environmental microbiology reports

Environmental Microbiology Reports (2014) **6**(6), 756–766 doi:10.1111/1758-2229.12200

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.*

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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 H_2 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 strive 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 Ca^{2+}-

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 hydrothermale*, *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 Hollergschwandner, 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. kuznetso*vii 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 phosphotransfer 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-

Table 1. Putative sporulation histidine kinases and SpoIIQ homologues in *Desulfotomaculum* species.

Putative sporulation histidine kinases			
Da	Dtox_3007*	Dtox_3081	Dtox_3426
Dal	H569DRAFT_02773*	H569DRAFT_00816	
Dc	Desca_0990**	Desca_2032***	Desca_1338*
Dg	Desgi_1001	Desgi_0716	Desgi_939
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*

Possible SpoIIQ homologues			
	Synteny	CD0125 BLASTp best hit	BSU36550 BLASTp best hit
Da	Dtox_4161	Dtox_3964	Dtox_3964
Dal	–	H569DRAFT_01960	H569DRAFT_02364
Dc	Desca_2625	Desca_2625	Desca_2716
Dg	–	Desgi_4627	Desgi_4686
Dh	DESHY_30129	DESHY_30129	DESHY_110533
Dk	Desku_3419	Desku_3503	Desku_3503
Dn	DesniDRAFT_2215	DesniDRAFT_1904	DesniDRAFT_1893
Dred	Dred_3146	Dred_0798	Dred_0798
Dru	Desru_3684	Desru_1027	Desru_3871

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.

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 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_2657)	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_3463)	D	D	G	V	M	E	D	T	G	K	P	F	D	L
Dn (DesniDRAFT_2247)	D	D	G	V	L	E	D	T	G	K	P	F	D	L
Dred (Dred_3178)	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

Amino acid residues are colour-coded: in orange, the catalytic residues; in green, the hydrophobic pocket residues; in blue, residues involved in the interaction with Spo0B which are likely to discriminate Spo0F from response regulators belonging to other families (Varughese, 2002) and are thus crucial for specific mating with Spo0B. Additional Spo0F residues involved in interaction with Spo0B are listed in Table S7. Bs: *B. subtilis*; Da: *D. acetoxidans*; Dal: *D. alcoholivorax*; Dc: *D. carboxydivorans*; Dg: *D. gibsoniae*; Dh: *D. hydrothermale*; Dk: *D. kuznetsovii*; Dn: *D. nigrificans*; Dred: *D. reducens*; Dru: *D. ruminis*. Da and Dal include two putative Spo0F homologues, thus we included both in this table. The locus tag of each putative Spo0F orthologue is indicated in parenthesis. The asterisk indicates that the protein was correctly annotated in the genome. These putative Spo0F orthologues were identified by BLASTp and synteny analysis, and by verification of the presence of the response regulator receiver domain characteristic of Spo0F (Pfam PF00072).

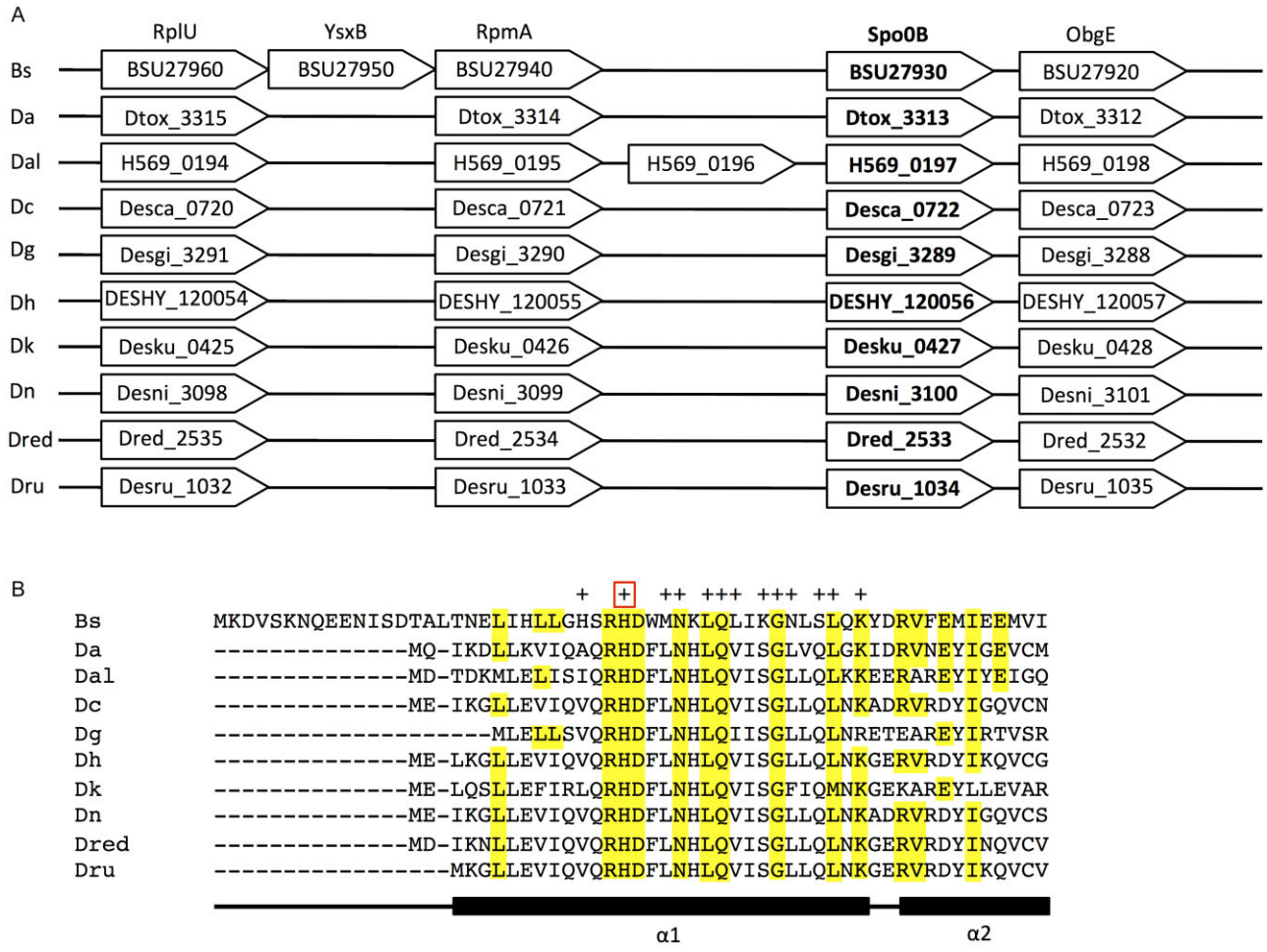

Fig. 1. (A) Synteny is conserved almost perfectly among *B. subtilis* Spo0B and its putative orthologues in *Desulfotomaculum* spp. rplU encodes for a ribosomal protein L21, rpmA for a ribosomal protein L27 and obgE for Obg family GTPase. Conservation of these functions in the neighbours of Spo0B putative orthologues is confirmed by gene annotation. Locus tags for *D. alcoholivorax* and *D. nigrificans* were contracted from H569DRAFT_ to H569_, DesniDRAFT_ to Desni_. (B) Multiple sequence alignment of the α1-helix region in *B. subtilis* Spo0B and its putative orthologues in *Desulfotomaculum* species. + symbols above the sequences indicate *B. subtilis* Spo0B residues known to be important for interaction with Spo0F and Spo0A. The red square indicates the active H residue. Letters shaded in yellow indicate residues conserved relative to the *B. subtilis* Spo0B. Acronyms: Bs: *B. subtilis*; Da: *D. acetoxidans*; Dal: *D. alcoholivorax*; Dc: *D. carboxydivorans*; Dg: *D. gibsoniae*; Dh: *D. hydrothermale*; Dk: *D. kuznetsovii*; Dn: *D. nigrificans*; Dred: *D. reducens*; Dru: *D. ruminis*.

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 *Desulfotomaculum* 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 Spo0F and Spo0A (Stephenson and Hoch, 2002).

The identification of Spo0B homologues in desulfotomacula 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 desulfotomacula are AbrB, CodY and σ^H, 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 desulfotomacula has two potential explanations. Either

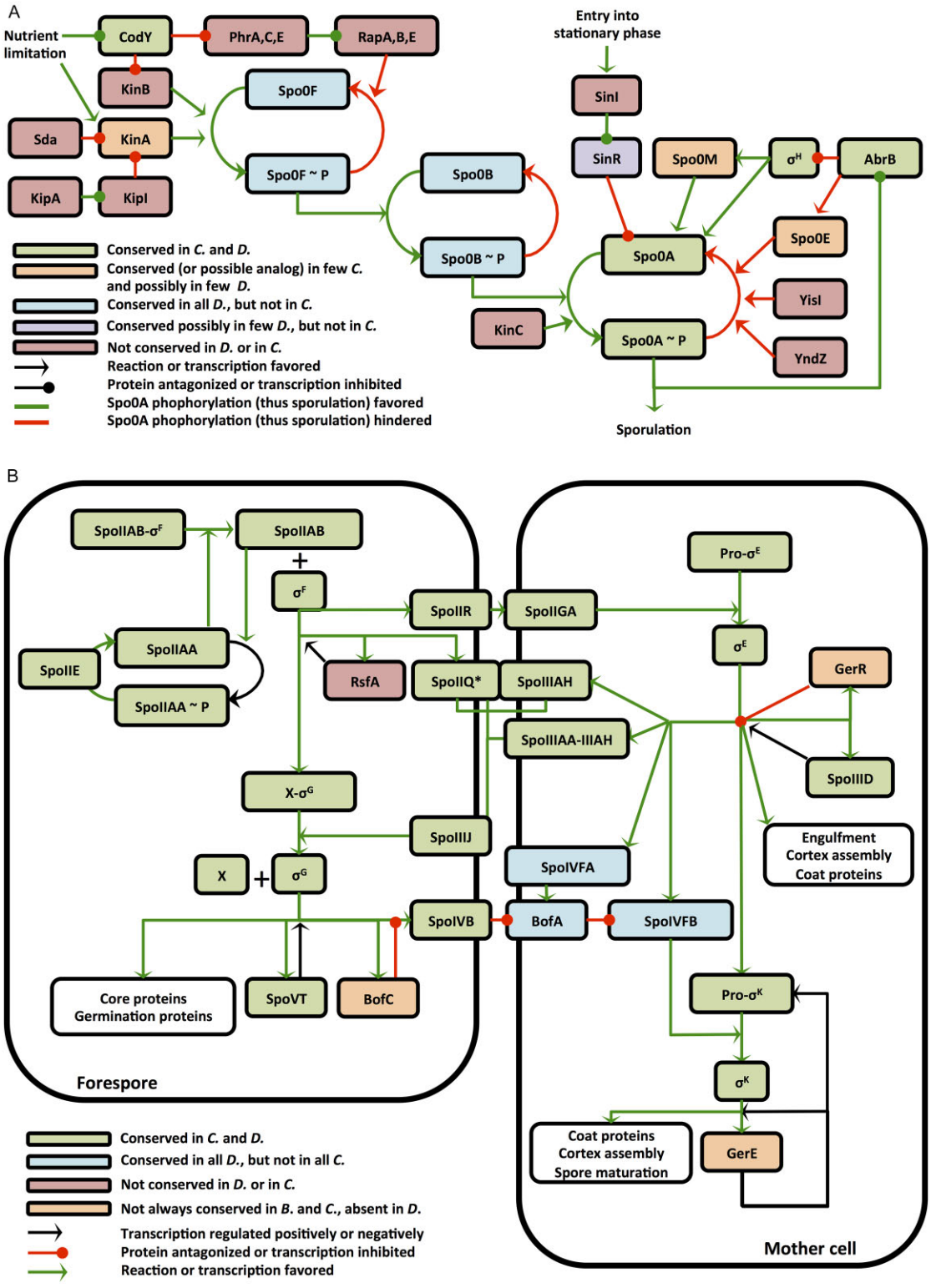

Fig. 2. (A) Schematic of the phosphorelay that leads to the phosphorylation of Spo0A, which initiates sporulation, based on the *B. subtilis* model. Colour-coding indicates the level of conservation among *Desulfotomaculum* spp. (D.) and *Clostridium* spp. (C.), based on our analysis and on previously published work (Stragier, 2002; Paredes *et al.*, 2005; Galperin *et al.*, 2012). (B) This is a simplified model of the sporulation criss-cross regulation cascade that follows the accumulation of Spo0A~P, based on the *B. subtilis* model. 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 *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 *Desulfotomaculum* spp. and *B. subtilis*.

The core regulatory proteins are conserved

The main regulatory proteins that control sporulation (Spo0A, σ^F, σ^E, σ^G, σ^K) 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 *Desulfotomaculum* spp. we investigated. The only *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 *Desulfotomaculum* spp. auxiliary regulators are significantly different from those in *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 *B. subtilis*, this protein forms a complex with SpoIIIAH (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 *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 *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 *Clostridium* spp. indicated that this is not the case. Notably, in contrast to *B. subtilis*, in *Clostridium difficile*, the activation of σ^G and of σ^K is independent of σ^E and σ^G respectively; lack of dependency of σ^G activation on σ^E was also observed in *Clostridium acetobutylicum*; in *Clostridium perfringens* and *Clostridium botulinum*, σ^K is activated upstream of σ^E; in addition, some differences in the morphologic stages affected by each of the sigma factors were observed in different clostridia with respect to *B. subtilis* (Fimlaid *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 *Desulfotomaculum* spp. have homologues for SpoIVB, BofA, SpoIVFB and SpoIVFA while the latter three are absent from *C. difficile* and SpoIVFA is absent from many clostridia (Stragier, 2002; Paredes *et al*., 2005; Fimlaid *et al*., 2013; Pereira *et al*., 2013; Saujet *et al*., 2013). This suggests that the proteolytic activation of σ^K, through the protease SpoIVFB (kept inactive in a complex with SpoIVFA and BofA), and its dependency on σ^G, through the signal protein SpoIVB, may be conserved in *Desulfotomaculum* spp. relative to the *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, Spo0J, 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 *Desulfotomaculum* species have homologues for FtsZ, SpoIIE, Spo0J and Soj, and all except for *D. kuznetsovii* 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 *Desulfotomaculum*, since in *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

SpoIIIE, SpoVG, SpoIIB, the DPM complex (SpoIID, SpoIIP and SpoIIM) and the Q-AH complex (Rudner and Losick, 2001; Hilbert and Piggot, 2004; Broder and Pogliano, 2006; De Hoon *et al*., 2010; Morlot *et al*., 2010; Higgins and Dworkin, 2012). We identified orthologues for all of these proteins except for SpoIIB and SpoIIQ (discussed above) in all the *Desulfotomaculum* species (Tables S1 and S2).

Desulfotomaculum kuznetsovii displays differences in cell division. Using *B. subtilis* DivIVA and its *Desulfotomaculum* orthologues as queries, we were unable to identify a putative orthologue in *D. kuznetsovii*. This finding is difficult to explain without experimental support because the absence of a DivIVA orthologue in this microorganism would signify there is a different mechanism of vegetative cell division and of cell compartmentalization. The possibility that this species has a different cell division system as compared with *B. subtilis* and the other desulfotomacula is bolstered by the absence of MinJ, a protein involved in mid-cell septal localization during binary fission. Interestingly, all *Desulfotomaculum* spp., including *D. kuznetsovii*, have *C. difficile* MinE homologues, a protein absent in *B. subtilis*, which is unrelated, but plays a similar role, to DivIVA in binary fission in Gram-negative bacteria, and possibly in clostridia. Therefore, it is conceivable that, in *D. kuznetsovii*, polar recruitment of the chromosomes is directed by the MinE orthologue rather than DivIVA. However, this possibility would imply a major divergence of *D. kuznetsovii* in both symmetrical and asymmetrical cell division from the *B. subtilis* model; furthermore, in the presence of MinE, an *Escherichia coli*-like oscillating system for cell division can be established in *B. subtilis*, but this appears to negatively affect sporulation (Jamroskovic *et al*., 2012).

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* SpoIVA) 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 intercompartmental 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).

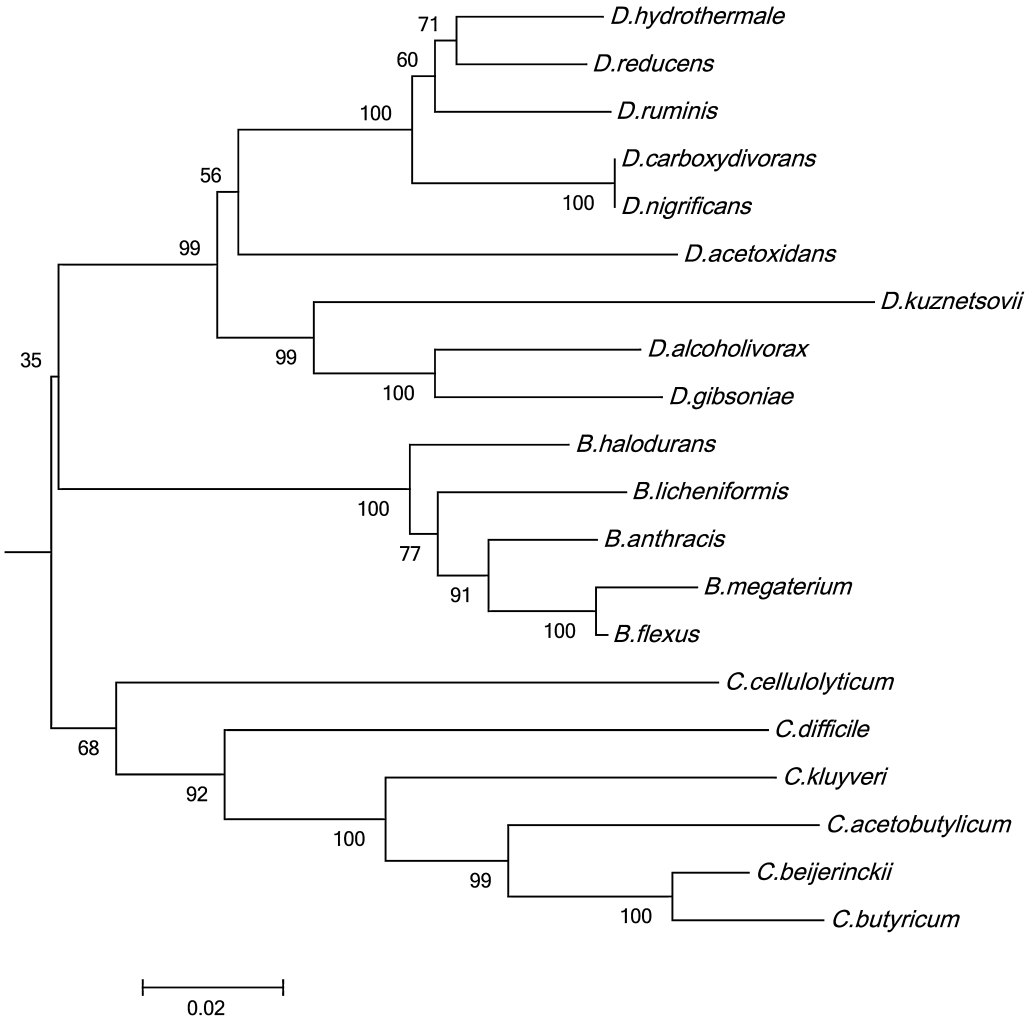

Fig. 3. Neighbour joining 16S rRNA gene tree of the *Desulfotomaculum* species considered in this study, and a few *Bacillus* and *Clostridium*, non-pathogenic spp., *B. anthracis* str. Sterne and *C. difficile* str. 630. Bootstrap values are indicated at the nodes. The scale bar represents 2% sequence difference. The 16S rRNA gene sequence of *Escherichia coli* was used as an outgroup, but was cropped. 16S rRNA gene sequences were obtained from the European Nucleotide Archive (ENA) (Leinonen *et al.*, 2011). We aligned the 16S rRNA gene sequences from the different species with MUSCLE (Edgar, 2004) and constructed a phylogenetic tree with MEGA 5 (Tamura *et al.*, 2011) using a neighbour joining method, with a bootstrap value of 1000.

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.

Acknowledgements

Research was funded by grants of the Earth and Life Sciences division (ALW) of the Netherlands Science Foundation (NWO) (project 819.02.014), ERC (project 323009) and the Netherlands Ministry of Education, Culture and Science

(Gravitation grant 024.002.002), and by the Swiss National Science Foundation (Grants Nos. 315230_127546/1 and 315230_146568). The authors would like to acknowledge Alexandre Bagnoud for contributing to the construction of the phylogenetic tree.

References

Abecasis, A.B., Serrano, M., Alves, R., Quintais, L., Pereira-Leal, J.B., and Henriques, A.O. (2013) A genomic signature and the identification of new sporulation genes. *J Bacteriol* **195:** 2101–2115. doi:10.1128/JB.02110-12.

Abhyankar, W., Hossain, A.H., Djajasaputra, A., Permpoonpattana, P., Ter Beek, A., Dekker, H.L., *et al*. (2013) In pursuit of protein targets: proteomic characterization of bacterial spore outer layers. *J Proteome Res* **12:** 4507–4521.

Adams, D.W., and Errington, J. (2009) Bacterial cell division: assembly, maintenance and disassembly of the Z ring. *Nat Rev Microbiol* **7:** 642–653.

Arigoni, F., Guérout-Fleury, A.-M., Barák, I., and Stragier, P. (1999) The SpoIIE phosphatase, the sporulation septum and the establishment of forespore-specific transcription in *Bacillus subtilis*: a reassessment. *Mol Microbiol* **31:** 1407–1415.

Barák, I., and Youngman, P. (1996) SpoIIE mutants of Bacillus subtilis comprise two distinct phenotypic classes consistent with a dual functional role for the SpoIIE protein. *J Bacteriol* **178:** 4984–4989.

Ben-Yehuda, S., and Losick, R. (2002) Asymmetric cell division in B. subtilis involves a spiral-like intermediate of the cytokinetic protein FtsZ. *Cell* **109:** 257–266.

Broder, D.H., and Pogliano, K. (2006) Forespore engulfment mediated by a ratchet-like mechanism. *Cell* **126:** 917–928.

Camp, A.H., and Losick, R. (2009) A feeding tube model for activation of a cell-specific transcription factor during sporulation in *Bacillus subtilis*. *Genes Dev* **23:** 1014–1024.

Chumsakul, O., Takahashi, H., Oshima, T., Hishimoto, T., Kanaya, S., Ogasawara, N., and Ishikawa, S. (2011) Genome-wide binding profiles of the *Bacillus subtilis* transition state regulator AbrB and its homolog Abh reveals their interactive role in transcriptional regulation. *Nucleic Acids Res* **39:** 414–428.

De Hoon, M.J.L., Eichenberger, P., and Vitkup, D. (2010) Hierarchical evolution of the bacterial sporulation network. *Curr Biol* **20:** R735–R745.

Doan, T., Morlot, C., Meisner, J., Serrano, M., Henriques, A.O., Moran, C.P., Jr, and Rudner, D.Z. (2009) Novel secretion apparatus maintains spore integrity and developmental gene expression in *Bacillus subtilis*. *PLoS Genet* **5:** e1000566.

Dürre, P., and Hollergschwandner, C. (2004) Initiation of endospore formation in *Clostridium acetobutylicum*. *Anaerobe* **10:** 69–74.

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* **19:** 1792–1797.

Fabret, C., Feher, V.A., and Hoch, J.A. (1999) Two-component signal transduction in *Bacillus subtilis*: how one organism sees its world. *J Bacteriol* **181:** 1975–1983.

Fimlaid, K.A., Bond, J.P., Schutz, K.C., Putnam, E.E., Leung, J.M., Lawley, T.D., and Shen, A. (2013) Global analysis of the sporulation pathway of *Clostridium difficile*. *PLoS Genet* **9:** e1003660.

Fujita, M., and Losick, R. (2005) Evidence that entry into sporulation in *Bacillus subtilis* is governed by a gradual increase in the level and activity of the master regulator Spo0A. *Genes Dev* **19:** 2236–2244.

Galperin, M.Y., Mekhedov, S.L., Puigbo, P., Smirnov, S., Wolf, Y.I., and Rigden, D.J. (2012) Genomic determinants of sporulation in Bacilli and Clostridia: towards the minimal set of sporulation-specific genes. *Environ Microbiol* **14:** 2870–2890.

Goorissen, H.P. (2002) Thermophilic methanol utilization by sulfate reducing bacteria. Doctoral Thesis, University of Groningen.

Henriques, A.O., and Moran, C.P., Jr (2007) Structure, assembly, and function of the spore surface layers. *Annu Rev Microbiol* **61:** 555–588.

Higgins, D., and Dworkin, J. (2012) Recent progress in *Bacillus subtilis* sporulation. *FEMS Microbiol Rev* **36:** 131–148.

Hilbert, D.W., and Piggot, P.J. (2004) Compartmentalization of gene expression during *Bacillus subtilis* spore formation. *Microbiol Mol Biol Rev* **68:** 234–262.

Hoch, J.A. (2000) Two-component and phosphorelay signal transduction. *Curr Opin Microbiol* **3:** 165–170.

Jamroskovic, J., Pavlendova, N., Muchova, K., Wilkinson, A.J., and Barak, I. (2012) An oscillating Min system in *Bacillus subtilis* influences asymmetrical septation during sporulation. *Microbiol* **158:** 1972–1981.

Lawley, T.D., Croucher, N.J., Yu, L., Clare, S., Sebaihia, M., Goulding, D., *et al*. (2009) Proteomic and genomic characterization of highly infectious *Clostridium difficile* 630 spores. *J Bacteriol* **191:** 5377–5386.

Lee, P.S., and Grossman, A.D. (2006) The chromosome partitioning proteins Soj (ParA) and Spo0J (ParB) contribute to accurate chromosome partitioning, separation of replicated sister origins, and regulation of replication initiation in *Bacillus subtilis*. *Mol Microbiol* **60:** 853–869.

Leinonen, R., Akhtar, R., Birney, E., Bower, L., Cerdeno-Tarraga, A., Cheng, Y., *et al*. (2011) The European nucleotide archive. *Nucleic Acids Res* **39:** D28–D31.

McBride, S.M. (2014) More than one way to make a spore. *Microbe* **9:** 153–157.

McKenney, P.T., Driks, A., Eskandarian, H.A., Grabowski, P., Guberman, J., Wang, K.H., *et al*. (2010) A distance-weighted interaction map reveals a previously uncharacterized layer of the *Bacillus subtilis* spore coat. *Curr Biol* **20:** 934–938.

McKenney, P.T., Driks, A., and Eichenberger, P. (2013) The *Bacillus subtilis* endospore: assembly and functions of the multilayered coat. *Nat Rev Microbiol* **11:** 33–44.

Mattoo, A.R., Saif Zaman, M., Dubey, G.P., Arora, A., Narayan, A., Jailkhani, N., *et al*. (2008) Spo0B of *Bacillus anthracis* – a protein with pleiotropic functions. *FEBS J* **275:** 739–752.

Morlot, C., Uehara, T., Marquis, K.A., Bernhardt, T.G., and Rudner, D.Z. (2010) A highly coordinated cell wall degradation machine governs spore morphogenesis in *Bacillus subtilis*. *Genes Dev* **24:** 411–422.

Onyenwoke, R.U., Brill, J.A., Farahi, K., and Wiegel, J. (2004) Sporulation genes in members of the low G + C Gram-

type-positive phylogenetic branch (Firmicutes). *Arch Microbiol* **182:** 182–192.
Paredes, C.J., Alsaker, K.V., and Papoutsakis, E.T. (2005) A comparative genomic view of clostridial sporulation and physiology. *Nat Rev Microbiol* **3:** 969–978.
Paredes-Sabja, D., Shen, A., and Sorg, J.A. (2014) Clostridium difficile spore biology: sporulation, germination, and spore structural proteins. *Trends Microbiol* **22:** 406–416.
Perego, M. (1998) Kinase–phosphatase competition regulates *Bacillus subtilis* development. *Trends Microbiol* **6:** 366–370.
Pereira, F.C., Saujet, L., Tomé, A.R., Serrano, M., Monot, M., Couture-Tosi, E., *et al*. (2013) The spore differentiation pathway in the enteric pathogen *Clostridium difficile*. *PLoS Genet* **9:** e1003782.
Permpoonpattana, P., Tolls, E.H., Nadem, R., Tan, S., Brisson, A., and Cutting, S.M. (2011) Surface layers of *Clostridium difficile* endospores. *J Bacteriol* **193:** 6461–6470.
Permpoonpattana, P., Phetcharaburanin, J., Mikelsone, A., Dembek, M., Tan, S., Brisson, M.-C., *et al*. (2013) Functional characterization of *Clostridium difficile* spore coat proteins. *J Bacteriol* **195:** 1492–1503.
Putnam, E.E., Nock, A.M., Lawley, T.D., and Shen, A. (2013) SpoIVA and SipL are *Clostridium difficile* spore morphogenetic proteins. *J Bacteriol* **195:** 1214–1225.
Rudner, D.Z., and Losick, R. (2001) Morphological coupling in development: lessons from prokaryotes. *Dev Cell* **1:** 733–742.
Saujet, L., Pereira, F.C., Serrano, M., Soutourina, O., Monot, M., Shelyakin, P.V., *et al*. (2013) Genome-wide analysis of cell type-specific gene transcription during spore formation in *Clostridium difficile*. *PLoS Genet* **9:** e1003756.
Sonenshein, A.L. (2000) Control of sporulation initiation in *Bacillus subtilis*. *Curr Opin Microbiol* **3:** 561–566.
Stackebrandt, E., Sproer, C., Rainey, F.A., Burghardt, J., Päuker, O., and Hippe, H. (1997) Phylogenetic analysis of the genus *Desulfotomaculum*: evidence for the misclassification of *Desulfotomaculum guttoideum* and description of *Desulfotomaculum orientis* as *Desulfosporosinus orientis* gen. nov., comb. nov. *Int J Syst Bacteriol* **47:** 1134–1139.
Steiner, E., Dago, A.E., Young, D.I., Heap, J.T., Minton, N.P., Hoch, J.A., and Young, M. (2011) Multiple orphan histidine kinases interact directly with Spo0A to control the initiation of endospore formation in *Clostridium acetobutylicum*. *Mol Microbiol* **80:** 641–654.
Stephenson, K., and Hoch, J.A. (2002) Evolution of signalling in the sporulation phosphorelay. *Mol Microbiol* **46:** 297–304.
Stragier, P. (2002) A gene odyssey: exploring the genomes of endospore- forming bacteria. In *Bacillus Subtilis and Its Closest Relatives From Genes to Cells*. Sonenshein, A.L., Hoch, J.A., and Losick, R. (eds). Washington, DC, USA: ASM Press, pp. 519–526.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28:** 2731–2739.
Tan, I.S., and Ramamurthi, K.S. (2014) Spore formation in *Bacillus subtilis*. *Environ. Microbiol. Rep.* **6:** 212–225.
Traag, B.A., Pugliese, A., Eisen, J.A., and Losick, R. (2013) Gene conservation among endospore-forming bacteria reveals additional sporulation genes in *Bacillus subtilis*. *J Bacteriol* **195:** 253–260.
Varughese, K.I. (2002) Molecular recognition of bacterial phosphorelay proteins. *Curr Opin Microbiol* **5:** 142–148.
Widdel, F. (2006) The genus *Desulfotomaculum*. In *Prokaryotes*. Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K-H., and Stackebrandt, E. (eds). New York, USA: Springer, pp. 787–794.
Wu, L.J., and Errington, J. (2003) RacA and the Soj-Spo0J system combine to effect polar chromosome segregation in sporulating *Bacillus subtilis*. *Mol Microbiol* **49:** 1463–1475.
Yutin, N., and Galperin, M.Y. (2013) A genomic update on clostridial phylogeny: Gram-negative spore formers and other misplaced clostridia. *Environ Microbiol* **15:** 2631–2641.

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 *Desulfotomaculum* 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 *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 S3. This table summarizes the results of the BLASTp search of the *B. subtilis* sporulation proteins against the *Desulfotomaculum* spp. proteomes. Different criteria were used to evaluate putative conservation of each protein in the desulfotomacula: 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 eval (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 *Desulfotomaculum* 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 IIIA [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* HK. *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, sulfhydryl 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. carboxydivorans*; Dg: *D. gibsoniae*; Dh: *D. hydrothermale*; Dk: *D. kuznetsovii*; 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 αß-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; Permpoonpattana *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.