Biochemical Characterization of VlmL, a Seryl-tRNA Synthetase Encoded by the Valanimycin Biosynthetic Gene Cluster*§

Ram P. Garg§, Jose M. Gonzalez§, and Ronald J. Parry††

From the §Department of Chemistry, Rice University, Houston, Texas 77005 and ††Departamento de Microbiología y Biología Celular, Facultad de Farmacia, Universidad de La Laguna, E-38206 La Laguna, Tenerife E-38206, Spain

Previous studies have shown that the valanimycin producer Streptomyces viridifaciens contains two genes encoding proteins that are similar to seryl-tRNA synthetases (SerRSs). One of these proteins (SvsR) is presumed to function in protein biosynthesis, because it exhibits a high degree of similarity to the single SerRS of Streptomyces coelicolor. The second protein (VlmL), which exhibits a low similarity to the S. coelicolor SerRS, is hypothesized to play a role in valanimycin biosynthesis, because the vlmL gene resides within the valanimycin biosynthetic gene cluster. To investigate the role of VlmL in valanimycin biosynthesis, VlmL and SvsR have been overproduced in soluble form in Escherichia coli, and the biochemical properties of both proteins have been analyzed and compared. Both proteins were found to catalyze a serine-dependent exchange of 32P-labeled pyrophosphate into ATP and to aminoacylate total E. coli tRNA with L-serine. Kinetic parameters for the two enzymes show that SvsR is catalytically more efficient than VlmL. The results of these experiments suggest that the role of VlmL in valanimycin biosynthesis is to produce seryl-tRNA, which is then utilized for a subsequent step in the biosynthetic pathway. Orthologs of VlmL were identified in two other actinomycetes species that also contain orthologs of the S. coelicolor SerRS. The significance of these findings is herein discussed.

The aminoacyl tRNA synthetases (AARSs) are a well studied family of enzymes that ligate a specific amino acid to the cognate tRNA for subsequent incorporation into protein (1). The ligation process involves a two-step reaction sequence. The first step proceeds by reaction of the amino acid with ATP to generate an aminoacyl adenylate AA-AMP. This step is followed by transfer of the amino acid to the 2'- or 3'-hydroxyl group of the 3'-terminal ribose of the tRNA.

The AARSs are divided into two unrelated classes. The enzymes of each class share a characteristic catalytic folding pattern, identifiable sequence motifs, and distinctive mechanistic features (2, 3). Structural studies have shown that the active site of class I synthetases contains a Rossmann dinucleotide binding fold, whereas members of the class II family of enzymes exhibit a seven-stranded antiparallel β-sheet flanked by three α-helices in a barrel-like structure (4). All class I enzymes attach their amino acid substrate to the 2'-hydroxyl group of the tRNA-terminal adenosine, whereas class II AARSs (except PheRS) attach the amino acid substrate to the 3'-hydroxyl group (2).

Investigations over the last two decades have shown that some AARSs participate in biochemical processes besides direct involvement with protein biosynthesis (5, 6). These alternate functions are of two types, those that involve the synthesis of an aminoacylated tRNA and those that do not. An example of the former is the conversion of Glu-tRNA\textsuperscript{Gln} and Asp-tRNA\textsuperscript{Asn} into their glutaminylated and asparaginylated versions by amidotransferases (1). Examples of the latter type of function include AARSs that participate in translational regulation, promote the splicing of mitochondrial cytochrome genes, and serve as precursors of species with cytokine-like activity (4, 6).

The antibiotic valanimycin (see Fig. 1) is a naturally occurring azoxy compound isolated from the fermentation broth of Streptomyces viridifaciens MG456-hF10 by Yamato et al. (7). In addition to antibacterial activity, valanimycin exhibits potent antitumor activity against in vitro cell cultures of mouse leukemia L1210, P388/S (doxorubicin-sensitive), and P388/ADR (doxorubicin-resistant) (7). As a naturally occurring azoxy compound, valanimycin is a member of a growing family of natural products that now includes the cycad toxins macrozamin and cycasin (7–10), the carcinogen elaiomycin (7–11), the antifungal agents maniwamycins A and B (14), the nematocidal compounds jetcins A and B (15), and the antifungal agent azyxybacilin (16). Precursor incorporation experiments have established that valanimycin is derived from L-valine and L-serine via the intermediary of isobutylamine and isobutylhydroxylamine (Fig. 1) (17). Furthermore, the azoxy nitrogen atoms in valanimycin have been shown to originate from the nitrogen atoms of isobutylamine and serine (17). Enzymatic and genetic investigations (18–20) led to the cloning and sequencing of the valanimycin biosynthetic gene cluster, which was found to contain 14 genes (21). The functions of five proteins encoded by these genes have been elucidated. These functions include a valine decarboxylase (VlmD) (21), a two-component flavin monoxygenase that converts isobutylamine
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![Diagram of biosynthetic pathway for the antibiotic valanimycin]

FIGURE 1. Biosynthetic pathway for the antibiotic valanimycin. The pathway proceeds from L-valine and L-serine through the intermediacy of isobutylhydroxylamine.

for Western blotting. Protein concentrations were determined with the Advanced Protein Assay Reagent from Cytoskeleton Inc. (Denver, CO) or by use of the BCA reagent from Pierce. Bovine serum albumin was used as a standard.

DNA Manipulations—Genomic DNA was prepared from S. viridifaciens MG456-hF10 using DNAZOL reagent (MRC Inc., Cincinnati, OH) after pulverization of mycelium frozen with liquid nitrogen. Plasmid DNA was purified with a QIAprep spin plasmid kit (Qiagen, Valencia, CA). DNA fragments were isolated from agarose gels with a QIAquick gel extraction kit (Qiagen). PCR products were separated on agarose gels and purified from the gels. Digestion with restriction endonucleases and ligation experiments were carried out by standard procedures under conditions recommended by the manufacturers. Automated DNA sequencing was performed at Lone Star Sequencing Laboratories (Houston, TX) using universal and synthetic oligonucleotide primers.

Computational Analyses—Primary sequence alignments were performed using The Gene Inspector, version 1.5, software (Textco, Inc., West Lebanon, NH) run on a Macintosh computer. BLAST (basic local alignment search tool) analyses were performed at the National Center for Biotechnology Information web site. GraphPad Prism, version 4.0 (GraphPad Software Inc., San Diego, CA), was used for the nonlinear regression analysis of all enzyme assay data. Sequencher, version 4.1 (Gene Codes Corporation, Ann Arbor, MI), was used to compile DNA sequence data. Phylogenetic analysis of SerRSs was carried out by the creation of a multiple sequence alignment with ClustalW (24). A tree was then generated based on a Kimura distance matrix and the neighbor-joining method using the PHYLIP software package (25).

Cloning of vlmL and svsR—vlmL was amplified from cosmid pVal35 (19) by PCR using an N-terminal primer containing an NdeI site (bold) (5'-TATTATCATGATGACCTTACACGAGTT-3') and a C-terminal primer with an EcoRI site (bold) (5'-TTGATGACAGACATGGTTCCTG-3'). The vlmL PCR product was cloned into a PCR3.2 TOPO TA vector (Invitrogen) and then subcloned into the NdeI and EcoRI sites of pFLAG-CTC to produce pFLAG-CTC-VlmL. The svsR gene was amplified from the genomic DNA of S. viridifaciens using primers designed from the nucleotide sequence of the S. coelicolor seryl-tRNA synthetase gene svvR. The N-terminal primer (5'-TATTATCATGATGACCTTACACGAGTT-3') contained an NdeI site, whereas the C-terminal primer (5'-TTGATGACAGACATGGTTCCTG-3') contained an EcoRI site. A mixture of Vent and TaqDNA polymerase was used for amplification in standard PCR buffer with annealing at 66 °C for 30 s. The 1.3-kb PCR product was purified after separation on an agarose gel, cloned into pGEM-T (Promega), and sequenced. The gene was then subcloned into the NdeI and EcoRI sites of pFLAG-CTC to produce pFLAG-CTC-SvsR. The N-terminal PCR primers for vlmL and svsR were both designed to replace the native GTG start codon of each gene with an ATG codon to improve expression in E. coli.

Overexpression, Purification, and Characterization of Proteins—Overproduction and purification of VlmL and SvsR from pFLAG-CTC-VlmL and pFLAG-CTC-SvsR were carried out according to the protocols outlined in the FLAG vector instruc-

EXPERIMENTAL PROCEDURES

General—Unless otherwise indicated, all reagents used in this study were purchased from Sigma, Roche Applied Science, Bio-Rad, or G. E. Healthcare. Oligonucleotides were obtained from the Sigma Genosys. Restriction enzymes were obtained from either Rad, or G. E. Healthcare. Oligonucleotides were obtained from the study were purchased from Sigma, Roche Applied Science, Bio-Rad, or G. E. Healthcare. Oligonucleotides were obtained from the study were purchased from Sigma, Roche Applied Science, Bio-Rad, or G. E. Healthcare. Oligonucleotides were obtained from the study were purchased from Sigma, Roche Applied Science, Bio-Rad, or G. E. Healthcare.

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Cloning and Sequence Analysis of VlmL and SvsR—Previous work identified a housekeeping seryl-tRNA synthetase gene in *S. viridifaciens* in addition to *vlmL* (21). In the current studies, the *S. viridifaciens* housekeeping SerRS (*svsR*) and *vlmL* were amplified using primers that contained restrictions sites to facilitate the cloning of each gene into the protein expression vector pFLAG-CTC. The amino acid translation product of each gene was analyzed by BLAST. SvsR and VlmL both display a strong degree of similarity to other seryl-tRNA synthetases, and both proteins display the three motifs that are associated with class II AARSs (2) (see supplemental Fig. 6). SvsR exhibits >90% identity to the housekeeping SerRSs of other *Streptomyces* strains, but only 38% identity to VlmL. BLAST analysis reveals that two other actinomycetes, *Streptomyces avermitilis* and *Frankia* sp. EAN1pec, each contain two genes that encode SerRSs. One of the two SerRS genes (GenBank™ BAC70005, ZP_00567562) present in each of these organisms encodes a protein that exhibits a high degree of identity to VlmL. Fig. 2
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Frankia sp. EAN1024 (TZ_00567562)

Psychrobacter arcticus 273-4 (AAZ19466)

Vibrio parahaemolyticus RIMD 2210633 (BAC53971)

Methylcococcus capsulatus Bath (AAM12005)

Aquifex aeolicus VF5 (AAC06595)

Bacillus subtilis 168 (CAC1789)

P. propionicius DSM 2379 (ZP_005677148)

Geobacter metallireducens GS-15 (ABB33733)

Solibacter usitatus Ellin6076 (ZP_00521250)

Desulfotalea psychrophila LSV54 (CAG34693)

Desulfovibrio desulfuricans G20 (ABB36997)

Streptomyces avermitilis MA-4680 (BAC70005)

Streptomyces viridifaciens MG456-hf10 (AAN10249; VlmL)

Arthrobacter sp. FB24 (ZP_00411331)

Brevibacterium linens BL2 (ZP_00380504)

Mycobacterium avium K-10 (NP_959131)

Nocardiia farcinica IMF 10152 (BAD54982)

C. glutamicum ATCC 13032 (BAC00287)

Thermotoga fusca YX (AAZ54069)

Streptomyces coelicolor A3(2) (CAA22233)

Streptomyces viridifaciens MG456-hf10 (AAM77028; SvsR)

Streptomyces avermitilis MA-4680 (NP_825421)

FIGURE 2. Phylogenetic tree for bacterial SerRSs. The figure displays an unrooted distance matrix tree of phylogenetic relationships between VlmL, SvsR, and other bacterial SerRs. The tree was created from a multiple sequence alignment in ClustalW using the PHYLIP software package with the Kimura distance matrix and the neighbor-joining method. The topology of the phylogenetic tree was evaluated by performing a bootstrap analysis with 100 replicates. The numbers at the nodes are bootstrap values >50%. The scale bar represents the Kimura distance (number of amino acid substitutions/site).

displays a phylogenetic tree created with PHYLIP software (25) relating these and other bacterial SerRs to one another. The phylogenetic tree suggests that VlmL (GenBankTM AAN10249), BAC70005, and ZP_00567562 are more closely related to the SerRs of Gram-negative bacteria than to the SerRs of Gram-positive bacteria. An alignment between the amino acid sequences of VlmL and the SerRSs of S. coelicolor, S. avermitilis, and Frankia sp. EAN1024 is shown in supplemental Fig. 6.

Protein Overexpression and Characterization—VlmL and SvsR were overexpressed in E. coli as C-terminal FLAG-tagged proteins using the pFLAG-CTC vector in which expression is driven from the inducible trc promoter. Both proteins were overproduced largely in soluble form with yields of ~5 mg/liter culture broth. The maximum amounts of protein were observed after 2 h of induction at 37 °C. Longer incubation at 37 °C or the use of lower temperatures resulted in proteolysis and reduced yields. On SDS-PAGE analysis, both VlmL and SvsR exhibited the molecular weights expected for the denatured proteins and appeared to be >95% pure (data not shown). The native molecular weights of VlmL and SvsR were determined by gel filtration in TBS buffer on a Superose 6 column calibrated with standard protein molecular weight markers. The elution volume of VlmL corresponded to a molecular weight of ~90,000, indicating that VlmL exists as a dimer. This observation is consistent with the behavior exhibited by other SerRSs (3, 28). SvsR displayed two major fractions on gel filtration, with total E. coli tRNA and 1-[3H(G)]serine and the [3H]seryl-tRNA produced from the pool of tRNA was measured by trichloroacetic acid precipitation on Whatman filter paper. Intriguingly, both VlmL and SvsR catalyzed the aminoacylation of tRNA by l-serine. The rates of tRNA aminoacylation catalyzed by VlmL and
SvsR were linear with time (Fig. 4), and omission of enzyme, ATP, or tRNA resulted in the complete lack of precipitated radioactivity (data not shown). The aminoacylation reactions catalyzed by VlmL and SvsR followed Michaelis-Menten kinetics under conditions in which the concentration of L-serine or tRNA (Fig. 5) was varied. The maximal rates for the conversion of radiolabeled serine into radiolabeled seryl-tRNA by VlmL and SvsR were calculated to provide a comparison of the efficiency of the aminoacylation reaction catalyzed by each enzyme. The calculations show that these two enzymes catalyze the aminoacylation reaction at comparable rates.

**Acid Urea Gel Analysis of Aminoacylation of tRNA**—In order to confirm that [3H]seryl-tRNA was produced in the aminoacylation reactions catalyzed by both VlmL and SvsR, the labeled seryl-tRNA produced in the enzymatic incubations was separated by PAGE using acidic, urea-containing gels, and the labeled seryl-tRNA was visualized by autoradiography. It was observed that the amount of labeled seryl-tRNA produced by both enzymes increased with time in a manner that was consistent with the increase in labeled seryl-tRNA observed by trichloroacetic acid precipitation (Fig. 4, insets). Acidic urea PAGE analysis of control incubations carried out in the absence of tRNA confirmed that the radioactive product observed by PAGE in the complete incubations was tRNA-dependent (data not shown). Additional control experiments verified that the formation of radiolabeled seryl-tRNA was also dependent on the presence of ATP and the presence of VlmL or SvsR (data not shown).

**DISCUSSION**

Previous investigations have shown that the valanimycin producer *S. viridifaciens* contains two genes that appear to code for SerRSs (21). Sequence analysis suggests that one of these genes...

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**TABLE 1**

| Parameter                  | VlmL         | SvsR          |
|----------------------------|--------------|---------------|
| [32P]PPi exchange assay     |              |               |
| Vmax                      | 409.0 ± 11.6 pmol min⁻¹ ml⁻¹ | 721.6 ± 42.9 pmol min⁻¹ ml⁻¹ |
| Km                        | 79.28 ± 5.94 mM | 274.4 ± 28.5 mM |
| kcat/Km                   | 1.01 min⁻¹ | 1721.6 ± 1721.6 min⁻¹ |
| kcat/Km                   | 12.74 min⁻¹·m⁻¹ | 55.19 min⁻¹·m⁻¹ |
| Aminoacylation assay       |              |               |
| Vmax                      | 130.7 ± 11.6 pmol min⁻¹ ml⁻¹ | 112.7 ± 6.4 pmol min⁻¹ ml⁻¹ |
| Km                        | 2.99 ± 0.43 mM | 6.48 ± 0.26 mM |
| kcat/Km                   | 7.91 min⁻¹ | 3.37 min⁻¹ |
| kcat/Km                   | 3.26 min⁻¹·m⁻¹ | 0.52 min⁻¹·m⁻¹ |
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AARS ⋅ AA − AMP + tRNA ⇌ AARS + AA − tRNA + AMP

(Eq. 2)

Michaelis-Mentel-like behavior was observed for both proteins in this assay (Fig. 5). The data (Table 1) show that both proteins catalyze the aminoacylation reaction with comparable efficiency. From these observations, we hypothesize that the function of VlmL in valanimycin biosynthesis is to produce seryl-tRNA, which then serves as the substrate for a subsequent step in the biosynthetic pathway. Precedent for involvement of an aminoacyl tRNA in a biochemical pathway unrelated to protein biosynthesis is provided by the formation of 5-aminolevulinic acid from l-glutamyl-tRNA in chloroplasts and some bacteria (29). Although the details of the later stages in the valanimycin biosynthetic pathway are presently unknown, several steps will clearly be required to produce valanimycin from seryl-tRNA and isobutylhydroxylamine. These include N–N bond formation, a formal four-electron oxidation of the N–N bond to the level of an azoxy group, and dehydration of the serine-derived hydroxyl group. The most novel of these processes is clearly N–N bond formation. It is possible that the ester moiety of the seryl-tRNA is somehow required for this reaction. Prior investigations have shown that the oxygen of isobutylhydroxylamine is not the source of the valanimycin azoxy oxygen atom (23). This observation limits the number of mechanisms that can be envisioned for formation of an N–N bond. An interesting issue raised by our hypothesis is the degree of cross-talk that occurs between the seryl-tRNA produced by SvsR for use in protein biosynthesis and the seryl-tRNA generated by VlmL for valanimycin biosynthesis. Previous studies have shown (21) that disruption of the vlmL gene strongly reduces, but does not eliminate, valanimycin production. The phenotype of the vlmL mutant suggests that seryl-tRNA produced by SvsR can be used for valanimycin production, although the process appears to be inefficient. This inefficiency might result from compartmentalization of the valanimycin pathway. Alternatively, VlmL may utilize only a single tRNASer isoacceptor as a substrate in contrast with SvsR, which would be expected to utilize all of the isoaccepting tRNASer species. Future studies will examine this question as well as the role played by seryl-tRNA in valanimycin biosynthesis.

Acknowledgments—We thank Dr. S. Martinis for critical reading of the manuscript and Amy Williams for the protocols for tRNA aminoacylation and acidic urea PAGE analysis. We also thank Dr. Robin Couch for the PP, exchange protocol and for preliminary PP, exchange assays of VlmL.
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