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Influence of *a Streptomyces lividans* SecG functional analogue on protein secretion

Carmen Palomino, Rafael P. Mellado*

National Center for Biotechnology, CSIC, Autonomous University of Madrid, Cantoblanco, Spain

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Summary. The membrane protein complex translocase mediates the translocation of bacterial proteins. In this complex, the SecY, SecE, and SecG proteins constitute an integral membrane domain. Sequence comparison revealed a potential *secG*-like gene in the gram-positive soil bacterium *Streptomyces lividans*. Chromosomal deletion of this gene resulted in a sporulation defect and an overall deficiency in secretion. The SecG-depleted strain was able to overproduce and secrete α-amylase, but the appearance of the oversynthesized protein outside the cell was delayed compared to the protein produced by the wild-type strain. SecG deficiency was found to result in more pronounced effects in *S. lividans* than in *Bacillus subtilis* or *Escherichia coli*. [**Int Microbiol** 2008; 11(1):25-31]

Key words: *Streptomyces lividans* · translocase · SecG · protein secretion

Introduction

Seven genes (*secA, B, D, E. F, G*, and *Y*) involved in the export of secretory pre-proteins across the *Escherichia coli* inner membrane have been described [12,28,31]. Three of these proteins are encoded by the *secD* operon (SecD, SecF, and YajC). They form an integral membrane heterotrimeric complex that associates with the other heterotrimeric membrane-embedded complex formed by proteins SecE, SecG, and SecY. Together, these six proteins constitute the hexameric integral membrane domain known as the translocase holoenzyme. Pre-protein translocation is mediated by the ATP-dependent action of SecA, which drives membrane insertion of the pre-protein as well as its translocation. SecG subsequently stimulates SecA insertion, which in turn is stabilized by SecDFYajC. Proteins encoded by the *secD* operon can functionally replace SecG and genetic studies have shown that *secG* is not, in fact, an essential gene in *E. coli* [4].

Gram-positive soil bacteria such as *Bacillus subtilis* and *Streptomyces lividans* are highly efficient in producing extracellular degradative enzymes and both species have been used extensively in industry for the production of homologous and heterologous exoenzymes. Comparisons of the genomic sequence of the *B. subtilis* and *Streptomyces coelicolor* (a strain whose genome is highly homologous to that of *S. lividans*) chromosomes [2,16] with the *E. coli* chromosome have facilitated the identification of genes equivalent to those of the translocase complex (*secA, D, E. F, G*, and *Y*) as well as the absence of a *secB* equivalent gene. It has been demonstrated that in *S. lividans* the signal recognition particle (SRP) functionally substitutes for the non-existent chaperone SecB [20,21] in transporting both membrane and secretory proteins to the membrane, and it presumably does so as well in *B. subtilis* [8]. Alternative mechanisms for membrane translocation have been identified in bacteria. Among these, the TAT (twin-arginine translocation) pathway, involved in the secretion of a small number of secretory proteins, has been shown to be comparatively more relevant in *Streptomyces* than in *E. coli* or *B. subtilis* [32].

***Corresponding author:** R.P. Mellado
Centro Nacional de Biotecnología, CSIC
Universidad Autónoma de Madrid, Cantoblanco
28049 Madrid, Spain
Tel. +34-915854547. Fax +34-915854506
E-mail: rpmellado@cnb.csic.es

The *B. subtilis* gene *yvaL* encodes a protein that is functionally homologous to the *E. coli* SecG protein, and sequence similarity between *yvaL* and s*ecG* has been shown [30]. Chromosomal disruption of *yvaL* results in cold-sensitive growth defects that are more moderate than those resulting from the depletion of *secG* in some *E. coli* strains [18]. Moreover, the effects of *yvaL* disruption become even more apparent following overexpression of a secretory enzyme in bacteria carrying the disrupted gene [30]. Specifically, at low growth temperatures protein secretion was altered in a *yvaL*-depleted *B. subtilis* mutant strain, whereas the yield of secreted proteins was similar to that of the wild-type at the standard growth temperature [30]. Furthermore, the cold-sensitive phenotype associated with the *secG* deletion in *E. coli* was found to be the effect of an additional mutation located in the *glpR* gene [5,6]. In the present study, we report the identification in the *S. lividans* chromosome of an open reading frame encoding a SecG-like protein. Deletion of this protein produced an associated delayed-sporulation phenotype in addition to a defect in extracellular protein secretion in bacterial cell cultures grown at the standard temperature. These observations show that deletion of the SecG-like protein results in the lack of a cold-sensitive phenotype. Additionally, overproduction of the model secretory enzyme α-amylase was delayed in the *S. lividans* SecG-deficient strain.

Materials and methods

Bacterial strains, plasmids and media. *S.lividans* TK21 [10], used as the wild-type strain, was cultured in liquid NMMP medium or solid R5 or MS media as indicated [11]. Plasmid pAMI11 is a pIJ486 derivative [13] that carries the *S. lividans* α-amylase gene *amlB* a frame-shift-mutated thiostrepton resistance gene [19]. Thiostrepton (5 μg/ml), kanamycin (5 μg/ml), ampicillin (100 μg/ml), or aparamycin (5 μg/ml) was added to the media when required**.** *E. coli* DH5a and *E. coli* ET12567 [17] were cultured in Luria broth (LB) [26] and used for plasmid propagation. *E. coli* ET12567, containing the conjugative plasmid pUZ8002 [16], was used in the *E. coli–S. lividans* conjugation experiments. Cosmid Ct54, containing the SecG coding sequence, came from a *S. coelicolor* gene library [25].

A 1324-bp DNA fragment containing *secG* was PCR-amplified from the *S. lividans* TK21 chromosome using primers G5 (5′-GCGGAATTCCGT GAACGCCAAGGTCAAGGCCGCCC-3′) and G8 (5′-CCCAAGCTTGC CTCGCCGTCCGCGTCACTGCGGCGG-3′) and inserted into pIJ486 through their unique *Eco*RI and *Hin*dIII sites to generate plasmid pSG10. The amplified DNA resulting from three different PCR reactions was mixed and used as starting material for the cloning experiment, to minimize the selection of potential PCR mutations. The sequence of the cloned gene was deposited in the GenBank database (accession no. AF182189). Plasmid pSG10 was used to complement the SecG deficiency in the *secG*-depleted strain. Plasmid pFDXlnC is a pFD666 derivative containing the *S. lividans* XlnC coding sequences [8].

DNA manipulation and PCR amplification. General recombinant DNA manipulation was carried out as described [11,26]. Restriction endonucleases and DNA modifying enzymes were obtained from Boehringer Mannheim, Promega, and Ecogene. *S.lividans* TK21 chromosomal DNA was used as the template for PCR amplification under the following conditions: 95ºC for 3 min, followed by 30 cycles of incubation at 95ºC (1 min), 45ºC (1 min), and 72ºC (2 min), with a final extension cycle of 10 min at 72ºC.

Real-time PCR. Real-time PCR was carried out using SYBR Green technology in an ABI Prism 700 Sequence Detection System (Applied Biosystems). Samples were initially denatured by heating at 95ºC for 10 min. A 40-cycle amplification and quantification program was then followed (95ºC for 15 s and 60ºC for 1 min), with a single fluorescence measurement per cycle, according to the manufacturer's recommendations. The final extension cycle was 72ºC, 1 min. Target DNAs (1–50 ng) from the experimental and reference samples were amplified in quadruplicate in separate PCRs using 0.3 mM of each primer. All PCR products were between 50 and 100 bp long. A standard curve was made from a dilution series (50–0.01 ng) of the reference DNA sample (A_{600} = 0.5) in order to quantify the abundance of a particular amplified DNA relative to the total amplified DNA obtained under each condition. Melting curve analysis was conducted after amplification to distinguish the targeted PCR products from the non-targeted ones. The melting curves were obtained by slow heating at temperatures ranging from 60 to 95ºC at a rate of 0.2ºC/s, with continuous fluorescence scanning. Oligonucleotides HDRBD (5′-CGCGGCATGCTCTTCCT-3′) and HDRBR (5′-AGGTGGCGTACGTGGAGAAC-3′) were used for amplification of the *hdrB* transcript, carried as an internal control to quantify the relative expression of target genes [22]. Oligonucleotides amlBF (5′-TGTTCGAGTGG AAGTTCACC-3′) and amlBR (5′-TCGACCATGCTCTTGAACTG-3′) were used to amplify the *amlB* transcript, and oligonucleotides xlnCF (5′-TA TAGCACCCAGTGGACCAA-3′) and xlnCR (5′-GGGTTGAAGTAGC CGT TGTAG-3′) to amplify the *xlnC* transcript.

S1 nuclease protection experiments. High-resolution S1 nuclease protection experiments were carried out as described [23] using 50 mg of total RNA. A 354-bp DNA fragment containing part of the SecG coding sequence was obtained by PCR amplification of the *S. lividans* TK21 genomic DNA using oligonucleotide primers SecG S1RE (5′-GTGATCCG GTCGAGGTTG-3′) and SecG S1FR (5′-TCGGACGAGTTCGTCAAGAT-3′). This amplified DNA fragment was used as a template to obtain a 354-nucleotide (nt) single-stranded DNA probe via an asymmetric PCR amplification in which the synthetic oligonucleotide SecG S1RE, radioactively labeled at its 5′ end, was used as a primer. In addition, the same 345-bp DNA fragment was treated with diphenylamine to generate a G+A sequence ladder [1].

Construction of the *secG* mutant. The ReDirect methodology was used as described previously [9]. The disruption cassette encompassing *oriT* and the apramycin resistance gene (Am^r) contained in plasmid pIJ773 [9] was generated by PCR amplification using oligonucleotide primers Sco1944F (5′-TACGTGCACAAGTGTGTGACGGGGCGACGGGACCC CTCATGTAGGCTGGAGCTGCTTC-3′) and Sco1944R (5′-TGCGCTAG CGGCGATACGTCGTACCCTTGCGGGGGCATGATTCCGGGGATC CGTCGACC-3′), both of which carried 39-nt DNA extensions around the start and stop codons of the SecG coding sequence. The resulting amplified DNA fragment was gel-purified and electroporated into *E. coli* BW25113 carrying cosmid StC54 and the compatible plasmid pIJ790, which harbors the RED functions necessary for linear recombination events [9]. The selected recombinant cosmid was propagated in *E. coli* ET12567 (pUZ8002) and then introduced into *S. lividans* TK21 by conjugation, as described previously [9]. Double-crossover mutants were selected for their resistance to apramycin and sensitivity to kanamycin. The selected Δ*secG* mutant strain (*S. lividans* TK21 Δ*secG*) was further analyzed by PCR amplification using oligonucleotide primers SecG Forward (5′-CGCGTCCAGTATTTGGAGTT-3′) and SecG Reverse (5′-TCGGACGCGTTCGTCAAGAT-3′) to amplify the inserted apramycin resistance gene. Chromosomal DNA from the *secG*-deleted strain was fragmented with *Sst*I restriction nuclease and the presence of the apramycin resistance gene was further confirmed by Southern blot analyses using the Sco1944F-Sco1944R cassette containing the apramycin resistance coding sequences (not shown).

Protein analysis and Western blot experiments. Supernatants from the indicated phases of *S. lividans* TK21 growth in cell cultures were concentrated by precipitation with 10% (v/v) TCA, washed with a buffer containing 70% (v/v) acetone, 10% (v/v) ethanol, and 10 mM Tris-HCl (pH 7.5), and re-suspended in 10 mM Tris-HCl (pH 7.5). Extracellular proteins were fractionated by SDS-PAGE in a 12% (w/v) acrylamide gel and visualized by Coomassie-blue staining [15].

In the Western blot analysis, proteins were fractionated by SDS-PAGE in a 10% (w/v) acrylamide gel [15] and transferred to immobilon polyvinylidene difluoride membranes (Millipore), as described [29]. The protein concentrations in the different samples were determined, as described [3], using standard I bovine gamma globulin (BioRad). The transferred material was incubated with rabbit polyclonal antibodies raised against *S. lividans* TK21 α-amylase (AmlB; a gift from C. Isiegas), or xylanase (XlnC; a gift from J. Anné), followed by incubation with HRP-conjugated protein A (Zymed Laboratories) diluted 1:10,000 in PBS containing 5% (w/v) skimmed milk for 40 min at room temperature [24]. Peptides reacting with the antibodies were revealed by enhanced chemiluminescence (Amersham Biosciences) after a 1-min incubation. Membranes were exposed to Agfa Curix RP2 film. The Quantity One software (BioRad) was used to quantify the amount of protein reacting with the antibody.

Subtilisin inhibitor activity. To determine extracellular activities, supernatants from 20-ml aliquots of bacterial cell cultures at the indicated phases of growth were concentrated by precipitation with ammonium sulfate brought to 80% saturation; the precipitated protein was collected by centrifugation at 13,000 ×*g* for 30 min and dissolved in 0.1 M Tris-HCl (pH 8). The total amount of protein present in the assay was determined using the BioRad protein determination kit, as indicated by the supplier. To assay the extracellular presence of the subtilisin inhibitor, aliquots were brought to a final volume of 250 ml with 0.1 M Tris HCl (pH 8.6) in the presence of 2.85×10^{-4} U subtilisin (Sigma) and 0.25 mM *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (sAAPF-pNA; Sigma) as substrate. The mixture was incubated at 25ºC until a yellow color developed, as described [14].

Results and Discussion

Identification of a *secG* homologue in *Streptomyces lividans*. To better understand the cellular mechanisms involved in the transport and secretion of extracellular proteins in the gram-positive bacterium *Streptomyces lividans*, *secG*, a gene encoding a homologous protein component of the precursor protein translocase, was cloned, its DNA sequence determined, and its identification assessed on the basis of sequence similarities with its gram-negative and gram-positive counterparts.

The genome sequence of the closely related bacterium *S. coelicolor* (GenBank accession no. AL645882) revealed the presence of a *secG*-like gene in *S. lividans*: The DNA sequence of the *S. coelicolor* gene was therefore used to derive oligonucleotide primers G5 and G8, which in turn were used to amplify the corresponding DNA sequence from the *S. lividans* chromosome, as described in Materials and methods. The resulting 1324-bp fragment was sequenced and the derived amino acid sequence was compared with the sequences of other SecG proteins (Fig. 1A). Accordingly, it was determined that the *S. lividans* SecG sequence resembled that of other SecG proteins of bacterial origin. Transcription initiation of the *secG* mRNA was mapped by S1 nuclease protection experiments. The results indicated that transcription initiation took place 71 bp upstream of a valine-encoding GTG triplet, strongly suggesting that the *S. lividans secG* gene is transcribed from its own promoter (Fig. 1B).

Characterization of SecG function. To investigate the function of the *secG* product in the translocase apparatus, a Δ*secG* deletion strain was constructed, as detailed in Materials and methods. The mutant strain showed a marked defect in sporulation when grown in two different media at 30ºC (Fig. 2A), in contrast to previous findings in *B. subtilis* [29]. To determine the effect of the *secG* mutation on the secretion pattern of extracellular proteins, *S. lividans* TK21 and *S.lividans* TK21Δ*secG* were incubated at 30ºC in NMMP medium supplemented with 0.5% (w/v) mannitol as carbon source and total extracellular proteins present in the culture broths were separated by SDS-PAGE on a 12% acrylamide gel. While no significant differences in the growth of the bacterial cell cultures were observed (Fig. 2B), extracellular proteins accumulated to a lesser extent in the *S.lividans* TK21Δ*secG* culture than in the wild-type bacterial cell culture (Fig. 2C), whereas the *B. subtilis* profile of extracellular proteins showed that only certain bands were affected by deletion of the *secG*-equivalent gene [29]. Considerable differences were observed in the secretion of subtilisin inhibitor, a major *S. lividans* extracellular protease [5], between the wild-type and the mutant strains (Fig. 2D), as determined by measuring subtilisin activity remaining after incubation with the corresponding extracellular protein extracts. This result confirmed the observed reduced secretory capacity of the *secG* mutant. Propagation of the *secG* gene in the mutant restored the sporulation deficiency (Fig. 2A), the extracellular protein pattern (Fig. 2C), and the capacity to inhibit subtilisin activity (Fig. 2D), strongly suggesting that SecG plays an important role in protein secretion. The *S. lividans* gene, rather than the *E. coli* or *B. subtilis* equivalents, was used to complement the mutation because of the different codon usage in *S. lividans*, which is biased due to the high G+C content of its genome (72.12%) [2]. In *S. lividans* this could render inefficient the synthesis of proteins encoded by A+T-rich DNA, as is the case for the *E. coli* or *B. subtilis* genomes.

Effect of *secG* depletion on α-amylase overproduction. In order to correlate the secretion defect of the SecG mutant strain with a potential limiting step in the translocation of pre-proteins, recombinant plasmid pAMI11 containing the *S. lividans amlB* gene, encoding α-amylase,

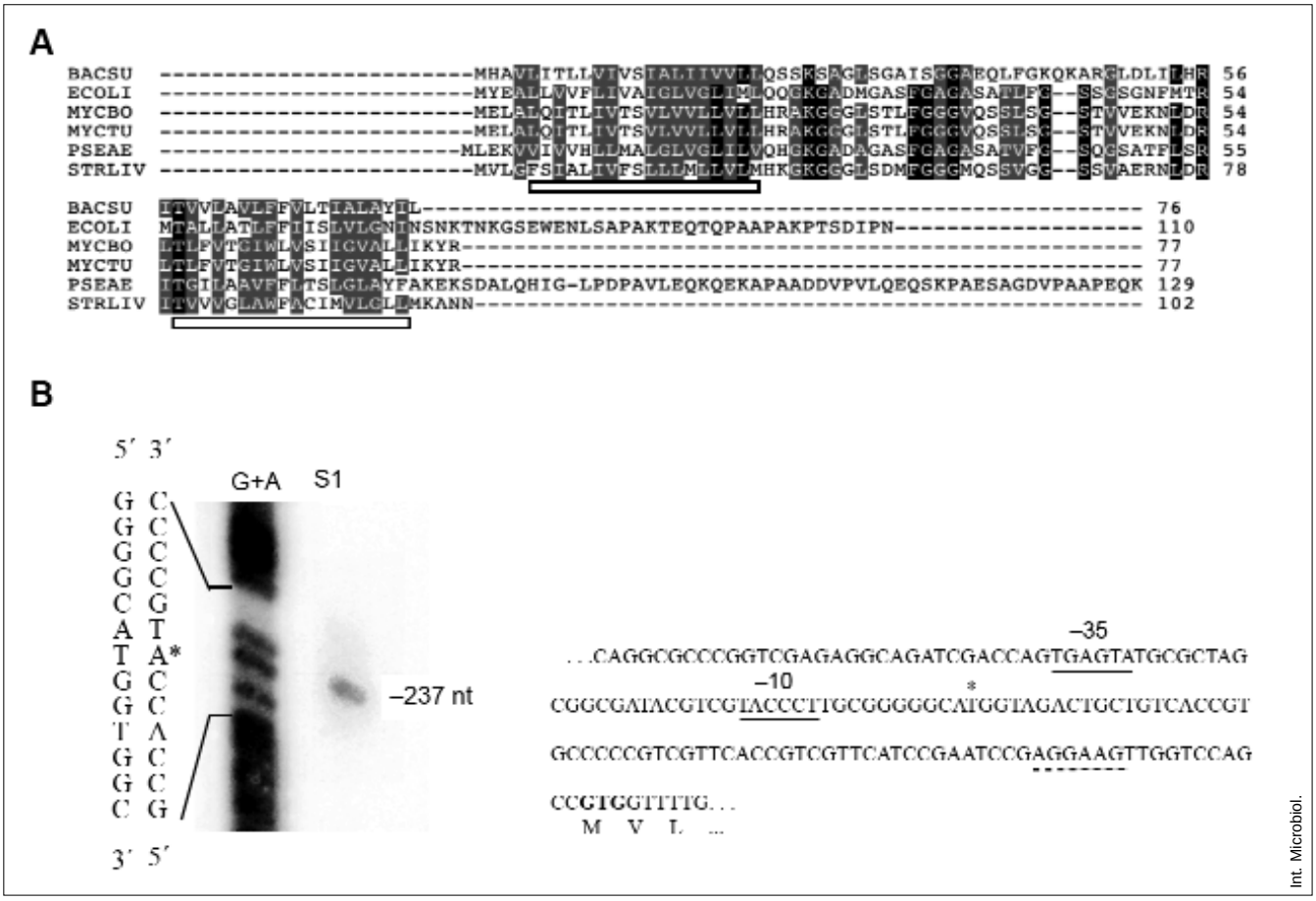

Fig. 1. Identification of the *Streptomyces lividans secG* gene. (**A**) Amino acid sequence comparison of SecG and potential homologues: Listed bacteria are as follows: BACSU, *Bacillus subtilis* (EMBL: Z9912), ECOLI, *Escherichia coli* (EMBL: D16463); MYCBO, *Mycobacterium bovis* (EMBL: BX248339); MYCLE, *Mycobacterium leprae* (EMBL: U00013); PSEAE, *Pseudomonas aeruginosa* (EMBL: AE004091); STRLIV, *Streptomyces lividans* (EMBL: AF182189). Conserved residues are shaded according to the number of sequences in which the residue is present. White boxes indicate the predicted transmembrane segments. (**B**) Transcriptional analysis of the *secG* gene. The *secG* transcription initiation site was determined by high-resolution S1 nuclease mapping using 50 mg total RNA from *S. lividans* TK21 (pSG10) cultures. RNA was extracted at the transition between the exponential and stationary phase of growth (left panel). The size (in nt) of the protected fragment is indicated. G+A indicates the guanine and adenine sequence ladder generated by diphenylamine treatment of the radioactively labeled probe, run in parallel. The asterisk indicates the nucleotide where transcription starts. In the depicted DNA sequence, putative –35 and –10 promoter regions are underlined. The discontinued line marks a potential ribosome-binding site. The predicted GTG triplet for translation initiation is indicated in bold.

was propagated in *S. lividans* TK21 and in the mutant strain. Western blotting with anti-AmlB serum, used to monitor AmlB secretion, showed that the extracellular presence of α-amylase occurred later in the mutant strain than in the wild-type (Fig. 3A). Specifically, the maximal detected level of AmlB secreted by the wild-type (2297 arbitrary integration units) at 24 h of growth was similar to that of the mutant strain (2399 arbitrary integration units) at 36 h of growth. A similar, but more pronounced phenomenon was found to occur when *S. lividans* is depleted of its major type I signal peptidase, SipY, which results in impaired translocase function and thus a transitory delay in the translocation of secretory proteins [21].

The *S. lividans* gene *xlnC* encodes a xylanase that is one of the few secretory proteins transported and translocated outside the bacterial cell by the bacterial twin-arginine translocation (TAT) pathway, rather than by translocase [27]. Plasmid pFDXlnC carries the *xlnC* gene and can be propagated in multiple copies in *S. lividans* strains. *S. lividans* TK21 and *S. lividans* TK21Δ*secG* strains were transformed with plasmid pFDXlnC, and Western blotting assays with anti-XlnC serum were carried out to monitor the extracellular appearance of XlnC in the wild-type and in the SecG-depleted strain. The results showed that extracellular xylanase was present at similar levels in the two strains (Fig. 3B), as expected for a secretory protein not targeted to the translocase. No significant

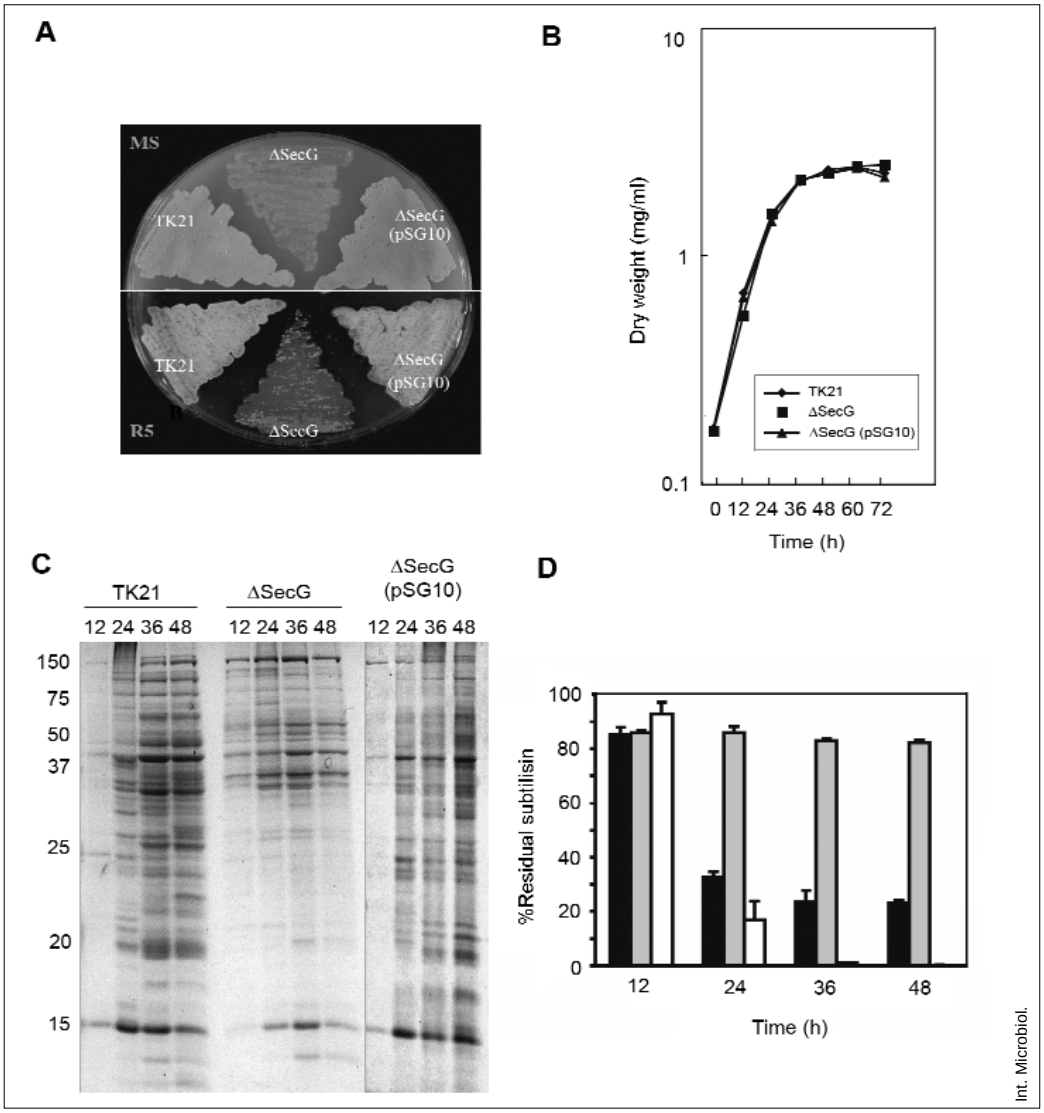

Fig. 2. Characterization of SecG function. (**A**) Mycelium pigmentation of *S. lividans* TK21, *S. lividans* TK21 Δ*secG*, and *S. lividans* TK21Δ*secG* (pSG10) strains grown on R5 or MS plates. (**B**) Growth curve of the bacterial cell cultures of *S. lividans* TK21, *S. lividans* TK21Δ*secG*, and *S. lividans* TK21Δ*secG* (pSG10) strains grown in NMMP medium. (**C**) Total extracellular proteins present in *S. lividans* TK21, *S. lividans* TK21Δ*secG*, and *S. lividans* TK21Δ*secG* (pSG10) cell cultures grown in NMMP after 12, 24, 36, and 48 h of growth were analyzed by SDS-PAGE on a 12% acrylamide gel. (**D**) Subtilisin inhibitor activities present in bacterial cell cultures of *S. lividans* TK21 (black rectangle), *S. lividans* TK21Δ*secG* (gray rectangle), and *S. lividans* TK21Δ*secG* (pSG10) (white rectangle) strains grown in NMMP medium. Activity is shown as a percentage of residual subtilisin activity under assay conditions.

differences in the transcriptional levels of *xlnC* or *amlB* were detected between the wild-type and mutant strains, as determined by real-time PCR analyses (Fig. 3C,D). These experiments indicated that, while transport and translocation of the overproduced pre-AmlB took place in both the wild-type and the *secG* deleted strain, translocation of the pre-protein was faster in the wild-type, reflecting the seemingly different pace at which this enzyme complex functions in the absence of SecG.

SecG is a non-essential component of the *E. coli* or *B. subtilis* precursor protein translocase, such that SecG depletion has an almost negligible effect on translocation at the standard growth temperature of these bacteria. This is in contrast to what was observed in *S. lividans*, since the mutant strain showed a clear sporulation deficiency, impaired extracellular protein secretion, and delayed overproduction of a model secretory enzyme. Interestingly, a similar phenotype is exhibited by a *S. lividans* strain deficient in the major type I signal

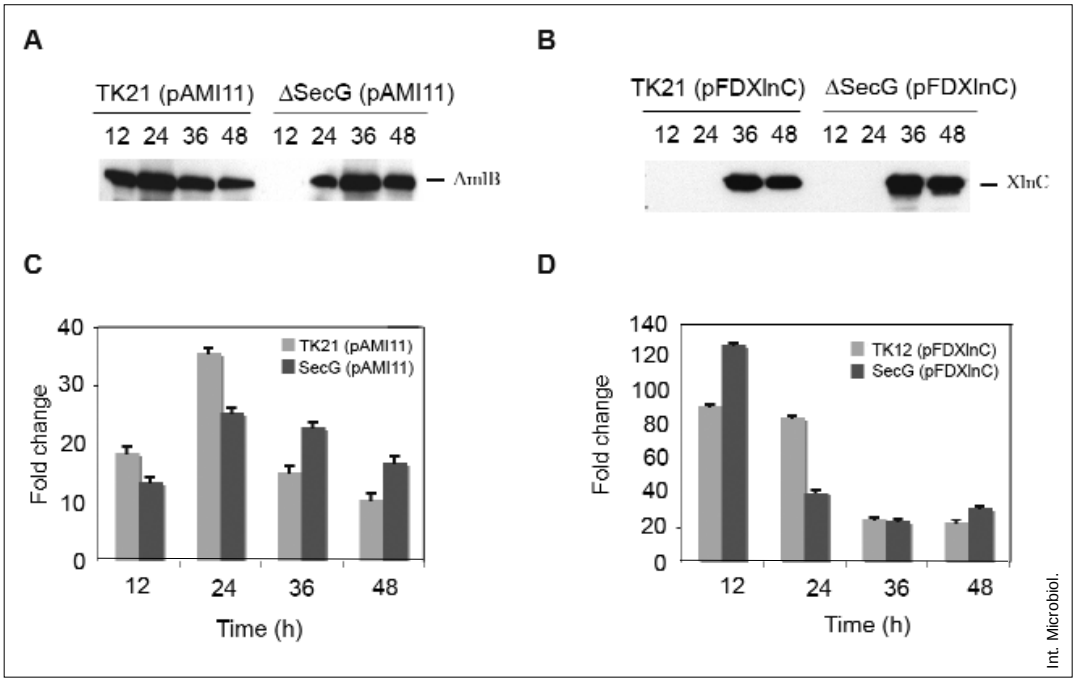

Fig. 3. The XlnC and AmlB secretion pattern of *S. lividans* strains. *S. lividans*TK21 and *S. lividans*TK21Δ*secG* strain cell cultures, carrying plasmids pAMI11 or pFDXlnC, respectively, were grown in NMMP. The amounts of extracellular xylanase and α-amylase present after 12, 24, 36 and 48 h of of growth were determined by Western blotting. (**A**) Antibodies raised against AmlB were used with *S. lividans* TK21(pAMI11) and *S. lividans* TK21Δ*secG* (pAMI11) strains. (**B**) Antibodies raised against XlnC were used with *S. lividan*s TK21(pFDXlnC) and *S. lividans* TK21Δ*secG* (pFDXlnC) strains. Real-time PCR analyses for the *amlB* transcripts (**C**) and *xlnC* transcripts (**D**) are shown.

peptidase. Such deficiencies may reflect the malfunction or temporal blockage of the translocation apparatus, due to the slow pace at which secretory proteins are cleaved in these mutant strains [5,19,21]. Genomic and proteomic studies are needed to ascertain to what extent depletion of SecG or SipY leads to the same or similar effects in the bacterial cell. *S. lividans secG* depletion is a non-essential mutation, as is also the case for *B. subtilis* and *E. coli*, although the phenotype is more pronounced in *S. lividans* than in these other species of bacteria. Nonetheless, as has been shown with respect to many other functions, essential processes are largely conserved in bacteria regardless of their evolutionary differences [7].

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