JOURNAL OF BACTERIOLOGY, Dec. 1988, p. 5890–5894
0021-9193/88/125890-05$02.00/0

Vol. 170, No. 12

Molecular Cloning and High-Level Expression of a Bromoperoxidase Gene from *Streptomyces aureofaciens* Tü24

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Received 9 March 1988/Accepted 29 August 1988

A bromoperoxidase gene was cloned from *Streptomyces aureofaciens* Tü24 into *Streptomyces lividans* TK64 by using the promoter-probe vector pIJ486. Subcloning of DNA from the original, unstable clone allowed the gene to be localized to a 1.7-kilobase (kb) fragment of DNA. Southern blotting showed that the cloned 1.7-kb insert hybridized to a 4.3-kb fragment in an *Sst*I digest of *S. aureofaciens* Tü24 total DNA. The 1.7-kb insert was shown to code for a protein with the electrophoretic properties of the subunits of the nonheme bromoperoxidase isolated from *S. aureofaciens* Tü24. The protein produced by *S. lividans* TK64 transformed with pHM621, which contained an 8.0-kb insert, was shown to be identical to the *S. aureofaciens* Tü24 bromoperoxidase in terms of its electrophoretic mobility on denaturing and nondenaturing polyacrylamide gels and its NH_2-terminal amino acid sequence. The bromoperoxidase was overproduced (up to 180 times) by *S. lividans* TK64 containing pHM621. Based on the heat stability of the *S. aureofaciens* Tü24 bromoperoxidase, a new and simple purification procedure with very high yields was developed.

Streptomyces aureofaciens Tü24 produces the antibiotic 7-chlorotetracycline. The incorporation of chlorine during the biosynthesis of 7-chlorotetracycline is believed to be catalyzed by a chloroperoxidase (12). However, only a brominating enzyme, a bromoperoxidase, could be isolated from this strain (18). Bromoperoxidases have been isolated from other chlorometabolite-producing bacteria as well (16, 17, 20). The only bacterial chloroperoxidase known until now was detected in *Pseudomonas pyrrocinia* (2). This enzyme was detected as a bromoperoxidase by the monochlorodimedone assay developed by Hager et al. (2). Monochlorodimedone was not chlorinated by the chloroperoxidase from *P. pyrrocinia*; however, when indole was used as a substrate, it was chlorinated to 7-chloroindole (21), a substance which is known to be produced by *P. pyrrocinia* (8). Therefore, it was thought to be possible that the bromoperoxidase isolated from *S. aureofaciens* Tü24 could be a chloroperoxidase, too, but only if a more "natural" substrate than monochlorodimedone was used. To check this, large amounts of the enzyme are needed, and thus I decided to try to clone the bromoperoxidase gene of *S. aureofaciens* Tü24. This report describes the cloning of the *S. aureofaciens* Tü24 bromoperoxidase gene in *S. lividans* TK64.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. lividans* TK64 and *S. lividans* TK24 containing the promoter-probe plasmid vector pIJ486 (19) were kindly supplied by D. A. Hopwood, Norwich, England, and *S. aureofaciens* Tü24 was provided by H. Zähner, Tübingen, Federal Republic of Germany (FRG). These strains were cultured on agar plates containing 2% (wt/vol) soybean flour and 2% (wt/vol) mannitol. This medium was also used as a liquid medium when cells were grown for the production of bromoperoxidase. Other media used were yeast extract-malt extract plus 34% (wt/vol) sucrose (YEME) (1); R2YE medium (14); P medium (13); LB medium (10); and the minimal medium (MM) described by Hopwood (3). Thiostrepton, kindly donated by E. J. Squibb and Sons Inc., Princeton, N.J., was added to solid (final concentration, 50 μg/ml) and liquid (5 μg/ml) media when indicated.

DNA preparations. Digestions with restriction endonucleases or treatment with alkaline phosphatase and ligation experiments were carried out by standard procedures (10). *S. lividans* TK64 protoplasts were prepared and transformed as described by Hopwood et al. (4). *S. aureofaciens* Tü24 protoplasts were prepared from mycelium by incubation at 37°C for 2 h in P medium containing lysozyme (2 mg/ml). Transformation was performed as described for *S. lividans*. After 16 h of growth, transformants were selected by flooding the plates with 1 ml of a thiostrepton suspension in water (200 μg/ml).

Shotgun cloning experiments. The cloning vector used in this experiment was pIJ486 (19). *S. aureofaciens* Tü24 total DNA (20 μg) was partially digested with *Sau*3A. Following digestion and heat inactivation of the restriction enzyme, the DNA was size-fractionated by agarose gel electrophoresis. Fragments in the range of 8 to 20 kilobases (kb) were extracted from the agarose gel (15). pIJ486 (4 μg) was digested with *Bam*HI and treated with 0.5 U of calf intestinal alkaline phosphatase. After inactivation of the phosphatase at 65°C for 45 min, the mixture was extracted twice with phenol-chloroform-isoamyl alcohol, and the DNA was precipitated with ethanol. Ligation was carried out with 5 μg of total DNA, 1 μg of vector DNA, and 1 U of T4 DNA ligase at 12°C for 16 h. DNA was collected from the ligation mixture by isopropanol precipitation in the presence of tRNA and dissolved in 30 μl of TE buffer (10) in preparation for transformation.

Subcloning in *S. lividans* TK64 and *Escherichia coli* TG2. For subcloning in *S. lividans* TK64, the promoter-probe vector pIJ486 was used. Plasmid DNA was isolated as described by Kieser (5), digested with restriction enzyme, fractionated by agarose gel electrophoresis, cut out, extracted from the agarose gel, and ligated with phosphatase-treated pIJ486. The *Pst*I fragments were first ligated with *Pst*I-cut pUC18, and the ligation mixture was then used to transform competent *E. coli* TG2 cells (9). The plasmid DNA isolated from *E. coli* TG2 was cut with *Hin*dIII, fractionated by agarose gel electrophoresis, extracted from the gel, and ligated with pIJ486 which had been cut with *Hin*dIII, and the

ligation mixture was used to transform protoplasts of *S. lividans* TK64.

Hybridization studies. Biotinylated DNA fragments were prepared with the nick translation reagent kit from BRL, Eggenstein, FRG, according to the manufacturer's instructions. DNA fragments separated by agarose gel electrophoresis were denatured in alkali, neutralized, and transferred to a Hybond nylon filter (Amersham-Buchler, Braunschweig, FRG) according to the manufacturer's instructions. The filter was preincubated at 65°C for 2 h in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.7% skim milk and 0.05% sodium dodecyl sulfate (SDS), which had previously been heated to 95°C for 10 min to inactivate DNases. Hybridization was performed in glass tubes. DNA hybridization was carried out at 68°C in 6× SSC–0.7% skim milk–0.05% SDS. The filter was stringently washed twice in 0.004× SSC–1% SDS at 50°C for 15 min. The filter was protein blocked in 6× SSC–0.7% skim milk–0.05% SDS at 40°C before being incubated with a complex of streptavidin-biotin-alkaline phosphatase (Detec-I-alk; Enzo Biochemicals Inc., New York, N.Y.) as recommended by the manufacturer. The hybridized probe was visualized by incubation of the filter with a substrate for alkaline phosphatase (Nitro Blue Tetrazolium [0.33 mg/ml] plus 5-bromo-4-chloro-3-indolyl phosphate [0.16 mg/ml]).

Screening of transformants and enzyme assay. Thiostrepton-resistant colonies were picked onto soybean flour-mannitol agar plates containing thiostrepton and incubated for 7 days at 30°C. The colonies from two plates (88 colonies) were washed off with liquid soybean flour-mannitol medium and transferred into a 250-ml flask containing 100 ml of liquid soybean flour-mannitol medium plus thiostrepton. The flasks were incubated at 30°C for 5 days with shaking. The mycelium was harvested by centrifugation and washed with 0.1 M ammonium acetate buffer, pH 6.8. The cells were suspended in twice their volume of 0.1 M sodium acetate buffer, pH 5.5, and disrupted by sonication for 20 min with 5-s bursts. After centrifugation, the supernatant was diluted 1:10 with water and applied to a DEAE-cellulose DE52 column (7.5 by 1.5 cm), equilibrated with 10 mM sodium acetate buffer, pH 5.5. The column was washed with 100 ml of 0.25 M NaCl in 10 mM sodium acetate buffer, pH 5.5, and then eluted with 20 ml of 0.6 M NaCl in 10 mM sodium acetate buffer, pH 5.5. Fractions (2.5 ml) were collected, heat treated at 80°C for 30 min, and assayed for brominating activity by the monochlorodimedone assay as described earlier (18). One unit of bromoperoxidase activity was defined as the formation of 1 μmol of monobromomonochlorodimedone per min.

SDS-PAGE and sequencing. SDS-polyacrylamide gel electrophoresis (PAGE) experiments were performed by the method of Laemmli (6) with a 10% acrylamide gel (pH 8.3) containing 0.1% SDS. The samples were denatured by boiling for 5 min. The protein standards used were D-lactalbumin (M_r, 14,400), soybean trypsin inhibitor (20,100), carbonic anhydrase (30,000), ovalbumin (43,000), bovine serum albumin (67,000), and phosphorylase *b* (94,000). PAGE under nondenaturing conditions at pH 7.5 was performed in 7.5% polyacrylamide gels by the method of Maurer (11). The NH_2-terminal amino acid sequence was determined with an Applied Biosystems model A gas-phase protein sequencer.

Protein determination. Protein concentrations were measured by the method of Lowry et al. (7) with bovine serum albumin as the standard.

Purification of bromoperoxidase. The crude extract was prepared by suspending 1 part of cells (wet weight) in 2 parts of 0.1 M sodium acetate buffer, pH 5.5, and disrupting them with a Branson sonifier (J-17 A) for 20 30-s periods. The cell debris was removed by centrifugation for 30 min at 22,100 × *g* and 2°C. The supernatant was heated to 80°C for 30 min, and the precipitated proteins were removed by centrifugation. The resulting supernatant was diluted 1:20 with water and applied to a column (23.0 by 3.0 cm) of Bio-Gel HTP hydroxylapatite, equilibrated with 5 mM sodium phosphate buffer, pH 7.0. The sample was washed onto the column with 500 ml of 5 mM sodium phosphate buffer, pH 7.0, and then eluted with 400 ml of a linear gradient (5 to 50 mM) of sodium phosphate buffer, pH 7.0. Fractions (2.5 ml) were assayed for protein (A_{280}) and brominating activity. Fractions having more than 10% of the activity of the most active fraction were pooled and concentrated over an Amicon PM-30 membrane.

RESULTS

Cloning of the bromoperoxidase gene from *S. lividans* TK64. By using the shotgun procedure described in Materials and Methods, a total of 2,795 thiostrepton-resistant transformants were obtained. As *S. lividans* TK64 itself produced a bromoperoxidase, this enzyme had to be inactivated by heat treatment at 80°C for 30 min.

The sib selection technique used allowed the ultimate isolation of a single colony producing a bromoperoxidase which was still active after heat treatment. This isolate contained a plasmid (pHM620) which had an 18.5-kb insert. However, this plasmid was unstable in *S. lividans* TK64 and gave rise to a smaller plasmid (4 kb). Colonies which harbored only the smaller plasmid did not produce the heat-stable bromoperoxidase.

Subcloning of the bromoperoxidase gene. The originally obtained insert had two sites for the restriction enzyme *Bgl*II, and digestion with *Bgl*II resulted in insertion fragments of 8.0, 6.2, and 4.3 kb. These fragments were isolated and introduced into the *Bam*HI site of pIJ486 by using *S. lividans* TK64 as a host. Colonies harboring the 8.0-kb insert (pHM621) produced high levels of heat-stable bromoperoxidase. The insert of pHM621 had a single *Hin*dIII site. Digestion with *Hin*dIII gave rise to two fragments of 10.2 and 4.0 kb. The 10.2-kb fragment consisted of pIJ486 (6.2 kb) plus a 4.0-kb insert fragment. This 10.2-kb fragment led to the production of heat-stable bromoperoxidase when religated (pHM622) and used to transform *S. lividans* TK64. When pHM622 was digested with *Sst*I and religated, a plasmid (pHM623) which had a 1.7-kb insert was obtained. Digestion of pHM622 with *Bam*HI or *Pst*I and religation gave rise to plasmids containing a 2.1-kb insert (pHM624) or a 2.4-kb insert (pHM625). All these plasmids led to the expression of heat-stable bromoperoxidase.

Restriction mapping of pHM621 and Southern blotting of total DNA digests. A partial restriction map of pHM621 is shown in Fig. 1. The insert contained no sites for the endonucleases *Eco*RI, *Eco*RV, and *Xba*I. In order to confirm the origin of the cloned bromoperoxidase gene, Southern hybridization of the 1.7-kb insert of pHM623 was performed with *Sst*I-digested total DNA from *S. aureofaciens* Tü24 and *S. lividans* TK64. As shown in Fig. 2, the biotinylated 1.7-kb fragment of pHM623 hybridized with a single 4.3-kb fragment of *S. aureofaciens* Tü24 total DNA but not with *S. lividans* TK64 total DNA. This result indicates that the cloned bromoperoxidase gene came from total *S. aureofaciens* Tü24 DNA.

Enzyme activity of bromoperoxidase-positive clones. The enzyme activities of different subclones were determined in crude extracts prepared from mycelium grown in soybean

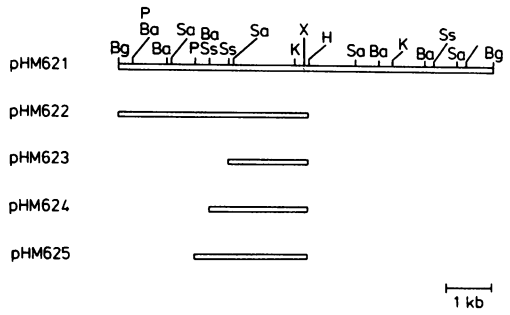

FIG. 1. Partial restriction maps of bromoperoxidase-producing subclones. The abbreviations Ba, Bg, H, K, P, Sa, Ss, and X indicate *Bam*HI, *Bgl*II, *Hin*dIII, *Kpn*I, *Pst*I, *Sal*I, *Sst*I, and *Xho*I, respectively.

TABLE 1. Bromoperoxidase activity in crude extracts[a]

Strain	Plasmid	Bromoperoxidase	
		Sp act (U/mg of protein)	Total activity (U/ml)
S. aureofaciens Tü24	None	—[b]	—
	pHM622	0.35	0.85
S. lividans TK64	None	—	—
	pHM621	2.18	2.74
	pHM622	1.82	1.0
	pHM623	1.19	0.59
	pHM624	1.05	0.8
	pHM625	1.55	2.63

[a] Cells were grown at 30°C for 5 days in soybean flour-mannitol medium in the presence of 5 μg of thiostrepton per ml.
[b] —, Could not be determined.

flour-mannitol medium. As the original clone was unstable, it was not included in this study. All subclones obtained, as well as *S. aureofaciens* Tü24 transformed with pHM622, overproduced bromoperoxidase, with *S. lividans* TK64 harboring pHM621 producing up to 180 times more enzyme than *S. aureofaciens* Tü24. The specific activities and the total activities are shown in Table 1. Figure 3 shows the protein pattern of crude extracts prepared from *S. aureofaciens* Tü24 and *S. lividans* TK64 with and without recombinant plasmids.

Purification of bromoperoxidase from *S. lividans* TK64 (pHM621) and NH_2-terminal amino acid sequence. As *S. lividans* TK64 containing pHM621 produced bromoperoxidase at very high levels, a new purification procedure was developed, based on the unusual heat stability of the *S. aureofaciens* Tü24 bromoperoxidase compared with that of most *S. lividans* TK64 proteins. The purification procedure is summarized in Table 2. The two-step purification had a yield of 116%. The bromoperoxidase isolated from *S. lividans* TK64(pHM621) was identical to the *S. aureofaciens* Tü24 bromoperoxidase in its electrophoretic mobility on denaturing and nondenaturing polyacrylamide gels (Fig. 4).

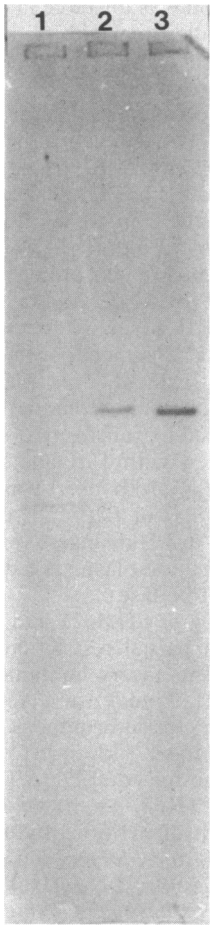

FIG. 2. Southern blotting of DNA digests. The hybridization probe used was the 4.3-kb insert of pHM623. Lane 1, *Sst*I-digested *S. lividans* TK64 total DNA; lane 2, *Sst*I-digested *S. aureofaciens* Tü24 total DNA; lane 3, *Sst*I-digested pHM621.

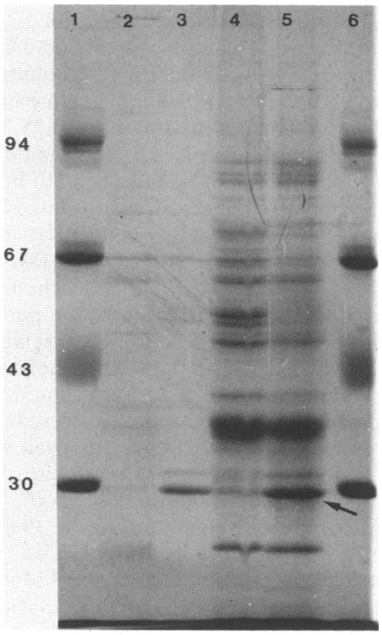

FIG. 3. SDS-PAGE of crude extracts from *S. lividans* TK64 (lane 2), *S. lividans* TK64 containing pHM621 (lane 3), *S. aureofaciens* Tü24 (lane 4), and *S. aureofaciens* Tü24 containing pHM622 (lane 5). Lanes 1 and 6 contained molecular weight standards (shown to the left in thousands). A 100-μg amount of protein was run on each lane on a 10% polyacrylamide–SDS gel. Bromoperoxidase subunits are indicated by an arrow.

TABLE 2. Purification of bromoperoxidase produced by *S. lividans* TK64(pHM621)

Purification step	Volume (ml)	Protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)
Crude extract[a]	24	96	86.8	0.90	100
Heat treatment	21	83	81.7	0.98	94
Bio-Gel HTP	10	18	100.5	5.58	116

[a] Prepared from 10.3 g of cells (wet weight).

The NH_2-terminal amino acid sequences of the purified enzymes were determined by automated Edman degradation and proved to be identical. The sequence for the first 20 amino acids was NH_2-Pro-Phe-Ile-Thr-Val-Gly-Gln-Glu-Asn-Ser-Thr-Ser-Ile-Asp-Leu-Tyr-Tyr-Glu-Asp-His.

DISCUSSION

Bacterial haloperoxidases usually cannot be detected in crude extracts. This was thought to be due to the presence of catalase in these extracts (17). However, catalase can be inhibited by azide, whereas the nonheme haloperoxidases are not inhibited by azide (18). As bromoperoxidase activity is not always detectable in crude extracts of *S. aureofaciens* Tü24 even in the presence of azide (18), a procedure was developed to remove the inhibiting substance from the brominating enzyme. Although *S. lividans* TK64 was not reported to produce halogenated metabolites, brominating activity could be measured in extracts of this strain after ion-exchange chromatography. To remove this activity, the extracts had to be incubated at 80°C for 30 min. Thus, extracts of thiostrepton-resistant colonies were subjected to ion-exchange chromatography to remove the unknown inhibitor which was also present in *S. lividans* TK64 and then incubated at 80°C for 30 min to inactivate the *S. lividans* TK64 bromoperoxidase. By using this procedure to screen the thiostrepton-resistant colonies obtained, a single clone was isolated that produced a heat-stable bromoperoxidase. This clone was unstable and rapidly lost the ability to produce the heat-stable bromoperoxidase. Subcloning with different restriction enzymes led to stable clones which produced the enzyme in high amounts. The overproduction of bromoperoxidase by *S. lividans* TK64 containing pHM621 (up to 180 times more than by *S. aureofaciens* Tü24) led to a new and simple purification procedure with very high yields. The fact that the total activity was over 100% is probably due to the removal of inhibitor. *S. aureofaciens* Tü24 transformed with pHM622 also produced much higher amounts of the enzyme (up to 60 times) than untransformed cells of *S. aureofaciens* Tü24. In contrast to *S. lividans* TK64, however, *S. aureofaciens* Tü24 produced a number of proteins which cannot be precipitated by heat treatment at 80°C. Therefore, *S. lividans* TK64 harboring pHM621 was used to produce up to 180 mg of homogeneous bromoperoxidase per liter of medium. To isolate the same amount of enzyme from *S. aureofaciens* Tü24 by the procedure described earlier (18), mycelium from a 2,100-liter culture would be needed.

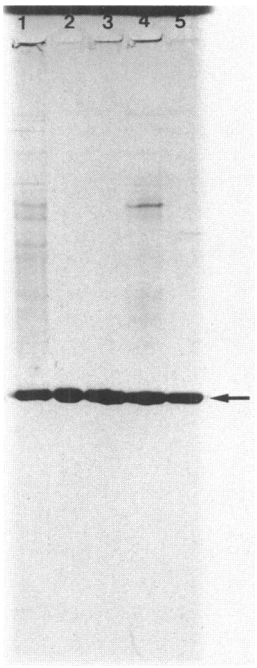

FIG. 4. Polyacrylamide gel run under nondenaturing conditions of different stages of the purification of bromoperoxidase produced by *S. lividans* TK64 containing pHM621. Lane 4, 100 μg of crude extract; lane 3, 100 μg of heat-treated extract; lane 2, 20 μg of protein after chromatography on hydroxylapatite; lane 1, 100 μg of crude extract of *S. lividans* TK64(pHM625); lane 5, 15 μg of bromoperoxidase purified from *S. aureofaciens* Tü24. The position of bromoperoxidase is indicated by an arrow.

ACKNOWLEDGMENTS

I am grateful to F. Lingens and J. Cullum for helpful discussions, and to B. Hauer for determination of the partial amino acid sequence.

This research was supported by a scientific and technical sectorial grant from the Commission of the European Communities and by a grant from the Deutsche Forschungsgemeinschaft.

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