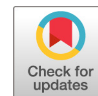
AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

COMMENTARY



Genetic Suppression Meets Structure Prediction: Probing a Spore Germination Receptor Complex

Anne Moir,[a] David L. Popham[b]

[a]School of Biosciences, University of Sheffield, Western Bank, Sheffield, United Kingdom
[b]Department of Biological Sciences, Virginia Tech, Blacksburg, Virginia, USA

ABSTRACT Despite the thousands of spore germinant receptor operons identified in genomes of bacilli and clostridia, understanding how the three essential receptor components act as a signal transduction machine in germination remains limited. The paper by Amon et al. in this issue uses the classical genetic approach of suppression to define a region of likely interaction between the GerAA and GerAB proteins: it provides a first glimpse into potential events within the receptor complex (J. D. Amon, L. Artzi, and D. Z. Rudner, J Bacteriol 204:e00470-21, 2022, https://doi.org/10.1128/JB.00470-21).

KEYWORDS Bacillus, germination, receptor interaction, spore, structure prediction, suppression

In bacilli and most clostridia, initiation of spore germination requires the action of three proteins, usually coexpressed in an operon; together, they constitute a receptor in the inner membrane of the spore, which somehow detects and responds to a nutrient germinant. This triggers, via unknown mechanisms, release of monovalent ions, release of Ca^{2+} and dipicolinic acid (DPA), and activation of cortex lytic enzymes. The inner membrane, in a semicrystalline state in dormant spores (1), regains permeability, and the spore core rehydrates culminating in exit from dormancy and outgrowth. Many species possess multiple receptors that respond to different germinant stimuli (2–4). The functions of players involved in downstream events in germination, such as the SpoVA proteins responsible for DPA release, and the cortex lytic enzymes SleB and CwlJ, have been defined, although the details of their mechanism of activation remain unclear. A recent review admirably summarizes the current limits to our understanding of the spore germination process (5).

The three proteins that constitute a single receptor are the A, B, and C subunits: GerAA, GerAB, and GerAC for the GerA receptor. The A and B subunits are integral membrane proteins with membrane surface-exposed domains, while the C subunit is a membrane-anchored lipoprotein. The A subunit is predicted to possess four to six transmembrane helices, with large N-terminal and small C-terminal surface-exposed domains, although there has been disagreement on whether these domains are intracellular or extracellular. Structural analysis of this domain of the *Bacillus megaterium* GerKA protein revealed a fold similar to that of a transporter substrate-binding domain and suggested that it may be involved in germinant binding (6). This would require this domain to be exterior to the spore inner membrane, a position that has not been definitively demonstrated and is not strongly supported by the protein sequence. The B subunit appears to possess 10 transmembrane helices with no large surface-exposed domains (7–9). The B subunits are distant relatives of the APC superfamily of transporters (10, 11) and several studies have suggested that the B proteins function as the germinant recognition subunits (7, 9, 11–13). The C subunit is a globular protein that is membrane anchored via a diacyl-glycerol lipid. Structural analysis of this *Bacillus subtilis* GerBC revealed a novel protein fold (14). The precise required role for the C protein within the receptor is still unclear.

Editor Tina M. Henkin, Ohio State University
Copyright © 2022 American Society for Microbiology. All Rights Reserved.
Address correspondence to David L. Popham, dpopham@vt.edu.
The authors declare no conflict of interest.
For the article discussed, see https://doi.org/10.1128/JB.00470-21.
The views expressed in this article do not necessarily reflect the views of the journal or of ASM.
Accepted manuscript posted online 6 December 2021
Published 15 February 2022

Downloaded from https://journals.asm.org/journal/jb on 29 June 2022 by 134.174.140.59.

Previously described mutations affecting the GerAA subunit of the alanine receptor in *B. subtilis* included site-directed mutations in a region within its hydrophobic domain containing two highly conserved proline residues. Of these, the GerAA (P326S) mutant phenotype was particularly striking and unexpected, as the spores released at the end of sporulation were phase dark and heat sensitive (15). Amon et al. (16) have demonstrated that this substitution in the GerAA receptor protein results in a hyperactive germinant receptor complex (GerA*), causing premature germination of spores in response to endogenous low levels of alanine during spore formation, and showed that it is dominant over wild type.

Using the classical genetic approach of suppression and a strain in which the only germinant receptor was GerA*, they applied serial enrichment cycles for spores surviving a heat challenge, recovering strains carrying suppressor mutations that prevent or reduce this premature germination, yielding a relatively high proportion of mature, heat-resistant spores that could germinate on rich medium and be recovered as colonies. This improved "viability" provided an easily measurable parameter of suppression and was combined with microscopy, scoring the percentage of phase-bright mature spores at the end of sporulation. The outcome of multiple experiments was the isolation of 83 suppressed strains; six of these reversed the original amino acid substitution, and the others altered 37 different codons across all three GerA receptor genes—a rich haul for further investigation. Not all of these have yet been explored in detail; this paper concentrates on suppressors of the GerAA (P326S) mutation that are located in the GerAB protein.

When introduced into a strain carrying wild-type GerAA and GerAC genes, most of these GerAB suppressor mutations on their own result in the ability of spores to form colonies on LB agar that was 15 to 40% that of wild type; this weakened (hypomorphic) response to germinant could explain suppression as a dampening of the A* response in the developing sporangium.

Two suppressors, however, showed very poor colony-forming ability in a wild-type background. However, these would increase spore "viability" (recovery of heat-resistant mature spores) of a GerA* receptor strain. By preparing washed, phase-bright spores and examining germination with L-alanine by the fall in optical density of spore suspensions, these could be studied in detail. One, GerAB (E105K), did not respond to L-alanine at all in an otherwise wild-type background, but in an A* receptor, spores in the population germinated very gradually, with a mean lag time of ~4 h, independent of alanine. Their interpretation is that this GerAB mutant protein cannot recognize L-alanine as a germinant and that the A* receptor now shows its basal spontaneous, germinant-independent triggering rate.

The second of these mutations was particularly revealing: for GerAB (F259S), the L-alanine germination behavior in an A* receptor mimicked the wild type, with only a very, very slow response without L-alanine. A model is proposed in which GerAB F259S protein is indeed capable of detecting alanine, but this AB protein cannot transmit the signal to GerAA unless the latter is in the A* conformation. The two mutations AA (P326S) and AB (F259S) thus appear compensatory and likely reflect a site of functional interaction. Introduction of the F259S mutation also blocked the germination signal from a hyperactive GerAB, isolated in a recent study of GerAB from the same laboratory (13), confirming that its block is downstream of nutrient sensing. From these genetic data, Amon et al. (16) have built a hypothetical diagram of receptor subunits with an interaction site between AA and AB, which would be at or close to residues 326 in GerAA and 259 in GerAB and which would represent a site of signal transduction from GerAB to GerAA.

Using multiple sequence alignments from the many thousands of Ger receptor operon homologs in genome sequences to identify coevolving residue positions, the Rudner lab generated a model that located regions of potential intermolecular contact between the three GerA subunits (13). Amon et al. have applied the AlphaFold 2.0 program, a new and potentially powerful tool to predict protein structure, to GerA (16). Strikingly, it predicts the close positioning of the first extracellular loop of GerAA, which is anchored by proline 326, and the fourth extracellular loop of GerAB, containing phenylalanine 259, the predicted sites of interaction from the suppressor analysis (Fig. 5 of Amon et al. [16]). These two loop

Downloaded from https://journals.asm.org/journal/jb on 29 June 2022 by 134.174.140.59.

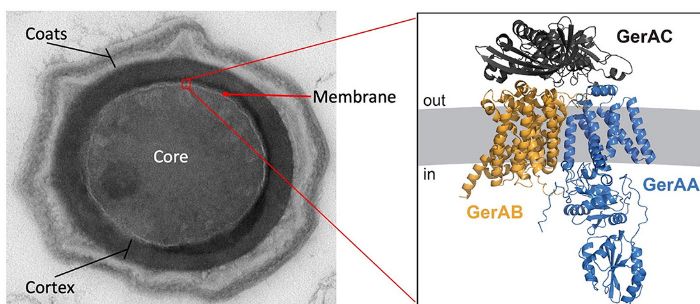


FIG 1 Spore germinant receptor location and predicted structure. The dormant endospore core is surrounded by a membrane and then cortex peptidoglycan and protein coat integument layers. Nutrient germinants can penetrate the integuments to interact with Ger receptors that are embedded in the membrane. The protein ribbon model indicates the predicted structures and orientations of the *B. subtilis* GerA receptor proteins. The electron micrograph was provided by M. Laue and MJ. Flores; the protein model is reproduced from Amon et al. (16).

regions are also the sites of several of the other P326S-suppressing amino acid substitutions in the GerAA and GerAB proteins.

How close this overall three-component structure (Fig. 1) is to reality remains to be further tested, and the more precise positioning of residues remains to be defined. Nevertheless, it seems plausible, and its predictions for receptor subunits would in general match their predicted membrane organization. The GerAB protein would have 10 membrane-spanning helices, as generally predicted. The extracellular GerAC lipoprotein anchored in the membrane would be in contact with both GerAA and GerAB. GerAA merits further discussion, because its predicted topology is less clear. Both AlphaFold 2.0 and a recent analysis of GerAA (13) would place N-terminal and C-terminal hydrophilic domains of GerAA intracellularly, as did a previous topological study of a *Bacillus anthracis* germinant receptor, GerH (17). In the AlphaFold 2.0 prediction, the hydrophobic domain would have six membrane-spanning helices. In *B. anthracis* GerH, four of these six transmembrane helices were predicted, but their analysis lacked green fluorescent protein (GFP) fusions to test the final two (17). A limited analysis, using *phoA* fusions expressed transiently in *Escherichia coli*, also suggested that fusion points at positions 327 in GerAA and 254 in GerAB, in loops that are the currently proposed sites of interaction, are located periplasmically (18).

Because the synthesis and assembly of the receptor takes place during sporulation before dehydration, predictions of topology based on normal membrane behavior would appear reasonable. Eventually, the inner membrane in mature spores is in a semicrystalline state (1), and it is possible that it might support or sequester the Ger receptors in a different conformation. Nevertheless, the genetic evidence for interaction between the two extracellular loops certainly applies in mature, dormant spores. To complicate the situation further, in Bacilli, the receptors, even those responding to different germinants, are usually all clustered in one or two foci in the spore membrane, as a germinosome, in combination with the GerD protein (19), although they can function in a less sensitive manner when dispersed, in the absence of GerD protein.

The experiments of Amon et al. (16) suggest one site of intersubunit signaling within the receptor complex. There are likely to be others, as yet unexplored. Neither do they tell us how the signal is transduced from the receptor to any downstream components—all suppressors are within the receptor genes themselves—this could be because no other protein is required for an initial biophysical change or because suppressing interactions with downstream proteins could be missed as a result of the experimental conditions of the enrichments. Some published evidence indicates direct interaction between the *B. subtilis* GerA receptor and the SpoVA Ca^{2+}-DPA channel (20), which could be a pathway for signaling to break the dehydrated dormant spore state. However, no evidence of such direct signaling has been developed.

Amon et al. have demonstrated the power of a genetic approach that requires no structural assumptions (16). Functional analysis of the remaining suppressor mutants and the

Downloaded from https://journals.asm.org/journal/jb on 29 June 2022 by 134.174.140.59.

extension of the suppressor approach should predict more functionally important interaction sites. Coupled with their comparison to the predicted structural model, there is finally some hope of shedding at least partial light on germinant receptor dynamics.

REFERENCES

1. Cowan AE, Olivastro EM, Koppel DE, Loshon CA, Setlow B, Setlow P. 2004. Lipids in the inner membrane of dormant spores of *Bacillus* species are largely immobile. Proc Natl Acad Sci U S A 101:7733–7738. https://doi.org/10.1073/pnas.0306859101.
2. Moir A. 2006. How do spores germinate? J Appl Microbiol 101:526–530. https://doi.org/10.1111/j.1365-2672.2006.02885.x.
3. Ireland JA, Hanna PC. 2002. Amino acid- and purine ribonucleoside-induced germination of *Bacillus anthracis* DeltaSterne endospores: *gerS* mediates responses to aromatic ring structures. J Bacteriol 184:1296–1303. https://doi.org/10.1128/JB.184.5.1296-1303.2002.
4. Barlass PJ, Houston CW, Clements MO, Moir A. 2002. Germination of *Bacillus cereus* spores in response to L-alanine and to inosine: the roles of *gerL* and *gerQ* operons. Microbiology 148:2089–2095. https://doi.org/10.1099/00221287-148-7-2089.
5. Christie G, Setlow P. 2020. *Bacillus* spore germination: knowns, unknowns and what we need to learn. Cell Signal 74:109729. https://doi.org/10.1016/j.cellsig.2020.109729.
6. Li Y, Jin K, Perez-Valdespino A, Federkiewicz K, Davis A, Maciejewski MW, Setlow P, Hao B. 2019. Structural and functional analyses of the N-terminal domain of the A subunit of a *Bacillus megaterium* spore germinant receptor. Proc Natl Acad Sci U S A 116:11470–11479. https://doi.org/10.1073/pnas.1903675116.
7. Blinker S, Vreede J, Setlow P, Brul S. 2021. Predicting the structure and dynamics of membrane protein GerAB from *Bacillus subtilis*. Int J Mol Sci 22:2793. https://doi.org/10.3390/ijms22073793.
8. Christie G, Lazarevska M, Lowe CR. 2008. Functional consequences of amino acid substitutions to GerVB, a component of the *Bacillus megaterium* spore germinant receptor. J Bacteriol 190:2014–2022. https://doi.org/10.1128/JB.01687-07.
9. Christie G, Lowe CR. 2008. Amino acid substitutions in transmembrane domains 9 and 10 of GerVB that affect the germination properties of *Bacillus megaterium* spores. J Bacteriol 190:8009–8017. https://doi.org/10.1128/JB.01073-08.
10. Jack DL, Paulsen IT, Saier MH. 2000. The amino acid/polyamine/organocation (APC) superfamily of transporters specific for amino acids, polyamines and organocations. Microbiology 146:1797–1814. https://doi.org/10.1099/00221287-146-8-1797.
11. Moir A, Corfe BM, Behravan J. 2002. Spore germination. Cell Mol Life Sci 59:403–409. https://doi.org/10.1007/s00018-002-8432-8.
12. Christie G, Gotzke H, Lowe CR. 2010. Identification of a receptor subunit and putative ligand-binding residues involved in the *Bacillus megaterium* QM B1551 spore germination response to glucose. J Bacteriol 192:4317–4326. https://doi.org/10.1128/JB.00335-10.
13. Artzi L, Alon A, Brock K, Green AG, Tam A, Ramirez-Guadiana FH, Marks D, Kruse A, Rudner AD. 2021. Dormant spores sense amino acids through the B subunits of their germination receptors. Nat Commun 12:6842. https://doi.org/10.1038/s41467-021-27235-2. https://doi.org/10.1038/s41467-021-27235-2.
14. Li Y, Setlow B, Setlow P, Hao B. 2010. Crystal structure of the GerBC component of a *Bacillus subtilis* spore germinant receptor. J Mol Biol 402:8–16. https://doi.org/10.1016/j.jmb.2010.07.018.
15. Mongkolthanaruk W, Cooper GR, Mawer JS, Allan RN, Moir A. 2011. Effect of amino acid substitutions in the GerAA protein on the function of the alanine-responsive germinant receptor of *Bacillus subtilis* spores. J Bacteriol 193:2268–2275. https://doi.org/10.1128/JB.01398-10.
16. Amon JD, Artzi L, Rudner DZ. 2022. Genetic evidence for signal transduction within the *Bacillus subtilis* GerA germinant receptor. J Bacteriol 204:e00470-21. https://doi.org/10.1128/JB.00470-21.
17. Wilson MJ, Carlson PE, Janes BK, Hanna PC. 2012. Membrane topology of the *Bacillus anthracis* GerH germinant receptor proteins. J Bacteriol 194:1369–1377. https://doi.org/10.1128/JB.06538-11.
18. Cooper G. 2009. Structure/function relationships in the GerA spore germination receptor of *Bacillus subtilis*. PhD thesis. University of Sheffield, Sheffield, England.
19. Griffiths KK, Zhang J, Cowan AE, Yu J, Setlow P. 2011. Germination proteins in the inner membrane of dormant *Bacillus subtilis* spores colocalize in a discrete cluster. Mol Microbiol 81:1061–1077. https://doi.org/10.1111/j.1365-2958.2011.07753.x.
20. Vepachedu VR, Setlow P. 2007. Analysis of interactions between nutrient germinant receptors and SpoVA proteins of *Bacillus subtilis* spores. FEMS Microbiol Lett 274:42–47. https://doi.org/10.1111/j.1574-6968.2007.00807.x.

Downloaded from https://journals.asm.org/journal/jb on 29 June 2022 by 134.174.140.59.