The twin-arginine signal peptide of PhoD and the TatA<sub>d</sub>/C<sub>d</sub> Proteins of Bacillus subtilis Form an Autonomous Tat Translocation System

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Ovidiu Pop‡§, Ulrike Martin¶, Christian Abel‡, and Jörg P. Müller‡§

From the Institutes of Molecular Biology and Virology, Jena University, Winzerlaer Str. 10, D-07745 Jena, Germany

The bacterial twin-arginine translocation (Tat) pathway has been recently described for PhoD of Bacillus subtilis, a phosphodiesterase containing a twin-arginine signal peptide. The expression of phoD is co-regulated with the expression of tatA<sub>d</sub> and tatC<sub>d</sub> genes localized downstream of phoD. To characterize the specificity of PhoD transport further, translocation of PhoD was investigated in Escherichia coli. By using gene fusions, we analyzed the particular role of the signal peptide and the mature region of PhoD in canalizing the transport route. A hybrid protein consisting of the signal peptide of β-lactamase and mature PhoD was transported in a Sec-dependent manner indicating that the mature part of PhoD does not contain information canalizing the selected translocation route. Pre-PhoD, as well as a fusion protein consisting of the signal peptide of PhoD (SP<sub>PhoD</sub>) and β-galactosidase (LacZ), remained cytosolic in the E. coli. Thus, SP<sub>PhoD</sub> is not recognized by E. coli transport systems. Co-expression of B. subtilis tatA<sub>d</sub>/C<sub>d</sub> genes resulted in the processing of SP<sub>PhoD</sub>-LacZ and periplasmic localization of LacZ illustrating a close substrate specificity of the TatA<sub>d</sub>/C<sub>d</sub> transport system. While blockage of the Sec-dependent transport did not affect the localization of SP<sub>PhoD</sub>-LacZ, translocation and processing was dependent on the pH gradient of the cytosolic membrane. Thus, the minimal requirement of a functional Tat-dependent protein translocation system consists of a twin-arginine signal peptide-containing Tat substrate, its specific TatA/C proteins, and the pH gradient across the cytosolic membrane.

The existence of a protein export pathway structurally and mechanistically similar to the ΔpH-dependent pathway used for importing chloroplast proteins into the thylakoid has been shown for a variety of bacteria (1–3). Despite the fact that the mechanism of targeting and the transport of folded proteins via the ΔpH/Tat<sup>1</sup> route is not yet understood, some common features characterize these translocation systems (for reviews, see Refs. 4 and 5). It has been shown that the Escherichia coli Tat system involves four proteins with calculated membrane-spanning domains (6–8). TatA/TatE and TatB are sequence-related proteins that are homologous to Tha4 and Hcf106 of the ΔpH-dependent thylakoid import pathway (7–11). Chloroplast cp-TatC has been described recently as the ortholog of E. coli TatC (12). While TatB and TatC appear to play a pivotal role in the Tat-dependent protein translocation in E. coli, TatA and TatE seem to fulfill complementary functions as the deletion of TatA or TatE does not block export, while the TatA/TatE double deletion drastically inhibits export (7). Expression studies suggested that tatE may be a cryptic gene duplication of tatA (13). An in vitro reconstituted translocation system demonstrated the necessity of TatA, TatB, and TatC for a functional E. coli Tat-dependent translocation system (14). The information about the structure of the Tat translocase is contradictory. While Bolhuis et al. (15) suggested that TatB and TatC proteins form a functional and structural unit of the E. coli Tat translocase, a recent report from Sargent et al. (16) demonstrated a double-layered ring structure with a central cavity of a complex consisting of TatA and TatB.

The presence of genes encoding TatA- and TatC-like proteins as well as the synthesis of exported proteins containing twin-arginine signal peptides are strong indications for the existence of the Tat pathway in eubacteria (17). While most of the bacteria contain one copy of tatA and one copy of tatC (5) sequencing of bacilli genomes (i.e. Bacillus subtilis, Bacillus halodurans, and Bacillus stearothermophilus) indicated the presence of multiple TatA and TatC proteins. In particular, B. subtilis contains two tatC- and three tatA-like genes (18). Both tatC genes are localized directly downstream from a tatA gene (19). A TatB-like protein appears to be absent from bacilli.

The recently described transport of PhoD of B. subtilis revealed that TatC could act as a specificity determinant for this process. While the inactivation of the tatC<sub>d</sub> completely inhibited the secretion of PhoD, the inactivation of the second tatC gene (tatC<sub>s</sub>) had no effect on the secretion of PhoD (19). This observation was the first indication for the existence of multiple Tat pathways in a single bacterial cell with separate substrate specificity. PhoD is a secretory protein with a twin-arginine signal peptide. We have shown previously that it is efficiently transported across the cytosolic membrane but only inefficiently processed. Slow processing of the enzymatically active precursor was shown to keep the protein at the outer surface of the cell envelope (20). The tatAltatC gene pair (designated tatA<sub>d</sub>/C<sub>d</sub>) localized downstream from phoD, is co-regulated with the expression of phoD (19).

To investigate the specificity of the PhoD transport further, we analyzed its transport in E. coli. By using gene fusion technology the particular role of the signal peptide and the mature region of PhoD in canalizing the transport was investigated. A fusion protein consisting of the signal peptide of β-lactamase (Bla) and mature PhoD was transported in a Sec-dependent manner. PhoD, as well as the fusion protein consisting of the signal peptide of PhoD (SP<sub>PhoD</sub>) and LacZ, was shown...
to be export-incompetent in *E. coli*. The co-expression of the *phoD*-associated *B. subtilis* gene pair *tatA°/C°* resulted in the processing and the translocation of SP *PhoD-LacZ*. This translocation, agarose gel electrophoresis, and transformation of an *E. coli* weight marker was from Amersham Biosciences, Inc..

For pulse-chase labeling experiments, M9 minimal medium was prepared as described previously (21). When required, media were supplemented with [35S]methionine and chased with unlabeled methionine, and then samples were taken at the times indicated immediately followed by precipitation with trichloroacetic acid (0°C). After cell lysis proteins

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### EXPERIMENTAL PROCEDURES

#### Plasmids, Bacterial Strains, and Media—Table I lists the plasmids and bacterial strains used. TY medium (tryptone/yeast extract) contained Bacto tryptone (1%), Bacto yeast extract (0.5%), and NaCl (1%). For pulse-chase labeling experiment M9 minimal medium was prepared as described previously (21). When required, media were supplemented with ampicillin (100 μg/ml), kanamycin (40 μg/ml), chloramphenicol (20 μg/ml), tetracycline (12.5 μg/ml), arabinose (0.2%), isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM), nigericin (1 μM), and/or sodium azide (3 mM). F[35S]Methionine was provided by Hartman Analytic (Braunschweig, Germany), and the [-C4-Labeled molecular weight marker was from Amersham Biosciences, Inc., Avenham, Germany.

**DNA Techniques**—Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of *E. coli* were carried out as described in Sambrook et al. (22). Restriction enzymes were from MBI Fermentas. PCR was carried out with the VENT DNA polymerase (New England Biolabs).

**To construct plasmid pAR3phoD, the phoD gene including its ribosome binding site was amplified from the chromosome of *B. subtilis* strain 168 by PCR using the primers P1 (5'-GAG GAT CAT CCA GGA GAG AGG GGA TCT TGA ATG GCA TAC GAC-3') containing a *BamHI* site and P2 (5'-CGA TCC TGC ACC TCA TCG GAG TTC-3') containing a *PstI* site. The amplified fragment was cloned with *BamHI* and *PstI* and cloned in the corresponding sites of pUC19. The resulting plasmid pAR3phoD was confirmed by DNA sequencing and by its ability to complement the arabinose-inducible expression of the *phoD-LacZ* fusion.

To construct a gene fusion containing the signal sequence of *phoD* and *lacZ*, a DNA fragment encoding the signal peptide of PhoD and the translational start site of *phoD* was amplified by PCR with primer P1 containing an unique *PstI* site and primer P2 containing a unique *BamHI* site. The amplified fragment was cloned with *BamHI* and *PstI* and cloned in the corresponding sites of *phoD*.

**To obtain a plasmid that mediates an inducible overexpression of *tatA°/tatC°* in *B. subtilis*, the DNA region containing these genes including their ribosome binding sites was amplified by PCR with the primer T1 (5'-CGA TCC TGC ACC TCA TCG GAG TTC-3') containing a *BamHI* site and primer T2 (5'-GGT CTC GAG TCC ACC TCA TCG GAG TTC-3') containing an *EcoRI* site and subsequently inserted into the corresponding sites of pORI24. The resulting plasmid pORI24 was isolated and inserted into the multiple cloning site downstream of the P_{E������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������������daleph promoter and the tetracycline resistance gene of pORI24 was inserted and cloned into the corresponding sites of pORI24 resulting in plasmid pORI24. The amplified fragment was amplified using primer L1 (5'-GAG GAT CAT CCA GGA GAG AGG GGA TCT TGA ATG GCA TAC GAC-3') containing a *BamHI* site and primer L2 (5'-GGT CTC GAG TCC ACC TCA TCG GAG TTC-3') containing an *EcoRI* site and subsequently inserted into the corresponding sites of pORI24 resulting in plasmid pORI24. The resulting plasmid pORI24 was linearized with *BamHI* and inserted into pAR3 cleaved with *BamHI*. The resulting plasmid pAR3phoD-lacZ allows the arabinose-inducible expression of the *phoD-lacZ* fusion.

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RESULTS

PhoD Is Not Transported in E. coli—The initial aim was to test whether PhoD could be exported by the Tat pathway in E. coli. For this purpose we placed the gene encoding this peptide under the control of the P_{BAD} promoter of Salmonella typhimurium localized at plasmid pAR3 (27). The resulting plasmid allowed the arabinose-inducible enzymatically active production of PhoD in E. coli TG1 (data not shown). Since phosphodiesterase is highly toxic to E. coli after induction of PhoD synthesis cell growth immediately ceased. To assay transport of PhoD in E. coli TG1(pARphoD) pulse-chase experiments were performed. As shown in Fig. 1A no processing of the wild type pre-PhoD was observed even 60 min after chase, indicating that pre-PhoD was not translocated by the E. coli Tat machinery. Localization of PhoD was further analyzed by in vivo protease mapping. As shown in Fig. 1B pre-PhoD was not accessible to proteinase K at the outer side of the cytosolic membrane, demonstrating that PhoD remained in a cytosolic localization.

PhoD Can Be Transported via the Sec-dependent Protein Translocation Pathway—Absence of pre-PhoD processing in E. coli could be due to inefficient recognition of the signal peptide of PhoD by the E. coli Tat machinery or due to the nature of the mature part of the PhoD peptide. This E. subtilis protein could have unexpected folding characteristics or the necessity of cofactors not present in E. coli. To address this question, the DNA encoding the mature peptide of PhoD was fused to the region encoding the signal peptide of β-lactamase (SP_{Bla}). The resulting gene fusion was cloned into the pMUTIN2 vector containing an IPTG-inducible P_{BAD} promoter allowing the synthesis of the SP_{Bla}-PhoD peptide. The transport and processing of this fusion protein was analyzed by immunoblotting of whole cell extracts of E. coli strain TG1(pMUTIN2bla-phoD). As shown in Fig. 2A, lane 2, SP_{Bla}-PhoD was completely converted to a protein with a molecular weight of mature PhoD indicating the efficient transport of the protein. To elucidate the export path used for SP_{Bla}-PhoD translocation, Sec-dependent transport was selectively inhibited by addition of sodium azide. While the presence of sodium azide abolished conversion of SP_{Bla}-PhoD to PhoD, addition of nigericin did not retard processing of SP_{Bla}-PhoD (Fig. 2A, lanes 3 and 4). To analyze the Sec dependence of SP_{Bla}-PhoD transport in a more detailed manner, expression of bla-phoD in E. coli TG1(pMUTIN2bla-phoD) was induced in the presence or absence of sodium azide, pulse-labeling with [35S]-methionine was carried out, and PhoD was subsequently immunoprecipitated. Fig. 2B demonstrates the kinetics of conversion of SP_{Bla}-PhoD to mature PhoD. The presence of sodium azide significantly retarded maturation of SP_{Bla}-PhoD (Fig. 2C). To demonstrate that azide was effective in inhibiting Sec-dependent translocation, the processing of pro-OmpA was monitored from the same cultures. While in untreated culture pro-OmpA was quickly converted into its mature form, in the azide-treated culture processing of pro-OmpA was efficiently retarded (Fig. 2, D and E). These data indicate that PhoD can be transported in E. coli in a Sec-dependent manner. Thus, it can be concluded that the mature PhoD peptide is not canalizing the export route and does not prevent efficient transport or processing.

The Signal Peptide of PhoD Cannot Mediate Transport of LacZ in E. coli Wild Type Cells—It has been shown that signal peptides containing a twin-arginine motif can canalize translocation of pro-OmpA into the cytoplasm (26). To demonstrate that azide was effective in inhibiting Sec-dependent translocation, the processing of pro-OmpA was monitored from the same cultures. While in untreated culture pro-OmpA was quickly converted into its mature form, in the azide-treated culture processing of pro-OmpA was efficiently retarded (Fig. 2, D and E). These data indicate that PhoD can be transported in E. coli in a Sec-dependent manner. Thus, it can be concluded that the mature PhoD peptide is not canalizing the export route and does not prevent efficient transport or processing.

In Vivo Protease Mapping—In vivo protease mapping was carried out according to Kiefer et al. (26). For spheroplast formation, cells were grown in M9 minimal medium to early logarithmic phase. 1 h prior to labeling expression of phoD was induced with IPTG (1 mM). Cells were labeled for 1 min with [35S]-methionine after which nonradioactive methionine was added. Samples were withdrawn at chase times 10, 20, 40, and 60 min and subjected to immunoprecipitation with monospecific antibodies against PhoD followed by SDS-PAGE using a 10% polyacrylamide gel and fluorography. M, molecular mass marker; Glu, uninduced control. B, in vivo protease mapping of PhoD in E. coli TG1(pAR3phoD). Cells were converted to spheroplasts and treated with proteinase K or with proteinase K and Triton X-100 or remained untreated as indicated. Localization of pre-PhoD is indicated. Accessibility of proteinase K to the cytosol was analyzed by monitoring SecB in a 15% polyacrylamide gel. PhoD and SecB were detected by monospecific antibodies.

were precipitated with specific antibodies against PhoD (20), OmpA, β-galactosidase, β-lactamase (5 Prime—3 Prime, Inc.). Relative amounts of radioactivity were estimated by using a phosphorimaging system (Fuji) and the associated image analytical software PC-BAS.

The Signal Peptide of PhoD Cannot Mediate Transport of LacZ in E. coli Wild Type Cells—It has been shown that signal peptides containing a twin-arginine motif can canalize transport of heterologous proteins via the Tat-dependent translocation route (for a review, see Ref. 5). The signal peptide of the E. coli trimethylamine-N-oxide reductase (TorA) has been successfully used to mediate Tat-dependent translocation of the thykaloid protein 23K, the glucose-fructose oxidoreductase of Zymomonas mobilis and green fluorescent protein (3, 7, 28, 29). To test whether the signal peptide of PhoD is recognized by the E. coli Tat machinery and could canalize the transport of a protein in E. coli, we constructed a gene fusion consisting of the DNA region encoding the signal peptide of PhoD (SP_{phoD}) and the lacZ gene encoding β-galactosidase as a reporter protein. The gene hybrid was inserted into plasmid pAR3 resulting in plasmid pAR3phoD-lacZ. Induction of production of the SP_{phoD}-LacZ fusion protein in E. coli TG1 resulted in LacZ^- colonies (data not shown). Hence, correct folding and tetramerization of the peptide as a prerequisite for its activity does occur in E. coli.

To analyze whether the signal peptide of PhoD could mediate translocation of LacZ into an extracytosolic localization, we studied localization of LacZ by using in vivo protease mapping. As shown in Fig. 3A no processing of SP_{phoD}-LacZ could be observed. The SP_{phoD}-LacZ fusion protein was not susceptible to...
protease digestion in spheroplasts. When spheroplasts were destroyed by addition of Triton X-100, the unprocessed SP\textsubscript{phoD}-LacZ protein became protease-sensitive. The reliability of the method was verified by using the cytosolic protein SecB as internal control. In spheroplasts SecB was resistant to proteinase K but was digested after solubilizing the spheroplasts with Triton X-100.

Export of SP\textsubscript{phoD}-LacZ Fusion Protein in E. coli Needs the Presence of the B. subtilis Tat\textsubscript{A} and Tat\textsubscript{Cd} Transport Components—The data demonstrated above indicate that the Tat system of E. coli does not mediate transport of pre-PhoD or of the SP\textsubscript{phoD}-LacZ fusion protein. Absence of translocation could be due to the necessity of additional components for the translocation of PhoD present only in B. subtilis or due to the specificity of recognition of pre-PhoD as a Tat-dependent substrate. To test the latter hypothesis, the B. subtilis tat\textsubscript{A} and tat\textsubscript{Cd} gene pair was coexpressed in E. coli strains TG1(pARphoD) and TG1(pARphoD\textunderscore lacZ).

To study the effect of Tat\textsubscript{A}/\textsubscript{Cd} proteins on localization of PhoD, strain TG1(pARphoD, pREP4, pQE9\textunderscore tat\textsubscript{A}/\textsubscript{Cd}) was grown in TY medium to logarithmic phase. 1 h prior to labeling, expression of phoD was induced with IPTG (1 mM). While one culture remained untreated (B and D), the other was treated with sodium azide (3 mM, lane 4) or remained untreated (lane 2). Samples were taken 20 min after induction of SP\textsubscript{bl}a-PhoD and lysed, and cell extracts were analyzed by SDS-PAGE using a 10% polyacrylamide gel. Expected bands representing SP PhoD-LacZ, LacZ, and PhoD are indicated.

Since processing of the translocation product is an indication of membrane translocation but does not necessarily prove that export of the protein has occurred, we examined whether LacZ was localized in the periplasmic space in TG1(pARphoD\textunderscore lacZ) and TG1(pARphoD\textunderscore LacZ) expressing either plasmid pARphoD\textunderscore lacZ (A) or plasmids pARphoD\textunderscore LacZ, pREP4, and pQE9\textunderscore tat\textsubscript{A}/\textsubscript{Cd} (B) were grown in TY medium to exponential growth phase, and expression of phoD\textunderscore lacZ and tat\textsubscript{A}/\textsubscript{Cd} was induced for 1 h with arabinose (0.2%) and IPTG (1 mM), respectively. Subcellular localization of SP\textsubscript{phoD}-LacZ was detected by in vivo protease mapping according to Fig. 1B. SP\textsubscript{phoD}-LacZ and SecB were monitored by antisera against LacZ and SecB. Bands representing SP\textsubscript{phoD}-LacZ, LacZ, and SecB are indicated.
requirement of a specific Tat-dependent protein translocation with the help of the export signals of Sec-dependent strains revealed that transport was independent of the Tat machinery. Transport of SP PhoD-LacZ in an \( \text{tatABCDE} \) strain did result in accumulation of pro-OmpA but did not affect the translocation-ATPase activity of the SecA protein. Treatment of the culture with sodium azide, \( \text{NaN}_3 \), did not prevent transport in an \( \text{tatACD} \) strain. As shown in Fig. 6 in the absence of the TatC \( \text{E. coli} \) strain, TatC \( \text{E. coli} \) Tat Components—To exclude co-operative action of \( \text{B. subtilis} \) and \( \text{E. coli} \) Tat proteins, \( \text{E. coli} \) strain TG1 was deleted for \( \text{tatABC} \) genes and subsequently transformed with plasmids \( \text{pARphoD-LacZ} \), \( \text{pREPM} \), and \( \text{pQEstatA(C)} \). Processing and localization of the SP PhoD-LacZ fusion protein was analyzed under identical conditions as described for the \( \text{E. coli} \) tat \( \text{A} \) strain. As shown in Fig. 6 in the absence of the \( \text{E. coli} \) tat \( \text{ABC} \) genes, most of SP PhoD-LacZ was protease-accessible demonstrating the extracytosolic localization of the fusion protein. The resistance of SecB to the proteolytic digestion demonstrated the stability of the spheroplasts (Fig. 6). Surprisingly, no processing of the SP PhoD-LacZ fusion protein could be observed in the absence of tat \( \text{ABC} \).

**DISCUSSION**

In the present report we have shown that the export signals of PhoD, a Tat-dependent transported phosphodiesterase of \( \text{B. subtilis} \), is incompatible with the Tat machinery of \( \text{E. coli} \). While the mature part of PhoD could be exported efficiently with the help of the export signals of Sec-dependent \( \beta \)-lactamase, wild type PhoD or a fusion protein consisting of the signal peptide of PhoD and LacZ remained cytosolic. The co-expression of the \( \text{phoD} \)-associated genes \( \text{tatA} \) and \( \text{tatC} \) mediated the Tat-dependent translocation of the SP PhoD-LacZ fusion protein. Since transport of SP PhoD-LacZ was blocked in the presence of spermine but not in the presence of sodium azide, it can be concluded that SP PhoD-LacZ is transported in a Sec-independent manner. Transport of SP PhoD-LacZ in an \( \text{E. coli} \) \( \text{tatABCDE} \) strain revealed that transport was independent of the \( \text{E. coli} \) Tat components. These data show that the minimal requirement of a specific Tat-dependent protein translocation system is consisting of a pair of TatA and TatC proteins, a signal peptide specifically recognized by these Tat components, and the existence of the \( \Delta \text{PH} \) gradient across the cytosolic membrane.

PhoD as well as SP PhoD-LacZ is not recognized by the \( \text{E. coli} \) Tat system. Our previous results obtained in \( \text{B. subtilis} \) revealed that transport of PhoD is mediated by TatC but is independent of the TatCp protein. These observations implied that \( \text{B. subtilis} \) contains at least two specific routes for Tat translocation. Further, \( \text{E. coli} \) tat \( \text{A} \) strains could not be complemented by its \( \text{B. subtilis} \) Tat proteins. Finally, absence of \( \text{E. coli} \) tat \( \text{A} \) genes did not prevent TatCp-mediated transport of SP PhoD-LacZ in \( \text{E. coli} \). These data strongly implicated that transport of hybrid peptides consisting of the signal peptide of PhoD, the reporter protein, and the TatA\( \text{Cp} \) protein pair form an autonomous Tat translocation system, and the recognition of Tat substrates is a selective process determined by multiple special protein-protein interactions between a given Tat substrate and its specific Tat proteins. Most of the twin-arginine signal peptide-containing \( \text{E. coli} \) proteins, and the existence of the \( \Delta \text{PH} \) gradient across the cytosolic membrane.

![Image](http://www.jbc.org/DownloadedFrom)
Overexpression of pre-PhoD could be due to slower transport kinetics of the Tat blocked. Most likely cytosolic phosphodiesterase activity was immediately after induction of PhoD synthesis cell growth was impaired for the cell viability preventing further protein synthesis of PhoD or raising protease degradation of the heterologous peptide. The induction of the SP-PhoD-LacZ fusion protein was not lethal for the E. coli cell. Several signal peptide-LacZ fusion proteins were previously used for studies of the Sec-dependent protein transport in E. coli. These fusion proteins were usually not transported through the cytosolic membrane. High level induction of these proteins was frequently detrimental for E. coli due to jamming of the Sec machinery (32–36). Induction of production of SP-PhoD-LacZ was not toxic for E. coli either in the absence or in the presence of B. subtilis TatA/Cd proteins. Since SP-PhoD is not recognized by the E. coli Sec or Tat machinery the fusion protein remained cytosolic. Co-induction of B. subtilis TatA/Cd proteins transported SP-PhoD-LacZ independent of E. coli-specific transport paths. Therefore, its translocation did not interfere with essential export functions of the cell. Tat-mediated export of LacZ is consistent with the capacity of the Tat translocation system to transport proteins that are probably folded prior to translocation.

Despite the fact that the SP-PhoD-LacZ was partially processed in E. coli TG1(pARphoD-lacZ, pREP4, pQE9tatA/Cd), the protein was entirely protease K-accessible. This observation indicates that the TatA/Cd components transport the protein efficiently through the cytosolic membrane, but cleavage of the signal peptide by E. coli LepB was inefficient. In E. coli TG1 ΔtatABCΔpARphoD-lacZ, pREP4, pQE9tatA/Cd), no processing of SP-PhoD-LacZ could be observed. At the moment there is no experimental knowledge about whether, when, and how the E. coli leader peptidase LepB cleaves signal peptides of Tat substrates. The presence of E. coli Tat components could be a prerequisite for cleavage of twin-arginine leader peptides by LepB.

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The Twin-arginine Signal Peptide of PhoD and the TatA_{d/Cd} Proteins of *Bacillus subtilis* Form an Autonomous Tat Translocation System

Ovidiu Pop, Ulrike Martin, Christian Abel and Jörg P. Müller

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