In bacteria, three different types of signal peptide-dependent translocation have been described: the Sec-dependent pathway, the signal recognition particle-dependent pathway, and the recently discovered twin-arginine-dependent pathway or Tat pathway, which is structurally and mechanistically related to the DeltaH-dependent pathway in chloroplast thylakoid membranes (11, 32).

The bacterial Tat pathway has been extensively studied in Escherichia coli. At least four proteins—TatA, TatB, TatC, and TatE (7, 36, 43)—are involved in Tat-dependent translocation. The genes coding for the first three components are part of a polycistronic operon, while tatE is located separately on the chromosome. TatA, TatB, and TatC are sequence-related proteins encoded by homologs of hcf106, a structural gene of the thylocoid DeltaH-dependent pathway (38). They possess a single N-terminal transmembrane helix, followed by a cytoplasmically located amphipatic helix. The TatC protein contains six transmembrane helices. In E. coli, TatB and TatC are essential for Tat-dependent translocation, while TatA and TatE are components since protein transport is only fully blocked in a tatAE double mutant (7, 36, 37, 43). Very recently, it was shown that the Tat complex contains only TatA, TatB, and TatC and that TatB and TatC act as a unit in both structural and functional terms (8). The precise function of the different components in the translocation of precursor proteins is still to be explained.

Bacterial precursor proteins translocated via the Tat pathway have an unusually long signal peptide with a conserved twin-arginine sequence S/T-R-R-x-D or D that is essential for transport (1, 29). The main energy source that drives the translocation process is the proton motive force instead of nucleoside triphosphates, as in the case of Sec-dependent translocation (35). A striking feature of this newly discovered translocation system is its ability to transport folded proteins eventually bound to a cofactor before export (33, 35). In addition to E. coli, the functionality of the Tat pathway has been demonstrated for Zymomonas mobilis (16), Ralstonia eutropha (3), and Bacillus subtilis (17).

We describe here the analysis of the Tat pathway in Streptomyces lividans. This species has a naturally high secretion capacity and is used as an efficient host for heterologous protein production (5, 23, 40). First, the tatA, -B, and -C genes of S. lividans were identified. To demonstrate the functionality of the Tat pathway, we constructed a tatC deletion mutant and tested the translocation of two putative Tat-dependent precursor proteins, the Streptomyces antibioticus tyrosinase and the chimeric pre-TorA-23K, shown to be translocated via the Tat pathway in E. coli (7).

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** S. lividans TK24 and derivatives were grown at 27°C with continuous shaking at 300 rpm in phage medium (21) or NM medium (41). For solid medium, R2 medium was used (29). Where necessary, thiotrepton (at 50 µg/ml in solid medium or at 10 µg/ml in liquid medium), apramycin (50 µg/ml), or kanamycin (50 µg/ml) was added. Proteolysis formation and transformation of S. lividans was carried out as described by Kieser et al. (19).

E. coli TG1 was used as host for cloning purposes. Cultures were grown in Luria-Bertani medium at 37°C (300 rpm) supplemented with ampicillin (50 µg/ml), tetracycline (15 µg/ml), apramycin (50 µg/ml), kanamycin (50 µg/ml), or chloramphenicol (25 µg/ml), if necessary. Plasmids used in this work are listed in Table 1.

**DNA techniques and vector constructions.** All DNA manipulations were performed by using standard techniques (34), and the restriction enzymes were from Life Technologies (Rockville, Md.). DNA sequence analysis was carried out according to the dideoxy chain termination method with the Thermo Sequenase Primer Cycle Sequencing Kit 7-Deaza-dGTP on an ALFexpress apparatus (Amersham Pharmacia Biotech, Rainham, United Kingdom).

PCR for isolation of tatA, tatB, and tatC was performed with Pfu polymerase (Stratagene, La Jolla, Calif.) in the presence of 10% dimethyl sulfoxide with the primers tat1 (5'-GAAGCGGCTGAAACCGGCCAC) and tat2 (5'-CCACGTTCCCATTCGTGCG) for the amplification of tatA, tat3 (5'-GGGGCGGATGC...
**RESULTS**

Cloning and sequencing of the *S. lividans* tatA, -B, and -C genes. In the *S. coelicolor* genome bank (http://www.sanger.ac.uk), we found a tatA, tatB, and tatC homolog. The tatA and tatC gene are clustered (cosmid SCI41), while tatB is located elsewhere on the chromosome (cosmid SCP8). With primers based on the *S. coelicolor* sequence, we were able to amplify the corresponding genes from *S. lividans* TK24. tatA and tatC were isolated on a 1,617-bp fragment and are separated by 92 bp. tatB, on the other hand, was isolated as a 580-bp fragment. While the *S. lividans* and *S. coelicolor* tatA genes are identical, the tatC and tatB genes of both strains show 99.8 and 99.6% identities based on the *S. coelicolor* sequence, we were able to amplify the *S. lividans* tatA, tatB, and tatC. While the tatA, tatB, and tatC are located on the chromosome of *S. lividans*, we found a 580-bp fragment. As the expression of pre-TorA-23K in *S. lividans*, the gene was placed under control of the *Streptomyces venezuelae* subtilisin inhibitor (vsI) promoter. Therefore, a PBSKAN fragment containing pre-TorA-23K was isolated from pMW18 and cloned into PBS-CBSS (23). From this construct, a BamHI/SalI fragment containing the vsI promoter and the downstream pre-TorA-23K coding region was cloned into the streptomyces muticopy plasmid pHJ486 (23) to give a PBSKAN derivative containing the *S. venezuelae* vsI promoter.

**Immunoblot analysis.** Western blot analysis was performed to check the translation of pre-TorA-23K in *S. lividans*. Extracellular fractions of 30-h *S. lividans* cultures were obtained by centrifugation (10 min, 4,200 × g, 0°C). Proteins in the growth medium were precipitated with trichloroacetic acid (final concentration of 20%) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (22). Transfer of proteins onto a nitrocellulose Porablot membrane (Macherey-Nagel, Düren, Germany) was performed by using a Bio-Rad Transblot semidry transfer cell (Bio-Rad, Hercules, Calif.) according to the manufacturer's recommendations. The 23K derivatives were visualized with rabbit anti-23K antibodies and alkaline phosphatase-conjugated goat anti-rabbit antibodies (Sigma, St. Louis, Mo.).

**Activity assays.** The tyrosinase activity was measured using the dopachrome assay procedure with l-3,4-dihydroxyphenylalanine (l-DOPA) as a substrate (18). We used 48-h precultures in phage medium (21) to inoculate 5-mL cultures of tyrosinase production medium (NM medium supplemented with 0.0375% l-tyrosine, 0.75% tiger milk [19], and 2 μM CuSO4) that were subsequently cultivated for 6 h. Supernatants were diluted in the assay buffer (0.1 M sodium phosphate buffer; pH 6.2), and the tyrosinase activity was measured immediately. The intracellular amount of tyrosinase was determined on cell lysates obtained by sonication (2 min, 20,000 Hz, 0°C) of the mycelia suspended in assay buffer. One unit of tyrosinase was defined as the amount of enzyme that converts 1 μmol of l-DOPA into dopachrome/min.

The inhibitory activity of subtilisin inhibitor was determined in the presence of the substrate N-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide as described by Kojima et al. (20). Cultures were grown in tyrosinase production medium, and supernatants and intracellular fractions were obtained in the same way as described for the sampling of the tyrosinase test. One unit of subtilisin inhibitor activity was defined as the percentage of inhibition of 5 μg of subtilisin/ml during 10 min of incubation at 25°C.
at the amino acid level, respectively. CLUSTALW similarity scores with analogous proteins of *E. coli* and *B. subtilis* are rather low (19 to 24% for *E. coli* and 22 to 27% for *B. subtilis*). All DNA sequences were submitted to the EMBL bank (accession numbers AJ251128, AJ251129, and AJ251149 for tatA, -B, and -C, respectively).

**Construction of a tatC deletion mutant.** TatC is an essential component of the *E. coli* Tat pathway (7). To prove the functionality of the twin-arginine translocation pathway in *S. lividans*, we therefore decided to knock out tatC. To do this, a 730-bp internal fragment of tatC comprising the six transmembrane domains was replaced with the neo gene in the *S. lividans* chromosome by using the integrative shuttle vector pGMtatC. ΔtatC mutants differed phenotypically from the wild type. Colonies grown on R2 medium (30) lacked red pigmentation, and the mycelium grew very dispersed in liquid medium in contrast to the mycelial aggregates of the wild-type strain.

**Translocation of pre-TorA-23K is tatC dependent.** Pre-TorA-23K is a chimeric construct that consists of the signal peptide and the first six amino acids of the mature *E. coli* trimethylamine N-oxide reductase (TMAO) fused to the mature part of the 23-kDa subunit of the plant photosystem II oxygen-evolving complex (7). It has clearly been proven that the pre-TorA-23K protein is translocated via the Tat pathway in *E. coli* (7) and via the ΔpH pathway in thylakoid membranes (44).

To investigate whether pre-TorA-23K is also Tat dependent in *S. lividans*, the translocation efficiency was compared in pVsiTor23K transformants of *S. lividans* TK24 and its ΔtatC mutant. Western blot analysis of extracellular protein fractions from the wild-type strain revealed an immunoreactive band of 26 kDa (Fig. 1), somewhat larger than expected for the fusion protein. On the other hand, no 23K-specific immunoreactive band was detected in the supernatant of the ΔtatC mutant containing pVsiTor23K (Fig. 1). These results showed that pre-TorA-23K could not be translocated in the ΔtatC mutant. Intracellular accumulation of pre-TorA-23K in the ΔtatC mutant could not be detected. To prove that *S. lividans* ΔtatC(pVsiTor23K) could express but not secrete pre-TorA-23K, a plasmid was constructed to complement the disrupted tatC gene. To do this, wild-type tatC transcribed from its own promoter was inserted into the integrative plasmid pSET152, resulting in pSETtatC. tatC is transcribed from its own promoter and does not constitute an operon with tatA, as revealed by transcription analysis experiments (data to be published). Western blot analysis of supernatants of 30-h cultures of *S. lividans* ΔtatC(pVsiTor23K) transformed with pSETtatC showed that the 26-kDa band appeared again (Fig. 1). The result indicated that the integrated copy of tatC restored the translocation of pre-TorA-23K in ΔtatC, and tatC was thus essential for translocation of pre-TorA-23K. The 23K-specific immunoreactive band observed in the supernatant of the tatC complemented strain was less intense than in the wild-type strain. Therefore, it is obvious that the integrated tatC copy complemented the deleted tatC, but secretion was not at the same level as in the original wild-type strain.

**Translocation of *S. antibioticus* tyrosinase is tatC dependent.** In addition to the heterologous pre-TorA-23K, we also tested the tatC dependence of the *S. antibioticus* tyrosinase secretion. Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase that catalyzes the formation of melamin pigment from tyrosine (25). The *S. antibioticus* tyrosinase is encoded by melC2, the second open reading frame of the melin operon (melC). The upstream gene melC1 encodes a transactivator protein with a twin-arginine signal peptide that was demonstrated to be essential for both tyrosinase activation and secretion (10, 26).

To test the Tat dependence of tyrosinase secretion, *S. lividans* TK24 and its ΔtatC mutant were transformed with plasmid pIJ702 (18) encoding the *S. antibioticus* melC2 operon, and the tyrosinase activity was assayed. Figure 2A shows the tyrosinase activities in cell lysates and supernatants of cultures grown for 6 h in tyrosinase production medium. At this growth stage, the activities reached a maximum value. Thereafter, decreasing values were measured, probably as a consequence of a lack of copper ions in the medium, since after the addition of extra Cu²⁺ the tyrosinase activity increased again (data not shown). The activity of tyrosinase in the supernatant of the wild-type strain was ca. 112 U/mg (dry weight) (Fig. 2A). In contrast, only trace amounts of tyrosinase activity could be detected in ΔtatC supernatant, indicating that tyrosinase was not translocated in this mutant. The rather low intracellular tyrosinase activity detected in the mutant and as a consequence, a much lower total amount of activity (ca. 11% of the wild type), indicated that there was no intracellular accumulation of tyrosinase in the ΔtatC strain (Fig. 2A), a similar phenomenon as that observed for pre-TorA-23K. In the wild-type strain, 92.7% of the tyrosinase was secreted in contrast to almost 0% in the mutant (Fig. 2B).

After complementation of ΔtatC(pIJ702) with pSETtatC, the tyrosinase secretion was restored, although only partially. As mentioned above, this phenomenon was also observed for pre-TorA-23K translocation when ΔtatC was complemented with the intact tatC. The tyrosinase activity in the supernatant was only ca. 15 U/mg (dry weight) (Fig. 2A, set 3), but the efficiency of secretion was restored to 61.9% (Fig. 2B). The lack of tyrosinase secretion in the *S. lividans* ΔtatC mutant and the complementation experiments confirmed the tatC dependence of the tyrosinase secretion. Therefore, it could be concluded that *S. antibioticus* tyrosinase is a substrate of the Tat pathway in *S. lividans*.

Because it has already been shown that the two components of tyrosinase, MelC1 and MelC2, form a complex before export (10, 26), our results show that, similar to *E. coli* and some
other species (3, 15, 35, 40), the Tat pathway of Streptomyces can translocate folded proteins.

**tatC is not involved in Sec-dependent secretion.** As a supplementary proof that *S. lividans* /H9004 tatC is a specific mutant that only affects Tat-dependent translocation and not the Sec-dependent secretion process, we investigated the secretion of the *S. lividans* subtilisin inhibitor, a Sec pathway-dependent substrate (unpublished data). Therefore, *S. lividans* TK24 and its ΔtatC strain were cultured in tyrosinase production medium. After 15 and 24 h of growth, subtilisin inhibition activity in the culture medium was measured. Values of ca. 26 and 45 U/mg (dry weight) were obtained after 15 and 24 h, respectively, both for wild-type *S. lividans* and the ΔtatC mutant (Fig. 3). These results showed that a disrupted TatC does not affect the Sec-dependent secretion of subtilisin inhibitor.

**DISCUSSION**

The experiments described in this report demonstrate that the translocation of two tested precursor proteins containing a twin-arginine signal peptide, i.e., the heterologous chimeric pre-TorA-23K and the *S. antibioticus* tyrosinase, is blocked in a tatC deletion mutant of *S. lividans* TK24. As a consequence, these results prove the existence of a functional twin-arginine-dependent translocation pathway in *S. lividans*.

The chimeric pre-TorA-23K, known to be translocated via the Tat pathway in *E. coli* (7), is also translocated through this pathway in *S. lividans*. Moreover, although the homologous components of the Tat complexes of *E. coli* and *S. lividans* have rather low similarity scores (ranging from 19 to 24%), the signal peptide of the *E. coli* TMAO-reductase can direct Tat-dependent transport in *S. lividans*. This is an indication of the conserved nature of the Tat mechanism. Also, the signal peptides of the Desulfovibrio vulgaris [NiFe]-hydrogenase (29) and of the high potential iron-sulfur protein of Chromatium vinosum (9) can direct Tat-dependent transport in *E. coli*. Moreover, signal peptides are functionally interchangeable between the bacterial Tat pathway and the ΔpH pathway in thylakoid membranes of chloroplasts (15, 27). An example of species-
specific recognition, however, is observed in *Zymomonas mobilis* (6). It was shown for this species that the Tat-dependent glucose-fructose oxidoreductase (GFOR) could only be exported to the periplasm in *E. coli* when its own signal peptide was replaced with an *E. coli* Tat signal peptide. It was suggested that specific recognition events should exist between Tat signal peptides and one or more components of the Tat translocase. As a consequence, signal peptides of Tat-dependent precursors would be optimally adapted specifically to their cognate export apparatus.

On the other hand, Cristóbal et al. (12) showed that not only the signal peptide but also the mature part of the precursor can be a determinant for Tat-dependent transport. The fusion between the TorA signal peptide and the Sec-dependent leader peptidase, for instance, did not reroute the translocation to the Tat pathway, indicating that Sec-targeting signals in Lep could override the Tat-targeting information in the TorA signal peptide.

The secretion of *S. antibioticus* tyrosinase has previously been investigated but was not fully understood (26). The melanin operon (*melC*) consists of two genes coding for MelC1, a transactivator protein, and the apotyrosinase MelC2. Only MelC1 has a signal peptide and can mediate MelC2 activation by a binary complex formation followed by copper insertion (10). A functional MelC1 is also essential for tyrosinase secretion (26). How the MelC1/MelC2 complex could disrupt the membrane was not evident, because Sec and SRP pathways cannot translocate folded proteins. As experimentally proven in this study and already suggested by Berks et al. (2), tyrosinase is translocated via the Tat pathway. No secretion was detectable in the tatC mutant, while in the wild-type *S. lividans* secretion of tyrosinase was very efficient and probably occurred cotranslationally or immediately after translation, since only very low amounts of tyrosinase could be detected intracellularly.

The translocation of folded proteins in *Streptomyces* can now be explained by the functionality of the Tat pathway. *E. coli* hydrogenase 2 is also encoded by an operon with two genes, of which only one is coding for a protein containing a signal peptide. This type of “hitchhiker” mechanism is also used by *S. lividans* to cotranslocate the two subunits of tyrosinase.

The complementation of *ΔtatC* with a wild-type copy was not complete. The reason for this effect is not yet understood. Gene dose effect cannot be the reason because the disrupted *tatC* gene was replaced by one intact copy. However, positional effects of integration or a lack of regulatory sequences present in the neighborhood of the wild-type *tatC* gene might result in a different transcription efficiency.

For both pre-TorA-23K and tyrosinase, an intracellular accumulation of the preprotein was not observed when the secretion process was blocked. As a consequence, the total amount of pre-TorA-23K or tyrosinase in the Δ*tatC* cultures was much lower than in those of the wild type. This effect was also noticed by Jongbloed et al. (17), who investigated the secretion of PhoD in a *tatCd* mutant of *B. subtilis*. These authors postulated that the intracellular amount of preprotein is not folded and therefore not stable. In the case of tyrosinase, it has been described that the two subunits of tyrosinase form an intracellular complex (10). The proteolytic breakdown of this complex is unlikely. However, if the total amount of the respective components should be unequal in the *S. lividans* Δ*tatC*, as already observed with some signal peptide mutants of MelC1 (26), it might be that the component in excess becomes sensitive to proteases. When Leu et al. (26) demonstrated low intracellular accumulation of tyrosinase, they agreed with the hypothesis suggested by Oliver (31) and Gennity et al. (14) that a coupling between secretion and translation exists. A blocked secretion apparatus would have a negative feedback on translocation of the protein. The results obtained in this study and in our previous work with Sec-dependent translocated proteins (23, 24) are in agreement with a negative feedback on the translation, or even transcription, of the precursor as a consequence of inhibiting secretion. The mechanism of this negative feedback has to be further investigated.

The TatC component of the Tat complex in *S. lividans* is essential for the translocation of the two precursor proteins under investigation, i.e., *S. antibioticus* tyrosinase and chimeric pre-TorA-23K. In *E. coli*, the unique *tatC* copy is essential for the translocation of all Tat pathway precursors studied this far (7). However, in *B. subtilis*, two *tatC* copies were detected but only one of these is essential for the Tat-dependent translocation of PhoD, indicating that *tatC* is a specificity determinant for protein secretion via the Tat pathway (17). In the fully sequenced genomes of *Mycobacterium tuberculosis* and *Mycobacterium leprae*, two species classified to be in the same order of the *Actinomycetales* as *Streptomyces*, only one *tatC* copy was identified. The *S. coelicolor* genome project is now finished, but no second *tatC* homolog was detected; neither was one observed by hybridization experiments (data not shown). Further studies are required to characterize the components of the *S. lividans* Tat system at the molecular level and to investigate the interactions between the Tat translocon components and between these proteins and the Tat-dependent precursors. In addition, the underlying reason for the altered phenotype observed for the Δ*tatC* mutant, indicating that the mutation has a pleiotropic effect, as also noticed for *E. coli*, where it resulted in an impaired cell separation and a defective cell envelope (39), is not understood.

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K.S. and M.S. contributed equally to this study.

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