Cyclophilin and Trigger Factor from *Bacillus subtilis* Catalyze in Vitro Protein Folding and Are Necessary for Viability under Starvation Conditions†

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Received May 27, 1998; Revised Manuscript Received July 16, 1998

ABSTRACT: Cyclophilin (the product of the *ppiB* gene) and the trigger factor (the product of the *tig* gene) are the only cytosolic peptidyl−prolyl cis−trans isomerases that are known in *Bacillus subtilis*. Both enzymes catalyze the in vitro refolding of ribonuclease T1, a reaction that is limited in rate by a prolyl cis/trans isomerization. The efficiency of cyclophilin as a folding catalyst is only modest with a k_{cat}/K_M value of 3.8×10^4 M^{-1} s^{-1}, but the trigger factor shows an almost 40-fold higher specific activity with a k_{cat}/K_M value of 1.4×10^6 M^{-1} s^{-1}. This high catalytic activity originates from the tight binding to the protein substrate as reflected in both the low K_M value of 0.5 μM and in the strong inhibition of the trigger factor by unfolded proteins. By use of a protein-folding assay, the concentrations of cyclophilin and the trigger factor in the cytosol of *B. subtilis* could be determined as 26 and 35 μM, respectively. Together they account for the entire folding activity that is detectable in crude extracts of wild-type *B. subtilis* cells. The genes encoding cyclophilin and the trigger factor in the *B. subtilis* chromosome were disrupted individually and simultaneously. Even in combination, these disruptions had no effect on cell viability in rich medium or under several stress conditions, such as heat, osmotic, or oxidative stress. However, in poor medium and, in particular, in the absence of amino acids, the growth of the double mutant strain was strongly decelerated, indicating that the prolyl isomerases become essential for growth under starvation conditions. It is not yet known whether this function relates to the catalysis of the proline-limited folding of essential proteins.

Cis/trans isomerizations of peptidyl−prolyl bonds are intrinsically slow reactions, which often determine the rates of protein-folding reactions in vitro and presumably also in vivo (*1, 2*). Peptidyl−prolyl cis−trans isomerases (prolyl isomerases) catalyze this interconversion of Xaa−Pro bonds both in peptides and in proteins (*3*).

Prolyl isomerases are ubiquitous enzymes, which belong to three unrelated families: the cyclophilins, the FK506 binding proteins (FKBPs), and the parvulins (*4*−*6*). The cyclophilins and FKBPs bind to the immunosuppressive drugs cyclosporin A and FK506, respectively (*3, 4*). Most organisms contain several members of each family. *Saccharomyces cerevisiae* contains eight cyclophilins, four FKBPs, and one parvulin homologue (*7*).

From *Bacillus subtilis*, only two prolyl isomerases have been isolated to date, cyclophilin, which is the product of the *ppiB*[1] gene and the trigger factor, the product of the *tig* gene. The cyclophilin from *B. subtilis* resembles the eukaryotic cyclophilins in its high affinity for cyclosporin A (*8*), but its catalytic activity toward tetrapeptides is comparatively low (*9*). From a *ppiB* null mutant of *B. subtilis* an additional prolyl isomerase could be purified, which was identified as the trigger factor (*10*).

In *Escherichia coli* the trigger factor is an abundant protein. Originally, it was suggested to be involved in the export of secretory proteins (*11*), and it was found associated with the ribosome (*12*). More recently the trigger factor was identified as a prolyl isomerase, which catalyzes proline-limited protein folding with a very high efficiency (*6*) and can bind to nascent polypeptide chains (*13, 14*). The trigger factor consists of three domains. The amino-terminal domain is required for binding to the ribosome (*15*), and the middle domain harbors a FKBP-type module (*16*), which carries the prolyl isomerase activity (*17, 18*). The function of the carboxy-terminal domain is not yet known. However, the high affinity of the trigger factor for unfolded protein chains (*19, 20*) and, as a consequence, the efficient catalysis of proline-limited protein-folding reactions requires the presence of all three domains (*21*). The trigger factor was also suggested to play a role in the GroEL-dependent proteolytic degradation of misfolded proteins (*22*).

In contrast to the whole host of information about the in vitro functions, the roles of the prolyl isomerases in the cell remain enigmatic. In *Sacharomyces cerevisae*, null mutants strains of FKBP12 and Cpr7 (a homologue of Cyclophilin

† This work was supported by the Deutsche Forschungsgemeinschaft (SFB 395) and the Fonds der Chemischen Industrie.

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[1] Abbrevations: *tig*, gene of the *B. subtilis* trigger factor; *ppiB*, gene of the *B. subtilis* cyclophilin; PPIase, peptidyl prolyl cis−trans isomerase; RCM-T1, reduced and carboxymethylated form of the S54G/P55N variant of the ribonuclease T1; RCM-La, reduced and carboxymethylated form of bovine α-lactalbumin; Ni^{2+}-NTA, nickel-nitrilotriacetate-agarose; Suc-, succinyl-; PMSF, phenylmethylsulfonyl fluoride; AEBSF, (aminoethyl)benzenesulfonyl fluoride; cat, chloramphenicol acetyltransferase; kan, kanamycin resistance cassette; OD_{600}, optical density at 600 nm.

S0006-2960(98)01253-7 CCC: $15.00
Published on Web 09/01/1998

40) exhibit slow growth (*23, 24*). Furthermore, it could be demonstrated that the mitochondrial cyclophilin Cpr3 of *Saccharomyces cerevisiae* is required for mitochondrial function at elevated temperatures (*25*) and accelerates protein refolding after mitochondrial import (*26*). However, under favorable growth conditions, in *Saccharomyces cerevisiae* all eight cyclophilins and four FKBPs, individually and collectively, seem to be dispensable for viability (*7*). The absence of a significant phenotype of PPIase null mutants might indicate that under standard growth conditions prolyl isomerases are not essential for viability (*27*).

In this work we analyze the prolyl isomerase activities of the soil bacterium *Bacillus subtilis* and show that only two enzymes, the cyclophilin and the trigger factor, contribute to this activity. We analyzed the catalytic activity of both isomerases in protein folding in vitro and explored their physiological role in vivo. We find that the two prolyl isomerases can complement each other functionally in mutants that are defective in one of the two prolyl isomerases. A mutant with disruptions in both *ppiB* and *tig* shows severe growth defects during starvation.

MATERIALS AND METHODS

Bacterial Strains and Materials. Bacterial strains used in this work are *Bacillus subtilis* JH642 (*28*) (trpC2 pheA1); *Bacillus subtilis* MH1 (*27*) (*trpC2 pheA1 ppiB::cat; cat*: chloramphenicol resistance cassette); *Bacillus subtilis* SG1 (*trpC2 pheA1 tig::kan*, this work; *kan*: kanamycin resistance cassette); *Bacillus subtilis* SG2 (*trpC2 pheA1 tig::kan ppiB::cat*, this work); *Escherichia coli* XL-1 Blue. As complete medium we used 2xYT medium (*29*) and SM medium as minimal medium (*30*). SM medium was supplemented with tryptophan and phenylalanine (each 0.1% (w/v)). The substrate tetrapeptide Suc-Ala-Xaa-Pro-Phe-4-nitroanilide (Xaa: Ala, Phe, Glu, Lys) was obtained from Bachem (Basel, Switzerland). Nickel-nitrilotriacetate-agarose (Ni^{2+}-NTA) and tip20 columns for plasmid preparation were from Qiagen. Restriction endonucleases and other enzymes were obtained from Amersham. Protein concentrations were determined according to Gill and von Hippel (*31*).

Overexpression and Purification of Cyclophilin, Trigger Factor, the Internal FKBP12 Fragment, and RCM-T1. The cyclophilin was overexpressed and purified in a two-step protocol as described previously (*9, 27*). Trigger factor and the internal FKBP12 fragment were constructed, overexpressed, and purified as His-tag fusion proteins (*10*). The (S54G, P55N)-RNaseT1 was purified, reduced, and carboxymethylated (RCM-T1) as described (*32, 33*).

Cloning and Expression of the yacD Gene and Purification of the Gene Product YacD. The *yacD* gene was amplified by PCR using chromosomal DNA of *B. subtilis* strain JH642 and two specific primers with the restriction sites (*Sph*I and *Bam*HI) corresponding to the 5′and 3′ regions of the *yacD* gene: 5′-CATGGCATGCAATCAAGAACAATCTGGA-3′ and 5′-CTTGGATCCTTTTTCCCCATAAAACC-3′, respectively. After digestion of the amplified PCR product, the DNA fragment was ligated into the vector pQE70 (Qiagen). The plasmid was used to transform *E. coli* strain XL1Blue (Stratagene) followed by plasmid preparation and restriction analysis of the constructed plasmid. pQE70 containing the *yacD* gene was then transformed in the *E. coli* strain SG13009 [pREP4].

The following conditions for the overexpression of *yacD* as histidine tagged protein were used to obtain soluble protein: 1 L of 2xYT medium, containing 100 μg/mL of ampicillin and 25 μg/mL of kanamycin, was inoculated with an overnight culture (30 °C) and incubated at 30 °C to an OD_{600} of 0.9. After induction with isopropyl β-D-thiogalactoside (0.3 mM), cultures were grown for a further 1 h at 30 °C. Cells were centrifuged (4000*g*, 4 °C, 15 min), resuspended in buffer A (50 mM HEPES pH 8.0, 300 mM NaCl), and passed through a french press. Brocken cells were centrifuged at 20 000*g* (4 °C, 30 min) and the supernatant applied to a FPLC−Ni^{2+}-NTA column equilibrated with buffer A. Bound protein was eluted by a linear gradient of 0−0.25 M imidazole. The purity of YacD was judged by Coomassie-brilliant-blue stained SDS−PAGE analysis.

Measurements of Prolyl Isomerase Activity in the Peptide Assays. In the protease-coupled assay, the chromogenic peptide Suc-Ala-Phe-Pro-Phe-4-nitroanilide and chymotrypsin were used (*34*); 910 μL of 0.1 M Tris/HCl (pH 8.0) and 50 μL of a 600 μM solution of α-chymotrypsin (Boehringer-Mannheim, Germany) were mixed in the spectrophotometer cell and preincubated at 15 °C for 10 min. Then 30 μL of the solution to be assayed was added and after 5 min the assay was initiated by adding 10 μL of a 7.8 mM solution of the assay peptide in trifluoroethanol that additionally contained 0.45 M LiCl (*35*). The cis → trans isomerization of the Phe−Pro bond, coupled with the chymotryptic cleavage of the trans peptide, was followed by the increase in absorbance at 390 nm in a HP 8452 diode array spectrophotometer. Monoexponential functions were fit to the progress curves, and the activity was calculated from the observed rate constants (*34*).

In the protease-free assay also, Suc-Ala-Phe-Pro-Phe-4-nitroanilide was used as a substrate; 955 μL of 0.1 M Tris/HCl (pH 8.0) and 30 μL of the enzyme under investigation were incubated in the spectrophotometer cell at 15 °C for 10 min. The assay was started by adding 15 μL of a 7.8 mM solution of the assay peptide in trifluoroethanol/0.45 M LiCl. The final peptide concentration was 120 μM. The cis/trans isomerization of the Phe−Pro peptide bond was followed by the small decrease of the absorbance at 330 nm caused by the rearrangement of the nitroanilide moiety (*36*).

Folding Experiments. The reduced and carboxymethylated form of the S54G/P55N variant of RNase T1 (RCM-T1) was unfolded by incubating the protein in 0.1 M Tris/HCl (pH 8.0) at 15 °C for at least 1 h. Refolding at 15 °C was initiated by a 40-fold dilution of the unfolded protein to final conditions of 2.0 M NaCl and the desired concentrations of the purified trigger factor, cyclophilin or *B. subtilis* crude extracts. The crude extracts that were assayed for folding activity contained 2 mM EDTA, 0.2 mM AEBSF ((aminoethyl)benzenesulfonyl fluoride), and 1 mM PMSF (phenylmethylsulfonyl fluoride) to inhibit the proteolytic degradation of both the refolding protein molecules and the prolyl isomerases. The refolding reaction was followed by the increase in protein fluorescence at 320 nm (10 nm bandwidth) after excitation at 268 nm (1.5-nm bandwidth) when purified trigger factor was used and at 295 nm when the activities of crude cell extracts were investigated. Measurements were performed with a Hitachi F4010 fluorescence spectrometer, and the rate constants were determined using the program Grafit 3.0 (Erithacus Software, Staines, UK).

To analyze the enzyme kinetics of catalyzed folding, we measured the kinetics of folding in the presence of 10 nM trigger factor and 0.05−16 μM RCM-T1 under the folding conditions described above. Both uncatalyzed and catalyzed folding occur in these experiments. The initial velocities of catalyzed folding were determined from the measured progress curves by using the procedure originally developed for the analysis of enzyme-catalyzed prolyl isomerization in a peptide (*37*) as adapted for the analysis of the catalyzed folding of RCM-T1 (*19*).

Interruption of the Chromosomal Copy of the tig Gene in B. subtilis Strains JH642 and MH1. The *tig* gene was amplified by PCR using chromosomal DNA of the *Bacillus subtilis* strain JH642 and two specific primers with restriction sites (*Apa*I and *Kpn*I) for cloning the *tig* gene in the pBluescript vector (Stratagene). The following primers, corresponding to the 5′ and the 3′ regions of the *tig* gene, were used: 5′-ATAGGGCCCCCGACACAACCTGAATA-GAT-3′ and 5′-ATAGGTACCAAGGCACGAATGATTCG-3′. After purification of the PCR product and digestion with the restriction enzymes *Apa*I and *Kpn*I, the DNA fragment was ligated in the pBluescript SK^+ vector and the resulting plasmid was transformed into the XL1 blue strain of *E. coli*. The isolated plasmid was digested with the restriction enzyme *Bgl*II, and the *kan* gene (*kan*: kanamycin resistance cassette), localized on the 1.3 kb *Bam*HI/*Bgl*II fragment of the plasmid pDG792 (*38*), was ligated in the compatible *Bgl*II site. The resulting plasmid ptig-kan containing *tig::kan* was amplified in *E. coli* XL1 blue cells. The *tig::kan* gene was amplified by PCR, and about 2 μg of the PCR fragment was transformed in competent *B. subtilis* cells. The *tig::kan* fragment was transformed in *B. subtilis* strain JH642 resulting in strain SG1 (*tig::kan*) as well as in *B. subtilis* strain MH1 (*ppiB::cat*) resulting in strain SG2 (*tig::kan ppiB::cat*). The *tig* deletion mutants were analyzed by Southern and Western blot analysis as well as by PCR. For the Southern blot analysis the *tig* gene was used as a probe.

Analysis of the PPIase Lacking Strains. We used 2xYT as rich medium, SM medium as minimal medium, and DSM as sporulation medium for the growth kinetics (*30*). Several stress conditions, such as heat, cold, or salt stress, were applied as described previously (*39, 40*).

To analyze the strains JH642, SG1, MH1, and SG2 for adaptation to SM medium, cells were grown in 2xYT complete medium to an OD_{600} of 0.8, centrifuged, and washed in SM medium, and the washed cells were used to inoculate 200 mL of SM medium that, in addition to tryptophan and phenylalanine, contained no other amino acids. To test nutrient limitation, cells were cultivated in SM medium containing amino acids (0.1% (w/v)) to OD_{600} = 0.8, washed in SM medium without amino acids, and then used for the growth kinetics.

To analyze the residual PPIase activity in the different strains (JH642, SG1, MH1, SG2), each strain was grown in 400 mL of 2xYT medium to an opical density of one (OD_{600} = 1). The centrifuged cells were resuspended in 5 mL of buffer A (50 mM Tris/HCl (pH 7.5), 1 mM DTT, 2mM EDTA, 0.2 mM AEBSF, 1 mM PMSF) and the suspension was sonicated at intervals for a total of 3 min. The broken cells were centrifuged for 30 min at 15 000*g*.

RESULTS

Catalytic Properties of the Trigger Factor and of Cyclophilin from Bacillus subtilis. Similar to its eukaryotic counterparts, cyclophilin from *B. subtilis* is active toward chromogenic tetrapeptides, such as Suc-Ala-Ala-Pro-Phe-4-nitroanilide, in a protease-linked activity assay for prolyl isomerases (*34*). At 10 °C a k_{cat}/K_M value of 2×10^6 M^{-1} s^{-1} was obtained (*9*), which is about 10-fold lower than the respective values for cytosolic cyclophilins of eukaryotes (*3, 4*).

The efficiencies of prolyl isomerases as catalysts of slow proline-limited protein-folding reactions can be determined conveniently by using the reduced and carboxymethylated form of a variant of ribonuclease T1 (RCM-T1) (*41, 42*) as a folding substrate. In the native state RCM-T1 contains a single cis prolyl bond (Tyr38−Pro39), and its folding mechanism is simple and well characterized in molecular detail (*43, 44*). Of all RCM-T1 molecules, 85% fold in a monophasic and reversible reaction, which is limited in rate by the slow trans → cis isomerization at Pro39. This folding reaction is catalyzed by many prolyl isomerases albeit with strongly different efficiencies (*1, 45, 46*).

The catalysis of folding of RCM-T1 in the presence of various concentrations of cyclophilin is shown in Figure 1A. With a k_{cat}/K_M value of 3.8×10^4 M^{-1} s^{-1} (Figure 1B), cyclophilin catalyzes prolyl isomerization in this protein-folding reaction about 50-fold less efficiently than prolyl isomerization in the tetrapeptide. With these catalytic properties it resembles the mammalian cytosolic cyclophilins18 (*41*). The measured rate of catalyzed folding depends linearly on RCM-T1 concentration, and we could not detect an inhibition of cyclophilin by permanently unfolded proteins, such as reduced and carboxymethylated α-lactalbumin (RCM-La). This suggests that cyclophilin has only a moderate affinity for unfolded protein substrates.

Unlike most cyclophilins and FKBPs, the trigger factors of both *E. coli* (*17*) and *B. subtilis* are sensitive to proteolytic cleavage, probably due to their modular organization. Therefore, the possibility of using the protease-linked peptide assays to measure the catalytic activity is fairly limited. Instead, we employed a variant of the peptide-based assay, in which the coupling with isomer-specific proteolysis is avoided (*36*). This assay exploits the small decrease in absorbance at 330 nm of the nitroanilide moiety upon Phe-Pro cis → trans isomerization to monitor its kinetics in the uncleaved assay peptide Suc-Ala-Phe-Pro-Phe-4-nitroanilide. With this assay we determined a k_{cat}/K_M value of 0.71 ± 0.08 μM^{-1} s^{-1} for the trigger factor from *B. subtilis*. This value resembles the respective value for the trigger factor of *E. coli* (k_{cat}/K_M = 0.8 μM^{-1} s^{-1} (*6*)). In contrast, when measured by the protease-coupled assay, a specifity constant of only 0.01 ± 0.002 μM^{-1} s^{-1} was obtained.

In the folding of RCM-T1, the trigger factor of *B. subtilis* catalyzes prolyl isomerization with a very high efficiency (Figure 2). The folding of 0.1 μM RCM-T1 is already 4-fold accelerated when 4 nM of trigger factor is added (Figure 2A). The first-order rate constant of the enzyme-catalyzed refolding of RCM-T1 increases in a linear fashion with the concentration of the trigger factor (Figure 2B). From the slope in Figure 2B a value of 1.4×10^6 M^{-1} s^{-1} is obtained for k_{cat}/K_M. This value is significantly higher than k_{cat}/K_M

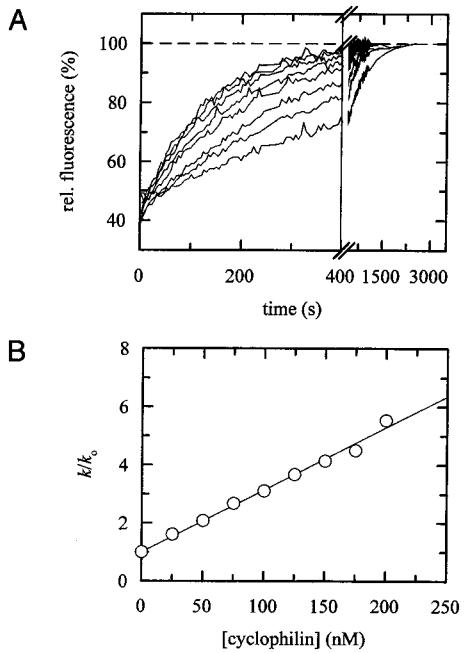

FIGURE 1: (A) Refolding kinetics of RCM-T1 in the presence of increasing concentrations of cyclophilin. The kinetics of refolding of 0.1 μM RCM-T1 in 0.1 M Tris/HCl pH 8.0; 2 M NaCl at 15 °C in the presence of various concentrations of *B. subtilis* cyclophilin. (B) Ratio of the observed rate constants of slow folding in the presence, k, and the absence, k_0, of cyclophilin as a function of the cyclophilin concentration. From the slope a k_{cat}/K_M value of 3.8×10^4 M^{-1} s^{-1} was determined. Refolding of RCM-T1 in 0.1 M Tris/HCl pH 8.0 was initiated by a 40-fold dilution to 2.0 M NaCl in the same buffer and followed by the increase in fluorescence at 320 nm.

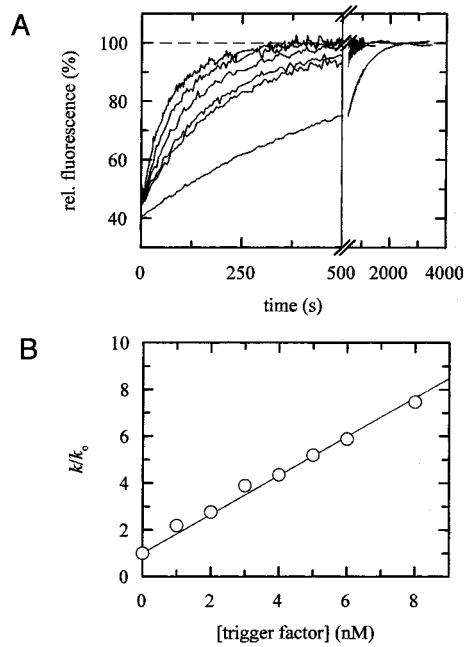

FIGURE 2: Refolding kinetics of RCM-T1 in the presence of increasing concentrations of trigger factor at 15 °C. (A) The kinetics of refolding of 0.1 μM RCM-T1 in 0.1 M Tris/HCl pH 8.0; 2 M NaCl at 15 °C, in the presence of various concentrations of trigger factor. (B) Ratio of the observed rate constants of slow folding in the presence, k, and the absence, k_0, of trigger factor as a function of the trigger factor concentration. From the slope a k_{cat}/K_M value of 1.4×10^6 M^{-1} s^{-1} was determined. The refolding experiments were performed as in Figure 1.

for prolyl isomerization in the tetrapeptide and almost 40-fold higher than the k_{cat}/K_M of cyclophilin.

The very high activity of the trigger factor in protein folding originates from its good binding to the unfolded protein substrate. Figure 3A shows the initial rate of catalyzed folding of RCM-T1 as a function of the substrate concentration. The analysis of these data by the Michaelis−Menten equation gives a K_M value of 0.45 ± 0.02 μM. This suggests that the affinity of the trigger factor of *B. subtilis* is similarly high as observed previously for the trigger factors of *E. coli* and *Mycoplasma genitalium* (*20*). The high affinity for unfolded protein chains is also reflected in the strong inhibition of catalyzed folding by permanently unfolded proteins, such as RCM-lactalbumin and the RCM form of the P39A variant of RNase T1 (Figure 3B). Under the conditions used, catalyzed folding is 50% inhibited by about 0.4 μM of RCM-P39A-RNase T1 or 0.7 μM of RCM-La, respectively.

The trigger factor contains an internal 12-kDa domain with significant sequence similarity to the FKBPs (*10, 17, 18*). Even though this domain harbors the prolyl isomerase activity, its k_{cat}/K_M value for the catalyzed refolding of RCM-T1 is decreased by almost 3 orders of magnitude from 1.4×10^6 M^{-1} s^{-1} (intact trigger factor) to 3.0×10^3 M^{-1} s^{-1} (FKBP fragment).

Construction of a Chromosomal tig and tig/ppiB Gene Disruption Mutant. To find evidence for the physiological function of the *tig* and *ppiB* gene products in vivo, the coding genes within the *B. subtilis* chomosome were interrupted by homologous recombination using antibiotic resistance markers (Figure 4A). In previous studies the *ppiB* gene was interrupted by the chloramphenicol resistance cassette, the *cat* gene (*ppiB::cat*, strain MH1 (*27*)). The *tig::kan* mutant (kanamycin resistance casette) constructed in this study was combined with that of *ppiB::cat* to generate the *tig/ppiB* double disruption mutant SG2 (Figure 4A). The disruption of both genes was verified by Southern hybridization, PCR, and Western blot analysis.

As shown by Western blot analysis (Figure 4B), the *ppiB* gene disruption mutant MH1 had no influence on the protein concentration of the trigger factor, and in analogy we have also analyzed the effect of *tig* gene disruption mutation on cyclophilin synthesis and also found no effect (data not shown).

A two-dimensional polyacrylamide gel electrophoresis analysis of the four *B. subtilis* strains (JH642, SG1, SG2, and MH1) revealed that the absence of the trigger factor and of cyclophilin had no influence on total protein synthesis (data not shown).

Prolyl Isomerase Activities in the tig/ppiB Disruption Mutant. Most organisms contain a multitude of prolyl isomerases. To examine whether cyclophilin and the trigger factor indeed account for the prolyl isomerase activity in wild type *B. subtilis*, we measured the activities in the crude extracts of wild type *B. subtilis* of the two single-disruption mutants MH1 and SG1 and of the double-disruption mutant SG2.

These activities cannot be measured by the conventional peptide-based assays. In the protease-coupled assay, sensitive prolyl isomerases, such as the trigger factor, are rapidly degraded by the helper enzyme. The protease-free assay cannot be used either, because the very small change in

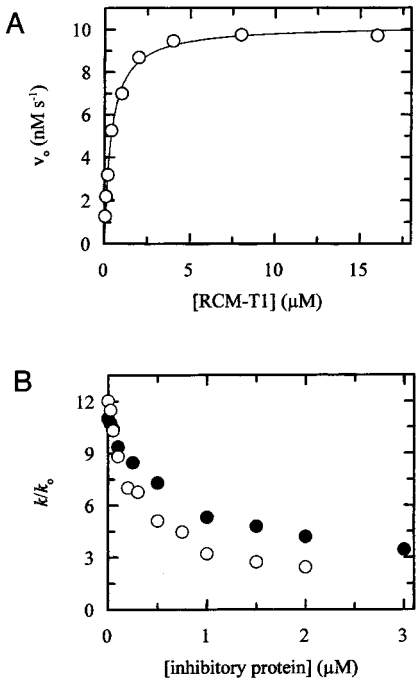

FIGURE 3: Enzyme kinetics of the trigger-factor-catalyzed refolding of RCM-T1. (A) Initial velocity of catalyzed refolding reaction at 15 °C as a function of the concentration of RCM-T1. The trigger factor concentration was 10 nM and the buffer was 0.1 M Tris/HCl, 2.0 M NaCl, pH 8.0, in all experiments. Values of $k_{cat}/K_M = 0.45 \times 10^{-6}$ M and $k_{cat} = 1.0$ s^{-1} were obtained from the analysis of the data (as shown by the continuous line). The initial folding rates were determined and analyzed as described by Scholz et al. (1997). (B) Inhibition of the trigger factor by unfolded RCM-La (●) and by RCM(P39A)T1 (○). The relative rate of catalyzed refolding (k/k_0) of 0.1 μM RCM-T1 is shown as a function of the concentrations of the two inhibitors. The conditions were as in panel A.

absorbance upon isomerization becomes buried underneath the very large changes that occur when the peptide is cleaved by trace amounts of proteases as are always present in crude extracts.

To overcome these problems, we developed an assay that is based on the folding activity of the prolyl isomerases toward RCM-T1. To avoid proteolytic degradation of this protein substrate, we added a mixture of protease inhibitors (see Materials and Methods) to the crude extracts prior to the assay. The results are shown in Figure 5. The extract from wild-type cells contains a high activity, and the addition of 10 μL of crude extract leads to a 8.5-fold acceleration of the folding of RCM-T1. This folding activity is only slightly lower (7.9-fold acceleration) in the extract from the MH1 cells, which lack cyclophilin. This indicates that the trigger factor accounts for the great majority of the folding activity in *B. subtilis*, as could be expected from the very high activity of the purified trigger factor. In fact, the extract from the SG1 cells, which lacks the trigger factor, shows only a small, but significant residual activity, which can be ascribed to cyclophilin. Finally, the SG2 cells, which carry a disruption in both genes *ppiB* and *tig*, do not show a detectable prolyl isomerase activity, and the kinetics of folding of RCM-T1 follow the same time course as the uncatalyzed folding in the control experiment. This close coincidence also confirms that the refolding RCM-T1 molecules are not degraded by proteases in the extract during the assay. In such a case the fluorescence amplitude would be decreased and the measured

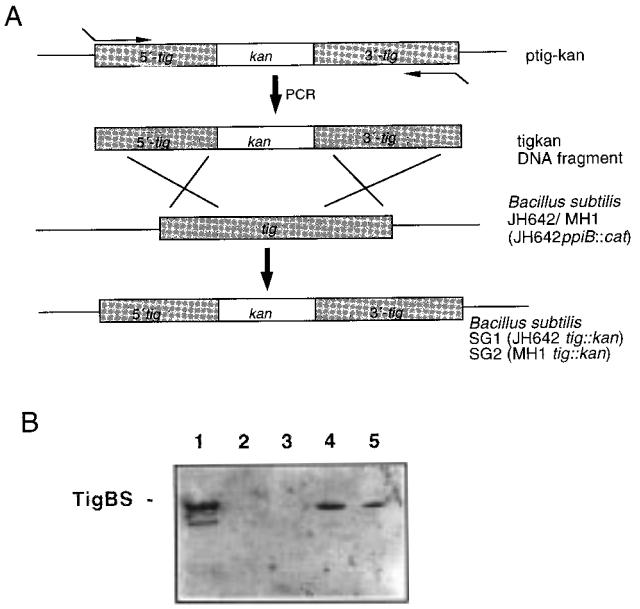

FIGURE 4: (A) Construction of the *tig* and *tig/ppiB* disruption mutants SG1 and SG2 by a double-crossover recombination event. The kanamycin resistance marker *kan* (white box) was cloned into the *Bgl*II site of *tig* (gray boxes) resulting in the plasmid ptig-kan. The *tig* gene was amplified by PCR (tig::kan), and the resulting fragment was transformed in competent cells for a double-crossover recombination event with chromosomal DNA of *B. subtilis* JH642 and MH1. (B) Western blot analysis of the resulting PPIase-lacking strains with antibodies against the trigger factor. Lane 1, purified trigger factor as control; lane 2, crude extract of SG2; lane 3, crude extract of SG1; lane 4, crude extract of MH1; lane 5, crude extract of JH642.

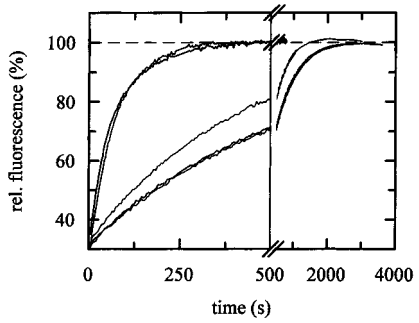

FIGURE 5: PPIase activities of PPIase-deficient strains. The refolding of 1.0 μM RCM-T1 was measured in 0.1 M Tris/HCl, 2.0 M NaCl, pH 8.0, at 15 °C. The lowest trace represents the control in the absence of cell extracts. It almost coincides with the trace observed in the presence of 10 μL of crude extract of SG2 (*ppiB*$^-$/*tig*$^-$). The third curve represents the activity of 10 μL of crude extract of SG1 (*tig*$^-$), and the two highest traces represent the activities found for MH1 (*ppiB*$^-$) and JH642 (wild-type). All crude extracts had the same protein concentration (12 mg/mL).

rate of folding increased. The sensitivity of this assay is such that about 1% of the folding activity of the trigger factor in wild-type *B. subtilis* cells can be detected reliably. The assay is performed in the presence of 2 M NaCl. Therefore, it would not detect prolyl isomerases that are inactive at high salt concentration.

Together, the results in Figure 5 suggest that at least 99% of the catalytic activity that can be measured by our protein folding assay in the crude extract of *B. subtilis* originates from only two prolyl isomerases: the cyclophilin (17.6 kDa) and the trigger factor (47.3 kDa), which contributes more than 90% of the overall activity. From the data in Figure 5

Table 1: Stress Conditions for Cell-Growth Analysis of the PPIase Disruption Mutants

	medium	stress condition	phenotype
1	2xYT		no difference in growth between wild-type (JH642) and PPIase lacking strains SG1, SG2, and MH1
2	DSM sporulation medium		as in 1
3	SM		as in 1
4	2xYT	cold shock: temp shift from 37 to 15 °C	as in 1; protein concentration of trigger factor and cyclophilin was 2-fold enhanced
5	SM	cold shock: as in 4	as in 1 and 4
6	heat shock: temp, shift from 37 to 48 °C	as in 1	
7	SM	salt stress: 0.8 M (w/v)	as in 1
8	SM	ethanol stress: 4% (v/v)	as in 1
9	SM	oxidative stress	as in 1
10	SM	amino acid limitation	mutant strains SG1 and MH1 showed a lag period in growth of about 5 h compared to wild-type.The double mutant SG2 doubled only once in 30 h.
11	SM	glucose starvation	like 1

and from the known specific activities, intracellular concentrations of 26 μM can be estimated for cyclophilin and 35 μM for the trigger factor.

We searched the entire genome of *B. subtilis* (available in the SubtiList Databank) for additional PPIases, and found only two further genes which showed significant sequence homologies to the *E. coli* parvulin, namely, *prsA* and *yacD*. *prsA* encodes a membrane-bound protein, 32.5 kDa in size, which is involved in secretion of extracellular proteins such as α-amylase (*47*, *48*). PrsA shows prolyl isomerase activity, which is, however, about 100- to 1000-fold lower than the activity of *E. coli* parvulin.[2] The function of the 34.1-kDa *yacD* gene product is not yet known.

To analyze YacD activity, the *yacD* gene was cloned into the expression vector pQE70, and the His-tagged YacD protein was purified. The protein had no prolyl isomerase activity tested with the substrates Suc-Ala-Xaa-Pro-Phe-4-nitroanilide, with Ala, Phe, Glu, and Lys as Xaa. It seems that, in fact, the prolyl isomerase activity that could be detected by our prolyl isomerization assay in *B. subtilis* originates from only two proteins, i.e., the trigger factor and cyclophilin.

Growth Defects of the Prolyl Isomerase Disruption Mutants. To elucidate the physiological role of the prolyl isomerase activity for the viability of *B. subtilis* cells, we exposed the strain SG2 which exhibits no measurable cytosolic PPIase activity to a variety of growth conditions (Table 1). In rich media (such as 2xYT and 37 °C), no

[2] Matti S., and Fischer G., personal comunication.

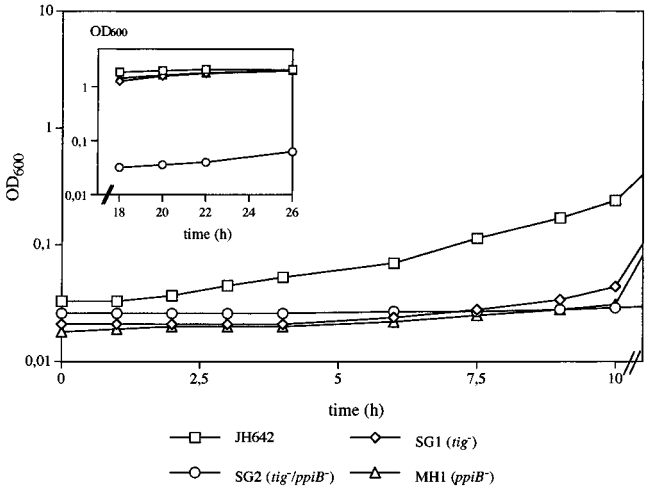

FIGURE 6: Growth kinetics of the strains JH642, SG1, SG2, and MH1 at 37 °C after a shift from SM medium containing all amino acids to SM medium containing no amino acids (exept tryptophan and phenylalanine needed by the parent strain). The inset shows the later time points in the growth kinetics.

difference in growth could be observed between wild-type and mutant strains. Therefore, we performed several stress tests including heat, cold, ethanol, oxidative, or salt stress, which are known to be sublethal for *B. subtilis* (*39*). Under most of these stress conditions (see Table I), no differences in growth could be detected for the strains JH642, SG1, SG2, and MH1. When, however, the cells were transferred from a rich medium to a minimal medium (SM medium), the SG2 strain, which lacks both prolyl isomerases, shows a significant growth retardation, indicating an important role of PPIases under these stringent conditions.

To further elucidate the conditions under which the PPIases are essential for cell viability, we performed growth experiments in minimal medium (SM) on glucose and amino acid limitation. Starvation for glucose had no effect on the growth of the strains that are deficient in prolyl isomerases. In contrast, when the supply with amino acids was limiting, strong differences in growth could be observed. As shown in Figure 6, the *tig/ppiB* double mutant could not fairly grow in SM medium that lacked all amino acids (exept Trp and Phe, which were needed by the parental strain). The single PPIase mutants MH1 and SG1 also showed retarded growth under these conditions, but after a lag period of 8 h (relative to the wild-type cells), both of them could resume growth. On solid minimal media, lacking amino acids, the double mutant is also significantly retarded in growth, a finding that may allow a search for suppressor mutations

The growth defect observed in the double mutant could not be rescued by adding back single amino acid to the SM medium, indicating a multiple requirement, next to tryptophan and phenylalanine which are needed by the parental strain. In further experiments, we therefore tested whether growth could be restored when the minimal medium was supplemented by different pools of amino acids (all at a final concentration of 50 μg/mL), which were grouped according to the six biosynthetic families in the following way: 1, Asp, Asn, Met, Thr, Lys, Ile; 2, Ala, Val, Leu; 3, His; 4, Tyr, 5, Glu, Gln, Pro, Arg; 6, Ser, Cys, Gly. As shown in Figure 7, the double mutant (SG2) could not resume growth in media containing amino acid sets 1, 3, 4, or 6. It could only grow when set 2 (the combination of Ala, Val, and Leu) or

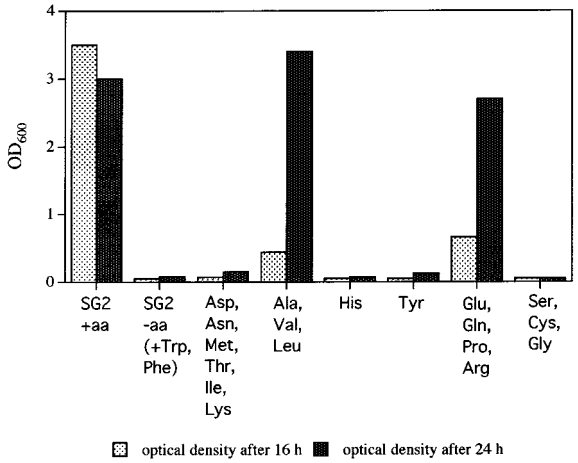

FIGURE 7: Growth of strain SG2 in SM medium containing different pools of amino acids. As a control, SG2 was grown in SM medium containing all amino acids (+aa) and no amino acids (−aa, except tryptophan and phenylalanine). Cells of strain SG2 were grown in SM medium with 0.1% casamino acids and these cells were used to inoculate an overnight culture of SM medium containing a combination of amino acids (50 μg/mL) mentioned in the legend of the figure. After 16 and 24 h, respectively, the OD_{600} (optical density at 600 nm) was measured.

set 5 (the combination of Glu, Gln, Pro, and Arg) were added. This growth was, however, much slower than in the presence of all amino acids in the medium (Figure 7), and the final optical density in the stationary phase (OD_{600} = 3) was reached 8 h later.

DISCUSSION

Only two prolyl isomerases, cyclophilin and the trigger factor, contribute to the measurable folding activity in crude extracts of *B. subtilis*. The cyclophilin of *B. subtilis* resembles the other prolyl isomerases of this family in its modest activity in protein folding. This low activity originates from the poor affinity of cyclophilins for unfolded protein chains. In contrast, the trigger factor of *B. subtilis* shows a high affinity for protein substrates and a very high catalytic activity in the folding of our model protein RCM-T1. In its enzymatic properties it resembles the homologous trigger factors from *E. coli* and *Mycoplasma genitalium* (*19*, *20*). More than 90% of the folding activity in the crude extract of *B. subtilis* originates from the trigger factor.

Mutants in which both the *ppiB* and the *tig* gene were disrupted showed no residual prolyl isomerase activity in a protein folding assay. This suggests that the other two prolyl isomerases that could be identified in the genome of *B. subtilis* (the products of the *prsA* and *yacD* genes) are inactive or show a very low prolyl isomerase activity, as was found for the purified PrsA (Fischer, G., et al., unpublished observation) and YacD proteins (this work).

The individual or the simultaneous disruption of the *ppiB* and *tig* genes did not affect the viability of *B. subtilis* in the cold, although the synthesis of both cyclophilin and the trigger factor is enhanced after a cold shock (*40*). A mutant of *E. coli* with a reduced expression of the trigger factor was reported to show reduced viability in the cold (*49*).

Differences in growth could also not be found for our mutants of *B. subtilis* under a variety of other stress conditions, such as heat, high salt concentrations, or oxidative conditions. Growth was, however, significantly affected when the wild-type and the mutant strains were transferred from rich to minimal medium. Unlike the wild-type strain and the single disruption mutants, the double *tig/ppiB* null mutant could not grow in minimal medium when certain amino acids were absent. This defect could be rescued completely by adding all amino acids to the medium or in part when the following two limited groups of amino acids (Ala, Val, Leu or Glu, Gln, Pro, Arg) were present. However, the phenotype in growth recovery could not be reduced to a single amino acid biosynthetic pathway. The two groups of amino acids mentioned above belong to two different branches of the well-known six amino acid biosynthetic families in bacteria. The remaining four amino acid biosynthetic families were found to be unable to rescue the growth retardation of the *tig/ppiB* null mutant in minimal medium (see Figure 7). At present we cannot advance a simple explanation for the cause of this phenotype (the first to be observed for a procaryotic prolyl isomerase mutant) or for the specific amino acids requirement of the double mutant. Therefore, further studies are needed to elucidate the possible physiological role of PPIases in *B. subtilis* growth under amino acid limitation. It may be noteworthy that SurA, a protein of the *E. coli* periplasm that is essential for survival in the stationary phase (*50*), shares some sequence similarity with the parvulins, a novel familiy of PPIases (*5*). It is also interesting to note that in yeast FKBP12 physically interacts with the aspartokinase, an enzyme of the biosynthetic pathway to Thr and Met in a regulatory manner (*51*).

Our data provide clear evidence that prolyl isomerases can become essential for the growth of a bacterium when the supply with nutrients is poor and that the two prolyl isomerases cyclophilin and trigger factor can substitute for each other functionally. It is still unclear whether this function relates to the acceleration of folding of some crucial enzymes of amino acid metabolism. Nonproductive folding or the aggregation of folding intermediates probably competes with uncatalyzed folding and thus decreases the yield of correctly folded proteins. These side reactions occur probably all the time. It is possible that they are tolerated in the cells under good growth conditions, but not when the supply with proteins or protein precursors is very poor. This is reminiscent of the chaperone Hsp70, which becomes essential only under heat shock conditions when the amount of inactive or aggregated proteins increases. Protein biosynthesis in bacteria may have evolved such that under good growth conditions most of the proteins can fold spontaneously and in sufficient amounts in the absence of catalysts or chaperones. When, however, the supply of nutrients, and in particular of building blocks for proteins, is severely limited, substantial misfolding may no longer be tolerable, and now the functions of folding catalysts and chaperones become essential. These harsh conditions should be the rule, rather than the exception, for many bacteria in their natural habitats.

B. subtilis is well suited to investigate the cellular functions of prolyl isomerases, because, unlike, e.g., yeast, it contains only very few prolyl isomerases. Similar to cyclophilin and the trigger factor of *B. subtilis*, the many different prolyl isomerases in higher organisms might very well replace each other functionally, which complicates the analyses of mutants.

ACKNOWLEDGMENT

We thank G. Stoller and G. Fischer (MPI zur Förderung der Wissenschaften, Halle, Germany) for initial measurements of the kinetic properties of the trigger factor with the protease free peptide assay.

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BI981253W