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TatAc, the Third TatA Subunit of *Bacillus subtilis*, Can Form Active Twin-Arginine Translocases with the TatCd and TatCy Subunits

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Two independent twin-arginine translocases (Tat) for protein secretion were previously identified in the Gram-positive bacterium *Bacillus subtilis*. These consist of the TatAd-TatCd and TatAy-TatCy subunits. The function of a third TatA subunit named TatAc was unknown. Here, we show that TatAc can form active protein translocases with TatCd and TatCy.

Protein transport from the cytoplasm to different bacterial compartments or the external milieu is facilitated by dedicated molecular machines (6). Among these protein translocases, the twin-arginine translocases (Tat) stand out because they permit the passage of tightly folded proteins across the cytoplasmic membrane. The proteins translocated by Tat are synthesized with signal peptides that contain a well-conserved twin-arginine (RR) motif for specific targeting to a membrane-embedded Tat translocase (13, 17, 23). The Tat translocases of Gram-negative bacteria, such as *Escherichia coli*, are composed of three subunits named TatA, TatB, and TatC (4, 18). The formation of an active protein-conducting channel is believed to require the formation of a supercomplex composed of a TatABC heterotrimeric complex and homo-oligomeric TatA complexes (1, 8). In contrast, most Gram-positive bacteria possess minimized Tat translocases that contain only TatA and TatC subunits. Nevertheless, various studies indicate that these TatAC translocases employ a mechanism similar to that of the TatABC translocases of Gram-negative bacteria (10, 17).

The Gram-positive bacterium *Bacillus subtilis* is a well-known "cell factory" for secretory protein production (20, 21). In this organism, two Tat translocases are known to operate in parallel. The TatAdCd translocase consists of the TatAd and TatCd subunits, and the TatAyCy translocase consists of the TatAy and TatCy subunits (11, 12, 15). While the TatAdCd translocase is produced mainly under conditions of phosphate starvation (12, 14, 15), the TatAyCy translocase is expressed under all conditions tested (12, 14). Interestingly, *B. subtilis* produces a third TatA subunit named TatAc (12). The function of TatAc has remained enigmatic due to the fact that no phenotype was so far detectable for *tatAc* mutant *B. subtilis* cells (11, 12, 20). Therefore, the present studies were aimed at determining whether TatAc can actually form active translocases in combination with TatCd or TatCy. This possibility was tested by expressing the respective *tat* genes in *E. coli*, because the activity and assembly of *Bacillus* Tat translocases can be assayed more readily in this organism than in *B. subtilis* (2). For this purpose, the *tatAc* gene was amplified from the *B. subtilis* genome (GenBank/EMBL/DDBJ accession number AL009126) and cloned into plasmid pBAD24, resulting in pBAD-Ac. Next, the *tatCd* and *tatCy* genes were PCR amplified such that the respective proteins contain a C-terminal StrepII tag. The amplified *tatCd*-StrepII and *tatCy*-StrepII genes were cloned into pBAD-Ac, resulting in pBAD-AcCd-Strep and pBAD-AcCy-Strep, respectively. These vectors were subsequently used to transform *E. coli* Δ*tatABCDE* cells, which lack all *E. coli tat* genes. Next, the resulting strains were tested for their ability to transport the previously identified *E. coli* Tat substrates TorA, AmiA, and AmiC (3, 5, 9). To monitor TorA export to the periplasm, *E. coli* cells were grown anaerobically until mid-exponential growth phase, and these cells were then subjected to subcellular fractionation as previously described (16). The periplasmic, cytoplasmic, and membrane fractions thus obtained were separated on a 10% native polyacrylamide gel that was subsequently assayed for trimethylamine *N*-oxide (TMAO) reductase activity as described previously (5, 19). The results in Fig. 1 show that the Δ*tatABCDE* cells producing TatAc plus TatCd or TatAc plus TatCy were capable of transporting active TorA to the periplasm. In contrast, Δ*tatABCDE* cells expressing only *tatAc*, *tatCy*, or *tatCd* were not able to export active TorA to this subcellular location. This showed for the first time that TatAc was able to form active translocases in combination with TatCd or TatCy. To further investigate the activity of these translocases, we tested the export of AmiA and AmiC, which are both required for cell wall biosynthesis in *E. coli* (3, 9). Cells that do not export these molecules to the periplasm grow in long chains, as is observed for the *E. coli* Δ*tatABCDE* strain (Fig. 2A and B) (9). As shown by phase-contrast microscopy, the bacteria producing TatAc plus TatCd or TatAc plus TatCy showed the wild-type phenotype, although some slightly longer chains were still detectable (Fig. 2C and D). This is indicative of active export of AmiA and/or AmiC to the periplasm, providing further support for the idea that active TatAcCd and TatAcCy complexes can be formed in *E. coli*.

To demonstrate the formation of TatAcCd and TatAcCy complexes, a blue native (BN) PAGE analysis was performed. For this purpose, membranes were isolated from cells expressing TatCd-StrepII, TatCy-StrepII, TatAc-TatCd-StrepII, or TatAc-TatCy-StrepII. In addition, cells producing TatAc-StrepII from plasmid pBAD24 were included in the analyses. Upon solubilization in 2% digitonin, membrane proteins were separated by BN PAGE, fol-

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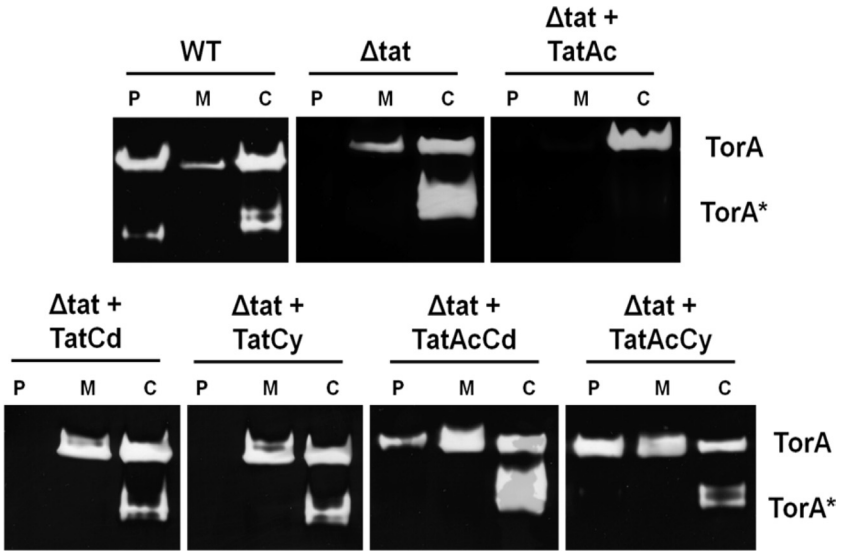

FIG 1 *B. subtilis* TatAcCd and TatAcCy facilitate TorA export in *E. coli*. Cells of *E. coli* Δ*tatABCDE* were subjected to subcellular fractionation. Proteins in the periplasmic (P), membrane (M), and cytoplasmic (C) fractions obtained were separated by native PAGE, and the gels were subsequently analyzed for TMAO reductase (TorA) activity. Strains used in this analysis were *E. coli* MC4100 (WT), *E. coli* Δ*tatABCDE* (Δtat), or *E. coli* Δ*tatABCDE* expressing *B. subtilis* TatAc from plasmid pBAD-Ac-Strep (Δtat + TatAc), *B. subtilis* TatCd from plasmid pBAD-Cd-Strep (Δtat + TatCd), *B. subtilis* TatCy from plasmid pBAD-Cy-Strep (Δtat + TatCy), *B. subtilis* TatAcCd from plasmid pBAD-AcCd-Strep (Δtat + TatAcCd), or *B. subtilis* TatAcCy from plasmid pBAD-AcCy-Strep (Δtat + TatAcCy). The position of active full-length TorA is indicated. TorA* indicates a faster-migrating form of TorA (22).

lowed by immunoblotting with antibodies against the StrepII tag. As shown in Fig. 3, TatCd-StrepII and TatCy-StrepII alone formed bands of ~66 kDa. In addition, TatCd-StrepII formed a minor band of ~100 kDa. TatAc-StrepII expressed by itself formed a small homogeneous complex of ~100 kDa. Importantly, when TatAc (nontagged) was coexpressed with either TatCd-StrepII or TatCy-StrepII, bands of ~230 kDa or ~200 kDa, respectively, were observed. This showed that TatAc does indeed form membrane-embedded complexes with TatCd and TatCy.

In conclusion, our present studies document for the first time that the hitherto enigmatic third TatA subunit of *B. subtilis* known as TatAc can engage in the formation of active TatAC-type trans-

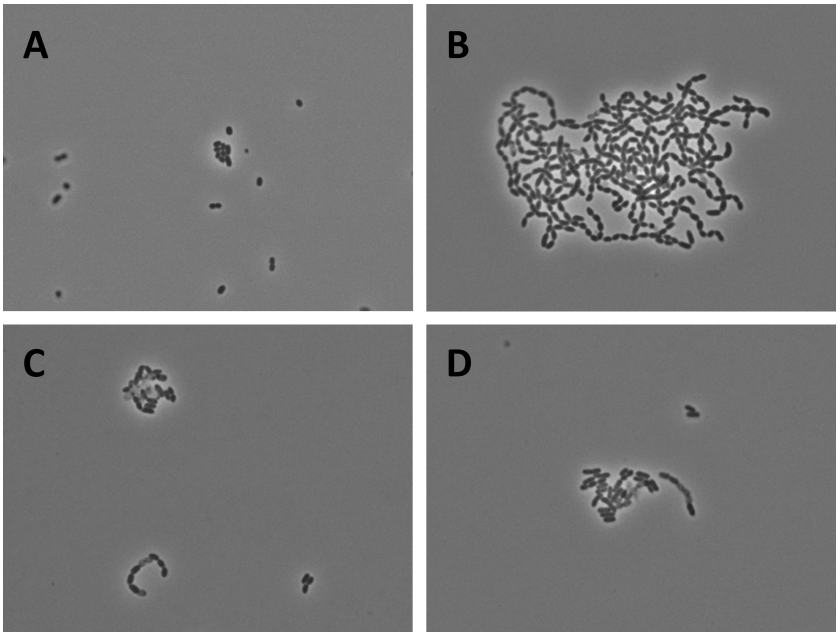

FIG 2 *B. subtilis* TatAcCd and TatAcCy facilitate AmiA and AmiC export in *E. coli*. The export of AmiA and AmiC in *E. coli* was assayed indirectly by assessing the chain length of exponentially growing cells. (A) *E. coli* MC4100 (WT); (B) *E. coli* Δ*tatABCDE* forms long chains due to the mislocalization of AmiA and AmiC (9); (C) *E. coli* Δ*tatABCDE* producing TatAcCd from plasmid pBAD-AcCd-Strep; (D) *E. coli* Δ*tatABCDE* producing TatAcCy from plasmid pBAD-AcCy-Strep. As evidenced by the significantly reduced chain length, the export of AmiA and AmiC in *E. coli* Δ*tatABCDE* is at least partially restored by the production of TatAcCd or TatAcCy.

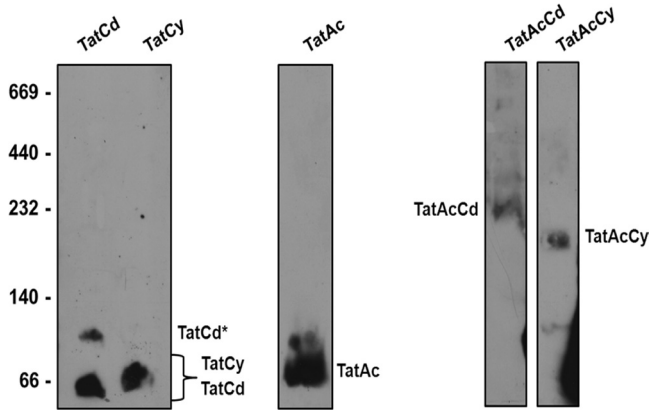

FIG 3 TatAcCd and TatAcCy form discrete complexes. To investigate complex formation, TatCd-StrepII, TatCy-StrepII, TatAc-StrepII, TatAc-TatCd-StrepII, or TatAc-TatCy-StrepII were expressed in *E. coli* Δ*tatABCDE*. Next, membranes from the respective cells were isolated. Membrane proteins were then solubilized in 2% digitonin and separated by blue native PAGE. The gels were immunoblotted using StrepII-specific antibodies and a secondary anti-mouse IgG-horseradish peroxidase conjugate. The EZ-ECL detection kit was used to visualize bound antibodies. The mobility of molecular mass markers (left panel) and StrepII-tagged proteins and complexes is indicated (kDa). TatCd* indicates a TatCd complex with higher molecular weight.

locases. The results also show that TatAc has no particular preference for partnering with TatCd or TatCy. It is noteworthy that the identified TatAcCd and TatAcCy complexes appear to be homogeneous and relatively small (~230 to 200 kDa). Interestingly, previous studies in *B. subtilis* have shown that the coexpression of TatAc and TatCd or TatAc and TatCy does not facilitate the export of the known Tat substrates PhoD and YwbN (7). Furthermore, a recent tiling array analysis across 104 conditions has shown that *tatAc* is expressed under most conditions (14). These previous observations together with our present findings suggest that TatAcCd and TatAcCy translocases could be involved in the specific export of as-yet-unidentified Tat substrates in *B. subtilis*. However, it has to be noted here that our present observations on TatAc function were made upon heterologous expression in *E. coli* and that it remains to be assessed whether TatAc fulfills the same functions in *B. subtilis*.

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