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Use of degenerate primers and touchdown PCR to amplify a halogenase gene fragment from *Streptomyces venezuelae* ISP5230

M Piraee and LC Vining

Biology Department, Dalhousie University, Halifax, NS, Canada B3H 4J1

Consensus amino acid sequences of $FADH_2$-dependent bacterial halogenases were used to design PCR primers amplifying a halogenase gene fragment from the chloramphenicol producer *Streptomyces venezuelae* ISP5230. The sequence-specific degenerate primers (MPF1 and MPR2) were used with a touchdown PCR procedure in the first PCR-assisted cloning of a halogenase gene fragment. In the region of the 290-bp PCR product containing the reverse primer, the deduced amino acid sequence exhibited characteristics of a $\beta-\alpha-\beta$ fold present in FAD-binding sites of certain monooxygenases. When used to probe Southern blots of restriction-enzyme-digested DNA, the [α-^{32}P]dCTP-labeled PCR product hybridized specifically with DNA fragments from genomic DNA of *S. venezuelae* ISP5230. Primers MPF1 and MPR2 also allowed amplification by PCR of approximately 290-bp DNA fragments from several other streptomycetes. The fragments from *Streptomyces aureofaciens* NRRL2209 and *Streptomyces coelicolor* A3(2) showed sequence identity with halogenase genes from these species. Thus, the PCR primers are of potential value for amplification and subsequent isolation of actinomycete halogenase genes.
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Keywords: PCR; FAD-binding site; gene cloning; halometabolite

Introduction

Enzyme-catalyzed transfers of halogen are common biological reactions, and generate a large variety of halogenated secondary metabolites in microorganisms and marine plants. Halogenation is less frequent in animals, but synthesis of the thyroid hormones is a noteworthy example of halogen transfer in this kingdom. Because halogenated metabolites are often biologically active [5], the halogenating enzymes involved in their synthesis have been studied for more than three decades [25]. In organisms producing halogenated secondary metabolites four different types of enzymes have been implicated. The most widely known are the haloperoxidases and perhydrolases, two types of enzymes now known to lack the substrate specificity associated with biosynthesis of many naturally occurring halometabolites [26]. The third type of enzyme yielding a halogenated product is the *S*-adenosylmethionine methyltransferase detected in cell-free extracts of the fungus *Phellinus pomaceus* [30]. Although significant in being responsible for the large amount of biologically generated CH_3Cl in the environment, this type of enzyme cannot account for the diversity of known halometabolites. The fourth type of halogenating enzyme is the group of $FADH_2$-dependent halogenases [12,26]. These enzymes exhibit substrate and regioselectivity [27], and after partial purification require FAD as well as NADH [17]. They could potentially be used in the production of fine chemicals to replace current chemical halogenation processes producing unacceptable amounts of by-products, but relatively few halogenases have been isolated. Information about their genes could facilitate expression and isolation of new enzymes.

Until now halogenase genes have been obtained by adapting methodology developed for cloning the genes of secondary metabolite biosynthesis. For example, the halogenase genes (*prnA* and *prnC*) responsible for chlorination reactions during pyrrolnitrin biosynthesis in *Pseudomonas fluorescens* [18], and those (*pltA*, *pltM*, and *pltD*) associated with pyoluteorin biosynthesis in *P. fluorescens* Pf-5 [20], were initially detected by Tn5 mutagenesis of the antibiotic-producing strains. By selecting for mutants blocked in antibiotic production, Hammer *et al* [9] identified a gene cluster containing the two halogenase genes needed for pyrrolnitrin biosynthesis. The genomic region containing the halogenase genes of pyoluteorin biosynthesis was isolated similarly; each gene was insertionally inactivated to verify its function [9,18,20]. Shotgun cloning was used to obtain the gene responsible for halogenation in chlortetracycline biosynthesis; chlortetracycline-sensitive *Streptomyces lividans* TK23 was transformed with a genomic library of *Streptomyces aureofaciens* DNA fragments. Because self-defense genes are usually clustered with genes for antibiotic biosynthesis, chlortetracycline-resistant colonies were isolated, and their plasmid DNA was used to probe a cosmid library. The halogenase gene (*chl*) was found with the resistance genes; its function was confirmed by complementing an *S. aureofaciens* mutant blocked in the chlorination step [1].

New insights into halometabolite biosynthesis have developed from the recent conclusion [12] that most halogenated secondary metabolites arise from the action of halogenases and not, as was earlier believed, from attack by an electron-depleted halogen cation generated through haloperoxidase activity [25].

Abbreviations: Cm, chloramphenicol; dNTP, deoxynucleoside triphosphate; PCR, polymerase chain reaction; PTS, potential target sites

Correspondence: Dr Leo C Vining, Biology Department, Dalhousie University, Halifax, NS, Canada B3H 4J1

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In halogenase-catalyzed reactions, a halide anion is introduced into a cationic site formed in the substrate by a specific oxidation requiring both NADH and FADH2 as cofactors [12,17]. Although the enzymes act on disparate substrates and are substrate-specific, they nevertheless share a distinctive reaction mechanism that should provide a target for cloning a variety of halogenase genes from the range of known halometabolite-producing organisms. The polymerase chain reaction (PCR) [19] is a powerful tool for isolating structurally similar genes from distantly related organisms, but so far there is no example of its use to amplify halogenase genes. Absence of homology between some cloned halogenase genes has been reported [18].

Chloramphenicol (Cm) is an *N*-dichloroacetylated phenylpropanoid metabolite that originates in the shikimate pathway of some actinomycetes. Several biosynthetic intermediates have been characterized [11], but information about the chlorination reaction remains incomplete. Here we describe degenerate primers capable of amplifying a 290-bp halogenase gene fragment from the Cm producer *Streptomyces venezuelae* ISP5230, and use of the PCR product as a labeled probe to detect the corresponding genomic DNA sequence. The primers were tested for their ability to amplify fragments of appropriate size from the genomic DNA of other *Streptomyces* species.

Materials and methods

Deduced amino acid sequences of halogenases PrnC [9], PltA, PltM, and PltD [20], and Chl [1] retrieved from the GenBank database were aligned, and PCR primers were designed from two conserved regions (Table 1). To increase the opportunity for successful matches between primers and target sites the primer sequences took account of codon usage in *Streptomyces* species [29]. The complexity of the forward primer pool was reduced by using deoxyinosines [14] at two positions where there was high amino acid variability and the codon usage bias did not indicate a clear base preference. Oligonucleotides were synthesized by MWG-Biotech (High Point, NC).

Preliminary tests of the effect of different formamide concentrations (0.5%, 1%, 2%, and 4% v/v) [24] on PCR amplification of *S. venezuelae* ISP5230 DNA showed that 1% was optimum. Final concentrations of ingredients in 25-μl reaction mixtures were 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 1.25 mM $MgCl_2$, 1% v/v formamide, 0.08% Nonidet P40 (MBI Fermentas, Burlington, ON, Canada) and 0.2 mM for each deoxynucleoside triphosphate

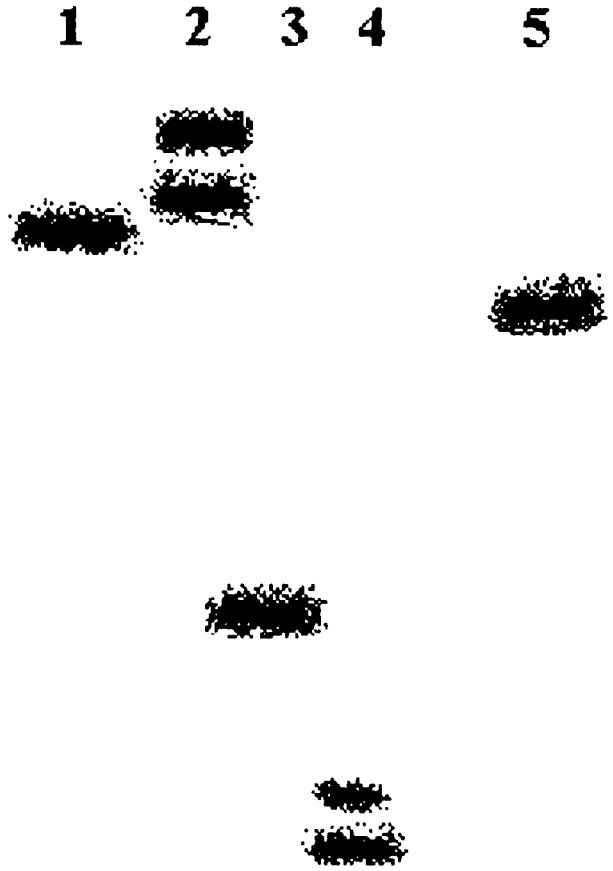

Figure 1 Hybridization of a ^{32}P-labeled PCR product of *S. venezuelae* ISP5230 with restriction endonuclease-digested genomic DNA from the organism. Estimated sizes of hybridization targets in the digests were: lane 1, *Bam*HI (13 kb); lane 2, *Kpn*I (>20 kb, 15 kb); lane 3, *Sal*I (1.5 kb); lane 4, *Pvu*I (0.6, 0.8 kb); lane 5, *Pst*I (8 kb). The >20-kb band in lane 2 corresponded to undigested genomic DNA.

Table 1 Halogenase amino acid sequences used to design primers MPF1 and MPR2, and specifications of the primers used to amplify halogenase gene fragments

Halogenase	For forward primer	For reverse primer
Amino acid sequences of consensus regions used		
PfPrnC	(246) EGGWLWVIP	(338) IDPLFSRGL
Chl	(247) ERGWMWIIP	(341) IDPLFLRGL
PltM	(234) EEGWLWVIP	(325) IDPLFSRGL
PltD	(241) DGGWVGVIP	(331) NDLLFSRKL
PltA	(234) TNTWVWQIP	(320) VDPIFSSGV
Derived primer sequences and their characteristics		
MPF1: 5′-S IRS RSS TGG ITS KGS VWS ATC CCS-3′	MPR2: 5′-S ASV YYS SRS VRG AAS AKS RGG TCS-3′	
25 mer; GC content, 64.6%;	25 mer; GC content, 65.3%	
Tm, 69.8°C; degeneracy 12,288-fold;	Tm, 70.1°C; degeneracy 147,456-fold;	
PTS*, 196,608	PTS, 147,456	

PfPrnC, halogenase PrnC from pyrrolnitrin producer *P. fluorescens* [18]; Chl, halogenase from chlortetracycline producer *S. aureofaciens* [1]; PltM, PltD, and PltA, halogenases from pyoluteorin producer *P. fluorescens* Pf-5 [20]. The numbers in parentheses show the position of the first amino acid of each consensus region in the overall sequence.
*PTS, potential target sites; K=G+T, R=G+A, S=G+C, V=G+C+A, W=A+T, Y=C+T, I=inosine.

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Aohal     GCKRLPAPVSG------NILSVAFDSGWFWYIPLSDTLTSVGAVVRREDAEKIQGDREK-
AmHal     GGKRLPEPVSG------NILSVAFDSGWFWYIPLSDTLTSVGAVVRREDAEKIQGDREK-
SlHal     NGKRMPEPNRF------NILCVAFESGWFWYIPLSDTLTSVGAVVRREMAEKIQGDPEK-
MfPrnC    DIFKPKGQRWR---WHEGTLHEMFHGGWLWVIPFNNHSRATNGLVSVGLQLDPRIHPKT-
Chl       DEVDMPAELRPPVPWNDGTMHHIFERGWMWIIPFNNHPGATNPLCSVGIQLDERRYPARP
PCR       -------------------------KGWVWVIPIKDDLYSVGLVVDRSKSAEVREQGADA
PltA      NFDRKSATQSPDKVDYIFIHFLPMTNTWVWQIPITETITSVGVVTQKQNYTNS-------
ZoHal     GVTRRPGEDAG------NISIYRHAEGWMWLIPLPEDIMSVGAVCYPEYMKTRKGD----
                                   *.* **: :   :.. :

Aohal     ALNALIAECPLISEYLANATRVITGKYGELRVRKD-YSYQQETYWRPGMILIGDAACFVD
AmHal     ALNTLIAECPLISEYLADATRVTTGRYGELRVRKD-YSYQQETYWRPGMILVGDAACFVD
SlHal     ALRALIDECPMIADYLSDATRVTEGQYGEIRVRKD-YSYHHTTFTRPGMMLVGDAACFVD
MfPrnC    EIPAQQEFDEFLARFPTIAAQFKDARPVRDWVKSDRLQYSSKSTVGDRYCLMLHAAGFID
Chl       DLIPEEEFRSHVDRFPAVQRQLKGARSVREWVRTDRMQYSSSRTVGERWCLMSHAAGFID
PCR       FYSSTLAKCAKAMDILGGAEQVDEVRIVQD------WSYDTEVFSADRFPLCGDAACFID
PltA      DLTYEEFFWEAVKTRENLEDALKASEQVRPFKKEADYSYGMKEVCGDSFVLIGDAARFVD
ZoHal     ---SEAFLMRTLALNPELNARMLDAERVAPVHATGNYAYECTRMAGPRWLMLGDAYTFVD
                          .    .               *             :  .*  * *

Aohal     PVFSSGVHLATYSALLAARSINSVLAGDLDEKTALNEFEMRYRREYGVFYEFLVSFYQMN
AmHal     PVFSSGVHLATYSALLAARSINSVLAGDLDEKTALNEFELRYRREYGVFYEFLVSFYQMN
SlHal     PVFSSGVHLATYSSLLAARSLNSVLEGKVDEDAAMKEFEARYRREYGVFYEFLVSFYEMH
MfPrnC    PLFSRGLENISVTIHALAARLIKALRDDDFSPERFEYIDRLQQKLLEHNDDFVSCCYTAF
Chl       PLFLRGLSNTCEIINALSWRLMAALREDDFAVERFAYVEELEQGLLDWNDKLVNNSFISF
PCR       PIFRRG------------------------------------------------------
PltA      PIFSSGVSVALNSARIASGDIIEAVKNNDFSKSSFTHYEGMIRNGIKNWYEFITLYYRLN
ZoHal     PMFSSGVFLAMEGAERGAAMVDAALRAPQSEAKLQRALQRELTRGVDEFIFSLSHRTSLV
          *:*   *
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Figure 2 Partial sequences of halogenases aligned with the amino acid sequence encoded by the PCR product amplified from *S. venezuelae* ISP5230 DNA. The sequences compared were: Aohal, halogenase homologue from *Amycolatopsis orientalis* [28]; AmHal, halogenase from *A. mediterranei* [21]; SlHal, putative halogenase from *S. lavendulae* (GenBank accession no. AAK81830); MfPrnC, halogenase PrnC from *M. fulvus* [8]; PCR, the deduced amino acid sequence of *S. venezuelae* ISP5230 PCR product; ZoHal, putative halogenase from *Xanthomonas oryzae* pv. *oryzae* (GenBank accession no. AAG38844). * indicates amino acids identical or conserved in all sequences, : indicates conserved substitutions, and . indicates semiconserved substitutions. Other abbreviations are as shown in Table 1.

(dNTP). Each reaction contained 2.5 ng of genomic DNA, 12.5 pmol of each primer and 1.5 U *Taq* DNA polymerase (MBI Fermentas). The thermal cycler (PTC-150 MiniCycler, MJ Research, Watertown, MA) was programmed for touchdown PCR [4], in which denaturation at 96°C for 2 min before addition of *Taq* polymerase was followed by 36 cycles of three 1-min steps: denaturation at 96°C, annealing at 65–55°C and extension at 72°C; the initial annealing temperature of 65°C was reduced by 2°C after every six cycles to reach 55°C for the final six cycles. The PCR ended with an extra extension of 8 min at 72°C to facilitate cloning in the pCR2.1TOPO vector (Invitrogen, Carlsbad, CA) by completing the *Taq* polymerase-catalyzed addition of single dATPs to the 3′-ends of products.

When genomic DNA of *S. venezuelae* ISP5230 was used as the template, electrophoresis of 20-μl samples in 1.2% agarose gels containing ethidium bromide (1 μg ml^{-1}) detected a unique 290-bp PCR product. This was excised, purified (UltraClean 15 DNA extraction kit, Mo Bio Labs, Solana Beach, CA), and cloned using the TOPO TA cloning kit (Invitrogen), giving pJV501. The cloned DNA was sequenced from the M13 reverse primer by the dideoxynucleotide chain-termination method [23] with an ABI Prism model 373 DNA sequencer. Nucleotide and amino acid sequence homology searches used the BLAST network service at the National Center for Biotechnology Information. Multiple sequences were aligned with Pileup [6] or a web-based Clustal W program (http://www2.ebi.ac.uk/clustalw/). The web-based FramePlot 2.3 program [15] (http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl) was used to analyze percent G+C content in the PCR product, and to identify the incomplete ORF. DNA was manipulated using standard protocols [22].

In Southern analyses to detect the halogenase gene of *S. venezuelae* ISP5230, restriction digests of genomic DNA subjected to electrophoresis in agarose gels were transferred to nylon membranes (NEN Life Science Products, Boston, MA), and probed with the PCR product labeled by the random primer method with [α-^{32}P]dCTP (Amersham Pharmacia Biotech, Baie d'Urfé, QC, Canada). After hybridization at 67°C in a solution containing 6× SSC (SSC is 8.76 g NaCl and 4.41 g sodium citrate per liter, adjusted to pH 7.0), 5× Denhardt's solution [2], 0.5% (w/v) sodium dodecylsulfate (SDS), and denatured salmon sperm DNA (100 μg ml^{-1}), the membranes were washed twice at room temperature with 2× SSC and then with 1× SSC containing 0.1% (w/v) SDS; they were finally washed at 67°C for 1 h with 0.1× SSC containing 0.1% (w/v) SDS. Hybridization signals were detected with a Bio-Rad CS phosphor-imaging screen (Hercules, CA) and scanned in a Bio-Rad GS525 Molecular Imager.

In tests with primers MPF1 and MPR2 for amplification of DNA from other streptomycetes (*Streptomyces armentosus* UC2862, *S. aureofaciens* NRRL2209, *S. coelicolor* A3(2), *S. griseoviridis*, *S. glaucescens*, *S. akiyoshiensis*, and *S. parvulus*), genomic DNA was isolated by the procedure of Hopwood *et al* [13] for use as PCR templates. The PCR product amplified as described for *S. venezuelae* from the DNA of *S. aureofaciens* NRRL2209 was sequenced directly from primers MPF1 and MPR2. The product from *S. coelicolor* A3(2) was cloned in the vector pCR 2.1-TOPO using protocols provided in the TOPO TA cloning kit (Invitrogen)

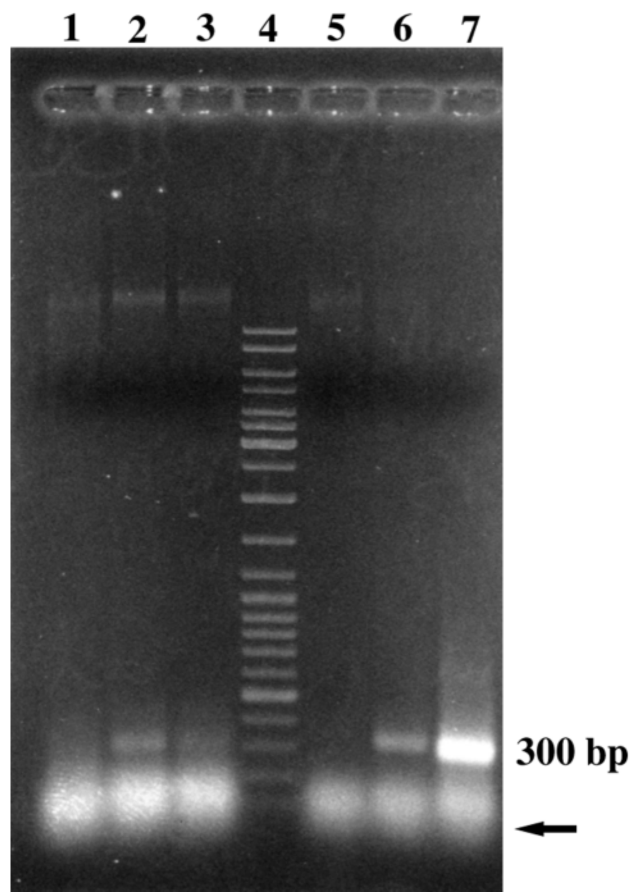

Figure 3 Agarose gel electrophoresis of PCR products from genomic DNA of six streptomycete strains amplified with primers MPF1 and MPR2. Left to right: *Streptomyces akiyoshiensis* (−), *S. griseoviridis* (+), *S. glaucescens* (+), DNA Ladder Mix (MBI Fermentas), *S. parvulus* (−), *S. armentosus* UC2862 (+), and *S. venezuelae* ISP5230 (+). No amplification is indicated as (−); amplification of a ~300-bp product is indicated by (+). The arrow points to excess primers.

to give pJV540. The cloned fragment was sequenced from vector primers.

Results

Primers for PCR were designed from consensus sequences recognized in two conserved regions after aligning the deduced amino acid sequences of five halogenase genes reported in GenBank (Table 1). The primers amplified a unique 290-bp DNA fragment from *S. venezuelae* ISP5230 genomic DNA. Frame analysis of the nucleotide sequence of this fragment detected a partial ORF with biased codon usage characteristic of streptomycete genes (93.7 mol% G+C in the third codon position) [29]. Genomic DNA of *S. venezuelae* ISP5230 digested with restriction enzymes and probed with the [α-^{32}P]-labeled PCR product gave hybridization signals confirming that the PCR product originated from *S. venezuelae* ISP5230 (Figure 1). A BLASTP search of the amino acid sequence deduced from 288 nucleotides of the partial ORF showed 34% identity to a halogenase involved in biosynthesis of a vancomycin-group antibiotic [28], 33% to PltA from *P. fluorescens* Pf-5 [20], 30% to PrnC from *Myxococcus fulvus* [8], and 25% identity to Chl from *S. aureofaciens* and the three other halogenases reported in GenBank (Figure 2). The PCR primers used with *S. venezuelae* ISP5230 DNA amplified fragments from genomic DNA of all but two (*S. parvulus* and *S. akiyoshiensis*) of the seven other streptomycetes tested (see Materials and Methods). The size of each PCR product (Figure 3) corresponded to the approximately 290-bp separation of the two consensus regions in the halogenases (Table 1). The sequence of the PCR product amplified from genomic DNA of *S. aureofaciens* NRRL2209 was completely identical to a region of the halogenase gene (*chl*) involved in chlortetracycline biosynthesis, and previously described from that organism [1]. The PCR product amplified from *S. coelicolor* A3(2) and cloned in pJV540 showed sequence identity to the putative halogenase gene reported (GenBank accession no. AL390188) in the *S. coelicolor* A3(2) genome.

Discussion

Amplification of putative halogenase genes with primers MPF1 and MPR2 was successful not only with genomic DNA from *S. venezuelae* ISP5230 but also with DNA from the halometabolite producers *S. armentosus* UC2862, *S. griseoviridis*, and *S. aureofaciens* NRRL2209, which produce, respectively, the chlorinated secondary metabolites armentomycin [16], roseophilin [10], and chlortetracycline [1]. Two other streptomycetes, *S. glaucescens* and *S. coelicolor* A3(2), not reported to produce halometabolites [7] also gave products when amplified by PCR with our primers. The product from *S. coelicolor* A3(2) was not unexpected since a halogenase gene homologue was recognized on cosmid 2G18 (GenBank accession no. AL390188) during genome sequencing of *S. coelicolor* A3(2) (http://www/sanger.uk/projects/s/coelicolor). The PCR products obtained from *S. aureofaciens* NRRL2209 and *S. coelicolor* A3(2) were confirmed by analysis of their sequences to have originated by amplification of genomic DNA as predicted. No halogenated metabolites have been recorded from *S. parvulus* and *S. akiyoshiensis* [7], and consistent with expectations no product was amplified from their DNA.

The ability of the primers to amplify a halogenase fragment from *S. venezuelae* ISP5230 is noteworthy because the primer sequences were based on a consensus from five halogenases that act on different substrates. Moreover, these halogenases catalyze chlorination of an aromatic carbon, whereas the carbon chlorinated in Cm resides in a nonaromatic acyl group. Of interest also, the halogenase gene fragment was the only product amplified from *S. venezuelae* ISP5230, and PCR products amplified from the genomes of other streptomycetes were similar to it in size. Hybridization of the amplified fragment at high stringency to *S. venezuelae* ISP5230 genomic digests confirmed the source of the PCR product. Failure of genomic DNA from two of the streptomycetes used in this study to give rise to PCR products, and the absence of nonspecific priming at related sites (such as those of monooxygenase genes), imply that amplification of products with primers MPF1 and MPR2 is a useful indicator of halogenase genes in a streptomycete genome. However, extensive optimization of PCR reactions may be needed to reach a final conclusion on primer specificity.

Specific amplification of a halogenase gene fragment from primers based on consensus sequences in enzymes acting on different types of substrates implies that the primer sequences are associated with shared catalytic activity. Consistent with this, a sequence similar to the FAD-binding sites in such FAD-dependent monooxygenases as the *p*-hydroxybenzoate hydroxylases (PobA) from *Acinetobacter calcoaceticus* and *P. fluorescens* [3] can be recognized in the deduced amino acid sequence of the region of the PCR product amplified from the reverse primer (Figure 4). The GDA and P residues in the GxxxxxGDAxHxxxPxxxxGxxxxxxD consensus for the FAD-binding site, which are conserved in the sequence deduced from the *S. venezuelae* ISP5230 PCR product, constitute the motif for a putative $\beta-\alpha-\beta$ fold [3]. This FAD-binding site appears to be conserved among other halogenases (Figure 4). In the reaction mechanism proposed for $FADH_2$-dependent halogenases [12], a flavin cofactor reduced by NAD(P)H and flavin reductase reacts with oxygen to form a flavin hydroperoxide. The flavin hydroperoxide is used to oxidize the organic substrate to an epoxide. Nucleophilic attack on the epoxide by a halide anion and specific removal of water yield the halogenated product. The presence of an FAD-binding site in a halogenase may well be an important factor in halogenation by these enzymes

This is the first report that PCR techniques can be used to amplify halogenase gene fragments. The results suggest that the

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AmHal   (228)   PGMILVGDAACFVDPVFSSG
PltA    (326)   DSFVLIGDAARFVDPIFSSG
Chl     (314)   ERWCLMSHAAGFIDPLFLRG
MfPrnC  (297)   DRYCLMLHAAGFIDPLFSRG
PltM    (309)   DRFCLLPQATGFIDPLFSRG
AcPobA  (252)   GKLFLAGDAAHIVPPTGAKG
PfPobA  (281)   GRLFLAGDAAHIVPPTGAKG
PCR             DRFFLCGDAACFTDPIFRRG
                     *  .*: :: *    *

FAD binding consensus: GxxxxxGDAxHxxxPxxxxGxxxxxxD [3]
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Figure 4 Alignment of putative FAD-binding sites in the *S. venezuelae* ISP5230 PCR product amplified from reverse primer MPR2, with the flavin-binding sites identified in other halogenases and in PobA (hydroxylase) proteins. Bold letters are consensus amino acids. AcPobA and PfPobA, hydroxylases from *A. calcoaceticus* and from *P. fluorescens* [3]. The number in parentheses indicates the position of the first amino acid in the sequence. The PCR sequence is not a full-length halogenase and no number is shown. Other abbreviations are as shown in Table 1.

primers may be useful for amplifying such fragments from additional *Streptomyces* species and from other bacteria. In the latter context, redesigning the primers to take account of the specific codon usage of the organism would likely improve primer selectivity and efficiency. The PCR method is more direct and should be faster than the Tn5 mutagenesis or shotgun cloning procedures used so far to clone halogenase genes.

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