SipY Is the *Streptomyces lividans* Type I Signal Peptidase Exerting a Major Effect on Protein Secretion

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Received 20 December 2001/Accepted 10 June 2002

Most bacteria contain one type I signal peptidase (SPase) for cleavage of signal peptides from secreted proteins. The developmental complex bacterium *Streptomyces lividans* has the ability to produce and secrete a significant amount of proteins and has four different type I signal peptides genes (*sipW*, *sipX*, *sipY*, and *sipZ*) unusually clustered in its chromosome. Functional analysis of the four SPases was carried out by phenotypical and molecular characterization of the different individual *sip* mutants. None of the *sip* genes seemed to be essential for bacterial growth. Analysis of total extracellular proteins indicated that SipY is likely to be the major *S. lividans* SPase, since the sip*Y* mutant strain is highly deficient in overall protein secretion and extracellular protease production, showing a delayed sporulation phenotype when cultured in solid medium.

Bacterial preproteins exported by the general secretion pathway (Sec pathway) contain a signal peptide required for correct translocation across the cytoplasmic membrane (9, 26, 34, 35); upon translocation, a type I signal peptidase (SPase) removes the signal peptide so that the mature protein is released from the membrane (8). Prokaryotic type I SPases, also known as leader peptidases (Lep), process the majority of exported preproteins. Most organisms contain only one type I SPase which seems to be essential, as is the case with *Escherichia coli* (28, 33) or yeast (3). There are other organisms containing two paralogous type I SPases, such as *Synechocystis* sp. strain PCC 6803 (28) and most eukaryotic species (9). At least two SPases have been described in the bacterium *Bacillus amyloliquefaciens* (14, 22) and *Staphylococcus aureus* (7); three have been found in *Deinococcus radiodurans* (36) and in the archaeon *Archaeoglobus fulgidus* (17). Seven SPases have been described for the gram-positive bacterium *Bacillus subtilis*, where the genes corresponding to five of them (*SipS, SipT, SipU, SipV, and SipW*) are widespread on the chromosome (33, 30) and two other genes (*SipP*) have been found in plasmids (22).

Gram-positive bacteria belonging to the *Streptomyces* genus are soil bacteria with mycelial growth that undergo a complex biochemical and morphological differentiation prior to the formation of exosporous chains (6). Streptomyces produce and secrete large quantities of proteins (12), and *S. lividans* is the most studied model organism for extracellular protease production, showing a delayed sporulation phenotype when cultured in solid medium.

Expression and purification of the N-terminally hexahistidine-tagged SipY protein for antibody preparation. Expression and purification of the N-terminally hexahistidine-tagged SipY protein was performed as described previously (11). Purified SPases were used to raise polyclonal antibodies in rabbits. Purified SPase preparations (50 μg in 500 μl) were mixed with 500 μl of complete Freund adjuvant and injected intramuscularly (twice, 1 ml each time) in a Holland rabbit (Pfd:HOL) with an interval of 3 weeks. At 2 weeks after applying the last injection, a blood sample was taken and the serum collected by centrifugation (5 min, 150 × g) was prepared as described previously (10). Polyclonal antibodies against agarase were obtained as described previously (25).

Materials and Methods

Bacterial strains, plasmids, and media. *S. lividans* TK21 (15) used as the wild-type strain was cultured in liquid NMMP medium or solid R5 medium as indicated (15). Thiostrepton (5 μg/ml) or kanamycin (10 μg/ml) was added to the media when required. *S. lividans* TK21W26, *S. lividans* TK21X516, *S. lividans* TK21Y62, and *S. lividans* TK21Z1 are the sip*W*, sip*X*, sip*Y*, and sip*Z* mutant strains, respectively. *E. coli* K514 (23) and *E. coli* ET12567 (21) were cultured in Luria broth (LB) (32) and were used for plasmid propagation. Ampicillin (100 μg/ml), tetracycline (10 μg/ml), or chloramphenicol (30 μg/ml) was added to the media when needed. Plasmids pSN425 and pSN426 are pUC18 derivatives containing the cluster of *S. lividans sip* genes and were used to construct sip*Y* and sip*X* mutants, respectively. Plasmid pSN408 is a pUC18 derivative containing the sip*W* gene and was used to construct the sip*W* mutant. Plasmid pAC301 (obtained from F. Malpartida), a pUC18 (37) derivative carrying a 1,060-bp long BclI DNA fragment encoding the thiostrepton resistance gene (*tsr* gene), was used to construct the sip*Z* mutant. The *tsr* marker was constituted by a 1,060-bp EcoRI-BclI fragment obtained from plasmid pGM9 (24). Multicopy plasmid pAGAs is a pAGAs1 (25) derivative containing the *S. coelicolor* agarase gene (*dagA*); the *dagA* gene of pAGAs was inactivated by a frameshift mutation so that pAGAs could be propagated in the different *S. lividans* sip*Y* mutant strains.

DNA manipulation and PCR amplification. General recombinant DNA manipulation was carried out as described previously (15, 27). Restriction endonucleases and DNA modifying enzymes were obtained from Boehringer Mannheim, Promega, and Ecodeme. *S. lividans* chromosomal DNA was used as a template for PCR amplification by incubation at 95°C for 3 min, followed by 30 cycles of incubation at 95°C (1 min), 45°C (1 min), and 72°C (2 min), with a final extension cycle of 10 min at 72°C.

Expression and purification of the hexahistidine-tagged Sip proteins for antibody preparation. Expression and purification of the N-terminally hexahistidine-tagged Sip proteins was performed as described previously (11). Purified SPases were used to raise polyclonal antibodies in rabbits. Purified SPase preparations (50 μg in 500 μl) were mixed with 500 μl of complete Freund adjuvant and injected intramuscularly (twice, 1 ml each time) in a Holland rabbit (Pfd:HOL) with an interval of 3 weeks. At 2 weeks after applying the last injection, a blood sample was taken and the serum collected by centrifugation (5 min, 150 × g) was prepared as described previously (10). Polyclonal antibodies against agarase were obtained as described previously (25).
Construction of mutants. Plasmids pSN425 and pSN426 were used to obtain the appropriate deletion mutants by inserting a 1,060-bp EcoRI-XhoI DNA fragment encoding the thiostrepton resistance gene depleted of its transcription termination sequence (tsr [15]), thereby generating plasmids pSN-Y and pSN-X, respectively. Plasmid pSN408 was used to produce the sipW mutant by inserting the 1,060-bp EcoRI-XhoI fragment containing the tsr gene, thereby generating plasmid pSN-W. Plasmid pSN425 is a pUC18 derivative carrying the 3.45-kb oligonucleotide sn30 (5'-CTGGCGGAGCCTGCGCCGCGAAGGC-3')-SphI, with a DNA fragment comprising the four sip genes and ending at the SphI site located right behind sipZ (26). Plasmid pSN426 is a pUC18 derivative that carries a 3.06-kb DNA fragment spanning from the BbrP site within the sipW coding sequence to the SphI site downstream of sipZ (26). Plasmid pSN408 is a pUC18 derivative carrying a 1,847-bp Ncol DNA fragment encoding sipW (26).

To delete sipY and inactivate sipX and sipW, S. lividans TK21 was transformed with plasmids pSN-Y, pSN-X, and pSN-W, respectively. In pSN-Y, the pSN425 399-bp PstI fragment (P2-P3, comprising SipY boxes D and E; Fig. 1) was replaced by the tsr marker; in pSN-X, tsr was inserted at the Stul site of pSN426 (St2, between SipX boxes C and D; Fig. 1); and in pSN-W, tsr was inserted at an MluI site of pSN408 (Ml, between SipW boxes C and D; Fig. 1). For sipZ insertional inactivation, a 315-bp sipZ internal DNA fragment (comprising SipZ box B to the middle of sipZ box E) was PCR amplified from the S. lividans chromosomal DNA by using the oligonucleotides sn49 (5'-CGCGGATCCGTTGCGCCGAGCAG-3') and sn3 (5'-GACCAGCTCGAATGACGCCGACG-3') as forward and reverse primers, respectively; the amplified DNA fragment was digested with restriction endonuclease BbrI and inserted into the StuI site of pSN426 (St2, between SipX boxes C and D; Fig. 1) and in pSN-W, S. lividans TK21 was transformed with plasmids pSN408 to give mutant strain (21) used as a host to propagate pSN plasmids.

Mutations in sipW, sipX, and sipY genes were constructed by transformation of S. lividans TK21 protoplasts with linearized and purified plasmids pSN-W, pSN-X, and pSN-Y containing the respective tsr-disrupted sip genes. The correct integration of linearized DNA fragments or plasmid pSN-Z in the chromosome of S. lividans that gave rise to mutant strains S. lividans TK21W26, S. lividans TK21X516, S. lividans TK21Y62, and S. lividans TK21Z1, respectively (Fig. 1), was verified by PCR and Southern blot hybridization analysis (not shown).

FIG. 1. Chromosomal organization of disrupted S. lividans sip genes. Relevant restriction endonuclease sites are indicated: BamHI (B), BstPI (B), BstUI (B), MluI (M), NcoI (N), PstI (P), SstI (S), Stul (St), SphI (Sp). Conserved type I SPase boxes (Al, B, C, D, E, and All [26]) are indicated.

**Extracellular protein analysis and Western blot experiments.** Total extracellular proteins were visualized by Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% polyacrylamide gel (19). For SPase Western blot analysis, intracellular proteins were fractionated by SDS-12.5% PAGE and transferred to Immobilon polyvinylidene difluoride membranes (Millipore Corp.) as described previously (29). Half of the transferred material was stained with 1% (wt/vol) Coomassie brilliant blue R-250 in 50% (vol/vol) methanol and 20% (vol/vol) acetic acid for 15 min. The other half of the transferred material was incubated with antibodies raised against SPases, followed by a further incubation with 0.1 μCi of 125I-labeled protein A from Staphylococcus aureus (Amersham, Plc.) ml−1, revealed peptides reacting with the antibodies, as described previously (29). Membranes were exposed to Agfa Curix RP2 film at −70°C. The protein concentrations in the different samples were determined as described previously (4) by using standard I bovine gamma globulin (Bio-Rad).

**Enzyme activities.** To determine the 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 8.0). The total amount of protein present in the assay was determined by using the Bio-Rad protein determination kit, as indicated by the supplier. To assay protease activities, different aliquots were brought to a 1-ml final volume of 0.1 M imidazole-HCl (pH 7.2) in the presence of 7 mg of Hide Powder Azure (Sigma Chemical Co.) and incubated at 37°C until the blue color developed as described previously (25). One enzyme unit was defined as the amount of enzyme that hydrolyzes 1 mg of Hide Power Azure in a 30-min incubation at 30°C (13). To assay the extracellular presence of the subtilisin inhibitor, aliquots were brought to a 250-μl final volume of 0.1 M Tris-HCl (pH 8.6) in the presence of 2.85 × 10−4 U of subtilisin (Sigma Chemical Co.) and a 0.25 mM concentration of the N-succinyl-Ala-Ala-Pro-Val-Phe-p-nitroanilide (SAP-125-NPA) (Sigma Chemical Co.) as the substrate, and the mixture was incubated at 25°C until the yellow color developed as described previously (18). The presence of the subtilisin inhibitor was referred to as a percentage of subtilisin activity remaining after the incubation period.

To determine isocitrate dehydrogenase (ICDH) activity, bacterial cells present in 20-ml aliquots of bacterial cell cultures at the indicated phases of growth were harvested and lyzed (15). Different aliquots from the lysates were brought to a 1-ml final volume with potassium phosphate buffer (pH 8) containing 250 mM...
MgCl₂ and 8 mM NADP; the reaction was started by the addition of 100 mM isocitrate (pH 7) and was followed by incubation at 30 °C (28). One ICDH unit was defined as the amount of protein that produced an increase of 0.00622 U in absorbance measured at 340 nm per min of incubation and per mg of protein (13).

RESULTS AND DISCUSSION

The sip individual mutations are not essential and do not affect the synthesis of the nonmutated SPases. None of the mutated genes (S. lividans TK21W26, S. lividans TK21X516, S. lividans TK21Y62, and S. lividans TK21Z1) were shown to be essential for bacterial growth; mutant strain S. lividans TK21Y62 showed a delayed sporulation phenotype with a whitish aerial mycelium on an orange substrate mycelium in contrast to the typical dark gray color of the properly sporulated wild-type strain and the remaining mutant strains when cultured in solid R5 medium (Fig. 2A). S. lividans TK21Y62 also showed an altered mycelium morphology when grown in minimal solid medium, as visualized by phase-contrast microscopy (Fig. 2B).

Western blot analysis was carried out to identify the sip gene products and to investigate whether the different mutations affected the synthesis of the nonmutated SPases, particularly those forming part of an operon. Submerged cultures of S. lividans TK21, S. lividans TK21W26, S. lividans TK21X516, S. lividans TK21Y42, or S. lividans TK21Z1 were incubated at 30°C in NMMP medium supplemented with 0.5% (wt/vol) mannitol as carbon source. They grew exponentially with a doubling time of ca. 4.2 h. The transition to stationary phase occurred at 25 to 30 h after inoculation at biomass dry weights of ca. 2.5 mg/ml. Cell-associated proteins were separated by SDS–12.5% PAGE; transferred to Immobilon-P membranes; incubated with antibodies raised against SipW, SipX, SipY, and SipZ; and visualized with ¹²⁵I-labeled protein A as described in Materials and Methods. The results showed specific protein bands reacting with each antibody with relative molecular masses of 27.5, 34, 36.5, and 27 kDa for SipW, SipX, SipY, and SipZ, respectively, in accordance with theoretical molecular masses of 27.6, 34.5, 35.8, and 26.5 kDa for SipW, SipX, SipY, and SipZ, respectively (26). All sip mutant strains were able to express the remaining intact sip genes, as deduced from the specific protein bands obtained with antibodies to the non-deleted proteins (Fig. 3). Disruption of the sipW or sipX gene did not result in polar effects on the expression of the genes located downstream of the sip operon due to the absence of a transcription terminator at the end of the tsr gene that was inserted in the construction of these two mutants so that the transcription could follow through tsr to terminate at the end of the operon.

FIG. 2. Sporulation of sip mutants. (A) Mycelium pigmentation of S. lividans TK21, S. lividans W26, S. lividans X516, S. lividans Y62, and S. lividans Z1 strains grown in R5 plates. (B) Phase-contrast microscopy of aerial hyphae from S. lividans TK21 (a), S. lividans W26 (b), S. lividans X516 (c), S. lividans Y62 (d), and S. lividans Z1 (e).

FIG. 3. Sip proteins present in the sip mutant strains. The different SPases present in S. lividans wild-type and the different sip mutant strain cell cultures after 36 h of growth in NMMP medium were analyzed by Western blotting, with antibodies raised against SipW (A), SipX (B), SipY (C), and SipZ (D).
Effect of sip depletion on overall secretion. To check the effect of the different sip mutations on the extracellular protein secretion pattern, *S. lividans* TK21, *S. lividans* TK21W26, *S. lividans* TK21X516, *S. lividans* TK21Y62, or *S. lividans* TK21Z1 was incubated at 30°C in NMMP medium supplemented with 0.5% (wt/vol) mannitol as the carbon source, and the total extracellular proteins from culture broths were separated by SDS–15% PAGE. Although no significant differences in the growth of the bacterial cell cultures were observed (Fig. 4A), the accumulation of extracellular proteins in the *S. lividans* TK21Y62 culture was severely diminished compared to that in the other bacterial cell cultures (Fig. 4B). The total extracellular protease activity was determined, and the presence of the extracellular subtilisin inhibitor was monitored in all cases. All mutants showed a reduced accumulated extracellular protease activity in comparison to that of the wild type (ca. 15, 30, and 45% for *sipX*, *sipZ*, and *sipW* mutants, respectively), with *SipY* inactivation having the strongest effect, thus causing extracellular protease activity to fall below detection limits. No great differences were observed upon secretion of the subtilisin inhibitor between the wild type (*S. lividans* TK21) and the different *sip* mutants, except for the *sipY* mutant (Fig. 5), as determined by measuring the subtilisin activity remaining after incubation with the corresponding extracellular protein extracts, thereby confirming the observed diminished secretory capacity of the *sipY* mutant (Fig. 4B), as well as strongly suggesting that *SipY* plays a major role in protein secretion. The measured extracellular ICDH activity appeared to be very

![Graph](image-url)

**FIG. 4.** Overall secretion pattern of *S. lividans* sip mutant strains. (A) Growth in NMMP of the wild-type and the different mutant strain cell cultures. (B) Total extracellular proteins present in *S. lividans* TK21, *S. lividans* W26, *S. lividans* X516, *S. lividans* Y62, and *S. lividans* Z1 cell cultures grown in NMMP after 12 h (1), 22 h (2), 36 h (3), and 45 h (4) of growth were analyzed by SDS–15% PAGE.
small compared to the respective intracellular activity in all cases, clearly indicating that the accumulation of extracellular proteins at the late phases of growth (Fig. 4B) was not due to lysis of the bacterial cell cultures (not shown).

**Effect of sip depletion on agarase overproduction.** In order to correlate the secretion defect of the sipY mutant strain with a defect in the processing of preproteins, recombinant plasmid pAGAs5 containing the *S. coelicolor* dagA gene was propagated in *S. lividans* TK21 and in the different sip mutant strains. DagA synthesis was monitored by Western blotting with anti-DagA serum. No cell-associated agarase was detected in the wild-type strain or in the sipW, sipX, and sipZ mutant strains, whereas pre-DagA and mature-cell-associated agarase were clearly detected in the sipY mutant strain (Fig. 6), thus not only showing that sipY depletion confers a major defect in preprotein processing to the cell but also indicating that the remaining SPases could compensate for the deficiency, allowing secretion of the overproduced agarase.

The construction of different combinations of the possible mutants is needed in order to obtain a further insight into the study of compensatory effects among the different sip genes and to confirm whether any combination of mutations that includes sipY may become essential. Thus far, the construction of double mutants containing a sipY mutation has not been possible, and attempts to produce a quadruple mutant have failed as well.

In the released sequence of the *S. coelicolor* genome, a putative fifth incomplete sip gene has been annotated in which the coding sequence for one of the transmembrane anchor domains is missing. The existence of a fifth sip gene in the *S. lividans* genome cannot be ruled out, although our attempts at finding this gene by screening of *S. lividans* genomic libraries have always produced negative results.

**B. subtilis** SipW is required for the efficient processing of the precursor of a spore-associated protein, pre-TasA (28). Apart from the specific activity of SipW, all *B. subtilis* Sip proteins apparently have overlapping substrate specificities (30, 31). From the results obtained it can be concluded that individual mutations in the different *S. lividans* sip genes, except for sipY, do not seem to have a severe effect on protein secretion, probably because of the compensatory effects of the minor SPases. Due to this compensatory effect, double and triple mutants of the sip genes need to be produced in order to assess substrate specificity for the different Sip proteins and two-dimensional gel electrophoresis, coupled with mass spectrometry, is needed to identify the differences in extracellular protein patterns between *S. lividans* TK21 and the different sip mutant strains.

**ACKNOWLEDGMENTS**

This research was supported by grants BIO97-0650-C02-01 and BIO2000-0907 from the Spanish CICYT and by European Union grant QLK3-2000-00122. N.G. is a fellow of the IWT. A.P. and V.P. contributed equally to this study.

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