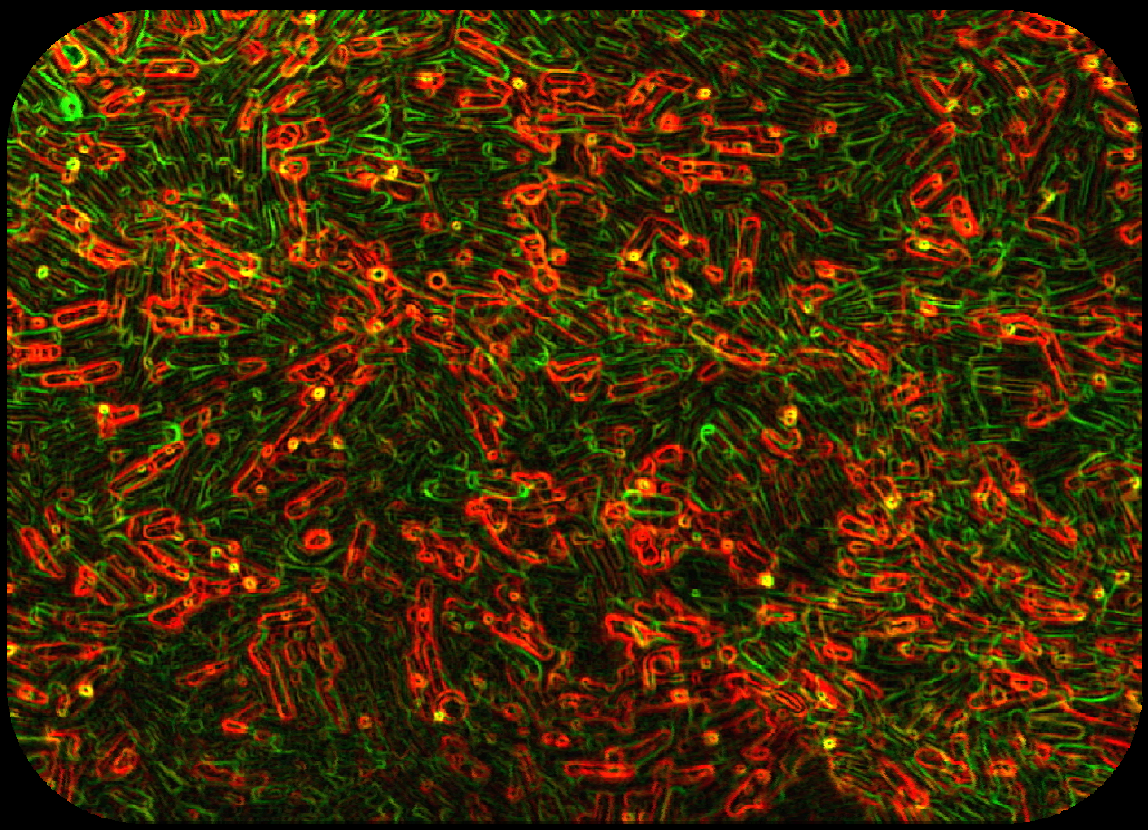
Dissecting the function of the SpoIIIJ and YqjG membrane protein insertases during bacterial spore development

Luísa Côrte



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
May, 2013

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Knowledge Creation

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The cover contains a treated fluorescence microscopy image of *Bacillus subtilis* cells stained with the membrane dye FM4-64 (red) or expressing a lipoprotein version of YqjG-GFP (green).

Supervisor: Prof. Adriano O. Henriques

Examiners: Prof. Mário A. Santos, Dr. Paulo Tavares, Prof. Isabel de Sá-Nogueira, Dr. Mónica Serrano, and Prof. Luís Paulo Rebelo

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ABSTRACT

SpoIIIJ and YqjG are the two members of the evolutionarily conserved YidC/Oxa1/Alb3 family of membrane protein insertases present in the Gram-positive bacterium *Bacillus subtilis*. Whilst either is capable of supporting viability, only SpoIIIJ is functional during sporulation, a carefully regulated developmental process that culminates in the formation of a dormant and highly resistant structure, the endospore. This partial functional overlap contrasts with the general picture of the family, for which trans-domain complementation is well documented. As a first approach to seek the basis for this differential behaviour we performed heterologous complementation with family members from other *Bacillus* species, most of which exhibited limited complementation capacity. Site-directed mutagenesis of conserved residues always produced fully functional proteins; random mutagenesis only resulted in reduced functionality when dramatic changes such as truncations or frameshifts were generated. These results seem to indicate that no essential amino acid residues exist in SpoIIIJ, similarly to the case of other YidC/Oxa1/Alb3 family members, highlighting the importance of overall conformation over primary structure. We also analysed the signal peptide regions of SpoIIIJ and YqjG, which we have shown to be different: whilst SpoIIIJ is a lipoprotein and cleaved by signal peptidase II, YqjG is cleaved by type I signal peptidases. However, sporulation levels remained unchanged upon converting YqjG into a lipoprotein. By analysing SpoIIIJ and YqjG, either devoid of their signal peptides or bearing non-functional ones, we show that the signal peptides of either protein are dispensable for their functions in viability and sporulation. Next, we turned our efforts towards YqjG, for which a secondary cleavage event was observed at the onset of the stationary phase and that we suggest to be linked to its inactivity during sporulation. The region of cleavage was circumscribed to amino acid residues 39-49 based on the combined information obtained from distinct data sets: chimeras of SpoIIIJ and YqjG, AMS labelling, and

deletion of a YqjG region combined with *in silico* analyses. We observed that *yqjG* is induced in the absence of *spoIIIJ*, most likely due to a post-transcriptional regulation mechanism, though YqjG only replaces SpoIIIJ during growth. Due to a combination of approaches we obtained a YqjG variant that was able to sustain wild-type sporulation levels, namely via increased stability conveyed by fusion to GFP and two separate amino acid substitutions that improve interaction with a SpoIIIJ sporulation-specific substrate, SpoIIIAE. Another aspect here analysed was the oligomerisation of SpoIIIJ and the involvement of its Cys134 residue *in vitro* and *in vivo*. Oligomerisation was found to be impaired in the presence of DTT or in a C134A variant. Our results indicate a role for Cys134 as part of an oligomerisation interface and in maintaining the potential for activity of the σ^G factor of the RNA polymerase during sporulation. We propose a model in which SpoIIIJ dimers are sustained by disulphide bonds and non-covalent interactions. This work also reinforces the membership of SpoIIIJ in the YidC/Oxa1/Alb3 family, given its malleability, oligomerisation ability and partial functional specialisation shared with YqjG.

SUMÁRIO

SpoIIIJ e YqjG são os dois membros da família evolutivamente conservada YidC/Oxa1/Alb3 de insertases de proteínas membranares presentes na bactéria Gram-positiva *Bacillus subtilis*. Qualquer das duas proteínas tem a capacidade de suportar viabilidade mas apenas SpoIIIJ é funcional durante a esporulação, um processo de desenvolvimento meticulosamente regulado que culmina na formação de uma estrutura celular de repouso altamente resistente, o endósporo. Esta sobreposição de funções parcial contrasta com o quadro geral da família, para a qual complementação entre domínios distintos está fortemente documentada. Numa primeira abordagem na tentativa de compreender a base para este comportamento diferencial realizámos estudos de complementação heteróloga com membros desta família provenientes de outras espécies do género *Bacillus*, a maioria dos quais exibiu uma capacidade de complementação limitada. Mutagénese dirigida de resíduos conservados produziu sempre proteínas totalmente funcionais; mutagénese aleatória apenas conduziu a uma redução de funcionalidade aquando da geração de proteínas truncadas ou *frameshifts*. Estes resultados parecem indicar a ausência de resíduos aminoacídicos essenciais nesta proteína, à semelhança de outros membros da família YidC/Oxa1/Alb3, sublinhando a importância da conformação global relativamente à sequência primária. Também analisámos os péptidos sinal de SpoIIIJ e YqjG, que se revelaram distintos: enquanto SpoIIIJ é uma lipoproteína e é clivado pela peptidase de sinal II, YqjG é clivado por peptidases de sinal de tipo I. No entanto, os níveis de esporulação mantiveram-se inalterados aquando da conversão de YqjG numa lipoproteína. Ao analisar SpoIIIJ e YqjG, quer contendo péptidos sinal não funcionais quer desprovidos dos mesmos, mostramos que os péptidos sinal das duas proteínas são dispensáveis para as suas funções em viabilidade e esporulação. Em seguida, focámo-nos na proteína YqjG, que se verificou ser clivada secundariamente ao corte da peptidase de sinal no início da fase estacionária, clivagem que sugerimos estar ligada à

sua inactividade durante a esporulação. A região de clivagem foi circunscrita aos resíduos aminoacídicos 39-49 com base na informação obtida a partir de vários conjuntos de dados: quimeras de SpoIIIJ e YqjG, marcação com AMS e deleção de uma região, combinados com análises *in silico*. Verificámos que o gene *yqjG* é induzido na ausência de *spoIIIJ*, provavelmente devido a um mecanismo de acção pós-transcricional, apesar de YqjG apenas substituir SpoIIIJ durante o crescimento. Graças a uma combinação de abordagens obtivémos uma variante de YqjG que suporta níveis selvagens de esporulação através de um aumento da estabilidade conferida pela sua fusão à GFP e de uma de duas substituições aminoacídicas distintas que melhoram a interacção com um substrato de SpoIIIJ específico da esporulação. Outro aspecto aqui analisado foi a oligomerização de SpoIIIJ e o envolvimento do resíduo Cys134 *in vitro* e *in vivo*. Verificámos que a oligomerização é reduzida na presença de DTT ou numa variante C134A. Os nossos resultados apontam para um papel para a Cys134 na interface de oligomerização e na manutenção do potencial necessário para a actividade do factor σ^G da polimerase de RNA durante a esporulação. Propomos um modelo no qual dímeros de SpoIIIJ são mantidos por ligações persulfureto e ligações não-covalentes. Este trabalho também reforça a filiação de SpoIIIJ na família YidC/Oxa1/Alb3, dada a sua maleabilidade, capacidade de oligomerização e especialização funcional parcial partilhada com YqjG.

TABLE OF CONTENTS

THESIS OUTLINE

This Thesis is divided into six chapters. Chapter I provides an introduction to *Bacillus subtilis*, including an overview of the process of sporulation, with emphasis on transcriptional regulation. This chapter also includes an overview of protein transport machinery in *Bacillus subtilis* and in other organisms, including the Oxa1 family of proteins.

Chapter II describes our analysis of the paradox regarding the ability of proteins of the Oxa1 family to accommodate substitutions, which contrasts with an only partial functional overlap of SpoIIIJ and YqjG in *B. subtilis*. SpoIIIJ was shown to be resilient to primary sequence alterations apart from changes as severe as truncations; however, Oxa1-like proteins from close members, namely other *Bacillus* species, exhibited limited complementation capacity.

Chapter III reports studies centred on YqjG, in which full complementation of a *spoIIIJ* mutant for sporulation is achieved with YqjG variants and/or under certain conditions. The inability of YqjG to support sporulation is suggested to be linked to proteolytic inactivation observed for YqjG at the onset of stationary phase during sporulation.

In the work described in Chapter IV we have pursued the analysis of the role of the signal peptides of YqjG and SpoIIIJ. Based on genetic studies, the proteins were shown to bear signal peptides from distinct classes. In addition, conversion of YqjG's signal peptide into the same class as SpoIIIJ's did not enhance its functionality during sporulation. We also show that their signal peptides can be deleted without loss of function regarding viability and sporulation, suggesting that the key determinants for viability and sporulation lie outside the signal peptide regions of these proteins.

In Chapter V, we describe our analysis on the oligomerisation of SpoIIIJ, both *in vitro* and during sporulation. We show that SpoIIIJ is able to oligomerise *in vitro*. The putative interface contains a cysteine residue that should be involved in oligomerisation, since it became impaired upon

substitution by an alanine residue or addition of the reducing agent DTT. This cysteine residue is also shown to have a role during sporulation when SpoIIIJ is present in low amounts.

Finally, Chapter VI presents a general discussion of the results and outlook.

Chapter I

General Introduction

Presenting *Bacillus subtilis*

Bacillus is a genus of ample significance in human history. The first antibacterial vaccine consisted of attenuated *Bacillus anthracis*, produced by Louis Pasteur in 1881. Robert Koch used this organism to demonstrate for the first time that a living organism could cause an infectious disease in 1876 (Barth *et al.*, 2004 and references therein). *Bacillus* members have been fairly used in a wide range of industrial processes, exploiting both their secretion capacity and the remarkable resistance properties of their spores. Some examples of commercially important products in medical, cosmetics, textile and food industries are alpha-amylase (Palva, 1982), riboflavin (Stahmann *et al.*, 2000), hyaluronic acid (Widner *et al.*, 2005), human interleukin-3 (Westers *et al.*, 2006), insecticides (Smouse and Nishiura, 1997), and peptide antibiotics (Stein, 2005). Spores are used as probiotics for animal and human consumption (Cutting, 2011), in the display of bioactive proteins on the spore surface (Isticato *et al.*, 2001; Potot *et al.*, 2010), being attractive vehicles of vaccine delivery, and also as genetically engineered biosensors of specific compounds (Su *et al.*, 2011). A potential application of bacterial spores from members of the genus *Bacillus* has recently been proposed, namely as self-healing agents in concrete, the currently most used construction material worldwide (Wiktor and Jonkers, 2011).

Members of the genus *Bacillus* are Gram-positive, rod-shaped, non-pathogenic and endospore-forming aerobic bacteria normally found in the soil (Priest, 1993), although they are ubiquitous in nature, having also been isolated from environments as diverse as freshwater, saline water, plants, animals and air (Maughan and Van der Auwera, 2011). *Bacillus subtilis* was initially identified by Ehrenberg in 1835 and later by Cohn in 1872 (Tindall and Garrity, 2008 and references therein), currently being one of the best-studied organisms and a model organism for Gram-positive bacteria, second only to *Escherichia coli* among bacteria. Several characteristics make *B. subtilis* an organism of choice for the study of basic

cell processes: i) the ease of genetic manipulation, superior even to that of *Escherichia coli* in respect to the inactivation of chromosomal genes and gene fusion construction (Cutting and Vander Horn, 1990); ii) non-pathogenicity; iii) sequencing of the whole genome (Kunst *et al.*, 1997), which provided a large body of information that opened major questions and possibilities in fundamental and applied studies; iv) the possibility of using sporulation as bacterial model for cell differentiation.

The endospore is a cell type that can survive for extended periods without nutrients, but is able to monitor its environment and readily revive if nutrients become available (Fig. 1; (Moir and Smith, 1990; Piggot and Losick, 2002)). It is thrilling to realise that *Bacillus* spores have been revived from extinct bees preserved in amber for 25 to 40 million years, and also from another *Bacillus* species with 250 million years from a salt crystal, although this latter finding is controversial (Cano and Borucki, 1995; Vreeland *et al.*, 2000; Nickle *et al.*, 2002). Such was achieved as spores can bear extreme environmental insults that include wet and dry heat, UV and gamma radiation, extreme desiccation (including vacuum), toxic chemicals, high pressure and oxidising agents. The levels of resistance are such that spores have been suggested as candidates for interplanetary transfer of life (Nicholson *et al.*, 2000; Nicholson *et al.*, 2005; Setlow, 2006, 2007).

An overview of sporulation

Cell differentiation is a fundamental biological process. A relatively simple case is spore formation in *B. subtilis*, which became a paradigm for the study of cell differentiation in bacteria. The process that gives rise to heat-resistant spores from vegetative cells of *B. subtilis* requires about 8 h at 37ºC and follows well-defined morphological stages (Piggot and Coote, 1976; Piggot and Hilbert, 2004; Fig. 1).

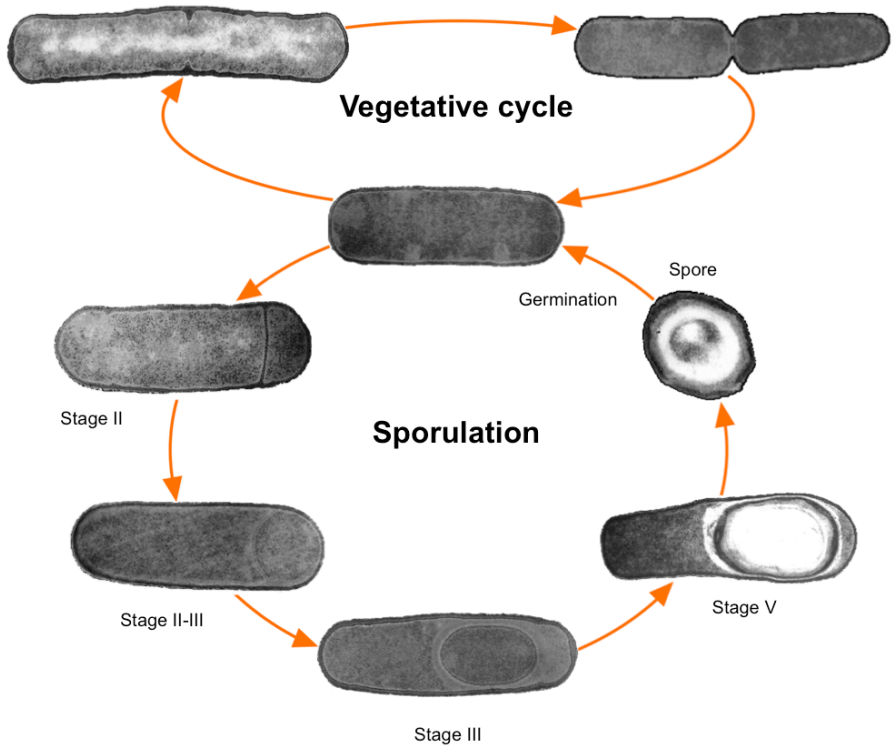

Figure 1. The sporulation and vegetative cycles of *Bacillus subtilis*. A vegetatively growing cell is defined as stage 0. Key morphological changes during sporulation are the asymmetric division (stage II), engulfment of the prespore by the mother cell (stages II-III), assembly of the protective layers (stages IV-V), spore maturation (stage VI), lysis of the mother cell and release of the mature spore (stage VII). In favourable conditions, the spore germinates and the vegetative cycle is restored.

Sporulation starts from a vegetative cell containing two copies of the chromosome (Stage 0). Both copies of the chromosome in the pre-divisional cell are remodelled into an elongated filament that stretches across the long axis of the cell, forming the axial filament (Stage I) (Hilbert and Piggot, 2004; Piggot and Hilbert, 2004). Asymmetric division is the emblematic morphological stage of spore formation that marks the point of no return, after which cells are committed to completing the process (Stage II) (Dworkin and Losick, 2005). A direct consequence of this event is the formation of unequally sized adjacent daughter cells: the smaller prespore (or forespore), which will become the mature spore, and the larger mother cell, which will ultimately lyse and release the spore. A transient genetic asymmetry is created between both compartments as the

asymmetric positioning of the septum traps two-thirds of the prespore chromosome on the mother cell side, which are then returned to the prespore by a septal DNA translocase (Wu and Errington, 1997; Frandsen *et al.*, 1999). Next, engulfment of the prespore by the mother cell takes place. This involves septal peptidoglycan degradation and migration of the mother cell membrane around the prespore, producing a free-floating protoplast in the mother cell. The engulfed prespore is thus isolated from the external medium as it is surrounded by two membranes, one derived from each of the two cells (Stage III) (reviewed in Hilbert and Piggot, 2004). Engulfment is followed by the deposition of two peptidoglycan layers between the two prespore membranes, the primordial germ cell wall and the cortex (Stage IV). The former is a thin layer adjacent to the inner prespore membrane that is thought to act as a precursor to the new cell wall upon germination, whereas the thicker cortex is assembled at the outer prespore membrane (Foster and Popham, 2002; Henriques and Moran Jr, 2007). The developing spore later sees deposition of a multiprotein coat around the outer membrane. Four layers that compose the spore coat can be observed by thin-sectioning electron microscopy: an amorphous undercoat, a lamellar inner coat, the more peripherally located striated and electron-dense outer coat (Stage V) and the recently detected crust as the outermost layer (reviewed in Henriques and Moran Jr, 2007; McKenney *et al.*, 2010). Maturation corresponds to Stage VI, when the spore develops its full resistance properties, conferred by the coat (to exogenous lytic enzymes, some chemicals, predatory microorganisms), the inner membrane (chemicals), the cortex (spore mineralisation and the development and maintenance of a reduced water content in the core, both required for thermal resistance; the latter also involved in protection against UV radiation), the core's dipicolinic acid and α/β-type small acid-soluble proteins (both contributing to thermal resistance and UV protection). The mature spore is released into the environment by lysis of the mother cell (Stage VII). The dormant spore is able to monitor its environment and when conditions are favourable for growth, germination

and outgrowth occur, followed by a resumption of the vegetative growth cycle (Setlow, 2003; Hilbert and Piggot, 2004).

The genetic regulation of sporulation

Entry into sporulation

Despite assuring survival under deteriorating environmental conditions, sporulation seems to be the last resort adaptation as it is a long and energy-consuming process that involves major physiological and morphological alterations (Errington, 1993; Errington, 2003). Before succumbing to sporulation, *B. subtilis* may explore a range of post-exponential growth responses, including chemotaxis and associated motility (Aizawa *et al.*, 2002), the secretion of degradative enzymes and antibiotic production (Ferrari *et al.*, 1993; Stachelhaus *et al.*, 2002), genetic competence (Dubnau and Lovett Jr, 2002), biofilm formation (Hamon and Lazazzera, 2001) and a cannibalistic behaviour in which early sporulating cells may delay or even prevent their full commitment to sporulation by killing and feeding on non-sporulating siblings (González-Pastor *et al.*, 2003). After ruling out these responses, *B. subtilis* initiates sporulation in response to nutritional starvation, high cell density and adequate cell cycle progression signals (reviewed in Errington, 1993; and in Hilbert and Piggot, 2004). These signals are integrated by a highly complex regulatory network, the phosphorelay, which regulates the activity of Spo0A (Fig. 2). Spo0A is one of the master regulators of development in *B. subtilis*, which controls some of the responses mentioned above and is instrumental in the initiation of sporulation. Activating signals are sensed by kinases (KinA to E) that introduce phosphoryl groups into the relay via Spo0F, which are then transferred to Spo0B and ultimately to Spo0A, producing the active form, Spo0A~P (phosphorylated Spo0A) (Burbulys *et al.*, 1991). Inactivating signals are introduced into the phosphorelay by members of the Spo0E and RAP families of phosphatases (Perego *et al.*, 1994;

Grossman, 1995).

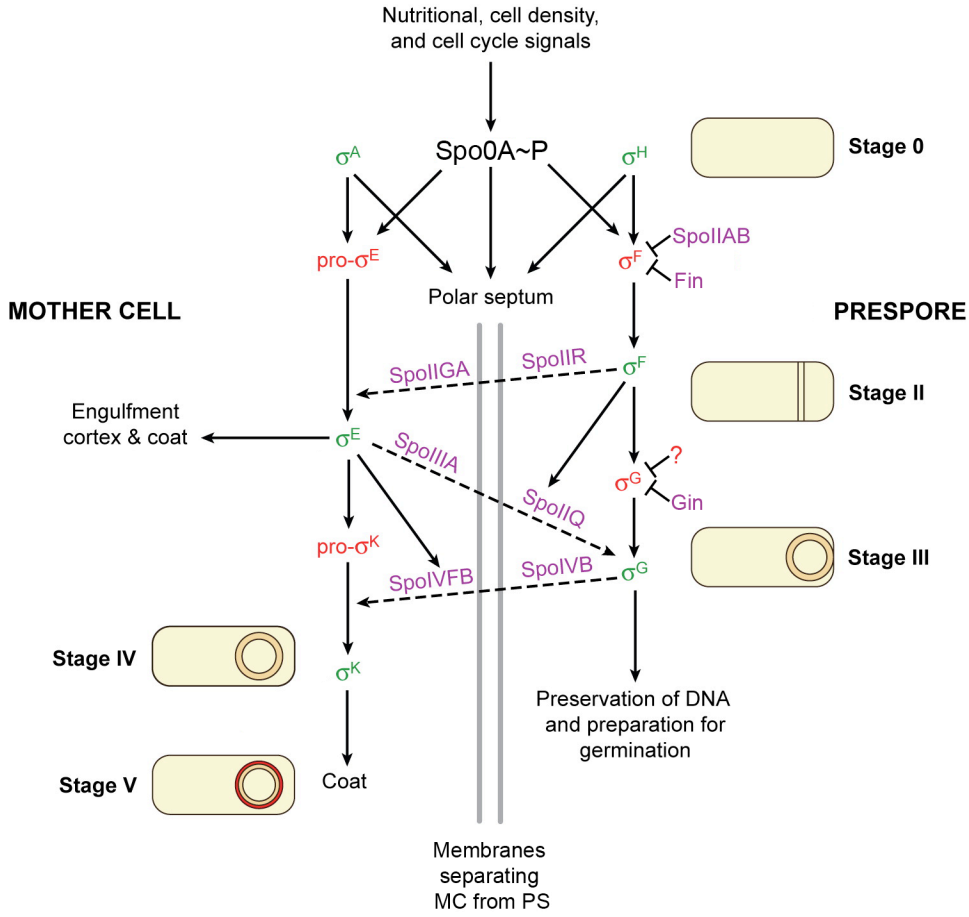

Figure 2. Regulatory network during *Bacillus subtilis* sporulation. Solid arrows indicate dependence relationships between σ factors (*green* means active and *red* means inactive) and target genes whose products bring about morphological change (described in words and simplified representations of the stages of sporulation). The grey vertical lines represent the two membranes that separate the mother cell (MC) from the prespore (PS) after the polar septum forms. Dashed arrows indicate signalling pathways between the two cell types. Key regulatory proteins are shown in purple. Adapted from Kroos, 2007, Camp *et al*., 2011, and Serrano *et al*., 2011.

Both Spo0A production and activity are tightly regulated. Spo0A~P influences its own production and activity: upon entry into stationary phase Spo0A~P stimulates the transcription of its own gene, of the phosphorelay component *spo0F*, and indirectly stimulates the transcription of *spo0H* (encoding σ^H) and of the phosphorelay phosphatase *spo0E* (reviewed in Lopez *et al.*, 2009).

Depending on the levels of Spo0A phosphorylation, a range of

physiological outcomes is possible. Since Spo0A~P-dependent genes with a direct role in sporulation possess low affinity for Spo0A~P, only in the presence of high levels of Spo0A-P is sporulation initiated (Fujita *et al.*, 2005).

Compartmentalisation of gene expression

At the beginning of sporulation, Spo0A~P cooperates with σ^A and σ^H in directing the transcription of *spoIIA*, *spoIIG* and *spoIIE* which encode key developmental regulators, among other genes (Errington, 2003; Hilbert and Piggot, 2004; Piggot and Hilbert, 2004). Spo0A~P and σ^H trigger the redirection of septum formation to a polar position, which results in the key event of asymmetric division (Kroos and Yu, 2000; Piggot and Hilbert, 2004). After asymmetric division, distinct but interdependent transcriptional programmes take place in each of the two cells. Their rigorous orchestration depends mainly on the differential usage of several sigma subunits by the RNA polymerase, directing it to specific classes of promoters (Hilbert and Piggot, 2004). Such is achievable due to the transient association between the RNA polymerase and the sigma subunits (Travers and Burgess, 1969).

Each of the four compartment-specific sigma subunits (σ^F, σ^E, σ^G and σ^K) is inactive at the time of its synthesis and requires subsequent activation. The activation of each sigma factor at the proper location and time is coupled to landmark morphological events by control mechanisms named checkpoints (Losick and Shapiro, 1993; Kroos *et al.*, 1999). Asymmetric division triggers the activation of σ^F in the prespore and σ^E in the mother cell, both shaping the initial stages of sporulation. Later, the completion of engulfment of the prespore by the mother cell leads to the activation of σ^G in the prespore and σ^K in the mother cell (Helmann and Moran Jr., 2002; Hilbert and Piggot, 2004). Intercompartmental signaling ensures coordination of the genetic programmes of both compartments (Piggot and Losick, 2002; Piggot and Hilbert, 2004).

Developmental checkpoints

Prespore line of gene expression

σ^F checkpoint

σ^F, encoded by *spoIIAC*, is the first compartment-specific sigma factor of sporulation. It is synthesised and kept inactive in the pre-divisional cell until asymmetric division takes place, being selectively activated in the prespore (Margolis *et al.*, 1991; Lewis *et al.*, 1996).

Confining of σ^F activity to the prespore involves a partner-switching mechanism involving SpoIIAB, SpoIIAA and SpoIIE. SpoIIAB switches between SpoIIAA and σ^F as binding partners (Alper *et al.*, 1994; Duncan *et al.*, 1995). SpoIIAB is an anti-sigma factor that binds to σ^F and prevents its association with the RNA polymerase (Duncan and Losick, 1993). SpoIIAA is an anti-anti-sigma factor that is inactive when phosphorylated. The SpoIIE phosphatase produces dephosphorylated SpoIIAA that counteracts the inhibitory effect of SpoIIAB by binding to the SpoIIAB-σ^F complex, causing the release of active σ^F (Duncan and Losick, 1993; Duncan *et al.*, 1995).

SpoIIE is a membrane protein that localises at the polar septum (Barák *et al.*, 1996). SpoIIE is a bifunctional protein, being required for σ^F activation and for proper asymmetric division (Barák and Youngman, 1996; Feucht *et al.*, 1996). SpoIIE is considered instrumental in the coupling of gene expression and morphogenesis, despite the precise mechanisms for preferential desphosphorylation of SpoIIAA in the prespore being still a matter of debate (reviewed in Barák and Wilkinson, 2005).

Another important contribution to the compartmentalisation of σ^F activity results from the transient exclusion of the gene encoding SpoIIAB from the prespore generated by polar division (Dworkin and Losick, 2001), and from degradation of free SpoIIAB in the prespore (Pan *et al.*, 2001).

σ^F was found to drive the expression of 48 genes (Wang *et al.*, 2006) including genes required for σ^E and σ^K activation in the mother cell, *spoIIR* and *spoIVB*, respectively (Hofmeister *et al.*, 1995; Karow *et al.*, 1995;

Londoño-Vallejo and Stragier, 1995; Gomez and Cutting, 1996), genes involved in the regulation of prespore-specific gene expression, *spoIIIG*, encoding the late prespore sigma factor σ^G (Karmazyn-Campelli *et al.*, 1989; Sun *et al.*, 1989), *spoIIQ*, required for both expression of *spoIIIG* and σ^G activity, and involved in engulfment (Londoño-Vallejo *et al.*, 1997; Sun *et al.*, 2000; Camp and Losick, 2008). Several σ^F-dependent genes seem to be transcribed by σ^G as well (Wang *et al.*, 2006).

The switch from σ^F to σ^G was proposed to require a putative anti-sigma factor named Fin (F inhibiting) that is related to the anti-sigma factor for σ^G, Gin, in combination with an unknown Fin-independent pathway (Camp *et al.*, 2011; Serrano *et al.*, 2011).

σ^G checkpoint

σ^G is the late sigma factor present in the prespore (Karmazyn-Campelli *et al.*, 1989; Sun *et al.*, 1989). Co-transcription of *spoIIIG* (encoding σ^G) and *spoIIG* (encoding σ^F and some of its regulators) before asymmetric division occurs with Spo0A and σ^A but σ^G is not produced due to a stem-loop structure that blocks the ribosome-binding site (Masuda *et al.*, 1988). Translation occurs later, from transcripts produced by σ^F and σ^G from a promoter located immediately upstream of the *spoIIIG* coding region (Sun *et al.*, 1989; Sun *et al.*, 1991). Transcription of *spoIIIG* in the prespore is delayed relative to other σ^F-dependent genes as it depends on a poorly understood signal transduction pathway requiring the action of σ^E in the mother cell (Partridge and Errington, 1993; Evans *et al.*, 2004). Transcription of *spoIIIG* also requires expression of the σ^F-controlled gene *spoIIQ* (Sun *et al.*, 2000). σ^G is autoregulatory and thus, once activated, can recognise its own promoter and maintain its own synthesis (Karmazyn-Campelli *et al.*, 1989; Sun *et al.*, 1991).

Following synthesis, σ^G only becomes active after engulfment of the prespore by the mother cell (reviewed in Hilbert and Piggot, 2004). Activity of σ^G requires the assembly of a novel type of secretion system composed of eight mother cell proteins (AA through AH) encoded by the

σ^E-controlled *spoIIIA* operon, and of the prespore-specific, σ^F-dependent SpoIIQ (Illing and Errington, 1991; Kellner *et al.*, 1996; Londoño-Vallejo *et al.*, 1997; Camp and Losick, 2008; Meisner *et al.*, 2008; Camp and Losick, 2009), aided by the membrane protein insertase SpoIIIJ (Errington *et al.*, 1992; Murakami *et al.*, 2002; Serrano *et al.*, 2003; Tjalsma *et al.*, 2003; Camp and Losick, 2008; Serrano *et al.*, 2008). This channel, which is formed between the prespore and the mother cell (Camp and Losick, 2008; Meisner *et al.*, 2008), was proposed to function as a "feeding tube" that allows the mother cell to nurture the prespore by providing small molecules needed for biosynthetic activity. The channel would be required for general macromolecular synthesis, rather than specifically activating σ^G (Camp and Losick, 2009), and to maintain prespore integrity (Li *et al.*, 2004; Doan *et al.*, 2009).

Three negative regulators of σ^G are known, the LonA protease and the anti-sigma factors CfsB and SpoIIAB. The latter also inhibits σ^F prior to asymmetric division (Rather *et al.*, 1990; Duncan and Losick, 1993; Schmidt *et al.*, 1994; Kellner *et al.*, 1996; Karmazyn-Campelli *et al.*, 2008). Both LonA and SpoIIAB are responsible for the inhibition of σ^G activity under conditions that do not favour sporulation and also in the mother cell during sporulation, contrary to the suggestion that SpoIIAB would have a role in the prespore (Rather *et al.*, 1990; Schmidt *et al.*, 1994; Kellner *et al.*, 1996; Serrano *et al.*, 2001; Serrano *et al.*, 2004; Chary *et al.*, 2005). CsfB (controlled by sigma F), also known as Gin (G inhibitor), is an anti-sigma factor that shows specificity to σ^G, unlike SpoIIAB, and is present in the prespore at early times (Decatur and Losick, 1996; Chary *et al.*, 2007; Karmazyn-Campelli *et al.*, 2008; Rhayat *et al.*, 2009). CsfB is also under σ^G control thus limiting its own ectopic activation in non-sporulating cells. It is also under σ^G control in pre-divisional cells with σ^G engineered with a substitution in the conserved residue N45 that reduces inhibition from CsfB (Serrano *et al.*, 2011). None of the regulators *per se* seems to have a decisive role in regulating the activation of the σ^G, but it seems to rather result from the conjugation of their combined action with both the

transcriptional control of *spoIIIG* and the nurturing by the SpoIIIA-SpoIIQ channel (Camp and Losick, 2008; Meisner *et al.*, 2008; Camp and Losick, 2009; Doan *et al.*, 2009; Serrano *et al.*, 2011).

The σ^G regulon includes genes involved in the regulation of prespore-specific gene expression, as its own gene, *spoIIIG*, and *spoVT* (Karmazyn-Campelli *et al.*, 1989; Bagyan *et al.*, 1996); genes involved in the activation of the late mother cell sigma factor σ^K, as *spoIVB* (Cutting *et al.*, 1991a); genes involved in spore maturation, as the *spoVA* operon, and the *ssp* genes (Helmann and Moran Jr., 2002; Tovar-Rojo *et al.*, 2002); and in germination, as the *gerA* and *gerB* operons, and *pdaA* (Paidhungat and Setlow, 2001; Fukushima *et al.*, 2002).

Mother cell line of gene expression

σ^E checkpoint

σ^E is the first mother cell-specific sigma factor during sporulation. σ^E is synthesised as pro-σ^E, an inactive precursor, and is activated by cleavage of the 27 amino acid residue N-terminal "Pro" sequence (LaBell *et al.*, 1987; Stragier *et al.*, 1988; Miyao *et al.*, 1993). This N-terminal sequence is also responsible for tethering Pro-σ^E to the membrane (Ju *et al.*, 1997; Fujita and Losick, 2002). Proprotein processing is carried out by the membrane-bound SpoIIGA protease (Peters and Haldenwang, 1994; Imamura *et al.*, 2008). σ^E and SpoIIGA are encoded by the two-gene *spoIIG* operon which is expressed in the pre-divisional cell (Kenney and Moran Jr, 1987). However, activation of the SpoIIGA protease occurs only upon receiving a signal from the prespore, the σ^F-dependent SpoIIR**.** SpoIIR is predicted to be secreted into the space between the septal membranes and then activate SpoIIGA proteolytic activity towards Pro-σ^E, thus tying σ^E activation to both σ^F activation and (indirectly) to asymmetric division (Hofmeister *et al.*, 1995; Karow *et al.*, 1995; Londoño-Vallejo and Stragier, 1995; Rubio and Pogliano, 2004). Additional mechanisms that promote compartmentalisation of σ^E activity are: i) selective persistent transcription of the *spoIIG* operon in the mother cell (by Spo0A, which becomes the first

mother cell-specific transcription factor (Fujita and Losick, 2002, 2003); ii) selective degradation of σ^E in the prespore (Ju *et al.*, 1998; Fujita and Losick, 2002).

The σ^E regulon has been defined by microchip array in two independent studies that found 171-253 genes under the control of σ^E (Eichenberger *et al.*, 2003; Feucht *et al.*, 2003). σ^E-dependent expression is required to prevent a second division at the distal pole which produces cells with two DNA-containing prespore compartments and an anucleate mother cell that subsequently fail to sporulate, the so-called abortively disporic phenotype (Lewis *et al.*, 1994). Three genes under the control of σ^E are required for the inhibition of this second division, and also for prespore engulfment: *spoIID*, *spoIIM* and *spoIIP* (Lopez-Diaz *et al.*, 1986; Smith *et al.*, 1993; Smith and Youngman, 1993; Frandsen and Stragier, 1995; Pogliano *et al.*, 1999). σ^E also controls the expression of genes involved in σ^G activation, the *spoIIIA* operon (Illing and Errington, 1991); genes required for initiating cortex synthesis and spore coat assembly, as *spoVE*, *spoIVA*, *cotE* and *spoVID* (Zheng *et al.*, 1988; Roels *et al.*, 1992; Beall *et al.*, 1993; Miyao *et al.*, 1993); and to direct synthesis of the late mother cell-specific factor σ^K, the *sigK* composite gene, *spoIVCA*, *spoIVF* and *spoIIID* (Kunkel *et al.*, 1989; Kunkel *et al.*, 1990; Cutting *et al.*, 1991b; Sato *et al.*, 1994).

Replacement of σ^E by σ^K in the mother cell involves a σ^K-dependent negative feedback loop that inhibits σ^A-dependent transcription, including that of the *spoIIGB* gene encoding σ^E, and that requires transcriptionally active σ^K (Zhang *et al.*, 1999).

σ^K checkpoint

The final sigma factor to be activated in the sporulation cascade is the late mother cell-specific factor, σ^K, and it is regulated at multiple levels. First, *sigK*, the gene encoding σ^K, is interrupted by the *skin* (sigK-intervening) element, a 48 kbp DNA element comprising an integrated prophage (Stragier *et al.*, 1989; Kunkel *et al.*, 1990; Takemaru *et al.*, 1995). Excision of the *skin* element joins the two coding pieces of *sigK* together in frame and

is carried out by the σ^E-dependent site-specific recombinase SpoIVCA that is encoded in the *skin* element (Kunkel *et al.*, 1990; Sato *et al.*, 1990; Popham and Stragier, 1992; Sato *et al.*, 1994). Second, the *sigK* gene is under the control of σ^E and of σ^K itself (Kunkel *et al.*, 1988; Kroos *et al.*, 1989). Third, like its mother cell predecessor, the σ^K protein is translated as an inactive precursor that requires proteolytic removal of an amino-terminal pro-sequence (Kroos *et al.*, 1989). Similarly to the case of σ^E, the "Pro" sequence prevents interaction with the RNA polymerase and localises the proprotein in the membrane (Zhang *et al.*, 1998). SpoIVFB is the metalloprotease responsible for the proteolytic processing of σ^K and its activation (Cutting *et al.*, 1991b; Resnekov and Losick, 1998; Rudner *et al.*, 1999). SpoIVFB is negatively regulated by two other membrane proteins, SpoIVFA and BofA (Bypass-of-forespore), all under the control of σ^E and present in a complex (Cutting *et al.*, 1990; Cutting *et al.*, 1991b; Ricca *et al.*, 1992; Rudner and Losick, 2002). SpoIVFA anchors the complex in the mother cell membrane that surrounds the prespore and acts as a platform bringing BofA and SpoIVFB together, whereby BofA inhibits SpoIVFB processing until a signal has been received from the prespore (Resnekov and Losick, 1998; Rudner and Losick, 2002). Such signal relies on the production of SpoIVB in the prespore under the control of σ^G (Cutting *et al.*, 1990; Gomez *et al.*, 1995). SpoIVB is a serine protease that is secreted into the space between the prespore membranes (Wakeley *et al.*, 2000). SpoIVB cleaves SpoIVFA, resulting in an alteration in the complex and in the release of SpoIVFB from BofA's inhibition, being able to process and activate σ^K (Dong and Cutting, 2003). A second serine protease, CtpB (Pan *et al.*, 2003), is also able to cleave SpoIVFA and trigger σ^K activation (Campo and Rudner, 2006). Activation of σ^K is coupled to σ^G activity and engulfment via SpoIVB, which is transcribed by σ^G and fails to accumulate when engulfment is impaired (Cutting *et al.*, 1990; Gomez and Cutting, 1996; Doan and Rudner, 2007).

The σ^K regulon includes genes involved in the formation of the spore coat, *cot* genes (Driks, 2002; Henriques and Moran Jr, 2007), in spore maturation

and germination, as *spoVK*, *spoVD* and *gerP* (Fan *et al.*, 1992; Daniel *et al.*, 1994; Behravan *et al.*, 2000), and in the regulation of σ^K-dependent transcription, as *gerE* (Zheng and Losick, 1990).

Protein transport in *B. subtilis* and other organisms

Protein transport systems

In all domains of life, the lipid membrane is a central feature that preserves the integrity of the cell. The membrane acts not only as a physical barrier, in the maintenance of the composition and concentration of molecules inside the cell, but is also involved in the controlled swap of substances and information between the cell and its surroundings, and with organelles if present. Such was allowed by the emergence of specific protein transport devices that include proteins that either span membranes to create selective pores or that bind the cytosolic face of membranes to create transport vesicles (Pohlschröder *et al.*, 2005b; Odorizzi and Rehling, 2009). In addition, the transport of proteins permitted the appearance of cellular compartments, as the periplasm of Gram-negative bacteria, or the components of the secretory pathway in the cytoplasm of eukaryotes (Pohlschröder *et al.*, 2005b).

Several specialised translocation/insertion apparatuses are known, as the conserved Sec pathway, the major route for protein translocation across and into the cytoplasmic membrane, that includes SecAYEG and, in some cases, SecB. The Tat pathway translocates folded proteins and that typically have bound metal cofactors. It is also involved in the biogenesis of some bacterial membrane proteins. Gram-negative bacteria exhibit a great diversity of secretion systems. Another important pathway is centred on the YidC/Oxa1/Alb3 family of membrane protein insertases (Driessen and Nouwen, 2008; Xie and Dalbey, 2008; Saller *et al.*, 2012) (Fig. 3).

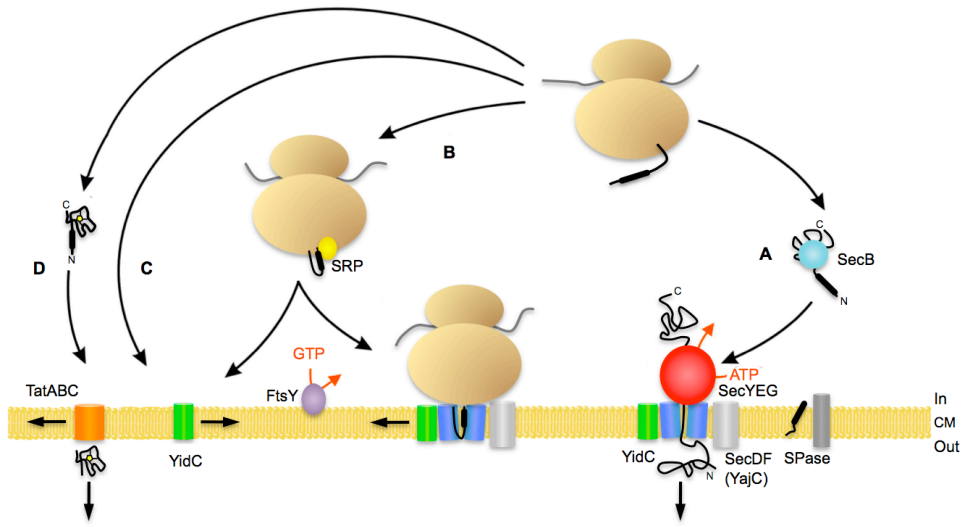

Figure 3. Schematic representation of bacterial protein targeting. The bacterial Sec translocon (blue) is composed of SecY, SecE, and SecG. SecA (red) acts as a peripheral motor protein on the cytoplasmic side. Signal peptides are cleaved by a signal peptidase (SPase). Other ancillary proteins are YidC (green) and SecDF/YajC (light grey). **(A)** Proteins synthesised at the ribosome (light brown) destined for secretion are mostly post-translationally targeted to the Sec translocase by a targeting sequence which is recognised by SecA. Alternatively, targeting to the translocon can be carried out by the molecular chaperone SecB (light blue). **(B)** Co-translational targeting of the ribosome with the nascent chain to the translocase complex is attained by the binding of the signal peptide of some preproteins or the signal anchor sequence of membrane proteins by the signal recognition particle (SRP) (yellow), then to the SRP receptor FtsY (purple). Membrane proteins with large hydrophilic periplasmic domains require the presence of SecA. YidC interacts with transmembrane segments as they emerge from the proposed lateral gate of SecYEG. SRP can also deliver proteins directly to YidC. **(C)** A subset of membrane proteins can insert into the cytoplasmic membrane via YidC after targeting of the ribosome nascent chain to YidC. **(D)** Translocation of folded precursor proteins occurs via the Tat translocase (orange). For *B. subtilis*, the majority of the scenario appears to be valid, with the exception of SecB, which does not exist and its function seems to carried out by CsaA. CM, cytoplasmic membrane. Adapted from Natale *et al.*, 2008, and Du Plessis *et al.*, 2011.

Targeting signals

In early cells diffusion may have been sufficient for proteins to reach protein transport devices. However, as cells became increasingly complex, specific piloting factors that enabled more efficient targeting to several protein translocation apparatuses have evolved (Pohlschröder *et al.*, 2005b).

Both secretory and membrane proteins are synthesised in the cytoplasm and are directed to their correct location by targeting signals encoded within their sequence. Membrane proteins contain topogenic signals that

are required for their proper insertion and topology. The cells possess targeting factors that recognise the signals embedded in the proteins and enable targeting to the correct transport apparatus. Several types of targeting/topogenic signals have been identified: (a) signal anchors (type II signal anchor) that initiate translocation of the carboxyl-terminal region of a membrane protein remaining as a membrane anchor with N_{in} C_{out} orientation; (b) reverse signal anchors (type I signal anchor) that initiate the translocation of the amino-terminal region of the protein and remain as a membrane-spanning region of N_{out} C_{in} orientation; (c) stop-transfer sequences which allow translocation arrest and lateral release from the translocation channel, remaining as membrane anchors with N_{out} C_{in} orientation; (d) helical hairpins, two closely spaced hydrophobic regions that insert in a folded manner, having both amino and carboxyl termini in the cytoplasm (Xie *et al.*, 2007; Driessen and Nouwen, 2008; Xie and Dalbey, 2008); (e) signal peptides, which are usually present at the amino-terminus of the precursor protein. Signal peptides are cleaved off from the mature protein and further degraded by signal peptide peptidases (Ichihara *et al.*, 1984; Bolhuis *et al.*, 1999). The existence of an N-terminal region that directed proteins to the correct transport machinery was initially suggested by Blobel and Sabatini in the early 1970s (reviewed in Leslie, 2005). Signal peptides were shown to also perform a role beyond targeting: they are also allosteric activators of the SecYEG translocase by binding to SecA and lowering its activation energy state (Gouridis *et al.*, 2009).

Signal peptides vary in size depending on the machinery they will direct the protein to, and also in different organisms (von Heijne and Abrahmsén, 1989) but they share a basic tripartite structure: a positively charged N-domain, a hydrophobic H-domain and a polar C-domain (Von Heijne, 1990). The positive charge of the N-domain was suggested to interact with the translocation machinery and the negatively charged membrane phospholipids (Akita *et al.*, 1990; De Vrije *et al.*, 1990). The hydrophobic residues forming the H-domain may adopt an α-helical

conformation upon insertion in the lipid membrane (Briggs *et al.*, 1986). Helix-breaking residues such as glycine or proline are often found in the middle of this domain, allowing the formation of a hairpin-like structure that facilitates membrane insertion (De Vrije *et al.*, 1990). The C-domain specifies the cleavage site for the signal peptidase (SPase) (von Heijne and Abrahmsén, 1989). Based on the cleavage sites and the export pathways that are expected to be utilised, five major types of signal peptides can be defined for *B. subtilis* (Figs. 4-5): (a) secretory (Sec-type or type I); (b) twin arginine (RR/KR); (c) lipoprotein (type II); (d) pseudopilin; (e) bacteriocin and pheromone signal peptides.

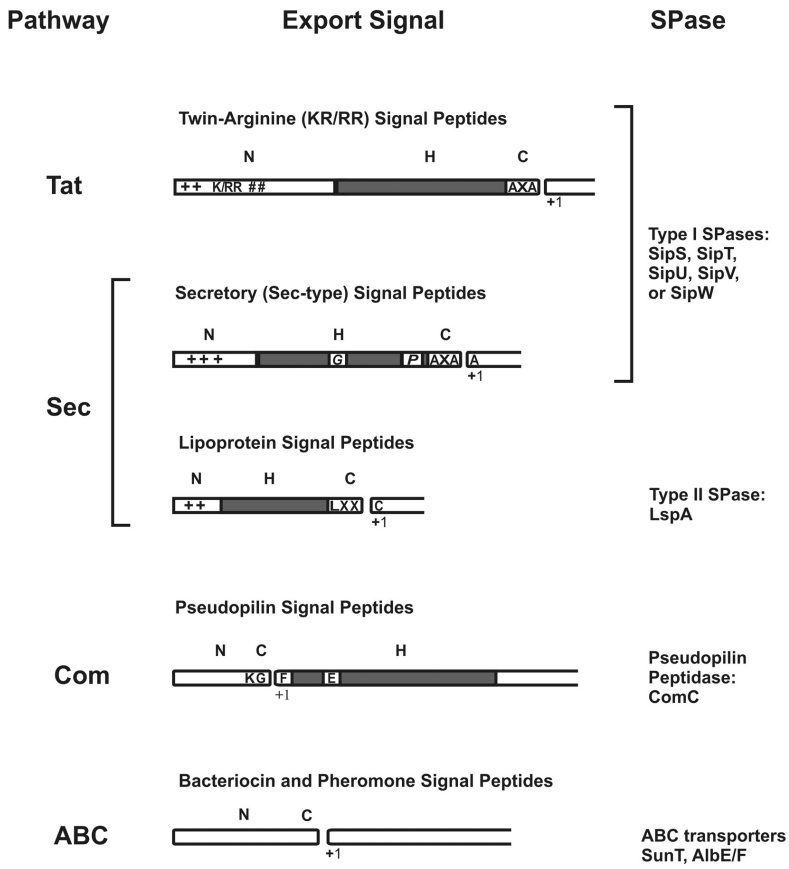

Figure 4. Classification of cleavable N-terminal signal peptides. Five classes were defined based on signal peptidase (SPase) cleavage sites and the export pathways via which the proteins are exported. The pathways and responsible SPases are indicated. N, H and C refer to the three domains present in most signal peptides. Positive residues are indicated by + and letters contained in the domains represent amino acids frequently found in those positions. The frequently occurring first amino acid of the mature protein (+1) is indicated. Adapted from Tjalsma *et al*., 2004.

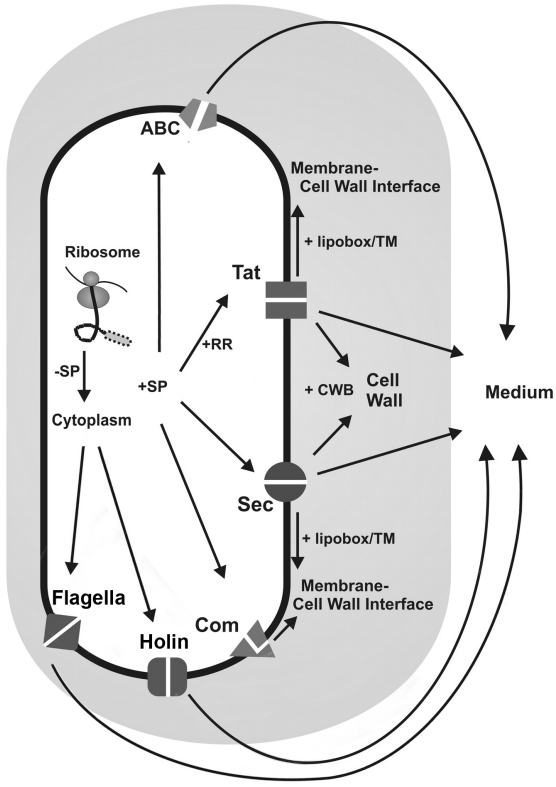

Figure 5. Protein export pathways in *B. subtilis.* Proteins can be sorted to various destinations depending on the presence (+SP) or absence (-SP) of an N-terminal signal peptide and specific retention signals. Proteins devoid of a signal peptide are either retained in the cytoplasm or may escape via the flagellar export machinery, the holin systems, or other unidentified systems. Proteins that are retained at the extracytoplasmic side of the membrane can either contain a transmembrane segment (TM) or a lipid modification (+lipobox) and are exported via the Sec or Tat pathways. Proteins that are retained in the cell wall can be exported via either the Sec or Tat pathway. These proteins are retained in the cell wall if their mature parts contain cell wall-binding repeats (+CWB). Proteins can be secreted into the medium via the Sec or Tat pathway or by ABC transporters. Pseudopilins are exported by the Com system. Adapted from Tjalsma *et al*., 2004.

Signal peptidases are enzymes that catalyse protein maturation by cleaving the signal peptide during or shortly after transport (reviewed in Tjalsma *et al*., 2004). Signal peptide types (a) and (b) are cleaved by type I SPases, whereas type (c) use LspA (SPase II). Type I SPases recognise a consensus sequence of A-X-A|A at positions -3 to +1 relative to the cleavage site. The vertical line represents the cleavage site. While the *E. coli* genome encodes only one type I SPase, in *B. subtilis* five of these genes exist, although only *sipT* or *sipS* have to be present to ensure viability (Silver and Wickner, 1983; Tjalsma *et al*., 1998).

The Sec-type (a) constitutes the classical example of signal peptides sharing the basic tripartite structure. Similar in structure to these are the twin-arginine signal peptides, which serve to direct proteins to the Tat pathway (b) (Fig. 4). This denomination arises from the fact that the consensus sequence in their N-domain usually contains two consecutive

arginines (in *B. subtilis*, R/K-R-X-#-#, # being a hydrophobic amino acid residue and X any residue). On average, the N-domain of Tat signal peptides is longer than Sec-type N-domains (Tjalsma *et al.*, 2004; Natale *et al.*, 2008). The H-domain tends to be less hydrophobic than that of Sec-type signal peptides in *E. coli* (Cristóbal *et al.*, 1999) but similar in *B. subtilis* (Tjalsma *et al.*, 2000). The C-domain may contain a so-called Sec-avoidance signal, composed of positively charged residues, that prevents interaction with Sec components (Blaudeck *et al.*, 2003).

Lipoprotein signal peptides (c) are lipid-modified by the dyacylglyceryl transferase encoded by *lgt*, an essential step for cleavage by LspA (type II SPase) both in *E. coli* and in *B. subtilis* (Sankaran and Wu, 1994 and references therein; Prágai *et al.*, 1997; Leskelä *et al.*, 1999). SPase II recognises a consensus sequence called "lipobox", usually L-(A/S)-(A/G)|C at positions -3 to +1 relative to the cleavage site, cleaving before the invariable cysteine residue. In *E. coli*, but not in *B. subtilis*, the cysteine residue is further modified by the apolipoprotein N-acyl transferase Lnt (Gupta and Wu, 1991; Kunst *et al.*, 1997).

Prepilin signal peptides were initially identified in bacterial type IV prepilin subunits (reviewed in Strom and Lory, 1993). Pseudopilin signal peptides (d) present a variation on the previously described general theme, as the cleavage site precedes the hydrophobic stretch that becomes thus part of the mature protein (reviewed in Pohlschröder *et al.*, 2005a). In *B. subtilis* four proteins bear this class of signal peptides (Tjalsma *et al.*, 2000), which are required for the binding and uptake of exogenous DNA during genetic competence (reviewed in Dubnau, 1997). They are transported via the specific Com pathway and cleaved by ComC (reviewed in Tjalsma *et al.*, 2000).

Still another class of signal peptides can be observed in ribosomally-synthesised bacteriocins and pheromones (e) that are exported by ABC (ATP-binding cassette) transporters. Removal of the signal peptide may be carried out by a subunit of the ABC transporter, by specific SPases or by general proteases (Wandersman, 1998). Sublancin168 and ComX from

B. subtilis bear such signal peptides (reviewed in Tjalsma *et al.*, 2004). The lack of the H-domain is the most distinctive feature of these signal peptides, being thus composed of N- and C-terminal domains (Tjalsma *et al.*, 2000).

Transport of proteins can also occur independently of signal peptides via holins, which form pores in the membrane through which extracellular proteins may reach the cell wall, and by dedicated machinery for the assembly of flagella (Namba *et al.*, 1989; Young and Bläsi, 1995).

Targeting factors

Proper targeting is a critical matter for proteins. When exiting the ribosomal tunnel during synthesis, polypeptides meet several aiding factors, namely chaperones, folding catalysts and targeting factors. Their function is to prevent proteins from aggregating and misfolding and to ensure proper routing to the translocation/membrane insertion machinery (Dalbey *et al.*, 2011).

In *E. coli*, trigger factor appears to be the default chaperone for nascent chains, preventing their premature folding, and it also interacts with the ribosome (Ullers *et al.*, 2003 and references therein). Trigger factor is also present in *B. subtilis* and was show to catalyse *in vitro* protein folding (Göthel *et al.*, 1998). Another aiding factor is the signal recognition particle (SRP), which mediates the transport of secretory and membrane proteins to the cytoplasmic membrane or to the endoplasmic reticulum, in bacteria, archaea and eukaryotes (reviewed in Yuan *et al.*, 2010). The SRP competes with trigger factor for the ribosome-nascent chain complexes (RNCs), both in *E. coli* and in *B. subtilis* (Ullers *et al.*, 2003; Zanen *et al.*, 2005; Knoops *et al.*, 2012 and references therein). Nascent polypeptides harbouring more hydrophobic targeting sequences enter the SRP pathway, in *E. coli* and in *B subtilis* (reviewed in Luirink and Sinning, 2004; Zanen *et al.*, 2005). Upon binding, SRP drives the RNCs to the Sec translocon by docking at its membrane-bound receptor, FtsY (Valent *et al.*, 1998). SRP has also been reported to direct proteins to YidC (Facey *et al.*, 2007; Welte *et al.*, 2012).

The SRP is a widely conserved ribonucleoprotein that is composed of a protein part with GTPase activity (the bacterial Ffh, for "fifty-four homologue" of the eukaryotic 54 kDa protein subunit and an RNA part (7S RNA in eukaryotes, 4.5 S RNA in *E. coli* and the *B. subtilis* small cytoplasmic RNA) (Walter and Blobel, 1982; Brown and Fournier, 1984; Nakamura *et al.*, 1992). In the latter organism the particle contains still another protein, HBsu (Nakamura *et al.*, 1999). The SRP pathway in archaea appears to be an intermediate between that of eukaryotes and bacteria (reviewed in Yuan *et al.*, 2010). The SRP and its membrane-bound receptor FtsY are essential for cell viability both in *E. coli* and *B. subtilis* (Brown and Fournier, 1984; Nakamura *et al.*, 1992; Phillips and Silhavy, 1992; Luirink *et al.*, 1994; Nakamura *et al.*, 1999; Kobayashi *et al.*, 2003).

SecB is a cytosolic chaperone present in many Gram-negative bacteria. When a signal peptide does not display a high level of hydrophobicity it is bound by SecB, which keeps the preprotein in an unfolded state (reviewed in Driessen and Nouwen, 2008). SecB routes preproteins to the Sec pathway by binding to the SecA ATPase. This interaction is strengthened by binding of the signal peptide to SecA (Fekkes *et al.*, 1998). SecB does not exist in *B. subtilis*, although an analogue may exist (CsaA) (Müller *et al.*, 2000). Thus, both organisms may share a common general mechanism involving co-translational targeting via SRP and post-translational targeting with SecB/CsaA (Tjalsma *et al.*, 2000).

The Sec pathway

The major route for protein transport across and into the cytoplasmic membrane is the Sec pathway. An important step in understanding this pathway was the identification of its major components as well as their structures. Another important contribution came from the functional *in vitro* reconstitution of the translocation reaction with purified components (Brundage *et al.*, 1990; reviewed in Driessen and Nouwen, 2008).

The core of the Sec pathway is the highly conserved heterotrimeric channel composed of three integral membrane proteins (SecYEG,

Sec61αβγ and SecYEβ, in bacteria, eukaryotes and archaea, respectively) (Brundage *et al.*, 1990; Görlich and Rapoport, 1993; Pohlschröder *et al.*, 1997). SecY is a highly hydrophobic protein that spans the membrane ten times in *M. jannaschii*, as well as in *E. coli* and *B. subtilis* (Akiyama and Ito, 1987; Nakamura *et al.*, 1990; Van den Berg *et al.*, 2004). SecY forms an hourglass-shaped channel through which the substrate is translocated. The interior of the channel is mostly hydrophilic with the exception of the hydrophobic waist that makes contact with the polypeptide. The pore in SecY has a clamshell-like structure that may open towards the lipid bilayer (Van den Berg *et al.*, 2004; Cannon *et al.*, 2005). SecE has three, one, or two transmembrane (TM) segments in *E. coli*, *B. subtilis* and *M. jannaschii*, respectively (Schatz *et al.*, 1989; Jeong *et al.*, 1993; Van den Berg *et al.*, 2004). In *E. coli a*nd *M. jannaschii*, SecE embraces the two SecY halves in a supportive manner, acting like a molecular clamp (Breyton *et al.*, 2002; Van den Berg *et al.*, 2004). SecG localises at the periphery of the complex and makes little contact with SecY (Breyton *et al.*, 2002). SecG has two TM segments both in *E. coli* and *B. subtilis* (Nishiyama *et al.*, 1993; van Wely *et al.*, 1999) and is not strictly required for function in either organism (Nishiyama *et al.*, 1994; van Wely *et al.*, 1999; Breyton *et al.*, 2002).

The oligomeric status of the translocon is a controversial matter. Different techniques suggest that the translocon can be found in a dynamic equilibrium between monomers, dimers or even higher-order oligomers, in eukaryotes, archaea and in bacteria (reviewed in Driessen and Nouwen, 2008; and in Du Plessis *et al.*, 2011).

In terms of subcellular localisation, several Sec components have been seen to form helices both in the rod-shaped *E. coli* and *B. subtilis* (Campo *et al.*, 2004; Shiomi *et al.*, 2006), although reports of uniform distribution exist for *E. coli* (Brandon *et al.*, 2003; Rubio *et al.*, 2005). Interestingly, in the coccoid bacterium *Streptococcus pyogenes,* the Sec system localised to a single microdomain, the ExPortal, adjacent to where a new cell division septum will form (Rosch and Caparon, 2004).

Several ancillary proteins that facilitate transport interact with the

channel, such as SecDFYajC and YidC/SpoIIIJ in bacteria (Bolhuis *et al.*, 1998; Nouwen and Driessen, 2002; Saller *et al.*, 2009), SecDF and possibly YidC in archaea (Eichler, 2003; Pohlschröder *et al.*, 2005a), and Sec62/Sec63/Sec71/Sec72 and TRAM (translocation-associated membrane protein) in eukaryotes (reviewed in Rapoport, 2007).

The energy required for transport is provided by peripheral components, such as the translating ribosome in the case of co-translational membrane insertion in bacteria, eukaryotes and archaea. Post-translational translocation is operated by Sec62/Sec63 and BiP in the endoplasmic reticulum of eukaryotes (reviewed in Du Plessis *et al.*, 2011); Although reports of post-translational translocation in archaea exist, its powering mechanism is not know (Rapoport, 2007 and references therein). In bacteria, the peripherally associated SecA ATPase and proton motive force energise post-translational translocation (Driessen, 1992; Economou and Wickner, 1994). SecA is also required during co-translational insertion of some membrane proteins, both in *E. coli* and in *B. subtilis* (e.g. Andersson and von Heijne, 1993; Bunai *et al.*, 2005).

The Tat pathway

The twin-arginine translocation (Tat) pathway catalyses the translocation of proteins in their folded state and/or containing cofactors both into and across membranes (reviewed in Natale *et al.*, 2008). This pathway is present in the three domains of life, being found in bacteria, archaea as well as in chloroplast thylakoid membranes in eukaryotes (reviewed in Natale *et al.*, 2008; and in Yuan *et al.*, 2010). Homologues were also found in mitochondria although not for the complete pathway (Bogsch *et al.*, 1998; Yen *et al.*, 2002).

Substrates are directed to the Tat machinery by an N-terminal signal peptide containing a conserved pattern of two almost invariant arginines within a Z-R-R-X-#-# consensus motif, where Z stands for a polar residue and # for a hydrophobic residue, present in both bacterial and thylakoidal substrates (Natale *et al.*, 2008).

The composition of the Tat translocase is variable amongst organisms. In *E. coli* and in plant thylakoids the minimal Tat translocase is formed by TatA, TatB and TatC (reviewed in Berks *et al.*, 2005). TatA and TatB are homologues but perform distinct functions (Sargent *et al.*, 1999). The TatBC complex functions in the binding of substrates, with TatC specifically recognising the consensus motif in the signal peptide and TatB interacting with the signal peptide and mediating transfer of the substrate from TatC to the pore (Alami *et al.*, 2003). In one model, TatA complexes constitute the protein-conducting channel of the Tat translocase, where the number of TatA protomers would change to match the size of the substrate (Gohlke *et al.*, 2005). A model with TatA and TatB forming the pore was also proposed (Sargent *et al.*, 2001). In *B. subtilis*, TatC and TatA constitute the minimal translocase. TatA is bifunctional in this organism as it performs the functions of both TatA and TatB of *E. coli* and thylakoids. Interestingly, three Tat translocases were found in *B. subtilis*, with distinct substrate specificities (Jongbloed *et al.*, 2004; Monteferrante *et al.*, 2012). Similarly to most Gram-positive bacteria, TatB is also absent in archaea and multiple copies of Tat components exist (reviewed in Yuan *et al.*, 2010).

Protein translocation by the Tat apparatus is energised exclusively by the proton motive force, both in plant thylakoids and in bacteria (Yahr and Wickner, 2001; Braun *et al.*, 2007).

In *E. coli*, Tat components seem to localise uniformly throughout the cytoplasmic membrane, although some punctations at the poles were observed, the significance of which is unclear (Ray *et al.*, 2005). TatCy and the three TatA proteins of *B. subtilis* showed a dual localisation pattern, both evenly in the membrane as well as in foci, which were more abundant at the cell poles and/or division sites (Meile *et al.*, 2006; Ridder *et al.*, 2009).

Other secretion systems

Gram-negative bacteria exhibit an ample diversity of extracellular protein

secretion systems that export proteins through their multilayered cell envelope and in some cases into host cells, requiring the crossing of three membranes in the latter case. Most of these systems are present in Gram-negative bacteria although some are found in Gram-positive bacteria, which also harbour specific systems (Economou *et al.*, 2006).

At least six major systems have been discovered so far (T1SS to T6SS, for Type X Secretion System) (Fig. 6) (reviewed in Economou *et al.*, 2006).

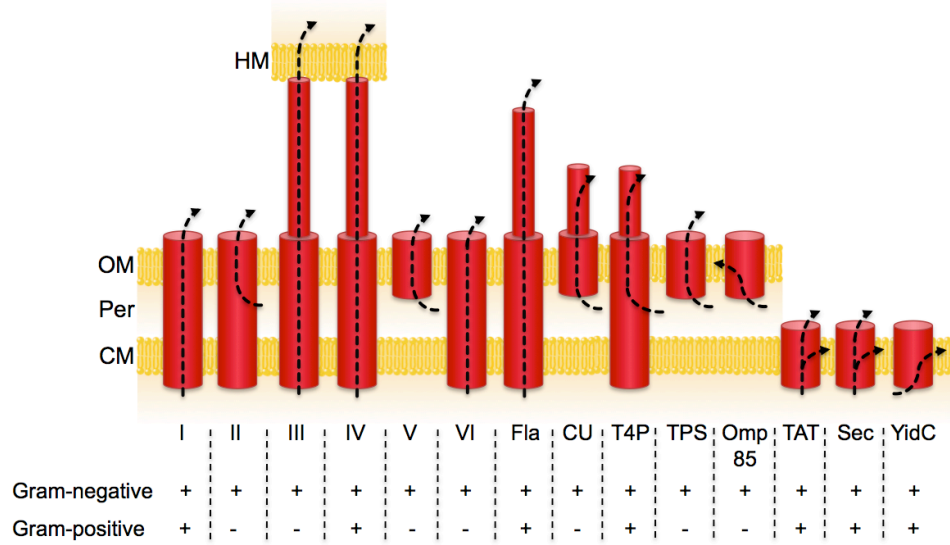

Figure 6. The bacterial protein export and secretion systems zoo. The arrows indicate the path that is taken by the exported protein. Arrows that initiate in the periplasm indicate that Sec (or rarely Tat)-dependent translocation across the cytoplasmic membrane is a necessary first step for these systems. I-VI, secretion systems from types 1 to 6; CM, cytoplasmic membrane; CU, chaperone-usher pathway; Fla, flagellum; HM, host cell membrane; OM, outer membrane; Omp85, also known as YaeT; Per, periplasm; TPS, two-partner secretion; T4P, type IV pili. Adapted from Papanikou *et al.*, 2007, and Economou *et al.*, 2006.

Such secretion systems can be classified as "one step" or "two step" mechanisms: in the first case, transport occurs directly through "tunnels" from the cytoplasm to the cell surface and external medium (e.g. T1SS and T6SS) or into the target cell cytoplasm (e.g. T3SS and T4SS), bypassing the inner membrane systems. T2SS and T5SS transport proteins in two steps, the first step occurring through Sec or Tat into the periplasm (reviewed in Desvaux *et al.*, 2009). Many more protein transport systems have been identified (reviewed in Holland, 2010) and this great diversity emphasises

the importance of the additional outer membrane barrier that has to be negotiated in Gram-negative bacteria.

The YidC/Oxa1/Alb3 family

Members of the evolutionarily conserved YidC/Oxa1/Alb3 family of proteins have been shown to perform critical roles in membrane insertion and assembly of energy-transducing complexes in eukaryotic organelles (mitochondria and chloroplast) and in bacteria (for reviews see Kol *et al.*, 2008; Saller *et al.*, 2012). The presence of Oxa1-related proteins in archaea is strongly suggested by genome sequencing (Bonnefoy *et al.*, 2009 and references therein).

The signature feature of this family of proteins is a group of five transmembrane (TM) segments (Yen *et al.*, 2001; Saller *et al.*, 2012) (Fig. 7).

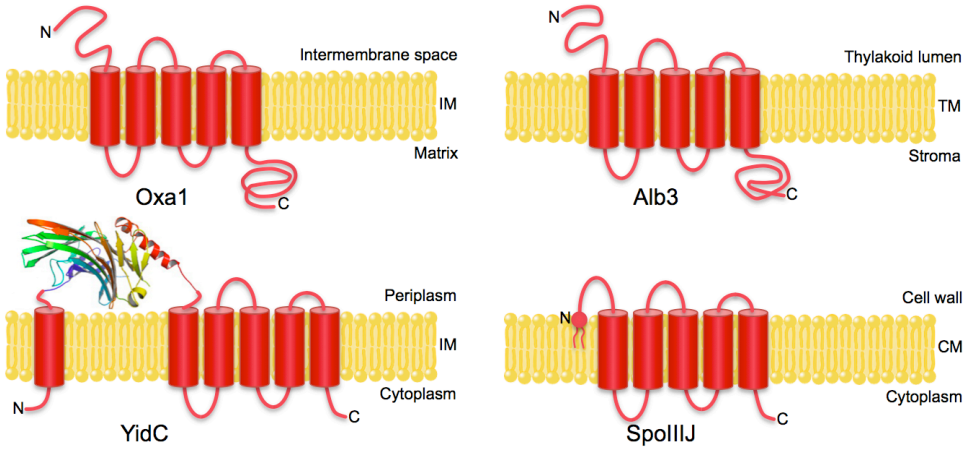

Figure 7. Topology of YidC/Oxa1/Alb3 family of proteins. Topology was mapped with several techniques or predicted for the various proteins herein depicted (for reviews see Yen *et al.*, 2001; Wang and Dalbey, 2011; Saller *et al.*, 2012). N and C, amino and carboxyl termini, respectively; IM, inner membrane; TM, thylakoid membrane; CM, cytoplasmic membrane. The structure of the periplasmic domain has been determined for YidC (Oliver and Paetzel, 2008; Ravaud *et al.*, 2008). The N terminus of SpoIIIJ is most likely lipid-modified. Adapted from Oliver and Paetzel, 2008, Tjalsma *et al.*, 2003, and Wang and Dalbey, 2011.

Oxa1 and Cox18

Oxa1 (oxidase assembly 1), present in the inner mitochondrial membrane of *Saccharomyces cerevisiae*, is the founding member of this family (Bonnefoy *et al.*, 1994; Kermorgant *et al.*, 1997). Oxa1 plays a critical role in

the insertion and assembly of energy-transducing respiratory complexes. In particular, Oxa1 directly mediates the co-translational insertion of proteins from the mitochondrial matrix, as the subunit II of cytochrome *c* oxidase, Cox2, (Hell *et al.*, 2001) and the assembly of both cytochrome *c* oxidase and of the membrane sector of the F_1F_0 ATPase (He and Fox, 1997; Hell *et al.*, 2001; Jia *et al.*, 2007).

Mitochondria typically contain proteins from two Oxa1 subfamilies – Oxa1 and Cox18. Cox18 (or Oxa2) was suggested to play a specialised role in the membrane biogenesis of cytochrome *c* oxidase in several organisms (Wang and Dalbey, 2011; Saller *et al.*, 2012). Cox18 lacks a C-terminal domain present in Oxa1, which was shown to interact with the large ribosomal unit, allowing Oxa1 to act as a ribosome receptor (Jia *et al.*, 2003). Oxa1 was suggested to form dimeric insertion pores on translating ribosomes (Kohler *et al.*, 2009).

Alb3 and Alb4

In chloroplasts of plants and algae Alb3 (*albino3*) plays an important role in the integration of members of the nuclear-encoded LHCP (light-harvesting chlorophyll-binding protein) family into the thylakoid membrane of chloroplasts (Sundberg *et al.*, 1997; Moore *et al.*, 2000; Bellafiore *et al.*, 2002). *alb4* is a homologue of *alb3* and it may also be present (Gerdes *et al.*, 2006). The C-terminal domain of Alb3 recruits chloroplast SRP and it is absent in Alb4, which cannot replace Alb3 (Falk *et al.*, 2010). Whilst both Alb3 and Alb4 are important for chloroplast membrane protein biogenesis, Alb4 was suggested to be required for assembly and/or stability of the F_1F_0 ATP synthase complex, being thus functionally more closely related to YidC and Oxa1 than Alb3 (Benz *et al.*, 2009).

Homologues in Archaea

Genes for YidC homologues were found in Euryarchaeota but not in the genomes of other archaea. These proteins are predicted to lack two TM

segments (corresponding to TM4 and 5 of YidC in *E. coli*), with TMs 2, 3 and 4 being homologous to TMs 2, 3 and 6 of YidC in *E. coli*, respectively) (Yuan *et al.*, 2010 and references therein).

YidC

The best-studied Oxa1 homologue in bacteria is YidC from *E. coli*. YidC is essential for viability and constitutes a key component in the biogenesis of membrane proteins (Samuelson *et al.*, 2000). YidC depletion results in a global change in cell physiology (Price *et al.*, 2010; Wang *et al.*, 2010; Wickström *et al.*, 2011). YidC was considered the missing bacterial insertase, as it facilitates the insertion of some Sec-independent proteins that were thought to insert spontaneously (Samuelson *et al.*, 2000). YidC can function independently but also in conjunction with the Sec translocase. In this context, YidC was suggested to transfer polypeptide segments from the SecAYEG complex into the lipid bilayer (Scotti *et al.*, 2000). YidC interacts with the Sec translocase by binding to SecD and SecF (Nouwen and Driessen, 2002). Proteins known to insert via the YidC-only pathway are the F_0c subunit of the F_1F_0 ATPase, MscL and the M13 and Pf3 phage coat proteins. YidC has been shown to be required for the membrane insertion of several Sec substrates, including the F_0a subunit of the F_1F_0 ATPase, NuoK (NADH dehydrogenase I subunit K) and subunit II of cytochrome *o* oxidase (CyoA) (reviewed in Wang and Dalbey, 2011). YidC has also been implicated in the folding of proteins following their insertion by the Sec translocase, as observed for MalF, belonging to the maltose transport complex, and the LacY lactose permease (Nagamori *et al.*, 2004; Wagner *et al.*, 2008). YidC also has a role in the assembly of multimeric complexes (van der Laan *et al.*, 2004; Kol *et al.*, 2008). YidC and FtsH have been suggested to have a linked role in the quality control of inner membrane proteins (van Bloois *et al.*, 2008).

Cooperation of YidC with the SRP pathway has been observed for both Sec-dependent and -independent substrates (reviewed in Dalbey and Kuhn, 2004). YidC itself is targeted by the SRP to the Sec-YidC translocon

for insertion (Urbanus *et al.*, 2002) and was shown to integrate TatC and MscL delivered by SRP *in vitro* (Welte *et al.*, 2012).

YidC revealed a predominantly polar localisation when fused to GFP (Urbanus *et al.*, 2002).

YidC possesses the five TM segments that are conserved in the Oxa1 family plus an additional N-terminal one, linked by a periplasmic loop that is not required for YidC activity. The five conserved C-terminal domains are critical for function, despite being remarkably tolerant to mutations (Sääf *et al.*, 1998; Jiang *et al.*, 2003). Cross-linking studies have shown that TM3 of YidC is in the proximity of the substrate during membrane biogenesis (Klenner *et al.*, 2008; Yu *et al.*, 2008). In addition, TM2 and TM3 were suggested to interact (Yuan *et al.*, 2007). Like Oxa1, YidC was proposed to form dimeric insertion pores on translating ribosomes. TM2 and TM3 of both monomers would form the core of the pore (Kohler *et al.*, 2009). Previously, YidC had already appeared as a monomer and a dimer in Blue Native PAGE (van der Laan *et al.*, 2001).

Conflicting reports exist regarding the ability of YidC to contact ribosomes, as it lacks the C-terminal ribosome-binding domain found in Oxa1. Recent work suggests that YidC does bind to ribosomes (Kohler *et al.*, 2009; reviewed in Price and Driessen, 2010; Welte *et al.*, 2012).

Complementation studies

The YidC/Oxa1/Alb3 family of proteins performs similar functions despite exhibiting some species-specific differences. Alb3, Oxa1 and Cox18 can replace YidC in *E. coli* and function in membrane protein insertion (Jiang *et al.*, 2002; van Bloois *et al.*, 2005; van Bloois *et al.*, 2007). YidC can functionally replace both Oxa1 and Cox18 in membrane protein insertion in mitochondria. In the case of Oxa1, its C-terminus matrix domain of had to be attached to YidC, unlike Cox18 (Preuss *et al.*, 2005). YidC1 and YidC2 of *Streptococcus mutans* can functionally substitute for YidC in *E. coli*, and YidC from *E. coli* is able to partially substitute for YidC2 (Dong *et al.*, 2008). Studies in *S. mutans* and *S. cerevisiae* show that

YidC2 and Oxa1 can partially complement each other (Funes *et al.*, 2009). Regarding *B. subtilis*, both SpoIIIJ and YqjG can functionally complement YidC in *E. coli* (Saller *et al.*, 2009).

SpoIIIJ and YqjG

Many Gram-positive bacteria, in contrast to Gram-negative bacteria, have two YidC homologues (reviewed in Yen *et al.*, 2001; Funes *et al.*, 2009). In *B. subtilis* they are called SpoIIIJ and YqjG (Errington *et al.*, 1992; Murakami *et al.*, 2002; Tjalsma *et al.*, 2003). Whilst deletion of either *spoIIIJ* or *yqjG* does not result in cell death, the absence of both is lethal. In addition, SpoIIIJ is required for sporulation, a function that YqjG cannot fulfil (Errington *et al.*, 1992; Murakami *et al.*, 2002; Tjalsma *et al.*, 2003). YqjG was shown to be involved in genetic competence development contrary to SpoIIIJ (Saller *et al.*, 2011). *spoIIIJ* is part of a bicistronic operon with *jag* (*spoIIIJ* associated gene). Jag is predicted to be a cytoplasmic protein containing single stranded nucleic acid-binding domains and it is dispensable for sporulation and growth (Errington *et al.*, 1992; Grishin, 1998; Tjalsma *et al.*, 2003). Similarly to *spoIIIJ*, *yqjG* is part of a bicistronic operon. *mifM* (for membrane protein insertion and folding monitor, or *yqzJ*) is the first gene of the operon and it is a sensor of SpoIIIJ activity that causes increased translation initiation of *yqjG* under SpoIIIJ-limiting conditions (Chiba *et al.*, 2009). SpoIIIJ and YqjG are uniformly distributed in the membrane during growth and sporulation (Rubio *et al.*, 2005).

SpoIIIJ and YqjG were implicated in protein secretion and in membrane protein biogenesis (Tjalsma *et al.*, 2003; Saller *et al.*, 2009). Reduced stability of the secreted AmyQ, LipA and PhoA was observed (Tjalsma *et al.*, 2003). The membrane proteins CtaC and QoxA, two homologues of Cox2, as well as FtsH, exhibited reduced stability (although different groups obtained different results) (Tjalsma *et al.*, 2003; Saller *et al.*, 2011). In cells depleted of SpoIIIJ and YqjG the majority of proteins exhibiting

reduced amounts were membrane proteins (Saller *et al.*, 2011). SpoIIIJ and YqjG facilitate insertion of subunit F_0c of the ATP synthase and were found to associate with the whole complex, suggesting a role in its assembly (Saller *et al.*, 2009; Saller *et al.*, 2011). More members of the Oxa1 family are in involved the formation of the F_1F_0 ATP synthase (reviewed in Wang and Dalbey, 2011; see above).

In the absence of *spoIIIJ*, sporulation is blocked after the completion of prespore engulfment. Mutations in *spoIIIJ* abolish the transcription of prespore-specific genes that use the σ^G sigma factor of the RNA polymerase but not transcription of the *spoIIIG* gene encoding σ^G (Errington *et al.*, 1992). σ^G accumulates in a *spoIIIJ* mutant but is mostly inactive (Serrano *et al.*, 2003). Expression of *spoIIIJ* in the prespore is sufficient for σ^G activity and efficient sporulation (Serrano *et al.*, 2003). It was suggested that the activation of σ^G after engulfment completion involves the combined action of the *spoIIIA*-encoded products from the mother cell together with SpoIIIJ from the prespore (Serrano *et al.*, 2003). Indeed, σ^G activation requires the formation of a channel between both compartments that is composed of two interacting proteins, SpoIIQ from the prespore and SpoIIIAH from the mother cell, as well as the remaining *spoIIIA* proteins, including SpoIIIAE (Blaylock *et al.*, 2004; Camp and Losick, 2008; Meisner *et al.*, 2008; Doan *et al.*, 2009). Genetic evidence points to a functional interaction between SpoIIIAE and SpoIIIJ (Serrano *et al.*, 2008). In addition, SpoIIIAE and SpoIIIJ were shown to directly interact in the membrane, linking the function of the *spoIIIJ* and *spoIIIA* loci in the activation of σ^G. Such was suggested to take place in the context of the Sec translocon by directing the final stages of insertion and/or folding of SpoIIIAE (Camp and Losick, 2008; Serrano *et al.*, 2008). SpoIIIAE also interacts with YqjG although the interaction appears to be non-functional, blocking YqjG (Serrano *et al.*, 2008). Suppressor mutants that partially bypass the dependence of σ^G activation on *spoIIIJ* were isolated. Interestingly, suppression was more potent regarding σ^G activation than spore formation, which is suggestive of another role for

SpoIIIJ later in sporulation (Camp and Losick, 2008). The suppressor mutations mapped to *pbpG*, *yqjG*, and *spoIIIAE*. PbpG is a peptidoglycan biosynthetic enzyme involved in cortex formation (McPherson *et al.*, 2001). The PbpG variant that partially bypassed the loss of SpoIIIJ is likely to impair and/or delay cortex synthesis between the membranes surrounding the prespore, leading to partial σ^G activation and spore formation (Camp and Losick, 2008). YqjG variants might have acquired some of the sporulation-specific functionality of SpoIIIJ, possibly by rendering the interaction with SpoIIIAE more productive (Camp and Losick, 2008; Serrano *et al.*, 2008). SpoIIIAE variants may assemble into the membrane on their own and/or may have acquired the ability to be recognised as a substrate by YqjG (Camp and Losick, 2008).

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GOALS OF THIS WORK

Bacillus subtilis cells lacking a functional *spoIIIJ* gene exhibit a blockage in the process of spore formation but no discernible effect in vegetative growth. SpoIIIJ and YqjG are both members of the YidC/Oxa1/Alb3 family of insertases present in *B. subtilis*, but only SpoIIIJ is functional during sporulation. One of the goals of this work was to explore the paradox regarding the lack of exchangeability between SpoIIIJ and YqjG given the numerous examples of heterologous complementation between members of this family (Chapter II). A second aspect of this work was focused on the exploration of specific differences that might be responsible for the partial function overlap shared by SpoIIIJ and YqjG. We thus sought to determine the basis for this behaviour and to overcome it (Chapter III). We also analysed the role of the signal peptides of SpoIIIJ and YqjG in terms of their contribution to the differential functioning of these proteins during sporulation (Chapter IV). Finally, we addressed several aspects concerning the oligomeric state of SpoIIIJ (Chapter V), as it is known to vary among members of the YidC/Oxa1/Alb3 family. We analysed SpoIIIJ's oligomerisation both *in vitro* and *in vivo*, and the role of a conserved cysteine residue (Cys134) in this process, specifically in the maintaining the potential for activity of the late prespore sigma factor σ^G during sporulation in *B. subtilis*.

Chapter II

Genetic plasticity *versus* species-specific requirements: the Spo11J paradox

All experiments were performed by the author of this Thesis except for the *in vivo* protein cross-linking and purification experiments, including the construction of associated strains, and the construction of pMS193.

Abstract

The YidC/Oxa1/Alb3 family is present in all domains of life, being required in several steps during the biogenesis of membrane proteins. SpoIIIJ and YqjG are two members from this family present in the Gram-positive spore-forming bacterium *Bacillus subtilis*. While either must be present to ensure viability, only SpoIIIJ supports efficient sporulation. This lack of interchangeability contrasts with the general picture observed for this family of proteins. As an initial approach to seek the basis for this differential behaviour we constructed chimeras of SpoIIIJ and YqjG. Three functional regions were defined in SpoIIIJ, ordered in terms of their contribution to spore formation. We also performed heterologous complementation. We show that the majority of Oxa1-like proteins from other *Bacillus* species here tested are unable to efficiently complement a *spoIIIJ* mutant. We also present biochemical evidence that indicate that SpoIIIJ(Bh) is able to interact with SpoIIIJ's substrate SpoIIIAE in *Escherichia coli*, despite not fully complementing a *spoIIIJ* mutant. We suggest that Oxa1-like proteins from *Bacillus* species are intrinsically able to interact with SpoIIIAE, being prevented to do so by other factors. Alternatively, SpoIIIJ is also required later during sporulation, when more refined interactions with its putative substrate are required. Data from both site-directed mutagenesis of conserved residues and from random mutagenesis suggest that, similarly to the case of other family members, no essential amino acid residues exist. We suggest that the overall conformation, rather than primary structure, is determinant for the function of the highly ductile YidC/Oxa1/Alb3 proteins. Our results support the view that the members of this family are able to lodge large alterations of primary structure while maintaining functionality.

Introduction

SpoIIIJ and YqjG are two members of the YidC/Oxa1/Alb3 family present in *Bacillus subtilis* (Errington *et al.*, 1992; Murakami *et al.*, 2002; Tjalsma *et al.*, 2003). This family of membrane protein insertases is ubiquitous, comprising members from bacteria to humans. Its members perform essential roles, e.g. in the biogenesis of energy-transducing membrane complexes. Specifically, they aid the insertion and folding of membrane proteins as well as their assembly into multimeric membrane complexes (Wang and Dalbey, 2011; Saller *et al.*, 2012). The *oxa1* gene was discovered in *Saccharomyces cerevisiae* whilst screening for proteins required for the assembly of the cytochrome oxidase complex (Bauer *et al.*, 1994; Bonnefoy *et al.*, 1994a), *alb3* in a genetic screen for albino (pigmentation-deficient) *Arabidopsis* mutants (Sundberg *et al.*, 1997), and YidC from *Escherichia coli* as an *oxa1* homologue (Scotti *et al.*, 2000). The number of genes from this family present per organism is quite variable: while Gram-negative bacteria generally possess only one (YidC, best studied in *E. coli*), Gram-positive bacteria usually contain two (e.g. SpoIIIJ and YqjG, in *B. subtilis*; YidC1 and YidC2, in *Streptococcus mutans*); yeast also has two homologues (Oxa1 and Cox18) and *Arabidopsis* harbours six such genes. The signature feature of this family is a hydrophobic core, which constitutes a functional unit and comprehends five transmembrane segments (Yen *et al.*, 2001; Saller *et al.*, 2012).

Generically speaking, proteins from this famlily may act alone or in conjunction with the Sec translocase, a major route for both insertion of membrane proteins as well as secretion (Samuelson *et al.*, 2000; Yen *et al.*, 2001; Klostermann *et al.*, 2002; Saller *et al.*, 2009; Saller *et al.*, 2012). Exceptions are Oxa1 and Cox18, mitochondrial proteins which, when fused to an initial region of *E. coli* YidC, complement a *yidC* mutant in its Sec-independent function only, which is not surprising as yeast mitochondria do not contain Sec homologues. These findings suggest that the Sec-independent function of Oxa1-related proteins is essential and conserved (Glick and Von Heijne, 1996; van Bloois *et al.*, 2005; van Bloois

et al., 2007). Interestingly, YidC is able to complement *oxa1* and *cox18* deficiency, provided that in the former case it has the C-terminus of Oxa1 appended but not in the latter (Preuss *et al.*, 2005). Other examples of trans-domain complementation exist, namely for Alb3 and Oxa1: Alb3, when fused to the first 57 residues of YidC was also able to complement an *E. coli yidC* depletion mutant, inserting both Sec-dependent and -independent proteins (Jiang *et al.*, 2002). The defect caused by the absence of YidC2 in *S. mutans* is overcome by the production of Oxa1 from *S. cerevisiae* and vice-versa (Funes *et al.*, 2009). Regarding complementation between more closely related organisms, both Oxa1 and Cox18 from *Neurospora crassa* complement the corresponding mutations in yeast (Nargang *et al.*, 2002; Funes *et al.*, 2004). Mitochondrial Oxa1 proteins from plants, humans and fission yeast are able to complement the corresponding mutation in yeast (Bonnefoy *et al.*, 1994b; Hamel *et al.*, 1997; Bonnefoy *et al.*, 2000), and also successful complementation of an *E. coli yidC* depletion mutant occurred with either homologue in *B. subtilis*, SpoIIIJ and YqjG (Saller *et al.*, 2009), emphasising the extensive interchangeability occurring between members of this family.

SpoIIIJ, one of the two insertases belonging to the YidC/Oxa1/Alb3 family present in *B. subtilis*, is absolutely required for one of the hallmarks of *B. subtilis* lifestyle, the ability to sporulate. A key morphological sign of sporulation is the formation of an asymmetric septum, which divides the cell into two compartments: a smaller one, the prespore, and the larger mother cell, that will engulf and nurture the prespore and eventually lyse, releasing the mature spore (Hilbert and Piggot, 2004). Proper spore formation requires meticulous coordination of a specific sequence of events, achieved with regulatory linkages between morphogenesis and gene expression. A major type of regulation occurs at the transcriptional level, through the differential use in time and space of alternate sigma factors of the RNA polymerase (Fig. 1A) (Hilbert and Piggot, 2004).

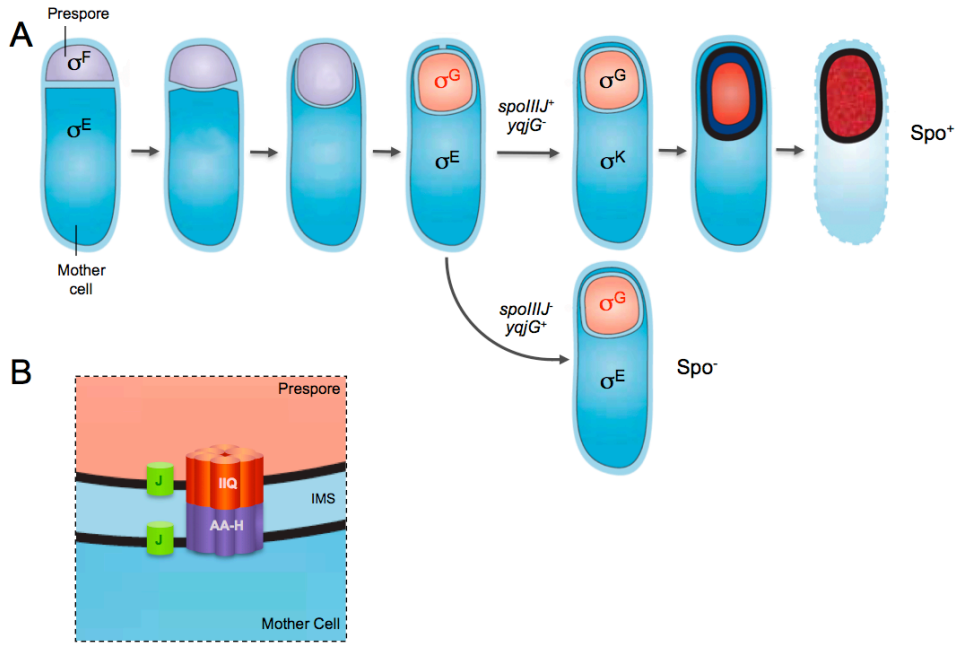

Figure 1. σ^G activity requires the action of SpoIIIJ in cooperation with SpoIIIA but not of YqjG. (A) An RNA polymerase sigma factor cascade regulates critical events during sporulation, where SpoIIIJ has an essential role in maintaining the potential for σ^G activity. **(B)** SpoIIIJ is required for the biogenesis of one or more SpoIIIA proteins, all of them required for σ^G activity. IMS, intermembrane space. Adapted from Errington, 2003, and Doan *et al.*, 2009.

Spore formation requires SpoIIIJ but not YqjG, most likely for the biogenesis of SpoIIIAE, also demanded along with the seven other σ^E-dependent SpoIIIA proteins for σ^G activity, the late prespore sigma factor (Fig. 1B) (Illing and Errington, 1991; Errington *et al.*, 1992; Kellner *et al.*, 1996; Serrano *et al.*, 2003; Camp and Losick, 2008; Serrano *et al.*, 2008). SpoIIIJ and YqjG are mostly transcribed during growth and form a synthetic lethal pair: at least one has to be present to ensure viability (Errington *et al.*, 1992; Murakami *et al.*, 2002; Tjalsma *et al.*, 2003), being thus partially redundant in their function.

Here we show that SpoIIIJ is a protein resilient to alterations apart from drastic changes such as conversion into stop codons or frameshifts, a property also observed for YidC in *E. coli*. These results are in accordance with the almost fully generalised ability of Oxa1-like proteins to complement the function of another member in quite distant organisms.

However, most of the tested Oxa1-like proteins from other *Bacillus* species failed to complement a *spoIIIJ* mutant for sporulation. An exception was YqjG from *B. halodurans*, which was shown to interact with SpoIIIAE from *B. subtilis*. We conclude that SpoIIIJ is quite malleable, being able to accommodate point mutations but paradoxically, with limited complementation capacity among close members.

Material and Methods

Media, bacterial strains and general techniques. The *B. subtilis* strains used in this work (listed in Table 1) are congenic derivatives of the Spo^+ strain MB24 (*trpC2 metC3*). Other *Bacillus* species here used are also listed. Luria-Bertani (LB) medium was used for growth or maintenance of *E. coli* and *B. subtilis*, and sporulation was induced in Difco sporulation medium (DSM) (Henriques *et al.*, 1995; Henriques *et al.*, 1998).

Construction of SpoIIIJ-YqjG chimeras. To construct pLC13 (chimera A) and pLC15 (B), $P_{spoIIIJ}$ and part of *spoIIIJ*'s coding region was amplified from chromosomal DNA of MB24 with the forward primer PIIIJD and either reverse primer J380R or J588R, respectively, producing J13 and J15. The *yqjG* fragments were synthesised with either spoIIIJ-yqjG-Y or spoIIIJ-yqjG-Pn as forward primers, and yqjG1256R as reverse primer, yielding Y13 and Y15. Joining of J13+Y13 and of J15+Y15 occurred through PCR using the external primers PIIIJ and yqjG1256R through splicing by overlap extension (SOE) (Horton *et al.*, 1989). Each fragment was digested with *Eco*RI and *Bam*HI and introduced between the same sites of pDG364 (Cutting and Vander Horn, 1990). To obtain pLC14 (chimera C) and 16 (D), the forward primer J-Y-prom and either yqjG586R or yqG811R as reverse primers were used to produce truncated *yqjG* genes with an upstream tail containing part of $P_{spoIIIJ}$ from DNA of MB24. These were fused by PCR to $P_{spoIIIJ}$ (amplified with primers PIIIJD and J200R) with the corresponding external primers, yielding Y14 and Y16, respectively.

Finally, the *spoIIIJ* fragments J14 and J16 were synthesised with either forward primer yqjG-spoIIIJ-Y or yqjG-spoIIIJ-Pn and the reverse primer J1039R, and joined to the corresponding $P_{spoIIIJ}$-*yqjG'* fragments with the external primers PIIIJ and J1039 (by SOE). The final PCR products were digested with *Eco*RI and *Bam*HI and introduced into similarly digested pDG364. Transformation of *Sca*I-linearised pLC13 through pLC16 into JOB44 (Δ*spoIIIJ::km*, a non-polar mutant, Serrano *et al*., 2003) yielded strains AH5045 through AH5048; into JOB9 (Δ*yqjG::sp*; Serrano *et al*., 2008) produced strains AH5062 through AH5065. Transformation of MB24 with pLC13 through pLC16 yielded strains AH5057 through AH5060. pFV4 and pFV5 contain a His-tag fused to the C-terminus of either '*spoIIIJ* or '*yqjG*, respectively (Chapter IV; Serrano *et al*., 2008). Strains AH5045 and AH5047 were transformed with pFV4, producing AH5232 and AH5234, respectively, through a single-reciprocal (Campbell-type) crossover event; AH5046 and AH5048 with pFV5, producing AHA5233 and AH5235, respectively. Linkage analysis and PCR testing were performed to confirm that the His-tag had introduced at the *amyE* locus. To construct pMS193, the *spoIIIJ* promoter region was PCR-amplified with primers J34D and J200R, and the *yqjG* coding region with spoIIIJ-yqjG-Pn and yqjG1256R, from DNA of MB24. The two PCR fragments were joined by PCR using the external primers (by SOE), digested with *Eco*RI and *Bam*HI and ligated to pDG364 similarly digested.

Random mutagenesis of *spoIIIJ*, *yqjG* and chimera D. *spoIIIJ* mutants were isolated by transforming the plasmid pJO49 (Serrano *et al*., 2003) into the DNA repair-deficient strain XL1-Red (Stratagene). Following growth, plasmid DNA was isolated, transformed into the *B. subtilis* strain JOB44 (Δ*spoIIIJ::km*), and plated on DSM agar plates at 37ºC. A control with only DNA (no cells) was included. Spo$^-$ *spoIIIJ* mutants were identified and tested for the Amy$^-$ phenotype. Chromosomal DNA was isolated for linkage analysis and DNA sequencing to identify the mutations. AmyE testing was also performed. Chimera D (pLC16) and $P_{spoIIIJ}$-*yqjG* (pMS193)

were subjected to a similar process, into JOB44 at 37ºC, looking for Spo$^+$ colonies. The mutagenised plasmid pJO49 was also transformed into AH5432 (Δ*yqjG*::P_{xylA}-*yqjG* Δ*spoIIIJ::km*) using competence-inducing medium containing 1 % xylose and lacking glucose, which could inhibit growth. DSM plates containing spectinomycin, kanamycin and chloramphenicol and 1 % xylose were incubated at 30ºC, 37ºC and 42ºC.

To check if P_{xylA} still responded to glucose, chromosomal DNA from ten colonies was transformed into JOB44 (Δ*spoIIIJ::km*) and plated with spectinomycin, kanamycin and either 0.5 % xylose or 0.2/0.5 % glucose, yielding plates with large and identical numbers of colonies in every case, suggesting that P_{xylA} had suffered mutations that converted it into a constitutive promoter in those Spo$^-$ colonies. A different strain for mutagenesis was obtained as follows: the MLS^r cassette, conferring erythromycin resistance, was removed from pDG1664 (Guérout-Fleury *et al.*, 1996) through *PagI* digestion, followed by fill-in with Klenow and *Eco*RI digestion, and cloned between the *Sma*I and *Eco*RI sites of pMLK83 (Karow *et al.*, 1995), producing pLC150. The region containing P_{spac}-*spoIIIJ* and *lac*I was recovered from pLC28 (see Chapter IV) through treatment with *Bam*HI, Klenow and *Eco*RI. This fragment was ligated to pLC150, which was freed from the *neo* cassette by digestion with *Not*I, treatment with Klenow and subsequently with *Eco*RI, producing pLC151. AH5009 (Table 1) was transformed with *Sca*I-linearised pLC151, replacing the chloramphenicol resistance with erythromycin resistance, producing AH5379. This strain was transformed with chromosomal DNA from JOB9 in the presence of IPTG, required for its viability, yielding AH5380.

We estimated the mutagenesis efficiency obtained with the XL1-Red mutator strain. ~0.81 % of the recovered colonies were Spo$^-$, and 16 of those were sequenced (2357 colonies counted). 5 of the 16 sequenced clones contained nonsense mutations, ~0.25 % of total colonies (5/16*0.81). 3 out of 64 codons in the genetic code are stop codons and, to simplify, assuming that substitution rates are similar for each base, we estimated that single amino acid residue substitutions without phenotypic

effect comprise a total of ~5.04 % (60/3*0.25). Thus, the overall mutagenesis rate should be ~5.84 % (assuming ≤1 mutation per gene), corresponding to 1 in each 17 random clones bearing some kind of mutation, and 1 in 20 containing substitutions that yield Spo^+ clones.

Site-directed mutagenesis. Single mutations predicted to be relevant for the function of *spoIIIJ* were obtained at positions 33, 53, 64, 254, and 238 (from the N-terminus) with the QuikChange site-directed mutagenesis system (Stratagene). pJO49 was used as template with primer pairs IIIJD33K-D and IIIJD33K-R, IIIJV53F-D and IIIJV53F-R, IIIJS64A-D and IIIJDS64A-R, IIIJQ254K-D and IIIJQ254K-R, IIIJQ238A-D and IIIJQ238A-R, producing pLC53 through pLC56, and pLC70, respectively. pLC60 was produced using pLC55 as a template with primer pairs IIIJS64A-D and IIIJDS64A-R. All plasmids were sequenced and, following *Sca*I-digestion, they were transformed into JOB44, producing strains AH5142 through AH5145, AH5183, and AH5160, respectively.

Construction of truncated *spoIIIJ* mutants. PCR-amplified *spoIIIJ* fragments using pJO49 as template and primers PIIIJD with either J936stop243R or J960stop250R, were digested with *Bam*HI and *Eco*RI, and ligated to similarly digested pDG364, to produce pLC66 and pLC67, respectively. Plasmids were confirmed by restriction analysis and sequenced, and, subsequently to *Sca*I-linearisation, were transformed into JOB44, producing AH5175 and AH5176, respectively.

Heterologous complementation. *spoIIIJ* and *yqjG* genes from *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus anthracis*, *Bacillus cereus* and *Bacillus halodurans* were PCR-amplified from chromosomal DNA of the corresponding species with the following primer pairs, respectively: (spoIIIJ-yqjG-Pn, yqjG1256R), *spoIIIJ*(Ba) (PBsBa, JBa1149R); *spoIIIJ*(Bc) (PBsBa, JBc1161R); *spoIIIJ*(Bh) (PBsJBh, JBh1186R); *yqjG*(Bh) (PBsYBh, YBh1281R); *spoIIIJ*(Bl) (PBsJBlich, JBlich1443R); *yqjG*(Bl) (PBsYBlich,

YBlich1436R). All genes were expressed from the *Bacillus subtilis spoIIIJ* promoter region, produced with primers PIIIJD and J200R. These hybrid PCR products were produced with PIIIJ and the corresponding species-specific reverse primer by SOE and subsequently cloned into pDG364, which inserts the genes at the non-essential *amyE* locus. $P_{spoIIIJ}$-*spoIIIJ*(Ba), $P_{spoIIIJ}$-*spoIIIJ*(Bc), $P_{spoIIIJ}$-*yqjG*(Bs), $P_{spoIIIJ}$-*spoIIIJ*(Bl), $P_{spoIIIJ}$-*yqjG*(Bl) were digested with *Bam*HI and *Eco*RI and ligated to pDG364, similarly digested, producing pLC39, pLC40, pLC64, pLC101 and pLC102, respectively. pLC41 and pLC42 were produced by digesting $P_{spoIIIJ}$-*spoIIIJ*(Bh) and $P_{spoIIIJ}$-*yqjG*(Bh) with *Eco*RI and ligating to pDG364 *Bam*HI-digested followed by fill-in with Klenow fragment and subsequent *Eco*RI digestion. *spoIIIJ*(Bc) was removed from pLC40 through *Eco*RI and *Bam*HI digestion and ligated to pDG1731 (Guérout-Fleury *et al.*, 1996) similarly digested, producing pLC61. $P_{spoIIIA(Bs)}$-*spoIIIAE*(Bs) and $P_{spoIIIA(Bs)}$-*spoIIIAE*(Bc), corresponding to pLC24 and pLC83, were constructed as follows: promoterless *spoIIIAE* was removed from pMS217 (Serrano *et al.*, 2008) through digestion with *Eco*RI and *Hin*dIII and ligated to pDG364 similarly digested, producing pLC23. Primers IIIA5383D and IIIA5658RBam were used to amplify $P_{spoIIIA}$ from pMS217. Both the promoter and pLC23 were digested with *Hin*dIII and *Bam*HI and ligated, producing pLC24; to obtain pLC83, the *spoIIIAE*(Bc) fragment was PCR-amplified from *B. cereus* chromosomal DNA with primers IIIAEBc247F and IIIAEBc1448R and ligated to pGEM T-Easy (Promega), yielding pLC51. *spoIIIAE*(Bc) was recovered from pLC51 digested with *Nco*I and *Sal*I. The vector part with $P_{spoIIIA}$ was rescued from pLC24 through digestion with *Eco*RI and *Hin*dIII, producing a vector with $P_{spoIIIA}$. Both the vector and the insert were Klenow filled-in, the vector dephosphorylated with CIAP and ligated, forming pLC83. All plasmids were confirmed by restriction analysis and sequencing. JOB44 was transformed with *Sca*I-digested pLC39-40, pLC41-42, pLC64, and pLC101-102, producing strains AH5120-1, AH5125-6, AH5161, and AH5290-1, respectively. MB24 was transformed with *Sca*I-digested pLC39-42, producing strains AH5151-4. JOB20 (Serrano *et al.*, 2003) was

transformed with chromosomal DNA from AH5151-4, producing strains AH5155-8, respectively. pLC61 was linearised with *Sca*I and transformed into JOB44 producing AH5159. pLC24 and pLC83 were linearised with *Sca*I and transformed into AH2468 (Δ*spoIIIAE*, Serrano *et al*., 2008) producing AH5435 and AH5208, respectively. AH5208, upon transformation with DNA from AH5159 resulted in AH5210 (Δ*spoIIIAE* Δ*amyE*::$P_{spoIIIA}$-*spoIIIAE*(Bc) Δ*thrC*::*spoIIIJ*(Bc)). This strain was transformed with DNA from JOB44, producing AH5213, to which *sspE-lacZ* (from AH1042) was added, yielding AH5219.

Construction of plasmids for cross-linking in *Escherichia coli*. Primers pairs J293D with Jhis, and G295D with Ghis were used to PCR-amplify *spoIIIJ* and *yqjG* from DNA of *Bacillus halodurans*, respectively. *spoIIIJ*(Bh), *yqjG*(Bh) and pETDuet-1 (Novagen, Darmstadt, Germany) were digested with *XhoI* and *NdeI* and ligated to the vector, producing pMS330 and pMS331, respectively. Both plasmids were introduced in *E. coli* C43(DE3) cells for overproduction.

***In vivo* protein cross-linking and purification of His-tagged protein complexes.** *E. coli* C43(DE3) cells expressing *spoIIIJ*(Bs)-*his*, *spoIIIJ*(Bh)-*his*, *yqjG*(Bh)-*his*, or *spoIIIAE*, alone or in combination, were grown in LB to an A_{600} of 0.7, and induced with 1 mM IPTG for 3 h. After induction, *E. coli* (10 ml) cells were centrifuged and washed with 150 mM NaCl, 20 mM $NaPO_4$ pH 7.2. Cells were concentrated 10-fold in the same buffer and Dithiobis(succinimidylpropionate) (DSP) was added to 0.2 mM. DSP has a fixed spacer arm of 12 Å and a cleavable disulphide bond (Pierce, Rockford, IL). Cross-linking was carried out for 30 min at 37ºC and was quenched with 20 mM Tris-HCl pH 7.5. Cells were harvested, resuspended in 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), and the suspension was passed twice through a French pressure cell at 19 000 lb/in^2. Membranes were isolated by a 60 min centrifugation at 100 000 x *g*, then solubilised in 8 M

urea, 10 mM Tris-HCl, 100 mM NaH_2PO_4, 1 % Triton X-100, 0.2 % Sarkosyl, adjusted to pH 8.0, mixed with 200 µl 50 % Ni-NTA resin (Qiagen, Hilden, Germany) for each 10 ml of suspension, and incubated for 1 h with mixing. The resin was washed three times for 10 min with 0.5 M NaCl, 20 mM Tris, 5 mM imidazole, 0.1 % sodium dodecyl sulfate (SDS), pH 8.0. Bound proteins were eluted with 8 M urea, 50 mM Tris, 2 % SDS, 0.4 M imidazole, pH 6.8. The cross-linker was reduced with 5 % β-mercaptoethanol in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) sample buffer (2 % SDS, 6.25 mM Tris-HCl, pH 6.8, 5 % glycerol, 0.025 % bromophenol blue), at 100°C for 5 min.

Whole-cell lysates and immunoblot analysis. Cultures of *B. subtilis* strains were grown in DSM to an A_{600} of 0.6 and samples collected one hour (T_{-1}) before the end of the exponential phase of growth, (defined as the onset of sporulation, or T_0), and at the indicated times thereafter. Samples of 15 ml were harvested by centrifugation. The cell pellets were resuspended in 1 ml of 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM PMSF, 0.1 mM DTT and lysed with a French pressure cell at 19 000 lb/in^2. Proteins (30 µg) were electrophoretically resolved through SDS-PAGE. The resolved proteins were transferred to nitrocellulose membranes which were first incubated for 30 min in phosphate buffered saline (PBS) containing 5 % low-fat milk and then overnight in PBS containing 0.5 % low-fat milk and a either mouse anti-His-tag antibody (Novagen, Darmstadt, Germany) for the detection of SpoIIIJ-His_6 or YqjG-His_6, or with rabbit antibodies raised against peptides derived from SpoIIIAE or SpoIIIJ (residues 66 to 80 and 247 to 261, respectively; Eurogentec, Seraing, Belgium). Proteins were visualised using the corresponding anti-mouse or anti-rabbit secondary antibodies with the ECL detection system (Amersham Biosciences) as described by the manufacturer.

Results

Defining functional regions in SpoIIIJ. A strong investment has been made in the study of various members of the widespread YidC/Oxa1/Alb3 family of protein insertases, being most evident in Oxa1 and YidC from *Saccharomyces cerevisiae* and *Escherichia coli*, respectively (Wang and Dalbey, 2011; Saller *et al*., 2012). Two members of the well-known YidC/Oxa1/Alb3 family of proteins required for the biogenesis of membrane proteins exist in *B. subtilis*, SpoIIIJ and YqjG. Whilst either SpoIIIJ or YqjG is required for viability, only SpoIIIJ supports spore formation. Specifically, it is required for the activity of the RNA polymerase sporulation-specific sigma factor σ^G (Fig. 1A) (Errington *et al*., 1992; Murakami *et al*., 2002; Tjalsma *et al*., 2003). In an initial attempt to isolate the singularities that empower SpoIIIJ in its role during spore formation, we constructed chimeras of *spoIIIJ* and *yqjG* and expressed them from the *spoIIIJ* promoter at the non-essential *amyE* locus in a *spoIIIJ*::*km* background (Fig. 2A-B). One chimera was able to support nearly wild-type levels of sporulation (B, AH5047), closely followed by chimera C (AH5046). Chimeras A and D provided lower sporulation titres (Fig. 2B; Table 3). Next, we investigated the accumulation of chimeras fused to a His-tag. Interestingly, non-functional chimera A accumulated at higher levels than chimera B (the most functional form here obtained) as visualised through immunoblot analysis with anti-His antibodies (AH5232 and AH5234, respectively; Fig. 2C). Chimera C (AH5233) was not observable, although more functional than chimera A, both with anti-His and anti-SpoIIIJ antibodies (not shown). Expression of these chimeras in MB24 or *yqjG*::*sp* backgrounds (AH5057-60 and AH5062-65, respectively) did not interfere with sporulation (Table 3). Based on these results, by comparing chimera pairs A and B, and C and D, we broadly defined three regions in SpoIIIJ and ordered them in terms of contribution for its function: region II, corresponding to the second quarter of the protein seems to be the most relevant one, followed by region III (second

half of the protein) and finally by region I, comprising the first quarter of SpoIIIJ.

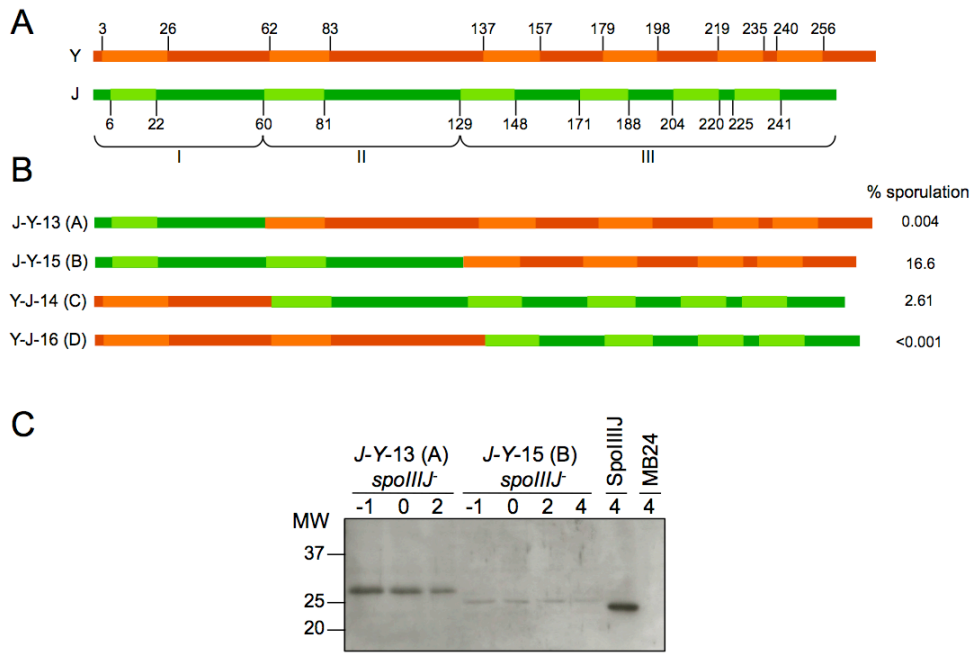

Figure 2. Defining functional regions in SpoIIIJ. Schematic representation of **(A)** the two native proteins, YqjG (orange) and SpoIIIJ (green), and of **(B)** four chimerical proteins. Light-coloured regions represent transmembrane segments and the numbers indicate the amino acid residues at their boundaries. **(C)** Strains AH5232 (Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ-yqjG-13-his* Δ*spoIIIJ::km*, lanes 1-3), AH5234 (Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ-yqjG-15-his* Δ*spoIIIJ::km*, lanes 4-7), AH9218 (Δ*spoIIIJ::spoIIIJ-his*, lane 8) and MB24 (wild-type, lane 9) were grown in DSM and samples were collected at the indicated times (in hours) relative to the onset of sporulation. The cells were lysed and the whole-cell extracts were electrophoretically resolved by SDS-PAGE and subjected to immunoblot analysis using antibodies against the His-tag.

Only YqjG from *Bacillus halodurans* supports wild-type sporulation levels. To look for specific residues that might provide some insight regarding the functional differences between YqjG and SpoIIIJ we tested the ability of several SpoIIIJ homologues from other *Bacillus* species to complement a *spoIIIJ*⁻ mutant for sporulation. An alignment of the SpoIIIJ and YqjG proteins from the organisms here tested, with several levels of shading according to the degree of conservation, and a phylogenetic tree based on that alignment are depicted in Figs. 3 and 4, respectively.

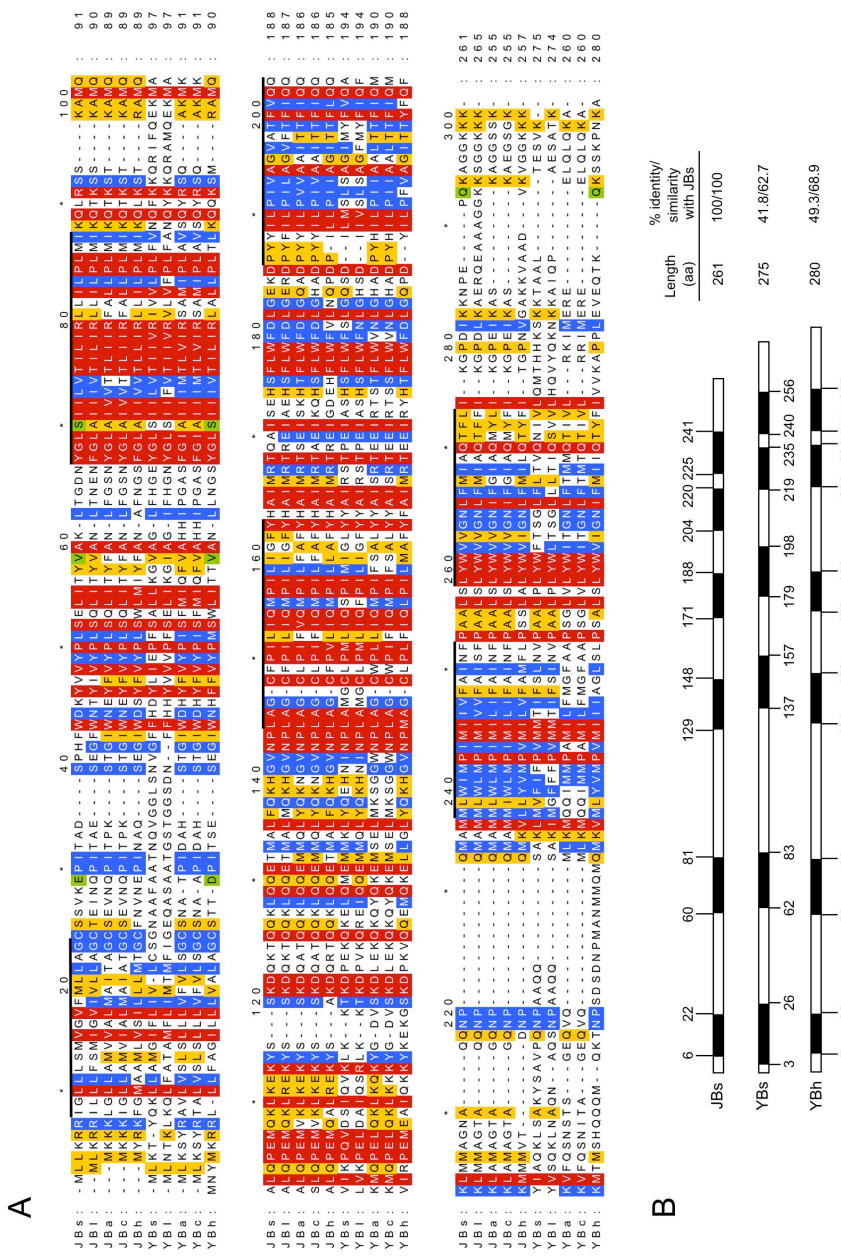

Figure 3. Alignment of YqjG and SpoIIIJ proteins from selected organisms. (A) Alignments of homologues of SpoIIIJ (J) and YqjG (Y) proteins from several *Bacillus* species. Bs, *B. subtilis*; Bl, *B. licheniformis*; Ba, *B. anthracis*; Bc, *B. cereus*; Bh, *B. halodurans*. Amino acid residue colouring was performed with GeneDoc (http://www.nrbsc.org/gfx/genedoc/) using the conservation mode with four shading levels, corresponding to a degree of conservation of 100 % (red), between 80 and 100 % (blue), between 60 and 80 % (yellow) and lower than 60 % (white). Similar residues (conservative substitutions) were considered as identical for purposes of determining the degree of conservation within columns of the alignment. Amino acid residues that are conserved mostly in SpoIIIJ from *B. subtilis* and YqjG from *B. halodurans* are depicted in green. A black line indicates the position of transmembrane segments. **(B)** Schematic

representation of SpoIIIJ from *B. subtilis* and YqjG from *B. subtilis* and *B. halodurans*. Dark regions correspond to transmembrane segments and the numbers indicate the amino acid residues at their boundaries. The length of each protein (in number of residues) and the percentage of identity and similarity relative to SpoIIIJ from *B. subtilis* are indicated on the panel on the right.

Each gene was expressed under the control of the *spoIIIJ* promoter from *B. subtilis* at the *amyE* locus. We defined as *spoIIIJ* the homologues with *jag* (*spoIIIJ*-associated gene) next to them (Fig. 4) (Errington *et al.*, 1992). We verified that *yqjG*(Bh) (AH5126) was the only one providing wild-type sporulation levels among those tested (Table 3), namely *spoIIIJ* genes from *B. anthracis* and *B. cereus*, and *spoIIIJ* and *yqjG* genes from *B. halodurans* and *B. licheniformis* (AH5120-1, AH5125-6, AH5290-1, respectively). Similar results were obtained in a *spoIIIJ*::*sp* background for the genes tested (*spoIIIJ*(Ba), *spoIIIJ*(Bc), *spoIIIJ*(Bh), *yqjG*(Bh), AH5155-8, respectively); in addition, strains expressing these genes in a wild-type background (AH5151-4) exhibited a spore titre similar to that of MB24. The sequence alignment, phylogenetic tree and percentage of similarity (Figs. 3 and 4; not shown) show that the homologues that more efficiently complement a *spoIIIJ*$^-$ strain are usually the ones with greatest similarity to SpoIIIJ, although not always in a direct proportion, note for instance the case of *yqjG*(Bh) relative to *spoIIIJ*(Bl). SpoIIIAE is a sporulation-specific substrate of SpoIIIJ (Camp and Losick, 2008; Serrano *et al.*, 2008). Interestingly, *spoIIIAE*(Bc) complements the *spoIIIAE* in-frame deletion, similarly to *spoIIIAE*(Bs) (AH5208 and AH5435, respectively), in contrast with the case of *spoIIIJ*(Bc) expressed from the *thrC* locus in *spoIIIJ*::*km* (AH5159) or from the *amyE* locus in *spoIIIJ*::*km* or *spoIIIJ*::*sp* backgrounds (AH5121 and AH5156, respectively). Combining the relevant genotypes from AH5159 and AH5208 (in AH5219, $P_{spoIIIAE(Bs)}$-*spoIIIAE*(Bc) Δ*spoIIIAE* *thrC*::$P_{spoIIIJ(Bs)}$*spoIIIJ*(Bc) *spoIIIJ*::*km*) did not result in enhanced sporulation levels relative to AH5159. These results suggest that *spoIIIJ*(Bs) interacts adequately with *spoIIIAE*(Bc) but that this should not be the sole function of SpoIIIJ during sporulation.

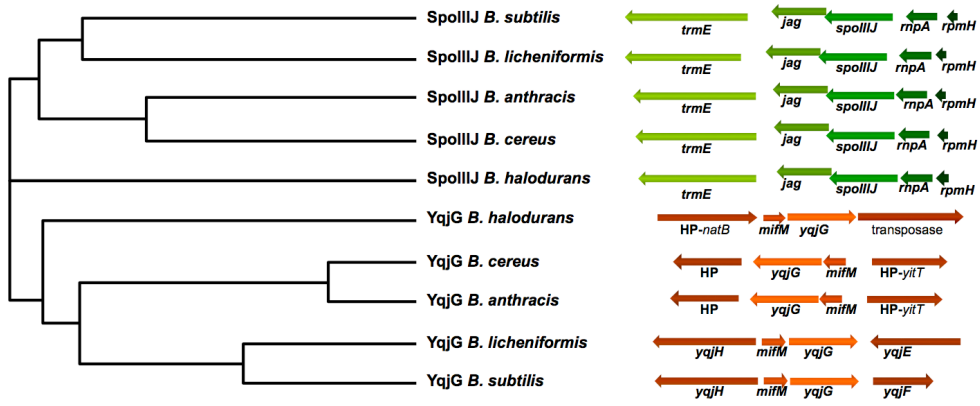

Figure 4. Phylogenetic tree and genomic regions of several *Bacillus*. Phylogenetic tree constructed with ClustalW2 - Phylogeny (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/) from a ClustalO (http://www.ebi.ac.uk/Tools/msa/clustalo/) alignment of several SpoIIIJ and YqjG proteins from several *Bacillus* species. The tree was complemented with the genomic regions comprising either *spoIIIJ* or *yqjG*, based on data from NCBI databases (www.ncbi.nlm.nih.gov) and Chiba *et al*., 2009. HP, conserved protein.

Both SpoIIIJ and YqjG from *Bacillus halodurans* form membrane-bound complexes with SpoIIIAE from *Bacillus subtilis* in *Escherichia coli*. SpoIIIAE was shown to interact with SpoIIIJ and YqjG, and were suggested to do so directly (Serrano *et al*., 2008). This prompted us to investigate if the distinct levels of sporulation obtained from the complementation of a *spoIIIJ*⁻ null mutant with SpoIIIJ homologues from other *Bacillus* species (see above) were linked to their ability to interact with SpoIIIAE from *B. subtilis*. We chose SpoIIIJ and YqjG from *B. halodurans* as they showed distinct complementation levels (Table 3).

We combined *in vivo* cross-linking and affinity chromatography to probe for interactions in *E. coli* cells, in which co-production of SpoIIIAE and either SpoIIIJ(Bh) or YqjG(Bh) took place. Cells of the several *E. coli* strains were treated with DSP, lysed, the membrane proteins solubilised, subjected to affinity purification of the His-tag-containing complexes from the extracts and treated with a reducing agent to cleave the cross-linker. Proteins in the total membrane preparations and the isolated complexes were resolved by SDS-PAGE and identified by immunoblot analysis with antibodies against the His-tag (upper panels in Fig. 5) or SpoIIIAE (lower

panels). SpoIIIJ(Bs)-His_6, SpoIIIJ(Bh)-His_6 and YqjG(Bh)-His_6 (Fig. 5A, upper panel, lane 1, lanes 3 and 4, and lanes 5 and 6, respectively) were readily pulled-out by Ni^{2+}-affinity chromatography in a single step. We observed that SpoIIIAE co-purified with SpoIIIJ(Bh)-His_6 and YqjG(Bh)-His_6 (Fig. 5A, lower panel, lanes 4 and 6, respectively), but did not purify independently of them, as expected (lane 2). SpoIIIAE also co-purified with the positive control SpoIIIJ-His_6 (lane 1). In Fig. 5B, non-purified extracts were processed as for Fig. 5A, as a control.

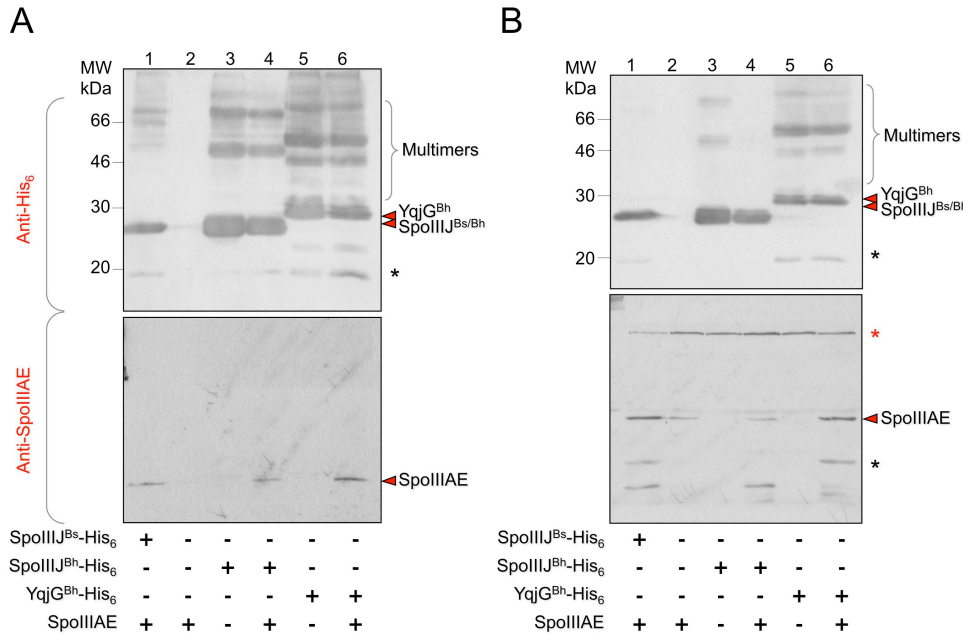

Figure 5. *In vitro* interaction of SpoIIIJ and YqjG from *Bacillus halodurans* with SpoIIIAE from *Bacillus subtilis*. Pull-down assays were performed with extracts from *E. coli* C43(DE3) strains grown in LB medium and induced to produce SpoIIIJ(Bs)-His_6, SpoIIIJ(Bh)-His_6, YqjG(Bh)-His_6 and SpoIIIAE(Bs) with pMS266 (Serrano *et al.*, 2008), pMS330, pMS331, or pMS267 (Serrano *et al.*, 2008), respectively). Following *in vivo* cross-linking at the end of exponential growth by incubation with dithiobis (succinimidyl propionate) (DSP), membranes were isolated, proteins resolved by SDS-PAGE under reducing conditions (which cleave the spacer arm (12 Å) of the cross-linker) and identified using antibodies against the His-tag or SpoIIIAE (upper and lower panels, respectively) as indicated, either after **(A)** or before purification **(B)**. The positions of SpoIIIAE(Bs), SpoIIIJ-His_6, SpoIIIJ(Bh)-His_6, YqjG(Bh)-His_6 (red arrowheads), degradation products (black asterisks) and cross-reactive species (red asterisk) are indicated. The position of molecular weight markers (in kDa) is shown.

In resemblance to the interaction observed between SpoIIIAE and SpoIIIJ/YqjG proteins from *B. subtilis* (Serrano *et al.*, 2008), here also (except if a highly conserved protein is involved) the results suggest that,

like SpoIIIJ(Bs), SpoIIIJ(Bh) and YqjG(Bh) interact directly with SpoIIIAE. Hence, the lack of complementation observed for SpoIIIJ(Bh) is not due to an intrinsic inability to interact with SpoIIIAE(Bs).

Site-directed mutagenesis of *spoIIIJ* produces functional genes. We performed site-directed mutagenesis of several amino acid residues in SpoIIIJ(Bs) based on a comparison between the fully functional proteins and the some of the less functional ones (YqjG(Bh) and SpoIIIJ(Bs) versus SpoIIIJ(Bh), SpoIIIJ(Bc), (SpoIIIJ(Ba) and YqjG(Bs)) (Fig. 3). SpoIIIJ(Bs) variants containing the substitutions D33K, V53F, S64A, Q254K, or V53F combined with S64A were expressed from the *amyE* locus in a *spoIIIJ*::*km* background (AH5142-5 and AH5160, respectively; Fig. 6, yellow). Q254G was never obtained. Interestingly, Q254K was only obtained when accompanied by other mutations, namely a frameshift from K249 on, or two close-by amino acid substitutions (AH5145). However, none of the engineered mutations had any effect on the ability of SpoIIIJ(Bs) to support sporulation, suggesting that this protein easily accommodates point mutations (Table 3). In addition, the Q238 residue, which is absolutely conserved in Firmicutes (not shown), can also be converted into an alanine without loss of function during spore formation in a *spoIIIJ*::*km* background (AH5183, Table 3).

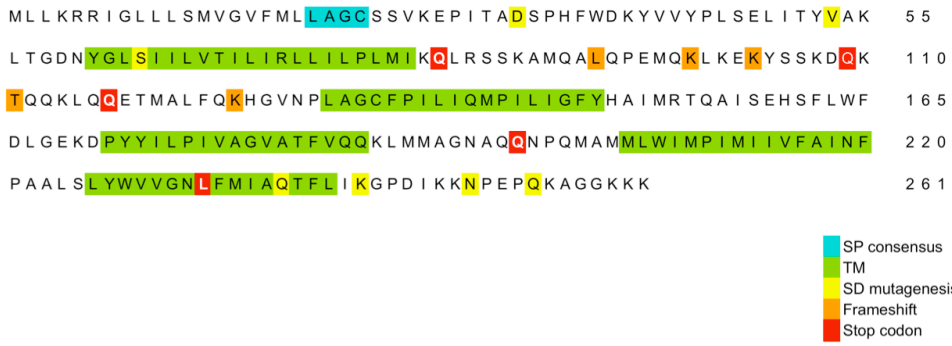

Figure 6. Map of SpoIIIJ substitutions as obtained from random and site-directed mutagenesis. Altered SpoIIIJ forms recovered after XL1-Red mutagenesis cause a Spo$^-$ phenotype due to frameshifts (orange) or conversion into stop codons (red). Predicted transmembrane segments are defined in green and the signal peptide (SP) consensus region is depicted in blue. Site-directed (SD) mutations are depicted in yellow (Q238A, K243stop, N250stop, D33K, V53F, S64A, Q254K).

SpoIIIJ comfortably accommodates point mutations. In order to find amino acid residues relevant for SpoIIIJ's function during sporulation we performed a screen for Spo$^-$ colonies using a library of mutated plasmids based on pJO49 (Serrano *et al*., 2003), using the XL1-Red mutator strain (Stratagene), and inserted it into a *spoIIIJ*$^-$ strain (JOB44). We estimated the mutagenesis efficiency obtained with the XL1-Red mutator strain to be ~5.84 % (see Materials and Methods for details). All the mutations that originated the desired phenotype (Fig. 6) consisted of either frameshifts (orange) or conversion into stop codons (red), again suggesting that SpoIIIJ is a malleable protein concerning the specificity of the residues required for its proper functioning. In addition, we verified that the C-terminal region of SpoIIIJ is not required for sporulation, as strains AH5175 and AH5176, expressing *spoIIIJ* truncated at residues 242 and 249, respectively, were able to complement a *spoIIIJ* mutant for sporulation (Table 3). We observed a mutational hotspot around residue T111, where seven of the sixteen frameshifts and missense mutations occurred. Fourteen mutations fell in the first cytoplasmic loop, although its significance is unclear. Recovery of Spo$^+$ colonies through random mutagenesis of pMS193 ($P_{spoIIIJ}$-*yqjG*) and pLC16 (chimera D) was also attempted but the desired phenotype was not obtained.

In an attempt to isolate amino acid substitutions that affect SpoIIIJ's ability to support sporulation but not viability we produced a strain expressing *spoIIIJ* from its own promoter from the *amyE* locus, in a *spoIIIJ*::*km* mutant background (JOB44) and having *yqjG* under the control of a xylose-inducible promoter, P_{xylA}. Competence of this strain was induced in glucose-free medium, cells were transformed with XL1-Red-mutagenised pJO49 and plated at 30ºC, 37ºC and 42ºC with 0.2 % glucose and appropriate antibiotics. However, the inducible promoter might be accumulating mutations leading to constitutive *yqjG* expression. Such was evaluated by transforming JOB44 with chromosomal DNA from ten Spo$^-$ colonies whilst selecting for P_{xylA}-*yqjG* either in the presence of glucose (0.2 and 0.5 %) or xylose (0.5 %). In both cases, large and similar numbers of

colonies were obtained, for the ten cases tested, which led us to conclude that *yqjG* was now being constitutively expressed. To select for sporulation-specific mutations more purposely, we proposed to produce a strain expressing *spoIIIJ* from its own promoter at the *amyE* locus, in a *spoIIIJ* and *yqjG* mutant background. Very few colonies were recovered when transforming AH5011 with chromosomal DNA from JOB9 (*yqjG*::*sp*); more were obtained when kanamycin was not included in the plates; in addition, as a control, we verified that the same amount of JOB9 chromosomal DNA was able to provide large numbers of colonies when introduced into the wild-type MB24. We interpret this as *spoIIIJ* expressed at the *amyE* locus from its 421 bp promoter region here used not being able to support viability when both *spoIIIJ* and *yqjG* loci are disrupted. We constructed yet another strain, expressing *spoIIIJ* from the IPTG-inducible promoter P_{spac} at the *amyE* locus, in the absence of both *spoIIIJ* and *yqjG*. This strain (AH5380) required IPTG for viability as expected. It is to note that previous constructs of P_{spac}-*spoIIIJ* and P_{xylA}-*spoIIIJ* always failed to yield Spo^- colonies in the absence of inducer (not shown), strongly suggesting that high amounts of SpoIIIJ are needed to support viability but that the amount produced due to leakiness of the promoter is sufficient to support sporulation. AH5380 was transformed with XL1-Red-mutagenised pLC28 (P_{spac}-*spoIIIJ*) and few Spo^- colonies were recovered. Upon re-transformation of either AH5380 or JOB44 with chromosomal DNA of three of the Spo^- clones, Spo^+ colonies were always obtained, suggesting that for those three clones the Spo^- phenotype was not linked to the mutagenised plasmids, and strengthening the view that loss-of-function substitutions are particularly difficult to obtain for SpoIIIJ.

Discussion

YqjG and SpoIIIJ are the two members of the YidC/Oxa1/Alb3 family of protein insertases present in *B. subtilis*. They share 41 % identities and 62 % similarities in terms of primary sequence but only SpoIIIJ supports efficient sporulation, despite the fact of either being able to support viability by itself (Errington *et al.*, 1992; Murakami *et al.*, 2002; Serrano *et al.*, 2003; Tjalsma *et al.*, 2003). In order to better understand the mechanisms underlying this behaviour we employed several approaches. The results herein obtained highlight the importance of the overall structure over the precise nature of amino acid residues for the function of SpoIIIJ. Our efforts to randomly mutagenise *yqjG* and chimera D for recovery of gain-of-function mutants during sporulation were unsuccessful; regarding SpoIIIJ, no single amino substitutions were found that produced a Spo$^-$ phenotype, which was only caused by nonsense mutations and frameshifts. A related experiment with YidC permitted the isolation of two cold-sensitive mutants (Yuan *et al.*, 2007); however, we did not succeed in obtaining single amino acid substitutions leading to a Spo$^-$ phenotype when incubating at 37ºC, 30ºC or 42ºC. Additionally, an attempt to force the recovery of mutations that affected sporulation but not viability (by inserting the pool of mutagenised *spoIIIJ* genes into a *spoIIIJ* null mutant with *yqjG* under the control of a xylose-inducible promoter) led only to the isolation of P_{xylA} promoters that no longer responded to glucose, reinforcing the notion that single amino acid substitutions with drastic phenotypic effects are considerably difficult to isolate for SpoIIIJ. This case is reminiscent of what has been observed for other members of the YidC/Oxa1/Alb3 family of protein insertases, as mutations recovered in Oxa1 (*S. cerevisiae*) and in YidC (*E. coli*) when changed into an alanine no longer provoked an altered phenotype (Jiang *et al.*, 2003; Mathieu *et al.*, 2010). This test was performed in more detail in YidC, for which it was shown that no amino acid residue is absolutely essential for its function (Jiang *et al.*, 2003). This scenario correlates with the remarkable complementation of functions that occurs even with

members with low sequence identity from evolutionarily distant organisms (Bonnefoy *et al.*, 1994b; Jiang *et al.*, 2002; Preuss *et al.*, 2005; van Bloois *et al.*, 2005; van Bloois *et al.*, 2007; Funes *et al.*, 2009). However, in this work we observed that the levels of complementation with proteins from closely related organisms were lower than expected (Table 3), given the above-mentioned examples found in literature. In particular, SpoIIIJ(Bh) (but not YqjG(Bh)) failed to sustain wild-type levels of heat-resistant spores (AH5125-6, respectively, Table 3), despite both interacting with SpoIIIAE(Bs), most likely in a direct way, in cross-linking experiments performed in *E. coli* (Fig. 5). One explanation may be that Oxa1-like proteins from *Bacillus* species have an intrinsic ability to interact with SpoIIIAE(Bs), and that lack of function may arise, at least in part, by prevention of their interaction. Another possibility is that proteins from this family may be interchangeable for functions that are essential and thus kept throughout evolution, but not necessarily for non-essential processes like sporulation, the one evaluated in this work. It is noteworthy that the homologue of *spoIIIJ* in *B. subtilis*, *yqjG*, when mutated in specific amino acid residues sees its ability to complement a *spoIIIJ* mutant augmented, although not to full levels (but see Chapter III). This is most likely due to improved interaction with SpoIIIAE, which is required for efficient sporulation at the same stage as SpoIIIJ itself (Camp and Losick, 2008). This hints that, although the precise sequence is not critical, a few residues are likely to be important for interacting with very specific non-essential substrates; the interaction may be occurring but in a non-productive manner due to requiring specific amino acid contacts. Interestingly, SpoIIIAE from *B. cereus* fully complemented a *spoIIIAE* in-frame deletion mutant, suggesting that it interacts properly with SpoIIIJ from *B. subtilis*. However, the situation is not paralleled for SpoIIIJ: SpoIIIJ from *B. cereus* does not fully complement a *spoIIIJ* mutant for sporulation, indicating that this gene should be required for still more function(s) other than regarding SpoIIIAE during sporulation, also suggested by others (Camp and Losick, 2008; Serrano *et al.*, 2008).

A case sharing many characteristics with ours is that of FtsL from *B. subtilis*, for which no essential amino acid residues were found as well: proteins subjected to site-directed mutagenesis of conserved residues were still able to complement a null mutation, as we observed for SpoIIIJ also, and random mutagenesis did not produce single amino acid substitutions provoking the desired phenotype, similar again to our case; an exception was the occurrence of a spontaneous PCR-related base substitution which resulted in the conversion of a leucine into a proline residue, likely abolishing function by altering the structure of the protein (Sievers and Errington, 2000). However, and dissimilar to what was observed for SpoIIIJ, the expression of heterologous *ftsL* genes with sequence identity values as low as 34 % were able to complement a null mutant (Sievers and Errington, 2000), but the reason for this discrepancy is not well understood.

Using chimerical proteins of SpoIIIJ and YqjG we broadly defined three functional regions in SpoIIIJ, the most important being the second quarter of SpoIIIJ, according to their performance in supporting sporulation. We also showed that the C-terminus of SpoIIIJ from the amino acid residue 243 on is dispensable for sporulation (Table 3). This was assessed with truncated proteins engineered based on data obtained from random mutagenesis (Fig. 6). Interestingly, the Q254K substitution was only obtained accompanied by other alterations, namely a frameshift from K249 on, or two close-by substitutions. This suggests that although this C-terminal region is dispensable for sporulation, certain residues may negatively affect some processes related to viability, at least in *E. coli*.

More important than the primary sequence itself are the interactions between specific regions, which seem to be the pivotal point for the function of members of the Oxa1-like proteins. The specific sequence of the corresponding regions in Oxa1 and YidC was shown not to be essential for function (TM 3 and 4, and 4 and 5, respectively). However, specific contacts between those regions are required for proper functioning: between TM 4 of Oxa1 and both the first external loop and

TM5 (Mathieu *et al.*, 2010); in YidC, an interaction between TM2 and 3 in YidC is also required for function (Yuan *et al.*, 2007). Interestingly, TM3 of YidC (corresponding to TM2 of SpoIIIJ/YqjG) seems to be quite important as four of the six serine substitutions that impaired function fell on this domain (Jiang *et al.*, 2003). In addition, TM3 interacts with both Sec-dependent and -independent substrates (Yu *et al.*, 2008) and was shown to be the most important TM in substrate binding (Klenner and Kuhn, 2012). This prompts the idea of a general mechanism for substrate recognition, and opens the possibility of specific determinants for the interaction with specific substrates lying outside the generic interaction domain in this family of proteins. Thus, different substrates could interact with different regions of the insertase, also suggested by Yuan *et al*. (2007) and Chen *et al*. (2003). In addition to this work, several reports suggest a second function of SpoIIIJ during sporulation (Camp and Losick, 2008; Serrano *et al.*, 2008). The identification of the second function, of more substrates and the characterisation of their distinct requirements in terms of SpoIIIJ domains and activity, especially of SpoIIIAE, are major goals for future work.

Acknowledgements

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Tables

Table 1. Bacterial strains.

Strain	Relevant Genotype/Phenotype[a, b]	Origin/ Reference
B. subtilis		
MB24	*trpC2 metC3*	Laboratory stock
JOB44	Δ*spoIIIJ::km* / Km^r	Serrano *et al.*, 2003
JOB9	Δ*yqjG::sp* / Sp^r	Serrano *et al.*, 2008
JOB20	Δ*spoIIIJ::sp* / Sp^r	Serrano *et al.*, 2003
AH1042	Δ*sspE::sspE-lacZ* / Erm^r	Laboratory stock
AH2468	Δ*spoIIIAE*	Serrano *et al.*, 2008
AH5009	Δ*spoIIIJ::km* Δ*amyE::spoIIIG-lacZ* / Km^r Cm^r	Serrano *et al.*, 2003
AH5011	Δ*spoIIIJ::km* Δ*amyE::spoIIIJ* / Km^r Cm^r	Serrano *et al.*, 2003
AH5045	Δ*spoIIIJ::km* Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ-yqjG-13* (chimera A) / Km^r Cm^r	This work
AH5046	Δ*spoIIIJ::km* Δ*amyE*::$P_{spoIIIJ}$-*yqjG-spoIIIJ-14* (chimera C) / Km^r Cm^r	«
AH5047	Δ*spoIIIJ::km* Δ*amyE*:: $P_{spoIIIJ}$-*spoIIIJ-yqjG-15* (chimera B) / Km^r Cm^r	«
AH5048	Δ*spoIIIJ::km* Δ*amyE*:: $P_{spoIIIJ}$-*yqjG-spoIIIJ-16* (chimera D) / Km^r Cm^r	«
AH5057	Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ-yqjG-13* / Cm^r	«
AH5058	Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ-yqjG-14* / Cm^r	«
AH5059	Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ-yqjG-15* / Cm^r	«
AH5060	Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ-yqjG-16* / Cm^r	«
AH5062	Δ*yqjG::sp* Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ-yqjG-13* / Cm^r Sp^r	«
AH5063	Δ*yqjG::sp* Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ-yqjG-14* / Cm^r Sp^r	«
AH5064	Δ*yqjG::sp* Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ-yqjG-15* / Cm^r Sp^r	«
AH5065	Δ*yqjG::sp* Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ-yqjG-16* / Cm^r Sp^r	«
AH5120	Δ*spoIIIJ::km* Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ*(Ba) / Km^r Cm^r	«
AH5121	Δ*spoIIIJ::km* Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ*(Bc) / Km^r Cm^r	«
AH5125	Δ*spoIIIJ::km* Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ*(Bh) / Km^r Cm^r	«
AH5126	Δ*spoIIIJ::km* Δ*amyE*::$P_{spoIIIJ}$-*yqjG*(Bh) / Km^r Cm^r	«
AH5142	Δ*spoIIIJ::km* Δ*amyE::spoIIIJ*(D33K) / Km^r Cm^r	«
AH5143	Δ*spoIIIJ::km* Δ*amyE::spoIIIJ*(V53F) / Km^r Cm^r	«
AH5144	Δ*spoIIIJ::km* Δ*amyE::spoIIIJ*(S64A) / Km^r Cm^r	«
AH5145	Δ*spoIIIJ::km* Δ*amyE::spoIIIJ*(Q254K) [c] / Km^r Cm^r	«
AH5151	Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ*(Ba) / Cm^r	«
AH5152	Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ*(Bc) / Cm^r	«
AH5153	Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ*(Bh) / Cm^r	«
AH5154	Δ*amyE*::$P_{spoIIIJ}$-*yqjG*(Bh) / Cm^r	«
AH5155	Δ*spoIIIJ::sp* Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ*(Ba) / Cm^r Sp^r	«
AH5156	Δ*spoIIIJ::sp* Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ*(Bc) / Cm^r Sp^r	«
AH5157	Δ*spoIIIJ::sp* Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ*(Bh) / Cm^r Sp^r	«
AH5158	Δ*spoIIIJ::sp* Δ*amyE*::$P_{spoIIIJ}$-*yqjG*(Bh) / Cm^r Sp^r	«
AH5159	Δ*spoIIIJ::km* Δ*thrC*::$P_{spoIIIJ}$-*spoIIIJ*(Bc) / Km^r Sp^r	«
AH5160	Δ*spoIIIJ::km* Δ*amyE::spoIIIJ*(V53F, S64A) / Km^r Cm^r	«
AH5161	Δ*spoIIIJ::km* Δ*amyE*::$P_{spoIIIJ}$-*yqjG*(Bs) / Cm^r Km^r	«
AH5175	Δ*spoIIIJ::km* Δ*amyE::spoIIIJ*-K243stop / Km^r Cm^r	«
AH5176	Δ*spoIIIJ::km* Δ*amyE::spoIIIJ*-N250stop / Km^r Cm^r	«
AH5183	Δ*spoIIIJ::km* Δ*amyE::spoIIIJ*(Q238A) / Km^r Cm^r	«
AH5208	Δ*spoIIIAE* Δ*amyE::* $P_{spoIIIA}$-*spoIIIAE*(Bc) / Cm^r	«
AH5213	Δ*spoIIIAE* Δ*amyE::* $P_{spoIIIA}$-*spoIIIAE*(Bc) Δ*spoIIIJ::km* Δ*thrC*::$P_{spoIIIJ}$-*spoIIIJ*(Bc) / Cm^r Km^r Sp^r	«

AH5219	Δ*spoIIIJ::km* Δ*spoIIIAE* Δ*amyE::* $P_{spoIIIA}$-*spoIIIAE*(Bc) Δ*sspE::sspE-lacZ* / Cm^r Km^r Sp^r	«
AH5232	Δ*spoIIIJ::km* Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ-yqjG-13-his* / Km^r Cm^r Sp^r	«
AH5233	Δ*spoIIIJ::km* Δ*amyE*::$P_{spoIIIJ}$-*yqjG-spoIIIJ-14-his* / Km^r Cm^r Sp^r	«
AH5234	Δ*spoIIIJ::km* Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ-yqjG-15-his* / Km^r Cm^r Sp^r	«
AH5235	Δ*spoIIIJ::km* Δ*amyE*::$P_{spoIIIJ}$-*yqjG-spoIIIJ-16-his* / Km^r Cm^r Sp^r	«
AH5290	Δ*spoIIIJ::km* Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ*(Bl) / Km^r Cm^r	«
AH5291	Δ*spoIIIJ::km* Δ*amyE*::$P_{spoIIIJ}$-*yqjG*(Bl) / Km^r Cm^r	«
AH5379	Δ*spoIIIJ::km* Δ*amyE::* P_{spac}-*spoIIIJ* / Km^r Erm^r	«
AH5380	Δ*spoIIIJ::km* Δ*amyE*::P_{spac}-*spoIIIJ* Δ*yqjG::sp* / Km^r Erm^r Sp^r	«
AH5433	Δ*spoIIIAE* Δ*amyE::spoIIIAE* / Cm^r	«
AH5432	Δ*spoIIIJ* Δ*yqjG*::P_{xylA}-*yqjG* / Km^r Sp^r	«
AH9218	Δ*spoIIIJ::spoIIIJ-his* / Sp^r	Chapter IV
B. licheniformis ATCC14580		D. Ziegler[d]
B. anthracis "Ames ancestor"		R. Zilhão[e]
B. cereus ATCC10987		R. Zilhão[e]
B. halodurans C-125		T. Sato[f]

[a] All *B. subtilis* strains are *trpC2 metC3*. Km, kanamycin; Cm, cloramphenicol; Erm, erythromycin; Sp, spectinomycin; Neo, neomycin.
[b] Ba, *Bacillus anthracis*; Bs, *B. subtilis*; Bc, *B. cereus*; Bh, *B. halodurans*; Bl, *B. licheniformis*.
[c] Containing two extra mutations (see text).
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[f] Takako Sato, Bio-Nano Electronics Research Centre, Toyo University, Kawagoe, Saitama, Japan.

Table 2. Oligonucleotides used in this study.

Primer	Sequence (5´to 3´)[a]
PIIIJD	GTTGAATTCCGCCAGTTTGTCTTATATACGC
J200R	TTCCTCCTATAATTAATCTTTACACTC
J380R	GTTATCTCCCGTCAATTTCGCTAC
J588R	TGGATTGACACCATGCTTTTGG
J34D	ATAAGAATTCTTCGTCAAAGTAGGC
spoIIIJ-yqjG-Pn	CCAAAAGCATGGTGTCAATCCA*CTTGCGATGGGCTGTCTTCC*
yqjG-spoIIIJ-Pn	GGAGCATAATATCAACCCG*TTGGCGGGATGTTTCCCGATTTTG*
spoIIIJ-yqjG-Y	GCGAAATTGACGGGAGATAAC*TACGGACTCTCTATTATCC*
yqjG-spoIIIJ-Y	GCTGTTTCACGGAGAA*TACGGGCTTTCAATTATTC*
J-Y-prom	GATTAATTATAGGAGGGA*TTGTTAAAAACATATCAAAAAC*
yqjG586R	TTCTCCGTGAAACAGCCCGGC
yqjG811R	CGGGTTGATATTATGCTCCTG
J1039R	CAATCCGGATCCTGTACTGCTTCATCGACATTTCGCCC
yqjG1256R	CCCCGGATCCTTTTTGCACGGGGTTGC
IIIJD33K-D	AAAGAGCCGATCACTGCA**AAA**AGTCCGCATTTCTGGGAC
IIIJD33K-R	GTCCCAGAAATGCGGACT**TTT**TGCAGTGATCGGCTCTTT
IIIJV53F-D	CATTGTCTGAACTCATTACGTAT**TTT**GCGAAATTGACGGGAGATAAC
IIIJV53F-R	GTTATCTCCCGTCAATTTCGC**AAA**ATACGTAATGAGTTCAGACAATG
IIIJS64A-D	GGGAGATAACTACGGGCTT**GCG**ATTATTCTAGTTACCATTTTAATTC
IIIJS64A-R	GAATTAAAATGGTAACTAGAATAAT**CGC**AAGCCCGTAGTTATCTCCC
IIIJQ254K-D	GATATTAAAAAAAATCCTGAGCCG**AAG**AAAGCCGGAGGAAAGAAAAAG
IIIJQ254K-R	CTTTTTCTTTCCTCCGGCTTT**CTT**CGGCTCAGGATTTTTTTTAATATC
IIIJQ238A-D	GGTAACTTGTTTATGATTGCG**GCA**ACTTTCCTCATTAAAGGAC
IIIJQ238A-R	GTCCTTTAATGAGGAAAGT**TGC**CGCAATCATAAACAAGTTACC
J960stop250R	GCGGATCCTGGAT**TCA**TTTTTTAATATC
J936stop243R	CAGGATCCT**TCA**AATGAGGAAAGTTTGC
PBsBa	GATTAATTATAGGAGGAA*TTGAAAAAGAAATTAGGTTTAC*
JBa1149R	CTCGGATCCTTTGAAACATTTAGTTGC

JBc1161R	CTACTGGATCCTTTGATGTATTTAATTGCC
PBsJBh	GATTAATTATAGGAGGAA*GTGTATAGAAAGTTTGGAATGG*
JBh1186R	CTTTTCGGATCCTCCACGACTGTATAC
PBsYBh	GATTAATTATAGGAGGAA*TTGAATTATATGAAACGTCG*
YBh1281R	CAGCTCGGATCCTTTCTTTGTTTCTC
PBsJBlich	GATTAATTATAGGAGGAA*TTGTTGAAGAGGCGAATTTTATTATTG*
JBlich1443R	GCAGGATCCGATGACCGAAAATGCCG
PBsYBlich	GATTAATTATAGGAGGAA*GTGTTGAACACGAAACTAAAGC*
YBlich1436R	GTTGCGGATCCTTTCAGCTCTTAGAGC
J293D	GAACCATATGTATAGAAAGTTTGGAATGG
Jhis	CCGCTCGAGTCAGTGGTGGTGGTGGTGGTGCTTTTTCTTTCCTCCCACC
G295D	GGACATATGAATTATATGAAACGTCG
Ghis	CCGCTCGAGTCAGTGGTGGTGGTGGTGGTGAGCTTTGTTAGGTTTCGATG
IIIA5383D	CATTCAAGCTTGAGCCTCCTCCTTTCTACCG
IIIA5658RBam	CAATTGTCATGCTTGGATCCAACGATGTC

[a] Restriction sites are underlined, mutations in bold, second gene in italics; italics and underline indicate regions common to *spoIIIJ* and *yqjG*.

Table 3. Heat resistance of various strains.

Strain	Relevant Genotype[a]	Viable cell count[b]	Heat[R] cell count[b]	Spo%[b]
MB24	wild-type	$2.7x10^8$	$1.9x10^8$	70.4
JOB44	Δ*spoIIIJ::km*	$7.2x10^7$	0	0
JOB9	Δ*yqjG::sp*	$6.6x10^8$	$4.7x10^8$	71.2
JOB20	Δ*spoIIIJ::sp*	$1.1x10^8$	$7.0x10^1$	<0.0001
AH5011	Δ*spoIIIJ::km* Δ*amyE::spoIIIJ*	$2.9x10^8$	$2.6x10^8$	89.7
AH5045	Δ*spoIIIJ::km* Δ*amyE::spoIIIJ-yqjG-13* (A)	$1.9x10^8$	$8.6x10^3$	0.004
AH5046	Δ*spoIIIJ::km* Δ*amyE::yqjG-spoIIIJ-14* (C)	$1.7x10^8$	$4.3x10^6$	2.61
AH5047	Δ*spoIIIJ::km* Δ*amyE::spoIIIJ-yqjG-15* (B)	$2.2x10^8$	$3.6x10^7$	16.6
AH5048	Δ*spoIIIJ::km* Δ*amyE::yqjG-spoIIIJ-16* (D)	$1.9x10^8$	$1.4x10^3$	0.0007
AH5120	Δ*spoIIIJ::km* Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ*(Ba)	$9.8x10^7$	$6.8x10^5$	0.69
AH5121	Δ*spoIIIJ::km* Δ*amyE*::$P_{spoIII-J}$*spoIIIJ*(Bc)	$5.3x10^7$	$9.3x10^4$	0.18
AH5125	Δ*spoIIIJ::km* Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ*(Bh)	$1.0x10^8$	$1.9x10^5$	0.09
AH5126	Δ*spoIIIJ::km* Δ*amyE*::$P_{spoIIIJ}$-*yqjG*(Bh)	$2.4x10^8$	$1.1x10^8$	45.8
AH5142	Δ*spoIIIJ::km* Δ*amyE::spoIIIJ*(D33K)	$3.2x10^8$	$2.8x10^8$	86.3
AH5143	Δ*spoIIIJ::km* Δ*amyE::spoIIIJ*(V53F)	$2.1x10^8$	$1.9x10^8$	88.2
AH5144	Δ*spoIIIJ::km* Δ*amyE::spoIIIJ*(S64A)	$3.3x10^8$	$3.3x10^8$	100
AH5145	Δ*spoIIIJ::km* Δ*amyE::spoIIIJ*(Q524K) [c]	$2.3x10^8$	$1.5x10^8$	75.8
AH5151	Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ*(Ba)	$3.8x10^8$	$2.9x10^8$	76.3
AH5152	Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ*(Bc)	$5.4x10^8$	$2.2x10^8$	40.7
AH5153	Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ*(Bh)	$4.1x10^8$	$2.6x10^8$	63.4
AH5154	Δ*amyE*::$P_{spoIIIJ}$-*yqjG*(Bh)	$3.9x10^8$	$2.1x10^8$	53.8
AH5155	Δ*spoIIIJ::sp* Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ*(Ba)	$1.4x10^8$	$1.8x10^6$	1.29
AH5156	Δ*spoIIIJ::sp* Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ*(Bc)	$9.0x10^7$	$4.6x10^5$	0.51
AH5157	Δ*spoIIIJ::sp* Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ*(Bh)	$1.1x10^8$	$3.3x10^6$	3.67
AH5158	Δ*spoIIIJ::sp* Δ*amyE*::$P_{spoIIIJ}$-*yqjG*(Bh)	$8.5x10^8$	$5.8x10^8$	3.00
AH5159	Δ*spoIIIJ::km* Δ*thrC*::$P_{spoIIIJ}$-*spoIIIJ*(Bc)	$1.0x10^8$	$1.1x10^4$	0.011
AH5160	Δ*spoIIIJ::km* Δ*amyE::spoIIIJ*(V53F S64A)	$1.5x10^8$	$1.3x10^8$	86.0
AH5161	Δ*spoIIIJ::km* Δ*amyE*::$P_{spoIIIJ}$-*yqjG*(Bs)	$8.3x10^7$	0	0
AH5175	Δ*spoIIIJ::km* Δ*amyE::spoIIIJ*(K243stop)	$2.4x10^8$	$1.8x10^8$	75.0
AH5176	Δ*spoIIIJ::km* Δ*amyE::spoIIIJ*(N250stop)	$2.2x10^8$	$1.4x10^8$	63.6
AH5183	Δ*spoIIIJ::km* Δ*amyE::spoIIIJ*(Q238A)	$2.1 x10^8$	$1.5 x10^8$	71.4

AH5208	Δ*spoIIIAE* Δ*amyE::* $P_{spoIIIAE(Bs)}$-*spoIIIAE*(Bc)	2.4 x10^8	1.6 x10^8	66.6
AH5219	Δ*spoIIIAE* Δ*amyE*::$P_{spoIIIAE(Bs)}$-*spoIIIAE*(Bc) Δ*spoIIIJ::km* Δ*thrC*::$P_{spoIIIJ(Bs)}$-*spoIIIJ*(Bc)	8.2x10^7	1.9x10^4	0.023
AH5290	Δ*spoIIIJ::km* Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ*(Bl)	1.7x10^8	1.7x10^7	10.0
AH5291	Δ*spoIIIJ::km* Δ*amyE*::$P_{spoIIIJ}$-*yqjG*(Bl)	1.4x10^8	0	0

[a] Ba, *Bacillus anthracis*; Bs, *B. subtilis*; Bc, *B. cereus*; Bh, *B. halodurans*; Bl, *B. licheniformis*.
[b] The titre of viable and heat-resistant colony forming units was measured 24 hours after the onset of sporulation in DSM (see Material and Methods). Spo, sporulation.
[c] Containing two extra mutations (see text).

Table 4. Plasmids used in this study.

Plasmid	Relevant features	Antibiotic resistance[a]	Source
pFV4	*'yqjG-his*	*bla, sp*	Serrano *et al.,* 2008
pFV5	*'spoIIIJ-his*	*bla, sp*	Chapter IV
pJO49	Δ*amyE::spoIIIJ*	*bla, cat*	Serrano *et al.,* 2003
pMS193	Δ*amyE::*$P_{spoIIIJ}$-*yqjG*	*bla, cat*	This work
pMS217	Δ*amyE::*$P_{spoIIIA}$-*spoIIIAE*	*bla, neo*	Serrano *et al*., 2008
pMS266	Overproduction of SpoIIIJ-His_6	*bla*	Serrano *et al*., 2008
pMS267	Overproduction of SpoIIIAE	*bla*	Serrano *et al*., 2008
pMS330	Overproduction of SpoIIIJ(Bh)-His_6	*bla*	This work
pMS331	Overproduction of YqjG(Bh)-His_6	*bla*	«
pDG364	*amyE insertion*	*bla, cat*	Cutting and Vander Horn, 1990
pDG1664	*thrC insertion*	*bla, erm*	Guérout-Fleury *et al*., 199*6*
pDG1731	*thrC insertion*	*bla, sp*	Guérout-Fleury *et al*., 1996)
pMLK83	*amyE insertion*	*bla, neo*	Karow and Piggot, 1995
pLC13	Δ*amyE::chimera A*	*bla, cat*	This work
pLC14	Δ*amyE::chimera C*	*bla, cat*	«
pLC15	Δ*amyE::chimera B*	*bla, cat*	«
pLC16	Δ*amyE::chimera D*	*bla, cat*	«
pLC23	Δ*amyE::spoIIIAE, promoterless*	*bla, cat*	«
pLC24	Δ*amyE::spoIIIAE*	*bla, cat*	«
pLC28	Δ*amyE*::P_{spac}-*spoIIIJ+lacI*	*bla, cat*	Chapter IV
pLC39	Δ*amyE::spoIIIJ*(Ba)	*bla, cat*	This work
pLC40	Δ*amyE::spoIIIJ*(Bc)	*bla, cat*	«
pLC41	Δ*amyE::spoIIIJ*(Bh)	*bla, cat*	«
pLC42	Δ*amyE::yqjG*(Bh)	*bla, cat*	«
pLC51	*spoIIIAE*(Bc)	*bla*	«
pLC53	Δ*amyE::spoIIIJ*(D33K)	*bla, cat*	«
pLC54	Δ*amyE::spoIIIJ*(V53F)	*bla, cat*	«
pLC55	Δ*amyE::spoIIIJ*(S64A)	*bla, cat*	«
pLC56	Δ*amyE::spoIIIJ*(Q254K)[b]	*bla, cat*	«
pLC60	Δ*amyE::spoIIIJ*(V53F S64A)	*bla, cat*	«
pLC61	Δ*thrC::spoIIIJ*(Bc)	*bla, sp*	«
pLC64	Δ*amyE*::$P_{spoIIIJ}$*yqjG*(Bs)	*bla, cat*	«
pLC66	Δ*amyE::spoIIIJ*(K243stop)	*bla, cat*	«
pLC67	Δ*amyE::spoIIIJ*(N250stop)	*bla, cat*	«
pLC70	Δ*amyE::spoIIIJ*(Q238A)	*bla, cat*	«
pLC83	Δ*amyE*::$P_{spoIIIA}$-*spoIIIAE*(Bc)	*bla*	«

pLC101	Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ*(Bl)	*bla, cat*	«
pLC102	Δ*amyE*::$P_{spoIIIJ}$-*yqjG*(Bl)	*bla, cat*	«
pLC111	Δ*amyE::his*	*bla, cat*	Chapter IV
pLC151	Δ*amyE*:: P_{spac}-*spoIIIJ, lacI*	*bla, erm*	This work

[a] *bla*, ampicillin; *cat*, cloramphenicol; *erm*, erythromycin; *sp*, spectinomycin; *neo*, neomycin.
[b] Containing two extra mutations.

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Chapter III

Suppression of the developmental defect of a *spoIIIJ* null mutant

All experiments were performed by the author of this Thesis except for the construction of AH5434, pTC1-3 and AH1901-3.

Abstract

A conserved trait of *Bacillus* species is the presence of two members of the YidC/Oxa1/Alb3 family of membrane protein insertases, YqjG and SpoIIIJ. The presence of at least one of these proteins is required for growth and viability but surprisingly, given the well-documented ability of even trans-domain complementation observed between proteins of this family, only SpoIIIJ supports sporulation. Both SpoIIIJ and YqjG have cleavable signal peptides, but here we show that YqjG undergoes at least a second cleavage event downstream of the signal peptide cleavage site. This cleavage takes place after the onset of stationary phase, and was estimated to occur between amino acids 39 and 49 of YqjG, based on the combined information obtained from distinct data sets: chimeras of SpoIIIJ and YqjG, AMS labelling, and deletion of a YqjG region combined with *in silico* analyses. Fusion of GFP to YqjG reduces the extent of cleavage, and increases the efficiency of sporulation, which could be completely restored to the YqjG-GFP producing strain at 30ºC, and at 37ºC in the presence of the A238S or G247V substitutions. These results suggest the existence in *B. subtilis* of a protease capable of degrading YqjG. Our results further suggest that the inability of YqjG to substitute for SpoIIIJ during sporulation is in great measure due to its proteolytic inactivation after the onset of stationary phase, more than because of any other sequence-specific differences between the two proteins.

Introduction

SpoIIIJ and YqjG are members of the YidC/Oxa1/Alb3 in *Bacillus subtilis* that are involved in membrane protein biogenesis and in protein secretion (Tjalsma *et al.*, 2003; Saller *et al.*, 2009). The members of this family of protein insertases share a conserved topology of five transmembrane segments (Yen *et al.*, 2001; Saller *et al.*, 2012). Proteins of this family are involved in various processes such as insertion and folding of membrane proteins, and in the assembly of multisubunit protein complexes, in all domains of life (Wang and Dalbey, 2011; Saller *et al.*, 2012). Other components that participate in the translocation and/or membrane insertion of proteins include the Sec and Tat pathways, both present in bacteria, archaea and eukaryotes (Natale *et al.*, 2008).

B. subtilis is capable to withstand extremely harsh environmental challenges if in the form of a dormant spore which germinates upon perceiving auspicious external conditions (Hilbert and Piggot, 2004; Setlow, 2006). The developmental process of spore formation requires communication between the two distinct cellular compartments generated by the formation of an asymmetric septum, the smaller prespore, which will become the mature spore, and the mother cell, responsible for its nurturing but ultimately lysing and releasing a fully developed spore (Hilbert and Piggot, 2004). This intercompartmental communication is accomplished by coupling distinct programmes of gene expression to morphological events. Asymmetric cell division is coupled to the activation of two of the four sigma factors of the RNA polymerase here involved (σ^F and σ^E, in the prespore and mother cell, respectively), whilst prespore engulfment completion is linked to the activation of σ^G and σ^K (in the prespore and mother cell, respectively) (Hilbert and Piggot, 2004).

SpoIIIJ is required for σ^G activity as *spoIIIJ* mutants exhibit transcription of *spoIIIG* (encoding σ^G) but not σ^G-dependent activity. It is transcriptionally linked to *jag* (SpoIIIJ-associated gene), which has no obvious function (Errington *et al.*, 1992). Despite SpoIIIJ and YqjG both being transcribed vegetatively, SpoIIIJ can also support efficient sporulation if produced

solely in the prespore, but not vegetatively-produced YqjG (Errington *et al.*, 1992; Murakami *et al.*, 2002; Serrano *et al.*, 2003; Tjalsma *et al.*, 2003). Either YqjG or SpoIIIJ must be present to sustain viability, thus partially superposing functionally (Murakami *et al.*, 2002; Tjalsma *et al.*, 2003). The latest reports point towards the existence of a channel that maintains prespore physiology thereby allowing σ^G activity. This channel is composed of the prespore-bourne SpoIIQ and the SpoIIIA proteins from the mother cell, with the biogenesis of one of them being supported by SpoIIIJ (Fig. 1) (Camp and Losick, 2008; Serrano *et al.*, 2008; Camp and Losick, 2009; Doan *et al.*, 2009 and references therein).

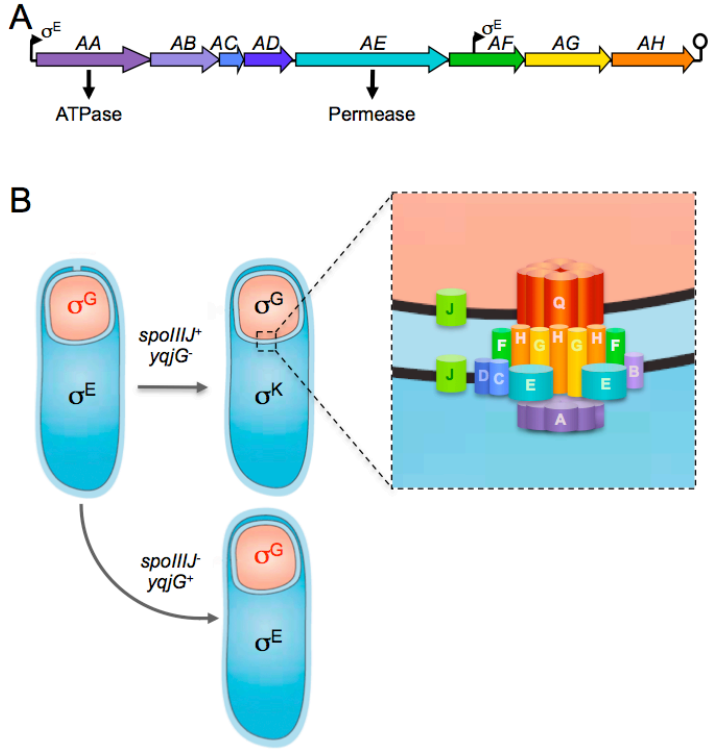

Figure 1. σ^G activity requires the action of SpoIIIJ in cooperation with SpoIIIA but not of YqjG. (A) Genetic organisation of the *spoIIIA* operon of *B. subtilis*. Two σ^E promoters (arrows) and a transcriptional terminator (stem-loop structure) are indicated, in addition to the predicted functions of two proteins, SpoIIIAA and SpoIIIAE. **(B)** SpoIIIJ is required for the biogenesis of SpoIIIAE, which is part of the SpoIIIA-SpoIIQ channel that acts a feeding tube, nurturing the prespore and allowing σ^G activity. The actual stoichiometry of proteins in the complex in unknown. SpoIIQ (Q), SpoIIIAA-H (A-H) and SpoIIIJ (J) are shown. Adapted from Errington, 2003, Guillot *et al*., 2007, and Doan *et al*., 2009.

Both SpoIIIJ and YqjG harbour signal peptides that are cleaved by signal peptidases, producing mature proteins (see Chapter IV). Signal peptidases are an example of a broader group of proteases that recognise specific sequences and process them therein; most proteases are relatively nonspecific (e.g. proteinase K); others recognise defined sequences but the precise sequence of residues where cleavage occurs is located elsewhere, and may not be critical (Keiler and Sauer, 1996; López-Otín and Bond, 2008; Rawson, 2008 and references therein). In general terms, proteolysis is an absolutely essential mechanism in all living organisms, controlling multiple biological processes (López-Otín and Bond, 2008).

Here we document a secondary cleavage event suffered solely by YqjG, one of the two members of the YidC/Oxa1/Alb3 family in *B. subtilis*, which may be responsible for the diminished functionality of this protein during sporulation relative to the absolutely required SpoIIIJ. In support of this vision are the increased sporulation titres supported by YqjG when stabilised by GFP, together with the effect of two separate amino acid substitutions that improve interaction with the acknowledged SpoIIIJ substrate, SpoIIIAE.

Material and Methods

Media, bacterial strains and general techniques. The *B. subtilis* strains used in this work (listed in Table 1) are congenic derivatives of the Spo^+ strain MB24 (*trpC2 metC3*). Luria-Bertani (LB) medium was used for growth or maintenance of *E. coli* and *B. subtilis*, and sporulation was induced in Difco sporulation medium (DSM) (Henriques *et al.*, 1995; Henriques *et al.*, 1998).

Construction of His_6 fusions. Basic molecular cloning methods were employed to construct fusions of several genes to a His-tag. To produce *yqjG* with an internal deletion, primers pairs PYqjG-460D and yqjGdel_R, and yqjGdel_D and YqjG_His_R were used to amplify from MB24 (wild-

type) chromosomal DNA two *yqjG* regions that were linked by SOE (splicing by overlap extension) (Horton *et al.*, 1989) using the external primers in a subsequent PCR amplification. Both the final PCR product and pLC111 (see Chapter IV) were *Bam*HI-digested and ligated, producing pLC122. Proper orientation of the fragment was tested with restriction enzymes. Transformation of JOB44 (Δ*spoIIIJ*::*km*, a non-polar mutant; Serrano *et al.*, 2003), with pLC122 after linearisation with *Sca*I resulted in AH5333 (Δ*amyE*::*yqjG-del-his* Δ*spoIIIJ*::*km*). To construct cysteine derivatives of *yqjG*, the procedure mentioned above was followed using primer pairs PYqjG-460D with yqjGA50C_R or yqjGH60C_R, and yqjGA50C_D or yqjGH60C_D with YqjG_His_R, yielding pLC138 and pLC139, respectively. These plasmids were *Sca*I-linearised and inserted into JOB44 producing strains AH5358 (Δ*amyE*::*yqjG*(A50C)-*his* Δ*spoIIIJ*::*km*) and AH5359 (Δ*amyE*::*yqjG*(H60C)-*his* Δ*spoIIIJ*::*km*), respectively. pMS254 (Serrano *et al.*, 2008) was inserted into MB24 selecting for cloramphenicol, producing AH5244 (Δ*yqjG*::*yqjG-his*); chromosomal DNA from this strain was used to transform JOB20 (Δ*spoIIIJ*::*sp*; Serrano *et al.*, 2003), yielding AH5246 (Δ*yqjG*::*yqjG-his* Δ*spoIIIJ*::*sp*). To produce AH9203 (Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjG-his*) chromosomal DNA from AH9246 (see Chapter IV) was used to transform JOB44.

Construction of GFP fusions. A *spoIIIJ-gfp* fusion at its locus was accomplished by introducing pLC3 (Serrano *et al.*, 2003) into MB24 through a Campbell-type recombination event, producing AH5000; correct insertion was confirmed by PCR analysis. To construct a *yqjG-gfp* fusion, *gfpmut2* and a C-terminal fragment of *yqjG* were amplified by PCR with primer pairs gfp30D/gfpR and yqjG692D/yqjGgfp using pEA18 (Cormack *et al.*, 1996) and MB24 chromosomal DNA as templates, and joined by SOE (see above) with the external primers yqjG692D and gfpR. '*yqjG-gfp* was cloned into pCR2.1 from the TOPO cloning kit (Invitrogen) as described by the manufacturer, yielding pLC8. The desired orientation was chosen and subsequently a spectinomycin cassette obtained from

pAH256 (Henriques *et al.*, 1997) that was recovered with *Eco*RV and *Xho*I was introduced in pLC8 similarly digested, producing pLC9. Transformation of MB24 with pLC9 yielded AH5006, which was confirmed by PCR analysis. Chromosomal DNA of this strain was used to transform JOB44 (Δ*spoIIIJ*::*km*), producing AH5007 (Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjG-gfp*). AH5284 resulted from the transformation of AH5007 with chromosomal DNA from Δ*spo0A*::*erm* (AH3531, a gift from Gonçalo Real). pLC97 was constructed by removing '*yqjG-gfp* from pLC8 and cloning into pMS38 (Zilhão *et al.*, 2004), both digested with *Eco*RV and *Xho*I. Transformation of MB24 with pLC97 produced AH5267, and its chromosomal DNA was used to transform JOB44 and JOB20, yielding AH5268 (Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjG-gfp*) and AH5269 (Δ*spoIIIJ*::*sp* Δ*yqjG*::*yqjG-gfp*), respectively. Plasmids expressing '*yqjG-gfp* harbouring the A238S or G247V substitutions were obtained by amplifying a similar region of *yqjG* fused to gfp from pLC97 whilst inserting the mutations corresponding to those substitutions. For each mutation, two PCR products were generated and subsequently ligated by SOE (see above) using the external primers. The first half of the fusion was obtained with yqjG701D and the reverse primers yqjGA238S_R or yqjGG247V_R; the second with yqjGA238S_D or yqjGG247V_D and gfpBglII_R. Both '*yqjG*(A238S)-*gfp* and '*yqjG*(G247V)-*gfp* were digested with *Bgl*II, and pMS38 with *Bam*HI and *Eco*RV and subsequently ligated, to produce pLC166 and pLC167, respectively, which upon linearisation with *Sca*I and transformation of MB24 yielded AH5421 (Δ*yqjG*::*yqjG*(A238S)-*gfp*) and AH5422 (Δ*yqjG*::*yqjG*(G247V)-*gfp*), respectively. Chromosomal DNA from AH5421 and AH5422 was used to transform either JOB44 or JOB20 producing AH5423 and AH5424 (Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjG*(A238S)-*gfp*; Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjG*(G247V)-*gfp*, respectively), AH5429 and AH5430 (Δ*spoIIIJ*::*sp* Δ*yqjG*(A238S)-*gfp*; Δ*spoIIIJ*::*sp* Δ*yqjG*(G247V)-*gfp*, respectively).

Construction of promoter-exchange strains. P_{yqjG}-*spoIIIJ* was constructed as follows: P_{yqjG} and promoterless *spoIIIJ* were PCR-amplified with primer

pairs yqjG-17D and yqjG400R, and with PyqjG-spoIIIJD and spoIIIJ1106R from wild-type chromosomal DNA, respectively, and fused together by SOE using the external primers. This PCR product was ligated to pGEM T-easy (Promega) as described by the manufacturer yielding pLC86, chosen according to the suitable orientation of the fragment. P_{yqjG}-*spoIIIJ* was recovered from pLC86 digested with *Bam*HI and *Hin*cII, being subsequently inserted into pDG364 (Cutting and Vander Horn, 1990) digested with *Hin*dIII, filled-in with the Klenow fragment of DNA polymerase, and digested with *Bam*HI, producing pLC92. Transformation of Δ*spoIIIJ*::*km* with pLC64 (see Chapter II) and pLC92 after linearisation with *Sca*I resulted in AH5161 (Δ*spoIIIJ*::*km* Δ*amyE*::$P_{spoIIIJ}$-*yqjG*) and AH5230 (Δ*spoIIIJ*::*km* Δ*amyE*::P_{yqjG}-*spoIIIJ*), respectively. C-terminal fusion to His_6 was achieved by transformation of AH5161 with pFV5 (see Chapter IV) and of AH5230 with pFV4 (Serrano *et al.*, 2008), resulting in AH5178 (Δ*spoIIIJ*::*km* Δ*amyE*::$P_{spoIIIJ}$-*yqjG-his*) and AH5231 (Δ*spoIIIJ*::*km* Δ*amyE*::P_{yqjG}-*spoIIIJ-his*), respectively. Linkage analysis and PCR testing were used to confirm that the His-tag was introduced at the expected location.

Construction of strains containing *sspE-lacZ* fusions. Several strains containing *sspE-lacZ* fusions were produced by transformation with chromosomal DNA of AH1042 (Table 1) of JOB44, AH5434 (see below), AH5268, AH5311 and AH5312 (see below), producing AH5166 (Δ*spoIIIJ*::*km* Δ*sspE*::*sspE-lacZ*), AH5310 (Δ*lonB*::*sp* Δ*sspE*::*sspE-lacZ*), AH5315 (Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjG-gfp* Δ*sspE*::*sspE-lacZ*), AH5313 (Δ*lonB*::*sp* Δ*spoIIIJ*::*km* Δ*sspE*::*sspE-lacZ*) and AH5314 (Δ*yqjG*::*yqjG-gfp* Δ*lonB*::*sp* Δ*spoIIIJ*::*km* Δ*sspE*::*sspE-lacZ*), respectively. AH5434 (Δ*lonB*::*sp*) was obtained by transformation of MB24 with chromosomal DNA of BSM105 (Serrano *et al.*, 2001). AH5311 and AH5312 were constructed sequentially: the former resulted from transformation of AH5434 with chromosomal DNA of JOB44, and transformation of this strain (AH5311, Δ*lonB*::*sp* Δ*spoIIIJ*::*km*), with chromosomal DNA from AH5267 resulted in AH5312 (Δ*lonB*::*sp* Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjG-gfp*).

β-Galactosidase assays. β-galactosidase activity was determined with the substrate *o*-nitrophenol-β-D-galactopyranoside, and enzyme activity was expressed in Miller units as described previously (Henriques *et al.*, 1995; Serrano *et al.*, 1999).

Fluorescence microscopy. Single colonies of AH5268-9 (Δ*yqjG*::*yqjG-gfp* Δ*spoIIIJ*::*km* and Δ*yqjG*::*yqjG-gfp* Δ*spoIIIJ*::*sp*, respectively), AH5423-4 (Δ*yqjG*::*yqjG*(A238S)-*gfp* Δ*spoIIIJ*::*km* and Δ*yqjG*::*yqjG*(G247V)-*gfp* Δ*spoIIIJ*::*km*, respectively), and AH5439-40 (Δ*yqjG*::*yqjG*(A238S)-*gfp* Δ*spoIIIJ*::*sp* and Δ*yqjG*::*yqjG*(G247V)-*gfp* Δ*spoIIIJ*::*sp*, respectively), were grown in DSM over two days at 37ºC and resuspended in 0.2 ml of phosphate-buffered saline (8 mM sodium phosphate [pH 7.5], 150 mM NaCl). 2 μl of cell suspensions were mounted on agarose pads (1.7 % in H_2O) before microscopic observation. Phase contrast and fluorescence images were acquired with a Leica DMRA2 Microscope equipped with a 63X magnification objective and a CoolSNAP™ HQ Photometrics camera (Roper Scientific). Images were acquired with Leica FW4000 software.

Whole-cell lysates and immunoblot analysis. *B. subtilis* strains were grown in DSM and samples collected one hour (T_{-1}) before the end of the exponential phase of growth, at the end of the growth phase (defined as the onset of sporulation, or T_0), then two and four hours thereafter. Cells were resuspended in 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mM DTT and lysed with a French pressure cell at 19 000 lb/in^2. Proteins (30 μg) were electrophoretically resolved in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) or in 16.5 % tricine polyacrylamide gels (Tricine-SDS-PAGE) (Schägger and von Jagow, 1987) overnight. The resolved proteins were transferred to nitrocellulose membranes which were first incubated for 30 min in phosphate buffered saline (PBS) containing 5 % low-fat milk and then overnight in PBS containing 0.5 % low-fat milk and a either mouse anti-His_6 antibody

(Novagen, Darmstadt, Germany) for the detection of SpoIIIJ-His_6 or YqjG-His_6, or a rabbit anti-GFP antibody (Eurogenetec, Seraing, Belgium). The proteins were visualised with the ECL detection system (Amersham Biosciences) as described by the manufacturer.

AMS assays. Strains were grown in DSM and samples were collected 4 h after the onset of sporulation. Cells were centrifuged and resuspended in 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM PMSF, 1 mM TCEP and lysed with a French pressure cell at 19 000 lb/in^2. 30 μg of total protein were incubated for 1 h at 37°C with 2 mM 4-acetomido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS, Invitrogen), and run overnight in Tricine-SDS-PAGE gels.

Topology prediction programs. The following programs were used to predict the localisation of Cys142 of YqjG (cytoplasmic/exterior/ in the membrane): TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html) and TOP PRED (http://mobyle.pasteur.fr/cgibin/portal.py?form=toppred).

Results

***yqjG* expression is post-transcriptionally regulated.** YqjG and SpoIIIJ are part of a wide family of protein insertases present in all three domains of life (Yen *et al.*, 2001; Saller *et al.*, 2012). SpoIIIJ, but not YqjG, is required for σ^G activity and efficient sporulation (Errington *et al.*, 1992; Murakami *et al.*, 2002; Tjalsma *et al.*, 2003) (Fig. 1A). *spoIIIJ* and *yqjG* are both transcribed during growth, being strongly reduced as the cells reach the stationary phase. However, transcription of *spoIIIJ* was observed to be either similar (Tjalsma *et al.*, 2003) or four- to five-fold higher than that of *yqjG* (Murakami *et al.*, 2002). In terms of accumulation of GFP fusions to either protein we observed an outcome similar to the latter report, since SpoIIIJ-GFP (black arrowhead) accumulates to higher levels relative to YqjG-GFP (grey arrowhead, Fig. 2A–B); this was assessed using a band which is also

present in the wild-type strain (asterisk) as an internal control for accumulation, in cells grown in DSM sampled at the times (in hours) indicated in Fig. 2 relative to the onset of sporulation, T_0. Whole-cell extracts were resolved by SDS-PAGE (see Materials and Methods) and subjected to immunoblot analysis with anti-GFP antibodies. We observed that in the absence of SpoIIIJ, both YqjG-GFP and YqjG-His_6 showed increased accumulation (Fig. 2C-D), suggesting that *yqjG* is induced in the absence of *spoIIIJ* as part of a backup mechanism. Such could take place via regulation at the transcriptional level or subsequently. To investigate this further, we swapped promoters for *yqjG* and *spoIIIJ* and checked the accumulation of each His-tagged protein through immunoblot analysis.

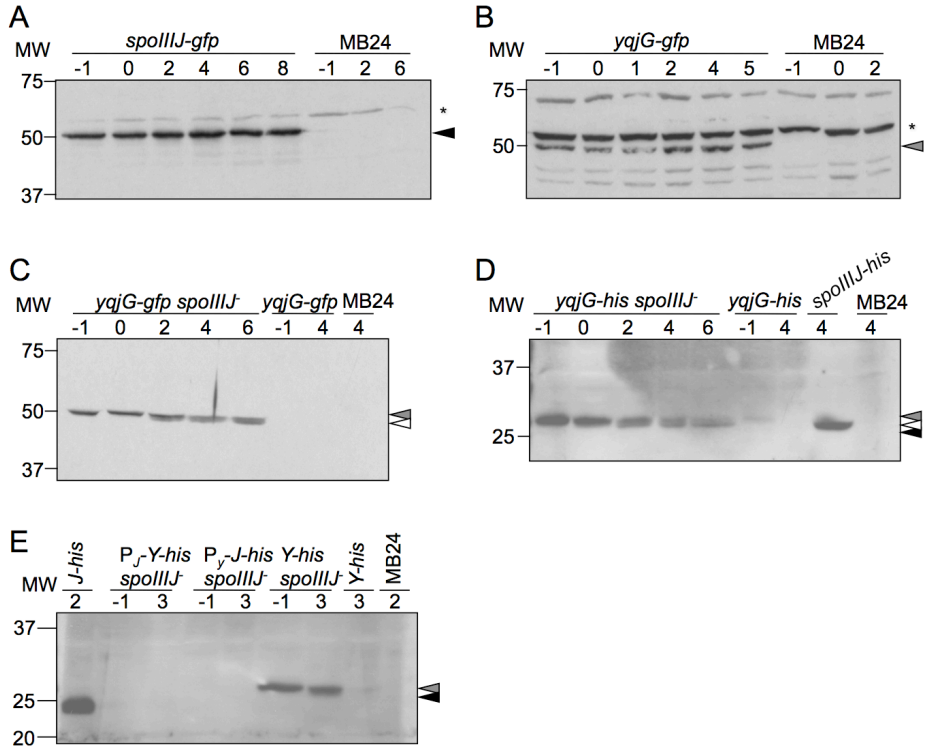

Figure 2. Accumulation of SpoIIIJ and YqjG. (A) Whole-cell extracts of *B. subtilis* DSM cultures (withdrawn at the indicated points after the onset of sporulation) of a strain expressing SpoIIIJ-GFP (AH5000, Δ*spoIIIJ*::*spoIIIJ-gfp*) and of MB24 (wild-type) were electrophoretically resolved by SDS-PAGE and subjected to immunoblot analysis using antibodies against GFP. **(B)** As in (A), for YqjG-GFP (AH5006, Δ*yqjG*::*yqjG-gfp*). **(C)** As in (A), for AH5006 and AH5007 (Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjG-gfp*). **(D)** As in (A), for AH9203 (Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjG-his*), AH9246 (Δ*yqjG*::*yqjG-his*), and AH9218 (Δ*spoIIIJ*::*spoIIIJ-his*) with anti-His_6 antibodies. **(E)** As in (D), for strains producing SpoIIIJ-His_6 or YqjG-His_6

from their native loci (AH9218 and AH9246, respectively), SpoIIIJ-His_6 or YqjG-His_6 from the promoter of the other homologous gene from the non-essential *amyE* locus in a *spoIIIJ*⁻ background (AH5231 and AH5178, respectively), YqjG-His_6 from its locus in a *spoIIIJ*⁻ background (AH9203), and MB24. Black arrowhead: SpoIIIJ-GFP (A) or SpoIIIJ-His_6 (D, E); grey: mature YqjG-GFP (B, C) or YqjG-His_6 (D, E); white: faster-migrating species of YqjG-GFP (C) or YqjG-His_6 (D); asterisk, non-specific cross-reactive species. The position of molecular weight markers (in kDa) is shown.

Unexpectedly, we verified that YqjG-His_6 was no longer visible in a *spoIIIJ*::*km* background when produced from our standard *spoIIIJ* promoter region (AH5178, *amyE*::$P_{spoIIIJ}$-*yqjG-his spoIIIJ*::*km*, Fig. 2E; 421 bp-long region, see Chapter IV), as opposed to when produced from its own promoter at its locus (AH9203, *yqjG*::*yqjG-his spoIIIJ*::*km*, Fig. 2D-E) or from a 749 bp-long promoter region at the non-essential *amyE* locus (AH5324, *amyE*::P_{yqjG}-*yqjG-his spoIIIJ*::*km*, Figs. 5B and 7C). In addition, SpoIIIJ-His_6 was also not visible through immunoblot analysis when produced from a 411 bp-long *yqjG* promoter region (AH5231, *amyE*::P_{yqjG}-*spoIIIJ-his spoIIIJ*::*km*, Fig. 2E), as opposed to when produced from its promoter at its locus (AH9218, *spoIIIJ*::*spoIIIJ-his*, Fig. 2D-E) or from its promoter at the non-essential *amyE* locus (AH5326, see Chapter IV). Given that the promoter region used in AH5178 is our standard $P_{spoIIIJ}$, we expect that the promoter is being recognised; also, AH5231 is viable and produces wild-type levels of heat-resistant spores (Table 3) even though it does not accumulate to detectable levels through immunoblotting. Taken together, these results exclude regulation at the transcriptional level, pointing to a mechanism operating at a later stage of expression.

A YqjG-GFP fusion shows increased sporulation and reduced accumulation of a faster-migrating species. When the ability to form heat-resistant spores of the YqjG-GFP fusion in two *spoIIIJ*⁻ backgrounds was tested (AH5268 and AH5269, *yqjG*::*yqjG-gfp* in *spoIIIJ*::*km* and *spoIIIJ*::*sp* backgrounds, respectively) we verified that it had increased approximately six orders of magnitude relative to the *spoIIIJ*⁻ mutant alone when grown at 37ºC in DSM until 24 h after the onset of sporulation (Table 3). Also, when examining colonies from plates of each strain

incubated over two nights at 37ºC under the microscope we observed high amounts of spores (Table 3; Fig. S1). In an attempt to mimic in liquid medium one of the features of growth on plates, slow growth rate, we grew the same strains at 30ºC in DSM. Wild-type sporulation levels were obtained for AH5269 after two-day growth (Table 3); the same was observed when performing heat tests from plates incubated over two nights at 37ºC, showing that under certain growth conditions YqjG is indeed able to fully support sporulation (Table 3). These observations prompted us to investigate whether the fusion to GFP could be stabilising YqjG. For that, strains AH5246 (*yqjG*::*yqjG-his spoIIIJ*::*sp*) and AH5269 (*yqjG*::*yqjG-gfp spoIIIJ*::*sp*) were grown in DSM at either 30ºC or 37ºC and samples were collected at the times (in hours) indicated in Fig. 3 relative to the onset of sporulation, T_0. Whole-cell extracts were resolved with Tricine-SDS-PAGE (see Materials and Methods) and subjected to immunoblot analysis with either anti-GFP (Fig. 3A) or anti-His_6 antibodies (Fig. 3B).

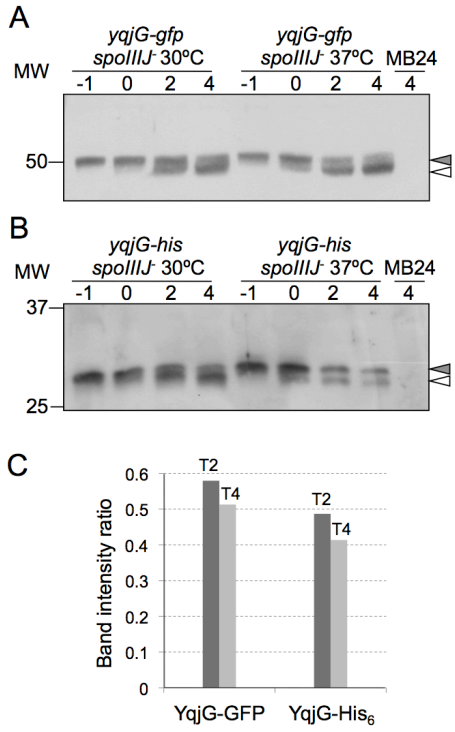

Figure 3. Relative accumulation of mature and faster-migrating bands of YqjG-GFP and YqjG-His_6. (A) Strains AH5269 (Δ*yqjG*::*yqjG-gfp* Δ*spoIIIJ*::*sp*) and MB24 (wild-type) were grown in DSM and samples were collected at the indicated hours relative to the onset of sporulation. The cells were lysed, and the whole-cell extracts were electrophoretically resolved by Tricine SDS-PAGE and subjected to immunoblot analysis using antibodies against GFP. **(B)** As in (A), for AH5246 (Δ*yqjG*::*yqjG-his* Δ*spoIIIJ*::*sp*), and using antibodies against the His_6 tag. **(C)** Ratios of the intensity of the slower-migrating (mature) band relative to the sum of the intensity of the slower- and faster-migrating bands, of AH5269 (YqjG-GFP, left bars) and of AH5246 (YqjG-His_6, right bars) at T_2 (dark grey) and T_4 (light grey) grown at 30ºC. Grey arrowhead, mature band; white arrowhead, faster-migrating band.

We confirmed the existence of a faster-migrating band already observed previously (Fig. 2C-D) for both YqjG-GFP and YqjG-His_6. At 37ºC we observed that the intensity of the upper band (grey arrowhead) persisted to a greater extent at T_2 and T_4 in the strain expressing *yqjG-gfp* when compared to the strain expressing *yqjG-his*, although the absolute levels of YqjG-GFP and of YqjG-His_6 are unknown (Figs. 2-3). Since high sporulation levels were obtained at 30ºC for AH5269 (Table 3), we also compared the accumulation of each fusion protein at 30ºC and 37ºC. The upper band of YqjG-GFP appears to be even more persistent throughout time at 30ºC when compared to the accumulation of the upper band from YqjG-GFP at 37ºC, suggesting an association between high spore titre and amount of mature YqjG-GFP (Fig. 3A, upper band, grey arrowhead). Furthermore, we quantified the intensity of the upper and lower bands of YqjG fused to either His_6 or GFP at 30ºC, both at T_2 and T_4, and calculated the ratio of the intensity of the upper band relative to the sum of the intensity of upper and lower bands, for AH5269 (Fig. 3C, YqjG-GFP, left bars) and for AH5246 (YqjG-His_6, right bars), at T_2 (dark grey bars) and T_4 (light grey bars). In YqjG-GFP, the upper band is present in higher amounts relative to the sum of both bands than in the analogous case for YqjG-His_6, suggesting that mature YqjG has increased stability when fused to GFP at 30ºC, which correlates with higher spore titre.

Either substitution in YqjG-GFP, A238S or G247V, is able to restore sporulation to wild-type levels at 37°C. Camp and Losick (2008) reported the isolation of two YqjG variants that partially bypassed the need for SpoIIIJ, exhibiting higher σ^G activity and sporulation titre by means of improved interaction with SpoIIIJ's putative substrate, SpoIIIAE (Camp and Losick, 2008; Serrano *et al.*, 2008). We hypothesised that YqjG might fulfil SpoIIIJ's function(s) completely during sporulation with the aid of two joined forces, namely an enhanced stability of the mature form of YqjG combined with an improved functional interaction with SpoIIIAE. We thus produced GFP-tagged YqjG versions bearing either substitution,

A238S or G247V, introduced them into *spoIIIJ*::*km* (producing AH5423 and AH5424, respectively) and into *spoIIIJ*::*sp* (producing AH5429 and AH5430, respectively) and verified that they localise similarly to their wild-type counterparts (Fig. S1). We observed an increment in the titre of heat-resistant spores of at least one order of magnitude when compared with the corresponding wild-type counterparts (AH5268 and AH5269), and even wild-type levels were obtained for AH5423 and AH5430 (Table 3). Thus, for the first time, and due to a combination of approaches, YqjG variants were able to fully complement a *spoIIIJ* null mutant for sporulation under standard growth conditions, at 37^{o}C.

YqjG exhibits the second species independently of Spo0A. Whilst analysing the accumulation of YqjG, we verified that a second species was recognised in both anti-GFP and anti-His_6 immunoblots after vegetative growth that is not present in the wild-type strain (Fig. 2C-D, respectively, white arrowhead; AH5007 (*yqjG*::*yqjG-gfp spoIIIJ*::*km*), AH9203 (*yqjG*::*yqjG-his spoIIIJ*::*km*)). This was observed in DSM but not in LB medium (Fig. 4A; white arrowhead: faster-migrating species, in AH9203, at the indicated times relative to T_0) suggesting that it is a sporulation-dependent event. However, that should not be the case since in a *spo0A*$^{-}$ background there is still accumulation of a second band of YqjG-GFP (Fig. 4B, AH5284 (*yqjG*::*yqjG-gfp spoIIIJ*::*km spo0A*::*erm*) at the times depicted relative to T_0; white arrowhead). Proteolysis seems a more attractive candidate to explain this phenomenon than alternative translational start (see Discussion and Fig. 8D).

The LonB protease is not involved in enhanced sporulation via YqjG-GFP. As substantiated above, a presumed inactivation of YqjG might be linked to its proteolysis. We examined the contribution of the LonB protease and verified that the sporulation levels of a strain expressing *yqjG-gfp* in a *spoIIIJ*$^{-}$ background were not enhanced in the absence of *lonB* (Table 3, compare AH5314 (*yqjG*::*yqjG-gfp lonB*::*sp spoIIIJ*::*km sspE-lacZ*)

and AH5315 (*yqjG*::*yqjG-gfp spoIIIJ*::*km sspE-lacZ*)). Yet, we considered that σ^G could be still more active in AH5314 relative to AH5315 even if more heat-resistant spores were not being formed and decided to monitor ß-galactosidase activity throughout time using a σ^G-dependent *sspE-lacZ* reporter fusion. The *lonB* mutant bearing the *sspE-lacZ* fusion behaves similarly to the corresponding wild-type strain although the increase in ß-galactosidase activity occurs at a slightly lower rate (Fig. 5A, compare AH5310 (*lonB*::*sp sspE-lacZ*, open circles) with AH1042 (*sspE-lacZ*, open diamonds)). The levels of ß-galactosidase activity were low for the remaining strains and, despite a slight increase observed upon removal of *lonB* (compare AH5314 (closed squares) and AH5315 (closed diamonds) in the inset of Fig. 5A), the overall results suggest that LonB is not involved in the turnover of YqjG-GFP.

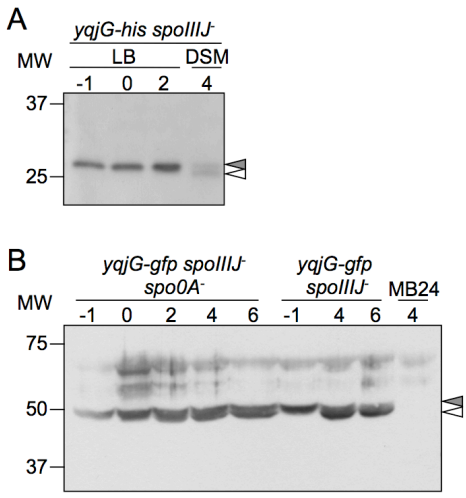

Figure 4. YqjG suffers the cleavage event post-exponentially and independently of Spo0A. (A) AH9203 (Δ*yqjG*::*yqjG-his* Δ*spoIIIJ*::*km*) was grown in either LB or DSM and samples were collected at the indicated hours relative to the end of exponential grown (LB) or to the onset of sporulation (DSM). The cells were lysed, and the whole-cell extracts were electrophoretically resolved by SDS-PAGE and subjected to immunoblot analysis using antibodies against the His_6 tag. **(B)** As in (A) but grown in DSM only and Tricine-SDS-PAGE was employed, before using antibodies against GFP, for AH5284 (Δ*yqjG*::*yqjG-gfp* Δ*spoIIIJ*::*km* Δ*spo0A*::*erm*), AH5007 (Δ*yqjG*::*yqjG-gfp* Δ*spoIIIJ*::*km*) and MB24 (wild-type). Grey arrowhead: mature YqjG-His_6 (A) or YqjG-GFP (B); white: faster-migrating band. The position of molecular weight markers (in kDa) is shown.

The YmfG, YyxA and AprX proteases are not involved in the processing of YqjG-His_6. In an attempt to inhibit cleavage and possibly obtain a non-cleaved and fully functional version of unmodified YqjG in spore formation, several proteases were selected and tested by immunoblotting for the absence of the faster-migrating species of YqjG-His_6. We introduced each of the *ymfG*, *yyxA* or *aprX* mutations into a strain

expressing *yqjG-his* in a *spoIIIJ*::*km* background producing strains AH5376, AH5377 and AH5378, respectively. These strains and the controls AH5324 (*amyE*::*yqjG-his spoIIIJ*::*km*) and AH5325 (*amyE*::*yqjG*(A26K)-*his spoIIIJ*::*km*) were grown in DSM at 37ºC and samples were collected at the times (in hours) indicated in Fig. 5B relative to the onset of sporulation, T_0. Whole-cell extracts were resolved by Tricine-SDS-PAGE and subjected to immunoblot analysis with anti-His_6 antibodies. None of the proteases tested (YmfG, YyxA and AprX) showed a significant involvement in the appearance of the faster-migrating species of YqjG-His_6 (Fig. 5B) nor affected sporulation efficiency (Table 3).

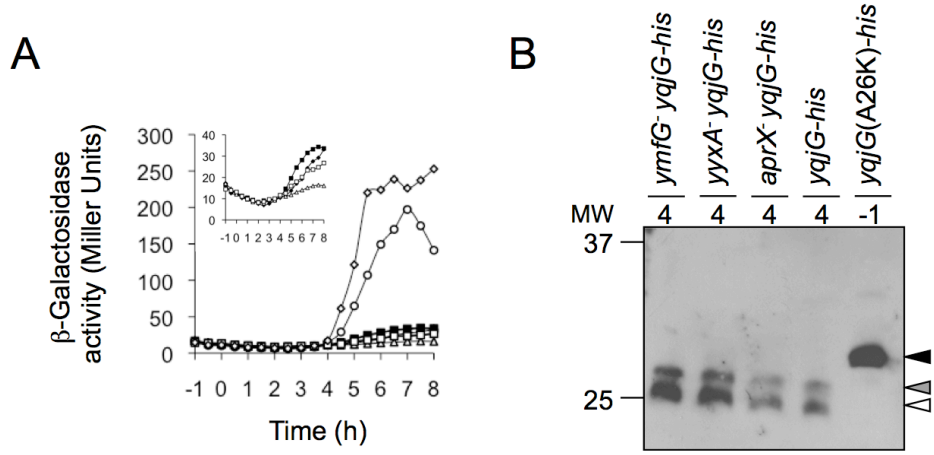

Figure 5. Testing for proteases possibly affecting YqjG. (A) Expression of *sspE-lacZ* was monitored during sporulation in *B. subtilis* strains AH1042 (Δ*sspE*::*sspE-lacZ*, open diamonds), AH5166 (Δ*spoIIIJ*::*km* Δ*sspE*::*sspE-lacZ*, open triangles), AH5310 (Δ*lonB*::*sp* Δ*sspE*::*sspE-lacZ*, open circles), AH5313 (Δ*lonB*::*sp* Δ*spoIIIJ*::*km* Δ*sspE*::*sspE-lacZ*, open squares), AH5314 (Δ*yqjG*::*yqjG-gfp* Δ*lonB*::*sp* Δ*spoIIIJ*::*km* Δ*sspE*::*sspE-lacZ*, closed squares) and AH5315 (Δ*yqjG*::*yqjG-gfp* Δ*spoIIIJ*::*km* Δ*sspE*::*sspE-lacZ*, closed diamonds). The complete relevant genotypes of the strains are given in Table 1. The inset shows ß-galactosidase activity for strains AH5313-5 and AH5166 in a different scale. Strains were grown in DSM, and samples were taken at 30-min intervals as indicated, relative to the onset of sporulation (T_0) and assayed for ß-galactosidase activity. ß-galactosidase activity is given in Miller units (see Materials and Methods). **(B)** Samples were withdrawn from DSM-grown cultures at the indicated times relative to the onset of sporulation, T_0. Cells from strains AH5324 (Δ*amyE*::*yqjG-his* Δ*spoIIIJ*::*km*), AH5325 (Δ*amyE*::*yqjG*(A26K)-*his* Δ*spoIIIJ*::*km*), AH5376 (Δ*amyE*::*yqjG-his* Δ*spoIIIJ*::*km* Δ*ymfG*::*sp*), AH5377 (Δ*amyE*::*yqjG-his* Δ*spoIIIJ*::*km* Δ*yyxA*::*sp*) and AH5378 (Δ*amyE*::*yqjG-his* Δ*spoIIIJ*::*km* Δ*aprX*::*sp*) were lysed and the whole-cell extracts were electrophoretically resolved by Tricine-SDS-PAGE and subjected to immunoblot analysis using antibodies against the His-tag. Black arrowhead, unprocessed YqjG-His_6 (see Chapter IV); grey arrowhead: mature YqjG-His_6; white arrowhead, faster-migrating band of YqjG-His_6. The position of molecular weight markers (in kDa) is shown on the left.

Chimeras of SpoIIIJ and YqjG place the secondary cleavage site upstream of Y63. As shown above, a second species of YqjG appears during sporulation, starting downstream of the cleavage site used by type I signal peptidases (since the His-tag is placed at the C-terminus).

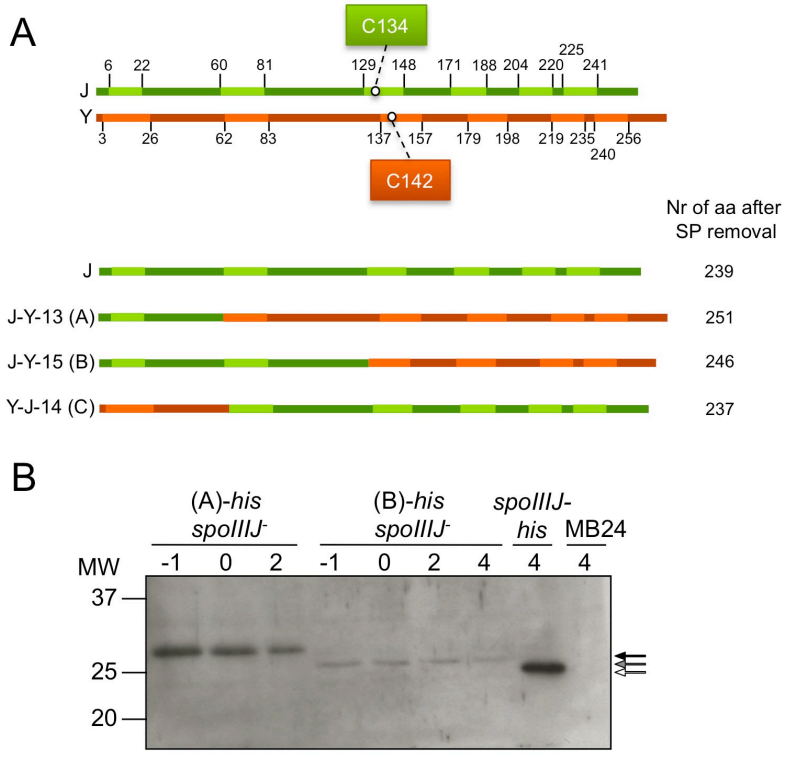

Figure 6. Mapping the secondary cleavage site of YqjG with chimerical proteins. (A) Schematic representation of three chimeras of SpoIIIJ (green) and YqjG (orange) and of the two native proteins. Light-coloured regions represent transmembrane segments and the numbers indicate the aminoacidic residues in their boundaries. White circles pinpoint the location of a conserved cysteine residue. The number of amino acid residues in each protein after removal of their signal peptide regions is indicated on the right. **(B)** Strains AH5232 (Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ-yqjG-13-his* Δ*spoIIIJ*::*km*), AH5234 (Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ-yqjG-15-his* Δ*spoIIIJ*::*km*), AH9218 (Δ*spoIIIJ*::*spoIIIJ-his*) and MB24 (wild-type) were grown in DSM and samples were collected at the indicated times (in hours) relative to the onset of sporulation. Cells were lysed and whole-cell extracts were electrophoretically resolved by SDS-PAGE and subjected to immunoblot analysis using antibodies against the His-tag. Black arrow: SpoIIIJ-YqjG-13-His_6 (AH5232); dark grey arrow: SpoIIIJ-YqjG-His_6 (AH5234); white arrow: SpoIIIJ-His_6 (AH9218). The position of molecular weight markers (in kDa) is shown.

In order to mark out the boundaries of the region encompassing the cleavage site several approaches were employed. In a first approach, the use of chimerical proteins of SpoIIIJ and YqjG permitted narrowing down

the region of cleavage to the first 62 amino acid residues since in chimera SpoIIIJ-YqjG-13 (A) (Fig. 6A), expressing a YqjG-His_6 variant in which the first external loop was replaced with the equivalent region from SpoIIIJ, the second species was not detected (Fig. 6B). This panel is repeated from Fig. 2 in Chapter II. A second band also does not appear in SpoIIIJ-YqjG-15 (B), as expected, given that the first half is composed of *spoIIIJ*. Interestingly, despite accumulating to lower levels, chimera SpoIIIJ-YqjG-15 (B) produces higher levels of heat-resistant spores relative to chimera SpoIIIJ-YqjG-13 (A) (AH5045 and AH5047, respectively, Table 3). Although chimera YqjG-SpoIIIJ-14 (C) produced much higher sporulation levels than chimera-SpoIIIJ-YqjG-13 (A), it did not accumulate to visible levels (AH5046 and AH5045, respectively, Table 3; Fig. 6B, and data not shown) so a more precise notion of the cleavage site could not be inferred by this method.

AMS mapping refines the position of the secondary cleavage site. Another approach employed to narrow down the cleavage site involved the use of AMS (4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid), a membrane-impermeable thiol-reactive reagent commonly employed in the labelling of cysteine residues. The rationale for this experiment is that, depending on the position of the binding site for AMS (e.g. an artificially introduced cysteine residue), a differential behaviour in terms of gel migration will occur: if the secondary cleavage site is downstream of the introduced cysteine residue only the slower-migrating band increases in molecular weight (MW); if it is upstream both bands do (Fig. 7). We thus constructed two YqjG-His_6 cysteine derivatives with the substitutions A50C and H60C (AH5358 (*amyE*::*yqjG*(A50C)-*his spoIIIJ*::*km*) and AH5359 (*amyE*::*yqjG*(H60C)-*his spoIIIJ*::*km*), respectively) and checked for an increase in MW of the faster-migrating species. Cultures of AH5358, AH5359, AH5324 and MB24 were sampled at hour 4 after the onset of sporulation (when the two bands are plainly visible, see Fig. 3B) and subjected to immunoblot analysis with anti-His_6 antibodies.

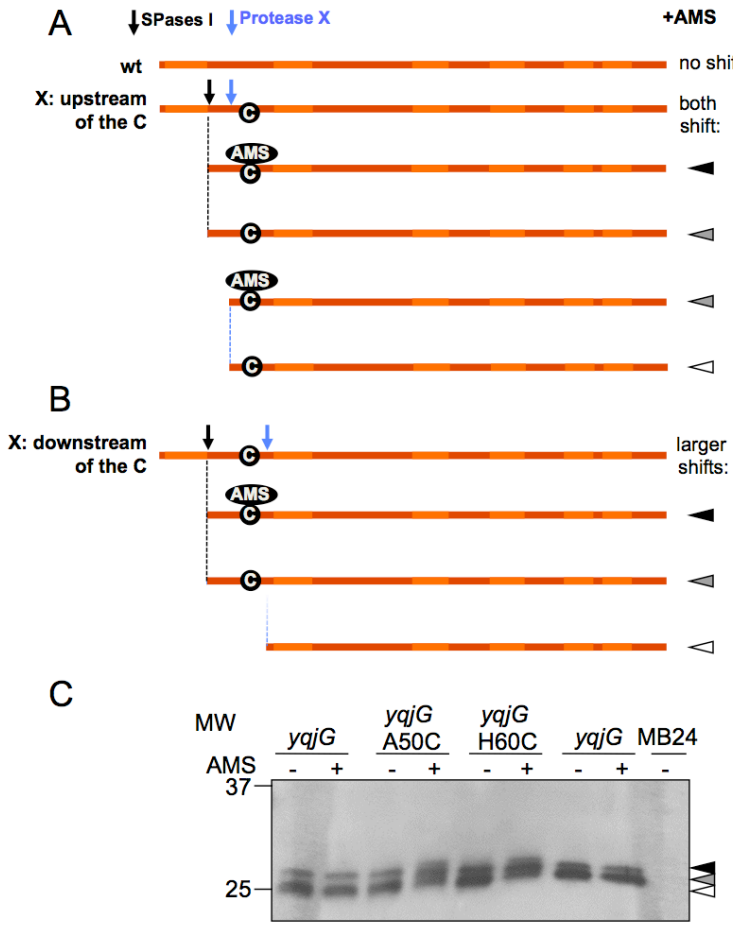

Figure 7. AMS-based mapping of the secondary cleavage site of YqjG. (A, B) Schematic representation of the rationale of the strategy employed for AMS based-mapping. The light-coloured regions of YqjG (orange) represent transmembrane segments. Cleavage of full-length YqjG (depicted as "wt") by type I SPases (black arrow) (see Chapter IV) generates a mature YqjG species; cleavage by protease X (blue arrow) generates a faster-migrating YqjG species. Incubation with the thiol-reactive reagent AMS leads to an increase in molecular weight as it binds the artificially introduced cysteine residue. If the secondary cleavage site (blue arrow) is upstream of this cysteine **(A)**, the migration of both species is delayed upon AMS addition; if present downstream **(B)** of the cysteine residue, the faster-migrating species remains unchanged. The migration of the mature band is delayed in both cases upon AMS addition. The arrowheads are colour-coded as in (C), indicating which species correspond to the observed bands. **(C)** Strains AH5358 (Δ*amyE*::*yqjG*(A50C)-*his* Δ*spoIIIJ*::*km*), AH5359 (Δ*amyE*::*yqjG*(H60C)-*his* Δ*spoIIIJ*::*km*), AH5324 (Δ*amyE*::*yqjG-his* Δ*spoIIIJ*::*km*) and MB24 were grown in DSM and sampled 4 h after the onset of sporulation. Cells were lysed and whole-cell extracts were incubated with 2 mM AMS at 37ºC for 1 h and subjected to Tricine-SDS-PAGE followed by immunoblot analysis with antibodies against the His-tag. Black arrowhead, AMS-shifted YqjG-His_6; grey arrowhead: signal peptidase I-processed YqjG-His_6 and AMS-shifted faster-migrating band of YqjG-His_6; white arrowhead, faster-migrating band of YqjG-His_6.

Note that the endogenous Cys142 residue should not be labeled as it is predicted to be in the membrane, according to several topology prediction programs (Fig. 6A; see Materials and Methods). Accordingly, there is no shift in MW in the wild-type YqjG-His_6 (AH5324) when AMS is added (compare the first two lanes in Fig. 7C). As expected, we detected a third (upper) band (black arrowhead in Fig. 7C) derived from AMS binding to the introduced cysteine residue in mutants AH5358 and AH5359, but not AH5324, the corresponding wild-type (Fig. 7C). In addition, the faster-migrating band (white arrowhead) exhibits reduced intensity whilst the middle band (grey arrowhead) sees it augmented in the AMS-added lanes compared to the AMS-free ones, for AH5358 and AH5359. We interpret this as both species suffering a shift in migration, both in AH5358 and AH5359, suggesting that the cleavage site is located upstream of the A50 residue (Fig. 7A).

A deletion in YqjG places the secondary cleavage site downstream of the G38 residue. In a third approach we deleted part of the region we expected to contain the cleavage site as deduced from the results obtained with the program "Compute pI/Mw tool" (http://expasy.ch/tools/pi_tool.html); here we also converted the G32 residue of YqjG into a histidine to make it more similar to SpoIIIJ (black box in Fig. 8A and D). Strains AH5333 (*amyE*::*yqjG-del-his spoIIIJ*::*km*) and AH5324 (*amyE*::*yqjG-his spoIIIJ*::*km*) were grown in DSM and sampled and the times in hours relative to the onset of sporulation as indicated in Fig. 8B. The faster-migrating species still appears, indicating that the cleavage site is not included in this region. Sporulation levels were similar to those of the *spoIIIJ* mutant alone (Table 3). Together, these observations suggest YqjG is usually cleaved during sporulation in a region comprising amino acid residues 39-49 (Fig. 8D, red box), which leads to its inactivation and inability to support efficient sporulation in the absence of SpoIIIJ.

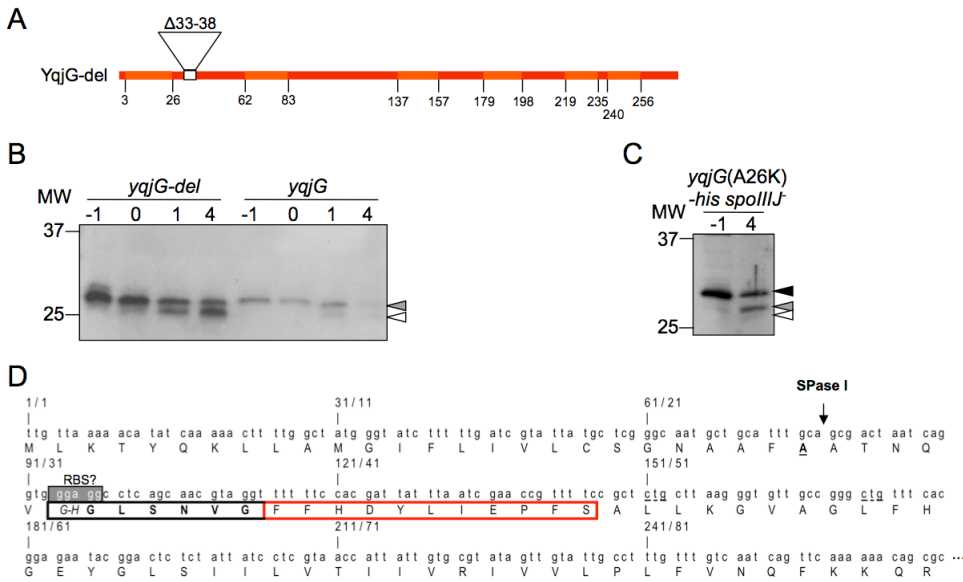

Figure 8. Faster-migrating band of YqjG results from cleavage downstream of G38 rather than from alternative translation. (A) YqjG is depicted in orange with the light-coloured regions representing transmembrane segments. The G32H substitution and deleted region are depicted to scale. The positions of the amino acid residues delimitating the boundaries of the transmembrane segments are indicated. **(B)** AH5324 (Δ*amyE*::*yqjG-his* Δ*spoIIIJ*::*km*) and AH5333 (Δ*amyE*::*yqjG-del-his* Δ*spoIIIJ*::*km*) were grown in DSM and samples were collected at the indicated time points (in hours) relative to the onset of sporulation. The cells were lysed, and the whole-cell extracts were electrophoretically resolved by Tricine-SDS-PAGE and subjected to immunoblot analysis using antibodies against the His-tag. Grey arrowhead, mature YqjG-del-His_6; white arrowhead, faster-migrating band. **(C)** As in (B), for AH5325 (Δ*yqjG*::*yqjG*(A26K)-*his* Δ*spoIIIJ*::*km*). Black arrowhead: YqjG(A26K)-His_6 unprocessed band; grey: mature YqjG(A26K)-His_6; white: faster-migrating band. The position of molecular weight markers (in kDa) is shown. **(D)** First 90 amino acid residues of YqjG. A26 is depicted in bold and underlined, and the cleavage site used by type I SPases is indicated with an arrow (see Chapter IV); the altered region in YqjG-del-His_6 is in a black box, indicated in bold (deletion) and italics (conversion of a Gly to a His). The grey box encompasses the putative RBS corresponding to the presumptive start codons CUG of L51 and L58 (underlined). The red box encompasses the region predicted to contain the secondary cleavage site. Numbers above the sequence indicate nucleotides (on the left) and amino acid residues (on the right).

***In silico* analyses support the placement of the secondary cleavage site between residues 39-49.** We were able to detect size differences of at least ~0.5 kDa, which was the difference predicted for chimera SpoIIIJ-YqjG-15 and SpoIIIJ devoid of their signal peptides (Compute pI/Mw tool), readily observed in Fig. 6B. This is less than the difference between the mature and faster-migrating bands of YqjG (approximately 1.4-1.67 kDa, see below), which supports the notion that chimera SpoIIIJ-YqjG-13 does not

suffer a secondary cleavage event and places the cleavage site upstream of Y63 (Fig. 6).

The difference in MW between the mature and faster-migrating species was estimated to be ~1.40 and ~1.67 kDa by the following approaches, respectively: i) direct observation of the three bands of YqjG(A26K)-His_6 (Fig. 8C) suggests such value to be roughly half of the difference between mature and unprocessed forms, which constitutes the signal peptide (~2.800 kDa, using the "Compute pI/Mw tool"); ii) plotting the relative electrophoretic mobility values of the MW standards against their Log_{10} values to derive calibration curves; linear trendlines were obtained and used to determine the MW of the bands in question. This was performed for two independent immunoblots containing five lanes with YqjG(A26K)-His_6 extracts (e.g. Fig. 8C). The difference in MW between mature and faster-migrating bands was estimated in ~1.67 kDa by this method; as a control, we also investigated the MW of the signal peptide, which was estimated to be ~2.64 kDa. These MW values (~1.40 and ~1.67) fall within the interval (1.10-2.50 kDa) predicted with the "Compute pI/Mw tool" in case the secondary cleavage event of the mature form of YqjG takes place between residues 39 and 49, respectively.

Discussion

The results herein described link the inability of YqjG to complement a *spoIIIJ* mutant during sporulation to the inactivation of its mature form through limited proteolysis. YqjG experiences a cleavage event secondary to the one exerted by type I signal peptidases (SPases) (see Chapter IV). This second cut in YqjG occurs in DSM independently of Spo0A (the master regulator for entry in sporulation), but not in LB medium (Fig. 4). The lack of cleavage in this medium was observed even when sampling four hours after the end of the exponential phase (not shown), contrasting with the appearance of the second band in DSM at that time point (Fig. 4). Possibly, a putative protease that cleaves YqjG may be regulated similarly

to the *ald* gene, which is activated at the onset of sporulation but in a Spo0A- and σ^H-independent manner by an unknown mechanism (Siranosian *et al.*, 1993). Alternatively, transcription of the gene coding for the putative protease might be Spo0A-independent but σ^H-dependent, as with $spoVGp_1$ and $citGp_2$ (Weir *et al.*, 1991 and references therein).

The cleavage site is predicted to occur outside the cell or in the intermembrane space between the prespore and the mother cell. The eight proteases responsible for most of the extracellular proteolytic activity (*aprE*, *nprE*, *bpr*, *epr*, *mpr*, *nprB*, *vpr* and *wprA*) are dependent on Spo0A and therefore should not be involved in the cleavage of YqjG (Kodama *et al.*, 2007). The *B. subtilis* genome encodes two Lon proteases, LonA and LonB, both of them ATP-dependent (Riethdorf *et al.*, 1994; Serrano *et al.*, 2001). LonA is stress-induced and involved in preventing σ^G activity under non-sporulation conditions (Schmidt *et al.*, 1994), whilst LonB does not interfere with the activities of σ^F or σ^G in a wild-type strain (Serrano *et al.*, 2001). Both LonA-GFP and LonB-GFP localise to the prespore during sporulation, and the latter initially localises in the prespore membrane (Simmons *et al.*, 2008). We observed that removal of *lonB* had little or no effect on the levels of σ^G activity of a *yqjG-gfp spoIIIJ*$^-$ strain (Fig. 5A). Other proteases were tested (*aprX*, *ymfG*, *yyxA*) without an observable effect on the inactivation of YqjG, either through immunoblot analysis or spore titre determination (Fig. 5B; Table 3). From the set of proteases here studied none was responsible for the cleavage event observed in YqjG. Nevertheless, the the *B. subtilis* genome encodes many proteases with at least 31 candidates having been described (Kunst *et al.*, 1997), as well as at least 17 sigma factors that are involved in the regulation of a wide range of processes (Helmann and Moran Jr., 2002). Hence, many candidates exist that may be responsible for the appearance of the secondary band of YqjG. This scenario may be even more complex, as it is possible that it is not the protease itself that has its expression regulated independently of Spo0A but an adaptor protein that binds the substrate and redirects the proteases' activity towards this substrate. An example is MecA, an

adaptor protein that regulates the ClpCP protease complex specificity in *B. subtilis* (Hengge and Bukau, 2003).

YqjG is cleaved by type I signal peptidases after the amino acid residue A26 (see Chapter IV). The second proteolytic event here observed was predicted to occur between amino acid residues 39-49, based on the combined information obtained from distinct data sets: chimeras of SpoIIIJ and YqjG (before residue 63), AMS labelling experiments (before residue 50), and deletion of a YqjG region (after residue 38) combined with *in silico* analyses (Figs. 6-8). Also, we observed that YqjG from *B. halodurans*, which shows low similarity with YqjG from *B. subtilis* in the predicted cleavage area, supports efficient sporulation and does not suffer the secondary processing (see Chapter II). The YqjG-del variant (Δ33-38) still produced the faster-migrating species, suggesting that the cleavage site lies outside of the affected region. It is nevertheless possible that cleavage usually occurs in that region, regardless of the sequence: in several reports a specific region was required outside of the cleavage site itself (Keiler and Sauer, 1996; Rawson, 2008 and references therein).

We conjecture that the faster-migrating species is not generated by alternative translation. Although CUG codons in L51 and L58 could potentially serve as initiation codons (Ambulos Jr. *et al.*, 1990; Snyder and Champness, 2003), they lack a Shine-Dalgarno sequence in the YqjG internal deletion variant (Fig. 8D, grey box), which still gives rise to the faster-migrating species. Alternative translation seems thus unlikely, proteolytic processing being a much more attractive hypothesis to explain the appearance of the faster-migrating band. Interestingly, the region we believe to comprise the cleavage site (between 39-49) does not match any recognition sites for known bacterial proteases, possibly allowing the establishment of a novel consensus sequence.

Our results herein presented show that YqjG can, under certain conditions, substitute for its homologue also during sporulation. Accordingly, we detected an increase in the expression level of YqjG-GFP and YqjG-His_6 upon deletion of *spoIIIJ* (Fig. 2). We propose that high

amounts of uncleaved YqjG may enable more efficient sporulation. Our results led us to propose that *yqjG* induction occurs via a mechanism that occurs post-transcriptionally. This is in accordance with the results by Murakami *et al*., (2002), who stated that deletion of either gene does not influence the homologue's transcriptional profile. These results were further substantiated by the work of Pogliano's and Driessen's labs (Chiba *et al.*, 2009; Saller *et al.*, 2011). Fusion of YqjG to GFP increased spore titre when compared to tagless YqjG or YqjG-His_6, which might be attributed to some level of protection against proteolysis offered by a bulky tag like GFP. Increased stability bestowed by GFP was noted before for others (Elkind *et al.*, 2000; Guéneron *et al.*, 2000; Rudner and Losick, 2002) and might be explained by the compact structure of GFP, which is stable under several conditions, e.g. treatment with proteases (Ormö *et al.*, 1996). Fully functional versions of YqjG were obtained by combining our stabilisation strategy with improved interaction with SpoIIIJ's substrate SpoIIIAE provided by the substitutions isolated by Camp and Losick (2008). The identification of the protease that cleaves YqjG, the cleavage site itself and consequences for cell physiology are major goals for future work.

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Tables

Table 1. Bacterial strains.

Strain	Relevant Genotype/Phenotype[a]	Origin/ Reference
MB24	*trpC2 metC3*	Laboratory stock
JOB44	Δ*spoIIIJ*::*km* / Km^r	Serrano *et al*., 2003
JOB20	Δ*spoIIIJ*::*sp* / Sp^r	Serrano *et al*., 2003
AH1042	Δ*sspE*::*sspE-lacZ* / Erm^r	Laboratory stock
AH1901	Δ*ymfG*::*sp* / Sp^r	Costa *et al*., unpublished
AH1902	Δ*yyxA*::*sp* / Sp^r	«
AH1903	Δ*aprX*::*sp* / Sp^r	«
AH3531	Δ*spo0A*::erm / Erm^r	Real *et al*., unpublished
AH5000	Δ*spoIIIJ*::*spoIIIJ-gfp* / Sp^r	This work
AH5006	Δ*yqjG*::*yqjG-gfp* / Sp^r	«
AH5007	Δ*yqjG*::*yqjG-gfp* Δ*spoIIIJ*::*km* / Km^r	«
AH5045	Δ*spoIIIJ*::*km* Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ-yqjG*-13 (chimera A) / Km^r Cm^r	Chapter II
AH5046	Δ*spoIIIJ*::*km* Δ*amyE*::$P_{spoIIIJ}$-*yqjG-spoIIIJ*-14 (chimera C) / Km^r Cm^r	«
AH5047	Δ*spoIIIJ*::*km* Δ*amyE*:: $P_{spoIIIJ}$-*spoIIIJ-yqjG*-15 (chimera B) / Km^r Cm^r	«
AH5161	Δ*spoIIIJ*::*km* Δ*amyE*:: $P_{spoIIIJ}$-*yqjG* / Km^r Cm^r	This work
AH5166	Δ*spoIIIJ*::*km* Δ*sspE*::*sspE-lacZ* / Km^r Erm^r	Chapter II
AH5178	Δ*spoIIIJ*::*km* Δ*amyE*:: $P_{spoIIIJ}$-*yqjG-his* / Km^r Cm^r Sp^r	This work
AH5230	Δ*spoIIIJ*::*km* Δ*amyE*:: P_{yqjG}-*spoIIIJ* / Km^r Cm^r	«
AH5231	Δ*spoIIIJ*::*km* Δ*amyE*:: P_{yqjG}-*spoIIIJ-his* / Km^r Cm^r Sp^r	«
AH5232	Δ*spoIIIJ*::*km* Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ-yqjG-13-his* (chimera A) / Km^r Cm^r	Chapter II
AH5233	Δ*spoIIIJ*::*km* Δ*amyE*::$P_{spoIIIJ}$-*yqjG-spoIIIJ-14-his* (chimera C) / Km^r Cm	«
AH5234	Δ*spoIIIJ*::*km* Δ*amyE*:: $P_{spoIIIJ}$-*spoIIIJ-yqjG-15-his* (chimera B) / Km^r Cm^r	«
AH5244	Δ*yqjG*::*yqjG-his* / Cm^r	This work
AH5246	Δ*spoIIIJ*::*sp* Δ*yqjG*::*yqjG-his* / Sp^r Cm^r	«
AH5267	Δ*yqjG*::*yqjG-gfp* / Cm^r	«
AH5268	Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjG-gfp* / Km^r Cm^r	«
AH5269	Δ*spoIIIJ*::*sp* Δ*yqjG*::*yqjG-gfp* / Sp^r Cm^r	«
AH5284	Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjG-gfp* Δ*spo0A*::*erm* / Km^r Sp^r Erm^r	«
AH5310	Δ*lonB*::*sp* Δ*sspE*::*sspE-lacZ* / Sp^r Erm^r	«
AH5311	Δ*lonB*::*sp* Δ*spoIIIJ*::*km* / Sp^r Km^r	«
AH5312	Δ*lonB*::*sp* Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjG-gfp* / Sp^r Km^r Cm^r	«
AH5313	Δ*lonB*::*sp* Δ*spoIIIJ*::*km* Δ*sspE*::*sspE-lacZ* / Sp^r Km^r Erm^r	«
AH5314	Δ*lonB*::*sp* Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjG-gfp* Δ*sspE*::*sspE-lacZ* / Sp^r Km^r Cm^r Erm^r	«
AH5315	Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjG-gfp* Δ*sspE*::*sspE-lacZ* / Km^r Cm^r Erm^r	«
AH5324	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG-his* / Km^r Cm^r	Chapter IV
AH5325	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG*(A26K)-*his* / Km^r Cm^r	«
AH5333	Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjG-del-his* / Km^r Cm^r	This work
AH5358	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG*(A50C)-*his* / Km^r Cm^r	«
AH5359	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG*(H60C)-*his* / Km^r Cm^r	«
AH5376	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG-his* Δ*ymfG*::*sp* / Km^r Cm^r Sp^r	«
AH5377	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG-his* Δ*yyxA*::sp / Km^r Cm^r Sp^r	«
AH5378	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG-his* Δ*aprX*::*sp* / Km^r Cm^r Sp^r	«

AH5421	Δ*yqjG*::*yqjG*(A238S)-*gfp* / Cm^r	«
AH5422	Δ*yqjG*::*yqjG*(G247V)-*gfp* / Cm^r	«
AH5423	Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjG*(A238S)-*gfp* / Km^r Cm^r	«
AH5424	Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjG*(G247V)-*gfp* / Km^r Cm^r	«
AH5429	Δ*spoIIIJ*::*sp* Δ*yqjG*::*yqjG*(A238S)-*gfp* / Sp^r Cm^r	«
AH5430	Δ*spoIIIJ*::*sp* Δ*yqjG*::*yqjG*(G247V)-*gfp* / Sp^r Cm^r	«
AH5434	Δ*lonB*::*sp* / Sp^r	Serrano *et al.*, unpublished
AH9203	Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjG-his* / Km^r Sp^r	This work
AH9218	Δ*spoIIIJ*::*spoIIIJ-his* / Sp^r	«
AH9246	Δ*yqjG*::*yqjG-his* / Sp^r	«

[a] All *B. subtilis* strains are *trpC2 metC3*. Km, kanamycin; Cm, cloramphenicol; Erm, erythromycin; Sp, spectinomycin; Neo, neomycin.

Table 2. Oligonucleotides used in this study.

Primer	Sequence (5´to 3´)[a]
YqjG_His_R	GAGGATCCTTTCACCGACTCAGTAAGAGCG
gfp30D	AGTAAAGGAGAAGAACTTTTCACTGGAG
gfpBglII_R	GAAGATCTTCTTATTTGTATAGTTCATCCATGCG
yqjG-17D	GGAAAATGAATTCGGCTCTTTCCC
yqjG400R	TTTTGTTCTCCTCCTTTTTATAAATGCG
PyqjG-spoIIIJD	TATAAAAAGGAGGAGAACAAAAatgttgttgaaaaggagaatag
PYqjG-460D	AGAGCGGGATCCCTGTATGGTGTATCG
spoIIIJ1106R	GAAACCCTTTGGATCCCTCTTCAATAAC
yqjGA50C_D	GATTATTTAATCGAACCGTTTTCC**TGC**CTGCTTAAGGGTGTTGCC
yqjGA50C_R	GGCAACACCCTTAAGCAG**GCA**GGAAAACGGTTCGATTAAATAATC
yqjGH60C_D	GGGTGTTGCCGGGCTGTTT**TGC**GGAGAATACGGACTCTCTATTATC
yqjGH60C_R	GATAATAGAGAGTCCGTATTCTCC**GCA**AAACAGCCCGGCAACACCC
yqjGdel_D	GCGACTAATCAGGTGCACTTTTTCCACGATTATTTAATCG
yqjGdel_R	CGATTAAATAATCGTGGAAAAAGTGCACCTGATTAGTCGC
yqjG_692D	GTTATTAAAGCTTAGGTTGACAGTATCCAGG
yqjG_701D	CCTCAGGTTGACAGTATCCAG
yqjGgfp	GTTCTTCTCCTTTACTtttcaccgactcagtaagagcggctg
gfpR	GGCGAATTCTTATTTGTATAGTTCATCCATGC
yqjGA238S_D	TTCTCGCTTAATGTGCCGGCA**T**CCCTTCCGCTGTACTGGTTTAC
yqjGA238S_R	GTAAACCAGTACAGCGGAAGGGATGCCGGCACATTAAGCGAGAA
yqjGG247V_D	CGCTGTACTGGTTTACAAGCG**T**ACTGTTTTTGACAGTGCAAAAC
yqjGG247V_R	GTTTTGCACTGTCAAAAACAGT**A**CGCTTGTAAACCAGTACAGCG

[a] Restriction sites are underlined, mutations in bold, fusions in different case.

Table 3. Heat resistance of various strains.

Strain	Relevant Genotype	Viable cell count[a]	$Heat^R$ cell count[a]	Spo%[a]
MB24	wild-type	$2.7x10^8$	$1.9x10^8$	70.4
JOB44	Δ*spoIIIJ*::*km*	$7.2x10^7$	0	0
JOB20	Δ*spoIIIJ*::*sp*	$1.1x10^8$	$7.0x10^1$	<0.0001
AH1042	Δ*sspE*::*sspE-lacZ*	$6.4x10^8$	$4.1x10^8$	64.0
AH5000	Δ*spoIIIJ*::*spoIIIJ-gfp*	$4.4x10^8$	$3.0\ x10^8$	68.2
AH5006	Δ*yqjG*::*yjqG-gfp*	$1.1x10^8$	$2.0x10^8$	18.2
AH5045	Δ*spoIIIJ*::*km* Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ-yqjG-13* (chimera A)	$1.9x10^8$	$8.6x10^3$	0.004
AH5046	Δ*spoIIIJ*::*km* Δ*amyE*::$P_{spoIIIJ}$-*yqjG-spoIIIJ-14* (chimera C)	$1.7x10^8$	$4.3x10^6$	2.61
AH5047	Δ*spoIIIJ*::*km* Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ-yqjG-15* (chimera B)	$2.2x10^8$	$3.6x10^7$	16.6

AH5161	Δ*spoIIIJ*::*km* Δ*amyE*::$P_{spoIIIJ}$-*yqjG*	$8.3x10^7$	0	0
AH5166	Δ*spoIIIJ*::*km* Δ*sspE*::*sspE-lacZ*	$9.5x10^7$	$1.0x10^1$	<0.0001
AH5230	Δ*spoIIIJ*::*km* Δ*amyE*::P_{yqjG}-*spoIIIJ*	$2.0x10^8$	$1.1x10^8$	55.0
AH5244	Δ*spoIIIJ*::*sp* Δ*yqjG*::*yqjG-his*	$3.5x10^8$	$1.7x10^8$	48.6
AH5246	Δ*spoIIIJ*::*sp* Δ*yqjG*::*yqjG-his*	$1.6x10^8$	$3.0x10^1$	<0.0001
AH5268	Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjG-gfp*	$8.1x10^7$	$1.2x10^6$	1.10
AH5269	Δ*spoIIIJ*::*sp* Δ*yqjG*::*yqjG-gfp*	$6.3x10^7$	$1.9x10^6$	2.34
AH5310	Δ*lonB*::*sp* Δ*sspE*::*sspE-lacZ*	$4.6x10^8$	$4.3x10^8$	93.5
AH5313	Δ*lonB*::*sp* Δ*spoIIIJ*::*km* Δ*sspE*::*sspE-lacZ*	$1.2x10^8$	$4.8x10^2$	0.0004
AH5314	Δ*lonB*::*sp* Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjG-gfp* Δ*sspE*::*sspE-lacZ*	$1.1x10^8$	$2.3x10^6$	2.10
AH5315	Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjG-gfp* Δ*sspE*::*sspE-lacZ*	$1.6x10^8$	$3.6x10^6$	2.30
AH5324	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG-his*	$7.8x10^7$	$1.0x10^1$	<0.0001
AH5325	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG*(A26K)-*his*	$5.0x10^7$	0	0
AH5333	Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjGdel-his*	$8.1x10^7$	$1.0x10^1$	<0.0001
AH5358	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG*(A50C)-*his*	$6.9x10^7$	$1.3x10^3$	0.0002
AH5359	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG*(H60C)-*his*	$9.4x10^7$	$1.0x10^1$	<0.0001
AH5376	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG-his* Δ*ymfG*::*sp*	$1.4x10^8$	0	0
AH5377	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG-his* Δ*yyxAG*::*sp*	$2.1x10^8$	0	0
AH5378	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG-his* Δ*aprX*::*sp*	$1.9x10^8$	0	0
AH5423	Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjG*(A238S)-*gfp*	$1.8x10^8$	$9.0x10^7$	50.0
AH5424	Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjG*(G247V)-*gfp*	$8.3x10^7$	$1.2x10^7$	14.5
AH5429	Δ*spoIIIJ*::*sp* Δ*yqjG*::*yqjG*(A238S)-*gfp*	$5.8x10^7$	$1.4x10^7$	24.1
AH5430	Δ*spoIIIJ*::*sp* Δ*yqjG*::*yqjG*(G247V)-*gfp*	$7.0x10^7$	$4.1x10^7$	58.6
AH9203	Δ*spoIIIJ*::*km* Δ*yqjG*::*yqjG-his*	$1.1x10^8$	0	0
AH9218	Δ*spoIIIJ*::*spoIIIJ-his*	$5.6x10^8$	$3.9x10^8$	69.6
AH9246	Δ*yqjG*::*yqjG-his*	$3.8x10^8$	$5.2x10^7$	13.7
30ºC				
JOB20	Δ*spoIIIJ*::*sp*	$2.5x10^8$	$1.2x10^5$	0.05
AH5269	Δ*spoIIIJ*::*sp* Δ*yqjG*::*yqjG-gfp*	$2.2x10^8$	$7.3x10^7$	33.2
MB24	wild-type	$3.9x10^8$	$2.8x10^8$	71.8
30ºC over two nights				
JOB20	Δ*spoIIIJ*::*sp*	$1.0x10^8$	$1.5x10^5$	0.15
AH5269	Δ*spoIIIJ*::*sp* Δ*yqjG*::*yqjG-gfp*	$2.5x10^8$	$1.5x10^8$	60.0
MB24	wild-type	$1.0x10^9$	$7.2x10^8$	72.0
Plates (37ºC)[b]				
JOB20	Δ*spoIIIJ*::*sp*	$1.0x10^8$	$2.7x10^5$	0.27
AH5269	Δ*spoIIIJ*::*sp* Δ*yqjG*::*yqjG-gfp*	$3.1x10^8$	$1.7x10^8$	54.8
MB24	wild-type	$3.3x10^8$	$1.8x10^8$	54.5

[a] The titre of viable and heat-resistant colony forming units was measured 24 hours after the onset of sporulation in DSM (see Material and Methods). Spo, sporulation.
[b] Sporulation was measured from the resuspension and serial dilution of a single colony (incubated over two nights at 37ºC) in 160 µl of DSM.

Table 4. Plasmids used in this study.

Plasmid	Relevant features	Antibiotic resistance[a]	Source
pLC3	*'spoIIIJ-gfp*	*bla, sp*	This work
pLC8	*'yqjG-gfp*	*bla*	«
pLC9	*'yqjG-gfp*	*bla, sp*	«
pLC64	Δ*amyE*::$P_{spoIIIJ}$-*yqjG*	*bla, cat*	Chapter II
pLC86	P_{yqjG}-*spoIIIJ*	*bla*	This work
pLC92	Δ*amyE*::P_{yqjG}-*spoIIIJ*	*bla, cat*	«
pLC97	*'yqjG-gfp*	*bla, cat*	«

pLC111	Δ*amyE*::*his*	*bla, cat*	Chapter IV
pLC122	Δ*amyE*::*yqjG-del-his*	*bla, cat*	This work
pLC138	Δ*amyE*::*yqjG*(A50C)-*his*	*bla, cat*	«
pLC139	Δ*amyE*::*yqjG*(H60C)-*his*	*bla, cat*	«
pLC166	Δ*yqjG*::*yqjG*(A238S)-*gfp*	*bla, cat*	«
pLC167	Δ*yqjG*::*yqjG*(G247V)-*gfp*	*bla, cat*	«
pTC1	Δ*ymfG*	*bla, sp*	Costa *et al.*, unpublished
pTC2	Δ*yyxA*	*bla, sp*	«
pTC3	Δ*aprX*	*bla, sp*	«
pMS38	Δ*yqjG*::P_{spac}-Δ*SPyqjG*	*bla, cat*	Zilhão *et al.*, 2004
pFV4	*'spoIIIJ-his*	*km, sp*	Serrano *et al.*, 2008
pFV5	*'yqjG-his*	*km, sp*	Chapter IV
pMS254	*vector with Cm^r cassette*	*km, cm*	Serrano *et al.*, 2008
pAH256	*vector with Sp^r cassette*	*bla, sp*	Henriques *et al.*, 1997
pEIA18	*gfp*	*bla, sp*	Cormack *et al.*, 1996
pDG364	*amyE insertion vector*	*bla, cat*	Cutting and Vander Horn, 1990

[a] *bla*, ampicillin; *cat*, cloramphenicol; *sp*, spectinomycin; *km*, kanamycin.

Supplemental Data

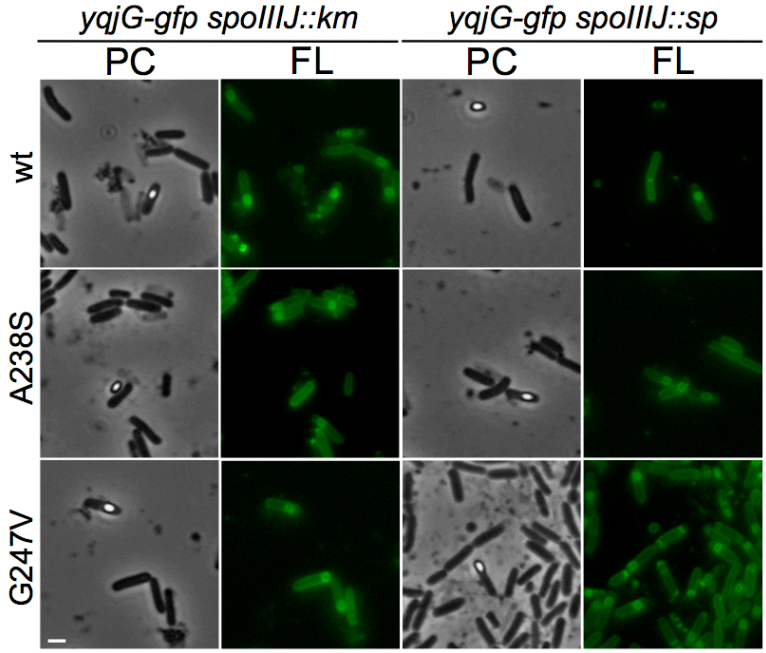

Figure S1. The YqjG-GFP A238S and G247V variants exhibit a localisation similar to their wild-type counterpart. Strains AH5268-9 (Δ*yqjG*::*yqjG-gfp* Δ*spoIIIJ*::*km* and Δ*yqjG*::*yqjG-gfp* Δ*spoIIIJ*::*sp*, respectively), AH5423-4 (Δ*yqjG*::*yqjG*(A238S)-*gfp* Δ*spoIIIJ*::*km* and Δ*yqjG*::*yqjG*(G247V)-*gfp* Δ*spoIIIJ*::*km*, respectively), and AH5439-40 (Δ*yqjG*::*yqjG*(A238S)-*gfp* Δ*spoIIIJ*::*sp* and Δ*yqjG*::*yqjG*(G247V)-*gfp* Δ*spoIIIJ*::*sp*, respectively), were grown in solid DSM over two days at 37ºC and single colonies were observed by phase contrast (PC) and fluorescence (FL) microscopy. Scale bar, 2 μm.

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Chapter IV

The two partially redundant membrane protein insertases of *Bacillus subtilis*, SpoIIIJ and YqjG, have different but dispensable signal peptides

The author of this Thesis performed all the experiments and constructed all the plasmids and strains required except for pMS176-8, pFV5, AH5074 and AH5420. The results present in this Chapter are to be submitted for publication.

Abstract

The genomes of spore-forming bacteria of the genus *Bacillus* and related organisms encode two members of the YidC/Oxa1/Alb3 family of membrane protein insertases. One of these two proteins, YqjG or SpoIIIJ, is required for growth and viability, but only SpoIIIJ supports sporulation. YqjG has a putative signal peptide recognised by type I signal peptidases (SPases), whereas SpoIIIJ's is similar to the ones found in many lipoproteins, which are cleaved by type II SPases. We show that processing of YqjG is insensitive to a mutation on the gene encoding SPase II (*lspA*) and on the gene for the lipoprotein diacylglyceryl transferase (*lgt*), required for SPaseII to act. However, processing is greatly reduced by the substitution of an alanine by a lysine (at position 26) that is part of the presumptive consensus sequence recognised by type I SPases. This suggests that YqjG is cleaved by type I SPases. In contrast, cleavage of SpoIIIJ is dependent on Lgt and LspA, and substitution of a cysteine by a lysine (at position 23) at the predicted processing site of its presumptive type II signal peptide abolished processing. This suggests that SpoIIIJ is a lipoprotein. Surprisingly, YqjG can be converted into an Lgt- and LspA-dependent protein and conversely, SpoIIIJ into an Lgt- and LspA-independent protein, without affecting growth, viability or sporulation. Furthermore, the signal peptide of SpoIIIJ can be deleted without affecting sporulation, and deletion of the signal peptide of YqjG did not affect viability in a *spoIIIJ*$^-$ background. Therefore, the signal peptides of YqjG or SpoIIIJ are not key determinants for viability or sporulation.

Introduction

In all cellular life forms, membranes establish a physical barrier indispensable for maintaining the composition and concentration of molecules and to promote their controlled swap between the cytoplasm, external medium and organelles, if present, with the aid of specialised transport systems (Pohlschröder *et al.*, 2005; Odorizzi and Rehling, 2009). Specialised machinery has to discriminate between cytoplasmic proteins and those to be exported, to distinguish between secretory and membrane proteins, whilst targeting them with specificity and accuracy, and to prevent the rapid folding of proteins destined for translocation across membranes or membrane insertion (Papanikou *et al.*, 2007).

Membrane and secreted proteins normally possess targeting and topogenic signals of several kinds, including uncleaved signal anchors, either type I or II, that initiate translocation of their N- or C-terminus, respectively (not to be confused with type I and II signal peptides, described below). Other signals include stop-transfers, that arrest translocation initiated by a preceding signal peptide, helical hairpins that insert in a folded manner, and N-terminally cleavable signal peptides which are molecular equivalents of a "zip code" and aid in directing the protein outside the cell or into a membrane, such as those of the endoplasmic reticulum, mitochondria, chloroplast and bacterial envelope. In these signal peptides three regions can be recognised: a polar N-region with a positive net charge, a hydrophobic core (H-region), and a polar C-region containing the cleavage site (Tjalsma *et al.*, 2000; Tjalsma *et al.*, 2004; Wickner and Schekman, 2005). In bacterial cells, these signals route the proteins through the Sec, Tat, or YidC/Oxa1/Alb3 pathways (Du Plessis *et al.*, 2011; Robinson *et al.*, 2011; Saller *et al.*, 2012). Eukaryotic cells also contain several organelles that require specific proteins, only a few of which being synthesised within the organelles themselves (e.g. chloroplasts and mitochondria), the others being synthesised in the cytoplasm and targeted to the endoplasmic reticulum, mitochondria or chloroplasts (Wang and Dalbey, 2011).

The Sec pathway exists in eukaryotes, archaea and in bacteria. Within the latter group, the Sec pathway has been most extensively studied in the model organisms *Escherichia coli* and *Bacillus subtilis*. In this pathway, proteins are translocated through the SecYEG channel. Distinct piloting factors recognise and bind signal peptides of nascent pre-proteins according to the hydrophobicity of such signals, guiding them to the SecYEG channel. One such factor is the signal recognition particle (SRP), mainly utilised by membrane proteins and in a co-translational manner. The ribosome-nascent pre-protein-SRP complex reaches SecYEG by docking at its membrane-bound receptor, FtsY. Another factor is the cytosolic chaperone SecB (possibly CsaA in *B. subtilis*) that interacts with secretory proteins and hands them over to SecA. The latter powers transport and is peripherically associated with the core translocase (Tjalsma *et al.*, 2000; Papanikou *et al.*, 2007; Rapoport, 2007; Natale *et al.*, 2008). Proteins that utilise the Sec pathway possess signal peptides that are usually processed by type I signal peptidases (five chromosomally encoded in *B. subtilis*), which cut in the consensus sequence A-S-A at positions -3 to -1 relative to the cleavage site, after the invariable alanine at -1. Lipoproteins are processed by a type II signal peptidase encoded by *lspA* (consensus L-(A/S)-(A/G)-C, the so-called lipobox), which cuts off the signal peptide before the imperative cysteine residue. This step requires *lgt* (lipoprotein diacylglyceryl transferase). Neither *lgt* nor *lspA* is required for viability (Prágai *et al.*, 1997; Leskelä *et al.*, 1999; reviewed in Tjalsma *et al.*, 2004). In *E. coli*, a subsequent step is performed by Lnt (lipoprotein N-acetyltransferase) but no obvious equivalent exists in *B. subtilis* (Gupta and Wu, 1991; Kunst *et al.*, 1997).

The twin-arginine (Tat) pathway has been found in archaea, chloroplasts, and in bacteria, and homologues were found in plant mitochondria although not for the complete pathway (Bogsch *et al.*, 1998; Yen *et al.*, 2002). The signal peptides of its substrates typically possess two consecutive arginines. Its most distinctive feature is to export folded proteins, including those with already bound metal cofactors, multimeric

proteins that assemble into a complex prior to export, and also some membrane proteins (Lee *et al.*, 2006; Xie and Dalbey, 2008).

Oxa1 from *Saccharomyces cerevisiae* was the first of the YidC/Oxa1/Alb3 protein family be to linked to the insertion of both mitochondrially- and nuclearly-encoded proteins in the mitochondrial inner membrane. This evolutionarily conserved pathway has been found in archaea, eukaryotic organelles and in bacteria. Proteins of the YidC/Oxa1/Alb3 family share a conserved topology of five transmembrane segments (Yen *et al.*, 2001; Wang and Dalbey, 2011; Saller *et al.*, 2012), and conduct the insertion and folding of membrane proteins, assembly into complexes and secretion (Tjalsma *et al.*, 2003; Kol *et al.*, 2008; Wagner *et al.*, 2008). One of the best-studied is YidC from *E. coli* that functions with the Sec pore or independently of it in the insertion of proteins in the inner membrane (Wang and Dalbey, 2011; Saller *et al.*, 2012). The genome of *B. subtilis*, like many Gram-positive bacteria, contains two Oxa1-like genes, *spoIIIJ* and *yqjG* (Kunst *et al.*, 1997; Yen *et al.*, 2001; Saller *et al.*, 2012). At least *spoIIIJ* is present in all spore-formers, generally in an operon with *jag* (*spoIIIJ*-associated gene). Whilst either is required for viability, being transcribed during vegetative growth and decreasing around the onset of sporulation, only *spoIIIJ* efficiently supports sporulation (Errington *et al.*, 1992; Murakami *et al.*, 2002; Serrano *et al.*, 2003; Tjalsma *et al.*, 2003; Rubio *et al.*, 2005). Several stresses may trigger sporulation, promoting conversion of a vegetative cell into a spore (Hilbert and Piggot, 2004). Careful regulation arises from the interplay of succeeding sigma factors of the RNA polymerase that thus transcribes different batches of genes, linking gene expression to key morphological events (Hilbert and Piggot, 2004). Note the powerful analogy between a *Bacillus* spore and a eukaryotic organelle, making sporulation an interesting but relatively simpler model for the study of protein targeting, secretion and membrane insertion. SpoIIIJ might cooperate with Sec in the biogenesis of its substrate SpoIIIAE, activity of the sigma factor σ^G (Serrano *et al.*, 2003; Camp and Losick, 2008). A fusion of GFP to the SRP receptor FtsY shows that it is transiently

enriched in invaginating septa and in complete non-engulfing septa; in contrast, as SecDF-GFP, SpoIIIJ- and YqjG-GFP localise uniformly in the membrane (Rubio *et al.*, 2005). SpoIIIJ and YqjG function similarly to other members, namely in the biogenesis of membrane proteins but may have acquired a new function, namely in the stability of secreted proteins (Tjalsma *et al.*, 2003; Saller *et al.*, 2009).

Here we show that SpoIIIJ and YqjG possess different types of signal peptides. SpoIIIJ has a type II signal peptide, typical of lipoproteins, which is recognised and cleaved by a type II signal peptidase, whereas YqjG possesses a type I signal peptide, but this is not the reason for their partial redundancy. We show that being a lipoprotein is neither necessary nor sufficient for a member of the YidC/Oxa1/Alb3 family to support sporulation, since non-lipoprotein SpoIIIJ variants support efficient sporulation but YqjG converted into a lipoprotein still does not. We also report that the signal peptides may be deleted without loss of function in viability and sporulation. We have therefore established that these signal peptides are not essential for function and hence that the determinants for viability or sporulation lie outside the signal peptide regions of either protein.

Materials and Methods

Media, bacterial strains and general techniques. The *B. subtilis* strains used in this work (listed in Table 1) are congenic derivatives of the Spo^+ strain MB24 (*trpC2 metC3*). Luria-Bertani (LB) medium was used for growth or maintenance of *E. coli* and *B. subtilis*, and sporulation was induced in Difco sporulation medium (DSM) (Henriques *et al.*, 1995; Henriques *et al.*, 1998).

Fusions of *spoIIIJ* and *yqjG* to a His-tag. C-terminal fusions to a His-tag were accomplished in two ways. In the first approach, a spectinomycin resistance cassette was isolated from pAH250 (Henriques *et al.*, 1998) with *Eco*RI and *Sal*I and ligated to the *yqjG*-containing plasmid pFV1 (Serrano

et al., 2008) digested with the same enzymes, resulting in pFV5. pFV4 (Serrano *et al.*, 2008) and pFV5 were transformed into MB24 and integrated via a single-crossover (Campbell-type) event at the corresponding locus (*spoIIIJ* and *yqjG*, respectively) producing AH9218 and AH9246, respectively. pFV4 and pFV5 were also recombined into *yqjG* or *spoIIIJ* genes previously introduced at the non-essential *amyE* locus. pLC103 was constructed by amplifying *yqjG* from wild-type chromosomal DNA from MB24 with primers PyqjG-460D and yqjG1256R, digesting with *Bam*HI and ligating to pDG364 (Cutting and Vander Horn, 1990) also digested with *Bam*HI. pLC103 was *Sca*I-linearised and transformed into JOB44 (Δ*spoIIIJ*::*km*; Serrano *et al.*, 2003) yielding AH5298, which produces AH5300 (Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG-his*) upon transformation with pFV5. For mutant *yqjG* genes, a PCR fragment was synthesised with PyqjG-460D and a reverse primer, which contains the desired mutation. The other fragment was amplified with a forward primer with that same mutation, and with yqjG1256R. pLC108 (see below) was used as template. The fragments were joined through splicing by overlap extension (SOE) (Horton *et al.*, 1989) using the external primers. The following primer pairs containing the chosen mutations, YqjGA26K_D and YqjGA26K_R, YqjGC19A_R, and YqjGC19A_D, YqjGlipoD and YqjGlipoR were used to obtain pLC104, pLC106 and pLC108, respectively, upon ligation of *Bam*HI-digested PCR products and pDG364. pLC104 and pLC106 were *Sca*I-linearised and introduced into JOB44 producing strains AH5299 and AH5307, respectively. AH5301 (Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG*(A26K)-*his*) and AH5309 (Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG(*C19A)-*his*) were obtained by transformation of AH5299 and AH5307 with pFV5, respectively, and AH5302 (Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG* Δ*yqjG*::*sp*) resulted from transformation of AH5298 with chromosomal DNA of JOB9 (Serrano *et al.*, 2008). To obtain the *spoIIIJ* C23A and C23K variants, this gene was amplified as two separate pieces with the following primer pairs: for C23A, PIIIJD with SpoIIIJC23A_R and SpoIIIJC23A_D with IIIJ1106R; for C23K, PIIIJD with SpoIIIJC23K_R and

SpoIIIJC23K_D with the reverse primer IIIJ1106R. Next, the fragments were joined using the external primers (PIIIJD and IIIJ1106R). Lastly, the two final products were digested with *Bam*HI and *Eco*RI and introduced between the same sites of pDG364 to produce pLC105 (bearing *spoIIIJ*(C23A)) and pLC109 (with *spoIIIJ*(C23K)). Transformation of JOB44 with *Sca*I-linearised pLC105 produced AH5306, which yielded AH5308 upon recombination of pFV4. Another strategy for obtaining gene fusions to histidine tags made use of pLC111, which was constructed with a PCR fragment encompassing the His-tag from pET-30c (Novagen) with primers HisD and HisR, digested with *Bam*HI and *Bgl*II, and introduced into *Bam*HI-cut pDG364; the correct orientation was verified by PCR analysis. The wild-type *yqjG* and *spoIIIJ* genes were amplified from chromosomal DNA of MB24 with primers PyqjG-460D and YqjG_His_R or PIIIJD and SpoIIIJ_His_R, cleaved with either *Bam*HI or with *Bam*HI and *Eco*RI (for the *yqjG* or *spoIIIJ* genes, respectively) and introduced into pLC111 similarly digested to produce pLC115 and pLC117, respectively. The mutated *yqjG* and *spoIIIJ* genes were constructed as their wild-type counterparts but using pLC104, pLC105, pLC109 and pLC108 as templates, yielding pLC116, pLC118, pLC119 and pLC120. Transformation of *Sca*I-linearised pLC115 through 120 into JOB44 yielded strains AH5324 through AH5329. AH5331 was obtained with AH5325 and chromosomal DNA from JOB9. AH5350 was produced by transformation of AH5329 with chromosomal DNA from AH5136 (see below). Strains AH5401 and AH5402 resulted from the transfer of either the *lgt* or *lspA* mutations from LUH102 (Bengtsson *et al.*, 1999) or ΔlspA (Antelmann *et al.*, 2001) into MB24, respectively. Strains AH5326 through 29 and AH5324 were transformed with chromosomal DNA from AH5401 and AH5402 yielding strains AH5403 through AH5412.

Signal peptide deletions. Primers pairs PIIIJD and spoIIIJ200R, and PyqjG-460D and yqjG400R were used to PCR-amplify the *spoIIIJ* ($P_{spoIIIJ}$) and *yqjG* (P_{yqjG}) promoter regions from chromosomal DNA of MB24. These

products were fused through PCR to versions of the *spoIIIJ* or *yqjG* genes lacking their signal peptides (SP), in turn produced with primers dSPIIIJD and SpoIIIJ-His-R, or with dSPyqjGD and YqjG-His-R, respectively. The final PCR products were digested either with *Eco*RI and *Bam*HI or only *Bam*HI and cloned into pLC111 to create pLC141 and pLC142, respectively. Transformation of JOB44, with these linearised plasmids produced strains AH5360 and AH5361, respectively.

Fusions of P_{spac} to *spoIIIJ*. *spoIIIJ* was amplified from chromosomal DNA of MB24 with forward primers SpoIIIJ172D or PspacdSPIIIJ and the reverse primer IIIJ1106R. The PCR products were digested with *Spe*I and *Bam*HI and introduced between the *Xba*I and *Bgl*II sites of pDH88 (Henner, 1990). This produced pLC27 (bearing a fusion of P_{spac} to the wild-type *spoIIIJ* gene) and pLC158 (P_{spac} fused to *spoIIIJ* deleted for the signal peptide). Both plasmids were then digested with *Eco*RI and *Bam*HI and a fragment containing *lacI* plus either P_{spac}-*spoIIIJ* allele was isolated and introduced between the same sites of pDG364, producing pLC28 and pLC159, respectively. JOB44 was transformed with either plasmid to produce AH5373 and AH5392, respectively. To place a wild-type *spoIIIJ* gene or an allele lacking the SP at its locus, pMS177 and pLC162 were used, respectively. For pMS177, a fragment derived from the 5'-end of *spoIIIJ* was PCR-amplified with primers spoIIIJ174D and spoIIIJ623R from chromosomal DNA of MB24, digested with *Sph*I and *Bgl*II and inserted into pDH88 similarly digested. To produce pLC162, the *spoIIIJ* gene was amplified from pLC117 with primers PspacdSPIIIJ and spoIIIJ610R, the PCR product was digested with *Spe*I and introduced between the *Bgl*II (Klenow filled-in) and *Xba*I sites of pDH88. MB24 was transformed with pMS177 and pLC162 with a single crossover event producing AH5074 and AH5413, respectively.

Chimeras of SpoIIIJ and YqjG. To construct pLC47 (chimera I) and pLC48 (J), $P_{spoIIIJ}$ and part of its coding region was amplified from

chromosomal DNA of MB24 with primer PIIIJD and either IIIJ269R or IIIJ272R, producing J47 and J48. The *yqjG* fragments were synthesised with either SPIIIJY-A or SPIIIJY-AS as forward primers, and yqjG1256R as reverse primer, yielding Y47 and Y48, respectively. Joining of J47 with Y47, and of J48 with Y48 occurred by PCR using the external primers PIIIJ and yqjG1256R. The PCR products JY47 and JY48 were digested with *Eco*RI and *Bam*HI and introduced between the same sites of pDG364, producing pLC47 and pLC48, respectively. To obtain pLC26-1 (chimera F), primers spoIIIJ-yqjG-prom and Ypep2R were used to amplify a *yqjG* fragment using pLC16 as a template (Chapter II). The *spoIIIJ* regions were amplified using PIIIJD with spoIIIJ200R ($P_{spoIIIJ}$), and Ypep2F with spoIIIJ1039R (C-terminus) from chromosomal DNA of MB24. The coding regions were joined by PCR with primers Ypep2F and spoIIIJ1039R, and the resulting product was linked to $P_{spoIIIJ}$ with the most external primers. The final PCR fragment was digested with *Bam*HI and *Eco*RI and inserted between the same sites of pDG364. pLC26-1 was subjected to Quikchange (Stratagene) with primers SPyqjG3F and SPyqjG3R, yielding pLC26-2 (chimera G), which contains the addition of the amino acid residues LAGCS. Transformation of *Sca*I-linearised pLC26-1 and -2, and pLC47-8 into JOB44 yielded strains AH5091, AH5119, AH5129 and AH5139, respectively. pFV4 and pFV5 contain a His-tag fused to the C-terminus of either '*spoIIIJ* or '*yqjG*, respectively; transformation of AH5091 and AH5119 with pFV4, and of AH5129 with pFV5 produced AH5236-7 and AH5239 (chimeras F, G and I fused to a His_6 tag), respectively, by single-crossover event. Linkage analysis and PCR testing were performed to confirm that the His-tag had been introduced at the *amyE* locus.

An *lgt* deletion mutant. First, an internal fragment derived the *lgt* gene was PCR-amplified with primers lgt288F and lgt675R from chromosomal DNA of MB24. Second, the *lgt* fragment was cleaved with *Eco*RI and *Bam*HI and cloned between the same sites of pMUTIN4 (Vagner *et al.*, 1998) to produce pLC50. Finally, pLC50 was transferred to the *lgt* locus of

MB24 by means of a single-crossover, as verified by PCR, to give strain AH5136. Chromosomal DNA from this strain was used to transform AH9218, producing AH5320 (Δ*spoIIIJ*::*spoIIIJ-his* Δ*lgt*::*erm*).

A *yqjG* conditional mutant. The P_{xylA}-*yqjG*-containing plasmid pMS178 was constructed in two steps, as follows: first, the P_{xylA} promoter was cleaved off from pGR40 (Real and Henriques, 2006) with *Sma*I and *Eco*RI and inserted in pUS19 (Benson and Haldenwang, 1993) similarly digested, yielding pMS176. A fragment derived from the 5' end of *yqjG* was PCR-amplified with primers yqjG362D and yqjG787R from chromosomal DNA of MB24. The *yqjG* fragment was cleaved with *Spe*I and *Eco*RI and cloned between the same sites of pMS176 to produce pMS178. pMS178 was transferred to the *yqjG* locus of MB24 by means of a reciprocal crossover, as verified by PCR, to give strain AH5420. Transformation of AH5009, AH5011, AH5360-1, AH5324, AH5373 and AH5392 with chromosomal DNA from AH5420 (Δ*yqjG*::P_{xylA}-*yqjG*) yielded strains AH5056, AH5352, AH5368-9, AH5372, AH5374 and AH5393.

Whole-cell lysates and immunoblot analysis. *B. subtilis* strains were grown in DSM and samples collected one hour (T_{-1}) before the end of the growth phase (defined as the onset of sporulation, or T_0), and 2 and 4 hours thereafter. Cells were resuspended in 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mM DTT and lysed with a French pressure cell at 19 000 lb/in^2. Proteins (30 μg) were electrophoretically resolved in 16.5 % tricine polyacrylamide gels (Tricine-SDS-PAGE) (Schägger and von Jagow, 1987) overnight. The resolved proteins were transferred to nitrocellulose membranes which were first incubated for 30 min in phosphate buffered saline (PBS) containing 5 % low-fat milk and overnight in PBS containing 0.5 % low-fat milk and a mouse anti-His_6 antibody for the detection of SpoIIIJ-His_6 or YqjG-His_6. Proteins were visualised with the ECL detection system (Amersham Biosciences) as described by the manufacturer.

Results

SpoIIIJ is a lipoprotein. σ^A drives transcription of both *spoIIIJ* and *yqjG* (Errington *et al.*, 1992; Murakami *et al.*, 2002). SpoIIIJ is required during sporulation for σ^G activation and when expressed solely in the prespore from a σ^F-dependent promoter it is able to support efficient sporulation (Errington *et al.*, 1992; Serrano *et al.*, 2003). Both genes are part of bicistronic operons: *spoIIIJ* with *jag* (*spoIIIJ*-associated gene) and *yqjG* with *mifM* (membrane protein insertion and folding monitor). Only SpoIIIJ is essential for efficient sporulation, unlike Jag and YqjG (Errington *et al.*, 1992; Murakami *et al.*, 2002; Serrano *et al.*, 2003; Tjalsma *et al.*, 2003).

To gain insight into the genetic organisation of *spoIIIJ* and *yqjG* genes in several bacterial genomes, we performed a BLAST search (www.ncbi.nlm.nih.gov). We observed that, in general, clostridial species possessed only one member of the YidC/Oxa1/Alb3 family, while *Bacillus* species possessed two. We reasoned that the gene next to *jag* should be considered as "*spoIIIJ*" and the homologous gene as "*yqjG*".

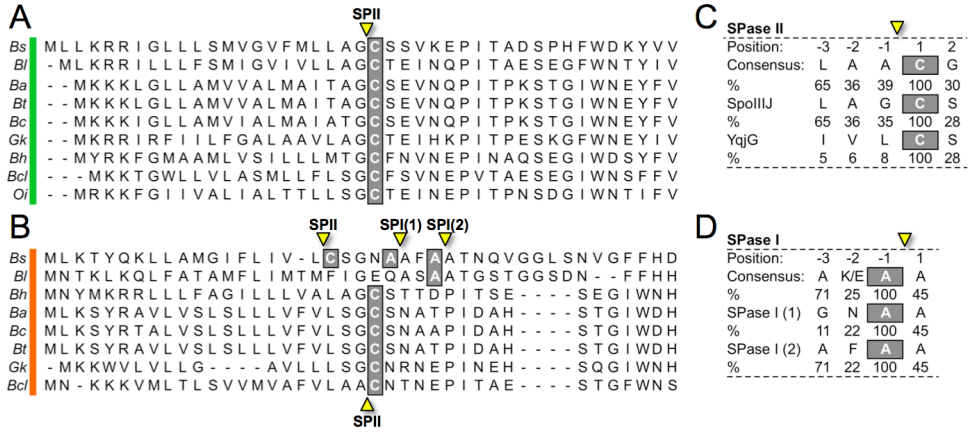

Figure 1. The signal peptides of SpoIIIJ and YqjG. Alignments containing the signal peptide regions of SpoIIIJ **(A)** and YqjG **(B)** proteins of selected organisms is shown. Putative cleavage sites for either type I (SPI) or type II (SPII) SPases are represented by arrowsheads; the conserved C or A residues are highlighted in grey. Note that two possible cleavage sites for SPI (sites 1 and 2) are indicated for YqjG from *B. subtilis*. Panels **(C)** and **(D)** show the consensus sequences for type II and type I SPases, respectively, according to Tjalsma *et al.* (2004), and compares the incidence of residues around the predicted cleavage sites present in SpoIIIJ or YqjG to the consensuses. Bs, *Bacillus subtilis*; Bl, *B. licheniformis*; Ba, *B. anthracis*; Bt, *B. thuringiensis*; Bc, *B. cereus*; Gk, *Geobacillus kaustophilus*; Bh, *B. halodurans*; Bcl, *B. clausii*; Oi, *Oceanobacillus iheyensis*.

By comparing the primary sequences of the SpoIIIJ and YqjG proteins with the predicted consensus for signal peptidases (SPases) I and II (cleaving Sec-type and lipoprotein signal peptides, respectively) (Tjalsma *et al.*, 2004) we observed that most SpoIIIJs (Fig. 1A) and YqjGs (Fig. 1B) from *Bacillus* species appear to have consensus sequences for cleavage by SPase II (lipobox) (Fig. 1C), suggesting that they are lipoproteins. This was supported by an analysis with DOLOP (Database of bacterial lipoproteins, http://www.mrc-lmb.cam.ac.uk/genomes/ dolop/). In a first approach to test whether *B. subtilis* SpoIIIJ is a lipoprotein we changed the cysteine residue (demanded by SPase II to cut) at position 23 into either an alanine (AH5308, Fig. 2A) or a lysine (AH5328, Fig. 2B). These substitutions were predicted to interfere strongly with cleavage by SPase II.

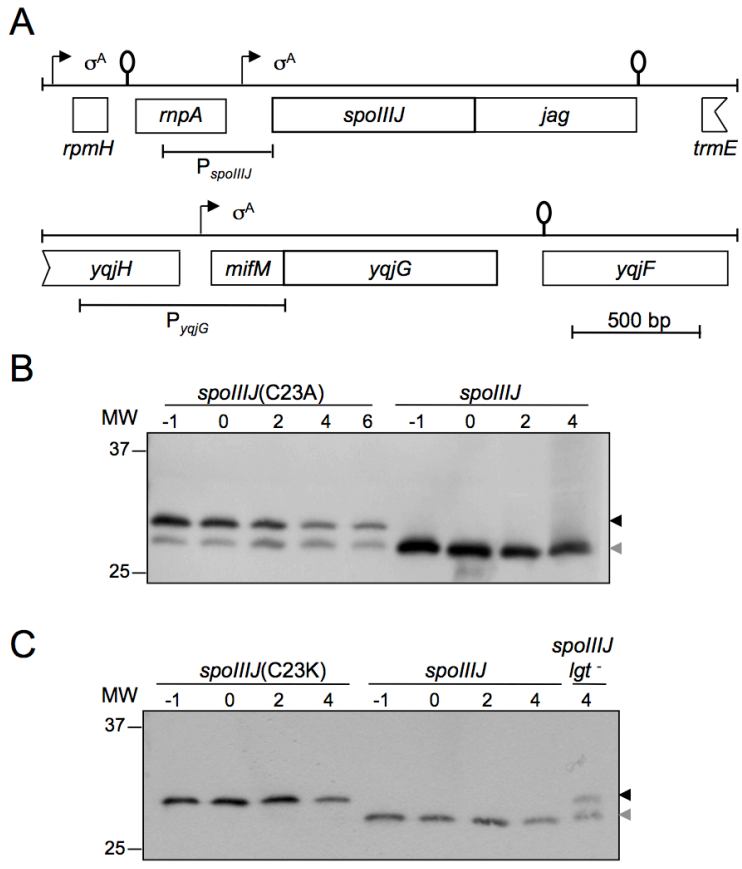

Figure 2. SpoIIIJ has a lipobox. (A) The genetic organisation of the *spoIIIJ-jag* and *mifM-yqjG* regions is depicted. An alternate name for *mifM* is *yqzJ*. The stem-loop structures indicate the position of possible transcription terminators. The positions of the promoters

are marked by arrows. Strains producing **(B)** SpoIIIJ(C23A)-His_6 (AH5308) and SpoIIIJ-His_6 (AH9218) or **(C)** SpoIIIJ(C23K)-His_6 (AH5328), SpoIIIJ-His_6 (AH5326), and SpoIIIJ-His_6 in the absence of *lgt* (AH5320), were grown in liquid DSM and samples withdrawn at the indicated times (in hours) relative to the onset of sporulation (T_0). Whole-cell extracts were then prepared, the proteins fractionated by Tricine-SDS-PAGE and immunoblotted using anti-His_6 antibodies. Black and grey arrowheads represent unprocessed and processed SpoIIIJ-His_6, respectively. The position of molecular weight markers (in kDa) is shown.

The *spoIIIJ* mutant genes were expressed from the *spoIIIJ* promoter at the non-essential *amyE* locus (in a *spoIIIJ*$^-$ background) except for AH9218 (*spoIIIJ*::*spoIIIJ-his*); wild-type sporulation levels were obtained for all strains (not shown). Cells were grown in DSM at 37ºC and samples were collected at the times indicated in Fig. 2 relative to the onset of sporulation, T_0. Whole-cell extracts were resolved by Tricine-SDS-PAGE and subjected to immunoblot analysis with anti-His_6 antibodies. The C23A substitution in the putative lipobox of SpoIIIJ caused the appearance of a higher molecular weight (MW) band relative to that of wild-type SpoIIIJ-His_6 (AH9218, Fig. 2A, grey arrowhead); this higher MW band most likely corresponds to a full-length, unprocessed form of SpoIIIJ-His_6 (AH5308, black arrowhead in Fig. 2A). However, a band of approximately the same MW as the one of wild-type SpoIIIJ-His_6 persists in this mutant at the time points tested. In Fig. 2B the C23K mutant also produces a higher MW band (AH5328, black arrowhead), as in the C23A mutant, when compared to the wild-type SpoIIIJ-His_6 (AH5326). However, in SpoIIIJ(C23K)-His_6, contrary to SpoIIIJ(C23A)-His_6, no band migrating at the level of the wild-type SpoIIIJ-His_6 is visible in the lanes containing SpoIIIJ(C23K)-His_6 (Fig. 2B). In the last lane both processed and unprocessed bands of SpoIIIJ-His_6 (in an *lgt* background, see below) are visible. We thus conclude that the cysteine 23 is part of a true lipobox.

In a second approach we disrupted the *lspA* and *lgt* genes, coding for SPase II and lipoprotein diacylglyceryl transferase, respectively. Both are required for the efficient cleavage of lipoproteins, with SPase II requiring previous action of Lgt (Prágai *et al.*, 1997; Leskelä *et al.*, 1999). Again, we examined the expression of wild-type SpoIIIJ-His_6 (grey arrowhead in Fig. 3A); in the absence of *lgt*, a higher MW band of SpoIIIJ-His_6 appears,

which should correspond to the unprocessed form of SpoIIIJ-His$_6$ (AH5403, black arrowhead in Fig. 3A), suggesting that SpoIIIJ is a lipoprotein. However, some processing still occurs in the absence of *lgt* since a band with an identical MW as the one present in the wild-type SpoIIIJ-His$_6$ persists in this mutant. SpoIIIJ(C23K)-His$_6$ was included as a control for the migration of the upper band (last lane).

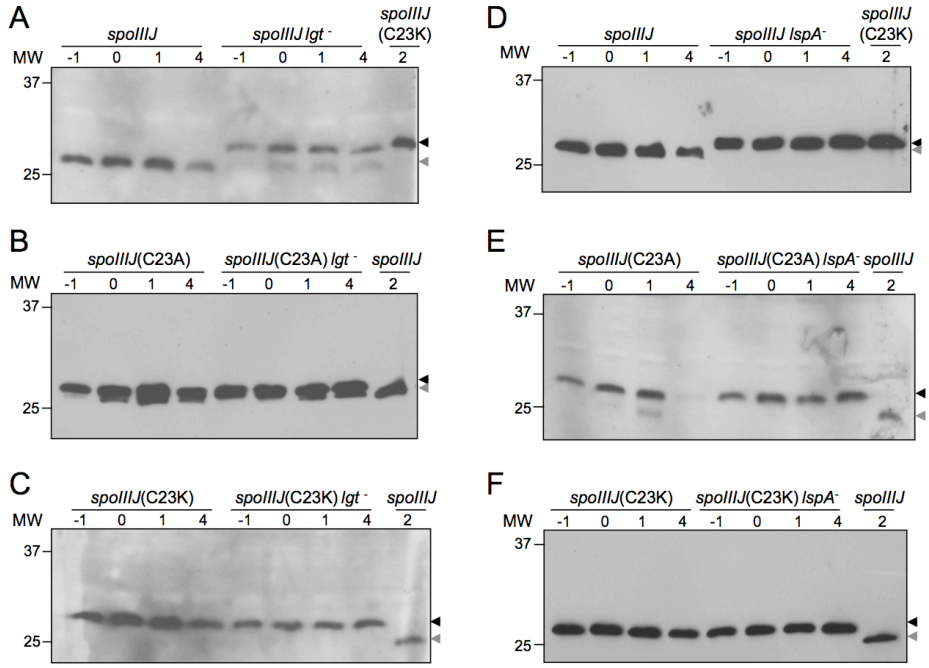

Figure 3. SpoIIIJ is cleaved by SPase II. (A) Strains producing SpoIIIJ-His$_6$ (AH5326), SpoIIIJ-His$_6$ in the absence of *lgt* (AH5403) and SpoIIIJ(C23K)-His$_6$ (AH5328) were grown in liquid DSM and samples withdrawn at the indicated times (in hours) relative to the onset of sporulation (T_0). Whole-cell extracts were then prepared, the proteins fractionated by Tricine-SDS-PAGE and immunoblotted using anti-His$_6$ antibodies. **(B)** through **(F)**: as in (A), except that the following additional strains were analysed, as indicated: AH5327 (producing SpoIIIJ(C23A)-His$_6$), AH5405 (SpoIIIJ(C23A)-His$_6$ in the absence of *lgt*), AH5328 (SpoIIIJ(C23K)-His$_6$), AH5407 (SpoIIIJ(C23K)-His$_6$ in the absence of *lgt*), AH5404 (SpoIIIJ-His$_6$ in the absence of *lspA*), AH5406 (SpoIIIJ(C23A)-His$_6$ in the absence of *lspA*), and AH5408 (SpoIIIJ(C23K)-His$_6$ in the absence of *lspA*). All strains bear a deletion of the *spoIIIJ* locus and the indicated His-tagged alleles of *spoIIIJ* at the *amyE* locus. The last lane of each panel is an internal control for the identification of the unprocessed (black arrowheads) or processed forms (grey arrowheads) of SpoIIIJ-His$_6$. The position of molecular weight markers (in kDa) is shown.

When testing the SpoIIIJ(C23A)-His$_6$ form we observe that again a higher MW band appears (black arrow in Fig. 3B) relative to the band obtained for the wild-type SpoIIIJ-His$_6$ (last lane, grey arrowhead), indicating that

the cleavage site for SPase II was affected, although only partial inhibition is occurring. The absence of *lgt* in the SpoIIIJ(C23A)-His_6 variant yields the same result of partial inhibition. When testing the SpoIIIJ(C23K)-His_6 variant we observed that only the unprocessed band accumulates (Fig. 3C, black arrowhead) when compared to the band indicated by the grey arrowhead in the last lane, containing wild-type SpoIIIJ-His_6. The absence of the *lgt* gene does not alter the phenotype of the SpoIIIJ(C23K)-His_6 variant, as in SpoIIIJ(C23A)-His_6 (Fig. 3B). The wild-type form was included in Fig. 3B-C for comparison (last lane in both cases).

With respect to the *lspA* mutation, we observed the expression of wild-type SpoIIIJ-His_6 in the absence of *lspA*, for which a higher MW band of SpoIIIJ-His_6 also appears (black arrowhead, Fig. 3D), as in the *lgt* mutant (Fig. 3A). This band, which migrates at the same level as the one from SpoIIIJ(C23K)-His_6 (last lane) should once more correspond to the unprocessed form of SpoIIIJ-His_6 suggesting that SpoIIIJ is a lipoprotein. Contrary to the *lgt* mutant (Fig. 3A), no processing was observed in the *lspA* mutant (Fig. 3D). For the SpoIIIJ(C23A)-His_6 form we observe that, contrary to the phenotype of this variant in the *lgt* mutant (Fig. 3B), the absence of *lspA* in the SpoIIIJ(C23A)-His_6 variant yields complete inhibition of processing (Fig. 3E). Concerning the SpoIIIJ(C23K)-His_6 variant we again observed that only the unprocessed band accumulates (Fig. 3C and F, black arrowhead) when compared with the band indicated by the grey arrowhead in the last lane, containing wild-type SpoIIIJ-His_6. The absence of *lgt* or of *lspA* (Fig. 3C and F, respectively) does not alter the phenotype of the SpoIIIJ(C23K)-His_6 variant. Whilst the C23K substitution in SpoIIIJ and *lspA* completely abolished processing at all tested time points (AH5328 in Fig. 3C and F; *lspA* mutants AH5404/6/8 in Fig. 3D, E and F; the black arrowhead indicates the unprocessed band, grey arrowhead indicates the processed form), the C23A substitution and *lgt* mutations produced an identical phenotype of only partial inhibition of cleavage (AH5327 in Fig. 3B and E; *lgt* mutants AH5403/5/7 in Fig. 3A, B and C). Surprisingly, SpoIIIJ(C23K)-His_6 is functional since this form

complements the null *spoIIIJ* mutant for sporulation (not shown) suggesting that being a lipoprotein is not required for this function.

YqjG has a type I signal peptide. YqjG was previously suggested to be a lipoprotein (Tjalsma *et al.*, 2003), but when we compared the N-terminus of YqjG with consensus sequences for SPase II and I it appears that YqjG is more likely to harbour a type I signal peptide (Fig. 1C-D). To investigate which signal peptide type YqjG possesses (either type I or a lipoprotein signal peptide), we designed several mutations aiming to destroy the potential cleavage sites. The mutant constructs were expressed from the *yqjG* promoter at the *amyE* locus in a *spoIIIJ*$^-$ background and subjected to immunoblot analysis as described above. For *yqjG*(C19A)-*his* (Fig. 4A, AH5309 at hours -1 to 6 relative to T_0) no difference was observed in the mobility of the bands when compared with the corresponding wild-type *yqjG-his* (Fig. 4A, AH5300, at hours -1 and 4), for which we observe two bands: the mature form, indicated by a grey arrowhead, and a faster-migrating band which appears during sporulation and is indicated by a white arrowhead. No higher MW band appears, indicating that C19 is not part of a lipobox. However, in *yqjG*(A26K)-*his* (AH5301 in Fig. 4A, hours -1 and 4) a shift in MW is clearly visible at both time points analysed (black arrowhead) indicating that the A26 residue is required for cleavage, and most probably by type I SPases. It is conceivable that the reduction in affinity of type I SPases for YqjG(A26K)-His_6 is mild and the only reason why we see the upper form is due to competition with the robust wild-type form present at its natural locus in this strain. We verified that in the absence of the native YqjG, inhibition of cleavage still occurred in AH5331 (*yqjG*(A26K)-*his spoIIIJ*$^-$ *yqjG*$^-$) (Fig. 4B, hours -1 through 4) similarly to the YqjG(A26K)-His_6 variant in a *spoIIIJ*$^-$ background (AH5325 in Fig. 4B), confirming that A26K strongly inhibits cleavage by type I SPases, also in the absence of wild-type YqjG. Also, sporulation levels were identical in AH5301, AH5331 and the corresponding wild-type AH5324 (not shown).

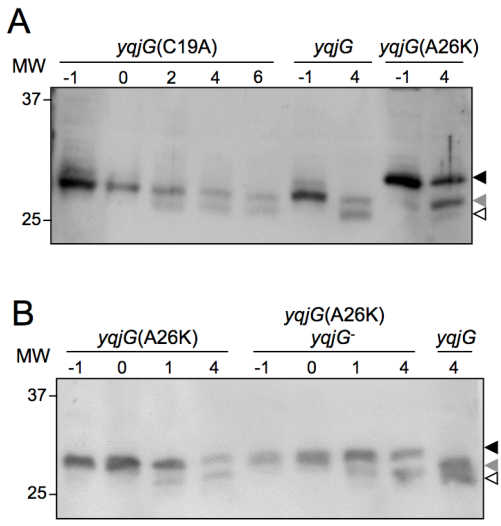

Figure 4. YqjG-His$_6$ is cleaved by type I SPases. Strains producing **(A)** YqjG(C19A)-His$_6$ (AH5309), YqjG-His$_6$ (AH5300), and YqjG(A26K)-His$_6$ (AH5301), or **(B)** YqjG(A26K)-His$_6$ (AH5325), YqjG(A26K)-His$_6$ in the absence of wild-type *yqjG* (AH5331), and YqjG(A26K)-His$_6$ (AH5324), were grown in liquid DSM and samples withdrawn at the indicated times (in hours) relative to the onset of sporulation (T_0). Whole-cell extracts were then prepared, the proteins fractionated by Tricine-SDS-PAGE and immunoblotted using anti-His6 antibodies. Note that all strains bear a deletion of the *spoIIIJ* gene. Black and grey arrowheads represent unprocessed and processed YqjG-His$_6$, respectively. White arrowheads point to a YqjG-His$_6$ product resulting from secondary processing. The position of molecular weight markers (in kDa) is shown.

Conversion of YqjG into a lipoprotein does not increase sporulation levels. As we have shown, YqjG and SpoIIIJ differ in their signal peptide type. We wanted to inspect if the conversion of YqjG into a lipoprotein would enable it to function during sporulation, therefore replacing SpoIIIJ. For that, we modified selected residues of the signal peptide of YqjG – A24L/F25A/A27C – with the aim of reproducing the most frequent lipobox sequence (see Fig. 1C; Tjalsma *et al.*, 2004); this substituted form will be referred to as YqjG-*lipo*. Analysis of YqjG and YqjG-*lipo* with DOLOP indicates that only the latter is a lipoprotein. Samples withdrawn from cultures grown in DSM were subjected to immunoblot analysis as described above. YqjG-*lipo*-His$_6$ accumulated both mature and faster-migrating bands, as expected (Fig. 5C, AH5329, hours -1 to 4 relative to the onset of sporulation, T_0). However, the appearance of the mature band could be due to the utilisation of the upstream potential SPI(1) cleavage site by type I SPases (see Fig. 1B and D). We reasoned that removing *lgt* and *lspA* should inhibit processing of the putative lipoprotein YqjG form, resulting in the appearance of an unprocessed, slower-migrating band. In both *spoIIIJ*$^-$ *lgt*$^-$ and *spoIIIJ*$^-$ *lspA*$^-$ backgrounds this upper form of YqjG-*lipo*-His$_6$ appears (Fig. 5C, AH5350 (*yqjG-lipo-his*

lgt$^-$), and 5D, AH5410 (*yqjG-lipo-his lspA*$^-$), black arrowhead) but not in the corresponding wild-type *yqjG-his* (Fig. 5A-B, AH5324, grey arrowhead), indicating that YqjG was indeed converted into a lipoprotein. In this view, we then tested whether YqjG-*lipo*-His$_6$ would indeed support efficient sporulation, replacing SpoIIIJ in this function. Remarkably, in heat tests this YqjG lipoprotein form was not able to complement a *spoIIIJ* mutant for sporulation (not shown).

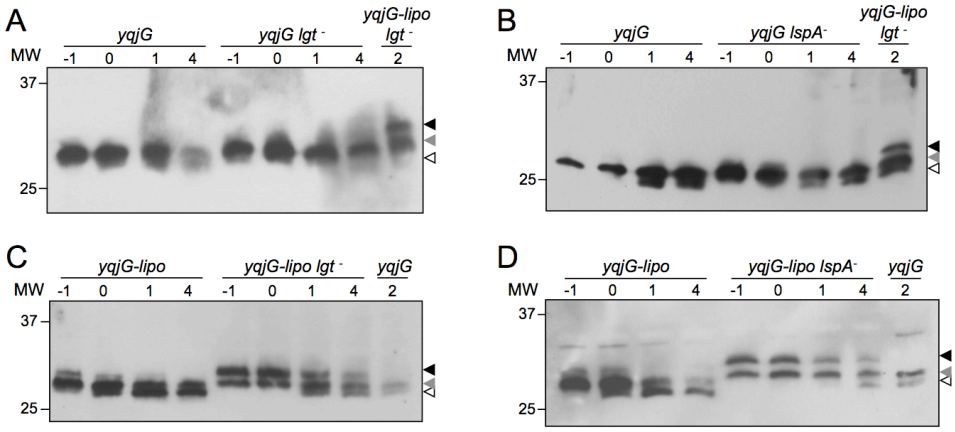

Figure 5. Conversion of YqjG into a lipoprotein. (A) Strains producing YqjG-His$_6$ (AH5324), YqjG-His$_6$ in the absence of *lgt* (AH5411) and YqjG-*lipo*-His$_6$, i.e., YqjG-His$_6$ bearing the A24L, F25A, and A27C substitutions (see text) in the absence of *lgt* (AH5409), were grown in DSM and samples collected at the indicated times relative to the onset of sporulation (T_0). Whole-cells extracts were prepared, proteins resolved by Tricine-SDS-PAGE and immunoblotted with anti-His$_6$ antibodies. **(B)** through **(D)**: as in (A) except that the following additional strains were used: AH5412, producing YqjG-His$_6$ in the absence of *lspA*; AH5329, producing YqjG-*lipo*-His$_6$; AH5350, producing YqjG-*lipo*-His$_6$ in the absence of *lgt*; and AH5410, producing YqjG-*lipo*-His$_6$ in the absence of *lspA*. All strains bear a deletion of the *spoIIIJ* gene and produce the His-tagged proteins from the *amyE* locus. Black arrowhead, unprocessed YqjG-His$_6$; grey arrowhead, processed YqjG-His$_6$; white arrowhead, YqjG-His$_6$ product of a secondary processing event. The position of molecular weight markers (in kDa) is shown.

The signal peptides of SpoIIIJ and of YqjG are dispensable for viability and sporulation. Since inhibition of cleavage does not seem to disturb the function of SpoIIIJ during sporulation (in the C23K *spoIIIJ*$^-$ mutant) and of YqjG during viability (in the A26K *yqjG*$^-$ *spoIIIJ*$^-$ mutant) we deleted the signal peptides of both SpoIIIJ and YqjG and tested the performance of those variants for viability and sporulation. Samples of DSM cultures were taken, resolved by Tricine-SDS-PAGE and subjected to immunoblot

analysis. Both ΔSPSpoIIIJ-His_6 and ΔSPYqjG-His_6 accumulate at levels similar to the wild-type forms (Fig. 6A, AH5360 (ΔSP*spoIIIJ-his spoIIIJ*$^-$) and AH5361 (ΔSP*yqjG-his spoIIIJ*$^-$), respectively, in hours -1 to 4 in both strains relative to T_0; not shown) so an eventual lack of function should not be due to unavailability of either protein. YqjG(A26K)-His_6 was used as a control for migration. Next, we tested the capacity of ΔSPYqjG-His_6 (AH5369, ΔSP*yqjG-his spoIIIJ*$^-$ P_{xylA}-*yqjG*) in sustaining viability, in LB supplemented with the appropriate antibiotics and either 0.1 % xylose to induce P_{xylA}-*yqjG* or 0.2 % glucose to repress it (Fig. 6C). This strain supported viability, behaving similarly to its wild-type counterpart possessing the signal peptide (AH5372), growing in both conditions. MB24 also grew in both conditions, as expected. In contrast, the negative control (*amyE*::*spoIIIG-lacZ spoIIIJ*::*km* P_{xylA}-*yqjG* (AH5056)) grew in the presence of xylose but not of glucose. In solid medium, plates containing DSM were incubated at 37°C for 20 h under repressing (Fig. 6B, 0.2 % glucose, upper panel) or inducing conditions (0.2 % xylose, middle panel). The results here obtained were identical to those obtained in liquid medium for strains AH5369, AH5372, AH5056, and AH5300; AH5300 is used as a positive control (Δ*amyE*::*yqjG-his* Δ*spoIIIJ*::*km*) instead of MB24, since the latter strain does not grow in the presence of the antibiotic here used to maintain P_{xylA}-*yqjG* introduced via a single crossover event.

Regarding ΔSPSpoIIIJ-His_6, its aptness in supporting viability was tested in the same manner as described for ΔSPYqjG-His_6, in liquid LB supplemented with either 0.2 % glucose (Fig. 7B, filled shapes) or 0.1 % xylose (Fig. 7C). In the presence of xylose, all strains grew similarly except for a reproducible difference observed for MB24. Both the wild-type and the SP-less forms of SpoIIIJ, when expressed from the *spoIIIJ* promoter (AH5352, *amyE*::$P_{spoIIIJ}$-*spoIIIJ spoIIIJ*::*km* P_{xylA}-*yqjG*, and AH5368, *amyE*::$P_{spoIIIJ}$-Δ*spoIIIJ-his spoIIIJ*::*km* P_{xylA}-*yqjG*, respectively) grew to suboptimal levels in the presence of glucose, although better than the negative control, AH5056 (Fig. 7B, groups III and IV, respectively), and grew normally under inducing conditions (Fig. 7C).

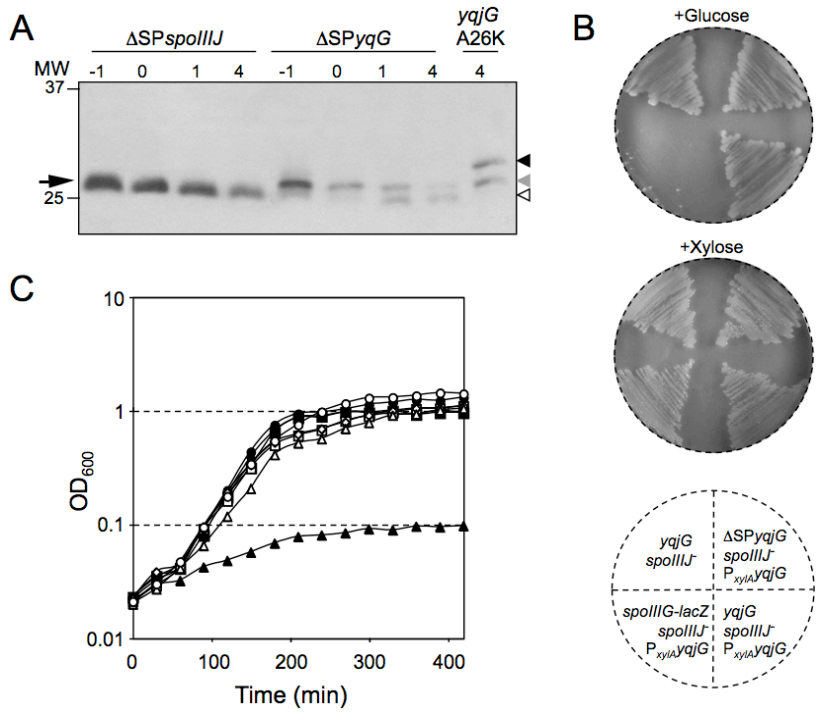

Figure 6. Functionality of signal peptide-less YqjG. (A) Strains producing ΔSPSpoIIIJ-His$_6$ (AH5360), ΔSPYqjG-His$_6$ (AH5361) and YqjG(A26K)-His$_6$ (AH5325) were grown in liquid DSM and samples withdrawn at the indicated times (in hours) relative to the onset of sporulation (T_0). Whole-cell lysates were prepared, fractionated by Tricine-SDS-PAGE and the proteins immunoblotted using anti-His$_6$ antibodies. All the His-tagged proteins were expressed from the *amyE* locus; all strains bear a *spoIIIJ* deletion, with other alterations as indicated. The black arrow (left) shows the position of ΔSPSpoIIIJ-His$_6$. Black and grey arrowheads correspond to unprocessed and processed YqjG-His$_6$, respectively. The white arrowhead indicates a YqjG-His$_6$ product of a secondary processing event. The position of molecular weight markers (in kDa) is shown. **(B)** Different strains were grown on DSM plates supplemented with either glucose (0.2 %) or xylose (0.5 %) as indicated, to test for the functionality of ΔSPYqjG-His$_6$; clockwise from the top left: AH5300 (Δ*amyE*::*yqjG-his* Δ*spoIIIJ*::*km*), AH5369 (Δ*amyE*::ΔSP*yqjG-his* Δ*spoIIIJ*::*km* Δ*yqjG*::P$_{xylA}$-*yqjG*), AH5372 (Δ*amyE*::*yqjG-his* Δ*spoIIIJ*::*km* Δ*yqjG*::P$_{xylA}$-*yqjG*), and AH5056 (Δ*amyE*::*spoIIIG-lacZ* Δ*spoIIIJ*::*km* Δ*yqjG*::P$_{xylA}$-*yqjG*). All plates contained the same antibiotics and were incubated at 37ºC for 20 h. **(C)** Growth curve in LB supplemented with either 0.2 % glucose (filled shapes) or 0.1 % xylose (open shapes) for the following strains: AH5056 (triangles), AH5369 (squares), AH5372 (diamonds) and MB24 (circles).

This altered phenotype was more drastic in solid medium for both AH5352 and AH5368 (Fig. 7A, left panels). We reasoned that maybe the amount of SpoIIIJ produced when expressed from the 421 bp *spoIIIJ* promoter was not sufficient to support viability, although sufficient to sustain efficient sporulation.

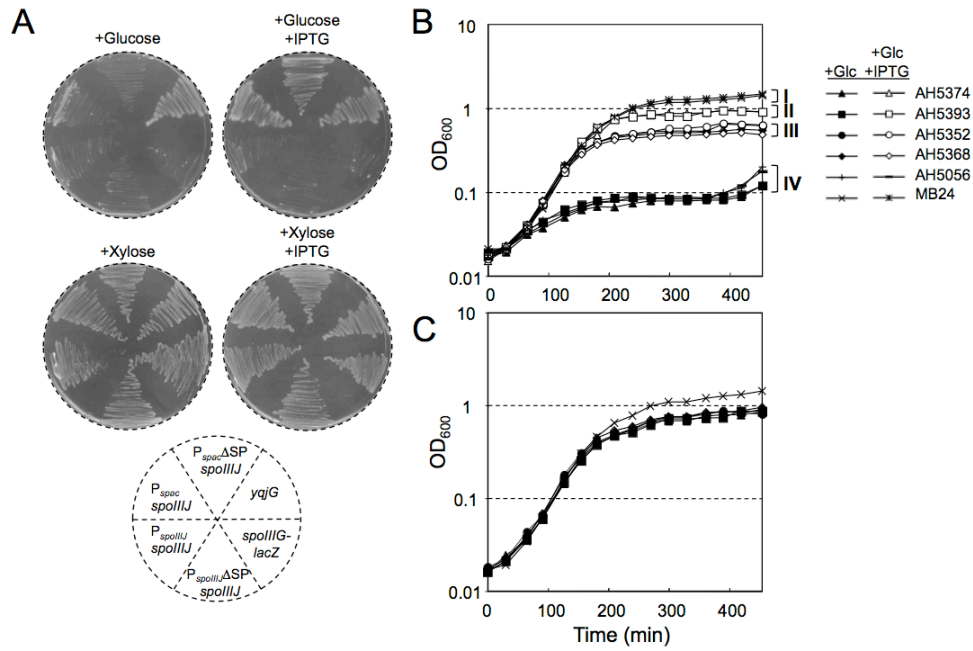

Figure 7. Deletion of the signal peptide of SpoIIIJ does not affect growth or viability. (A) Different strains were grown on DSM plates supplemented with either glucose (0.2 %), glucose and IPTG (1 mM), xylose (0.5 %), or xylose and IPTG as indicated, to test the functionality of the *spoIIIJ-his* and ΔSP*spoIIIJ-his* alleles. The following strains were tested (clockwise from the top middle): AH5393 (Δ*amyE*::P_{spac}-ΔSP*spoIIIJ-his* Δ*spoIIIJ*::*km* Δ*yqjG*::P_{xylA}-*yqjG*), AH5302 (Δ*amyE*::*yqjG-his* Δ*spoIIIJ*::*km* Δ*yqjG*::*sp*), AH5056 (Δ*amyE*::*spoIIIG-lacZ* Δ*spoIIIJ*::*km* Δ*yqjG*::P_{xylA}-*yqjG*), AH5368 (Δ*amyE*::$P_{spoIIIJ}$-ΔSP*spoIIIJ-his* Δ*spoIIIJ*::*km* Δ*yqjG*::P_{xylA}-*yqjG*), AH5352 (Δ*amyE*::$P_{spoIIIJ}$-*spoIIIJ-his* Δ*spoIIIJ*::*km* Δ*yqjG*::P_{xylA}-*yqjG*), and AH5374 (Δ*amyE*::P_{spac}-*spoIIIJ-his* Δ*spoIIIJ*::*km* Δ*yqjG*::P_{xylA}-*yqjG*). All plates contained the same antibiotics and were incubated at 37ºC for 20 h. Panels B and C show growth curves in LB supplemented with either **(B)** 0.2 % glucose (filled shapes), 0.2 % glucose and 1 mM IPTG (open shapes) or **(C)** 0.1 % xylose, for the following strains: Panel B - AH5056 (vertical line and rectangles for glucose and glucose + IPTG, respectively), AH5352 (circles), AH5368 (diamonds), AH5374 (triangles), AH5393 (squares) and MB24 (crosses and asterisks for glucose and glucose + IPTG, respectively); Panel C - AH5056 (triangles), AH5369 (squares), AH5372 (diamonds) and MB24 (circles). Group I: MB24 in the presence of glucose or glucose + IPTG; group II: AH5393 and AH5374 with glucose + IPTG; group III: AH5368 and AH5352 either with glucose + IPTG or glucose alone; group IV: AH5056 with glucose + IPTG or glucose alone, and AH5393 and AH5374 with glucose.

To inspect this hypothesis we expressed wild-type and SP-less *spoIIIJ* from the P_{spac} IPTG-inducible promoter in properly supplemented liquid medium. We verified that when glucose was present, only with IPTG was growth supported by either SpoIIIJ form (Fig. 7B; AH5374, *amyE*::P_{spac}-*spoIIIJ spoIIIJ*::*km* P_{xylA}-*yqjG*; AH5393, *amyE*::P_{spac}-ΔSP*spoIIIJ spoIIIJ*::*km* P_{xylA}-*yqjG*; these strains fall into group II in the presence of glucose + IPTG, and

into group IV when only in the presence of glucose). This phenotype was confirmed in solid DSM plates containing the appropriate antibiotics incubated at 37ºC for 20 h under P_{xylA}-*yqjG* repression (Fig. 7A, 0.2 % glucose, upper-left panel; 0.2 % glucose with 1 mM IPTG to induce P_{spac}, upper-right panel) or inducting conditions (Fig. 7A, 0.5 % xylose, bottom-left panel). A plate with both 0.1 % xylose and 1 mM IPTG was included as a control (Fig. 7A, bottom-right panel). All strains share a *spoIIIJ*⁻ P_{xylA}-*yqjG* background except for AH5302, here used as a positive control instead of MB24, since the latter strain does not grow in the presence of the antibiotic here used to maintain P_{xylA}-*yqjG* introduced via a single crossover event. Sporulation levels for AH5011, AH5360, AH5373 and AH5392 ($P_{spoIIIJ}$-*spoIIIJ*, $P_{spoIIIJ}$-ΔSP*spoIIIJ*, P_{spac}-*spoIIIJ* and P_{spac}-ΔSP*spoIIIJ* at the non-essential *amyE* locus, respectively, all in a *spoIIIJ*⁻ background) were similar to those of MB24 (not shown), even in the absence of IPTG for the two latter strains (not shown), confirming that low levels of SpoIIIJ are sufficient to sustain sporulation but not viability.

The effects of changes in the signal peptide region of chimerical proteins. Another strategy used in the attempt to elucidate the relevance of the signal peptide region in the distinct functionality of SpoIIIJ and YqjG during sporulation made use of chimerical proteins fused at different points, whilst trying to minimise changes in the structure of the final proteins (Fig. 8). These were expressed from the non-essential *amyE* locus from the *spoIIIJ* promoter in a *spoIIIJ*⁻ background. Chimeras F and G (AH5091 and AH5119, respectively, Fig. 8B) consist of the N-terminal region of YqjG up to the first hydrophobic region joined to the corresponding remainder SpoIIIJ, differing in three amino acid residues: chimera F contains the sequence "LIVLCSSV" and chimera G has an insertion of LAG before the C, creating the consensus site for cleavage by signal peptidase II present in SpoIIIJ (Tjalsma *et al.*, 2004). This produced a two-log difference in the titre of heat-resistant spores yielded by each strain (F and G, 10^5 ml^{-1} and 10^7 ml^{-1}, respectively), despite neither protein

being visible in anti-His_6 immunoblots (AH5236-7; not shown). Other chimeras were made (I, J), of a C-terminal YqjG fragment fused to the N-terminal region of SpoIIIJ including its signal peptide (AH5129 and AH5139, respectively; Fig. 8B). It is interesting to note that chimera I (containing VFMLLAGCATNQ) provided a titre of 10^5 ml^{-1} whilst chimera J (possessing an S after LAGC, which should improve the consensus) and YqjG-*lipo* (containing SGNALAACTNQ), which was confirmed to be a lipoprotein (Figs. 4-5), produced 10^1 ml^{-1} or no heat-resistant spores at all, respectively. Chimera I accumulates to very low levels (AH5239; not shown), and such was not tested for J. Regarding Y-*lipo*, note that LAAC is the most frequent consensus for signal peptidase II, immediately followed by that of SpoIIIJ. We can thus observe that very subtle changes, at least in the signal peptide region, may have profound effects on the functionality of chimeras, suggesting that if present, the signal peptide is of importance for the function of chimerical proteins during sporulation.

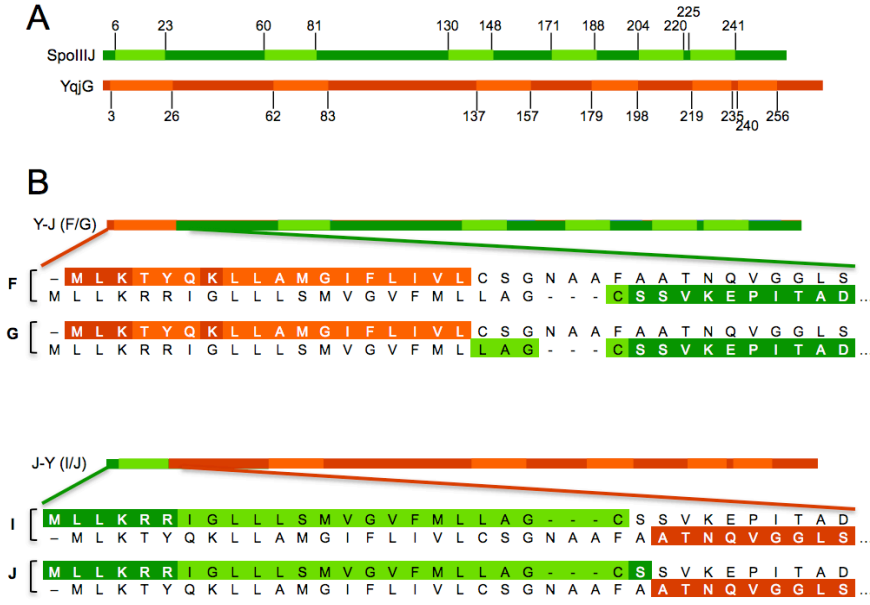

Figure 8. Chimerical proteins of SpoIIIJ and YqjG. (A) Schematic representation of SpoIIIJ (green) and YqjG (orange). Light-coloured regions represent transmembrane segments and the numbers indicate the amino acid residues in their boundaries. **(B)** Four chimeras of SpoIIIJ and YqjG (F, G, I, J) with the signal peptide region (SP) expanded. The SP regions are depicted as alignments of SpoIIIJ and YqjG. The residues that compose each chimera are highlighted in the same colour code as the native proteins.

Discussion

Here we have analysed the relevance of the signal peptides of both SpoIIIJ and YqjG in their function during vegetative growth, and also in sporulation in the case of SpoIIIJ. One of the major differences between SpoIIIJ and YqjG appeared to be the signal peptide type, as emerged from direct comparison of their sequences with the known consensus for cleavage by type I and II SPases (Tjalsma *et al.*, 2000; Tjalsma *et al.*, 2004). If authentic, could this difference at the level of the signal peptide be responsible for the dissimilar functionality of SpoIIIJ and YqjG during sporulation? To test this hypothesis several mutations were constructed, in the signal peptides themselves as well as in *lgt* and *lspA*, both genes being required for lipoprotein processing. *lspA* encodes SPase II, and requires the action of Lgt (prolipoprotein diacylglyceryl transferase) to cleave lipoprotein signal peptides. Curiously, the phenotype of *lgt*$^-$ and *lspA*$^-$ strains regarding the extracellular proteome of *B. subtilis* was reported to be quite different (Antelmann *et al.*, 2001) so we tested our mutant proteins in both backgrounds. In an *lgt* mutant, processing of both YqjG-lipo-His_6 and SpoIIIJ-His_6 is only partially inhibited, whereas in an *lspA* mutant only the processing of SpoIIIJ-His_6 is completely abolished, contrary to YqjG-lipo-His_6. This indicates that in *lgt* and *lspA* mutants at least some lipoproteins may suffer an alternative N-terminal processing (e.g. proteolytic "shaving"), and that uncleaved proteins may still retain activity, being or not shed into the medium (Tjalsma *et al.*, 2004; Tjalsma *et al.*, 2008).

Regarding SpoIIIJ, we observed that the C23K substitution blocks processing of any kind in each of the backgrounds here tested, but C23A only suffers complete inhibition of processing in the absence of SPase II. This discourages us from believing that type I SPases (for which SpoIIIJ(C23A) exhibits a reasonable closeness to the consensus sequence) are responsible for the partial processing as the absence of the cysteine residue should render the protein insensitive to a mutation in SPase II. Tjalsma and co-workers (1999) reported an alternative processing of pre-

PrsA, not dependent on type I SPases, which may be occurring here also for SpoIIIJ(C23A). SpoIIIJ and SpoIIIJ(C23A) could be alternatively processed N-terminally in an *lgt* mutant but not in *lspA*$^-$, as the lipid modification might inhibit the alternative processing. The proteases acting on SpoIIIJ and YqjG should be distinct: the K23 residue in SpoIIIJ may disrupt any possibility of contact between SpoIIIJ and the protease, allowed by an alanine, explaining the differential cleavage of both forms; yet, YqjG(A26K)-His_6 is cleaved. Also, YqjG-*lipo*-His_6 is partially cleaved in *lspA*$^-$, contrary to every SpoIIIJ form in the same background, again suggesting a different protease for YqjG, here for the lipoprotein form. In some polytopic proteins (CtaC) the lack of cleavage by SPase II resulted in protein instability (Bengtsson *et al.*, 1999), but in *Lactococus lactis* it was shown for some lipoproteins that processing by SPase II is not absolutely required for their activity (Venema *et al.*, 2003) and also in *B. subtilis* (Tjalsma *et al.*, 1999). Similarly, in the conditions in which SpoIIIJ was here tested, loss of the cysteine did not lessen its function.

Several cases have been described of proteins inserting into the membrane in the absence of a signal peptide. The ribosome-bound SRP was shown to identify long, highly hydrophobic signal peptides but also transmembrane anchors (Tjalsma *et al.*, 2004; Papanikou *et al.*, 2007). The mature part of a protein deprived of its signal peptide may contain export signals. Some uncleaved signal sequences combine two functions: targeting and membrane anchoring, being called "signal-anchors" (Martoglio and Dobberstein, 1998; Xie and Dalbey, 2008), and that might explain how SpoIIIJ and YqjG reach the membrane in the absence of their signal peptides. Alternatively, SpoIIIJ and YqjG may belong to a class of transmembrane proteins that can bypass the SRP pathway (Tjalsma *et al.*, 2004), or possibly remain partially unfolded being then targeted to the translocase, as in *prlA* mutants (Prinz *et al.*, 1996). Yet, the signal peptide region was observed to be sensitive to minor alterations, if present, despite being dispensable, at least in chimerical proteins. Chimera J contains an additional serine residue relative to chimera I that was

predicted to improve the consensus for cleavage by SPase II, but instead reduced sporulation levels. Chimera F does not contain strong signal peptide consensus sequences and provides a titre of 10^5 ml^{-1}, which shows a two-log increase in spore titre upon creation of a consensus for SPase II (chimera G) (Fig. 8). These results suggest that if present, the signal peptide region might be important for the function of SpoIIIJ and YqjG.

As mentioned above, both *spoIIIJ* and *yqjG* are transcribed during vegetative growth by RNA polymerase with σ^A and *spoIIIJ* is co-transcribed with *jag* from a promoter without a -35 region that would thus require an activator, as suggested by Errington and co-workers (1992). They also suggest the possibility of transcription occurring from an upstream promoter due to two observations: that no terminator exists between *rnpA* and *spoIIIJ*, and the presence of weak higher molecular weight bands in primer extension experiments. Possibly, some transcription occurs from the ribosomal protein L34 promoter (which generates at least an *rpmH-rpnA* transcript), needed in high amounts in the cell. However, *rpmH* mRNA levels are approximately 90-fold higher than those of *rnpA*, probably due to post-transcriptional regulation, since no obvious promoter exists between *rpmH* and *rnpA* (Gössringer *et al.*, 2006 and references therein). Nevertheless, any transcription of *spoIIIJ* occurring from this promoter, even in small quantities relative to L34 transcription, may explain the lack of growth in solid medium observed when *spoIIIJ* is expressed from its nearest promoter only. We examined the genetic organisation of *spoIIIJ* and *yqjG* regarding the presence of each gene and their relative localisation in the genome of several *Bacillus* species. The *mifM-yqjG* operon is localised quite differently in several species whilst *spoIIIJ-jag* is located near the origin of replication (not shown), exhibiting high synteny, possibly due to the close proximity to the crucial ribosomal protein L34. Regarding the signal peptide region, most of the homologues of SpoIIIJ/YqjG in *Bacillus* are predicted to be lipoproteins, with the exception of YqjG from *B. subtilis* and *B. licheniformis*. Inhibition of cleavage or even the total absence of the signal

peptides appears not to disturb SpoIIIJ function during sporulation (C23K variant and SP-less SpoIIIJ) and of YqjG during viability (YqjG(A26K) producing strain devoid of native YqjG; SP-less YqjG). Supporting the idea that the lipoprotein signal peptide is not needed for sporulation are the results of Camp and Losick (2008) and our results (Chapter III), regarding substitutions in YqjG that increase sporulation levels most likely by improving its interaction with SpoIIIAE, normally accomplished by SpoIIIJ. In addition, SpoIIIJ homologues of the genus *Clostridium* do not possess a signal peptide and are actually devoid of the corresponding region in *Bacillus* homologues (not shown), which indicates it is not required for function(s) in those organisms. Besides the case of *B. subtilis*, other examples in which only one of the homologues acts in a specific function exist in *Schizosaccharomyces pombe* (Bonnefoy *et al.*, 2000), *Chlamydomonas reinhardtii* (Göhre *et al.*, 2006) and in *Streptococcus mutans* (Funes *et al.*, 2009). We speculate that in these species the homologues are acquiring new functions, allowed by the presence of a functional homologue in the genome.

We conclude that the signal peptides of both SpoIIIJ and YqjG are essentially dispensable for viability and sporulation in *B. subtilis*. Determining which characteristics make SpoIIIJ able to support sporulation relative to YqjG and the mechanism through which it does so are major goals for future work.

Acknowledgements

The author of this Thesis performed all the experiments and constructed all the plasmids and strains required except for pMS176-8, AH5074 and AH5420 (by Mónica Serrano), and pFV5 (by Filipe Vieira). The author thanks Michael Hecker for the gift of LUH102 and of ΔlspA, and thanks Jan Maarten van Dijl for helpful discussions. The author is the recipient of a Ph.D. fellowship (SFRH/BD/6489/2001) from the Fundação para a Ciência e a Tecnologia.

Tables

Table 1. Bacterial strains.

Strain	Relevant Genotype[a]	Origin/ Reference
MB24	*trpC2 metC3*	Laboratory stock
JOB9	Δ*yqjG*::*sp* / Sp^r	Serrano *et al*., 2008
JOB44	Δ*spoIIIJ*::*km* / Km^r	Serrano *et al*., 2003
AH5009	Δ*spoIIIJ*::*km* Δ*amyE*::*spoIIIG-lacZ* / Km^r Cm^r	«
AH5011	Δ*spoIIIJ*::*km* Δ*amyE*::*spoIIIJ* / Km^r Cm^r	«
LUH102	*trpC2 lgt*::pMUTIN2, Δ*lgt*::*lgt-lacZ* / Erm^r	Bengtsson *et al*., 1999
ΔlspA	*trpC2 lgt*::pMUTIN2, Δ*lspA*::*lspA-lacZ* / Erm^r	Antelmann *et al*., 2001
AH5056	Δ*spoIIIJ*::*km* Δ*amyE*::*spoIIIG-lacZ* Δ*yqjG*::P$_{xylA}$-*yqjG* / Km^r Cm^r Sp^r	This work
AH5074	Δ*spoIIIJ*::P$_{spac}$-*spoIIIJ* / Cm^r	«
AH5091	Δ*spoIIIJ*::*km* Δ*amyE*::P$_{spoIIIJ}$-*yqjG-spoIIIJ*-26-1 / Km^r Cm^r	«
AH5119	Δ*spoIIIJ*::*km* Δ*amyE*::P$_{spoIIIJ}$-*yqjG-spoIIIJ*-26-2 / Km^r Cm^r	«
AH5129	Δ*spoIIIJ*::*km* Δ*amyE*::P$_{spoIIIJ}$-*yqjG-spoIIIJ*-47 / Km^r Cm^r	«
AH5136	Δ*lgt*::*erm* / Erm^r	«
AH5139	Δ*spoIIIJ*::*km* Δ*amyE*::P$_{spoIIIJ}$-*yqjG-spoIIIJ*-48 / Km^r Cm^r	«
AH5236	Δ*spoIIIJ*::*km* Δ*amyE*::P$_{spoIIIJ}$-*yqjG-spoIIIJ*-26-1-*his* / Km^r Cm^r Sp^r	«
AH5237	Δ*spoIIIJ*::*km* Δ*amyE*::P$_{spoIIIJ}$-*yqjG-spoIIIJ*-26-2-*his* / Km^r Cm^r Sp^r	«
AH5239	Δ*spoIIIJ*::*km* Δ*amyE*::P$_{spoIIIJ}$-*yqjG-spoIIIJ*-47-*his* / Km^r Cm^r Sp^r	«
AH5298	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG* / Km^r Cm^r	«
AH5299	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG*(A26K*)* / Km^r Cm^r	«
AH5300	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG-his* / Km^r Cm^r Sp^r	«
AH5301	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG*(A26K)-*his* / Km^r Cm^r Sp^r	«
AH5302	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG-his yqjG*::*sp* / Km^r Cm^r Sp^r	«
AH5306	Δ*spoIIIJ*::*km* Δ*amyE*::*spoIIIJ*(C23A) / Km^r Cm^r	«
AH5307	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG*(C19A) / Km^r Cm^r	«
AH5308	Δ*spoIIIJ*::*km* Δ*amyE*::*spoIIIJ*(C23A)-*his* / Km^r Cm^r Sp^r	«
AH5309	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG*(C19A)-*his* / Km^r Cm^r Sp^r	«
AH5320	Δ*spoIIIJ*::*spoIIIJ-his* Δ*lgt*::*erm* / Sp^r Erm^r	«
AH5324	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG-his* / Km^r Cm^r	«
AH5325	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG*(A26K)-*his* / Km^r Cm^r	«
AH5326	Δ*spoIIIJ*::*km* Δ*amyE*::*spoIIIJ-his* / Km^r Cm^r	«
AH5327	Δ*spoIIIJ*::*km* Δ*amyE*::*spoIIIJ*(C23A)-*his* / Km^r Cm^r	«
AH5328	Δ*spoIIIJ*::*km* Δ*amyE*::*spoIIIJ*(C23K)-*his* / Km^r Cm^r	«
AH5329	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG*(A24L/F25A/A27C)-*his* / Km^r Cm^r	«
AH5331	Δ*spoIIIJ*::*km* Δ*yqjG*::*sp* Δ*amyE*::*yqjG*(A26K)-*his* / Km^r Sp^r Cm^r	«
AH5350	Δ*spoIIIJ*::*km* Δ*lgt*::*erm* Δ*amyE*::*yqjG*(A24L/F25A/A27C)-*his* / Km^r Erm^r Cm^r	«
AH5352	Δ*spoIIIJ*::*km* Δ*amyE*::P$_{spoIIIJ}$-*spoIIIJ-his* Δ*yqjG*::P$_{xylA}$-*yqjG* / Km^r Cm^r Sp^r	«
AH5360	Δ*spoIIIJ*::*km* Δ*amyE*::P$_{spoIIIJ}$-ΔSP*spoIIIJ-his* / Km^r Cm^r	«
AH5361	Δ*spoIIIJ*::*km* Δ*amyE*::P$_{spoIIIJ}$-ΔSP*yqjG-his* / Km^r Cm^r	«
AH5368	Δ*spoIIIJ*::*km* Δ*amyE*::P$_{spoIIIJ}$-ΔSP*spoIIIJ-his* Δ*yqjG*::P$_{xylA}$-*yqjG* / Km^r Cm^r Sp^r	«
AH5369	Δ*spoIIIJ*::*km* Δ*amyE*::P$_{yqjG}$-ΔSP*yqjG-his* Δ*yqjG*::P$_{xylA}$-*yqjG* / Km^r Cm^r Sp^r	«
AH5372	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG-his* Δ*yqjG*::P$_{xylA}$-*yqjG* / Km^r Cm^r Sp^r	«

AH5373	Δ*spoIIIJ*::*km* Δ*amyE*::P_{spac}-*spoIIIJ* / Km^r Cm^r	«
AH5374	Δ*spoIIIJ*::*km* Δ*amyE*::P_{spac}-*spoIIIJ* Δ*yqjG*::P_{xylA}-*yqjG* / Km^r Cm^r Sp^r	«
AH5392	Δ*spoIIIJ*::*km* Δ*amyE*::P_{spac}-ΔSP*spoIIIJ* / Km^r Cm^r	«
AH5393	Δ*spoIIIJ*::*km* Δ*amyE*::P_{spac}-ΔSP*spoIIIJ* Δ*yqjG*::P_{xylA}-*yqjG* / Km^r Cm^r Sp^r	«
AH5401	Δ*lgt*::pMUTIN2, Δ*lgt*::*lgt-lacZ* / Erm^r	«
AH5402	Δ*lspA*::pMUTIN2, Δ*lspA*::*lspA-lacZ* / Erm^r	«
AH5403	Δ*spoIIIJ*::*km* Δ*amyE*::*spoIIIJ-his* Δ*lgt*::pMUTIN2, Δ*lgt*::*lgt-lacZ* / Km^r Cm^r Erm^r	«
AH5404	Δ*spoIIIJ*::*km* Δ*amyE*::*spoIIIJ-his* Δ*lspA*::*lspA*::pMUTIN2, Δ*lspA*::*lspA-lacZ* / Km^r Cm^r Erm^r	«
AH5405	Δ*spoIIIJ*::*km* Δ*amyE*::*spoIIIJ*(C23A)-*his* Δ*lgt*::*lgt*::pMUTIN2, Δ*lgt*::*lgt-lacZ* / Km^r Cm^r Erm^r	«
AH5406	Δ*spoIIIJ*::*km* Δ*amyE*::*spoIIIJ*(C23A)-*his* Δ*lspA*::*lspA*::pMUTIN2, Δ*lspA*::*lspA-lacZ* / Km^r Cm^r Erm^r	«
AH5407	Δ*spoIIIJ*::*km* Δ*amyE*::*spoIIIJ*(C23K)-*his* Δ*lgt*::*lgt*::pMUTIN2, Δ*lgt*::*lgt-lacZ* / Km^r Cm^r Erm^r	«
AH5408	Δ*spoIIIJ*::*km* Δ*amyE*::*spoIIIJ*(C23K)-*his* Δ*lspA*::*lspA*::pMUTIN2, Δ*lspA*::*lspA-lacZ* / Km^r Cm^r Erm^r	«
AH5409	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG*-(A24L/F25A/A27C)-*his* Δ*lgt*::*lgt*::pMUTIN2, Δ*lgt*::*lgt-lacZ* / Km^r Cm^r Erm^r	«
AH5410	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG*-(A24L/F25A/A27C)-*his* Δ*lspA*::*lspA*::pMUTIN2, Δ*lspA*::*lspA-lacZ* / Km^r Cm^r Erm^r	«
AH5411	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG-his* Δ*lgt*::*lgt*::pMUTIN2, Δ*lgt*::*lgt-lacZ* / Km^r Cm^r Erm^r	«
AH5412	Δ*spoIIIJ*::*km* Δ*amyE*::*yqjG-his* Δ*lspA*::*lspA*::pMUTIN2, Δ*lspA*::*lspA-lacZ* / Km^r Cm^r Erm^r	«
AH5413	Δ*spoIIIJ*::P_{spac}-ΔSP*spoIIIJ* / Cm^r	«
AH5420	Δ*yqjG*::P_{xylA}-*yqjG* / Sp^r	«
AH9218	Δ*spoIIIJ*::*spoIIIJ-his* / Sp^r	«
AH9246	Δ*spoIIIJ*::*yqjG-his* / Sp^r	«

[a] All *B. subtilis* strains are *trpC2 metC3*. Km, kanamycin; Cm, cloramphenicol; Erm, erythromycin; Sp, spectinomycin.

Table 2. Oligonucleotides used in this study.

Primer	Sequence (5´to 3´)[a]
lgt288F	GGGGAATTCTCGGGCTGTGGATAGC
lgt675R	CAGGATCCTAAAAAGGCTCTGCTGACAGC
yqjGC19A-D	CTTTTTGATCGTATTA**GCT**TCGGGCAATGCTGC
yqjGC19A-R	GCAGCATTGCCCGA**AGC**TAATACGATCAAAAAG
spoIIIJC23A-D	CATGCTTTTGGCTGGA**GCT**TCGAGTGTGAAAGAG
spoIIIJC23A-R	CTCTTTCACACTCGA**AGC**TCCAGCCAAAAGCATG
SpoIIIJ-His-R	GAGGATCCCTTTTTCTTTCCTCCGGCTTTTTGC
YqjG-His-R	GAGGATCCTTTCACCGACTCAGTAAGAGCG
HisD	CTGGATCCCACCACCACCACCACCACTGAG
HisR	ACAGATCTCGTCCCATTCGCCAATCCGGATATAG
SpoIIIJC23K-D	ATGCTTTTGGCTGGA**AAA**TCGAGTGTGAAAGAG
SpoIIIJC23K-R	TCTTTCACACTCGA**TTT**TCCAGCCAAAAGCATG
PyqjG-460D	AGAGCGGGATCCCTGTATGGTGTATCG
yqjG400R	TTTTGTTCTCCTCCTTTTTATAAATGCG
yqjG362D	CATCTGGCATGCAAAATCGGGAAACATCCCG
yqjG787R	CAGCTTCATGAATTCCATTTGCAGCTC
YqjGA26K-D	TATGCTCGGGCAATGCTGCATTT**AA**AGCGACTAATCAGGTGGGAGG
YqjGA26K-R	CCTCCCACCTGATTAGTCGCT**TT**AAATGCAGCATTGCCCGAGCATA

yqjG1256R	CCCCGGATCCTTTTTGCACGGGGTTGC
PIIIJD	GTTGAATTCCGCCAGTTTGTCTTATATACGC
spoIIIJ172D	AACTAGTGTAAAGATTAATTATAGGAGGAAATG
spoIIIJ174D	GAGTGTAAAGATCTAATTATAGGAGG
spoIIIJ200R	TTCCTCCTATAATTAATCTTTACACTC
IIIJ1039R	CAATCCGGATCCTGTACTGCTTCATCGACATTTCGCCC
spoIIIJ-yqjG-prom	GATTAATTATAGGAGGGAttgttaaaaacatatcaaaaac
IIIJ1106R	GAAACCCTTTGGATCCCTCTTCAATAAC
spoIIIJ610R	ATCGGGAATTCATCCCGCCAATGG
spoIIIJ623R	CATCTGGCATGCAAAATCGGGAAACATCCCG
YqjGlipoD	GCTCGGGCAAT**GCTTTGGCT**GCA**TGC**ACTAATCAGGTGGGAG
YqjGlipoR	CTCCCACCTGATTAGT**GCA**TGC**AGCCAAAGC**ATTGCCCGAGC
dSPIIIJD	GATTAATTATAGGAGGAAATGAGTGTGAAAGAGCCGATCACTG
dSPyqjGD	CGCATTTATAAAAAGGAGGAGAACAAAATTGGCGACTAATCAGGTGGGAG
PspacdSPIIIJ	AACTAGTGTAAAGATTAATTATAGGAGGAAatgagtgtgaaagagccgatcactg
IIIJ269R	GCATCCAGCCAAAAGCATGAATACG
IIIJ272R	CGAGCATCCAGCCAAAAGCATGAATACG
SPIIIJY-A	CATGCTTTTGGCTGGATGC*GCG*actaatcaggtgggagg
SPIIIJY-AS	CATGCTTTTGGCTGGATGCtcggcgactaatcaggtggg
SPyqjG3F	GGTATCTTTTTGATCGTATTA**TTGGCTGGA**TGCTCGAGTGTGAAAGAGC
SPyqjG3R	GCTCTTTCACACTCGAGCA**TCCAGCCAA**TAATACGATCAAAAAGATACC
Ypep2F	CTTTTTGATCGTATTatgctcgagtgtgaaagagccgatc
Ypep2R	CGAGCATAATACGATCAAAAAGATACC

[a] Restriction sites are underlined, mutations in bold, fusions in different case.

Table 3. Plasmids.

Plasmid	Relevant features	Antibiotic resistance[a]	Source
pAH250	Sp^r cassette	*bla, sp*	Henriques *et al*., 1998
pDG364	Δ*amyE*::*cat*	*bla, cat*	Cutting and Vander Horn, 1990
pGR40	P_{xylA}	*bla, neo*	Real and Henriques, 2006
pUS19	Sp^r vector	*bla, sp*	Benson and Haldenwang, 1993
pMUTIN4	P_{spac}	*bla, erm*	Vagner *et al*., 1998
pDH88	P_{spac}	*bla, cat*	Henner, 1990
pLC16	Δ*amyE*::$P_{spoIIIJ}$-*yqjG*	*bla, cat*	Chapter II
pFV1	*'yqjG-his*	*km*	Serrano *et al*., 2008
pFV4	*'spoIIIJ-his*	*km, sp*	«
pFV5	*'yqjG-his*	*km, sp*	This work
pMS176	P_{xylA}	*bla, sp*	«
pMS177	P_{spac}-*spoIIIJ*	*bla, cat*	«
pMS178	P_{xylA}-*yqjG*	*bla, sp*	«
pLC26-1	Δ*amyE*::*yqjG-spoIIIJ-26-1 (chimera F)*	*bla, cat*	«
pLC26-2	Δ*amyE*::*yqjG-spoIIIJ-26-2 (chimera G)*	*bla, cat*	«
pLC27	P_{spac}-*spoIIIJ*	*bla, cat*	«
pLC28	Δ*amyE*::P_{spac}-*spoIIIJ*	*bla, cat*	«
pLC47	Δ*amyE*::*spoIIIJ-yqjG-47 (chimera I)*	*bla, cat*	«
pLC48	Δ*amyE*::*spoIIIJ-yqjG-48 (chimera J)*	*bla, cat*	«
pLC50	Δ*lgt*::*erm*	*bla, erm*	«
pLC103	Δ*amyE*::*yqjG*	*bla, cat*	«
pLC104	Δ*amyE*::*yqjG*(A26K)	*bla, cat*	«
pLC105	Δ*amyE*::*spoIIIJ*(C23A)	*bla, cat*	«
pLC106	Δ*amyE*::*yqjG*(C19A)	*bla, cat*	«

pLC108	Δ*amyE*::*yqjG*(A24L/F25A/A27C)-*his*	*bla*, *cat*	«
pLC109	Δ*amyE*::*spoIIIJ*(C23K)	*bla*, *cat*	«
pLC111	Δ*amyE*::*his*	*bla*, *cat*	«
pLC115	Δ*amyE*::*yqjG-his*	*bla*, *cat*	«
pLC116	Δ*amyE*::*yqjG*(A26K)-*his*	*bla*, *cat*	«
pLC117	Δ*amyE*::*spoIIIJ-his*	*bla*, *cat*	«
pLC118	Δ*amyE*::*spoIIIJ*(C23A)-*his*	*bla*, *cat*	«
pLC119	Δ*amyE*::*spoIIIJ*(C23K)-*his*	*bla*, *cat*	«
pLC120	Δ*amyE*::*yqjG*(A24L/F25A/A27C)-*his*	*bla*, *cat*	«
pLC141	Δ*amyE*::ΔSP*spoIIIJ-his*	*bla*, *cat*	«
pLC142	Δ*amyE*::ΔSP*yqjG-his*	*bla*, *cat*	«
pLC158	P_{spac}-ΔSP*spoIIIJ*	*bla*, *cat*	«
pLC159	Δ*amyE*::P_{spac}-ΔSP*spoIIIJ*	*bla*, *cat*	«
pLC162	Δ*spoIIIJ*::P_{spac}-ΔSP*spoIIIJ*	*bla*, *cat*	«

[a] *bla*, ampicillin; *cat*, cloramphenicol; *erm*, erythromycin; *sp*, spectinomycin; *km*, kanamycin; neo, neomycin.

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Chapter V

The conserved Cys134 residue of *Bacillus subtilis* SpoIIIJ is important for its dimerisation and endospore development

The author of this Thesis conducted all the *B. subtilis* experiments and constructed the *B. subtilis* strains and plasmids except for pFiV2-5. The results present in this Chapter are to be submitted for publication.

Abstract

During sporulation in *Bacillus subtilis*, the onset of activity of the late prespore-specific sigma factor σ^G coincides with completion of prespore engulfment by the mother cell. At this stage, the prespore becomes a free protoplast, surrounded by the mother cell cytoplasm and separated from it by two membranes that derive from the asymmetric division septum. Continued gene expression in the prespore, isolated from the surrounding medium, relies on the SpoIIIA-SpoIIQ secretion system assembled from proteins synthesised both in the mother cell and in the prespore. The membrane protein insertase SpoIIIJ, of the widely spread YidC/Oxa1/Alb3 family, is involved in the assembly of the SpoIIIA-SpoIIQ complex. Here we show that SpoIIIJ exists as a mixture of monomers and dimers stabilised by a disulphide bond. We show that residue Cys134 within transmembrane segment 2 (TM2) of SpoIIIJ is important to stabilise the protein in the dimeric form. Labelling of Cys134 with a Cys-reactive reagent could only be achieved under stringent conditions, suggesting a tight association, at least in part through TM2, between monomers in the membrane. Substitution of Cys134 by an Ala results in a higher proportion of the monomer and reduces SpoIIIJ function *in vivo*. Therefore, SpoIIIJ activity *in vivo* appears to require dimer formation.

Introduction

Regulation of protein activity is critical for cell metabolism and physiology. One way of achieving this is through oligomerisation. This term describes the association of molecules that may or not be different; the type of bond established may vary as well as its avidity and duration (Ali and Imperiali, 2005). Oligomerisation offers several advantages over a plain monomeric existence. Large proteins are more stable against denaturation and degradation, having a reduced surface area exposed to the solvent when compared to monomeric proteins; oligomers also offer improved error control during synthesis, coding efficiency (minimising genome size), control over the accessibility and specificity of active sites, and regulation of assembly. However, production of non-native oligomers can be deleterious, generating pathogenic states (Goodsell and Olson, 2000; Marianayagam *et al.*, 2004; Ali and Imperiali, 2005). Interestingly, protein oligomerisation in the membrane reduces unfavourable lipid-protein interactions (reviewed in Cymer and Schneider, 2012).

Protein secretion and membrane protein insertion are fundamental processes in all living organisms, and several pathways are known to serve those purposes, namely the Sec, Tat, and YidC/Oxa1/Alb3 pathways (Natale *et al.*, 2008; Du Plessis *et al.*, 2011; Saller *et al.*, 2012). These pathways have been extensively studied in bacteria, being well studied in the model organisms *Escherichia coli* and *Bacillus subtilis*. The most prominent one is the Sec pathway. Its core is the SecYEG channel, which is utilised both for secretion and membrane protein insertion, using distinct piloting factors that guide each class of proteins to SecYEG. The SecB chaperone in *E. coli* (possibly CsaA in *B. subtilis*) guides proteins to be secreted. In contrast, nascent membrane proteins are delivered to SecYEG by the signal recognition particle (SRP) (reviewed in Tjalsma *et al.*, 2000; Du Plessis *et al.*, 2011). The twin-arginine translocation (Tat) pathway has the peculiarity of permitting the transport of previously folded proteins (containing two consecutive arginine residues in the signal peptide region) across or into membranes (reviewed in Natale *et al.*,

2008). The YidC/Oxa1/Alb3 family members, which are present in archaea, bacteria and in eukaryotic organelles, participate in the biogenesis of membrane proteins. The first protein from this family to be identified was Oxa1 in mitochondria of *Saccharomyces cerevisiae*. Proteins from this family are polytopic membrane proteins that share an architecture of five transmembrane (TM) segments. They perform critical roles in membrane insertion and folding and also in the assembly of energy-transducing multimeric membrane complexes (reviewed in Saller *et al.*, 2012). The best-studied proteins from this family in bacteria are YidC (*E. coli*) and SpoIIIJ (*B. subtilis*), functioning with the above-mentioned Sec channel or independently of it. The Sec-independent function of these proteins is conserved and similar to that of Oxa1 in mitochondria, which lack the Sec system (Glick and Von Heijne, 1996; Saller *et al.*, 2009; Saller *et al.*, 2012). The large periplasmic region of YidC (absent in SpoIIIJ and from the conserved core that characterises the family) was found to be monomeric (Oliver and Paetzel, 2008; Ravaud *et al.*, 2008). However, full-length YidC was observed as a dimer through cryo-electron microscopy with a resolution of 14.4 Å (Kohler *et al.*, 2009) and in a projection map with a resolution of with 10 Å (Lotz *et al.*, 2008), suggesting that the determinants for dimerisation lie within the conserved regions of the protein. SpoIIIJ is required for spore formation in *B. subtilis* (Errington *et al.*, 1992). The conversion of vegetative cells into highly resistant spores is one of the responses of this organism that allows coping with highly stressful situations (Hilbert and Piggot, 2004). Besides SpoIIIJ, another member of this family exists in *B. subtilis*, YqjG. Although they can substitute for each other during growth, when they are most actively transcribed, only SpoIIIJ supports efficient sporulation (Errington *et al.*, 1992; Murakami *et al.*, 2002; Serrano *et al.*, 2003; Tjalsma *et al.*, 2003; Rubio *et al.*, 2005). Spore differentiation takes place in a cell divided into two unequally-sized compartments, between which successive waves of gene expression in one compartment or the other are activated in a coordinated manner and in register with the course of morphogenesis (Hilbert and

Piggot, 2004). Transcription is here controlled by the successive activation of sigma factors that confer specificity to the RNA polymerase. From the prespore, σ^F signals the activation of σ^E in the larger mother cell, which is in turn required for σ^G activity in the prespore following engulfment completion. σ^G is in turn responsible for the activation of σ^K in the mother cell. The onset of σ^G activity is coupled to the morphological signal of engulfment completion of the prespore by the mother cell, which is a key process of endosporulation (Kroos *et al.*, 1999; Hilbert and Piggot, 2004). SpoIIIJ is needed specifically during this step of sporulation for σ^G activity, possibly by participating in the biogenesis of at least one of the eight mother cell-encoded proteins in the *spoIIIA* operon. These proteins were suggested to be part of the SpoIIIA-SpoIIQ channel that is required for general macromolecular synthesis in the prespore and maintaining its physiology, thereby allowing σ^G activity (Errington *et al.*, 1992; Camp and Losick, 2008; Serrano *et al.*, 2008; Doan *et al.*, 2009).

Here we present a genetic and biochemical characterisation of the SpoIIIJ membrane protein insertase. We have purified this polytopic membrane protein from *E. coli* cells and shown that it forms a dimer. We also provide evidence that a cysteine residue predicted to be in the second TM segment of SpoIIIJ facilitates dimer formation by establishing a disulphide bond at the dimer interface. Replacement of the cysteine by an alanine residue results in a higher proportion of the monomer and under certain conditions impairs the activity of σ^G and reduces sporulation. Our results suggest a model in which a disulphide bond contributes, with additional non-covalent interactions, to the formation of a SpoIIIJ dimer, and that this dimer is important for SpoIIIJ's activity during sporulation.

Materials and Methods

Media, bacterial strains and general techniques. The *B. subtilis* strains used in this work (Table 1) are congenic derivatives of the Spo^+ strain MB24 (*trpC2 metC3*). Luria-Bertani (LB) medium was used for growth or maintenance of *E. coli* and *B. subtilis*, and sporulation was induced in Difco

sporulation medium (DSM) (Henriques *et al.*, 1995; Henriques *et al.*, 1998).

Strains and plasmids. A *spoIIIJ*(C134A) allele for expression in *E. coli* was constructed in two steps: an initial amplification from chromosomal DNA of the wild-type *B. subtilis* strain MB24 using primers pairs J112D with JC134A_D, and JC134A_R with Jhis (Table 2); next, the PCR fragments were joined using the external primers through splicing by overlap extension (SOE) (Horton *et al.*, 1989). The final PCR product was cleaved with *Nco*I and *Eco*RI, ligated to pETDuet-1 (Novagen, Darmstadt, Germany) similarly digested to obtain pFiV1, resulting in a fusion to a His_6 tag at the 3' of *spoIIIJ*(C134A). Wild-type *spoIIIJ* was expressed from pMS266 (Serrano *et al.*, 2008). Both plasmids were introduced into the *E. coli* C43(DE3) derivative of BL21(DE3), which allows higher expression of membrane proteins (Miroux and Walker, 1996).

For controlled expression of His_6 fusions to *spoIIIJ* and *spoIIIJ*(C134A) in *B. subtilis* from a promoter that can be induced with isopropyl-β-D-thiogalactopyranoside (IPTG), chromosomal DNA of MB24 was amplified with primers J174D and JhisR (for wild-type *spoIIIJ*) and primer pairs J174D with JC134A_R, and JC134A_D with JhisR, being the last two products joined by PCR (SOE) with the external primers (for the mutant *spoIIIJ*(C134A)). The final PCR products were digested with *Bgl*II and *Sph*I and ligated to pDH88 (Henner, 1990) similarly digested, generating pFiV2 and pFiV3, respectively. Both pFiV2 and pFiV3 were digested with *Eco*RI and *Bam*HI, the inserts were recovered and ligated to pDG1664 (Guerout-Fleury *et al.*, 1996) similarly digested to produce pFiV4 and pFiV5, respectively; these plasmids were inserted at the non-essential locus *thrC* of JOB44 (Δ*spoIIIJ*::*km*) (Serrano *et al.*, 2003) through a double recombination event, producing AH5425 (Δ*spoIIIJ*::*km* Δ*thrC*::P_{spac}-*spoIIIJ*-*his*) and AH5426 (Δ*spoIIIJ*::*km* Δ*thrC*::P_{spac}-*spoIIIJ*(C134A)-*his*), respectively. Either strain was transformed with Δ*yycR*::P_{sspE}-*cfp* from AH6566 (Serrano *et al.*, 2008) (constructed with DNA from BTD2633 (kindly provided by D. Rudner)) whilst selecting for Cm^r, resulting in AH5431 and AH5432,

respectively (Table 1). JOB44 was transformed with DNA of BTD2633 resulting in AH5433 (Δ*spoIIIJ*::*km* Δ*yycR*::P_{sspE}-*cfp*).

To construct pLC138, two PCR products were obtained with primer pairs PYqjG-460D with YA50C_R, and YA50C_D with YqjG-His-R, which were joined by PCR (SOE) using the external primers. Both the final PCR product and pLC111 (see Chapter IV) were digested with *Bam*HI and ligated. To construct the *yqjG*(A50C C142A)-*his* derivative, primers PYqjG-460D with YC142A_R were used to amplify the first half of the gene from pLC138, and primers YC142A_D with YqjG-His-R for the second half, using pLC115 (see Chapter IV) as a template. The products were joined by PCR (SOE) with the external primers, digested with *Bam*HI and cloned into pLC111 similarly digested, resulting in pLC155. Transformation of JOB44 with pLC155 resulted in AH5382 (Δ*spoIIIJ*::*km* Δ*amyE*::P_{yqjG}-*yqjG*(A50C C142A)-*his*).

Production of SpoIIIJ-His_6 and of SpoIIIJ(C134A)-His_6. *E. coli* strain C43(DE3) bearing the plasmids pMS266 or pFiV1 expressing *spoIIIJ-his* or *spoIIIJ*(C134A)-*his*, respectively, from a T7*lac* promoter, were grown in LB and protein induction with IPTG occurred at 37ºC for 3 h, as described previously (Miroux and Walker, 1996). Cells were broken three times with a French pressure cell at a pressure of 19 000 lb/in^2 in lysis buffer composed of 20 mM Tris-HCl pH 7.6, 50 mM NaCl, 20 % glycerol, 1 mM phenylmethylsulphonyl fluoride (PMSF) and DNAse I. The whole-cell extract was separated in soluble and membrane extracts through ultracentrifugation at 100 000 x *g* during 45 minutes at 4ºC. Membrane proteins were extracted from the pellet with 2 % dodecyl maltoside (DDM), 20 mM Tris-HCl pH 7.6, 20 % glycerol, 0.5 M NaCl and 10 mM imidazole, for 1 h on ice with mild agitation, and again ultracentrifuged as described above. Purification proceeded with a Ni^{2+}-NTA affinity column (Qiagen, Hilden, Germany) to which already solubilised SpoIIIJ-His_6 was applied. The resin was previously equilibrated with a buffer composed of 0.5 M NaCl, 0.1 % DDM 20 mM Tris-HCl pH 8.0 with 20 mM imidazole,

and then loaded. The column was washed five times with the same buffer. Bound protein was eluted with a similar buffer containing 25 mM, 50 mM, or 100 mM imidazole.

Circular dichroism. Purified SpoIIIJ-His_6 or SpoIIIJ(C134A)-His_6 were first dialysed in 20 mM Tris-HCl pH 7.6, 10 % glycerol, 0.5 M NaCl, 0.1 % DDM. Far-UV CD spectra were measured at 20ºC on a Jasco J-815D CD spectrometer using a quartz polarised 1 mm path length cuvette, from 200 to 260 nm.

Size exclusion chromatography. SpoIIIJ-His_6 or SpoIIIJ(C134A)-His_6 purified from *E. coli* were loaded onto a Superose 12 HR 10/30 column (GE Healthcare) previously equilibrated with a buffer composed of 20 mM Tris-HCl pH 7.6, 10 % glycerol, 0.5 M NaCl, 0.1 % DDM, and 100 mM imidazole. The column was calibrated with the gel filtration molecular markers Dextran Blue, aldolase (158 kDa), ovalbumin (43 kDa), chymotryspinogen A (25 kDa), and lysozyme (14 kDa), in the above-mentioned buffer at 0.3 ml min^{-1}. Fractions were subjected to immunoblot analysis.

BN-PAGE. Blue-native polyacrylamide gel electrophoresis (BN-PAGE) was performed using gradient gels (5-15 %) under conditions described previously (Schägger and von Jagow, 1991). An elution fraction of 9 μg of His-tagged purified SpoIIIJ containing 100 mM imidazole and 500 mM NaCl was used. HMW-Native (Amersham Biosciences) was used as molecular weight marker.

Fluorescence microscopy. Samples (0.6 ml) of DSM cultures were collected at 4 and 6 h after the onset of sporulation, either non-induced or induced with 0.5 mM IPTG, and resuspended in 0.2 ml of phosphate-buffered saline (8 mM sodium phosphate [pH 7.5], 150 mM NaCl) supplemented with the lipophilic membrane dye FM 4-64 (Molecular

Probes) at a final concentration of 10 μg ml^{-1}, and with the DNA dye 4-,6-diaminodino-2-phenylindole (DAPI) at 0.2 μg ml^{-1}, which were used for sporulation stage evaluation (Setlow *et al.*, 1991; Pogliano *et al.*, 1999). For the quantitative analysis of the P_{sspE}-*cfp* expression at least 200 cells were scored for the fluorescence patterns designated by low (class a) or high (class b). ImageJ (http://imagej.nih.gov/ij/) was used for quantification.

MalPEG labelling of *B. subtilis* SpoIIIJ-His_6. Cells grown in LB were resuspended in 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM PMSF, 1 mM Tris(2-carboxyethyl)phosphine (TCEP) and lysed with a French pressure cell at 19 000 lb/in^2. Membranes were isolated by a 60 min centrifugation at 100 000 x *g*, resuspended in 50 mM Tris-HCl pH 6.8, 1 mM TCEP and further incubated either at 37ºC or 80ºC, with or without methoxy-polyethylene glycol 5000 maleimide (malPEG), containing 0, 1, or 2 % SDS. Overnight dialysis was performed. To dilute malPEG, which distorts migration, an extra volume of 1 x LB containing 1 mM TCEP was added. Other conditions for labelling of SpoIIIJ-His_6 were tested: 10 % SDS; 2 % SDS + 8 M Urea; 1 % Triton + 4 M GdnHCl. Note that GdnHCl and SDS form a precipitate and could not be used in conjunction for this analysis; the following range of temperatures was tested: 4ºC, 37ºC, 60ºC and 80ºC.

Whole-cell lysates and immunoblot analysis. *B. subtilis* strains were grown in DSM and samples collected one hour (T_{-1}) before the end of the growth phase (defined as the onset of sporulation, or T_0), and 2 and 4 h thereafter. Cells were resuspended in 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM PMSF, 0.1 mM dithiothreitol (DTT) and lysed with a French pressure cell as above. Proteins (30 μg) were electrophoretically resolved in 12.5 % polyacrylamide gels (SDS-PAGE) (Laemmli, 1970), transferred to nitrocellulose membranes and subjected to immunoblot analysis with a mouse anti-His_6 antibody (Novagen, Darmstadt, Germany) for the detection of SpoIIIJ-His_6 and YqjG-His_6. The proteins were visualised with the ECL detection system (Amersham Biosciences) as described by the

manufacturer.

Topology prediction: SpoIIIJ's Cys134. TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html) and TOP PRED (http://mobyle.pasteur.fr /cgibin/portal.py?form=toppred) were used to predict the localisation of SpoIIIJ's Cys134 (cytoplasmic/ exterior/ in the membrane).

Results

A role for Cys134 in the oligomerisation of SpoIIIJ. SpoIIIJ is known to be required for spore formation in *B. subtilis*, but the complete elucidation of its mechanism of action remains elusive. Previous work performed in our lab has identified a possible interaction between SpoIIIJ and SpoIIIAE (Serrano *et al.*, 2008). SpoIIIJ is a polytopic membrane protein with five predicted transmembrane (TM) segments, an architecture shared by many members of the YidC/Oxa1/Alb3 family of membrane protein insertases (Saller *et al.*, 2012), and a predicted molecular mass of 27 kDa (Fig. 1A). In agreement, analysis by far-UV circular dichroism indicates that the purified and detergent-solubilised SpoIIIJ-His_6 corresponds to a folded α-helical-rich protein (Fig. S1). We purified SpoIIIJ-His_6 from cells of *E. coli* C43(DE3). SpoIIIJ-His_6 was solubilised with dodecyl maltoside (DDM) from the membrane fraction obtained by ultracentrifugation and further purified over a Ni^{2+}-column (Fig. S2A). About 0.8 mg/L of essentially pure SpoIIIJ-His_6, as judged by SDS-PAGE analysis, were obtained (Fig. S2A). The most abundant form of the protein migrates with an apparent mass of 23 kDa (Fig. 1B) (see also Serrano *et al.*, 2003). In addition to the monomer, higher molecular weight (MW) species (bands "a" and "b", with apparent sizes of 30 and 45 kDa, respectively) were detected upon migrating purified SpoIIIJ in Coomassie-stained SDS-PAGE gels and in anti-His_6 immunoblots; these and shorter forms of the protein are discussed below. The higher MW forms were not detected upon treatment

of the sample with the reducing agent DTT (Fig. 1B), which suggested that disulphide bonds between two cysteine (Cys) residues of the monomers could be responsible for the formation of oligomeric forms of SpoIIIJ.

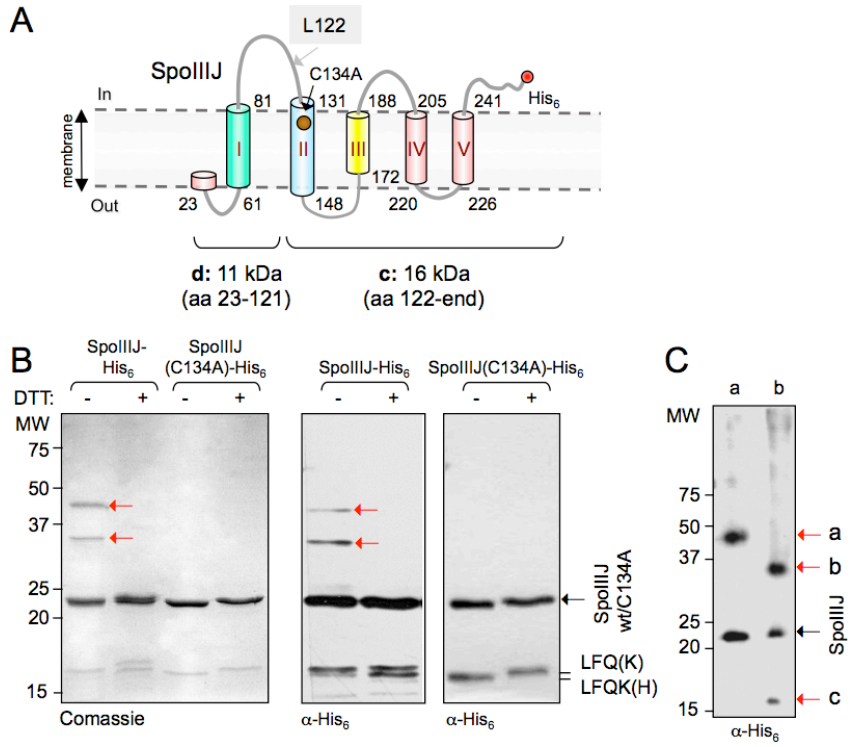

Figure 1. Dimerisation of SpoIIIJ-His$_6$ purified from *E. coli*. (A) Topological model of SpoIIIJ in the membrane plane. The numbers refer to the amino acid residues that delimitate the transmembrane (TM) segments, which are depicted as cylinders. The modified Cys23 of the signal peptide is indicated, as well as Cys134 (dark red circle). Red circle, His$_6$ tag. The expected molecular weight for regions "c" and "d" of SpoIIIJ are depicted. **(B)** SDS-PAGE and Coomassie-staining (left panel) and immunoblotting (right) with an anti-His$_6$ antibody of purified SpoIIIJ-His$_6$ and SpoIIIJ(C134A)-His$_6$ in loading buffer with (100 mM) or without dithiothreitol (DTT). The N-terminal sequence of two processing products of SpoIIIJ-His$_6$ is indicated. **(C)** Immunoblot analysis of SpoIIIJ-His$_6$ species "a" and "b", isolated from the gel in panel (B) (left), using an anti-His$_6$ antibody. In panels (B)-(C), the position of molecular weight markers (in kDa) is shown; black arrows show the position of full-length SpoIIIJ-His$_6$ and red arrows the position of other SpoIIIJ forms.

SpoIIIJ-His$_6$ has two Cys residues. One is located in the lipoprotein-type signal peptide and is modified during insertion of the protein into the membrane (Côrte *et al.*, manuscript in preparation, see Chapter IV). The other one, unmodified in mature SpoIIIJ-His$_6$, is Cys134, predicted to be located in TM2 (Fig. 1A). Cys134 was found to be highly conserved, as shown in the alignment of the TM2 segments of SpoIIIJ and YqjG proteins

from several *Bacillus* species and of TM3 of the related YidC protein from *E. coli* (Fig. S3A). A cysteine residue is found at the homologous position of YqjG (Cys142), the other member of the YidC/Oxa1/Alb3 family found in *B. subtilis*. As shown with several membrane topology prediction programs (see Materials and Methods), this residue seems to be always located within a TM segment (Fig. S3A). A helical wheel projection of the second TM segment of SpoIIIJ (Fig. S3B) showed it is a typical hydrophobic helix (Bowie, 1997; von Heijne, 2011). The Cys134 residue was the likely candidate to be involved in the formation of a disulphide bond between SpoIIIJ monomers. To test this, we overproduced and purified a C-terminal His-tagged SpoIIIJ(C134A) variant (Fig. S2B). In contrast to the wild-type, no higher MW bands of SpoIIIJ(C134A)-His_6 were detected either by Coomassie-staining or immunoblotting of SDS-PAGE gels in the presence or absence of DTT, an indication that the substitution rendered this SpoIIIJ variant insensitive to DTT (Fig. 1B), reinforcing the involvement of Cys134 in oligomerisation. Far-UV CD analysis showed that the C134A substitution is not deleterious for the SpoIIIJ fold, as the spectrum obtained for this purified and detergent solubilised variant is essentially identical to that of the wild-type protein (Fig. S1A-B). Nevertheless, whereas reduction of SpoIIIJ-His_6 with DTT yields minor secondary structure interconversions compatible with structural rearrangements upon reduction of the presumed Cys134-Cys134 disulphide, the same is not observed in the case of SpoIIIJ(C134A)-His_6 (Fig. S1C). Therefore, Cys134 is required for the formation of a DTT-sensitive bond (presumably a disulphide) that stabilises a multimeric form of SpoIIIJ.

In addition to the higher MW forms of SpoIIIJ-His_6 visualised in Fig. 1B (bands "a" and "b"), species migrating closely together at around 15 kDa were also seen (Fig. 1B). The apparent MW of band "a" is compatible with a dimer of full-length mature SpoIIIJ (that is, cleaved by signal peptidase II, see Chapter IV). Band "b" could, in turn, represent a heterodimer formed by full-length mature SpoIIIJ-His_6 and one of the species found

around the 15 kDa region of the gel. To test this hypothesis, bands "a" and "b" were excised from an SDS-PAGE gel and re-run separately. In both cases, the original higher MW bands were detected along with the monomeric form of SpoIIIJ (Fig. 1C, red and black arrows, respectively). Band "a" seems to be composed of two SpoIIIJ monomers, whilst band "b" is suggested to correspond to a monomer plus a truncated form of SpoIIIJ. Subfractionation of band "b" also revealed the presence of a lower MW band "c", that seems to correspond to the slower-migrating species of the doublet previously observed (Fig. 1B). N-terminal sequencing revealed their start at L122, where proteolytic cleavage possibly occurs. Cleavage at this position would generate fragments of 11 (from Cys23 to part of the first cytoplasmic loop (including TM1), fragment "d" in Fig. 1A) and 16 kDa (the remainder of that loop to the end of the protein (including TM2), fragment "c" in Fig. 1A). Possibly, the difference in mobility of the doublet is caused by some modification of the proteolytic product of SpoIIIJ-His_6. In any event, these observations support the idea that SpoIIIJ-His_6 purified from *E. coli* forms dimeric species. Moreover, they suggest that SpoIIIJ-His_6 can form a heterodimer with a form of the protein lacking TM1.

Analysis of the oligomeric state of SpoIIIJ-His_6 and SpoIIIJ(C134A)-His_6. Our results point to the involvement of Cys134 in the oligomerisation of SpoIIIJ (Fig. 1). To assess its oligomeric state, blue-native (BN) PAGE and size exclusion chromatography (SEC) were employed. With BN-PAGE, three species were observed that exhibited molecular weights compatible with a monomer, a dimer (the most represented species) and traces of a possible hexamer (Fig. 2A). SEC of purified SpoIIIJ-His_6 revealed two main peaks (Fig. 2B, top), both containing the protein (as shown by the immunoblot of the peak fractions; insert in Fig. 2B, top). The larger peak corresponds to a species with a calculated size (75 kDa) closer to that of a SpoIIIJ-His_6 dimer (Fig. 2B, top). The smaller peak is caused by a species with a calculated size (47 kDa)

closer to that of monomeric SpoIIIJ-His_6. The differences in these sizes from that estimated from BN-PAGE analysis probably result from the presence of DDM, the amount of which we were unable to estimate by SEC-MALLS (not shown). Peaks with the same estimated sizes were also observed for SpoIIIJ(C134A)-His_6 but, in this case, the size of peak B, corresponding to the smaller species, increased relative to peak A (Fig. 2B, bottom). Quantification of the peak areas shows ratios of B to A of 1:9 for SpoIIIJ-His_6 and 1:2.5 for SpoIIIJ(C134A)-His_6. Formation of the most represented species (peak A) does not require Cys134 but taken together our results suggest that Cys134 stabilises a multimeric (possibly dimeric) form of SpoIIIJ-His_6, in line with the suggestion that Cys134 is involved in formation of a disulphide bond between two monomers of the protein.

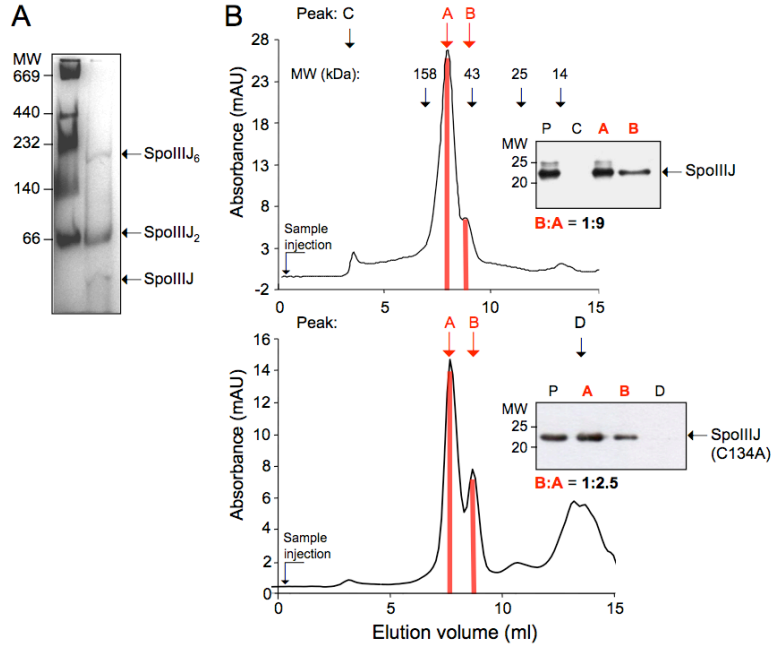

Figure 2. Oligomeric state of SpoIIIJ-His_6 and SpoIIIJ(C134A)-His_6. (A) Blue-Native PAGE of purified SpoIIIJ-His_6. Presumed hexameric ($SpoIIIJ_6$), dimeric ($SpoIIIJ_2$) and monomeric (SpoIIIJ) species are indicated. MW markers are shown in kDa. **(B)** Size exclusion chromatography of SpoIIIJ-His_6 (top) or SpoIIIJ(C134A)-His_6 (bottom) in the presence of 0.1 % DDM and 500 mM NaCl at pH 7.6 (see the Material and Methods section for details). Vertical black arrows indicate the elution volumes of the size standards and additional peaks. The inserts show the immunoblot analysis of the peaks indicated in the two panels. Purified SpoIIIJ-His_6 (top) or SpoIIIJ(C134A)-His_6 (bottom) were included as a migration control (lane P). The areas of the A and B peaks in the two panels were estimated using the ImageJ software (http://imagej.nih.gov/ij/) and their ratio indicated.

Cys134 of SpoIIIJ is part of a protein-protein interface in *B. subtilis*. Cys134 is predicted to occur in the membrane plane (Fig. 1A and S3). Furthermore, incubation of SpoIIIJ purified from *E. coli* with DTT was shown to abolish oligomer formation (Fig. 1), pointing to a role for Cys134 in a disulphide bond between SpoIIIJ-His_6 monomers. We wanted to examine whether Cys134 is located within the membrane and if it could be part of a protein-protein interaction interface. The membrane fraction of whole-cell lysates prepared from *B. subtilis* cultures was isolated by ultracentrifugation and the available Cys residues in the proteins present in the sample were labelled with the Cys-modifying reagent methoxypolyethylene glycol 5000 maleimide (malPEG for simplicity). This reagent binds free sulfhydryl groups in cysteine residues forming thioether bonds, whilst adding an extra 5 kDa per available cysteine residue. As malPEG induces distortion in gel migration, the minimum concentration required to obtain appreciable labelling was determined (1 mM, not shown). As malPEG is membrane-impermeable, SDS was also used to solubilise the membranes. If the cysteine is in the membrane plane, labelling was expected only in the presence of SDS. To increase the availability of free sulfhydryl groups by reducing eventual disulphide bonds, Tris(2-carboxyethyl)phosphine (TCEP) was used; unlike DTT, TCEP does not compete with the cysteine residues for malPEG. As a positive control for labelling we used a substituted form of YqjG, harbouring the A50C substitution. As for the highly similar SpoIIIJ (Fig. 1A), this position is predicted to be located outside the cytoplasmic membrane and facing the extracellular space (Fig. 3A); this residue should be more readily labelled with malPEG than a residue located in the plane of the membrane. Cys142 of YqjG (Fig. 3A) is homologous to Cys134 of SpoIIIJ and was substituted by an alanine to facilitate interpretation of the labelling pattern. Labelling of SpoIIIJ with malPEG was not detected even in the presence of 2 % SDS (Fig. 3B, lanes 1-2) or higher concentrations (not shown), whereas labelling of the positive control was clearly evident with 1 % SDS and also in its absence (Fig. 3B, lanes 3-4). This shift in

molecular weight was larger than the expected 5 kDa, which has also been reported previously (Jefferies and Forgac, 2008). The combined action of detergents and denaturants has been shown to disclose normally hidden residues (Koide *et al.*, 2007; Neale *et al.*, 2007). However, the combinations herein tested (GdnHCl, urea, Triton and SDS) did not result in labelling of Cys134 of SpoIIIJ (see Materials and Methods). Next, we further tested a wide range of temperatures (4ºC, 37ºC, 60ºC and 80ºC) in an attempt to modulate the availability of the cysteine residues. SpoIIIJ was labelled only at 80ºC and in the presence of SDS (Fig. 3C, lane 5). In contrast, incubation at 37ºC did not ensure labelling, despite the presence of SDS (lanes 1-4). The level of SpoIIIJ in mixtures incubated in the absence of SDS at 80ºC is strongly reduced (Fig. 3C, lanes 6 and 8), as the protein failed to enter the gel (not shown). Thus, high temperature and the presence of a detergent are required for labelling Cys134 with malPEG. These results are compatible with the view that Cys134 is located within the membrane plane and part of a tight protein-protein interface.

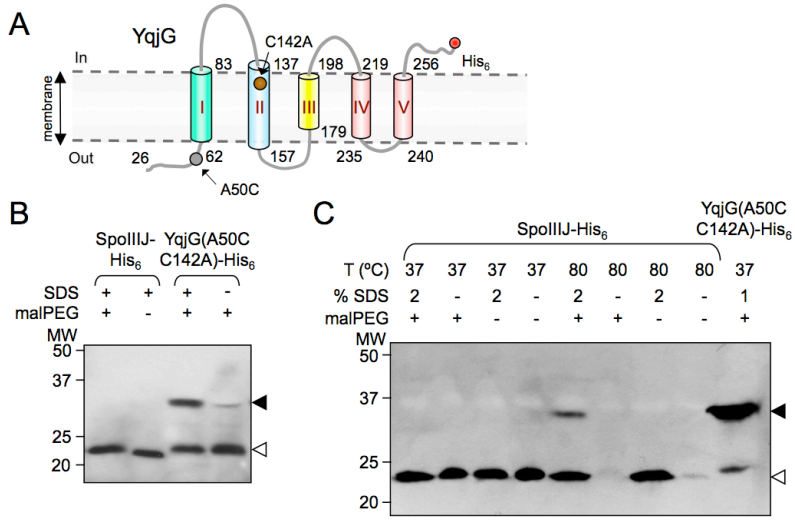

Figure 3. Labelling of YqjG(A50C C142A)-His$_6$ and SpoIIIJ-His$_6$ with malPEG. (A) Topological model of YqjG in the membrane. The numbers refer to the amino acid residues that delimit the transmembrane (TM) segments I-V. The positions of the A50C and C142A substitutions are indicated. **(B)** Strains producing SpoIIIJ-His$_6$ (AH9218, lanes 1-2) and YqjG(A50C C142A)-His$_6$ in a *spoIIIJ* mutant background (AH5382, lanes 3-4) were grown in liquid LB and samples were withdrawn. Cells were resuspended in a buffer containing 1 mM TCEP, lysed and membranes isolated. Membranes were resuspended in the presence of 1 mM TCEP and further incubated at 37ºC with (1 mM) or without malPEG (lanes 1 and 2, respectively, for AH9218; always with malPEG for AH5382) in the

presence of 2 % SDS (AH9218, lanes 1-2), 1 or 0 % SDS (AH5382, lanes 3 and 4, respectively). Proteins (30 µg) were electrophoretically resolved by SDS-PAGE and immunoblotted with an anti-His_6 antibody for the detection of SpoIIIJ-His_6 and YqjG(A50C C142A)-His_6. **(C)** Samples from cultures of strains producing SpoIIIJ-His_6 (AH9218, lanes 1-8) and YqjG(A50C C142A)-His_6 (AH5382, lane 9) were withdrawn at the onset of the stationary growth phase on LB medium. Samples were treated as in (B), except that no TCEP was added to the French press buffer. Incubation proceeded at the temperatures indicated (ºC) in the presence of 1 mM TCEP, ± malPEG and 0, 1 or 2 % SDS, as indicated. White arrowhead: bands from SpoIIIJ-His_6 or YqjG(A50C C142A)-His_6 in LB; SpoIIIJ-His_6 migrates slightly faster. Black arrowhead: SpoIIIJ-His_6 or YqjG(A50C C142A)-His_6 labelled reaction products. The position of MW markers (in kDa) is shown.

Accumulation of wild-type and substituted SpoIIIJ during sporulation in *B. subtilis*. *Bacillus subtilis* is able to sporulate when placed in hostile conditions. *spoIIIJ* is known to be required for sporulation (Errington *et al.*, 1992). To evaluate the ability of SpoIIIJ(C134A) to support efficient sporulation, both the wild-type and the mutagenised *spoIIIJ* variant were expressed from the IPTG-inducible promoter P_{spac}, placed at the non-essential *thrC* locus (Fig. 4A). When measured 24 h after the onset of sporulation in DSM, the non-induced mutant strain produced very low levels of heat-resistant spores when compared to its wild-type counterpart (AH5426 and AH5425, respectively) (Fig. 4B and Table 4). Importantly, the level of heat-resistant spores of AH5425 is similar to that of the wild-type MB24. In contrast, upon addition of IPTG both the wild-type and the mutant exhibited sporulation levels similar to those of MB24. Similar results were obtained at T_8 (Table 3). The fact that a low spore titre was only observed in the non-induced mutant suggested that under those conditions the SpoIIIJ(C134A)-His_6 form accumulates to lower levels or is less functional than the wild-type form. To distinguish between these possibilities, we analysed the levels of each protein under inducing and non-inducing conditions. When induced, both the wild-type (AH5431, *thrC*::P_{spac}-*spoIIIJ-his spoIIIJ*$^-$) and mutagenised (AH5432, *thrC*::P_{spac}-*spoIIIJ*(C134A)-*his spoIIIJ*$^-$) SpoIIIJ forms accumulate to levels similar to those of SpoIIIJ produced from its natural locus (AH9218) (Fig. 4C). When non-induced, the levels of both the wild-type and the mutant are extremely reduced, but similar to one another (Fig. 4C). Hence, it seems unlikely that different expression levels account for the difference in spore

titre observed for the two forms of the protein (Fig. 4B; Tables 3 and 4). Overall, these results suggest that SpoIIIJ(C134A) is less functional than the wild-type protein and that this reduced functionality emerges at low expression levels of the corresponding alleles.

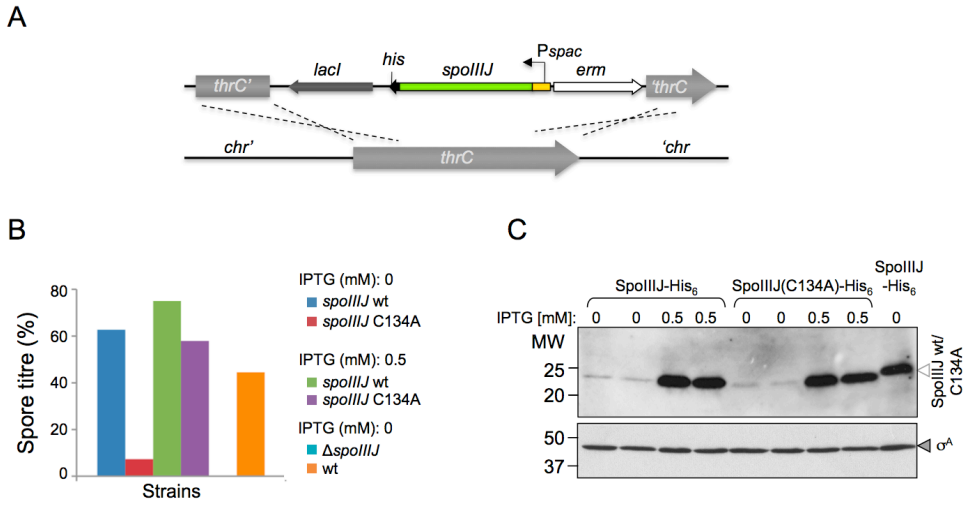

Figure 4. SpoIIIJ-His$_6$ and SpoIIIJ(C134A)-His$_6$ expressed from an inducible promoter accumulate at similar levels. (A) Schematic representation of the double crossover integration of a P$_{spac}$-*spoIIIJ-his* fusion at the non-essential *thrC* locus. The figure includes the position of the regulator *lacI*. **(B)** The titre of heat-resistant spores was assessed 24 h after the onset of sporulation with 0.5 mM or without the addition of IPTG to strains expressing either SpoIIIJ-His$_6$ (AH5425; non-induced, dark blue; induced, green) or SpoIIIJ(C134A)-His$_6$ (AH5426; non-induced, red; induced, purple) from an IPTG-inducible promoter in a *spoIIIJ* mutant background. JOB44 and MB24 (in light blue and orange, respectively) were included as controls. **(C)** Strains expressing SpoIIIJ-His$_6$ (AH5431, lanes 1-4), SpoIIIJ(C134A)-His$_6$ (AH5432, lanes 5-8) and SpoIIIJ-His$_6$ from its natural locus (AH9218, lane 9) were grown in liquid DSM with 0.5 mM or without the addition of IPTG as indicated and samples withdrawn at 4 and 6 h (odd and even lanes, respectively) relative to the onset of sporulation (T$_0$). Proteins in the whole-cell extracts were subjected to immunoblot analysis with an anti-His$_6$ (upper panel) or an anti-σ^A antibody (lower panel). With the exception of the strain producing SpoIIIJ-His$_6$ from its normal locus (AH9218), all strains carry a *spoIIIJ* deletion and produce SpoIIIJ-His$_6$ forms from the P$_{spac}$ promoter (as shown in panel (A)). The position of molecular weight markers (in kDa) is shown. The positions of SpoIIIJ-His$_6$ (wild-type or bearing the C134A substitution) and σ^A are indicated.

Requirement of Cys134 of SpoIIIJ for σ^G activity. The developmental process of sporulation begins with an asymmetric division that produces two compartments, followed by the engulfment of the smaller compartment, the prespore, by the larger mother cell (Fig. 5A). Since SpoIIIJ is required for the activity of the late prespore RNA polymerase

sigma factor σ^G (Errington *et al.*, 1992), we wanted to assess the impact of the C134A substitution on the activity of σ^G. We used the σ^G-controlled transcriptional fusion P_{sspE}-*cfp* (Doan *et al.*, 2009) that allows monitoring of σ^G activity in single cells by fluorescence microscopy (Fig. 5B).

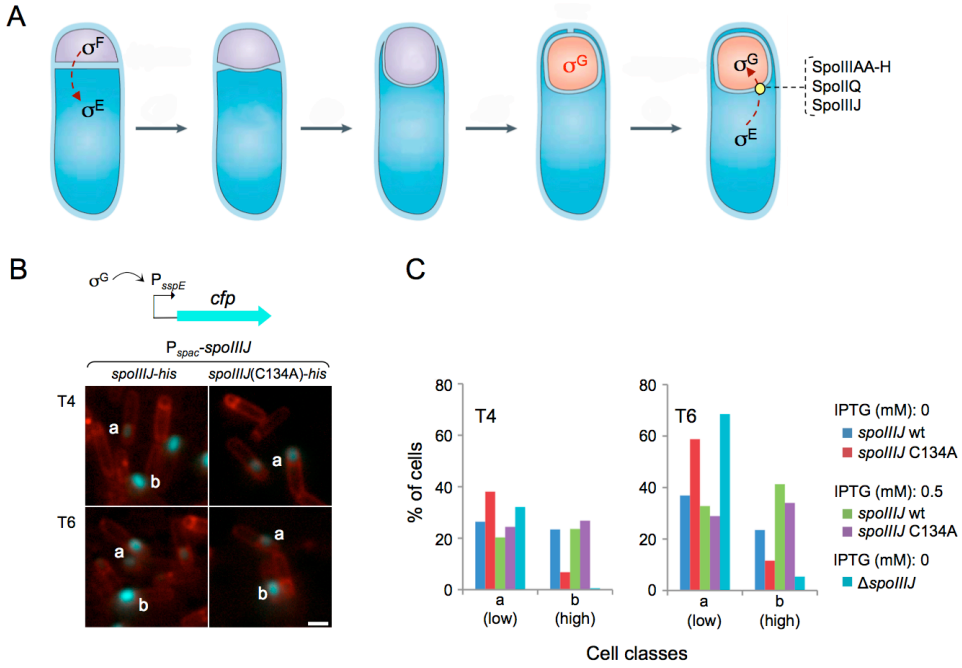

Figure 5. Cys134 of SpoIIIJ is required for proper functioning at low levels. (A) The main stages of engulfment and the periods of activity of the two main prespore-specific transcriptional regulators σ^F and σ^G are depicted. σ^F is activated in prespore soon after asymmetric division of the sporulating cell and drives activation of σ^E in the mother cell. Septal thinning and migration of the mother cell membrane around the prespore occurs, releasing it free in the mother cell cytoplasm. σ^G is only activated following engulfment completion, requiring σ^E and several proteins present in the membranes delimitating the prespore. Adapted from Errington, 2003, and Camp and Losick, 2008. **(B)** Expression of a P_{sspE}-*cfp* fusion, used as a reporter for σ^G activity, in DSM cultures of strains expressing wild-type *spoIIIJ-his* (AH5431) or the *spoIIIJ*(C134A)-*his* allele (AH5432) from the P_{spac} promoter at the *thrC* locus. Samples were collected at the indicated times (in hours) after the onset of sporulation, stained with the membrane dye FM 4-64 and observed by fluorescence microscopy. CFP and FM 4-64 fluorescence is shown in light blue and red, respectively. Scale bar, 1 μm. **(C)** Quantitative analysis of P_{sspE}-*cfp* expression, at hours 4 and 6 of sporulation. Strains are as follows: AH5431 (non-induced, dark blue; P_{spac}-*spoIIIJ-his*), and induced with 0.5 mM IPTG (green), AH5432 (non-induced, red; P_{spac}-*spoIIIJ*(C134A)-*his*) and induced with 0.5 mM IPTG (purple) and JOB44 (light blue; Δ*spoIIIJ*::*km*). This last strain was not included in panel B for simplicity, and no fluorescence was detected for the Δ*sigF* mutant AH9335 (not shown). CFP images were acquired and processed with identical parameters to permit direct comparison of fluorescence intensity among samples. At least 200 cells were scored for the fluorescence patterns designated by low (class a) or high (class b) (see the Material and Methods section for details).

DSM cultures of strains expressing either *spoIIIJ* or *spoIIIJ*(C134A) from P_{spac} in a P_{sspE}-*cfp* and *spoIIIJ* mutant background (AH5431 and AH5432, respectively) were sampled at hours 4 and 6 after the onset of sporulation. The cells were observed by fluorescence microscopy, after staining with the membrane dye FM 4-64 and the nucleoid stain DAPI (see Materials and Methods) to identify and score the morphological stage of sporulation. Sporulating cells were assigned to three classes according to the intensity of the signal obtained from CFP: non-fluorescent, with low (a) and with high (b) fluorescence (Fig. 5B-C). High-fluorescence cells completed engulfment and activated σ^G, thus leading to high expression of the reporter gene fusion P_{sspE}-*cfp*; on the other hand, low fluorescence mainly results from the weaker utilisation of the *sspE* promoter by σ^F, the preceding factor in the prespore line of gene expression (Serrano *et al.*, 2008).

A large proportion of the non-induced cells bearing the wild-type P_{spac}-*spoIIIJ* construct (AH5431) were scored as highly fluorescent, an indication of active σ^G; in contrast, the *spoIIIJ*(C134A) expressing strain (AH5432) under non-inducing conditions showed a large proportion of low-fluorescence cells, whilst the high-fluorescence cells were scarce (Fig. 5B-C). A similar distribution was obtained with the null *spoIIIJ* mutant harbouring the reporter fusion (AH5433), which also exhibited a large proportion of low-fluorescence cells (Fig. 5C) as expected since it contains active σ^F but not active σ^G (Errington *et al.*, 1992). Upon addition of IPTG (to 0.5 mM), similar percentages of highly fluorescent cells were obtained for the strains expressing either *spoIIIJ*(C134A) or its wild-type counterpart (Fig. 5C). Altogether, these results show that the C134A substituted form of SpoIIIJ is less active than the wild-type protein, though the lower activity of SpoIIIJ(C134A) was evident *in vivo* only when lower than normal levels of the two proteins were produced.

Discussion

Oligomerisation is a common mechanism known to regulate protein function (Ali and Imperiali, 2005). In this work we have shown that *B. subtilis* SpoIIIJ, a member of the YidC/Oxa1/Alb3 family of insertases, is able to dimerise and that its Cys134 residue has a role in this process, presumably by establishing a disulphide bond. Several lines of evidence favour the existence of an intramembrane disulphide bond involving Cys134 that promotes formation of, or stabilises a SpoIIIJ dimer. The use of DTT, a reducing agent that disrupts disulphide bonds between sulfhydryl groups of cysteine residues, was shown to alter the oligomeric state of SpoIIIJ. By SDS-PAGE and only upon addition of DTT we observed the disappearance of two higher molecular weight forms of SpoIIIJ (relative to the monomer); strikingly, these were not detected for SpoIIIJ(C134A) even in the absence of DTT (Fig. 1B). Also, by circular dichroism spectroscopy analysis, we verified that DDM-solubilised SpoIIIJ is a folded, mainly α-helical protein (Fig. S1); interestingly, the addition of DTT caused an alteration in the content of α-helices and anti-parallel β-sheets in the wild-type SpoIIIJ but not in the SpoIIIJ(C134A) variant. This change in secondary structure may be explained by a change in the oligomeric state of SpoIIIJ. In addition, the C134A variant of SpoIIIJ, which is unable to establish disulphide bonds, exhibited an altered monomer:dimer ratio relative to the wild-type SpoIIIJ with a higher proportion of the monomer, as estimated by SEC (Fig. 2). Overall, these data sets strongly support that disulphide bond formation plays a role in the dimerisation of SpoIIIJ.

The Cys134 residue is predicted to occur within the membrane plane (see Materials and Methods). It is often assumed that the membrane environment is not appropriate for the formation of disulphide bonds but there are an increasing number of reports that argue in favour of the existence of such intramembrane disulphide bonds (e.g. Rutledge *et al.*, 1992; Arien *et al.*, 2003; Sousa *et al.*, 2003; Chiang and Knowles, 2008). The experiments using malPEG were designed to test whether a free cysteine

residue was accessible for modification in SpoIIIJ. We have found that labelling with malPEG only took place with the concomitant use of high temperature and the detergent SDS (Fig. 3). The harsh conditions required for labelling of Cys134 suggest that this residue is present at a tight interface, as the bulky malPEG may have its access to the cysteine residue limited, as previously suggested (Koide *et al.*, 2007). This may contribute to the incomplete labelling observed (Fig. 3). Since the labelling experiments were implemented with extracts prepared from *B. subtilis* cultures, another contributing factor could be the existence of a mixed population of SpoIIIJ in several oligomeric states and/or belonging to distinct proteins complexes that allow different degrees of malPEG accessibility.

Distinct oligomeric states are described for several members of the YidC/Oxa1/Alb3 family of membrane protein insertases. Oxa1 from *Neurospora crassa* was suggested to form tetramers (Nargang *et al.*, 2002) and Oxa1 from *S. cerevisiae* was observed to form only dimers (Kohler *et al.*, 2009), only tetramers (Reif *et al.*, 2005), or dimers and tetramers (Krüger *et al.*, 2012). Alb3 from *Arabidopsis thaliana* is able to form dimers (Dünschede *et al.*, 2011). Finally, YidC from *E. coli* was reported to form dimers (Nouwen and Driessen, 2002; Lotz *et al.*, 2008; Boy and Koch, 2009; Kohler *et al.*, 2009) and was also recovered as a mixture of monomers and dimers (van der Laan *et al.*, 2001; van der Does *et al.*, 2003). Our results support a scenario in which SpoIIIJ dimerises and the dimer is held by a disulphide bond established between the Cys134 residues of two SpoIIIJ monomers (Fig. 6). This nicely agrees with the results of the homologous protein in *E. coli*, YidC (van der Laan *et al.*, 2001; Nouwen and Driessen, 2002; van der Does *et al.*, 2003; Lotz *et al.*, 2008; Boy and Koch, 2009; Kohler *et al.*, 2009). Note that SpoIIIJ(C134A) exhibits an altered monomer:dimer peak ratio relative to the wild-type through SEC, but both peaks are still detected (Fig. 2). Thus, we conclude that despite being relevant, the cysteine is not absolutely required for dimerisation. We suggest that the dimer is sustained by disulphide bonds between cysteine residues along

with other interactions, possibly non-covalent bonds. The presence of an interchain disulphide bond may still be advantageous as it might enable coping with naturally occurring mutations (Bolliger and Johansson, 1999). We cannot exclude that forms of SpoIIIJ of order higher than the dimer are formed and have physiological relevance. The BN-PAGE, for example, suggests that a SpoIIIJ hexamer can be formed, and at least part of the dimer population could serve as an intermediate for the formation of this species. Conservation of a Cys residue at a homologous position in the YqjG paralogue, and among SpoIIIJ orthologues, underscores the importance of this residue, and presumably of disulphide bond formation for optimal functionality.

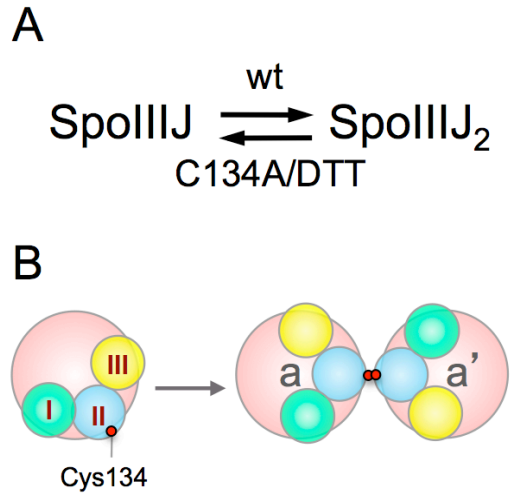

Figure 6. Model for the dimerisation of SpoIIIJ. (A) SpoIIIJ occurs as a monomer and a dimer. The dimer is thought to be stabilised by a disulphide bond involving residue Cys134. However, this residue is not essential for dimer formation. **(B)** A disulphide bond between cysteine residues (red circles), along with other interactions, promotes the formation and/or maintenance of the dimeric form of SpoIIIJ. TM segments 1, 2 and 3 are depicted as green, blue, and yellow circles, respectively.

A role for TM1 of SpoIIIJ (corresponding to TM2 of YidC) in SpoIIIJ-SpoIIIJ interactions is hinted from the fact that a heterodimer composed of a full-length (though mature, Côrte *et al.*, manuscript in preparation; see Chapter IV) and a truncated SpoIIIJ monomer (lacking TM1) was observed but not a homodimer of two truncated SpoIIIJ monomers (Fig. 1). This role for TM1 may be direct or indirect. In the former, TM1 itself could be involved in protein-protein interactions; in the latter, TM1 would

maintain the regions directly interacting (possibly TM2, with Cys134) in the correct position for proper interaction to take place. Interestingly, TM2 of YidC (corresponding to SpoIIIJ's TM1) was suggested to have a structural role as well, in helix-helix interactions (Klenner and Kuhn, 2012). In addition, an interaction between TM2 and 3 of YidC (corresponding to TM1 and 2 of SpoIIIJ, respectively) was also suggested (Yuan *et al.*, 2007).

Both SpoIIIJ and YidC function in a Sec-dependent and -independent manner (reviewed in Wang and Dalbey, 2011; Saller *et al.*, 2012). For instance, SpoIIIJ participates in the biogenesis of the SpoIIIAE polytopic membrane protein, one of the components of the secretion system also required for σ^G activity after engulfment completion during sporulation in *B. subtilis*, possibly in conjunction with the Sec pathway (Camp and Losick, 2008; Serrano *et al.*, 2008). Depending on either mode of action, in conjunction with the Sec pathway or not, SpoIIIJ might be functional in distinct oligomeric states. The precise role fulfilled by SpoIIIJ during membrane protein biogenesis regarding different substrates could also be related to its oligomeric state, either specifically in membrane protein insertion or later, in the folding or the assembly of membrane protein complexes (Saller *et al.*, 2009). We observed that the SpoIIIJ(C134A) variant is less functional during sporulation in *B. subtilis*: at low expression levels, SpoIIIJ(C134A), but not the wild-type protein, shows a reduced ability to support sporulation, specifically exhibiting a lower titre of heat-resistant spores and a lower fraction of cells activating σ^G (Figs. 4 and 5; Tables 3 and 4). Nevertheless, when expressed at wild-type levels, the SpoIIIJ(C134A) supported efficient σ^G activity and heat-resistant spore formation (Figs. 4 and 5; Tables 3 and 4). These results favour a scenario in which a higher concentration of SpoIIIJ(C134A) (relative to the wild-type form) is needed to promote dimer formation, sustained by non-covalent interactions, since the disulphide bond cannot be formed.

We propose that the Cys134 residue has a role in the dimerisation of SpoIIIJ and that such is required for proper functioning of SpoIIIJ during

spore formation in *B. subtilis*, in particular at the level of σ^G activity, in the assembly of the SpoIIIA-SpoIIQ secretion system. The basis for the observation that SpoIIIJ but not YqjG supports efficient sporulation is intriguing. Since a positional homologue of Cys134 is found in the YqjG paralogue of SpoIIIJ, it seems possible that this residue is also involved in disulphide bond formation in this protein.

Acknowledgements

All experiments were performed by the author of this Thesis except for the protein overproduction in *E. coli*, circular dichroism, size exclusion chromatography and BN-PAGE, including the construction of associated strains and pFiV plasmids (by Filipa Valente). The author thanks D. Rudner for the gift of BTD2633, Cláudio Gomes for his help with the circular dichroism experiments and critical reading, and Charles P. Moran Jr. for helpful suggestions and critical reading. The author is the recipient of a Ph.D. fellowship (SFRH/BD/6489/2001) from the Fundação para a Ciência e a Tecnologia.

Tables

Table 1. Bacterial strains.

Strain	Relevant Genotype/Phenotype[a]	Origin/ Reference
MB24	*trpC2 metC3*	Laboratory stock
JOB44	Δ*spoIIIJ::km* / Km^r	Serrano *et al.*, 2003
AH5382	Δ*spoIIIJ::km* Δ*amyE::yqjG*(A50C C142A)-*his* / Km^r Cm^r	This work
AH5425	Δ*spoIIIJ::km thrC*::P_{spac}-*spoIIIJ-his* / Km^r Erm^r	«
AH5426	Δ*spoIIIJ::km thrC*::P_{spac}-*spoIIIJ*(C134A)-*his* / Km^r Erm^r	«
AH5431	Δ*spoIIIJ::km thrC*::P_{spac}-*spoIIIJ-his* Δ*yycR*::P_{sspE}-*cfp* / Km^r Cm^r Erm^r	«
AH5432	Δ*spoIIIJ::km* Δ*thrC*::P_{spac}-*spoIIIJ*(C134A)-*his* Δ*yycR*::P_{sspE}-*cfp* / Km^r Cm^r Erm^r	«
AH5433	Δ*spoIIIJ::km* Δ*yycR*::P_{sspE}-*cfp* / Km^r Cm^r	«
AH6566	Δ*yycR*::P_{sspE}-*cfp* / Cm^r	Serrano *et al.*, 2008
AH9218	Δ*spoIIIJ::spoIIIJ-his* / Sp^r	Chapter IV
AH9335	Δ*spoIIAC*::*erm* Δ*yycR*::P_{sspE}-*cfp* / Erm^r Cm^r	Serrano *et al.*, 2008
BTD2633	Δ*yycR*::P_{sspE}-*cfp* / Cm^r	D. Rudner

[a] All *B. subtilis* strains are *trpC2 metC3* except BTD2633. Km, kanamycin; Cm, cloramphenicol; Erm, erythromycin; Sp, spectinomycin.

Table 2. Oligonucleotides used in this study.

Primer	Sequence (5´to 3´)[a]
J112D	GGAGGCCATGGTGTTGAAAAGGAGAATAGGG
Jhis	GGAATTCTCA**GTGGTGGTGGTGGTGGTG**CTTTTTCTTTCCTCCGGCTTTTTGCGGC
JC134A_D	GTCAATCCATTGGCGGGA**GCT**TTCCCGATTTTGATCCAG
JC134A_R	CTGGATCAAAATCGGGAA**AGC**TCCCGCCAATGGATTGAC
J174D	GAGTGTAAAGATCTAATTATAGGAGG
JhisR	ACATGCATGCTCA**GTGGTGGTGGTGGTGGTG**CTTTTTCTTTCCTCCGGCTTTTTGCGGC
YqjG-His-R	GAGGATCCTTTCACCGACTCAGTAAGAGCG
PYqjG-460D	AGAGCGGGATCCCTGTATGGTGTATCG
YA50C_D	GATTATTTAATCGAACCGTTTTCC**TGC**CTGCTTAAGGGTGTTGCC
YA50C_R	GGCAACACCCTTAAGCAG**GCA**GGAAAACGGTTCGATTAAATAATC
YC142A_D	ATCAACCCGCTTGCGATGGGC**GCT**CTTCCAATGCTGATTCAGTCTC
YC142A_R	GAGACTGAATCAGCATTGGAAG**AGC**GCCCATCGCAAGCGGGTTGAT

[a] Restriction sites are underlined, mutations in bold.

Table 3. Efficiency of sporulation of strains bearing *spoIIIJ* alleles measured at T_8.

Strain	Relevant Genotype	Viable cell count[a]	Heat^R cell count[a]	Spo[a] (%)
AH5425	*spoIIIJ* Δ*spoIIIJ::km* 0[b]	$1.8x10^8$	$1.0x10^6$	1.00
AH5426	*spoIIIJ*(C134A) Δ*spoIIIJ::km* 0	$1.5x10^8$	$2.1x10^4$	0.014
AH5425	*spoIIIJ* Δ*spoIIIJ::km* 0.5	$3.4x10^8$	$2.5x10^7$	7.35
AH5426	*spoIIIJ*(C134A) Δ*spoIIIJ::km* 0.5	$2.2x10^8$	$4.3x10^6$	1.96
JOB44	Δ*spoIIIJ::km*	$1.2x10^8$	0	0
MB24	wild-type	$4.6x10^8$	$1.8x10^7$	3.91

[a] The titre of viable cells and heat-resistant spores were measured 8 h after the onset of sporulation in DSM (see Materials and Methods). Spo, sporulation. [b] IPTG concentration in mM.

Table 4. Efficiency of sporulation of strains bearing *spoIIIJ* alleles measured at T_{24}.

Strain	Relevant Genotype	Viable cell count[a]	Heat^R cell count[a]	Spo[a] (%)
AH5425	*spoIIIJ* Δ*spoIIIJ::km* 0[b]	$1.5x10^8$	$9.4x10^7$	62.7
AH5426	*spoIIIJ*(C134A) Δ*spoIIIJ::km* 0	$1.1x10^8$	$8.0x10^6$	7.27
AH5425	*spoIIIJ* Δ*spoIIIJ::km* 0.5	$1.6x10^8$	$1.2x10^8$	75.0
AH5426	*spoIIIJ*(C134A) Δ*spoIIIJ::km* 0.5	$1.9x10^8$	$1.1x10^8$	57.9
JOB44	Δ*spoIIIJ::km*	$7.6x10^7$	0	0
MB24	wild-type	$2.7x10^8$	$1.2x10^8$	44.5

[a] The titre of viable cells and heat-resistant spores were measured 24 h after the onset of sporulation in DSM (see Materials and Methods). Spo, sporulation. [b] IPTG concentration in mM.

Table 5. Plasmids.

Plasmid	Relevant features	Antibiotic resistance[a]	Origin/Reference
pFiV1	pETDUET-1+*spoIIIJ*(C134A)-*his*	*bla*	This work
pFiV2	pDH88+*spoIIIJ-his*	*bla, cat*	«
pFiV3	pDH88+*spoIIIJ*(C134A)-*his*	*bla, cat*	«

pFiV4	Δ*thrC*::P_{spac}-*spoIIIJ-his*	*bla, erm*	«
pFiV5	Δ*thrC*::P_{spac}-*spoIIIJ*(C134A)-*his*	*bla, erm*	«
pMS266	pETDUET-1+*spoIIIJ-his*	*bla*	Serrano *et al*., 2008
pLC111	Δ*amyE::his*	*bla, cat*	Chapter IV
pLC115	Δ*amyE::yqjG-his*	*bla, cat*	«
pLC138	Δ*amyE::yqjG*(A50C)-*his*	*bla, cat*	This work
pLC155	Δ*amyE::yqjG*(A50C C142A)-*his*	*bla, cat*	«
pDH88	P_{spac}	*bla, cat*	Henner, 1990
pDG1664	*thrC* insertion	*bla, erm*	Guerout-Fleury *et al*., 1996

[a] *bla*, ampicillin; *cat*, cloramphenicol; *erm*, erythromycin.

Supplemental Data

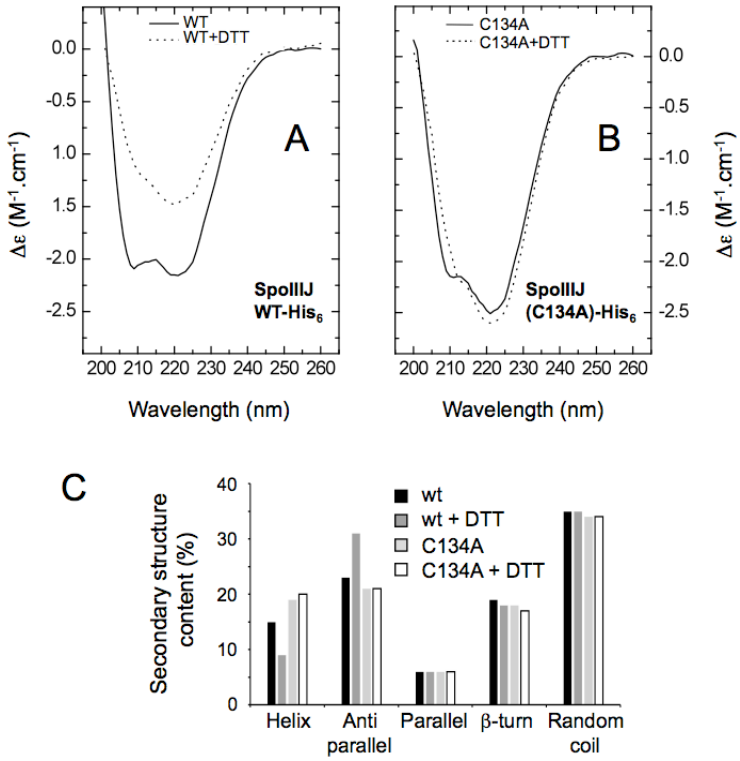

Figure S1. **Circular dichroism spectroscopy of SpoIIIJ-His$_6$ and SpoIIIJ(C134A)-His$_6$.** Far UV-CD spectra of purified SpoIIIJ-His$_6$ **(A)** or SpoIIIJ(C134A)-His$_6$ **(B)** (0.2 mg ml^{-1} of purified protein in 20 mM Tris-HCl pH 8, 0.1 M NaCl, 10 % glycerol) in the presence or the absence of 1 mM DTT, dotted and solid lines, respectively. The spectra are typical of folded α-helical-rich proteins with minima at 208 and 222 nm, consistent with the predicted structure of the SpoIIIJ. Addition of DTT affects the spectrum of SpoIIIJ-His$_6$, but has no effect on the spectrum of SpoIIIJ(C134A)-His$_6$. **(C)** Relative estimates of the secondary structure of SpoIIIJ-His$_6$ (with or without 1 mM DTT, dark grey and black bars, respectively) and SpoIIIJ(C134A)-His$_6$ (with or without 1 mM DTT, white and light grey bars, respectively). Addition of DTT decreases the α-helix content by 6 % and increases the content of anti-parallel β-sheets (more 8 %) of SpoIIIJ-His$_6$, but does not significantly alter SpoIIIJ(C134A)-His$_6$.

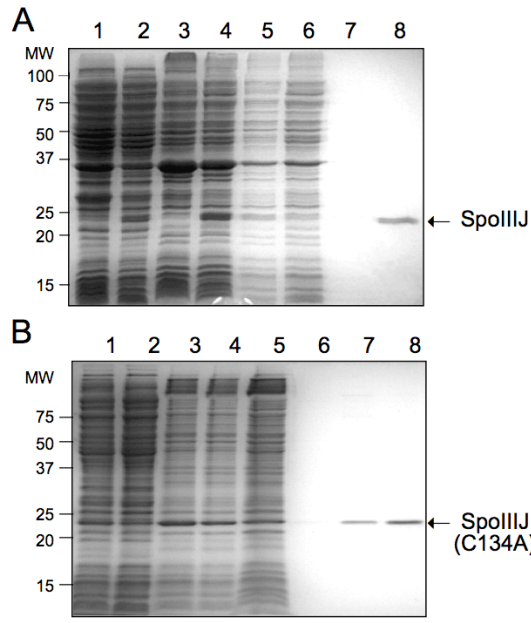

Figure S2. Overproduction and purification of SpoIIIJ-His$_6$ and SpoIIIJ(C134A)-His$_6$. Overproduction and purification of SpoIIIJ-His$_6$ (A) or and SpoIIIJ(C134A)-His$_6$ (B) from *E. coli* strain C43(DE3) carrying pMS266 or pFiV1, respectively. The cells were grown in LB to mid log phase, split into two cultures, and one was induced with IPTG. The cells were lysed and fractionated into a soluble and a membrane fraction. Proteins in the membrane fraction were solubilised with 2 % DDM and the extract applied onto a Ni^{2+}-NTA column. **(A)** The fractions analysed by SDS-PAGE are as follows: lanes 1 and 2, crude extract of non-induced and induced cells, respectively; lanes 3 and 4, membrane fraction extracted with 2 % DDM from non-induced and induced cells, respectively; lane 5, column flow-through; lane 6, column wash; lane 7, 50 mM imidazole elution fraction; lane 8, 100 mM imidazole fraction. **(B)** Lanes 1 and 2, crude extract of non-induced and induced cells, respectively; lane 3, membrane extract solubilised with 2 % DDM; lane 4, column flow-through; lane 5, column wash; lane 6, 25 mM imidazole elution fraction; lanes 7 and 8, 50 mM imidazole elution. The position of molecular weight markers (in kDa) is shown; arrows show the position of full-length SpoIIIJ-His$_6$ or SpoIIIJ(C134A)-His$_6$.

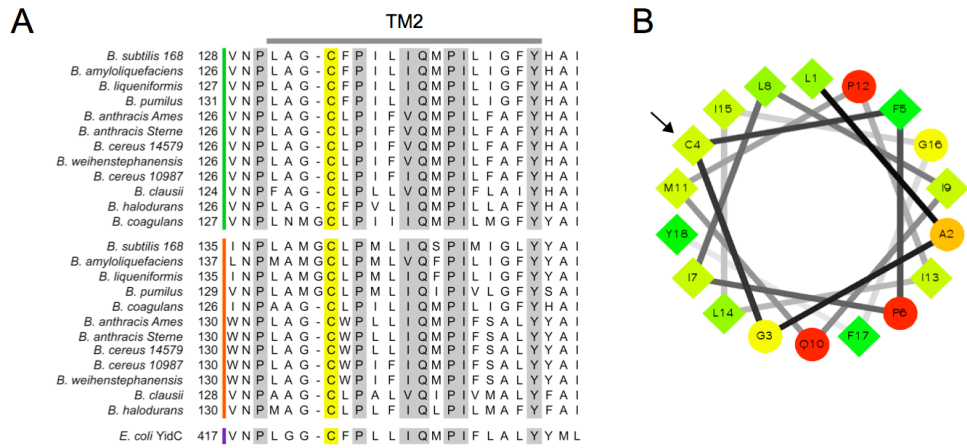

Figure S3. Conservation of Cys134 among SpoIIIJ homologues. (A) Alignment of the transmembrane (TM) segment 2 of SpoIIIJ (green) and YqjG (orange) proteins from several *Bacillus* species and of TM3 of YidC (purple) from *E. coli*. Conserved residues are shaded in grey except for the cysteine (yellow). The sequences were aligned with ClustalW (Thompson *et al.*, 1994). **(B)** Helical wheel projection of TM2 of SpoIIIJ from *B. subtilis* (http://rzlab.ucr.edu/scripts/wheel/wheel.cgi). Circles denote hydrophilic residues and diamonds hydrophobic ones. A colour code was used: green, hydrophobic residues, with the amount of green decreasing proportionally to the hydrophobicity; yellow, zero hydrophobicity; red, the most hydrophilic (uncharged) residue, the amount of red decreasing proportionally to the hydrophilicity.

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Chapter VI

General Discussion

The work presented in this Thesis focused on the role of the homologous proteins SpoIIIJ and YqjG during the developmental process of sporulation in *Bacillus subtilis*. Our results highlight the importance of secondary structure over primary structure in SpoIIIJ, a distinctive feature of the YidC/Oxa1/Alb3 family. Furthermore, they link a previously unidentified proteolytic event to the inactivity of YqjG during spore formation. Our data also show that SpoIIIJ and YqjG harbour distinct signal peptide types, and reveal that those signal peptides are dispensable for their roles during sporulation and viability. Finally, they show that SpoIIIJ oligomerises and that impaired oligomerisation can be detrimental for sporulation.

The malleability of SpoIIIJ

Previous work established a role for SpoIIIJ during spore formation. Disruption of the *spoIIIJ* locus blocks sporulation at stage III, after completion of prespore engulfment (Errington *et al.*, 1992). The genome of *B. subtilis* encodes a SpoIIIJ homologue, YqjG. Whilst the presence of either is sufficient to ensure viability, only SpoIIIJ is functional during sporulation (Murakami *et al.*, 2002; Tjalsma *et al.*, 2003). This prompted us into investigating the basis for the different functioning of these proteins. SpoIIIJ and YqjG share 41 % identities and 62 % similarities, as analysed *in silico* (Chapter II). Many examples of cross-complementation between members of this family exist, even involving proteins from organisms belonging to different domains and with low identity levels. Many examples are documented, as the case of human Oxa1 in *S. cerevisiae* (Bonnefoy *et al.*, 1994), Alb3 in *E. coli* and in *S. cerevisiae* (Sundberg *et al.*, 1997; Jiang *et al.*, 2002), YidC2 of *S. mutans* in *oxa1*-deficient *S. cerevisiae* and Oxa1 in *S. mutans* lacking YidC2 (Funes *et al.*, 2009), Oxa1 and Cox18 from *S. cerevisiae* in *E. coli* (van Bloois *et al.*, 2005; van Bloois *et al.*, 2007), and YidC in *oxa1*- or *cox18*-deficient *S. cerevisiae* (Preuss *et al.*, 2005). Following this trend, both SpoIIIJ and YqjG from *B. subtilis* were able to

functionally compensate for the defects caused by YidC depletion in *E. coli* (Saller *et al.*, 2009). Given this panorama, we decided to seek potentially important amino acid residues for the role of SpoIIIJ during sporulation by expressing several *spoIIIJ* and *yqjG* genes from close species, namely from other *Bacillus* species, in a *spoIIIJ* mutant background. Unexpectedly, most heterologous genes failed to substitute for *spoIIIJ* during sporulation regarding the ability form of heat-resistant spores (Table 3, Chapter II). We chose the *B. halodurans* proteins for cross-linking studies since it harbours a homologue that fully supports sporulation (YqjG(Bh)) and one that only exhibits a low level of complementation (SpoIIIJ(Bh)). Strikingly, YqjG(Bh) exhibits lower sequence identity and similarity than both SpoIIIJ(Bl) and SpoIIIJ(Bh) relative to SpoIIIJ(Bs), despite being the one that is fully functional (Table 1, Chapter VI; Table 3, Chapter II). As we could expect, YqjG(Bh) was shown to interact with SpoIIIAE(Bs). Interestingly, SpoIIIJ(Bh) was also found to interact with SpoIIIAE(Bs) in *E. coli* (see Chapter II), similarly to SpoIIIJ(Bs) and YqjG(Bs) (Serrano *et al.*, 2008). So, how to explain the low level of complementation supported by SpoIIIJ(Bh) relative to YqjG(Bh)? Taken in conjunction, these results suggest two non-mutually exclusive scenarios: 1) SpoIIIJ-like proteins are intrinsically able to interact with SpoIIIAE; 2) SpoIIIJ has a second function in sporulation. In the first scenario, an interaction would be prevented by external factors, such as a physical block. Another possibility is that an interaction takes place but is non-productive, as observed for YqjG(Bs) with SpoIIIAE(Bs) (Serrano *et al.*, 2008). In the second scenario, the heterologous SpoIIIJ-like proteins could fulfil their SpoIIIAE-related role but fail to interact with the putative second substrate. A second function for SpoIIIJ during sporulation has been suggested previously, as the restoration of σ^G activity levels by *spoIIIJ* suppressors was much more potent than that of the heat-resistant spore titre (Camp and Losick, 2008). More evidence for this line of thought comes from the fact that SpoIIIAE(Bc) fully complements a *spoIIIAE* in-frame deletion in terms of heat-resistant spore titre, but does not restore

wild-type sporulation levels to a strain expressing SpoIIIJ(Bc) in a *spoIIIJ* mutant background (AH5208 and AH5219, Table 3 in Chapter II). SpoIIIJ(Bc) might interact properly with SpoIIIAE(Bs) but not with one or more other SpoIIIA proteins that would require SpoIIIJ for proper functioning, but functional interactions between *B. subtilis* SpoIIIJ and the remaining SpoIIIA proteins were not detected (Camp and Losick, 2008; Serrano *et al.*, 2008). In order to isolate *spoIIIJ* mutants blocked in a putative second σ^G-independent function, a genetic screen of a *spoIIIJ* mutated library for *sspE-lacZ* positive colonies that were still Spo$^-$ and negative for another reporter gene fused to a late sporulation promoter could be performed.

Table 1. Identity and similarity percentages of tested SpoIIIJ and YqjG proteins from other *Bacillus* species relative to SpoIIIJ(Bs).

Sequence[a]	% identity	% similarity
SpoIIIJ(Bl)	81.1	91.5
SpoIIIJ(Ba)	69.9	83.7
SpoIIIJ(Bc)	69.1	82.1
SpoIIIJ(Bh)	61.8	78.4
YqjG(Bs)	41.8	62.7
YqjG(Bl)	42.9	60.5
YqjG(Bh)	49.3	68.9

[a] Bl, *Bacillus licheniformis*; Ba, *B. anthracis*; Bc, *B. cereus*; Bh, *B. halodurans*; Bs, *B. subtilis*. Identity and similarity percentages were obtained with EMBOSS 6.3.1 (http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::matcher)

Site-directed mutagenesis of SpoIIIJ was also employed in the pursuit of amino acid residues required for sporulation. Several conserved residues were changed, including the absolutely conserved Q238 among Firmicutes (not shown), as well as several residues conserved in the two functional proteins, SpoIIIJ(Bs) and YqjG(Bh), with no change in the ability of supporting heat-resistant spore formation. Additionally, we found that the C-terminal tail of SpoIIIJ is not required, at least for

sporulation (from residue 243 onwards) (see Chapter II). The elongated and positively charged C-terminus of Oxa1 of *S. cerevisiae* and YidC2 of *S. mutans* is responsible for ribosome binding (Jia *et al.*, 2003; Szyrach *et al.*, 2003; Funes *et al.*, 2009). The shorter (20 residue-long) and positively charged C-terminus of YidC was shown to enhance binding to ribosomes (Kohler *et al.*, 2009), although a C-terminally truncated YidC retained activity *in vivo* (Jiang *et al.*, 2003). It is thus tempting to speculate that the also positively charged and 20 residue-long C-terminus of SpoIIIJ might enhance ribosome binding, despite its absence not affecting sporulation efficiency. It is possible that the extra substitutions we obtained near Q254 compensate for a negative impact that this substitution would exert on ribosome binding. Nevertheless, as suggested for YidC, the C-terminus of SpoIIIJ may enhance ribosome binding but other contributions should exist, both from other regions of the protein and from the substrate nascent polypeptide chain (Kohler *et al.*, 2009; Klenner and Kuhn, 2012).

Data from random mutagenesis of *spoIIIJ* also support the notion of the this gene's tolerance to mutations regarding its function: only drastic mutations (nonsense and frameshifts) were recovered when selecting for Spo$^-$ clones in a *spoIIIJ::km* background transformed with a mutagenised *spoIIIJ* library. In the ten Spo$^-$ clones tested, the xylose-inducible promoter P_{xylA} became constitutive when the mutagenised *spoIIIJ* library was inserted in a *spoIIIJ::km* P_{xylA}-*yqjG* background in the presence of glucose; this was assessed by transforming *spoIIIJ::km* with chromosomal DNA not in excess from ten clones and plating in the presence of spectinomycin, kanamycin and either 0.5 % xylose or 0.2 % glucose, which resulted in large and similar numbers of colonies for both cases (see Chapter II).

In toto, the results from random mutagenesis, site-directed mutagenesis, heterologous complementation (see Chapter II), as well as the possibility to delete the signal peptide without affecting viability or sporulation (see Chapter IV), all suggest that SpoIIIJ is a malleable protein that can accommodate dramatic amino acid substitutions, a case reminiscent of that seen for YidC for which no absolutely essential amino acid residue

was found (Jiang *et al.*, 2003; Klenner and Kuhn, 2012). Loss of activity and/or lack of growth only occurred when structure was perturbed, either by introducing protease sites that alter the conformation of YidC, making it temperature-sensitive, or by converting specific residues into serines; note that for the latter, substitution by other residues resulted in functional YidC variants (Chen *et al.*, 2003; Jiang *et al.*, 2003; Klenner and Kuhn, 2012). In addition, loss of function occurred mostly upon combining mutations, either of serine substitutions (Jiang *et al.*, 2003; Klenner and Kuhn, 2012) or of engineered protease sites (Chen *et al.*, 2003), again indicative of a remarkable tolerance to changes. Mutagenesis conditions that allow several alterations to accumulate in the same gene might lead to the isolation of important residues in SpoIIIJ. We suggest that the overall structure of these proteins is more relevant than their precise sequence. Possibly, like the case of YidC, there may also be a minimum hydrophobicity threshold for the transmembrane (TM) regions to maintain proper topology and the correct structure of the *B. subtilis* Oxa1-like proteins (Jiang *et al.*, 2003). However, certain regions of SpoIIIJ/YqjG may be required for the fulfilling of specific functions towards different substrates. Two separate substitutions in YqjG that raise the titre of heat-resistant spore in the absence of *spoIIIJ* were proposed to improve the interaction with SpoIIIAE, a substrate of SpoIIIJ (Camp and Losick, 2008; Serrano *et al.*, 2008; Chapter III). In addition, data from SpoIIIJ/YqjG chimeras suggest the second quarter of SpoIIIJ to be the most important region of the ones here analysed for its function during sporulation. Another view is that the chimeras composed of regions that interact better might be more functional and provide higher spore titres. For both YidC and Oxa1, certain residues were shown to be required for the proper interaction of different regions of the protein and for its function (Yuan *et al.*, 2007; Mathieu *et al.*, 2009). It was also proposed that different substrates require different regions of YidC for their membrane insertion, as different YidC variants were able to insert different substrates (Chen *et al.*, 2003; Yuan *et al.*, 2007) and some YidC mutants were unable

to support growth but retained the ability to insert the substrate in question and vice-versa (Jiang *et al.*, 2003). This is consistent with the idea of a general docking site in Oxa1 proteins (Yu *et al.*, 2008), leaving room for specific interactions with specific substrates to take place on the remaining regions of the protein. It is also conceivable that the Sec-dependent and -independent functions of YidC/SpoIIIJ, as well as the distinct functions proposed for this family of proteins (membrane insertion, folding, and assembly of complexes) exhibit a differential degree of necessity for different regions of the protein. This emphasises the need to find and analyse more substrates regarding their behaviour with respect to different SpoIIIJ variants.

On the functionality of YqjG during sporulation

The two members of the YidC/Oxa1/Alb3 family of protein insertases present in *B. subtilis* constitute an essential gene pair, as at least one must be present to ensure viability (Murakami *et al.*, 2002; Tjalsma *et al.*, 2003; Thomaides *et al.*, 2007). Both SpoIIIJ and YqjG have complementary functions in membrane protein insertion and assembly. They were both implicated in the biogenesis of the F_1F_0 ATP synthase (Saller *et al.*, 2009; Saller *et al.*, 2011) and in the stability of secreted proteins (Tjalsma *et al.*, 2003). However, only SpoIIIJ is functional in the developmental process of spore formation (Errington *et al.*, 1992; Murakami *et al.*, 2002; Tjalsma *et al.*, 2003), specifically in a post-insertional stage of the biogenesis of SpoIIIAE (Serrano *et al.*, 2008). In this work we have rendered YqjG able to fully replace SpoIIIJ in its role during sporulation (see Chapter III). We suggest that such occurs by virtue of the combined effect of increased stability of YqjG and improved interaction with SpoIIIAE.

On YqjG stability, we show that both YqjG-GFP and YqjG-His_6 exhibit a faster-migrating species that most likely arises from proteolysis (Fig. 2C-D, Chapter III). Cleavage takes place downstream of the signal peptidase I processing site (Fig. 4B, Chapter IV) and is exclusive of YqjG, not being

observed for SpoIIIJ. Fusion of YqjG to GFP but not to a His-tag leads to a higher heat-resistant spore titre and increased accumulation of the mature upper species as visualised by immunoblotting, suggesting that cleavage reduces functionality (Fig. 4; AH5269 in Table 3, Chapter III). Increased stability as a result of fusing proteins to GFP was noted before (Elkind *et al.*, 2000; Guéneron *et al.*, 2000; Rudner and Losick, 2002). The cleavage site was estimated to be present between amino acid residues 39 and 49 based on several data sets: AMS-labelling, chimeras of SpoIIIJ and YqjG, *in silico* analyses and an internal deletion in YqjG (see Chapter III). To get finer details on this proteolytic event, further experiments are suggested. Fusion of this region to a known protein would allow assessing if it is necessary and sufficient for cleavage. Also, a region downstream of residue 49 could be deleted in YqjG, resulting in three possible outcomes: i) it contains the recognition sequence (separate from the cleavage sequence) and cleavage is prevented; ii) it does not contain the recognition sequence and the precise sequence between residues 39-49 is important for cleavage, resulting in a smaller protein; iii) it does not contain the recognition sequence and the precise sequence between residues 39-49 is irrelevant for cleavage, producing a protein of the same size. We investigated several proteases but none was found to be responsible for the cleavage event (Fig. 5, Chapter III).

On the improved interaction with a sporulation-specific substrate, we tested the effect of the two different amino acid substitutions isolated by Camp and Losick (2008) that were reported to increase the ability of YqjG to support a higher heat-resistant spore titre. However, it was still lower than the wild-type's and it was only obtained in the presence of other genetic alterations. We succeeded in engineering YqjG to fully complement a *spoIIIJ* mutant, obtaining higher levels of heat-resistant spores than the corresponding wild-type strain (AH5423-4 relative to AH5268 and AH5429-30 relative to AH5269). Furthermore, the fully functional YqjG variants harbouring either substitution were obtained in an otherwise wild-type genetic background (AH5423 and AH5430, Table 3

in Chapter III). These results support the idea that even though Oxa1-like proteins are malleable in terms of amino acid sequence, specific interactions with certain substrates might be required to fully fulfil their functions. The G247V and A238S substitutions are suggested to improve interaction with SpoIIIAE (Camp and Losick, 2008). These substitutions could be engineered into chimeras composed of the first region of SpoIIIJ and the rest of YqjG (chimeras A and B in Fig. 2, Chapter II); if the titre of heat-resistant spores were raised, it would again be suggestive of certain contacts being needed for different substrates. On the other hand, overproducing YqjG-GFP might contribute to elucidate whether an excess of this protein is able to bypass the need for specific interactions.

Curiously, the secondary cleavage of YqjG occurs before stage III of sporulation, when SpoIIIJ is required for the activation of σ^G (Errington *et al.*, 1992), and in the absence of SpoIIIJ. Why such a proteolytic inactivation mechanism would arise is a matter of debate, as we did not observe any detrimental effects caused by the presence of YqjG variants capable of replacing SpoIIIJ in its fundamental role during sporulation. Possibly, the presence of mature YqjG is detrimental under other (Spo0A-independent) stress conditions but its inactivation is nonetheless kept during sporulation for some presently unknown reason. Alternatively, this non-processive proteolytic event may correspond to a second maturation of YqjG that allows it to play (or the cleaved peptide) an as yet unknown role.

Expression of SpoIIIJ and YqjG

During the course of this work, we verified that the amount of SpoIIIJ produced from its promoter region here employed, from the non-essential *amyE* locus, was sufficient to support sporulation but not viability. Repression of *yqjG* expression (from a xylose-inducible promoter) in a *spoIIIJ* mutant results in strains that depend on SpoIIIJ for viability, whether produced from a *spoIIIJ* 429 bp-long promoter region or from the

IPTG-inducible P_{spac}. Strains depending on P_{spac}-*spoIIIJ* grew normally only in the presence of the inducer, as opposed to the suboptimal growth of strains depending on SpoIIIJ produced from $P_{spoIIIJ}$ at the *amyE* locus, displaying an even more drastic phenotype in solid medium (Fig. 7, Chapter IV). On the other hand, SpoIIIJ produced from the same promoter region expressed at the *amyE* locus supports sporulation and is readily detected in immunoblots; however, when expressed from P_{yqjG}, it is not detected through immunoblot analysis but still sustains wild-type sporulation levels (see Chapter III). In addition, all of our constructs of P_{spac}-*spoIIIJ* and P_{xylA}-*spoIIIJ* always failed to yield Spo$^-$ colonies in the absence of inducer (not shown), strongly suggesting that high amounts of SpoIIIJ are needed to support viability but the amount produced due to leakiness of the promoter is sufficient to support sporulation. When expressed from its native locus, additional transcription is suggested to arise from the upstream ribosomal protein L34 promoter (Errington *et al.*, 1992) (Fig. 1, Chapter VI).

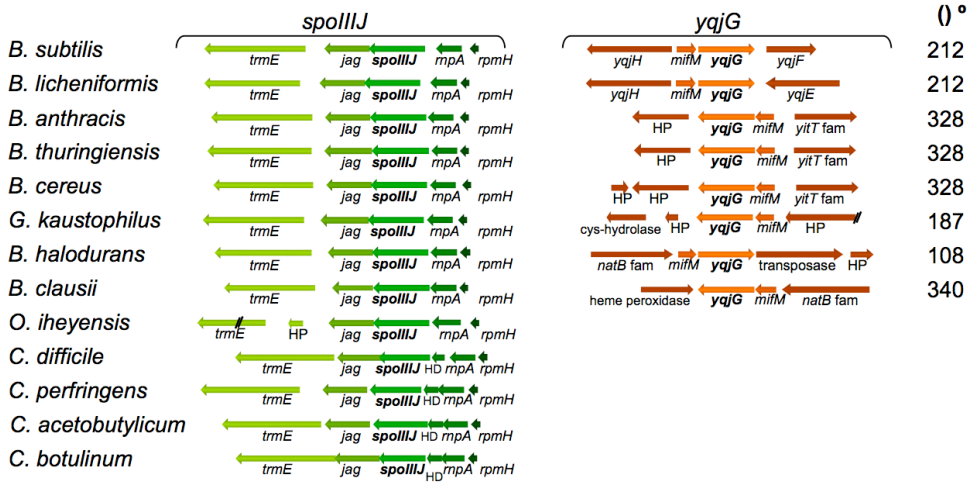

Figure 1. The genetic environment around *spoIIIJ* is conserved but not around *yqjG*. Genomic regions comprising either *spoIIIJ* or *yqjG* in several *Bacillus*, *Geobacillus*, *Oceanobacillus* and *Clostridium* species. *spoIIIJ* genes are located at 359º, whereas *yqjG* genes are distributed as indicated. HP, hypothetical protein; fam, family; HD, haemolytic domain. Double tilted lines, interruption in the DNA sequence.

The relationship between SpoIIIJ and YqjG goes beyond similarity of their primary sequence, temporal expression and localisation patterns, as in the absence of *spoIIIJ* we observe increased levels of both YqjG-GFP and YqjG-His_6 relative to those of the corresponding strains containing *spoIIIJ* (Fig. 2C-D, Chapter III). In addition, fluorescence of YqjG-GFP is much stronger in a *spoIIIJ*$^-$ background (not shown). We analysed the expression of *spoIIIJ* and *yqjG* by exchanging promoters and observing the accumulation of each protein when produced from the non-essential *amyE* locus in a *spoIIIJ*::*km* background. We observe accumulation of YqjG-His_6 when produced from its natural locus (i) (Fig. 3, Chapter III), as well as from the non-essential *amyE* locus from its own promoter (ii) (e.g. Figs. 4-6, Chapter IV; note that we used the same 749 bp-long promoter region as described in Chiba *et al*. (2009)). In contrast, when *yqjG-his* is under the control of $P_{spoIIIJ}$ at the *amyE* locus (iii), accumulation is no longer observed, but SpoIIIJ-His_6 is visible under the same conditions (iv) (Fig. 2, Chapter III; Figs. 2-3, Chapter IV, respectively). The main difference between constructs (ii) and (iii), which should be responsible for the difference in accumulation between those constructs, is the promoter region and the 5' end of the resulting mRNA up to the start codon. We hypothesise that an essential putative 5'-stabiliser (see below) for *yqjG* is absent from the hybrid mRNA produced from construct (iii), leading to reduced protein accumulation. Altogether, these results are in line with the work of Chiba *et al*. (2009), who revealed that this induction event is due to MifM, which constitutes a novel ribosome-nascent chain sensor mechanism in *B. subtilis* that is responsible by monitoring SpoIIIJ activity and that, in its absence, induces *yqjG* translation. *mifM* is the first gene of the bicistronic operon containing *yqjG* (Fig. 2A, Chapter IV; Fig. 1 Chapter VI). In the absence of SpoIIIJ, the translation of the *mifM-yqjG* mRNA is arrested. This positions the stalled ribosome over the 5′ end region of a hairpin that would otherwise block the initiation of *yqjG* translation by occluding its Shine-Dalgarno site (Fig. 2, Chapter VI).

Curiously, YqjG-His_6 and SpoIIIJ-His_6 were overproduced in *E. coli* at similar levels, which might be explained by overall differences in the mechanisms regulating mRNA stability between both organisms. Both in *E. coli* and *B. subtilis,* the 5'-end of transcripts is a major determinant of mRNA stability (reviewed in Condon, 2003). However, the absence of a 5'-stabiliser might have a greater effect in *B. subtilis*, in which 5' to 3' exoribonucleolytic degradation exists; in addition, roadblocks such as bound or stalled ribosomes, bound proteins or secondary structures near the 5' end of mRNAs have significant positive effects on the stability of the downstream portion, up to several kilobases, whereas in *E. coli*, other mechanisms in addition to 5'-end secondary structures regulate mRNA stability (Condon, 2003, 2007; Bechhofer, 2011).

SpoIIIJ-His_6 was also not observed in immunoblots of AH5231 when expressed from a shorter *yqjG* promoter region at the *amyE* locus in the absence of *spoIIIJ* (Fig. 2E, Chapter III). One possibility is that SpoIIIJ-His_6 may be down-regulating itself, as *mifM* senses the presence of SpoIIIJ-His_6 and the Shine-Dalgarno sequence becomes occluded. Another possibility is that such is due to the shorter promoter region (411 bp), which might be insufficient even though it contains *mifM* and 88 bp upstream of its start codon. However, based on several programs (not shown) and the DBTBS database (http://dbtbs.hgc.jp/) a promoter region is predicted to exist 50 bp upstream of the translational start; in addition, wild-type heat-resistant spores titres were obtained for AH5231, showing that the promoter region used is functional and that efficient sporulation can occur with diminished levels of SpoIIIJ-His_6.

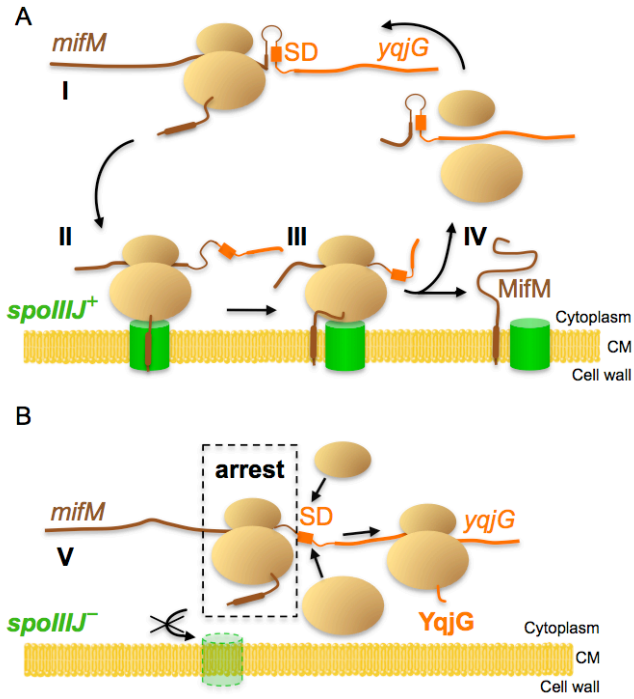

Figure 2. Model for *yqjG* regulation. (A) The mRNA stem-loop masks the *yqjG* Shine-Dalgarno (SD) site, resulting in a blockage of *yqjG* translation (I). When MifM inserts into the membrane, the translational arrest is transient or does not occur, thereby allowing only transient unfolding of the mRNA secondary structure by the ribosome (II–IV). **(B)** When membrane insertion of MifM is impaired, *mifM* translation is arrested and the ribosome unfolds the mRNA, allowing ribosomes to recognise the *yqjG* Shine-Dalgarno and initiate translation (V). Adapted from Chiba *et al*., 2009.

We examined the genetic organisation of *spoIIIJ* and *yqjG* in terms of the presence of each gene and their relative localisation in the genome. *spoIIIJ* and its downstream neighbour gene *jag* are mostly in an operon and located near the origin of replication, whilst the *mifM-yqjG* operon is localised quite differently in several species, as observed from the coordinates obtained from the National Center for Biotechnology Information (NCBI) databases (Fig. 1, Chapter VI). In organisms containing only *spoIIIJ*, *jag* is genetically linked to it as observed in species of at least ten genera belonging to Firmicutes; exceptions are some genera that possess two homologues but not *jag* (*Staphylococcus*) or just *spoIIIJ*, without *jag* or *yqjG* (*Mycoplasma*).

The higher conservation of *spoIIIJ*'s genetic environment and location in the genome may be connected its higher cellular relevance, further

supported by the fact that a mechanism exists that allows *yqjG* to act as a backup. Interestingly, YqjG was found to have a role in genetic competence in *B. subtilis* that SpoIIIJ cannot fulfil (Saller *et al.*, 2011). Such is in agreement with the notion of Thomaides *et al.*, (2007) that essential gene pairs directly contribute to the fitness of the organism, whilst perhaps offering the possibility of acquiring mutations that fine-tune or extend protein functions.

The signal peptides

Bacteria possess numerous systems that enable membrane protein insertion and secretion across the cytoplasmic membrane, of which the Sec pathway, the twin-arginine (Tat) pathway and YidC are fine examples. Targeting of a protein to these transport apparatuses relies on signals usually embedded in N-terminus of the protein itself, the signal peptides (Tjalsma *et al.*, 2004; Holland, 2010; Yuan *et al.*, 2010).

The N-terminal protein sequences of both SpoIIIJ and YqjG from several organisms were compared with known consensus sequences recognised by signal peptidases (SPases), which catalyse the removal of the signal peptides (Tjalsma *et al.*, 2004). All SpoIIIJs and most YqjGs exhibited a strong consensus for SPase II, encoded by *lspA* in *B. subtilis*, whereas YqjGs from *B. subtilis* and *B. licheniformis* displayed a consensus for type I SPases of which five exist in *B. subtilis* (Prágai *et al.*, 1997; Tjalsma *et al.*, 1998) (Fig. 1, Chapter IV). This deviation might hint some functional specialisation of YqjG in these organisms. In *B. subtilis*, YqjG was shown to be involved in competence (Saller *et al.*, 2011), but the role of its signal peptide in this process was not investigated.

We reasoned that if the signal peptide regions were distinct, that could be responsible for the differential functionally of SpoIIIJ and YqjG during sporulation, prompting us to further explore the matter. We verified that SpoIIIJ is indeed a lipoprotein, as substitutions of the invariable cysteine residue of the consensus sequence for SPase II inhibit cleavage (C23A and

C23K, AH5308 and AH5328, respectively, in Fig. 2, Chapter IV). In addition, we observed that the processing of SpoIIIJ was inhibited by mutations in *lspA* and *lgt* (the latter being required for SPase II activity (Leskelä *et al*., 1999)) (Fig. 3, Chapter IV). The *lgt*$^{-}$ background was also analysed as the extracellular proteomes for *lspA* and *lgt* mutants are quite distinct (Antelmann *et al*., 2001). As for YqjG, we observed that the A26K substitution affecting the consensus for cleavage by type I SPases did indeed inhibit cleavage (AH5301 and AH5325, similar promoter and coding regions but obtained differently (Materials and Methods) in Fig. 4, Chapter IV). Inhibition of cleavage was not observed for YqjG containing the C19A substitution that would affect a putative weak consensus for SPase II (AH5309 in Fig. 4), further reinforcing the notion that YqjG is cleaved by type I SPases.

After establishing that SpoIIIJ and YqjG harbour distinct signal peptides, we sought to obtain a lipoprotein version of YqjG in an attempt to increase its functionality during sporulation. Our results strongly suggest that we successfully converted YqjG into a lipoprotein, as this variant's cleavage was affected by mutations in *lgt* and *lsp* (Fig. 5 in Chapter IV). However, it produced a similar heat-resistant spore titre to that of native YqjG, indicating that if being a lipoprotein is required to support sporulation, it is not sufficient. In support of the idea that a lipoprotein signal peptide is dispensable for sporulation are the results concerning YqjG variants that promote higher heat-resistant spore titres most likely via improved interaction with SpoIIIAE (Camp and Losick, 2008), and the results regarding increased sporulation levels in the presence of YqjG-GFP, present in Chapter III.

An indication that the cleavage of the signal peptides of SpoIIIJ and YqjG might not to be required for function came from strains AH5328 (*amyE*::*spoIIIJ*(C23K)-*his spoIIIJ*::*km*) and AH5331 (*amyE*::*yqjG*(A26K)-*his spoIIIJ*::*km yqjG*::*sp*). The former exhibits efficient sporulation that is supported by an uncleaved SpoIIIJ variant; the latter is viable, with growth being supported by uncleaved YqjG(A26K)-His_6. However, these

functions might still be supported by SpoIIIJ/YqjG that had suffered alternative N-terminal processing, even if not visible in immunoblots (as observed for AH5231 (*amyE*::P_{yqjG}-*spoIIIJ-his spoIIIJ*::*km*) which sporulates efficiently despite not accumulating to visible amounts; Chapter III). Possibly, the signal peptides themselves could be dispensable for function. That was shown to be the case, as signal peptide-less forms of YqjG and SpoIIIJ support viability, and also sporulation in the case of SpoIIIJ (Figs. 6 and 7, Chapter IV).

Interestingly, we still observe the presence of a faster-migrating species both in YqjG(A26K)-His_6 and in YqjG that has been converted into a lipoprotein (Figs. 4 and 5, respectively, Chapter IV), which migrates at the same level as the faster-migrating band in wild-type YqjG-His_6. In addition, this species is observed even for ΔSPYqjG-His_6 (Fig. 6). These results suggest that the secondary cleavage event of YqjG is not related to the type of signal peptide it possesses and does not even require its presence.

How do the polytopic SpoIIIJ and YqjG reach the membrane? Several cases of proteins inserting into the membrane in the absence of a signal peptide have been reported. The mature part of the proteins may still contain signals, for instance, TM segments that can be recognised by the ribosome-bound SRP (reviewed in Papanikou *et al.*, 2007). It was also suggested that non-cytoplasmic proteins might fold slowly and that proteins in that state are a target for transport (Prinz *et al.*, 1996). Additionally, SpoIIIJ proteins from the genus *Clostridium* do not possess the signal peptide region, indicative of membrane insertion taking place in its absence.

The oligomerisation of SpoIIIJ

An increasing number of cases of oligomer formation in the YidC/Oxa1/Alb3 family of insertases have been reported. Oxa1 from *Neurospora crassa* was suggested to form homo-oligomers, most likely

tetramers (Nargang *et al.*, 2002); Oxa1 from *Saccharomyces cerevisiae* was suggested to form just dimers (Kohler *et al.*, 2009), dimers and tetramers (Krüger *et al.*, 2012), and just tetramers (Reif *et al.*, 2005); Alb3 from *Arabidopsis thaliana* is able to form dimers (Dünschede *et al.*, 2011); YidC from *E. coli* has been shown to form dimers (Nouwen and Driessen, 2002; Lotz *et al.*, 2008; Boy and Koch, 2009; Kohler *et al.*, 2009), and it has also been recovered as a mix of monomers and dimers (van der Laan *et al.*, 2001; van der Does *et al.*, 2003). Numerous examples of oligomerisation of proteins belonging to other transport systems exist, such as the Sec system (Du Plessis *et al.*, 2011), the Tat system (Gohlke *et al.*, 2005), and the variety of transport systems present in Gram-negative bacteria (Holland, 2010).

In this work we performed a genetic and biochemical characterisation of the SpoIIIJ insertase from *B. subtilis*. We purified this membrane protein overproduced in *E. coli* and observed several oligomeric species. The involvement of the cysteine residue 134 of SpoIIIJ in dimerisation via disulphide bond formation is supported by the impairment in dimer formation observed upon addition of a reducing agent (DTT) and also upon replacement of the cysteine by an alanine residue (Figs. 1 and 2, Chapter V*).* In addition, the CD spectra of wild-type SpoIIIJ±DTT show some differences whilst those of SpoIIIJ(C134A)±DTT are nearly identical; this differential sensitivity to DTT of only the wild-type form suggests the existence of a disulphide bond that, upon breaking, produces a conformational change.

Disulphide bonds have been described in the membrane plane for several proteins (e.g. Rutledge *et al.*, 1992; Arien *et al.*, 2003; Sousa *et al.*, 2003; Chiang and Knowles, 2008). It has been suggested that the presence of residues that create a polar environment within the membrane would favour the requisite formation of the cysteinyl anion intermediate for disulphide bonding (Rutledge *et al.*, 1992). In the TM segment of interest, SpoIIIJ possesses two polar amino acid residues, including a glutamine. Interestingly, this is one of the single polar residues that drove oligomerisation of artificial TM segments (Zhou *et al.*, 2001).

We investigated the environment surrounding Cys134, which is predicted to belong to the second TM segment of SpoIIIJ, using the sulfhydryl-reactive label malPEG. Labelling of Cys134 was only observed when performed at a high temperature. This suggests that the cysteine belongs to a protein interface that requires harsh conditions to be loosened sufficiently to allow access to the bulky malPEG. Accordingly, Koide *et al*., (2007) have found that the bulkiness of this compound prevented labelling when compared to a smaller sulfhydryl-reactive label. These results suggest that Cys134 is involved in a disulphide bond located at an interface of dimerisation in SpoIIIJ.

In our model (Fig. 6, Chapter V), the cysteines are part of a dimerisation interface, forming disulphide bonds that sustain the dimeric form along with non-covalent interactions between other amino acid residues. This is supported by the altered monomer:dimer ratio observed in the size exclusion chromatography (SEC) profile of SpoIIIJ(C134A), for which we observe a higher proportion of the monomer relative to that of the wild-type protein.

An additional species containing SpoIIIJ was visualised through SDS-PAGE: a heterodimer composed of a "full-length" (mature) monomer and a truncated one was observed in *E. coli* only (Fig. 1, Chapter V), suggesting that SpoIIIJ is susceptible to cleavage by a protease absent in *B. subtilis*; alternatively, the cleaved region is somehow protected from that protease in *B. subtilis*. Some insight on the regions required for oligomerisation may arise from the fact that heterodimers but not homodimers of the truncated form were observed. We conclude that the first TM segment is either directly involved in oligomer formation or indirectly. In the latter, TM1 would maintain the involved region(s) (e.g. TM2) in an adequate conformation required for oligomerisation. This idea is in line with the structural role of the corresponding TM segment in *E. coli*'s YidC (TM2) and with its interaction with TM3 (corresponding to SpoIIIJ's TM2) (Yuan *et al*., 2007; Klenner and Kuhn, 2012). In addition,

YidC from *E. coli* harbours a cysteine residue (Cys423) that corresponds to Cys134 of SpoIIIJ from *B. subtilis*. Cys423 belongs to the substrate-interacting face of TM3 but was suggested to play a structural role, specifically in helix-helix interaction (Yuan *et al.*, 2007; Yu *et al.*, 2008; Klenner and Kuhn, 2012). This is in agreement with our data that also suggest a structural role for Cys134, specifically in the dimerisation of SpoIIIJ.

A role for Cys134 during sporulation

A channel bridging the two compartments of the developing sporangium was suggested to be required for maintaining the engulfed prespore's integrity and physiology, including activity of the late prespore sigma factor, σ^G. The SpoIIIA-SpoIIQ channel is composed of nine different proteins, SpoIIQ from the prespore along with the eight SpoIIIA proteins from the mother cell, one of them being SpoIIIAE (Fig. 3, Chapter VI) (Camp and Losick, 2009; Doan *et al.*, 2009). SpoIIIJ was suggested to be required for the proper biogenesis of the Sec-dependent SpoIIIAE (Camp and Losick, 2008; Doan *et al.*, 2009), specifically in a post-insertional step (Serrano *et al.*, 2008). As *E. coli*'s YidC was shown to be involved in the folding of at least two Sec-dependent proteins but not in their insertion (Nagamori *et al.*, 2004; Wagner *et al.*, 2008), and SpoIIIJ was suggested to participate in the assembly of the F_1F_0 ATP synthase complex (Saller *et al.*, 2009), we suggest a role for SpoIIIJ in SpoIIIAE's folding and/or assembly onto the SpoIIIA-SpoIIQ channel. In any case, SpoIIIJ's requirement for sporulation is justified, via SpoIIIAE, specifically for σ^G activity after engulfment completion (Errington *et al.*, 1992) and in maintaining prespore integrity (Li *et al.*, 2004).

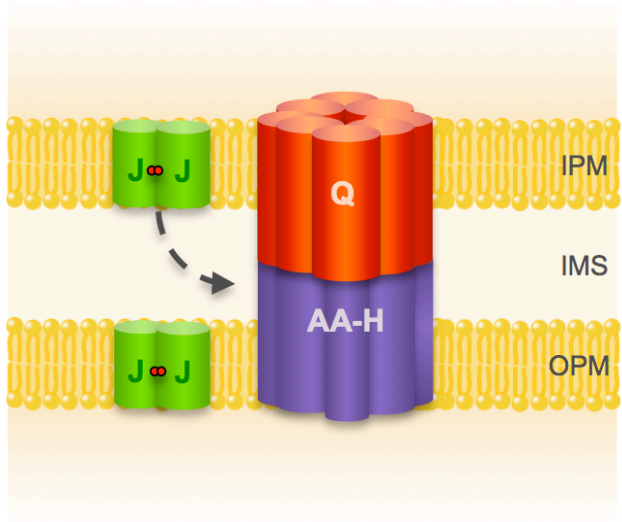

Figure 3. Dimeric SpoIIIJ participates in the biogenesis of the SpoIIIA-SpoIIQ channel. SpoIIIJ, here depicted as a dimer joined by a disulphide bond between cysteine residues (red circles) along with other interactions, is present in both the inner and outer prespore membranes (IMP and OPM, respectively). SpoIIIJ is required for the biogenesis of SpoIIIAE, which is part of the SpoIIIA-SpoIIQ channel that allows nurturing of the prespore by the mother cell after engulfment completion and thus σ^G activity. Adapted from Doan *et al.*, 2009.

We found a role for Cys134 of SpoIIIJ both in the dimerisation of SpoIIIJ and in maintaining the potential for σ^G activity during spore formation. This suggests that dimerisation, promoted by Cys134 might be important for SpoIIIJ to fully fulfil its function(s), at least during sporulation. This view is strengthened by the fact that Cys134 is critical during σ^G activation when SpoIIIJ is present in low amounts only: we suggest that under these conditions Cys134 of wild-type SpoIIIJ enhances dimerisation, which is required for sporulation, but the C134A variant fails to dimerise as efficiently, resulting in a drop of the sporulation titre and in the proportion of cells with active σ^G (Fig. 5 and Tables 3-4, Chapter V).

SpoIIIJ likely exists as a mixed population of distinct oligomeric states that may be related to which function is required of SpoIIIJ for different substrates, such as membrane protein insertion, folding or assembly, and on whether or not such occurs in cooperation with the Sec pathway.

Interestingly, SpoIIIJ was found to participate in the formation of the F_1F_0 ATP synthase in superstoichiometric amounts (Saller *et al.*, 2009). One

possibility is that such is due to the presence of oligomers of SpoIIIJ. In addition, multiple locations in the complex with the F_1F_0 ATP synthase may require SpoIIIJ.

According to our model (Fig. 6, Chapter V), SpoIIIJ forms disulphide-linked dimers. We cannot exclude the existence of a hexameric species, as suggested by BN-PAGE, in which basic dimeric units would trimerise into a hexamer. Hexamers composed of trimers of dimers have been observed previously, e.g. for a chemotaxis receptor in *E. coli* (Kim *et al.*, 1999) and for the HIV-1 capsid protein (Tsiang *et al.*, 2012). Further analysis of the oligomeric state of SpoIIIJ may involve determining the absolute molecular mass of its several oligomeric forms through analytical ultracentrifugation and static light scattering techniques (DeGrado *et al.*, 2003; Slotboom *et al.*, 2008). Other techniques also commonly used to investigate the oligomeric state of transmembrane proteins are fluorescence resonance energy transfer (FRET) spectroscopy and *in vivo* chemical cross-linking (DeGrado *et al.*, 2003; Bruce, 2012).

The transfer of Cys134 to another position in the α-helix might enable more efficient labelling, also suggestive of Cys134 being part of an interface. Also, a direct interaction between two TM2 segments of SpoIIIJ might be obtained via a yeast two-hybrid assay.

An interesting experiment would be to perform SEC-LS (coupled to static light scattering) (Slotboom *et al.*, 2008) of a mixture of purified SpoIIIJ and SpoIIIJ(C134A) in different proportions and examine if the ratio of monomer:dimer peak varies as expected. Another possibility is to test each protein individually in the presence or absence of the reducing agent DTT and check the elution profiles for diminished dimerisation of the wild-type SpoIIIJ.

The effect of the C134A substitution in dimer stability could also be studied by comparing wild-type and substituted SpoIIIJ purified in the presence of a mild detergent. Either protein would then be_subjected to increasing amounts of a harsher detergent that destroys the native structure, followed by cross-linking and SDS-PAGE (Cymer and

Schneider, 2012).

In order to achieve a more accurate reflection of how SpoIIIJ behaves in *B. subtilis*, both the already employed and the above-mentioned techniques should be performed with SpoIIIJ purified from *B. subtilis*. Interestingly, YqjG also possesses cysteine residue at a homologous position (Cys142), hinting that it may also oligomerise.

Overall, this work explores the relationship between SpoIIIJ and YqjG and highlights the key role of SpoIIIJ during sporulation. Although these proteins share a common essential role during vegetative growth, our results indicate that the presence of two homologues in the genome of *B. subtilis* allowed functional specialisation whilst maintaining common traits of their family such as malleability and the ability to oligomerise.

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