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Responses of *Bacillus subtilis* to Hypotonic Challenges: Physiological Contributions of Mechanosensitive Channels to Cellular Survival[▽]

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Mechanosensitive channels are thought to function as safety valves for the release of cytoplasmic solutes from cells that have to manage a rapid transition from high- to low-osmolarity environments. Subsequent to an osmotic down-shock of cells grown at high osmolarity, *Bacillus subtilis* rapidly releases the previously accumulated compatible solute glycine betaine in accordance with the degree of the osmotic downshift. Database searches suggest that *B. subtilis* possesses one copy of a gene for a mechanosensitive channel of large conductance (*mscL*) and three copies of genes encoding proteins that putatively form mechanosensitive channels of small conductance (*yhdY*, *yfkC*, and *ykuT*). Detailed mutational analysis of all potential channel-forming genes revealed that a quadruple mutant (*mscL yhdY yfkC ykuT*) has no growth disadvantage in high-osmolarity media in comparison to the wild type. Osmotic down-shock experiments demonstrated that the MscL channel is the principal solute release system of *B. subtilis*, and strains with a gene disruption in *mscL* exhibited a severe survival defect upon an osmotic down-shock. We also detected a minor contribution of the SigB-controlled putative MscS-type channel-forming protein YkuT to cellular survival in an *mscL* mutant. Taken together, our data revealed that mechanosensitive channels of both the MscL and MscS types play pivotal roles in managing the transition of *B. subtilis* from hyper- to hypo-osmotic environments.

The gram-positive soil bacterium *Bacillus subtilis* has to frequently manage the transition from high- to low-osmolarity surroundings due to drying and flooding of the upper layers of the soil (7, 35). To fend off the negative effects of high osmolarity on cellular water content and cell physiology, *B. subtilis* accumulates compatible solutes through either synthesis or uptake from the environment (7, 20). Compatible solutes are highly soluble organic osmolytes (e.g., proline and glycine betaine) that can be amassed by the cell to exceedingly high levels without interfering with cell physiology (12). In this way, the water content of the cell is balanced with the prevalent osmolarity of the environment and cell turgor is stabilized. Consequently, cell growth can occur under osmotically unfavorable conditions.

The massive accumulation of compatible solutes permits cell survival under hypertonic circumstances. However, the very same compounds become a threat to the integrity of the cell when *B. subtilis* is suddenly exposed to low-osmolarity surroundings via rainfall or washout into fresh water sources (8, 35). The compatible solutes accumulated by the cell increase the osmotic potential of the cytoplasm and thereby instigate an osmotically controlled water influx (36). This water influx drives up turgor to nonphysiological high values and in extreme cases can lead to cell lysis (4, 6). Various researchers have observed that a number of cytoplasmic solutes, including proline, glycine betaine, potassium, glutamate, trehalose, and ATP, are rapidly released from osmotically downshifted cells (3, 9, 29, 30). It is generally thought that mechanosensitive channels located in the cytoplasmic membrane are responsible for the rapid release of the various compounds. Mechanosensitive channels thus appear to act as safety valves against a strong buildup of turgor, and their gating activities prevent cell lysis upon a severe osmotic down-shock (4, 5, 22, 25, 27).

The best-characterized bacterial mechanosensitive channels are the channels of large conductance (MscL) and small conductance (MscS): for both of these, crystal structures have been obtained (2, 10). In response to changes in the lipid bilayer tension, the MscL and MscS channel proteins gate and they open transiently to form large aqueous pores, through which both solutes and solvents can pass rapidly. Consequently, the concentration of water-attracting solutes inside the cells is lowered, turgor is reduced, and cell lysis is prevented (5, 22, 25). The structural genes for the MscL and MscS channel-forming proteins have been initially identified in *Escherichia coli* (21, 31), and homologues of these genes have been found in a variety of microbial species (27). Disruption of either the *E. coli mscL* or the *mscS* gene displays no obvious phenotype, but in an elegant study, Levina et al. (21) demonstrated that an *mscL mscS* double mutant cannot effectively manage the transition from hyperosmotic to hypo-osmotic conditions and most of the cells lyse.

Mechanosensitive channel activity in *B. subtilis* was originally discovered by Zoratti et al. (37) and Szabo et al. (32), using patch clamp experiments with giant protoplasts. Evidence for the presence of ion channels in the *B. subtilis* plasma membrane was also obtained by Alcayaga et al. (1) in electrophysiological studies using fusions of plasma membrane vesicles with planar lipid bilayers. The channels detected in these studies showed a wide range of conductances and kinetic behaviors, suggesting that more than one channel-forming protein operates in *B. subtilis*. The activity of the stretch-activated channels of *B. subtilis* could be inhibited by gadolinium ions (3), an

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effective blocker of stretch-activated channels in eucaryotes. The *B. subtilis* genome contains one copy of the *mscL* gene. Heterologous expression of *mscL* in *E. coli* and analysis of the recombinant strain by patch clamp studies revealed that the *B. subtilis mscL* gene encodes a functional mechanosensitive channel (24). Recent data by Wahome and Setlow (34) strongly suggest an important role for the MscL channel in the management of the transitions of log-phase cells of *B. subtilis* from high- to low-osmolarity surroundings. Database searches conducted by Pivetti et al. (27) revealed the existence of three genes (*yfkC*, *ydhY*, and *ykuT*) in the *B. subtilis* genome whose deduced protein products display homology to the *E. coli* MscS protein (21). No functional analysis of the *yfkC*, *ydhY*, and *ykuT* genes in the *B. subtilis* osmoadaptation response has been reported.

To study the adaptation reaction of *B. subtilis* in response to a rapid transition from hyperosmotic to hypo-osmotic circumstances, we constructed a comprehensive set of single and multiple gene disruption mutations in the *mscL* gene and the three genes (*yfkC*, *ydhY*, and *ykuT*) encoding the putative mechanosensitive channels of small conductance (MscS type) and analyzed their properties.

MATERIALS AND METHODS

Media, growth conditions, and chemicals. The various *B. subtilis* strains were grown in Spizizen's minimal medium (SMM), with 0.5% (wt/vol) glucose as the carbon source. The medium was supplemented with L-tryptophan (20 mg liter^{-1}), L-phenylalanine (18 mg liter^{-1}), and a solution of trace elements (16). Cells were routinely grown aerobically at 37°C in 100-ml Erlenmeyer flasks with a culture volume of 20 ml in a shaking water bath set at 230 rpm. The growth of the bacterial cultures was monitored spectrophotometrically at a wavelength of 578 nm (OD_{578}). The salinity of bacterial cultures was raised by adding appropriate volumes of NaCl from a 5 M stock solution. The osmolarities of growth media were determined with a vapor pressure osmometer (model 5500; Wescor). Antibiotics for *B. subtilis* strains carrying antibiotic resistance cassettes integrated into the genome were used at the following concentrations: chloramphenicol, 5 μg ml^{-1}; spectinomycin, 100 μg ml^{-1}; tetracycline, 15 μg ml^{-1}; and erythromycin, 0.4 μg ml^{-1}.

[1-^{14}C]glycine betaine (2.035 GBq mmol^{-1}; 3.7 MBq ml^{-1}) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). Glycine betaine was obtained from Sigma (Deisenhofen, Germany).

Survival assays of *B. subtilis* mutant strains exposed to osmotic downshifts. The cells were cultured in SMM with 0.8 M NaCl until they reached mid-exponential growth phase (OD_{578} = 0.6 to 0.8). To determine the number of viable cells, samples were withdrawn from the cultures and appropriate dilutions were plated onto SMM plates containing 0.8 M NaCl to avoid an osmotic down-shock of the cells. After incubation for 48 h at 37°C, colonies were counted and this total cell number was set at 100% of preshocked cells for each individual experiment. To perform a hypo-osmotic shock experiment, 1 ml of the cell culture (grown in SMM with 0.8 M NaCl) was withdrawn and the cells were subjected to an osmotic down-shock by diluting the culture into 19 ml of prewarmed (37°C) SMM containing various concentrations of NaCl. These cells were then incubated with shaking at 37°C for 30 min to allow adjustment to the new osmotic conditions. Subsequently, 100-μl aliquots were withdrawn from the cultures, serial dilutions were prepared in isotonic growth medium, and the cells were plated onto SMM plates containing appropriate amounts of NaCl to avoid a further osmotic downshift. The plates were incubated for 2 days at 37°C before the colonies were counted.

Bacterial strains, plasmids, and construction of *B. subtilis* mutants. The *B. subtilis* strains used in this study are all derivatives of the wild-type strain JH642 (*trpC2 pheA1*) (BGSC 1A96), a kind gift from J. Hoch (La Jolla, CA). Chromosomal mutations in the *mscL* (*ywpC*), *ydhY*, *yfkC*, and *ykuT* genes were constructed by marker replacement via homologous recombination with plasmid-encoded gene disruption mutations carrying antibiotic resistance markers (11). For the construction of these mutants, plasmids pSM33 (*mscL*::*spc*), pSM41 [Δ(*yfkC*::*tet*)], pSM44 [Δ(*ydhY*::*ery*)], and pSM47 [Δ(*ykuT*::*cat*)] (see Fig. 2) were linearized by cutting with a restriction enzyme, the *B. subtilis* wild-type strain JH642 was then transformed with linear plasmid DNA, and the desired *B. subtilis* mutants were selected by plating the transformation mixture onto LB plates containing the appropriate antibiotic. This yielded the following mutant strains: SMB53 (*mscL*::*spc*), SMB58 [Δ(*ydhY*::*ery*)], SMB62 [Δ(*yfkC*::*tet*)], and SMB63 [Δ(*ykuT*::*cat*)]. The correct physical structures of the chromosomal gene disruptions in these strains were verified by Southern hybridization using probes derived from plasmids pSM31 (*mscL*$^+$), pSM38 (*yfkC*$^+$), pSM39 (*yhdY*$^+$), and pSM40 (*ykuT*$^+$). These plasmids were constructed as follows. In each case, the target gene or gene fragments were amplified by PCR using appropriately chosen primers (data not shown). Plasmid pSM31 is a derivative from pBSK$^-$ (Stratagene, Heidelberg, Germany) carrying the *'ywpB-mscL-'ywpD* region; plasmid pSM38 is a derivative of pBSK$^-$ carrying the *'yfkB-yfkC-'yfkD* region; plasmid pSM39 is a derivative of pPD100 (13, 15) carrying the *'yhdX-yhdY-yhdZ-'yheN* region, and plasmid pSM40 is a derivative of pBSK$^-$ carrying the *ykuU'-ykuT-'ykuS* region. Into these plasmids, antibiotic resistance cassettes were inserted as summarized in Fig. 2. Cassettes conferring resistance to spectinomycin (*spc*), tetracycline (*tet*), erythromycin (*ery*), and chloramphenicol (*cat*) were cut by restriction digests out of plasmids pDG1726 (15), pDG1515 (15), pBKB80 (B. Kempf and E. Bremer, unpublished data), and pJMB1 (M. Jebbar and E. Bremer, unpublished data), respectively.

TABLE 1. Strains with mutations in putative channel-forming genes

Strain	Presence of mutation in:			
	mscL	*yhdY*	*ykuT*	*yfkC*
JH642	+	+	+	+
SMB53	−	+	+	+
SMB58	+	−	+	+
SMB62	+	+	+	−
SMB63	+	+	−	+
SMB64	−	−	+	+
SMB65	−	+	+	−
SMB66	−	+	−	+
SMB67	+	−	+	−
SMB76	+	−	−	−
SMB80	−	−	−	−

For Southern hybridization, a nonradioactive digoxigenin DNA labeling and detection kit (Roche, Mannheim, Germany) was used for the labeling of restriction fragments with digoxigenin-dUTP. Chromosomal DNA was isolated from the various *B. subtilis* mutant strains and transformed into appropriate chosen recipient *B. subtilis* strains to construct combinations of gene disruption mutations. The collection of strains constructed in this way is listed in Table 1. To construct a mutant that was simultaneously defective in *mscL* and *sigB*, we isolated chromosomal DNA from the *B. subtilis* mutant BLOB22 (*sigBΔ2*::*cat*) (33) and transformed strain SMB53 (*mscL*::*spc*). Chloramphenicol-resistant colonies were selected, and one of these colonies was designated strain THB7 (*mscL*::*spc sigBΔ2*::*cat*).

Release of radiolabeled glycine betaine from osmotically down-shocked cells. The various strains were cultured at 37°C in a shaking water bath in 100-ml flasks containing 40 ml SMM supplemented with 0.8 M NaCl to raise the osmolarity until the cells reached mid-exponential growth phase (OD_{578} = 0.6 to 0.8). Glycine betaine (1 mM) and 1 μM [^{14}C]glycine betaine were then added to the culture (corresponding to a specific activity of 40.7 kBq per culture), and the cells were further grown for 120 min to allow glycine betaine uptake. During this period, glycine betaine uptake activity reached saturation (Fig. 1A). Subsequently, 4-ml aliquots of the cells were distributed to several Falcon tubes, the OD_{578} was determined, and the cells were then exposed to osmotic downshifts of various degrees by diluting the culture with SMM containing various amounts of NaCl. To monitor uptake and expulsion of [^{14}C]glycine betaine by the *B. subtilis* cells, 300-μl aliquots were withdrawn from the cultures at time intervals and filtered through Millipore membranes (0.45 μm). The cells retained by the filters were washed twice with 3 ml of an isotonic SMM medium, and the radioactivity remaining in the cells was determined by scintillation counting.

RESULTS AND DISCUSSION

Release of glycine betaine upon a hypo-osmotic shock. *B. subtilis* cells growing under hyperosmotic conditions can accumulate large amounts of the compatible solute glycine betaine

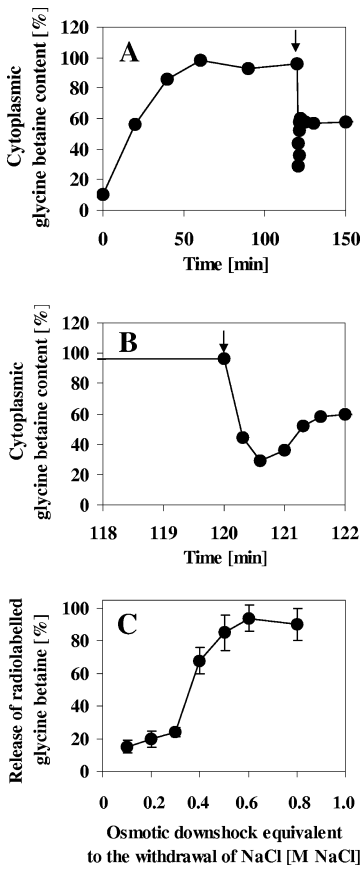

FIG. 1. Release of glycine betaine from osmotically down-shocked cells. The *B. subtilis* wild-type strain JH642 was grown in SMM with 0.8 M NaCl in the presence of 1 mM glycine betaine and 1 μM [^{14}C]glycine betaine until the uptake of glycine betaine reached a steady state. Cells were then subjected to osmotic downshifts of various degrees by dilution into SMM containing the appropriate amounts of NaCl. (A) Representative time course of the release and reaccumulation of glycine betaine in cells subjected to an osmotic down-shock equivalent to the withdrawal of 0.38 M NaCl. The arrow marks the time point of the down-shock. (B) To visualize the time course of the release of glycine betaine from the cells immediately after the osmotic downshift, the measured data points shown in panel A were replotted on a larger time window. (C) The release of glycine betaine is dependent on the degree of the down-shock. The amount of glycine betaine released by the cells immediately after the osmotic downshift was calculated in relation to the maximal glycine betaine concentration accumulated before the osmotic down-shock that was set as 100%. The data given in panel C are the means of three independent experiments.

from environmental sources via the osmoregulated OpuA, OpuC, and OpuD transport systems (17–19). This leads to intracellular glycine betaine pools that are linearly correlated with the external osmolarity and reach values of 1.2 M to 1.3 M in cells cultivated in SMM with 1 M NaCl (S. Moses, E. P. Bakker, and E. Bremer, unpublished results).

We were interested in the release of glycine betaine from cells grown at high osmolarity that subsequently experienced an osmotic down-shock. To monitor expulsion of glycine betaine, we grew the *B. subtilis* wild-type strain JH642 to mid-exponential growth phase (OD_{578} = 0.6 to 0.8) in a minimal medium (SMM) whose osmolarity had been increased with 0.8 M NaCl (final osmolarity, 1,900 mosmol/kg of water) to trigger uptake of glycine betaine from the medium. A mixture of radiolabeled glycine betaine (1 μM) and nonlabeled glycine betaine (1 mM) was added to the cells, and the culture was further grown for 120 min until glycine betaine uptake activity reached saturation (Fig. 1A). We then subjected these actively growing cells to an osmotic down-shock equivalent to the withdrawal of 0.38 M NaCl from the culture. The amount of radiolabeled glycine betaine remaining in the cells was measured after the osmotic down-shock over a 20-min time period by scintillation counting. Immediately after the osmotic down-shock, the cells ejected about 70% of the initially accumulated radiolabeled glycine betaine (Fig. 1A and B). However, they quickly reaccumulated part of the released glycine betaine until a new steady-state level was reached (Fig. 1A and B).

We then performed a series of experiments of this type where the culture was suddenly diluted into SMM supplemented with different concentrations of NaCl (0 to 0.8 M NaCl), thereby generating osmotic down-shocks of different degrees. The release of radiolabeled glycine betaine from the cell immediately after the osmotic down-shock was plotted versus the degree of the osmotic downshift (Fig. 1C). With osmotic decreases equivalent to the withdrawal of 0.285 M NaCl, there was only a minor overall release of glycine betaine from the cells, indicating that *B. subtilis* cells can withstand moderate osmotic downshifts without losing much of the previously accumulated compatible solutes. A further increase in the strength of the osmotic down-shock triggered a substantial efflux of glycine betaine. The amount of glycine betaine released from the cells was correlated with the severity of the osmotic down-shock (Fig. 1C), thereby allowing the cell a graded response to osmotic changes in its environment. Almost the entire amount of preaccumulated glycine betaine was released from the cells when the osmotic downshift was equivalent to the withdrawal of at least 0.57 M NaCl (Fig. 1C). Release of glycine betaine from the osmotically downshifted *B. subtilis* cells was not caused by cell lysis, since the wild-type strain JH642 can sustain an osmotic down-shock equivalent to the withdrawal of 0.76 M NaCl with only marginal loss of cell viability (see below and Fig. 3). It is thus apparent from the experiments whose results are documented in Fig. 1 that osmoregulating *B. subtilis* cells sensitively readjust their intracellular compatible solute pool in tune with decreases in medium osmolarity.

Construction of mutations in genes for putative mechanosensitive channel-forming proteins. *B. subtilis* contains one MscL-type mechanosensitive channel (24), which is encoded by the *mscL* (*ywpC*) gene located at position 319.7° on the genetic map of *B. subtilis*. Database searches and phylogenetic analysis performed by Pivetti et al. (27) suggest the existence of three homologues of the *E. coli* MscS (YggB) channel protein in *B. subtilis*. These are the *yhdY* gene at position 88.7°, the *yfkC* gene at position 74.1°, and the *ykuT* gene at position 127.4° on the *B. subtilis* chromosome. The *B. subtilis* YkuT (267 amino acids) and YfkC (280 amino acids) proteins are of sizes similar to that of the *E. coli* MscS (YggB) protein (286 amino acids) and exhibit moderate protein sequence identities of 22% and 14%, respectively, to MscS. The YkuT and YfkC proteins have predicted topologies similar to that known for the MscS protein (2, 23), with three membrane-embedded protein segments. The YkuT and YfkC proteins exhibit 28% protein se-

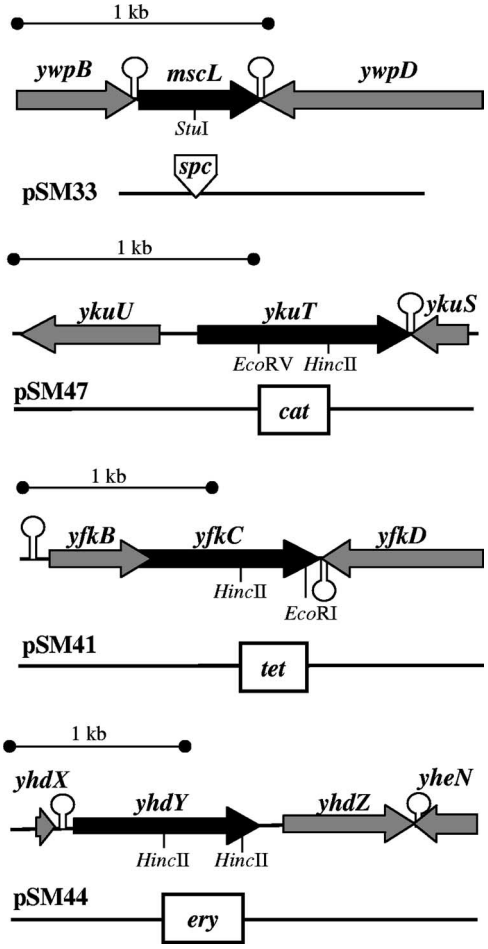

FIG. 2. Genetic organization of the *B. subtilis mscL*, *ykuT*, *yhdY*, and *yfkC* genes. The genetic organization of the *mscL*, *ykuT*, *yhdY*, and *yfkC* loci on the *B. subtilis* chromosome are shown. The names of the plasmids used for chromosomal marker replacement are given. The positions of the restriction sites relevant for the construction of gene disruptions are indicated. The antibiotic resistance cassettes used to disrupt the *mscL*, *ykuT*, *yhdY*, and *yfkC* genes are given; the sizes of the antibiotic resistant cassettes are not drawn to scale.

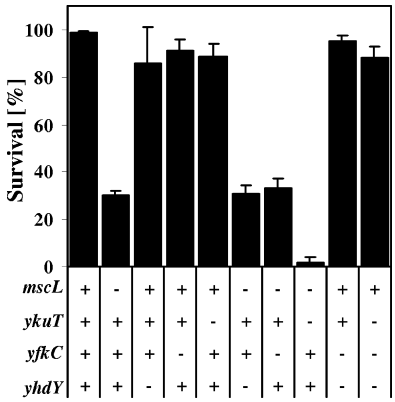

FIG. 3. Survival of *mscL*, *yfkC*, *yhdY*, and *ykuT B. subtilis* mutant strains following an osmotic downshift. The cells were pregrown in SMM with 0.8 M NaCl until they reached an OD_{578} of 0.6 to 0.8. The cultures were then subjected to an osmotic down-shock by a 20-fold dilution in SMM without salt (resulting in an osmotic down-shock equivalent to the withdrawal of 0.76 M NaCl). Cellular survival of the osmotic down-shock is expressed in percentages relative to the viable cell number before the osmotic down-shock. The data given are the means of three independent experiments.

quence identity to each other, and major regions of sequence identity occur in the predicted membrane-spanning segments. A third MscS-related protein, YhdY, has been noted in *B. subtilis* by Pivetti et al. (27). However, YhdY (370 amino acids) is clearly different from YkuT and YfkC since YhdY contains five predicted membrane-spanning sequences and a very long carboxy-terminal tail (191 amino acids), which is predicted to face the outside of the cytoplasmic membrane. Its overall sequence identities to YkuT (13%) and YfkC (14%) from *B. subtilis* and MscS (6%) from *E. coli* are modest.

To study the physiological functions of the of *B. subtilis mscL* (*ywpC*), *ykuT*, *yfkC*, and *yhdY* genes, we constructed an isogenic set of mutants derived from the wild-type strain JH642 that carried different antibiotic resistance markers in each of these genes (Fig. 2). The antibiotic resistance markers used for these strain constructions were chosen in such a way that the various mutations in the putative channel-forming genes could be readily combined by genetic transformation of the wild-type *B. subtilis* strain with chromosomal DNA to give appropriate double-, triple-, and quadruple-mutant strains (Table 1).

To test whether mutations in the putative channel-forming genes *mscL*, *ykuT*, *yfkC*, and *yhdY* influence cell growth at high osmolarity, we used the quadruple-mutant strain SMB80 (Table 1) and compared its growth properties to those of the wild-type strain JH642 in a minimal medium with 1.2 M NaCl. The mutant strain SMB80 exhibited the same growth characteristics as the parent strain JH642 (data not shown), demonstrating that the channel protein MscL and the putative MscS-type channel proteins YkuT, YfkC, and YhdY are dispensable for growth of *B. subtilis* in high-osmolarity environments. This quadruple-mutant strain (SMB80) and the single-mutant strains also did not exhibit any growth phenotype when they were cultured in SMM minimal medium without added salt (data not shown).

Physiological responses of mechanosensitive channel mutant strains to severe osmotic down-shocks. We first tested the survival of the *B. subtilis mscL* (*ywpC*), *yhdY*, *yfkC*, and *ykuT* single-mutant strains upon a severe osmotic downshift. For these experiments, the cells were grown to mid-exponential phase (OD_{578} = 0.6 to 0.8) in SMM with 0.8 M NaCl and were suddenly diluted to low-osmolarity conditions (SMM with 0.04 M NaCl). This drastic drop in osmolarity did not impair the colony-forming ability of strain JH642 (Fig. 3), indicating that a *B. subtilis* wild-type strain can sustain severe osmotic downshifts with only a marginal effect on cell viability. This also has been independently observed by Wahome and Setlow (34). The single-mutant strains SMB58 (*yhdY*), SMB62 (*yfkC*), and SMB63 (*ykuT*) exhibited the same survival capacity as the wild-type strain JH642 upon a drastic osmotic downshift. In contrast, approximately 70% of the cells of the *mscL* (*ywpC*) mutant SMB53 were killed by such a down-shock (Fig. 3), demonstrating that the integrity of the MscL channel-forming protein is critical for the effective management of a hypo-osmotic shock by the *B. subtilis* cells. This situation is similar to

that found by Folgering et al. (14) in *Lactococcus lactis*, where also an MscL-type channel functions as the main solute release system upon an osmotic downshift. This differs from the situation observed in *E. coli* where an *mscL* mutant by itself has no obvious phenotype (21).

We also constructed an *yhdY yfkC* double mutant (strain SMB67) and an *yhdY yfkC ykuT* triple mutant (SMB76) and assessed their survival upon a severe osmotic downshift. Neither the double mutant nor the triple mutant was affected by the osmotic down-shock (Fig. 3). Hence, *B. subtilis* can effectively manage a rapid transition from high to low osmolarity as long as the MscL channel is intact.

We then individually combined the *mscL* (*ywpC*) mutation with single mutations in *ydhY*, *yfkC*, and *ykuT* and tested the sensitivity of these double-mutant strains to an osmotic downshift. The double-mutant strains SMB64 (*yhdY mscL*) and SMB65 (*yfkC mscL*) exhibited the same phenotype as the *mscL* (*ywpC*) single-mutant strain SMB53 (Fig. 3). However, when the *ykuT* mutation was combined with the *mscL* mutation (strain SMB66), there were essentially no surviving cells subsequent to an osmotic down-shock. Hence, while the *ykuT* mutation by itself (strain SMB63) has no significant negative effect on cell survival (Fig. 3), its combination with an *mscL* mutation enhanced the osmosensitivity of *mscL* mutants subsequent to a hypo-osmotic shock.

***mscL* mutants are sensitive to osmotic stress rather than salt stress.** To test if the killing of strains carrying *mscL* mutations by osmotic downshifts was specific to cultures grown in the presence of the ionic osmolyte NaCl or represented a true osmotic effect, we grew the various mutant strains in SMM in the presence of 0.9 M sucrose and then subjected these cultures to osmotic downshifts. The same phenotype as that in the above-reported experiments with NaCl-grown cultures was observed, indicating that the sensitivity of *mscL* mutants to an osmotic down-shock is related to osmotic and not ionic strength.

Killing of the *mscL ykuT* double mutant SMB66 in response to graded hypo-osmotic challenges. The above-presented data on the release of glycine betaine from osmotically downshocked cells suggest that the mechanosensitive channels from *B. subtilis* open when the cultures are subjected to an osmotic down-shock equivalent to the withdrawal of >0.285 M NaCl (Fig. 1C). To further corroborate this finding, we carried out a series of osmotic downshift experiments where we graded the degree of osmotic down-shocks in small increments. For these experiments, we focused on the *mscL ykuT* double-mutant strain SMB66 since it exhibited the strongest phenotype of all strains that we tested in response to osmotic downshifts (Fig. 4). SMB66 tolerated osmotic downshifts equivalent to the withdrawal of 0.333 M NaCl without significant loss of viability. However, when the osmotic down-shock exceeded this value, the survival of SMB66 decreased in parallel with increases in the magnitude of the osmotic downshift (Fig. 4). We therefore conclude from these data that *B. subtilis* can readily withstand an osmotic down-shock equivalent to the withdrawal of 0.333 M NaCl in the absence of the MscL and YkuT proteins, but larger drops in the osmolarity are detrimental to cell viability.

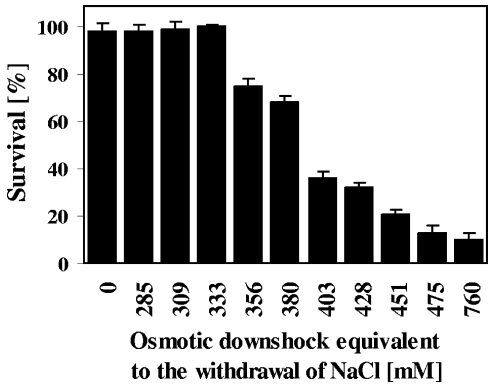

FIG. 4. Survival of the *mscL ykuT* double-mutant strain SMB66 in response to hypo-osmotic down-shocks of different degrees. A culture of strain SMB66 (*mscL ykuT*) was pregrown in SMM with 0.8 M NaCl until the culture had reached an OD_{578} of 0.6 to 0.8. Osmotic down-shocks of various degrees were then performed by diluting 1 ml of culture in 19-ml volumes of SMM media containing various concentrations of NaCl, resulting in osmotic down-shocks equivalent to withdrawals of between 0 and 760 mM. Cellular survival of strain SMB66 subsequent to the osmotic down-shock is expressed in percentages relative to the viable cell number before the osmotic down-shock. The data given are the means of three independent experiments.

Influence of the alternative sigma factor SigB on cell viability after a hypo-osmotic shock. Among the three MscS-type putative channel-forming proteins (YkuT, YfkC, and YhdY) of *B. subtilis* (27), the YkuT protein attracted our special interest because its structural gene has been shown to be under the control of the alternative sigma factor SigB (26), which regulates the general stress regulon of *B. subtilis* (28). To analyze the influence of a *sigB* mutation on cell survival subsequent to an osmotic down-shock, we monitored the cell survival of the *sigB* mutant BLOB22 (*sigB*Δ2::*cat*). In comparison to that of its isogenic wild-type parent strain JH642, survival of the *sigB* mutant is not impaired by a sudden osmotic downshift equivalent to the withdrawal of 0.760 M NaCl (Fig. 5). Since the transcription of the *ykuT* gene is under the control of SigB (26), we introduced the *sigB* mutation present in BLOB22 (*sigB*Δ2::*cat*) into the *mscL* mutant strain SMB53, yielding strain THB7. We compared the survival of THB7 (*mscL sigB*) with that of SMB66 (*mscL ykuT*) and found that both strains were highly sensitive to an osmotic downshift (Fig. 5). Hence, the combination of an *ykuT* mutation or a *sigB* mutation with an *mscL* mutation causes hypersensitivity to an osmotic downshock. These observations indicate that the sensitivity of a *sigB mscL* double mutant (THB7) is caused by blocking the transcription of the SigB-controlled *ykuT* gene.

Conclusions. Since their discovery in bacteria, it has been proposed that mechanosensitive channels function as safety valves to prevent cell lysis when a bacterium is rapidly shifted from high-osmolarity to low-osmolarity environments (4, 6). The data presented in this study on the physiological response of *B. subtilis* toward osmotic downshifts support this notion. We demonstrate in this contribution that the MscL channel functions as the main mechanosensitive solute release system of *B. subtilis*. The SigB-controlled MscS-type channel formed by YkuT makes a modest contribution to solute release in *B.*

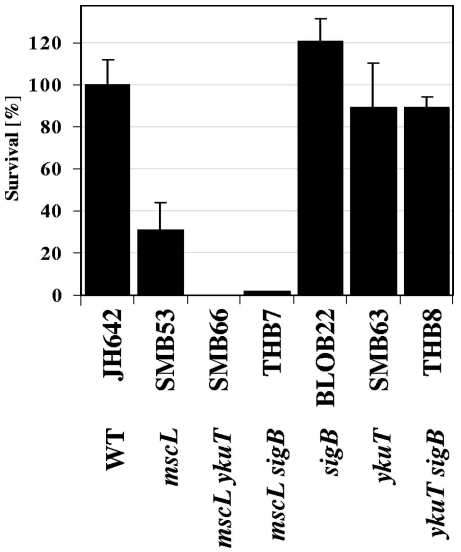

FIG. 5. Survival of *B. subtilis* wild-type (WT) and *ykuT* and *sigB* mutant cells challenged by an osmotic downshift. The cells were pregrown in SMM with 0.8 M NaCl until they reached an OD_{578} of 0.6 to 0.8. The cultures were then subjected to an osmotic down-shock by a 20-fold dilution in SMM without salt (resulting in an osmotic down-shock equivalent to the withdrawal of 0.76 M NaCl). Cellular survival of the osmotic downshifted strains is expressed in percentages relative to the viable cell number before the osmotic down-shock. The data given are the means of three independent experiments.

subtilis, but its combination with an *mscL* mutation enhances the sensitivity of *B. subtilis* to a drop in the external osmolarity. The channel formed by YkuT might make important contributions to solute release from the cells under special growth circumstances because its structural gene is controlled by the central regulator (SigB) of the general stress regulon of *B. subtilis* that responds to various environmental cues. The sensitivity of *mscL* and *mscL ykuT* mutants to osmotic downshifts unambiguously shows that mechanosensitive channels play a pivotal role in the management of *B. subtilis* cells in the transition from a hypo- to a hypertonic environment.

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