Proteins YlaJ and YhcN contribute to the efficiency of spore germination in Bacillus subtilis

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One sentence summary: The YlaJ and YhcN proteins help Bacillus subtilis spores to germinate.

ABSTRACT

The YlaJ and YhcN spore lipoproteins of Bacillus subtilis contain a common domain, and are of unknown function. Homologues of YlaJ or YhcN are widespread in Bacilli and are also encoded in those Clostridia that use cortex lytic enzymes SleB and CwlJ for cortex hydrolysis during germination. In B. subtilis, we report that single and double mutants lacking YlaJ and/or YhcN show a reduced rate of spore germination in L-alanine, with a delay in loss of heat resistance, release of dipicolinic acid and OD fall. If B. subtilis spores lack the cortex lytic enzyme CwlJ, spore cortex degradation and subsequent outgrowth to form colonies is strictly dependent on the other cortex lytic enzyme SleB, allowing a test of SleB function; in a cwlJ mutant background, the combined loss of both ylaJ and yhcN genes resulted in a spore population in which only 20% of spores germinated and outgrew to form colonies, suggesting that SleB activity is compromised. YlaJ and YhcN have a role in germination that is not yet well defined, but these proteins are likely to contribute, directly or indirectly, to early events in germination, including effective SleB function.

Keywords: spore; germination; Bacillus; YlaJ; YhcN

INTRODUCTION

Bacillus subtilis spores germinate in response to nutrient germinants by interaction of those germinants with cognate receptors in the inner membrane of the spore (Moir 2006; Cooper and Moir 2011; Mongkolthanaruk et al. 2011; Paredes-Sahja, Setlow and Sarker 2011). Heat resistance is lost, and ions and dipicolinic acid (DPA) released from the spore. The enzymes responsible for hydrolysis of the peptidoglycan of the spore cortex, SleB and CwlJ, are then activated (Setlow 2013). The CwlJ enzyme can be activated for germination by the addition of exogenous CaDPA, and is likely to respond to CaDPA released from the core during nutrient germination (Paidhungat, Ragkousi and Setlow 2001); in contrast, the mechanism of activation of the SleB protein remains unknown, although the YpeB protein, frequently encoded in the same operon as SleB, is required for its localisation in the spore (Chirakkal et al. 2002; Bernhards and Popham 2014), and is likely to have an additional role in its regulation (Li et al. 2013).

Bacillus subtilis proteins YhcN and YlaJ are spore proteins that contain a common domain of unknown function (InterPro 019076), found exclusively in Firmicutes; YlaJ is distinguished by its acidic, low complexity, 40 amino acid C-terminal domain; an alignment of their amino acid sequences is shown in Fig. S1, Supporting Information. Homologues of these two proteins are found in the genomes of Bacilli and some Clostridia. Both YlaJ and YhcN are predicted lipoproteins, and are forespore expressed and sigmaG-dependent (Bagyan et al. 1998; Johnson 2005; Steil et al. 2005). The YhcN protein has been detected in the inner spore membrane (Bagyan et al. 1998), and a yhcN mutant was reported by these authors as slower to outgrow. Both YhcN and YlaJ are reported as abundant proteins in the
spore inner membrane proteome (Zheng et al. 2016); both have also been extracted from spore outer layers (Kuwana et al. 2002), suggesting that they may be present in both locations. Notably, a processed form of YhcN, lacking the N-terminal 39 amino acids, has been detected as a major protein component in the germination exudate of *B. subtilis* spores (Chirakkal et al. 2002). Although the yla genes are very frequently monocistronic, in the *B. cereus/anthracis/thuringiensis* family, the yla gene is located as a third gene in the sleB ypeB operon. The aim of this study was to explore in more detail the spore germination behaviour of mutants of *B. subtilis* lacking one or both of these proteins.

**METHODS**

**Bacterial strains**

These are listed in Table 1. Mutations in yla (inactivated by pMUTIN4 insertion (Kobayashi et al. 2003)) and yhcN (by replacement of most of the gene by a spectinomycin cassette (Bagyan et al. 1998)) were introduced into the genetic background of our laboratory strain AM1604 by transformation. Strain constructions were verified by colony PCR.

**Spore germination assays**

Spores were heat activated at 70°C for 30 min, and stored on ice for up to 3 h. The OD₄₉₀ of spore suspensions was measured during incubation at 37°C in 10 mM Tris–HCl, pH 8.4, 10 mM KCl, following addition of L-alanine (concentration as described in the text), in a Wallac Victor 1420 Multilabel counter, at an initial OD of 0.3. Germination assays were carried out in triplicate, on several independent spore preparations. Representative data are shown.

Germination in CaDPA was performed as described by Jaye and Ordal (1965), without prior spore heat activation. For measurement of loss of heat resistance in response to CaDPA and in nutrient germination, aliquots of spore suspensions were serially diluted in water, heat challenged (70°C for 30 min), plated on Nutrient Agar and counted after overnight incubation at 37°C. The percentage of spores that had germinated was calculated by comparing the number of heat-resistant survivors to that of equivalently incubated spores that had not been exposed to germinant.

<table>
<thead>
<tr>
<th>Table 1. Bacterial strains.</th>
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<tbody>
<tr>
<td>1604</td>
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<tr>
<td>BSF1229</td>
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<tr>
<td>IB345</td>
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<tr>
<td>HC101</td>
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<tr>
<td>CJ01</td>
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<tr>
<td>CJ06</td>
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<tr>
<td>CJ11</td>
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<td>CJ17</td>
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<td>CJ18</td>
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<td>CJ19</td>
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<tr>
<td>ER221</td>
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<td>CJ14</td>
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<td>CJ15</td>
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<td>CJ16</td>
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</table>

Figure 1. Spore germination in L-alanine at (A), 10 mM and (B), 0.25 mM, measured by the decrease in OD of spore suspensions after addition of germinant. Symbols: (closed square) parental strain 1604; (closed circle) ylaJ; (closed diamond) yhcN; (closed triangle) ylaJ yhcN; (open diamond) no germinant.

**Measurement of DPA**

DPA released during germination was measured in filtered supernatants by the method of Scott and Ellar (1978), and compared to the total DPA extracted from dormant spores by boiling for 20 min.

**RESULTS**

**Germination behaviour**

Spores of mutants carrying ylaJ or yhcN mutations, singly or in combination, in the otherwise isogenic background of our laboratory strain, AM1604, germinated more slowly in L-alanine than did the wild-type parent, as estimated by the loss of OD of a spore suspension (Fig. 1A and B). The average lag before germination was longer at a saturating germinant concentration (10 mM) and further extended in response to 250 μM L-alanine. The germination defect of double mutants lacking both ylaJ and yhcN was also delayed; however, the germination defect was not more severe than for the single mutants.

The OD fall of spore suspensions is considered as reflecting both early and late events in germination, full OD loss reflecting rehydration of the spore core. Therefore, we also examined an early event, loss of heat resistance (Luu and Setlow 2014) (Fig 2). Single and double mutant spore suspensions were slower than wild type to lose heat resistance as well as to lose OD, suggesting that early events, too, were delayed. DPA release, measured at limiting alanine concentrations for maximum clarity, was also delayed (Fig. 3).
Germination in exogenously added CaDPA

The germination response to CaDPA is dependent on the CwlJ cortex lytic enzyme, but not on SleB or germinant receptors. The single and double mutants germinated well with CaDPA, if slightly slower than the parental strain (Fig. 4), suggesting that YlaJ and YhcN are not required for germination in CaDPA.

Table 2. Colony-forming ability of spores.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Colonies from spores (×10⁸)</th>
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<tbody>
<tr>
<td>HC101</td>
<td>cwlJ</td>
<td>1.59 ± 0.16</td>
</tr>
<tr>
<td>CJ17</td>
<td>cwlJ ylaJ</td>
<td>1.26 ± 0.14</td>
</tr>
<tr>
<td>CJ18</td>
<td>cwlJ yhcN</td>
<td>1.28 ± 0.05</td>
</tr>
<tr>
<td>CJ19</td>
<td>cwlJ ylaJ yhcN</td>
<td>0.4 ± 0.03</td>
</tr>
<tr>
<td>ER221</td>
<td>cotE</td>
<td>1.52 ± 0.1</td>
</tr>
<tr>
<td>CJ14</td>
<td>cotE ylaJ</td>
<td>0.42 ± 0.03</td>
</tr>
<tr>
<td>CJ15</td>
<td>cotE yhcN</td>
<td>0.47 ± 0.05</td>
</tr>
<tr>
<td>CJ16</td>
<td>cotE ylaJ yhcN</td>
<td>0.52 ± 0.06</td>
</tr>
</tbody>
</table>

All spore suspensions were at OD₄₉₀ = 1.0

The defect is not overcome by increasing spore permeability to germinants

Mutants lacking proteins of the gerP operon show reduced germination, apparently because these proteins are required for rapid access of germinants to their receptors in the inner spore membrane. The rate of germination of spores of gerP mutants can be improved by introduction of a cotE mutation, which results in a defective outer spore coat and increased permeability of spores (Behravan et al. 2000). A cotE mutation was therefore introduced into the ylaJ, yhcN and double mutants, to test whether germination would be improved; in fact, the resulting spores showed a further decrease in germination, as measured by OD loss in alanine (data not shown). This is as would be expected in an otherwise wild-type cotE mutant where later stages of germination are slowed, as CwlJ is absent (Bagyan and Setlow, 2002). The defect in ylaJ and yhcN mutant spores is therefore not due to a reduction in the spore coat’s permeability to germinants.

Is SleB function normal in the mutants?

A cwlJ sleB double mutant of Bacillus subtilis has a very low colony forming efficiency (<0.05%), due to the inability of the dormant spore to lyse the cortex region before returning to vegetative growth (Setlow, Melly and Setlow 2001). Spores that lack only CwlJ are able to form colonies normally on rich medium, as the activity of the other germination-specific cortex lytic enzyme, SleB (Boland et al. 2000), is sufficient for cortex hydrolysis; the proteins are at least partially redundant in function. An effect on SleB function, therefore, might be detected in a cwlJ mutant background.

The colony-forming ability of a cwlJ mutant (Table 2) was the same as that of the laboratory parent strain 1604 (1.5–1.6 × 10⁸/ml), as was that of CJ01 (ylaJ::pMUTIN4), CJ06 (yhcN::spc) and CJ11 (ylaJ ΔyhcN). The negative control, a sleB cwlJ double mutant lacking both germination-specific cortex lytic enzymes, was not recovered at the dilution tested (equivalent to a plating efficiency reduced by greater than 10³-fold; data not shown). Combination of a cwlJ mutation with either ylaJ or yhcN mutations reduced colony formation only slightly. However, the absence of both YlaJ and YhcN proteins in spores that also lack CwlJ reduced colony formation by 80% (Table 2). Although this effect is much less dramatic than that which would result from complete loss of SleB, it does suggest that loss of the two proteins causes a significant reduction in SleB function.

Western blotting (Johnson 2005; Fig. S2, Supporting Information) indicated that both SleB and YpeB proteins were present at normal levels in the bulk spore population in single and double...
ylaj, yhcN mutants, suggesting that the effect is likely on activity rather than amounts of these proteins. Spores of a cotE mutant have an incomplete outer spore coat, resulting in a loss of the coat-located CwlJ (Bagyan and Setlow 2002). Introducing a cotE mutation into these mutant backgrounds also affected colony formation, but in this case loss of either of YlaJ or YhcN was sufficient to reduce the colony-forming efficiency to 20%. The defect may be more severe in the cotE yhcN mutant, for which the colonies obtained were heterogeneous in size after 16 h at 37°C, though the small colonies (25%-30% of the total) were normal in size after a further 24 h incubation; this suggests particularly slow germination and/or outgrowth of some spores. The plating behaviour of the triple cotE yhcN ylaJ mutant was identical to that of the cotE yhcN mutant.

DISCUSSION

The precise roles of YlaJ and YhcN proteins in germination remain elusive, but they clearly are involved in some way with SleB function in Bacillus subtilis, from the data presented here. A defect in spore outgrowth was reported in a yhcN mutant (Bagyan et al. 1998), although their data, on closer examination, also suggest reduced OD fall during germination in rich media.

The presence of both ylaJ and yhcN, and often multiple homologues, in genomes of Bacilli adds to the complexity of analysis. Although ylaJ is monocistronic in B. subtilis, its presence in an operon with sleB and ypeB in the B. anthracis/cereus/thuringiensis family suggests the possibility of some functional relationship with these proteins. Whilst a null mutant in ylaJ (BAS2560) in B. anthracis displayed no apparent germination defect in rich medium (Bernhards and Popham 2014), B. anthracis does encode a number of ylaJ/yhcN homologues which may compensate functionally for the single deletion. The work presented here reveals a role for the B. subtilis paralogues in L-alanine germination; in preliminary experiments, (Johnson 2005) spores of the ylaJ yhcN double mutant, but not the single mutants, germinated more slowly than wild type in the combination of germinants asparagine, glucose, fructose and KCl (AGFK), a process mediated by the GerB and GerK receptors. However, all spores, including wild type, were relatively slow to germinate in these experiments, compared to alanine germination, and this needs to be more thoroughly examined under conditions of optimal, more extensive, heat activation (Luu et al. 2015). Whether or not such germination defects are also relevant to other Bacillus species would require further investigation.

The observation that suggests a role for proteins of this family in SleB function in B. subtilis is that approximately three-quarters of the spore population of mutants lacking both YhcN and YlaJ proteins are dependent on CwlJ cortex lytic enzyme for completion of germination to form colonies on agar. One possibility is that SleB enzyme activation is not achieved to the normal extent in the majority of spores, even after prolonged exposure to germinants. It is also possible that the effects of YhcN and YlaJ on spore germination are because they operate in some way during sporulation to influence spore properties, such as the local environment of the SleB protein, or even cortex synthesis/structure.

The increased defect in cotE mutants, where the absence of either one of YlaJ or YhcN results in a similarly reduced ability of spores to germinate as measured by colony formation, cannot be explained, but may possibly reflect that not only CwlJ, but also the integument-located fraction of SleB, YlaJ and YhcN proteins, are likely to be absent, leaving only the function of their inner-membrane associated components.

The putative role for YlaJ/YhcN in SleB function is consistent with the observation that in Clostridia, species that encode SleB as a cortex lytic enzyme (such as Clostridium botulinum, tetani, kluwyeri, and novyi) also encode a YlaJ/YhcN-like protein, whereas species that use the alternative system involving a proteolytically activated SleC type cortex lytic enzyme (such as C. perfringens, difficile, acetobutylicum, phytofermentans) do not appear to encode a YlaJ/YhcN homologue.

However, the role of the YlaJ/YhcN proteins may not be limited to the effect on SleB function. Considering the germination defect in L-alanine in not only double but also single mutants in an otherwise wild-type background, each mutation alone reduces not only the rate of OD fall of spore suspensions, but also the rate of loss of spore heat resistance, and the rate of DPA release, suggesting a role in germination events earlier than cortex hydrolysis. The defect in a double mutant is no worse than that in the single mutants, measured in this way, suggesting that both proteins may influence the same early process, in a non-additive fashion.

Two more lipoproteins in B. subtilis, YucT and YrbB (CoxA), also contain a YhcN/YlaJ domain, and are also present in the inner membrane fraction of dormant spores (Zheng et al. 2016). Whether absence of these proteins would further influence spore germination behaviour remains to be explored. It is also of interest that a yhcN/ylaJ homologue is present in a recently described transposon, within an operon also containing spoVA genes, spoVA260, that contributes to higher levels of spore heat resistance and reduced germination in B. subtilis strains (Berendsen et al. 2016; Krawczyk et al. 2016).

Although the mechanistic function of the YhcN and YlaJ protein family remains to be determined, they are clearly worthy of further investigation and may yet provide additional insights into our very limited understanding of the signal transduction process in spore germination.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

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Conflict of interest. None declared.

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