Identification of key residues in *Bacillus subtilis* germinant receptor protein GerAB involved in spore water uptake

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Abstract

Some species in the *Bacillales* and *Clostridiales* bacterial orders form spores in unfavorable environments. These spores are metabolically dormant, and resistant to harsh conditions. However, upon triggering by a variety of small molecule nutrients, termed germinants, spores may germinate, increasing their core water content from 25%-45% in spores to ~80% in growing cells, thus restoring rapid metabolism and leading to outgrowth and then vegetative growth. Recent structural modeling and mutagenesis studies showed that a subunit of the prototypical *Bacillus subtilis* spore germinant receptor (GR) GerA, the integral inner membrane (IM) protein GerAB, is involved in spore germination initiated by L-alanine. Notably, a previous molecular simulation study showed the formation of a putative water channel in GerAB. In the current study we employed Steered Molecular Dynamics (SMD) simulations to force a single water molecule through GerAB. Examining the simulations revealed three key amino acid residues, Y97, L199 and F342, that interfere with water passage. These three residues were altered to alanine, followed by assays of germination. In the Y97A, L199A, F342A single mutants and the Y97A, L199A and F342A triple mutant (triA), all four types of mutant spores barely responded to L-alanine, while wild type spores germinated almost 100% under the same conditions. Y97A spores expressed the GerAA protein as determined by western blot analysis, corroborating the presence of the GerA GR in this mutant. However, for the other three mutants, the existence of GerA receptor in the spore could not be confirmed. In conclusion, Y97 plays a key role in GerAB mediated germination and suggests that GerAB indeed facilitates water passage and further indicating that water passage through GerAB is a crucial event in *B. subtilis* spore germination.

Introduction

Several species within the *Bacillales* and *Clostridiales* bacterial orders can form dormant spores in harsh environments. These spores are metabolically inert, extremely resistant to a variety of harsh treatments, and dormancy can last for decades. However, cells derived from some of these spores can cause food spoilage, infectious diseases, intoxications and foodborne illnesses[1]. This holds true for spores of *Bacillus anthracis*, *Clostridiodes difficile* and *Bacillus cereus,* respectively. Consequently, the biology of spore-forming bacteria has been intensively studied for decades. A major feature of dormant spores is their very low core water content, 25%-45% of wet weight, in spores of the model organism *Bacillus subtilis* compared to ~80% of wet weight. in growing cells. However, how water is taken up into the dormant spore core is not clear, as *B. subtilis* lacks known water pores such as aquaporins[2,3].

The loss of spore dormancy and resumption of its metabolism and cell-like structure requires spore germination, in which spore core water content increases at least 45% in wet weight, and small molecule nutrients, termed germinants, including specific monosaccharides, monovalent cations, and amino acids, can trigger spore germination with no germinant

catabolism involved[4]. Germinant receptors (GRs) play a crucial role in kick-starting germination. In the model organism *B. subtilis*, the major GR, GerA, is composed of three subunits, A, B, and C. The B subunit GerAB is a transmembrane protein belonging to the APC transporter superfamily that mediates L-alanine triggered germination[5–7]. The three subunits work closely together and all three are essential for functional GerA formation[1,4]. Blinker *et al*. identified a putative water channel in GerAB using molecular dynamics (MD) simulations[8]. A recent study by Gao *et al.* used a combination of AlphaFold[9] structure prediction and mutagenesis to show that GerA forms an oligomeric membrane channel which releases monovalent cations upon sensing L-alanine[10]. However, to date, the function and mechanism of spore water intake during germination and the possible role that GerAB plays in this process has not been fully clarified.

In order to investigate the molecular mechanism of GerAB mediated water uptake, MD simulation studies coupled with experimental mutagenesis and spore germination assays have been carried out. First, water molecules were pulled through GerAB from the outside of the inner membrane (IM) to its inside in Steered Molecular Dynamics (SMD) simulations. In these simulations, the termini of GerAB are assumed to be located on the inside of the spore[10] (Figure 1). During these SMD simulations, the work required for pulling a water molecule through GerAB was measured. Visual inspection of the SMD trajectories showed three amino acid residues Y97, L199 and F342 interfere with the passage of water through GerAB. The sidechains of these residues are close to one another and act as obstacles to water passage. To verify the role of these residues, spores of mutants Y97A, L199A, F342A and triA (Y97A, L199A and F342A triple mutant) were prepared, and the germination efficiencies of the wild type and mutant spores were measured. With L-alanine as germinant, wild type spores germinated almost 100% while all four mutant spores germinated poorly (no more than 20%) under the same conditions. Western blotting verified both wild type and Y97A spores contained GerAA, while the other three mutants did not, indicating that only the wild type and Y97A mutant spores could assemble the complete GerA GR. These observations collectively demonstrate that GerAB facilitates water passage, with Y97 playing a critical role in this process. Consequently, this study confirms that Y97 is essential for *B. subtilis* germination, as it is crucial for water transport through GerAB.

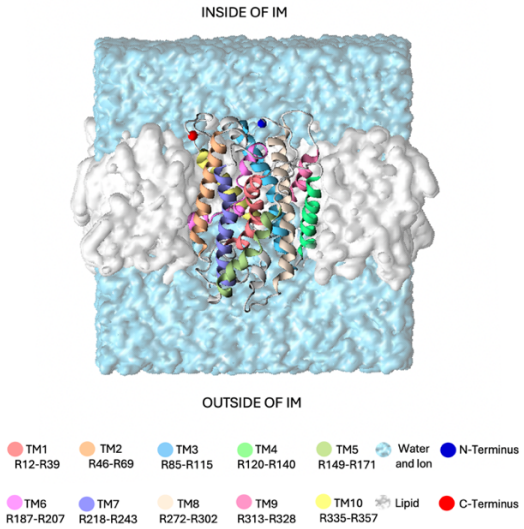

Figure 1. Simulation system used in this study. The GerAB protein is embedded in a lipid bilayer membrane (shown as a white surface), with its N- and C-termini oriented towards the interior of the spore's inner membrane (IM). The protein is displayed as a ribbon, with the 10 transmembrane helices (TMs) individually color-coded. Residues assigned to each TM follow the prediction by Blinker *et al*.[8] The surrounding water, containing 20 mM KCl, is represented as a light blue surface.

Materials and Methods

1. Simulations and analysis

One 100 ns molecular dynamics (MD) simulation of a protein structure model based on *Methanocaldococcus jannaschii* ApcT (PDB: 3GI8)[11] generated by the structure prediction tool raptorX[12] was conducted using the same starting structure and settings as those described by Blinker *et al*[8]. The simulation was performed with GROMACS 2020.4[13], using the CHARMM36[14] forcefield. In a cubic periodic box with starting dimensions of 10 by 10 by 11 nm, the system was solvated by TIP3P water molecules[15]. The simulation was maintained at a temperature of 298 K using the Bussi velocity rescaling thermostat[16] and at constant pressure of 1.0 bar with the Parrinello-Rahman barostat[17,18]. Long-range electrostatic interactions were treated using the Particle Mesh Edward[19] method with a maximum grid spacing at 1.6 Å. For short-range electrostatic and Van-der-Waals interactions the cutoff radius was set to 12 Å. The production run lasted 100 ns with a timestep of 2 fs, with coordinates saved every 2 ps. The term "(free) MD simulation" refers to the MD simulation production run conducted for this research.

Steered Molecular Dynamics (SMD) simulations were performed to pull a water molecule through GerAB protein. To extract suitable starting structure from MD simulation trajectory, the protein was first positioned at the center of the simulation box (with its center of mass between 4.76 nm and 4.85 nm along the x- and y-coordinate, and membrane boundaries at 3.0 nm < z < 7.0 nm). The entrance of the water was defined within the region 3.5 nm < x < 6.5 nm, 3.5 nm < y < 6.5 nm, and 4.6 nm < z < 5.0 nm. Ten frames, each containing a single water molecule within the defined entrance region, were extracted as the starting configurations for steered molecular dynamics (SMD) simulations. The selected water molecule was then subjected to the pulling force during the simulation. The steered MD simulations were conducted using PLUMED 2.7.0[20–22] embedded with GROMACS 2020.4, where the steering collective variable (CV) was the position in the z-direction of the oxygen atom of the pulled water molecule. In all SMD simulations, the selected water molecule was pulled 4 nm over 100 ns along the z-coordinate towards the inside of the inner membrane (IM), following the direction of water intake during germination. Over the 100 ns simulation, a moving harmonic potential kept the water oxygen constrained to z with a force constant $\kappa = 10000$ kJ mol^{-1} nm^{-2}. The harmonic restraint moved at a constant velocity of 0.04 nm/ns. In addition, the water oxygen atom was constrained in the x and y direction with a harmonic potential with a force constant $\kappa = 10$ kJ mol^{-1} nm^{-2}, to keep the water molecule inside the protein. The SMD simulations were performed with the same settings as the MD simulation. The work performed to constrain the water molecule along the z coordinate in the SMD simulations was computed as the cumulative force applied to the water molecule over the simulation time. In total, 10 SMD runs were performed. Visual inspection of the simulation trajectories was conducted using Visual Molecular Dynamics (VMD) 1.9.4a53 version[23].

123 2. Mutagenesis
124 All strains were derived from *Bacillus subtilis* PY79. The *gerA* operon was cloned by PCR,
125 inserted in plasmid vector pUC19 with the Gibson Assembly Master Mix kit (New England
126 BioLabs, NEB # E2611S). Mutagenesis was carried out with the QuikChange Lightning Site-
127 Directed Mutagenesis Kit (Agilent Technologies, Cat # 210518-5). Mutant *gerAB* sequences
128 were integrated into the original *gerAB* locus by a double crossover along with an erythromycin
129 (erm) resistance cassette. The precision of the mutagenesis in the *gerAB* locus was confirmed
130 by Sanger sequencing of all mutants. Strains involved in this study are listed in Table 1.
131
132 Table 1. Strains constructed in this study.

Strain	Strain	Source
***gerAB* Wild Type (wt)**	*Bacillus subtilis* PY79	Constructed in this study
***gerAB* Y97A Erm**	*Bacillus subtilis* PY79	Constructed in this study
***gerAB* L199A Erm**	*Bacillus subtilis* PY79	Constructed in this study
***gerAB* F342A Erm**	*Bacillus subtilis* PY79	Constructed in this study
***gerAB* triA (Y97A L199A F342A triple mutant) Erm**	*Bacillus subtilis* PY79	Constructed in this study
Δ*gerA*	*Bacillus subtilis* FB20 [24]	Prof. Peter Setlow lab, UConn Health

133
134 3. Sporulation and spore purification with liquid culture
135 All strains were initially grown to give single colonies on an LB-agar plate. A single colony was
136 transferred to 5 ml LB medium (pH 7.5) and grown at 37°C; after the OD_{600} reached 0.3-0.4,
137 serial 10-fold dilutions were incubated overnight in 8 mM MOPS minimal medium (K_2HPO_4 1.32
138 mM, $MgCl_2$ 0.4 mM, K_2SO_4 0.276 mM, $FeSO_4$ 0.01 mM, $CaCl_2$ 0.14 mM, MOPS 8 mM, Tricine 4
139 mM, pH 7,5)[25]. 1 ml of the dilution with OD_{600} at ~ 0.3-0.4 was further diluted 20-fold in
140 prewarmed MOPS minimal medium (pH 7.5). The culture was kept at 37°C while shaking at 200
141 rpm until the OD_{600} reached 0.3-0.4. To obtain the spore crop, 1% of the obtained (v/v) pre-
142 culture was inoculated into the final sporulation MOPS (8 mM) minimal medium described
143 above and incubated for three days at 37°C while shaking at 200 rpm. The culture was then
144 pelleted by centrifugation at 5000 rpm for 15 min at 4°C. The pellet was resuspended in sterile
145 milliQ (MQ) water and centrifuged at 5000 rpm for 15 min at 4°C. This washing step was
146 repeated 2-3 times. To remove vegetative cells the spores were suspended in 20% Histodenz
147 and loaded onto 50% Histodenz. The loaded samples were centrifuged at 15000 rpm for 60
148 min (4°C) and the supernatant was discarded. This step was then repeated to remove any
149 germinated spores. Again, the supernatant was discarded, and the pellet was resuspended in
150 MQ water and washed 2-3 times. Finally, the retrieved spores were diluted to an OD_{600} of
151 approximately 50 in 1 mL MQ water and samples were stored at -80°C.
152
153 4. Sporulation and spore purification with agar plates
154 Bacterial strains were streaked onto LB agar plates with appropriate antibiotics and incubated
155 overnight to avoid stationary phase; a single colony was inoculated into liquid LB with
156 antibiotics. Once OD_{600} reached 1.0-2.0, 200 µL liquid culture was spread onto 2xSG agar
157 plates (Difco Nutrient Broth 16 g/L, KCl 26 mM, $MgSO_4$ 2 mM, $MnCl_2$ 0.1 mM, $FeSO_4$ 1.08 µM,
158 $Ca(NO_3)_2$ 1 mM, Glucose 5.5 mM, Agar 15g/L) without antibiotics. Plates were incubated
159 upside down in plastic bags at 37°C for 2 to 5 days to allow sporulation, monitored by phase-
160 contrast microscopy. After sporulation, the plates were dried on the benchtop for
161 approximately 2 days to allow cell lysis. Spores were scraped from the plates and transferred
162 to 50 mL centrifuge tubes with cold MQ water. The spore suspensions from 2–3 plates were

combined, sonicated for 1 minute at full power, cooled on ice, and centrifuged at ~8,000 rpm for 20 minutes to remove debris. The purity of spore preparation was checked with phase contrast microscopy.

5. Germination assay with phase contrast microscopy

Purified phase-bright spores at OD_{600} of 5 in MQ water were heat-activated at 70°C for 30 min followed by 20 min incubation on ice. Before the addition of the germinants, the germinant solutions were kept on ice. A final concentration of 1 mM L-alanine or 10 mM AGFK solution (10 mM each of L-asparagine, D-glucose, D-fructose and KCl)[7,26] was added prior to slide preparation and imaging according to Wen et al.[25] In essence, to prepare a slide for wide field imaging, slides and two types of coverslips (size 22mm round and size 22mm*30mm rectangular) were cleaned with 70% EtOH and air dried in a vertical position minimizing dust collection. Two rectangular coverslips were prewarmed for several seconds on a 70°C heating block, 65 µl 2% agar was deposited on top of one coverslip, while the other coverslip was placed on top of it, spreading the agar in between. The agar patch was dried for approximately 10 min, after which the coverslips were slid off each other. The agar patch was cut into a 1x1cm section to which 0.4 µl of purified spore suspension with germinant added. The patch was transferred onto a round coverslip by placing it onto the patch and sliding it off. A G-frame was stuck onto the air-dried slide, onto which the coverslip was placed, closing all corners of the frame, and completing the slide for microscopy. For wide field time-lapse experiments, we used a Nikon Eclipse Ti equipped with a Nikon Ti Ph3 phase contrast condenser. Connected to it were a Nikon Plan Apo Plan Apo λ Oil Ph3 DM lens (100X, NA=1.49, T = 23°C), a Lumencor Spectra Multilaser (470 nm, 555 nm) using Lambda 10-B filter blocks, a NIDAQ Lumencor shutter, a Ti XY-and Z drive, and a Hamamatsu C11440-22C camera. All hardware was connected to a computer running NIS-Elements AR 4.50.00 (Build 1117) Patch 03. Before every use the condenser was adjusted to the optimal setting bringing the pixel size to 0.07 µm. A 100-minute time lapse video was recorded for every slide with a 30 sec interval between frames. The slide chamber was kept at 37°C, and germination was analyzed with SporeTrackerX[27]. The percentages of germinated spores at the end of the time-lapsed session were defined as germination efficiency.

6. Germination assay with optical density

Purified phase-bright spores, normalized to an OD_{600} of 1.2 in 25 mM HEPES buffer (pH 7.4), were heat-activated at 70 °C for 30 minutes, followed by incubation on ice for 20 minutes. 100 µL of the heat-activated spores were added, and L-alanine solutions were dispensed into a 96-well plate to a final concentration of 1mM. The OD_{600} was monitored every 10 minutes for 60 minutes using a plate reader, with the plate maintained at 37°C and agitated between measurements. The percentage of OD drop is measured against the initial OD.

7. Germination assay with DPA release

CaDPA release was measured using a fluorescence plate reader following the method outlined by Yi and Setlow. [5] Spores, at an OD_{600} of 0.5, were germinated with 1 mM L-alanine in 25 mM K-HEPES buffer (pH 7.4) at 45°C without prior heat activation. At various time points, 190 µL of the sample was mixed with 10 µL of 1 mM $TbCl_3$, and relative fluorescent units (RFU) were recorded using a plate reader.

8. SDS-PAGE and immunoblotting

To prepare samples for western blotting, the spores were first decoated and then lysed as follows. 50 ODs of spores were pelleted by centrifugation. The pelleted spores were resuspended in 1 mL TUDSE buffer (8 M Urea, 50 mM Tris-HCl, pH 8.0, 1% SDS, 50 mM DTT, 10 mM EDTA) and incubated for 45 min at 37˚C, centrifuged (3 min, max rpm, room temperature) and resuspended in 1 mL TUDS (8 M Urea, 50 mM Tris-HCl, pH 8.0, 1% SDS) buffer. The suspension was then incubated for another 45 min at 37˚C, centrifuged (3 min, max rpm, room temperature) and then washed six times by centrifugation (3 min, max rpm, room temperature) and then all suspended in 1 mL TEN buffer (10 mM Tris-HCl pH 8, 10 mM EDTA, 150 mM NaCl). The final decoated spores were resuspended in 1mL water if samples were not immediately given further treatment. 50 OD of the decoated spore preparation was treated with 1mg lysozyme in 0.5mL TEP buffer (50 mM Tris-HCl pH 7.4, 5 mM EDTA) containing 1mM phenylmethylsulfonyl fluoride (PMSF), 1µg RNase, 1 µg DNase I, and 20 µg of $MgCl_2$ at 37°C for 6 to 8 min, and then put on ice for 20 min. Glass disruptor beads (0.10 to 0.18 mm, 100 mg) were added to each sample and spores were disrupted with three bursts of sonication (micro probe, medium power for 10 sec, and placed on ice for 30 sec between bursts). Following the final sonication, samples were allowed to settle for 15 sec and 100 µl of the upper liquid was withdrawn and added to 100 µl 2x Laemmli sample buffer (BIO-RAD cat. #1610737) containing 5% (v/v) 2-mercaptoethanol and 1 mM $MgCl_2$ and incubated at 90-95°C for 3 min. This was saved as the total lysate and ready to run on SDS-PAGE. 20 µg of total protein was loaded onto each lane of SDS-PAGE on a 10% acrylamide gel (BIO-RAD cat. #4561034) and run 45 min at 60 V followed by 45 min at 110 V. After SDS-PAGE, proteins on the gel were transferred to a 0.22 µm PVDF membrane. The membrane was then blocked for 30 min by 2.5% low fat milk in TBST buffer (20 mM Tris, 150 mM NaCl, 0.1% Tween 20) and incubated with 1:3000 anti-GerAA antibody overnight[28]. After washing three times with TBST, the membrane was incubated with 1:2500 goat anti rabbit HRP antibody (BIO-RAD cat. #1706515) for an hour before visualization.

Results

1. Key residue identification

In the SMD simulations, water molecules were pulled through GerAB from the outside of the spore to the inside of the spore, as indicated in Figure 2A. For each individual SMD run, the cumulative work as a function of the z coordinate was calculated. For all runs, the exerted work increases after z = 5 – 5.5 nm until a z-coordinate between 6.5 and 7.5 nm. This means that the water molecule does not go spontaneously through the protein at the pulling speed. At z > 7.5 nm, the cumulative works stays approximately constant, which means that no additional work is required to pull the water molecule through bulk water after it has exited the protein (Figure 2B). This observation shows that the SMD does not suffer from artefacts caused by pulling the water too fast, as pulling the water molecule through bulk water happens at a velocity typical for water diffusion at 298 K[29]. This indicates that the pulling speed is sufficiently slow.

The pulling work profiles all have a similar shape but differ in when the plateau is reached. To quantify this variation, the difference of the cumulative work is defined as $\Delta w = w_{end} - w_{start}$. Δw ranged from Δw_{min}= 42 kJ/mol to Δw_{max}= 275 kJ/mol (Figure 2B). According to the Boltzmann distribution, the process requiring the least amount of work is the most likely to occur during the process of water passage[30]. Consequently, we visually inspected the run with the smallest Δw, labeled Δw_{min}. In this simulation, a group of three amino acids with bulky side chains

(termed the triad, consisting of Tyr97, Leu199, and Phe342, Figure 2C) blocked the movement of the water molecule along the z-axis. To continue moving through GerAB, the water molecule bypassed the triad between TM3 and TM6 where Tyr97 and Leu199 are located, respectively (Figure 2D). On the contrary, in all the other SMD simulation trajectories, the water was pulled through the three bulky side chains of the triad, with run Δw_{max} as an example (Figure 2E).

In summary, the bulky side chain triad interfered with water passage in all the SMD simulations trajectories. We also observed that the pulling work varied between the runs, and that the water paths are different for the lowest work simulation Δwmin and the other simulations. These results indicate that the triad may play a role in water passage. To verify the function of the triad in vivo, we hypothesized that by mutating the triad residues to alanine, the bulky side chains were removed, and therefore we had constructed mutant spores with an aberrant water passage profile, such as for example a leaky channel which would lead to a spontaneous germinating phenotype.

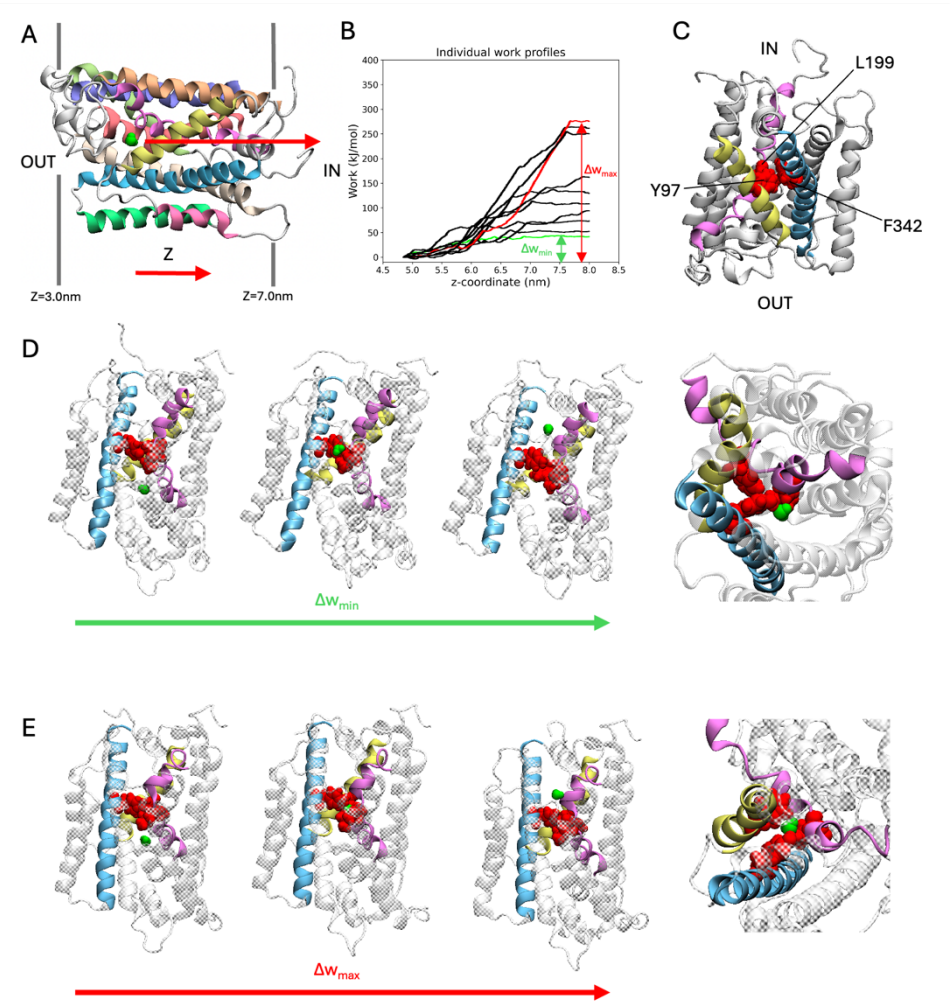

Figure 2. SMD Simulations of water molecules pulled through GerAB. (A) Example of the starting frame for the SMD simulations. The water molecule which is pulled is shown in a green space filling representation. The arrow indicates the pulling direction, from the outside of the spore IM to the inside. Dark grey lines flanking the protein indicate the membrane. (B) Work profiles of the SMD simulations along the z-axis. The work profiles Δw_{max} and Δw_{min} are shown in red and green,

respectively. (C) A triad of Tyr97, Leu199, and Phe342 (all in red spacefilling representation) are located on TM3, TM6, and TM10, respectively. Only TMs with the triad are colored, according to the coloring scheme in Figure 1. Protein regions not part of the triad are shown in white. (D), (E), Water molecule passing the triad in the SMD simulation with Δw_{min} and Δw_{max}, respectively. From left to right: snapshots of simulation for water oxygen at z = 4.84 nm, 5.89 nm, 6.65 nm (for Δw_{min}), and z = 5.07 nm, 6.21 nm, 7.84 nm (for Δw_{max}), showing the water before, during, and after passing the triad, followed by a closeup of the triad with the water molecule during water molecule passing the triad. The closeup is shown from the inside of the spore.

2. Y97 is important for GerAB function in *B. subtilis* spore germination

Four mutant strains of *B. subtilis* PY79 were successfully constructed and verified by sequencing. Residues in the triad were replaced by alanine, one at a time, to understand if removal of the bulky side chains could affect the water intake of the spore upon L-alanine or AGFK-induced spore germination. The mutant strains include: Y97A, L199A, F342A, triA (Y97A, L199A and F342A triple mutant).

Differences in germination efficiency of each strain with L-alanine or the AGFK (mixture of L-asparagine, D-glucose, D-fructose and K^+ ions)[26] shows that each of the mutated residues have an important role in L-alanine mediated germination due to the inability to respond to L-alanine effectively, either because the mutation altered the mechanism of water passage through GerAB, or the residue plays important role in GerAB and thus the GerA GR integrity. Compared to the wild type, where approximately all the spores germinated, all the mutant spores were only able to germinate with low efficiency with L-alanine (no more than 20%) (Figure 3A, C).
In addition to analyzing single-spore germination of spores prepared in liquid media, we also examined how wt and mutant spores (Y97A and L199A) responded when prepared on solid surfaces (2 × SG agar plates). Y97A and L199A were chosen as they displayed the lowest and highest residual germination capabilities, respectively. The germination kinetics of wt and both mutant spores in response to 1 mM L-alanine are shown in Figure 4. Interestingly, in both OD_{600} drop (Figure 4A) and DPA release (figure 4B), solid-prepared Y97A spores exhibited partial germination in response to 1 mM L-alanine matching ~25% of that of wt spores. However, solid-prepared L199A spores barely responded to L-alanine. As it is the water uptake that causes changes in the OD of the spores, this suggests that Y97A spores took up more water than L199A spores, although less than wt spores. And DPA release was correspondingly higher in Y97A than L199A spores. Compared to liquid-prepared spores, these results of solid-prepared spores highlighted a difference in spore properties, as the germination behaviour of solid-prepared spores is opposite to that of liquid-prepared ones. This difference may be attributed to variations in inner membrane (IM) fluidity between liquid- and solid-prepared spores. Since both the GerA receptors (involved in water uptake) and SpoVA channels (responsible for DPA release) are located in the spore IM, differences in IM fluidity could significantly impact the germination kinetics of spores prepared by different methods, as discussed by Yu *et al.* [31]

We next performed Western blot analysis of mutant spore proteins with a purified GerAA antibody, in which only wild type spores and Y97A mutant spores exhibited GerAA signals, albeit the level in the Y97A mutant is lower than that in wild type spores (Figure 5). This means that the low response of L-alanine of L199A, F342A and triA mutant spores was due to the

absence of GerA GR[32], while in Y97A mutant spores, with GerA GR present, this mutant could alter the germination efficiency of the spores and providing strong evidence that GerAB can facilitate water passage during germination. In addition, only spores of the Y97A mutant germinated with AGFK comparably to that of wt spores, while an approximately 10%-20% drop of germination efficiency with AGFK was observed with other mutant spores (Figure 3B, D). As AGFK is sensed by the GerB and GerK, GRs with no involvement of GerAB, consistent with the GerA GR only present in the Y97A mutant, this indicates that the absence of the GerA GR could affect the ability of the GerB and GerK GRs to respond to AGFK. This is not surprising since different GRs in *B. subtilis* spores work synergistically[33,34]. However, the germination defect in Y97A mutant spores does not support an initial hypothesis, and instead of creating a large cavity in GerAB and increasing its permeability, this mutation lowered the capability of GerAB to facilitate water passage.

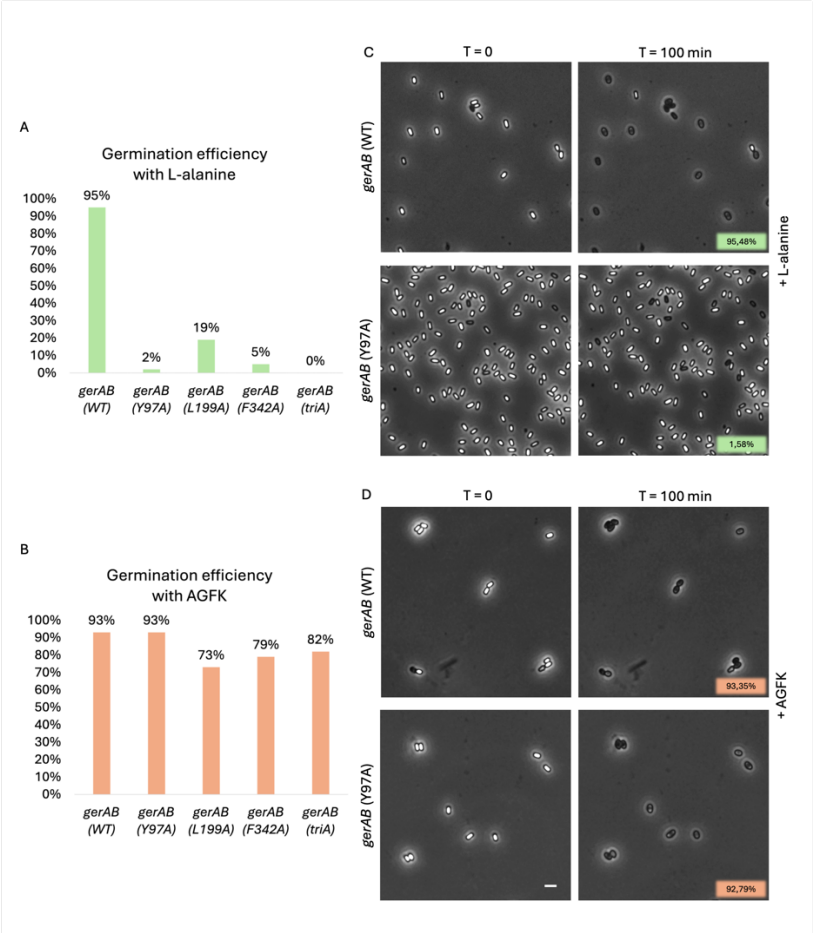

Figure 3. Germination assay of wt and different spore variants. Germination efficiency of spores with different GerAB variants with L-alanine (A) or AGFK (B). Wild type spores reacted almost 100% to both germinant conditions, while all mutants responded only poorly to L-alanine. Only Y97A spores responded to AGFK to a comparable level of wild type spores, while other mutant spores exhibit a 10% to 20% drop of germination efficiency to AGFK in comparison. (C), (D) Representative phase-contrast images of wt and Y97A spores before and after 100 min incubation with L-alanine or AGFK, respectively. One typical germination experiment data is shown for each strain under each condition. Representative large field time-lapse videos can be found in Supplementary data. Scale bar, 2 μm.

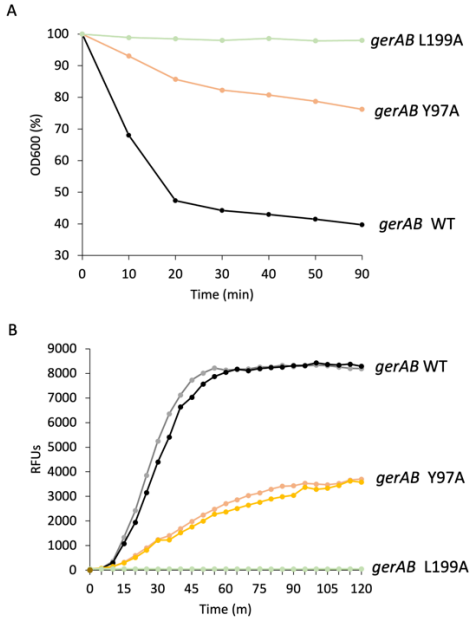

Figure 4. Germination assay of solid prepared spores with 1mM L-alanine. Solid prepared Y97A mutant spores showed partial germination around 25% of that of wt spores in both OD_{600} drop (A) and DPA release (B). Solid prepared L199A spores barely germinated in respond to L-alanine, as verified by both OD_{600} drop (A) and DPA repease (B). One typical experiment result was shown for OD_{600} drop experiment. The DPA release experiment was done in duplicate for each strain.

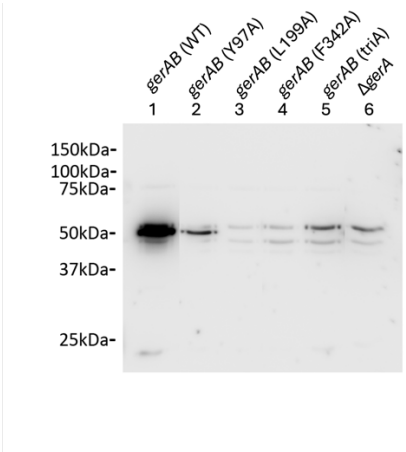

Figure 5. Western blot of *B. subtilis* PY79 wt and mutant spore proteins with GerAA antibody. Both *gerAB* (wt) and *gerAB* (Y97A) sample show a clear band with the GerAA antibody at ~50 kDa, while the other mutants exhibit minimal if any GerAA. Notably, *gerAB* (L199A), *gerAB* (F342A) and *gerAB* (triA) samples exhibited only the same bands as the Δ*gerA* GerAA deletion control sample. All samples were taken from the same blot; irrelevant lanes were removed.

Discussion

In this study, the function of GerAB was studied by both simulations and mutagenesis. By pulling a single water molecule through GerAB in SMD simulations, we discovered that the side

368 chains of Y97, L199 and F342, named triad in this study, interfere with water passage. The SMD
369 further revealed two different ways of interaction between water molecule and the triad. In the
370 simulation with the lowest pulling work (Δw_{min}= 42 kJ/mol), the water molecule moved through
371 GerAB bypassing the triad between TM3 and TM6. The simulation with the highest pulling work
372 (Δw_{max}= 275 kJ/mol) showed the water molecule passing through the three bulky residue
373 sidechains of the triad. Note that these observations are qualitative, to point out different
374 mechanisms of water passing through GerAB. For better quantitative results, *i.e.* a free energy
375 profile, several more SMD simulations need to be carried out, to reduce the variance.
376
377 On the other aspect of our work, mutagenesis of GerAB followed by western blot analysis, we
378 verified that Y97 is a key residue in GerAB mediated germination as while the GerA GR was
379 present in this mutant's spores, albeit at lower levels than in the wt spores, there was almost
380 no L-alanine germination. However, upon preparing Y97A spores by a different method, namely
381 on agar plates[35], these Y97A mutant spores exhibited L alanine induced germination of ~ 25%
382 of that of the wt spores. This might be due to the rigidity of spores' IM varying between spores
383 made by different methods with attendant effects on IM proteins, which requires further
384 investigation. Also, to understand the exact role of Y97 in the function of GerAB, mutating Y97
385 to different residues might give us further insight. Moreover, future simulation work could
386 provide detailed understanding of the mechanism of the interaction between water and GerAB,
387 and how these interactions contribute to the chain of molecular events during spore
388 germination.
389
390
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395
396 **Author contributions**
397 L.C. performed simulations and analysis, conducted experiments on sporulation in liquid
398 culture, carried out germination assays with phase contrast microscopy, analyzed the
399 experimental data, and wrote the manuscript. H.B. conducted simulations and analysis. C.B.
400 carried out mutagenesis experiments. G.V. performed sporulation in liquid culture and
401 germination assays. G.K. conducted sporulation on agar plates, germination assays with OD
402 measurements, DPA release experiments, and Western Blot analyses. J.V. provided
403 supervision and guidance on the simulations. P.S. and S.B. provided supervision and oversight
404 on spore biology. All corresponding authors contributed to the study design, and secured
405 funding, and critical revisions and feedback on the manuscript.
406
407 **Declaration of interests**
408 The authors declare no competing interests.
409
410 **Supplementary data**
411 Input files for the SMD simulations reported here and representative large-field time-lapse
412 videos are deposited on FigShare with https://doi.org/10.6084/m9.figshare.27225249.v1 and
413 https://doi.org/10.6084/m9.figshare.27226959.v1, respectively.
414

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