A spectinomycin resistance determinant from the spectinomycin producer *Streptomyces flavopersicus*

Dimitrina Lyutskanova,¹† Jürgen Distler² and Josef Altenbuchner¹

Author for correspondence: Josef Altenbuchner. Tel: +49 711 685 7591. Fax: +49 711 685 6973.
e-mail: joe@genius.biologie.uni-stuttgart.de

¹ Institut für Industrielle Genetik, Universität Stuttgart, Allmandring 31, 70569 Stuttgart, Germany

² FB9-Mikrobiologie, Bergische Universität-GH, 42097 Wuppertal, Germany

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 (Schermerhorn *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 & Ito, 1982). *In vivo* feeding experiments have recently indicated the key roles of glutamine-aminocyclitol aminotransferases in biosynthesis of Sp and the other aminoglycoside antibiotics (Walker, 1995).

The genes required for the biosynthesis of an antibiotic

† **Present address:** Institute of Microbiology, Bulgarian Academy of Sciences, Acad. G. Bonchev str., BL26, Sofia 1113, Bulgaria.

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* NRRL2820 was the donor of the *Sp* resistance genes (Oliver *et al.*, 1962). *Streptomyces lividans* TK64 (Hopwood *et al.*, 1983) was used as a *Sp*- and thiostrepton-sensitive recipient in shotgun cloning experiments. *Escherichia coli* JM109 (Yanisch-Perron *et al.*, 1985) was the host for transformations in subcloning experiments. Plasmid pIJ699 (Kieser & Melton, 1988) was used as a vector in *S. lividans* and pIC20R, pIC20H (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 pBTac1 (Brosius *et al.*, 1981) was modified. The *NdeI* 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 *NdeI* site with an ATG start codon were inserted to give pJOE2453 (oligonucleotide 1: 5'-AATTCTTAAGAAG-GAGATATACATATGAAATCTCCCGCCCTGGCCG-3'; oligonucleotide 2: 5'-GATCCGGCCAGGGCGGGAGATTTCATATGTATATCTCCTTCTTAAG-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 MgCl and 0.5% glycine (Hopwood *et al.*, 1985). For protoplast regeneration and to get spores from *S. lividans*, R2YE agar plates (Hopwood *et al.*, 1985) were used, supplemented with 40 µg thiostrepton ml⁻¹ when necessary. *E. coli* strains were grown at 37 °C in 2 × YT 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* NRRL2820, chromosomal DNA was digested with *BamHI* or *BglII* and ligated to the vector pIJ699 cleaved with *BglII* 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 thiostrepton 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 pIC20R (pDGL21-2; pDGL21-5). Various deletions were introduced by using restriction enzymes cutting in the polylinker sequence of pIC20R and in the insert or by subcloning small fragments in pIC20R. 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 BLASTN, 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 searcher 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'-ACACATATGGAAGATCTT-CCTGAGAA-3'; 3'-end of *spcN*: 5'-ACCAAGCTTCAG-CTCGGTAATTCGG-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 *PstI* 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*-*SphI* fragment and a 1.25 kb *PvuII*-*NcoI* fragment from pDGL21-2 after blunt-ending with Klenow polymerase between the two *SmaI* 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 10⁷ 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 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 2 × YT containing 100 µg ampicillin ml⁻¹ and grown at 37 °C in a waterbath shaker to OD₆₀₀ 0.4. Then 0.1 mM 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 Feinbiochemica). Proteins from the low-M_r 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 OD₅₆₀ 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 [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 Spherisorb 80 SAX 5 µm; Knauer). The detection of phosphorylated products was carried out as described previously (Neumann *et al.*, 1996). The radioactivity was measured on-line 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 A₂₆₀.

RESULTS

Isolation of Sp-resistant *S. lividans* transformants

*Bgl*II-digested chromosomal DNA of *S. flavopersicus* NRRL2820 was ligated to *Bgl*II-digested and alkaline-phosphatase-treated pIJ699 DNA. *S. lividans* TK64 was transformed with the ligation mixture and thiostrepton-resistant (Ts^R) colonies were selected. About 8000 Ts^R transformants were obtained and replica-plated to M40 agar containing Sp. There were no colonies showing resistance to Sp (later we found a *Bgl*II site in the N-terminal region of the resistance gene explaining this negative result). The experiment was repeated with chromosomal DNA digested with *Bam*HI. After replica-plating of the Ts^R transformants (about 10000) to Sp-containing M40 agar plates, four colonies were found to be resistant to Sp (Sp^R). 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 Ts^R 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 *Hind*III fragment, labelled with DIG and hybridized to the chromosomal DNA cut with *Bam*HI, *Bgl*II and *Eco*RI. The fragment hybridized with a 3.65 kb *Bam*HI-, an 8.7 kb *Bgl*II- and a 6.6 kb *Eco*RI 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 Sp^R determinant and analysis of the deduced proteins

To obtain more information about the cloned DNA fragment conferring Sp resistance, the 3.65 kb *Hind*III 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 (Sedlmeier & 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 *Streptomyces 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 RBSs 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 *orfI* is deleted in its C-terminal part. This may have been caused by an insertion sequence (IS). An ORF (*orfII*) was found in opposite orientation to *orfI* starting 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 *orfII* 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

repeats of IS112 in 14/15 out of 18 bp and might be one end of this IS112-derivative in *S. flavopersicus*.

Localization of the *Sp^R* 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.

Fig. 2. Comparison of the deduced SpcN and SpcR proteins with StrN and StrR from the Sm biosynthesis operon of *S. griseus*. The dot plot analysis shows the collinear alignments of (a) SpcN and StrN compared in a window of 25 residues and a stringency of 14, and (b) SpcR and StrR compared in a window of 25 and a stringency of 18.

Fig. 3. Alignments of (a) the polypeptide sequence ORFI and the amidinotransferase ADT1 (*strB1*) from *S. griseus* (Distler et al., 1987) and (b) of ORFII and the putative transposase InsA of IS112 (Rodicio et al., 1991). Identical aa are marked by vertical lines, the ends of ORFI and ORFII by asterisks.

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 pBTac1 (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 *lacI^q* gene in JM109. In accordance with this, a new weak protein band of *M_r* 36000 (calculated *M_r* of SpcN is 36091) was seen in crude extracts of *E. coli* JM109(pJOE2686) in comparison to JM109(pJOE2453) on denaturing SDS-PAGE. The intensity of this band was increased severalfold in crude extracts of 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

Fig. 4. Physical map and deletion analysis of the 3651 bp *Bam*HI 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 5 mm filter disc). Inhibition zones (mm) were determined after 48 h growth on GME plates. Symbols: (+) resistant; (-) sensitive.

(Fig. 6b–e, g). Most likely these products are phosphorylated 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 ^{32}P -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-adenylation 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 C_{1192} 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 (Pissowatzky *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) Nhyhyhy (7–9x) hyhyDhy (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 with the first aspartate changed to a glutamate residue. In the Sm producer *S. griseus* the major resistance enzyme is the aminoglycoside 6-phosphotransferase APH (6) encoded by *strA* (*aphD*, *sph*). StrN is thought to be involved in Sm biosynthesis since expression of the gene in *E. coli* does not confer resistance to Sm (unpublished). In contrast, the homologous SpcN is probably the major Sp resistance enzyme of *S. flavopersicus*. No other fragment mediating resistance to Sp was obtained in the cloning experiments, even when using other restriction enzymes and other cloning vectors (not shown). Furthermore, cloning of the Sp resistance genes from the Sp producer *Streptomyces spectabilis* revealed a gene highly homologous to SpcN (unpublished).

No putative Sp biosynthesis gene was found on the 3651 bp fragment from *S. flavopersicus*, but the presence of an *strR* homologous gene near *spcN* is a good indication that this gene is part of the Sp biosynthesis cluster. In *S. lividans* *spcN* seems to be constitutively expressed. No other gene was necessary for expression as shown by deletion analysis. Maybe this is caused by the location of *spcN* on a high-copy plasmid in *S. lividans*, whereas in the chromosome of *S. flavopersicus* *spcR* is needed for regulation of *spcN*. A short palindromic sequence upstream of *spcN* might be involved in regulation. The *spcN* promoter should be located at this region within about 65 bp upstream of the trans-

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_r 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.

lation initiation sequence of *spcN* according to the deletion analysis. No *E. coli*-like σ^{70} 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

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 $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. 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.

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 auto-inducer produced by *S. griseus* and other streptomycetes. In the presence of A-factor, transcription of *strR* is induced and StrR finally binds to a specific nucleotide sequence present at several locations in the Sm gene 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 *spcR* and *spcN*. Future studies will show if *spcR* is regulated similarly to *strR* and if A-factor or related γ -butyrolactones are involved in regulation.

A very interesting and surprising finding was the presence of an incomplete amidinotransferase gene upstream of *spcN*. The deduced polypeptide was nearly 90% identical with the N-terminal amino acid sequence of the Sm biosynthesis amidinotransferases encoded by *strB1* and *strB2* (Distler *et al.*, 1987; Pissowatzki *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 ORFI 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 *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 (*strB* genes) and adaptation of genes by mutations to new functions (*spcN*).

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