Scientific Research Publishing

Advances in Microbiology, 2017, 7, 315-327
http://www.scirp.org/journal/aim
ISSN Online: 2165-3410
ISSN Print: 2165-3402

Activation without Proteolysis of Anti-σ Factor RsiV of the Extracytoplasmic Function σ Factor σ^V in a Glucolipid-Deficient Mutant of *Bacillus subtilis*

Takahiro Seki, Kouji Matsumoto, Satoshi Matsuoka, Hiroshi Hara*

Department of Biochemistry and Molecular Biology, Graduate School of Science and Engineering, Saitama University, Saitama, Japan
Email: *hhara@mail.saitama-u.ac.jp

How to cite this paper: Seki, T., Matsumoto, K., Matsuoka, S. and Hara, H. (2017) Activation without Proteolysis of Anti-σ Factor RsiV of the Extracytoplasmic Function σ Factor σ^V in a Glucolipid-Deficient Mutant of *Bacillus subtilis*. *Advances in Microbiology*, **7**, 315-327.
https://doi.org/10.4236/aim.2017.74026

Received: March 22, 2017
Accepted: April 25, 2017
Published: April 30, 2017

Abstract

Extracytoplasmic function (ECF) σ factors are a crucial link in the process of bacterial response to environmental stresses, in which bacteria transmit information across the cytoplasmic membrane. Among the seven ECF σ factors of *Bacillus subtilis* σ^V, which is sequestered by transmembrane anti-σ factor RsiV under normal growth conditions, responds to lysozyme. When *B. subtilis* cells are challenged by lysozyme, the lysozyme-bound RsiV undergoes two successive proteolysis steps, by a signal peptidase and RasP protease, and releases σ^V. An unchallenged *B. subtilis ugtP* mutant lacking glucolipids exhibited higher σ^V activity than wild type. However, the activation occurred in the absence of RasP, and no proteolysis of RsiV was observed. It is likely that a conformational change, not proteolysis, of RsiV leads to this activation of σ^V in the absence of glucolipids. Replacement of the C-terminal region of RsiV with that of RsiW, the cognate σ factor of which, σ^W, is not activated in the *ugtP* mutant, indicated that the C-terminal extracytoplasmic region of RsiV was necessary for the response to glucolipid deficiency.

Keywords

Anti-σ Factor, *Bacillus subtilis*, ECF σ Factor, Glucolipid, RsiV, σ^V, UgtP

1. Introduction

Extracytoplasmic function (ECF) σ factors are a group of bacterial σ factors that direct transcription of genes involved in tasks such as maintenance of cell surface integrity in response to environmental stresses. *Bacillus subtilis* has seven

ECF σ factors: σ^M, σ^V, σ^W, σ^X, σ^Y, σ^Z and σ^{YlaC}. Except for 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 and bind RNA polymerase core enzyme to form holoenzymes, which transcribe the respective regulon genes. The genes for the σ factors and the anti-σ factors form operons whose transcription is directed by the cognate σ factors; the activities of the promoters for ECF σ factor genes are thus indicative of the activities of the respective ECF σ factors [1] [2] [3].

Among these ECF σ factors, σ^V primarily responds to lysozyme [4]. It is activated by stepwise proteolytic destruction of the anti-σ factor RsiV via regulated intramembrane proteolysis (RIP) [5]. Lysozyme directly binds to RsiV and induces the cleavage of its C-terminal extracytoplasmic portion by signal peptidase (site-1 cleavage). Subsequently the N-terminal portion is processed by the intramembrane protease RasP (site-2 cleavage), and σ^V is released [6] [7].

The *B. subtilis* membrane contains the glucolipids (monoglucosyldiacylglycerol, diglucosyldiacylglycerol and triglucosyldiacylglycerol) in addition to phospholipids (phosphatidylethanolamine, phosphatidylglycerol, cardiolipin and lysylphosphatidylglycerol) [8] [9]. Glucolipids are synthesized by the gene product of *ugtP*, which processively transfers glucose from UDP-glucose to diacylglycerol [10]. UgtP protein is implicated in nutrient-dependent cell size control [11]. *ugtP* null mutants which completely lack glucolipids have been found to show abnormal cell morphology [12] [13] and constitutive activation of three ECF σ factors σ^M, σ^V and σ^X [13] [14].

When the *ugtP* gene with a hexahistidine tag-coding sequence was placed under the control of the isopropyl-β-D-thiogalactoside (IPTG)-inducible P_{spac} promoter and integrated into the chromosomal *aprE* locus in the Δ*ugtP* mutant, the activities of σ^M, σ^V and σ^X monitored by the expression of *lacZ* placed under the control of the respective σ factor gene promoter and integrated into the chromosomal *amyE* locus decreased with increasing IPTG concentration [15]. A *ugtP* mutant gene whose 18th codon was changed from His to Ala produced a normal level of the protein but showed no glucolipid synthetic activity. When P_{spac}-*ugtP*$_{H18A}$-His_6 was integrated into the *aprE* locus instead of P_{spac}-*ugtP*-His_6, the activities of σ^M, σ^V and σ^X did not change with increasing IPTG concentration. Thus, the lack of glucolipids, not the absence of UgtP protein, activates these ECF σ factors [16].

Escherichia coli has a much simpler membrane lipid composition than *B. subtilis*. The membrane contains three kinds of phospholipids (phosphatidylethanolamine, phosphatidylglycerol and cardiolipin), except for the outer leaflet of the outer membrane, which is composed of lipopolysaccharide [17]. It does not contain glucolipid. When the genes for *B. subtilis* σ^M, σ^V and σ^X and their anti-σ factors were introduced into *E. coli* cells, *lacZ* fused to the ECF σ factor-regulated promoters was expressed. Additional introduction and expression of *B. subtilis ugtP* gene in the *E. coli* cells led to the synthesis of small amounts of glucolipids, and the activities of *B. subtilis* σ^M and σ^V were partially repressed,

but the activity of σ^X was unaffected [15]. This result indicates that in heterologous *E. coli* cells the lack of glucolipids activates *B. subtilis* σ^M and σ^Y.

We wondered if *B. subtilis* σ^Y is activated by an RIP mechanism in *E. coli* cells. Although *E. coli* has a signal peptidase and a site-2 protease RseP, which functions in RIP of the anti-σ factor to ECF σ factor σ^E [18] [19] [20], we thought it possible that the activation of σ^Y might not involve RIP. In the same vein, we suspected that the activation of σ^Y in a *B. subtilis* Δ*ugtP* mutant lacking glucolipid [15] might not involve RIP either. It seemed possible that a conformational change of anti-σ^Y activates σ^Y weakly; σ^Y activation by the lack of glucolipids is only two- to three-fold, whereas treatment with a high concentration (100 μg/mL) of lysozyme causes ~65-fold activation [5]. In this study we explored the question of how σ^Y is activated in the *B. subtilis* Δ*ugtP* mutant.

2. Materials and Methods

2.1. Bacterial Strains, Plasmids and Culture Media

The strains and the plasmids used for this study and DNA primers used for strain construction are listed in **Tables 1-3**, respectively. The marker-free deletion of *rsiV* (SBS352) was obtained as previously described [14] [21] and confirmed by polymerase chain reaction (PCR). Luria-Bertani (LB) medium was used. For plates, media were solidified with 1.5% agar. Tryptose blood agar (Difco) was used for preparation of *B. subtilis* strains for transformation. Synthetic media CI and CII were used for competence development for transformation [22]. When appropriate, antibiotics were included at the following concentrations (in μg/mL): ampicillin (100), chloramphenicol (5), erythromycin (0.5), neomycin (10), spectinomycin (100) and tetracycline (10). Lysozyme used was Wako Pure Chemical's lysozyme hydrochloride from egg white. Cell growth was monitored with a mini photo 518R photometer (TAITEC) equipped with a 530-nm interference filter.

2.2. Construction of Chimeric Anti-σ Factors

RsiV and RsiW were divided into N-terminal regions ($RsiV_{1\text{-}36}$ and $RsiW_{1\text{-}82}$), transmembrane regions ($RsiV_{37\text{-}59}$ and $RsiW_{83\text{-}105}$) and C-terminal regions ($RsiV_{60\text{-}285}$ and $RsiW_{106\text{-}208}$), respectively, based on the prediction of the transmembrane regions by TMHMM (URL: http://www.cbs.dtu.dk/services/TMHMM/). Chimeric anti-σ factors, a chimera of the N-terminal region of RsiV and the transmembrane and C-terminal regions of RsiW ($RsiV_N$-$RsiW_{TMC}$) and a chimera of the N-terminal and transmembrane regions of RsiV and the C-terminal region of RsiW ($RsiV_{NTM}$-$RsiW_C$), were designed. These chimeras were constructed by PCR and Gibson Assembly Cloning Kit (New England Biolabs) on a vector pSG1729 [23] digested with KpnI and BamHI. The chimeric genes were transferred together with ribosome-binding site (RBS) on the vector pSG1729 by PCR and Gibson Assembly Cloning Kit to a vector pAPNC213 [24] digested with SalI. The resultant chimeric genes under

Table 1. Bacterial strains used for this study.

Strain	Relevant genotype	Construction, source or reference
E. coli		
DH5	*recA1 endA1 hsdR17*	Laboratory collection
B. subtilis		
168	*trpC2*	Laboratory collection
YTB019	168 Δ*ugtP*::*kan*	[34]
YTB040	YTB019 *amyE*::(P_{sigV}-*lacZ cat*)	[14]
YTB041	168 *amyE*::(P_{sigV}-*lacZ cat*)	[14]
1012 *rasP*	*leuA8 metB5 trpC2 hsrM1 rasP*::*tet*	[30] [35]
SBS100	168 *rasP*::*tet*	1012 *rasP* →[a] 168
SBS101	YTB041 *rasP*::*tet*	SBS100 → YTB041
SBS102	YTB040 *rasP*::*tet*	SBS100 → YTB040
SBS200	168 *rsiV*::(pMUTIN-P_{spac}-*flag-rsiV erm*)	K. Asai
SBS201	SBS200 Δ*ugtP*::*kan*	YTB019 → SBS200
SBS204	SBS200 *amyE*::(P_{sigV}-*lacZ cat*)	YTB041 → SBS200
SBS205	SBS204 Δ*ugtP*::*kan*	YTB019 → SBS204
TMO310	168 *aprE*::(*lacI* P_{spac}-*mazF spc*)	[21]
SBS303	168 *sigV*::(*lacI* P_{spac}-*mazF spc*)	This study
SBS304	168 Δ*sigV*	This study
SBS313	SBS304 *amyE*::(P_{sigV}-*lacZ cat*)	YTB041 → SBS304
SBS314	SBS313 Δ*ugtP*::*kan*	YTB019 → SBS313
SBS351	168 *rsiV*::(*lacI* P_{spac}-*mazF spc*)	This study
SBS352	168 Δ*rsiV*	This study
SBS361	SBS352 *amyE*::(P_{sigV}-*lacZ cat*)	YTB041 → SBS352
SBS362	SBS361 Δ*ugtP*::*kan*	YTB019 → SBS361
SBS500	SBS361 *aprE*::(*lacI* P_{spac}-RBS-$rsiV_N$-$rsiW_{TMC}$ *spc*)	pAP-$RsiV_N$-$RsiW_{TMC}$ → SBS361
SBS501	SBS500 Δ*ugtP*::*kan*	YTB019 → SBS500
SBS502	SBS361 *aprE*::(*lacI* P_{spac}-RBS-$rsiV_{NTM}$-$rsiW_C$ *spc*)	pAP-$RsiV_{NTM}$-$RsiW_C$ → SBS361
SBS503	SBS502 Δ*ugtP*::*kan*	YTB019 → SBS502

a →, transformation with chromosomal or plasmid DNA.

P_{spac} and RBS were integrated into the chromosomal *aprE* locus. Constructs were verified by sequencing.

2.3. Genetic and Recombinant DNA Procedures

These were based on standard methods [25] [26]. The cycle number and the annealing temperatures for PCR were 40 cycles and 40˚C - 65˚C according to the T_m values of primers, respectively.

2.4. Biochemical Procedures

The β-galactosidase assay method using *o*-nitrophenyl-β-D-galactoside as sub-

Table 2. Plasmids used for this study.

plasmid	Description	Construction or reference
pSG1729	*amyE' spc* P_{xyl} *gfpmut1 'amyE bla*	[23]
pAPNC213	*aprE' spc lacI* P_{spac} *'aprE bla*	[24]
pSG-$RsiV_N$-$RsiW_{TMC}$	pSG1729 P_{xyl}-*rsiV*$_N$-*rsiW*$_{TMC}$	This study
pSG-$RsiV_{NTM}$-$RsiW_C$	pSG1729 P_{xyl}-*rsiV*$_{NTM}$-*rsiW*$_C$	This study
pAP-$RsiV_N$-$RsiW_{TMC}$	pAPNC213 P_{spac}-RBS-*rsiV*$_N$-*rsiW*$_{TMC}$	This study
pAP-$RsiV_{NTM}$-$RsiW_C$	pAPNC213 P_{spac}-RBS-*rsiV*$_{NTM}$-*rsiW*$_C$	This study

Table 3. PCR primers used for strain construction.

Primer	Sequence (5′→3′)	T_m (˚C)	Purpose
sigVA-F	CATTATTGCGTACGGAGAC	58.1	For marker-free deletion of *sigV*
sigVA-R	TGCAATAAAGGGCTCCT	58.3	
sigVB-F2	CAAAAAGGAGCCCTTTATTGCAATGGATAAGAGATTACAGC	76.8	
sigVB-R	GCTTGAGTCAATTCCGCTGTCGGCTTTGTGTGTAGGAAG	82.5	
sigVC-F	CAAAATTAACGTACTGATTGGGTAGGATCCGCGCAGGTTGGCTTTCAGTTATG	84.8	
sigVC-R	CGTGTTTTCTCCTGTAATTTCC	61.2	
rsiVA-F	CTAGGTAACAGCCTACG	50.2	For marker-free deletion of *rsiV*
rsiVA-R	TAAGAAAGATCCTCCTTCG	56.0	
rsiVB-F	GTTGACGAAGGAGGATCTTTCTTAATCCATCTAAGATTATGGATG	76.4	
rsiVBmazF-R	GCTTGAGTCAATTCCGCTGTCGGTTCTTCAATCGCCAGCGAC	87.6	
mazFrsiVC-F	CAAAATTAACGTACTGATTGGGTAGGATC	87.6	
rsiVC-R	GATAGAGAGTAAATCTGGCGTGTC	61.9	
pAPNC-F	CGACAGCGGAATTGACTCAAGC	70.0	
chpA-R	CGCGGATCCTACCCAATCAGTACGTTAATTTTG	75.7	
sigVcheck-F	CACCTTTAACAGGCTATGCC	61.0	For confirmation of marker-free deletion of *sigV*
rsiVcheck-F	CCTTCATGATATGAGCAG	53.7	For confirmation of marker-free deletion of *rsiV*
rsiVcheck-R	CACAAGCAATGATATCAC	51.6	For confirmation of marker-free deletion of *sigV* and *rsiV*
RsiVNup	GGAGATTCCTAGGATGGACTACAAAGACGACGACGACAAAATGGATAAGAGATTACAGCAATTAAGAG	78.1	For construction of chimeric anti-σ factors $RsiV_N$-$RsiW_{TMC}$ and $RsiV_{NTM}$-$RsiW_C$
RsiVNdown	GGATGGGTTCTGAACCATTTCTTTTTTGGTTCTTGCTG	79.2	For construction of chimeric anti-σ factor $RsiV_N$-$RsiW_{TMC}$
RsiVNTMdown	GTCATTATGCCAGCTGTTGATATTAACAAGCGCAGTG	77.4	For construction of chimeric anti-σ factor $RsiV_{NTM}$-$RsiW_C$
RsiWTMCup	TGGTTCAGAACCCATCCCGTTATCG	73.7	For construction of chimeric anti-σ factor $RsiV_N$-$RsiW_{TMC}$
RsiWTMCdown	TCGAGGGGGGGCCCGTGTTACTCTTCTCCGTTCGGAT	87.2	For construction of chimeric anti-σ factors $RsiV_N$-$RsiW_{TMC}$ and $RsiV_{NTM}$-$RsiW_C$
RsiWCup	AACAGCTGGCATAATGA	60.1	For construction of chimeric anti-σ factor $RsiV_{NTM}$-$RsiW_C$
pAPNC-SalI-up	CTGCAGGCATGCCTGCAGGGAGATTCCTAGGATGGACTAC	84.4	For construction of chimeric anti-σ factors
pAPNC-SalI-down	CCGGGGATCCTCTAGAGTCGAGGGGGGGCCCGTG	88.6	
pAPNCcheckF	ACAAGGTGTGGCATAATGTG	60.9	For sequence confirmation of chimeric anti-σ factors
pAPNCcheckR	GCTATATTGGCCGCTTCCTG	65.5	

strate and the unit definition were as described by Wang and Doi [27]. Proteins were separated by SDS gel electrophoresis with 15% polyacrylamide gel. Luminata Forte Western HRP Substrate (Millipore) was used for immunodetection according to the manufacturer's instructions. For immunoblotting, anti-DYKDDDK tag (FLAG tag) monoclonal antibody (Wako Pure Chemicals) and anti-σ^A antiserum (a gift from K. Asai) were used. The secondary antibodies were peroxidase-conjugated anti-mouse IgG (H+L) and anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories). Immuno-stained bands were detected by C-Digit scanner (LI-COR), and the band intensities were measured by Image Studio software (LI-COR).

3. Results

3.1. Lack of Glucolipids Affects σ^V Activity through the Anti-σ Factor RsiV

The activity of σ^Y was monitored by β-galactosidase activity expressed from P_{sigV}-*lacZ* integrated into the chromosomal *amyE* locus. A *ugtP* deletion mutant showed an approximately two-fold activation of σ^Y compared to wild type (**Figure 1(a)**). In Δ*sigV* and Δ*sigV* Δ*ugtP* mutants the activity was almost abolished, indicating that P_{sigV}-*lacZ* expression was dependent on σ^Y activation. Deletion of the *rsiV* gene encoding anti-σ^Y factor led to high σ^Y activity, but additional introduction of a *ugtP* deletion mutation did not lead to additional activation of σ^Y (**Figure 1(a)**). The *rsiV* deletion mutant was insensitive to the lack of glucolipids, which is consistent with the idea that the lack of glucolipids affects σ^Y activity through RsiV.

3.2. 1,10-Phenanthroline Inhibited Activation of σ^V Caused by Addition of Lysozyme But Not the Activation by Lack of Glucolipids

The zinc chelator 1,10-phenanthroline (PT) inhibits the site-2 protease RasP [5], as it does many other site-2 metalloproteases [28]. When *ugtP*$^+$ cells were challenged by hen egg white lysozyme (HEWL) at low concentration (100 ng/mL), σ^Y was activated several-fold. Addition of 10 mM PT returned the σ^Y activity to the basal level (**Figure 1(b)**), most probably via inhibition of RasP. Approximately two-fold activation of σ^Y in the Δ*ugtP* mutant compared to wild type was not changed by addition of 10 mM PT (**Figure 1(b)**), suggesting that the inhibition of RasP by PT did not affect activation. It seems that the activation of σ^Y by the lack of glucolipids does not depend on the function of the site-2 protease RasP. Addition of HEWL to the PT-treated Δ*ugtP* cells did not increase the σ^Y activity (**Figure 1(b)**). The σ^Y activation by HEWL seems to depend on RasP in the Δ*ugtP* cells as well as in the *ugtP*$^+$ cells.

3.3. A *rasP* Mutation Abolished the σ^V Activation Caused by the Addition of Lysozyme But Not the Activation by the Lack of Glucolipids

When wild-type cells were treated with increasing concentrations (25, 50 and

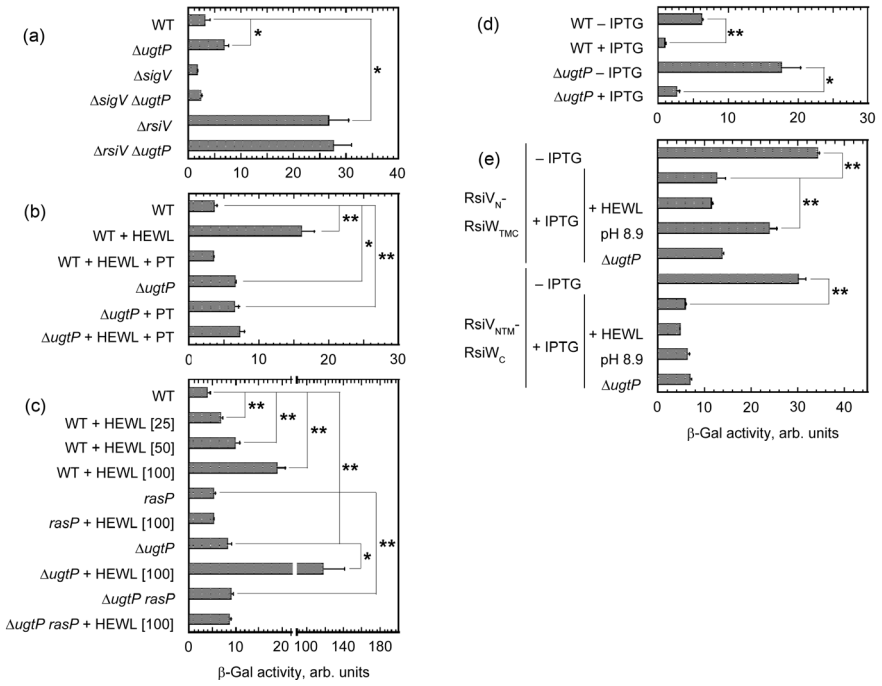

Figure 1. Activation levels of σ^V monitored via P_{sigV}-*lacZ* transcriptional fusion. Cells were grown in LB medium to mid-exponential phase (mini photo 518R reading of 0.35 - 0.45) unless otherwise noted, and β-galactosidase activity was measured. The means and standard deviations of at least three measurements are shown. Asterisks indicate significant differences as determined by Student's *t*-test: $*P < 0.05$, $**P < 0.01$. (a) Effect of Δ*sigV* and Δ*rsiV* on the σ^V activity. Strains YTB041, YTB040, SBS313, SBS314, SBS361 and SBS362 were analyzed; (b) Effect of PT on the σ^V activity. Strains YTB041 and YTB040 were analyzed. For HEWL treatment of YTB041 the cells were grown to early-exponential phase (mini photo reading of 0.20), and HEWL was added to 100 ng/mL, followed by 30 min shaking, after which the culture reached mid-exponential phase. For PT treatment of YTB040 the cells were grown to mid-exponential phase (mini photo reading of 0.45), and PT was added to 10 mM, followed by 20 min shaking. For HEWL and PT treatment the cells were grown to mid-exponential phase (mini photo reading of 0.45), and PT was added to 10 mM, which stopped the turbidity increase; after 20 min shaking HEWL was added to 100 ng/mL, followed by 30 min shaking; (c) Effect of a *rasP* mutation on the σ^V activity. Strains YTB041, SBS101, YTB040 and SBS102 were analyzed. The cells were treated with HEWL at the concentrations (in ng/mL) indicated in the square brackets as described for YTB041 in (b); (d) FLAG-RsiV is functional. Strains SBS204 and SBS205 were analyzed. The cells were grown in the presence or absence of 1 mM IPTG; (e) Anti-σ activities of $RsiV_N$-$RsiW_{TMC}$ and $RsiV_{NTM}$-$RsiW_C$. Strains SBS500, SBS501, SBS502 and SBS503 were analyzed. One millimolar IPTG was added or withheld from the beginning of the culture. SBS500 and SBS502 cells were subjected to 100 ng/mL HEWL as described for YTB041 in (b). For alkali treatment of SBS500 and SBS502 the cells were grown to early-exponential phase (mini photo reading of 0.20) and 5.5 μl of 5 N NaOH was added to the 5-mL cultures, which brought the medium pH to ca. 8.9; this was followed by 30 min shaking.

100 ng/mL) of HEWL, the activity of σ^V increased accordingly (Figure 1(c)). A *rasP*::*tet* mutant showed a basal level σ^V activity in the absence of HEWL (Figure 1(c)). Whereas the addition of 100 ng/mL HEWL activated σ^V several-fold in *rasP*$^+$ cells, the σ^V activity level in the *rasP*::*tet* mutant did not change in the

presence of 100 ng/mL HEWL (Figure 1(c)), indicating that the activation of σ^Y by HEWL requires RasP [5]. On the other hand, the approximately two-fold activation of σ^Y in the Δ*ugtP* mutant did not change when, in addition, *rasP* was disrupted (Figure 1(c)), indicating that RasP has nothing to do with the σ^Y activation by the lack of glucolipids. Addition of 100 ng/mL HEWL to the Δ*ugtP* mutant resulted in a remarkably high activation of σ^Y (Figure 1(c)). The Δ*ugtP* mutant seems to become sensitized to the stress from HEWL addition. Addition of HEWL to Δ*ugtP rasP*::*tet* mutant cells did not change the σ^Y activity (Figure 1(c)). The σ^Y activation by HEWL required RasP in the Δ*ugtP* cells as well as in the *ugtP*$^+$ cells.

3.4. RsiV Was Not Degraded in the Δ*ugtP* Cells

RsiV is degraded by RIP in response to lysozyme. N-terminally FLAG tag-fused RsiV was expressed and analyzed by immunoblotting (Figure 2). When *ugtP*$^+$ cells were treated with 25, 50 and 100 ng/mL HEWL, the amount of intact FLAG-RsiV protein was reduced to about 90%, about 70% and about 40%, respectively, compared with cells that were not treated with HEWL. This is consistent with the increasing activity of σ^Y with increasing concentrations of HEWL (Figure 1(c)). In contrast, Δ*ugtP* cells showed no decrease in the amount of intact FLAG-tagged RsiV compared with the wild type not treated with HEWL. This result indicates that site-1 cleavage did not occur in Δ*ugtP* cells. Site-1 cleavage is a prerequisite for site-2 cleavage, and RasP cleavage is constitutive once site-1 cleavage has occurred [5]. The idea that RasP has nothing to do with the σ^Y activation by lack of glucolipids fits into this scenario. When 100 ng/mL HEWL was added to Δ*ugtP* cells, the amount of intact FLAG-RsiV protein was reduced to about 7% (Figure 2). This corresponds well with the remarkably high activation of σ^Y in the Δ*ugtP* mutant when 100 ng/mL HEWL was added (Figure 1(c)). The FLAG-tagged RsiV was functional: when the expression of P_{spac}-*flag-rsiV* was induced with IPTG, σ^Y activity was repressed in both *ugtP*$^+$ and Δ*ugtP* cells (Figure 1(d)).

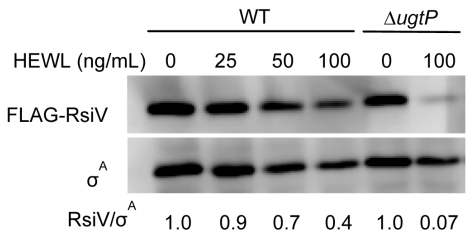

Figure 2. Degradation of RsiV. Strains SBS200 and SBS201 were analyzed. The cells were grown in LB medium containing 1 mM IPTG to mid-exponential phase (mini photo reading of 0.40 - 0.45), and 250 μg/mL chloramphenicol and HEWL of the indicated concentrations were added, followed by 10 min shaking. Harvested samples were sonicated and subjected to SDS-PAGE. The sample amount per lane was normalized by the culture turbidity. FLAG-RsiV and σ^A (an internal control) were detected by immunoblotting. The relative ratio of the amount of Flag-RsiV to that of σ^A is shown under each lane. The ratio in SBS200 (WT) in the absence of HEWL was set to 1.0.

3.5. The Behavior of RsiV-RsiW Chimeric Anti-σ Factors Indicated That the C-Terminal Extracytoplasmic Region of RsiV Was Necessary for the Response to Lack of Glucolipids

Chimeric anti-σ factors, $RsiV_N$-$RsiW_{TMC}$ and $RsiV_{NTM}$-$RsiW_C$, were constructed (see Materials and Methods). RsiW is an anti-σ factor for σ^W [1] [2]. Whereas σ^W is activated by RIP of RsiW [29] [30] in response to a variety of signals, including an alkaline shock [31], it is not activated by lack of glucolipids [13] [14].

Both chimeric anti-σ factors are functional as anti-σ^Y: when their expression was induced with IPTG in a Δ*rsiV* background, σ^Y activity was repressed (**Figure 1(e)**). The N-terminal region of RsiV seems to be sufficient to sequester σ^Y.

In cells expressing either chimeric anti-σ factor, σ^Y was not activated by the addition of 100 ng/mL HEWL (**Figure 1(e)**). This is consistent with reports that the C-terminal region of RsiV binds HEWL [6] [7].

We found that σ^Y was activated when the cells expressing $RsiV_N$-$RsiW_{TMC}$ suffered alkaline stress (pH 8.9) (**Figure 1(e)**). Most probably the transmembrane and C-terminal extracytoplasmic regions of σ^W underwent RIP in response to the alkaline stress, which led to degradation of the chimeric anti-σ factor and to release of σ^Y. In contrast, in the cells expressing $RsiV_{NTM}$-$RsiW_C$ σ^Y was not activated in response to alkaline stress (**Figure 1(e)**). The C-terminal extracytoplasmic region of RsiW may undergo site-1 cleavage, but it does not seem to lead to the site-2 cleavage of the fused transmembrane region of RsiV.

The expression of the chimeric anti-σ factors in Δ*ugtP* cells resulted in σ^Y activity at the basal level of the *ugtP*$^+$ cells (**Figure 1(e)**). This indicates that the C-terminal extracytoplasmic region is necessary for σ^Y to respond to the lack of glucolipids.

We also constructed chimeric anti-σ factors, $RsiW_N$-$RsiV_{TMC}$ and $RsiW_{NTM}$-$RsiV_C$, but they were not functional as anti-σ^W: IPTG induction of their expression in a Δ*rsiW* background even from multicopy plasmids did not lead to repression of σ^W activity (data not shown); the reason is unknown.

4. Discussion

B. subtilis σ^Y is activated primarily in response to lysozyme [4]. The activation depends on the RIP of the anti-σ factor RsiV [5] [6] [7]. By contrast, in a Δ*ugtP* mutant that lacks glucolipids, σ^Y is activated without RIP of RsiV. The activation was not inhibited by the zinc chelator PT, an inhibitor of the site-2 protease RasP, nor was it abolished in a *rasP* mutant. No proteolysis of RsiV was observed in the Δ*ugtP* mutant, indicating that site-1 cleavage did not occur. Most probably a conformational change of RsiV without proteolysis is responsible for the release of σ^Y from RsiV in the Δ*ugtP* mutant.

Some relevant information concerning the activation of *B. subtilis* ECF σ factors is available. It is known that σ^W is activated by RIP. Under alkaline stress, the anti-σ factor RsiW is first degraded by the site-1 protease PrsW, trimmed by several peptidases, and then degraded by the site-2 protease RasP [29]. There are

two anti-σ factors, YhdL and YhdK, involved in the regulation of σ^M, where YhdL has the major function of binding σ^M directly. Under salt stress, σ^M is activated but no proteolysis of N-terminally hexahistidine-tagged YhdL is observed [32]. This suggests that σ^M is probably released by a conformational change of YhdL.

The activation of σ^Y in the Δ*ugtP* mutant cells upon addition of 100 ng/mL HEWL was higher than in *ugtP*$^+$ cells, and degradation of RsiV was correspondingly enhanced in the Δ*ugtP* mutant cells. This may be due to the proposed conformational change of RsiV in the Δ*ugtP* mutant cells.

Addition of Mg^{2+} suppressed the σ^Y activation in the absence of glucolipids [16]. Thus, it seems unlikely that direct interaction of glucolipids with RsiV is required for its sequestering of σ^Y. The lack of glucolipids probably affects the membrane properties, which may then lead to a conformational change of RsiV and to release of σ^Y. One line of reasoning might be as follows: glucolipids have no charge in their polar head groups. It is possible that the change in the charge distribution on the membrane surface brought about by their absence affects the conformation of RsiV. We note that synthesis of *Acholeplasma laidlawii* monoglucosyldiacylglycerol suppressed, albeit only partially, the σ^Y activation in the Δ*ugtP* mutant [16]. This too, may be caused by a change in the charge characteristics of the membrane surface. In this regard, it is also noteworthy that the membrane surface charge characteristics are an important topological determinant of *E. coli* LacY protein [33].

Experiments with chimeric anti-σ factors indicated that the C-terminal region of RsiV is necessary for the response to glucolipid deficiency. It seems that the C-terminal extracytoplasmic region of RsiV is important for it to be sensitive to the alteration in charge characteristics of the membrane surface.

We conclude that, in glucolipid-lacking mutants of *B. subtilis*, σ^Y can be activated without proteolysis of anti-σ factor RsiV and that this is most likely brought about by a conformational change of RsiV due to the alteration of the membrane surface charge characteristics.

Acknowledgements

We are grateful to Thomas Wiegert for providing the chromosomal DNA of the strain 1012 *rasP* and to Kei Asai for providing the strain SBS200 and anti-σ^A antiserum. T. S. is a research fellow of Japan Society for the Promotion of Science (JSPS). This work was supported in part by JSPS KAKENHI (Grant-in-Aid for JSPS Research Fellow) Grant Number JP16J10629 to T. S.

References

[1] Helmann, J.D. (2002) The Extracytoplasmic Function (ECF) Sigma Factors. *Advances in Microbial Physiology*, **46**, 47-110.

[2] Yoshimura, M., Asai, K., Sadaie, Y. and Yoshikawa, H. (2004) Interaction of *Bacillus subtilis* Extracytoplasmic Function (ECF) Sigma Factors with the N-Terminal Regions of Their Potential Anti-Sigma Factors. *Microbiology*, **150**, 591-599. https://doi.org/10.1099/mic.0.26712-0

[3] Asai, K., Matsumoto, T. and Sadaie, Y. (2005) ECF (Extracytoplasmic Function) Sigma Factors of *Bacillus subtilis*. In: Yamada, M., Ed., *Survival and Death in Bacteria*, Research Signpost, Kerala, 143-153.

[4] Ho, T.D., Hastie, J.L., Intile, P.J. and Ellermeier, C.D. (2011) The *Bacillus subtilis* Extracytoplasmic Function Sigma Factor σ^V Is Induced by Lysozyme and Provides Resistance to Lysozyme. *Journal of Bacteriology*, **193**, 6215-6222. https://doi.org/10.1128/JB.05467-11

[5] Hastie, J.L., Williams, K.B. and Ellermeier, C.D. (2013) The Activity of σ^V, an Extracytoplasmic Function σ Factor of *Bacillus subtilis*, Is Controlled by Regulated Proteolysis of the Anti-σ Factor RsiV. *Journal of Bacteriology*, **195**, 3135-3144. https://doi.org/10.1128/JB.00292-13

[6] Hastie, J.L., Williams, K.B., Sepulveda, C., Houtman, J.C., Forest, K.T. and Ellermeier, C.D. (2014) Evidence of a Bacterial Receptor for Lysozyme: Binding of Lysozyme to the Anti-σ Factor RsiV Controls Activation of the ECF σ Factor σ^V. *PLoS Genetics*, **10**, e1004643. https://doi.org/10.1371/journal.pgen.1004643

[7] Hastie, J.L., Williams, K.B., Bohr, L.L., Houtman, J.C., Gakhar, L. and Ellermeier, C.D. (2016) The Anti-Sigma Factor RsiV Is a Bacterial Receptor for Lysozyme: Co-Crystal Structure Determination and Demonstration That Binding of Lysozyme to RsiV Is Required for σ^V Activation. *PLoS Genetics*, **12**, e1006287. https://doi.org/10.1371/journal.pgen.1006287

[8] Matsumoto, K., Matsuoka, S. and Hara, H. (2012) Membranes and Lipids. In: Sadaie, Y. and Matsumoto, K., Eds., *Escherichia coli and Bacillus subtilis*: *The Frontier of Molecular Microbiology Revisited*, Research Signpost, Kerala, 61-91.

[9] De Mendoza, D., Schujman, G.E. and Aguilar, P.S. (2002) Biosynthesis and Function of Membrane Lipids. In: Sonenshein, A.L., Hoch, J.A., and Losick, R., Eds., *Bacillus Subtilis and Its Closest Relatives*, ASM Press, Washington DC, 43-55. https://doi.org/10.1128/9781555817992.ch5

[10] Jorasch, P., Wolter, F.P., Zahringer, U. and Heinz, E. (1998) A UDP Glucosyltransferase from *Bacillus subtilis* Successively Transfers up to Four Glucose Residues to 1,2-Diacylglycerol: Expression of *YpfP* in *Escherichia coli* and Structural Analysis of Its Reaction Products. *Molecular Microbiology*, **29**, 419-430. https://doi.org/10.1046/j.1365-2958.1998.00930.x

[11] Weart, R.B., Lee, A.H., Chien, A.C., Haeusser, D.P., Hill, N.S. and Levin, P.A. (2007) A Metabolic Sensor Governing Cell Size in Bacteria. *Cell*, **130**, 335-347.

[12] Price, K.D., Roels, S. and Losick, R. (1997) A *Bacillus subtilis* Gene Encoding a Protein Similar to Nucleotide Sugar Transferases Influences Cell Shape and Viability. *Journal of Bacteriology*, **179**, 4959-4961. https://doi.org/10.1128/jb.179.15.4959-4961.1997

[13] Matsuoka, S., Chiba, M., Tanimura, Y., Hashimoto, M., Hara, H. and Matsumoto, K. (2011) Abnormal Morphology of *Bacillus subtilis UgtP* Mutant Cells Lacking Glucolipids. *Genes & Genetic Systems*, **86**, 295-304. https://doi.org/10.1266/ggs.86.295

[14] Hashimoto, M., Seki, T., Matsuoka, S., Hara, H., Asai, K., Sadaie, Y. and Matsumoto, K. (2013) Induction of Extracytoplasmic Function Sigma Factors in *Bacillus subtilis* Cells with Defects in Lipoteichoic Acid Synthesis. *Microbiology*, **159**, 23-35. https://doi.org/10.1099/mic.0.063420-0

[15] Seki, T., Mineshima, R., Hashimoto, M., Matsumoto, K., Hara, H. and Matsuoka, S. (2015) Repression of the Activities of Two Extracytoplasmic Function Sigma Factors, σ^M and σ^V, of *Bacillus subtilis* by Glucolipids in *Escherichia coli* Cells. *Genes & Genetic Systems*, **90**, 109-114. https://doi.org/10.1266/ggs.90.109

[16] Matsuoka, S., Seki, T., Matsumoto, K. and Hara, H. (2016) Suppression of Abnormal Morphology and Extracytoplasmic Function Sigma Activity in *Bacillus subtilis UgtP* Mutant Cells by Expression of Heterologous Glucolipid Synthases from *Acholeplasma laidlawii*. *Bioscience, Biotechnology, and Biochemistry*, **80**, 2325-2333. https://doi.org/10.1080/09168451.2016.1217147

[17] Shibuya, I. (1992) Metabolic Regulations and Biological Functions of Phospholipids in *Escherichia coli*. *Progress in Lipid Research*, **31**, 245-299.

[18] Alba, B.M. and Gross, C.A. (2004) Regulation of the *Escherichia coli* σ^E-Dependent Envelope Stress Response. *Molecular Microbiology*, **52**, 613-619. https://doi.org/10.1111/j.1365-2958.2003.03982.x

[19] Kanehara, K., Ito, K. and Akiyama, Y. (2002) YaeL (EcfE) Activates the σ^E Pathway of Stress Response through a Site-2 Cleavage of Anti-σ^E, RseA. *Genes & Development*, **16**, 2147-2155. https://doi.org/10.1101/gad.1002302

[20] Kanehara, K., Ito, K. and Akiyama, Y. (2003) YaeL Proteolysis of RseA Is Controlled by the Pdz Domain of YaeL and a Gln-Rich Region of RseA. *EMBO Journal*, **22**, 6389-6398. https://doi.org/10.1093/emboj/cdg602

[21] Morimoto, T., Ara, K., Ozaki, K. and Ogasawara, N. (2009) A New Simple Method to Introduce Marker-Free Deletions in the *Bacillus subtilis* Genome. *Genes & Genetic Systems*, **84**, 315-318. https://doi.org/10.1266/ggs.84.315

[22] Anagnostopoulos, C. and Crawford, I.P. (1961) Transformation Studies on the Linkage of Markers in the Tryptophan Pathway in *Bacillus subtilis*. *Proceedings of the National Academy of Sciences of the United States of America*, **47**, 378-390. https://doi.org/10.1073/pnas.47.3.378

[23] Lewis, P.J. and Marston, A.L. (1999) GFP Vectors for Controlled Expression and Dual Labelling of Protein Fusions in *Bacillus subtilis*. *Gene*, **227**, 101-110.

[24] Morimoto, T., Loh, P.C., Hirai, T., Asai, K., Kobayashi, K., Moriya, S. and Ogasawara, N. (2002) Six GTP-Binding Proteins of the Era/Obg Family Are Essential for Cell Growth in *Bacillus subtilis*. *Microbiology*, **148**, 3539-3552. https://doi.org/10.1099/00221287-148-11-3539

[25] Miller, J.H. (1992) A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

[26] Sambrook, J. and Russell, D.W. (2001) Molecular Cloning: A Laboratory Manual. 3rd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

[27] Wang, P.Z. and Doi, R.H. (1984) Overlapping Promoters Transcribed by *Bacillus subtilis* σ^{55} and σ^{37} RNA Polymerase Holoenzymes during Growth and Stationary Phases. *Journal of Biological Chemistry*, **259**, 8619-8625.

[28] Feng, L., Yan, H., Wu, Z., Yan, N., Wang, Z., Jeffrey, P.D. and Shi, Y. (2007) Structure of a Site-2 Protease Family Intramembrane Metalloprotease. *Science*, **318**, 1608-1612. https://doi.org/10.1126/science.1150755

[29] Heinrich, J., Hein, K. and Wiegert, T. (2009) Two Proteolytic Modules Are Involved in Regulated Intramembrane Proteolysis of *Bacillus subtilis* RsiW. *Molecular Microbiology*, **74**, 1412-1426. https://doi.org/10.1111/j.1365-2958.2009.06940.x

[30] Schöbel, S., Zellmeier, S., Schumann, W. and Wiegert, T. (2004) The *Bacillus subtilis* σ^W Anti-Sigma Factor RsiW Is Degraded by Intramembrane Proteolysis through YluC. *Molecular Microbiology*, **52**, 1091-1105. https://doi.org/10.1111/j.1365-2958.2004.04031.x

[31] Wiegert, T., Homuth, G., Versteeg, S. and Schumann, W. (2001) Alkaline Shock Induces the *Bacillus subtilis* σ^W Regulon. *Molecular Microbiology*, **41**, 59-71.

https://doi.org/10.1046/j.1365-2958.2001.02489.x

[32] Inoue, H. (2013) Analysis of Extracytoplasmic Stress Response Mechanisms Contributing to Cell Surface Integrity in *Bacillus subtilis*. PhD Thesis, Saitama University, Saitama. (In Japanese)

[33] Dowhan, W. and Bogdanov, M. (2009) Lipid-Dependent Membrane Protein Topogenesis. *Annual Review of Biochemistry*, **78**, 515-540. https://doi.org/10.1146/annurev.biochem.77.060806.091251

[34] Matsuoka, S., Hashimoto, M., Kamiya, Y., Miyazawa, T., Ishikawa, K., Hara, H. and Matsumoto, K. (2011) The *Bacillus subtilis* Essential Gene *DgkB* Is Dispensable in Mutants with Defective Lipoteichoic Acid Synthesis. *Genes & Genetic Systems*, **86**, 365-376. https://doi.org/10.1266/ggs.86.365

[35] Zellmeier, S., Schumann, W. and Wiegert, T. (2006) Involvement of Clp Protease Activity in Modulating the *Bacillus subtilis* σ^{W} Stress Response. *Molecular Microbiology*, **61**, 1569-1582. https://doi.org/10.1111/j.1365-2958.2006.05323.x