The Tat (twin-arginine protein translocation) system initially discovered in the thylakoid membrane of chloroplasts has been described recently for a variety of eubacterial organisms. Although in Escherichia coli four Tat proteins with calculated membrane spanning domains have been demonstrated to mediate Tat-dependent transport, a specific transport system for twin-arginine signal peptide containing phosphodies-terase PhoD of Bacillus subtilis consists of one TatA/ TatC (TatAd/TatCd) pair of proteins. Here, we show that TatAd was found beside its membrane-integrated localization in the cytosol where it was interacted with prePhoD. prePhoD was efficiently co-immunoprecipitated by TatAd. Inefficient co-immunoprecipitation of mature PhoD and missing interaction to Sec-dependent and cytosolic peptides by TatAd demonstrated a particular role of the twin-arginine signal peptide for this interaction. Affinity of prePhoD to TatAd was interfered by peptides containing the twin-arginine motif but remained active when the arginine residues were substituted. The selective binding of TatAd to peptides derived from the signal peptide of PhoD elucidated the function of the twin-arginine motif as a target site for pre-protein TatAd interaction. Substitution of the binding motif demonstr-ated the pivotal role of basic amino acid residues for TatA binding. These features suggest that TatA interacts prior to membrane integration with its pre-protein substrate and could therefore assist targeting of twin-arginine pre-proteins.

Bacteria have two distinct pathways for the export of proteins across the cytoplasmic membrane. The majority of the periplasmic proteins are exported in an unfolded conformation via the Sec pathway, which is promoted by ATP hydrolysis (1–4). Proteins translocated by this pathway are targeted to the membrane-embedded proteinaceous Sec pore by soluble targeting factors (1). The transport is mediated by N-terminal signal peptides that are similar structurally but do not show sequence conservation (5).

Several proteins use an alternate translocation pathway. Because of its highly conserved twin-arginine sequence motif present in the signal peptides of proteins using this way, it is called Tat1 (twin-arginine translocation) pathway (6–8). It was originally identified in chloroplasts (9, 10) and has been described recently (9, 11) for Escherichia coli. The currently only known driving force of the translocation is the pH gradient at the membrane (9, 12). Because most of the Tat substrates require the incorporation of cofactors or subunit association (13–15), the Tat pathway appears to be responsible for proteins incompatible with the Sec pathway (16). By a yet identified control mechanism the Tat system exports only Tat substrates that attained a native conformation (15–17).

The currently best characterized Tat system of E. coli consists of four proteins with calculated membrane spanning domains (10, 18, 19). Sequence analysis predicts that TatA, TatB, and TatE are proteins that comprise a transmembrane N-terminal α-helix followed by an amphipathic α-helix at the cytoplasmic side of the membrane (20, 21). TatC, the fourth protein known to be involved in the Tat system of E. coli, has six predicted transmembrane helices (20). Topology determination revealed that TatC contains four transmembrane helices (18). Although tatB and tatC appear to play a pivotal role in the Tat-dependent protein translocation in E. coli (11, 19, 20), TatA and TatE can, at least partially, functionally substitute each other (20). By analyzing the presence of Tat substrates and components of the Tat machinery surveys of prokaryotic genomes indicate that the Tat pathway is widespread among bacteria and archaea (8, 22). The standard Tat systems (such as in E. coli) consist of one TatC, at least one copy of TatAE, and TatB (23). In certain prokaryotes a TatB homolog appears to be absent.

Currently there exist only weak ideas about the targeting of the twin-arginine precursors to the translocase unit and the structure of the Tat transport system. In vitro work with the plant thylakoid Tat system demonstrated that no soluble factors are required of Tat-dependent export (24) and that TatA is required for the transport steps following precursor recognition (25, 26). The TatA/B/E proteins have been predicted to act as membrane receptors for Tat substrates (22) or to form the export channel itself (27–31). The targeting of Tat-dependent

The abbreviations used are: Tat, twin arginine translocation; IPTG, isopropyl-β-D-thiogalactopyranoside; PBS, phosphate-buffered saline.
iron-sulfur protein HiPIP to the membrane appears to be independent of the Tat components (15). The use of the Tat system by redox proteins in many bacteria or preferably non-redox proteins in other species, as well as the different composition of the Tat proteins, indicates that the Tat system is of heterogeneous nature (8, 22, 23).

Analysis of Tat-like proteins in Bacillus subtilis revealed that the genome encodes three TatA and two TatC-like proteins (31). TatB-like proteins appear to be absent of B. subtilis and of other sequenced bacilli (22, 32). Translocation of the B. subtilis protein PhoD containing a twin-arginine signal peptide was shown to be dependent on the expression of a tatA tatC pair (33). These genes (designated tatAd and tatCd) are co-localized with phoD in one operon resulting in co-regulated expression of phoD and tatAd/tatCd. A second copy of tatC (tatCm) was not required for PhoD export (31). The PhoD-specific transport system was functionally active in E. coli. Although PhoD or a fusion consisting of the signal peptide of PhoD and LacZ was not recognized by the E. coli Tat components, co-expression of B. subtilis tatAd/tatCd resulted in translocation of SPprePhoD-LacZ. This transport was shown to be pH-dependent. These studies revealed that the minimal requirement of a Tat transport system consists of a TatA/TatC pair, a twin-arginine signal peptide, and the pH gradient at the bacterial cytoplasmic membrane (33). PhoD is the only known substrate of the Tat system by redox proteins in many bacteria or preferably non-redox proteins in other species.

The B. subtilis phoD gene encodes a secretory enzyme with alkaline phosphatase and phosphodiesterase activities (36). Slow processing maintains the protein in a cell wall-associated localization before release (37). It has been demonstrated that PhoD is a member of the so-called Pho regulon of B. subtilis (38). The Pho regulon comprises a group of genes that are induced in response to the depletion of inorganic phosphate in the growth medium and is regulated by the two component signal transduction system PhoR/PhoP (39). A phoR12 mutation in B. subtilis strain GCH871 was shown to be functionally active under phosphate replete conditions resulting in the induction of Pho regulon genes (40).

To investigate the selectivity and specificity of the PhoD transport system further, we analyzed the localization and affinity of TatA by combining genetic and in vitro approaches in B. subtilis and E. coli. Unexpectedly, we found TatA in the cytoplasmic membrane, as well as in the cytosol. By using purified TatA and prePhoD we demonstrated the interaction of both peptides. Inefficient binding of TatA to mature PhoD or Sec-dependent or cytosolic proteins demonstrated the particular role of the twin-arginine-containing signal peptide of PhoD in the recognition process. The selective affinity of TatA to peptides derived from the signal peptide of PhoD showed that the twin-arginine motif acts as a binding site of TatA. Substitution of amino acid residues of the binding motif elucidated the role of particular amino acids of this motif for TatA recognition.

**EXPERIMENTAL PROCEDURES**

### Plasmids, Bacterial Strains, and Media—Table I lists the plasmids and bacterial strains used. TY medium (tryptone/yeast extract) contained Bacto tryptone (1%), Bacto yeast extract (0.5%), and NaCl (1%). For pulse labeling of proteins M9 minimal medium was used (41). To induce or repress the pho operon in B. subtilis 168 from low or high phosphate defined medium, as described (40). B. subtilis phoR12 was cultivated in absence of isopropyl-β-D-thiogalactopyranoside (IPTG). Induction of phosphate starvation response was monitored by determining alkaline phosphatase activity as described previously (39). When required, media were supplemented with ampicillin (80 μg/ml), kanamycin (20 μg/ml), chloramphenicol (20 μg/ml or 5 μg/ml), tetracycline (12.5 μg/ml), erythromycin (5 μg/ml), arabinose (0.2%), or IPTG (1 mM).

**DNA Techniques—**Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of E. coli were carried out as described by Sambrook et al. (42). Restriction enzymes were from MBI Fermentas. PCR was carried out with the VENT DNA polymerase (New England Biolabs) using chromosomal DNA of B. subtilis 168 as template. To construct pQE9phoD, the phoD gene lagging the 5′-terminal region encoding the signal sequence was amplified from the chromosone of B. subtilis strain 168 by PCR using the primers P1 (5′-GTA GGA TCC GCC CCT AAC TTC TCA AGC-3′) containing a BamHI site and P2 (5′-CGA TAC TCC AGG ACC TCA TCA GAT GTC-3′) containing a PstI site. The amplified fragment was cleaved with BamHI and PstI and cloned in the corresponding sites of pQE9. The resulting plasmid pQE9phoD allowed the IPTG-inducible synthesis of N-terminally His₆-tagged mature PhoD in E. coli. To overexpress tatAd and tatCd genes in E. coli, genes were amplified...
using primers A₁ (5'-GGT GGA TTC ATT GGA ATT GG-3') containing a BamHI site and primer A₂ (5'-CTG CAG CAT TCA GCC CGC G-3') containing a PstI site and C₁ (5'-TTT CCA TGG ATA AAA AAG AAA CCC G-3') containing a NcoI restriction site and C₂ (5'-CCG GGA TCC GCC CGC CGC TGT TTC TCC-3') containing a BamHI restriction site, respectively. The tat₄C gene was cleaved with BamHI and PstI and cloned in the corresponding sites of pQE9. The resulting plasmid pQE9-tat₄C allowed the IPTG-inducible synthesis of Hist₄-Tat₄C.

TatAd-mediated Targeting

**Construction of mutant strains of B. subtilis tat₄C-TAP.** Schematic presentation of the phoD operon of B. subtilis 168 (A), 168::pORI22-tat₄C-TAP (B), and 168::pORI22-tat₄C'-TAP (C). By a Campbell-type integration of the pORI22-derivative plasmids were integrated into the chromosome of B. subtilis 168. B. subtilis 168::pORI22-tat₄C-TAP or 168::pORI22-tat₄C'-TAP allowed the phosphate starvation-inducible synthesis of fusion proteins consisting of Tat₄C and TAP epitopes or the ATG start codon of Tat₄C and TAP, respectively. The tat₄C gene in the latter strains was placed under the control of the in B. subtilis constitutive repC promoter.

**Fig. 1. Construction of mutant strains of B. subtilis tat₄C-TAP.** To synthesize epitope-tagged Tat₄C, the DNA region encoding phoD and tat₄C was amplified from the chromosome of B. subtilis 168 using primer P1 (33) and primer T1 (5'-CGT AAT ATG TTA ATT ATT TTG-3') containing a BamHI site and primer Y₁ (5'-TAA AAT ATT ATT ATT G-3') incorporating a BamHI restriction site, primer Y₂ (5'-TAA AAT ATT ATT ATT G-3') incorporating a HindIII restriction site, respectively. The resulting plasmid pQE9-tat₄C allowed the IPTG-inducible synthesis of Hist₄-Tat₄C. Amplifying the DNA fragment encoding the mature part of YvaY primer Y₃ (5'-CAG GAT CCA AAG AAA ACC ATA CAT TT-3') incorporating a HindIII restriction site, and primer Y₂ were used. For amplification of the DNA fragment encoding the mature part of YvaY primer Y₃ (5'-CAG GAT CCA AAG AAA ACC ATA CAT TT-3') incorporating a HindIII restriction site, and primer Y₂ were used. For amplification of the DNA fragment encoding the mature part of YvaY primer Y₃ (5'-CAG GAT CCA AAG AAA ACC ATA CAT TT-3') incorporating a HindIII restriction site, and primer Y₂ were used. For amplification of the DNA fragment encoding the mature part of YvaY primer Y₃ (5'-CAG GAT CCA AAG AAA ACC ATA CAT TT-3') incorporating a HindIII restriction site, and primer Y₂ were used. The PCR fragments were digested with BamHI and HindIII and inserted 3' to the HindIII restriction site of the pQE9 digest as described. The resulting plasmids pQE9-tat₄C and pQE9-tat₄C' were transformed into E. coli TG1(pREP4).

**Post-embedding Labeling of B. subtilis**—Phosphate-starved B. subtilis cells were embedded in Lowicryl K4M as described. Ultrathin sections of Lowicryl-embedded cells were mounted on nickel grids coated with polylaminating and subsequently labeled with specific rabbit antibodies and goat anti-rabbit IgG (10 nm gold) or alkaline phosphatase-conjugated goat anti-rabbit IgG (10 nm gold) and incubated overnight. The replicates were washed four times in PBS buffer and treated with PBS containing 1% bovine serum albumin for 30 min. Next they were placed in PBS containing bovine serum albumin (0.5%) and monospecific antibodies against Tat₄C, SecY, or DnaK (dilution 1:200). Subsequently the replicas were washed four times with PBS and placed on a 1:50 diluted solution of the secondary gold-conjugated antibody (goat anti-rabbit IgG with 10 nm gold). Britisch Biocell International (Cardiff, UK) in PBS containing 0.5% bovine serum albumin for 1 h. After immunogold labeling, the replicas were immediately rinsed several times in PBS, fixed with 0.5% glutaraldehyde in PBS for 10 min at room temperature, washed four times in distilled water, and finally picked onto Formvar-coated grids for viewing in an EM 902 electron microscope (Zeiss, Oberkochen, Germany). Freeze-etch immunogold labeling was performed as described. Freeze-fracture replicas were transferred to blotting solution (2.5% SDS in 10 mM Tris buffer, pH 8.3, and 30 mM sucrose) and incubated overnight. The replica were washed four times with PBS buffer and treated with PBS containing 1% bovine serum albumin for 30 min. Next they were placed in PBS containing bovine serum albumin (0.5%) and nonmonospecific antibodies against Tat₄C, SecY, or DnaK (dilution 1:200) for 1 h. Subsequently the replicas were washed four times with PBS and placed on a 1:50 diluted solution of the secondary gold-conjugated antibody (goat anti-rabbit IgG with 10 nm gold). The replicas were washed four times with PBS and placed on a 1:50 diluted solution of the secondary gold-conjugated antibody (goat anti-rabbit IgG with 10 nm gold). The replicas were washed four times with PBS and placed on a 1:50 diluted solution of the secondary gold-conjugated antibody (goat anti-rabbit IgG with 10 nm gold). The replicas were washed four times with PBS and placed on a 1:50 diluted solution of the secondary gold-conjugated antibody (goat anti-rabbit IgG with 10 nm gold).

**Cell Fractionation of B. subtilis and E. coli**—B. subtilis cells were harvested and resuspended in PBS buffer (140 mM NaCl, 2.7 mM KCl, 1.3 mM KHPO₄, 10 mM Na₂HPO₄, pH 7.3) containing 5 mM phenylmethylsulfonyl fluoride. Cell suspension was passed three times through a French press at 16,000 lb/min. Unbroken cells were removed by centrifugation at 10,000 x g for 10 min. To obtain membrane-free cytosolic protein cell lysate was centrifuged at 150,000 x g for 4 h. Pellets contained cytosolic and membrane fractions of E. coli were obtained from spheroplasted cells. Spheroplasts were washed in 50 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, and subsequently centrifuged at 100,000 x g for 1 h. Pellets contained membrane fraction and supernatant cytosolic protein.

**SDS-PAGE and Western Blot Analysis**—SDS-PAGE and Western Blot Analysis were prepared as described. After separation by SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) (48). Proteins were visualized using monospecific antibodies against PhoD (37), Tat₄C, or TatC₄ and alkaline phosphatase-conjugated goat anti-rabbit antibodies (Sigma) according to the manufacturer’s instructions.
Purification of His-tagged Proteins—His-tagged proteins were prepared from IPTG-induced E. coli strain TG1(pREP4, pQE60tatAd), TGI(pREP4, pQE60tatCp), TG1(pREP4, pQE9phoDp), TG1(pREP4, pQE9phoAp), TG1(pREP4, pQE60CP), TG1(pREP4, pQE9vac), and TGI(pREP4, pQE9vac) cultures as abundant proteins and purified by nickel-nitritotriacetic acid affinity chromatography. The purification of TatCp, CopR, PhoD, and YvaY proteins were carried out under denaturing conditions. Soluble His6-TatA was purified under native conditions with sodium phosphate buffer under standard conditions (Qiagen).

Preparation and Purification of Antibodies against TatAd—Purified His6-TatA or TatCp-His6 emulsified in MPL + TDM + CWS adjuvant (catalog number M6661, Sigma) at 100 µg/ml were used to immunize New Zealand White rabbits (Charles River, Hilden, Germany). Specific antibodies were affinity-purified from the sera by adsorption to and elution (with 0.1 M glycine at pH 2.5) from nitrocellulose (37). As proved by Western blotting, monospecific antibodies specifically cross-reacted with TatA or TatCp.

In Vivo Labeling of His-tagged Proteins—[35S]Labeled His-tagged proteins were obtained by pulse labeling of E. coli cultures. Strains were grown in M9 minimal medium, expression of genes of interest was induced for 15 min, and cultures were labeled in 50 µCi of [35S]methionine for 5 min. Subsequent purification using nickel-nitritotriacetic acid affinity chromatography was carried out essentially as described above.

Co-immunopurification—Purification of TAP proteins was carried out following standard procedures (43). Cytosolic fractions of B. subtilis cell lysates were prepared in purification buffer IPP150 (43) as described above.

Co-immunoprecipitation—Binding of His6-tagged [35S]-labeled proteins to unlabelled TatA was measured as follows: [35S]-labeled proteins were incubated with 10 µg excess of His6-TatA (total 0.4 µg) at room temperature in 50 µl of PBS buffer supplemented with 0.5% n-octyl-β-D-glucopyranoside if indicated. For competition experiments synthetic peptides QNTFDKRKFQGGAGKIAG or QNTFDAAAQFQGGIK (34 st) were added to the reaction mixture. After 60 min, 50 µl of PBS buffer containing 1 µl of monospecific antibodies against the unlabelled protein, pre-complexed with 10 µl of Dynabeads (Dynal, Oslo, Norway), were added, and the mixture was further incubated for 60 min while shaking. Subsequently, the protein A Dynabeads were washed five times with 500 µl of PBS buffer. [35S]-Labeled proteins bound to protein A beads were counted in scintillation liquid. Immunoprecipitation experiments were carried out at least three times.

Synthesis and Screening of the Cellulose-bound Peptide Arrays—Peptide arrays were prepared by automated spot synthesis using the AMS SPOT-robot (Abimed, Langenfeld, Germany) (49, 50). Before screening, the membranes were washed in methanol for 10 min, three times in TBS buffer (50 mM Tris, 137 mM NaCl, 27 mM KCl, pH 8.0), and subsequently incubated in blocking buffer (10% GENOSYS SU-07-250; 5% sucrose, TBS buffer (TBS with 0.05% Tween) for 3 h. After washing with TBS buffer peptide arrays were incubated with [35S]-labeled His6-TatA (50 ng/ml; 10,000 cpm/ml) in blocking buffer for 16 h at room temperature with gentle shaking. Unbound protein was washed out with TBS buffer. Amount of retained [35S]-labeled protein was quantified using phosphorimage analysis. Relative amounts of radioactivity were estimated by using a phosphorimag (Fuji) and associated image analytical software PC-BAS.

RESULTS

Immuno gold Labeling of TatA—the E. coli Tat system has the ability to transport folded proteins and enzyme complexes across the cytoplasmic membrane (22, 27). To transport these folded peptides would result in a translocation pore with a maximum diameter of 5 nm (51). We have shown that the TatA/Cp transport system of B. subtilis is able to transport PhoD with a molecular mass of 62.7 kDa, as well as a hybrid protein consisting of the signal peptide of PhoD and LacZ, resulting in a molecular mass of 120 kDa (31, 33), resulting in a necessarily similar sized translocation pore. Because freeze-fracture cytochemistry is known to be a powerful technique to study macromolecular architecture of biomembranes (46, 52) we used this method to investigate the cytoplasmic membrane of B. subtilis and E. coli containing TatA/Cp proteins that could form detectable structures. Immuno gold labeling of TatA with monospecific antibodies was carried out to localize TatA and to identify visible ultrastructures and TatA-containing translocation particles. Immuno gold labeling of TatA in freeze-fractured membranes of B. subtilis 168 cells grown under phosphate starvation revealed an even distribution of TatA in the cell envelope. Interesting, most immuno gold labels appeared to be clustered both at the protoplasmic (PF) and the exoplasmic side (EF) of the cytoplasmic membrane (Fig. 2, A and B). Absence of immuno gold labeling of freeze fractures of B. subtilis 168 wild type cells grown under phosphate replete conditions demonstrated specificity of labeling (Supplemental Fig. 1C). Because no labeling of gold-conjugated anti rabbit antibody could be observed in absence of primary antibodies, unspecific binding of the secondary antibody could be excluded (data not shown). To rule out that the monospecific TatA antibodies cross-reacted with another phosphate starvation-induced cytosolic protein, we compared the immunodetection of soluble B. subtilis proteins between cell extracts obtained from cells grown under phosphate replete and phosphate depleted conditions. No other proteins, except for TatA, could be detected in the Western blot (data not shown). Unexpectedly, a substantial amount of gold particles was localized at the cytosol indicating that B. subtilis TatA is not an exclusively membrane integrated protein (Fig. 2A). Artificial induction of the phosphate starvation response by using B. subtilis 168 phoR12 resulted in similar distribution of TatA except for a higher expression level of TatA (Supplemental Fig. 1, A, B, and D). Again clusters of TatA-labeled protein could be observed but were not linked to vesicle-like structures in the membrane (Supplemental Fig. 1A). To validate immuno gold labeling for protein localization, freeze-fractured cells were immunogold-labeled with antibodies against the chaperone DnaK and integral membrane protein SecY, a part of the Sec-translocase unit (3). As expected, the chaperone DnaK could be detected in the cytosol only (Supplemental Fig. 2B), and immuno gold labeling of SecY indicated that SecY was predominantly localized in the cytoplasmic membrane (Supplemental Fig. 2A).

Localization of TatA was further elucidated by immuno gold labeling of ultrathin sections of B. subtilis 168 cells grown under phosphate starvation. Again beside the expected membrane associated localization of the gold particles, about 50% could be detected in the cytosol of the cell (Fig. 3). As a control DnaK and SecY protein were immunogold-labeled in B. subtilis 168. Although DnaK was found in the cytosol, SecY was membrane-associated (Supplemental Fig. 3). To elucidate whether the localization of TatA was depending on the presence of prePhoD, we analyzed its localization in a B. subtilis phoD strain. Strain MH5444 deleted for phoD was transformed with plasmid pREP9tatA/Cp allowing the IPTG-
inducible synthesis of the TatA\textsubscript{d}/TatC\textsubscript{d} proteins. MH5444-(pREP\textsubscript{tatA\textsubscript{d}}/C\textsubscript{d}) was grown to phosphate starvation, expression of Tat proteins was induced, and localization of TatA\textsubscript{d} was detected using immunogold labeling of freeze-fracture cells essentially as described above. No TatA\textsubscript{d} could be detected in the cytosol of the cell (data not shown).

Because the PhoD-specific TatA\textsubscript{d}/TatC\textsubscript{d} translocation system has been demonstrated to be functionally active in \textit{E. coli} (33), we analyzed the localization of TatA\textsubscript{d} in \textit{E. coli}. Cells of \textit{E. coli} TG1(pREP4, pQE9\textsubscript{tatA\textsubscript{d}}/C\textsubscript{d}) expressing \textit{B. subtilis} TatA\textsubscript{d}/TatC\textsubscript{d} proteins were analyzed by freeze-fracture technique with subsequent immunogold labeling of TatA\textsubscript{d}. Like in \textit{B. subtilis}, immunologically detected TatA\textsubscript{d} was localized at the cytoplasmic membrane, as well as in the cytosol (Supplemental Fig. 4).

TatA\textsubscript{d} Is Localized in the Cytosolic Membrane and the Cytosol—The above results indicated that TatA\textsubscript{d} is not exclusively localized in the membrane. Immunological detection of TatA\textsubscript{d} in cytosolic cell fractions of \textit{B. subtilis} strains 168, GCH871, and TG1(pREP4, pQE9\textsubscript{tatA\textsubscript{d}}/C\textsubscript{d}) confirmed that a substantial amount of the protein was localized in the cytosol (Fig. 4). Absence of membrane proteins in the cytosolic fraction was monitored by detecting TatC\textsubscript{d}. No TatC\textsubscript{d} could be detected in the cytosolic fractions either of \textit{B. subtilis} strains or of \textit{E. coli} TG1(pREP4, pQE9\textsubscript{tatA\textsubscript{d}}/C\textsubscript{d}) (Fig. 4).

A kinetic study revealed that cytosolic localization of TatA\textsubscript{d} in phosphate-starved \textit{B. subtilis} 168\textsubscript{d} was variable. At the onset of phosphate starvation substantial amounts of TatA\textsubscript{d} was detected in the cytosol. Upon ongoing starvation cytosolic TatA\textsubscript{d} decreased (data not shown). The fraction of TatA\textsubscript{d} that was co-purified with membranes, resisted carbonate extraction, indicating that this protein is membrane-integrated (data not shown).

Co-purification of TatA\textsubscript{d} with prePhoD—\textit{In vivo} synthesized TAP epitope-tagged proteins have been successfully used to demonstrate the interaction between proteins in yeast, plants, and mammalian cells (53–55). To elucidate the affinity of TatA\textsubscript{d} for its substrate prePhoD, TAP-tagged TatA\textsubscript{d} was synthesized in \textit{B. subtilis}. Strains 168::pORI22-tatA\textsubscript{d}-TAP and 168::pORI22-tatC\textsubscript{d}-TAP were grown to phosphate starvation, and TAP peptides were subsequently purified from the cytosolic fraction using IgG-Sepharose beads (43). Both TatA\textsubscript{d}–TAP and TatA\textsubscript{d}–TAP peptides could be detected in the cytosolic fraction. Strain 168::pORI22-tatA\textsubscript{d}–TAP mediated the synthesis of the N-terminal methionine of TatA\textsubscript{d} with the TAP epitope (TatA\textsubscript{d}–TAP) showed inducible synthesis of a protein with the molecular weight of the TAP epitope (Fig. 5, lane 2). Strain 168::pORI22-tatA\textsubscript{d}–TAP produced a protein according to the molecular weight of TatA\textsubscript{d}–TAP. TatA\textsubscript{d}–TAP was detected by protein A (Fig. 5, lane 1), as well as TatA\textsubscript{d} antibodies (data not shown). IgG-purified TAP peptides were assayed for presence of PhoD. Although prePhoD was co-purified with TatA\textsubscript{d}–TAP, no PhoD could be co-purified with TatA\textsubscript{d}–TAP (Fig. 5). This result demonstrated the \textit{in situ} interaction of TatA\textsubscript{d} and prePhoD in the cytosol of the cell.

TatA\textsubscript{d} Has Affinity for prePhoD—After demonstrating interaction of TatA\textsubscript{d} with prePhoD in the cytosol, co-immunoprecipitation experiments were carried out to further investigate specificity of this interaction \textit{in vitro}. Complex formation of purified [\textsuperscript{35}S]-labeled His\textsubscript{c}-prePhoD (Table II) with purified His\textsubscript{c}-TatA\textsubscript{d} was assessed by immunoprecipitation with TatA\textsubscript{d}-specific antibodies. Taking into account that soluble TatA\textsubscript{d} forms high molecular weight homomultimers,\textsuperscript{2} 10−mM excess of TatA\textsubscript{d} was used to co-immunoprecipitate peptides. His\textsubscript{c}-prePhoD could be co-immunoprecipitated with His\textsubscript{c}-TatA\textsubscript{d}, whereas only low levels of His\textsubscript{c}-prePhoD were immunoprecipitated when either the TatA\textsubscript{d} or the TatA\textsubscript{d} antibodies were omitted from the mixture (Table II).

To analyze the specificity of TatA\textsubscript{d} we investigated affinity to

\textsuperscript{2} O. Pop and J. P. Müller, unpublished data.
The affinity to TatAd. Essentially similar results were obtained
when antibodies or TatAd were omitted, only weak interaction
was observed for the wild type-derived peptide (data not shown).

The cellulose-bound peptides were incubated with [35S]-labeled His6-TatAd.
TatAd showed selective affinity to peptides containing the twin-
arginine motif (Fig. 6A). Binding was most pronounced when
these residues were localized at the N-terminal flexible end of
the peptide and gradually decreased when they moved to the
C-terminal end of the peptides. Optimal affinity was observed
for the peptide 26 containing R27K28 at its N-terminal end.

Amounts of bound mature [35S]-His6-PhoD, to the precursor and mature
Sec-containing peptides were purified from the cytosolic fraction by
using IgG beads. Purified TAP proteins were separated via SDS-PAGE
and subsequently detected by using Western blotting with monospecific
antibodies against protein A and PhoD. Bands representing TatA-
TAP, TatA'-TAP (TAP), IgG, and prePhoD are indicated.

**Table II**

| His8 protein | TatAd | pAb TatAd | Bound [35S]protein |
|--------------|-------|-----------|--------------------|
| prePhoD      | +     | +         | 58.6               |
| PhoD         | +     | +         | 13.0               |
| preYvaY      | +     | +         | 21.0               |
| YvaY         | +     | +         | 25.0               |
| preYvaY      | +     | +         | 3.3                |
| CopR         | +     | +         | 12.0               |
| CopR         | +     | +         | 8.5                |
| CopR         | +     | +         | 2.9                |
| CopR         | +     | +         | 17.7               |
| CopR         | +     | +         | 12.9               |
| CopR         | +     | +         | 2.9                |
| CopR         | +     | +         | 23.4               |

*pAb, polyclonal antibodies.

Radiolabeled mature PhoD, to the precursor and mature Sec-
dependent *B. subtilis* protein YvaY (56), as well as CopR, a
cytoplasmic protein involved in replication control of plasmids
(57). His8-tagged proteins were purified as abundant proteins
(Supplemental Fig. 5). As shown in Table II only 25% of mature
His8-PhoD could be co-immunoprecipitated by His8-TatAd
compared with 58% of His8-prePhoD (Table II). Because the
amount of bound mature [35S]-His8-PhoD was hardly higher
when antibodies or TatAd were omitted, only weak interaction
can be concluded. Amounts of co-immunoprecipitated [35S]-
labeled His8-preYvaY, His8-YvaY, and His8-CopR fairly pro-
tрудed the level of bound protein when TatAd or TatAd anti-
body was omitted, demonstrating no interaction with Sec-
dependent or cytoplasmic proteins. These data indicate that the
signal peptide of PhoD contains specific information mediating
the affinity to TatAd. Essentially similar results were obtained
when octylglucoside was omitted from the reactions.

**TatA Specifically Binds to Twin-arginine Peptides of the Signal Peptide of PhoD**—Cellulose-bound peptide arrays have been used successfully to characterize substrate binding motifs
of proteins (58). To determine the sequence-specific information
necessary for binding of TatAd to prePhoD, we screened a
cellulose-bound peptide scan of the N-terminal region of pre-
PhoD for TatAd binding. The peptide scan was composed of
20-mer peptides that overlap by 19 residues over the sequence
of 60 N-terminal localized amino acids and covering the 56-
amino acid residue-long signal peptide of PhoD. The cellulose-
bound peptides were incubated with [35S]-labeled His8-TatAd.
TatAd showed selective affinity to peptides containing the twin-
arginine motif (Fig. 6A). Binding was most pronounced when
these residues were localized at the N-terminal flexible end of
the peptide and gradually decreased when they moved to the
C-terminal end of the peptides. Optimal affinity was observed
for the peptide 26 containing R27K28 at its N-terminal end.

Amounts of bound prePhoD was quantified by counting in scintillation
liquid. Bound prePhoD is indicated in percent of input. Data were
obtained from a representative experiment.

Several components with one or six calculated membrane
spanning domains have been described to mediate the trans-
port of twin-arginine signal peptide-containing proteins (22).
Therefore, Tat-dependent protein export is believed to be inde-
pendent of soluble cytosolic factors (60). In the present study
we demonstrate that a substantial fraction of TatAd protein of
the PhoD-specific *B. subtilis* translocation system can be found
beside its expected membrane-integrated localization in the
cytosol. Soluble TatAd was functional active as demonstrated
by its affinity to its substrate prePhoD and sequence-specific
interaction with twin-arginine containing peptides. Therefore,
we currently favor the thesis that TatAd on its way to the
cytosolic membrane could fulfill a function as targeting
factor for Tat substrate prePhoD.
We have shown previously (33) that the transport of PhoD can be mediated by only two Tat proteins, one similar to TatA and one similar to TatC of *E. coli*. To investigate the function of TatA proteins further, we studied the distribution of the TatAd protein in *B. subtilis* and in *E. coli* by using freeze-fracture technique and subsequent immunogold labeling. In *B. subtilis*, membrane-bound TatAd was equally distributed at both sides of the freeze-fractured cytoplasmic membrane. Immunogold-labeled protein was found to be aggregated with proteinaceous structures with a size of less than 5 nm. Absence of larger structures stimulates the hypothesis that the transport of proteins is independent of vesicle formation, which would be visible in the freeze-fractured membranes (22). Freeze-fractured membranes of *E. coli* cells overexpressing TatAd/TatCd demonstrated the uniform distribution of TatAd over the surface of the cell envelope.

In addition to its expected membrane-associated localization, freeze fractures through the cytosol indicated that a substantial amount of TatAd was localized in the cytosol in both bacterial systems. Ultrathin sections of *B. subtilis* cells confirmed an abundant localization of TatAd in the cytosol. Immunogold labeling of reference proteins demonstrated the reliability of both freeze-fracture analysis and labeling of ultrathin sections.

The cytosolic chaperone DnaK could be detected in the cytosol, and SecY was detected predominately in the cytoplasmic membrane. Cytosolic localization of TatAd was first observed in *E. coli* TG1(pREP4, pQE9 tatAd) cells overexpressing His6-TatAd. An abundant amount of His6-TatAd was soluble after sonication of the cells under native buffer conditions (data not shown). This unexpected observation was of crucial interest, because soluble TatAd is functional as it could be demonstrated to bind prePhoD and prePhoD-derived peptides specifically. Identification of TatAd in membrane-free cell extracts of *B. subtilis* confirmed the cytosolic localization of TatAd. Absence of TatC in the cytosolic fractions of *B. subtilis*, as well as *E. coli*, demonstrated absence of membrane proteins in the cytosolic fraction. Interestingly, the amount of soluble TatAd varied in dependence of the induction time of the phoD operon. Because the amount of cytosolic TatAd decreased during prolonged phosphate starvation, presence of soluble TatAd might...
depend on the availability of newly synthesized prePhoD substrate to be targeted to the membrane. Absence of cytosolic TatA\textsubscript{d} in a strain not producing prePhoD confirmed this thesis. Thus, transient presence of cytosolic homomultimeric TatA\textsubscript{d} is linked to its substrate prePhoD. In addition, this observation points out that TatA\textsubscript{d} interacts specifically with prePhoD.

Co-immunoprecipitation demonstrated preferred binding of TatA\textsubscript{d} to prePhoD. The weak co-purification of mature PhoD indicated that secondary, but obviously less important binding sites, might be present in the mature part of the protein. The Sec-dependent transported \textit{B. subtilis} protein preVyaY, mature YvaY, or cytosolic CopR were not recognized by TatA\textsubscript{d}. It can therefore be concluded that the twin-arginine signal peptide is the preferred binding site of TatA\textsubscript{d}. At the moment we can not quantify the stoichiometric ratio of the TatA\textsubscript{d}-prePhoD complexes. Gel filtration of purified TatA\textsubscript{d} indicated that the soluble protein forms complexes bigger than 100 kDa indicating that functional TatA\textsubscript{d} acts as a homomultimeric protein.\textsuperscript{3}

Affinity of TatA\textsubscript{d} to a 20-mer peptide library of the N-terminal region of PhoD elucidated sequence specificity of TatA\textsubscript{d} recognition. Selective binding to peptides containing the twin-arginine motif could be observed. The different binding pattern of SecB to the peptide library demonstrated that binding was motif-specific. Recognition of the sequence motif was position-dependent. Localization of the motif at the flexible N-terminal end resulted in stronger TatA\textsubscript{d} peptide interaction and gradually decreased when the motif moved to the C-terminal attachment site of the peptide. Accessibility of the recognition motif for TatA\textsubscript{d}, especially because we observed that TatA\textsubscript{d} forms large homomultimeric complexes, might be sterically hampered if it is localized close to the inflexible C terminus of the peptide. Therefore, preferred binding of peptides with N-terminal localized RRK can be explained. The use of 20-mer peptides might underestimate the role of the secondary and tertiary structures of the signal peptide of PhoD for TatA\textsubscript{d} recognition. Still, selective binding to twin-arginine motif-containing peptides indicated that the twin-arginine motif is the target site recognized by TatA\textsubscript{d} and could play, in general, an essential role for the interaction of the Tat-translocase component TatA with its substrates. This result is consistent with the observation that TatA\textsubscript{d} binds preferably prePhoD, but less efficiently the mature PhoD as shown by co-immunoprecipitation.

Additional evidence about the role of particular amino acids for TatA\textsubscript{d} binding was obtained by substitution of 10 N-terminal amino acid residues of peptide DRRKFlQGAGKi-AglslglT\textsubscript{5–41} localized in the signal peptide of PhoD. Substitution of the twin-arginine motif unambiguously demonstrated the essence of these residues for TatA\textsubscript{d} recognition. Beside substitution of the arginine residues, replacement of the lysine residues had similar consequences for TatA\textsubscript{d} binding. Therefore, it can be speculated that this third basic residue belongs to the recognition motif of TatA\textsubscript{d}. Surprisingly, binding of TatA\textsubscript{d} to peptides with altered RRK cluster was mainly charge-dependent. Although conservative substitution hardly reduced binding, uncharged amino acids reduced and acidic amino acid residues abolished TatA\textsubscript{d} binding. Remarkably, variation of amino acids localized C-terminal or N-terminal of the RRK cluster had only inferior effects on TatA\textsubscript{d} binding indicating that these amino acid residues are not essentially involved in the TatA\textsubscript{d} recognition. Based on these data we tend to speculate that RRK\textsubscript{26–29} is involved in the TatA\textsubscript{d} recognition motif. This indicates that the proposed conserved (S/T)RRX-FLK sequence motif of the Tat signal peptides (27) might be involved in recognition of other Tat components. Moreover, it cannot be excluded that TatA\textsubscript{d}, functionally active in the cytosol of the \textit{B. subtilis} cell, is acting different from TatA proteins of other organisms and therefore shares different recognition specificities. Absence of a TatB-like protein indicates that the Tat export in \textit{B. subtilis} might be functionally different from \textit{E. coli}. Most of the bacterial and plant Tat signal peptides studied demonstrated that both arginine residues of the consensus motif were critically important for the Tat transport (7, 13, 61–64). However, it has been demonstrated recently that a single lysine substitutions for arginine either naturally occurring (65) or replaced experimentally (61) were still transported in a Tat-dependent manner. Our data confirm that one of the arginine residues and, in addition, the lysine residue can be substituted by another positively charged amino acid residue and will still be recognized by TatA\textsubscript{d}. The physiological relevance of this observation is currently being studied in more detail.

Interference of twin-arginine peptides with Tat substrates for interaction with Tat components has been first demonstrated in an \textit{in vitro} translocation system by Alami and co-workers (66). We demonstrated that co-immunoprecipitation of prePhoD by TatA\textsubscript{d} was interfered by addition of a peptide containing the RRK motif. Substitution of this motif by alanine residues resulted in a far lesser extent of this interference. These data confirmed that interaction of soluble peptides is sequence-specific, and co-immunoprecipitation experiments reflect in \textit{vivo} function of TatA\textsubscript{d}. The discrepancy between the efficient interference of peptide QNNTFRRKFLQKAGKI in the co-purification of prePhoD by TatA\textsubscript{d} and the inefficient recognition in the cellulose-bound peptide library can be explained by a higher flexibility of soluble peptide.

Despite the fact that TatA has an unusual and not very hydrophobic predicted structure in which only the extreme N terminus has the potential to form a classic hydrophobic transmembrane helix, TatA\textsubscript{d} of \textit{E. coli} has been found membrane-associated only (67, 68). Tha\textsubscript{4}, the plant TatA orthologue, was localized entirely in the membrane of thylakoids (51, 69). Other proteinaceous factors having affinity to TatA\textsubscript{d} proteins could possibly mediate the targeting of selected substrates (70). \textit{In vitro} translocation systems established for the \textit{E. coli} Tat system demonstrated that no soluble factors are necessary to obtain protein translocation into inverted membrane vesicles (66, 71). Vesicle-based transport systems might underestimate the role of peptide-mediated targeting. In addition, because Tat substrates fold prior to translocation, the necessity for a fast targeting process to maintain export competence might be reduced compared with the Sec translocation system. Cytosolic localization of TatA\textsubscript{d} could reflect that Tat translocation in \textit{B. subtilis} acts functionally different from other systems. Although soluble TatA\textsubscript{d} could mediate targeting of newly synthesized prePhoD to the translocation site, membrane-integrated TatA\textsubscript{d} could be involved in the translocation process. Structural and functional data of homomultimeric TatA\textsubscript{d} complexes present in the cytosol, as well as in the membrane,\textsuperscript{3} will help to uncover the relevance of the dual localization of TatA\textsubscript{d}.

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\textsuperscript{3} M. Westermann, unpublished data.
