Plasmid-encoded genes influence exosporium assembly and morphology in Bacillus megaterium QM B1551 spores

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One sentence summary: Essential components of the outermost shell that envelopes Bacillus megaterium spores are encoded on plasmid-located genes.

ABSTRACT

Spores of Bacillus megaterium QM B1551 are encased in a morphologically distinctive exosporium. We demonstrate here that genes encoded on the indigenous pBM500 and pBM600 plasmids are required for exosporium assembly and or stability in spores of this strain. Bioinformatic analyses identified genes encoding orthologues of the B. cereus-family exosporium nap and basal layer proteins within the B. megaterium genome. Transcriptional analyses, supported by electron and fluorescent microscopy, indicate that the pole-localized nap, identified here for the first time in B. megaterium QM B1551 spores, is comprised of the BclA1 protein. The role of the BxpB protein, which forms the basal layer of the exosporium in B. cereus spores, is less clear since spores of a null mutant strain display an apparently normal morphology. Retention of the localized nap in bxpB null spores suggests that B. megaterium employs an alternative mechanism to that used by B. cereus spores in anchoring the nap to the spore surface.

Keywords: Bacillus; spores; exosporium; indigenous plasmids; hairy nap

INTRODUCTION

Bacterial members of the orders Bacillales and Clostridiales form environmentally resistant endospores in response to nutrient limitation. Spores of all species are encased in a proteinaceous outer shell, or coat, comprising upwards of 50 or more distinct proteins (Henriques and Moran 2007; McKenney, Driks and Eichenberger 2013). Much attention has been paid to the regulation and assembly of the spore coat, particularly in Bacillus subtilis, where significant progress has been made in elucidating the genetic networks and key morphogenetic proteins that control and underpin the assembly of this macromolecular structure (McKenney et al. 2010; McKenney and Eichenberger 2012). Spores of some species, however, including B. cereus and B. megaterium, contain an additional structure—the exosporium—which is morphologically distinct from the spore coat, and forms the outermost layer of the spore. Cryo-electron microscopy structural studies have revealed that the exosporium in B. cereus group members is formed of a crystalline lattice, or distinct layers of defined crystal types, that probably serve as environmental molecular sieves i.e. permitting ingress of small germinant molecules while excluding potentially harmful lytic enzymes and other macromolecules (Ball et al. 2008; Kailas et al. 2011).

While insights to the identity and assembly of a number of exosporium components have been achieved for B. cereus and B. anthracis spores (Todd et al. 2003; Redmond et al. 2004), little is...
known of the orthologous structure in \textit{B. megaterium} spores. The latter species forms one of the major clades in the genus \textit{Bacillus} and comprises a number of morphologically distinct strains. The best studied, particularly in the areas of spore germination and spore structure, is the \textit{QM B1551} strain, which carries ~11% of its genome on seven indigenous plasmids (Rosso and Vary 2005; Eppinger et al. 2011). Despite also frequently carrying genetic information on plasmids, exosporium-encoding genes have not been identified as being plasmid borne in \textit{B. cereus} family members. Accordingly, the purpose of this study was to examine whether plasmid-encoded genes influence the morphology of the \textit{B. megaterium} \textit{QM B1551} exosporium.

\section*{MATERIALS AND METHODS}

\subsection*{Bacterial strains and preparation of spores}

\textit{Bacillus megaterium} strains employed in this study (Table 1) were all isogenic with the \textit{QM B1551} strain. All strains were cultured on LB medium at 30 °C, with antibiotics where appropriate (Table 1). Mutant strains were constructed by polyethylene glycol-mediated transformation of protoplasts. Transformant colonies that had undergone single or double crossover recombination events were isolated and verified by PCR, essentially as described previously (Gupta et al. 2013). Spores were prepared by nutrient exhaustion and purified as described previously (Gupta et al. 2013).

\subsection*{Molecular biology procedures}

Transcriptional analysis from loci of interest was examined by RT-PCR, using gene-specific primers designed to amplify ~400 bp DNA fragments (Ramirez-Peralta et al. 2013). \textit{Bacillus megaterium} strains bearing C-terminal GFP and mCherry fusions to proteins of interest were constructed as described previously (Manetsberger, Hall and Christie 2014). A plasmid designed to facilitate the construction of a strain bearing a truncated version of \textit{BclA1} (BMQ_pBM50077), in which the predicted N-terminal domain of the protein was fused at the C-terminus to GFP (i.e. omitting the collagen-like region of the protein) was prepared by cloning a 477 bp DNA fragment, encompassing codons 1-159 of the \textit{bclA1} gene, into pVLG6. Additionally, a number of strains were prepared in which genes of interest were placed under the control of an IPTG-inducible promoter on the episomal pH7254 plasmid. Essentially, PCR was used to prepare DNA fragments that encoded either the entire open reading frame (minus stop codons) or a defined fragment of the gene of interest, with a gfp ampiclon fused in-frame at the 3’ end. PCR amplicons were ligated with linearized pH7254, and the resultant plasmids used to transform \textit{B. megaterium} to chloramphenicol resistance. Transformant clones were verified by PCR, and inducible protein expression initiated in sporulating cultures (upon entry to stationary phase) via addition of 1 mM IPTG.

A \textit{B. megaterium} \textit{bxpB} (BMQ_pBM60048) null mutant strain was created via allelic exchange with a kanamycin resistance cassette flanked by 200-bp fragments from the 5’ and 3’ ends of the \textit{bxpB ORF}, employing procedures described previously (Gupta et al. 2013). Sequence information for all oligonucleotides used in this work is available upon request. Cloning and propagation of plasmids was achieved using \textit{Escherichia coli} DH5α (NEB, Hitchin, UK), cultured typically in LB medium supplemented with 50 μg mL⁻¹ carbenicillin.

\subsection*{Microscopy}

Fluorescence and thin-section transmission electron microscopy (TEM) analyses were conducted as described previously (Manetsberger, Hall and Christie 2014). Negatively stained intact spores were imaged using a Philips CM100 transmission electron microscope operated at an accelerating voltage of 100 kV. Micrographs were collected at a size of 1024 × 1024 pixels using a Gatan Multiscan 794 1k × 1k CCD camera and analysed with Gatan Digital Micrograph software (DM, Gatan Inc.).

\begin{table}[h!]
\centering
\caption{\textit{Bacillus megaterium} strains used in this study.}
\begin{tabular}{|l|l|l|}
\hline
Strain & Relevant genotype & Source \\
\hline
\textit{QM} B1551 & Wild-type strain & P.S. Vary \\
PV361 & ΔpBM100, 200, 300, 400, 500, 600, 700 & P.S. Vary \\
PV202 & ΔpBM100, 600, 700 & P.S. Vary \\
PV203 & ΔpBM100, 300, 400, 600, 700 & P.S. Vary \\
PV203-F & ΔpBM100, 300, 400, 500, 600 & P.S. Vary \\
PV208 & ΔpBM300, 400, 500, 600, 700 & P.S. Vary \\
JM100 & ΔpBM400, 700 & This study \\
Null mutant strain & & \\
JM411 & Δ\textit{bxpB}:Km & This study \\
GFP/mCherry fusion strains & & \\
JM412 & bxpB::pVLG6 (\textit{gfp}) Cm & This study \\
JM413 & \textit{bclA1}:pVLG6 (\textit{gfp}) Cm & This study \\
JM414 & \textit{bclA2}:pVLG6 (\textit{gfp}) Cm & This study \\
JM415 & \textit{bclA1}:pVLG6 (\textit{gfp}) Cm & This study \\
JM416 & pH7254-\textit{bxpB}-\textit{gfp} Cm & This study \\
JM417 & pH7254-\textit{bclA1} (codons 1-159)-\textit{gfp} Cm & This study \\
JM418 & pH7254-\textit{bclA1} (codons 1-159)-\textit{gfp} Cm & This study \\
JM419 & \textit{Alr}:pVLG6 (\textit{gfp}) Cm & This study \\
JM708 & scl::pVLG6 (\textit{gfp}) Cm & Manetsberger, Hall and Christie (2014) \\
JM709 & cotX::pVLG7 (mCherry) Cm & Manetsberger, Hall and Christie (2014) \\
\hline
\end{tabular}
\end{table}

\footnotesize{Abbreviations for antibiotics: \textit{Km}, kanamycin resistance (5 μg mL⁻¹); \textit{Cm}, chloramphenicol resistance (5 μg mL⁻¹).

\textsuperscript{†}This strain was constructed in the Δ\textit{bxpB}:Km background.}
RESULTS

Electron microscopy analysis of *B. megaterium* QM B1551 spores

TEM was used to investigate the ultrastructure of *B. megaterium* QM B1551 spores. Thin-sectioned TEM images revealed the typical *B. megaterium* QM B1551 spore architecture i.e. a central spore core (protoplasm) surrounded by the electron translucent peptidoglycan cortex, with contiguous layers of lamellar coat material and then the outermost ‘walnut-like’ exosporium (Fig. 1a) (Beaman, Pankratz and Gerhardt 1972). The extended poles of the exosporium are revealed in images of negatively stained intact spores to result from a flattened rim of exosporium material that surrounds the densely stained inner-spore integuments (Fig. 1b). Analysis of intact spores revealed a novel morphological feature not previously associated with *B. megaterium* QM B1551 spores. Discernible filament-like structures, which we assume to be analogous to the hair-like nap evident on *B. cereus* family spores, are localized to a projected ring or sheath of exosporium material located at one of the spore poles (Fig. 1b and c). To our knowledge, these images confer the first evidence for the presence of a nap on *B. megaterium* QM B1551 spores.

Influence of plasmids on *B. megaterium* QM B1551 spore morphology

*Bacillus megaterium* QM B1551 carries seven indigenous plasmids that range in size from the 5.5 kb pBM100 to the 165 kb pBM700. In order to assess the impact of plasmid-encoded genes on spore morphology, we obtained a number of plasmid-cured strains that had been characterized previously for plasmid content by plasmid purification and gel electrophoresis (Stevenson, Lach and Vary 1993). We reexamined these strains, and isolated from our own laboratory, using a more reliable PCR-based method that utilized defined plasmid-specific oligonucleotides (Table 1 and Fig. S1, Supporting Information). Having established plasmid profiles for the different strains, TEM was then employed to characterize spore morphology. These analyses revealed that spores of the PV361 strain, which lacks all seven plasmids, lack an exosporium, although the coat and cortex appear normal (Fig. 2). In support of the latter, resistance to wet heat and lysozyme in all mutant strains was comparable to wild-type spores (data not shown). Wild-type spores are, however, considerably more hydrophobic and adhere to a range of materials much more tightly than exosporium-less PV361 spores, indicating that at least one function of the *B. megaterium* exosporium concerns the attachment of spores to surfaces (Ke Xu Zhou, unpublished results). Similarly, several other strains were observed to have lost their exosporium (PV202, PV203, PV203-F, PV208), while retaining apparently normal underlying layers. A common feature of these strains is that they lack the pBM600 plasmid. The collective presence of plasmids pBM200 through to pBM500 (strain PV202) is evidently insufficient to support exosporium development, whereas pBM400 and pBM700 are dispensable (strain JM100). Hence, based on the relatively limited analysis of mutant strains described above, it seems that essential components of the *B. megaterium* QM B1551 exosporium are encoded on plasmid pBM600. Additionally, since we have yet to isolate a strain that lacks pBM500 but retains pBM600 we cannot rule out an essential role for pBM500 in exosporium formation.

Plasmid-associated exosporium genes

Homology searches were conducted against the *B. megaterium* QM B1551 genome using the NCBI Translated BLAST tblastn program using established exosporium proteins from *B. cereus* and *B. anthracis* as seeds (Sylvestre, Couture-Tosi and Mock 2002; Todd et al. 2003; Redmond et al. 2004). Of the four hits that were returned, two orthologues of the *B. cereus*/anthracis hairy-nap protein, BcIA, were identified as being encoded on plasmid pBM500 (BMQ_pBM50077 and BMQ_pBM50081; Fig. S2, Supporting Information). BMQ_pBM50077 encodes a 954-amino-acid protein with an estimated molecular mass of 87 kDa. It incorporates 220 GXT repeats in its sequence (where X is normally I or A), characteristic for collagen-like proteins, and has been assigned the name BcIA1. BMQ_pBM50081 has a predicted molecular weight of 44 kDa, including 40 GXX repeats, and has been assigned the name BcIA2. In addition to the characteristic collagen-like regions, both proteins are predicted to have globular N- and C-terminal domains. The N-terminal domains appear to differ markedly from the orthologous domain in *B. anthracis* BcIA i.e. the *B. megaterium* BcIA1 N-terminal domain comprises 159 residues, compared to 38 residues in *B. anthracis* BcIA. Furthermore, neither N-terminal domain appears to contain the sequence motif required for incorporation of the orthologous BcIA/BcIB proteins into the *B. anthracis* exosporium (Thompson and Stewart 2008; Tan and Turnbough 2010) (Fig. S3, Supporting Information). In contrast, the C-terminal domains both comprise ~135 residues (134 in *B. anthracis*) and share 17% sequence identity (30% similarity).
Homology searches yielded also an orthologue of the *B. anthracis* BxpB exosporium basal layer protein (ExsFA in *B. cereus*), which is encoded on plasmid pBM600 at locus BMQ_pBM60048 (Fig. S2, Supporting Information). *Bacillus megaterium* BxpB has a predicted molecular mass of 17 kDa, and shares 26% sequence identity (45% similarity) at the amino acid level with *B. anthracis* BxpB. The only other putative exosporium protein identified from tblastn searches as being encoded within the *B. megaterium* QM B1551 genome is an orthologue of alanine racemase (Alr), which is encoded on the chromosome (BMQ_0226) and is predicted to share 39% sequence identity with the *B. anthracis* protein.

**Expression and localization of *B. megaterium* BclA1, BclA2 and BxpB**

Reverse transcription polymerase chain reaction (RT-PCR) was conducted on cDNA derived from cellular samples collected throughout sporulation in order to examine transcription from the *bclA1*, *bclA2* and *bxpB* loci (Fig. 3). These analyses were compromised to an extent by the relative asynchronicity of sporulation in SNB medium; however, the low abundance of PCR products associated with the *bclA2* locus indicates that the BclA2 protein is expressed at only very low levels.

Expression of the longer collagen-like protein, BclA1, was more readily detected throughout the course of sporulation (PCR products of low abundance detected prior to entry to stationary phase presumably reflect asynchronous sporulation). Based on the abundance of PCR products, expression of BclA1 appears highest during the early stages of sporulation, an observation that is consistent with a potential $\sigma^E$ consensus sequence identifiable upstream of the *bclA1* ORF (data not shown). RT-PCR products indicate that the BxpB protein is expressed predominantly during the mid to later stages of sporulation, although less abundant PCR products are evident early in sporulation also. Sequence analysis revealed poorly conserved potential $\sigma^E$ and
et al. BclA1 is involved in localizing the nap to the developing bacillus mega sppores revealed the retention of the 2005 promoter sequences upstream of the predicted hasnoeffectontheassembly 4 bclA1 B. megaterium B. cereus gene was disrupted by allelic exchange with an antibi- sporesandinter- sporesinasimilarmanner a strain engineered to express a C-terminal GFP fusion did not show any appreciable fluorescence, and no further analyses ever, a strain engineered to express a C-terminal GFP fusion did not show any appreciable fluorescence, and no further analyses were conducted.

BxpB null mutant spores

The bxpB gene was disrupted by allelic exchange with an antibiotic resistance cassette in order to examine the role of the BxpB protein in exosporium assembly. The orthogonal protein forms the exosporium basal layer in B. cereus/anthracis spores and interacts with BclA and BclB to anchor the nap proteins to the spore surface (Sylvestre, Couture-Tosi and Mock 2005; Thompson et al. 2011). However, TEM analyses revealed apparently normal looking spor architecture in B. megaterium bxpB spores, including an intact exosporium (Fig. 5a). Unexpectedly, TEM analysis of negatively stained ΔbpB spores revealed the retention of the localized nap on mutant spores (Fig. 5b and c). Similarly, fluorescence microscopy revealed a similar pattern of localization of the truncated BclA1-GFP protein in bxpB spores to that observed in the wild-type background (Fig. 5d). Fluorescence microscopy also revealed that deletion of bxpB has no effect on the assembly of the Sle inner coat protein (Fig. S4, Supporting Information). The putative outer coat/exosporium protein, CotX2, was also observed to localize around bxpB spores in a similar manner to that observed in the wild-type background (Fig. S4, Supporting Information). In this case, however, shards of fluorescent material were observed to detach from the surface of some spores within the population, indicating perhaps that the loss of BxpB has compromised the structural integrity of the exosporium.

DISCUSSION

The present study has revealed several new insights to the structure and assembly of the outermost layers of B. megaterium QM B1551 spores. First, we provide evidence that essential exosporium gene products are plasmid encoded. To our knowledge, this is the first demonstration of such an association between exosporium formation and plasmid-borne genes within the Bacilli. A common genotype in all exosporium-less strains examined in the course of this work was the absence of the 67-kb pBM500 and 100-kb pBM600 plasmids, indicating that proteins crucial to exosporium assembly, structural integrity and/or stability are encoded on these plasmids. Neither plasmid is essential for viability, so presumably selective pressure in the environmental niche originally inhabited by this strain outweighs the metabolic burden associated with plasmid maintenance.

Second, TEM analyses of negatively stained intact spores revealed the presence of a hair-like nap, localized to one of the spore poles. Bioinformatic, transcriptional and fluorescence/electron microscopy analyses indicate that the nap is composed of fibres formed by the BclA1 protein, which is encoded on pBM500, one of the plasmids essential for exosporium assembly. Unfortunately, we have been unable to corroborate this hypothesis by examination of BclA1 null mutant spores since several attempts aimed at constructing this strain have failed. However, despite lacking the consensus sequence identified within the orthogonal B. anthracis protein, we have been able to demonstrate that the N-terminal domain of B. megaterium BclA1 is involved in localizing the nap to the developing

Figure 4. Fluorescence micrographs showing expression and localization of putative B. megaterium exosporium basal layer and nap proteins. (a) Diffuse mother cell localized fluorescence associated with expression of BxpB-GFP; (b) Localization of BclA1-GFP to the forespore during sporulation; (c) C-terminally truncated BclA1 (residues 1–159) retains the ability to localize to the developing forespore.
forespore. Surprisingly, the protein appears to localize around the entire surface of the developing spore, rather than being localized to one of the poles. However, subsequent loss of fluorescence during spore maturation may reflect detachment of the protein from the bulk of the spore surface, with only pole-localized BclA1 fibres remaining. Whether the nap-associated pole represents an analogous structure to the 'bottle-cap' from which B. anthracis cells emerge during spore germination (Stichelen, Kearney and Turnbough 2007) remains to be ascertained.

Finally, the role of the BxpB protein—a key structural component in the exosporium of members of the B. cereus family—has been examined in this work. The orthologous B. megaterium protein is encoded on pBM600, the second of the indigenous plasmids essential for exosporium assembly in B. megaterium. Despite RT-PCR and (to a lesser extent) fluorescence microscopy analyses supporting the expression of this protein during sporulation, TEM analyses of bxpB null spores indicate that the protein has only a minor role in the assembly of the B. megaterium exosporium. Localization of the BclA1 protein in bxpB null spores also suggests that the mechanism of nap assembly in B. megaterium spores also differs to that observed in B. cereus family spores. The absence of additional BxpB orthologues encoded within the B. megaterium genome, and apparent differences in the time of optimal expression, confers further evidence that the nap proteins are anchored to the spore surface via a BxpB-independent mechanism. Indeed, bioinformatic analyses using known B. cereus family exosporium proteins as seeds revealed distinct orthologues of only three putative exosporium proteins encoded within the B. megaterium QM B1551 genome, indicating that the protein composition of the exospora may differ substantially. The future challenge will be in identifying those essential plasmid-encoded protein(s) involved in nap localization and basal layer assembly in the B. megaterium exosporium.

SUPPLEMENTARY DATA
Supplementary data are available at FEMSLE online.

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