APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1996, p. 570–577 Vol. 61, No. 2
0099-2240/96/$04.00+0

The Peptide Synthetase Gene *phsA* from *Streptomyces viridochromogenes* Is Not Juxtaposed with Other Genes Involved in Nonribosomal Biosynthesis of Peptides

D. SCHWARTZ,[1] R. ALIJAH,[2] B. NUßBAUMER,[2] S. PELZER,[1] AND W. WOHLLEBEN[1,2]*

Mikrobiologie/Biotechnologie, Eberhard-Karls-Universität Tübingen, D-72076 Tübingen,[1] and Genetik, Universität Bielefeld, D-33501 Bielefeld,[2] Germany

Received 9 May 1995/Accepted 6 December 1995

By complementation of a previously described non-phosphinothricin tripeptide (PTT)-producing mutant, NTG1, which is blocked in nonribosomal synthesis of the peptide, a DNA fragment including the putative peptide synthetase gene *phsA* was isolated (W. Wohlleben, R. Alijah, J. Dorendorf, D. Hillemann, B. Nußbaumer, and S. Pelzer, Gene 115:127–132, 1992). Sequence analysis of *phsA* revealed that it encodes a protein of 622 amino acids with regions which are highly similar to core motifs characteristic for peptide synthetases. PhsA represents one functional domain of a peptide synthetase which is necessary for activation and condensation of one amino acid, probably *N*-acetyl-demethyl-phosphinothricin. With regard to the arrangement of the flanking genes, *phsA* is the first peptide synthetase gene which is not in the direct neighborhood of additional peptide synthetase genes involved in the formation of peptide antibiotics. Gene disruption mutants with internal fragments of *phsA* subcloned in temperature-sensitive pGM vectors were generated. Integration occurred either into the chromosomal copy of *phsA* or into a gene outside the known *phsA* locus, resulting in two classes of non-PTT-producing mutants. In cofeeding experiments the former *phsA* mutants showed the same phenotype as did NTG1, which confirmed participation of *phsA* in nonribosomal synthesis of PTT. A truncated *phsA* gene was overexpressed in *Escherichia coli*, and the resulting protein of 593 amino acids was purified for raising antibodies. By performing immunoblotting experiments, the expression of *phsA* could be detected in *Streptomyces viridochromogenes* Tü494 in the stationary-growth phase after 4 days of incubation.

The structurally identical peptide antibiotics phosphinothricin tripeptide (PTT) and bialaphos are produced in *Streptomyces viridochromogenes* (4) and *Streptomyces hygroscopicus* (21), respectively. PTT consists of two molecules of L-alanine and one molecule of the unusual amino acid phosphinothricin (PT). The bioactive compound PT shows bactericidal, fungicidal, and herbicidal properties. In agriculture, Basta (the ammonium salt of PT) and Herbiace (the tripeptide bialaphos) are used as broad-spectrum herbicides. Basta-resistant transgenic plants were generated with the *S. viridochromogenes* PTT resistance gene (*pat*) (48, 51), which is part of the PTT biosynthetic cluster.

Two features make the study of PTT biosynthesis an interesting subject: first, it possesses a C—P—C bond, which is very rare in natural compounds, and second, its three amino acids are linked nonribosomally. The nonribosomal synthesis of peptide antibiotics is catalyzed by multifunctional peptide synthetases (for a review, see reference 27) by the thiol template mechanism (26, 50). The main steps of this process are activating of substrate amino acids as aminoacyl-adenylates, binding of the activated amino acids covalently to the protein template in the form of thioesters, and through the action of the covalentely attached 4′-phosphopantetheine, condensing the amino acids to the reaction product. Peptide synthetases are usually multifunctional enzymes consisting of several amino acid-activating domains. One domain has a size of 600 to 700 amino acids. Each domain shows highly conserved core regions of 3 to 10 amino acid residues which are essential for amino acid activation, thioester formation, and condensation (27). Large peptide synthetases, for example, gramicidin S synthetase B, which activate and condense two and more amino acids, consist of several activating domains interrupted by spacer regions of approximately 500 amino acids (47).

The biosynthesis of bialaphos in *S. hygroscopicus* has been investigated by analysis of intermediates accumulated and converted by non-PTT-producing mutants. Biosynthetic genes and a resistance gene were isolated and cloned. Various genes of the biosynthetic cluster were mapped (14, 35). At least five genes which are supposed to be involved in the tripeptide-forming alanylation step were identified (13).

Comparison of the organization of the biosynthetic genes in *S. hygroscopicus* and *S. viridochromogenes* resulted in the conclusion that the pathways and the arrangements of the genes are very similar in both strains (14, 52).

Non-PTT producing mutants of *S. viridochromogenes* Tü494 were generated by treatment with UV and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (1). The mutant NTG1 was blocked in the alanylation step (1). By complementation experiments, a DNA fragment containing a putative peptide synthetase gene, *phsA*, was isolated: *phsA* restored PTT production to NTG1 (52).

In this paper, we report the sequence of the *phsA* gene, the analysis of its deduced product, gene disruption experiments with *phsA*, and an optimized integration protocol with temper-

* Corresponding author. Mailing address: Mikrobiologie/Biotechnologie, Eberhard-Karls-Universität Tübingen, Auf der Morgenstelle 28, D-72076 Tübingen, Germany. Phone: 49 7071 29-6944. Fax: 49 7071 29-4634. Electronic mail address: wowo@molbio.biol.biologie.uni-tuebingen.de.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Source or reference
S. viridochromogenes		
Tü494	PTT-producing wild type	H. Zähner (4)
NTG1	Non-PTT-producing, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutant	1
SP91	Integration of pSP91 in *phsA*, non-PTT producing	This study
SP92	Integration of pSP92 in *phsA*, non-PTT producing	This study
SP62/1	Integration of pSP62 in *phsA*, non-PTT producing	This study
SP62/2	Integration of pSP62 outside *phsA*, non-PTT producing	This study
E. coli XL1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F' *proAB lacI*^{q}*ZΔM15* Tn*10*(Tet^r)]	6
Plasmids		
pK19	*aphII*	34
pUC18	*bla*	49
pDS1	1.9-kb *Xmn*I-*Pma*C1 fragment of 4-kb *Bam*HI fragment (1), containing *phsA*, cloned in the *Sma*I site of pUC18	This study
pDS2	pK19 containing 1.8-kb *Sal*I-*Bam*HI fragment of pDS1 (1.7-kb fragment of *phsA*)	This study
pQE30	Expression vector	Qiagen
pDS3	pQE30 containing a 1.8-kb *Sph*I-*Kpn*I fragment of pDS2 (1.7-kb fragment of *phsA*)	This study
pDS4	pQE30 containing a 1.9-kb *Eco*RI-*Hin*dIII fragment of pDS1 (*phsA*)	This study
pGM9	*tsr* (thiostrepton resistance gene), temperature-sensitive *Streptomyces* vector	31
pSP91	pGM9 containing an internal 1.1-kb *Pst*I-*Bgl*II fragment of *phsA*	This study
pSP92	pGM9 containing an internal 0.45-kb *Sal*I-*Sma*I fragment of *phsA*	This study
pGM160	*tsr bla*, temperature-sensitive *Streptomyces-E. coli* shuttle vector	31
pSP62	pGM160 derivative containing an internal 0.45-kb *Sal*I-*Sma*I fragment of *phsA*	This study
pHC79	*bla tet*	16
pPtcos1	44-kb genomic DNA fragment of *S. viridochromogenes* Tü494 containing parts of the PTT biosynthetic cluster, cloned in *Bam*HI site of pHC79	1

ature-sensitive pGM vectors to verify the involvement of *phsA* in nonribosomal synthesis of peptides. Furthermore, the expression of *phsA* in *S. viridochromogenes* Tü494 and heterologously in *Escherichia coli* was examined.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

DNA sequencing and analysis. The sequence of the 4-kb *Bam*HI fragment was determined by standard techniques (28, 39). The DNA fragment was examined for open reading frames by applying the codon usage program of Staden and McLachlan (42). The programs BLAST (2) and FASTA (33) were used for homology searches.

Gene disruption procedure for *S. viridochromogenes*. Disruption of the chromosomal *phsA* gene was performed by cloning internal fragments of the *phsA* gene into the temperature-sensitive pGM vectors (31). The hybrid plasmids were transformed into *S. viridochromogenes* Tü494 as described previously (43). Liquid YM medium (10 g of malt extract, 4 g of yeast extract, and 4 g of glucose per liter [pH 7.2]) containing 50 μg of thiostrepton per ml was inoculated with 10^7 spores or 2 ml of homogenized mycelium of plasmid-carrying *S. viridochromogenes*. The culture was incubated in 100 ml of selective liquid medium in an orbital shaker (200 rpm) at 28°C in 500-ml Erlenmeyer flasks with steel springs. After 3 days of incubation, 5 ml of *S. viridochromogenes* was homogenized and 0.2 ml was diluted (500-fold with liquid medium) and further incubated at 40 or 28°C. At different times, samples were homogenized, diluted in P buffer (18), plated in parallel on selective (thiostrepton-containing) and nonselective media, and incubated at 40°C. After 3 days, the numbers of cells growing on antibiotic-containing and antibiotic-free media were determined and the ratio of plasmid-containing cells was calculated. Similar experiments, in which the incubation temperature after plating was 28°C instead of 40°C, were performed. In order to prove whether cells which grow on selective media contained the plasmid integrated into the chromosome, Southern hybridizations were performed.

In all these experiments, solid and liquid YM media were used. Plates contained 16 g of agar per liter.

Southern hybridization. Samples of genomic DNA from *S. viridochromogenes* Tü494, SP61/1, and SP62/2 were digested with *Bam*HI. The fragments were fractionated by electrophoresis on a 1% agarose gel in TAE buffer and transferred to Hybond-N nylon membranes (Amersham, Little Chalfont, United Kingdom) by the Southern procedure (38). *Sau*3A-digested cosmid pPtcos1 containing parts of the PTT biosynthetic cluster was labeled with (DIG)-dUTP by the random-priming procedure. The hybridization and the colorimetric detection were carried out according to the manufacturer's standard protocols (Boehringer, Mannheim, Germany).

***phsA** expression and protein purification.** The expression plasmid pDS3 (Table 1) was introduced into *E. coli* XL1 Blue by transformation (38). For protein purification, cultures of *E. coli* were inoculated with a 100-fold dilution of an overnight preculture and grown at 37°C (150 rpm) in Luria-Bertani medium containing 150 μg of ampicillin per ml. The expression of the protein was induced at an optical density of A_{580} from 0.6 to 0.7 by addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 2 mM. The incubation was carried out for 3 h. Cells were harvested by centrifugation at 4,000 × *g* at 4°C for 10 min. The cell pellet was resuspended in 0.1 M phosphate buffer, pH 8.0 (3 ml/g of wet cells). DNase I was added to a final concentration of 0.1 mg/ml of cell suspension. Cells were broken down with a French press. The insoluble protein fraction was harvested by centrifugation at 15,000 × *g* for 30 min. The protein was purified from the pellet by metal chelate affinity chromatography with nickel-nitrilotriacetic acid resin according to the denaturing protocol provided by the manufacturer (Qiagen, Hilden, Germany). Collected fractions were analyzed by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (38), and fractions containing PhsA* were pooled.

SDS-PAGE and immunoblotting. Protein extracts were separated by SDS-PAGE in 12.5% Laemmli (23) gels. Electrophoretic transfer of polypeptides to nitrocellulose (Schleicher & Schuell, Dassel, Germany) was performed with the MilliBlot Graphit Electroblotter I (Millipore Inc., Bedford, Mass.) blotting apparatus. Gels were stained with Coomassie brillant blue (Sigma, St. Louis, Mo.). Immunoblotting was performed as described by Sambrook et al. (38). Immunological characterization of PhsA was carried out with polyclonal antibodies against the proline-activating domain of gramicidin synthetase B, kindly provided by M. Marahiel (Marburg, Germany). In addition, polyclonal antibodies against PhsA* were used. Anti-rabbit immunoglobulin G-AP (Boehringer) was used as the second antibody.

Preparation of crude extracts of *S. viridochromogenes* Tü494 and *E. coli* XL1 Blue. Cells of *S. viridochromogenes* Tü494 were incubated in YM medium at 30°C. Samples were taken after 3, 4, 5, 6, and 7 days. Cells were resuspended in disintegration buffer (50 mM phosphate buffer [pH 8.0], 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride), harvested by centrifugation (5,000 × *g*, 10 min, 4°C), and broken down with a French press. The insoluble fraction was separated by centrifugation at 15,000 × *g* for 30 min at 4°C. Induced *E. coli* XL1 Blue cells (induction at 37°C with 1 mM IPTG for 3 h) carrying plasmids pDS3 and pDS4 were treated in the same way. The soluble fractions of the crude extracts were examined for existence of PhsA protein by immunoblotting experiments.

Nucleotide sequence accession number. The nucleotide sequence data reported have been assigned the accession no. X65195 in the EMBL data library.

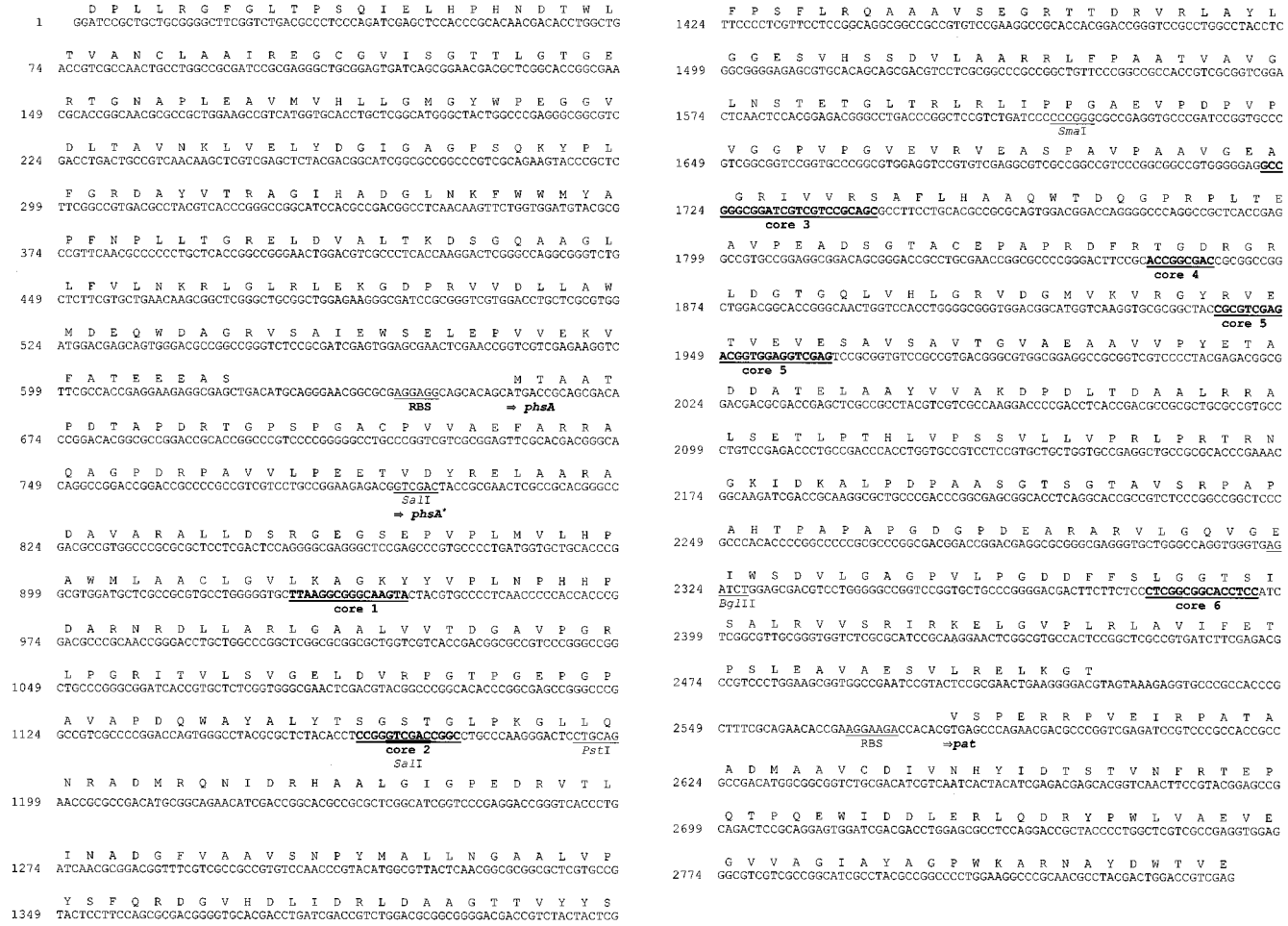

FIG. 1. Nucleotide sequence of the 2.8-kb *Bam*HI-*Sal*I subfragment containing *phsA*. The nucleotide sequence of the *phsA* gene and parts of the flanking genes is presented. The deduced amino acid sequence is given in single-letter code. A putative Shine-Dalgarno sequence (RBS) and conserved core motifs of peptide synthetases are indicated (core motifs 1 to 6). The putative translational start sites of *phsA*, *phsA**, and *pat* are marked by arrows. Restriction sites used in subcloning experiments are also marked.

RESULTS

DNA sequence analysis of *phsA*. By complementation of the non-PTT-producing mutant NTG1 (1), a 4-kb *Bam*HI fragment containing the putative peptide synthetase gene *phsA* (52) was isolated. The DNA sequence of the fragment was determined (accession no., X65195). In Fig. 1, the nucleotide sequence of the 2,823-bp *Bam*HI-*Sal*I DNA fragment containing the complete *phsA* gene is shown. *phsA* has a size of 1,866 bp and encodes a protein of 622 amino acids. A putative Shine-Dalgarno site (5′ AGGAGG 3′) is located 8 bp in front of the ATG start codon. The gene is flanked upstream by the 3′ regions of the biosynthetic gene (*pms*) encoding the phosphinomethylmalic acid synthase (52) and downstream by the PTT resistance gene, *pat* (44). The arrangement of the genes is presented in Fig. 2. The distance between the stop codon of the preceding *pms* gene and the start codon of *phsA* gene was determined to be 33 bp. No significant promoter structure showing similarities to *Streptomyces* (45) or *E. coli promoters* (15) was found upstream of the *phsA* start codon. A rare TTA codon, until now only found in *Streptomyces* genes expressed in the late-growth phase (7), could be detected at amino acid position 222. The presence of this TTA codon was confirmed by sequencing different DNA fragments by different techniques (28, 39). The G+C content of 75.9 mol% is characteristic for *Streptomyces* DNA (5).

Analysis of the deduced PhsA protein. The deduced amino acid sequence of *phsA* was compared with those of all proteins deposited in databases. It showed significant similarity to peptide synthetases, in particular with regard to the functional domains of these enzymes. All conserved core motifs of peptide synthetases (27) could be identified in the deduced amino acid sequence of *phsA* (Fig. 3).

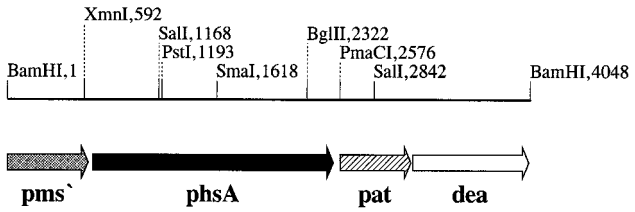

FIG. 2. Arrangement of genes on the 4-kb *Bam*HI DNA fragment. The localizations of incomplete *pms* (*pms′*) (phosphinomethylmalic acid synthase gene), *phsA* (PTT synthetase gene), *pat* (PT *N*-acetyltransferase gene), and *dea* (deacetylase gene) are given. Restriction sites used in subcloning experiments are marked.

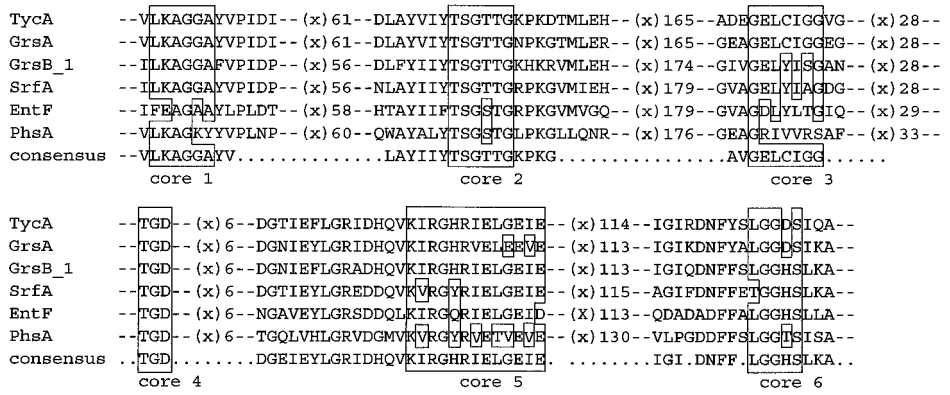

FIG. 3. Alignment of peptide synthetases sequences, including that of PhsA. Conserved regions including core motifs (shaded and boxed) of different peptide synthetases are presented (47). The distances between the conserved regions are indicated. The consensus sequence was postulated by Turgay et al. (47). TycA, tyrocidine synthetase A (29); GrsA, gramicidin S synthetase A (22); GrsB_1, proline-activating domain of gramicidin S synthetase B (22); SrfA, surfactin synthetase A (8), EntF, enterobactin synthetase (37); PhsA, PTT synthetase.

The PhsA protein contains the highly conserved core motifs 2 and 4. These motifs were found in carboxyl-activating enzymes and are essential parts in ATP binding and hydrolysis (47): the SGTTG motif (core 2) forms a phosphate binding loop, which is found in all guanosine and some adenosine nucleotide-binding proteins (40). The TGD motif (core 4) is widespread among cation ATPases (11). Furthermore, core motif 5, participating in aminoacyl adenylation, was found to be conserved in PhsA. The first conserved glycine residue was identified by mutant enzyme analysis and site-specific mutagenesis as a requirement for adenylate formation within the proline-activating domain of gramicidin S synthetase B (46). This conserved glycine residue and its conserved flanking region only detected in enzymes catalyzing aminoacyl-adenylate formation (46) were also found in the amino acid sequence of PhsA. The structural features of core motif 4 and the adjacent core motif 5 imply a possible role in the coordination of the magnesium cation in Mg^{2+}-ATP (32).

The carboxy-terminal end of PhsA contains the peptide synthetase-specific core motif 6. This motif is only found in enzymes catalyzing the formation of the covalent amino acid enzyme intermediate. The conserved serine residue of the motif is the putative binding site of the cofactor 4′-phosphopantetheine, which is involved in thioester formation with the amino acid substrate (41). In addition, the conserved core motif 1, whose function is unknown, was identified in the amino acid sequence of PhsA.

Since PhsA shows this significant similarity to peptide synthetases and the size of PhsA (622 amino acids) is in the range of one typical amino acid-activating domain, PhsA is probably sufficient for the activation and addition of one amino acid. Thus, further enzymes are necessary to catalyze tripeptide formation. In the case of all peptide synthetases described until now, the genes encoding the peptide synthetase complex are juxtaposed. *phsA* is flanked by *pms* and *pat*, and therefore further PTT peptide synthetase genes are unlikely to be located next to *phsA*.

But until now, it could not safely be ruled out that *phsA* does not encode a PTT peptide synthetase but that *phsA* encodes a protein with a high degree of similarity to the desired PTT peptide synthetase. Complementation of the PTT peptide synthetase-deficient mutant NTG1 might be achieved by the overproduction of PhsA, whose function may correspond to that of a peptide synthetase. Something similar is known for example for acyl carrier proteins in polyketide synthesis (20). We therefore intended to construct a *phsA* disruption mutant to analyze its phenotype and to compare it with NTG1.

Improvement of the gene disruption procedure with *S. viridochromogenes*. In order to increase the efficiency of the pGM vectors in the gene disruption experiments, we first optimized the disruption protocol. In principle, it has been shown that pGM vectors based on the temperature-sensitive replicon of pSG5 (30) could be used in gene disruption experiments with *S. viridochromogenes* (31). In the gene disruption procedure, plasmid elimination is the most critical step. In the standard procedure (31), mycelia containing pGM related vectors are plated on selective solid media and elimination of free plasmids is attained by incubation at a temperature of 39°C. By this method, about 1.0% of the colonies still contained freely replicating plasmids. This ratio can be further diminished if the cells containing plasmids are first incubated in liquid media at a nonpermissive temperature of 40°C for 3 days. By the gene disruption protocol described in Materials and Methods, the effectivity of plasmid loss was determined. Fewer than 0.01% of the colonies were antibiotic resistant after treatment according to the modified procedure (Table 2). Further tests revealed that resistance of these cells was not stably propagated.

Incubation in liquid media for more than 3 days at 40°C did not result in a measurable improvement in plasmid elimination. We determined the incubation time of 3 days to be optimal, and therefore it was used in further gene disruption experiments. Since plasmid elimination was much more effective if plated cells were incubated at 40°C instead of 28°C (Table 2), selection of integration mutants was performed routinely at 40°C.

TABLE 2. Ratios of resistant cells after different incubation times[a]

Incubation time (day) at 40°C	% CFU plating at:	
	28°C	40°C
0	88.24	8.33
1	6.00	0.386
2	1.89	0.013[b]
3	1.42	0.008[b]
4	0.33	0.004[b]
5	0.35	0.003[b]

[a] Cells containing pGM vectors were grown in liquid media for 0 to 5 days, plated, and incubated for 3 days.
[b] Growing cells did not result from plasmid integration.

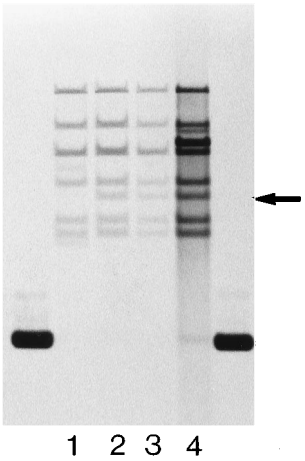

FIG. 4. Examination of the integration locus of pSP62 in gene disruption mutants SP62/1 and SP62/2 of *S. viridochromogenes* Tü494. *Bam*HI-digested genomic DNAs of *S. viridochromogenes* Tü494 and of the two gene disruption mutants SP62/1 and SP62/2 were separated by electrophoresis on a 1% agarose gel. In the standard Southern hybridization experiments described in Materials and Methods, the pattern of the hybridizing DNA fragments was determined. The position of the 4-kb *Bam*HI DNA fragment containing *phsA* is marked by an arrow. Lane 1, SP62/1, integration of pSP62 in *phsA*; lane 2, SP62/2, integration of pSP62 outside *phsA*; lane 3, *S. viridochromogenes* Tü494; lane 4, *Bam*HI-digested pPtcos1.

Gene disruption mutagenesis in *phsA*. Two plasmids were constructed for gene disruption of *phsA*, both based on pGM9 (31): pSP91 contained the internal 1.1-kb *Pst*I-*Bgl*II fragment (Fig. 1; position, bp 1193 to 2322), and pSP92 contained the internal 0.45-kb *Sal*I-*Sma*I fragment (Fig. 1; position, bp 1168 to 1618). Both plasmids were introduced into *S. viridochromogenes* Tü494 by transformation (18). By the optimized method for integration mutagenesis, gene disruption mutants in *phsA* were constructed. Integration of pSP91 in *S. viridochromogenes* SP91 and pSP92 in *S. viridochromogenes* SP92 resulted in mutated *phsA* genes: *S. viridochromogenes* SP91 synthesized a truncated PhsA protein which lacked the 4′-phosphopantetheine binding site (core motif 6), whereas *S. viridochromogenes* SP92 produced a truncated PhsA protein which lacked core motifs 4, 5, and 6. On the basis of plasmid-mediated resistance, the integration rates of plasmid pSP91 and pSP92 were determined to be 83 and 99%, respectively. The correct plasmid integration was examined by Southern hybridization (data not shown). Integration of single or multiple copies in tandem orientation were found.

Phenotype of *phsA* gene disruption mutants SP91 and SP92. The gene disruption mutants *S. viridochromogenes* SP91 and *S. viridochromogenes* SP92 have lost the ability to produce PTT. This was determined by an antibiotic production test with *Bacillus subtilis* as a PTT-sensitive indicator bacterium. In cofeeding tests with PTT-null mutants (1), the phenotypes of the integration mutants SP91 and SP92 were determined to be identical to that of the previously described mutant NTG1. The latter was shown to be defective in the alanylation step. Thus, the direct involvement of the *phsA* gene in nonribosomal formation of tripeptides was demonstrated.

Isolation of a non-PTT-producing gene disruption mutant defective in a gene outside the *phsA* locus. In addition to pSP91 and pSP92, a further disruption plasmid, pSP62, was constructed by cloning of the internal 0.45-kb *Sal*I-*Sma*I fragment (see pSP92) into a pGM160 vector (Table 1). With pSP62 as the integration plasmid, two types of non-PTT-producing mutants were obtained: one type (SP62/1) showed the same cofeeding behavior as NTG1, SP91, and SP92. Analogous to the mutants SP91 and SP92, pSP62 was integrated into the *phsA* gene. The other type (SP62/2) could not be assigned to one of the characterized mutant classes according to its cofeeding behavior (1). The integration site was examined by Southern hybridization experiments with genomic DNA and DIG-labeled PTT biosynthetic genes (pPtcos1 [Table 1]) as the probe. The results are presented in Fig. 4. Correct integration of pSP62 into the 4-kb *Bam*HI DNA fragment containing the *phsA* gene resulted in two hybridizing DNA fragments (2.9 and 5 kb) based on the additional *Bam*HI site of the integrated vector. In the case of the second mutant, SP62/2, this DNA fragment remained unchanged. Integration of pSP62 has therefore occurred in a gene other than *phsA*. The described results were confirmed in additional Southern hybridization experiments (data not shown) with other restriction endonucleases, e.g., *Bgl*II or *Pvu*II.

Production of antibodies against PhsA. No practicable enzyme assay is available to detect PhsA activity. We therefore intended to produce antibodies against PhsA protein to analyze the expression of *phsA*. Since no overexpression of the native *phsA* gene could be detected, a truncated *phsA* gene lacking 135 bp at the amino terminus was fused to a six-histidine affinity tag coding sequence in the expression vector pQE30, resulting in vector pDS3. The lacking DNA fragment does not contain any of the conserved core motifs described above. *E. coli* XL1 Blue cells harboring the expression plasmid were shown to overproduce a fusion protein (PhsA*) when grown under inducing conditions. PhsA* consisted of 577 amino acids of the PhsA protein, preceded by a 16-residue linker including the six-histidine tag at the amino terminus. The insoluble protein was purified by metal chelate affinity chromatography with nickel-nitrilotriacetic acid resin under denaturing conditions. The results of the protein purification are presented in Fig. 5. Refolding experiments with stepwise dialysis did not result in soluble protein. The denatured purified PhsA* protein was used to raise antibodies.

Examination of expression of *phsA* in *S. viridochromogenes* Tü494 and *E. coli*. By performing immunoblotting experiments, the expression of *phsA* was examined in *S. viridochromogenes* Tü494 and in *E. coli* XL1 Blue carrying the *phsA*

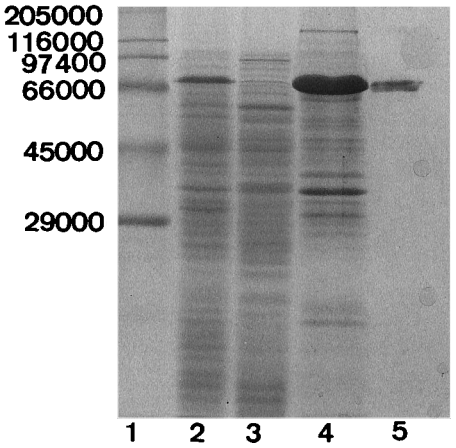

FIG. 5. Analysis of PhsA* purification by SDS-PAGE and staining with Coomassie brillant blue. Lane 1, SDS molecular weight standards (MW-SDS-200; Sigma); lane 2, crude extract; lane 3, soluble protein fraction of crude extract; lane 4, insoluble protein fraction of crude extract resuspended in 8 M urea–0.1 M phosphate buffer–0.01 M Tris-HCl, pH 8.0; lane 5, purified enzyme.

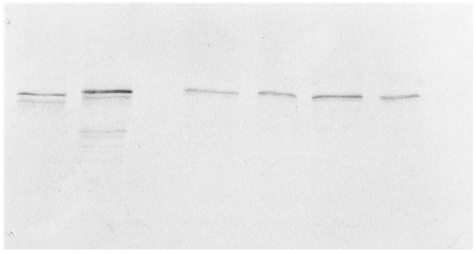

FIG. 6. Expression of *phsA* in *S. viridochromogenes* Tü494 and in *E. coli* XL1 Blue. Soluble protein fractions of the crude extracts of *S. viridochromogenes* Tü494 and *E. coli* XL1 Blue carrying *phsA* expression plasmids pDS4 and pDS3, respectively, were examined for existence of complete or truncated PhsA protein by immunoblotting experiments with polyclonal antibodies against PhsA*. Lane 1, crude extract of induced *E. coli* XL1 Blue carrying plasmid pDS3, which contains the truncated *phsA* gene (*phsA**); lane 2, crude extract of induced *E. coli* XL1 Blue carrying *phsA* expression plasmid pDS4; lane 3, empty; lanes 4 to 8, crude extracts of *S. viridochromogenes* Tü494 after incubation for 7 to 3 days, respectively.

expression plasmid pDS4. The results are presented in Fig. 6. For the expression of the complete *phsA* gene in *E. coli*, a 1.9-kb DNA fragment, containing the gene with its native *Streptomyces* ribosome binding site (RBS), was cloned in the expression vector pQE30, resulting in pDS4. By this cloning strategy (Table 1), the histidine affinity tag coding sequence (including the RBS) of the vector was removed. In immunoblotting experiments, a low-level expression of *phsA* was detected in induced cells of *E. coli* XL1 Blue carrying the *phsA* expression plasmid pDS4.

In *S. viridochromogenes* Tü494, no production of PhsA was detectable until 3 days of incubation. After 3 days, the expression of *phsA* could be observed. This correlated with the onset of PTT production, which was measured in an antibiotic production test with *B. subtilis* as a PTT-sensitive microorganism. The polyclonal antibodies were found to be specific for PhsA; no cross-reaction with other *S. viridochromogenes* proteins was observed.

Further biochemical characterization of PhsA will be achieved by using the polyclonal antibodies for monitoring the purification of the protein from *S. viridochromogenes* Tü494 and from *E. coli.*

DISCUSSION

In subcloning experiments, a 3.0-kb *Bam*HI-*Bgl*II DNA fragment of the PTT biosynthetic cluster was shown to complement the non-PTT-producing mutant NTG1, which is blocked in nonribosomal synthesis of the tripeptide (52). In addition to the PTT resistance gene (*pat*), the fragment contains one complete open reading frame (*phsA*). The deduced PhsA protein revealed high levels of similarity to peptide synthetases which are characterized by the presence of conserved functional domains. According to its length and the presence of the highly conserved core motifs, PhsA should be able to activate one amino acid.

Peptide synthetases involved in the activation of one amino acid, for example, tyrocidine synthetase A, gramicidin synthetase A, and the first domain of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase, did not show a conserved spacer motif at the amino-terminal end (10). The lack of this motif was only found in peptide synthetase domains participating in the initiation of peptide synthesis. Interestingly, the spacer motif is also lacking in the amino acid sequence of PhsA. So, it can be suggested that PhsA takes part in the initiation reaction of tripeptide formation in PTT biosynthesis. In this case, PhsA should activate the amino acid *N*-acetyl-demethyl-PT.

In order to confirm our conclusions drawn from the sequence information, we constructed gene disruption mutants in the *phsA* gene with temperature-sensitive pGM vectors. The integration rates observed in these experiments reached surprisingly high levels of 83% (pSP91) and 99% (pSP92). The high integration rates and the unexpected result that pSP92, containing the small, internal 0.45-kb fragment of *phsA*, revealed higher integration rates than did pSP91 are perhaps attributable to the small number of measurements, which allows no statistical interpretation, and by the uncertainties of working with homogenized *Streptomyces* mycelia. But these high values for integration rates agree with results described for integration mutagenesis of other *Streptomyces* strains with pGM vectors, such as *Streptomyces coelicolor* A3(2) (19).

Integration of pSP91 resulted in a non-PTT-producing mutant which showed the same phenotype as mutant NTG1. In the case of vector pSP91, integration should result in the production of a predicted PhsA protein lacking 65 amino acids at the carboxy terminus. This part of the protein contains core motif 6, with the conserved binding site of 4′-phosphopantetheine. Lack of this part of PhsA seems to abolish its function as a peptide synthetase. This observation agrees with the results of Dieckmann et al. (9), who described the enzymatic properties of a truncated tyrocidine synthetase A lacking the 4′-phosphopantetheine binding site. Therefore, the resulting protein could not catalyze nonribosomal synthesis of peptides.

Gene replacement mutants of *phsA* which are characterized by an exchange of an internal fragment of *phsA* for a thiostrepton resistance cassette (33a) have not lost the ability to activate the amino acid alanine (18a). We therefore postulated that PhsA participates in the activation of *N*-acetyl-demethyl-PT. This confirms the assumption based on sequence analysis (see above).

Since the *phsA* gene is flanked by the *pms* and *pat* genes, *phsA* is a peptide synthetase gene which is involved in the biosynthesis of peptide antibiotics and is not juxtaposed to the other cooperating peptide synthetase genes. This is in contrast to the biosynthesis of gramicidin S (22), surfactin (8), or tyrocidine (29), in which the peptide synthetase genes are located next to each other.

In front of *phsA*, no promoter sequence could be found, which possibly suggests that *phsA* is part of an operon. In the course of studying the regulation of bialaphos production genes in *S. hygroscopicus*, Holt et al. (17) isolated a 10-kb DNA fragment containing at least six biosynthetic genes, including *pms*, *bar* (*pat*), and *bah* (*dea*) (Fig. 2). Considering the similar organizations of the PTT biosynthetic clusters in *S. viridochromogenes* Tü494 and in *S. hygroscopicus* (14), the analogous *phsA* gene of *S. hygroscopicus* should be located on this fragment. Holt et al. speculated that these genes may be coordinately expressed by transcription from one promoter (17). This promoter should be located in front of a gene upstream of *phsA*. This would be in agreement with our observation for *S. viridochromogenes* that there was no recognizable promoter in front of *phsA*. The expression in *S. hygroscopicus* was regulated by the gene product of the regulatory gene *brpA* (3). Possible regulation by the corresponding *brpA* gene in *S. viridochromogenes* should be tested in further examinations.

In *S. coelicolor* A3(2), TTA codons are supposed to be involved in regulation of secondary metabolic genes (24, 25). Mutants (*bldA*) which cannot translate the rare leucin UUA codon are defective in antibiotic production and morphological differentiation in *S. coelicolor* A3(2), *Streptomyces lividans*,

and *Streptomyces griseus* (7). Until now, the TTA codon was found only in the genes of secondary metabolism, particularly in regulatory genes such as *actII-orf*4, a transcriptional activator of actinorhodin biosynthesis (7). The presence of *bldA* tRNA determines the translation of this regulator and consequently the antibiotic production. The role of *bldA* in secondary metabolism and differentiation seems to be widespread among streptomycetes (7). Interestingly, *phsA* contains a single TTA codon (Leu-222), making it possibly *bldA* dependent. The role of PhsA as peptide synthetase in PTT biosynthesis makes it unlikely that PhsA is a regulator of this biosynthesis.

For biochemical characterization of the PhsA protein, we intended to express the *phsA* gene in *E. coli*. The *phsA* gene was cloned behind the *lacZ* or *aphII* promoters in *E. coli* vectors such as pUC19 or pK18 and in pT7 expression vectors, respectively (data not shown). Under optimal conditions for induction, overexpression of *phsA* was not observed by SDS-PAGE.

Since the structure and distance of the RBS of *phsA* show high similarities to those of the RBS in *E. coli* (36), the RBS of *phsA* should not limit the gene expression. Poor expression could also result from codon usage of streptomycetes because of its G+C moles percent content. Compared with *E. coli* and *Bacillus* sp. genes such as *tycA* or *grsA*, which could be effectively expressed in *E. coli* (11), *phsA* shows a significantly higher G+C moles percent content (75 mol% in comparison with 50 mol%). It has been shown that overexpression of polyketide biosynthesis genes from *Streptomyces glaucescens* is influenced by the G+C moles percent content of the first nine codons in *E. coli* (12). The existence of *Streptomyces*-specific codons also found at this position in the *phsA* gene could remarkably decrease the expression of *Streptomyces* genes.

For detection of *phsA* expression, we intended to raise antibodies for use in immunoblotting experiments. Antibodies against another peptide synthetase domain (the proline-activating domain of GrsB) showed no cross-reaction with PhsA* (data not shown). Therefore, the purified truncated PhsA protein (PhsA*), showing all the functional parts of peptide synthetases, was used to immunize a rabbit. With these antibodies in immunoblotting experiments, it was possible to show that despite the use of typical *Streptomyces* codons in the *phsA* gene a low-level expression of the complete gene occurred in induced cells of *E. coli* XL1 Blue carrying the *phsA* expression plasmid pDS4.

The production of secondary metabolites is characterized by transcription and translation of biosynthetic genes in the stationary-growth phase (7). In *S. viridochromogenes* Tü494, the expression of *phsA* showed the same characteristic. The production of the protein could be detected after 4 days of incubation, which most likely indicates the beginning of the idiophase of growth.

During the inactivation experiments of the *phsA* gene with the integration plasmid pSP62, the non-PTT-producing mutant SP62/2 was isolated. Surprisingly, in this mutant the plasmid was integrated outside of the known *phsA* locus. It is likely that pSP62 integrated into a region which possesses a high degree of sequence similarity to the cloned *phsA* fragment, since we have never observed integration via pGM vector fragments. Integration may have occurred via semispecific recombination, which was also observed during the construction of gene disruption mutants, in the bialaphos resistance gene *bar* from *S. hygroscopicus*, with the internal fragment of the PTT resistance gene *pat* from *S. viridochromogenes* (31a). The homology at the DNA level between the two resistance genes was determined to be 87% (51). Considering the conserved peptide synthetase domains which possess significant homologies at the DNA level in conserved regions, we may have created an insertion mutation in an unknown PTT biosynthetic gene which is a peptide synthetase gene or shows similarities to this kind of gene. In further examinations, the insertion locus of the plasmid pSP62 and role of the mutated gene in the PTT biosynthesis has to be examined.

These results demonstrate that "semispecific integration" may offer an interesting approach for mutating and subsequently isolating genes which possess areas highly homologous to cloned fragments. In addition to creating the possibility of constructing specifically truncated genes by integration of vectors carrying defined gene fragments (see above), this approach further broadens the applicability of integration vectors such as the pGM vectors.

ACKNOWLEDGMENTS

This research was supported by a grant from the Deutsche Forschungsgemeinschaft (Wo 485/2-1).

The technical assistance of Anja Pielsticker is gratefully acknowledged. We thank D. F. Brolle and G. Muth for critical readings of the manuscript, M. Marahiel for providing antibodies, and U. Keller for communicating results prior to publication. The antibiotic thiostrepton was kindly provided by S. J. Lucania, Squibb, New York, N.Y.

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