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A Minimal Tat System from a Gram-positive Organism

A BIFUNCTIONAL TatA SUBUNIT PARTICIPATES IN DISCRETE TatAC AND TatA COMPLEXES

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James P. Barnett1,2, Robyn T. Eijlander1,2, Oscar P. Kuipers1, and Colin Robinson1,2

From the 4Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, United Kingdom and the 5Department of Molecular Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

The Tat system transports folded proteins across bacterial and thylakoid membranes. In Gram-negative organisms, a TatABC substrate-binding complex and separate TatA complex are believed to coalesce to form an active translocon, with all three subunits essential for translocation. Most Gram-positive organisms lack a tatB gene, indicating major differences in organization and possible differences in mode of action. Here, we have studied Tat complexes encoded by the tatAdCd genes of Bacillus subtilis. Expression of tatAdCd in an Escherichia coli tat null mutant results in efficient export of a large, cofactor-containing E. coli Tat substrate, TorA. We show that the tatAd gene complements E. coli mutants lacking either tatAE or tatB, indicating a bifunctional role for this subunit in B. subtilis. Second, we have identified and characterized two distinct Tat complexes that are novel in key respects: a TatAdCd complex of ~230 kDa that is significantly smaller than the analogous E. coli TatABC complex (~370 kDa on BN gels) and a separate TatAd complex. The latter is a discrete entity of ~270 kDa as judged by gel filtration chromatography, very different from the highly heterogeneous E. coli TatA complex that ranges in size from ~50 kDa to over 600 kDa. TatA heterogeneity has been linked to the varying size of Tat substrates being translocated, but the singular nature of the B. subtilis TatA complex suggests that discrete TatAC and TatA complexes may form a single form of translocon.

The Tat (twin arginine translocation) pathway is involved in the transport of proteins across the chloroplast thylakoid membrane and the plasma membranes of a wide range of bacteria (1–6). It differs fundamentally from the other export pathway, the Sec pathway, in its ability to translocate prefolded proteins over the plasma membrane. This process appears not to require energy in the form of ATP hydrolysis but is instead dependent on the presence of the proton motive force across the membrane (1, 7, 8). Substrates for this translocation pathway bear N-terminal signal peptides that contain a highly conserved, characteristic twin-arginine motif in the N-terminal domain of the signal peptidase (9–11).

In Escherichia coli and other Gram-negative organisms, the above studies have identified three genes as being essential for Tat-dependent protein translocation: tatA, tatB, and tatC (usually in the form of a tatABC operon). The three tat genes of E. coli encode membrane proteins of 9.6, 18.4, and 28.9 kDa in size, respectively. The TatA and TatB proteins are both predicted to contain a single transmembrane spanning domain, whereas the larger TatC protein is predicted to contain six transmembrane-spanning domains (12). These three proteins have been shown to form two main types of complex within the cytoplasmic membrane of E. coli. The first is a TatABC complex of ~370 kDa (13, 14), which is thought to be involved in substrate recognition and binding; in particular, substrates appear to interact initially with the TatB and TatC subunits (15, 16). The second form of Tat complex appears to contain only TatA molecules, and whereas the E. coli TatABC complex is both discrete and stable, the TatA complexes range in size from less than 100 kDa to over 500 kDa (17, 18). Single-particle electron microscopy reveals the TatA to form ring-like structures of varying diameter (18), prompting suggestions of a role in formation of the translocation pore.

Tat systems in other Gram-negative organisms appear (so far) to conform to a similar pattern, whereas Gram-positive organisms contain very different Tat systems. In virtually every case, with the exception of Streptomyces species (19), these organisms contain only tatAC genes, indicating fundamental differences between the two types of Tat apparatus. Because TatA and TatB proteins share a degree of sequence homology, one possibility is that the TatA proteins in Gram-positive bacteria are bifunctional, performing the functions of both the TatA and TatB proteins of E. coli (20). Alternatively, TatB may perform a function in E. coli that is not required in Gram-positive organisms, or the two types of system may differ in other key respects.

In this report we have set out to characterize in detail a Tat system from a Gram-positive organism, Bacillus subtilis. This is a nonpathogenic, Gram-positive soil bacterium that secretes high levels of extracellular proteins. B. subtilis contains three variants of the tatA gene, denoted tatAd, tatAy, and tatAc, together with two variants of tatC, denoted tatCd and tatCy; it lacks a tatB gene. Recently studies have shown the presence of two distinct translocases, one involving TatAd and TatCd and the other involving TatAy and TatCy. TatAdCd and TatAyCy display differing substrate specificities (21). The secretion of

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†2 These authors contributed equally to this work.
‡1 To whom correspondence should be addressed. Tel: 44-2476-523557; Fax: 44-2476-523568; E-mail: colin.robinson@warwick.ac.uk.
PhoD, a protein with phosphodiesterase and alkaline phosphatase activity, is completely TatAdCd-dependent (22), and the three genes are part of the pho-regulon that is only expressed under phosphate-limiting conditions. No other TatAdCd-dependent proteins have been identified to date. TatAyCy has been shown to form an active translocase for the translocation of YwbN, an iron-dependent DyP-peroxidase (22, 23). The function of the third TatA component, TatAc, remains unknown.

Little is currently known about the organization of the B. subtilis Tat proteins within the membrane. Here, we analyze the organization and nature of the complexes formed by the components of the B. subtilis TatAdCd pathway. We show that these subunits form an active translocase when expressed in an E. coli background, and we furthermore show that TatAd is indeed bifunctional. Finally, we show that the B. subtilis Tat complexes have unexpected properties that suggest a new model for Tat-dependent protein transport, involving a single defined translocon rather than a spectrum of size variants.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions**—All of the strains and plasmids used are listed in Table 1. E. coli MC4100 (24) was used as the parental strain, and ΔtatABCDE (4) has been described previously. Arabino- and PhoD-resistant derivatives were used as described previously (13). E. coli was grown aerobically in Luria broth at 37 °C. E. coli was grown anaerobically in TY (trypton/yeast extract) medium, consisting of bactotryptone (1%; w/v), Bacto yeast extract (0.5%; w/v), and NaCl (1%; w/v), unless indicated otherwise. The media were supplemented with ampicillin to a final concentration of 100 µg/ml and arabinose to 0.5 mM when required. B. subtilis was grown in TY (trypton/yeast extract) medium, consisting of bacto-tryptone (1%; w/v); Bacto yeast extract (0.5%; w/v); and NaCl (1%; w/v), unless indicated otherwise. The media were supplemented with kanamycin (10 µg/ml), chloramphenicol (5 µg/ml), and/or spectinomycin (100 µg/ml).

**DNA Techniques**—All of the cloning techniques and transformation of E. coli were performed as described by Sambrook et al. (25). All of the enzymes used were from Roche Applied Science or Fermentas Life Sciences. The PCR was performed using Expand DNA polymerase (Roche Applied Science). For arabinose-inducible overproduction of B. subtilis TatAdCd, plasmid pBADCd was constructed as follows. The tatAdCd operon was amplified from B. subtilis 168 chromosomal DNA with primers RTEAdF (5′-CGC GTC TCG CAT GTT TTC AAA CAT TGG AAT ACC GGG CTT G-3′) and JJdstrep02 (5′-ATG TCC TAG ATT TTT CAA TCT ACT GTC GGT GCG ACC AAT TCG AAG CGG CCG CCG CTG TTT CTT CC-3′). RTEAdF was designed as such that restriction of the generated tatAdCd-strep PCR-amplified fragment with dovetail enzyme Esp3I would create an NcoI overhang to ensure direct cloning in the vector pBAD24. JJdstrep02 was constructed in such a way that a C-terminal Strep-tag II (26) (underlined) would be directly attached to tatAd during the PCR amplification. pBADCd was constructed by ligating an Esp3I- and XbaI-cleaved PCR-amplified fragment of tatAdCd-strep into NcoI- and XbaI-cleaved pBAD24. For isopropyl-β-D-thiogalactopyranoside-inducible overproduction of B. subtilis TatAdCd, tatAdCd-strep was cut out of pBADCd with Nhel and Xbal and ligated into Nhel/XbaI-cut pEXT22 to construct pEXT-AdCd.

For arabinose-inducible overproduction of B. subtilis TatAd, plasmid pBAD-His was constructed as follows. The tatAd gene was amplified from B. subtilis 168 chromosomal DNA with primers RTEAdF (5′-CGC GTC TCG CAT GTT TTC AAA CAT TGG AAT ACC GGG CTT G-3′) and TatAdhis-Xba-R (5′-ATG TCC TAG ATT TTT CAA TCT ACT GTC GGT GCG ACC AAT TCG AAG CGG CCG CCG CTG TTT CTT CC-3′). TatAdhis-Xba-R was constructed in such a way that a C-terminal His6 tag (underlined) would be directly attached to tatAd during the PCR amplification. pBAD-His was constructed by ligating an Esp3I- and XbaI-cleaved PCR-amplified fragment of tatAdCd-strep into NcoI- and XbaI-cleaved pBAD24.

**Expression and Purification of the TatAdCd Complex and TatAd Complex**—E. coli ΔtatABCDE cells containing plasmid pBADCd or pBAD-His were grown aerobiologically to mid-exponential phase with induction of tatAdCd on plasmid pBADCd and tatAd on plasmid pBAD-His using 0.5 mM arabinose. The membranes were isolated as described previously and solubilized in 2% digitonin (13). Solubilized membranes were incubated with 2 µg/ml aqvin to block any boitin-containing proteins before application to an equilibrated 2 ml Streptactin affinity column (Institut fur Bioanalytik). The column was washed with eight column volumes of equilibration buffer containing Tris-HCl, pH 8.0, 10% glycerol, 150 mM NaCl, and 0.1% digitonin. Bound protein was eluted from the column in six 1.0-ml fractions.

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### TABLE 1

| Plasmids and strains | Relevant properties | Reference |
|----------------------|--------------------|-----------|
| **Plasmids**         |                    |           |
| pBAD-ABC             | pBAD24 derivative containing the E. coli tatABC operon; Amp' | Ref. 13 |
| pBADCd               | pBAD24 derivative containing the B. subtilis tatAdCd-strep operon; Amp' | This study |
| pEX2-AdCd            | pEX22 derivative containing the B. subtilis tatAdCd operon; Kan' | This study |
| pBAD-his             | pBAD24 derivative containing the B. subtilis tatAd-his gene; Amp' | This study |
| **Strains**          |                    |           |
| E. coli              |                    |           |
| MC1061               | F-; araD139; Δ(ara-leu)7696; Δ(lac)X74; galL; galK; lacY1; mcrA; mcrB1; rpsL; rpsL9 | Ref. 42 |
| MC4100               | F- ΔlacI169 araD139 rpsL150 relA1 ptsF rbs flbB5301 | Ref. 24 |
| B. subtilis          |                    |           |
| tatACDE              | F- ΔlacI169 araD139 rpsL150 relA1 ptsF rbs flbB5301 | Ref. 43 |
| 168                  | trpC2              |           |

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3 The abbreviations used are: TMAO, trimethylamine N-oxide; GFP, green fluorescent protein; BN, blue native.
using the same buffer as above but containing 3 mM desthiobiotin (Sigma). Elution fractions were pooled and concentrated to 250 μl using Vivaspin-4 centrifugal concentrators (molecular weight cut-off, 10,000; Vivasience). The concentrated sample was loaded onto a Superose-6HR gel filtration column (Amersham Biosciences) and was eluted with the equilibration buffer described above. For complete purification of the TatAdCd complex, the sample of solubilized membrane was subjected to Q-Sepharose chromatography before the Streptactin and Superose-6HR chromatography steps; the method was as described in Ref. 13.

**SDS-PAGE and Western Blotting**—The proteins were separated using SDS-polyacrylamide gel electrophoresis and immunoblotted using specific antibodies to TatAd (kindly provided by J. Müller (27)) and goat anti-rabbit IgG horseradish peroxidase conjugate. The Strep-tag II on TatCd was detected directly using a Streptactin-horseradish peroxidase conjugate (Institut fur Bioanalytik). SufI, a Tat-dependent substrate of E. coli, was visualized using specific antibodies (kindly provided by T. Palmer). GFP was detected using a specific anti-GFP antibody (Living Colors) followed by goat anti-rabbit IgG horseradish peroxidase conjugate. An ECL detection kit (Amersham Biosciences) was used to visualize the proteins.

**Blue Native Polyacrylamide Gel Electrophoresis**—Blue native polyacrylamide gel electrophoresis was performed as described previously (28). The membranes were prepared as described above and solubilized in 50 mM Bis-Tris, pH 7.0, 750 mM 6-aminocaproic acid, and 2% (w/v) digitonin. Solubilized membranes and purified protein were loaded and separated on a polyacrylamide gradient gel (5–13%). The proteins were detected by immunoblotting as described above.

**TMAO Reductase Activity and TorA-GFP Assays**—TMAO reductase activity assay was performed as described previously (13, 29). E. coli cells were grown anaerobically until mid-exponential growth phase prior to fractionation into periplasm, cytoplasmic, and membrane fractions. The cell fractions were loaded and separated on a 10% native polyacrylamide gel that was subsequently assayed for TMAO reductase activity as described previously. For TorA-GFP export assays, a construct comprising the TorA signal peptide linked to GFP (30) was expressed using the pBAD-24 plasmid as previously described (31). For these experiments, TatAdCd was expressed from the compatible pEXT22 vector.

**Microscopy**—Images were recorded with a Leica DMRE microscope equipped with a Leica TCS SP2 confocal unit and an argon laser. The 488-nm laser line was selected, and the images were recorded with a photomultiplier using the Leica confocal software. The samples were visualized with a 63× oil immersion objective (numerical aperture, 1.4; Leica). The image size is 512 × 512 pixels, and the images were averaged from four successive scans.

**RESULTS**

**Overexpressed TatAdCd Forms an Active Translocation Pathway in E. coli**—We set out to determine whether overexpressed TatAdCd can form an active translocation pathway in E. coli, to use a single defined background for comparing Tat systems from Gram-negative and Gram-positive organisms. The tatAdCd genes were expressed in an E. coli tat null mutant (ΔtatABCDE), and we conducted a TMAO reductase activity assay as described under “Experimental Procedures.” TMAO reductase (TorA) is a periplasmic protein required for growth of E. coli on a minimal TMAO and glycerol medium. It is a known E. coli Tat substrate that is frequently used for Tat export assays because its presence can be detected through an assay using methyl-viologen-linked reduction on a native polyacrylamide gel. TatAdCd was overexpressed from plasmid pBADCd in ΔABCDE cells under anaerobic conditions, after which the cells were fractionated, and TorA was localized as shown in Fig. 1.

The right-hand sets of lanes in Fig. 1 show control tests in which the wild type E. coli strains, MC1061 and MC4100 (from which the ΔABCDE strain was derived) were fractionated into membrane, cytoplasm, and periplasm samples (lanes M, C, and P, respectively). The data show that the TorA activity is localized primarily in the periplasm as expected, with low levels found in the cytoplasm as is often observed (13). In the ΔABCDE strain (denoted Δtat), no periplasmic activity is detected, and TorA is found exclusively in the cytoplasm. Expression of plasmid-borne E. coli TatABC in this strain (from the pBAD-ABC vector) restores export activity as expected. The important point is that expression of B. subtilis TatAdCd, using the same vector, also leads to efficient export of TorA. Clearly, TatAdCd is able to form an active translocation pathway in the E. coli ΔABCDE strain, and this is the first demonstration that a TatAC-type system from a Gram-positive organism can function in a Gram-negative background and export authentic E. coli Tat substrates. Moreover, because TatB has been found to be essential for TorA translocation in E. coli (32), this strongly suggests that B. subtilis TatAd displays both TatA and TatB activity.

**Overexpression of TatAdCd Does Not Fully Complement E. coli ΔABCDE Cells in Tat-dependent Substrate Translocation**—Although repeated tests consistently showed TatAdCd to be capable of efficiently exporting TorA, it was considered important to analyze its ability to also export other substrates. We first tested whether the cells can export a fusion protein comprising the TorA signal peptide linked to GFP. This fusion is exported by the Tat pathway in E. coli (30, 31). We expressed TorA-GFP in E. coli ΔABCDE cells and coexpressed tatAdCd or E. coli tatABC on the compatible pEXT22 plasmid...
contrast, the fluorescence is purely cytoplasmic when the construct is expressed in ΔtatABCDE cells. 

Fig. 2B also illustrates an important point regarding the ability of the TatAdCd system to export other substrates. Two Tat substrates in E. coli, amidase A and C, have been shown to be involved in cytokinesis (33). These substrates are mislocalized in a ΔABCDE deletion strain causing a distorted cell division phenotype; as a result the cells grow in long chain-like filaments (34). The wild type phenotype is clearly restored by complementing the ΔABCDE strain with plasmid-borne TatABC (TatAc image). However, expression of B. subtilis TatAdCd also restores the wild type cell division phenotype (TatAdCd panel), strongly suggesting that the TatAdCd complex of B. subtilis is able to translocate one or both of the amidase A and C proteins.

We also analyzed the translocation of SufI, another authentic Tat substrate, in the E. coli ΔABCDE deletion strain complemented with B. subtilis TatAdCd (Fig. 3). The mature size, 50-kDa SufI protein is clearly detected in the periplasm (P) of wild type MC4100 cells with some precursor protein apparent in the cytoplasmic fraction (C). An additional band slightly smaller than mature SufI is also detected in the membrane fraction (M), although the significance of this band is unclear. As expected, deletion of the E. coli Tat machinery resulted in accumulation of SufI in the cytoplasm (Δtat panel). In this case, export could not be restored by expression of TatAdCd of B. subtilis (Δtat/pBAdCd panel), with no SufI detected in the periplasm. We conclude that not all Tat-dependent substrates of E. coli can be translocated by this AC-type translocase.

TatAd Is Bifunctional and Able to Complement Both the ΔtatAE and ΔtatB E. coli Mutants—The absence of a tatB gene in most Gram-positive organisms suggests that the Gram-positive TatA subunits are able to carry out both the TatA and TatB roles involved in Tat systems from Gram-negative organisms (20). This important point has not been addressed experimentally, and we therefore expressed B. subtilis TatAd in the E. coli ΔtatAE and ΔtatB strains to test whether it can complement the defects. The results (Fig. 4) show that TatA activity is transported to the periplasm when His-tagged TatAd is expressed in either the ΔtatAE or ΔtatB strains, clearly demonstrating that this subunit is able to execute both the TatA and TatB roles. Note that the signal intensity of the periplasmic TorA band appears to be low when TatAd is expressed in the ΔtatAE strain, but this assay is not quantitative, and from other experiments TatAd complements the two strains to an apparently equal
**B. subtilis Tat Complexes**

**FIGURE 4.** *B. subtilis* TatA complements both the ΔtatAE and ΔtatB *E. coli* mutants. His-tagged TatA was expressed using the pBAD-His vector (“Experimental Procedures”) in the *E. coli* ΔtatAE, ΔtatB and ΔtatABCDE mutants as shown. After induction for 3 h in the presence of 0.1 mM arabinose, the cells were fractionated into membrane, cytoplasm, and periplasm samples (*M, C, and P*, respectively), which were analyzed on a native gel that was stained for TatA activity. Similar fractionations were made using ΔtatAE and ΔtatB cells in control experiments.

In the control sample, no periplasmic TatA activity is detected when TatA is expressed in a *tat* null mutant strain (ΔtatABCDE; the panel is overexposed to detect the cytoplasmic and membrane-bound TatA activity in the *P* and *M* lanes). No export of TatA is evident in the ΔtatAE or ΔtatB strains either, because Tat activity is absolutely dependent on the presence of TatB plus either TatA or the TatA homolog, TatE (Ref. 4 and data not shown).

**Separation of distinct *B. subtilis* TatAdCd and TatAd-containing complexes, and purification of the *B. subtilis* TatAdCd complex.** A, membranes were prepared from ΔtatABCDE cells expressing *B. subtilis* TatAdCd (from plasmid pBAdCd as above), solubilized in digitonin and applied to a Streptactin affinity column as detailed under “Experimental Procedures.” The figure shows immunoblots of wash (washes 1–8) and elution (elutions 1–6) fractions using antibodies to the Strept-tag II on the C terminus of TatCd (protein is denoted as TatCd-strep) and to TatAd. Mobilities of molecular mass markers (in kDa) are shown on the left. B, purification of the TatAdCd complex to homogeneity. Solubilized membranes from cells expressing TatAdCd were subjected to Q-Sepharose, Streptactin, and Superose-6HR gel filtration chromatography as detailed under “Experimental Procedures.” Samples of each eluate were analyzed on a silver-stained gel (right panel) or blotted with antibodies to TatAd (left panel). The mobilities of molecular mass markers (in kDa) are shown.

**Highly Discrete TatAdCd and TatAd Complexes**—The sizes of the two forms of *B. subtilis* Tat complex were analyzed using calibrated gel filtration chromatography. Digitonin-solubilized membranes from cells expressing TatAdCd-strep were applied to a calibrated Superose-6 gel filtration column as described under “Experimental Procedures,” and elution fractions were immunoblotted using antibodies against TatAd and TatCd-strep (Fig. 6A). The elution profiles of the TatAdCd and TatAd complexes were quantified by densitometry of the bands, and the graphs are shown in Fig. 6A. We also expressed, solubilized, and fractionated the membrane-bound *E. coli* TatABC and TatA complexes under exactly the same conditions for comparison, and we analyzed the TatA-type complexes extracted from the cytoplasm (also indicated in Fig. 6B). The data show that TatCd elutes in fractions 20–27, and TatAd elutes in fractions 22–26. Quantification of the intensity of the bands reproducibly shows that TatCd is eluting with a peak in fraction 23 corresponding to a molecular mass of ~350
B. subtilis Tat Complexes

**FIGURE 6.** TatAdCd and TatAd are discrete complexes that differ from their *E. coli* counterparts. A, digitonin-solubilized membranes from cells expressing TatAdCd (as in above figures) or *E. coli* TatABC were applied to a calibrated Superose-6HR column. Peak elution fractions were analyzed by immunoblotting using antibodies to the Strep-tag II (on TatCd) or TatAd as indicated. The immunoblots were analyzed by densitometry (of the entire set of elution fractions), and the intensities of the bands are shown plotted against fraction number. TatAdCd and TatAd elution are denoted by black diamonds and black triangles, respectively. The elution of *E. coli* TatA complexes was analyzed under identical conditions and is shown in the same graph (squares). B, fractionation of membrane-bound and cytosolic TatAd. Upper graph, TatAdCd was expressed as in A and membranes were solubilized in 1% digitonin as in A, or 1% octyl glucoside or 1% dodecyl maltoside and subjected to Superose 6 chromatography in the same detergent. The graph shows the elution of TatAd in digitonin (black triangles), octyl glucoside (white triangles), or dodecyl maltoside (white squares). The elution of digitonin-solubilized *E. coli* TatA is also illustrated for comparative purposes (black squares). The lower graph of B shows the fractionation of cytosolic TatAd. Here, TatAdCd were expressed as in A (with TatAd untagged), or with a His₆ tag attached to the C terminus of TatAd using the vector pBAD-his. After lysis of the cells, the cytosolic fraction was subjected to Superose 6 gel filtration chromatography as in A but without detergent present at any step. The fractions were immunoblotted for TatAd, and the graph illustrates the elution profiles for untagged and His-tagged TatAd (white diamonds and black triangles, respectively).

The lower graph (B) shows the fractionation of cytosolic TatAd. Here, TatAdCd were expressed as in A (with TatAd untagged), or with a His₆ tag attached to the C terminus of TatAd using the vector pBAD-his. After lysis of the cells, the cytosolic fraction was subjected to Superose 6 gel filtration chromatography as in A but without detergent present at any step. The fractions were immunoblotted for TatAd, and the graph illustrates the elution profiles for untagged and His-tagged TatAd (white diamonds and black triangles, respectively).

Digitonin forms particularly large micelles, so for a more accurate estimation of the size of the TatAd complex, we expressed TatAd alone and solubilized the membranes in either dodecyl maltoside or octyl glucoside prior to fractionation in the same detergent (Fig. 6B, upper graph, white squares or triangles, respectively). In both cases the TatAd complex elutes later than when solubilized in digitonin (black triangles), presumably because of the much smaller micelle size. In both dodecyl maltoside and octyl glucoside, TatAd elutes at a position that corresponds to a mass of 160 kDa, which we assume to be a more accurate reflection of the complex size (although the micelle will still contribute to a degree).

**Cytosolic TatAd Is Present as Very Large Aggregates**—Although studies on the *E. coli* and thylakoid systems have focused on roles of membrane-bound TatABC proteins, recent studies on the *B. subtilis* TatAdCd system have suggested a completely different mode of action in which the precursor of PhoD is initially recognized by TatAd in the cytosol (27, 35, 36). This would differ dramatically from the *E. coli* and thylakoid systems, where substrates bind to a membrane-bound TatBC dimer (15, 16). Having characterized the size properties of membrane-bound TatAd when expressed in *E. coli*, we therefore tested whether TatAd is also present in the cytosol. This was done after expression of two forms of TatAd, an untagged version of TatAd (using the plasmid pBADCd as in the above studies) and one with a His₆ tag present on the C terminus of TatAd (pBAD-His plasmid). The two forms were expressed using the pBAD24 vector, and the cytosolic fraction was subjected to Superose 6 gel filtration chromatography (Fig. 6B, lower graph). The data show that the untagged TatAd is present as very large multimeric forms. The protein elutes in fraction 14 (white diamonds), which corresponds to an average size of ~2 MDa. Surprisingly, the presence of a His tag causes a huge change of structure, and the protein elutes much later from the column, at a position corresponding to a size of ~200 kDa (similar to that observed in Ref. 27). These data do raise questions about the significance of the cytosolic TatAd, and this issue is discussed below.

**Blue Native Gel Electrophoresis Shows the Presence of a ~230-kDa TatAdCd Complex**—To analyze the purified TatAdCd and TatAd complexes further, blue native (BN) polyc-
rylamide gel electrophoresis was utilized according to the method described under "Experimental Procedures" (Fig. 7). Gel filtration chromatography provides a reasonable estimate of complex size, but it is heavily affected by the size of detergent micelles. To circumvent this effect and obtain a more accurate description of the complexes, purified TatAdCd and solubilized membranes expressing TatAdCd were run on a blue native polyacrylamide gel. This gel system has been specifically developed for the analysis of membrane protein complexes because it effectively removes the bias caused by the detergent micelles. Digitonin-solubilized membranes from cells expressing B. subtilis TatAdCd-strep (lanes Bs), E. coli TatAdCd (lanes Ec), or purified B. subtilis TatAdCd-strep (lanes AdCd) were loaded onto a BN gel. The gels were subjected to Western blotting followed by immunodetection with antibodies against the Strep-tag II (Fig. 7A), E. coli TatA (Fig. 7B), and B. subtilis TatAd (Fig. 7C).

Reproducing earlier findings (17), the E. coli TatABC complex was visualized as a band comigrating with the marker band of 440 kDa using antibodies against the Strep-tag II (Fig. 7A, lane Ec). Previous studies have shown that, after careful calibration of this gel system, the actual size of this complex is ~370 kDa as judged by this technique (28). Analysis of purified B. subtilis TatAdCd complex (prepared as described above) reveals a band that comigrates with the 232-kDa marker. A band of the same size is detected when the same blot is probed with antibodies to TatAd (Fig. 7C), confirming that this band corresponds to a TatAdCd complex of ~230 kDa. A band of similar size is evident in samples of whole membranes taken from cells expressing TatAdCd (lane Bs), although it is less clear, and some smearing is evident. However, because essentially all of the TatAdCd complex binds to the Streptactin affinity matrix, and only the ~230-kDa TatAdCd complex is detected, we are confident that this is the major species of TatAdCd complex present in the membrane.

Fig. 7B clearly shows the “ladder” of differently sized E. coli TatA complexes, but it has not been possible to study the B. subtilis TatAd complex using BN gels. Although the TatAd antibodies clearly detect the purified TatAdCd complex in Fig. 7C (lane AdCd), there is no clear additional band in the whole membrane sample (lane Bs) from cells expressing TatAdCd. It is possible that the TatAd complex is too small for accurate analysis using the BN system under these conditions.

DISCUSSION

A wealth of data have emerged from studies on the TatABC and TatA complexes of Gram-negative bacteria (particularly E. coli) as well as the corresponding plant thylakoid complexes (Hcf106-TatC complex and homo-oligomeric Tha4 complexes). In most respects, the E. coli and plant systems appear to operate by broadly similar mechanisms in which two key points are considered to be noteworthy. First, cross-linking data point to the presence of a membrane-bound substrate-binding complex in which TatB and TatC play critical roles (15, 16). A tight association between these components has been confirmed by one study that showed a translational TatB-TatC fusion to be active (13). Second, the available evidence indicates that a TatA (or Tha4) complex assembles and exists as a separate entity and appears to be recruited by the TatABC (or plant Hcf106-TatC complex) only at the point of translocation (37).

In this study we have sought to study Tat complexes from the Gram-positive organism B. subtilis. The absence of tatB genes in the majority of Gram-positive organisms clearly indicates that their Tat systems differ significantly from the E. coli model, and it is important to identify similarities and differences (particularly any mechanistic differences) between the two types of system. In this context, our data are important because they show for the first time that an authentic E. coli Tat substrate, TorA, can be effectively transported by the B. subtilis TatAdCd system. This indicates that there are no system-specific factors in B. subtilis that are required for this system to operate, which could be absent in Gram-negative organisms. This focuses attention on the TatAdCd subunits as the critical components.

The absence of a tatB gene in B. subtilis raises the possibility that TatA is bifunctional in Gram-positive bacteria, fulfilling the roles of both TatA and TatB from Gram-negative organisms (20). This possibility is backed up by the recent demonstration (38) that selective mutations in E. coli TatA can enable it to carry out both TatA and TatB functions. In this report we have provided experimental confirmation of the bifunctional nature of TatAd; this subunit can complement both the ΔtatAE and ΔtatB E. coli mutants. TatA and TatB play completely different roles in the E. coli Tat system (1, 2, 13, 15, 18), which clearly indicates a dual role for TatAd in the B. subtilis TatAC-type mechanism.

When compared with the E. coli Tat complexes, the B. subtilis Tat complexes exhibit important common features but also significant differences. The primary common feature is the presence of two distinct Tat complexes. We have shown that expression of the B. subtilis tatAdCd genes results in the presence of separate membrane-bound TatAdCd and TatAd complexes, and these results are very reminiscent of findings on the E. coli system where separate TatABC and TatA complexes are present at steady state (13, 17, 18).

However, the B. subtilis Tat complexes encoded by tatAdCd differ from their E. coli counterparts in three important
respectively. One of these has already been noted: the absence of TatB in the core substrate-binding complex; its position appears to be occupied by TatAd. The second relates to the nature of the TatAdCd complex; like the E. coli TatABC complex, the TatAdCd purifies as a highly discrete entity, but our data indicate that the B. subtilis TatAdCd complex is significantly smaller than its E. coli counterpart: 230 kDa compared with 370 kDa for the E. coli complex as estimated previously by BN gels (17). This may have implications for the number and/or organization of the TatC-containing domains, although it should be noted that an individual TatAdCd domain is smaller than an equivalent E. coli TatABC domain (~35 kDa compared with 56 kDa, assuming equimolar stoichiometry as observed for TatBC in the E. coli complex (13)).

The third and perhaps most significant difference concerns the TatA complexes. The E. coli TatA complex exists as a remarkably heterogeneous set of complexes that range in size from ~50 kDa to well over 500 kDa (17, 18), and this heterogeneity can be clearly visualized as a striking ladder of bands on BN gels. In the above studies it was proposed that this heterogeneity could provide the flexibility for this system to assemble active translocons of differing sizes, which would be logical if a wide range of substrates are to be accommodated without compromising membrane integrity. In contrast, the B. subtilis TatA complex is highly discrete in nature; there is no evidence for heterogeneity among the complexes isolated. Nevertheless, this combination of TatAdCd and TatAd complexes is able to transport substrates of very differing sizes, such as authentic TorA (85 kDa) and GFP (25 kDa), as well as its original PhoD substrate in B. subtilis (62.5 kDa). On the basis of these data, we believe that the active translocon may be a singular unit capable of accommodating substrates that differ dramatically in terms of size and shape. This would, of course, require real flexibility within the active translocation complex. This scenario would raise questions about the functional significance of the E. coli TatA heterogeneity but would be consistent with our observation that the TatA size spectrum is identical in cells that are completely unable to export proteins via this pathway (39).

The TatAdCd complex is furthermore surprisingly small; the size exclusion chromatography estimate is ~160 kDa, and the detergent micelle probably accounts for some of this estimate. These data have interesting implications for the translocation mechanism because TatAdCd is capable of exporting TorA, which is one of the larger Tat substrates (85 kDa). If, as proposed, the active Tat translocon is generated by the coalescence of a TatABC and TatA complex, the sizes of the E. coli complexes have been deemed to be consistent with this model (for example, an active translocon of up to 1 MDa in size could be generated by fusion of one of the largest TatA complexes with the 370-kDa TatABC complex). It is less clear whether a substrate of this size can be transported in a folded state by a translocon comprising a 270-kDa TatAdCd complex and 160-kDa TatAd complex. Therefore, it may be the case that the actual translocation process is more complicated than currently imagined, perhaps involving the recruitment of several TatA or TatAd (or even several TatABC or TatAdCd) complexes in both Gram-negative and Gram-positive bacteria.

Finally, it is useful to consider these data in the light of other studies on Tat systems from Gram-positive organisms. Recent studies on the B. subtilis TatAdCd system (27, 35, 36) suggest a translocation mechanism that is very different from any current model for the E. coli or thylakoid Tat system. These studies showed that TatAd can be detected as cytoplasmic oligomers of ~250 kDa, and these oligomers were shown to be able to bind the precursor form of PhoD, the natural substrate of TatAdCd (a membrane-bound form of TatAd is also present in B. subtilis). The authors proposed that cytosolic TatAd acts as a soluble receptor for substrate, after which the membrane-bound TatCd recognizes this cytosolic assembly and assists in its membrane integration. More confusingly still, studies on Streptomyces lividans (one of the few Gram-positive organisms containing tatABC genes) have shown that both TatA and TatB can be detected in the cytoplasm, and the authors proposed that a cytosolic TatAB complex binds substrates and delivers them to TatC (40). These models are fundamentally different in key respects to current models for the Tat system in Gram-negative bacteria and plant thylakoids, where the Tat components are only active in the membrane (13, 15–18).

Although our aim in this study was to analyze the potentially bifunctional TatAd protein and the complexes formed by TatAdCd expression, our data do have relevance to this ongoing debate about Tat mechanism(s) and whether they do indeed differ so diametrically in Gram-negative and Gram-positive organisms. First, we have shown that the TatAd subunit carries out both TatA-like and TatB-like roles in E. coli, and because these subunits are believed to function in the membrane in E. coli, it is logical to assume that TatAd likewise carries out these functions as a transmembrane protein. Second, we have shown that the TatAdCd system can transport TorA and a TorA-GFP fusion protein, and there is firm evidence that a basically similar TorA fusion protein interacts with a membrane-bound TatBC-containing complex in E. coli. Cross-linking studies with a construct comprising the TorA signal peptide linked to a thylakoid lumen passenger protein (41) have shown that the TorA signal peptide specifically binds to a membrane-bound TatBC heterodimer as previously shown for another Tat precursor protein, SufI (15). We have shown that TorA-GFP is capable of using the B. subtilis TatAdCd apparatus instead, and it thus seems probable that it interacts with a membrane-bound TatAdCd complex (which we have shown to be present) rather than following a completely different export pathway involving the binding of a soluble TatAd complex. Nevertheless, these are indirect forms of evidence and different, more direct approaches are required to determine whether TatAd functions in the membrane, the cytoplasm, or both.

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