TatC Is a Specificity Determinant for Protein Secretion via the Twin-arginine Translocation Pathway*

Received for publication, June 6, 2000, and in revised form, September 27, 2000 Published, JBC Papers in Press, September 27, 2000, DOI 10.1074/jbc.M004887200

The recent discovery of a ubiquitous translocation pathway, specifically required for proteins with a twin-arginine motif in their signal peptide, has focused interest on its membrane-bound components, one of which is known as TatC. Unlike most organisms of which the genome has been sequenced completely, the Gram-positive eubacterium Bacillus subtilis contains two tatC-like genes denoted tatCd and tatCy. The corresponding TatCd and TatCy proteins have the potential to be involved in the translocation of 27 proteins with putative twin-arginine signal peptides of which 6–14 are likely to be secreted into the growth medium. Using a proteomic approach, we show that PhoD of B. subtilis, a phosphodiesterase belonging to a novel protein family of which all known members are synthesized with typical twin-arginine signal peptides, is secreted via the twin-arginine translocation pathway. Strikingly, TatCd is of major importance for the secretion of PhoD, whereas TatCy is not required for this process. Thus, TatC appears to be a specificity determinant for protein secretion via the Tat pathway. Based on our observations, we hypothesize that the TatC-determined pathway specificity is based on specific interactions between TatC-like proteins and other pathway components, such as TatA, of which three paralogues are present in B. subtilis.

The Gram-positive eubacterium Bacillus subtilis is known to secrete a great variety of proteins into the growth medium (1). Together with components of the protein secretion pathways of B. subtilis, these native secreted proteins form the so-called TatC-like genes denoted tatCd and tatCy. The corresponding TatCd and TatCy proteins have the potential to be involved in the translocation of 27 proteins with putative twin-arginine signal peptides of which 6–14 are likely to be secreted into the growth medium. Using a proteomic approach, we show that PhoD of B. subtilis, a phosphodiesterase belonging to a novel protein family of which all known members are synthesized with typical twin-arginine signal peptides, is secreted via the twin-arginine translocation pathway. Strikingly, TatCd is of major importance for the secretion of PhoD, whereas TatCy is not required for this process. Thus, TatC appears to be a specificity determinant for protein secretion via the Tat pathway. Based on our observations, we hypothesize that the TatC-determined pathway specificity is based on specific interactions between TatC-like proteins and other pathway components, such as TatA, of which three paralogues are present in B. subtilis.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† These authors contributed equally to this work.

‡ Supported by Grant 805-33.605 from the Stichting Levensmiddelendraga (8, 9).

§ Supported by the Deutsche Forschungsgemeinschaft.

∥ Supported by Genencor International (Leiden, The Netherlands).

¶ To whom correspondence should be addressed. Tel.: 31-50-3633079; Fax: 31-50-3636908; E-mail: j.m.van.dijl@farm.rug.nl.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† These authors contributed equally to this work.

‡ Supported by Grant 805-33.605 from the Stichting Levensmiddelendraga (8, 9).

§ Supported by the Deutsche Forschungsgemeinschaft.

∥ Supported by Genencor International (Leiden, The Netherlands).

¶ To whom correspondence should be addressed. Tel.: 31-50-3633079; Fax: 31-50-3636908; E-mail: j.m.van.dijl@farm.rug.nl.

The recent discovery of a ubiquitous translocation pathway, specifically required for proteins with a twin-arginine motif in their signal peptide, has focused interest on its membrane-bound components, one of which is known as TatC. Unlike most organisms of which the genome has been sequenced completely, the Gram-positive eubacterium Bacillus subtilis contains two tatC-like genes denoted tatCd and tatCy. The corresponding TatCd and TatCy proteins have the potential to be involved in the translocation of 27 proteins with putative twin-arginine signal peptides of which 6–14 are likely to be secreted into the growth medium. Using a proteomic approach, we show that PhoD of B. subtilis, a phosphodiesterase belonging to a novel protein family of which all known members are synthesized with typical twin-arginine signal peptides, is secreted via the twin-arginine translocation pathway. Strikingly, TatCd is of major importance for the secretion of PhoD, whereas TatCy is not required for this process. Thus, TatC appears to be a specificity determinant for protein secretion via the Tat pathway. Based on our observations, we hypothesize that the TatC-determined pathway specificity is based on specific interactions between TatC-like proteins and other pathway components, such as TatA, of which three paralogues are present in B. subtilis.

The recent discovery of a ubiquitous translocation pathway, specifically required for proteins with a twin-arginine motif in their signal peptide, has focused interest on its membrane-bound components, one of which is known as TatC. Unlike most organisms of which the genome has been sequenced completely, the Gram-positive eubacterium Bacillus subtilis contains two tatC-like genes denoted tatCd and tatCy. The corresponding TatCd and TatCy proteins have the potential to be involved in the translocation of 27 proteins with putative twin-arginine signal peptides of which 6–14 are likely to be secreted into the growth medium. Using a proteomic approach, we show that PhoD of B. subtilis, a phosphodiesterase belonging to a novel protein family of which all known members are synthesized with typical twin-arginine signal peptides, is secreted via the twin-arginine translocation pathway. Strikingly, TatCd is of major importance for the secretion of PhoD, whereas TatCy is not required for this process. Thus, TatC appears to be a specificity determinant for protein secretion via the Tat pathway. Based on our observations, we hypothesize that the TatC-determined pathway specificity is based on specific interactions between TatC-like proteins and other pathway components, such as TatA, of which three paralogues are present in B. subtilis.
B. subtilis (see Fig. 1B). Interestingly, each of the two tatC genes of B. subtilis was preceded by a tatABE-like gene. These observations are consistent with the identification of genes for (putative) exported proteins with RR signal peptides in B. subtilis. Strikingly, however, the WapA and WprA proteins, which are synthesized with potential RR signal peptides, were recently shown to be secreted in an Ffh- and SecA-dependent manner (21). As the transport of proteins via the Tat pathway of E. coli was shown to be independent of SecA and largely independent of Ffh (22), this observation raised the question whether a functional Tat pathway exists in B. subtilis. On the contrary, the observation that B. subtilis contains two paralogous tatC genes, each with an upstream tatA gene, might even suggest that two parallel routes for twin-arginine translocation exist in this organism. This idea was, to some extent, also suggested by the observation that one set of tatAC genes of B. subtilis was preceded by the phoD gene, which specifies a secreted phosphodiesterase (i.e., PhoD) (23) with an RR signal peptide (see Table I) and which is expressed only under conditions of phosphate starvation (23). In the present study, we show that the latter tatC gene, denoted tatCd, is expressed only under conditions of phosphate starvation. Moreover, it seems to be specifically required for the secretion of PhoD, a process that was almost completely blocked when the tatCd gene was disrupted, but not when the tatCy gene was disrupted. These observations show that the TatCd protein of B. subtilis is a specificity determinant for Tat-dependent protein secretion.

EXPERIMENTAL PROCEDURES

Plasmids, Bacterial Strains, and Media—Table II lists the plasmids and bacterial strains used. TY medium (Tryptone/yeast extract) contained Bacto-Tryptone (1%), Bacto-yeast extract (0.5%), and NaCl (1%). Minimal medium was prepared as described (24). Schaeffer’s sporulation medium was prepared as described (25). High phosphate and low phosphate (LPDM)3 defined media were prepared as described (26). To test anaerobic growth, S7 medium was prepared as described (27, 28) and bacterial strains used. TY medium (Tryptone/yeast extract) contained Bacto-Tryptone (1%), Bacto-yeast extract (0.5%), and NaCl (1%).

DNA Techniques—Procedures for DNA purification, restriction, ligation, gel electrophoresis, and transformation of E. coli were carried out as described (29). Enzymes were from Roche Molecular Biochemicals. B. subtilis was transformed as described (30). PCR was carried out with the Pwo DNA polymerase (New England Biolabs Inc.) as described (31).

To construct B. subtilis tatCd, the 5′-region of the tatCd gene was amplified by PCR with primers JJ14dT (5′-CCC AAG CTT ATG AAA GGG AGG GCT TTT TTG AAT GG-3′, containing a HindIII site) and JJ15t (5′-GGG GAT CCA ATG ACC ATG ATG CC-3′, containing a BamHI site). The amplified fragment was cleaved with HindIII and BamHI and cloned in the corresponding sites of pMic21 (32), resulting in pMicCd. B. subtilis tatCd was obtained by a Campbell-type integration (single crossover) of pMicCd into the tatCd region of the chromosome.

To construct B. subtilis tatCy, the 5′-region of the tatCy gene was amplified by PCR with primers JJ103Cy (5′-CCCC AAC CTT ATG AAA AAG GGA GAT CAG TAA GGT AGG ATG-3′, containing a HindIII site) and JJ104Cy (5′-GGG GAT CCA AGT CTT GAG AAC TCC G-3′, containing a BamHI site). The amplified fragment was cleaved with HindIII and BamHI and cloned in the corresponding sites of pMic21, resulting in pMicCy. B. subtilis tatCy was obtained by a Campbell-type integration (single crossover) of pMicCy into the tatCy region of the chromosome.

To construct B. subtilis tatCd, the tatCd gene was amplified by PCR with primers JJ33Cd (5′-GGA ATT CTT GGG GCT ACC-3′, containing an EcoRI site and 5′-sequences of tatCd) and JJ34Cd (5′-CGG GAT CCA TCA TGC GAA GCG-3′, containing a BamHI site and 3′-sequences of tatCD). Next, the PCR-amplified fragment was cleaved with EcoRI and BamHI and ligated into the corresponding sites of pUC21, resulting in pJCy3. Plasmid pJCy2 was obtained by replacing an internal BclI-AccI fragment of the tatCd gene in pJCy1 with a pdG792-derived kanamycin resistance marker, flanked by BamHI and ClAI restriction sites. Finally, B. subtilis ΔtatCd was obtained by a double crossover recombination event between the disrupted tatCd genes in pJCy2 and the chromosomal tatCd gene.

To construct B. subtilis ΔtatCy, the tatCy gene was amplified by PCR with primers JJ29Cy (5′-GGG GAT CCA AAC GAA GCT TCA TGC G-3′, containing a KpnI site and 5′-sequences of tatCy) and JJ30Cy (5′-GGG GAT CCA TGC GCT AGC ATC GG-3′, containing a BamHI site) and amplified by PCR with primers Kpn1 and BamHI and ligated into the Asp718 and BamHI sites of pUC21, resulting in pJCy4. Plasmid pJCy2 was obtained by ligating a pdG1726-derived spectinomycin resistance marker, flanked by PstI restriction sites, into the unique PstI site of the tatCy gene in pJCy1. Finally, B. subtilis ΔtatCy was obtained by a double crossover recombination event between the disrupted tatCy gene of pJCy2 and the chromosomal tatCy gene.

ΔtatCd-ΔtatCy double mutants were constructed by transforming the ΔtatCy mutant with chromosomal DNA of the ΔtatCd or ΔtatCd mutant strain. Correct integration of plasmids or resistance markers into the chromosome of B. subtilis was verified by Southern blotting. The BLAST algorithm (33) was used for protein comparisons in the GenBank2/EBI Data Bank. Protein sequence alignments were carried out with the ClustalW program (34) using the Blosum matrices. Version 6.7 of the PCGene Analysis Program (IntelliGenetics Inc.) putative transmembrane segments and their membrane topologies were predicted with the TopPred2 algorithm (35, 36).

Competence and Sporulation—Competence for DNA binding and uptake was determined by transformation with plasmid or chromosomal DNA (37). The efficiency of sporulation was determined by overnight growth in Schaeffer’s sporulation medium, killing of cells with 0.1

**Table I**

Predicted twin-arginine signal peptides of B. subtilis

| Protein | Signal peptide |
|---------|---------------|
| AmyX | M-K-A-A-A-A-A-A-A- |
| AmyY | K-A-A-A-A-A-A-A- |
| AmyZ | A-A-A-A-A-A-A- |
| ApoB | B-A-A-A-A-A-A-A- |
| LDH | H-A-A-A-A-A-A- |
| Opp | P-A-A-A-A-A-A-A- |
| PhoB | B-A-A-A-A-A-A-A- |
| QsrA | S-A-A-A-A-A-A-A- |
| Tma | M-A-A-A-A-A-A-A- |
| WapA | A-A-A-A-A-A-A-A- |
| WprA | P-A-A-A-A-A-A-A- |
| YepA | A-A-A-A-A-A-A-A- |
| YebF | F-A-A-A-A-A-A-A- |
| YebK | K-A-A-A-A-A-A-A- |
| YamT | T-A-A-A-A-A-A-A- |
| YamV | V-A-A-A-A-A-A-A- |
| YaeC | E-A-A-A-A-A-A- |
| YaeU | U-A-A-A-A-A-A- |

2 The abbreviations used are: LPDM, low phosphate defined medium; IPTG, isopropyl-β-D-thiogalactopyranoside; PCR, polymerase chain reaction.
volume of chloroform, and subsequent plating.

**Enzyme Activity Assays**—The assay and calculation of β-galactosidase units (expressed as units/A absorbance) were carried out as described (38). Overnight cultures were diluted 100-fold in fresh medium, and samples were taken at hourly intervals for A absorbance readings and β-galactosidase activity determinations. Induction of the phosphate starvation response was monitored by alkaline phosphatase activity determinations as described (39).

**Western Blot Analysis and Immunodetection**—To detect PhoB and PhoD, *B. subtilis* cells were separated from the growth medium by centrifugation (14,000 rpm, 2 min, room temperature). Proteins in the growth medium were concentrated 20-fold upon precipitation with trichloroacetic acid, and samples for SDS-polyacrylamide gel electrophoresis were prepared as described previously (40). After separation by SDS-polyacrylamide gel electrophoresis, proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) as described (41). PhoB and PhoD were visualized with specific antibodies (42) and alkaline phosphatase-conjugated goat anti-rabbit antibodies (Sigma) according to the manufacturer’s instructions.

**Two-dimensional Gel Electrophoresis of Secreted Proteins**—*B. subtilis* strains were grown at 37°C under vigorous agitation in 1 liter of a synthetic medium (43, 44) containing 0.16 mM KH2PO4 to induce a phosphate starvation response. After 1 h of post-exponential growth, cells were separated from the growth medium by centrifugation. The secreted proteins in the growth medium were precipitated with ice-cold 10% trichloroacetic acid and collected by centrifugation (40,000 × g, 2 h, 4°C). The pellet was washed three times with 96% ethanol; dried; and resuspended in 400 μl of rehydration solution containing 2 μl thiourea, 8 μl urea, 1% Nonidet P-40, 20 mM dithiothreitol, and 0.5% Pharmalyte (pH 3–10). Cells were disrupted by sonication as described (45), and cellular proteins were resuspended in rehydration solution as described above. Samples of secreted or cellular proteins in rehydration solution were used for the re-swelling of immobilized pH gradient strips (pH 3–10; Amersham Pharmacia Biotech). Next, protein separation in the immobilized pH gradient strips (first dimension electrophoresis) was performed as recommended by the manufacturer. Electrophoresis in the second dimension was performed as described (46). The resulting two-dimensional gels were stained with silver nitrate (47) or Coomassie Brilliant Blue R-250.

**Protein Identification**—In-gel tryptic digestion of proteins, separated by two-dimensional gel electrophoresis, was performed using a peptide-collecting device (48). For this purpose, 0.5 μl of peptide solution was mixed with an equal volume of a saturated α-cyano-4-hydroxycinnamic acid solution in 50% acetonitrile and 0.1% trifluoroacetic acid. The resulting mixture was applied to the sample template of a matrix-assisted laser desorption/ionization mass spectrometer (Voyager DE-STR, PerSeptive Biosystems). Peptide mass fingerprints were analyzed using MS-Fit software.

**RESULTS**

**Identification of tat Genes of B. subtilis**—To investigate whether *B. subtilis* contains a potential Tat pathway, a search for homologues of *E. coli* Tat proteins was performed using the complete sequence of the *B. subtilis* genome (3). First, sequence comparisons revealed that *B. subtilis* contains three paralogous genes (i.e., *yczB*, *ydiI*, and *ynzA*) that specify proteins with sequence similarity to the three paralogous *E. coli* TatA, TatB, and TatE proteins. Specifically, the *ydiI* gene (57 residues), which was renamed TatAy, showed the highest degree of sequence similarity to the *E. coli* TatA protein (58% identical residues and conservative replacements); the *yczB* protein (70 residues), which was renamed TatAd, showed the highest degree of sequence similarity to the *E. coli* TatB protein (58% identical residues and conservative replacements); and the *ynzA* protein (62 residues), which was renamed TatAc, showed the highest degree of sequence similarity to the *E. coli* TatE protein (53% identical residues and conservative replacements). All three *B. subtilis* proteins were renamed TatA to avoid possible misinterpretations with respect to their respective functions, which are presently unknown. Like TatA, TatB, and TatE of *E. coli*, the three TatA proteins of *B. subtilis* appear to have one amino-terminal membrane-spanning domain (Fig. 1A), and the carboxyl-terminal parts of these proteins are predicted to face the cytoplasm. Even though TatAc, TatAd, and TatAy of *B. subtilis* show significant similarity to TatA, TatB, and TatE of *E. coli* when the amino acid sequences of these proteins are compared pairwise, only a limited number of residues are conserved in all six amino acid sequences (17% identical residues and conservative replacements) (Fig. 1A).

Second, in contrast to *E. coli*, which contains a unique *tatC* gene (11), *B. subtilis* was shown to contain two paralogous *tatC*-like genes (i.e. *yczB* and *ydiJ*). The *yczB* protein (245 residues), which was renamed TatCd, and the *ydiJ* protein (254 residues), which was renamed TatCy, showed significant similarity to the *E. coli* TatC protein (57% identical residues and conservative replacements in the three aligned sequences) (Fig. 1B). Like TatC of *E. coli*, TatCd and TatCy of *B. subtilis* have six potential transmembrane segments (Fig. 1B), and the amino termini of these proteins are predicted to face the cytoplasm (data not shown).

In contrast to *E. coli*, in which the *tatA*, *tatB*, and *tatC* genes form one operon and the *tatE* gene is monocistronic (10), the *tat* genes of *B. subtilis* are located at three distinct chromosomal regions. Two of these regions contain adjacent *tatA* and *tatC* genes, whereas the *tatB* and *tatCy* genes are located independently upstream of the *tatCd* and *tatCy* genes, respectively (Fig. 2). Strikingly, the *tatAd* and *tatAy* genes, which map at 24.4° on the *B. subtilis* chromosome, are located immediately downstream of the *phoD* gene, specifying a secreted protein with a putative RR signal peptide (Table I). Furthermore, the *tatAy* and *tatCy* genes are located at 55.3° on the *B. subtilis* chromosome, within a cluster of genes with unknown function (Fig. 2), and the *tatAc* gene is located at 162.7° on the *B. subtilis* chro-
Tat Pathway Specificity

A. B. subtilis

\[ \text{ycbR} \rightarrow \text{phoD} \rightarrow \text{tatAd} \rightarrow \text{tatCd} \rightarrow \text{pcp} \]

B. E. coli

\[ \text{yigR} \rightarrow \text{tatA} \rightarrow \text{tatB} \rightarrow \text{tatC} \rightarrow \text{tatD} \rightarrow \text{rjAI} \]

**Fig. 2. tatAC regions of B. subtilis and E. coli.** A, chromosomal organization of the B. subtilis tatAd-tatCd and tatAy-tatCy regions (adapted from the SubeList Database). Note that the tatAd and tatCd genes are located downstream of the phoD gene, B. chromosomal organization of the E. coli tatABC region (adapted from the Colibri Database).

mosome (data not shown), immediately downstream of the cotC gene, specifying a spore coat protein (49).

Taken together, these observations strongly suggest that B. subtilis has a Tat pathway for the translocation of proteins with RR signal peptides across the cytoplasmic membrane. Furthermore, the observation that the tatAd and tatCd genes are located downstream of the phoD gene, which is a member of the pho regulon (23), suggests that the tatAd and tatCd genes might be exclusively expressed under conditions of phosphate starvation.

**TatC-dependent Secretion of the PhoD Protein**—To investigate whether an active Tat pathway exists in B. subtilis, various single and double tatC mutants were constructed. For this purpose, the tatCd gene was either disrupted with a kanamycin resistance marker or placed under the control of the IPTG-dependent P_{spac} promoter of plasmid pMutin2, resulting in the B. subtilis strains ΔtatCd and ΔtatCdC, respectively (Fig. 3, A and B). Similarly, the tatCy gene was either disrupted with a spectinomycin resistance marker or placed under the control of the IPTG-dependent P_{spac} promoter of plasmid pMutin2, resulting in the B. subtilis strains ΔtatCy and ΔtatCyC, respectively (Fig. 3, A and C). tatCdΔtatCy double mutants were constructed by transforming the ΔtatCy mutant with chromosomal DNA of the ΔtatCd or ΔtatCdC mutant strain.

The fact that tatCdΔtatCy double mutants could be obtained shows that TatC function is not essential for viability of B. subtilis, at least not when cells are grown aerobically in TY or minimal medium at 37 °C or anaerobically in S7 medium supplemented with NaNO\(_3\) (0.2%) and glycerol (2%) at 37 °C (data not shown). Furthermore, the ΔtatCd-ΔtatCy double mutation did not inhibit the development of competence for DNA binding and uptake, sporulation, and the subsequent spore germination (data not shown), showing that these primitive developmental processes do not require TatC function.

The effects of single and double tatC mutations on protein secretion via the Tat pathway were studied using PhoD as a native reporter protein. For this purpose, tatC mutant strains were grown under conditions of phosphate starvation in LPDM. As shown by Western blotting, the secretion of PhoD was strongly reduced in the ΔtatCd mutant strain and the ΔtatCdΔtatCy double mutant, whereas it was not affected or even improved in the ΔtatC mutant strain (Fig. 4A). In contrast, the secretion of the alkaline phosphatase PhoB, which is dependent of the major (Sec) pathway for protein secretion (50), was not affected in the tatC mutants of B. subtilis (Fig. 4B). Notably, in some experiments, very low amounts of PhoD were detectable in the growth medium of B. subtilis ΔtatCd (data not shown), but never in that of the ΔtatCdΔtatCy and ΔtatCdC-ΔtatCdC double mutants (Fig. 4, A and C). As exemplified with the B. subtilis ΔtatCdC-ΔtatCy double mutant strain, the cells of all tatC mutant strains contained similar amounts of pre-PhoD, which were comparable to those in the parental strain 168 (Fig. 4C; data not shown). Finally, two-dimensional gel electrophoresis of proteins in the medium of phosphate-starved cells of B. subtilis ΔtatCdΔtatCy or the parental strain 168 showed that PhoD is the only protein of which the secretion is detectably affected by the tatC double mutation under conditions of phosphate starvation (Fig. 5). As expected, the secretion of proteins lacking an RR signal peptide, such as the glycerol phosphoryl-diester phosphodiesterase GlpQ, the pectate lyase Pel, the alkaline phosphatases PhoA and PhoB, the phosphate-binding protein PstS; the minor extracellular serine protease Vpr; and the protein with unknown function, YncM) was not significantly affected by the tatC double mutation. Surprisingly, however, the secretion of YdbF, a phosphate starvation-inducible protein of unknown function (44), and the 2’,3’-cyclic

**Fig. 3. Construction of tatC mutant strains of B. subtilis.** A, schematic presentation of the construction of B. subtilis ΔtatCd and ΔtatCdC. The chromosomal tatCd gene was disrupted with a kanamycin resistance marker (Km\(^\text{r}\)) by homologous recombination. For this purpose, B. subtilis 168 was transformed with plasmid pCy2, which cannot replicate in B. subtilis and contains a mutant copy of the tatCy gene with a spectinomycin resistance marker in the Pet site. Only restriction sites relevant for the construction are shown. ΔtatCd, 5’-end of the tatCd gene; ΔtatCdC, 3’-end of the tatCd gene; ΔtatCy, 5’-end of the tatCy gene; ΔtatCyC, 3’-end of the tatCy gene. B, schematic presentation of the tatCd region of B. subtilis ΔtatCdC. By a Campbell-type integration of the pMutin2 derivative pMICd1 into the B. subtilis 168 chromosome, the tatCd gene was placed under the control of the IPTG-dependent P_{spac} promoter, which can be repressed by the product of the lacI gene. Simultaneously, the spoVG-lacz reporter gene of pMutin2 was placed under the transcriptional control of the tatCd promoter region. PCR-amplified regions are indicated by black bars. ori pBR322, replication functions of pBR322; Ap\(^\ast\), ampicillin resistance marker; Em\(^\ast\), erythromycin resistance marker; Tac, 3’-truncated tatCd gene; T\(_T\), transcriptional terminators on pMutin2. C, schematic presentation of the tatCy region of B. subtilis ΔtatCyC. By a Campbell-type integration of the pMutin2 derivative pMICy1 into the B. subtilis 168 chromosome, the tatCy gene was placed under the control of the IPTG-dependent P_{spac} promoter. Simultaneously, the spoVG-laczZ reporter gene of pMutin2 was placed under the transcriptional control of the tatCy promoter region. tatCyC, 3’-truncated tatCy gene.
nucleotide 2'-phosphodiesterase YkN, 3 which are both synthesized with potential RR signal peptides (Table I), was also not affected by the disruption of tatCd and tatCy (Fig. 5). Similarly, comparable WprA-derived protein spots could be demonstrated in the medium fractions of the B. subtilis ΔtatCd-ΔtatCy and

3 Please note that, accidentally, the YkN spot was previously designated XkD (49).
the H-region is an important determinant that allows the cell to discriminate between Sec-type and RR signal peptides. Notably, the predicted RR motifs of WapA, WprA, YdhF, and YfkN are also different from previously described RR signal peptides because they contain either a His residue at position +2 relative to the twin arginines or a Lys or Ser residue at position +3 (Table I). In fact, hydrophilic residues are completely absent from positions +2 and +3 relative to the twin arginines of known RR signal peptides (5, 6, 10, 14, 18, 22). If low overall hydrophobicity and the presence of hydrophobic residues at positions +2 and +3 are used as criteria for the prediction of RR signal peptides, the total number of predicted B. subtilis signal peptides of this type can be reduced from 27 to 11. Of these 11 preproteins, four contain additional transmembrane segments, and one lacks a signal peptidase cleavage site. Thus, based on these more stringent criteria, one would predict that merely six proteins of B. subtilis (i.e. AlbB, LipA, PhoD, YkuE, YusC, and YwvN) are secreted into the growth medium via the Tat pathway. This would explain why the secretion of only one protein, PhoD, was detectably affected in B. subtilis ΔtatCd ΔtatCy under conditions of phosphate starvation. In this respect, it is important to note that TatC-dependent secretion of some other proteins with (predicted) RR signal peptides may have remained unnoticed in the present study because they are expressed at very low levels under conditions of phosphate starvation. Furthermore, it is conceivable that other TatC-dependent proteins were missed in the two-dimensional gel electrophoretic analysis due to their poor separation in the first dimension.

Interestingly, the YdhF protein was also predicted to be a lipoprotein (Table I) (4). The fact that YdhF was found in the growth medium suggests either that this prediction was wrong or that YdhF is released into the growth medium via a secondary processing event that follows cleavage by the lipoprotein-specific (type II) signal peptidase (52). Such secondary processing events have been described previously for other Bacillus lipoproteins (see Ref. 4). In fact, the latter possibility most likely explains why the phosphate-binding protein PstS, which is a typical lipoprotein (previously known as YggG) (4, 53), was found in the growth medium. As expected for lipoproteins, significant amounts of PstS were also present in a cell-assoc-
Homologues of B. subtilis (Bsu) PHOD were identified by amino acid sequence similarity searches in the GenBank™/EBI Data Bank using the BLAST algorithm. SP1 (Sco), gene SCS75A.32e of Streptomyces coelicolor (accession number CAB61732); SP2 (Sco), gene SCF43A.18 of S. coelicolor (accession number CAB48905); SP3 (Sco), gene SCG6.37 of S. coelicolor (accession number CAB51460); SP4, phoD gene of Streptomyces tendae (accession number CAB25656). Conserved residues of the twin-arginine consensus sequence are indicated in boldface. The hydrophobic H-region is shaded. Signal peptide I recognition sequences predicted with the SignalP algorithm (62, 63) are underlined.

**TABLE IV**

| proteus         | signal peptide |
|-----------------|----------------|
| PhOD            | MAPRQGDSQVFLFSGFPRRLACGQKARSLYIVLHFLAEGAEQLKYILQKQSFEP       |
| Bsu             | MAPRQGDSQVFLFSGFPRRLACGQKARSLYIVLHFLAEGAEQLKYILQKQSFEP       |
| SP1 (Sco)       | MAPRQGDSQVFLFSGFPRRLACGQKARSLYIVLHFLAEGAEQLKYILQKQSFEP       |
| SP2 (Sco)       | MAPRQGDSQVFLFSGFPRRLACGQKARSLYIVLHFLAEGAEQLKYILQKQSFEP       |
| SP3 (Sco)       | MAPRQGDSQVFLFSGFPRRLACGQKARSLYIVLHFLAEGAEQLKYILQKQSFEP       |
| SP4 (Sco)       | MAPRQGDSQVFLFSGFPRRLACGQKARSLYIVLHFLAEGAEQLKYILQKQSFEP       |

of Berks et al. (19), in which, on the basis of theoretical considerations, it was proposed that the TatABE proteins form a protein-conducting channel, whereas the TatC protein acts as an RR signal peptide receptor. Alternatively, it is still conceivable that certain proteins with RR signal peptides are recognized by TatA-like proteins, provided that a specific TatC-like partner protein is present. A third possibility would be that specific TatA- and TatC-like partner proteins are jointly involved in substrate recognition. The facts that neither TatAc nor TatAd of B. subtilis was able to complement tatA, tatB, or tatE mutations in E. coli and that TatCd of B. subtilis was unable to complement the E. coli tatC mutation suggest that the TatC-determined pathway specificity, as described in the present study, is based on specific interactions between TatA- and TatC-like proteins. If so, this implies that B. subtilis contains two parallel routes for twin-arginine translation, one of which involves the TatCd protein. As shown in the present study, the TatCd-dependent translocation appears to be activated specifically under conditions of phosphate starvation, perhaps with the sole purpose of translocating PhoD. Similar to the situation in B. subtilis, parallel routes for twin-arginine translocation may be present in other organisms, such as *Archaeboglobus fulgidus*, which was shown to contain two paralogous tatC-like genes (19, 61). In our ongoing research on protein secretion in *B. subtilis*, we are trying to challenge this hypothesis.

**Acknowledgments**—We thank Dr. A. J. M. Driessen for providing sera against PhoB and Drs. A. Bolhuis, C. Robinson, and M. L. van Roosmalen and other members of the ExporterRPs Consortium for useful discussions.

**REFERENCES**

1. Simonen, M., and Palv, I. (1993) Microbiol. Rev. 57, 109–137
2. Tjalsma, H., Bolhuis, A. Jongbloed, J. D. H., Bron, S., and van Dijl, J. M. (2000) Microbiol. Mol. Biol. Rev. 48, S15–S57
3. Kunst, F., Ogawa, K., Mosher, J., Albertini, A. M., Aloni, G., Aveneto, V., Bertero, M. G., Besier, P., Bolotin, A., Borchert, S., et al. (1997) Nature 390, 249–256
4. Tjalsma, H., et al. (1999) *J. Biol. Chem.* 274, 1498–1507
5. Berks, B. C. (1996) *Mol. Microbiol.* 22, 393–404
6. Brink, S., et al. (1998) *FEBS Lett.* 434, 425–430
7. Cristobel, S., de Gier, J. W., Nielsen, H., and von Heijne, G. (1999) *EMBO J.* 18, 2982–2990
8. Settles, A. M., and Martiennsen, R. (1998) *Trends Cell Biol.* 8, 494–501
9. Deley, B. C., and Robinson, C. (1999) *Trends Biochem. Sci.* 24, 17–22
10. Sargent, F., et al. (1998) *J. Biol. Chem.* 273, 18003–18006
11. Forys, R. M., and Robinson, C. (1991) *J. Biol. Chem.* 266, 12189–12193
12. Robinson, C., et al. (1999) *J. Biol. Chem.* 274, 2715–2722
13. Wester, M., Sargent, F., et al. (1999) *J. Biol. Chem.* 274, 674–676
14. Chaddock, A. M., et al. (1999) *J. Biol. Chem.* 275, 16717–16722
15. Coffer, J. P., et al. (1998) *J. Biol. Chem.* 273, 5465–5469
16. Weimer, D. J., et al. (1998) *Cell* 93, 95–101
17. Chanda, A., et al. (1998) *Mol. Microbiol.* 29, 674–676
18. Sargent, F., et al. (1998) *J. Biol. Chem.* 273, 26073–26082
19. Berks, B. C., et al. (1998) *Mol. Microbiol.* 29, 260–274
20. Settles, A. M., et al. (1998) *Microbiology* 144, 2041–2047
21. Horige, I., Sano, K., et al. (1998) *Microbiology* 144, 2041–2047
22. Santini, C. L., et al. (1998) *J. Biol. Chem.* 273, 101–112
23. Rieger, S. L., et al. (1998) *Microbiology* 144, 2041–2047
24. Tjalsma, H., et al. (1998) *J. Biol. Chem.* 273, 101–112
25. Rieger, S. L., et al. (1998) *Microbiology* 144, 2041–2047
26. Muller, J. F., et al. (1998) *Microbiology* 144, 2041–2047
27. van Dijl, J. M., de Jong, A., et al. (1999) *J. Gen. Microbiol.* 137, 2073–2083
28. de Jong, A., et al. (1999) *J. Gen. Microbiol.* 137, 2073–2083
28. van Dijl, J. M., de Jong, A., Smith, H., Bron, S., and Venema, G. (1991) *Mol. Gen. Genet.* **227**, 40–48.
29. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
30. Tjalsma, H., Noback, M. A., Bron, S., Venema, G., Yamane, K., and van Dijl, J. M. (1997) *J. Biol. Chem.* **272**, 25983–25992.
31. van Dijl, J. M., de Jong, A., Venema, G., and Bron, S. (1995) *J. Biol. Chem.* **270**, 3611–3618.
32. Vagner, V., Dervyn, E., and Ehrlich, S. D. (1998) *Microbiology* (Read.) **144**, 3097–3104.
33. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) *Nucleic Acids Res.* **25**, 3389–3402.
34. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22**, 4673–4680.
35. Sipos, L., and von Heijne, G. (1993) *Eur. J. Biochem.* **213**, 1333–1340.
36. Cserzo, M., Wallin, E., Simon, I., von Heijne, G., and Elofsson, A. (1997) *Protein Eng.* **10**, 673–676.
37. Bron, S., and Venema, G. (1972) *Mutat. Res.* **15**, 1–10.
38. Miller, J. H. (1982) *Experiments in Molecular Biology*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
39. Hulett, F. M., Bookstein, C., and Jensen, K. (1990) *J. Bacteriol.* **172**, 735–740.
40. Laemmli, U. K. (1970) *Nature* **227**, 680–685.
41. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 4350–4354.
42. Muller, J. P., and Wagner, M. (1999) *FEBS Microbiol. Lett.* **180**, 287–296.
43. Antelmann, H., Engelmann, S., Schmid, R., Sorokin, A., Lapidus, A., and Hecker, M. (1997) *J. Bacteriol.* **179**, 7251–7256.
44. Antelmann, H., Scharf, C., and Hecker, M. (2000) *J. Bacteriol.* **182**, 4478–4490.
45. Koyama, C., Mach, H., Harwood, C. R., and Hecker, M. (1996) *Microbiology* (Read.) **142**, 3163–3170.
46. Bernhardt, J., Volker, U., Volker, A., Antelmann, H., Schmid, R., Mach, H., and Hecker, M. (1997) *Microbiology* (Read.) **143**, 999–1017.
47. Blum, H., Beier, H., and Gross, H. J. (1987) *Electrophoresis* **8**, 93–99.
48. Otto, A., Thiede, B., Muller, E. C., Scheler, C., Wittmann-Liebold, B., and Jungblut, P. (1996) *Electrophoresis* **17**, 1643–1650.
49. Donovan, W., Zheng, L. B., Sandman, K., and Losick, R. (1987) *J. Mol. Biol.* **196**, 1–10.
50. Swaving, J., van Wely, K. H., and Driessen, A. J. (1999) *J. Bacteriol.* **181**, 7021–7027.
51. Bolhuis, A., Sorokin, A., Azevedo, V., Ehrlich, S. D., Braun, P. G., de Jong, A., Venema, G., Bron, S., and van Dijl, J. M. (1996) *Mol. Microbiol.* **22**, 605–618.
52. Praigai, Z., Tjalsma, H., Bolhuis, A., van Dijl, J. M., Venema, G., and Bron, S. (1997) *Microbiology* (Read.) **143**, 1237–1333.
53. Qi, Y., and Hulett, F. M. (1998) *J. Bacteriol.* **180**, 4007–4010.
54. Rodrigue, A., Chanaia, A., Beck, K., Muller, M., and Wu, L. F. (1999) *J. Biol. Chem.* **274**, 13223–13228.
55. Bogaeh, E., Brink, S., and Robinson, C. (1997) *EMBO J.* **16**, 3851–3859.
56. Hynds, P. J., Robinson, D., and Robinson, C. (1998) *J. Biol. Chem.* **273**, 34868–34874.
57. Yu, J., Hederstedt, L., and Piggot, P. J. (1995) *J. Bacteriol.* **177**, 6751–6760.
58. Stephenson, K., and Harwood, C. R. (1998) *Appl. Environ. Microbiol.* **64**, 2875–2881.
59. Bolhuis, A., Tjalsma, H., Stephenson, K., Harwood, C. R., Venema, G., Bron, S., and van Dijl, J. M. (1999) *J. Biol. Chem.* **274**, 15865–15868.
60. Bolhuis, A., Tjalsma, H., Smith, H. E., de Jong, A., Meima, R., Venema, G., Bron, S., and van Dijl, J. M. (1999) *Appl. Environ. Microbiol.* **65**, 2934–2941.
61. Klenk, H. P., Clayton, R. A., Tomb, J. F., White, O., Nelson, K. E., Ketchum, K. A., Dodson, R. J., Gwinn, M., Hickey, E. K., Peterson, J. D., Richardson, D. L., et al. (1997) *Nature* **390**, 364–370.
62. Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) *Protein Eng.* **10**, 1–6.
63. Nielsen, H., Brunak, S., and von Heijne, G. (1999) *Protein Eng.* **12**, 3–9.
64. Vieira, J., and Messing, J. (1990) *Gene (Amst.*) **100**, 189–194.
65. Guerout-Fleury, A. M., Shazand, K., Frandsen, N., and Stragier, P. (1995) *Gene (Amst.*) **167**, 335–336.
66. Wirtman, K. F., Wyman, A. R., and Botstein, D. (1986) *Cell* **49**, 253–262.