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Streptomyces Genes Involved in Biosynthesis of the Peptide Antibiotic Valinomycin

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We have identified genes from *Streptomyces levoris* A-9 involved in the biosynthesis of the peptide antibiotic valinomycin. Two segments of chromosomal DNA were recovered from genomic libraries, constructed by using the low-copy-number plasmid pIJ922, by complementation of valinomycin-deficient (*vlm*) mutants of *S. levoris* A-9. One set of plasmids restored valinomycin production to only one mutant, that carrying *vlm*-1, whereas a second set of plasmids restored productivity to seven *vlm* mutants, those carrying *vlm*-2 through *vlm*-8. Additional complementation studies using subcloned restriction enzyme fragments showed that the *vlm-1*$^+$ gene was contained within a 2.5-kilobase (kb) DNA region, whereas alleles *vlm-2*$^+$ through *vlm-8*$^+$ were contained in a 12-kb region, representing at least three genes. Physical mapping experiments based on the isolation of cosmid clones showed that the two *vlm* loci were 50 to 70 kb apart. Southern hybridization experiments demonstrated that the *vlm-2*$^+$ gene cluster was highly conserved among other valinomycin-producing *Streptomyces* strains, whereas the *vlm-1*$^+$ gene was ubiquitous among *Streptomyces* species tested. Increasing the copy number of the *vlm-2*$^+$ gene cluster in *S. levoris* A-9 by the introduction of low-copy-number recombinant plasmids resulted in a concomitant increase in the level of valinomycin production.

Peptide antibiotics represent a unique class of bioactive molecules produced by both procaryotic and eucaryotic microorganisms (16). In most cases, these antibiotics are synthesized from enzymatic complexes rather than from ribosome-dependent systems (14, 25–27). Extensive biochemical studies indicated that these complexes consist of several large polypeptides, ranging in molecular mass from 100 to 440 kilodaltons (kDa) and containing many thiol-active catalytic sites (17). Collectively, these complexes serve as a template to activate amino acids, racemize amino acids from L to D forms, and direct their sequential condensation to form the peptide antibiotic; the enzymology of this unique biochemical process has been extensively reviewed (16, 18, 19, 24).

Despite the wealth of biochemical knowledge, the structure and regulation of the genes that encode the polypeptide enzymes are just beginning to be understood. These studies have been greatly facilitated with the recent availability of cloned biosynthetic genes for bacitracin (13, 20), gramicidin (21–23), and tyrocidine (32, 34) synthesis, permitting investigation into the organization and regulation of peptide antibiotic biosynthetic genes at the molecular level (33).

We are interested in isolating the genes involved in the biosynthesis of the peptide antibiotic valinomycin (4, 5). Valinomycin is a cyclic dodecadepsipeptide molecule consisting of L-lactic acid, D-hydroxyisovaleric acid, and both the L AND D forms of valine (29), the synthesis of which is reported to occur from a multienzyme complex (1, 37). Active as a potassium (K^+) ionophore (9), valinomycin is used commercially as an insecticidal-nematocidal agent (W. Patterson, U.S. Patent no. 3,520,973, 1970) and in the biomedical industry as a key component of K^+ measurement instruments (7). Here we report the cloning of valinomycin biosynthetic genes by complementation of blocked *vlm* mutants and by chromosomal walking in *Escherichia coli*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The valinomycin-producing strain *Streptomyces levoris* A-9, a recent soil isolate, was used as the source for valinomycin biosynthetic genes; valinomycin-deficient strains of *S. levoris* A-9 are listed in Table 1 (S. K. Guterman et al., unpublished data). Strains *Streptomyces tsusimaensis* (ATCC 15141) and *Streptomyces* sp. (ATCC 23836), two other valinomycin-producing strains, were obtained from the American Type Culture Collection. Strains *Streptomyces lividans* 1326, *Streptomyces griseus* 3404, and *Streptomyces coelicolor* A332 were kindly provided by D. Hopwood. Strain ED8767 (*supE*, *supF*, *hsdS*, *met*, *recA56*) was the *E. coli* host used to prepare cosmid gene libraries. Plasmids pIJ922 (28), pIJ702 (15), and pIJ486 (41) were also obtained from D. Hopwood. pIJ922, passaged through *S. levoris* A-9, was used as the source for vector in plasmid gene library constructions. Plasmid pIJ922Δ_1 containing a spontaneous 7-kilobase (kb) deletion was isolated from *S. levoris* A-9(pIJ922) bacteria. The *E. coli* plasmid used to generate cosmid gene libraries was pHC79 (Boehringer Mannheim Biochemicals; 10).

Media and growth conditions. Medium R2YE (40) was used for routine culturing, preparation of spores, and regeneration of *Streptomyces* protoplasts. In some cases, *S. lividans* 1326 was cultured in YEME (40). When assaying for valinomycin production, tryptone soya broth (TSB) medium (Oxoid) was used to culture bacteria both in broth and agar plates. Luria-Bertani (LB) medium (2) prepared without glucose was used for culturing of *E. coli* bacteria. Selection for thiostrepton resistance (Thio^r) was made at a drug concentration of 50 μg/ml in agar plates or 25 μg/ml in broth

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TABLE 1. Characteristics of valinomycin-deficient (*vlm*) mutants of *S. levoris* A-9

Strain designation	Genotype[a]
2-1	*vlm-1 sul osm*
5-2b	*vlm-2*
4-3d	*vlm-3*
1-15	*vlm-4*
8-3	*vlm-5 liv*
7-1	*vlm-6*
1-4	*vlm-7*
4-2a	*vlm-8*

[a] *sul*, Requirement for cysteine, methionine, and vitamin B_2; *osm*, sensitivity to high concentrations of sucrose, salts, and detergents; *liv*, requirement for leucine, isoleucine, and valine.

cultures. Selection for ampicillin resistance (Ap^r) was made at 100 μg/ml.

Isolation of *vlm* mutations. Valinomycin-deficient (*vlm*) strains of *S. levoris* A-9 were obtained by subjecting independent pools of spores to three rounds of *N,N*-methylnitrosoguanidine mutagenesis, using standard methods (11), and testing recovered colonies for the absence of valinomycin production (see below). Genetic linkages were determined relative to the *vlm-1* mutation by using standard protoplast-polyethylene glycol (PEG) fusion mapping methods (11); all mutations were closely linked to *vlm-1*. Characteristics of mutations are summarized in Table 1. Experimental details will be presented elsewhere (Guterman et al., unpublished data).

Isolation and analysis of chromosomal and plasmid DNA. Total DNA was isolated from *S. levoris* A-9 and *S. lividans* by the lysozyme-sodium dodecyl sulfate lysis procedure described by Hopwood et al. (11). Chromosomal DNAs of other *Streptomyces* species were isolated from bacteria grown to late log phase in TSB broth, lysed with Sarkosyl, and treated with pronase as previously described (42). Both large- and small-scale preparations of plasmid DNA from *S. lividans* 1326, *S. levoris* A-9, and *E. coli* bacteria were isolated by the alkaline lysis procedure of Birnboim and Doly (3). Restriction digestions were carried out with enzymes purchased from Boehringer Mannheim and were analyzed by electrophoresis, using standard procedures (38).

Preparation and transformation of *Streptomyces* protoplasts. For the preparation of *S. levoris* A-9 protoplasts, bacteria were grown in TSB medium containing 0.5% glycine for 42 to 48 h and treated with lysozyme at a final concentration of 2 mg/ml as previously described (11). To prepare protoplasts of *S. lividans* 1326, YEME growth medium was substituted for TSB. Protoplasts were transformed by using a slightly modified PEG-assisted procedure of Hopwood et al. (11). DNA (up to 20 μl) was added to 0.5×10^9 to 1.0×10^9 protoplasts per ml, and the DNA-protoplast-PEG mixture was immediately plated onto R2YE agar plates. After 17 h (*S. levoris* A-9) or 20 to 22 h (*S. lividans* 1326) of incubation, regenerating protoplasts were overlaid with 3.0 ml of R2YE soft agar containing selecting levels of antibiotic.

Valinomycin assays. To identify valinomycin-producing bacteria, spores or mycelia were transferred by toothpick to TSB medium contained in wells of microdilution plates (Falcon). After 2 days of growth at 30°C, mycelia were overlaid with nutrient soft agar (Difco Laboratories) containing *Sarcina lutea* (Carolina Biologicals, Inc.) grown to log phase in LB medium. Valinomycin-producing bacteria generated a circular inhibitory zone or halo after 16 h of incubation at 30°C.

Bioautography was used to assay for intracellular valinomycin. Cultures of *Streptomyces* bacteria were grown for 5 days in 25 ml of TSB medium at 30°C. Unless otherwise specified, mycelium was collected and extracted with methanol (8 ml of methanol per g [wet weight] of mycelium) by vortexing for 1 min at room temperature. The methanol extract was filtered by using 3-mm Whatman paper, and samples were spotted on thin-layer chromatography plates (Schleicher & Schuell, Inc.) and chromatographed, using ethyl acetate as the liquid phase. The chromatogram was air dried and blotted for 90 min onto nutrient agar plates overlaid with nutrient soft agarose containing *Sarcina* bacteria and 0.7% agarose (SeaKem, Inc.) as the solid support medium. After overnight incubation at 30°C, antibiotic was detected by the appearance of a circular inhibitory zone or halo at the same R_f as authentic valinomycin (CalBiochem-Behring). Valinomycin concentration was determined by two methods: (i) high-performance liquid chromatography (HPLC) (Beckman Instruments, Inc.) using a reverse-phase microbore RP-8 5-μm analytical column (100 by 4.6 mm; Brownlee, Inc.) and measurement and integration of the valinomycin peak at a wavelength of 412 nm, and (ii) bioautography, by comparing the area of inhibition of diluted samples with a standard curve representing the log of known valinomycin quantities versus area of their inhibition zone. Individual colonies were assayed for valinomycin by transferring a sporulating colony into a 1.5-ml Eppendorf tube, extracting with 0.1 ml methanol, and subjecting the methanol extract to thin-layer chromatography.

Hybridization methods. Nick translation of plasmid DNAs and Southern blotting were performed by standard procedures (36, 39). In some experiments, DNA restriction fragments to be used as ^{32}P-labeled probes were purified from agarose gels by electroelution or by NaI-glass beads (Cleangene kit; Bio101, Inc.). Hybridizations were carried out in 5× SSC (0.75 M NaCl, 0.075 M sodium citrate, pH 7.0), 50% formamide, 1× Denhardt solution, and 10 mg of salmon testes DNA (Sigma Chemical Co.) per ml at 42 to 45°C; filters were washed four times with 2× SSC–sodium dodecyl sulfate 0.1% at 22°C and once with 0.1% SSC at 22 or 42°C.

Construction of *Streptomyces* plasmid gene library. To prepare donor DNA, 600 μg of high-molecular-weight A-9 chromosomal DNA was partially digested with *Sau*3A restriction endonuclease to yield DNA fragments ranging from 10 to 30 kb; fragments of this size were then purified by sucrose (10 to 40%) rate zonal centrifugation. The *Streptomyces* plasmid vector used was pIJ922, a SCP2* derivative that carries a thiostrepton resistance gene (*tsr*) derived from *Streptomyces azureus* and contains a unique *Bam*HI restriction site useful for cloning large DNA fragments generated by the *Bam*HI-*Bcl*I-*Bgl*II-*Sau*3A (*Mbo*I) family of restriction endonucleases (28, 30). This plasmid also has a moderate host range which successfully allowed introduction of pIJ922 into *S. levoris* A-9 by protoplast transformation; the plasmid was stably maintained in A-9 bacteria under drug selection at one to five copies per cell. In a typical shotgun cloning experiment, 1 μg of plasmid isolated from A-9 bacteria was digested to completion with *Bam*HI. The *Bam*HI termini of the plasmid molecules were dephosphorylated with calf intestine alkaline phosphatase (Boehringer Mannheim), using conditions recommended by the manufacturer. After several phenol and ethyl ether extractions to remove the phosphatase, the DNA was precipitated with ethanol, collected by centrifugation, and dissolved in TE buffer. Approximately 0.5 μg of 10- to 30-kb size-selected *Sau*3A fragments of A-9 DNA was added to 0.1 μg of *Bam*HI-cut,

dephosphorylated pIJ922 DNA (5:1 molar ratio of ends) in six replicated samples, and the DNAs were incubated with 1 μl of T4 DNA ligase in a volume of 15 μl for 16 h at 15°C. Afterward, the ligation reactions were pooled and the DNA was concentrated by ethanol precipitation. Ligated DNA equivalent to 0.1 μg of the vector was used to transform *vlm-1* and *vlm-2* valinomycin-deficient hosts of *S. levoris* A-9 and Thio^r transformants screened for valinomycin production (Vlm$^+$ complementation).

Construction of *E. coli* cosmid gene library. Donor DNA was prepared by digesting 600 μg of high-molecular-weight *S. levoris* A-9 chromosomal DNA to completion with *Eco*RI or *Sau*3A enzyme to yield DNA fragments ranging from 30 to 40 kb. Fragments of this size were isolated from both digests by sucrose (10 to 40%) rate zonal centrifugation, and the termini of the DNA molecules were dephosphorylated with calf intestine alkaline phosphatase enzyme under the conditions recommended by the manufacturer. These fragments were cloned in *E. coli* by using cosmid pHC79, which carries both unique *Bam*HI and *Eco*RI restriction cloning sites (10). In a typical experiment, 0.5 μg of pHC79 was digested to completion with either *Bam*HI or *Eco*RI enzyme and ligated to 7.0 μg of dephosphorylated 30- to 40-kb size-selected (*Sau*3A or *Eco*RI) DNA fragments (1:1 molar ratio of ends), using 1 μl of T4 DNA ligase in a volume of 15 μl for 16 h at 15°C. Afterward, 4 μl of ligated DNA was packaged in vitro into λ bacteriophage particles, using commercially prepared extracts (Stratagene, Inc.). Transduction was carried out according to conventional procedures, using *E. coli* ED8767 as the recipient host (35). Recombinants carrying relevant DNA were detected by probing Ap^r colonies with a mixture of ^{32}P-labeled 5.0-kb *Bgl*II-*Eco*RI (*vlm-2*$^+$) and 2.8-kb *Bam*HI (*vlm-5*$^+$) fragments of pVB6 or a mixture of two ^{32}P-labeled 8.0-kb *Bam*HI (*vlm-1*$^+$) fragments of pVB3, using hybridization methods of Grunstein and Hogness (8).

Physical mapping of *vlm* mutants. Plasmids pIJ702, pIJ922, pIJ922Δ_1, and pIJ486, isolated from *S. levoris* A-9, served as vectors to subclone restriction fragments containing one or more *vlm* alleles. To facilitate recovery of recombinant plasmids, *vlm*-containing restriction fragments were purified from agarose gels and ligated to the enzyme-cut, dephosphorylated vector. pVB7 was constructed by ligating the 4.3-kb *Pst*I fragment of pVB3 into *Pst*I-cut pIJ702. pVB9 was constructed by ligating the 7.5-kb *Pst*I-*Bgl*II fragment of pVB3 into *Pst*I-*Bgl*II-cut pIJ702. pVB10 was constructed by ligating the 8.0-kb *Bam*HI fragment of pVB3 into *Bam*HI-cut pIJ922Δ_1. pVB11 was isolated by ligating together the *vlm*$^+$, rep-containing 23-kb *Bgl*II and the *tsr*-containing 7.5-kb *Bgl*II fragments of pVB5. pVB13 was constructed by ligating the 9-kb *Bgl*II fragment of pVB5 into *Bam*HI-cut pIJ922Δ_1. pVB17 was constructed by dilute ligation of *Bam*HI-cut pVB6. The 8.7-kb *Bam*HI fragment of pVB6 was ligated into *Bam*HI-cut pIJ922Δ_1 to generate pVB16. The 2.8-kb fragment of pVB6 was ligated to *Bam*HI-cut pIJ486 to generate pVB28. pVB30 and pVB31 were constructed by first inserting the 9-kb *Bgl*II fragment of pVB6 into the *Bam*HI site of pIJ486 in both orientations and then cutting either plasmid with *Eco*RI and religating under dilute DNA concentrations. Minipreparations of the recombinant plasmids were used to transform *vlm* mutants and Thio^r transformants screened for the Vlm$^+$ phenotype.

RESULTS

Cloning of two independent sets of *vlm* biosynthetic genes. Our experimental strategy to isolate *vlm* biosynthetic genes was to prepare a gene library of *S. levoris* A-9 DNA, using the SCP2* plasmid derivative pIJ922 as a cloning vector, and then to screen for recombinant plasmids that complemented mutants of *S. levoris* A-9 blocked in valinomycin production. Two mutants, *S. levoris* 2-1 (*vlm-1 osm-1 sul-1*) and 5-2b (*vlm-2*) that contained the most widely separated of linked *vlm* mutations as judged by recombination frequencies (Guterman et al., unpublished data) were used as recipient cloning hosts to ensure that the entire *vlm* pathway was isolated. The primary method used to detect Vlm$^+$ colonies from the Vlm$^-$ library background was their ability to kill *Sarcina lutea* bacteria. Since variants of *S. levoris* A-9, which transiently overproduced another minor antibiotic (nonactin), appeared at a frequency of 0.5% when the strain underwent protoplast formation and regeneration, only Thio^r transformants from the library that cleared the *Sarcina* indicator in three or more successive experiments were analyzed further for plasmid content and valinomycin production by bioautography.

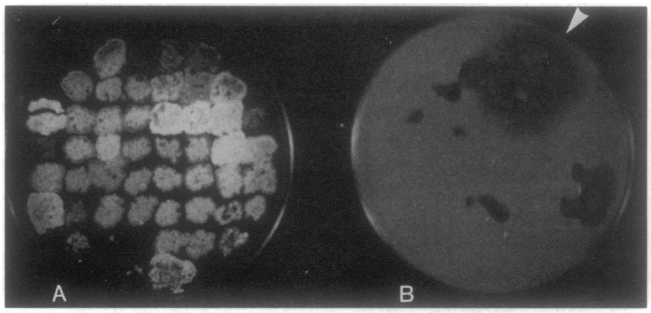

FIG. 1. Complementation test identifying a Vlm$^+$ recombinant colony from a gene library of *S. levoris* A-9 DNA in the *vlm-1* mutant strain. (A) Fifty random colonies from gene library grown on R2YE agar medium containing 50 μg of thiostrepton per μl. (B) Same colonies grown on TSB agar medium and then overlaid with indicator *Sarcina lutea* bacteria as described in Material and Methods; arrowhead indicates valinomycin killing halo generated by recombinant colony (clearing halo at bottom right is caused by a spontaneous variant of the *vlm-1* mutant strain that overproduces nonactin antibiotic).

Using this criterion, three *vlm-1*$^+$-complementing and two *vlm-2*$^+$-complementing Thio^r transformants were detected from three gene libraries prepared by using the shotgun procedures described in Materials and Methods. The *Sarcina*-killing phenotype used to identify one Vlm$^+$ Thio^r recombinant transformant is shown in Fig. 1. The production of valinomycin by mycelia of each Thio^r Vlm$^+$ transformant was subsequently confirmed by bioautography (data not shown). Plasmids pVB1, pVB2, and pVB3 isolated from *vlm-1*$^+$ colonies contained inserts of 11, 13, and 24 kb, respectively, whereas, *vlm-2*$^+$ colonies contained plasmids pVB5 and pVB6 with inserts of 16.5 and 16.8 kb, respectively. The *vlm*$^+$ inserts of pVB3 and pVB6 were shown to be derived from a single continuous segment of the A-9 chromosome by probing chromosomal restriction digest with ^{32}P-nicked translated pVB3 and pVB6 (data not shown). Regions of DNA identity within the *vlm-1*$^+$ and *vlm-2*$^+$ sets of cloned DNA were revealed by restriction endonuclease mapping and plasmid cross-hybridization experiments. The *vlm-1*$^+$ DNAs contained a single region in common covering approximately 5.5 kb (Fig. 2A); both *vlm-2*$^+$ DNA inserts were virtually identical across their lengths (Fig. 2B). No homology between DNAs containing *vlm-1*$^+$ and *vlm-2*$^+$ was detected. Thus, the cloned DNA containing these genes

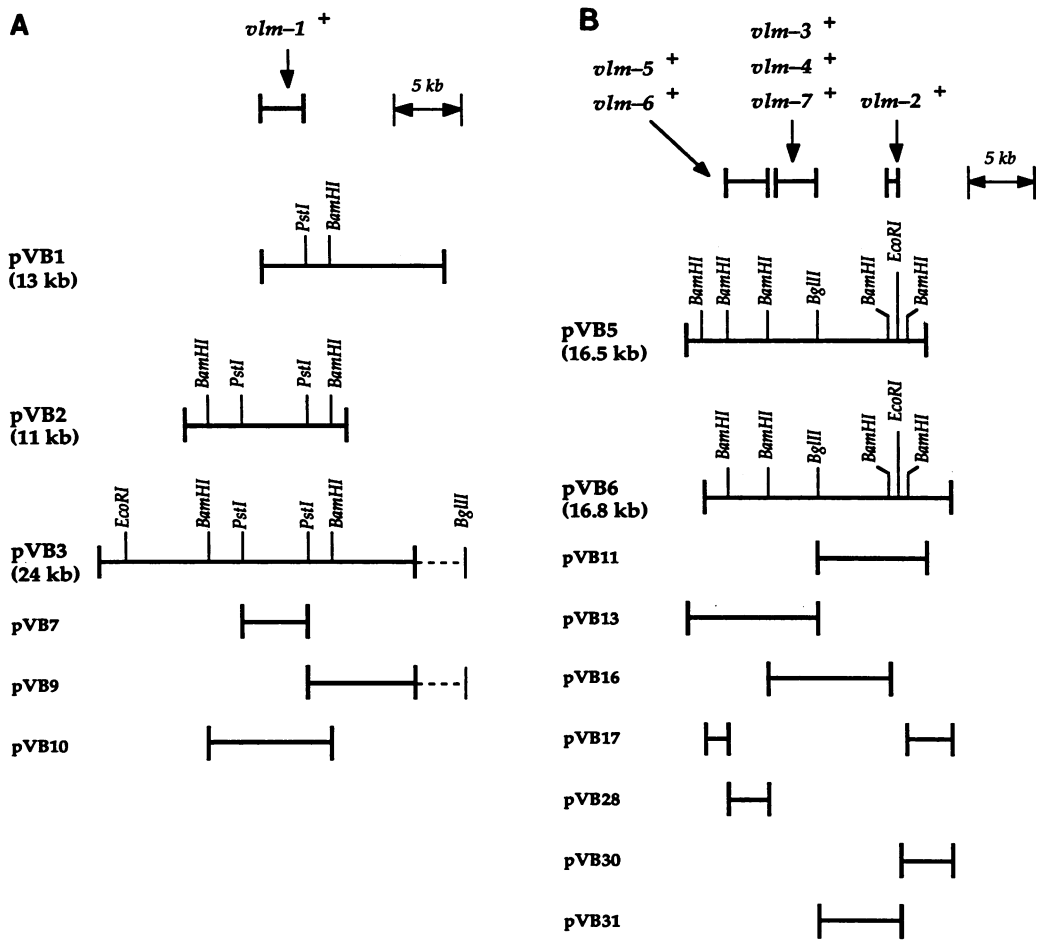

FIG. 2. Restriction endonuclease maps of two valinomycin gene clusters and location of *vlm* alleles. Recombinant plasmids pVB1-3 (A) and pVB5 and pVB6 (B) were recovered from gene library by complementation to either *vlm-1* or *vlm-2* mutant strains, respectively. Plasmid subclones, generated from segments of either pVB3 or pVB5 and pVB6 insert DNA as described in Material and Methods, are indicated below each set of restriction maps. Dashed line represents adjacent vector sequences. Locations of *vlm* alleles, indicated above the restriction maps, were determined by testing plasmid subclones for the ability to restore valinomycin production in each *vlm* mutant strain (Table 2); the position of *vlm-8*$^+$ could not be accurately obtained and is not indicated.

originated from nonoverlapping segments of the *S. levoris* A-9 chromosome.

To examine whether the cloned DNA encoded additional *vlm* genes, the *vlm-1*$^+$ and *vlm-2*$^+$ plasmids were introduced into additional *vlm*-blocked mutants of *S. levoris* A-9 by transformation, and Thio^r transformants were analyzed for the Vlm$^+$ phenotype. The cloned DNAs restored valinomycin production to all eight *vlm* mutants tested (Table 2). Plasmids pVB1 to pVB3 complemented only one mutant *vlm-1*, the original cloning host. However, both pVB5 and pVB6 restored valinomycin production to the remaining seven mutants, *vlm-2* through *vlm-8*, suggesting that the bulk of the genes essential for valinomycin biosynthesis were contained in these two plasmids. The Vlm$^+$ phenotype of *vlm-8* transformants deserves comment. Unlike the other six *vlm* mutants, *vlm-8* isolates containing pVB5 and pVB6 did not produce wild-type levels of antibiotic, but instead these colonies exhibited a variety of different-size halos on *Sarcina*-containing agar plates. This variability of valinomycin production was confirmed by bioautography. The most likely explanation of these results was that the cloned DNAs of pVB5 and pVB6 contained only a portion of the gene defined by the *vlm-8*$^+$ allele and hence the Vlm$^+$ phenotype was being restored by marker rescue recombination instead of by complementation (31).

Physical mapping of *vlm* alleles. To map the various *vlm* mutations on the cloned DNAs, complementation-marker rescue experiments were performed with subcloned DNAs. Figure 2 shows the various restriction fragments that were subcloned into pIJ702, pIJ486 or pIJ922Δ_1 (a spontaneous 7-kb deletion derivative of pIJ922) by procedures described in Materials and Methods. The *vlm* mutations were assigned map positions by transforming each subclone into the *vlm* mutants and testing Thio^r transformants for valinomycin production, using bioautography. The *vlm-1*$^+$ complementation activity was localized to a 2.5-kb region within the 4.3-kb *Pst*I fragment of pVB3 (Fig. 2A and Table 2). This gene, therefore, must encode a protein of no more than 90,000 Da according to standard calculations.

Mapping the seven *vlm* alleles within pVB5 and pVB6 cloned DNA was more complicated, involving the construction of nine subclones which together subdivided the cloned DNA into seven sectors (Fig. 2B and Table 2). Results indicated that six of seven *vlm* alleles mapped to three restriction fragments covering a 12-kb region: *vlm-5*$^+$ and *vlm-6*$^+$ to the 2.8-kb *Bam*HI fragment; *vlm-3*$^+$, *vlm-4*$^+$, and

TABLE 2. Complementation or marker rescue recombination activity of *vlm* mutants by plasmids containing primary cloned DNA or subcloned restriction fragments

Plasmid[a]	Valinomycin production by transformants containing donor plasmid DNA[b]							
	vlm-1	*vlm-2*	*vlm-3*	*vlm-4*	*vlm-5*	*vlm-6*	*vlm-7*	*vlm-8*
pVB1	+	—	—	—	—	ND	—	—
pVB2	+	—	—	—	—	ND	—	—
pVB3	+	—	—	—	—	—	—	—
pVB5	—	+	+	+	+	+	+	+/−
pVB6	—	+	+	+	+	ND	+	+/−
pVB7	+	ND	ND	ND	ND	ND	ND	ND
pVB9	—	ND	ND	ND	ND	ND	ND	ND
pVB10	+	ND	ND	ND	ND	ND	ND	ND
pVB11	ND	+/−	—	—	—	—	—	—
pVB13	ND	—	+/−	+/−	+	+/−	+/−	+/−
pVB16	ND	—	+/−	+/−	—	—	+/−	—
pVB17	ND	—	—	—	—	ND	—	—
pVB28	ND	—	—	—	+	+/−	—	—
pVB30	ND	−	ND	ND	ND	ND	ND	ND
pVB31	ND	+/−	ND	ND	ND	ND	ND	ND

[a] See Fig. 2 for structure of vlm^+ cloned DNA.

[b] Plasmids isolated from *S. levoris* A-9 were transformed into protoplasts of the indicated mutants. Ability to produce valinomycin was determined by two methods: scoring at least 24 $Thio^r$ transformants from each experiment for valinomycin halo by using plate bioassay and detection of authentic valinomycin in individual $Thio^r$ Vlm^+ transformants by bioautography (see Materials and Methods). +, Wild-type production of valinomycin; +/−, variable production of valinomycin; —, no production of valinomycin; ND, not determined.

vlm-7$^+$ to the 4.0-kb *Bam*HI-*Bgl*II fragment; and *vlm-2*$^+$ to a 0.45-kb segment within the 0.9-kb *Bam*HI fragment. A tentative identification of gene transcription units was possible by determining whether subclone-containing transformants produced wild-type levels of antibiotic (complementation) or whether transformants produced variable levels of antibiotic (marker rescue recombination; 31). On the basis of this analysis, five *vlm* alleles appeared to be organized in one large gene or transcriptional unit of approximately 9 kb (*vlm-2*$^+$, *vlm-3*$^+$, *vlm-4*$^+$, *vlm-6*$^+$, and *vlm-7*$^+$). A sixth *vlm* allele, *vlm-5*$^+$, appeared to be located in a separate gene or transcriptional unit beginning on the 2.8-kb *Bam*HI fragment, since introduction of this *Bam*HI fragment into the *vlm* mutant gave wild-type levels of valinomycin (Fig. 2B and Table 2). The *vlm-8*$^+$ allele, which may represent a truncated third gene or transcription unit, was more difficult to map. The *vlm-8*$^+$ activity was detected on a 9.0-kb *Bgl*II fragment representing the left half of the cloned DNA. Subcloning smaller *Bam*HI fragments within this region completely abolished Vlm^+ activity, suggesting that this allele mapped very close to one of two *Bam*HI sites located at the left end of the insert.

Isolation of *S. levoris* A-9 DNA linking *vlm-1*$^+$ and *vlm-2*$^+$ to *vlm-8*$^+$ loci by chromosomal walking. To join physically the cloned *vlm-1*$^+$ and *vlm-2*$^+$ to *vlm-8*$^+$ DNAs, two cosmid gene libraries were prepared by using different restriction enzymes as described in Materials and Methods. One library consisted of 30- to 40-kb size-selected partial *Sau*3A fragments of *S. levoris* A-9 DNA, which were inserted into the *Bam*HI site of pHC79. The second library contained *Eco*RI fragments of *S. levoris* A-9 DNA. Since both *vlm-1*$^+$ and *vlm-2*$^+$ to *vlm-8*$^+$ sets of cloned DNA contained single *Eco*RI sites (Fig. 2) and Southern chromosomal hybridization experiments revealed that *Eco*RI fragments extending in either direction from the *vlm-2*$^+$ to *vlm-8*$^+$ loci and in one direction from the *vlm-1*$^+$ locus were between 30 and 40 kb in size (data not shown), it was possible to clone these fragments from a cosmid gene library containing fragments generated by a complete *Eco*RI digest of *S. levoris* A-9 DNA. Recombinant plasmids were identified by colony hybridization, using ^{32}P-labeled fragment isolated from either end of pVB3 (*vlm-1*$^+$) or pVB6 (*vlm-2*$^+$ to *vlm-8*$^+$). The structure of cloned DNA from several recombinant plasmids was determined by probing *Bam*HI and *Eco*RI digestion products with either a *vlm-2*$^+$-specific 5.0-kb *Bgl*II-*Eco*RI fragment, *vlm-5*$^+$-specific 2.8-kb *Bam*HI, or a *vlm-1*$^+$-specific 4.3-kb *Pst*I fragment. The results revealed that the positive Ap^r colonies contained plasmid with one or the other end (or both) of existing *vlm* cloned DNA together with an extension of additional chromosomal DNA, terminating at either an *Eco*RI or a *Sau*3A site. Additional Southern hybridization experiments showed that the cloned DNAs were colinear with the A-9 chromosome, a finding that indicated that no sequences were rearranged during cloning (data not shown). Preliminary physical maps of cloned DNA from several recombinant cosmids, positioned relative to previously cloned DNA, are depicted in Fig. 3.

Several of the recombinant cosmids were analyzed to determine whether *vlm-1*$^+$ DNA was linked to *vlm-2*$^+$ to *vlm-8*$^+$ DNA. Plasmid pVB32 contained a 40-kb *Eco*RI insert originating in *vlm-2*$^+$ to *vlm-8*$^+$ DNA that extended leftward approximately 25 kb from the end of pVB6. Plasmids pVB37 and pVB38, isolated from the *Sau*3A cosmid library, contain a 35-kb insert that originated in *vlm-1*$^+$ DNA and extended rightward 20 kb beyond the end of pVB3. Since these DNAs contained *Eco*RI sites near the end of the right-end inserts, we speculated that the distal *Eco*RI sites of pVB32 and of pVB37 and pVB38 cloned DNAs were the same. To test this, the 40-kb *Eco*RI fragment was purified, labeled with ^{32}P, and used to probe *Bam*HI digests of these two *Sau*3A-generated cosmids. Radioactive bands were detected with only these *vlm-1*$^+$ cosmids (Fig. 4A). Conversely, two *vlm-2*$^+$-containing cosmids from the *Sau*3A cosmid library, pVB35 and pVB36, were also found to share sequences with *vlm-1*$^+$ pVB37 and pVB38 by using the 6.6-kb *Eco*RI fragment of pVB37 as a probe (Fig. 4B). Similar results were obtained by probing *Eco*RI-digested cosmid DNAs (data not shown). These data were used to generate an *Eco*RI restriction enzyme map of the *S. levoris* A-9 chromosome surrounding the *vlm* loci as depicted in Fig. 3. We calculate that approximately 120 kb of overlapping chromosomal DNA was recovered from the cosmid banks and that the distance between *vlm-1*$^+$ and *vlm-2*$^+$ is approximately 70 kb.

***vlm* DNA is partially homologous to DNA in other valinomycin-producing *Streptomyces* species.** Valinomycin is produced by several *Streptomyces* species, including *S. tsusimaensis* and *Streptomyces* sp. strain ATCC 23836. To determine whether the DNA cloned from *S. levoris* A-9 was unique to valinomycin producers, we hybridized the cloned DNA of *S. levoris* A-9 to chromosomal DNA isolated from both valinomycin-producing and nonproducing *Streptomyces* species. Chromosomal DNA was isolated from the valinomycin-producing strains *S. tsusimaensis* and *Streptomyces* sp. strain ATCC 23836 and the nonproducing strains *S. lividans* 1326, *S. coelicolor*, and *S. griseus* and digested with *Bam*HI enzyme; the fragments were separated by electrophoresis and transferred to nitrocellulose filters, and the filters were hybridized under stringent conditions, using a mixed probe of ^{32}P-labeled *vlm-5*$^+$-specific (2.8-kb *Bam*HI) and *vlm-2*$^+$-specific (5.0-kb *Bgl*II-*Eco*RI) DNA fragments. The *vlm-2*$^+$–*vlm-5*$^+$ probe hybridized strongly to only DNA

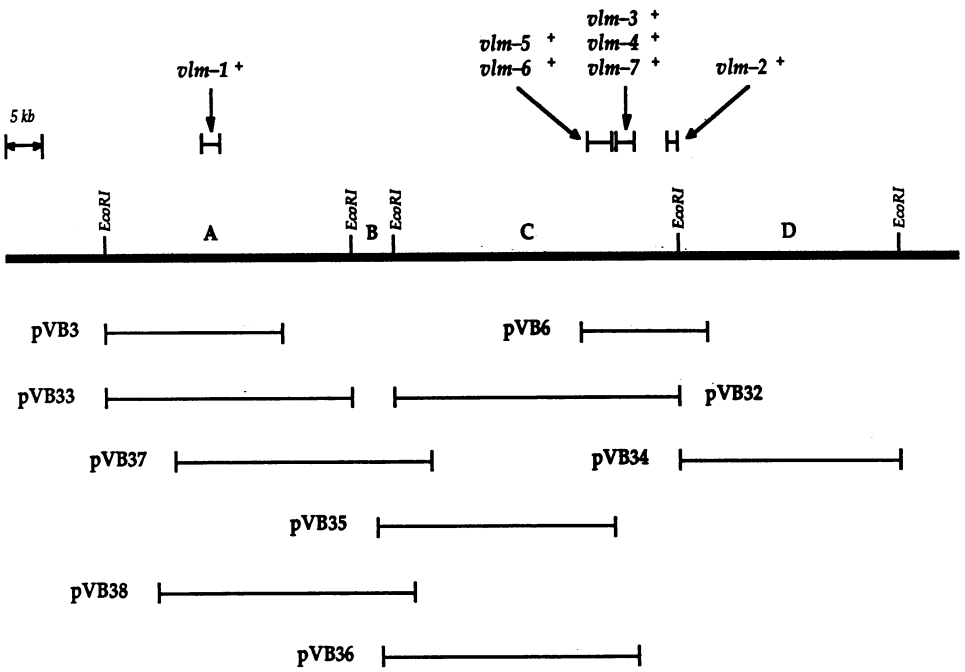

FIG. 3. Chromosomal map of *vlm*$^+$ gene clusters. The top line represents the chromosome of *S. levoris* A-9, showing the tentative order of *Eco*RI restriction fragments: A, 34 kb; B, 6.6 kb; C, 40 kb; D, 30 kb. Positions of primary *vlm* recombinant plasmids and recombinant cosmids are indicated below the chromosomal map. Positions of *vlm* alleles are indicated above the chromosomal map. The distance between *vlm-1*$^+$ and *vlm-2*$^+$ is approximately 70 kb.

fragments of valinomycin producers *S. tsusimaensis* and *Streptomyces* sp. strain ATCC 23836 (Fig. 5A). The fingerprint patterns of several labeled fragments, however, differed somewhat from the *S. levoris* A-9 DNA control. For example, in the *S. levoris* A-9 control, the probe hybridized to three *Bam*HI fragments: 8.7, 2.8, and 0.9 kb. The *Bam*HI digest of *S. tsusimaensis* also contained labeled fragments of 8.7- and 2.8-kb but was missing the 0.9-kb fragment; instead, a new band appeared at 8 kb. The ATCC 23836 DNA digest contained only the 2.8-kb *Bam*HI fragment and a new band of 20 kb. Assuming that the changes in fragment patterns were due to loss of *Bam*HI-cut sites, the restriction maps were prepared and aligned to pVB6 fragment map to identify regions of homology (Fig. 6). This comparison revealed that *Bam*HI sites were conserved everywhere except the region surrounding the left end, in the vicinity of *vlm-2*$^+$. Similar results were obtained when *Eco*RI enzyme was used.

These hybridization experiments were repeated with *vlm-1*$^+$-specific 4.3-kb *Pst*I fragment used as a probe (Fig. 5B). Surprisingly, this *vlm-1*$^+$ probe hybridized to DNAs from both valinomycin producers and nonproducers, generating in many cases different fingerprint patterns of labeled *Bam*HI fragments, suggesting that the *vlm-1*$^+$ gene product may not be uniquely involved in valinomycin biosynthesis. Similar results were obtained by using smaller *Sma*I fragments (1.7 and 0.7 kb) contained within the 2.5-kb *vlm-1*$^+$ region, making it unlikely that hybridization was due to non-*vlm* sequences carried on the 4.3-kb *Pst*I probe (data not shown).

Since certain DNA sequences were conserved only among valinomycin-producing *Streptomyces* species, it was possible to obtain a rough estimate of the location of *vlm*-specific genes within the 120-kb region cloned in the cosmids by probing chromosomal digests with the larger *Eco*RI cloned fragments (40, 30, 34, and 6.6 kb) of *S. levoris* A-9 DNA and

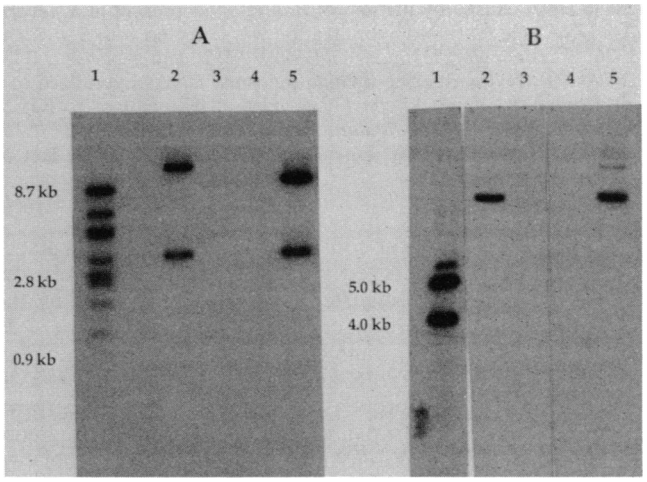

FIG. 4. Physical linkage of *vlm-1*$^+$- and *vlm-2*$^+$-containing cosmids by Southern hybridization. *Sau*3A-generated (1 μg) cosmid DNAs and *S. levoris* A-9 chromosomal DNA (10 μg) were digested with *Bam*HI; the products were transferred to nylon filters, after electrophoresis in 0.6% agarose, and then incubated with denatured ^{32}P-labeled nick-translated restriction fragments. (A) *vlm-1*$^+$ cosmid DNAs hybridized with a ^{32}P-labeled 40-kb *Eco*RI fragment of pVB32. Lanes: 1, A-9 chromosomal DNA; 2 and 5, pVB37 and pVB38, respectively, containing *vlm-1*$^+$ and flanking sequences extending rightward; 3 and 4, two additional *vlm-1*$^+$ cosmids containing nonoverlapping sequences. (B) *vlm-2*$^+$ cosmid DNAs hybridized with a ^{32}P-labeled 6.6-kb *Eco*RI fragment of cosmid pVB37. Lanes: 1, A-9 chromosomal DNA; 2 and 5, pVB35 and pVB36, respectively, containing *vlm-2*$^+$ through *vlm-8*$^+$ and flanking sequences extending leftward; 3 and 4, two additional *vlm-2*$^+$ cosmids containing nonoverlapping sequences. Structures of cosmid DNAs are diagrammed in Fig. 3. Phage λ *Hin*dIII standards were used to determine molecular weights of detected fragments.

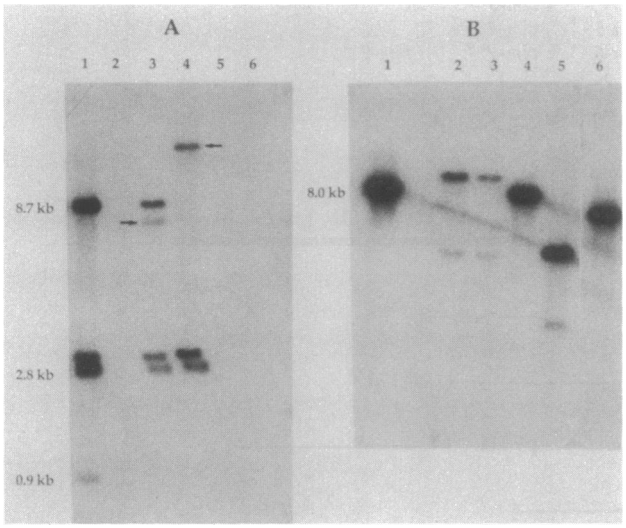

FIG. 5. Determination of conserved chromosomal restriction fragments among valinomycin-producing and nonproducing *Streptomyces* species by Southern hybridization. (A) Nylon filter containing *Bam*HI-digested chromosomal DNA (10 μg) isolated from valinomycin producers *S. levoris* A-9 (lane 1), *S. tsusimaensis* (lane 3), and *Streptomyces* sp. strain ATCC 23836 (lane 4) and from nonproducers *S. lividans* 1326 (lane 2), *S. coelicolor* A332 (lane 5), and *S. griseus* 3404 (lane 6) hybridized to a mixture of ^{32}P-labeled 2.8-kb *Bam*HI (*vlm-5*$^+$) and 5.0-kb *Bgl*II-*Eco*RI (*vlm-2*$^+$) restriction fragments of pVB6. The doublet band of 2.8 kb is an artifact caused by slippage of the filter during Southern blotting. Arrows indicate the appearance of new labeled bands in *S. tsusimaensis* and ATCC 23836 DNA caused by the loss of *Bam*HI sites present in *S. levoris* A-9 DNA (see text). No hybridization was detected with *S. lividans* 1326, *S. coelicolor* 1326, *S. coelicolor* A332, and *S. griseus* 3404 DNAs. (B) Nylon filter containing the same DNAs hybridized to a ^{32}P-labeled 4.3-kb *Pst*I (*vlm-1*$^+$) restriction fragment of pVB3: *S. levoris* A-9 (lane 1), *S. lividans* 1326 (lane 2), *S. coelicolor* A332 (lane 3), *S. tsusimaensis* (lane 4), ATCC 23836 (lane 5), and *S. griseus* 3404 (lane 6).

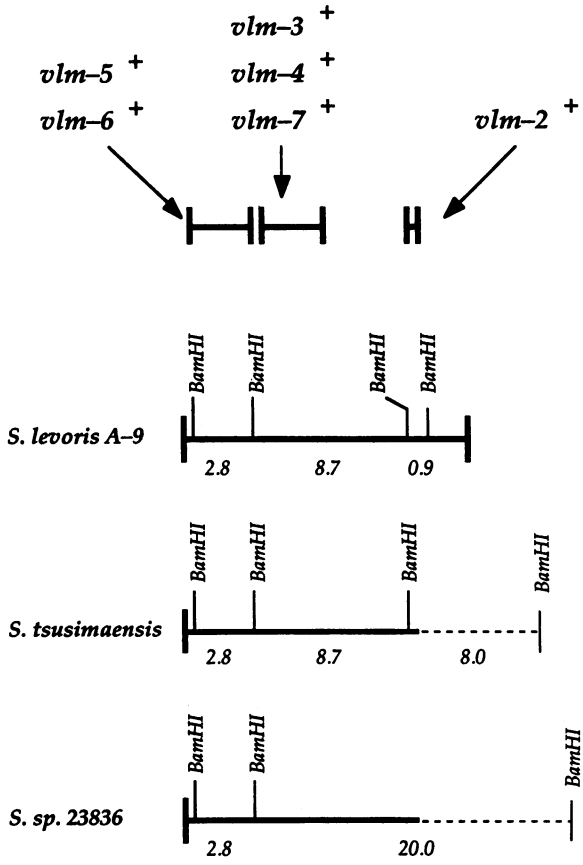

FIG. 6. Restriction endonuclease maps of conserved DNA regions in the vicinity of the *vlm-2*$^+$ gene cluster among valinomycin producers *S. levoris* A-9, *S. tsusimaensis*, and ATCC 23836. Regions that have either retained or lost *Bam*HI sites are indicated by solid or dashed lines, respectively, as determined by Southern hybridization (Fig. 5); molecular weights of restriction fragments are indicated in kilobase pairs. Position of alleles *vlm-2*$^+$ through *vlm-7*$^+$ of *S. levoris* A-9 are indicated above the restriction maps.

assessing the number of conserved restriction fragments. A high number of conserved restriction fragments were detected when *Bam*HI-digested chromosomal DNA of the three valinomycin producers was probed with the ^{32}P-labeled 40-kb *Eco*RI fragment (Table 3). Both the *vlm-1*$^+$-containing 34-kb *Eco*RI and the 30-kb *Eco*RI (located to the right of *vlm-2*$^+$) probes produced diverging fingerprint patterns, whereas, surprisingly, the 6.6-kb *Eco*RI fragment of *S. levoris* A-9 failed to hybridize to DNA of the other two valinomycin producers. These results indicate that the genes for the entire valinomycin biosynthetic pathway are contained on the 40-kb *Eco*RI fragment and raise questions as to the role of the *vlm-1*$^+$ gene in valinomycin biosynthesis.

***vlm-2*$^+$ to *vlm-8*$^+$ cloned DNA enhances valinomycin production in *S. levoris* A-9.** Amplification of cloned *Streptomyces* antibiotic biosynthetic genes has resulted, in some cases, in a concomitant increase in antibiotic production (6, 12). We first sought to determine whether the *vlm* cloned DNA on the pIJ922 replicon would also stimulate valinomycin production when introduced into the *S. levoris* A-9 host. In 25-ml shake flask cultures, bacteria containing pIJ922 produced 6 to 16 μg of valinomycin per (wet weight) of mycelia, as determined by quantitative bioautography and reverse-phase HPLC (see Materials and Methods). Assuming that the *vlm* cloned DNA in pVB3, pVB5, and pVB6 was amplified no more than one to five copies per cell based on the copy number of pIJ922 (27), we could except plasmid-containing mycelia to yield at least two- to sixfold more valinomycin. Plasmid DNAs of pVB3 (*vlm-1*$^+$) and pVB5 and pVB6 (*vlm-2*$^+$ to *vlm-8*$^+$) were isolated from mutant hosts and transformed into *S. levoris* A-9 protoplasts, selecting for Thio^r. Mycelia of several transformants harboring pVB5 or pVB6 contained 4- to 10-fold more valinomycin than did mycelia harboring pIJ922 (Table 4), a finding that suggested that the *vlm-2*$^+$ to *vlm-8*$^+$ cloned DNA carried a gene(s) whose product is rate limiting for valinomycin synthesis; no enhancement of valinomycin was detected with pVB3-containing A-9 transformants (data not shown).

DISCUSSION

The synthesis of valinomycin is likely to be mediated by a multienzyme complex consisting of several *thio* (—SH) template-containing polypeptides ranging in molecular size from 100 to 440 kDa. We have demonstrated by several independent tests that genes encoding these large polypeptides are likely contained within a 120-kb region of *Streptomyces* DNA that has been cloned on a series of overlapping DNA fragments. When the cloned DNA was transformed into several independent *vlm* mutants, valinomycin produc-

TABLE 3. Determination of the approximate boundaries of the *vlm* gene cluster by chromosomal Southern analysis of valinomycin-producing and -nonproducing *Streptomyces* species

Plasmid, probe	No. of hybridizing fragments[a]					
	S. levoris A-9	*S. lividans*	*S. coelicolor*	*S. tsusimaensis*	*Streptomyces* sp. strain ATCC 23836	*S. griseus*
pVB34, 30-kb *Eco*RI	8	3 (0/8)	3 (0/8)	7 (0/8)	6 (0/8)	6 (0/8)
pVB32, 40-kb *Eco*RI	10	—	—	6 (5/10)	4 (2/10)	—
pVB37, 6.6-kb *Eco*RI	2	—	—	—	—	—
pVB33, 34-kb *Eco*RI	7	6 (0/7)	5 (0/7)	6 (2/7)	7 (1/7)	4 (0/7)

[a] Number of *Bam*HI restriction fragments that strongly hybridized to the specified probe. Shown in parentheses is the fraction of labeled restriction fragments with the same approximate size as labeled *S. levoris* A-9 fragments; —, no significant hybridization.

tion was restored in all mutants either by complementation or by marker rescue recombination. Moreover, it also appeared that the bulk of the *vlm* biosynthetic genes (representing seven of eight *vlm* alleles) were contained within a 12-kb region, since this DNA could enhance valinomycin production when transformed into a wild-type host and since subcloned fragments from this region cross-hybridized only to chromosomal DNA isolated from valinomycin-producing *Streptomyces* species.

Although we were unable to determine the exact boundaries of all of the biosynthetic genes necessary for valinomycin synthesis, some preliminary information concerning the number, size, and organization of several *vlm* genes or transcription units could be deduced. Results from a complementation (functional) study of subcloned restriction fragments indicated that the 12-kb region carried by pVB5 and pVB6 contained at least three genes: a 9-kb "gene" represented by alleles *vlm-2*$^+$, *vlm-3*$^+$, *vlm-4*$^+$, *vlm-6*$^+$, and *vlm-7*$^+$, a gene of less than 2.8 kb represented by *vlm-5*$^+$, and a gene of unknown size represented by *vlm-8*$^+$. Although it is formally possible that the 9-kb "gene" really represents a transcription unit composed of more than one gene, we believe that this is not likely, since this putative gene would encode a polypeptide of approximately 300,000 Da, well within the predicted size range of known biosynthetic enzymes involved in peptide antibiotic synthesis.

What also remains unclear is the function of the *vlm-1*$^+$ gene. We were intrigued to discover the *vlm-1*$^+$ mapped 50 to 70 kb from the main body of all other *vlm* alleles. It is unlikely that *vlm-1* defines a second locus of valinomycin-specific biosynthetic genes, since results of cross-hybridization experiments showed that DNA within and surrounding this locus was present in both valinomycin-producing and nonproducing *Streptomyces* species. Therefore, we speculate that *vlm-1*$^+$ does not encode a peptide in the multienzyme complex but instead encodes either a regulatory protein or an enzyme involved in the synthesis of a component required for valinomycin synthesis. Further analysis of DNA encoding *vlm-1*$^+$ and the *vlm* biosynthetic genes should add to the understanding of peptide antibiotic gene organization and regulation.

TABLE 4. Enhancement of valinomycin production by *vlm*$^+$-containing plasmids pVB5 and pVB6 in *S. levoris* A-9

Strain	Relevant genotype	Valinomycin (mg/liter)[a]	
		HPLC	Bioassay
A-9(pIJ922)	*vlm*$^+$	6	16
A-9(pVB5)	*vlm*$^+$/*vlm*$^+$	59	86
A-9(pVB6)	*vlm*$^+$/*vlm*$^+$	50	70

[a] Values are averages for duplicate 25-ml TSB cultures grown for 48 h in the presence of thiostrepton (25 μg/ml). Mycelia were collected and extracted with methanol (5 ml) and analyzed for valinomycin by HPLC or bioassay as described in Materials and Methods.

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