A spectinomycin resistance determinant from the spectinomycin producer Streptomyces flavopersicus

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The spectinomycin (Sp) resistance determinant from Streptomyces flavopersicus was cloned into Streptomyces lividans using the plasmid vector pIJ699. A plasmid, pDGL15, with a 3.65 kb insert from S. flavopersicus conferring resistance to Sp was isolated. DNA sequence analysis of the 3651 bp DNA insert revealed four open reading frames (ORFs). The amino acid sequence deduced from one ORF (SpCN) showed a high degree of similarity to an aminoglycoside phosphotransferase (StrN) and from a second one (SpCR) to a regulatory protein (StrR) of the streptomycin biosynthesis gene cluster from S. griseus. The two other ORFs were incomplete and the deduced amino acid sequences showed similarities to an amidinotransferase encoded in the streptomycin biosynthesis gene cluster of S. griseus and to the transposase of IS112, respectively. Expression of the spCN gene in E. coli under the control of tac promoter conferred Sp resistance to the cells. An enzymic assay confirmed that the gene product of spCN is an ATP-dependent aminoglycoside phosphotransferase which phosphorylates Sp and actinamine, the aminocyclitol moiety of Sp.

Keywords: Streptomyces flavopersicus, spectinomycin resistance, spectinomycin phosphotransferase, aminoglycoside antibiotics

INTRODUCTION

The genus Streptomyces is an economically important group of Gram-positive bacteria that produce a wide variety of unusual metabolites including antibiotics and other pharmacologically active agents. However, the enzymes and the mechanisms of biosynthesis and the regulation of the production of these compounds are often poorly understood. Elucidation of the biosynthesis pathways and the regulation is of great importance for increasing antibiotic productivity and designing new antibiotics with hybrid structures and properties.

Spectinomycin (Sp), formerly actinospectacin (Mason et al., 1961), is an aminoglycoside broad-spectrum antibiotic which lacks oto- and nephrotoxicity. It is used in gynaecology as an alternative to penicillin for treatment of Neisseria gonorrhoeae infections (Pederson et al., 1972) and for veterinary purposes (Shermuhrorn et al., 1995). It blocks the translocation step of protein synthesis by inhibiting the binding of elongation factor G to the ribosome (Brink et al., 1994). It has a unique structure in which a single sugar component, actinospectose, is linked to the diaminocyclitol moiety actinamine by β-glycosidic and hemiketal bonds (Wiley et al., 1963). Sp is produced by several Streptomyces species such as Streptomyces spectabilis (Mason et al., 1961) or Streptomyces hygroscopicus (Yamamoto et al., 1974). We are interested in the Sp producer Streptomyces flavopersicus NRRL2820 (Oliver et al., 1962). Not much is known about the enzymic reactions involved in Sp biosynthesis (Mitscher et al., 1971; for a review see Okuda & lto, 1982). In vivo feeding experiments have recently indicated the key roles of glutamine-amidinocyclitol amidotransferases in biosynthesis of Sp and the other aminoglycoside antibiotics (Walker, 1993). The genes required for the biosynthesis of an antibiotic

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Abbreviations: DIG, digoxigenin; PTS, phosphotransferase.
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in streptomycetes are usually clustered and tightly linked to the genes encoding resistance to the antibiotic and genes involved in the regulation of biosynthesis of that antibiotic. Isolation of the antibiotic resistance gene(s) is therefore a straightforward first step in studying the antibiotic biosynthesis and regulation genes. For studying Sp biosynthesis we isolated and analysed Sp resistance genes in S. flavopersicus.

**METHODS**

**Bacterial strains and plasmids.** S. flavopersicus NRR2820 was the donor of the Sp resistance genes (Oliver et al., 1962). S. lividans TK64 (Hopwood et al., 1983) was used as a Sp- and thioestrepton-sensitive recipient in shotgun cloning experiments. Escherichia coli JM109 (Yanisch-Perron et al., 1985) was the host for transformations in subcloning experiments. Plasmid pJ699 (Kieser & Melton, 1988) was used as a vector in S. lividans and pCl20R, pCl20H (Marsh et al., 1984) and pJOE930 (Altenbuchner et al., 1992) for subcloning in E. coli. The shuttle vector pJOE875 (Altenbuchner et al., 1992) was used in both hosts. For expression of spcN the expression vector pBTacI (Brosius et al., 1981) was modified. The Ndel site was filled in by Klenow (large fragment) polymerase and between EcoRI and BamHI site complementary oligonucleotides containing the RBS of T7 gene 10 (Rosenberg et al., 1987) and an Ndel site with an ATG start codon were inserted to give pJOE2453 (oligonucleotide 1: 5'-AATTCTTAAGAGGAGATATACATATGCAGGCGCCCTGGCCG-3'; oligonucleotide 2: 5'-GATCCGGCCAGGGCGGGAGATTTCATATGTATCCTCCTCTTAAAG-3').

**Media and culture conditions.** S. flavopersicus cultures were grown at 32°C in SGGP medium (Yamamoto et al., 1986). S. lividans was grown at 30°C in YEME medium supplemented with 27% (w/v) sucrose, 10 mM MgCl2 and 0.5% glycine (Hopwood et al., 1983). For protoplast regeneration and to get spores from S. lividans, R2YE agar plates (Hopwood et al., 1985) were used, supplemented with 40 μg thioestrepton ml−1 when necessary. E. coli strains were grown at 37°C in 2YT liquid medium and on agar plates (Sambrook et al., 1989) supplemented with 100 μg ampicillin ml−1. Minimal medium M40 (Polsinelli & Beretta, 1966) supplemented with 100 μg Sp ml−1 (Sigma) was used for selection of Sp resistant S. lividans TK64 transformants.

**DNA manipulations.** Chromosomal DNA from S. flavopersicus was isolated as described by Sedlmeier & Altenbuchner (1992). Plasmid DNA from S. lividans and E. coli was isolated by the method of Kieser (1984). For construction of a genomic library of S. flavopersicus NRR2820, chromosomal DNA was digested with BamHI or BglII and ligated to the vector pJ699 cleaved with BglIII and treated with alkaline phosphatase. S. lividans protoplasts were transformed with the ligation mixtures and transformants were selected by overlaying the R2YE plates, after 16 h incubation, with 3 ml soft R2YE containing thioestrepton to a final concentration 40 μg ml−1 (Hopwood et al., 1985). Transformation of E. coli JM109 was carried out according to Chung et al. (1989). Restriction enzymes, T4 ligase and alkaline phosphatase were purchased from Boehringer Mannheim and used in accordance with the recommendation of the manufacturer.

**DNA sequencing.** The DNA was sequenced by the chain-termination method (Sanger et al., 1977) on an ALF DNA Sequencer (Pharmacia) using the fluorescent-labelled universal and reverse primers from the AutoRead Sequencing kit (Pharmacia). The 3-65 kb HindIII fragment from pDGL15 was inserted in pCl20R (pDGL21-2; pDGL21-5). Various deletions were introduced by using restriction enzymes cutting in the polylinker sequence of pCl20R and in the insert or by subcloning small fragments in pCl20R. The plasmids were prepared with the Qiagen DNA preparation kit (QUIAwell-8 plasmid kit) and used as templates for sequencing. Sequence analysis was done with the GCG software (Devereux et al., 1984). Database searches were run with the programs BLAST, BLASTP and TBLASTN (Altschul et al., 1990) on the BLAST electronic mail server from the National Center for Biotechnology Information, Bethesda, MD, USA. Patterns in protein were searched with the BLOCKS search based on the Blocks database version 9.1 (Henikoff & Henikoff, 1994).

**PCR of spcN.** The spcN gene was amplified by PCR with primers (5'-end of spcN: 5'-ACACATATGGAGAAGTTTCCCTGAGAA-3'; 3'-end of spcN: 5'-ACCAAGCTTCCGATCCTGTACCCTCGG-3') and Taq polymerase (Pharmacia) in 30 cycles from plasmid pDGL21-2 as described by Piendl et al. (1994).

**Southern hybridization.** Hybridization experiments were performed using the nonradioactive digoxigenin (DIG) DNA labelling and detection kit (Boehringer Mannheim) following the manufacturer's recommendations. Blotting of DNA from agarose gels on nylon membranes was described by Smith & Sommers (1980).

**Deletion analysis and antibiotic diffusion assay.** For construction of pJOE2328 the 3-65 kb HindIII fragment from pDGL15 was inserted between the two inverted repeated terminator sequences of the shuttle vector pJOE875 replacing the corresponding HindIII fragment (encoding melanin biosynthesis). The plasmid pJOE2327 was obtained by inserting a 3-01 kb SacI fragment from pDGL21-2 in pJOE875, replacing the corresponding SacI fragment. Plasmid pJOE2619 was generated by first deleting a 1 kb PsiI fragment of pDGL21-1 and inserting the remaining 2-6 kb as an XbaI fragment in pJOE875. The plasmids pJOE2494, pJOE2495 and pJOE2618 were constructed by inserting a 1-37 kb PvuII-XbaI, a 1-31 kb PvuII-SphiI fragment and a 1-25 kb PvuII-Ncol fragment from pDGL21-2 after blunt-ending with Klenow polymerase between the two Smal sites of the vector pJOE930 and then as HindIII fragments in pJOE875. The plasmids were transformed into S. lividans and spore suspensions of transformants prepared in 20% glycerol. About 106 spores were plated on GME plates (Sedlmeier & Altenbuchner, 1992). A filter disc (5 mm diameter) bearing 1 mg Sp, 2 mg streptomycin (Sm), 0.5 mg kanamycin, 0.3 mg gentamicin or 0.5 mg neomycin was laid onto the agar plates. The diameters of inhibition zones (less than 5 mm filter) were determined after 48 h incubation at 30°C.

**Expression of spcN in E. coli for SDS-PAGE analysis.** The expression vectors pJOE2453 (control) and pJOE2686 (containing spcN) were used to transform E. coli JM109. Overnight cultures of transformants were diluted (OD600 0-05) in 2x YT containing 100 mg ampicillin ml−1 and grown at 37°C in a waterbath shaker to OD600 0.4. Then 0.1 ml IPTG was added and the cells further incubated for 4 h. Crude extracts were prepared by sonication as described by Pelletier & Altenbuchner (1995) and protein separated on a 12% SDS-PAGE using the discontinuous buffer system of Laemmli (1970). Gels were stained with Serva Blue R (Serva Feinbiochecma). Proteins from the low-M, calibration kit.
from Bio-Rad (phosphorylase b, BSA, ovalbumin, carbonic anhydrase, trypsin inhibitor and lysozyme) were used as standards. Protein concentrations were determined at 595 nm using the method of Bradford (1976) with BSA as standard.

**Sp phosphotransferase (PTS) assay.** *E. coli* JM109(pJOE2686) and JM109(pJOE2453) were grown to OD600 0·6 in 100 ml LB supplemented with 100 µg ampicillin ml⁻¹ at 37 °C. The expression of *spcN* was induced by adding IPTG to a final concentration of 1 mM. After 2 h the cells were collected by centrifugation (5000 g, 10 min) and washed twice with buffer A (50 mM Tris/HCl, 10 mM MgCl₂, 1 mM DTT, pH 7·5). For the preparation of cell-free extracts the cells were resuspended in 2 ml buffer A and disintegrated at 140 MPa in a French press. The cell debris was removed by centrifugation at 20000 g (20 min, 4 °C). The supernatant was used as cell-free extract and stored at −70 °C. The PTS assay was performed according to the method of Distler & Piepersberg (1985) with the following modifications: the enzyme assays were performed with 40 µg protein at 30 °C in a volume of 100 µl of 50 mM Tris/HCl pH 7·5, 10 mM MgCl₂, 24 mM ATP and 1 µCi [γ-³²P]ATP (specific activity >5000 Ci mmol⁻¹/185 TBq mmol⁻¹; Amersham). Sp, Sm (Sigma), actinamine (J. Davies, University of British Columbia, Vancouver, Canada), streptidine (W. Piendl, University of Innsbruck, Austria), streptamine and s-inosamine were added at a final concentration of 8 mM. The reaction was stopped by an incubation for 5 min at 95 °C. The precipitated protein was removed by centrifugation (10000 g, 5 min) and 20 µl of the supernatant was used for HPLC analysis (column Spherosorb 80 SAX 5 µm; Knauer). The detection of phosphorylated products was carried out as described previously (Neumann et al., 1996). The radioactivity was measured online using a solid-phase scintillator unit (LS371, Beckmann).

Radiolabelled ADP was identified by co-chromatography with a standard solution containing 50 pmol unlabelled ADP and on-line determination of A₂₅₀.

**RESULTS**

**Isolation of Sp-resistant *S. lividans* transformants**

BgII-digested chromosomal DNA of *S. flavopersicus* NRRL2820 was ligated to BgII-digested and alkaline-phosphatase-treated pJ699. *S. lividans* TK64 was transformed with the ligation mixture and thioestrepton-resistant (Tsr) colonies were selected. About 8000 Tsr transformants were obtained and replica-plated to M40 agar containing Sp. There were no colonies showing resistance to Sp (later we found a BgII site in the N-terminal region of the resistance gene explaining this negative result). The experiment was repeated with chromosomal DNA digested with BamHI. After replicating the Tsr transformants (about 10000) to Sp-containing M40 agar plates, four colonies were found to be resistant to Sp (SpR). Restriction analysis of the plasmid DNA isolated from these transformants revealed that all of them contained an identical insert of 3·65 kb. One of the plasmids, named pDGL15, was further characterized.

Plasmid pDGL15 was re-transformed into *S. lividans* TK64 and all Tsr transformants showed resistance to Sp. To confirm the origin of the cloned fragment in plasmid pDGL15, Southern blot hybridization with the chromosomal DNA of *S. flavopersicus* was carried out. The 3·65 kb insert of pDGL15 was isolated as a HindIII fragment, labelled with DIG and hybridized to the chromosomal DNA cut with BamHI, BgIII and EcoRI. The fragment hybridized with a 3·65 kb BamHI-, an 87 kb BgIII- and a 6·6 kb EcoRI fragment of *S. flavopersicus* chromosomal DNA, but not with that from *S. lividans* TK64 (data not shown).

**Nucleotide sequence of the cloned DNA fragment containing the SpR determinant and analysis of the deduced proteins**

To obtain more information about the cloned DNA fragment conferring Sp resistance, the 3·65 kb HindIII fragment was inserted into the *E. coli* vector pIC20R in both orientations (pDGL21-2 and pDGL21-5). These plasmids and deletion derivatives were used for sequencing the complete fragment on both strands. Fig. 1 presents the nucleotide (nt) sequence of the 3651 bp fragment together with the deduced amino acid (aa) sequences of potential genes. Positions of ORFs are depicted schematically in Fig. 4. The DNA fragment showed a mean high G + C content of 67 mol % typical for *Streptomyces* DNA. Potential coding sequences were determined according to the highly biased codon usage in *Streptomyces* genes using the CODONPREFERENCE program (Devereux et al., 1984) and a Streptomyces codon usage table (Sedlmeyer & Altenbuchner, 1992). Four ORFs were identified on the 3651 bp fragment. One ORF begins at nt 3530 with an ATG start codon and ends at nt 2538 with a TGA stop codon (*spcN*, Fig. 1). The predicted gene product SpcN of 330 aa shares 33% identity with StrN (Fig. 2), a putative aminoglycoside PTS encoded by the Sm biosynthesis gene cluster of *S. griseus* (Pissowotzki et al., 1991). Another ORF (*spcR*) transcribed in the opposite direction to *spcN* starts at nt 1317 with GTG as translation initiation codon and ends at nt 2309 with a TAA stop codon. The gene product SpcR had the same number of amino acids (330) and shared 46% identity with StrR (Fig. 2), a transcriptional regulator protein of Sm biosynthesis (Distler et al., 1987). No RBs were present upstream of *spcN* and *spcR* within a reasonable distance (3–12 nt).

A region of about 900 nt upstream of *spcR* contained no other ORF having the highly biased codon usage of streptomycetes. Further upstream, two such ORFs were identified. *orfI* transcribed in the same direction as *spcR* and contained a stop codon at nt 272. The translational start codon was apparently outside the cloned fragment. The first 80 aa of the deduced 90 aa sequence showed a very high aa sequence identity (85%) to the N-terminal region (position 11–90, Fig. 3) of the amidinotransferase ADT1 of the Sm biosynthesis gene cluster of *S. griseus* (Distler et al., 1987; Tohyama et al., 1987). The *S. griseus* ADT1 consists of 347 aa and very likely starts at nt 399.
with an ATG translation initiation codon and ending at nt 127 with a TAA codon, hereby overlapping orfI. The first 47 aa of the deduced 80 aa sequence (Fig. 3) are 79% identical to the N-terminal end of the putative transposase InsA of IS112 (Rodicio et al., 1991). InsA of IS112 is 253/256 aa in size and therefore orfI might also be the result of a deletion fusing orfI and orfII somewhere between nt 240 and nt 260 (Fig. 1). A sequence at nt 1026–1043 is identical to the inverted repeat of IS112 in 14/15 out of 18 bp and might be one end of this IS112-derivative in S. flavopersicus.

**Localization of the SpR gene**

The only possible gene conferring Sp resistance was spcN according to the nucleotide sequence analysis. To prove that, deletions were made in the 3.65 kb fragment and a 15 bp sequence (IR) with high similarity to the putative transposase InsA of IS112 (Rodicio et al., 1991). The regions of ORF1 and ORFII with high similarity to ADTl of IS112 (Rodicio et al., 1991) are underlined and in bold.

**Fig. 1.** Nucleotide sequence and deduced polypeptide sequences of the 3651 bp BamIII fragment from S. flavopersicus encoding an Sp resistance gene. Polypeptides of genes transcribed from left to right are written below the corresponding nt sequence and the ones with opposite direction above. The ends of the polypeptides are marked by asterisks. Recognition sites of restriction enzymes are marked by an ATG translation initiation codon and ending at nt 127 with a TAA codon, hereby overlapping orfI. The first 47 aa of the deduced 80 aa sequence (Fig. 3) are 79% identical to the N-terminal end of the putative transposase InsA of IS112 (Rodicio et al., 1991). InsA of IS112 is 253/256 aa in size and therefore orfI might also be the result of a deletion fusing orfI and orfII somewhere between nt 240 and nt 260 (Fig. 1). A sequence at nt 1026–1043 is identical to the inverted repeat of IS112 in 14/15 out of 18 bp and might be one end of this IS112-derivative in S. flavopersicus.

**Localization of the SpR gene**

The only possible gene conferring Sp resistance was spcN according to the nucleotide sequence analysis. To prove that, deletions were made in the 3.65 kb fragment and tested in S. lividans by an antibiotic diffusion assay.
Furthermore, the gene was amplified by PCR and expressed in *E. coli.*

For deletion analysis, various restriction fragments (Fig. 4) were first subcloned in pIC20H (Marsh et al., 1984) and in a second step inserted in the *E. coli–* *S. lividans* shuttle vector pJOE875. The plasmids pJOE2327 and pJOE2619 had deletions of *orfI* and *orfII,* the plasmids pJOE2494, pJOE2495 and pJOE2618 had deletions of *orfI,* *orfII* and *spcR.* In addition, pJOE2495 had a deletion ending 62 bp upstream of the translation initiation codon ATG of *spcN* and in pJOE2618 the upstream deletion ended within this gene. *S. lividans* TK64 was transformed with the plasmids and inhibition zones were determined on agar plates with filter discs containing 1 mg Sp. As shown in Fig. 4, *orfI, orfII* and *spcR* are not essential for Sp resistance and the 62 bp sequence upstream of *spcN* is enough to allow expression of *spcN,* whereas the deletion ending in *spcN* leads to Sp sensitivity. No increase of resistance to Sm, kanamycin, gentamicin or neomycin was seen in *S. lividans* TK64(pJOE2328) carrying the complete 3-65 kb fragment in comparison to cells with the plasmid pJOE875 (data not shown).

For expression in *E. coli,* *spcN* was amplified by PCR with primers complementary to the N- and C-terminal encoding parts of the gene. In addition, nucleotides forming an *NdeI* site and overlapping the ATG start codon as well as a *HindIII* site following the TGA stop codon were added to the 5′ ends of the primers. The amplified DNA was cleaved with *NdeI* and *HindIII* and inserted into pJOE2453 cut with the same restriction enzymes. The plasmid pJOE2453 is a derivative of the expression vector pBTacl (Brosius et al., 1981). In this vector the original *NdeI* site was removed and complementary oligonucleotides containing an *NdeI* site and the RBS of T7 gene 10 to improve expression of *Streptomyces* genes in *E. coli* were inserted downstream of the *E. coli* tac promoter. *E. coli* JM109 with the corresponding plasmid pJOE2686 (Fig. 5) was resistant and grew on 2× YT agar plates supplemented with 100 μg Sp ml⁻¹. No IPTG for induction of the tac promoter had to be added, presumably due to the high basal expression of this promoter even in the presence of the lac promoter in *JM109.* In accordance with this, a new weak protein band of M, 36000 (calculated M, of SpcN is 36091) was seen in crude extracts of *E. coli* JM109(pJOE2686) when cells were induced with IPTG (Fig. 5).

### spcN encodes a PTS

Cell-free protein extracts of IPTG-induced *E. coli* JM109 containing the *spcN* expression vector pJOE2686 were prepared and analysed for a Sp-phosphorylating activity. With this extract and [γ-³²P]ATP an additional peak of radioactivity was detected by HPLC analysis with either Sp or actinamine as phosphate acceptors.
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phorylated derivatives of Sp and actinamine, as they were only formed by protein extracts containing SpcN and with Sp or actinamine added to the reaction mixture. Sp phosphate and actinamine phosphate had retention times of 3.5 and 4.2 min, respectively (Fig. 6). Two additional 32P-labelled compounds also present or formed by protein extracts of E. coli missing SpcN, were identified as ATP and ADP. A third phosphorylated product which eluted with a retention time of 14 min was not identified. This product was also generated without a phosphate acceptor added. There was no phosphorylation of Sm, streptidine, streptamine nor s-inosamine by SpcN protein (data not shown).

**DISCUSSION**

Resistance to aminoglycoside or aminocyclitol antibiotics in clinical isolates of bacteria is usually mediated by enzymes encoded by plasmids or transposons. The enzymes modify the antibiotics by O-phosphorylation, O-adenylylation or N-acetylation (Benveniste & Davies, 1973; Haas & Dowding, 1975) in a way that they can no longer interact with their target sites. In addition, mutations of the target sites, usually of ribosomal proteins and the rRNA or modification of specific nucleotides of the target sites can lead to resistance (Skeggs et al., 1987). For Sp resistance two mechanisms have been described so far, target site mutations, changing for example the residue C1129 of the E. coli 16S rRNA (Brink et al., 1994), and plasmid-encoded adenylyltransferases which modify Sp and Sm. To our knowledge an ATP-dependent phosphorylation of Sp by a PTS as observed with SpcN from S. flavopersicus has only been reported once for a Campylobacter strain and was not further investigated (Pinto-Alphandary et al., 1990). Since both Sp and actinamine were substrates for SpcN it is most likely that Sp is phosphorylated at one of the hydroxyl groups at position 2 or 6 of the actinamine moiety (Fig. 7).

SpcN shows similarity to StrN mainly in its C-terminal part, a putative enzyme involved in Sm biosynthesis. StrN was postulated to be a PTS (Pissowotzky et al., 1991) according to its similarity to known PTSs like APH (3') and APH (6) from S. griseus (Heinzel et al., 1988; Distler et al., 1987), especially in a structural motif II/III with the consensus amino acid sequence HxD(4x)NhyhyDhy (hy = hydrophobic residues). This motif is suggested to be involved in substrate binding and transferase activity and can also be identified at a homologous position in SpcN (aa 223-243) but at a homologous position in SpcN (aa 223-243) but

![Fig. 4. Physical map and deletion analysis of the 3651 bp BamHl fragment. Fragments indicated below the restriction map by black lines were inserted in pJOE875, brought into S. lividans TK64 by transformation and the recombinants tested by the antibiotic diffusion assay for resistance to Sp (1 mg Sp on a 6 mm filter disc). Inhibition zones (mm) were determined after 48 h growth on GME plates. Symbols: (+) resistant; (−) sensitive.](image-url)
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**Fig. 5.** Restriction map of the *spcN* expression plasmid pJOE2686 (left) and SDS-PAGE analysis of crude extracts from *E. coli* JM109(pJOE2453) and JM109(pJOE2686) (right). Lanes: M, M, standards; 1 and 2, JM109(pJOE2453); 3 and 4, JM109(pJOE2686). Symbols: +, cells were induced with IPTG 4 h before crude extracts were prepared; −, noninduced cells.

**Fig. 6.** Measurement of PTS activity in cell-free extracts of *E. coli* JM109(pJOE2686). Extracts were incubated with Sp (b-e), actinamine (g) and with no phosphate acceptor (f) for 0 (b), 20 (c), 40 (d) and 60 (e, g) min, and analysed by HPLC. Line a shows the HPLC elution profile of [*γ-32P*]ATP. The peaks representing ADP and ATP are indicated.

**Fig. 7.** Chemical structure of Sp. Possible positions for O-phosphorylation in the actinamine moiety are indicated by arrows.

Spectinomycin resistance initiation sequence of *spcN* according to the deletion analysis. No *E. coli*-like σ70 promoter sequence could be identified. Furthermore, *spcN* is lacking an RBS. This is not unusual, at least 11 genes isolated from actinomycetes, mainly antibiotic resistance genes, have leaderless mRNAs. Transcription of the mRNA and translation start at the same nucleotide (Janssen, 1993). Primer extension and S1 mapping experiments should prove this hypothesis and help to localize the *spcN* promoter.

*spcR* found on the 3.65 kb fragment is divergently oriented relative to *spcN*. Two palindromic sequences between the two genes might be transcriptional terminators, one for each gene (Fig. 1). The homologous gene product, *StrR*, is a positive regulator of the Sm biosynthesis pathway in *S. griseus* with a helix-turn-helix motif typical of DNA-binding proteins (Retzlaff & Distler, 1995). This regulatory gene is the final target of
a complicated regulatory cascade involving many pleiotropic and specific factors. One of the essential molecules in this regulatory network is the A-factor, an autoinducer produced by \textit{S. griseus} and other streptomycetes. In the presence of A-factor, transcription of \textit{strR} finally binds to a specific nucleotide cluster (Vujaklija et al., 1993). A helix-turn-helix motif could also be identified in SpcR (Fig. 1) using the blocks searcher (Henikoff & Henikoff, 1994) but no StrR homologous DNA-binding sites (Retzlaff & Distler, 1995) were found upstream of \textit{spcR} and \textit{spcN}. Future studies will show if \textit{spcR} is regulated similarly to \textit{strR} and if A-factor or related \textit{γ}-butyrolactones are involved in regulation.

A very interesting and surprising finding was the presence of an incomplete amidinotransferase gene upstream of \textit{spcN}. The deduced polypeptide was nearly 90\% identical with the N-terminal amino acid sequence of the Sm biosynthesis amidinotransferases encoded by \textit{strB1} and \textit{strB2} (Distler et al., 1987; Pissowotzki et al., 1991). The deduced protein sequence of a divergently oriented and overlapping ORF again was nearly identical to the N-terminal end of the putative IS112 transposase (Rodicio et al., 1991). The similarity of ORF1 to amidinotransferases and of ORFII to IS112 transposase ended within the overlapping region of the two ORFs, between nt 240–260 (Fig. 1). This is a clear indication for a deletion between an amidinotransferase gene and a copy of an IS112-derivative fusing the two genes as observed on the 3.65 kb fragment. Since the two truncated ORFs were not completely identical to ADT1 of \textit{S. griseus} and InsA of IS122, the exact fusion point could not be determined or the possibility of more than one deletion event excluded.

Amidinotransferases are necessary for the synthesis of the related antibiotics Sm and bluensomycin but not for Sp. It is therefore tempting to speculate that the Sp biosynthesis pathway evolved from the Sm or related biosynthesis pathway by inactivation of genes (\textit{strB} genes) and adaptation of genes by mutations to new functions (\textit{spcN}).

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