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MicroMeeting

From fundamental studies of sporulation to applied spore research

Imrich Barák,[1]* Ezio Ricca[2] and Simon M. Cutting[3]
[1]*Institute of Molecular Biology, Slovak Academy of Sciences, Dubravska cesta, 845 51 Bratislava 45, Slovak Republic.*
[2]*Department of General and Environmental Physiology, University Federico II, via Mezzocannone 16, 80134 Naples, Italy.*
[3]*School of Biological Sciences, Royal Holloway University of London, Egham, Surrey TW20 0EX, UK.*

Summary

Sporulation in the Gram-positive bacterium, *Bacillus subtilis*, has been used as an excellent model system to study cell differentiation for almost half a century. This research has given us a detailed picture of the genetic, physiological and biochemical mechanisms that allow bacteria to survive harsh environmental conditions by forming highly robust spores. Although many basic aspects of this process are now understood in great detail, including the crystal and NMR structures of some of the key proteins and their complexes, bacterial sporulation still continues to be a highly attractive model for studying various cell processes at a molecular level. There are several reasons for such scientific interest. First, some of the complex steps in sporulation are not fully understood and/or are only described by 'controversial' models. Second, intensive research on unicellular development of a single microorganism, *B. subtilis*, left us largely unaware of the multitude of diverse sporulation mechanisms in many other Gram-positive endospore and exospore formers. This diversity would likely be increased if we were to include sporulation processes in the Gram-negative spore formers. Spore formers have great potential in applied research. They have been used for many years as biodosimeters and as natural insecticides, exploited in the industrial production of enzymes, antibiotics, used as probiotics and, more, exploited as possible vectors for drug delivery, vaccine antigens and other immunomodulating molecules. This report describes these and other aspects of current fundamental and applied spore research that were presented at European Spores Conference held in Smolenice Castle, Slovakia, June 2004.

Accepted 19 October, 2004. *For correspondence. E-mail imrich.barak@savba.sk; Tel. (+42) 1 259 307 418; Fax (+42) 1 259 307 416.

Introduction

Some Gram-positive bacteria belonging to the *Bacillus* and *Clostridia* genera can undergo a complex developmental cell differentiation process what allows them to adapt to changing environmental conditions by producing highly resistant spores. This process, called sporulation, involves progression through different stages including initiation, chromosome segregation, sporulation-specific cell division (asymmetric in rod-shaped bacteria), differential gene expression and specific signal transduction mechanisms. The return pathway, leading to vegetative cell growth, involves spore germination followed by outgrowth of the germinated spore. All of these aspects of sporulation have been studied for many years in great detail and have had both a substantial impact on our understanding of many other basic cell processes and have started to fuel applied spore research with new ideas.

The European Spores Conference enabled scientists working not only with model organism *Bacillus subtilis* but also with other spore formers to discuss all aspects of spore research. The emphasis was on recent research developments important for understanding mechanisms of sporulation and germination and on the use of spores in applied research. This report highlights some of most exciting reports presented at this conference.

Initiation of sporulation

Bacteria that are able to sporulate have a sophisticated and sensitive signal transduction pathway to monitor and respond rapidly to environmental signals by activating a phosphorelay mechanism (Hoch, 1993). The crucial component of the phosphorelay is Spo0A, the master

response regulator of sporulation initiation that is activated when phosphorylated (Spo0A~P). Spo0A is a transcription factor consisting of two domains, an N-terminal phosphoacceptor domain connected to a C-terminal DNA-binding domain by a flexible hinge. The phosphoacceptor domain mediates dimerization of Spo0A upon phosphorylation (Lewis *et al*., 2002). Phosphorylation-induced dimerization increases the affinity of Spo0A for tandemly repeated 0A-boxes within the promoter regions of target genes. The importance of Spo0A as an activator or repressor of transcription of many stationary-phase and sporulation-specific genes is highlighted by the finding that expression of more than 500 genes is dependent on Spo0A (Molle *et al*., 2003). The complex view of Spo0A structure–function relationship was presented by Katarína Muchová (I. Barák's laboratory, Institute Mol. Biol., Bratislava, Slovakia and A.J. Wilkinson's laboratory, University of York, UK). The study was based on the crystal structures of the receiver domain (N-Spo0A) in both phosphorylated and unphosphorylated form (Lewis *et al*., 1999; 2000a) as well as the effector domain (C-Spo0A) (Lewis *et al*., 2000b). Comparison of the structure of N-Spo0A~P with the structures of unphosphorylated response regulators suggested that major structural changes occur upon phosphorylation. Mutational analysis was used to evaluate the contribution of specific amino acid residues to signalling in Spo0A. This analysis, together with biochemical data, suggested that dimerization and signal transduction between the two domains of Spo0A are mediated principally by the $\alpha_4\beta_5\alpha_5$ signalling surface in the receiver domain (Fig. 1) (Muchová *et al*., 2004). Jan-Willem Veening (Molecular Genetics group, Groningen, the Netherlands) presented an interesting study of sporulation gene expression heterogeneity in individual *B. subtilis* cells (Veening *et al*., 2004). Within a sporulating culture of *B. subtilis*, some cells initiate this developmental programme while other cells do not. The underlying mechanisms responsible for this heterogeneity in sporulation gene expression are unknown. Veening showed that only cells that have reached the threshold level of active Spo0A, the key sporulation regulator, initiate sporulation gene expression. By fluorescence microscopy and flow cytometric analyses, Veening demonstrated that the autostimulatory loop of Spo0A is responsible for the observed heterogeneity. This feedforward loop to generate heterogeneity in gene expression leading to phenotypic variation is a quick and energy-efficient genetic system to cope with the ever-changing circumstances in the natural environment of *B. subtilis*.

Chromosome segregation and cell division during sporulation

Sporulation in *B. subtilis* is intimately tied to the cell cycle. At the onset of sporulation, a specific chromosome partitioning mechanism ensures that the *oriC* regions of the two chromosomes migrate away from the cell centre and towards the cell poles (Glaser *et al*., 1997; Lin *et al*., 1997). This produces an elongated structure, called the axial filament, which requires the Spo0J, Soj, DivIVA and RacA proteins (Quisel *et al*., 1999; Thomaides *et al*., 2001; Ben Yehuda *et al*., 2003). Interestingly DivIVA is required both for vegetative growth, by ensuring that MinCD is localized to the cell poles, and in sporulation, when it participates in the attachment of the chromosome to the cell pole via the DNA-binding protein RacA. Consequently, both in vegetative growth and during sporulation the subcellular location of DivIVB at the pole has been considered essential for its function. David H. Edwards (Ninewells Medical School, Dundee, UK) pre-

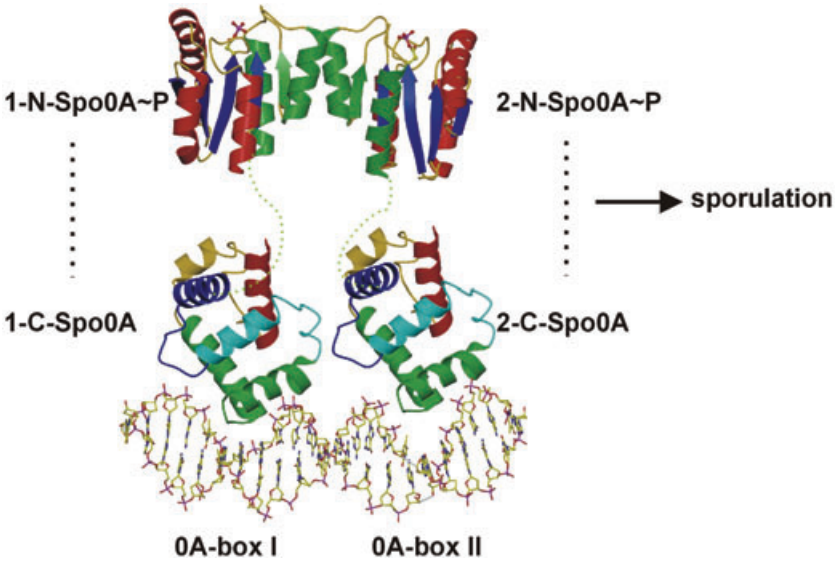

Fig. 1. Model of activated (phosphorylated) two molecules of Spo0A protein bound to two 0A-boxes. The model is derived from X-ray structures of the individual domains.

sented the results of a mutational analysis of DivIVA aimed at understanding the relationship between its localization and function. Mutations were analysed for their effect on localization, vegetative growth and sporulation efficiency. One of the mutations resulted in an altered protein, $DivIVA_{R18C}$, that could not localize to the cell pole but, surprisingly, retained its ability to support both vegetative growth and high sporulation efficiency. Further analysis of $DivIVA_{R18C}$ revealed that it colocalized with the cell nucleoid in a Spo0J/Soj-dependent manner and participated in the orientation of the chromosomes at the onset of sporulation. The authors proposed an interesting model in which localization to the cell division site, and not the cell pole, is crucial for DivIVA function. They also suggested that DivIVA, Spo0J/Soj and MinD all participate in chromosome organization at the onset of sporulation. Eva Kutejová (I. Barák's laboratory, Institute Mol. Biol., Bratislava, Slovakia; H. Stahlberg's laboratory, UC-Davis, USA and A.J. Wilkinson's lab, University of York, UK) presented cryo-negative stain transmission electron microscopy studies that demonstrated that functional DivIVA forms an elongated particle with lateral expansion at both ends producing a form that resembles a 'doggy-bone' (Stahlberg *et al*., 2004). These DivIVA oligomers serve as building blocks in the formation of higher-order assemblies, giving rise to strings, wires and finally two-dimensional lattices in a time-dependent manner. The oligomerization and lattice-forming properties exhibited by DivIVA might be significant for the biological function of wild-type protein. These results suggest a possible structural explanation for how DivIVA recognizes the cell poles.

A DivIVA homologue also exists in the exospore former *Streptomyces coelicolor*. Klas Flärdh (Uppsala University, Sweden) reported that the function of the *S. coelicolor* DivIVA homologue ($DivIVA_{SC}$) differs substantially from its *B. subtilis* counterpart. $DivIVA_{SC}$ is important in hyphal tip growth. In contrast to most other bacteria, the *Streptomyces* cell wall is polymerized at one extremity, the hyphal tip. This pronounced tip extension is analogous to apical growth of filamentous fungi but the underlying mechanisms in streptomycetes are different and largely unknown. Interestingly, $DivIVA_{SC}$ localizes to hyphal tips and nascent lateral branches. It is essential for growth, and partial depletion produces a phenotype strikingly similar to tip growth or nuclear migration mutants in fungi. Abnormally high levels of $DivIVA_{SC}$ have dramatic effects on shape determination, leading to conspicuous swollen and pear-shaped cells. In addition, $divIVA_{SC}$ overexpression in pre-formed hyphae rapidly gives rise to multiple new sites of wall growth from which branch-like lateral outgrowths emerge. Before this, DivIVA redistributes to form new foci along the lateral wall, at which new growth seems to be triggered. Thus, $DivIVA_{SC}$ not only targets tips and affects morphogenesis, but is also instrumental in the establishment of new tips (Flärdh, 2003).

Bacillus and *Clostridia* species undergo medial division during vegetative growth and asymmetric (polar) division at the onset of sporulation. Both septation events use essentially the same proteins with the exception of sporulation-specific SpoIIE protein. Also the hierarchy of their assembly appears similar for both vegetative and sporulating cells. Goncalo Real (Universidade Nova de Lisboa, Portugal) reported that DivIB protein is required for asymmetric cell division even under genetic conditions making it dispensable for medial division. DivIB is a small bitopic membrane protein that is only essential for vegetative cell division at high temperatures, in part because of its stabilization of the otherwise unstable FtsL. Overexpression of *ftsL* bypasses the need for DivIB for vegetative division. Real's studies showed that *ftsL* overexpression does not restore asymmetric cell division in a *divIB* mutant strain. Consequently, these cells fail to activate σ^F and are unable to sporulate. Presumably there is a more stringent requirement for DivIB during asymmetric cell division, or the protein plays an as yet undiscovered function specifically in sporulation septum assembly.

The cell division process in the Gram-positive soil bacterium *S. coelicolor* has several unusual features. Although homologues of many components of the *Escherichia coli* and *B. subtilis* cell division machineries have been found in *Streptomyces*, several, including FtsA and MinC, are apparently absent. Like *B. subtilis*, streptomycetes undergo two kinds of cell division. Both require the FtsZ protein, but they are differentially regulated in time, space and cell-type specificity. Nina Grantcharova (K. Flärdh's laboratory, Uppsala University, Sweden) addressed the question of how development modulates the differential performance of the same cell division machinery in the different cell-types of *S. coelicolor*. She and colleagues visualized the Z ring assembly *in vivo* and found that the synchronized sporulation septation involved dynamic remodelling of FtsZ polymers with formation of spiral-shaped FtsZ intermediates that led to Z rings and initiation of septation. The proper assembly of FtsZ during sporulation was defective in the non-sporulating *ftsZ17*(Spo) mutant (Grantcharova *et al*., 2003), in which (altered) FtsZ is present at normal levels and capable of making vegetative septa. These results indicate that cytokinesis in *S. coelicolor* A3(2) might be developmentally controlled at the level of the dynamic remodelling of FtsZ polymers.

Differential gene expression

The asymmetric division of the sporulating cell produces two unequal progeny, the prespore and the much larger mother cell. Immediately following asymmetric division,

different but interdependent programmes of gene expression are initiated first in the prespore then in the mother cell, through the compartment-specific activation of transcription factors σ^F and σ^E respectively. σ^F is the first prespore-specific sigma factor to be activated and is regulated by three proteins: SpoIIAB, SpoIIAA and SpoIIE. In the presence of ATP, the anti-sigma factor SpoIIAB can either form an inactive complex with σ^F or act as a protein kinase to phosphorylate SpoIIAA. SpoIIE is a specific protein phosphatase that hydrolyses SpoIIAA~P. The resulting SpoIIAA interacts with σ^F–SpoIIAB complexes to release σ^F. SpoIIAA is phosphorylated once more during this interaction (for further details, see Errington, 2003; Hilbert and Piggot, 2004). Joanna Clarkson (M. Yudkin's laboratory, University of Oxford, UK) described *in vitro* experiments using a modified σ^F that has a reproducible fluorescence response on binding to SpoIIAB but is otherwise indistinguishable from the wild-type protein. She and colleagues used this fluorescence response to measure the amount of σ^F that remains bound to SpoIIAB over time after the addition of SpoIIAA. Their results showed that within a 10-fold increase in the SpoIIAA concentration, σ^F switched from being almost entirely bound to SpoIIAB to being free in solution. Additional experiments showed that with a ratio of SpoIIAA to SpoIIAB similar to that in the cell, σ^F switches from being predominantly bound to SpoIIAB to being predominantly free in solution over a 10-fold increase in SpoIIE activity. Moreover, continued activity of SpoIIE allowed σ^F to remain free for relatively long periods of time (σ^F needs to be active for about 1 h in the sporulating cell). Taken together, the results from this study reveal three features of this system that are important for its cellular function. First, there is an all-or-nothing response within a narrow range of SpoIIE activity, such that only a small increase in SpoIIE-specific activity is required to activate σ^F. Second, the system allows for a rapid response to dephosphorylated SpoIIAA, so that σ^F activity increases very soon after there is a change in the specific activity of SpoIIE. Finally, the system has evolved to be efficient in maintaining σ^F activity over the time period required during sporulation. These observations have a profound impact on our understanding of the asymmetric activation of σ^F specifically in the prespore compartment.

The RNA polymerase containing sigma factor σ^F governs transcription of *spoIIIG*, encoding the late prespore-specific regulator σ^G. σ^G is initially maintained in an inactive form. Its activation coincides with completion of engulfment of the prespore by the mother cell and requires expression of the s*poIIIA* and *spoIIIJ* loci. Several lines of evidence suggest that σ^G is kept inactive until engulfment is completed or in *spoIIIA* or *spoIIIJ* mutants by forming a complex with the anti-sigma factor SpoIIAB (Kellner *et al.*, 1996). This general model was questioned in the report of Adriano Henriques (Universidade Nova de Lisboa, Portugal), who showed that in *spoIIIA* mutants, a mutant form of σ^G with an E156K substitution that affects its interaction with SpoIIAB only allows σ^G-directed transcription about 2 h later than engulfment completion. The prespore membranes of the *spoIIIA* mutant show signs of instability at this time. Moreover, expression of σ^G-dependent genes in the *spoIIIA*/s*poIIIGE156K* double mutant is not confined to the prespore but rather takes place in both compartments. These and other results suggest that SpoIIAB either is redundant or has no major role in the regulation of σ^G in the prespore. In contrast, the presented results support the view that SpoIIAB, together with the LonA protease, is important for the negative regulation of σ^G in the mother cell. Luisa Corte (Universidade Nova de Lisboa, Portugal) reported a study about activation of σ^G in the prespore. Activation of σ^G requires expression of the *spoIIIJ* gene, which has a paralogue, the *yqjG* gene. Both genes encode membrane proteins of the Oxa1p/YidC family, with six transmembrane (TM) domains. At least one of the paralogues must be present to sustain growth and viability, but only *spoIIIJ* supports σ^G activation and efficient sporulation. The basis for this delineation is unclear, as both genes show similar temporal expression profiles, and both proteins localize to the membranes that surround the prespore. Presumably, specific amino acids or regions within SpoIIIJ account for its capacity to promote sporulation. Corte and colleagues assessed the viability and spore-forming ability of cells producing several chimaeras between SpoIIIJ and its homologue YqjG to identify SpoIIIJ domains critical for sporulation. Authors defined functionally important regions in SpoIIIJ from which only signal peptide, the first TM domain and the following hydrophilic loop are critical for sporulation.

Spore morphogenesis

The session on spore morphogenesis was mainly focused on the coat, the protein structure encasing the spore and playing a most important role for the resistance of spores to toxic chemicals and for their efficient germination. Recent studies have shown that specific coat components have enzymatic activities (Martins *et al.*, 2002; Costa *et al.*, 2004) and that the coat surface presents peculiar structures (ridges) that seem to be formed when the spore volume decreases (during sporulation) and to disappear when the spore swells (during germination) (Chada *et al.*, 2003). These findings suggest that the coat of *B. subtilis* spores is a dynamic structure that might sense the external environment through active enzymes present on its surface and adapt to changes in the spore volume by expanding and contracting in response to dehydration and rehydration (Driks, 2003).

A heterogeneous group of over 30 polypeptides forms

the coat which, by electron microscopy analysis, appears to be composed from three main structural layers: a diffuse undercoat, a laminated inner layer and a thick electron-dense outer coat. Several of these polypeptides have been studied and their structural (*cot*) genes identified. Expression of all *cot* genes is governed by a cascade of four transcription factors, acting specifically in the mother cell compartment of the sporangium in the sequence σ^E > SpoIIID > σ^K > GerE, with σ^E and σ^K being RNA polymerase σ factors and SpoIIID and GerE being DNA-binding proteins acting in conjunction with RNA polymerase associated with σ^E ($E\sigma^E$) and σ^K ($E\sigma^K$) (Henriques *et al*., 2004). In addition to the transcriptional control, a variety of post-translational modifications have been shown to occur during coat formation with some coat-associated polypeptides that appear to be cross-linked, glycosylated, proteolytically processed or assembled in the mature coat in either homo- or heteromultimeric forms (Isticato *et al*., 2004; Zilhao *et al*., 2004). The occurrence of cross-links within the coat was suggested because *o,o*-dityrosine cross-links were found in purified coat material (Henriques *et al*., 2004). The presence in the coat of ε-(-γ-glutamyl)lysyl isopeptide cross-links, catalysed by a transglutaminase (TGase), was also proposed and a TGase activity detected in sporulating cells of several *Bacillus* species (Kobayashi *et al*., 1998). A gene (*tgl*) coding for a spore-associated TGase was identified and cloned (Kobayashi *et al*., 1994; 1998).

Rachele Isticato (Universidade Nova de Lisboa, Portugal and University of Naples, Italy) reported a detailed study on the effects of *tgl* mutations on the assembly and function of the spore coat. In the TGase-mediated cross-linking reaction, a Gln-containing substrate binds to a cysteine present in the active site of the enzyme, releasing ammonia. Then, a second Lys-containing substrate binds to the enzyme–substrate intermediate and a ε-(-γ-glutamyl)lysyl isopeptide bond is formed between the Gln residue of the first substrate and the Lys of the second substrate. As such bonds are stable and resistant to proteolysis, they most probably contribute to the resistance of the *B. subtilis* spore. The construction and subsequent analysis of deletion and point mutations in *tgl* showed that (i) the coat-associated protein GerQ, as well as a group of at least three other coat polypeptides, was more readily extracted from the coats of *tgl* null mutant spores and (ii) a C116A substitution in *B. subtilis* Tgl protein, known to interfere with its activity *in vitro* (Kobayashi *et al*., 1998), did not prevent Tgl assembly within the coat but caused an increased yield in the extraction of GerQ and the other coat proteins *in vivo*. Therefore, in strains that do not have TGase activity, either because of a null or because of a point mutation in *tgl*, the association of GerQ or the three other coat proteins with the spore is not prevented and their extraction yield is increased. This suggests that the Tgl-dependent cross-link is likely to occur at the surface of the developing spore. Based on these results, GerQ was proposed as a specific substrate for Tgl but whether GerQ undergo inter- or intramolecular cross-linking is still unknown (R. Isticato *et al*., submitted). In addition, while some Tgl molecules were assembled on the forming spore, some others persisted in the mother cell, suggesting a role for Tgl in the mother cell.

The initial stages of coat assembly occur early after the onset of sporulation and involve functional interactions between at least two morphogenetic proteins, SpoIVA and CotE, both of which are made under σ^E control. Initially, SpoIVA localizes at the outer forespore membrane and then directs the assembly of CotE into a ring-like structure that surrounds the forespore. Assembly of CotE generates a gap between the SpoIVA and the CotE rings (Henriques *et al*., 2004). This gap is thought to become the site of assembly of the inner coat components. In contrast, the outer coat proteins are assembled on the outside of the CotE structure (Driks, 2003; Henriques *et al*., 2004). Three additional proteins, SpoVID, SafA and CotH, have a morphogenetic role and are needed for coat formation. Teresa Costa (A. Henriques's lab, Universidade Nova de Lisboa, Portugal) reported an elegant study of the interaction of two of these proteins: SpoVID and SafA. SpoVID is a highly acidic protein produced under σ^E control that has the dual role of maintaining the CotE ring around the forespore and directing SafA to the forming spore (Henriques *et al*., 2004). SafA, also produced in the mother cell under σ^E control, has a role not only in coat morphogenesis but also in the development of lysozyme resistance and germination properties of mature spores (Henriques *et al*., 2004). A cell wall-binding (CWB) motif is present at the N-terminus of SafA as well as at the C-terminus of SpoVID and both proteins localize to the coat–cortex interface (Henriques *et al*., 2004). The C-terminal half of SafA contains a PYYH motif that is needed for the interaction between SafA and SpoVID (Henriques *et al*., 2004). In addition to the PYYH motif, residues just downstream of the CWB domain of SafA were shown to be essential for interaction with SpoVID. Deletion of these residues of SafA did not prevent normal accumulation of SafA in sporulating cells, but resulted in the formation of spores that were highly susceptible to lysozyme, therefore indicating that the interaction between SpoVID and SafA is essential for the construction of a lysozyme-resistant spore. It was also reported that SafA specifically interacts with the N-terminal part of SpoVID, leading the authors to conclude that SpoVID–SafA interaction occurs through the N-terminal regions of the two proteins.

The third morphogenetic protein, CotH, plays a role in the assembly of various outer coat components, such as CotB, CotC and CotG. In addition, CotH also has a role in the development of the lysozyme resistance of the

mature spore and, in conjunction with CotE, is required for efficient spore germination (Henriques *et al*., 2004). Recent studies have shown that the previously observed role of CotH in the assembly of the outer coat components CotC, CotG and CotB is to stabilize CotC (Isticato *et al*., 2004) and CotG, which in turn is needed for dimerization and assembly of CotB (Zilhao *et al*., 2004). Loredana Baccigalupi (E. Ricca's laboratory, University of Naples, Italy) analysed expression of the *cotH* gene and the effects of its deregulation on the spore coat and on the assembly of the outer coat component CotC. *cotH* is transcribed under σ^K control and is negatively regulated by the transcriptional regulator GerE. A GerE binding site between the *cotH* promoter and the translational start site was shown to be needed for the GerE-mediated control of *cotH* expression. A mutation in this GerE box, causing increased and prolonged *cotH* expression, was used to analyse the effects of the GerE-independent expression of *cotH* on the structure and/or function of the spore coat. While no major defects were found by electron microscopy or by analysing the resistance and germination properties of wild-type and mutant spores, CotC, a CotH-controlled outer coat component, was found to accumulate in the mother cell compartment. CotC assembles into the coat generating at least four different oligopeptides, all dependent on the expression of the *cotH* gene (Isticato *et al*., 2004). The authors proposed that (i) CotC is normally produced in the mother cell compartment of the sporulating cell in excess of the amount that can be assembled and (ii) the number of CotC molecules that can be assembled on the forming spore is somehow regulated (Baccigalupi *et al*., 2004).

Anne Moir (University of Sheffield, UK) presented a study of a poorly understood spore structure, the exosporium, which appears as a loose-fitting sac that encases the endospore (Fig. 2). The exosporium is not found in *B. subtilis* but is present in most other *Bacillus* species, notably in members of the *Bacillus cereus sensu lato* group that includes two important human pathogen, *Bacillus anthracis* and *B. cereus*. Moir has shown that the exosporium can be removed with little damage to the spore, that is, spores stripped of the exosporium maintain most of their resistance properties and can germinate normally. The exosporium of *B. cereus* contains one or more surface glycoproteins, as well as perhaps around 20 other proteins. Some of these exosporium genes, including that encoding the major surface glycoproteins BclA, and ExsF, are clustered, whereas others are scattered throughout the chromosome. Interestingly, relatives of some *B. subtilis* coat proteins (including homologues of CotY/Z, CotG and CotB) are present in the exosporium, although their function has not yet been established. The role of the exosporium remains intriguing. Is it important for adhesion and establishment of biofilms or is it a means to evading phagocytosis when spores enter a mammalian host?

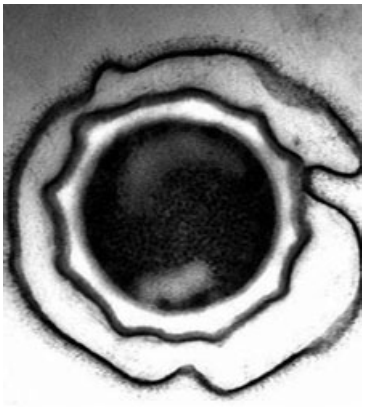

Fig. 2. The electron micrograph of thin section of *B. cereus* exosporium.

Spore germination

The heat resistance of bacterial spores and their ability to germinate to form vegetative cells is a major cause of food spoilage and safety problems. Bart Keiser, Andrea O'Brien and Remco Kort (S. Brul's laboratory, University of Amsterdam and Unilever Research and Development, the Netherlands) presented different aspects of germination, spore heat resistance and monitoring in the food processing industry. *B. cereus* is an important cause of food poisoning, producing both diarrhoea and emetic-type syndromes. Food poisoning is caused by the ingestion of large numbers of spores present in contaminated food ($>10^5$ g^{-1}). *B. cereus* is believed to undergo rapid germination in the small intestine. The acidity of the stomach might play a role in activating germination. Luc Hornstra (Wageningen Centre for Food Science, the Netherlands) reported work in which they systematically inactivated *B. cereus* germination genes with homologues in *B. subtilis* with the long-term aims of elucidating the nutritional signals required that stimulate germination in this important food pathogen. Hornstra has shown the importance of one germination locus, *gerR*, in the germination response and has proposed this to encode a dominant receptor able to respond to alanine, cysteine, threonine, inosine and adenosine.

Following germination, *B. cereus* is able to colonize the gastrointestinal tract briefly, probably through the formation of mixed biofilms on food particles as well as on the mucosal epithelium. Thus, understanding what controls germination and how this organism colonizes the gut is important for understanding the underlying nutritional signals that promote food poisoning. Philippe Schmitt (UMR A408, France) has made detailed studies on what makes *B. cereus* produce enterotoxins. The Hbl and Nhe toxins

associated with the diarrhoeal-type syndrome are well characterized. Schmitt has shown that *B. cereus* needs oxygen to produce these toxins. This is interesting because although *B. cereus* can grow anaerobically, one would expect oxygen to be present at ever decreasing levels as the bacterium passes through the gastrointestinal tract. Schmitt also revealed that Hbl production was increased at low growth rates and as glucose levels decreased. The take-home message from these studies is that the physiological conditions within the gut can substantially affect the ability to produce food poisoning. The ability of *B. cereus* to secrete enterotoxins depends very much on its ability to gain a foothold in the small intestine and this is dependant on the amount of spore germination as well as the microenvironment within the small intestine and might explain, in part, why not everyone eating contaminated food gets food poisoning.

One area of *Bacillus* biology that is becoming of interest is the role of *Bacillus* species in the environment, rather than simply as lab models of unicellular differentiation. Recent studies coming out of the Losick and Kolter laboratories (Branda *et al*., 2001) have shown evidence of multicultural behaviour in *B. subtilis* with the formation of fruiting bodies that act as preferential sites of spore formation in biofilms. Another characteristic of *Bacillus* species is the ability to swarm on solid surfaces. Swarming of *B. subtilis*, *B. cereus* and *Bacillus thuringiensis* is a flagella-driven process that is promoted on nutrient-rich media. Francesco Celandroni and co-workers (S. Senesi's laboratory, University of Pisa, Italy) have begun to investigate the nature of swarming in *B. subtilis* and, starting from a hyper-motile mutant, have characterized a genetic locus, *ifm*, which appears to control swarming behaviour. The *ifm* locus comprises two genes, *ifmA* and *ifmB*, both of which are expressed in solid and liquid media. Interestingly, Celandroni proposes that these genes are cryptic in the laboratory or 'domesticated' strain of *B. subtilis* but active in natural variants. Clearly, understanding the life of *Bacillus* species in their natural state is only just beginning but will offer many valuable insights into how bacteria interact with themselves and with their hosts.

Spore-related applications

The session on the Emerging Application of Bacterial Spores was closely related to that on spore coat structure, as it focused on the use of *B. subtilis* spores as vaccine vehicles displaying heterologous antigens incorporated into coat surface. This is a new application of bacterial spores, as a genetic system to present heterologous antigens on the spore surface (Isticato *et al*., 2001) and tests of recombinant spores as oral vaccines are very recent developments (Duc *et al*., 2003; Ciabattini *et al*., 2004; Mauriello *et al*., 2004). Many potential advantages are connected with the utilization of spores as vaccine delivery systems: (i) heat stability, ensured by the well-documented resistance of the bacterial spore (Ricca and Cutting, 2003), (ii) safety record, established through the common use of spores of several species as probiotics (Senesi 2004) and (iii) simple and economic production of large amounts of spores, based on the commonly used procedures for industrial-scale production and commercialization of spore-based products. In addition, the diffuse use of *Bacillus* spores as probiotics for both humans and animals (Senesi, 2004) might facilitate their eventual licensing as oral vaccines. Although this field is only now emerging, initial proof of principle studies have been recently reviewed (Cutting, 2004).

The development of a spore surface display system was initially based on the use of CotB, a *B. subtilis* spore coat component, as a fusion partner to express a highly immunogenic tetanus toxin fragment C (TTFC) on the spore surface (Isticato *et al*., 2001). More recently, another coat component, CotC, was also tested as fusion partner for the expression of TTFC and of the B subunit of the heat-labile toxin of *E. coli* (LTB) (Mauriello *et al*., 2004). Emilia Mauriello (E. Ricca's laboratory, University of Napoli, Italy) reported recent developments in the display of heterologous antigens on the *B. subtilis* spore surface. The same two antigens independently exposed on the spore surface were simultaneously exposed on the surface of the same recombinant spore (E.M.F. Mauriello and E. Ricca, in preparation). These new developments point to the possibility of obtaining a recombinant spore presenting multiple antigens on its surface and therefore capable of acting as a multivalent, heat-stable vaccine. Moreover, the simultaneous expression of two antigens, TTFC and LTB, respectively, suggests the view of the spore surface as a dynamic structure that can accommodate relatively large heterologous molecules without apparent effects on its structure and functionality. In addition to TTFC and LTB, viral epitopes have also been successfully presented on the spore surface, both individually and in combination (E.M.F. Mauriello and E. Ricca, in preparation). Mice dosed orally with recombinant spores expressing TTFC or LTB spores were shown to develop systemic IgG and mucosal sIgA responses (Duc *et al*., 2003; Mauriello *et al*., 2004). Most importantly, mice orally immunized with spores expressing TTFC were protected against challenge with a 20 LD_{50} dose of tetanus toxin (Duc *et al*., 2003). Recombinant spores were also tested in a mucosal priming-parenteral boosting vaccination strategy. Mice were orally immunized with recombinant spores expressing TTFC and then subcutaneously boosted with soluble TTFC. Two weeks after boosting, a significantly higher serum TTFC-specific IgG response was stimulated in mice primed with recombinant spores compared with those inoculated with wild-type spores, suggesting that a

combined mucosal/parenteral strategy can be used to stimulate both local and systemic antigen-specific immune responses (Ciabattini *et al*., 2004).

Le H. Duc (S. Cutting's laboratory, Royal Holloway University, UK) examined the immunogenicity and intracellular fate of *B. subtilis* endospores in a murine model. In contrast with the old view that spores ingested as food contaminants quickly transit the animal gut, it now appears that spores have a complex interaction with the gut-associated lymphoid tissue (GALT). A proportion of ingested spores germinate in the intestine and undergoes limited rounds of growth before sporulating again. Spores can disseminate to the GALT and are even likely to persist briefly within phagocytic cells in the Peyer's patches. This persistence might elicit cellular responses against spores as well as enhancing systemic humoural and local responses. *In vitro* studies with a macrophage-like cell line demonstrated that spores could germinate efficiently in macrophages, initiate gene expression as well as induce proinflammatory cytokines. Analysis of cytokine mRNA in GALT and lymphoid organs showed early induction of interferon (IFN)-γ, a Th1 cytokine, as well as the proinflammatory cytokine TNF-β (Duc *et al*., 2004).

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