# GENETICS OF VALINE CATABOLISM, ITS REGULATION IN *STREPTOMYCES COELICOLOR* AND THE ROLE OF VALINE CATABOLITES AS PRECURSORS FOR THE MACROLIDE ANTIBIOTIC BIOSYNTHESIS

by

LI TANG

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# Abbreviations

Am ... apramycin

Ap ... Ampicillin

bp ... base pairs

C ... carbon

CoA ... coenzyme A

dig-AP ... digoxigenin alkaline phosphatase

h ... hours

Hg ... hygromycin

*hmmA* ... homologous to mmsA

*hyg* ... hygromycin resistance gene

IR ... inverted repeats

kb ... kilobase

MM ... minimal medium

MW ... molecular weight

*mmsA* ... methylmalonate-semialdehyde dehydrogenase gene

N ... nitrogen

$NH_4^+$ ... ammonium ion

nt ... nucleotides

orf ... open reading frame

PCR ... polymerase chain reaction

RT ... room temperature

ss DNA ... single strand DNA

Th ... thiostrepton

ts ... temperature sensitive

tsp ... transcriptional start piont

*tsr* ... thiostrepton resistance gene

Vdh ... valine (branched-chain amino acid) dehydrogenase

*vdh* ... valine dehydrogenase gene

vut ... valine utilization

XylE ... catechol dioxygenase

*xylE* ...catechol dioxygenase gene

WT ... wild-type

## Abstract

The gene encoding the valine (branched-chain amino acid) dehydrogenase (Vdh) from *Streptomyces coelicolor* was characterized as follows. The *vdh* gene was identified by hybridization to a specific oligodeoxynucleotide that was synthesized according to the N-terminal amino acid sequence of the purified Vdh. Nucleotide sequence analysis predicts that the *vdh* gene contains a 364 amino acid open reading frame that should produce an $M_r$ 38,305 protein. The deduced amino acid sequence of the Vdh protein is significantly similar to several other amino acid dehydrogenases, especially leucine and phenylalanine dehydrogenase from *Bacillus* spp. The *vdh* gene is apparently transcribed from a single major transcriptional start point, separated by only 8 base pairs (bp) from the 5' end of a divergent transcript, which is located 63 bp upstream from the *vdh* translational start. 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 caused an approximately 32% and 80% increase in actinorhodin production in the *vdh*$^+$ and *vdh*$^-$ strains, respectively.

Expression of the *vdh* gene is regulated by valine, glucose and ammonium ion ($NH_4^+$) at the transcriptional level. The results of assays for the level of accumulated *vdh* mRNA in the S. *coelicolor* J802 strain by primer extension experiments and for the level of XylE activity in *S. coelicolor* J802(*vdh*::*xylE*) transformants show that transcription of the *vdh* gene is induced approx. 2.5-fold by valine compared to asparagine as the sole nitrogen source. This induction is repressed by glucose, compared to glycerol, and by $NH_4^+$. Glucose repression is released in *S. coelicolor* M480, a glucose kinase (*glkA*) deletion mutant, and this suggests that glucose repression of *vdh* and carbohydrate metabolism are due to the same mechanism in *S.*

*coelicolor*, which involves glucose kinase. Deletion of the sequence upstream of the *vdh* promoter region from -47 or -96 nucleotide (nt) relative to the transcription start point at +1 results in uninducible *vdh* promoter activity. The presence of *vdh* sequence between -47 to +205 nt on a multicopy plasmid results in decreased transcription from the chromosomal *vdh* gene in the presence of valine, possibly by titration of a transcriptional activator, and the presence of *vdh* sequence between -96 to +205 nt that contains a dyad repeat sequence results in increased transcription from the chromosomal *vdh* gene in the absence of the valine inducer, possibly by titration of a repressor. Using DNA gel mobility shift assays with total protein extracts, protein binding was detected to 2 different regions upstream of the *vdh* gene and the -10/-35 region of the *vdh* promoter. These binding sites correspond to the upstream activation and repression sequences predicted from the results of the plasmid titration assays.

Targeted inactivation of the *vdh* gene was used to study the role of valine catabolism in the production of spiramycin in *Streptomyces ambofaciens*. The deduced product of the *vdh* gene, cloned and sequenced from *S. ambofaciens* ATCC 15154 shows 96% identical over a span of the first N-terminal 107 amino acids with the deduced product of the *S. coelicolor vdh* gene. The organization of the regions flanking the *vdh* gene is the same as in *S. coelicolor*. Inactivation of the genomic copy of the *vdh* gene in *S. ambofaciens* by insertion of a hygromycin resistance gene (*hyg*) caused loss of the Vdh activity, and thus only one enzyme is responsible for the Vdh activity in this organism as in *S. coelicolor*. Analysis of the culture broths by bioassay revealed that the *vdh*::*hyg* mutants produce an approx. 4-fold lower level of spiramycin compared with the wild-type *S. ambofaciens* strain, while maintaining essentially identical growth in a defined miminal medium with glucose and asparagine as the carbon and nitrogen sources. The addition of the valine catabolites, propionate or isobutyrate, and introduction of the wild-type *vdh* gene into the *vdh*::*hyg* mutant reversed the negative effect of the *vdh* mutation on spiramycin producion. These data show that the catabolism of valine is a

major source of fatty acid precursors for the biosynthesis of a typical macrolide under a defined growth condition and imply that amino acid catabolism is a vital source of certain antibiotic precursors in actinomycetes.

A homolog of the *mmsA* gene of *Pseudomonas aeruginosa*, which encodes methylmalonate-semialdehyde dehydrogenase and is involved in valine catabolism in pseudomonads and mammals, was cloned and sequenced from S. *coelicolor*. There are two orfs in the 4.0 kb sequenced region, which were convergently transcribed and separated by a 62 nt noncoding region. The deduced amino acid sequence of the *hmmA* gene (homlogous to *mmsA*) is significantly similar to a variety of prokaryotic and eukaryotic aldehyde dehydrogenases (NAD+), particularly to the MmsA protein from *P. aeruginosa* (approx. 47% identical overall by GAP analysis). However, no significant homology was found between the deduced product of ORF1, the second gene, and known proteins in the databases.

A method for Tn*5096* transposon mutagenesis of *S. coelicolor* for the isolation of valine utilization (vut) mutants was developed. About 10 vut mutants were classified by DNA hybridization and growth in minimal medium containing valine as the sole N or N and C sources. Among these mutants, WMH1603 had Tn*5096* insertions in the *vdh* locus. One class (two vut mutants) had Tn*5096* inserted at a locus different than the *vdh* gene and their characteristics was investigated by cloning and sequence analysis. In this class, Tn*5096* did insert into an ORF in one region, but we could not deduce its function by comparison of its deduced product with known proteins in the databases.

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# Chapter 1

## Sequence, Transcriptional and Functional Analysis of the Valine (Branched-chain Amino Acid) Dehydrogenase Gene of *Streptomyces coelicolor*

A modified version of this paper was published in J. Bacteriol. (1993, 175:4176-4185) with Dr. C. R. Hutchinson and myself as the sole authors.

# INTRODUCTION

Branched-chain amino acids are normally catabolized in bacteria by an initial dehydrogenation step, followed by oxidative decarboxylation of the resulting 2-keto acid (13). Most studies of the catabolism of branched-chain amino acids have been done with species of *Pseudomonas*. 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, the complete catabolism of a branched-chain amino acid requires the cooperation of two sequential series of reactions. The enzymes in the first series comprise a common pathway and catalyze the conversion of leucine, valine and isoleucine 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-CoA metabolites formed subsequent to the common pathway are catabolized by three separate series of enzymes, one specific for each initial amino acid (13).

In *Streptomyces* spp., valine degradation takes a somewhat different course than in other bacteria (24, 26). C-2 of valine or C-1 of 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). These bacteria also have an enzyme that catalyzes the reversible intramolecular rearrangement of the 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 catalyses the oxidative deamination of branched 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 amino acids), is repressed by glucose and $NH_4^+$, 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 ($NH_4^+$) (20). Superior chemically defined media for tylosin and monensin production contain L-valine as a major carbon and nitrogen source for this reason (16, 26). The branched-chain amino acid dehydrogenases may also provide the starter units for the synthesis of iso- and anteiso-fatty acids that predominate in the cellular lipids of actinomycetes (12).

Four L-valine dehydrogenases (16, 23, 34, 35) have been purified and characterized from *Streptomyces* spp. We chose to study the Vdh from *Streptomyces coelicolor* because a subsequent investigation of the genetics of valine catabolism could lead to data comparable with the properties of the other catabolic enzymes and their genes that have been studied in this genetically well-characterized organism (for example, see refs. 3 and 9). The *S. coelicolor* Vdh is a dimeric enzyme consisting of 2 identical subunits with a $M_r$ 40,000. Its activity is induced by D- or L-valine and repressed in the presence of glucose and $NH_4^+$ (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 that of a divergently transcribed gene (ORF1) of unknown function, which 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. Disruption of the *vdh* gene in the *S. coelicolor* J802 strain blocks growth on branched-chain amino acids as the sole N source

but does not lower the production of actinorhodin, it is principal acetate-derived antibiotic.

## MATERIALS AND METHODS

**Strains, plasmids and culture conditions.** *Escherichia coli* strains DH-5α (25), GM2929 (*dam*-, *dcm*-), obtained from Doug MacNeil, Merck, Sharp & Dohme Co., and JM105 (25), used as hosts for plasmids or for M13 DNA sequencing, were grown at 37°C on LB and 2xYT media (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 modified MM: MGV, containing 1% L-valine instead of asparagine; and MV, containing 1% L-valine without asparagine and glucose. Media for actinorhodin production were: SMM, a minimal medium consisting of 5% PEG 6000 (BDH), 5 mM $MgSO_4$, 25 mM TES (Sigma) pH 7.2, 1 mM phosphate, 1% wt/vol glucose and 0.2% wt/vol casamino acids (33); and SMV, 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 (Th; obtained from S. G. Lucania, The Squibb Institute for Medical Research, Princeton, NJ) 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 performed as described by Morelle (14). Single-stranded (ss) M13 DNA 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

Table 1. Plasmids and strains used in this work

| Plasmid or strain | Characteristics[a] | References |
|---|---|---|
| Plasmids | | |
| pXH106 | *bla tsr hyg*; Tn*5099*; ts | 8 |
| pWHM1050 | N-terminal of *vdh* gene from A3(2) strain on pUC18 | This study |
| pWHM1051 | *vdh*, ORF1 and ORF2 genes from J802 strain on pUC18 | " |
| pWHM1065 | 6.0 kb *Hin*dIII fragment of pXH106 was religated | " |
| pWHM1057 | An 1.6 kb *Bam*HI fragment of the *vdh* region from pWHM1051 was subcloned into pUC18 | " |
| pWHM1058 | The 1.7 kb *Sma*I-*Eco*RV fragment of the *hyg* gene from pXH106 was inserted into the *Apa*I site of pWHM1057. A 3.3 kb *Bam*HI fragment was isolated from the resulting plasmid and subcloned into the *Bam*HI site of pWHM1065 | " |
| pWHM1059 | A 2.1 kb *Sal*I-*Sph*I fragment of the ORF1 region from pWHM1051 was subcloned into pUC18 | " |
| pWHM1060 | The 1.7 kb *Sma*I-*Eco*RI fragment of the *hyg* gene from pXH106 was inserted into the *Bss*HII sites of pWHM1059. A 3.6 kb *Hin*dIII-*Bsp*HI fragment was isolated from the resulting plasmid and cloned into the *Hin*dIII-*Nco*I sites of pWHM1065 | " |
| Strains | | |
| WMH1505 | *vdh*::*hyg* gene disrupted mutant of *S. coelicolor* J802 | This study |
| WMH1506 | ORF1::*hyg* gene disrupted mutant of *S. coelicolor* J802 | " |

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

separation on agarose gels, followed by treatment with the USBioclean® MP kit (United States Biochemicals (USB), Cleveland, OH) according to the manufacturer's directions. Streptomycete genomic DNA was isolated by the lysozyme-SDS method of Hopwood et al. (10). Oligodeoxynucleotides for hybridization probes or sequencing primers were synthesized by an Applied Biosystems Model 391 DNA synthesizer and purified according to the manufacturer's protocols.

A 50-mer oligodeoxynucleotide, 5'-ACCGACGTCAACGGCGCCCCIGCCGACGT-CCTICACACCCTITTCCACTC-3', representing the coding sequence of the first 17 N-terminal residues [TDVNGAPADVLHTLFHS] of the purified Vdh from *S. coelicolor* A3(2) and containing inosine (I) substitutions at especially degenerate codon positions (18), was end-labeled with $^{32}P$ by standard methods (25) and used to probe several restriction enzyme digestions of the *S. coelicolor* genomic DNA. Southern-blot hybridization showed that 1.15 kilobase (kb) *Bam*HI-*Sph*I and 7.0 kb *Pst*I-*Sst*I DNA fragments hybridized with the [$^{32}P$]-labeled probe, although the signal was much weaker with the latter fragment. *S. coelicolor* J802 genomic DNA was double-digested with *Bam*HI-*Sph*I and *Pst*I-*Sst*I, size-fractionated by electrophoresis on a 1% agarose gel, and 1.0 to 1.5 kb *Bam*HI-*Sph*I DNA fragments and 6.5 to 7.5 kb *Pst*I-*Sst*I 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 base pair (bp) *Sph*I-*Kpn*I DNA segment from pWHM1050 to clone the *vdh* gene as pWHM1051.

**DNA hybridization and sequencing**. DNA was size-fractionated by electrophoresis and transfered to Hybond-N membranes (Amersham, Arlington Heights, IL) by capillary transfer or electrotransfer (25). Prehybridization and hybridization to [$\alpha$–$^{32}P$]-dCTP labeled DNA fragments or [$\gamma$–$^{32}P$]-dATP labeled oligonucleotides were carried out at

42°C in 50% formamide, 5x SSC (25), 2x Denhardt solution (25), 100 µg of denatured salmon sperm DNA per ml, and 0.1% sodium dodecyl sulfate (SDS) (10, 25). The dig-AP labeling, hybridization, and detection were done with the Genius® kit, following the manufacturer's protocols (Boehringer Mannheim, Indianapolis, IN). The blot was washed two times with 1x SSC-0.1% SDS for 30 min at 42°C and then two times in 0.1x SSC-0.1% SDS for 30 min at 68°C, except for the oligonucleotide probing which was washed two times with 1x SSC-0.1% SDS for 30 min at 42°C and two times in 0.5x SSC-0.1% SDS for 15 min at 60°C .

DNA segments for sequencing were purified and subcloned into M13mp18 or M13mp19 (36). Exonuclease III-S1 nuclease deletions were prepared by the Erase-a-Base system (Promega Biotech, Madison, WI). 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 (USB), following the manufacturer's instructions. 7-Deaza-dGTP was used in the 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 using the DNASTAR (Madison, WI) 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 databases by using the FASTA and TFASTA programs (6).

**RNA isolation.** Cultures of *S. coelicolor* were grown in R2YE medium for 2 to 3 days, 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 media, or MM containing inducers, and grown another 24 or 48 h. RNA was isolated from the recovered cells essentially by the method of Fisher and Wray (7), except that cells were lysed by grinding with a mortar and

pestle in the presence of liquid nitrogen, followed by the addition of the guanidinium isothiocyanate buffer and centrifugation in cesium chloride solution. RNA concentration was determined by measuring the $A_{260}$ of an mRNA solution in diethyl pyrocarbonate (DEPC) treated $H_2O$.

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

**High resolution S1 nuclease and primer extension mapping**. M13mp18 and -mp19 subclones containing a portion of the 5'-end of either the *vdh* or the ORF1 genes were used to prepare radiolabeled ssDNA 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; 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 labelling mixture, using 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 and eluted by the crush and soak method (25), then hybridized with 40 µg of RNA and treated with S1 nuclease. The reaction products

were analyzed on a 6% polyacrylamide-8 M urea-12% formamide gel along with dideoxy DNA sequencing ladders made with the same primer.

A primer extension experiment was conducted by a modification of the method of Stein et al. (29). The same primers as used in S1 mapping were annealed to 20 µg of RNA in the reverse transcriptase reaction buffer (supplied by BRL) in a 50 µl vol. Primer extension was performed by adding 2.5 µl [$^{35}$S]-dCTP, 5.0 µl labelling mix (10 mM dGTP, dTTP, dATP), 5.0 µl 0.1M DTT, 0.5 µl of a 1% (wt/vol) solution of actinomycin D (Sigma), and 200 U Superscript M-MLV reverse transcriptase (BRL). The reaction was incubated for 30 min at 42°C, then 0.5 µl of unlabeled 100 mM dCTP was added and the reaction 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.** To construct the plasmid used for *vdh* gene inactivation, an 1.6 kb *Bam*HI DNA fragment of the *vdh* coding region from pWHM1051 was subcloned into *Bam*HI-digested pUC18 to give 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 this plasmid and subcloned into the *Bam*HI site of a modified pXH106 (pWHM1065), which was treated with *Hin*dIII and ligated, resulting in deletion of a portion of the Tn*5099* transposon, to give plasmid pWHM1058 (Fig. 5). For the 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 give 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 *Hind*III-*Bsp*HI fragment was isolated from the resulting plasmid and cloned into the *Hin*dIII-*Nco*I sites of pWHM1065 resulting in plasmid pWHM1060 (Fig. 5).

pWHM1058 and pWHM1060 were introduced into *S. coelicolor* J802 by transformation, $Th^R$ transformants were selected on plates and grown in R2YE liquid plus 200 U/ml hygromycin (Hg; Sigma) at 28°C for 3 days. These cultures were homogenized then 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/ml Hg and incubated at 39°C for 4 to 5 days, then spores were collected and screened for $Hg^RTh^S$ clones.

**Actinorhodin production and Vdh activity measurements.** For these determinations, *S. coelicolor* cultures were grown at 30°C in 25 ml of R2YE for 48 h with shaking at 200 rpm. The mycelial cells were harvested by centrifugation, washed 2x with MM without glucose and asparagine (Mm) and suspended in 5 ml of Mm. Equal portions of this cell suspension were added to the MM and MGV media, and the cultures were incubated at 30°C for 24 h prior to assaying the Vdh specific activity or determining the protein concentration of cell aliquots. Vdh activities were determined as described by Navarrete et al. (16). Culture samples (300 µl) were added to 300 µl 1 N NaOH and incubated at 37°C for 24 h, then the protein concentrations were determined in aliquots of these samples by the method of Bradford (5) by using bovine serum albumin as the standard. Actinorhodin content in the SMM and SMV media was estimated spectrophotometrically by measuring the $OD_{612}$ 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 from 1 to 3007 nucleotides described in this paper have been deposited at EMBL and GenBank with the

accession number L13455.

## RESULTS

**Cloning the *S. coelicolor vdh* gene.** The *vdh* gene was cloned by hybridization to an oligodeoxynucleotide constructed in accord with the N-terminal amino acid sequence of the purified Vdh from *S. coelicolor* A3(2) (16). Screening of plasmid DNA isolated from a minilibrary consisting of 1.0 to 1.5 kb *Bam*HI-*Sph*I DNA fragments of *S. coelicolor* A3(2) genomic DNA yielded one positive clone, pWHM1050, which was shown to contain a 1.15 kb *Bam*HI-*Sph*I DNA fragment (see Figs. 1 and 2). Southern analysis showed that the 50-mer oligodeoxynucleotide probe hybridized to only one and the same 1.15 kb *Bam*HI-*Sph*I DNA fragment in the genomic and cloned DNA (Fig. 1). By using a [$^{32}$P]-labeled 350 bp *Sph*I-*Kpn*I fragment from pWHM1050 DNA that contains part of the N-terminal region of Vdh as a probe to screen the *Pst*I-*Sst*I 6.5 to 7.5 kb minilibrary of *S. coelicolor* J802 DNA (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. 2.

Using the 350 bp *Sph*I-*Kpn*I fragment as the probe, we detected homologous DNA sequences in hybridization experiments (Materials and Methods) with genomic Southern blots of *Streptomyces lividans* TK24, the salinomycin-producing *Streptomyces albus* ATCC 21838, the monensin-producing *Streptomyces cinnamonensis* WMH585, the tetracenomycin-producing *Streptomyces glaucescens* GLA.O, the tylosin-producing *Streptomyces fradiae* C373.1, the spiramycin-producing *Streptomyces ambofaciens* ATCC 15154 and the midecamycin-producing *Streptomyces mycarofaciens* 1748, but not in the erythromycin-producing *Saccharopolyspora erythraea* WMH22 (Fig. 3).

**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 using a combination of restriction fragments and exonuclease III deletions. Restriction sites were overlapped as

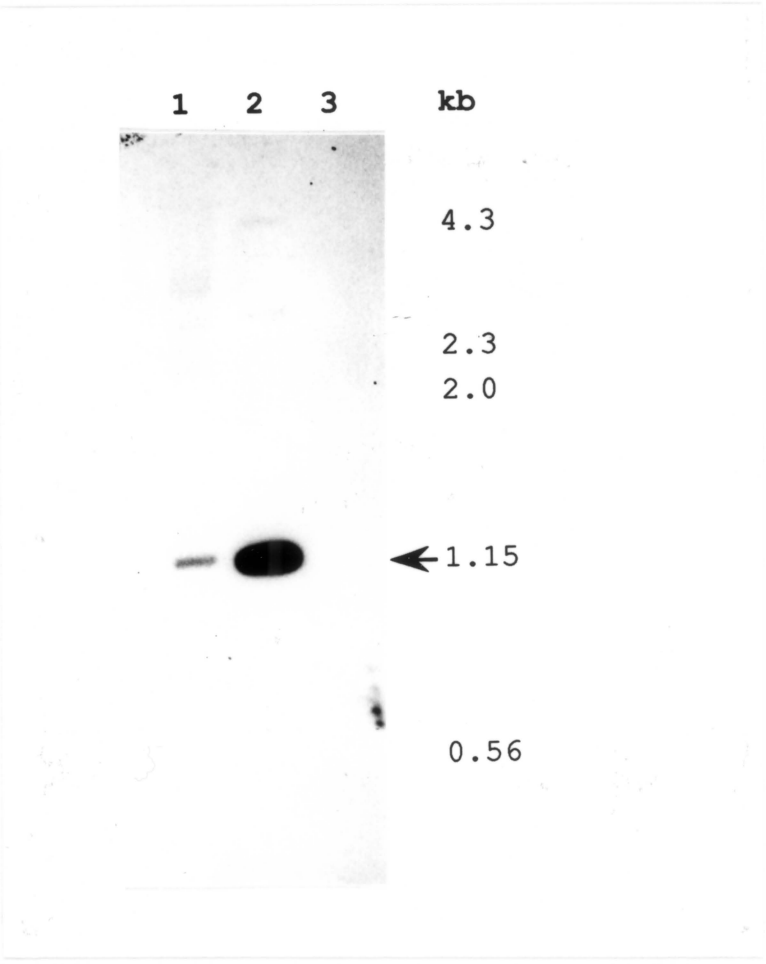



FIG. 1. Southern-blot analysis of *S. coelicolor* J802 chromosomal DNA and pWHM1050 DNA hybridized with a 50-mer oligodeoxynucleotide probe. Lane 1, *S. coelicolor* total DNA digested with *Bam*HI-*Sph*I. Lane 2, pWHM1050 DNA digested with *Bam*HI-*Sph*I. Lane 3, λ phage DNA digested with *Hin*dIII.

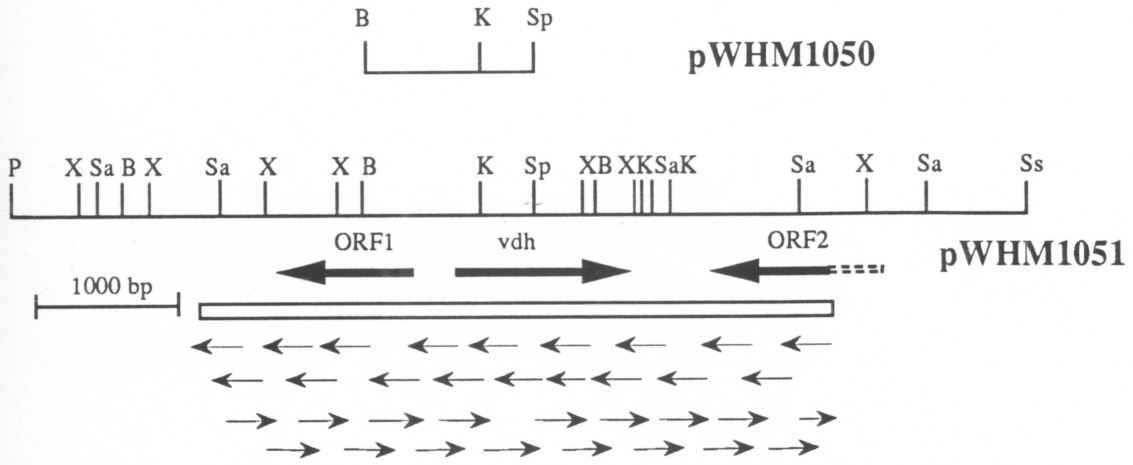



FIG. 2. Restriction map and DNA sequencing strategy for the 4.27 kb DNA fragment cloned in pWHM1051. The thick bold arrows indicate the direction of the transcription for the three orfs. The open box beneath them indicates the region that was sequenced. The thinner arrows beneath this box illustrate the sequencing strategy. Restriction enzyme abbreviations: B, *Bam*HI; K, *Kpn*I; Sp, *Sph*I; Sa, *Sal*I; P, *Pst*I; Ss, *Sst*I; X, *Xma*I;

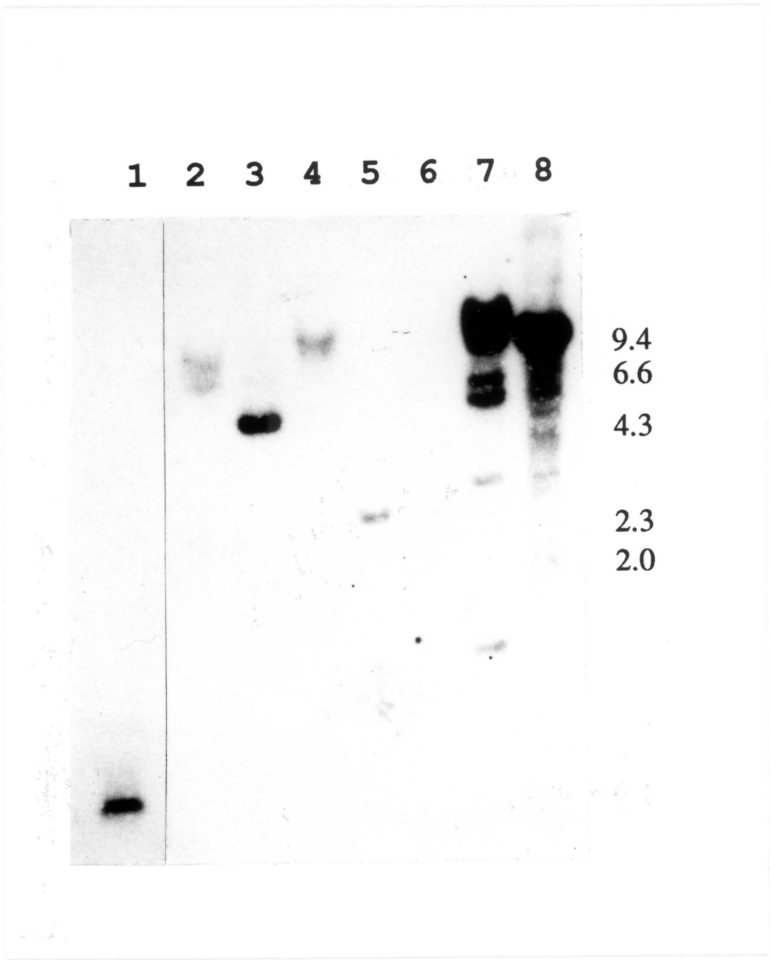
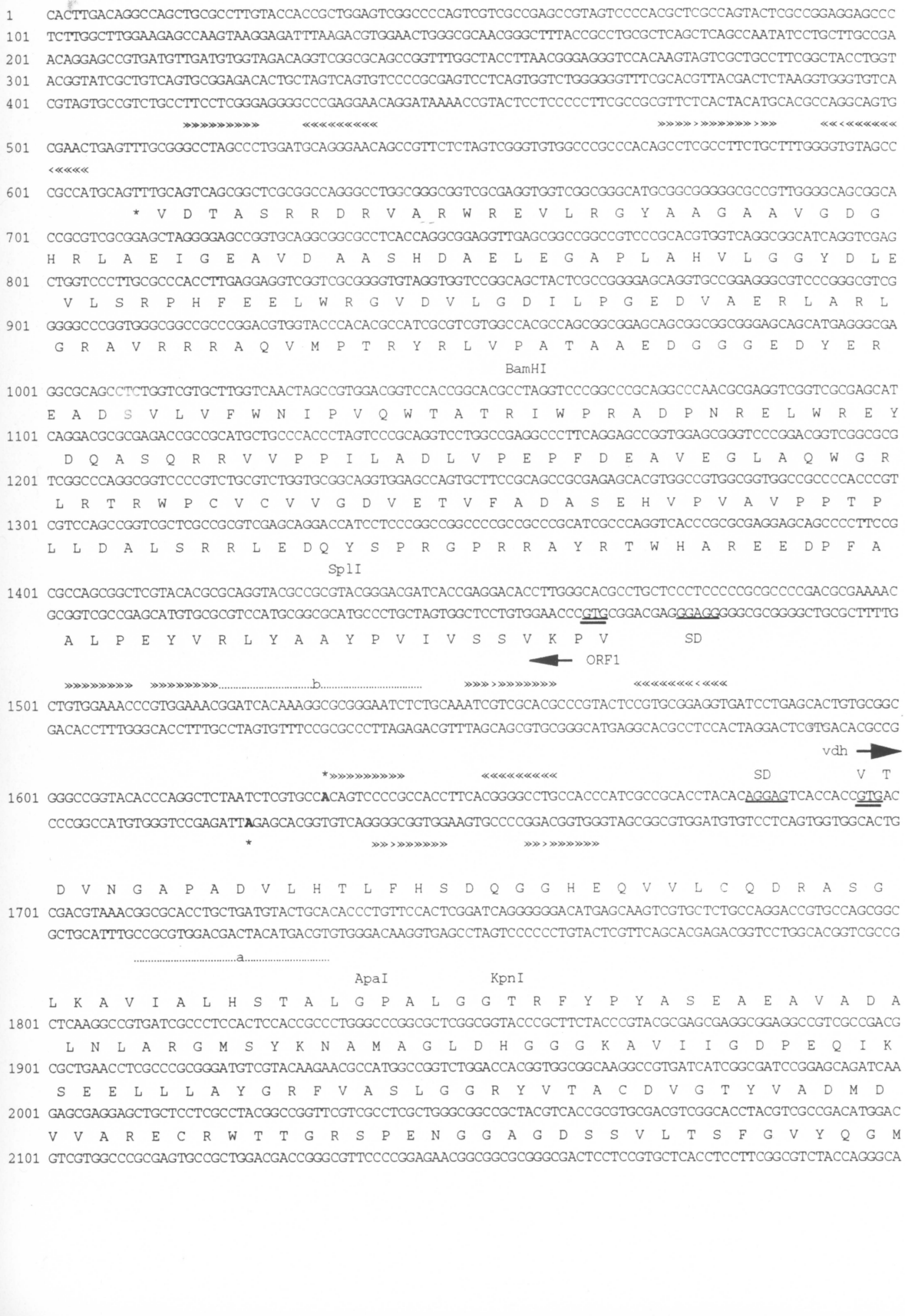



FIG. 3. Southern-blot analysis of *vdh* homologous DNA sequences in genomic digests of the tetracenomycin-producing *Streptomyces glaucescens* GLA.O (lane 1), the spiramycin-producing *Streptomyces ambofaciens* ATCC 15154 (lane 2), the monensin-producing *Streptomyces cinnamonensis* WMH585 (lane 3), and the midecamycin-producing *Streptomyces mycarofaciens* 1748 (lane 4), the tylosin-producing *Streptomyces fradiae* C373.1 (lane 5), the erythromycin-producing *Saccharopolyspora erythraea* WMH22 (lane 6), *Streptomyces lividans* TK24 (lane 7), and the salinomycin-producing *Streptomyces albus* ATCC 21838 (lane 8).

necessary and each DNA strand was sequenced at least three times. CODON PREFERENCE analysis (6) showed that there are two complete and one partial open reading frames (orf) in this region (Fig. 5), having the characteristic codon usage pattern for *Streptomyces* DNA (1). The *vdh* gene is sandwiched between orfs reading in the opposite direction, and its sequence is shown in Fig. 4. The 1092 nt *vdh* orf begins with a GTG at position 1696 and terminates in 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 fMet) coincides with the N-terminal amino acid sequence of the purified Vdh enzyme, except residue 19, where A is replaced by Q. Since the identical sequence was obtained from the same region in pWHM1050 DNA (Fig. 2), we believe that the result shown in Fig. 4 is 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. 4) has a significant degree of complementarity to the 3' end of 16*S* rRNA of *Streptomyces lividans* [5'-GAUCACCUCCUUUCU-3'] (2) and should serve as the ribosome binding site. A search of the GenEMBL databases as of June 1991 using 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 by GAP (6) analysis).

Another orf was found upstream of the *vdh* gene on the opposite strand, which is temporarily designated as ORF1. This 855 nt orf is likely to begin with GTG at position 1466 and terminate in 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. 4). No significant homology was found between the deduced product of ORF1 and known proteins


BamHI
SplI
SD
ORF1
vdh
ApaI
KpnI

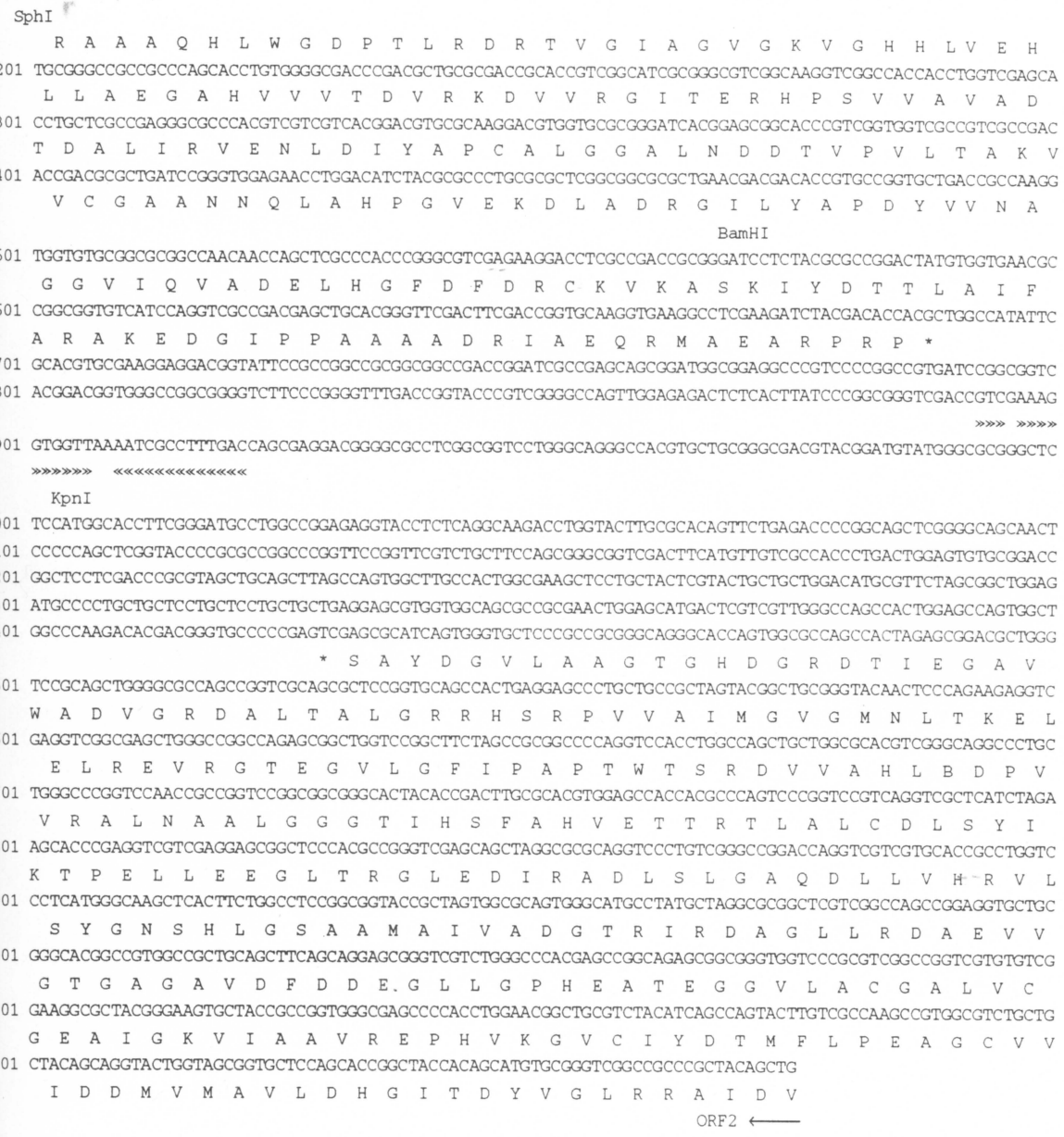



FIG. 4. Nucleotide sequence of the *vdh* region. Both strands are shown from nt 1401 to 1800. Only the bottom strand is shown from nt 1 to 1400 and 3001 to 4275, the top strand is shown from nt 1801 to 3000. Selected restriction enzyme sites are listed above their recognition sequences. The translational start sites of the *vdh* and ORF1 genes are double underlined. The transcriptional start points of *vdh* and ORF1 are indicated by bold face letters with an asterisk. SD indicates the presumed Shine-Dalgarno ribosome binding sites. Dotted lines show regions where synthetic primers were used to identify transcriptional starts. Dyad and tandem repeats in the sequence are indicated by arrow heads showing the direction of repeated sequence.

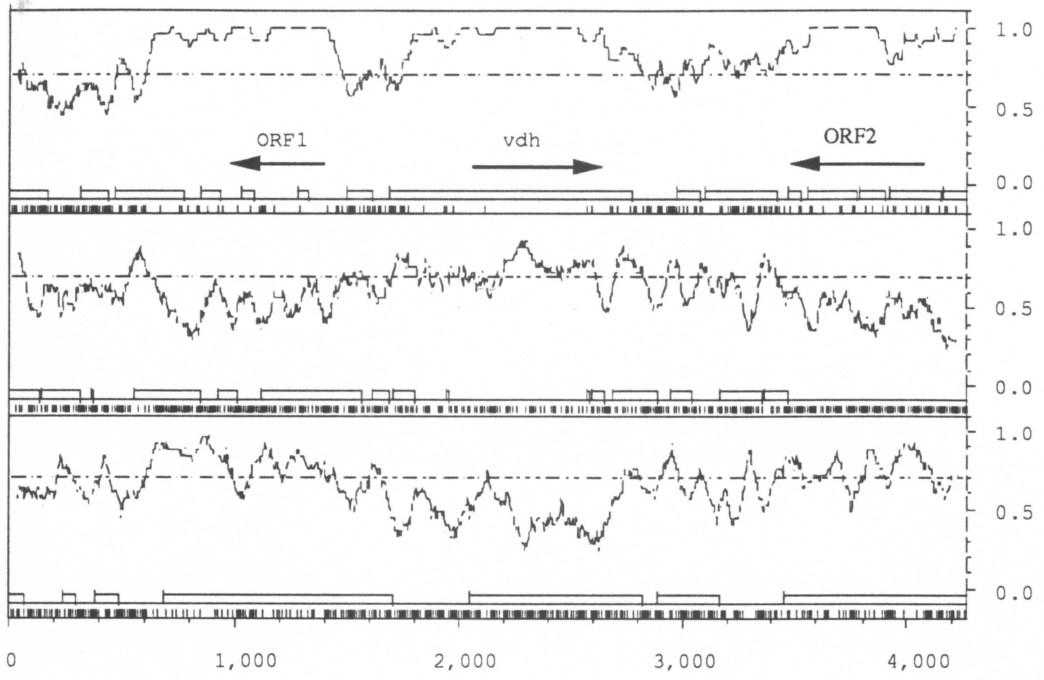



FIG. 5. CODON PREFERENCE analysis (6) of the sequence from the *vdh* region in the 5' to 3' direction. The mole fraction of G and C nucleotides at the third position of each codon in the three possible reading frames (within a window of 25 consecutive codons) is plotted versus location in the sequence. A third position G+C content of greater than 90% is characteristic of protein coding sequences in *Streptomyces* spp.. Rectangles below each plot indicate orfs that start with either ATG or GTG codons. Tick marks on the abscissa correspond to rare codons (less than 10% utilization, on average, in *Streptomyces* spp.). The analysis reveals two complete orfs with features typical of *Streptomyces* genes in the top reading frame, and the end of a third orf around nt 3420 in this same frame. The arrows above each orf indicate its direction of transcription.

by database searching as of July 1992.

A third orf, downstream of *vdh* and also on the opposite strand (Fig. 2), was detected. This ORF2 gene has not been sequenced completely, but its 3' end most likely is 629 nt downstream of the *vdh* gene (Fig. 4). We could show by TFASTA analysis that the 282 amino acid protein deduced from the 847 nt of sequenced DNA has a significant similarity to the product 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 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 or MV media were annealed to different ssDNAs made from the *vdh* region and treated with S1 nuclease. The protected DNA was analysed by Southern-blot hybridization, using the 2.88 kb *Sal*I fragment containing the entire *vdh* gene (Fig. 6) as a [$^{32}$P]-labeled probe. A rightward transcript was found to protect approx. 220 nt of clone A (800 nt *Bam*HI-*Kpn*I region) (Fig. 6, lanes 5 to 7), 720 nt of clone B (720 nt *Kpn*I-*Bam*HI region) (Fig. 6, lane 10), and 350 nt of clone C (420 nt *Bam*HI-*Kpn*I region) (Fig. 6, 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. 6, it appears that the same amount of RNA was accumulated in the 24 and 48 h MV cultures, but that a different amount of RNA was accumulated in the 24 h cultures of the R2YE and MV media. A leftward transcript, representing ORF1, was detected that protected approx. 560 bp of clone D (Fig. 6, lanes 3 and 4). This result indicates that the 5'-end of the transcript was about 150 bp upstream of the

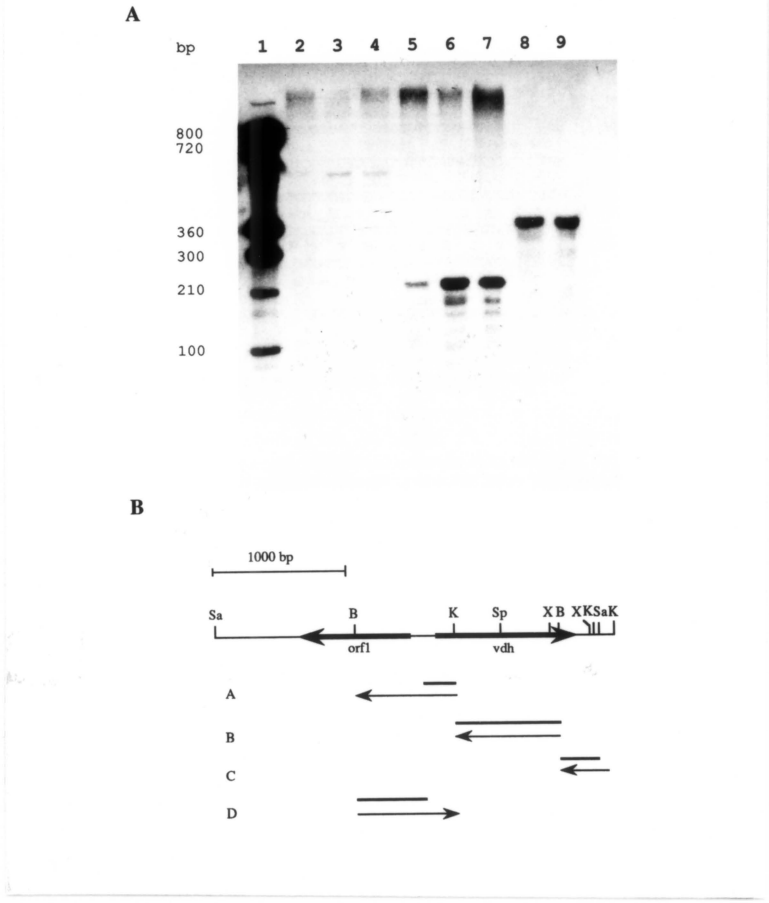
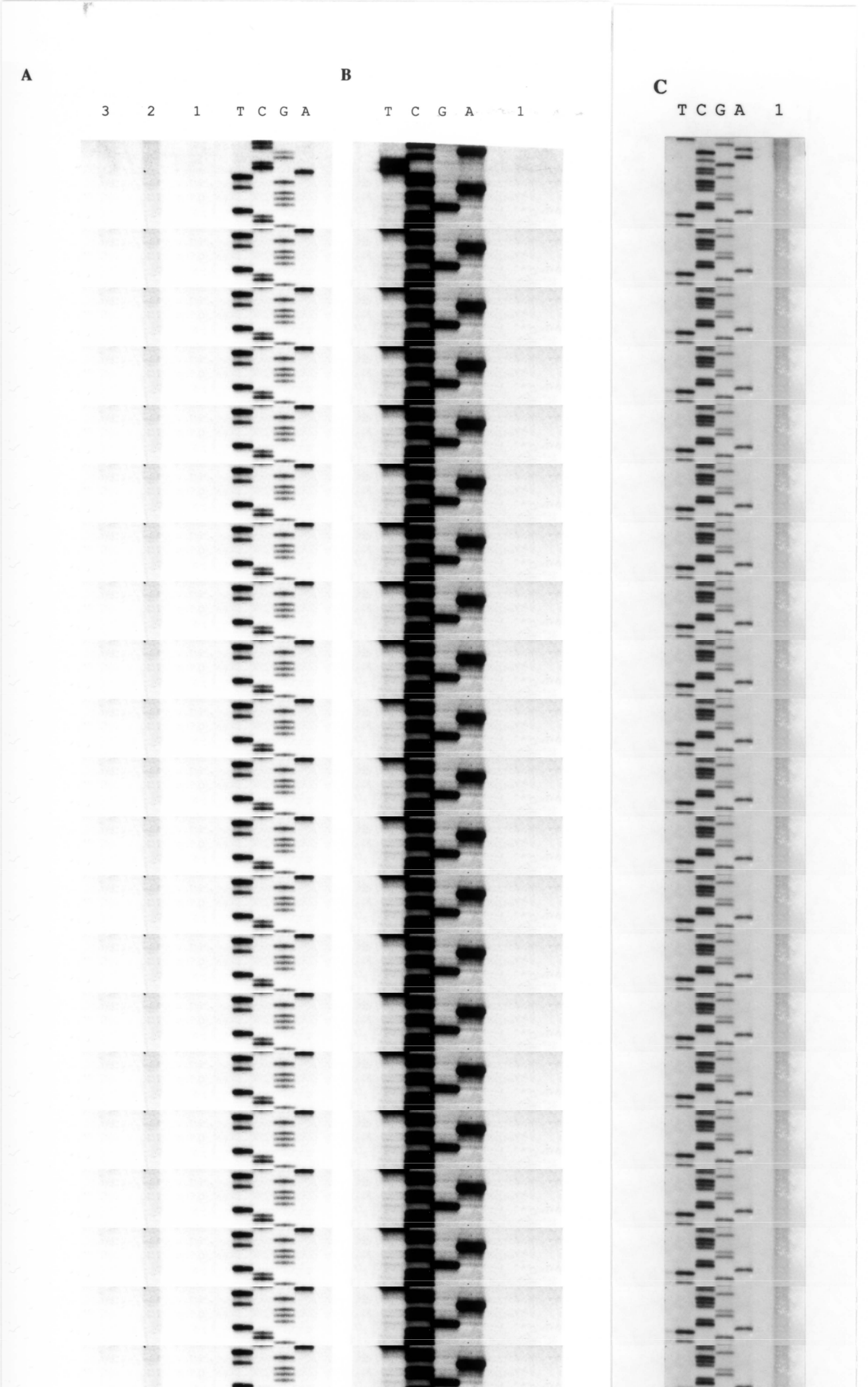



FIG. 6. Low resolution S1 protection analysis of the *vdh* region. (A) Autoradiograph of protected DNA molecules. Lane 1, digested probe fragments as MW marker. Lane 2, RNA from a 24 h culture grown in R2YE after treatment with DNA fragment D. Lane 3, as in lane 2, except the culture was grown in MV medium. Lane 4, as in lane 3, except a 48 h culture. Lane 5, RNA from a 24 h culture grown in R2YE after treatment with DNA fragment A. Lane 6, as in lane 5, but the culture was grown in MV medium. Lane 7, as in lane 6, but a 48 h culture. Lane 8, RNA from a 24 h culture grown in MV after treatment with DNA fragment C. Lane 9, as in lane 8, but a 48 h culture. DNA fragments A, B, C, D are the ssDNA produced from M13 clones shown in (B). (B) Strategy for S1 analysis. Thick bars above DNA fragments A, B, C, D (arrows) show the positions of S1-protected segments in relation to a partial restriction map of the region with the *vdh* and ORF1 genes.

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 culture. No significant difference was seen in the amount of accumulated ORF1 mRNA between the 24 vs. 48 h samples. The apparent level of the accumulated ORF1 message from the MV culture was significantly lower than that of the *vdh* message and almost the same as that of the *vdh* message in the R2YE culture.

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 *Bam*HI-*Kpn*I 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. 4. These [$^{35}$S]-labeled ssDNAs were hybridized to total RNA from *S. coelicolor* J802 grown in the MV medium, followed by treatment with S1 nuclease and electrophoretic analysis (Fig. 7C for *vdh*, data not shown for orf1). We used the same primers for primer extension experiments with RNA extracted from *S. coelicolor* J802 grown in the MV medium for ORF1, and in MV, MM and MGV media for *vdh*. The primer extension products were electrophoresed beside dideoxy sequence ladders generated with the same primers. Figure 7A and 7B shows the results of the primer extension experiments for the *vdh* and ORF1 genes. (The apparent tsp's determined for these two genes were the same in the high resolution S1 mapping experiments). These data indicate that the *vdh* tsp is at an "A" 63 nt upstream of the GTG translational start codon, and the ORF1 tsp is at an "A" 158 nt upstream of the presumed translational start. The relative levels of the accumulated *vdh* mRNA detected by primer extension in the RNA isolated from cells grown in the MM, MGV and MV media 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. 11) were consistent


A
3 2 1 T C G A
B
T C G A 1
C
T C G A 1

with the relative levels measured by S1 nuclease protection experiments; *vdh* mRNA appeared to accumulate to the highest amount in the MV medium (Fig. 6A, lane 6 and Fig. 8A, lane 8).

**Induction of *vdh* expression in *S. coelicolor* J802**. To gain an initial insight about the regulation of *vdh* expression by alternative carbon (C) and nitrogen (N) sources, we determined the level of accumulated *vdh* mRNA by low resolution S1 nuclease protection assay in different growth media. Total RNA was isolated from 24 h cultures grown in different media and the relative amounts of accumulated *vdh* message are shown in Fig. 8. These results suggest that the maximum *vdh* expression was reached in the MV medium with valine as the sole N and C sources, as shown in Fig. 8, lane 8. Not suprisingly, other branched-chain L-amino acids also apparently increase the level of accumulated *vdh* transcript compared with the level seen in the R2YE and in MM media with asparagine as the N source. (Fig. 8, lanes 2 and 3 vs. 5 to 11). Thus, the expression of *vdh* in *S. coelicolor* is inducible by the branched-chain L-amino acids or α-aminobutyric acid, and the transcription of *vdh* may be regulated in response to the availability of branched chain L-amino acids. Since reduced levels of *vdh* mRNA appeared to accumulate in the presence of glucose, it seems that the induction of *vdh* expression is partially repressed by glucose.

***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 give pWHM1058 and pWHM1060 (Table 1). After the J802(pWHM1058) and J802(pWHM1060) transformants were grown at 39°C to eliminate the plasmid, we chose 2 $Hg^{R}Th^{S}$ transformants isolated from each of the J802 strains containing pWHM1058 or pWHM1060; the *S. coelicolor* WMH1505 and WMH1506 strains (Fig. 9) for further study. Chromosomal DNA was isolated from both of these strains as well as the J802

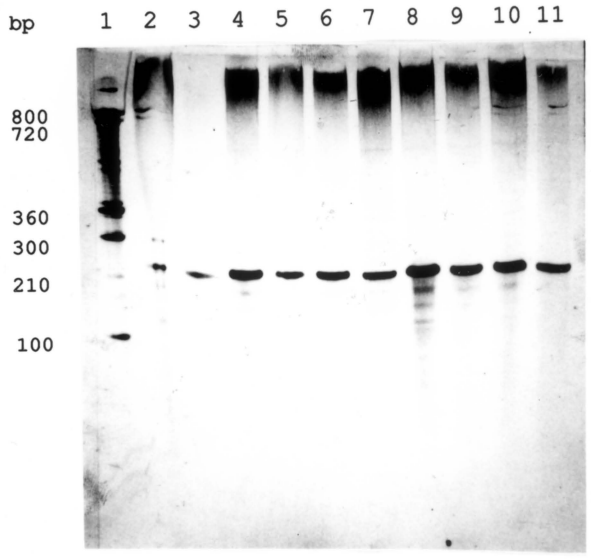



FIG. 8. Low resolution S1 analysis of the regulation of *vdh* gene expression by alternative C and N sources with ssDNA fragment A (Fig. 4) as the protecting DNA. Lane 1, digested probe fragments as MW marker. Lane 2, 40 µg RNA from 24 h R2YE culture. Lane 3, 40 µg RNA from 24 h MM culture. Lane 4, 40 µg RNA from 24 h MGV culture. Lane 5, 40 µg RNA from 24 h MM culture with 1% L-aminobutyrate in place of asparagine as sole N source. Lane 6, same as lane 5 with 1% L-isoleucine instead of asparagine. Lane 7, same as lane 5 with 1% L-leucine instead of asparagine. Lane 8, 40 µg RNA from 24 h MV culture. Lane 9, 40 µg RNA from 24 h MM culture with 1% L-α-aminobutyrate instead of asparagine and glucose as N and C sources. Lane 10, same as lane 9 with 1% isoleucine as sole C and N sources. Lane 11, same as lane 9 with 1% leucine as sole C and N sources.

strain and used for Southern analysis to analyze the region about the *vdh* and ORF1 genes. The dig-AP labelled 2.1 kb *Sph*I-*Sal*I fragment used as a probe (Fig. 9) hybridized to a 3.3 kb *Bam*HI fragment in strain WMH1505 in place of the 1.6 kb *Bam*HI fragment in the J802 strain (Fig. 10, lanes 3 and 4 vs. 9). Digestion with *Sal*I gave 1.3 and 3.3 kb hybridizing bands in place of a 2.9 kb band (Fig. 10, lanes 7 and 8 vs. 10). Digestion of the WMH1506 strain with *Bam*HI showed a 3.1 kb hybridizing band in place of the 1.6 kb band in the J802 strain (Fig. 10, lanes 1 and 2 vs. 9), and digestion with *Sal*I gave 1.8 and 2.6 kb hybridizing bands in place of the 2.9 kb fragment (Fig. 10, lanes 5 and 6 vs. 10). These data confirm that the *vdh* and ORF1 genes were indeed disrupted through the expected double crossover recombinations.

The ORF1::*hyg* mutants showed almost the same Vdh activity as the J802 strain under the growth conditions tested to date: 2.24±0.51 and 5.72±0.64 U/mg protein (16) for the respective 24 h MM and MGV cultures of the J802 strain versus 2.07±0.43 and 4.48±0.27 U/mg protein for the respective 24 h MM and MGV cultures of the WMH1506 strain. 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 medium with valine, isoleucine, leucine or α–aminobutyrate as the sole nitrogen source. The specific rates of growth of the *vdh*::*hyg* mutant and J802 strains were very similar in the MM medium, but the WMH1505 strain did not grow in the MGV medium (Fig. 11).

**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 12 shows the growth and actinorhodin production by *S. coelicolor* J802 and the WMH1505 strains in the SMM and valine containing SMV media, adapted from the work of Takano et al. (33). The

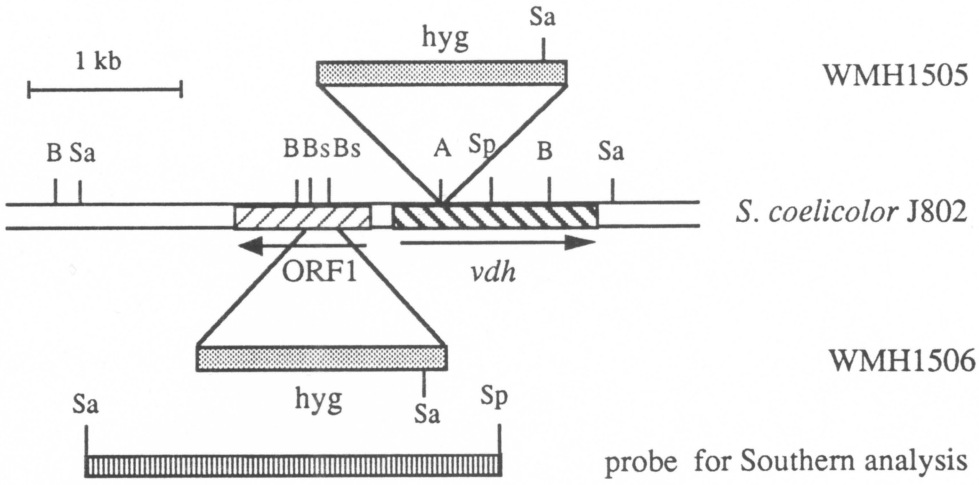



FIG. 9. The genomic structure of strains with disrupted *vdh* or ORF1 genes. *S. coelicolor* Strain WMH1505 has a *hyg* gene inserted into the *Apa*I site of the *vdh* gene and strain WMH1506 has a *hyg* gene inserted into the *Bss*HII sites of the ORF1 gene. Abbreviations: hyg, hygromycin resistance gene; restriction sites of interest, Ap, *Apa*I; B, *Bam*HI; Bs, *Bss*HII; Sa, *Sal*I; Sp, *Sph*I.

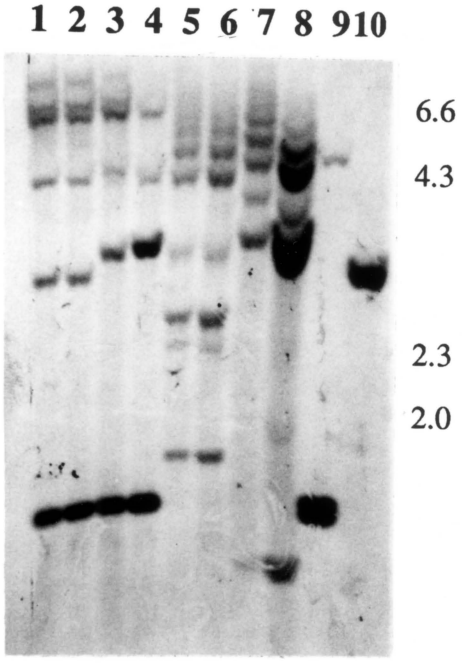



FIG. 10. Southern-blot analysis of *S. coelicolor* J802, WMH1505 and WMH1506 genomic DNA hybridized with 2.1 kb *Sph*I-*Sal*I fragments (Fig. 9). Lanes 1 and 2, WMH1506 total DNA digested with *Bam*HI. Lanes 3 and 4, WHM1505 total DNA digested with *Bam*HI. Lanes 5 and 6, WHM1506 total DNA digested with *Sal*I. Lanes 7 and 8, WMH1505 total DNA digested with *Sal*I. Lane 9, J802 total DNA digested with *Bam*HI. Lane 10, J802 total DNA digested with *Sal*I.

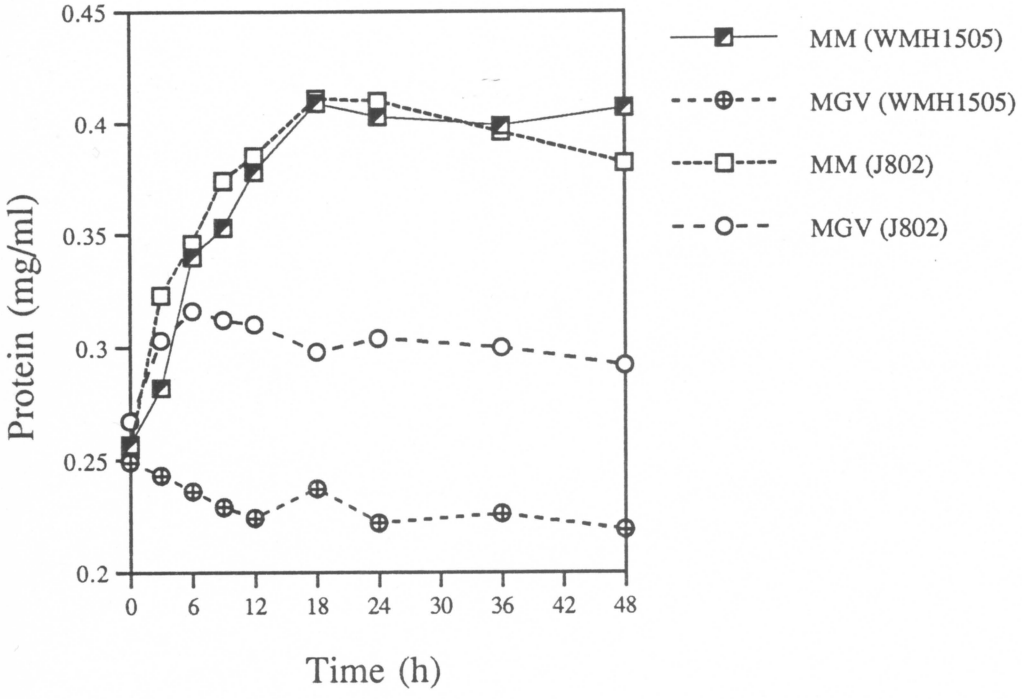



FIG. 11. Growth kinetics of the *S. coelicolor* J802 and WMH1505 strains in MM and MGV media. The data are the average for two separate experiments, using the average values of duplicate samples from each separate experiment, and represent an average variation of $\leq \pm 1.2\%$.

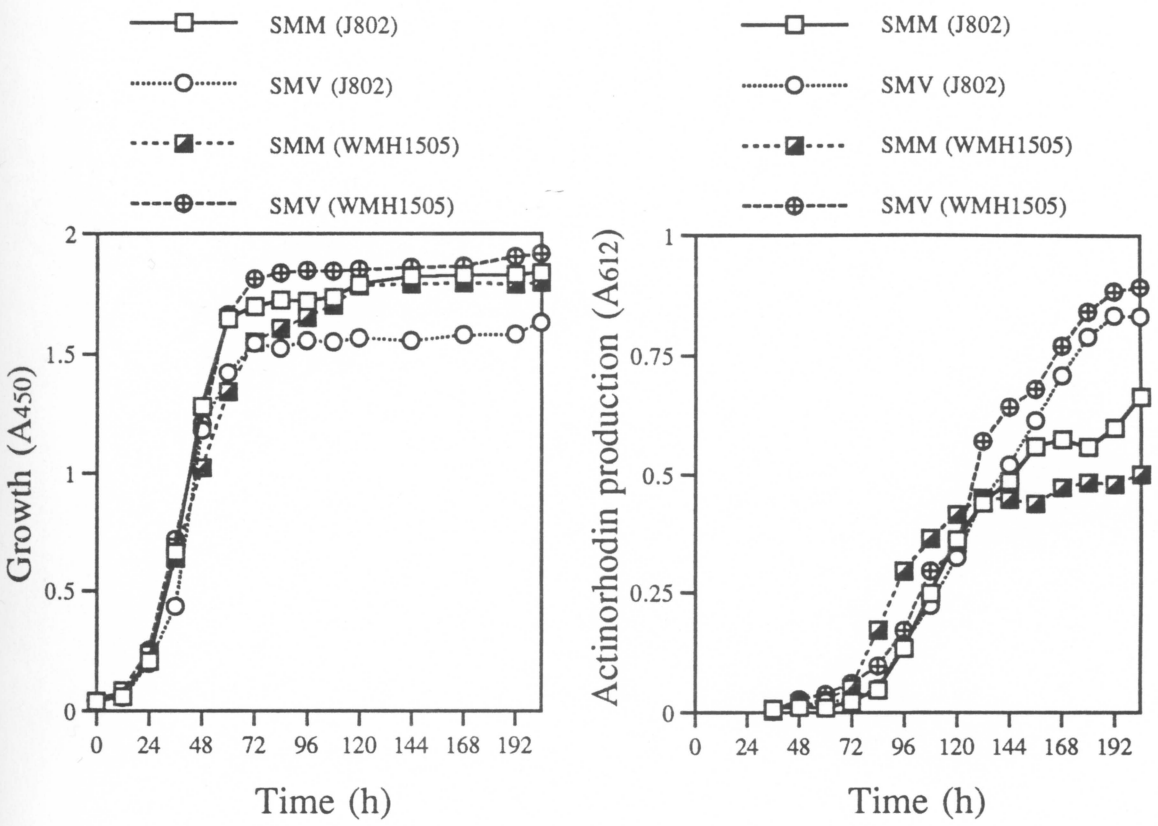



FIG. 12. Growth and actinorhodin production by the *S. coelicolor* J802 and WMH1505 strains in the SMM and SMV media. The data are the average for two separate experiments, using the average values of duplicate samples from each separate experiment, and represent an average variation of $\leq \pm 1.5\%$.

*vdh*::*hyg* mutation does not significantly effect the growth or actinorhodin production, although the 0.2% L-valine added to the SMM medium caused an approximately 32% and 80% increase of actinorhodin production in the J802 and WMH1505 strains, respectively. Since the WMH1505 strain cannot metabolize valine to provide the acetate precursor of actinorhodin synthesis, valine must increase actinorhodin synthesis by some indirect effect.

## 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 (32). Sequence analysis has established the identity of the *vdh* locus in the *S. coelicolor* A3(2) wild-type and J802 strains (32), thus justifying further use of J802 in our work.

The amino acid sequence of the *S. coelicolor* Vdh enzyme has significant similarity with several other NAD(P)+ dependent dehydrogenases. It displays the conserved nicotinamide coenzyme binding region (Fig. 13), 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). The 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 requires NAD+ for the oxidative deamination of valine in vitro (16). Finally, this Vdh contains a lysine residue adjacent to a Gly-rich region towards the N-terminus (Fig. 13) that also has been proven to be essential for enzyme activity in such dehydrogenases (22), but it lacks the Lys 6 residue further from this region towards the C-terminus ('+' at 100 in Fig. 13) that is conserved in the leucine and phenylalanine dehydrogenases of *Bacillus* spp (15).

The nucleotide sequence data show that the *vdh* gene is flanked by two divergently

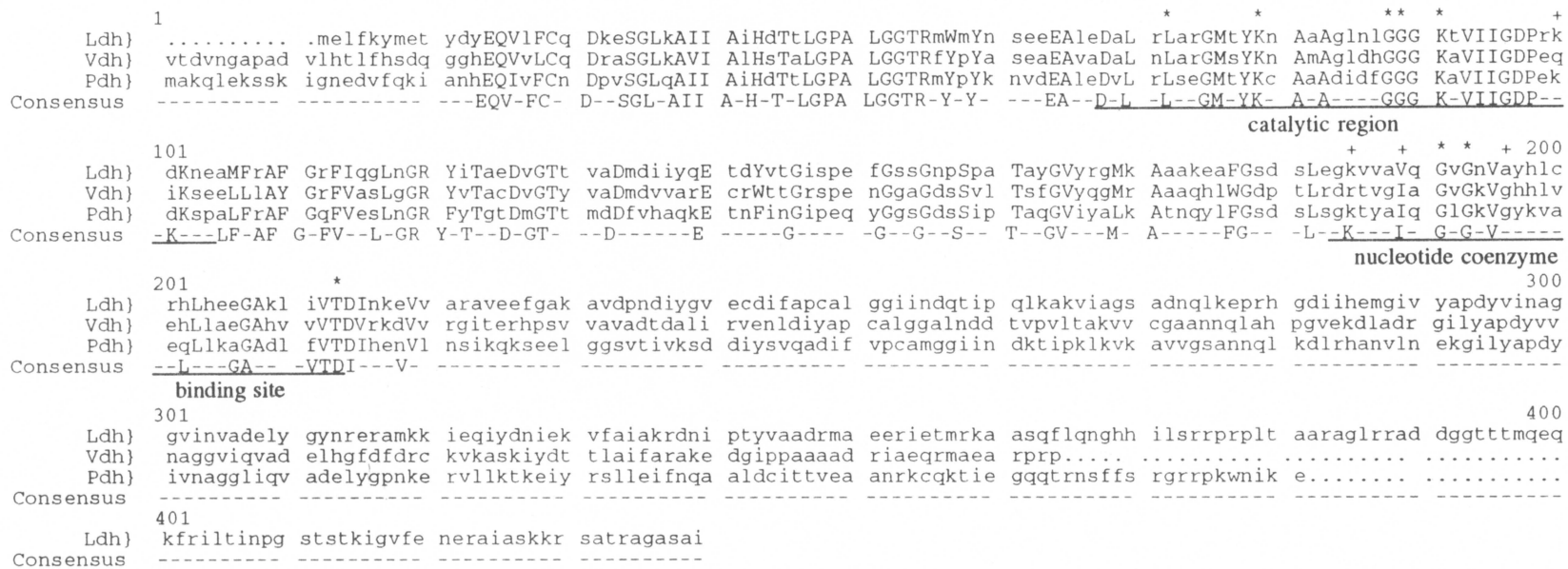



FIG. 13. PILEUP (6) comparison of the deduced *S. coelicolor* Vdh protein with leucine and phenylalanine dehydrogenases from *Bacillus* spp. The consensus line was determined with the PRETTY program (6); a capital letter indicates 3 identical residues or conservative substitutions in each of the vertical columns. Conserved residues that are thought to be important for coenzyme binding and the catalytic region are indicated by "*" and "+". Abbreviations: leucine dehydrogenase, Ldh; phenylalanine dehydrogenase, Pdh; valine dehydrogenase, Vdh.

transcribed genes. The downstream ORF2 gene is a homolog of the *purM* genes from *E. coli* (27) and *Bacillus* spp. The role of the ORF1gene is unknown, although our results suggest that it is not essential for *vdh* function under the conditions tested.

Earlier studies have shown that all four L-valine dehydrogenases 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, which is separated by only 8 base pairs (bp) from the 5' end of the divergent ORF1 transcript (Fig. 4). This bidirectional overlapping promoter region contains a group of repeat sequences that we know from studies of deletion mutants are important for *vdh* expression (32) and might be binding sites for proteins involved in transcription in addition to RNA polymerase. Futhermore, from the results of our studies of the regulation of *vdh* expression that are in progress, we have found that it is specifically induced by valine or the other branched-chain amino acids, when compared to growth on asparagine as the sole N source, and that its induction is repressed by glucose and $NH_4^+$ (32).

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# Chapter 2

## Regulation of the Expression of the Valine (Branched-chain Amino Acid) Dehydrogenase Gene from *Streptomyces coelicolor*

A modified version of this paper has been submitted to J. Bacteriol. with Dr. C. R. Hutchinson and myself as the sole authors.

# INTRODUCTION

Streptomycetes have unusual physiology: not only do they produce a wide range of secondary metabolites, commonly during nutrient-limited growth, but the regulation of some aspects of their primary metabolism differs from that of other bacteria. For instance, studies of carbon (C) assimilation indicate that C catabolite control in *Streptomyces coelicolor* does not depend on cAMP and involves glucose kinase in some way (1, 15, 19) in the transcriptional repression of different genes, including those for glycerol (34, 35), galactose (9, 18, 41) and agarose (14) utilization. Direct repeat sequences in the promoter region have been implicated in the regulation of the galactose operon (24) and chitinase genes (6), but further insight into the mechanism of C catabolite control is lacking.

In enteric bacteria, several amino acid catabolism systems also are known to be subject to C and N catabolite repression (5, 11, 32). The utilization of branched-chain amino acids by *Pseudomonas* spp., which involves a branched-chain 2-keto acid dehydrogenase (Bkd), the multienzyme complex that catalizes the second step of branched-chain amino acid metabolism, appears to be regulated by induction of the *bkd* operon by branched-chain 2-keto acids and repression by glucose and $NH_4^+$ (22, 36). cAMP appears not to be involved in C catabolite repression in this organism also (36).

We are investigating the regulation of valine catabolism in streptomycetes as a way to gain insights about the regulation of secondary metabolism at the level of precursor supply, since the catabolism of branched-chain amino acids can provide propionate and 2-methylmalonate, key precursors of macrolide, polyene and polyether antibiotics. Because the first step in this process is governed by valine dehydrogenase (Vdh) (23, 27), there is the possibility that $NH_4^+$ could alter *vdh* gene expression and/or Vdh enzyme activity as the principal reason for the negative effect of $NH_4^+$ on macrolide antibiotic production (21, 28-

30). The *S. coelicolor vdh* gene responsible for valine, leucine and isoleucine catabolism has been cloned and found to be transcribed from a single major transcriptional start point (tsp) separated by only 8 base pairs (bp) from the 5' end of the divergent ORF1 transcript (37). The bidirectional and overlapping *vdh*/ORF1 promoter region contains a group of repeat sequences that might be binding sites for proteins involved in *vdh*/ORF1 transcription in addition to RNA polymerase (37).

We report here the results of a study of the regulation of *vdh* expression in *S. coelicolor* J802 that aims to establish the basis for subsequent research on the underlying molecular biology and physiology. Although *S. coelicolor* is not known to produce antibiotics made from propionate and 2-methylmalonate, information about branched-chain amino acid utilization in *S. coelicolor* can be integrated into the growing knowledge about the genetics of nutrient utilization in this organism and can serve as a model for the macrolide, polyene and polyether antibiotic producers. Using the level of accumulated *vdh* mRNA in the *S. coelicolor* J802 strain and the level of XylE activity in *S. coelicolor* J802(*vdh*::*xylE*) transformants, we show that *vdh* expression is regulated by valine, glucose and $NH_4^+$ at the level of transcription, and that the sequence immediately upstream of the *vdh* promoter is involved in the regulatory mechanism. Interestingly, *vdh* transcription is induced by valine, and this induction is strongly repressed by glucose and $NH_4^+$. Analysis of the induction process by DNA deletion, plasmid titration and DNA gel retardation assays reveal that regions upstream of the *vdh* tsp are binding targets for putative regulatory proteins.

## MATERIALS AND METHODS

**Strains and culture conditions, plasmid isolation and transformation.** *E. coli* strains DH-5α (33) and GM2929 (*dam*-, *dcm*-), obtained from Doug MacNeil, Merck, Sharp & Dohme Co., were used as hosts for plasmids during gene cloning experiments and

were grown at 37°C on LB medium (33). *S. coelicolor* J802 (*dagA1, agaA7*) (13), obtained from D. A. Hodgson and used in most experiments, was grown on R2YE solid or in R2YE liquid media (16) for the isolation of spores and for the seed medium. The MM minimal medium of Hopwood et al. (16) and the MGV medium (37), which contain 1% glucose and 0.05% asparagine (MM) or 1% L-valine (MGV), were used for the enzyme activity assays. *S. coelicolor* transformation was performed as described by Hopwood et al. (16) with plasmid DNA isolated from *E. coli* GM2929, and transformants were selected on R2YE plates supplemented with 25 μg of thiostrepton (Th) (obtained from S. G. Lucania, The Squibb Institute for Medical Research, Princeton, NJ) per ml. Small-scale preparations of *E. coli* plasmid DNA were performed as described by Morelle (25). The plasmids used in this study are listed in Table 1.

**Construction of *xylE* transcriptional fusions to the *vdh* and ORF1 promoters.** To construct pWHM1055 (Table 1), an 400 bp *Spl*I-*Apa*I DNA fragment of plasmid pWHM1051 (37), containing the *vdh* and ORF1 promoter sequence, was filled-in with Klenow DNA polymerase, ligated to *Bam*HI linkers, and digested with *Bam*HI. The recovered 0.4 kilobase (kb) *Bam*HI DNA fragment was ligated to *Bam*HI-digested pIJ2839 (4) upstream of the promoterless *xylE* gene and resulted in plasmids pWHM1052 and pWHM1053. These two plasmids contain the promoter region of the *vdh* and ORF1 gene in the two possible orientations that generate a transcriptional fusion between the *vdh* or ORF1 promoter and a promoterless *xylE* gene contained in pIJ2839 (Fig. 1). pWHM1052 was digested with *Pst*I and *Hin*dIII and ligated with a gel-purified *Pst*I-*Hin*dIII fragment from pIJ2840 (4) containing the transcriptional terminator of phage fd (tfd) to give pWHM1054. pWHM1054 was digested with *Pst*I and *Hpa*I, the ends were filled-in with Klenow polymerase, and the resulting 1.05 kb fragment was gel purified and cloned into the unique

Table 1. Plasmids used in this work

| Plasmid | Characteristics[a] | References |
|---|---|---|
| pWHM1051 | *vdh*, ORF1, ORF2 genes from *S.coelicolor* cloned on pUC18 | 37 |
| pIJ2839 | *bla tsr*; promoterless *xylE*; SCP2 origin | 4 |
| pIJ2840 | *bla tsr;* tfd; promoterless *xylE*; SCP2 origin | 4 |
| pXH106 | *bla tsr hyg*; promoterless *xylE*; Tn*5099*; ts origin | 10 |
| pWHM1065 | 6.0 kb *Hin*dIII fragment of pXH106 was religated; ts; *tsr* | 37 |
| pWHM1052 | 400 bp *Spl*I-*Apa*I fragment of *vdh* promoter cloned in pIJ2839 to generate a *vdh*::*xylE* transcriptional fusion | This study |
| pWHM1053 | 400 bp *Spl*I-*Apa*I fragment of ORF1 promoter cloned in pIJ2839 to generate an ORF1::*xylE* transcriptional fusion | This study |
| pWHM1054 | 400 bp *Spl*I-*Apa*I fragment of *vdh* promoter cloned in pIJ2840 to generate a *vdh*::*xylE* transcriptional fusion | This study |
| pWHM1055 | 400 bp *Spl*I-*Apa*I fragment cloned in pXH106 to generate a *vdh*::*xylE* transcriptional fusion | This study |
| pWHM1064 | 320 bp *Tfi*I-*Apa*I fragment with the promoterless *xylE* and *hyg* genes cloned in pWHM1065 to generate a *vdh*::*xylE* transcriptional fusion | This study |
| pWHM1067 | 270 bp *Dde*I-*Apa*I fragment with the promoterless *xylE* and *hyg* genes cloned in pWHM1065 to generate a *vdh*::*xylE* transcriptional fusion | This study |

[a]*bla*, ampicillin resistance gene; *tsr*, thiostrepton resistance gene; *hyg*, hygromycin resistance gene; tfd, transcriptional terminator of phage; ts, temperature sensitive.

*Stu*I site of pXH106 (10) to give pWHM1055 (Fig. 1).

**Deletion of sequences upstream of the *vdh* promoter.** The 420 bp *Spl*I fragment of pWHM1051 containing the *vdh* promoter region was gel purified and digested with *Dde*I or *Tfi*I, and the ends of each of the resulting fragments were filled-in with Klenow polymerase, followed by digestion with *Kpn*I. The resulting approximately 270 bp *Dde*I-*Kpn*I or 320 bp *Tfi*I-*Kpn*I DNA fragments were gel purified and cloned into pUC19 (40) between the *Hinc*II and *Kpn*I sites. The desired DNA segments were removed from the resulting pUC19 derivatives by digestion with *Hin*dIII and *Bst*XI and ligated with the 2.7 kb *Bst*XI-*Avr*II fragment of pWHM1055 and the 6.0 kb *Hin*dIII-*Xba*I fragment of pWHM1065 (37) to give plasmids pWHM1067 and pWHM1064 (Fig. 1).

**Enzyme assays and cell growth measurement.** For the quantitative measurement of catechol dioxygenase (XylE) or Vdh activities in nutritional shift experiments, cultures were grown in 250 ml baffled flasks at 30°C in 25 ml of R2YE with 200 units (U) hygromycin (Hg) (Sigma) per ml for 48 h with shaking at 200 rpm. The mycelial cells were harvested by centrifugation and washed 2 times with MM without glucose and asparagine (Mm) and suspended in 5 ml of Mm. Equal portions of this cell suspension were added to 25 ml of the media described in the text but without Hg, and the cultures were incubated as before prior to assaying the XylE or Vdh specific activity or determining the protein concentration of cell aliquots as a measure of growth. These conditions were developed to permit rapid growth of the finely dispersed mycelia so that experiments could be repeated expediently, but we do not know if they achieve true steady-state conditions. The dynamics of *S. coelicolor* growth with or without plasmid were measured for each set of enzyme activity assays and all of the growth curves were very similar to the one shown in Fig. 3B. XylE activity was determined spectrophotometrically as described by Ingram et al. (18), and Vdh activity was determined as described by Navarrete et al. (27). Protein concentrations were determined according to Tang

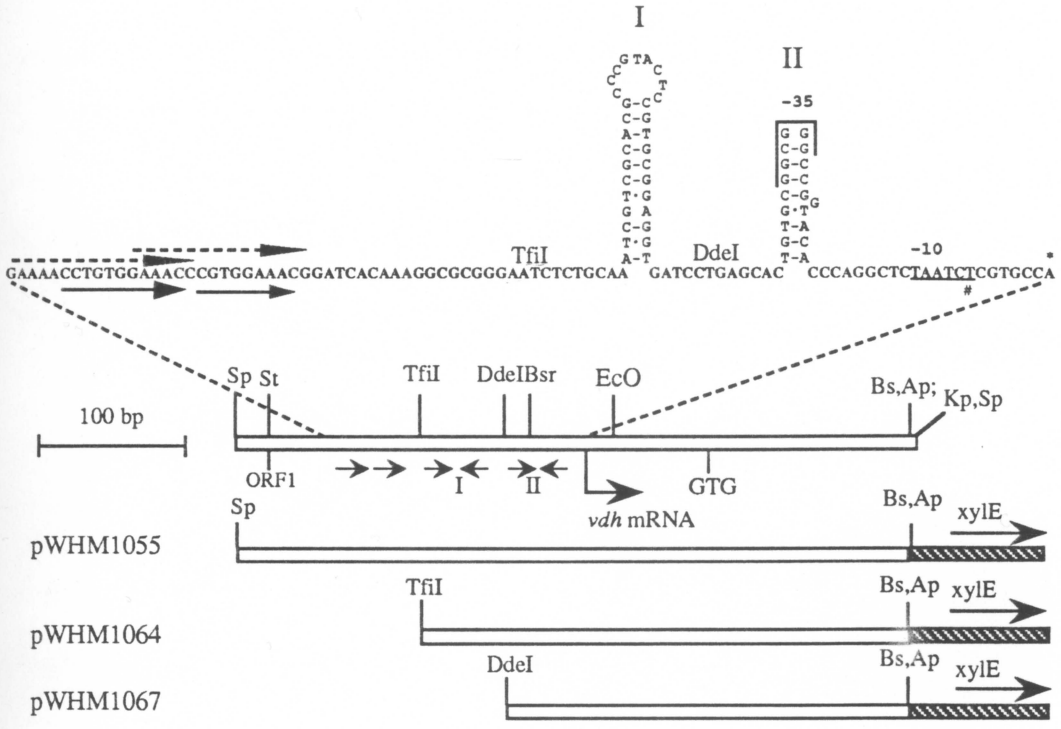



FIG. 1 Organization of the promoter region of the *S. coelicolor vdh* gene cloned on the promoter probe vector pXH106 containing a promoterless *xylE* gene. The short arrows below the DNA map indicate the direct and dyad repeat sequences mentioned in the text. The transcriptional start sites of the *vdh* and ORF1 genes are indicated by the * and #, respectively. The -35 and -10 regions of the *vdh* promoter are overlined and underlined respectively. GTG and ORF1 indicate the respective translational start sites for the Vdh and Orf1 genes. Restriction site abbreviations: Sp, *Spl*I; Ap, *Apa*I; Kp, *Kpn*I; Bs, *Bst*XI; St, *Sty*I; EcO, *Eco*O109I; Bsr, *Bsr*FI.

and Hutchinson (37) by the method of Bradford (3) by using bovine serum albumin as the protein standard.

**RNA isolation and primer extension experiments.** *S. coelicolor* J802 cultures for RNA isolation were grown as described above for the XylE and Vdh activity assays. RNA extraction and primer extension experiments were done as described previously (37), using a primer 5'-GTGCAGTACACCAGCAGGTGCGCC-3' beginning 39 bp downstream of the *vdh* GTG translational start codon and complementary to the *vdh* mRNA (37). RNA concentration was determined by measuring the $A_{260}$ of an mRNA solution and the same amount of total RNA (10 μg) was used for all primer extension reactions. After overnight exposure to Kodak X-Omat AR X-ray film, the film was scanned on a digital densitometer (Molecular Dynamics ImageQuant 3.0 and Model 300A) and the optical density values computed in relative units were plotted.

**Integration of a *vdh*::*xylE* transcriptional fusion into the *S. coelicolor* J802 chromosome.** pWHM1055 transformants were grown in liquid R2YE plus 25 μg/ml Th, homogenized, and plated on R2YE agar plus 200 U Hg to obtain 20 to 100 isolated colonies per plate. The plates were incubated at 29°C for 2 to 3 days and then incubated at 39°C for 10 to 14 days. Cells containing pWHM1055 integrated into the *S. coelicolor* genome continued to grow at 39°C and formed sectors. Sectors were collected and plated on R2YE plus 200 U Hg then incubated at 39°C to confirm their Hg resistance.

**DNA labeling and Southern-blot hybridization.** The dig-Ap labeling of plasmid pXH106 and detection of hybridizing DNA by Southern analysis were done with the Genius® kit, following the manufacturer's protocols (Boehringer Mannheim, Indianapolis, IN). Hybridization was performed at 42°C overnight and the blots were washed 2 times with 1x SSC-0.1% SDS (33) for 15 min at 42°C and then 2 times in 0.1x SSC-0.1% SDS for 15

min at 68°C.

**Electrophoretic gel mobility shift assays.** To prepare cell extracts, *S. coelicolor* J802 cultures were grown at 30°C in MM or MGV media for 6 h, according to the nutritional shift protocol described above. Cells were collected by centrifugation and washed with 10% aqueous glycerol, then resuspended in TGED buffer (10 mM Tris pH 7.9, 10% glycerol (v/v), 0.1 mM EDTA pH 8.0, 0.1 mM DTT, 0.5 mM PMSF). The mycelium was disrupted by sonification, and the cell-free crude extract was obtained by centrifugation at 25,000 x *g* for 45 min. The proteins in the crude extract were precipitated with polyethylenimine (Sigma) at 0.7% (v/v) final concentration. The pellet was washed with 1/2 vol TGED buffer plus 0.05 M KCl twice, then the proteins were eluted from this pellet with equal amount of TGED buffer plus 1 M KCl by centrifugation at 25,000 x *g* for 15 min. The proteins in the supernatant fraction were precipitated with ammonium sulfate at 80% saturation and collected by centrifugation at 100,000 x *g* for 30 min. The precipitate was resuspended in 0.5 ml TGED buffer with 0.05 M KCl and dialyzed against 1 liter of the same buffer at 4°C overnight with two changes of buffer.

DNA-protein binding assays were done by the gel mobility shift method of Vujaklija et al. (39). Poly(dI-dC):(dI-dC), 1.5 µg; 0.5 to 5 ng of DNA fragments that had been $^{35}$S- or $^{32}$P-dCTP end-labeled with dNTP and Klenow polymerase, and 25 µg of proteins were mixed in 25 µl of TGED with 50 mM KCl. After incubation at 30°C for 20 min, protein-bound and free DNA were resolved on 5% nondenaturing polyacrylamide gels run in a high-ionic-strength buffer containing 50 mM Tris, 380 mM glycine and 2 mM EDTA, pH 8.5, or the standard TBE buffer (33). The gels were dried at 60°C under vacuum and exposed to Kodak X-ray film overnight.

## RESULTS

**Valine induction of *vdh* expression in *S. coelicolor*.** The promoter of the *S. coelicolor vdh* gene, which is transcribed as a monocistronic mRNA from a single major tsp, separated by only 8 bp from the 5'-end of the divergent ORF1 transcript (37), contains a putative RNA polymerase binding site with a recognition sequence very similar to that observed in the promoter of the *S. coelicolor gylR* regulatory gene from the glycerol utilization operon (34), and in the *gal*P1 promoter of the galactose utilization operon (9, 18, 24) of *S. lividans* and *S. coelicolor* (Fig. 2). The latter promoter is glucose catabolite controlled and uses a novel form of σ factor (24, 41). All three of these promoters have a simlar -35 region with a string of G residues and a possible *E. coli* $\sigma^{70}$-like -10 region (Fig. 2).

Nutritional shift experiments were used to determine the effect of valine on *vdh* expression in *S. coelicolor*. Vdh specific activity, measured as a function of time in cell-free extracts of the J802 strain grown for 48 h in the R2YE medium, then transfered to the MM (with 1% glucose and asparagine) or MGV (with 1% glucose and valine) media, increased steadily within 6 to 10 h after transfer of washed mycelium to fresh MGV medium, then fell to a lower level over the next 20 h (Fig. 3). In contrast, the Vdh activity increased only slightly within 12 h after transfer of washed cells to fresh MM medium, then remained constant over the same period (Fig. 3). These data were highly reproducible and reveal that the Vdh activity was induced approx. 3.5-fold at 12 h specifically by the valine in the MGV medium compared to asparagine in the MM medium. Previous work from our laboratory (27) reported that *vdh* expression was induced approx. 1.9-fold at 24 h by valine compared to histidine instead of asparagine in the MM medium and was induced approx. 13-fold in MM with valine as the sole C and N source.

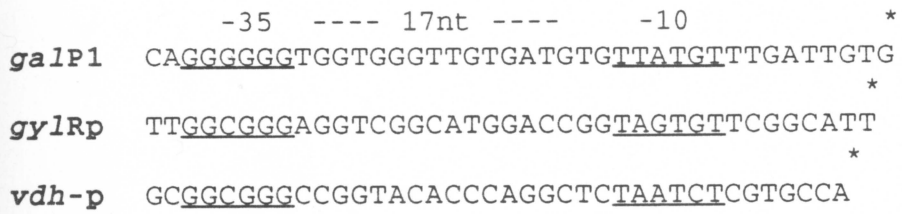



FIG. 2. Comparison of *S. coelicolor gylR* and *gal*P1 promoter sequences with the *vdh* promoter sequence. The transcriptional start site is indicated by an asterisk and the underlined sequences indicate the -35 and -10 regions.

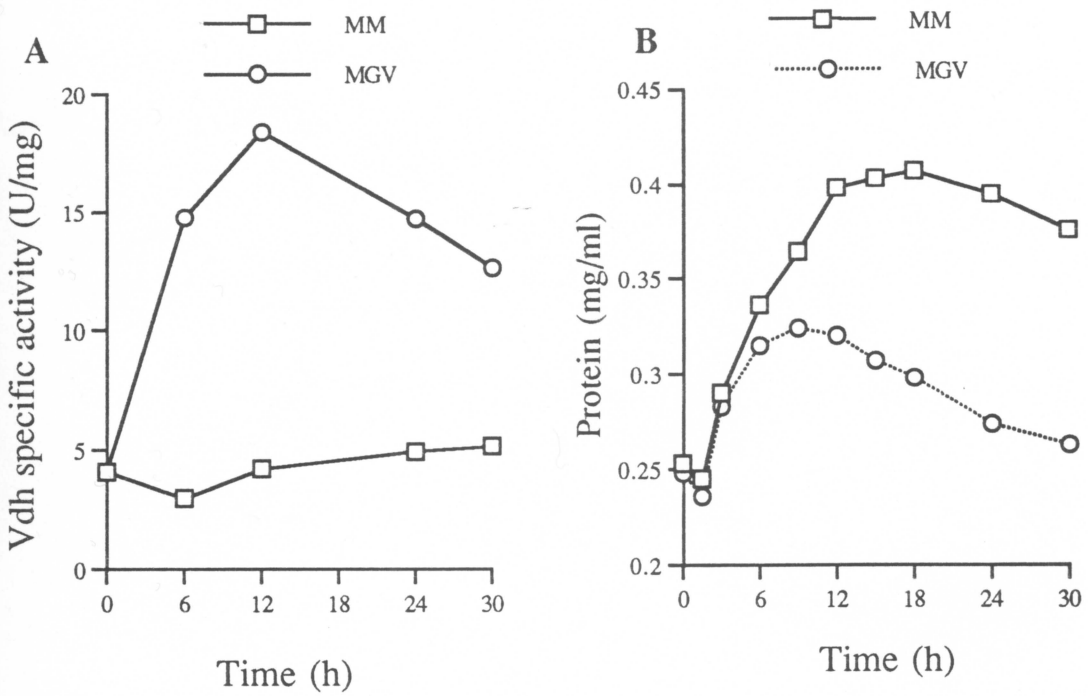



FIG. 3. Specific activity of Vdh (A) and growth (B) of *S. coelicolor* J802 cells during growth in the MM and MGV media after nutritional shift at 0 h. The Vdh specific activity data are the average for two separate experiments, using the average values of duplicate samples from each separate experiment, and represent an average variation of less than 5%. The growth data are the average for two separate experiments, using the average values of three samples from each separate experiment, and represent an average variation of less than 2.5%.

For initial information about the regulation of *vdh* expression, primer extension analyses were performed to determine the level of accumulated *vdh* mRNA. Total RNA was isolated from J802 cells grown under inducing (MGV) or non-inducing (MM) conditions at various time points in the nutritional shift experiments. Although the same level of *vdh* mRNA was observed in the washed cells at 0 h, the level increased markedly at 6 h after the shift to MGV medium but increased only slightly at 12 h after the shift to MM medium, then fell to a low level at 24 h (Fig. 4). Comparison of the apparent level of *vdh* mRNA at 6 h in the two experiments shows a 3 to 4-fold induction of *vdh* transcription by valine compared to asparagine.

As a further way to examine the induction of *vdh* expression, we used a *vdh*::*xylE* transcriptional fusion and measured the XylE specific activity as a function of culture age, again in nutritional shift experiments. The *xylE* gene has been used as a convenient means of monitoring the expression of *Streptomyces* genes (18), most often in various promoter-probe vectors (4, 18). In our work, an 400 bp *Spl*I-*Apa*I fragment containing the *vdh* and ORF1 promoter region (Fig. 1) was fused with a promoterless *xylE* gene downstream of the fd phage transcriptional terminator (tfd) on the high copy plasmid pXH106 (10), as described in Materials and Methods, to give plasmid pWHM1055 (Table 1 and Fig. 5). We integrated pWHM1055 into the *vdh* locus in the chromosome via homologous recombination as follows. *S. coelicolor*(pWHM1055) transformants were grown in R2YE at 39°C to eliminate the free temperature-sensitive plasmid as described in Materials and Methods, following the work of Hahn et al. (10), to obtain strain WMH1600. Southern analysis of genomic DNA from two representative Hg and Th resistant WMH1600 recombinant strains and pWHM1055, both digested with *Not*I or *Nco*I-*Eco*RI and probed with dig-AP labeled pXH106 DNA, clearly showed that pWHM1055 had integrated into the *S. coelicolor* J802 chromosome by a single crossover between the homologous *vdh* regions in pWHM1055 and the chromosome (Fig.

5A). The WMH1600 strains generated three *Not*I fragments (2.4, 6.4 and 8.5 kb) in place of the two *Not*I fragments (5.0 and 6.4 kb) in free plasmid pWHM1055 (Fig. 5B, lanes 4 *vs* 5 and 6). Therefore, this strain should also generate six *Nco*I-*Eco*RI fragments but since only five of them are present in the pXH106 probe, only five of the bands should appear on Southern analysis. The sizes of four of the five hybridization bands in the two WMH1600 strains analyzed matched the sizes of the corresponding four pXH106 *Nco*I-*Eco*RI fragments (1.0, 1.7, 3.3 and 4.3 kb), and there is a faint band at 1.1 kb (the weak signal is due to the limited amount of homology) resulting from the 1.1 kb *Nco*I fragment containing the tfd and flanking chromosomal sequence (Fig. 5B, lanes 1 *vs* 2 and 3), which replaces the 1.05 kb *Nco*I-*Eco*RI segment of pXH106 (Fig. 5A). Consequently, both the *vdh* and *xylE* genes are transcribed under the control of *vdh* promoters located in the chromosome (Fig. 5A). It is surprising that the *vdh*::*xylE* fusion was not integrated into the *S. coelicolor* J802 chromosome by transposition of Tn*5099,* which contains the *vdh*::*xylE* fusion cloned in pWHM1055 (Fig. 5A), to give a Th sensitive and Hg resistant strain, as expected from the behavior of this element in other streptomycetes (10, 38).

The XylE specific activity of WHM1600 cell extracts, measured with cells grown in the MM and MGV media as above, showed that the *vdh* gene was induced approx. 2.5-fold at 6 h by valine compared to asparagine (Fig. 6). The changes in XylE activity in the two media mirror those shown for the mRNA in Fig. 4. These results suggest that induction of *vdh* expression and Vdh activity by valine occurs at the transcriptional level.

**Repression of *vdh* induction by $NH_4^+$.** Since *vdh* expression is likely to be regulated by N sources that can be assimilated more easily than the amino acids, asparagine and valine, used in the MM and MGV media, respectively, we measured the effect of $NH_4^+$ on *vdh* expression by primer extension analysis and by a *vdh*::*xylE* fusion. The relative level of the accumulated *vdh* mRNA detected by primer extension in the RNA isolated from cells

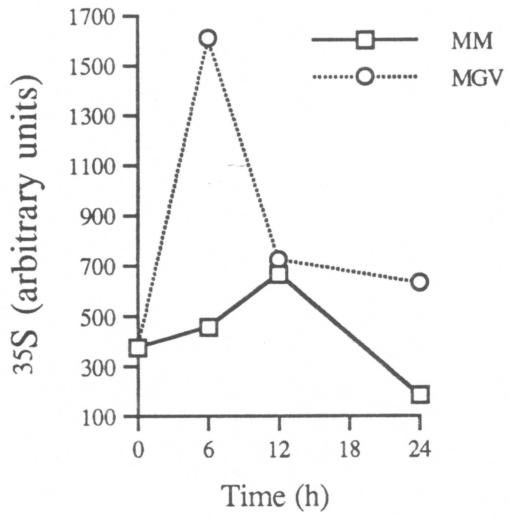
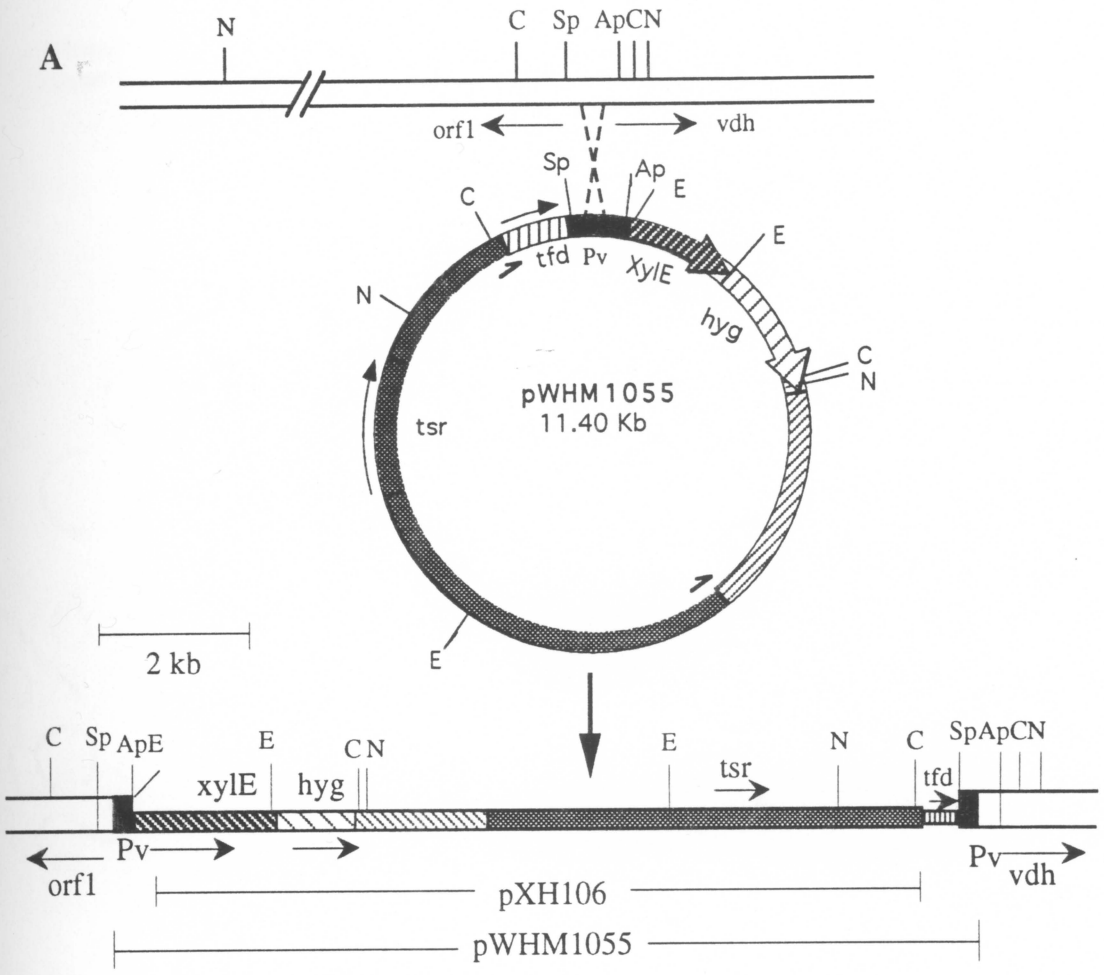



FIG. 4. Primer extension analysis of *S. coelicolor* J802 *vdh* mRNA prepared from cells grown in MM and MGV at 0, 6, 12 and 24 h after nutritional shift. The autoradiogram was scanned by a densitometer and the relative intensity units were plotted.


A
N
C Sp ApCN
orf1
vdh
Sp
Ap
E
C
tfd Pv
XylE
E
N
hyg
C
N
pWHM1055
11.40 Kb
tsr
2 kb
E
C Sp ApE
E
CN
xylE
hyg
E
tsr
N
C
SpApCN
tfd
Pv
Pv
orf1
vdh
pXH106
pWHM1055

B

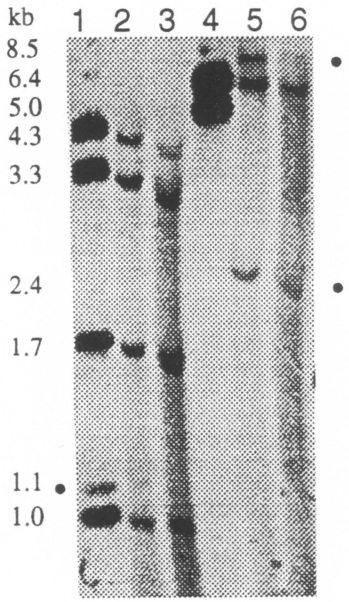

kb
1 2 3 4 5 6
8.5
6.4
5.0
4.3
3.3
2.4
1.7
1.1
1.0

FIG. 5. (A) Schematic representation of integration of pWHM1055 into the *S. coelicolor* J802 chromosome by a single crossover as shown. Restriction site abbreviations: E, *Eco*RI; C, *Nco*I; N, *Not*I. The half-arrows in the circular form of pWHM1055 designate the two ends of Tn*5099*. (B) Southern analysis of chromosomal DNA of the WMH1600 strain (lanes 2, 3, 5 and 6) and plasmid pWHM1055 (lanes 1 and 4) with vector pXH106 as the probe. DNA was digested with *Not*I (lanes 4 to 6) or *Nco*I and *Eco*RI (lanes 1 to 3). Faint bands not visible in the scanned photograph are indicated by a '•'.

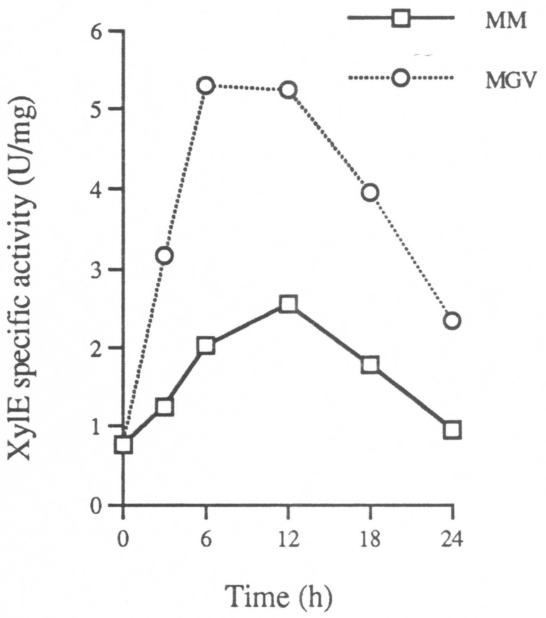



FIG. 6. The XylE specific activity of *S. coelicolor* WMH1600 cells containing pWHM1055 integrated into the chromosome during growth in the MM and MGV media. The XylE specific activity data are the average for two separate experiments, using the average values of duplicate samples from each separate experiment, and represent an average variation of less than 5%.

grown in the MGV medium supplemented with 25 mM ammonium chloride (MGVN) was approx. 2-fold lower than from the MGV medium (Fig. 7A). This result was corroborated by the results of XylE specific activity determinations for cell extracts prepared from *S. coelicolor* J802(pWHM1055) transformants grown in the MGV medium supplemented with 0, 25 (N25), 50 (N50) or 100 (N100) mM ammonium chloride. The addition of $NH_4^+$ to the MGV medium markedly decreased the XylE specific activity from the highest value reached, 22.6 U/mg of protein, in the cells from the MGV cultures vs. only 11, 9, or 6 U/mg of protein in the MGV medium with 25, 50 and 100 mM ammonium chloride, respectively (Fig. 7B). These data demonstrate that $NH_4^+$ repressed the induction of *vdh* transcription in the presence of valine. ("Repression is used here only to mean inhibition of expression and does not imply that the mechanism is similar to the classical *lac* operon model.) Our previously published work (27) indicated that Vdh activity in *S. coelicolor* was repressed by $NH_4^+$ and glucose, but that conclusion was based on data obtained from cells grown in the absence, not in the presence, of the inducer valine.

**Repression of *vdh* induction by glucose.** Since C catabolite repression appears to be a global mechanism controlling important physiology in streptomycetes and is thought to involve glucose kinase as a regulator (2, 19), we determined the effect of glucose on *vdh* expression. Primer extension analysis was performed with RNA isolated from *S. coelicolor* J802 and *S. coelicolor* M480, a prototrophic glucose kinase (*glkA*) deletion mutant provided by M. J. Bibb, grown on minimal medium containing either 1% glucose-valine (MGV, repressing condition) or 1% glycerol-valine (MRV, derepressing condition) as C and N sources. The tsp of the *vdh* gene was the same in the *S. coelicolor* M480 and J802 strains, but the relative level of the primer extension product was much higher from J802 cells grown in MRV than in MGV (Figs. 8C *vs* 8B or 8A); an approx. 2-fold increase relative to the fully

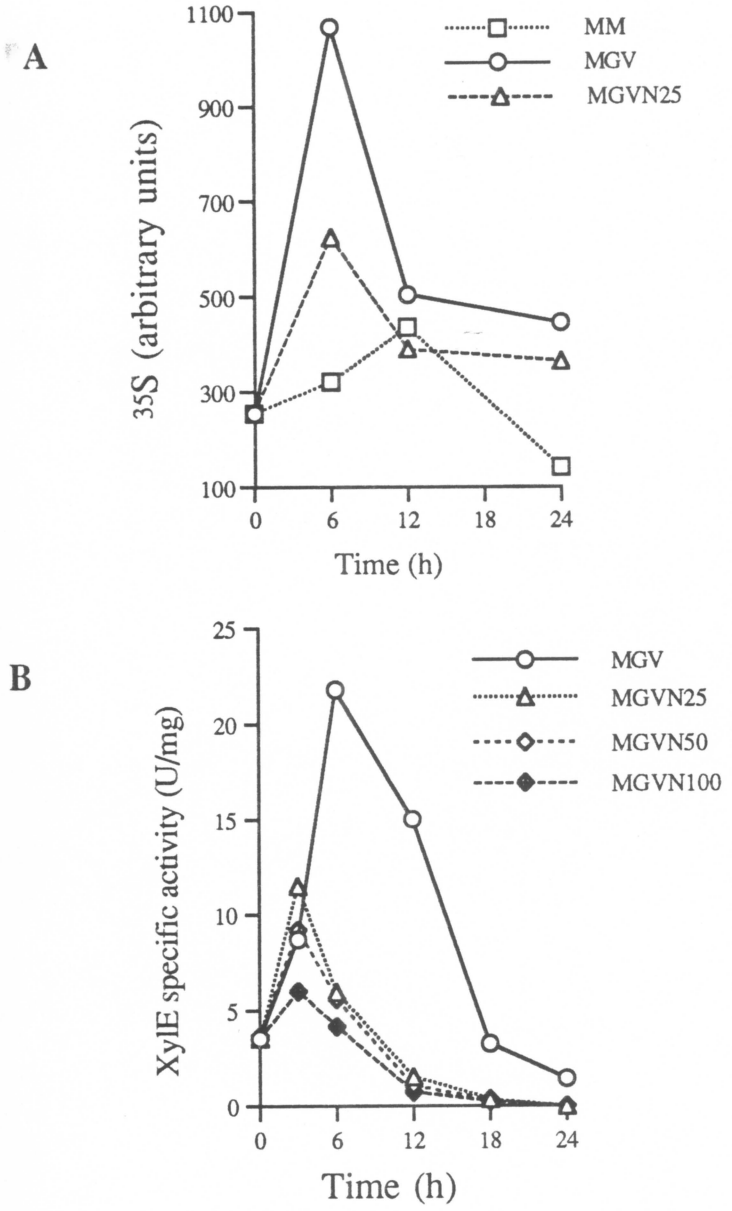



FIG. 7. (A) Primer extension analysis of *S. coelicolor* J802 *vdh* mRNA prepared from cells grown in the MM, MGV or MGV media supplemented with 25 mM ammonium chloride (MGVN) after nutritional shift. The autoradiogram was scanned by a densitometer and the relative intensity units were plotted. (B) The XylE specific activity of *S. coelicolor* J802 cells containing pWHM1055 during growth in the MGV medium with 0 (MGV), 25(MGVN25), 50 (MGVN50) and 100 mM (MGVN100) ammonium chloride. The XylE specific activity data are the average for two separate experiments, using the average values of duplicate samples from each experiment, and represent an average variation of less than 10%.

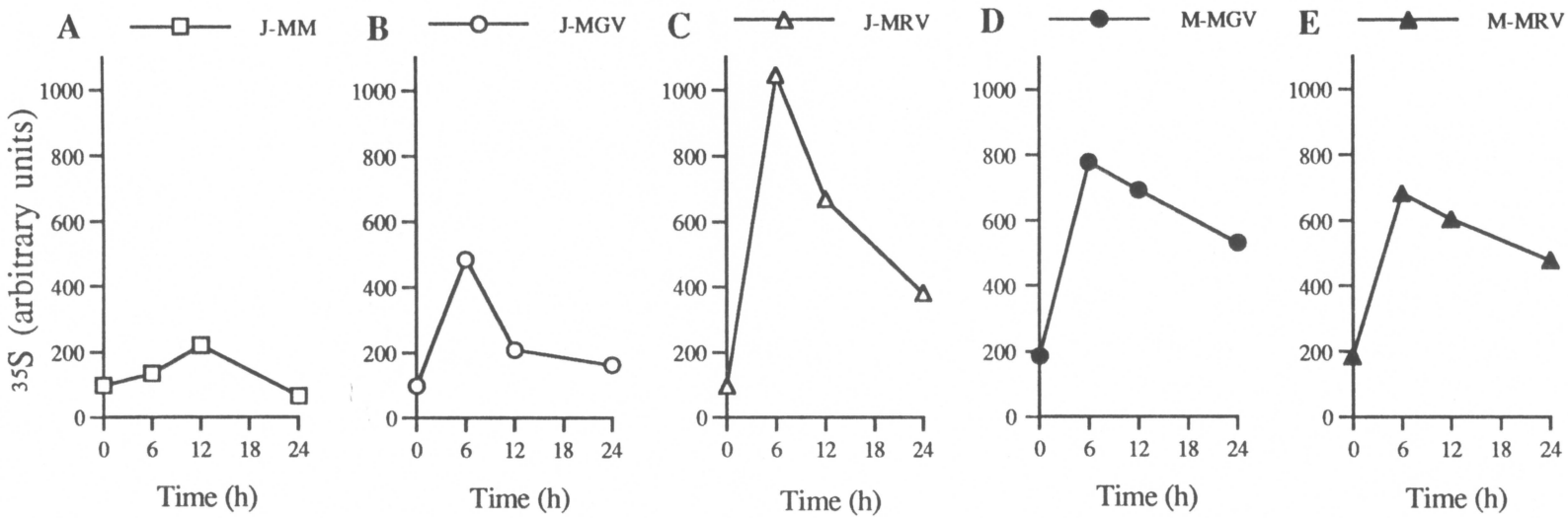



FIG. 8. Primer extension analysis of accumulated *vdh* mRNA from the *S. coelicolor* J802 (open symbol) and M480 (closed symbol) strains prepared from cells grown in MM, MGV and MRV media. The autoradiogram was scanned by a densitometer and the relative intensity units were plotted.

induced level of expression in glucose was observed in the presence of glycerol and approx. 5-fold increase in MRV (glycerol-valine) compared to that in MM (glucose-asparagine). No significant difference was observed in the level of the accumulated mRNA from the M480 cells grown in the MGV and MRV media (Figs. 8D and 8E), whereas the maximum induction of *vdh* mRNA was reproducibly lower in the *glkA* mutant M480 than in the J802 strain grown in the glycerol medium (Figs. 8E *vs* 8C). These results are in agreement with the work M. J. Bibb and colleagues who have reported that the *glkA* mutation relieves glucose repression and also appears to affect induction of the agarase *dagA*p4 promoter (2). Recent results reported by Angell et al. (2) suggest that the *glkA* protein plays a regulatory role in mediating glucose repression by interacting with protein(s) that presumably bind to the promoter regions of glucose-repressible genes. These data together show that glucose appears to repress *vdh* induction at the transcriptional level and suggest that this occurs by the same mechanism as glucose repression of carbohydrate metabolism in *S. coelicolor* (2).

**Multicopy effect of a plasmid *vdh*::*xylE* fusion on *vdh* induction.** Since Westpheling and colleagues (24) have successfully used a plasmid-borne copy of *xylE* as a reporter gene to investigate the effects of glucose and galactose on expression of the *gal* operon in *Streptomyces* spp., we investigated whether a plasmid-borne *vdh*::*xylE* fusion would faithfully reproduce some of the results described above. Plasmid pWHM1055 (Table 1 and Fig. 5) is a derivative of the pSG5-derived plasmid vectors (10) that are reported to have a copy number of 40 to 50 per chromosome (26). We did not measure the copy number of pWHM1055 or the other plasmids used in the following work and assume that variations in the copy number were possible during growth, even though all of the data obtained were highly reproducible. XylE specific activity was assayed quantitatively in cell extracts prepared from *S. coelicolor* J802(pWHM1055) transformants grown under the nutritional shift conditions described above. The activity increased to the maximium level within 6 h after

transfer in both the MM and MGV media, then diminished rapidly over the following 24 h (Fig. 9). The maximal XylE activity was about the same in both the MM and MGV media whereas the XylE activity was below the limit of detection for cells containing pXH106 without the *vdh* promoter. Thus, the XylE specific activity observed with the plasmid-borne *vdh*::*xylE* fusion did not correlate well with the XylE specific activity observed with the chromosomally integrated *vdh*::*xylE* fusion and the relative amount of *vdh* mRNA accumulated in cells grown in the MM or MGV media. The maximum XylE activity in the MGV medium for the integrated *vdh*::*xylE* fusion was about 5-fold lower than that observed for the plasmid-borne fusion, and the XylE activity in the MM medium for the integrated fusion was much lower than for the plasmid-borne fusion (Figs. 6 *vs* 9). These observations suggest the possibility that a regulatory factor required for valine induction was titrated out by multiple copies of the *vdh* promoter region or was unable to interact properly with the plasmid-borne *vdh* promoter region in pWHM1055.

**Upstream regions of the *vdh* promoter have a cis-acting effect on the regulation of *vdh* expression.** Before investigating whether pWHM1055 can bind factors involved in *vdh* expression, we examined the role of the sequence upstream of the *vdh* promoter by determining the effect of deletions constructed between the *Spl*I and *Tfi*I or *Dde*I sites immediately upstream of the -35 region of the *vdh* promoter (Fig. 1) on expression of the plasmid-borne *vdh*::*xylE* fusion, because cis-acting sequences are known to regulate the expression of a *Streptomyces* chitinase gene (4) and the *S. coelicolor* galactose operon (24). Deletion of the 95 bp *Spl*I-*Tfi*I segment (cloned as pWHM1064) removes a region with two sets of direct repeats compared with the 400 bp segment cloned in pWHM1055, and deletion of the 142 bp *Spl*I-*Dde*I segment (cloned as pWHM1067) removes these sequences in addition to a region with an inverted repeat sequence (I) closer to the -35 region (Fig. 1). Each of these DNA segments was inserted upstream of the *xylE* gene cloned on the high copy pXH106-

derivative and introduced into the *S. coelicolor* J802 strain by transformation. The XylE specific activities determined for *S. coelicolor* J802 strains containing pWHM1055, pWHM1064 and pWHM1067, and grown in the MGV medium as described above, are shown in Fig. 10. The strain with pWHM1064 showed no increase in *vdh* promoter activity upon transfer of the cells to the MGV medium, and that with pWHM1067 showed only a slight increase at 3 h compared to the data obtained for the pWHM1055 culture at 3 and 6 h (Fig. 10). Thus, the region upstream of the *vdh* promoter between -200 to -96 nt relative to the tsp at +1 is important for *vdh* regulation. Although these results are undoubtedly influenced by the same factors that cause the aberrant behavior of the plasmid-borne *vdh* promoter noted above, we believe that the differences in the apparent level of *vdh* promoter activity between the strains with pWHM1055, pWHM1064 or pWHM1067 suggest that the region between -200 to -96 nt containing the two direct repeat sequences (Fig. 1) might constitute a site for positive regulation, because no significant increase in XylE activity was caused by valine when these direct repeats were deleted. In contrast, the dyad repeat sequences I between -96 to -47 nt (Fig. 1) with a calculated $\Delta G_{25^{o}C}$ = - 13.5 kcal/mol (38a) might involve some form of negative regulation of the *vdh* gene, since the XylE activity increased approx. 2-fold at 3 h when the region with this dyad repeat I was deleted in addition to the region with two direct repeats: compare the effect of pWHM1067 with pWHM1064 in Fig. 10 (the differences in XylE activities between pWHM1064 and pWHM1067 are significant and reproducible). The results obtained in the two sets of experiments just described led to a genetic and biochemical investigation of trans-acting factors that bind to these regions.

**Trans-acting effect of sequences upstream of the *vdh* promoter region.** To determine whether certain regions upstream of the *vdh* promoter could titrate regulatory factors required for induction by valine, we tested the effect of plasmids that can increase the

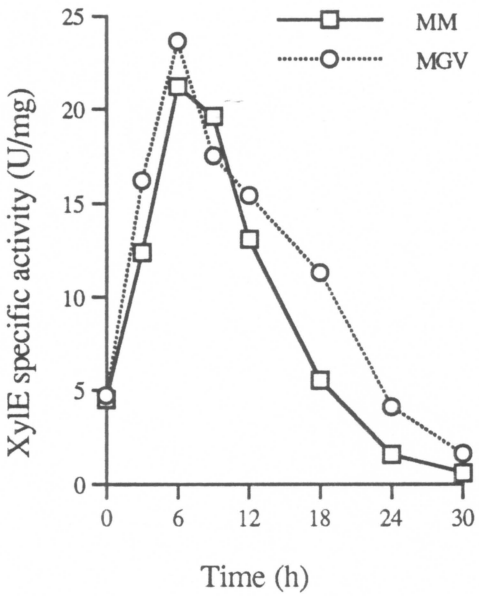



FIG. 9. XylE specific activity of the *S. coelicolor* J802 cells containing plasmid pWHM1055 during growth in the MM and MGV media. The XylE specific activity data are the average for at least 2 separate experiments, using the average values of duplicate samples from each experiment, and represent an average variation of less than 10%.

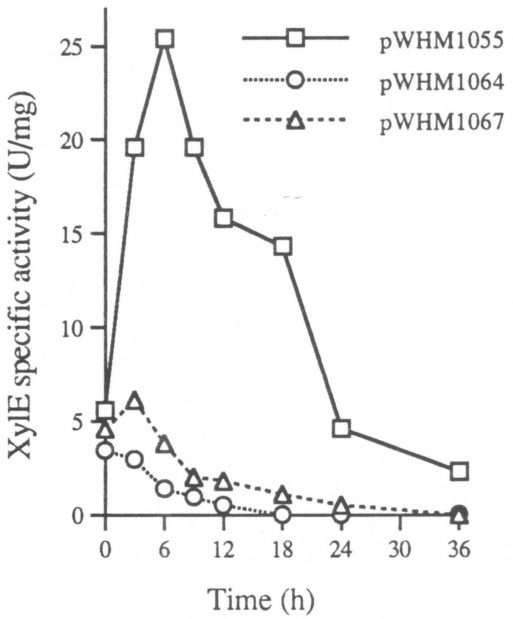



FIG. 10. The XylE specific activity of *S. coelicolor* J802 cells containing plasmid pWHM1055, pWHM1064 or pWHM1067 during growth in the MGV medium. The XylE specific activity data are the average for two separate experiments, using the average values of duplicate samples from each separate experiment, and represent an average variation of less than 10%.

number of copies of the *vdh* promoter region in the cell on Vdh activity due to the chromosomal *vdh* gene. If the cells have a limited amount of a regulatory protein that binds to the upstream regions, then the presence of multiple copies of the binding region should create a competition between the plasmid-borne and chromosomal regions for the regulatory protein. This competition could titrate the regulatory protein and prevent its interaction with the chromosomal promoter, resulting in an increase or decrease in *vdh* expression if a repressor or activator protein were operating on the *vdh* gene. We monitored the Vdh activity because this is a convenient way to determine the possible trans-acting effect of multiple copies of the plasmid-borne *vdh* promoter region on transcription of the *vdh* gene. In the presence of pWHM1055 (which contains the region with two direct repeats and both dyad repeats shown in Fig. 1) or pWHM1067 (which contains only the region with dyad repeat II in the -35 region of the promoter), Vdh activity decreased about 2-fold at 12 h in cells grown in the MGV medium (Figs. 11B and 11D), compared with the Vdh activity seen for the plasmid-free control (Fig. 11A). However, the Vdh activity varied the same way as in the control when the cells containing pWHM1055 or pWHM1067 were grown in the MM medium (Figs. 11A, 11B and 11D). These results imply that extra copies of the upstream regions cloned in pWHM1067 or pWHM1055 reduce the level of Vdh induction by valine, possibly by titration of a transciptional activator. When the region with only the two dyad repeats I and II is present in extra copies, as in cells containing pWHM1064, Vdh activity was induced by valine to the full level observed in the control cells (Figs. 11C *vs* 11A). Moreover, *vdh* expression was also significantly higher at 12 h (about 1.9-fold) when cells with pWHM1064 were grown in the MM medium (Fig. 11C *vs* 11A). This might be due to titration of a transcriptional repressor, resulting in a partially constitutive phenotype under the repressing condition (MM).

The observations made in the experiments on the cis- and trans-effects of the upstream regions point to the existence of sites that might bind an activator and a repressor of *vdh*

expression. These sites may or may not involve the direct and dyad repeats noted in the DNA sequence. Moreover, proteins that interact with these sites may interact with each other by DNA looping so as to enhance or reduce *vdh* expression. Possible explanations of the complex effects of pWHM1055, pWHM1064 and pWHM1067 on *vdh* expression are discussed below, following the presentation of direct evidence for protein binding to the region upstream of the *vdh* promoter.

**DNA binding proteins interact with the *vdh* promoter region.** For additional evidence that the region upstream of the *vdh* promoter contains targets for the binding of transcriptional regulators, we performed gel retardation experiments using $^{35}$S- or $^{32}$P-dCTP end-labeled fragments derived from the *vdh* promoter region (Fig. 12A) in the presence of a large molar excess of poly(dI-dC):(dI-dC) to eliminate non-specific effects on DNA-protein binding. Mobility shifts were first examined by polyacrylamide gel electrophoresis in the low ionic strength TBE buffer (25) with a cell-free crude extract of *S. coelicolor* J802 cells grown in the MM or MGV media using $^{35}$S-dCTP end-labeled fragments (Fig. 12B). The data revealed a specific DNA-protein binding of fragments F3, F5 and F6, all of which contain the -10/-35 region of promoter sequence (Fig. 12B, lanes 8, 9, 13, 14 and 16). This binding was abolished by an excess amount of unlabeled template F3 and F5 (Fig. 12B, lanes 10 and 11), which suggests that the -10/-35 region is the binding target.

Since the sensitivity of the above assay was insufficient to detect the DNA binding of fragments F1 and F2 that contain sequences upstream of the *vdh* promoter, excluding the -10/-35 region, we fractionated the crude cell extract by precipitating DNA-protein complexes with polyethylenimine (see Materials and Methods) and dissected the *vdh* promoter region into smaller fragments to enhance the sensitivity and specificity of the binding assay. When the gel retardation experiment was repeated with $^{32}$P-dCTP end-labeled fragments and the proteins obtained from the polyethylenimine fraction from the MGV culture, a mobility shift was

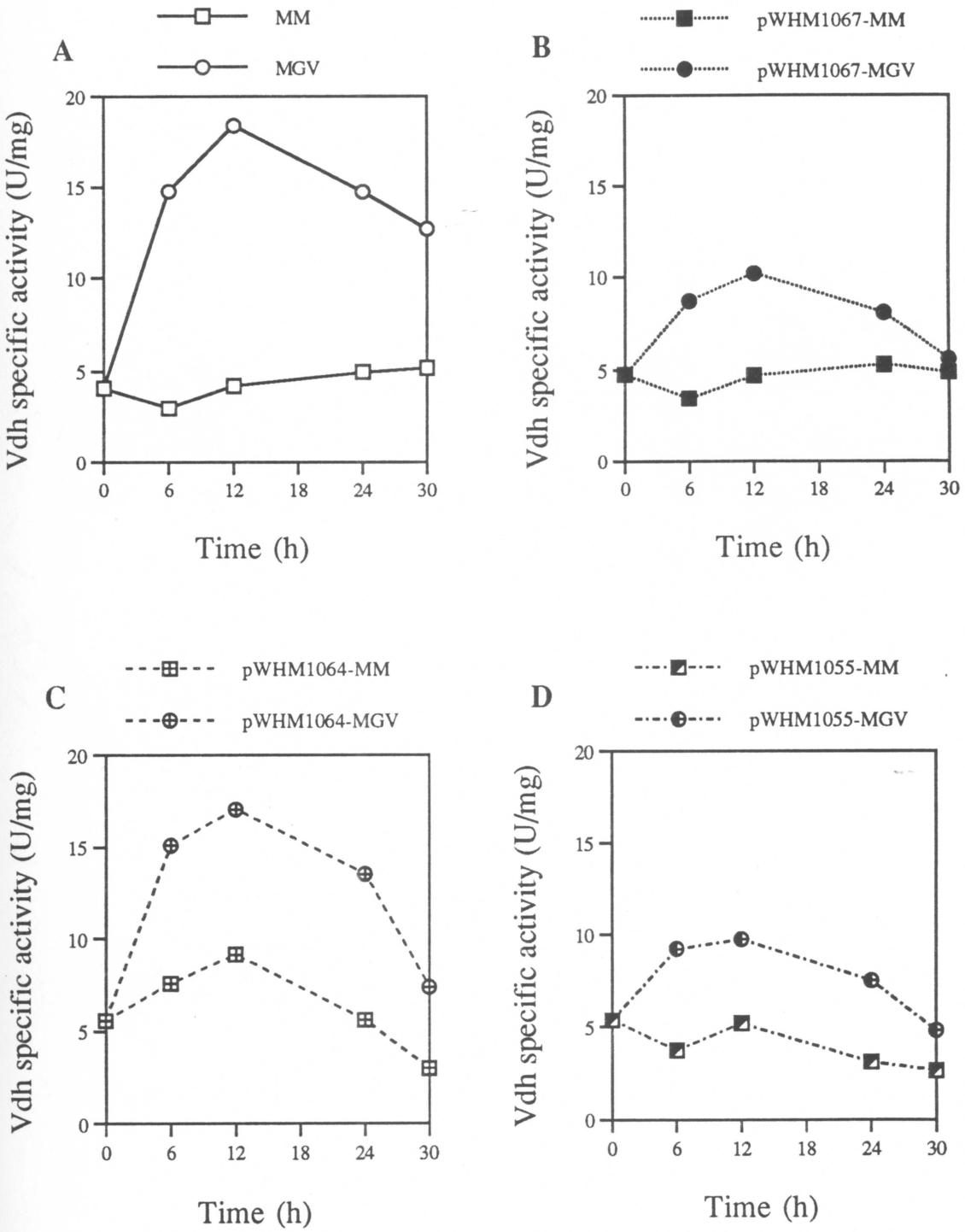
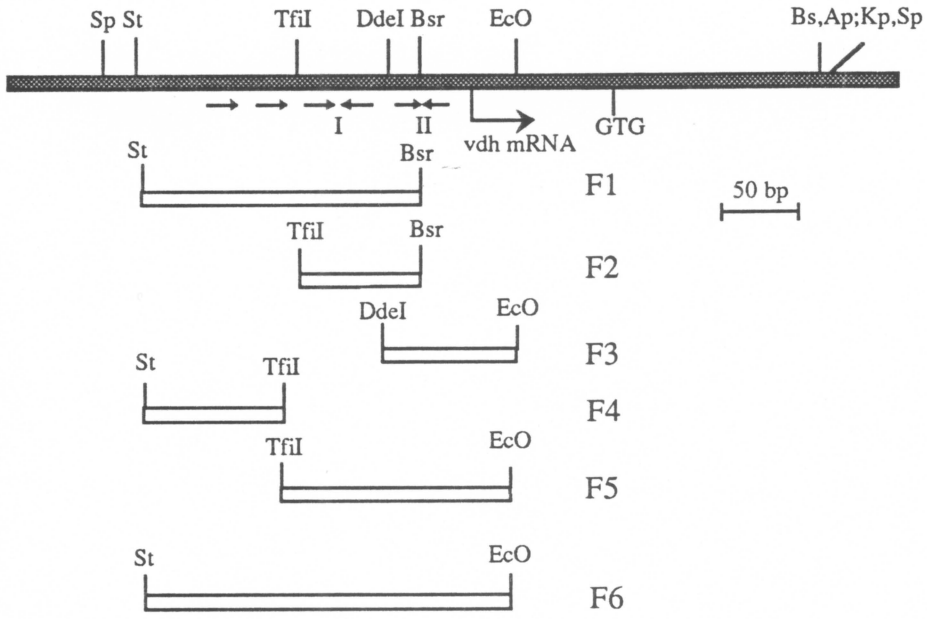

A
MM
MGV
Vdh specific activity (U/mg)
Time (h)
B
pWHM1067-MM
pWHM1067-MGV
Vdh specific activity (U/mg)
Time (h)
C
pWHM1064-MM
pWHM1064-MGV
Vdh specific activity (U/mg)
Time (h)
D
pWHM1055-MM
pWHM1055-MGV
Vdh specific activity (U/mg)
Time (h)

FIG. 11. The Vdh specific activity of *S. coelicolor* J802 and J802 cells containing plasmid pWHM1055, pWHM1064 or pWHM1067 during growth in the MM and MGV media. The Vdh specific activity data are the average for two separate experiments, using the average values of duplicate samples from each experiment, and represent an average error of less than 5%. The differences observed between apparent repression, derepression and activation of the chromosomal *vdh* gene by plasmid-borne *vdh* promoter regions were reproducible.

FIG. 12. (A) Restriction map of the fragments surrounding the *vdh* promoter region that were used as templates in the gel retardation assays. (B) Gel retardation analysis in a 5% polyacrylamide gel run in TBE buffer with $^{35}$S-dCTP end-labeled DNA fragments in the presence of 1.5 μg of poly(dI-dC):(dI-dC) and the cell-free extracts of *S. coelicolor* J802 grown in the MM and MGV media. Lanes 1, 4, 7, 12 and 15: protein free template as indicated; lanes 2, 5, 8 and 13: addition of the cell-free crude extract from the MM culture; lanes 3, 6, 9, 14 and 16: addition of the cell-free crude extract from the MGV culture; lane 10: the same as lane 9, except that a 20-fold excess of cold F5 fragment was added; lane 11: the same as lane 9, except that a 20-fold excess of cold F3 fragment was added. (C) Gel retardation analysis in a 5% polyacrylamide gel run in a high-ionic strength buffer with $^{32}$P-dCTP end-labeled DNA fragments in the presence of 1.5 μg of poly(dI-dC):(dI-dC) and the polyethylenimine precipitated protein fraction from cell-free extracts of *S. coelicolor* J802 grown in the MM and MGV media. Lanes 1, 4 and 7: protein free template as indicated; lanes 2, 5 and 8: addition of the protein fraction from the MGV culture; lanes 3, 6 and 9: addition of the protein fraction from the MM culture. ['*' indicates bands due to an impurity of partially digested template.]

A


Sp St
TfiI
DdeI Bsr
EcO
Bs,Ap;Kp,Sp
I
II
vdh mRNA
GTG
St
Bsr
F1
50 bp
TfiI
Bsr
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F3
St
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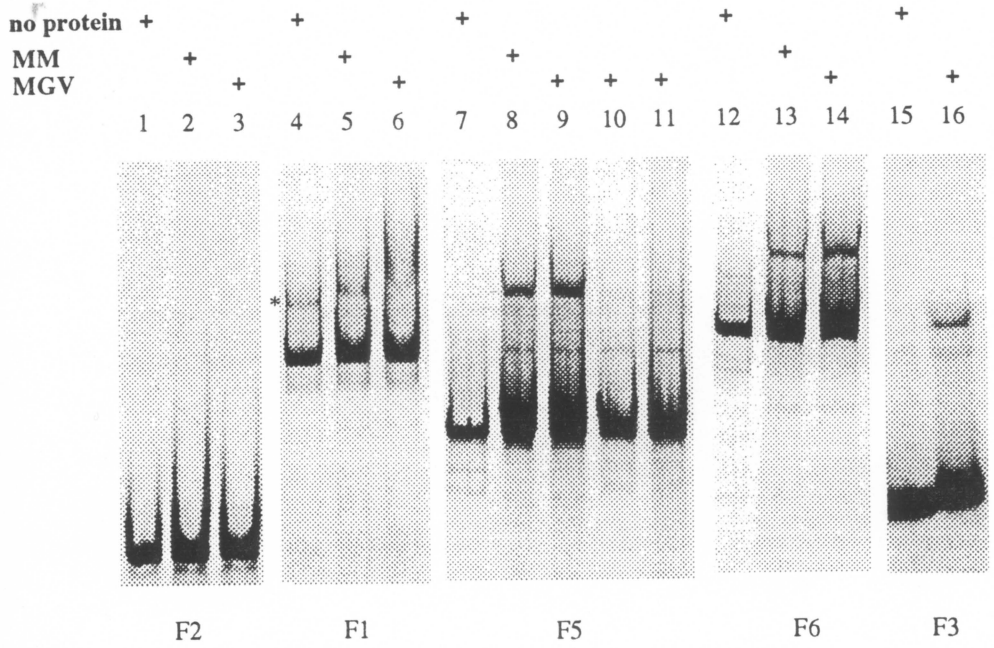
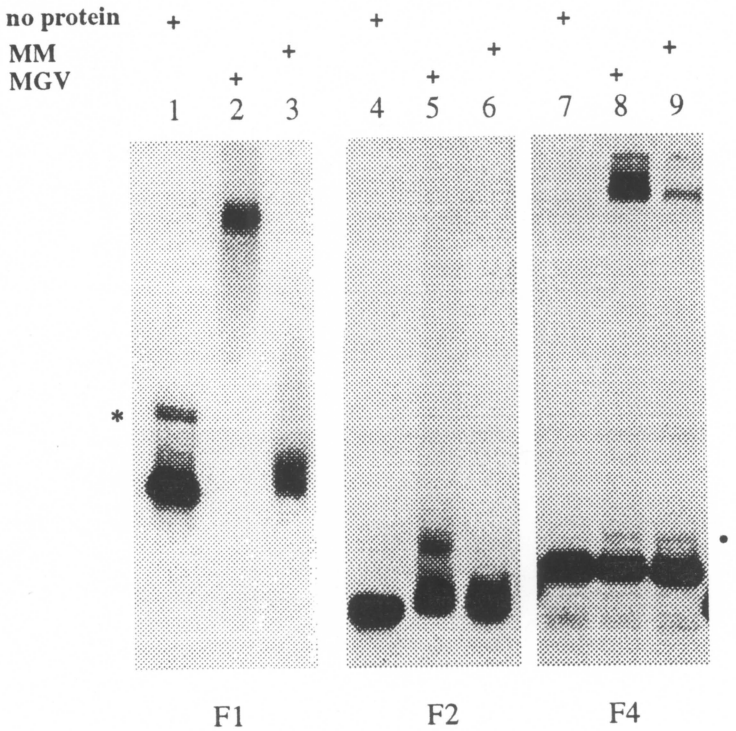
TfiI
EcO
F5
St
EcO
F6

**B**


no protein
MM
MGV
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
F2
F1
F5
F6
F3

**C**


no protein
MM
MGV
1 2 3 4 5 6 7 8 9
F1
F2
F4

observed for fragments F1, F2 and F4 (Fig. 12C, lanes 2, 5 and 8). A similar mobility shift was observed with the protein fraction from the MM culture only for fragment F4, but the amount of this complex was much less (Fig. 12C, lane 9). No significant retardation was seen with fragment F3 (data not shown). A faint band seen in the lower part of lanes 8 and 9 for the F4 fragment (dotted in Fig. 12C) suggests that this fragment binds two different proteins or the same protein in different stoichiometries. We used the same amount of total protein from the MM and MGV cultures (25 μg protein) in all of the gel retardation assays, but the amount of DNA-protein complex was clearly less from the MM culture than from the MGV culture (Figs. 12B, lanes 8 and 13; 12C, lane 9), and no binding was detected to fragment F1 and F2 with protein from the MM culture (Fig. 12C, lanes 3 and 6). From these experiments, we conclude that the -10/-35 region of the *vdh* promoter and the upstream regions that include the direct repeats and the dyad repeat I interact with DNA sequence-specific binding proteins, and that the binding sites correspond to the upstream activation and repression sequences predicted from the results of the plasmid titration assays.

## DISCUSSION

The information we have uncovered about the molecular genetics of the regulation of *S. coelicolor vdh* gene expression indicates that valine utilization is likely to be controlled by systems that regulate C and N utilization globally, since *vdh* expression is strongly influenced by the type of C and N source available in the growth medium. Extensive knowledge about the molecular genetics and biochemistry of nutrient utilization in several other bacteria, most notably enterobacteria like *E. coli* and *Klebsiella aerogenes*, has led to detailed models for the mechanism of C catabolite repression (31) and N assimilation (32). An equivalent depth of understanding has not been developed for streptomycetes, although important advances have been made recently in the areas of C catabolite control involving glucose kinase (2, 15) and its

effect on the utilization of agarose (2, 14), galactose (41) and chitin (6); and N assimilation involving glutamine synthetase and glutamate dehydrogenase (7, 8) or histidine (20).

This lack of insight hinders our ability to explain the observations about the behavior of the *vdh* promoter as a function of the C and N source reported here. Nonetheless, the following hypothesis provides a framework to guide further experimentation. Since substitution of valine for asparagine in a minimal medium with glucose as the C source induces *vdh* expression at the transcriptional level (Figs. 4 and 6) and results in notable changes in DNA-protein interactions (Fig. 12), it is likely that valine elicits changes in DNA-binding factors that regulate *vdh* transcription. Whether this is due to activation or derepression of the *vdh* gene by one or more of the proteins detected by the DNA mobility shift assays is not yet clear. The changes in Vdh activity seen in the plasmid titration experiments (Fig. 11) suggest a possible role of the -10/-35 region even though it alone clearly is not sufficient for full *vdh* induction based on the data shown for pWHM1067 in Fig. 10 and does not contain direct repeat sequences like those implicated in regulation of the *Streptomyces* chitinase [6] or galactose utilization [24] genes. However, the mechanism of *vdh* induction may involve the -35 GGCGGG site within the dyad repeat II sequence as a means for an activator to contact RNA polymerase or for binding of a specialized RNA polymerase holoenzyme formed in limited amount in response to a changing N or C source. We do not have any data that directly bear on this issue, yet note that the promoter region of the glucose catabolite controled *Aspergillus nidulans alcA* gene also contains a GCGGGGC sequence that is thought to be a binding site for CreA, a glucose-responsive repressor protein (12). Since the plasmid-borne -10/-35 region has a negative effect in trans on *vdh* induction (compare Figs. 11B and 11A), it may be that the dyad repeat II in this region is a binding site for an activator, resulting in a lower Vdh activity in the presence of pWHM1067. The results of the titration experiments also imply that the region with the dyad repeat I could be a binding site for a repressor since

pWHM1064 relieved the negative effect on *vdh* expression under the non-inducing condition and removed the negative effect of pWHM1067 (due to activator titration) on the Vdh level under the inducing condition (Fig. 11C). pWHM1064 may titrate both the activator and repressor but repression predominates due to lack of the region with the direct repeats. The fact that pWHM1055 caused less induction of Vdh activity compared to the control (Fig. 11D) does not necessarily invalidate this idea since pWHM1055 with both the direct repeats and dyad repeat I might bind both the activator and repressor, but not bind the repressor as well as pWHM1064 since the Vdh level did not increase in the MM medium in Fig. 11D the same way as shown in Fig. 11C. This analysis also could explain why the XylE activity for the pWHM1055 transformants had the same level in the presence of asparagine or valine (Fig. 9). Since the data in Figs. 4 and 6 clearly show that asparagine causes a much lower level of *vdh* expression, which peaks about 6 h later than that due to valine induction, we assume that the behavior of the *vdh::xylE* fusion in pWHM1055 reflects increased *vdh* "induction" by asparagine because the plasmid-borne *vdh* promoter region binds more of an activator or less of a repressor than the chromosomal *vdh* promoter region in MM medium with asparagine, giving the false impression that the *vdh* gene is induced by asparagine as well as by valine. A possible effect of the region downstream of the *vdh* tsp is ignored in the foregoing analysis since all of the plasmids tested contained it, along with the -10/-35 region of the *vdh* promoter.

If we assume that the results of the plasmid titration experiments are due to removal of limiting amounts of transcriptional regulators by a complex regulatory region that operates with a dynamic balance between derepression and activation, then the behavior of the plasmid-borne *vdh::xylE* fusions shown in Fig. 10 is consistent with the foregoing hypothesis for the following reasons. Compared with pWHM1055 which contains all three regions with sets of direct and/or dyad repeat sequences, the lack of *vdh* induction by valine with pWHM1064 could be due to the absence of the direct repeat sequences, which normally facilitate an

enhancer to assist activator binding to the -10/-35 region. The weak induction by valine in the case of pWHM1067, which lacks the region with the dyad repeat I sequence, could be due to relief of residual repression seen for pWHM1064 and lack of the enhancer effect of the direct repeats. Full induction by valine then must require the region with the direct repeat sequences upstream of the dyad repeats I and II.

It would be premature to illustrate these ideas by a mechanism for *vdh* induction that shows the interaction of specific transcriptional factors with the three upstream regions highlighted in Fig. 1. Further work is needed to identify the number of proteins involved and to test the role of the repeat sequences by site-specific mutation, before a model can be presented. Nonetheless, the results of the DNA deletion, plasmid titration and DNA:protein binding experiments presented here point to clear ways that such information can be obtained.

The divergent transcribed ORF1 gene, whose promoter region overlaps that of the *vdh* gene (Fig. 1), is apparently not required for *vdh* function since the *S. coelicolor* WMH1506 ORF1::*hyg* mutant with an insertionally inactivated ORF1 gene grows as well as the J802 strain and exhibits the same level of Vdh activity when grown on valine as the sole N source (data not shown). The results of preliminary nutritional shift experiments to test the promoter activity of the ORF1 gene, which were done with *S. coelicolor* J802 transformants containing a low copy version of the ORF1*::xylE* fusion (pWHM1053, Table 1), revealed a very low promoter activity when transformants were grown in either the MM or MGV media (data not shown).

Our principal interest in valine catabolism in streptomycetes is to find out whether branched-chain amino acid metabolism provides propionate and 2-methylmalonate for the biosynthesis of macrolide, polyene and polyether antibiotics. We now have cloned the *vdh* genes from the spiramycin-producing *Streptomyces ambofaciens* (38) and the tylosin-producing *Streptomyces fradiae* (38), and have determined that the organization of the *vdh* and

ORF1 genes in this region is the same as that in *S. coelicolor* (37). Furthermore, we have found that insertional inactivation of the *vdh* genes in each of these organisms causes a 4- to 6-fold decrease in spiramycin or tylosin production in a glucose-asparagine medium, and that antibiotic production by the *vdh::hyg* mutants is restored nearly to the wild-type level by adding propionate to the culture medium or by introducing the wild-type *vdh* gene on a plasmid vector (38). Consequently, we plan to extend the study of the regulation of *vdh* expression to these macrolide antibiotic producers to learn if the *vdh* gene is a principal point where macrolide production is regulated by C and N sources in the culture medium. These results, and the recent work of Hsieh and Kolattukudy on the role of the *Saccharopolyspora erythraea eryM* gene in supplying propionyl-coenzyme A for erythromycin biosynthesis (17), imply that macrolide production is regulated physiologically at the level of precursor supply.

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# Chapter 3

## Amino Acid Catabolism and Antibiotic Synthesis: Valine is a Source of Precursors for Macrolide Biosynthesis in *Streptomyces ambofaciens*

A modified version of this chapter as part of a paper, "Amino acid catabolism and antibiotic synthesis: valine is a source of precursors for macrolide biosynthesis in *Streptomyces ambofaciens* and *Streptomyces fradiae*", has been submitted to J. Bacteriol. with Dr. C. R. Hutchinson, Y. X. Zhang and myself as the sole authors.

## INTRODUCTION

Many studies have shown that antibiotic production is influenced by the type and level of the N and C sources in the growth medium (2, 5). A critical component of understanding the basic biological processes of secondary metabolism involves dissecting the molecular mechanisms that control carbon flow from primary to secondary metabolic pathways. In the case of substrate availability for the biosynthesis of the macrolide antibiotics like tylosin and spiramycin, it is known that the carbon framework is made from the coenzyme A (CoA) esters of acetate, propionate and butyrate plus their 2-carboxy derivatives (18). The propionyl- and 2-methylmalonyl-CoA substrates for macrolide biosynthesis could have multiple metabolic origins (8): the catabolism of odd-numbered fatty acids, reduction of acrylate, rearrangement of succinyl-CoA and the catabolism of methionine, threonine or valine. The latter two processes are likely to be the primary routes to 2-methylmalonyl- and propionyl-CoA under typical growth conditions.

Isotopic labeling experiments have been used to show that valine catabolism can supply the n-butyrate, propionate and 2-methylmalonate units for the biosynthesis of typical macrolide antibiotics (19, 24, 25). The negative effect of $NH_4^+$ on macrolide production (12, 20) and the strong correlation between macrolide production and the level of Vdh (12, 19, 21) favor the idea that branched-chain amino acid catabolism could be an important source of the macrolide building blocks like propionyl- and 2-methylmalonyl-CoA, when sufficient propionate is not available directly from the nutrient medium. This view is supported by the facts that addition of valine or isoleucine into the medium resulted in about a 3- to 5-fold higher level of protylonolide, the macrolide precursor of tylosin, than addition of succinate or asparagine (7, 21). Furthermore, 50 mM $NH_4^+$ strongly inhibits the incorporation of valine (20, 21) and the formation of tylosin and spiramycin (12, 21), and this negative effect largely is suppressed by

the addition of isobutyrate to the medium (12, 19, 21). These results led to the idea that Vdh is the site for inhibition of macrolide production by $NH_4^+$. We in fact have shown that the *S. coelicolor vdh* gene, which encodes the first enzyme of the valine catabolism pathway (14, 17), is induced by valine and repressed by $NH_4^+$ (27).

To better understand the correlation between macrolide production and branched-chain amino acid catabolism, we cloned the *vdh* gene from the spiramycin-producer *Streptomyces ambofaciens*, so that we could determine whether the inability to catabolize valine affects macrolide production under a defined growth condition. Disruption of the *vdh* gene by insertion of the *hyg* gene inhibited the biosynthesis of spiramycin, and the addition of propionate or isobutyrate to the medium or the introduction of the *vdh* gene into the *vdh*::*hyg* mutants reversed the negative effect of the *vdh*::*hyg* mutation on spiramycin production.

## MATERIALS AND METHODS

**Strains, plasmids and culture conditions.** Plasmids and strains made in this work are listed in Table 1. *Escherichia coli* strains DH-5α (23) and JM105 (23), used as hosts for plasmids or for M13 DNA sequencing, were grown at 37°C on LB and 2xYT media (23), respectively. Plasmid pWHM1051 (26) containing the *vdh* gene from *S. coelicolor* J802 was used as a probe in Southern analyses and colony hybridization. The spiramycin-producer *S. ambofaciens* ATCC 15154 was grown on TSB (Trypticase Soy broth, BBL) or R2YE plates (10) at 30°C for general use. Transformation of *S. ambofaciens* protoplasts was done by standard procedures (10), and transformants were selected on R2YE plates supplemented with 25 μg of thiostrepton (Th) or 200 U hygromycin (Hg, Sigma) per ml. For spiramycin production, *S. ambofaciens* cultures were grown in TSB medium at 30°C for 3 days, with shaking at 300 rpm, then the mycelial cells were harvested by centrifugation and used for seed

inoculate or stored in 20% glycerol at -80oC. A 0.2 ml portion of this mycelial suspension was used to inoculate 25 ml seed medium (3% glucose, 1% peptone, 1% yeast extract, 0.3% malt extract and 0.3% Casamino acid, pH 7.0) and the culture was incubated for 3 days. The resulting seed cultures were used to inoculate the desired fermentation medium (4% vol/vol). All fermentation cultures were conducted in 300 ml baffled flasks containing 50 ml medium at 30°C with shaking at 300 rpm. The fermentation medium contained per liter: 1 g $MgSO_4 \cdot 7H_2O$, 15 mg $ZnSO_4 \cdot 7H_2O$, 2 g $KH_2PO_4$, 15 g MOPS (morpholinopropane sulphonic acid, sodium salt), 5 g $CaCO_3$, 20 g NaCl, 0.3 mg $CoCl_2$, 20 g glucose and 1 g asparagine, pH 7.0.

**DNA preparation, plasmid construction, and screening of minilibraries.** Small-scale preparations of *E. coli* plasmid DNA were performed as described by Morelle (16). ss M13 DNA was isolated from JM105 as described by Sambrook et al. (23), 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, followed by treatment with the USBioclean® MP kit (United States Biochemicals (USB), Cleveland, OH) according to the manufacturer's directions. Streptomycete genomic DNA was isolated by the lysozyme-SDS method of Hopwood et al. (10).

An 1.8 kilobase (kb) fragment from pWHM1051 containing the *S. coelicolor vdh* gene (26) was dig-AP labeled as described below and used to probe several restriction enzyme digestions of *S. ambofaciens* genomic DNA. The 0.7, 1.8 and 2.0 kb *Sst*I DNA fragments from *S. ambofaciens* that hybridized with the labeled probe were size-fractionated by electrophoresis and isolated from a 1% agarose gel, and each fragment was cloned separately in pUC18 (30). These DNA minilibraries were screened with the *S. coelicolor vdh* probe by colony hybridization to obtain clones pWHM1071, pWHM1072, pWHM1073 (Fig. 1).

**DNA hybridization and sequencing.** Southern blot-hybridization and colony

Table 1. Plasmids and strains used in this work

| Plasmid/strain | Constructions and characteristics[a] | References |
|---|---|---|
| Plasmids | | |
| pXH106 | *bla tsr hyg*; ts; multicopy | 9 |
| pWHM1051 | *vdh*, ORF1 and ORF2 genes from *S.coelicolor* J802 in pUC18 | 26 |
| pWHM1065 | 6.0 kb *Hin*dIII fragment of pXH106 was religated | 26 |
| pWHM1071 | pUC19 with 0.7 kb *Sst*I fragment from *S. ambofaciens* | This work |
| pWHM1072 | pUC19 with 1.8 kb *Sst*I fragment of *S. ambofaciens*; ORF1 | " |
| pWHM1073 | pUC19 with 2.0 kb *Sst*I fragment of *S. ambofaciens*; *vdh* | " |
| pWHM1074 | The 0.85 kb *Sst*I-*Sal*I fragment from pWHM1073 was subcloned in pUC19. The resulting plasmid was digested with *Sst*I and ligated with the 0.7 kb *Sst*I fragment from pWHM1071 | " |
| pWHM1075 | pWHM1072 was digested with *Sst*I and partially digested with *Sal*I to give the 1.35kb *Sst*I-*Sal*I fragment which was subcloned in pUC19 | " |
| pWHM1076 | pWHM1074 was digested with *Hin*dIII and partially digested with *Sst*I, then the resulting 1.55 kb *Hin*dIII-*Sst*I fragment and the 1.3 kb *Sst*I-*Hin*dIII fragment from pWHM1075 were ligated into *Hin*dIII site of pWHM1065; *vdh* and ORF1 gene | " |
| pWHM1077 | The 1.85 kb *Bam*HI and *Hin*dIII fragment from pWHM1076 was cloned in pUC19. The resulting plasmid was digested with *Apa*I and the ends were filled-in then the 1.7 kb *Sma*I-*Eco*RV fragment of the *hyg* gene was clone into the blunt-end fragment. A 3.55 kb *Hin*dIII-*Bam*HI fragment was isolated from the resulting plasmid and cloned into the *Hin*dIII-*Bam*HI sites of pWHM1065; *vdh*::*hyg* | " |
| Strains | | |
| WMH1608 | *vdh*::*hyg* gene disrupted mutant of *S. ambofaciens* ATCC 15154 | This work |

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

hybridization were performed with Hybond-N membranes (Amersham, Arlington Heights, IL) by standard techniques (23). The dig-AP labeling, hybridization, and detection were done with the Genius® kit, following the manufacturer's protocols (Boehringer Mannheim, Indianapolis, IN). Hybridization was performed at 42°C overnight and the blot was washed 2 times with 1X SSC-0.1% SDS (23) for 5 min at room temperature and 2 times in 0.1X SSC-0.1% SDS for 15 min at 68°C .

DNA segments for sequencing were purified and subcloned into M13mp18 or M13mp19 (30). The nucleotide sequence of the resulting ssDNA was determined by the dideoxy chain termination method with the M13 -40 primers and a Sequenase® 2.0 kit (USB), following the manufacturer's instructions. 7-Deaza-dGTP was used in the 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 using the DNASTAR (Madison, WI) software and digitizer. The GCG software (6) version 7.0 was used for sequence analysis. Nucleotide sequence-deduced amino acid sequence data were compared with Vdh and ORF1 proteins from *S. coelicolor* by using the GAP program (6).

**Disruption of the *vdh* gene and Vdh activity assays.** To construct the plasmid used for *vdh* gene replacement in *S. ambofaciens*, an 1.85 kb *Bam*HI-*Hin*dIII segment of the *vdh* coding region from pWHM1076 was subcloned into the *Bam*HI-*Hin*dIII sites of pUC18, the resulting plasmid 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 (9), containing the *hyg* gene. A 3.5 kb *Bam*HI-*Hin*dIII fragment was isolated from this plasmid and subcloned into the *Bam*HI-*Hin*dIII sites of pWHM1065 to give plasmid pWHM1077 (Fig. 1). pWHM1077 was introduced into *S. ambofaciens* by transformation, then Th$^{R}$Hg$^{R}$ transformants were selected on R2YE plates and grown in R2YE liquid plus 200

U/ml Hg at 28°C for 3 days.

The mycelial cells from *S. ambofaciens*(pWHM1077)cultures were homogenized and further incubated at 39°C for 3 to 5 days to eliminate the autonomously replicating plasmid. The mycelia cells were plated on R2YE agar plus 200 U/ml Hg and incubated at 39°C for 5 to 7 days, then spores were collected and screened for $Hg^RTh^S$ clones.

Vdh specific activity was measured as described previously (26), using cells from *S ambofaciens* culures grown in TSB medium for 24 or 48 h.

**Antibiotic production.** Spiramycin titers of the culture broth was estimated by the agar plate diffusion assay, with *Bacillus subtilis* as test microorganism in Antibiotic Medium 1 (DIFCO, MI) adjusted to pH 8.0, from the standard curve line by seperately averge the zone diameters of the standard and the zone diameters of the samples tested on each set of three plates. Spiramycin (Sigma) was used as standard to prepare the standard curve. Identification of antibiotic producton was carried out by thin-layer chromatograph (TLC): A 5 ml portion of 6 or 7 day fermentation cultures of *S. ambofaciens* wild type (wt) and *vdh* mutant strains was extracted with 2.5 ml ethyl acetate. The ethyl acetate layer was dried and resuspended in 20 µl of fresh ethyl acetate and 1 µl of this extract was spotted on silica gel TLC plates, which were developed in chloroform-methanol (4:1 vol/vol) and bioautographed with *B. subtilis*. Growth of cultures was measured by dried cell weight (DCW).

## RESULTS

**Cloning and sequence analysis of the *vdh* gene from *S.ambofaciens*.** The *vdh* gene from *S. ambofaciens* was cloned by hybridization to the *vdh* gene from *S. coelicolor* J802 (26) as described in the Materials and Methods. Clones pWHM1071, pWHM1072, pWHM1073 were isolated from *S. ambofaciens* and a restriction map of the

cloned region is showed in Fig. 1. A 2.8 kb *Sal*I fragment (which contains the entire *vdh* and ORF1 genes) was recovered from the 3 clones and religated into pWHM1065 (26) to give pWHM1076 (Fig. 1, Table 1).

The sequence of an approx. 1.0 kb segment of the *S. ambofaciens vdh* gene in pWHM1076 was determined (Fig. 2). CODON PREFERENCE and GAP analysis (6) showed that there are two partial open reading frames (orfs) transcribed divergently in this region with the characteristic codon usage pattern for *Streptomyces* DNA (1). The amino acid sequence deduced from the two partial orfs exhibited significant sequence similarities with the Vdh and ORF1 proteins from *S. coelicolor* (26) showing approx. 96 and 90% identity over a span of 107 and 132 amino acids, respectively. Putative ribosome binding sites (RBS) were found at a suitable distance from the predicted GTG start codons (Fig. 2).

***vdh* gene inactivation.** To test the idea that valine catabolism, initiated by Vdh-catalyzed hydrolysis to produce $NH_4^+$ and 2-ketoisovaleric acid (which is further catabolized to isobutyrate, 2-methylmalonate and propionate [17, 24]), provides essential precursors of macrolide antibiotics, we disrupted the *vdh* gene in *S. ambofaciens* by insertion of the *hyg* gene into the respective coding regions. The *vdh*::*hyg* construct was cloned in a temperature-sensitive *Streptomyces* plasmid pWHM1065 (26) to give pWHM1077 (Table 1). After the *S. ambofaciens*(pWHM1077) transformants were grown at 39°C to eliminate the temperature-sensitive plasmid, 4 $Hg^RTh^S$ transformants (the WMH1608 strains) were isolated from the *S. ambofaciens*(pWHM1077) strain for further study. Chromosomal DNA was isolated from two representative WMH1608 strains and the wild-type strain and the DNA was used to analyze the region about the *vdh* gene by Southern analysis. The dig-AP labelled 1.15 kb *Bam*HI-*Sph*I fragment from pWHM1076 (Fig. 1) hybridized to a 2.9 kb *Bam*HI-*Sph*I fragment in strain WMH1608 in place of the 1.15 kb *Bam*HI-*Sph*I fragment in the wild-type strain (Fig. 3, lanes 2 and 3 *vs* 1). Digestion with *Sal*I gave 1.25 and 1.65 kb hybridizing bands in place of

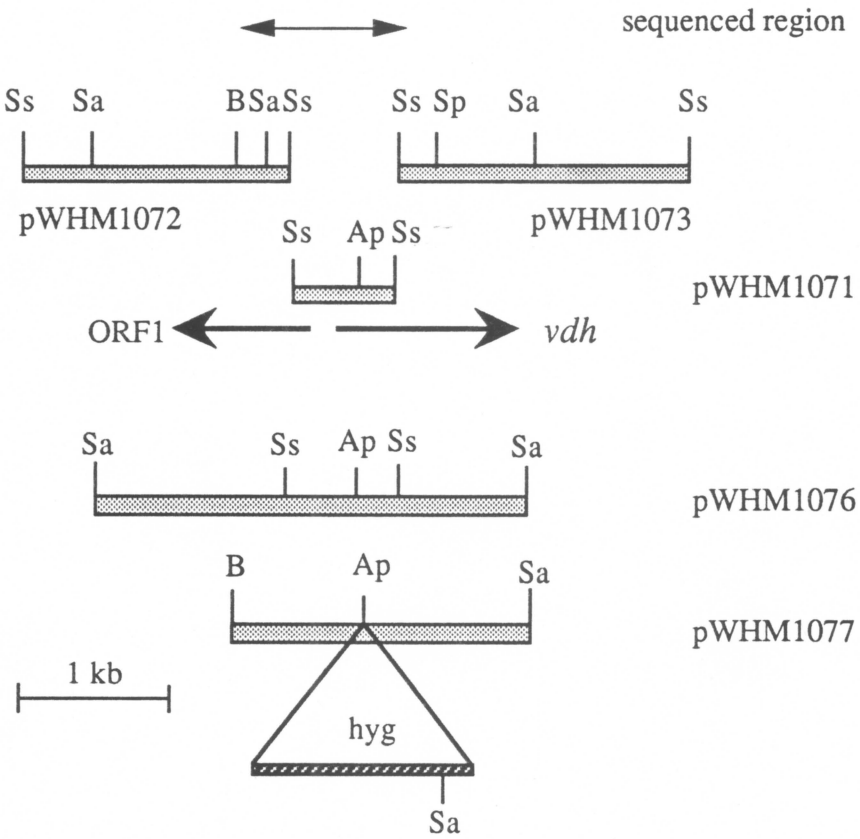


FIG. 1. Restriction map of DNA clones from *S. ambofaciens*. The thick arrows indicate the direction of gene transcription. The thinner arrows at both ends illustrate the sequenced region. Abbreviations: Ap, *Apa*I; B, *Bam*HI; Sp, *Sph*I; Sa, *Sal*I; Ss, *Sst*I; hyg, hygromycin resistance gene.

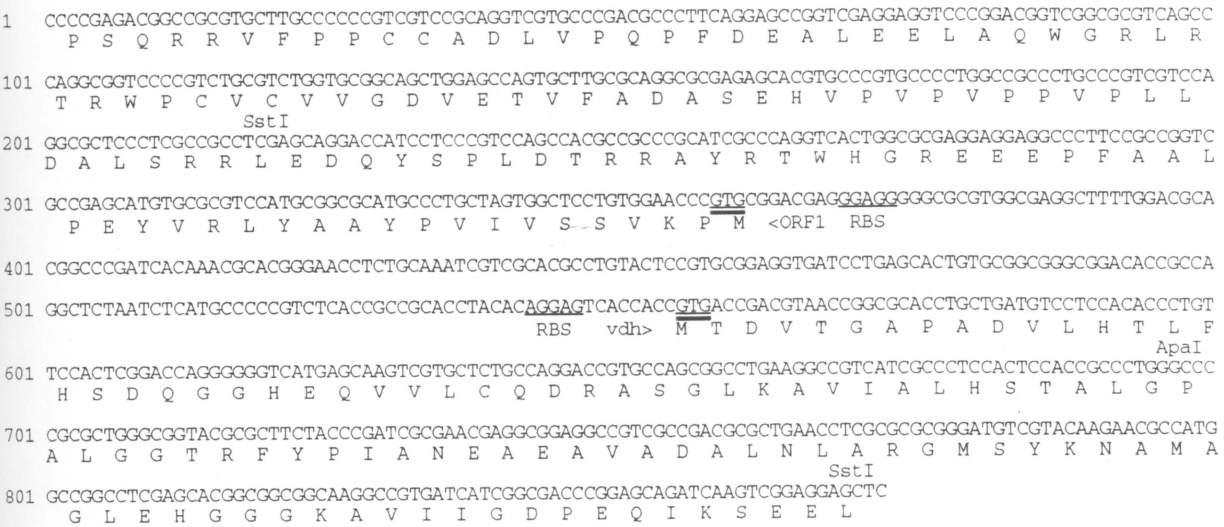


FIG. 2. Nucleotide sequence of the *vdh* region from *S. ambofaciens*. Only the bottom strand is shown from nt 1 to 400 and the top strand is shown from nt 401 to 872. Selected restriction enzyme sites are listed above their recognition sequences. The predicted translational start sites of the *vdh* and ORF1 genes are double underlined. RBS indicates the presumed ribosome binding sites (underlined).

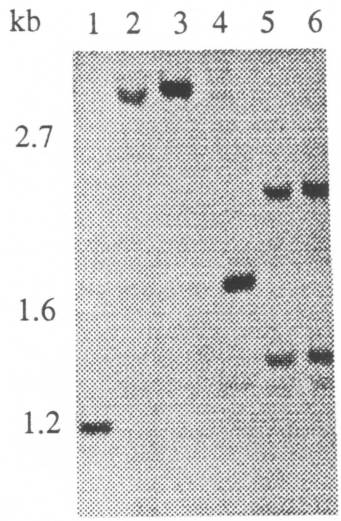


FIG. 3. Southern blot-hybridization analysis of chromosomal DNA of the *S. ambofaciens* wild-type strain (lanes 1 and 4) and *vdh* disrupted mutants WMH1608 (lanes 2, 3, 5 and 6). Genomic DNA was digested with *Bam*HI-*Sph*I (lanes 1 to 3) and *Sal*I (lanes 4 to 6).

1.25, 1.35 and 2.4 kb bands (Fig. 3, lanes 5 and 6 *vs* 4). These data confirm that the *vdh* gene was disrupted through the expected double crossover recombination.

As expected, the *vdh*::*hyg* mutants had no detectable Vdh activity in cell extracts of TSB-grown cultures, whereas the parental strain exhibited considerable Vdh activity at 24 or 48 h (Fig. 4) that was induced 2 to 3-fold by valine (data not shown). This result revealed that *S. ambofaciens* has only one gene encoding Vdh as in *S. coelicolor* (26). The *vdh* disrupted mutants WMH1608 therefore are suitable for testing the role of Vdh in antibiotic production.

**The *vdh*::*hyg* mutation greatly lower spiramycin production.** Spiramycin production of the parental strains and the *vdh* disrupted mutants WMH1608 under a defined growth conditions were compared by bioassay to detemine whether the inability to catabolize valine (or other branched-chain amino acids [28]) affects macrolide production. As shown in Fig. 5, the *S. ambofaciens* WMH1608 *vdh* mutants produced an approx. 4-fold lower level of spiramycin than the wild-type strain in the glucose-asparagine medium. The wild-type and *vdh*::*hyg* mutant strains maintain essentially identical growth profiles. As determined by TLC and bioautography assays, the *S. ambofaciens* WMH1608 *vdh*::*hyg* mutants as well as the parental wild-type strain mainly produced spiramycin (data not shown).

**Addition of propionate or isobutyrate compensates for the negative effect of the *vdh*::*hyg* mutation on spiramycin production.** To test whether the intermediates of valine catabolism, propionate and isobutyrate, could reverse the decrease in spiramycin production in *vdh*::*hyg* mutants, the level of spiramycin production by the WMH1608 mutants and parental strain was determined in the presence of 25 mM propionate or isobutyrate. Upon addition of propionate to the glucose-asparagine medium at time 0 h, spiramycin production by the WMH608 mutants was restored to a level comparable to that of the wild-type strain (Figs. 6 *vs* 5). Addition of exogenous propionate to the *S. ambofaciens* wild-type strain caused only a slight increase in spiramycin production (Fig. 6). However, the

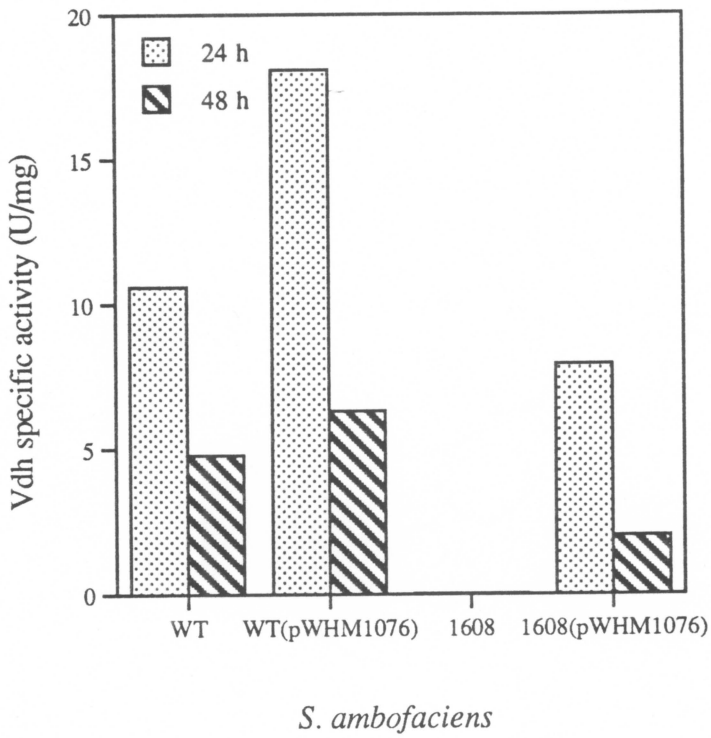


FIG. 4. The Vdh specific activity of the *S. ambofaciens* wild-type strain (WT), WMH1608 *vdh* mutant strains and their pWHM1076 transformants grown in TSB medium for 24 or 48 h.

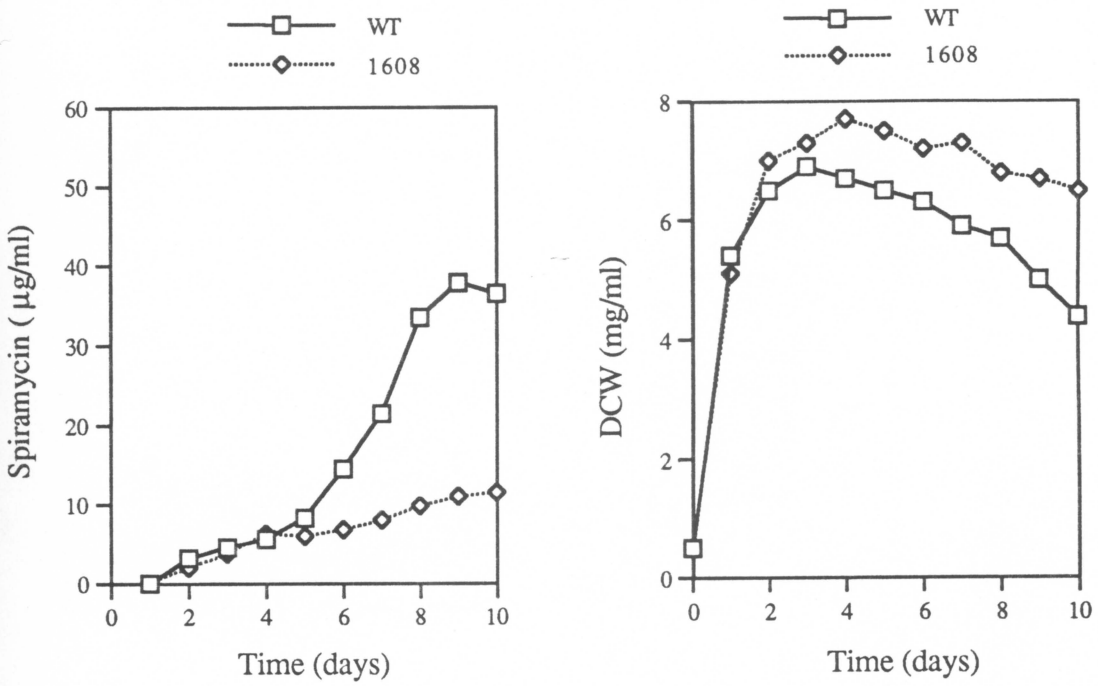


FIG. 5. Spiramycin production and growth (DCW) of the *S. ambofaciens* wild-type (WT) and WMH1608 *vdh* mutant strains grown in the glucose-asparagine medium. The spiramycin production data are the average for at least two separate experiments, using the average values of duplicate samples from each separate experiment, and the data for mutant WMH1608 strains are the average from two individual ThSHgR transformants. The average variation was less than 10%. The growth data represent an average error of less than 5%.

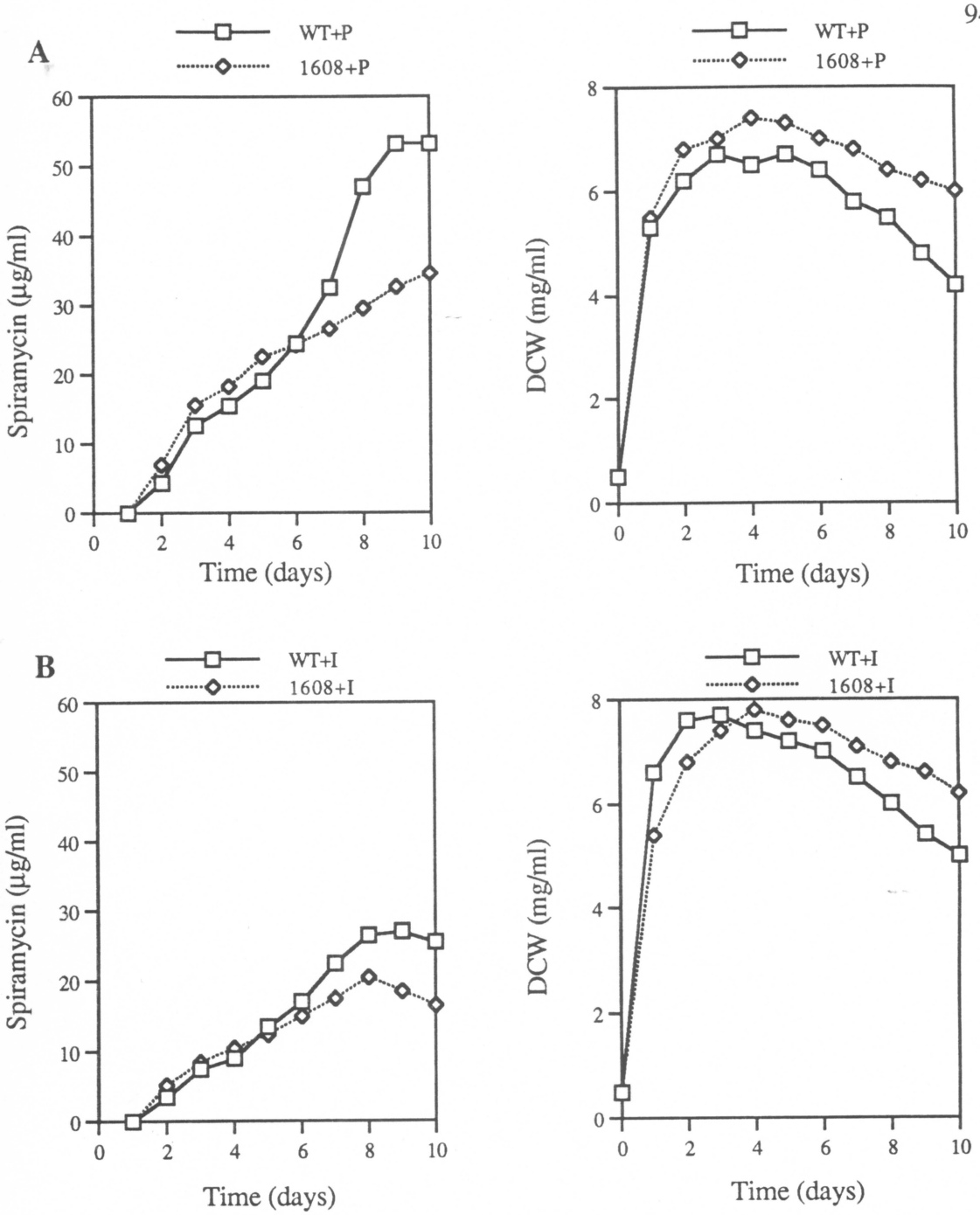


FIG. 6. Spiramycin production and growth (DCW) of the *S. ambofaciens* wild-type (WT) and WMH1608 mutant strains grown in the glucose-asparagine medium in the presence of 25 mM propionate (P) or isobutyrate (I) as indicated. The spiramycin production data are the average for at least two separate experiments, and the data for mutant WMH1608 strains are the average from two individual $Th^{S}Hg^{R}$ transformants. These data represent an average variation of less than 10% and the growth data represent an average variation of less than 5%.

addition of 25 mM isobutyrate caused a slightly lower level of spiramycin production in the wild-type strain and very little increase (approx. 2-fold) in the mutant strains (Fig. 6). Although the addition of propionate or isobutyrate did not appreciable modify the growth, the onset of spiramycin production was much earlier (Figs. 6 *vs* 5).

**Introduction of the *vdh* gene into the *vdh*::*hyg* mutants produces a notable increase in spiramycin production.** To examine the effect of introducing the *vdh* gene back to the *vdh*::*hyg* mutants on spiramycin production, the level of spiramycin production and the Vdh activity of the *S. ambofaciens*(pWHM1076) and WMH1608(pWHM1076) transformants were determined. A 2.85 kb fragment containing the *vdh* and ORF1 genes from *S. ambofaciens* was cloned on the multicopy plasmid pWHM1065 to give plasmid pWHM1076 (Table 1), then pWHM1076 was introduced into *S. ambofaciens* ATCC 15154 and WMH1608 *vdh*::*hyg* strains by transformation. The Vdh specific activity of the two types of pWHM1076 transformants was assayed in TSB grown cells at 24 or 48 h and found to increase in the wild-type strain and to return to near wild-type level in the WMH1608 mutants (Fig. 4). The WMH1608(pWHM1076) transformants produced an approx. 2-fold higher level of spiramycin than the WMH1608 mutants, although the level did not reach a value similar to that of the wild-type strain (Figs. 7 *vs* 5). Wild-type *S. ambofaciens*(pWHM1076) transformants produced significantly lower levels of spiramycin than the wild-type strain without the plasmid (Figs. 7 *vs* 5). Negative effects of plasmid vectors have been observed previously with other antibiotic-producing streptomycetes (4, 13, 29, 31).

## DISCUSSION

Our work reported here on the *vdh* gene from the spiramycin-producer *S. ambofaciens* strain was carried out in parallel with the tylosin-producer *S. fradiae* strain (31) to provide a

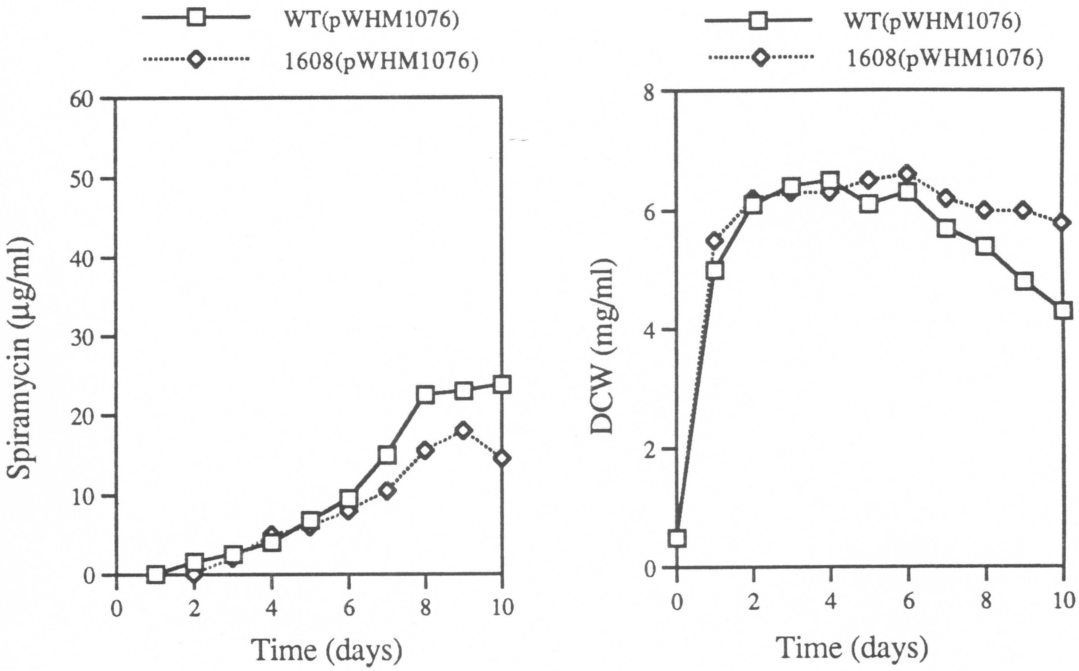


FIG. 7. Spiramycin production and growth (DCW) of the *S. ambofaciens* (pWHM1076) and WMH1608(pWHM1076) transformants grown in the glucose-asparagine medium. The spiramycin production data are the average for at least 2 separate experiments, and the data for the wild-type and *vdh* mutants are the averge from 2 individual pWHM1076 transformants. These data represent an average variation of less than 10% and the growth data represent an average variation of less than 5%.

thorough assessment of *vdh* structure and function, with respect to a possible role of the *vdh* gene in macrolide production. We found that the region surrounding the *vdh* gene has the same organization in *S. ambofaciens* as in *S. coelicolor* (26), tylosin producer *S. fradiae* (31) and polyether producer *Streptomyces albus* (28). Furthermore, the deduced amino acid sequences of the *vdh* and orf1 genes from *S. ambofaciens* are significantly similar to the products of the corresponding *S. coelicolor* genes (26) and *S. fradiae* genes (31). The *vdh* disrupted mutants from *S. ambofaciens* have no detectable Vdh activity, confirming the finding, first reported for *S. coelicolor* (26) and then *S. fradiae* (31), that there is only one gene for the Vdh activity in these organisms and presumably in other streptomycetes.

Parallel work also has been done by Zhang et al. (31) for the tylosin production in the *S. fradiae*. We cloned and sequenced the *vdh* gene from the tylosin-producer *S. fradiae* C373.1 and this *vdh* gene should encode a 363 amino acid protein with a calculated molecular mass of 37,917 Da. This would provide an enzyme subunit with a similar mass to those of the Vdh enzyme from *S. coelicolor* (17, 26) or *Streptomyces cinnamonensis* (22). Similar results were obtained for the effect of *vdh* mutation on tylosin production as for spiramycin production. The *S. fradiae* WMH1610 *vdh*::*hyg* mutant produced an approx. 6-fold lower level of tylosin than the parental wild-type strain in the defined glucose-$NH_4^+$ medium. Addition of 15 mM propionate to the glucose-$NH_4^+$ medium at time 0 h (the *S. fradiae* wild-type and WMH1610 mutant strains grew poorly in 25 mM propionate or isobutyrate) caused a significant increase in tylosin production by the WMH1610 mutant as well as the *S. fradiae* wild-type strain (31). Addition of 10 mM isobutyrate to the *S. fradiae* wild-type strain did not cause a significant increase in the production of tylosin but did restore tylosin production by the WMH1610 mutant to the level of the wild-type strain (31). We also have found that by introducing the *S. fradiae* wild-type *vdh* gene on plasmid pWHM1086 into the *vdh*::*hyg* disrupted mutant, the Vdh activity was restored (31). Furthermore, WMH1610(pWHM1086)

transformants have about the same level of Vdh as in the wild-type strain with pWHM1086, although the specific activity was increased approx. 3-fold compared to the plasmid free wild-type strain (31). The *S. fradiae* WMH1610(pWHM1086) transformants produced an approx. 4-fold higher level of tylosin than the WMH1610 mutant (31), whereas the *S. fradiae*(pWHM1086) transformants produced slightly lower levels of tylosin than the plasmid free wild-type strain.

The lack of a functional *vdh* gene reduces macrolide production significantly in the spiramycin- and tylosin-producing organisms when they are grown in a defined glucose-asparagine or glucose-$NH_4^+$ media. Since antibiotic production was not entirely lost but was restored by exogenous propionate or isobutyrate, valine catabolism (Fig.8) (17) is an important but not exclusive source of fatty acid precursors for macrolide biosynthesis. In the defined minimal media, glucose and/or asparagine should be converted to TCA cycle intermediates such as succinyl-CoA from which 2-methylmalonyl- and propionyl-CoA can be formed (8) and become precursors of the aglycone portions of macrolide antibiotics. However, since the work described here proves that valine catabolism is another source of such precursors, we speculate that once the cells enter the stationary phase, proteins are broken down to amino acids for conversion to the fatty acid precursors of macrolides when an alternative source (the TCA cycle for instance) of substrates like propionyl-CoA or 2-methylmalonyl-CoA is limited. Indeed, evidence from nutritional studies suggests that macrolide precursors are more directly derived from branched-chain amino acids than from other amino acids or succinyl-CoA (7). This idea is supported by our findings. Nevertheless, succinyl-CoA also appears to be an important source of propionyl-CoA according to the recent work of Hsieh and Kolattukudy (11). These workers suggest that in *Sacchoropolyspora erythraea* a malonyl-CoA decarboxylase can generate the propionyl-CoA needed for erythromycin biosynthesis from the (2R)-2-methymalonyl-CoA formed from succinyl-CoA by

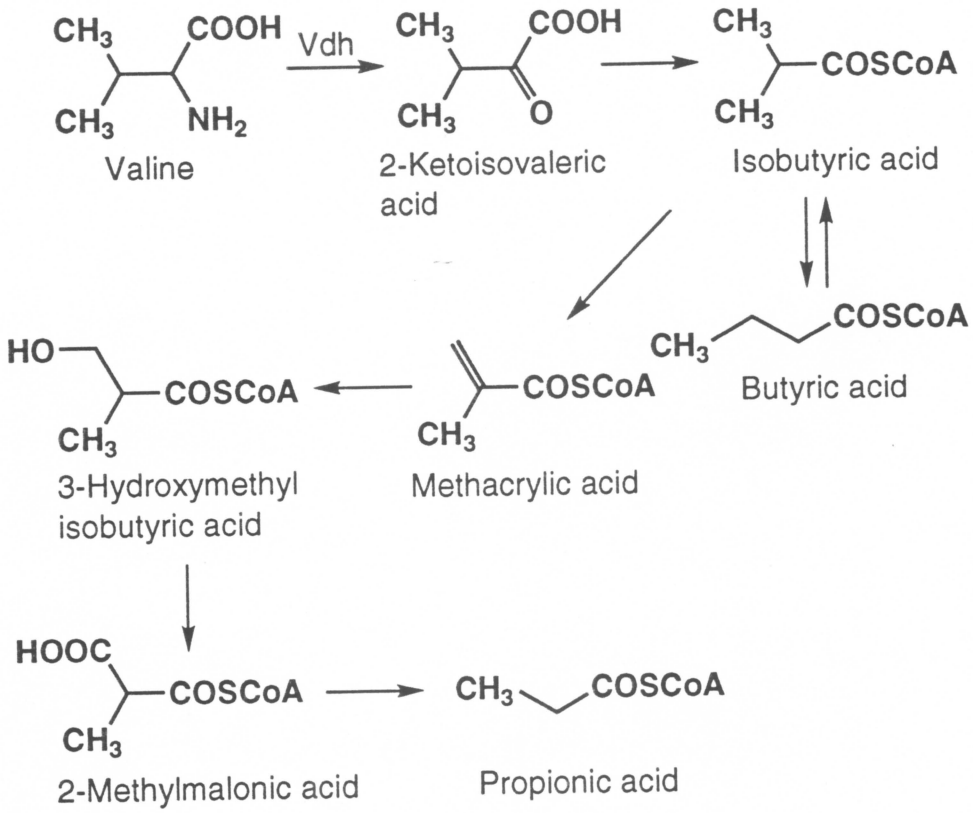


FIG. 8. The pathway for valine catabolism in streptomycetes. Intermediates in the pathway beyond 2-ketoisovaleric acid are arbitrarily shown as their CoA esters.

methylmalonyl-CoA mutase. Their hypothesis is based on the facts that disruption of the *S. erythraea eryM* gene for a malonyl-CoA decarboxylase lowered production of the macrolide antibiotic erythromycin by resting cells about 2.5-fold in a sucrose-alanine medium (where the organism did not grow) and that production was restored to the wild-type level by the addition of propionate to the culture medium.

Our results furthermore imply that macrolide production is regulated physiologically at the level of precursor supply because the apparent lack of valine-derived propionate causes a considerable decrease in spiramycin and tylosin production. Spiramycin production also was reduced in the *S. ambofaciens* wild-type strain when 25 mM isobutyrate was added to the fermentation medium, although this valine catabolite increased spiramycin production approx. 2-fold in the *S. ambofaciens* WMH1608 *vdh*::*hyg* mutants. The negative effect on spiramycin production in the wild-type strain was probably due to feedback repression of cellular metabolism by excess isobutyrate. A similar reason may explain why addition of 25 mM valine to the fermentation medium significantly decreased spiramycin and tylosin production in both the wild-type strains and *vdh* disrupted mutants, whereas addition of 10 mM valine resulted in a slight increase on spiramycin and tylosin production in both types of strains (data not shown).

The connection between amino acid catabolism and macrolide antibiotic production described here may be a logical consequence of the switch from vegetative growth to the formation of aerial mycelia, thence spores, in the morphogenesis of streptomycetes on solid medium. Hardisson and coworkers have shown that the vegetative substrate mycelium undergoes considerable degradation in *Streptomyces antibioticus* at the onset of aerial myceliation and that substances released from the lysing cells enter the aerial hyphae (15). Chater and Merrick, in fact, had suggested earlier that streptomycetes cannibalize one cell type in order to form a new type during morphogenesis, and that the production of antibiotics at this

time could protect the lysing cells from invasion by bacteria and preserve them as a source of nutrients for aerial growth (3). Antibiotic production thus could make use of the protein-derived amino acids as a source of essential precursors, for instance in the biosynthesis of the macrolide oleandomycin in *S. antibioticus*.

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# Chapter 4

## A homolog of the *mmsA* gene of *Pseudomonas aeruginosa* PAO is present in *Streptomyces coelicolor*

### INTRODUCTION

Streptomycetes produce a phenomenal variety of secondary metabolites including antibiotics and numerous gene clusters involved in the formation of antibiotics have been intensively studied, leading to considerable understanding of the genetic elements governing the biosynthesis of these compounds. However, comparatively little attention has been devoted to the mechanisms that operate in the transition from primary to secondary metabolism and control the carbon flow for the required building blocks. Several studies have shown that valine catabolism plays an important role in supplying fatty acid precursors for macrolide and polyether antibiotic formation (6, 9, 11, 12, 16).

Valine catabolism has been investigated in microbes and higher animals (7, 14, 15, 17). There is general agreement on the initial events of valine catabolism which include oxidative decarboxylation to yield isobutyryl-CoA. It is known that valine is degraded to propionate by some microorganisms and mammals, and that ß-hydroxyisobutyryl-CoA and 2-methylmalonate-semialdehyde are intermediates in this pathway. The aldehyde carbon of 2-methylmalonate-semialdehyde, which comes from C-3' of ß-hydroxyisobutyrate, becomes the carboxyl of propionate in this system as shown in Fig. 1b. However, the results of isotope labelling experiments (11) suggest that streptomycetes metabolize isobutyrate through a

different route (Fig. 1a), since C-1 of propionate comes from the carboxyl carbon of isobutyrate (C-2 of valine). This pathway may involve the direct conversion of hydroxyisobutyrate to methylmalonyl-CoA and subsequent decarboxylation of methylmalonyl-CoA to yield propionyl-CoA, but this idea has never been validated by experimental data.

We are interested in elucidating the pathway of valine catabolism in streptomycetes and characterizing the structural genes involved in the route leading to methylmalonyl-CoA and propionyl-CoA. As a first step, we used the *mms* operon DNA from *Pseudomonas* spp. (13) as a probe to look for the homologous sequences in streptomycetes. The *mms* operon contains *mmsA*, which encodes methylmalonate-semialdehyde dehydrogenase, and *mmsB*, which encodes 3-hydroxyisobutyrate dehydrogenase, has been cloned and characterized (13). Methylmalonate-semialdehyde dehydrogenase, an enzyme involved in the distal pathway of valine catabolism in Pseudomonads and mammals, catalyzes the direct conversion of methylmalonate-semialdehyde to propionyl-CoA with the concurrent reduction of NAD. The 3-hydroxyisobutyrate dehydrogenase, which catalyzes the $NAD^+$-dependent oxidation of 3-hydroxyisobutyrate to methylmalonate-semialdehyde, has been purified from rabbit liver. In this chapter, we report the molecular cloning and nucleotide sequencing of a homolog of the methylmalonate-semialdehyde dehydrogenase gene of *Pseudomonas aeruginosa* from *Streptomyces coelicolor.*

## MATERIALS AND METHODS

**Strains, plasmids and culture conditions.** *E. coli* strains DH-5α (10) and JM105 (10), used as hosts for plasmids or for M13 DNA sequencing, were grown at 37°C on LB and 2xYT media (10), respectively. *S. coelicolor* J802 (*dagA1, agaA7*) (4), obtained from David Hodgson, was grown on R2YE plates (5) at 30°C for general use. Plasmid pSJR78, kindly provided by John R. Sokatch, Univ. of Oklahoma, was used as a probe.

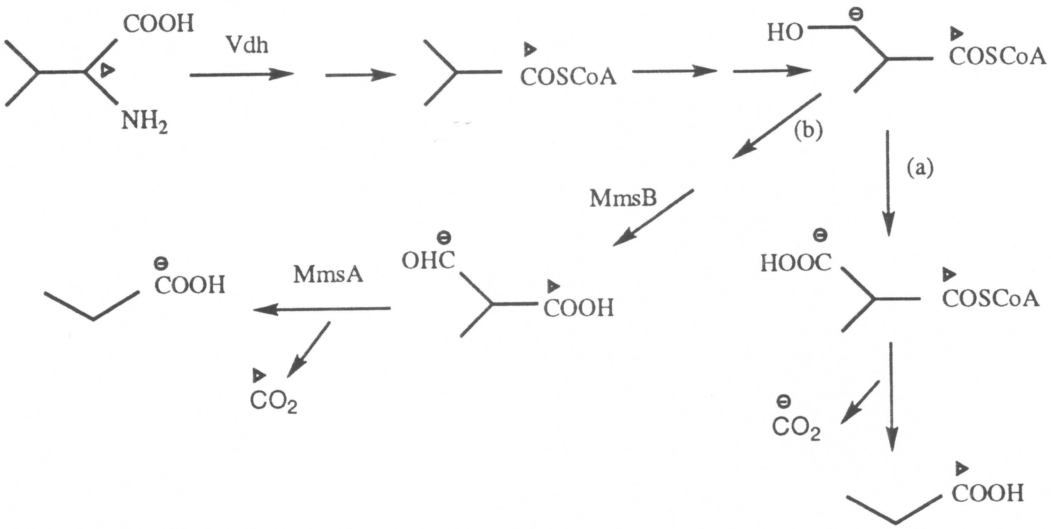



FIG. 1. Catabolism of valine in streptomycetes (a) and in other organisms (b). The symbols indicate the predicted relationships between carbons that are retained or lost during the catabolism.

**DNA preparation and construction, and screening of minilibraries.** Small-scale preparations of *E. coli* plasmid DNA were performed as described by Morelle (8). ssM13 DNA was isolated from JM105 as described by Sambrook et al. (10), 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, followed by treatment with the USBioclean® MP kit (United States Biochemicals (USB), Cleveland, OH) according to the manufacturer's directions. Streptomycete genomic DNA was isolated by the lysozyme-SDS method of Hopwood et al. (5). Oligodeoxynucleotides for PCR primers were synthesized by an Applied Biosystems Model 391 DNA synthesizer and purified according to the manufacturer's protocols.

The approx. 1.3 kb *Nar*I-*Kpn*I and 0.6 kb *Sph*I-*Stu*I fragments of pJRS78 were dig-AP labeled as probes for the *mmsA* and *mmsB* genes, respectively, and used to probe several restriction enzyme digestions of the *S. coelicolor* genomic DNA. Southern-blot hybridization showed that a 5.5 kb *Sst*I-*Sph*I DNA fragment hybridized with the *mmsA* probe. *S. coelicolor* J802 genomic DNA was double-digested with *Sph*I-*Sst*I, size-fractionated by electrophoresis on a 1% agarose gel, and 5.0 to 6.0 kb *Sph*I-*Sst*I DNA fragments were cloned in pUC18 (18). The DNA minilibrary was screened with the PCR product of the *mmsAB* probe to obtain clone pWHM1070.

**Polymerase chain reaction (PCR) product of *mmsAB*.** The primers for the 5'-end of the *mmsA* gene and 3'-end of *mmsB* gene used in the PCR experiment were oligo #1: CCCAGTCCGCCACCTCATCGCGG and oligo #2: GGATGCTGGAGAAGTCCAGC. 0.5 µg of each primer was incubated with 30 ng of the 2.0 kb *Hin*dIII-*Eco*RI fragment of pSJR78, which contains the *mmsAB* genes, in 20 mM Tris-HCl (pH 8.3), 1.2 mM $MgCl_2$, 20 mM KCl, 0.1% Triton X-100, 100 mg/ml BSA, 5% formamide (v/v) and 120 mM dCTP and

dGTP, 80 mM dATP and dTTP in a 99 µl total volume. The reaction was carried out in a DNA thermal cycler 480 (Perkin Elmer Cetus). After overlaying with mineral oil, the reaction mixture was boiled for 5 min and then cooled to 70°C and 4.5 U of Taq polymerase (Sigma) were added. Amplification was achieved with 25 cycles of denaturation at 96°C for 50 sec, followed by annealing/extension at 70°C for 3.5 min, with the addition of an extra 4.5 U of Taq polymerase at cycles 6, 12, 18. The resulting PCR product was purified by an Ultrafree-MC unit (Millipore Co.) and analyzed by restriction enzyme digestion.

**DNA hybridization and sequencing**. Southern hybridization and colony hybridization were performed with Hybond-N membranes (Amersham, Arlington Heights, IL) by standard techniques (10). The dig-AP labeling, hybridization, and detection were done with the Genius® kit, following the manufacturer's protocols (Boehringer Mannheim, Indianapolis, IN). The blot was washed 2 times with 1x SSC-0.1% SDS for 5 min at RT and then 2 times in 0.5x SSC-0.1% SDS for 15 min at RT. When the plasmid pWHM1070 was used as a probe, the blot was washed 2 times with 1x SSC-0.1% SDS for 5 min at RT and 2 times in 0.1x SSC-0.1% SDS for 15 min at 68°C .

DNA segments for sequencing were purified and subcloned into M13mp18 or M13mp19 (18). Nucleotide sequence of the resulting ss DNA was determined by the dideoxy chain termination method with the M13 -40 primer and a Sequenase® 2.0 kit (USB), following the manufacturer's instructions. 7-Deaza-dGTP was used in the 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 using the DNASTAR (Madison, WI) software and digitizer. The GCG software (2) version 7.0 was used for sequence analysis. Nucleotide sequence-deduced amino acid sequence data were compared with available databases by using the FASTA and TFASTA

programs (2).

## RESULTS AND DISCUSSION

**Cloning the homolog of the *P. aeruginosa* PAO *mmsA* gene from *S. coelicolor* J802.** The DNA probes containing the *P. aeruginosa mmsA* or *mmsB* genes were dig-AP labeled and used to identify cross-hybridizing bands in a Southern-bloting experiment with *S. coelicolor* J802 genomic DNA digested with various restriction enzymes. The high GC content of *Pseudomonas* spp. (about 67%) and *Streptomyces* spp. chromosomal DNA (about 70%) was expected to make it possible to probe *S. coelicolor* DNA sucessfully with the *mmsAB* genes. Bands were detected at low stringency (washing with 0.5x SSC-0.1% SDS at RT) with the *mmsA* probe, indicating the possible presence of closely a related sequence in the *S. coelicolor* genome, but not with the *mmsB* probe (data not shown). Approx. 5.0 to 6.0 kb *Sst*I-*Sph*I genomic DNA fragments corresponding in size to the band of interest were excised from an agarose gel, purified and ligated to *Sst*I-*Sph*I digested pUC18. Since the *mmsA* and *mmsB* probes derived from pJRS78, when excised from an agarose gel, gave strong hybridization bands with pUC18 at low stringency, a dig-AP labeled PCR product of the *mmsAB* genes (Fig. 2) was used as the probe to identify a positive clone, designated pWHM1070, by colony hybridization experiments. Restriction mapping (Fig. 2) demonstrated that it represented approx. 5.5 kb of genomic DNA from *S. coelicolor*.

Southern analysis using a dig-AP labeled probe derived from pWHM1070 DNA indicated that a corresponding *hmmA* gene (homologous to *mmsA*) is also presence in chromosome of *Streptomyces ambofaciens* ATCC 15154 and *Streptomyces fradiae* C373.1, although the intensity of the signal was much weaker in *S. fradiae* (data not shown).

**Nucleotide sequencing of the *hmmA* gene and flanking region.** The DNA sequence of a 4.0 kb fragment from pWHM1070 containing the *hmmA* locus and flanking

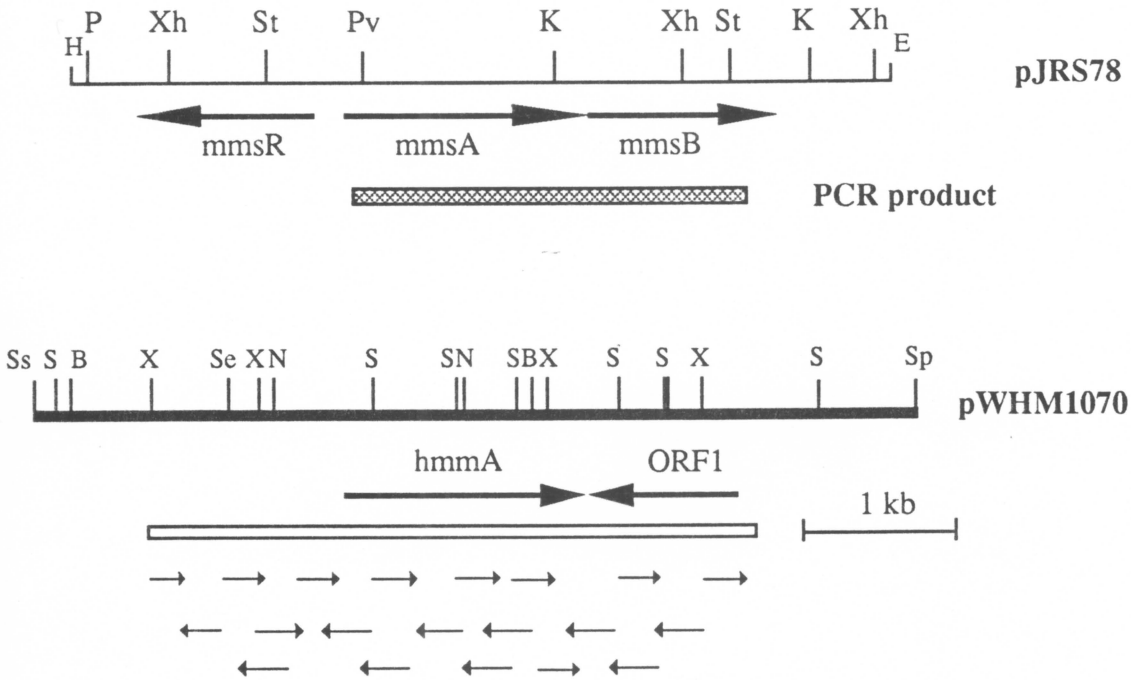



FIG. 2. Restriction maps of pJRS78 and pWHM1070 and DNA sequencing strategy for the 4.0 kb DNA fragment cloned in pWHM1070. The thick bold arrows indicate the direction of the transcription for the orfs. The open box beneath pWHM1070 indicates the region that was sequenced. The thinner arrows beneath this box illustrate the sequencing strategy. Restriction enzyme abbreviations: B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nsp*I; Pv, *Pvu*II; Sp, *Sph*I; S, *Sal*I; Se, *Spe*I; St, *Stu*I; P, *Pst*I; Ss, *Sst*I; X, *Xma*I; Xh, *Xho*I.

sequence was determined. CODON PREFERENCE analysis (2) showed that there are two major orfs in this region (Fig. 4), having the characteristic codon usage pattern for *Streptomyces* DNA (1). The convergently transcribed *hmmA* and ORF1 genes are separated only by a 62 nt noncoding region (Fig. 3). The 1500 nt *hmmA* orf begins with an ATG at position 1342 and terminates in a TGA at position 2842 and should encode a 500 amino acid protein. A region centered about 10 nt 5' to the first codon of the *hmmA* gene (Fig. 3) has a significant degree of complementarity to the 3' end of 16*S* rRNA of *S. lividans* and should serve as the ribosome binding site. This orf is followed by a potential transcription termination site ($\Delta G_{25°C}$ = -54 kcal/mol) that may be shared with ORF1.

Another orf was found downstream of the *hmmA* gene on the opposite strand, which is temporarily designated as ORF1. This 855 nt orf is likely to begin with ATG at position 3759 and terminate in TGA at position 2904, and to encode a 285 amino acid protein. A putative ribosome binding site was found at a suitable distance from the ATG start codon (Fig. 3).

A search of the GenEMBL databases using FASTA and TFASTA (2) revealed significant sequence similarities between the deduced HmmA protein and a variety of prokaryotic and eukaryotic aldehyde dehydrogenases ($NAD^+$-dependent), particularly with the MmsA protein from *P. aeruginosa*. But no significant homology was found between the deduced product of ORF1 and known proteins by database searching. The nucleotide and amino acid sequences of *P. aeruginosa mmsA* gene and the *S. coelicolor hmmA* gene were compared; as shown in Fig. 5, this comparison revealed approx. 63% and 47% identity between the nucleotide (B) and deduced amino acid (A) sequences, respectively, of the two genes, but no significant homology between ORF1 and *mmsB* or *mmsR* at the DNA or protein levels.

Methylmalonate-semialdehyde dehydrogenase is an usual enzyme in the aldehyde

```
        XmaI
1       CCCGGGGTCCCACGAGGGTCCGGAAGGTGCAGCCGCGGGCCGCCGGGTCCCACTCGTACAGGACGACCTCGCCGACGAGTTCGCCGGTGGCGCGGTCGGT

101     GACGGCCAGGTCGAGCCGGTCGGGATCGGCGGTGCGCATGCCGTACCAGGAGCGCAGTCCGTCCAGGGTGAGTTCGGTGGAGGGCTCGAAGGTGAAGCGG

201     ACCACCTCGGGGTCGTTGACGATCTCCCACATCGTGTCGGCGTCCTCGGCGGTGAACGGCCGCAGGACGGTCTGTCGCCGTGAGCACGTTCTCGAGATGC

301     ATGCACGCACTGTGCCACCGCGTGGGCACTGACGTTTGCCTCCGCCCTCCGGCCCTCCGTGCCTGCGCCCTCCGTCGTCCCGTCGCTACCGGTTGCGGTT

401     GCGGGGGCGGACGCCACCAGGCCGGACCGTGCTCCGCGTACCAGTACGCGCTTTCCGCCCGTCCACGTGGTCGCGCGGGGGCAGCAGCCCCGTGCTTGCG

501     GTACGACCGAACGTGTCCGGCTGGACCTTGATGTGGGCCGCGATGTCCTTGTAGGACCAGAGCCTTCGGTCGGTCACTAGTCGCACCTCCCTGCGCGCGC

601     CGCGGCGGCGGCCGGGGACGGCCGCCGGGGGGAGCCGGGCGCTGCGCTGGCGATCACCAAGCCTGTTCCGGGTGAACGACGCGGGGTGACGGACGGTCAG

        XmaI
701     GGCCCGTTCCCGGGTGTGACGGAAGACCCGCGTGCGCGTGACATGTGTGACGCGGCGGGCGCTTTTGTGACACGAGTGCCACACAGGCGTACGCCGTCGT

801     TCAGGCGCGGCGCGTTGACTGCGGCGGACGGAGGTGTTCCGCCGTACGCCCCTCCACCGCCACTCGGACTACGTCGTCCGAATGTCGGGACAAAACATTG

901     ACAGGCCGCACGCACGGGGCTAGAAACCGGGGTGACGCAAGGGCGCGGGCAAGGAGGGAACGCGATGGCGCACGACCCGACGGCACACGGCCGGGGCGCC

1001    GGTTCCCCACCGGAACGCCTCCGGCGCGGGAAGGCGGCCTCGCCTGGCAGGAGCGAGGCACGAGCGCCAACTCCCGCTCGCACCGGCTCGGCGGAGGTCC

1101    ACTGGGTCCGCCACGCGGCCGCCCACACTGCCGCAGTGTCTCGCGACACCAACCGCAGACACAGTGTCGGCGCGCCCGCGGCGCCCGGCTGCTGGGATGG

1201    AGTCATGGCCGAGACCGCGACCCGACCGTCCCCGGTCCCGGCCCGTGAGCAGCGCGAACAGCGTCTGCCGCGCGAACTGCGTCAGCTGCACCTACGGCAC

                                SD  hmmA >  M  T  N  I  V  N  H  W  I  G  G  K  T  A  E  G  A  S  G  T
1301    GTCTCGACCCGTCGCCGCCATCTGTGAAGGAGTTCTCGGTCATGACGAACATCGTCAACCACTGGATCGGCGGCAAGACCGCCGAAGGCGCCTCGGGCAC

          Y  G  P  V  T  N  P  A  T  G  E  V  T  T  K  V  A  F  A  S  V  D  E  V  D  A  A  V  A  A  A  R  E
1401    GTACGGGCCGGTGACGAACCCGGCGACCGGCGAGGTCACCACCAAGGTCGCCTTCGCCTCCGTCGACGAGGTGGACGCCGCGGTCGCCGCCGCCAGGGAG

          A  Y  L  T  W  G  Q  S  S  L  A  Q  R  T  S  I  L  F  R  F  R  A  L  L  D  A  H  R  D  E  I  A  E  L
1501    GCGTACCTGACCTGGGGGCAGTCCTCGCTGGCGCAGCGGACCTCGATCCTCTTCCGCTTCCGGGCGCTGCTGGACGCGCACCGCGACGAGATCGCCGAGC

            I  T  A  E  H  G  K  V  H  S  D  A  L  G  E  V  A  R  G  L  E  I  V  D  L  A  C  G  I  N  V  Q  L
1601    TGATCACCGCCGAGCACGGCAAGGTGCACTCCGACGCGCTCGGCGAGGTCGCGCGCGGCCTGGAGATCGTGGACCTGGCCTGCGGCATCAACGTACAGCT

          K  G  E  L  S  T  Q  V  A  T  R  V  D  V  S  S  I  R  Q  P  L  G  V  V  A  G  I  T  P  F  N  F  P
1701    CAAGGGCGAGCTGTCGACGCAGGTCGCGACCCGCGTGGACGTGTCCTCGATCCGGCAGCCGCTCGGTGTCGTCGCGGGCATCACGCCGTTCAACTTCCCG

          A  M  V  P  L  W  M  F  P  I  A  I  A  C  G  N  T  F  V  L  K  P  S  E  K  D  P  S  A  A  V  K  V  A
1801    GCGATGGTGCCGCTGTGGATGTTCCCGATCGCCATCGCCTGCGGCAACACCTTCGTGCTCAAGCCGAGCGAGAAGGACCCGTCGGCCGCCGTGAAGGTCG

            E  L  L  S  E  A  G  L  P  D  G  V  F  N  V  V  H  G  D  K  V  A  V  D  R  L  L  E  H  P  D  V  K
1901    CCGAGCTGCTCTCCGAGGCGGGTCTGCCGGACGGCGTCTTCAACGTCGTGCACGGCGACAAGGTGGCCGTGGACCGACTCCTGGAGCACCCGGACGTCAA

          A  V  S  F  V  G  S  T  P  I  A  R  Y  I  H  T  T  A  S  A  N  G  K  R  V  Q  A  L  G  G  A  K  N
2001    GGCGGTCTCCTTCGTCGGGTCGACCCCGATCGCCCGCTACATCCACACCACCGCCTCCGCCAACGGCAAGCGCGTGCAGGCGCTCGGCGGCGCCAAGAAC

          H  M  L  V  L  P  D  A  D  L  D  A  A  A  D  A  A  V  S  A  A  Y  G  S  A  G  E  R  C  M  A  I  S  A
2101    CACATGCTGGTGCTGCCCGACGCCGACCTCGACGCGGCGGCCGACGCGGCGGTCAGCGCGGCCTACGGCTCGGCCGGCGAGCGCTGCATGGCGATCTCCG

            V  V  A  V  G  A  V  G  D  E  L  V  E  K  I  R  E  R  A  E  K  I  K  I  G  P  G  T  D  P  S  S  E
2201    CGGTCGTGGCGGTCGGCGCCGTCGGCGACGAACTGGTGGAGAAGATCCGCGAGCGCGCCGAGAAGATCAAGATCGGCCCCGGCACCGACCCGTCCTCCGA

          M  G  P  L  I  T  K  A  H  R  D  K  V  A  S  Y  V  A  G  A  A  A  E  G  C  E  V  V  L  D  G  T  G
2301    GATGGGCCCGCTGATCACCAAGGCGCACCGCGACAAGGTCGCCTCCTACGTCGCGGGCGCGGCGGCCGAGGGCTGCGAGGTCGTCCTGGACGGCACCGGC

          Y  T  V  D  G  H  E  E  G  H  W  I  G  I  S  L  L  E  R  V  P  T  T  A  K  A  Y  Q  D  E  I  F  G  P
2401    TACACGGTCGACGGCCACGAGGAGGGGCACTGGATCGGCATCTCCCTGCTCGAGCGGGTGCCGACCACCGCGAAGGCGTACCAGGACGAGATCTTCGGCC

                                                                                                    XmaI
            V  L  C  V  L  R  A  D  T  Y  E  E  G  V  A  L  I  N  A  S  P  F  G  N  G  T  A  I  F  T  R  D  G
2501    CCGTCCTGTGCGTGCTGCGCGCCGACACGTACGAGGAGGGCGTGGCCCTGATCAACGCCTCGCCGTTCGGCAACGGCACCGCGATCTTCACCCGGGACGG

          G  A  A  R  R  F  Q  L  E  I  E  A  G  M  V  G  V  N  V  P  I  P  V  P  V  G  Y  H  S  F  G  G  W
2601    CGGCGCGGCCCGCCGCTTCCAGCTGGAGATCGAGGCCGGCATGGTCGGCGTGAACGTGCCGATCCCGGTCCCGGTGGGCTACCACTCCTTCGGTGGCTGG

        K  D  S  L  F  G  D  H  H  I  Y  G  N  D  G  T  H  F  Y  T  R  G  K  V  V  T  T  R  W  P  D  P  A  D
2701    AAGGACTCGCTCTTCGGCGACCACCACATCTACGGCAACGACGGCACGCACTTCTACACCCGCGGCAAGGTCGTCACCACCCGCTGGCCGGACCCGGCCG

          A  P  A  G  V  D  L  G  F  P  R  N  H  *                  >>>>>>>>>>>  >>>>>>>          <<<<<<<  <<<<<<<<<<<
2801    ACGCCCCGGCGGGAGTGGACCTGGGCTTCCCGCGCAACCACTGAGGCGGCGCGCACGCCCCCGGCCCGCCGAGCGGCACGGCGGACCGGGGGCGTCGCGG

2901    GTCACTGGCCGATAGCCGCGTCCCGCTGCTTCGTGACCTGCTCGGTGAGGTCGCCGGTCTCGGCGGCGGGCGGAGCCGTGATCTCCTTGGTGGCCCCGAA
        CAGTGACCGGCTATCGGCGCAGGGCGACGAAGCACTGGACGAGCCACTCCAGCGGCCAGAGCCGCCGCCCGCCTCGGCACTAGAGGAACCACCGGGGCTT
          *  Q  G  I  A  A  D  R  Q  K  T  V  Q  E  T  L  D  G  T  E  A  A  P  P  A  T  I  E  K  T  A  G  F
```

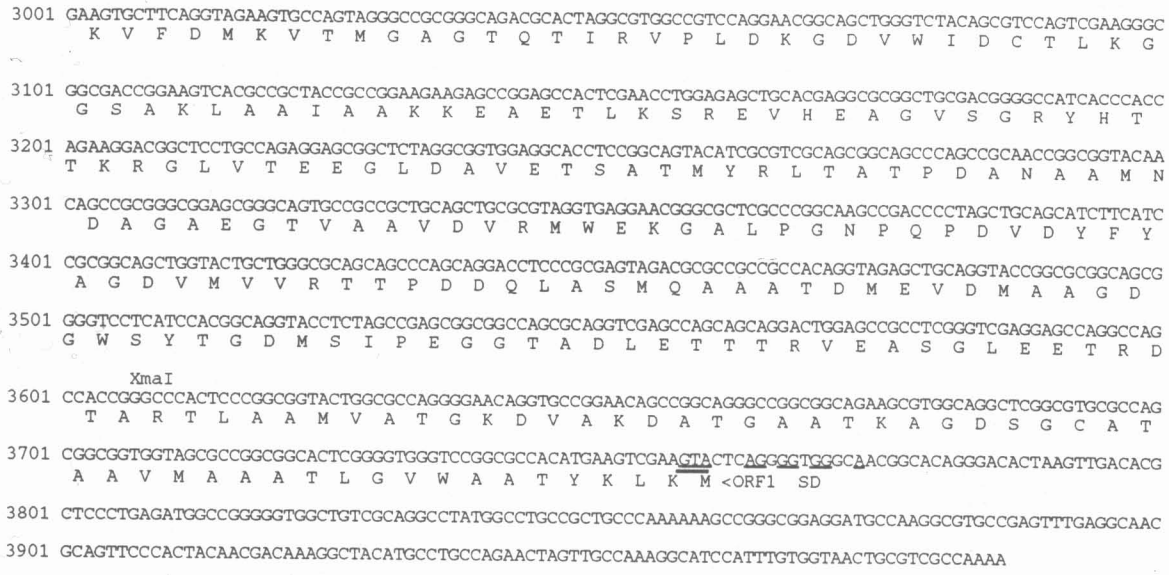



FIG. 3. Nucleotide sequence of the *hmmA* region. Both strands are shown from nt 2901 to 3000. Only the bottom strand is shown from nt 3001 to 3985, the top strand is shown from nt 1 to 2900. Selected restriction enzyme sites are listed above their recognition sequences. The ATG translational start sites of the *hmmA* and ORF1 genes are double underlined. SD indicates the presumed Shine-Dalgarno ribosome binding sites (underlined). Dyad repeats in the sequence are indicated by arrowheads showing the direction of repeated sequence.

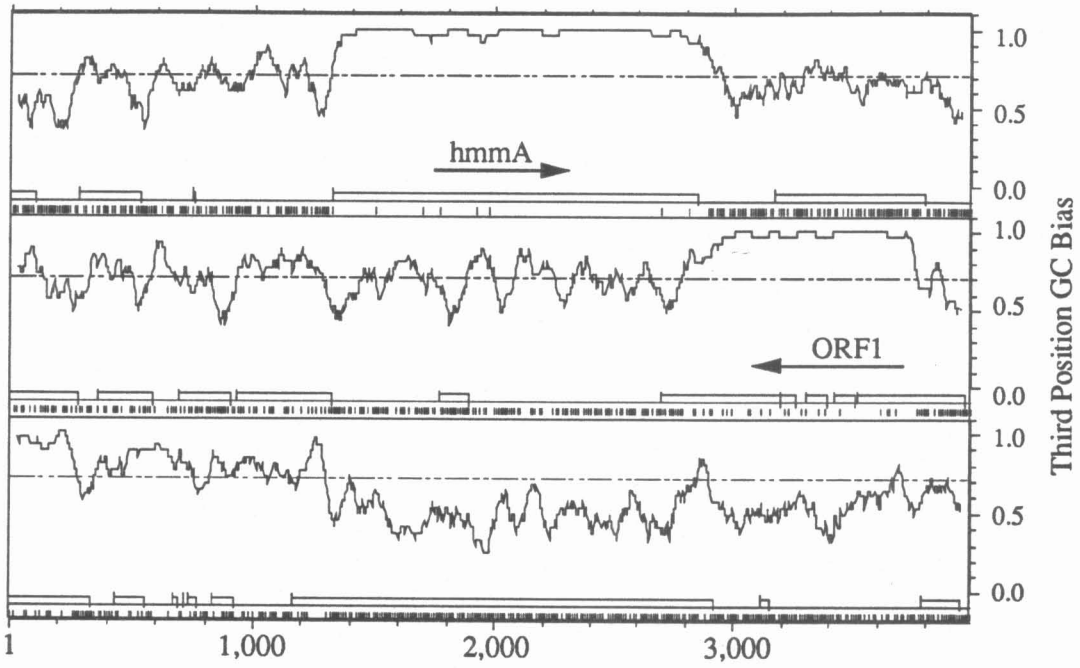



FIG. 4. CODON PREFERENCE analysis (2) of the sequence from the *hmmA* region in the 5' to 3' direction. The mole fraction of G and C nucleotides at the third position of each codon in the three possible reading frames (within a window of 25 consecutive codons) is plotted versus location in the sequence. A third position G+C content of greater than 90% is characteristic of protein coding sequences in *Streptomyces* spp. Rectangles below each plot indicate orfs that start with either ATG or GTG codons. Tick marks on the abscissa correspond to rare codons (less than 10% utilization, on average, in *Streptomyces* spp.). The analysis reveals two complete orfs with features typical of *Streptomyces* genes. The arrows above each orf indicate the direction of transcription.

**A**

```
HmmA 1 MTNIVNHWIGGKTAEGASGTYGPVTNPATGEVTTKVAFASVDEVDAAVAA 50
       |.  |.|:|:|  .|| ::   ||.|| .... ..:| ||.::|:.|||.
MmsA 1 MSVPVRHLIAGAFVEGLGAQRIPVSNPLDNSTLAEIACASAEQVEQAVAS 50

    51 AREAYLTWGQSSLAQRTSILFRFRALLDAHRDEIAELITAEHGKVHSDAL 100
       |||.: .| :..:.:|..:::|:.|||..|:||:|.::..| ||. .||
    51 ARETFASWKETPVSERARVMLRYQALLKEHHDEIAKIVSSELGKTFEDAK 100

   101 GEVARGLEIVDLACGINVQLKGELSTQVATRVDVSSIRQPLGVVAGITPF 150
       |:| ||:|:|: ||.:   |.||  ..|| .:|. || |||||..|||||
   101 GDVWRGIEVVEHACNVPSLLMGETVENVARNIDTYSITQPLGVCVGITPF 150

   151 NFPAMVPLWMFPIAIACGNTFVLKPSEKDPSAAVKVAELLSEAGLPDGVF 200
       |||||:||||||:||||||.|:|||||. | ..|::|||: ||| |.||:
   151 NFPAMIPLWMFPLAIACGNAFILKPSEQVPLTSVRLAELFLEAGAPKGVL 200

   201 NVVHGDKVAVDRLLEHPDVKAVSFVGSTPIARYIHTTASANGKRVQALGG 250
       .||||:| .||.||.||:|||||||||.:::.|:. |:.|:.||||.::|
   201 QVVHGGKEQVDQLLKHPQVKAVSFVGSVAVGQYVYHTGTAHNKRVQSFAG 250

   251 AKNHMLVLPDADLDAAADAAVSAAYGSAGERCMAISAVVAVGAVGDELVE 300
       |||||:::|||| ... .. |:|. |.||:||||||..| ||| : |::.
   251 AKNHMVIMPDADKAQVISNLVGASVGAAGQRCMAISVAVLVGA.AREWIP 299

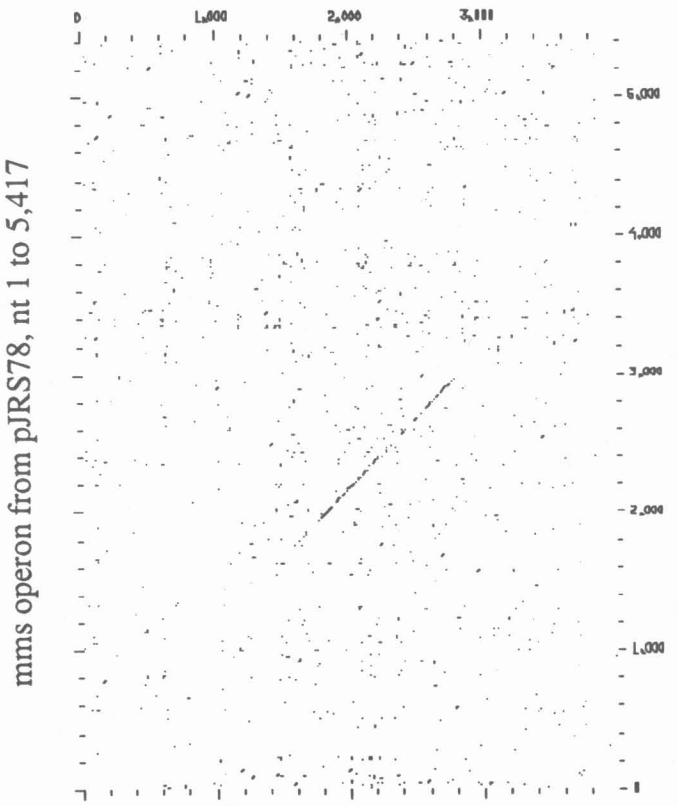
   301 KIRERAEKIKIGPGTDPSSEMGPLITKAHRDKVASYVAGAAAEGCEVVLD 350
       .||: .|:: || .|.:.. ||:|... :.::...::.:..||.:::||
   300 EIRDALAKVRPGPWDDSGASYGPVINPQAKARIERLIGQGVEEGAQLLLD 349

   351 GTGYTVDGHEEGHWIGISLLERVPTTAKAYQDEIFGPVLCVLRADTYEEG 400
       | ||.|:|..:|:|:| .|:. |...   |.:|:|||||:  .|..|::
   350 GRGYKVEGYPDGNWVGPTLFAGVRPDMAIYREEVFGPVLCLAEVDSLEQA 399

   401 VALINASPFGNGTAIFTRDGGAARRFQLEIEAGMVGVNVPIPVPVGYHSF 450
       : |||.||:|||.|||..|:||| || .||.| ||:|:|||||:.: ||
   400 IRLINESPYGNGTSIFTSSGAAARTFQHHIEVGQVGINIPIPVPLPFFSF 449

   451 GGWKDSLFGDHHIYGNDGTHFYTRGKVVTTRWPDPADAPAGVDLGFPRNH 500
       .|||:|::|| | ||.:|.:||| .|.||.|| | .|..||.::::...
   450 TGWKGSFYGDLHAYGKQGVRFYTETKTVTARWFD.SDSVAGTNFSIQMR. 497
```

**B**




*hmmA* region from pWHM1070, nt 1 to 3,986

FIG. 5. (A) Comparison of amino acid sequences of MmsA from *P. aeruginosa* and HmmA from *S. coelicolor*. (B) DOTPLOT of nucleotide sequence of *mmsA* and flanking region from *P. aeruginosa* *vs. hmmA* from *S. coelicolor*. Window = 21; Stringency = 15.

dehydrogenase superfamily since it catalyzes the formation of a thioester and also has malonate-semialdehyde dehydrogenase activity (3). This enzyme is involved in the catabolism of valine, thymine and compounds catabolized by the way of ß-alanine, including uracil and cytidine, in mammals (3). The role of the *hmmA* gene present in *Streptomyces* spp. is not known but its characterization is being studied by gene disruption and enzyme activity assays in our laboratory. This study may suggest that the *S. coelicolor hmmA* gene is not involved in the catabolism of valine or that the HmmA protein may not have its primary activity as a methylmalonate-semialdehyde dehydrogenase and be involved in oxidizing 3-hydroxyisobutyrate to methylmalonyl-CoA. On the other hand, since a homolog of the *mmsB* gene was not found by hybridization to the *mmsB* probe of *P. aeruginosa* or adjacent to the *hmmA* gene by sequence analysis, the *hmmA* gene may encode a methylmalonate-semialdehyde dehydrogenase for valine degradation in *Streptomyces* spp. that is able to oxidize 3-hydroxyisobutyrate directly to methylmalonyl-CoA.

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# Chapter 5

## Transposon mutagenesis for the study of valine utilization in *Streptomyces coelicolor*

## INTRODUCTION

Utilization of amino acids like valine as a C or N source in bacteria requires processes for uptake and catabolism. Valine catabolism has been studied in *E. coli* (1) and extensively in *Pseudomonas* spp.(7, 9, 17), but in *Streptomyces* spp., the valine catabolic enzyme that has been studied almost to the exclusion all others, is the first enzyme of the pathway (Vdh) (4a, 18). Definitive information is lacking about regulation of valine utilization by *Pseudomonas* spp. (8) and *Streptomyces* spp.(4a, 19). The definition and characterization of valine utilization is important not only to provide an understanding of the primary metabolism of streptomycetes but also to facilitate the manipulation of precursor supply for secondary metabolism (13, 18). A search for *S. coelicolor* mutants deficient in valine utilization (Vut) following UV or NTG mutagenesis (21) revealed that such mutants are quite unstable and revert to the Vut$^+$ phenotype at too high a frequency for our experimental studies.

Transposable elements have been widely exploited in the genetic analysis and molecular manipulation of bacteria. The characteristics of only a few streptomycetes insertion elements have been studied in detail. IS*110* isolated from *S. coelicolor* A3(2) appears to have a strong target preference (2). Tn*4556*, a class II transposable element from *S. fradiae*, has been

shown to transpose into many sites (12), though not entirely randomly (22). IS*493*, isolated from *S. lividans*, is a 1.6 kb class I insertion sequence containing inverted repeats at its ends and two orfs (16). Tn*5096*, an IS*493* derivative containing the *aac(3)IV* (apramycin resistance gene) transposed into many sites in the *S. griseofusus* chromosome (15). Since transposon mutagenesis normally produces stable mutants, we investigated the two *Streptomyces* transposons Tn*5096* and Tn*4556* (12), and transposon mutagenesis of *S. coelicolor* J802 with Tn*5096* was then used to study the genetic basis of valine utilization further. The work described in this chapter is part of an investigation into the genetic basis of valine catabolism leading to understanding of the regulation of secondary metabolism at the level of precursor supply for the biosynthesis of macrolide antibiotics.

## MATERIALS AND METHODS

**Strains, plasmids and culture conditions.** *Escherichia coli* strains DH-5α (14), GM2929 (*dam*-, *dcm*-), obtained from Doug MacNeil, Merck, Sharp & Dohme Co., and JM105 (14), used as hosts for plasmids or for M13 DNA sequencing, were grown at 37°C on LB and 2xYT media (14). *S. coelicolor* J802 (*dagA1, agaA7*) (5), obtained from David Hodgson, was grown on R2YE plates (6) at 30°C for general use, and on minimal medium (MM) (6) or the following modified MM, MGV (containing 1% L-valine instead of asparagine) and MV (containing 1% L-valine without asparagine and glucose) to screen for Vut mutants. *Streptomyces* transformation was performed as described by Hopwood et al. using plasmid DNA isolated from *E. coli* GM2929 or *S. lividans*. (6), and transformants were selected on R2YE plates supplemented with 25 μg of Th (obtained from S. G. Lucania, The Squibb Institute for Medical Research, Princeton, NJ) per ml. Plasmid pCZA168 (Fig. 3), a pGM160 (11) derivative containing Tn*5096*, was used for transposition. Plasmid pWHM1051

(18) containing the *vdh* gene from *S.coelicolor* was used as a probe in Southern analysis.

**DNA preparation and analysis.** Small-scale preparations of *E. coli* plasmid DNA were performed as described by Morelle (10). ssM13 DNA was isolated from JM105 as described by Sambrook et al. (14), except that the supernatant containing the phage was extracted three times with neutral phenol:chloroform (3:1 vol/vol). Streptomycete genomic DNA was isolated by the lysozyme-SDS method of Hopwood et al. (6). Individual DNA restriction fragments were purified by separation on agarose gels, followed by treatment with the USBioclean® MP kit (United States Biochemicals (USB), Cleveland, OH) according to the manufacturer's directions.

For Southern-blot hybridizations, genomic or plasmid DNA samples were electrophoresed in a 1% agarose gel and DNA was transfered to Hybond-N membranes (Amersham, Arlington Heights, IL) by capillary action (14). The following dig-AP labeled probes were used: Tn*5096* was a 3.09 kb *Hin*dIII fragment of pCZA168 and the *vdh* probe was an 1.05 kb *Sal*I-*Apa*I fragment of pWHM1051. The dig-AP labeling, hybridization, and detection were done with the Genius® kit, following the manufacturer's protocols (Boehringer Mannheim, Indianapolis, IN). The blot was washed 2 times with 1x SSC-0.1% SDS for 15 min at 42°C and 2 times in 0.1x SSC-0.1% SDS for 15 min at 68°C .

**Isolation of Tn*5096* transposition and valine utilization mutants.** Transposition of Tn*5096* was obtained from the temperature sensitive plasmid pCZA168. *S. coelicolor* J802 containing plasmid pCZA168 was grown in liquid R2YE plus 25 mg/ml Th, homogenized, and plated on R2YE agar plus 25 mg/ml apramycin (Am, Sigma) to obtain 20 to 100 isolated colonies per plate. The plates were incubated at 29°C for 2 to 3 days and then incubated at 39°C for 8 to 14 days to eliminate the autonomously replicating ts plasmid. At 39°C, cells containing transpositions of Tn*5096* into the *S. coelicolor* genome continued to grow and formed sectors. Cells from the sectors were picked and screened for the $Th^{S}Am^{R}$

phenotype to identify strains containing transpositions, and then the DNA from several sectors was analyzed by Southern-blot hybridization. Each of the Tn*5096* transposition strains was screened by comparing its growth on MM, MGV or MV plates.

**Cloning and sequencing of the Tn*5096* transposition locus from Vut mutant WMH1604.** The Vut mutant WMH1604 genomic DNA was digested with *Bam*HI, size-fractionated by electrophoresis on a 1% agarose gel. The resulting 4.0 to 5.0 kb *Bam*HI DNA fragments, which hybridized to the Tn*5096* probe and contain a part of Tn*5096* sequence including the *aac(3)VI* gene and the flanking region, were ligated to pGEM 7Zf(+) (Promega, WI) at the *Bam*HI site which had been treated with calf intestine alkaline phosphatase (CIAP; Biolabs, New England). The ligation mixture was transferred into *E. coli* DH-5α and plated on LB agar plus 25 mg/ml Am and 100mg/ml Ampicillin (Ap, Sigma). Two identical $Am^RAp^R$ colonies were obtained and the isolated plasmid was named pWHM1069. It was shown to contain approx. 4.5 kb *Bam*HI fragment by restriction enzyme digestion.

DNA segments for sequencing were purified and subcloned into M13mp18 or M13mp19 (23). Nucleotide sequence was determined by the dideoxy chain termination method with the M13 -40 primer and a Sequenase® 2.0 kit (USB), following the manufacturer's instructions. 7-Deaza-dGTP was used in the 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 using the DNASTAR (Madison, WI) software and digitizer. The GCG software (3) version 7.0 was used for sequence analysis. Nucleotide sequence-deduced amino acid sequence data were compared with available databases by using the FASTA and TFASTA programs (3).

## RESULTS AND DISCUSSION

**Transposition of Tn*5096*.** The *S. coelicolor vdh*::*hyg* mutant is blocked in the

first step in the catabolism of valine and the other branched-chain amino acids (18), but neither it nor the *S. coelicolor vdh*$^+$ strain can grow on 2-ketoisovaleric, isobutyric or propionic acid as the sole C source. This obviates the simplest approach to cloning additional genes of valine catabolism, screening for *vut* mutants that cannot utilize one of these intermediates of valine catabolism. Therefore, transposon mutagenesis with Tn*5096* was used to generate *vut* mutants as a way to study the genetic basis of valine catabolism beyond the first step governed by the *vdh* gene. The generation of Am$^R$ sectors by Tn*5096* transposition during growth at 39$^o$C for 8 to 14 days was used to isolate a large number of independent transposition events. Usually, one or two sectors per colony were evident. Approximately 85% of the sectors picked were Am$^R$Th$^S$. The DNA from eight *S. coelicolor* strains with transpositions of Tn*5096* was analyzed by Southern-blot hybridization of *Bam*HI genomic digests with Tn*5096* as a probe. All eight mutants contained Tn*5096* inserted in a different location (Fig. 1).

**Characterization of Vut mutants.** Approximately 4000 sectors containing Tn*5096* transposition were screened by comparing their growth in MM, MM containing valine as the sole N (MGV) or as the sole N and C sources (MV). Approx. 10 *vut* mutants that could not use valine as the sole N or N and C source were analyzed by Southern-blot hybridization with Tn*5096* and *vdh* probes (Fig. 2). It seems that mutants WMH1604 and WMH1605 as well as WMH1606 and WMH1607 may have identical transpositions on the basis of the Southern analysis of *Bam*HI or *Pst*I (Fig. 2A and 2B) genomic DNA digests. WMH1603 appears to have a Tn*5096* insertion in the *vdh* locus (Fig. 2C, lane 5), while each of other strains may contain the transposon at different loci.

By Southern analysis and nucleotide sequencing of DNA flanking the insertions of Tn*5099* (4), another IS*493* derivative, in the *S. coelicolor* genome, Zhang et al. (24) showed that the vector pGM160 (11) has a sequence homologous to the IS*493* terminal inverted repeat

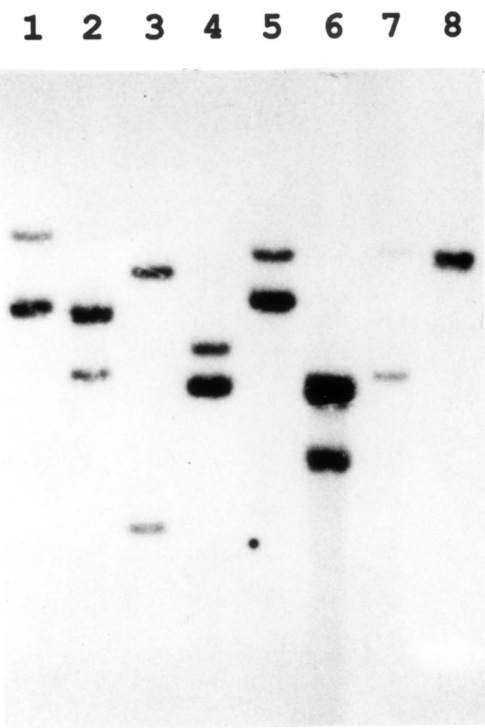


FIG. 1. Southern-blot hybridization analysis of Tn*5096* transposition from independent clones. *S. coelicolor* genomic DNA from $Am^{R}Th^{S}$ clones was digested with *Bam*HI and probed with Tn*5096*. Since Tn*5096* contains one internal *Bam*HI site, usually two bands are seen.

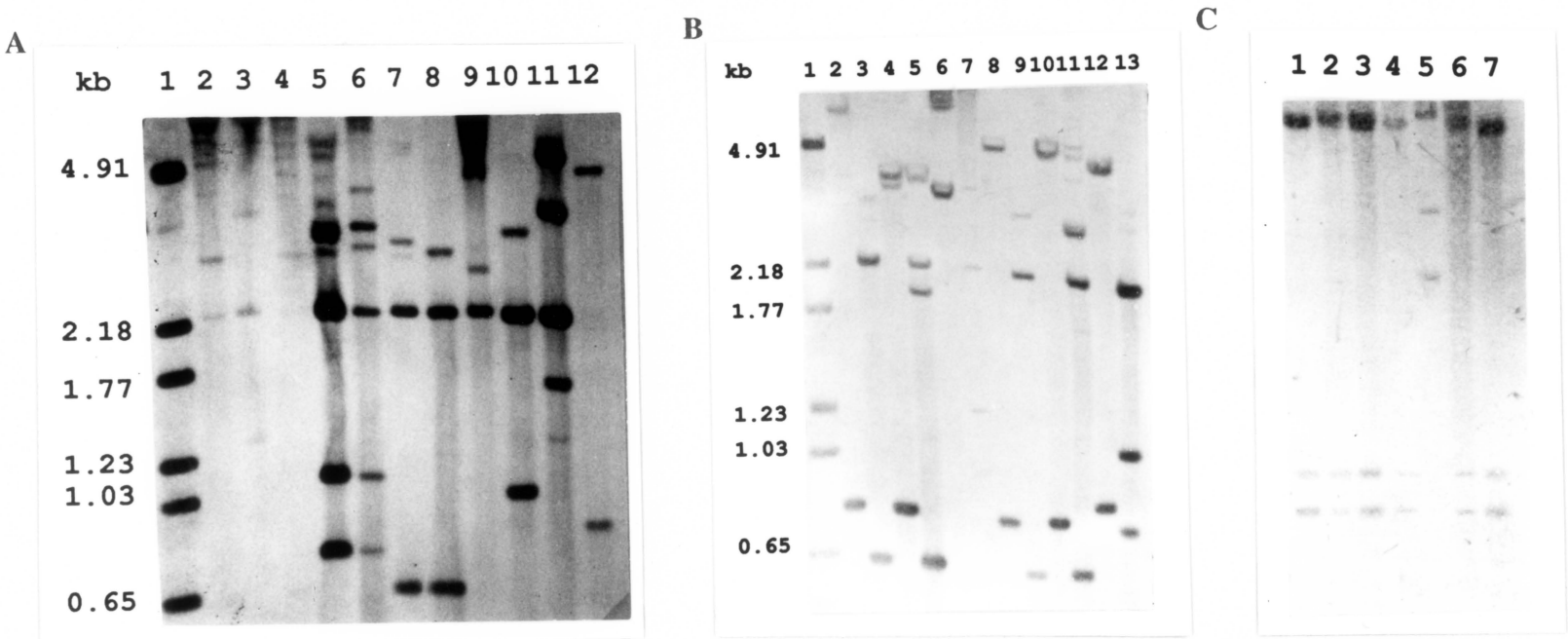


FIG. 2. Southern-blot hybridization analysis of Tn*5096* transposition in *S. coelicolor* Vut mutants. Genomic DNA was digested with *Bam*HI or *Pst*I and probed with Tn*5096* (Fig. 2A and 2B) or digested with *Kpn*I and probed with the *vdh* gene fragment from *S. coelicolor* (Fig. 2C). (A). Lane 1, molecular weight (MW) markers; lane 2, *Pst*I digested genomic DNA of WMH1601 strain; lane 3, *Pst*I digested genomic DNA of WMH1603 strain; lane 4, *Pst*I digested genomic DNA of WMH1602 strain; lane 5, *Pst*I digested genomic DNA of WMH1604 strain; lane 6, *Pst*I digested genomic DNA of WMH1605 strain; lane 7, *Pst*I digested genomic DNA of WMH1606 strain; lane 8, *Pst*I digested genomic DNA of WMH1607 strain; lane 9, *Pst*I digested genomic DNA of WMH1611 strain; lane 10, *Pst*I digested genomic DNA of WMH1612 strain; lane 11, *Pst*I digested genomic DNA of WMH1613 strain; lane 12, *Bam*HI digested genomic DNA of WMH1605 strain. (B). Lane 1, MW marker; lane 2, *Bam*HI digested genomic DNA of WMH1601 strain; lane 3, *Pst*I digested genomic DNA of WMH1601 strain; lane 4, *Bam*HI digested genomic DNA of WMH1602 strain; lane 5, *Pst*I digested genomic DNA of WMH1602 strain; lane 6, *Bam*HI digested genomic DNA of WMH1603 strain; lane 7, *Pst*I digested genomic DNA of WMH1603 strain; lane 8, *Bam*HI digested genomic DNA of WMH1606 strain; lane 9, *Pst*I digested genomic DNA of WMH1606 strain; lane 10, *Bam*HI digested genomic DNA of WMH1607 strain; lane 11, *Pst*I digested genomic DNA of WMH1607 strain; lane 12, *Bam*HI digested genomic DNA of WMH1604 strain; lane 13, *Pst*I digested genomic DNA of WMH1604 strain. (C). *Kpn*I digested genomic DNA in lane 1, J802 strain; lane 2, WMH1604 strain; lane 3, WMH1605 strain; lane 4, WMH1602 strain; lane 5, WMH1603 strain; lane 6, WMH1606 strain; lane 7, WMH1607 strain.

sequence (IR) that can apparently be recognized by transposase proteins of IS*493*. We also found that although the majority (85%) of sectors isolated are $Am^RTh^S$, which were obtained by using either plasmid pCZA168 or pXH106 (4), a small number of these contained about 2 kb of the plasmid vector sequence at the IR end flanking the site where the the transposon had integrated into the chromosome (Fig. 3). Since the 0.76 kb *Bam*HI or 0.6 kb *Pst*I band derived from plasmid pCZA168 (Figs. 2 and 3) were detected in all four of these strains, it looks like mutants WMH1601, -1602, -1606 and -1607 also contain the 2.0 kb plasmid vector sequence at the IR-A end flanking the site of Tn*5096* integration into the chromosome.

**Cloning and sequence analysis of the flanking region from the WMH1604 Vut mutant.** Since the $Am^R$ gene in Tn*5096* also expresses in *E. coli*, it can be used to select for transposition events in streptomycetes and to recover the transposon-tagged region by plasmid rescue in *E. coli*. To characterize a representative Vut mutant, a 4.5 kb *Bam*HI fragment of the *S. coelicolor* WMH1604 genome that contains part of the Tn*5096* sequence and the flanking region was cloned in pGEM7Zf(+) using the $Am^R$ of Tn*5096* as a convenient marker. A restriction map of clone pWHM1069 is shown in Fig. 4. The sequence of 1.05 kb of the junction fragment (Figs. 4 and 5A) and CODON PREFERENCE (3) analysis of the sequence data indicated the existence of an incomplete ORF (Fig. 5B). No obvious similarities could be detected between the translated sequence of this orf and known sequences in the available database by FASTA and TFASTA analysis (3).

A method for transposon mutagenesis of *S. coelicolor* for the isolation of valine utilization mutants using Tn*5096*, a broad-host-range genetic element (15), was developed. But lack of much knowledge about the genetics and physiology of C and N metabolism in streptomycetes and insufficient information about any of these strains we are studying makes this risky work. However, application of promoter probe transposon may facilitate genetic analysis of valine utilization. A related approach to identifying genes involved in valine

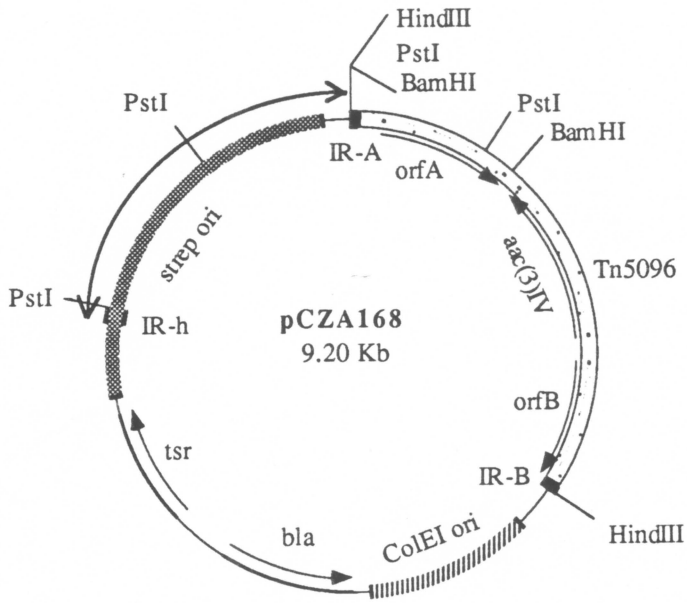


FIG. 3. Restriction map of plasmid pCZA168. IR-A and IR-B (blank boxes) indicate the inverted repeat sequence of Tn*5096*; The double arrowhead region represent the part of vector integrated into chromosome with Tn*5096* or Tn*5099*; IR-h indicates the homologous IR sequence on plasmid vector pGM160; orfA and orfB, transposase genes; aac(3)IV, apramycin resistance gene; bla, ampicillin resistance gene; tsr, thiostrepton resistance gene; strep ori and ColEI ori, replicating origins in streptomycetes and *E. coli*, respectively. pXH106 is the same vector containing Tn*5099* instead of Tn*5096*.

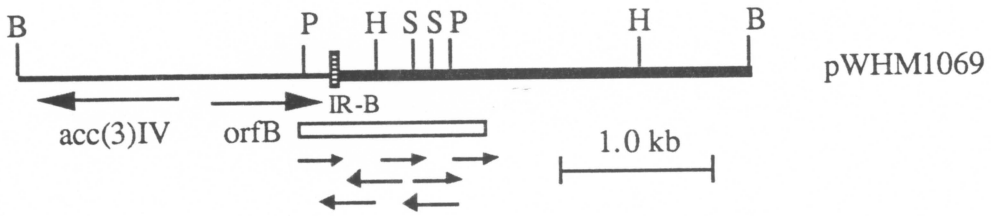


FIG. 4. Restriction map and DNA sequencing strategy for the 1.05 kb DNA junction fragment cloned in pWHM1069. The thickened dark line indicates the genomic region cloned in pWHM1069. The open box beneath the map indicates the region that was sequenced. The thinner arrows beneath this box illustrate the sequencing strategy. Restriction enzyme abbreviations: B, *Bam*HI; Sa, *Sal*I; P, *Pst*I; H, *Hin*dIII. IR-B, Tn*5096* inverted repeat sequence (Fig. 3); aac(3)IV, apramycin resistance gene; orfB, transposase gene *orfB* of Tn*5096* (Fig. 3).

A

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1    CTCAGCTTGAGGATGGAAAACGCTCACTGTTCCGAATACATCGCGAGCTTCAAAGAGCACGGGAACCCCGCCGAGGAATACAGCGACGAACTGGGCAGCA
          IR-B end of Tn5096 H  C  S  E  Y  I  A  S  F  K  E  H  G  N  P  A  E  E  Y  S  D  E  L  G  S  K

101  AGGACCTGCGTGACGTTTTTGGAGAGTTTTCAGACACCTGGAAGAAGACTCGAAAGAAACTCATGAAGGACATCGAGAATCTGGCGAAGTTCACCAAGAC
       D  L  R  D  V  F  G  E  F  S  D  T  W  K  K  T  R  K  K  L  M  K  D  I  E  N  L  A  K  F  T  K  T

201  CGCGGCGGACACGTACGACGTAGTCGATCACAAGCTTGCCGAGGCCCTCCGGGATGCCCGCAAGAAGTCGAAGGGGAAGAAGTGAGTTCGCGGCCACGCG
       A  A  D  T  Y  D  V  V  D  H  K  L  A  E  A  L  R  D  A  R  K  K  S  K  G  K  K  S  V  R  G  H  A
301  ATTGGGAACCCCTCCACGACGGCGACCCCATACCCGGAGACCCCTACGAGGTTGCCGGACTCGGCAAGAAGCTCCGCAAGATGGCGGACGAGATCGACAA
       I  G  N  P  S  T  T  A  T  P  I  P  G  D  P  Y  E  V  A  G  L  G  K  K  L  R  K  M  A  D  E  I  D  K

401  ACAGTCTCGAAACATCAGAGCCCTGTCGTCCGTCGACGGATGGGACAGCGATGCCGGGCGCGCCTTTCACGAGATAGCGGACGGCGCGTCGGGTCGCCTC
       Q  S  R  N  I  R  A  L  S  S  V  D  G  W  D  S  D  A  G  R  A  F  H  E  I  A  D  G  A  S  G  R  L

501  AAGCGAGCGTTCGATCGCTATGACGAAGCGGCCAAGGCGTTGGGCACGAAGGTTGTCGACGGCGAGGAATCAAAAGAGTACGCCAGCGAGTTGCACCGGG
       K  R  A  F  D  R  Y  D  E  A  A  K  A  L  G  T  K  V  V  D  G  E  E  S  K  E  Y  A  S  E  L  H  R

601  CTCAAAAGGTCGCGGACAAGGCTCTGCAGAAGTACCGCGAGGCGGAGACGGACCACAAAACCGCCATCGGCGACCTCAAGCAGTACGAAGGGACCGTTCC
       A  Q  K  V  A  D  K  A  L  Q  K  Y  R  E  A  E  T  D  H  K  T  A  I  G  D  L  K  Q  Y  E  G  T  V  P

701  GTCACGCGACGACGTGACGGACCGCACTCGTCTCGAGAAGAAGCGTGACGCCGCCATGGGTGTCTACCGCGAGTGCCACAGCGAAATCGGCC
       S  R  D  D  V  T  D  R  T  R  L  E  K  K  R  D  A  A  M  G  V  Y  R  E  C  H  S  E  I  G
```

B

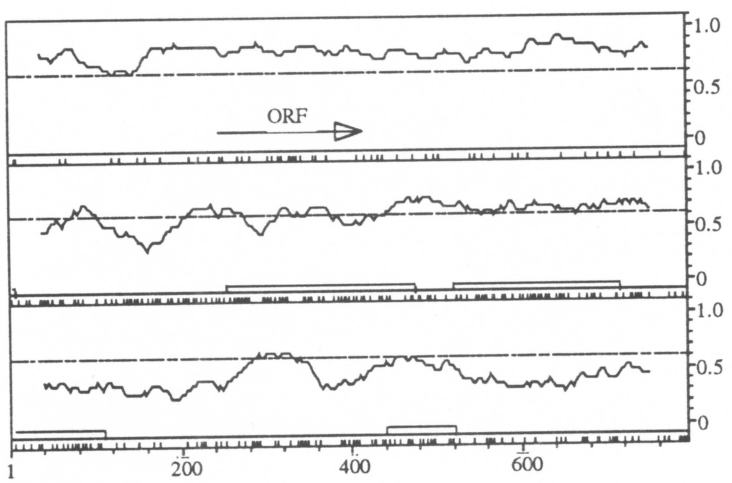


FIG. 5. (A). Nucleotide sequence of the region flanking Tn*5096* in pWHM1069. Only the top strand is shown from the Tn*5096* IR - B (underlined). (B). CODON PREFERENCE analysis (7) of the sequence in the 5' to 3' direction. The mole fraction of G and C nucleotides at the third position of each codon in the three possible reading frames (within a window of 25 consecutive codons) is plotted versus location in the sequence. The analysis reveals one incomplete ORF with features typical of *Streptomyces* genes in the top reading frame. The arrow indicates the direction of the transcription for this partial ORF.

assimilation involves screening Tn*5099* (4) insertions of *S. coelicolor* strain for clones that exhibit altered expression of the promoterless *xylE* gene adjacent to the one IR-end of Tn*5099*. After transposition from this plasmid to a new location, expression of the *xylE* gene can be brought under the control by the chromosomal promoter, which can be detected by the appearance of a yellow color after the colonies sprayed with 0.5 M catechol. We have been able to identify Tn*5099* transposition mutants that display altered levels and timing of XylE activity in response to valine when their growth on a MM with glucose as the C source and asparagine *vs* valine as the N source is compared (24). These results suggest that valine metabolism regulated promoters can be identified by using a promoter probe transposon.

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