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Chloroperoxidase from *Streptomyces lividans*: Isolation and Characterization of the Enzyme and the Corresponding Gene

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For the first time, a halogenating enzyme which is not known to produce halogenated metabolites has been isolated from a bacterial strain. The gene encoding the nonheme chloroperoxidase (CPO-L) from *Streptomyces lividans* TK64 was cloned, and its gene product was characterized. *S. lividans* TK64 produced only very small amounts of the enzyme. After cloning of the gene into *Streptomyces aureofaciens* Tü24-88, the enzyme was overexpressed up to 3,000-fold. Based on the overexpression, a simple purification procedure using acid precipitation and hydrophobic interaction chromatography was developed. Thus, 54 mg of homogeneous CPO-L could be obtained from 27 g (wet weight) of mycelium. The native enzyme has a molecular weight of 64,000 and consists of two identical subunits. The enzyme does not exhibit an absorption peak in the Soret region of the optical spectrum. X-ray fluorescence spectroscopy revealed that the enzyme does not contain any metal ions in equimolar amounts. CPO-L showed cross-reaction with antibodies raised against the nonheme chloroperoxidase from *Pseudomonas pyrrocinia* but not with antibodies raised against CPO-T from *S. aureofaciens* Tü24. CPO-L exhibits substrate specificity only for chlorination, not for bromination. Therefore, monochlorodimedone is only brominated by CPO-L, whereas indole is brominated and chlorinated. The functional chloroperoxidase gene was located on a 1.9-kb *SalI* DNA fragment. DNA sequence analysis revealed an open reading frame encoding a predicted polypeptide of 276 amino acids. The overall identity of the amino acid sequence to that of chloroperoxidase from *P. pyrrocinia* was 71%, whereas that to bromoperoxidase BPO-A2 from *S. aureofaciens* ATCC 10762 was only 42%.

Halogenated products of natural origin are widely distributed. Bacteria produce many chlorinated compounds with antibiotic activity. For example, 7-chlorotetracycline is produced by *Streptomyces aureofaciens* (7), chloramphenicol is produced by *Streptomyces venezuelae* (8), and pyrrolnitrin is produced by *Pseudomonas pyrrocinia* (3). The introduction of chlorine at certain steps during the biosynthesis is believed to be catalyzed by chloroperoxidases (42).

According to their prosthetic group or cofactor, three groups of haloperoxidases are distinguished: heme type, eukaryotic nonheme type, and bacterial nonheme type. The well-studied heme haloperoxidases usually contain protoporphyrin IX as a prosthetic group (5, 18, 24, 25, 35). A representative of this group is the chloroperoxidase from the fungus *Caldariomyces fumago* (23). The enzyme shows a lack of substrate specificity and stereospecificity and is unstable under reaction conditions. Haloperoxidases of the second group contain vanadium instead of heme (37, 40). They have been isolated, for example, from the marine algae *Ascophyllum nodosum* (40) and *Corallina pilulifera* (19). In contrast to heme haloperoxidases, they are not inhibited by azide and have no peroxidase activity in the absence of bromide or iodide.

In the last few years, a number of prokaryotic nonheme haloperoxidases have been purified from different bacteria and characterized (36, 39, 42). In these enzymes, which represent the third group of haloperoxidases, no prosthetic groups or metal ions are detectable. Very little is known about the reaction mechanism and the active site of bacterial nonheme haloperoxidases. Haag et al. (11) postulated a reaction mechanism for these haloperoxidases with methionine at the active site.

All bacterial nonheme haloperoxidases catalyze the bromination but not the chlorination of monochlorodimedone. Therefore, they were isolated as bromoperoxidases. While the bromination of organic compounds is very unspecific, a substrate specificity exists for the chlorination. Appropriate substrates such as indole or phenyl pyrrole derivatives were chlorinated by different bacterial haloperoxidases (4, 41, 42). The substrate specificity which is shown by this group of haloperoxidases contrasts to the lack of substrate specificity of heme haloperoxidases.

Halogenating enzymes are believed to be responsible for the formation of naturally occurring halogenated compounds (24). So far, haloperoxidases have been isolated only from bacteria, which are known to produce halogenated metabolites. Thus, it was surprising that in *Streptomyces lividans*, which is frequently used as a cloning host, a halogenating activity had been detected during the cloning of the haloperoxidase gene from *S. aureofaciens* Tü24 (33), as no halogenated metabolites have been found in this strain until now. Therefore, it was of considerable interest to determine whether the haloperoxidase present in *S. lividans* was similar to the enzymes isolated from halometabolites-producing strains. As *S. lividans* produces only very low amounts of the enzyme, the haloperoxidase gene was cloned and was overexpressed in a suitable host.

MATERIALS AND METHODS

Materials. Hydrogen peroxide (30%) was purchased from Merck (Darmstadt, Germany). Monochlorodimedone, phenol red, and lysozyme were supplied by Sigma Chemie (Deisenhofen, Germany). Ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and isopropyl-β-D-thiogalactopyr-

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anoside (IPTG) were obtained from Boehringer (Mannheim, Germany). Restriction enzymes and T4 ligase used in the cloning experiments were from Pharmacia-LKB Biotechnology (Uppsala, Sweden). All other reagents were of analytical grade.

Bacterial strains, culture conditions, and plasmids. For cloning the chloroperoxidase gene *cpoL* from *S. lividans* TK64, we used *Escherichia coli* TG2 (9), *S. lividans* TK64 (16), and *S. aureofaciens* Tü24-88 (12) as hosts for recombinant plasmids. For DNA sequencing, plasmid DNA was prepared from *E. coli* JM109 (45).

The vector used for construction of the gene library was pUC19 (45), and pIJ486 (38) was used in subcloning experiments.

Cultures of *Streptomyces* strains were grown in medium containing 2% (wt/vol) soybean flour and 2% (wt/vol) mannitol at 30°C for 5 days. Cultures of *Streptomyces* strains harboring recombinant plasmids were grown in soybean flour-mannitol medium containing thiostrepton (kindly donated by E. R. Squibb & Sons Inc., Princeton, N.J.). Liquid media contained 5 μg of thiostrepton ml^{-1}, and agar media contained 50 μg of thiostrepton ml^{-1}.

E. coli cells were grown in LB medium. Transformants carrying pUC19 and derivative plasmids were selected on agar media containing 100 μg of ampicillin ml^{-1}, 40 μg of X-Gal ml^{-1}, and 0.2 mM IPTG.

Enzyme assays and protein determination. All assays were performed at 25°C. Brominating activity was measured spectrophotometrically by the method of Hewson and Hager (14) with monochlorodimedone as the substrate as described previously (27).

The pH optimum was determined with the standard assay by replacing the sodium acetate buffer with ammonium acetate buffer in the pH range from 6 to 8.

To screen the *Streptomyces* recombinant clones in the cloning experiments for expression of brominating activity, transformants were overlaid with lysozyme solution (2 mg of lysozyme ml^{-1} in 0.3 M sucrose, 25 mM Tris [pH 8], 25 mM EDTA [pH 8], 5% Triton X-100) for 1 h at 37°C. The lysozyme solution was then exchanged for the substrate solution. This solution contained phenol red (0.002%), sodium bromide (1 M), sodium azide (10 mM), and H_2O_2 (17.6 mM) in 1 M sodium acetate buffer (pH 5.5). Brominating activity was detected by the conversion of phenol red to bromophenol blue.

Protein concentrations were determined by the method of Lowry et al. (20), using bovine serum albumin as the standard.

Preparation of crude extracts. For the preparation of crude extracts, frozen cells of *S. lividans* TK64 were thawed and suspended in 100 mM ammonium acetate buffer (pH 6.8). Those of *S. aureofaciens* Tü24-88 were suspended in 100 mM sodium acetate buffer (pH 4.5). The suspension was subjected to ultrasonic treatment for 15 min with an ultrasonic disintegrator (Branson Sonifier 450; 160 W, 50% duty cycle, 4°C). The cell debris was removed by centrifugation at 22 100 × *g* and 4°C for 30 min.

Partial purification of nonheme chloroperoxidase (CPO-L) from *S. lividans* TK64. The crude extract was dialyzed against 10 mM sodium acetate buffer (pH 5.5) for 15 h. After centrifugation (22,100 × *g*, 45 min, 4°C), precipitated proteins were removed.

The supernatant was applied to a DEAE-Sephacel column (5.5 by 12 cm). The column had been equilibrated with 0.1 M NaCl in 10 mM sodium acetate buffer (pH 5.5). Proteins were eluted with an 800-ml linear NaCl gradient (0.1 to 0.8 M NaCl) in 10 mM sodium acetate buffer (pH 5.5). Fractions of 4 ml were collected and assayed for protein (A_{280}) and brominating activity. Fractions containing brominating activity were pooled and concentrated in an ultrafiltration cell (YM-10 membrane; Amicon, Witten, Germany).

The concentrated protein solution was dialyzed against 10 mM ammonium acetate buffer (pH 6.8) containing 0.8 M $(NH_4)_2SO_4$ for 15 h. The dialyzed solution was applied to a column of Butyl-Sepharose (2.5 by 15 cm) equilibrated with 10 mM ammonium acetate buffer (pH 6.8)–0.8 M $(NH_4)_2SO_4$. Proteins were eluted with a 600-ml linear gradient from 0.8 to 0 M $(NH_4)_2SO_4$ in 10 mM ammonium acetate buffer (pH 6.8). Fractions of 4 ml were collected, and those with brominating activity were pooled.

The pooled fractions from the Butyl-Sepharose column were dialyzed against 10 mM potassium phosphate buffer (pH 6.8) for 15 h. The calcium tartrate gel (ctg 35 [1]) column (2.5 by 7 cm) was equilibrated with the same buffer. The dialyzed extract was applied to the column, and proteins were eluted with a 400-ml linear gradient from 10 to 50 mM potassium phosphate buffer (pH 6.8). Fractions of 4 ml were collected.

The final purification step was performed with an FPLC-Superdex Hiload 200 16/60 column, equilibrated with 200 mM ammonium acetate buffer (pH 6.8) at room temperature. Chloroperoxidase-containing fractions from the adsorption chromatography were pooled, concentrated to 1 ml by ultrafiltration, and applied to the column. Fractions of 1 ml were assayed for enzyme activity, and those containing CPO-L were pooled and concentrated. After the buffer was changed by ultrafiltration to 10 mM sodium acetate buffer (pH 5.5), the protein solution was stored at −20°C.

Purification of CPO-L from *S. lividans* TK64 produced in *S. aureofaciens* Tü24-88. The crude extract was dialyzed against 10 mM sodium acetate buffer (pH 4.5) for 15 h. After centrifugation (22,100 × *g*, 45 min, 4°C), the supernatant solution was dialyzed against 0.6 M $(NH_4)_2SO_4$ in 10 mM ammonium acetate buffer (pH 6.8) for 15 h.

The dialyzed solution was applied to a column of Butyl-Sepharose (2.5 by 10 cm), equilibrated with 0.6 M $(NH_4)_2SO_4$ in 10 mM sodium acetate buffer (pH 6.8). Proteins were eluted with a 300-ml linear gradient from 0.6 to 0 M $(NH_4)_2SO_4$ in 10 mM ammonium acetate buffer (pH 6.8). Fractions of 3 ml were collected. Fractions with brominating activity were pooled, concentrated, and stored at −20°C.

Electrophoresis. Purification of CPO-L was monitored by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described by Schägger and von Jagow (31), using a vertical apparatus with 0.5- or 1-mm gels. The low-molecular-weight calibration kit (Pharmacia-LKB) was used as a standard.

Analytical isoelectric focusing was performed in rehydrated gels as recommended by Pharmacia-LKB (28), using a horizontal apparatus (Pharmacia-LKB) with a 0.5-mm polyacrylamide gel (5%, wt/vol) and carrier ampholytes (Pharmacia-LKB) in the pH range of 2.5 to 5. The pI was determined by comparing the migration rate with those of standard proteins (Pharmacia-LKB) in the pI range of 2.5 to 6.

Gels were stained in 0.2% (wt/vol) Coomassie brilliant blue R250, dissolved in aqueous 50% (vol/vol) methanol–10% (vol/vol) acetic acid, and destained in 20% (vol/vol) methanol–10% (vol/vol) acetic acid.

Determination of the amino-terminal sequence. The subunits of the partially purified CPO-L were separated from other proteins on an SDS-polyacrylamide gel (10%). Semidry electroblotting onto Immobilon polyvinylidene difluoride membranes (Millipore, Eschborn, Germany) was performed as recommended by Pharmacia-LKB (29). The amino-terminal amino acid sequence was determined by automated Edman

degradation in a 471A gas-phase protein sequencer with a 140A solvent delivery system (Applied Biosystems, Weiterstadt, Germany).

DNA preparations and general manipulations. Total DNA from *S. lividans* TK64 was isolated using 2× Kirby mix (15). Plasmids were isolated by the alkaline lysis method (17). *Streptomyces* protoplasts were prepared and transformed as described by Hopwood et al. (15). The protoplasts were regenerated on R2YE (15) 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}).

DNA digestions with restriction enzymes, dephosphorylation with shrimp alkaline phosphatase (U.S. Biochemical, Bad Homburg, Germany), and ligation were performed as described by Maniatis et al. (22). To remove and purify DNA from agarose gels, the Geneclean-II kit (Dianova, Hamburg, Germany) was used according to the manufacturer's instructions.

Transfers and hybridization. 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 to Hybond-N^+ nylon membranes (Amersham-Buchler, Braunschweig, Germany) according to the manufacturer's instructions.

Hybridization experiments using a mixed 17-base oligonucleotide as the probe were carried out overnight at 47°C. The oligonucleotide used corresponded to amino acid residues 12 to 17 of the amino terminus of chloroperoxidase (CPO-P) from *P. pyrrocinia* (44). The oligonucleotide was labelled with digoxigenin-ddUTP as instructed by the manufacturer (Boehringer).

Hybridization and luminescent detection with 3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)-phenyl-1,2-dioxetane (AMPPD) was performed with the DIG luminescent detection kit from Boehringer. The filters were stringently washed twice in 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% SDS at 47°C for 10 min. Luminescence was documented by a short exposure (15 to 25 min) to X-ray films.

Construction of an enriched gene library of *S. lividans* TK64 in *E. coli* and screening for clones containing the chloroperoxidase gene. Chromosomal DNA of *S. lividans* TK64 was digested with *Bam*HI and fractionated by agarose gel electrophoresis. Fragments in the range of 4.3 kb were extracted from the agarose gel and ligated into *Bam*HI-digested and dephosphorylated pUC19. The ligation mixture was then used to transform competent *E. coli* TG2 cells by the method of Mandel and Higa (21). Pools of 15 recombinant *E. coli* TG2 clones were used to inoculate 25 ml of LB medium containing ampicillin. After incubation at 37°C for 16 h, the recombinant plasmids were isolated and digested with *Bam*HI, and the DNA fragments were separated on a 0.7% agarose gel. After Southern blotting, the filters were hybridized by using the digoxigenin-labelled oligonucleotide as a probe. The individual clones of positive pools were incubated separately and treated in the same way.

Nucleotide sequencing. Deletions and subcloning of the 1.9-kb *Sal*I fragment containing the chloroperoxidase gene from *S. lividans* were done with the restriction endonucleases *Aat*II, *Apa*I, *Apa*LI, *Hin*dII, *Not*I, *Sac*II, *Sfi*I, *Sma*I, *Sph*I and *Xho*I and plasmids pUC18 and pUC19 (45). The sequencing reactions were carried out with double-stranded plasmid DNA, using the AutoRead sequencing kit and procedure B of the instruction booklet provided with the kit, and the DNA was analyzed on a 6% polyacrylamide gel run at 50°C in an automated laser fluorescent A.L.F. sequencer (Pharmacia Biotechnology, Freiburg, Germany).

Computer analysis. The nucleotide sequence was analyzed on a MicroVAX workstation 3200, using programs of the University of Wisconsin Genetics Computer Group package, version 7.3 (6). For the codon preference program (10), a codon usage table from eight *Streptomyces* genes was generated as described previously (27). Data base searches were run with the programs blastn, blastp, blastx, and tblastn (2) on the BLAST electronic mail server from the National Center for Biotechnology Information, Bethesda, Md. The program CLUSTAL for multiple sequence alignment was kindly provided by the German Cancer Research Center, Heidelberg, in the software package HUSAR.

Ouchterlony gel diffusion assay. Agarose (0.7%, wt/vol) with 0.05% (wt/vol) sodium azide in 50 mM potassium phosphate buffer (pH 7) was used. The assay was done as described by Ouchterlony (26).

Determination of metal content. The metal content of CPO-L was determined by using X-ray fluorescence spectrometry (system 77; Finnigan International Inc.). The sample contained 115 μg of CPO-L in 5 mM sodium acetate buffer (pH 5.5). Buffer was used for background correction.

M_r determination. The M_r value of purified CPO-L was estimated by gel filtration through a Superdex 200 preparative-grade 16/60 (Pharmacia-LKB) column, equilibrated with 200 mM ammonium acetate buffer (pH 6.8). The calibration proteins were gamma globulin (M_r, 168,000), bovine serum albumin (M_r, 68,000), ovalbumin (M_r, 43,000), α-chymotrypsinogen A (M_r, 25,000), and RNase A (M_r, 13,700). The molecular weights of the CPO-L subunits were estimated by SDS-PAGE under denaturing conditions, using the low-molecular-weight standard proteins from Pharmacia.

Absorption spectra. Protein absorption spectra were recorded on a Uvikon 930 spectrophotometer (Kontron Instruments, Neufahrn, Germany) in 10 mM sodium acetate buffer (pH 5.5). The concentration of the chloroperoxidase was 0.6 mg ml^{-1}.

Chlorination of indole by CPO-L. Indole was used as the substrate to demonstrate the chlorinating activity of CPO-L. The reaction mixture contained indole (0.3 mM), sodium chloride (100 mM), H_2O_2 (35 mM), sodium azide (10 mM), and chloroperoxidase with a brominating activity of 1 U in 1 M sodium acetate buffer (pH 4.5). The total volume was 10 ml. The reaction mixture was incubated at room temperature for 6 h. A blank without enzyme was treated in the same way.

After incubation, the reaction mixture was extracted with 10 ml of ethyl acetate and dried over anhydrous sodium sulfate, and the solvent was evaporated in vacuo. The products were redissolved in 50 μl of methanol and analyzed by gas chromatography (GC) and GC-mass spectrometry (MS).

GC was performed with a Packard model 427 gas chromatograph (Packard Instrument Co., Warrenville, Ill.), using a SE 30 capillary column (Macherey & Nagel, Düren, Germany). The injector temperature was 250°C. The oven temperature was run from 100 to 240°C (10°C min^{-1}), and the detector temperature was 300°C.

For GC-MS, a Varian model 3700 gas chromatograph with a SE 30 column (Varian, Bremen, Germany) was used. The gas chromatograph was connected to a MAT 44 S mass spectrometer (Finnigan, Bremen, Germany).

Nucleotide sequence accession number. The GenBank accession number for the *S. lividans* chloroperoxidase gene is U02635.

TABLE 1. Partial purification of CPO-L from *S. lividans* TK64

Purification step	Total protein (mg)	Total activity (U)	Sp act (U mg^{-1})	Purification (fold)	Recovery (%)
Crude extract[a]	7,250	—[b]	—	—	—
Acid precipitation	4,460	22.3	0.005	1	100
DEAE-Sephacel	560	21.8	0.039	8	98
Butyl-Sepharose	28	14.0	0.49	100	63
Calcium tartrate gel	2	10.8	5.4	1,080	43
Superdex 200	0.3	5.6	18.7	3,740	25

[a] Prepared from 400 g (wet weight) of cells.
[b] —, could not be determined.

TABLE 2. Purification of CPO-L from *S. lividans* TK64 produced by *S. aureofaciens* Tü24-88 plus pRB882

Purification step	Total protein (mg)	Total activity (U)	Sp act (U mg^{-1})	Purification (fold)	Recovery (%)
Crude extract[a]	734	3,010	4.1	1	100
Acid precipitation	69	2,208	32	7.8	73
Butyl-Sepharose	54	2,044	38	9.3	68

[a] Prepared from 27 g (wet weight) of cells.

RESULTS

Partial purification of CPO-L from *S. lividans* TK64. No brominating activity was detected in the crude cell extracts of *S. lividans*. After acid precipitation of the crude extract, bromination but not chlorination of monochlorodimedone could be measured. The enzyme was enriched 3,740-fold by a five-step procedure. The recovery after the last purification step was 25%. The results of a typical purification procedure are summarized in Table 1. Electroblotting onto Immobilon membranes showed that the enzyme had not been purified to homogeneity.

Determination of the amino-terminal amino acid sequence. Blotted CPO-L was subjected to Edman degradation. The amino-terminal amino acid sequence was NH_2Gly/Ala-Thr-Val-Thr-Thr-Ser-Asp-Gly-Thr-Asn-Ile-**Phe-Tyr-Lys-Asp-Trp-Gly**-Pro-X-Asp. Amino acids 12 to 17 (boldface) of the amino-terminal end of CPO-L from *S. lividans* and chloroperoxidase (CPO-P) from *P. pyrrocinia* (43) were identical. A mixed 17-base oligonucleotide derived from these amino acid residues (44) was used to screen the enriched gene library of *S. lividans* TK64 in *E. coli*.

Cloning and identification of the chloroperoxidase gene. The enriched gene library in *E. coli* was prepared with total DNA from *S. lividans*. The oligonucleotide derived from the amino-terminal amino acid sequence hybridized with a 4.3-kb *Bam*HI fragment of chromosomal DNA from *S. lividans* (data not shown). *Bam*HI-digested total DNA was separated on an agarose gel; DNA in the range of 4.3 kb was isolated and ligated into pUC19. After transformation of *E. coli*, 500 white colonies containing recombinant plasmids were obtained on LB agar plates containing X-Gal and IPTG. When these clones were screened as described in Materials and Methods, colonies which hybridized with the probe were isolated. The plasmids of these colonies contained a 4.3-kb insert. *E. coli* clones harboring the recombinant plasmids did not show any brominating activity.

Expression of the *cpoL* gene in *S. lividans* and subcloning. As the gene was not expressed in *E. coli*, subcloning was carried out in *S. lividans* TK64. One *E. coli* clone was selected, and the recombinant plasmid was isolated. The *Bam*HI 4.3-kb insert was ligated into pIJ486. After transformation, the clones were screened for brominating activity on agar plates as described in Materials and Methods for enzyme assay. The rapid conversion of phenol red to bromophenol blue by the positive clones showed an overexpression of the chloroperoxidase gene compared with nontransformed colonies.

Several transformants containing recombinant plasmids were examined for the expression of the chloroperoxidase gene by measuring the brominating activity in crude extracts and after acid precipitation. In contrast to the untransformed strain, brominating activity could be measured in the crude extract. The total activity after the acid precipitation step was 40-fold higher than that found in *S. lividans* TK64 harboring the chloroperoxidase gene on the chromosome. The activity had increased from 55 mU to 2.2 U g (wet weight) of cells^{-1}.

To localize the chloroperoxidase gene more precisely, the 4.3-kb *Bam*HI fragment was digested with *Sal*I, and the fragment containing the chloroperoxidase gene was identified by Southern blot hybridization. A 1.9-kb *Sal*I fragment hybridizing with the oligonucleotide was subcloned into pUC19. From this, the 1.9-kb insert was obtained as a *Bam*HI-*Hin*dIII fragment and ligated into pIJ486. The recombinant plasmid carrying this fragment was designated pRB882. The gene was expressed in *S. lividans* TK64, but not at such a high rate as described for other bacterial nonheme haloperoxidases (27, 33, 44). Therefore, a mutant of *S. aureofaciens* Tü24 with a deletion of its own chloroperoxidase gene (12) was transformed with pRB882. After transformation of *S. aureofaciens* Tü24-88 protoplasts, screening was carried out as described above.

Purification of CPO-L produced by *S. aureofaciens* Tü24-88 harboring pRB882. *S. aureofaciens* Tü24-88 containing the cloned chloroperoxidase gene from *S. lividans* TK64 produced the enzyme at a very high level. This allowed the development of a new and simple purification procedure. This procedure was based on the high pH stability of the chloroperoxidase compared with that of most *S. aureofaciens* Tü24-88 proteins.

The brominating activity in the crude extract of *S. aureofaciens* Tü24-88 containing pRB882 was 2,000- to 3,000-fold higher than that found after the first purification step in *S. lividans* TK64 with the chromosomal gene. Acid precipitation at pH 4.5 yielded a 7.8-fold purification with 73% recovery. After chromatography on Butyl-Sepharose, 54 mg of homogeneous chloroperoxidase was isolated from 27 g (wet weight) of cells. This two-step purification resulted in a yield of 68%. The purification procedure is summarized in Table 2. The purified enzyme gave a single band on SDS-PAGE (Fig. 1). The enzyme showed brominating activity in the presence of sodium azide, indicating that it belongs to the group of nonheme haloperoxidases.

M_r values and isoelectric point. The M_r value of purified native CPO-L estimated by gel filtration on Superdex 200 (preparative grade) was 64,000. SDS-PAGE showed a single protein band corresponding to an M_r for the subunits of 32,000 ± 2,000 (Fig. 1). Comparison with the M_r value of the native CPO-L suggested a subunit structure of two identical subunits, as confirmed by the analysis of the N terminus.

The pI was determined by isoelectric focusing to be at pH 3.6.

Absorption spectrum and metal content. The absorption spectrum of the native enzyme did not exhibit any absorption bands in the visible region of the spectrum (Fig. 2). The metal content was determined by X-ray fluorescence spectroscopy. The chloroperoxidase did not contain any metals in equimolar amounts. Assuming a molecular weight of 64,000, the content

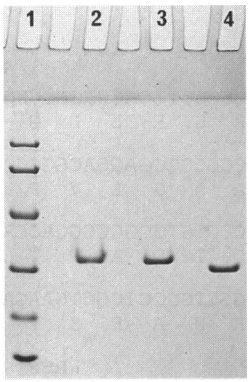

FIG. 1. SDS-PAGE of different nonheme haloperoxidases. Lanes: 1, SDS-PAGE molecular weight marker proteins (phosphorylase *b* [94,000], bovine serum albumin [67,000], ovalbumin [43,000], carbonic anhydrase [30,000], trypsin inhibitor [20,100], and α-lactalbumin [14,400]); 2, 2 μg of CPO-L from *S. lividans* TK64; 3, 2 μg of CPO-P from *P. pyrrocinia*; 4, 2 μg of CPO-T from *S. aureofaciens* Tü24. The gel was stained with Coomassie brilliant blue R250.

of iron was calculated to be 0.02 atom per molecule of enzyme. Other metals like vanadium, titanium, manganese, chromium, nickel, copper, and selenium were not present in the enzyme preparation. Therefore, the enzyme belongs to the nonmetal, nonheme haloperoxidases.

Catalytic properties. CPO-L catalyzed the bromination but not the chlorination of monochlorodimedone. The specific brominating activity in the standard enzyme assay was 38 U mg of protein^{-1}. The pH optimum for the brominating activity in 1 M acetate buffer was between pH 5 and 5.5.

The stability of the enzyme was examined under various conditions. Chloroperoxidase could be stored in 20 mM sodium acetate buffer (pH 3.5 to 6) and ammonium acetate buffer (pH 6 to 8) at 6°C for weeks without loss of activity. Below pH 3, the enzyme was inactivated. The enzyme could be stored in sodium acetate buffer (pH 5.5) at −20°C for months without any loss of activity.

From Lineweaver-Burk plots, the apparent K_m values for H_2O_2 (2.1 to 2.3 mM) and bromide (11 mM) were estimated.

Immunological investigations. Purified CPO-L was compared by Ouchterlony gel diffusion assay with BPO-A1 and BPO-A2 from *S. aureofaciens* ATCC 10762 (39), CPO-T from *S. aureofaciens* Tü24 (36), and CPO-P from *P. pyrrocinia* ATCC 15958 (42) with antisera raised against homogeneous CPO-T and CPO-P. The chloroperoxidase produced by *S. lividans* TK64 showed partial cross-reaction with CPO-P (Fig. 3) but not with the other enzymes.

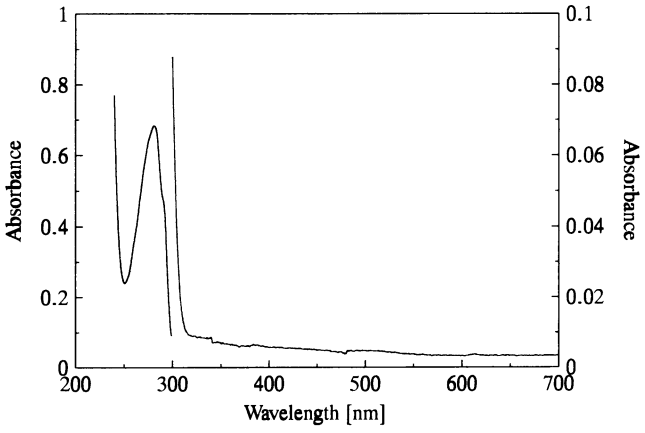

FIG. 2. Absorption spectrum of native CPO-L. The spectrum was recorded in 10 mM sodium acetate buffer (pH 5.5) at scales of 0.1 (700 to 300 nm) and 1.0 (300 to 240 nm).

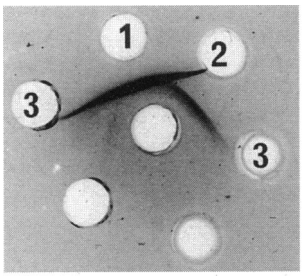

FIG. 3. Ouchterlony gel diffusion assay. The central well contained antiserum raised against CPO-P from *P. pyrrocinia*. Other wells: 1, CPO-P from *P. pyrrocinia*; 2, CPO-L from *S. lividans* TK64; 3, CPO-T from *S. aureofaciens* Tü24.

Chlorination of indole. Chlorinating activity of CPO-L was confirmed by chlorination of indole. GC-MS analysis showed the formation of three products. The molecular ion of the major product appeared as a doublet at *m/z* 151/153, split into 3:1 distribution as a result of the different isotopes of chlorine (*m/z* 35/37). This is characteristic for a monochloro-substituted compound and indicates monochlorination of the substrate by chloroperoxidase. The second product also appeared as a doublet. It showed a molecular ion at *m/z* 195/197. The intensity ratio was 1:1, which is characteristic for a monobrominated compound. The formation of bromoindole is due to the contamination of the reaction mixture with bromide and the much higher brominating than chlorinating activity. The product which was also produced in the absence of enzyme showed a molecular ion at *m/z* 133. The spectral data and the retention time by GC were identical with those from an authentic sample of oxindole. The formation of oxindole during the enzymatic chlorination was due to a nonenzymatic oxidation of indole by hydrogen peroxide. This reaction can be repressed by using lower hydrogen peroxide concentrations. These findings correspond to the results obtained by Wiesner et al. (41) for the chlorination of indole by CPO-P from *P. pyrrocinia*.

Nucleotide sequence analysis. The nucleotide sequence of the 1.9-kb *Sal*I fragment containing the *cpoL* gene is shown in Fig. 4. Analysis of the DNA sequence with the codon preference program revealed a 828-bp open reading frame (ORF) matching the codon usage of *Streptomyces* spp. The 828-bp ORF starting with an ATG codon at nucleotide 544 encodes a deduced polypeptide of 276 amino acids. The calculated M_r of 29,914 of this polypeptide is in good agreement with that determined experimentally for the CPO-L subunits (32,000 ± 2,000). Furthermore, the predicted N-terminal amino acid (underlined in Fig. 4) is identical to the one obtained by protein sequence analysis of the CPO-L purified from *S. lividans* and confirms the identity of this ORF and *cpoL*. Upstream of the ATG start codon, a DNA sequence complementary to the 3′ end of the 16S rRNA of *S. lividans* (32) might act as a ribosomal binding site. In addition, a DNA sequence matching the consensus sequence of an *E. coli*-like *Streptomyces* promoter (32) could be identified. Between this putative promoter region and the beginning of the *cpoL* are two 10-bp

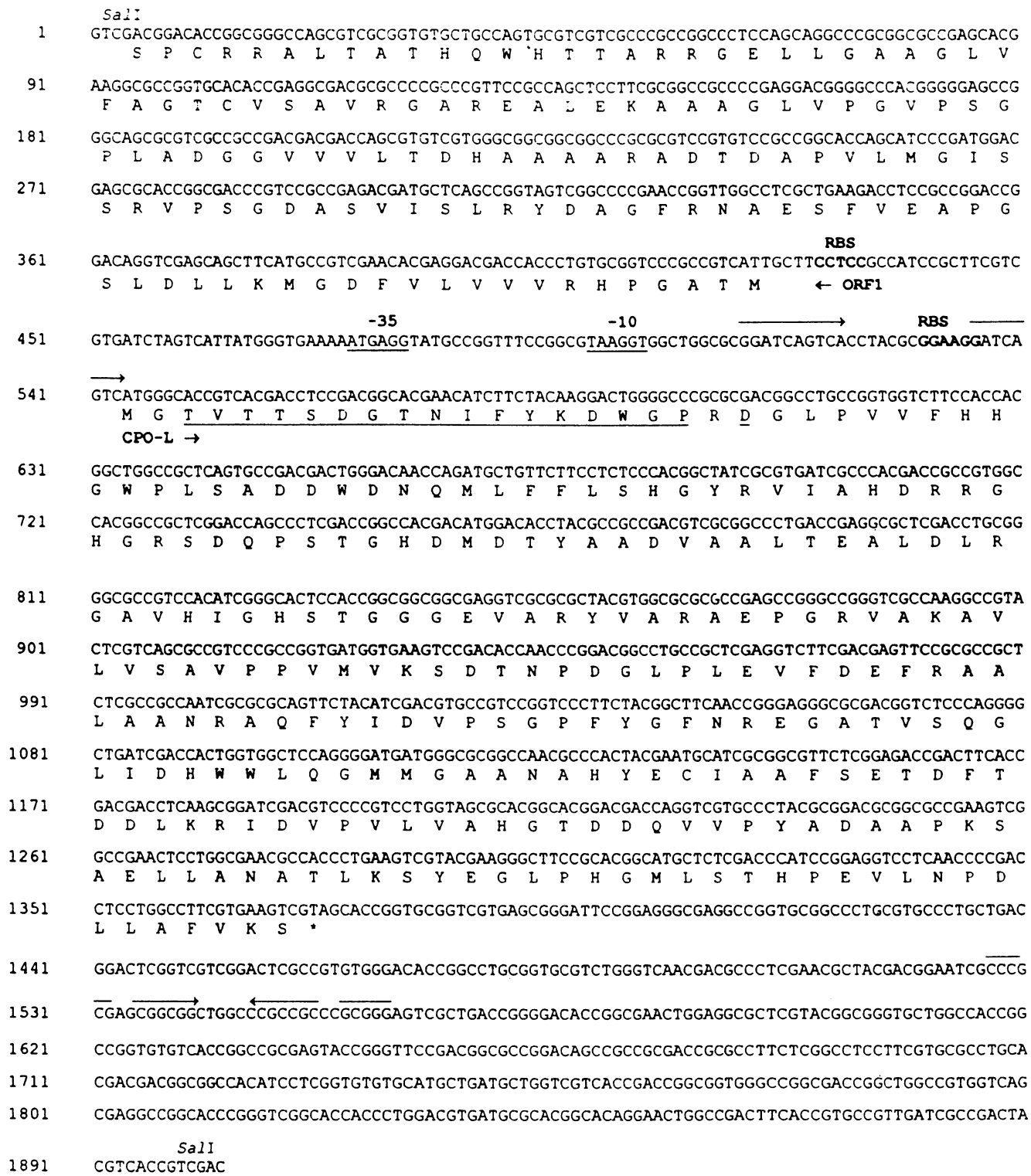

FIG. 4. Nucleotide sequence of the 1.9-kb *Sal*I fragment containing the *cpoL* gene from *S. lividans* and the deduced amino acid sequence of CPO-L and ORF1 in the one-letter code. The underlined amino acids of CPO-L were determined by Edman degradation. Putative promoter sequences are underlined; ribosomal binding sites (RBS) are shown in boldface. Direct and inverted repeats are marked by arrows above the nucleotide sequence.

```
                .         .         .         .         .
CPO-L       MGTVTTSDG----TNIFYKDWGPRDGLPVVFHHGWPLSADDWDNQMLFFLSHGYRVIAHD
CPO-P       MPYVTTKDN----VEIFYKDWGPKDAQPIVFHHGWPLSGDDWDAQMLFFVQKGYRVIAHD
BPO-A2      MPFITVGQENSTSIDLYYEDHGT--GQPVVLIHGFPLSGHSWERQSAALLDAGYRVITYD
            *   *            * * *     * *  ** ***   *  *       *****  *

CPO-L       RRGHGRSDQPSTGHDMDTYAADVAALTEALDLRGAVHIGHSTGGGEVARYVARAE--PGR
CPO-P       RRGHGRSAQVSDGHDMDHYAADAFAVVEALDLRNAVHIGHSTGGGEVARYVAKHGQPAGR
BPO-A2      RRGFGQSSQPTTGYDYDTFAADLNTVLETLDLQDAVLVGFSMGTGEVARYVSSYG--TAR
            *** * * *   * * *  ***     * ***  **  * * * *******        *

CPO-L       VAKAVLVSAVPPVMVKSDTNPDGL-PLEVFDEFRAALAANRAQFYIDVPSGPFYGFNRE-
CPO-P       VAKAVLVSAVPPLMLKTESNPEGL-PIEVFDGFRKALADNRAQFFLDVPTGPFYGFNRA-
BPO-A2      IAKVAFLASLEPFLLKTDDNPDGAAPQEFFDGIVAAVKADRYAFYTGFFND-FYNLDENL
             **        *   *   ** *  * * **    *    *  *        **

CPO-L       GATVSQGLIDHWWLQGMMGAANAHYECIAAFSE--TDFTDDLKRIDVPVLVAHGTDDQVV
CPO-P       GATVHQGVIRNWWRQGMEGSAKAHYDGIKAFSE--TDQTEDLKSITVPTLVLHGEDDQIV
BPO-A2      GTRISEEAVRNSWNTAASGG---FFAAAAAPTTWYTDFRADIPRIDVPALILHGTGDRTL
            *           *     *          *     **   *   * ** *  **  *

CPO-L       PYADAAPKSAELLANATLKSYEGLPHGMLSTHPEVLNPDLLAFVKS
CPO-P       PIADAALKSIKLLQNGTLKTYPGYSHGMLTVNADVLNADLLAFVQA
BPO-A2      PIENTARVFHKALPSAEYVEVEGAPHGLLWTHAEEVNTALLAFLAK
            *    *      *          *  ** *       *  ****
```

FIG. 5. Alignment of CPO-L from *S. lividans*, CPO-P from *P. pyrrocinia*, and BPO-A2 from *S. aureofaciens* ATCC 10762. Amino acids common to all three proteins are marked by asterisks. (Parameters for CLUSTAL pairwise similarity scores: K-tuple length = 1, gap penalty = 3, window size = 10; multiple alignment, gap penalty = 10).

direct repeated sequences, which might be involved in the regulation of *cpoL* expression. Finally, an inverted repeated sequence (ΔG = −42 kcal [ca. −176 kJ]) which might be involved in transcription termination was found 153 bp downstream of the *cpoL* stop codon.

Besides the *cpoL* gene, further ORFs with a typical *Streptomyces* codon usage were identified on the 1.9-kb *Sal*I fragment. ORF1 is upstream and divergently orientated to *cpoL* and starts with an ATG codon at nucleotide 425. Its C-terminal end lies outside the sequenced 1.9-kb fragment. There is a potential ribosomal binding site in front of ORF1 at the right distance to the start codon, but no *E. coli*-like −10, −35 promoter sequence was found. Another ORF (ORF2) with the same orientation as *cpoL* begins 13 bp downstream of the *cpoL* stop codon at nucleotide 1388 with a GTG start codon and ends outside the sequenced fragment (not shown in Fig. 4). No consensus sequence indicating a Shine-Dalgarno sequence or promoter was found in front of the GTG codon or other possible start codons following in frame downstream in the ORF. Furthermore, the inverted repeat mentioned above lies within ORF2, and therefore it seems unlikely that this ORF is indeed a transcribed gene.

Protein sequence comparison. The deduced amino acid sequence of *cpoL* was compared with sequences of the other two sequenced nonheme-type haloperoxidases by using the BESTFIT and CLUSTAL programs. There is a striking similarity of 71% identical amino acids between CPO-L and the chloroperoxidase CPO-P from *P. pyrrocinia* (Fig. 5). A lower but still highly significant similarity was found to the bromoperoxidase BPO-A2 (42% identity), an enzyme from *S. aureofaciens* ATCC 10762, indicating a common ancestor of this type of haloperoxidases.

The deduced amino acid sequences of the two additional ORFs identified on the 1.9-kb fragment were compared with entries in protein data bases provided by GenBank, using the blastp program. No similarity to any of the protein sequences deposited in the data bases could be identified for ORF1 and ORF2.

DISCUSSION

Until now, no halogenated compounds from *S. lividans* were known. However, during the cloning of the *cpoT* gene from *S. aureofaciens* Tü24, brominating activity had been detected in this strain. Like other previously isolated haloperoxidases, CPO-L was not detected in crude extracts (36, 39, 41) but only after dialysis or ion-exchange chromatography. This phenomenon may be due to one or several inhibitory substances of unknown structure. The ability to remove these inhibitory compounds by dialysis suggests that they are of low molecular weight. After cloning of the *cpoL* gene and overexpression in *S. lividans* TK64 and *S. aureofaciens* Tü24-88, activity could be measured in crude extracts, probably because of the increased levels of CPO-L compared with the levels of inhibitory compounds.

Like other *Streptomyces* haloperoxidases (27, 33), chloroperoxidase from *S. lividans* was not expressed in *E. coli*. Therefore, subcloning was carried out in the original host. *S. lividans* TK64 transformed with the *cpoL* gene produced up to 40-fold-higher amounts of the enzyme. Compared with *S. lividans* TK64 containing haloperoxidase genes cloned from *S. aureofaciens* ATCC 10769 (27) and from *S. aureofaciens* Tü24 (33), this was still only a moderate level of expression. The reason might be repression of transcription of *cpoL* by a regulatory protein or plasmid instability caused by homologous recombination of the plasmid and the genomic DNA of *S. lividans* TK64. To avoid these problems, a mutant of *S. aureofaciens* Tü24, which had lost the *cpoT* gene (12), was used. The overproduction of chloroperoxidase by *S. aureofaciens* Tü24-88 containing pRB882 is up to 3,000 times higher than that by untransformed cells of *S. lividans* TK64. This high expression cannot be explained only by a gene dosage effect of the high-copy-

number plasmid pIJ486; it also indicates a derepressed transcription of *cpoL*.

Although the enzyme was isolated as a bromoperoxidase and does not chlorinate monochlorodimedone, it is able to chlorinate a more appropriate substrate such as indole. A substrate specificity for chlorination has been demonstrated for the chloroperoxidases from *S. aureofaciens* Tü24 (4) and *P. pyrrocinia* (4, 41, 42). This is different from that of the heme chloroperoxidase from the fungus *C. fumago*, which catalyzes the bromination and chlorination of monochlorodimedone (13).

Since CPO-L was not inhibited by azide, it belongs to the class of nonheme haloperoxidases. The spectrum of the purified protein showed, unlike those of the heme-containing haloperoxidases (34), no absorption in the visible region. X-ray fluorescence spectroscopy demonstrated that the chloroperoxidase did not contain any metals in equimolar amounts.

In heme haloperoxidases, the electron transfer from the halide ion to the peroxide proceeds via a prosthetic group (24); in eukaryotic nonheme haloperoxidases, it proceeds via the cofactor vanadium (30). In both cases, enzymatically produced HOBr and HOCl are the halogenating species. The bacterial nonheme haloperoxidases contain neither a cofactor nor a prosthetic group (36, 42). Therefore, the electron transfer must take place at an amino acid. Haag et al. (11) postulated a reaction mechanism with a methionine at the active site. Here only HOBr but no HOCl is formed enzymatically as the halogenating species. Chlorine can be transferred directly from the enzyme only onto certain organic substrates. This results in the observed substrate specificity for the chlorination reaction.

Comparison of the chloroperoxidase from *S. lividans* with other nonheme haloperoxidases revealed a high identity in amino acid sequence (71%) to CPO-P from *P. pyrrocinia* (43) and to a lesser extent of identity (42%) to BPO-A2 from *S. aureofaciens* ATCC 10762 (27). The multiple alignment of the deduced amino acid sequences of CPO-L, CPO-P, and BPO-A2 showed several conserved regions. However, there was no methionine residue that was conserved in all three sequences (27, 43). The amino acid sequence of BPO-A2 from *S. aureofaciens* ATCC 10762 contains only one methionine residue, at position 102; CPO-P from *P. pyrrocinia* contains five, and CPO-L from *S. lividans* contains six, methionine residues. CPO-L and CPO-P both have methionine residues at positions 46, 76, 134, 197, and 268 but none at position 102. The additional methionine residue of CPO-L is at position 198. Site-directed mutagenesis of the genes and the elucidation of the three-dimensional structure of the enzyme should help to identify the methionine residues necessary for activity.

In contrast to *S. aureofaciens* Tü24 and *S. aureofaciens* ATCC 10762, which both produce 7-chlorotetracycline, and *P. pyrrocinia*, which produces pyrrolnitrin, *S. lividans* has not been reported to produce any halogenated metabolites. In *Streptomyces* spp., genes of biosynthetic pathways are usually clustered in large operons. The *cpoL* gene might be part of such a gene cluster, producing a so far unidentified halogenated metabolite, but it is unlikely that it is transcribed with other genes into a polycistronic mRNA. Immediately upstream of *cpoL*, an *E. coli*-like promoter sequence was identified, followed by an ORF in opposite orientation to *cpoL*. Downstream of *cpoL*, another ORF with the same orientation as *cpoL* and a typical *Streptomyces* codon usage is present, but there was no sequence in front of several possible start codons fitting the consensus sequence of a ribosomal binding site. Because there is an inverted repeat within this ORF, it is more likely that this inverted repeat is a transcription terminator ending transcription about 200 bp downstream of the *cpoL* gene.

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