How do spores wake up? Proteins involved in the first stages of spore germination
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3 Bacillus subtilis spore protein SpoVAC functions as a mechanosensitive channel*

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A critical event during spore germination is the release of Ca-DPA (calcium in complex with dipicolinic acid). The mechanism of release of Ca-DPA through the inner membrane of the spore is not clear, but proteins encoded by the Bacillus subtilis spoVA operon are involved in the process. We cloned and expressed the spoVAC gene in Escherichia coli and characterized the SpoVAC protein. We show that SpoVAC protects E. coli against osmotic downshift, suggesting that it might act as a mechanosensitive channel. Purified SpoVAC was reconstituted in unilamellar lipid vesicles to determine the gating mechanism and pore properties of the protein. By means of a fluorescence-dequenching assay, we show that SpoVAC is activated upon insertion into the membrane of the amphiphiles lysoPC and dodecylamine. Patch clamp experiments on E. coli giant spheroplast as well as giant unilamellar vesicles (GUVs) containing SpoVAC show that the protein forms transient pores with main conductance values of about 0.15 and 0.1 nS respectively. Overall, our data indicate that SpoVAC acts as a mechanosensitive channel and has properties that would allow the release of Ca-DPA and amino acids during germination of the spore.

3.1 Introduction

The genera Bacillus and Clostridium are the best-studied endospore-forming bacteria, which are characterized by extreme resistance to unfavourable environmental conditions such as heat, radiation, and chemical agents\textsuperscript{15,34}. The bacterial spore is composed of a number of compartments that are termed (i) exosporium, (ii) coat, (iii) outer membrane, (iv) cortex, (v) germ cell wall, (vi) inner membrane, and (vii) central core. (i) The exosporium is the most external layer but is not found in all species of Bacillus. It is composed of glycoproteins and may provide resistance to chemical and enzymatic treatments, providing the spore with the ability to adhere to surfaces\textsuperscript{26,27}. (ii) In Bacillus subtilis the coat is formed by up to 70 different proteins. The function of the coat has not been completely addressed; however, it is implicated in spore resistance to chemicals and predation\textsuperscript{28}. (iii) The outer membrane is an essential structure during sporulation and it might play a role as a permeability barrier\textsuperscript{26,28}. (iv) The cortex is composed of peptidoglycan (PG) and essential for the formation of a dormant spore. During spore germination, the cortex is degraded to allow the expansion of

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The core and further outgrowth. (v) The germ cell wall is also composed of PG and forms the cell wall of the outgrowing spore. (vi) The inner membrane is a strong permeability barrier playing a major role in resistance to a wide range of chemicals. It contains proteins that are important in germination such as the nutrient germinant receptors (GRs) and the SpoVA proteins. (vii) The central core plays an important role in spore resistance, containing most of the spore enzymes, DNA, ribosomes and tRNAs. It has unique molecules such as pyridine-2,6-dicarboxylic acid (DPA) and small acid-soluble spore proteins (SASP), which are crucial for spore resistance[28,34].

The ability to form spores makes the cells resilient to environmental contaminants as well as mild food-processing regimes and antiseptic procedures. Spores are able to develop into growing cells in response to specific nutrients (germinants) in the environment such as amino acids, sugars, nucleosides or mixtures of nutrients[14,15,91]. This process is called germination and it is transduced into a cascade of events, wherein the GRs located in the spore’s inner membrane mediate the first step(s)[14,40,91]. Upon nutrient binding to specific GRs, a series of physiological events is initiated, including the rapid release of monovalent cations and (dipicolinic acid, DPA) together with Ca$^{2+}$ ions. The concomitant water/Ca-DPA exchange results in an increase of the hydration of the spore core and the hydrolysis of the spore’s peptidoglycan cortex. The posterior swelling of the spore core through further water intake and expansion of the germ cell wall allows enzyme action and germination of the spore[15,40,91].

A crucial event during spore germination is the release of Ca-DPA, which takes place within the first minutes of the germination process[15,70,72–74]. The mechanism of release of Ca-DPA across the inner membrane of the spore is not clear. Previous studies suggested that proteins encoded by the spoVA operon are involved in Ca-DPA release[73,74]. This release of Ca-DPA is triggered not only by nutrients but also by agents such as the cationic surfactant dodecylamine, which bypasses the GRs[75,114]. The molecular mechanism of this effect is not well understood, but it is possible that surfactants modify the tension (lateral pressure profile) of the membrane with the concomitant activation of an associated protein channel. The effect would be reminiscent of the activation by amphipaths of mechano-sensitive (MS) channel proteins involved in cellular osmoregulation[86,115–117]. The bacterial MS channels sense and respond to membrane tension and act as emergency release valves under conditions of hypo-osmotic stress[118]. Thus, the insertion of amphipaths in one of the membrane leaflets causes a change in the lateral pressure profile and gates MS channels such as MscL[115,116].

Although the notion of Ca-DPA release through a MS channel during germination is appealing, previous reports suggested that B. subtilis homologues of known MS channels are not involved in sporulation or germination. Setlow specifically looked at mutants of MscL and MscS, which showed identical sporulation and germination properties compared to the wild type[89,90,119]. On the basis of genetic studies, the SpoVA proteins are the most likely candidates for the release of Ca-DPA. SpoVA could behave either as a channel or as a regulator of channel activity[74]. During spore germination, temperature-sensitive spoVA mutants do not release Ca-DPA at non-permissive temperature[70,73,114]. However, how Ca-DPA is released in germinating spores is not known.

In B. subtilis the SpoVA proteins are encoded by a heptacistronic operon that comprises spoVAA, spoVAB, spoVAC, spoVAD, spoVAEa, spoVAEb and spoVAF. Of these, SpoVAA, SpoVAB, SpoVAC, SpoVAEb and SpoVAF are predicted to be membrane proteins, with two to five membrane spanning regions and most likely present in the inner membrane
of the spore\cite{12,68–70}. Mutations in any of the first six cistrons of the spoVA operon eliminate Ca-DPA uptake during sporulation\cite{69,71,72}. The biochemical characterization of SpoVA proteins is limited to one study of Li \textit{et al.}\cite{120} (http://www.rcsb.org/pdb/; PDB code 3LM6), who found that the structure of SpoVAD is similar to that of \( \beta \)-ketoacyl synthase and polyketide synthases. In addition, Ca-DPA has been shown to bind to SpoVAD and mutations in the binding pocket eliminate Ca-DPA uptake by developing spores\cite{120}. SpoVAD is located at the outer surface of the spore inner membrane, possibly in complex with other SpoVA membrane proteins\cite{55,59}. Thus, SpoVAD could be the receptor component that acts in conjunction with a membrane-bound component(s) in the uptake of Ca-DPA in maturating spores. Overall, there is strong evidence that one or more SpoVA proteins are involved in Ca-DPA release\cite{28}.

We cloned and expressed several SpoVA genes in \textit{Escherichia coli} and \textit{Lactococcus lactis}. Best expression was obtained for SpoVAC in \textit{E. coli} and the protein produced with a C-terminal myc epitope and 6His tag. We purified SpoVAC from membrane vesicles and reconstituted it into synthetic lipid vesicles. We show that SpoVAC has channel like properties that would allow the release of Ca-DPA during germination of the spore.

### 3.2 Experimental Procedures

#### 3.2.1 \textit{E. coli} strains used in this study

MC1061 (\textit{araD139, \Delta (ara-Ieu)7697, \Delta (lac)X74, galU\textsuperscript{-}, galK\textsuperscript{-}, hsdR\textsuperscript{-}, rpsL})\cite{121} was used for cloning and expression of SpoVAC. MJF641, \( \Delta 7 (\text{mscS}\textsuperscript{-}, \text{mscK}\textsuperscript{-}, \text{ybdG}\textsuperscript{-}, \text{ybiO}\textsuperscript{-}, \text{yjeP}\textsuperscript{-}, \text{gnaI}\textsuperscript{-} \) and \( \text{mscL}\textsuperscript{-} \))\cite{122} and MJF465 (Frag1 \( \Delta \text{mscL::cm, \Delta mscS, \Delta mscK::kan} \))\cite{123} was used for cell viability experiments after osmotic downshock, and MJF455 (Frag1 \( \Delta \text{mscL::cm, \Delta mscS} \))\cite{124} was used for electrophysiology experiments.

#### 3.2.2 Cloning and expression of 10His-SpoVAC

\textit{SpoVAC} was cloned in the pBAD vector\cite{125} and expressed in \textit{E. coli} strain MC1061. The primers for amplification of \textit{spoVAC} were designed to allow LIC (ligation-independent cloning), as described by Geertsma \textit{et al.}\cite{96}. The \textit{spoVAC} gene was amplified using genomic DNA of \textit{B. subtilis} 168 as template and the following primers were used:

\begin{verbatim}
SpoVACfwdnLic
5’-ATGGGTAGAATTACTATTTTCAGGTAACAAAAATTACAACCTGA-3’
SpoVACrevnLic
5’-TGGGAGGGTGGGTATTTTCATGACATCGTTTCATAAAGCAAACCCG-3’
\end{verbatim}

#### 3.2.3 Cloning and expression of SpoVAC-myc-6His

The \textit{spoVAC} was cloned in pBAD/Myc-His B expression vector (pBAD 24 derivative; Invitrogen). The protein SpoVAC-myc-6His was expressed in \textit{E.coli} strain MC1061\cite{121}. The primers for amplification of SpoVAC-myc-6His (see below) were designed to allow LIC (ligation-independent cloning), as described by Geertsma \textit{et al.}\cite{96}, but with a slight modification; the reverse primer which has one nucleotide extra to allow cloning in the
pBAD/Myc-His B plasmid. The primers used to amplify spoVAC-myc-6His are the following:

**Forward**

5’-ATGGTGAGAATTTATATTTTCAAGGTACAAACATAAAAGAAAATTACAAATCA-3’

**Reverse**

5’-TGGGAGGGTGGGATTTTCATTAATGACATCAGTTTCTCAAAAGCAAACC-3’

The SpoVA proteins containing an N-terminal 10His tag and TEV cleavage site (10His-SpoVAC) or C-terminal myc epitope and 6His tag (SpoVAC-myc-6His) were grown aerobically in Luria–Bertani (LB) medium and supplemented with 100 μg ml$^{-1}$ ampicillin. For large-scale cultures, a 2 or 10 l batch reactor (Applikon Biotechnology, Delft) was used and operated at 37 °C, pH 7.5, > 30% air (flow console, Applikon Biotechnology, Delft). When the cells had reached OD$$_{600}$$ ~1.8, the temperature was lowered to 25 °C. Once this temperature was reached (at OD$$_{600}$$ ~2), 10–3% (w/v) L-arabinose was added; 0.2% (v/v) of glycerol was added to obtain a higher biomass. After 2 h of induction, E. coli cells were harvested by centrifugation at 5000 g, 15 min, 4 °C, followed by resuspension in 50 mM potassium phosphate, pH 8, to OD$$_{600}$$ ~200. The cell suspension was flash frozen in liquid nitrogen and stored at -80°C.

### 3.2.4 Analysis of cell viability

To analyse the viability of cells after osmotic downshift, E. coli MJF641, with seven genes encoding mechanosensitive channels deleted$^{[122]}$ or MJF465 with three of the major MS channels deleted$^{[123]}$ was transformed with pBADnLIC-10his-spoVAC or pBAD-spoVAC-myc-6His respectively, pBAD-mscL or empty plasmid. Freshly streaked colonies were grown overnight at 37 °C in LB [per litre: 5 g of yeast extract, 10 g of bactotryptone (Difco labs Detroit, MI), and 5 g of NaCl (0.085 M)]. The overnight culture was diluted 1:20 in 10 ml of LB and grown for 1 h. The culture was then diluted to an OD$_{600}$ of 0.05 in 10 ml of the same medium supplemented with 0.5 M NaCl. The cultures were grown to an OD$_{600}$ of 0.2–0.25, at which stage expression of spoVAC or mscL was induced for 1 h with 5 × 10–3% (w/v) of L-arabinose. The induced cultures were diluted 1:20 into LB (osmotic downshift) or LB plus 0.5 M NaCl. Cells were grown with shaking at 37 °C for 15 min, and then serially diluted into medium containing either no additional salt (osmotic downshift) or 0.5 M NaCl (iso-osmotic). The diluted cultures were plated in triplicate and grown overnight at 37 °C on LB-ampicillin agar plates. The colony-forming units were counted and averaged per experiment. Each experiment was carried out as true biological replicate in triplicate.

### 3.2.5 Membrane vesicle preparation

The frozen cells were thawed at room temperature and supplemented with 1 mM PMSF, 1 mM MgSO$_4$ and DNase (~50μg ml$^{-1}$). The cells were lysed by single passage through the Constant Systems Ltd cell disrupter, operated at 25 kPsi and 5 °C. Cell debris was removed by centrifugation (20 min, 18 500 g, 4 °C), and subsequently the membrane vesicles were collected by ultracentrifugation (90 min, 150 000 g at 4 °C). The membrane vesicles were kept on ice and resuspended in 50 mM potassium phosphate, pH 8.0, and the total protein content was measured with a BCA protein assay (Thermo Scientific Pierce). Finally, the membrane vesicles were aliquotted, flash frozen in liquid nitrogen and stored at −80 °C.
3.2.6 Purification and membrane reconstitution of SpoVAC-myc-6His

Membrane vesicles at 5 mg ml\(^{-1}\) of total protein were solubilized in buffer A containing 50 mM KPi pH 8.0, 300 mM KCl, 10% (v/v) glycerol, 0.5% (w/v) DDM and 15 mM of imidazole for 30 min on ice with occasional gentle mixing. Then the mixture was centrifuged (20 min, 325 000 \(g\), 4 °C) to separate the soluble from non-soluble materials. For purification, 0.250 ml resin of Ni\(^{2+}\)-Sepharose (Amershan Bio-sciences) was placed in a disposable column (Bio-Rad) and washed with 10 column volume (CV) of MilliQ water and equilibrated with 10 CV of buffer A. The solubilized material was added to the Ni\(^{2+}\)-Sepharose, and the mixture was incubated for 1 h at 4 °C and gently rotated. Subsequently, the resin was washed with 30 CV of buffer A supplemented with 50 mM of imidazole plus 0.04% (w/v) DDM. The SpoVAC-myc-6His proteins were eluted from the column by adding subsequently 200 µl, 300 µl and 300 µl of buffer A supplemented with 500 mM of imidazole plus 0.04% (w/v) DDM; most of SpoVAC-myc-6His eluted in the 300 µl of the second elution fraction. A second purification step was performed on a Superdex 200 10/300 GL size-exclusion column (Amershan Biosciences), using 50 mM KPi, pH 8.0, 150 mM KCl plus 0.2% (v/v) Triton X-100. The purity of the protein samples was confirmed by SDS-PAA gel electrophoresis and Coomassie Brilliant Blue staining of the gels.

The purified proteins were reconstituted in liposomes according to Geertsma et al.\[^{100}\]. Liposomes composed of Azolectin (Soy total extract lipid; Avanti Polar Lipids) or \textit{E. coli} polar lipid plus egg phosphatidylcholine in a ratio 3:1 (wt/wt) at 20 mg ml\(^{-1}\) of total lipid in 150 mM KCl, 10 mM potassium phosphate, pH 8.0, were homogenized by extrusion 11 times through a 400 nm filter. The liposomes were destabilized by the stepwise addition of Triton X-100 as described previously\[^{96}\]. Protein and lipids were mixed at 1:100 weight ratio and incubated for 30 min at 50 °C for Azolectin lipids or at room temperature for \textit{E. coli} polar lipids plus egg phosphatidylcholine. Subsequently, Biobeads (SM-2 Absorbents; Bio-Rad) were added in steps to remove the detergent\[^{100}\].

The proteoliposomes were converted to giant-unilamellar vesicles (proteoGUVs) by electroformation, that is rehydration of the lipid film in an oscillating electrical field using a vesicle PrepChamber (Nanion Technologies). Therefore, 0.8–1 mg ml\(^{-1}\) of proteoliposomes were spotted and dried on two indium tin oxide coated glass slides (ITO-slides). A chamber was built from the two ITO-slides separated by a spacer, creating a reservoir filled with 500 mM sorbitol. A voltage of 1.2 V at 10 Hz was applied for at least 3.5 h through electrodes sealed onto the glass plates. The resulting giant-unilamellar vesicles (GUVs), 5–50 µm in diameter, were used for electrophysiology measurements.

3.2.7 Fluorescence dequenching assay

For the fluorescence dequenching assay, 200 mM of calcein, in 10 mM NaPi, pH 8.0 plus 150 mM KCl was included into the vesicle lumen of large-unilamellar vesicles (LUVs), according to Koçer et al.\[^{126}\]. To remove the external calcein, a Sephadex G50 size-exclusion column was equilibrated with buffer B (10 mM KPi, pH 8.0, 150 mM KCl plus 1 mM Na\(_3\)EDTA). The proteoliposomes with reconstituted SpoVAC or empty liposomes were applied onto the column to remove the free calcein dye. All elution fractions were assayed in a Varian Cary Eclipse Fluorimeter at an excitation wavelength of 495 nm and recording the emission at 515 nm; the slit width was 5 nm. For monitoring the efflux of calcein, 2.5 to 5 µl of calcein-filled (proteo)liposomes were diluted into 2100 µl buffer B. Following 5 min of equilibration at room temperature, lysophosphatidylcholine (LysoPC) (Avanti Polar lipids) or dodecylamine
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**Figure 3.2.1.** Expression of N-terminal tagged SpoVAC and rescuing of *E. coli* from osmotic downshift conditions. A. Expression of 10His-SpoVAC in *E. coli* MC1061. Lane 1: Total protein (60 μg) from cells carrying pBADnLIC-10his-spoVAC and induced with 5 x 10^-3% (w/v) of L-arabinose. The sample was run in a 15% SDS-PAA gel and the protein was visualized by Coomassie Brilliant Blue staining. Lane 2: Immunoblot of the same gel, using an anti-His antibody. The arrow indicates the band that corresponds to SpoVAC. B. Viability of *E. coli* MJF641 with vector control, or expressing MscL or 10His-SpoVAC, following exposure to osmotic downshift. The P-value for the difference in viability of the vector control and 10His-SpoVAC was 0.056 as determined by the Student’s t-test, indicating a low significance. The immunoblot below panel B shows the relative levels of expression of MscL and SpoVAC. C. Viability of *E. coli* MJF465 with vector control or expressing MscL or SpoVAC-myc-6His, following exposure to osmotic downshift. All experiments were performed in triplicate with three independent biological replicates; the standard error (SE) is shown. The data are presented as percentage of the viability under iso-osmotic conditions (LB supplemented with 0.5 M NaCl). The immunoblot below panel B shows the relative levels of expression of MscL and SpoVAC.

(SIGMA-ALDRICH) was added at final concentrations of 4 and 32 μM respectively. The fluorescence was measured continuously, and the 100% signal was determined by the addition of 0.5% (v/v) Triton X-100 (complete lysis of the liposomes), typically after 15 min. The release of the calcein self-quenching dye is referred as % of release and equals (I_t − I_0) × 100, where I_t is the fluorescence intensity at a given time, I_0 is the initial fluorescence intensity, and I_100 is the fluorescence intensity after the addition of Triton X-100.

**3.2.8 Electrophysiology studies**

*Escherichia coli* giant spheroplast and giant-unilamellar vesicles containing SpoVAC-myc-6His were prepared as previously described [127]. For spheroplast preparation, cephalixin treatment was done for 1 h and when the cell had sufficiently elongated (microscopic inspection), SpoVAC-myc-6His expression was initiated with 0.1% of L-arabinose for 45–60 min. The expression was checked by Western blot.

For patch clamp measurements, 5 μl of spheroplasts or giant-unilamellar vesicles containing SpoVAC-myc-6His were transferred to a sample chamber containing a ground electrode and 160 μl of patch clamp buffer: 5 mM HEPES-KOH, pH 7.2, 200 mM KCl, 90 mM MgCl_2, 10 mM CaCl_2. As negative control, we used giant-unilamellar vesicles without SpoVAC-myc-6His. In case of *E. coli* spheroplasts obtained from strain MJF455, we used the same buffer but with KCl replaced by NaCl to suppress MscK activity. Channel activity was recorded using an Axopatch 200A amplifier and a digital converter, and Axoscope software was used for the data analysis (Axon Instruments, Foster City, CA). Data were acquired at
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A sampling rate of 33 kHz and filtered at 10 kHz. The presented traces were additionally filtered to decrease electronic noise, using Clampfit 10.3 software (Axon Instruments) with the low pass Boxcar filter at smoothing point 21. Offline analysis was performed using PClamp 10.3 software (Axon Instruments).

3.3 Results

3.3.1 Expression and complementation studies: 10His-SpoVAC

Initially, we expressed in E. coli MC1061 SpoVAC with an N-terminal 10-His tag and the recombinant protein was detected after immunoblotting and using an anti-His antibody (Fig. 3.2.1-A). 10His-SpoVAC migrated at approximately 16 kDa, which is consistent with its calculated molecular mass (18.4 kDa) and the tendency of very hydrophobic proteins not to unfold completely. We used the in vivo osmotic downshift assay to assess the functionality of 10His-SpoVAC. The MJF641 strain, constructed in the Booth laboratory[122], lacks detectable MS channel activity and when these cells are grown in LB plus 0.5 M NaCl and subsequently diluted into LB then the majority of cells lyse. When MJF641 is complemented in trans with the mscL gene, all the cells survive the osmotic downshift. For 10His-SpoVAC, we obtain significant but lower survival than with MscL (Fig. 1B, upper panel), which most likely reflects the much lower expression of SpoVAC in MJF641 as compared to MscL (Fig. 3.2.1-C, lower panel). Overall, these data provide the first indication that SpoVAC may function as mechanosensitive channel, and release osmolytes at a high rate. To carry out more detailed biochemical studies, we needed to increase the expression level of SpoVAC.

3.3.2 Expression and complementation studies: SpoVAC-myc-6His

We tested numerous conditions (inducer concentration, temperature, medium, and growth phase for induction) to boost the expression of 10His-SpoVAC in E.coli or L. lactis; however,
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the amounts of protein that we could purify remained very low. Then we made modifications in the recombinant protein, and constructs with a modified N-terminus and extended C-terminus (myc epitope plus C-terminal 6-His tag) expressed significantly better than 10His-SpoVAC; hereafter, this protein is named SpoVAC-myc-6His. Figure 1C shows that SpoVAC-myc-6His rescues *E. coli* MJF465 from osmotic downshift to the same extent as MscL.

### 3.3.3 Purification of SpoVAC-myc-6His

*Escherichia coli* MC1061 cells carrying pBAD-spoVAC- myc-6His were grown in a 10 l bioreactor with pH of 7.5 and oxygen control above 30%. The membrane fraction was isolated and the protein was solubilized and purified in DDM (Fig. 3.3.1-A) as described under Experimental procedures. The SpoVAC-myc-6His protein was observed in Coomassie blue stained gels and its identity confirmed by immunoblot and mass spectrometry analysis. The sample was highly enriched in SpoVAC-myc-6His after Ni-Sepharose chromatography but numerous contaminants were present as well. After size-exclusion chromatography (Fig. 3.3.1-B), we observed a major peak around 12 ml corresponding to an oligomer of SpoVAC plus bound detergent. The two purification steps allowed us to obtain sufficient amounts of highly pure SpoVAC (Fig. 3.3.1-A, lane 8), which was used later on for functional studies in proteoliposomes and proteoGUVs.

### 3.3.4 Calcein release assay

To further test the functionality of SpoVAC-myc-6His, the protein was reconstituted in lipid vesicles composed of azolectin lipids. The proteoLUVs were loaded with the fluorescent dye calcein, which was incorporated at a ‘self- quenching’ concentration of 100 mM. Upon addition of lysoPC a rapid increase in fluorescence (dequenching) was observed, which reflects the release of calcein from the vesicle lumen (Fig. 3.3.2-A). The release data were fitted to a first-order rate equation:

\[
[F]_t = [F]_\infty (1 - e^{-(k \cdot t)})
\]

(3.3.1)

The first-order rate constant \(k\) was 1.8 min\(^{-1}\); \([F]_t\) represents the total fluorescence; \([F]_\infty\), the fluorescence at infinity; and \(t\) the time of reaction. The release was strictly dependent on the addition of lysoPC and 100% of the liposomes released their cargo. Amphipathic molecules such as lysoPC intercalate into one of the bilayer leaflets and thereby create mismatch in the lateral pressure profile between the two leaflets. Importantly, we also observed specific release of calcein upon addition of dodecylamine (Fig. 3.3.2-B), the cationic surfactant that has been shown to trigger the release of Ca-DPA from *B. subtilis* spores in *vivo*. We find that SpoVAC responds to lysoPC (and dodecylamine) in a manner similar to MscL, at least *in vitro* (Fig. 3.3.2-C), and the mechanistic basis for its activation might be similar to that of mechanosensitive channels involved in osmoregulation. Contrary to the activation of spores *in vivo*, presumably by acting on SpoVAC, there is yet no information that MS channels like MscL are activated by amphipaths *in vivo.*

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3.3.5 Electrophysiology characterization of SpoVAC

Patch clamp recordings of E. coli giant spheroplasts and inside-out patches of proteoGUVs were carried out to determine the conductance properties of SpoVAC. Most of the recordings were made at +/-30 mV or +/-40 mV. We observed little channel activity with negative pipette pressures of 65–80 mmHg for both E. coli giant spheroplasts and proteoGUVs (Fig. 3.4.1 A and B). However, raising the pressure to values close to 100 mmHg or higher (close to the lytic membrane tension), we frequently observed channel activity that we attribute to SpoVAC (level 1 in Fig. 3.4.1-A). The unitary conductance of SpoVAC in E. coli giant spheroplasts is 0.15 ± 0.004 nS (standard error of the mean is given; n = 3). A conductance of 0.91 ± 0.0023 nS was also found (level 2 in Fig. 3.4.1-A) but it may correspond to one or more endogenous E. coli channels (Li et al., 2002), as the E. coli MJF455 strain only lacks MscL and MscS, the dominant mechanosensitive channels (Fig. 3.4.1-A). To unambiguously resolve which conductance corresponds to SpoVAC, we purified the protein and reconstituted SpoVAC in liposomes composed of E. coli polar lipids plus egg phosphatidylcholine in a ratio 3:1 (wt/wt) (Fig. 3.4.1-B). In membrane patches from SpoVAC-containing GUVs, we observed a ~0.11 ± 0.002 nS conductance as the dominant channel activity. The negative control, GUVs without SpoVAC, did not show channel activity when subjected to similar pressures (Fig. 3.4.1-C), indicating that the observed conductance is not due to membrane disruption.

All-points amplitude histograms show the main levels of conductance of SpoVAC in E. coli giant spheroplasts (Fig. 3.4.2-A) and proteoGUVs (Fig. 3.4.2-B). We also calculated the open dwell times of SpoVAC in patches derived from E. coli giant spheroplasts (Fig. 3.4.2-C) and proteoGUVs (Fig. 3.4.2-D). The channel open time (dwell) is shown in histograms and the best fit was with two-component exponential function. The dwell times for E. coli giant spheroplasts are 0.87 ± 0.14 ms and 3.7 ± 0.31 ms, and for proteoGUVs the dwell times are 0.85 ± 0.1 ms and 7.9 ± 0.13 ms. Taken collectively, we conclude that SpoVAC
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3.4 Discussion

We have purified and membrane-reconstituted SpoVAC from \textit{B. subtilis}. To obtain sufficient protein, we used SpoVAC-myc-6His for most of our studies; 10His-SpoVAC was expressed at least an order of magnitude less well, but the protein was functional. Our results provide for the first time clear biophysical and biochemical evidence for channel-like activity of SpoVAC. Our data indicate that SpoVAC has mechanosensitive channel-like properties, like MscL or MscS from \textit{E. coli}\textsuperscript{[122,129]}. SpoVAC provides protection against hypo-osmotic stress in \textit{E. coli} and responds to the asymmetric insertion into the membrane of the amphiphile lysoPC and dodecylamine, and its electrophysiological behaviour is typical of that of mechanosensitive channels.

3.4.1 Characteristics of SpoVAC

Prokaryotic MS channels function as safety valves that open when cells are exposed to severe osmotic downshift, that is, when the membrane tension gets too high due to excessive water intake. Upon activation of MS channels cytoplasmic osmolytes are released, the turgor pressure is lowered and membrane damage is prevented\textsuperscript{[88]}. When expressed in \textit{E. coli} SpoVAC provides protection against osmotic downshift. In addition, in proteoLUVs SpoVAC releases calcein upon asymmetric insertion of lysoPC or dodecylamine into the membrane. Calcein is a 623 Da anionic fluorophore, which suggests that SpoVAC most likely mediates the passage of a wide range of low-molecular-weight solutes (ions, nutrients). Taken collectively, we conclude that SpoVAC acts as channel that facilitates the efflux down the concentration gradient of osmolytes up to a mass of at least 600 Da. Our results are in accordance with in vivo studies in \textit{B. subtilis}, showing the early release of molecules in this mass range (e.g. divalent cations, arginine, glutamic acid and DPA)\textsuperscript{[74,130]}.

The unitary conductance of the most frequent open state of SpoVAC in \textit{E. coli} giant spheroplast is somewhat higher than in proteoGUVs. Similar differences have been observed previously\textsuperscript{[127]} and may relate to difference in the membrane environment; the spheroplast
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Figure 3.4.2. Electrophysiological characterization of SpoVAC-myc-6His. A and C. All points-amplitude histograms showing the main levels of conductance from recordings on SpoVAC-myc-6His containing spheroplasts and GUVs at −30 mV and +40 mV respectively. B and D. The corresponding dwell time analysis of the first level of conductance of SpoVAC-myc-6His in spheroplasts and GUVs. The continuous lines are the fit of the two-component exponential function to the data and the dashed lines show the individual components of these functions. The fit of the data yielded dwell times of 0.874 and 3.69 ms for spheroplast and 0.89 and 7.9 ms for GUVs. The y-axis shows the square root of the counts (N); the fit had a correlation coefficient near 1.
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system is obviously more complex and crowded and differs in lipid composition from the GUVs. The conductance of SpoVAC falls in the category of MS channels with a relatively low conductance, like MscM (0.3–0.4 nS) and YnaI (0.1 nS)\textsuperscript{[122]}. The diameter of the SpoVAC channel in the open state was estimated using the Hille equation\textsuperscript{[131]}:

\[
d = \frac{\rho g}{\pi} \left( \frac{\pi^2}{4} + \frac{4\pi l}{\rho g} \right)
\]

(3.4.1)

where \(d\) is the pore diameter, \(\rho = \) the buffer resistivity (25.3 Ω cm), \(g = \) conductance and \(l = \) the membrane spanning length (40 Å). For \(g = 0.15\) nS, this would correspond to a pore diameter of 4.6 Å, i.e. on the assumption that the pore has a symmetric cylindrical shape. The value of the pore calculated on the basis of the conductance of \(E. coli\) giant spheroplasts is somewhat smaller than the dimension of calcein (5 × 7 × 13 Å)\textsuperscript{[132]}. We do not have information on the geometry and surface properties of the SpoVAC pore, and thus the pore diameter should be regarded as gross estimate.

The dwell times of SpoVAC indicate that the channel generally opens and closes quickly as was described by Moe et al. for MscL from \(Staphylococcus aureus\)\textsuperscript{[128]}. However, we occasionally observed channel activity with very long dwell-times as shown in Fig. 3.4.1-B, which is atypical for MS channels. Another important observation is that the open probability of SpoVAC increases with membrane tension. It is well known that ultrahigh pressures may induce germination of bacterial spores, but the mechanistic basis is not known\textsuperscript{[84]}. Indeed, it has been shown that high pressures (500 MPa) by-pass germinant receptors during spore germination\textsuperscript{[83,85]}. However, it is difficult to estimate how these pressures translate to tension changes in the inner spore membrane, and how these relate to changes in membrane tension modulated by pipette pressure in proteoGUVs (\textit{vide infra}).

3.4.2 Gating mechanism and role of lipids

The open probability of SpoVAC increases upon increasing of the pipette pressure in membrane patches derived from \(E. coli\) giant spheroplasts and in proteoGUVs. These results are in line with the observation that SpoVAC protects \(E. coli\) against osmotic downshift, which is indicative for gating by membrane tension. In our experimental set up, the electrophysiology assays were performed either in \(E. coli\) giant spheroplast or SpoVAC-reconstituted in \(E. coli\) polar lipids (≈ 57.5% phosphatidylethanolamine, ≈ 15.1% phosphatidylglycerol, ≈ 9.8% cardiolipin and ≈ 17.6% of unknown lipids), plus egg phosphatidylcholine 3:1 (wt/wt). We note that the \(B. subtilis\) spore inner membrane has a different lipid headgroup composition with 12% phosphatidylethanolamine, 35%, phosphatidylglycerol, 50% cardiolipin and 3% diglucosyl diacylglycerol\textsuperscript{[113]}. Thus, the lipid composition used in our ‘membrane models’ may not be totally mimicking the endogenous membrane environment. As a consequence, the kinetic properties of channel gating may differ from those in the in vivo situation. Next to the lipid headgroup composition, the physical state of the membrane is crucial for membrane protein function. We determined the activation of SpoVAC in membranes in a hydrated, liquid-crystalline state, whereas germination initiates in spores in which the water content is extremely low. Biophysical studies of the inner spore membrane indicated that the lipids are largely immobile, but upon germination the mobile fraction is increased\textsuperscript{[30]}. Cowan \textit{et al.} suggested that the restricted mobility of lipids is due to the dehydrated state of the spore, implying that the lipid bilayer is in a gel-like state\textsuperscript{[30]}. The lateral pressure profiles and thus the forces acting
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on the protein will be different under conditions that the membrane is in a liquid-crystalline or gel-like state. Thus, next to a different headgroup composition, a different (more fluidic) state of the membrane may have contributed to the gating behaviour and conductance state of SpoVAC in our in vitro experiments.

3.4.3 A role for SpoVAC in germination

SpoVA proteins have been implicated in the Ca-DPA release that takes place in the first minutes of spore germination, but still there is no strong evidence that these proteins can associate and form a channel to release Ca-DPA\textsuperscript{[28,120]}. Our data provide the first biochemical evidence that one of the SpoVA family proteins is sufficient to act as a non-selective solute channel. We cannot rule out the possibility that in complex with other SpoVA proteins SpoVAC has an altered activity. We tried to express the other membrane components of the spoVA gene cluster in \textit{E. coli} and \textit{L. lactis}, but in general the proteins expressed poorly or failed to express at all.

In conclusion, we make an important step in the elucidation of the action mechanism of one of the SpoVA proteins, SpoVAC, that can act as a mechanosensitive channel, conceivably facilitating the release of CaDPA and other low-molecular-weight compounds during germination \textit{in vivo}. Possible roles of other SpoVA proteins in channel activity and that of germinant receptors in signalling and germination triggering \textit{in vivo} remain to be elucidated.