

Characterization of a Novel Member of the DegS-DegU Regulon Affected by Salt Stress in *Bacillus subtilis*

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As a soil bacterium also found in estuarine and marine habitats, *Bacillus subtilis* has evolved various sensing and adaptation systems in order to face salt stress conditions. Among these regulatory mechanisms is the DegS-DegU signal transduction system, which was previously shown to be stimulated by high salt concentrations. A search for promoters regulated in response to salt stress led to the identification of *wapA*, encoding a wall-associated protein, which is strongly expressed at low salt concentrations and almost completely repressed in the presence of 0.7 M disodium succinate. Repression of *wapA* transcription by salt stress was shown to require the phosphorylated form of DegU. Moreover, DegU-mediated repression of *wapA* occurred only in high-salt medium. Alignment between the control region of *wapA* and other DegU-regulated promoters allowed the identification of a putative DegU target sequence, AGAAN₁₁TTCAG. Mutation/deletion analyses of the *wapA* promoter region confirmed the role of the putative DegU control site in repression of *wapA* transcription at high salt concentrations and revealed a second site of repression located downstream from the transcription start site. Since residual negative control was observed at this second site in the absence of DegU, it seems likely that an additional repressor acts on the *wapA* control region to further downregulate *wapA* transcription under salt stress conditions.

In its natural environment, *Bacillus subtilis* spends most of its life in a starving or nongrowing state because of different growth-limiting and stress conditions. As a soil bacterium, *B. subtilis* is exposed to runoff into the sea, and it is largely found in coastal waters, estuarine sediments and other saline habitats. It dominates the marine flora to such an extent that it could be considered a primary inhabitant of the oceans (3, 27). To adapt to drastic variations of environmental conditions including increasing saline concentrations, *B. subtilis* has developed a highly sophisticated regulatory network that involves transcriptional modulation of large sets of genes controlling cellular differentiation (26) and the induction of a set of proteins called general stress proteins (GSPs) or stress-specific proteins (11).

At least three distinct mechanisms of salt stress induction have been identified in *B. subtilis*. Transcription of the so-called class II heat shock genes, encoding GSPs, is activated by the alternative sigma factor σ^B . This regulation involves two separate pathways which respond either to environmental stresses, including salt stress, or energy limitation, through a dual multicomponent network (15, 32). Class II heat shock genes encode a σ^B -independent group of GSPs including FtsH (5) and the ClpP, ClpC, and Lon proteases (16, 28, 33). Among these proteins involved in stress response, ClpC was clearly shown to be required for tolerance to salt stress (16). The DegS-DegU two-component system was suggested to be involved in sensing salt stress, as exemplified by the effect of high salt concentration on the production of degradative enzyme synthesis and the expression of *comG*, a late competence gene, known to be controlled by DegU (18). In contrast to the first two mechanisms, which provide nonspecific stress resistance,

the DegS-DegU regulatory pair seems to be subjected to salt-specific induction. Interestingly, the DegS-DegU signal transduction system also plays a key role in the complex network that governs post-exponential-phase responses under growth-limiting conditions (21).

As a first step toward a better understanding of *B. subtilis* behavior in saline environments, we have sought promoters that are differentially expressed in low-salt and high-salt conditions. Here, we report the isolation of the *B. subtilis* *wapA* promoter, from which expression is relatively strong in low-salt medium and completely repressed by high salt concentrations. The *wapA* gene encodes a wall-associated protein which belongs to a large family of high-molecular-weight, surface-associated proteins involved in various cellular processes, including surface hydrophobicity, pathogenicity, wall metabolism, secretion, and cell adhesion (34). We further show that salt stress repression of *wapA* is mediated by the DegS-DegU two-component system and propose a tentative target site for DegU-mediated regulation based on mutation/deletion analysis of the *wapA* regulatory region together with comparison of promoters known to be controlled by DegU.

MATERIALS AND METHODS

Bacterial strains and genetic techniques. All strains used in this study are listed in Table 1.

Escherichia coli transformations were performed by electroporation of the K-12 strain TG1 [*F'* *traD36 lacI^q Δ(lacZ)M15 proA⁺B⁺/supE Δ(hsdM-mcrB)5 (r_K⁻ m_K⁻ McrB⁻) thi Δ(lac-proAB)*] (9), using a Bio-Rad Gene Pulser as specified by the supplier. Selection was done on LB broth supplemented when appropriate with ampicillin (100 μg ml⁻¹).

B. subtilis strains were transformed with chromosomal or plasmid DNA as follows. Cells were grown in LB liquid medium until they reached transition from exponential growth to the stationary phase. The culture was diluted 20-fold in GE medium, containing 1% glucose, 0.2% potassium L-glutamate, 100 mM potassium phosphate buffer (pH 7.0), 3 mM trisodium citrate, 3 mM MgSO₄, 22 mg of ferric ammonium citrate per liter, and 50 mg of L-tryptophan per liter. After dilution, incubation was continued for 4 h at 37°C and DNA was added. Selection was carried out on erythromycin (1 μg ml⁻¹; 10 μg ml⁻¹ for pHT304

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TABLE 1. *B. subtilis* strains used in this study

Strain	Genotype or description ^a	Source ^b or reference
168	<i>trpC2</i>	Laboratory stock
QB136	<i>trpC2 leuA8 degU32(Hy)</i>	17
QB4414	<i>trpC2 degU146</i>	4
QB4487	<i>trpC2 degUΔBclI-EcoRI::erm</i>	T. Msadek
QB4871	<i>trpC2 amyE::(wapA'-lacZ aphA3)</i>	This work
QB4883	<i>trpC2 amyE::(wapA'-lacZ aphA3) ΔdegU::erm</i>	QB4487→QB4871
QB4950	<i>trpC2 amyE::(wapA'-lacZ aphA3)</i>	pWP252→168
QB4951	<i>trpC2 wapA::(wapA'-lacZ cat)</i>	pWP253→168
QB4955	<i>trpC2 amyE::(wapA'-lacZ aphA3) degU146</i>	QB4871→QB4414
QB4959	<i>trpC2 amyE::(wapAΔD'-lacZ aphA3)</i>	pWP263→168
QB4961	<i>trpC2 amyE::(wapA[A₋₃₈G]'-lacZ aphA3)</i>	pWP259.15→168
QB4962	<i>trpC2 amyE::(wapA[A₋₃₈G]ΔD'-lacZ aphA3)</i>	pWP265→168
QB4963	<i>trpC2 amyE::(wapA[TT_{-23/-22}AA]ΔD'-lacZ aphA3)</i>	pWP267→168
QB4964	<i>trpC2 amyE::(wapAΔD'-lacZ aphA3) ΔdegU::erm</i>	QB4487→QB4959
QB4965	<i>trpC2 amyE::(wapA[TT_{-23/-22}AA]ΔD'-lacZ aphA3) ΔdegU::erm</i>	QB4487→QB4963
QB4966	<i>trpC2 amyE::(wapA[A₋₃₈G]ΔD'-lacZ aphA3) ΔdegU::erm</i>	QB4487→QB4962
QB4967	<i>trpC2 amyE::(wapAΔA'-lacZ aphA3)</i>	pWP259→168
QB4968	<i>trpC2 amyE::(wapA[TT_{-23/-22}AA]ΔD'-lacZ aphA3) degU32(Hy)</i>	pWP267→QB136
QB4998	<i>trpC2 amyE::(wapAΔB'-lacZ aphA3)</i>	pWP280→168

^a *cat* is the chloramphenicol acetyltransferase gene from pC194 (14), *aphA3* is the *Streptococcus faecalis* kanamycin resistance gene (30), and *erm* is the constitutive *erm* gene from Tn1545 (31).

^b → indicates construction by transformation.

derivatives), chloramphenicol (5 μg ml⁻¹), kanamycin (5 μg ml⁻¹) and spectinomycin (100 μg ml⁻¹).

Growth media. LSM medium contains 1.7% Bacto Agar (Difco), 0.2% casein hydrolysate (Oxoid), 0.5% glucose, 100 mM potassium phosphate buffer (pH 7.0), 3 mM MgSO₄, 22 mg of ferric ammonium citrate per liter, and 50 mg of L-tryptophan per liter, supplemented with 80 mg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per liter to detect β-galactosidase activity. HSM medium is LSM medium containing 0.7 M disodium succinate added from a 30% disodium succinate solution (pH 7.0). MB liquid medium contains 100 ml of a 10 × MB solution (tryptone, 100 g per liter; yeast extract, 50 g per liter) per liter, 3 mM MgSO₄, and 50 mg of L-tryptophan per liter, supplemented with 100 mM disodium succinate (low-salt MB) or 0.7 M disodium succinate (high-salt MB). Bacterial growth was followed by measuring the optical density at 600 nm in liquid cultures.

Nucleic acid manipulations. The *B. subtilis* genomic library used in this study was constructed as follows. Chromosomal DNA from wild-type strain 168 was partially digested with *Sau3A*, and fragments between 0.2 and 1.8 kb in size were ligated at the dephosphorylated *Bam*HI site of the *B. subtilis* integrative vector pAZ7 to create transcriptional fusions with the *lacZ* reporter gene. *E. coli* TG1 was transformed with the ligation mixture, and plasmid DNA was extracted from a pool of 4,000 clones. After transformation of *B. subtilis* 168, integration into the chromosome at the *amyE* locus and screening on low-salt and high-salt media, the insert of the unique plasmid of interest was recovered by PCR using oligonucleotides complementary to regions located on each side of the insert and containing *Bam*HI restriction sites. The resulting fragment was then cloned and sequenced.

Sequencing reactions were carried out on both strands of double-stranded DNA purified with QIAprep spin columns (Qiagen Inc., Chatsworth, Calif.), using a dideoxy-chain termination sequencing kit from Pharmacia and synthetic primers.

Plasmid constructions. The following *lacZ* transcriptional fusions were constructed by using the pJM783 vector for integration at the original locus of the fused promoter and the pJM115 vector for double-crossover integration at the *amyE* locus (23). Plasmids pJM783 and pJM115 are chloramphenicol-resistant and kanamycin-resistant derivatives of pDH32, which harbors the *lacZ* reporter gene translated from the *spoVG* ribosome binding site (25). Plasmids pWP252 and pWP253 were obtained by subcloning the *wapA* promoter region and 5' end of the coding sequence comprised between oligonucleotides OMD26 (5'-GAA TGAATTCGCTGAGGGTACGGATATTG-3') and OMD28 (5'-GTAGGGA TCCCTAGTACATCGGCTGGCAC-3') at the *Eco*RI and *Bam*HI sites (underlined) of pJM115 and pJM783, respectively. Plasmid pWP259 is a variation of pWP252 that carries a shorter insert, ending near the *wapA* start codon, which was obtained by PCR with the oligonucleotides OMD26 and WP3BA (5'-GC AAGGATCCTTTAAAGTTTCGCCTCTTC-3'). Plasmid pWP259.15 is a pWP259 derivative harboring the A₋₃₈G mutation isolated from pWP266.15 after PCR random mutagenesis (see below). A *wapA'-lacZ* fusion devoid of sequences located downstream of the transcription start site (pWP263) was constructed in pJM115 by using oligonucleotides OMD26 and WP+1 (5'-AAAGG GATCCTTACTCAATAATCTTAAGTAG-3'). Plasmid pWP265 is pWP263 carrying the A₋₃₈G mutation. Finally, plasmid pWP280 carries the *wapA* pro-

moter region devoid of sequences located upstream from the putative DegU target site, obtained with oligonucleotides OMD261 (5'-TCGCGAATCTTAA AATAAGATAAATTTCTAGAAA-3') and WP3BA.

Site-directed and PCR random mutagenesis of the *wapA* 5' regulatory region. Site-directed mutagenesis of the putative DegU binding site in the promoter region of *wapA* was carried out by PCR. A 150-bp upstream fragment (A) ending at the mutation site (TT_{-23/-22}AA) was amplified by using oligonucleotides OMD26 and 5'-TTAAGAAGACTGTTATATCATTACAATATTTTTC-3' and digested with *Eco*RI and *Bbs*I to liberate 5'-TTAT-3' asymmetric overhang carrying the mutations to be introduced. The downstream fragment (B) was amplified with the oligonucleotides 5'-TATTGAAGACATATAACAGTCTAG TTAACATTATTG-3' and WP3BA. Digestion with *Bam*HI and *Bbs*I liberated 5'-ATAA-3' cohesive ends. A three-way ligation was performed between the pJM115 integrational vector containing the *lacZ* reporter gene (23) cleaved with *Eco*RI and *Bam*HI and fragments A and B described above. The recombinant clone was designated pWP267, and the insert was sequenced to verify that no additional mutation had been introduced in the course of PCR amplification and cloning processes.

Random mutations in the control region of *wapA* leading to constitutive expression at high salt concentrations were isolated by PCR under conditions that reduce the fidelity of DNA synthesis by the *Taq* DNA polymerase (19). First, the *wapA* 5' regulatory region was subcloned between the *Eco*RI and *Bam*HI sites of pBluescriptSK, using oligonucleotides OMD26 and WP3BA, to give plasmid pWP258. PCR reactions were then carried out in the presence of limiting dATP or dGTP (1:10 ratio), using the universal and reverse primers and pWP258 as the template, to obtain a collection of potentially mutated fragments. These were digested with *Hind*III and *Bam*HI and ligated in front of *lacZ* in the replicative vector pHT304-18Z (1). *E. coli* TG1 was transformed by electroporation with the ligation mixture, and plasmid DNA was extracted from a pool of 12,000 clones. *B. subtilis* 168 was transformed with the resulting library, and transformants were screened on HSM medium. In parallel, the control plasmid pWP266 was obtained by subcloning the wild-type *wapA* promoter region in pHT304-18Z, using oligonucleotides OMD26 and WP3BA. The mutant derivatives were designated pWP266.1 to pWP266.48S.

β-Galactosidase assay. *B. subtilis* strains harboring *lacZ* fusions were assayed for β-galactosidase activity as previously described (20).

Mapping of mRNA start site by primer extension. The synthetic oligonucleotide OMD25 (5'-GCCAACACTAAAATGCTGCAATGAACC-3') was 5'-end labeled with [γ-³²P]ATP (110 TBq mmol⁻¹) by using T4 polynucleotide kinase. Forty micrograms of RNA and 1 pmol of labeled primer were annealed in a total volume of 18 μl of reverse transcriptase buffer (50 mM Tris-HCl, 8 mM MgCl₂, 30 mM KCl, 1 mM dithiothreitol [pH 8.5]). The mixture was incubated for 3 min at 60°C and then slowly cooled at room temperature. One microliter (25 U) of avian myeloblastosis virus (Boehringer) reverse transcriptase and 1 μl of a deoxynucleoside triphosphate solution (20 mM each) were added. After 30 min at 37°C, reactions were stopped by the addition of 5 μl of 95% formamide, 10 mM EDTA, 0.3% xylene cyanol, and 3.3% bromophenol blue. Two microliters of each reaction was loaded on a 6% polyacrylamide sequencing gel.

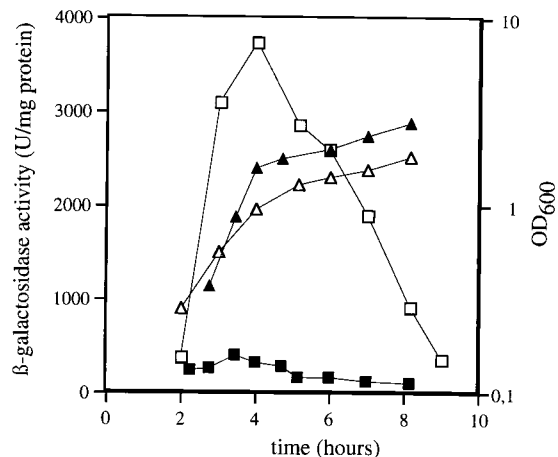


FIG. 1. Effect of salt stress on β -galactosidase expression driven from a *wapA'*-*lacZ* transcriptional fusion integrated at the *amyE* locus. *B. subtilis* strain QB4950 was grown in MB medium (1% tryptone, 0.5% yeast extract, 50 μ g of tryptophan per ml, 1 mM $MgSO_4$) containing 100 mM disodium succinate (\square) or 0.7 M disodium succinate (\blacksquare). The corresponding growth curves are shown with open (high salt) and closed (low salt) triangles. OD, optical density at 600 nm.

RESULTS

Transcription of *wapA* is repressed under salt stress conditions. A *B. subtilis* genomic library was constructed by using the pAZ7 integrative vector that contains the *lacZ* reporter gene and genomic fragments between 0.5 and 1.8 kb in size to obtain a promoter library (see Materials and Methods for details). A preliminary screening of 150 clones was performed on high-salt and low-salt media to detect promoters affected by salt stress. One of the clones (strain QB4871) appeared to carry a promoter strongly expressed at low salt concentration as observed on LSM medium (minimal medium containing 100 mM disodium succinate) while significantly repressed in HSM (0.7 M disodium succinate). After PCR rescue and sequencing, the corresponding insert was found to harbor the promoter region and 5' end of the coding sequence of *wapA*, encoding a wall-associated protein (8). Analysis of the *wapA* and adjacent chromosomal regions indicated that *wapA* is preceded by an open reading frame (ORF1) ending 165 bp upstream of the *wapA* start codon and that the ORF1-*wapA* intergenic region contained a putative σ^A -dependent promoter. To determine whether the observed phenotype was conferred by a promoter located directly upstream of the *wapA* ORF, *wapA'*-*lacZ* fusions were made by using a PCR-amplified fragment encompassing the ORF1-*wapA* intergenic region. The resulting transcriptional fusions, carried on plasmids pWP252 and pWP253, were integrated in the chromosome of the wild-type strain 168 at the *amyE* and *wapA* loci to give strains QB4950 and QB4951, respectively. In both cases, *wapA*-driven expression of β -galactosidase was significantly decreased under salt stress conditions, as observed on HSM compared to LSM. This was further confirmed by monitoring the β -galactosidase activity of strain QB4950 grown in low-salt and high-salt MB liquid media. The results shown in Fig. 1 indicate that *wapA* is transcribed from a promoter located in the ORF1-*wapA* intergenic region and subjected to a drastic repression under high-salt conditions. The transcription start site, determined by primer extension analysis, was found to be located at nucleotide position -35 relative to the *wapA* start codon (Fig. 3, lane 1). Putative -35 and -10 boxes typical of σ^A promoters were detected in the corresponding regions.

wapA belongs to the DegS-DegU regulon affected by salt stress. To determine whether *wapA* regulation could be categorized as one of the three salt stress induction mechanisms described above, expression of the *wapA'*-*lacZ* fusion in low-salt and high-salt media was tested in *sigB*, *clpC*, and *degU* deletional mutants. Only inactivation of the *degU* response regulator had an effect on the expression of *wapA*. More precisely, either deleting *degU* or replacing *degU* with the *degU146* allele, which produces a nonphosphorylatable form of DegU, caused a strong derepression of *wapA* in high-salt medium (Fig. 2). These results confirmed that the DegS-DegU two-component system is involved in adaptation to salt stress and also indicated that transcription of *wapA* is repressed by the phosphorylated form of DegU under high-salt conditions. Interestingly, none of these two *degU* mutations (*degU* or *degU146*) had a significant effect on the expression of *wapA* in LB or low-salt medium (data not shown), indicating that DegU-mediated control of *wapA* occurs mostly under conditions of salt stress. These observations were confirmed by primer extension analysis using mRNA isolated from cultures of the wild-type and *degU* mutant strains in low-salt and high-salt media. As shown in Fig. 3, deletion of *degU* had no effect on either the position of the transcription start site or the apparent amount of *wapA* transcript under low-salt conditions (lanes 1 and 2). In agreement with the β -galactosidase results shown in Fig. 1 and 2, the apparent amount of *wapA* transcript in the wild-type strain was strongly decreased by high salt concentrations whereas *wapA* transcription in the *degU* null mutant was independent of the salt concentration.

A consensus sequence upstream of DegU-regulated genes?

In an attempt to define a putative target sequence for DegU control, the promoter regions of genes known to be regulated by DegS and DegU were aligned, and the consensus sequence AGAAN₁₁TTCAG was inferred (Fig. 4). To validate our observations, site-directed mutagenesis of the *wapA* promoter region was undertaken to transform the TTCAG conserved motif, which lies between the -35 and -10 boxes (Fig. 5), into AACAG, thereby modifying two invariant nucleotides in the putative DegU target site. The corresponding *wapA*[TT_{-22/-23}AA]-*lacZ* fusion, carried on plasmid pWP267, was integrated at the *amyE* locus of strain 168 and tested for its ability to be repressed under salt stress conditions. The β -ga-

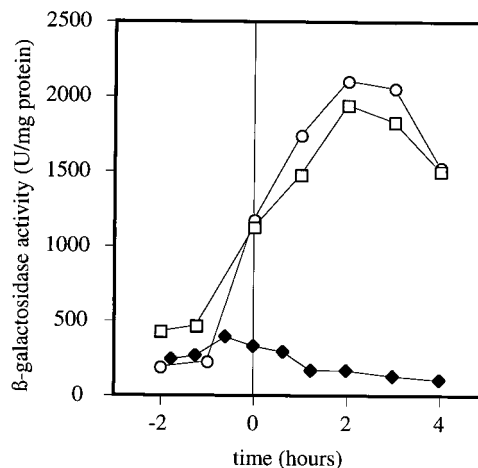


FIG. 2. Effects of *degU* mutations on the expression of *wapA'*-*lacZ* under salt stress conditions. *B. subtilis* QB4871, QB4883, and QB4955 were grown in high-salt MB medium. Symbols: \blacklozenge , QB4871 (wild type); \square , QB4883 (*degU::erm*); \circ , QB4955 (*degU146*).

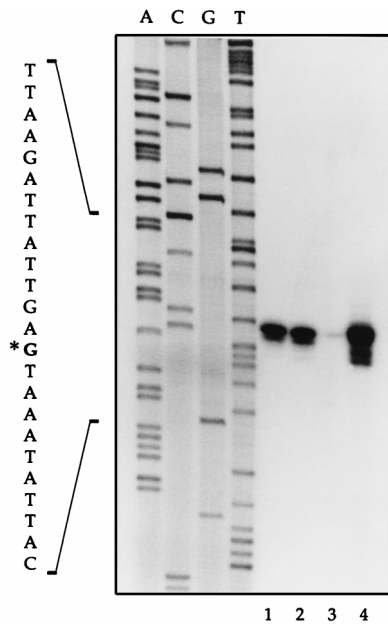


FIG. 3. Determination of the transcription start site of *wapA* by primer extension analysis using an oligonucleotide complementary to the DNA sequence from positions +59 to +33 relative to the *wapA* start codon. The products of a sequencing reaction generated with the same oligonucleotide were run in parallel. mRNA was isolated from low-salt (lanes 1 and 2) and high-salt (lanes 3 and 4) cell cultures of the wild-type strain 168 and the *degU* strain QB4487, respectively. Equivalent amounts of total RNA were used in all primer extension experiments.

lactosidase phenotype observed on HSM solid medium as well as β -galactosidase assay in liquid high-salt MB medium revealed that expression of *wapA*[TT_{-22/-23}AA]-*lacZ* was significantly derepressed in high-salt medium compared to the wild-type fusion (Table 2), indicating that the TT_{-23/-22}AA mutations prevented full repression of *wapA* transcription under salt stress conditions. However, the *degU32*(Hy) mutation, which encodes a highly stable phosphorylated form of DegU (4), was able to restore full repression whereas inactivation of *degU* led to an additional derepression (Table 2), suggesting that the putative DegU target site had been only partially altered by the TT_{-23/-22}AA modification and/or that another site within the *wapA* regulatory region is involved in DegU-mediated regulation.

Isolation of random mutations that prevent repression of *wapA* by salt stress. To confirm the results obtained by site-directed mutagenesis and further define the target of *wapA* repression by salt stress, a PCR random mutagenesis was performed on the 5' regulatory region of *wapA* as described in Materials and Methods. A library of potentially mutated PCR fragments was obtained and subcloned in the replicative vector pHT304-18Z (1) in front of the *lacZ* reporter gene. The resulting library was transferred into *B. subtilis* 168 and screened for clones that were no longer sensitive to high-salt repression and had therefore retained a Lac⁺ phenotype on HSM solid medium. Among 12,000 clones examined, over 50 colonies displayed a light to dark blue color; by comparison, the control strain harboring the wild-type *wapA* regulatory region (plasmid pWP266) remained white on HSM. Plasmid DNA was isolated from the positive clones, designated pWP266.1 to pWP266.48S; the corresponding inserts were sequenced, and only those clones that carried a single mutation were retained. Further screening allowed us to distinguish two classes of mu-

tants. The majority (26 of 30) were specifically derepressed on HSM, while their expression remained unchanged on low-salt or sporulation medium. On the other hand, 4 clones of 30 exhibited a nonspecific increase of expression on all media tested. Accordingly, these mutants appeared to carry a promoter-up mutation (TAAGAT→TATGAT) that improved the -10 box recognized by σ^A . A compilation of these mutations is presented in Fig. 5. Interestingly, the high-salt-specific mutations appeared to fall into two separate clusters: one corresponding to the conserved sequence thought to be involved in DegU binding, and a second focus of high mutation frequency located downstream from the transcription start site within the 5' untranslated region (Fig. 5). Examination of the DNA sequence encompassing the second cluster of mutations allowed the detection of a direct repeat of two 6-bp motifs (TATTAC) separated by a three-nucleotide spacer. Shortening of this spacer by 1 bp (mutant 3S) also led to a derepressed phenotype. We selected a subset of representative mutants of each class in each region and determined the corresponding β -galactosidase activities in low-salt and high-salt media. The results presented in Table 3 and Fig. 6 confirm the phenotypes observed on plates. It must be noted that pWP266 plasmids are pHT304 derivatives which are present at about four copies per equivalent chromosome (2).

In an attempt to better elucidate the role of each of the two apparent regulatory regions in DegU-mediated regulation, we designed several *lacZ* fusions to (i) remove sequences located upstream of the two clusters of random mutations, (ii) alter the upstream site (site I), and/or (iii) delete the downstream site (site II) as schematically represented in Fig. 6 and further described in Materials and Methods. They were integrated as a single copy at the *amyE* locus of strains 168, QB4883 (*degU*), and QB136 [*degU32*(Hy)], and the resulting strains were assayed for β -galactosidase activity in high-salt medium. Our data first indicate that the region starting from -65, relative to the transcription start site, and extending downstream the initiation codon (pWP280) is repressed under high-salt conditions except in the absence of phosphorylated DegU, indicating that pWP280 carries the determinant(s) for DegU-mediated repression under salt stress. Second, altering or deleting site I or site II leads to a partial derepression of *wapA* transcription in high-salt medium, and deleting *degU* causes an additional derepression of the corresponding constructs (plas-

<i>wapA</i>	-41	AAAA	ATAT	TGTAATGATAT	TTCAG	TCT
<i>sacB</i>	-128	GAAA	AGAA	ACAGATAGATTTT	TTTAG	TTC
	-118	AGAT	AGAT	TTTTTAGTTTC	TTTAG	GCC
<i>sacXY</i>	+245	ATGA	ACAA	GCCGACTTTTC	TTCAG	CAA
<i>aprE</i>	-188	ACCT	AAAA	AAGCATGACAT	TTCAG	CAT
<i>degU</i>	(214)	GAGA	AGAA	AAACAAATCA	TTCGG	CTT
<i>degR</i>	-286	ATCG	AGAG	CTGCCAGCTC	TTCAG	ATT
<i>degQ</i>	-173	ATTA	AGAA	TCTTTCTCAAT	TTCAG	CAC
<i>srfAB</i>	(168)	TGAA	AAAA	CAGTACAAACA	TTCAG	CCC
<i>yxjII</i>	(347)	AAAA	AGAA	ATCCAGCCCAT	TTCAA	AAA
<i>comG</i>	-94	TGCC	AGAA	AGAATTGGTTC	TTCAG	CAT
<i>comC</i>	-100	CGGC	AGAA	TCAAAAAAGA	TTCGT	CCG
<i>comEA</i>	(284)	AAAT	AAAA	ATCATCTCGTT	TTCAG	AAA
<i>mecA</i>	-85	AAAT	AGAA	ATACAAAGGAA	TTCAC	ACT
consensus:			AGAA	N ₁₁	TTCAG	

FIG. 4. Comparison of nucleotide sequences located upstream of genes reported to be regulated by DegU. Numbers preceding the sequences indicate positions of the leftmost nucleotide relative to the transcriptional start site; those in parentheses correspond to positions relative to the translational start site. The *yxjII* operon was sequenced by Glaser et al. (10) in the framework of the genome sequencing project, and its transcription was shown to be repressed by DegU (4a).

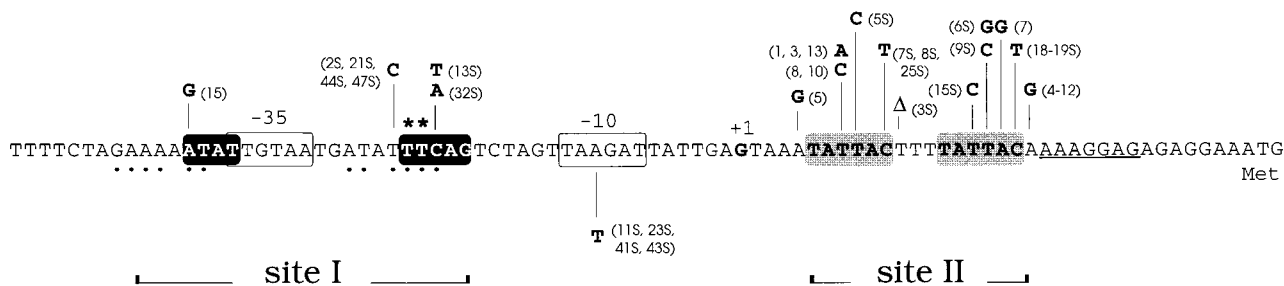


FIG. 5. Localization of random mutations leading to high-salt-resistant expression of *wapA*. The mutations indicated above the nucleotide sequence conferred specific derepression under salt stress conditions, while mutations that exhibited a nonspecific increase of expression in both low- and high-salt media are indicated below the sequence. Asterisks mark nucleotides modified by site-directed mutagenesis. The black-boxed sequences correspond to the conserved motif found upstream of genes known to be regulated by DegU, also referred to as the upstream site or site I. The dots under nucleotides in the upstream region indicate a potential dyad symmetry. The gray boxes highlight the 6-bp direct repeats encompassing the second focus of mutations (site II).

mids pWP263, -267, and -259.15, [Fig. 6]). Third, combining a mutation in site I with the deletion of site II led to a strong derepression of *wapA*, indicating that negative control at the two sites is cumulative. In the *degU32*(Hy) mutant, a drastic repression of *wapA* transcription was observed for each construct tested. The efficiency of this negative control was reduced when both sites I and II were altered.

DISCUSSION

As a widespread soil bacterium also found in coastal and estuarine sediments, as well as in marine and freshwater habitats (27), *B. subtilis* must be able to adapt to transient or permanent high salt concentrations in its environment. Extensive studies on general and salt stress-specific proteins (see reference 11 for a review) together with the recent advances in functional analysis of the newly sequenced *B. subtilis* genome indeed indicate that *B. subtilis* modulates the expression of an impressive number of genes in response to salt stress (2a). However, the molecular mechanisms that govern salt stress-specific regulation remain to be fully elucidated. Recently, expression of genes that are controlled by the DegS-DegU two-component system was shown to be affected under salt stress conditions, such as 1 M NaCl or 1 M KCl. The stimulatory or inhibitory effect proved to be DegS-DegU dependent, as well as salt specific since it could not be obtained during growth at high osmolarity such as 0.5 M lactose (18). A similar effect was also obtained when NaCl was replaced with disodium succinate, indicating that chloride anions were not involved in this salt-mediated regulation (16a).

In the course of a screening for novel promoters modulated by salt stress, the *wapA* gene has been isolated and shown to undergo negative regulation by the DegS-DegU regulatory pair in high-salt medium. Since inactivation of *degU* had no effect on *wapA* transcription at low salt concentrations (data

not shown), we propose that the negative control exerted on *wapA* under salt stress conditions is mediated at least partially by DegU. We also observed that the DegU(Hy)32 protein, of which the phosphorylated form is highly stable, remains able to repress *wapA* transcription at low salt concentrations (data not shown). This finding supports the hypothesis that salt stress increases the amount of phosphorylated DegU in the cell, by either increasing its synthesis or stimulating DegS-mediated phosphorylation of DegU. As already pointed out, DegU is a pleiotropic regulator involved in various post-exponential-phase responses. It positively controls the synthesis of certain exoenzymes such as the *sacB*-encoded levansucrase, which produces branched polymers called levans (17). Accordingly, it has been observed that salt stress increases *sacB* transcription (18). In addition, DegU is a positive regulator of competence and DNA uptake. Thus, the DegS-DegU signal transduction system could be viewed as a means to modulate the synthesis, degradation, or entry into the cells of various macromolecules, thereby participating to osmoregulation and adaptation upon osmotic upshock. In the case of *B. subtilis* WapA, no precise function could be assigned to the protein based on the absence of any particular phenotype of a *wapA* null mutant (8). Nevertheless, wall-associated proteins are generally involved in pathogenicity (e.g., the major antigen A from *Streptococcus mutans*) (7), wall metabolism, secretion, adhesion, or other cell surface-associated properties (34). It could be hypothesized that WapA increases cell wall permeability by functioning as a sieve, or an ion pore or channel, and that turning off its synthesis may prevent excessive exchanges between the outside medium and the cytoplasmic compartment.

Based on a conserved region previously detected by Dubnau

TABLE 2. Effect of TT_(-23/-22)AA and $\Delta degU$ mutations on the repression of *wapA* transcription by salt stress^a

Strain	Relevant genotype	β -Galactosidase sp act (U/mg of protein) ^b
QB4950	<i>wapA'</i> - <i>lacZ</i>	100
QB4883	<i>wapA'</i> - <i>lacZ degU</i>	1,900
QB4963	<i>wapA</i> [TT _{-23/-22} AA]'- <i>lacZ</i>	520
QB4965	<i>wapA</i> [TT _{-23/-22} AA]'- <i>lacZ degU</i>	1,950
QB4968	<i>wapA</i> [TT _{-23/-22} AA]'- <i>lacZ degU32</i> (Hy)	<10

^a Cells were grown in MB liquid medium containing 0.7 M disodium succinate.
^b Determined 3 h after the end of the exponential growth phase.

TABLE 3. Effects of PCR random mutations on the repression of *wapA* transcription by salt stress

Plasmid ^a	β -Galactosidase sp act (U/mg of protein) ^b	
	High salt	Low salt
pWP266 (wild type)	400	3,700
pWP266.2S (T ₋₂₄ C)	1,325	4,180
pWP266.3S (T ₊₁₂)	2,460	4,780
pWP266.7S (C ₊₁₁ T)	3,570	4,780
pWP266.19S (C ₊₂₀ T)	4,500	2,800
pWP266.32S (C ₋₂₁ A)	2,125	3,900

^a See Fig. 5 for positions of the mutations.
^b Cells were grown in MB liquid medium containing 100 mM (low salt) or 0.7 M (high salt) disodium succinate. β -Galactosidase activities were determined 3 h after the end of the exponential growth phase.

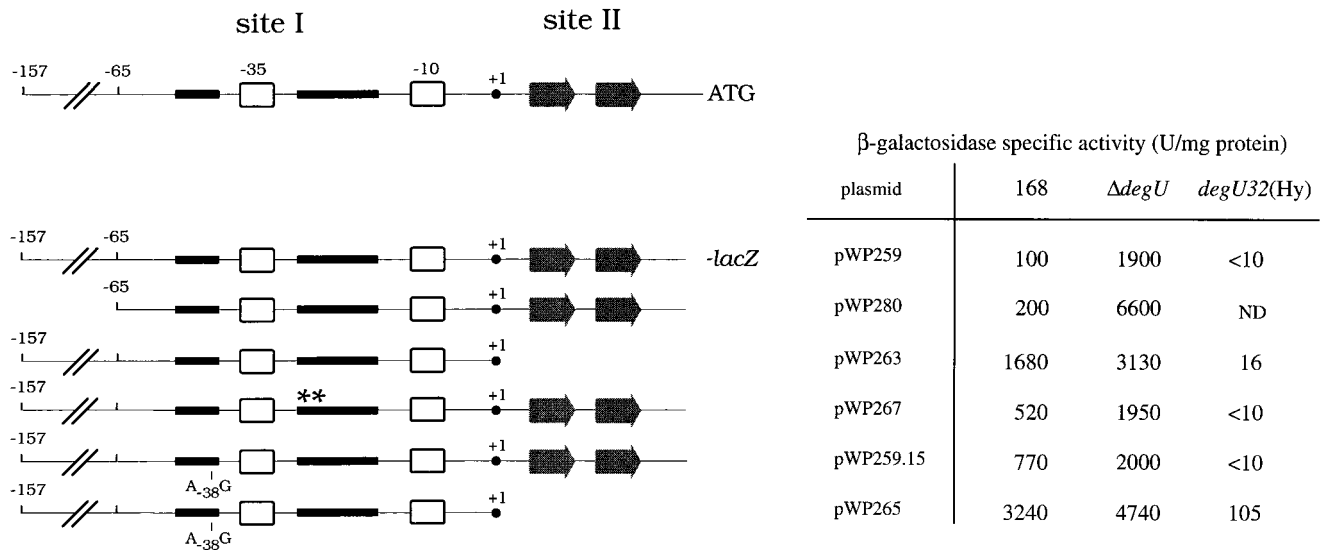


FIG. 6. Schematic representation of the *wapA* regulatory region together with the different constructs designed to alter or delete each one or both of the putative sites involved in repression by salt stress. Plasmid pWP259 contains the entire wild-type 5' regulatory region of *wapA* transcriptionally fused to *lacZ*. In pWP280, sequences located upstream of the two clusters of random mutations have been removed. Plasmid pWP263 is a pWP259 derivative that lacks the downstream site (site II). Plasmid pWP267 harbors the TT_{-22/-23}AA site-directed mutation, pWP259.15 carries the A₋₃₈G mutation isolated in site I by random mutagenesis, and pWP265 combines deletion of site II and mutation A₋₃₈G in site I. Each plasmid was integrated at the chromosomal *amyE* locus of the wild-type, *degU*, and *degU32*(Hy) strains. The corresponding β-galactosidase activities were determined in high-salt liquid cultures as described for Table 3 and are shown at the right. The data relative to the *wapA*[TT_{-22/-23}AA] mutant obtained by site-directed mutagenesis are shown for easier comparison.

upstream from the *comC*, *comG*, and *recA* genes (6), alignment of several promoters known to be controlled by the DegS-DegU system allowed the identification of the conserved sequence AGAAN₁₁TTCAG. It must be pointed out that in the case of *sacB*, the two detected motifs (Fig. 4) are centered around a 20-bp region shown to be strictly required for DegU-mediated activation, by successive 5' deletions in the control region of *sacB* (4a, 12). A computer search of the *B. subtilis* genome has revealed sequences that perfectly match the consensus presented above in promoter regions not known to be regulated by DegU to date, such as *rapA*, encoding an aspartate phosphatase that functions to prevent sporulation (24), and *paiB*, a negative regulator of sporulation and degradative enzyme synthesis (13). Since both *rapA* and *paiB* null mutants are able to sporulate in glucose-enriched medium (13, 22) as is the *degU32*(Hy) mutant, one could hypothesize that phosphorylated DegU lies upstream of *rapA* and *paiB* in the regulatory cascade leading to catabolite repression of sporulation.

Site-directed mutagenesis of the putative DegU target, by changing the two invariant T residues in the TTCAG motif to obtain AACAG, led to a partial derepression of *wapA* transcription in high-salt medium compared to the transcriptional levels of the wild-type *wapA'*-*lacZ* in a *degU* null mutant (about 1900 Miller units). Deleting *degU* in the wild-type or *wapA'* [TT_{-23/-22}AA] *wapA'*-*lacZ* strain had a similar effect, supporting the hypothesis that the elevated levels observed in strain QB4963 (*wapA*[TT_{-23/-22}AA]) were due to a decreased efficiency of DegU repression. At least two reasons can be envisioned to explain the partial effect of the TT_{-23/-22}AA mutation: (i) it may only slightly decrease the binding capability of DegU to the modified target or (ii) DegU could bind to some additional site(s) located in the *wapA* control region to further downregulate its transcription. The observation that DegU (Hy)32, a mutant version of DegU that exhibits a hyperstable phosphorylated state, remains able to fully repress transcription of *wapA* [TT_{-23/-22}AA] is compatible with either of these explanations. Complete elimination or drastic modification of

the putative target of DegU proved impossible since it overlaps the -35 box that specifies σ^A binding.

Random PCR mutagenesis of the control region of *wapA* confirmed the role of the putative DegU binding site (site I) in repression of *wapA* transcription at high salt concentrations and allowed the detection of a second site of repression located downstream from the transcription start site (site II [Fig. 5]). The relatively low occurrence of mutations that were obtained in site I could be due to possible interference of such mutations with the binding of σ^A to the -35 promoter box. Using constructs designed to alter site I and/or delete site II, we have shown that the negative control at the two sites is cumulative in the wild-type background as well as in the *degU* null mutant. This suggests the existence of a second mechanism of repression by salt stress occurring at site II and DegU independent, since deletion of site II causes an additional derepression in a *degU* background (compare β-galactosidase activities conferred by constructs pWP259 and pWP263 or by pWP259.15 and pWP265 in the *degU* strain). Furthermore, derepression observed in the absence of the downstream operator site proved to be salt specific and could not be observed at low salt concentrations (data not shown). It therefore seems unlikely that the removal of site II, located downstream from the transcription start site, generally affects transcription efficiency by altering the 5' untranslated sequence of the *wapA* transcript in pWP263 and derived plasmids. The hypothesis of a second repressor is also reinforced by the observation that transcriptional levels of *wapA'*-*lacZ* in low-salt medium (Fig. 1) are twofold higher than those observed at high salt concentrations in the absence of active DegU (Fig. 2), suggesting the existence of residual negative control in the absence of DegU.

However, the *degU32*(Hy)-encoded protein remained able to fully repress transcription of *wapA'*-*lacZ* fusions that carried either an intact or a modified site I (Fig. 6). In addition to the *wapA* mutant promoters presented in Fig. 6, regulation by the *degU32*(Hy)-encoded protein was examined on each PCR mutant in the putative operator site I (pWP259.21S, -13S, and

-32S [Fig. 5]). None of these mutations was able to eliminate or otherwise affect the ability of DegU(hy) to repress *wapA* transcription at high salt concentrations. Since DegU(Hy) only partially represses *wapA* at low salt concentrations (not shown), the effect of each mutation located in site I was examined in low-salt medium in the *degU32(Hy)* background. A slight resistance to DegU(Hy) repression could be observed with pWP259.21S and 32S compared to the wild-type promoter (twofold decrease), suggesting that at least those two mutations affect indeed the ability of DegU(Hy) to repress *wapA*. On the other hand, deletion of site II also appeared to slightly affect repression by the DegU(Hy) protein (Fig. 6). Although the significance of these last data might be questionable, the possibility that DegU itself is involved directly or indirectly in regulation at the downstream site cannot be ruled out. A computer search of the *B. subtilis* genome revealed sequences that match the TATTACN₃TATTAC motif upstream from *aprE*, *srfA*, and *sacX*, suggesting that the site I-site II combination may not be unique to the *wapA* regulatory region. Transposon mutagenesis will be undertaken in an attempt to identify an additional repressor and characterize the second mechanism of salt stress repression exerted on *wapA* in combination with DegU.

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