Two Conserved Histidine Residues Are Critical to the Function of the TagF-like Family of Enzymes

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The TagF protein from Bacillus subtilis 168 is the poly(glycerol phosphate) polymerase responsible for the synthesis of wall teichoic acid and is the prototype member of a poorly understood family of similar teichoic acid synthetic enzymes. Here we describe in vitro and in vivo characterization of TagF, which localizes the active site to the carboxyl terminus of the protein and identifies residues that are critical for catalysis. We also establish the first mechanistic link among TagF and similar proteins by demonstrating that the identified residues are also critical in the function of TagB, a homologous enzyme implicated as the glycerophosphotransferase responsible for priming poly(glycerol phosphate) synthesis (Bhavsar, A. P., Truant, R., and Brown, E. D. (Sept. 2, 2005) J. Biol. Chem. 36691–36700). We investigated the dependence of TagF activity on pH and showed that deprotonation of a residue with a pK<sub>a</sub> near neutral is critical for proper function. Alteration of histidine residues 474 and 612 by site-directed mutagenesis abolished TagF activity in vitro (5000-fold reduction in k<sub>cat</sub>/K<sub>m</sub>) while variants in four other conserved acidic residues showed minimal loss of activity. Complementation using H474A and H612A mutant alleles failed to suppress a lethal temperature-sensitive defect in vivo despite confirmation of robust expression by Western blot. When corresponding mutations were made to the homologous tagB gene, these alleles were unable to suppress a tagB temperature-sensitive lethal phenotype. These results extend the mechanistic observations for TagF across a wider family of enzymes and provide the first biochemical evidence for the relatedness of these two enzymes.
analysis of the TagF enzyme and provide the first information about its structure and mechanism. We extend these results to TagB through in vivo complementation studies using corresponding mutants and provide a mechanistic and functional link between the two enzymes and the broader TagF-like family. In conjunction with the accompanying report (21), these observations also serve to support the contention that TagB is the Tag primase.

**MATERIALS AND METHODS**

**General Methods**—Strains used in this study are listed in TABLE ONE. Oligonucleotides and plasmids used in this work are described in supplemental TABLES IS and IIS, respectively. All cultures were grown in LB medium (22) supplemented with antibiotics or xylose where necessary. Antibiotics were used at the following concentrations: 75 μg/ml ampicillin, 10 μg/ml chloramphenicol, and 25 μg/ml kanamycin. Cloning was performed in the *E. coli* strain Novable (Novagen) according to established protocols (22). Transformations in *B. subtilis* were carried out according to procedures described previously (23, 24). Restriction enzymes, T4 DNA ligase, and Vent polymerase were from New England Biolabs (Beverly, MA). Hotstar Taq polymerase was from Qiagen (Mississauga, Ontario, Canada). The Gateway cloning system was from Invitrogen. [U-14C]Glycerol-3-phosphate, Ni2+ chelating columns, and Superdex 200 columns were from Amersham Biociences. High-performance liquid chromatography columns and scintillation fluid were from Waters (Mississauga, Ontario, Canada). Chromatography was carried out using either an Amersham Biociences Äkta fast-protein liquid chromatography system or Waters High-performance liquid chromatography system. Filters were from Millipore (Nepean, Ontario, Canada). Mouse α-His antibodies were from Amersham Biosciences, donkey α-mouse-horseradish peroxidase and donkey α-rabbit-horseradish peroxidase antibodies were from Bio/Can Scientific (Mississauga). Polyclonal rabbit α-TagF and α-TagB antibodies were raised for us by Cocalico Biologicals (Reamstown, PA) using purified full-length protein. Dithiothreitol, isopropyl-β-D-thiogalactopyranoside, imidazole, MOPS, and ampicillin were from Bioshop (Burlington, Ontario, Canada). Potassium phosphate was from EM Science (Darmstadt, Germany). Protease Inhibitor Mixture Set III was from Calbiochem.

**Mutational Analysis of the TagF and TagB Proteins**

### TABLE ONE

| Strain             | Description | Source/Ref. |
|--------------------|-------------|-------------|
| **B. subtilis**    |             |             |
| EB6                | hisA1 argC4 metC3 | L5087 (41) |
| EB247              | leu8 tagF1   | BGSC* (LA486) |
| EB311              | leu8 tagF1 amyE:xyrP₆ₓₓₓ₆₆ tagF cat86 | (20) |
| EB486              | pheA1 purA16 hisA35 trpC2 tag-1 | L5058 (42) |
| EB527              | pheA1 purA16 hisA35 trpC2 tag-1 amyE:xyrP₆ₓₓₓ₆₆ tagB cat86 | This work |
| EB893              | EB486 with pRBl374 | This work |
| EB894              | EB486 with pRBtagRo2rbs | This work |
| EB984              | leu8 tagF1 amyE:xyrP₆ₓₓₓ₆₆ tagF(H474A) cat86 | This work |
| EB985              | leu8 tagF1 amyE:xyrP₆ₓₓₓ₆₆ tagF(H612A) cat86 | This work |
| EB1010             | EB486 with pRBtagBl(H126A) | This work |
| EB1011             | EB486 with pRBtagBl(H253A) | This work |
| EB1012             | EB486 with pRBtagBl(D271A) | This work |
| EB1089             | EB486 with pRBtagBl(E245A) | This work |
| EB1090             | EB486 with pRBtagBl(D279A) | This work |
| EB1091             | EB486 with pRBtagBl(D285A) | This work |
| EB1216             | pheA1 purA16 hisA35 trpC2 tag-1 amyE:xyrP₆ₓₓₓ₆₆ tagBl(H126A) cat86 | This work |
| EB1217             | pheA1 purA16 hisA35 trpC2 tag-1 amyE:xyrP₆ₓₓₓ₆₆ tagBl(H253A) cat86 | This work |
| EB1218             | pheA1 purA16 hisA35 trpC2 tag-1 amyE:xyrP₆ₓₓₓ₆₆ tagBl(D271A) cat86 | This work |
| EB1226             | pheA1 purA16 hisA35 trpC2 tag-1 amyE:xyrP₆ₓₓₓ₆₆ tagBl(E245A) cat86 | This work |
| EB1227             | pheA1 purA16 hisA35 trpC2 tag-1 amyE:xyrP₆ₓₓₓ₆₆ tagBl(D279A) cat86 | This work |
| EB1229             | pheA1 purA16 hisA35 trpC2 tag-1 amyE:xyrP₆ₓₓₓ₆₆ tagBl(D290A) cat86 | This work |
| EB1230             | pheA1 purA16 hisA35 trpC2 tag-1 amyE:xyrP₆ₓₓₓ₆₆ tagBl(D285A) cat86 | This work |
| EB1240             | pheA1 purA16 hisA35 trpC2 tag-1 amyE:xyrP₆ₓₓₓ₆₆ tagBl(D290A) cat86 | This work |
| EB1249             | EB486 with pRBtagBl(D290A) | This work |
| EB1275             | leu8 tagF1 amyE:xyrP₆ₓₓₓ₆₆ tagBl(F604A) cat86 | This work |
| EB1276             | leu8 tagF1 amyE:xyrP₆ₓₓₓ₆₆ tagBl(F630A) cat86 | This work |
| EB1277             | leu8 tagF1 amyE:xyrP₆ₓₓₓ₆₆ tagBl(F639A) cat86 | This work |
| EB1278             | leu8 tagF1 amyE:xyrP₆ₓₓₓ₆₆ tagBl(F645A) cat86 | This work |
| EB1279             | leu8 tagF1 amyE:xyrP₆ₓₓₓ₆₆ tagBl(F650A) cat86 | This work |
| E. coli            |             |             |
| BL21(DE3)          | F- ompT hsdSB (rB- mB-) gal dcm (DE3) | Novagen |
| Novablue           | endA1 hisdR7 (v₅₄₂ m₆K₂₂) sup44 thr-1 recA1 gyrA96 relA1 lac(F’ proA B lacZAM15:s-Tn10(Tcr)] | Novagen |

*a Bacillus Genetic Stock Center (Ohio State University, Columbus, OH).
at the carboxyl terminus of the protein. The enzyme was then purified as previously described (20). The variant enzymes described in this work were all similarly purified.

Tag Polymerase Assay—Polymerase reactions were carried out as previously described (20). Briefly, 250-μl reactions containing 3.33 mg of protein/ml of B. subtilis membranes and 50 nm purified TagF protein were initiated upon addition of CDP-glycerol to 150–4000 μM. Each reaction contained 0.2 μCi of CDP-[U-14C]glycerol. Reactions were allowed to proceed at room temperature for 20 min before being quenched by the addition of urea to 4 M. Membranes were then separated from the supernatant by ultracentrifugation and washed twice in reaction buffer. Finally, all supernatants and pellets were analyzed by liquid scintillation counting.

pH Effects for TagF—Steady-state kinetic parameters were determined for the native enzyme between pH 5.5 and 8.0 using the following overlapping buffers: 25 mM MES-NaOH (pH 5.5–6.5), 25 mM MOPS-NaOH (pH 6.5–7.5), and 25 mM EPPS-NaOH (pH 7.5–8.0). The ionic strength of each buffer solution was calculated using the Henderson-Hasselbalch equation and was adjusted to 20 mM through the addition of NaCl. At overlapping pH levels (i.e. pH 6.5 and pH 7.5) the enzyme was assayed in both buffer systems, and all data were included. The data were analyzed using Sigma Plot 8.0 (SPSS Scientific, Chicago, IL) and were fit using non-linear regression to the following equation for a single ionization,

$$\nu = \frac{V_{\text{max}} \lim}{1 + 10^{0.1 \left(H_0 - \text{pH} \right)}} \quad (\text{Eq. 1})$$

where $\nu$ represents the first order ($k_{\text{cat}}$) or second order ($k_{\text{cat}}/K_m$) rate constant, and $V_{\text{max}} \lim$ is the pH-independent value.

Site-directed Mutagenesis—The tagF mutants described were generated using the QuikChange® method (Stratagene, La Jolla, CA). Primers JS35–JS41 and their reverse complements were used as primer pairs in the reactions. pDEST14-tagF was used as template. Mutant alleles were then amplified by PCR using primers JS04 and JS05 and cloned into pSWEET (26) for in vivo complementation. Each clone was confirmed by nucleotide sequencing.

To generate equivalent mutations in tagB, the gene was first amplified by PCR from the chromosome of B. subtilis 168 using primers tagB-optbtsfor and tagBrev3 and cloned into the EcoRV site of pBluescript II SK+ (Stratagene, La Jolla, CA). Single crossover PCR was then used to introduce the desired mutations. 27-mer primer pairs encompassing four codons flanking the desired mutagenic codon were used to amplify tagB in two fragments using the T7 and T3 primers (the latter two primers bind to plasmid sequence). Following amplification, parental plasmid was digested using DpnI. Both tagB fragments were gel-purified and used as template for a second PCR reaction using T7 and T3 primers. Full-length product was gel-purified and cloned into pRBS74 (27) and pSWEET using appropriate restriction sites. All clones were confirmed by nucleotide sequencing.

Native Proteolysis—TagF protein (10 μg) was incubated in proteolysin buffer (50 mM HEPES, 40 mM KCl, 10 mM MgCl₂, pH 7.3) containing 2 μg/ml trypsin or chymotrypsin for 0, 5, 15, or 30 min. Reactions were quenched by the addition of phenylmethylsulfonyl fluoride to 0.5 mM followed by the addition of boiling Laemmli buffer (28). Samples were then analyzed by discontinuous SDS-PAGE using 3% stacking and 12% separating gels visualized with Coomassie Blue stain. Protease protection by the substrate was tested in the presence of CDP-glycerol (2 mM).

Conditional Complementation—tagF and tagB mutant alleles were cloned into the pSWEET chromosomal integration vector. The plasmids were linearized by restriction digest, and the linear DNA fragments containing the mutant alleles under the control of the Pαf promoter were transformed into strains EB247 or EB486. Successful chromosomal integrants (containing both the temperature-sensitive allele and a complementing tagF/tagB mutant allele) were grown in the presence of 2% xylose at 47 °C. Cultures were grown to A600 = 1.0, normalized for cell density, and serially diluted. 2 μl of each diluted sample were spotted onto LB-2% xylose agar and grown at 47 °C.

Western Analysis of Complementation Strains—Cell lysates were analyzed by Western blot to test for expression of the variant proteins. Expression of TagB from chromosomally integrated pSWEET was undetectable by Western blot, so strains harboring the mutant tagB alleles on pRB374 were used to test expression of these variants. 4-ml cultures of each strain were grown at 30 °C under conditions for expression from the mutant alleles. Cultures were grown to A600 = 0.5 (0.3 for tagF), pelleted, and resuspended in TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0). Samples were then treated with lysozyme (1 mg/ml) and finally boiled in Laemmli buffer (28). The resulting samples were then analyzed by Western blot using α-TagF or α-TagB polyclonal antibody and α-FtsY polyclonal antibody as loading control.

RESULTS

Carboxyl-terminal His₆ Tag Improves Yield of TagF Protein—Previously we reported the purification and characterization of amino-terminally His₆-tagged TagF protein (20). It was noted that this protein was difficult to purify to a substantial yield. Wild-type and mutant tagF alleles described in this study were cloned into pDEST-14 and engineered to be expressed with a carboxyl-terminal His₆ tag. Using this strategy, we were able to achieve an increase of ~10-fold in protein yield versus the amino-terminally tagged enzyme (~0.25 mg/liter versus ~2.5 mg/liter of culture). Steady-state kinetics showed no difference between the two tagged versions of TagF (data not shown). Carboxyl-terminally tagged enzyme was stable when stored at ~80 °C for several months with no appreciable loss in activity.

pH Dependence of TagF Activity—Nothing about the TagF reaction mechanism was known or could be inferred a priori from similarity to any known enzyme. We assayed the enzyme at various pH levels to identify any ionizable groups that might be important for catalysis. Steady-state kinetic parameters were determined for the native enzyme between pH 5.5 and 8.0 using a series of overlapping buffer systems (see “Materials and Methods”). Fig. 1 shows the relationships between the apparent first ($k_{\text{cat}}$) and second ($k_{\text{cat}}/K_m$) order rate constants for the TagF reaction versus pH. No value is reported at pH 5.5 in the plot of $\log(k_{\text{cat}})$ versus pH, because at this pH the underlying velocity versus substrate concentration curve showed a linear relationship through all substrate concentrations tested. Therefore a value for $k_{\text{cat}}$ could not be extracted, although $k_{\text{cat}}/K_m$ could still be determined from the slope of that line. The plots of $\log(k_{\text{cat}})$ versus pH and $\log(k_{\text{cat}}/K_m)$ versus pH both show an increase with pH with a slope of one followed by a plateau at higher pH. When fit to the equation for a single ionization (Equation 1), both relationships gave a pKₐ for the ionizable group near neutral (6.7 ± 0.2 and 7.0 ± 0.1, respectively). This suggests the presence of a group that must be deprotonated for function (i.e. activity is inhibited by high [H⁺]) in both the free enzyme (or the substrate) and the enzyme-substrate complex (29).

Mutational Analysis of TagF—The identification of an important ionizable group with a pKₐ near neutral implicated histidine as a possible critical residue in the function of TagF. Fig. 2 shows an amino acid sequence alignment of the carboxyl-terminal region of TagF against several other enzymes predicted to be involved in teichoic acid biosyn-
Mutational Analysis of the TagF and TagB Proteins

thesis in *B. subtilis* strains 168 and W23 and *Staphylococcus epidermidis*. It is noteworthy that these strains produce different teichoic acids (poly(glycerol phosphate) for strain 168 and *S. epidermidis* and poly(ribitol phosphate) for strain W23) yet express enzymes with a high degree of sequence homology to the Tag polymerase in our model *B. subtilis* strain 168. Two absolutely conserved histidine residues were identified at positions 474 and 612 of TagF. In addition, five other conserved acidic residues were deemed interesting, because the Tag polymerase has previously been shown to require Mg$^{2+}$ (or Ca$^{2+}$) ions for catalysis (17, 18). In a survey of known crystal structures, magnesium (and calcium) ions were found preferentially bound to hard oxygen ligands (30) which, in the absence of any structural information, justified the selection of the conserved Glu-604, Asp-630, Glu-639, Asp-645, and Asp-650 residues for further study. The seven identified residues were individually substituted to alanine by site-directed mutagenesis and subsequently expressed and purified to examine their effects on catalysis.

Each of the TagF variants purified in this study was tested for activity using the Tag polymerase assay (see "Materials and Methods"). TABLE TWO presents the results of these experiments in comparison to the wild-type enzyme. The Asp→Ala and Glu→Ala substitutions resulted in only minor changes in the second order rate constant $k_{cat}/K_m$. These variants displayed 4-fold or smaller reductions in $k_{cat}/K_m$ with no individual change in $k_{cat}$ or $K_m$ being greater than 2- or 4-fold, respectively. These effects were likely due to small changes in local environment accompanying the non-conservative amino acid substitutions. The D650A variant did not express in *E. coli*, which suggests that this residue may play a significant role in maintaining TagF structure. In contrast to the acidic variants, neither histidine variant exhibited any detectable activity under standard assay conditions. To assay the intrinsically low activity of the H474A variant, the enzyme concentration was increased to 500 nM, and 0.4 μCi of CDP-[U-14C]glycerol was used in a 60-min reaction. Under these conditions, we were able to extract the first order rate constant $V_{max}/K_m$ from the slope of a linear velocity versus substrate concentration plot for the H474A variant, but the H612A variant still exhibited no detectable activity. The dramatic effects of the histidine substitutions on enzyme activity (~5000-fold reduction in $k_{cat}/K_m$, TABLE TWO) suggested that these residues were important for polymerase activity.

Identification of a Protease-sensitive Site within the Carboxyl-terminal Region of TagF—TagF was incubated in the presence of trypsin and chymotrypsin in an attempt to gain some information about its domain structure and to identify a protease fingerprint associated with properly folded protein. Digestion of TagF by trypsin resulted in the generation of two fragments, which were stable for at least 30 min under reaction conditions (Fig. 3A). A nearly identical pattern was observed after digestion with chymotrypsin (data not shown), which suggested the presence of a protease-hypersensitive region within the enzyme’s structure. Western analysis of the digestion products using α-His$_6$ antibody showed that the smaller 21-kDa fragment contained the carboxyl terminus of the protein (data not shown) and placed the theoretical site of cleavage within the region of high similarity among TagF-like proteins. The carboxyl-terminal fragment was transferred to polyvinylidene difluoride membrane, excised, and subjected to amino-terminal sequencing. The amino-terminal sequence of the 21-kDa fragment was identified as YKFDL. This sequence is unique within the TagF primary sequence and is identified by an arrow in Fig. 2.

CDP-glycerol Protects TagF from Protease Digestion—The effects of substrate binding on protease sensitivity of TagF were tested by subjecting the enzyme to trypsin digestion in the presence of 2 mM CDP-glycerol. In the presence of its substrate, native TagF was significantly protected from trypsin digestion (Fig. 3A). Control experiments showed that the addition of CDP-glycerol had no effect on the digestion of BSA (data not shown). These results suggested that binding of CDP-glycerol to TagF either physically blocked the cleavage site or caused a conformational rearrangement that rendered the site no longer accessible. Together, the protease fingerprint of the free enzyme combined with protease protection in the presence of substrate provided us with a robust test that we could use to assess proper folding of the TagF variants. Each variant enzyme was tested in this way and found to be indistinguishable from the wild-type enzyme (Fig. 3B shows the results for the H474A variant).

Conditional Complementation of the tagF1 Defect Using Mutant tagF Alleles—The mutant *tagF* alleles were cloned into pSWEET, integrated into the chromosome of strain EB247 (*tagF1* temperature-sensitive strain), and tested for their ability to complement the lethal defect. We have shown previously (20) that wild-type *tagF* is capable of full complementation of this defect (strain EB311), thus confirming that the *tagF1* mutation alone is responsible for the observed phenotype and providing a manner in which to test proper *in vivo* function. Fig. 4A shows the results of dilution experiments performed on each of the conditionally complemented mutant strains to observe whether they were capable of suppressing the lethal *tagF1* phenotype. Successive 10-fold dilutions of cells were plated on LB-agar containing 2% xylose and grown at 47 °C. At this temperature, only the gene products expressed from pSWEET could be expected to carry out the essential Tag polymerase activity, because the mutant *tagF1* gene product is incapable of supporting
growth at this temperature (31–33). It can clearly be seen from Fig. 4A that the organism could not survive when either the H474A or H612A alleles (strains EB984 and EB985, respectively) were provided to complement the tagF1 defect, whereas the wild-type allele restored robust growth (strain EB311). These results indicate that disruption of either critical histidine residue results in both catastrophic reduction of TagF activity as measured in vitro and elimination of the enzyme’s ability to perform its function in vivo. Interestingly, we observed that the D650A

### TABLE TWO

| Name  | k\_cat | K\_m  | k\_cat/K\_m | (k\_cat/K\_m)\_wild type/(k\_cat/K\_m)\_variant |
|-------|--------|-------|-------------|---------------------------------------------|
| WT    | 14     | 230   | 5.9×10^4    | 4900                                        |
| H474A | 1.2×10^4 | <1.2×10^4 | >4900       |
| H612A | 4.5×10^4 | 1.5×10^4 | 3.9         |
| E604A | 1.8×10^4 | 1.7×10^4 | 3.5         |
| D630A | 700    | 750   | 3.9         |
| E639A | 10     | 700   | 3.5         |
| D645A | 13     | ND    | ND          |
| D650A | ND     | ND    | ND          |

*ND, not determined.

**FIGURE 2.** Alignment of TagF with proteins predicted to be involved in teichoic acid biosynthesis. Amino acid sequence alignment of the carboxyl-terminal region of TagF from *B. subtilis* strain 168 (168) against the TagB sequence from *S. epidermidis* (SE), two organisms which contain poly(glycerol phosphate) teichoic acids. The TagF sequence was also compared with the TarB, TarF, TarK, and TarL sequences from *B. subtilis* strain W23 (W23), a poly(ribitol phosphate) teichoic acid containing strain. Sequence alignment was performed using the ClustalW algorithm. Black highlights denote identical amino acids, whereas conservative substitutions are highlighted in gray. The amino acids at positions marked by asterisks were individually substituted to alanine in this study in order to examine their importance for catalysis. The arrow marks the site of trypsin cleavage within TagF.

**FIGURE 3.** Trypsin digestion and protease protection. Wild-type TagF and all variants produced in this study were tested for their sensitivity to digestion by trypsin using native proteolysis. Shown are the results for the wild-type enzyme (A) and the H474A variant (B). The enzyme (10 μg) was incubated with 2 μg/ml trypsin for 0-, 5-, 15-, and 30-min time intervals. Native proteolysis was also carried out in the presence of the substrate CDP-glycerol (2 mM). The positions of molecular weight markers (kDa) appear to the left of each gel and the time intervals (min) are listed across the top of each gel.
Mutational Analysis of the TagF and TagB Proteins

The Tag polymerase is a key enzyme in the biosynthesis of peptidoglycan in bacterial cell walls. This study focuses on the analysis of the TagF and TagB proteins, which are involved in this process. The researchers generated mutant alleles of these proteins and studied their effects on the growth of the bacterial strain.

They observed that a mutant allele (strain EB1279) was also incapable of complementation at 47 °C. This suggests that the histidine residues at positions 474 and 612 are important for the activity of the Tag polymerase.

The study also indicated that the histidine mutant alleles were unable to complement the temperature-sensitive defect at 47 °C, whereas the wild-type enzyme restored robust growth. This supports the functional importance of these residues.

The researchers also investigated the pH dependence of the TagF activity. They found that while the wild-type enzyme has a near neutral pH dependence, the histidine mutant enzymes showed a pH dependence of approximately 5-6. This is consistent with the presence of an active site base that must be protonated for activity.

In conclusion, the study highlights the importance of specific histidine residues in the activity of the TagF protein. These findings provide insights into the mechanism of action of the Tag polymerase and may guide future investigations into the role of specific residues in catalysis or metal binding.

**FIGURE 4.** Complementation studies using wild type and mutant tagF and tagB alleles. The wild type and mutant tagF (A) and tagB (B) alleles were integrated into the chromosome of their respective temperature-sensitive strains at the amyF locus using the pSWEET system. Shown is the growth of wild-type B. subtilis and the temperature-sensitive tagF and tagB strains (EB274 and EB486, respectively) engineered to express the identified enzyme variants. Each strain was grown in liquid culture at 30 °C to allow for the survival of all strains, normalized for cell density, and lysed. Samples were probed using polyclonal α-tagF or α-tagB antibody. Each sample was also probed using polyclonal α-FtsY antibody to ensure equal loading.

**FIGURE 5.** Expression of TagF and TagB variant enzymes. Expression of the TagF (A) and TagB (B) variant enzymes in each of the strains used for conditional complementation experiments was confirmed by Western blot. Strains were grown at 30 °C (to allow for the survival of all strains), normalized for cell density, and lysed. Samples were probed using polyclonal α-tagF or α-tagB antibody. Each sample was also probed using polyclonal α-FtsY antibody to ensure equal loading.
In addition to the histidine mutants, it was observed that the D650A (D290A in tagB) mutant allele was also unable to complement the temperature-sensitive defect. The TagF D650A variant did not express in E. coli and could not be purified to test in vitro activity. Consequently, it was also impossible to examine the proteolysis fingerprint of this variant or test for proteolysis protection in the presence of substrate, two experiments that were performed on all other variants to assess proper folding. However, the fact that the D650A variant and the corresponding TagB D290A variant did express in B. subtilis, yet failed to suppress their respective temperature-sensitive defects, raises the possibility that the failure to complement was due to the loss of Asp-650 specifically and not simply gross protein misfolding leading to degradation. Asp-650 sits within one of the most highly conserved regions of TagF (LITD<sup>650</sup>YSSV) and clearly warrants further study, along with His-474 and His-612, to determine what roles these residues play in the enzyme’s activity. Work is ongoing in our laboratory toward this end.

With this work we also provide the first insight into the physical structure of the TagF enzyme. Sequence alignments suggested that the active site of the polymerase could possibly be found within the carboxyl-terminal region. We examined variant enzymes containing amino acid substitutions within this region and identified two histidine residues that were critical to function both in vitro and in vivo and one aspartate residue whose alteration affected function in vivo. We further identified a protease sensitive site within the carboxy-terminal region of TagF that was significantly protected by the addition of substrate. The catalytically impaired histidine variants showed proteolysis protection that was indistinguishable from wild type suggesting that substrate binding was not drastically impaired in these variants and that the carboxyl-terminal region contained residues that were involved in catalysis. Together, these experiments confirm the location of the active site within the carboxy-terminal region of TagF and may give further information about catalysis. The location of the cleavage site is interesting in the context of the multiple sequence alignment because it falls within a region that is not universally conserved and, because of its susceptibility to proteases, is likely surface-exposed. Protection from proteolysis may have come as a result of CDP-glycerol binding to TagF and physically blocking the cleavage site. This would be consistent with the presence of the active site near the carboxyl terminus of the protein. However, an intriguing possibility is that the binding of the substrate may have caused a conformational rearrangement that rendered the cleavage site no longer accessible. Many processive enzymes contain structures that partially or completely encircle their polymeric substrates/products (reviewed in Ref. 37).

Mutational Analysis of the TagF and TagB Proteins

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Mutational Analysis of the TagF and TagB Proteins

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