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

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Targeted inactivation of the valine (branched-chain amino acid) dehydrogenase gene (*vdh*) was used to study the role of valine catabolism in the production of tylosin in *Streptomyces fradiae* and spiramycin in *Streptomyces ambofaciens*. The deduced products of the *vdh* genes, cloned and sequenced from *S. fradiae* C373.1 and *S. ambofaciens* ATCC 15154, are approximately 80% identical over all 363 amino acids and 96% identical over a span of the first N-terminal 107 amino acids, respectively, to the deduced product of the *Streptomyces coelicolor vdh* gene. The organization of the regions flanking the *vdh* genes is the same in all three species. Inactivation of the genomic copy of the *vdh* gene in *S. fradiae* and *S. ambofaciens* by insertion of a hygromycin resistance (*hyg*) gene caused loss of the valine dehydrogenase (Vdh) activity, and thus only one enzyme is responsible for the Vdh activity in these organisms. Analysis of the culture broth by bioassay revealed that the *vdh*::*hyg* mutants produce an approximately sixfold-lower level of tylosin and an approximately fourfold-lower level of spiramycin than the wild-type *S. fradiae* and *S. ambofaciens* strains, while maintaining essentially identical growth in a defined minimal medium with either 25 mM ammonium ion or 0.05% asparagine as the nitrogen source. The addition of the valine catabolite, propionate or isobutyrate, and introduction of the wild-type *vdh* gene back to each *vdh*::*hyg* mutant reversed the negative effect of the *vdh*::*hyg* mutation on spiramycin and tylosin production. These data show that the catabolism of valine is a major source of fatty acid precursors for macrolide biosynthesis under defined growth conditions and imply that amino acid catabolism is a vital source of certain antibiotic precursors in actinomycetes.

Many studies have shown that antibiotic production in actinomycetes is influenced by the type and level of the nitrogen (N) and carbon (C) sources in a growth medium (3, 6). 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 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 (19). The propionyl- and 2-methylmalonyl-CoA substrates for macrolide biosynthesis could have multiple metabolic origins (9): catabolism of odd-numbered fatty acids, reduction of acrylate, rearrangement of succinyl-CoA, and 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 *n*-butyrate, propionate, and 2-methylmalonate units for the biosynthesis of typical macrolide antibiotics (22, 25, 26). The negative effect of ammonium ion (NH_4^+) on macrolide production (13, 20) and the strong correlation between macrolide production and the level of valine dehydrogenase (Vdh) (13, 21, 22) favor the idea that branched-chain amino acid catabolism could be an important source of the macrolide building blocks propionyl- and 2-methylmalonyl-CoA when sufficient propionate is not available directly from the nutrient medium. This view is supported by the fact that addition of valine or isoleucine to the medium resulted in a three- to fivefold-higher level of protylonolide, the macrolide precursor of tylosin, than addition of succinate or asparagine (8, 21). Furthermore, 50 mM NH_4^+ strongly inhibited the incorporation of valine (20, 21) and the formation of tylosin and spiramycin (13, 21), and this negative effect was largely suppressed by the addition of isobutyrate to the medium (13, 20, 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 *Streptomyces coelicolor vdh* gene, which encodes the first enzyme of the valine catabolism pathway (15, 18), is induced by valine and repressed by NH_4^+ (28).

To better understand the correlation between macrolide production and branched-chain amino acid catabolism, we cloned the *vdh* genes from the spiramycin producer *Streptomyces ambofaciens* and the tylosin producer *Streptomyces fradiae* so that we could determine whether the inability to catabolize valine affects macrolide production under defined growth conditions. Disruption of the *vdh* gene by insertion of the hygromycin resistance (*hyg*) gene inhibited the biosynthesis of spiramycin and tylosin, 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 macrolide production.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. Plasmids and strains made in this work are listed in Table 1. *Escherichia coli* DH5α (24) and JM105 (24), used as hosts for plasmids or for M13 DNA sequencing, were grown at 37°C on LB and 2× YT media (24), respectively. Plasmid pWHM1051 (27), containing the *vdh* gene from *S. coelicolor* J802, was used as a probe in

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TABLE 1. Plasmids and strains used in this work

Plasmid or strain	Construction and characteristics[a]	Reference
Plasmids		
pXH106	*bla tsr hyg*; ts; multicopy	10
pKC1139	*bla Am*; ts; *oriT*; multicopy	2
pWHM1051	*vdh*, ORF1 and ORF2 genes from *S. coelicolor* J802 in pUC18	27
pWHM1065	6.0-kb *Hin*dIII fragment of pXH106 was religated	27
pWHM1071	pUC19 with 0.7-kb *Sst*I fragment from *S. ambofaciens* ATCC 15154	This study
pWHM1072	pUC19 with 1.8-kb *Sst*I fragment from *S. ambofaciens*; ORF1	This study
pWHM1073	pUC19 with 2.0-kb *Sst*I fragment from *S. ambofaciens*; *vdh*	This study
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	This study
pWHM1075	pWHM1072 was digested with *Sst*I and partially digested with *Sal*I to give the 1.35-kb *Sst*I-*Sal*I fragment, which was subcloned in pUC19	This study
pWHM1076	pWHM1074 was digested with *Hin*dIII and partially digested with *Sst*I, and 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 the *Hin*dIII site of pWHM1065; *vdh* and ORF1 gene from *S. ambofaciens*	This study
pWHM1077	The 1.85-kb *Bam*HI-*Hin*dIII fragment from pWHM1076 was cloned in pUC19; the resulting plasmid was digested with *Apa*I and the ends were filled in, and then the 1.7-kb *Sma*I-*Eco*RV fragment of the *hyg* gene was cloned into the blunt-ended 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*	This study
pWHM1081	pUC19 with 1.7-kb *Sal*I-*Sph*I fragment from *S. fradiae* C373.1	This study
pWHM1082	pUC19 with 1.8-kb *Sph*I-*Bam*HI fragment from *S. fradiae* C373.1	This study
pWHM1083	A 0.8-kb *Sph*I-*Kpn*I fragment from pWHM1082 subcloned in pUC19	This study
pWHM1084	The 0.8-kb *Sph*I-*Eco*RI fragment from pWHM1083 and the 1.7-kb *Sal*I-*Sph*I fragment from pWHM1081 ligated into the *Sal*I-*Eco*RI sites of pGEM-9zf(−)	This study
pWHM1085	pWHM1084 was digested with *Sph*I, the ends were filled in, and the resulting blunt-ended fragment was ligated with the 1.7-kb *Sma*I-*Eco*RV fragment of the *hyg* gene; the resulting plasmid was digested with *Bam*HI and partially digested with *Eco*RI to give a 3.3-kb *Bam*HI-*Eco*RI fragment that was cloned into the *Bam*HI-*Eco*RI sites of pKC1139; *vdh*::*hyg*	This study
pWHM1086	A 2.5-kb *Sal*I-*Eco*RI fragment from pWHM1084 was cloned into pKC1139	This study
Strains		
WMH1608	*vdh*::*hyg* gene-disrupted mutant of *S. ambofaciens* ATCC 15154	This study
WMH1610	*vdh*::*hyg* gene-disrupted mutant of *S. fradiae* C373.1	This study

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

Southern analyses and colony hybridization. The spiramycin producer *S. ambofaciens* ATCC 15154 and the tylosin producer *S. fradiae* C373.1, obtained from Eli Lilly and Co., were grown on Trypticase soy broth (TSB) (BBL) or R2YE plates (11) at 30°C for general use. Transformation of *S. ambofaciens* protoplasts was done by standard procedures (11), and transformants were selected on R2YE plates supplemented with 25 μg of thiostrepton or 200 U of hygromycin (Sigma) per ml. Conjugal transfer of DNA from *E. coli* to *S. fradiae* was performed as described by Bierman et al. (2), and exconjugants were selected on R2YE plates supplemented with 25 μg of apramycin (Sigma) or 200 U of hygromycin per ml. For macrolide production, *S. fradiae* and *S. ambofaciens* cultures were grown in TSB medium at 30°C for 1 or 3 days, respectively, with shaking at 300 rpm, and the mycelial cells were harvested by centrifugation and used for seed inoculum or stored in 20% glycerol at −80°C. A 0.2-ml portion of the mycelial suspension was used to inoculate 25 ml of seed medium (3% glucose, 1% peptone, 1% yeast extract, 0.3% malt extract, and 0.3% Casamino Acids, 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 fermentations were conducted in 300-ml baffled flasks containing 50 ml of medium at 30°C with shaking at 300 rpm. The fermentation medium for spiramycin contained, per liter, 1 g of $MgSO_4 \cdot 7H_2O$, 15 mg of $ZnSO_4 \cdot 7H_2O$, 2 g of KH_2PO_4, 15 g of MOPS (morpholinopropane sulfonic acid, sodium salt), 5 g of $CaCO_3$, 20 g of NaCl, 0.3 mg of $CoCl_2$, 20 g of glucose, and 1 g of asparagine (pH 7.0). The fermentation medium for tylosin contained per liter, 20 g of starch, 5 g of glucose, 2.7 g of sodium lactate, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of K_2HPO_4, 3 g of $CaCO_3$, 1.7 g of $(NH_4)_2SO_4$, and 3 ml of trace elements (11) (pH 7.0).

DNA preparation, plasmid construction, and screening of minilibraries. Small-scale preparations of *E. coli* plasmid DNA were made as described by Morelle (17). Single-stranded M13 DNA was isolated from *E. coli* JM105 as described by Sambrook et al. (24), 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, Cleveland, Ohio) according to the manufacturer's directions. Streptomycete genomic DNA was isolated by the lysozyme-sodium dodecyl sulfate (SDS) method of Hopwood et al. (11).

A 1.8-kb fragment from pWHM1051 containing the *S. coelicolor vdh* gene (27) was digoxigenin-AP labeled as described below and used to probe several restriction enzyme digestions of *S. ambofaciens* or *S. fradiae* genomic DNA. The 0.7-, 1.8-, and 2.0-kb *Sst* 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 (33). These DNA minilibraries were screened with the *S. coelicolor vdh* gene by colony hybridization to obtain clones pWHM1071, pWHM1072, and pWHM1073 (Fig. 1A). The 1.7-kb *Sal*I-*Sph*I and 1.8-kb *Sph*I-*Bam*HI fragments from *S. fradiae* C373.1 that hybridized with the labeled probe were treated in the same

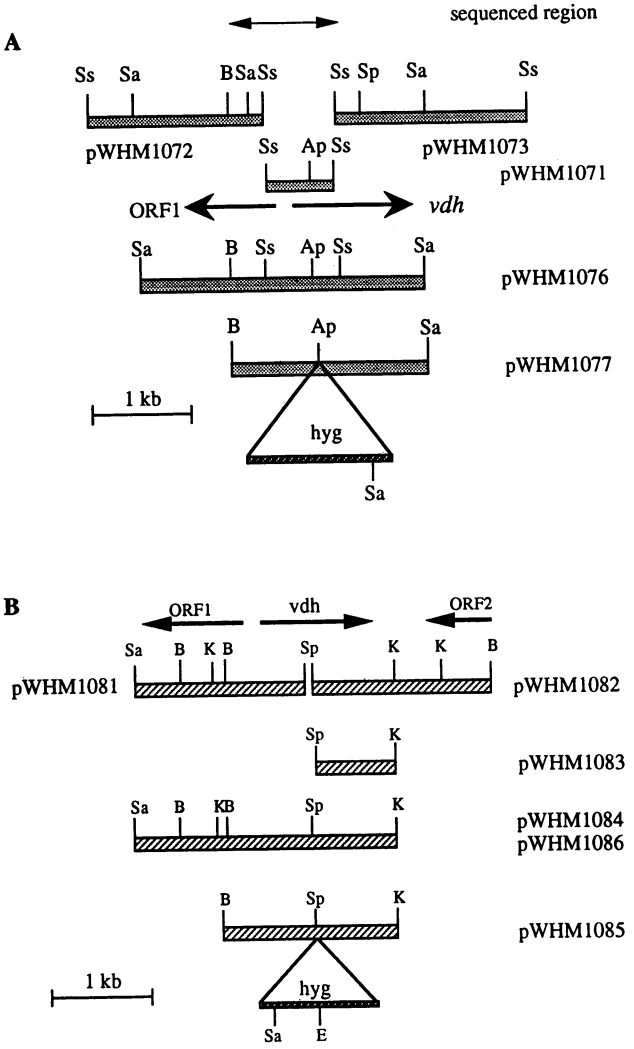


FIG. 1. Restriction maps of DNA clones from *S. ambofaciens* (A) and *S. fradiae* (B). The thick arrows indicate the direction of gene transcription. The junction of the original clones pWHM1081 and pWHM1082 at the *Sph*I site was confirmed by sequence analysis of a suitable PCR product. Abbreviations: Ap, *Apa*I; B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; Sp, *Sph*I; Sa, *Sal*I; Ss, *Sst*I; hyg, hygromycin resistance gene.

manner, and DNA minilibraries were screened by colony hybridization to obtain clones pWHM1081 and pWHM1082 (Fig. 1B).

DNA hybridization and sequencing. Southern blot hybridization and colony hybridization were performed with Hybond-N membranes (Amersham, Arlington Heights, Ill.) by standard techniques (24). The digoxigenin-AP labeling, hybridization, and detection were done with the Genius kit (Boehringer Mannheim, Indianapolis, Ind.), according to the manufacturer's protocols. Hybridization was performed at 42°C overnight, and the blot was washed two times with 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS (24) for 5 min at room temperature and two times in 0.1× SSC–0.1% SDS for 15 min at 68°C.

DNA segments for sequencing were purified and subcloned into M13mp18 or M13mp19 (33). The nucleotide sequence of the resulting single-stranded DNA was determined by the dideoxy chain termination method with the M13 −40 primers and a Sequenase 2.0 kit (United States Biochemicals) according to 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 by using the DNASTAR (Madison, Wis.) software and digitizer. The Genetics Computer Group software (7), version 7.0, was used for sequence analysis. Nucleotide sequence-deduced amino acid sequence data were compared with the sequences of the Vdh and ORF1 proteins from *S. coelicolor* by using the GAP program (7).

Disruption of the *vdh* gene and Vdh activity assays. To construct the plasmid used for *vdh* gene replacement in *S. ambofaciens*, a 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 (10), which contains the *hyg* gene. A 3.55-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. 1A). pWHM1077 was introduced into *S. ambofaciens* by transformation, and then thiostrepton- and hygromycin-resistant (Th^r Hg^r) transformants were selected on R2YE plates and grown in R2YE liquid plus 200 U of hygromycin per ml at 28°C for 3 days.

To construct the plasmid used for *vdh* gene inactivation in *S. fradiae* C373.1, pWHM1084 was digested with *Sph*I, and the ends were filled in with Klenow polymerase and ligated to the 1.7-kb *Sma*I-*Eco*RV fragment containing the *hyg* gene. The resulting plasmid was digested with *Bam*HI and partially digested with *Eco*RI, and the resulting 3.4-kb *Bam*HI-*Eco*RI fragment was subcloned into the *Bam*HI-*Eco*RI sites of pKC1139 (2) to give plasmid pWHM1085 (Fig. 1B). pWHM1085 was introduced into *S. fradiae* by conjugation, and then apramycin-resistant (Am^r), Hg^r exconjugants were selected on R2YE plates and grown in TSB liquid plus 200 U of hygromycin per ml at 28°C for 3 days.

The mycelial cells from *S. ambofaciens*(pWHM1077) and *S. fradiae*(pWHM1085) cultures were homogenized and further incubated at 39°C for 3 to 5 days to eliminate the autonomously replicating plasmid. The mycelial cells were plated on R2YE agar plus 200 U of hygromycin per ml and incubated at 39°C for 5 to 7 days, and then spores were collected and screened for Hg^r Th^s or Hg^r Am^s clones.

Vdh specific activity was measured as described previously (27), using cells from *S. ambofaciens* or *S. fradiae* cultures grown in TSB medium for 24 or 48 h.

Antibiotic production. Spiramycin and tylosin titers of the culture broths were estimated by the agar plate diffusion assay, with *Bacillus subtilis* as the test microorganism in antibiotic medium 1 (Difco Laboratories, Detroit, Mich.) adjusted to pH 8.0, from the standard curve line by separately averaging the zone diameters of the standard and the zone diameters of the samples tested on each set of three plates. Spiramycin and tylosin (Sigma) were used as standards to prepare the standard curve. Identification of antibiotic production was carried out by thin-layer chromatography, as follows. A 5-ml portion of 6- or 7-day fermentation cultures of *S. ambofaciens* or *S. fradiae* wild-type and *vdh* mutant strains was extracted with 2.5 ml of ethyl acetate. The ethyl acetate layer was dried, the residue was suspended in 20 μl of fresh ethyl acetate, and 1 μl of this extract was spotted on silica gel thin-layer chromatography plates, which were developed in chloroform-methanol (4:1,

FIG. 2. Nucleotide sequences of the *vdh* regions from *S. ambofaciens* (A) and *S. fradiae* (B). The sequence reported was fully determined on both strands. In panel A, both strands are shown from nt 376 to 525, only the bottom strand is shown from nt 1 to 375, and the top strand is shown from nt 526 to 872. In panel B, both strands are shown from nt 997 to 1225 and from nt 2976 to 3025, only the bottom strand is shown from nt 1 to 996 and from nt 3026 to 3600, and only the top strand is shown from nt 1226 to 2975. 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 are underlined. Dyad repeats in the sequence (B) indicate potential transcriptional termination sites.

vol/vol) and bioautographed with *Bacillus subtilis*. Growth of cultures was measured by dried cell weight.

Nucleotide sequence accession numbers. The DNA sequence data for the *vdh* genes from *S. ambofaciens* and *S. fradiae* described in this paper have been deposited at EMBL and GenBank with accession numbers L33871 and L33872, respectively.

RESULTS

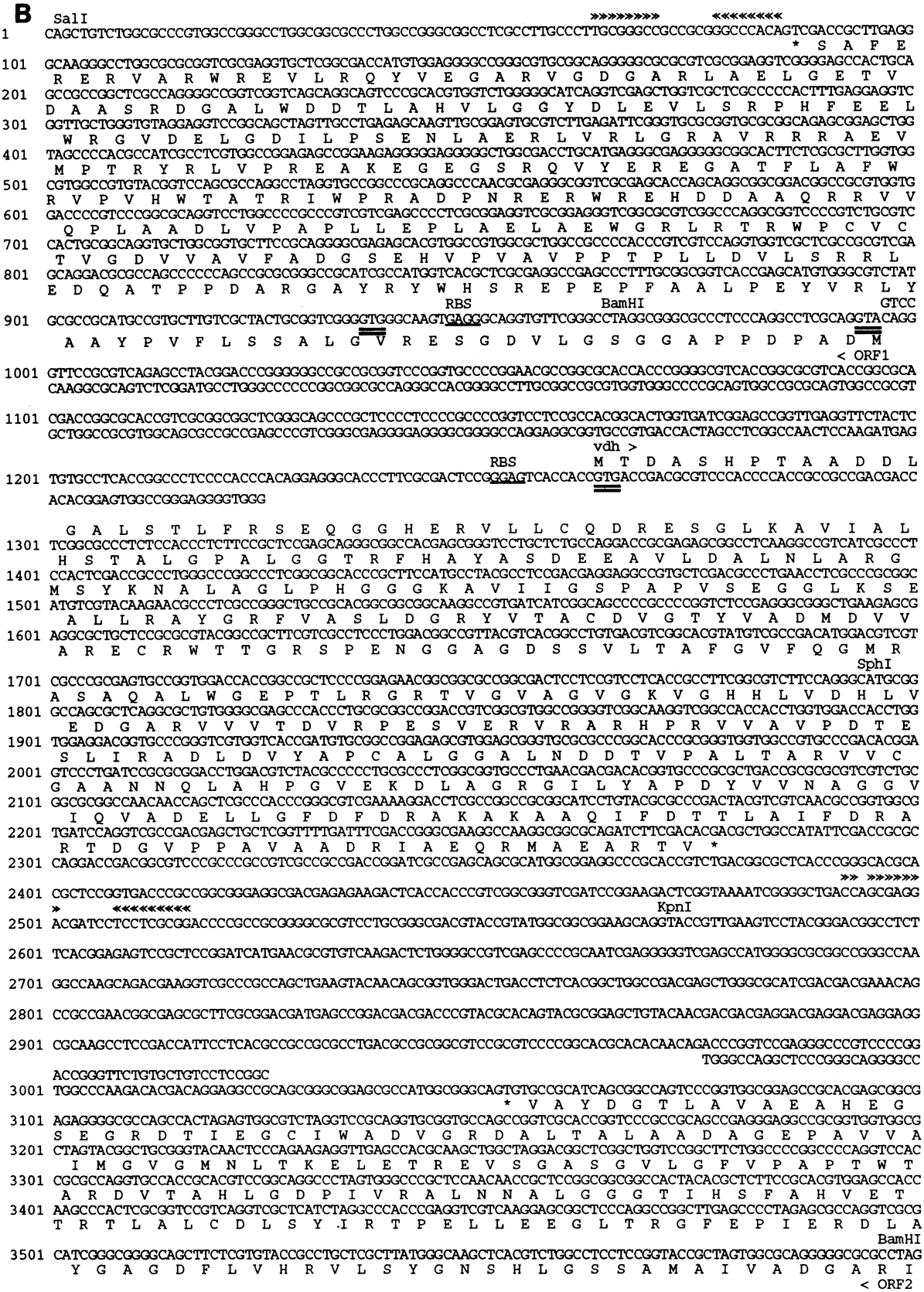
Cloning and sequence analysis of the *vdh* genes from *S. ambofaciens* and *S. fradiae*. The *vdh* genes from *S. ambofaciens* and *S. fradiae* were cloned by hybridization to the *vdh* gene from *S. coelicolor* J802 (27) as described in Materials and Methods. Clones pWHM1071, pWHM1072, and pWHM1073 were isolated from *S. ambofaciens*, and a restriction map of the cloned region is shown in Fig. 1A. A 2.85-kb *Sal*I fragment (which contains the entire *vdh* and ORF1 genes) was recovered from the three clones and religated into pWHM1065 (27) to give pWHM1076 (Fig. 1A; Table 1).

The sequence of an approximately 1.0-kb segment of the *S. ambofaciens vdh* gene in pWHM1076 was determined (Fig. 2A). CODON PREFERENCE and GAP analysis (7) showed that there are two partial open reading frames (*orf*s) transcribed divergently in this region with the characteristic codon usage pattern for *Streptomyces* DNA (1). The amino acid sequences deduced from the two partial *orf*s exhibited significant sequence similarities with the Vdh and ORF1 proteins from *S. coelicolor* (27), showing approximately 96 and 90% identity over a span of 107 and 132 amino acids, respectively. Both *orf*s are preceded by putative ribosome binding sites (RBS) on the basis of complementarity to a region close to the 3′ end of the 16S rRNA of *S. lividans* (Fig. 2A).

The *vdh* gene from *S. fradiae* was also cloned in a similar manner, resulting in clones pWHM1081 and pWHM1082 (Fig. 1B). A 2.5-kb *Sal*I-*Kpn*I fragment from the two clones, which contains the entire *vdh* and ORF1 genes, was cloned in pGEM-9zf(−) (Promega, Madison, Wis.) to give pWHM1084 (Fig. 1B; Table 1).

The sequence of an approximately 3.6-kb segment of pWHM1084 from *S. fradiae* was determined (Fig. 2B). CODON PREFERENCE and GAP analyses (7) showed that in this region there are two complete *orf*s and one partial *orf* with the characteristic codon usage pattern for *Streptomyces* DNA (1). The 1,113-nucleotide (nt) *vdh* gene, which is sandwiched between *orf*s reading in the direction opposite to that in *S. coelicolor* (27), begins with a GTG at position 1264, terminates in a TGA at position 2379, and has a putative RBS at a suitable distance from the GTG start codon (Fig. 2B). It is followed by an imperfect dyad repeat (calculated $\Delta G_{25°C}$ = −19 kcal [ca. −79 kJ]/mol [31]) that is a potential transcription termination site. The *vdh* gene should encode a 371-amino-acid protein with a calculated molecular mass of 38,734 Da.

Another *orf* was found upstream of the *vdh* gene on the opposite strand and is temporarily designated ORF1. This 909-nt *orf* may begin with the ATG at position 996, terminate in the TGA at position 87, and encode a 303-amino-acid protein. No RBS was found immediately upstream of the ATG


B
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RBS
BamHI
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vdh >
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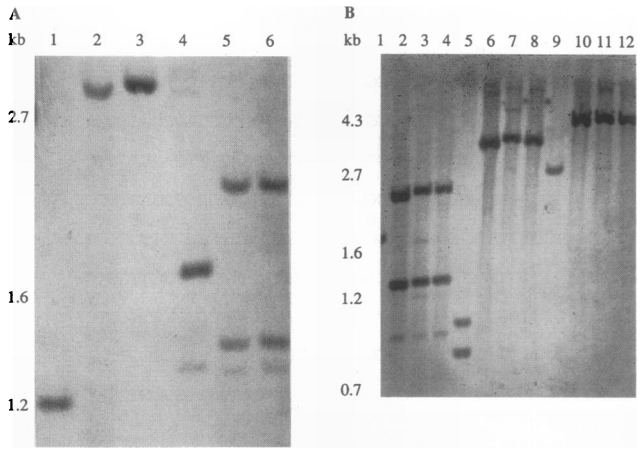


FIG. 3. Southern blot-hybridization analysis of chromosomal DNAs of the *S. ambofaciens* (A) wild-type strain (lanes 1 and 4) and *vdh*-disrupted mutants WMH1608 (lanes 2, 3, 5, and 6) and the *S. fradiae* (B) wild-type strain (lanes 1, 5, and 9) and *vdh*-disrupted mutants WMH1610 (lanes 2 to 4, 6 to 8, and 10 to 12). Genomic DNA was digested with *Bam*HI-*Sph*I (panel A, lanes 1 to 3), *Sal*I (panel A, lanes 4 to 6), *Sal*I-*Kpn*I (panel B, lanes 1 to 4), *Kpn*I-*Sph*I (panel B, lanes 5 to 8), and *Bam*HI (panel B, lanes 9 to 12).

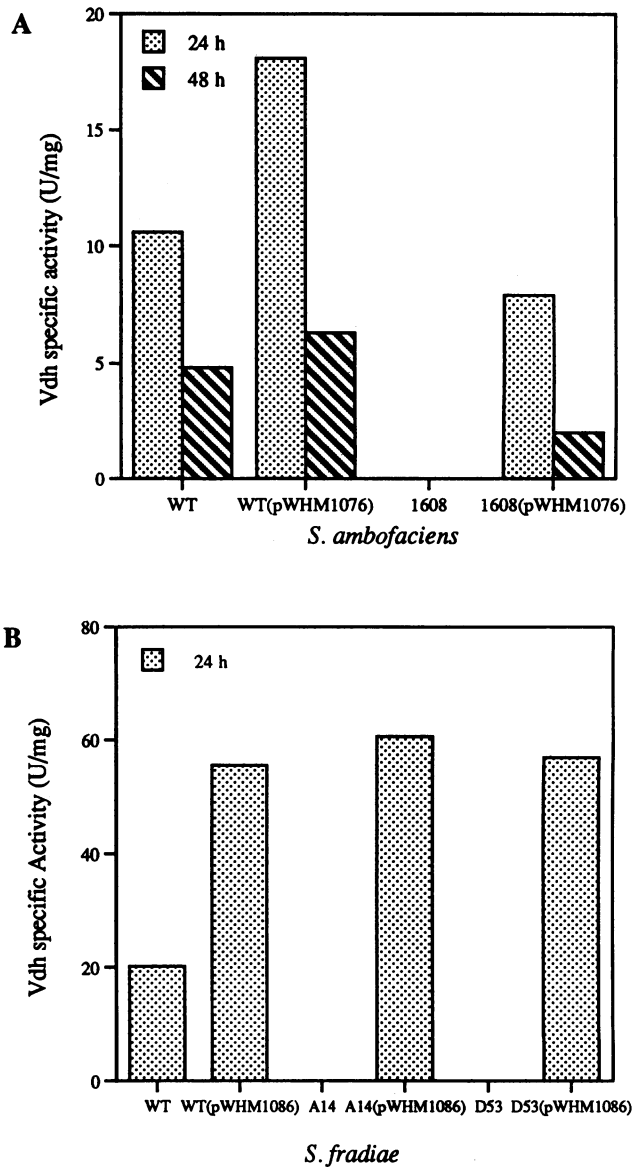


FIG. 4. Vdh specific activities of the *S. ambofaciens* wild-type (WT) strain, WMH1608 *vdh* mutant strains, and their pWHM1076 transformants (A) and of the *S. fradiae* WT strain, WMH1610 *vdh* mutant strains (A14 and D53), and their pWHM1086 transformants (B) grown in TSB medium for 24 or 48 h.

start codon, but a putative RBS is present at a suitable distance from the GTG at position 936 (Fig. 2B), which corresponds to the predicted ORF1 start codon in *S. coelicolor* (27) or *S. ambofaciens* (Fig. 2A). The amino acid sequences deduced from the *vdh* and ORF1 genes have significant similarities with those of the Vdh and ORF1 proteins from *S. coelicolor*: approximately 80 and 73% identity overall by GAP analysis, respectively.

A third *orf*, detected downstream of *vdh* and also on the opposite strand (Fig. 2B), was designated ORF2. The entire gene has not been sequenced completely, but the amino acid sequence deduced from the available data has significant similarity to that of the product of the ORF2 gene from *S. coelicolor*, which is homologous to the *purM* gene from *E. coli* (27).

***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 [18, 25]), provides essential precursors of macrolide antibiotics, we disrupted the *vdh* genes in *S. ambofaciens* and *S. fradiae* by insertion of the *hyg* gene into the respective coding regions. For *S. ambofaciens*, the *vdh*::*hyg* construct was cloned in the temperature-sensitive *Streptomyces* plasmid pWHM1065 (27) to give pWHM1077 (Table 1). After the *S. ambofaciens* (pWHM1077) transformants were grown at 39°C to eliminate the plasmid, four Hg^r Th^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 around the *vdh* gene by Southern analysis. The digoxigenin-AP-labeled 1.15-kb *Bam*HI-*Sph*I fragment from pWHM1076 (Fig. 1A) hybridized to a 2.9-kb *Bam*HI-*Sph*I fragment in the WMH1608 strains in place of the 1.15-kb *Bam*HI-*Sph*I fragment in the wild-type strain (Fig. 3A; compare lanes 2 and 3 with lane 1). Digestion with *Sal*I gave 1.25- and 1.65-kb-hybridizing bands in place of 1.25-, 1.35-, and 2.4-kb bands (Fig. 3A; compare lanes 5 and 6 with lane 4). These data confirm that the *vdh* gene was disrupted through the expected double crossover.

For *S. fradiae*, the *vdh*::*hyg* construct was cloned in the temperature-sensitive *Streptomyces* conjugative plasmid pKC1139 (2) to give pWHM1085 (Table 1). After the *S. fradiae* (pWHM1085) exconjugants were grown at 39°C to eliminate the plasmid, we chose three Hg^r Am^s exconjugants (the WMH1610 strains) from the *S. fradiae* transformants for further study. Chromosomal DNA was isolated from these three WMH1610 strains and the wild-type strain, and the DNA was used to analyze the region around the *vdh* gene by Southern analysis. The digoxigenin-AP-labeled 1.5-kb *Bam*HI-*Kpn*I fragment from pWHM1084 (Fig. 1B) hybridized to 1.2- and 2.3-kb *Sal*I-*Kpn*I fragments in the WMH1610 strains in

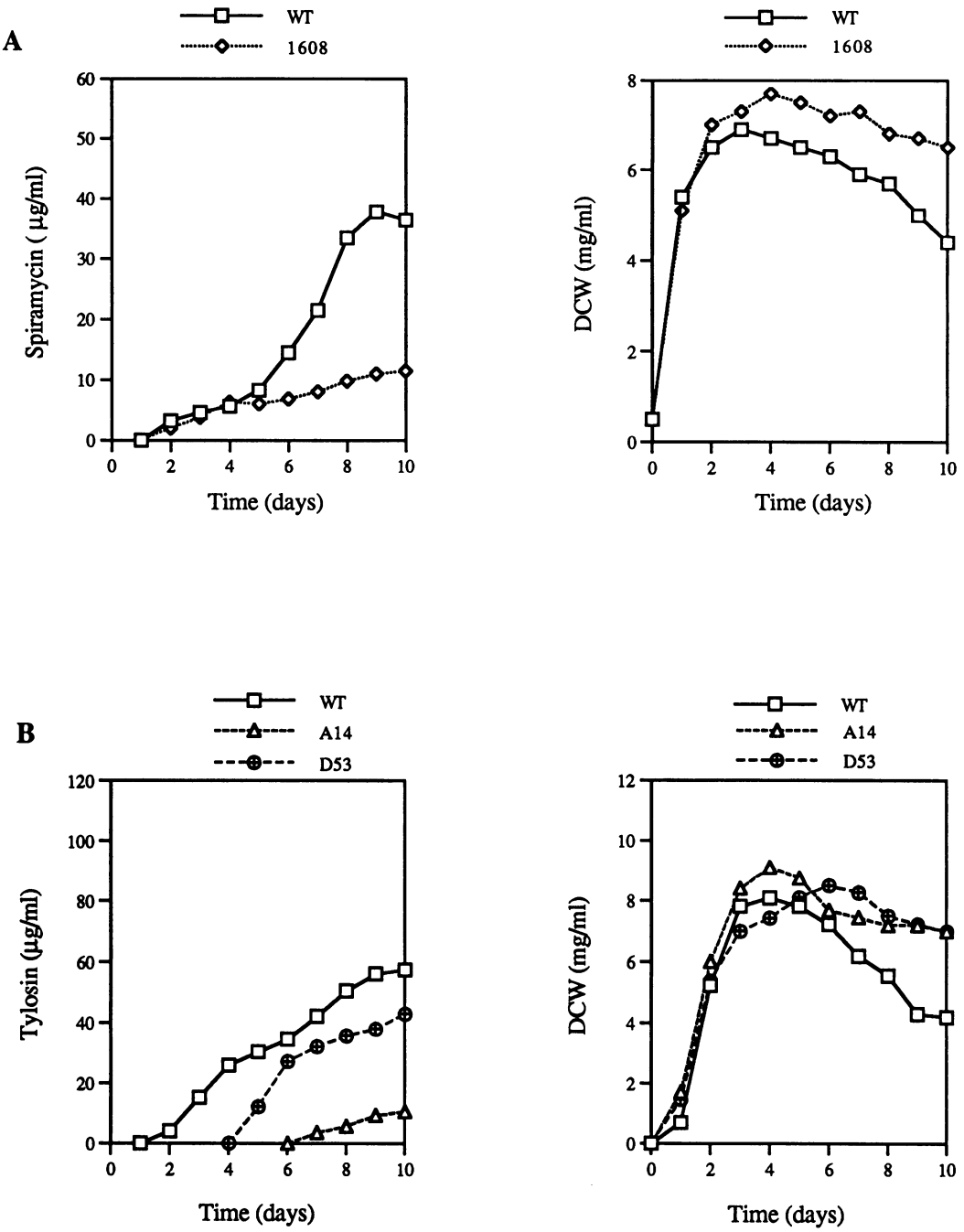


FIG. 5. (A) Spiramycin production and growth (dried cell weight [DCW]) of the *S. ambofaciens* wild-type (WT) and WMH1608 *vdh* mutant strains grown in the glucose-asparagine medium. The spiramycin production data are averages for at least two separate experiments, using the average values of duplicate samples from each separate experiment, and for mutant WMH1608 are averages from two individual Th^s Hg^r transformants. The average error was less than 10%. The growth data represent an average error of less than 5%. (B) Tylosin production and growth (DCW) of the *S. fradiae* WT and WMH1610 mutant strains (A14 and D53) grown in the glucose-NH_4^+ medium. The tylosin production data are averages for at least two separate experiments and represent an average error of less than 10% for the *S. fradiae* wild-type and mutant A14 strains and 15% for the mutant D53 strain. The growth data represent an average error of less than 5%.

place of the 1.7-kb *Sal*I-*Kpn*I fragment in the wild-type strain (Fig. 3B; compare lanes 2 to 4 with lane 1). Digestion with *Kpn*I-*Sph*I gave a 3.5-kb-hybridizing band in place of 0.8- and 0.9-kb bands in the wild-type strain (Fig. 3B; compare lanes 6 to 8 with lane 5). Digestion with *Bam*HI gave a 4.4-kb-hybridizing band in place of the 2.6-kb band in the wild-type strain (Fig. 3B; compare lanes 10 to 12 with lane 9). These data confirm that the *vdh* gene was disrupted through the expected double crossover.

As expected, the *vdh*::*hyg* mutants of both species had no detectable Vdh activity in cell extracts of TSB-grown cultures, whereas the parental strains exhibited considerable Vdh activ-

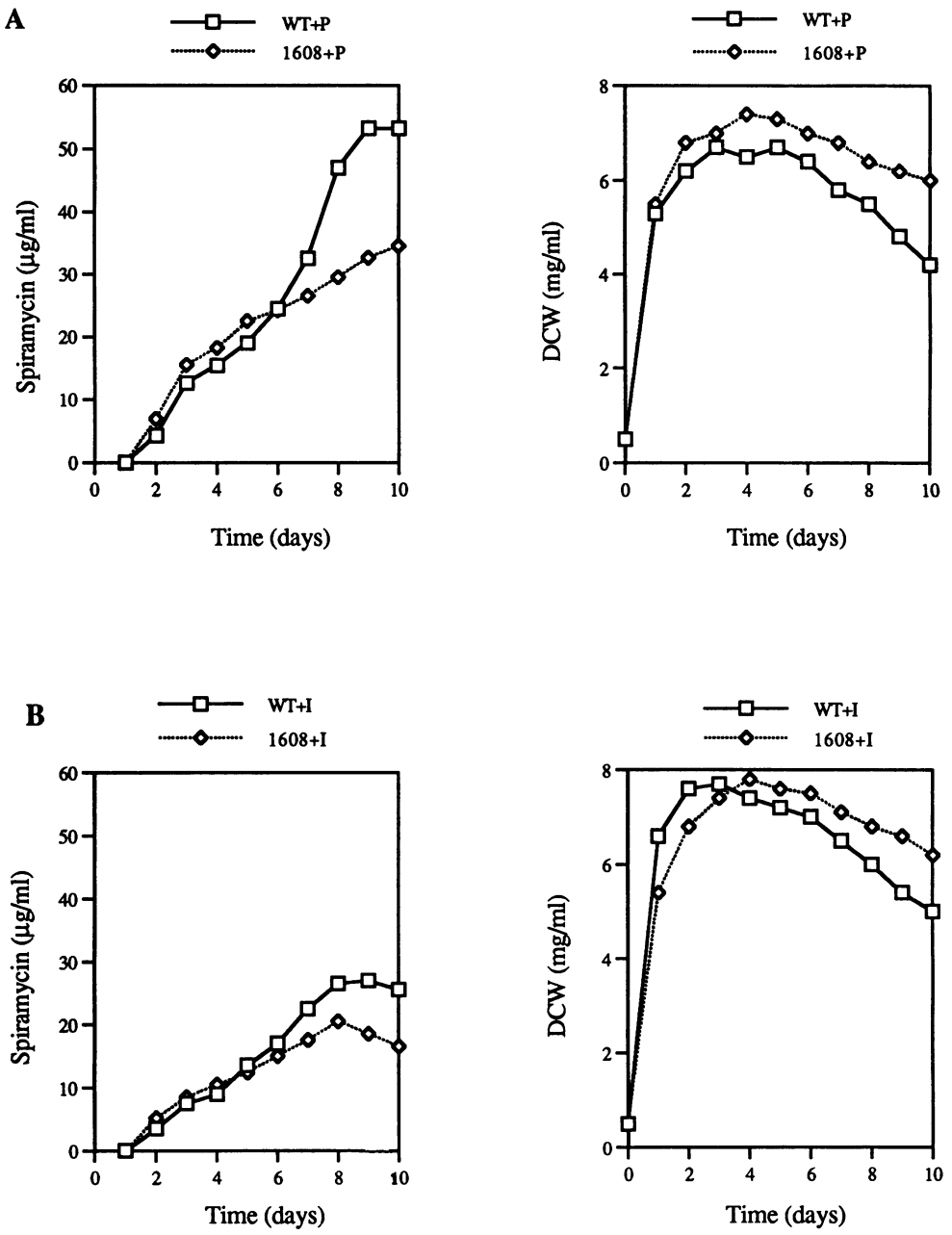
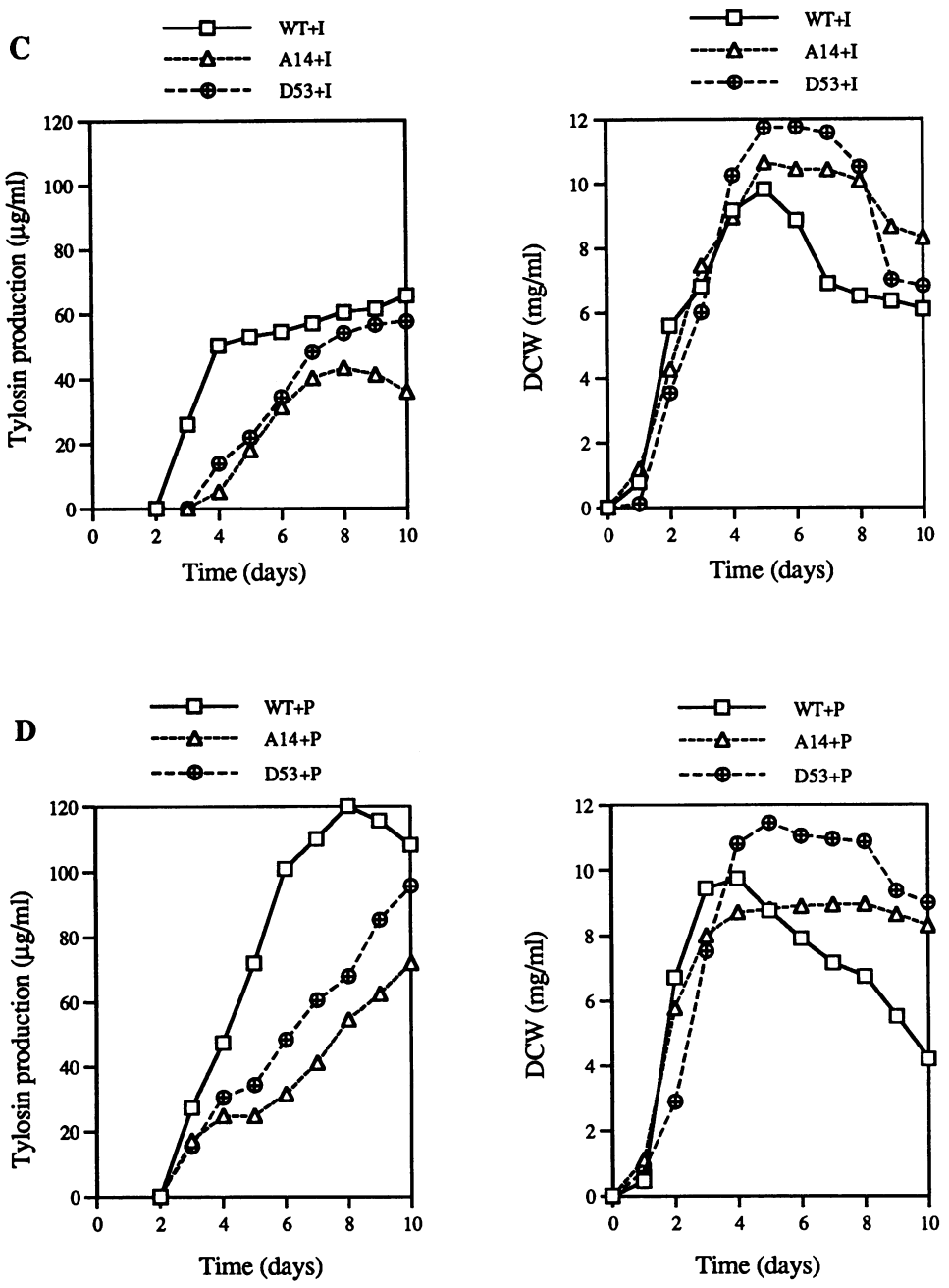


FIG. 6. (A and B) Spiramycin production and growth (dried cell weight [DCW]) of the *S. ambofaciens* wild-type (WT) and WMH1608 *vdh* mutant strains grown in the glucose-asparagine medium in the presence of 25 mM propionate (P) or isobutyrate (I). (C and D) Tylosin production and growth (DCW) of the *S. fradiae* WT and WMH1610 *vdh* mutant strains (A14 and D53) grown in the glucose-NH_4^+ medium in the presence of 12.5 mM P or 10 mM I. The antibiotic production data are averages for at least two separate experiments, and the data for mutant WMH1608 are the averages from two individual Th^s Hg^r transformants. These data represent an average error of less than 10%, and the growth data represent an average error of less than 5%.

ity at 24 or 48 h (Fig. 4), which was induced two- to threefold by valine (data not shown). Consequently, both *S. ambofaciens* and *S. fradiae* have only one *vdh* gene, as in *S. coelicolor* (27). The *vdh*-disrupted mutants WMH1608 and WMH1610 therefore are suitable for testing the role of Vdh in antibiotic production.

The *vdh*::*hyg* mutations greatly lower spiramycin and tylosin production. Spiramycin and tylosin production by the parental strains and the *vdh*-disrupted mutants WMH1608 and WMH1610 under defined growth conditions were compared by bioassay to determine whether the inability to catabolize valine (or other branched-chain amino acids [27]) affects macrolide production. As shown in Fig. 5, the *S. ambofaciens* WMH1608 *vdh* mutants produced an approximately fourfold-lower level of spiramycin than the wild-type strain in the glucose-asparagine medium (Fig. 5A), and the *S. fradiae* WMH1610-A14 mutant produced an approximately sixfold-lower level of tylosin than the parental strain in the glucose-NH_4^+ medium (Fig. 5B).



Two individually isolated *S. fradiae vdh::hyg* mutants, WMH1610-A14 and WMH1610-D53, showed a significant difference in the level of tylosin production (Fig. 5B). Both the A14 and D53 mutants had the *hyg* gene inserted into the chromosomal *vdh* gene, as revealed by an identical Southern blot hybridization profile (Fig. 3B, lanes 2, 6, and 10 for mutant A14 and lanes 4, 8, and 12 for mutant D53), and had no detectable Vdh activity (Fig. 4B). The wild-type and *vdh::hyg* mutant strains had essentially identical growth profiles (Fig. 5). As determined by thin-layer chromatography and bioautography assays, the *S. ambofaciens* WMH1608 and *S. fradiae* WMH1610 *vdh::hyg* mutants as well as the parental wild-type strains mainly produced spiramycin or tylosin, respectively (data not shown).

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

tyrate caused a significantly lower level of spiramycin production in the wild-type strain and very little increase (approximately twofold) in the mutant strains (Fig. 6B). Although the addition of propionate or isobutyrate did not appreciably modify cell growth, the onset of spiramycin production was much earlier (compare Fig. 6 and 5). Similar results were obtained for tylosin production. Addition of 15 mM propionate to the glucose-NH_4^+ medium at 0 h (the wild-type and WMH1610 *vdh* mutant strains grew poorly in 25 mM propionate or isobutyrate) caused a significant increase in tylosin production by the WMH610-A14 and WMH1610-D53 strains as well as by the *S. fradiae* wild-type strain (compare Fig. 6D and 5B). Addition of 10 mM isobutyrate to the *S. fradiae* wild-type and WMH1610-D53 mutant strains did not cause an increase in the production of tylosin but did restore tylosin production by the WMH1610-A14 strain to the level in the wild-type strain (compare Fig. 6C and 5B).

Introduction of the *vdh* gene into the *vdh*::*hyg* mutants produces a notable increase in macrolide production. To examine the effect of introducing the *vdh* gene back to the *vdh*::*hyg* mutants on macrolide production, the levels of spiramycin production and the Vdh activities 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 (27) to give plasmid pWHM1076 (Table 1), and 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 was found to increase in the wild-type strain and to return to near the wild-type level in the WMH1608 mutants (Fig. 4A). The WMH1608(pWHM1076) transformants produced an approximately twofold-higher level of spiramycin than the WMH1608 mutants, although the level did not reach a value similar to that in the wild-type strain (compare Fig. 7A and 5A). Wild-type *S. ambofaciens*(pWHM1076) transformants produced significantly lower levels of spiramycin than the wild-type strain without the plasmid (compare Fig. 7A and 5A). Negative effects of plasmid vectors have been observed previously with other antibiotic-producing streptomycetes (5, 14, 30).

Similar results were found with tylosin production. A 2.5-kb fragment containing the *vdh* and ORF1 genes from *S. fradiae* was cloned in the multicopy plasmid pKC1139 to give plasmid pWHM1086 (Table 1), and then pWHM1086 was introduced into *S. fradiae* C373.1 and *vdh*::*hyg* mutants WMH1610-A14 and WMH1610-D53 by conjugative transfer. The Vdh specific activities of the three types of pWHM1086 transformants were assayed at 24 h and found to be about the same in the *vdh*::*hyg* strains as in the wild-type strain, although the specific activity was increased approximately threefold compared with that in the plasmid-free wild-type strain (Fig. 4B). The WMH1610-A14(pWHM1086) transformants produced an approximately fourfold-higher level of tylosin than the WMH1610-A14 mutant (Fig. 7B), whereas the *S. fradiae*(pWHM1086) transformants produced slightly lower levels of tylosin than the plasmid-free wild-type strain. Introduction of pWHM1086 into the WMH 1610-D53 strain did not have any effect on tylosin production (Fig. 7B). However, the respective pWHM1076 and pWH M1086 transformants of the *vdh*::*hyg* mutants WMH1608 and WMH1610 produced almost the same amount of macrolide antibiotic as the parental strains. This indicates that introduction of the wild-type *vdh* gene can reverse the negative effect of the *vdh* mutation on spiramycin and tylosin production.

DISCUSSION

Our work on the *vdh* genes from the macrolide-producing *S. ambofaciens* and *S. fradiae* strains was carried out in parallel to provide a thorough assessment of *vdh* structure and function, with respect to a possible role of Vdh in macrolide production. The region surrounding the *vdh* gene has the same organization in *S. ambofaciens* and *S. fradiae* as in *S. coelicolor* (27) and the polyether-producer *Streptomyces albus* (29). Furthermore, the deduced amino acid sequences of the *vdh*, ORF1, and ORF2 genes from *S. ambofaciens* and *S. fradiae* are significantly similar to those of the products of the corresponding *S. coelicolor* genes (27). The *vdh* gene from *S. fradiae* should encode a 371-amino-acid protein with a calculated molecular mass of 38,734 Da. This would provide an enzyme subunit with a mass similar to those of the Vdh enzymes from *S. coelicolor* (18, 27), *S. albus* (29), and *Streptomyces cinnamonensis* (23). Surprisingly, the Vdh protein isolated from another tylosin-producing *S. fradiae* strain is reported to have a native molecular mass of 215,000 Da and to be composed of 12 subunits of 18,000 Da each (32). The reason for this large size discrepancy is not known, but it is not likely due to multiple *vdh* genes, since the *vdh*-disrupted mutants from *S. ambofaciens* and *S. fradiae* have no detectable Vdh activity, confirming the finding, first reported for *S. coelicolor* (27), that there is only one gene for the Vdh activity in these organisms and presumably in other streptomycetes.

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^+ medium. Since antibiotic production was not entirely lost but was restored by exogenous propionate or isobutyrate, valine catabolism (Fig. 8) (18) 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 tricarboxylic acid cycle intermediates, such as succinyl-CoA, from which 2-methylmalonyl-CoA and propionyl-CoA can be formed (9) 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 tricarboxylic acid cycle, for instance) of substrates like propionyl-CoA or 2-methylmalonyl-CoA is limited. Indeed, evidence from nutritional studies suggests that macrolide precursors are derived more directly from branched-chain amino acids than from other amino acids or succinyl-CoA (8). 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 (12). These workers suggest that in *Saccharopolyspora erythraea* a malonyl-CoA decarboxylase can generate the propionyl-CoA needed for erythromycin biosynthesis from the (2*R*)-2-methylmalonyl-CoA formed from succinyl-CoA by 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

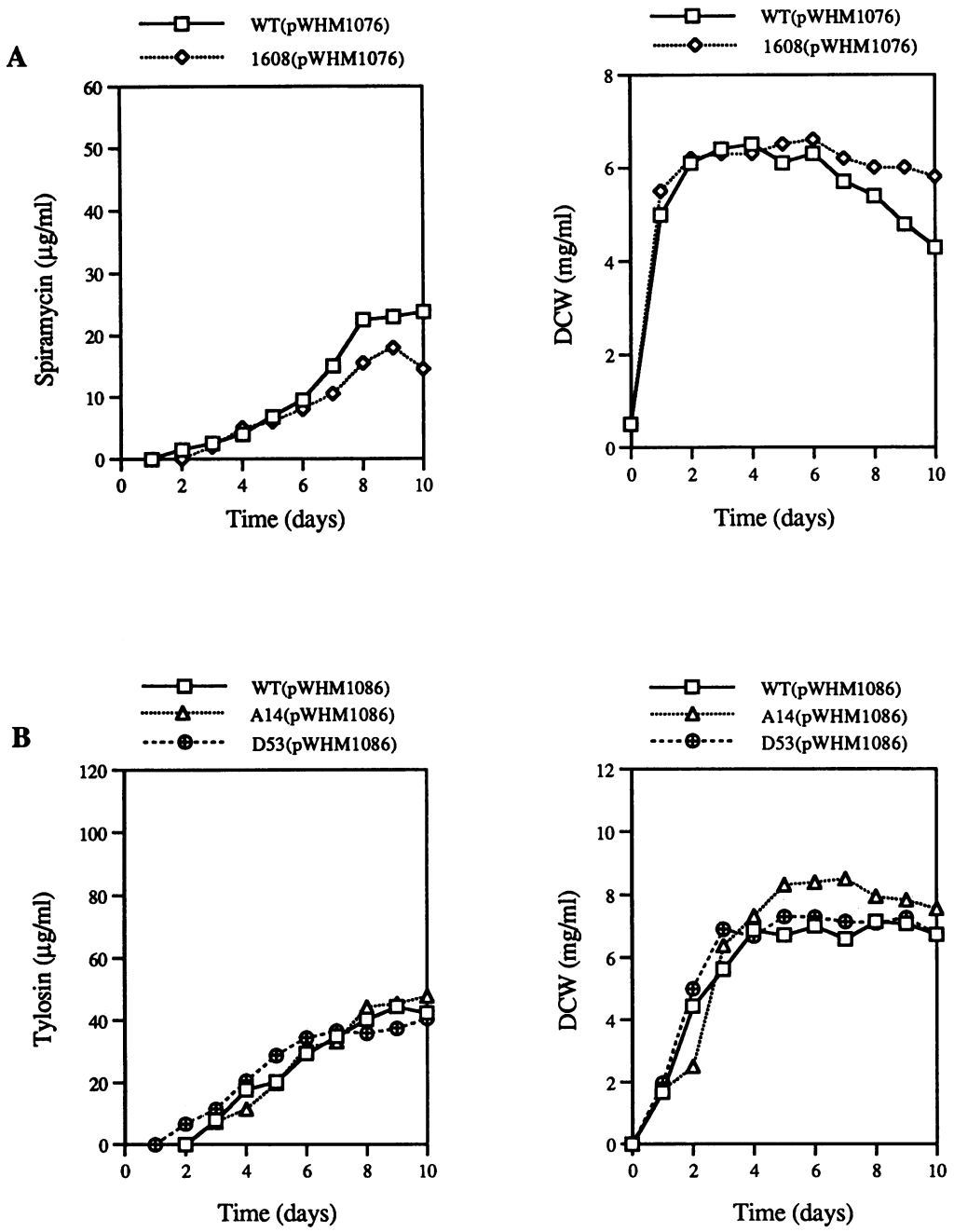


FIG. 7. (A) Spiramycin production and growth (dried cell weight [DCW]) of the *S. ambofaciens*(pWHM1076) (WT) and WMH1608(pWHM1076) transformants grown in the glucose-asparagine medium. (B) Tylosin production and growth (DCW) of *S. fradiae*(pWHM1086) (WT), WMH1610-A14(pWHM1086), and WMH1610-D53(pWHM1086) transformants grown in the glucose-NH_4^+ medium. The antibiotic production data are the averages for at least two separate experiments, and the data for the wild-type and *vdh* mutants are the averages from two individual pWHM1076 or pWHM1086 transformants. These data represent an average error of less than 10%, and the growth rate data represent an average error of less than 5%.

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 approximately twofold 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 of spiramycin and tylosin production in both types of strains (data not shown).

The noticeably different behavior of the *S. fradiae* WMH1610-A14 and WMH1610-D53 *vdh*::*hyg* mutants cannot be explained

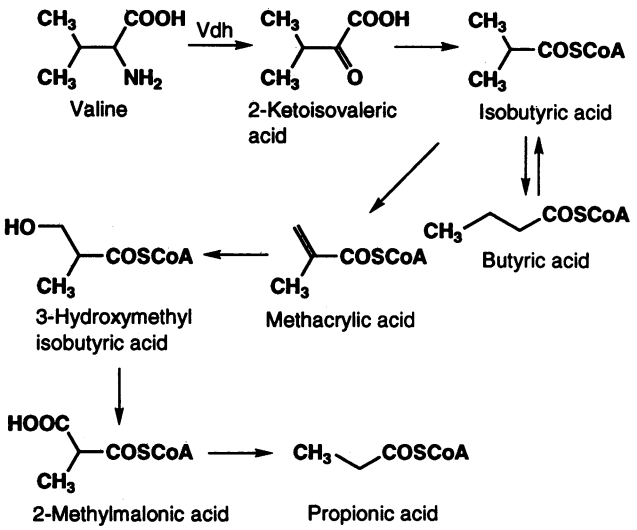


FIG. 8. Proposed pathway for valine catabolism in streptomycetes (18). Intermediates in the pathway beyond 2-ketoisovaleric acid are arbitrarily shown as their CoA esters.

by the available data, since both mutants appear to have the same genotype and no Vdh activity. Mutant WMH1610-A14 was isolated directly as the product of a double crossover recombination, but mutant WMH1610-D53 was isolated first as a single crossover recombinant, which was followed by subsequent rounds of propagation on R2YE plates with 200 U of hygromycin at 39°C to obtain the double crossover recombinant. Mutant WMH1610-D53 initially produced a very low level of tylosin, like mutant WMH1610-A14, but after one transfer the WMH1610-D53 strain showed a level of production that was variable but higher than that of mutant WMH1610-A14. This suggests that the WMH1610-D53 strain could have a second-site mutation affecting tylosin production.

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, and thence spores, in the morphogenesis of streptomycetes on solid medium. Mendez 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 (16). 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 to preserve them as a source of nutrients for aerial growth (4). 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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