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YlaC is an Extracytoplasmic Function (ECF) Sigma Factor Contributing to Hydrogen Peroxide Resistance in *Bacillus subtilis*

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In this study, we have attempted to characterize the functions of YlaC and YlaD encoded by *ylaC* and *ylaD* genes in *Bacillus subtilis*. The GUS reporter gene, driven by the *yla* operon promoter, was expressed primarily during the late exponential and early stationary phase, and its expression increased as the result of hydrogen peroxide treatment. Northern and Western blot analyses revealed that the level of *ylaC* transcripts and YlaC increased as the result of challenge with hydrogen peroxide. A YlaC-overexpressing strain evidenced hydrogen peroxide resistance and a three-fold higher peroxidase activity as compared with a deletion mutant. YlaC-overexpressing and YlaD-disrupted strains evidenced higher sporulation rates than were observed in the YlaC-disrupted and YlaD-overexpressing strains. Analyses of the results of native polyacrylamide gel electrophoresis of recombinant YlaC and YlaD indicated that interaction between YlaC and YlaD was regulated by the redox state of YlaD *in vitro*. Collectively, the results of this study appear to suggest that YlaC regulated by the YlaD redox state, contribute to oxidative stress resistance in *B. subtilis*.

Keywords: *Bacillus subtilis*, *ylaC*, *ylaD*, ECF, anti-sigma, hydrogen peroxide

Bacillus subtilis spores are formed in response to a variety of physical and chemical factors, including nutrient limitation, cell density, DNA synthesis and damage, Krebs cycle, manganese, and hydrogen peroxide. Spore formation is also modulated by a variety of alternative sigma factors (σ^H, σ^E, σ^F, σ^K, and σ^G) (Haldenwang, 1995; Stragier *et al*., 1996; Phillips *et al*., 2002), which appear to control the expression of general stress proteins (σ^B) (Haldenwang, 1995), as well as chemotaxis, autolysin expression, and motility (σ^D). The sigma factor recognizes its specific promoter with RNA polymerase, and this complex has been demonstrated to induce gene expression.

The extracytoplasmic function (ECF) subfamily of sigma factors comprises a structurally special group of proteins, which regulates gene expression in response to extracytoplasmic stimuli, including oxidative stress, high salt concentration, and heat (Lonetto *et al*., 1994). By bacterial genome sequencing, ECF sigma factors have been located within a wide range of gram-positive and gram-negative bacteria, including 7 open reading frames (ORFs) in *B. subtilis* (Kunst *et al*., 1997) and 10 ORFs in *Mycobacterium tuberculosis* (Cole *et al*., 1998). For example, ECF sigma factors have been shown to regulate the expression of genes associated with nickel and cobalt resistance responses in *Alcaligenes eutrophus* (Liesegang *et al*., 1993), carotenoid biosynthesis in *Myxococcus xanthus* (Gorham *et al*., 1996), oxidative stress responses and thioredoxin system expression in *Streptomyces coelicolor* (Paget *et al*., 1998; Kang *et al*., 1999; Paget *et al*., 2001), and cytochrome c_2 expression in *Rhodobacter sphaeroides* (Newman *et al*., 2001). The genes encoding ECF sigma factors are autoregulated, and are coupled to the expression of a cognate anti-sigma factor.

Anti-sigma factors are regulators which negatively regulate transcription via interaction with the cognate sigma factor. In the absence of an external signal, the majority of ECF sigma factors are held in an inactive complex with an anti-sigma factor. Extracytoplasmic stimuli activate transcriptional responses via the dissociation of sigma factors from anti-sigma factors (Lonetto *et al*., 1994). Well-known anti-sigma factors include CarR in *M. xanthus* (Gorham *et al*., 1996), RsrA in *S. coelicolor* (Paget *et al*., 1998; Kang *et al*., 1999; Paget *et al*., 2001) and ChrR in *R. sphaeroides*

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(Newman *et al*., 2001). The majority of anti-sigma factors are localized within the cytoplasmic membrane, in which they interact with their regulatory ligands (Braun, 1997).

The complete genome sequence of *B. subtilis* revealed the presence of seven ECF sigma factors, and the functions of some of these factors have already been delineated. The σ^X mutant exhibits an elevated sensitivity to heat and oxidative stress (Huang *et al*., 1997). σ^X is expressed at the late logarithmic phase and has been shown to play a role in the modulation of cell wall structure (Huang *et al*., 1998). σ^W is regulated by the anti-sigma factor, RsiW, and autoregulated. σ^W has a detoxification function and protects against antimicrobials. It is activated during the early stationary phase (Huang *et al*., 1998). σ^M is essential for growth under high salt concentration conditions (Horsburgh *et al*., 1999). The entire *ylaABCD* operon is induced by YlaC and harbors a YlaC-dependent distal promoter and an internal Spx-dependent promoter, which responds to oxidative stress (Matsumoto *et al*., 2005). YlaB and YlaD have been shown, via yeast two-hybrid analysis, to engage in direct interaction (Yoshimura *et al*., 2004). In this study, we identified the ECF sigma factor, YlaC, and the anti-sigma factor, YlaD, and demonstrated that the YlaC was required for resistance against oxidative stress, most notably hydrogen peroxide exposure. We also determined that the interaction between YlaC and YlaD was regulated by the redox state of YlaD *in vitro*.

Materials and Methods

Bacterial strains and culture conditions

The bacterial strains used in this study are shown in Table 1. *Escherichia coli* was grown at 37°C in Luria-Bertani (LB) medium and *B. subtilis* was grown in antibiotic medium 3 (Difco) or 2 × SG medium, consisting of 1.6% nutrient broth (w/v), 0.05% $MgSO_4$ (w/v), 0.2% KCl (w/v), 0.1% glucose (w/v), 1 μM $FeSO_4$, 1 mM $CaCl_2$, and 100 μM $MnCl_2$. The media used for the transformation of *B. subtilis* were LB agar plates containing 50 μg/ml ampicillin in *E. coli* or 10 μg/ml neomycin in *B. subtilis*, or Schaeffer agar medium (0.8% nutrient broth, 0.1% KCl, 0.012% $MgSO_4 \cdot 7H_2O$, and 1.5% agar) and MMG medium (1 × Spizizen's medium, 0.5% glucose, and 5 mM $MgSO_4$)

Cloning of ylaC and ylaD genes and transformation of B. subtilis

In order to effect the *ylaC* and *ylaD* genes over-

Table 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Characteristic	Sources or reference
B. subtilis		
PS832	Wild type Trp^+ revertant of strain 168	Peter Setlow
HB001	PS832 containing pRB374 [a]Nm^r	This study
HBC01	PS832 containing pRB374::*ylaC* Nm^r	This study
HBC02	PS832 containing pMLK117::internal *ylaC* Nm^r	This study
HBD01	PS832 containing pRB374::*ylaD* Nm^r	This study
HBD02	PS832 containing pMLK117::internal *ylaD* Nm^r	This study
HBP01	PS832 containing pMLK83 Nm^r	This study
HBP02	PS832 containing pMLK83::P_{yla} Nm^r	This study
E. coli		
DH5α	F⁻ Δ*lacU169(ϕ80lacZΔM15)endA1 rec1hsdR17 deoR supE44 thi-1 λ⁻gyrA96 relA1*	Hanahan,1983
BL21AI	F⁻*ompT hsd*$S_B(r_B\text{-}m_B\text{-})$*gal dcm araB*::T7RNAP-tetA	Novagen
Plasmids		
pRB374	*neo bla ble* Nm^r [b]Amp^r	P. J. Piggot
pMLK117	*LacI gus neo*, *bla* Nm^r Amp^r	P. J. Piggot
pMLK83	*AmyE gusA neo bla* Nm^r Amp^r	P. J. Piggot

[a]Nm^r, neomycin resistance
[b]Amp^r, ampicillin resistance

expression in *B. subtilis* harboring the pRB374 vector (Reinhold Brückner,1992), the following primers, predicated on the complete genome of *B. subtilis*, were synthesized and used: *ylaC*:pYC-OF, 5'-GGCCA TTTGCATGCCGGTGTGG-3' and pYC-OR 5'-TTTCT AGAAAGCAGGTCATATC-3' (the underlined sequences indicate the *Sph*I and *Xba*I sites, respectively) and *ylaD*:pYD-OF, 5'-TAAAGCTTAAGAAAAACATGAC-3' and pYD-OR, 5'-CTTGCCGGATCCGGACAAGCGC-3' (the underlined sequences indicate the *Hin*dIII and *Bam*HI sites, respectively). For the disruption of *ylaC* and *ylaD* genes in *B. subtilis* harboring the pMLK117 vector (Karow and Piggot, 1995), the following primers were used: *ylaC*:pYC-DF, 5'-TATTCAGAA GGATCCATCAT-3' and pYC-DR, 5'-TTAACTTCAG GAGCTCCTGC-3' and *ylaD*:pYD-DF, 5'-AAGAGAC CTGGATCCTCTGT-3' and pYD-DR, 5'-AATCCATAG GAGCTCTGTTT-3' (the underlined sequences indicate the *Bam*HI and *Sac*I sites, respectively). The resultant plasmids were serially transformed into *E. coli* and *B. subtilis* by natural competency according to the method proposed by Dubnau and Davidoff-Abelson (1971), with some modifications.

Construction and analysis of gusA fusion reporter strain

In our investigation of *yla* operon expression, we first constructed the P_{yla}-*gusA* fusion reporter in *B. subtilis* PS832, using the pMLK83 vector (Karow and Piggot, 1995). The putative promoter region was amplified by PCR using the following primers: pCDP-F, 5'-ATAC TGAAAGCTTTATATTG-3' and pCDP-R, 5'-ACGAAC AAGGATCCTTTACT-3' (the underlined sequences indicate the *Hin*dIII and *Bam*HI sites, respectively). β-Glucuronidase (βGlu) activity was measured as previously described (Harwood and Cutting, 1990; Karow and Piggot, 1995). Unit was calculated according to the following formula: $1000 \times A_{420}$/reaction time (min) × OD_{595} of culture.

Northern blot analysis

Total RNA was isolated from *B. subtilis* using the 'Modified Kirby Mix', which was designed by Van Dessel *et al*. (2004). Northern blot analysis was adopted from the general method developed by Kenney and Moran (1987). The probe was amplified by PCR and labeled with [α-^{32}P]-dATP.

Enzyme assay

In order to measure peroxidase activity, cells were prepared from exponentially growing strains that had been treated with 2 mM hydrogen peroxide for 10 minutes, and then collected by centrifugation. The cells were then resuspended in lysis buffer [20 mM Tris-HCl (pH 8.0), 1 mM phenylmethylsulfonyl fluoride, 5 mM ethylenediaminetetraacetic acid, and 0.5 mg/ml lysozyme] and incubated for 10 additional minutes at 37°C. After centrifugation, 100 μl of the supernatants were added to 1 ml of 20 mM sodium acetate buffer (pH 5.5) containing 0.5 mM *o*-dianisidine and 1 mM H_2O_2. The reaction was run for 10 minutes at 30°C. One unit (U) of enzyme activity was defined as the quantity of enzyme required to increase the A_{460} by 0.1 under the above assay conditions.

Determination of survival and sporulation rate

The ratio of spores to total viable cells, which indicates the sporulation rate, was determined on the basis of the resistance of spores to heat treatment (Milhaud and Balassa, 1973; Shin *et al.*, 2005). The survival rate was calculated as a comparison of viable cell numbers before and after hydrogen peroxide treatment.

Overproduction and purification of YlaC and YlaD

ylaC genes was cloned in pET-15b (Novagen) from the complete *ylaC* gene of *B. subtilis* and over-expressed in *E. coli*. The recombinant protein with an N-terminal hexa-histidine sequence was purified in accordance with the manufacturer's (Novagen) recommendations. The YlaD was overproduced in *E. coli* from the partial *ylaD* gene of *B. subtilis* cloned in pGEX 4T-1 (Amersham Bioscience), then purified in accordance with the manufacturer's recommendations. Following thrombin treatment for the removal of glutathione S-transferase (GST), YlaDN was concentrated by ultrafiltration (Amicon membrane, 3 kDa cut-off size) and purified further with Superdex^{TM} 75 (Amersham Bioscience) in TNG buffer [20 mM Tris-HCl, 200 mM NaCl, and 20% glycerol, (pH 8.0)]. The recombinant YlaC with an N-terminal hexa-histidine sequence was then concentrated in the same buffer. The purified protein was confirmed using 12% SDS-polyacrylamide gel and 15% tricine/polyacrylamide gel.

Analysis of YlaC-YlaD interaction

YlaC (4 μM) and YlaDN (4 and 8 μM) in TNG buffer were incubated in the presence of 1 mM dithiothreitol (DTT) or 2.5 mM H_2O_2 for 30 minutes at 37°C. The samples were electrophoretically separated in native 12% polyacrylamide gel at 4 mA for 12-13 hours. The running gel and buffer included 2 mM and 10 mM DTT, respectively (Kang *et al*., 1999; Li *et al*. 2003).

Results

Sequence analysis of yla operon

Analysis of the DNA sequence of *yla* operon via

sequencing of the *B. subtilis* genome revealed four ORFs (*ylaA*, *ylaB*, *ylaC,* and *ylaD*) (Kunst *et al*., 1997) (Fig. 1A). The functions of the YlaA and YlaB were unknown. Searches against the entire protein database also failed to identify any homologues of YlaA and YlaB. The YlaC and YlaD have been identified as an ECF sigma factor and its cognate anti-sigma factor, respectively. Unlike *ylaA* and *ylaB*, the *ylaC* and *ylaD* start codons overlapped the *ylaB* and *ylaC* stop codons, respectively. *ylaC* gene encoded for a polypeptide of 173 amino acids, with a molecular mass of 19 kDa, and YlaC evidenced 42%, 32%, and 28% identity with σ^H in *Pseudomonas aeruginosa*, σ^X in *B. subtilis*, and σ^R in *S. coelicolor*, respectively. These proteins have previously been identified as ECF sigma factors.

ylaD gene encoded for a polypeptide of 97 amino acids, with a molecular mass of 11 kDa. The deduced amino acid sequences evidenced a high degree of identity with OrfH from *M. tuberculosis*, OrfE from *M. xanthus,* and the RsrA of *S. coelicolor* (Fig. 2A). These proteins are known to function as anti-sigma factors. The YlaD harbored transmembrane helices at the C-terminal amino acids, composed of GLLIMKA ACWFGAAVMNLIIKLLI (Fig. 2B), and the conserved motif HX_3CXXC at the N-terminal region. Each of the anti-sigma factors harbored these conserved residues, including the HX_3CXXC motif, which serves as a zinc binding site, and is known to participate in the formation of disulfide bonds (Newman *et al*., 2001; Paget *et al*., 2001). The HX_3CXXC motif of RsrA in *S. coelicolor* appears to function in interactions with the ECF sigma factor, σ^R, by redox exchange (Kang *et al*., 1999; Li *et al*., 2002, 2003;

A

B

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     1  TATGAAAGTATACCTGTCTTCCATGTATATCTGTTTCATTTTATTGGCCATTTGGATGAC
    61  GGTGTGGTTTTATTTTTAATGAAGCATAGGGATTCCATTGAGGACTTGTATCGGCAGTAT
                          M  K  H  R  D  S  I  E  D  L  Y  R  Q  Y
   121  TATCAAGAAATTTTAAATTATTTATTCAGAAGGACTCATCATCTTGAAACAGCCAAGGAC
        Y  Q  E  I  L  N  Y  L  F  R  R  T  H  H  L  E  T  A  K  D
   181  TTAGCGCAGGACACGTTTGTAAAAGCTCTTAACGGTCTGGCTTCGTTTAGGGGGCATTCT
        L  A  Q  D  T  F  V  K  A  L  N  G  L  A  S  F  R  G  H  S
   241  TCCATCAGAACATGGCTCTACACCATTGCGCATCATACCTTTATCAATTGGTACCGAAGG
        S  I  R  T  W  L  Y  T  I  A  H  H  T  F  I  N  W  Y  R  R
   301  GATGTCAAATACCAATTTACTGAAATCAGCAAAAATGAAGGGTTAACGCAAACAACTTAT
        D  V  K  Y  Q  F  T  E  I  S  K  N  E  G  L  T  Q  T  T  Y
   361  GACCAGCCTGAACAGTATCTGTCACGGACGGTGAAAAGCGAAACACTGCGGCAGGAGCTC
        D  Q  P  E  Q  Y  L  S  R  T  V  K  S  E  T  L  R  Q  E  L
   421  CTGAAGTTAAAAGATCAGCATCAATCCGTTTTGATTTTAAGAGAATTCCAAGAGCTTTCT
        L  K  L  K  D  Q  H  Q  S  V  L  I  L  R  E  F  Q  E  L  S
   481  TATGAAGAAATCGCTGAGATATTAGGATGGAGTCTTTCTAAGGTGAATACCACATTGCAC
        Y  E  E  I  A  E  I  L  G  W  S  L  S  K  V  N  T  T  L  H
   541  CGGGCTAGATTAGAGCTAAAGAAAAACATGACGAAAAGTAGAGAGGAGGAGCGGATATGA
        R  A  R  L  E  L  K  K  N  M  T  K  S  R  E  E  E  R  I  *
                                                              M  T
   601  CCTGCTTTCTAGTAAGAGACCTGCTTCCTCTGTATCTTGAAGGTGATTGTAAAAGAGAAA
          C  F  L  V  R  D  L  L  P  L  Y  L  E  G  D  C  K  R  E  T
   661  CGGAACACGTTATAGAAGAGCATTTAAAAATGTGCAGCAGCTGCAGAGACATGTATGACA
          E  H  V  I  E  E  H  L  K  M  C  S  S  C  R  D  M  Y  D  T
   721  CGATGGCTGAGCCATTTGAATTGGAAAGCGAACAGGCCGTTGAGGAGGCTTATCTGCCGG
          M  A  E  P  F  E  L  E  S  E  Q  A  V  E  E  A  Y  L  P  E
   781  AAGAAGAACTGCGTTTTAAACAGAGGTACTATGGATTACTGATCATGAAAGCTGCCTGCT
          E  E  L  R  F  K  Q  R  Y  Y  G  L  L  I  M  K  A  A  C  W
   841  GGTTTGGAGCGGCGGTTGCCATGATGCTGATCATCAAACTGCTGATATAAAAAAAGCGCT
          F  G  A  A  V  A  M  M  L  I  I  K  L  L  I  *
   901  TGTCCGATTCCGGCAAGCGCTTTTTTACATTAAGCCTGGGGATGAATCATATCGTAAATA
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Fig. 1. Diagram of *yla* operon and the deduced amino acid sequences of *ylaC* and *ylaD* genes. (A) The coding regions of *ylaA*, *ylaB*, *ylaC* and *ylaD* genes are indicated by thick arrows. Promoter region is marked by a thin arrow. (B) The nucleotide sequences and deduced amino acid sequences of *ylaC* and *ylaD* genes. Start codons are represented in bold type and asterisks indicate stop codons. Ribosome-binding site and HX_3CXXC motif are underlined and boxed, respectively. An inverted repeat sequence functioning as a transcriptional terminator for the *yla* operon is indicated by a double underline.

A

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BsuYlaD           MTCFLVRDLLPLYLEGDCKRETEHVIEEHLKMCSSCRDMYDT---MA  44
MtuOrfH           MGCAEVIAEVWTLLDGECTPETRERLRRHLEACPGCLRHYG----LE  43
MxaOrfE           MNCQDLERLLYPYLDGEFQPEERVDLETHLSGCADCRRRAEEEKQMQ  47
ScoRsrA   MSCGEPHETDCSEILDHLYEFLDKEMPDSDCVKFEHHFEECSPCLEKYG----LE  51
                     *  :   :   *: :   .    :. *:. *. *         :
BsuYlaD   EPFELESEQAVEEAYLPEEELRFKQRYYGLLIMKAACWFGAAVAMMLIIKLLI    97
MtuOrfH   ERIKALIGTKCRGDRAPEGLRERLRLEIRRTTIIRGGP                  81
MxaOrfE   QALRRAARHSVSGMRAPASLRAGIQVGLKQEQRRVQFGVWLRAGAMALVVVTVGG 102
ScoRsrA   QAVKKLVKRCCGQDDVPGDLRAKVMGRLDLIRS-GQSVPEHDVAAAPSSSAPQES 105
          : ..           *
```

B

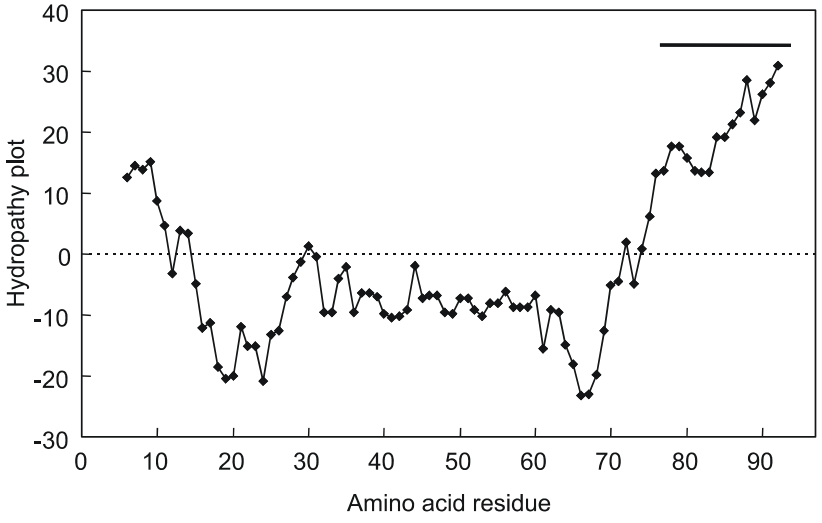

Fig. 2. Alignment of the amino acid sequences of YlaD and YlaD-related proteins and hydropathy plot for the structural prediction of YlaD. (A) The deduced amino acid sequence of YlaD (BsuYlaD) was aligned with those of *M. tuberculosis* OrfH (MtuOrfH), *M. xanthus* OrfE (MxaOrfE) and *S. coelicolor* RsrA (ScoRsrA). The positions of the conserved amino acids are marked by asterisks and colons for identical and similar matches, respectively. (B) The hydropathy of each of the amino acid residues was calculated via the methods developed by Kyte and Doolittle, using a window size of 11 residues. The solid bar in the plot indicates the segment predicted to form transmembrane helices.

Bae *et al*., 2004).

Analysis of P_{yla}-gusA fusion reporter strain under various conditions

Many ECF sigma factors function as cell adaptation systems in diverse environments. The σ^M of *B. subtilis* was found to be essential for growth under high salt concentration conditions (Horsburgh and Moir, 1999). The σ^X mutant conferred a slight elevation in sensitivity to heat and oxidative stress (Huang *et al*., 1997), and σ^W was shown to perform a role in detoxification and protection against antimicrobials (Huang *et al*., 1998). σ^R and its cognate anti-sigma factor, RsrA, in *S. coelicolor* have been demonstrated to function in oxidative stress resistance (Paget *et al*., 1998).

In order to evaluate the expression of P_{yla} containing the upstream of the initiation site (TTG) of *ylaA* gene during the cell growth phase and under diverse conditions, the strain harboring the pMLK83 vector (HBP01) and the strain harboring the P_{yla}-*gusA* fusion reporter (HBP02) were prepared, as was described in Materials and Methods. β-glucuronidase (βGul) activity was not detectable in the HBP02 strain during cell growth, except in the late exponential and early stationary phases. However, βGul activity levels were slightly increased during the late exponential and early stationary phases (data not shown). The βGul activity of the HBP02 strain was measured under a variety of conditions. Ethanol and heat treatment were found to have no effects on the βGul activity of the HBP02 strain. However, although the βGul activity of the HBP02 strain in the absence of hydrogen peroxide treatment was slightly elevated at 20 minutes, the addition of hydrogen peroxide resulted in a dramatic (approximately four-fold) increase in the βGul activity of the HBP02 strain (Fig. 3). The *ylaABCD* operon contained the YlaC- dependent distal promoter and the internal Spx-dependent promoter, which function in responses to oxidative stress. When the transcription of the entire operon was induced by the YlaC-dependent distal promoter, YlaC was detected, but no

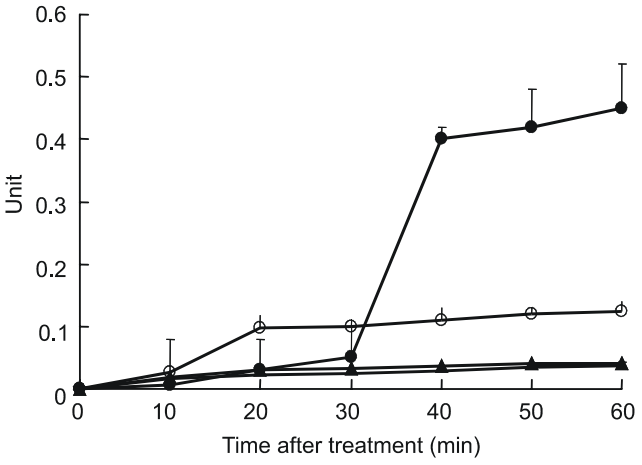

Fig. 3. βGul activities in HBP01 and HBP02 strains. Samples were prepared from exponentially growing strains (open symbols). For the 1 mM hydrogen peroxide treatment (closed symbols), samples were prepared from the same phase. βGul activity was measured as described in Materials and Methods. βGul activity of HBP01 strain without and with the treatment of hydrogen peroxide is indicated by △ and ▲. In the HBP02 strain, this is indicated by ○ and ●, respectively. Units were calculated according to the formula: 1000 × A_{420}/reaction time (min) × OD_{595} of culture.

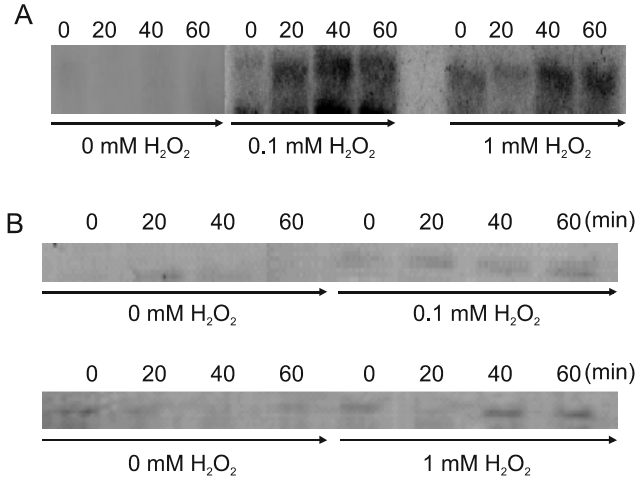

Fig. 4. The Northern blot (A) and Western blot (B) of *ylaC*. Exponentially growing *B. subtilis* PS832 was treated with 0.1 and 1 mM hydrogen peroxide. Total RNA (25 μg) and crude cells (20 μg) were prepared from *B. subtilis* PS832 at the indicated times and detected via Northern hybridization and Western blotting, using probes specific to *ylaC* and anti-YlaC antibody, respectively.

Table 2. Survival rates of *B. subtilis* and mutants against hydrogen peroxide

Hydrogen peroxide	HB001	HBC01	HBC02	HBD01	HBD02
1 mM	0.35	34.70	0.13	0.05	8.00
2 mM	0.05	3.00	0.10	0.03	0.40

YlaC was detected during diamide-induced transcription from the internal promoter (Matsumoto *et al.*, 2005). In order to verify the results regarding the βGul activity of the HBP02 strain in response to hydrogen peroxide treatment, we conducted Northern and Western blot analyses. In the exponentially growing *B. subtilis*, *ylaC* transcript levels increased after the administration of hydrogen peroxide (Fig. 4A). Also, *ylaC* transcripts were detected in the distal and internal promoters, by S1 nuclease analysis. However, they were detected in the distal promoter only during earlier stages, and were not detected later. However, the *ylaC* transcript levels increased continuously from the internal promoter (data not shown). Although YlaC was not detected during diamide-induced transcription from the internal promoter, YlaC levels clearly increased after challenge with hydrogen peroxide (Fig. 4B).

YlaC increased hydrogen peroxide resistance and sporulation rate

Reactive oxygen species are known to inflict damage upon DNA, RNA, and proteins. Therefore, cells carry superoxide dismutase, peroxidase, and catalase, in order to protect the cell against the deleterious effects of such reactive oxygen species (Nunoshiba *et al*., 1999).

In order to determine the function of YlaC, the strain harboring the pRB374 vector (HB001), the YlaC-overproducing strain (HBC01), and the YlaC-disrupted strain (HBC02) were prepared, as was described in Materials and Methods. The YlaD-overproducing strain (HBD01) and the YlaD-disrupted strain (HBD02) were also prepared via the same method.

The HB001, HBC02, and HBD01 strains manifested similar growth patterns on media containing 0～1 mM hydrogen peroxide, but did not grow on 2 mM hydrogen peroxide. However, the HBC01 and HBD02 strains evidenced clear growth on the media containing 2 mM hydrogen peroxide. In order to verify the hydrogen peroxide resistance functions of YlaC and YlaD, we evaluated the survival rates of mutants in the presence of hydrogen peroxide: 1 or 2 mM hydrogen peroxide was added to each of the strains during exponential phase. The survival rates were then calculated, as was described in Materials and Methods. The HBC01 and HBD02 strains evidenced survival rates superior to those of the HBC02 and HBD01 strains in the presence of hydrogen peroxide. About 35% and 3% of the HBC01 cells survived after treatment with 1 and 2 mM hydrogen peroxide, and 8% and 0.4% of the HBD02 cells survived under identical conditions. However, the HBC02 and HBD01 strains rarely survived under the same conditions (Table 2). Therefore, we assessed the peroxidase and catalase activity levels of the cells, in an attempt to

Table 3. Peroxidase activity of *B. subtilis* and mutants (HBC01 and HBC02)

Strains	Peroxidase activity (A_{460})	Specific activity (U/mg)
HB001	0.239	1
HBC01	0.518	2.9
HBC02	0.223	0.93

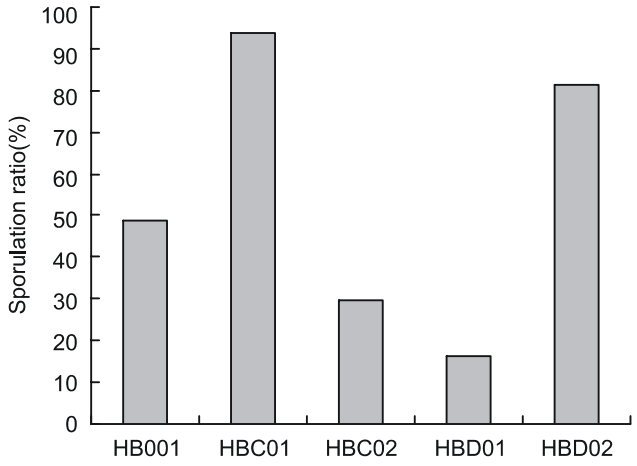

Fig. 5. Sporulation of HB001 and mutants. Viable and spore cells of HB001 and mutants were counted via spore test, as was described in Materials and Methods, after the cells were incubated for 24 hours. The sporulation ratio is the percentage of spore cells to total viable cells.

determine how the HBC01 cells were able to survive after treatment with 2 mM hydrogen peroxide, as compared with the HB001 strain. The HB001, HBC01, and HBC02 strains evidenced similar catalase activity after treatment with 2 mM hydrogen peroxide. However, the HBC01 strain exhibited approximately three-fold higher peroxidase activity than was evidenced by the HB001 and HBC02 strains (Table 3). This indicates that the HBC01 cells were able to survive after treatment with 2 mM hydrogen peroxide by virtue of an increase in their peroxidase activity levels, as compared with the HB001 strain.

In order to investigate the relationship existing between the YlaC and spore formation, we evaluated the sporulation rate of *B. subtilis* that had survived heat treatment (Milhaud and Balassa, 1973). Approximately 80～100% of the HBC01 and HBD02 strains were found to have sporulated at the late stationary phase, manifesting a sporulation rate significantly higher than that of the HB001 strain. However, 20～30% of the HBC02 and HBD01 strains sporulated, which is a lower sporulation rate than was observed in the HB001 strain (Fig. 5). Although the precise mechanisms exploited by the YlaC during sporulation remain to be elucidated, these results indicated that the overproduction of the YlaC may exert some influence with regard to sporulation efficiency.

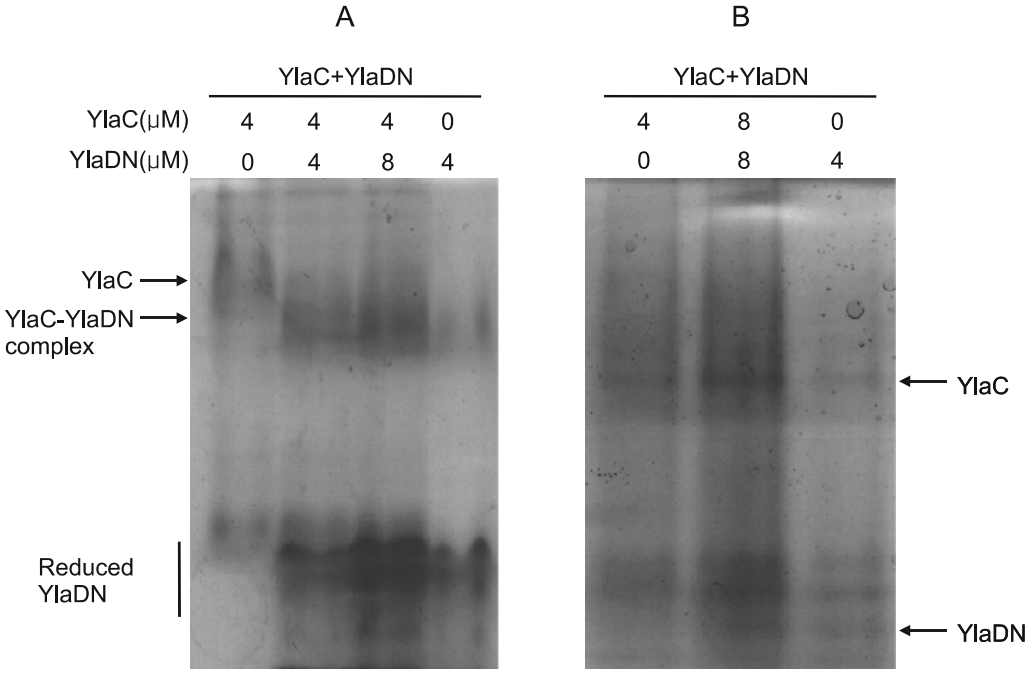

Fig. 6. Formation of the YlaC-YlaDN complex monitored by 12% native PAGE. (A) YlaC and YlaDN were incubated in TNG buffer containing 1 mM DTT, as was described in Materials and Methods. The running gel and buffer included 2 mM and 10 mM DTT, respectively. (B) Samples were incubated in TNG buffer containing 2.5 mM hydrogen peroxide. DTT was omitted from the running gel and buffer.

Interaction of YlaC and YlaD

To characterize the interaction between YlaC and YlaD, YlaC was overproduced in *E. coli* and purified from the soluble fractions of the cell extracts. However, high YlaC concentrations and the removal of imidazole by dialysis resulted in the precipitation of His-tagged YlaC. His-tagged YlaC was soluble at >50 μM concentration in TNG buffer [20 mM Tris-HCl, 200 mM NaCl, 20% glycerol, (pH 8.0)]. The complete YlaD harboring the transmembrane helices could not be overproduced in *E. coli*. Therefore, we prepared a recombinant protein from partial YlaD, designated YlaDN, which harbors the active site HX_3CXXC and lacks the C-terminal region containing the transmembrane helices.

According to the results of our analyses of βGul activity and hydrogen peroxide treatment survival rates in each of the mutant strains, it was hypothesized that hydrogen peroxide might exert some influence on the interaction between YlaC and YlaD. In order to test this possibility, recombinant YlaC and YlaDN were incubated under different redox state conditions. Purified YlaDN was incubated for 30 minutes at 37°C in the presence of 1 mM DTT. Then, purified YlaC (4 μM) and reduced YlaDN (4 μM and 8 μM) were incubated together in the presence of 1 mM DTT, and run on 12% native polyacrylamide gel, as was described in Materials and Methods. As is shown in Fig. 6A, a YlaC-YlaDN complex was detected as a new band (lane 2-3) in this case. However, when the purified YlaC and YlaDN were incubated in the presence of 2.5 mM hydrogen peroxide, no complex was detected (Fig. 6B). Also, no complex was detected in the absence of DTT (data not shown). This data suggests that the interaction between YlaC and YlaDN is regulated via the formation of an intramolecular disulfide bond in YlaD *in vitro*.

Discussion

The extracytoplasmic function (ECF) sigma factor has a distinctive structure and function, and appears to regulate gene expression in response to extracytoplasmic stimuli. Many ECF sigma factors are regulated by their cognate anti-sigma factors, and participate in autoregulation, as well as gene expression (Brown and Hughes, 1995; Missiakas and Raina, 1998; Helmann, 1999). Among the seven ECF sigma factors identified in *B. subtilis*, σ^X, σ^W, and σ^M have been fairly thoroughly studied. The physiological roles of other ECF sigma factors have been very difficult to determine, as deletion mutants do not manifest clear phenotypes. In this study, although YlaC and YlaD mutants also did not manifest clear phenotypes, we showed that YlaC, an ECF sigma factor in *B. subtilis*, contributed to oxidative stress resistance along with its cognate anti-sigma factor, YlaD, and that the interaction between YlaC and YlaD was regulated by the redox state of YlaD.

The transcriptions of the *ylaABCD* operon from the distal and internal promoters were initiated by YlaC and diamide-induced oxidative stress, respectively (Matsumoto *et al.*, 2005). We assessed βGul activity in the HBP02 strain during the late exponential and early stationary phases, as well as in response to hydrogen peroxide treatment (Fig. 3). Although no YlaC was detected subsequently to the addition of diamide, the *ylaC* transcripts from the distal and internal promoter and YlaC were detected after hydrogen peroxide treatment.

When *B. subtilis* is grown to late stationary phase or exposed to hydrogen peroxide, a specific set of genes is expressed (Dowds *et al*., 1987; Bol and Yasbin, 1990). For example, *B. subtilis* exhibits an adaptation to hydrogen peroxide which involves the induction of *katA* (catalase), *ahpCF* (alkyl hydroperoxide reductase), and *mrgA* (DNA-binding protein) genes (Dowds, 1994; Chen and Helmann, 1995; Chen *et al*., 1995; Antelmann *et al*., 1996; Bsat *et al*., 1996). These genes appear to be induced via the entry of grown cells into stationary phase. We conducted Northern blot analyses with probes of *katA*, *aphC*, *mrgA*, and *trxA* (thioredoxin) genes in the HBC01 and HBC02 strains (data not shown). The results of the Northern blot analyses revealed slightly different patterns in the levels of these gene transcripts. However, the HBC01 strain exhibited a constant level of *trxA* transcripts, and this level was higher than that observed in the HBC02 strain. The HBC01 and HBD02 strains were able to grow on media containing 2 mM hydrogen peroxide, and evidenced survival rates higher than those of the HBC02 and HBD01 strains. In the upstream region of the *trxA* gene encoding for thioredoxin, two promoters, S_A and S_B, occurred, and only the S_A promoter was bound to sigma factor SigA after treatment with hydrogen peroxide and puromycin (Scharf *et al.*, 1998). Transcription from the internal promoter of the *ylaABCD* operon was induced by oxidative stress, and may have been dependent on SigA (Matsumoto *et al.*, 2005). This data suggests that YlaC influences gene expression in response to hydrogen peroxide, and is related to SigA. From our results regarding the survival rate of mutants against hydrogen peroxide, we were able to infer that YlaC and YlaD were related to hydrogen peroxide response.

Reactive oxygen species are known to induce cell damage to DNA, RNA, and proteins. Therefore, cells carry superoxide dismutase, peroxidase, and catalase, all of which function to protect the cell against the

deleterious effects associated with reactive oxygen species (Nunoshiba *et al*., 1999). In this study, we demonstrated that the HBC01 strain was able to survive after treatment with 2 mM hydrogen peroxide, by virtue of an increase in cellular peroxidase activity, as compared with that of the HB001 strain (Table 3). Thioredoxin may also contribute to YlaC-mediated resistance capabilities, as the *trxA* levels in the HBC01 strain were higher than those of the HBC02 strain.

In *B. subtilis,* sporulation is triggered by a variety of stimuli, and requires sporulation-specific sigma factors, including σ^E, σ^F, σ^G, and σ^K (Haldenwang, 1995). In order to determine whether YlaC and YlaD are involved in sporulation, we evaluated the sporulation efficiency of the *ylaC* and *ylaD* mutants. The HBC01 and HBD02 strains evidenced higher sporulation efficiency than was seen in the HBC02 and HBD01 strains. However, spore formation in the HBC02 and HBD01 strains also proceeded normally (Fig. 5), thereby indicating that YlaC and YlaD affected sporulation in a pattern similar to that associated with hydrogen peroxide resistance. Although YlaC and YlaD are dispensable with regard to sporulation, it appears likely that the expression of these factors also influences development in *B. subtilis*, either directly or indirectly.

In *B. subtilis,* the interaction of ECF sigma factor with anti-sigma factor in response to extracytoplasmic stimuli has yet to be clearly elucidated. The interaction between σ^R and RsrA, harboring the conserved motif HX_3CXXC, is regulated in *S. coelicolor* by changes in the redox state. RsrA functions as a redox-sensitive and σ^R-specific anti-sigma factor (Kang *et al*., 1999), and is a metalloprotein which contains zinc, which is released upon the oxidation of RsrA, concomitant with disulfide bond formation (Bae *et al*., 2004). YlaD is a membrane protein that harbors transmembrane helices at its C-terminal region, and the conserved HX_3CXXC motif within its N-terminal region. YlaD has a structure that is so reminiscent of the structure of RsrA that it has been suggested to function similarly to RsrA. Although no interaction was detected between YlaC and YlaD in the yeast two-hybrid system experiment (Yoshimura *et al.*, 2004), the formation of a YlaC-YlaDN complex was observed in the presence of 1 mM DTT, but not detected with 2.5 mM hydrogen peroxide or without DTT *in vitro* in 12% native polyacrylamide gel (Fig. 6). This shows that the formation of the YlaC-YlaD complex may be regulated by the redox state of YlaD. Under reduced conditions, YlaD exists in a reduced form, and in a complex with YlaC. Exposure to oxidative stress induced the oxidation of YlaD, which resulted in its dissociation from the YlaC-YlaD complex, thereby allowing free YlaC to recognize its specific promoter, and subsequently induce the transcription of oxidative stress-associated genes.

In this study, we have shown that the YlaC may influence gene expression in response to oxidative stresses, namely hydrogen peroxide exposure, and may also be related to spore formation. We also determined that the redox state of YlaD may play a significant role in the aforementioned functions. Detailed studies into the YlaC-YlaD complex and gene expression in response to oxidative stress are expected to uncover more interesting features of the ECF sigma factors.

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