Molecular cloning and sequencing of a non-haem bromoperoxidase gene from *Streptomyces aureofaciens* ATCC 10762

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A bromoperoxidase gene (*bpoT*), recently cloned from *Streptomyces aureofaciens* Tü24, was used as a probe in Southern blot hybridization of total DNA from *S. aureofaciens* ATCC 10762. A single *Sst*I fragment of 5.4 kb was detected, which was cloned via an enriched gene library into *Escherichia coli*. The functional bromoperoxidase gene was located on a 2.1 kb *Bam*HI–*Hind*III fragment by subcloning into *S. lividans* TK64, using the multicopy plasmid pIJ486. The enzyme was overproduced in *S. lividans* TK64 (up to 30 000 times compared to *S. aureofaciens* ATCC 10762) and showed the same electrophoretic and immunological properties as the bromoperoxidase BPO-A2 purified from *S. aureofaciens* ATCC 10762. DNA sequence analysis revealed an open reading frame encoding a predicted polypeptide with the same *M_r* and N-terminal amino acid sequence as the purified subunit of BPO-A2.

Introduction

Haloperoxidases, enzymes that can use halide ions in the presence of hydrogen peroxide and a halogen acceptor for the catalytic formation of carbon–halogen bonds, are widely distributed. They are divided into three classes: haem type, eukaryotic non-haem type and bacterial non-haem type haloperoxidases. The haem type enzymes usually have protoporphyrin IX as prosthetic group (Olsen & Little, 1984; Hewson & Hager, 1980; van Pée & Lingens, 1985) and show halogenating activity as well as peroxidase and catalase activity. The reaction mechanism of these enzymes has been thoroughly investigated (Neidleman & Geigert, 1986). Haloperoxidases of the second class contain vanadium instead of haem (De Boer *et al.*, 1986), whereas the third class, the bacterial non-haem haloperoxidases, do not require metal ions or any other cofactors (van Pée *et al.*, 1987; Wiesner *et al.*, 1988).

Studies of the bacterial non-haem enzymes are just beginning. Comparison of the bromoperoxidase from

Streptomyces aureofaciens Tü24 with the chloroperoxidase from *Pseudomonas pyrocinia* allowed us to gain the first insights into the reaction mechanism of bacterial non-haem bromoperoxidases (Haag *et al.*, 1991). However, very little is known about the active site of these enzymes. Investigation of their biochemical features, comparison of their amino acid sequences and x-ray analysis should give more information about the active site.

S. aureofaciens Tü24 and *S. aureofaciens* ATCC 10762 produce both 7-chlorotetracycline and tetracycline. The first bacterial non-haem bromoperoxidase (BPO-T) was isolated from *S. aureofaciens* Tü24 by van Pée *et al.* (1987). Surprisingly, *S. aureofaciens* ATCC 10762 produces two non-haem bromoperoxidases, called BPO-A1 and BPO-A2 (formerly BPO 1 and BPO 2; Weng *et al.*, 1991). Antibodies raised against BPO-T revealed that BPO-T cross-reacts with BPO-A2, but not with BPO-A1.

In this paper, we describe the cloning and DNA sequence analysis of one of the two bromoperoxidase genes of *S. aureofaciens* ATCC 10762. The protein expressed in *Streptomyces lividans* TK64 from the cloned gene is identical to BPO-A2, according to its biochemical and immunological properties. The purposes of the cloning were first, to overproduce the enzyme to facilitate its purification and to obtain high yields for crystallization, and second, to get insights into the primary structure of the enzyme and the relationship to other haloperoxidases.

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Abbreviation: BPO, bromoperoxidase.

The nucleotide sequence data reported in this paper (*bpoA2*; Fig. 2) have been submitted to GenBank and have been assigned the accession number M84990.

Methods

Chemicals. Hydrogen peroxide (30%) was purchased from Merck and monochlorodimedone was from Sigma. Streptavidin-alkaline-phosphatase complex was purchased from Gibco/BRL.

Bacterial strains, plasmids, phages and culture conditions. *S. aureofaciens* ATCC 10762, *S. aureofaciens* Tü24 and *S. lividans* TK64 containing the cloned bromoperoxidase gene from *S. aureofaciens* Tü24 on a plasmid (pHM623) (van Pée, 1988) were used. For cloning the bromoperoxidase gene, *E. coli* TG2 (Gibson, 1984) and *S. lividans* TK64 (Hopwood *et al.*, 1983) were used as hosts for recombinant plasmids. Plasmids pUC18 (Yanisch-Perron *et al.*, 1985) and pIJ486 (Ward *et al.*, 1986) were used in the construction of the gene library and in subcloning experiments. For DNA sequencing, phages M13mp18 and M13mp19 (Yanisch-Perron *et al.*, 1985) were used and propagated in *E. coli* strain TG1 (Maniatis *et al.*, 1982). Cultures of *S. lividans* TK64 harbouring recombinant plasmids were grown in soybean flour/mannitol medium. Liquid media contained 5 µg thioestrepton ml⁻¹ and agar media contained 50 µg thioestrepton ml⁻¹ (kindly donated by E. R. Squibb & Sons Inc., Princeton, NJ, USA). *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 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) ml⁻¹ and 0.2 mM-isopropyl β-D-thiogalactopyranoside (IPTG). Recombinant M13 phages were identified, after infection of *E. coli* TG1, as white plaques in LB soft agar containing X-Gal and IPTG.

Enzyme assays. Brominating activity was measured spectrophotometrically (Hewson & Hager, 1980) with monochlorodimedone (48 µM), H₂O₂ (8.8 mM), bromide (100 mM), sodium azide (10 mM) and a suitable amount of enzyme in 1 M-sodium acetate buffer (pH 5.5). Absorbance changes at 290 nm were measured at 25 °C. One unit of bromoperoxidase activity is defined as the amount catalysing the formation of 1 µmol monobromomonochlorodimedone min⁻¹. 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) using a vertical apparatus with gels of 1 mm thickness (Phase GmbH). Proteins from the low molecular mass calibration kit (Pharmacia-LKB) were used as standards. Gels were stained for proteins with Serva Blue R. Brominating activity was detected by conversion of phenol red to bromophenol blue (Loo *et al.*, 1964). The reagent contained 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.

DNA preparations and general DNA manipulations. Total DNA from *S. aureofaciens* ATCC 10762 was isolated using '2 × Kirby mix' as described by Hopwood *et al.* (1985). Plasmids were isolated using 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 thioestrepton suspension in water (200 µg ml⁻¹). Phage DNA of M13 derivatives was extracted as described by Maniatis *et al.* (1982). Most of the enzymes required for cloning experiments were from Gibco/BRL. DNA digestion with restriction enzymes, dephosphorylation with calf intestinal alkaline phosphatase (CIAP; Boehringer Mannheim) and ligation were performed as described by Maniatis *et al.* (1982).

Hybridization studies. DNA fragments separated by agarose gel electrophoresis were denatured in alkali, neutralized, and transferred to a Nytran N nylon filter (Schleicher & Schuell) according to the

manufacturer's instructions. Prehybridization and hybridization were performed as described previously (van Pée, 1988). The 1.7 kb insert of pHM623 containing the bromoperoxidase gene isolated from *S. aureofaciens* Tü24 (van Pée, 1988) was biotinylated with the nick-translation kit from Gibco/BRL according to the manufacturer's instructions.

Construction of an enriched gene library of *S. aureofaciens* ATCC 10762 and screening. Chromosomal DNA was digested with *Sst*I and separated by agarose gel electrophoresis. Fragments in the 5.4 kb range were extracted from the agarose gel by the 'freeze-squeeze' method (Tautz & Renz, 1983). pUC18 digested with *Sst*I and treated with calf intestinal alkaline phosphatase was ligated with the chromosomal DNA fragments at 12 °C for 16 h; the ligation mixture was then used to transform competent *E. coli* TG2 cells (Mandel & Higa, 1970). Pools of 16 recombinant *E. coli* TG2 clones were used to inoculate 25 ml of LB medium containing ampicillin. After incubation at 37 °C for 16 h, plasmids were isolated (Kieser, 1984), digested with *Sst*I, and separated on a 0.7% agarose gel. After Southern blotting the filters were hybridized using the biotinylated 1.7 kb *Sst*I insert of pHM623 as a probe. The 16 colonies were then incubated separately and treated in the same way.

Expression of the bromoperoxidase gene and subcloning. The insert DNA of recombinant plasmids hybridizing with the 1.7 kb insert of pHM623 was ligated with alkaline-phosphatase-treated pIJ486 and the mixture was then used to transform *S. lividans* TK64 protoplasts. Thioestrepton-resistant colonies were incubated in 100 ml of soybean flour/mannitol medium at 30 °C for 5 d. The mycelium was harvested by centrifugation and washed with 0.1 M-ammonium acetate buffer (pH 6.8). The cells were resuspended in twice their volume of 0.1 M-sodium acetate buffer (pH 5.5) and disrupted by sonication (twenty 30 s bursts, 70 W, 4 °C). The crude extract was heated at 70 °C for 15 min, clarified by centrifugation and assayed for brominating activity. For subcloning in *S. lividans* TK64, plasmid DNA from one positive clone was digested with *Bam*HI-*Hind*III. The fragments were isolated from agarose gels, ligated with *Bam*HI- or *Bam*HI-*Hind*III-digested, alkaline-phosphatase-treated pIJ486, and *S. lividans* TK64 protoplasts were transformed with the ligation mixtures. Colonies containing the bromoperoxidase gene were identified as described above.

Nucleotide sequence analysis. The 2.1 kb *Bam*HI-*Hind*III fragment containing the bromoperoxidase was subcloned into M13mp18 and M13mp19 to obtain the fragment in both orientations (according to the origin of single strand DNA synthesis). After preparation of single strand DNA, the DNA sequence was determined by the M13-dideoxy chain termination method of Sanger *et al.* (1977), in an automated DNA sequencing system (ALF-Sequencer; Pharmacia-LKB) using fluorescent labelled primers (Ansorge *et al.*, 1987). Sequencing reactions were performed at 37 °C using Sequenase (purchased from Pharmacia and used as recommended by the manufacturer), and alternatively at 65 °C using *Bst*-polymerase (purchased from Bio-Rad) according to the manufacturer's instructions, in order to reduce the formation of secondary structures. To resolve compressions, the reactions were carried out in the presence of deoxy-7-deaza-GTP instead of dGTP (Mizusawa *et al.*, 1986) and the run on the 6% polyacrylamide gel was performed at 50 °C. Fluorescent oligonucleotides were synthesized on a Milligen DNA-synthesizer (Cyclone Plus DNA Synthesizer) by phosphoramidite chemistry. The fluorescent label to the 5'-end of the primers was added in two steps: addition of an amino linker amidite, followed by the attachment of fluorescein isothiocyanate, according to the procedures given in the AutoPrimer Synthesis kit (Pharmacia).

Computer analysis. The DNA sequence was analysed using programs from the sequence analysis package version 6.0 of the University of Wisconsin Genetics Computer Group run on a Microvax work station

3200. For the statistical analysis of codon utilization, using the codon preference program (Gribkov *et al.*, 1984), a codon usage table for *Streptomyces* was generated by analysis of eight genes isolated from *Streptomyces*: thiostrepton-resistance (*tsr*), viomycin-phosphotransferase (*vph*) and aminoglycoside-phosphotransferase (*aph*) (Bibb *et al.*, 1985); orf438, tyrosinase (*mel*) and endoglycosidase H (*endoH*) (Bernan *et al.*, 1985); streptomycin-phosphotransferase (*sph*) and amidinophosphotransferase (*apt*) (Tohyama *et al.*, 1987).

Protein purification and analysis. Purification of BPO-A1 and BPO-A2 from *S. aureofaciens* ATCC 10762, and Ouchterlony gel diffusion assays were done as described by Weng *et al.* (1991). Bromoperoxidase BPO-A2 was prepared from extracts of *S. lividans* TK64 harbouring the plasmids encoding BPO-A2 in the following way. Cells (16 g wet wt) were resuspended in 30 ml 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 48 400 g. The supernatant solution was incubated at 75 °C for 30 min and precipitated proteins were removed by centrifugation. The solution was applied to a calcium tartrate column (7.5 × 2.6 cm) equilibrated with 10 mM-potassium phosphate buffer (pH 6.8) (Akhrem & Drozhdenyuk, 1989). The column was washed with the same buffer and proteins were eluted with 400 ml of a linear gradient (10–50 mM) of potassium phosphate buffer (pH 6.8). Fractions (3 ml) with brominating activity were combined and concentrated by ultrafiltration with a PM-10 membrane.

Results

Cloning of the *bpoA2* gene from *S. aureofaciens* ATCC 10762

The 1.7 kb insert of pHM623 containing the bromoperoxidase gene from *S. aureofaciens* Tü24 hybridized with a 5.4 kb *Sst*I fragment of chromosomal DNA from *S. aureofaciens* ATCC 10762. Thus *Sst*I-digested total DNA was separated on an agarose gel. DNA in the 5.4 kb region was cut out and ligated into pUC18. After transformation of *E. coli*, 500 white colonies were obtained on LB agar containing X-Gal and IPTG. When these clones were screened using the 'sib selection' technique described in Methods, one colony which hybridized with the biotinylated probe was isolated. The *E. coli* clone harbouring the plasmid pOP2854 did not show any brominating activity.

Expression of the *bpoA2* gene in *S. lividans* TK64

The 5.4 kb insert of pOP2854 was isolated and ligated to *Sst*I-digested pIJ486. Transformation of *S. lividans* TK64 with the ligation mixture gave 750 thiostrepton-resistant colonies. When 160 clones were screened for brominating activity using the sib selection technique, two clones with high brominating activity were detected. The two positive clones contained the same 5.4 kb insert in both orientations (pOP2654 and pOP2655).

Fig. 1. Comparison of partial restriction maps of the *Sst*I fragments that contain bromoperoxidase genes from (a) *S. aureofaciens* ATCC 10762 (pOP2654, 5.4 kb) and (b) *S. aureofaciens* Tü24 (pHM621, 4.8 kb; van Pée, 1988). The abbreviations Ba, H, K, N, Sa, Ss and X indicate *Bam*HI, *Hind*III, *Kpn*I, *Nco*I, *Sal*I, *Sst*I and *Xho*I, respectively. The smallest subclones exhibiting bromoperoxidase activity are marked with bold lines. The arrow in (a) indicates the bromoperoxidase gene as determined from the nucleotide sequence.

Subcloning and restriction mapping of pOP2654

Digestion of pOP2654 with *Bam*HI and *Hind*III resulted in five fragments, four insert fragments of 2.1 kb, 1.6 kb, 1.0 kb and 0.3 kb, and a 6.5 kb fragment, consisting of the 6.2 kb fragment from the vector pIJ486 and 0.3 kb from the insert. The *Bam*HI and *Bam*HI–*Hind*III fragments were ligated with pIJ486 and *S. lividans* TK64 protoplasts were transformed. Only those colonies of *S. lividans* TK64 harbouring the 2.1 kb *Bam*HI–*Hind*III fragment (pOP2621) produced heat-stable bromoperoxidase. A partial restriction map of pOP2654 is shown in Fig. 1. The insert contained no restriction sites for the endonucleases *Bcl*II, *Bgl*III, *Eco*RV, *Nco*I and *Xba*I.

DNA sequencing

The 2.1 kb *Bam*HI–*Hind*III fragment that still leads to expression of the bromoperoxidase was cloned into M13mp18 replicative form DNA cut with the same enzymes, as well as into M13mp19 to get the opposite strand. The DNA sequence was determined starting from the universal primer in both phage DNAs, and additional primers were designed with the newly obtained sequence informations, using a walking strategy.

Preliminary sequence data revealed the presence of an open reading frame (ORF) terminating near the *Hind*III site of the *Bam*HI–*Hind*III fragment. Therefore, only 1.7 kb upstream of the *Hind*III site were sequenced on both strands (Fig. 2).

1 *SmaI*
GTCGCCGAGCCCGGCCACCCGGGTGGCGGCGTCCGCGACGTCGCGGAGGATGGTCTCGTGCGCGTCCGAGGAGCCGCGAGA
CAGCGGCTCGGGCCGGTGGGCCACCGCCGACGCGCTGCAGCGCTCCTACCAGAGCACGCGCAGCTCCTCGGCGCTCT
D G L G A V R T A A D R V D A L I T E H A D L L R S L

81
GCATCGAGTGACGTCCTCGATGCCGTCCGGCGGGCGCGGCACCCCGGTGATCCGCGCGGCGTGCCGGGGGTGCGCCACG
CGTAGCTCACGTGCAGGAGCTACGGCAGGCCGCCCGCGCGTGGGGCCACTAGGCGCGCCGACGCGCCCCAGCGGGTGC
M S H V D E I G D P P R P V G T I R A A H R P D G V

161 *SalI*
GCGACCCCGCGAGCGTCTGCACCCGTTCCGGCGAGCGCGTCGACCAGCTCCAGTTGCTCCTGGGCGTGCTTGTGCAAGAG
CGCTGGGGCGGCTCGCAGACGTGGGCAAGCCGCTCGCGCAGCTGGTCGAGGTCAACGAGGACCCGACGAACAGCTTCTC
A V G G L T Q V R E A L A D V L E L Q E Q A H K D F L

241 *SphI*
CAGGTGCAGCTGGTATGCGGTCCGCCCGCGCATGCCCCAGTGGCACTTCTTGACAGCGCGTACAGGAACCTGGGTGTCCG
GTCCACGTCCGACATACGCCAGCCGGGCGCGTACGGGGTCCACGTGAAGAACATGTCCGCGCATGTCTTGACCCACAGGC
L H L Q Y A T P G R M G W H C K K Y L A Y L F Q T D A

321
CGAGGACGCGGTTGAGCCGCTGGCACGAGTACTGCCGGGCGTCCGTGGGAGAGCGCGATCCGGCAGCTGGGTGACGGTGCCG
GCTCCTGCGCAACTCGGCGACCGTGCTCATGACGGCCCGCAGCACCTCTCGCGCTAGCCGTCGACCCACTGCCACGGC
L V R N L R Q C S Y Q R A D H S L A I P L Q T V T G

401 -35
AACTCCTGGATCTCGGCGGCGTGTGATGAAGGTGTGGCTGGTGGTTCATGGCGCTCCTCGGGACATCTGGATCGTTCGG
TTGAGGACCTAGAGCCGCCGACAACCTACTTCCACACCGACCGACCACTACCGCGAGGACCCCTGTAGACCTAGCAAGCC
F E Q I E A A H Q H L H P Q S T M RBS
<-----
ORF1

481 -10 1 1
GGGATCAGGCTAGGAACGCGCGGGCCACGGCCTGTTCCGACCGCGCACTTCTGCGGTCCCTTCGCGCACCCGCGCGGG
CCCTAGTCCGATCCTTGCGCGCCCGGTGCCGACAAGGCTGGCGCGTGAAGGACGCCAGGAAGCGCGTGGGCGCGCCC
2

561 *DraIII*
AGTTGAGCCCGTGTGAGCCGCGGGCGCACGCGGAGCACTCTCGGACGACACCGGGTGCTCTCCGAGGCAACAAGCCGGC
TCAACTCGGGCACACTCGGCGGCCCGCGTGCCTCGTGAAGAGCCTGCTGTGGCCACGAGAGGCTCCGTTGTTCCGCGC
3 2 -10 -35

641 RBS *bpoA2* >
GACGGCCGCCCTAGCGTGAGCCCATCGCAGCCACACGCTGTCCGGAAGATAGGAAGAAACCCCATGCCGTTTCATCACC
CTGCCGCGGGGATCGCACTCGGGGTAGCGTGGTGTGCGACAGGCCTTCTATCCTTCTTTGGGTACGGCAAGTAGTGG
3 M P F I T

721
GTCGGCCAGGAGAACTCCACCTCCATCGACCTCTACTACGAGGACCACGGCACTGGGCAGCCGGTCTGCTCCTCATCCACGG
CAGCCGGTCTCTTGAGGTGGAGGTAGCTGGAGATGATGCTCCTGGTGGCGTGACCCGTCGGCCAGCAGGAGTAGGTGCC
V G Q E N S T S I D L Y Y E D H G T G Q P V V L I H G

801
GTTCCCGTGAGCGGGCACTCCTGGGAGCGGCAGAGCGCCGCGTGTCTGACGCGCGCTACCGGGTCATCACCTACGACC
CAAGGGCGACTCGCCCGTGAGGACCCTCGCCGTCTCGCGCGCGACGAGCTGCGGCCGATGGCCAGTAGTGGATGCTGG
F P L S G H S W E R Q S A A L L D A G Y R V I T Y D R

881
 GCCGCGGGCTTCGGCCAGTCTCCAGCCCACCACGGCTACGACTACGACACCTTCGCCGCGGACCTCAACACCGTGTG
 CGGCGCCGAAGCCGGTCAGGAGGGTCGGGTGGTGGCCGATGCTGATGTGTGGAAGCGGCGCTGGAGTTGTGGCACGAC
 R G F G Q S S Q P T T G Y D Y D T F A A D L N T V L

961
 GAGACCTCGACCTCCAGGACGCCGTGCTGGTCGGCTTCTCGATGGGCACCGGCGAGGTCGCCCCGGTACGTGTCTCCTA
 CTCTGGGAGCTGGAGGTCCTGCGGCACGACCAGCCGAAGAGCTACCCGTGGCCGCTCCAGCGGGCCATGCACAGGAGGAT
 E T L D L Q D A V L V G F S M G T G E V A R Y V S S Y

1041 *NarI*
 CGGCACTGCCCGCATCGCCAAAGTTCGCCTTCTGTCCTCCCTGGAGCCGTTCTGCTGAAGACCGACGACAACCCGGACG
 GCCGTGACGGGCGTAGCGGTTCCAGCGGAAGGACCGGAGGGACCTCGGCAAGGACGACTTCTGGCTGCTGTTGGCCCTGC
 G T A R I A K V A F L A S L E P F L L K T D D N P D G

1121
 GCGCCGCCCCGAGGAGTTCTTCGACGGCATCGTCGCCGCCGTGAAGGCCGACCGCTACGCCTTCTACACCGGCTTCTTC
 CGCGCGGGGCGTCTCAAGAAGCTGCCGTAGCAGCGCGGCACTTCCGGCTGGCGATGCGGAAGATGTGGCCGAAGAAG
 A A P Q E F F D G I V A A V K A D R Y A F Y T G F F

1201
 AACGACTTCTACAACCTGGACGAGAACCTCGGCACCCGGATCAGCGAGGAGGCCGTCGCAACAGCTGGAACACCGCGGC
 TTGCTGAAGATGTTGGACCTGCTCTTGGAGCCGTGGGCCCTAGTCGCTCCTCCGGCAGGCGTTGTGACCTTGTGGCGCGC
 N D F Y N L D E N L G T R I S E E A V R N S W N T A A

1281
 GAGCGGCGGCTTCTTCGCCCGCGCGGCCGCCACCACTGGTACACCGACTTCCGCGCGGACATCCCCCGCATCGACG
 CTCGCCGCCGAAGAAGCGGCGCGCGCGGGTGGTGGACCATGTGGCTGAAGGCGCGGCTGTAGGGGGCGTAGCTGC
 S G G F F A A A A A P T T W Y T D F R A D I P R I D V

1361 *SmaI*
 TCCCCGCGTGATCCTGCACGGCACCGGCGACCGCACCTGCCCATCGAGAACACCGCCCGGCTTCCACAAGGCGCTG
 AGGGGCGCGACTAGGACGTGCCGTGGCCGCTGGCGTGGGACGGGTAGCTCTTGTGGCGGGCCAGAAGGTGTTCCGCGAC
 P A L I L H G T G D R T L P I E N T A R V F H K A L

1441
 CCCTCCGCGGAGTACGTGGAGGTCGAGGGCGCGCCGACGGCCTGCTCTGGACCCACGCGGAGGAAGTGAACACCGCGCT
 GGGAGGCGGCTCATGCACCTCCAGCTCCCGCGCGGCGTCCGGACGAGACCTGGGTGCGGCTCCTTCACTTGTGGCGCGA
 P S A E Y V E V E G A P H G L L W T H A E E V N T A L

1521 *NruI* *KpnI*
 GCTCGCTTCTCGCGAAGTGAACCGGACGGCGCGCTTCCCCGACCGCGGCGCTCCGATCGCGGTACCGCGCTC
 CGAGCGGAAGGAGCGCTTCACTGGGCGTCCCGCGCAAGGGGGCTGGCGCCGGCAGGCAGGGTAGCGGCCATGGCGGCAG
 L A F L A K ***

1601 *KpnI* *XhoI*
 ACCGCCCCGGGTCCGGACGGCGACGGCGGTACCGACACACACCGCGCCCGGAGCCCGCTCGAGCGGGCTCCGGGCGCG
 TGGCGGGGCCAGGCCTGCCGCTGCCGCCATGGCTGGTGTGTGGGCGCGGCCTCGGCGAGCTCGCCGAGGCCCGCGC

1681 *HindIII*
 AACCATGACCAGGAGGTACCCGTGAAGCTT
 TTGGTACTGGTCTCCAGTGGGCACTTCGAA

Fig. 2. Nucleotide sequence of the 1.7 kb fragment containing the *bpoA2* gene and the truncated ORF1, divergently oriented, and the corresponding amino acid sequences. The amino acid sequence shown in bold letters was determined by the Edman degradation procedure. Both translational start sites are underlined and the direction of transcription of both genes is indicated by arrows. The putative Shine-Dalgarno sequences found upstream of both ATG codons are designated RBS. Two potential -10 and -35 promoter sequences, which could be involved in the transcription of *bpoA2* and ORF1, are underlined in the intergenic region. Three pairs of direct repeat sequences (indicated by 1, 2 and 3) and a long palindromic sequence are also displayed in the intergenic region. In the 3' flanking region of *bpoA2*, three sequences with dyad symmetry, presumably encoding a transcriptional terminator, are indicated by opposite arrows.

Fig. 3. Codon preference plot of the DNA sequence of the 1.7 kb fragment, upstream of the *Hind*III site. The statistical codon preference program (Gribskov *et al.*, 1984) identifies potential genes, according to a low rare codon content in agreement with typical codon usage of *Streptomyces*. The figure shows the distribution of rarely used codons (displayed as a plot above the dashed line, which indicates a random codon utilization), ORFs (open boxes) and rare codons (short vertical lines) for each of the three reading frames in both orientations. The location of *bpoA2* in the first frame and the divergently oriented ORF1 in the fifth frame is indicated.

Nucleotide sequence analysis

Analysis of the sequence data from the 1.7 kb fragment allowed the identification of an 834 bp ORF, matching the codon usage of *Streptomyces* (Fig. 3). The 834 bp ORF, starting with an ATG start codon encodes a predicted polypeptide of 278 amino acids. The N-terminal sequence of the deduced protein corresponds exactly to the amino terminal sequence previously determined from the purified enzyme (Weng *et al.*, 1991) and the calculated M_r (30384) is in very good agreement with the size of the purified subunit (M_r 31000), determined by SDS-PAGE (Fig. 4). These results confirm the identity of this ORF and *bpoA2*. The overall average G+C composition of the *bpoA2* coding sequence is 69%, well within the range (62–74%) for previously sequenced genes isolated from *Streptomyces*,

and the G+C compositions of the codon positions 1, 2 and 3 are respectively 64%, 45% and 98%, showing that the high G+C content is reflected mainly at the third position. The ATG start codon is preceded by the sequence AGATAGGAaG, showing high complementarity with the 3'-end of the 16S rRNA of *S. lividans* (Bibb & Cohen, 1982), and might act as a typical Shine-Dalgarno ribosome-binding site. The stop codon of *bpoA2* is followed by a GC-rich region containing three long palindromic sequences, which might act as a transcriptional terminator. The scan of the 1.7 kb fragment by the codon preference program identified a second ORF upstream of *bpoA2*, which should be transcribed in the opposite direction to *bpoA2* (Fig. 3). This potential 450 bp coding sequence, designated ORF1, truncated at its 3'-end, is preceded by a putative ribosome-binding site. The 250 bp intergenic region,

Fig. 4. SDS-PAGE of the bromoperoxidase produced by *S. lividans* TK64(pOP2654), and of BPO-A1 and BPO-A2 from *S. aureofaciens* ATCC 10762. Lanes: 1, SDS marker proteins; 2, 3.2 µg BPO-A2; 3, 3.2 µg bromoperoxidase produced by *S. lividans* TK64(pOP2654); 4, 3.2 µg BPO-A1. The gel was stained with Serva Blue R.

Fig. 5. Native PAGE of bromoperoxidase produced by *S. lividans* TK64(pOP2654), and of BPO-A1 and BPO-A2 from *S. aureofaciens* ATCC 10762. (a) Activity staining with phenol red for brominating activity; (b) protein staining with Serva Blue R. Lanes: 1, 3.2 µg BPO-A2; 2, 3.2 µg bromoperoxidase produced by *S. lividans* TK64(pOP2654); 3, 3.2 µg BPO-A1.

Table 1. Purification of *S. aureofaciens* ATCC 10762 BPO-A2 produced by *S. lividans* TK64(pOP2654)

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg ⁻¹)	Purification (-fold)	Recovery (%)
Crude extract*	234	296	1.3	1.0	100
Heat treatment	200	286	1.4	1.1	97
Calcium tartrate column	30	266	8.9	6.8	90

* Prepared from 16 g (wet wt) cells.

delineated by the initiation codon of both *bpoA2* and ORF1, contains three pairs of direct repeats and a long imperfect palindromic region (free energy -175 kJ), which are good candidates for regulatory sequences.

Purification of BPO-A2 from *S. lividans* TK64(pOP2654) and comparison of the cloned bromoperoxidase with the two enzymes from *S. aureofaciens* ATCC 10762

Since *S. lividans* TK64 containing pOP2654 produced bromoperoxidase at high levels, a simplified purification procedure was developed (Table 1). The two-step procedure resulted in a 90% recovery of bromoperoxidase.

The bromoperoxidase of *S. lividans* TK64(pOP2654) and the two enzymes from *S. aureofaciens* ATCC 10762 were compared by PAGE under native and denaturing conditions. The cloned bromoperoxidase was identical to BPO-A2 from *S. aureofaciens* ATCC 10762 in its electrophoretic mobility on nondenaturing and denaturing polyacrylamide gels, but displayed large differences from BPO-A1 on native polyacrylamide gels (Fig. 5).

Partially purified crude extract of *S. lividans* TK64(pOP2654) was compared with purified BPO-A1 and BPO-A2 from *S. aureofaciens* ATCC 10762 by the Ouchterlony gel diffusion assay, with antiserum raised against homogeneous BPO-T. The bromoperoxidase produced by *S. lividans* TK64(pOP2654) showed identical cross-reaction with BPO-A2, as well as with BPO-T, but not with BPO-A1 (data not shown).

Discussion

Comparative investigations with the two bromoperoxidases from *S. aureofaciens* ATCC 10762 showed that the bromoperoxidase expressed from the gene cloned in *S. lividans* TK64(pOP2654) is identical to BPO-A2.

The partial restriction maps of the cloned 5.4 kb *Sst*I-fragment from *S. aureofaciens* ATCC 10762 containing the *bpoA2* gene and the corresponding 4.8 kb *Sst*I-fragment from *S. aureofaciens* Tü24 are very similar. The *bpoT* gene is located between the *Nco*I and *Hind*III restriction sites. Since the *bpoA2* gene has been localized upstream of the *Hind*III site by sequence analysis, it can be concluded that both genes have the same location. Although the cloned *bpoA2* gene and *bpoT* show high homology (90% or more) by Southern hybridization, and although the two bromoperoxidases are immunologically identical, they have different properties on native polyacrylamide gels and different temperature stability (Weng *et al.*, 1991). Furthermore, BPO-A2 gives crystals more suitable for x-ray diffraction analysis than BPO-T (Sobek *et al.*, 1991).

The coding sequence of *bpoA2* from *S. aureofaciens* ATCC 10762 was located by subcloning the 5.4 kb *Sst*I fragment in *S. lividans* TK64. The 2.1 kb *Bam*HI–*Hind*III fragment was shown to contain all the information for expression of BPO-A2 in *S. lividans* TK64. Analysis of the nucleotide sequence revealed a 834 bp ORF, identified as *bpoA2*, by agreement between the predicted amino acid sequence of the N-terminal part and that determined from purified BPO-A2 protein. The overall G + C content of 69% reflects a very biased use of synonymous codons, primarily due to the preference for those with G or C at the third position. Immediately downstream of the *bpoA2* translational stop codon, three inverted repeat sequences are found, which are presumably involved in transcriptional termination. Nucleotide sequence analysis revealed the presence of a second ORF, called ORF1, in the opposite direction to *bpoA2*. The identification of this additional gene is based on the presence of a translational start codon, preceded by a potential Shine–Dalgarno sequence, and by agreement with the coding features of other *Streptomyces* genes. Preliminary results from promoter-probe studies indicate the presence of two divergently arranged promoters in the region between *bpoA2* and ORF1, further confirming the existence of this putative gene. Primer extension experiments are underway to determine the beginning of both transcripts and to confirm the location of both the promoter sequences identified in the intergenic region.

Searching for similarities between *bpoA2*- or ORF1-encoded proteins with other protein sequences for which data are available in the EMBL and GenBank databases revealed a high degree of identity (38%) between the BPO-A2 bromoperoxidase from *S. aureofaciens* ATCC 10762 and a chloroperoxidase from *Pseudomonas pyrocinia* (data not shown) over their overall amino acid sequences (278 amino acids), suggesting that they belong to the same family of non-haem haloperoxidases. However, no significant similarities were found for the ORF1-encoded polypeptide. The relationship of BPO-A2 with the putative ORF1-encoded protein, corresponding to the divergently transcribed arrangement, remains to be elucidated.

S. lividans TK64 harbouring the cloned *bpoA2* gene did not produce BPO-A1. Therefore, it must be concluded that a second *bpo* gene is present in this strain. Cloning of this *bpoA1* gene and comparison of the sequences of the three *bpo* genes should produce more information about their relationship.

The overproduction of BPO-A2 by *S. lividans* TK64 and the remarkable heat stability of this enzyme lead to a simple purification procedure with very high yields. *S. lividans* TK64 harbouring pOP2654 produces 2 mg of homogeneous bromoperoxidase per gram (wet wt) of

cells. To isolate the same amount of enzyme from *S. aureofaciens* ATCC 10762, 30 kg of cells would be needed (Weng *et al.*, 1991). This allows further investigation of the properties of this enzyme. Information about the tertiary structure had already been obtained by crystallization and preliminary x-ray analysis (Sobek *et al.*, 1991). The availability of high amounts of the bromoperoxidase will allow further insights to be obtained into the reaction mechanism of the bacterial non-haem haloperoxidases.

S. aureofaciens Tü24 and *S. aureofaciens* ATCC 10762 produce both 7-chlorotetracycline and tetracycline. Since in *S. aureofaciens* Tü24 only the bromoperoxidase that is homologous to BPO-A2 could be detected, it is very likely that BPO-A2 and the immunologically identical bromoperoxidase from *S. aureofaciens* Tü24 are the chlorinating enzymes involved in 7-chlorotetracycline biosynthesis. Although these enzymes were isolated as bromoperoxidases and do not chlorinate monochlorodimedone they can chlorinate a more appropriate substrate (unpublished results), as already shown for the chloroperoxidase from *Pseudomonas pyrocinia* (Wiesner *et al.*, 1988). Further investigations on the biological function of these enzymes should show whether they are actually involved in 7-chlorotetracycline biosynthesis.

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