JOURNAL OF BACTERIOLOGY, Feb. 1996, p. 1216–1218 Vol. 178, No. 4
0021-9193/96/$04.00+0

Cloning and Heterologous Expression of the Entire Set of Structural Genes for Nikkomycin Synthesis from *Streptomyces tendae* Tü901 in *Streptomyces lividans*

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Received 6 September 1995/Accepted 11 December 1995

A genomic library from *Streptomyces tendae* raised in shuttle cosmid vector pKC505 was screened with a previously isolated 8-kb DNA fragment containing the *orfP1* gene, which is involved in nikkomycin biosynthesis. The entire set of structural genes for nikkomycin synthesis was heterologously expressed in *S. lividans* TK23 by introducing recombinant cosmids p24/32 and p9/43-2, carrying inserts of about 31 and 27 kb, respectively, overlapping by 15 kb. *S. lividans* transformants synthesized nikkomycins X, Z, I, and J, which were identified by high-pressure liquid chromatography analyses of culture filtrates.

Nikkomycins are peptidyl nucleoside antibiotics and act as strong competitive inhibitors of chitin synthetases from fungi and insects (3, 4, 16). A series of nikkomycins have been isolated from the culture filtrates of wild-type and mutant *Streptomyces tendae* Tü901 and by mutasynthesis (for a review, see reference 8). Nikkomycins X and Z, produced as main components by *S. tendae* Tü901, are the biologically most active structures (Fig. 1). They are composed of an aminohexuronic acid with *N*-glycosidically bound 4-formyl-4-imidazoline-2-one and uracil and the peptidically linked unusual amino acid 4-hydroxy-pyridyl homothreonine (nikkomycin D) (10). Nikkomycins I and J, synthesized in minor amounts, are structurally analogous to nikkomycins X and Z and contain glutamic acid peptidically bound to the 6′-carboxyl group of aminohexuronic acid (Fig. 1; 9). The biosynthesis of nikkomycins has been investigated by incorporation experiments with labelled precursors. The base of nikkomycin originates from L-histidine and uracil (5, 7, 18). Furthermore, L-lysine has been identified as the precursor of the pyridyl residue and the attached carbon atom of nikkomycin D (19). The biosynthesis of the aminohexuronic acid moiety is thought to be similar to that of the related polyoxins (12, 13).

Recently, we identified cellular proteins involved in nikkomycin production in *S. tendae* by investigating gene expression (15). By two-dimensional gel electrophoresis of cellular proteins, we detected 10 proteins (P1 to P10) that were synthesized when nikkomycin was produced. By reverse genetic analysis of proteins P1/2 and P6, an 8-kb *Bam*HI fragment from the *S. tendae* genome has been cloned that contains the *orfP1* gene encoding proteins P1 and P2 by translational initiation at two start codons. *orfP1* gene disruption mutants lost the ability to produce nikkomycin, indicating that proteins P1 and/or P2 are essential for nikkomycin production. The recognition site for the oligonucleotide probe designed from protein P6 maps directly upstream of the *orfP1* gene, imply that the isolated DNA fragment contains a part of the nikkomycin gene cluster. To isolate nikkomycin biosynthetic genes, a genomic library of *S. tendae* Tü901/8c constructed in shuttle cosmid vector pKC505 was screened with the 8-kb *Bam*HI fragment as a probe. In this communication, we describe the heterologous expression of the nikkomycin gene cluster in *S. lividans* TK23.

An *S. tendae* Tü901/8c (obtained from H. Zähner, University of Tübingen, Tübingen, Germany) gene library was prepared in cosmid pKC505 (provided by S. Kuhstoss, Eli Lilly, Indianapolis, Ind.) as described by Richardson et al. (17) by using *Escherichia coli* DH5α for transfection and screened by colony hybridization with the digoxigenin 11-dUTP-labelled 8-kb *Bam*HI fragment (0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate, 68°C). From about 5,000 apramycin-resistant *E. coli* cells, 20 colonies hybridized to the probe. Restriction analyses of the isolated cosmids revealed that about 65 kb of genomic *S. tendae* DNA had been cloned on overlapping inserts that were 25 to 33 kb long. To localize the genes encoding proteins P4, P5, and P8, which are implicated in nikkomycin biosynthesis (15), oligonucleotide probes VM4, VM5, and VM8, deduced from their N-terminal amino acid sequences {VM4, a mixed 50-mer corresponding to amino acids 1 to 17 of protein P4 [5′-ATGA A(AG)GC(CG)GA(AG)CA(AG)GG(ACGT)CC(CG)TTCC T(CG)CT(CG)CT(CG)AA(CT)AC(CG)AA(AG)GA(AG)A TCGT-3′]; VM5, a mixed 27-mer corresponding to amino acids 11 to 19 of protein P5 [5′-GT(CG)CT(CG)GA(CT) GC(CG)GG(ACGT)CT(CG)ATCGG(ACGT)ATG-3′]; and VM8, a mixed 32-mer corresponding to amino acids 4 to 14 (VM8) of protein P8 [5′ GT(CG)AA(CT)GA(AG)AA(CT) GG(ACGT)ATGTACTTCGT(CG)CC(CG)GC-3′]}, were hybridized to the isolated cosmids as described by Möhrle et al. (15). The membranes were washed for 10 min at 54, 50, and 35°C, respectively, when VM4, VM5, and VM8 were used as probes. All probes strongly hybridized to cosmid p24/32 (Fig. 2). Probe VM4 hybridized to a 3.8-kb *Sst*I fragment, a 2.9-kb *Bam*HI fragment, and a 1-kb *Bam*HI-*Pvu*II fragment of p24/32, whereas no signals were obtained with p9/43 (Fig. 2). Probes VM5 and VM8 hybridized to a 7-kb *Bam*HI fragment of p9/43, while there were no signals with cosmid pA3. Since VM5 hybridized to the 2.8-kb *Pvu*II fragment and VM8 hybridized to the 1.1-kb *Pvu*II fragment of p9/43, the recognition sites for VM4, VM5, and VM8 mapped within a region of 8 kb (Fig. 2). The hybridizing sites for probes VM1/2 and VM6, deduced from proteins P1/P2 and P6, respectively, had previously been mapped on the 8-kb *Bam*HI fragment used as probe to screen the *S. tendae* gene library (15; Fig. 2). Consequently, all of the recognition sites for oligonucleotide

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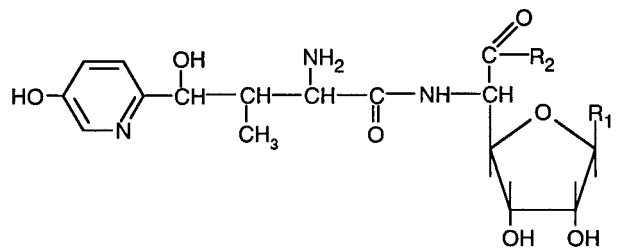

Nikkomycin	R_1	R_2
X	A	OH
Z	B	OH
I	A	Glu
J	B	Glu

FIG. 1. Structures of nikkomycins.

probes designed on the basis of the N-terminal amino acid sequences of proteins implicated in nikkomycin synthesis according to gene expression studies (15) mapped within a 15.5-kb region on cosmid p24/32.

S. lividans TK23 was used for transformation (11) with p24/32 and DNAs from 13 of the other isolated cosmids by using 50 μg of apramycin (kindly provided by Eli Lilly) per ml for selection. Culture filtrates from the resulting transformants grown for 6 days in liquid production medium (2) were examined for the presence of nikkomycins; none (20 μl) caused inhibition of the test organism *Mucor miehei* in agar diffusion tests (1). High-pressure liquid chromatography (HPLC) analyses (6) revealed unknown compounds in the culture filtrates of TK23 p24/32 (data not shown) that exhibited absorbance spectra characteristic for nikkomycins. These substances might be precursors or shunt metabolites of nikkomycin synthesis. We expected a clustered organization of all of the nikkomycin biosynthetic genes that is generally found for genes involved in the production of a particular antibiotic (14) and supposed that none of the isolated cosmids contained the entire nikkomycin cluster. Therefore, cosmids p24/32 and p9/43, carrying inserts of about 31 and 27 kb, respectively, 15 kb of which overlapped (Fig. 2), were selected to be transformed together into *S. lividans* TK23. To facilitate selection for both plasmids, the apramycin resistance gene of p9/43 was removed by restriction with *Xho*I (17), which did not cut within the insert, and replaced with a 1.3-kb *Hin*dIII-*Sma*I fragment containing the *aph* gene from Tn*5* (isolated from plasmid pJoe865, obtained from J. Altenbuchner, University of Stuttgart, Stuttgart, Germany), yielding p9/43-2. Upon selection with neomycin and apramycin (each at 50 μg/ml), 100 transformants grew on solid HA medium (2) and were tested for antifungal activity after cultivation for 6 days at 30°C by transferring agar plugs on plates seeded with *M. miehei* (1). Six transformants gave inhibition zones against *M. miehei*. These transformants were grown in liquid production medium (2) containing apramycin and neomycin (each at 25 μg/ml). All transformants produced nikkomycins X, Z, I, and J, identified by HPLC analysis of the culture filtrates with a photodiode array detector (6). The retention times and spectra of the corresponding peaks were identical to those of reference samples of nikkomycins X, Z, I, and J (Fig. 3). Five of the transformants produced about 10 mg of nikkomycins per liter. In contrast, transformant TK23/A16 synthesized about 400 to 500 mg of nikkomycins per liter, which was only 50% less than *S. tendae* Tü901/8c. In the culture filtrate of the control strain, *S. lividans* pKC505, nikkomycins were not detected (Fig. 3). Large-size (>50-kb) plasmid DNA was detected in total DNA from *S. lividans* A16 separated by pulsed-field electrophoresis (1% agarose, 0.5 × *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], 150 V, 10-s pulse, 22 h, 14°C; data not shown), imply that recombination events occurred between the homologous regions of the plasmids, the segments of vector pKC505, and/or the homologous regions of the inserts. Southern hybridization experiments with total DNA from *S. lividans* TK23 with p24/32 and p9/43 as probes (0.1× SSC, 0.1% sodium dodecyl sulfate, 68°C) did not reveal hybridization signals (data not shown), indicating that *S. lividans* did not have DNA homologous to the introduced cosmids. This finding eliminates the possibility of

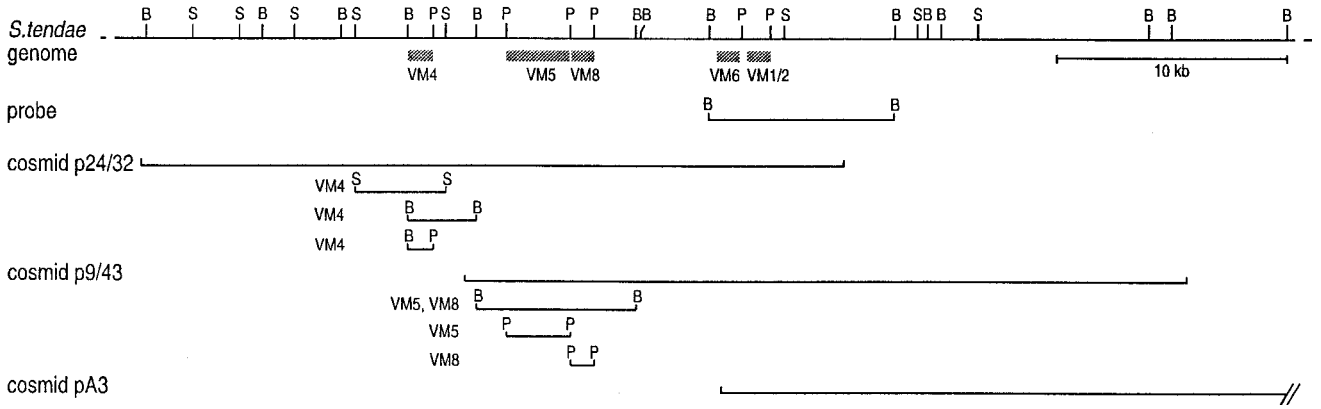

FIG. 2. Restriction map of the part of the *S. tendae* genome containing nikkomycin genes and localization of the recognition sites for oligonucleotide probes deduced from proteins P4, P5, and P8. The shaded boxes below the genomic map indicate the recognition sites for oligonucleotide probes VM4, VM5, and VM8 deduced from proteins P4, P5, and P8. The hybridizing sites for probes VM1/2 and VM6, deduced from proteins P1/2 and P6 and determined in an earlier study (15), are also marked. The cosmids used for mapping are shown; below, the fragments hybridizing to the probes are indicated. The hybridization probe used to screen the *S. tendae* gene library is also indicated. B, *Bam*HI; S, *Sst*I; P, *Pvu*II. *Sst*I and *Pvu*II are not mapped to the right from the *Sst*I site on the right site of the map.

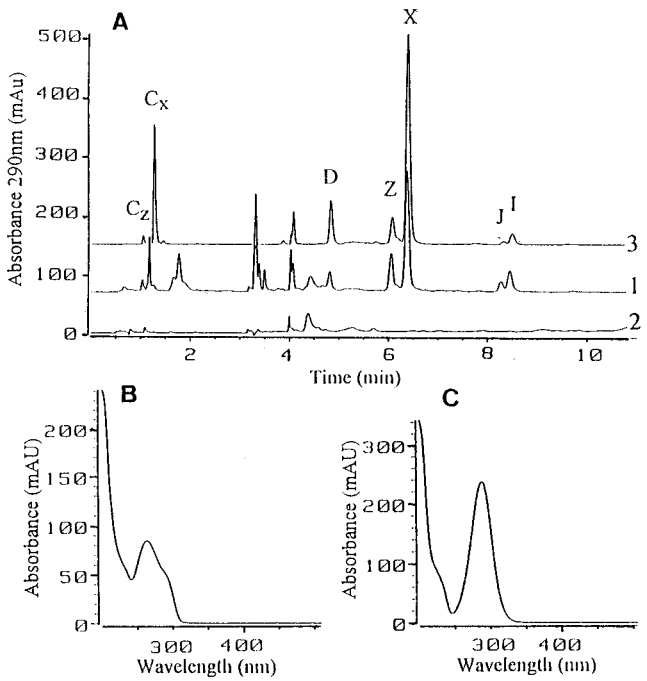

FIG. 3. HPLC analyses of culture filtrates from *S. lividans* TK23/A16 (1) and TK23(pKC505) (2) and of a nikkomycin standard (3) containing nikkomycins C_z, C_x, D, Z, X, J, and I (A). The strains were cultivated for 6 days in production medium containing apramycin and neomycin, each at a concentration of 25 μg/ml (TK23/A16), or 25 μg of apramycin per ml [TK23(pKC505)]. The spectrum of the HPLC peak of the culture filtrate from TK23/A16 appearing at 6.1 min (B) and at 6.5 min (C); the spectra of the peaks appearing at 8.3 and 8.5 min are identical to those in B and C, respectively. mAU, milli absorbance units.

either activation or complementation of cryptic genes in the host. Thus, all of the structural genes required for nikkomycin synthesis occur in the insert of p24/32 and p9/43. Southern hybridizations revealed that nonproducing *S. tendae* Tü901 mutants (2, 15) had deletions of at least 65 kb spanning all of the structural genes for nikkomycin synthesis. Nonproducing mutants occur at a frequency of about 3% among mutagenized spores (2). That is relatively high in comparison with mutants blocked in specific steps of nikkomycin biosynthesis (0.005%), imply that the nikkomycin gene cluster is located in an unstable genomic region.

Characterization of all of the genes involved in nikkomycin biosynthesis will be useful in detecting homologous genes from other streptomycetes, such as from species producing the related polyoxins (13), and subsequent construction of novel hybrid antibiotics.

We thank Mulugeta Nega and Hans-Peter Fiedler for HPLC analyses and Gerd Helm for mapping the binding sites of oligonucleotide probes VM4 and VM5. We thank Reinhold Brückner and Wolfgang Wohlleben for critical comments on the manuscript and Karen A. Brune for editing the manuscript.

This work was supported by Deutsche Forschungsgemeinschaft grants Bo 978/2-1 and Bo 978/2-2.

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