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Sequence, Transcriptional, and Functional Analyses of the Valine (Branched-Chain Amino Acid) Dehydrogenase Gene of *Streptomyces coelicolor*

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The gene encoding the valine (branched-chain amino acid) dehydrogenase (Vdh) from *Streptomyces coelicolor* has been characterized as follows. The *vdh* gene was identified by hybridization to a specific oligodeoxynucleotide that was synthesized on the basis of the N-terminal amino acid sequence of purified Vdh. Nucleotide sequence analysis predicts that the *vdh* gene contains a 364-amino-acid open reading frame that should produce a $38,305-M_r$ protein. The deduced amino acid sequence of the Vdh protein is significantly similar to those of several other amino acid dehydrogenases, especially the leucine and phenylalanine dehydrogenases from *Bacillus* spp. The *vdh* gene is apparently transcribed from a single major transcriptional start point, separated by only 8 bp from the 5' end of a divergent transcript and located 63 bp upstream from the *vdh* translational start point. Mutants with a disrupted *vdh* gene have no detectable Vdh activity and have lost the ability to grow on valine, leucine, or isoleucine as the sole nitrogen source. This *vdh* mutation does not significantly affect growth or actinorhodin production in a minimal medium, yet the addition of 0.2% L-valine to the medium provokes approximately 32 and 80% increases in actinorhodin production in *vdh*+ and *vdh* strains, respectively.

Branched-chain amino acids are normally catabolized in bacteria first by dehydrogenation and then by oxidative decarboxylation of the resulting 2-keto acid (13). Most studies of the catabolism of branched-chain amino acids have been done with *Pseudomonas* spp. Three active transport systems for the uptake of branched-chain amino acids are known, and in all of these, the genes for the transport components are organized as a single operon (11). Following its uptake, a branched-chain amino acid requires for complete catabolism the cooperation of two sequential series of reactions. The enzymes in the first series constitute a common pathway and catalyze the conversion of Leu, Val, and Ile to their respective 2-keto acids (13). Branched-chain 2-oxoacid dehydrogenase, which catalyzes the second step in this initial process, is a multienzyme complex involved in the oxidation of the 2-keto acid derivatives of all three branched-chain amino acids (31). The acyl coenzyme A metabolites formed subsequent to the common pathway are catabolized by three separate series of enzymes, one specific for each initial amino acid (13).

Valine degradation takes a somewhat different course in *Streptomyces* spp. than in other bacteria (24, 26). Carbon 1 of valine and its isobutyric acid and malonic acid semialdehyde catabolites becomes the carboxy group of propionate, instead of being lost as CO_2, as in *Pseudomonas* spp. (26). *Streptomyces* spp. also have an enzyme that catalyzes the reversible intramolecular rearrangement of isobutyrate produced from L-valine to *n*-butyrate (24), thereby possibly forging a direct link between the pathways of straight-chain and branched-chain fatty acid metabolism.

Valine dehydrogenase (Vdh) from streptomycetes is an NAD+-dependent enzyme that catalyzes the oxidative deamination of branched-chain L-amino acids to the corresponding 2-keto acids. The enzyme has been assumed to

play a role in the bacterial utilization of such amino acids, since it is induced by valine (and other branched-chain amino acids), is repressed by glucose and NH4+, and is the first enzyme of the valine catabolic pathway (13, 16, 23). Thus, the regulation of its level and/or activity could be the predominant factor in the regulation of valine utilization. Valine catabolism can supply *n*-butyrate, 2-methylmalonate, and propionate units for the biosynthesis of the aglycones of the macrolide antibiotics tylosin (20) and leucomycin (21) and the polyether antibiotics monensin A (28) and lasalocid A (26). This process is inhibited by high concentrations of ammonium ion (20). Superior chemically defined media for tylosin and monensin production contain L-valine as a major carbon and nitrogen source for this reason (16). The branched-chain amino acid dehydrogenases may also provide the starter units for the synthesis of the iso- and anteiso-fatty acids that predominate in the cellular lipids of actinomycetes (12).

Four L-Vdh enzymes (16, 23, 34, 35) have been purified from *Streptomyces* spp. and characterized. We chose to study the Vdh from *Streptomyces coelicolor* because a subsequent investigation of the genetics of valine catabolism could lead to data comparable to the properties of the other catabolic enzymes and their genes that have been studied in this genetically well-characterized organism (for examples, see references 1 and 9). The *S. coelicolor* Vdh is a dimeric enzyme consisting of two identical subunits each with an M_r of 40,000. Its activity is induced by D- or L-valine and repressed in the presence of glucose and NH4+ (16).

We now report the cloning and sequencing of the *vdh* gene from *S. coelicolor* and the characterization of its transcriptional organization. We have found that the *vdh* promoter overlaps the promoter of a divergently transcribed gene (open reading frame [ORF] 1 [ORF1]) that has an unknown function and that apparently is not essential for the function of *vdh*. A homolog of the *Escherichia coli purM* gene (27) is just downstream of the *vdh* gene and transcribed towards it.

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TABLE 1. Plasmids and strains used in this work

Plasmid or strain	Characteristic(s)[a]	Source or reference
Plasmids		
pXH106	*bla tsr hgr*; Tn5099; ts	8
pWHM1050	N terminus of *vdh* gene from strain A3(2) on pUC18	This study
pWHM1051	*vdh*, ORF1, and ORF2 genes from strain J802 on pUC18	This study
pWHM1065	Religated 6.0-kb HindIII fragment of pXH106	This study
pWHM1057	1.6-kb BamHI fragment of the *vdh* region from pWHM1051 subcloned into pUC18	This study
pWHM1058	1.7-kb SmaI-EcoRV fragment of the *hyg* gene from pXH106 inserted into the ApaI site of pWHM1057; 3.3-kb BamHI fragment isolated from the resulting plasmid and subcloned into the BamHI site of pWHM1065	This study
pWHM1059	2.1-kb SalI-SphI fragment of the ORF1 region from pWHM1051 subcloned into pUC18	This study
pWHM1060	1.7-kb SmaI-EcoRI fragment of the *hyg* gene from pXH106 inserted into the BssHII sites of pWHM1059; 3.6-kb HindIII-BspHI fragment isolated from the resulting plasmid and subcloned into the HindIII-NcoI site of pWHM1065	This study
Strains		
WMH1505	*vdh::hyg* gene-disrupted mutant	This study
WMH1506	ORF1::*hyg* gene-disrupted mutant	This study

[a] *bla*, ampicillin resistance gene; *tsr*, thiostrepton resistance gene; *hyg*, hygromycin resistance gene; ts, temperature sensitive.

Disruption of the *vdh* gene in *S. coelicolor* J802 blocks growth on branched-chain amino acids as the sole N source but does not lower the production of actinorhodin, its principal acetate-derived antibiotic.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. *E. coli* DH-5α (25), GM2929 (*dam dcm*) (obtained from Doug MacNeil, Merck Sharp & Dohme), and JM105 (25), used as hosts for plasmids or for M13 DNA sequencing, were grown at 37°C on LB or 2×YT medium (25). *S. coelicolor* J802 (*dagA1 agaA7*) (9), obtained from David Hodgson, was grown on R2YE plates (10) at 30°C for general use and on minimal medium (MM) (10) or the following media (modified from MM): MGV, containing 1% L-valine instead of asparagine; and MV, containing 1% L-valine but no asparagine and glucose. Media used for actinorhodin production were SMM, which contains 5% polyethylene glycol 6000 (BDH), 5 mM MgSO₄, 25 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) (Sigma) (pH 7.2), 1 mM potassium phosphate, 1% (wt/vol) glucose, and 0.2% (wt/vol) Casamino Acids (32); and SMV, which is SMM containing 0.2% L-valine. *Streptomyces* transformation was performed as described by Hopwood et al. (10), and transformants were selected on R2YE plates supplemented with 25 μg of thiostrepton (obtained from S. G. Lucania, The Squibb Institute for Medical Research, Princeton, N.J.) per ml. *S. coelicolor* was transformed only with plasmid DNA isolated from *E. coli* GM2929 or *S. lividans*. Plasmids and strains made in this work are listed in Table 1.

DNA preparation and construction and screening of minilibraries. Small-scale preparations of *E. coli* plasmid DNA were made as described by Morelle (14). M13 single-stranded DNA (ssDNA) was isolated from JM105 as described by Sambrook et al. (25), except that the supernatant containing the phage was extracted three times with neutral phenol-chloroform (3:1 [vol/vol]). Individual DNA restriction fragments were purified by separation on agarose gels and then treated with the USBioclean MP kit (United States Biochemicals, Cleveland, Ohio) in accordance with the

manufacturer's directions. Streptomycete genomic DNA was isolated by the lysozyme-sodium dodecyl sulfate (SDS) method of Hopwood et al. (10). Oligodeoxynucleotides for hybridization probes or sequencing primers were synthesized by use of an Applied Biosystems model 391 DNA synthesizer and purified in accordance with the manufacturer's protocols.

A 50-mer oligodeoxynucleotide, 5'-ACCGACGTCAACGG CGCCCCIGCCGACGTCCTICACACCCTITTCCACTC-3', representing the coding sequence of the first 17 N-terminal residues (TDVNGAPADVLHTLFHS) of purified Vdh from *S. coelicolor* A3(2) and containing inosine (I) substitutions at especially degenerate codon positions (18), was end labeled with ³²P by standard methods (25) and used to probe several restriction enzyme digests of the *S. coelicolor* genomic DNA. Southern blot hybridization showed that 1.15-kb BamHI-SphI and 7.0-kb PstI-SstI DNA fragments hybridized with the ³²P-labeled probe, although the signal was much weaker with the latter fragment. *S. coelicolor* genomic DNA was doubly digested with BamHI-SphI and PstI-SstI and size fractionated by electrophoresis on a 1% agarose gel, and 1.0- to 1.5-kb BamHI-SphI DNA fragments and 6.5- to 7.5-kb PstI-SstI DNA fragments were cloned separately in pUC18 (36). These DNA minilibraries were screened with the oligodeoxynucleotide probe to obtain clone pWHM1050 and, subsequently, with a 350-bp SphI-KpnI DNA segment from pWHM1050 to clone the *vdh* gene as pWHM1051.

DNA hybridization and sequencing. DNA was size fractionated by electrophoresis and transferred to Hybond-N (Amersham, Arlington Heights, Ill.) membranes by capillary transfer or electrotransfer (25). Prehybridization and hybridization to [α-³²P]dCTP-labeled DNA fragments or [γ-³²P] dATP-labeled oligonucleotides were carried out at 42°C in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (25)–2× Denhardt solution (25)–100 μg of denatured salmon sperm DNA per ml–0.1% SDS (10, 25). Digoxigenin-alkaline phosphatase labeling, hybridization, and detection were done with the Genius kit in accordance with the manufacturer's protocols (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The blot was washed two times with 1× SSC–0.1% SDS for 30 min each time at 42°C and then two times with 0.1× SSC–0.1% SDS for 30 min

each time at 68°C, except for the oligonucleotide probing blot, which was washed two times with 1% SSC–0.1% SDS for 30 min each time at 42°C and two times with 0.5% SSC–0.1% SDS for 15 min each time at 60°C.

DNA segments for sequencing were purified and subcloned into M13mp18 or M13mp19 (36). Exonuclease III-S1 nuclease deletions were prepared by use of the Erase-a-Base system (Promega Biotec, Madison, Wis.). The nucleotide sequence was determined by the dideoxy chain termination method with either the M13 −40 primers or specifically synthesized sequencing primers and a Sequenase 2.0 kit (United States Biochemicals) in accordance with the manufacturer's instructions. 7-Deaza-dGTP was used in place of dGTP to reduce the number of sequencing artifacts. [^{35}S]dCTP-labeled samples were run on 6% polyacrylamide–8 M urea—12% formamide wedge gels. Sequence data were read from dried gels with the DNASTAR (Madison, Wis.) software and digitizer. The GCG software (6), versions 6.2 and 7.0, were used for sequence analysis. Nucleotide sequence-deduced amino acid sequence data were compared with available data bases by use of the FASTA and TFASTA programs (6).

RNA isolation. Cultures of *S. coelicolor* were grown in R2YE medium for 2 to 3 days, and then a single 50-ml culture was split into 10-ml aliquots and centrifuged. The recovered mycelia were used to inoculate R2YE, MM, MGV, and MV, and the cultures were grown for another 24 or 48 h. RNA was isolated from the recovered cells essentially by the method of Fisher and Wray (7), except that the cells were lysed by grinding with a mortar and pestle in the presence of liquid nitrogen, guanidinium isothiocyanate buffer was added, and the cells were centrifuged in cesium chloride solution. The RNA concentration was determined by measuring the A_{260} of an mRNA solution in diethyl pyrocarbonate-treated H_2O.

Low-resolution S1 nuclease protection assay. In accordance with the method described by Neal and Chater (17), ssDNA (1 μg) was coprecipitated and hybridized with 40 μg of total RNA. The hybridization samples were treated with 130 U of S1 nuclease for 30 min at 37°C, and then the protected DNA fragments were fractionated on a 4% denaturing polyacrylamide–8 M urea gel and electrotransferred to a Hybond-N membrane for hybridization. The blot was probed with a 2.88-kb *SalI* DNA fragment containing the *vdh* gene with its 5′-noncoding region.

High-resolution S1 nuclease protection and primer extension mapping. M13mp18 and M13mp19 subclones containing a portion of the 5′ end of either the *vdh* or the ORF1 gene were used to prepare radiolabeled ssDNAs as probes to identify the apparent start of transcription. Two 24-mer synthetic oligodeoxynucleotides were used as primers: primer a was 5′-GTGCAGTACATCAGCAGGTGCGCC-3′, beginning 39 bp downstream of the *vdh* GTG translation initiation codon and complementary to the *vdh* mRNA; and primer b was 5′-GGATCACAAAGGCGCGGGAATCTC-3′, beginning 54 bp upstream of the presumed ORF1 GTG start codon and complementary to the ORF1 mRNA. Primers were annealed to ssDNA templates prepared from the M13 subclones, and complementary DNA strands were generated with [^{35}S]dCTP in the labeling mixture by use of the Sequenase 2.0 kit. After restriction enzyme digestion and heat denaturation, the labeled ssDNA samples were size fractionated on a 4% polyacrylamide–8 M urea gel, eluted by the crush-and-soak method (25), hybridized with 40 μg of RNA, and treated with S1 nuclease. The reaction products were analyzed on a 6% polyacrylamide–8 M urea—12% forma-

mide gel along with dideoxy DNA sequencing ladders made with the same primers.

A primer extension experiment was conducted by a modification of the method of Stein et al. (29). The same primers as those used for S1 mapping were annealed to 20 μg of RNA in reverse transcriptase reaction buffer (supplied by Bethesda Research Laboratories, Inc., Gaithersburg, Md.) in a 50-μl volume. Primer extension was performed by the addition of 2.5 μl of [^{35}S]dCTP, 5.0 μl of labeling mixture (10 mM each dGTP, dTTP, and dATP), 5.0 μl of 0.1 M dithiothreitol, 0.5 μl of a 1% [wt/vol] solution of actinomycin D [Sigma], and 200 U of Superscript Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories). The reaction mixture was incubated for 30 min at 42°C, 0.5 μl of unlabeled 100 mM dCTP was added, and the reaction mixture was incubated for another 45 min. The reaction was stopped and the RNA was removed as described by Stein et al. (29), but no phenol extraction was used. The resulting DNA fragments were analyzed by electrophoresis as described above.

***vdh* and ORF1 gene replacement.** For construction of the plasmid used for *vdh* gene inactivation, a 1.6-kb *Bam*HI DNA fragment of the *vdh* coding region from pWHM1051 was subcloned into *Bam*HI-digested pUC18 to yield pWHM1057. pWHM1057 was digested with *Apa*I, and the ends were filled in by treatment with Klenow polymerase and ligated to the 1.7-kb *Sma*I-*Eco*RV fragment of pXH106 (8), containing the hygromycin resistance gene (*hyg*). A 3.3-kb *Bam*HI fragment was isolated from the resulting plasmid and subcloned into the *Bam*HI site of modified pXH106 (pWHM1065), which was treated with *Hin*dIII and ligated, resulting in deletion of a portion of the Tn*5099* transposon, to yield plasmid pWHM1058 (see Fig. 5). For inactivation of the ORF1 gene, a 2.1-kb *Sph*I-*Sal*I DNA fragment of the ORF1 region from pWHM1051 was subcloned into *Sph*I-*Sal*I-digested pUC18 to yield pWHM1059. pWHM1059 was digested with *Bss*HII, resulting in the deletion of about 0.2 kb, and the ends were filled in by treatment with Klenow polymerase and ligated to the 1.7-kb *Sma*I-*Eco*RV fragment containing the *hyg* gene. A 3.6-kb *Hin*dIII-*Bsp*HI fragment was isolated from the resulting plasmid and cloned into the *Hin*dIII-*Nco*I site of pWHM1065, resulting in plasmid pWHM1060 (see Fig. 5).

pWHM1058 and pWHM1060 were introduced into *S. coelicolor* J802 by transformation, and thiostrepton-resistant (Th^r) transformants were selected on R2YE plates and grown in R2YE liquid medium plus 200 U of hygromycin (Sigma) per ml at 28°C for 3 days. These cultures were homogenized, and the mycelial cells were further incubated at 39°C for 3 days to eliminate the autonomously replicating plasmid. The mycelial cells were plated on R2YE agar plus 200 U of hygromycin per ml and incubated at 39°C for 4 to 5 days, and spores were collected and screened for hygromycin-resistant (Hg^r) Th^s clones.

Actinorhodin production and Vdh activity measurements. For actinorhodin production and Vdh activity determinations, *S. coelicolor* cultures were grown at 30°C in 25 ml of R2YE medium for 48 h with shaking at 200 rpm. The mycelial cells were harvested by centrifugation, washed twice with MM without glucose and asparagine (Mm), and suspended in 5 ml of Mm. Equal portions of this cell suspension were added to MM and MGV, and the cultures were incubated at 30°C for 24 h prior to assay of Vdh specific activity or determination of the protein concentrations in cell aliquots. Vdh activities were determined as described by Navarrete et al. (16). Culture samples (300 μl) were added to
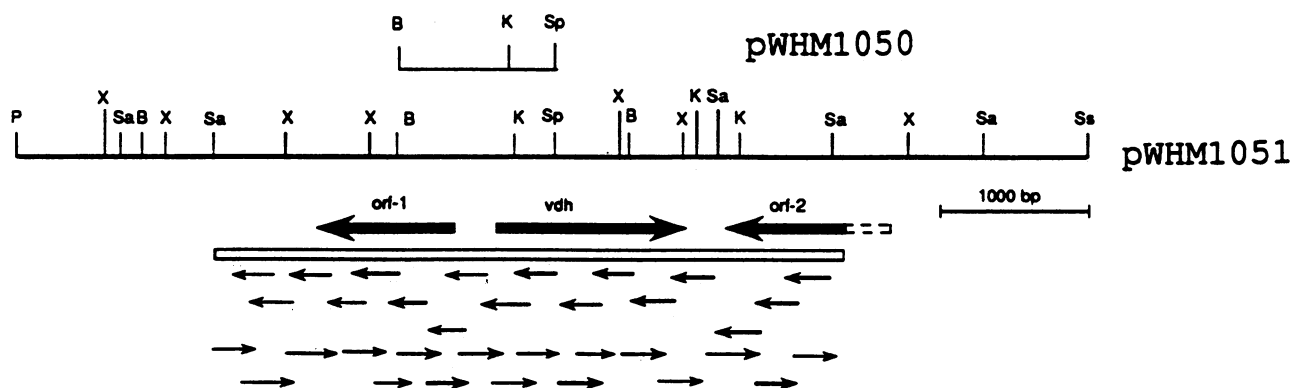
FIG. 1. Restriction map and DNA sequencing strategy for the 4.26-kb DNA fragment cloned in pWHM1051. The thick bold arrows indicate the direction of transcription for the three ORFs. The open box beneath them indicates the region that was sequenced. The thin arrows beneath this box illustrate the sequencing strategy. Restriction enzyme abbreviations: B, BamHI; K, KpnI; Sp, SphI; Sa, SalI; P, PstI; Ss, SstI; X, XmaI.

300 μl of 1 N NaOH and incubated at 37°C for 24 h, and the protein concentrations were determined in aliquots of these samples by the method of Bradford (5) with bovine serum albumin as the standard. The actinorhodin contents in SMM and SMV were estimated spectrophotometrically by measuring the optical density at 612 nm of cell-free culture supernatants that had been adjusted to pH 12 as described by Strauch et al. (30).

Nucleotide sequence accession number. The DNA sequence data described in this paper have been deposited at EMBL and GenBank under accession number L13455.

RESULTS

Cloning of the S. coelicolor vdh gene. The vdh gene was cloned by hybridization to an oligodeoxynucleotide constructed on the basis of the N-terminal amino acid sequence of purified Vdh from S. coelicolor A3(2) (16). Screening of plasmid DNA isolated from a minilibrary consisting of 1.0- to 1.5-kb BamHI-SphI DNA fragments of S. coelicolor A3(2) genomic DNA yielded one positive clone, pWHM1050, which was shown to contain a 1.15-kb BamHI-SphI DNA fragment (Fig. 1). Southern analysis showed that the 50-mer oligodeoxynucleotide probe hybridized to only one and the same 1.15-kb BamHI-SphI DNA fragment in the genomic and cloned DNAs (data not shown). By using a ^{32}P-labeled 350-bp SphI-KpnI fragment from pWHM1050 DNA that contains part of the N-terminal region of Vdh as a probe to screen the 6.5- to 7.5-kb PstI-SstI minilibrary of S. coelicolor J802 DNA (see Materials and Methods) by colony hybridization, we cloned an approximately 7-kb DNA fragment as pWHM1051. A restriction map of the cloned region is shown in Fig. 1.

Using the 350-bp SphI-KpnI fragment as a probe, we detected homologous DNA sequences in hybridization experiments (see Materials and Methods) with genomic Southern blots of Streptomyces lividans TK24, salinomycin-producing Streptomyces albus ATCC 21838, monensin-producing Streptomyces cinnamonensis WMH585, tetracenomycin-producing Streptomyces glaucescens GLA.O, tylosin-producing Streptomyces fradiae WMH574, spiramycin-producing Streptomyces ambofaciens ATCC 15154, and midecamycin-producing Streptomyces mycarofaciens 1748 but not erythromycin-producing Saccharopolyspora erythraea WMH22 (data not shown).

Sequence analysis of the vdh region. The sequence of a 4.26-kb segment of pWHM1051 DNA that contains the entire vdh gene was determined by use of a combination of restriction fragments and exonuclease III deletions. Restriction sites were overlapped as necessary, and each DNA strand was sequenced at least three times. CODON PREFERENCE analysis (6) showed that there are two complete ORFs and one partial ORF in this region, having the characteristic codon usage pattern for Streptomyces DNA (3). The vdh gene is sandwiched between ORFs reading in the opposite direction, and its sequence is shown in Fig. 2. The 1,092-nucleotide (nt) vdh ORF begins with a GTG at position 1696 and terminates with a TGA at position 2788. This ORF is followed by a potential transcription termination site and should encode a 364-amino-acid protein with a calculated molecular mass of 38,305 daltons. The first 20 amino acids of this ORF (minus the initiating formylmethionyl) coincide with the N-terminal amino acid sequence of the purified Vdh enzyme, except for residue 19, at which A is replaced by Q. Since the identical sequence was obtained for the same region of pWHM1050 DNA (Fig. 1), we believe that the results shown in Fig. 2 are correct. The codon usage of the vdh gene is atypical for the first 18 residues, which contain a noticeably high percentage of rare codons above the 0.1% threshold (6). A region centered about 9 nts 5' to the first codon of the vdh gene (Fig. 2) has a significant degree of complementarity to the 3' end of 16S rRNA of S. lividans (5'-GAUCACCUCCUUUCU-3') (2) and should serve as the ribosome binding site. A search of the GenBank and EMBL data bases as of June 1991 with FASTA and TFASTA (6) revealed significant sequence similarities between the deduced Vdh protein and several other NAD(P)$^+$-dependent dehydrogenases, particularly the leucine (15) and phenylalanine (19) dehydrogenases from Bacillus spp. (55 to 60% identity overall, as determined by GAP [6] analysis; data not shown).

Another ORF was found upstream of the vdh gene on the opposite strand; this ORF is temporarily designated ORF1. This 855-nt ORF is likely to begin with GTG at position 1466, to terminate with TGA at position 611, and to encode a 285-amino-acid protein. A putative ribosome binding site was found at a suitable distance from the GTG start codon (Fig. 2). No significant homology was found between the deduced product of ORF1 and known proteins by data base searching.

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   1  CACTTGACAGGCCAGCTGCGCCTTGTACCACCGCTGGAGTCGGCCCCAGTCGTCGCCGAGCCGTAGTCCCCACGCTCGCCAGTACTCGCCGGAGGAGCCC
 101  TCTTGGCTTGGAAGAGCCAAGTAAGGAGATTTAAGACGTGGAACTGGGCGCAACGGGCTTTACCGCCTGCGCTCAGCTCAGCCAATATCCTGCTTGCCGA
 201  ACAGGAGCCGTGATGTTGATGTGGTAGACAGGTCGGCGCAGCCGGTTTGGCTACCTTAACGGGAGGGTCCACAAGTAGTCGCTGCCTTCGGCTACCTGGT
 301  ACGGTATCGCTGTCAGTGCGGAGACACTGCTAGTCAGTGTCCCCGCGAGTCCTCAGTGGTCTGGGGGGTTTCGCACGTTACGACTCTAAGGTGGGTGTCA
 401  CGTAGTGCCGTCTGCCTTCCTCGGGAGGGGCCCGAGGAACAGGATAAAACCGTACTCCTCCCCCTTCGCCGCGTTCTCACTACATGCACGCCAGGCAGTG
           >>>>>>>>>>>        <<<<<<<<<<<<                              >>>>>>>>>>>>>>>>>> >>>>    <<<<<<<<<<<<<<
 501  CGAACTGAGTTTGCGGGCCTAGCCCTGGATGCAGGGAACAGCCGTTCTCTAGTCGGGTGTGGCCCGCCCACAGCCTCGCCTTCTGCTTTGGGGTGTAGCC
        <<<<<<<
 601  CGCCATGCAGTTTGCAGTCAGCGGCTCGCGGCCAGGGCCTGGCGGGCGGTCGCGAGGTGGTCGGCGGGCATGCGGCGGGGGCGCCGTTGGGGCAGCGGCA
         *  V  D  T  A  S  R  R  D  R  V  A  R  W  R  E  V  L  R  G  Y  A  A  G  A  A  V  G  D  G
 701  CCGCGTCGCGGAGCTAGGGGAGCCGGTGCAGGCGGCGCCTCACCAGGCGGAGGTTGAGCGGCCGGCCGTCCCGCACGTGGTCAGGCGGCATCAGGTCGAG
       H  R  L  A  E  I  G  E  A  V  D  A  A  S  H  D  A  E  L  E  G  A  P  L  A  H  V  L  G  G  Y  D  L  E
 801  CTGGTCCCTTGCGCCCACCTTGAGGAGGTCGGTCGCGGGGTGTAGGTGGTCCGGCAGCTACTCGCCGGGGAGCAGGTGCCGGAGGGCGTCCCGGGCGTCG
       V  L  S  R  P  H  F  E  E  L  W  R  G  V  D  V  L  G  D  I  L  P  G  E  D  V  A  E  R  L  A  R  L
 901  GGGGCCCGGTGGGCGGCCGCCCGGACGTGGTACCCACACGCCATCGCGTCGTGGCCACGCCAGCGGCGGAGCAGCGGCGGCGGGAGCAGCATGAGGGCGA
       G  R  A  V  R  R  R  A  Q  V  M  P  T  R  Y  R  L  V  P  A  T  A  A  E  D  G  G  G  E  D  Y  E  R
                                                                                            BamHI
1001  GGCGCAGCCTCTGGTCGTGCTTGGTCAACTAGCCGTGGACGGTCCACCGGCACGCCTAGGTCCCGGCCCGCAGGCCCAACGCGAGGTCGGTCGCGAGCAT
       E  A  D  S  V  L  V  F  W  N  I  P  V  Q  W  T  A  T  R  I  W  P  R  A  D  P  N  R  E  L  W  R  E  Y
1101  CAGGACGCGCGAGACCGCCGCATGCTGCCCACCCTAGTCCCGCAGGTCCTGGCCGAGGCCCTTCAGGAGCCGGTGGAGCGGGTCCCGGACGGTCGGCGCG
       D  Q  A  S  Q  R  R  V  V  P  P  I  L  A  D  L  V  P  E  P  F  D  E  A  V  E  G  L  A  Q  W  G  R
1201  TCGGCCCAGGCGGTCCCCGTCTGCGTCTGGTGCGGCAGGTGGAGCCAGTGCTTCCGCAGCCGCGAGAGCACGTGGCCGTGGCGGTGGCCGCCCCACCCGT
       L  R  T  R  W  P  C  V  C  V  V  G  D  V  E  T  V  F  A  D  A  S  E  H  V  P  V  A  V  P  P  T  P
1301  CGTCCAGCCGGTCGCTCGCCGCGTCGAGCAGGACCATCCTCCCGGCCGGCCCCGCCGCCCGCATCGCCCAGGTCACCCGCGCGAGGAGCAGCCCCTTCCG
       L  L  D  A  L  S  R  R  L  E  D  Q  Y  S  P  R  G  P  R  R  A  Y  R  T  W  H  A  R  E  E  D  P  F  A
                                                      SplI
1401  CGCCAGCGGCTCGTACACGCGCAGGTACGCCGCGTACGGGACGATCACCGAGGACACCTTGGGCACGCCTGCTCCCTCCCCCGCGCCCCGACGCGAAAAC
      GCGGTCGCCGAGCATGTGCGCGTCCATGCGGCGCATGCCCTGCTAGTGGCTCCTGTGGAACCC GTGCGGACGAGG GGAGG GGGCGCGGGGCTGCGCTTTTG
       A  L  P  E  Y  V  R  L  Y  A  A  Y  P  V  I  V  S  S  V  K  P  V        SD
                                                                       < orf1
           >>>>>>>>>>  >>>>>>>>>>......................b...........................    >>>>>>>>>>>>  <<<<<<<<<<<<<<<
1501  CTGTGGAAACCCGTGGAAACGGATCACAAAGGCGCGGGAATCTCTGCAAATCGTCGCACGCCCGTACTCCGTGCGGAGGTGATCCTGAGCACTGTGCGGC
      GACACCTTTGGGCACCTTTGCCTAGTGTTTCCGCGCCCTTAGAGACGTTTAGCAGCGTGCGGGCATGAGGCACGCCTCCACTAGGACTCGTGACACGCCG
                                                                                                   vdh >
              *>>>>>>>>>>        <<<<<<<<<<<<               SD        V  T
1601  GGGCCCGGTACACCCAGGCTCTAATCTCGTGCCACAGTCCCCGCCCACCTTCACGGGGCCTGCCACCCATCGCCGCCACCTACAC AGGAG TCACCACC GTGAC
      CCCGGGCCATGTGGGTCCGAGATTAGAGCACGGTGTCAGGGGCGGTGGAAGTGCCCCGGACGGTGGGTAGCGGCGTGGATGTGTCCTCAGTGGTGGCACTG
                     *                        >>> >>>>>>>>>      >>> >>>>>>>>>>

       D  V  N  G  A  P  A  D  V  L  H  T  L  F  H  S  D  Q  G  G  H  E  Q  V  V  L  C  Q  D  R  A  S  G
1701  CGACGTAAACGGCGCACCTGCTGATGTACTGCACACCCTGTTCCACTCGGATCAGGGGGGACATGAGCAAGTCGTGCTCTGCCAGGACCGTGCCAGCGGC
      GCTGCATTTGCCGCGTGGACGACTACATGACGTGTGGGACAAGGTGAGCCTAGTCCCCCCTGTACTCGTTCAGCACGAGACGGTCCTGGCACGGTCGCCG
              ...........................a...............
                      ApaI        KpnI
       L  K  A  V  I  A  L  H  S  T  A  L  G  P  A  L  G  G  T  R  F  Y  P  Y  A  S  E  A  E  A  V  A  D  A
1801  CTCAAGGCCGTGATCGCCCTCCACTCCACCGCCCTGGGCCCGGCGCTCGGCGGTACCCGCTTCTACCCGTACGCGAGCGAGGCGGAGGCCGTCGCCGACG
       L  N  L  A  R  G  M  S  Y  K  N  A  M  A  G  L  D  H  G  G  G  K  A  V  I  I  G  D  P  E  Q  I  K
1901  CGCTGAACCTCGCCCGCGGGATGTCGTACAAGAACGCCATGGCCGGTCTGGACCACGGTGGCGGCAAGGCCGTGATCATCGGCGATCCGGAGCAGATCAA
       S  E  E  L  L  L  A  Y  G  R  F  V  A  S  L  G  G  R  Y  V  T  A  C  D  V  G  T  Y  V  A  D  M  D
2001  GAGCGAGGAGCTGCTCCTCGCCTACGGCCGGTTCGTCGCCTCGCTGGGCGGCCGCTACGTCACCGCGTGCGACGTCGGCACCTACGTCGCCGACATGGAC
       V  V  A  R  E  C  R  W  T  T  G  R  S  P  E  N  G  G  A  G  D  S  S  V  L  T  S  F  G  V  Y  Q  G  M
2101  GTCGTGGCCCGCGAGTGCCGCTGGACGACCGGGCGTTCCCCGGAGAACGGCGGCGCGGGCGACTCCTCCGTGCTCACCTCCTTCGGCGTCTACCAGGGCA
      SphI
       R  A  A  A  Q  H  L  W  G  D  P  T  L  R  D  R  T  V  G  I  A  G  V  G  K  V  G  H  H  L  V  E  H
2201  TGCGGGCCGCCGCCCAGCACCTGTGGGGCGACCCGACGCTGCGCGACCGCACCGTCGGCATCGCGGGCGTCGGCAAGGTCGGCCACCACCTGGTCGAGCA
       L  L  A  E  G  A  H  V  V  V  T  D  V  R  K  D  V  V  R  G  I  T  E  R  H  P  S  V  V  A  V  A  D
2301  CCTGCTCGCCGAGGGCGCCCACGTCGTCGTCACGGACGTGCGCAAGGACGTGGTGCGCGGGATCACGGAGCGGCACCCGTCGGTGGTCGCCGTCGCCGAC
       T  D  A  L  I  R  V  E  N  L  D  I  Y  A  P  C  A  L  G  G  A  L  N  D  D  T  V  P  V  L  T  A  K  V
2401  ACCGACGCGCTGATCCGGGTGGAGAACCTGGACATCTACGCGCCCTGCGCGCTCGGCGGCGCGCTGAACGACGACACCGTGCCGGTGCTGACCGCCAAGG
       V  C  G  A  A  N  N  Q  L  A  H  P  G  V  E  K  D  L  A  D  R  G  I  L  Y  A  P  D  Y  V  V  N  A
                                                                                                   BamHI
2501  TGGTGTGCGGCGCGGCCAACAACCAGCTCGCCCACCCGGGCGTCGAGAAGGACCTCGCCGACCGCGGGATCCTCTACGCGCCGGACTATGTGGTGAACGC
       G  G  V  I  Q  V  A  D  E  L  H  G  F  D  F  D  R  C  K  V  K  A  S  K  I  Y  D  T  T  L  A  I  F
2601  CGGCGGGTGTCATCCAGGTCGCCGACGAGCTGCACGGGTTCGACTTCGACCGGTGCAAGGTGAAGGCCTCGAAGATCTACGACACCACGCTGGCCATATTC
       A  R  A  K  E  D  G  I  P  P  A  A  A  E  Q  R  M  A  E  A  R  P  R  P  *
2701  GCACGTGCGAAGGAGGACGGTATTCCGCCGGCCGCGGCGGCCGACCGGATCGCCGAGCAGCGGATGGCGGAGGCCCGTCCCCGGCCGTGATCCGGCGGTC
2801  ACGGACGGTGGGCCGGCGGGGTCTTCCCGGGGTTTGACCGGTACCCGTCGGGGCCAGTTGGAGAGACTCTCACTTATCCCGGCGGGTCGACCGTCGAAAG
                                                                                          >>>>  >>>>>
2901  GTGGTTAAAATCGCCTTTGACCAGCGAGGACGGGGCGCCTCGGCGGTCCTGGGCAGGGCCACGTGCTGCGGGCGACGTACGGATGTATGGGCGCGGGCTC
       >>>>>>>   <<<<<<<<<<<<<<<<
3001  AGGTACC
       KpnI
```

FIG. 2. Nucleotide sequence of the *vdh* region. Both strands are shown from nts 1401 to 1800. Only the bottom strand is shown from nts 1 to 1400, and only the top strand is shown from nts 1801 to 3007. Selected restriction enzyme sites are listed above their recognition sequences. The translational start sites of the *vdh* and ORF1 genes are underlined twice. The tsp of *vdh* and ORF1 are indicated by boldface letters and an asterisk. SD, presumed Shine-Dalgarno ribosome binding site. Dotted lines show regions for which synthetic primers were used to identify tsp. Dyad and tandem repeats in the sequence are indicated by arrowheads showing the direction of the repeated sequence.

A third ORF, downstream of *vdh* and also on the opposite strand (Fig. 1), was detected, but its sequence data are not shown in Fig. 2. This ORF, ORF2, has not been sequenced completely, but its 3' end most likely is 629 nts downstream of the *vdh* gene. We found by TFASTA analysis that the 282-amino-acid protein deduced from the 847 nts of sequenced DNA shows significant similarity to the products of the *purM* genes from *E. coli* and *Bacillus* spp., which encode a phosphoribosylformylglycinamidine cyclo-ligase that catalyzes the fifth step of purine biosynthesis (27). The values ranged from 41 to 48% identity over a span of about 282 amino acids, as determined by GAP analysis (6).

Transcriptional analysis of the *vdh* region. Since the DNA sequence data suggested that the *vdh* gene would have a monocistronic transcript, RNA samples prepared from cultures of *S. coelicolor* J802 grown for different lengths of time in R2YE medium or MV were annealed to different ssDNAs made from the *vdh* region and treated with S1 nuclease. The protected DNA was analyzed by Southern blot hybridization with the 2.88-kb *SalI* fragment containing the entire *vdh* gene (Fig. 3) as a ^{32}P-labeled probe. A rightward transcript was found to protect approximately 220 nts of clone A (800-nt *BamHI-KpnI* region) (Fig. 3, lanes 5 to 7), 720 nts of clone B (720-nt *KpnI-BamHI* region) (data not shown), and 350 nts of clone C (420-nt *BamHI-KpnI* region) (Fig. 3, lanes 8 and 9). These results indicate that the entire *vdh* message was within the region defined by clones A to C and that the 5' end of this transcript was about 60 bp upstream of the *vdh* translational start codon. From the relative intensities of the signals shown in lanes 4 to 9 of Fig. 3, it appears that the same amounts of RNA accumulated in the 24- and 48-h MV cultures but that a different amount of RNA accumulated in the 24-h cultures with R2YE medium and MV. A leftward transcript that represented ORF1 and that protected approximately 560 nts of clone D was detected (Fig. 3, lanes 3 and 4). This result indicates that the 5' end of the transcript was about 150 bp upstream of the ORF1 translational start codon. This mRNA was detected in the RNA from the 24- and 48-h samples of the MV cultures, but a much weaker band appeared in the RNA from the R2YE medium culture. No significant difference was seen in the amounts of accumulated ORF1 mRNA between the 24- and 48-h samples. The apparent level of the accumulated ORF1 message in the MV cultures was significantly lower than that of the *vdh* message and almost the same as that of the *vdh* message in the R2YE medium cultures.

High-resolution S1 nuclease mapping and primer extension experiments were then performed to locate the apparent transcriptional start points (tsp) of the vdh and ORF1 genes precisely. The 800-bp *BamHI-KpnI* fragment carrying the divergent promoter region for *vdh* and ORF1 was cloned in M13mp18 and M13mp19, and the ^{35}S-labeled complementary strands were synthesized from the ssDNA templates with the two 24-mer oligodeoxynucleotide primers, a and b, shown in Fig. 2. These ^{35}S-labeled ssDNAs were hybridized to total RNA from *S. coelicolor* J802 grown in MV, treated with S1 nuclease, and analyzed by electrophoresis. We used the same primers for primer extension experiments with RNA extracted from *S. coelicolor* J802 grown in MV for ORF1 and in MV, MM, and MGV for *vdh*. The primer extension products were electrophoresed beside dideoxy sequencing ladders generated with the same primers. Figure 4 shows the results of the primer extension experiments for the *vdh* and ORF1 genes. (The apparent tsp determined for these two genes were the same in the high-resolution S1 nuclease mapping experiments [data not shown].) These
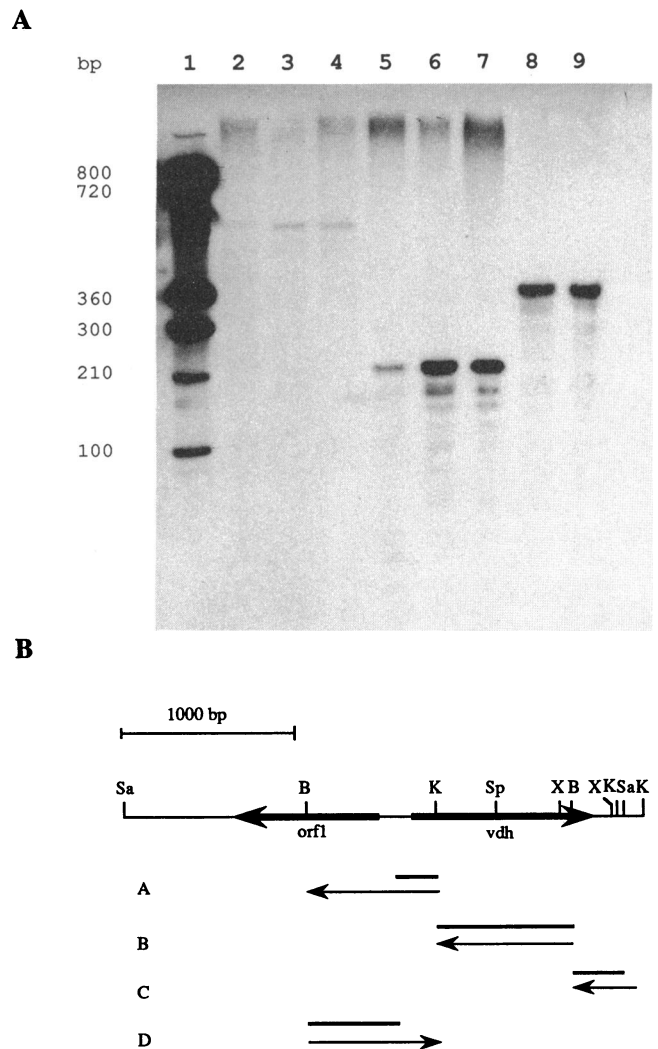

FIG. 3. Low-resolution S1 protection analysis of the *vdh* region. (A) Autoradiograph of protected DNA molecules. Lanes: 1, digested probe fragments as molecular size markers; 2, RNA from a 24-h culture grown in R2YE medium after treatment with DNA fragment D; 3, as in lane 2, except that the culture was grown in MV; 4, as in lane 3, except that the culture was a 48-h one; 5, RNA from a 24-h culture grown in R2YE medium after treatment with DNA fragment A; 6, as in lane 5, except that the culture was grown in MV; 7, as in lane 6, except that the culture was a 48-h one; 8, RNA from a 24-h culture grown in MV after treatment with DNA fragment C; 9, as in lane 8, except that the culture was a 48-h one. DNA fragments A, B, C, and D are the ssDNAs produced from the M13 clones shown in panel B and indicated (arrows). (B) Strategy for S1 analysis. Thick arrows above DNA fragments A, B, C, and D show the positions of S1-protected segments in relation to a partial restriction map of the region with the *vdh* and ORF1 genes. Restriction enzymes are as defined in the legend to Fig. 1.

data indicate that the *vdh* tsp is at an A 63 nts upstream of the GTG translational start codon and that the ORF1 tsp is at an A 158 nts upstream of the presumed translational start codon. The relative levels of the accumulated *vdh* mRNA detected by primer extension in the RNA isolated from cells grown in MM, MGV, and MV at 24 h (this time corresponds to the early stationary phase of growth in MM and the mid-stationary phase of growth in MGV or MV; see Fig. 6)

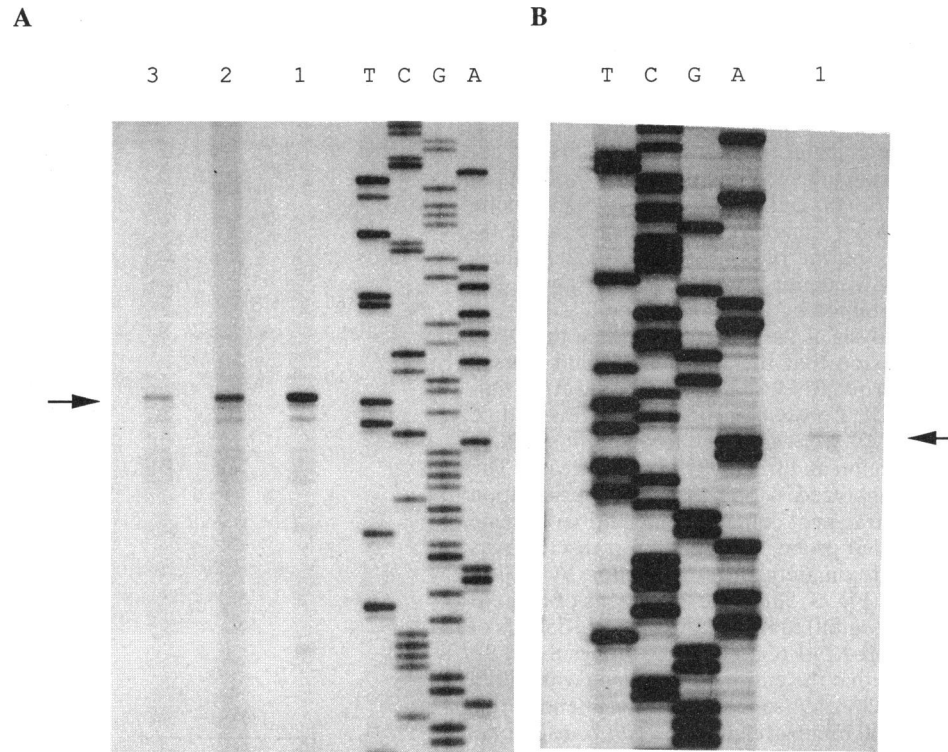A   B

FIG. 4. (A) Primer extension analysis of *vdh*. Twenty micrograms of RNA from a 24-h culture was hybridized to primer a (Fig. 2) (5'-GTGCAGTACATCAGCAGGTGCGCC-3') in each experiment. Lanes: 1, RNA from an MV culture; 2, RNA from an MGV culture; 3, RNA from an MM culture. Lanes labeled TCGA are sequencing reactions generated with the same primer as that used for the primer extension experiments. The arrow indicates the bands for the tsp found in both primer extension and high-resolution S1 nuclease mapping experiments (data not shown). (B) Primer extension analysis of ORF1. Lanes: 1, 20 μg of RNA from a 24-h MV culture was hybridized to primer b (Fig. 2) (5'-GGATCACAAAGGCGCGGGAATCTC-3'). Lanes labeled TCGA are sequencing reactions generated with the same primer. The arrow indicates the probable tsp.

were consistent with the relative levels measured in S1 nuclease protection experiments; *vdh* mRNA appeared to accumulate to the highest level in MV (Fig. 4A, lane 1).

vdh and ORF1 gene inactivation. We disrupted the *vdh* and ORF1 genes in *S. coelicolor* J802 by insertion of the *hyg* gene into their respective coding regions. The *vdh::hyg* and ORF1::*hyg* constructs were cloned in a temperature-sensitive *Streptomyces* plasmid (pWHM1065; Table 1) to yield

pWHM1058 and pWHM1060 (Table 1). After the J802 (pWHM1058) and J802(pWHM1060) transformants were grown at 39°C to eliminate the plasmid, we isolated two Hg^r Th^s transformants from each of the J802 strains containing pWHM1058 or pWHM1060; *S. coelicolor* WMH1505 and WMH1506 (Fig. 5) were chosen for further study. Chromosomal DNA was isolated from both of these strains as well as strain J802 and used for Southern analysis to analyze the
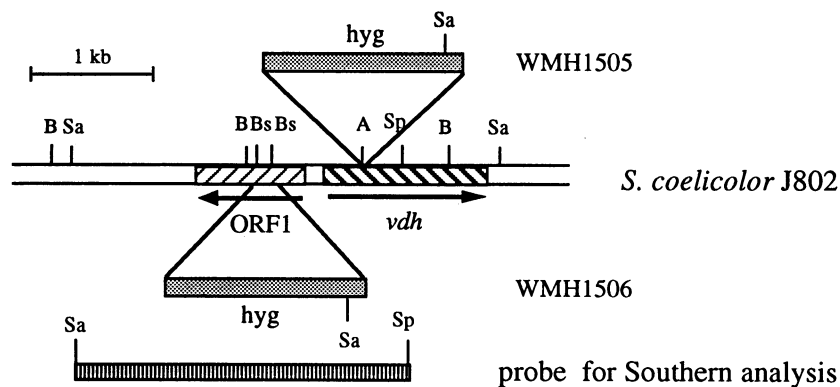
FIG. 5. Genomic structures of strains with disrupted *vdh* or ORF1 genes. Strain WMH1505 has an *hyg* gene inserted into the *Apa*I site of the *vdh* gene, and strain WMH1506 has an *hyg* gene inserted into the *Bss*HII sites of the ORF1 gene. Abbreviations: *hyg*, hygromycin resistance gene; Ap, *Apa*I; B, *Bam*HI; Bs, *Bss*HII; Sa, *Sal*I; Sp, *Sph*I.
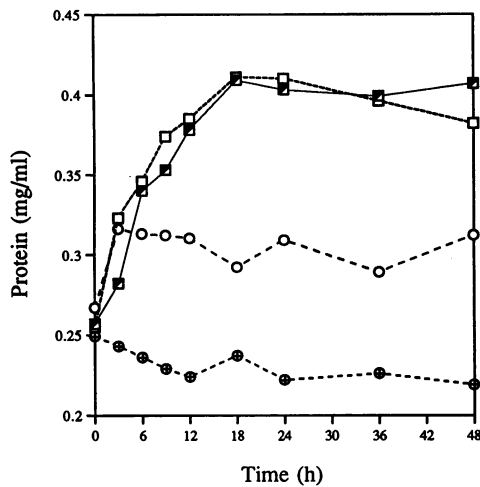
FIG. 6. Kinetics of growth of *S. coelicolor* J802 (□ and ○) and WMH1505 (▨ and ⊕) in MM (▨ and □) and MGV (⊕ and ○). The data are the averages for two separate experiments each with duplicate samples and represent an average error of ≤ ±1.2%.

region near the *vdh* and ORF1 genes. The digoxigenin-alkaline phosphatase-labeled 2.1-kb *Sph*I-*Sal*I fragment used as a probe (Fig. 5) hybridized to a 3.3-kb *Bam*HI fragment in strain WMH1505 instead of the 1.6-kb *Bam*HI fragment, as in strain J802. Digestion with *Sal*I yielded 1.3- and 3.3-kb hybridizing bands in place of a 2.9-kb band. Digestion of strain WMH1506 with *Bam*HI yielded a 3.1-kb hybridizing band in place of the 1.6-kb band, as in strain J802, and digestion with *Sal*I yielded 1.8- and 2.6-kb hybridizing bands in place of the 2.9-kb band. These data (not shown) confirm that the *vdh* and ORF1 genes were indeed disrupted through the expected double-crossover recombinations.

The ORF1::*hyg* mutant showed almost the same Vdh activity as strain J802 under the growth conditions tested to date: 2.24 ± 0.51 and 5.72 ± 0.64 U/mg of protein (16) for the respective 24-h MM and MGV cultures of strain J802 versus 2.07 ± 0.43 and 4.48 ± 0.27 U/mg of protein for the respective 24-h MM and MGV cultures of strain WMH1506. Thus, the ORF1 gene product apparently is not essential for *vdh* function. As expected, the *vdh*::*hyg* mutant had no detectable Vdh activity in cell extracts and lost the ability to grow on MGV with valine, isoleucine, leucine, or α-aminobutyrate as the sole nitrogen source (data not shown). The specific rates of growth of the *vdh*::*hyg* mutant and strain J802 were very similar in MM, but strain WMH1505 did not grow in MGV (Fig. 6).

Effect of *vdh* inactivation on actinorhodin production. Since valine can be metabolized to acetate by a lengthy but documented route (26, 28), we were interested in the possible differences in actinorhodin production in the *vdh*⁺ and *vdh* strains. Figure 7 shows the growth of and actinorhodin production by *S. coelicolor* J802 and WMH1505 in SMM (32) and SMV. The *vdh*::*hyg* mutation did not significantly affect growth or actinorhodin production, although 0.2% L-valine added to SMM caused approximately 32 and 80% increases in actinorhodin production in strains J802 and WMH1505, respectively. Since strain WMH1505 cannot metabolize valine to provide the acetate precursor of actinorhodin synthesis, valine must increase actinorhodin synthesis by some indirect effect.
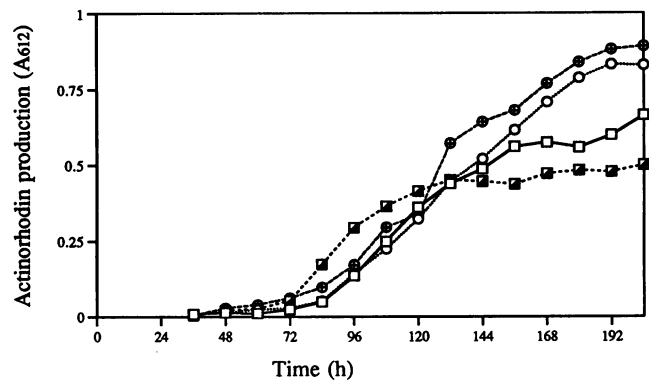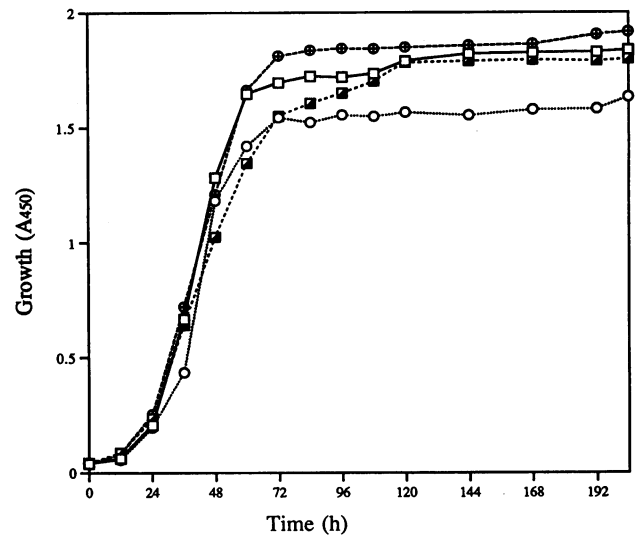

FIG. 7. Growth of and actinorhodin production by *S. coelicolor* J802 (□ and ○) and WMH1505 (▨ and ⊕) in SMM (□ and ▨) and SMV (○ and ⊕). The data are the averages for two separate experiments each with duplicate samples and represent an average error of ≤ ±1.5%.

DISCUSSION

S. coelicolor J802 (*agaA7 dagA1*) was chosen for the genetic analysis of valine utilization (Vut) because it cannot grow on agar as the sole carbon source, a property that has proven useful in the isolation of Vut⁻ mutants by Tn*5096* mutagenesis (33). Sequence analysis has established the identity of the *vdh* locus in *S. coelicolor* A3(2) (wild type) and J802 (33), thus justifying the further use of J802 in our work.

The amino acid sequence of the *S. coelicolor* Vdh enzyme shows significant similarity to those of several other NAD(P)⁺-dependent dehydrogenases. It displays the conserved nicotinamide coenzyme binding region (Fig. 8), which contains the hexapeptide sequence GXG(A)XXG(A), plus a basic residue (usually Lys) 5 to 6 residues and a hydrophobic residue (usually Val) 2 residues before the hexapeptide (4). An aspartic acid 18 residues after the hexapeptide is also found in this region and is thought to be important in coenzyme binding (4). These data are consistent with the fact that the *S. coelicolor* Vdh enzyme requires NAD⁺ for the oxidative deamination of valine in vitro (16). Finally, this Vdh contains adjacent to a Gly-rich region

```
         1                                                              *         *       **   *        +
Ldh}     .......... .melfkymet ydyEQVlFCq DkeSGLkAII AiHdTtLGPA LGGTRmWmYn seeEAleDaL rLarGMtYKn AaAglnlGGG KtVIIGDPrk
Vdh}     vtdvngapad vlhtlfhsdq gghEQVvLCq DraSGLkAVI AlHsTaLGPA LGGTRfYpYa seaEAvaDaL nLarGMsYKn AmAgldhGGG KaVIIGDPeq
Pdh}     makqlekssk ignedvfqki anhEQIvFCn DpvSGLqAII AiHdTtLGPA LGGTRmYpYk nvdEAleDvL rLseGMtYKc AaAdidfGGG KaVIIGDPek
Consensus ---------- ---------- ---EQV-FC- D---SGL-AII A-H-T-LGPA LGGTR-Y-Y- ---EA--D-L -L--GM-YK- A-A----GGG K-VIIGDP--
                                                                                             catalytic region
         101                                                        +       +    *  *   + 200
Ldh}     dKneaMFrAF GrFIqgLnGR YiTaeDvGTt vaDmdiiyqE tdYvtGispe fGssGnpSpa TayGVyrgMk AaakeaFGsd sLegkvvaVq GvGnVayhlc
Vdh}     iKseeLLlAY GrFVasLgGR YvTacDvGTy vaDmdvvarE crWttGrspe nGgaGdsSvl TsfGVyqgMr AaaqhlWGdp tLrdrtvgIa GvGkVghhlv
Pdh}     dKspaLFrAF GqFVesLnGR FyTgtDmGTt mdDfvhaqkE tnFinGipeq yGgsGdsSip TaqGViyaLk AtnqylFGsd sLsgktyaIq GlGkVgykva
Consensus -K---LF-AF G-FV--L-GR Y-T--D-GT- --D------E -----G---- -G--G--S-- T--GV---M- A-----FG-- -L--K---I- G-G-V-----
              binding site                                                              nucleotide coenzyme
         201        *                                                                              300
Ldh}     rhLheeGAkl iVTDInkeVv araveefgak avdpndiygv ecdifapcal ggiindqtip qlkakviags adnqlkeprh gdiihemgiv yapdyvinag
Vdh}     ehLlaeGAhv vVTDVrkdVv rgiterhpsv vavadtdali rvenldiyap calggalndd tvpvltakvv cgaannqlah pgvekdladr gilyapdyvv
Pdh}     eqLlkaGAdl fVTDIhenVl nsikqkseel ggsvtivksd diysvqadif vpcamggiin dktipklkvk avvgsannql kdlrhanvln ekgilyapdy
Consensus --L---GA-- -VTDI---V- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
              binding site
         301                                                                                       400
Ldh}     gvinvadely gynreramkk ieqiydniek vfaiakrdni ptyvaadrma eerietmrka asqflqnghh ilsrrprplt aaraglrrad dggtttmqeq
Vdh}     naggviqvad elhgfdfdrc kvkaskiydt tlaifarake dgippaaaad riaeqrmaea rprp..... ......... ......... ..........
Pdh}     ivnaggliqv adelygpnke rvllktkeiy rslleifnqa aldcittvea anrkcqktie gqqtrnsffs rgrrpkwnik e........ ..........
Consensus ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
         401
Ldh}     kfriltinpg ststkigvfe neraiaskkr satragasai
Consensus ---------- ---------- ---------- ----------
```

FIG. 8. PILEUP (6) comparison of the deduced *S. coelicolor* Vdh protein with leucine dehydrogenase (Ldh) and phenylalanine dehydrogenase (Pdh) from *Bacillus* spp. The consensus line was determined with the PRETTY program (6). A capital letter indicates 3 identical residues or conservative substitutions in each of the vertical columns. Conserved residues that are thought to be important for coenzyme binding and the catalytic region are indicated by * and +.

towards the N terminus (Fig. 8) a lysine residue that also has been proven to be essential for enzyme activity in such dehydrogenases (22), but it lacks further from this region towards the C terminus (+ at 100 in Fig. 8) the Lys-6 residue that is conserved in the leucine and phenylalanine dehydrogenases from *Bacillus* spp. (15).

The nucleotide sequence data show that the *vdh* gene is flanked by two divergently transcribed genes. The downstream, ORF2, gene is a homolog of the *purM* genes from *E. coli* (27) and *Bacillus* spp. The role of the ORF1 gene is unknown, although our results suggest that this gene is not essential for *vdh* function under the conditions tested.

Earlier studies showed that all four Vdh enzymes purified from *Streptomyces* spp. have similar K_m values for 2-oxoisovaleric acid and NAD(H), pH optima in the oxidative and reductive directions, and the ability to oxidatively deaminate valine, isoleucine, leucine, and α-aminobutyrate, although at different relative rates (16, 23, 34, 35). In the present study, we have found that *S. coelicolor* J802 can utilize these branched-chain amino acids as the sole N and C sources for growth. Since a *vdh* mutant isolated as a *vdh::hyg* gene disruption could not use valine, isoleucine, leucine, or α-aminobutyrate as a sole N source and had no detectable Vdh activity in cell extracts, *S. coelicolor* must have a single gene for the oxidative deamination of branched-chain amino acids.

Certain features of the *vdh* gene suggest that the regulation of its expression may be complex. Our results show that the *vdh* gene is expressed as a monocistronic mRNA and is transcribed from a single major promoter, separated by only 8 bp from the 5' end of the divergent ORF1 transcript (Fig. 2). This bidirectional overlapping promoter region contains a group of repeat sequences that we know from studies of deletion mutants are import for *vdh* expression (33) and might be binding sites for proteins involved in transcription (in addition to RNA polymerase). Furthermore, from the results of our studies in progress of the regulation of *vdh* expression, we have found that *vdh* is not specifically

induced by valine or the other branched-chain amino acids, when compared with growth on asparagine as the sole N source, whereas Vdh activity is increased approximately threefold at 14 h by valine, and that its expression is affected in a complex way by carbon sources (33).

REFERENCES

1. **Bibb, M., G. Jones, R. Joseph, M. J. Buttner, and J. M. Ward.** 1987. The agarase gene (*dagA*) of *Streptomyces coelicolor* A3(2): affinity purification and characterization of the cloned gene product. J. Gen. Microbiol. 133:2089–2096.
2. **Bibb, M. J., and S. N. Cohen.** 1982. Gene expression in *Streptomyces*: construction and application of promoter-probe plasmid vectors in *Streptomyces lividans*. Mol. Gen. Genet. 187:265–277.
3. **Bibb, M. J., P. R. Findlay, and M. W. Johnson.** 1984. The relationship between base composition and codon usage in bacterial genes and its use for the simple and reliable identification of protein coding sequences. Gene 30:157–166.
4. **Birktoft, J. J., and L. J. Banaszak.** 1984. Structure-function relationships among nicotinamide-adenine dinucleotide dependent oxidoreductases. Pept. Protein Rev. 4:1–46.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.
6. **Devereux, J., P. Haeberli, and O. Smithies.** 1988. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12:387–395.
7. **Fisher, S., and L. Wray.** 1989. Regulation of glutamine synthetase in *Streptomyces coelicolor*. J. Bacteriol. 171:2378–2383.
8. **Hahn, D. R., P. J. Solenberg, and R. H. Baltz.** 1991. Tn*5099*, a *xylE* promoter probe transposon for *Streptomyces* spp. J. Bacteriol. 173:5573–5577.

9. Hodgson, D. A., and K. F. Chater. 1981. A chromosomal locus controlling extracellular agarase production by *Streptomyces coelicolor* A3(2) and its inactivation by chromosomal integration of plasmid SCP1. J. Gen. Microbiol. 124:339–348.

10. Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf. 1985. Genetic manipulation of *Streptomyces*. A laboratory manual. John Innes Institute, Norwich, United Kingdom.

11. Hoshino, T., and K. Kose. 1990. Genetic analysis of the *Pseudomonas aeruginosa* PAO high-affinity branched-chain amino acid transport system by use of plasmids carrying the *bra* genes. J. Bacteriol. 172:5540–5543.

12. Kaneda, T. 1991. Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. Microbiol. Rev. 55:288–302.

13. Massey, L. M., J. R. Sokatch, and R. S. Conrad. 1976. Branched-chain amino acid catabolism in bacteria. Bacteriol. Rev. 40:42–54.

14. Morelle, G. 1988. A plasmid extraction procedure on a miniprep-scale. Focus 11:7–8.

15. Nagata, S., K. Tanizawa, N. Esaki, Y. Sakamoto, T. Ohshima, H. Tanaka, and K. Soda. 1988. Gene cloning and sequence determination of leucine dehydrogenase from *Bacillus stearothermophilus* and structural comparison with other NAD(P)$^+$ dependent dehydrogenases. Biochemistry 27:9056–9062.

16. Navarrete, R. M., J. A. Vara, and C. R. Hutchinson. 1990. Purification of an inducible L-valine dehydrogenase of *Streptomyces coelicolor* A3(2). J. Gen. Microbiol. 136:273–281.

17. Neal, R. J., and K. F. Chater. 1991. Bidirectional promoter and terminator regions bracket *mmr*, a resistance gene embedded in the *Streptomyces coelicolor* A3(2) gene cluster encoding methylenomycin production. Gene 100:75–83.

18. Ohtsuka, E., S. Matsuki, M. Ikehara, Y. Takahoshi, and K. Matsubara. 1985. An alternative approach to deoxyoligonucleotides as hybridization probes by insertion of deoxyinosine at ambiguous codon positions. J. Biol. Chem. 260:2605–2608.

19. Okazaki, N., Y. Hibino, Y. Asano, M. Ohmori, N. Numao, and K. Kondo. 1988. Cloning and nucleotide sequencing of phenylalanine dehydrogenase gene of *Bacillus sphaericus*. Gene 63:337–341.

20. Omura, S., Y. Tanaka, H. Mamada, and R. Masuma. 1984. Effect of ammonium ion, inorganic phosphate and amino acids on the biosynthesis of protylonolide, a precursor of tylosin aglycone. J. Antibiot. 37:1362–1369.

21. Omura, S., K. Tsuzuki, Y. Tanaka, H. Sakakibara, M. Aizawa, and G. Lukacs. 1983. Valine as a precursor of the n-butyrate unit in the biosynthesis of macrolide aglycones. J. Antibiot. 36:614–616.

22. Piszkiewicz, D., M. Landon, and E. L. Smith. 1970. Bovine liver glutamate dehydrogenase. J. Biol. Chem. 245:2622–2626.

23. Priestly, N. D., and J. A. Robinson. 1989. Purification and catalytic properties of L-valine dehydrogenase from *Streptomyces cinnamonensis*. Biochem. J. 261:853–861.

24. Reynolds, K. A., D. O'Hagan, D. Gani, and J. A. Robinson. 1988. Butyrate metabolism in Streptomycetes. Characterization of an intramolecular vicinal interchange rearrangement linking isobutyrate and butyrate in *Streptomyces cinnamonensis*. J. Chem. Soc. Perkin Trans. 1 1988:3195–3208.

25. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

26. Sherman, M. M., S. Yue, and C. R. Hutchinson. 1986. Biosynthesis of lasalocid A. Metabolic interrelationships of carboxylic acid precursors and polyether antibiotics. J. Antibiot. 39:1135–1143.

27. Smith, J. M., and H. A. Daum III. 1986. Nucleotide sequence of the *purM* gene encoding 5′-phosphoribosyl-5-aminoimidazole synthetase of *Escherichia coli* K12. J. Biol. Chem. 261:10632–10636.

28. Sood, G. R., D. M. Ashworth, A. A. Ajaz, and J. A. Robinson. 1988. Biosynthesis of the polyether antibiotic monensin A. Results from the incorporation of labeled acetate and propionate as a probe of the carbon chain assembly process. J. Chem. Soc. Perkin Trans. 1 1988:3183–3194.

29. Stein, D. S., K. J. Kendall, and S. N. Cohen. 1989. Identification and analysis of transcription regulatory signals for the *kil* and *kor* genes of *Streptomyces* plasmid pIJ101. J. Bacteriol. 171:5768–5775.

30. Strauch, E., E. Takano, H. A. Baylis, and M. J. Bibb. 1991. The stringent response in *Streptomyces coelicolor* A3(2). Mol. Microbiol. 5:289–298.

31. Sykes, P. J., G. Burns, J. Menard, K. Hatter, and J. R. Sokatch. 1987. Molecular cloning of genes encoding branched-chain keto acid dehydrogenase of *Pseudomonas putida*. J. Bacteriol. 169:1619–1625.

32. Takano, E., H. C. Gramaji, E. Strauch, N. Andres, J. White, and M. J. Bibb. 1992. Transcriptional regulation of the *redD* transcriptional activator gene accounts for growth phase dependent production of the antibiotic undecylprodigiosin in *Streptomyces coelicolor* A3(2). Mol. Microbiol. 6:2797–2804.

33. Tang, L., and C. R. Hutchinson. Unpublished data.

34. Vancura, A., I. Vancurora, J. Volc, S. M. Fussey, M. Flieger, J. Neuzil, J. Marsalek, and V. Behal. 1988. Valine dehydrogenase from *Streptomyces fradiae*: purification and properties. J. Gen. Microbiol. 138:3213–3219.

35. Vancurora, I., A. Vancura, J. Volc, J. Neuzil, M. Flieger, G. Basarova, and V. Behal. 1988. Isolation and characterization of valine dehydrogenase from *Streptomyces aureofaciens*. J. Bacteriol. 170:5192–5196.

36. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–119.