Functional analysis of 11 putative essential genes in *Bacillus subtilis*

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Systematic inactivation of *Bacillus subtilis* genes has previously revealed that 271 are indispensable for growth. In the present study, 11 of these (*yacA*, *ydiB*, *ydiC*, *ykqC*, *ylaN*, *yloQ*, *ymdA*, *yneS*, *yqel*, *yqjK* and *ywlC*) were identified as genes encoding proteins of unknown function. By analysing the effects of protein depletion, and examining the subcellular localization of these proteins, a start has been made in elucidating their functions. It was found that four of these genes (*ydiB*, *yloQ*, *yqel* and *ywlC*) were not required for *B. subtilis* viability. Analysis of the localization of YkqC suggests that it co-localizes with ribosomes, and it is proposed that it is involved in processing either rRNA or specific mRNAs when they are associated with the ribosome. The results suggest that other novel essential proteins may be involved in lipid synthesis and control of cell wall synthesis.

INTRODUCTION

The emergence of drug-resistant pathogens has led to a demand for new antimicrobial compounds. One strategy to combat this problem is to identify and exploit new molecular targets. Any such target should be: (i) essential for viability of the bacterium, so that a compound that acts on the target will inhibit growth; (ii) absent from humans; and (iii) conserved in a range of pathogenic bacterial species (Allsop, 1998). Current antimicrobial compounds typically act against a limited number of proteins involved in key processes (synthesis of cell walls, DNA, RNA and proteins). By identifying essential proteins that carry out novel functions, this list of targets can be expanded. In recent years, the availability of complete bacterial genome sequences has facilitated drug discovery by identifying the entire protein-coding capability of a bacterium, and has also allowed the development of new strategies to identify potential drug targets. Bioinformatic analysis can aid the comparison of different bacterial genomes, and determine the extent to which particular proteins are conserved between species. Such information allows the potential spectrum of a new antimicrobial compound to be predicted.

Several genome-wide inactivation studies have been carried out to identify essential genes in different bacterial species. Targeted gene disruptions have been used in *Escherichia coli*

(Arigoni *et al.*, 1998; Freiberg *et al.*, 2001) and *Streptococcus pneumoniae* (Thanassi *et al.*, 2002; Zalacain *et al.*, 2003). Transposon mutagenesis in *Haemophilus influenzae* (Akerley *et al.*, 2002) and *Mycoplasma* sp. (Hutchison *et al.*, 1999) has identified genes that are not required for survival, whilst RNA interference strategies have been utilized for *Staphylococcus aureus* (Forsyth *et al.*, 2002; Ji *et al.*, 2001) and *Streptococcus mutans* (Wang & Kuramitsu, 2003). Although inactivation programmes may identify which proteins are potential targets, they do not provide any information as to their role. Some understanding of the function of a protein is generally required in order to exploit it as an antibacterial target, so that appropriate assays and screening methods can be developed to find a compound that acts upon it.

Following large-scale inactivation of *Bacillus subtilis* genes, 271 have been reported to be essential for viability (Kobayashi *et al.*, 2003). In this study, we took the 11 genes (*yacA*, *ydiB*, *ydiC*, *ykqC*, *ylaN*, *yloQ*, *ymdA*, *yneS*, *yqel*, *yqjK* and *ywlC*) that have been identified as those encoding essential proteins of unknown function, and attempted to determine their roles. In contrast to Kobayashi *et al.* (2003), we found that four of these genes can be disrupted and, therefore, are not required for viability of *B. subtilis*. By constructing conditional alleles of each of the essential genes, we were able to examine the effects of depleting their products on cell growth and morphology. In addition, we used green fluorescent protein (GFP) fusions to analyse the subcellular localization of these novel proteins. In combination, such approaches proved to be valuable for suggesting functions for some proteins.

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Abbreviations: 5FU, 5-fluorouracil; GFP, green fluorescent protein.

While this work was in progress, the functions of some of these proteins have been established. *yqjK* (renamed *rnz*) has been shown to encode an endoribonuclease, RNase Z, and is responsible for processing the 3' end of CCA-less tRNAs (Pelligrini *et al.*, 2003). *yacA* encodes an RNA-modifying enzyme, TilS (Soma *et al.*, 2003). YkqC and its homologue YmfA have also been identified as two novel endoribonucleases, RNase J1 (YkqC) and RNase J2 (YmfA) (Even *et al.*, 2005). Both enzymes are able to cleave the leader sequences of *thrS* and *thrZ* (encoding threonyl-tRNA synthetases) mRNAs, and additional data suggest that RNase J1 may also contribute to global mRNA degradation (Even *et al.*, 2005).

yloQ has recently been shown to encode a GTPase, and is proposed to have a role in ribosome maturation and assembly (Campbell *et al.*, 2005). How our data correlate with the information on these proteins, and with the functions proposed for homologous proteins in other species, is discussed.

METHODS

Bacterial strains, plasmids and growth conditions. The *B. subtilis* strains and plasmids used in this study are listed in Table 1. *B. subtilis* strains were cultured in casein hydrolysate (CH) medium

Table 1. *B. subtilis* strains and plasmids used in this study

Strain or plasmid	Relevant genotype*	Construction, source or reference
Strains		
168	<i>trpC2</i>	Laboratory stock
168 Δ <i>upp</i>	<i>trpC2</i> Δ <i>upp</i>	Fabret <i>et al.</i> (2002)
1049	<i>trpC2</i> Ω <i>amyE</i> ::(<i>P</i> _{xyI} - <i>rpsB</i> - <i>gfpmut1</i> <i>spc</i>)	Lewis <i>et al.</i> (2000)
3551	<i>trpC2</i> Ω <i>ydiB</i> :: <i>pSG5351</i> (<i>ydiB'</i> - <i>lacZ</i> <i>ermC</i> <i>P</i> _{spac} -' <i>ydiB</i>)	This work
3552	<i>trpC2</i> Ω <i>yloQ</i> :: <i>pSG5352</i> (<i>yloQ'</i> - <i>lacZ</i> <i>ermC</i> <i>P</i> _{spac} -' <i>yloQ</i>)	This work
3553	<i>trpC2</i> Ω <i>ywlC</i> :: <i>pSG5353</i> (<i>ywlC'</i> - <i>lacZ</i> <i>ermC</i> <i>P</i> _{spac} -' <i>ywlC</i>)	This work
3554	<i>trpC2</i> Δ <i>upp</i> Ω <i>amyE</i> ::(<i>P</i> _{xyI} - <i>ylnA</i> <i>cat</i>)	This work
3555	<i>trpC2</i> Δ <i>upp</i> Ω <i>amyE</i> ::(<i>P</i> _{xyI} - <i>yqeI</i> <i>cat</i>)	This work
3556	<i>trpC2</i> Δ <i>upp</i> Ω <i>ylnA</i> :: <i>upp</i> - <i>phleo</i> Ω <i>amyE</i> ::(<i>P</i> _{xyI} - <i>ylnA</i> <i>cat</i>)	This work
3557	<i>trpC2</i> Δ <i>upp</i> Ω <i>yqeI</i> :: <i>upp</i> - <i>phleo</i> Ω <i>amyE</i> ::(<i>P</i> _{xyI} - <i>yqeI</i> <i>cat</i>)	This work
3558	<i>trpC2</i> Δ <i>upp</i> Δ <i>ylnA</i> Ω <i>amyE</i> ::(<i>P</i> _{xyI} - <i>ylnA</i> <i>cat</i>)	This work
3559	<i>trpC2</i> Δ <i>upp</i> Ω <i>yqeI</i> :: <i>upp</i> - <i>phleo</i>	This work
3560	<i>trpC2</i> Ω <i>yacA</i> :: <i>pSG5356</i> (<i>yacA'</i> - <i>lacZ</i> <i>ermC</i> <i>P</i> _{spac} - <i>yacA</i>)	This work
3561	<i>trpC2</i> Ω <i>ydiC</i> :: <i>pSG5357</i> (<i>ydiC'</i> - <i>lacZ</i> <i>ermC</i> <i>P</i> _{spac} - <i>ydiC</i>)	This work
3562	<i>trpC2</i> Ω <i>yneS</i> :: <i>pSG5358</i> (<i>yneS'</i> - <i>lacZ</i> <i>ermC</i> <i>P</i> _{spac} - <i>yneS</i>)	This work
3563	<i>trpC2</i> Ω <i>yqjK</i> :: <i>pSG5359</i> (<i>yqjK'</i> - <i>lacZ</i> <i>ermC</i> <i>P</i> _{spac} - <i>yqjK</i>)	This work
3564	<i>trpC2</i> Ω <i>ykqC</i> :: <i>pSG5360</i> (<i>P</i> _{spacHY} - <i>ykqC</i> <i>cat</i>)	This work
4043	<i>trpC2</i> Ω <i>ymdA</i> :: <i>pSG1802</i> (<i>P</i> _{spacHY} - <i>ymdA</i> <i>cat</i>)	This work
3565	<i>trpC2</i> Ω <i>amyE</i> ::(<i>P</i> _{xyI} - <i>gfpmut1</i> - <i>ykqC</i> <i>spc</i>)	This work
3566	<i>trpC2</i> Ω <i>amyE</i> ::(<i>P</i> _{xyI} - <i>yneS</i> - <i>gfpmut1</i> <i>spc</i>)	This work
3567	<i>trpC2</i> Ω <i>amyE</i> ::(<i>P</i> _{xyI} - <i>gfpmut1</i> - <i>ylnA</i> <i>spc</i>)	This work
3569	<i>trpC2</i> Ω <i>amyE</i> ::(<i>P</i> _{xyI} - <i>ymdA</i> - <i>gfpmut1</i> <i>spc</i>)	This work
Plasmids		
pMUTIN4	<i>bla</i> <i>P</i> _{spac} <i>lacZ</i> <i>lacI</i> <i>ermC</i>	Vagner <i>et al.</i> (1998)
pJQ43	<i>bla</i> <i>P</i> _{spacHY} <i>P</i> _{pen} - <i>lacI</i> <i>cat</i>	Quisel <i>et al.</i> (2001)
pSG1154	<i>bla</i> <i>amyE</i> :: <i>P</i> _{xyI} - <i>gfpmut1'</i> <i>spc</i>	Lewis & Marston (1999)
pSG1729	<i>bla</i> <i>amyE</i> :: <i>P</i> _{xyI} -' <i>gfpmut1</i> <i>spc</i>	Lewis & Marston (1999)
pSG5351	<i>bla</i> <i>P</i> _{spac} -' <i>ydiB</i> '- <i>lacZ</i> <i>lacI</i> <i>ermC</i>	This work
pSG5352	<i>bla</i> <i>P</i> _{spac} -' <i>yloQ</i> '- <i>lacZ</i> <i>lacI</i> <i>ermC</i>	This work
pSG5353	<i>bla</i> <i>P</i> _{spac} -' <i>ywlC</i> '- <i>lacZ</i> <i>lacI</i> <i>ermC</i>	This work
pSG5354	<i>bla</i> <i>P</i> _{spac} - <i>yacA'</i> - <i>lacZ</i> <i>lacI</i> <i>ermC</i>	This work
pSG5355	<i>bla</i> <i>P</i> _{spac} - <i>ydiC'</i> - <i>lacZ</i> <i>lacI</i> <i>ermC</i>	This work
pSG5356	<i>bla</i> <i>P</i> _{spac} - <i>yneS'</i> - <i>lacZ</i> <i>lacI</i> <i>ermC</i>	This work
pSG5357	<i>bla</i> <i>P</i> _{spac} - <i>yqjK'</i> - <i>lacZ</i> <i>lacI</i> <i>ermC</i>	This work
pSG5358	<i>bla</i> <i>P</i> _{spacHY} - <i>ykqC</i> <i>P</i> _{pen} - <i>lacI</i> <i>cat</i>	This work
pSG1802	<i>bla</i> <i>P</i> _{spacHY} - <i>ymdA</i> <i>P</i> _{pen} - <i>lacI</i> <i>cat</i>	This work

*Resistance gene abbreviations as follows: *aph*-A3, kanamycin; *bla*, ampicillin; *ermC*, erythromycin and lincomycin; *cat*, chloramphenicol; *neo*, kanamycin; *phleo*, phleomycin; *spc*, spectinomycin. Other abbreviations: Δ , deletion; Ω , insertion of integrated plasmid.

(Sterlini & Mandelstam, 1969), Difco antibiotic medium 3 (PAB) or S medium (Sharpe *et al.*, 1998) supplemented, where required, with 0.2 mM IPTG and/or 0.5% xylose. *B. subtilis* strains were transformed according to the method of Anagnostopoulos & Spizizen (1961). Transformants were selected on nutrient agar (Oxoid) supplemented, where required, with erythromycin (1 µg ml⁻¹), lincomycin (50 µg ml⁻¹), chloramphenicol (5 µg ml⁻¹), spectinomycin (50 µg ml⁻¹), phleomycin (1 µg ml⁻¹), kanamycin (5 µg ml⁻¹), 1 mM IPTG or 0.5% xylose. Transformation of *E. coli* DH5α was carried out according to standard methods (Sambrook *et al.*, 1989). *E. coli* strains were grown in Luria-Bertani (LB) broth supplemented, where required, with ampicillin (100 µg ml⁻¹).

Construction of in-frame deletions of *ylaN* and *yqeI*. In-frame deletions of *ylaN* and *yqeI* were constructed by replacing the *ylaN* or *yqeI* coding sequence with a cassette carrying a phleomycin resistance gene and the *upp* gene, using a modified version of the method of Fabret *et al.* (2002). Regions extending from just inside *ylaN* or *yqeI* to 2 kb upstream or downstream of each gene were amplified by PCR. The primer overlapping the 3' end of each gene had a 5' 21 bp region homologous to the 'b-end' of the *upp-phleo* cassette, and a 3' region of ~25 bp homologous to the chromosome. The primer overlapping the 5' end of each gene had a 5' 21 bp region homologous to the 'a-end' of the *upp-phleo* cassette, and a 3' region of ~25 bp homologous to the chromosome. The primers were designed so that the first and last 15 bp of each gene were retained. The *upp-phleo* cassette was amplified by a PCR using the primers Phleo3 and Phleo5 (Fabret *et al.*, 2002), which are homologous to the 'a-end' and 'b-end' of the *upp-phleo* cassette, respectively. The *upp-phleo* cassette and the two flanking regions were joined together and amplified in a PCR reaction, employing the outermost primers that were used to amplify the regions flanking each gene. The resulting PCR products were used to transform a Δupp *B. subtilis* strain containing a xylose-inducible copy of either *ylaN* (strain 3554) or *yqeI* (strain 3555) at an ectopic locus (*amyE*). Selection for phleomycin resistance and 5-fluorouracil (5FU) sensitivity in the presence of xylose gave rise to strains carrying the *upp-phleo* cassette in place of the chromosomal copy of *ylaN* (strain 3556) or *yqeI* (strain 3557). To remove the cassette and leave an unmarked in-frame deletion, the flanking regions of each gene were re-amplified by PCR. This time each of the primers bordering *ylaN* or *yqeI* comprised a 3' region homologous to one end of the gene adjacent to the deletion site, and a 5' region homologous to the chromosome on the other side of the cassette. The two DNA fragments were then joined as before and transformed into either 3556 or 3557. Transformants were selected on glutamate minimal medium (Lemon & Grossman, 1998) containing 10 µM 5FU, 1 µg thiamine hydrochloride ml⁻¹ and 0.5% xylose. 5FU-resistant, phleomycin-sensitive colonies were checked by PCR and sequenced to confirm the deletion.

Depletion of essential proteins. To deplete any of the essential proteins, the relevant cultures were grown overnight in CH, PAB or S medium containing 0.2 mM IPTG or 0.5% xylose. The cells were washed twice with fresh medium and diluted back to OD₆₀₀ 0.05 in medium with or without the appropriate inducer. Most of the depletion strains required a second dilution back to OD₆₀₀ 0.05 after 4 h growth, before any effects on growth or morphology could be observed.

Construction of GFP fusions. N- and C-terminal GFP fusions were constructed using the plasmids pSG1729 and pSG1154 (Lewis & Marston, 1999). Each gene was amplified by PCR and cloned into the *Apal/XhoI* sites of pSG1729 and pSG1154. Transformation of *B. subtilis* 168 with these plasmids resulted in the integration of the GFP fusion protein, under the control of the xylose-inducible *P_{xyI}* promoter, into the *amyE* gene. Strains carrying GFP fusions were grown at 30 °C, and expression of the fusion proteins was induced

by the addition of 0.2% xylose. To compare the localization of RpsB-GFP and GFP-YkqC, strains 1049 and 3565 were grown in CH medium at 30 °C to mid-exponential phase in the presence of 1 and 0.2% xylose, respectively. The cultures were rediluted to OD₆₀₀ 0.1. Tetracycline (final concentration 3 µg ml⁻¹) or rifampicin (final concentration 0.05 µg ml⁻¹) was added as required, and growth was continued at 30 °C. Cells were observed 60 and 120 min after addition of the inhibitors.

Microscopy. Live cells for microscopic analysis were mounted onto a thin film of 1.2% agarose in water, as described previously (Glaser *et al.*, 1997). To visualize the nucleoid, 1 µl 4',6-diamidino-2-phenylindole (DAPI) (1 µg ml⁻¹; Sigma) was added to 10 µl culture for 1 min before viewing. To visualize the cell membrane, 1 µl Nile red (100 µg ml⁻¹; Sigma) was added to 10 µl culture for 1 min before viewing. Image acquisition was performed as described previously (Lewis & Errington, 1997) using a Sony CoolSnap HQ cooled CCD camera (Roper Scientific) attached to a Zeiss Axiovert 200M microscope. Digital images were acquired and analysed with METAMORPH version 6 software.

Analysis of antibiotic resistance of *ykqC*. Strain 3564 was grown in CH medium containing 0.2 mM IPTG to OD₆₀₀ 0.5. The culture was washed twice in fresh CH medium, and rediluted to OD₆₀₀ 0.05. Samples of 10 µl were spotted onto nutrient agar plates containing varying concentrations of either trimethoprim (Sigma) or sulfamethoxazole (Sigma), in the presence or absence of 1 mM IPTG. Plates were incubated at 30 °C for 24 h.

RESULTS AND DISCUSSION

Seven genes of unknown function are essential

The *Bacillus* Functional Analysis Program defines genes to be essential, based on the inability to inactivate the gene by insertion of pMUTIN4 into the coding region (Kobayashi *et al.*, 2003). To confirm whether the 11 genes classed in the unknown function category are indeed essential, we repeated the original study and attempted to inactivate each of the genes by insertion of pMUTIN4. A small (200–400 bp), internal, near N-terminal coding fragment of each gene was cloned into pMUTIN4. Integration of the resulting plasmid into the *B. subtilis* chromosome by a single crossover event disrupted the coding sequence, and resulted in fusion of the promoter sequence and N-terminal region of the gene to *lacZ*. Expression of downstream genes can be driven by the IPTG-inducible *P_{spac}* promoter. Two of the proposed essential genes, *ylaN* and *yqeI*, were too small to be inactivated by pMUTIN4; therefore, in-frame deletions were attempted for these genes instead (see below). Inactivations using pMUTIN4 were achieved for three of the reported essential genes: *ydiB* (strain 3551), *yloQ* (strain 3552) and *ywlC* (strain 3553). Each of these strains was checked by PCR to ensure that the plasmid had integrated into the correct site. Inactivation of the remaining genes (*yacA*, *ydiC*, *ykqC*, *ymdA*, *yneS* and *yqjK*) was attempted at least three times, but no disruptions were obtained.

Deletions of *ylaN* and *yqeI* were constructed using a modified version of the method of Fabret *et al.* (2002) (see Methods). Each of the genes was replaced by a cassette containing a phleomycin resistance gene and the *upp* gene,

which can be used as a counter-selectable marker to allow removal of the cassette, leaving an unmarked deletion. To determine whether these genes are essential, chromosomal DNA of strains 3556 and 3557 was used to transform wild-type *B. subtilis* 168 with selection for phleomycin in the presence of xylose. Analysis of the resistance markers of the transformants showed that 100% of transformants that had received DNA from strain 3556 had also inherited the copy of *ylaN* at *amyE*, whereas only 2% of transformants receiving DNA from strain 3557 had inherited a copy of *yqeI* at *amyE* (200 transformants analysed), indicating that *ylaN* is essential and *yqeI* is non-essential. Removal of the *upp-phleo* cassette from strain 3556 resulted in strain 3558, which carried an in-frame deletion of *ylaN* at the native chromosomal locus, and an inducible copy of *ylaN* at *amyE*. We were unable to remove the *upp-phleo* cassette from the *yqeI* locus in strain 3557; therefore, strain 3559, which carries the *upp-phleo* cassette in place of the native copy of *yqeI*, and no additional copy of *yqeI* at *amyE* (obtained by transforming strain 168 with chromosomal DNA of strain 3557), was used for further analysis.

In summary, out of the 11 reported essential genes of unknown function, we were able to disrupt four of them (*ydiB*, *yloQ*, *yqeI* and *ywlC*), indicating that these genes are in fact non-essential, but we confirmed that the other seven genes (*yacA*, *ydiC*, *ykqC*, *ylaN*, *ymdA*, *yneS* and *yqjK*) are essential for viability. All of the disruption strains are slow-growing, and this could account for the discrepancy between

the original study and our own. Our results are summarized in Table 2.

Phenotypes of mutants disrupted for the non-essential genes

Growth of each of the disruption mutants was followed in rich (CH) and poor (S) media, and the cells were observed by microscopy (Fig. 1). Each of the disruption strains was able to grow in rich and poor media, although they grew at a slower rate compared to that of strain 168 (Fig. 1A). No changes in cell morphology were observed for the *ydiB* or *ywlC* disruptions.

Analysis of the sequence of YdiB showed that it is a member of a family of uncharacterized P-loop ATPases. Its homologues in *E. coli* and *H. influenzae* (YjeE) have been studied more extensively than YdiB (Teplyakov *et al.*, 2002; Allali-Hassani *et al.*, 2004). *yjeE* has been shown to be essential in *E. coli* (Freiberg *et al.*, 2001; Allali-Hussani *et al.*, 2004), and it has been proposed that YjeE is involved in cell wall synthesis, based on its conservation in all bacteria except *Mycoplasma* and *Ureaplasma*, and the position of the gene next to *amiB* (a cell wall amidase) in *E. coli* (Teplyakov *et al.*, 2002). Depletion of YjeE in *E. coli* results in a morphology that is consistent with defective cell wall synthesis (J. Handford, personal communication). However, we found that disruption of *ydiB* had only a mild effect on growth in *B. subtilis*, and cell morphology was unaffected

Table 2. Summary of the analysis of the 11 genes in this study

Gene	Essential for viability	Morphology following disruption/depletion	GFP localization	Function (predicted)	References
<i>yacA</i>	Yes	Elongated cells	Cytoplasmic	tRNA ^{Ile} -lysine synthetase, TisS	Soma <i>et al.</i> (2003)
<i>ydiB</i>	No	Wild-type	Cytoplasmic	(ATPase)	Teplyakov <i>et al.</i> (2002); Allali-Hassani <i>et al.</i> (2004)
<i>ydiC</i>	Yes	Wild-type	Cytoplasmic	(Protease, chaperone)	
<i>ykqC</i>	Yes	Elongated cells, anucleate cells, bisected chromosomes, similar to a defective DNA replication morphology	Ribosomal-like	RNase J1, <i>thrS/thrZ</i> mRNA processing, may affect global mRNA stability	Even <i>et al.</i> (2005)
<i>ylaN</i>	Yes	Abnormal cell width, some shorter cells	Single focus	Unknown	
<i>yloQ</i>	No	Curved cells, uneven edges	Cytoplasmic	GTPase, may be involved in ribosome maturation/assembly, or in mRNA translation	Campbell <i>et al.</i> (2005)
<i>ymdA</i>	Yes	Elongated cells, anucleate cells, minicells	Cell periphery	(RNase)	Aravind & Koonin (1998); Condon (2003)
<i>yneS</i>	Yes	Shorter cells	Cell periphery	(Fatty acid or phospholipid synthesis)	
<i>yqeI</i>	No	Some abnormal cell lengths	Cytoplasmic	(RNA-binding protein)	Ostheimer <i>et al.</i> (2002); Willis <i>et al.</i> (2002)
<i>yqjK</i>	Yes	Wild-type	Cytoplasmic	RNase Z, tRNA processing	Pelligrini <i>et al.</i> (2003)
<i>ywlC</i>	No	Wild-type	Cytoplasmic	(RNA-binding protein)	Teplova <i>et al.</i> (2000)

Fig. 1. Effects of disruptions of non-essential genes on growth and cell morphology. (A) Growth curves of wild-type strain 168, and strains containing $\Delta ydiB$, $\Delta ywlC$, $\Delta yloQ$ or $\Delta yqeI$ grown in CH medium. (B–E) Cells of strain 168 grown in (B) S and (D) CH media, $\Delta yloQ$ in (C) S medium and $\Delta yqeI$ in (E) CH medium, stained with Nile red.

in the *ydiB* deletion strain. Therefore, the functions of YdiB and its homologue in *E. coli* may differ.

The crystal structure of YrdC, the *E. coli* homologue of YwlC, suggests that it may be a nucleic acid-binding protein. Nucleic acid-binding studies have shown that YrdC binds preferentially to dsRNA (Teplova *et al.*, 2000).

In CH medium, the $\Delta yloQ$ mutant cells were indistinguishable from those of the wild-type. However, in S medium, the mutant cells were often curved and had rough edges (Fig. 1C). The cells also appeared longer than those of strain 168 (Fig. 1B). These observations are consistent with the results of Campbell *et al.* (2005), who also showed that *yloQ* is dispensable for growth in *B. subtilis*.

Disruption of *yqeI* resulted in cells of variable length (Fig. 1E). However, although there were more cells at both extremes of length, the mean length of *yqeI* cells was not dissimilar to that of strain 168; 3.0 ± 1.0 μm compared with 2.9 ± 0.67 μm , respectively (> 500 cells measured for each

strain; data not shown). The slow growth and abnormal cell length could be complemented by an ectopic copy of *yqeI* alone, showing that the phenotype was not due to polar effects of the disruption on the downstream genes *yqeJKLM*. Crystal structures of YqeI homologues from *E. coli* (YhbY) and *H. influenzae* (YhbY) have shown that these proteins possess a fold that is common to several RNA-binding proteins (Ostheimer *et al.*, 2002; Willis *et al.*, 2002), and in *E. coli*, YhbY has been found to co-sediment with free 50S ribosomal subunits (Ostheimer *et al.*, 2002).

Depletion of essential proteins

To analyse the effects of removing each of the essential proteins, depletion constructs were made using pMUTIN4. For these constructs a 200–400 bp fragment containing the ribosome-binding site and the N-terminal portion of each gene was cloned into pMUTIN4 to create pSG5354–7. These plasmids were transformed into strain 168 to generate strains 3560 (*P_{spac}-yacA*), 3561 (*P_{spac}-ydiC*), 3562 (*P_{spac}-yneS*) and 3563 (*P_{spac}-yqjK*). Integration of each plasmid into the chromosomal copy of the gene by a single crossover event resulted in the N-terminal portion of the gene under the control of its native promoter fused to *lacZ*, and a full-length copy of the gene under the control of the inducible *P_{spac}* promoter. Again, each of the resulting strains was checked by PCR to verify that the plasmid had integrated correctly. To deplete YlaN, we used strain 3558, in which the gene was controlled by the xylose-dependent *P_{xyI}* promoter. In the presence of the appropriate inducer, the cells should grow like those of the wild-type. In the absence of inducer, the expression of the protein should be minimal, and the effects of removing the essential protein can be established.

The YneS depletion strain showed an immediate effect on growth in the absence of inducer. For the majority of the depletion strains, the uninduced cultures grew similarly to the induced cultures, and there were no obvious effects on cell morphology. For this reason, all of the depletion strains, except for 3552 (*P_{spac}-yneS*), were rediluted back to a low OD in fresh medium after 4 h of growth in the presence and absence of inducer. After this redilution, defects in growth and morphology became apparent for all of the depletion strains. In the absence of inducer, *yacA* and *ylnA* showed very little increase in OD (Fig. 2A, B). Cells depleted of YdiC or YneS continued to grow, but much more slowly than the corresponding induced cultures (Fig. 2C, D). The growth of cells depleted of YneS also tended to plateau at a relatively low OD. The YqjK-depleted cells maintained growth at almost the same rate as that of the induced culture (Fig. 2E). We found that growth of the pMUTIN4-based YkqC and YmdA depletion strains was abnormally slow, even in the presence of 1 mM IPTG, and that the YkqC depletion strain also showed misshapen cells in the presence of inducer. We assumed that these constructs were not able to provide sufficient expression of *yqkC* or *ymdA* in the presence of inducer to allow the cells to grow properly. To overcome this, alternative depletion constructs were made (pSG5358, *yqkC*; pSG1802, *ymdA*) using pJQ43 (Quisel *et al.*, 2001).

Fig. 2. Effects of depletion of essential proteins on growth. Growth curves of *P_{spac}-yacA* (A), *P_{xyI}-ylnN* (B), *P_{spac}-ydiC* (C), *P_{spac}-yneS* (D), *P_{spac}-yqjK* (E), *P_{spacHY}-ykqC* (F) and *P_{spacHY}-ymdA* (G) grown in CH medium, in the presence (■) or absence (□) of the appropriate inducer. All strains, with the exception of *P_{spac}-yneS*, were grown for 4 h in the presence or absence of inducer before being diluted back to OD₆₀₀ 0.05. Time 0 for these strains represents the start of growth after redilution. All strains were grown at 37 °C, except for *P_{spacHY}-ykqC* and *P_{spacHY}-ymdA*, which were grown at 30 °C.

The *P_{spacHY}* promoter gave a higher level of expression in the presence of IPTG. However, although the resulting strains (3564, *ykqC*; 4043, *ymdA*) had wild-type growth and morphology in the presence of IPTG, in the absence of inducer, the phenotypes were much less severe than those with the pMUTIN4-based depletions. Depletion of YkqC or YmdA in these strains at 30 instead of 37 °C overcame this problem. It appears that the *P_{spacHY}* promoter was more tightly regulated at 30 °C, resulting in less leaky expression. In the absence of inducer, growth of *P_{spacHY}-ykqC* was slower than that in the induced culture, and also reached a plateau at a much lower OD (Fig. 2F). A similar response was observed following depletion of YmdA (Fig. 2G).

Presumably the variability in response to the removal of inducer between the different depletion strains is due to the stability of individual proteins. It is also likely that there is some expression from *P_{spac}* and *P_{xyI}*, even in the absence of inducer, and for some of the essential proteins, a small amount may be enough to allow growth.

Effects on morphology following depletion of essential proteins

Each of the depletion strains was observed by light and fluorescence microscopy to detect effects on cell morphology. Cells depleted for YdiC or YqjK showed no change in

cell size, shape or nucleoid morphology; therefore, this experiment gave no clues as to the function of YdiC. YdiC is homologous to the *O*-sialoglycoprotein endopeptidase Gcp (YdiE), although it lacks the two conserved histidine residues that bind Zn^{2+} . In Gram-positive organisms, *ydiB*, *ydiC*, *ydiD* and *gcp* (*ydiE*) are conserved within an operon, whereas in *E. coli*, their homologues are dispersed throughout the chromosome. The YdiC and Gcp homologues in *E. coli* (YeaZ and YgjD, respectively) have been shown to form a complex (Butland *et al.*, 2005), and it is possible that members of the *ydiBCDE* operon also interact in *B. subtilis*.

Depletion of YacA or YneS resulted in an abnormal cell length distribution; YacA-depleted cells were, on average, longer than cells expressing *yacA* (mean length 4.1 ± 1.2 μm , compared with 2.5 ± 0.6 μm) (Figs 3A, B and 4A). YneS-depleted cells were, on average, shorter than the induced cells (mean length 1.8 ± 0.4 μm , compared with 2.5 ± 0.6 μm) (Figs. 3C, D and 4B). It is not obvious why the depletion of YacA, a tRNA-modifying enzyme, should lead to an increase in cell length.

The small-cell phenotype of the *yneS* mutant resembles the effects of either treating cells with cerulenin (an inhibitor that targets the β -ketoacyl-ACP synthase FabF), or depleting PgsA (phosphatidylglycerophosphate synthase) (Thomaides, 1999), which catalyses the synthesis of phosphatidylglycerolphosphate from CDP-diacylglycerol during phospholipid biosynthesis. It has been proposed that *yneS* has a role in chromosome separation, as it is divergently clustered with *parC* and *parE* (encoding the subunits of topoisomerase IV) (Zalacain *et al.*, 2003). We found no evidence of a defect in chromosome separation following depletion of YneS, either on its own, or in combination with a *spo0J* null mutation (data not shown). Additionally, no direct interaction was found between YneS and either ParC or ParE in a bacterial two-hybrid system (data not shown). Given the morphological phenotype of cells depleted for YneS, and their similarity to cells that are defective in phospholipid or fatty acid synthesis, we propose that YneS is involved in one of these pathways.

YlaN is a small protein (93 aa), showing homology only to other uncharacterized proteins, and it is conserved in a very limited number of Gram-positive bacteria (*Bacillus*, *Listeria*, *Staphylococcus* and *Enterococcus*). Depleting YlaN resulted in cells that had a non-uniform cell width (Fig. 3E, F). These cells were quite often wider at one pole than at the other, and many cells also appeared to be shorter than normal. Within a chain of cells, it was common to see wider cells in the middle of the chain, whilst towards the ends of the chain the cells became normal in width but shorter in length. The shape defect was seen when cells were depleted in CH or PAB medium, but not in S medium. Recently, we have reported that normal growth of *mreB*, *mreC* and *mreD*, which are required for controlling cell shape, can be restored by the addition of magnesium to the medium (Formstone & Errington, 2005; Leaver & Errington, 2005). However, this is not the case for *ylnA*, suggesting that cells depleted for YlaN

have lost the ability to control some aspect of growth or cell wall synthesis that is distinct from that operated on by the Mre proteins.

Depletion of YmdA resulted in several effects on cell and chromosome morphology (Fig. 3G, H). First, the cell length distribution was aberrant, with many unusually short cells and almost spherical cells, reminiscent of the minicells produced by *minC* or *minD* mutants (arrowheads, Fig. 3H). There were also a few unusually longer cells. Secondly, chromosome segregation appeared to be seriously deranged, with a number of obvious effects. Some chromosomes had an elongated, dispersed appearance, and the minicells were usually associated with the cell regions deficient in DNA. Some longer anucleate cells were also evident (arrow, Fig. 3H).

The phenotypic effects observed could have been due to a primary defect in chromosome segregation, leading to aberrant positioning of division septa, or they might have been due to abnormal positioning of the cell division site, leading to cells with abnormally positioned chromosomes. To try to identify the primary cell cycle defect, we combined the *ymdA* conditional mutations with various other mutations affecting cell cycle processes. First, the *ymdA* mutation was introduced into cells bearing mutations in the cell division genes *ftsZ* and *divIB*. The filamentous cells formed following depletion of either FtsZ or DivIB showed a more or less regular distribution of nucleoids in the *ymdA*⁺ background, whereas in the *ymdA* mutant background, the nucleoid distribution was clearly aberrant (data not shown). These results suggested that the nucleoid positioning defect was not due to incorrect positioning of division septa. We then made a *minD ymdA* double mutant. *minD* mutants make minicells (small anucleate cells), due to a failure to block the inherent ability of the division machinery to divide close to the cell poles (reviewed by Rothfield *et al.*, 2005). The double mutant made strikingly high numbers of minicells (data not shown). The simplest explanation for this effect is that *ymdA* perturbs nucleoid positioning, and that this then increases the likelihood that the subpolar region of the cell will be deficient in DNA. The reduced DNA content then allows division to occur more frequently in these subpolar positions, due to decreased nucleoid occlusion (see Rothfield *et al.*, 2005).

If *ymdA* had a chromosome-segregation defect, this might be exacerbated by combination with other chromosome-segregation defects. To test this, we combined *ymdA* mutation with mutations in the *spo0J*, *smc* and *spoIIIE* genes. None of the resultant double mutants seemed to have a substantially worse phenotype than that which would be expected based on combination of the two parental phenotypes (data not shown). Also, none of the double mutants exhibited a significantly slower growth rate than that of the *ymdA* single mutant. Thus, if *ymdA* does have a primary defect in chromosome segregation, this does not seem to be exacerbated by loss of any of the well-characterized chromosome-segregation genes.

Fig. 3. Effects of depletion of essential proteins on cell morphology. Cells of *P_{spac}-yacA* grown in the presence (A) and absence (B) of IPTG; *P_{spac}-yneS* grown in the presence (C) and absence (D) of IPTG; *P_{xyl}-ylnN* grown in the presence (E) and absence (F) of xylose; *P_{spacHY}-ymdA* grown in the presence (G) and absence (H) of IPTG; and *P_{spacHY}-ykqC* grown in the presence (I) and absence (J) of IPTG are shown. (A–F) Cell membranes were stained with Nile red; (G–J) overlay of images showing the cell membranes stained with Nile red (red) and the nucleoids stained with DAPI (blue). Strains were grown in CH medium and depleted as described in the legend to Fig. 2.

The amino acid sequence of YmdA contains a stretch of 21 predominantly hydrophobic residues beginning at residue 4. There are no charged residues at the N terminus, but the hydrophobic region is immediately followed by two basic residues (R, K). According to the positive inside rule of von Heinje (1992), these features are strongly suggestive of a single transmembrane region with the C-terminal part of the protein located in the cytosol. YmdA is predicted to contain an N-terminal KH (ribonucleoprotein K homology) domain, which is found in a variety of nucleic acid-binding proteins, and an HD (His–Asp-containing phosphohydrolase) domain towards the C terminus (Pfam; <http://www.sanger.ac.uk/Software/Pfam>). The HD domain is characteristic of a family of metal-dependent phosphohydrolases, many of which have a role in nucleic acid metabolism or signal transduction (Aravind & Koonin, 1998). Due to the presence of these domains, YmdA has been highlighted as a

potential RNase (Aravind & Koonin, 1998; Condon, 2003). The highly conserved histidine and aspartate residues within members of this family indicate that coordination of divalent cations is essential for their activity (Aravind & Koonin, 1998). Analysis of point mutations created throughout YmdA showed that residue H368, corresponding to the histidine of the conserved His–Asp doublet, was important for the function of YmdA; a strain carrying an H368A mutation showed a similar phenotype to that in cells depleted for YmdA (data not shown). Although the morphological phenotype of YmdA-depleted cells suggests a defect in chromosome segregation, if YmdA is an RNase, it is likely that the cell cycle deficiency is an indirect, rather than primary defect in these cells.

Cells that were depleted for YkqC showed a very striking morphology (Fig. 3I, J), in which the cells tended to be

Fig. 4. Cell length distributions of cells depleted for YacA or YneS. Cell length measurements of *P_{spac}-yacA* (A) and *P_{spac}-yneS* (B) grown in CH medium in the presence and absence of IPTG, as described in the legend to Fig. 2, are shown.

elongated, and the distribution of the nucleoids was affected, with large regions at one or both poles of the cell devoid of DNA. Anucleate cells were frequent (arrowhead, Fig. 3H) and there were occasional cells in which the cell division septum had bisected a nucleoid (arrow, Fig. 3H). The overall morphology was very similar to that observed when DNA replication is blocked, either by depleting the replication initiation protein DnaA, or by treating cells with the replication inhibitor HPUra [6-(*p*-hydroxy-phenylazo)-uracil]. However, YkqC has been shown to be an RNase, the depletion of which results in an increase in the global half-life of mRNA (Even *et al.*, 2005). Therefore, it is again likely that the primary morphological phenotype is an indirect effect arising from a primary deficiency in RNA processing or degradation.

Depletion of YkqC results in increased resistance to antibiotics that target folate synthesis

As part of this study, we tested the response of YkqC-depleted cells to certain antibiotics, to see whether there was any difference in the sensitivity of the strain. Surprisingly, we found that depleting YkqC resulted in cells that were much more resistant to trimethoprim. Trimethoprim targets DNA synthesis by inhibiting dihydrofolate reductase, an enzyme in the synthesis pathway of the cofactor folate. Fig. 5 shows that induced cells were unable to grow in concentrations $>0.1 \mu\text{g trimethoprim ml}^{-1}$, whereas the cells depleted for YkqC were able to grow in $2.5 \mu\text{g trimethoprim ml}^{-1}$. As this was an unexpected result, we also tested the growth of induced and uninduced cultures in sulfamethoxazole, which inhibits another step in the folate pathway. Again, the uninduced cells showed increased resistance; the depleted cells were able to grow in $100 \mu\text{g sulfamethoxazole ml}^{-1}$, whereas the induced culture barely grew in $>20 \mu\text{g sulfamethoxazole ml}^{-1}$ (Fig. 5). We have no explanation for this curious effect.

Subcellular localization of the uncharacterized proteins

Gene fusions that produce N- and C-terminal GFP fusions to each of the 11 proteins were constructed and placed at the *amyE* locus under the control of the xylose-inducible *P_{xyI}*

Fig. 5. Increased resistance of YkqC-depleted cells to trimethoprim and sulfamethoxazole. Strain 3564 containing *P_{spacHY}-ykcQ* was spotted onto nutrient agar containing either trimethoprim or sulfamethoxazole, in the presence or absence of IPTG.

promoter. In each of the resulting strains, the native copy of the gene remained intact. For most of the fusion proteins, the fluorescence signal was dispersed uniformly over the entire cell (seen for YacA, YdiB, YdiC, YloQ, YqeI, YqjK and YwlC). To test whether the fusion proteins were functional, the GFP fusion constructs were combined with the corresponding disruptions. The GFP fusions of YdiB, YloQ, YqeI and YwlC complemented their respective disruptions, and the strains showed wild-type growth in the presence of xylose (and the fluorescence pattern was unchanged), indicating that these fusions are functional, and that the proteins function within the cytoplasm (data not shown). Immunoblots using an anti-GFP antibody confirmed that the fusion proteins were intact in all of the GFP strains, with the exception of the N-terminal fusion to YneS (data not shown).

The C-terminal YneS fusion (strain 3566) appeared to be associated with the cell periphery (Fig. 6A). This localization pattern is in agreement with the predicted topology of YneS as a transmembrane protein containing five transmembrane-spanning domains, with an extracellular N terminus and a cytoplasmic C terminus (SOSUI structure prediction of membrane proteins; <http://sosui.proteome.bio.tuat.ac.jp/>).

The GFP fusions to YlaN gave differing localization patterns: the C-terminal fusion gave an even fluorescence all over the cell, whereas the N-terminal fusion generally produced one bright focus per cell (Fig. 6B). Time-lapse microscopy showed that these foci moved randomly around the cell, moving the length of the cell around twice per minute. Complementation of a *ylaN* deletion strain by the GFP-YlaN, but not the YlaN-GFP, construct suggested that the N-terminal fusion of YlaN was functional, whilst the C-terminal fusion was not. Treatment of the cells with either protein synthesis inhibitors (kanamycin or chloramphenicol) or with sodium azide did not perturb the movement of the foci. It is possible that these foci are inclusion bodies containing the majority of GFP-YlaN, and do not represent the true localization of YlaN within the cell.

The C-terminal GFP fusion to YmdA exhibited a peripheral GFP signal (Fig. 6C), consistent with the protein being associated with the membrane, and with the predicted membrane topology of YmdA. This fusion protein was functional and was able to complement a *ymdA* null mutation.

N- and C-terminal fusions of YkqC were both localized towards the cell poles and at future mid-cell division sites (Fig. 6D). This pattern was similar to that of a ribosomal protein (RpsB)-GFP fusion (Lewis *et al.*, 2000). Combination of each of the YkqC-GFP fusion proteins with a deletion of the native copy of *ykqC* showed that the N-terminal GFP fusion was functional. The localization of the GFP-YkqC fusion protein to areas that were occupied by ribosomal proteins indicates that YkqC might have a role in protein synthesis.

Fig. 6. Localization of novel proteins. Fluorescent images of cells expressing YneS-GFP (A), GFP-YlaN (B), YmdA-GFP (C) and GFP-YkqC (D) are shown. Strains were grown in CH medium at 30 °C in the presence of 0.2% xylose.

GFP-YkqC always localizes to the areas of the cell occupied by ribosomes

GFP fusions can be used as valuable markers of specific structures or complexes within the cell. The localization of these structures can be perturbed by the addition of certain inhibitors (A. Hunt & J. Errington, unpublished observations). As described earlier, ribosomes are predominantly excluded from the area occupied by the nucleoid, and are therefore concentrated towards the cell poles (Lewis *et al.*, 2000). By treating the cells with different inhibitors, the areas occupied by the nucleoid and ribosomes can be altered. In order to determine how tightly the localization of YkqC was associated with the localization of ribosomes in the cell, we examined the effects of different inhibitors on the localization of a GFP fusion to a ribosomal protein (RpsB), and compared this to the localization of the GFP-YkqC fusion under the same conditions. Treatment of *B. subtilis* with several protein synthesis inhibitors caused the nucleoid to compact; therefore, it occupies a smaller area in the centre of the cell. Fig. 7 shows a typical response to tetracycline. Under these conditions, RpsB-GFP was

still located at the cell poles, but extended further towards mid-cell, into the area that had previously been occupied by

Fig. 7. Localization of GFP-YkqC corresponding to the localization of the ribosomal protein RpsB. Fluorescent images of cells expressing RpsB-GFP (A–I) and GFP-YkqC (J–R), in the presence of tetracycline (D–F and M–O) and rifampicin (G–I and P–R), showing GFP fluorescence (green; A, D, G, J, M and P), nucleoids stained with DAPI (red; B, E, H, K, N and Q) and overlays of GFP and DAPI images (C, F, I, L, O and R).

the nucleoid (Fig. 7D). In the presence of tetracycline, the GFP-YkqC fusion also spread further towards the middle of the cell (Fig. 7M). Treatment of *B. subtilis* with an inhibitor of transcription (rifampicin) had the opposite effect: the nucleoid expanded and formed a diffuse structure that extended over the whole cell (Fig. 7H). Expansion of the nucleoid was accompanied by dispersion of the ‘ribosome zone’ throughout the cell, as shown by the diffuse spreading of RpsB-GFP throughout the cell (Fig. 7G). Again, a similar effect was observed for localization of GFP-YkqC (Fig. 7P). These results are consistent with a model in which YkqC is associated with ribosomes or other components of the translational machinery. During their study, Even *et al.* (2005) were able to purify YkqC and YmfA from a high-salt ribosome wash, which correlates with our findings on the localization of YkqC.

A role for YkqC in processing ribosomal or ribosomally associated RNA?

Given the co-localization of YkqC with ribosomes, and its identification as an RNase, we propose that the major target of YkqC is ribosome-associated RNA. YkqC may be required to process rRNA, and may therefore contribute to ribosome assembly or degradation. Alternatively, YkqC may act upon mRNAs when they are associated with the ribosome. It is possible that the phenotypes that we observed with the YkqC-depleted strain are an indirect effect of one or more transcripts not being processed fully. For example, one unprocessed transcript could lead to the replication-deficient phenotype, and another to the increase in resistance to trimethoprim and sulfamethoxazole.

Large-scale inactivation studies have been useful in identifying the proteins essential for viability in different bacteria. We revisited the essentiality of 11 genes of unknown function that were highlighted by the *Bacillus* Functional Analysis Program (Kobayashi *et al.*, 2003), and attempted to characterize these genes. For many proteins, analyses of mutant cell morphology and protein localization are effective methods for investigating function. By using a combination of these approaches, we have been able to propose tentative functions for a number of *B. subtilis* genes.

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