this putative gene has been determined. In the

neighbourhood of *bpoA1*, the nearest ORF(*orf1*) that can be recognized based on the highly biased codon usage of *Streptomyces* has the same orientation as *bpoA1* and terminates 700 bp upstream of this gene. Furthermore, it appears to encode a protein sharing sequence similarity with autolysins. The distance between *orf1* and *bpoA1*, the presence of a potential transcriptional terminator in the non-coding region between *orf1* and *bpoA1* and the presence of a putative promoter region upstream of *bpoA1* make it unlikely that these genes are part of the same operon. In the non-coding sequences downstream of the genes, *bpoA2* showed three very distinct inverted repeats (Pfeifer *et al.*, 1992), whereas *bpoA1* has a single quite imperfect inverted repeat.

The putative promoter regions of *bpoA1* and *bpoA2* have no apparent sequence similarities, but the coding sequences share 62% identical nucleotides. A comparison of the amino acid sequences of the enzymes reveals 40% identity, indicating a probable evolutionary relationship. However, BPO-A1 displays an even higher level of identical amino acids (49%) with CPO-P from *P. pyrrocinia*.

Of special interest was the distribution of the methionine residues in BPO-A1. Incubation of CPO-T from *S. aureofaciens* Tü24 and CPO-P from *P. pyrrocinia* in the presence of cyanide, bromide and hydrogen peroxide leads to cleavage of the proteins into two peptides. Based on this observation, Haag *et al.* (1991) have postulated that a methionine is involved in the catalytic cycle of these haloperoxidases. In contrast, BPO-A2 contains, in addition to the formyl-methionine, only one methionine residue at position 102 within the overall primary sequence, whereas CPO-P contains five methionine residues; none of these methionines is at the position homologous to Met102. BPO-A1 also contains five methionine residues, but in this case one is exactly equivalent to Met102, in a region highly conserved in all haloperoxidases. Site-directed mutagenesis, now in progress, should clarify the catalytic role of this methionine in halogenation.

The function of BPO-A1 in the cell remains unclear. If haloperoxidases are responsible for the chlorination step in the biosynthesis of 7-chlorotetracycline, this task is very likely fulfilled by BPO-A2 because an immunologically identical enzyme is present in *S. aureofaciens* Tü24, whereas a counterpart to BPO-A1 is absent. We can assume that *S. aureofaciens* ATCC 10762 produces another halogenated compound not yet identified, or that the enzyme has lost its function as part of a biosynthetic pathway due to DNA rearrangement.

The over-production of BPO-A1 by *S. lividans* TK64 is probably due to the high copy number of pIJ487 (Ward *et al.*, 1986), the resistance of bacterial non-haem haloperoxidases to proteolysis (K.-H. van Pée, unpublished results) and the lack of a regulatory element on the plasmid. The over-production and remarkable heat stability of the enzyme led to a simple purification procedure with very high yields. *S. lividans* TK64 harbouring pOP1738 produces 143 µg of homogeneous bromo-

peroxidase per 1 g (wet wt) of cells. To isolate the same amount of enzyme from *S. aureofaciens* ATCC 10762, 2800 g of cells would be needed (Weng *et al.*, 1991). The availability of the bromoperoxidase will allow experiments yielding further insights into the mechanism of bacterial non-haem haloperoxidase reactions. Crystallization and X-ray analysis should give an opportunity for comparative studies with BPO-A2, which has already been crystallized and from which preliminary X-ray data have already been obtained (Sobek *et al.*, 1991).

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