Conservation and Variation between *Rhodobacter capsulatus* and *Escherichia coli* Tat Systems^{∇†}

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The Tat system allows the translocation of folded and often cofactor-containing proteins across biological membranes. Here, we show by an interspecies transfer of a complete Tat translocon that Tat systems are largely, but not fully, interchangeable even between different classes of proteobacteria. The Tat apparatus from the α -proteobacterium *Rhodobacter capsulatus* was transferred to a Tat-deficient *Escherichia coli* strain, which is a γ -proteobacterium. Similar to that of *E. coli*, the *R. capsulatus* Tat system consists of three components, rc-TatA, rc-TatB, and rc-TatC. A fourth gene (*rc-tatF*) is present in the *rc-tatABCF* operon which has no apparent relevance for translocation. The translational starts of *rc-tatC* and *rc-tatF* overlap in four nucleotides (ATGA) with the preceding *tat* genes, pointing to efficient translational coupling of *rc-tatB*, *rc-tatC*, and *rc-tatF*. We show by a variety of physiological and biochemical assays that the *R. capsulatus* Tat system functionally targets the *E. coli* Tat substrates TorA, AmiA, AmiC, and formate dehydrogenase. Even a Tat substrate from a third organism is accepted, demonstrating that usually Tat systems and Tat substrates from different proteobacteria are compatible with each other. Only one exceptional Tat substrate of *E. coli*, a membrane-anchored dimethyl sulfoxide (DMSO) reductase, was not targeted by the *R. capsulatus* Tat system, resulting in a DMSO respiration deficiency. Although the general features of Tat substrates and translocons are similar between species, the data indicate that details in the targeting pathways can vary considerably.

The twin-arginine translocation (Tat) system is known to translocate folded proteins across bacterial energy-transducing membranes (26). Three functionally distinct components of Tat systems have been identified, namely, TatA, TatB, and TatC. TatA and TatB resemble each other, and single-amino-acid exchanges can render TatA to a TatB-substituting component (6). Some organisms contain paralogs of individual components, and many organisms contain only one TatA/B-like component (13). Substrates of the Tat system are synthesized with an N-terminal signal sequence which contains the eponymous twin-arginine pattern (26). As the individual Tat substrates differ between species, it appears possible that Tat systems preferentially recognize their respective substrates. This view has been supported by studies on the transport of glucose:fructose oxidoreductase (GFOR) from *Zymomonas mobilis* in *Escherichia coli* (7). GFOR was not compatible with the Tat system from *E. coli* but became compatible when the signal sequence was exchanged with that from *E. coli* trimethylamine N-oxide (TMAO) reductase. Also, a *Bacillus subtilis* PhoD signal LacZ fusion was not translocated by the *E. coli* Tat system but became translocated by the PhoD-specific *Bacillus* Tat system (30). On the other hand, the Tat substrates alkaline phosphatase from *Thermus thermophilus* (1), high-potential iron-sulfur protein (HiPIP) from *Allochromatium vinosum* (9), and PlcH from *Pseudomonas aeruginosa* (42) were compatible with the *E. coli* Tat translocon, and *E. coli* TorA

was compatible with Tat systems from *Salmonella enterica* serovar Typhimurium and *Agrobacterium tumefaciens* (27).

In this study, we intended to use a heterologous Tat system to address the unclear aspects of substrate specificity. The Tat system from *Rhodobacter capsulatus* was transferred to a Tat-deficient *E. coli* strain, and the functionality of the Tat transport was analyzed with physiological and biochemical assays. The Tat system from *R. capsulatus* fully complemented most established phenotypes of Tat deficiency. The fourth gene product of the *R. capsulatus* *tat* operon *rc-tatF* was not required for this complementation. Despite a high degree of compatibility between the tested Tat systems, the typical and well-studied Tat substrate DmsA was not membrane targeted by the heterologous *R. capsulatus* Tat system. These observations point to variations between Tat systems which can lead to exceptional rejections of certain Tat substrates.

MATERIALS AND METHODS

Strains and growth conditions. The *E. coli* strain MC4100 (10) and its *tat*-deficient derivative DADE (MC4100 Δ *tatABCDE*) (49) were used for functional analyses as well as for expression of *E. coli* *tatABCdstrep*. The strain BL21-DE3 was used for expression of *rc-tatF*, and the strain XL1-Blue Mrf⁺ Kan (Stratagene) was used for all cloning steps. *E. coli* was grown at 37°C on LB medium (1% tryptone, 1% NaCl, 0.5% yeast extract) in the presence of the appropriate antibiotics (100 μ g/ml ampicillin, 12.5 μ g/ml tetracycline). TMAO or dimethyl sulfoxide (DMSO) respiration was tested by anaerobic growth on M9 medium supplemented with 0.5% glycerol, trace elements (SL12 [28]), and 1.1% (wt/vol) TMAO or 0.7% (vol/vol) DMSO, respectively. *R. capsulatus* strain B10S (22) was grown aerobically on 0.15% Bacto peptone, 0.15% yeast extract, 0.8 mM MgCl₂, and 0.5 mM CaCl₂.

Plasmids and genetic methods. The *tat* operon of *R. capsulatus* B10S was amplified from chromosomal DNA using the primers *Rc-tat*-HindIII-F (5' GTT CGA AGC TTT CGT TGC GTG TCA TCG 3') and *Rc-tat*-XbaI-R (5'-GCC GCT CTA GAG GCC TTT GCA CTG CCC-3'). The PCR fragment was restricted with HindIII and XbaI and cloned behind the *lac* promoter into the corresponding sites of the low-copy RK2 *oriV*-containing plasmid pRK415 (21),

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resulting in pRK-*rc-tatABC*. An *rc-tatABC* complementation vector was constructed by amplification of *rc-tatABC*, using the primers *Rc-tat-HindIII-F* (see above) and *Rc-tatABC-XbaI* (5' TCG GGT CTA GAG TGC CAC AAA AAC GCA TCC 3'), restriction of the PCR fragment with HindIII and XbaI, and cloning into the corresponding sites of pRK415, resulting in pRK-*rc-tatABC*.

For expression of *rc-tatF*, the gene was amplified from chromosomal DNA using the primers F-NdeI-F (5' GAC GAC ATA TGA CCG ATC CCC TGA CGC GGA TTG 3') and F-HindIII-R (5' CGT CAA AGC TTC AGC CCG TGC CGC CCT GCC AG 3'). The PCR fragment was restricted with NdeI and HindIII and cloned into the corresponding sites of pET22-b(+) (Novagen), resulting in pEX-*rc-tatF-H6*. For expression of *ec-tatABCDstrep*, the complete *tat* operon of *E. coli* was amplified from chromosomal DNA, using the primers *Ptat-XbaI-F* (5' ATT CTA GAT TAA AGA TCA GGT CGG TAT TCC TGC 3') and *tatD-XhoI-R* (5' TAT CTC GAG CGC AAT CCC AAA CAG TGT TTT GAC 3'), restricted with XbaI and XhoI, and cloned into the corresponding sites of pABS-*tatABCstrep* (5), resulting in pABS-*tatABCDstrep*. All constructs were confirmed by sequencing and restriction analyses.

For rhamnose-regulated expression of *hip* encoding HiPIP from *Allochroa-tium vinosum*, the gene was NdeI/HindIII excised from pEXH5 (8) and cloned into the corresponding sites of pBW22 (50).

Biochemical methods. For purification of rc-TatF, *E. coli* BL21-DE3 was transformed with pEX-*rc-tatF-H6* and grown at high aeration at 37°C to an optical density at 600 nm of 1, induced for 2 h with 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside), and harvested. Cells were resuspended in precooled 20 mM Tris HCl, pH 8.0, 400 mM NaCl, and 5 mM imidazole (5 ml buffer/g wet cells) and disintegrated by two French pressure cell passages at 20,000 lb/in². Cell debris was removed by centrifugation (18,000 $\times$ g, 30 min, 4°C), the supernatant was further centrifuged at 100,000 $\times$ g (30 min, 4°C), and the soluble proteins were loaded on a 2-ml Ni-nitrilotriacetic acid His bind resin (Merck) equilibrated with the above buffer. After being washed with a further 12 ml of buffer, rc-TatF was eluted with 20 mM Tris HCl, pH 8.0, 400 mM NaCl, and 200 mM imidazole, dialyzed against 20 mM Tris HCl, pH 8.8, and 0.5% Tween 20, and loaded on a Superose 6 gel filtration column (flow rate of 0.4 ml/min, equilibrated with 20 mM Tris HCl, pH 8.8, and 0.5% Tween 20; Pharmacia).

The chain formation phenotype was assessed by differential contrast microscopy as described previously (5). Complementation of the sodium dodecyl sulfate (SDS) sensitivity of the Tat mutant strain DADE was quantified as described by Ize et al. (19). Cell fractionation, SDS-polyacrylamide gel electrophoresis, and Western blotting were carried out as described previously (9). TMAO reductase was detected in native gels as described elsewhere (41). HiPIP detection was carried out as described previously (9). Formate dehydrogenase (FDH) activity was quantified by 2,6-dichlorophenolindophenol reduction, with phenazine methosulfate as the mediator, as described elsewhere (12).

Prediction of Tat substrates. The genome sequence and the amino acid sequences of all predicted proteins from *R. capsulatus* strain SB1003 were obtained via ERGO Light (Integrated Genomics). The sequences were analyzed by TATFIND 1.2 as described previously (13, 31). As a slight modification, the Tat substrate was not allowed to be translationally initiated at position (X^{-1}) of the RR pattern (X^{-1}) (R^0) (R^{+1}) (X^{+2}) (X^{+3}) (X^{+4}) (X^{+5}), nor to have a P at position (X^{+3}), a Q at position (X^{+4}), or an I/L/C at position (X^{+5}). With these additional restrictions, obvious false positives in *R. capsulatus* were eliminated. Four further likely Tat substrates, such as the well-characterized Tat substrate NosZ (18), could be identified by nonautomated proteome analysis. The reason for the nondetection of these Tat substrates by TATFIND 1.2 was the restriction to only a subset of amino acids at position (X^{+2}). TatP (3), a second Tat substrate identification program, recognized all but four of the listed putative Tat substrates, which were different from the four not recognized by TATFIND 1.2. At least one of the four unrecognized substrates, the cytochrome *bc*₁ complex Rieske subunit, has been clearly established as a Tat substrate in other organisms (25).

RESULTS

It is proposed that Tat translocons are adapted to their natural set of substrates (7). Only single natural Tat substrates have been tested for their compatibility with Tat translocons from a different organism (1, 7, 9, 27, 42). To test in vivo the adaptation of a Tat translocon to its substrates, we decided to exchange the Tat translocon of *Escherichia coli* with that of *Rhodobacter capsulatus*. With this strategy, all *E. coli* Tat substrates are left in their native environment, where they fulfill

FIG. 1. Organization of the *tat* genes in *R. capsulatus* and *E. coli*. The length of the genes (in nucleotides) is indicated below the corresponding gene arrow. The position of the predicted stem-loop structure behind *tatC* is marked with a stem-loop sign.

their natural role, thus allowing physiological assessments of Tat substrate functionality with certain substrates. *E. coli* is best suited for such an experiment, as various physiological assays for Tat functionality are established for this organism (5, 19, 43). Moreover, a mutant strain of this γ -proteobacterium is available which is devoid of all known Tat components (49). The Tat system of the α -proteobacterium *R. capsulatus* was chosen, as it differs significantly from that of *E. coli* with respect to its predicted set of Tat substrates, whereas it is similar to that of *E. coli* with respect to translocon composition.

The set of Tat substrates in *R. capsulatus*. The genome of *R. capsulatus* has been sequenced by Integrated Genomics and made publicly available via the ERGO Light homepage (<http://www.ergo-light.com/ERGO/>). Using the programs TATFIND and TatP (3, 31), we could identify genes for 29 likely Tat substrates in the genome of *R. capsulatus* (see Table S1 in the supplemental material). Among these are six predicted to be cofactor-containing redox enzymes, eight periplasmic binding proteins, two hydrolases, five enzymes involved in biogenesis pathways (such as N₂O reductase maturation, periplasmic disulfide formation, and glucan biosynthesis), and eight hypothetical proteins without functional assignments. One known Tat substrate from *R. capsulatus*, a DMSO reductase with high similarity to *E. coli* TMAO reductase (40), was not detected in the available genome data of the sequenced strain. The high number of transporter-associated binding proteins is unexpected and in marked contrast to that in *E. coli*, which has only one predicted Tat substrate among its binding proteins. Only 6 out of the 29 predicted *R. capsulatus* Tat substrates have homologs among the Tat substrates of *E. coli*. Thus, the *E. coli* system differs considerably in its substrate range from that of *R. capsulatus*.

The Tat translocon components in *R. capsulatus*. The Tat translocon components from *R. capsulatus* resemble those of *E. coli*. We identified by BLAST analyses one operon which encodes orthologs of TatA, TatB, and TatC (in the following termed rc-TatA, rc-TatB, and rc-TatC, respectively). The corresponding genes are organized together with a fourth gene, *rc-tatF* (Fig. 1). This situation resembles that of *E. coli*, which has a *tatABC* operon connected with a fourth gene, *tatD*, which

is a nuclease with no relevance for translocation (49). *E. coli* contains two TatA paralogs, TatA and TatE, of which TatE is encoded by a monocistronic gene (38). rc-TatA, rc-TatB, and rc-TatC show significant sequence identity to their orthologs in *E. coli*. As expected from phylogenetic studies (51), TatA/E and TatB orthologs from both species resemble each other, with 20 to 37% overall identity and certain conserved boxes with over 40% identity. rc-TatA has the highest similarity to TatA (37% overall identity, 50% in residues 6 to 43). rc-TatB has the highest similarity to TatB (27% overall identity, 69% in residues 1 to 26, and 42% in residues 122 to 145). The TatC proteins from both organisms show similar overall sequence identities (27%), and there are three conserved boxes with an identity higher than 40% (61% in residues 8 to 25, 43% in residues 89 to 143, and 45% in residues 186 to 238). The fourth gene product of the *R. capsulatus* *tatABC* operon, rc-TatF, has no detectable sequence homology to any gene product of *E. coli*. The *rc-tatC* stop codon overlaps with the start codon of *rc-tatF* (ATG ACC), strongly suggesting a translational coupling of the two genes. An efficient translational coupling by this four-nucleotide overlap is very likely, as the genes for rc-TatB and rc-TatC show the same overlap (ATG ACG), and it is known that TatB and TatC form stable complexes (26). In the only other sequenced *Rhodobacter* species, *R. sphaeroides*, the two orthologs are in the same position and share the same four nucleotides, suggesting that the translational coupling of the *tatF* and *tatC* orthologs is conserved within the genus.

Sequence analyses revealed that the *rc-tatABC* operon allows the formation of a RNA hairpin structure downstream of *rc-tatC*. This is similar to the *tatABCD* operon from *E. coli* (49), in which a hairpin is found in an untranslated region between *tatC* and *tatD* (Fig. 1). In the case of *R. capsulatus*, there is no untranslated region between *rc-tatC* and *rc-tatF*, and the hairpin is predicted to be encoded 54 bases downstream of *rc-tatC* within *rc-tatF* (CCG CCC CCG AGTTTT CGG GGG CGG). As it is known that such hairpins can block 3' to 5' exonuclease activities (23), this hairpin is likely to stabilize the *rc-tatABC* portion of the transcript. Therefore, although *rc-tatF* is translationally coupled to *rc-tatC*, it is likely that the gene product TatF is less abundant than TatABC.

In summary, the Tat translocon components from *R. capsulatus* resemble those of *E. coli*. However, the fourth component encoded by the *rc-tat* operon, rc-TatF, is unrelated to components from *E. coli*.

The *R. capsulatus* Tat translocase is functional in *E. coli*. To test the functionality of the Tat translocon from *R. capsulatus*, the complete *rc-tat* operon was transferred on a low-copy vector to an *E. coli* mutant strain (DADE) which is devoid of all known endogenous Tat components (49). In a first approach, Tat system functionality was assessed by the so-called chain formation phenotype which is observed when the two Tat substrates AmiA and AmiC are not translocated. These two proteins are murein amidases which are important for the hydrolysis of the cell division septum and thus for the separation of cells after cell division (17). When AmiA and AmiC are not translocated into the periplasm of Tat-deficient bacteria, cell chains can be readily observed by microscopy (4, 19).

The Tat-deficient strain DADE carrying only the empty vector formed the expected cell chains (Fig. 2A). The *R. capsulatus* Tat system as well as the *E. coli* Tat system completely

FIG. 2. Complementation of cell chain formation and SDS sensitivity phenotypes of *tat*-deficient *E. coli* by recombinant Tat systems from *R. capsulatus* and *E. coli*. Phase-contrast micrographs of (A) *E. coli* DADE (MC4100 Δ *tatABCDE*)/pRK415 (empty vector control) (B) *E. coli* DADE (MC4100 Δ *tatABCDE*)/pRK-*ec-tatABC* (positive control), and (C) *E. coli* DADE (MC4100 Δ *tatABCDE*)/pRK-*rc-tatABC* (complementation with *R. capsulatus* Tat system). (D) SDS sensitivity as expressed in percent survival after 3 h of growth in LB medium containing the indicated SDS concentrations (19). Diamonds, *E. coli* DADE/pRK415 (empty vector control); squares, *E. coli* DADE/pRK-*ec-tatABC* (complementation with *E. coli* Tat system, positive control); filled circles, *E. coli* DADE/pRK-*rc-tatABC* (complementation with *R. capsulatus* Tat system). Error bars indicate the standard deviations as derived from triplicate assays.

cured this morphological defect, resulting in single cells with wild-type shape (Fig. 2B and C). As the absence of AmiA or AmiC alone already results in short cell chains, the full complementation indicates that both Tat substrates are localized properly (17). Therefore, the heterologous *R. capsulatus* Tat system translocates AmiA and AmiC in *E. coli*.

The transport block of AmiA and AmiC also contributes to a cell envelope defect which renders the cells sensitive towards detergents such as SDS, and an SDS sensitivity assay has been established which quantifies the tolerance of the growing bacteria towards this detergent (19). We thus tested the SDS sensitivity of the heterologous translocon-containing strain (Fig. 2D). In agreement with the morphological observations, the complementation of this phenotype was complete, indicating that the *R. capsulatus* Tat translocon can translocate AmiA and AmiC.

Another physiological assay for Tat system functionality is growth by TMAO respiration. The TMAO reductase TorA, which is the terminal reductase of this anaerobic respiration, is a bis(molybdopterin guanine dinucleotide) molybdenum cofactor-containing Tat substrate which requires Tat system functionality for its translocation into the periplasm (36). *E. coli* thus does not grow on TMAO media with glycerol as a non-fermentable carbon source in the absence of a functional Tat system. We therefore tested the functionality of the *R. capsulatus* Tat system by complementation of the TMAO respiratory growth of the Tat-deficient *E. coli* strain DADE. TMAO growth of the strain containing the empty vector was tested as a negative control, and the complementation by the *E. coli* Tat system was tested as a positive control. As shown in Fig. 3A, the *R. capsulatus* Tat system fully complemented the TMAO growth deficiency of the complete *tat* knockout strain. As expected, the growth deficiency was also complemented by the *tatABC* genes from *E. coli*. The vector-only negative control strain did not grow, indicating that the growth conditions were selective.

TorA export was also monitored by detection of TorA in the periplasm of cells shifted to anaerobic growth in the presence of TMAO. TorA activity can be detected in native gels soaked with dithionite-reduced methyl viologen. When the dark-blue gel is transferred into a TMAO-containing solution, it is locally destained at the TorA bands, due to TMAO-dependent oxidation of reduced methyl viologen. Subcellular fractions from various strains were thus tested for the presence of TorA activity (Fig. 3B). TorA was equally active in the corresponding subcellular fractions from strains containing either the *E. coli* or the *R. capsulatus* Tat system but was absent from the periplasm and the membrane fraction in the strain which lacked a Tat system (Fig. 3B). The absence of TorA activity from these fractions indicated that the Tat mutant strain did not translocate TorA. In contrast, strains containing the *R. capsulatus* or the *E. coli* Tat systems translocated this enzyme equally well. Significant activity was detected in the cytoplasm and most likely corresponded to a precursor or TorA, as has been reported earlier (36). Interestingly, TorA activity was also detected in the membrane fraction, and here, it depended on the presence of a functional Tat system. This indicates that at least a significant portion of TorA associates with the membrane after translocation, probably by the specific interaction with the membrane-bound pentaheme *c*-type cytochrome TorC, which has been clearly demonstrated (16). As TorA is the inducible TMAO reductase of *E. coli*, we also checked for the inducibility of the activity in our assays. Bacteria were grown anaerobically on LB-0.5% glycerol-0.4% nitrate to an optical density at 600 nm of about 0.5, and TorA was induced by the addition of 1.1% TMAO. After 1 h of continued growth,

FIG. 3. Assessment of Tat system functionality by TMAO respiratory growth and TorA translocation analyses. (A) *rc-tatABC* complements TMAO growth deficiency of *tat*-deficient *E. coli*. Anaerobic growth in minimal medium with glycerol as the only carbon and electron source and TMAO as the terminal electron acceptor is monitored by an optical density (OD) at 600 nm which is semilogarithmically plotted against time. Diamonds, *E. coli* DADE/pRK415 (empty vector control); triangles, *E. coli* DADE/pRK-*ec-tatABC* (complementation with *E. coli* Tat system, positive control); filled circles, *E. coli* DADE/pRK-*rc-tatABC* (complementation with *R. capsulatus* Tat system). Error bars indicate the standard deviations from three cultures. (B) Detection of TorA activity in subcellular fractions after induction of TMAO respiration. no Tat, *E. coli* DADE/pRK415 (empty vector control); R.c. Tat, *E. coli* DADE/pRK-*rc-tatABC* (complementation with *R. capsulatus* Tat system); E.c. Tat, *E. coli* DADE/pRK-*ec-tatABC* (complementation with *E. coli* Tat system, positive control); P, periplasm; M, membrane; C, cytoplasm. The right-side gel shows inducibility of TorA activity by the addition of TMAO (see Materials and Methods).

periplasm was prepared and analyzed by the described activity staining (Fig. 3B, right). The data show that the detected activity was indeed inducible by TMAO. Together, these results confirm that periplasmic and membrane-associated TMAO reductase activity by TorA depends on a functional Tat system and show that the *R. capsulatus* Tat system functions well with TorA.

We also analyzed the Tat-dependent targeting of formate dehydrogenase by means of its activity. *E. coli* contains three formate dehydrogenases. Two of them, FDH-N and FDH-O, are present under anaerobic conditions during growth with nitrate as the final electron acceptor (39). The periplasmic subunits of these two formate dehydrogenases are Tat substrates which are translocated as heterodimers of nearly 150 kDa and thus are so far the largest Tat substrates known (37). These homologous enzymes are attached to a membrane anchor subunit after translocation. *R. capsulatus* does not encode a formate dehydrogenase Tat substrate in its sequenced genome. We thus measured formate dehydrogenase activity in membrane and soluble fractions after anaerobic growth on nitrate. The specific FDH activity was 173 ± 18 U/mg (mean $\pm$ standard deviation) in the wild-type strain MC4100 carrying

the empty vector, 11 ± 4 U/mg in the Tat-deficient strain DADE carrying the empty vector, 172 ± 4 U/mg in the strain DADE with a complementing *E. coli* Tat system, and 62 ± 10 U/mg in the strain DADE with a complementing *R. capsulatus* Tat system. Targeting of formate dehydrogenase to the membrane fraction thus clearly depended on a functional Tat system. While the targeting deficiency of a Tat-deficient strain was fully compensated by expression of the *tatABC* genes in *trans* from pRK-*tatABC*, the targeting efficiency was near 35% of the wild-type level when the *E. coli* Tat system was exchanged with that of *R. capsulatus*. Therefore, the *R. capsulatus* Tat system can translocate formate dehydrogenases.

To test whether the observed function of the heterologous *R. capsulatus* Tat translocon was independent of rc-TatF, we tested TMAO respiration with a shortened operon lacking *rc-tatF*. The absence of rc-TatF had no influence on the functional complementation of TMAO respiration (data not shown). Similarly, TatF was not relevant for AmiA and AmiC transport, as monitored by SDS sensitivity and complementation of chain formation (data not shown). TatF is therefore not essential for the translocation activity of the Tat translocon of *R. capsulatus* in *E. coli*.

The location of the *rc-tatF* gene behind *rc-tatC* in *R. capsulatus* is analogous to the position of *tatD* behind the *tatC* gene in *E. coli*. Although the two gene products, TatD and rc-TatF, do not exhibit any sequence similarity, it was possible that they could resemble each other functionally. As TatD is a DNase, one thus could imagine that rc-TatF exhibits DNase activity, too. To test this, rc-TatF was tagged with a C-terminal His₆ tag and purified from *E. coli* BL21 DE3 by affinity chromatography, followed by a gel filtration step. The thus highly enriched His-tagged rc-TatF migrated in SDS-polyacrylamide gel electrophoresis as a single band at 30 kDa, which is in agreement with the calculated molecular mass of 32 kDa (data not shown). rc-TatF was stable at pH 8.5 in buffers containing 0.5% Tween 20. Purified rc-TatF had no nuclease activity (data not shown). DNase activity, which was present after His tag affinity chromatography, was not due to rc-TatF, as the DNase eluted prior to rc-TatF in the gel filtration chromatography purification step. Unless the tag affects activity, this observation indicates that rc-TatF is not only structurally but also functionally unrelated to TatD of *E. coli*.

The *R. capsulatus* Tat translocase does not target *E. coli* DmsA to the membranes. Like TMAO respiration, respiratory growth on DMSO is also dependent on the functionality of the Tat translocon. DmsA, the catalytic subunit of the membrane-anchored DMSO reductase, is Tat dependently targeted as a heterodimer together with the DmsB subunit (33, 44, 48). Therefore, a nonfunctional Tat system results in the absence of DmsA from the membrane fraction and consequently in a deficiency of DMSO respiration. We tested DMSO respiration and found that the *R. capsulatus* translocon did not support this growth mode (Fig. 4A). This was in marked contrast to the *E. coli* Tat system, which supported DMSO respiratory growth. It was therefore possible that the Tat system of *R. capsulatus* was not compatible with DmsA targeting. Alternatively, DMSO reductase was membrane targeted but nonfunctional.

We resolved this question by assessment of the presence of DmsA in the membranes by Western blotting, using antiserum against DmsA (Fig. 4B). As a negative control, we analyzed

FIG. 4. Analysis of DMSO respiratory growth and DmsA targeting. (A) The *R. capsulatus* Tat system does not support DMSO growth of *E. coli*. Anaerobic growth in minimal medium with glycerol as the only carbon and electron source and DMSO as the terminal electron acceptor is monitored by an optical density (OD) at 600 nm which is semilogarithmically plotted against time. Diamonds, *E. coli* DADE/pRK415 (empty vector control); squares, *E. coli* DADE/pRK-*ec-tatABC* (complementation with *E. coli* Tat system, positive control); filled circles, *E. coli* DADE/pRK-*rc-tatABC* (complementation with *R. capsulatus* Tat system). Error bars indicate the standard deviations from three cultures. (B) Western blot detection of DmsA in membrane fractions from *E. coli* strain MC4100 (MC) (wild type) and its Tat-deficient derivative DADE, which express, as indicated, the *R. capsulatus* *tatABC* genes (*rc-tat*), the *E. coli* *tatABC* genes (*ec-tat*), or no *tat* genes (—) in *trans*. The position of the DmsA band is indicated on the left.

membranes from the Tat-deficient strain DADE, and as a positive control, we used membranes from the parental strain MC4100 or membranes from DADE complemented with the *E. coli* *tatABC* genes in *trans*. DmsA was not detectable in the membranes in the absence of a Tat system (DADE), but it was present in the membranes of all those strains which contained the *E. coli* Tat system. When the Tat system from *R. capsulatus* was present in one strain together with the *E. coli* Tat system, there was no difference compared to what was observed in a strain lacking the *R. capsulatus* Tat system. Importantly, when only the *R. capsulatus* Tat system was present, DmsA targeting to the membranes was completely abolished. Therefore, DmsA and DmsB are not functionally targeted to the membrane by the *R. capsulatus* Tat system, which causes a proteolytic degradation of DMSO reductase and consequently leads to a DMSO respiration deficiency. These results indicate that the tested heterologous Tat system of *R. capsulatus*, although func-

FIG. 5. Analysis of translocation of the heterologous Tat substrate HiPIP from *A. vinosum* by the heterologous Tat system from *R. capsulatus* in the *E. coli* host. The detection of HiPIP in subcellular fractions of *E. coli* containing either the recombinant *E. coli* Tat system or the recombinant *R. capsulatus* Tat system is shown. Expression was from pBW-*hip* in either DADE/pRK-*ec-tatABC* (*E. coli* Tat system) or DADE/pRK-*rc-tatABC* (*R. capsulatus* Tat system), induced with 0.4% rhamnose for 2 hours. HiPIP was detected by Western blotting using specific antibodies. P, periplasm; M, membrane; C, cytoplasm. The positions of precursor (pre) and mature (mat) forms of HiPIP are indicated.

tional with TorA, FDH, AmiA, or AmiC, is not functional with DmsA.

Translocation of HiPIP from *Allochrochromatium vinosum* by the Tat translocase of *R. capsulatus* in *E. coli* demonstrates low species specificity of the Tat systems. The functionality of our heterologous Tat system was also assessed with the heterologous Tat substrate HiPIP from *Allochrochromatium vinosum*. In *E. coli*, HiPIP can be translocated only by the Tat system (9). HiPIP was indeed translocated by the *R. capsulatus* Tat system (Fig. 5). As is usually observed with recombinant Tat substrates, the transport was limiting, which caused significant accumulation of precursor proteins with protease-sensitive signal sequences in the cytoplasm (2, 19, 45). The actual translocation efficiency with the *R. capsulatus* Tat system was only slightly lower than that observed with the recombinant *E. coli* Tat system. The data show for the first time that a heterologous Tat substrate can be translocated by a heterologous Tat system in a third organism, which underlines the rather low species specificity of Tat systems and the exceptionality of the observed DmsO reductase mistargeting.

DISCUSSION

In this work, the exchange of a complete Tat translocon was used to study the possibility of translocon substrate selectivity. Substrate selectivity may be caused by (i) species-specific variations in the recognition of Tat determinants or (ii) the absence of translocon-specific targeting factors in the heterologous system. The idea of species-specific Tat substrate selectivity led to the finding that the Tat substrate GFOR of the α -proteobacterium *Zymomonas mobilis* is not accepted by the *E. coli* Tat system (7). However, when the signal sequence of TorA was fused to the mature part of GFOR, translocation in *E. coli* became possible. Therefore, it was not the mature domain but rather the signal sequence of GFOR which was incompatible with the *E. coli* Tat system. The mature domain of Tat substrates indeed appears not to contain species-specific recognition signals for their respective Tat translocons. This is exemplified by many artificial Tat substrates which are Tat dependently translocated as a result of an N-terminal fusion to a natural Tat signal sequence, most frequently that of the *E. coli* TMAO reductase TorA (6, 11, 15, 20, 24, 34, 35, 46, 47).

Tat translocons recognize a broad range of signal sequences

which varies between species. Only little has been done to understand the proposed species specificity of Tat substrate signal sequences. We used a "whole-translocon exchange" experiment to study the acceptance of various Tat substrates by a heterologous translocon. This approach preserves the natural expression and folding conditions of Tat substrates. If some Tat substrates are selectively not translocated by a heterologous translocon, this is evidence of some selectivity of the translocation machinery.

The results of this study demonstrate that the Tat translocon of *R. capsulatus* does not require species-specific auxiliary factors. The heterologous system is compatible with the majority of Tat substrates, demonstrating a high degree of flexibility with regard to accepted Tat substrates. Only one Tat substrate, DmsA, was neither targeted to the membrane fraction nor supportive of DMSO growth. This contrasts the translocation of TorA, AmiA, AmiC, and formate dehydrogenases. Moreover, the efficient translocation of the heterologous Tat substrate HiPIP demonstrates that substrates from one organism can be translocated by a Tat system from a second organism in the cellular background of a third organism. The data thus clearly indicate that the compatibility between Tat translocons and Tat substrates is the rule and that the substrate DmsA is an exception.

DmsA is a well-established *E. coli* Tat substrate, and the absence of DmsA from the membrane fraction indicates a deficiency in the Tat translocon (48). It has to be noted that the DMSO reductase DorA of *R. capsulatus* is not a membrane protein, such as DmsABC of *E. coli*. Instead, DorA belongs to the family of *E. coli* TorA, which is nicely translocated by the *R. capsulatus* Tat system (Fig. 3). The exact topology of DmsABC has been extensively studied. Two opinions exist, one placing DmsA on the periplasmic face of the cytoplasmic membrane and the other on the cytoplasmic face. It has been shown that DmsA and DmsB are translocated together across the membrane in a Tat-dependent manner when the membrane anchor subunit DmsC is absent and that the membrane-impermeable substrate TMAO can be specifically reduced by the DmsABC system (44). This contrasts a variety of experiments, such as immunogold-labeling techniques and proteolytic accessibility assays, which suggested that DmsAB is only membrane targeted by the Tat system but not translocated (32). Despite this controversy, it is interesting that DMSO reductase was not targeted by the Tat system of *R. capsulatus*. The data could be explained by the requirement of a specific interaction of a DmsAB translocon targeting factor with the corresponding Tat system, such as has been suggested for this enzyme (29). Generally speaking, the biogenesis of DmsABC is likely to require additional features of the Tat translocon which are absent in the Tat system of *R. capsulatus*.

This study shows that Tat signal sequences from very different *E. coli* Tat substrates are accepted by the *R. capsulatus* Tat system. The substrates are translocated and functional, thus allowing physiological processes which can be monitored by various assay systems. Therefore, this study underlines that Tat translocons from α - and γ -proteobacteria share basic signal sequence recognition characteristics. Some variations may occur with respect to preferred sequence patterns, and some Tat substrates, such as DmsA in this study, may not be targeted correctly. A complete abolishment of the targeting process may

reflect exceptional targeting requirements of rare substrates, such as the membrane targeting of DmsA. Another example from the literature is GFOR from *Zymomonas mobilis* (7).

Prospects of whole-translocon exchange approaches. Heterologous systems for physiological Tat system functionality assays are powerful tools, as they can rapidly provide strong positive evidence. However, our results indicate that if a negative outcome of one physiological assay is observed, it does not necessarily mean that the Tat system is not working. Other physiological assays may turn out to be positive, which proves functionality. In fact, these negative exceptions are the interesting results, as they provoke explanatory studies which lead to new insights. Thereby, one may find additional translocon-specific factors or characteristics which are required for the targeting of a certain substrate, thus abolishing translocation by a heterologous system. One may also consider that different routes could exist for the targeting to the translocon and that heterologous systems may differ in that respect. Certainly, the translocon exchange approach is rapid for the evaluation of the functionality of a proposed Tat translocon of some organism.

TatF: a novel component associated with Tat and Sec systems? An interesting aspect of this study is rc-TatF, the fourth gene product of the *rc-tatABCF* operon. rc-TatF is not required for translocation, but there is some evidence which argues for a functionality in association with protein translocation machineries. rc-TatF is the first analyzed member of a new family of proteins found in α -, β -, and δ -proteobacteria. Within the α -proteobacteria, the closest homolog is that of *R. sphaeroides*. It is intriguing that the start codons of the *tatF* genes from *R. capsulatus* as well as those from *R. sphaeroides* overlap with the stop codon of the corresponding *tatC* genes, indicating a strict coupling of translation. In other TatF-containing α -proteobacteria (e.g., *Agrobacterium*, *Bradyrhizobium*, *Brucella*, *Mesorhizobium*, *Rhodopseudomonas*, and *Sinorhizobium* species), the *Rhizobiales*, *tatF* is localized directly adjacent to the genes encoding YajC and SecDF. The TatF homologs of β - and δ -proteobacteria do not show this proximity to protein translocation-related genes. The strong conservation of *yajC* and *secDF* proximity to *tatF* in *Rhizobiales* suggests that the TatF homologs may also function somehow in protein translocation in these organisms, albeit in the Sec system and not in the Tat system. TatF also neighbors the YajC homolog in *Caulobacter crescentus*, a member of the *Caulobacteriales* and another α -proteobacterium. The YajC and SecDF components form a complex which improves the SecA-dependent translocation at the SecYEG translocon (14). One might speculate that the association of TatF orthologs with Sec or Tat protein translocation systems in many organisms may not be coincidental. TatF homologs generally show motifs of ATPases associated with various cellular functions (AAA family). They all belong to an uncharacterized protein family (Pfam entry DUF815 at Sanger). As many AAA family proteins show ATP-dependent chaperone functions, TatF could play a role in the translocation of certain Sec or Tat substrates, depending on the organism, which could explain the differential association of the corresponding genes with the two systems.

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