Short Communication

Expressions of alkaline phosphatase genes during phosphate starvation are under positive influences of multiple cell wall hydrolase genes in *Bacillus subtilis*

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Alkaline phosphatases (APases) are hydrolases that remove phosphate groups from various molecules. During phosphate starvation, the two-component system PhoPR of *Bacillus subtilis* activates transcription of APase genes (*phoA*, *phoB*, and *phoD*) to generate free phosphate groups for uptake and utilization (Allenby et al., 2005; Antelmann et al., 2000). Among the vegetatively expressed cell wall hydrolase genes *lytC*, *lytD*, *lytE*, *lytF*, *cwlO*, and *cwlS* of *B. subtilis*, only *lytE* and *cwlO* are not regulated by SigD (Chen et al., 2009; Huang et al., 2013; Noone et al., 2014; Tseng et al., 2011). It has been reported that *LytE* and *CwlO* are required for cell elongation and that cells lacking both enzymes exhibit a synthetic lethal phenotype (Bisicchia et al., 2007; Hashimoto et al., 2012). *lytC* encodes a major vegetative autolysin and is regulated by both SigA and SigD (Kuroda and Sekiguchi, 1991; Lazarevic et al., 1992).

The monocistronic genes *lytE* and *phoA* of *B. subtilis* are adjacent and divergently transcribed (Barbe et al., 2009). Given the co-induction of *lytE* and *phoA* by heat stress (Helmann et al., 2001; Huang et al., 2013; Tseng et al., 2011) and the co-inhibition of the levels of *LytE* and *PhoA* by *Spo0A* in *B. subtilis* (Hulett et al., 1994; Jensen et al., 1993; Kodama et al., 2007), we wondered whether there would be other correlation(s) between expression of *lytE* and *phoA*. Firstly, effect of mutation of *lytE* on transcription of *phoA* was investigated by using PCR and the primer pair A64 plus A65 (Table S1) to construct the pDL-based integrative plasmid pGS2095 (Table 1), which carries a transcriptional fusion of the regulatory region of *phoA* with *bgaB*. The resulting plasmid was then introduced into *B. subtilis* cells by the method of Contente and Dubnau (1979) followed by integration at the *amyE* locus of the wild-type *B. subtilis* or the *lytE* mutant BNB11 to generate strains BM1670 and BM1671, respectively. Cor-

rect integrants were screened by a method described previously (O’Kane et al., 1986). Since it is known that *phoA* is expressed in response to phosphate starvation (Hulett et al., 1990), we cultured *B. subtilis* cells in the slightly modified low-phosphate defined medium, which consisted of 3 mM (NH₄)₂SO₄, 35 mM MgSO₄, 2 mM CaCl₂, 0.68 mM sodium citrate, 0.3 mM KH₂PO₄, 3 μM FeSO₄, 1 μM MnSO₄, 10 μM ZnSO₄, and 50 mM Tris-HCl (pH 7.4), supplemented with 50 μg ml⁻¹ tryptophan plus 0.4% glucose. Activity of the β-galactosidase *BgaB* was measured as described previously (Schroegel and Allmansberger, 1997). It was found that mutation of *lytE* led to a considerable downregulation in expression of *phoA-bgaB* (Fig. 1A). The *phoA-bgaB* fusion was also integrated at the *amyE* loci of the *lytD* mutant BNB12 and the *lytF* mutant BM1713 to generate the strain BM1691 and BM1719, respectively. Construction of the *lytF* mutant BM1713 by integration of the plasmid pGS2136 onto the chromosome through a single-crossover recombination was performed as described previously (Fedhila et al., 2002). The pRN5101-based plasmid pGS2136 was constructed by PCR and the primer pair A284 plus A285. The result showed that mutation of either *lytD* or *lytF* had no effect on expression of *phoA-bgaB* (Fig. 1B).

We next examined whether mutation of *lytE* would have any effect on transcription of other APase genes during phosphate starvation. The plasmids pGS2137 (using the primer pair A338 plus A339) and pGS2738 (B409 plus B410), which carry a transcriptional fusion of the regulatory region of *phoB* or *phoD* with *bgaB*, respectively, was constructed and integrated individually at the *amyE* locus of the wild-type *B. subtilis* or the *lytE* mutant BNB11. It was found that mutation of *lytE* also caused a downregulation of expression of *phoB-bgaB* or *phoD-bgaB*

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Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Source or reference
<i>Escherichia coli</i>		
DH5 α	F' ϕ 80dlacZAM15 Δ (lacZYA-argF) <i>recA1 gyrA endA1 relA1 supE44 hsdR17</i>	Laboratory stock
<i>Bacillus subtilis</i>		
168	<i>trpC2</i>	Laboratory stock
BKE34800	<i>trpC2</i> Δ cwlO::erm	Bacillus Genetic Stock Center
BKE35620	<i>trpC2</i> Δ lytC::erm	Bacillus Genetic Stock Center
BNB11	<i>trpC2</i> <i>lytE</i> ::pENB11	(Tseng et al., 2011)
BNB12	<i>trpC2</i> <i>lytD</i> ::pENB12	(Tseng et al., 2011)
BNB13	<i>trpC2</i> <i>lytE</i> ::pENB11 <i>amyE</i> ::pENB14	(Tseng et al., 2011)
BM1670	<i>trpC2</i> <i>amyE</i> ::pGS2095	This study
BM1671	<i>trpC2</i> <i>lytE</i> ::pENB11 <i>amyE</i> ::pGS2095	This study
BM1691	<i>trpC2</i> <i>lytD</i> ::pENB12 <i>amyE</i> ::pGS2095	This study
BM1692	<i>trpC2</i> <i>amyE</i> ::pGS2117	This study
BM1693	<i>trpC2</i> <i>lytE</i> ::pENB11 <i>amyE</i> ::pGS2117	This study
BM1711	<i>trpC2</i> <i>amyE</i> ::pGS2137	This study
BM1712	<i>trpC2</i> <i>lytE</i> ::pENB11 <i>amyE</i> ::pGS2137	This study
BM1713	<i>trpC2</i> <i>lytF</i> ::pGS2136	This study
BM1719	<i>trpC2</i> <i>lytF</i> ::pGS2136 <i>amyE</i> ::pGS2095	This study
BM1750	<i>trpC2</i> <i>amyE</i> ::pENB14	This study
BM2546	<i>trpC2</i> <i>amyE</i> ::pGS2738	This study
BM2550	<i>trpC2</i> <i>lytE</i> ::pENB11 <i>amyE</i> ::pGS2738	This study
BM2587	<i>trpC2</i> Δ lytC::erm <i>amyE</i> ::pGS2137	This study
BM2588	<i>trpC2</i> Δ cwlO::erm <i>amyE</i> ::pGS2095	This study
BM2589	<i>trpC2</i> Δ lytC::erm <i>amyE</i> ::pGS2095	This study
BM2590	<i>trpC2</i> Δ cwlO::erm <i>amyE</i> ::pGS2137	This study
BM2591	<i>trpC2</i> Δ cwlO::erm <i>amyE</i> ::pGS2738	This study
BM2593	<i>trpC2</i> Δ lytC::erm <i>amyE</i> ::pGS2738	This study
Plasmids		
pDL	Ap ^r , Cm ^r ; <i>amyE</i> integration vector for generation of transcriptional fusions to <i>bgaB</i>	(Yuan and Wong, 1995)
pRN5101	Ap ^r , Em ^r ; integration vector used for gene disruption	(Fedhila et al., 2002)
pENB11	pRN5101 carrying an internal region close to the N terminus of <i>lytE</i> (+17 to +372 relative to the translational start site of <i>lytE</i>)	(Tseng et al., 2011)
pENB12	pRN5101 carrying an internal region close to the N terminus of <i>lytD</i> (+67 to +427 relative to the translational start site of <i>lytD</i>)	(Tseng et al., 2011)
pENB14	pDL carrying the regulatory and coding regions of <i>lytE</i> (−327 to +1079)	(Tseng et al., 2011)
pGS2095	pDL carrying the regulatory region of <i>phoA</i> (−276 to +89)	This study
pGS2117	pDL carrying the regulatory region of <i>phoP</i> (−350 to +112 relative to the translational start site of <i>phoP</i>)	This study
pGS2136	pRN5101 carrying an internal region close to the N terminus of <i>lytF</i> (+55 to +348 relative to the translational start site of <i>lytF</i>)	This study
pGS2137	pDL carrying the regulatory region of <i>phoB</i> (−320 to +37 relative to the translational start site of <i>phoB</i>)	This study
pGS2738	pDL carrying the regulatory region of <i>phoD</i> (−470 to −16 relative to the translational start site of <i>phoD</i>)	This study

in the low-phosphate defined medium (Figs. 1C and 1D).

It was previously shown that PhoA and PhoB constitute about 98% of the APase activity expressed during phosphate starvation (Hulett et al., 1994). To further confirm the influence of *lytE* on expression of APase genes, we measured the levels of APase activity in the wild-type *B. subtilis* and the *lytE* mutant cultured in the low-phosphate defined medium. Activities of APase were determined according to the method described previously (Pragai and Harwood, 2002). One unit of APase was defined as the amount of enzyme that hydrolyzes one nanomole of *p*-nitrophenolphosphate (PNPP) in one minute at 37°C. Specific activity of APase was calculated as units of activity per milliliter of culture divided by optical density at 600 nm. The result showed that the levels of APase activity in the *lytE* mutant were remarkably lower than those in the wild-type *B. subtilis* (Fig. 2A), confirming the positive influence of *lytE* on expression of APase genes. In contrast, mutation of either *lytD* or *lytF* had no significant influence on the levels of APase activity (Fig. 2B).

For a complementation analysis, we integrated the plasmid pENB14, which carries an intact *lytE* gene plus its regulatory region (Table 1), at the *amyE* locus of the *lytE* mutant. It was found that the levels of APase activity were partially restored in this complemented mutant (Fig. 2A). We have no satisfactory explanation why the levels of

APase activity could not be completely restored. We also integrated the plasmid pENB14 at the *amyE* locus of the wild-type *B. subtilis* to generate strain BM1750. It was found that the wild-type *B. subtilis* carrying an extra copy of the *lytE* gene exhibited higher levels of APase activity than the wild-type control (Fig. 2A). Taken together, these results support the notion that expression of APase genes during phosphate starvation is under the positive influence of *lytE*.

It is known that the response regulator PhoP directly activates transcription of *phoA*, *phoB*, and *phoD* during phosphate starvation (Allenby et al., 2005; Antelmann et al., 2000). We attempted to explore whether the influence of *lytE* on expression of APase genes might be mediated through PhoP. We first determined the effect of mutation of *lytE* on transcription of *phoP*. The plasmid pGS2117 (using the primer pair A267 plus A268), which carries a transcriptional fusion of the regulatory region of *phoP* with *bgaB*, was constructed and integrated at the *amyE* locus of the wild-type *B. subtilis* or the *lytE* mutant. The result showed that mutation of *lytE* led to an upregulation rather than a downregulation of *phoP*-*bgaB* expression in the low-phosphate defined medium (Fig. 2C), suggesting that the influence of *lytE* on expression of APase genes is, at least in part, not mediated through PhoP and may involve other regulatory mechanism.

Fig. 1. Expression of APase genes in various *B. subtilis* strains.

(A) The *phoA-bgaB* fusion (pGS2095) in the wild-type *B. subtilis* (BM1670: solid circles) or the *lytE* mutant (BM1671: open squares). Cells were cultured in the low-phosphate defined medium to an OD₆₀₀ of 0.3 (approximately 12 h) and then continued to grow for various time periods (as indicated) followed by harvesting for BgaB activity assays. (B) The *phoA-bgaB* fusion in the wild-type *B. subtilis* (BM1670: solid circles), the *lytD* mutant (BM1691: open squares), or the *lytF* mutant (BM1719: open triangles). (C) The *phoB-bgaB* fusion (pGS2137) in the wild-type *B. subtilis* (BM1711: solid circles) or the *lytE* mutant (BM1712: open squares). (D) The *phoD-bgaB* fusion (pGS2738) in the wild-type *B. subtilis* (BM2546: solid circles) or the *lytE* mutant (BM2550: open squares). Each value shown in (A) to (D) is the mean $\pm$ SD of three independent experiments.

Fig. 2. Comparison of the levels of APase activity in various *B. subtilis* strains and effect of *lytE* mutation on expression of the *phoP-bgaB* fusion.

(A) The wild-type *B. subtilis* (solid circles), the *lytE* mutant (BNB11: solid squares), the *lytE* mutant complemented with an intact *lytE* gene (BNB13: open squares), and the wild-type *B. subtilis* carrying an extra copy of the *lytE* gene (BM1750: open circles). (B) The wild-type *B. subtilis* (solid circles), the *lytD* mutant (BNB12: open squares), and the *lytF* mutant (BM1713: open triangles). (C) The *phoP-bgaB* fusion (pGS2117) in the wild-type *B. subtilis* (BM1692: solid circles) or the *lytE* mutant (BM1693: open squares). Cells were grown in the same way as described in Fig. 1A. Each value shown in (A) to (C) is the mean $\pm$ SD of three independent experiments.

Fig. 3. Effect of *cwI* deletion on expression of various APase genes.

(A) The *phoA-bgaB* fusion (pGS2095) in the wild-type *B. subtilis* (BM1670: solid circles) or the *cwI* mutant (BM2588: open squares). (B) The *phoB-bgaB* fusion (pGS2137) in the wild-type *B. subtilis* (BM1711: solid circles) or the *cwI* mutant (BM2590: open squares). (C) The *phoD-bgaB* fusion (pGS2738) in the wild-type *B. subtilis* (BM2546: solid circles) or the *cwI* mutant (BM2591: open squares). Cells were grown in the same way as described in Fig. 1A. Each value shown in (A) to (C) is the mean $\pm$ SD of three independent experiments.

Fig. 4. Effect of *lytC* deletion on expression of various APase genes.

(A) The *phoA-bgaB* fusion (pGS2095) in the wild-type *B. subtilis* (BM1670: solid circles) or the *lytC* mutant (BM2589: open squares). (B) The *phoB-bgaB* fusion (pGS2137) in the wild-type *B. subtilis* (BM1711: solid circles) or the *lytC* mutant (BM2587: open squares). (C) The *phoD-bgaB* fusion (pGS2738) in the wild-type *B. subtilis* (BM2546: solid circles) or the *lytC* mutant (BM2593: open squares). Cells were grown in the same way as described in Fig. 1A. Each value shown in (A) to (C) is the mean $\pm$ SD of three independent experiments.

Given that *LytE* and *CwI* have partially overlapping functions in cell wall elongation (Bisicchia et al., 2007; Hashimoto et al., 2012), we also tested the effect of *cwI* deletion on expression of various APase genes. The re-

sults showed that deletion of the monocistronic gene *cwIO* likewise led to a downregulation of expression of *phoA*-, *phoB*-, and *phoD-bgaB* in the low-phosphate defined medium (Figs. 3A to 3C). The major autolysin gene *lytC* is the last gene of the *lytABC* operon. The effect of deletion of *lytC* on expression of various APase genes was also investigated. The results revealed that, in the *lytC* mutant, expression of *phoA*-, *phoB*-, and *phoD-bgaB* in the low-phosphate defined medium was also downregulated (Figs. 4A to 4C). How multiple cell wall hydrolase genes affect expressions of APase genes during phosphate starvation is an interesting question and remains to be further clarified.

In conclusion, this study demonstrates that expressions of alkaline phosphatase genes in *B. subtilis* during phosphate starvation are under the influences of multiple cell wall hydrolase genes, which appear to have positive contributions to the phosphate starvation response.

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Supplementary Materials

Table S1. Primers used in this study.

Supplementary table is available in our J-STAGE site (<http://www.jstage.jst.go.jp/browse/jgam>).

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