Universidade de Lisboa
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Departamento de Biologia Vegetal

Deciphering the genetic makeup of the *Bacillus subtilis* Esat-6-like secretion system

Master Thesis
Hugo Manuel Condessa Barreto

Mestrado em Microbiologia Aplicada
2013

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This thesis was fully performed at the Center of Molecular Pathogenesis-Unit of Retroviruses and Associated Infections (CPM-URIA), Faculty of Pharmacy of the University of Lisbon under the direct supervision of Prof. Dr. Carlos São-José.

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Abstract

The locus yukEDCBuyeBC of the Gram-positive model bacterium Bacillus subtilis was recently shown to encode a functional Esat-6-like secretion system (Ess), in natural isolates of this bacterium, with YukE being a typical secretion substrate of the WXG100 superfamily. This genetic locus has been renamed BsEss. Type VII/Ess secretion systems are widespread among bacteria of the phyla Actinobacteria and Firmicutes and in some species they play an important role in bacterial pathogenesis, as in Mycobacterium tuberculosis and Staphylococcus aureus. Thus, the BsEss is viewed as an attractive model to study molecular details of this important secretion pathway. Interestingly, the system is impaired in the classical B. subtilis lab strain 168, although carrying an intact BsEss locus.

There are several reported mutations in the genome of strain 168, when compared to ancestral or undomesticated strains like NCIB 3610 and ATCC 6051, all of them affecting genes involved in Deg-regulated cellular processes. The BsEss is known to be activated by the phosphorylated state of DegU, so in this work we aimed to identify genetic mutations present in strain 168 that could account for the defective operation of the secretion system, and thus to contribute to the understanding of its functioning and regulation. Among the studied mutations, the ones that mostly impaired BsEss operation were those affecting the expression of genes sfp and degQ. Sfp is necessary for surfactin production, a requisite for B. subtilis swarming motility, functioning also as a signaling molecule in biofilm formation. DegQ was shown to enhance phosphotransfer from DegS~P to DegU, being also necessary to robust biofilm development. By complementing these two mutations in strain 168 we could restore YukE secretion to the levels observed in undomesticated strains.

Other genes were included in this study, related to the DegS-DegU regulon. Among these we found swrB, a gene involved in the synthesis of flagella, as being important for YukE export. B. subtilis YeeF, a homologue of the S. aureus Ess-associated protein EsaD, seemed also to be important for BsEss functioning.

Finally, in this work we have further explored previous results that suggested a negative role of BsEss expression in competence development, in an effort to uncover potential cellular functions for this secretion system.
Resumo

Foi recentemente demonstrado que o locus genómico yukEDCByueBC da bactéria Gram-positiva *Bacillus subtilis* codifica para um sistema de secreção do tipo Esat-6 (Ess). Este sistema é funcional em isolados naturais desta bactéria, sendo YukE um substrato de secreção típico da superfamília WXG100. Este locus foi recentemente renomeado *BsEss*. Os sistemas de secreção do Tipo VII/Ess são bastante prevalentes em bactérias dos filos *Actinobacteria* e *Firmicutes*, sendo que em algumas espécies desempenham um papel importante na patogénese bacteriana, nomeadamente em *Mycobacterium tuberculosis* e *Staphylococcus aureus*. Assim, o *BsEss* é visto como um modelo atractivo para estudar os detalhes moleculares deste importante sistema de secreção.

Curiosamente, este sistema parece inibido na estirpe 168 de *B. subtilis*, a estirpe laboratorial mais utilizada em trabalhos de investigação, apesar de esta possuir o locus *BsEss* intacto. São conhecidas várias mutações na estirpe 168 quando comparada com estirpes ancestrais ou não domesticadas, como por exemplo os isolados NCIB 3610 e ATCC 6051. Essas mutações afectam genes envolvidos em processos celulares regulados pelo sistema de dois componentes DegS-DegU. Sabe-se que o *BsEss* é activado pela forma fosforilada de DegU, pelo que neste trabalho pretendeu-se identificar mutações presentes na estirpe 168 responsáveis pela inibição do *BsEss*, ao mesmo tempo contribuindo para uma melhor compreensão do seu funcionamento e regulação. Entre as mutações reportadas para a estirpe 168, verificámos que aquelas que mais influenciam negativamente a função do *BsEss* são as que afectam os genes *sfp* e *degQ*. *Sfp* é necessário para a produção de surfactina, um surfactante essencial para a motilidade de *B. subtilis* em superfícies sólidas ("swarming"), para além de funcionar também como molécula sinalizadora no desenvolvimento de biofilmes. *DegQ* aumenta a fosforilação de DegU através de DegS e está também envolvido na formação de biofilmes. Ao complementar ambas a mutações na estirpe 168, observou-se um restauro da secreção de YukE para níveis semelhantes aos das estirpes não domesticadas.

Outros genes foram incluídos neste estudo, relacionados com o regulão DegS-DegU. De entre estes, descobrimos *swrB*, um gene envolvido na síntese dos flagelos, como sendo importante para a exportação de YukE. A proteína YeeF de *B. subtilis*, a qual é homóloga à proteína EsaD do Ess de *S. aureus*, revelou-se também importante para o funcionamento do BsEss.

Finalmente, num esforço para desvendar possíveis funções celulares para este sistema de secreção, foi ainda explorado com mais detalhe resultados anteriores que sugeriam uma influência negativa da expressão de *BsEss* no desenvolvimento da competência de *B. subtilis*. 
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I – Introduction

Bacteria form a very wide diversity of biotic associations, ranging from biofilms to mutualistic or pathogenic interactions with larger host organisms[1]. Bacterial transport systems are of major interest because of their essential roles in bacterial lifestyles. By mediating transport of molecules across the cell envelope they allow bacteria to perceive, respond and adapt to environmental challenges[2]. They are also fundamental in some processes, such as cell differentiation, horizontal gene transfer, nutrients uptake, motility, intercellular communication and in the establishment of pathogenic interactions with eukaryotic cells, in a context of bacterial infection[1,3].

In this work we have studied the genetics of a recently discovered protein secretion pathway, the Type VII/Esat-6 secretion system (T7SS/ESS), in the Gram-positive bacterium Bacillus subtilis. The next sections will provide a brief introduction to the best studied bacterial secretion systems and a more detailed description of the key features of T7SS/ESS.

I.1 – Bacterial protein secretion systems

Among the different bacterial transport systems, those involved in protein traffic have been intensively studied. Bacteria use different systems to translocate and insert proteins across and into the cytoplasmic membrane. They can be divided in the widely conserved Sec, Tat and YidC pathways and Type I to Type VII secretion systems, which can transport proteins to different cellular compartments. Gram-negative bacteria contain at least four subcellular locations (cytoplasm, inner membrane, periplasm, and outer membrane), while Gram-positive bacteria contain at least three subcellular locations (cytoplasm, membrane and cell wall)[4]. Proteins can be targeted from their site of synthesis to their correct intracellular destinations or the extracellular space[4].

I.1.1 – The general secretion, twin-arginine and YidC pathways

The general secretion pathway (Sec) and the twin-arginine (Tat) pathways are universal to bacteria and responsible for the secretion of proteins across the single plasma membrane in Gram-positive bacteria and the export of proteins into the periplasm in Gram-negative bacteria[1].

I.1.1.1 – The Sec and YidC pathway

The Sec pathway is essential to cell viability[5] and is a multi-stage reaction that occurs mainly post-translationally, which can be divided in three main steps: protein sorting and
targeting; translocation; release and maturation. The machinery of the Sec pathway recognizes a hydrophobic N-terminal leader sequence on nascent proteins destined for secretion, and translocates proteins in an unfolded state. The nascent proteins are recognized by the ribonucleoprotein signal-recognition particle (SRP) or the SecB chaperone. The proteins recognized by SRP are mainly targeted to the inner membrane. Then, the nascent proteins are targeted to the translocase at the membrane. For SRP, this is achieved by docking to its membrane receptor FtsY and for SecB by docking to the SecA subunit of the translocase (Fig.1).

In *Escherichia coli* the Sec translocase is comprised of the SecYEG translocation channel and the accessory components SecA, SecDFYajC, and YidC. The cytoplasmic SecA subunit hydrolyzes ATP to drive translocation, SecDFYajC improves protein translocation and membrane protein insertion and YidC is required for membrane insertion of certain Sec-dependent substrates and for membrane insertion of Sec-independent substrates. In the YidC pathway, YidC functions as a membrane insertase without the Sec machinery (Fig.1).

In comparison to *E. coli*, the main difference in *B. subtilis* Sec pathway is the lack of the chaperone SecB and the existence of other two chaperones, GroE and DnaK.

### I.1.1.2 – The Tat pathway

The machinery of the Tat secretion pathway recognizes a typical twin-arginine motif in the N-terminal domain and translocates the protein in a folded state.

In *E. coli*, the Tat machinery consists of TatA, TatB, TatC and TatE proteins but its variable in other bacteria. Thus, the minimal Tat translocase is composed of one TatA and one TatC subunit. TatA, TatB and TatC are integral membrane proteins that are inserted in the inner membrane in *E. coli*, forming a “core complex” (Fig.1). TatC functions in the recognition of targeted proteins, while TatA is thought to be the major pore-forming subunit.

To avoid the translocation of Tat substrates by the Sec pathway, typically the C-terminal region of Tat substrates carries a basic residue, the “Sec-avoidance” motif that, in combination with the twin-arginine motif in the N-region, avoids the translocation of Tat substrates by the Sec pathway.
I.1.2 – Type I to Type VI secretion systems

This type of classification evolved from the studies on secretion pathways of Gram-negative bacteria and some authors argue that this classification should apply only to systems that translocate proteins across both the cytoplasmic and outer membranes. However, it is unquestionable that some of these pathways are at least partially conserved in Gram-positive bacteria. Some secreted proteins are exported across the inner and outer membranes in a single step, whereas other are first exported into the periplasmic space via the universal Sec or Tat pathways mentioned above and then translocated across the outer membrane\textsuperscript{[1]} (Fig.2).

Fig. 2 – Summary of Type I to Type VI secretion systems. HM, host membrane; OM, outer membrane; IM, inner membrane; OMP, outer membrane protein; MFP, membrane fusion protein. ATPases and chaperones are shown in yellow. Adapted from \textsuperscript{[1]}. 
I.1.2.1 – Type I secretion system (T1SS)

The T1SS or ATP-binding cassette (ABC) transporters are heterotrimeric complexes consisting of an inner membrane ABC exporter, a membrane fusion protein and a pore-forming, outer-membrane protein[3] (Fig.2).

In the α-hemolysin (HlyA) secretion of *E. coli*, the T1SS complex is composed by TolC, HlyD and HlyB. TolC is an integral membrane protein on the outer membrane; HlyD is a membrane fusion protein and HlyB is an inner membrane ABC exporter[16]. Most protein substrates possess a C-terminal signal sequence characterized by loosely conserved secondary structures[17], which is not cleaved off during secretion and must be presented to the ABC exporter in an unfolded state[16].

I.1.2.2 – Type II secretion system (T2SS) and type V secretion system (T5SS)

The T2SS and T5SS are only found in Gram-negative bacteria and take proteins that were exported by the Sec pathway or, in a few cases, the Tat pathway, into the periplasmic space, remove the signal peptides and translocate them in a folded state across the outer membrane[18, 19] (Fig.2).

The T2SS consists in 12 core components: an outer membrane secretin; a cytoplasmic ATPase; an inner membrane protein; the major and minor pseudopilins; the pre-pseudopilin peptidase/metiltransferase; and a protein that might be involved in substrate recognition and/or secretin interactions[20, 21]. These proteins of the T2SS assemble to form a complex that spans the entire gram-negative cell envelope and is composed by a component in the cytoplasm, an inner membrane sub-complex that reaches into the periplasmic compartment and a secretion pore in the outer membrane[19]. This complex is responsible for the multi-protein assembly in the periplasm and their translocation across the outer membrane[19].

In the T5SS, after the proteins are exported to the periplasm by the Sec or Tat pathway, the secreted proteins are translocated across the outer membrane via a transmembrane pore, formed by a beta barrel[22] and with no energy coupling or accessory factors required for the translocation[23].

There are three sub-classes of the T5SS machinery: the T5aSS; the T5bSS; the T5cSS. The T5aSS is also named the autotransporter system. The proteins are synthetized with an N-terminal signal peptide and exported to the periplasm via the Sec pathway like T5bSS and T5cSS. The C-terminal domain forms a beta barrel that is required for translocation into the extracellular space[1]. The T5bSS, also known as two-partner secretion pathway[24] consists of pairs of proteins in which one partner carries the beta barrel domain, and the other partner is the secreted protein, needing to interact with each other to be translocated to the extracellular medium[25]. Finally, in the T5cSS the proteins are trimeric and the beta barrel is formed from contributions from all three polypeptides[1] (Fig.2).
I.1.2.3 – Type III secretion system (T3SS)

The T3SS are also restricted to Gram-negative bacteria and form complex, supramolecular structures which span the inner membrane, the periplasmic space, the outer membrane, the extracellular space and the host cellular membrane[3] (Fig.2). This machinery is termed the injectisome and delivers effector proteins across the bacterial and host membranes into the cytosol of host cells in a Sec-independent manner[1, 26].

The substrates of this system are divided in two classes: the components of the machinery itself and substrates that are translocated to the target cell[18]. The Type III secretion machinery is highly conserved, consisting of more than 20 proteins, many of them sharing homology to flagellar export factors[27].

The injectisomes are composed of a series of basal rings that span the bacteria inner and outer membranes, connected to an hollow needle, filament or pilus, tipped with a translocation pore that is inserted into the plasma membrane of the target cell[1, 26]. Two oligomeric rings form a ring complex called “basal body” inserted in the inner membrane with an ATPase bounded in the cytoplasmic face of the basal body, energizing the protein translocation[3].

I.1.2.4 – Type IV secretion system (T4SS)

The T4SS are found in Gram-negative and Gram-positive bacteria, and are characterized by its unique ability to translocate nucleic acids and complexes of proteins into plant and animal cells, generally by a contact-dependent mechanism and to receive foreigner DNA (transformation)[1, 3, 18].

T4SS can be divided in three groups: with the ability to mediate the conjugative transfer of plasmid DNA or transposons; with the ability of mediate DNA uptake from and release it into the extracellular medium; with the ability to deliver effector macromolecules into eukaryotic cells during the course of infection[28].

The machinery of T4SS is composed by a substrate receptor, an envelope-spanning translocation channel and an extracellular pilus or surface filament (Fig.2).

I.1.2.5 – Type VI secretion system (T6SS)

The T6SS were first described by Pukatzki in 2006[29], and are characterized by a membrane-penetrating structure, delivering the effector proteins directly into the cytoplasm of host cells, or as a channel for protein translocation without the requirement of hydrophobic N-terminal signal sequences[3, 29, 30].

The machinery of T6SS shows similarities to the T4SS components and a model is proposed to include a cytoplasmic chaperone with ATPase activity, a channel bridging from the inner membrane to the outer membrane and a needle tipped with a pore-forming protein[31] (Fig.2).
Recently it has been shown that phage tails and T6SS are structurally, functionally and evolutionary related\[^32\]. It seems that T6SS is a mechanism that can be adapted by individual bacterial species to interact with other prokaryotes and eukaryotes\[^33\].

I.2 – The Type VII/ESAT-6 secretion system (T7SS/ESS)

The T7SS/ESS is a general designation for a protein secretion system with wide distribution among Gram-positive bacteria and some Actinobacteria such as mycobacteria\[^34\]. Apart from a few core elements, the remaining components of this secretion pathway can be completely unrelated among the different bacterial species\[^35\].

The system was first described in *Mycobacterium tuberculosis*, which has a complex cell envelope that includes a peculiar outer membrane enriched in mycolic acids\[^36\]. For this reason, following the classification scheme in Gram-negative bacteria this secretion pathway was coined as T7SS. Although with some controversy\[^2, 37\] this is the nomenclature currently used in *Actinobacteria*, whereas in Gram-positive bacteria, which have only a cytoplasmic membrane and a cell wall, the most common adopted designation is Esat-6-like secretion system (ESS) or WXG100 secretion system (WSS)\[^2, 38\] (see below).

Unlike bacterial T3SS or T4SS, secretion of T7SS/ESS substrates in *M. tuberculosis* occurs in *in vitro* grown cultures and seems not to require host cell target interaction for translocation of the secreted proteins\[^39\].

I.2.1 – Discovery of the T7SS/ESS in *M. tuberculosis*

The first clues for a new T7SS/ESS came from the *in silico* analysis of the *M. tuberculosis* virulence effectors ESAT-6 (early secreted antigen target, 6 kDa) and CFP-10 (culture filtrate protein, 10 kDa), encoded respectively by *esxA* and *esxB* genes in the region of difference 1 (RD1)\[^35\]. These effectors are known to be secreted without a recognizable secretion signal\[^40, 41\] and interact with each other forming a tight 1:1 heterodimer\[^42, 43\].

The RD1 region, which contributes to the pathogenesis of *M. tuberculosis*\[^44\], is deleted in the vaccine strain *Mycobacterium bovis* (BCG), being this one of the reasons for the attenuated virulence of BCG\[^45\]. When the complete RD1 region was reintroduced in the BCG strain the secretion of ESAT-6 and CFP-10 was restored\[^46\], revealing the first evidences for a new secretory system.

The ESAT-6 and CFP-10 proteins are important T-cell antigenic targets\[^47\] and belong to the WXG100 superfamily, whose members were initially defined as sharing a central tryptophan-variable-glycine (WXG) motif, a protein length of ~100 residues\[^35, 48\] and the tendency to cluster with genes for membrane proteins, ATPases and/or chaperones\[^40, 41, 48\]. *M. tuberculosis* has up to five T7SS/ESS, termed ESX-1 to ESX-5, being the ESX-1 the best studied and the responsible for the secretion of ESAT-6 and CFP-10\[^49\].
There are other substrates of the T7SS/ESS of *M. tuberculosis*, such as the PE and PPE proteins that form heterodimeric complexes\[^{50}\] and are associated with virulence\[^{51}\] like ESAT-6 and CFP-10. Distantly related gene clusters were also found in the genome of pathogenic and non-pathogenic Gram-positive bacteria such as *Staphylococcus aureus* and *B. subtilis*\[^{1, 35}\] (Fig. 3).

**Fig. 3 – Schematic representation of the gene clusters encoding core components and substrates of T7SS-like systems in *M. tuberculosis* (ESX-1), *S. aureus* (Ess) and *B. subtilis* (BsEss).** The nomenclature of ESX-1 genes is that propose by Bitter et al.\[^{37}\]. For simplicity, the genes located immediately or far upstream of *M. tuberculosis* eccA1 are not represented. Genes described as essential or important for secretion of cognate WXG100 proteins, or of other specific substrates, are marked with the letter “E”. Genes coding for products sharing conserved domains are depicted with the same color code, whereas those specific of each system are colored in white. Features of conserved gene products are indicated below. TMD, transmembrane domain.\[^{52}\]

**I.2.2 – Molecular composition of T7SS/ESS**

In *M. tuberculosis*, the five T7SS/ESS share a number of highly conserved components, named Ecc (ESX-conserved-component)\[^{37}\]. Five of these components are predicted to be membrane proteins and could form a translocation channel. Two other components show homology to proteins with known function: MycP, a protease that belongs to the subtilisin protein family; and EccC which is a member of the FtsK/SpoIIIE family of ATPases\[^{53}\].

In *M. tuberculosis*, the membrane complex is thought to be composed of the membrane proteins EccB, EccC, EccD and EccE, that likely form a translocation channel\[^{49}\]. This complex has a size of ~1500 kDa and might consist of six copies of EccB, EccC and EccD and three copies of EccE, being EccE only absent in the ESX-4\[^{49}\]. The six copies of the ESX-5 EccC are in accordance to members of the FtsK/SpoIIIE family which forms hexamers\[^{54}\]. Recently, it has been proposed two models for the topology of the T7SS/ESS membrane complex (Fig. 4).
Fig. 4 – The two proposed models for the T7SS/ESS membrane channel. EccBCDE are embedded in the cytosolic membrane where they assemble into a large membrane complex. The three nucleotide-binding domains of EccC are likely involved in energizing translocation of substrates through the translocation channel. In (A) is a two-step process and in (B) a one-step process. IM, inner membrane; PG, peptidoglycan; AG, arabinogalactan; OM, mycobacterial outer membrane.

I.2.3 – Mechanism of action of T7SS/ESS

Although the substrates and the membrane complex have been studied intensively, the mechanism of action is still unclear. It is known that all the substrates in ESX-1 are mutually dependent upon each other for secretion and in mycobacteria they seem to be preferentially secreted as heterodimers. It has been identified a CFP-10 secretion signal in its C-terminus, more specifically a YxxxD/E motif that appears to be shared by all known classes of T7SS/ESS substrates in mycobacteria, except for PPE and ESAT-6-like. These last proteins are secreted as a complex with a motif-containing substrate and can be used to predict putative unknown T7SS/ESS substrates, although the signal does not discriminate among the various ESX pathways.

Recently it has been added the function of EspG, a protein that may maintain the PE/PPE proteins in a stable conformation, possibly directing them to the membrane-embedded secretion machinery where the YxxxD/E motif is potentially recognized by EccE and then translocated across the mycomembrane.

I.2.4 – Role on pathogenic interaction with eukaryotic host

As it was mentioned above, ESAT-6 and CFP-10 act as virulence effectors of *M. tuberculosis*, inducing a strong T-cell response during infection. Among all the different proposed functions for ESAT-6, CFP-10 and ESX-1, the most frequently reported observations...
points towards a function linked to lysis of cells and/or membranes\[^{39, 44}\], induced apoptosis, translocation to the cytosol and phagosomal rupture in the host cell\[^{59, 60, 61}\]. The immune responses induced by ESX-1 antigens are generated at both the innate and adaptive levels to limit mycobacterial replication, and an ESAT-6-specific T-cell response is obtained only when ESAT-6 and CFP-10 are expressed and secreted\[^{39}\].

It is known that ESAT-6 plays a crucial role in \textit{M. tuberculosis} infection\[^{62}\], namely by interacting and disintegrating the host cell membrane\[^{63, 64}\], by modulating the activation of the macrophage\[^{65}\] and interacting with the host signaling pathway\[^{62}\]. It is also known that the RD1 is involved in the translocation of \textit{M. tuberculosis} from the phagosome into cytoplasm of the host cell at later stages of infection\[^{66}\], which results in the induction of cytotoxicity and in the end, host cell death\[^{61}\].

Altogether, this data shows that the T7SS/ESS of \textit{M. tuberculosis} is clearly involved in pathogenicity and host-cell infection.

\textbf{I.2.5 – DNA transfer mediated by T7SS/ESS}

The T7SS of \textit{Mycobacterium smegmatis}, which is homologous to the \textit{M. tuberculosis} ESX-1, is involved in DNA transfer by a conjugation-like mechanism\[^{67}\]. Interestingly, T7SS has opposite impact in donor and recipient strains: donor secretion negatively regulates transfer whereas in recipient is essential\[^{68}\]. Such opposed effects cannot be explained by the nature of the secreted substrates (ESAT-6/CFP-10 homologues) as these are identical in donor and recipient strains\[^{68}\]. Thus, secretion by T7SS and DNA transference appear to have a close interplay in \textit{M. segmatis}, suggesting that this relation can also occur in other bacteria that possess Esat-6-like secretions systems.

\textbf{I.3 – The \textit{S. aureus} Esat-6-like secretion system (Ess)}

As mentioned before, \textit{S. aureus} possesses a gene cluster, the \textit{ess} locus (Fig.3), encoding an Esat-6-like secretion system with some elements displaying homology to those of T7SS/ESS of \textit{M. tuberculosis}.

The Ess proved to be functional and its WXG100 substrates required for the pathogenicity of \textit{S. aureus}\[^{38}\]. The component EssC is a member of the FtsK/SpoIIIE family, whose function is essential for secretion of EsxA and EsxB, which in their turn are two secreted substrates of the WXG100 superfamily\[^{38}\]. EsxA and EsxB depend on each other to be secreted. In addition, EsaC is a polypeptide that is also secreted by the \textit{S. aureus} Ess\[^{69}\].

Recently it has been shown that EsaD and EssB are involved in the secretion of Ess substrates\[^{70, 71}\]. EsaD is a polypeptide located in the staphylococcal membrane, is part of the Ess and supports the secretion of EsxA, being therefore involved in the pathogenesis of staphylococcal infections\[^{70}\]. EsaD also shows homology to the \textit{B. subtilis} YeeF\[^{70}\]. EssB is a
membrane protein, required for EsxA secretion and an essential component of the Ess translocon. It probably interacts with itself and other machinery components\textsuperscript{[71]}. EssB is homologous to \textit{B. subtilis} YukC\textsuperscript{[71]}. The Ess was proved to interfere with host immune responses and to favor the establishment of persistent infections\textsuperscript{[69]}.

I.4 – The \textit{B. subtilis} Esat-6-like secretion system (\textit{BsEss})

The Gram-positive non-pathogenic model bacteria \textit{B. subtilis} has a gene cluster, the yuk locus that is positively regulated by phosphorylated DegU\textsuperscript{[72]} and which carries some components homologous to elements of the \textit{M. tuberculosis} T7SS and \textit{S. aureus} Ess\textsuperscript{[48]}.

This \textit{B. subtilis} locus is composed by an FtsK/SpolIIE-like ATPase encoded by yukB, an ubiquitin-like protein YukD\textsuperscript{[73]}, the YukC protein with a pseudokinase-like fold\textsuperscript{[74]}, the membrane proteins YueB and YueC and the putative WXG100 substrate YukE. YueB was previously known as the receptor essential for phage SPP1 infection\textsuperscript{[75]}. Recently, YukE was shown to be secreted as a homodimer, being its export absolutely dependent on elements of the Yuk cluster and on the phosphorylated form of the response regulator DegU\textsuperscript{[52]}.

In this work the yuk locus was renamed \textit{BsEss} (\textit{B. subtilis} Esat-6-like secretion system). The only conserved elements among the T7SS/ESS of \textit{M. tuberculosis}, \textit{S. aureus} and \textit{B. subtilis} are the FtsK/SpolIIE-like ATPase (YukB in \textit{B. subtilis}) and the WXG100 substrates (YukE in \textit{B. subtilis})\textsuperscript{[38, 48]}. In addition to these elements, \textit{S. aureus} and \textit{B. subtilis} share esaA/yueB, essA/yueC, esaB/yukD and essB/yukC\textsuperscript{[52]} (Fig.3).

\textit{BsEss} operation was demonstrated in the undomesticated strain \textit{B. subtilis} ATCC 6051 when entering the stationary growth phase. Genes of the \textit{BsEss} locus were required for YukE stable production, secretion and accumulation in culture supernatants and their activation depended on high levels of phosphorylated DegU\textsuperscript{[52]}. It is known that the classic lab strain \textit{B. subtilis} 168 has accumulated several mutations during is domestication that seem to diminish DegU phosphorylation or its action as transcriptional activator\textsuperscript{[72, 76]}, thus possibly affecting the expression of the \textit{BsEss} locus. In strain ATCC 6051, where YukE is secreted\textsuperscript{[52]}, these mutations are not present\textsuperscript{[77]}.

I.5 – Thesis goals

The T7SS/ESS secretion systems are widespread among bacteria of the phyla \textit{Actinobacteria} and \textit{Firmicutes} and in some species they play an important role in bacterial pathogenesis. Due to the amenability and availability of tools for the genetic manipulation of the \textit{B. subtilis} lab strain 168 and its derivatives, the \textit{BsEss} could be an attractive model to study molecular details of this important secretion pathway.

However, as mentioned above, secretion of YukE is strongly diminished in this domesticated strain. Strain 168 is known to have mutations affecting genes \textit{epsC}, \textit{sfp}, \textit{swrA} and \textit{degQ}\textsuperscript{[77, 78, 79]},
all of them involved in Deg-regulated cellular processes like swarming and biofilm formation\textsuperscript{[72, 79, 80, 81, 82]}. In addition, strain 168 is also cured from a plasmid that has strong influence in biofilm architecture of ancestral strains such as NCIB 3610\textsuperscript{[79]}. This plasmid was recently shown to be responsible for the low competence for transformation of undomesticated strains\textsuperscript{[83]}.

In this work, we aimed to study if genetic mutations reported in \textit{B. subtilis} 168 could account for the defective operation of BsEss in this domesticated strain, at the same time contributing to the understanding of the functioning and regulation of this secretion pathway. The effect of other genes such as \textit{swrB, aprE, degR} and \textit{yeeF}, the latter of which is homologue to \textit{S. aureus} EssD, shown to support Ess functioning\textsuperscript{[70]}, was also studied. As an attempt to uncover cellular functions of the BsEss (none is presently known) we have also explored in this work previous results that linked the BsEss cluster to the development of competence for DNA uptake (C. Baptista, unpublished).
II – Results

II.1 – Why is *B. subtilis* strain 168 impaired in *BsEss* functioning?

II.1.1 – Study of well-known 168 mutations in an undomesticated background

As previously mentioned, the genome of the *B. subtilis* reference strain 168 (GenBank NC_000964) carries mutations affecting genes *epsC*, *swrA*, *sfp* and *degQ*, when compared to undomesticated strains such as NCIB 3610 (GenBank NZ_CM000488) and ATCC 6051 (GenBank NC_020507)\(^{[77,79]}\).

Mutations in *swrA* and *sfp* correspond to frameshift mutations\(^{[79,82]}\), whereas that impairing *epsC* is a missense mutation\(^{[79]}\). The mutation affecting *degQ* lies in the -10 box of the promoter sequence, thus decreasing its transcription\(^{[79,81]}\). These mutations impair swarming and biofilm development, which are cellular processes under the control of one major cell-fate regulator, DegU\(^{[72,84]}\). In addition, the 3610 strain carries an ~84-kb plasmid encoding functions that stimulate and repress biofilm formation and competence development, respectively\(^{[79,83]}\).

Our lab as recently confirmed that the *BsEss* is part of the DegS-DegU regulon, being its expression and functioning highly enhanced by the phosphorylated form of DegU, DegU\(\sim P\)\(^{[52]}\). The same study showed that strain 168 secreted very low levels of YukE when compared to strain 6051, suggesting a deficient activation and/or functioning of the *BsEss* in the domesticated strain. Given the link between the 168 mutations referred above and the Deg regulon, we have decided to study the involvement of the respective genes on *BsEss* functioning. Gene *epsC* was not included in this study since the mutation reported in strain 168 seems to impair a specific step of exopolysaccharide biosynthesis\(^{[79]}\), and thus we have considered it unlikely to be involved in *BsEss* operation.

We have started by checking YukE production and secretion in the stationary growth phase of strains derived from the 6051 or 3610 backgrounds, which carried mutations affecting the genes altered in strain 168. In terms of their chromosomal sequences strains 3610 and 6051 are very similar\(^{[78]}\) and in our lab conditions the level of yukE accumulation in culture supernatants is indistinguishable between the two ancestral strains (not shown). The different mutant strains used in this study are described in the Materials and Methods section.

Culture samples taken 2 hours after entry in the stationary phase were processed for precipitation of total proteins present in cell-free supernatants. After solubilization of the precipitates, total protein was quantified by the Bradford method, which seemed to give overestimates of protein content, probably due to the colored PRMM precipitates (see
The precipitates were analyzed by SDS-PAGE and Coomassie blue staining to monitor the quality and relative protein quantities before transfer for Western blot analyses (see methods).

YukE secretion in a 3610 derivative lacking the 84-kb plasmid (strain DS2569) seemed similar to that observed in the WT strain (Fig.5). This result indicates that the absence of this plasmid in strain 168 should not be responsible for the malfunction of the BsEss. In contrast, a degQ knockout mutant of strain 6051 (strain W105) resulted in a marked decrease of YukE secretion (Fig.5). Complementation of the degQ mutant by inserting in the dispensable thrC locus a WT copy of degQ under the control of its native promoter (strain HCB1, see methods) restored high levels of YukE secretion (Fig.5).

![Immunodetection of YukE in supernatant precipitates of different B. subtilis mutants derived from undomesticated strains 6051 or 3610.](image)

**A**

- 3610
- Plasmid cured
- degQ
- sfp
- degQ/thrC::P<sub>degQ</sub>-degQ
- sfp/amyE::P<sub>sfp</sub>-sfp
- swrA

Each lane was loaded with 20µg total protein.

**B**

Coomassie blue-stained gel to show even protein loading.

In absence of *swrA* (strain DS2415) YukE secretion seemed to be unaffected (Fig.5), suggesting that the *swrA* mutation described in strain 168 should not be affecting *BsEss* operation. The *sfp* mutation (strain DS3337) seemed to cause significant reduction of the YukE signal (Fig.5). However, when we tried to complement the *sfp* mutation by inserting *P<sub>sfp</sub>*-*sfp* in the *amyE* locus of strain DS3337 (HCB2, see methods) we did not obtain the expected increase in YukE secretion (Fig.5). At this point the results were inconclusive concerning the real contribution of *sfp* to the *BsEss* pathway (see discussion).
In summary, by introducing in undomesticated backgrounds genetic modifications that mimic those known to occur in strain 168 we have concluded that the 84-kb plasmid and gene swrA have no major role in BsEss functioning. On the contrary, mutations affecting degQ and eventually sfp seem to produce an impact on BsEss operation, as judged by the observed reduction in YukE secretion.

II.1.2 – Restoring BsEss functionality in B. subtilis strain 168

The results from the previous section suggested that the mutations known to affect degQ and sfp expression in strain 168 could be responsible for the defective BsEss functioning in this genetic background. We have confirmed by sequencing that strain 168 from our collection carried the reported mutations (not shown). To check the role of these mutations in the BsEss defective phenotype of strain 168 we have tested if their complementation resulted in an increase of YukE secretion. Complementation of the degQ mutation was carried out by inserting the wild-type cassette $P_{degQ}$-degQ in the thrC locus of strain 168, resulting in the derivative HCB3 (see methods). This strain showed a clear increase in YukE secretion when compared to the parental strain 168, yet YukE signal was still much smaller than that observed in an undomesticated strain (Fig. 6). This result suggested that increasing degQ expression in strain 168 was not sufficient to fully restore BsEss operation and/or YukE accumulation in culture supernatants.

![Fig. 6 – Immunodetection of YukE in supernatant precipitates of different B. subtilis mutants derived from the domesticated strain 168.](image)

For details of strains and mutations see section Material and Methods. 3610 (wild-type strain NCIB 3610); 168 (L16601 strain); 168 degQ$^+$ (HCB3 strain); 168 sfp$^-$ (HCB4 strain); 168 degQ$^+$sfp$^+$ (HCB5 strain). Each lane was loaded with 20µg total protein. B. Coomassie blue-stained gel to show even protein loading.
A similar result was obtained with a 168 derivative (HCB4) carrying \(P_{spf}\)-\(spf\) in the \(amyE\) locus (Fig.6). Interestingly, a double-complemented 168 strain (HCB5) showed levels of YukE secretion comparable to those observed in ancestral strains (Fig.6), indicating that the correction of these two mutations are necessary and sufficient to fully restore the \(BsEss\) functioning in strain 168. In summary, the impairment of \(BsEss\) in strain 168 seems to be due to the mutations reported in the promoter region of \(degQ\) and in \(spf\).

### II.2 – Other genes involved in \(BsEss\) functioning or regulation

Several studies on other T7SS/ESS systems and on the Deg regulon suggested that other \(B. subtilis\) genes could be involved in the operation and/or regulation of the \(BsEss\). Among these we have selected to study genes \(swrB\), \(degR\), \(aprE\) and \(yeeF\). When growing in rich liquid media, \(B. subtilis\) populations are majorly composed of swimming (motile) cells thanks to the activity of SwrA that stimulates the \(fla/che\) operon. In addition to be involved in flagella synthesis and chemotaxis functions, the \(fla/che\) operon also encodes \(\sigma^D\) and its activator SwrB, which are responsible for stabilizing the motility state by driving the expression of other genes\(^{[85]}\). It is also known that \(swrB\) is required for swarming motility, probably by stimulating the assembly and number of cell surface flagella\(^{[82]}\). \(DegR\) expression is driven by \(\sigma^D\)\(^{[86]}\), and \(DegR\) has a positive effect on phosphorylated \(DegU\) by stabilizing their phosphorylated state\(^{[87]}\). The gene product of \(aprE\) is the extracellular protease subtilisin\(^{[88]}\), which is representative of a large subset of serine proteases (PFAM family Peptidase_S8, PF00082). Expression of \(aprE\) is stimulated by phosphorylated \(DegU\), which binds to the \(aprE\) promoter\(^{[89, 90]}\). Interestingly, MycP1, a subtilisin-related mycosin has been shown to have a role in \(M. tuberculosis\) ESX-1 functioning, probably by cleaving specific domains of ESX-1 components\(^{[91, 92]}\).

Given the link of all these genes to the DegS-DegU regulon, which as shown before is essential to the activation of the \(BsEss\)\(^{[52]}\), we have probed secretion of YukE in an undomesticated strain individually affected in these genes. In an \(aprE\) mutant (strain W103) secretion of YukE seemed to be unaffected when compared to strain 3610, whereas in a \(degR\) mutant (strain W128) it was only slightly reduced (Fig.7). These results suggested that YukE and DegR are not crucial for \(BsEss\) function. Somewhat surprisingly (see discussion), YukE secretion was much reduced in a \(swrB\) deletion mutant (strain DS2509), suggesting a role for this gene in \(BsEss\) operation and/or YukE extracellular accumulation. Unfortunately, YukE secretion levels could not be restored in a complementation strain (DS2522) carrying native \(swrB\), under the control of the operon \(fla/che\) promoter, in the \(amyE\) locus of the parental DS2509 (Fig.7), a result that claims for additional confirmatory studies.
Fig. 7 – Immunodetection of YukE in supernatant precipitates B. subtilis mutants derived from ancestral strains. 3610 (wild-type NCIB 3610 strain); aprE (W103 strain); degR (W128 strain); yeeF (HCB6 strain); swrB (DS2509 strain); swrB/amyE::P_{fla/che}-swrB (DS2522 strain). Each lane was loaded with 20µg total protein. B. Coomassie blue-stained gel to show even protein loading.

Recently, it has been shown that EsaD, an homologue of B. subtilis yeeF, is required for normal secretion of EsxA by the S. aureus Ess\cite{70}. Therefore, we have decided to test if yeeF disruption would produce some impact on YukE secretion. yeeF was disrupted through integration of a pMutin4 derivative. The advantage of this strategy is that, in principle, only the target gene is affected. This is achieved by two main properties of the vector\cite{93}: i) vector encoded transcriptional terminators block the transcription initiated upstream of the integration site, and ii) the vector-borne, IPTG-inducible promoter P_{spac} allows expression downstream of the inactivated gene, thus bypassing polar effects in case of operon structures. In the undomesticated strain with yeeF disrupted (HCB6; see methods) the culture samples were obtained in the presence of IPTG, to avoid polar effects. YukE secretion in this strain was reduced (Fig.7), suggesting that yeeF has a similar role to his homologue EsaD. For a full confirmation of these results, the complementation of this strain with yeeF expressed ectopically shall be made in future works.
II.3 – On the track of *BsEss* cellular function: does *BsEss* affect competence development?

At this moment there is no known cellular function for the *BsEss*. In this work we have decided to follow a lead from preliminary results that seemed to implicate the *BsEss* cluster in a reduction of natural competence for DNA transformation (C. Baptista, unpublished). This lead came from a global analysis that aimed to study the effect of *BsEss* deletion in general cellular processes like competence development, sporulation and secretion of degradative enzymes. Interestingly, these results were obtained in the lab strain 168, where *BsEss* expression/functioning seem to be repressed when compared to undomesticated strains (see above). We should note that competence development is very difficult to induce in undomesticated strains in lab conditions\[^{[77, 83]}\], making reliable quantitative measurements of competence efficiency very difficult to perform. An additional motivation to study the effect of *BsEss* in competence development was the fact that DegU is also known to be involved in the regulation of competence genes\[^{[94]}\], in addition to the master competence regulator ComK\[^{[95, 96]}\].

As the regulation of *BsEss* and of competence genes involves DegU, *BsEss* functioning could somehow interfere with the development of competence. Although impaired in YukE secretion in LB medium, strain 168 is still able to transcribe *BsEss* genes at different levels, depending on growth conditions\[^{[75, 97]}\].

We have studied development of competence in the control strain L16601.amy::CM, which is a 168 derivative with a chloramphenicol cassette in the *amyE* locus, and in strain L.Del6, which carries a deletion covering the promoter region of the *BsEss* cluster located upstream *yukE* and downstream genes until *yueB*\[^{[98]}\]. Construction of this deletion mutant implied the insertion of a chloramphenicol resistance cassette in gene *yukF*, which lies immediately upstream and is divergently transcribed relatively to *yukE*. To control a putative effect derived from *yukF* inactivation, a 168 derivative strain carrying *yukF* disrupted by a chloramphenicol cassette (L.cat86) was also tested for transformation efficiency.

The protocol followed for developing *B. subtilis* competence was essentially that described by Yasbin et al.\[^{[99]}\] (see methods). Literature is a bit ambiguous about the time necessary for optimal competence development after culture dilution in GM2 medium and about the minimal quantity of transforming DNA for maximal efficiency of transformation\[^{[99, 100, 101, 102]}\]. We have thus, in a first step, conducted experiments to optimize the protocol in order to have the highest transformation efficiency possible. The results showed a peak of transformation efficiency 90 minutes after dilution and incubation in GM2 (Fig.8A), which roughly corresponded to the time when cultures re-gained exponential growth (Fig.8B).
Fig. 8 – *B. subtilis* transformation efficiency in GM2. GM1 cultures that had been in stationary growth phase for 90 min were 10-fold diluted in GM2 and assayed for their efficiency of transformation at different time points post-dilution. **A.** Strain 168 showed the highest transformation efficiency 90 minutes after dilution in GM2. **B.** Strain 168 re-entered in exponential growth phase after 90 minutes in GM2.

Next we determined the efficiency of transformation for three concentrations of transforming DNA (1, 5 and 25 µg/ml). With 5 µg/ml of DNA the number of transformants per total cell number was clearly higher than with 1 µg/ml, but only slightly lower than with 25 µg/ml of DNA (not shown). Therefore, in all subsequent experiments we have used for transformation 5 µg/ml of DNA and 90 minutes of incubation in GM2.

Strain L.Del6 showed transformation efficiency about fourteen times higher than the control strain (Fig.9), suggesting that the deletion of the *BsEss* cluster and/or the disruption of *yukF* play a role in the development of competence. In an attempt to clarify this question, we tested
strain L.cat86, which supposedly only carried yuKF disrupted by a chloramphenicol resistance cassette. Surprisingly, the results showed a transformation efficiency similar to that of strain L.Del6 (Fig. 9). According to Baptista\(^6\), the construction of strain L.cat86 implied several amplifications of the sequences covering the 5' end of yuKF, the BsEss promoter region, yuKE, yuKD and the 5' end of yuKC. Although this strain showed the expected DNA structure and behaved like the parental 168 in terms of SPP1 infection\(^8\), the presence of eventual point mutations in BsEss genes, due to amplification errors, was never ruled out. So, for clarifying the role of yuKF in the transformation efficiency, a strain with yuKF disrupted by single crossover plasmid integration (L.yuKF, see methods), and therefore more reliable than L.cat86, was equally tested. The results showed transformation efficiency similar to the control strain, in clear contrast to the results observed for L.cat86.

![Fig. 9 – Relative transformation efficiency of 168 mutant strains.](image)

In summary, the results were contradictory in terms of the effect of disrupting yuKF in competence development, making it difficult to draw any conclusion about the results obtained with the BsEss deletion strain, which also carried inactivated yuKF. We are currently constructing a strain only deleted for the BsEss operon in order to elucidate these results.
Recently, it was proved that the Esat-6-like secretion system of *B. subtilis*, encoded by the *BsEss* gene cluster, was functional in an undomesticated strain and dependent of high levels of phosphorylated DegU\[^{[52]}\]. In this work we sought to understand the genetic bases for the deficient functioning of the *BsEss* in the domesticated *B. subtilis* lab strain 168. In addition to the advantages of having a fully operational *BsEss* in strain 168, which is much more amenable to genetic manipulation when compared to ancestral strains, with this work we also wanted to get further insight on the genetic circuits involved in the regulation and/or molecular mode of action of this secretion system.

*B. subtilis* 168 has accumulated during its domestication several mutations that seem to diminish DegU phosphorylation or its action as a transcriptional activator, namely mutations blocking *degQ* and *swrA* expression. Other differences between strain 168 and the 6051 or 3610 ancestral strains include mutations blocking *sfp* and *epsC* and the absence of a plasmid in strain 168. The mutation in *epsC* seems to specifically affect the production of an exopolysaccharide important for biofilm formation\[^{[79]}\], and thus we have decided not to include it in our study.

Based on the literature, mutations affecting *degQ* and *swrA* would be the ones more probable to affect the function of *BsEss*. DegQ stimulates production of DegU-P, a major activator of *BsEss* and it also enhance its own transcription and of yukC\[^{[72]}\]. Expression of *degQ* is in its turn modulated by the two-component regulatory system ComP-ComA, and also to some extent by DegS-DegU, upon different nutritional stimuli\[^{[103]}\]. DegQ is required to swarming motility and biofilm formation\[^{[79, 82]}\]. SwrA had been previously described as a positive modulator of DegU function as transcriptional activator\[^{[76]}\]. DegQ and SwrA seem to be functionally “linked” as mutations in their genes affect the same cellular phenomena such as swarming and biofilm formation\[^{[79, 82]}\]. In our genetic analysis we have confirmed the expected positive contribution of *degQ* to *BsEss* operation, both in an undomesticated background and in strain 168, but not for SwrA.

Intriguingly, we found that an in frame deletion of *swrB*, a gene belonging to the SwrA-stimulated operon *fla/che*, resulted in a drastic decrease of YukE secretion. Unfortunately, ectopic expression of a native *swrB* was not successful in reverting the YukE secretion phenotype, which calls for a re-evaluation of these results. In any case, if we confirm the effect of *swrB* mutation, the apparent contradictory *swrA/swrB* results can be explained if we assume that: i) *swrB* is still expressed to some level in *swrA* mutants and ii) the range of SwrB upregulated genes may extend beyond those involved in stabilizing motility\[^{[82, 85]}\]. In fact, it appears that SwrA is required to express *swrB* and the upstream gene *sigD* above a certain threshold in a subpopulation of *B. subtilis* cultures, being *swrB* still expressed at considerable levels in a *swrA*-independent manner in the other fraction of the population (D. Kearns, personal communication).
Another gene that seems important to BsEss functioning is sfp. Its inactivation in an undomesticated strain resulted in an apparent decrease of YukE secretion, whereas its ectopic expression in strain 168, which is defective for sfp, resulted in a clear increase of extracellular YukE. Sfp is necessary for the production of the lipopeptide antibiotic surfactin\textsuperscript{[104]}. Surfactin acts also as biosurfactant to reduce solid surface tension, which is an essential requisite for swarming motility\textsuperscript{[82, 105, 106, 107]}. The mechanism by which sfp function stimulates BsEss is presently unknown. In addition of being an essential component of surfactin synthesis, sfp was also suggested to be involved in the regulation of the surfactant biosynthesis at the transcriptional level\textsuperscript{[104]}. Moreover, surfactin itself seems to have the ability to function as modulator of gene expressing as it happens in biofilm formation, in which surfactin acts as an autoinducer or a quorum-sensing signal leading to the derepression of genes involved in matrix synthesis\textsuperscript{[108]}. It will be interesting to test whether the simple addition of commercially available surfactin to media is sufficient to stimulate YukE secretion.

Disruption of gene yeeF inhibited YukE secretion, similarly to what happens when its homologue, the \textit{S. aureus} Ess protein EsaD is disrupted\textsuperscript{[70]}. EsaD is located in the staphylococcal membrane and is proposed to contribute to the selection of secretion substrates and/or interact with the Ess secretion machine, supporting the secretion of EsxA\textsuperscript{[70]}. A similar role can be envisaged for YeeF. Further studies with ectopically expressed yeeF in HCB6 are still necessary to confirm its role in BsEss.

Other genes involved in Deg-regulated processes also tested in this work were aprE and degR. Their individual inactivation produced no major impairment in YukE secretion, despite a slight and somewhat expectable reduction observed with the degR mutant.

Overall, our genetic analysis indicates that correction of mutations affecting degQ and sfp in strain 168 are required, and should be sufficient, to restore YukE secretion to levels similar to those observed in ancestral strains. Other genes that seem to be involved in the activation and/or functioning of the BsEss are swrB and yeeF. These results, allied to the already known role of phosphorylated DegU in the activation of this system\textsuperscript{[52]} are summarized in Figure 10.

In another part of this work we aimed to uncover a possible cellular function of this secretion system, based on the previous results that linked the BsEss cluster to the development of competence (C. Baptista, unpublished). At least in \textit{M. smegmatis} the T7SS equivalent to the \textit{M. tuberculosis} ESX-1 has been previously shown to be involved in DNA transfer\textsuperscript{[68]}. By testing this hypothesis in the 168 strain, it was observed a ~14-fold increase of the transformation efficiency in a strain deleted of genes yukE to yueB of the BsEss cluster (L.Del6). As result of the construction strategy, this strain also carried yukF, the gene immediately upstream of yukE, disrupted by a cat86 cassette (confers chloramphenicol resistance), an so we had to rule out this mutation as being involved in the observed phenotype. Unfortunately, we obtained opposite results when using two strains that supposedly only carried yukF disrupted: L.cat86 behaved essentially as L.Del6, whereas strain L.yukF displayed competence levels similar to control strain L16601.amy::CM. Since the steps used to construct L.cat86 might have inadvertently introduced point mutations in some genes of the BsEss cluster (see section II.3), we tend to
believe that results with strain L. *yukF* are the trustable ones, that is, that simple *yukF* disruption produces no effect on competence development. In that scenario, which of course needs further confirmation, it is the lack of *BsEss* that is the responsible for the increased competence in L.del6. When *yukF* is inactivated in an undomesticated background by the same strategy used to generate L.*yukF*, no effect on YukE secretion is observed (C. Baptista, unpublished).

The development of competence is related to sporulation, both processes sharing a lot of regulatory and essential genes\[^{109}\]. Interestingly, recently *yukF* was renamed *adeR* and shown to be required for normal sporulation of *B. subtilis*, being a transcriptional activator that mediates *ald* expression, also required for normal sporulation, in response to alanine availability\[^{110}\]. Our results allied with these recent findings suggest that *yukF* and the *BsEss* cluster play a role in sporulation and competence, respectively. Further studies with a more “clean” strain only deleted for *BsEss* will be conducted in a near future.

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**Fig. 10 – Model for activation and regulation of *B. subtilis* *BsEss*.** Note that the mechanism by which Sfp, SwrB and YeeF exert their positive contribution to YukE secretion is completely unknown (dashed arrows).
IV - Concluding remarks

One major goal of this work was to elucidate the genetics underlying the defective BsEss operation in *B. subtilis* lab strain 168. We believe this was achieved as we found that mutations affecting *degQ* and *sfp* are sufficient to explain the defective YukE secretion in this strain. This is a valuable knowledge as it will permit to construct a marker-less derivative of strain 168 with fully functional *BsEss*. Such derivative might be advantageous to further dissect *BsEss* molecular details in a more amenable genetic background. As result of this work, two additional genes were identified, *swrB* and *yeeF*, which seem to contribute positively to *BsEss* functioning. Although the real contribution of these genes still requires formal confirmation by gene complementation assays, we are convinced that these preliminary findings will lead to new insights on this secretion system.

Unfortunately, we could not also obtain definitive conclusions about the interplay between *BsEss* and *B. subtilis* competence, but our interpretation of the results tend to favor the previously raised hypothesis that *BsEss* expression negatively affects competence development. This is a topic that certainly deserves further investigation.
V – Materials and Methods

V.1 – Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this work are listed in Table 1. *E. coli* and *B. subtilis* strains were usually grown in Luria-Bertani (LB) medium\textsuperscript{[111]} with aeration at 37°C, except for *E. coli* transformants harboring pMutin4 derivatives, which were selected and propagated at 30°C. Agar was added to LB medium at a final concentration of 1.5% (wt/vol) for bottom plates. *E. coli* strains carrying vectors or recombinant plasmids were grown in the presence of ampicillin (100µg/ml), while erythromycin (1µg/ml), chloramphenicol (5µg/ml), spectinomycin (100µg/ml) or lincomycin (200µg/ml) were used for the selection of *B. subtilis* transformants. *B. subtilis* transformants expressing β-galactosidase were selected in X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) supplemented (0.02%, wt/vol) LB plates.

Table 1 – Strains and plasmids used in this work.

<table>
<thead>
<tr>
<th>Bacterial strains and plasmids</th>
<th>Genotype or relevant features</th>
<th>Source or reference</th>
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</thead>
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<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
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<td>TG1</td>
<td><em>supE hsdΔ5 thi Δ(lac-proAB) F'[traD36 proAB\textsuperscript{+} lacI\textsuperscript{q} lacZΔM15]</em></td>
<td>Stratagene</td>
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<td><strong>B. subtilis strains</strong></td>
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<td><em>B. subtilis</em> 168 strain</td>
<td>[112]</td>
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<td>BGSC* [78]</td>
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<td>ATCC** [78]</td>
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<td>NCIB 3610 derivative, Δ<em>swrB</em></td>
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<td>NCIB 3610 derivative, <em>swrB</em> amyE::P\textsubscript{fla/che}-swrB cm\textsubscript{R}</td>
<td>Daniel Kearns, unpublished</td>
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<td>NCIB 3610 derivative, plasmid cured</td>
<td>[79]</td>
</tr>
<tr>
<td>DS3337</td>
<td>NCIB 3610 derivative, <em>sfp::mls</em> ery\textsubscript{R} linc\textsubscript{R}</td>
<td>[80]</td>
</tr>
<tr>
<td>DS5758</td>
<td><em>B. subtilis</em> 168 derivative, amyE::P\textsubscript{sfp}-sfp cm\textsubscript{R}</td>
<td>[80]</td>
</tr>
<tr>
<td>W103</td>
<td>ATCC 6051 derivative, aprE; cm\textsubscript{R}</td>
<td>[72]</td>
</tr>
<tr>
<td>W105</td>
<td>ATCC 6051 derivative, degQ; cm\textsubscript{R}</td>
<td>[72]</td>
</tr>
<tr>
<td><strong>W128</strong></td>
<td>ATCC 6051 derivative, \textit{degR}; \textit{cm}^R</td>
<td>Kazuo Kobayashi, unpublished</td>
</tr>
<tr>
<td><strong>HCB1</strong></td>
<td>W105 derivative, \textit{degQ} \textit{thrC}:P_{\text{degQ}}-\text{degQ}; \textit{cm}^R \textit{spec}^R</td>
<td>This work</td>
</tr>
<tr>
<td><strong>HCB2</strong></td>
<td>DS3337 derivative, \textit{sfp}:\textit{mls} \textit{amyE}:P_{\text{sfp}}-\textit{sfp} \textit{ery}^R \textit{linc}^R \textit{cm}^R</td>
<td>This work</td>
</tr>
<tr>
<td><strong>HCB3</strong></td>
<td>L16601 derivative, \textit{thrC}:P_{\text{degQ}}-\text{degQ}; \textit{spec}^R</td>
<td>This work</td>
</tr>
<tr>
<td><strong>HCB4</strong></td>
<td>L16601 derivative, \textit{amyE}:P_{\text{sfp}-\text{sfp}}; \textit{cm}^R</td>
<td>This work</td>
</tr>
<tr>
<td><strong>HCB5</strong></td>
<td>HCB3 derivative, \textit{thrC}:P_{\text{degQ}}-\text{degQ} \textit{amyE}:P_{\text{sfp}}-\textit{sfp}; \textit{cm}^R</td>
<td>This work</td>
</tr>
<tr>
<td><strong>HCB6</strong></td>
<td>NCIB 3610 derivative, \textit{yeeF}pHCB6; \textit{ery}^R</td>
<td>This work</td>
</tr>
<tr>
<td><strong>L.\textit{yeeF}</strong></td>
<td>L16601 derivative, \textit{yeeF}pHCB6; \textit{ery}^R</td>
<td>This work</td>
</tr>
<tr>
<td><strong>L16601.\textit{amy::CM}</strong></td>
<td>1A772 derivative; \textit{cm}^R</td>
<td>[98] BGSC*</td>
</tr>
<tr>
<td><strong>L.\textit{cat86}</strong></td>
<td>L16601 derivative with \textit{cat86} inserted in the 5' end of \textit{yukF}; \textit{cm}^R</td>
<td>[98]</td>
</tr>
<tr>
<td><strong>L.\textit{Del6}</strong></td>
<td>L16601 derivative deleted of \textit{BsEss}; \textit{cm}^R</td>
<td>[98]</td>
</tr>
<tr>
<td><strong>L.\textit{yukF}</strong></td>
<td>L16601 \textit{yukF}pCB5; \textit{ery}^R</td>
<td>[113]</td>
</tr>
</tbody>
</table>

### Vectors and plasmids

| **pDG1731** | Integration vector; allows ectopic integration into \textit{thrC} locus; \textit{amp}^R \textit{spec}^R | [114] |
| **pMutin4** | Integration vector used for gene inactivation; \textit{amp}^R \textit{ery}^R | [93] |
| **pHCB3** | pHCB1 derivative carrying NCIB 3610 P_{\text{degQ}}-\text{degQ} | This work |
| **pHCB6** | pHCB1 derivative carrying a PCR product internal to \textit{yeeF} | This work |

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**American Type Culture Collection

### V.2 – General recombinant-DNA techniques

\textit{B. subtilis} genomic DNA was obtained from 2-ml culture samples. Cells were recovered in 0.2 ml of TEG buffer supplemented with 2 mg of fresh lysozyme/ml, and after an incubation period of 10 min at 37ºC, cells were lysed by the addition of 0.6 ml of GES-\textbeta. The lysate was then extracted three times with 0.8 ml of a phenol-chloroform-isoamyl alcohol mixture (25:24:1) and once with 0.8 ml of a chloroform-isoamyl alcohol solution (24:1) before DNA precipitation.
with 0.8V of 2-propanol. The DNA precipitate was washed with 1 ml of ethanol (70%, v/v), dried and finally resuspended in Tris-EDTA buffer\cite{111} supplemented with 20 µg of RNase A/ml.

Restriction endonuclease digestions, DNA ligations, and conventional agarose gel electrophoresis were performed essentially as described by Sambrook and Russel\cite{111}. PCR amplification of DNA fragments was carried out in a Biometra T1 thermocycler using DNA polymerases as recommended by the suppliers. The reaction settings were decided based on the oligonucleotides melting temperature, amplified product size and manufacturer’s indications for DNA polymerase. All the oligonucleotides used in this work were purchased from Invitrogen and are listed in Table 2. Highly specific PCR products were directly purified from PCR mixtures using the commercial kit “High Pure PCR Purification Kit” (Roche Applied Science). Plasmid DNA extraction and purification was carried out using “PureLink™ Quick Plasmid Miniprep Kit” (Invitrogen). The pretended plasmids were extracted by the alkaline extraction method as in Birnboin and Doly\cite{116}. Transformations of *E. coli* and *B. subtilis* with recombinant plasmids were performed essentially as described by Chung et al.\cite{117} and Yasbin et al.\cite{99} (see section V), respectively.

Table 2 – Primers used in this work. Restriction endonucleases sites are underlined. All primers were acquired from Invitrogen (Life Technologies).

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>degQFwEcoRI</td>
<td>CCTGAATTCTACAAGGACCTATTGAGATTGC</td>
</tr>
<tr>
<td>degQRvBamHI</td>
<td>AAAGGATCCTTTGTTTCCAAGTCTTTTTTCACG</td>
</tr>
<tr>
<td>lacZR1</td>
<td>GTGCTGCAAGGCGATTAAGTT</td>
</tr>
<tr>
<td>pDG1731-1</td>
<td>TCTTGCCAGTCACGTAGTT</td>
</tr>
<tr>
<td>pMutin-1</td>
<td>TTCTACATCCAGAACAACCTC</td>
</tr>
<tr>
<td>thrC-Fw2</td>
<td>TTGCCCTTGTCAACTCAGTCA</td>
</tr>
<tr>
<td>yeeF-Bam</td>
<td>CCGGGATCCTGATTTGTTGTTGTCAGACA</td>
</tr>
<tr>
<td>yeeF-Eco</td>
<td>AAAGAATTCAGCAACATCAAGGCATTCTATC</td>
</tr>
<tr>
<td>yeeF-Rv1</td>
<td>TTGGAGCTTTTGAAAGTGCGA</td>
</tr>
</tbody>
</table>

V.3 – Construction of *B. subtilis* mutants

Strain HCB1 was constructed by transforming strain W105 with chromosomal DNA of strain HCB3 (see below) and selected for chloramphenicol and spectinomycin resistance. Strains HCB2 and HCB4 were obtained via transformation of DS3337 and L16601, respectively, with DS5758 chromosomal DNA. HCB2 was selected for chloramphenicol, erythromycin and lincomycin resistance, whereas HCB4 for chloramphenicol resistance.
For construction of HCB3, \textit{degQ} and its promoter sequences were PCR amplified (using primer pair degQFwEcoRI/degQRvBamHI) from NCIB 3610 genome: The PCR product was double digested with \textit{EcoRI} and \textit{BamHI} and ligated to similarly digested pDG1731\textsuperscript{[114]} (Fig. 11).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig11.png}
\caption{Fig. 11 – Schematic representation of pDG1731. \textit{thrC}' and 'thrC, 5' and 3' segments of the \textit{B. subtilis} 168 \textit{thrC} gene, respectively; hom, 3' end of the \textit{B. subtilis} 168 hom gene (part of the \textit{thrC} operon); \textit{thrB}, 5' end of the \textit{B. subtilis} 168 \textit{thrB} gene (part of the \textit{thrB} operon); spc, encodes spectinomycin adenyltransferase, selectable in either \textit{E. coli} or \textit{B. subtilis}; \textit{erm}, encodes rRNA adenine N-6-methyltransferase, selectable in \textit{B. subtilis}; \textit{bla}, encodes β-lactamase, selectable in \textit{E. coli} only. Image obtained from BGSC.}
\end{figure}

The resulting recombinant plasmid (pHCB3) was obtained in \textit{E. coli} strain TG1 and then used to transform \textit{B. subtilis} L16601. The transformants with the ectopic integration of \textit{P}_{\textit{degQ}}-\textit{degQ} into the \textit{B. subtilis} \textit{thrC} locus (Fig. 12) were screened for sensitivity to erythromycin and spectinomycin resistance to confirm that a double-crossover recombination has occurred, rather than a Campbell-type insertion. The ectopic desired DNA structure was confirmed by PCR using pDG1731-specific primers (pDG1731-1 and thrC-Fw2) and by DNA sequencing.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig12.png}
\caption{Fig. 12 – Schematic representation of HCB3 final structure. The pDG1731-derivative integrates by a double crossing-over event between homologues sequences. \textit{P}_{\textit{degQ}}, promoter of \textit{degQ}\textsuperscript{[79]}.}
\end{figure}
Strain HCB5 was obtained via transformation of HCB3 with chromosomal DNA of strain HCB4 and selected for chloramphenicol and spectinomycin resistance.

For construction of a NCIB 3610 derivative with disrupted yeeF (strain HCB6) a ~400 bp DNA fragment internal to yeeF of strain L16601 was PCR amplified (using primer pair yeeF-Eco/yeeF-Bam), double digested with EcoRI and BamHI, and ligated to similarly digested pMutin4 (Fig.13).

![Fig.13 – Schematic representation of pMutin4. spoVG-lacZ, E. coli lacZ gene fused to the B. subtilis 168 spoVG ribosome binding site; Pspac, hybrid promoter, inducible by IPTG; T1T2T0, transcriptional terminator; lacI, encodes lac repressor, with modified ribosome binding site for Gram-positive expression; erm, encodes rRNA adenine N-6-methyltransferase, selectable in B. subtilis for erythromycin; bla, encodes β-lactamase, selectable in E. coli only for ampicillin. Image obtained from BGSC.](image)

The resulting recombinant plasmid (pHCB6) was obtained in E. coli strain TG1 and then used to transform B. subtilis L16601, because of its higher transformation efficiency. The integrant (L.yeeF) resulting from the Campbell-type recombination between the chromosomal locus and the cloned sequence was selected for erythromycin resistance and blue color in X-Gal-LB plates. The DNA from strain L.yeeF was then used to transform NCIB 3610, given rise to strain HCB6. Gene disruption resulting from the integration event was confirmed by PCR using pMutin4-specific primers (pMutin-1 and lacZR1) and a primer flanking the target loci (yeeF-Rv1). In the resulting strain HCB6 (Fig.14), the yeeF gene was disrupted and this strain was always grown in the presence of IPTG to avoid polar effects downstream.
Fig. 14 – Schematic representation of HCB6 final structure. The target region is represented in white. The pMutin4-derivative integrates by a single crossing-over event between homologous sequences. RapHp, promoter for rapH and phrH[118]; P1, putative promoter for yeeF.

V.4 – Production of Protein Extracts and Western Blot Analysis

Thirty milliliter samples of B. subtilis cultures were collected for preparation of protein extracts two hours after entry in stationary phase (T2). Strain HCB6 was grown in presence of 1 mM IPTG to guarantee expression downstream of the inactivated gene yeeF. The samples were centrifuged (5000rpm, 10min, 4°C) for cell recovery and the supernatants filtrated through 0.2 µm membranes to eliminate the remaining cells. Proteins from cell-free supernatants were precipitated with an equal volume of PRMM solution (0.05 mM pyrogallol red, 0.16 mM sodium molybdate, 1.0 mM sodium oxalate, 50.0 mM succinic acid, 20% [vol/vol] methanol in H₂O, adjusted to pH 2.0 with HCl), essentially as described by Caldwell and Lattemann[119]. After two washes with cold acetone, protein precipitates were solubilized in a buffer containing 50 mM Tris.Cl pH 8.8, 7 M urea and 2 M thiourea and stored at 4 °C. Protein quantification was performed by the Bradford method (Bio-Rad Laboratories).

For SDS-PAGE analysis samples from these protein extracts were heated at 37°C (not boiled) for up to 10 min and then supplemented with 1X SDS-PAGE sample buffer from a 10-fold concentrated solution that had been previously heated at 95°C for 5 min. SDS-PAGE used in this work was composed by a resolution gel of 15% [vol/vol] (National Diagnostic) supplemented with 5% sucrose. The molecular size marker used was BenchMark™ Pre-stained Protein Ladder (Invitrogen) and the electrophoresis was conducted in the Mini-Protean Cell system (Bio-Rad Laboratories). After the run, gels were either stained with Coomassie blue (Bio-Rad Laboratories) for 45 minutes at room temperature and gentle shaking, to monitor the quality and relative protein quantities, or transferred to 0.2 µm nitrocellulose membranes (Bio-Rad Laboratories) for two hours at 100V using Mini Transblot Module system (Bio-Rad Laboratories) for Western Blot analyses.

For YukE immunodetection, membranes were blocked overnight at 4°C in a TBS (50 mM Tris.Cl pH 7.5, 150 mM NaCl) blocking solution (1% [vol/vol] Western Blocking Reagent Solution, Roche Applied Science). In the next day, membranes were washed twice (10 min each washing) with TBS-T (TBS supplemented with 0.2% [vol/vol] Tween20), followed by two 10-min washes with TBS. Then, the membrane was incubated with a 1:5000 dilution of a Rabbit polyclonal antibody raised against pure YukE-His[52] in TBS with 0.5% [vol/vol] blocking reagent for one hour. The subsequent procedures to detect antigen/antibody complexes were according to the instructions of Chemiluminescence Western Blotting Kit (Roche Applies Science),
except that the substrate for the chemiluminescence reaction was Luminata™ Forte (Western HRP substrate, Milipore). Chemiluminescent bands were recorded in Amersham Hyperfilm ECL (GE Healthcare). Film developing was conducted in a dark room using D-19 Developer and Kodak Fixer (Carestream® Kodak® Processing Chemicals, Sigma-Aldrich, St. Louis).

V.5 – Study of competence development in *B. subtilis*

Competence media GM1 and GM2 were prepared essentially as described in Yasbin et al. [99], being GM1 composed of 1.24% [wt/vol] K₂HPO₄, 0.76%[wt/vol] KH₂PO₄, 0.1% [wt/vol] Trisodic citrate, 0.6% [wt/vol] (NH₄)₂SO₄, 0.025% [wt/vol] MgSO₄(7H₂O), 0.5% [wt/vol] glucose, 0.1% [wt/vol] yeast extract and 50 µg/ml of the aminoacids tryptophan, arginine, lysine, methionine, glycine, histidine, valine, threonine and aspartate. GM2 had the same composition of GM1, but with the addition of 0.5 mM CaCl₂ and 2.5 mM MgCl₂. The induction of competence was done using a method similar to that described by Boylan[120]. 2 ml of *B. subtilis* cells in LB were grown overnight (30°C, 200 rpm) and in the following morning diluted in GM1 to an OD₆₀₀ between 0.5 and 0.1. Growth was monitored by plotting OD₆₀₀ data each 30 min. Ninety minutes after reaching the end of exponential growth (Fig.15), cultures were diluted 10 fold in GM2 and incubated (37°C, 200 rpm) for additional 90 minutes.

![Fig.15 – Growth curve of L16601.amyE::CM in GM1 at 37°C. A fitting curve for the exponential growth phase is presented. Growth curves of each experiment showed no significant differences (not shown). The arrow indicates the time point correspondent to the end of the exponential phase.](image)

At this point, a sample of the cultures was serially diluted in LB and plated for determination of total CFU/ml. For transformation, 0.5 ml culture samples were supplemented with transforming DNA (5 µg/ml) and incubated for another 30 minutes (200 rpm, 37 ºC), after which different dilutions were plated onto LB agar with the selected antibiotics. The transformation efficiency was calculated with the formula: Transformants/(Cfu/ml) x 100.
V.6 – DNA/protein sequencing and Bioinformatics analyses

DNA sequencing services were purchased to Macrogen (Amsterdam). DNA and protein sequences were analyzed with Gene Runner (version 3.05). Multiple protein sequence alignments were performed with ClustalW2[^121].


44. Simeone, R., Bottai, D., Brosch, R. (2009) ESX/type VII secretion systems and their role in host-pathogen interaction. *Current Opinion in Microbiology* 12: 4-10


