Characterization of the *Bacillus subtilis* GTPase YloQ and its role in ribosome function

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We present an analysis of the cellular phenotype and biochemical activity of a conserved bacterial GTPase of unknown function (YloQ and YjeQ in *Bacillus subtilis* and *Escherichia coli* respectively) using a collection of antibiotics of diverse mechanisms and chemical classes. We created a *yloQ* deletion strain, which exhibited a slow growth phenotype and formed chains of filamentous cells. Additionally, we constructed a conditional mutant in *yloQ*, where growth was dependent on inducible expression from a complementing copy of the gene. In phenotypic studies, depletion of *yloQ* sensitized cells to antibiotics that bind at the peptide channel or peptidyl transferase centre, providing the first chemical genetic evidence linking this GTPase to ribosome function. Additional experiments using these small-molecule probes *in vitro* revealed that aminoglycoside antibiotics severely affected a pre-

viously characterized ribosome-associated GTPase activity of purified, recombinant YjeQ from *E. coli*. None of the antibiotics tested competed with YjeQ for binding to 30 or 70 S ribosomes. A closer examination of YloQ depletion revealed that the polyribosome profiles were altered and that decreased expression of YloQ led to the accumulation of ribosomal subunits at the expense of intact 70 S ribosomes. The present study provides the first evidence showing that YloQ/YjeQ may be involved in several areas of cellular metabolism, including cell division and ribosome function.

Key words: antibiotic, gene deletion, GTPase, polyribosome profile, ribosome, YloQ.

INTRODUCTION

GTPases constitute a functionally diverse group of proteins that are involved in essential cellular functions including protein synthesis, cell cycle and differentiation, transmembrane signalling, protein trafficking and secretion, cytoskeletal organization and motility [1]. In an analysis of 42 completely sequenced prokaryotic genomes, 497 G-proteins were identified [2]. Of these, 177 were of unknown function and a subset of 23 showed no similarity to the currently known GTPase subfamilies [2]. *Bacillus subtilis* YloQ represents a broadly conserved group of orthologous GTPases lacking similarity to known G-proteins. We have shown the *Escherichia coli* orthologue, YjeQ, to possess low intrinsic GTPase activity and an unusual connectivity [3]. In contrast with the usual G1-G2-G3-G4 connectivity, the GTPase domain of YjeQ is circularly permuted to G4-G1-G2-G3. Additionally, YjeQ is flanked by an N-terminal OB-fold (oligonucleotide/oligosaccharide binding fold) domain and a C-terminal zinc knuckle-like cysteine cluster [3–5]. Similarly, YloQ has been shown to have low intrinsic GTPase activity [5]. Recently, the crystal structures of *Thermotoga maritima* YjeQ and *B. subtilis* YloQ have been solved [4,5]. Structurally, the two proteins are almost identical and implicate the same key residues as those necessary for proper molecular function [4,5]. *In vitro* studies have demonstrated that YjeQ is able to bind to the ribosome and that its GTPase activity is stimulated by this interaction [6]. Efforts to further characterize this protein have focused on the dispensability of YjeQ and YloQ for the growth of *E. coli* and *B. subtilis* respectively. The essential nature of YloQ is elusive since it has been reported to be essential [5,7,8], while its *E. coli* orthologue is dispensable [9,10]. We sought in the present study to confirm the dispensability and to understand further the physiological function of this unique GTPase in bacteria.

Classical genetic approaches have traditionally been used to address gene/protein function in the context of cellular phenotype. More recently, chemical genetics has emerged as a new approach to study protein function, where small molecules are used as probes of gene function by mimicking the cellular effects of genetic mutations [11,12]. In the present study, we have taken advantage of the wealth of mechanistic information that exists for using conventional antibiotics to probe the function of the conserved bacterial GTPase YloQ, both *in vivo*, at the level of cellular phenotype, and *in vitro*, through biochemical interactions. Specifically, we sought to probe its potential role in translation, given the recent discovery of the capacity of YjeQ to associate with ribosomes *in vitro* [6].

In the present study, we report the first *yloQ* deletion strain, which definitively proves that YloQ is dispensable in *B. subtilis*. The deletion strain resulted in slow growth and the cells showed a predominant phenotype of filamentous chains, indicating a defect in cell division. To investigate further the function of YloQ, we made use of 28 antibacterial agents with well-annotated functions and looked for antibiotics that showed increased efficacy against cells that were depleted for the gene product of *yloQ* and have referred to the interaction as chemical synthetic lethality. We conducted these experiments in the model Gram-positive organism *B. subtilis* since it has permeability characteristics that are superior to the model Gram-negative *E. coli* and is susceptible to antibiotics of a wider variety of chemical classes and mechanisms. The work revealed synthetic lethal interactions with antibiotics that bind at the peptide channel or peptidyl transferase centre of the ribosome. To probe further the protein function, we focused on the *E. coli* orthologue, YjeQ, which has been well-characterized biochemically in our laboratory, including the characterization of a ribosome-stimulated GTPase activity *in vitro* [3,6]. *In vitro* biochemical experiments reported here revealed

Abbreviations used: LB medium, Luria–Bertani medium; MIC, minimum inhibitory concentration.

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Table 1 Strains, plasmids and primers used in the present study

	Description
Strains	
Novablue	General <i>E. coli</i> cloning strain
EB6	Wild-type <i>B. subtilis</i> (<i>hisA1 argC2 metC3</i>)
EB508	<i>yloQ</i> diploid strain, derived from EB6 (<i>hisA1 argC2 metC3 amyE::P_{xyI}-yloQ cat</i>)
EB611	<i>yloQ</i> conditional deletion strain, derived from EB508 (<i>hisA1 argC2 metC3 yloQ::spec amyE::P_{xyI}-yloQ cat</i>)
EB1256	<i>yloQ</i> deletion strain, derived from EB6 (<i>hisA1 argC2 metC3 yloQ::spec</i>)
Plasmids	
pSWEET	Facilitates <i>B. subtilis</i> chromosomal integration of a gene under the control of a xylose inducible promoter at the <i>amyE</i> locus [15]
pUS19	A source for a spectinomycin resistance cassette, used to replace <i>B. subtilis</i> genes
Oligonucleotides	
<i>yloQ</i> -F	5'-GGGATTAATTAAGAATAACAAGGAGGGAAAATATGCCTGAGGGC-3'
<i>yloQ</i> -R	5'-GGGAGCTAGCGCTAATACCTCGGCTTTCTGTCTTTAATCTCCG-3'
<i>yloQ</i> -a	5'-GAAGAACGGCAAAAGAAAAAGTGGC-3'
<i>yloQ</i> -b	5'-TAATCATGGTCATAGCTGTTTCTGTTCTGTTATTTCATCTTCGG-3'
<i>yloQ</i> -c	5'-GAAATAAATGCATCTGTATTGAATGAAGGAGGTAGCATAATGATAAAGGTTGC-3'
<i>yloQ</i> -d	5'-GCCCAATCAAGGGCGAGGTCTAAATC-3'
<i>spec</i> -F	5'-CACAGGAAACAGCTATGACCATGATTAGGCGAATGGCGATTTCGTTTCG-3'
<i>spec</i> -R	5'-CATTCAAATACAGATGCATTTTATTTCCCATTTTTCATTTTATAATTTTAAATC-3'

that a subset of aminoglycosides impeded the stimulation of the GTPase activity of YjeQ by both 30 and 70 S ribosomes, but had no impact on ribosome association. Finally, we found that depletion of YloQ *in vivo* leads to accumulation of ribosomal subunits. The present study provides initial observations that YloQ/YjeQ may be involved in multiple areas of cellular metabolism, including cell division and ribosome function.

EXPERIMENTAL

General methods

Strains, plasmids and primers used are listed in Table 1. *E. coli* and *B. subtilis* strains were grown in LB (Luria–Bertani) medium. Ampicillin was used at a concentration of 50 µg/ml. Antibiotic concentrations for *B. subtilis* were chloramphenicol (10 µg/ml) and SPEC (spectinomycin; 150 µg/ml). HotStarTaq PCR reagents, gel extraction kits and plasmid mini-prep kits were obtained from Qiagen (Mississauga, ON, Canada) and the Expand PCR system was from Roche Diagnostics (Laval, PQ, Canada). Cloning was performed in the *E. coli* cloning strain Novablue (Novagen, Madison, WI, U.S.A.) according to established methods [13]. Preparation and transformation of *E. coli* electrocompetent cells was performed following the electroporator manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, U.S.A.), whereas *B. subtilis* competent cells and transformations were performed according to established methods [14]. Restriction enzymes were obtained from New England Biolabs (Beverly, MA, U.S.A.). All antibiotics were obtained from Sigma–Aldrich (Oakville, ON, Canada). Hepes was from Bioshop Canada (Burlington, ON, Canada). Malachite Green and ammonium molybdate were from Sigma–Aldrich.

Deletion of *yloQ*

A strain containing an additional copy of *yloQ* at the *amyE* locus on the chromosome was created using the plasmid pSWEET as described previously [15]. Primers *yloQ*-F and *yloQ*-R were used to PCR-amplify the gene and clone into pSWEET. Subsequent transformation of this linearized plasmid into wild-type *B. subtilis* resulted in the creation of strain EB508 (two chromosomal copies of *yloQ*). To produce a linear DNA fragment required for transformation of strain EB508 or EB6 for creation of the deletion strains, a double crossover PCR strategy was used. Primers *yloQ*-a

and *yloQ*-b, *yloQ*-c and *yloQ*-d, and *spec*-F and *spec*-R were used with the Roche Expand PCR system to amplify chromosomal DNA or plasmid DNA in the latter case. The PCR products were purified and all three products were used as templates in a final reaction with primers *yloQ*-a and *yloQ*-d. The resulting DNA fragment contained 1 kb flanking sequences for the gene *yloQ* with a spectinomycin resistance cassette in between. The 3 kb PCR product was transformed into strain EB6 to create a *yloQ* deletion strain (EB1256) and into strain EB508 to create a conditionally complemented *yloQ* deletion strain (EB611).

Growth curves for the *yloQ* deletion strains

Strains EB6, EB611 and EB1256 were grown overnight on LB medium at 30 °C. The cells were resuspended in 2 ml of sterile saline and used to inoculate 200 µl of LB medium in a 96-well microtitre plate at an initial absorbance A_{600} of 0.005. All strains were grown in triplicate with and without xylose. Samples were incubated at 30 °C with shaking at 250 rev./min and the A_{600} was recorded.

To monitor the effect of *yloQ* depletion on growth rate, the conditional *yloQ* deletion strain (EB611) was grown overnight on LB medium at 30 °C. The cells were resuspended in 2 ml of sterile saline and used to inoculate 100 ml of medium at an initial A_{600} of 0.005. The conditional deletion strain (EB611) was grown in LB medium/xylose (2, 0.2, 0.063, 0.02, 0.002 and 0 %). The samples were incubated at 30 °C with shaking at 250 rev./min and the A_{600} was recorded.

Light microscopy

Samples to be examined were prepared by growing the cells on LB agar plates overnight at 30 °C with no xylose. The cells were resuspended from the plates in saline and applied to poly-lysine-treated microscope slides. Images were captured using a Q-color 3 camera (Olympus, Mississauga, ON, Canada).

MICs (minimum inhibitory concentrations) of antibacterial agents for *B. subtilis*

Wild-type (EB6), the diploid strain (EB508) and the conditional deletion strain for *yloQ* (EB611) were grown overnight on LB medium at 30 °C. Cells were resuspended from the plates in saline and added to LB medium at an initial concentration of 25 000

colony-forming units/100 μ l. Media containing xylose and cells (200 μ l) and antibiotics (5 μ l) were added to each well of a 96-well microtitre plate using a Bio-mek FX liquid handling system (Beckman, Mississauga, ON, Canada). Strain EB6 was tested in the absence of xylose, and strain EB508 was tested in both the presence (0.2%) and absence of xylose. Xylose concentrations tested for strain EB611 were 0.2, 0.063, 0.02, 0.002 and 0%. The plates were incubated at 30 °C with shaking at 250 rev./min. After 22 h, the absorbance for each well of the plate was determined with a Spectra-max Plus instrument (Molecular Devices, Sunnyvale, CA, U.S.A.). MIC was assigned as the lowest concentration of drug that completely inhibited the growth of a particular strain. All samples were analysed in duplicate.

Impact of protein synthesis inhibitors on binding of YjeQ to ribosomes and ribosome-mediated GTPase stimulation

The purification of whole 70 S ribosomes and 30 S subunits was performed as described previously [6]. The impact of protein synthesis inhibitors was assessed by measuring GTPase activity in the presence and absence of different concentrations of the antibiotic (20, 40 and 80 μ M with 70 S ribosomes and 80, 160 and 320 μ M with 30 S ribosomes). The samples (50 μ l) consisted of equimolar amounts (500 nM) of YjeQ and ribosome preincubated [10 μ l in 10 mM Tris/HCl (pH 7.5 at 4 °C), 10 mM magnesium acetate, 60 mM NH₄Cl and 3 mM 2-mercaptoethanol] for 15 min with the antibiotic in assay buffer (50 mM Hepes, pH 7.5, 10 mM MgCl₂ and 2.5 mM GTP). Reactions were performed in quadruplicate for 1 h at 30 °C. The samples were analysed for phosphate content using a Malachite Green/ammonium molybdate colorimetric assay as described previously [6]. Absorbances were measured at 660 nm with a Spectra-max Plus spectrophotometer (Molecular Devices, Sunnyvale, CA, U.S.A.). The impact of protein synthesis inhibitors on the binding of YjeQ to ribosomes was assayed using a previously described ribosomal pelleting assay [6]. The above reactions were performed with the following modifications: addition of 100 μ M GMP-PNP (guanosine 5'-[β , γ -imido]triphosphate) instead of 2.5 mM in the reaction mixtures and the assays were performed in the presence and absence of antibiotics (80 μ M with 70 S ribosomes and 320 μ M with 30 S ribosomes). After the reaction mixtures were incubated for 1 h at 30 °C, they were separated into supernatant and pellet fractions by ultracentrifugation at 150 000 *g* for 2 h. Pellets were resuspended in an identical volume as the supernatant for analysis. Samples were subjected to SDS/PAGE (15% polyacrylamide) and immunoblotting was performed using a rabbit polyclonal antibody specific for YjeQ and a horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody. Blots were developed using the Western Lightning Chemiluminescence Reagent Plus kit (PerkinElmer, Boston, MA, U.S.A.).

Polyribosome profiles

Wild-type (EB6) and the conditional deletion strain for *yloQ* (EB611) were grown overnight on LB medium at 30 °C. The cells were resuspended in 2 ml of sterile saline and used to inoculate 1 litre of medium at an initial *A*₆₀₀ of 0.005. The cells were grown to an *A*₆₀₀ of 0.15, pelleted at 15 000 *g* for 20 min and resuspended in gradient buffer [20 mM Tris (pH 7.5 at 4 °C), 10.5 mM magnesium acetate, 300 mM NH₄Cl, 0.5 mM EDTA and 3 mM 2-mercaptoethanol]. Cells were lysed by three passages through the French press at 10 000–12 000 p.s.i. (1 p.s.i. = 6.9 kPa) and the lysates were clarified by spinning at 31 000 rev./min for 45 min. The absorbance of the clarified lysates at 260 nm was determined and 60 absorbance units were loaded on to 10–35% sucrose gradients in the gradient buffer followed by

Figure 1 Growth of *yloQ* deletion strains on solid media

(A) Strains were grown overnight at 30 °C in the presence of 2% (w/v) xylose (left panel) and in its absence (right panel). (B) Growth of the *B. subtilis yloQ* deletions after 48 h. The plates from (A) were left to grow for a total of 48 h at 30 °C in the presence of xylose (B, left panel) and in its absence (B, right panel).

ultracentrifugation at 43 000 *g* using an SW28 rotor in a Beckman ultracentrifuge at 4 °C for 16 h. The sucrose gradients were fractionated by upward displacement with 70% (v/v) glycerol and analysed by reading the absorbance at 260 nm.

RESULTS AND DISCUSSION

Creation of a *B. subtilis yloQ* deletion strain

We set out to characterize the biological function of YloQ with the construction of a *B. subtilis* strain that conditionally expressed *yloQ*. Using methods developed in our laboratory [15], a copy of *yloQ* was placed under the control of a tightly regulated xylose-inducible promoter at the *amyE* locus. Subsequently, the wild-type copy of *yloQ* was replaced with a spectinomycin resistance cassette. Initial observations showed the *yloQ* depletion strain, EB611, to be capable of slow growth in the absence of the inducer, xylose. These results implied that *yloQ* was dispensable for the growth of *B. subtilis*, which is contradictory to previously published results stating that it was essential [5,7,8]. To investigate this possibility, we attempted to make a *yloQ* deletion in the absence of complementation. Our efforts resulted in the creation of strain EB1256, an uncomplemented deletion of *yloQ*, and definitively proved that YloQ is dispensable for the growth of *B. subtilis*. These results are in agreement with recently published results showing that *yjeQ* is dispensable in *E. coli* [10].

To investigate the growth phenotypes of our *yloQ* deletion strains, they were grown on both solid and liquid media. Figure 1(A) illustrates the growth of cells on LB agar overnight at 30 °C. In the presence of xylose (Figure 1A, left panel), strains EB6 (wild-type), EB508 (*yloQ* diploid) and EB611 (conditionally complemented deletion) grow well, with the formation of single colonies. Strain EB1256 (*yloQ* deletion) shows very little growth overnight and is only visible where a heavy inoculum is plated with no single colonies. In the absence of xylose strains, EB6 and EB508 show a similar good growth, whereas strain EB611

Figure 2 Growth of the *B. subtilis* *yloQ* deletion strains in liquid media

(A) Cells were grown in LB medium at 30 °C. Closed symbols indicate samples grown with 2% xylose and open symbols indicate samples grown in the absence of xylose: (●, ○) strain EB6, (▼, ▽) strain EB611 and (■, □) strain EB1256. (B) Strain EB611 was grown in LB medium at 30 °C with different xylose concentrations: 2% (●), 0.2% (○), 0.063% (▼), 0.02% (▽), 0.002% (■) and 0% xylose (□).

resembles that of EB1256. In the absence of xylose, *yloQ* expression will be turned off in EB611, leading to the same phenotype as having no *yloQ* present as in EB1256. Figure 1(B) shows the same plates incubated at 30 °C for 48 h. In both the presence and absence of xylose, strain EB1256 has grown to a greater extent and shows the formation of single colonies. Similarly, EB611 has grown in the absence of xylose and shows colony morphology very similar to that of EB1256. These results illustrate that YloQ is dispensable to *B. subtilis* and that, in its absence, there is a slow growth phenotype. To investigate further this slow growth, we examined growth curves of the cells in liquid medium.

Figure 2(A) depicts a growth curve in liquid medium for strains EB6, EB611 and EB1256. Strain EB6 grows equally well in both the presence and absence of xylose, and EB611 mirrors this growth in the presence of xylose, indicating that YloQ expression can restore normal growth. Strain EB1256 grows marginally faster in the presence of xylose, probably due to the presence of an additional carbon source enriching the medium. In the absence of xylose, strains EB1256 and EB611 exhibit an almost identical growth curve during the time course of this experiment. It is evident that, in the absence of YloQ, there is an increase in the lag phase and a decrease in the exponential growth rate of the cells. Figure 2(B) shows that this decrease in growth rate is directly related to *yloQ* expression, since as the level of xylose is decreased

Figure 3 Characterization of conditional *B. subtilis* *yloQ* deletion strains by microscopy

Strains EB611 (A) and EB1256 (B) were grown in the absence of xylose and visualized by light microscopy. Scale bars, 10 µm.

from 2 to 0%, there is an increase in lag phase and a concomitant decrease in the exponential growth rate. It is clear from these results that, although dispensable for growth, in the absence of YloQ expression, there is a significant decrease in growth rate, indicating impairment of a key cellular function. To investigate further the effects of this slow growth phenotype, we looked at the cell morphology of strains EB611 and EB1256.

Morphological effects of YloQ depletion

In an attempt to gain insight into the cellular processes affected by YloQ depletion, the morphology of depleted cells was examined using microscopy. Strains EB611 and EB1256 exhibit similar cellular morphologies, which is in agreement with their similar growth rates in both liquid and solid media. The predominant phenotype associated with depletion of YloQ in strain EB611 (Figure 3A) is filamentous cells. Additionally, these filamentous cells tend to form chains that are not seen with cells that are wild-type in appearance. It was found that approx. 15% of the cells showed an altered morphology from wild-type. When grown in the presence of xylose, strain EB611 has all wild-type-looking cells (results not shown). As seen with EB611, strain EB1256 also showed a predominant phenotype of filamentous cells (Figure 3B). Nearly all cells display an abnormal morphology compared with only a small fraction for EB611. We believe that the difference in the number of altered cells is probably the result of low levels of expression due to leak from the xylose promoter in strain EB611 in the absence of xylose. It has been observed that strain EB1256 requires 36 h to reach saturation in liquid culture, whereas strain EB611 is saturated after overnight growth (results

not shown). As with EB611, EB1256 exhibited a tendency to form chains of filaments and an additional phenotype of cell curvature is present, resulting in long wavy cells or short curved rods. The chains of cells may be indicative of a cell-wall defect preventing cell separation after division [16,17], whereas rod curvature implies a problem in the maintenance of cell shape. Similar phenotypes were observed with depletion of Obg and Bex (Era orthologue) in *B. subtilis* [18,19]. Obg is an essential GTP-binding protein that has been suggested to function in the stress response, chromosome partitioning, DNA repair, cell division and ribosome assembly [20–25]. The depletion of Obg resulted in cell elongation and cell curvature, and it is suggested that this is due to cell-division defects [19]. Era is also an essential GTPase that is believed to be involved in 16 S rRNA maturation, ribosome assembly, carbon metabolism and cell division [26–28]. Depletion of the Era orthologue, Bex, was associated with chains of elongated cells and, as with Obg depletion, a cell-division defect is thought to be responsible [18]. Given the similarity between depletion of YloQ and depletion of Obg and Era, it is possible that YloQ may similarly be involved in cell division. To investigate further the physiological function of YloQ, we used an annotated collection of antibiotics as probes of cellular phenotype and biochemical function.

Chemical synthetic lethality to investigate biological function

Chemical genetic methods typically employ small molecules to mimic genetic mutations, circumventing the need to create a deletion strain for a particular gene [29]. We decided to use the conditional *yloQ* deletion strain to look for chemical genetic interactions with antibiotics of different mechanisms and chemical class on depletion of YloQ. We have referred to this method as chemical synthetic lethality. Classically, synthetic lethality refers to the combination of two genetic defects that together prevent growth and imply a common or interacting function for the two genes [30]. Here, the primary defect is a lesion at *yloQ*, while the other defect is the chemical impact of an antibacterial agent. We hypothesize that depletion of YloQ should result in the sensitization of our mutant to antibiotics, targeting it or the pathway(s) in which it functions. A large number of protein synthesis inhibitors were included in our screen since previous *in vitro* biochemical experiments have shown an interaction of YjeQ (YloQ orthologue in *E. coli*) with the ribosome [6]. Also included were inhibitors of metabolism, membrane function etc. We asked whether the MIC, defined as the concentration of drug required for complete inhibition of growth, for these particular compounds changed when cells were depleted for YloQ. We set an arbitrary threshold of more than 2-fold sensitization for compounds to be considered as showing a chemical genetic interaction in this assay. We were searching for compounds that showed a noteworthy interaction with the *yloQ* mutant and not artifacts that might report on the strain's compromised state as a result of the YloQ depletion.

Table 2 reports the MIC values for each level of xylose tested with the conditional deletion strain. Figure 4 depicts our analysis of the sensitization (calculated as fold decrease in MIC) of the *yloQ* mutant to these antibiotics over a range of inducer concentrations. Out of seven cell-wall-active compounds tested, only one, phosphomycin, resulted in a significant sensitization (Figure 4A). Similarly, from a group of six nucleic acid synthesis inhibitors, cells were sensitized only to the RNA synthesis inhibitor rifampicin. Phosphomycin exerts its antibacterial effects through inhibition of MurA [31,32], whereas rifampicin is an inhibitor of RNA polymerase [33]. Although YloQ-depleted cells are clearly sensitized to phosphomycin, it seems unlikely that YloQ has a primary role in cell-wall biosynthesis since all other inhibitors

Table 2 Minimum inhibitory concentrations for antibiotics used in the chemical synthetic lethality screen against strain EB611 at different xylose concentrations

Antibiotic	Minimum inhibitory concentration ($\mu\text{g/ml}$)				
	0.2 % Xylose	0.063 % Xylose	0.02 % Xylose	0.002 % Xylose	0 % Xylose
Ampicillin	0.5	0.5	0.37	0.25	0.25
Azlocillin	0.125	0.094	0.094	0.062	0.062
Bacitracin	128	128	96	96	96
Cycloserine	32	32	32	24	48
Phosphomycin	64	64	32	24	8
Ramoplanin	0.25	0.125	0.125	0.125	0.125
Vancomycin	0.125	0.125	0.125	0.125	0.125
Ciprofloxacin	0.031	0.031	0.031	0.031	0.031
Nalidixic acid	48	64	128	96	96
Norfloxacin	0.25	0.25	0.25	0.125	0.125
Novobiocin	0.5	0.5	0.5	0.5	0.5
Proflavine	16	16	16	16	8
Rifampicin	1	1	0.75	0.25	0.312
Cetrimide	1	1	0.5	0.5	0.75
Furazolidone	0.75	0.75	0.5	0.25	0.25
Rhodamine	0.75	0.75	0.75	0.25	0.25
Trimethoprim	0.875	0.75	0.438	0.625	0.781
Kanamycin	2.4	2.4	2	2	1.6
Neomycin	0.25	0.25	0.188	0.125	0.125
Puromycin	16	16	16	16	16
Tetracycline	2	2	1.5	1.5	1
Fusidic acid	0.25	0.25	0.188	0.188	0.188
Synercid	0.5	0.5	0.5	0.375	0.25
Thiostrepton	0.5	0.5	0.375	0.25	0.25
Clindamycin	0.75	0.25	0.125	0.062	0.031
Erythromycin	0.188	0.125	0.078	0.027	0.023
Tylosin tartrate	1	0.5	0.25	0.25	0.25
Linezolid	1	0.5	0.25	0.125	0.125

tested from this category failed to show any synergy with the *yloQ* mutant. It is conceivable that phosphomycin has an additional cellular target other than MurA or that the lesion in *yloQ* exerts secondary effects on this target. Rifampicin is the only RNA synthesis inhibitor tested in this study, and YloQ-depleted cells were sensitized to this agent, implying that YloQ may play a role in RNA synthesis or maturation. Finally, from a group of compounds targeting other cellular functions, two displayed minor sensitization, namely furazolidone and rhodamine. The sensitization seen towards these antibacterial agents was very low and could represent a non-specific result.

Figure 4(B) depicts the results with protein synthesis inhibitors and reveals significant interactions with inhibitors of protein synthesis. These findings substantiate previous *in vitro* experiments with the *E. coli* orthologue, YjeQ, which revealed a selective interaction with the ribosome in pelleting assays [6]. Interestingly, chemical genetic interactions with the *yloQ* lesion were only evident with a subset of these compounds. Depletion of YloQ resulted in a sensitization towards inhibitors that, when bound, block the peptide channel or peptidyl transferase centre on the ribosome. In contrast, no interactions were seen with protein synthesis inhibitors that act at the A-site on the ribosome. Our chemical synthetic lethality experiments thus provide the first chemical genetic evidence that the *in vivo* function of YloQ/YjeQ is associated with the ribosome and, more specifically, that this function shows interactions with agents targeting the peptide channel or peptidyl transferase centre on the ribosome.

Looking more closely at the inhibitors tested in the present study, we searched to see if there was a common mechanism of action among these compounds, which might point towards a

Figure 4 Sensitivity of *B. subtilis* YloQ depleted cells to various antibacterial agents

Fold sensitization in MIC is calculated with respect to the complemented deletion strain (0.2%). Bars from left to right: 0.2, 0.063, 0.02, 0.002 and 0% xylose. **(A)** Antibacterial agents targeting cellular functions other than protein synthesis. **(B)** Protein synthesis inhibitors. A-site drugs indicate agents that block the A-site by direct binding or by preventing tRNA release. Peptide channel or peptidyl transferase centre drugs bind at or near these locations on the ribosome.

specific function for YloQ/YjeQ. Erythromycin and tylosin belong to the macrolide class of protein synthesis inhibitors [33]. Macrolides are believed to bind near the P-site at the entrance to the tunnel through which nascent peptide chains are channelled, blocking the progression of the growing peptide chain [34]. Tylosin shows a similar mode of binding, but is also capable of inhibiting the peptidyl transferase reaction [34]. Clindamycin belongs to the lincosamide family of antibiotics [33] and interacts with both the A- and P-sites on the ribosome and has been shown to block sterically the movement of the nascent peptide towards the tunnel [34]. Clindamycin and macrolides bind competitively with one another to the ribosome. Linezolid belongs to the oxazolidinone class of antibiotics, which target the P-site on ribosomes [35]. Linezolid inhibits the binding of initiator tRNA to the P-site, thereby inhibiting the formation of the first peptide bond [36].

Although the macrolides and clindamycin share some overlap in their mechanism of action, each of the drugs has distinct features where the unifying trait is binding at the peptide channel or peptidyl transferase centre.

Our chemical synthetic lethality data clearly illustrate that depletion of YloQ results in sensitization towards protein synthesis inhibitors, implying that YloQ functions in conjunction with the ribosome *in vivo*. A similar chemical synthetic lethal relationship was observed in a deletion strain of the non-essential *ssrA* gene [37]. This gene codes for tmRNA that is known to play a role in translation by interacting with and releasing stalled ribosomes [37]. The observation that YloQ exhibits a nearly identical chemical synthetic lethal pattern with a previously characterized ribosome protein is strong evidence that YloQ also functions at the ribosome.

Table 3 Comparisons of kinetic parameters for YjeQ GTP hydrolysis in the presence and absence of 30 S ribosomes

Enzyme $\pm$ stoichiometric amounts of ribosomes	K_m (μ M)	k_{cat} (h^{-1})	k_{cat}/K_m ($\times 10^4$ $M^{-1} \cdot h^{-1}$)
YjeQ	210.1 ± 30.9	3.1 ± 0.4	1.5 ± 0.2
YjeQ + 30 S ribosomes	195.3 ± 50.4	530.4 ± 37.2	271.6 ± 70.1

Figure 5 Impact of protein synthesis inhibitors on the ribosome-stimulated GTPase activity of YjeQ *in vitro*

(A) Impact of aminoglycosides on the stimulation of YjeQ GTPase activity by 70 S ribosomes. The level of GTPase stimulation resulting from interaction of YjeQ (500 nM) with the 70 S ribosome (500 nM) in the absence of antibiotic [6] was set as a reference (0% inhibition) for reactions tested with three concentrations of antibiotic: 20 μ M (dark-grey bar), 40 μ M (white bar) and 80 μ M (black bar). (B) Impact of aminoglycosides on the stimulation of YjeQ GTPase activity by 30 S ribosomes. The level of GTPase stimulation resulting from interaction of YjeQ (500 nM) with the 30 S ribosome (500 nM) in the absence of antibiotic [6] was set as a reference (0% inhibition) for reactions tested with three concentrations of antibiotic: 80 μ M (dark-grey bar), 160 μ M (white bar) and 320 μ M (black bar).

Effect of protein synthesis inhibitors on *in vitro* ribosome binding and GTPase stimulation of YjeQ

To follow up on the chemical genetic interactions observed, we further investigated the impact of protein synthesis inhibitors on the *in vitro* GTPase stimulation and interaction of YjeQ with ribosomes using assays that we have described previously [6]. Table 3 indicates the catalytic activity of the ribosome-stimulated and unstimulated YjeQ GTPase in the absence of inhibitors. We have shown this ribosomal stimulation to be specific for YjeQ by creating an N-terminal truncation variant (YjeQ 114–350), which retained intrinsic GTPase activity, but was unable to bind to ribosomes and showed no stimulation of GTPase activity in the

Figure 6 *In vitro* ribosome-binding assays to probe the impact of protein synthesis inhibitors on YjeQ association with 70 and 30 S ribosomes

1, No antibiotic; 2, neomycin; 3, streptomycin; 4, spectinomycin; and 5, tetracycline. Supernatant (S) and pellet (P) fractions were Western blotted and probed with an anti-YjeQ antibody. (A) Association with 70 S ribosomes tested at 80 μ M drugs and (B) association with 30 S ribosomes tested at 320 μ M drugs.

presence of ribosomes [6]. Of the 13 drugs tested, preincubation of ribosomes with aminoglycosides showed a significant dose-dependent reduction of GTPase stimulation with 70 S ribosomes (Figure 5A), whereas inhibitors that bind at the peptide channel or peptidyl transferase centre had no effect on the ribosome-stimulated GTPase activity. To control for non-specific interactions of aminoglycosides with YjeQ, the inhibition of intrinsic YjeQ GTPase activity was tested. The aminoglycosides were found to have no impact on YjeQ GTPase activity in the absence of ribosomes (results not shown).

Assays with 70 S ribosomes revealed that, at the lowest concentration of aminoglycoside tested, inhibition of ribosome-stimulated GTPase activity was 92, 22 and 8% with neomycin, streptomycin and spectinomycin respectively and increased with increase in drug concentration. To validate further the effects of antibiotics on the GTPase activity of YjeQ, we tested a subset of 30 S-specific protein synthesis inhibitors (neomycin, streptomycin, spectinomycin and tetracycline) with 30 S ribosomal preparations. Figure 5(B) illustrates similar results as observed for 70 S ribosomes. Neomycin again showed the greatest level of inhibition of GTPase stimulation. It is worth mentioning that, to investigate the effect of the 30 S-specific antibiotics on YjeQ GTPase activity, we had to increase the concentration of drugs up to 4-fold when compared with the concentrations used in assays with 70 S ribosomes. This is probably due to the fact that YjeQ is capable of a much tighter interaction with 30 S subunits when compared with whole 70 S ribosomes [6]. With the 70 and 30 S ribosomes, a dose-dependent increase in inhibition of ribosome-stimulated YjeQ GTPase activity was observed with aminoglycoside antibiotics, so we next investigated whether aminoglycosides and YjeQ competed for binding to the ribosome.

Using a previously established *in vitro* binding assay for YjeQ and ribosomes [6], we examined the binding of YjeQ to 70 S in the presence of saturating GMP-PNP. These assays utilized the highest concentration for all aminoglycosides identified in our GTPase assays, with tetracycline as a control (Figure 6A). The specificity of the ribosome pelleting assay used in these experiments was previously determined in our laboratory through

Figure 7 Polyribosome profiles of *B. subtilis* YjeQ depleted cells at different inducer concentrations

(A) EB611, 2 % xylose; (B) EB611, 0.2 % xylose; (C) EB611, 0.063 % xylose; (D) EB611, 0.02 % xylose; (E) EB611, 0.0063 % xylose; and (F) EB611, 0.002 % xylose.

stringency analysis and the use of truncation variants incapable of binding to the ribosome [6]. None of the antibiotics affected the binding of YjeQ to 70 S ribosomes. The doublet of cross-reactive bands seen in the binding assay with 70 S ribosomes has been observed previously and probably results from some small level of proteolytic activity contaminating the 70 S ribosomal preparations [6]. An equivalent experiment was performed using 30 S subunits (Figure 6B) and again there was no competition for binding. Interestingly, neomycin was the only drug that showed any impact on ribosome association, resulting in a slight increase in YjeQ binding to 30 S subunits. This increase in binding could be a result of alterations in the ribosome that lock YjeQ in a GTP-bound form, preventing hydrolysis and dissociation from the ribosome. Taken together, these results imply that YjeQ binds at a distal site on the ribosome from all of the antibiotics used in the present study and that inhibition of GTPase activity by aminoglycosides is an allosteric effect and not due to direct competition for the same or overlapping binding sites.

Our finding that neomycin had the most significant impact on the *in vitro* ribosome-associated GTPase activity of YjeQ, followed by streptomycin and spectinomycin, is contradictory to results obtained by another group, where spectinomycin was not observed to have any effect on the ribosome-associated GTPase

activity of YjeQ at concentrations up to 500 μ M [10]. The authors suggest the neomycin and streptomycin inhibition is due to competitive binding of YjeQ with antibiotics that bind at the A-site; however, when we tested for competitive binding, we found that YjeQ was capable of full binding to both 70 and 30 S ribosomes in the presence of all aminoglycosides.

It is noteworthy that the level of inhibition varies among the aminoglycoside probes used. Neomycin, the most efficacious inhibitor in our assay, is a 4,5-deoxystreptamine that binds to helix 44 of A-site 16 S rRNA [38,39]. It is well documented that aminoglycosides interacting with helix 44 cause a remarkable movement of the helix and the displacement of two adenines A1492 and A1493 into the A-site that alters the binding affinity of tRNAs in this site, resulting in a decrease in translational fidelity, ultimately leading to mistranslation and growth cessation owing to defects associated with the translated products [39,40]. From the results presented in this study, it is conceivable that the remarkable movement of helix 44 promotes a conformation that allows for binding of the GTP-bound form of YjeQ to the ribosome, but is inhibitory to hydrolysis and suggests that GTPase activity is coupled with release from the ribosome. Indeed, the function of YjeQ may be regulated by A-site occupancy or allosterically through structural changes originating in the A-site. These results

provide further evidence that YloQ/YjeQ function is related to the ribosome.

The *in vivo* genetic data and the *in vitro* biochemical results highlight different inhibitors related to YloQ/YjeQ function on the ribosome (YjeQ has been shown to be capable of binding to 30, 50 and 70 S ribosomes and may function in one or all of the ribosomal forms [6]). This result is not surprising since the *in vivo* work points towards physiological effects of GTPase depletion, whereas the *in vitro* work highlights the biochemistry of its function in the context of the ribosome. Additionally, *in vivo*, the full complement of translation factors will be present, whereas *in vitro*, only purified ribosomes and YjeQ are at hand.

Polyribosome profiles of YloQ-depleted cells

Both our *in vivo* and *in vitro* results support the hypothesis that YloQ/YjeQ function is associated with the ribosome. To further investigate this relationship, we examined the ribosome profiles of the conditionally complemented *yloQ* deletion strain. As the inducer level is decreased, there is an accumulation of unassembled 30 and 50 S ribosomal subunits with a concomitant decrease in intact 70 S ribosomes (Figure 7). Wild-type control cells grown in the presence of the same level of xylose did not show any accumulation of ribosomal subunits (results not shown), indicating that this effect is directly related to YloQ function *in vivo*. A similar result was observed in a *yjeQ* deletion strain [10] and for other proteins that are involved in translation or subunit assembly. Deletion of *rrmJ* (*fisJ*), an rRNA methyltransferase required for the methylation of 23 S rRNA at position 2552, resulted in the accumulation of 30 and 50 S subunits and a decrease in 70 S ribosomes [24,41]. This ribosome defect is believed to be a result of impaired ribosome assembly or stability of 70 S ribosomes [24]. Interestingly, cells depleted for Obg or Era, which have already been found to have similar cellular morphology with depletion of YloQ, also show the same ribosome profile with an accumulation of 30 and 50 S subunits [27,42]. Another similarity between YjeQ and Era is that depletion of both these proteins results in the accumulation of a 16 S rRNA precursor, 17 S rRNA [10,27]. The accumulation of both 17 S rRNA and 30 and 50 S subunits upon depletion of YloQ/YjeQ leads us to propose that YloQ/YjeQ may be involved in ribosome maturation and assembly.

In this paper, we have presented a variety of cellular effects associated with depletion of YloQ. Although dispensable in *B. subtilis* and *E. coli*, the growth rate is affected severely enough to implicate a function in important areas of cellular metabolism. Examination of cellular morphologies implicated YloQ in cell division, whereas antibiotic probes and ribosome profiles reveal a role in ribosome assembly or maturation. Recent studies with the Era and Obg proteins have also revealed a role for these GTPases of unknown function in multiple areas of cellular metabolism [43]. From this work, it appears that YloQ/YjeQ may fall into this same category. The next step will be to determine whether there is a link between these distinct cellular functions of YloQ/YjeQ. These results clearly show that depletion of YloQ diminishes the assembly of, or alters the stabilization of, intact 70 S ribosomes, which could explain the growth defect associated with YloQ depletion.

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