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Protein secretion in *Streptomyces griseus* N2-3-11: characterization of the *secA* gene and its growth phase-dependent expression

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Abstract

The chromosomal region encoding the *secA* gene of *Streptomyces griseus* N2-3-11 was cloned and analyzed. The *secA* gene encodes a polypeptide of 939 aa with a molecular mass of 105 kDa. The growth defect of temperature sensitive *Escherichia coli secA* mutants was not restored by the *S. griseus* SecA. The *secA* promoter was analyzed and the transcriptional start point of the gene was determined. Northern blot and Western blot analyses revealed a growth phase dependent *secA* expression. The integration of an additional copy of the *S. griseus secA* gene into the genome of *S. lividans* TK23 had no visible effect on the efficiency of protein secretion.

Keywords: *Streptomyces griseus*; Protein secretion; *secA* gene; *secA* expression

1. Introduction

Streptomycetes secrete large amounts of secondary metabolites such as antibiotics, fungicides or herbicides as well as a number of proteins and hydrolytic enzymes, e.g. proteases, chitinases, amylases or cellulases [1,2]. The metabolic diversity of these indigenous soil microorganisms has been widely exploited in industry and agriculture. Taking into account the increasing biotechnological applications of streptomycetes for the expression and secretion of heterologous proteins, more detailed information is needed concerning the components involved in protein secretion.

The general secretory pathway (GSP) is well characterized in the Gram-negative bacterium *Escherichia coli* [3]. Genetic studies have identified at least seven genes involved in protein translocation across the cytoplasmic membrane. The central component of the GSP is the SecA protein which interacts with the preprotein either bound to SecB or unbound [4], the phospholipid layer, and the integral membrane proteins SecY, SecE, and SecG [5,6]. In its homodimeric form [7] SecA catalyzes the translocation of precursor proteins across the membrane by undergoing ATP-driven cycles of membrane insertion and deinsertion [8]. SecA homologues have been identified in higher plants, algae, Gram-negative and Gram-positive bacteria. The SecA proteins of Gram-positive bacteria appear to be structurally and functionally similar to SecA of *E. coli*. Although

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the *secA* gene of *Streptomyces lividans* had recently been identified by two independent groups [9,10], the regulation of *secA* gene expression in Streptomycetes or other Gram-positive bacteria has not been studied as yet. In this study we report on the cloning, functional characterization and transcriptional regulation of the *secA* gene from *S. griseus* N2-3-11.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

The strains and plasmids used are listed in Table 1. The methods used for the cultivation of *Streptomyces* species and *E. coli* strains were described elsewhere [11–13]. The *E. coli secA* mutants were grown as described [14]. Antibiotics were used at the following concentrations: ampicillin (Ap) 100 μg/ml, hygromycin (Hy) 200 μg/ml, and thiostrepton (Th) 25 μg/ml. XylE activities of cells grown in TSB medium were assayed as described by Ingram et al. [15].

2.2. DNA and RNA manipulations

DNA and RNA manipulations were performed as described elsewhere [11–13]. RNA was isolated using the Qiagen RNA/DNA Midi Kit (Qiagen, Hilden, Germany). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out ac-

Table 1
Bacterial strains, bacteriophages and plasmids

Strain/plasmid	Relevant genotype	Source
Strains		
Streptomycetes		
S. lividans TK23		D.A. Hopwood
S. griseus N2-3-11	Streptomycin (Sm) producer	Kakem Chem. Co., Tokyo, Japan
E. coli		
DH5α	(*thi*-1, *endA1*, *gyrA96*, *hsdR17*, *relA1*, *recA1*, *supE44*, Δ(*lacZYA-argF*)*U169*, ϕ80 *lacZ*ΔM15	[28]
NM539	*supF, hsdR lacY* (P2)	Promega, Heidelberg, Germany
JM109 (DE3)	(*thi*-1, *endA1*, *gyrA96*, *hsdR17*, *relA1*, *recA1*, *supE44*, Δ(*lac-proAB*), F′ *traD36 proAB lacI*^{q} ZΔM15) λ(DE3)	Promega, Heidelberg, Germany
MM52	(F^{-}, *araD139*, Δ(*argF-lac*)*U169*, *rpsL150*, *relA1*, *flbB5301*, *deoC1, ptsF25, secA51*^{ts})	[29]
BA13	(F^{-}, *araD139*, Δ(*argF-lac*)*U169*, *rpsL150*, *relA1*, *flbB5301*, *deoC1*, *ptsF25*, *secA13*^{am}, *supF*^{ts})	[30]
MM66	(F^{-}, *araD139*, Δ(*argF-lac*)*U169*, *rpsL150*, *relA1*, *flbB5301*, *deoC1*, *ptsF25*, *geneX*^{am}, *supF*^{ts})	[31]
Plasmids/bacteriophages		
λEMBL3 library	*S. griseus* N2-3-11 gene library in λEMBL3	[32]
λsecA8	λEMBL3 phage containing the complete *S. griseus secA* gene	this work
pBluescriptSKII^{+}	Ap^{R}	Stratagene, Heidelberg, Germany
pKSA2	pBluescript SK II^{+} with *secA* of *S. griseus* (3 kb *Apa*I insert)	this work
pT7-7	Ap^{R}, P$_{T7\phi10}$	[33]
pT7A2	pT7-7 containing the *secA* gene as a *Nde*I-*Eco*RI fragment	this work
pTRC99A	Ap^{R}, P$_{trc}$	[34]
pTRA2	pTRC99A containing the *secA* gene as a *Bam*HI-*Xba*I fragment isolated from pT7A2	this work
pWKD13	low copy number, Th^{R}, promoterless *xylE* gene	[35]
pWKDA2	pWKD13 *Hin*dIII/PolIk-*Eco*RI digested containing a 406 bp *Kpn*I/PolIk-*Eco*RI fragment from pKSA2 which comprises 79 bp of the *secA* upstream region	this work
pWKDAN2	pWKD13 *Eco*RI-*Hin*dIII/PolIk digested containing a 300 bp *Xho*II-*Nco*I/PolIk fragment of λsecA8 which comprises 243 bp of the *secA* upstream region	this work
pHM8a	mini-circle, Hy^{R}, P$_{E}$	[36]
pHMA2	pHM8a containing the *secA* gene as a *Nde*I-*Bam*HI fragment isolated from pT7A2	this work

cording to the protocol of Laemmli [16]. Genomic DNA from *S. griseus* N2-3-11 was isolated using the modified CTAB method as described previously [17]. For hybridization assays DNA probes were labelled with [^{32}P]dCTP using the Mega Prime Kit (Amersham Buchler, Braunschweig, Germany). Primer extension experiments were performed with the oligonucleotide xylE1 (5′-CGGTCGCATTACACC-TTTGTTCAT-3′OH) which is complementary to the 5′-end of the *xylE* gene.

2.3. PCR and nucleotide sequencing

PCR experiments were performed using a Personal Thermocycler (Biometra, Göttingen, Germany) and either *Taq* DNA polymerase (Life Technologies, Eggenstein, Germany) or Vent DNA polymerase (NEN Biolabs, Schwalbach, Germany) in the presence of 10% (v/v) dimethylsulfoxide. Oligonucleotides were synthesized on an ABI DNA synthesizer model 381A.

The degenerate oligonucleotides secA1 (5′-GTS-CACGTSGTSACSGTSAACGA-3′OH) and secA4r (5′-CCSGCCATGTTSGTSGCGAT-3′OH) were used for the amplification of the internal *secA* fragment from genomic DNA of *S. griseus*. The PCR primers secANde (5′-GACCAGCCCATATGTCC-GTCTTC-3′OH) and secAEco (5′-ACCCTGAATT-CACTTCTTGCGACG) were used for generating *Nde*I and *Eco*RI restriction sites at the 5′- and 3′-end of the gene, respectively. The sequence of the PCR product was determined and corresponded to the original sequence.

Nucleotide sequences were determined using an ALF automated DNA sequencer (Pharmacia, Freiburg, Germany), fluorescein-labelled primers and the dideoxy chain termination procedure [18].

2.4. Immunoblot analysis and production of antiserum

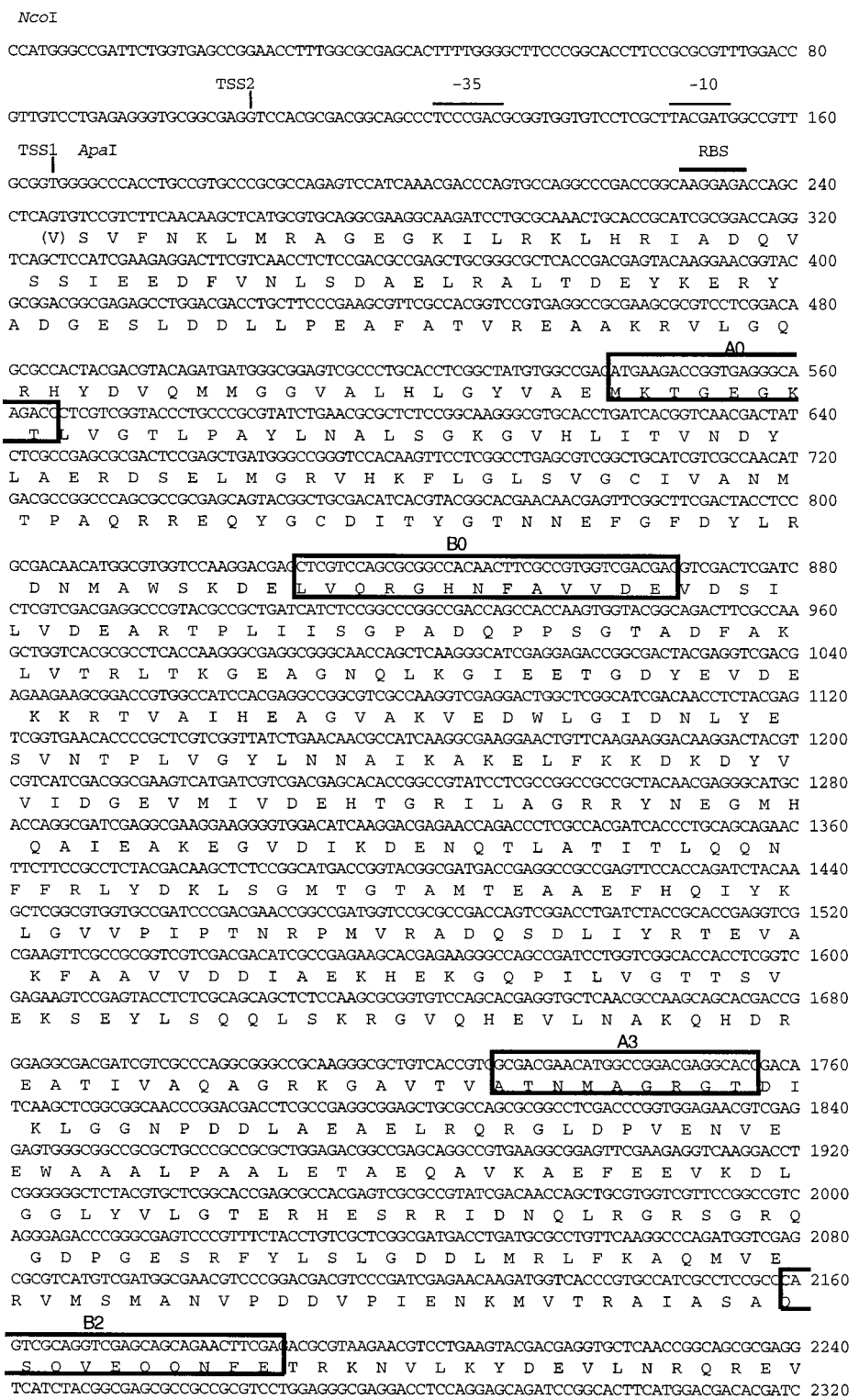
Proteins were transferred electrophoretically from SDS-PAGE gels to Hybond C$^+$ membranes (Amersham Buchler, Braunschweig, Germany) and stained with Ponceau S. The blots were blocked in Tris buffered saline containing 0.1% (v/v) Tween-80 and 0.2% (w/v) non-fat dried milk. Antisera raised against the *Bacillus subtilis* SecA protein were obtained from R. Freudl and M. Klein, Jülich, Germany. Immunoblot assays were performed with antisera diluted 1:1000 or 1:5000 following standard protocols [12]. Immunodetection was performed with the BM Chromogenic Western Blotting Kit from Boehringer-Mannheim, Germany. The *S. griseus* SecA protein was overproduced in *E. coli* JM109 (DE3)/pT7A2 as insoluble inclusion bodies. The inclusion bodies were enriched and the SecA protein was purified by preparative SDS-PAGE followed by electroelution. The antiserum raised against the purified SecA was obtained from Eurogentec (Ougrée, Belgium) and used in a dilution of 1:10 000.

3. Results and discussion

3.1. Cloning and sequencing of the secA gene from S. griseus N2-3-11

Two degenerate oligonucleotides were derived from highly conserved regions (VHVVTVNE and IATNMAG, corresponding to aa 126–132 and 502–508, respectively, of the *E. coli* SecA protein) within the SecA proteins of *E. coli* [19], *B. subtilis* [20], and *Staphylococcus carnosus* [21]. Using these primers and chromosomal DNA of *S. griseus* as a template, a 1.1 kb fragment was amplified in PCR assays. This fragment contained the expected *secA* region of *S. griseus* N2-3-11.

The internal *secA* fragment was used as a probe for the screening of a DNA library of *S. griseus* in λEMBL3. Four of the 10 000 recombinant phages tested hybridized with the probe. Further analyses led to the cloning of a 3 kb *Apa*I fragment containing the complete *secA* gene from *S. griseus* (Fig. 1). The *secA* gene (2820 bp) starts with a GTG start codon located 79 nt downstream of the *Apa*I site. The coding region has a G+C content of 65.5% with 93% G or C in the third position of the codon, which is typical for streptomycete genes [22]. A putative ribosomal binding site precedes the proposed GTG start codon (AGGAGA, nt 64–69). The deduced protein (939 aa, estimated M_r 105 044) has 47%, 50%, 48%, and 85% aa similarity to the respective SecA proteins of *E. coli*, *B. subtilis*, *S. carnosus*, and *S. lividans*. Although the SecA proteins of *S. griseus* and *S. lividans* are very similar, they differ remarkably around aa 380. In this otherwise highly


NcoI
TSS2
-35
-10
TSS1
ApaI
RBS
A0
B0
A3
B2

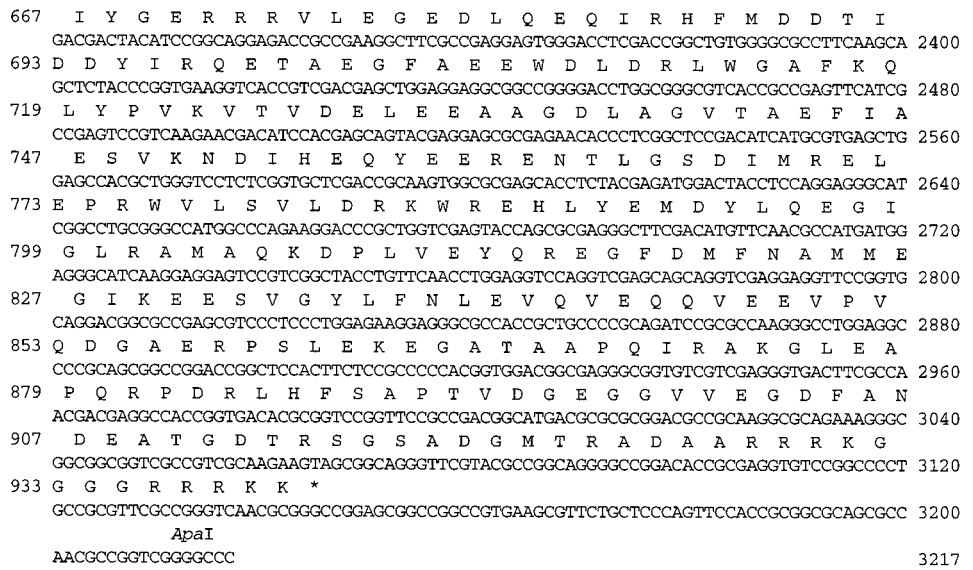


Fig. 1. Nucleotide sequence of the *S. griseus* N2-3-11 *secA* region. The determined DNA sequence extends from the *Nco*I site located 243 nt upstream of the postulated *secA* start codon to the *Apa*I site 150 nt downstream of the TAG stop codon, the deduced aa sequence is given. The transcriptional start sites (TSS1 and TSS2) are indicated. The postulated −35 and −10 regions of TSS1 and the putative ribosomal binding site (RBS) are marked. The postulated high and low affinity ATP binding sites A0, B0, and A3, B2 are boxed. Position numbers on the left and the right side mark the protein and the nucleotide sequence, respectively. (V) indicates a methionine encoded by the GTG start codon. The sequence is available from the EMBL nucleotide database under accession number Y10980.

conserved region among all SecA proteins an insertion of 10 aa is present in the *S. lividans* protein [9,10], but missing in the *S. griseus* and in the *S. galbus* SecA proteins (our unpublished data). Two Walker A motifs (A0, A3) and one Walker B motif (B0) responsible for the binding of ATP [23], which were characterized in the SecA proteins of *E. coli* and *B. subtilis* [24], are also present in the *S. griseus* protein. An additional Walker A-box-like sequence motif is located at the N-terminus (aa 11–14, aa sequence GEGK) of the *S. griseus* SecA protein. This motif is also conserved in the SecA protein of *S. lividans*. Whether this additional motif plays an essential role in ATP binding and hydrolysis has to be tested.

3.2. Expression of the S. griseus SecA protein in temperature sensitive E. coli secA mutants

Plasmid pTRA2 was transformed into the *E. coli* *secA*^{ts} mutants MM52 (L43P), BA13 (*secA*^{am}), and MM66 (*geneX*^{am}). Growth of the mutants was not restored by the SecA protein from *S. griseus* under any condition tested. The expression of the SecA protein by the various constructs used for the complementation assays was confirmed by immunoblot analysis using an antiserum raised against *B. subtilis* SecA. Complementation of *E. coli secA*^{ts} mutants with the SecA protein of *S. lividans* or *S. carnosus* failed, too [9,10,21]. However, the SecA protein of *S. lividans* was shown to complement the azide resistant (Az^{R}) *secA*4 mutants of *E. coli* [9].

3.3. Analysis of the putative secA gene promoter region

The plasmids pWKD13, pWKDA2, pWKDAN2 were used for promoter activity tests in *S. lividans* TK23. While no XylE activity was observed in *S. lividans* TK23 harboring pWKD13 (control) and pWKDA2, the fragment cloned in pWKDAN2 led to the expression of the *xylE* reporter gene (Fig. 2A). The XylE activity was determined at different growth phases. The promoter activity kept at a constant level during the exponential growth phase and decreased in the stationary growth phase (Fig. 2A).

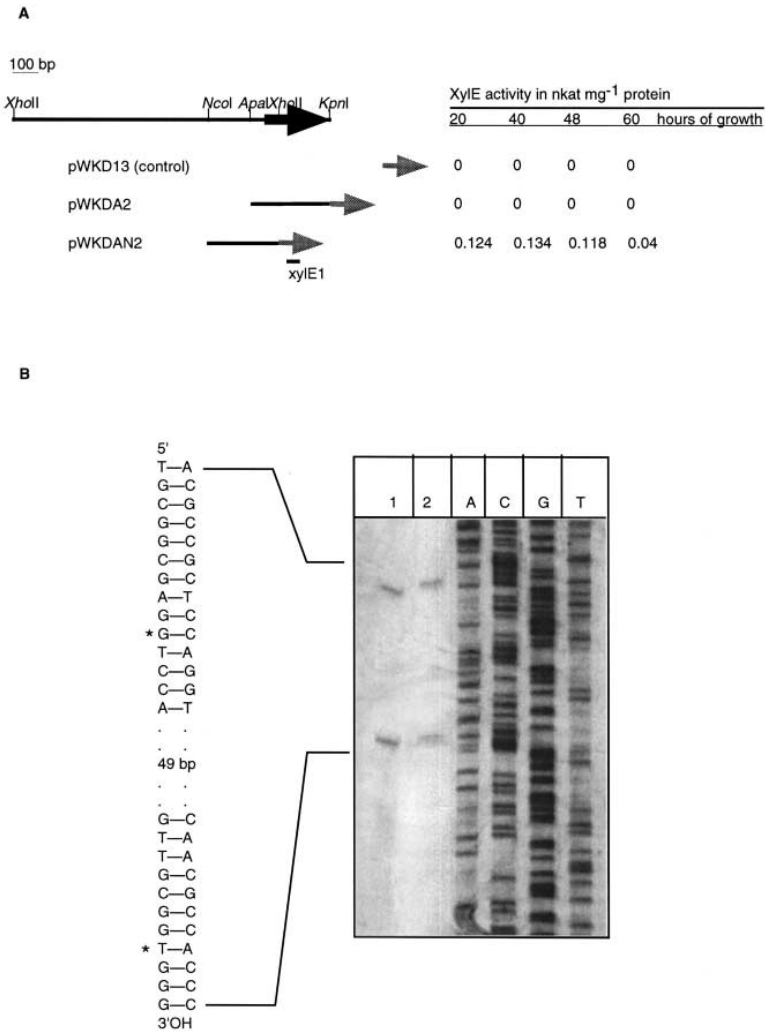


Fig. 2. Analyses of the *secA* pomoter region. A: Promoter probe assays using the promoterless *xylE* gene as a reporter gene. Selected restriction sites of the *secA* promoter region (upper lines) and the start of the *secA* gene (black arrow) are indicated. The fragments (black line) which were fused to the *xylE* gene (gray arrow) are shown and the respective plasmid name is given. The black bar shows the binding site of the oligonucleotide named xylE1 which was used for the primer extension analysis. XylE activities of *S. lividans* TK23 harboring the respective promoter probe plasmids pWKD13, pWKDA2 or pWKDAN2 are given. The data represent mean results of three experiments. B: Primer extension analysis of the 5′-end of the *secA* mRNA. Lanes 1 and 2 represent the primer extension products obtained in reverse transcriptase reactions performed at 42°C (lane 1) or 50°C (lane 2). The lanes labelled A, C, G, T show the DNA sequence obtained with the same primer (xylE1). Only the relevant part of the sequence is given. The apparent transcriptional start sites (TSS) are indicated by asterisks.

These data correlate with the results obtained in Northern blot assays (see Section 3.4).

For the determination of the transcriptional start site (TSS) of the *secA* gene we used RNA of *S. lividans* TK23/pWKDAN2 isolated from cells in the late exponential growth phase. Primer extension analysis (Fig. 2B) revealed two transcriptional start points. The postulated −10 and −35 regions of TSS1 are similar to *E. coli* $E\sigma^{70}$-like promoters (Fig. 1). These findings were supported by the fact that *E. coli* strains harboring pWKDAN2 exhibited XylE activity whereas strains harboring pWKD13 or

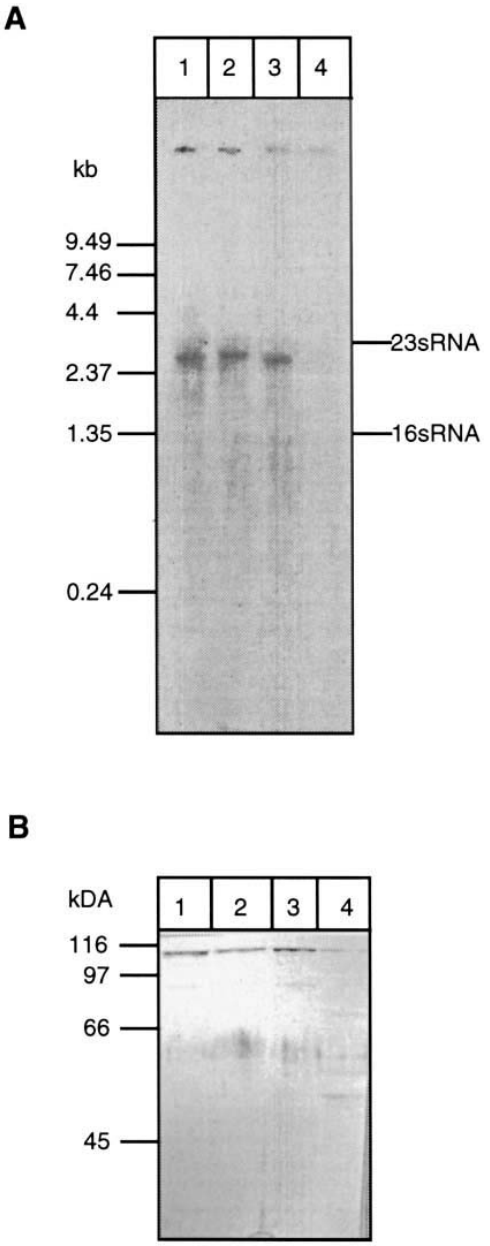


Fig. 3. Growth phase dependent expression of *secA*. A: Northern blot analysis of total RNA isolated from *S. griseus* N2-3-11 in the early log phase (OD_{560} 0.2, lane 1), mid log phase (OD_{560} 0.6, lane 2), late log phase (OD_{560} 2, lane 3) and stationary phase (OD_{560} 12, lane 4). 5 μg/lane of RNA was applied to a 1.4% agarose/0.22 M formaldehyde gel and electrophorized in 0.22 M formaldehyde/MOPS running buffer. RNA Ladder (Life Technologies, Eggenstein, Germany) was used as length standard. The RNA was transferred to a Hybond-N^+ membrane (Amersham Buchler, Braunschweig, Germany) by capillary blotting using 20×SSC. Hybridization was performed at 50°C in high-SDS buffer (Boehringer Mannheim, Germany). An internal 1.1 kb *Pst*I fragment of the *secA* gene was used as a probe. Filters were washed twice in 2×SSC/0.1% SDS at 50°C. On the left the migration of the respective marker RNA bands is shown, on the right the location of the 16S rRNA and the 23S rRNA, respectively. B: Western blot analysis using a polyclonal antiserum raised against the SecA protein from *S. griseus* N2-3-11. 15 μg crude cell extract proteins of *S. griseus* from different growth phases in TSB medium (cf. Fig. 2A, lanes 1–4 represent the same growth phases as in A) were seperated on an 9% SDS-PAGE gel and transferred to a nitrocellulose membrane.
←

pWKDA2 did not (data not shown). Most of the streptomycete 'housekeeping' genes are controlled by $E\sigma^{70}$-like promoters [25]. The region upstream of TSS2 shares a GGGTG motif with the *xylA* and *bar* promoters of *S. rubiginosus* and *S. coelicolor*, respectively [26,27].

3.4. Growth phase dependent expression of the secA gene

The expression of the *secA* gene during different growth phases was determined by Northern blot analyses. A transcript of approximately 2.9 kb corresponding to the expected size of a monocistronic *secA* mRNA was detected in all exponential growth phases. No *secA* mRNA was detected when RNA isolated from cells in the stationary growth phase was analyzed (Fig. 3A).

We also monitored the growth phase dependent production of SecA by immunoblot analyses. Since the *S. griseus* SecA protein was not detectable with the *B. subtilis* antiserum in wild-type cells, an antiserum was raised against purified *S. griseus* SecA protein. The intracellular SecA level was nearly constant during the exponential growth phase (Fig. 3B). With protein of cells in the stationary growth phase a strong proteolytic degradation of SecA was observed (Fig. 3B). From these data we conclude that the degradation of the SecA protein and the breakdown of the secretion machinery might be stimulated by the onset of sporulation. Similar degradation products were detected in immunoblots with crude protein extracts of *E. coli* strains overproducing the *S. griseus* SecA protein (data not shown).

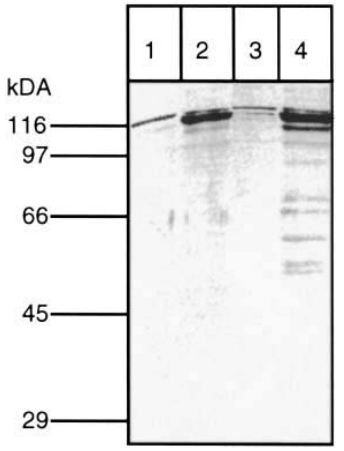


Fig. 4. Western blot analysis using the *S. griseus* SecA antiserum and 15 μg of crude cell extracts from *S. lividans* TK23 (lanes 1 and 3) and *S. lividans* TK23/pHMA2 (lanes 2 and 4) grown in TSB medium. Cells were harvested after 18 h (lanes 1 and 2) and 42 h (lanes 3 and 4) of growth. On the left the migration of the marker protein bands is shown.

3.5. *Expression of the secA gene from S. griseus in S. lividans TK23*

The *S. griseus secA* gene was inserted into the vector pHM8a. With the resulting construct (pHMA2) *secA* is expressed from the *ermE* promoter. pHMA2 was integrated into the genome of *S. lividans* TK23. The integration was confirmed by Southern blot analysis (data not shown). In SDS-PAGE analysis of crude extracts from *S. lividans* TK23/pHMA an additional band corresponding to the expected size of *S. griseus* SecA was visible. This protein was clearly distinguishable from the *S. lividans* SecA protein by Western blot analysis (Fig. 4). The additional SecA had no visible effect on efficiency of protein secretion in this strain. The secretory activity was tested by expression of heterologous extracellular enzymes (lipases and proteases, data not shown). We speculate that the expression of the heterologous genes rather than the secretion of the heterologous products is the limiting factor.

Acknowledgments

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