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RESEARCH ARTICLE

# Phosphoserine Phosphatase Is Required for Serine and One-Carbon Unit Synthesis in *Hydrogenobacter thermophilus*

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**ABSTRACT** *Hydrogenobacter thermophilus* is an obligate chemolithoautotrophic bacterium of the phylum *Aquificae* and is capable of fixing carbon dioxide through the reductive tricarboxylic acid (TCA) cycle. The recent discovery of two novel-type phosphoserine phosphatases (PSPs) in *H. thermophilus* suggests the presence of a phosphorylated serine biosynthesis pathway; however, the physiological role of these novel-type metal-independent PSPs (iPSPs) in *H. thermophilus* has not been confirmed. In the present study, a mutant strain with a deletion of *pspA*, the catalytic subunit of iPSPs, was constructed and characterized. The generated mutant was a serine auxotroph, suggesting that the novel-type PSPs and phosphorylated serine synthesis pathway are essential for serine anabolism in *H. thermophilus*. As an autotrophic medium supplemented with glycine did not support the growth of the mutant, the reversible enzyme serine hydroxymethyltransferase does not appear to synthesize serine from glycine and may therefore generate glycine and 5,10-$CH_2$-tetrahydrofolate (5,10-$CH_2$-THF) from serine. This speculation is supported by the lack of glycine cleavage activity, which is needed to generate 5,10-$CH_2$-THF, in *H. thermophilus*. Determining the mechanism of 5,10-$CH_2$-THF synthesis is important for understanding the fundamental anabolic pathways of organisms, because 5,10-$CH_2$-THF is a major one-carbon donor that is used for the synthesis of various essential compounds, including nucleic and amino acids. The findings from the present experiments using a *pspA* deletion mutant have confirmed the physiological role of iPSPs as serine producers and show that serine is a major donor of one-carbon units in *H. thermophilus*.

**IMPORTANCE** Serine biosynthesis and catabolism pathways are intimately related to the metabolism of 5,10-$CH_2$-THF, a one-carbon donor that is utilized for the biosynthesis of various essential compounds. For this reason, determining the mechanism of serine synthesis is important for understanding the fundamental anabolic pathways of microorganisms. In the present study, we experimentally confirmed that a novel phosphoserine phosphatase in the obligate chemolithoautotrophic bacterium *Hydrogenobacter thermophilus* is essential for serine biosynthesis. This finding indicates that serine is synthesized from an intermediate of gluconeogenesis in *H. thermophilus*. In addition, because glycine cleavage system activity and genes encoding an enzyme capable of producing 5,10-$CH_2$-THF were not detected, serine appears to be the major one-carbon donor to tetrahydrofolate (THF) in *H. thermophilus*.

**KEYWORDS** amino acid biosynthesis, carbon metabolism, thermophiles

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*Hydrogenobacter thermophilus*, which belongs to the order *Aquificales*, is a strictly thermophilic hydrogen-oxidizing chemolithoautotroph that fixes carbon dioxide through the reductive tricarboxylic acid (TCA) cycle (1, 2). Recently, two novel-type phosphoserine phosphatases (PSPs; EC 3.1.3.3), metal-independent PSP iPSP1 and iPSP2, were identified and characterized following the purification of proteins with

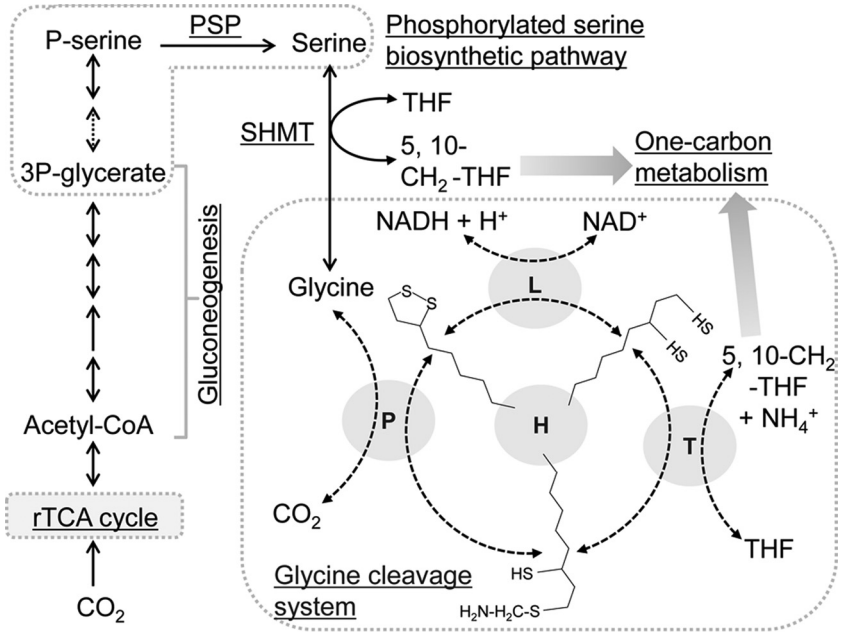


**FIG 1** Metabolic pathway related to serine biosynthesis in *H. thermophilus*. The unbroken arrows indicate the biochemically confirmed reactions, and dashed arrows indicated predicted reactions based on genomic analyses. CoA, coenzyme A; rTCA, reductive TCA.

phosphoserine phosphatase activity from *H. thermophilus* cells (3–5). iPSP1 is a homodimer of PspA subunits, whereas iPSP2 is a heterodimer of PspA and PspB. Although PspA and PspB share 37% identity and exhibit phosphatase activity, only the PspA subunit recognizes phosphoserine as a substrate (4). The discovery of iPSP1 and iPSP2 in *H. thermophilus* strongly suggests the presence of a phosphorylated serine biosynthesis pathway, in which serine is produced from the gluconeogenesis intermediate 3-phosphoglycerate in a three-step conversion process (Fig. 1) (6). Despite this speculation, the synthesis of serine from phosphoserine by the catalysis of iPSP in *H. thermophilus* has not been experimentally confirmed, and it is unclear whether other serine synthesis pathways are active in this bacterium.

In addition to iPSPs, *H. thermophilus* also possesses the enzyme serine hydroxymethyltransferase (SHMT), which has the potential to synthesize serine by catalyzing the reversible conversion of glycine using tetrahydrofolate (THF) as a one-carbon carrier (Fig. 1) (7). Methanotrophic and methylotrophic bacteria use SHMT for serine synthesis (8). Higher plants also synthesize a portion of serine from glycine (6). A one-carbon donor, 5,10-$CH_2$-tetrahydrofolate (5,10-$CH_2$-THF), which is used for this direct conversion reaction, is provided by the glycine cleavage system (9) or through the direct fixation of a one-carbon compound to THF (8, 10). Numerous organisms use SHMT for glycine synthesis from serine (11, 12). The one-carbon units produced in this direct reaction are used for the synthesis of several important compounds, including methionine, purine, and pyrimidine (13, 14). Therefore, identifying the components of the serine biosynthesis pathway is necessary to determine how an organism provides the one-carbon donor 5,10-$CH_2$-THF for the biosynthesis of various fundamental compounds, including serine, methionine, purines, and pyrimidines.

Here, we analyzed the serine biosynthesis pathway of *H. thermophilus* using a *pspA* deletion mutant (Δ*pspA*). A component involved in the uptake of serine and the main reaction by which 5,10-$CH_2$-THF is produced in *H. thermophilus* are also discussed.

## RESULTS

**Physiological characterization of *pspA* and *pspB* deletion mutants.** The constructed *pspA* deletion mutant of *H. thermophilus* was cultivated in a medium supple-

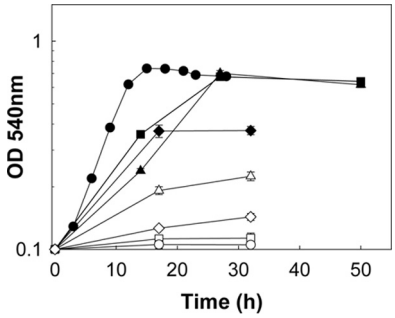


**FIG 2** Growth profiles of the Δ*pspA* mutant cultured in autotrophic medium supplemented with various serine concentrations. Growth of the Δ*pspA* mutant was monitored in an autotrophic medium not supplemented with serine (○) or supplemented with 10 μM (□), 25 μM (◇), 50 μM (△), 100 μM (◆), 300 μM (●), 400 μM (■), or 500 μM (▲) serine. The data are expressed as the mean ± standard error (SE) ($n = 3$).

mented with various concentrations of L-serine, and the cell densities at stationary phase were compared (Fig. 2). Clear growth was observed in medium supplemented with serine at concentrations of 50 μM or higher, confirming that the Δ*pspA* mutant is a serine auxotroph. The final cell yields were comparable between the cultures supplemented with 300, 400, and 500 μM L-serine; however, the Δ*pspA* mutant cells cultured in medium supplemented with 300 μM serine grew faster than those in the medium containing 400 or 500 μM serine (Fig. 2). Therefore, 300 μM serine was selected as the optimum concentration for the growth of the Δ*pspA* mutant. The serine concentration in the medium decreased with increasing growth of the Δ*pspA* mutant (Fig. 3A), indicating that serine was incorporated into the cells. In contrast, the supplementation of medium with serine had no detectable effects on cell growth of a *pspB* deletion (Δ*pspB*) mutant of *H. thermophilus* (Fig. 3B) or the wild type (Fig. 3C). No clear serine uptake by wild-type cells was observed either (Fig. 3C).

To confirm whether compounds derived from serine or 5,10-$CH_2$-THF (15–17) could support the growth of the Δ*pspA* mutant, Δ*pspA* cells were cultivated in inorganic medium supplemented with 300 μM glycine, L-tryptophan, methionine, histidine, or formate (Fig. 3A). No growth was detected in the first 24 h of culture (Fig. 3A). In contrast, the growth of wild-type cells was not inhibited, indicating that these compounds were not incorporated into cells, or that they were incorporated into cells but could not compensate for the inability of the Δ*pspA* mutant to synthesize serine.

**Evaluation of serine transport.** The uptake of serine by the Δ*pspA* mutant indicated the existence of a serine transporter in *H. thermophilus*. However, homologs of known serine transporters in *Escherichia coli* (18, 19, 34) ($Na^+$/serine threonine symporter [accession no. P0AGE4.1], $H^+$/threonine serine symporter [accession no.

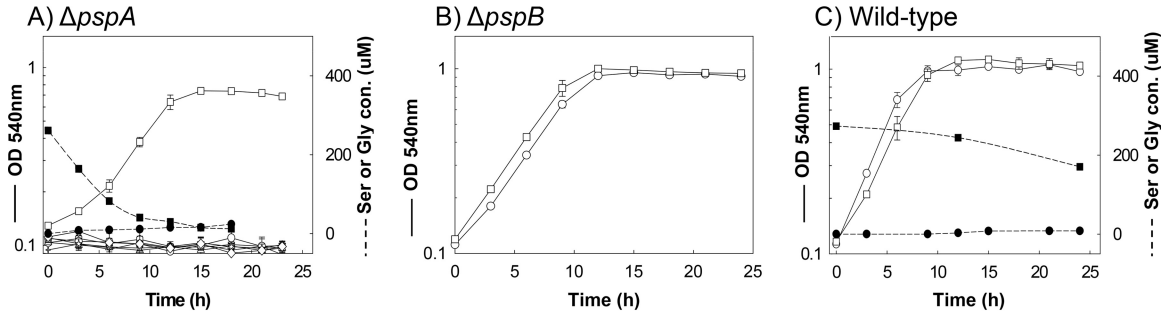


**FIG 3** Growth profiles and serine uptake by the Δ*pspA* mutant (A), Δ*pspB* mutant (B), and wild-type (C) strains. Growth was monitored in an autotrophic medium not supplemented (○) or supplemented with 300 μM Ser (□), Gly (△), Trp (◇), Met (⌔), His (†), or formate (☆). Serine and glycine concentrations (con.) within the medium supplemented with serine are shown with closed squares and closed circles, respectively. The data are expressed as the mean ± SE ($n = 3$).

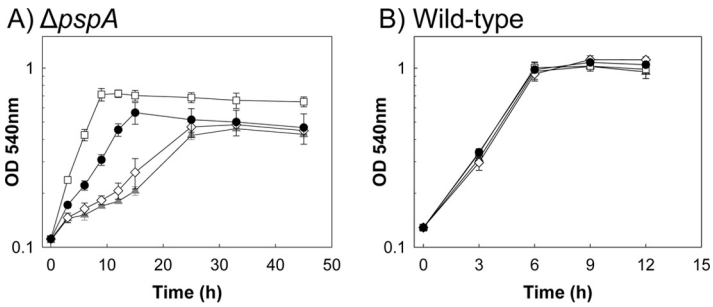


**FIG 4** Inhibitory effect of Leu, Ile, and Val on the growth of Δ*pspA* (A) and wild-type (B) cells. A 300 $\mu$M concentration of Val (●), Ile (◇), or Leu (△) or nothing (control, □) was added to autotrophic medium not supplemented or supplemented with 300 $\mu$M Ser for the wild-type and Δ*pspA* mutant strains, respectively. The data are expressed as the mean ± SE ($n$ = 3).

CDH66785], and serine transporter [accession no. ADX49536.1]) were not found in the *H. thermophilus* genome. In contrast, homologs of all five proteins that comprise the leucine-isoleucine-valine (LIV) transport system, which has been proposed to function as a serine transporter (18, 20), were detected in the genome. The candidates of the *H. thermophilus* LIV system components showed amino acid identities ranging from 26% to 38% and E values of $10^{-13}$ to $10^{-48}$ with those of *E. coli* (see Table S1 in the supplemental material).

Inhibition tests using leucine, isoleucine, and valine were performed to explore the possibility that the LIV system of *H. thermophilus* functions as a serine transporter. Growth inhibition of the Δ*pspA* mutant was detected when 300 $\mu$M leucine, isoleucine, or valine was added to medium containing 300 $\mu$M serine (Fig. 4A). In contrast, no growth inhibition was observed when leucine, isoleucine, or valine was added to the autotrophic medium of the wild-type strain (Fig. 4B). These data suggest that serine uptake is competitively inhibited by leucine, isoleucine, or valine and therefore indicate that the LIV system may function as a serine transporter in *H. thermophilus*.

**Detection of the glycine cleavage system.** A homology search was performed for *E. coli* homologs of the glycine cleavage system in *H. thermophilus*. Homologs of all protein components (P, H, T, and L proteins) of the *E. coli* glycine cleavage system were found. Notably, the N- and C-terminal domains of the *E. coli* P protein exist as two separate proteins in *H. thermophilus*. The candidate proteins shared 30% to 48% identity and had E values of $10^{-32}$ to $10^{-70}$ with those in *E. coli* (Table S2). Notably, the glycine cleavage system proteins were not highly conserved in the phylum *Aquificae*. For example, organisms in the families *Desulfurobacteriaceae* and *Hydrogenothermaceae* and several species in *Aquificaceae* lack at least one component of the glycine cleavage system (Table 1).

To confirm whether the glycine cleavage system is functional in *H. thermophilus*, the glycine cleavage activity in cell extract was assayed by measuring both the THF and $NAD^+$- or $NADP^+$-dependent decrease of glycine and the THF-dependent reduction of benzyl viologen (BV), an artificial electron acceptor alternative to $NAD(P)^+$. However, no activity (less than 20 nmol glycine cleaved by 1 mg of protein per min) was detected under the present experimental conditions (data not shown).

## DISCUSSION

In this study, the physiological role of novel-type PSPs in *H. thermophilus* was investigated using *pspA* and *pspB* deletion strains. The Δ*pspB* mutant did not show apparent phenotypic change under our standard autotrophic cultivation conditions. A physiological role of PspB is unclear so far, because previous biochemical analyses failed to identify a physiological substrate of PspB, while it seems to have relatively low phosphatase activity against *p*-nitrophenyl phosphate (3). Further studies are required to understand the reason why iPSP2, a heteromer of PspA and PspB, exists at the same level as iPSP1, a homodimer of PspAs in *H. thermophilus* (3). In contrast, a deletion

**TABLE 1** Homolog search of glycine cleavage system proteins in *Aquificae*[a]

| | | P protein N-terminal domain | | P protein C-terminal domain | | T protein | | H protein | | L protein | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Family | Organism | % identity | Accession no. | % identity | Accession no. | % identity | Accession no. | % identity | Accession no. | % identity | Accession no. |
| *Aquificaceae* | *Aquifex aeolicus* VF5 | 33 | WP_010880695 | 39 | WP_010881244 | 38 | O67441.2 | 49 | WP_010880694 | 28 | WP_010880444 |
| | *Hydrogenobacter thermophilus* TK-6 | 35 | WP_012964165 | 38 | WP_012963212 | 37 | WP_012963253 | 48 | WP_012964164 | 30 | WP_012963843 |
| | *Hydrogenobaculum* sp. strain Y04AAS1 | ND | | ND | | ND | | 37 | WP_012514115 | 27 | WP_012513374 |
| | *Thermocrinis albus* DSM 14484 | 37 | WP_012991979 | 38 | WP_012992260 | 37 | WP_012991224 | 45 | WP_012991980 | 30 | WP_012992228 |
| *Hydrogenothermaceae* | *Persephonella marina* EX-H1 | ND | | ND | | ND | | 44 | WP_012675418 | 35 | WP_012676035 |
| | *Sulfurihydrogenibium* sp. strain YO3AOP1 | ND | | ND | | ND | | 48 | WP_012459599 | 30 | WP_012459600 |
| *Desulfurobacteriaceae* | *Desulfurobacterium thermolithotrophum* | ND | | ND | | ND | | ND | | ND | |
| | *Thermovibrio ammonificans* HB-1 | ND | | ND | | ND | | ND | | ND | |

[a]Proteins which showed an E value of $<10^{-10}$ against queries are listed as possible candidates of glycine cleavage (GCV) proteins. *E. coli* genes (GenBank accession numbers NP_417379, NP_417381, NP_417380, and NP_414658 for GCV P, T, H, and L proteins, respectively) were used as queries (33). ND, not determined.

mutant of the PSPs' catalytic subunit, the Δ*pspA* mutant, was revealed to require serine for growth. Based on this finding, PspA and, most likely, the phosphorylated serine synthesis pathway, appear to be essential for serine synthesis in *H. thermophilus*.

The observed decrease in serine in the culture medium of the Δ*pspA* mutant suggests that this obligate autotroph possesses a serine uptake system. *H. thermophilus* lacks homologs of all three serine uptake systems in *E. coli* but encodes homologs of all *E. coli* LIV transport system components. In addition, growth impairment of Δ*pspA* cells was observed in cultures supplemented with branched-chained amino acids. The transport of serine by the *E. coli* LIV system has not been conclusively determined (18, 20, 21); however, the present results suggest that this system is involved in the uptake of serine. In contrast to the case of the Δ*pspA* mutant, the wild-type strain did not show clear uptake of serine. Therefore, quantitative real-time reverse transcription-PCR analysis was performed to see if the presence or absence of serine uptake is due to the presence or absence of the LIV transporter using *livK*, the first gene of the operon of the LIV transporter genes. Statistically significant differences were observed neither between the expression levels in the wild-type strains cultivated with and without serine nor the wild-type strain and Δ*pspA* mutant cultivated with serine (data not shown). These results suggest that the uptake of serine is regulated not by the expression level of the LIV transporter but by the intracellular serine concentration, although further studies are required to confirm this speculation.

*H. thermophilus* possesses SHMT, a reversible enzyme with the potential to synthesize serine from glycine and 5,10-$CH_2$-THF (7, 22). If *H. thermophilus* has the ability to synthesize glycine from compounds other than serine, such as glyoxylate, and can produce 5,10-$CH_2$-THF without an SHMT reaction, we speculated that the Δ*pspA* mutant would be able to grow without exogenously added serine. However, the Δ*pspA* mutant required supplemented serine to grow. This result seems to be reasonable because *H. thermophilus* has neither genes encoding the enzymes for the glyoxylate cycle nor enzymatic activities for the metabolism (23). Furthermore, if *H. thermophilus* possesses an active glycine cleavage system, supplementation of the culture medium with glycine may support the growth of the Δ*pspA* mutant, even if cells cannot produce sufficient glycine for growth without functional iPSPs. However, neither the growth of the Δ*pspA* mutant in autotrophic medium supplemented with glycine nor glycine cleavage system activity was observed. These results suggest that SHMT catalyzes the generation of glycine and 5,10-$CH_2$-THF in *H. thermophilus*.

THF-based one-carbon units are essential for fundamental metabolic processes, including nucleic acid biosynthesis and amino acid and vitamin metabolism (24). In the case of prokaryotes, serine cleavage by SHMT, glycine cleavage by the glycine cleavage system, and the direct fixation of formate to THF by formate-THF ligase (EC 6.3.4.3; also called formyltetrahydrofolate synthetase) are the major reactions that produce THF-based one-carbon donors (10, 25, 26). Although several glycine cleavage system candidates were detected in the genome of *H. thermophilus*, this system does not appear to be operative. As the components of this system are not highly conserved among members of the order *Aquificales*, and because truncated homologs of P protein and low-identity L proteins were detected in *H. thermophilus*, these proteins might have other functions in *H. thermophilus*. Formate-THF ligase is used for $CO_2$ fixation in the Wood-Ljungdahl pathway (10, 27) or one-carbon compounds by methylotrophs (28). However, *H. thermophilus*, which fixes $CO_2$ using the reductive TCA cycle, lacks a homolog of this enzyme. Taken together, these findings indicate that SHMT is the major provider of one-carbon units in *H. thermophilus*.

If SHMT is the major provider of one-carbon units and a glycine cleavage system is absent, the amount of glycine produced and one-carbon units in biosynthesis are stoichiometrically coupled. Therefore, excess glycine needs to be excreted or further converted. Since no clear accumulation of glycine in the medium was observed when the Δ*pspA* mutant and the wild type were cultivated with supplemented serine (Fig. 3A and C), excess glycine should be further catabolized *in vivo*. We are expecting that glycine is converted into glyoxylate and then used as a carbon source, because *H.*

*thermophilus* possesses at least 13 transaminase genes and a glycine oxidase (EC 1.4.3.19) candidate gene.

The present results demonstrate that iPSPs are essential for the synthesis of serine in *H. thermophilus*. This finding, together with the lack of detectable glycine cleavage system activity in this autotrophic bacterium, strongly suggests that serine is the major THF-based one-carbon donor in *H. thermophilus*.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** *H. thermophilus* TK-6 (IAM 12695, DSMZ 6534) was cultivated at 70°C under a gas phase of $H_2$-$O_2$-$CO_2$ (75:10:15 [vol/vol/vol]) in a 100-ml vial containing 10 ml of autotrophic medium, as described previously (2). A *pspA* deletion mutant of strain TK-6 (Δ*pspA*) was maintained in the autotrophic medium supplemented with 300 $\mu$M L-serine. To monitor the growth of *H. thermophilus* and the Δ*pspA* mutant, cells in early stationary phase were inoculated into fresh medium to give an optical density at 540 nm ($OD_{540}$) of approximately 0.1. For solid culture, autotrophic medium containing 300 $\mu$M L-serine and 10 $\mu g \cdot ml^{-1}$ $CuSO_4 \cdot 5H_2O$ was solidified with 1.0% (wt/vol) Gelrite (Wako Pure Chemical Industries, Osaka, Japan). When required, 100 $\mu g \cdot ml^{-1}$ ampicillin and 500 $\mu g \cdot ml^{-1}$ kanamycin were added to the medium for *Escherichia coli* and *H. thermophilus*, respectively.

**Deletion of *pspA* or *pspB* in *H. thermophilus*.** The *pspA* and *pspB* genes were deleted in *H. thermophilus*, using a previously established homologous recombination method (29), with pEX18Ap used as a vector in place of pUC19. To construct plasmids pEX18Ap-dpspA and pEX18Ap-dpspB, approximately 1.1-kbp fragments of the *pspA* and *pspB* genes were PCR amplified from *H. thermophilus* chromosomal DNA using the primer pairs *pspA*1_F (5′-TAT Gga att cGT AAG CCT GGA GAG-3′) and *pspA*1_R (5′-Gag gta ccA AGA AAA AGA CAC TGG-3′), and *pspA*2_F (5′-CAA ggt acc TAG CCT GCT TTT TAC-3′) and *pspA*2_R (5′-TAG tct aga GGA AGC TCT GGG AAG-3′) (lowercase letters indicate EcoRI, KpnI, or XbaI restriction endonuclease sites) for pEX18Ap-dpspA; and *pspB*1_F (5′-GCT gag ctc GTT TTG CCT ACA CC-3′) and *pspB*1_R (5′-TAA ggt acc TTT GCC ACA CAA GAC-3′), and *pspB*2_F (5′-GAG ggt acc GCA TAT CAC TTA TGG-3′) and *pspB*2_R (5′-AGC gga tcc TTT ACA GAA GAG GAG-3′) (lowercase letters indicate SacI, KpnI, or BamHI restriction endonuclease site) for pEX18Ap-dpspB. A thermostable kanamycin nucleotidyltransferase gene (*htk*) (30) was amplified using the primers htk_F (5′-AAA ggt acc ACA TTC GGT GAG AAG CTA CAG G-3′) and htk_R (5′-AAA ggt acc GGT CAT CGT TCA AAA TGG TAT G-3′) (lowercase letters indicate KpnI sites). These three fragments were digested with the appropriate restriction enzymes and cloned into pEX18Ap as a fused sequence.

After transfecting the constructed pEX18Ap-dpspA or pEX18Ap-dpspB into *H. thermophilus* by electroporation, positive transformants (designated Δ*pspA* and Δ*pspB* mutants) were selected on a solid medium supplemented with kanamycin. Disruption of the genes was confirmed by checking the length and sequence of PCR-amplified DNA fragments containing the *pspA* or *pspB* and *htk* genes.

**Quantification of serine and glycine.** Serine and glycine were detected and quantified using high-performance liquid chromatography with a reverse-phase column after phenylthiocarbamyl derivatization, as described previously (31, 32). Alanine was used as an internal standard.

**Preparation of cell extract.** *H. thermophilus* cells were harvested during the early stationary phase by centrifugation (10,000 × *g* for 15 min). The collected cells were resuspended in a 4-fold volume of 20 mM Tris-HCl (pH 8.0) and disrupted by sonication (Ultrasonic disruptor UD-201; Tomy, Japan). After centrifugation at 100,000 × *g* and 4°C for 1 h, the supernatant was collected and used as the cell extract.

**Glycine cleavage system assay.** Glycine cleavage system activity was assayed using two methods. In the first method, the decrease in glycine was monitored in 150 $\mu$l of a reaction mixture consisting of 20 mM Tris-HCl (pH 7.5 at 70°C), 5 mM glycine, 3.5 mM THF, 5 mM $NAD^+$ or $NADP^+$, 10 mM 2-mercaptoethanol, and cell extract (80 $\mu$g of protein) during incubation at 70°C. The second method utilized an artificial electron acceptor, benzyl viologen (BV), in place of $NAD(P)^+$ to monitor the reduction of BV. Four hundred microliters of the reaction mixture, which contained 20 mM Tris-HCl (pH 7.5 at 70°C), 5 mM glycine, 3.5 mM THF, 5 mM BV, 0.125 mM dithiothreitol, and cell extract (200 $\mu$g of protein), was incubated at 70°C in argon gas. The reduction of BV was monitored photometrically at 578 nm.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00409-17.

**SUPPLEMENTAL FILE 1,** PDF file, 0.1 MB.

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