Cloning, sequencing and disruption of a bromoperoxidase-catalase gene in *Streptomyces venezuelae*: evidence that it is not required for chlorination in chloramphenicol biosynthesis

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Genomic DNA libraries of *Streptomyces venezuelae* ISP5230 and of a mutant blocked at the chlorination step of chloramphenicol biosynthesis were probed by hybridization with a synthetic oligonucleotide corresponding to the N-terminal amino acid sequence of a bromoperoxidase-catalase purified from the wild-type strain. Hybridizing fragments obtained from the two strains were cloned and sequenced. Analysis of the nucleotide sequences demonstrated that the fragments contained the same 1449 bp open reading frame with no differences in nucleotide sequence. The deduced polypeptide encoded 483 amino acids with a calculated *M_r* of 54200; the N-terminal sequence was identical to that of the bromoperoxidase-catalase purified from wild-type *S. venezuelae*. Comparison of the amino acid sequence predicted for the cloned bromoperoxidase-catalase gene (*bca*) with database protein sequences showed a significant similarity to a group of prokaryotic and eukaryotic catalases, but none to other peroxidases or haloperoxidases. Replacement of the *bca* gene in the wild-type strain of *S. venezuelae* with a copy disrupted by insertion of a DNA fragment encoding apramycin resistance did not prevent chloramphenicol production. The results suggest that the role of the enzyme in *S. venezuelae* is related to its activity as a catalase rather than as a halogenating agent.

Keywords: *Streptomyces venezuelae*, bromoperoxidase-catalase, chloramphenicol, gene disruption

INTRODUCTION

Many micro-organisms produce chlorinated compounds with antibiotic activity (Strunz, 1984), but in only a few has the biochemical mechanism for introducing chlorine been examined in detail. An extensive study of chlorination in the fungus *Caldariomyces fumago*, which produces caldariomycin through the action of a chloroperoxidase (Morris & Hager, 1966), led to a general expectation that similar enzymes would prove to be responsible for the

introduction of chlorine into most natural products (Neidleman & Geigert, 1986). *Streptomyces venezuelae* ISP5230 produces the antibiotic chloramphenicol (Vining & Westlake, 1984), in which two atoms of chlorine are contained within an *N*-dichloroacetyl substituent. Although incorporation of chlorine during the biosynthesis of chloramphenicol has been attributed to a chloroperoxidase (Neidleman & Geigert, 1986), only bromoperoxidases have been isolated from this strain (Knoch, 1988). The properties of one such enzyme, designated bromoperoxidase I, are similar to those of a haem-containing bromoperoxidase found in *Streptomyces phaeochromogenes* (van Pée & Lingens, 1984, 1985), which also produces chloramphenicol. A second halogenating enzyme in *S. venezuelae* resembles bromoperoxidase I in

Abbreviation: *bca*, gene encoding bromoperoxidase-catalase

The GenBank accession number for the nucleotide sequence of the wild-type gene reported in this paper is X74791.

containing haem and in being able to brominate but not to chlorinate monochlorodimedone; however, it is distinctly different in having strong catalase but very low peroxidase activity. This bromoperoxidase-catalase (M_r 127 000–136 000) consists of two identical subunits of apparent M_r 61 000 (Knoch *et al.*, 1989). In contrast to other haem haloperoxidases and like typical catalases (Nadler *et al.*, 1986), it shows catalase activity that is pH-independent in the range pH 5–10, is inhibited by 3-amino-1,2,4-triazole, is stable to treatment with a mixture of ethanol/chloroform and is not inactivated by sodium dithionite (Knoch *et al.*, 1989).

In *S. venezuelae* cml-2, a chloramphenicol-nonproducing mutant that forms corynecins (unhalogenated analogues of chloramphenicol) and is presumed to be blocked in the chlorination step of the biosynthetic pathway (Doull *et al.*, 1985), the bromoperoxidase-catalase was inactive (Knoch *et al.*, 1989). Since chloroperoxidases catalyse the oxidation of Br^- and Cl^- , the concurrent loss of both chlorinating and brominating activity suggested that the *S. venezuelae* bromoperoxidase-catalase would function as a chlorinating enzyme with the appropriate substrate. We report here the cloning and sequencing of the bromoperoxidase-catalase gene (*bca*) from *S. venezuelae* ISP5230 and show that it is unaltered in the mutant cml-2. By disrupting the gene and introducing the disrupted copy into *S. venezuelae* to replace the native gene, we demonstrated that the bromoperoxidase-catalase is not required for chloramphenicol production.

METHODS

Bacteria, plasmids and culture conditions. *Streptomyces venezuelae* ISP5230 (ATCC 10712; Stuttard, 1982) and the mutant cml-2 were maintained on MYM medium as described by Doull *et al.* (1985). Cultures of *Streptomyces lividans* TK64 (Hopwood *et al.*, 1983) harbouring recombinant plasmids were grown at 30 °C in SM medium (2%, w/v, soybean flour, 2%, w/v, mannitol) containing thiostrepton (5 $\mu\text{g ml}^{-1}$ in liquid media, 50 $\mu\text{g ml}^{-1}$ in agar media; thiostrepton was kindly donated by E. R. Squibb and Sons). Cultures of *Escherichia coli* TG1 (Carter *et al.*, 1985) and *E. coli* 12567 (MacNeil *et al.*, 1992) were grown at 37 °C in LB medium (Sambrook *et al.*, 1989). Enriched genomic libraries were constructed in the high-copy-number plasmid vector pUC18 (Yanisch-Perron *et al.*, 1985). Plasmids pIJ486 (Ward *et al.*, 1986) and pHJL400 (Larson & Hershberger, 1986) were used in subcloning for heterologous expression and gene disruption. Transformants carrying pUC18 and derivative plasmids were selected on agar media containing ampicillin (100 $\mu\text{g ml}^{-1}$), 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal; 40 $\mu\text{g ml}^{-1}$) and 0.2 mM isopropyl- β -D-thiogalactopyranoside (IPTG). Recombinant plasmids in *E. coli* TG1 were identified as white colonies in LB soft agar containing X-Gal and IPTG.

Transformation and DNA manipulation. *S. lividans* TK64 protoplasts were prepared, transformed and regenerated on R2YE agar; transformants were selected by overlaying the agar with 1 ml of a thiostrepton suspension (200 $\mu\text{g ml}^{-1}$) in water (Hopwood *et al.*, 1985). Protoplasts of *S. venezuelae* were prepared and transformed as reported by Aidoo *et al.* (1990). Procedures for transforming *E. coli* and for recovering *E. coli* plasmid and phage DNA were as described by Sambrook *et al.*

(1989). Genomic DNA was isolated from *Streptomyces* strains as described by Hopwood *et al.* (1985). Plasmids were obtained from *S. lividans* transformants by the alkaline lysis procedure of Kieser (1984). DNA fragments were isolated from agarose gels with the GeneClean II kit (Dianova).

N-terminal amino acid sequence and oligonucleotide synthesis. Bromoperoxidase-catalase was isolated from cultures of *S. venezuelae* ISP5230 as reported previously (Knoch *et al.*, 1989). After further purification by nondenaturing PAGE, the protein was transferred to a polyvinylidene difluoride (Immobilon, Millipore) membrane. The bromoperoxidase-catalase band detected with Coomassie blue was excised and used to determine the N-terminal amino acid sequence of the protein by automated Edman degradation (Applied Biosystems protein sequencer). A 48-mer deoxyinosine-containing oligodeoxynucleotide probe with fourfold degeneracy was synthesized by the Regional DNA Synthesis Laboratory, University of Calgary, Canada (Applied Biosystems 380B DNA synthesizer), purified by gel electrophoresis and end-labelled with digoxigenin-dideoxyuridine triphosphate.

Cloning of the bromoperoxidase-catalase gene. Southern hybridization of *S. venezuelae* ISP5230 genomic DNA using the oligodeoxynucleotide probe detected a 2.2 kb hybridizing fragment in *Bam*HI digests. Fragments of about 2.2 kb were extracted from the agarose gel after electrophoresis of a digest, and ligated to pUC18 that had been digested with *Bam*HI and treated with shrimp alkaline phosphatase (US Biochemical). Transformation of *E. coli* TG1 with the ligation mixture yielded an enriched genomic minilibrary. The library was screened by colony hybridization at 50 °C with the oligodeoxynucleotide probe (Sambrook *et al.*, 1989). Nylon membranes were washed (15 min) twice at 50 °C in 2 $\times$ SSC, once at 50 °C in 1 $\times$ SSC, and once at 65 °C with 1 $\times$ SSC. All wash solutions contained 0.1% sodium dodecyl sulphate; 1 $\times$ SSC consisted of 150 mM NaCl and 15 mM sodium citrate, pH 7.0. Hybridizing DNA fragments were detected with the DIG luminescence detection kit (Boehringer Mannheim).

With the same oligodeoxynucleotide probe, Southern hybridization of genomic DNA from *S. venezuelae* mutant cml-2 digested with *Bgl*II and *Pst*I detected *bca* in a 3.5 kb fragment. Fragments about 3.5 kb in size were retrieved from the digest and ligated to pUC18 linearized with *Bam*HI and *Pst*I to generate an enriched genomic minilibrary in *E. coli* as above. The 3.5 kb fragment was detected by colony hybridization and isolated by the procedures described.

Sequence determination and analysis. DNA fragments were cloned in M13mp18 or M13mp19 (Yanisch-Perron *et al.*, 1985), and sequenced using a T7 sequencing kit (Pharmacia) by the chain-termination method of Sanger *et al.* (1977). Nucleotide sequences were analysed with the GENMON program (Gesellschaft für Biotechnologische Forschung, Braunschweig, FRG); they were compared with the EMBL nucleotide sequence data library. Amino acid sequences were compared with the SWISS-PROT protein database. The CLUSTAL program for multiple sequence alignment was kindly provided by the German Cancer Research Center, Heidelberg, in the software package HUSAR.

Construction of replacement vectors. The *Streptomyces*–*E. coli* bifunctional vector pHJL400 (Larson & Hershberger, 1986), which is segregationally unstable in *S. venezuelae*, was modified to obtain the replacement vectors pDQ402 and pDQ403 by inserting in both orientations a DNA fragment carrying *bca* disrupted at its *Sa*I site with a gene conferring apramycin resistance (*Apr*^R). To construct the insert, pSF2201 was linearized by digestion with *Xba*I and *Pst*I, and the single-strand

overhangs were filled by incubation with the Klenow fragment and deoxynucleotide triphosphates. Ligation of the blunt ends yielded pDQ401, in which the *Sa*I site of the pUC18 multiple cloning sequence in pSF2201 had been deleted. A DNA fragment conferring *Apr*^R was then inserted within the *bca* sequence in pDQ401. The fragment conferring *Apr*^R was obtained from pKC462a (Stanzak *et al.*, 1986). A 1.5 kb *Bam*HI–*Pst*I fragment carrying the gene was excised and cloned in pBluescriptII SK(+), from which it was then retrieved as a *Bam*HI–*Hind*III fragment. This was end-filled by Klenow treatment, and ligated to pDQ401, which had been linearized at its unique *Sa*I site (within *bca*) and also end-filled by Klenow treatment. The plasmids extracted from *E. coli* TG1 transformants were digested with *Bam*HI to yield 3.7 kb fragments in which the DNA (in its two orientations) conferring *Apr*^R was flanked by *S. venezuelae* DNA. One such fragment was recloned in each orientation in the *Bam*HI site of pHJL400 to give the replacement vectors pDQ402 and pDQ403.

Protein electrophoresis, enzyme assays and protein measurements. For native enzymes, 7.5% (w/v) polyacrylamide gels (pH 7.5) were used (Maurer, 1964). Brominating activity was detected by the conversion of phenol red to bromophenol blue as described by Knoch *et al.* (1989). Gels were stained for proteins with Coomassie blue R250. Catalase activity was measured spectrophotometrically at 240 nm (Beers & Sizer, 1952) with 6.5 mM H₂O₂ (in 100 mM sodium acetate buffer, pH 5.5) as the substrate. One unit of catalase converted 1 µmol H₂O₂ to H₂O in 1 min at 25 °C. Protein was determined by the Lowry method, using a bovine serum albumin standard.

Partial purification of bromoperoxidase-catalase expressed from cloned *bca*. The enzyme was kept at 4 °C throughout the purification. Solutions were buffered with sodium acetate at pH 5.5. The 2.2 kb *Bam*HI insert of pSF2201 was recloned in *Bam*HI-digested and dephosphorylated pIJ486; the recombinant vector was used to transform *S. lividans* TK64 protoplasts. Transformants grown at 30 °C for 6 d in 100 ml SM medium supplemented with thiostrepton were harvested by centrifugation, resuspended in twice their volume of 100 mM buffer and disrupted by sonication for 20 min (Branson Sonifier 450, 160 W, 50% duty cycle). The supernatant obtained by centrifugation (22 100 g, 30 min) of the sonicate was dialysed against 100 mM buffer for 15 h, clarified by centrifugation as before, and applied to a DEAE cellulose column (Whatman DE52, 5.5 × 11 cm) equilibrated with 150 mM buffer. Proteins were eluted with a gradient (600 ml) of 150–700 mM buffer. Fractions (4 ml) with at least 25% of the catalase activity of the most active fraction were pooled and concentrated by ultrafiltration (Amicon PM-30 membrane).

Measurement of chloramphenicol production. Cultures were incubated in 250 ml Erlenmeyer flasks on a rotary shaker (220 r.p.m.) at 27 °C. For chloramphenicol production, portions (25 ml) of glucose-isoleucine medium (Doull *et al.*, 1985) were inoculated (4%, v/v) from a vegetative culture prepared by incubating *S. venezuelae* spores for 24 h in 10 ml GNY medium (Malik & Vining, 1970). The chloramphenicol production cultures were examined after 3 d growth by extracting a 3 ml sample of the clarified broth with an equal volume of ethyl acetate. The extract, after evaporation, was taken up in 0.1 ml methanol/water (3:1), and a sample (20 µl) was analysed by high-performance liquid chromatography (HPLC) using a C₁₈ reverse-phase column (80 × 4.0 mm) and stepped linear gradients from 0 to 25% (v/v) (1 min), 25 to 50% (5 min) and 50 to 100% (1 min) of methanol/water, followed by 100% methanol for 1 min, and a gradient returning to 100% water (1 min). Chloramphenicol was eluted at 6.05 min; its concentration was

estimated by comparing the peak area with peak areas given by reference samples.

RESULTS

Isolation of *bca* from *S. venezuelae* ISP5230

Edman analysis of the bromoperoxidase-catalase purified from *S. venezuelae* ISP5230 identified 27 amino acids in the N-terminal sequence (Fig. 1a). This information was used to synthesize a 48-mer oligonucleotide corresponding to amino acids 2–17. Degeneracy was minimized by taking account of the biased codon usage in streptomycetes (Wright & Bibb, 1992). To further reduce the complexity of the mixed probe population, deoxyinosines were used at seven ambiguous positions where the codon usage bias was insufficient (see Fig. 1a). Probing a *Bam*HI digest of genomic DNA from *S. venezuelae* ISP5230 with the oligonucleotide detected a 2.2 kb hybridizing fragment. This fragment was isolated as a *Bam*HI insert (Fig. 1b) in pSF2201 by screening an enriched minilibrary of *S. venezuelae* genomic DNA in pUC18. A colony of *E. coli* TG1 containing pSF2201 was identified by hybridization with the oligodeoxynucleotide probe.

Fig. 1. (a) Sequence of amino acids at the N-terminus of the bromoperoxidase-catalase of *S. venezuelae* ISP5230, determined by the Edman procedure. The synthetic oligodeoxynucleotide probe (shown below) based on the sequence of amino acids 2–17 contained deoxyinosine (I) at positions where codon degeneracy prevented preferential assignments. Asterisks indicate nucleotides that did not match the *bca* sequence determined later. (b) Restriction maps of the fragments of *S. venezuelae* chromosomal DNA cloned in pSF2201 from the wild-type strain (ISP5230), and in pFG3.5 from the chlorination-deficient mutant (cml-2). Only restriction sites used for sequence analysis are shown. The arrows locate *bca* as determined from the nucleotide sequence: B, *Bam*HI; Bg, *Bgl*II; P, *Pst*I; X, *Xho*I; S, *Sal*I; Ss, *Sst*I; Sa, *Sau*3A1.

5'-	
GGATCCTATAGATCCGGGCCCTGATCGCGTTTCCGGACGCAACCGGACGACCCCGGGCGCAACAGTAAAGCCCCGCCG	80
CGGGCGGGGCTTCTGTGTGGCGTCTCAGGCGCTGCCGGCGCAGCTGGGGCAGATGCCTCGGTAGGTGACCTCGACGTTT	160
GAGATGGTGAAGCCGAAGCGCTCGGTGTCCGGCAGGTCCGCCAGCGGGTCGCCGCCGGATGCACATCGCGGATCGCGCC	240
GCAGCGGCCGAGACCAGGTGCTGGTGCGGCCGGTGGGCGTTGGGGTCGTAGCGCTTGGCGCGGCCGTCGGTTCGCGACCT	320
CGATGACCTCGCCGAGGTGACCATCTCGCCGAGCGTGTGTAGACGGTCGCGCGCAGATCTCGGGCAGGCGCGTCACG	400
GCGCGCGCGTGGACCTCGTCGGCGGTGAGGTGTACGTGGTCCCCGTCCAGGACCTCCGCCACCACACGCCGCTGCGCGGT	480
CATCCGCCAGCCGCTCCGCCGAGTCGTTCCAACAGGTCACTCATGAGGGCCAGCCTAACAGTCGGGGGCGCCGCTTCCC	560
GAACCGGTGTGACTTTGGATGTTCACTT AGACT TAGACATTGTCTATCGT TAGGAT CGAGTACGGCACGCAGGACAGGACGC	640
<u>M T Q G P L T T E A G A P V A D N Q N S E T A</u>	23
<u>AGGAGGCGCACGTGACGCAGGACCGCTCACCAGGAGGCCGGCGCCGGTCGCCGACAACAGGACGAGACGGCG</u>	720
<u>G V G G P V L V Q D Q L L L E K L A H F N R E R I P E</u>	50
GGCGTAGGCGGTCCGGTCTCGTCCAGGACAGCTGCTGCTCGAGAAGCTCGCCCACTTCAACCGTGAGCGCATCCCGGA	800
R V V H A R G A G A Y G T F T L T R D V S R W T R A	76
GCGCGTGGTCCACGCGCGCGGTGCGGGCGCCTACGGCACCTTCACGTCACCCGTGACGTCTCGCGGTGGACGCGGGCCG	880
A F L S E V G K R T E T F L R F S T V A G S L G A A D	103
CCTTCTCTCCGAGGTCCGCAAGCGGACCGAGACCTCCCTGCGCTTCTCCACCGTCGCGGGCAGCCTCGGCGCGGCCGAC	960
A V R D P R G W A L K F Y T E E G N Y D L V G N N T P	130
GCGGTGCGGATCCGCGCGGTGGGCGCTGAAGTTCTACACCGAGGAGGGCAACTACGACCTCGTCGGAACAACACCCC	1040
V F F I K D A I K F P D F I H T Q K R D P Y T G S Q	156
GGTGTCTTCATCAAGGACGCCATCAAGTTCCCGACTTCATCCACACCCAGAAGCGCGACCCGTACACGGGCTCGCAGG	1120
E A D N V W D F W G L S P E S T H Q V T W L F G D R G	183
AGGCGGACAACGTCTGGGACTTCTGGGGCCTGTGCGCGGAGTCGACCCACAGGTGACCTGGCTGTTGCGCGACCGCGGC	1200
I P A S Y R H M N G Y G S H T Y Q W N N E A G E V F W	210
ATCCCGCCTCGTACCGCCACATGAACGGCTACGGCTCGCACAGTACCAGTGGACCAACGAGGCCGCGAGGTCTTCTG	1280
V K Y H F K T D Q G I K N L T Q D E A N R L A G E D	236
GGTCAAGTACCCTTCAAGACCGACCGGGGATCAAGAACCTCACGCAGGACGAGGCCAACCGGCTGGCGGCGAGGACC	1360
P D S H Q R D L R E A I E R G D F P T W T V Q V Q I M	263
CGGACTCGCACCAGCGCGACTGCGCGAGGCCATCGAGCGCGGCGACTTCCCGACCTGGACCGTGCAGGTCCAGATCATG	1440
P A A D A G A G G Y R F N P F D L T K V W P H E D Y P P V	290
CCCGCGCCGCGGCGGCTACCGCTTCAACCCCTTCGACCTCACCAAGGTCTGGCCGACGAGGACTACCCGCGCGGT	1520
E I G T L E L N R N P E N I F A E V E Q S I F S P A	316
CGAGATCGGCACCTTGGAGTCAACCGCAACCCGAGAACATCTTCGCCGAGGTGAGCAGAGCATCTTCTCCCGCGCGC	1600
H F V P G I G P S P D K M L Q G G R L F A Y G D A H R Y	343
ACTTCGTGCCCGGATCGGGCGTCCCGGACAAGATGCTCAGGGCGTCTCTTCGCGTACGGCGACGCGCACCGCTAC	1680
R V G I N A D H L P V N R P H A T E A R T H S R D G F	370
CGCGTCGGCATCAACGCCGACCACTGCCGGTGAACCGCCGACGCCACCGAGGCGCGGACGCACTCCCGTGACGGCTT	1760
L Y D G R H K G A K N Y E P N S F G G P V Q T D R P	396
CCTCTACGACGGGCCCAAGGGCGCGAAGAACTACGAGCCAACTCCTTCGGCGGACCGGTCCAGACGGACCGCCCGC	1840
L W Q P T P V T G V T G D H A A P S H A E D D D F T Q	423
TGTGGCAGCCACCCCGGTACCGGGCTCACCGGCGACCGCGGCCCGTTCGACGCGGAGGACGACGACTTCACGCAG	1920
A G D L Y R L M S E D E K G R L I D N L S G F I A K V	450
GCGGGCGACCTCTACCGGTGATGTCGGAGGACGAGAAGGGCCGCTGATCGACAATCTGTCCGGCTTCATCGCCAAGGT	2000
S R D D I A E R A I G N F R R A D E D F G K R L E A	476
CTCGCGCAGACATCGCGGAGCGCGATCGGCAACTTCCGCCGGGCGGACGAGGACTTCGCAAGCGGCTGGAGGCCG	2080
A V Q A L R G ***	483
CGGTCCAGGCCCTGCGCGGT TGA AGGGCGCTTGCCGTAGAGAGGGAGCGGGTCGGATTCCGTACGGATTCCGGCCCCGTCC	2160
CGCGTTCACAGGGGCCGTGCCCCCGCTCAGCTCACAACGCTGTGCACGGGGATCC	2216
-3'	

Fig. 2. Nucleotide and deduced amino acid sequence of the *S. venezuelae* *bca* gene. Amino acids that correspond to the experimentally determined N-terminal sequence of the bromoperoxidase-catalase are underlined. A probable Shine-Dalgarno sequence is doubly underlined. Potential promoter hexamers are boxed. Translational start (GTG) and termination (TGA) codons are in boldface. Arrows locate an inverted repeat.

Fig. 3. Alignment of the bromoperoxidase-catalase from *S. venezuelae* (bca_strve) with *Bacillus subtilis* vegetative catalase (catv_bacsu), bovine liver catalase (cata_bovin), mouse catalase (cata_mouse) and human catalase (cata_human). Asterisks identify amino acids common to all five proteins. Similar amino acids are marked by dots. Hyphens indicate gaps introduced to increase similarity. Arrows indicate amino acids in the active centre. Amino acids on the distal side of the haem-binding site are enclosed with solid lines, those on the proximal side with dashed lines. Parameters for CLUSTAL pairwise similarity scores are: K-tuple length = 1, gap penalty = 3, window size = 5, multiple alignment gap penalty = 10.

Nucleotide sequence and sequence analysis of *bca*

The DNA sequence of the insert in pSF2201 (Fig. 2) and a codon usage analysis of the sequence established that an open reading frame (ORF) was present (see Fig. 1b). The G + C content of the 1449 bp ORF was 70 mol %, and the frequency of codons containing G or C in the third position was 97.5 %. These values are consistent with the high percentage of G + C in the genomic DNA of streptomycetes (Enquist & Bradley, 1971; Wright & Bibb, 1992). The sequence at the 5'-end is close (only two

of the 48 bases are mismatched) to that of the mixed synthetic oligonucleotide probe used for hybridization (see Fig. 1a). The translational initiation codon at nt 652–654 is GTG, which in the G + C-rich DNA of streptomycetes is not uncommon as a start codon. Five base pairs upstream from this GTG is a putative Shine–Dalgarno sequence (SD; Shine & Dalgarno, 1974) for ribosome binding [5'-AGGAGG-3', ΔG –9.4 kcal mol⁻¹ (39.3 kJ mol⁻¹); Tinoco *et al.*, 1973] that matches the consensus SD for *S. lividans* (Bibb & Cohen, 1982). A potential promoter sequence comprising –35 (5'-TTGACT-3'; nt 587–592) and –10 (5'-TAGGAT-3'; nt 609–614) hexamers separated by 16 bp is located upstream of the initiation codon. The –35 hexamer shows 5/6 homology with the consensus –35 sequences of both *E. coli* and *E. coli*-like *Streptomyces* promoters (Strohl, 1992); in the –10 region, the homology with the consensus sequences for *E. coli* and *E. coli*-like *Streptomyces* promoters is 4/6 and 6/6, respectively. An imperfect inverted repeat sequence (nt 2122–2141 and 2142–2161) representing a putative termination signal is present 18 bp downstream of the TGA termination codon at nt 2101–2103.

Characteristics of the deduced amino acid sequence

The ORF encodes a 54 kDa polypeptide with 483 amino acids. The deduced amino acid sequence at the N-terminus precisely matches that of the purified bromoperoxidase-catalase determined by Edman degradation. In a search of protein databases (SWISS-PROT, EMBL), no appreciable sequence similarity was found between the predicted ORF product and known haloperoxidases. However, significant similarity was found to several catalases (Fig. 3), notably *Bacillus subtilis* vegetative catalase (59.2 % identity; Bol & Yasbin, 1991), mouse catalase (51.3 % identity; Shaffer *et al.*, 1990), bovine liver catalase (51.3 % identity; Schroeder *et al.*, 1982) and human kidney catalase (50.9 % identity; Bell *et al.*, 1986). The three amino acids (His-74, Ser-113 and Asn-147) participating in the active site of bovine liver catalase (Fita & Rossmann, 1985) are conserved in the *S. venezuelae* bromoperoxidase-catalase as His-54, Ser-93 and Asn-127 (see Fig. 3). Furthermore, the four amino acids (Val-73, Thr-114, Phe-152 and Phe-160) interacting with the distal side of the haem group of bovine liver enzyme, and the three (Pro-335, Arg-353 and Tyr-357) interacting on the proximal side, have counterparts (Val-53, Thr-94, Phe-132, Phe-140, Pro-315, Arg-333 and Tyr-337, respectively), in the bromoperoxidase-catalase sequence.

Expression of the cloned *S. venezuelae* *bca* in *S. lividans*

To confirm that the DNA insert cloned in pSF2201 encodes the bromoperoxidase-catalase purified from *S. venezuelae*, the *Bam*HI fragment was excised and recloned in pIJ486. The recombinant plasmid (pSF2202) was used to transform the heterologous host *S. lividans* TK64. Extracts of the transformant, as well as of *S. lividans* TK64(pIJ486), were examined for haloperoxidase activity. As this activity usually cannot be detected in crude

Fig. 4. Polyacrylamide gel electrophoresis of partially purified bromoperoxidase-catalase from *S. venezuelae* and *S. lividans* under non-denaturing conditions. (a) Gel stained for brominating activity with phenol red. (b) Gel stained for protein with Coomassie blue. Lanes: 1, protein (35 µg) from *S. lividans* TK64(pIJ486) after ion-exchange chromatography; 2, protein (35 µg) from *S. lividans* TK64(pSF2202) after ion-exchange chromatography; 3, bromoperoxidase-catalase (15 µg) from *S. venezuelae* ISP5230 after hydrophobic-interaction chromatography. The position of the bromoperoxidase-catalase is indicated by an arrow.

extracts of streptomycete mycelium (van Pée, 1988), the extracts were partially purified to remove inhibiting substances before electrophoresis on a non-denaturing polyacrylamide gel. Staining with phenol red showed two blue bands due to brominating activity in *S. lividans* TK64(pIJ486), whereas the pSF2202 transformant gave three blue bands (Fig. 4). The third band coincided with that obtained with partially purified bromoperoxidase-catalase from *S. venezuelae* ISP5230. Protein staining of the gel visualized a band in the *S. lividans* TK64(pSF2202) extract that also matched the band in the *S. venezuelae* ISP5230 extract, but was absent from the extract of *S. lividans* TK64(pIJ486).

Isolation and nucleotide sequence of *bca* from mutant *cml-2*

Probing a *Bgl*II/*Pst*I digest of genomic DNA from *S. venezuelae* *cml-2* with the synthetic oligodeoxynucleotide shown in Fig. 1(a) detected a 3.5 kb hybridizing fragment. The fragment was obtained in pFG3.5 by screening an enriched genomic minilibrary of *S. venezuelae* *cml-2* DNA in pUC18, using the colony hybridization procedure described earlier for isolating pSF2201. The inserts cloned in pFG3.5 and pSF2201 were shown to be in the same region of chromosomal DNA by restriction mapping (see Fig. 1b). Sequencing demonstrated that the insert contained a 1449 bp ORF identical in all respects to *bca* cloned in pSF2201 from the wild-type *S. venezuelae*.

Disruption of the bromoperoxidase-catalase gene

A replacement vector was prepared by inserting the *Apr*^R gene from pKC462a (Stanzak *et al.*, 1986) into the *Sal*I site internal to the *bca* insert in pDQ401 (pSF2201 modified by deleting *Sal*I from the multiple cloning site). The disrupted *bca* construct was re-cloned in both orientations in the *Bam*HI site of the shuttle vector pHJL400 to give pDQ402 and pDQ403 (Fig. 5a). To reduce the severity of the apparent restriction barrier against methylated DNA in *S. venezuelae* (Han *et al.*, 1994), replacement vectors were isolated from the *E. coli* *dam dcm hsdM* strain ET12567 (MacNeil *et al.*, 1992). With this DNA, *S. venezuelae* gave (per µg of the vector used) approximately 100 *Apr*^R Thio^R transformants. Two of these (VS661 and VS662), with the

*Bam*HI insert in opposite orientations, were examined. Southern hybridization with pDQ402 as the probe indicated that the plasmid had integrated into the chromosome of each strain by single homologous recombination. The integrants were propagated on thiostrepton-free medium for two generations to allow vector excision and elimination (Paradkar *et al.*, 1993); progeny screened for loss of thiostrepton resistance yielded strains with an *Apr*^R Thio^S phenotype. Southern hybridization using pDQ402 to probe chromosomal DNA from these strains (e.g. VS663 derived from VS661, and VS664 derived from VS662; Fig. 5b) indicated that the vector DNA had been excised by a second crossover, resulting in replacement of the native *bca* with the disrupted copy from pDQ402 or pDQ403, respectively.

Measurement of chloramphenicol production by the strains in which *bca* had been modified by either vector integration or gene replacement gave titres of 60–75 µg ml⁻¹, within the range of wild-type values under the growth conditions used. The product was indistinguishable from authentic chloramphenicol when co-chromatographed by HPLC, and differed in retention time from reference samples of corynecins. The results indicated that the intact bromoperoxidase-catalase gene is not required for the chlorination reaction in chloramphenicol biosynthesis.

DISCUSSION

The close similarity between the oligodeoxynucleotide probe sequence and 5' sequences of the ORFs within the fragments cloned from *S. venezuelae* ISP5230 and its *cml-2* mutant indicate that the gene for the bromoperoxidase-catalase isolated from the wild-type strain by Knoch *et al.* (1989) was correctly targeted by the synthetic oligonucleotide. This is supported by the complete identity of the N-terminal amino acid sequence deduced for the cloned gene with the N-terminal amino acid sequence obtained from the purified bromoperoxidase-catalase. Further evidence is provided by the detection of an *S. venezuelae*-like bromoperoxidase-catalase in *S. lividans* transformants carrying the cloned gene from *S. venezuelae* ISP5230. The two bands of brominating activity detected after electrophoresis of an extract of *S. lividans* transformed

Fig. 5. Disruption and replacement of *bca* in *S. venezuelae*. (a) Transformation of *S. venezuelae* ISP5230 with the replacement vectors pDQ402 and pDQ403, and selection for both Thio^R and Apr^R gave VS661 and VS662. Propagation of these transformants without thio streptomycin selection and screening for Thio^S Apr^R progeny yielded VS663 and VS664. (b) Southern hybridization using pDQ402 to probe *S. venezuelae* chromosomal DNA digested with *Bam*HI and electrophoresed in a 0.7% agarose gel. Lanes: 1, VS663; 2, VS664; 3, VS661; 4, VS662; 5, ISP5230. The relatively high intensity of the hybridizing bands at 3.7 and 5.8 kb is due to integration of multiple copies of the plasmid vector into the chromosome of single-crossover strains VS661 and VS662, as observed previously in *S. venezuelae* ISP5230 (Paradkar *et al.*, 1993; Han *et al.*, 1994).

with the vector alone are consistent with earlier results indicating that *S. lividans* contains at least one non-haem haloperoxidase (van Pée, 1988; Bantleon *et al.*, 1994). As far as we know, *S. lividans* TK64 harbouring pIJ486 does not produce any halogenated metabolites, and thus the role of these enzymes is not clear.

Since *bca* is intact in the *cml-2* mutant of *S. venezuelae*, the absence of the chlorinated metabolite from cultures, and of halogenating activity in the protein corresponding to the bromoperoxidase-catalase in fractionated cell extracts from *cml-2* (Knoch *et al.*, 1989), are clearly not caused by a mutation within *bca*. The *cml-2* mutation may affect an ancillary reaction needed to generate an active enzyme; if so, the same reaction is presumably involved not only in introducing chlorine into chloramphenicol, but also in the activity of other halogenating enzymes, such as the

bromoperoxidase-catalase reported here. It may be significant that the M_r of 54 200 calculated for the product of *bca* is below the expected size for a dimeric protein in a native enzyme determined by gel filtration to have an M_r of 127 000–136 000 (Knoch *et al.*, 1989). The ability of strains in which *bca* had been replaced by a disrupted gene to continue making chloramphenicol confirms that *bca* is not essential for biosynthesis of the antibiotic.

The coding sequence of the *S. venezuelae* gene is preceded by a plausible SD sequence and by a promoter-like structure resembling the consensus promoter sequences of *E. coli* genes and *E. coli*-like *Streptomyces* genes recognized by the vegetative $E\sigma^{70}$ -type RNA polymerase holoenzyme. Since genes associated with either primary or secondary metabolism in streptomycetes can be transcriptionally controlled by such promoters (Baltz, 1990;

Strohl, 1992), a role for *bca* in primary metabolism cannot be inferred from this information. Of interest, however, is the close similarity reported recently between the amino acid sequence of the *S. venezuelae* bromoperoxidase-catalase and the sequences of several peptides from the single catalase in *Streptomyces coelicolor* A3(2) (Walker *et al.*, 1995). This enzyme and the major catalase in a second *S. coelicolor* strain (Kim *et al.*, 1994) are potentially involved in the response to oxidative stress. Both are tetrameric proteins with subunits of M_r $55\,000 \pm 2000$ (Kim *et al.*, 1994; Walker *et al.*, 1995). In this they resemble the catalase of *Haemophilus influenzae* (Bishai *et al.*, 1994), and for the *S. coelicolor* A3(2) protein the resemblance extends to the amino acid sequence (Walker *et al.*, 1995).

The amino acid sequence deduced for the *S. venezuelae* bromoperoxidase-catalase has strong similarity to that of the oxygen-responsive catalase in the anaerobe *Bacteroides fragilis* (Rocha & Smith, 1995). The two proteins belong to a group of dimeric catalases with subunit sizes of approximately 55 000 Da; the group includes the catalases from *Mycobacterium tuberculosis* (Dias & Wayne, 1974), *Comamonas compransoris* (Nies & Schlegel, 1982) and *Klebsiella pneumoniae* (Goldberg & Hochman, 1989). It shares sequence similarity with the tetrameric catalases of similar subunit size, and with a number of other catalases from Gram-positive and Gram-negative bacteria (Rocha & Smith, 1995). The evolutionary relationship indicated by sequence similarities between the *S. venezuelae* bromoperoxidase-catalase and catalases from animals as well as bacteria, and the functional relationship implied by the conservation of amino acids established from the crystal structure of bovine liver catalase to be structurally and catalytically important residues (Fita & Rossmann, 1985), suggest that the catalase activity of the bromoperoxidase-catalase may determine the role of this enzyme in *S. venezuelae*.

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