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# Function of a Principal $Na^+/H^+$ Antiporter, ShaA, Is Required for Initiation of Sporulation in *Bacillus subtilis*

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**ShaA (sodium/hydrogen antiporter, previously termed YufT [or NtrA]), which is responsible for $Na^+/H^+$ antiporter activity, is considered to be the major $Na^+$ excretion system in *Bacillus subtilis*. We found that a *shaA*-disrupted mutant of *B. subtilis* shows impaired sporulation but normal vegetative growth when the external $Na^+$ concentration was increased in a low range. In the *shaA* mutant, $\sigma^H$-dependent expression of *spo0A* ($P_S$) and *spoVG* at an early stage of sporulation was sensitive to external NaCl. The level of $\sigma^H$ protein was reduced by the addition of NaCl, while the expression of *spo0H*, which encodes $\sigma^H$, was little affected, indicating that posttranscriptional control of $\sigma^H$ rather than *spo0H* transcription is affected by the addition of NaCl in the *shaA* mutant. Since this mutant is considered to have a diminished ability to maintain a low internal $Na^+$ concentration, an increased level of internal $Na^+$ may affect posttranscriptional control of $\sigma^H$. Bypassing the phosphorelay by introducing the *sof-1* mutation into this mutant did not restore *spo0A* ($P_S$) expression, suggesting that disruption of *shaA* affects $\sigma^H$ accumulation, but does not interfere with the phosphorylation and phosphotransfer reactions of the phosphorelay. These results suggest that ShaA plays a significant role at an early stage of sporulation and not only during vegetative growth. Our findings raise the possibility that fine control of cytoplasmic ion levels, including control of the internal $Na^+$ concentration, may be important for the progression of the sporulation process.**

All living cells actively extrude sodium ions and maintain an inwardly directed gradient of sodium concentration (33). Sodium extrusion is important as a detoxification process, because internal sodium inhibits many metabolic activities when present at high concentrations (29, 41). The major $Na^+$-extruding mechanism in most bacterial cells is the $Na^+/H^+$ antiporter, which extrudes $Na^+$ in exchange for $H^+$ (32, 42). This process is driven by an electrochemical gradient of proton across the cytoplasmic membrane, which is established by the respiratory chain or the $H^+$-translocating ATPase (55). Besides its role in $Na^+$ extrusion, the $Na^+/H^+$ antiporter plays important roles in pH homeostasis (3, 32), cell volume regulation (17), and establishment of an electrochemical potential of $Na^+$ (33).

Most of the $Na^+/H^+$ antiporters reported to date are encoded by single genes (6, 26, 27, 39, 44, 49, 52, 53). However, recent reports have demonstrated the existence of a novel type of cation/$H^+$ antiporters usually encoded by a cluster of seven genes (20, 25, 45). Hiramatsu et al. (20) have recently reported the features of the *mnh* (multisubunit $Na^+/H^+$ antiporter) locus encoding an $Na^+/H^+$ antiporter in *Staphylococcus aureus*. All seven genes (*mnhA* to *mnhG*) are required for the antiporter activity, suggesting that the Mnh antiporter consists of seven kinds of subunits and forms a huge ion transport complex. Homologues of the *mnh* locus have been found in alkaliphilic *Bacillus* sp. strain C-125, *Rhizobium meliloti*, and *Bacillus subtilis*. These may be considered to be members of a multisubunit antiporter family. This gene family was first discovered in alkaliphilic *Bacillus* sp. strain C-125 (18). The homologue first identified in *Bacillus* sp. strain C-125 is related to the $Na^+/H^+$ antiporter and is required for pH homeostasis in an alkaline environment (18). The *pha* (pH adaptation) locus of *R. meliloti* contains a whole set of the seven corresponding genes and is required for invasion of nodule tissue to establish nitrogen-fixing symbiosis (45). *pha* mutants show sensitivity to $K^+$, but not to $Na^+$, in their growth and are deficient in diethanolamine-induced $K^+$ efflux (45). It seems that the *pha* locus in *R. meliloti* may encode a $K^+/H^+$ antiporter which is involved in pH adaptation during the infection process (45).

A whole set of the seven genes has also been found in the gram-positive endospore-forming *B. subtilis* (25). We have recently shown that disruption of the first gene, *yufT*, results in a decrease in $Na^+/H^+$ antiporter activity and impaired growth when the external sodium concentration is increased, indicating that *yufT* encodes a $Na^+/H^+$ antiporter which has a dominant role in the extrusion of cytotoxic sodium (31). Ito et al. have more recently shown that the same set of seven genes is transcribed as an operon and that the operon is responsible for cholate resistance and pH homeostasis as well as sodium resistance (25).

Endospore formation in *B. subtilis* has been extensively studied at the molecular level as a simple model system with which to understand cellular differentiation. Only a few studies have focused on the role of ions or ion transport in sporulation of *B. subtilis*. $Mn^{2+}$ and $Fe^{2+}$ are known to be essential for sporulation (5, 13), and transport of $Mn^{2+}$ and $Ca^{2+}$ is activated during sporulation (46). It has been shown as well that the activities of several proteins related to sporulation, including KinA (16), SpoIIE (11), and RapB (51), are dependent on divalent cations. However, little is known about the role of monovalent cations in sporulation. In the present study, we

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

| Strain | Relevant genotype | Source or reference |
|---|---|---|
| UOT1285 | *trpC2 lys 1 aprEΔ3 nprE18 nprR2* | Laboratory stock |
| SK6 | *trpC2 lys 1 aprEΔ3 nprE18 nprR2 shaA*::*neo* | This study |
| RIK10 | *trpC2 lys 1 aprEΔ3 nprE18 nprR2 amyE*::*spo0A* ($P_S$)-*bgaB cat* | 38 |
| RIK51 | *trpC2 lys 1 aprEΔ3 nprE18 nprR2 amyE*::*spoVG-bgaB cat* | 1 |
| RIK50 | *trpC2 lys 1 aprEΔ3 nprE18 nprR2 amyE*::*spo0H-bgaB cat* | 38 |
| SK610 | SK6 *amyE*::*spo0A* ($P_S$)-*bgaB cat* | SK6 DNA→RIK10[a] |
| SK651 | SK6 *amyE*::*spoVG-bgaB cat* | SK6 DNA→RIK51[a] |
| SK650 | SK6 *amyE*::*spo0H-bgaB cat* | SK6 DNA→RIK50[a] |
| RIK62 | *trpC2 lys 1 aprEΔ3 nprE18 nprR2 sof-1 spo0FΔS amyE*::*spo0A* ($P_S$)-*bgaB cat* | This study |
| SK662 | *trpC2 lys 1 aprEΔ3 nprE18 nprR2 sof-1 spo0FΔS amyE*::*spo0A* ($P_S$)-*bgaB cat shaA*::*neo* | SK6 DNA→RIK62[a] |

[a] RIK10, RIK51, RIK50, and RIK62 were each transformed with chromosomal DNA from strain SK6 to construct SK610, SK651, SK650, and SK662, respectively.

found that disruption of *yufT* leads to a diminished $Na^+$ excretion capacity (31), which entails sporulation defects, when the external sodium concentration is increased. To identify the stage at which the function of the $Na^+/H^+$ antiporter is required, we examined the expression of early sporulation genes in the *yufT* mutant, and our findings suggest the possibility that intracellular $Na^+$ levels may play a role in posttranscriptional regulation of $\sigma^H$, an alternative sigma factor required for an initial process in the course of sporulation in *B. subtilis*. This report is the first to provide evidence of a relationship between $Na^+$ and sporulation in *B. subtilis*.

We have previously proposed renaming *yufT* as *ntrA* ($Na^+$ transporter) (31). However, to avoid confusion with *ntr* of nitrogen regulation, we have again renamed the operon by including *yufT* as *sha* (sodium/hydrogen antiporter).

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The *B. subtilis* strains used in this study are listed in Table 1. All strains were derived from UOT1285, used as the wild-type strain in our laboratory. The *shaA*::*neo* allele contains a neomycin-resistant cassette inserted into the *Eco*RV site in *shaA* as described previously (31).

*B. subtilis* cells were routinely cultivated in LB1/2 (10 g of Difco tryptone, 5 g of Difco yeast extract, and 5 g of NaCl per liter [pH 7.0]) in the case of $shaA^+$ strains or LBK1/2 (10 g of Difco tryptone, 5 g of Difco yeast extract, and 5 g of KCl per liter [pH 7]) in the case of *shaA* mutant strains. Neomycin (7.5 μg/ml) and chloramphenicol (5 μg/ml) were added for selection when necessary. The nutrient sporulation medium used was modified 2× SG medium [16 g of Difco nutrient broth per liter, 1 g of KCl per liter, 1 mM $MgSO_4$, 1 mM $Ca(NO_3)_2$, 1 μM $FeSO_4$, 10 μM $MnCl_2$, 1 g of glucose per liter] (34). S7K minimal sporulation medium supplemented with 0.1% (wt/vol) glucose is identical to S7 medium (14), except that sodium glutamate was replaced with potassium glutamate. Cultures were incubated at 37°C with vigorous shaking. Growth was monitored by measuring the optical density at 660 nm ($OD_{660}$).

**Transformation of *B. subtilis*.** Competent *B. subtilis* cells were prepared and transformed by the method previously described (10). When isolating *shaA*::*neo* strains, cells were grown in modified CI (CIK) medium [14 g of $K_2HPO_4$ per liter, 6 g of $KH_2PO_4$ per liter, 2 g of $(NH_4)_2SO_4$ per liter, 1 g of tripotassium citrate dihydrate per liter, 5 mM $MgSO_4$, 5 g of glucose per liter, 50 μg of L-tryptophan per ml, 50 μg of L-lysine per ml, 1 g of yeast extract per liter] until early stationary phase. A 0.5-ml portion of the culture was centrifuged, and the cell pellet was resuspended in 1 ml of modified CII (CIIK) medium [14 g of $K_2HPO_4$ per liter, 6 g of $KH_2PO_4$ per liter, 2 g of $(NH_4)_2SO_4$ per liter, 1 g of tripotassium citrate dihydrate per liter, 5 mM $MgSO_4$, 5 g of glucose per liter, 25 μg of L-tryptophan per ml, 25 μg of L-lysine per ml, 0.5 g of yeast extract per liter]. A 0.1-ml portion of the suspension of competent cells was mixed with DNA and incubated at 37°C for 90 min with shaking. Then, 0.3 ml of CIK medium was added, and the mixture was further incubated for 60 min. An appropriate volume of the culture was then spread on a CIK plate containing 7.5 μg of neomycin per ml.

**Assay of spore formation.** Cells were grown in 2× SG medium, and spores were assayed 22 h after the end of the exponential phase ($T_{22}$). The number of viable cells per ml of culture was determined as the total number of CFU on LB1/2 ($shaA^+$ strains) or LBK1/2 (*shaA* mutant strains) plates. The number of spores per ml of culture was determined as the number of CFU after heat treatment (80°C, 10 min).

**Assay of β-galactosidase activity.** The expression of transcriptional *bgaB* fusions was monitored by measuring thermostable β-galactosidase activity (21, 22). Cells were grown in 2× SG medium, and 50- to 500-μl samples of the cultures were removed at appropriate times for the assay. The cell pellets were toluenized in 0.5 ml of Z buffer (60 mM $Na_2HPO_4$, 40 mM $NaH_2PO_4$, 10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol). After preincubation at 62°C, reactions were started by adding 0.2 ml of 4 mg of *o*-nitrophenyl-β-D-galactoside (ONPG) per ml to the samples and stopped by adding 0.5 ml of 1 M $Na_2CO_3$. After centrifugation, the $A_{420}$ of the supernatants was measured. The specific activity was expressed as 1,000 × ($A_{420}$ per min of incubation per ml of culture per $OD_{660}$ unit).

**Western blot analysis.** Western blot analysis was performed as described by Asai et al. (2). Cells were grown in 2× SG medium, and the same amount of cells (the culture volume × $OD_{660}$ = 5) was collected in each instance at appropriate times. The cell pellets were resuspended in 50 μl of lysis buffer A (50 mM Tris-HCl, 1 mM EDTA, 50 g of glycerol per liter, 0.1 M NaCl, 0.1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 2 mg of lysozyme per ml [pH 7]) and incubated at 37°C for 6 min to allow cell lysis to occur. Then, 1.5 μl of lysis buffer B (330 mM $MgCl_2$, 6.6 mg of DNase I per ml, 16.6 mg of RNase A per ml) was added, and the mixture was further incubated for 6 min. Aliquots of the whole-cell extracts (15 μg of total protein) were diluted in sodium dodecyl sulfate (SDS) sample buffer, boiled for 5 min, and subjected to electrophoresis on an SDS–12% polyacrylamide gel. Thereafter, the proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad) by using a Mini Trans-Blot transfer cell (Bio-Rad) in a semidry condition. The blotted membrane was incubated with primary anti-$\sigma^H$ antibody (2) and secondary goat anti-rabbit immunoglobulin G alkaline phosphatase conjugate (Sigma) and finally reacted with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium salt (both supplied by Boehringer Mannheim) to detect signals.

## RESULTS

**Sporulation of the *shaA* mutant is impaired with an increase in external sodium concentration.** As shown previously, the *shaA*-disrupted strain shows diminished $Na^+/H^+$ antiport activity and impaired growth when the external NaCl concentration was increased, and it is therefore considered to have a diminished $Na^+$ excretion capacity (31). A 2× SG sporulation medium containing 0.1% glucose was found to have a pH of 6.2 to 6.4, and it contained 18 mM $Na^+$ as a contaminant, as determined by atomic absorption analysis. Under these conditions, SK6, a *shaA*-disrupted derivative of UOT1285, grew exponentially at a rate almost equal to that of the wild type, as shown in Fig. 1. This finding was consistent with results reported previously (31). As shown in Table 2, the numbers of viable cells at $T_{22}$ were the same when comparing SK6 and the wild type (both ~$10^8$ cells/ml). The number of spores formed at $T_{22}$, however, was somewhat lower in the case of SK6 (~$10^7$ spores/ml) than that of the wild type (~$10^8$ spores/ml), as shown in Table 2. When 30 mM NaCl was added to the medium, sporulation of SK6 was severely affected, resulting in less than 10 spores per ml (Table 2), whereas vegetative growth of SK6 was little affected (Fig. 1). On the other hand, addition of NaCl at concentrations up to 200 mM affected neither vegetative growth nor sporulation of the wild type (Table 2) (some data not shown).

We considered that 18 mM $Na^+$ present as a contaminant in the 2× SG medium may have weakly inhibited the sporulation

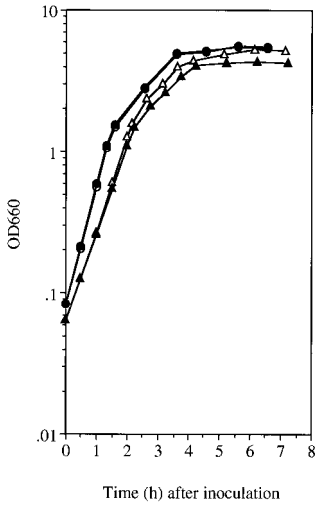


FIG. 1. Growth in 2× SG medium containing 0.1% glucose. Strains UOT1285 (*shaA*$^+$ [circles]) and SK6 (*shaA*::*neo* [triangles]) were grown at 37°C in the absence (open symbols) and presence (solid symbols) of 30 mM NaCl. Growth was monitored by measuring the $OD_{660}$.

of SK6. In order to eliminate the effect of the contaminating $Na^+$, the sporulation of SK6 was also examined in S7K medium, which contained less than 1 mM $Na^+$ as a contaminant. SK6 produced $10^8$ spores per ml, the same level as that of the wild type, in S7K medium without added NaCl. The sporulation of SK6 was clearly impaired when the external NaCl concentration was increased. When 50 mM NaCl was added to S7K medium, the SK6 cells reached stationary phase at a lower cell density (~$10^7$ cells per ml versus ~$10^8$ cells per ml without NaCl), and produced $10^5$ spores per ml, 1,000-fold less than that in the absence of added NaCl. Moreover, the addition of 100 mM NaCl severely blocked the sporulation of SK6 (Table 2). The effect of the added NaCl on SK6 sporulation was apparently due to the increase in $Na^+$ concentration, rather than being a nonspecific effect of increased ionic strength and/or increased osmolarity, because 50 mM KCl had no effect on sporulation (Table 2) and 25 mM $Na_2SO_4$ reduced the spore titer to ~$10^5$ per ml, the same level as that observed in the presence of 50 mM NaCl (data not shown). The effect of NaCl on sporulation of SK6 was more severe in 2× SG medium than in S7K medium. Both 2× SG medium with 30 mM NaCl added and S7K medium with 50 mM NaCl contain ~50 mM $Na^+$; however, SK6 produced less than 10 spores in the former and ~$10^5$ spores in the latter.

The primary environmental signal for initiation of sporulation is nutrient depletion (12). The severe effects on sporulation are often linked to a decrease in growth rate or growth yield. The vegetative growth rate of SK6 was little affected by the addition of 30 mM NaCl (Fig. 1), but we cannot exclude the possibility that the cells do not fully metabolize components of the medium that may act as inhibitors of sporulation. To exclude this possibility, we examined whether the sporulation of SK6 is inhibited by NaCl when added after the end of exponential growth ($T_0$). SK6 cells were grown in 2× SG medium without added NaCl, and NaCl was added at a final concentration of 30 mM at the times indicated in Fig. 2. The sporulation of SK6 was severely inhibited by NaCl when added just at $T_0$ or before $T_3$ (3 h after the end of exponential growth), but was not affected when added at $T_3$ and after. These results clearly indicate that the addition of 30 mM NaCl specifically affects sporulation events in SK6 which precede $T_3$, but not vegetative growth.

In 2× SG medium with 30 mM NaCl added, freshly isolated SK6 produced less than 10 spores, but the strain produced $10^4$ to $10^6$ spores under the same conditions after the cells had been repeatedly cultured on LBK1/2 (including 7.5 μg of neomycin per ml) plates. There is a possibility that additional mutations occurred in the repeatedly cultured SK6 cells. Thus, we always used cells freshly prepared from a stock culture of this strain in the following experiments.

**Induction of *spo0A* ($P_S$) expression at the onset of sporulation is blocked by external NaCl in the *shaA* mutant.** Whether or not *B. subtilis* cells initiate sporulation is believed to be decided by the intracellular level of the phosphorylated form of Spo0A (Spo0A-P), which is the key transcription factor that regulates early sporulation genes positively or *abrB* negatively (23). Phosphorylation of Spo0A occurs through a multicomponent phosphorelay that involves three sensor kinases (KinA,

TABLE 2. Sporulation of the *shaA* mutant and the wild type

| Strain | Relevant genotype | Medium[a] | No. (CFU/ml) of: | | Frequency (%) |
|---|---|---|---|---|---|
| | | | Viable cells | Spores | |
| UOT1285 | *shaA*$^+$ | 2× SG | $1.3 \times 10^9$ | $5.3 \times 10^8$ | 41 |
| UOT1285 | *shaA*$^+$ | 2× SG + 30 mM NaCl | $7.8 \times 10^8$ | $5.0 \times 10^8$ | 64 |
| UOT1285 | *shaA*$^+$ | 2× SG + 50 mM NaCl | $7.7 \times 10^8$ | $5.5 \times 10^8$ | 71 |
| SK6 | *shaA*::*neo* | 2× SG | $5.2 \times 10^8$ | $1.1 \times 10^{7b}$ | 2.1 |
| SK6 | *shaA*::*neo* | 2× SG + 30 mM NaCl | $4.5 \times 10^8$ | <10 | $<2 \times 10^{-6}$ |
| SK6 | *shaA*::*neo* | 2× SG + 50 mM NaCl | $1.4 \times 10^8$ | <10 | $<7 \times 10^{-6}$ |
| UOT1285 | *shaA*$^+$ | S7K | $3.6 \times 10^8$ | $2.5 \times 10^8$ | 69 |
| UOT1285 | *shaA*$^+$ | S7K + 20 mM NaCl | $3.6 \times 10^8$ | $2.1 \times 10^8$ | 58 |
| UOT1285 | *shaA*$^+$ | S7K + 50 mM NaCl | $3.4 \times 10^8$ | $2.0 \times 10^8$ | 59 |
| UOT1285 | *shaA*$^+$ | S7K + 100 mM NaCl | $4.1 \times 10^8$ | $2.0 \times 10^8$ | 49 |
| UOT1285 | *shaA*$^+$ | S7K + 50 mM KCl | $3.0 \times 10^8$ | $2.7 \times 10^8$ | 90 |
| SK6 | *shaA*::*neo* | S7K | $3.0 \times 10^8$ | $1.1 \times 10^8$ | 37 |
| SK6 | *shaA*::*neo* | S7K + 20 mM NaCl | $3.2 \times 10^8$ | $3.5 \times 10^7$ | 11 |
| SK6 | *shaA*::*neo* | S7K + 50 mM NaCl | $1.1 \times 10^7$ | $6.8 \times 10^5$ | 6.2 |
| SK6 | *shaA*::*neo* | S7K + 100 mM NaCl | $1.5 \times 10^7$ | $5.0 \times 10$ | $2.9 \times 10^{-4}$ |
| SK6 | *shaA*::*neo* | S7K + 50 mM KCl | $2.2 \times 10^8$ | $1.3 \times 10^8$ | 59 |

[a] The 2× SG liquid medium (0.1% glucose) contains 18 mM contaminating $Na^+$. The S7K liquid medium (0.1% glucose) contains less than 1 mM contaminating $Na^+$.

[b] The spore production fluctuated between $10^5$ and $10^7$.

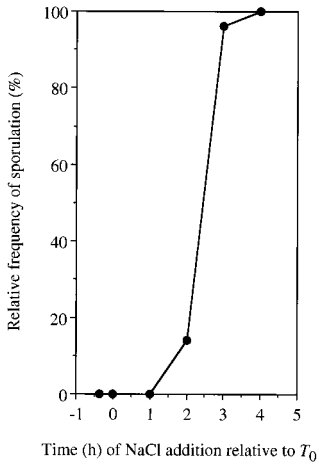


FIG. 2. Effect of NaCl on sporulation of a *shaA*::*neo* strain. SK6 (*shaA*::*neo*) cells were grown in 2× SG medium containing 0.1% glucose without added NaCl, and 30 mM NaCl was added to the medium at the indicated times after the end of exponential growth ($T_0$). Relative frequency of sporulation is the spore production per milliliter relative to that of SK6 when grown in 2× SG without added NaCl ($4.8 \times 10^6$ spores/ml).

KinB, and KinC), a response regulator (Spo0F), and a phosphotransferase (Spo0B) (4). Transcription of *spo0A* is regulated by two promoters, $P_V$ and $P_S$, which are, respectively, recognized by E-$\sigma^A$ during vegetative growth and by E-$\sigma^H$ at an early stage of sporulation (7). Transcription of *spo0A* from the $P_S$ promoter also requires its own product, Spo0A-P (47). Since sporulation of SK6 was impaired when the external $Na^+$ concentration was increased, it seemed likely that one or more steps in the sporulation process may become sensitive to $Na^+$. We first examined the effect of the disruption of *shaA* on the expression of *spo0A* from $P_S$ at the time of the onset of sporulation.

As shown in Fig. 3A, transcription of *spo0A* from $P_S$ was induced in the wild type 1 h after $T_0$ in the presence or absence of 30 mM NaCl. On the other hand, in the absence of 30 mM NaCl, the rate of *spo0A* ($P_S$) induction was lower in the *shaA* mutant than that of the wild type. Moreover, *spo0A* ($P_S$) induction was almost completely blocked in the *shaA*-disrupted mutant by the addition of 30 mM NaCl. The expression of *spo0A* ($P_S$) in the *shaA* mutant was also blocked by 30 mM NaCl when added just at $T_0$, the same as when the mutant was grown in 2× SG with 30 mM NaCl (data not shown). It is, therefore, most likely that the *shaA* mutation blocks spore development by affecting the formation of Spo0A-P and/or the active $\sigma^H$-containing RNA polymerase at an early stage of sporulation.

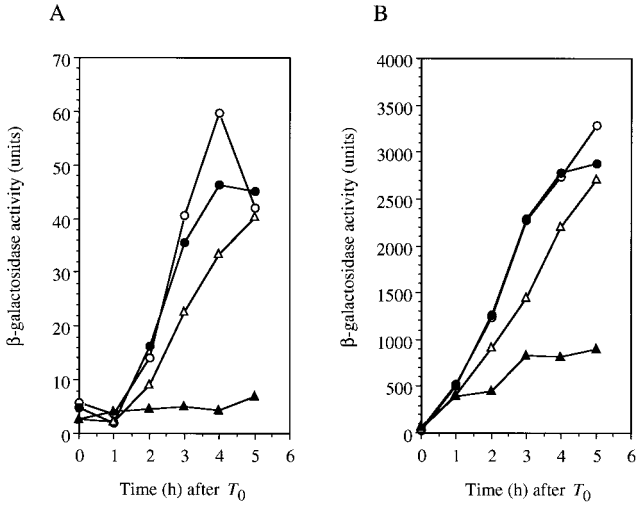


FIG. 3. Expression of $\sigma^H$-dependent genes in *shaA*$^+$ (RIK10 and RIK51) and *shaA*::*neo* (SK610 and SK651) strains using the *bgaB* gene coding for a heat-stable β-galactosidase as a reporter. Cells of *shaA*$^+$ (circles) and *shaA*::*neo* (triangles) strains carrying a *spo0A* ($P_S$)-*bgaB* (A) or *spoVG*-*bgaB* (B) fusion were grown at 37°C in 2× SG medium containing 0.1% glucose in the absence (open symbols) and presence (solid symbols) of 30 mM NaCl. Samples were taken at the indicated time to determine the extent of growth and to measure β-galactosidase activity as described in Materials and Methods.

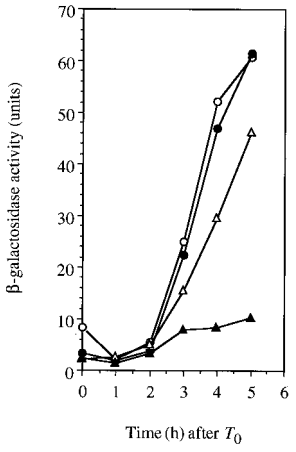


FIG. 4. Effect of the *sof-1* mutation on *spo0A* ($P_S$) transcription in a *shaA*::*neo* strain. Cells carrying a *spo0A* ($P_S$)-*bgaB* fusion were grown at 37°C in 2× SG medium containing 0.1% glucose in the absence (open symbols) and presence (solid symbols) of 30 mM NaCl. Circles, RIK62 (*sof-1 spo0FΔS shaA*$^+$); triangles, SK662 (*sof-1 spo0FΔS shaA*::*neo*).

**The effect of the *sof-1* mutation on the *shaA* mutant.** Spo0A-P is generated through an active phosphorelay system, and it induces the transcription of the components of the system, *spo0F* as well as *spo0A* itself, from their $\sigma^H$-dependent $P_S$ promoters at the time of the onset of sporulation (47, 48). This allows the stimulation of the phosphorelay in a positive feedback manner and then an increase in the level of Spo0A-P at an early stage of sporulation (48). Since the addition of 30 mM NaCl severely blocked the induction of *spo0A* ($P_S$) expression in the *shaA* mutant, it may be that the phosphorelay becomes stagnant under these conditions because of an insufficient supply of Spo0A protein. If disruption of *shaA* affects Spo0A-P production by inhibiting the phosphorelay, it is expected that *spo0A* ($P_S$)-*bgaB* expression would be restored when Spo0A is phosphorylated independent of the phosphorelay. We therefore introduced the *sof-1* mutation (24, 28), which is a mutation within the *spo0A* locus and which serves to bypass the need for *spo0F* in the phosphorylation of Spo0A, into the *shaA* mutant and examined whether *spo0A* ($P_S$) induction was restored or not in the presence or absence of 30 mM NaCl.

As shown in Fig. 4, the rate of *spo0A* ($P_S$) induction in the *shaA sof-1* double mutant was lower than that in the wild type in the absence of added NaCl, and the induction was blocked by the addition of 30 mM NaCl. Moreover, sporulation of the *shaA sof-1* double mutant occurred with the same yield of spores produced as that in the case of the *shaA* mutant (~$10^5$ to $10^7$ spores per ml in 2× SG medium and less than 10 spores per ml in 2× SG medium with 30 mM NaCl at $T_{22}$).

**A posttranscriptional regulation of $\sigma^H$ is affected in the *shaA* mutant.** The finding that the *sof-1* mutation failed to

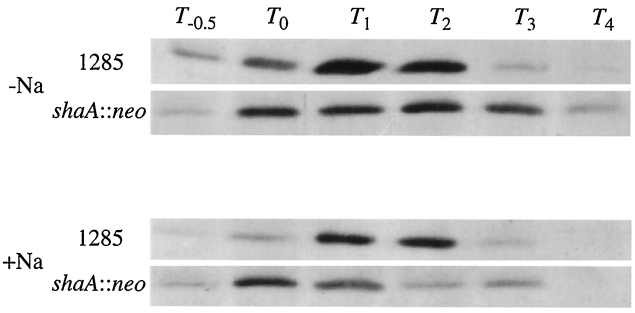


FIG. 5. Western blot analysis of $\sigma^H$ protein from whole-cell extracts of UOT1285 (*shaA*$^+$) and SK6 (*shaA*::*neo*) cells. Cells were grown at 37°C in 2× SG medium containing 0.1% glucose with or without 30 mM NaCl and harvested at the time indicated. Aliquots of cell lysates (15 μg of total protein) were electrophoresed and analyzed as described in Materials and Methods.

restore *spo0A* ($P_S$) induction in the *shaA* mutant suggested that the defect in *spo0A* ($P_S$) expression in the *shaA* mutant may involve the loss of $\sigma^H$ function. We therefore examined the expression of another $\sigma^H$-dependent gene, *spoVG*, in the *shaA* mutant. As shown in Fig. 3B, induction of *spoVG* expression was severely inhibited by the addition of 30 mM NaCl, as in the case of *spo0A* ($P_S$) expression. These results clearly showed that disruption of *shaA* resulted in $Na^+$ sensitivity of $\sigma^H$-dependent transcription during sporulation.

It is expected that the defects in $\sigma^H$-dependent transcription are the result of a decrease in the intracellular level of $\sigma^H$ protein and/or a decrease in E-$\sigma^H$ transcription activity. We therefore assayed the level of $\sigma^H$ protein in extracts of cells grown in 2× SG medium in the presence or absence of 30 mM NaCl by Western blot analysis with anti-$\sigma^H$ antibody. As shown in Fig. 5, the level of $\sigma^H$ in the wild-type cells began to increase at $T_0$, reached a maximum at $T_2$, and then rapidly decreased after $T_3$, which was in good agreement with results reported previously (38, 40). On the other hand, the level of $\sigma^H$ in the *shaA* mutant increased from $T_0$ through $T_2$ and began to decrease at $T_3$ in the absence of added NaCl. The $\sigma^H$ level at $T_1$ and $T_2$ was slightly lower than that of the wild type, which may explain the lower rate of induction of the $\sigma^H$-dependent genes. In the presence of 30 mM NaCl, $\sigma^H$ accumulated at $T_0$, but the level of $\sigma^H$ decreased after $T_1$ in the *shaA* mutant. The addition of NaCl did not affect $\sigma^H$ accumulation in the wild type. Thus, we concluded that the defects in $\sigma^H$-dependent transcription in the *shaA* mutant are the result of impaired accumulation of $\sigma^H$ protein at an early stage of sporulation.

It has been shown that the intracellular level of $\sigma^H$ protein increases at the onset of sporulation through both transcriptional and posttranscriptional mechanisms, and then the $\sigma^H$-dependent genes are induced (2, 19, 54). Since the addition of NaCl inhibited the accumulation of $\sigma^H$ in the *shaA* mutant, we next determined the level of expression of *spo0H*, which encodes $\sigma^H$. Its promoter is recognized by E-$\sigma^A$ and is repressed by AbrB during the vegetative phase (54). As shown in Fig. 6, in the absence of added NaCl, the induction of *spo0H* in the *shaA* mutant showed normal timing and the level of expression of *spo0H* was somewhat higher than that in the wild type. In the presence of 30 mM NaCl, the *spo0H* expression level in the *shaA* mutant was lower than that in the absence of NaCl, but it was not significantly lower compared with that in the wild type. In particular, the induction rates from $T_0$ through $T_2$ were almost the same for the *shaA* mutant and the wild type. These results indicated that the decrease in the $\sigma^H$ level in the *shaA* mutant that occurred with the addition of NaCl was not due to a decrease in *spo0H* transcription. These results suggested that posttranscriptional regulation of $\sigma^H$ was mainly affected in the *shaA* mutant by the addition of 30 mM NaCl.

## DISCUSSION

We showed here that disruption of the $Na^+/H^+$ antiporter, ShaA, results in a $Na^+$-sensitive sporulation-deficient phenotype. It is not the growth defect in the *shaA* mutant that causes the sporulation defect. Since ShaA is the major $Na^+$ extrusion mechanism in *B. subtilis* (31), the *shaA*-disrupted mutant is considered to have a diminished ability to maintain a low internal sodium concentration. The defect in sporulation resulting from the disruption of *shaA* is, therefore, considered to be due to inhibition of some steps in the sporulation process by an increased level of internal $Na^+$. Furthermore, such steps sensitive to $Na^+$ seem to be limited at early stages (before $T_3$).

To understand which step or steps in sporulation are affected by $Na^+$, we examined the expression of early sporulation genes in the *shaA* mutant. We found that the $\sigma^H$-dependent expression of both *spo0A* ($P_S$) and *spoVG* was severely affected in the *shaA* mutant by the addition of 30 mM NaCl. At the same time, the level of $\sigma^H$ protein was diminished in the mutant cells. The accumulation of $\sigma^H$ protein was already observed at $T_0$ in SK6 cells, in the presence or absence of 30 mM NaCl. Asai et al. (2) found a similar effect on $\sigma^H$ accumulation when glucose and glutamine were in excess. Introduction of the *sof-1* mutation into the *shaA* mutant to bypass the phosphorelay did not restore *spo0A* (Ps) expression, suggesting that the primary defect in sporulation conferred by the *shaA*::*neo* mutation is not directly related to phosphorylation of Spo0A.

It is likely that $\sigma^A$-dependent transcription is not affected by the *shaA* mutation, as shown by the absence of any defect in the vegetative growth. The fact that *spo0H* transcription, which depends on $\sigma^A$, was not affected in the *shaA* mutant shows that the *shaA* mutation affects some steps in $\sigma^H$ control subsequent to *spo0H* transcription. The expression of *spo0H* is repressed by the repressor AbrB during the vegetative phase of growth and is induced at the onset of sporulation by repression of *abrB* transcription by Spo0A-P (54). Since less Spo0A-P is needed for the repression of *abrB* than for the activation of *spo0A* ($P_S$) or stage II genes (8, 37, 50), it is likely that a small amount of

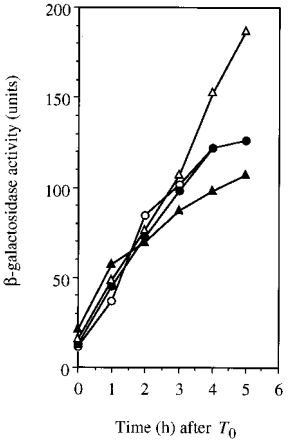


FIG. 6. Expression of a *spo0H-bgaB* transcriptional fusion in *shaA*$^+$ (RIK50 [circles]) and *shaA*::*neo* (SK650 [triangles]) strains. Cells were grown at 37°C in 2× SG medium containing 0.1% glucose in the absence (open symbols) and presence (solid symbols) of 30 mM NaCl.

Spo0A-P, but enough to allow *spo0H* expression through the AbrB pathway, would be produced in the *shaA* mutant.

The existence of a posttranscriptional regulatory mechanism(s) governing the level of $\sigma^H$ has been clearly demonstrated (9, 15, 19), but the detailed features of the mechanism remain to be elucidated. Liu et al. (35) have recently reported that $\sigma^H$ is subject to additional levels of posttranslational control involving the ATP-dependent protease Lon and the regulatory ATPase ClpX. Ohashi et al. (40) have recently isolated *spo0H*(Ts) mutants in which the level of $\sigma^H$ is decreased at the nonpermissive temperature. Based on the finding that mutations within *rpoB* encoding the β subunit of RNA polymerase restore the level of $\sigma^H$ in these temperature-sensitive mutants, they suggest that holoenzyme formation contributes to the stabilization of $\sigma^H$ at the start of sporulation (40). Our observation that an increased level of internal $Na^+$ may affect posttranscriptional regulation of $\sigma^H$ leads us to speculate that a reaction(s) or interaction(s) sensitive to $Na^+$ may be involved in the posttranscriptional regulation of $\sigma^H$. It seems that $\sigma^H$ is unable to stably associate with RNA polymerase to form the holoenzyme under conditions in which the internal $Na^+$ concentration is high. Alternatively, the impaired accumulation of $\sigma^H$ in the *shaA* mutant may be the consequence of abnormal induction of the Lon- or ClpX-dependent proteolytic regulation of $\sigma^H$ under conditions in which the internal $Na^+$ concentration is high.

There is another report that offers an important suggestion concerning posttranscriptional control of the $\sigma^H$ protein. Cosby and Zuber (9) showed that an increase in the pH of the culture medium from ~5 to 7 results in an increase in the level of expression of $\sigma^H$-dependent genes and, thus, an increase in the level of $\sigma^H$ protein, whereas *spo0H* transcription is not fully increased, under conditions in which glucose and glutamine are present in excess. An active tricarboxylic acid (TCA) cycle seems to be required for the induction of $\sigma^H$-dependent gene expression caused by elevation of the pH (9). It is unknown, however, how elevation of the external pH causes an increase in the internal $\sigma^H$ level. We think that the increase in pH itself rather than the absolute value of external pH may be important in stabilization of $\sigma^H$ based on the following considerations. Cytoplasmic pH is not maintained entirely constant, but it changes far less than the external pH; that is, there is substantial pH homeostasis (3, 32, 43). Since the magnitude of the proton motive force (Δp) is relatively independent of external pH, the composition of Δp (the chemical component ΔpH versus the electrical component $\Delta\Psi$) has to change in response to external pH to provide such pH homeostasis (43). Under acidic conditions, a large inwardly directed ΔpH mainly contributes to Δp (30). Thus, it is likely that transient elevation of the external pH under the above conditions would result in a decrease in ΔpH, and, therefore, Δp. As a consequence, the TCA cycle and respiration would be activated in order to support recovery of the Δp. The improved Δp would activate several secondary membrane transporters, including $Na^+/H^+$ antiporters, and thereby change internal ion levels, which would be expected to result in the stabilization of $\sigma^H$. Thus, the results reported by Cosby and Zuber (9) indicating that an increase in external pH results in stabilization of $\sigma^H$ and our results indicating that disruption of *shaA* leads to an increased level of internal $Na^+$ which affects the accumulation of $\sigma^H$ may raise the possibility that posttranscriptional regulation of $\sigma^H$ is influenced by internal ion levels, including the internal pH and/or the internal $Na^+$ concentration.

Many studies on bacterial $Na^+/H^+$ antiporters have focused on pH homeostasis or sodium extrusion during vegetative growth. We have shown here that the function of the ShaA antiporter is required for the initiation of sporulation. Considering that a subtle change in internal pH and/or ion concentrations can alter cellular reactions or interactions, fine control of cytoplasmic ion levels is likely to be very important for cellular functions that involve many complicated reactions and steps, such as sporulation.

It has been shown recently that *shaA* and the six genes adjacent to it are transcribed as an operon (25). According to our preliminary findings in primer extension analyses, three bands of reverse transcripts were detected, suggesting that there are three transcriptional start sites in the regulatory region of the *sha* operon (unpublished results). In the region upstream of each start site, two apparent $\sigma^A$-dependent promoters and a $\sigma^B$-like promoter were found. However, the primer-extended band corresponding to the $\sigma^B$-like promoter did not disappear in the case of a *sigB*-null mutant, suggesting that expression of the *sha* operon may depend on other sigma factors, such as the extracytoplasmic function-type sigma factors (36). The regulatory mechanism controlling transcription of the *sha* operon during both vegetative growth and sporulation is the subject of future study.

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