Anti-sigma factor-mediated cell surface stress responses in *Bacillus subtilis*

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(Received 1 October 2017, accepted 30 October 2017; J-STAGE Advance published date: 17 January 2018)

Proteins belonging to the sigma factor family in eubacteria initiate transcription by associating with RNA polymerase. A subfamily, the extracytoplasmic function (ECF) sigma factors, which form a widely distributed bacterial signal transduction system comprising a sigma factor and a cognate membrane-embedded anti-sigma factor, regulates genes in response to stressors that threaten cell envelope integrity including the cell wall and membrane. The Gram-positive soil bacterium *Bacillus subtilis* provides a valuable model for investigation of the ECF sigma factors. This review focuses on the function and regulation of ECF sigma factors in *B. subtilis*, in which anti-sigma factors play a role in connecting an external stimulus with gene regulation. As representative examples, the regulon and regulatory mechanism of σ^W are closely associated with membrane-active stressors, whereas σ^M is strongly induced by conditions that impair peptidoglycan synthesis. These studies demonstrate that the mechanisms of ECF-dependent signaling are divergent and constitute a multi-layered hierarchy, and provide useful insights into the elucidation of unknown mechanisms related to ECF sigma factors.

Key words: anti-sigma factor, *Bacillus subtilis*, cell surface, sigma factor, stress response

INTRODUCTION

Bacteria are roughly classified into two groups, enteric bacteria that reside within the animal or human gut and environmental bacteria that live in natural habitats globally. The former situation is relatively stable because the bacteria depend on their host environment, which is under homeostatic control. In contrast, the latter situation ranges from benign to extremely hostile surroundings and can change dynamically and suddenly. In order to adapt to and survive such hostile conditions, bacterial cells have developed complex stress response mechanisms. The surface of a cell is directly exposed to the external environment, and functions as a rampart to protect the cell interior. The cell surface also acts as a sensor, which perceives the external state and transduces the information across the surface, thus allowing adaptation. Through this process, physical information (such as external stressors) is converted to biological signals, which causes alteration of gene expression, fundamentally at the transcriptional level. The cell sur-

face of free-living bacteria is basically composed of a cell wall and membrane. The sensing machinery, in many cases, resides within the cell membrane as membrane-embedded proteins.

The genes required for stress responses are not constantly transcribed during cell growth. In bacteria, transcription initiation is regulated almost entirely by sigma factors, which are components of the RNA polymerase complex. Sigma factors recognize specific promoter sequences and allow genes to be transcribed. A particular group of sigma factors is activated only when they are needed for a particular response, resulting in directed gene transcription specific to the response. In this regard, some sigma factors are regulated by cognate anti-sigma factors, which impedes RNA binding, and the so-called ECF (extracytoplasmic function) sigma factors are representative of such sigma factors. The ECF subfamily was first designated in a study on *Streptomyces coelicolor* σ^E , a sigma factor controlling the extracellular agarase gene (Lonetto et al., 1994). Characteristically, σ^E of *S. coelicolor* lacks several domains that are conserved among the major family of sigma factors, and its structurally homologous sigma factors also regulate functions related to the cell surface response to extracytoplasmic stimuli. To date, advances in genome sequenc-

Edited by Kenji Ichiyanagi

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DOI: <http://doi.org/10.1266/ggs.17-00046>

ing have revealed that environmental bacteria especially possess multiple ECF sigma factors and that numerous sigma factors expressed by diverse bacteria are categorized in the ECF subfamily. The anti-sigma factor is a membrane-embedded protein composed of an external domain displayed on the cell surface and an internal domain located within the cell. The internal domain binds a cognate sigma factor and thus sequesters it from RNA polymerase. The external domain senses an environmental signal and transmits it to the internal domain, resulting in the release of the sigma factor, which can then bind to RNA polymerase. Although many ECF sigma factor-anti-sigma factor pairs are found in genome databases, little is known about the molecular mechanism of regulation of ECF sigma factors by their anti-sigma factors - for instance, how anti-sigma factors receive and sense signals, and how they release the sigma factors.

One of the commonest Gram-positive and environmental soil bacteria, *Bacillus subtilis*, has been studied for a century as a model organism in many fields of biology. When the surrounding nutrition is exhausted, these bacteria produce a spore within the cell, which is highly dehydrated, tolerant to long storage and resistant to unfavorable conditions. Additionally, *B. subtilis* possesses response mechanisms for adapting to various environmental stresses such as high temperature, high osmolality and oxidative conditions. *Bacillus subtilis* has seven genes that encode ECF sigma factors, of which six are apparently regulated by individual anti-sigma factors as the genes encoding ECF sigma and anti-sigma factors constitute an operon (Fig. 1); these six are also involved in similar mechanisms for stress response (Table 1). An important approach to unraveling the cellular function of sigma factors is to identify the genes whose transcription is under direct control of these sigma factors, using comprehensive analyses such as microarray analysis (Asai et al., 2003), and many other investigations have been undertaken to date. A highly detailed and general description about the structure and function

of ECF sigma factors is provided in three recent reviews (Mascher, 2013; Paget, 2015; Helmann, 2016). In this issue, I focus on regulatory aspects of anti-sigma factor activity, especially based on the cell surface (i.e., cell membrane and cell wall) dynamics, mainly in *B. subtilis*, compared to the representative model proposed in other bacteria.

Characteristic features of ECF sigma factors and their anti-sigma factors in *B. subtilis* Nineteen sigma factors are encoded in the *B. subtilis* genome;

Fig. 1. Gene organization around the ECF sigma factor genes. The ECF sigma factor genes and their cognate anti-ECF sigma factor genes are indicated in black (arrows and bold letters) and gray (arrows and bold letters), respectively. The locations of promoters (P) are indicated with hooked arrows, and the subscript after 'P' indicates the last letter of the sigma factor that recognizes the promoter. The subscript 'A' indicates SigA, a housekeeping and essential sigma factor in *B. subtilis*.

Table 1. Summary of stress sigma factors in *Bacillus subtilis*

σ	Anti- σ	Activation stress signals	Function	Reference
σ^A	—	—	housekeeping	
σ^M	YhdL/YhdK	salt, cell wall antibiotics, ethanol, heat, acid, superoxide stress	cell wall homeostasis	Thackray and Moir (2003)
σ^W	RsiW	membrane-active agents, alkali	cell membrane homeostasis	Helmann (2002)
σ^X	RsiX	cationic antimicrobial peptide	cell surface homeostasis	Helmann (2002)
σ^V	RsiV	lysozyme	lysozyme resistance	Ho et al. (2011)
σ^Y	YxlC/YxlD	nitrogen starvation	unknown	Tojo et al. (2003)
σ^{YlaC}	YlaD	oxidative stress	unknown	Matsumoto et al. (2005)
σ^Z	unknown	salt	unknown	Asai et al. (2005)
σ^B	RsbW	energy stress, environmental stress	general stress response	Hecker et al. (2007)

among these, seven belong to the ECF sigma factor sub-family and are called σ^M , σ^V , σ^W , σ^X , σ^Y , σ^Z and σ^{YlaC} (Table 1). Figure 1 shows their gene organizations with the location of their promoters and their anti-sigma factor genes. The genes for a sigma factor and its anti-sigma factor constitute an operon in most cases. Generally, the N-terminal portion of the anti-sigma factor, which is flanked by the predicted membrane-spanning region, resides in the cytosolic space and interacts with the partner sigma factor. In the case of RpoE and RseA, which are an ECF sigma factor and its anti-sigma factor, respectively, in *Escherichia coli*, the cytosolic domain of RseA was revealed to be sandwiched between the so-called region 2 and region 4 domains of RpoE, based on crystal structure analysis, thus blocking the interaction between RNA polymerase and the sigma factor (Campbell et al., 2003). In contrast, with the cobalt and nickel resistance-regulating ECF sigma factor CnrH and its anti-sigma factor CnrY in the β -proteobacterium *Cupriavidus metallidurans*, the cytoplasmic domain of CnrY embraces the region 2 and region 4 domains of CnrY, thus blocking RNA polymerase from promoter recognition (Maillard et al., 2014). The direct interaction of sigma factors with their anti-sigma factors in *B. subtilis* was examined using a yeast two-hybrid system (Yoshimura et al., 2004). This analysis showed that the sigma factors bind their cognate anti-sigma factors exclusively, indicating that the interaction of each pair is highly specific. The amino acid sequences of anti-sigma factors are evolutionarily unrelated (although well-conserved counterparts are found among closely related species) (Helmann, 2002). This evoked the idea that the pairs of a sigma factor and an anti-sigma factor might have co-evolved and been inherited through vertical transfer. This notion also correlates with the fact that the environmental signals to which the anti-sigma factors respond are not unified even though

they sometimes overlap, and the regulatory mechanisms that control their function, most of which are not uncovered, are potentially diverse.

Typically, the transcription of each operon is regulated by the sigma factor encoded within the operon (Fig. 1). Therefore, the activities of sigma factors are measured by the strength of transcription directed by promoters that they themselves recognize. Basically, disruption of the anti-sigma factor leads to activation of the cognate sigma factor (Asai et al., 2003). The effect of disruption of the anti-sigma factor genes except for *yhdL-yhdK* and *yslC-yslD* on several promoters was tested in a round-robin system (Table 2). Inactivation of *yhdL-yhdK* and *yslC-yslD*, the anti-sigma factors of σ^M and σ^Y , respectively, was not successful, suggesting that abnormally high expression of σ^M and σ^Y is toxic to the cell (Horsburgh and Moir, 1999; Tojo et al., 2003). Although the promoter sequences recognized by ECF sigma factors are very similar, as shown in Fig. 2, promoter activity increases only when the corresponding sigma factor is activated by deletion of its cognate anti-sigma factor gene. Simultaneous

Table 2. Effects of mutation of anti-sigma factor genes on the promoter activity of the ECF sigma factor operon

Mutation	Promoter fused with <i>lacZ</i> gene					
	<i>PsigM</i>	<i>PsigV</i>	<i>PsigW</i>	<i>PsigX</i>	<i>PsigY</i>	<i>PylaA</i>
Wild	1.00	1.00	1.00	1.00	1.00	1.00
<i>rsiV</i> ⁻	0.79	248	0.41	0.63	0.94	4.14
<i>rsiW</i> ⁻	0.72	0.87	2.08	0.85	0.53	1.54
<i>rsiX</i> ⁻	0.70	0.84	0.59	1.26	0.62	2.20
<i>ylaD</i> ⁻	0.79	0.53	0.77	0.96	0.52	1.42

The activities of β -galactosidase are shown as relative values compared to the activity in wild type. Figures in bold indicate activities caused through self-regulation, as determined in previous studies.

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-35          . . . . . 17bp . . . . . -10
sigM : GTAATGTGCAACTTTAAACCTTTCTTATGCGTGTAATACATA
sigW : AAAAATTGAAACCTTTTGAAAC GAAGCTCGTATACATACAGA
sigX : TTGTAATGTAACTTTTCAAGCTATTCATACGACAAAAAAGTG
sigV : TTCTGCGCAAACTATTTTTTCAGCCTGCCGCGTCTATTCCTAGG
sigY : AAAAAGATGAAACGCTTTTGAATCCGGTGTCGTCTCATAAGGC

ylaA : TCATCTTGAAACTTTTTGAAAA GTCCGCTGTCTAACCGAATG
yrgG : TTTAATCGTAAACAAAGAGCAAACAATTGTTTGAATCAATTGTA
Consensus      TG · AAC                                CGTCTA

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Fig. 2. Promoter sequences of the ECF sigma factor genes. The first five promoters are located just upstream of the sigma factor genes and are recognized by the sigma factors themselves. In the last two promoters, *ylaA* is the first gene of the operon including *ylaC* encoding for ECF sigma factor σ^{YlaC} . This promoter is recognized with σ^{YlaC} . The gene *yrgG* is located upstream of *sigZ* (encoding the ECF sigma factor σ^Z), in the opposite orientation, and its promoter is recognized by σ^Z .

induction of multiple ECF sigma factors by a single ECF sigma factor induced with a stimulus to the cell seems to be avoided. As mentioned above, these observations support the idea that each sigma factor might have emerged independently even though ECF sigma factors are categorized as one large subfamily of sigma factors based on their structural similarities.

The signals to which ECF sigma factors respond tend to reflect their function. For instance, transcription of *sigV* is induced by lysozyme, and σ^V induces genes that confer lysozyme resistance to the cells (Ho et al., 2011). The operon containing the *sigY* gene is induced under nitrogen-starved conditions, but the physiological significance of this sigma factor is not clear (Tojo et al., 2003). σ^{YlaC} resides within an operon comprising four genes, in which transcription of *ylaC-ylaD* is induced when cells are exposed to oxidative stress dependent on Spx (Matsumoto et al., 2005). No detailed study on σ^Z , which is an orphan sigma factor (the anti-sigma factor is unknown), has been performed to date. In contrast, σ^M ,

σ^W and σ^X are expressed to some extent during normal growth and, together with σ^V , their function and regulatory mechanism have been well studied. These three or four sigma factors can play crucial roles in stress responses in *B. subtilis* (Asai et al., 2005; Helmann, 2016), acting like “The Three Musketeers or Four”. Each of the seven ECF sigma factors is individually dispensable for growth (Kobayashi et al., 2003), but they are involved in response mechanisms to various external stresses. Moreover, these seven genes can also be simultaneously deleted from the genome (Asai et al., 2008; Luo et al., 2010). Although the regulatory mechanisms comprising the sigma factor and anti-sigma factor are diversified, the signals that stimulate these ECF sigma factors in *B. subtilis* and their regulon genes partly overlap (Mascher et al., 2007), indicating that they fight cooperatively against external stress. The northern blots in Fig. 3A show the transcripts of indicated genes after cells were exposed to a high temperature for 30 min. The *ypuA* gene was induced by heat treatment (lanes 1 and

Fig. 3. Effect of sigma factor deletion on the activity of the remaining sigma factors. (A) Northern blotting analysis visualized using probes hybridizing with the genes indicated on the left. Total RNA was extracted from the cells before heat treatment (37 °C) (lanes 1 to 5) and after heat treatment (42 °C) for 30 min (lanes 6 to 10). The genotypes of the strains used in this experiment are as follows. Lanes 1 and 6: parental strain (*B. subtilis* 168); lanes 2 and 7: σ^M ; lanes 3 and 8: σ^M and σ^V ; lanes 4 and 9: σ^M and σ^W ; and lanes 5 and 10: σ^M and σ^X . (B) The cells harboring the promoter recognized by σ^W fused to *lacZ* (Kosono et al., 2004) were harvested at the indicated times and β -galactosidase activity was measured. Circles: wild type; squares: σ^M , σ^V , σ^X , σ^Y , σ^Z and *ylaC*; triangles: σ^M , σ^V , σ^W , σ^X , σ^Y , σ^Z and *ylaC* (Asai et al., 2008).

6). This induction was weakened by inactivation of *sigM* (lane 7) and almost abolished by simultaneous deletion of *sigM* and either *sigW* or *sigX* (lanes 9 and 10), suggesting that these three sigma factors are involved in the transcription of *ypuA*. In contrast, double deletion of *sigM* and *sigV* or *sigX* (lanes 8 and 10) restored the downshift of the expression of *yjbC* and *ywoA* to the level of the *sigM*⁺ strain, and this restoration was abolished in the *sigW*⁻ strain (lane 9). These results suggest that σ^W , which is not drastically induced by heat in wild type cells, was activated to cope with the crisis in the absence of σ^M , σ^X and σ^V . Thus, the role of σ^W in heat stress may be obscured by σ^M , σ^X and σ^V . Notably the expression of *sigW* was constitutively higher than that in the wild type strain when the six ECF sigma factor genes other than *sigW* were deleted and σ^W was the sole ECF sigma factor in *B. subtilis* (Fig. 3B). This suggests that the cells perceive a crisis from the state of less responsive mechanisms and exert the best possible measures to overcome it using their remaining resources. The robustness of cells against stresses that threaten their existence may have been accomplished through such multilayered and overlapping mechanisms (Radeck et al., 2017).

Regulation of σ^W by the anti- σ^W protein RsiW The environmental signals that activate ECF sigma factors through the anti-sigma factors are expected to stimulate the cells physically and modulate the proteins related to the ECF sigma factor-responding system. The best-studied mechanisms that regulate the intracellular behavior of anti-sigma factors are observed with σ^W and RsiW (Fig. 4). After exposure of cells to stress conditions, the

transmembrane segment of RsiW is cleaved at site 1 near the boundary of the extracellular region by a membrane-embedded protease, PrsW (protease responsible for activating sigma-W; formerly YpdC) (Ellermeier and Losick, 2006), and is then cleaved at site 2 near the boundary of the intracellular region by another membrane protease, RasP (regulating anti-sigma factor protease; formerly YluC) (Schobel et al., 2004). The intracellular portion (anti-sigma factor binding) released through this proteolytic action is eventually degraded by the ClpXP protease complex (Zellmeier et al., 2006), resulting in the complete disengagement of σ^W from RsiW. This mechanism is similar to the regulation of RseA, the anti-sigma factor of σ^E in *E. coli*, by DegS and RseP proteases (Ades et al., 1999; Alba et al., 2002; Kanehara et al., 2002). This sequential cleavage of a protein within a membrane is called regulated intramembrane proteolysis (RIP) and was first described in the regulation of mammalian cholesterol metabolism by proteolysis of the membrane-bound transcription factor SREBP, which is also cleaved sequentially by two membrane proteases (Brown and Goldstein, 1997). In contrast to the fact that the cleaved portion of the anti-sigma factor is not useful for cellular activity, SREBP is a precursor that is cleaved to its active form as a transcriptional factor. The principle of RIP is employed in two different types of such transcriptional regulation in mammals and bacteria. This fact evokes the idea that the bacterial two-component-type system, composed separately of a transcriptional factor and a regulatory anti-factor, developed into a one-component-type system through fusion. ECF sigma factors classified as group ECF41 contain a C-terminal extension, which seems to

Fig. 4. Model of the mechanisms of RsiW degradation resulting in σ^W activation in *B. subtilis*. The details are described in the text. Still-unresolved function, interaction, signal and mechanism are indicated by question marks.

be a fused anti-sigma-like domain and is also required for sigma factor activity (Wecke et al., 2012). The activity of CorE-like sigma factors classified as group ECF44 is also positively or negatively regulated by a C-terminal extension (Gómez-Santos et al., 2011). A putative ECF sigma factor gene prolonged with a putative anti-sigma factor gene in a single open reading frame is occasionally found in the bacterial genome database (e.g., OB_RS12020 in *Oceanobacillus iheyensis* HTE831).

When RasP was the only known protease that regulated the proteolysis of RsiW in *B. subtilis*, many studies were undertaken to identify a protease functioning upstream of RasP. We tested the effect of inactivating all the annotated protease genes encoded in the *B. subtilis* genome on σ^W -dependent transcription. As PrsW was a novel family of membrane proteases and was not annotated as a protease at the time, we overlooked it. However, we found that inactivation of *ypuD*, encoding a protein of unknown function, led to a slight decrease in σ^W activity (K. A., unpublished results). Sequence similarity searches indicate that YpuD is a putative intramembrane metalloprotease with four transmembrane domains and is a member of the so-called CPBP (CAAX proteases and bacteriocin-processing enzymes) family, a large group of diverse proteins including the type II CAAX prenyl endopeptidases (Pei and Grishin, 2001). The involvement of YpuD in the regulation of RsiW has not yet been analyzed in detail.

Other investigations using transposon-mediated random mutagenesis were performed to elucidate the unknown

regulatory factors of RsiW, including still-undiscovered proteases. In this screening, PrsW was not detected for some unknown reason, although σ^W activity was found to be impaired in a strain harboring a mutation in the *ecsA* gene (Heinrich et al., 2008; K. A., unpublished results). The mutation in *ecsA* was also revealed to block the proteolysis of RsiW by RasP. The gene *ecsA* exists in a putative operon of three genes, *ecsA*, *ecsB* and *ecsC*, which is predicted to encode a so-called ABC transporter complex, although the function of *ecsC* is still unknown. Inactivation of *ecsA* and/or *ecsB* (but not *ecsC*) in the operon caused a similar deficiency of SigW activity, suggesting that the transport ability of EcsA/EcsB was responsible for RasP regulation (Fig. 4). It was previously reported that *ecs* mutants showed pleiotropic phenotypes including decreased exoamylase (or possibly exoenzymes) and deficiencies of competence and sporulation in *B. subtilis* (the name of the operon is derived from these pleiotropic phenotypes) (Leskela et al., 1996). To date, the role of EcsABC, an ABC transporter involved in multiple events occurring after the logarithmic growth phase, in the regulation of RasP has not been elucidated precisely. One possible role is suggested from the experiment shown below. In the *rsiW*-deleted mutant, σ^W activity was higher than that in the wild type strain (Fig. 5A). Truncation of *rsiW* is also expected to cause loss of RsiW function, resulting in an increased level of σ^W activity. Unexpectedly, when the C-terminal, extracytosolic segment of RsiW was sequentially deleted, yielding ΔRsiW^{72} and ΔRsiW^{55} , which still possessed 72 and 55

Fig. 5. Altered expression of *sigW* under various conditions. Vertical axes indicate the β -galactosidase activities of the *lacZ* gene fused with the promoter sequence, which is recognized by σ^W . (A) Effect of RsiW deletion on its function of sequestering σ^W as an anti-sigma factor. Cells harboring the *lacZ* fusion and a progressively truncated series of RsiW were harvested at the indicated times and β -galactosidase activities were measured. Closed circles: *rsiW*⁺ (RsiW¹⁰³); open circles: *rsiW*⁻ (ΔRsiW^0); open diamonds: ΔRsiW^{72} ; open squares: ΔRsiW^{55} ; and open triangles: ΔRsiW^7 . The superscript indicates the number of amino acids in the extracytoplasmic region, predicted by the PSORT program. (B) Effect of the addition of Zn ions on *sigW* expression. Cells of wild type (circles) and *rasP* mutant (triangles) in the absence (open symbols) and presence of 100 mM ZnCl₂ (closed symbols) were harvested at the indicated times and the β -galactosidase activities were measured.

amino acids in the extracytosolic space (in contrast to 103 in wild type RsiW), the σ^W activity of cells decreased to levels lower than that of the wild type strain, indicating that the truncated RsiW strongly suppressed σ^W activity. On the other hand, further deletion, yielding Δ RsiW⁷, caused σ^W activity to increase, suggesting that Δ RsiW⁷ is unstable and susceptible to proteases. This observation suggested that the truncated RsiWs (Δ RsiW⁷² and Δ RsiW⁵⁵) did not suffer further truncation with proteolysis, resulting in their being stabilized and rigidly binding to σ^W . It is proposed that the very end of the C-terminal region of RsiW would be required for interaction with PrsW, or that the peptide released from RsiW, cleaved at site 1 by PrsW, could induce further proteolysis at site 2 by RasP. Thus, in the latter case, the released peptide could be transported by or attached to EcsAB and this event would activate RasP (Fig. 4), although further analysis is still needed.

In *S. coelicolor* A3(2), the ECF sigma factor σ^R regulates the thioredoxin system in response to changes in the cytoplasmic thiol-disulfide status, and is controlled by an anti-sigma factor, RsrA, whose cytoplasmic (sigma factor binding) domain contains the CysX_nHisX₃CysX₂Cys (CHCC) motif for the coordination of a zinc ion (Kang et al., 1999; Paget et al., 2001; Campbell et al., 2007). Under thiol-oxidizing conditions, RsrA forms intramolecular disulfide bonds and cannot bind σ^R , and thus discharges it. Therefore, this anti-sigma factor family is called the ZAS (zinc-binding anti-sigma factor) family. RsiW and YlaD (the putative anti- σ^{YlaC}) of *B. subtilis* have a CHCC motif in the N-terminal domain and are classified as ZAS family proteins (Paget et al., 2001). Although transcription of the *ylaABCD* operon depends on the ECF sigma factor σ^{YlaC} , and YlaD acts as an anti-sigma factor for σ^{YlaC} (Fig. 1), oxidative stress did not induce the transcription of *ylaABCD* (Matsumoto et al., 2005). Moreover, the interaction between σ^W and RsiW was not changed by oxidation of the CHCC motif in RsiW and the expression of *sigW* is unrelated to thiol-oxidizing conditions, suggesting that the RsiW activity for σ^W suppression is not regulated by the state of zinc coordination (Devkota et al., 2017). In *B. subtilis*, these CDCC motifs may have become silenced during the process of evolution, one (in YlaD) by changing into a pseudomotif and the other (in RsiW) by acquisition of additional mechanisms. According to our unpublished results, expression of *sigW* was higher in the presence of 100 μ M ZnCl₂ in the medium than in the absence of excess Zn ions (Fig. 5B). However, this elevation was not observed in the *rasP* mutant, suggesting that proteolysis of RsiW by proteases is regulated by zinc availability (possibly excess zinc stress).

The anti- σ^V , RsiV, is controlled by RIP, in which RasP also cleaves RsiV at site 2, whereas cleavage of RsiV at site 1 is performed by an unknown protease other than PrsW. Exposure of cells to the inducer lysozyme acti-

vates site 1 proteolysis, resulting in further truncation at site 2 by RasP (Hastie et al., 2013). It has been reported that *E. coli* outer membrane proteins unfolded by extracellular stresses bind to DegS, thus allowing DegS to cleave RseA (anti- σ^E) (Walsh et al., 2003). The molecular mechanisms that activate these controlling proteins are not yet understood precisely in *B. subtilis*, with the exception that the anti- σ^W factor RsiW is known to be regulated by multiple proteins in response to extracellular stresses. Since the structure of the cell surface in Gram-positive *B. subtilis* is distinct from that in Gram-negative *E. coli*, the underlying mechanisms are likely to be different. Expression of *sigW* is induced by various extracellular stresses including alkaline stress, cell wall-active antibiotics, antimicrobial peptides produced by other bacteria, and especially membrane stresses (Ho and Ellermeier, 2012; Helmann, 2016). The involvement of these different kinds of extracellular stresses in σ^W activation might explain the complexity of the regulatory proteins related to RsiW. The fact that almost all factors, like RsiW, PrsW, RasP and EcsAB, that are involved in the mechanism are membrane proteins raises the possibility that membrane destabilization, which is caused by the σ^W -activating stresses, leads to activation of these factors and then triggers the cleavage of RsiW by PrsW.

Regulation of σ^M in response to extracellular cell wall stresses

σ^M is another well-studied ECF sigma factor in *B. subtilis*. The *sigM* operon is composed of three genes, *sigM*, *yhdL* and *yhdK*, and the latter two encode the putative membrane proteins YhdL and YhdK (Fig. 1). YhdL acts as the anti- σ^M and YhdK is predicted to interact only with YhdL and not with σ^M (Yoshimura et al., 2004). Abnormal overexpression of *sigM* caused by inactivation of *yhdL* provokes inhibition of cell growth (Horsburgh and Moir, 1999). Additionally, inactivation of *yhdK* causes retardation of cell growth, which is milder than that induced by *yhdL* inactivation, suggesting that YhdK binds to the σ^M -YhdL complex and supports YhdL function, e.g., by stabilization of the complex. The operon structure of *sigM* and *yhdL* is conserved in several bacterial species belonging to Firmicutes, but in some species *yhdK* is missing, indicating an auxiliary function for YhdK. It has been reported that more than 60 genes are regulated by σ^M (Asai et al., 2003; Eiamphungporn and Helmann, 2008). Notably, these include genes encoding the central components of cell division (e.g., *div* genes, *min* genes) and cell wall biosynthesis (e.g., *mur* genes, *mre* genes), which are essential for cell growth. This is consistent with the idea that disordered expression of these essential genes along with elevated expression of σ^M leads to lethality of the cell.

Expression of the *sigM* operon is upregulated in response to high salt, cell wall-active antibiotics, ethanol, heat, acidic pH and superoxide stress (Thackray and Moir,

2003). These stressors seem to affect cell wall integrity or function, whereas the stress signals against σ^W , including alkaline pH, seem to impair cell membrane stability. This discrimination is partly proven by the experiment shown in Fig. 6. The expression of *sigM* and *sigW* was induced when cells were exposed to acidic pH (6.0) and alkaline pH (8.0), respectively. The *sigW* expression induced by pH upshift was repressed by pH downshift, and the inversely repressed expression of *sigW* by pH downshift was immediately induced by pH upshift. In contrast, *sigM* expression induced by pH downshift was slightly repressed by pH upshift even though *sigM* expression repressed by pH upshift was not reversed by pH downshift. This discrepancy between σ^M and σ^W may reflect a difference in their mechanisms of regulation by the anti-sigma factor. In any case, these reversible and contrasting effects of pH shift on their expression may reflect a difference in a cell structure retaining the machinery that senses a stress signal between the two factors; namely, the structure is the cell wall for σ^M and the cell membrane for σ^W .

The overall processes of peptidoglycan synthesis are illustrated in the left part of Fig. 7. The basic units of a cell wall (N-acetylmuramic acid, N-acetylglucosamine and pentapeptide) are formed in the cytosolic space and assembled onto undecaprenyl phosphate, forming the so-called lipid II. Lipid II is then flipped to the outer surface of the cell membrane. Lipid II polymerization into a nascent peptidoglycan strand occurs via a glycosyltransferase-mediated peptidoglycan polymerization reaction. Cross-linking of peptide units in adjacent glycan strands occurs via a transpeptidase-mediated action (Lovering et al., 2012). Several steps are specifically

inhibited by cell wall-active antibiotics. For example, fosfomycin blocks construction of the basic unit of the cell wall at the very first stage, moenomycin inhibits glycosyltransferase, vancomycin inhibits transpeptidase and bacitracin blocks the dephosphorylation of undecaprenyl diphosphate, resulting in defective recycling of undecaprenyl phosphate (Fig. 7). Such cell wall-active antibiotics are known to induce σ^M and several other genes, which catalyze steps of peptidoglycan synthesis and are included in the σ^M regulon. One of the σ^M regulon genes, *bcrC* (formerly *ywoA*), encodes a predicted undecaprenyl-diphosphate phosphatase (the target of bacitracin) (Ohki et al., 2003; Bernard et al., 2005). The cells of *sigM*-knockout mutants bulge out at the division site, which is indicative of cell wall defects (Horsburgh and Moir, 1999). As mentioned above, these observations strongly indicate that σ^M contributes to cell wall homeostasis against extracellular stress.

To discover novel factors that influence the regulation of σ^M activity, transposon-mediated random mutagenesis was employed as in the case of σ^W . It was found that when *yfhO*, encoding a presumed bactoprenol-linked sugar translocase, is disrupted, expression of *CsbB*, which was already known to be transcribed with σ^B (Table 1) and σ^X -containing RNA polymerases in response to extracellular stress (Akbar and Price, 1996; Huang and Helmann, 1998) and encodes a putative undecaprenol glycosyltransferase, causes constitutive activation of σ^M (Inoue et al., 2013). As shown in the right part of Fig. 7, it is proposed that accumulation of glycosylated undecaprenol diphosphate catalyzed by *CsbB* reduces the undecaprenol pool unless the undecaprenol is recycled after consumption of the sugar loaded on it by the action of *YfhO*. Short-

Fig. 6. Effect of pH shift on the expression of *sigM* and *sigW*. The vertical axes indicate the β -galactosidase activities of the *lacZ* gene fused with the promoter sequence recognized by σ^M (A) and σ^W (B). Cells harboring the *lacZ* fusion were cultured in LB medium at various pHs, harvested at the indicated times, and the β -galactosidase activities were measured. Closed circles: pH 7.0 (normal condition); open circles: pH 6.0 (A) and pH 8.0 (B) (induction condition); closed squares: pH 8.0 to pH 6.0 after 30 min; closed triangles: pH 6.0 to pH 8.0 after 30 min. The experiments were performed repeatedly and the standard errors are indicated.

Fig. 7. Diagram of peptidoglycan synthesis (left) and the regulation of σ^M by YhdL/K (right) in *B. subtilis*. The details are described in the text. The inhibition sites of some cell wall antibiotics are indicated by flat arrows. GlcNAc: N-acetylglucosamine; MurNAc: N-acetylmuramic acid.

age of available undecaprenol within a cell induces σ^M activity. The well-characterized system known as Gtr, including undecaprenol glucosyltransferases, determines serotype conversion (O-antigen glucosylation) in Gram-positive enteric bacteria (Korres et al., 2005). In *B. subtilis*, there are two CsbB homologs, YkcC and YkoT, in which essential amino acid residues residing in the catalytic domain are also conserved. The genes encoding YkcC and YkoT are preceded by genes encoding YkcB and YkoS, which possess a putative transmembrane region (14 and eight passes, respectively). Transcription of *ykcBC* is regulated by two-component regulatory systems of unknown function, YrkQ/P and YclJ/K (Ogura et al., 2008, 2010). YkcB and YkoS have no significant similarity in amino acid sequence with YfhO, which possesses 13 transmembrane domains. Nevertheless, similar to the case of CsbB and YfhO, deletion of *ykcB* and induction of *ykcC* leads to σ^M activation, whereas deletion of *ykoS* and induction of *ykoT* does not (K. A., unpublished results). In Gram-positive bacteria, peptidoglycan is further modified with carbohydrate-based anionic polymers such as lipoteichoic acid and wall teichoic acid, which plays an important role in cell envelope integrity. These polymers are decorated with sugar and phosphate, which may be modified after presentation on the cell surface by this presumed Gtr-like system in *B. subtilis*.

Experiments testing whether already-known proteases that decompose the anti-sigma factors are involved in proteolysis of YhdL and YhdK did not show a definitive result. Our preliminary data suggested that the intracellular concentration of epitope-tagged YhdL was unchanged even when the cells were exposed to

σ^M -inducing stressors such as high salt. As shown in Fig. 6, σ^W activity changed instantly, increasing and decreasing with pH exchange, indicating nimble turnover of RsiW via proteolysis; in contrast, σ^M activity could not respond immediately to pH shift, suggesting the rather stable behavior of YhdL. These observations suggest that a conformational change in YhdL structure would be caused by extracellular stresses, resulting in release of σ^M (Fig. 7). Even so, it remains unknown whether direct sensing of cell wall damage by the C-terminal extension of YhdL protruding from the cell membrane (and possibly sensing the availability or abundance of undecaprenol or the lipid II intermediate via the transmembrane region of YhdL), and/or cell membrane degeneration caused by cell wall impairment, would lead to a conformational change in YhdL structure.

Universal effects of membrane degeneration on anti-sigma factors The molecular mechanisms regulating ECF sigma factors other than σ^W , σ^V and σ^M in *B. subtilis* have not been elucidated, although a few reports indicate that a broad effect on the composition and status of the cell membrane leads to global activation of ECF sigma factors. The processes of cell membrane biosynthesis, including lipoteichoic acid and glucolipids and their relationship with ECF sigma factors, are explained precisely in another review in this issue. Induction of σ^M and σ^V activities is observed when the lipid composition of membranes is altered by *pgsA* mutation, which causes a drastic reduction in phosphatidylglycerol content, whereas the others are not involved (Hashimoto et al., 2009). This demonstrates a specific relationship

between the alteration of lipid composition in membranes and anti-sigma factor function. Lipoteichoic acid and glucolipids in the cell membrane are also required for maintenance of cell surface structure, and all the genes encoding ECF sigma factors except *sigZ* are induced in lipoteichoic acid synthesis-deficient cells (Hashimoto et al., 2013), while σ^M , σ^V and σ^X were found to be activated in glucolipid-lacking cells (Matsuoka et al., 2011). In a mutant strain with a disrupted *shaA* gene, which encodes the sodium-hydrogen antiporter, expression of σ^M and σ^W increased whereas that of σ^X was reduced. It was postulated that disruption of *shaA* damaged the proton gradient across the membrane, resulting in superoxide generation and cell membrane damage (Kosono et al., 2004). A counterpart of σ^W -RsiW exists in *O. iheyensis*, OI σ^W -OIRsiW. Heterologous expression of OI σ^W -OIRsiW in *B. subtilis* has a lethal effect on the cell, suggesting OIRsiW dysfunction and toxicity of the abnormally expressed OI σ^W (note that overexpression of σ^W is not toxic). The function of OIRsiW is restored by extension of the alpha helix in the transmembrane segment, suggesting that proper thickness of the cell membrane is required for the translocation and/or function of membrane proteins like RsiW (Yano et al., 2011). The transcription of *fabF*, which resides in an operon for fatty acid synthesis comprising *fabHa* and *fabF*, is regulated by σ^W through a promoter that exists within the *fabHa* gene (Kingston et al., 2011). Thus, upregulation of FabF via transcription induced with this promoter increases the average fatty acid chain length, resulting in a reduction of membrane fluidity and an increase of resistance to stress response. These observations indicate that the activity of ECF sigma factors is sometimes regulated by an anti-sigma factor via the universal effects of membrane degeneration; it is thus plausible that multiple stress response systems should be launched rapidly and simultaneously in order to confront an immediate and diverse environmental and internal exchange. Notably, glucose addition leads to increased levels of acetylated CshA, which is known to associate with RNA polymerase, and RNA polymerase bound to acetylated CshA could preferentially bind σ^X and σ^M (Ogura and Asai, 2016). This is a unique model of a regulatory link between ECF sigma factors and intracellular nutritional signals as well as extracellular stress signals, independent of anti-sigma factors.

Concluding remarks The subfamily of ECF sigma factors was discovered recently after the emergence of whole-genome sequencing technology, in contrast to the gradual accumulation of studies about other sigma factors, even though the ECF sigma factors comprise a large proportion of all sigma factors found to date. This implies a particular physiological feature of ECF sigma factors; that is, these sigma factors are activated only under certain conditions, which rarely occur in laboratory

conditions, and their functions are redundant in a single microorganism. Therefore, it is difficult to unearth these genes by means of typical genetic analyses because a representative phenotype emerging from the inactivation of an ECF sigma factor cannot easily be determined.

ECF sigma factors constitute the largest and most diverse subfamily within the sigma factor family. On average, most eubacteria possess a dozen ECF sigma factors. Protein subfamilies such as ECF sigma factors are predicted to be derived from a primary (housekeeping and essential) sigma factor and to have diverged by duplication and accumulation of mutations (Wösten, 1998). Comprehensive analysis of the phylogenetic relationship of ECF sigma factors was performed by comparative genomic prediction to seek an insight into their evolutionary history, and suggested that the signal transduction system with which the ECF sigma factors are involved is far more complex and diverse than had been previously observed and postulated (Staron et al., 2009). Some ECF sigma factors are widely distributed in diverse bacterial phyla, indicating that they appeared before the individual phyla separated. However, the majority of ECF sigma factors are phylum-specific, implying that they have evolved individually to allow the species to specifically adapt to their environmental or cellular conditions.

As emphasized in this issue, diversity, hierarchy, specificity, complexity, and many other keywords of scientific interest in the field of molecular evolution and biology are included within the regulatory systems of transcription by ECF sigma factors, such as robustness acquired during the process of adaptation, interaction between ECF sigma factors and their cognate anti-sigma factors, and the molecular mechanisms of responses to the external environment via cell surface structures. Quests for answering how ECF sigma factors emerged and how they work will contribute to a comprehensive understanding of the biological regulatory network of gene expression.

I thank Fujio Kawamura, Naotake Ogasawara and Yoshito Sadaie for helpful discussions. This work was supported by JSPS Grants-in-Aid for Scientific Research (24580101 and 15K07350), and a JSPS Grant-in-Aid for Scientific Research on Innovative Areas (24119502).

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