2 Biochemical characterization of the ABC subunits of germinant receptor GerA from *Bacillus subtilis*

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Bacillus spores can survive for extended periods of time in their dormant state, but spore germination can be triggered upon binding of nutrient germinants to spore-specific protein complexes, the so-called germinant receptors. Despite the large amount of genetic evidence supporting a role for germinant receptors in the initiation of germination, there are no studies showing that purified germinant receptors bind specific germinants *in vitro*. In this work, we study the GerA family receptor of *Bacillus subtilis*. We successfully cloned and overexpressed *gerAA*, *AB* and *AC* genes in *Lactococcus lactis*, with the subsequent isolation, purification and incorporation of GerAA, AB and AC proteins into artificial membranes. We characterize the proteins by performing intrinsic and extrinsic fluorescence assays to monitor conformational changes of GerAA, AB or AC upon addition of potential ligands. Unfortunately, we have not observed any changes in fluorescence that would characterize such a ligand as germinant. The stability and folding of GerAC was studied with differential scanning calorimetry and circular dichroism. Moreover, membrane reconstitution of GerAA and GerAB in liposomes and transport assays were performed using radiolabelled substrate. We did not observe detectable transport of the substrate. In summary, GerA receptor proteins isolated with our experimental approach are not very stable after purification, which was a limitation for biochemical characterization. Improvements of purification and reconstitution of GerA receptors proteins will help to understand the function of these proteins.

2.1 Introduction

Bacterial spores are formed under conditions of nutrient limitation. They have the ability to remain dormant for long periods of time and are resistant to heat, desiccation, extreme pH changes and toxic chemicals[34]. However, upon exposure to specific nutrients they rapidly enter into a growing cell stage, a process called germination[15,61]. During germination a series of events occur, wherein the germinant receptors, located in the spore's inner membrane, mediate the first step(s)[14,40,91]. To start the germination process, 'germinants' should activate these receptors. It is not clear how the germinants reach the inner membrane of the spore and to what extent the permeability of the spore coat is sufficient for passage of exogenous germinants. There is some evidence that GerP could be involved in facilitating the access of germinants to the inner membrane of the spore. However, the underlying mechanism is not clear yet[28,46–48].

Upon germination, the cell releases monovalent cations and dipicolinic acid (DPA) complexed with Ca^{2+} ions. The concomitant influx of water results in an increase of the hydration of the spore core and subsequent hydrolysis of the spore's peptidoglycan cortex. Next, the spore core takes in more water and the germ cell wall expands, allowing enzymatic action and full germination of the spore[15,40,91].

Bacillus subtilis expresses three major families of germinant receptors: GerA, GerB and GerK. However, GerA family is the most studied receptor, mediating L-Alanine- or L-Valine-triggered germination. Arranged in a tricistronic operon, the GerA receptors comprise three genes: *gerAA*, *gerAB* and *gerAC*[53]. Disruption of any of these cistrons abolishes germination[54–56].

Previous reports suggest that GerAA is an integral membrane protein with 4 to 6 transmembrane (TM) segments with a large N-terminal hydrophilic domain and a small hydrophilic C-terminal domain[29,58,59]. GerAB is predicted to be an integral membrane protein containing 10 to 12 TM segments, flanked by short hydrophilic termini[58,59]. GerAC is predicted to contain a pre-lipoprotein signal sequence, suggesting that the C-subunit is anchored to the outer surface of the membrane via an N-terminally attached lipid moiety[40,60,61]. Mutational analysis indicates that lipidation of GerAC is essential for germination (18, 19). The crystal structure of *B. subtilis* GerBC (homolog of GerAC) has been solved, but its function is still matter of debate[34,63].

Mutations of highly conserved residues in GerAA, GerAB and GerAC have been shown to affect germinant receptor function[15,28,56,61,62,64,65]. GerAB has been proposed to be involved in the recognition of the germinant[14,40,62,91–93]. GerAB protein has a certain homology with a superfamily of transmembrane amino acid permeases[28,46–48,67]. Previous reports of GerAB homologs in *B. megaterium* QM 1551 suggest that germinant specificity varies when alternate B subunits (GerUB and GerVB) are used[53,93]. In addition, point mutations in TM 9 and 10 of GerVB protein from *B. megaterium* reduces the affinity and specificity for germinants[54–56,94]. Furthermore, it has been suggested that the C-terminus of the B subunits is involved either in the formation of a binding pocket or affects the conformation of nearby functionally-important regions[29,58,59,95]. A recent study, using site-directed mutations in *B. subtilis gerAB*, shows that the residues modified in the transmembrane domain affect the function of GerAB and the whole GerA complex[58,59,62].

There is yet little understanding of how germinant-receptor interactions trigger Ca-DPA release, an essential event in the first step of spore germination. Vepachedu et al[40,60,61,74] suggest that germinant receptors interact with SpoVA proteins that are involved in Ca-DPA release[60,62,74]. Recent *in vitro* studies demonstrated that SpoVAC can act as a mechanosensitive channel, which would allow release of low molecular weight molecules from the spore during germination[38].

Most of the conclusions about the function of germinant receptors are based on genetic experiments, but biochemical characterization of the proteins is lacking. One of the bottlenecks in the characterization of germinant receptors is the difficulty to overexpress and purify the protein complexes[61]. Here, we report the cloning and overexpression of the *gerAA*, *gerAB* and *gerAC* genes, and the purification and membrane reconstitution of the respective GerAA, GerAB and GerAC proteins. We performed several biochemical assays including ligand binding and transport studies but could not unequivocally assign a function to the GerA proteins.

2.2 Material and Methods

2.2.1 Cloning and expression of GerA genes

GerAA, *gerAB* and *gerAC* were amplified by PCR, using the *Bacillus subtilis* 168 genome as template and Phusion polymerase, according to the manufacturer's instructions (Fermentas Life Sciences (Burlington, CA). Full-length *gerAA* and *gerAB* were cloned in frame in the pRE-NLIC vector (described by Geertsma)[96]. On the other hand, *gerAC* was cloned without the nucleotide sequence coding for the signal sequence (*gerAC*Δss). The PCR products were cloned in pRE-NLIC vector and converted into *Lactococcus lactis* expression vectors, using vector-backbone exchange system[96].

All the primers used in this work are described here:

gerAA and *gerAB* fwd nLic

5' ATGGTGAGAATTTATATTTTCAAGGTGAACAAACAGAGTTTAAGGAATATATA 3'

gerAA and *gerAB* rev nLic

5' TGGGAGGGTGGGATTTTCATTTTGTTGTAATCCTCCTCTTGAGAGC 3'

*gerAC*Δss fwd nLic

5' ATGGTGAGAATTTATATTTTCAAGGTTGGGACAGTGAGAATATCGAGGAATTA 3'

*gerAC*Δss rev nLic

5' TGGGAGGGTGGGATTTTCATTTGTTTGCGCCTTTCGTTCCGAAGTC 3'

GerAA and GerAB proteins containing N- and C-terminal His_{10} tag, respectively[96], and GerAC containing a N-terminal His_{10} tag followed by TEV cleavage site were produced in *L. lactis* NZ9000, grown semi-anaerobically in M17 medium[97] and supplemented with 1% (w/v) of glucose and 5 μg l^{-1} chloramphenicol. For large-scale protein production, *L. lactis* was grown in 2 or 10 litter batch reactors (Applikon Biotechnology, Delft) at 30 °C. The pH was kept constant at 6.5 by titration with 1M KOH. The cells were induced with 0.1 % (v/v) culture supernatant of the nisin A-producing strain NZ9700. After 2 hours of induction the cells were harvested at 5,000×g for 15 min at 4 °C and the pellet of 1 L of cells was resuspended in 10 ml of 50 mM KPi at pH 7.5. The cells were frozen in liquid nitrogen and stored at -80 °C.

2.2.2 Cell lysis and membrane vesicle preparation

Frozen cells expressing GerAA, GerAB or GerAC were thawed at room temperature and supplemented with 1 mM PMSF, 1 mM MgSO4 and DNase (∼50 μg ml^{-1}). The cells were lysed using two passages through a Constant System Ltd cell disrupter, operated at 39 kpsi and 5 °C. Cell debris was removed by centrifugation (20 min 18,500×g, at 4 °C), and subsequently membrane vesicles containing both GerAA and GerAB were collected by ultracentrifugation (90 min, 150,000×g at 4 °C); the supernatant was discarded. Membrane vesicles were kept resuspended in 50 mM potassium phosphate, pH 8.0 and store at -80 °C, following flash freezing of 1ml aliquots in liquid nitrogen. The total protein content was measured with a BCA protein assay (Thermo Scientific Pierce).

To isolate the water-soluble GerAC protein, the supernatant after ultracentrifugation of lysed cells was collected and kept on ice for purification of the protein; here, the pellet was discarded.

2.2.3 Purification of GerAA, GerAB and GerAC

For purification of GerAA and GerAB, membrane vesicles (40 mg of total protein) were thawed and diluted to approximately 5 mg ml^{-1} total protein in buffer A (50 mM KPi pH 7.5, 200 mM KCl, 20% (v/v) glycerol, 0.5% (w/v) *n*-dodecyl-β-D-maltoside (DDM) and 15 mM of imidazole) plus 1 mM of dithiotreitol (DTT) for 30 min on ice with occasional gentle mixing. Next, the mixture was centrifuged (20 min, 325,000×g, 4 °C) to separate the soluble from non-soluble material. For purification, 0.8 ml of Ni^{2+} - Sepharose resin (Amershan Biosciences) was placed in a disposable column (BioRad) and washed with 10 column volumes (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 hour at 4 °C under gentle mixing. Subsequently, the resin was washed with 20 CV of buffer A supplemented with 50 mM of imidazole plus 0.05% (w/v) DDM. The His-tagged proteins were eluted from the column by adding subsequently 640 µl, 960 µl and 960 µl of buffer A supplemented with 500 mM of imidazole plus 0.05% (w/v) DDM; most of GerAA and GerAB eluted in the 960 µl fraction. DTT (1 mM) was present in all the steps of purification.

In case of GerAC, 0.5 ml of Ni^{2+} – Sepharose resin (Amersham Biosciences) was mixed with 40 ml of supernatant from lysed cells. The mixture was incubated overnight by rotation at 4 °C in buffer A (50mM KPi, 300 mM NaCl, 10% (v/v) glycerol, pH 7.5) supplemented with 15 mM imidazole. The next day, the sample was collected in a polypropylene column and the flow through was discarded. Subsequently, the resin was washed with 40 column volumes of buffer A containing 70 mM of imidazole. The protein was eluted in fractions of 400 µl, 600 µl and 600 µl, using elution buffer containing 500 mM of imidazole. The second elution fraction was concentrated, using the 30 kDa cut-off Vivaspin concentrators with PES membrane from Sartorius, and GerAC was purified further on a Superdex 200 10/ 300 GL size exclusion column (Amersham Bioscience), using 50 mM KPi plus 200 mM NaCl at pH 7.5. Fractions of pure protein were kept on ice and used immediately for further studies. The purity of the protein samples was confirmed by SDS-PAA gel electrophoresis and Coomassie Brilliant Blue staining.

2.2.4 Multi-angle laser light scattering (SEC-MALLS)

To determine the oligomeric state of GerAC, size-exclusion chromatography coupled to multi-angle laser light scattering (SEC-MALLS) was used. 200 µl of ∼0.5 mg ml^{-1} GerAC was applied on a Superdex 200 10/300 GL gel filtration column (GE health care) in 50 mM Kpi and 200 mM NaCl pH 7.5, using an Agilent 1200 series isocratic pump (flow rate of 0.5 ml min^{-1}) at room temperature. Detectors were used for absorbance at 280 nm (Agilent), static light scattering (miniDawn TREOS Wyatt) and differential refractive index (Optilab Rex Wyatt). The ASTRA software package version 5.3.2.10 (Wyatt) was used for data analysis[98,99].

2.2.5 Membrane reconstitution of GerAA and GerAB

Purified GerAA and GerAB were reconstituted in *E. coli* polar lipids plus egg phosphatidylcholine (PC) in a ratio 3:1 (w/w), according to Geertsma et al[100], at 20 mg ml-1 of total lipid in 50 mM KPi, pH 8.0, and homogenized by extrusion through a 400-nm filter. For detergent-mediated reconstitution, the liposomes were destabilized by

the stepwise addition of Triton X-100 as described previously[100]. Protein and lipids were mixed at 1:50, 1:100 and 1:250 (w/w). Subsequently, Biobeads (SM-2 Absorbents; Bio-Rad) were added in steps to remove the detergent[100].

2.2.6 Intrinsic fluorescence of GerAA, GerAB and GerAC

Measurements of fluorescence were performed on a Spex Fluorolog 322 fluorescence spectrophotometer (Jobin Yvon) at 25 °C in a 500 µl stirred cuvette. The excitation and emission wavelengths were 280 and 340 nm, with slit widths of 1 and 2 nm, respectively. Solutions of substrate were added in 4 µL steps. We tested all amino acids, either individually or as mixtures at concentrations ranging from 1 µM to 10 mM. As a control, titrations with buffer (50 mM kPi plus 200 mM NaCl, pH 7.5) in the presence of protein were performed.

2.2.7 ANS binding to GerAC

To monitor conformational changes via an extrinsic fluorophore, GerAC was labeled with 8-anilino-1-naphthalene sulfonate (ANS). 100 µM final concentration of ANS was used for labelling. The GerAC-ANS complex was excited at 297 nm and the fluorescence emission was monitored between 300 and 550 nm[101]. Solutions of substrate were added in 4 µL steps. We tested at all amino acids individually or as mixtures at concentration ranging from 0.2 µM to 100 µM.

2.2.8 Homology modeling of GerAC

The tertiary structure of GerAC was modeled theoretically using MODELLER, a program for protein structure modeling[102], using as a template the structure of GerBC[63].

2.2.9 Circular dichroism (CD) of GerAC

To determine (changes in) the secondary structure of GerAC, Far-UV circular dichroism spectra were recorded on a Jasco J-815 CD spectrometer (Jasco, UK) at room temperature, using a quartz cell of 1 mm path length between 198 and 250 nm and scanning every 2 nm. A buffer containing 50 mM kPi plus 200 mM NaCl (pH 7.5) was used as a reference. In order to estimate GerAC secondary structure from CD spectra, we used K2D2 method currently available on line[103,104].

2.2.10 Differential scanning calorimetry (DSC) of GerAC

The stability of GerAC without and with amino acids was measured by DSC on a VP-DSC (MicroCal) at 25 °C and at 25 psi. 600 µL of GerAC (7.5 µM) in buffer containing 50 mM kPi plus 200 mM NaCl (pH 7.5) was added to the cell. To prevent the formation of air bubbles, protein samples and buffers were degassed before injection into the cell. For temperature scans in the presence of L-Ala, the samples were equilibrated for 10 min at 25 °C prior to the temperature ramps. A scan rate of 60 °C/hour and a temperature range of 30 to 65 °C were used. Data was analyzed by the MicroCal software[105].

2.2.11 Unfolding and refolding of GerAC

The fluorescence emission spectrum of the native GerAC was obtained with emission scans from 300 to 400 nm and following excitation of 280 nm. For (partial) unfolding, GerAC was treated with 3 M of urea, and, subsequently, the protein was refolded by dilution of urea (the concentration of urea remained was 0.6 mM) in the absence or presence of amino acids.

2.2.12 Counterflow assay with GerAA and GerAB reconstituted in liposomes

GerAA and GerAB reconstituted in *E. coli* polar lipids plus egg PC in a ratio 3:1 (w/w) at 1:50, 1:100 and 1:250 protein to lipid ratio (w/w) was assayed for amino acid counterflow activity. To preload the vesicles with amino acid, the proteoliposomes were frozen and thawed 3 × in 50 mM KPi, pH 7.5, 50 mM NaCl and 10 mM L-Ala. The samples were frozen in liquid nitrogen and thawed slowly at room temperature. After 11× extrusion through a 400-nm filter, the proteoliposomes were centrifuged (15 min at 280,000×g, 4 °C) and resuspended in 30 µl of buffer (50 mM KPi, pH 7.5, 50 mM NaCl and 10 mM L-Ala) to about 6.6, 3.3, and 1.3 mg ml^{-1} of protein and 333.3 mg ml^{-1} of lipid.

To determine putative binding or transport of L-Ala, aliquots of 3 µl of proteoliposomes were diluted into 293 µl of 50 mM KPi, pH 7.5 plus 50 mM NaCl, containing 5 µM of [^{14}C] L-Ala (105 µM, final concentration). The uptake of [^{14}C] L-Ala was stopped at different time intervals by dilution of the sample with 2 ml of ice-cold 0.1 M LiCl and rapid filtering on 0.45-nm cellulose nitrate filters (Schleicher & Schuell). Empty liposomes were taken as a negative control.

2.2.13 Counterflow experiment using fused membranes

The membrane vesicles expressing GerAA and GerAB were thawed at room temperature and subsequently fused with liposomes composed of *E.coli* polar lipids plus egg PC as was described previously by Driessen et al[106]. 1 mg of membrane vesicles and 10 mg of liposomes were mixed by pipetting. The suspension was rapidly frozen and stored in liquid nitrogen. Fused membranes were equilibrated with 1 or 5 mM of L-Ala and 5 mM of $MgSO_4$ for 3 or 5 hours at 25 °C. Then the sample was extruded through 400 nm and subsequently through 200 nm polycarbonate filters. The fused membranes was washed by centrifugation (15 min at 280,000×g, 4 °C) in 50 mM KPi, pH 7.5 plus 5 mM $MgSO_4$ and containing 1 or 5 mM L-Ala; the sample was resuspended at final protein concentration of 25 mg ml^{-1}. For counterflow assays, 4 µl of sample was rapidly diluted into 200 µl of KPi pH 6.0 plus $MgSO_4$ and 3 µM [^{14}C] L-Ala (103 µM, final concentration).

2.3 Results

2.3.1 Cloning, expression and purification of GerAA, GerAB and GerAC

The Vector-Backbone Exchange system (VBEx), described by Geertsma et al[96], gave good results for the overexpression of GerA receptors proteins in L. lactis. The *gerAA* and *AB* genes were cloned in tandem in the same construct and *gerAC* was

cloned as a soluble protein without the lipid anchor (described in Materials and Methods). We were able to co-purify GerAA and GerAB with a final yield of 0.03 mg and 0.3 mg respectively from 40 mg (total membrane protein); these values were calculated from gel using ImageJ, Image processing and analysis in java[107]. In case of GerAC we purified 0.5 mg from 200 mg (total soluble protein) (Figure 2.3.1).

The GerAA and GerAB proteins were expressed at different levels (Figure 2.3.1-A). The predicted molecular weights of GerAA and GerAB are 55 and 44 kDa, respectively. GerAA migrates in the gel around 55 KDa and GerAB migrates around 32 kDa. The apparent increased mobility of GerAB can be explained by the tendency of very hydrophobic proteins not to unfold completely. The levels of GerAA were low and barely visible after Ni^{2+}-Sepharose chromatography (Figure 2.3.1-A).

Figure 2.3.1-B shows the purification profile of GerAC after Ni^{2+}-Sepharose and size-exclusion chromatography. A monomeric state of GerAC was confirmed by static-light scattering measurements, yielding a molecular weight of 45 kDa (Figure 2.3.1-C), which is close to the predicted molecular weight of 42.7 kDa. Thus, of the three GerA receptors proteins, we could purify GerAB and GerAC to a high degree of purity.

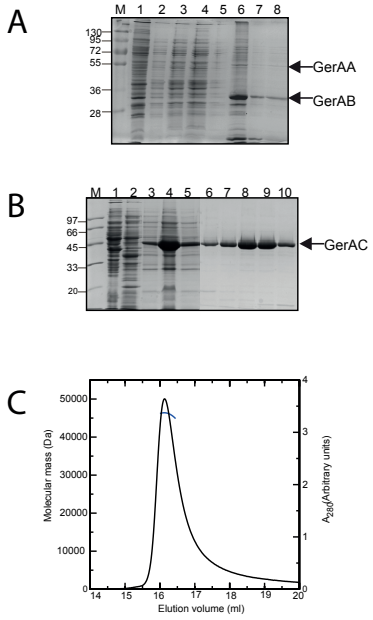

Figure 2.3.1. Expression and purification of GerAA, GerAB and GerAC. (A) Protein samples from different purification steps of GerAA and GerAB expressed in *L. lactis*. Membrane vesicles were used as starting material for the protein purification. Lane 1: DDM-insoluble fraction (∼10 µg of total protein loaded), lane 2: DDM-soluble fraction, lane 3: flow-through of the Ni^{2+}-Sepharose column, lane 4: wash fraction of the Ni^{2+}-Sepharose column, lanes 5-7: three elution fractions of the Ni^{2+}-Sepharose column, lane 8: GerAA and GerAB reconstituted in liposomes. (B) Protein samples from the GerAC purification, using the cell lysate as starting material. Lane 1: flow-through of the Ni^{2+}-Sepharose column, lane 2: wash fraction of the Ni^{2+}-Sepharose column, lanes 3-5: three elution fractions of the Ni^{2+}-Sepharose column. Lanes 6-10: peak elution fractions (∼0.5 mg ml^{-1}) of the size-exclusion chromatography. The peak elution fraction shown in lane 9 was applied to a Superdex 200 column for SEC coupled to multi-angle laser light scattering SEC-MALLS measurements. (C) Molecular weight determination by SEC-MALLS. The molecular mass was calculated through the eluting peaks and is indicated in blue line. The protein samples were run on a 12.5 % SDS-PAA gel electrophoresis and stained with Coomassie Brilliant Blue.

2.3.2 Fluorescence studies

Intrinsic fluorescence of GerAA, AB and GerAC

In order to study the binding of L-Ala (proposed germinant) or mixtures of amino acids to GerA subunits, we performed an intrinsic protein fluorescence assay either in the presence or absence of potential substrates as described in the Materials and Methods. GerAA, GerAB and GerAC show a high peak of emission spectra around 345 nm in the absence of substrate (Figure 2.3.2-A and 2.3.2-B), and we did not observe any change in emission spectra upon addition of L-Ala or a mixture of amino acids compared to the control

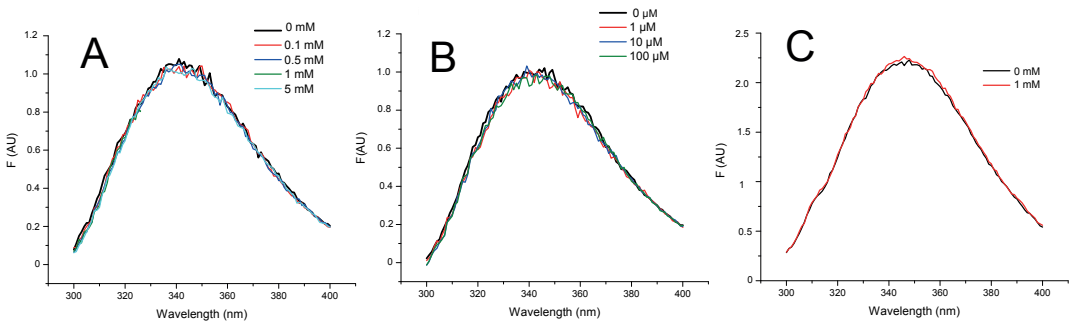

Figure 2.3.2. Effect of L-Alanine (L-Ala) or mixtures of amino acids on the intrinsic fluorescence of GerAA and GerAB and GerAC. (A). Emission spectra of GerAA and GerAB were recorded in the absence (black line) and presence of L-Ala (red, blue, green and light blue lines correspond to increasing concentrations of L-Ala). (B) Emission spectra of GerAA and GerAB in the absence (black line) or presence of mixture of all amino acids (red, blue and green indicate increasing concentrations). (C). Emission spectra of GerAC in the absence (black line) and presence of 1mM of L-Ala (red line).

(addition of buffer). Also, in case of GerAC
(Figure 2.3.2-C), no clear difference was observed after the addition of 1mM of L-Ala. These preliminary results suggest that the proteins do not exhibit a recordable binding of L-Ala or other amino acids under our experimental set up.

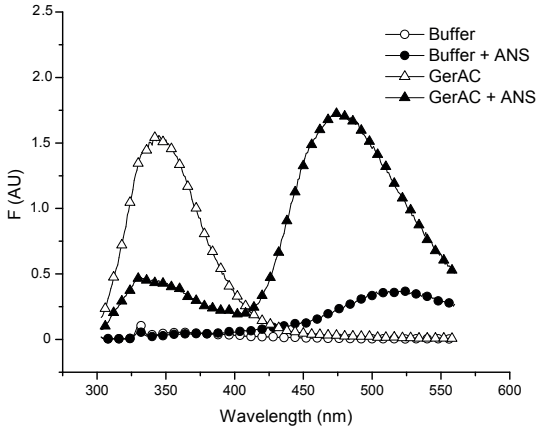

Figure 2.3.3. Fluorescence emission spectra of ANS in the absence and presence of GerAC. The excitation wavelength for the four samples was 297 nm, and the emission spectra were recorded between 300 and 600 nm. The measurements were performed in 50 mM KPi (pH 7.5). Buffer only (open circles), buffer plus 100 µM ANS (closed circles), 3 µM GerAC in buffer (open triangles) and ANS in the presence of 3 µM GerAC (closed triangle)

Changes in intrinsic protein fluorescence require a fraction of aromatic residues, most notably Tryptophan (Trp), to change environment upon binding of ligand, which does not always happen in proteins binding a particular ligand. We thus performed an alternative fluorescence-based assay to probe ligand-binding activity, using ANS as extrinsic probe for GerAC. We obtained a successful binding of ANS to GerAC, as can be seen from the resonance energy transfer from the protein (decrease in peak at around 345 nm) to ANS (increase in peak at around 475. nm) (Figure 2.3.3).

Figure 2.3.4 shows the effect of subsets of amino acids (neutral, basic and acid in Figure 2.3.4-B, 2.3.4-C and 2.3.4-D, respectively) in the fluorescence of GerAC-ANS. Clearly, the protein did not exhibit a significant and reproducible change in the profile of the fluorescence upon the addition of the amino acid mixtures, i.e. when compared to the control sample (buffer). In fact, a decrease of the fluorescence was

observed for every case, disregarding the nature of the amino acids, and the effect was similar in the control sample (Figure 2.3.4-A). Taking together, the protein did not clearly exhibit specificity for any of the substrates tested in our experiments.

Unfolding and refolding of GerAC with Urea

The fluorescence maxima of a native protein is strongly correlated with the polarity of the environment of the Trp residues and can range from 308-350 nm, with aromatic residues in apolar microenvironments having blue emission and residues in polar environments having red emission. Unfolding of a protein almost always leads to a red shift in the emission to wavelength maxima of around 345-355 nm[108,109].

A feature found in the previous experiments with GerAC protein clearly shows that the emission spectra of unlabelled GerAC have a maximum at 345 nm. Apparently this behaviour can be attributed to high degree of solvent accessibility to aromatic residues (e.g. Trp). This indicates that a significant fraction of aromatic residues of recombinantly-expressed and purified GerAC are (partially) exposed to solvent with emission in the red.

Next, we tested whether the fluorescence spectra of GerAC could be shifted upon (partial) denaturation and refolding. Firstly, we treated the protein with 3M of urea. Unfolding of the protein was confirmed by the emission spectra with a peak near 360 nm. After that, we attempted to refold GerAC by dilution of urea with buffer and observed an emission maximum at 350 nm (Figure 2.3.6-A), indicating that GerAC did not go back to its native structure. Posteriorly, we added the amino acid mixture but did not observe any change in the intrinsic protein fluorescence.

Homology modelling of GerAC

In order to understand the molecular details of our previous fluorescence experiments, we analysed the X-ray structure of the highly homologous GerBC (33 % of sequence identity, PDB: 3N54). The structure reveals that tryptophan residues are indeed exposed to the environment (Figure 2.3.5-B). To depict the structural organization of GerAC more clearly, we modelled the tertiary structure followed by a relaxation and 1 μs molecular dynamics simulation. Figure 2.3.5-A depicts the average structure, revealing a clear exposure of the aromatic residues to the solvent. Further calculation of the surface area accessible to solvent of tryptophans revealed a total of 8 nm^2 and 11 nm^2 for GerBC and GerAC respectively. In fact, this result is attributed to the extra aromatic residue present in GerAC, increasing the area of exposure.

Overall, our results suggest that GerAC shows a partially red shifted emission spectrum, similar to GerBC, which structure shows that most of the residues are exposed to the solvent[63]. Moreover, the aromatic residues in GerAC are prone to be solvent exposed, which is in agreement with our fluorescence measurements.

2.3.3 Circular dichroism (CD)

In order to validate the results from the fluorescence assays, we performed circular dichroism (CD) measurements to determine the secondary structure of unfolded and refolded GerAC. We observed that GerAC contains a mixture of α-helix and β-sheet structures with predicted values of 24.05 % and 22.54 %, respectively[104] (Figure 2.3.6-B). These results are in agreement with those for GerBC. As described previously, GerBC adopts an uncharacterized

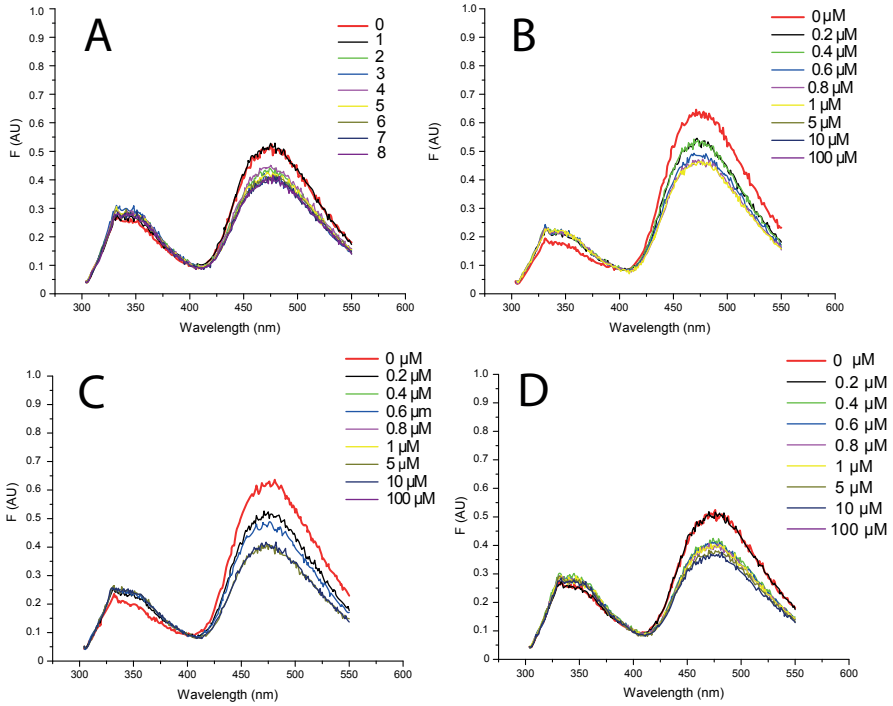

Figure 2.3.4. Fluorescence Spectra of GerAC-ANS complex titrated with amino acid mixture. (A) Titration of GerAC-ANS with buffer (1 to 8 represent, buffer addition of 4 µl). (B) Titration of GerAC-ANS with 0 to 100 µM of neutral amino acids (mixture). (C) Titration of GerAC-ANS with 0 to 100 µM of basic amino acids (mixture). (D) Titration of GerAC-ANS with 0 to 100 µM of acid amino acids (mixture). For each addition 4 µl of solution was used in all the experiments.

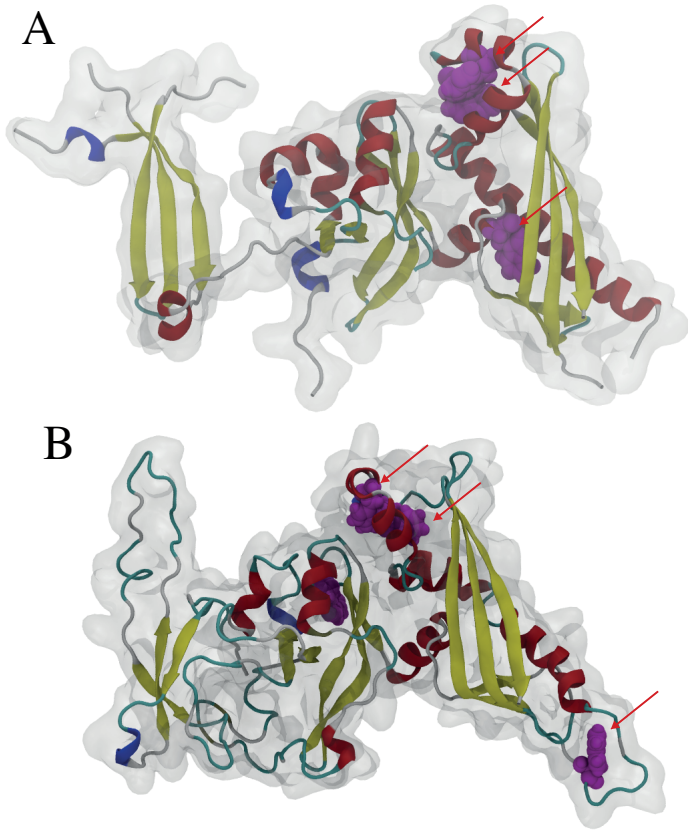

Figure 2.3.5. Homology modelling of GerAC. (A) GerAC homology model; (B) GerBC crystal structure; data taken from Li et al [63]. The red arrows show Trp residues that are depicted in purple color.

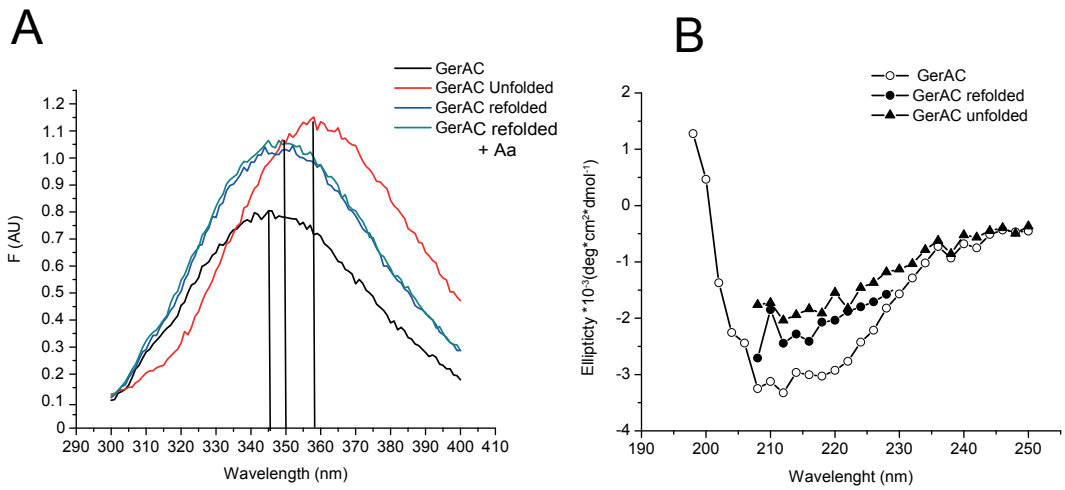

Figure 2.3.6. Unfolding and refolding of GerAC. Emission spectra scans from 300 to 400 nm was measured. GerAC in the absence of urea is shown as black line; GerAC in the presence of 3M urea is shown as red line; GerAC refolded upon dilution of urea is shown as blue line; refolded GerAC in the presence of amino acid mixture is shown as green line. (B) CD spectra of unfolded and refolded GerAC. GerAC without urea (open circles); GerAC with 3 M of urea (closed triangles) and GerAC refolded (closed circles). The concentration of GerAC used in both experiments was 0.3 µM. Both experiments (A) and (B) were done the same day with the same protein preparation.

type of protein fold consisting of three distinct domains containing β—sheets surrounded by multiple α-helices[63].

We were unable to record CD spectra below 205 nm upon incubation with 3M of urea, however, we observed changes in the CD signals at either 222 or 225 nm, which are essential to assess protein unfolding[110,111]. The observed shift in ellipticity of α-helix and β-sheet towards random coil structures indicates that the protein was indeed unfolded. Upon urea dilution, the original secondary structure was not totally recovered, indicating that the protein does not refold well.

2.3.4 Differential Scanning Calorimetry (DSC)

DSC was used to study the thermal stability of GerAC without or with added amino acids. Figure 2.3.7 shows thermograms of GerAC in the presence or absence of 1 mM of amino acid mixture. We determined a melting temperature of around 47 °C and a very low heat capacity (Cp), which is indicative of a largely unstable or unfolded protein.

2.3.5 Binding or transport of GerAA and GerAB triggered by L-Ala

Because other studies indicate that GerAB could be involved in binding of germinants and conceivably transport L-alanine[62], we purified and reconstituted GerAA and GerAB in *E.coli* polar lipids plus egg PC and probed the counterflow uptake of L-Ala. The counterflow uptake is a useful assay to measure the inward flux of an isotopic-labelled solute ([^{14}C] L-Ala) for the outward flux of an unlabelled compound (L-Ala or other amino acid).

Proteoliposomes were loaded with 10 mM of L-Ala at pH 7, and uptake of [^{14}C] L-Ala at 5 µM was measured. Three protein-to-lipid ratios (1:20, 1:50 and 1:100, w/w) were tested, however, we did not find significant uptake of radiolabeled L-Ala (data not shown). In addition, we fused membrane vesicles from *L. lactis* expressing GerAA and GerAB with liposomes composed of *E.coli* polar lipids plus egg PC as described in Materials and Methods, but again no uptake above background was observed (data not shown).

2.4 Discussion

2.4.1 Cloning and overexpression of GerA receptor proteins

We have successfully cloned and overexpressed *gerAA*, *gerAB* and *gerAC* genes in *L. lactis*, and purified the GerA receptor proteins. GerAB and GerAC were obtained in higher amounts than GerAA, but the isolated GerAA and GerAB protein products were sufficient for biochemical studies such as reconstitution of the proteins in *E. coli* polar lipids and egg PC.

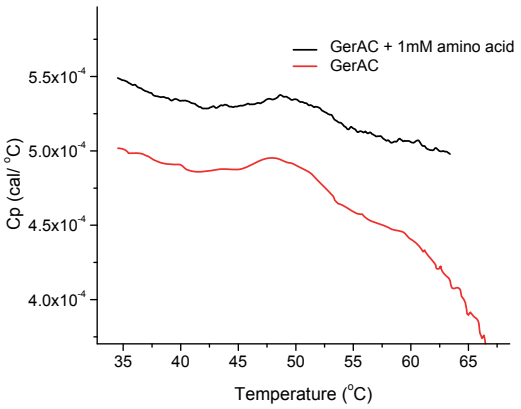

Figure 2.3.7. Differential scanning calorimetry of GerAC in the absence and presence of amino acids mixture (1 mM final concentration). The concentration of GerAC was 7.5 µM.

2.4.2 Spectroscopic and calorimetric analysis of GerA receptor proteins

Tryptophan fluorescence emission spectroscopy is a standard method to monitor conformational changes in proteins upon binding of ligands[112]. We have not observed any changes in fluorescence upon the addition of amino acids to GerAA, GerAB and GerAC, implying that i) the (individual) proteins do not bind amino acids. ii) a complex of all three is necessary for the binding. iii) one or more of the proteins are misfolded, which may affect the overall activity. The latter was specifically studied for GerAC, using extrinsic probe-protein fluorescence, DSC and CD spectroscopy. Overall, our results indicate that the secondary structure of GerAC and GerBC protein are similar[63], and both proteins expose several aromatic residues (tryptophans) to the solvent. Thus, the localization of the aromatic residues in the GerBC protein suggests that they are not part of a proper binding site to allow screening of ligand binding by fluorescence changes.

2.4.3 Binding or transport mediated by GerAA and GerAB

GerAA and GerAB were reconstituted in liposomes composed of *E.coli* polar lipids plus egg PC, and counterflow assays have been performed, to probe for potential transport of radiolabeled L-Ala. However, no additional influx of labeled L-Ala was observed in pre-loaded liposomes compared to the empty (control) liposomes. This outcome may have been affected by experimental limitations. Firstly, in our binding assays the proteins were 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). The in vivo composition of the *B. subtilis* spore inner membrane lipids is different[113], which may have affected potential transport activity. Secondly, it is conceivable that the purified GerAA and GerAB were not sufficiently stable in the detergent-solubilized state. Increased stability may be obtained by using other types of detergent, increase the amount of glycerol, modification of the ionic strength of the buffer, and/or adding lipids to the solubilized protein(s). Thirdly, germinant receptors may cooperatively work as a complex, not only among GerA family (GerAA, AB and AC) but also with other operons such as GerB and GerK (which were not present in this study) as was suggested by Atluri et al and Li et al[65,66]. Notably, we managed to co-reconstitute the three different GerA proteins: GerACs (with signal sequence for lipid modification),GerAA and GerAB according to a previously described method by Geerstma et al[100]. We were unable to monitor any binding and/or transport activity with the three GerA proteins (data not shown). We faced several shortcomings in expression and purification of the proteins (e.g. low amount of GerACs, poor detergent-solubilization and aggregation), which may have prohibited the detection of ligand binding or transport activity.