Investigation of sporulation in the *Desulfotomaculum* genus: a genomic comparison with the genera *Bacillus* and *Clostridium*

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Summary

The genus *Desulfotomaculum*, belonging to the Firmicutes, comprises strictly anaerobic and endospore-forming bacteria capable of dissimilatory sulfate reduction. These microorganisms are metabolically versatile and are widely distributed in the environment. Spore formation allows them to survive prolonged environmental stress. Information on the mechanism of sporulation in *Desulfotomaculum* species is scarce. Herein, this process was probed from a genomic standpoint, using the *Bacillus subtilis* model system as a reference and clostridial sporulation for comparison. *Desulfotomaculum* falls somewhere in between the *Bacillus* and *Clostridium* in terms of conservation of sporulation proteins. Furthermore, it showcased the conservation of a core regulatory cascade throughout genera, while uncovering variability in the initiation of sporulation and the structural characteristics of spores from different genera. In particular, while in *Clostridium* species sporulation is not initiated by a phosphorelay, *Desulfotomaculum* species harbour homologues of the *B. subtilis* proteins involved in this process. Conversely, both *Clostridium* and *Desulfotomaculum* species conserve very few *B. subtilis* structural proteins, particularly those found in the outer layers of the spore. *Desulfotomaculum* species seem to share greater similarity to the outer layers of *Clostridium difficile*.

Introduction

The genus *Desulfotomaculum* comprises Gram-positive, low GC content bacteria belonging to the order Clostridiales. They are strict anaerobes, endospore formers and are distinct from their close *Clostridium* relatives in that they are capable of dissimilatory sulfate reduction (Widdel, 2006). Desulfotomacula are physiologically and ecologically versatile. They can use a wide variety of organic substrates such as organic acids, alcohols, sugars and amino acids as electron donors for sulfate reduction. Additionally, some species are able of autotrophic growth with H2 or CO as electron donors for sulfate reduction. *Desulfotomaculum* spp. are found in diverse environments, including soils, marine and fresh waters, wastewater, thermal springs and animal guts. Despite their requirement for anoxic conditions for growth, they can be found in environments with alternating redox regimes (Stackebrandt *et al.*, 1997; Widdel, 2006). One of the strategies they employ to thrive in diverse conditions is the formation of endospores (henceforth called spores). Some of the species form extremely heat-resistant spores: 10% of *Desulfotomaculum kuznetsovii* spores survived 15 min at 140°C (Goorissen, 2002).

Spores are metabolically inactive cells formed in response to environmental stress. Spore formation is a successful survival strategy due to their remarkable resistance to harsh conditions such as nutrient limitation, high temperatures, UV irradiation, desiccation and toxic molecules (Hilbert and Piggot, 2004; Higgins and Dworkin, 2012; McKenney *et al.*, 2013; Tan and Ramamurthi, 2014). Spore resistance is achieved by, among other factors, the condensation of the chromosome in a tight bundle protected by specific proteins in a partially dehydrated core, surrounded by a sequence of compact layers. Assembly of this concentric cell morphology requires a tightly regulated sequence of events that begins with the recognition of environmental stress signals. Once sporulation is initiated, the cell undergoes asymmetric division by which the original cell (the mother cell, MC) develops a small compartment, called the forespore (FS). The FS is transformed to a mature spore through a series of structural amendments: a modified peptidoglycan layer, the cortex, is formed; small acid soluble proteins (SASPs) are synthesized and Ca2+-
dipicolinic acid (Ca-DPA) is recruited to the core to protect the chromosome; and during late stages of sporulation, the outer layers (the coat and, in some cases, the exosporium) are assembled (Rudner and Losick, 2001; Higgins and Dworkin, 2012). Subsequently, the MC lyses and the mature spore is released to the environment, where it remains dormant until favourable conditions for growth resume.

The exact regulatory system and the precise composition of each spore layer that leads to the formation of this special cell type has been extensively studied in a model bacterium, *Bacillus subtilis*. It is much less well characterized in other spore-forming bacteria (Paredes et al., 2005; Galperin et al., 2012; Higgins and Dworkin, 2012). In particular, little information is available about sporulation in *Desulfotomaculum* species, despite the environmental and metabolic relevance of this genus. The genomes of nine species from this genus have become available recently (*Desulfotomaculum acetoxidans*, *Desulfotomaculum alcoholivorax*, *Desulfotomaculum carboxydivorans*, *Desulfotomaculum gibsoniae*, *Desulfotomaculum kuznetsovii*, *Desulfotomaculum nigrificans*, *Desulfotomaculum reducens*, *Desulfotomaculum ruminis*; Table S1), prompting the present genomic investigation of sporulation. We used the *B. subtilis* sporulation process and the information available on *Clostridium* spp. as models for our investigation and attempted to identify putative homologues of sporulation proteins in the desulfotomacula. Similar genomic studies have been previously carried out with the overall goal of identifying a core set of spore-forming bacteria-specific proteins (Stragier, 2002; Onyenwoke et al., 2004; Galperin et al., 2012; Abecasis et al., 2013; Traag et al., 2013). In these studies, however, only one or two *Desulfotomaculum* spp. were included, i.e. *D. reducens* (Galperin et al., 2012; Abecasis et al., 2013; Traag et al., 2013) and *D. acetoxidans* (Galperin et al., 2012) respectively. Here, we aimed at identifying similarities of sporulation in *Desulfotomaculum* spp. to the *Bacillus* and *Clostridium* models.

**Results and discussion**

We searched for putative orthologues of 288 *B. subtilis* proteins involved in sporulation of the nine above-mentioned *Desulfotomaculum* species (Table S1). The BLASTp results are listed in Table S2 and summarized in Table S3. In the following paragraphs, we discuss the most relevant findings. In particular, we address the level of conservation of the regulatory and morphogenetic proteins involved in the initiation of sporulation, the core regulatory proteins and the proteins of the most outer spore layers. In contrast, we do not discuss proteins pertaining the inner layers (i.e. those involved in cortex synthesis, DPA synthesis and import to the core, and SASPs) because they are overall very well conserved (Tables S2–S4).

*Desulfotomaculum* spp. encode for putative orthologues of phosphorelay proteins

The bacilli enter sporulation following a four component phosphorelay that is initiated by the autophosphorylation of a sensory histidine kinase (HK), proceeds with the sequential transfer of the phosphoryl group to Spo0F and Spo0B, and culminates with the accumulation of Spo0A-P, the master regulator of sporulation (Perego, 1998; Hoch, 2000; Fujita and Losick, 2005; Higgins and Dworkin, 2012). The phosphorelay is highly conserved among the bacilli, but homologues of its components could not be found in *Clostridium* species, except for Spo0A, which conserves its master regulator role. This suggests that the clostridia enter sporulation through direct phosphorylation of Spo0A by sensory HKs, essentially a basic two-component system (Dürre and Hollerschwandner, 2004; Paredes et al., 2005; De Hoon et al., 2010; Steiner et al., 2011; Galperin et al., 2012). In contrast, we found that, in all the desulfotomacula, putative homologues of sporulation HKs, Spo0B and Spo0F were readily identifiable, suggesting a phosphorelay system akin to that in bacilli.

First, we screened the desulfotomacula genomes for putative class IIIB orphan HKs (Fabret et al., 1999), as five such HKs (KinA, KinB, KinC, KinD, KinE) are known to be capable of phosphorylating Spo0F in *B. subtilis* (Fujita and Losick, 2005; Higgins and Dworkin, 2012). We targeted those HKs that were the most conserved across different species (based on synteny analysis and inter-*Desulfotomaculum* BLASTp) (see Tables S5 and S6, for further detail) and identified three orphan class IIIB HKs per organism (except *D. kuznetsovii* and *D. alcoholivorax*, for which we could identify only one and two HKs respectively) (Table 1).

Second, we identified proteins with high similarity to *B. subtilis* Spo0F in all the strains. For *D. alcoholivorax* and *D. acetoxidans*, two candidate Spo0F orthologues were identified, but discrimination of the functional homologue is not possible without experimental support. All the catalytic residues involved in the phosphorytransfer activity of Spo0F are largely conserved throughout the desulfotomacula (Table 2), as are most of the residues known to be crucial for interaction with Spo0B (see also Table S7) (Stephenson and Hoch, 2002; Varughese, 2002).

Third, a BLASTp analysis-based search for Spo0B homologues identified significant alignments (E-values between 1e-5 and 1e-9) for all *Desulfotomaculum* species except for *D. kuznetsovii*, although identities and cover-
age were generally low (Tables S2 and S3). This level of conservation was also observed among bacilli (Stephenson and Hoch, 2002; Mattoo et al., 2008). To support the putative orthology of the best BLASTp hits to B. subtilis Spo0B, we used synteny analysis and found that the genes surrounding the putative spo0B are conserved relative to B. subtilis (Fig. 1A) (Stragier, 2002). We used this information to identify a putative Spo0B orthologue in D. kuznetsovii, since the BLASTp search was not successful and found a protein annotated as ‘signal transduction histidine kinase regulating citrate/malate metabolism’. Many of the identified Spo0B

| Table 1. Putative sporulation histidine kinases and SpoIIQ homologues in Desulfotomaculum species. |
|---------------------------------------------------|---------------------------------------------------|---------------------------------------------------|
| Da   | Dtox_3007* | Dtox_3081 | Dtox_3426 |
| Dal  | H569DRAFT_02773* | H569DRAFT_00816 |  |
| Dc   | Desca_0990* | Desca_2032*** | Desca_1338* |
| Dh   | Desgi_1001 | Desgi_0716 | Desgi_039 |
| Dh   | DESHY_90002 | DESHY_60174*** | DESHY_110073* |
| Dk   | Desku_2564 |  |  |
| Dn   | DesniDRAFT_1018** | DesniDRAFT_1367*** | DesniDRAFT_0665* |
| Dred | Dred_1269 | Dred_1139*** | Dred_1745* |
| Dru  | Desru_3403 | Desru_2967*** | Desru_2230* |

| Table 2. Conservation of Spo0F crucial residues (for phosphotransfer activity and for interaction with residues in the amino acid sequence of Spo0B in B. subtilis). |
|---------------------------------------------------|---------------------------------------------------|---------------------------------------------------|
| Spo0F   | 10 | 11 | 14 | 15 | 18 | 21 | 54 | 82 | 85 | 104 | 105 | 106 | 107 | 108 |
| Bs (BSU37130) | D | D | G | I | L | E | D | T | G | K | P | F | D | I |
| Da (Dtox_3740) | D | D | G | V | L | I | D | T | G | K | P | F | D | I |
| Da (Dtox_0055) | D | D | G | V | L | I | D | T | G | K | P | F | D | V |
| Dal (H569DRAFT_02855) | D | D | G | V | L | I | D | T | G | K | P | F | D | V |
| Dal (H569DRAFT_01679) | D | D | G | V | L | E | D | T | S | K | P | F | D | L |
| Dc (Desca_2625) | D | D | G | V | L | E | D | T | G | K | P | F | D | L |
| Dg (Desgi_4633) | D | D | G | V | L | E | D | T | G | K | P | F | D | L |
| Dh (DESHY_30097)* | D | D | G | V | L | E | D | S | G | K | P | F | D | L |
| Dk (Desku_3419) | D | D | G | V | L | E | D | T | G | K | P | F | D | L |
| Dn (DesniDRAFT_2215) | D | D | G | V | L | E | D | T | G | K | P | F | D | L |
| Dred (Dred_3146) | D | D | G | V | L | E | D | T | G | K | P | F | D | L |
| Dru (Desru_3715) | D | D | G | V | L | E | D | T | G | K | P | F | D | L |

The upper part of this table lists the putative sporulation histidine kinases that we identified. They were selected from the orphan class IIIB HKs listed in Table S6, based on their sequence conservation and synteny. Each of the three columns groups HKs which share great sequence similarity; the *, ** or *** indicate synteny. The proteins shaded in grey share significant sequence similarity among each other, but not with the other proteins in the same column and are not syntenic. The lower part of the table lists potential SpoIIQ homologues. Three methods were used to identify them: synteny analysis (closeness to SpoIID), BLASTp of B. subtilis SpoIIQ (CD0125) and BLASTp of C. difficile SpoIIQ (BSU36550). Details on the best alignments are listed in Table S8. Discrimination of the functional homologue of SpoIIQ will require experimental work.
putative homologues in the Desulfotomacula are annotated in the same way, and it has been suggested that Spo0B has evolved from an ancestral citrate/malate kinase (Mattoo et al., 2008). Thus, we propose that the D. kuznetsovii protein identified by synteny conservation (Desku_0427) may be a Spo0B homologue. Furthermore, all putative Spo0B orthologues identified in Desulfovibrio species contain a Spo0B-type α-helix domain (PFAM14689), which is significantly conserved relative to the B. subtilis Spo0B (Fig. 1B). In B. subtilis, the α-helix domain contains the residues responsible for the interaction with SpoOF and SpoOA (Stephenson and Hoch, 2002).

The identification of Spo0B homologues in Desulfovibrio is novel, since none of the proteins identified had been previously recognized as involved in the sporulation phosphorelay; in addition, Spo0B was previously presumed missing in D. acetoxidans and D. reducens (Galperin et al., 2012).

In contrast to the conservation of its main players, we were unable to identify most of the effectors of the phosphorelay (Fig. 2A). The only elements for which we found significant homology in all Desulfovibrio species are AbrB, CodY and σH, which is consistent with the importance of these regulators for other physiological states (Fig. 2A) (Perego, 1998; Sonenshein, 2000; Hilbert and Piggot, 2004; Chumsakul et al., 2011; Higgins and Dworkin, 2012). The significant lack of homologues of the B. subtilis phosphorelay regulators in Desulfovibrio has two potential explanations. Either
Fig. 2. (A) Schematic of the phosphorelay that leads to the phosphorylation of Spo0A, which initiates sporulation, based on the *B. subtilis* model. Colour-coding indicates the level of conservation among *Desulfotomaculum* spp. (D.) and *Clostridium* spp. (C.), based on our analysis and previously published work (Stragier, 2002; Paredes et al., 2005; Galperin et al., 2012). (B) This is a simplified model of the sporulation cross-cross regulation cascade that follows the accumulation of Spo0A in *B. subtilis*. Colour coding indicates different levels of conservation across the genera *Bacillus* (B.), *Clostridium* (C.) and *Desulfotomaculum* (D.), based on our analysis and previously published works (Paredes et al., 2005; De Hoon et al., 2010; Galperin et al., 2012; Fimlaid et al., 2013).
entry into sporulation is significantly less controlled than in the bacilli, or the regulatory and phosphorylation controls over each component of the phosphorelay differ significantly from those in \textit{B. subtilis}. If the latter is the case, this may reflect that desulfotomacula occupy different environmental niches than bacilli, and thus the signals that trigger sporulation, and their associated proteins, are distinct in \textit{Desulfotomaculum} spp. and \textit{B. subtilis}.

The core regulatory proteins are conserved

The main regulatory proteins that control sporulation (Spo0A, σ\(^5\), σ\(^6\), σ\(^3\), σ\(^8\)) and the proteins involved in their activation were shown to be widely conserved across spore-forming bacteria (Stragier, 2002; Onyenwoke et al., 2004; De Hoon et al., 2010; Galperin et al., 2012; Abecasis et al., 2013; Traag et al., 2013). We confirmed this in all the \textit{Desulfotomaculum} spp. we investigated. The only \textit{B. subtilis} proteins of the sporulation regulatory cascade that are not conserved in this genus are the transcription regulators GerE, GerR and RfsA, and BofC, the non-transcriptional regulator of SpoIVB. These proteins were already reported to be poorly conserved even among Bacillus species (De Hoon et al., 2010). This suggests either that the \textit{Desulfotomaculum} spp. auxiliary regulators are significantly different from those in \textit{B. subtilis} (i.e. are genus or species specific) or that regulation is less tightly controlled (requiring fewer feed-forward loops) than for bacilli.

Another poorly conserved protein, essential for sporulation, is SpoIIQ. In \textit{B. subtilis}, this protein forms a complex with SpoIIAH (Q-AH) that is bifunctional: it contributes to FS engulfment and it forms a feeding channel that allows nutrients (and possibly regulatory signals) to be transferred from the MC to the FS during spore assembly (Broder and Pogliano, 2006; Camp and Losick, 2009; Doan et al., 2009; Higgins and Dworkin, 2012). The SpoIIQ amino acid sequence has already been reported to be poorly conserved even among bacteria of the same genus, and it has been suggested that this may be due to non-orthologous gene replacement events (Onyenwoke et al., 2004; Galperin et al., 2012; Abecasis et al., 2013). Thus, the lack of an obvious SpoIIQ orthologue in \textit{Desulfotomaculum} spp. was not entirely unexpected. However, we propose candidate orthologues based on synteny and BLASTp searches (Table 1; Table S8).

Figure 2B shows the \textit{B. subtilis} regulatory cascade model, highlighting the level of conservation of each protein across different genera. Until recently, this model was considered valid for all spore-forming bacteria, but recent transcriptional profiling investigations in \textit{Clostridium} spp. indicated that this is not the case. Notably, in contrast to \textit{B. subtilis}, in \textit{Clostridium difficile}, the activation of σ\(^5\) and of σ\(^k\) is independent of σ\(^6\) and σ\(^8\) respectively; lack of dependency of σ\(^6\) activation on σ\(^8\) was also observed in \textit{Clostridium acetobutylicum}; in \textit{Clostridium perfringens} and \textit{Clostridium botulinum}, σ\(^k\) is activated upstream of σ\(^5\); in addition, some differences in the morphologic stages affected by each of the sigma factors were observed in different clostridia with respect to \textit{B. subtilis} (Filmaid et al., 2013; Pereira et al., 2013; Saujet et al., 2013; McBride, 2014; Paredes-Sabja et al., 2014). Transcriptomic investigations will be needed to reveal the details of sporulation regulation. However, based on genomic information alone, it is notable that \textit{Desulfotomaculum} spp. have homologues for SpoIVB, BofA, SpoIVFB and SpoIVFA while the latter three are absent from \textit{C. difficile} and SpoIVFA is absent from many clostridia (Stragier, 2002; Paredes et al., 2005; Filmaid et al., 2013; Pereira et al., 2013; Saujet et al., 2013). This suggests that the proteolytic activation of σ\(^6\), through the protease SpoVF (kept inactive in a complex with SpoIVFA and BofA), and its dependency on σ\(^5\), through the signal protein SpoIVB, may be conserved in \textit{Desulfotomaculum} spp. relative to the \textit{B. subtilis} model.

Morphogenesis of Desulfotomaculum spores

Asymmetric cell division and FS engulfment. The morphologic hallmark of commitment to sporulation is the formation of the polar septum and consequent conversion of the cell to the two-compartment (i.e. FS and MC) sporangium (Rudner and Losick, 2001). The major proteins involved in cell compartmentalization are FtsZ and SpoIIE, essential for correct septum assembly, and DivIVA, SpoOJ, RacA and Soj, which are involved in the migration of the two chromosome copies to the cell poles (Barák and Youngman, 1996; Arigoni et al., 1999; Ben-Yehuda and Losick, 2002; Adams and Errington, 2009). All \textit{Desulfotomaculum} species have homologues for FtsZ, SpoIIE, SpoOJ and Soj, and all except for \textit{D. kuznetsovi}i have a DivIVA homologue, while no obvious homologue of RacA could be identified in any of the species (Tables S2 and S3). It is possible that Soj could be sufficient to fulfill the chromosome anchoring to the poles in \textit{Desulfotomaculum}, since in \textit{B. subtilis}, this protein appears to play a partially redundant role with RacA in targeting the chromosomes to the cell poles in a DivIVA-dependent manner (Wu and Errington, 2003; Lee and Grossman, 2006). Alternatively, a non-orthologous functional analogue of RacA could be present in this genus.

Septum formation, is followed by complete translocation of one of the chromosomes to the FS and engulfment of the FS by the MC membrane, through the action of
Sporulation in the Desulfotomaculum genus

The outer layers of Desulfotomaculum spores share greater similarity with C. difficile than Bacillus spp.

Desulfotomaculum spp. display a proteinaceous coat as well as an outermost proteinaceous layer: the exosporium. The latter is present in B. anthracis while in B. subtilis, it is replaced by a spore crust (McKenney et al., 2010). Thus, we used B. subtilis coat and B. anthracis exosporium proteins to identify putative components of the Desulfotomaculum spore outer layers.

Of the 97 B. subtilis coat proteins and 17 B. anthracis exosporium proteins we considered, we only identified 15 and 3 putative homologues, respectively, in Desulfotomaculum (Tables S9 and S10). Limited spore outer layer conservation was also observed in the clostridia, relative to the bacilli (Henriques and Moran, 2007; Paredes-Sabja et al., 2014). We observed that most, but not all, of the Bacillus proteins conserved in Clostridium are also conserved in Desulfotomaculum, and those that are not are also not conserved in C. difficile (this organism has recently been proposed to be renamed Peptoclostridium difficile, but here we refer to it by its traditional name) (Henriques and Moran, 2007; Yutin and Galperin, 2013). Therefore, we used recent proteomic studies of C. difficile (Lawley et al., 2009; Permpoonpattana et al., 2011; 2013; Abhyankar et al., 2013; Putnam et al., 2013; Paredes-Sabja et al., 2014) to search for putative homologues in Desulfotomaculum species. We found that Desulfotomaculum have putative homologues for 23 of the 57 C. difficile spore surface proteins we tested (Tables S9 and S10). Remarkably, among the C. difficile proteins for which homologues were identified are two morphogenetic proteins SpoIVA (a homologue of B. subtilis SpolVA) and SipL (a previously uncharacterized protein, first identified in the C. difficile spore proteome), and some of the proteins with defined enzymatic activity, i.e. superoxide dismutase, manganese catalase, peroxiredoxin reductase (Table S9) (Lawley et al., 2009; Permpoonpattana et al., 2011; 2013; Putnam et al., 2013; Paredes-Sabja et al., 2014). These observations, and the lack of detectable homologues of B. subtilis morphogenetic proteins (except for SpoIVA), suggest that the composition and assembly mechanism of the Desulfotomaculum spore outer layers may share greater similarity to C. difficile than to the Bacillus species.

Summary

Our investigation confirmed that the main proteins of the core regulatory cascade are conserved in the desulfotomacula, as was already observed for the bacilli and the clostridia. With the currently available information, however, it is impossible to establish whether the Desulfotomaculum regulatory pathway, in terms of temporal progression of sigma factor activation and inter-compartmental signalling, resembles more closely the Bacillus or one of the Clostridium spp. models.

In contrast to the clostridia, the desulfotomacula appear to have the components required to enter sporulation in an analogous way to the bacilli. Furthermore, the inner layers of the spore are morphologically well conserved relative to B. subtilis, while the coat is significantly different and shares a somewhat greater similarity to the C. difficile coat. It is, however, most likely that Desulfotomaculum spp. have an as of yet unidentified set of species- or genus-specific coat proteins, reflecting the diverse environments they occupy.

Altogether, these observations indicate that Desulfotomaculum sporulation is, in some respects, closer to Clostridium, which is expected, due to the fact that these genera belong to the same class, and are distinct from the Bacilli class (Stackebrandt et al., 1997; Widdel, 2006).
However, there are some aspects for which Desulfotomaculum is closer to Bacillus. The Desulfotomaculum and the Bacillus genera are descendants from the Clostridium, and may have a common ancestor (Fig. 3). In certain processes, such as sporulation, and in particular its initiation, the desulfotomacula could have evolved a more complex mechanism than their clostridial ancestors.

Our results are of particular interest because they represent a focused study of sporulation in the Desulfotomaculum genus, which is of great environmental relevance and comprises species, i.e. D. kuznetsovii, whose spores exhibit exceptional heat resistance (can survive several autoclave cycles). Our findings, based purely on bioinformatic approaches, could not establish genes responsible for this unique heat resistance, although they indicate that with respect to cell division, D. kuznetsovii may also be distinct from the other members of its genus. Even though experimental verification of our observations is needed, this work provides an exhaustive overview of the current state of knowledge and a starting point for experimental work, once a genetic system for these bacteria is available.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Table S1. Summary of the main features of the Desulfitomaculum species for which the genome sequence is available, and which were used for our investigation.

Table S2. Here all the BLASTp best hit results are indicated for all the B. subtilis sporulation proteins we identified against the Desulfitomaculum species proteomes. For each best alignment, the following information are included: best hit annotation, E-value of the alignment, query coverage (%), subject coverage (%), identities (%).

Table S3. This table summarizes the results of the BLASTp search of the B. subtilis sporulation proteins against the Desulfitomaculum spp. proteomes. Different criteria were used to evaluate putative conservation of each protein in the desulfitomaculum: E-value < threshold (criterium name: eval); E-value < threshold and query coverage > 70% (eval + query coverage); E-value < threshold, query and subject coverage > 70% (eval + query and subject coverage); E-value < threshold, query and subject coverage > 70%, identities > 40 % (eval + query & subject coverage + ids); query coverage > 70%, identities > 40 % (query coverage + ids); query and subject coverage > 70%, identities > 40 % (query & subject coverage + ids). Two E-values were used as thresholds: 1e-2 and 1e-5. When the criteria are satisfied, the cells contains a ‘1’ and are coloured in red; while cells that do not satisfy the criteria are white and contain a ‘-’. We used the hits that satisfied the least stringent condition (eval<1e-2) as queries to perform a bidirectional BLASTp search against B. subtilis. The results of this search are reported to the right of the table. When the hits are bidirectional best hits, the ‘1’ in the (1e-2) section are in black text instead of blue.

Table S4. List of small acid soluble proteins, responsible for binding and protecting the chromosome in the spore core, extracted from the proteomes of the Desulfitomaculum species. The JGI ID, locus tag and annotation are indicated, as well as the best BLASTp hit against the B. subtilis
proteome, and the relative alignment E-value, identities (%), coverage of subject and query (%).

Table S5. Potential HKs in Desulfotomaculum species. These HKs were selected based on their belonging to the IIIB class, or to the closely related IIIB [based on the similarity of the residues that surround the reactive H, see Table S3 (Fabret et al., 1999)], and on the fact that they are probably orphans: the only potential mate-TR (identified by gene proximity) are significantly different from Spo0F, so they are unlikely partners of IIIB-type HKs. BLASTp best hits against B. subtilis are reported to support the similarity of these HKs to the sporulation HKs of B. subtilis (only in one case, highlighted in green, this was not the case). They are marked in bold if they are reciprocal best BLASTp hits with the relative Desulfotomaculum species. *Desku_2572 (a Spo0F type TR) is most likely mated to the HK Desku_2571, and not to the putative sporulation HK Desku_2564. This HKs list was further screened for highly conserved HKs (see Table 1 in the main text).

Table S6. Multiple sequence alignment of the residues surrounding the reactive H in IIIB type HKs of B. subtilis and the putative sporulation HKs in the Desulfotomaculum species listed in Table S2. These residues are highly conserved and characterize these HKs as IIIB type, since in other HK classes the residues surrounding the reactive H are significantly different (Fabret et al., 1999). Colour-coding indicates residues with similar properties. Red: small and hydrophobic residues (AVFPMILW); blue: acidic (DE); magenta: basic (RK); green: hydroxyl, sulphydryl and amine residues (STYHCNGQ).

Table S7. Additional Spo0F residues involved in interaction with Spo0B (although the exact contribution of these residues is not clear). This table completes Table 2, with less relevant residues. The first line indicates the position of each residue in the amino acid sequence of Spo0F in B. subtilis. The other lines indicate the residue present at each position in B. subtilis and in the Desulfotomaculum species. Acronyms: Bs: B. subtilis; Da: D. acetoxidans; Dal: D. alcoholivorax; Dc: D. carboxydvorans; Dg: D. gibsoniae; Dh: D. hydrothermale; Dk: D. kuznetsovi; Dn: D. nigrificans; Dred: D. reducens; Dru: D. ruminis. In Da and Dal there are two putative Spo0F homologues, thus we included both in this figure: in parenthesis, the locus tag id is indicated. A few residues are very poorly conserved across the desulfotomacula, in particular the residues corresponding to B. subtilis A34, N35, L37, Q38. This, however, is not surprising given that these residues in B. subtilis interact with the αβ-fold domain of Spo0B, which we found not to be conserved in the putative Spo0B homologues in the Desulfotomaculum species (see main text).

Table S8. BLASTp best hit results for C. difficile and B. subtilis SpoIIQ, i.e. CD0125 and BSU36550, respectively, against the Desulfotomaculum species proteomes. For each best alignment the following information are included: best hit annotation, E-value of the alignment, query coverage (%), subject coverage (%), identities (%).

Table S9. Spore coat and exosporium proteins from B. subtilis, B. anthracis and C. difficile putatively conserved in Desulfotomaculum. A best reciprocal BLASTp hit approach was used to define putative homologues of query proteins in each Desulfotomaculum sp., and this table indicates the proteins for which putative homologues were identified in at least five out of nine of the species considered (proteins included in this table). The locus tags of the Desulfotomaculum reciprocal best hits are indicated in the last column; the column ‘Loc.’ indicates the localization of the protein in the outer layers of B. subtilis, B. anthracis or C. difficile: C = coat, E = exosporium, ND = not determined, although it pertains the outer layers of the spore. Detailed BLASTp results, including spore outer layer proteins for which we did not identify homologues in Desulfotomaculum, are listed in Tables S2 and S10. These proteins were identified or reviewed in previous works (Henriques and Moran, 2007; Lawley et al., 2009; Permpongopattana et al., 2011; 2013; Abhyankar et al., 2013; Putnam et al., 2013; Paredes-Sabja et al., 2014).

Table S10. BLASTp best hit results for B. anthracis exosporium, C. difficile spore outer layers and B. subtilis coat proteins against the Desulfotomaculum species proteomes. For each best alignment the following information are included: best hit annotation, E-value of the alignment, query coverage (%), subject coverage (%), identities (%). The results are summarized in columns Da to Dru, indicating with ‘+’ that no alignment with an E-value < 0.01 was found, with ‘1’ (red coloured cells) that such an alignment was identified; cells with ‘1’ are in black text if the alignment is a best reciprocal BLASTp hit, in blue if not.