Activation of extracytoplasmic function sigma factors upon removal of glucolipids and reduction of phosphatidylglycerol content in *Bacillus subtilis* cells lacking lipoteichoic acid

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(Received 30 August 2018, accepted 2 November 2018; J-STAGE Advance published date: 9 April 2019)

In *Bacillus subtilis*, extracytoplasmic function (ECF) sigma factors are activated by reduction of phosphatidylglycerol (PG) content, absence of glucolipids, or absence of lipoteichoic acid (LTA). LTA is synthesized by polymerization of the glycerophosphate moiety of PG onto diglucosyldiacylglycerol (DGLcDG), a major glucolipid in *B. subtilis*, in the plasma membrane. Thus, reduction of PG content or absence of glucolipids might cause some changes in LTA, and hence we investigated whether reduction of PG content or absence of glucolipids induces the activation of ECF sigma factors independently from an ensuing change in LTA. Disruption of *ugtP*, responsible for glucolipid synthesis, in cells lacking LTA caused an additive increase of activation levels of σ^M , σ^X , σ^V and σ^Y (3.1-, 2.2-, 2.1- and 1.4-fold, respectively), relative to their activation levels in cells lacking LTA alone. Reduction of PG content (by repressing P_{spac} -*pgsA*) in the cells lacking LTA caused an additive increase of activation levels of σ^M , σ^W and σ^V (2.3-, 1.9- and 2.2-fold, respectively). These results suggested that absence of glucolipids or reduction of PG alone, not the possible secondary alteration in LTA, leads to changes that affect the regulation systems of some ECF sigma factors in the plasma membrane.

Key words: ECF sigma factor, lipoteichoic acid, glucolipids, phosphatidylglycerol, lipid composition

INTRODUCTION

Bacillus subtilis has seven extracytoplasmic function (ECF) sigma factors, σ^M , σ^V , σ^W , σ^X , σ^Y , σ^{YlaC} and σ^Z , which are a group of bacterial sigma factors that direct transcription of genes involved in tasks such as maintenance of cells upon external stresses. Except σ^Z , these are regulated directly by their respective cognate transmembrane anti- σ factors, which sequester the σ factors and keep them inactive. Under stress conditions the σ factors are released from the anti- σ factors, form RNA polymerase holoenzymes and transcribe the respective regulon genes. The genes encoding σ and anti- σ factors form operons whose transcription is autoregulated. Acti-

vation of ECF sigma factors can result from a number of stresses on the cell envelope. For example, σ^M is activated in response to high salt, cell wall antibiotics, ethanol, heat, acid and superoxide stresses (Thackray and Moir, 2003; Asai et al., 2005; Luo and Helmann, 2012); σ^V is strongly and specifically activated in response to lysozyme (Guariglia-Oropeza and Helmann, 2011; Ho et al., 2011); σ^W is activated in response to salt, alkali, antibiotics inhibiting cell wall biosynthesis and oxidative stresses (Petersohn et al., 2001; Helmann, 2002; Cao et al., 2003; Asai et al., 2005; Butcher and Helmann, 2006); and σ^X is activated by inhibitors of peptidoglycan and wall teichoic acid (WTA) biosynthesis, and serves to alter cell envelope properties to protect against cationic antimicrobial peptides (Helmann, 2002; Cao and Helmann, 2004; Murray and Stanley-Wall, 2010). Among these ECF sigma factors, it is clear that σ^V and σ^W are activated by proteolysis of anti-sigma factors via regulated intramembrane proteolysis (Schobel et al., 2004; Zellmeier et al., 2006; Heinrich et al., 2009; Hastie et al., 2013, 2014, 2016).

Edited by Kei Asai

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DOI: <http://doi.org/10.1266/ggs.18-00046>

As shown in Fig. 1, *B. subtilis* membranes contain various lipids, including glucosylated diacylglycerol, monoglucosyldiacylglycerol (MGlcDG), diglucosyldiacylglycerol (DGlcDG) and triglucosyldiacylglycerol (TDGlcDG), and a complex glycerophospholipid, lysylphosphatidylglycerol (LysylPG), in addition to the glycerophospholipids found in *Escherichia coli* membranes, phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL) (Matsumoto et al., 2015). All genes responsible for synthesis of final lipid products are dispensable, except for *pgsA*, which encodes phosphatidylglycerophosphate synthase and is responsible for the committed step in PG synthesis. Lipoteichoic acid (LTA), a polymer of glycerol-1-phosphate derived from PG attached to DGlcDG, by which it is anchored on the membrane, is present on the outer leaflet of the membrane (Neuhaus and Baddiley, 2003; Reichmann and Gründling, 2011).

ugtP encodes a glucosyltransferase that processively transfers glucose from UDP-glucose to diacylglycerol (Jorasch et al., 1998). Cells with a deletion of *ugtP* that are completely lacking glucolipids show abnormal cell morphology and activation of three ECF sigma factors, σ^M , σ^V and σ^X (Salzberg and Helmann, 2008; Matsuoka et al., 2011; Hashimoto et al., 2013). The phenotype is complemented by expression of heterologous glucolipid synthase from *Acholeplasma laidlawii* (Matsuoka et al., 2016). Moreover, we reported that σ^V is activated without proteolysis of RsiV in the $\DeltaugtP$ cells (Seki et al., 2017). Cells harboring *P_{spac}-pgsA* in place of the wild-type *pgsA* allele have a PG content reduced by half (from

28.1% to 14.1%), arrested cell growth, and induction of σ^M and σ^V at 60 min after removal of inducer (Hashimoto et al., 2009).

Bacillus subtilis has four LTA synthase genes homologous to *LtaS* in *Staphylococcus aureus*: *ltaS* (formerly *yflE*), *yfnI*, *yqgS* and *yvgJ* (Gründling and Schneewind, 2007a; Schirner et al., 2009). All LTA synthesis genes can be simultaneously deleted and therefore LTA is dispensable for *B. subtilis* growth (Schirner et al., 2009; Wörmann et al., 2011). Finally, six ECF sigma factors, σ^M , σ^X , σ^W , σ^{YlaC} , σ^V and σ^Y , are induced in cells lacking LTA because of disruption of all four LTA synthase genes (Hashimoto et al., 2013). However, the exact contributions of these membrane components to the activation of ECF sigma factors in *B. subtilis* cells are still unclear.

In the case of *S. aureus*, deletion of *yfpP*, which is a homolog of *B. subtilis* *ugtP*, induces an alteration in the anchor of LTA from DGlcDG to DG (Kiriukhin et al., 2001; Gründling and Schneewind, 2007b). Therefore, it is believed that reduction of PG content and absence of glucolipids in *B. subtilis* cause abnormalities in LTA, as a result of which some ECF sigma factors are induced in cells lacking glucolipids or having reduced PG content. However, the pattern of activation of ECF sigma factors differs between *B. subtilis* cells with a deletion of some LTA synthase genes and those without glucolipids or with reduced PG content (Hashimoto et al., 2009, 2013). To address this difference, we investigated the effect of PG reduction and absence of glucolipids on LTA profile and activity of ECF sigma factors in this study.

Fig. 1. Biosynthetic pathways for phospholipids in *B. subtilis*. The compiled pathways are from Matsumoto et al. (2015). The gene product catalyzing each step is indicated. PG, Phosphatidylglycerol; PE, phosphatidylethanolamine; CL, cardiolipin; lysylPG, lysylphosphatidylglycerol; PA, phosphatidic acid; CDP-DG, CDP-diacylglycerol; MGlcDG, monoglucosyldiacylglycerol; DGlcDG, diglucosyldiacylglycerol; TGlcDG, triglucosyldiacylglycerol; DG, diacylglycerol; LTA, lipoteichoic acid.

MATERIALS AND METHODS

Bacterial strains, media and bacterial growth The *B. subtilis* and *E. coli* strains and plasmids used in this study are listed in Table 1. Luria-Bertani (LB) medium contained 1% tryptone (Difco), 0.5% yeast extract (Difco) and 1% NaCl. Synthetic media CI and CII were used for competence development for transformation of *B. subtilis* cells (Anagnostopoulos and Crawford, 1961). When required, the following supplements were added to the media (ml^{-1}): 50 or 100 μg spectinomycin (Sigma), 0.5 μg erythromycin (Sigma), 10 μg neomycin (Wako), 5 μg chloramphenicol (Sigma), 5 μg tetracycline (Sigma) or 100 μg ampicillin (Wako). Isopropyl- β -D-1-thiogalactopyranoside (IPTG) (Sigma) was added at a final concentration of 1 mM when required. Cells were grown at 37 °C, and growth was monitored by measuring turbidity with a TAITEC photometer, miniphoto518R (530 nm filter). For solid media, 1.5% agar (Difco) was included.

Genetic and recombinant DNA procedures These were based on standard methods (Miller, 1992; Sambrook and Russell, 2001).

SDS-PAGE and immunoblot analysis of LTA Cells of 168 (wild-type), YTB019 (ΔugtP) and SLD08 (ΔltaS ΔyfnI ΔyqgS ΔyvgJ) were grown to $\text{OD}_{530} = 0.5$ at 37 °C in 5 ml LB medium and then diluted 1/10 into 5 ml of fresh LB medium and grown to $\text{OD}_{530} = 0.5$. MHB001 ($\text{pgsA}::\text{P}_{\text{spac}}\text{-pgsA}$) cells were cultivated overnight at 37 °C in 5 ml LB medium containing 1 mM IPTG and then washed three times using fresh LB medium without IPTG and diluted to $\text{OD}_{530} = 0.05$ into 5 ml fresh LB medium without IPTG. Cells were grown to the point at which turbidity stopped increasing ($\text{OD}_{530} = 0.5$) and 5 ml of culture was collected. Bacterial pellets were suspended in 2 $\times$ protein sample buffer normalized for OD_{530} readings; that is, 25 μl of 2 $\times$ sample buffer was used per ml culture of $\text{OD}_{530} = 0.5$. Samples were boiled for 45 min, centrifuged at 19,000 g for 5 min at 4 °C, and 10 μl of the supernatant was loaded on a 15% Extra PAGE One Precast Gel

Table 1. Bacterial strains and plasmids used in this study

Strain	Genotype	Source
<i>B. subtilis</i>		
168	<i>trpC2</i>	Laboratory stock
YTB019	168 $\Delta\text{ugtP}::\text{kan}$	Matsuoka et al., 2011
MHB001	168 $\text{pgsA}::\text{P}_{\text{spac}}\text{-pgsA}$ <i>erm</i>	Hashimoto et al., 2009
MBS12	168 $\Delta\text{ltaS}::\text{spc}$ $\Delta\text{yfnI}::\text{tet}$	Hashimoto et al., 2013
SLD06	168 $\Delta\text{ltaS}::\text{spc}$ $\Delta\text{yfnI}::\text{tet}$ ΔyqgS	Hashimoto et al., 2013
SLD07	168 $\Delta\text{yfnI}::\text{tet}$ ΔyqgS ΔyvgJ	Hashimoto et al., 2013
SLD08	168 $\Delta\text{ltaS}::\text{spc}$ $\Delta\text{yfnI}::\text{tet}$ ΔyqgS ΔyvgJ	Hashimoto et al., 2013
SLD09	SLD07 $\Delta\text{ugtP}::\text{kan}$	This study
SLD10	SLD08 $\Delta\text{ugtP}::\text{kan}$	This study
SLD11	SLD07 $\text{pgsA}::\text{P}_{\text{spac}}\text{-pgsA}$ <i>erm</i>	This study
SLD12	SLD08 $\text{pgsA}::\text{P}_{\text{spac}}\text{-pgsA}$ <i>erm</i>	This study
<i>E. coli</i>		
UE54	MG1655 <i>lpp-2</i> Δara714 <i>rscF::mini-Tn10</i> <i>cam</i> $\text{pgsA}::\text{FRT-kan-FRT}$	Shiba et al., 2004
UE83	MC4100 <i>lpp-2</i> Δara714	Nagahama et al., 2006
FT01	UE83 $\text{pgsA}::\text{FRT-kan-FRT}$	This study
Plasmid		
pFZY1	<i>F'</i> lac replicon, <i>lacZY</i> <i>bla</i>	Koop et al., 1987
pFZY1- P_{sigM}'	pFZY1 $\text{P}_{\text{sigM}}'\text{-lacZY}$	Seki et al., 2015
pFZY1- P_{sigV}	pFZY1 $\text{P}_{\text{sigV}}'\text{-lacZY}$	Seki et al., 2015
pFZY1- P_{sigX}'	pFZY1 $\text{P}_{\text{sigX}}'\text{-lacZY}$	Seki et al., 2015
pBR322	<i>bla</i> <i>tet</i>	Balbás et al., 1986
pBR-sigM-anti-sigM	pBR322 $\text{P}_{\text{sigM}}'\text{-sigM-yhdL-yhdK}$ <i>tet</i>	Seki et al., 2015
pBR-sigV-anti-sigV	pBR322 $\text{P}_{\text{sigV}}'\text{-sigV-rsiV}$ <i>tet</i>	Seki et al., 2015
pBR-sigX-anti-sigX	pBR322 $\text{P}_{\text{sigX}}'\text{-sigX-rsiX}$ <i>tet</i>	Seki et al., 2015

(Nacalai Tesque). LTA-specific primary antibody (clone 55; HyCult Biotechnology) and peroxidase-conjugated AffiniPure goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories) were used at a dilution of 1:1000 for LTA detection. Incubation with antibody was performed in TBS-T buffer containing 2% BSA (Sigma). Luminata Forte Western HRP substrate (Millipore) was used for immunodetection in Western blotting experiments according to the manufacturer's instructions. Western analysis bands were detected by a C-DiGit scanner (LI-COR) and signal intensities were analyzed by ImageStudio (LI-COR).

Real-time RT-PCR Cells of 168, YTB019, SLD08, SLD10 (Δ ltaS Δ yfnI Δ yqgS Δ yvgJ Δ ugtP), MHB001 and SLD12 (Δ ltaS Δ yfnI Δ yqgS Δ yvgJ pgsA::Pspac-pgsA) were grown under the same conditions described above for the SDS-PAGE experiments. A 500- μ l aliquot of each sample was collected, and RNA was then extracted using a Total RNA Purification Kit (Jena Bioscience) and an RNase-free DNase set (Roche Applied Science) according to the manufacturer's recommendations. The DNase-treated RNA was reverse-transcribed using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO) according to the manufacturer's recommendations. Real-time RT-PCR with SYBR Green I was performed using Thunderbird SYBR qPCR Mix (TOYOBO). Real-time RT-PCR was carried out as described previously, using the pairs of primers for *sigA*, *sigM*, *sigV*, *sigW*, *sigX*, *sigY* and *ylaC* listed in Supplementary Table S1 (Hashimoto et al., 2013). Each real-time RT-PCR was performed in at least quintuplicate with *sigA* as the internal standard.

Lipid analysis [14 C]-labeled membrane lipids of 168, YTB019, SLD08, SLD10, MHB001 and SLD12 were obtained as previously described (Hashimoto et al., 2009, 2013). Lipid fractions were subjected to 2D thin layer chromatography on silica gel (no. 60, Merck); the first dimension was with chloroform/methanol/water (65:25:4, by volume) and the second was with chloroform/methanol/acetic acid (65:25:10, by volume) as described previously (Kawai et al., 2004). Spots for [14 C]-labeled lipids were visualized and quantified with an FLA-7000 bioimaging analyzer (Fujifilm). The molar percentage of each component (evaluated from the relative number of carbon atoms) was calculated.

β -galactosidase assay in *E. coli* The *E. coli* cells with plasmids were grown to mid-log phase and 1-ml aliquots of the culture were withdrawn, and β -galactosidase activity in the cells was assayed as described previously (Wang and Doi, 1984).

RESULTS AND DISCUSSION

Lack of glucolipids affects the structure of LTA To reveal the effect of an absence of glucolipids and reduction of PG content on the LTA profile in *B. subtilis*, cell extracts from a mid-log phase culture ($OD_{530} = 0.5$) of wild-type cells (168 strain, hereafter called WT cells), cells lacking glucolipids (YTB019 strain, hereafter called Δ GLs cells), cells with a reduced PG content (MHB001 strain, hereafter called PG^{Red.} cells) and cells lacking LTA (SLD08 strain, hereafter called Δ LTA cells) were prepared and LTA content analyzed by Western blot. A signal in the 15-kDa area was detected in WT cells but not in Δ LTA cells (Fig. 2), indicating that it is an LTA-specific signal. In Δ GLs cells the signal was shifted to the 20-kDa area (Fig. 2). This reduced LTA migration on SDS-PAGE observed in *B. subtilis* cells with a deletion of *ugtP* suggests that *ugtP* deletion induces the alteration of the LTA anchor from DGlCDG to DG, as seen in the case of the *S. aureus* *ypfP* deletion (Gründling and Schneewind, 2006). However, no change of signal pattern was observed in PG^{Red.} cells. These results suggest that not the reduction of PG, but the absence of DGlCDG affected the LTA profile. In addition, a broad signal

Fig. 2. LTA detection by Western blotting. LTA was extracted from *B. subtilis* strains 168 (wild-type; WT), YTB019 (Δ ugtP; Δ GLs), MHB001 (pgsA::P_{spac}-pgsA; PG^{Red.}) and SLD08 (Δ ltaS Δ yfnI Δ yqgS Δ yvgJ; Δ LTA). The samples were normalized based on culture OD_{530} values, separated by 15% SDS-PAGE, and detected with an LTA-specific antibody by C-DiGit scanner. The positions of protein molecular mass markers (in kDa) are indicated on the left.

around the 35-kDa area, which is a WTA signal caused by LTA antibody cross-reacting with WTA (Wörmann et al., 2011), was observed in Δ LTA cells (Fig. 2). Although the signal around 35 kDa was not observed in WT cells or PG^{Red.} cells, a weak signal was detected in Δ GLs cells. The change of LTA profile as well as the lack of LTA may reflect an increase of the WTA content to compensate for the LTA abnormality.

Lack of glucolipids activates ECF sigma factors independent of abnormalities in LTA To determine whether or not the lack of glucolipids activates ECF sigma factors independent of the change in LTA profile observed in Δ GLs cells (Fig. 2), we deleted *ugtP* from Δ LTA cells and analyzed the activation levels of ECF sigma factors. The mRNA level of ECF sigma factors was regarded as the activation level, because the transcription of each ECF sigma factor is autoregulated. The mRNA levels of the genes for six ECF sigma factors in Δ GLs cells, Δ LTA cells and cells lacking both LTA and glucolipids (SLD10 strain, hereafter called Δ LTA Δ GLs cells) were determined by real-time RT-PCR to estimate the activation levels of sigma factors. In Δ GLs cells the mRNA levels of the four ECF sigma factor genes *sigM*, *sigX*, *sigV* and *sigY* were increased 2.3-, 1.5-, 2.7- and 1.5-fold, respectively,

compared with those of WT cells (Fig. 3). In this work a novel activation of σ^Y was observed. In Δ LTA cells the mRNA levels of *sigM*, *sigX*, *sigW*, *ylaC*, *sigV* and *sigY* were increased (Fig. 3). In Δ LTA Δ GLs cells the mRNA levels of *sigM*, *sigX*, *sigV* and *sigY* were increased 4.5-, 1.6-, 4.1- and 3.7-fold, respectively, compared with those of WT cells, but the levels of *sigW* and *ylaC* were not changed (Fig. 3). This result shows that the effects of a lack of both glucolipids and LTA on the activation of three sigma factors (*sigM*, *sigV* and *sigY*) were additive in Δ GLs Δ LTA cells, suggesting that the removal of glucolipids activates the three ECF sigma factors σ^M , σ^V and σ^Y independent of the absence of LTA.

To investigate whether the additive increase of activation levels of σ^M , σ^V and σ^Y observed in Δ LTA Δ GLs cells was caused by a secondary effect on the lipid composition induced by simultaneous deletion of the *ugtP* and LTA synthase genes, the lipid composition of cells lacking glucolipids and/or LTA was examined (Table 2). In Δ LTA cells, CL, CDP-DG and DG were accumulated (2.3-, 6.9- and 2.8-fold, respectively), and DGlcdG and LysylPG were reduced (4.0- and 1.5-fold) compared with their content in WT cells. In Δ GLs cells, the CL content of Δ GLs cells was increased (2.7-fold) over WT cells and CDP-DG content was decreased (8.0-fold). In Δ LTA Δ GLs cells, the CDP-

Fig. 3. Activation levels of ECF sigma factors in *B. subtilis*. Strains used were *B. subtilis* 168 (wild-type; WT) and its derivatives, SLD08 (Δ taS Δ yfnI Δ yqgS Δ yvgJ; Δ LTA), YTB019 (Δ ugtP; Δ GLs), SLD10 (Δ taS Δ yfnI Δ yqgS Δ yvgJ Δ ugtP; Δ LTA Δ GLs), MHB001 (*pgsA*::*P*_{spac}-*pgsA*; PG^{Red.}) and SLD12 (Δ taS Δ yfnI Δ yqgS Δ yvgJ *pgsA*::*P*_{spac}-*pgsA*; Δ LTA PG^{Red.}). WT cells, Δ LTA cells, Δ GL cells and Δ LTA Δ GLs cells were cultivated in LB medium to exponential growth phase (OD₅₃₀ = 0.5) and total RNA was extracted and used for preparation of cDNA samples. Cultures of PG^{Red.} cells and Δ LTA PG^{Red.} cells were grown in LB medium supplemented with 1 mM IPTG to exponential growth phase, transferred into fresh medium and cultivated without IPTG to the point at which turbidity reached an OD₅₃₀ of 0.5, and then total RNA was extracted and used for preparation of cDNA samples. The cDNA samples were then subjected to real-time RT-PCR analysis with the appropriate primer pairs for *sigM*, *sigV*, *sigW*, *sigX*, *sigY* and *ylaC*, with the primer pair for *sigA* as the internal control. Mean values $\pm$ SD, calculated from six measurements excluding the highest and lowest values obtained from eight PCR assays, of the level of each sigma factor mRNA in the mutant cells, relative to those of WT cells (dotted line), are presented. Asterisks indicate significant differences as determined by Student's *t*-test: **P* < 0.01.

Table 2. Lipid compositions of mutants

Strain	% of total lipid										
	PG	PE	CL	LysylPG	PA	CDP-DG	MGlcDG	DGlcDG	TGlcDG	DG	Others
168	36.2±7.7	23.8±2.7	8.3±3.9	11.6±0.5	1.9±0.2	1.6±0.4	1.2±0.7	8.0±0.7	0.9±0.4	4.9±0.8	2.1±0.7
Δ LTA	20.9±2.3	16.2±1.2	19.2±7.5	7.6±0.5	2.7±2.5	11.0±3.2	0.6±0.2	2.0±0.5	0.6±0.3	13.8±3.2	5.4±2.8
Δ GLs	26.7±4.2	21.1±1.7	22.4±8.4	12.8±0.7	1.9±0.6	0.2±0.04	N.D.	N.D.	N.D.	7.6±1.5	7.4±1.9
Δ LTA Δ GLs	35.4±6.5	18.0±1.4	9.5±3.2	13.5±1.4	4.6±2.1	1.6±0.4	N.D.	N.D.	N.D.	14.8±1.7	2.7±1.7
PG ^{Red.}	3.4±1.0	47.6±1.8	3.7±0.4	6.8±0.9	3.5±0.7	2.1±0.3	0.6±0.4	6.8±0.8	0.1±0.05	19.2±0.6	6.2±0.4
Δ LTA PG ^{Red.}	5.7±0.7	38.9±2.2	6.6±3.1	4.4±0.9	2.5±0.2	7.1±1.7	0.6±0.1	2.5±1.1	0.2±0.05	24.7±1.6	6.8±2.6

Data shown are mean $\pm$ SD values from three different cultures. N.D.: not detected.

Table 3. Relationship between activation of ECF sigma factors and lipid composition

Cells	ECF sigma factor	Lipid composition
Δ LTA	σ^M , σ^X , σ^W , σ^{YlaC} , σ^V , σ^Y	GL (-), CL (+), CDP-DG (+), LysylPG (-), DG (+)
Δ GL	σ^M , σ^X , σ^V , σ^Y	GL (N.D.), CL (+), CDP-DG (-)
Δ LTA Δ GL	σ^{M*} , σ^X , σ^W , σ^{YlaC} , σ^{V*} , σ^{Y*}	GL (N.D.)
PG ^{Red.}	σ^M , σ^W , σ^{YlaC} , σ^V	PG (-), PE (+), CL (-), LysylPG (-), DG (+)
Δ LTA PG ^{Red.}	σ^{M**} , σ^{W**} , σ^{YlaC**} , σ^{V**} , σ^Y	PG (-), PE (+), CL (-), GL (-), CDP-DG (+), LysylPG (-), DG (+)

* Asterisks indicate that the ECF sigma factor was activated additively in the Δ LTA Δ GL cells compared with activation in Δ LTA cells and Δ GL cells.

** Double asterisks indicate that the ECF sigma factor was activated additively in the Δ LTA PG^{Red.} cells compared with activation in Δ LTA cells.

(+): increase; (-): decrease; N.D.: not detected.

DG content was also decreased (6.9-fold) compared with that of Δ LTA cells, indicating that *ugtP* deletion induces the reduction of CDP-DG content. Furthermore, the CL content was decreased (2.0-fold) in Δ LTA Δ GLs cells compared with that in Δ LTA cells. Since CL was accumulated in Δ GLs cells, the decrease of CL content observed in the Δ LTA Δ GLs cells was not caused by *ugtP* deletion. Thus, the change of CL content in the Δ LTA Δ GLs cells may be caused by a secondary effect of simultaneous deletion of the *ugtP* and *LTA* synthase genes. Table 3 shows the relationship between the activated ECF sigma factors and the lipid content, which were changed in each mutant cell. In Δ LTA Δ GLs cells, only glucolipid content was changed. Nevertheless, σ^M , σ^V and σ^Y were additively activated. If the change of CL and CDP-DG content activates σ^M , σ^V and σ^Y , the activation levels of these ECF sigma factors should be decreased in the Δ LTA Δ GLs cells, because these lipids were back to wild-type levels. However, the activation levels of σ^M , σ^V and σ^Y were additively increased in the Δ LTA Δ GLs cells (Fig. 3). Therefore, the change of CL and CDP-DG content is not the cause of the activation of σ^M , σ^V and σ^Y .

Taken together, these results show that a lack of glucolipids activates σ^M , σ^V and σ^Y independent of the changes in *LTA* structure and other lipid composition caused by deletion of *ugtP*. The results also suggest that σ^M , σ^V and σ^Y sensed the lack of glucolipids and of *LTA* as distinct stresses, but that σ^X sensed them as a

single stress. Although there are no reports that a lack of glucolipids is induced by environmental stresses in *B. subtilis*, we found that glucolipid content decreased when *B. subtilis* cells were grown in high salt condition (S. Matsuoka, unpublished results). Therefore, the membrane of cells without glucolipids may be mimicking that of cells exposed to high salt stress.

Reduction of phosphatidylglycerol content induces ECF sigma factors independent of a possible change in *LTA*

The reduction of PG content by restriction of *pgsA* expression did not result in a change of *LTA* profile (Fig. 2). This suggests the possibility that reduction of PG content directly induces ECF sigma factors irrespective of *LTA*. To determine whether the reduction of PG content can induce the ECF sigma factors in the absence of *LTA*, *pgsA* expression in Δ LTA cells was restricted and we analyzed the activation levels of ECF sigma factors in the Δ LTA cells with a reduced PG content (SLD12 strain, hereafter called Δ LTA PG^{Red.} cells). The mRNA levels of the genes for six ECF sigma factors in PG^{Red.} and Δ LTA PG^{Red.} cells were determined by real-time RT-PCR to estimate the activation levels of the six sigma factors. WT cells were used as a control for comparison of mRNA levels. In PG^{Red.} cells the levels of mRNA from the four ECF sigma factor genes *sigM*, *sigW*, *ylaC* and *sigV* were increased 3.7-, 3.5-, 1.5- and 4.5-fold, respectively, compared with those of WT cells (Fig. 3). In this work the

activation of σ^W and σ^{YlaC} was observed in addition to σ^M and σ^V . Moreover, the mRNA level of *sigX* was decreased 3.3-fold in PG^{Red.} cells. In Δ LTA PG^{Red.} cells the mRNA levels of *sigM*, *sigW*, *ylaC*, *sigV* and *sigY* were increased 3.4-, 3.5-, 1.3-, 4.4- and 1.6-fold, respectively, compared with those of WT cells, but the level of *sigX* was decreased 2.0-fold (Fig. 3). The activation levels of σ^M , σ^W and σ^V in Δ LTA PG^{Red.} cells were higher than those in Δ LTA cells, indicating that the reduction of PG content activated these ECF sigma factors even without LTA. Therefore, these results suggest that the reduction of PG content induces σ^M , σ^W and σ^V directly.

The activation levels of four ECF sigma factors, except σ^X and σ^Y , in Δ LTA PG^{Red.} cells corresponded with those in PG^{Red.} cells (Fig. 3). To test whether the correspondence of the activation levels of the four ECF sigma factors (σ^M , σ^W , σ^{YlaC} and σ^V) between Δ LTA PG^{Red.} cells and PG^{Red.} cells was caused by insufficient reduction of PG content in Δ LTA PG^{Red.} cells, the lipid composition of PG^{Red.} cells and Δ LTA PG^{Red.} cells was examined (Table 2). In PG^{Red.} cells CL and lysylPG, which are positioned downstream of PG in the lipid biosynthesis pathway (Fig. 1), were also reduced (2.2- and 1.7-fold, respectively) in addition to PG (10.6-fold) compared with their levels in WT cells. Moreover, the PE and DG content of PG^{Red.} cells was increased (2.0- and 3.9-fold, respectively). In Δ LTA PG^{Red.} cells PG, CL and lysylPG levels were decreased (3.7-, 2.9- and 1.7-fold, respectively) and PE and DG levels were increased (2.4- and 1.8-fold, respectively) compared with those in Δ LTA cells. The reduction level of PG compared to that in WT cells (6.4-fold) in Δ LTA PG^{Red.} cells was comparable with that seen in PG^{Red.} cells. This indicates that the

correspondence of the activation levels of the four ECF sigma factors between Δ LTA PG^{Red.} cells and PG^{Red.} cells was not caused by insufficient reduction of the PG content of Δ LTA PG^{Red.} cells. Table 3 shows that in Δ LTA PG^{Red.} cells, the effects of deletion of LTA synthase genes and restriction of *pgsA* expression on lipid composition were additive. Nevertheless, the effects of deleting LTA synthase genes on activation levels of σ^M , σ^W , σ^{YlaC} and σ^V were masked. Therefore, the correspondence suggests that the activation mechanisms of σ^M , σ^W , σ^{YlaC} and σ^V sensed the reduction of PG content and lack of LTA as the same stress. As in the case of *E. coli* LacY lactose permease, proper distribution of negative charge on the cell membrane may be required for folding and function of anti-sigma factors (Dowhan and Bogdanov, 2009; Bogdanov et al., 2010). The effect of a lack of LTA on membrane negative charge density may be weak enough to be masked by the reduction of PG content, because LTA, which is localized to the outer leaflet of the cell membrane through its linkage to DGlCDG, is farther from the membrane than PG, which is a component of the membrane. Hence, the effect of the absence of LTA on activities of ECF sigma factors in Δ LTA PG^{Red.} cells may be masked owing to the reduction in PG levels. Since PG is an essential lipid molecule for *B. subtilis* growth, significant reduction of PG content is not induced by environmental stress. On the other hand, the PG content of a daptomycin-resistant strain is reportedly low, similar to that of PG^{Red.} cells (Hachmann et al., 2011). σ^M and σ^W are also activated in the daptomycin-resistant strain, suggesting that these ECF sigma factors are activated by a reduction of PG content.

Fig. 4. Effect of activities of σ^M , σ^V and σ^X on PG using an *E. coli* heterologous expression system. (A) *E. coli* UE83 (WT) and its derivative strain FT01 (Δ *pgsA*; Δ PG), harboring pFZY1-P_{sigM} and pBR-sigM-anti-sigM (*sigM* operon), pFZY1-P_{sigV} and pBR-sigV-rsiV (*sigV* operon), pFZY1-P_{sigX} and pBR-sigX-rsiX (*sigX* operon), were used. (B) UE83 (WT) and FT01 (Δ PG), harboring pFZY1-P_{sigM} (*sigM*), pFZY1-P_{sigV} (*sigV*) or pFZY1-P_{sigX} (*sigX*), and pBR322 (void) were used as control assays. Overnight cultures were diluted 100-fold in LB medium and cultivated with shaking at 30 °C. At OD₅₃₀ = 0.3 on the miniphoto518R photometer, β -galactosidase activity was measured. The means and standard errors of three measurements are shown.

Fig. 5. Summary of relationship between activation of ECF sigma factors and changes of lipid composition. Lack of GLs (ΔGLs) and lack of lipoteichoic acid (ΔLTA) activated σ^{M} and σ^{V} independently, whereas ΔLTA and reduction of PG content ($\text{PG}^{\text{Red.}}$) were not distinguishable in their activation of σ^{M} and σ^{V} . Therefore, ΔGLs and $\text{PG}^{\text{Red.}}\text{-}\Delta\text{LTA}$ activate σ^{M} and σ^{V} as distinct stresses. σ^{Y} was activated by ΔGLs and ΔLTA independently but not activated by $\text{PG}^{\text{Red.}}$. σ^{X} was not additively activated by ΔGLs and ΔLTA . Hence, these activate σ^{X} as the same stress. ΔLTA and $\text{PG}^{\text{Red.}}$ were not distinguishable in their activation of σ^{W} and σ^{YlaC} . Hence, these activate σ^{W} and σ^{YlaC} as the same stress.

Induction of σ^{M} and σ^{V} in *E. coli* cells lacking PG

Escherichia coli can lack PG due to deletion of *pgsA* (Kikuchi et al., 2000; Suzuki et al., 2002). To confirm that the activities of σ^{M} , σ^{V} and σ^{X} were altered by the absence of PG, the activation levels of σ^{M} , σ^{V} and σ^{X} were examined using an *E. coli* heterologous expression system. We deleted the *pgsA* gene in a strain with a defect in *lpp* and introduced two plasmids: one was a pBR322 derivative having an operon of an ECF sigma factor and anti-sigma factor (either *sigM-yhdLK*, *sigV-rsiV* or *sigX-rsiX*) and the other an F' plasmid having a transcriptional fusion of a promoter recognized by an ECF sigma factor and *lacZ* ($P_{\text{sigM}}\text{-lacZ}$, $P_{\text{sigV}}\text{-lacZ}$ or $P_{\text{sigX}}\text{-lacZ}$) as a reporter, allowing us to monitor β -galactosidase activity as a proxy for the induction levels of σ^{M} , σ^{V} and σ^{X} . In the ΔpgsA cells the activities of σ^{M} and σ^{V} were significantly increased, by 13.7- and 2.3-fold, respectively, compared with those in WT cells (Fig. 4A), but were not increased in ΔpgsA cells lacking the *sigM-yhdLK* or *sigV-rsiV* operons (Fig. 4B), indicating that σ^{M} and σ^{V} are induced by the absence of PG in *E. coli* cells. These results support our hypothesis that the reduction of PG content activates σ^{M} and σ^{V} independent of the absence of LTA in $\Delta\text{LTA PG}^{\text{Red.}}$ cells of *B. subtilis* (Fig. 3). Unexpectedly, the *sigX-rsiX* operon did not work properly in *E. coli* ΔpgsA cells (Fig. 4A).

CONCLUSION

In this study, we found that the deletion of *ugtP* additively activated σ^{M} , σ^{V} and σ^{Y} in cells lacking LTA, and that the restriction of *pgsA* expression also additively activated σ^{M} , σ^{V} and σ^{W} (Fig. 3). Moreover, σ^{M} and σ^{V} responded directly to a lack of PG in *E. coli* as well as a lack of glucolipids (Fig. 4A) (see also Seki et al., 2015). Therefore, we concluded that a lack of glucolipids and a reduction of PG content directly activate some ECF sigma factors independently of LTA disorder in *B. subtilis*. Our data also suggested that σ^{M} and σ^{V} can sense ΔGLs and $\text{PG}^{\text{Red.}}\text{-}\Delta\text{LTA}$ as distinct stresses; σ^{X} can sense $\Delta\text{GLs-}\Delta\text{LTA}$ and $\text{PG}^{\text{Red.}}\text{-}\Delta\text{LTA}$ as distinct stresses; σ^{Y} can sense ΔGLs and ΔLTA as distinct stresses; σ^{W} and σ^{YlaC} can sense $\text{PG}^{\text{Red.}}\text{-}\Delta\text{LTA}$; and σ^{X} can sense $\Delta\text{GLs-}\Delta\text{LTA}$ (Fig. 5). This implies that these molecules have specific or overlapping functions on the membrane or cell surface. To determine the exact function of each lipid and LTA in the cells, further analysis of the activation mechanisms of each of the ECF sigma factors will be necessary.

We are grateful to K. C. Burtis for carefully reading the manuscript. T. S. is a research fellow of the Japan Society for the Promotion of Science (JSPS). This work was supported in part by JSPS KAKENHI (Grant-in-Aid for JSPS Research Fellows) Grant Numbers JP16J10629 to T. S. and 15K18664 to S. M.

CONFLICTS OF INTEREST

None declared.

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