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One of Two OsmC Homologs in *Bacillus subtilis* Is Part of the σ^B-Dependent General Stress Regulon

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In this report we present the identification and analysis of two *Bacillus subtilis* genes, *yklA* and *ykzA*, which are homologous to the partially RpoS-controlled *osmC* gene from *Escherichia coli*. The *yklA* gene is expressed at higher levels in minimal medium than in rich medium and is driven by a putative vegetative promoter. Expression of *ykzA* is not medium dependent but increases dramatically when cells are exposed to stress and starvation. This stress-induced increase in *ykzA* expression is absolutely dependent on the alternative sigma factor σ^B, which controls a large stationary-phase and stress regulon. *ykzA* is therefore another example of a gene common to the RpoS and σ^B stress regulons of *E. coli* and *B. subtilis*, respectively. The composite complex expression pattern of the two *B. subtilis* genes is very similar to the expression profile of *osmC* in *E. coli*.

σ^B was discovered in 1980 by Haldenwang and Losick and was the first alternative sigma factor of *Bacillus subtilis* identified (19). However, the function of the regulon controlled by σ^B remained a matter for speculation until 1993, when it was shown to be involved in the cellular response to stress. It has subsequently been demonstrated that expression of a large number of genes is induced in a σ^B-dependent manner by such different stimuli as heat shock, ethanol, acid, and salt stress, and starvation for oxygen, phosphate, and glucose (6, 7, 10, 11, 21, 22, 40, 43). Since induced expression of more than 50 genes is absolutely dependent on σ^B, it was tempting to assume that the gene products perform essential adaptive functions in *B. subtilis*. However, earlier studies had shown that a null *sigB* mutant strain was apparently not impaired in sporulation or response to stress compared to the wild type (9, 10, 21, 23, 39). This is unusual, since starving or stressed *B. subtilis* cells devote a considerable amount of their residual protein-synthesizing capacity to the synthesis of the members of the σ^B regulon (8). This apparently anomalous result has prompted a concerted effort to identify the genes of the σ^B regulon, to elucidate the function(s) of their gene products, and to establish their contributions to the cellular response to stress and starvation conditions.

Among others, genes encoding a catalase (*katE*), a nonspecific DNA-binding and protecting protein (*dps*), and an osmotically activated proline uptake system (*opuE*) have been shown to be subject to the control of σ^B in *B. subtilis* (5, 13, 44). Interestingly, in *Escherichia coli* genes like *katE*, *dps*, and *proP*, whose gene products perform similar functions, are subject to a RpoS-dependent regulation (28, 30, 34). RpoS directs the expression of a large group of genes whose expression is induced following starvation and stress (20, 33). Null mutations in *rpoS* result in a loss of stationary-phase-induced resistance against heat, acid, or oxidative stress and impairment in the ability to survive prolonged periods of starvation (20, 26, 27, 29). Therefore, it was tempting to speculate that σ^B-dependent stress proteins may provide the stressed or starved *B. subtilis* cell with a general multiple-stress resistance similar to that provided by the RpoS-dependent proteins of *E. coli*. This view is supported by the demonstration that *sigB* mutants are impaired in stationary-phase-induced resistance to oxidative stress, like *E. coli rpoS* mutants (4, 12). Recently, the Dps protein of *B. subtilis* has been shown to play a crucial role in the development of this nonspecific starvation-mediated resistance to oxidative stress (5). However, it is necessary to identify and investigate the physiological roles of additional general stress proteins to further support this hypothesis. Identification of general stress proteins by N-terminal sequencing (3, 8, 40, 41) has greatly benefited from the recent release of the complete sequence of the *B. subtilis* genome (25). In this paper we present an investigation of two genes, *yklA* and *ykzA*, identified in *B. subtilis* during the genome-sequencing project, which show homology to *osmC* from *E. coli*. The expression profile of *osmC* in *E. coli* is complex (16, 18). It has two independent promoters, which provide medium-, growth phase-, and stress-dependent expression. One of the promoters is partially controlled by RpoS. Although there are two genes in *B. subtilis* which have homology to *osmC*, the composite expression profile of the two genes (medium-, growth phase-, and stress-dependent expression) is very similar to that of the *E. coli* gene. In addition, expression of the homolog YkzA is directed by σ^B, the *B. subtilis* stress sigma factor.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *B. subtilis* wild-type strain 168 and its isogenic *sigB* mutant strain ML6 (*trpC2 sigB*::Δ*Hin*dIII-*Eco*RV::*cat* [21]) were cultivated at 37°C under vigorous agitation in Luria broth (LB) (31) or a synthetic medium described previously (37). The synthetic medium had to be used in order to achieve glucose limitation and in the experiments involving salt stress, in order to avoid the protective effects of osmoprotective substances present in LB. Stresses and starvation were imposed as described previously (40, 42).

Cloning, sequencing, and construction of the corresponding mutants. *B. subtilis* chromosomal DNA for sequencing was isolated from strain 168 by chromosomal walking with the integrating plasmid pDIA5304 as previously described (24). *E. coli* TP611 (*recBC hsdRM cya610 pcn*) was used for cloning large chromosomal DNA fragments (15). *E. coli* TG1 [K-12 Δ(*lac pro*) *supE thi hsdR* F′ *traD36 proAB lacI lacZ*ΔM15] was used for subcloning and for preparation of sequencing templates. *E. coli* strains were routinely grown in LB. The isolated chromosomal DNA from *B. subtilis* was sequenced by a shotgun strategy and by

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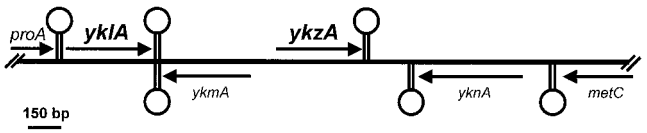

FIG. 1. Schematic diagram of chromosomal organization in the region containing the *yklA* and *ykzA* genes. Open reading frames are indicated by arrows, and their positions indicate whether they are encoded on the top (above) or the bottom (below) DNA strand. Terminators are indicated by "lollipops" on the top (above) or the bottom (below) strand. The terminator between *yklA* and *ykmA* is bidirectional.

a directed approach with oligonucleotides. Plasmid DNA (30 μg in 120 μl of Tris-EDTA buffer) was randomly sheared either by sonication (Braun Labsonic sonicator) (seven pulses, 0.22 cycles, 0.25 W) or by using DNase I in the presence of manganese (35). Sequencing reactions were carried out with fluorescent dye primer sequencing kits (GENPAK, Polegate, East Sussex, England) according to the manufacturer's instructions. Reactions were resolved on an ABI automated sequencer model 373A. Gaps in the sequence were filled with custom-synthesized oligonucleotides (PCR-Mate DNA synthesizer; Applied Biosystems) and the Applied Biosystems dye terminator sequencing kit.

Two oligonucleotide primer pairs were made (Genosys Biotechnologies Ltd., Cambridge, United Kingdom) and used to amplify fragments of DNA located within the *yklA* (139-bp) and *ykzA* (152-bp) open reading frames (the positions of the oligonucleotides are given in parentheses): YklA-14F, 5′-AAGCGACAAA TCCAGAGC-3′, and YklA-14R, 5′-GCTTCATCCTTTAACAGGC-3′ (33232 to 33371); and YkzA-16F, 5′-CCAAAAAAGAAGGACAAACCGG-3′, and YkzA-16R, 5′-ATCCTTCATGAGGCGACC-3′ (34245 to 34397). The DNA amplified with each pair of primers was isolated, the ends were polished, and the fragments were subcloned in pUC19 to give plasmids pLA004 and pNA005, respectively. The integrity of the cloned fragments was checked by sequencing. Insert DNA was excised from pLA004 and pNA005 with *Bam*HI and *Hin*dIII and directionally cloned into the plasmid pMutin4 (an integrating plasmid conferring resistance to erythromycin and containing a promoterless *lacZ* gene [a gift from V. Vagner and S. D. Ehrlich]) to give plasmids pMutin004 and pMutin005. Plasmids pMutin004 and pMutin005 were integrated into the chromosome of *B. subtilis* 168 through homology with the *yklA* and *ykzA* fragments by a Campbell-type event, which generates strains BFS1816 (carrying a *yklA-lacZ* transcriptional fusion) and BFS1818 (carrying a *ykzA-lacZ* transcriptional fusion). The location and structural integrity of the DNA at the integration site was verified by PCR with combinations of oligonucleotides external and internal to the integrated plasmid DNA.

B. subtilis transformation was carried out according to the method of Anagnostopoulos and Spizizen (2). *E. coli* transformation was carried out according to the method of Sambrook et al. (35).

RNA isolation and analysis of transcription. RNA was isolated with RNeasy cartridges from Qiagen as described previously (42). Northern blot analysis, hybridization, and quantification of specific hybrids were performed as described by Scharf et al. (36). For the preparation of the digoxigenin-labeled RNA probes, a DNA fragment encompassing the two *osmC*-homologous genes *yklA* and *ykzA* was amplified from chromosomal DNA of the wild-type strain 168 with the synthetic oligonucleotides UV114 (5′-GAGAGGATCCGTGAATAGCGGGGTAAT G-3′) and UV115 (5′-GAGAATCGATGTCCGACACCAAAAAACATC-3′). The PCR fragment was digested with *Bam*HI/*Cla*I and cloned into pBluescript KS(−) digested with the same enzymes. The resulting plasmid, pUV321, was digested with *Hin*cII and religated, yielding plasmid pUV521. pUV521 can be used for the production of a digoxigenin-labeled, *yklA*-specific RNA probe with T3 RNA polymerase after linearization with *Bam*HI. Digestion of pUV321 with *Bam*HI/*Bgl*II and religation of the remaining plasmid resulted in pUV520, which is devoid of *yklA* and can be used for the preparation of digoxigenin-labeled, *ykzA*-specific RNA probe with T3 RNA polymerase after linearization with *Sph*I.

Primer extension experiments were performed with synthetic oligonucleotides UV117 (5′-GACATCAAGCTCAAGAAC-3′) and UV116 (5′-CATTTGGCAT GAAATATC-3′), complementary to the regions encoding the N termini of *yklA* and *ykzA*, as described previously (45). A DNA-sequencing ladder prepared with the same primers and pUV320 plasmid DNA as a template was used to assign the 5′ end of the mRNAs.

Two-dimensional protein gel electrophoresis. Protein extracts were prepared by passage through a French press after cells had been harvested on ice. Equal amounts of protein (300 μg) were loaded. The proteins were separated with immobiline dry strips, pH 4 to 8, in the first dimension on the multiphor apparatus supplied by Pharmacia, equilibrated, loaded onto 12.5% polyacrylamide gels, and separated according to their molecular masses with the InvestigatorTM electrophoresis system of ESA Inc. (Chelmsford, Mass.).

Enzyme assays. Expression of *lacZ* was measured as described by Ferrari et al. (14) with the following modifications: activity units are expressed in nanomoles per minute per microgram of protein and the cells were lysed for 25 min in Z buffer containing 10 μg of lysozyme per ml, 1 mM dithiothreitol, 0.00025% Triton X-100, and 1 μg of DNase I per ml.

Computer sequence analysis. Sequence alignment and editing were performed with the XBAP program of the STADEN package. Conceptual translation of the sequence and other sequence analyses were performed with the NIP program of the STADEN package. The GenBank database was accessed with ACNUC (17), and homology searches of the database were performed with the TBLASTN program (1). Multiple sequence alignments were performed with CLUSTAL W (38).

RESULTS

Cloning and sequencing of the chromosomal region containing *yklA* and *ykzA*. Two homologs of *osmC* from *E. coli* were identified during the *B. subtilis* genome-sequencing project (25). They are called *yklA* and *ykzA* and are arranged as shown in Fig. 1 at approximately 105° on the chromosome. Their sequence can be found in GenBank entry AJ002571. Both genes are transcribed in the direction of chromosomal replication and are separated by *ykmA*, which is transcribed in the opposite direction. There is a putative σ^A promoter positioned upstream of *yklA* and a putative σ^B promoter located upstream of the *ykzA* gene. The paralogs YklA and YkzA differ in size by five amino acids (141 and 136, respectively) and are 49% identical (67% similar) to each other. Both YklA and YkzA are approximately 28% identical (42% similar) to OsmC from *E. coli*.

Transcriptional regulation of *yklA* and *ykzA*. Expression of the *osmC* gene from *E. coli* is subject to osmotic and growth phase-dependent regulation, which are partially dependent on the presence of the stress and stationary-phase sigma factor RpoS. Therefore, we wanted to determine if one or both of the *B. subtilis* homologous genes *yklA* and *ykzA* are similarly regulated by osmotic stress or starvation. Total RNA was prepared from exponentially growing cells and from bacteria which had been treated with sodium chloride or which entered the stationary phase as a result of the exhaustion of glucose. An analysis of the *ykzA* mRNA level revealed that the expression of this gene was strongly and rapidly induced by salt stress (Fig. 2). The induction was transient, reaching a maximum between 9 and 12 min after the imposition of stress. *ykzA* was

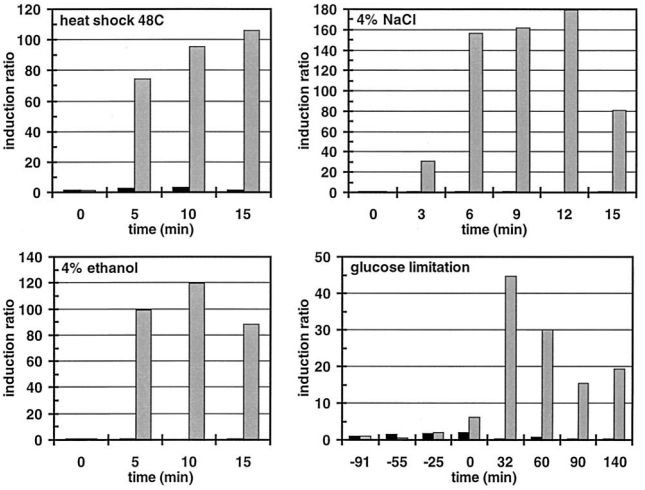

FIG. 2. Levels of *yklA*- and *ykzA*-specific mRNA before and after the imposition of different stresses. RNA was prepared from the wild-type strain 168 at the times indicated on the *x* axes. Specific RNAs were detected with digoxigenin-labeled RNA probes, and the intensities of the signals were quantified with a laser densitometer as described previously (36). The amount of RNA present during exponential growth was set to one. The induction ratios of *yklA* (solid bars) and *ykzA* (shaded bars) at the different time points are displayed. All stresses were applied at time zero with the exception of glucose limitation, where zero indicates the point at which the culture ceased to grow.

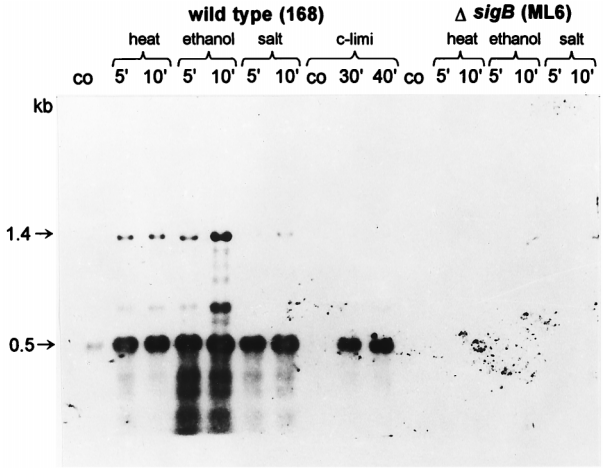

FIG. 3. Northern blot analysis of *ykzA*. Equal amounts of total RNA (10 μg) prepared from the wild-type 168 or the isogenic *sigB* mutant (ML6) before (co) and at different times (in minutes) after exposure to stress were separated on denaturing gels, transferred onto a positively charged nylon membrane, and hybridized with digoxigenin-labeled RNA probe specific for *ykzA*.

also induced during the exhaustion of glucose, but clearly the level of induction was less pronounced than during salt stress. Induction by stress was not confined to salt stress. A similar very strong and transient induction of *ykzA* was also measured following heat shock and ethanol stress (Fig. 2). Therefore, *ykzA* belongs to the group of general stress genes induced by multiple stimuli in *B. subtilis*. When the same RNA preparations were probed with a *yklA*-specific probe, we failed to detect significant changes in the expression of *yklA* in response to any of the stimuli examined (Fig. 2).

In view of its response to multiple stimuli, transcription of *ykzA* was analyzed in more detail by Northern blot analysis. A signal of 0.5 kb was observed, which is the expected size of a monocistronic transcript encoding only *ykzA*. However, two additional, but less intense, signals corresponding to transcripts of 0.8 and 1.4 kb were also detected (Fig. 3). Only the 0.5-kb transcript was detected during growth, but the intensities of all three transcripts increased upon stress or starvation. Northern analysis experiments with probes spanning the regions upstream and downstream of *ykzA* indicated that the signals corresponding to the two larger transcripts result from readthrough at the terminator downstream of *ykzA* (data not shown).

In *B. subtilis*, expression of most of the general stress genes induced by stress or starvation depends on the sigma factor σ^B. Since YkzA belongs to this family of general stress proteins and there is a putative σ^B-dependent promoter upstream of *ykzA*, it was decided to examine expression of *ykzA* in a *sigB* mutant strain. Total RNA isolated from *sigB* mutant strain ML6 after exposure to heat, ethanol, and salt was probed with a *ykzA*-specific probe by Northern analysis. No *ykzA*-specific signals were detected in this strain after exposure to any of the stresses (Fig. 3).

The promoters of *yklA* and *ykzA* were mapped by primer extension analysis. Primers were designed complementary to the DNA regions encoding the N termini of *yklA* and *ykzA* as outlined in Materials and Methods. A very weak signal was detected for *yklA*, which did not significantly increase upon stress. A signal of similar intensity was also obtained with RNA isolated from a *sigB* mutant (Fig. 4A). The size of this reverse transcript is consistent with expression of *yklA* being driven by the σ^A-type promoter which was identified by sequence analysis (TTGACA-17 nucleotides-TACAAT). Primer extension analysis with the *ykzA*-specific primer revealed a reverse transcript (Fig. 4B) which was barely detectable with RNA from exponentially growing bacteria, but its intensity increased dramatically with RNA isolated from cells which had been exposed to stress (Fig. 4B). No transcript was detected with RNA isolated from a similarly stressed *sigB* mutant strain (Fig. 4B). The point of initiation of transcription for *ykzA* is consistent with transcription being driven from the σ^B-type promoter (GTTTAA-12 nucleotides-GGGAAA) identified in the sequence analysis.

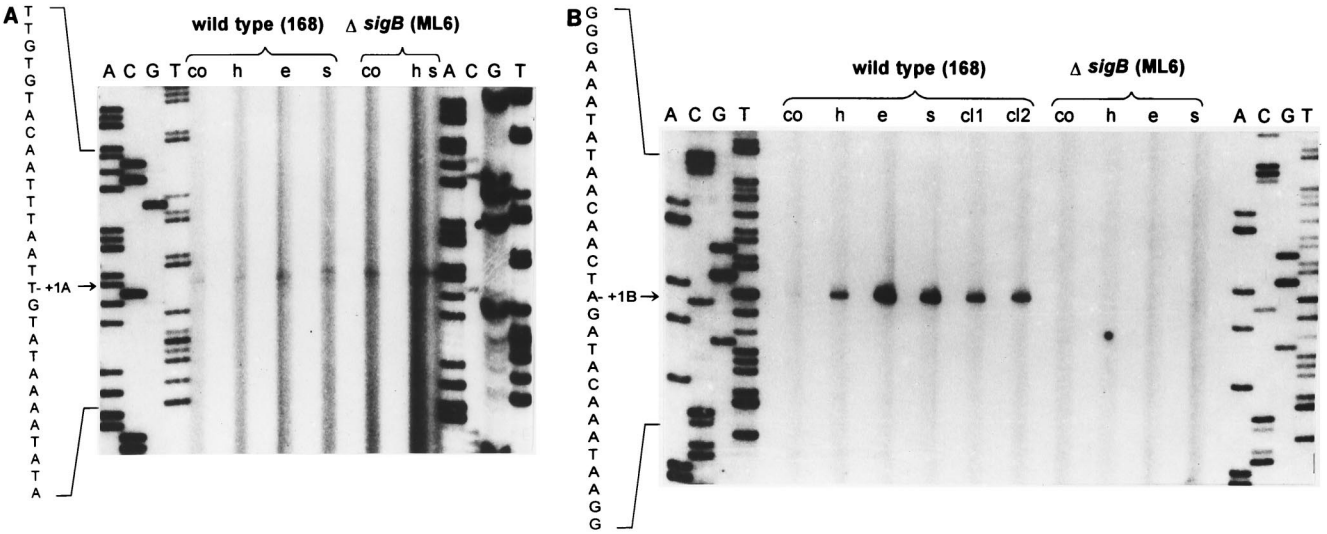

FIG. 4. Mapping of the 5′ ends of the *yklA* (A) and *ykzA* (B) mRNA during growth and after exposure to stress. Total RNA was isolated from the wild-type strain and its isogenic *sigB* mutant during exponential growth (co), 10 min after the imposition of the different stresses (h, heat shock; e, ethanol stress; s, salt stress), and 30 or 40 min after the limitation of glucose (cl1 and cl2, respectively). The primer extension analysis was performed as described in Materials and Methods. The 5′ ends of the transcripts were determined by comparison with a DNA-sequencing ladder generated with the same primer and run in parallel on the same gel (lanes A, C, G, and T).

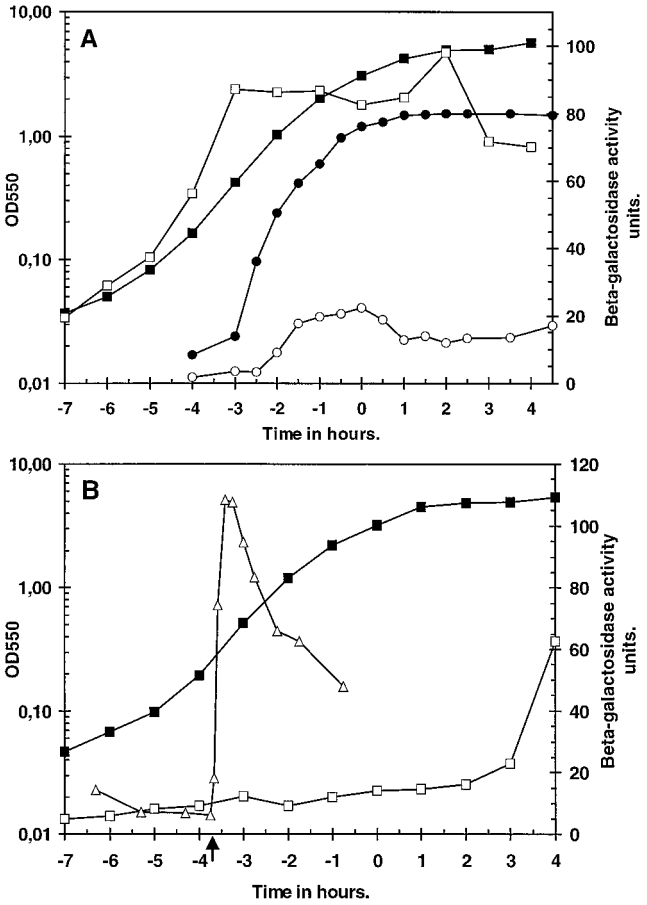

FIG. 5. Expression of the *yklA-lacZ* (A) and *ykzA-lacZ* (B) transcriptional fusions during the growth cycle in minimal medium or nutrient broth and after exposure to salt stress. Cells were grown as described in Materials and Methods, and samples were taken every 30 or 60 min as indicated. (A) solid symbols indicate growth of *B. subtilis* BFS1816, and open symbols indicate β-galactosidase activity. Circles, nutrient medium; squares, minimal medium. (B) Solid squares represent growth of *B. subtilis* BFS1818 in minimal medium, and open squares indicate β-galactosidase activity. The influence of the addition of salt during exponential growth on the accumulation of β-galactosidase is also indicated (open triangles). The point of salt addition is indicated by an arrow. OD550, optical density at 550 nm.

Expression of *yklA* and *ykzA* in minimal and rich medium during the growth cycle and after induction by salt. Two strains (BFS1816 and BFS1818), mutated in *yklA* and *ykzA*, respectively, were constructed by integration of pMutin004 and pMutin005 into the chromosome of *B. subtilis*. The pMutin-derived plasmids contained an internal fragment of the *yklA* and *ykzA* open reading frames, respectively, cloned immediately upstream of a promoterless *lacZ* gene, allowing the expression of each gene to be examined. The strains grew and sporulated normally both on minimal medium and on medium containing 0.3 M NaCl. Expression of *yklA-lacZ* and *ykzA-lacZ* was examined in nutrient broth and minimal medium throughout the growth cycle. When cells harboring *yklA-lacZ* were grown in nutrient medium, β-galactosidase activity reached approximately 20 U during exponential growth and decreased slightly at the onset of the stationary phase (Fig. 5A). When these cells were grown in minimal medium, β-galactosidase levels rose during the early period of the growth cycle. This accumulation reached a plateau of approximately 90 U by the midpoint of the growth cycle (T_{-3}), and this level was maintained for the remainder of the growth cycle (Fig. 5A). Addition of 0.3 M NaCl to exponentially growing cells containing *yklA-lacZ* did not affect the β-galactosidase activity (data not shown), confirming the results of the slot blot analysis, which showed that expression of *yklA* is not responsive to osmotic stress (Fig. 2).

When cells harboring *ykzA-lacZ* were grown in nutrient medium, the level of β-galactosidase remained at ≤20 U throughout the exponential and stationary phases of the growth cycle (data not shown). When these cells were grown in minimal medium the level of β-galactosidase also remained at ≤20 U until approximately 2 to 3 h into the stationary phase, when an increase in activity was discernible (Fig. 5B). When salt was added to exponentially growing cells containing *ykzA-lacZ* in minimal medium (to a final concentration of 0.3 M NaCl), there was a rapid 10-fold increase in β-galactosidase activity, which peaked approximately 20 min after the addition of salt. The β-galactosidase level declined during the subsequent 2.5 h of growth but still remained approximately three- to fivefold higher than that in unstressed cells at the end of this time interval (Fig. 5B). These results demonstrate that expression of these two paralogs is complex but complementary: expression of *yklA* is medium dependent but is not responsive to stress. In contrast, expression of *ykzA* is medium independent but is responsive to osmotic and other stresses (as shown by transcription analysis).

Identification of YkzA on two-dimensional protein gels; level of YkzA during exponential growth and after imposition of stress. We have determined the N-terminal sequences of general stress proteins of *B. subtilis* by microsequencing (3, 8, 40). When comparing these sequences with the sequences of YklA and YkzA, we discovered that the N-terminal sequence of the general stress protein Gsp17o (ALFTAKVTAR GGRAAHIT SD D) matched the amino acid sequence deduced from the *ykzA* DNA sequence (with the exception of the alanine residue at position 15). Therefore, the ATG codon at position 34145 of the DNA sequence is indeed the start codon of *ykzA*, and the N-terminal formyl-methionine is subsequently removed. Two-dimensional protein gel electrophoresis of crude protein extracts from cells harvested during exponential growth or after imposition of stress was used to show that levels of YkzA significantly increased following heat, salt, and ethanol stress and that the stress sigma factor σ^B was required for this increase to occur (Fig. 6). The intensity of a reference spot corresponding to the ribosomal protein RplJ did not increase during the same time interval (Fig. 6).

DISCUSSION

The expression profiles of two *B. subtilis* genes, *yklA* and *ykzA*, whose products are highly similar to the general stress protein OsmC first identified in *E. coli*, have been presented. The profiles are complex, with expression of the genes being growth phase, medium, and stress dependent. Expression of *yklA* is directed by a σ^A-type promoter, and it is maximally expressed during exponential growth. The expression of *yklA* is four- to fivefold higher in minimal medium than in a rich medium, and it is not induced by stress.

Expression of *ykzA*, in contrast, is directed by a promoter which requires σ^B, the so-called stress sigma factor of *B. subtilis*, for initiation of transcription. Expression of *ykzA* is not medium dependent and is very low throughout exponential growth. However, expression of *ykzA* is rapidly induced by salt and ethanol stress, heat shock, and starvation. Therefore, the expression patterns, although complex, appear complementary, with *yklA* being medium responsive whereas *ykzA* is stress and starvation responsive.

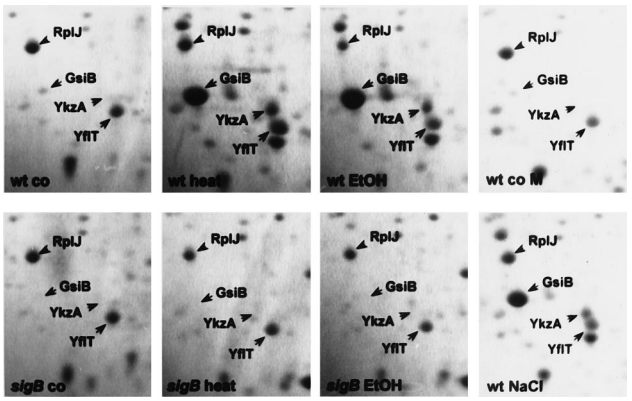

FIG. 6. Level of YkzA in the wild type (wt) and the *sigB* mutant (*sigB*) before (co) and after the imposition of stresses (heat, ethanol [EtOH], and NaCl). Bacteria were grown in LB (co, heat, and EtOH) or minimal medium (co M and NaCl) and harvested during exponential growth, 60 min after the imposition of heat shock or ethanol, or 90 min after exposure to NaCl. Crude protein extract (300 μg) was separated by two-dimensional gel electrophoresis and stained with Coomassie brilliant blue R-350. Besides YkzA, GsiB is indicated as an additional σ^B-dependent stress protein and RplJ is labeled as a vegetative marker protein. The identities of the labeled proteins were verified by microsequencing or mass spectrometry.

It is instructive to compare the organization and expression of *osmC* from *E. coli* with those of *yklA* and *ykzA* from *B. subtilis*. There is only one *osmC* gene in *E. coli*, whose expression is directed by two overlapping but apparently independent promoters (16, 18). In contrast, *B. subtilis* has two *osmC* homologs, each expressed from a single promoter. The $osmCp_1$ from *E. coli* is recognized by the housekeeping sigma factor Sigma70. Similarly, the *yklA* gene from *B. subtilis* seems to be recognized by the housekeeping sigma factor σ^A. Expression of *osmC* from the $osmCp_2$ promoter and expression of *ykzA* are directed by the stress sigma factors RpoS and σ^B, respectively. Induction of these genes, which can be effected by a variety of stresses, is dependent on these sigma factors. Expression directed by $osmCp_2$ also increases when the cells enter stationary phase, as does expression of *ykzA* in *B. subtilis*. Although only the σ^B-dependent promoter of *ykzA* is induced following salt stress in *B. subtilis*, both promoters of *osmC* of *E. coli* are salt responsive.

Nevertheless, despite the overt differences in gene organization between the two bacteria, the similarity of the composite expression profiles is striking. It is evident that both bacteria must regulate the level of the general stress protein with great precision, and expression must be responsive to growth, medium, and stress conditions. In *E. coli* this is achieved by the expression of one gene being directed by two promoters, whereas in *B. subtilis* it is achieved by having two genes each directed by a single promoter.

Our data clearly demonstrate that there are three RNA transcripts produced upon induction of *ykzA*. All three transcripts begin at the *ykzA* promoter. The major transcript is 0.5 kb in length, which is consistent with transcription ceasing at the putative terminator located immediately downstream of *ykzA*. The lengths of the other two transcripts are consistent with transcription proceeding through the *ykzA* terminator and ending at the putative terminators for the *ykoA* and *metC* genes, respectively, which are expressed from the strand opposite to *ykzA*. Therefore, it is evident from our data that there is a disparity between the strength of the *ykzA* promoter and that of the terminator.

Despite our extensive knowledge of the organization and control of expression of *osmC*, *yklA*, and *ykzA*, the functions of the proteins are unknown. It is evident that they play roles in cellular response to a variety of stressful conditions, but their precise functions remain to be established. No obvious phenotype is observed, even under stressful conditions, when *osmC* is inactivated in *E. coli* or when either gene is inactivated in *B. subtilis*. The occurrence of *osmC*-homologous genes among bacteria does not correlate with any bacterial group or ecological niche and so does not shed any light on its function. There are now 11 members of this gene family distributed among the following bacteria: *E. coli* (one copy), *B. subtilis* (two copies), *Mycoplasma pneumoniae* (one copy), *Mycoplasma genitalium* (one copy), *Acinetobacter calcoaceticus* (one copy), *Xanthomonas campestris* (one copy), *Pseudomonas aeruginosa* (two copies), and *Deinococcus radiodurans* (two copies). However, no member of this gene family has been identified in the complete genome sequences of *Haemophilus influenzae*, *Helicobacter pylori*, or *Synechocystis* sp. An alignment of the 11 proteins reveals four regions which are absolutely conserved (data not shown): (i) a glycine residue near the amino terminus, (ii) an NPEQ/EXL motif, (iii) a CF motif, and (iv) an AXXXCPXS motif. These motifs do not show similarity to any other motif in the database. However, conservation of the two cysteine residues is interesting, suggesting that perhaps the protein contains a disulfide bond which is required for activity. Alternatively, it may bind a metal ion or may participate in maintaining disulfide bonds in other proteins, i.e., a type of disulfide bond chaperone. At least the *ohr* gene of *X. campestris*, which is a member of the *osmC* family, is required for protection against organic hydroperoxides (32). A phylogenetic analysis of the eleven proteins (Fig. 7) shows that they can be grouped into three families: (i) the *E. coli* family, which includes, besides *osmC*, one each of the *D. radiodurans* and *P. aeruginosa* genes; (ii) the *Mycoplasma* family; and (iii) a family containing *yklA*

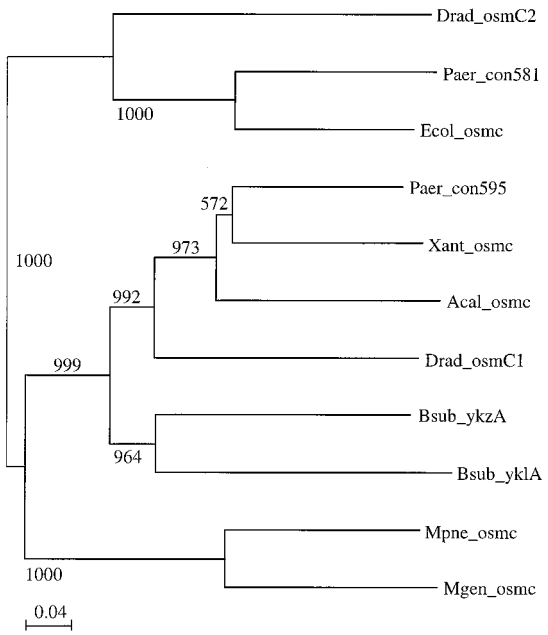

FIG. 7. Unrooted phylogenetic tree of the relationships between the 11 members of the *osmC* gene family produced from a multiple alignment by using the CLUSTAL W program. The data were bootstrapped 1,000 times, and the values are indicated on the horizontal axes. Drad, *D. radiodurans*; Paer, *P. aeruginosa*; Ecol, *E. coli*; Xant; *X. campestris*; Acal, *A. calcoaceticus*; Bsub, *B. subtilis*; Mpne, *M. pneumoniae*; Mgen, *M. genitalium*.

and *ykzA* from *B. subtilis*, one each of the *D. radiodurans* and *P. aeruginosa* genes, and the genes of *A. calcoaceticus* and *X. campestris*. The interesting feature of this tree is that *yklA* and *ykzA* are more closely related to each other than to any other member of the family. In contrast to *P. aeruginosa* and *D. radiodurans*, which also have two copies of *osmC*, the paralogs fall into distinct phylogenetic groups. This suggests that the duplicated genes in *B. subtilis* have not evolved to fulfill separate functions within the cell. Instead we propose that the duplication provides a mechanism for the *Bacillus* cell to regulate OsmC levels in response to a wide range of environmental and nutritional stimuli by placing each copy of the gene under the control of different but complementary expression signals. In *E. coli*, the environmental and nutritional conditions under which *osmC* is expressed are very similar to those in *Bacillus*. However, the mechanism through which this is achieved differs in that expression of a single gene is directed by two different but independent promoters.

The complexity of *osmC* gene expression in *E. coli* and *B. subtilis* suggests that it plays an important role in the response of the cells to stress. Since protection of stressed or starving cells from oxidative stress seems to be a premier function of the σ^B regulon, we are currently investigating whether YkzA and/or YklA is involved in establishing a protective resistance, as does Ohr of *X. campestris* (32).

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