Structure-Function Analysis of PrsA Reveals Roles for the Parvulin-like and Flanking N- and C-terminal Domains in Protein Folding and Secretion in Bacillus subtilis*

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The PrsA protein of Bacillus subtilis is an essential membrane-bound lipoprotein that is assumed to assist post-translational folding of exported proteins and stabilize them in the compartment between the cytoplasmic membrane and cell wall. This folding activity is consistent with the homology of a segment of PrsA with parvulin-type peptidyl-prolyl cis/trans isomerases (PPIases). In this study, molecular modeling showed that the parvulin-like region can adopt a parvulin-type fold with structurally conserved active site residues. PrsA exhibits PPIase activity in a manner dependent on the parvulin-like domain. We constructed deletion, peptide insertion, and amino acid substitution mutations and demonstrated that the parvulin-like domain as well as flanking N- and C-terminal domains are essential for in vivo PrsA function in protein secretion and growth. Surprisingly, none of the predicted active site residues of the parvulin-like domain was essential for growth and protein secretion, although several active site mutations reduced or abolished the PPIase activity or the ability of PrsA to catalyze proline-limited protein folding in vitro. Our results indicate that PrsA is a PPIase, but the essential role in vivo seems to depend on some non-PPIase activity of both the parvulin-like and flanking domains.

The rate-limiting steps in the folding of proteins containing cis-prolines are catalyzed by peptidyl prolyl cis/trans isomerases (PPIases).1 These ubiquitous proteins can be divided into three families, cyclophilins, FK506-binding proteins (FKBPs), and parvulins (1). Because cyclophilins and FKBPs are targets of immunosuppressive drugs, they are called immunophilins. No specific inhibitor is available for parvulins. Although PPIases catalyze the isomerization of prolyl peptide bonds in vitro, the physiological role of many of these proteins is still unclear. The effects of their mutational inactivation vary from no nontypical effect (2) to severe defects (3–6). PPIases may also act as chaperones either in a PPIase domain-dependent (7) or -independent (8) manner, and they may have essential overlapping functions with other PPIases and chaperones (4, 9, 10).

Members of the parvulin family have been found in all types of cells. The prototype member of the family is the parvulin of Escherichia coli (11). It is a small cytoplasmic protein comprising essentially a PPIase domain only. In other members a homologous PPIase domain is flanked by N- and C-terminal domains of various lengths (12). At least three subfamilies can be identified based on structural and functional differences. Pin1-type parvulins consist of an N-terminal WW domain followed by a PPIase domain. The plant members, however, lack the WW domain (12, 13). The crystal structure of the human Pin1 protein has been solved, and the PPIase domain has been shown to consist of a four-stranded, anti-parallel, flattened β-barrel that is surrounded by four α-helices (βαααβαβ) (13). The Pin1 protein binds specifically to a phosphorylated serine or threonine residue located N-terminal to proline. The WW domain, characteristically a module binding proline-rich sequence, and the PPIase domain are both involved in the binding of phosphorylated residues (14). The phosphate group is recognized by a conserved cluster of three positively charged residues located in the N terminus of the loop preceding α1 (13, 15). PPIases of this subfamily are involved in the regulation of mitosis (15), and linked to chromatin remodeling and general transcriptional regulation (5). In the second subfamily the PPIase domain is similar to that of hPar14; the α1-helix and the

1 The abbreviations used are: PPIase, peptidyl-prolyl cis/trans isomerases; FKBPs, FK506-binding proteins; AmyQ, α-amylase of B. amyloliquefaciens; PDB, Protein Data Bank; IPTG, isopropyl-1-thio-β-D-galactopyranoside; MES, 4-morpholineethanesulfonic acid; GST, glutathione S-transferase; WT, wild type; CsA, cyclosporin A.

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loop that precedes it are several residues shorter than in Pin1 (16). Furthermore, these enzymes have an insertion in the loop between α4 and β3. The biological function of hPar14-type PPIases is unknown. In the third subfamily, the PPIase domain is similar to that of the E. coli parvulin. It lacks the hPar14-type insertion and the Pin1-type cluster of positively charged residues involved in the binding of phosphoserine and -threonine. Many PPIases of this subfamily are bacterial proteins required for the folding and/or maturation of extracytoplasmic proteins (16, 17). The periplasmic SuraA and PspD, which have roles in the assembly of outer membrane proteins (4), belong to this subfamily.

We have previously identified in *Bacillus subtilis* a membrane-bound lipoprotein, PrsA, which according to sequence similarity belongs to the parvulin family of PPIases (18). In PrsA a stretch of 90 amino acids is 45% identical with *E. coli* parvulin. Furthermore significant homology is observed with parvulin-like or PPIase domains of all members of the parvulin family. This parvulin-like region is flanked by a 115- and 70-residue long N- and C-terminal domain, respectively, which are not homologous to any regions of other parvinus except for those of PrsA-like proteins of species related to *B. subtilis*. So far it is not known whether the parvulin-like region and the flanking regions form separate domains. The first amino acid residue at the N terminus of the mature PrsA is a cysteine, which is covalently linked to a diacylglycerol molecule that anchors the protein to the membrane. It has been suggested that PrsA catalyzes or assists the post-translational folding of extracytoplasmic proteins at the cytoplasmic membrane-cell wall interface (19, 20). The secretion rate of AmyQ-amylase, a model protein used in many of our studies, is linearly dependent on the cellular level of PrsA (6). PrsA is an essential protein in *B. subtilis*, because decreasing its cellular level below 200 molecules per cell results in cell lysis (6). The lethal effect is partially suppressed by the increased negative charge density of the cell wall matrix (21). A defect in PrsA results in the accumulation of misfolded AmyQ at the membrane-wall interface (19). The accumulation of misfolded protein in the cell envelope activates the CsdS/R two-component signal transduction pathway and consequently the synthesis of HtrA-type envelope activator, which is covalently linked to a diacylglycerol molecule that activates the CssS/R two-component signal transduction pathway and consequently the synthesis of HtrA-type envelope activator (22). The secretion rate of AmyQ at the cell wall interface (23) was used to construct the pKrH3327 gene was PCR-amplified with oligonucleotides 8874 and 8875 containing the ribosome-binding site and 444, 663, 780, 828, or 873 base pairs of the encoding region and fused using the SOE method. The resulting *prsA*mutant fragment was inserted into the unique HindIII site in the pDG148 vector. The resulting PrsA derivatives encoding truncated PrsA proteins, RNA fragments containing the ribosome-binding site and 444, 663, 780, 828, or 873 base pairs of the encoding region were PCR-amplified and cloned in pDG148. The constructions were verified by sequencing.

The insertion mutagenesis was carried out as follows. First, the *B. subtilis* pKrH3327 gene was PCR-amplified with oligonucleotides 8874 (5′-CACATCGTAAAGAATTGATGTA-3′) and 8875 (5′-CAAGTCG-AAGACCTCAGCAAGACTG-3′) using the chromosomal DNA of *B. subtilis* as a template. The resultant fragment, which contains the ribosome-binding site, the C-terminal domain, and the transcription termination site of *prsA*, was cloned into the pGemT-easy (Stratagene) vector. The plasmid obtained was mutagenized in vitro with the cat-Mu (NotI) transposon (28) using the Mutagenesis Generation System (Finzymes) as essentially described (28). A pool of mutated plasmids was transformed into *E. coli* cells and subjected to a series of manipulations including recloning of the transposon containing *prsA* genes into pUC19 (24) and elimination of the resistance marker gene (cat) by NotI digestion and religation. The protocol produced a pool of about 35,000 *prsA* mutants. Next, the *prsA* genes, which contained 15-bp insertions, were again PCR-amplified using the plasmid pool as a template. The PCR primers contained the same homologous region as 8874 and 8875 oligonucleotides. The PCR amplimers were inserted into HindIII and SaI sites of the pDG148 plasmid, placing the *prsA* genes under the transcriptional control of the P selv promoter. The recombinant plasmids were transformed into *E. coli* C600 cells (24). Plasmids from 75 transformants were isolated and subjected to HindIII-NotI and SaI-NotI double digestions to identify plasmids that contain only a single 15-bp random insert in the *prsA* gene. The total of 49 plasmids fulfilled this criterion. The exact location of 31 insertions in these plasmids was determined by sequencing.

**Complementation of prsA3 and Lethality of PrsA Depletion—** Complementation of prsA3 was examined by determining AmyQ secretion with a halo assay as previously described (29). One bacterial colony was inoculated on a plate in 0.5 ml of water and incubated for 24 h on LB-agar plates containing 3% starch with or without 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The plates were incubated for 16 h at 37°C followed by staining with iodine. The diameters of the halos around raised bacterial colonies were measured. The complementation
of prsA with the PrsA variants containing site-directed mutations were also determined in liquid cultures. α-Amylase secretion was determined with the Phadebas amylase test (Amersham Biosciences) as described previously (21).

To investigate the complementation of the lethality of PrsA deletion, the B. subtilis IH7211 (P<sub>acs</sub>-prsA) was transformed with pDG148 derivatives containing mutant prsA genes. In the resultant strains, the chromosomal wild-type prsA gene and a plasmid-carrier of the mutant prsA gene were both under the control of the P<sub>acs</sub> promoter. However, because the P<sub>acs</sub> in the plasmids is leaky and that in the chromosome is tight, we were able to determine the complementation. Cells grown to the density of Klett 100 in the presence of 1 mM IPTG were harvested and washed once with L-broth, diluted 1000-fold in 2× L-broth, and then cultivated for 10 h. Growth was measured with a Klett-Densometer.

**Purification of Lipomodified and Nonlipomodified PrsA Proteins**

For the purification of lipomodified PrsA proteins, B. subtilis cells were grown in buffered tryptone broth (1% tryptone, 100 mM potassium phosphate, pH 7.5, 2% glucose, 1 mM MgSO<sub>4</sub>, 3.5 mM FeSO<sub>4</sub>, 3.5 mM ZnSO<sub>4</sub>, 0.45 mM MnSO<sub>4</sub>, 40 mM CuSO<sub>4</sub>, and 7 mM Cr(OH)<sub>3</sub>). In the early stationary phase, the cells were harvested, treated with lysozyme (4 mg/ml) for 30 min, and then sonicated until the viscosity disappeared. The cell membranes were isolated by centrifugation at 44,000 × g for 1 h 15 min and solubilized in 2% n-octyl-β-D-glucopyranoside (OG) (Sigma-Aldrich). Insoluble material was removed by centrifugation, and the supernatant was passed through a DEAE-Sepharose CL-6B (Amersham Biosciences) column equilibrated with a buffer containing 10 mM Tris-HCl, pH 7.5, and 30 mM NaCl. The flow-through fraction containing PrsA was applied to a CM-Sepharose CL-6B (Amersham Biosciences) column equilibrated with a buffer containing 30 mM MES, pH 6.0, and 30 mM NaCl, and PrsA was eluted with 300 mM NaCl. From the solubilization step onward, all the solutions contained 1% OG.

In order to produce PrsA in secreted form, the type II signal peptide anchoring PrsA to the membrane was replaced with the type I signal peptide of AmyQ. The PrsA-secretting strain was grown in Terrific Broth (17 mM KH<sub>2</sub>PO<sub>4</sub>, 72 mM K<sub>2</sub>HPO<sub>4</sub>, 24 g liter<sup>−1</sup> yeast extract, 12 g liter<sup>−1</sup> tryptone, and 0.4% glucose) containing 5% glycerol at 37 °C to Klett 100 + 2 h. The cells were harvested by centrifugation for 1 h at 30 °C, and the cells were harvested by centrifugation for 2 h at 4°C. The supernatant was passed through a DEAE-Sepharose CL-6B column equilibrated with 0.1 M NaCl. The refolding was initiated by a 60-fold dilution with the refolding buffer (50 mM Tris-HCl, pH 7.8, 10 mM dithiothreitol, and 50 mM sodium thiocyanate) in the absence or presence of folding factors. In experiments with GroEL, 1 mM MgCl<sub>2</sub>, 10 mM KCl, and 1 mM ATP were added to the refolding buffer. Over 60 min of incubation, aliquots were withdrawn at several time points. Rhodanese activity of the refolding reaction samples was measured as described in Ref. 33. PrsA preparation was 5% GroE (2.5 μM) and bovine serum albumin (15 μM) were used as positive and negative controls, respectively.

**Extracellular Protease Activity**

**Production and Purification of GST-PrsA Fusion Proteins**

The GST gene fusion system was used to produce PrsA proteins in E. coli for purification. Cells were grown in 2× TY (1.6% tryptone, 1% yeast extract, 1% NaCl) to Klett 50–70, and 0.2–1 mM IPTG was added to induce the synthesis of GST-PrsA fusion proteins from pGEX-2T (Amersham Biosciences) derivatives containing prsA genes. After incubation for 2 h at 30 °C, the cells were harvested by centrifugation, suspended in phosphate-buffered saline, and disrupted with a French Press. Cell debris was removed by centrifugation prior to applying the cell lysate into a glutathione-Sepharose 4 fast flow (Amersham Biosciences) and applied to a Sepharose XL cation-exchange column (Amersham Biosciences) equilibrated with 30 mM MES, pH 6.0. PrsA bound to the column was eluted with 500 mM NaCl.

**Ribonuclease T1 Refolding and Protease-coupled PPIase Assays**

The refolding was assayed by ribonuclease T1 (RNase) essentially as has been previously described (30). The RNase was purchased from Sigma-Aldrich. Centricon 3 filters (Amicon) were used to remove the ammonium sulfate of the RNase solution. To the RNase, it was incubated at 20 μM concentration in a buffer containing 100 mM Tris-HCl, pH 7.8, 8 μM urea, and 1 mM EDTA for 1 h at room temperature. The refolding was initiated by diluting the solution 15-fold with a buffer containing 100 mM Tris-HCl, pH 7.8, and 1 mM EDTA (the tryptophan fluorescence was measured at 320 nm (5-nm bandwidth) with excitation at 288 nm (3-nm bandwidth) using a Shimadzu RF-5000 or Varian Cary Eclipse spectrofluorometer). PrsA was added prior to the dilution at the final concentrations of 1–8 μM. When the activity of lipomodified PrsA lipins was determined, 10% octylglucoside was added to the refolding buffer. The RNase refolding activity of nonlipomodified and lipomodified PrsA was measured at 10 and 12 °C, respectively. Cyclosporin A (CSA, Sigma-Aldrich) and FK506 (Calbiochem) were used at 5 μM concentrations to inhibit PPIase activity of putative contaminating cyclophilins and FKBP-type PPIases in the PrsA preparations, respectively. Juglone (Sigma-Aldrich), which is an inhibitor of numerous parvulins, was used at 7 μM concentrations as described (31). Cyclophilin, which was used as a positive control, was from calf thymus and was purchased from Sigma-Aldrich. PPIase activities were expressed as k<sub>p</sub>/K<sub>m</sub> (m<sup>−1</sup> s<sup>−1</sup>). A protease-coupled assay with α-chymotrypsin was used to determine PPIase activities with a tetrapeptide substrate (succinyl-Ala-Lys-Pro-Phe-4-nitroanilide) (Fischer et al., Ref. 40). The peptide concentration was 37.5 μM.

Refolding of Chymotrypsin—Unfolding and refolding of rhodanese was carried out essentially as previously described (32). Rhodanese (Sigma Aldrich) was incubated at 38 μM concentration in a buffer containing 50 mM Tris-HCl, pH 7.8, 6 mM GdnHCl, and 5 mM ZnCl<sub>2</sub> at 20 °C for 1 h. The refolding was initiated by a 60-fold dilution with the refolding buffer (50 mM Tris-HCl, pH 7.8, 10 mM dithiothreitol, and 50 mM sodium thiocyanate) in the absence or presence of folding factors. In experiments with GroEL, 1 mM MgCl<sub>2</sub>, 10 mM KCl, and 1 mM ATP were added to the refolding buffer. Over 60 min of incubation, aliquots were withdrawn at several time points. Rhodanese activity of the refolding reaction samples was measured as described in Ref. 33. PrsA concentration was 5 μM. GroELS (2.5 μM) and bovine serum albumin (15 μM) were used as positive and negative controls, respectively.

RESULTS

**The N-terminal, C-terminal, and Parvulin-like Regions Are All Essential for PrsA Function in Vivo**—Many parvulins consist of functionally and structurally independent domains (8, 13, 16, 35). In order to identify which regions (or domains) of the PrsA protein are required for protein secretion and viability in B. subtilis, a series of deletion mutations of prsA were constructed, and the mutations were characterized for their effects on in vivo PrsA activity (Fig. 1). The constructed dele-

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duction of the C-terminal region either deleted the whole region (PrsA-(1–202)), or part of it (PrsA-(1–256) and PrsA-(1–242)). We also constructed mutations that deleted the entire parvulin-like region (PrsA-(N+C)) or both the C-terminal and parvulin-like region (PrsA-(1–128)). Furthermore, PrsA-(33–258) molecules were designed that lacked the 32 N-terminal and 15 C-terminal amino acids. All mutant genes were expressed from an IPTG-inducible promoter in a multicopy plasmid.

All mutant PrsA proteins were detected as abundant pro-

teins by immunoblotting, indicating their in vivo stability. The cellular location of two proteins, PrsA-(1–242) and PrsA-(N+C), was studied by trypsin accessibility on the surface of B. subtilis. All PrsA as well as GroEL were degraded. These results indicate that the mutant PrsA proteins translocate across the cell membrane and become surface-exposed in a manner similar to the wild-type PrsA.

The in vivo activities of the mutant PrsA proteins were determined by two independent complementation assays.
First, we determined their ability to restore AmyQ secretion in the secretion-deficient prsA3 (D249N) mutant. Second, the ability of plasmid-coded mutant PrsA proteins to restore the growth of cells depleted of the chromosomally coded wild-type PrsA was studied. For the PrsA depletion, a strain in which the growth of cells depleted of the chromosomally coded wild-type PrsA is functional (Fig. 1). This indicates that the serine/threonine tail is dispensable. Deletions about half of the C-terminal region (PrsA-(1–242)) inactivated both functions of PrsA (Fig. 1), implying that the C-terminal region is essential for the PrsA function in vivo. Consistently, the truncation of the whole C-terminal region (PrsA-(1–202)) or both the C- and parvulin-like region (PrsA-(1–128)) resulted in inactive PrsA proteins. A mutant PrsA containing the N- and C-terminal regions but lacking the parvulin-like region (PrsA-(N+C)) was also unable to restore the growth of PrsA-depleted cells (Fig. 1). This mutant PrsA displayed, however, a weak activity in the AmyQ secretion assay, about 15% of the wild-type level. A short N-terminal deletion (PrsA-(33–258)) also fully inactivated PrsA.

The deletion mutant PrsA proteins were stable in vivo as revealed by immunoblotting, unlike other PrsA mutant proteins, such as PrsA3, which is largely degraded by cell membrane- and wall-associated quality control proteases (19). The finding suggests that they have a native-like fold and folding kinetics. The integrity of the native-like fold of PrsA(N+C) was also confirmed by circular dichroism spectroscopy (data not shown).

We also characterized PrsA by insertion mutagenesis. The aim was to obtain a large set of in-frame insertions of five amino acids at diverse positions in PrsA and to analyze the effects of the insertions on AmyQ secretion and cell viability in a manner similar to those outlined above. The mutagenesis was carried out using the in vitro strategy that is based on Mu transposition (28), and properties of 31 insertion mutants were characterized (Table I). The cellular level of all except one mutant PrsA protein remained unaffected (Table I), suggesting that the inserted pentapeptides did not significantly alter the PrsA fold. One mutant PrsA was truncated probably because of proteolytic degradation.

Numerous pentapeptide insertions in the N-terminal region (residues 1–128) either inactivated PrsA or deteriorated its activity severely (Table I). The effects of the insertions were comparable for both AmyQ secretion and viability. It is also notable that the insertions close to the N terminus of PrsA more severely affected the PrsA function than those close to the parvulin-like region. Insertion mutation 36 (Table I), which introduced a pentapeptide after Gin25 in the N-terminal domain, showed a surprising phenotype. The synthesis of PrsA36 in the prsA3 mutant caused a retardation in growth; the cells were aberrant and grew in tiny colonies on agar plates. No growth was observed when cells from the tiny colonies were replated on fresh plates. In contrast, in the wild-type strain, PrsA36 was not harmful to growth. Thus, it seems that the PrsA36 protein is synthetic lethal in the prsA3 mutant. The PrsA36 protein was also unable to rescue the growth of cells depleted of the wild-type PrsA (Table I), indicating that it is an inactive PrsA protein. The correct cellular location of two inactive insertion mutants, PrsA5 and PrsA113, was verified by trypsin accessibility using the method described above (data not shown). Furthermore, the signal peptide of all insertion mutant PrsA variants was cleaved off as revealed by immunoblotting (not shown), also suggesting that these mutant proteins were translocated across the cell membrane, lipomodified, and located at the cell membrane-wall interface in a manner similar to the wild-type PrsA.

In the parvulin-like and C-terminal regions pentapeptide insertions were mostly tolerated. However, two mutations (54 and 113) in the parvulin-like domain also fully disrupted the activity of PrsA in both the AmyQ secretion and viability assays (Table I). Furthermore, it was found that many mutants with an insertion in the parvulin-like or C-terminal region exhibited distinctly different activities in the two assays. None of the pentapeptide insertions apart from 54 and 113 conferred an effect on the complementation of the secretion defect in prsA3, but reduced by about half the activity in the viability assay.

**Molecular Modeling of the PPIase Domain of B. subtilis PrsA**—The structure-function analysis of PrsA was continued by modeling the three-dimensional structure of the parvulin-

| Complementation | prsA3 (AmyQ secretion) | PprA-prsA (Viability) |
|-----------------|------------------------|----------------------|
| PrsA1-273 (wt)  | N                      | Parvulin             | C                      |
| PrsA1-256       |                        |                      |                       |
| PrsA1-242       |                        |                      |                       |
| PrsA1-202       |                        |                      |                       |
| PrsA1-128       |                        |                      |                       |
| PrsA(N+C)       |                        |                      |                       |
| PrsA33-258      | + (15%)                |                      |                       |

**Fig. 1**. The ability of various deletion mutants of PrsA to restore AmyQ secretion in the prsA3 secretion mutant and rescue cells depleted of the wild-type PrsA from lethality. The schematic diagram shows the N-terminal domain (dark gray), the parvulin-like domain (gray), and the C-terminal domain (light gray). Full complementation (++) or, no complementation (--), and partial complementation (percent of full complementation) are shown. Because PrsA-(33–258) is a nonlipoprotein, unlike the other PrsA proteins, its complementation activity was compared with a nonlipomodified form of PrsA lacking only the N-terminal cysteine residue (PrsA-(2–273)). Although PrsA-(2–273) is secreted into the culture medium, its activity in the complementation assays was comparable to that of the lipomodified PrsA-(1–273).
like domain of *B. subtilis* PrsA, taking advantage of the known structure of a human parvin, hPar14 (16, 35). The parvin-like domain of PrsA, which contains 45% identical residues with hPar14, could be modeled to a typical parvin-type PPIase structure (Fig. 2). In the Pin1 structure, the peptidyl-prolyl bond isomerization is thought to be catalyzed by a cysteine, two histidines, and a serine (13). Both of the histidine residues are conserved in the PrsA structure, but aspartic acid and tyrosine substitute for the cysteine and serine, respectively. The hydrophobic pocket for the substrate proline is formed in both hPar14 and Pin1 by three amino acids, leucine, methionine, and phenylalanine (13, 16, 35). According to the model, all the proline residues (37, 38). The PPIase activities of both the lipomodified and nonlipomodified PrsA were examined. The nonlipomodified PrsA does not contain the N-terminal diacylglycerol moiety. Cyclophilin was used as a positive control.

As shown in Fig. 3A, PrsA indeed catalyzed the proline-limited folding in a concentration-dependent manner. The *k_cat/K_m* values were 143 mM\(^{-1}\) s\(^{-1}\) and 25 mM\(^{-1}\) s\(^{-1}\) for the lipomodified and nonlipomodified PrsA proteins, respectively (Table II). The catalytic activity of the nonlipomodified PrsA was the same whether it was produced in *E. coli* or *B. subtilis* (Table II). The catalytic activity of the nonlipomodified PrsA fusion protein or a secreted form (see "Materials and Methods") was the same whether it was produced in *E. coli* or *B. subtilis*. Cyclophilin was used as a positive control.

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The reason for this is unclear.

We confirmed the PPIase activity of PrsA using a tetrapeptide substrate (succinyl-Ala-Lys-Pro-Phe-4-nitroanilide) and the conventional protease-coupled PPIase assay (40). For these measurements, proteins were produced in *E. coli* as GST fusion proteins. Both the full-length PrsA and parvulin-like domain exhibited a PPIase activity with the tetrapeptide substrate, but again the activity of cyclophilin was significantly higher, by about 60-fold (parvulin-like domain) (Fig. 3C and Table II). PrsA-(N+C) was consistently inactive. Chymotrypsin, which is used in the PPIase assay to cleave the trans form of Lys-Pro, nicked or partially truncated the full-length PrsA protein during the assay (SDS-PAGE, not shown). However, chymotrypsin

![Image](https://example.com/image.png)

**Fig. 2.** The molecular model of the parvulin-like PPIase domain of PrsA and the mutations constructed in this study. A, structural conservation of the active site residues in PrsA (numbered), hPar14, and Pin1, indicated by magenta, green, and yellow, respectively. The backbone structure of Pin1 differs to some extent from that of the hPar14-based model, and for clarity the Ser154 of Pin1 at Tyr156 of PrsA is not shown. For the same reason, only side chain conformations of the other active site residues of Pin1 are shown. B, site-directed amino acid replacements in the parvulin-like domain. In addition to tyrosine, the Phe176 residue was also replaced with alanine. C, the ΔMet172-Lys182, ΔThr185-Lys202, and ΔAsp154-Gln171 in-frame deletions, and the secondary structures of the parvulin-like domain. D, locations of the various pentapeptide insertions in the PPIase domain structure. The amino acid residues of PrsA preceding the insertions are indicated. The mutant PrsA proteins that were partially defective in the complementation of lethality are indicated by white text.
The Catalytic Amino Acid Residues of the Predicted Active Site Are Dispensable for the PrsA Function in AmyQ Secretion and Growth—Taking advantage of the modeled structure of the parvulin-like domain, we wanted to determine whether the conserved amino acid residues in the predicted active site are needed for the PrsA function in vivo. The catalytically important residues, Met\(^{172}\) and Phe\(^{176}\), located in the proline-binding pocket, and His\(^{122}\), Asp\(^{154}\), Tyr\(^{196}\), and His\(^{189}\), located in the catalytic site, were replaced either with alanine or tyrosine (Fig. 2B). The residues Gly\(^{161}\) and Ile\(^{200}\) were mutated in the same way, because alanine substitutions of the corresponding residues in the E. coli PpiD parvulin are known to disrupt the PPIase activity as well as the in vivo function in the assembly of outer membrane proteins (4, 41). Residues Phe\(^{144}\), Ser\(^{152}\), Gly\(^{161}\) were also replaced with alanine because of their high conservation. The S156P mutation resulted accidentally from a PCR error. Additionally, three internal deletions in the parvulin-like domain were constructed. The shortest of the deletions, ΔMet\(^{172}\)-Lys\(^{182}\), removed the whole α4-helix (Fig. 2C), which includes residues Met\(^{172}\) and Phe\(^{176}\) of the proline-binding pocket. The deletion of ΔAsp\(^{154}\)-Gln\(^{171}\) led to loss of the α3-helix, β2-strand, and almost the whole loop following the β2-strand, whereas the deletion of ΔThr\(^{185}\)-Lys\(^{202}\) removed β3 and half of β4 (Fig. 2C). These mutant PrsA proteins were present in cells at near wild-type levels (immunoblotting, not shown), suggesting that the protein domains folded correctly to the native protease-resistant conformation, apart from three, PrsA-H199A, PrsA-(ΔAsp\(^{154}\)-Gln\(^{171}\)), and PrsA-(ΔThr\(^{185}\)-Lys\(^{202}\)), which were partially degraded and thus may be at least partially misfolded (Table III). The effects of the constructed mutations on the PrsA function in vivo were analyzed using the complementation assays.

Strikingly, the His\(^{122}\), Asp\(^{154}\), Met\(^{172}\), and Phe\(^{176}\) residues of the active site, and four other highly conserved residues, Phe\(^{144}\), Ser\(^{152}\), Gly\(^{161}\), and Ile\(^{200}\), could be substituted without effect on the PrsA function in vivo (Table III). The S156P mutation also did not affect the PrsA function. These mutant PrsA proteins restored AmyQ secretion in the prsA3 mutant to the wild-type level as well as rescued the growth of cells depleted of wild-type PrsA. Two proteins, PrsA-G197A and PrsA-H199A, also fully restored PrsA activity in the viability assay but displayed only partial activity in the AmyQ secretion assay (Table III). Conversely, the PrsA-Y196A substitution mutant fully restored AmyQ secretion in prsA3 but only partially restored growth in cells depleted of the wild-type PrsA. Despite these minor effects, the results indicate that none of the predicted active site residues are indispensable for the PrsA functions in AmyQ secretion and growth. It was surprising that even PrsA-(ΔMet\(^{172}\)-Lys\(^{182}\)), which lacks most of the likely proline-binding site, exhibited full PrsA activity in the viability assay, and even in the AmyQ secretion assay the activity was only moderately (70% of wild type) reduced (Table III). The longer internal deletions, ΔAsp\(^{154}\)-Gln\(^{171}\) and ΔThr\(^{185}\)-Lys\(^{202}\), however, inactivated PrsA (Table III), in a manner similar to the deletion of the whole parvulin-like domain (Fig. 1), consistent with an essential role for this domain.

The Influence of PrsA Depletion and Active Site Mutations of the PrsA PPIase on the Extracellular Proteome—In previous studies the extracellular proteome of B. subtilis wild type has been described (34). Taking advantage of the exoproteome analysis, we now investigated the effects of partial PrsA depletion and four of the active site mutations (H122A, D154A, Y196A, and ΔMet\(^{172}\)-Lys\(^{182}\)) on the exoproteome. These mutations were chosen either since they were partially defective in the above complementation assays. Y196A and ΔMet\(^{172}\)-Lys\(^{182}\) or the corresponding active site mutations in a Psn1-type eukaryotic parvulin have been shown to cause phenotypic effects (H122A and D154A) (5). IH7211 cells (P\(_{prsA}\)-prsA) were depleted of the wild-type PrsA protein by cultivating them in the absence of IPTG and thus the P\(_{prsA}\)-prsA activity was repressed but the initial conditions did not inhibit growth. Strains transformed with plasmids encoding the mutant PrsA proteins were at 300 μg/ml for 5 min did not affect the level of the PPIase activity (data not shown). The protein consisting of the parvulin-like domain was resistant to chymotrypsin.

FIG. 3. The dependence of PrsA-catalyzed proline-limited folding and prolyl isomerization on the parvulin-like PPIase domain. A, the refolding kinetics of chemically denatured ribonuclease T1 was followed spectrophotometrically in the absence of any foldase and in the presence of 1–3 μM PrsA. B, the catalytic activity of the mutant derivative of PrsA lacking the parvulin-like domain, and cyclophilin (positive control) was similarly determined as in A. The degree of refolding is indicated as a relative fluorescence in which 100% is the fluorescence of the native RNase T1. C, the catalysis of prolyl isomerization of the tetrapeptide succinyl-Ala-Lys-Pro-Phe-4-nitroanilide.
similarly partially depleted of the chromosome-coded wild-type PrsA. The strains were grown to the early stationary growth phase, followed by the sample preparation and two-dimensional gel electrophoresis as described under “Experimental Procedures.” For gel comparison we used the false-color image analysis and quantitation tool of the DECODON Delta two-dimensional program.

The partial PrsA depletion caused prominent alterations in the pattern of the extracellular proteome. First, strongly elevated amounts of cytoplasmic proteins were observed (Fig. 4). The quantification of 17 previously identified cytoplasmic proteins showed 5–20-fold higher relative amounts in the exopro-}

**TABLE II**

| PrsA protein | Proline-limited folding $k_{cat}/K_m$ | Prolyl isomerization $k_{cat}/K_m$ | Source of PrsA |
|--------------|-------------------------------------|----------------------------------|----------------|
| PrsA-(2–273) (full-length) | 25 $mM^{-1} s^{-1}$ | 6 $mM^{-1} s^{-1}$ | E. coli GST fusion |
| PrsA-(N+C) | BD | BD | E. coli GST fusion |
| PrsA-(PPI) | 28 | 15 | E. coli GST fusion |
| PrsA-(PPI-S156P) | Weak | 8 | E. coli GST fusion |
| PrsA-(PPI+C) | 21 | ND | E. coli GST fusion |
| PrsA-(33–241) | 22 | ND | E. coli GST fusion |
| Negative control | BD | BD | E. coli GST fusion |
| Cyclophilin | 1300 | 942 | |
| Cyclophilin +juglone | BD | ND | |
| PrsA-(2–273) + CasA +FK506 | 29 | ND | E. coli GST fusion |
| PrsA-(2–273) + juglone | 22 | ND | E. coli GST fusion |
| PrsA-(PPI-H122A) | Weak | BD | E. coli GST fusion |
| PrsA-(PPI-D154A) | Weak | 6 | E. coli GST fusion |
| PrsA-(PPI-ΔMet172-Lys182) | Weak | BD | E. coli GST fusion |
| PrsA-(1–273) (wt) | 143 | ND | B. subtilis lipoprotein |
| PrsA-(H122A) | 119 | ND | B. subtilis lipoprotein |
| PrsA-(D154A) | 138 | ND | B. subtilis lipoprotein |
| PrsA-(ΔMet172-Lys182) | 127 | ND | B. subtilis lipoprotein |
| PrsA-(2–273) | 25 | ND | B. subtilis soluble protein |

$^a$ Values are means of at least two measurements.

$^b$ Prolyl isomerization of the tetrapeptide succinyl-Ala-Lys-Pro-Phe-4-nitroanilide.

$^c$ PrsA proteins were produced either as GST fusion proteins in E. coli or as lipoproteins in B. subtilis. PrsA was cleaved from GST, and the product is devoid of the N-terminal cysteine and lipomodification of the native protein.

$^d$ BD, below detection limit.

$^e$ Close to the detection limit and therefore considerable variation from one experiment to another.

$^f$ ND, not determined.

$^g$ Preparation similar to GST-PrsA fusion protein but obtained from cells expressing GST not fused to PrsA.

$^h$ Bovine serum albumin.

$^i$ The PrsA lipoprotein contains a covalently linked diacylglycerol moiety at the N-terminal cysteine residue.

$^j$ Produced as a secretory protein and devoid of lipomodification.

**TABLE III**

| Mutation | PrsA$^a$ | AmyQ secretion$^b$ | Viability$^b$ |
|----------|---------|----------------|-------------|
| Substitutions | | | |
| H122A | WT | +++ | +++ |
| F144Y | WT | +++ | +++ |
| S152A | WT | +++ | +++ |
| D154A | WT | +++ | +++ |
| S156P | WT | +++ | +++ |
| G161A | WT | +++ | +++ |
| M172A | WT | +++ | +++ |
| F176A | WT | +++ | +++ |
| F176Y | WT | +++ | +++ |
| Y196A | WT | +++ | +++ |
| G197A | WT | 0.8 × WT | +++ |
| H198A | Partial degradation | 0.4 × WT | +++ |
| I200A | WT | +++ | +++ |
| Internal deletions | | | |
| ΔAsp$^{174}$-Gln$^{177}$ | Partial degradation | – | – |
| ΔMet$^{172}$-Lys$^{182}$ | WT | 0.7 × WT | +++ |
| ΔThr$^{185}$-Lys$^{202}$ | Partial degradation | – | – |

$^a$ Protein level detected by immunoblotting.

$^b$ Complementation of the AmyQ secretion defect of the prsA3 mutant. The range is from +++ , the WT level, to −, the prsA3 level.

$^c$ Complementation of the lethality of PrsA depletion. The range is from +++ , normal growth, to −, no growth.
PrsA depletion on the exoproteome (not shown). The exoproteome analysis of the parvulin domain mutants revealed that PrsA-H122A also restored the wild-type pattern of the exoproteome (Figs. 5A and 6). This is consistent with the complementation in the AmyQ secretion and viability assays above and indicates that PrsA-H122A is a fully functional PrsA protein in vivo. PrsA-Y196A also restored the wild-type pattern of the exoproteome despite the moderate defect in the viability assay (Fig. 6). Interestingly, the exoproteome of the mutant ΔMet^{172–182} resembled that of the partial PrsA depletion (Figs. 5B and 6). There was a decrease in secreted proteins (Fig. 6B) and indications of cell lysis as judged by the increased amount of proteins released into the culture medium. However, the contamination of the exoproteome by cytoplasmic proteins was less extensive than for PrsA depletion (Fig. 6A), suggesting partial complementation. A similar partial complementation was also seen with the D154A mutant construct (Fig. 6).

The PPlase Active Site Is Important for PrsA-catalyzed Folding and Prolyl Isomerization in Vitro—Several PrsA proteins with active site mutations (PrsA-H122A, PrsA-D154A, PrsA-ΔMet^{172–182}, PrsA-(PPI-H122A), PrsA-(PPI-D154A) and PrsA-(PPI-ΔMet^{172–182}) constructed in this study were subjected to RNase T1 refolding and protease-coupled PPlase assays. Also the catalytic activities of a PrsA variant with a change of the serine 156 to proline in the parvulin-like domain (PrsA-(PPI-S156P)) were determined. The mutant PrsA proteins were produced either as GST fusion proteins in *E. coli* or lipoproteins in *B. subtilis*. The integrity of the PrsA variants purified was confirmed by immunoblotting (not shown).

The PrsA-(PPI-S156P) protein catalyzed RNase T1 refolding (Table II) only marginally. A similar reduction in the catalytic activity was also observed when the active site residue His^{122} or Asp^{154} was mutated. The findings suggest that there are specific structural requirements for the parvulin-like domain in the catalysis of proline-limited protein folding. The PrsA-(PPI-ΔMet^{172–182}) protein was inactive. However, when these mutations were in the full-length, lipomodified PrsA, they had no effect on the catalytic activity (Table II). The activity of these lipomodified PrsA proteins determined in a detergent milieu was significantly higher (about 5-fold) than that of nonlipomodified PrsA in a non-detergent milieu.

The PPlase assay with the succinyl-Ala-Lys-Pro-Phe-4-nitroanilide tetrapeptide substrate revealed that PrsA-(PPI-H122A) is inactive (Table II), indicating that His^{122} is indispensable for the prolyl isomerization. The ΔMet^{172–182} deletion also inactivated the tetrapeptide isomerization. Although PrsA-(PPI-S156P) was strongly defective in catalyzing RNase T1 refolding, it exhibited a PPlase activity with the tetrapeptide substrate (Table II). The activity was about half of that of the wild-type parvulin-like domain (PrsA-PPI). The PrsA-(PPI-D154A) protein also displayed an activity similar to PrsA-(PPI-S156P).

In the protease-coupled PPlase assay chymotrypsin caused some degradation of PrsA-(PPI-S156P) and PrsA-(PPI-D154A) (not shown), and therefore the actual PPlase activities of these mutant PrsA proteins may be higher than what was observed (Table II). The rate of degradation of PrsA-(PPI-H122A) was similar to that of PrsA-(PPI-S156P) and PrsA-(PPI-D154A), implying that the degradation does not explain the PPlase-negative phenotype of this PrsA variant. The PrsA-(PPI-ΔMet^{172–182}) protein was more sensitive to proteolytic degradation than the other PrsA variants.

**PrsA Does Not Possess a General Chaperone Activity**—Since some PPlases are known to have a dual PPlase chaperone role in protein folding (7, 8), we determined whether PrsA has the properties of a molecular chaperone. We used chaperone assays in which the ability of PrsA to prevent either unfolded rhodanese or citrate synthase from aggregating during refolding.
was determined (see “Experimental Procedures” for details).

In the absence of a chaperone, only about 30% of the initial rhodanese activity was recovered (Fig. 7). GroEL/S chaperone increased the recovery up to 80%, whereas bovine serum albumin, which was used as a negative control, did not affect the recovery. Unlike GroEL/S, PrsA did not exhibit a chaperone activity (Fig. 7). The result was similar when the chaperone activity was determined using citrate synthase as a substrate, and the degree of aggregation was measured by light scattering (data not shown).

**DISCUSSION**

In this study we carried out a structure-function analysis of *B. subtilis* PrsA. The aim was to identify which regions or domains of PrsA are required for protein secretion and growth with the emphasis on the characterization of the parvulin-like domain, its putative peptidyl-prolyl cis/trans isomerase activity, and the role of the PPIase activity in vivo. PrsA indeed exhibited PPIase activity in a parvulin-like domain-dependent manner. Our results furthermore indicate that in vivo the parvulin-like PPIase domain as well as the flanking N- and C-terminal non-PPIase domains are essential, but the PPIase activity as such is non-essential.

The molecular modeling of PrsA revealed a Par14- or Pin1-type parvulin fold and structural conservation of the proline-binding and catalytic site residues. PrsA in vitro catalyzed the proline-limited folding of ribonuclease T1. However, the catalytic activity of PrsA was fairly weak. Much higher activities have been reported for the *E. coli* trigger factor, human Pin1 and *E. coli* PpiD (4, 13, 42), whereas the activity of *E. coli* SurA is comparable to that of PrsA (8). Because PrsA-PPI protein containing only the parvulin-like region catalyzed the proline-limited folding in a similar manner as the full-length PrsA, the parvulin-like region is clearly responsible for the activity. The activities of PrsA-(33–241) and PrsA-(PPI+C) are consistent with this conclusion. The PPIase activity of PrsA was confirmed with a tetrapeptide substrate. These results indicate that the parvulin-like region indeed forms a separate domain, which is able to fold correctly into the native enzymatically active conformation without any significant influence from the flanking regions. The replacement of the serine 156 in the parvulin-like domain to proline resulted in a mutant PrsA protein (PrsA-(PPI-S156P)) that was unable to catalyze the refolding of denatured RNase T1. Interestingly, however, PrsA-(PPI-S156P) catalyzed the prolyl isomerization of a tetrapeptide substrate. This result indicates that the catalysis of proline-limited folding of a complex protein substrate such as ribonuclease by PrsA also requires some other determinants in the parvulin-like domain than those required for the PPIase activity per se. Similar additional determinants have also been observed in the catalysis of the folding of a reduced and carboxymethylated RNase T1 variant by the parvulin-like domains of SurA (8). These determinants of PrsA are not important for the PrsA function in vivo, since the PrsA-(PPI-S156P) protein was fully active in the complementation assays. PrsA-(N+C), which does not contain the parvulin-like domain, was unable to catalyze prolyl isomerization, consistent with the conclusion that the PPIase activity resides in the parvulin-like domain.

In the eukaryotic parvulin Pin1, the His119 and Cys113 active site residues are highly important for the PPIase activity. The H122A substitution results in almost complete loss of the PPIase activity (15) and a replacement of the Cys113 decreases the PPIase activity to about 1% of wild-type level (13). Substitutions of the corresponding catalytic amino acids in the parvulin-like domain of PrsA, His122 and Asp154, also affected the PPIase activity as determined with the peptide substrate. The H122A substitution inactivated the PPIase function and
D154A decreased the activity to about half of the wild-type level. Furthermore, the deletion of the α4-helix resulted in a PrsA variant that did not catalyze prolyl isomerization. These mutant variants of the parvulin-like domain were also defective in catalyzing the proline-limited folding of denatured RNase T1. These results suggest that the predicted active site residues are indeed crucially important for the PPIase activity of PrsA. Despite the effect of the H122A substitution on the PPIase activity, this mutation did not affect the PrsA functions in the AmyQ secretion and growth, indicating that the PPIase domain and the flanking domains are able to fold correctly independently. 

None of the targeted amino acid replacements of the likely active site residues inactivated PrsA in the AmyQ secretion or growth. These functions also remained unaffected when Gly397 and Ile500 were mutated, in contrast to the corresponding mutations in PpiD, which have been shown to inactivate the PpiD function both in the assembly and stability of outer membrane proteins and in vitro-determined PPIase activity (4). However, the parvulin-like domain is most probably essential for the PrsA functions in vivo, as evidenced by the inability of the mutant PrsA proteins lacking the whole PPIase domain (PrsA-(N+C)) or a major part of it (PrsA-(ΔAsp154–Gln171)) and PrsA-(ΔThr185–Lys202) to catalyze AmyQ secretion and/or support growth, although the essential activity is not the PPIase activity. An alternative explanation could be that the N- and C-terminal domains in these mutant PrsA proteins are incorrectly folded and they are consequently inactive in vivo rather than the inactivation being due to the absence of the parvulin-like domain. Since PrsA-(ΔAsp154–Gln171) and PrsA-(ΔThr185–Lys202) were partially degraded in vitro, their conformation may be incorrect. Based on the structural model, ΔAsp154–Gln171 and ΔThr185–Lys202 deletions are likely to disrupt the conformation of the central β-sheet. PrsA-(N+C), however, was stable in vivo, suggesting that the N- and C-terminal domains in this mutant PrsA protein are in a native-like conformation. PrsA-(N+C) also exhibited partial PrsA activity in the AmyQ secretion assay, suggesting that the fold is proper. Furthermore, the CD spectroscopic analysis of PrsA-(N+C) and the full enzymatic activity of PrsA-PPI suggest that the PPIase domain and the flanking domains are able to fold correctly independently.

It is interesting that a similar parvulin domain deletion mutant of E. coli SurA as B. subtilis PrsA-(N+C) may be functional in the assembly and folding of outer membrane proteins (8), and thus in contrast to PrsA its in vivo function may be independent of the parvulin-like domain. The PrsA-like protein of Lactococcus lactis may also function in a manner independent of the parvulin-like domain (44). However, these results need to be confirmed. In addition to SurA, there is in the E. coli periplasm also another protein functionally to similar PPIase, PpiD, which may interfere with the complementation analysis of functions of SurA mutants (4, 8, 45). PrsA is the only essential PPIase at the cell membrane-wall interface of B. subtilis and therefore functionally similar PPIases do not disturb its functional analysis. In the parvulin-like domain, most peptide insertions that affected the PrsA function in vivo were found to be located in three spots around the active center (see Fig. 2D). This suggests that despite the high tolerance to substitutions of single amino acid residues and peptide insertions

FIG. 6. Relative amounts of abundant cytoplasmic proteins (A) and extracellular proteins with signal peptides (B) in the extracellular medium. The relative protein amounts were obtained by comparing the spot intensities in the extracellular proteomes of the strains IH7211 (pGlu-prsA) grown without versus with IPTG (bar 1), ΔMet172–Lys186 versus wild type (bar 2), H122A versus wild type (bar 3), D154A versus wt (bar 4), Y196A versus wild type (bar 5).
the active center of the parvulin-like domain most probably is important for the PrsA function.

It was peculiar that in the parvulin-like and C-terminal domains many peptidopeptide insertions partially disrupted the PrsA activity in the viability assay but did not at all affect the activity in the AmyQ secretion assay. We can put forward three possible explanations for this obvious discrepancy. (i) PrsA may be a multimeric protein and in the prsA3 mutant the insertion mutant PrsA proteins may interact with the PrsA3 protein, form functional heterodimeric or multimeric PrsA3-PrsA peptide complexes and thus restore AmyQ secretion in the prsA3 mutant by intermolecular complementation, but not the function essential for viability. (ii) An interaction of the insertion mutant PrsA proteins with PrsA3 may stabilize this unstable but functional PrsA protein and thereby restore AmyQ secretion in the prsA3 mutant. (iii) The insertion mutant PrsA proteins may interact with the quality-control proteases that degrade PrsA3 and prevent the degradation by competitive inhibition. The synthetic lethal phenotype of the PrsA36 protein in the prsA3 mutant supports the first hypothesis. The most likely explanation for the synthetic lethality is formation of functionally inactive dimeric or multimeric PrsA3-PrsA36 complexes and consequent inactivation of the PrsA3 protein and cell death.

In addition to the complementation of the AmyQ secretion defect in prsA3 and the growth defect of cells depleted of PrsA we also used exoproteome analysis to characterize the function of some of the mutant PrsA proteins. It was observed that when cells were partially depleted of the chromosomally coded wild-type PrsA the level of numerous secreted proteins in the exoproteome decreased, whereas the level of some other proteins remained about the same as that of the non-depleted cells. This result suggests that the stability and secretion of several exoproteins is dependent on PrsA. However, it is difficult to assess from our data, how much the PrsA depletion actually decreased the secretion of these PrsA-dependent exoproteins, since the PrsA depletion also caused significant cell lysis and increase of the level of contaminating cytoplasmic proteins in the exoproteome. The active site mutants PrsA-ΔMet172-Lys182 and PrsA-D154A were only partially able to restore the wild-type pattern of the exoproteome even though they fully restored growth in the PrsA-depleted cells, indicating that they are to some extent defective PrsA proteins. The underlying mechanism of the effects on the exoproteome is most probably independent of the PPlase activity, since the PrsA-H122A protein fully restored the wild-type pattern of the exoproteome. It has been shown that the FKBP-type PPlase domain of E. coli FkpA has a PPlase-independent chaperone activity in vitro (7). Also E. coli SurA parvulin (8) exhibits a general chaperone activity. Although our results indicated that PrsA does not have a general chaperone activity, a dedicated chaperone activity is not excluded.

Our results also showed that in addition to the PPlase domain, the N- and C-terminal domains are essential for the PrsA function in vivo. Hence, some non-PPlase activity of the flanking domains is required together with the PPlase domain activity. We propose at least three possible functional roles for the flanking domains. They may be interaction domains that bring the parvulin-like domain into contact with its substrates in the cell wall, in a manner similar to the flanking domains of some other PPlases they may mediate binding to other cellular components or substrates (3, 46, 47). Alternatively, PrsA might be a modular protein in which the parvulin-like domain and the flanking domains operate in completely unrelated essential functions. The synthetic lethal phenotype of PrsA36 in the prsA3 mutant suggests a third possible role. The flanking regions may be domains that are required for the putative formation of PrsA dimers or multimers in a manner similar to the dimerization of the Legionella pneumophila Mip protein, an FKBP-type PPlase (48).
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Structure-Function Analysis of PrsA Reveals Roles for the Parvulin-like and Flanking N- and C-terminal Domains in Protein Folding and Secretion in *Bacillus subtilis*

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