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Cloning, Analysis, and Overexpression of the Gene Encoding Isobutylamine *N*-Hydroxylase from the Valanimycin Producer, *Streptomyces viridifaciens*

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The flavoprotein isobutylamine *N*-hydroxylase (IBAH) catalyzes the oxidation of isobutylamine to isobutylhydroxylamine, a key step in the biosynthesis of the azoxy antibiotic valanimycin. By using oligonucleotide primers designed from peptide sequence information derived from native IBAH, a fragment of the gene (*vlmH*) encoding IBAH was amplified by PCR from a genomic library of the valanimycin-producing organism, *Streptomyces viridifaciens* MG456-hF10. The gene fragment was then employed as a probe to clone the entire *vlmH* gene from an *S. viridifaciens* genomic library. Overexpression of the *vlmH* gene in *Escherichia coli* gave a soluble protein that was purified to homogeneity. The purified protein exhibited the catalytic activity expected for IBAH. The deduced amino acid sequence of IBAH exhibited the greatest similarity to the Sox/DszC protein from *Rhodococcus* sp. strain IGT38, a flavoprotein involved in the oxidation of dibenzothiophene to the corresponding sulfone. Significant similarities were also found between the amino acid sequence of IBAH and those of the acyl coenzyme A dehydrogenases.

The antibiotic valanimycin (Fig. 1) is a naturally occurring azoxy compound isolated from the fermentation broth of *Streptomyces viridifaciens* MG456-hF10 by Yamato and coworkers (37). In addition to antibacterial activity, valanimycin exhibits potent antitumor activity against in vitro cell cultures of mouse leukemia L1210, P388/S (doxorubicin sensitive), and P388/ADR (doxorubicin resistant) (37). Preliminary evidence suggests that the biological activity of valanimycin may involve the damaging of DNA (37). As a naturally occurring azoxy compound, valanimycin is a member of a growing class of natural products which now includes the cycad toxins macrozamin and cycasin (11, 13, 16, 23), the carcinogen elaiomycin (25–27), the antifungal agents maniwamycins A and B (28), and the nematocidal compounds jietacins A and B (10). A characteristic structural feature of these naturally occurring azoxy compounds is the presence of an N-N bond. A wide variety of natural products have been reported to contain N-N linkages (14), but little is known at present about the biochemistry of N-N bond formation.

Investigations of the biosynthesis of valanimycin have shown that it is derived from L-serine and L-valine and that valine is incorporated via the intermediacy of isobutylamine and isobutylhydroxylamine (20) (Fig. 1). Evidence was also deduced for the intermediacy of a hydrazine derivative formed by the condensation of isobutylhydroxylamine with L-serine (18).

Parry and Li (19) have shown that two enzymes are involved in the conversion of isobutylamine to isobutylhydroxylamine. The first of these is an NADH:riboflavin 5′-phosphate oxidoreductase (flavin mononucleotide [FMN] reductase) that provides reduced FMN for the second enzyme, isobutylamine *N*-hydroxylase (IBAH). The hydroxylase utilizes reduced FMN and molecular oxygen to catalyze the hydroxylation of isobutylamine to isobutylhydroxylamine. Only the hydroxylase enzyme could be purified to homogeneity.

The study reported here describes the cloning, analysis, and overexpression of the structural gene (*vlmH*) coding for IBAH. As far as we are aware, this is the first report of the cloning of a gene that appears to be involved in the biosynthesis of an azoxy antibiotic.

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MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The bacterial strains, phages, and plasmids are listed in Table 1.

Media and bacteriological techniques. *S. viridifaciens* MG456-hF10 was maintained as frozen stock in 20% glycerol at −80°C and was grown as described by Yamato et al. (37). *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37°C. Selection was made with 100 μg of ampicillin or 30 μg of kanamycin per ml in LB agar or liquid media.

DNA methods. Genomic DNA was prepared from *S. viridifaciens* MG456-hF10 by the method of Sambrook et al. (24). Phage DNA was purified according to the method of Sambrook et al. (24). Plasmid DNA was purified with a QIAprep spin plasmid kit. DNA fragments were isolated from agarose gels with a QIAquick gel extraction kit. PCR products were separated on agarose gels and purified from the gel. Digestion with restriction endonucleases and ligation experiments were carried out by standard procedures (24) under conditions recommended by the manufacturers. Automated DNA sequencing was performed with an Applied Biosystems DNA sequencer at the Molecular Genetics Core Facility, University of Texas Houston Medical School by using universal and synthetic oligonucleotide primers. The sequence shown in Fig. 4 was determined by complete sequencing of both DNA strands, with multiple sequencing of some regions.

Transformations. Transformations were carried out with commercially available competent *E. coli* cells. The procedures followed the protocols recommended by the manufacturers.

Construction of an *S. viridifaciens* MG456-hF10 genomic library. Genomic DNA of *S. viridifaciens* MG456-hF10 was partially digested with *Sau*3AI and size fractionated by ultracentrifugation in a 15 to 40% (wt/vol) sucrose gradient. Fractions containing DNA fragments of 9 to 20 kb were used for library construction. The DNA fragments were ligated to *Bam*HI-digested Lambda DASH II arms (Stratagene). The recombinant phage DNA was packaged into phage heads in vitro with the Gigapack II Gold packaging kit (Stratagene). The packaged phages were then propagated in *E. coli* XL1-Blue MRA cells to a titer of 2×10^6 PFU/ml.

Oligonucleotide primers and probes. Native IBAH was subjected to N-terminal sequencing at the Biochemistry Core Facility at Baylor College of Medicine and to internal sequencing after tryptic digestion at the Harvard University Microchemical Facility. Table 2 displays the amino acid sequence obtained for the hydroxylase, the nucleic acid sequences of the degenerate PCR primers designed from the amino acid sequences, the degenerate nucleic acid sequence of probe WLA, and the actual nucleic acid sequence determined for IBAH. Synthetic oligonucleotides were purchased from Integrated DNA Technologies, Inc., Coralville, Iowa.

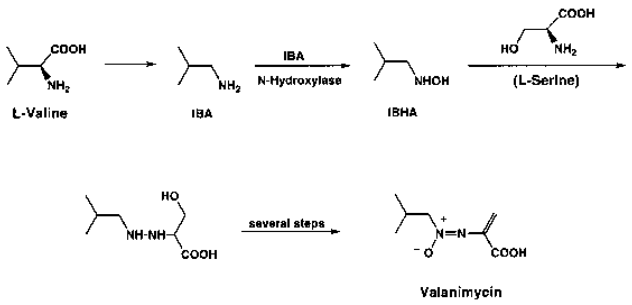

FIG. 1. Biosynthetic pathway for valanimycin. IBA, isobutylamine; IBHA, isobutylhydroxylamine.

PCR conditions. A 50-μl PCR reaction mixture contained 10 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, dNTP (each species, 200 μM), PCR primers (0.5 μM each) DNA template, and *Taq* DNA polymerase (1.25 U). PCR tubes (0.65 ml) containing template DNA and primers were incubated at 94°C in a PCR personal cycler (Biometra). A master mixture containing all the components except the template DNA and primers was preheated at 85 to 90°C for 2 min, and PCR was initiated by addition of the heated master mixture to the PCR tubes. Two temperature programs were used for the PCR experiments: touchdown (94°C for 3 min; 41 cycles of the following: 94°C for 1 min, 65 to 45°C [−0.5°C/cycle] for 2 min, and 72°C for 3 min; and 72°C for 7 min) and normal (94°C for 1 min; 30 cycles of the following: 94°C for 0.5 min, 65°C for 1 min, and 72°C for 2 min; and 72°C for 5 min).

Hybridizations. The degenerate probe WLA (Table 2) was radiolabeled with [γ-^{32}P]ATP by using the Promega DNA 5′-End Labeling System. The Lambda DASH II library of *S. viridifaciens* MG456-hF10 genomic DNA was plated on a lawn of *E. coli* XL1-Blue MRA cells grown on NZY agar plates (24) and was transferred to Hybond-N nylon membranes (Amersham) in accordance with the manufacturer's instructions. The membranes were probed at 35°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 5× Denhardt's solution and washed at 35°C in 6× SSC. Two likely positive clones were selected after secondary screening, and the phage DNA was prepared from these two phage clones by the method of Sambrook et al. (24). A sequence analysis of subclones indicated that neither of the phage clones contained the *vlmH* gene.

A nondegenerate 983-bp fragment of the *vlmH* gene was obtained by digestion of plasmid pGN62 with *Stu*I and *Xho*I. This fragment was radiolabeled with [α-^{32}P]dCTP and the New England Biolabs NEBlot kit. The genomic DNA library of *S. viridifaciens* MG456-hF10 in Lambda DASH II was plated on a lawn of *E. coli* XL1-Blue MRA cells grown on NZY agar plates and was transferred to Hybond-N nylon membranes. The membranes were probed at 65°C in 5× SSC and 5× Denhardt's solution and washed at 65°C in 5× SSC. Six positive clones were selected after secondary screening, and phage DNA was prepared from each clone.

Sequence analysis. DNA sequence assembly and restriction site analysis were performed with Sequencher, Macintosh version 3.0. A BLOCKS search was done through the e-mail BLOCKS Searcher at blocks@howard.fhcrc.org (9). Pattern analysis was performed with MacPattern version 3.4 (7). The computations for BLAST were performed at the National Center for Biotechnology Information by using the BLAST network service. All other sequence analyses were performed with the University of Wisconsin Genetics Computer Group package, version 8.1-UNIX, including MAP, BESTFIT, ISOELECTRIC, FASTA, TFASTA, BLAST, MOTIFS, COMPARE, PILEUP, and PRETTY. Alignment of amino acid sequences was achieved as follows. First, an initial multiple-sequence alignment was created with PILEUP. The alignment was then manually adjusted according to information obtained from BLOCKS and BLAST searches. The aligned sequences thus obtained were displayed using SeqVu, Macintosh version 1.01.

Construction of expression plasmid. In order to facilitate the cloning of the *vlmH* gene into the expression vector pET-24a(+), two PCR primers (IBANF and IBACR) were designed to allow the introduction of an *Nde*I site on the upstream side of the gene and an *Xho*I site on the downstream side (Fig. 2). The IBACR primer was also designed to convert the stop codon of *vlmH* from TGA to TAG in order to allow optional expression of the C-terminal His tag by use of a *supE E. coli* strain for expression. A 1,149-bp fragment containing the *vlmH* gene was amplified by PCR from pB634E3 with primers IBANF and IBACR and was then cloned into the PCR vector pGEM-T to give pGDIBAH. pGDIBAH was digested with *Nde*I and *Xho*I, and the resulting fragment was isolated from an agarose gel and cloned into *Nde*I-*Xho*I-digested pET-24a(+). The resulting recombinant plasmid, pEIBAH, was amplified in *E. coli* DH5α cells, and the insert was sequenced in order to ensure that the correct construction had been obtained. Finally, pEIBAH was introduced into *E. coli* BL21(DE3) cells for expression of IBAH.

Heterologous expression of *vlmH* in *E. coli*. LB broth (50 ml, 30 μg of kanamycin per ml) was inoculated with 2 ml of frozen stock of *E. coli* BL21(DE3) cells containing the recombinant expression vector pEIBAH and was incubated at 37°C at 300 rpm for about 2 h to reach an optical density at 600 nm of 0.8. IPTG (isopropyl-β-D-thiogalactopyranoside; 0.5 ml of a 100 mM solution) was added, and the culture was incubated at 37°C at 300 rpm for 3 h. The cells were then collected by centrifugation and washed once with buffer B (50 mM sodium phosphate buffer, pH 7.0, containing 15% [vol/vol] glycerol, 1 mM EDTA · Na_2, and 1 mM benzamidine · HCl). The washed cells were used immediately for protein purification.

Protein purification. Cells from 35 ml of culture were suspended in 4 ml of buffer B plus 1 mM phenylmethylsulfonyl fluoride and disrupted with 0.1-mm-diameter glass beads in a type BX-4 Mini-Bead-Beater cell disrupter (Biospec Products) for six 30-s cycles at 5,000 rpm. The mixture was centrifuged at 13,000 rpm for 10 min in an Eppendorf centrifuge, and the supernatant was collected. Solid ammonium sulfate was added to the supernatant, with constant stirring, to 70% saturation (43.6 g/100 ml), while the pH of the mixture was maintained at about 7 with 1 N NaOH. The mixture was stirred for 10 min and then centrifuged at 14,000 × *g* for 15 min. The resulting pellet was redissolved in 0.5 ml of buffer B plus 1 mM phenylmethylsulfonyl fluoride to give the ammonium sulfate fraction. The ammonium sulfate fraction was mixed with 0.4 volume of saturated cold ammonium sulfate solution (about 4 M) in 50 mM sodium phosphate (pH 7.0) to give a final ammonium sulfate concentration of about 1 M. The mixture was centrifuged at 14,000 × *g* for 10 min to remove any precipitate. The supernatant was then loaded onto a phenyl agarose column (10-ml bed volume, 25-mm inside diameter) equilibrated with 0.25 M ammonium sulfate in buffer B (pH 7.0)

TABLE 1. Bacterial strains, phages, and plasmids used

Strain, phage, or plasmid	Relevant properties	Source or reference
Strain		
E. coli XL1-Blue MRA	Δ(*mcrA*) 183 Δ (*mcrCB-hsdSMR-mrr*) 173 *endA1 supE44 thi-1 gyrA96 relA1 lac*	Stratagene
E. coli DH5α	F^-ϕ80d*lacZ* ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* (r_K^- m_K^+) *phoA supE44*λ$^-$ *thi-1 gyrA96 relA1*	Gibco BRL
E. coli BL21(DE3)	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Novagen
S. viridifaciens MG456-hF10	Valanimycin producer	37
Phage		
Lambda DASH II	Multicloning site vector	Stratagene
Plasmids		
pGEM-T	Multicloning site vector; Amp^r	Promega
pBlueScript II SK(−)	Multicloning site vector; Amp^r	Stratagene
pET-24a(+)	Multicloning site vector; Kan^r, T7*lac* promoter	Novagen
pGN62	0.98-kb PCR product PN2/62R in pGEM-T; Amp^r	This study
pB63E3	7-kb *Eco*RI-*Eco*RI insert from phage 6.3.4 in pBluescript II SK(−); Amp^r	This study
pGDIBH	1.1-kb PCR product from pB63E3 in pGEM-T; Amp^r	This study
pEIBAH	1.1-kb *Nde*I-*Xho*I insert from pGDIBAH in pET-24a(+); Kan^r, T7*lac* promoter	This study

TABLE 2. Sequences of peptide fragments of native IBAH and derived oligonucleotides

N terminus or internal peptide fragment[a]	Amino acid or nucleic acid sequence[b]
NT	
Amino acid	M R S L D A A R D T C E R L H P G L I K A L E E L P L L E R E A E G S P V L D I F R A H G
DNA	ATGCGTTCTCTCGATGCGGCCCGTGATACGTGCGAGCGCCTCCACCCCGGGCTGATCAAGGCGCTGGAGGAACTTCCCCTCCTGGAGCGGGAGGCGGAAGGCAGCCCAGTGCTCGACATCTTCCGGGCACATGGT
Probe WLA	5′-GACGCSGCSMGSGACACSTGCGAGMGSYTSCACCC-3′
Primer N1	5′-GACGCSGCSCGSGACACSTG-3′
Primer N2	5′-GARGGSWSSCCSGTSCTSGACATCTTC-3′
PT49	
Amino acid	A H G G A G L L V P S A Y G G H G A D A L D A V R
DNA	GCACATGGTGGGGCAGGCCTGCTCGTTCCCTCCGCGTACGGGGGGCACGGAGCCGACGCCCTGGATGCCGTGCGG
Primer 49R	3′-CGSATGCCSCCSGTRCCSC-5′
PT60	
Amino acid	A A L G I Q L E S A V G L T E G V A
DNA	GCTGCCCTCGGCATCCAGTTGGAATCCGCAGTCGGGCTGACAGAGGGCGTGGCC
Primer 60R1	3′-CGSCGSGASCCSTAGGTCGAC-5′
Primer 60R2	3′-TAGGTCGASCTYWSSCGSCASCCG-5′
PT62	
Amino acid	T S S S P H L V E Y F S G G P L E I
DNA	ACGAGTTCCTCGCCCCACTTGGTCGAGTACTTCAGCGGCGGTCCTTTGGAGATC
Primer 62R	3′-CTYATGAAGWSSCCSCCSGGSGASCTC-5′
PT65	
Amino acid	S P E L A Y L S S A L H P L A F H P P G R
DNA	TCGCCGGAACTCGCCTACCTGTCGTCCGCGCTGCATCCGCTGGCCTTCCACCCTCCCGGCCGC
Primer 65R	3′-CGSAAGGTRGGSGGSCCSG-5′

[a] NT, N terminus.
[b] S = G/C; W = A/T; R = G/A; M = C/A; Y = C/T. The nucleic acid sequence shown is the actual sequence of the *vlmH* gene.

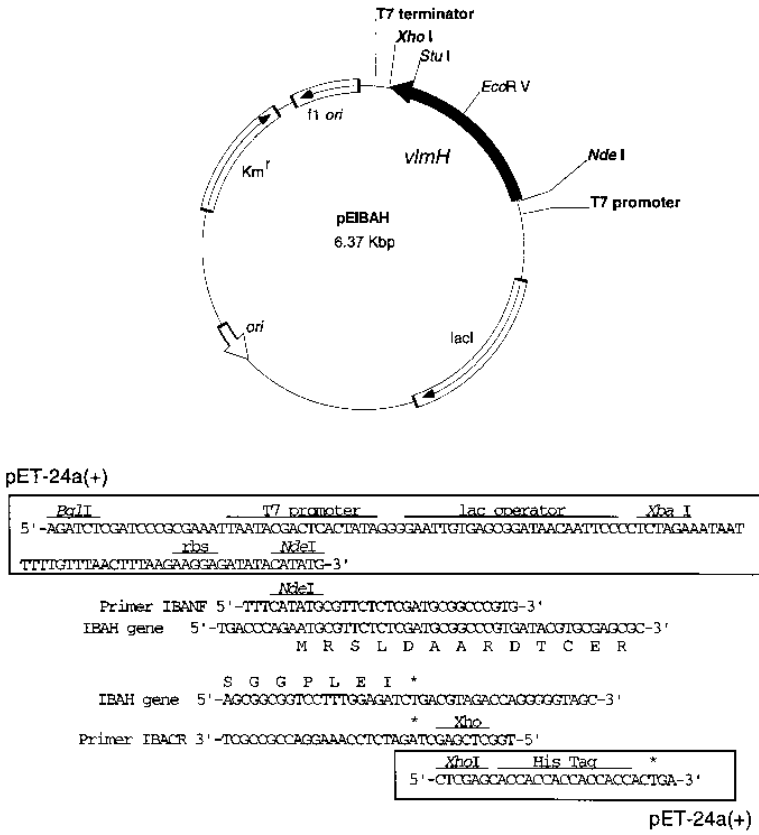

FIG. 2. Plasmid clone pEIBAH used to overexpress the *vlmH* gene in *E. coli*. The *vlmH* gene was flanked at the 5′ end with an *Nde*I site and at the 3′ end with an *Xho*I site and was cloned into *Nde*I-*Xho*I-cut pET-24a(+). rbs, ribosome binding site.

and was washed with 30 ml of 0.25 M ammonium sulfate in buffer B. The protein was then eluted with 30 ml of buffer B and concentrated with a YM 10 membrane to about 0.9 ml. Analysis of the protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) indicated that it had been purified to homogeneity. The details of the purification are summarized in Table 3.

Enzyme assay. IBAH activity was assayed in 100 mM bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane-amide–Tris–HCl buffer, pH 7.5, in the presence of 100 mM NaCl, 10 mM isobutylamine, 4 mM NADH, 10 μM FMN, 0.4 U of catalase per ml, 0.001 U of the NAD(P)H:FMN oxidoreductase of *Photobacterium fischeri* (Boehringer Mannheim) per μl, and a sample of the overexpressed hydroxylase. The incubation was for 30 min at 30°C. The formation of isobutylhydroxylamine was monitored by derivatization of the hydroxylamine with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (1) followed by high-pressure liquid chromatography analysis. The analysis was carried out on a Whatman Partisphere C_{18} reversed-phase column (4.6 by 235 mm; 5-μm particle size) which was eluted with a mixture of three parts methanol to seven parts 25 mM sodium phosphate buffer, pH 7.0, at a flow rate of 1 ml/min. Monitoring was carried out at 450 nm. Under these conditions, the isobutylhydroxylamine derivative exhibited a retention time of 12 min. The isobutylhydroxylamine concentration in the enzyme assay mixture was then calculated from the peak area by using a standard curve (19).

TABLE 3. Purification of recombinant IBAH[a]

Step	Total protein (mg)	Total units (nmol of IBHA/min)[b]	Sp act (nmol of IBHA/min/mg)	Purification factor
Crude extract	4.3	60.5	14.1	1.0
Ammonium sulfate	2.5	89.2	35.7	2.5
Phenyl agarose	0.77	39.4	51.1	3.6

[a] The purification used cells from 35 ml of an *E. coli* culture.
[b] IBHA, isobutyhydroxylamine.

Other methods. SDS-PAGE was performed according to the method of Laemmli (12). Protein concentrations were determined by the method of Bradford (5) with bovine serum albumin as the standard.

Nucleotide sequence accession number. The sequence presented has been assigned the accession number U76606.

RESULTS

Identification and cloning of the *vlmH* gene. Native IBAH was purified to homogeneity from cell extracts of *S. viridifaciens* MG456-hF10 (19), and the N-terminal sequence of the protein was obtained. Degenerate probe WLA was designed from the N-terminal sequence (Table 2). However, attempts to clone the *vlmH* gene from a Lambda DASH II library of *S. viridifaciens* MG456-hF10 genomic DNA by using this probe were unsuccessful. Therefore, the native protein was digested with trypsin and four peptide fragments (PT49, PT60, PT62, and PT65) were isolated from the tryptic digest and sequenced (Table 2). Seven degenerate PCR primers (N1, N2, 49R, 60R1, 60R2, 62R, 65R) were then designed from those peptide fragments (Table 2) taking into account the codon usage in *Streptomyces*, the strength of base pairing by the primers, and the possible formation of hairpins or dimers by the primers. Touchdown PCR was then carried out with genomic DNA of *S. viridifaciens* MG456-hF10 as the template. Of all the primer combinations, the primer pairs N2/49R, N2/60R2, N2/62R, and N2/65R each gave a single PCR product (PN2/49R, PN2/60R2, PN2/62R and PN2/65R) (Table 4). The results from nested PCR with PN2/60R2, PN2/62R, and PN2/65R as DNA templates suggested that these fragments were correct PCR prod-

TABLE 4. PCR reactions and their specific products

Template DNA[a]	Primer 1	Primer 2	PCR program	Product size (bp)	Product name	Clone name (in pGEM-T)
G-DNA	N2	49R	Touchdown	82	PN2/49R	
G-DNA	N2	60R2	Touchdown	768	PN2/60R2	pGN60
G-DNA	N2	62R	Touchdown	1,035	PN2/62R	pGN62
G-DNA	N2	65R	Touchdown	982	PN2/65R	pGN65
PN2/62R	N2	65R	Touchdown	982		
PN2/62R	N2	60R2	Touchdown	768		
PN2/65R	N2	60R2	Touchdown	768		
PN2/60R	N2	49R	Touchdown	82		
pB634E3	T7	R187	Touchdown	667		
pB634E3	T7	62R	Touchdown	1,515		
pB634E3	IBANF	IBACR	Normal	1,149	dibah	pGDIBAH

[a] G-DNA, genomic DNA.

ucts (Table 4). PN2/60R2, PN2/62R, and PN2/65R were then cloned into the PCR cloning vector pGEM-T. DNA sequencing of the clones revealed that the PCR products overlapped one another and that the amino acid sequences deduced from the nucleic acid sequence of PN2/62R matched the amino acid sequences determined for the N terminus and the four internal peptide fragments of IBAH. Plasmid pGN62 containing PCR product PN2/62R was digested with *Stu*I and *Xho*I, and the 983-bp fragment of the *vlmH* gene was recovered by gel electrophoresis. This fragment was then used to screen a library of *S. viridifaciens* genomic DNA in Lambda DASH II by plaque hybridization. Six positive clones were obtained from a total of 4×10^4 clones. Touchdown PCR experiments with the primer pair N2/62R using phage DNA from the six positive clones as a template gave an approximately 1.1-kb PCR product in each case, suggesting that all six phage clones contained the *vlmH* gene. One lambda clone, 6.3.4, was chosen for further analysis. Restriction digestion of DNA from clone 6.3.4 with *Eco*RI gave three insert-derived fragments with sizes of 11 kb (634E1), 9 kb (634E2), and 7 kb (634E3). Southern analysis using the 983-bp *Stu*I-*Xho*I fragment from pGN62 suggested that fragment 634E3 contained the *vlmH* gene. Fragment 634E3 was then cloned into pBlueScript II SK(−) to give pB634E3. Two specific primers, F748 and R187, were derived from the DNA sequence of PN2/62R. The sequence of F748 was 5′-CAGTTGGAATCCGCAGTCGGGC-3′, while the sequence of R187 was 5′-CGAAGAGCATCGCAGCAGTGAA G-3′. With pB634E3 as a template, touchdown PCR experiments using a T7 or T3 primer and an N2, F748, R187, or 62R primer were carried out to determine the relative position and orientation of the *vlmH* gene. Specific PCR products were obtained only when using the T7 and 62R primers (1,515 bp) and the T7 and R187 primers (667 bp). This suggested that (i) insert 634E3 contains the complete *vlmH* gene, (ii) the *vlmH* gene is near the T7 promoter of pB634E3, and (iii) the N terminus of the *vlmH* gene is near the T7 promoter in pB634E3 (Fig. 3). The nucleic acid sequence (Fig. 4) of the 634E3 insert in pB634E3 was then determined by primer walking. The *vlmH* gene was found to consist of 1,134 bp running from positions 310 to 1443 of the insert and to encode a polypeptide of 378 amino acids. The *vlmH* gene exhibited an overall G+C content of 66.6%, with an average G+C content at the third codon position of 82.1%. These values are somewhat lower than the average values for *Streptomyces* genes (36). A region centered about 13 nucleotides 5′ to the first codon of the *vlmH* gene has a significant degree of complementarity to the 3′ end of 16S rRNA of *S. lividans* (5′-GAUCACCUCCUUUCU-3′) and could serve as a ribosome binding site (3). The calculated

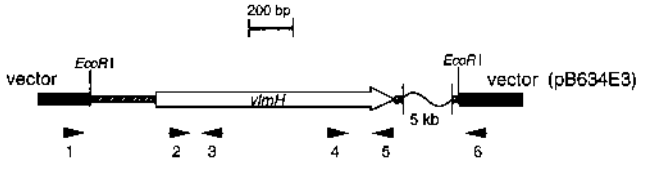

FIG. 3. PCR experiments using plasmid pB634E3 as a template. Numbered arrowheads indicate primers as follows: 1, T7; 2, N2; 3, R187; 4, F748; 5, 62R; 6, T3.

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-129 GCAACACTGACGGCACGTGCCGATAAGCGCGGCTAGTTCTGTGCCCGCTTCCGCACGGTC  -70
 -69 CCTTATCTGCTTCCTGTGCCTTATGCCTGGGAGCTGTCTTGTCCGACGGACGCTGGAGGT  -10
  -9 TGACCCAGAATGCGTTCTCTCGATGCGGCCCGTGATACGTGCGAGCCGCCTCCACCCCGGG  +50
 {1}          M  R  S  L  D  A  A  R  D  T  C  E  R  L  H  P  G    {17}

 +51 CTGATCAAGGCGCTGGAGGAACTTCCCCTCCTGGAGCGGGAGGCGGAAGGCAGCCCAGTG  +110
{18} L  I  K  A  L  E  E  L  P  L  L  E  R  E  A  E  G  S  P  V   {37}

+111 CTCGACATCTTCCCGGGCACATGGTGGGGCAGGCCTGCTCGTTCCCTCCGCGTACGGGGGG  +170
{38} L  D  I  F  R  A  H  G  G  A  G  L  L  V  P  S  A  Y  G  G   {57}

+171 CACGGAGCCGACGCCCTGGATGCCGTGCGGGTGACTCGGGCCCTGGGCGCTTGCTCCCCT  +230
{58} H  G  A  D  A  L  D  A  V  R  V  T  R  A  L  G  A  C  S  P   {77}

+231 TCGCTGGCGGCTGCTGCCACCATGCACAACTTCACTGCTGCGATGCTCTTCGCGCTCACG  +290
{78} S  L  A  A  A  A  T  M  H  N  F  T  A  A  M  L  F  A  L  T   {97}

+291 GACCGCGTGATCCCGCCCACCGACGAGCAGAAGAAACTGCTCGCTCGCGTGGCGCCAGAG  +350
{98} D  R  V  I  P  P  T  D  E  Q  K  K  L  L  A  R  V  A  P  E  {117}

+351 GGCATGCTCCTGGCCTCGGGGTGGGCCGAGGGCAGGACTCAGCAGGACATCCTGAATCCC  +410
{118} G  M  L  L  A  S  G  W  A  E  G  R  T  Q  Q  D  I  L  N  P  {137}

+411 TCGGTGAAGGCTACGCCCGTCGACGACGGCTTCATCCTCAACGGGTCGAAGAAGCCCTGC  +470
{138} S  V  K  A  T  P  V  D  D  G  F  I  L  N  G  S  K  K  P  C  {157}

+471 AGCCTGTCCGGTTCGATGGATATCCTCACCGCGAGCGTGATCCTCCCCGACGAGACCGGA  +530
{158} S  L  S  R  S  M  D  I  L  T  A  S  V  I  L  P  D  E  T  G  {177}

+531 CAGCAGTCGCTTGCTGTGCCTCTGATCATGGCTGACTCGCCGGGCATCTCCGTACACCCG  +590
{178} Q  Q  S  L  A  V  P  L  I  M  A  D  S  P  G  I  S  V  H  P  {197}

+591 TTCTGGGAAAGCCCTGTACTGGCCGGCTCGCAGAGCAATGAGGTCCGCCTGAAGGATGTC  +650
{198} F  W  E  S  P  V  L  A  G  S  Q  S  N  E  V  R  L  K  D  V  {217}

+651 CATGTCCCCGAGAAGCTGATCATCCGGGGCACGCCGGACGACCCCGGCCGACTCGACGAC  +710
{218} H  V  P  E  K  L  I  I  R  G  T  P  D  D  P  G  R  L  D  D  {237}

+711 CTGCAGACCGCGACCTTCGTATGGTTCGAGCTGCTCATCACCTCTGCATACGTAGGCGCC  +770
{238} L  Q  T  A  T  F  V  W  F  E  L  L  I  T  S  A  Y  V  G  A  {257}

+771 GCCAGCGCACTGACGGAGTTGGTGATGGAGCGGGACCGCGGCTCGGTGACCGACCGGGCT  +830
{258} A  S  A  L  T  E  L  V  M  E  R  D  R  G  S  V  T  D  R  A  {277}

+831 GCCCTCGGCATCCAGTTGGAATCCGCAGTCGGGCTGACAGAGGGCGTGGCCCGCGCAGTG  +890
{278} A  L  G  I  Q  L  E  S  A  V  G  L  T  E  G  V  A  R  A  V  {297}

+891 CGTGACGGTGTGTTCGGAGAGGAAGCCGTCGCTGCCGCCCTGACCGCCCGGTTCGCCGTG  +950
{298} R  D  G  V  F  G  E  E  A  V  A  A  A  L  T  A  R  F  A  V  {317}

+951 CAGAAGACCCTGGCCGCGATTTCCGACCAGGCCATCGAGTTGCTCGGCGGAATCGCGTTC +1010
{318} Q  K  T  L  A  A  I  S  D  Q  A  I  E  L  L  G  G  I  A  F  {337}

+1011 ATCAAGTCGCCGGAACTCGCCTACCTGTCGTCCGCGCTGCATCCGCTGGCCTTCCACCCT +1070
{338} I  K  S  P  E  L  A  Y  L  S  S  A  L  H  P  L  A  F  H  P  {357}

+1071 CCCGGCCGCACGAGTTCCTCGCCCCACTTGGTCGAGTACTTCAGCGGCGGTCCTTTGGAG +1130
{358} P  G  R  T  S  S  S  P  H  L  V  E  Y  F  S  G  G  P  L  E  {377}

+1131 ATCTGACGTAGACCAAGGGGTAGCTCAACCATGGATCAGCTCAACTCGTTCGAATCGCCC +1190
{378} I  *
```

FIG. 4. Nucleotide sequence of the *vlmH* gene. The first base of the first codon is numbered zero. The putative ribosomal binding site is underlined.

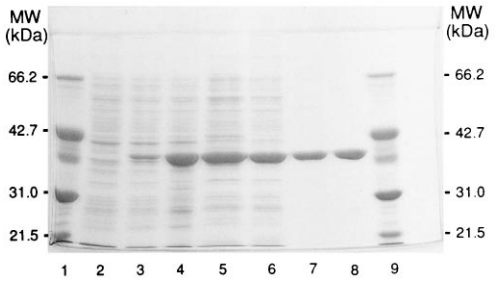

FIG. 5. SDS-PAGE analysis of IBAH overexpression and purification. Lanes 1 and 9, molecular weight markers bovine serum albumin (66,200), hen egg white ovalbumin (42,699), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500); lane 2, total protein from *E. coli* BL21(DE3)(pEIBAH) before IPTG induction; lane 3, total protein from *E. coli* BL21(DE3)(pEIBAH) 0.5 h after IPTG induction; lane 4, total protein from *E. coli* BL21(DE3)(pEIBAH) 3 h after IPTG induction; lane 5, crude extract from *E. coli* BL21(DE3)(pEIBAH); lane 6, saturated ammonium sulfate fraction; lane 7, phenyl agarose fraction; lane 8, purified native IBAH from *S. viridifaciens* MG456-hF10.

molecular mass of the *vlmH* gene product was 39,864 Da, and the calculated isoelectric point was 4.92. The values of these parameters obtained from the purified, native IBAH were 40 kDa and 5.12, respectively.

Overexpression of the *vlmH* gene product, IBAH. To establish that the putative *vlmH* gene did in fact encode a protein with IBAH activity, a DNA fragment containing the gene was amplified by PCR and subcloned into the expression vector pET-24a(+) and introduced into *E. coli* DH5α cells for amplification. PCR was also used to change the stop codon of *vlmH* from TGA to TAG in order to allow for optional expression of the C-terminal His tag by use of a *supE E. coli* strain for expression. The resulting construct, pEIBAH (Fig. 2), was sequenced in order to verify proper reading frame. pEIBAH was then introduced into *E. coli* BL21(DE3) cells, and expression was induced with IPTG. Periodic examination of total protein of the induced cells by SDS-PAGE indicated a protein of the correct size (40 kDa) had been produced after the induction (Fig. 5). Analysis by scanning densitometry of the SDS-polyacrylamide gel of the crude cell extracts 3 h after induction indicated that the overproduced protein constituted approximately 35% of the total soluble protein.

Purification and assay of IBAH. The protein produced by overexpression of the *vlmH* gene was purified from the crude bacterial lysate in two steps, i.e., ammonium sulfate fractionation and hydrophobic interaction chromatography on phenyl agarose (Table 3). The purified protein was shown to possess IBAH activity by incubation with isobutylamine, NADH, FMN, and the NAD(P)H:FMN oxidoreductase of *P. fischeri*. The specific activity of the IBAH produced by overexpression was found to be less than that of the native protein isolated from *S. viridifaciens* when assayed under identical conditions (19). This may be due to a lack of temperature control during the disruption of the recombinant *E. coli* cells with a Mini-Bead-Beater cell disrupter.

Sequence homology studies. Database searches with FASTA and TFASTA did not identify any proteins with significant similarities to IBAH. A search of the PROSITE pattern database with MOTIFS did not disclose any motifs in common with isobutylamine hydroxylase. However, a BLAST (2) search revealed a high degree of similarity between IBAH, the Sox/DszC protein of *Rhodococcus* sp. strain IGTS8, and several acyl coenzyme A (CoA) dehydrogenases. The Sox/DszC protein is an enzyme that catalyzes the oxidation of dibenzothiophene to dibenzothiophene sulfone (6, 21). Like IBAH, it requires $FMNH_2$ for activity (15). The acyl-CoA dehydrogenases are also known to be flavoproteins (32). A BLOCKS search also identified a strong similarity between IBAH and the acyl-CoA dehydrogenase family of proteins. Acyl-CoA dehydrogenases contain four short, ungapped, highly conserved amino acid sequences (blocks) (8, 9). All four blocks aligned with the sequence of IBAH in the correct order. Furthermore, the distances between the four blocks of the acyl-CoA dehydrogenase family are similar to the distances between the corresponding segments in the IBAH sequence. The probability that such a combination occurs by chance was calculated by the BLOCKS program to be less than 8.5×10^{-7}. Multiple sequence alignments of IBAH, SoxC, and four acyl-CoA dehydrogenases revealed strong similarities among them that extend throughout the entire protein region (Fig. 6). However, two acyl-CoA dehydrogenase signatures, which are found in blocks A and D of these proteins, do not occur in the sequences of IBAH and Sox/DszC, suggesting that there are important differences between IBAH and Sox/DszC and the acyl-CoA dehydrogenases. The BLOCKS search also identified a possible similarity between IBAH and the flavodoxins. The search with the IBAH sequence pointed to the first block (BL 00201A) of three flavodoxin blocks. There is a flavodoxin signature motif [LIV]-[LIVFY]-[FY]-x-[ST]-x(2)-[AGC]-x-T-x(3)-A-x(2)-[LIV] in block BL 00201A. The corresponding segment of the IBAH sequence contained a similar pattern (boldface) beginning at isoleucine 281: **I** Q L E **S** A V **G** L **T** E G V **A** R A **V**. A BESTFIT comparison between IBAH and a number of potentially related proteins is shown in Table 5. This analysis confirmed the findings of the BLAST analysis by indicating that IBAH is most closely related to the Sox/DszC protein and the acyl-CoA dehydrogenases.

DISCUSSION

The *vlmH* gene, which encodes the protein IBAH, has been cloned from *S. viridifaciens* MG456-hF10 and overexpressed in active form in *E. coli*. It appears likely that this gene is involved in valanimycin biosynthesis since precursor incorporation experiments strongly support the intermediacy of isobutylamine and isobutylhydroxylamine in the valanimycin biosynthetic pathway (18, 20). However, definitive proof for the role of this gene in valanimycin biosynthesis will require that a gene disruption experiment be performed. Studies with native IBAH purified from *S. viridifaciens* have shown that NADH, molecular oxygen, flavin adenine dinucleotide or FMN, and a second protein present in cell extracts of *S. viridifaciens* are required to catalyze the hydroxylation of isobutylamine to isobutylhydroxylamine (19). It has also been found that the function of the second protein can be fulfilled by the NAD(P)H:FMN oxidoreductase of *P. fischeri* (19). IBAH therefore appears to be a flavin monooxygenase that catalyzes the hydroxylation of isobutylamine to isobutylhydroxylamine via the intermediacy of a flavin 4a-hydroperoxide, while the role of the second protein is to supply the hydroxylase with a reduced form of the flavin cofactor.

N-hydroxylation reactions are also known to occur in the biosynthetic pathways leading to the siderophores pyoverdin in *Pseudomonas aeruginosa* and *Ustilago maydis* and aerobactin in *E. coli*. A key step in these pathways involves the N^5-hydroxylation of L-ornithine in the case of pyoverdin (17, 34) and the N^6-hydroxylation of L-lysine in the case of aerobactin (22, 29, 30). These hydroxylations are catalyzed by flavin monooxygenases. However, a BLAST search failed to reveal a close relationship between these proteins and IBAH, and a BESTFIT

FIG. 6. Multiple sequence alignment of IBAH, SoxC, and acyl-CoA dehydrogenases. Consensus residues are indicated by boxes. Shaded areas correspond to blocks in the acyl-CoA dehydrogenases. Abbreviations for protein sequences: SoxC, dibenzothiophene desulfurization enzyme; Acdb, acyl-CoA dehydrogenase; Acd, acyl-CoA dehydrogenase; Ivd, isovaleryl-CoA dehydrogenase; Acdl, long-chain acyl-CoA dehydrogenase. Abbreviations for species: Svf, *S. viridifaciens*; Rho, *Rhodococcus* sp. strain IGTS8; Bsu, *Bacillus subtilis*; Mlp, *Mycobacterium leprae*; Rat, *Rattus norvegicus*; Hum, *Homo sapiens*. Accession numbers for proteins are shown in Table 5.

analysis (Table 5) predicted only a weak similarity between IBAH and these proteins. A possible explanation for this may be the fact that the L-lysine and L-ornithine *N*-hydroxylases catalyze both the reduction of the flavin cofactor and the *N*-hydroxylation process.

Another enzyme system that appears similar to IBAH is that required for the final step of pristinamycin II_A biosynthesis in *Streptomyces pristinaespiralis*. This reaction, which involves the oxidation of a D-proline residue to a 2,3-dehydroproline residue, requires two enzymes, an FMN reductase encoded by the *snaC* gene and a heterodimeric oxidase encoded by the *snaA* and *snaB* genes (4, 31). The mechanism of the double-bond introduction process is unknown, although it has been shown to require molecular oxygen. A BLAST search did not suggest a close relationship between these proteins and IBAH, and a BESTFIT comparison of the *snaA* and *snaB* gene products with IBAH found only a weak similarity (Table 5).

TABLE 5. Comparison between IBAH and related proteins with BESTFIT program[a]

Protein[b]	Length (aa)[c]	% Similarity	% Identity	Z score	Accession no.[d]
Rho.SoxC	417	44.9	22.4	13.2	U08850
Bsu.acdb	379	38.7	16.4	8.9	P45857
Rat.ivd	424	42.6	18.5	8.2	P12007
Mlp.acd	389	41.1	18.9	7.2	P46703
Hum.acdl	430	41.2	16.6	3.5	P28330
Eco.lnh	426	35.9	17.1	1.2	P11295
Pag.onh	426	37.6	16.1	0.9	A49892
Sps.SnaA	422	36.5	16.5	0.4	U21215
Umd.onh	570	37.8	16.8	0.3	A47266
Sps.SnaB	277	39.9	17.0	0	U21215

[a] Gap weight, 4.0; length weight, 0.2.

[b] Abbreviations for proteins: Rho.SoxC, dibenzothiophene oxidase from *Rhodococcus* sp.; Bsu.acdb, *Bacillus subtilis* acyl-CoA dehydrogenase; Rat.ivd, rat isovaleryl-CoA dehydrogenase; Mlp.acd, *Mycobacterium leprae* acyl-CoA dehydrogenase; Hum.acdl, human acyl-CoA dehydrogenase; Eco.lnh, L-lysine N^6-hydroxylase from *E. coli*; Pag.onh, L-ornithine N^5-hydroxylase from *P. aeruginosa*; Sps.SnaA, pristinamycin II_B oxidase component from *S. pristinaespiralis*; Umd.onh, L-ornithine N^5-hydroxylase from *U. maydis*; Sps.SnaB, pristinamycin II_B oxidase component from *S. pristinaespiralis.*

[c] aa, amino acid.

[d] Numbers preceded by A are from the PIR database; numbers preceded by P are from the SWISS-PROT database; numbers preceded by U are from the GenBank database.

A BLAST search revealed a higher degree of similarity between IBAH, Sox/DszC, and the acyl-CoA dehydrogenases. This similarity is strongly supported by a BLOCKS search and is also indicated by a BESTFIT comparison of these proteins (Table 5) and by a multiple alignment of their amino acid sequences (Fig. 6). As the Sox/DszC reaction involves the flavin-catalyzed oxidation of a heteroatom, it probably proceeds via the formation of a flavin 4a-hydroperoxide. A significant relationship between IBAH and Sox/DszC is therefore understandable. The similarities found between IBAH and the acyl-CoA dehydrogenases (Fig. 6) are more surprising, since the mechanism of the reaction catalyzed by the acyl-CoA dehydrogenases does not bear a close resemblance to that of the reaction catalyzed by IBAH (32). In this case, the similarity between IBAH and the acyl-CoA dehydrogenases may reflect their requirement for a flavin cofactor, and it is conceivable that the sequence homology between these proteins is due in part to a common flavin binding region. A BLOCKS search also identified some similarity between IBAH and the flavodoxins. The similarity was restricted to the first block of the flavodoxins and was related to a flavodoxin signature motif that is believed to be involved in binding the phosphate group of FMN (35).

In summary, the first gene encoding an enzyme that appears to be involved in valanimycin biosynthesis has been cloned, characterized, and overexpressed in soluble form. The overexpressed protein, IBAH, exhibited the expected enzymatic activity, thereby confirming the identity of the protein. The

amino acid sequence of IBAH exhibited a strong degree of similarity to the Sox/DszC protein and the acyl-CoA dehydrogenases, which are also flavoproteins. Because antibiotic biosynthesis genes are generally clustered in *Streptomyces* (33), the successful cloning of the *vlmH* gene should allow other genes of the valanimycin biosynthetic pathway to be identified by genome walking.

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