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**The essential YycFG two-component system controls cell
wall metabolism in *Bacillus subtilis***

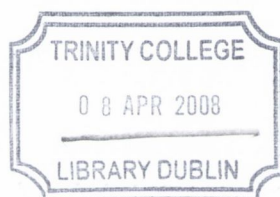
by Paola Bisicchia

A Thesis Submitted to the University of Dublin
for the Degree of Doctor of Philosophy

Supervised by Professor Kevin Devine

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October 2007



THESIS
8328

DECLARATION

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Paola Bisicchia

Paola Bisicchia

October 2007

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SUMMARY

The work presented in this thesis describes a comprehensive analysis of the YycFG two-component system (TCS) in *Bacillus subtilis*. TCSs are widespread mechanisms used by bacteria to sense and respond to the prevailing environmental conditions. YycFG is the only essential TCS encoded in the *B. subtilis* genome, but the nature of its essentiality has not been established. In addition, its regulon is poorly defined.

In order to get insights into the nature of the cellular processes controlled by this TCS, we carried out a comprehensive analysis of the YycFG regulon, based on microarray experiments performed on a strain depleted for YycFG, as well as by bioinformatics. The results clearly show that YycFG is a regulator of cell wall metabolism. We have identified five novel components of the YycFG regulon. Among them are three positively regulated genes, namely *lytE*, *yvcE* and *ydjM*. We show that a *yvcE lytE* double mutant is not viable and that an endopeptidase-type activity, performed either by LytE or by YvcE(CwlO), is required for elongation of the cell cylinder during growth. The gene *ydjM* is also predicted to have a role in cell wall metabolism. A triple mutant strain deleted in *yocH*, *yvcE* and *ydjM* was obtained at a low transformation frequency, displays severe morphological defects, as well as the inability to grow in liquid culture. This finding further supports a role for YdjM in cell wall metabolism, this protein being required for normal growth in the absence of the two autolysins *yocH* and *yvcE*. A genetic analysis shows that essentiality of YycFG in *B. subtilis* is polygenic in nature. This conclusion is derived from a number of observations. Firstly, although a double mutant deleted in both *lytE* and *yvcE* is not viable, we show that the combination of the two genes does not account for the essentiality of YycFG. Furthermore, the severe growth and morphological defects of the triple mutant deleted in *yocH*, *yvcE* and *ydjM* indicate that these three genes contribute to the essentiality of YycFG, but do not fully explain it. We therefore propose that the essentiality of YycFG is polygenic, and that the growth cessation and lysis phenotypes observed upon YycFG depletion are a manifestation of disrupted cell wall metabolism, caused by aberrant expression of a number of YycFG regulon genes. The microarray analysis also identifies four genes negatively regulated by YycFG: *yoeB* and *ykuP*, which both encode proteins of unknown function, *yocE* (*des*), a gene encoding a fatty acid desaturase and *yjeA*, which encodes a putative peptidoglycan deacetylase. Direct regulation is shown for *yoeB* and *yjeA*. Helmann and co-workers have shown that YoeB modulates autolysin activity and protects cells from autolysis (Salzberg and Helmann, 2007). We show that YjeA functions as a peptidoglycan deacetylase that modifies peptidoglycan thereby

altering the cell wall susceptibility to lysozyme digestion. The control of *yoeB* and *yjeA* by YycFG further demonstrates its involvement in cell wall metabolism, which occurs both at a transcriptional level, through direct regulation of teichoic acid biosynthetic genes and autolysins, and at a post-transcriptional level, by modulation of the level of acetylation of peptidoglycan, which controls autolysin activity, and by induction of a gene, *yoeB*, encoding a protein that protects cell walls from autolysis. We also show that cell walls from YycFG depleted cells are deficient in auto-digestion activity, however none of the cell wall-related genes *yocH*, *yvcE*, *lytE* or *yjeA* contributes to this effect. The presence of the *des* among the genes negatively regulated by YycFG in an indirect way suggests a link between YycFG and cell membrane metabolism, as previously observed in other bacteria (Martin *et al.*, 1999; Mohedano *et al.*, 2005; Ng *et al.*, 2005). Expression of the *des* gene is strictly controlled by the DesKR TCS, and is induced in response to reduced cell membrane fluidity (Aguilar *et al.*, 2001). Therefore the up-regulation of *des* upon depletion for YycFG indicates a perturbation at the level of membrane fluidity, which might be caused by a miss-regulation of cell membrane metabolism. In conclusion, this work greatly advances the knowledge on the physiological role of the essential YycFG TCS.

An additional project is also reported. The structural similarities shared by the three TCSs YycFG, PhoPR and ResDE, as well as the known interactions between the two pairs of TCSs YycFG and PhoPR, and ResDE and PhoPR prompted us to investigate the possibility of a network of interactions involving these three TCSs. We focused in particular on the TCSs YycFG and ResDE, and we found indications that such interactions may occur at different levels: our data suggests that the two HKs may sense the same signal, the compound NO, or alternatively the HK ResE may phosphorylate the non-cognate RR YycF. Furthermore, our results indicate that YycFG may participate in transcriptional regulation of the *resABCDE* promoter (encoding the TCS ResDE as well as three essential genes), and of other constituent genes of the ResDE regulon, namely *hmp* and *ctaA*. However, these results need further experimental validation.

PUBLICATIONS ARISING FROM THE WORK REPORTED IN THIS THESIS

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ABBREVIATIONS

A	Alanine
aa	Amino acids
APase	Alkaline phosphatase
ATP	Adenosine triphosphate
BgaB	β -galactosidase (<i>B. stearothermophilus</i> derived)
BLAST	Basic Local Alignment Search Tool
bp	Base pair
C	Carboxyl
D	Aspartate
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
G + C	Guanine + Cytosine
H	Histidine
HAMP	Histidine kinase, Adenylyl cyclase, Methyl accepting chemotaxis protein, Phosphatase
HK	Histidine kinase
HTH	Helix-turn-Helix
IPTG	Isopropyl β -D-1-thiogalactopyranoside
Kb	Kilobase pairs
BgaB/ <i>bgaB</i>	β -galactosidase protein/structural gene
LB	Luria Bertani
N	Amino
NO	Nitric oxide
OD	Optical density
ONPG	o-Nitrophenyl β -D-galactopyranoside
ORF	Open reading frame
P	Proline
PAS	Per-Arnt-Sim (subdomain)
PCR	Polymerase chain reaction
~P	Phosphorylated
RBS	Ribosome binding site
RNA	Ribonucleic acid
rpm	Revolutions per minute

RR	Response regulator
SDS	Sodium dodecyl sulfate
SM	Schaeffer's sporulation medium
SOE	Strand overlap extension
TCS	Two-component system

CHAPTER 1

INTRODUCTION

1.1. TWO-COMPONENT SIGNAL TRANSDUCTION SYSTEMS

Two-component signal transduction systems (TCSs) are a common mechanism used by bacteria to sense and respond to the prevailing environmental conditions. With a few exceptions, TCSs are present in all bacteria, where they have been regarded as the most widespread mechanism of signal transduction (Wolanin *et al.*, 2002). The number of TCSs can vary considerably in different bacterial species, ranging from 0 in *Mycoplasma* species to 80 in the cyanobacterium *Synechocystis* and 81 in *S. coelicor* (Kiil *et al.*, 2005; Mizuno *et al.*, 1996). Analysis of complete bacterial genomes revealed that the abundance of TCSs does not relate to the size of the genome or to the number of proteins encoded, but it rather correlates with the potential environmental diversity that a species may encounter within its habitat. Bacteria with a complex lifestyle like *Myxococcus xanthus*, or those which are found ubiquitously in varied environments such as *Pseudomonas*, tend to have the largest complements of TCSs, while parasitic bacteria like *Neisseria gonorrhoea* and *Helicobacter pylori*, which live in a relatively uniform habitat, have significantly fewer TCSs (Galperin, 2005; Rodrigue *et al.*, 2000). Indeed, the number of encoded TCSs could be used as a measure of the organism's ability to adapt to a changing environment: a sort of "sensory complement" (Galperin, 2005).

TCSs probably originated in bacteria, and then radiated into archaea and eukaryotes by lateral gene transfer (Koretke *et al.*, 2000). In accordance with this hypothesis, only a limited number of TCSs have been found in eukaryotes, where they represent a small portion of all signal transduction systems. TCSs have been identified in lower eukaryotes such as fungi (Catlett *et al.*, 2003; Santos and Shiozaki, 2001) and the amoeba *Dictyostelium*, and in plants, such as *Arabidopsis thaliana* and tomato (Urao *et al.*, 2000, 2001). No TCSs have been identified in the human, fly or worm genomes and they are thought to be absent from the whole animal kingdom (Wolanin *et al.*, 2002).

The absence of TCSs from the animal kingdom, together with their ubiquitous presence in bacteria and their involvement in important physiological processes including virulence and antibiotic resistance, makes them an attractive target for antimicrobial therapy (for reviews see in Stephenson and Hoch, 2002a, b; Matsushita and Janda, 2002).

1.1.1. Mechanism of two-component signal transduction systems

A typical TCS includes a membrane-bound sensor histidine protein kinase (HK), and its associated cytoplasmic response regulator (RR). For reviews see Hoch and Silhavy (1995) and Stock *et al.* (2000). A schematic representation the structure and functioning of a TCS is presented in Figure 1.1.

The HK usually consists of an amino-terminal “input domain”, and a carboxy-terminal “transmitter domain”, which can be subdivided into a kinase domain, containing a conserved histidine residue, and an ATP-binding domain. Binding of a ligand to the input domain induces the autokinase activity of the transmitter domain and the transfer of a phosphoryl group from ATP to the conserved histidine residue.

The RR consists of an amino-terminal receiver domain, which receives the phosphoryl group from the HK on an aspartate residue, and a carboxyl-terminal output domain, which is often a DNA-binding domain: the phosphorylation of the aspartate activates the function of the output domain. The level of phosphorylated RR ultimately determines the output response.

The transmitter and receiver domains are highly conserved among TCSs, reflecting the common phosphotransfer mechanism of signal transduction, while the input and output domains are widely divergent among TCSs, displaying the diversity of signals that can be sensed, and the specificity needed for adaptative responses.

In addition to the prototypical TCS described above, an alternative His-Asp phosphotransfer scheme, named multi-step phosphorelay, has been discovered both in prokaryotes and eukaryotes (Appleby *et al.*, 1996). In a typical phosphorelay (represented in Figure 1.1) the phosphoryl group is first transferred from the histidine residue of the kinase to the aspartate residue of a single-domain RR, then to a histidine-containing phosphotransfer domain (HPT) and finally to a cytoplasmic receiver domain (Stephenson and Hoch, 2002a). Alternatively, phosphorelay systems can involve hybrid-type HKs, containing both histidine and aspartate-containing domains (Zhang and Shi, 2005). In these cases the phosphoryl group is first transferred within the HK from its histidine to its aspartate residue, then to the HPT and finally to the RR. The HPT domain can exist either as a domain in the hybrid-type HK, or as a separate single protein, or as a domain contained in another protein. These expanded phosphorelay networks allow greater levels of control than the basic TCSs, thanks to a great number of potential regulatory sites and to the possibility to integrate multiple signals into a single output response. Phylogenetic analysis indicates that hybrid HKs originated by lateral recruitment of a receiver domain into a HK protein. Multi-step phosphorelays are predominant

in eukaryotes, but have been recently found to be present also in 56 of 156 complete prokaryotic genomes (Zhang and Shi, 2005).

A further variation from the prototypical TCS is the unorthodox TCS, in which the HK contains two histidine domains and one aspartate domain. Computer analysis has indicated that such a complex phosphorelay mechanism allows higher sensitivity to the external stimulus, and a more robust response to fluctuation of the stimulus compared to orthodox TCSs (Kim and Cho, 2006).

1.1.2. Structure of histidine kinases

A typical HK is composed of a highly variable transmembrane-spanning N-terminal input or sensing domain, and a more conserved cytoplasmic C-terminal output or “core” domain. The sensing domain often contains one or more extracytoplasmic loops, flanked by two transmembrane modules, although there are a number of variations from this general scheme (reviewed in Mascher *et al.*, 2006; Wolanin *et al.*, 2002).

The extra-cytoplasmic loop plays a crucial role in detecting the external stimulus in periplasmic-sensing HK (Mascher *et al.*, 2006; Wolanin and Stock, 2003). The transmembrane domains function as an anchor to the cytoplasmic membrane, but can also be implicated in signal perception (Mascher *et al.*, 2006).

A linker HAMP domain, approximately 50-aminoacids long, connects the periplasmic, ligand-binding domain to the cytoplasmic output domain (Aravind and Ponting, 1999). HAMP domains are found in histidine kinases, as well as adenylyl cyclases, methyl-accepting proteins and phosphatases (Aravind and Ponting, 1999), and are shown to play a role in the transmission of the signal between the input and output domains of HK, although the precise mechanism of signal transmission is still unknown. HKs usually function as dimeric proteins (Tomomori *et al.*, 1999): HAMP domains have been also implicated in HK dimerization. Two alternative models have been proposed for such a function; in both cases, the binding of the ligand to the input domain of the HK would alter the conformation of the two α -helices contained within the HAMP domain. This would result in a change in conformation of the bihelical dimerization domain contained in the HK, which would become “free” to align with the dimerization domain of another HK, allowing dimerization (Khorchid and Ikura, 2006; Williams and Stewart, 1999).

HK domains include the H-box motif, which contains the conserved histidine residue, as well as sequences responsible for the HK dimerization. Auto-phosphorylation is believed to occur *in trans*, with one subunit using ATP to phosphorylate the conserved histidine residue of

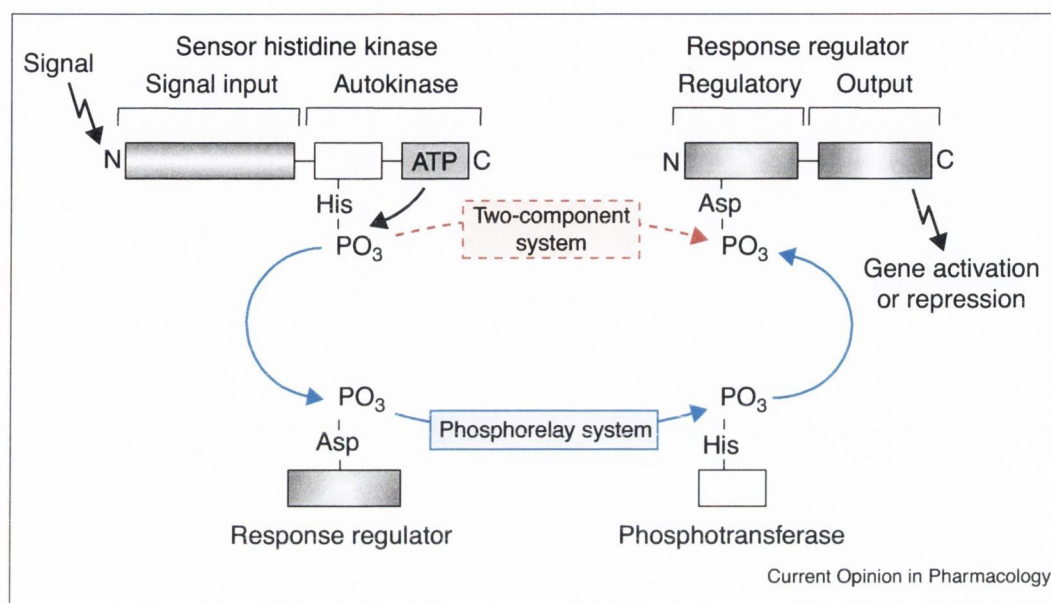


Figure 1.1. Mechanism of two-component and phosphorelay signal-transduction systems. Signal ligands stimulate autophosphorylation of the sensor kinase on a histidine residue. In a two-component system (red), the phosphoryl group is subsequently transferred to an aspartate residue of a two-domain response regulator to activate its transcriptional properties. In a phosphorelay (blue), the phosphoryl group is first transferred to an aspartate of a single-domain response regulator that lacks a DNA binding domain, then to a histidine residue of a phosphotransferase that subsequently serves as the phosphoryl donor to activate the transcriptional properties of the response regulator. This figure is taken from Stephenson and Hoch (2002a).

the partner within a dimer (Dutta *et al.*, 1999). The phosphate group is subsequently transferred to a conserved aspartate residue within the cognate response regulator. Many HKs also possess a phosphatase activity, that is the ability to de-phosphorylate the RR in its phosphorylated form (RR~P); such a phosphatase activity is often mediated by the dimerization domain (Perego *et al.*, 1996; Perego and Hoch, 1996). These bifunctional HKs, by limiting the response regulator's activation, achieve an additional level of control, which is required when the cellular response needs to be shut down quickly (Stock *et al.*, 2000).

A number of conserved sub-domains have been identified within the output region of HKs (Mascher *et al.*, 2006 and references therein): among them are the cytoplasmic signaling PAS domains. Such domains were initially identified in the *Drosophila* period circadian protein PER, the vertebrate aryl hydrocarbon receptor nuclear translocator ARNT, and the *Drosophila* single-minded protein SIM, and were later found in over 1100 sensor proteins from both prokaryotes and eukaryotes. (Khorchid and Ikura, 2006; Taylor and Zhulin, 1999). PAS domains are known to be involved in sensing light, oxygen, small ligands, the redox potential and the overall energy state of the cell (Taylor and Zhulin, 1999), and in some cases can act as sites of protein-protein interaction (Elsen *et al.*, 2003; Taylor and Zhulin, 1999; Wang *et al.*, 2001). The *B. subtilis* phosphorelay sensor kinase KinA possesses three PAS domains (A, B and C): PAS-B and PAS-C were shown to mediate dimerization, while PAS-A has been implicated in enzymatic activity (Wang *et al.*, 2001). PAS-A was shown to be required for auto-phosphorylation of the HK, and has been proposed to be involved in either signal sensing or structural integrity or both. In a further study PAS-A was shown to bind and hydrolyze ATP; it was proposed that energy from ATP hydrolysis might drive the conformational changes for activation or de-activation of the kinase in response to the external stimulus (Stephenson and Hoch, 2001). Signal sensing, therefore, does not seem to be the exclusive role for PAS domains in HKs. One intriguing hypothesis is that PAS domains might constitute signaling domains which would function as "checkpoints", enhancing or counteracting the primary signal sensed by the HK through its input domain (Stephenson and Hoch, 2002c).

1.1.3. Classification of histidine kinases

HKs have been classified by a number of groups according to different criteria. Classification cannot be accomplished on the basis of overall protein homology, because of the high variability of the input domains, which reflects the wide range of signals sensed by different kinases. Most classifications, therefore, were based on the highly conserved cytoplasmically located transmitter domain. One criterion used was the position of the

histidine-containing region (H-box region) with respect to the kinase domain in the primary sequence of the protein, which allowed identification of two classes of HKS (Dutta *et al.*, 1999). A more comprehensive analysis, based on comparison of six conserved boxes within the transmitter domain, allowed distinction of eleven different classes (Grebe and Stock, 1999). A recent classification was based on the sequence of the conserved H-box region and of the kinase domain, as well as on the predicted secondary structure of the H-box, and allowed grouping of bacterial and archeal HKs into five different classes (Kim and Forst, 2001). A classification based on the sequence of the region around the conserved histidine residue was proposed for *B subtilis*, and has found widespread use (Fabret *et al.*, 1999): HKs were grouped in five different classes, with a further sub-classification based on similar size and membrane configuration. According to this classification, the three kinases YycG, ResE and PhoR form a “sub-group” within the homology class IIIA, clearly indicating evolution from a common progenitor. Indeed all the above classifications, based on the features of the highly conserved transmitter domain, are likely to reflect the evolution of TCSs and their phylogenetic relationship. On the other hand, such criteria do not allow prediction of a HK biological function, which is defined by the input domain.

A number of conserved input sub-domains have been recently identified on the basis of comparative genomic analysis (Mascher *et al.*, 2006 and references therein). While such motifs are crucial in the understanding of the kinases biological function, overall classification of HKs based on the input domains is hampered by the high degree of sequence variability, which reflects the wide variety of signals sensed. Functional classification of HKs, therefore, requires an alternative approach, which cannot be based on sequence alignments alone. Intramembrane-sensing HKs have been recently classified on the basis of domain architecture, genomic context as well as sequence similarity (Mascher, 2006). A more comprehensive functional grouping of all HKs was based on their membrane topology, on the number of transmembrane domains, and on the sequential arrangement of conserved sensory sub-domains within the input domain (Mascher *et al.*, 2006). Such criteria, however, don't take into consideration phylogenetic aspects.

1.1.4. Structure of response regulators

A typical RR contains a conserved N-terminal receiver domain, which can be phosphorylated on an aspartate residue, and a variable C-terminal output domain; a flexible interdomain linker connects the two regions.

The receiver domain is approximately 120 amino acids long and adopts a conserved three-dimensional organization, with a central β -sheet (made of five parallel β -strands) surrounded by five α -helices. The active site, containing the conserved aspartic residue, is located at the C-terminal end of the β -sheet (Robinson *et al.*, 2000). Phosphorylation of the receiver domain occurs in a Mg^{2+} -dependent way and drives conformational changes which are needed for full activation of the protein (Cho *et al.*, 2001). In some cases phosphorylation is achieved through the utilization of small molecules, such as acetyl phosphate, as phosphodonors (Stock *et al.*, 2000).

The interlinker domain was shown to affect activation of the RR, most likely by playing a role in transmitting the signal from the receiver to the output domain (Walthers *et al.*, 2003).

Most RRs are transcription factors containing DNA-binding output domains. In these cases the cellular response is elicited through activation or repression of genes encoding for proteins that are required to respond to the specific signal sensed by the cognate HK. Structural analysis of various DNA-binding domains revealed a common helix-turn-helix (HTH) motif (Aravind *et al.*, 2005), but a wide diversity in their global architecture (Robinson *et al.*, 2000), which reflects both the variety and the specificity of the different cellular responses.

In addition to their phosphotransfer activity, RRs possess also a Mg^{2+} -dependent auto-phosphatase activity, which limits the lifetime of their phosphorylated state to a timeframe ranging from seconds to hours, depending on the specific needs of each cellular response (Cho *et al.*, 2001). In some cases, RRs can be de-phosphorylated by auxiliary phosphatases (Stock *et al.*, 2000). The above mechanisms, together with the phosphatase activity performed on RRs by some HKs mentioned in the previous section, all represent regulatory strategies to tightly control the level of phosphorylated RR, which ultimately determines the output response.

1.1.5. Classification of response regulators

RRs have been classified by several different criteria (Glaperin, 2006; Kojetin *et al.*, 2003 and references therein). In *B. subtilis* five families of regulators have been identified on the basis of the homology of their output domain and have been named with reference to known families of *E. coli* RRs (Fabret *et al.*, 1999). According to this criterion, the three RRs YycF, PhoP and ResD belong to the OmpR family. In an alternative classification, based on the surface characteristics of the receiver domains, YycF, ResD and PhoP, which share very similar surface features, have been classified as belonging to the sub-class A within the OmpR

family (Kojetin *et al.*, 2003). The three TCSs YycFG, PhoPR and ResDE, therefore, share a high degree of similarity both in their HK and in their RR components, which can be explained by the evolution from a common progenitor (Fabret *et al.*, 1999).

A more comprehensive classification of RRs has been recently performed on 200 bacterial and archaeal species whose genome has been completely sequenced (Galperin, 2006). Such classification was based on the domain architectures of the receiver domain, and of DNA-binding, enzymatic, ligand-binding and previously uncharacterized domains. This analysis indicated that a common evolutionary mechanism of RRs is the fusion of receiver domains to DNA-binding or enzymatic domains, which results in new RRs: virtually any fusion can occur, but only some of them are fixed by evolution.

1.2. YycFG: AN ESSENTIAL TWO-COMPONENT SYSTEM

Only a few TCSs have been found to be essential for cell viability among bacteria. Such a finding is not surprising, being consistent with the physiological role of TCSs in bacteria, which is to respond to specific signals that may not always be present in their habitat. An essential TCS, in fact, would seem to limit the range of conditions for bacterial survival.

One such rare essential TCS is YycG-YycF, which was first identified in *Bacillus subtilis*, and which is the object of this study. Among the 34 TCSs present in *B. subtilis* (Fabret *et al.*, 1999; Kunst *et al.*, 1997), YycFG is the only essential one, as revealed by direct gene knockout study of all the RRs (Fabret and Hoch, 1998). Unlike all the other TCS, therefore, YycFG seems to respond to conditions that are required for the survival of the cell.

Orthologues of YycFG have been identified in other low G+C Gram-positive bacteria such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Listeria monocytogenes*, *Enterococcus faecalis*, *Lactococcus lactis*, *Lactobacillus sakei*, *Streptococcus pyogenes*, *Streptococcus mutans*, *Clostridium acetobutylicum*, *Cyanobacterium*, *Bacillus cereus*, *Bacillus thuringensis* and *Bacillus anthracis* (Ashby and Houmard, 2006; Bhagwat *et al.*, 2001; de Been *et al.*, 2006; Ferretti *et al.*, 2001; Glaser *et al.*, 2001; Hancock and Perego, 2002; Kuroda *et al.*, 2001; Morel-Deville *et al.*, 1998; Nolling *et al.*, 2001; O'Connell-Motherway *et al.*, 1997; Qin *et al.*, 2006; Tettelin *et al.*, 2001).

In some cases, such as in *B. subtilis* and *S. aureus*, both the HK and the RR are essential (Fabret and Hoch, 1998; Martin *et al.*, 1999). In other cases, such as in *S. pneumoniae*, only the RR has been shown to be essential (Throup *et al.*, 2000; Wagner *et al.*, 2002), although the HK YycG is required for optimal growth when YycF levels are reduced (Ng *et al.*, 2003). In

most cases, however, YycF seems to be essential for growth. Clear evidence for YycF essentiality is given for *L. monocytogenes* and *E. faecalis* (Hancock and Perego, 2002; Kallipolitis and Ingmer, 2001; Williams *et al.*, 2005), as well as the above mentioned *B. subtilis*, *S. aureus* and *S. pneumoniae*. For some species, however, the literature reports conflicting data. In the bacterium *S. pyogenes*, Federle *et al.* (1999) were unable to inactivate YycF, while Liu *et al.* (2006) were able to construct both a YycF and a YycG insertional mutant. However, this YycF mutant was not stable *in vivo* and the authors failed to obtain a YycF deletion mutant. In *S. mutans* YycF was shown to be essential and YycG was found to be dispensable, but required for optimal growth, in a way similar to what observed in *S. pneumoniae* (Bhagwat *et al.*, 2001; Senadheera *et al.*, 2005). However, Lee *et al.*, (2004) were able to obtain a YycF knock-out mutant in *S. mutans*. These conflicting data do not allow a firm conclusion as to whether or not the YycFG TCS is essential in *S. pyogenes* and *S. mutans*. YycF seems to be dispensable for growth in *L. sakei* (Morel-Deville *et al.*, 1998), while in *L. lactis* both the YycG and the YycF proteins could be deleted (O'Connell-Motherway *et al.*, 2000).

The YycFG TCS is very highly conserved, with YycF RR sharing 70% amino acid identity on average (Howell *et al.*, 2003). This observation, along with its essential nature, has encouraged the study of YycFG as a novel therapeutic target in the treatment of diseases caused by Gram-positive pathogen bacteria, especially following the development in recent times of multi-drug resistant pathogens. For this purpose, studies have been carried out to identify inhibitors of the kinase YycG in *B. subtilis*, *S. aureus*, *E. faecalis* and *S. epidermidis* (Furuta *et al.*, 2005; Kitayama *et al.*, 2004; Kitayama *et al.*, 2007; Okada *et al.*, 2007; Qin *et al.*, 2006; Watanabe *et al.*, 2003b; Yamamoto *et al.*, 2001).

This TCS has been reported under different nomenclatures in different bacterial species: besides the largely used YycFG denomination, some reports use the MicB/MicA nomenclature to reflect its role in competence regulation in *S. pneumoniae*, while the name VicK/VicR is used in *S. pneumoniae* and *S. mutans* for its role in virulence and competence regulation (Echenique and Trombe, 2001; Senadheera *et al.*, 2005; Wagner *et al.*, 2002). A number of studies, performed by different groups in different model organisms, have investigated the cellular function of YycFG, the structure of the YycG HK and the YycF RR, the YycFG regulon and the nature of its essentiality. However, a complete understanding of the physiological role of this TCS still needs to be achieved: the aim of the present study is to elucidate the function of YycFG in *B. subtilis*.

1.2.1. Structure and classification of YycG histidine kinases in different bacteria

As anticipated above, the three HKs YycG, ResE and PhoR in *B. subtilis* have been classified on the basis of the sequence of the region around the conserved histidine residue as belonging to the homology class IIIA, where they form a “sub-group”, clearly indicating evolution from a common progenitor (Fabret *et al.*, 1999).

The kinase activity of purified YycG extracted from several bacteria has been studied *in vitro*. The cytoplasmic domains of the *B. subtilis*, *S. aureus* and *S. pneumoniae* YycG have been shown to possess autokinase activity and to be able to phosphorylate their cognate purified regulators (Dubrac and Msadek, 2004; Echenique and Trombe, 2001; Howell *et al.*, 2003). However, the crystal structure of YycG has not been resolved. Therefore structural studies on YycG have been performed solely on the basis of sequence analysis (Kiil *et al.*, 2005). A three-dimensional model is available only for the conserved ATP-binding subdomain, which has been reconstructed *in silico* for *S. epidermidis* as a basis for discovery of new inhibitors for YycG (Qin *et al.*, 2006). Conserved domains can be identified in all the YycG orthologues, as described in Figure 1.2: a HAMP (linker) domain, a PAS domain, a histidine kinase domain containing the conserved histidine residue, an ATPase domain and one or two transmembrane domains (Ng and Winkler, 2004).

PAS domains are typically involved in sensing signals like oxygen or light or cofactors that respond to redox state (Taylor and Zhulin, 1999): the presence of a PAS domain in YycG orthologues has suggested a role for YycG in sensing small molecules and ligands that reflect the metabolic state of the cell (Ng and Winkler, 2004). A mutational analysis of the signal-sensing domain of ResE has shown that the PAS domain is essential for sensing oxygen limitation and nitric oxide and for consequent transcriptional activation of ResDE-dependent genes (Baruah *et al.*, 2004). However, in the *B. subtilis* HK PhoR, the PAS domain does not seem to be involved in the sensing of the signal, because deletion of the whole N-terminal region, including the PAS domain, did not affect activation of the PhoP response (Shi and Hulett, 1999). The role of the PAS domain in the *B. subtilis* HK YycG, closely related to both ResE and PhoR, remains to be investigated.

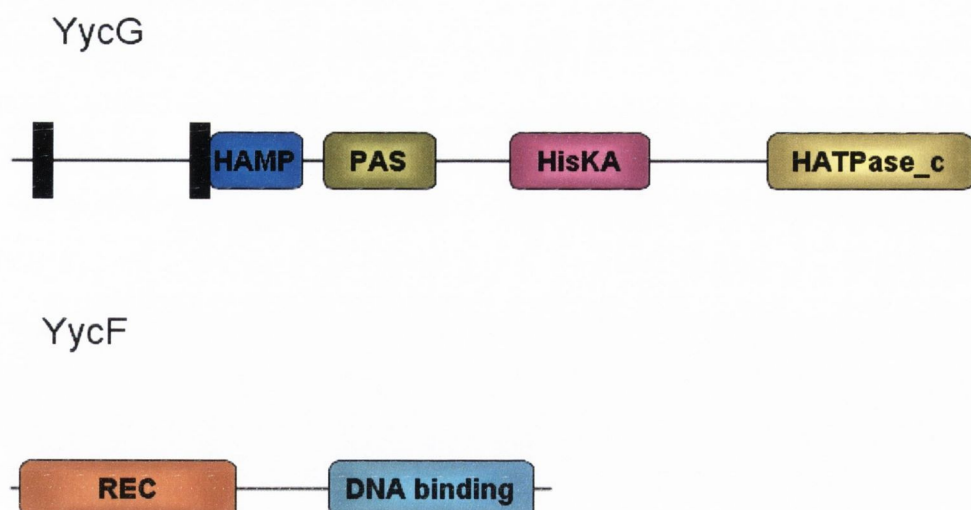


Figure 1.2. General domain organization of the HK YycG and the RR YycF. See text for details.

Interestingly, YycG is the only protein containing a PAS domain in *S. pneumoniae* (Echenique and Trombe, 2001). It has been postulated that in this bacterium YycG might be involved in sensing the cellular redox potential through its PAS domain, in order to coordinate membrane growth (Throup *et al.*, 2000). Residue L100 in the N terminal portion of the PAS domain was shown to be crucial for the YycFG-dependent regulation of competence *in vivo*, as well as for the autokinase activity of the pneumococcal YycG *in vitro* (Echenique and Trombe, 2001). These results show the importance of the PAS domain for the functionality of YycG in *S. pneumoniae*; however, the precise role of this domain and the signal sensed by it still remain to be investigated.

Despite the presence of conserved domains, there are clear structural differences among YycG orthologs, regarding the number of transmembrane domains and the presence and length of the extra-cytoplasmic loop. Most YycG orthologs contain a large extra-cytoplasmic loop, 142 to 154 amino acids long, flanked by two transmembrane domains. One exception is represented by the *Lactococcus lactis* YycG, where the extra-cytoplasmic loop is only 4 amino acids long. Furthermore, YycG orthologs in *Streptococcus* species lack the extra-cytoplasmic loop and contain a single transmembrane domain, with only 4 to 12 amino acids protruding from the transmembrane domain into the periplasmic space (Ng and Winkler, 2004).

The extra-cytoplasmic loop is known to play a crucial role in detecting the external stimulus in some HKs, while the transmembrane domains function as an anchor to the cytoplasmic membrane, but can also be implicated in signal perception (Mascher *et al.*, 2006). Therefore the structural differences among YycG orthologues are likely to reflect a variety of

signaling mechanisms: YycG might sense different signals in different organisms, in accordance with their specific metabolism and physiology. Consistent with this interpretation, a functional classification of HKs like the one proposed by Mascher *et al.* (2006) would colocate YycG orthologues from different bacteria into three separate classes. Most YycG homologues, which contain a long extra-cytoplasmic loop, could be classified as a “periplasmic-sensing histidine kinase” (Mascher *et al.*, 2006); the *Lactococcus lactis* YycG, with its short loop and two transmembrane domains, could be considered a “histidine kinase with sensing mechanisms linked to the transmembrane regions” (Mascher *et al.*, 2006); finally, Streptococcal YycG could be classified as a “cytoplasmic sensing histidine kinases” (Mascher *et al.*, 2006), where the PAS domain might function as a cytoplasmic input domain. However, the precise sensing mechanism and the role of the different potential signaling domains have not been fully elucidated.

There is an interesting correlation between the structure of YycG orthologues and their essentiality. The only species in which YycG was found to be dispensable were *Lactococcus lactis* (O'Connell-Motherway *et al.*, 2000) and *Streptococcus* species (Senadheera *et al.*, 2005; Throup *et al.*, 2000; Wagner *et al.*, 2002), which all lack a long extra-cytoplasmic loop. Although it is still unclear how such structural differences relate to the essentiality of YycG, this observation further confirms the existence of distinct classes of YycG HKs.

It has been suggested that the dispensability of YycG in *S. pneumoniae* could be explained by non-cognate phosphorylation (Clausen *et al.*, 2003). Consistent with this hypothesis, cross-phosphorylation was observed *in vitro* between the *S. aureus* and *S. pneumoniae* YycFG proteins (Clausen *et al.*, 2003); furthermore, the *E. faecalis* VanRS was shown to be able to phosphorylate the *S. pneumoniae* YycF *in vitro* (Wagner *et al.*, 2002). In addition, recent *in vitro* data have shown that acetyl phosphate is a phosphoryl group donor for purified *S. pneumoniae* YycF (Ng *et al.*, 2005). Acetyl phosphate is an important signaling molecule in some bacteria and its capacity to phosphorylate YycF could partly account for the dispensability of YycG in *S. pneumoniae*.

1.2.2. Structure and classification of the YycF response regulator

As anticipated above, the three RR YycF, PhoP and ResD have been classified according to different criteria as belonging to the OmpR family of RRs (Fabret *et al.*, 1999) or to the subclass A within the OmpR family (Kojetin *et al.*, 2003). Both classifications reflect evolution from a common progenitor.

YycF is a typical RR with an N-terminal receiver domain and a C-terminal DNA binding domain (see Figure 1.2). The receiver domain presents a conserved aspartate residue (D54 relative to YycF from *B. subtilis*), which is probably the phosphorylation site of the regulator. Although the crystal structure of whole YycF protein has not been resolved yet [only preliminary data are available to date for a mutant form of the pneumococcal YycF (D59A) (Riboldi-Tunnicliffe *et al.*, 2004)], the structure of the receiver domain and of the DNA binding domain have been resolved separately in *S. pneumoniae* and *E. faecalis* respectively (Bent *et al.*, 2004; Trinh *et al.*, 2007). The pneumococcal YycF N-terminal domain crystallized as a homodimer, and revealed the conserved three-dimensional organization typical of many RR receivers domains (Robinson *et al.*, 2000), with a central β -sheet (made of five parallel β -strands) surrounded by five α -helices (see Figure 1.3). As expected for such motifs, the active site, containing the conserved aspartic residue, was located at the C-terminal end of the β -sheet.

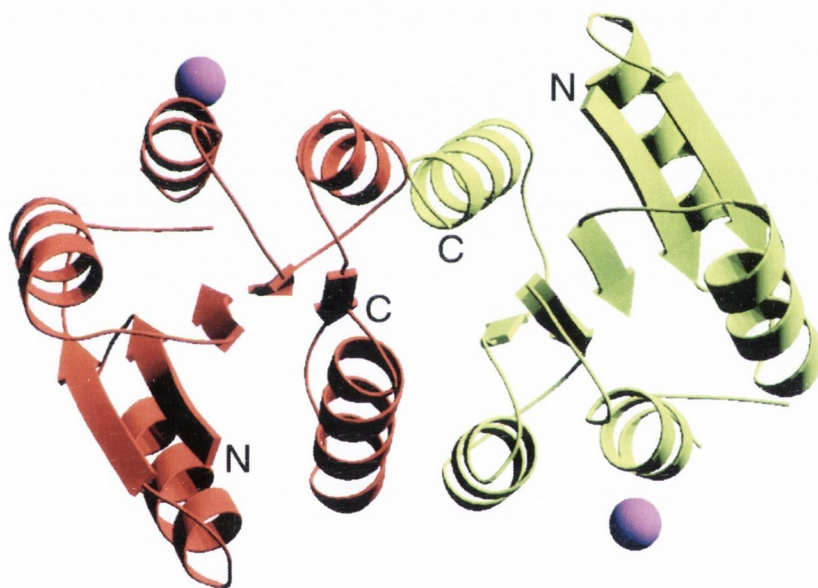


Figure 1.3. Ribbon diagram of the dimer obtained upon crystallization of the *S. pneumoniae* YycF RR (figure adapted from Bent *et al.*, 2004).

Comparison with the structures of receiver domains from other members of the OmpR family revealed an overall high degree of similarity, with observable differences only in the fine details within the β -4 and α -4 regions. A point mutation in the *yycF* coding sequence resulting in the substitution of the aspartate (D) residue at position 59 of YycF with an alanine (A) residue was shown to affect the stability of the phosphorylated form of the pneumococcal YycF *in vitro* (Echenique and Trombe, 2001). The D59 residue of YycF, originally identified

as a putative phosphorylation site, is actually located in the loop connecting β -3 and α -3 within the active site, as revealed by the crystal structure of RR02rec (Bent *et al.*, 2004; Echenique and Trombe, 2001). The two RR02rec monomers were shown to interact through their α -4, β -5 and α -4 regions, in a way which is different from what was found for PhoP, which was shown to utilize two distinct faces for interaction [α -2- α -3- α -4 on one molecule and α -4- β -5- α -5 on the second molecule (Birck *et al.*, 2003)]. Dynamic light scattering experiments showed formation of higher-order RR02rec oligomers, which must interact through surfaces different than those used by the dimer (Bent *et al.*, 2004).

The crystal structure of the DNA-binding domain has been elucidated for the YycF homologous in *E. faecalis* (Trinh *et al.*, 2007). It revealed a conserved motif belonging to the winged helix-turn-helix (HTH) family of DNA binding domains (Aravind *et al.*, 2005), which comprises a N-terminal β -sheet, a central bundle of three α -helices, and a C-terminal β -hairpin turn (see Figure 1.4).

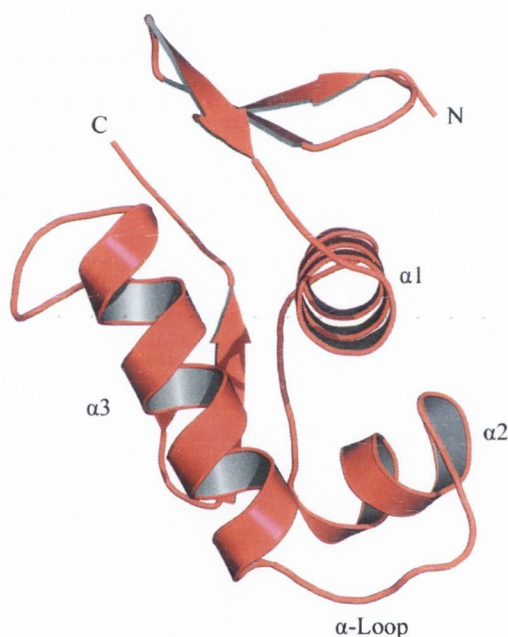


Figure 1.4. Ribbon diagram of the structure of the *E. faecalis* YycF DNA binding domain. Helices $\alpha 2$ and $\alpha 3$ form the helix-turn-helix motif, linked by the α -loop (figure and legend adapted from Trinh *et al.*, 2007).

When compared to other members of the OmpR family of response regulators, the main differences were found at the level of the “ α -loop”, which connects the second and third α -helices, and of the “second loop”, which links the third α -helix to the C-terminal β -hairpin. In particular, the α -loop in YycF was found to be similar to the one present in the *E. coli* PhoB, but shorter with respect to the one observed in the *E. coli* OmpR. The structural similarity to PhoB suggests that the α -loop in YycF might interact with the σ^A factor and might play a

crucial role in transcriptional activation (Trinh *et al.*, 2007). A model for a complex between the YycF DNA-binding domain and DNA was constructed *in silico* on the basis of the known structure of the *E. coli* PhoB-DNA complex (Blanco *et al.*, 2002). It was suggested that in YycF the second loop might play a role in DNA-protein interaction, as opposed to PhoP, in which the loop is too short to contact DNA (Trinh *et al.*, 2007).

This last prediction is in agreement with the results of a study performed on a mutated form of the *B. subtilis* YycF (Watanabe *et al.*, 2003a). In the mutated YycF protein the histidine (H) residue at position 215 is substituted with a proline (P). Residue 215 localizes in the second loop of YycF, and the H215P mutation was previously reported to cause temperature-sensitive growth (Fabret and Hoch, 1998). Circular dichroism spectroscopy revealed that the H215P mutation affected the secondary structure of YycF, as well as its ability to bind to the promoter of the YycFG-regulated genes *ftsAZ*, and its ability to dimerize. In contrast, neither phosphotransfer from auto-phosphorylated YycG nor interaction of YycF with YycG was affected (Watanabe *et al.*, 2003a). These findings point towards a role for the second loop in DNA-binding, as well as in dimerization. The role of the H215 residue in YycF dimerization might appear in conflict with the result obtained in the study performed on the pneumococcal YycF homologue mentioned above (Bent *et al.*, 2004). In this study, in fact, the site of interaction between monomers was shown to be the receiver domain. However, it is possible that both the C-terminal and the N-terminal domains are implicated in dimerization, with the second loop playing an indirect role on the interacting surfaces located in the receiver domain.

RRs typically bind DNA through the third α -helix within their HTH motifs (Aravind *et al.*, 2005). The YycF protein binds to two hexanucleotide direct repeats separated by five nucleotides. The following consensus (5'-TGT A/T A A/T/C-N5-TGT A/T A A/T/C-3') has been established in *B. subtilis* and *S. aureus* (Howell *et al.*, 2003; Dubrac and Msadek, 2004; Bisicchia *et al.*, 2007) with a slightly different consensus (5'-TGTNAN-N5-NGTNANA-3') defined in *S. pneumoniae* (Ng *et al.*, 2005). The target sequence for *B. subtilis* PhoP is very similar to the complement of the one for YycF, and consists of two direct repeats of 5'-TT(A/T/C)ACA-3, separated by a 5 $\pm$ 2 bp spacer (Eder *et al.*, 1999). This observation can be explained by the high degree of conservation of the α_3 -helix shared by YycF and PhoP. On the other hand, despite the high level of homology, there seem to be some differences in the structures of the PhoP and YycF DNA-binding domains, which might account for the specificity of their functions. Although the crystal structure of the PhoP DNA-binding site has not been resolved, alanine-scanning of its C-terminal portion indicated that PhoP has a long α -

loop and a short α_3 -helix, in a fashion similar to what observed for the *E. coli* OmpR (Chen *et al.*, 2004), but different from what observed for the *E. faecalis* YycF (Trinh *et al.*, 2007). Such differences might be crucial for their transcriptional activity and might influence their ability to interact with the RNA polymerase as well as the target DNA.

1.2.3. Genetic organization of the *yycFG* operon

Three genomic organizations of the *yycFG* operon have been observed in Gram-positive bacteria with low G+C content (Figure 1.5). A six cistron operon (*yycFGHIJK*-type operon) was found in *Bacillus* and *Listeria* species; *Staphylococci* and *Enterococci* lack the last cistron (*yycFGHIJ*-type operon), while a three cistron operon (*yycFGJ*-type operon) was found in *Streptococcus* and *Lactococcus* species (Bhagwat *et al.*, 2001; Fabret and Hoch, 1998; Martin *et al.*, 1999; Ng and Winkler, 2004; O'Connell-Motherway *et al.*, 2000; Wagner *et al.*, 2002).

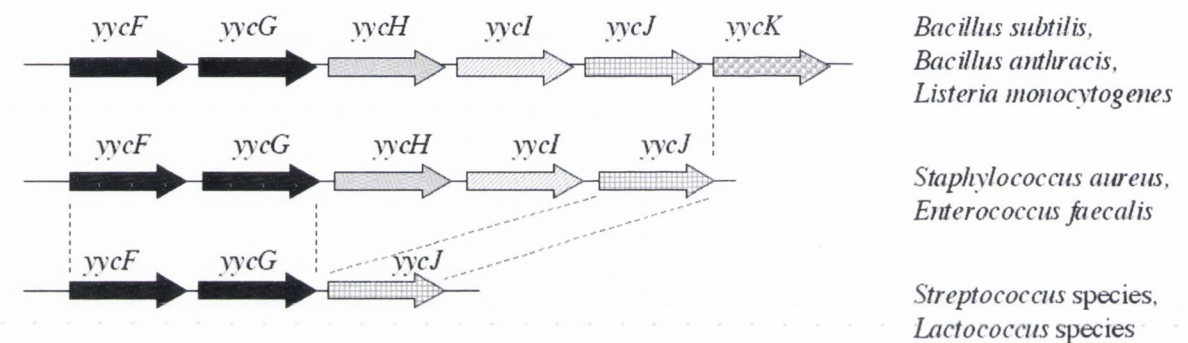


Figure 1.5. Genomic organization of the *yycFG* operon in different species.

There is an interesting correspondence between the type of *yycFG* operon organization and the features of the encoded YycG HK. The *yycH* and *yycI* genes are present only in bacteria having an essential YycG HK, with two transmembrane domains flanking an extra-cytoplasmic loop, while in species lacking the *yycH* and *yycI* genes the YycG HK is non-essential and has only one transmembrane domain (Ng and Winkler, 2004). The only exception is represented by *L. lactis*, which lacks the *yycH* and *yycI* genes but encodes a YycG homologue with two transmembrane domains. However in this species YycG kinase is dispensable and its extra-cytoplasmic loop is only four amino acids long (O'Connell-Motherway *et al.*, 2000).

YycH and YycI share no homology with other proteins in the database, apart from the corresponding orthologues found in the *yycFG* operon of different bacteria. Insights on their function and structure have been recently reported for *B. subtilis* (Santelli *et al.*, 2007;

Szurmant *et al.*, 2005; Szurmant *et al.*, 2006; Szurmant *et al.*, 2007c). Deletion of the *yycH* or the *yycI* gene resulted in aberrant regulation of the YycFG regulon, manifest by growth and cell wall defects and a 10-fold up-regulation of the YycFG-dependent gene *yocH*. YycH and YycI were found to localize in the periplasm of the cell, anchored to the membrane by a N-terminal transmembrane domain. Bacterial two hybrid assays showed that they form a ternary complex together with the YycG kinase. The combination of above data indicates that YycH and YycI interact with the periplasmic portion of YycG and play a role in modulating its activity, probably through the binding and integration of multiple signals. Despite a lack of significant sequence similarity, the crystal structure of YycH and YycI revealed a common fold, which suggests that they originated from a common ancestor and have since then diverged significantly. Based on their unusual tertiary structure, the two proteins were indicated as components of a novel class of HK regulatory proteins. Interestingly, the above findings provide a rationale for the absence of *yycH* and *yycI* in *Streptococci* and *Lactococci* species: YycH and YycI interact with the periplasmic portion of YycG in *B. subtilis*, and therefore would be unable to contact YycG kinases lacking a long extra-cytoplasmic loop (Szurmant *et al.*, 2007c). In addition, the absence of YycH and YycI from *Streptococcus* and *Lactococcus* species strongly suggests that the signal being sensed and the regulation of YycG activity differs in these bacteria, likely reflecting their different cell structure and physiology.

The only gene consistently found in association with *yycFG* in all bacteria possessing this TCS is *yycJ*. Although the function of the YycJ protein is unknown, its functional motifs indicate that it may be a member of the metallo-beta-lactamase superfamily (Senadheera *et al.*, 2007; Wagner *et al.*, 2002), suggesting a possible role of this protein in cell wall metabolism. While there are conflicting results concerning the essentiality of *yycJ* in different strains of *S. pneumoniae* (Echenique and Trombe, 2001; Ng *et al.*, 2003; Throup *et al.*, 2000; Wagner *et al.*, 2002), this gene was found to be dispensable in *B. subtilis* and *S. mutans* (Fabret and Hoch, 1998; Fukuchi *et al.*, 2000; Senadheera *et al.*, 2007). Deletion of *yycJ* in *B. subtilis* was shown to affect colony morphology and to induce additional mutations that resulted in sporulation deficient colonies (Szurmant *et al.*, 2007c). In *S. pneumoniae* strains inactivation of *yycJ* did not cause significantly altered growth rates in laboratory growth conditions (Ng *et al.*, 2003; Throup *et al.*, 2000; Wagner *et al.*, 2002). However, the pneumococcal YycJ was shown to be required for optimal growth when YycF amount was reduced (Ng *et al.*, 2003); in addition, constitutive expression of YycJ in a *yycG* mutant strain resulted in attenuation of the competence defect caused by *yycG* inactivation (Wagner *et al.*, 2002). YycJ seems also to be involved in virulence in *S. pneumoniae*, as over-expression of YycJ resulted in decrease

virulence in a mouse model (Wagner *et al.*, 2002), and a *yycJ* null mutation caused a strong attenuation of bacterial growth in a murine respiratory tract infection model (Throup *et al.*, 2000). In *S. mutans*, inactivation of *yycJ* caused a more pronounced effect: the mutant strain showed an increased doubling-time, an increase in biofilm formation, and was drastically compromised developing natural competence and was significantly less tolerant to oxidative stress (Senadheera *et al.*, 2007). While the molecular mechanisms underlying these phenotypic changes require further investigation, it is interesting to note that the absence of *yycJ* resulted in the same transformability and oxidative stress resistance defects found in a strain over-expressing YycFG; in addition, the *yycJ* mutation caused up-regulation of the *gftB/C* genes, previously shown to be positively regulated by YycF (Senadheera *et al.*, 2005; Senadheera *et al.*, 2007). In other words, the effects of *yycJ* deletion mimic the ones caused by YycFG over-expression, strongly suggesting that YycJ may exert a negative effect on YycF and/or YycG activation. Such a role would be consistent with the genomic localization of *yycJ* within the *yycFG* operon, and would allow listing YycJ besides YycH and YycI as the known regulators of the YycFG signal transduction pathway.

The last gene of the operon, *yycK* (also named *yyxA* in *B. subtilis*), is specific for *Bacillus* and *Listeria* species (see Figure 1.5). It encodes a protein similar to members of the HtrA serine protease family, showing 35% identity with HtrA from *E. coli* (Fabret and Hoch, 1998). YycK is predicted to be anchored in the bacterial membrane through its N-terminal transmembrane domain; this domain is followed by a trypsin like proteolytic domain and a PDZ domain, usually involved in interactions between proteins. The last two domains are found in combination in all the identified HtrA family members (Clausen *et al.*, 2002). HtrA proteases mediate proteolysis of abnormal proteins, and are involved in the oxidative and heat stress responses (Pallen and Wren, 1997). In *L. monocytogenes*, YycK was shown to be involved in resistance to oxidative stress and pathogenesis in a murine model of infection (Stack *et al.*, 2005).

1.2.4. Regulation of *yycFG* operon expression

In *B. subtilis* two transcripts, one extending through all six cistrons of the operon (*yycFGHIJK*), and a second shorter transcript encoding only *yycF* were detected during early exponential growth phase, while a third *yycK* transcript was detected in the late sporulation phase, indicating the presence of a major *yycFG* operon and a second promoter located upstream of *yycK* (Fukuchi *et al.*, 2000). This expression profile is supported by transcriptomic data: transcriptional fusion assays confirmed that the gene *yycK* is expressed

both during exponential phase from the *yycF* promoter, and during sporulation from its own promoter (Fabret and Hoch, 1998). In addition, transcriptional analysis of the *yycF* promoter region showed expression during early exponential phase, and a rapid shut off at the transition phase in sporulation medium; such a decrease was shown to be independent of the major sporulation factor Spo0A. In LB medium the rate of decrease was lower and levels of *yycF* expression remained higher during stationary phase (Fabret and Hoch, 1998). However, western-blot analysis showed that decreased expression of the operon did not result in decreased YycG levels during the stationary phase of the growth cycle in *B. subtilis* in cells grown in LB medium (Szurmant *et al.*, 2005) and in low phosphate medium (Howell, 2006). *yycF* levels of expression did not vary upon over-expression of the YycFG TCS, indicating that the operon is not subjected to auto-regulation (Fabret and Hoch, 1998).

In *S. pneumoniae* R6 a 2.9Kb transcript, corresponding to the predicted size of *yycFGJ*, was detected only in a strain over-expressing the operon (Wagner *et al.*, 2002). The literature reports conflicting data regarding the auto-regulation of the *yycFG* operon in *S. pneumoniae*. In a transcriptomic study *yycG* transcriptional levels were found to increase upon YycF over-expression, suggesting auto-regulation of the *yycFG* operon (Mohedano *et al.*, 2005). However, in a different study the two genes *yycG* and *yycJ* were not found to be differentially expressed in a strain carrying a null mutation in *yycF* (and expressing the essential gene *pcsB* constitutively); furthermore, *in vitro* gel shift assays failed to detect any interactions between the purified YycF protein of *S. pneumoniae* and the promoter region of the *yycFG* operon, strongly suggesting that YycFG does not regulate its own expression in this bacterium (Ng *et al.*, 2005).

Regulation of the *yycFG* operon has not been characterized to date in other bacteria.

1.2.5. The YycFG regulon

Assigning a physiological role to the TCS YycFG was hampered by its essential nature, which precludes the use of conventional genetic approaches based on the construction of strains containing null mutations in the HK and/or the RR. However, a combination of alternative approaches were employed, such as using a hybrid response regulator, YycFG depletion and overexpression through heterologous inducible expression systems and generation of conditional mutants, coupled with genetic, phenotypic and transcriptomic analysis of the resultant strains (Dubrac and Msadek, 2004; Dubrac *et al.*, 2007; Fabret and Hoch, 1998; Howell *et al.*, 2003; 2006; Liu *et al.*, 2006; Lee *et al.*, 2004; Mohedano *et al.*, 2005; Ng *et al.*, 2003; 2004; 2005; Senadheera *et al.*, 2005). Such approaches have yielded

insight into YycFG function and have allowed the identification of YycFG regulon members in different bacteria. Cumulative data suggest that YycFG plays a role in controlling cell wall and membrane metabolism, as well as cell division and virulence. However, significant differences were found among bacteria.

In *B. subtilis* a thermosensitive mutation in YycF caused filamentous growth throughout the growth cycle at the permissive temperature, as opposed to the parental strain in which chains of cells were observed only during early exponential growth; such a phenotype is suggestive of a defect in cell division. A temperature shift to 47°C caused growth cessation and lysis, and empty cells were observed within the chains (Fabret and Hoch, 1998; Watanabe *et al.*, 2003a). A very similar phenotype was observed upon YycFG depletion, which caused growth cessation, a drop in viability and the appearance of empty cells (Fukuchi *et al.*, 2000). Over-expression of either a wild type or a constitutively active mutant form of YycF caused production of a relatively high proportion of mini cells, and a decrease in the average cell length, indicating excessive cell division (Fukuchi *et al.*, 2000). On the basis of these findings, it was hypothesized that YycFG might play a role in promoting cell division, and that it might control the essential cell division *ftsAZ* operon. This hypothesis was verified, as direct up-regulation of the *ftsAZ* operon was observed upon YycFG over-expression. However, deletion of the *ftsAZ* promoter region containing the putative YycF-binding motif did not affect growth and did not alter the *ftsAZ* basal transcription level. For these reasons *ftsAZ* regulation cannot account for the essential nature of YycFG (Fukuchi *et al.*, 2000).

A combined hybrid regulator/transcriptome approach involving a chimeric PhoP'-YycF protein allowed the identification of *yocH*, encoding a putative autolysin, as a gene directly regulated by YycFG (Howell *et al.*, 2003). On the basis of the common features shared by the *ftsA* and *yocH* promoters, a potential consensus recognition sequence for YycF was defined and was used to identify further putative YycF-regulated genes. Purified YycF was shown to bind to the promoter regions of *yocH* and *ftsAZ*, as well as of the newly identified *ykvT*, encoding a potential autolysin. It also bound to the promoter regions of *tagAB/tagDE*, encoding essential genes involved in teichoic acid biosynthesis (Howell *et al.*, 2003). In a later work YycFG was shown to mediate repression of the *tagAB/tagDE* divergon in response to phosphate limitation: this finding indicates that the essential nature of YycFG is unlikely to be due to the transcriptional control of this divergon (Howell, 2006). Therefore, only a small number of genes regulated by the *B. subtilis* YycFG have been identified so far, and the nature of its essentiality has not been identified: establishing the YycFG regulon, its physiological

role and the reason for its essentiality in *B. subtilis* are the aims of the work presented in this thesis.

The high level of conservation between the YycF response regulators of *B. subtilis* and *S. aureus* (74% amino acid sequence identity), particularly within the DNA binding regions, allowed the use of the *B. subtilis* consensus YycF DNA-binding motif to identify YycFG-regulated genes in *S. aureus* (Dubrac and Msadek, 2004). Bioinformatic and transcriptional studies showed that YycFG positively regulates transcription of nine genes encoding proteins likely to be involved in cell wall metabolism. Among them are the genes encoding the two major *S. aureus* autolysins, AtlA and LytM; genes encoding two proteins with a transglycosylase domain, SceD and IsaA, and five genes encoding proteins with CHAP amidase domain (SsaA, SA0620, SA2097, SA2353, SA0710) (Dubrac and Msadek, 2004; Dubrac *et al.*, 2007). A direct interaction between YycF and the promoter regions of *ssaA*, *isaA* and *lytM* was demonstrated *in vitro*. The phenotypes observed upon altered YycFG expression or activity are consistent with defects in cell wall or membrane metabolism: a *S. aureus* strain carrying a temperature sensitive mutation in YycF displayed hypersusceptibility to MLS antibiotics, a feature commonly found in strains mutated in genes involved in maintaining the structural integrity of the cell envelope (Ling and Berger-Bachi, 1998; Martin *et al.*, 1999), and was hypersensitive to unsaturated fatty acids, which affect growth presumably by hampering the permeability of the cell membrane (Butcher *et al.*, 1976; Martin *et al.*, 1999). In addition, YycFG-depleted cells displayed a reduced rate of cell wall biosynthesis and turn-over, and resistance to tritonX-100 and lysostaphin with concomitant increases in peptidoglycan cross-linking and glycan chain length (Dubrac *et al.*, 2007). However, unlike in *B. subtilis*, depletion of YycFG in *S. aureus* does not lead to cell lysis (Dubrac and Msadek, 2004).

Similar to the situation found in *B. subtilis* and *S. aureus*, the pneumococcal YycFG was shown to be involved in regulation of cell wall and cell membrane metabolism. Transcriptome analysis showed that YycFG controls expression of genes involved in cell wall metabolism such as (i) *pcsB* and *lytB* encoding cell wall hydrolases, (ii) several genes encoding proteins with LysM cell wall binding domains, (iii) a gene encoding a phosphate transporter, (iv) *pspA* encoding a virulence factor and (v) *prtA* encoding a serine protease inhibitor (Ng *et al.*, 2003; 2004; 2005). In these studies, Winkler and colleagues show that expression of PcsB, an amidase with a CHAP domain typical of murein hydrolases, is activated by YycFG and that regulation of this gene accounts for YycFG essentiality in *S. pneumoniae* (Ng *et al.*, 2003; 2004; 2005). This is the only instance so far whereby the essentiality of the YycFG TCS can

be ascribed to regulation of a single essential gene. Consistent with the cell wall-related function of many YycFG-regulated genes, depletion for YycFG resulted in defects in cell morphology and murein synthesis in this bacterium (Ng *et al.*, 2004).

A body of evidence suggests the involvement of the pneumococcal YycFG in cell membrane metabolism, although the data present in the literature is not entirely consistent. In a first study YycF over-expression resulted in the induction of a cluster of 12 genes involved in fatty acids biosynthesis, had an impact on cell membrane composition, and displayed an increased ratio of 18-carbon fatty acids to 16-carbon fatty acids (Mohedano *et al.*, 2005). However, a second transcriptomic study did not support direct regulation of fatty acid biosynthesis by YycF, although a *yycF* null mutant (expressing the essential gene *pcsB* constitutively) was shown to require fatty acids for optimal growth in some conditions, indicating that the pneumococcal YycFG may be involved in mediating membrane integrity (Ng *et al.*, 2005).

Studies in *S. mutans* show that YycFG activates expression of *gtfB*, *gtfC* and *gtfD*, encoding glucosyltransferases, of *flf* encoding a fructosyltransferase, and of *gbpB*, encoding a glucan-binding protein. Direct regulation was shown for genes *gtfB*, *gtfC* and *ftf*. These genes are involved in the synthesis of extracellular polysaccharides for cellular adhesion in the formation of dental caries, thereby showing adaptation of the YycFG regulon to the environmental niche of this bacterium (Senadheera *et al.*, 2005). In addition, in a recent report the *S. mutans* YycFG TCS was shown to control expression of the major autolysin Atl, and of SMu0629, a putative thiol-disulfide oxidoreductase involved in the control of Atl production and maturation (Ahn and Burne, 2007). There is a further paper on YycFG in *S. mutans* by Lee *et al.* (2004). These authors have mis-identified YycFG as CovRS, but they found that it negatively regulates fructosyl-transferase expression and the production of a novel glucose and glucuronic acid-containing extracellular carbohydrate (Lee *et al.*, 2004). Therefore these findings seem to contradict the study by Senadheera *et al.* (2005). In addition, in this paper the authors claim they were able to obtain a YycF knock-out mutant in *S. mutans*, therefore contradicting two reports in which the *S. mutans* YycF was shown to be essential and YycG was found to be dispensable, but required for optimal growth (Bhagwat *et al.*, 2001; Senadheera *et al.*, 2005). In conclusion, the paper from Lee *et al.* (2004) is in clear contrast with what reported in Senadheera *et al.* (2005), but the mis-identification does not inspire confidence in the results.

A recurring theme emerging from the identification of genes regulated by YycFG in different bacteria is its involvement in cell wall and cell membrane metabolism. Therefore

regulation of cell envelope homeostasis can be used as a unifying theme with which to interpret the diverse range of phenotypes observed upon YycG mutation or YycFG depletion in *Streptococci*. These phenotypes include: in *S. pneumoniae*, defects in growth, competence and virulence (Wagner *et al.*, 2002; Kadioglu *et al.* 2003), and filamentation with irregular cell size and shape with empty cells (Ng *et al.*, 2003; 2004); in *S. mutans* defects in growth, altered biofilm formation with a decrease in CFU in dental plaque (Senadheera *et al.*, 2005). In *S. pyogenes*, in addition to a role in cell wall metabolism, YycFG plays a role in nutrient uptake and osmotic stress (Liu *et al.*, 2006).

1.2.6. The role of YycFG in virulence

Alteration of cell envelope structure, composition and metabolism under conditions of altered YycFG expression also present a unifying theme for considering on the role of this TCS in infection and virulence. A link between the YycFG TCS and virulence has been established in both *S. aureus* and *Streptococcal* species.

In *S. aureus* YycFG regulates expression of *sdrD* and *ebpS*, whose products are involved in interactions between *S. aureus* and host extracellular matrix proteins, and promote biofilm formation, an important capability for infection (Dubrac *et al.*, 2007).

In *S. pneumoniae* virulence genes such as *pspA* and *piaBCDA* encoding respectively a surface virulence factor involved in the evasion of complement during infection and an ABC transporter involved in iron uptake are members of the YycFG regulon (Mohedano *et al.*, 2005; Ng *et al.*, 2003; Ng *et al.*, 2005; Ren *et al.*, 2004a; Ren *et al.*, 2004b). Although these findings strongly suggest the involvement of the pneumococcal YycFG in virulence regulation, surprisingly null *yycG* mutants of *S. pneumoniae* R6 and of clinical isolates of serotype 3 and 22 displayed wild-type virulence when tested by murine intraperitoneal challenge or in a murine respiratory tract infection model (Kadioglu *et al.*, 2003; Throup *et al.*, 2000; Wagner *et al.*, 2002). However, the virulence of strains D39 and S6 was significantly attenuated by *yycG* mutation when tested after intranasal challenge (Wagner *et al.*, 2003). In addition, a *S. pneumoniae* mutant strain derived from clinical isolates of serotype 3 and 22 and over-expressing genes *yycG* and *yycJ* displayed decreased virulence when injected intraperitoneally into mice (Wagner *et al.*, 2002). These studies emphasize the importance of bacterial strain and animal model considerations when interpreting such results.

S. mutans is the major agent involved in dental caries, producing sucrose-metabolizing enzymes that are critical for cariogenesis. These enzymes include three glucosyltransferases (encoded by *gtfB*, *gtfC* and *gtfD*) and one fructosyltransferase (encoded by *ftf*) that catalyze the

cleavage of sucrose to synthesize glucan and fructan polymers that promote the capacity of this bacterium to attach to the tooth surface. As mentioned above, expression of *gftB*, *gftC*, *gftD*, and *ftf* is positively regulated by YycFG and it has been correlated to a positive control of *S. mutans* sucrose-mediated adherence by YycFG (Senadheera *et al.*, 2005). In group A *Streptococcus*, a *yycF* mutant has been shown to be impaired in its capacity to grow in human blood and to kill mice after subcutaneous infection (Liu *et al.*, 2006).

The essentiality of YycFG partially limits the study of the role of this TCS on virulence. Many of the studies in *Streptococcal* species are performed on *yycG* null mutant strains; however, in the absence of YycG it is not clear to what extent other kinases and metabolites such as acetyl phosphate may compensate for its absence. It is clear however that YycFG may play two roles in virulence: a non-specific one derived from the fundamental nature of the processes that it regulates, and a specific one in controlling expression of specific genes that encode virulence factors (such as in *S. mutans* above). Dissecting their relative contributions will require significant further investigation.

1.2.7. YycFG and antibiotic resistance

Recent reports indicate a link between the *S. aureus* YycFG and resistance to vancomycin and daptomycin. Vancomycin is an antibiotic that functions by inhibiting transglycosylation and transpeptidation by binding to the D-Ala-D-Ala residues of Lipid II, an intermediate in cell wall biosynthesis (Bhavsar and Brown, 2006; Kahne *et al.*, 2005). In a vancomycin intermediately resistant *S. aureus* (VISA) clinical isolate the YycFG TCS was found to be drastically up-regulated, due to an insertion mutation in the *yycFG* promoter which enhanced its efficiency (Jansen *et al.*, 2007). In agreement with this finding, whole-genome sequencing of *S. aureus* isogenic clinical isolates recovered periodically from a patient undergoing vancomycin treatment established a link between increased vancomycin resistance and a mutation in the *yycH* gene, whose product has been shown to down-regulate the activity of the YycFG system (Mwangi *et al.*, 2007; Szurmant *et al.*, 2007c). Both studies therefore link vancomycin resistance with increased YycFG levels or activity. Crucially, YycFG over-expression in a susceptible *S. aureus* strain was shown to result in decreased susceptibility to vancomycin, clearly indicating a role of YycFG in developing resistance to this antibiotic, probably through regulation of cell wall metabolism (Jansen *et al.*, 2007).

Daptomycin exerts its effect at the level of the cell membrane, by penetrating the membrane lipid layers and dissipating the proton motive force (Silverman *et al.*, 2003). Both laboratory-derived strains and clinical isolates with decreased susceptibility to daptomycin

were found to be mutated in the *ycg* gene (Friedman *et al.*, 2006). However from this study it is not clear how such mutations affect YycG activity (whether these mutations result in increased or decreased YycG activity) and whether the YycFG TCS plays a positive or negative role in daptomycin resistance. Interestingly, one mutation found in a daptomycin resistant clinical isolate was a single nucleotide insertion early in the *ycg* open reading frame, predicted to result in a truncated inactive YycG protein. This result seems to contradict the established essentiality of YycG in *S. aureus*: to reconcile these findings, the authors suggest the possibility of a YycG protein which may be functional even in the presence of the frameshift mutation, or the phosphorylation of the YycF regulator by an alternative HK (Friedman *et al.*, 2006). However, the possibility that increased YycG activity might lead to higher daptomycin resistance cannot be excluded. In any case, the established link between YycFG and resistance to daptomycin is in agreement with the observed role of this TCS in modulating cell membrane metabolism.

1.2.8. YycFG as a target for antimicrobial therapy

The emergence of multiple drug-resistant bacterial infections is an escalating problem facing public health. Antimicrobial molecules that are commonly used target relatively few cellular functions as cell wall integrity, nucleic acids and proteins synthesis. As a consequence, bacterial-acquired resistance to one antibiotic often leads to a multi-resistance pattern. Because of their central role in bacterial metabolism, two-component systems represent a valid target for antimicrobial therapy, with the potential of reducing the emergence of multiple drug resistance, which is unlikely to develop as quickly to multiple targets. In addition, TCS are absent from the animal kingdom, they are highly conserved and ubiquitous in bacteria, and they are often involved in important physiological processes including virulence and antibiotic resistance, all features that make them attractive targets for antimicrobials (Matsushita and Janda, 2002; Stephenson and Hoch, 2002a, b). The TCS YycFG not only shares all these characteristics, but in addition is essential and is very well conserved and specific to low G+C% Gram-positive bacteria, some of them known to develop drug-resistance patterns. Therefore inhibitors directed against YycFG would be expected to be active against a broad range of Gram-positive pathogens.

A first study, published in 2001, reports the use of imidazole and zerumbone derivatives as efficient inhibitors of the purified *B. subtilis* YycG autophosphorylation *in vitro*, and of *B. subtilis* growth (Yamamoto *et al.*, 2001). A stereoisomer of one such compound (the zerumbone ring-opening derivative 4) was later shown to possess a more efficient YycG

inhibitory effect *in vitro* (Kitayama *et al.*, 2004). A more recent study from the same research group reports the effects of zerumbone ring-opening derivatives on purified YycG *in vitro* and on growth of methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *E. faecalis* (VRE) (Kitayama *et al.*, 2007). Results from this study show that a zerumbone ring-opening derivative (compound 2) affects YycG auto-phosphorylation but does not inhibit growth of MRSA and VRE strains, while a tryptophan derivative of zerumbone 2 (compound 34) is capable of inhibiting YycG auto-phosphorylation and growth of MRSA and VRE strains. In addition, Watanabe *et al.* (2003) developed a screening method to identify compounds that inhibit YycG in *B. subtilis* and *S. aureus*. Such a system, based on a temperature sensitive *B. subtilis* *yycF* mutant that is supersensitive to HK inhibitors, allowed the identification of aranorosinol B, a known antimicrobial agent against Gram-positive bacteria, as a potent inhibitor of YycG auto-phosphorylation.

While the above studies aimed to the identification of compounds targeting YycG auto-phosphorylation, Furuta and co-workers (Furuta *et al.*, 2005) have developed a high-throughput system for targeting HK homodimerization, which is an essential step for bacterial signal transduction systems. This strategy led to the identification of a compound, I-8-15, capable of specifically inhibiting dimerization of the *S. aureus* YycG *in vitro* and causing *S. aureus* cell death *in vivo*. In addition, this compound was found to be active against growth of MRSA (methicillin-resistant *S. aureus*) and VRE (vancomycin-resistant *E. faecalis*).

In a recent report a number of novel inhibitors of the YycG kinase of *S. epidermidis* were identified following a structure-based virtual screening (SBVS) of a library of small molecular compounds (Qin *et al.*, 2006). Such molecules were shown to target YycG auto-phosphorylation *in vitro*, and to significantly affect growth of *S. epidermidis* and of other bacteria as *S. aureus*, *S. pyogenes* and *S. mutans*, without displaying any activity against mammalian cells or hemolytic effects. The SBVS technology appears as a promising tool that could be widely used for the discovery of new antimicrobial compounds against TCSs.

1.3. AIMS OF THIS WORK

The central aims of this work are to establish the function of the YycFG TCS in *B. subtilis*, to identify constituent genes of its regulon and to investigate the reason for its essentiality.

Only a few genes belonging to the YycFG regulon have been identified in *B. subtilis*. We posit that the set of regulated genes is larger than that discovered so far. In addition, none of the known members of the YycFG regulon accounts for its essentiality. This work aims not only to extend the YycFG regulon, but also to address the issue of the essentiality of this TCS. The chosen strategy is a genetic analysis involving the construction of strains containing single and multiple null mutations in each of the genes positively regulated by YycFG. A promoter analysis will also be performed on YycFG-regulated genes, in order to distinguish between direct and indirect regulation, and to dissect the features of promoters required for direct regulation by YycF. In addition, the functions of individual genes directly regulated by YycFG will be investigated, in order to get insights into the physiological processes modulated by this essential TCS.

CHAPTER 2

THE YycFG REGULON

2.1 INTRODUCTION

Bacteria use TCSs to adapt to the prevailing environmental and nutritional conditions. YycFG is the only essential TCS encoded in the *B. subtilis* genome, with both the HK and the RR being required for growth (Fabret and Hoch, 1998). Although an orthologous YycFG two-component system is present in other Gram-positive bacteria, the essentiality of the HK and the RR varies depending on the bacterium. Accumulated evidence coming from studies of the phenotype of YycFG-depleted cells and of the constituent genes of the YycFG regulon in different bacteria suggest a role for YycFG in controlling cell wall and membrane homeostasis, cell division and virulence (Dubrac and Msadek, 2004; Fukuchi *et al.*, 2000; Howell *et al.*, 2003; Martin *et al.*, 1999; Martin *et al.*, 2002; Mohedano *et al.*, 2005; Ng *et al.*, 2004; Ng *et al.*, 2003; Wagner *et al.*, 2002). YycFG-controlled genes that participate in cell wall homeostasis include those encoding the autolysins YkvT and YocH in *B. subtilis* (Howell *et al.*, 2003), LytM in *S. aureus* (Dubrac and Msadek, 2004) and PcsB in *S. pneumoniae* (Ng *et al.*, 2003). Further evidence for YycFG involvement in cell wall and membrane metabolism comes from its regulation of the teichoic acid biosynthetic operons *tagAB* and *tagDEF* in *B. subtilis* (Howell *et al.*, 2006) and of the fatty acid biosynthetic genes in *S. pneumoniae* (Mohedano *et al.*, 2005). Expression of the cell division genes *ftsAZ* is YycFG controlled in *B. subtilis* while 5 virulence genes of *S. aureus* are putative members of the YycFG regulon (Dubrac and Msadek, 2004; Fukuchi *et al.*, 2000).

The reason for YycFG essentiality has been established unambiguously only in *S. pneumoniae*, where it derives from its activation of *pcsB* expression, a gene that encodes a putative murein hydrolyase (Ng *et al.*, 2003). The nature of YycFG essentiality in *B. subtilis* has not been established beyond the recognition that it is not the result of YycFG control of a single essential gene. Furthermore, only a limited number of genes regulated by YycFG have been identified in *B. subtilis* so far, and a comprehensive analysis of the YycFG operon in *B. subtilis* has not been performed to date.

This study aims to obtain further insight into the nature of the cellular processes controlled by YycFG, to identify new constituent genes of the YycFG regulon and to determine the reason for its essentiality in *B. subtilis*. In order to identify YycFG-controlled genes, two alternative approaches were adopted. Firstly, a transcriptomic analysis was performed on *B. subtilis* cultures at the point of growth cessation caused by YycFG depletion. In addition, a bioinformatics approach was adopted in order to identify YycFG-regulated genes that might have been missed in the array analysis due to their RNA not being sufficiently depleted at the point of growth cessation. Having identified new members of the

YycFG regulon, a promoter analysis was undertaken in order to distinguish between direct and indirect regulation, and to dissect the features of promoters directly regulated by YycF. In addition, a genetic analysis was performed to determine which gene or combination of genes regulated by YycFG contributes to its essentiality. Finally, the phenotypes of strains deleted in such genes were analyzed in order to get insights into their function. The results derived from these studies are presented in the next section.

2.2. RESULTS

2.2.1. IDENTIFICATION OF NEW MEMBERS OF THE YycFG REGULON AND ANALYSIS OF THEIR PROMOTER REGIONS

2.2.1.1. Identification of additional members of the YycFG regulon

The work presented in this paragraph was performed by Professor Kevin Devine in collaboration with Thomas Jensen and Hanne Jarmer at the Center for Biological Sequence Analysis, Department of Biotechnology, The Technical University of Denmark. The northern blot analysis was performed by Doctor Alistair Howell in the laboratory of Professor Kevin Devine at the Smurfit Institute of Genetics, Trinity College Dublin, with the exception of the northern blot experiment on gene *yoeB*, displayed in Figure 2.2, which I performed.

In a previous study it was found that when a culture of strain AH9912 ($P_{spac} yycFGHIJK$) grown in LB containing 100 μ M IPTG was used to inoculate LB without IPTG, cells grew normally for approximately 4 generations, then growth ceased and cells subsequently lysed (Howell *et al.*, 2006). It was also found that the level of YycG decreased significantly during this growth period (Howell *et al.*, 2006). We reasoned therefore that a comparison between the transcriptomes of normally growing cells and cells harvested at the precise point of growth cessation due to YycFG depletion would identify not only constituent genes of the YycFG regulon, but also the genes responsible for the cessation of growth and hence for the essentiality of YycFG.

Two flasks of LB, one containing 1mM IPTG and the other without IPTG, were inoculated with an exponentially growing culture of strain AH9912 grown in presence of 100 μ M IPTG. A third flask of LB was inoculated with wild-type strain 168. All three cultures started growth at similar OD₆₀₀ values (0.025) and grew at the same rate for 100 minutes after inoculation, reaching OD₆₀₀ 0.412 (Figure 2.1A). However, growth of the YycFG depleted culture (strain AH9912 without IPTG) slowed substantially between 100 and 130 minutes

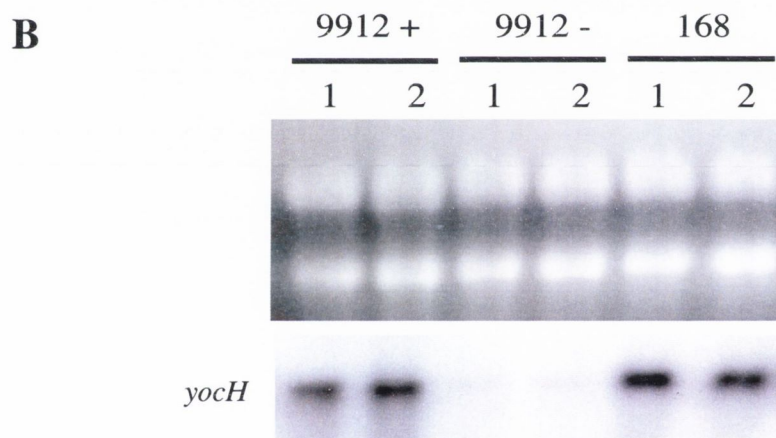
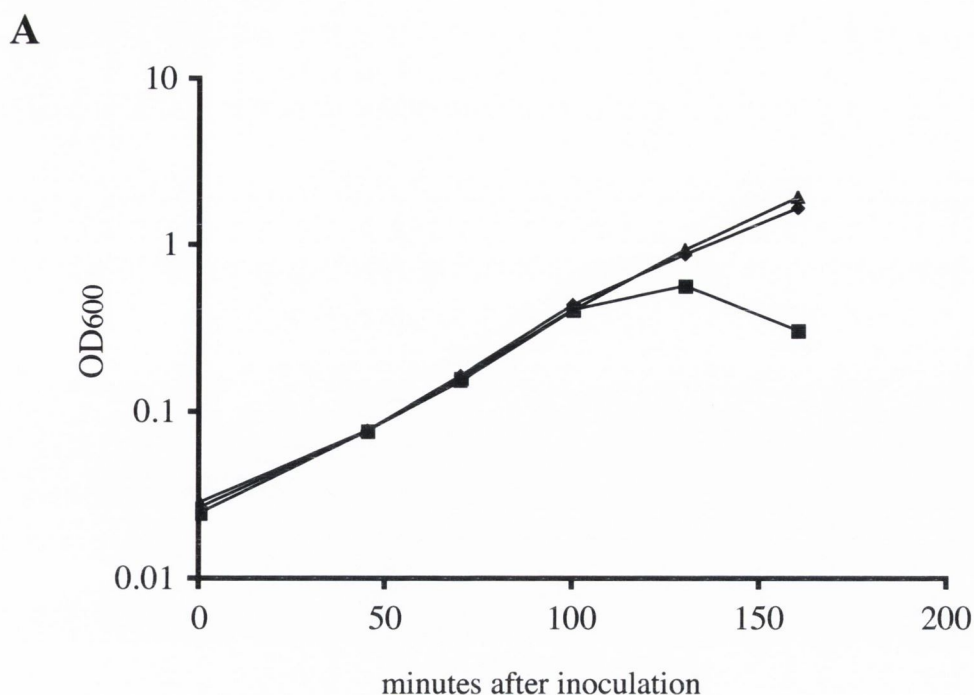


Figure 2.1. Growth profiles and analysis of RNA preparations prepared from strains 168 (wild type) and AH9912 ($P_{yxc} yycFGHIJK$) grown in the presence (+) and absence (-) of IPTG inducer.

(A) Growth profiles of strain 168 (triangles) and strain AH9912 ($P_{yxc} yycFGHIJK$) grown in the presence of 1 mM IPTG (diamonds) and in the absence of IPTG (squares). Strains were cultured in LB broth and growth was monitored turbidimetrically. Time is indicated in minutes after inoculation.

(B) Analysis of the RNA preparations used in the microarray experiment (see text). Total RNA was made from the cultures shown in Figure 1A at the 100 minutes time point. The gel used in the Northern blotting experiment was stained with ethidium bromide and showed a comparable pattern of ribosomal RNA bands in each lane (upper panel). The *yocH* transcriptional levels were analysed by Northern blotting (lower panel).

post-inoculation when compared to the two other cultures. Total RNA was made from cells harvested from each culture at the 100-minute time-point, since it is clear that these cells must already be limited for some YycFG-dependent function that prevents continued growth at the normal rate. Three experimental replicates were performed, each showing growth kinetics similar to that shown in Figure 2.1A.

To confirm that at the chosen time point cells harvested from strain AH9912 grown without IPTG were depleted for YycFG, all the RNA preparations were analyzed by Northern analysis, testing for the expression level of *yocH*, a gene known to be positively regulated by YycFG (Howell *et al.*, 2003). A representative picture of the stained gel (upper panel) and the level of *yocH* transcript (lower panel) is shown in Figure 2.1B. The ethidium stained gel shows that the RNA is of very good quality, based on the integrity of the ribosomal RNA bands, and that each lane is loaded to the same extent. It is clear from the bottom panel that the level of *yocH* transcript is approximately the same in the cultures of wild-type strain 168 and of strain AH9912 grown in the presence of 1mM IPTG. However, the level of *yocH* transcript is barely detectable in strain AH9912 grown in the absence of IPTG, a result obtained for all three experimental replicates. This experiment confirmed that the *yocH* gene, whose expression is activated by YycFG, is depleted at the 100-minute time-point, suggesting that a transcriptomic analysis of these RNA samples would identify additional genes of the YycFG regulon.

The RNA preparations from cultures of strain AH9912 grown in the presence of 1mM IPTG and in the absence of IPTG (three experimental replicates) were used for transcriptomic analysis using Affymetrix *B. subtilis* microarrays.

To assess the overall gene expression profile of YycFG depleted cells, a Pearson correlation coefficient was calculated comparing (i) expression of all genes and (ii) expression of 268 of the 271 genes reported to be essential in *B. subtilis* (Kobayashi *et al.* 2003) from YycFG replete and YycFG depleted cultures. The correlation coefficient for all genes was 0.9864, while that for the 268 essential genes was 0.9969. These correlation values indicate that global changes in RNA levels had not occurred in YycFG depleted cells at the point of harvesting. The *yycG*, *yycH*, *yycI* and *yycJ* genes (members of the *yycFGHIJK* operon whose expression is under the control of the IPTG-inducible P_{spac} promoter) showed the largest and most significant decrease in transcript level upon YycFG depletion, verifying that transcription of this operon is significantly reduced as expected. Interestingly, the *yycF* transcript was reduced only ~2-fold, while the transcripts of the other four genes were reduced between 15- and 24-fold (see Supplementary Material in Bisicchia *et al.*, 2007 for complete array data).

This difference could be explained by processing and/or differential stability of the operon transcript.

The 8 genes that show the highest differences in transcript levels between YycFG replete and YycFG depleted cells are listed in Table 2.1. Expression of three genes, *ydjM*, *yvcE* and *yocH*, was significantly reduced upon YycFG depletion: *ydjM* encodes a protein of unknown function that has no orthologue in current databases; *yvcE* encodes an endopeptidase-type autolysin previously characterised by Yamaguchi *et al.* (2004) and renamed *cwlO* and *yocH*, encoding a putative autolysin, was previously shown to be regulated by YycFG (Howell *et al.*, 2003). Five genes showed significantly increased expression upon YycFG depletion: *yjeA* encodes a putative peptidoglycan deacetylase; *yocE (des)* encodes a fatty acid desaturase (Aguilar *et al.*, 1998); *dhbF* encodes a protein that participates in siderophore biosynthesis (May *et al.*, 2001); *yoeB* encodes a protein that modulates autolysin activity (Salzberg and Helmann, 2007) and *ykuP* encodes a protein of unknown function.

Table 2.1. Genes that show differential expression upon YycFG depletion

Gene	Fold Change [§]	Pvalue [§]	Function	Reference
Downregulated upon YycFG depletion				
<i>ydjM</i>	-7.2	0.0071	Unknown function	Yamaguchi <i>et al.</i> (2004)
<i>yocH</i>	-4.3	0.071	Putative autolysin	
<i>yvcE</i>	-3.0	0.0081	CwlO autolysin	
Upregulated upon YycFG depletion				
<i>yjeA</i>	8.5	0.0031	Peptidoglycan deacetylase	This work
<i>yocE (des)</i>	6.9	0.0025	Fatty acid desaturase	Aguilar <i>et al.</i> (1998)
<i>dhbF</i>	6.0	0.006	Siderophore synthesis	May <i>et al.</i> , (2001)
<i>yoeB</i>	4.3	0.003	Autolysin activity modulation	Salzberg and Helmann (2007)
<i>ykuP</i>	4.3	0.01	Unknown function	

§ these values were derived using the Qspline normalisation described in Materials and Methods

Seven of the genes listed in Table 2.1 were chosen to confirm the array results by Northern analysis. New total RNA preparations were used and the transcription profile of each gene was established during growth. The results (Figure 2.2) show that *yocH*, *yvcE* and *ydjM* are expressed primarily during exponential growth in the wild-type strain 168, even though

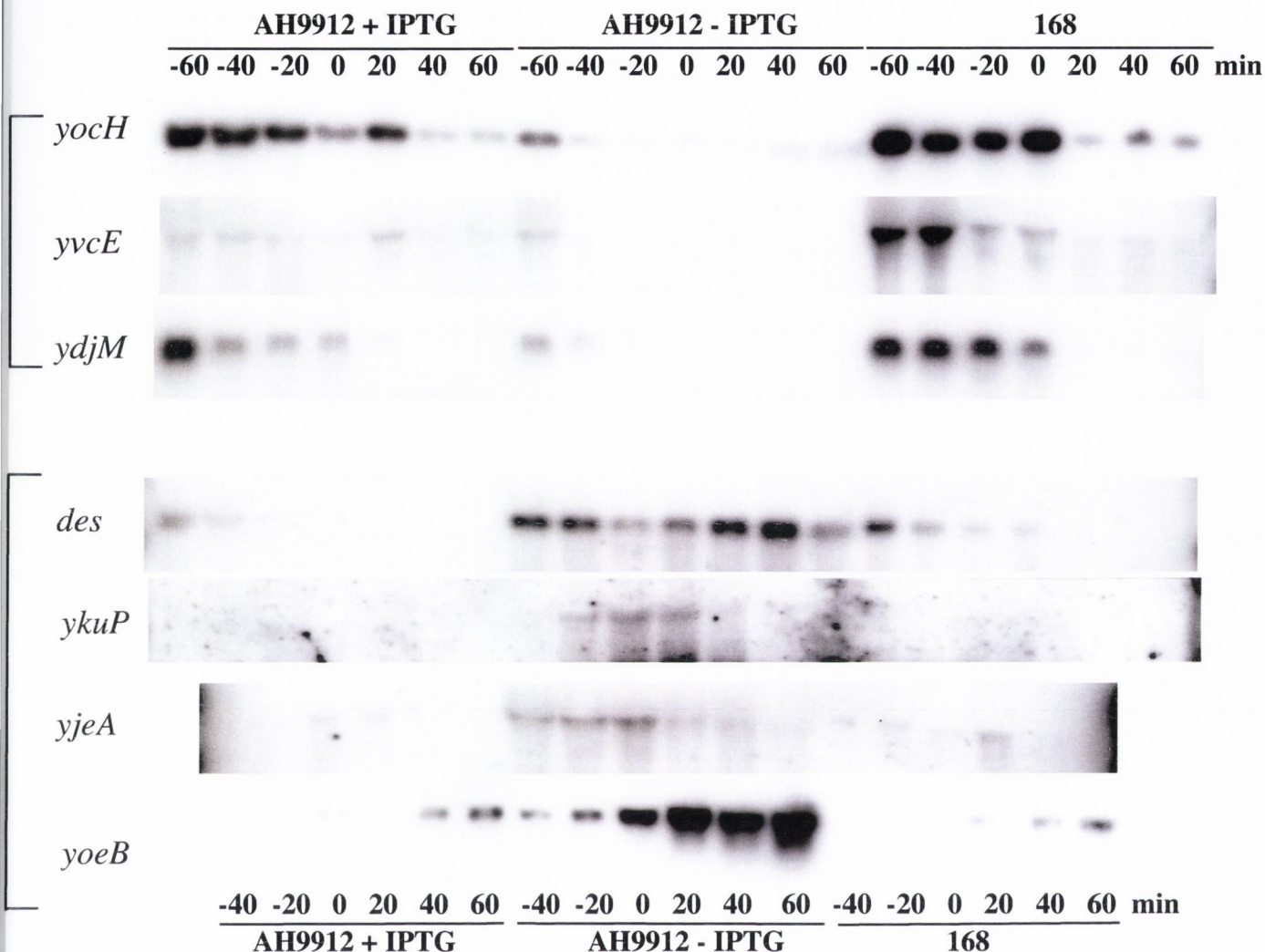


Figure 2.2. Northern analysis of YycFG controlled genes identified using microarrays. Total RNA was made from wild type strain (168) and from strain AH9912 ($P_{yyc}yycFGHIJK$) grown in the presence (+) and in the absence (-) of IPTG in LB medium. Cells were harvested at the time points indicated, numbered according to the transition phase T_0 . The expression profiles of genes positively (+) and negatively (-) regulated by YycFG are bracketed. Twenty-five micrograms of total RNA was loaded onto each lane.

their expression profiles differ somewhat during the growth cycle: *yocH* and *ydjM* transcript levels are high during exponential growth and low during the stationary phase of growth; *yvcE* transcript levels are high during early exponential phase, lower between T_{-20} and T_0 and undetectable during stationary phase. However, transcript levels of all three genes are significantly reduced in YycFG depleted cells (AH9912 -IPTG), especially during the period of exponential growth. Therefore *yvcE* and *ydjM* are positively regulated by YycFG in a manner similar to *yocH* (Howell *et al.*, 2003). The converse expression profile was observed for the *des*, *yjeA*, *yoeB* and *ykuP*: transcriptional levels were low or undetectable during exponential growth in wild-type strain 168 and in YycFG replete cells (AH9912+IPTG), but increased significantly upon YycFG depletion (AH9912 -IPTG), confirming that their expression is negatively regulated by YycFG. In summary, microarray analysis, confirmed by Northern blot analysis, allowed identification of 6 additional genes whose expression is responsive to the cellular YycFG level.

2.2.1.2. YycF directly regulates expression of *ydjM*, *yvcE*, *yjeA* and *yoeB*

All the work presented in this paragraph was performed by me, with the exception of the gel shift on the *htrA* promoter fragment, which was performed by Doctor David Noone. To test whether YycF regulates expression of the newly identified YycFG-regulated genes in a direct way, a gel shift analysis was performed (direct regulation of *yocH* was previously shown, Howell *et al.*, 2003). A biotin-labelled DNA fragment spanning the promoter region of each gene was prepared by PCR, and a fragment containing the *htrA* promoter was used as a negative control. Purified YycF protein was phosphorylated *in vitro* by addition of purified 'YycG HK, prior to the binding reaction. Each reaction contained the unspecific competitor DNA poly [d(I-C)] to ensure specificity of interaction. The results are shown in Figure 2.3. It is evident that YycF~P binds to the DNA fragments containing the promoter regions of *ydjM*, *yvcE*, *yjeA* and *yoeB* (Figure 2.3, lanes 2-4). In each case, YycF~P binding to the biotinylated DNA fragment could be competed by addition of a 100 fold excess of unlabelled DNA fragment, confirming the specificity of interaction (data not shown). No retardation of the fragment containing the *htrA* promoter was observed, as expected, even upon addition of 20 μ M YycF~P (*htrA*, lane 2). YycF~P did not bind to the *des* and *ykuP* promoters (data not shown). These data show that YycF regulates expression of *ydjM*, *yvcE*, *yoeB* and *yjeA* in a direct way.

2.2.1.3. Transcriptional analysis of the *ydjM*, *yvcE*, *yjeA* and *yoeB* genes

All the work presented in this paragraph was performed by me, with the exception of primer extension experiments and BgaB assays on the *yoeB* promoter region, which were performed by Doctor David Noone and Doctor Efthimia Lioliou respectively, in the laboratory of Professor Kevin Devine at the Smurfit Institute of Genetics, Trinity College Dublin.

A transcriptional analysis was undertaken in order to characterize the *ydjM*, *yvcE*, *yoeB* and *yjeA* gene promoters and to establish the effect of YycFG on their expression. A unique initiation point of transcription was established for *ydjM*, *yvcE*, *yoeB* and *yjeA* through primer extension analysis (shown underlined and in bold as +1 in Table 2.2). The promoter sequences of these genes were then aligned relative to their initiation points of transcription (Table 2.2). The YycF-regulated promoters of *yocH* (Howell *et al.*, 2003) and *ftsA* (Fukuchi *et al.*, 2000) are also included for comparison.

The *yocH* and *ftsA* promoters were previously classified as SigA-type promoters (Fukuchi *et al.*, 2000; Howell *et al.*, 2003). For the newly identified genes, the same classification is proposed, although for some of them the SigA recognition elements deviate from the consensus to some extent. The consensus binding sequence for the housekeeping sigma factor SigA in *B. subtilis* was identified as TTGaca(-35)-N₁₄-tgnTATAat(-10) (Helmann and Moran, 2002). Consensus -10 and -35 motifs are indicated by double underlines in Table 2.2. While all these promoters have consensus -10 motifs (one mismatch in the case of *yocH*) at the expected position, no putative -35 motifs are found at the canonical position in the *yvcE* and *ydjM* promoter regions. However, putative SigA-type -35 motifs can be identified within the *yvcE* and *ydjM* promoters (indicated with wavy underline in Table 2.2), although their positioning relative to the -10 motif deviates from that of a normal SigA-type promoter: there is an 11 base-pair spacing between the putative -10 and -35 regions of *yvcE*, while the distance is extended to 23 base-pairs for the *ydjM* gene. A putative -35 motif has been previously assigned within the *yocH* promoter at the canonical position (Howell *et al.*, 2003, double underlined in Table 2.2). However, there is also a consensus SigA -35 motif 3' to this sequence (shown with wavy underline in Table 2.2) that would give an 11 bp spacing, similar to that observed in the *yvcE* promoter.

In a previous study (Howell *et al.*, 2003), a potential YycF consensus recognition sequence was established, consisting of two hexanucleotide direct repeats separated by five nucleotides: 5'-TGT A/T A A/T/C N₅ TGT A/T A A/T/C-3'. Putative YycF consensus sequences were identified in the promoter regions of *yocH*, *yvcE* and *ydjM* (Howell *et al.*, 2003; this work), positioned upstream of the putative -35 promoter region, as expected for

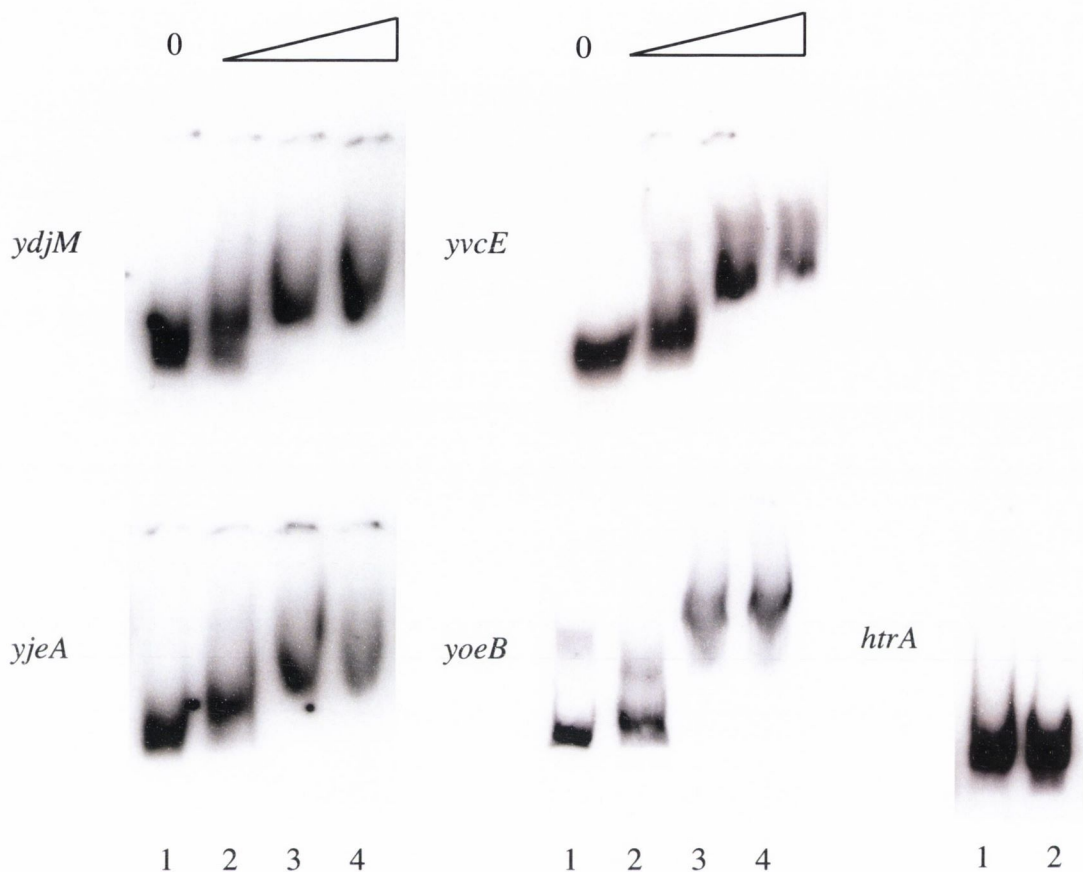


Figure 2.3. Gel shift mobility analysis of genes regulated by YycF. Promoter fragments for each gene were prepared as described in Materials and Methods. Purified YycF protein was phosphorylated *in vitro* prior to the binding reaction by addition of $3\mu\text{M}$ purified 'YycG histidine kinase. 2 ng of labelled promoter DNA were used in each reaction. The mobility of fragments without YycF~P addition is shown in lane 1 (0). For the *ydjM*, *yvcE*, *yjeA* and *yoeB* promoter fragments, lanes 2 to 5 show mobility after binding of 1, 5 and $10\mu\text{M}$ YycF respectively. For the *htrA* promoter fragment, no mobility shift was observed after binding with $20\mu\text{M}$ YycF~P (lane 2). The wedge represents increasing YycF~P levels.

Table 2.2. Alignment of promoters regulated by the YycFG two-component system.*Genes positively regulated by YycF:*

		<u>-35</u>	<u>-10</u>	<u>+1</u>
<i>ftsA</i>	CTGTTGTTATTTTTT <u>TGTTAC</u> ACACT <u>TGTAA</u> GCCACATT <u>CATTG</u> TATTGTTGTTCCGCAAATAATAGAAATG(26nt) ATG			
<i>yocH</i>	TCAT <u>TGTAA</u> AGGAAC <u>TGTAAT</u> CTAAATCATATCTTTTTGTTATTGATT <u>TGACAT</u> TTACGTGTGT <u>TACGAT</u> ATCACCTG(39nt) ATG			
<i>yocH1</i>	TCAC <u>ACA</u> AAAGGAAC <u>TGTAAT</u> CTAAATCATATCTTTTTGTTATTGATT <u>TGACAT</u> TTACGTGTGT <u>TACGAT</u> ATCACCTG(39nt) ATG			
<i>yocH2</i>	TCAT <u>TGTAA</u> AGGAAC <u>ACAAT</u> CTAAATCATATCTTTTTGTTATTGATT <u>TGACAT</u> TTACGTGTGT <u>TACGAT</u> ATCACCTG(39nt) ATG			
<i>yvcE</i>	ACTTGACATCTGTT <u>TGAA</u> ACATAAA <u>TGTAAT</u> AAAATTTATCGCTAATTTGTCGAAACTTGATGGTATAATGATTCTC(187nt) GTG			
<i>yvcE1</i>	ACTTGACATCTGTT <u>ACA</u> ACATAAA <u>TGTAAT</u> AAAATTTATCGCTAATTTGTCGAAACTTGATGGTATAATGATTCTC(187nt) GTG			
<i>ydjM</i>	AACTTTTTTGACTTT <u>TGTAAC</u> ATTAC <u>TGTAAG</u> GATATTGAAATAAAAAATAGCTGGTTGATCGTGATAATCTTCCTA(24nt) ATG			
<i>ydjM1</i>	AACTTTTTTGACTTT <u>TGTAAC</u> ATTAC <u>ACAAG</u> GATATTGAAATAAAAAATAGCTGGTTGATCGTGATAATCTTCCTA(24nt) ATG			
<i>ydjM2</i>	AACTTTTTTGACTTT <u>TGTAAC</u> ATTAC <u>TGTAAG</u> GATATTGAAATAAAAAATTTGATCGTGATAATCTTCCTA(24nt) ATG			
<i>ydjM3</i>	AACTTTTTTGACTTT <u>TGTAAC</u> ATTAC <u>ACAAG</u> GATATTGAAATAAAAAATTTGATCGTGATAATCTTCCTA(24nt) ATG			
<i>lytE</i>	TAGAACGAATAATTAAGAAATT <u>TGTCAC</u> ATGA <u>AGTCA</u> AGACTATTTCTGATGGGAATCTATCCTTATAATAGAAATC(62nt) ATG			

Genes negatively regulated by YycF:

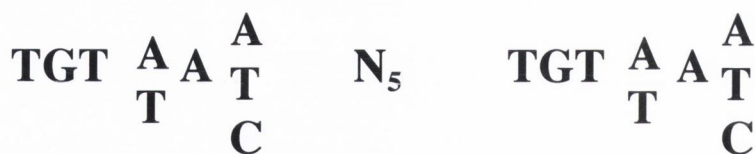
		<u>-35</u>	<u>-10</u>	<u>+1</u>
<i>yoeB</i>	CAAAAGTAT <u>TGTAAT</u> CTATCCGTAATTAT <u>TGTAAC</u> ATT <u>TGTAAC</u> ATAAGAGAAAGAGATTTTGAAGGAGAGAGCGATA. ATG			
<i>yoeB1</i>	CAAAAGTAT <u>TGTAAT</u> CTATCCGTAAATTAT <u>TGTAAC</u> ATT <u>ACAAC</u> ATAAGAGAAAGAGATTTTGAAGGAGAGAGCGATA. ATG			
<i>yoeB2</i>	CAAAAGTAT <u>TGTAAT</u> CTATC <u>ACAAT</u> TAT <u>TGTAAC</u> ATTTGTAA <u>CA</u> ATAAGAGAAAGAGATTTTGAAGGAGAGAGCGATA. ATG			
<i>yjeA</i>	AAAAGGATTGACATGAACTTCTAAATCTCATAGTATTACAAATGTGAAATAAA <u>TGTTACT</u> ACAATGT <u>TACAA</u> . (73 nt). TTG			
<i>yjeA1</i>	AAAAGGATTGACATGAACTTCTAAATCTCATAGTATTACAAATGTGAAATAAA <u>CACTACT</u> ACAAC <u>CACTACAA</u> . (73 nt). TTG			

Putative YycF recognition sequences are marked in red; mutated bases within YycF recognition boxes are marked in green; blue sequence is the 6 bp that is deleted between the -35 and -10 regions of the *ydjM* promoter to give promoters *ydjM2* and *ydjM3* that have the canonical 17 bp spacing between the promoter motifs; sequences with wavy underline are putative SigA -35 promoter motifs positioned abnormally relative to the -10 motif and initiation point of transcription (+1).

genes positively regulated by YycF (potential YycF binding sequences are indicated in red capitals in Table 2.2). Conversely, the positioning of YycF binding sites within the *yoeB* and *yjeA* promoters is characteristic of negative regulation: the putative YycF-binding sequences for the *yoeB* and *yjeA* genes are positioned either overlapping (*yoeB*) or downstream of (*yjeA*) the putative SigA-type promoters (Table 2.2). The *yoeB* gene has two putative YycF-binding sequences, which overlap the putative –35 and –10 regions of the promoter and the initiation point of transcription. The *yjeA* gene has one putative YycF-binding sequence, with one tandem repeat overlapping the initiation point of transcription and the other positioned downstream from it. A further single direct repeat can be identified within the *yjeA* promoter region 5 bp downstream from the putative YycF consensus. This finding deviates somewhat from what is currently known to constitute a YycF binding sequence: YycF is thought to contact DNA in the form of a dimer, each monomer binding to a single tandem repeat (or half-site). Although there is no direct evidence that YycF can bind to a triplet of direct repeats, evidence from *S. pneumoniae* indicates that YycF can form oligomers: dynamic light scattering experiments performed on the receiver domain of the *S. pneumoniae* YycF (RR02rec) showed formation of higher-order RR02rec oligomers (Bent *et al.*, 2004). Therefore the triplet of YycF binding direct repeats found in the *yjeA* promoter region might indicate the binding of three YycF molecules, forming an oligomer.

Having found new members of the YycFG regulon, we sought to establish whether the previously identified consensus sequence for the binding of YycF was confirmed. Figure 2.4 shows an updated version of the YycF binding motif, where the height of the letters is proportional to their frequency. The sequence logo was generated using the WebLogo program (Crooks *et al.*, 2004). As evident from Figure 2.4, the new version of the YycF-binding sequence is very similar to the one proposed by Howell *et al.* (2003), indicating that the previously identified consensus was accurate. However, it is interesting to note that variations from the consensus are possible, both in the composition of the tandem repeats, and in the spacing between them. For example, the first and second direct repeats are separated by four base pairs in the case of *lytE*, and by three base pairs in one of the two YycF binding motifs of the *yoeB* promoter region (see Table 2.2). These findings suggest that there might be additional genes in the YycFG regulon with diverged consensus sequences awaiting discovery. Variations from the YycF consensus sequence have also been previously observed in *S. pneumoniae* (Ng *et al.*, 2005).

Previously identified YycF recognition sequence (Howell *et al.*, 2003)



Updated version of the YycF recognition sequence (this work)

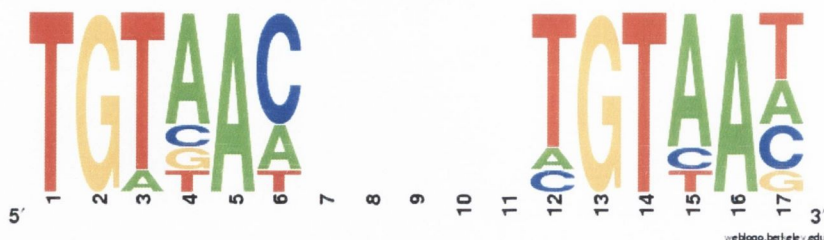


Figure 2.4. The YycF recognition sequence. The previously identified YycF recognition sequence (Howell *et al.*, 2003) is displayed above, and an updated version of the consensus sequence, derived from the newly identified members of the YycFG regulon, is displayed below. The height of the letters is proportional to their frequency. The sequence logo was generated using the WebLogo program (Crooks *et al.*, 2004).

A series of transcriptional *bgaB* fusions were constructed with wild-type and mutated promoter fragments of each gene to confirm that the putative YycF binding motifs participate in the observed YycF-mediated control of gene expression. The TGT sequences were changed to CAC (shown in green in Table 2.2), since this triplet is the most conserved within the YycF-binding motif. The results of the expression analysis are shown in Figure 2.5. Levels of expression of *ydjM* (squares, Figure 2.5A) and *yvcE* (squares, Figure 2.5B) are low during the exponential phase of growth and in both cases increase somewhat during stationary phase. These results might appear in conflict with the expression profiles observed for both *yvcE* and *ydjM* through Northern blot analysis, where transcript levels decreased upon entry into the stationary phase of growth (see Figure 2.2). However, the increased transcriptional activity observed during stationary phase in the BgaB experiment is likely to be due to continued *yvcE* and *ydjM* expression, although at a low level, in the absence of cell division, which may result in accumulation of the transcript and of the BgaB fusion protein within the cells. Such effect might be enhanced by the known stability of the BgaB protein in *B. subtilis* (Schroegel and Allmansberger, 1997). Mutation of the putative YycF binding motifs significantly reduced expression of both *ydjM* and *yvcE* transcriptional fusions, with less than 5 units of activity observed throughout the growth cycle in both cases (triangles, Figure 2.5). The converse result was obtained for the *yjeA* and *yoeB* transcriptional fusions. Approximately 5 units and 50 units of BgaB activity were observed for the wild-type *yjeA* and *yoeB* fusions during exponential

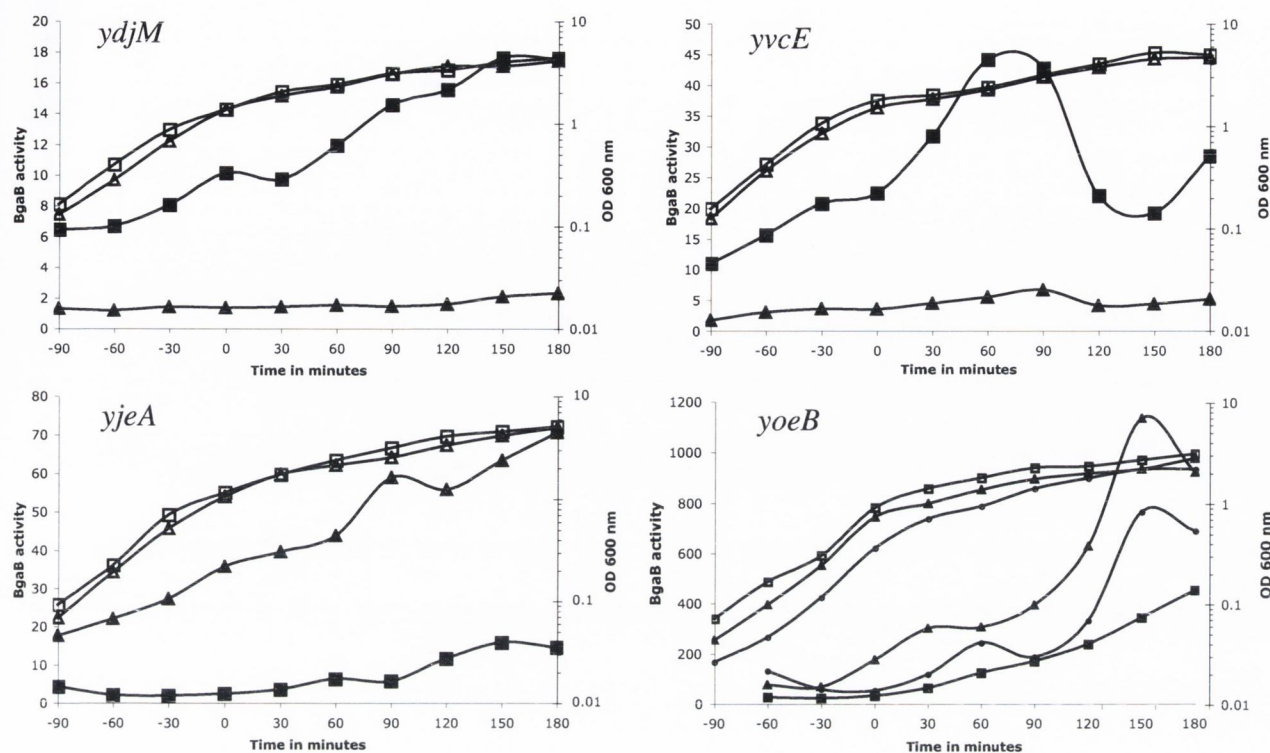


Figure 2.5. Growth and β -galactosidase expression profiles of strains carrying transcriptional *bgaB* fusions. The growth of strains containing wild-type (open squares) and mutated (open triangles and circles) promoter fusions is shown. Accumulation of β -galactosidase is shown for the wild-type promoter (closed squares) and for the promoter mutated in the YycF binding sequence (closed triangles and circle). The expression profiles shown are: *yjdM-bgaB* (squares); *yjdM1-bgaB* (triangles); *yvcE-bgaB* (squares); *yvcE1-bgaB* (triangles); *yjeA-bgaB* (squares); *yjeA1-bgaB* (triangles); *yoeB-bgaB* (squares); *yoeB1-bgaB* (triangles); *yoeB2-bgaB* (circles). The sequence of each promoter and the location and sequence of the mutations introduced (highlighted in red) are shown in Table 2.2. Three independent β -galactosidase experiments were performed for each strain, and representative profiles are shown.

growth, and levels increased during the stationary phase of the growth (squares, Figure 2.5). However, mutation of the putative YycF-binding motifs in the promoters of both genes (fusions *yjeA1-bgaB*, *yoeB1-bgaB* and *yoeB2-bgaB*, see Table 2.2) resulted in significantly higher expression throughout the growth cycle. Expression of the mutated *yjeA* promoter rose to 20-30 units during exponential growth and continued during stationary phase rising to ~60 units (triangles, Figure 2.5). The wild-type *yoeB* promoter was expressed at a level of approximately 50 units of activity during exponential growth, and expression rose significantly to 400 units at the end of the 3 hours stationary phase (squares, Figure 2.5). Two mutated *yoeB* promoter constructs were made, *yoeB1* and *yoeB2* (see Table 2.2). Expression of both constructs was significantly higher at all stages of the growth cycle, with expression of *yoeB1* being higher than that observed for *yoeB2* (triangles and circles, Figure 2.5). In summary these data confirm that the putative YycF binding motifs identified within the promoter regions of *yvcE*, *ydjM*, *yoeB* and *yjeA* do participate in YycFG controlled regulation of these genes.

2.2.1.4. The spacing between the YycF binding site and the -10 region of the *ydjM* promoter is crucial for YycFG-mediated regulation of expression

All the work presented in this paragraph was performed by me, with the exception of the construction of two strains, namely SQ1 and SQ3, which were made under my direction by Sarah Quigley, an undergraduate student I have supervised.

The genes *yvcE* and *ydjM*, positively regulated by YycFG, have SigA-type promoters with unusual features: while consensus -10 motifs are present in these promoters, no putative -35 motifs are found at the canonical position. However, putative -35 motifs are present at non-canonical positions relative to the -10 motif (there is an 11 base-pair spacing between the putative -10 and -35 regions of *yvcE*, and an extended 23 base-pairs spacing in the *ydjM* promoter, see Table 2.2). The absence of putative -35 motifs at the canonical position suggests that SigA-mediated transcription might require an additional factor, such as the transcriptional regulator YycF. According to this model, SigA would require YycF for initiation of transcription. Therefore the distance between the -10 motif and the YycF binding site would be a critical parameter to ensure a contact between SigA RNA polymerase and YycF. In this scenario, the unusual spacing between the -10 and -35 motifs would be a feature to ensure YycFG-mediated transcription.

To test this model and to evaluate the contributions of these unusual features to YycFG-mediated transcription, a mutational analysis on expression of the *ydjM* promoter was carried

out. Six base pairs (marked in blue, Table 2.2) were deleted between the -35 and -10 regions of the *ydjM-bgaB* promoter fusion (wild-type) and of the *ydjM1-bgaB* construct (mutated in the YycF binding site), generating promoter fusions *ydjM2-bgaB* and *ydjM3-bgaB* respectively. It is noteworthy that the 6 bp deletion results in two concomitant effects: a canonical spacing between the -10 and -35 motifs is restored, and the distance between the -10 motif and the YycF binding site is altered. The expression profiles of wild-type and mutated promoters are presented in Figure 2.6.

Expression of the wild-type promoter (squares, Figure 2.6) is low throughout the growth cycle and is further reduced when the YycF binding site is mutated (triangles, Figure 2.6), confirming previous results (see Figure 2.5). However, when a canonical 17 base pair spacing is restored in the wild-type promoter (fusion *ydjM2-bgaB*), expression increases significantly throughout the growth cycle (compare diamonds with squares, Figure 2.6). During exponential growth, the wild-type promoter is expressed at 6-8 units of activity whereas expression from the *ydjM2* promoter is approximately 20 units of activity. During the stationary phase, expression of the wild-type promoter increases from 8 to 16 units of activity while expression of the *ydjM2* promoter increases from 20 to ~70 units of activity. These results show that the putative -35 motif present in the *ydjM* promoter region is functional when placed at the canonical position.

To test whether expression of the *ydjM2-bgaB* construct, containing a canonical SigA-type promoter, is still mediated by YycF, one of the YycF binding direct repeats was mutated (shown in green type, Table 2.2), to give fusion *ydjM3-bgaB*. This mutation had no effect on expression: the expression profiles of *ydjM2-bgaB* (YycF binding site intact) and *ydjM3-bgaB* (YycF binding site mutated) were very similar (compare diamonds and circles, Figure 2.6). Since the same mutation was shown to abolish transcription in a wild type promoter (triangles, Figure 2.6), this result shows that the *ydjM* promoter expression is constitutive and independent of YycFG when in the context of a canonical SigA-type promoter.

In conclusion, these results show that in addition to the presence of an intact YycF binding site, the spacing between the YycF binding motif and the SigA-type -10 motif is also crucial for YycF-mediated control of expression of *ydjM*. Although a putative -35 motif can be identified within the *ydjM* promoter region, and appears to be functional when a canonical spacing is restored, whether it plays a role in normal promoter function remains to be investigated.

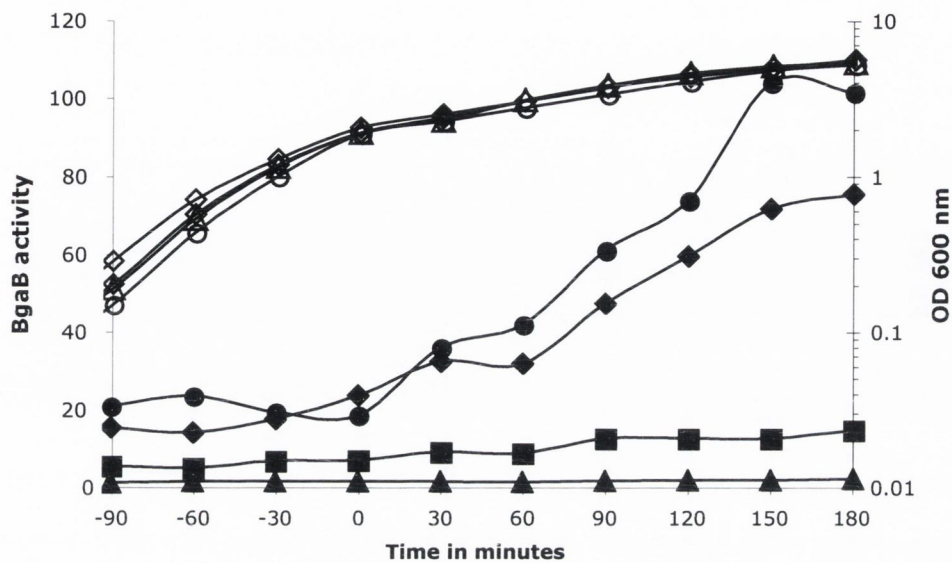


Figure 2.6. Analysis of the expression of wild-type and mutated *ydjM* promoters monitored by transcriptional fusions with the *bgaB* reporter gene. Growth of strains is shown by open symbols and β -galactosidase by closed symbols: squares, BP066 (*ydjM-bgaB*); triangles, BP075 (*ydjM1-bgaB*); diamonds, SQ1 (*ydjM2-bgaB*) and circles, SQ3 (*ydjM3-bgaB*). Time zero indicates the point of transition between the exponential and stationary phases of growth. This experiment was performed three times with comparable results, and a representative profile is shown.

2.2.1.5. Expression of the autolysin *LytE* is controlled by YycFG

In addition to the microarray analysis performed on YycFG-depleted cells, a bioinformatics approach was used to identify additional genes regulated by YycFG. It was reasoned that some YycF-controlled genes might have been missed in the array analysis if their transcript level was not sufficiently depleted at the point of growth cessation.

The bioinformatics analysis was based on three characteristics of YycFG regulated genes, which have been established in previous work as well as in this work: (i) the YycFG regulon comprises a significant number of genes involved in cell wall metabolism; (ii) promoter regions of YycF-activated genes often have a canonical SigA-type -10 motif but not a -35 at the canonical position and (iii) promoter regions of YycF-regulated genes have at least one copy of the YycF binding motif. Such criteria were used to locate typical YycF-controlled SigA-type promoters in genes involved in cell wall metabolism. The *lytE* gene, which encodes a DL-endopeptidase-type autolysin (Ishikawa *et al.*, 1998), was found to fulfill all three criteria: (i) it encodes a cell wall autolysin, implicated in regulation of cell wall metabolism (Carballido-Lopez *et al.*, 2006; Ishikawa *et al.*, 1998); (ii) its promoter region contains a canonical SigA -10 motif but a non-canonical -35 (CTGATG) motif with a 12 bp spacing between the two (Table 2.2); (iii) its promoter contains a putative YycF binding site, positioned upstream of the -35 region.

To test whether YycFG regulates *lytE* expression, a Northern blot analysis was performed on RNA harvested from cultures of wild-type strain 168 and of strain AH9912 (P_{spac} *yycFGHIJK*) grown in the presence and in the absence of IPTG. Strains were cultured in LB medium. The growth profiles of the three cultures are shown in Figure 2.7A. Northern analysis (Figure 2.7B) shows that in wild-type strain 168 *lytE* transcript levels are low during exponential growth (T_{60} and T_{80}) and increase upon entry into stationary phase (T_{120} and T_{140}). This profile differs from the one observed by Ishikawa *et al.* (1998) in wild type cells grown in SM sporulation medium, where expression was turned off upon entry into stationary phase. More recent publications have shown that the master regulator of sporulation, Spo0A, is a repressor of *lytE* expression (Fujita *et al.*, 2005; Kodama *et al.*, 2007; Molle *et al.*, 2003). Therefore the discrepancy observed between growth in LB and growth in sporulation medium is likely to be due to the activation of Spo0A upon entry into stationary phase in SM medium, which results in repression of *lytE* transcription. In strain AH9912 grown in the presence of 1mM IPTG, *lytE* transcript levels are increased when compared to the levels observed in wild-type cells during exponential growth (T_{60} and T_{80} , Figure 2.7B), indicating positive regulation by YycF. It is interesting to note that control of *yvcE* expression by YycFG is also most

evident during early exponential growth (see Figure 2.2). In strain AH9912 grown without IPTG, *lytE* transcript levels are very similar to those observed in wild-type cultures during exponential phase and transitional phase [compare $T_{60} - T_{100}$ in 168 and AH9912 (-IPTG), Figure 2.7B], but decrease during stationary phase [see time points T_{120} and T_{140} of AH9912 (-IPTG), Figure 2.7B]. A duplicate gel using the same RNA preparations showed a large increase in *yoeB* transcript levels in the AH9912 (-IPTG) culture in the samples where the *lytE* transcript levels had decreased, verifying that YycFG depletion had occurred and that lowered *lytE* transcript was not due to RNA degradation (data not shown). The *lytE* expression profile observed in this Northern Blot experiment is consistent with the result of the array analysis, where the gene did not display significant changes in transcript levels between YycFG replete and YycFG depleted conditions (for array data see Supplementary Material in Bisicchia *et al.*, 2007). The RNA used for microarray analysis was harvested at the point of growth cessation upon YycFG depletion. The Northern blot experiment shows that at this time point (corresponding to T_{100} in Figure 2.7B) *lytE* transcript is not decreased upon YycFG depletion [compare T_{100} in AH9912 (+IPTG) and AH9912 (-IPTG), Figure 2.7B], a result consistent with the microarray data.

During stationary phase *lytE* transcript levels are comparable in the wild-type strain and in the YycFG replete culture [compare $T_{120} - T_{140}$ in 168 and AH9912 (+IPTG), Figure 2.7B], but are lower in the YycFG depleted culture [see $T_{120} - T_{140}$ in AH9912 (-IPTG), Figure 2.7B]. This result can be interpreted in two different ways. On one hand, the difference in *lytE* transcriptional levels observed in strain AH9912 grown in the presence and in the absence of IPTG might indicate a YycFG-dependent induction of transcription during stationary phase. On the other hand, expression levels are comparable in the wild-type strain and in the strain over-expressing YycFG, suggesting that the observed induction of transcription upon entry into stationary phase might be YycFG-independent and might be due to a further unknown regulator that activates *lytE* during stationary phase. In this second scenario, the lower transcriptional levels observed in strain AH9912 grown without IPTG might be explained by the fact that the YycFG-depleted culture never reached the stationary phase (see Figure 2.7A, open circles). In order to discern between these two hypotheses and to test whether YycF controls *lytE* expression during stationary phase, expression of the *lytE* promoter was investigated using *lytE-bgaB* transcriptional fusions, both in a wild-type background (strain BP118) and in a strain expressing the *yycFG* operon from the IPTG inducible P_{spac} promoter (strain BP120). The results of the expression analysis are presented in Figure 2.7C. Transcriptional levels of *lytE* were very low during exponential growth in a wild-type

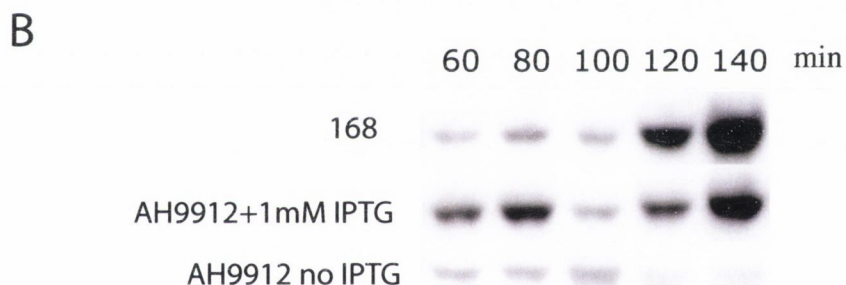
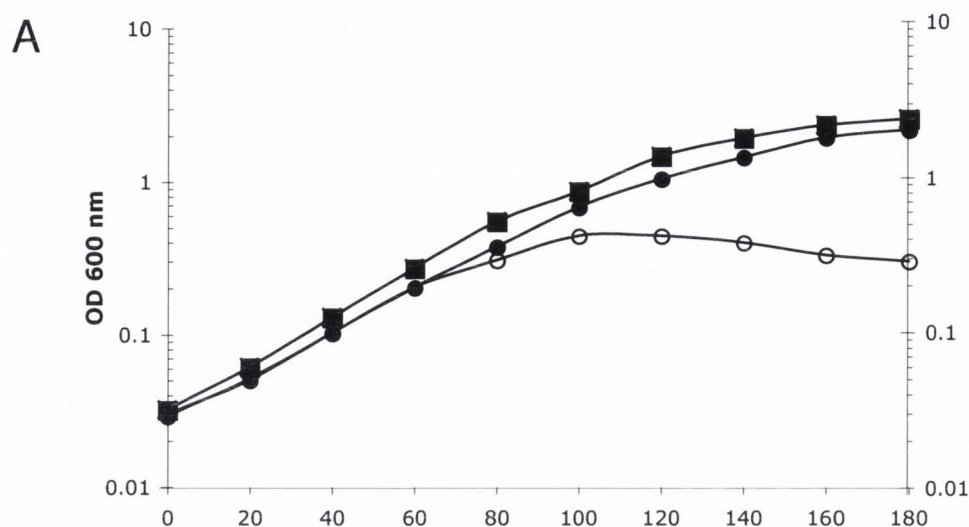
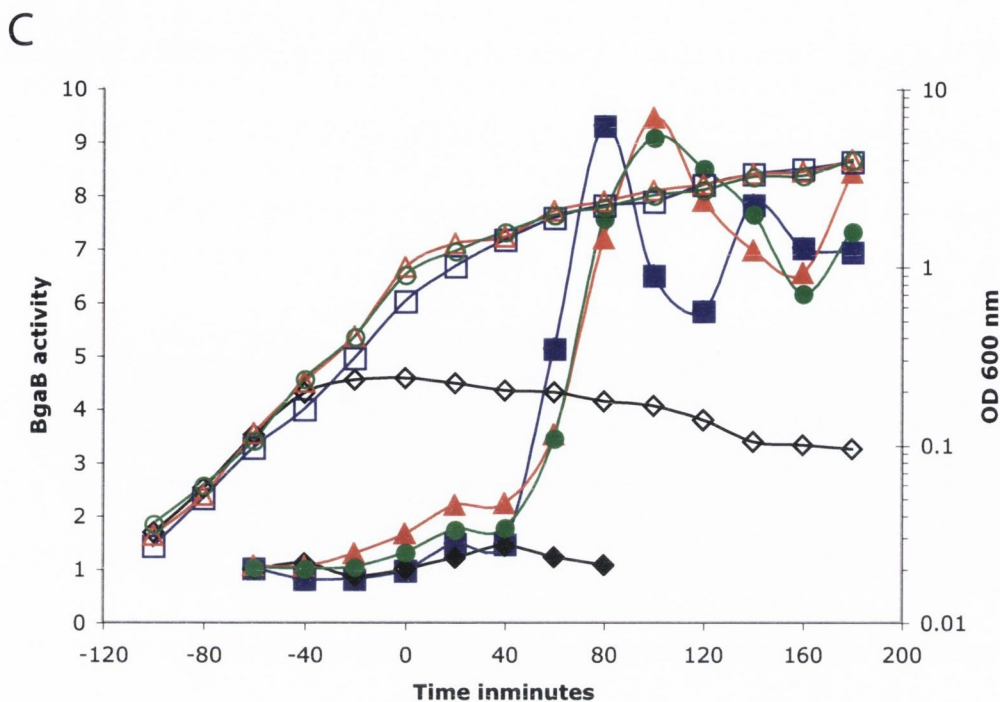


Figure 2.7A,B. Analysis of YycFG control of *lytE* expression. (A) Growth profiles of strains 168 (wild type, squares) and AH9912 (P_{spoC} *yycFGHIJK*) grown in the presence (closed circles) and absence (open circles) of 1mM IPTG. Strains were cultured in LB medium and growth was monitored turbidimetrically. Time is indicated in minutes after inoculation. (B) Northern analysis of *lytE* transcript levels: total RNA was made from the cultures shown in Figure 5A with cells harvested at the time points indicated (numbered according to the transition phase T_0). Twenty micrograms of total RNA was loaded onto each lane.



D



Figure 2.7C,D. (C) Growth and β -galactosidase expression profiles of strains carrying *lytE-bgaB* transcriptional fusions. Growth of strains is shown by open symbols and BgaB specific activity by closed symbols. Strain BP118 (*trpC2 amyE::PlytE::bgaB Cm^r*, squares) and strain BP120 [*trpC2 amyE::PlytE::bgaB Cm^r Δ yycF::pAH022 (P_{spc} yycFGHIJK; P_{yycF} lacZ *erm^r*)*] were cultured in LB broth. Strain BP120 was cultured in the absence of IPTG (black diamonds) and in the presence of 100 micromolar IPTG (red triangles) and 1mM IPTG (green circles). Time zero indicates the point of transition between the exponential and stationary phase of growth. This experiment was performed three times with comparable results, and a representative profile is shown. (D) Gel mobility shift analysis of the *lytE* promoter by purified phosphorylated YycF protein. 2ng of a DNA fragment containing the *lytE* promoter was incubated in the absence (lane 1) or presence of 1, 5 and 10 μ M YycF~P (lanes 2 to 4 respectively) and separated on an acrylamide gel. The wedge indicates increasing YycF~P concentrations.

background, but increased significantly during stationary phase (strain BP118, closed blue squares). Low levels of expression were observed in strain BP120 grown in the absence of IPTG throughout the growth (closed black diamonds); however in this case cells lysed before reaching the stationary phase. During stationary phase expression levels were comparable for strain BP118 (closed blue squares) and for the IPTG-dependent strain BP120 grown in the presence of 100 μ M IPTG (closed red triangles) or 1mM IPTG (closed green circles), showing that altered YycFG levels did not affect *lytE* expression during stationary phase. These results indicate that the post-exponential induction of *lytE* expression is YycFG-independent and that the decreased level of *lytE* transcript observed in the Northern analysis is due to the cultures not reaching a normal stationary growth phase.

To establish whether YycF regulates expression of *lytE* directly, gel shift analysis was performed on a biotinylated DNA fragment spanning the *lytE* promoter region. Increasing amounts of purified phosphorylated YycF proteins were used in the experiment, and poly [d(I-C)] was added to each binding reaction to ensure specificity of interaction. The results are shown in Figure 2.7D. The *lytE* promoter fragment shifted upon addition of the YycF~P protein, being almost completely shifted with 5 μ M YycF~P (lane 3, Figure 2.7D). YycF binding to the labeled DNA fragment could be competed by addition of a 100 fold excess of non-labelled DNA fragment, confirming the specificity of interaction (data not shown). In summary, these data show that YycFG directly regulates *lytE* expression during the exponential phase of growth.

2.2.2. INVESTIGATION OF THE NATURE OF YycFG ESSENTIALITY

The nature of YycFG essentiality in *B. subtilis* has not been established to date. Previous studies have shown that YycFG directly regulates the putative autolysin *yocH* (Howell *et al.*, 2003; 2006), the essential cell division genes *ftsAZ* (Fukuchi *et al.*, 2000) and the essential teichoic acid biosynthetic genes *tagAD* (Howell *et al.*, 2003; 2006). However, none of these genes accounts for the essential nature of YycFG (Fukuchi *et al.*, 2000; Howell *et al.*, 2006). In this work five additional members of the YycFG regulon have been identified using microarray and bioinformatics approaches: the positively regulated genes *yvcE*, *ydjM* and *lytE* and the negatively regulated *yjeA* and *yoeB*. A genetic analysis was undertaken to investigate whether the newly identified members of the YycFG regulon contribute to the essentiality of this TCS in *B. subtilis*, singly or in combination. The strategy adopted was to attempt to delete

each of the genes positively regulated by YycFG individually through a double cross-over event, and then to try and combine the different deletions.

2.2.2.1. Construction of strains mutated in one YycFG-regulated gene

It was possible to construct strains carrying null mutations in any **one** of the genes *yocH*, *ydjM*, *yvcE* and *lytE*, namely strains AH023 (*yocH::kan^r*), BP068 (*ydjM::tet^r*), BP079 (*yvcE::spc^r*) and L16638 (*lytE::Cm^r*) [see Strain Construction; strain AH023 was constructed by A. Howell; strain L16638, was a gift from Philippe Margot (Margot *et al.*, 1999)]. All the null mutant strains were viable, showing that none of these genes is essential, confirming previous results (Kobayashi *et al.*, 2003).

2.2.2.2. Construction of strains deleted in two YycFG-regulated genes

In order to determine whether more than one of the genes regulated by YycFG contributes to its essentiality, we attempted to generate strains carrying null mutations in two of these genes. If deletion of two genes is lethal to the cell, no colonies are expected to grow when a strain carrying a null mutation in one of the genes is transformed with chromosomal DNA from a strain carrying a null mutation in the second gene. However, a small number of colonies may be obtained: such colonies may arise from suppressor mutations, which occur at a very low frequency. Conversely, many transformants are expected when a combination of null mutations is not deleterious to the cell. We carried out transformation experiments in a manner that distinguished real transformants from strains carrying suppressor mutations. It is crucial to observe that a very small number of transformants can be indicative of two distinct situations: (i) a combination of null mutations that is lethal to the cell (in this case the transformants will be suppressors) or (ii) a recipient strain with very low competence levels. For this reason, competence is a critical parameter to be considered when performing these experiments. For each recipient strain, carrying a null mutation in one YycFG-regulated gene, we calculated both the competence level and the frequency of integration into the chromosome of a DNA construct engineered to delete a second gene. In order to do so, the following control experiments were carried out: (i) each strain carrying a null mutation was transformed with a DNA construct integrating at a chromosomal locus for which no adverse selection is expected. Chromosomal DNA extracted from strain BP081, which carries a *htrA::spc^r* construct that integrates at the non-essential *htrA* locus, was chosen as a control. Competence level of recipient strains was measured by comparing the number of colonies obtained when transforming with equal amounts of BP081 chromosomal DNA. (ii) Each strain carrying a null

mutation was also transformed with a plasmid or with chromosomal DNA containing a construct engineered to delete a different YycFG-controlled gene. The frequency of integration of the deletion construct was calculated by dividing the number of colonies obtained when transforming competent cells with such construct by the number of colonies obtained when transforming the same competent cells with the control BP081 chromosomal DNA. (iii) The wild-type strain 168 was transformed in parallel to strains containing null mutations in order to compare competence levels and frequencies of integration of deletion constructs.

Strains with the following combinations of null mutations were obtained: BP087 (*yocH::kan^r*, *yvcE::spc^r*); BP071 (*yocH::kan^r*, *ydjM::tet^r*); BP083 (*yvcE::spc^r*, *ydjM::tet^r*); BP122 (*lytE::Cm^r*, *yocH::kan^r*) and BP123 (*lytE::Cm^r*, *ydjM::tet^r*). Interestingly, competence level of the recipient strain AH023 (*yocH::kan^r*) was seven fold reduced with respect to the wild-type. However, all transformants were obtained at a frequency high enough to exclude the occurrence of strains carrying suppressor mutations. Many attempts to construct a strain with null mutations in both *yvcE* and *lytE* (both encoding endopeptidase-type autolysins) failed. These transformation experiments were conducted in a controlled way as described above, but because of the different antibiotic cassettes carried by the recipient strain, this time competence levels of the wild-type 168 strain and of the BP079 (*yvcE::spc^r*) strain were measured by transforming them with equal amounts of BP066 chromosomal DNA, which carries a *ydjM-bgaB::Cm^r* construct that integrates at the non-essential *amyE* locus. Repeated transformation experiments showed that strain BP079 (*yvcE::spc^r*) was 7 fold less competent than the wild-type, a result similar to what was observed for strain AH023 (*yocH::kan^r*). Attempts to generate a double mutant deleted in both *yvcE* and *lytE* by transformation of strain BP079 (*yvcE::spc^r*) with 100 nanograms of chromosomal DNA from strain L16638 (*lytE::Cm^r*) resulted in only three colonies. Such colonies were tested for congression. Results showed that the three colonies carried the incoming selected mutation, but that the null mutation in the recipient strain had reverted to wild-type, presumably by congression. This result indicates that either YvcE(CwlO) or LytE is required for cellular growth in *B. subtilis*.

2.2.2.3. Lateral cell wall synthesis and cell elongation of *B. subtilis* requires an essential endopeptidase-type activity, which can be performed either by LytE or by YvcE(CwlO)

In order to verify that the *yvcE* and *lytE* genes cannot both be inactivated, strain BP115 (*lytE::Cm^r* *P_{xyt}yvcE erm^r*) was constructed, where expression of *yvcE* is inducible by xylose in

a *lytE* null mutant background. This strain grew on LB agar only in the presence of xylose, showing that either *lytE* or *yvcE* is required for normal growth of *B. subtilis*.

Strain BP115 (*lytE*::*Cm^r* *P_{xyt}* *yvcE*) was grown also in LB broth in the presence (closed squares) and absence (open squares) of xylose: growth profiles are shown in Figure 2.8A. Strains 168 (diamonds), L16638 (*lytE*::*Cm^r*, triangles) and BP079 (*yvcE*::*spc^r*, circles) were grown in parallel as controls. This experiment was repeated three times with comparable results and a representative growth profile is displayed. Strain BP079 (*yvcE*::*spc^r*) grew similarly to the wild-type strain 168 (compare blue diamonds and circles) while strain L16638 (*lytE*::*Cm^r*) displayed a growth defect (red triangles). Strain BP115 (*lytE*::*Cm^r* *P_{xyt}* *yvcE*) grew similarly to strain L16638 (*lytE*::*Cm^r*) in the presence of 1% xylose, consistent with both being mutant for the *lytE* gene (compare red closed squares and triangles). However, when BP115 was grown in the absence of xylose inducer (red open squares), growth rapidly ceased but the cells did not lyse. This result showed that either *yvcE* or *lytE* is required for normal growth of *B. subtilis*, both on LB agar and in LB broth.

The phenotype of the cells from all cultures was examined by fluorescence microscopy: cells were harvested at the point of the growth curve indicated by an arrow (Figure 2.8A), fixed in paraformaldehyde and stained with 4',6-diamidino-2-phenylindole (DAPI) in order to visualize the nucleoids, as described in Materials and Methods. Figure 2.8B shows the cellular morphology of strain L16638 (*lytE*::*Cm^r*) and strain BP115 (*lytE*::*Cm^r* *P_{xyt}* *yvcE*) grown with and without xylose inducer. Strains L16638 and BP115 (grown with 1% xylose) displayed similar morphology, with regular rod-shaped cells of normal length (Figure 2.8B, panels a and c), all containing nucleoids, as viewed by DAPI staining (Figure 2.8B, panels b and d). Strain BP115 grown without xylose inducer displayed a very different phenotype, with very short cells (Figure 2.8B, panels e and f). Cell length was measured in 50 randomly chosen cells from all the cultured strains displayed in Figure 2.8A: cells from strain BP115 grown without inducer were 1.5 μm $\pm$ 0.33 long, while cells from all the other strains [(168 (wild-type), L16638 (*lytE*::*Cm^r*), BP079 (*yvcE*::*spc^r*) and BP115 grown with xylose inducer] were of similar length (3.5 μm $\pm$ 0.5). In addition, cells from strain BP115 grown without xylose were aberrant in that some were empty, flat looking and devoid of nucleoids, and some had a bent shape (Figure 2.8B, panels e and f). At a higher magnification this aberrant phenotype was more evident: a cluster of cell images is displayed in Figure 2.8B, panel g. Cells were irregularly shaped, with bulging and occasional bent cylinders, and some (indicated by arrows) were flat-looking and empty. This phenotype suggested that cells were defective in lateral cell wall synthesis and cell elongation, and that an endopeptidase-type activity, performed either

A

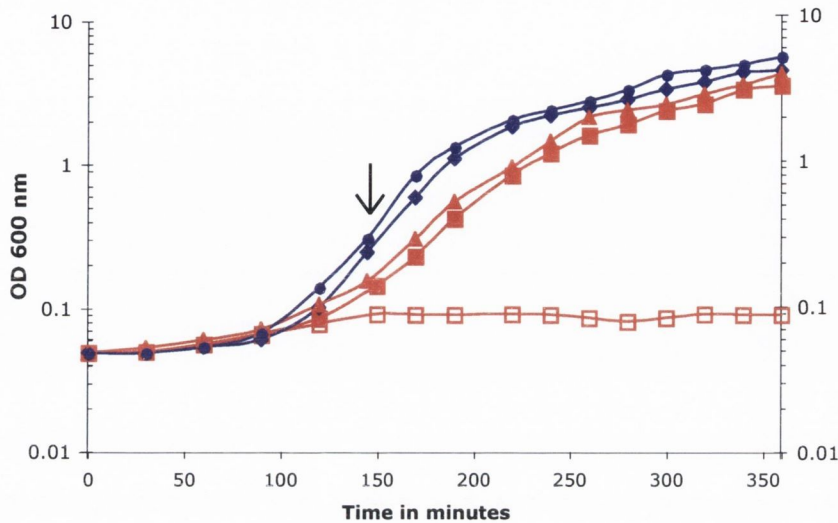


Figure 2.8 A,B. Analysis of the cellular roles of *LytE* and *YvcE* (CwlO).

(A) Growth profiles of strains depleted for *LytE*, *YvcE*(CwlO) or both autolysins: diamonds, 168 (wild type); triangles, L16638 (*lytE::Cm^r*); circles, BP079 (*yvcE::spc^r*); closed squares, BP115 (*P_{xy}l yvcE, lytE::Cm^r*) grown in the presence of 1% xylose; open squares, BP115 (*P_{xy}l yvcE, lytE::Cm^r*) grown in the absence of xylose. Strains were cultured in LB medium starting from freshly streaked colonies and growth was monitored turbidimetrically. This experiment was performed three times with comparable results and a representative profile is displayed. Time is indicated in minutes after inoculation. The arrow indicates the point at which cells were harvested for microscopic examination.

(B) Visualization of cell morphology and cell wall synthesis by microscopy. Cells were prepared as described in experimental procedures and viewed by phase contrast (panels a, c, e, g cluster) and by phase contrast with a DAPI stained image superimposed (panels b, d, f). Peptidoglycan synthesis was visualized by staining with fluorescein-labeled vancomycin (panels h, i, j). The red line indicates scale, with the length shown above.

B

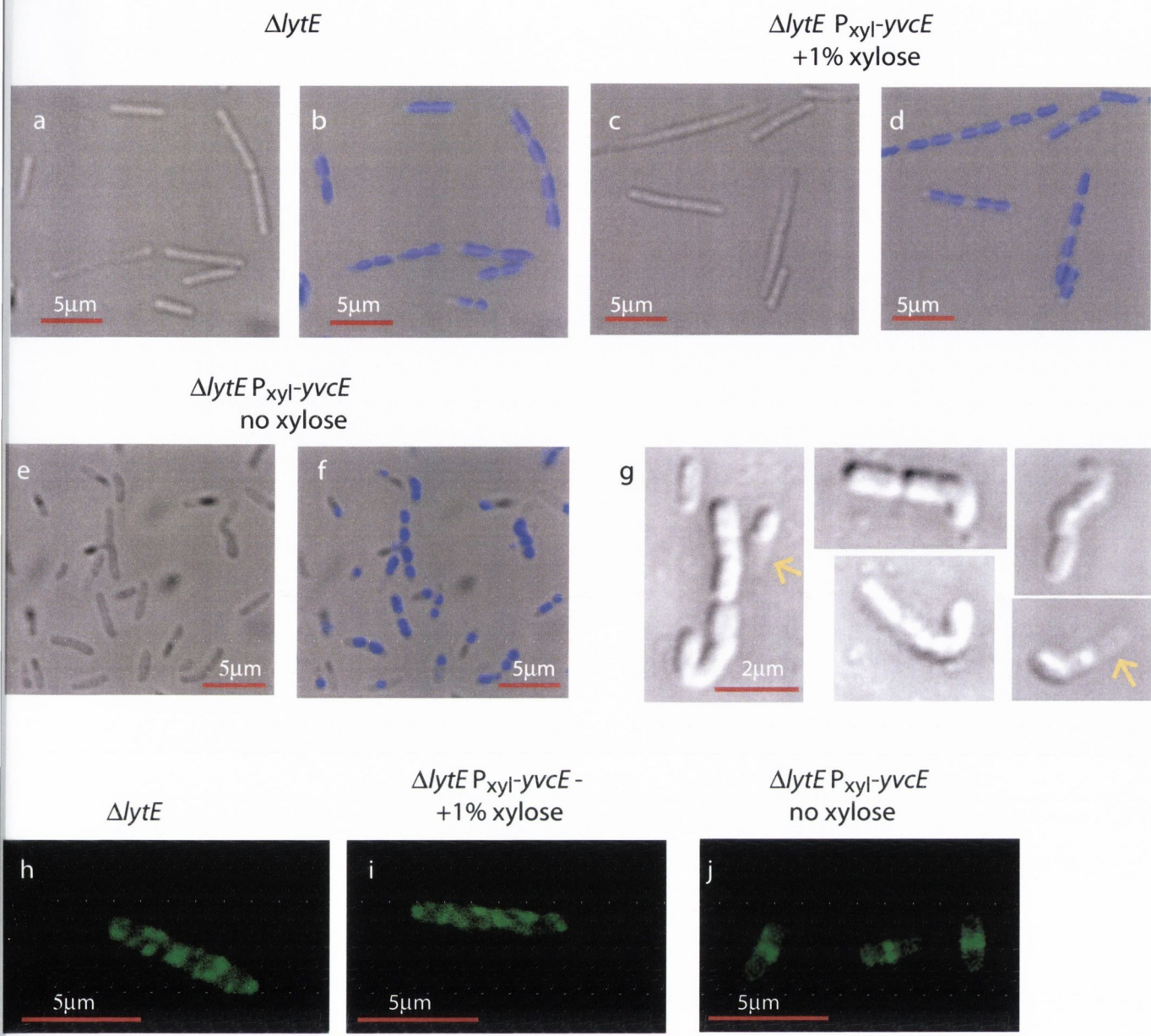


Fig 2.8B.

by LytE or by YvcE(CwlO), might be required for elongation of the cell cylinder during growth.

To test this hypothesis we examined cell wall synthesis in these strains using fluorescein-labelled vancomycin, a compound that binds to the D-Ala-D-Ala residues of Lipid II and blocks the transpeptidation reaction of peptidoglycan synthesis (Daniel and Errington, 2003; Kahne *et al.*, 2005). Cells of the parent strain L16638 and of strain BP115 grown with xylose inducer showed patches of fluorescent vancomycin perhaps arranged in a helical pattern along the length of the cell (Figure 2.8B, panels h and i), in a way similar to what has been observed in normally elongating wild-type cells (Daniel and Errington, 2003). However no labeling was observed along the truncated cell cylinders of strain BP115 grown without xylose, and peptidoglycan synthesis appeared to be confined to the septal region (Figure 2.8B, panel j). This result confirmed the involvement of LytE and YvcE(CwlO) in lateral cell wall synthesis and cell elongation.

From the above results we conclude that *B. subtilis* has an essential requirement for an autolytic endopeptidase-type activity which can be provided either by LytE or YvcE(CwlO), such activity playing an important role in lateral cell wall synthesis and cell elongation during growth.

2.2.2.4. YycFG-mediated control of *lytE* and *yvcE* transcription is not the sole reason for the essentiality of this TCS in *B. subtilis*

Having identified two genes positively regulated by YycFG, *yvcE* and *lytE*, one or other of which is required for growth, we reasoned that control of expression of these two genes might be the sole reason for YycFG essentiality. To test this hypothesis we adopted two alternative experimental approaches.

A first strategy involved attempting to delete the *yycFG* genes from a strain constitutively expressing both *yvcE* and *lytE*. Strain BP113 was constructed in which *yvcE* is expressed from the xylose inducible P_{xyl} promoter, and the *lytE* gene is expressed from the IPTG inducible P_{spac} promoter ($P_{spac}lytE P_{xyl}yvcE$). Crucially, from the data presented in the previous paragraphs it is clear that the P_{xyl} promoter is able to direct expression of the YvcE protein at levels which are high enough to sustain growth in the presence of inducer [see growth profile of strain BP115 (*lytE::Cm^r P_{xyl}yvcE*), Figure 2.8A]. Furthermore, the P_{spac} promoter appeared to produce the LytE protein at levels sufficient for growth even in the absence of IPTG inducer [see in previous paragraph construction of strains BP111 (*yvcE::spc^r P_{spac}lytE erm^r*) and BP112 (*yvcE::spc^r P_{spac}lytE erm^r pMAP65 kan^r neo^r*)]. Therefore if control of *yvcE* and *lytE*

expression were the sole reason for YycFG essentiality, it should be possible to delete the *yycFG* genes from strain BP113, which expresses *yvcE* under P_{xyl} control and *lytE* under P_{spac} control. To test this hypothesis strain BP113 was transformed with DNA extracted from strain BP056 (*yycFG::neo^r lacA::P_{spac}yycG amyE::P_{xyl}yycF*), selecting for *neo^r* and hence *yycFG* deletion. As a positive control, the same chromosomal DNA was transformed into strain BP053 (*lacA::P_{spac}yycG amyE::P_{xyl}yycF*), which expresses the *yycG* and *yycF* genes under IPTG and xylose inducible promoters. Competence levels of the two recipient strains were measured by transforming them with equal amounts of chromosomal DNA extracted from strain BP082 (*glu::kan^r*): the two strains were found to be equally competent. One, 10 or 100 ng of BP056 chromosomal DNA were used to transform both strains, and cells were plated on LB agar containing neomycin 3μg/ml, 1% xylose and 1mM IPTG. However, while ~200 transformants per ng of chromosomal DNA were obtained when transforming strain BP053, no colonies were obtained upon transformation of strain BP113 with up to 100 ng of DNA, indicating that the *yycF* and *yycG* genes cannot be deleted from the chromosome even when both *lytE* and *yvcE* are constitutively expressed. This result indicates that YycFG-controlled expression of *yvcE* and *lytE* is not the sole reason for its essentiality.

A second experimental approach to test the contribution of the autolysins LytE and YvcE to the essentiality of YycFG involved the construction of a strain expressing the *yvcE* gene under P_{xyl} control and the TCS *yycFG* under P_{spac} control. We reasoned that if YycFG-controlled expression of the combination of the two genes *yvcE* and *lytE* accounted for the essentiality of this TCS, it should be possible to deplete for YycFG when expressing one of the two genes constitutively. This second approach presented two main advantages. Firstly, rather than being based on negative data coming from a transformation experiment like the one described above, it was based on the analysis of the growth profile of a strain carrying *yycFG* under P_{spac} control, a profile which has been well characterized both in this and previous work (Figure 2.1 of this work, Fabret and Hoch, 1998; Howell *et al.*, 2003). Secondly, this experimental approach allowed us to assess the contribution of the *yvcE* gene alone to the essentiality of YycFG, by testing the effect of constitutive *yvcE* expression on the growth cessation and lysis phenotypes normally observed upon YycFG depletion. Strain BP125 (P_{spac} *yycFGIJK* P_{xyl} *yvcE*) was constructed by transforming strain BP080 (*thrC::P_{xyl}yvcE* *spc^r*) with plasmid pAH022 (P_{spac} *yycFGIJK* *erm^r*) and its growth profile was examined with and without xylose and IPTG inducers as appropriate (Figure 2.9). When cultures were grown with neither inducer, the usual growth cessation and cell lysis phenotypes were observed (Figure 2.9, triangles). However, when cultures were grown without IPTG but with increasing

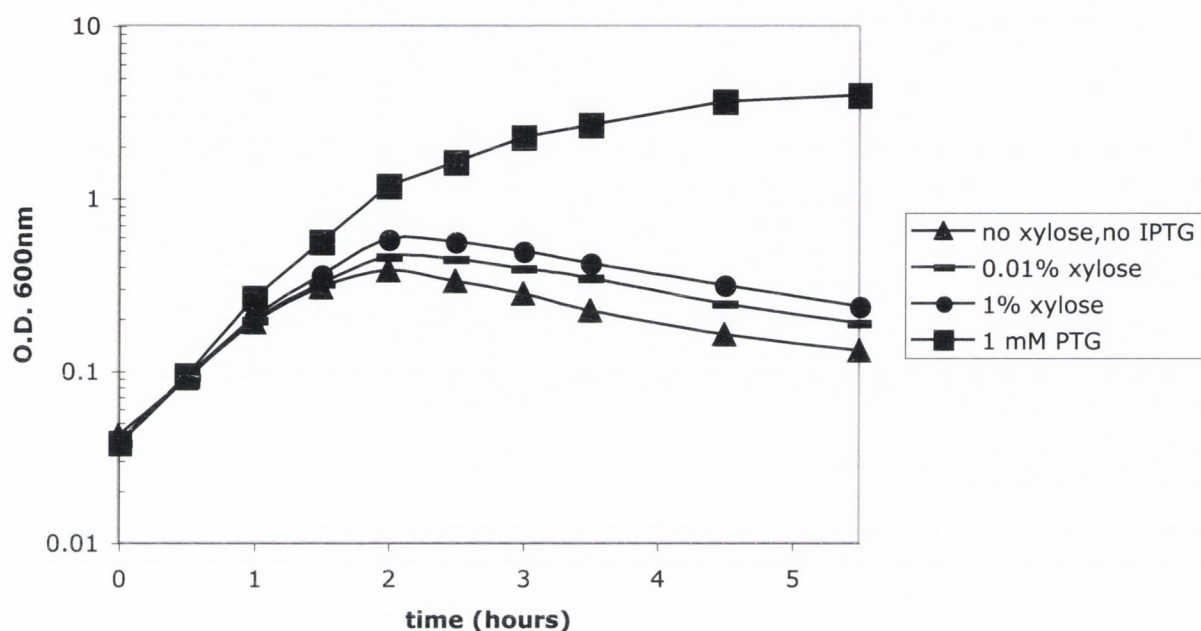


Figure 2.9. Effect of constitutive YvcE(CwlO) expression on the growth of cells upon YycFG depletion. Strain BP125 ($P_{gpx} yycFGHIJK$, $P_{xyI} yvcE$) was grown in LB medium in the presence of 1mM of IPTG only (squares); without IPTG or xylose (triangles); in the absence of IPTG but with 0.01% xylose (rectangles) and in the absence of IPTG but in the presence of 1% xylose (circles). Time is indicated in minutes after inoculation. This experiment was performed three times with comparable results and a representative profile is displayed.

concentrations of xylose, the phenotype was ameliorated, with growth cessation and lysis occurring later and to a lesser extent, in a xylose-dependent manner (compare rectangle and circles, Figure 2.9). This result confirmed that YycFG-mediated control of *yvcE* and *lytE* does not account for the essentiality of this TCS in *B. subtilis*; however, it is concluded that reduced expression of *yvcE* does contribute to the growth cessation and lysis phenotype of YycFG depleted cells, even though there must be additional contributory factors.

2.2.2.5. YycFG controlled expression of a triplet of genes, encoding the autolysins YocH and YvcE(CwlO) and the protein of unknown function YdjM, contributes to the essentiality of this TCS in *Bacillus subtilis*

The results presented in the previous paragraph have established the partial contribution of YycFG-mediated control of *yvcE* to the lysis phenotype observed upon YycFG-depletion. In order to assess what other YycFG-controlled genes might contribute to this phenotype and hence to the essentiality of YycFG in *B. subtilis*, the construction of strains carrying null mutations in three genes positively regulated by YycFG was attempted.

It was possible to construct strains carrying both the possible combinations of null mutations (excluding the ones where both *yvcE* and *lytE* are deleted). The two strains, namely BP090 (*yvcE::spc'* *ydjM::tet'* *yocH::kan'*) and BP124 (*lytE::Cm'* *ydjM::tet'* *yocH::kan'*) were obtained at very low transformation frequencies.

Strain BP090 was constructed by transforming strain BP083 (*ydjM::tet* *yvcE::spc'*) with chromosomal DNA extracted from strain AH023 (*yocH::kan'*), selecting for kanamycin resistance. Repeated transformation experiments showed that the recipient strain BP083 was four fold less competent than the wild-type 168 strain, as measured by transformation with chromosomal DNA extracted from strain BP082 (*glu::kan'*). Furthermore, the frequency of integration of the *yocH::kan'* construct into the BP083 recipient strain was up to 30 fold reduced with respect to the frequency of integration of the same construct into the wild-type strain 168, and the number of transformants obtained did not increase with increasing amounts of the transforming DNA. Nevertheless, a low number of transformants was obtained, characterized by a small colony phenotype (Figure 2.10).

These transformants were stabbed onto LB agar containing kanamycin, spectinomycin or tetracycline, in order to test for congression (Figure 2.10). Some colonies (8% of total transformants) were of normal size, and were found to have undergone congression, being resistant to only two of the three antibiotics (see Figure 2.10, 2nd day, colony number 1). Interestingly, all the transformants in which congression had occurred had acquired a wild-

type copy of the *ydjM* gene. The majority of transformants (92%) were confirmed to be resistant to all the three antibiotics. Colonies were small and irregularly shaped, with asymmetrical edges probably originated from individual colonies growing out of the stabs, a phenotype suggestive that the observed growth was due to accumulation of suppressor mutations (Figure 2.10, 2nd day). Colonies obtained on the second day were re-stabbed onto new LB agar plates containing the three antibiotics, and the aberrant morphology was retained (Figure 2.10, 3rd day).

Growth in LB broth was tested for strain BP090. Cells were harvested from colonies growing on LB agar, inoculated in LB broth and growth was monitored turbidimetrically. Results are presented in Figure 2.11. No growth was observed for up to 5-6 hours of culture in LB broth for either the primary transformants (Figure 2.11A) or the re-streaked colonies (Figure 2.11B). However, in many cases growth was observed subsequently indicating that the cells were not dead, but again suggesting accumulation of suppressor mutations (Figure 2.11A,B).

Cells were harvested from both the primary and re-streaked colonies, fixed, stained with DAPI and examined using confocal fluorescence microscopy. A comparison between BP090 (*yvcE::spc^r ydjM::tet^r yocH::kan^r*) cells and cells of AH9912 (*P_{spac} yycFGHIK*) grown with YycFG expression (1mM IPTG) and depleted for YycFG (no IPTG) is shown in Figure 2.12. In samples depleted for YycFG, many apparently empty cells were observed that were not seen in YycFG replete samples (see arrowed cells in Figure 2.12f and compare with Figure 2.12c), as previously reported (Fukuchi *et al.*, 2000). Cells from both the primary transformants and re-streaked colonies of strain BP090 displayed an aberrant phenotype: a representative picture of cells from the re-streaked colonies is displayed in Figure 2.12 g-i. Cells were very short and had an aberrant “lumpy”-type shape (Figure 2.12g). In addition, while many such cells contained nucleoids, a significant proportion was devoid of any DAPI stained material (see arrowed cells in Figure 2.12i), reminiscent of YycFG depleted cells (see arrowed cells in Figure 2.12f). When cells from the re-streaked colonies were streaked again onto fresh LB agar plates and grown for a third day, although the colonies retained their aberrant morphology (see Figure 2.10, 3rd day), cells from these colonies looked normal when observed by phase contrast microscopy, again suggesting the accumulation of suppressor mutations.

Cells from both primary transformants and re-streaked colonies of strain BP090 were stained with DAPI and Nile red (in DMSO) at the same time, in order to visualize the nucleoids as well as the cell membranes. Similar results were obtained in both cases, and a

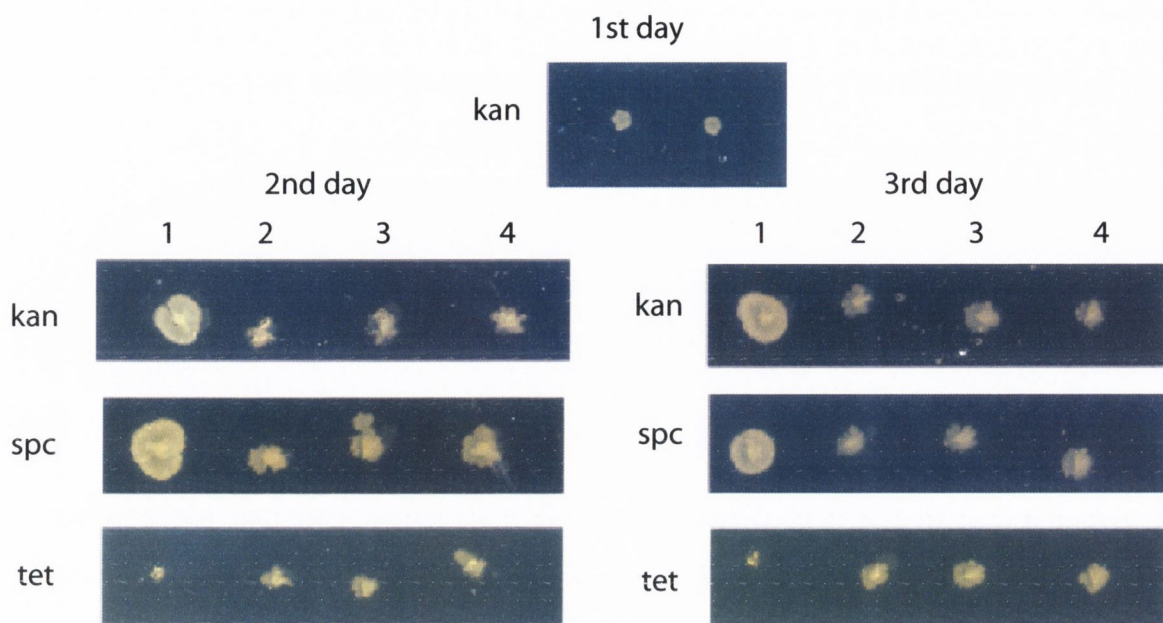


Figure 2.10. Colony morphology of strain BP090 (*yvcE::spc^r ydjM::tet^r yocH::kan^r*).

Transformants (1st day) were selected for resistance to kanamycin only. These colonies were stabbed onto three plates containing kanamycin, spectinomycin or tetracycline (2nd day). 8% of transformant colonies were of normal size, but were found to have undergone congression (see transformant 1 that is resistant to *kan* and *spc* but is sensitive to *tet*, indicating a congression that re-introduced the gene *ydjM*). The majority of transformants (92%) were resistant to the three antibiotics. The colonies were small and irregularly shaped, with asymmetrical edges originated from individual colonies growing out of the stabs. The colonies obtained on the 2nd day were re-stabbed onto new plates containing the three antibiotics, and the aberrant morphology was retained (3rd day). The displayed result is representative of three independent experiments.

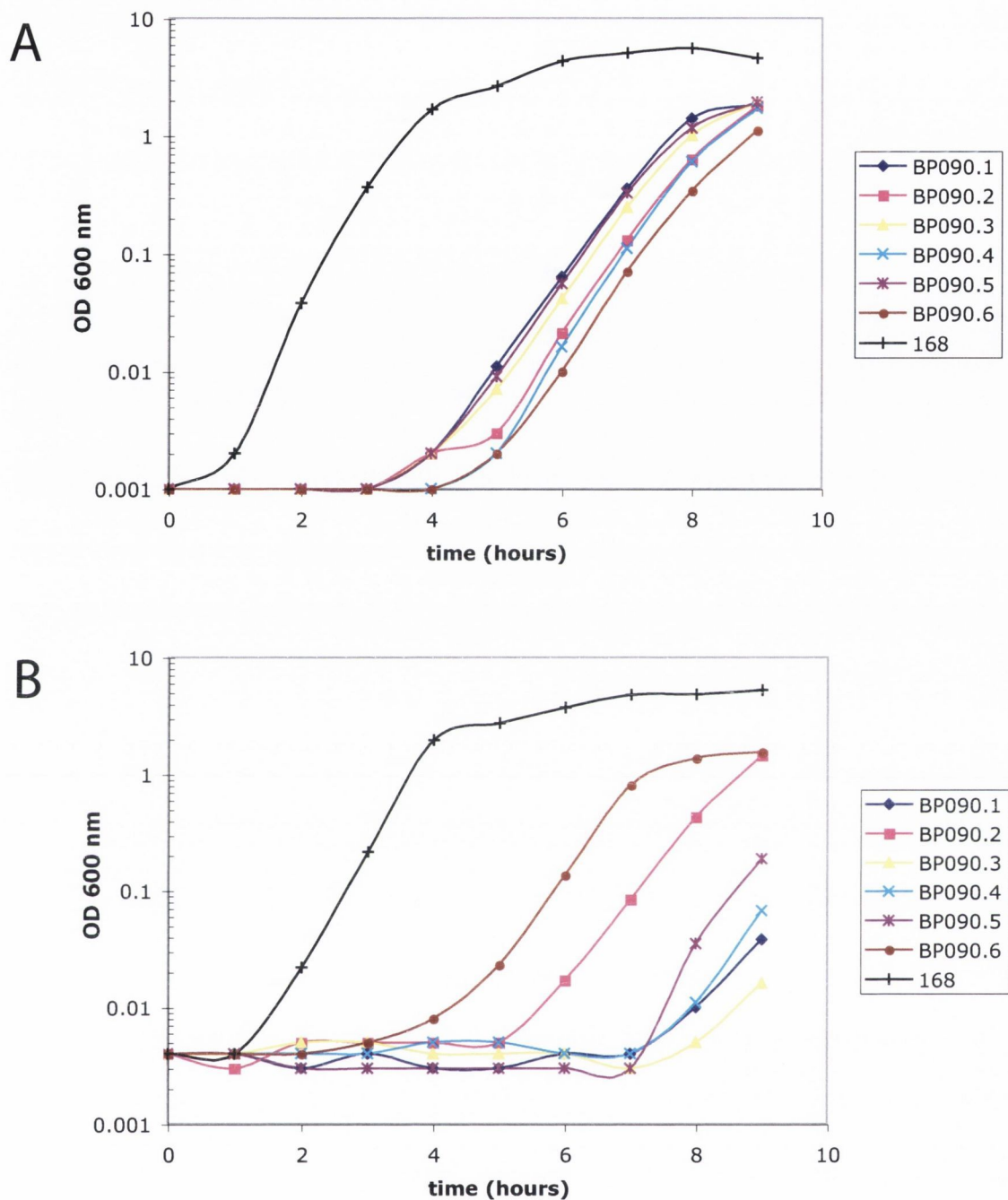


Figure 2.11. Growth in LB broth of strain BP090 (*yvcE::spc^r ydjM::tet^r yocH::kan^r*). Six independent transformants (BP090.1-BP090.6) were chosen for analysis and inoculated in liquid medium. The wild-type strain 168 was cultured in parallel as a control. Cells were taken from primary transformants (A) and from the same transformants stabbed on LB plates and grown for a further day (B). No growth was observed for up to 5-6 hours of culture. However in many cases growth was observed subsequently indicating that the cells were not dead but suggesting accumulation of suppressor mutations. Time is indicated in hours after inoculation.

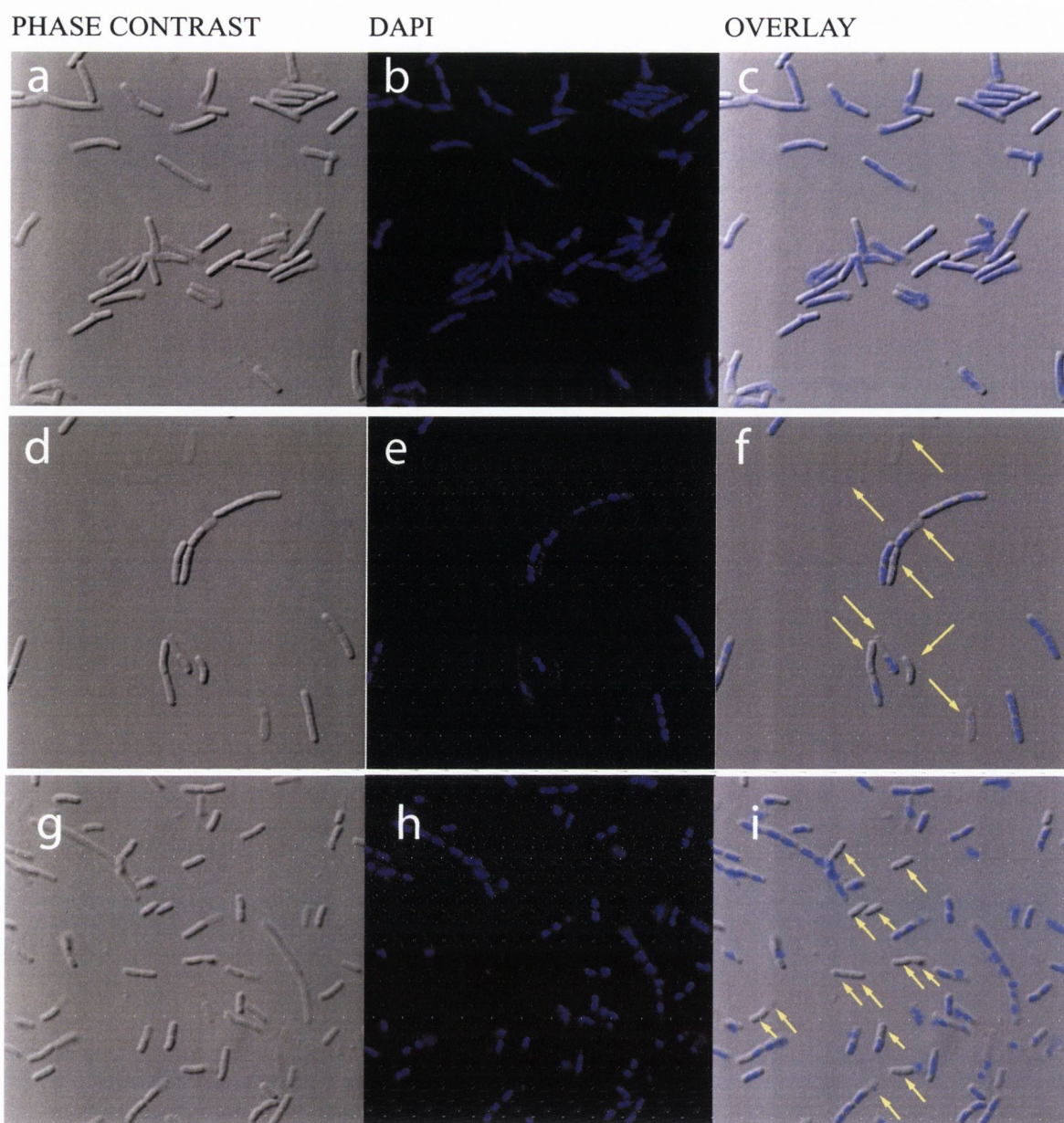


Figure 2.12. Fluorescence microscopic analysis of strains AH9912 ($P_{spac} yycFGHIJK$) and BP090 ($yvcE::spc^r ydjM::tet^r yocH::kan^r$). Cells from both the primary transformants of strain BP090 and from colonies obtained re-streaking them onto new LB plates showed similar morphologies. Cells from re-streaked colonies, displayed in this picture, were harvested directly from LB agar plates. Cells of strain AH9912, grown in the absence (-) of IPTG, were harvested at the point of growth cessation. Cells of strain AH9912 grown in the presence of IPTG were harvested at the same OD_{595} as the cells grown the absence of IPTG, as described in Materials and Methods. Cells were stained with DAPI to visualize the nucleoids. Phase contrast (a, d, g), fluorescence (b, e, h) and combined phase contrast-fluorescence (c, f, i) images are shown for the strains indicated. Arrows indicate cells that are devoid of nucleoids.

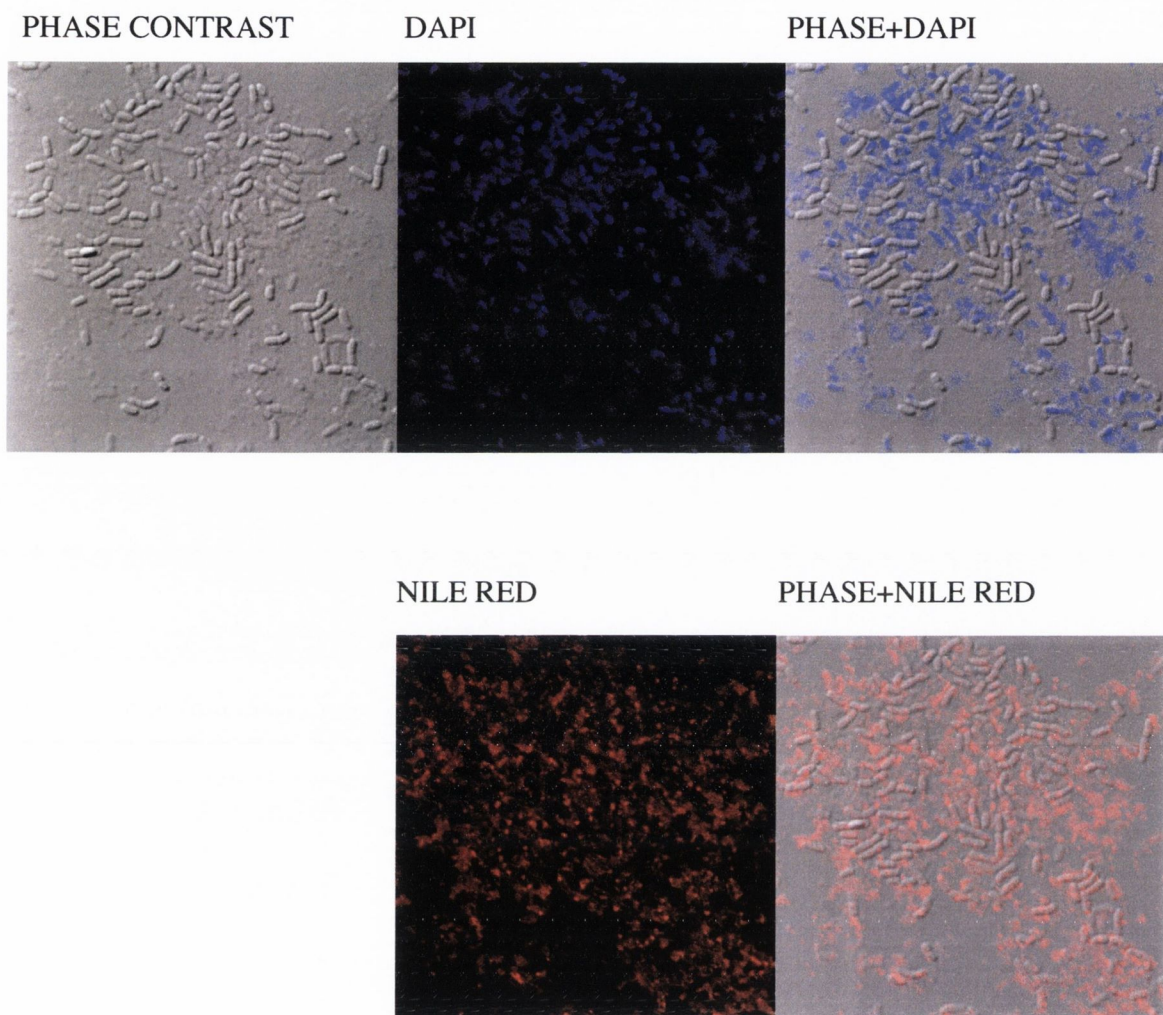


Figure 2.13. Fluorescence microscopic analysis of strain BP090 (*yvcE::spc^r ydjM::tet^r yocH::kan^r*) stained with DAPI in order to visualize the nucleoids and with Nile red in order to visualize the cell membranes. Cells from both primary transformants and from colonies obtained re-streaking them onto new LB agar plates were analyzed, and similar morphologies were observed. Cells from re-streaked colonies are displayed in this picture. Samples were collected from colonies growing on LB agar plates. Phase contrast, fluorescence and combined phase contrast-fluorescence images are shown as indicated.

representative image (from re-streaked colonies) is displayed in Figure 2.13. Smeared DAPI and Nile red staining were observed around the cells, indicating that the cells had lysed during the fixation and the preparation process. This feature was not observed in cell preparations from strain BP090 treated with DAPI only, where the empty cells were a minority of the population, and no smeared DNA material was observed around the cells (see Figure 2.12g-i). Therefore cell lysis is likely to be due to the presence of the solvent DMSO, which is known to affect the structural integrity of fixed cells (Malinin and Malinin, 2004). However, cell preparations from the wild-type strain 168 and from strain AH9912 ($P_{spac} yycFGHIJK$) grown with and without IPTG did not display the same defect when treated with both DAPI and Nile red (in DMSO), indicating that BP090 cells were especially fragile, and prone to lose their cytoplasmic and membrane contents.

The series of experiments to construct and analyse strain BP090 ($yvcE::spc^r ydjM::tet^r yocH::kan^r$) was performed twice, with identical results. In summary, microscopic analysis of the primary transformants of strain BP090 and of colonies re-streaked from them, revealed a complex phenotype. Cells had a “lumpy”-type shape; they were short, occasionally empty and exceptionally fragile and prone to lysis. It is interesting to note that both nucleoid free cells and cell lysis are typical of YycFG depleted cells (this work, Fukuchi *et al.*, 2000, Howell *et al.*, 2003). The fact that both phenotypes are evident in cells mutated in the genes *ydjM*, *yocH* and *yvcE*, coupled with the reduced frequency with which such transformants are obtained, with their inability to grow in LB broth, and with their tendency to accumulate suppressor mutations, suggests that YycFG-mediated control of expression of this triplet of genes during exponential growth contributes significantly to the essentiality of this TCS in *B. subtilis*.

A second strain carrying three null mutation, BP124 ($lytE::Cm^r ydjM::tet^r yocH::kan^r$), was constructed by transforming strain BP071 ($yocH::kan^r ydjM::tet^r$) with chromosomal DNA extracted from strain L16638 ($lytE::Cm^r$), selecting for chloramphenicol resistance. The recipient strain BP071 was found to be as competent as the wild-type strain 168: competence levels were checked by transformation with equal amounts of chromosomal DNA extracted from strain BP066, which carries a $ydjM-bgaB::Cm^r$ construct that integrates at the non-essential *amyE* locus. However, the frequency of integration of the $lytE::Cm^r$ construct into strain BP071 was 30 fold reduced with respect to the frequency of integration of the same construct into the wild-type strain 168. The transformants obtained were stabbed onto LB agar containing chloramphenicol, kanamycin or tetracycline, in order to test for congression mutations. 9% of all colonies were found to have undergone congression, being resistant to only two of the three antibiotics: 5% had acquired the wild-type copy of the *yocH* gene, while

4% had acquired the wild-type copy of the *ydjM* gene. However, the majority of transformants (91%) were confirmed to be resistant to all three antibiotics.

Cells from strain BP124 (*lytE::Cm^r ydjM::tet^r yocH::kan^r*) looked normal when observed by phase contrast microscopy. Therefore simultaneous deletion of the three genes *lytE*, *ydjM* and *yocH* did not have the same deleterious effects found in the triple mutant strain BP090 (*yvcE::spc^r ydjM::tet^r yocH::kan^r*), in which a different triplet of genes was deleted. Since the difference between the two strains is in the DL-endopeptidase encoding gene that is missing from chromosome (*lytE* in the case of BP124 and *yvcE* in strain BP090), this result suggests that the phenotypes observed in strain BP090 are specifically due to the absence of the gene *yvcE* when combined to *yocH* and *ydjM* null mutations. Therefore although the autolysins LytE and YvcE share the same enzymatic activity (they are both DL-endopeptidases, Smith *et al.*, 2000) and they are able to substitute for each-other in performing an essential function in the cell elongation process (this work), their cellular role does not exactly overlap, and they must differ in other functional aspects.

2.2.3. PHENOTYPIC ANALYSIS OF STRAINS MUTATED IN GENES DIRECTLY REGULATED BY YycFG

A phenotypic analysis was undertaken in order to investigate the function of genes directly regulated by YycFG. Growth on LB agar and in LB broth, cell length and sporulation were analyzed in single, double and triple mutant strains deleted in genes positively regulated by YycFG. Results are summarized in Table 2.3. The phenotypes observed in strain BP090 (*yvcE::spc^r ydjM::tet^r yocH::kan^r*), have been extensively discussed above (see Figures 2.11, 2.12 and 2.13) and will not be included in this section.

Insights into the function of the YycFG-controlled genes *yvcE* and *lytE* were obtained during the analysis performed to investigate the nature of the essentiality of YycFG, presented in the preceding section: *yvcE* and *lytE* were found to perform an essential redundant role in the processes of cell elongation and lateral peptidoglycan synthesis. A further analysis was undertaken to elucidate their role in cell wall homeostasis.

A functional analysis of the *yjeA* gene, negatively regulated by YycFG, is also presented.

Table 2.3. Phenotypic analysis of strains deleted in genes positively regulated by YycFG

Strain	AH023	BP079	L16638	BP068	BP071	BP083	BP123	BP124	BP122	BP087
Genotype	<i>yocH</i>	<i>yvcE</i>	<i>lytE</i>	<i>ydjM</i>	<i>ydjM</i> , <i>yocH</i>	<i>ydjM</i> , <i>yvcE</i>	<i>ydjM</i> , <i>lytE</i>	<i>ydjM</i> , <i>lytE</i> , <i>yocH</i>	<i>yocH</i> , <i>lytE</i>	<i>yocH</i> , <i>yvcE</i>
Colony morphology	wt	wt	wt	shiny	shiny	shiny	shiny, small and big	shiny, small and big	wt	lysis, suppressors
Growth in LB broth	wt	wt	impaired	impaired	impaired	impaired	ND	ND	ND	wt
Cell length	wt	wt	wt	impaired	impaired	impaired	ND	ND	ND	wt
Sporulation	wt	wt	wt	impaired	impaired	impaired	ND	ND	ND	wt

Genotypes of strains are schematically represented by the name of the gene(s) deleted from the chromosome. ND: not determined. wt: no phenotype observed (same as wild-type).

2.2.3.1. Phenotypes observed during growth on LB agar

Growth on LB agar and colony morphology were analyzed in strains deleted in genes positively regulated by YycFG. Such analysis was performed repeatedly with identical results. Results are summarized in Table 2.3.

Shiny colonies were observed in strains BP068 (*ydjM::tet^r*), BP071 (*yocH::kan^r ydjM::tet^r*), BP083 (*ydjM::tet yvcE::spc^r*), BP123 (*lytE::Cm^r ydjM::tet^r*) and BP124 (*lytE::Cm^r ydjM::tet^r yocH::kan^r*). All these strains carry a deletion in gene *ydjM*, which is likely to be responsible for the phenotype observed.

Strains BP123 (*lytE::Cm^r ydjM::tet^r*) and BP124 (*lytE::Cm^r ydjM::tet^r yocH::kan^r*) displayed a further phenotype, with a mixture of small and big colonies growing on LB agar. Cells from both strains were harvested from LB agar plates and analyzed by phase contrast microscopy: a mixed population of motile, rod-shaped cells and immotile chains of cells was observed in both cases. Cells from the wild-type strain 168 grown on LB agar were analyzed for comparison, and only motile rods were observed. To test a possible correspondence between the size of the colonies and the chaining phenotype in strains BP123 and BP124, cells from isolated small and big colonies were observed by phase contrast microscopy: small colonies contained only immotile chains of cells, while big colonies contained only motile cells. This finding is consistent with the ability of motile cells to move across the surface of the LB agar plates, and to reach longer distances, resulting in the formation of bigger colonies. This result suggests that deletion of *lytE* and *ydjM* causes cellular heterogeneity, a phenomenon that can occur in genetically identical cell populations (Longo and Hasty, 2006 and references therein). It is noteworthy that in this work cellular heterogeneity was not

observed during growth on LB agar in the wild-type strain or in any of the mutant strains containing a wild-type copy of *lytE* or *ydjM*. Therefore the absence of both the *lytE* and *ydjM* genes in strains BP123 and BP124 must account for the observed phenotype. This result suggests that *lytE* and *ydjM* might share a redundant function in preventing the formation of sub-populations of motile and sessile cells in wild-type *B. subtilis* during growth on LB agar.

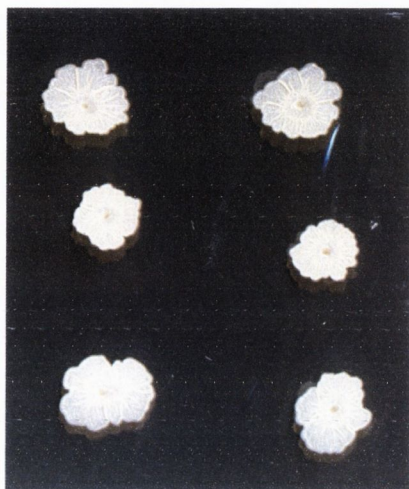
All the other single and double mutant strains analyzed displayed colony morphologies comparable to the wild-type (see Table 2.3), with the only exception of strain BP087 (*yocH::kan^r yvcE::spc^r*). This strain, mutated in the autolysins-encoding genes *yocH* and *yvcE*, initially formed colonies with a normal morphology on LB agar plates. However, these colonies became translucent after 5 days on LB agar and after 7 days many normal looking opaque colonies grew out from the edges of the original colony (Figure 2.14). This result suggests that cells carrying null mutations in both *yocH* and *yvcE* are prone to cell lysis, and that suppressor mutations can arise with a high frequency. This result is also consistent with the role of *yocH* and *yvcE* as autolysins: this class of enzymes is known to be involved in maintaining the structural integrity of the cell envelope and in preventing cell lysis, especially in hypotonic conditions (Smith *et al.*, 2000). Since strains mutated only in *yvcE* or in *lytE* did not display the lysis phenotype, this result suggests that YvcE and YocH might play a redundant role in prevention of cell lysis. Furthermore, this and previous work (Fukuchi *et al.*, 2000; Howell *et al.*, 2003) have established that cell lysis is a typical feature of YycFG-depleted cells: the fact that cells mutated in the two autolysins *yocH* and *yvcE* are prone to lysis suggests that the lowered *yocH* and *yvcE* expression levels that occur upon YycFG depletion might contribute to the lysis phenotype of YycFG-depleted cells. This result is also consistent with the phenotype observed in the triple mutant strain BP090 (*yvcE::spc^r ydjM::tet^r yocH::kan^r*), whose cells were particularly fragile and prone to lysis (see Figure 2.13).

2.2.3.2. Phenotypes observed during growth in LB broth

Growth in LB broth was monitored in strains deleted in genes positively regulated by YycFG. Results are summarized in Table 2.3.

Growth in LB broth of strain L16638 (*lytE::Cm^r*) has been analyzed during a previous experiment (see Figure 2.8A). This strain displayed a growth rate lower than the wild-type strain 168 (compare red triangles with blue diamonds, Figure 2.8A), and an average cell length comparable to the wild-type at the time point chosen for microscopic analysis (indicated by an arrow in Figure 2.8A). These findings appear in contrast with results obtained in previous

168 (wild-type)



BP087 (*yocH::kan^r yvcE::spc^r*)



Figure 2.14. Colony morphologies of strains 168 (wild-type) and BP087 (*yocH::kan^r yvcE::spc^r*) after seven days of incubation at room temperature on LB agar plates. Colonies of the wild-type strain 168 were opaque and never lysed, while colonies of strain BP087 became translucent after 5 days and many small opaque colonies started to grow from the edges of the original lysed colonies after 7 days.

works, where strain L16638 did not exhibit any growth defect (Margot *et al.*, 1998), and an equivalent *lytE* null mutant strain displayed cells twice as long as the wild-type (Ishikawa *et al.*, 1998). However, in both studies strains were grown in Schaeffer medium, while in this work strain L16638 was grown in LB medium. Therefore the different media compositions are likely to account for the discrepancies between this and previously published work.

Growth in LB broth was analyzed in the strains carrying null mutations in YycFG-regulated genes listed in Table 2.3. This analysis was performed twice with comparable results and representative growth profiles are displayed in Figure 2.15. Strain 168 (wild-type) was included in the analysis as a control. Exponentially growing cultures of each strain were used to inoculate 50 ml LB cultures at an $OD_{600} = 0.02$. Strains AH023 (*yocH::kan^r*), BP079 (*yvcE::spc^r*) and BP087 (*yocH::kan, yvcE::spc^r*) did not show any growth defect when compared to the wild-type strain 168 (compare blue squares, circles and plus symbols with black diamonds, Figure 2.15). Strains BP068 (*ydjM::tet^r*) and BP071 (*yocH::kan^r ydjM::tet^r*) exhibited reduced growth rates during the exponential phase and reached stationary phase at a lower optical density with respect to the wild-type strain 168 (compare red stars and triangles with black diamonds, Figure 2.15). The growth characteristics of strain BP083 (*ydjM::tet yvcE::spc^r*) were intermediate between the wild-type strain and strains BP068 and BP071 (red dashed lines, Figure 2.15). In summary, growth defects were observed in all the strains carrying a null mutation in *ydjM* (see Table 2.3). This suggests that deletion of this gene is perhaps responsible for the phenotypes observed.

The morphology of cells in these cultures was analyzed by phase contrast microscopy. Samples were harvested during the exponential, transition and stationary phases of growth (time points 1, 2 3 indicated in Figure 2.15) and observed using a confocal microscope (see Materials and Methods). Cell morphology of strains AH023 (*yocH::kan^r*), BP079 (*yvcE::spc^r*) and BP087 (*yocH::kan, yvcE::spc^r*) did not differ from the wild-type. These same strains did not show any defect in the growth profiles (see Figure 2.15). Strains BP068 (*ydjM::tet^r*), BP071 (*yocH::kan^r ydjM::tet^r*), and BP083 (*ydjM::tet yvcE::spc^r*), which were all defective in growth (see Figure 2.15), displayed a normal rod-shaped morphology, but a difference in cell length with respect to the wild-type strain. Cell length of the wild-type strain and of mutant strains carrying a wild-type copy of the gene *ydjM* was observed to vary throughout the growth curve: cells were longer during exponential phase (Figure 2.15, time point 1); cell length was reduced during transition phase (Figure 2.15, time point 2), and decreased to a further extent during stationary phase (Figure 2.15, time point 3). On the contrary, in strains deleted in *ydjM* (strains BP068, BP071 and BP083) cell length did not vary throughout the

growth and was comparable to the cell length observed in time point 2 (Figure 2.15) for the wild-type strain. This result might suggest an involvement of *ydjM* in regulating cell length variation during the different phases of growth.

Cells from the cultures displayed in Figure 2.15 were observed by phase contrast microscopy after 24 hours incubation in LB broth: a mixed population of rod-shaped cells and phase-bright spores was observed in the wild-type culture as well as in cultures of mutant strains carrying a wild-type copy of gene *ydjM* (strains AH023, BP079 and BP087). No spores were observed in cultures of strains carrying the *ydjM* null mutation (strains BP068, BP071 and BP083). This result indicates that deletion of *ydjM* might contribute to a sporulation defect.

2.2.3.3. Sporulation defects of a strain deleted in *ydjM*

To further investigate the sporulation defect observed in strains deleted in *ydjM*, the kinetics of alkaline phosphatase (APase) expression was tested in strain BP068 (*ydjM::ter'*) as described in Materials and Methods. Results are presented in Figure 2.16. Alkaline phosphatase is an enzyme that is induced at stage II of sporulation (which corresponds in time to two hours after the onset of the stationary phase) and peaks at five hours into stationary phase (Nicholson and Setlow, 1990). A peak of APase activity was seen in strain BP068 coincident with the peak observed in wild-type strain 168, but the level of activity observed in the *ydjM* null mutant strain was lower than the wild-type (compare closed circles and closed squares in Figure 2.16). This result suggests that the sporulation process is not blocked in strain BP068, but that sporulation efficiency might be reduced.

To validate this result, sporulation efficiency of strain BP068 was assayed by measuring the percentage of cells resistant to heat and chloroform, as described in Materials and Methods. Strains BP068 (*ydjM::ter'*) and 168 (wild-type) were grown in LB medium until the OD₆₀₀ reached a value of 0.3, then cultures were resuspended in Sterlini-Mandlestam medium: growth profiles are displayed in Figure 2.17A. Cells were harvested 4, 6, 8, 10, 25 and 52 hours after resuspension in Sterlini-Mandlestam medium and a four-fold reduction in the percentage of heat and chloroform resistant cells was observed in strain BP068 at the 10, 25 and 52 hour time points, indicating reduced sporulation efficiency (Figure 2.17B).

These results show that deletion of *ydjM* from the chromosome of *B. subtilis* results in a reduced sporulation efficiency. This finding might indicate an involvement of YdjM in the sporulation process; however the sporulation phenotype might also be a secondary effect due to the growth defect exhibited by strains deleted in *ydjM* cultured in LB broth (see Figure 2.15, red symbols).

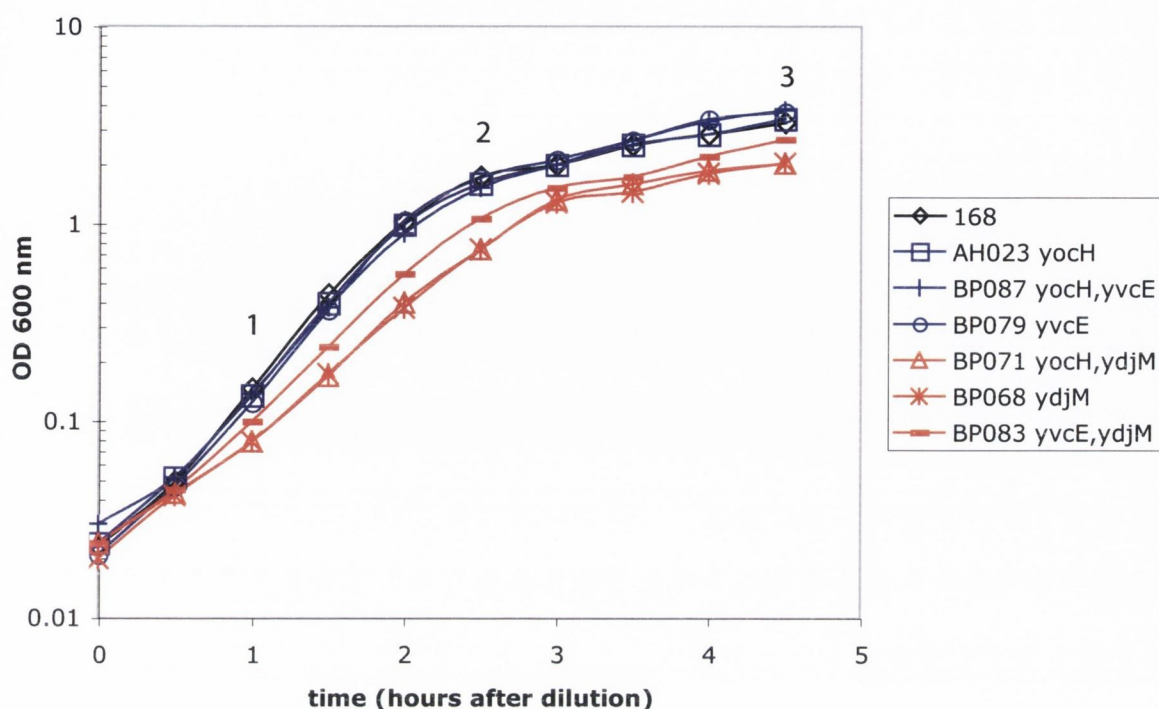


Figure 2.15. Growth profiles of strains carrying null mutations in genes positively regulated by YycFG. The wild-type strain 168 was cultured in parallel as a control. Starter cultures were inoculated from freshly streaked colonies on LB agar and grown in 10 ml of LB broth until OD₆₀₀ 1.00. These cultures were used to inoculate 50 ml LB cultures to an OD₆₀₀ of 0.02. Growth was monitored turbidimetrically and cells were harvested at the time points indicated for microscopic analysis. Growth profile of the control strain 168 is shown in black; growth profiles of strains carrying a deletion in gene *ydjM* are shown in red; growth profiles of mutant strains carrying a wild-type copy of *ydjM* are shown in blue. Strain 168 (wild-type): diamonds; strain AH023 (*yocH::kan^r*): squares; strain BP068 (*ydjM::tet^r*): stars; strain BP079 (*yvcE::spc^r*): circles; strain BP071 (*yocH::kan^r ydjM::tet^r*): triangles; strain BP083 (*ydjM::tet^r yvcE::spc^r*): dashed lines; strain BP087 (*yocH::kan^r, yvcE::spc^r*): plus symbols. This experiment was performed twice with comparable results and a representative profile is displayed.

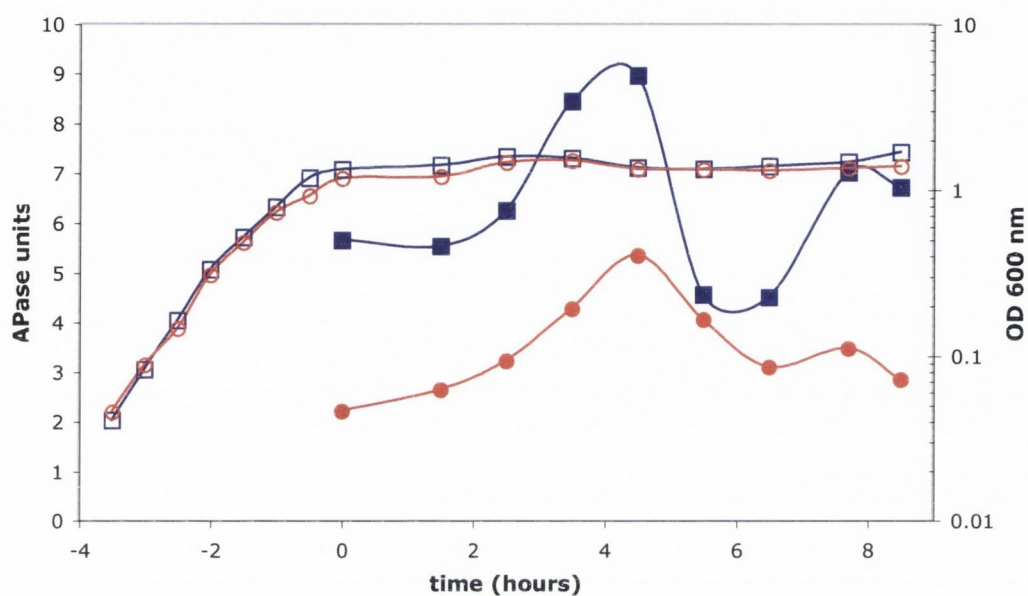


Figure 2.16. Analysis of alkaline phosphatase expression of strains 168 (wild type) and BP068 (*ydhM::tet'*). Cells were grown in Schaeffer's sporulation medium and growth was monitored turbidimetrically at OD₆₀₀. Growth of strains is shown by open symbols and APase activity by closed symbols: squares, 168 (wild-type); circles: BP068 (*ydhM::tet'*). Time zero indicates the point of transition between the exponential and stationary phase of growth. This experiment was performed twice with comparable results and a representative profile is shown.

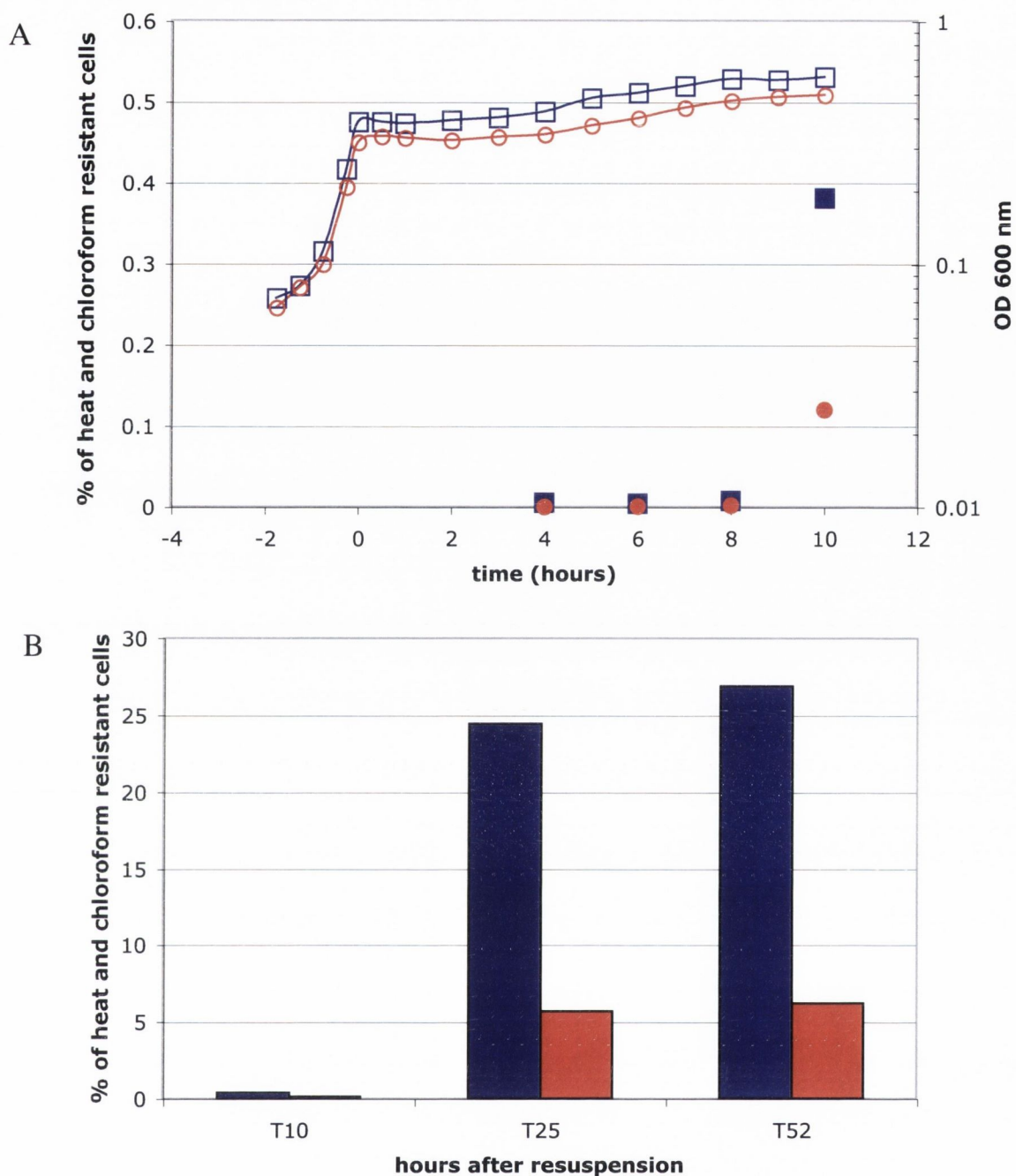


Figure 2.17. Analysis of sporulation efficiency of strains 168 (wild type) and BP068 (*yjdM::tet*^r). Cells were grown in LB medium until the OD₆₀₀ reached a value of 0.3 and cultures were resuspended in Sterlini-Mandlestam medium. Cells were harvested at 4, 6 8 10, 25 and 52 hours after resuspension and sporulation efficiency was assayed by measuring the percentage of cells resistant to heat and chloroform as described in Materials and Methods. The displayed result is representative of two independent experiments. (A) Growth profiles and percentage of heat and chloroform resistant cells of strains 168 (wild type, squares) and BP068 (*yjdM::tet*^r, circles). Growth of strains is shown by open symbols and sporulation efficiency by closed symbols. Time zero indicates the point of resuspension. Growth was monitored turbidimetrically for the first 10 hours after resuspension. (B) sporulation efficiency of strains 168 (in blue) and BP068 (in red) at 10, 25 and 52 hours after resuspension.

2.2.3.4. Mg^{2+} is capable of rescuing the growth and sporulation defects of a *ydjM* null mutant strain

It was noticed that the growth defect observed during culture of strain BP068 (*ydjM::tet'*) in LB broth (see Figure 2.15) was less evident during culture in Schaeffer's sporulation medium and Sterlini-Mandlestam medium (used in the APase assay and in the sporulation efficiency assay respectively, see Figures 2.16 and 2.17A). Both these media contain high concentrations of Mg^{2+} (Nicholson and Setlow, 1990; Schaeffer P, 1965), an ion that is capable of restoring almost wild-type growth and morphology to mutants impaired in cell wall homeostasis (Carballido-Lopez *et al.*, 2006; Formstone and Errington, 2005; Lazarevic *et al.*, 2005). It was reasoned that the amelioration of the growth defect of strain BP068 observed during growth in the two sporulation media might be due to high Mg^{2+} concentrations, and that this effect might suggest an involvement of YdjM in cell wall metabolism.

To verify the effect of Mg^{2+} on growth of strain BP068, this strain was grown in LB broth containing increasing $MgCl_2$ concentrations, as shown in Figure 2.18. Strain 168 (wild-type) was grown in parallel as a control. A starter culture was inoculated in LB broth from a freshly streaked colony on LB agar and grown until OD_{600} 1.00. This culture was used to inoculate 50 ml LB cultures containing different $MgCl_2$ concentrations, at a starting OD_{600} of 0.02. The growth defect exhibited by strain BP068 (green diamonds, Figure 2.18) was rescued by Mg^{2+} addition (red symbols, Figure 2.18), since the OD levels of these cultures were indistinguishable from the ones displayed by the wild-type strain 168 (black stars, Figure 2.18). Comparable growth profiles were observed when adding 25mM, 50mM and 100mM $MgCl_2$ (red circles, squares and triangles respectively, Figure 2.18). Although the mechanism by which high Mg^{2+} concentration can rescue mutants affected in the cell wall is not currently understood, this result suggests that *ydjM* participates in cell wall homeostasis.

To test the ability of Mg^{2+} to rescue the sporulation defect exhibited by strain BP068 in LB broth, the cultures shown in Figure 2.18 were grown for four days and samples were harvested after an overnight and after two and four days of incubation, and observed by phase contrast microscopy. After one overnight a mixed population of short, rod-shaped cells and phase-bright spores was observed in the wild-type culture, while no spores were observed in the strain deleted in *ydjM*, as previously observed. Cultures containing additional $MgCl_2$, however, showed a phenotype comparable to the wild-type. After two days the wild-type culture contained almost only phase-bright spores, while the culture of strain BP068 (*ydjM::tet'*) grown without $MgCl_2$ addition contained only a small minority of spores. Addition of $MgCl_2$ to cultures of strain BP068 (*ydjM::tet'*) rescued sporulation to wild-type levels. After

four days only spores and cell debris were observed in strain 168 and in strain BP068 containing MgCl_2 , while in the absence of Mg^{2+} strain BP068 (*ydjM::tet'*) showed a mixed population containing phase-bright spores as well as rod-shaped cells. Therefore Mg^{2+} addition is capable of rescuing the sporulation defect of strain BP068 (*ydjM::tet'*). Taken together, these data show that Mg^{2+} can rescue both the growth and the sporulation defects exhibited by a strain deleted in *ydjM*, strongly suggesting the involvement of YdjM in cell wall homeostasis.

2.2.3.5. Mg^{2+} addition does not significantly ameliorate the lysis phenotype of YycFG-depleted cells

Addition of Mg^{2+} to the growth medium has been shown to rescue the phenotypes of several mutants affected in cell wall metabolism (Carballido-Lopez *et al.*, 2006; Formstone and Errington, 2005; Lazarevic *et al.*, 2005). This and previous studies show that the YycFG regulon has a high proportion of genes involved in control of cell wall metabolism (Howell *et al.*, 2003; 2006). In addition, this work shows that the three genes *yvcE*, *yocH* and *ydjM* contribute to the essentiality of YycFG in *B. subtilis*, although they do not fully explain it, and that Mg^{2+} addition can rescue both the growth and the sporulation defects exhibited by a mutant strain deleted in *ydjM*, indicating the involvement of *ydjM* in cell wall homeostasis. Multiple evidence from this work indicate that YycFG essentiality is polygenic in nature, and is likely to derive from the control of a number of genes involved in cell wall homeostasis.

On the basis of the above observations, we reasoned that if the essentiality of YycFG is caused by its control of cell wall-related genes, increased Mg^{2+} might rescue the lysis phenotype observed in YycFG-depleted cells.

To test this hypothesis, strain AH9912 (P_{spac} *yycFGHIJK*) was grown with YycFG sufficiency (+IPTG) and YycFG depletion (-IPTG), in the presence of increasing MgCl_2 concentrations, as shown in Figure 2.19. A starter culture of strain AH9912 was grown in LB broth containing 100 μM IPTG to an OD_{600} of 1.00. This culture was used to inoculate four 50 ml LB cultures, at a starting OD_{600} of 0.02. One culture was grown in the presence of 1mM IPTG (closed squares), and three cultures in the absence of IPTG (open symbols) and in the presence of 0, 25mM and 50 mM MgCl_2 (squares, triangles and circles respectively). The lysis phenotype of the YycFG-depleted cells was not significantly ameliorated by MgCl_2 addition (compare black empty squares with red empty triangles and circles, Figure 2.19). This result was reproducible: comparable growth profiles were observed upon repetition of this experiment.

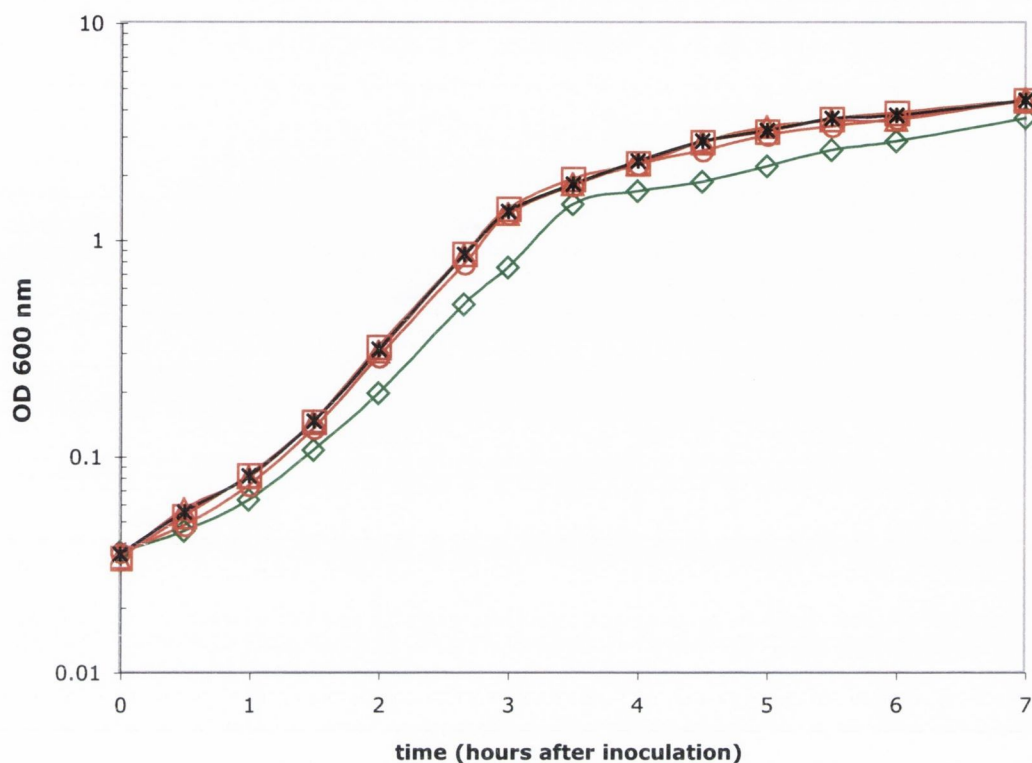


Figure 2.18. Analysis of the effect of Mg^{2+} addition on growth of strain BP068 (*ydhM::terf*). Strain BP068 (*ydhM::terf*) was grown in LB in the absence of $Mg^{2+}Cl_2$ (green diamonds) and in the presence of 25mM (red circles), 50mM (red squares) and 100mM (red triangles) $MgCl_2$. Strain 168 was grown in parallel as a control (black stars). Cultures were inoculated at OD_{600} 0.02 from starter cultures grown in LB broth to OD_{600} 1.00. Growth was monitored by measuring the optical density at 600 nm. Time is indicated in hours after inoculation. This experiment was performed twice with comparable results and a representative profile is shown.

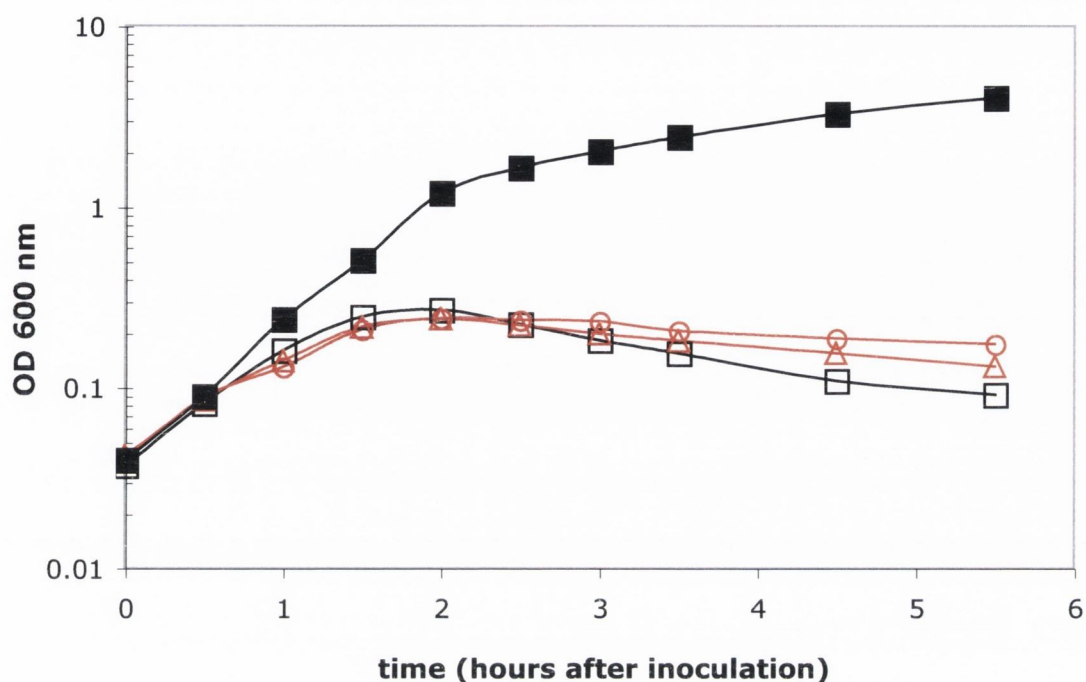


Figure 2.19. Analysis of the effect of Mg^{2+} addition on the lysis phenotype of YycFG-depleted cells. Strain AH9912 ($P_{yyc}yycFGHIJK$) was grown with YycFG sufficiency (1mM IPTG, black closed squares) and YycFG depletion (no IPTG, empty symbols) in the presence of increasing $MgCl_2$ concentrations. Cultures were inoculated at an OD_{600} of 0.02 from a starter culture grown in LB broth containing 100 μ M IPTG to an OD_{600} of 1.00. YycFG depleted cultures were grown in the absence of additional $MgCl_2$ (empty squares), and in the presence of 25mM $MgCl_2$ (empty triangles) and 50 mM $MgCl_2$ (empty circles). Time is indicated in hours after inoculation. Growth was monitored by measuring the optical density at 600 nm. This experiment was performed three times with comparable results and a representative profile is shown.

2.2.3.6. The YvcE and LytE autolysins participate in peptidoglycan synthesis and turnover respectively

This and previous studies show that control of cell wall metabolism is a primary function of the YycFG TCS in *B. subtilis* (Howell *et al.*, 2003; 2006). Among the genes regulated by YycFG, *yocH* is a potential autolysin, *ydjM* is likely to participate to cell wall homeostasis (this work) and the two endopeptidase-type autolysins *yvcE* and *lytE* share a redundant essential function in cell wall elongation and lateral cell wall synthesis (this work). To further elucidate the role of these genes in cell wall metabolism, we established their contributions to peptidoglycan synthesis and turnover during the exponential phase of the growth cycle.

Cell wall synthesis was analyzed in the wild-type strain 168 and in strains with null mutations in genes *lytE*, *yocH*, *yvcE* and *ydjM*, by measuring the rates of [¹⁴C]-N-acetylglucosamine incorporation into cell walls during exponential growth, as described in Materials and Methods. Results are shown in Figure 2.20A. The level of incorporation in wild-type strain 168 (circles) increased throughout the growth period, reaching a value of 18×10^4 CPM/OD₆₀₀. Similar incorporation levels were observed in cultures of strain L16638 (*lytE::Cm^r*) and AH023 (*yocH::kan^r*) showing that neither mutation significantly affected peptidoglycan synthesis under these conditions (data not shown). However lower levels of incorporation were observed in strain BP079 (squares, *yvcE::spc^r*): cell-associated radioactivity reached a value of $12\text{--}14 \times 10^4$ CPM/OD₆₀₀, which corresponds to a 23 – 33% reduction in radioactivity incorporation during the period tested. A similar lowered incorporation was observed in strain BP087 [(*yocH::kan^r* *yvcE::spc^r*), data not shown]. Lowered incorporation levels were found also in strain BP068 (*ydjM::tet^r*); however, this strain displays a growth defect in LB (see Figure 2.15), which may account for the observed reduction in cell wall synthesis. Indeed, when normalizing the data according to generation time, the profile of incorporation displayed by strain BP068 was very similar to the wild-type (data not shown). The profiles of incorporation were found to be highly reproducible for all strains during this experiment. Since incorporation decreased similarly in strains BP079 (*yvcE::spc^r*) and BP087 (*yocH::kan^r* *yvcE::spc^r*), but did not decrease in strain AH023 (*yocH::kan^r*), these data indicate that the incorporation defect is due to deletion of the *yvcE* gene and that the autolysin YvcE(CwlO) functions in peptidoglycan synthesis in *B. subtilis*.

The contributions of the *yvcE*, *lytE*, *yocH* and *ydjM* genes to peptidoglycan turnover were then determined by measuring the loss of [¹⁴C]-N-acetylglucosamine from the cell walls of strains carrying null mutations in *lytE*, *yocH*, *yvcE* and *ydjM* during exponential growth. Strain 168 was tested in parallel as a control. Cells grown for 2 generations in the presence of [¹⁴C]-

N-acetylglucosamine were harvested, washed and resuspended in pre-warmed media containing unlabelled N-acetylglucosamine. The amount of cell-associated radioactivity and radioactivity free in the medium was then determined for each harvested sample. When added together at each time point, these values always totalled 100% of the radioactivity that was present at the T_0 point of each experiment. The results are shown in Figure 2.20B. Strain 168 (wild-type, circles) displayed an 80% decrease in cell-associated radioactivity in the course of the experiment; the kinetics of turnover were similar to those observed by de Boer *et al.*, (1982), with an initial lag phase followed by a more rapid rate of radioactivity loss. A very similar profile was observed in strain BP087 (*yocH::kan^r yvcE::spc^r*, triangles). However, in strain L16638 (diamonds, *lytE::Cm^r*) the loss of cell-associated radioactivity was significantly slower (50% loss in ~51 minutes) than that observed for the wild-type or BP087 strains (50% loss in ~36 minutes). These data show that deletion of *lytE* results in a cell wall turnover defect. A reduction in cell wall turnover was also observed in strain BP068 (*ydjM::tet^r*). However, as mentioned above, in this strain the growth rate is slower than the wild-type in LB. It is known that rates of peptidoglycan turnover and cell growth of *B. subtilis* are correlated (Cheung *et al.*, 1983). To test whether the slower growth rate of strain BP068 accounted for the decrease in cell wall turnover, the data was normalized according to doubling time: in such format the cell wall turnover profile displayed by strain BP068 was very similar to the wild-type (data not shown), indicating that deletion of *ydjM* did not affect cell wall turnover.

In conclusion, the YvcE (CwlO) autolysin participates in cell wall synthesis and the LytE autolysin in cell wall turnover, while YocH and YdjM do not detectably affect either process under the conditions of the experiment.

2.2.3.7. YjeA deacetylates peptidoglycan, altering its susceptibility to lysozyme digestion

Among the newly identified members of the YycFG regulon, the *yjeA* gene, encoding a putative peptidoglycan deacetylase, is repressed by YycFG during exponential growth (see Figures 2.2, 2.5). This finding has addressed an observation I had made in 2004 but was unable to explain at that time. While performing β -galactosidase assays on strains carrying *yocH-bgaB* promoter fusions and expressing the *yycFG* operon under the control of the IPTG inducible P_{spac} promoter, I noticed that cell lysates prepared from cells grown under conditions of YycFG sufficiency (+IPTG) were always clear, but remained turbid when prepared from cells grown under conditions of YycFG depletion (-IPTG). It is known that deacetylation of the N-acetylglucosamine moiety of the peptidoglycan carbohydrate chain makes it more

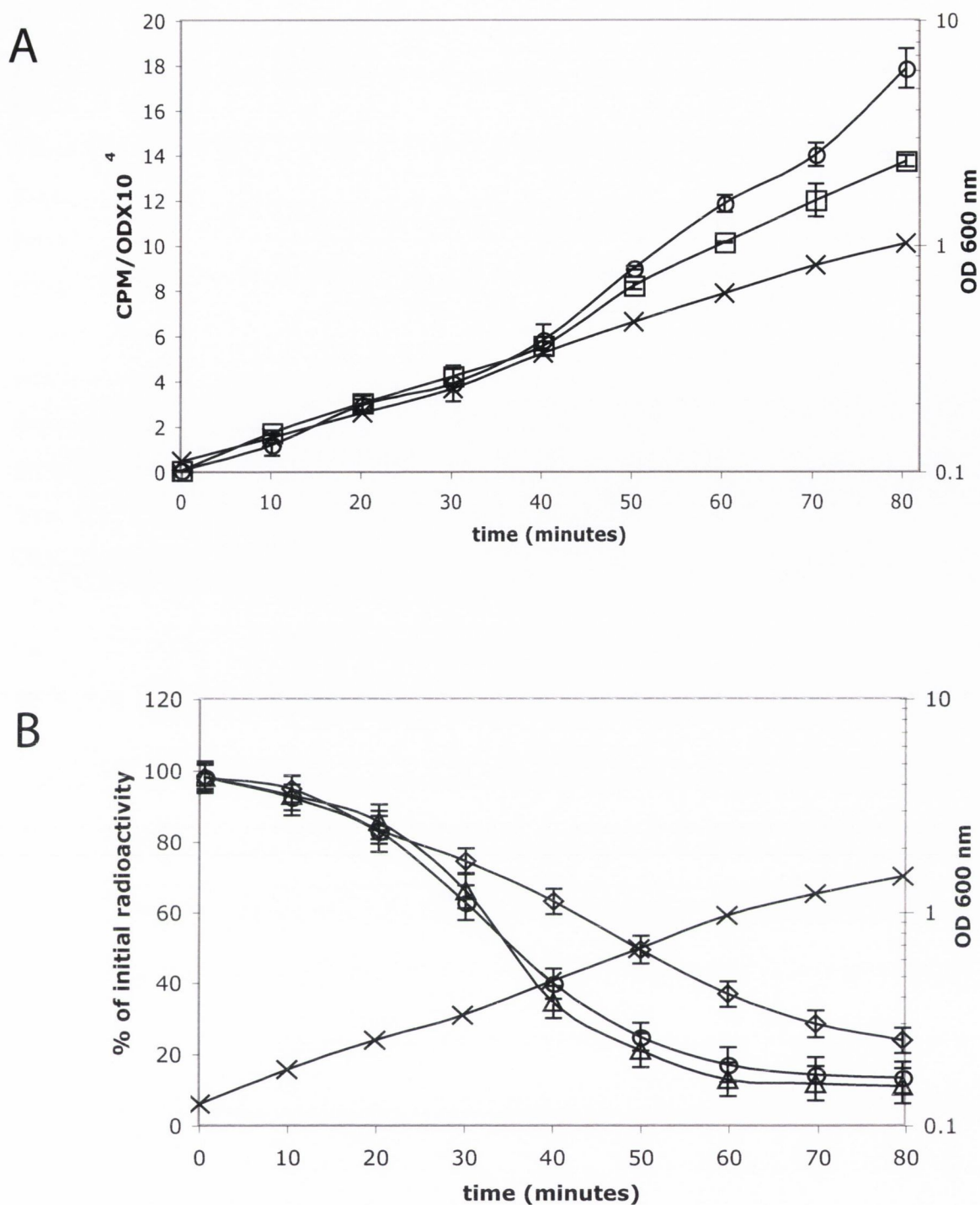


Figure 2.20. The contribution of YocH, YvcE (CwlO) and LytE to cell wall synthesis and turnover during exponential growth. (A) Incorporation of [^{14}C]-N-acetylglucosamine during exponential growth of strains: strain 168 (wild-type), circles; BP079 (*yvcE::spc'*), squares. Time is indicated in minutes after inoculation of an exponentially growing culture in media containing [^{14}C]-N-acetylglucosamine. Growth of all strains was virtually identical and a representative profile is shown (x symbol) and error bars indicate standard deviation. (B) Cell wall turnover monitored by loss of radiolabelled peptidoglycan from exponentially growing cultures of the following strains: strain 168 (wild-type), circles; diamonds, L16638 (*lytE::Cm'*) and triangles, BP087 (*yvcE::spc'* *yocH::kan'*). A representative growth profile is shown (x symbol) and error bars indicate standard deviation.

resistant to lysozyme degradation (De Las Rivas *et al.*, 2002; Vollmer and Tomasz, 2000). Therefore we hypothesized that YjeA is a peptidoglycan deacetylase and that increased *yjeA* expression in YycFG depleted cells (see Figure 2.2) results in the cell wall peptidoglycan becoming less acetylated and hence more resistant to lysozyme digestion, the first step in the preparation of cell lysates for β -galactosidase assays. To test this hypothesis, we examined the sensitivity to lysozyme digestion of cell walls prepared from wild-type strain 168, and from strain AH9912 ($P_{spac} yycFGHIJK$) grown with YycFG sufficiency (+IPTG) and YycFG depletion (-IPTG). In the YycFG-depleted culture (-IPTG), cells were harvested at the point of growth cessation, while in the YycFG-replete culture (grown with 1mM IPTG) and the wild-type culture, cells were harvested at the same OD₆₀₀ as the YycFG-depleted one (in all cases OD₆₀₀ = 0.8). Lysozyme was added to purified cell wall suspensions (OD₆₀₀ = 0.3) of each strain and digestion was monitored spectrophotometrically. This experiment was repeated four times using cell wall preparations from four biological replicates, and reproducible results were obtained. A representative profile is presented in Figure 2.21. The results show that cell walls prepared from a YycFG depleted culture (open squares) are more resistant to lysozyme digestion than cell walls from the wild-type strain 168 (open triangles) and from strain AH9912 over-expressing YycFG (open circles).

To confirm that the decreased lysozyme sensitivity observed in cell walls from YycFG depleted cells was caused by reduced acetylation of peptidoglycan, each cell wall preparation was chemically acetylated with acetic anhydride (see Materials and Methods) before lysozyme digestion. The susceptibility to lysozyme digestion of the chemically acetylated cell walls was increased for all three preparations (Figure 2.21, compare closed and open symbols). It is noteworthy that chemical acetylation increased the sensitivity to lysozyme digestion of cell walls prepared from YycFG depleted cells to a level that was very similar to the one observed in acetylated cell walls prepared from wild-type strain 168 (compare closed squares and triangles, Figure 2.21). Therefore the increased resistance to lysozyme digestion displayed by cell walls extracted from YycFG depleted cells could be counteracted by artificial acetylation, indicating that the chemical modification responsible for this phenotype is indeed de-acetylation of peptidoglycan.

We next set out to establish whether the increased resistance to lysozyme digestion of cell walls prepared from YycFG depleted cells was due to derepression of *yjeA*. The lysozyme digestion profile was established for cell walls prepared from strain BP070 (*yjeA::kan^r*), and from strain BP088 (*yjeA::kan^r P_{spac} yycFGHIJK*) grown under YycFG depleted conditions (cells were harvested at the point of growth cessation). Cell walls extracted from strain 168

(wild-type) and strain AH9912 ($P_{spac} yycFGHIJK$) grown in YycFG replete and YycFG depleted conditions were assayed in parallel as controls. The results are presented in Figure 2.22. Cell walls from YycFG depleted cells were more resistant to lysozyme digestion (closed squares), than those prepared from strain AH9912 grown under YycFG replete conditions (circles) and from wild-type strain 168 (triangles), as previously shown (see also Figure 2.21). The sensitivity to lysozyme digestion of cell walls extracted from strain BP070 ($yjeA::kan^r$, X symbols) was very similar to the one observed in wild-type cell walls (triangles). However, cell walls prepared from a culture of strain BP088 (which carries a null mutation in *yjeA*) grown under YycFG depleted conditions were no longer resistant to lysozyme, but were degraded at the same rate as that observed for wild type cells walls [compare rectangles (strain BP088 -IPTG) and triangles (strain 168) with closed squares (strain AH9912 -IPTG)]. These results show that the increased resistance to lysozyme degradation displayed by cell walls extracted from YycFG depleted cells is caused by de-repression of the peptidoglycan deacetylase YjeA.

2.2.3.8. YycFG-depleted cells are deficient in endogenous cell wall degrading activity, but the YvcE, LytE, YocH and YjeA proteins do not play a role in such activity

To further investigate the role of YycFG controlled genes in cell wall metabolism, we evaluated the contribution of YycFG-controlled autolysins YvcE, LytE and YocH and of the YjeA peptidoglycan deacetylase to endogenous cell wall degrading activity. Native cell walls were prepared as described in Materials and Methods, re-suspended at $OD_{600} = 0.3$ and auto-digestion was monitored spectrophotometrically. Results are shown in Figure 2.23. Cell walls prepared from wild-type strain 168 (diamonds) reached approximately 35% digestion during the 90 minutes incubation, while cell walls prepared from YycFG depleted cells (squares) showed virtually no auto-digestion during the same incubation period. This result shows that cell walls prepared from YycFG depleted cells are severely deficient in endogenous cell wall degrading activity. Cell walls prepared from strains AH9912 ($P_{spac} yycFGHIJK$) and BP088 ($yjeA::kan^r P_{spac} yycFGHIJK$), both grown under YycFG depleted conditions, displayed very similar auto-digestion profiles (compare squares and rectangles, Figure 2.23), indicating that the high level of YjeA deacetylase present in YycFG depleted cells has little effect on cell wall auto-digestion. Consistent with this result is the profile displayed by cell walls of strain BP070 ($yjeA::kan^r$), which is indistinguishable from the one displayed by wild-type cells walls (compare triangles and diamonds, Figure 2.23): this result indicates that *yjeA* does not have a major role in cell wall auto-digestion in the presence of physiological YycFG levels.

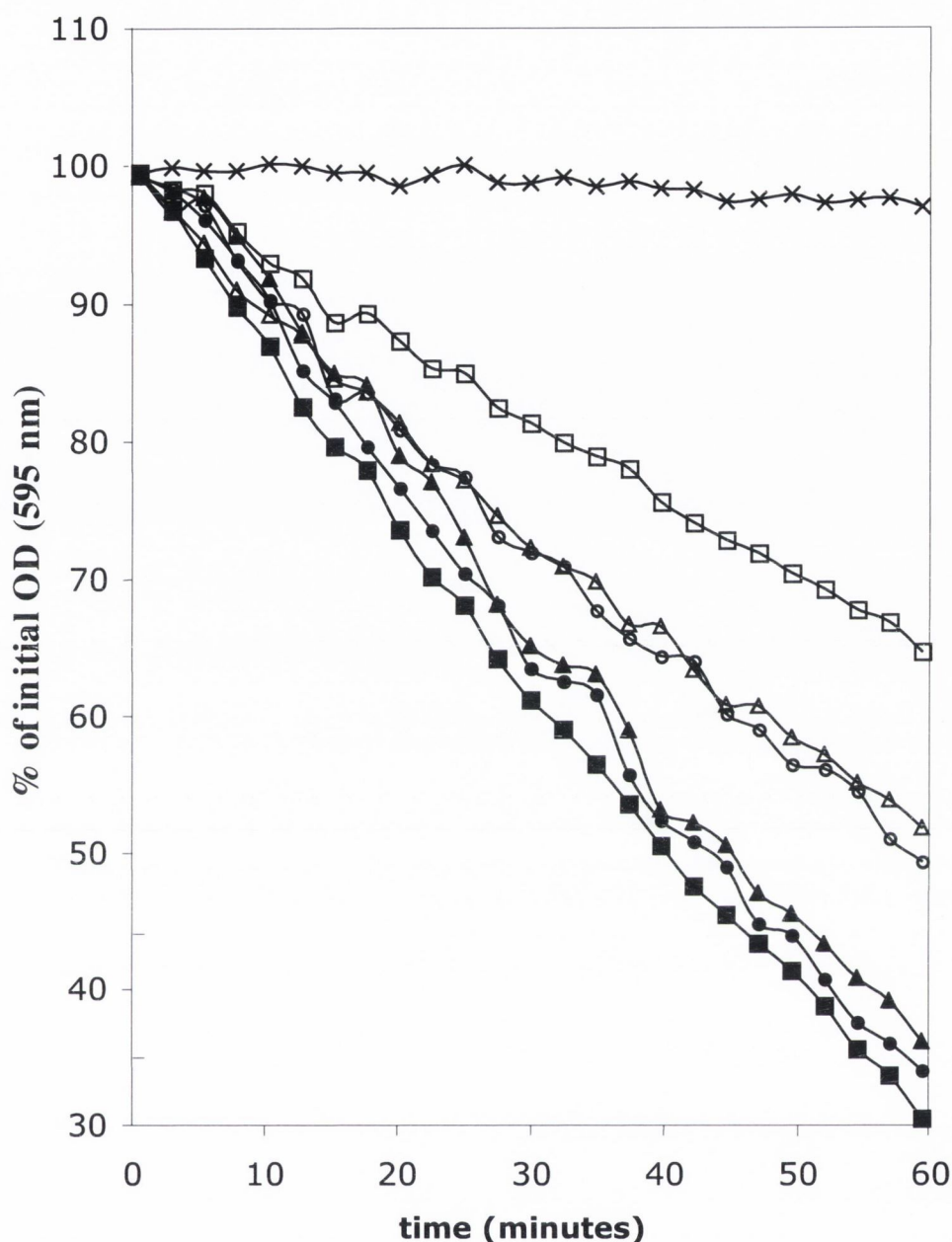


Figure 2.21. Effect of YycFG depletion and chemical acetylation on lysozyme digestion of *B. subtilis* cell walls. Open symbols represent cell walls treated with lysozyme (20 $\mu\text{g/ml}$); closed symbols represent chemically acetylated cell walls treated with lysozyme (20 $\mu\text{g/ml}$). No decrease in turbidity was observed without lysozyme addition (X symbol), this single line being representative of all such negative control digestions. Cell walls were extracted from the following strains: triangles, strain 168 (wild-type); squares, strain AH9912 ($P_{\text{spac}}\text{yycFGHIJK}$) grown in the absence of IPTG; circles, strain AH9912 ($P_{\text{spac}}\text{yycFGHIJK}$) grown in the presence of 1mM IPTG. This experiment was performed three times with comparable results and a representative profile is shown.

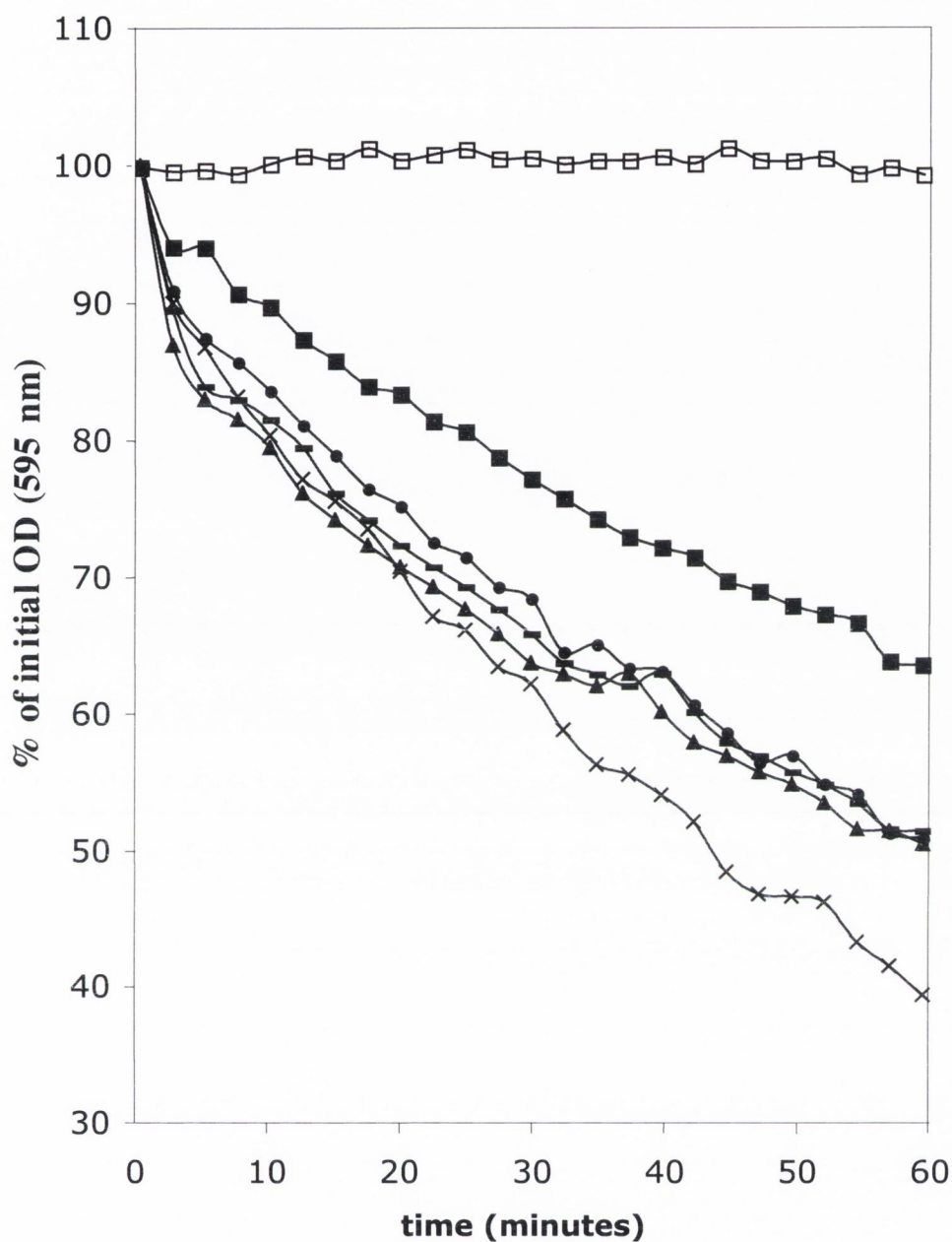


Figure 2.22. The contribution of YjeA to the decreased susceptibility to lysozyme digestion of cell walls from YycFG depleted cells. Open squares represent cell walls without lysozyme addition, this single line representing all such control digestions. Closed symbols represent cell wall preparations from the following strains treated with 20 $\mu\text{g/ml}$ lysozyme: triangles, strain 168 (wild-type); squares, strain AH9912 ($P_{yyc}yycFGHIJK$) grown in the absence of IPTG; circles, strain AH9912 ($P_{yyc}yycFGHIJK$) grown in the presence of 1mM IPTG; x symbols, strain BP070 ($yjeA::kan^r$) and rectangles, strain BP088 ($yjeA::kan^r$ $P_{yyc}yycFGHIJK$) grown in the absence of IPTG. This experiment was performed three times with comparable results and a representative profile is shown.

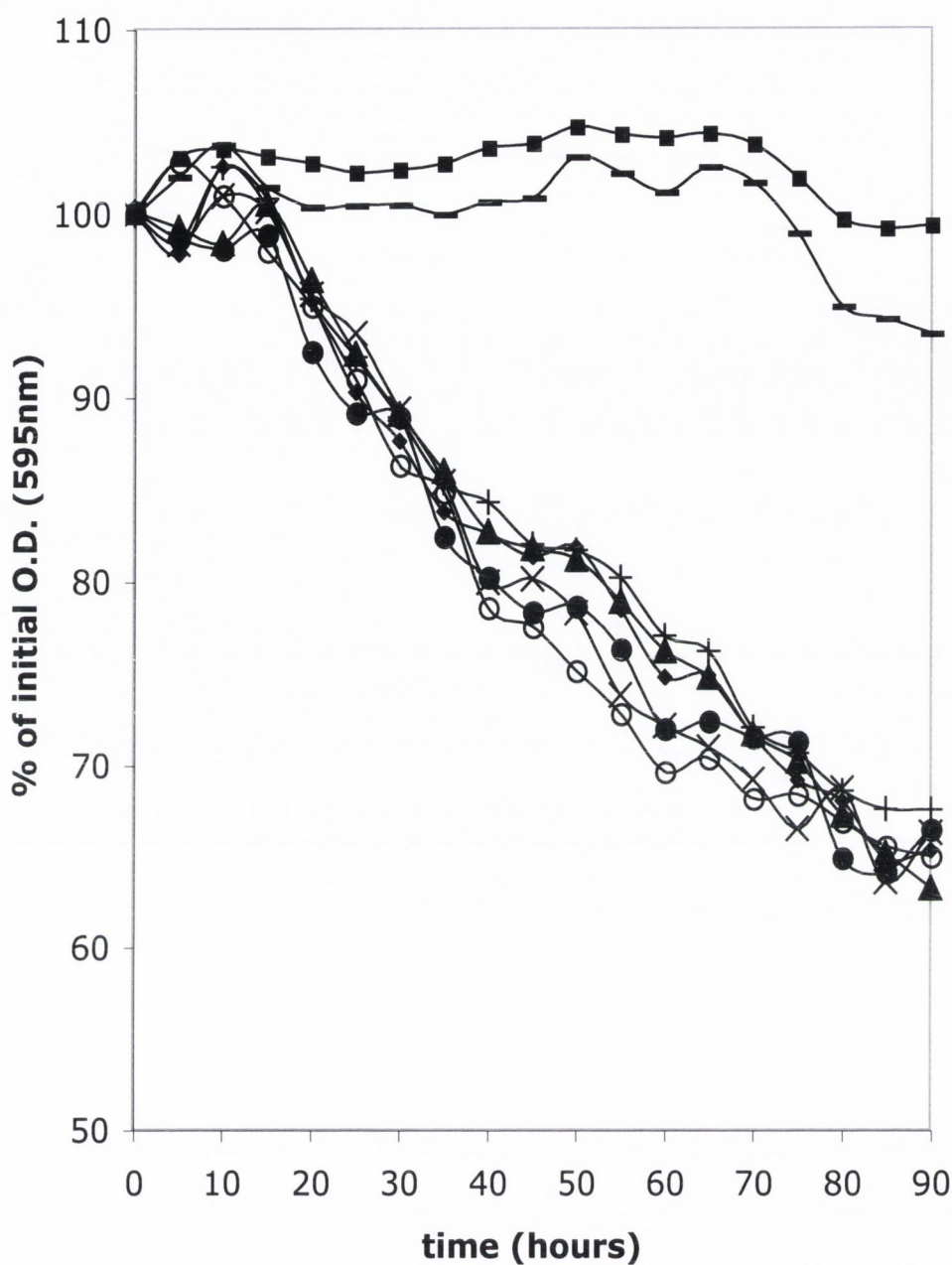


Figure 2.23. Digestion of native cell wall preparations by endogenous autolysins. Native cell walls were prepared from the following strains: diamonds, strain 168; squares, strain AH9912 ($P_{spc} yycFGHIJK$) grown without IPTG; closed circles, strain AH9912 ($P_{spc} yycFGHIJK$) grown with IPTG; triangles, strain BP070 ($yjcA::kan^r$); rectangles, strain BP088 ($yjcA::kan^r P_{spc} yycFGHIJK$), grown without IPTG; x symbol, strain BP088 ($yjcA::kan^r P_{spc} yycFGHIJK$) grown with IPTG; plus symbol, strain BP087 ($yocH::kan^r yvcE::spc^r$) and open circles, strain L16638 ($lytE::Cm^r$). Native cell walls were resuspended at an OD_{595} of 0.3 and autodigestion was monitored spectrophotometrically. This experiment was performed three times and a representative profile is shown.

Furthermore, none of the YycFG-controlled autolysins LytE, YvcE, or YocH appeared to play a major role in autolysis: the auto-digestion profiles of cell wall preparations from BP087 (plus symbols, *yocH::kan'*, *yvcE::spc'*) and L16638 (open circles, *lytE::Cm'*) were very similar to that observed in the wild-type strain 168 (diamonds).

In summary these data show that cell walls prepared from YycFG depleted cells are highly deficient in their auto-digestion capability, but surprisingly the three autolysins YocH, YvcE, and LytE, shown to be positively regulated by YycFG in this and previous studies (Howell *et al.*, 2003), do not seem to make a significant contribution to the endogenous cell wall degrading activity. In addition, the peptidoglycan deacetylase YjeA does not seem to influence the auto-digestion capability of native cell walls prepared from the wild type strain and from cells grown in YycFG replete or in YycFG depleted conditions.

2.3. DISCUSSION

2.3.1. Overview

The aim of this study was to establish the function of the TCS YycFG, to identify the constituent genes of its regulon and to investigate the nature of its essentiality in *B. subtilis*. There are five main conclusions from the work reported:

(1) In this work five new members of the YycFG regulon have been identified, all involved in cell wall metabolism: *yvcE(cwlO)* and *lytE*, both encoding murein hydrolases with a DL-endopeptidase activity (Smith *et al.*, 2000); *yjeA*, which encodes a peptidoglycan deacetylase (this work); *yoeB*, which modulates autolysin activity (Salzberg and Helmann, 2007) and *ydjM*, which has a cell wall associated function (this work).

(2) This work also shows that the two endopeptidase-type autolysins LytE and YvcE(CwlO) function in cell wall turnover and synthesis respectively, that one or other of these enzymes is required for cell growth and that they play a redundant essential role in lateral cell wall synthesis and cell elongation. A model to explain the different physiological roles of functionally homologous autolysins LytE and YvcE(CwlO) is proposed based on these results.

(3) In addition, the up-regulation of the fatty acid desaturase *des* upon YycFG depletion indicates a role for YycFG in controlling cell membrane metabolism.

(4) A genetic analysis performed in this work shows that YycFG essentiality is polygenic in nature, arising from the control of a number of genes involved in cell wall homeostasis.

(5) Finally, on the basis of this and previous studies, it is proposed that the essential TCS YycFG senses one or more signals, likely associated with cell wall or cell membrane metabolism, that indicate conditions favourable to growth; this results in activation of the YycFG regulon and hence in the expression of proteins that are required for the correct timing and location of cell wall synthesis and turnover.

2.3.2. YycFG controls expression of genes involved in cell wall metabolism

The evidence presented in this work shows that the TCS YycFG is a regulator of cell wall metabolism. An updated and complete listing of the constituent genes of its regulon is presented in Table 2.4 YycFG controls expression of autolysins [YocH, YkvT, YvcE(CwlO) and LytE], proteins that modify autolysin activity (YjeA and YoeB), teichoic acid biosynthetic proteins (TagAD) and other cell wall-associated proteins (YdjM).

Of the newly identified members of the YycFG regulon, some have been previously characterized: here a brief overview of the data present in the literature will be given for each gene, followed by the original contribution of this work to the understanding of their function and regulation. The previously identified member of the YycFG regulon *yocH* (Howell *et al.*, 2003, 2006), encoding a putative amidase autolysin, was included in our phenotypic analysis to gain insight into its function singularly or in combination with other YycFG-regulated genes.

Table 2.4. The YycFG regulon in *Bacillus subtilis*

Gene	Direct binding by YycF	Regulation by YycFG	Function
<i>yocH</i>	yes	+	Putative amidase autolysin
<i>yvcE (cwlO)</i>	yes	+	Endopeptidase-type autolysin
<i>ydjM</i>	yes	+	Secreted protein, cell-wall associated ^a
<i>ykvT</i>	yes	ND	Putative autolysin
<i>lytE</i>	yes	+	Endopeptidase-type autolysin
<i>tagAB</i>	yes	ND	Teichoic acid biosynthesis
<i>tagDEF</i>	yes	ND	Teichoic acid biosynthesis
<i>phoPR</i>	yes	ND	TCS activated by phosphate limitation
<i>yoeB</i>	yes	–	Modulator of autolysin activity ^b
<i>yjeA</i>	yes	–	Peptidoglycan deacetylase ^a
<i>ftsAZ</i> ^c	yes	+	Cell division

a. This work

b. Salzberg and Helmann (2007)

c. Fukuchi *et al.* (2000).

+, activation; –, repression; ND, not determined.

2.3.3. YvcE(CwlO) expression is YycFG-dependent; YvcE(CwlO) is involved in peptidoglycan synthesis

The YvcE(CwlO) protein has been classified as a member of the DL-endopeptidase autolysin family II (Smith *et al.*, 2000). This protein contains a C-terminal NlpC/P60-type peptidase domain (Yamaguchi *et al.*, 2004), which overlaps a CHAP domain (cysteine, histidine-dependent amidohydrolases/peptidase), a recently characterized motif that identifies a new superfamily of peptidoglycan hydrolases (Rigden *et al.*, 2003). The C-terminal portion of the protein was confirmed to possess a DL-endopeptidase activity and to be able to hydrolyze peptidoglycan *in vitro* (Yamaguchi *et al.*, 2004). YvcE(CwlO) has an N-terminal secretory (Sec-type) signal peptide (Tjalsma *et al.*, 2000) and is secreted into the growth medium *via* the Sec protein secretion machinery (Jongbloed *et al.*, 2002). The N-terminal portion of this autolysin seems to lack an obvious cell wall binding motif: only two coiled-coil domains, whose significance is uncertain, can be identified in this region (protein domain searches were performed using NCBI Conserved Domain Database, Pfam and SMART databases). However, although the YvcE(CwlO) N-terminal portion has no paralogue in *B. subtilis*, it exhibits high sequence similarity with a number of cell wall binding proteins from different organisms, and among them the N-terminal domain of the putative LytE endopeptidases from *B. cereus* and *B. anthracis* (searches were performed using the BLAST bioinformatics tool, Altschul *et al.*, 1997). It is possible, therefore, that cell wall binding domains are present in the N-terminal portion of YvcE(CwlO), although they cannot be detected by currently available bioinformatics tools. A proteomics study performed on secreted proteins as well as on cell wall binding proteins showed that YvcE(CwlO) was abundantly present in the extracellular fraction, but was absent from the cell wall proteome, suggesting that it might not be anchored to the cell wall or it might be loosely attached to it (Antelmann *et al.*, 2002). However, the possibility that YvcE(CwlO) was not properly extracted from the cell wall or was degraded during the extraction process cannot be excluded. Nevertheless, the presence of cell wall-bound proteins in the extracellular proteome is not an unusual feature in *B. subtilis*, and can be explained by the turnover of the cell wall, which results in the shedding of these proteins into the growth medium (Tjalsma *et al.*, 2004). The full-size YvcE(CwlO) protein (50.8 kD), together with two degradation products (both 30 kD in size), were found in the extracellular proteome of exponentially growing cells, while only smaller degradation products (20 kD in size) was present in the growth medium during stationary phase. The full-size product was found during stationary phase only in a protease-deficient strain, indicating that YvcE(CwlO) is subjected to proteolytic degradation, especially

during stationary phase (Antelmann *et al.*, 2002). In addition, the *yvcE* gene was shown to be expressed only during early exponential phase in sporulation medium (Yamaguchi *et al.*, 2004). YvcE(CwlO) expression, therefore, seems to be confined to the exponential phase of the growth, and to be tightly regulated both at the transcriptional and post-transcriptional levels.

In this study we show that the *yvcE(cwlO)* transcriptional profile is determined by the TCS YycFG. In the wild-type strain a high level of *yvcE(cwlO)* transcript is seen only during the early part of exponential growth, with a significant decrease observed even before the transition point (see Figure 2.1). This profile appears to be entirely dependent on YycFG since depletion of the TCS or mutation of the YycF binding sites greatly reduces expression (see Figures 2.1, 2.5). Furthermore, the unusual features of the *yvcE* promoter (the absence of a -35 motif at the canonical position, see Table 2.2) and the fact that YycFG is activated only during exponential growth (Howell *et al.*, 2003, 2006) may both contribute to restrict *yvcE* expression to the exponential period of the growth cycle.

This work also demonstrates the involvement of YvcE(CwlO) in peptidoglycan synthesis: in a *yvcE* null mutant the rate of peptidoglycan synthesis was diminished, while peptidoglycan turnover was not affected (see Figure 2.20A,B). This result is somewhat counter-intuitive, given the established function of YvcE(CwlO) as a cell wall degrading enzyme, and hence its expected involvement in cell wall turnover rather than cell wall synthesis. However, this and previous works show that deletion of a number of autolysins has no detectable effect in cell wall turnover: besides YvcE(CwlO), also the autolysins YocH and LytD seem not to be directly involved in cell wall turnover (this work, Figure 2.20B; Margot *et al.*, 1994). A possible explanation for the involvement of YvcE(CwlO) in peptidoglycan synthesis might derive from the currently proposed model for cell wall formation in bacteria: a number of studies indicate that cell wall synthesis may be carried out by a multienzyme complex, comprising murein hydrolases and synthases, as well proteins responsible for proper localization of the complex (Divakaruni *et al.*, 2005; Holtje, 1998; Kruse *et al.*, 2005; Leaver and Errington, 2005; van den Ent *et al.*, 2006; von Rechenberg *et al.*, 1996). Therefore it is conceivable that disruption of one component of such a complex may affect the efficiency of the whole peptidoglycan synthetic machinery. The autolysin YvcE(CwlO), although not a direct participant in the murein biosynthetic pathway, might be a component of such a complex, and its deletion might influence peptidoglycan synthesis in an indirect way. YvcE(CwlO) might interact with the other components of the complex through its N-terminal domain, which contains two coiled-coil motifs, often involved in protein-protein interactions.

However this hypothesis awaits experimental validation. It is noteworthy that although the model of a multienzyme complex for peptidoglycan synthesis has accumulated increasing experimental support in the last ten years, to date there is no direct evidence for the involvement of an autolysin as part of such complex. Therefore testing the hypothesis that the YvcE(CwlO) autolysin is a component of this complex is an attractive subject for future investigation, as it might provide the final proof of the presence of autolysins within the peptidoglycan synthetic multiprotein complex.

2.3.4. The autolysin YvcE(CwlO) and the putative autolysin YocH may be redundantly functional in protecting the structural integrity of the cell envelope

It is interesting to note that the YycFG-regulated putative autolysin YocH exhibits similar features to the ones displayed by YvcE(CwlO): (i) it is found in the extracellular proteome and not in the cell wall proteome, indicating that it is shed into the growth medium (Antelmann *et al.*, 2002); (ii) it is secreted during the exponential growth, but disappears from the medium upon entry into the stationary phase (Antelmann *et al.*, 2002), and (iii) its expression is restricted to the exponential phase of the growth in a YycFG-dependent-manner (this work; Howell *et al.*, 2003, 2006).

Such similarities may account for the phenotypes observed in this work in a strain deleted for both autolysins: this strain lysed on LB agar plates after five days of incubation at room temperature, and suppressors arose after seven days from the lysed colonies (see Figure 2.14). These phenotypes might indicate that YocH and YvcE(CwlO) are redundantly functional in protecting the structural integrity of the cell envelope. In addition, these phenotypes are reminiscent of the ones displayed during growth in LB broth by YycFG-depleted cells, suggesting that lowered expression of *yocH* and *yvcE(cwlO)* may partially contribute to the cell growth defects of YycFG-depleted cultures.

2.3.5. YycFG controls LytE expression during exponential phase; LytE is involved in peptidoglycan turnover

This work shows that the TCS YycFG regulates expression of two autolysins belonging to the DL-endopeptidase-type II family: YvcE(CwlO) and LytE. The two autolysins exhibit high levels of similarity in their C-terminal catalytic domains, both containing a NlpC/p60 domain overlapping with a CHAP motif (Rigden *et al.*, 2003; Yamamoto *et al.*, 2003). Furthermore, both proteins possess an N-terminal secretory (Sec-type) signal peptide (Tjalsma *et al.*, 2000). However YvcE(CwlO) and LytE differ in the remaining part of their N-terminal domain:

while no obvious cell wall binding motifs can be identified in YvcE(CwlO), LytE possesses three LysM motifs, which are thought to function as general peptidoglycan binding modules (Bateman and Bycroft, 2000; Yamamoto *et al.*, 2003). This structural difference may account for the different localization pattern of the two proteins: while YvcE(CwlO) was not found in the cell wall proteome of *B. subtilis*, LytE was identified in the cell wall binding protein fraction, indicating a stronger attachment to the cell wall (Antelmann *et al.*, 2007; Margot *et al.*, 1998; Yamamoto *et al.*, 2003).

This and previous work indicate that *lytE* is subjected to a fine transcriptional control from a number of positive and negative regulators. In previous work *lytE* was shown to be expressed only during exponential phase in SM sporulation medium (Ishikawa *et al.*, 1998). Such a profile differs from the one observed in this work during growth in LB, where *lytE* expression increases upon entry into stationary phase (Figure 2.7A). Recent work has shown that the master regulator of sporulation, Spo0A, is a repressor of *lytE* expression (Fujita *et al.*, 2005; Kodama *et al.*, 2007; Molle *et al.*, 2003); therefore it is likely that Spo0A, which is activated upon entry into stationary phase in sporulation medium, is responsible for turning off *lytE* expression in SM. Crucially, this work extends previous findings, by showing that YycFG controls *lytE* expression during exponential growth. In addition, we show that the induction of *lytE* transcription observed in LB medium during stationary phase is YycFG-independent, and is likely to be due to a further transcriptional regulator, yet to be identified.

Interestingly, LytE expression seems to be tightly regulated both at a transcriptional level and at a post-transcriptional level, being subjected to proteolytic degradation by the extracellular proteases WprA and Epr (Yamamoto *et al.*, 2003), in a way similar to that observed for YvcE(CwlO).

Although the LytE specific DL-endopeptidase enzymatic activity has not been confirmed, LytE was shown to possess a cell wall hydrolytic activity *in vitro* (Ishikawa *et al.*, 1998; Margot *et al.*, 1998). In addition, LytE was found to be involved in cell separation together with the endopeptidase LytF (Ohnishi *et al.*, 1999). Consistent with this finding, LytE was localized at cell separation sites and cell poles by immunofluorescence microscopy (Yamamoto *et al.*, 2003). However, recent work from Carballido-Lopez *et al.* (2006) extended the role of LytE, by showing that it is also positioned in a helical pattern along the cylindrical cell wall of growing cells, a distribution that requires the interaction between the cytoskeleton actin-like protein MreBH and LytE to target the autolysin to this location. The authors proposed that LytE is required for controlled cell wall maturation during growth, being inserted into newly synthesized peptidoglycan and participating in cell wall turnover.

Crucially, our results support their prediction, by showing that in a *lytE* null mutant the rate of peptidoglycan turnover is decreased (see Figure 2.20B).

2.3.6. Lateral cell wall synthesis and cell elongation have an essential requirement for an endopeptidase-type autolysin activity to which LytE or YvcE(CwlO) redundantly contribute

This work shows that a $\Delta lytE \Delta yvcE(cwlO)$ double mutant strain is not viable, and that a strain lacking *lytE* and expressing *yvcE* under the control of a xylose-inducible promoter grows only in the presence of the inducer (see Figure 2.8A). These findings indicate that one or the other gene is required for growth. Furthermore, depletion for both *lytE* and *yvcE* results in aberrant cells that are short, bent, bulging and occasionally empty (see Figure 2.8B). This very same phenotype has been reported for cells mutated in genes that participate in lateral cell wall metabolism and cell elongation *eg. mreC, mreD, mbl* and *lytE/mreBH* at low Mg^{2+} concentrations (Carballido-Lopez and Errington, 2003; Carballido-Lopez, 2006; Carballido-Lopez *et al.*, 2006; Daniel and Errington, 2003; Jones *et al.*, 2001; Leaver and Errington, 2005; Soufo and Graumann, 2003). In addition, cells depleted for both *lytE* and *yvcE* are deficient in lateral cell wall synthesis, as determined by visualization of newly synthesized peptidoglycan with fluorescein-labelled vancomycin (see Figure 2.8B). The combination of the above results shows that lateral cell wall synthesis and cell elongation have an essential requirement for an endopeptidase-type autolysin activity to which LytE or YvcE(CwlO) redundantly contribute.

These findings advance the current knowledge on bacterial cell wall synthesis and elongation, providing new insights on the role of autolysins in this process. Although the breakage of covalent bonds within the peptidoglycan is thought to be essential for cell wall expansion, so far a clear link between autolytic activity and cell elongation has not been established in *B. subtilis*. Strains with mutations in multiple autolysin-encoding genes have been constructed (Blackman *et al.*, 1998; Fukushima *et al.*, 2006; Ohnishi *et al.*, 1999; Smith *et al.*, 1996); however, none displayed a cell wall elongation defect. Two possible explanations have been postulated for the seeming lack of an essential autolysin activity: (i) the large number and possible functional redundancy of autolysins in *B. subtilis* has hampered the construction of a strain that reveals a role for autolysins in cell expansion, or (ii) elongation of the cell does not require autolysins, but occurs through the physical stretching of the outer peptidoglycan layers, that fractures the critical covalent bonds thereby allowing expansion (Smith *et al.*, 2000). This work allows discrimination between these two

explanations, showing that an enzymatic activity is required for cell elongation, that had not been identified so far because of the functional redundancy of the LytE and YvcE(CwlO) autolysins in *B. subtilis* (Smith *et al.*, 1996, 2000). Another possible contributing factor is that LytE and YvcE(CwlO) are defined as “minor” autolysins (Smith *et al.*, 2000), and for this reason they might not have been considered as likely candidates for such a crucial function. More importantly, this work presents for the first time a direct link between an essential autolytic function and cell elongation in *B. subtilis*, showing that a DL-endopeptidase-type murein hydrolase activity is an essential requirement for cell growth.

The only report in the literature of the involvement of an autolysin in cell elongation in *B. subtilis* is the work by Carballido-Lopez *et al.* (2006) on LytE. However, cell morphology defects indicative of an aberrant cell elongation were observed in a *lytE* null mutant strain only in conditions of depletion for Mg^{2+} , indicating that LytE is not required for cell elongation under normal growth conditions. In agreement with this result, our work shows that a *lytE* null mutant is capable of cell expansion during growth in rich medium, and it displays cell elongation defects only in a *yvcE* depleted background. Therefore our results extend previous findings, confirming LytE involvement in cell elongation, and showing that this autolysin contributes with YvcE(CwlO) to an essential function.

In addition, this work provides an insight into the requirements and mechanism of lateral cell wall synthesis and cell elongation during growth. A helical pattern of peptidoglycan insertion has been observed in the elongating cell wall in *B. subtilis* (Daniel and Errington, 2003). Current models propose that the bacterial actin homologs MreB, Mbl and MreBH, which form helical cables within the cell, are responsible for the spatial organization of the peptidoglycan synthetic machinery. The MreC and MreD membrane proteins would provide the bridge between the bacterial actin-like cytoskeleton and the extracellular cell wall synthetic complex, thereby resulting in a pattern of newly synthesized peptidoglycan [for reviews see Scheffers and Pinho (2005); Carballido-Lopez (2006)]. Carballido-Lopez *et al.* (2006) showed that LytE is located helically along the cell cylinder and interacts with MreBH, which targets it to its subcellular location. Our finding that LytE and YvcE(CwlO) contribute to an essential function in cell elongation suggests that the two proteins might have a similar pattern of localization and might be targeted to their final locations in a similar way. We hypothesize that YvcE(CwlO) might interact with one of the other actin isoforms MreB or Mbl, which would recruit it in the cytoplasm for insertion into the sites of wall assembly (model under investigation). Finally, these findings strongly support the vision of the TCS

YycFG as a general regulator of cell wall metabolism, showing its direct involvement in controlling the essential process of cell expansion during growth.

2.3.7. A model for the role of autolysins in peptidoglycan synthesis: functionally homologous autolysins may exert different functions by being targeted to different sub-cellular locations

This work shows that the two endopeptidase-type autolysins YvcE(CwlO) and LytE share an essential function in lateral cell wall synthesis and cell elongation, but at the same time are involved in different cell wall metabolic processes, playing a role in peptidoglycan synthesis and turnover respectively. It is conceivable that the differences in the N-terminal portion of the two proteins may account for their functional diversity. Indications for this hypothesis come from the different cellular localization of the two proteins. As anticipated above, YvcE(CwlO), which lacks cell wall binding domains, appears to be loosely attached to the cell wall, being found only in the supernatant and not in the cell wall extracts (Antelmann *et al.*, 2002; Antelmann *et al.*, 2007). On the contrary LytE, which possesses three lysM cell wall binding domains, was identified in the cell wall binding protein fraction, indicating a stronger attachment to the cell wall (Antelmann *et al.*, 2007; Margot *et al.*, 1998; Yamamoto *et al.*, 2003). As mentioned above, results from this work indicating a role for YvcE(CwlO) in cell wall synthesis might be explained by its involvement in the multienzyme peptidoglycan synthetic machinery. In addition, Carballido-Lopez *et al.* (2006) proposed that LytE, after being seeded into the newly synthesized peptidoglycan, moves outwards as new peptidoglycan layers are underlaid during cell elongation, and becomes active only on the fully stretched peptide bonds present in the outermost region of the wall: according to their model, LytE function would be to cleave the older stretched cell wall material at the periphery of the cell in order to balance the helical insertion of the new material at the membrane surface.

On the basis of the above models and observations it is tempting to speculate that the different roles of YvcE(CwlO) and LytE in cell wall homeostasis might derive from their different subcellular localization: LytE, being strongly bound to the cell wall, would remain attached to the newly synthesized peptidoglycan strands it and would exert its function while it moves outwards through the cell wall layers or, as suggested by Carballido-Lopez *et al.* (2006), only at the very periphery of the cell. On the other hand YvcE(CwlO), which lacks strong cell wall binding domains, may remain located in the periplasmic space, and may interact with the other components of the peptidoglycan synthetic machinery through its N-terminal coiled-coil domains, which are often involved in protein-protein interactions.

The finding that the two DL-endopeptidase-type autolysins YvcE(CwlO) and LytE can substitute each other in performing an essential task required for cell elongation might appear to conflict with the different roles they play in cell wall homeostasis, being involved in peptidoglycan synthesis and turnover respectively. However, not only can such an apparent contradiction be resolved, but it may also provide further insights into the roles of these two autolysins. The fact that cell elongation can occur in the absence of either YvcE(CwlO) or LytE means that either protein is capable of providing an essential mureinolytic endopeptidase activity that is required for insertion of new peptidoglycan units along the cell cylinder, thereby allowing cell expansion. This finding indicates that LytE can act not only at the very periphery of the cell wall, as proposed by Carballido-Lopez *et al.* (2006), to release the tension of the old stretched cell wall material, but also proximal to the cell wall biosynthetic complex at the membrane, to allow insertion of the newly synthesized peptidoglycan monomers into the growing peptidoglycan strands.

Interestingly, the finding that in a *yvcE* null mutant the rate of cell wall synthesis is diminished indicates that LytE can substitute for YvcE(CwlO) only to a certain extent. This result is entirely consistent with the model proposed: YvcE(CwlO), being part of the periplasmic cell wall synthetic multienzyme complex, would act more efficiently on the growing peptidoglycan strands newly synthesized by the complex, while LytE, after being seeded into the periplasm, would remain attached to the newly synthesized peptidoglycan only transiently and would then be dragged outward following its movement through the cell wall. Conversely, the finding that in a *lytE* null mutant the rate of cell wall turnover is significantly lowered, indicates that YvcE(CwlO) cannot substitute LytE in its function in peptidoglycan turnover: this result can be explained by the proposed periplasmic location of YvcE(CwlO), which, lacking strong cell wall binding motifs, would remain attached to the peptidoglycan synthetic complex and would not act on the outer cell wall layers.

Yeast two-hybrid experiments and pull-down experiments might be used in order to validate the model proposed and to test the presence of YvcE(CwlO) within (and the absence of LytE from) the multienzyme complex. Such analysis might contribute to define the different roles of autolysins, whose investigation has been largely hampered by their functional redundancy (Smith *et al.*, 2000). According to the proposed model, the subcellular localization of functionally equivalent autolysins, mediated by their different N-terminal motifs, would be the critical determinant of their distinct physiological functions.

2.3.8. YdjM has a cell wall associated function

YdjM is a protein of unknown function whose expression is activated by YycF and is highest during the exponential period of the growth cycle. This protein has no orthologue in current databases; it has an N-terminal secretory (Sec-type) signal peptide, hence it is predicted to be secreted into the growth medium *via* the Sec protein secretion machinery (Tjalsma *et al.*, 2000).

This work shows that strains carrying a *ydjM* null mutation have a shiny colony morphology, a reduced growth rate in LB broth, reach stationary phase at a lowered optical density, display a reduced sporulation frequency and the cells are shorter than wild-type during exponential growth. However, it was noted that the growth defect observed during culture of strain BP068 (*ydjM::tet'*) in LB was less evident when cultured in Schaeffer's and Sterlini-Mandelstam media. The latter media contain high concentrations of Mg^{++} (Nicholson and Setlow, 1990; Schaeffer P, 1965), an ion capable of restoring almost wild-type growth and morphology to mutants impaired in cell wall homeostasis (Carballido-Lopez *et al.*, 2006 and references therein). This work shows that addition of 25mM $MgCl_2$ to LB medium rescues both the growth and the sporulation defects exhibited by a *ydjM* null mutant strain, strongly suggesting that *ydjM* participates in cell wall homeostasis.

2.3.9. The YjeA protein functions to deacetylate peptidoglycan, altering its susceptibility to lysozyme digestion

The *yjeA* gene encodes a putative peptidoglycan deacetylase. Expression of *yjeA* is low during exponential growth of the wild-type strain, but increases in cells depleted for YycFG, and is therefore negatively regulated by this TCS. The YjeA protein is predicted to have a transmembrane domain in the amino terminal region, with the bulk of the protein located external to the cytoplasmic membrane. Two studies confirm that YjeA is a membrane protein, a location consistent with its proposed function, *ie.* deacetylation of the cell wall peptidoglycan (Eymann *et al.*, 2004; Tjalsma and van Dijk, 2005).

Numerous studies have shown that in several bacterial species deacetylation of the N-acetylglucosamine moiety of the peptidoglycan carbohydrate chain makes it more resistant to lysozyme degradation (Amano *et al.*, 1977; Araki *et al.*, 1980; Atrih *et al.*, 1999; De Las Rivas *et al.*, 2002; Psylinakis *et al.*, 2005; Veiga *et al.*, 2007; Vollmer and Tomasz, 2000, 2002; Westmacott and Perkins, 1979). This work shows that cell walls prepared from YycFG depleted cells display an increased resistance to lysozyme degradation that is dependent on YjeA activity. In other words, increased *yjeA* expression in YycFG depleted cells results in the

cell wall peptidoglycan becoming less acetylated and hence more resistant to lysozyme digestion. This result is consistent with the *yjeA* expression profile, the location of the protein and the known properties of peptidoglycan deacetylases.

Interestingly, the percentage of unsubstituted amino sugars in the cell walls of pathogenic species like *S. pneumoniae*, *B. cereus*, *B. anthracis* and *B. thuringiensis* is much higher than in the non-pathogenic *B. subtilis* (Atrih *et al.*, 1999; Psylinakis *et al.*, 2005; Vollmer and Tomasz, 2000; Zipperle *et al.*, 1984). This datum is consistent with lysozyme being a first line of defence of the host organism against infection (Vollmer and Tomasz, 2002): pathogenic bacteria have probably evolved highly de-acetylated peptidoglycan as a way to increase their resistance against the lysozyme of the human host. In *S. pneumoniae*, a strain carrying a null mutation in the N-acetylglucosamine deacetylase *pgdA* was shown to be hypersensitive to lysozyme digestion, to produce fully N-acetylated glycan and to cause a drastic reduction in virulence in a mouse model: these results allowed to indicate Pgda as the main enzyme responsible for pneumococcal peptidoglycan deacetylation in this bacterium (Vollmer and Tomasz 2000, 2002).

Of the six peptidoglycan deacetylase homologues that are present in the *B. subtilis*, three (*pdaA*, *pdaB* and *ylxY*) are involved in sporulation (Blair and van Aalten, 2005; Eichenberger *et al.*, 2004; Fukushima *et al.*, 2002, 2004, 2005; Gilmore *et al.*, 2004), while *yjeA* is the first deacetylase established to be active during exponential growth in *B. subtilis* (this work). This observation, together with the results from this work outlined above, suggests that YjeA might be the primary, if not the only enzyme responsible for de-N-acetylation of peptidoglycan during vegetative growth in *B. subtilis*, in a way similar to what observed for Pgda in *S. pneumoniae*. Nevertheless, cell walls prepared from a *yjeA* null mutant strain displayed a lysozyme digestion profile comparable to the wild-type (Figure 2.22), apparently indicating that YjeA does not have a major impact on the sensitivity to lysozyme digestion of *B. subtilis* cell walls. However, it is critical to observe that in a wild-type background *yjeA* transcriptional levels are very low, and increase significantly only in conditions of depletion for YycFG (this work, Figures 2.2, 2.5), and that unlike in *S. pneumoniae*, the level of glucosamine de-acetylation in *B. subtilis* cell walls is low, occurring only on only 17.3% of the total muropeptides (Atrih *et al.*, 1999). For these reasons it is conceivable that a *yjeA* null mutation does not have a detectable effect on the lysozyme digestion profile of *B. subtilis* cell walls, and this result does not exclude the possibility that YjeA might be the main peptidoglycan deacetylase that is active during vegetative growth in *B. subtilis*. In order to test this hypothesis, we are planning to compare the level of de-acetylation of cell walls taken from

wild-type cells and from *yjeA* null mutant cells. If YjeA is the main vegetative peptidoglycan deacetylase in *B. subtilis*, the de-acetylation levels of mucopeptides would be lower than the 17.3% observed by Atrih *et al.*, (1999).

Studies on *S. pneumoniae* and *L. lactis* have shown that changing the acetylation level of peptidoglycan alters its susceptibility not only to lysozyme digestion, but also to endogenous autolysins (De Las Rivas *et al.*, 2002; Veiga *et al.*, 2007; Vollmer and Tomasz, 2000). Consistent with these findings, it has been proposed that peptidoglycan deacetylases may play a role in controlling the activity of cell wall hydrolyzing enzymes, which may be differentially active towards substrates with different acetylation levels (Atrih *et al.*, 1999; Vollmer and Tomasz, 2000). This work shows that the auto-digestion capability of *B. subtilis* cell walls is not altered in a *yjeA* null mutant, and it is only slightly affected by the *yjeA* null mutation in a strain depleted for YycFG (see Figure 2.23). This result seems to indicate that peptidoglycan de-acetylation has little or no effect in controlling autolysins activity in *B. subtilis*. However, studies in *S. pneumoniae* have shown that altering the acetylation level of peptidoglycan can have opposite effects on different autolysins (De Las Rivas *et al.*, 2002; Vollmer and Tomasz, 2000). Therefore it is possible that the global effect of *yjeA* deletion on autolysins' activities derives from the sum of positive and negative effects, and that individual effects might be masked under our experimental conditions. It will be interesting to test the effect of peptidoglycan de-acetylation on the activity of individual autolysins, particularly on the ones whose expression is regulated by YycFG, namely YvcE, LytE and YocH.

Although the precise role of YjeA in controlling autolysin activity in *B. subtilis* remains to be established, the presence of this protein among the ones regulated by YycFG further exemplifies the role of this TCS in controlling cell wall metabolism. The *B. subtilis* genome encodes up to 35 autolysins expressed during growth, stationary phase, sporulation and phage induction (Smith *et al.*, 2000). Control of autolytic activity is crucial to ensuring cell growth and expansion is regulated and to prevent the cells from committing suicide by the inappropriate action of these potentially autolytic enzymes. In this study we add regulated expression by the TCS YycFG and post-transcriptional control of autolysin activity by the peptidoglycan deacetylase *yjeA* to the many regulatory mechanisms already established to control expression, localization and activities of autolysins.

Intriguingly, YjeA homologues occur only sporadically in bacteria that have a YycFG orthologue. There are 6 putative peptidoglycan deacetylases encoded in each of the *B. cereus* and *B. anthracis* genomes, but only two in the *Bacillus halodurans* genome (Psylinakis *et al.*, 2005). There are two orthologues in *S. mutans* and a single orthologue in *S. pneumoniae*

(Vollmer and Tomasz, 2000; 2002), *L. lactis* (Veiga *et al.*, 2007), *E. faecalis*, *S. pyogenes*, *S. agalactiae* and *Listeria* species. However there is no orthologue present in *S. aureus*, *S. epidermidis* or *Lactobacillus* species. Interestingly, potential YycF binding motifs can be identified within the promoter regions of some, but not all of the genes encoding peptidoglycan deacetylases in different species; however, the significance of such putative motifs is uncertain. These observations indicate that there is likely to be variability in the constituent genes of YycFG regulons in different bacterial species.

2.3.10. YycFG-depleted cells are highly deficient in auto-digestion activity, but none of the YycFG-controlled genes *yocH*, *yvcE*, *lytE* or *yjeA* contributes to this effect

This work shows that native cell walls prepared from growing cells have significant autodigestive capability, while those prepared from YycFG depleted cells harvested at the point of growth cessation are highly deficient in such activities (see Figure 2.23). Surprisingly, mutation of *YocH*, *YvcE*(CwlO), *LytE* or *YjeA* did not decrease the autodigestion capability, indicating that none of these YycFG-regulated proteins contributes to this effect.

There are two possible explanations for this result: (i) it is possible that YycFG controls additional autolysins or peptidoglycan modifying encoding genes that have not been identified in this study: mis-regulation of such genes might be responsible for the auto-digestion defect displayed by YycFG-depleted cells; (ii) the autolytic activity of YycFG-depleted cell walls may be inhibited by the elevated levels of *YoeB* that occur under these conditions, since Helmann and co-workers have shown that *YoeB* modulates autolysin activity and protects cells from autolysis (Salzberg and Helmann, 2007). It is also possible that a combination of the two effects is responsible for the observed result. Since our work shows co-regulation of the *YoeB* protein and the autolysins *YocH*, *LytE* and *YvcE*(CwlO), Salzberg and Helmann (2007) have proposed these autolysins as potential targets of *YoeB* action. Although this does not seem to be the case for *YocH* (Salzberg and Helmann, 2007), exploring this possibility for *LytE* and *YvcE*(CwlO) might be an interesting topic for future work.

It is noteworthy that despite cell walls from YycFG-depleted cells being deficient in auto-digestion capability, cells lyse when starved for YycFG (see Figure 2.1). A possible explanation for this paradox might be an unbalanced synthesis/turnover ratio in YycFG depleted cells. The maintenance of a stable balance between the rates of peptidoglycan synthesis and turnover is known to be crucial for the cell, particularly in conditions when the rate of growth is lowered abruptly. In such cases an uneven slowing down of the rates of wall synthesis and turnover may result in lysis of the cell (de Boer *et al.*, 1982; Koch, 2001). This

work demonstrates that two of the genes positively regulated by YycFG play a role in cell wall synthesis (*yvcE*) and turnover (*lytE*). In cells depleted for YycFG (and hence also depleted for YvcE and LytE) the balance between synthesis and turnover might be affected, and the rates of synthesis and turnover might decrease in an uneven way, resulting in lysis of the cell.

2.3.11. YycFG essentiality is polygenic in nature

One of the aims of this study was to establish the reason for YycFG essentiality in *B. subtilis*. Since all YycFG regulated genes can be individually mutated, it was evident that essentiality must be polygenic (this study; Kobayashi *et al.*, 2003). This work establishes that YycFG controls expression of LytE and YvcE(CwlO), the functions of which partially overlap since one or other protein is essential for cell growth. However, the level of *lytE* transcript at the point of growth cessation upon YycFG depletion is not lower than that seen in wild type cells, although it does decrease subsequently (see Figure 2.7B), therefore it is unlikely that growth cessation is caused solely by depletion for LytE and YvcE(CwlO). This hypothesis is supported by the observation that YycFG cannot be deleted from cells constitutively expressing LytE and YvcE(CwlO), thereby showing that YycFG controlled expression of *yvcE(cwlO)* and *lytE* is not the sole reason for its essentiality. Furthermore, heterologous expression of *yvcE(cwlO)* during YycFG depletion ameliorates, but does not prevent growth cessation and lysis, indicating that YvcE(CwlO) contributes to the essentiality of YycFG but it does not account for it. Interestingly, there is some degree of similarity between the phenotypes of YycFG-depleted and *yvcE(cwlO) lytE*-depleted cells, which in both cases are occasionally devoid of nucleoids: this observation suggests that lowered levels of both *yvcE(cwlO)* and *lytE* might be partly responsible for the lysis observed upon YycFG depletion, and that the two genes might contribute significantly to the essentiality of YycFG.

Additional evidence suggests that YycFG essentiality derives from the control of a number of genes involved in cell wall metabolism. Firstly, concomitant deletion of the two autolysins encoding genes *yvcE(cwlO)* and *yocH* results in fragile cells, which lyse on LB agar plates. We observe colonies of apparent normal phenotype growing from these lysed colonies, suggesting the emergence of bacteria carrying suppressor mutations. This result indicates that the two autolysins YvcE and YocH may contribute to the essentiality of YycFG. In addition, in strains where *lytE* or *yvcE(cwlO)* null mutations are combined with null mutations in both *yocH* and *ydjM*, the resulting triple mutant strains are obtained at extremely low frequency, and the strain mutated in *yvcE(cwlO)*, *yocH* and *ydjM* has severe growth and morphological defects. These results suggest that YycFG controlled expression of the gene triplet

yvcE(cwlO), *yocH* and *ydjM* contributes significantly to its essentiality. This view is supported by some aspects of the phenotype of this triple mutant which are reminiscent of YycFG depletion: cells are growth defective in LB broth, they are very fragile and prone to lyse and they form short stumpy cells, some of which are devoid of nucleoids.

Therefore it is concluded that YycFG essentiality is polygenic in nature, arising from abnormal cell wall metabolism due to aberrant expression of multiple genes under YycFG control. These effects can be direct, as in YycFG-controlled expression of cell wall metabolism-related genes, or indirect, as suggested by the observation that cell walls of YycFG depleted cells are refractory to digestion by endogenous autolysins.

It is interesting to note that the YycFG-controlled autolysin YvcE(CwlO) contains an amino-terminal domains with amino acid sequence similarity to that of the pneumococcal PcsB, which accounts for YycFG essentiality in *S. pneumoniae* (Ng *et al.*, 2004). Intriguingly, the phenotypes observed in PcsB-depleted cells are consistent with a role for PcsB as an autolysin that balances the extent of cell wall synthesis and turnover: PcsB under-expression leads to excess cell wall synthesis and to a cell separation defect, indicating that PcsB may act as a murein hydrolase that counterbalances cell wall synthesis and participates in cell division (Ng *et al.*, 2004). These same functions are controlled by the *B. subtilis* YycFG through transcriptional regulation of *yvcE(cwlO)* and *lytE*, which are involved in cell wall synthesis, cell wall turnover and cell separation. On the bases of these findings, we propose that while a correct balance between cell wall synthesis and turnover in *S. pneumoniae* is guaranteed by the YycFG-controlled PcsB, in *B. subtilis* such a balance is achieved through YycFG-mediated expression of a number of genes that function in concert, and among them *yvcE(cwlO)* and *lytE*. Variation in the complexity of YycFG essentiality among bacteria (evidenced by the observations in *B. subtilis* and *S. pneumoniae*) is likely to be due to differences in cell wall metabolism, perhaps reflecting different bacterial shapes.

2.3.12. YycFG-dependent expression of *ydjM* derives from an unusual promoter structure

Many of the SigA-type promoters that are activated by YycF have unusual features. Within the *yvcE*, *ydjM* and *lytE* promoter regions, in addition to the YycF binding sequence, there is a canonical SigA-type -10 motif, but no putative -35 sequence is found at the expected position (17 bp from the -10 motif). However, putative -35 motifs can be identified within the promoters of these genes, although located at non-canonical positions (23, 11 and 14 bp from the -10 motif for *yvcE*, *ydjM* and *lytE* respectively). Two putative -35 motifs are

present within the *yocH* promoter region: one at the canonical site (previously assigned by Howell *et al.*, 2003) and a second one located 11 bp upstream from the -10 motif.

In order to get insights into the functional meaning of such aberrant promoter structures, we performed a mutational analysis focussing on the *ydjM* promoter. Our results show that *ydjM* expression is almost completely abolished when the YycF binding motifs are mutated. Furthermore, higher expression levels are obtained upon a 6 bp deletion that generates a canonical 17 bp spacing between the SigA-type -35 and -10 putative motifs and that concomitantly alters the distance between the -10 motif and the YycF binding site. Crucially, such deletion results in *ydjM* expression becoming constitutive throughout the growth cycle and independent of YycF. These findings show that YycF dependent expression of *ydjM* is the result of its unusual promoter structure and that it requires not only an intact YycF binding motif, but also a correct spacing between the YycF binding site and the -10 region. It is noteworthy that although the putative SigA-type -35 motif is functional when placed at a canonical position within this promoter, whether it plays a role in expression under wild-type conditions remains to be established.

The aberrant promoter structure observed in many YycF-activated genes is reminiscent of the one displayed by the promoters of the *B. subtilis* genes *spoIIG* and *spoIIE*, and the *Bacillus amyloliquefaciens* gene *phyC* (Guzman *et al.*, 1988; Kenney and Moran, 1987; Makarewicz *et al.*, 2006). The *spoIIG* and *spoIIE* promoters have SigA-type -10 motifs, but no recognizable -35 motifs are present at the canonical position, while putative -35 motifs can be identified at unusual positions, separated 21 and 22 nucleotides respectively from the -10 motif (Guzman *et al.*, 1988; Kenney and Moran, 1987). The *spoIIE* and *spoIIG* genes were shown *in vivo* to require high levels of phosphorylated Spo0A for transcription, consistent with their activation at the outset of sporulation (Fujita *et al.*, 2005). *In vitro* experiments showed that phosphorylation of Spo0A increases its affinity for weak 'OA' boxes located within the promoters (Baldus *et al.*, 1994; Strauch *et al.*, 1990). This mechanism restricts expression of these genes to periods when Spo0A~P levels are high in the cell, *ie* to the initial stage of sporulation. Interestingly, the Sigma A RNA polymerase binds to the -10 motifs, but not to the putative -35 regions of *spoIIG* and *spoIIE* promoters: the deleterious effects of a mutation in the -10 region on gene expression could be suppressed by a specific mutation in SigA, while at least in the case of *spoIIE*, the effects of mutations in the -35 region couldn't be suppressed by any known SigA mutations (Kenney *et al.*, 1988; Kenney *et al.*, 1989; York *et al.*, 1992). Consistent with these findings, Spo0A was shown to bind to sites overlapping the -35 motifs *in vitro* (Satola *et al.*, 1991; York *et al.*, 1992). In addition, a number of studies

showed that Spo0A interacts directly with SigA (Seredick and Spiegelman, 2001 and references therein). On the basis of these results, it was proposed that the binding of Spo0A~P at the 'OA' boxes and its interaction with the RNA polymerase overcome the steric constraints caused by the aberrant spacing between the -35 and -10 motifs, allowing the SigA-containing RNA polymerase (RNAP) to recognize the promoter and initiate transcription. A very similar mechanism was suggested for transcriptional activation of the *Bacillus amyloliquefaciens* gene *phyC* by the phosphorylated form of the response regulator PhoP (Makarewicz *et al.*, 2006). In both cases the unusual promoter structure would function to restrict activation of gene expression to periods when the specific transcriptional regulator is present at high levels in its phosphorylated form. Since activation of gene expression by YycF is most evident during the exponential period of the growth cycle (see Figure 2.2), it will be interesting to establish whether activation of YycFG-induced genes occurs by a similar mechanism. Experiments are underway to test this hypothesis.

Intriguingly, studies on *spoIIIG* transcriptional activation showed that RNAP can bind to the *spoIIIG* promoter region, but cannot efficiently initiate transcription on a linear template in the absence of Spo0A~P (Bird *et al.*, 1993). In accordance with this finding, recent work has shown that RNAP binds transiently and highly reversibly to the *spoIIIG* promoter, and that it recruits Spo0A~P, which subsequently stabilizes a closed complex between RNAP and the *spoIIIG* promoter, until the contacts between the RNAP and the -10 motif induce strand separation and hence transcription (Seredick and Spiegelman, 2004, 2007). However, there are indications that in the case of the aberrantly structured *Bacillus amyloliquefaciens* *phyC* promoter, PhoP~P might activate transcription through a different mechanism: since purified RNAP does not bind the promoter (Makarewicz *et al.*, 2006), it is possible that PhoP~P stimulates *phyC* expression by enhancing the initial binding of RNAP to the DNA. Although the latter hypothesis needs further experimental validation, these data indicate that transcriptional regulators can activate abnormally spaced promoters in two possible ways: by recruiting RNAP to the DNA or by stimulating a post-recruitment step and stabilizing an intermediate complex during transcription initiation. It will be interesting to establish which of the two mechanisms is employed by YycF for transcriptional activation, in order to provide further insights into this issue.

2.3.13. YycFG depletion alters cell membrane homeostasis

This study shows that expression of *des*, encoding a fatty acid desaturase, increases upon YycFG depletion (see Figure 2.1), and is therefore negatively regulated by this TCS. There are

no putative YycF binding sites located within the *des* promoter, and YycF~P does not bind to the promoter region, indicating that *des* regulation by YycFG is indirect.

This finding is interesting in light of previous studies showing that YycFG is involved in membrane integrity and fatty acid biosynthesis in *S. aureus* and *S. pneumoniae*. These studies have been outlined in the introduction to this thesis. In *B. subtilis* expression of *des* is stringently and uniquely regulated by the DesKR two-component system and is induced in response to a decrease in temperature (Aguilar *et al.*, 1998, 2001). However, there is evidence that membrane fluidity, rather than growth temperature controls *des* transcription: *des* induction is observed both after a downward shift in temperature, which causes increased rigidity of the membrane, as well as under isothermal conditions upon growth of cells in the absence of isoleucine in strain JH642, which results in decreased membrane fluidity by reduction of the pool of branched-chain fatty acids of plasma membrane lipids (Cybulski *et al.*, 2002). Under both conditions the TCS DesKR senses a decrease in membrane fluidity and induces *des* transcription; in turn the desaturase Des introduces double bonds into the fatty acid moieties of membrane phospholipids in order to maintain the appropriate membrane fluidity (Mansilla and de Mendoza, 2005).

On the basis of the above observations, it is concluded that the increased expression of Des observed in this study is the result of decreased membrane fluidity occurring upon YycFG depletion. This effect might be explained by three possible scenarios: (i) YycFG might be directly involved in regulating transcription of fatty acid biosynthetic genes, although such genes have not been identified in our microarray analysis: mis-regulation of these genes might be responsible for the decreased membrane fluidity sensed by the TCS DesKR in YycFG-depleted cells; (ii) alternatively, YycFG might participate in regulating phospholipid metabolism, and hence membrane composition, consistent with the close relationship between YycFG and phosphate metabolism (Howell *et al.*, 2003, 2006); (iii) finally, YycFG might be involved in other aspects of cell membrane metabolism, yet to be identified. In all cases depletion for YycFG would result in cell membrane perturbation and hence DesKR activation.

Indications in support to the first model come from a crucial observation: *des* induction has so far been observed in *B. subtilis* under isothermal conditions only in strain JH642, and only during growth in minimal medium lacking isoleucine (Cybulski *et al.*, 2002). Therefore this work represents the first instance of *des* induction obtained in a 168-derivative strain (depleted for YycFG), during growth at 37°C in rich medium. It is known that *B. subtilis* can respond to a decrease in membrane fluidity through two distinct mechanisms: one is the *de novo* synthesis of unsaturated fatty acids, which have a lower melting point than saturated

fatty acids and therefore provide a higher degree of membrane fluidity (Klein *et al.*, 1999); the second mechanism is the *des*-mediated desaturation of already existing fatty acid moieties in order to increase the pool of unsaturated fatty acids in the cell membrane (Hunger *et al.*, 2004). Strain JH642 contains two point mutations within the *bkdR* gene (Mohamed A. Marahiel, personal communication). This gene encodes a regulator that controls isoleucine and valine utilization as sole nitrogen sources (Debarbouille *et al.*, 1999). Isoleucine plays a crucial role as a precursor molecule in the *de novo* branched fatty acid synthesis pathway (Kaneda, 1977, 1991). Therefore strain JH642 can synthesize unsaturated fatty acids only in the presence of an external isoleucine source, while in the absence of isoleucine this strain can adjust membrane fluidity only through DesKR-mediated induction of the *des* desaturase. The fact that *des* induction has so far been observed at 37°C only in strain JH642, defective in the *de novo* synthesis of unsaturated fatty acids, when grown in the absence of isoleucine, indicates that the induction of *des* observed in this work in a 168-derivative strain depleted for YycFG during growth in rich medium is likely to be caused by a defect of YycFG-depleted cells in the *de novo* branched fatty acids synthetic pathway. Exploring the possibility of YycFG direct regulation of genes involved in branched fatty acid biosynthesis might be a potential topic for future study.

2.3.14. Cell wall metabolism, cell envelope and the nature of the signal sensed by the YycG sensor kinase

This work shows that YycFG activates expression of three autolysins, YocH, YvcE(CwlO) and LytE, two of which are required for cell elongation, and of the cell wall-related protein YdjM, while YycFG depletion results in de-repression of two genes that modulate autolysins activity: *yjeA* and *yoeB*. Intriguingly, Salzberg and Helmann (2007) have recently shown that the cell wall associated protein YoeB is activated in response to cell envelope stress and functions to protect *B. subtilis* from autolysis. In addition, although the precise role of the peptidoglycan deacetylase YjeA remains to be established, it is known that cell wall deacetylation modulates autolysins activity (Atrih *et al.*, 1999; De Las Rivas *et al.*, 2002; Veiga *et al.*, 2007; Vollmer and Tomasz, 2000). In other words, YycFG promotes transcription of genes required for normal growth and cell elongation and for a physiological cell wall homeostasis, and repress genes that might play a protective function in conditions of cell wall stress.

On the basis of these findings it is tempting to propose that the HK YycG might monitor cell wall metabolism during normal cell growth and might sense one or more signals

indicative of “physiological conditions”, transducing this information into the cell for continued expression of genes required for cell elongation and for maintenance of a proper cell wall homeostasis. On the other hand, cell envelope perturbation might hamper YycG activation, leading to de-repression of YoeB, which in turn would protect the cell wall from autolysis. This model implies that in conditions of cell envelope perturbation the activity of cell wall hydrolyzing enzymes might be prematurely triggered unless stress-response mechanisms intervene, and that YoeB induction would function as an emergency response to protect the cell from autolysis. Given the known role of peptidoglycan deacetylation in controlling autolysins activity, it is conceivable that also *yjeA* de-repression might participate in protecting the cell wall from autolysis during cell envelope stress.

According to this model, the TCS YycFG would regulate cell wall metabolism not only in physiological conditions, by modulating expression of genes required for proper cell wall homeostasis, but also in conditions of cell envelope stress, by preventing cell autolysis through de-repression of YoeB and YjeA and concomitant repression of the cell wall hydrolyzing enzymes YocH, LytE and YvcE(CwlO). This mechanism would allow the cell to monitor conditions conducive to cell growth as well as growth-limiting conditions through the same TCS: in conditions of cell envelope stress YycFG de-activation would function as an emergency mechanism to protect the cell from auto-digestion. This scenario would represent the first instance of a TCS responding to cell envelope stress through de-activation, *ie.* in the absence of the input signal, rather than through signal-mediated activation.

2.3.15. YycFG and cell division: extending the current model of the YycFG function and localization

In the absence of a known activating signal for YycFG, sub-cellular localization of the YycG HK was carried out in order to gain insights as to where the TCS functions and therefore where it receives its signal input (Szurmant *et al.*, 2007b). The *B. subtilis* YycG sensor kinase was found to localize at cell division sites, as revealed by immunofluorescent staining techniques (Szurmant *et al.*, 2007b). In addition, YycG was shown to co-localize with the cell divisional master regulator FtsZ (unpublished data in Surmant *et al.*, 2007b), and to require FtsZ for localization to the incipient septum (Szurmant *et al.*, 2007a). The two proteins YycG and FtsZ co-immunoprecipitate, indicating that they may directly interact or that they may be both part of a multi-protein complex (Szurmant *et al.*, 2007a). These results are consistent with the established role of YycFG in controlling cell division processes through transcriptional activation of the essential cell division genes *ftsAZ* (Fukuchi *et al.*, 2000). This

observation prompted the authors to propose a model whereby the HK YycG “associates with the cell division apparatus for signal acquisition and serves to coordinate the formation of the cell wall and other structural constituents with DNA synthesis and septation” (Szurmant *et al.*, 2007a). This model focuses on one aspect of YycFG function, *i.e.* its role in controlling cell division. However, our recent results demonstrate the involvement of YycFG in regulating additional physiological processes in *B. subtilis*: we show that YycFG is a major regulator of wall metabolism, and that it positively controls transcription of genes required for lateral cell wall synthesis and cell elongation (Bisicchia *et al.*, 2007). Therefore we reason that the proposal of a sensor YycG kinase confined to the septal region and functioning solely in promoting cell division and in coordinating DNA synthesis and septation might not reflect all aspects of YycFG function. We propose a model that extends the one put forward by Hoch and co-workers (Szurmant *et al.*, 2007a,b), by integrating the two aspects of YycFG-mediated control of cell division and cell elongation. We suggest that the *B. subtilis*, sensor kinase YycG may be located at septa, but also along the sides of the cell walls, where it might sense one or more signals indicative of conditions conducive to growth, transducing this information into the cell for continued expression of genes required both for cell division, and for cell elongation and maintenance of a proper cell wall homeostasis. A precedent exists whereby a protein, the cell wall-associated LytE, was originally localized at septa but subsequently observed over the lateral wall (Carballido-Lopez *et al.*, 2006; Yamamoto *et al.*, 2003). We reason that technical limitations might have hampered the visualization of YycG over the cylindrical part of the cell.

It is noteworthy that although direct up-regulation of the *ftsAZ* operon was observed upon YycFG over-expression, deletion of the *ftsAZ* promoter region containing the putative YycF-binding motif did not affect growth and did not alter the *ftsAZ* basal transcription level (Fukuchi *et al.*, 2000). These data show that YycFG is not required for basal *ftsAZ* transcription, and suggest that YycFG might mediate *ftsAZ* induction only in the presence of high levels of the RR YycF. On the basis of these findings, it might be interesting to consider the possibility that YycFG may play a role in coordinating the switch between cell wall elongation and cell division in *B. subtilis*. At the beginning of the cell cycle YycFG might control cell elongation and cell wall homeostasis by inducing transcription of genes with cell wall-related functions at a low threshold; accumulation of higher levels of phosphorylated YycF within the cell would then result in induction of the essential cell division genes *ftsA* and *ftsZ*, thereby promoting the formation of the septal apparatus.

CHAPTER 3
INVESTIGATION OF POSSIBLE INTERACTIONS
BETWEEN THE TCSs YycFG AND ResDE IN *B. subtilis*

3.1. INTRODUCTION

As anticipated in the general introduction to this thesis, the TCSs YycFG, ResDE and PhoPR of *B. subtilis* are very closely related phylogenetically (Fabret *et al.*, 1999). The HK YycG, ResE and PhoR belong to the homology class IIIA, where they form a “sub-group” (Fabret *et al.*, 1999). They share a very similar structure, with two transmembrane domains, a large extracytoplasmic loop, an intermediate-length linker region and a PAS domain (for a schematic representation of the structure of the HK YycG see Figure 1.2). Similarly to the corresponding HKs, also the RRs YycF, PhoP and ResD show high levels of homology. The three RRs have been classified according to different criteria as belonging to the OmpR family of RRs (Fabret *et al.*, 1999) or to the sub-class A within the OmpR family (Kojetin *et al.*, 2003), both classifications reflecting their common phylogenetic origin.

The structural similarity displayed by the three TCSs YycFG, ResDE and PhoPR is likely to reflect evolution from a common progenitor (Fabret *et al.*, 1999), but may also have a functional and physiological significance. This hypothesis is supported by a number of studies, which demonstrate the existence of a network of interactions between the TCSs YycFG and PhoPR, and between the TCSs ResDE and PhoPR. This scenario represents an extension to the understanding of the way TCSs operate. According to a general model, an environmental signal is sensed by a HK and converted into a very specific response through the activation of the cognate RR. However, studies in *E coli* (Hagiwara *et al.*, 2003) and *B. subtilis* (Birkey *et al.*, 1998; Sun *et al.*, 1996a) show that such a response can be achieved through a signaling network involving different TCSs that function *in concert*.

We propose that YycFG is part of a signaling network comprising the three TCSs YycFG, ResDE and PhoPR, at least under certain conditions. In this work we set out to investigate in particular the possible interactions between the YycFG and ResDE components of such network. An outline of the theoretical basis for such investigation will be given below.

3.1.1. The TCS PhoPR

The TCS PhoPR controls the response to phosphate limitation: the PhoR kinase senses reduced levels of extracellular inorganic phosphate and responds by phosphorylating the RR PhoP. The activated PhoP regulator controls the expression of genes whose function is to respond to limited phosphate availability (Allenby *et al.*, 2005; Hulett, 2002; Ogura *et al.*, 2001).

There are a number of links between the TCSs YycFG and PhoPR in *B. subtilis*. The first is their structural similarity, which was demonstrated experimentally by construction of two functional hybrid response regulators, PhoP'-YycF and YycF'-PhoP, that could be phosphorylated by the cognate kinase of their receiver domain (Howell *et al.*, 2003). A second link is manifest in the finding that the sensor kinase PhoR is able to phosphorylate the non-cognate RR YycF *in vivo*, under phosphate starvation conditions, while YycG can only activate its cognate RR YycF (Howell *et al.*, 2006). A further connection between YycFG and PhoPR is established by the requirement of YycFG for induction of a PhoPR-mediated phosphate limitation response. The mechanism of this dependence has not been established, although the observation that YycF binds to the *phoPR* promoter at DNA:protein ratios similar to those of PhoP suggests that YycFG is required for induction and/or continued expression of the PhoPR TCS in *B. subtilis* (Howell *et al.*, 2006). Finally, expression of some genes (*e.g. phoPR, tagAB, tagDEF*) is controlled by both YycFG and PhoPR (Howell *et al.*, 2006). These observations imply a complex relationship between the YycFG and PhoPR TCSs and their respective regulons.

3.1.2. The TCS ResDE

The TCS ResDE is required for control of aerobic and anaerobic respiration (Sun *et al.*, 1996b) and is also involved in controlling fermentation (Nakano *et al.*, 1997). The HK ResE and the RR ResD are transcribed from a five cistron operon, *resABCDE*, which encodes three further proteins, namely ResA, ResB and ResD, which are essential and are similar to those involved in cytochrome c biogenesis (Le Brun *et al.*, 2000). Two transcriptional units have been identified by northern blot analysis, one extending through all five cistrons of the operon (*resABCDE*), and a second shorter one encoding only ResDE (see Figure 3.1); the strength of the intracistronic promoter was shown to be low if compared with that of the *resA* promoter (Sun *et al.*, 1996b).

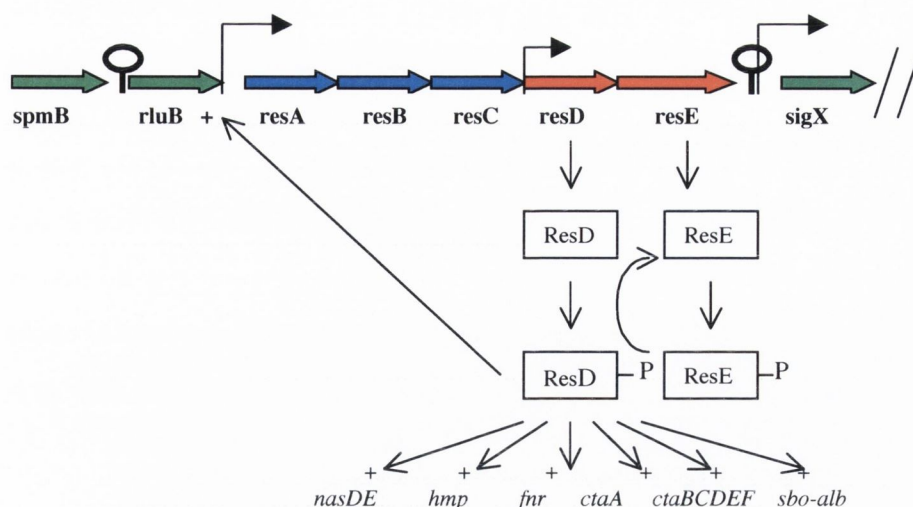


Figure 3.1. Diagram showing a layout of *resABCDE* operon. Block arrows indicate separate open reading frames and the direction of gene transcription. Gene names are displayed directly below the corresponding block arrows. A circle joined to a vertical line shows transcriptional terminators. Right-angled lines terminating in flattened arrowheads represent promoters and indicate the direction of transcription. Positive regulation is indicated by an arrow in conjunction with the '+' symbol.

Expression from the *resA* promoter is induced by phosphate starvation, nitrogen starvation, entry into stationary phase and oxygen limitation (Nakano and Zhu, 2001; Sun *et al.*, 1996b). As outlined in Figure 3.1, the *resABCDE* operon is positively autoregulated by ResD (Sun *et al.*, 1996b); furthermore, ResDE controls the expression of different target genes, such as *nasDE*, *hmp*, *fnr*, *ctaA*, *ctaBCDEF* and *sbo-alb*, which all encode proteins involved in the respiratory process (Nakano, 2002b).

A body of evidence, reported in three papers by Birkey and co-workers (Birkey *et al.*, 1998; Sun *et al.*, 1996a; Sun *et al.*, 1996b) proves the existence of a network of interactions linking the TCSs PhoPR and ResDE under phosphate starvation conditions. The critical findings of these studies are:

- (i) Mutations in *resE* and *resD* result in reduced levels of phosphate starvation-induced alkaline phosphatase (APase) specific activity (Sun *et al.*, 1996b). This finding implies that ResDE regulates expression of APase activity under phosphate starvation conditions, and suggests that ResDE controls the PhoPR regulon in a positive way.
- (ii) Such a positive effect is due to ResDE regulating transcription of the *phoPR* operon (Sun *et al.*, 1996a).
- (iii) Expression of the *resABCDE* operon is induced by phosphate starvation conditions (Sun *et al.*, 1996b). This effect is due to the direct binding of PhoP to the *resA* promoter region.

PhoP also binds to the internal promoter upstream to *resD*, with a negative effect on its expression (Birkey *et al.*, 1998).

Crucially, PhoPR is not required for *resABCDE* induction during anaerobic growth nor during stationary phase or under nitrogen limitation: therefore the interactions between PhoPR and ResDE described above seem to occur only under specific environmental conditions. A diagram illustrating the regulatory network of interactions between PhoPR and ResDE under phosphate limitation conditions is shown in Figure 3.2.

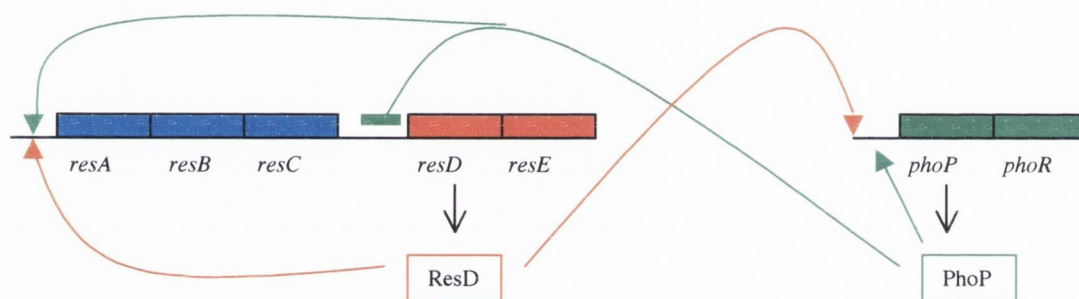


Figure 3.2. Schematic representation of the network of interactions between the ResDE and PhoPR TCS under conditions of phosphate limitation. Positive regulation is indicated by lines terminating in flattened arrowheads, negative regulation is indicated by a line terminating in a rectangle (adapted from Birkey *et al.*, 1998).

3.2. AIMS OF THIS WORK

On the basis of the above studies, which demonstrate a link between YycFG and PhoPR and between ResDE and PhoPR, we propose that YycFG is part of a signaling network in which the three related TCSs YycFG, ResDE and PhoPR function *in concert* to regulate gene expression in a coordinated way, at least under certain conditions.

Our objective therefore is to extend the model of interaction proposed by Birkey *et al.* (1998) and outlined in Figure 3.2 by introducing YycFG as a third player in this network. Interactions among the three TCS may occur at several levels:

- (i) at the signal level, whereby the same signal may be sensed by more than one kinase;
- (ii) at the kinase level, whereby a kinase may phosphorylate a non-cognate response regulator;
- (iii) at the regulator level, whereby a RR may be involved in the transcription of genes controlled by a different regulator.

This work focuses in particular on the possibility of an interaction between the TCS YycFG and ResDE. The main objectives of this study are:

- (a) to determine whether YycG is able to sense the same stimulus sensed by the kinase ResDE;
- (b) to investigate the possibility of a cross-talk between the sensor kinase YycG and the RR ResD, and between the kinase ResE and the RR YycF;
- (iii) to determine whether YycFG, in conjunction with ResDE, participates to the regulation of gene expression during aerobic/anaerobic growth, by controlling transcription of the *resABCDE* operon or of other constituent genes of the ResDE regulon.

Intriguingly, a link between YycFG and oxygen availability has been proposed in two studies performed in *S. pneumoniae* and *S. aureus*. In *S. pneumoniae* YycFG was shown to be involved in competence repression under oxygen limitation conditions, and was proposed to be able to sense oxygen availability through the PAS domain of the YycG kinase (Echenique and Trombe, 2001). In *S. aureus* a *yycF* mutant strain, which is hypersensitive to macrolide and lincosamide antibiotics, displayed reduced susceptibility when grown under oxygen limiting conditions, suggesting that the physiological function of YycFG may differ in aerobic versus anaerobic conditions (Martin *et al.*, 1999). These studies indicate the involvement of YycFG in sensing and responding to oxygen availability, and are therefore consistent with the hypothesis of an interaction between this TCS and the anaerobic growth regulator ResDE.

3.3. RESULTS AND DISCUSSION

3.3.1. The stimulus: does YycG sense anaerobic conditions?

As mentioned above, the three HK YycG, ResE and PhoR all possess a PAS domain. PAS domains are typically involved in sensing signals such as light, redox potential, oxygen, small ligands and the overall energy of the cell (Taylor and Zhulin, 1999). A mutational analysis of the signal-sensing domain of ResE showed that the PAS domain is essential for sensing oxygen limitation and nitric oxide and for consequent transcriptional activation of ResDE-dependent genes (Baruah *et al.*, 2004). The role of the PAS domain in the HK YycG has not been fully elucidated. However, in the *S. pneumoniae* YycG, residue L100 in the N terminal portion of the PAS domain was shown to be crucial for the YycFG-dependent regulation of competence *in vivo*, as well as for autokinase activity of the pneumococcal YycG *in vitro* (Echenique and Trombe, 2001).

On the basis of the structural similarity between ResE and YycG, we set out to investigate whether YycG is involved in sensing oxygen levels or nitric oxide. We tested this hypothesis by employing two different experimental approaches.

In a first approach the possibility of YycG sensing oxygen limiting conditions was tested by comparing the transcriptional levels of the YycFG-activated gene *yocH* in cultures grown under aerobic versus anaerobic conditions. Transcriptional activity was measured by performing β -galactosidase assays on a strain (BP019) carrying a promoter-fusion *yocH-bgaB*. We observed that *yocH* transcriptional levels were comparable under aerobic and anaerobic conditions, both during the exponential phase and during the stationary phase (see Figure 3.3). This result indicates that YycG is probably not differentially activated by anaerobic conditions.

This result also seems to exclude the possibility of a cross-talk between ResE and YycF. It is known that under anaerobic conditions ResE exerts a kinase activity towards ResD (Nakano and Zhu, 2001); the observation that *yocH* transcriptional levels are not induced by anaerobic conditions indicates that the HK ResE probably does not phosphorylate the non-cognate RR YycF.

However, results from these experiments should be interpreted with particular caution, because they are based on the comparison between two different growth conditions, characterized by different growth rates and optical densities. For this reason such results cannot be considered conclusive.

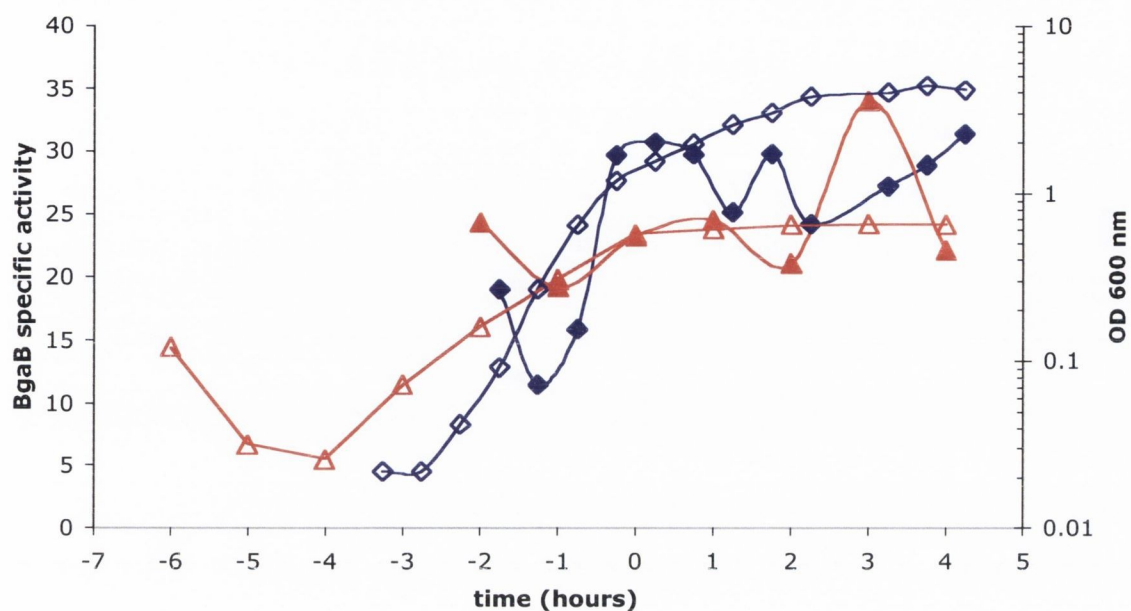


Figure 3.3. Analysis of the expression of the *yocH* promoter during aerobic and anaerobic growth. Growth of strain BP019 (*yocH-bgaB*) is shown by open symbols and β -galactosidase by closed symbols. Strain BP019 was grown in LB broth under aerobic (blue lines) and anaerobic (red lines) conditions. Time zero indicates the point of transition between the exponential and stationary phases of growth. This experiment was performed twice with comparable results and a representative profile is shown.

A second experiment involved the use of sodium nitroprusside (SNP), a chemical compound that is known to release nitric oxide (NO) in solution when exposed to visible light. Addition of SNP at a concentration of 100 or 500 μM to early to mid-log-phase aerobic cultures was previously found to induce transcription of ResDE-controlled genes (Nakano, 2002a). This effect is likely to be due to activation of ResE by NO, a probable ligand for this HK (Baruah *et al.*, 2004). SNP therefore can be used to induce the ResDE regulon during aerobic growth, “mimicking” anaerobic conditions. Crucially, SNP addition has only a marginal effect on growth of *B. subtilis* (see Figures 3.4, 3.5). Therefore this approach overcomes some of the limitations of the previous experiment because it allows comparing transcriptional activities of cultures growing at similar rates and reaching similar optical densities.

A control experiment was performed in order to verify induction of the ResDE regulon upon SNP addition under our experimental conditions. The gene *hmp*, encoding a flavohemoglobine that functions in the detoxification of NO, is strongly induced upon oxygen limitation (LaCelle *et al.*, 1996). Transcriptional activation of *hmp* is due to the binding of ResD to the *hmp* promoter (Nakano *et al.*, 2000), and this is stimulated upon phosphorylation of ResD by the ResE kinase. Therefore, measuring *hmp* expression upon addition of SNP to the medium is a way to monitor the level of activation of ResE. Strain BP032, carrying the promoter-fusion *hmp-bgaB*, was grown in LB in aerobic conditions to early log-phase (see Figure 3.4); the culture was split and SNP was added to one half to a final concentration of 250 μM . Cultures were then grown to stationary phase. Expression of *hmp-bgaB* fusion was induced 3 fold 30 minutes after the addition of SNP, and to a higher degree when the cells reached the stationary phase of growth. This result demonstrates that ResE is phosphorylated upon addition of SNP to the medium.

In order to test whether YycG is activated by the same signal sensed by ResE, we monitored the transcriptional variations of the YycFG-controlled gene *yocH* in response to addition of SNP to the medium. As shown in Figure 3.5, *yocH* transcriptional levels were comparable in the presence and in the absence of SNP during the exponential phase of growth, but they were significantly higher during the stationary phase in the culture containing SNP.

In order to interpret this result, it is crucial to observe that in the culture containing SNP the HK ResE is biased towards its kinase-active form throughout the growth cycle, as demonstrated by the induction of *hmp* transcription observed in the control experiment in the presence of SNP (see Figure 3.4). The absence of significantly altered *yocH* transcriptional levels during the exponential phase suggests that the kinase YycG does not sense NO, and that

there is not a cross-talk between ResE and YycF, confirming previous results (see Figure 3.3). However, the result obtained during the stationary phase of growth is not consistent with this interpretation: increased levels of *yocH* activity were observed upon SNP addition, apparently indicating the ability of YycG to sense NO in the medium, or, alternatively, a cross-talk between ResE and YycF. There are a number of possible explanations for this paradox.

One possibility is that the HK YycG may be capable of sensing NO, which may induce its kinase activity. The fact that SNP addition results in induction of *yocH* transcription only during the stationary phase of growth could be explained by the presence of multiple activating signals for YycG. During the exponential phase YycG might be activated by an unidentified signal, resulting in YycF phosphorylation. Such activation may occur to saturation: this would explain the absence of *yocH* induction during the exponential phase in the presence of the additional putative signal NO. It is noteworthy that during the stationary phase the RR YycF is probably de-phosphorylated, as indicated by the decrease in *yocH* transcriptional levels observed by northern blot analysis at the end of the exponential phase (Howell *et al.*, 2003). One possibility is that YycF is de-phosphorylated by its cognate kinase YycG. It is known, in fact, that a sensor HK can have a phosphatase activity toward the cognate RR (Pratt, 1995); YycG, therefore, may switch from a kinase to a phosphatase activity during the growth cycle, possibly thanks to the accumulation of particular factors in the medium, through a so-called “quorum sensing type mechanism”. The addition of the compound SNP may affect the phosphatase activity of YycG and may bias the HK YycG towards its kinase-active form, resulting in enhanced YycF phosphorylation and hence in induced *yocH* transcription.

An alternative explanation for the result observed in Figure 3.5 is that the HK ResE might be able to cross-phosphorylate YycF. Like in the previous case, the positive effect of SNP addition on *yocH* transcription would not be visible during the exponential phase of growth because the HK YycG may phosphorylate the cognate RR YycF to saturation. During stationary phase in the presence of SNP the HK ResE senses NO, which may stimulate its kinase activity both towards the cognate RR ResD (as demonstrated by *hmp* induction, see Figure 3.4) and towards the non-cognate RR YycF, resulting in *yocH* induction.

A further possibility is that SNP addition may interfere with YycF de-phosphorylation by the non-cognate HK ResE. As mentioned above, previous data indicate that YycF is probably

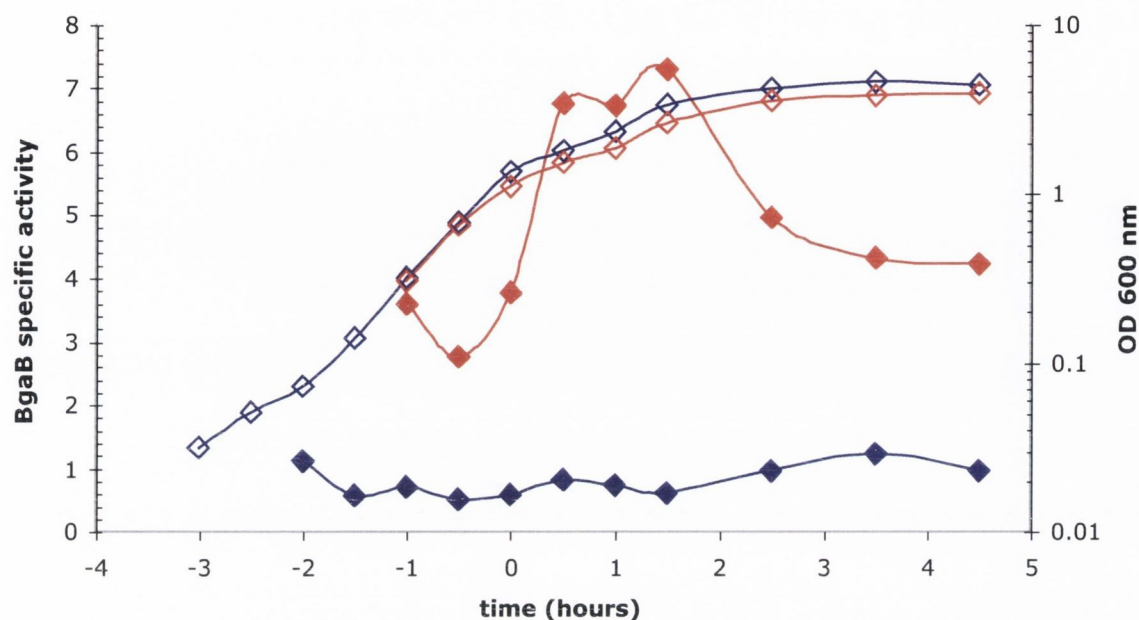


Figure 3.4. Analysis of the expression of the *hmp* promoter upon sodium nitroprusside (SNP) addition. Growth of strain BP032 (*hmp-bgaB*) is shown by open symbols and β -galactosidase by closed symbols. Strain BP032 was cultured in LB in aerobic conditions to early log-phase; the culture was then split and SNP was added to one half to a final concentration of 250 μ m; the growth was continued until stationary phase. Blue lines indicate aerobic conditions; red lines indicate aerobic conditions in the presence of SNP. Time zero indicates the point of transition between the exponential and stationary phases of growth. This experiment was performed twice with comparable results and a representative profile is shown.

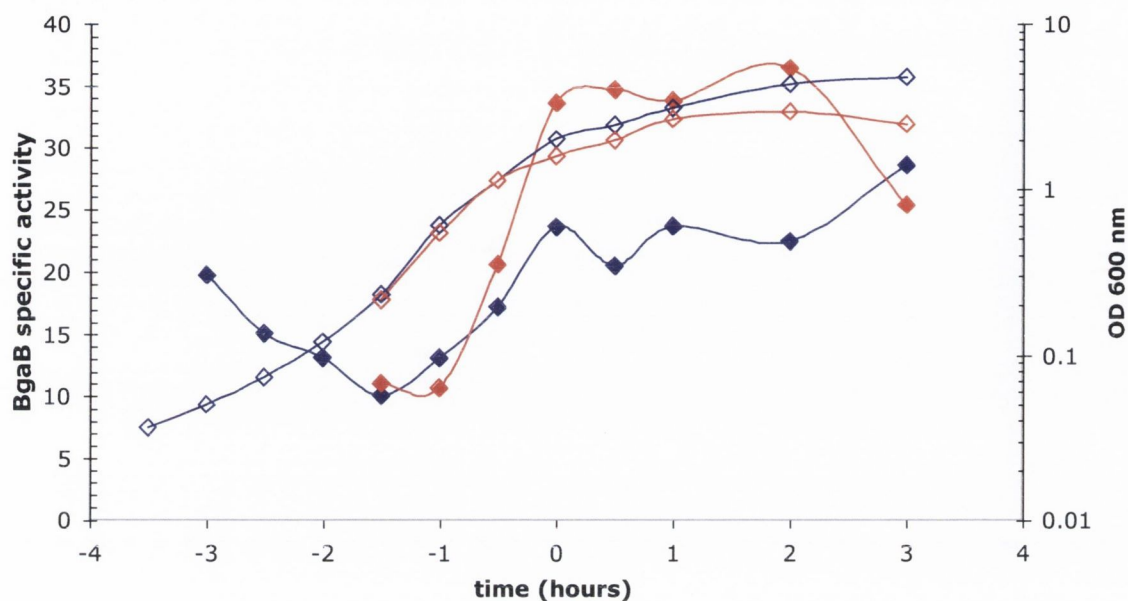


Figure 3.5. Analysis of the expression of the *yocH* promoter upon sodium nitroprusside (SNP) addition. Growth of strain BP019 (*yocH-bgaB*) is shown by open symbols and β -galactosidase by closed symbols. Strain BP019 was cultured in LB in aerobic conditions to early log-phase; the culture was then split and SNP was added to one half to a final concentration of 250 μ m; the growth was continued until stationary phase. Blue lines indicate aerobic conditions; red lines indicate aerobic conditions in the presence of SNP. Time zero indicates the point of transition between the exponential and stationary phases of growth. This experiment was performed twice with comparable results and a representative profile is shown.

de-phosphorylated during the stationary phase of growth (Howell *et al.*, 2003); however, the phosphatase responsible for such activity has not been identified: one possibility is that ResE may be partially responsible for YycF de-phosphorylation. It is known, in fact, that ResE functions both as a kinase and a phosphatase towards ResD, and that oxygen availability induces its phosphatase activity (Nakano and Zhu, 2001). ResE may therefore act as a phosphatase towards the non-cognate RR YycF in aerobic conditions, and SNP addition may interfere with such process, biasing ResE towards its kinase activity and hence resulting in *yocH* induction. However, if this were the case, *yocH* transcriptional levels would be expected to increase in anaerobic conditions, because of the predominant kinase activity of ResE, which would not be able to de-phosphorylate YycF. Such phenomenon was not observed, at least under our experimental conditions (see Figure 3.3), making this hypothesis rather weak.

In conclusion, the above results are consistent with the hypothesis of YycG sensing the same stimulus that activates ResE (the compound NO) or, alternatively, with the occurrence of a cross-talk between ResE and YycF.

3.3.2. YycF down-regulation: a quorum sensing-type mechanism?

As mentioned above, previous work (Howell *et al.*, 2003) showed a down regulation of *yocH* transcription at the end of the exponential phase of growth, presumably due to a de-phosphorylation of the RR YycF by a phosphatase. The fact that the down-regulation occurred at a certain stage of the growth cycle suggests that such a phosphatase activity could be induced by the accumulation in the medium of a compound, which might exert its effect only when it reaches a critical concentration, through a so called “quorum sensing-type mechanism”.

The protein acting as a phosphatase towards YycF could be the cognate HK YycG, or the HK ResE, or a different phosphatase, for example belonging to the Rap phosphatase family. Rap phosphatases are known to dephosphorylate specific RRs (Perego, 1997; Perego and Brannigan, 2001; Pratt, 1995), and their activity can be modulated by the accumulation in the medium of small peptides, as in the case of the *B. subtilis* RapA phosphatase, whose activity is directly inhibited by a pentapeptide (Perego, 1997).

To test if the down-regulation of YycF activity is subjected to a similar “quorum sensing type” mechanism, cells were grown in two types of Conditioned Minimal Medium (CondMM), a filter-sterilized minimal medium in which wild-type cells have been previously grown to different stages of the growth cycle, and which contains all the small molecules and peptides secreted from the cells at such stages. The ExpCondMM was prepared by growing

cells to $OD_{600} \approx 1.0$, while the StatCondMM was made by growing cells to stationary phase ($OD_{600} \approx 4.0$).

Strain BP019, carrying a promoter-fusion *yocH-bgaB*, was grown in New Minimal Medium (NewMM) to $OD_{600} \approx 1.0$ (exponential phase) and diluted 25-fold into NewMM and into the two different kinds of Conditioned Minimal Medium (ExpCondMM and StatCondMM). The growth of the starting culture was then followed to the stationary phase and *bgaB* activity levels were measured for all the four cultures.

As shown in Figure 3.6, the profile and the levels of expression for *yocH* obtained during growth in New MM are very different from the ones obtained in a culture grown in LB (see Figures 3.3 and 3.5). In LB *yocH* transcriptional activity is induced during the exponential phase up to 25-30 BgaB units, and remains at these levels during the stationary phase, while in a culture grown in NewMM (Figure 3.6) levels of activity are high (around 40 units) at the beginning of the growth cycle and rapidly decrease during the exponential phase, where they reach very low levels (around 5 units). A crucial observation is that when the cells are diluted into fresh NewMM no induction is observed during the exponential phase and the activity levels remain low. It is likely, therefore, that the high levels of expression observed at the beginning of the growth curve are the result of a carry-over from the overnight culture used as an inoculum. It is possible that the lower *yocH* transcriptional levels observed in NewMM with respect to LB are due to lower levels of expression of the response regulator YycF in this medium.

Crucially, *yocH* transcriptional levels were comparable in the cultures grown in New MM, ExpCondMM and StatCondMM, suggesting that YycF activity is not subjected to a “quorum sensing type mechanism” of regulation. In fact if the phosphatase responsible for YycF dephosphorylation was activated by the accumulation of soluble compounds in the medium, *yocH* transcriptional levels would decrease during growth in the conditioned medium. However, the levels of activity obtained for all the four cultures were very low, and close to the sensitivity limit of the assay. For this reason results from this experiment cannot be considered conclusive.

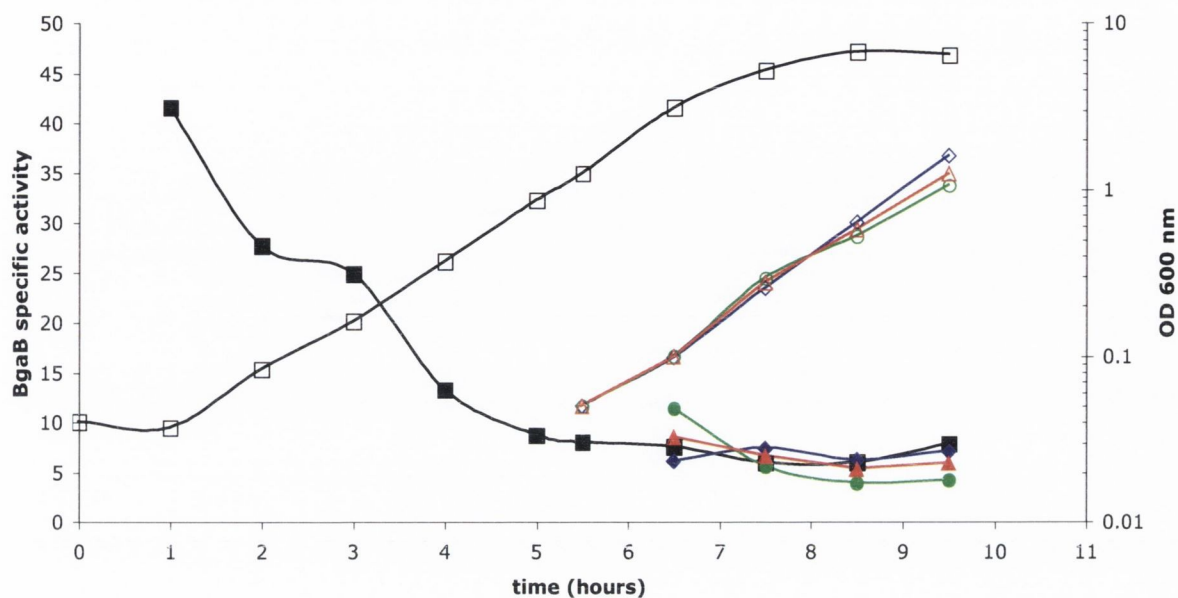


Figure 3.6. Analysis of the expression of the *yocH* promoter during growth in conditioned medium. Growth of strain BP019 (*yocH-bgaB*) is shown by open symbols and β -galactosidase by closed symbols. Strain BP019 was cultured in New Minimal Medium (black lines) to $OD_{600} \approx 1.0$ and diluted 25-fold into NewMM and into the two different kinds of Conditioned Minimal Medium (ExpCondMM, red lines and StatCondMM, green lines). The growth of the starting culture was followed to the stationary phase and *bgaB* activity levels were measured for all the four cultures. This experiment was performed twice with comparable results and a representative profile is shown.

3.3.3. Effect of YycFG constitutive expression on ResDE regulon

We then set out to test whether YycFG is involved in regulating gene expression under aerobic versus anaerobic conditions, separately or in conjunction with ResDE.

For this purpose we tested whether YycFG can regulate expression of the *resABCDE* operon, or of other constituent genes of the ResDE regulon. Transcriptional levels of *resA*, *ctaA*, *hmp* and *resD* were analyzed through BgaB assays in aerobic and in anaerobic conditions, in strains expressing physiological levels of YycFG and in strains expressing YycFG at constant levels throughout the growth cycle from the IPTG inducible P_{spac} promoter.

***resA* transcription.** Strain BP033, carrying a promoter-fusion *resA-bgaB*, was grown in LB broth under aerobic conditions. Strain BP033A, carrying the same promoter fusion and expressing the *yycFG* operon under the control of the IPTG-inducible P_{spac} promoter, was grown in parallel in LB medium containing no IPTG, 100 μ M IPTG and 1mM IPTG. In the absence of IPTG cells grew for a short time (around 2 hours) and then lysed, while in the cultures containing IPTG cells grew normally and at similar rates (see Figure 3.7). The IPTG concentration of 100 μ M is close to the minimal IPTG concentration (75 μ M) that allows normal growth in LB medium in a strain expressing YycFG from the IPTG inducible P_{spac} promoter (Howell *et al.*, 2003). In all cultures *resA* transcriptional levels were low during exponential growth, and were induced during stationary phase, as previously reported (Sun *et al.*, 1996b). Constitutive expression of the *yycFG* operon did not significantly affect *resA* transcriptional levels (compare strains BP033 and BP033A in Figure 3.7); however, a small but reproducible induction was observed upon YycFG overexpression in three duplicate experiments (compare strain BP033A grown in the presence of 100 μ M and 1mM IPTG in Figure 3.7), indicating that YycFG may participate in *resA* transcriptional regulation in a positive way.

A similar assay was carried out in anaerobic conditions (see Figure 3.8). Strains BP033 and BP033A were grown in the absence of aeration in LB broth, inoculating the cultures from frozen aliquots of cells. Strain BP033A was grown in LB medium containing no IPTG, 100 μ M IPTG and 1mM IPTG, and it grew only in the presence of IPTG. In the three growing cultures *resA* transcriptional levels were comparable during the exponential phase (see Figure 3.8), and reached higher levels than in aerobic conditions, as previously reported (Nakano and Zhu, 2001). A further induction was observed during the stationary phase; although values were slightly higher in strain BP033A grown in the presence of 1mM IPTG with respect to the

same strain grown with 100 μ M IPTG, such difference cannot be considered significant, as it was not observed in a number of duplicate experiments.

In conclusion, these data indicate that YycFG may participate in *resA* transcriptional regulation during growth in aerobic conditions, although the observed effect was subtle. If significant, it may have a physiological relevance resulting from a direct interaction between the RR YycF and the *resA* promoter region. Consistently with this interpretation, the *resA* promoter contains one putative YycF binding site, located within the region covered by PhoP and complementary to the consensus sequence for PhoP binding (Birkey *et al.*, 1998).

***ctaA* transcription.** Transcription of the ResDE-controlled gene *ctaA* was tested during aerobic growth in LB medium in a wild-type background and in a strain expressing YycFG under IPTG-inducible control. In all cases *ctaA* transcription was induced at the outset of the stationary phase, as previously observed (Zhang and Hulett, 2000). Strain BP031A, carrying a promoter-fusion *ctaA-bgaB* and expressing the *yycFG* operon under the control of the P_{spac} promoter, displayed significantly higher levels of activity when grown in the presence of 1mM IPTG than with 100 μ M IPTG (see Figure 3.9). This result indicates that YycFG may positively regulate *ctaA* transcription. Interestingly, the *ctaA* promoter region contains three putative YycF binding sites, which are contained within the three binding sites for ResD (Zhang and Hulett, 2000). YycFG regulation of *ctaA* transcription, therefore, may be direct.

***hmp* transcription.** Transcriptional activity of *hmp* was tested during growth in anaerobic conditions (see Figure 3.10). A strong induction of *hmp* transcription was observed at the late-exponential phase of the growth cycle, with even higher levels of activity during the stationary phase, as previously reported (LaCelle *et al.*, 1996; Nakano *et al.*, 2000; Nakano and Zhu, 2001). Transcriptional levels were around three fold higher in a strain expressing physiological levels of YycFG (strain BP032) with respect to a strain expressing YycFG at constant levels throughout the growth cycle from the IPTG inducible P_{spac} promoter (strain BP032A). Therefore YycFG constitutive expression exerts a negative effect on *hmp* transcription under anaerobic conditions. A putative YycF binding site is present within the *hmp* promoter region, in a position that overlaps the ResD binding sequence. The observed effect may therefore be direct: YycF may compete with ResD for the binding to the *hmp* promoter, hampering its transcription. Such effect may reflect a physiological phenomenon but it may also be artifactual, deriving from the non-physiological YycFG levels present in strain BP032A.

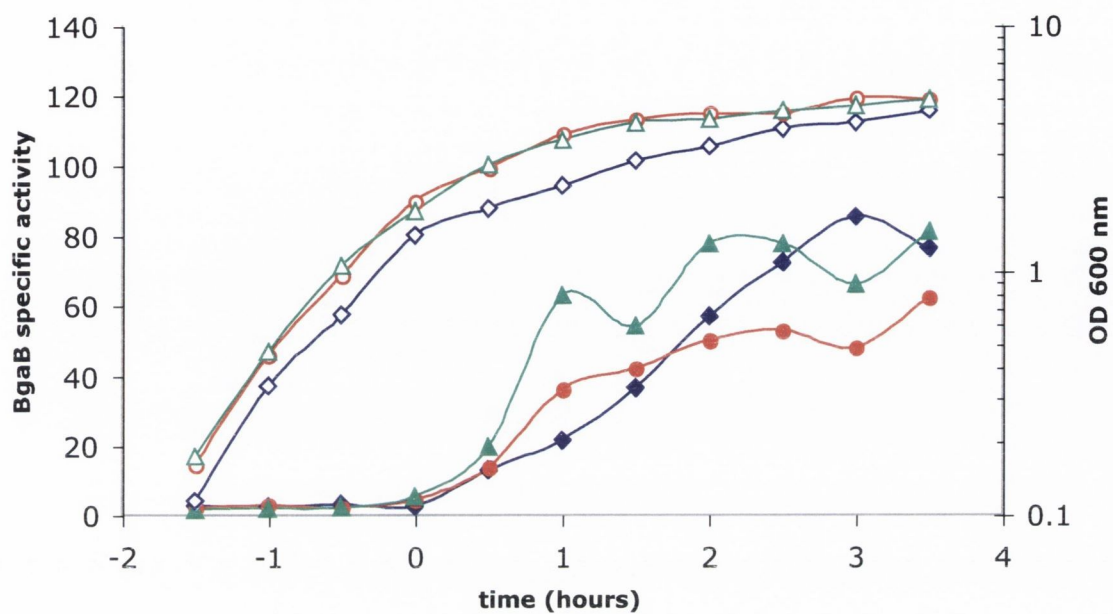


Figure 3.7. Analysis of the expression of the *resA* promoter during growth in aerobic conditions. Growth of *B. subtilis* strains is shown by open symbols and β -galactosidase by closed symbols. Strains BP033 (*resA-bgaB*, blue lines) and strain BP033A (*resA-bgaB*, P_{gpc} *yycFGHIJK*) were grown in LB broth under aerobic conditions. Strain BP033A was cultured in the presence of 100 μ M IPTG (red lines) and 1 mM IPTG (green lines). Time zero indicates the point of transition between the exponential and stationary phases of growth. This experiment was performed three times with comparable results and a representative profile is shown.

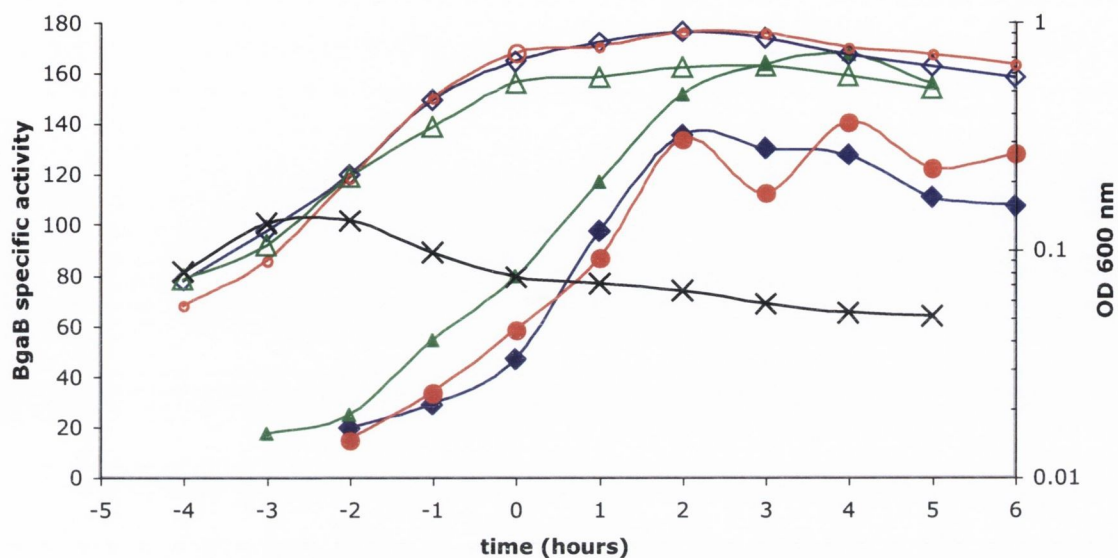


Figure 3.8. Analysis of the expression of the *resA* promoter during growth in anaerobic conditions. Growth of *B. subtilis* strains is shown by open symbols and β -galactosidase by closed symbols. Strains BP033 (*resA-bgaB*, blue lines) and strain BP033A (*resA-bgaB*, P_{yxc} *yycFGHIJK*) were grown in LB broth under anaerobic conditions. Strain BP033A was cultured in the absence of IPTG (black lines) and in the presence of 100 μ M IPTG (red lines) and 1 mM IPTG (green lines). Time zero indicates the point of transition between the exponential and stationary phases of growth. This experiment was performed three times with comparable results and a representative profile is shown.

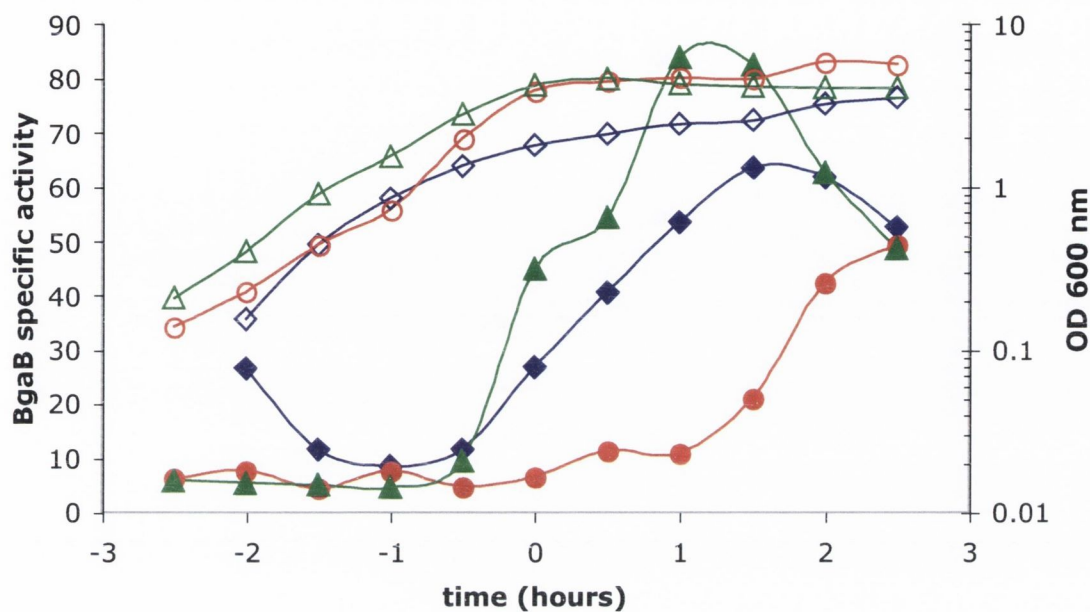


Figure 3.9. Analysis of the expression of the *ctaA* promoter during growth in aerobic conditions. Growth of *B. subtilis* strains is shown by open symbols and β -galactosidase by closed symbols. Strains BP031 (*ctaA-bgaB*, blue lines) and strain BP031A (*ctaA-bgaB*, P_{gac} *yycFGHIJK*) were grown in LB broth under aerobic conditions. Strain BP031A was cultured in the presence of 100 μ M IPTG (red lines) and 1 mM IPTG (green lines). Time zero indicates the point of transition between the exponential and stationary phases of growth. This experiment was performed three times with comparable results and a representative profile is shown.

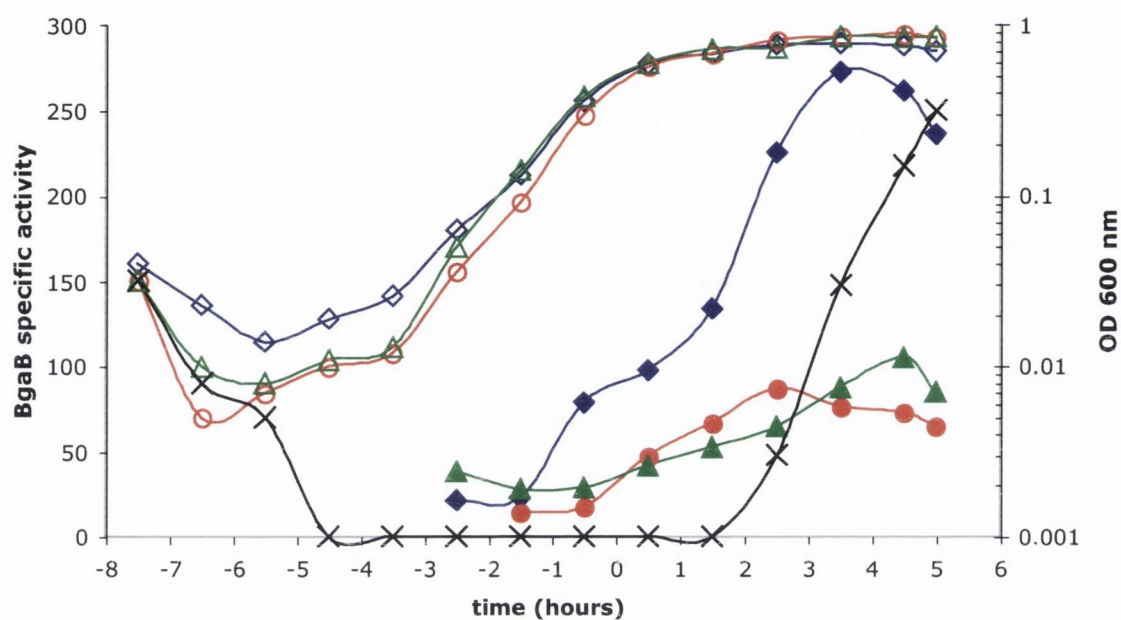


Figure 3.10. Analysis of the expression of the *hmp* promoter during growth in aerobic conditions. Growth of *B. subtilis* strains is shown by open symbols and β -galactosidase by closed symbols. Strains BP032 (*hmp-bgaB*, blue lines) and strain BP032A (*hmp-bgaB*, $P_{yyc} yycFGHIJK$) were grown in LB broth under aerobic conditions. Strain BP032A was cultured in the absence of IPTG (black lines) and the presence of 100 μ M IPTG (red lines) and 1mM IPTG (green lines). Time zero indicates the point of transition between the exponential and stationary phases of growth. This experiment was performed three times with comparable results and a representative profile is shown.

***resD* transcription.** Transcription of the *resD* gene, driven by the intracistronic promoter of the *resABCDE* operon (see Figure 3.1), was assayed both in aerobic and anaerobic conditions.

During growth in aerobiosis, *resD* transcriptional levels were low during exponential growth and higher during stationary phase in a wild-type background (see Figure 3.11). This profile is different from what reported by Sun and colleagues (Sun *et al.*, 1996b), who found an induction during the exponential phase and a subsequent repression during the stationary phase. Such discrepancy might be explained by the stability of the BgaB protein, which may result in an accumulation of this protein within the cell especially during the stationary phase, when the cells stop dividing. At first sight YycFG overexpression does not appear to have any effect on *resD* activity during growth in aerobiosis: *resD* transcriptional levels were comparable in a wild type background (strain BP034) and in a strain expressing YycFG under the control of the P_{spac} promoter (strain BP034A) grown in the presence of 100 μ M IPTG and 1mM IPTG (see Figure 3.11A). However, looking closely at the values obtained during the exponential phase, it may be noticed that in the strain expressing YycFG constitutively BgaB activity levels are somewhat lower than in the wild-type background, and that depletion for YycFG results in *resD* de-repression. It must be stated that the relevance of these results is problematic since activity values are very low and close to the sensitivity limit of the assay. However, a putative YycF binding site can be identified within the *resD* promoter region, upstream to the -10 and -35 Sigma A binding motifs: the observed very small effect, therefore, may be direct. In order to test this hypothesis, a *resD-bgaB* promoter fusion containing a mutated putative YycF binding sequence was constructed, and β -galactosidase assays were carried out in a wild-type background (strain BP035), and in a strain expressing YycFG from the P_{spac} promoter (strain BP035A). Mutation of the putative YycF binding motif did not affect *resD* activity levels (see Figure 3.11B), indicating that any effect of YycF may be indirect.

Analogous experiments were carried out in anaerobic conditions (see Figure 3.12). Transcriptional activities of the wild type and mutated *resD* promoters were assayed in strains expressing physiological YycFG levels (strains BP034 and BP035 respectively) and in strains expressing *yycFG* from the inducible P_{spac} promoter (strains BP034A and BP035A). Transcriptional levels of *resD* were low in all strains during the exponential phase of the growth. During stationary phase *resD* transcription was induced in all cases, but higher levels of expression were observed in the strains overexpressing YycFG, with comparable levels in the cultures grown in the presence of 100 μ M IPTG and 1mM IPTG (see Figures 3.12). Crucially, the induction of *resD* transcription detected during the stationary phase of the

growth cycle upon YycFG constitutive expression was not affected by the destruction of a putative binding site for YycF within the *resD* promoter region (compare strains BP034A and BP035A in Figures 3.12A and 3.12B) indicating that such effect is likely to be indirect.

It is known that under phosphate starvation conditions PhoP represses *resD* transcription by binding directly to *resD* promoter region (Birkey *et al.*, 1998), while it binds to *resA* promoter enhancing its transcription (see Figure 3.3). It's crucial to observe that these findings refer only to phosphate starvation conditions, and that PhoP is not required for the induction of the *resABCDE* operon under anaerobic conditions. However, the possibility that PhoP binds to the *resD* promoter under anaerobic conditions cannot be excluded. On the basis of these observations, and in the light of our working hypothesis of a network of interactions involving the three TCSs YycFG, ResDE and PhoPR, we hypothesized that YycFG might induce *resD* transcription indirectly by acting as a negative regulator towards the TCS PhoPR. This would result in reduced levels of phosphorylated PhoP, and hence in *resD* de-repression. In order to test this hypothesis, we adopted two different strategies.

1) Firstly, we constructed a *resD-bgaB* promoter fusion containing a mutated PhoP binding site, and we measured *resD* transcriptional activity during anaerobic growth, both in a wild-type background (strain BP040) and in a strain expressing YycFG from the P_{spac} promoter (strain BP040A). Crucially, no induction was observed upon YycFG over-expression (see Figure 3.13A). Therefore mutation of the PhoP binding site within the *resD* promoter region abolishes the induction of *resD* transcription observed upon YycFG over-expression (see Figure 3.12), indicating that such effect is probably mediated through PhoP.

2) An alternative strategy involved the construction of a strain containing a wild type *resD-bgaB* promoter fusion and a deletion in the *phoP* coding sequence (strain BP041). Similarly to what found in the previous experiment, no induction of *resD* activity was observed upon YycFG constitutive expression (strain BP041A, see Figure 3.13B). Results from both experiments, therefore, indicate that YycFG over-expression induces *resD* transcription probably through PhoP.

However, it is crucial to observe that in strains expressing physiological YycFG levels, *resD* transcriptional activity was not affected by deletion of the *phoP* gene or by the presence of a mutated PhoP binding site within the *resD* promoter fusion (compare strains BP034,

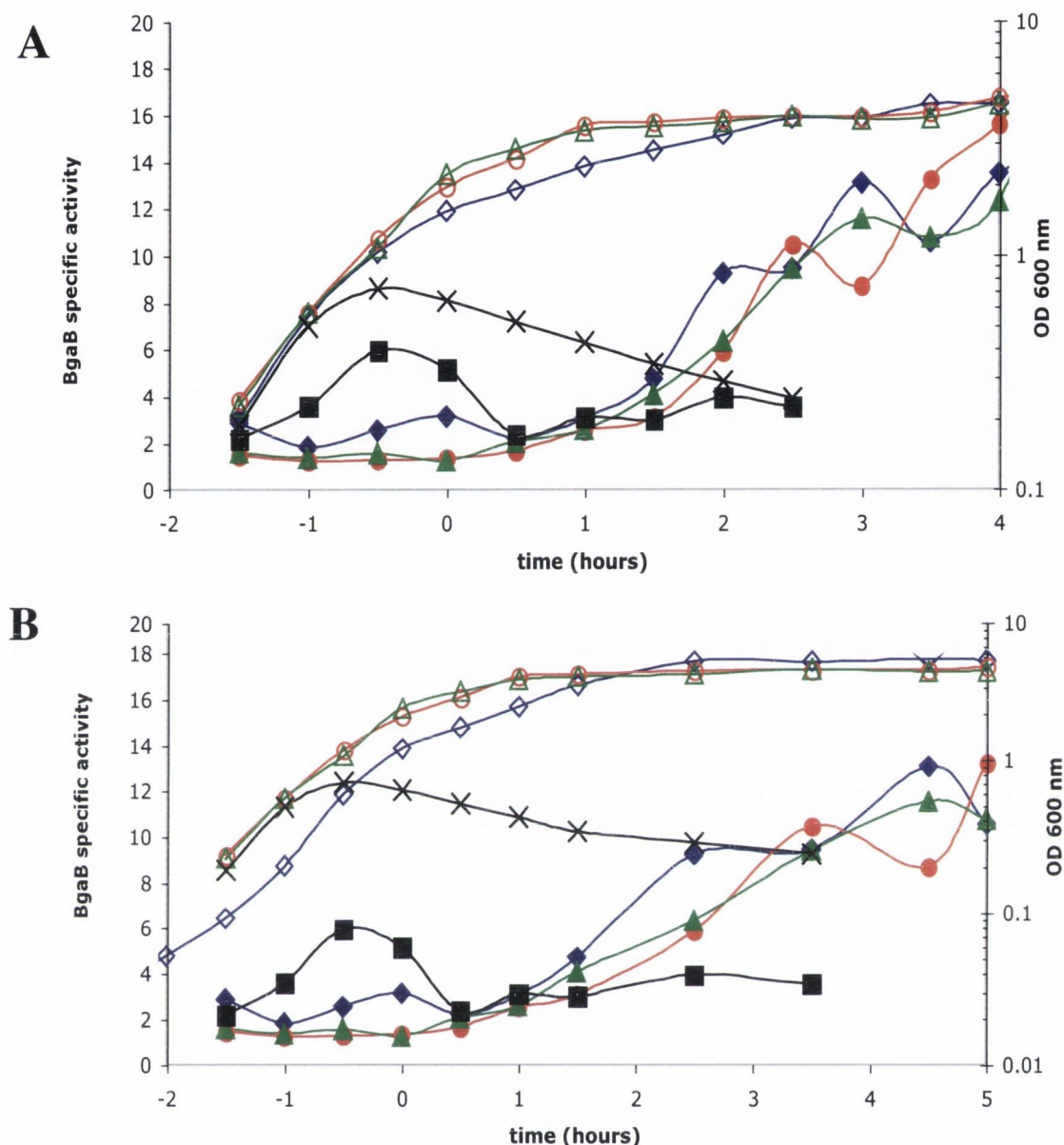


Figure 3.11. Analysis of the expression of wild-type and mutated *resD* promoter during growth in aerobic conditions. Growth of *B. subtilis* strains is shown by open symbols and β -galactosidase by closed symbols.

A. Strain BP034 (*resD-bgaB*, blue lines) and strain BP034A (*resD-bgaB*, P_{spx} *yycFGHIJK*) were grown in LB broth under aerobic conditions. Strain BP034A was cultured in the absence of IPTG (black lines) and the presence of 100 μ M IPTG (red lines) and 1mM IPTG (green lines).

B. Strain BP035 (blue lines), carrying a *resD-bgaB* promoter fusion containing a mutated YycF binding motif, and strain BP035A, carrying the same promoter fusion and expressing the *yycFGHIJK* operon under the control of the IPTG-inducible P_{spx} promoter, were grown in LB broth under aerobic conditions. Strain BP035A (*resD-bgaB*, P_{spx} *yycFGHIJK*) was cultured in the absence of IPTG (black lines) and the presence of 100 μ M IPTG (red lines) and 1mM IPTG (green lines). Time zero indicates the point of transition between the exponential and stationary phases of growth. The above experiments were performed three times with comparable results and representative profiles are shown.

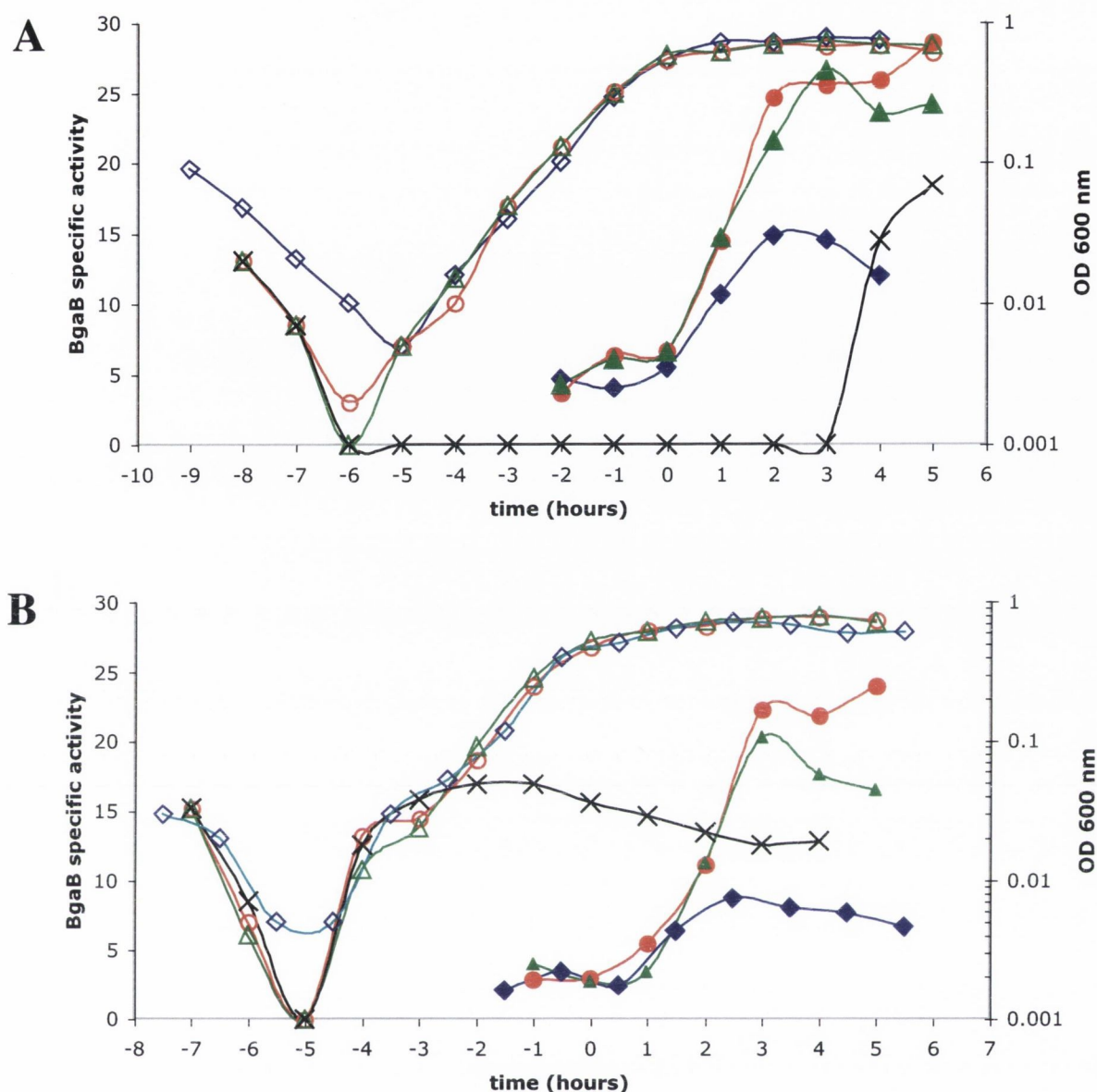


Figure 3.12. Analysis of the expression of wild-type and mutated *resD* promoter during growth in anaerobic conditions. Growth of *B. subtilis* strains is shown by open symbols and β -galactosidase by closed symbols.

A. Strain BP034 (*resD-bgaB*, blue lines) and strain BP034A (*resD-bgaB*, P_{spc} *yycFGHIJK*) were grown in LB broth under anaerobic conditions. Strain BP034A was cultured in the absence of IPTG (black lines) and the presence of 100 μ M IPTG (red lines) and 1mM IPTG (green lines).

B. Strain BP035 (blue lines), carrying a *resD-bgaB* promoter fusion containing a mutated YycF binding motif, and strain BP035A, carrying the same promoter fusion and expressing the *yycFGHIJK* operon under the control of the IPTG-inducible P_{spc} promoter, were grown in LB broth under anaerobic conditions. Strain BP035A (*resD-bgaB*, P_{spc} *yycFGHIJK*) was cultured in the absence of IPTG (black lines) and the presence of 100 μ M IPTG (red lines) and 1mM IPTG (green lines). Time zero indicates the point of transition between the exponential and stationary phases of growth. The above experiments were performed three times with comparable results and representative profiles are shown.

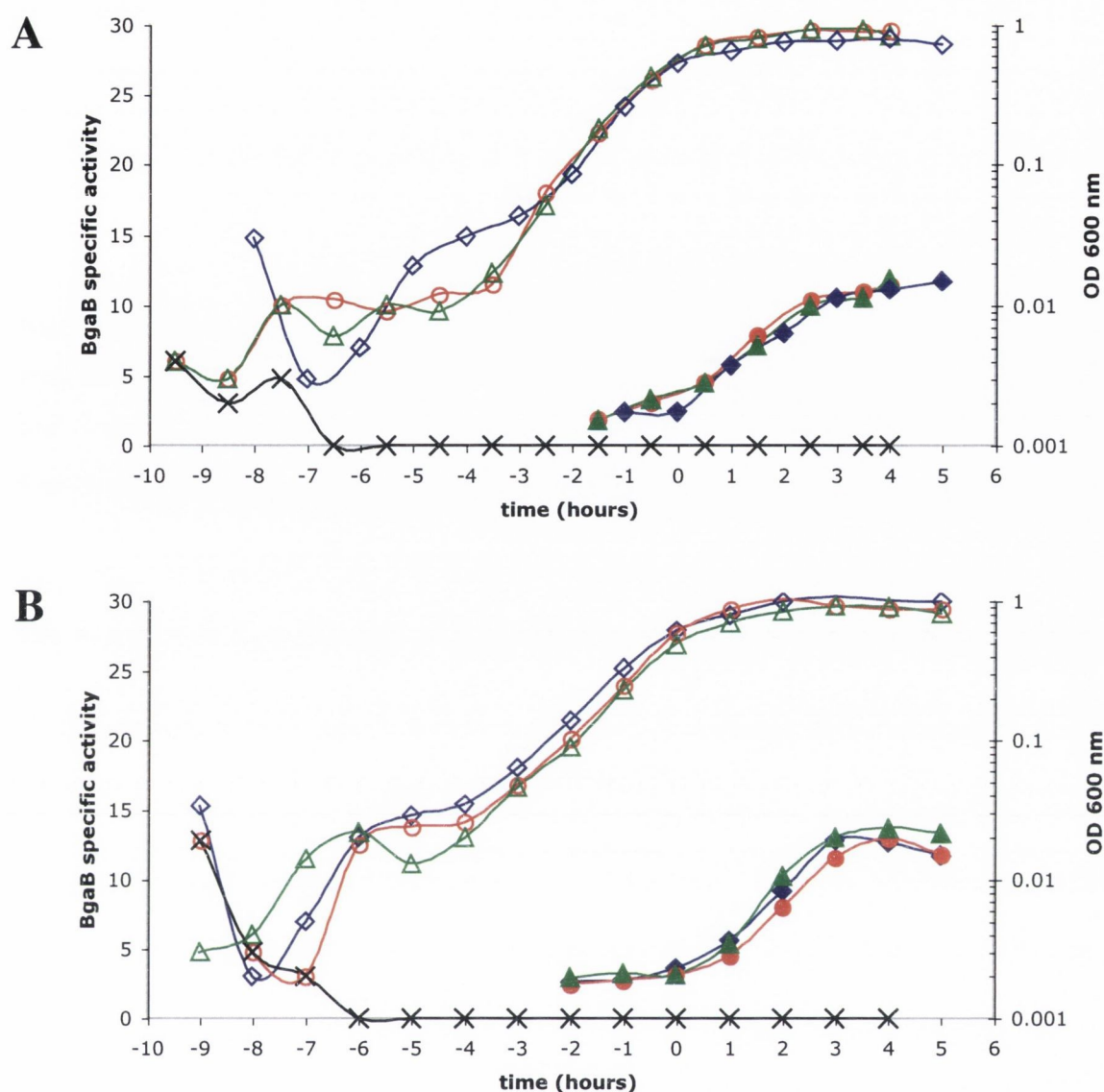


Figure 3.13. Analysis of the effect of PhoP on expression of the *resD* promoter during growth in anaerobic conditions. Growth of *B. subtilis* strains is shown by open symbols and β -galactosidase by closed symbols.

A. Strain BP040 (blue lines), carrying a *resD-bgaB* promoter fusion containing a mutated PhoP binding motif, and strain BP040A, carrying the same promoter fusion and expressing the *yycFGHIJK* operon under the control of the IPTG-inducible P_{yyc} promoter, were grown in LB broth under anaerobic conditions. Strain BP040A (*resD-bgaB*, P_{yyc} *yycFGHIJK*) was cultured in the absence of IPTG (black lines) and the presence of 100 μ M IPTG (red lines) and 1 mM IPTG (green lines).

B. Strain BP041 (blue lines), carrying a wild-type *resD-bgaB* promoter fusion containing a *phoP* null mutation, and strain BP041A, carrying the same promoter fusion and expressing the *yycFGHIJK* operon under the control of the IPTG-inducible P_{yyc} promoter, were grown in LB broth under anaerobic conditions. Strain BP041A (Δ *phoP*, *resD-bgaB*, P_{yyc} *yycFGHIJK*) was cultured in the absence of IPTG (black lines) and the presence of 100 μ M IPTG (red lines) and 1 mM IPTG (green lines). Time zero indicates the point of transition between the exponential and stationary phases of growth. The above experiments were performed three times with comparable results and representative profiles are shown.

BP040 and BP041 in Figures 3.12A, 3.13A and 3.13B). This result indicates that PhoP does not play a role in the induction of *resD* transcription observed during the stationary phase in anaerobic conditions, and that the positive effect observed upon YycFG constitutive expression may not be physiologically relevant, and may derive from interactions between YycFG and PhoPR which occur only in the presence of abnormal YycFG protein levels.

In summary, our transcriptional analysis suggests that YycFG might be involved in the regulation of transcription from the *resA* and the *resD* promoters of the *resABCDE* operon, as well as in transcriptional regulation of constituent genes of the ResDE regulon, such as *ctaA* and *hmp*. The presence of putative YycF binding sites within the promoter regions of *resA*, *ctaA* and *hmp* indicates that such regulation might be direct.

Our results indicate in particular that YycFG might positively regulate transcription of *resA* and *ctaA* during aerobic growth, while it might repress *hmp* transcription in anaerobic conditions. In order to interpret these results, it is interesting to observe that while *resA* and *ctaA* are expressed both in aerobic and anaerobic conditions (although at higher levels in anaerobiosis), the gene *hmp* is expressed at significant levels only during anaerobic growth. Such a difference might explain the opposite effects observed on the expression of these genes upon YycFG constitutive expression. It is tempting to speculate that YycFG may exert a positive effect on genes that are needed during both aerobic and anaerobic growth, like *resA* and *ctaA*, possibly initiating their transcription, while genes expressed exclusively during anaerobic growth like *hmp* may not require YycF for initial transcription and may be induced only by the phosphorylated form of ResD.

A number of additional observations points towards the involvement of the three RRs YycF, ResD and PhoP in the transcriptional regulation of *resA* and *ctaA*. (i) It is known that PhoP induces *resA* transcription in phosphate starvation conditions by binding directly to its promoter region (Sun *et al.*, 1996b). (ii) Interestingly, the consensus sequence for YycF corresponds almost exactly to the complement of the PhoP binding motif: consistent with this observation, the putative YycF binding site identified within the *resA* promoter overlaps with the complement of the PhoP binding motif; in addition, although transcription of the *ctaA* gene has not been shown to be regulated by PhoP, its promoter includes three putative PhoP/YycF binding sites, each contained within one of the three region covered by ResD. (iii) Furthermore, recent unpublished work from our laboratory indicates that YycF and PhoP are able to induce transcription of a number of YycFG-regulated genes by binding to the same promoter regions, although they act at different stages of the growth cycle and in different

growth conditions. On the basis of these observations, we propose that the three RRs YycF, PhoP and ResD may all contribute to expression of *resA* (and, possibly, of *ctaA* and of other members of the ResDE regulon), each of them inducing transcription at a specific stage of the growth cycle and in a specific environmental condition. YycF, which is present in its active form during the exponential phase, might initiate transcription of these genes; the activated form of PhoP might induce them during phosphate starvation, while during anaerobic growth the main transcriptional regulator might be ResD in its phosphorylated form. Intriguingly, this hypothesis might also contribute to explain the essential nature of the TCS YycFG, which might play a crucial role in initiating transcription of a number of genes, including the essential operon *resABCDE*.

As anticipated above, the negative effect observed upon YycFG constitutive expression on transcription of the *hmp* gene may be non-physiological, as it may derive from altered YycFG levels that may interfere with the normal transcriptional regulation of this gene. However, it is interesting to note that the *hmp* promoter contains a putative YycF binding motif, which is included within a ResD binding region: upon YycFG constitutive expression YycF may compete with ResD for the binding to the same region, resulting in *hmp* transcriptional repression. Although the effect observed under our experimental conditions may not be physiological *per se*, it may reflect a real phenomenon: in the presence of physiological YycFG levels, the RR YycF may bind to the *hmp* promoter, repressing its transcription, during growth in aerobiosis, while during anaerobic growth high levels of phosphorylated ResD may compete with YycF for the binding to the *hmp* promoter, and induce its transcription. However this hypothesis needs further experimental evidence.

Results on *resD* transcriptional activity support the model of a network of interactions involving the three TCSs YycFG, PhoPR and ResDE: *resD* transcription is induced upon YycFG constitutive expression in anaerobic conditions, and this phenomenon is mediated by the RR PhoP. However, deletion of *phoP* and mutation of the PhoP binding site do not affect *resD* induction during anaerobic growth, indicating that the observed effect might be artifactual, and might derive from interactions between YycFG and PhoPR that may occur only when *yycFG* is constitutively expressed.

3.3.4. Conclusions

The critical findings of this work can be summarized as follows:

(i) Expression of the YycFG-controlled gene *yocH* is induced upon SNP addition during the stationary phase of aerobic growth (see Figure 3.5). This result may reflect the ability of the kinase YycG to sense the compound NO (also sensed by ResE), or alternatively it may indicate a cross-talk between the kinase ResE and the regulator YycF.

(ii) YycFG overexpression induces transcription of the *resA* operon and of the *ctaA* gene during growth in aerobic conditions. Strains expressing *yycFG* from the P_{spac} promoter displayed higher levels of *resA* and *ctaA* transcriptional activity when grown with 1mM IPTG with respect to 100 μ M IPTG (see Figures 3.7 and 3.9). No effect on *resA* expression was observed upon YycFG constitutive expression during anaerobic growth (see Figure 3.8).

(iii) Transcription of the *hmp* gene is repressed upon YycFG constitutive expression during anaerobic growth (see Figure 3.10).

(iv) *resD* transcription might be indirectly repressed by YycF during the exponential phase of growth in aerobic conditions (see Figure 3.11). In addition, during anaerobic growth *resD* transcriptional activity is induced upon YycFG constitutive expression, such effect being mediated by the RR PhoP (see Figures 3.12 and 3.13).

These findings support the model of a network of interactions occurring among the TCSs YycFG, ResDE and PhoPR. However, the exact nature of such interactions remains to be established. Our results suggest that interactions among YycFG, ResDE and PhoPR may occur at the level of the signal, whereby YycG may be able to bind NO, the same signaling molecule sensed by ResE; at the kinase level, whereby ResE might be able to cross-phosphorylate YycF; and at the regulator level, whereby the three RR YycF, PhoP and ResD might be involved in transcriptional regulation of the same genes, although at different growth stages and in different environmental condition. Nevertheless, none of the above hypothesis has been unequivocally proven, and additional experiments should be carried out in order to discriminate among a number of possible models that might explain our results. In addition, the fact that most of the observed effects were subtle, indicates that they might derive from the

indirect interactions which are known occur between the two pairs of TCS YycFG and PhoPR, and ResDE and PhoPR. In other words, it is possible that perturbing the level or the activation of one of these TCSs (*i.e.* YycFG), may influence the activity of a second TCS (*i.e.* ResDE) indirectly, through its interaction with a third TCS (*i.e.* PhoPR). We reasoned that the effort required to identify the exact nature of the interactions occurring among these TCSs might be not worthwhile, especially in the light of the complexity of the systems involved. For this reason, and in view of the new exciting results obtained in the microarray analysis presented in the previous chapters, I focused on this second project, which became the main area of my research and which proved itself to be a more productive field of investigation.

CHAPTER 4

MATERIALS AND METHODS

4.1. Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 4.1. Details of strain construction are presented in the next paragraph. Strain TG1 was used for routine cloning in *Escherichia coli* (Sambrook *et al.*, 1989). *B. subtilis* strain 168 *trpC2* was used throughout to generate mutant strains and to establish expression patterns using transcriptional fusions. *E. coli* strains were grown in Luria-Bertani (LB) medium (Miller, 1972). *B. subtilis* strains were grown in Luria-Bertani (LB) medium (Miller, 1972), anaerobic rich medium (Marino *et al.*, 2001), New Minimal Medium and Conditioned Minimal Medium (Andersen, 2003). IPTG was added at concentrations specified in the text while antibiotics were added to cultures at the following concentrations per ml: ampicillin 100 µg; tetracycline 13µg; kanamycin 10 µg; spectinomycin 100µg; erythromycin 3µg; chloramphenicol 3µg.

Table 4.1. Bacterial strains and plasmids used in this work

Strain or plasmid	Genotype	Source or reference
<i>E. coli</i> strains		
TG-1	<i>supE hsdΔ5 thi Δ(lac-proAB) F'[traD6 proAB trpC2⁺ lacI^f lacZΔM15]</i>	Sambrook <i>et al.</i> (1989)
<i>B. subtilis</i> strains		
168	<i>trpC2</i>	Laboratory stock
AE04	<i>leuA8 metB5 trpC2 hsrM1 ΔyycFG::neo^r, lacA::P_{spac} yycG erm,^r amyE:: P_{xyI} yycF Cm^r</i>	Wolfgang Schumann, unpublished
MH5117	<i>PheA1 trpC2 mdh::tet^r phoPΔ-EcoRI</i>	Hulett <i>et al.</i> (1994)
AH023	<i>trpC2 ΔyocH::kan^r</i>	pAH025 →168
AH9912	<i>trpC2 ΔyycF::pAH022 (P_{spac} yycFGHIJyyxA; P_{yycF}-lacZ erm^r)</i>	Howell <i>et al.</i> (2003)
BP019	<i>trpC2 amyE::PyocH-bgaB Cm^r</i>	pBP008 →168
BP031	<i>trpC2 amyE::PctaA-bgaB Cm^r</i>	pBP022 →168
BP031A	<i>trpC2 amyE::PctaA-bgaB Cm^r, ΔyycF::pAH022 (P_{spac} yycFGHIJyyxA; P_{yycF}-lacZ erm^r)</i>	pAH022→BP031
BP032	<i>trpC2 amyE::Phmp-bgaB Cm^r</i>	pBP023 →168
BP032A	<i>trpC2 amyE::Phmp-bgaB Cm^r, ΔyycF::pAH022 (P_{spac} yycFGHIJyyxA; P_{yycF}-lacZ erm^r)</i>	pAH022→BP032

BP033	<i>trpC2 amyE::PresA-bgaB Cm^r</i>	pBP024 → 168
BP033A	<i>trpC2 amyE::PresA-bgaB Cm^r, ΔyycF::pAH022 (P_{spac} yycFGHIJyyxA; P_{yycF}-lacZ erm^r)</i>	pAH022→BP033
BP034	<i>trpC2 amyE::PresD-bgaB Cm^r</i>	pBP025 → 168
BP034A	<i>trpC2 amyE::PresD-bgaB Cm^r, ΔyycF::pAH022 (P_{spac} yycFGHIJyyxA; P_{yycF}-lacZ erm^r)</i>	pAH022→BP034
BP035	<i>trpC2 amyE:: PresD^{YycF} binding sites modified-bgaB Cm^r</i>	pBP026 → 168
BP035A	<i>trpC2 amyE::PresD^{YycF} binding sites modified-bgaB Cm^r, ΔyycF::pAH022 (P_{spac} yycFGHIJyyxA; P_{yycF}-lacZ erm^r)</i>	pAH022→BP035
BP040	<i>trpC2 amyE:: PresD^{PhoP} binding sites modified-bgaB Cm^r</i>	pBP032 → 168
BP040A	<i>trpC2 amyE::PresD^{PhoP} binding sites modified-bgaB Cm^r, ΔyycF::pAH022 (P_{spac} yycFGHIJyyxA; P_{yycF}-lacZ erm^r)</i>	pAH022→BP040
BP041	<i>PheA1 trpC2 mdh::tet^r phoPA-EcoRI, amyE:: PresD -bgaB Cm^r</i>	pBP025→MH5117
BP041A	<i>PheA1 trpC2 mdh::tet^r phoPA-EcoRI, amyE::PresD-bgaB Cm^r, ΔyycF::pAH022 (P_{spac} yycFGHIJyyxA; P_{yycF}-lacZ erm^r)</i>	pAH022→BP041
BP050	<i>trpC2 amyE::PyjeA-bgaB Cm^r</i>	pBP039→ 168
BP051	<i>trpC2 amyE::PyjeA1-bgaB Cm^r</i>	pBP040→ 168
BP053	<i>trpC2 lacA::P_{spac} yycG erm,^r amyE:: P_{xyI} yycF Cm^r</i>	AE04 chr DNA→ 168
BP056	<i>trpC2 ΔyycFG::neo^r, lacA::P_{spac} yycG erm,^r amyE::P_{xyI} yycF Cm^r</i>	AE04 chr DNA→ BP053
BP063	<i>trpC2 amyE::PyvcE-bgaB Cm^r</i>	pBP056→ 168
BP064	<i>trpC2 amyE::PyvcE1-bgaB Cm^r</i>	pBP057→ 168
BP066	<i>trpC2 amyE::PydjM-bgaB Cm^r</i>	pBP058→ 168
BP068	<i>trpC2 ΔyjdM::tet^r</i>	pBP061→ 168
BP070	<i>trpC2 ΔyjeA::kan^r</i>	pBP063→ 168
BP071	<i>trpC2 ΔyocH::kan^r ΔyjdM::tet^r</i>	pBP061→ AH023
BP075	<i>trpC2 amyE::PydjM1-bgaB Cm^r</i>	pBP064→ 168
BP079	<i>trpC2 ΔyvcE::spc^r</i>	pBP062→ 168
BP080	<i>trpC2 thrC:: P_{xyI} yvcE spc^r</i>	pBP069→ 168
BP081	<i>trpC2 htrA::pBP068 spc^r</i>	pBP068→ 168

BP082	<i>trpC2 glu::kan^r</i>	pBCJ310.1→168
BP083	<i>trpC2 ΔyjdM::tet^r ΔyvcE::spc^r</i>	pBP062→BP068
BP087	<i>trpC2 ΔyocH::kan^r ΔyvcE::spc^r</i>	BP079 chr DNA→AH023
BP088	<i>trpC2 ΔyjeA::kan^r ΔyycF::pAH022 (P_{spac} yycFGHIJyyxA; P_{yycF}-lacZ erm^r)</i>	pAH022→BP070
BP090	<i>trpC2 ΔyvcE::spc^r ΔyjdM::tet^r ΔyocH::kan^r</i>	AH023 chr DNA→BP083
BP110	<i>trpC2 ΔlytE::pBP088 (P_{spac} lytE) erm^r</i>	pBP088→168
BP111	<i>trpC2 ΔyvcE::spc^r ΔlytE::pBP088 (P_{spac} lytE) erm^r</i>	pBP088→BP079
BP112	<i>trpC2 ΔyvcE::spc^r ΔlytE::pBP088 (P_{spac} lytE) erm^r</i> <i>pMAP65 km^r neo^r</i>	pMAP65→BP111
BP113	<i>trpC2 thrC::P_{xyI} yvcE spc^r, ΔlytE::pBP088 (P_{spac} lytE) erm^r</i>	pBP088→BP080
BP114	<i>trpC2 ΔyvcE::pBP092 (P_{xyI} yvcE) erm^r</i>	pBP092→168
BP115	<i>trpC2 ΔlytE::Cm^r, ΔyvcE::pBP092 (P_{xyI} yvcE) erm^r</i>	pBP092→L16638
BP118	<i>trpC2 amyE::PlytE-bgaB Cm^r</i>	pBP093→168
BP120	<i>trpC2 amyE::PlytE-bgaB Cm^r ΔyycF::pAH022 (P_{spac} yycFGHIJyyxA; P_{yycF}-lacZ erm^r)</i>	pAH022→BP118
BP122	<i>trpC2 ΔyocH::kan^r, ΔlytE::Cm^r</i>	L16638 chr. DNA→AH023
BP123	<i>trpC2 ΔyjdM::tet^r, ΔlytE::Cm^r</i>	L16638 chr. DNA→BP068
BP124	<i>trpC2 ΔyocH::kan^r, ΔyjdM::tet^r, ΔlytE::Cm^r</i>	L16638 chr. DNA→BP071
BP125	<i>trpC2 thrC::P_{xyI} yvcE spc^r, yycF::pAH022 (P_{spac} yycFGHIJyyxA; P_{yycF}-lacZ erm^r)</i>	pAH022→BP080
EL014	<i>trpC2 amyE::PyoeB2-bgaB Cm^r</i>	pEL010.22→168
EL015	<i>trpC2 amyE::PyoeB1-bgaB Cm^r</i>	pEL011.34→168
EL016	<i>trpC2 amyE::PyoeB-bgaB Cm^r</i>	pEL012.43→168
EL019	<i>trpC2 yycF::pAH022 (P_{spac} yycFGHIJyyxA; P_{yycF}-lacZ erm^r)</i> <i>yocFG::km^r</i>	pAH022→EL017
L16638	<i>trpC2 ΔlytE::Cm^r</i>	Margot <i>et al.</i> (1998)
SQ1	<i>trpC2 amyE::PydjM2-bgaB Cm^r</i>	PSQF1.1→168
SQ3	<i>trpC2 amyE::PydjM3-bgaB Cm^r</i>	PSQF3.1→168

Plasmids

pBCJ310.1	Campbell integration vector for insertion at the beta glucanase locus, conferring kanamycin resistance	Jester BC, unpublished
pDL	Integration vector for the introduction of single copy transcriptional fusions to bgaB by double crossover at the <i>amy</i> locus (Ap ^r Cm ^r).	Yuan and Wong (1995)
pDG782	Vector for double crossover deletion and replacement of the deleted region by a kanamycin resistance cassette (Ap ^r Kan ^r).	Guerout-Fleury <i>et al.</i> (1995)
pDG1515	Vector for double crossover deletion and replacement of the deleted region by a tetracycline resistance cassette (Ap ^r Tet ^r).	Guerout-Fleury <i>et al.</i> (1995)
pDG1727	Vector for double crossover deletion and replacement of the deleted region by a spectinomycin resistance cassette (Ap ^r Spc ^r).	Guerout-Fleury <i>et al.</i> (1995)
pMAP65	Non-integrative vector encoding <i>lacI</i> for a tighter repression of the P _{spac} promoter in the absence of IPTG inducer.	Petit <i>et al.</i> (1998)
pMutin4	Integration vector for constructing transcriptional fusions to the <i>E. coli lacZ</i> gene and for inducible gene expression from the P _{spac} promoter (Ap ^r Erm ^r).	Vagner <i>et al.</i> (1998)
pXT	Vector for single copy gene expression under inducible control of the <i>B. subtilis xylA</i> promoter and integration at the <i>thrC</i> locus (Ap ^r Spc ^r Erm ^r).	Derré <i>et al.</i> (2000)
pHTxyl	Integration vector for inducible gene expression from the <i>B. subtilis xylA</i> promoter (Ap ^r Erm ^r).	Tarek Msadek (unpublished)
pAH022	pMUTIN4 derivative for inducible expression of the <i>yycF</i> operon. Contains a 257 bp fragment overlapping the RBS and 5' portion of <i>yycF</i> cloned into <i>EcoRI-BamHI</i> .	Howell <i>et al.</i> (2003)
pBP008	pDL derivative containing the <i>yocH</i> promoter region (Ap ^r Cm ^r).	This work
pAH025	pDG782 derivative for deletion of the <i>yocH</i> locus. Contains fragment partially upstream of <i>yocH</i> (nucleotides -147 to 118 of <i>yocH</i> ORF cloned into <i>XhoI-BglII</i>) and fragment	This work

	predominantly within <i>yocH</i> (a 538 bp fragment beginning at nucleotide 405 of <i>yocH</i> ORF cloned into <i>EcoRI-BamHI</i>) either side of a kanamycin resistance cassette.	
pBP022	pDL derivative containing the <i>ctaA</i> promoter region (Ap ^r Cm ^r).	This work
pBP023	pDL derivative containing the <i>hmp</i> promoter region (Ap ^r Cm ^r).	This work
pBP024	pDL derivative containing the <i>resA</i> promoter region (Ap ^r Cm ^r).	This work
pBP025	pDL derivative containing the <i>resD</i> promoter region (Ap ^r Cm ^r).	This work
pBP026	pDL derivative containing a <i>resD</i> promoter region with a modified putative YycF binding site (Ap ^r Cm ^r).	This work
pBP032	pDL derivative containing a <i>resD</i> promoter region with a modified PhoP binding site (Ap ^r Cm ^r).	This work
pBP039	pDL derivative containing the <i>yjeA</i> promoter region (Ap ^r Cm ^r).	This work
pBP040	pDL derivative containing the <i>yjeA1</i> promoter region (Ap ^r Cm ^r).	This work
pBP043	pMUTIN4 derivative for inducible expression of the <i>yvcE</i> gene. Contains a 518 bp fragment overlapping the RBS and 5' portion of <i>yvcE</i> cloned into <i>BamHI</i> .	This work
pBP056	pDL derivative containing the <i>yvcE</i> promoter region (Ap ^r Cm ^r).	This work
pBP057	pDL derivative containing the <i>yvcE1</i> promoter region (Ap ^r Cm ^r).	This work
pBP058	pDL derivative containing the <i>ydjM</i> promoter region (Ap ^r Cm ^r).	This work
pBP061	pDG1515 derivative for deletion of the <i>ydjM1</i> locus. Contains fragment upstream of <i>ydjM</i> (nucleotides -723 to -30 of <i>ydjM</i> ORF cloned into <i>PstI</i>) and fragment predominantly downstream of <i>ydjM</i> (a 833 bp fragment beginning at nucleotide +353 of <i>ydjM</i> ORF cloned into <i>HindIII</i>) at either	This work

	side of a tetracycline resistance cassette.	
pBP062	pDG1727 derivative for deletion of the <i>yvcE</i> locus. Contains fragment upstream of <i>yvcE</i> (nucleotides -893 to -224 with of <i>yvcE</i> ORF cloned into <i>Bam</i> HI) and fragment predominantly downstream of <i>yvcE</i> (a 918 bp fragment beginning at nucleotide +1389 of <i>yvcE</i> ORF cloned into <i>Xho</i> I) at either side of a spectinomycin resistance cassette.	This work
pBP063	pDG780 derivative for deletion of the <i>yjeA</i> locus. Contains fragment predominantly upstream of <i>yjeA</i> (nucleotides -889 to +59 with respect of <i>yjeA</i> ORF cloned into <i>Bam</i> HI) and fragment predominantly downstream of <i>yjeA</i> (a 918 bp fragment beginning at nucleotide +1347 of <i>yvcE</i> ORF cloned into <i>Xho</i> I) at either side of a kanamycin resistance cassette.	This work
pBP064	pDL derivative containing the <i>ydjMI</i> promoter region (Ap^r Cm^r)	This work
pBP068	pDG1727 derivative for Campbell integration at the <i>htrA</i> locus. Contains a 153 bp fragment from the N-terminal region of the PDZ domain of <i>htrA</i> cloned between the <i>Mlu</i> I and the <i>Eco</i> RI sites flanking a spectinomycin resistance cassette.	This work
pBP069	pXT derivative containing for inducible expression of the <i>yvcE</i> gene. Contains a 1524 bp fragment overlapping the RBS and the coding region of <i>yvcE</i> cloned into <i>Bam</i> HI.	This work
pBP088	pMUTIN4 derivative for inducible expression of the <i>lytE</i> gene. Contains a 552 bp fragment overlapping the RBS and 5' portion of <i>lytE</i> cloned into <i>Bam</i> HI.	This work
pBP092	pHTxyl derivative for inducible expression of the <i>yvcE</i> gene. Contains a 506 bp fragment overlapping the RBS and 5' portion of <i>yvcE</i> cloned into <i>Bam</i> HI.	This work
pBP093	pDL derivative containing the <i>lytE</i> promoter region (Ap^r Cm^r).	This work
pEL011.34	pDL derivative containing the <i>yoeB1</i> promoter region (Ap^r	This work

	Cm').	
pEL010.22	pDL derivative containing the <i>yoeB2</i> promoter region (Ap ^r Cm').	This work
pSQF1.1	pDL derivative containing the <i>ydjM2</i> promoter region (Ap ^r Cm').	This work
pSQF3.1	pDL derivative containing the <i>ydjM2</i> promoter region (Ap ^r Cm').	This work

4.2. Strain construction

Strain AH9912 was constructed as described previously (Howell *et al.*, 2003). Strain AH023 was generated by transforming strain 168 with linearized plasmid pAH025, selecting for kanamycin resistance. This plasmid was constructed by cloning a fragment upstream of *yocH* (amplified with primers *yocHKO*-1 and *yocHKO*-2) and a fragment downstream of *yocH* (amplified with primers *yocHKO*-3 and *yocHKO*-4) either side of the *kan^r* cassette in pDG782 (Guerout-Fleury *et al.*, 1995). Plasmid pDL (Yuan and Wong, 1995) was used to construct the transcriptional fusions to the *bgaB* reporter gene. *Pst*I linearised pDL constructs were transformed into appropriate *B. subtilis* strain 168 giving ectopic insertion at the *amyE* locus. Promoter fragments with mutated YycF recognition boxes (see Table 2.2) were constructed using the PCR-based method of strand overlap extension (Horton, 1989). Strain BP019 was constructed by transforming strain 168 with linearized plasmid pBP008. This plasmid was constructed by cloning a 613 bp fragment containing the *yocH* promoter region (amplified with primers *yocH* PM1 and *yocH* PM2) into pDL. Strain BP031 was constructed by transforming strain 168 with linearized plasmid pBP022. This plasmid was constructed by cloning a 314 bp fragment containing the *ctaA* promoter region (amplified with primers oBP015 and oBP016) into pDL. Strain BP031A was constructed by transforming strain BP031 with plasmid pAH022 (Howell *et al.*, 2003). Strain BP032 was constructed by transforming strain 168 with linearized plasmid pBP023. This plasmid was constructed by cloning a 343 bp fragment containing the *hmp* promoter region (amplified with primers oBP017 and oBP018) into pDL. Strain BP032A was constructed by transforming strain BP032 with plasmid pAH022 (Howell *et al.*, 2003). Strain BP033 was constructed by transforming strain 168 with linearized plasmid pBP024. This plasmid was constructed by cloning a 302 bp fragment containing the *resA* promoter region (amplified with primers *resAPM*1 and *resAPM*2) into pDL. Strain BP033A was constructed by transforming strain BP033 with plasmid pAH022

(Howell *et al.*, 2003). Strain BP034 was constructed by transforming strain 168 with linearized plasmid pBP025. This plasmid was constructed by cloning a 333 bp fragment containing the *resD* promoter region (amplified with primers oBP020 and oBP021) into pDL. Strain BP034A was constructed by transforming strain BP034 with plasmid pAH022 (Howell *et al.*, 2003). Strain BP035 contains a *resD-bgaB* promoter fusion carrying mutations in the putative YycF binding motif. Two PCR products (a fragment amplified with primers oBP020 and yycFRS2 and a fragment amplified with primers yycFRS1 and oBP021) were fused by the SOE method and cloned into plasmid pDL, resulting in plasmid pBP026. Strain BP035 was then constructed by transforming strain 168 with linearized plasmid pBP026. Strain BP035A was constructed by transforming strain BP035 with plasmid pAH022 (Howell *et al.*, 2003). Strain BP040 contains a *resD-bgaB* promoter fusion carrying mutations in the PhoP binding motif. Two PCR products (a fragment amplified with primers oBP020 and phoPRS2 and a fragment amplified with primers phoPR1 and oBP021) were fused by the SOE method and cloned into plasmid pDL, resulting in plasmid pBP032. Strain BP040 was then constructed by transforming strain 168 with linearized plasmid pBP032. Strain BP040A was constructed by transforming strain BP040 with plasmid pAH022 (Howell *et al.*, 2003). Strain BP041 contains a *resD-bgaB* promoter fusion and a deletion in the *phoP* coding region. It was constructed by transforming strain MH5117 (Hulett *et al.*, 1994) with linear plasmid pBP025, described above. Strain BP041A was constructed by transforming strain BP041 with plasmid pAH022 (Howell *et al.*, 2003). Strain BP050 was constructed by transforming strain 168 with linearized plasmid pBP039. This plasmid was constructed by cloning a 374 bp fragment containing the *yjeA* promoter region (amplified with primers oBP054 and oBP055) into pDL. Strain BP051 contains a *yjeA1-bgaB* promoter fusion with putative YycF binding sequences mutated as shown in Table 2.2. Two PCR products (a 240 bp fragment amplified with primers oBP054 and oBP056 and a 151 bp fragment amplified with primers oBP057 and oBP055) were fused by the strand overlap extension method and cloned into plasmid pDL, resulting in plasmid pBP040. Strain BP051 was then constructed by transforming strain 168 with linearized plasmid pBP040. Strain BP053 was constructed by transforming strain 168 with chromosomal DNA isolated from strain AE04 and selecting for chloramphenicol and erythromycin resistance. Strain BP056 was constructed by transforming strain BP053 with chromosomal DNA isolated from strain AE04 and selecting for neomycin resistance. Strain BP063 was constructed by transforming strain 168 with linearized plasmid pBP056. This plasmid was constructed by cloning a 512 bp fragment containing the *yvcE* promoter region (amplified with primers oBP104 and oBP105) into pDL. Strain BP064 contains a *yvcE1-bgaB* promoter fusion

in which the putative YycF binding sequences are mutated as shown in Table 2.2. Two PCR products (a 220 bp fragment amplified with primers oBP104 and oBP109 and a 307 bp fragment amplified with primers oBP108 and oBP105) were fused by the strand overlap extension method and cloned into plasmid pDL, resulting in plasmid pBP057. Strain BP064 was then constructed by transforming strain 168 with linearized plasmid pBP057. Strain BP066 was constructed by transforming strain 168 with linearized plasmid pBP058. This plasmid was constructed by cloning a 517 bp fragment containing the *ydjM* promoter region (amplified with primers oBP106 and oBP107) into pDL. Strain BP068 was constructed by transforming strain 168 with linearized plasmid pBP061, selecting for tetracycline resistance. This plasmid was constructed by cloning a fragment upstream of *ydjM* (amplified with primers oBP091 and oBP092) and a fragment predominantly downstream of *ydjM* (amplified with primers oBP116 and oBP103) either side of a *tet^r* cassette in pDG1515 (Guerout-Fleury *et al.*, 1995). Strain BP070 was constructed by transforming strain 168 with linearized plasmid pBP063, selecting for kanamycin resistance. This plasmid was constructed by cloning a fragment predominantly upstream of *yjeA* (amplified with primers oBP050 and oBP055) and a fragment predominantly downstream of *yjeA* (amplified with primers oBP115 and oBP053) either side of a *kan^r* cassette in pDG780 (Guerout-Fleury *et al.*, 1995). Strain BP071 was constructed by transforming strain AH023 with plasmid pBP061. Strain BP075 contains a *ydjM1-bgaB* promoter fusion carrying mutations in the putative YycF binding motifs as detailed in Table 2.2. Two PCR products (a 435 bp fragment amplified with primers oBP106 and oBP111 and a 101 bp fragment amplified with primers oBP110 and oBP107) were fused by the SOE method and cloned into plasmid pDL, resulting in plasmid pBP064. Strain BP075 was then constructed by transforming strain 168 with linearized plasmid pBP064. Strain BP079 was constructed by transforming strain 168 with linearized plasmid pBP062, selecting for spectinomycin resistance. This plasmid was constructed by cloning a fragment upstream of *yvcE* (amplified with primers oBP074 and oBP075) and a fragment predominantly downstream of *yvcE* (amplified with primers oBP112 and oBP090) either side of a *spc^r* cassette in pDG1727 (Guerout-Fleury *et al.*, 1995). Strain BP080 was constructed by transforming strain 168 with linearized plasmid pBP069. This plasmid was constructed by cloning a DNA fragment that overlaps the ribosome binding site and the coding region of *yvcE* (amplified with primers oBP130 and oBP131) into plasmid pXT (Derré *et al.*, 2000). Strain BP081 was constructed by transforming strain 168 with plasmid pBP068. This plasmid was constructed by cloning a fragment internal of *htrA* (amplified with primers htrAΔpdz91 and htrAΔpdz92) upstream of a *spc^r* cassette in pDG1727 (Guerout-Fleury *et al.*, 1995). Strain

BP082 was constructed by transforming strain 168 with plasmid pBCJ310.1 (Brian Jester, unpublished) and selecting for kanamycin resistance. Strain BP083 was constructed by transforming strain BP068 with linearized plasmid pBP062 (described above). Strain BP087 was constructed by transforming strain AH023 with chromosomal DNA extracted from strain BP079. Strain BP088 was constructed by transforming strain BP070 with plasmid pAH022 (Howell *et al.*, 2003). Strain BP090 was constructed by transforming strain BP083 with chromosomal DNA extracted from strain AH023. Strain BP110 was constructed by transforming strain 168 with plasmid pBP088. This plasmid was constructed by cloning a 552 bp DNA fragment, that overlaps the ribosome binding site and 5' portion of *lytE* (amplified with primers oBP182 and oBP183), into plasmid pMutin4 (Vagner *et al.*, 1998). Strain BP111 was constructed by transforming strain BP079 with plasmid pBP088. This plasmid was constructed as outlined above. Strain BP112 was constructed by transforming strain BP111 with plasmid pMAP65 (Petit *et al.*, 1998). Strain BP113 was constructed by transforming strain BP080 with plasmid pBP088. This plasmid was constructed as outlined above. Strain BP114 was constructed by transforming strain 168 with plasmid pBP092. This plasmid was constructed by cloning a 506 bp fragment overlapping the ribosome binding site and 5' portion of *yvcE* (amplified with primers oBP184 and oBP062) into plasmid pHTxyl (T. Msadek, unpublished). Strain BP115 was constructed by transforming strain L16638 with plasmid pBP092. Plasmid pBP092 was constructed as described above. Strain BP118 was constructed by transforming strain 168 with linearized plasmid pBP093. This plasmid was constructed by cloning a 274 bp fragment containing the *lytE* promoter region (amplified with primers oBP177 and oBP166) into pDL. Strain BP120 was constructed by transforming strain BP118 with plasmid pAH022 (Howell *et al.*, 2003) and screening for IPTG-dependent colonies. Strain BP122 was constructed by transforming strain AH023 with chromosomal DNA isolated from strain L16638 and selecting for chloramphenicol resistance. Strain BP123 was constructed by transforming strain BP068 with chromosomal DNA isolated from L16638, selecting for chloramphenicol resistance. Strain BP124 was constructed by transforming strain BP071 with chromosomal DNA isolated from L16638 and selecting for chloramphenicol resistance. Strain BP125 was constructed by transforming strain BP080 with plasmid pAH022 (Howell *et al.*, 2003). Plasmid pBP043 was constructed by cloning a 518 bp DNA fragment, that overlaps the ribosome binding site and 5' portion of *yvcE* (amplified with primers oBP061 and oBP062), into plasmid pMutin4 (Vagner *et al.*, 1998).

Plasmid pEL012.43 was constructed by cloning a 414bp *EcoRI*-*BamHI* PCR fragment corresponding to the *yoeB* promoter region (synthesized with primers yoeBELF1 and

yoeBELR1) into similarly restricted pDL vector. To introduce mutations into the *yoeB* promoter (shown in Table 2.2), plasmid pEL011.34 was constructed generating the *yoeB1* construct. Two PCR fragments were generated using primers yoeBELF1/yoeBELR4 and yoeBELF4/yoeBELR1. The two fragments were then fused using the strand overlap extension method, restricted with *EcoRI-BamHI* and then cloned into pDL. Similarly to generate the *yoeB2* promoter plasmid pEL010.22 was constructed by fusing two PCR fragments generated by primer pairs yoeBELF1/yoeBELR3 and yoeBELF3/yoeBELR1. The resultant fragment was digested with *EcoRI-BamHI* and cloned it into pDL. Strains EL016, EL015 and EL014 were obtained by transforming plasmids pEL012.43, pEL011.34 and pEL010.22 into 168, respectively, and selecting for chloramphenicol resistance.

The AKP21 strain (Aguilar *et al.*, 2001) used in this study carries a *amyE::P_{des}-lacZ Cm^r* and a *yocFG(desKR)::Km^r* locus. To separate the two loci, chromosomal DNA from AKP21 was transformed into 168. The transformants were screened for kanamycin resistance and chloramphenicol sensitivity and the separation of loci was verified by PCR. Thus strain EL017 was generated carrying only the *yocFG(desKR)::Km^r* locus. Strain EL019 was then obtained by transforming plasmid pAH022 (Howell *et al.*, 2003) into EL017. Strain SQF1.1 contains a *ydjM1-bgaB* promoter fusion carrying a deletion of 6 base pairs in between the SigA -10 motif and the putative SigA -35 motif as detailed in Table 2.2. Two PCR products (a 456 bp fragment amplified with primers oBP106 and ydjMOL2 and a 55 bp fragment amplified with primers ydjMOL1 and oBP107) were fused by the SOE method and cloned into plasmid pDL, resulting in plasmid pSQF1.1. Strain SQ1 was then constructed by transforming strain 168 with linearized plasmid pSQF1.1. Strain SQ3 contains a *ydjM1-bgaB* promoter fusion carrying the same deletion described above for strain SQ1 as well as the mutation in the putative YycF binding sequences described above for strain BP075. Strain SQ3 was constructed by transforming strain 168 with linearized plasmid pSQE3.1. Plasmid pSQE3.1. was constructed essentially as described above for plasmid pSQF1.1, with the difference that for the SOE PCR reaction plasmid pBP064 was used as a template. All constructs were confirmed by sequencing.

4.3. DNA manipulations

DNA manipulations were carried out by standard procedures as described by Sambrook *et al.* (1989).

PCR reactions were carried out in 25-50 µl final volumes according to the manufacturer protocols. *Taq* polymerase (Invitrogen; Paisley, UK) was used for screening procedures; *Pfu*

Turbo polymerase (stratagene; Amsterdam, The Netherlands) was used for high-fidelity cloning. Primers were synthesized to order by Sigma Genosys, Cambridge, UK. Strand overlap extension PCR was carried out as described by Horton *et al.*, (1989). The oligonucleotides used in this study are listed in Table 4.2.

Plasmid isolation was carried out according to the boiling lysis method (Holmes, 1981) followed by further purification with the use of Invitrogen DNA purification columns.

Bacterial transformation was carried out by standard procedures: *E. coli* TG1 was transformed by the calcium chloride method (Sambrook *et al.*, 1989) and *B. subtilis* strains were transformed as described by Leskela (Leskela *et al.*, 1996).

Table 4.2. Primers used in this work

Name	Sequence
oBP015	5'-CGCGGATCCTTAGCCTAACGTAACCTCCCTTAC-3'
oBP016	5'-CGCGGATCCACAAATGTCGTCAGAACACC-3'
oBP017	5'-CGCGGATCCGCTTGCAAATGCGCTCGG-3'
oBP018	5'-CGCGGATCCCCATGCTGTTGCAATACGGG-3'
oBP020	5'-CGCGGATCCGGGACCCTAAGGAAGTATG-3'
oBP021	5'-CGCGGATCCCTGGCTTCGTCATCAAC-3'
oBP050	5'-CGCGGATCCAAGCCATCACAAACGCGGTTC-3'
oBP053	5'-CCGCTCGAGGCAGCGTAAGGGTCAACAAGAG-3'
oBP054	5'-CCGGAATTCGGCTGTCTTTCATCATAAGGGG-3'
oBP055	5'-CGCGGATCCCCCACCACACATACAACTGC-3'
oBP056	5'-GTAGTGTTGTAGTAGTGTTCATTTTACATTTG-3'
oBP057	5'-CACTACTACAACACTACAAAAACATTTTC-3'
oBP061	5'-CCGGAATTCGATTCCAAATGAGGACAGGTTC-3'
oBP062	5'-CGCGGATCCAGTCGCACGAGAGATAAAGTCACC-3'
oBP074	5'-CGCGGATCCAAAGTCGAGTCGCAATATCCC-3'
oBP075	5'-CGCGGATCCCCATCAAGTTTCGACAAATTAGCG-3'
oBP090	5'-CCGCTCGAGCATTATCAAATGCTCTCGC-3'
oBP091	5'-AAAACCTGCAGCTCGACTACAACGCGGTCTCC-3'
oBP092	5'-AAAACCTGCAGGATTATACACGATCAACCAGC-3'
oBP103	5'-CCCAAGCTTTCGTTTTATGTTGCAAAGG-3'
oBP104	5'-CCGGAATTCGACACGAAAAAAGACACAGCGG-3'
oBP105	5'-CGCGGATCCGATGACGGAAGCCAAACCAAG-3'
oBP106	5'-CCGGAATTCGCTCCGTTTTTGGCTCTTCG-3'
oBP107	5'-CGCGGATCCCTTCAACATTTTCGTCCTCCCTG-3'
oBP108	5'-GACTTGACATCTGTTCAACATAAATG-3'
oBP109	5'-GTGAACAGATGTCAAGTCACACATC-3'
oBP110	5'-GACTTTGTAACATTACCACAAGGATATTG-3'
oBP111	5'-GTGGTAATGTTACAAAGTCAAAAAAGTTTCGTCCTCCCTG-3'
oBP112	5'-CCGCTCGAGTCCTTACTGGAAAGCAGC-3'
oBP115	5'-CCGCTCGAGCAATTGGTGACAGTTTCTCAGC-3'
oBP116	5'-CCCAAGCTTAAGCAAAGGTAAAGTAGC-3'
oBP130	5'-CGCGGATCCAGGTTACAGTAAAAGGG-3'

oBP131	5'-CGCGGATCCAAATGTATTCGAAAAGGG-3'
oBP165biotin	5'-GTGTGTGCAAATAACGCTCC-3'
oBP166	5'-GTGTGTGCAAATAACGCTCC-3'
oBP177	5'-CGCGGATCCGCACTCGTTTTTTGTCACC-3'
oBP182	5'-CGCGGATCCGAGTTAACATTTGGGGAGG-3'
oBP183	5'-CGCGGATCCAACCGTAGTGCCGTATTTCC-3'
oBP184	5'-CGCGGATCCCGATTCCAAATGAGGACAGGTC-3'
phoPRS1	5'-GCGACACAGTTCTCAAACCTTTGGGGCGATTTG-3'
phoPR1	5'-CAAATCGCCCCAAAGTTTGAGAACTGTGTCGC-3'
resAPM1	5'-CCGGAATTTCGCCAGAGAGTTACGCCTCACC-3'
resAPM2	5'-CGCGGATCCTTCGGTGCCTCGGACC-3'
ydjMOL1	5'-ATTGAAATAAAAAATTTGATCGTGTATAATCTTCC-3'
ydjMOL2	5'-GATTATACACGATCAAATTTTTTATTTCAATATCC-3'
yocHKO-1	5'-CCGCTCGAGTTCAGCAAGTCTTTCCATCAGAGAG-3'
yocHKO-2	5'-GAAGATCTAGATTCCCCAGAGCGTATCACCC-3'
yocHKO-3	5'-CCGGAATTCGCAAACAAACGCACCTCAAGC-3'
yocHKO-4	5'-CGCGGATCCTCCCCGTAGCCTTCAACATAGAC-3'
yocHPM1	5'-CCGGAATTCAGCCCCGTAAATGATGATG-3'
yocHPM2	5'-CGCGGATCCGTATATTGCCAGTCGTGGTTG-3'
yoeBELF1	5'-GGAATTCCAAGAGGTTCTTGTGATGTTTCC-3'
yoeBELR1	5'-CGGGATCCGCTTGTTTTTGTGATAAATCGCC-3'
yoeBELF3	5'-GTATTGTAATCTATCAACAATTATTGTAAC-3'
yoeBELR3	5'-GTTACAATAATTGTTGATAGATTACAATAC-3'
yoeBELF4	5'-CCGTAATTATTGTAACATTCAACAATAAG-3'
yoeBELR4	5'-CTTATGTTGTGAATGTTACAATAATTACGG-3'
yoeB PROBE-1	5'-CCGGAATTCCTGAAGGAGAGAGCGATAATG-3'
yoeB PROBE-2	5'-CGCGGATCCTAATACGACTCACTATAGGGTCGTATGTG ATTGTGCTGCC-3'
ykuP PROBE-1	5'-CCGGAATTCCTCATTAGAGGAGGAACAAGG-3'
ykuP PROBE-2	5'-CGCGGATCCTAATACGACTCACTATAGGGTGATCAGAC AGCGCACCCG-3'
yycFRS1	5'-CGGTTTTGCCCCTATTATGTGGGATTTAATATTCG-3'
yycFRS2	5'-CCCACATAATAGGGGGCAAACCGATTACGGCC-3'
des PM-1	5'-CCGGAATTCGAGAGGATACTTACTATG-3'
des PROBE-2	5'-CGCGGATCCTAATACGACTCACTATAGGGCCAGATTCTG TAAGGTATGTG-3'
ydjM PROBE-1	5'-CCGGAATTCGGACGAAATGTTGAAGAAAGTC-3'
ydjM PROBE-2	5'-CGCGGATCCTAATACGACTCACTATAGGGGGCTAACAT CCAGCACTGC-3'
yvcE PROBE-1	5'-CCGGAATTCGTTTGGCTTCCGTCATCGGG-3'
yvcE PROBE-2	5'-CGCGGATCCTAATACGACTCACTATAGGGCCGCTTCAG AATCTTCGAGC-3'
LYTEF1	5'-CTACGACAGCAGTTGTTTTAGGAGC-3'
LYTER1	5'-TGAAGACGATGAAGATGAAGCAGG-3'
htrAΔpdz91	5'-CGGAATTCGGACAAATTGAACGTCCATATATC-3'
htrAΔpdz92	5'-CGACGCGTGGCCTTTTCAGCAGGAGAGC-3'
ydjMbiotin	5'-CTATAACTTTTGATGTGAGTAATTCC-3'
ydjMGSR	5'-CCCTGTTTGTAAACATCTAGG-3'
desbiotin	5'-GCTATTTTCGGGTACATCACG-3'
desGSR	5'-GTTTAAAGCTGAATCAGGC-3'

yoeBbiotin	5'-CAAGAGGTTCTTGTGATGTTTCC-3'
yoeBGSR	5'-GCTTGTTTTTGTGATAAATCGCC-3'
ykuNbiotin	5'-TATTAGGTTATGAGCCTGCC-3'
ykuNGSR	5'-CATTTATCTCGACACAATCGATATCC-3'
yvcEbiotin	5'-TTAAAGAGTTGATTTATTTAATCG-3'
yvcEGSR	5'-CTCTTTCTCACTTTTATATCCTCC-3'
yjeAELF1	5'-AGGCTGTCTTTCATCATAAGGGG-3'
yjeAELR1	5'-CGTCATTACACAAGCGTTGCG-3'
htrAbiotin	5'-AGCTGTCTAGCGATCATATCC-3'
htrAGSR	5'-CGGGATCCCCCTGATCGTTCTCTTTCGT-3'
yvcEPE1	5'-CATACTCTACGAGGAATGATGG-3'
yvcEPE2	5'-CTCTTATTCTGGTTGTATAGCG-3'
ydjMPE1	5'-CGTTCACATGTTTCGCACTGGC-3'
ydjMPE2	5'-TTCCTACTAAGATAAAAGCGGC-3'
yoeBPE1	5'-CTTAATGACAGAATAAGTGCAATGG-3'

Underlined sequences indicate restriction sites added for cloning purpose.

4.4. Northern and primer extension analysis

Northern and primer extension analysis was carried out as described in Noone *et al.* (2000). Twenty five (25µg) micrograms of total RNA was used in each reaction. Probes for northern analysis were synthesized by PCR using primer pairs yoeB PROBE-1/yoeB PROBE-2 (for *yoeB*), ykuP PROBE-1/ykuP PROBE-2 (for *ykuP*), des PM-1/des PROBE-2 (for *des*), ydjM PROBE-1/ ydjM PROBE-2 (for *ydjM*), yvcE PROBE-1/ yvcE PROBE-2 (for *yvcE*) and LYTEF1/LYTER1 (for *lytE*). The *yocH* probe was as described in Howell *et al.*, 2003. Primers yvcEPE1, yvcEPE2, ydjMPE1 and ydjMPE2 were used for primer extension from 168 WT total RNA and primers yoeBGSR, yoeBPE1, yjeAGSR and oBP55 were used for primer extension analysis of total RNA isolated from strain AH9912 grown in the absence of IPTG.

4.5. Microarray analysis

The Affymetrix (Affymetrix Ltd., USA) *B. subtilis* Genome Array (antisense) was used for transcriptome analysis of strain AH9912 grown under conditions of YycFG repletion (1mM IPTG) and depletion (no IPTG). An overnight culture of strain AH9912 grown in 100µM IPTG was used to inoculate two LB cultures, one containing 1mM IPTG and the other without IPTG. A culture of wild-type strain 168 *B. subtilis* was similarly prepared as a control. Cells were harvested from the three cultures throughout the following 2.5-hour growth cycle and frozen in liquid nitrogen as described by (Eymann *et al.*, 2002). The point of growth cessation of the AH9912 culture grown without IPTG was determined from the growth curve.

Then total RNA was prepared from *B. subtilis* strain 168 and from strain AH9912 grown in the presence of 1mM IPTG and in the absence of IPTG at this point of the growth cycle of all three cultures and processed according to the method of Eymann *et al.*, (2002). Four separate experiments were performed yielding four total RNA preparations for AH9912 cells grown without IPTG and with IPTG and for wild-type strain 168. The quality of each RNA preparation and the extent to which the cells were YycFG-depleted were estimated using gel electrophoresis and Northern analysis, probing for *yocH* transcript levels, respectively. Three RNA preparations from cells grown under each condition (*ie* + and – IPTG) were chosen for use as probes in the microarray analysis. Biotinylated antisense RNA probe was prepared using the MessageAmpTM II-Bacteria Kit (Ambion Ltd) incorporating biotin-UTP and biotin-CTP. This was used to hybridize to the *B. subtilis* Affymetrix microarrays using standard procedures described for prokaryotic arrays. The arrays were washed in the Affymetrix GeneChip fluidics station 450 and scanned using the GeneChip 3000 scanner.

The raw probe intensities were normalized using the nonlinear-normalization method, Qspline (Workman *et al.*, 2002), and the logit-*t* method was applied to determine differentially expressed genes between the two categories (Lemon *et al.*, 2003). The gene expression index values used to determine the fold changes, were calculated by the use of the method developed by Li and Wong (2001), after applying the Robust Multi-arrays Average (RMA) method for background correction to the raw Perfect Match (PM) probe intensities (Irizarry *et al.*, 2003), and normalizing these using Qspline (Workman *et al.*, 2002).

4.6. Measurement of incorporation of ¹⁴C-labelled N-acetylglucosamine into *B. subtilis* cell walls

A *B. subtilis* culture was grown in LB medium at 37°C and 120 rpm until the OD₆₀₀ had reached a value of 0.5. A 2 ml aliquot of this culture was then added to 8 ml of pre-warmed LB medium containing 20µl of ¹⁴C-labelled N-acetylglucosamine (7.4 MBq/ml; Amersham) and 12.8 µl of unlabelled N-acetylglucosamine (5ng/ml; Sigma Chemical) to give a final concentration of 6.4 mg/L. Growth was monitored turbidimetrically at OD₆₀₀ and 400 µl samples of the cell suspension were harvested in duplicate every 10 minutes, transferred to micro-centrifuge tubes containing 400µl of 0.1% of sodium dodecylsulphate (SDS), pelleted by centrifugation at 15,000 rpm for 10 minutes and the supernatant was discarded. The cell pellets were resuspended in 400µl of distilled water and mixed with 5 ml of scintillation liquid (Ecoscint A, National Diagnostic) and the radioactivity was measured using a liquid scintillation counter (Packard Tri-Carb). To calculate incorporation of ¹⁴C-labelled N-

acetylglucosamine, the amount of radioactivity present in the T_0 samples was subtracted from the value obtained for each sample.

4.7. Measurement of cell wall turnover of *B. subtilis* cell walls

In order to obtain radiolabelled cell walls, 1ml of an exponentially growing culture (OD_{600} 0.25) was added to 9 ml of pre-warmed LB medium containing 20 μ l of 14 C-labelled N-acetylglucosamine (7.4 MBq/ml; Amersham) and incubated at 37°C, 120 rpm for a time corresponding to two generations (44 minutes for all strains with the exception of strain BP068, for which cells were incubated for 60 minutes). Cells were pelleted by centrifugation at 6,000 rpm for 5 minutes at 25°C, and washed twice with pre-warmed LB medium. The cells were then resuspended in 10ml of pre-warmed LB containing 100mg/L of cold N-acetylglucosamine and incubated at 37°C, shaking at 120 rpm. Growth was monitored turbidimetrically at OD_{600} and 400 μ l samples of the cell suspension were taken in duplicate every 10 minutes and transferred into micro-centrifuge tubes containing 400 μ l of 0.1% of sodium dodecylsulphate (SDS). Cells were pelleted by centrifugation at 15,000 rpm for 10 minutes and the radioactivity of both the supernatant and the pellet was measured as described above.

4.8. Protein purification

The YycF protein was purified according to the procedure described in Howell *et al.* (2006).

4.9. Gel mobility shift DNA binding assays

DNA fragments spanning the promoter regions of *yjeA* (340bp, YjeAbiotin/YjeAGSR), *ydjM* (419bp, YdjMbiotin/YdjMGSR), *yvcE* (385bp, YvcEbiotin/YvcEGSR), *lytE* (268bp, oBP177, oBP165biotin), *yoeB* (414bp, YoeBbiotin/YoeBGRS) and *htrA* (htrA biotin/htrAGSR) were generated by PCR using Phusion polymerase (NEB) and the indicated biotinylated oligonucleotide pairs. Biotinylated DNA fragments were gel purified and binding reactions, electrophoresis and detection were performed essentially as described in Howell *et al.* (2006), with the following modifications: 6% native polyacrylamide gels with a 80:1 acrylamide:bisacrylamide ratio were used with a Tris-acetate buffer system. All reactions contained 'YycG and were performed in the presence of 1mM ATP and poly [d(I-C)]. An aliquot of 2ng of biotinylated promoter fragment was used and where appropriate 100X excess of unlabelled competitor DNA.

4.10. Purification, acetylation and lysozyme digestion of *B. subtilis* cell walls

Four hundred ml cultures of *B. subtilis* strains AH9912 and BP088 (both have the *yycFGHIJK* operon under the control of the IPTG-inducible P_{spac} promoter) were grown in the presence and the absence of IPTG. In YycFG-depleted cultures (-IPTG), cells were harvested at the point of growth cessation while in YycFG-replete cultures (1 mM IPTG) cells were harvested at the same OD₅₉₅ as the YycFG-depleted ones (in both cases OD₅₉₅ ~ 0.8). Cells were pelleted by centrifugation at 16100 g for 5 min at 4°C and washed twice in 10 volumes of 10mM sodium phosphate buffer (pH 7). Cell suspensions were transferred to 2 ml screw cap tubes containing 0.5g of glass beads (Biospec; Bartlesville, OK, USA) and broken by vigorous shaking in a Fastprep bead beater (Bio101, Biospec; Bartlesville, OK, USA) for 45 seconds at speed 6.5. The process was repeated 10 times and the tubes were cooled on ice for 2 min in between. Unbroken cells and glass debris were removed by centrifugation at 5000 rpm for 2 min at 4°C and the supernatant was transferred to new tubes. Cell wall fragments were pelleted by centrifugation at 16100 g for 5 min at 4°C, resuspended in 2% SDS and incubated twice for 5 min at 100°C in 2% SDS to remove proteins. Deproteinized cell walls were then washed twice in distilled water, twice in 10mM sodium phosphate buffer (pH 7) and twice in distilled water and stored at -20°C. Cell walls were acetylated as described by Vollmer and Tomasz (2000). To determine lysozyme sensitivity, a suspension of cell wall (both non-acetylated and acetylated) was made in 10mM Tris/HCl pH8 and the turbidity was adjusted to OD₅₉₅ = 0.3. Lysozyme was added at a final concentration of 20µg/ml and digestion was estimated by measuring the decrease in OD₅₉₅ at 25°C. Preparations without added lysozyme were used as negative controls. Native cell walls were prepared essentially as described above but the boiling step in 2% SDS was omitted. Native cell walls were suspended in 10mM Tris/HCl to OD₅₉₅ = 0.3, incubated at 37°C and the decrease in optical density was measured at 595nm.

4.11. Microscopy

Cell samples for microscopic analysis were pelleted by centrifugation and fixed in 3% paraformaldehyde (in Spizizen's minimal salts, pH7.5) for 10 minutes on ice. Cells were washed twice in Spizizen's minimal salts containing 15mM sodium azide and mounted onto glass slides covered with 1.2% agarose in water. To visualize the nucleoids, 10µl of DAPI (0.1µg/ml in 50% v/v glycerol; Sigma) was added to the samples and incubated for 5 minutes in the dark before viewing. To visualize newly synthesized peptidoglycan, cells were grown in the presence of unlabelled and fluorescein-labelled vancomycin for 15 minutes (Molecular

Probes) and prepared for microscopic examination as described by Daniel and Errington (2003). Images were acquired using an Olympus Fluoview 100 Confocal Microscope and analyzed using Fluoview software version 1.3.

4.12. Beta-galactosidase assays

β -galactosidase assays were performed as described in Yuan and Wong (1995). Protein concentration was determined using the Bio-Rad microassay (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

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