Microbiology (2004), **150**, 591–599 DOI 10.1099/mic.0.26712-0

Interaction of *Bacillus subtilis* extracytoplasmic function (ECF) sigma factors with the N-terminal regions of their potential anti-sigma factors

Mika Yoshimura,[1] Kei Asai,[2] Yoshito Sadaie[2] and Hirofumi Yoshikawa[1]

[1]Department of Bioscience, Tokyo University of Agriculture, Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan

[2]Department of Biochemistry and Molecular Biology, Faculty of Sciences, Saitama University, Saitama 338-7507, Japan

Correspondence
Hirofumi Yoshikawa
hiyoshik@nodai.ac.jp

Received 15 August 2003
Revised 11 November 2003
Accepted 4 December 2003

Extracytoplasmic function (ECF) sigma factors constitute a diverse family of proteins, within the class of the sigma 70 subunit of RNA polymerase. Most members of the family studied to date are known to regulate gene expression in response to stress conditions. The *Bacillus subtilis* genome encodes at least 17 distinct sigma factors, seven of which are members of the ECF subfamily. Among these, five sigma factors, namely SigV, SigW, SigX, SigY and SigM, are encoded by the first genes of the cognate sigma operons. Disruption or repressed expression of the downstream gene(s) resulted in transcriptional activation of the cognate sigma operon. Moreover, *in vivo* protein–protein interaction analyses by yeast two-hybrid experiments indicated that these immediate downstream gene products bind the cognate ECF sigma factor, suggesting that they function as anti-sigma factors by capturing sigma factor on the inner surface of the cytoplasmic membrane. Interaction with other sigma factors was not observed. The results presented here also show that these anti-sigma factors interact with ECF sigma factors through their N-terminal region, implying that the N-terminal domain resides inside the cytoplasmic membrane.

INTRODUCTION

The extracytoplasmic function (ECF) sigma factors are found in a diverse range of bacteria and many are activated to transcribe their regulons in response to a change in environmental conditions (Helmann, 2002; Lonetto *et al.*, 1994; Raivio & Silhavy, 2001). For example, ECF sigma factors regulate iron uptake and heat-shock responses in *Escherichia coli* (Braun, 1997; De Las Penas *et al.*, 1997), alginate biosynthesis and exotoxin secretion in *Pseudomonas aeruginosa* (Hershberger *et al.*, 1995; Ochsner *et al.*, 1996), carotenoid biosynthesis in *Myxococcus xanthus* (Gorham *et al.*, 1996) and expression of the thioredoxin system in response to oxidative stress in *Streptomyces coelicolor* (Kang *et al.*, 1999; Paget *et al.*, 1998).

In many cases, the ECF sigma factor is co-transcribed with one or more negative regulators. Often, these include a trans-membrane protein with an extracytoplasmic sensory domain and an intracellular inhibitory domain functioning as an anti-sigma factor that binds and inhibits the cognate sigma factor. Although only a limited number of examples have been shown, direct interaction with sigma factor and anti-sigma factor has been reported for *E. coli* SigE and RseA (Campbell *et al.*, 2003; Raivio & Silhavy, 2001), FecI and FecR (Enz *et al.*, 2000), *P. aeruginosa* AlgU and MucA (Rowen & Deretic, 2000), *Rhodobacter sphaeroides* SigE and ChrR (Newman *et al.*, 2001), *S. coelicolor* SigR and RsrA (Li *et al.*, 2002) and *Myxococcus xanthus* CarQ and CarR (Browning *et al.*, 2003).

Bacterial genome sequencing has revealed numerous new members of this class of sigma factor including seven in *Corynebacterium diphtheriae* (http://www.sanger.ac.uk/Projects/C_diphtheriae/), 10 in *Mycobacterium tuberculosis* (Cole *et al.*, 1998), 16 in *Mesorhizobium loti* (Kaneko *et al.*, 2000) and 41 in *Streptomyces coelicolor* A3(2) (Bentley *et al.*, 2002). The ECF sigma factors retain many of the conserved domains of principal sigma factors, but show significant divergence from other members of the family. The functions and mechanisms of regulation for most of these newly described potential ECF sigma factors remain unknown.

In *Bacillus subtilis*, seven putative ECF sigma factors (SigV, SigW, SigX, SigY, SigZ, SigM and YlaC) have been identified (Kunst *et al.*, 1997). Among them, SigX has been reported to contribute in survival at high temperature (Huang *et al.*, 1997). Brutsche & Braun (1997) demonstrated and confirmed the anti-sigma activity and the predicted membrane localization of RsiX (YpuN), the product of the downstream gene. The *sigW* gene is co-transcribed with its downstream gene *ybbM* (Huang *et al.*, 1998) which

Abbreviation: ECF, extracytoplasmic function.

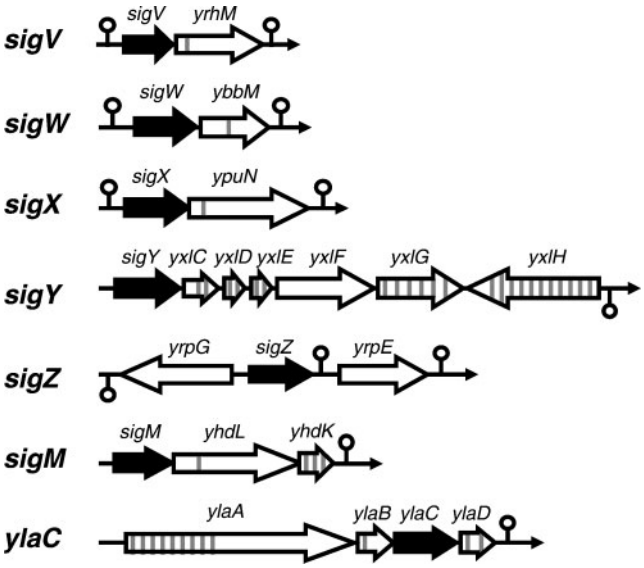

Fig. 1. Gene organization of the ECF sigma operons in *B. subtilis*. ECF sigma genes are shown as solid arrows. Stem and loop structures represent putative transcription terminators. Vertical grey lines in the genes indicate probable membrane-spanning domains, predicted by the program SOSUI (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html).

is postulated to be anti-sigma factor RsiW (Helmann, 2002), although no sufficient experimental evidence has been shown. Expression of *sigM* is up-regulated in cells growing in medium of high-salt concentration, and a *sigM* mutant failed to grow in such medium (Horsburgh & Moir, 1999). The two downstream genes *yhdL* and *yhdK* are known to negatively regulate the activity of SigM and, interestingly, *yhdL* is essential for normal growth (Horsburgh & Moir, 1999).

We employed a yeast two-hybrid analysis to examine specific interactions between putative anti-sigma proteins and their cognate sigma factors. Of the seven ECF sigma candidates, six genes are encoded within operons; *sigZ* is mono-cistronic (Sorokin *et al.*, 1997), as illustrated in Fig. 1. The *sigV*, *sigW* and *sigX* operons appear to be bi-cistronic (Liu *et al.*, 1997; Sorokin *et al.*, 1993, 1997), and the products of the downstream genes each contain one trans-membrane domain (see information described in the *Bacillus subtilis* Genome Database web site at http://bacillus.genome.ad.jp). The putative *sigM* operon includes three genes, of which two encode negative regulators (Horsburgh & Moir, 1999). The *sigY* gene is the first gene of a putative hexa-cistronic operon (Yoshida *et al.*, 1996). The *ylaC* gene is the third gene in a putative tetra-cistronic operon (Kunst *et al.*, 1997). We analysed the genes located in the putative ECF sigma operons, as well as *yrpG*, *yrpE* and *yxlH*, as primary candidates for screening of potential anti-sigma factors since these genes reside near the ECF sigma genes (Fig. 1). Here, we report at least five ECF sigma factors (SigV, SigW, SigX, SigY and SigM) that interact with the products of respective downstream genes as judged by a yeast two-hybrid analysis. The interactions appear to be highly specific as no cross-talk was observed.

METHODS

Construction of strains to assay for sigma-directed expression of *lacZ*. *B. subtilis* derivative strains of 168 (*trpC2*) harbouring the ECF sigma promoter–*lacZ* fusion in the *amyE* locus were constructed previously to monitor the expression of each sigma gene (Asai *et al.*, 2003). The cloned promoter region for each operon was −270 to +49 for *sigV*, −216 to +116 for *sigY* and −318 to +34 for *ylaA*, where +1 represents the first nucleotide of the initiation codon of each gene. The promoters were fused transcriptionally to *lacZ* through cloning into the *Eco*RI and *Bam*HI sites of pDL2, as described previously (Fukuchi *et al.*, 2000). The resulting strains, BSU32, BSU35 and BSU37, are summarized in Table 1.

Disruption or overexpression of anti-sigma factor candidate genes using pMUTIN derivatives. pMUTIN derivative plasmids pMUTINT3ΔZ and pMUTIN2ΔZ, which were devoid of the *E. coli lacZ* gene, were constructed from plasmids pMUTINT3 (Moriya

Table 1. Strains used in this study

Strains	Relevant properties	References
BSU32	*trpC2 amyE*::*sigV–lacZ* (Cm)	Asai *et al.* (2003)
BSU35	*trpC2 amyE*::*sigY–lacZ* (Cm)	Asai *et al.* (2003)
BSU37	*trpC2 amyE*::*ylaA–lacZ* (Cm)	Asai *et al.* (2003)
ASK431	*trpC2 yxlC*::pMUTIN2ΔZ (P_{spac}–*yxlC–D–E–F–G*)	Tojo *et al.* (2003)
ASK441	*trpC2 yrhM*::pMUTINT3ΔZ	This work
ASK446	*trpC2 ylaD*::pMUTINT3ΔZ	This work
ASK4001	*trpC2 amyE*::*sigV–lacZ yrhM*::pMUTINT3ΔZ	This work, BSU32→ASK441
ASK4002	*trpC2 amyE*::*ylaA–lacZ ylaD*::pMUTINT3ΔZ	This work, BSU37→ASK446
ASK4016	*trpC2 amyE*::*sigY–lacZ*(Cm::Sp) *sigY*::pX2 (P_{xyl}–*sigY–yxlC–D–E–F–G*)	This work
ASK4017	*trpC2 amyE*::*sigY–lacZ*(Cm::Sp) *sigY*::pX2 (P_{xyl}–*sigY*) *yxlC*::pMUTIN2ΔZ (P_{spac}–*yxlC–D–E–F–G*)	This work, ASK431→ASK4016

et al., 1998) and pMUTIN2 (Vagner *et al.*, 1998), through excision of *Cla*I–*Sac*I fragments. Putative anti-sigma factor genes *yrhM* and *ylaD* were disrupted with pMUTINT3ΔZ harbouring DNA fragments internal to the coding region, which were PCR-amplified using the primer sets listed in Table 2. Resulting strains were ASK441 and ASK446, respectively (Table 1). The cognate promoter activities for the *sigV* and *ylaA* operons were assayed with these disruptants by constructing strains ASK4001 and ASK4002 by using the chromosomal DNA of strains BSU32 and BSU37 (Table 1). Strain ASK431 was constructed for the overexpression experiment in which the *yxlC* gene was placed under the control of the *spac* promoter (P_{spac}) using pMUTIN2ΔZ through cloning of the 5′-upstream region of *yxlC*. PCR primers used to amplify the region are also listed in Table 2.

Xylose-inducible-promoter insertion. Using the primer set for *sigY* listed in Table 2, a PCR-amplified fragment containing the ribosome-binding site and the 5′ end of the *sigY* ORF was cloned into plasmid pX2 (Mogk *et al.*, 1997), an integrational vector harbouring a xylose-inducible promoter. The amplified fragment was treated with *Bam*HI prior to cloning in vector pX2. The chloramphenicol-resistance gene of strain BSU35, carrying the P_{sigY}–*lacZ* construct into the *amyE* locus, was substituted with a spectinomycin-resistance gene, using the integrational plasmid pCm::Sp (*Bacillus* Genetic Stock Center, OH, USA). Finally, this strain was transformed with the pX2-derived plasmid to construct a strain, ASK4016, which was chloramphenicol-resistant and expressed *sigY* under the control of a xylose-inducible promoter (Table 1 and Fig. 2a). To determine the effect of overexpression of putative anti-sigma factor YxlC and downstream genes on the transcription of the *sigY* operon, strain ASK4016 was transformed with chromosomal DNA of strain ASK431 to obtain strain ASK4017 (Table 1 and Fig. 2b). The genetic structures of these constructs were verified through PCR.

Cell culture conditions and β-galactosidase assay. An aliquot (1 ml) of cells growing in LB medium with or without IPTG or xylose was harvested by centrifugation, and the β-galactosidase activity was assayed according to the methods described by Youngman *et al.* (1985). Unit of enzyme activity is expressed as nmol ONPG hydrolysed min^{-1} $(mg\ protein)^{-1}$. Protein concentration was determined using a Bio-Rad Protein Assay Kit.

Yeast two-hybrid analysis. Yeast strains used in this analysis were PJ69-4A (*MAT***a** *trp1-901 leu2-3, 112 ura3-52 his-200 gal4*Δ *gal80*Δ *LYS2*::*GAL1–HIS3 GAL2–ADE2 met2*::*GAL7–lacZ*) and PJ69-4α (*MAT*α *trp1-901 leu2-3, 112 ura3-52 his-200 gal4*Δ *gal80*Δ *LYS2*::*GAL1–HIS3 GAL2–ADE2 met2*::*GAL7–lacZ*), obtained from Philip James (James *et al.*, 1996). Plasmid pGBTK, a GAL4 DNA-binding domain fusion vector, is a derivative of pGBT9 (Clontech) in which the ampicillin-resistance gene is replaced with the kanamycin-resistance gene of pGBKT7 (Clontech). Plasmid pGADT7, a GAL4 activation domain fusion vector, was purchased from Clontech. Oligonucleotides used for yeast two-hybrid analysis are listed in Table 2. Each fragment, except for YhdK–N, was PCR-amplified from *B. subtilis* genomic DNA using Proof Start DNA polymerase (Qiagen) and cloned into pGBTK or pGADT7. Since the fragment of YhdK–N is very small, the whole region was synthesized with restriction-site linkers (Table 2). Inserts of the resultant plasmids were verified by sequence determination.

Plasmid vectors pGBTK (*TRP1*) and pGADT7 (*LEU2*) were used to transform mating-type yeast strains PJ69-4A (for pGBTK) and PJ69-4α (for pGADT7) using the method essentially described by Gietz & Schiestl (1995) and as modified by Ito *et al.* (2000). Briefly, yeast strains were cultured in YPAD (1 % yeast extract, 2 % peptone, 0·00002 % adenine sulfate, 2 % glucose) medium, and competent cells were prepared without the addition of 10 % DMSO. Transformants were mated in appropriate liquid media in flat-bottomed 96-well plates. After mating, cultures were collected and washed with sterile water, then spotted onto a synthetic complete (SC) agar plate lacking Leu and Trp (SC−LW) for selection of *LEU2* and *TRP1* diploid cells. These cells were replica-plated onto selection media – SC lacking His (SC−LWH) and/or SC lacking adenine (SC−LWHA) – agar plates supplemented with 1 or 5 mM 3-aminotriazole, to inhibit auto-activation of the *HIS3* reporter gene.

RESULTS

Transcriptional regulation of ECF sigma operon by downstream gene(s)

As described above, some of the ECF sigma factors, SigW, SigX and SigM, have already been demonstrated to be regulated by their respective downstream gene products. Here, we analysed the remaining sigma factors, namely SigV, SigY and YlaC, but not SigZ, since it is mono-cistronic (Asai *et al.*, 2003). If each anti-sigma is inactivated and the corresponding sigma is derepressed, then the transcription of the cognate operon will be activated because the SigV, SigY and YlaC operons appear to be positively autoregulated by RNA polymerases bearing the corresponding sigma factor (Asai *et al.*, 2003). Thus, we tried to inactivate the downstream genes as primary candidates for anti-sigma factor genes through insertion of exogenous plasmid DNA, as described in Methods. Disruption mutant strains of *yrhM* and *ylaD* were constructed in which *sigV–lacZ* and *ylaA–lacZ* activity could be compared with that of the relevant wild-type strain (Table 3). As expected, inactivation of *yrhM* elevated the expression of a *sigV–lacZ* fusion (strain ASK4001). In contrast, disruption of *ylaD* had little effect on the expression of *ylaA–lacZ* (ASK4002), suggesting that YlaD is not a negative regulator of expression of *ylaC*. We carried out similar experiments for the *sigW*, *sigX* and *sigM* operons and confirmed previously reported results (data not shown).

In the case of the *sigY* operon, *yxlC* or *yxlD* gene disruptants showed abnormal colony morphology (data not shown). As an alternative approach, we inserted a xylose-inducible promoter upstream of the *sigY* gene to drive expression of the operon, which was not detectable under normal conditions, and we fused an IPTG-inducible promoter upstream of the *yxlC* gene to see the effect of expression of downstream genes on *sigY–lacZ* expression. Addition of xylose only slightly enhanced *sigY–lacZ* expression (Fig. 2a), but high levels of expression were obtained upon xylose induction when downstream gene expression was repressed (Fig. 2b). Addition of IPTG induced the expression of *yxlC* and downstream genes and resulted in total impairment in *sigY–lacZ* expression (Fig. 2b).

Specific interactions of SigW, SigX and SigY with their possible anti-sigma factors

To examine direct interactions between ECF sigma factors and other gene products, we adopted a yeast two-hybrid analysis. We first cloned the seven sigma genes into pGBTK

Table 2. Primer pairs and oligonucleotides used for cloning in this study

Restriction sites that were added artificially are underlined.

Yeast two-hybrid analysis	Sequence of primer pair (forward, reverse)
SigV	GCGAATTCATGAAGAAAAAACAAACAACAAAAGCGTTGC, GCGGATCCTTAAGAAAGATCCTCCTTCGTC
SigW	GCGAATTCATGGAAATGATGATTAAAAAAAGAATTAAAC, GCCTGCAGTTAAAGATCCCTTAATTGTT
SigX	GCGAATTCATGGAAGAAACCTTTCAATTATTATATGATAC, GCGGATCCACGATGGTCTTTAACTGCCG
SigY	GCGAATTCTTGGATACACAAGAAGAACAGCG, GCGGATCCCGGTATAGAGGGATTGGCTG
SigZ	GCGAATTCATGAATATAGAGGATCTATGGGATC, GCGGATCCAATAACTGACTATGTTCATCAG
SigM	GCGAATTCGTGACGATCGATGAAATTTACC, GCGGATCCCAGTCATTTCCTGGTCGCTC
YlaC	GCGGATCCTGAAGCATAGGGATTCCATTGAG, GCGTCGACAGAAAGCAGGTCATATCCGC
YrhM	GCGAATTCATGGATAAGAGATTACAGCAATTAAGAG, GCGGATCCTTGATAGCGGGGTTACGACC
YbbM	GCGAATTCATGAGCTGTCCTGAACAAATTGTGCAG, GCGGATCCACGCGCTTTACTCTTCTCCG
YpuN	GCGAATTCATGATGAAGTCAGAATGGAACGAAG, GCGGATCCTTAGTTTAAGGAAATCGGATTTGGAGCAG
YxlC	GCGAATTCATGAATAAAGAAAAGCTTTCTGATCATTTAAAG, GCGGATCCGTCATCCGCGTTTCACCTCGCA
YxlD	GCGAATTCATGACACAAACAGAAATTATCATAACGG, GCGGATCCCAGCTGTAATACAATCAGCG
YxlE	GCGAATTCATGAATATTTCTTGGGAAATGATTTTGCC, GCGGATCCGCTTACCTTTGCTTTCTGCC
YxlF	GCGAATTCATGCTGTCTATTGAATCATTATGC, GCGTCGACCATCATCACCTTCATGCGTG
YxlG	GCGAATTCATGAAGGTGATGATGGCGCT, GCGGATCCCGCCTTTTTTGTGATGCTCG
YxlH	GCGAATTCATGGAATTACAGCAGGACTTGATTC, GCGTCGACGCTTCCGGCGGTATGAAAGC
YrpE	GCGAATTCATGAACATTTTATTTTCAAAACGGCTAGGC, GCGGATCCTTAATGAGCCATCATCTCATGGG
YrpG	GCGAATTCTTGCGGGTGAGCCGTTTATG, GCGTCGACTATCACCAGGCGTATGCCTC
YhdL	GCGGATCCTGATGAATGAAGAATTTAAAAAGCG, GCGTCGACGATCCAGCCGAATACATTGTG
YhdK	GCGAATTCATGGAACTGGTAAGAATTTTTAAAGAACAC, GCGGATCCTTAAAAGCCAAACTGTAAATTCGTATCTACAG
YlaA	GCGAATTCTTGTTGTTCGTGTTGGAGTTATTCAG, GCGGATCCTCATCCTCTAGCTCTCCTTC
YlaB	GCGAATTCATGAATCATAAAGAAAAAGAGTCTG, GCGGATCCCCTCAATGGAATCCCTATGC
YlaD	GCGAATTCATGACCTGCTTTCTAGTAAGAGAC, GCGGATCCTTGCCGGAATCGGACAAGCG
YrhM–N	GCGAATTCATGGATAAGAGATTACAGCAATTAAGAG, GCGGATCCTCTTTTCTTTTTTGGTTCTTGCTG
YrhM–C	GCGAATTCCCGGACGCCGCTCAGGCTATG, GCGGATCCTTGATAGCGGGGTTACGACC
YbbM–N	GCGAATTCATGAGCTGTCCTGAACAAATTGTGCAG, GCGGATCCGAACCATCTTTTTACAGAAGC
YbbM–C	GCGAATTCTGGCATAATGACCACAATTTCAGC, GCGGATCCACGCGCTTTACTCTTCTCCG
YpuN–N	GCGAATTCATGATGAAGTCAGAATGGAACGAAG, GCGGATCCGACAGCGGGCTTATTTTTACG
YpuN–C	GCGAATTCTTTGATCAGGCACAGCCTC, GCGGATCCTTAGTTTAAGGAAATCGGATTTGGAGCAG
YxlC–N	GCGAATTCATGAATAAAGAAAAGCTTTCTGATCATTTAAAG, GCGGATCCTTTTTTTCTGTATTCAGCTTTCATTTGTG
YhdL–N	GCGGATCCTGATGAATGAAGAATTTAAAAAGCG, GCGTCGACATAAGACTTGCGTTTGCCGTAG
YhdL–C	GCGGATCCTCTATTACGGAATGGGTGG, GCGTCGACGATCCAGCCGAATACATTGTG
YlaA–C	GCGAATTCAAGCCAGCAAATGTCCATGATTCATTC, GCGGATCCTCATCCTCTAGCTCTCCTTC
YlaB–N	GCGAATTCATGAATCATAAAGAAAAAGAGTCTG, GCGGATCCATATCTGATTTGGCTTTCTTCTTCAGC
YlaD–M	GCGAATTCGATTGTAAAAGAGAAACGGAACACG, GCGGATCCATAGTACCTCTGTTTAAAACGCAG
YhdL–NM	GCGGATCCTGATGAATGAAGAATTTAAAAAGCG, GCGTCGACGTAGCTTCCCAGCGTACAAAGC
YhdL–MC	GCGGATCCTGCGAATTTCTGTGCTTGCC, GCGTCGACGATCCAGCCGAATACATTGTG
YhdK–N	AATTCATGGAACTGGTAAGAATTTTTAAAGAACACAATGTATTCG, GATCCGAATACATTGTGTTCTTTAAAAATTCTTACCAGTTCCATG
YhdK–NM	GCGAATTCATGGAACTGGTAAGAATTTTTAAAGAACAC, GCGGATCCCATTTGATAGGAATAAAACGTGACG
Gene disruption	
yrhMd	AAGAAGCTTGAGATTACAGCAATTAAGAG, GGAGGATCCCGGACAAAGCAGGTGT
ylaDd	AAGAAGCTTTCTAGTAAGAGACCTGC, GGAGGATCCGGCAGATAAGCCTCCTC
Xylose-inducible-promoter insertion	
sigYsd	GGAGGATCCATGGATACACAAGAAGAACAGC, GGAGGATCCAGCTTTAACAAGTATTTATATAAG
pMUTIN insertion	
yxlCsd	GAAGAATTCCAGCAAATCAGAAAGGAG, GGAGGATCCTTGAAGAAGTTTTTTTCTGTATTC

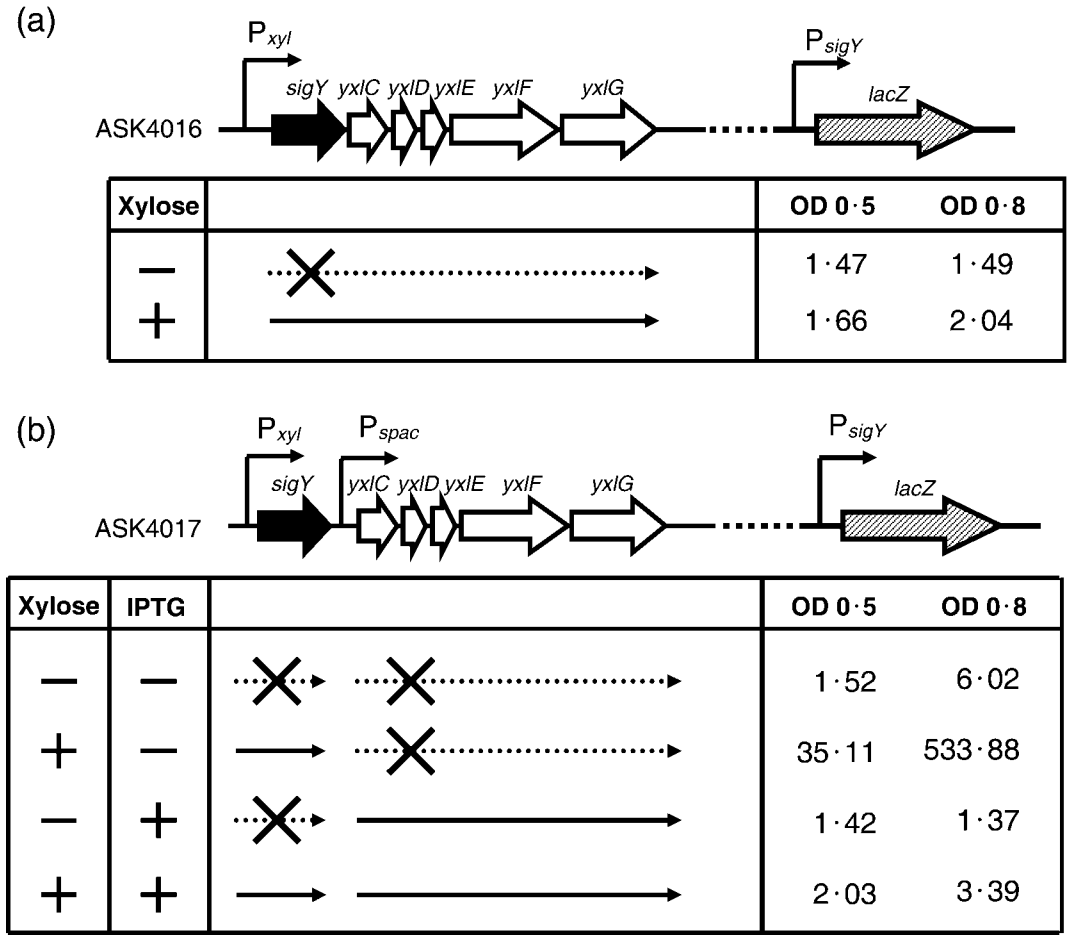

Fig. 2. Effect of overexpression of putative anti-sigma factor on transcription of its cognate sigma factor gene. SigY activity was monitored as β-galactosidase activity (Miller units) using P_{sigY}–*lacZ* inserted in the *amyE* locus, with the original *sigY* operon fused to the xylose-inducible promoter. Samples were withdrawn from the culture at two points during exponential growth (OD_{600} 0·5 and 0·8) and analysed (a). The downstream genes of *sigY* were directed by an IPTG-inducible promoter and the SigY activity was measured as above. Note that the expression of downstream genes was repressed without IPTG due to the terminator located in front of P_{spac} (b).

Table 3. Effect of mutation in putative anti-sigma factor genes on sigma-directed expression of *lacZ*

Strains	Genotype	β-Galactosidase activity* at:	
		OD_{600} 0·5	OD_{600} 0·8
amyE*::P_{sigV}–*lacZ			
BSU32	*yrhM* wild-type	6·0	1·7
ASK4001	*yrhM*Δ	1227·6	692·6
amyE*::P_{ylaA}–*lacZ			
BSU37	*ylaD* wild-type	0·97	1·11
ASK4002	*ylaD*Δ	1·45	1·50

*Values are expressed in Miller units. To measure β-galactosidase activity, aliquots were withdrawn from cultures of exponentially growing cells at points corresponding to OD_{600} 0·5 and 0·8. Experiments were repeated several times and representative results are shown.

to obtain DNA-binding domain fusions and cloned all the other genes shown in Fig. 1 into pGADT7 for activation domain fusion proteins. After mating, interactions between fusion proteins in yeast cells were screened through the expression of the reporter genes *HIS3* and/or *ADE2*. As shown in Fig. 3, positive interactions were clearly observed in pairs of SigW/YbbM, SigX/YpuN and SigY/YxlC. No other combinations yielded positive colonies.

ECF sigma factors interact with the N-terminal region of anti-sigma factors

In other organisms, most of the anti-sigma factors investigated so far are integral cytoplasmic membrane proteins (Helmann, 2002; Rowen & Deretic, 2000). If this is also the case for *B. subtilis*, then in at least either side of the membrane-spanning domain, anti-sigma proteins should possess a cytoplasmic domain where sigma factors interact.

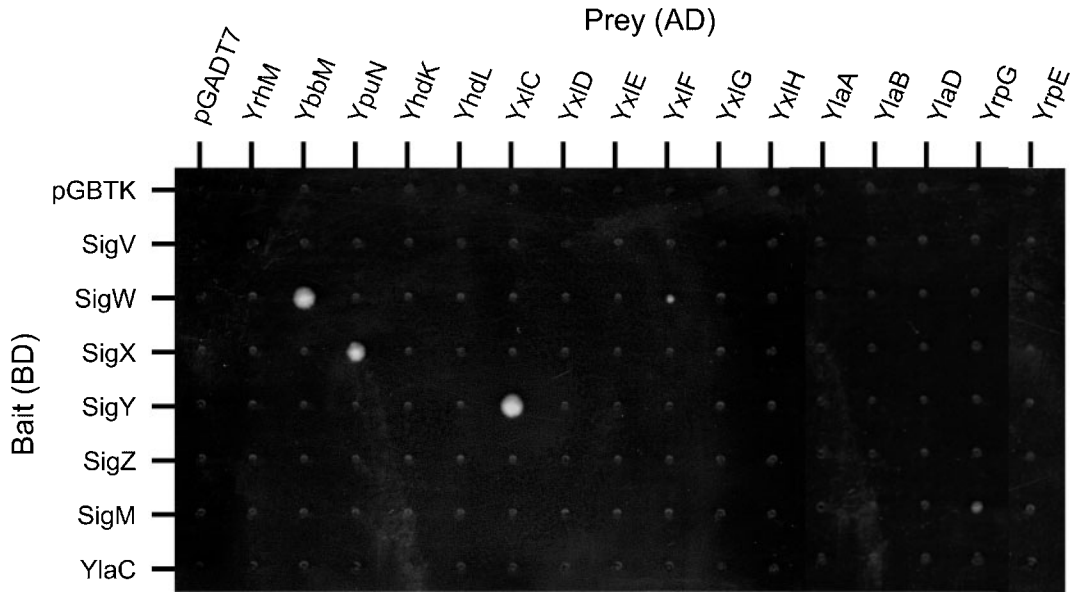

Fig. 3. Interaction of ECF sigma factors with possible anti-sigma factors. Diploid strains were constructed by mating PJ69-4A containing pGBTK or its derived plasmids harbouring ECF sigma factors with PJ69-4α containing pGADT7 or its derived plasmids harbouring anti-sigma factor candidates. Two-day-old cells that had been grown on a plate were suspended in culture medium, and 30 μl each of bait and prey plasmid-harbouring cells were mixed with 140 μl of media per well, allowing the cells to mate during overnight incubation at 30 °C. Cells were washed twice in distilled water and resuspended in 20 μl distilled water; a 5 μl aliquot was then spotted onto an SC−LW plate. Plates were incubated at 30 °C for 2–3 days, followed by replica plating of diploid cells onto selective SC−LWHA plates containing 5 mM 3-aminotriazole using a replicator and incubation at 30 °C for another 7 days. AD, Activation domain; BD, DNA-binding domain.

Therefore, we analysed separately the N-terminal and C-terminal domains of the possible anti-sigma factors without the membrane-spanning domain, to verify the interaction with ECF sigma factors, since a defined region often gives a clear result in yeast two-hybrid analysis. The N-terminal regions of YrhM, YbbM, YpuN, YxlC and YhdL each interacted with their cognate ECF sigma factor (Fig. 4). These results strongly suggest that the N-terminal region of these putative anti-sigma factors is localized in the cytoplasm to interact with the sigma factor.

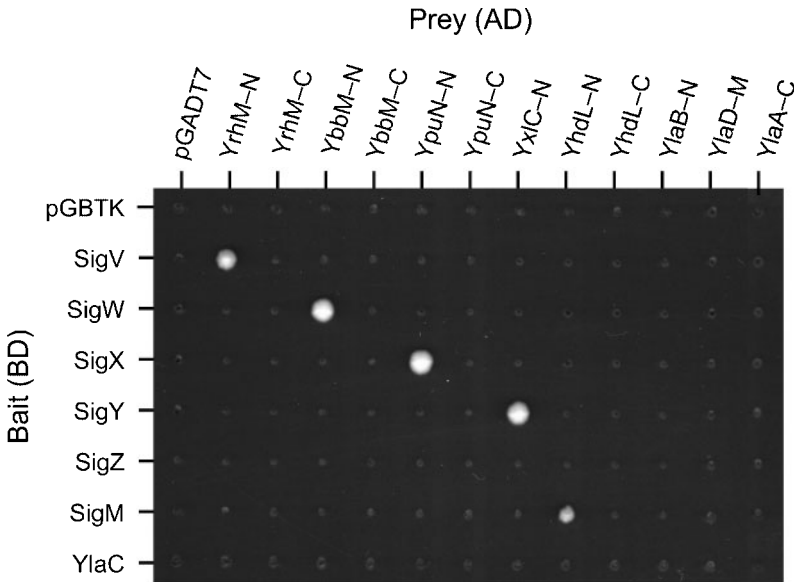

Fig. 4. Interaction of ECF sigma factors with fragments of possible anti-sigma factors devoid of the membrane-spanning domains. Bait constructs were the same as for Fig. 3. For the prey constructs, the N-terminal region or the C-terminal region of the anti-sigma candidates without putative membrane-spanning domains was cloned in pGADT7. The amino acid residues of the anti-sigma candidate protein regions are: YrhM–N, 1–37; YrhM–C, 61–285; YbbM–N, 1–84; YbbM–C, 108–208; YpuN–N, 1–45; YpuN–C, 69–368; YhdL–N, 1–71; YhdL–C, 95–358; YxlC–N, 1–49; YlaA–C, 278–648; YlaB–N, 1–64; and YlaD–M, 17–72. Diploid strains for testing were grown and spotted as described in Fig. 3 except that an SC−LWHA plate containing 1 mM 3-aminotriazole instead of 5 mM 3-aminotriazole was used. AD, Activation domain; BD, DNA-binding domain.

Interactions among *sigM* operon proteins

As shown in Fig. 4, we detected an interaction between SigM and the N-terminal region of YhdL. It has been postulated that the activity of SigM is negatively regulated by the downstream gene products YhdL and YhdK (Horsburgh & Moir, 1999). To determine the role of YhdK in the SigM–YhdL interaction, we analysed the possible interactions among SigM operon proteins and obtained evidence that YhdK interacts with YhdL (Fig. 5a). Since these proteins have integral membrane-spanning domains, in order to eliminate the possibility of the non-specific hydrophobic interaction frequently observed in the two-hybrid analysis, we tested YhdK and YhdL with other proteins containing one or two membrane-spanning regions. Results included in Fig. 5(a) demonstrate a highly specific interaction between YhdK and YhdL.

To investigate further the role of the membrane-spanning domain of these proteins, various fusion proteins with or without the membrane-spanning domain were constructed and analysed. As clearly summarized in Fig. 5(b, c), only proteins possessing a membrane-spanning domain exhibited positive interaction, implying that this domain might be an essential component for the interaction of these proteins to occur, probably in the cytoplasmic membrane.

DISCUSSION

It is widely believed that ECF sigma factors regulate the expression of their own gene/operon in response to external signals, i.e. so-called autoregulation (Helmann, 2002). This is also the case for *B. subtilis*, as shown by DNA microarray analyses of transcription stimulated by *B. subtilis* ECF sigma factors (Asai *et al.*, 2003). Taking these factors into consideration, our strategy was to monitor sigma factor activity through measuring the cognate promoter activity. Experimentally, we analysed the effects of anti-sigma factor gene disruption on the expression of the cognate promoter–*lacZ* fusion gene, measured as *β*-galactosidase activity. In the case of the bi-cistronic operons *sigV*, *sigW*

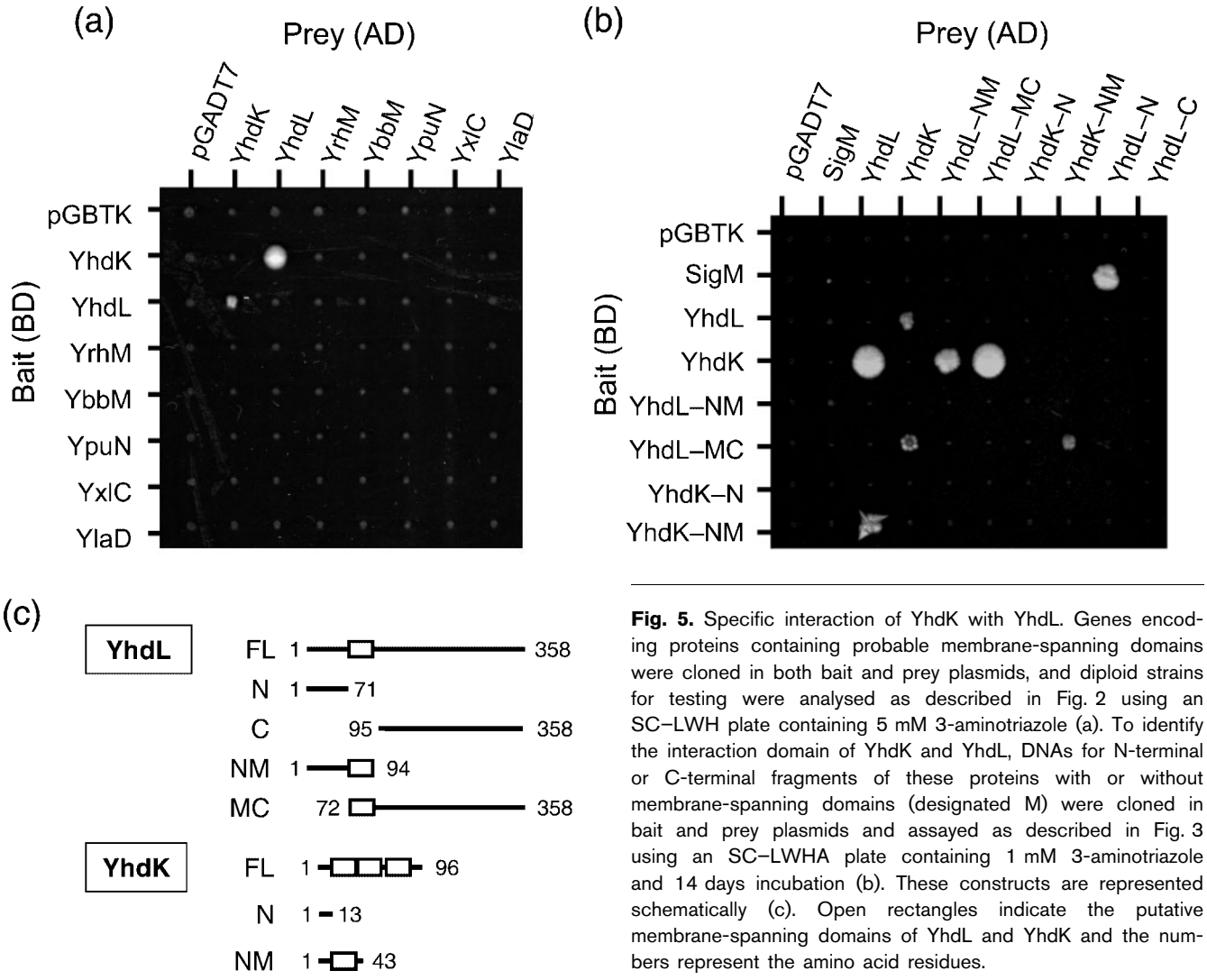

Fig. 5. Specific interaction of YhdK with YhdL. Genes encoding proteins containing probable membrane-spanning domains were cloned in both bait and prey plasmids, and diploid strains for testing were analysed as described in Fig. 2 using an SC−LWH plate containing 5 mM 3-aminotriazole (a). To identify the interaction domain of YhdK and YhdL, DNAs for N-terminal or C-terminal fragments of these proteins with or without membrane-spanning domains (designated M) were cloned in bait and prey plasmids and assayed as described in Fig. 3 using an SC−LWHA plate containing 1 mM 3-aminotriazole and 14 days incubation (b). These constructs are represented schematically (c). Open rectangles indicate the putative membrane-spanning domains of YhdL and YhdK and the numbers represent the amino acid residues.

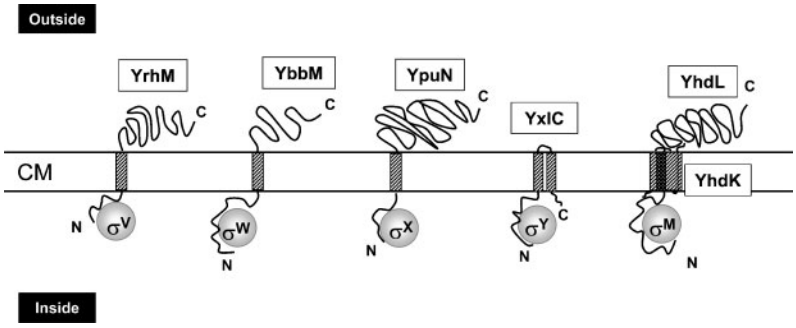

Fig. 6. Model of the interactions between ECF sigma factors and putative anti-sigma factors. Hatched boxes indicate predicted trans-membrane domains. Other interactions suggested in this article are also included. CM, cytoplasmic membrane.

and *sigX*, our transcriptional activation experiment for *sigV* and *sigW*, as well as previous reports on *sigX*, indicate that these three sigma factors are negatively regulated by the second gene products of the cognate operon (Table 3, and Huang *et al.*, 1997, 1998). On the other hand, we find the hexa-cistronic *sigY* operon to be rather complicated. Interaction with SigY was observed only against YxlC, but disruption of the *yxlD* gene produced a similar colony morphology to that of the *yxlC* disruptant (data not shown). This may suggest some co-operation between YxlC and YxlD. Cao *et al.* (2003) reported that *yxlC* could be disrupted and YxlD, not YxlC, might regulate SigY activity. Further investigation needs to be carried out.

Direct interaction between ECF sigma factor and anti-sigma factor has been demonstrated in several Gram-negative bacteria and *Streptomyces* (see Introduction). In this study, using a yeast two-hybrid system, we have shown that *B. subtilis* ECF sigma factors SigM, SigV, SigW, SigX and SigY interact with proteins encoded by the immediate downstream genes, an interaction highly specific only for those within the operon. SigM seems to be also bound to the N-terminal domain of YhdL, an observation that has been made for other bi-cistronic gene products. However, the third gene product, the small hydrophobic protein YhdK, appears to interact with the trans-membrane domain of YhdL, suggesting some specific role for YhdK in the anti-sigma function of YhdL. YxlC is a SigY-binding protein, but the function(s) of the other gene products in the cognate operon needs further investigation.

Interestingly, all interactions observed between sigma factors and anti-sigma factors occurred through the N-terminal regions of the latter, implying that the N-terminal domain is configured intracellularly (Fig. 6). The involvement of the N-terminal region has been reported in the above-mentioned Gram-negative examples, RseA, FecR and MucA (Campbell *et al.*, 2003; Enz *et al.*, 2000; Rowen & Deretic, 2000). The involvement of a number of membrane proteins in the ECF sigma operon, as seen in the *sigY*, *sigM* and *ylaC* operons, suggests participation of these proteins in the signal transduction (Figs 1 and 6). It is noteworthy that the *rpoE* operon of *Thermoanaerobacter tengcongensis*, and LMO2228 and the downstream genes of *Listeria monocytogenes* share homology, at least in part, with the *B. subtilis sigY* operon. YbbM and YlaD contain the conserved HxxxCxxC motif and have previously been classified as members of the ZAS family of anti-sigma factors by Paget *et al.* (2001). Moreover, some extended overall homologies are found among YbbM, *Bacillus halodurans* BH0264 and *T. tengcongensis* TTE0873, as well as between YhdL and *T. tengcongensis* TTE1558. More understanding of the functionalities of these anti-sigma factors may shed light on their evolutionary roles.

ACKNOWLEDGEMENTS

We are grateful to W. Schumann (University of Bayreuth) for providing plasmid pX2. We also thank N. Ogasawara (Nara Institute of Science and Technology) for critical reading of the manuscript. This work was supported by a Grant-in-Aid for Science, Research on Priority Area (C) of the Ministry of Education, Science, Sports and Culture, Japan.

REFERENCES

Asai, K., Yamaguchi, H., Kang, C. M., Yoshida, K., Fujita, Y. & Sadaie, Y. (2003). DNA microarray analysis of *Bacillus subtilis* sigma factors of extracytoplasmic function family. *FEMS Microbiol Lett* **220**, 155–160.

Bentley, S. D., Chater, K. F., Cerdeno-Tarraga, A. M. & 40 other authors (2002). Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* **417**, 141–147.

Braun, V. (1997). Surface signaling: novel transcription initiation mechanism starting from the cell surface. *Arch Microbiol* **167**, 325–331.

Browning, D. F., Whitworth, D. E. & Hodgson, D. A. (2003). Light-induced carotenogenesis in *Myxococcus xanthus*: functional characterization of the ECF sigma factor CarQ and antisigma factor CarR. *Mol Microbiol* **48**, 237–251.

Brutsche, S. & Braun, V. (1997). SigX of *Bacillus subtilis* replaces the ECF sigma factor FecI of *Escherichia coli* and is inhibited by RsiX. *Mol Gen Genet* **256**, 416–425.

Campbell, E. A., Tupy, J. L., Gruber, T. M., Wang, S., Sharp, M. M., Gross, C. A. & Darst, S. A. (2003). Crystal structure of *Escherichia coli* sigmaE with the cytoplasmic domain of its anti-sigma RseA. *Mol Cell* **11**, 1067–1078.

Cao, M., Salzberg, L., Tsai, C. S., Mascher, T., Bonilla, C., Wang, T., Ye, R. W., Marquez-Magana, L. & Helmann, J. D. (2003). Regulation of the *Bacillus subtilis* extracytoplasmic function protein sigma(Y) and its target promoters. *J Bacteriol* **185**, 4883–4890.

Cole, S. T., Brosch, R., Parkhill, J. & 39 other authors (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**, 537–544.

De Las Penas, A., Connolly, L. & Gross, C. A. (1997). The sigmaE-mediated response to extracytoplasmic stress in *Escherichia coli* is transduced by RseA and RseB, two negative regulators of sigmaE. *Mol Microbiol* **24**, 373–385.

Enz, S., Mahren, S., Stroeher, U. H. & Braun, V. (2000). Surface signaling in ferric citrate transport gene induction: interaction of the FecA, FecR, and FecI regulatory proteins. *J Bacteriol* **182**, 637–646.

Fukuchi, K., Kasahara, Y., Asai, K., Kobayashi, K., Moriya, S. & Ogasawara, N. (2000). The essential two-component regulatory system encoded by *yycF* and *yycG* modulates expression of the *ftsAZ* operon in *Bacillus subtilis*. *Microbiology* **146**, 1573–1583.

Gietz, R. D. & Schiestl, R. H. (1995). Transforming yeast with DNA. *Methods Mol Cell Biol* **5**, 255–269.

Gorham, H. C., McGowan, S. J., Robson, P. R. & Hodgson, D. A. (1996). Light-induced carotenogenesis in *Myxococcus xanthus*: light-dependent membrane sequestration of ECF sigma factor CarQ by anti-sigma factor CarR. *Mol Microbiol* **19**, 171–186.

Helmann, J. D. (2002). The extracytoplasmic function (ECF) sigma factors. *Adv Microb Physiol* **46**, 47–110.

Hershberger, C. D., Ye, R. W., Parsek, M. R., Xie, Z. D. & Chakrabarty, A. M. (1995). The *algT* (*algU*) gene of *Pseudomonas aeruginosa*, a key regulator involved in alginate biosynthesis, encodes an alternative sigma factor (sigma E). *Proc Natl Acad Sci U S A* **92**, 7941–7945.

Horsburgh, M. J. & Moir, A. (1999). Sigma M, an ECF RNA polymerase sigma factor of *Bacillus subtilis* 168, is essential for growth and survival in high concentrations of salt. *Mol Microbiol* **32**, 41–50.

Huang, X., Decatur, A., Sorokin, A. & Helmann, J. D. (1997). The *Bacillus subtilis* sigma(X) protein is an extracytoplasmic function sigma factor contributing to survival at high temperature. *J Bacteriol* **179**, 2915–2921.

Huang, X., Fredrick, K. L. & Helmann, J. D. (1998). Promoter recognition by *Bacillus subtilis* sigmaW: autoregulation and partial overlap with the sigmaX regulon. *J Bacteriol* **180**, 3765–3770.

Ito, T., Tashiro, K., Muta, S., Ozawa, R., Chiba, T., Nishizawa, M., Yamamoto, K., Kuhara, S. & Sakaki, Y. (2000). Toward a protein-protein interaction map of the budding yeast: a comprehensive system to examine two-hybrid interactions in all possible combinations between the yeast proteins. *Proc Natl Acad Sci U S A* **97**, 1143–1147.

James, P., Halladay, J. & Craig, E. A. (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics* **144**, 1425–1436.

Kaneko, T., Nakamura, Y., Sato, S. & 21 other authors (2000). Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*. *DNA Res* **7**, 331–338.

Kang, J. G., Paget, M. S., Seok, Y. J., Hahn, M. Y., Bae, J. B., Hahn, J. S., Kleanthous, C., Buttner, M. J. & Roe, J. H. (1999). RsrA, an anti-sigma factor regulated by redox change. *EMBO J* **18**, 4292–4298.

Kunst, F., Ogasawara, N., Moszer, I. & 148 other authors (1997). The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. *Nature* **390**, 249–256.

Li, W., Stevenson, C. E., Burton, N., Jakimowicz, P., Paget, M. S., Buttner, M. J., Lawson, D. M. & Kleanthous, C. (2002). Identification and structure of the anti-sigma factor-binding domain of the disulphide-stress regulated sigma factor sigma(R) from *Streptomyces coelicolor*. *J Mol Biol* **323**, 225–236.

Liu, H., Haga, K., Yasumoto, K., Ohashi, Y., Yoshikawa, H. & Takahashi, H. (1997). Sequence and analysis of a 31 kb segment of the *Bacillus subtilis* chromosome in the area of the *rrnH* and *rrnG* operons. *Microbiology* **143**, 2763–2767.

Lonetto, M. A., Brown, K. L., Rudd, K. E. & Buttner, M. J. (1994). Analysis of the *Streptomyces coelicolor sigE* gene reveals the existence of a subfamily of eubacterial RNA polymerase sigma factors involved in the regulation of extracytoplasmic functions. *Proc Natl Acad Sci U S A* **91**, 7573–7577.

Mogk, A., Homuth, G., Scholz, C., Kim, L., Schmid, F. X. & Schumann, W. (1997). The GroE chaperonin machine is a major modulator of the CIRCE heat shock regulon of *Bacillus subtilis*. *EMBO J* **16**, 4579–4590.

Moriya, S., Tsujikawa, E., Hassan, A. K., Asai, K., Kodama, T. & Ogasawara, N. (1998). A *Bacillus subtilis* gene-encoding protein homologous to eukaryotic SMC motor protein is necessary for chromosome partition. *Mol Microbiol* **29**, 179–187.

Newman, J. D., Anthony, J. R. & Donohue, T. J. (2001). The importance of zinc-binding to the function of *Rhodobacter sphaeroides* ChrR as an anti-sigma factor. *J Mol Biol* **313**, 485–499.

Ochsner, U. A., Johnson, Z., Lamont, I. L., Cunliffe, H. E. & Vasil, M. L. (1996). Exotoxin A production in *Pseudomonas aeruginosa* requires the iron-regulated *pvdS* gene encoding an alternative sigma factor. *Mol Microbiol* **21**, 1019–1028.

Paget, M. S., Kang, J. G., Roe, J. H. & Buttner, M. J. (1998). sigmaR, an RNA polymerase sigma factor that modulates expression of the thioredoxin system in response to oxidative stress in *Streptomyces coelicolor* A3(2). *EMBO J* **17**, 5776–5782.

Paget, M. S., Bae, J. B., Hahn, M. Y., Li, W., Kleanthous, C., Roe, J. H. & Buttner, M. J. (2001). Mutational analysis of RsrA, a zinc-binding anti-sigma factor with a thiol-disulphide redox switch. *Mol Microbiol* **39**, 1036–1047.

Raivio, T. L. & Silhavy, T. J. (2001). Periplasmic stress and ECF sigma factors. *Annu Rev Microbiol* **55**, 591–624.

Rowen, D. W. & Deretic, V. (2000). Membrane-to-cytosol redistribution of ECF sigma factor AlgU and conversion to mucoidy in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *Mol Microbiol* **36**, 314–327.

Sorokin, A., Zumstein, E., Azevedo, V., Ehrlich, S. D. & Serror, P. (1993). The organization of the *Bacillus subtilis* 168 chromosome region between the *spoVA* and *serA* genetic loci, based on sequence data. *Mol Microbiol* **10**, 385–395.

Sorokin, A., Bolotin, A., Purnelle, B., Hilbert, H., Lauber, J., Dusterhoft, A. & Ehrlich, S. D. (1997). Sequence of the *Bacillus subtilis* genome region in the vicinity of the *lev* operon reveals two new extracytoplasmic function RNA polymerase sigma factors SigV and SigZ. *Microbiology* **143**, 2939–2943.

Tojo, S., Matsunaga, M., Matsumoto, T., Kang, C. M., Yamaguchi, H., Asai, K., Sadaie, Y., Yoshida, K. & Fujita, Y. (2003). Organization and expression of the *Bacillus subtilis sigY* operon. *J Biochem (Tokyo)*, **134**, 935–946.

Vagner, V., Dervyn, E. & Ehrlich, S. D. (1998). A vector for systematic gene inactivation in *Bacillus subtilis*. *Microbiology* **144**, 3097–3104.

Yoshida, K., Shindo, K., Sano, H., Seki, S., Fujimura, M., Yanai, N., Miwa, Y. & Fujita, Y. (1996). Sequencing of a 65 kb region of the *Bacillus subtilis* genome containing the *lic* and *cel* loci, and creation of a 177 kb contig covering the *gnt–sacXY* region. *Microbiology* **142**, 3113–3123.

Youngman, P., Perkins, J. & Sandman, K. (1985). Use of Tn*917*-mediated transcriptional gene fusions to *lacZ* and *cat-86* for the identification and study of regulated genes in the *Bacillus subtilis* chromosome. In *Molecular Biology of Microbial Differentiation*, pp. 47–54. Edited by J. A. Hoch & P. Setlow. Washington, DC: American Society for Microbiology.