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The ClpY-ClpQ protease regulates multicellular development in *Bacillus subtilis*

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Abstract

ATP-dependent proteases play essential roles in both protein quality control and the regulation of protein activities in bacteria. ClpYQ (also known as HslVU) is one of several highly conserved ATP-dependent proteases in bacteria. The regulation and biological function of ClpYQ have been well studied in Gram-negative bacteria, but are poorly understood in Gram-positive species. In this study, we showed that in the Gram-positive bacterium *Bacillus subtilis*, the Δ*clpYQ* deletion mutant formed early and robust biofilms, while swarming motility was severely impaired. Colonies of the Δ*clpYQ* mutant were also much less mucoid on agar plates, indicating the loss of the production of secreted γ-poly-DL-glutamic acid (γ-PGA). Global proteomic analysis using isobaric tags for relative and absolute quantification (iTRAQ) confirmed that a number of proteins involved in motility, chemotaxis and the production of γ-PGA were less abundant in the Δ*clpYQ* mutant. The results from both iTRAQ and Western immunoblotting showed that levels of the biofilm master repressor SinR were modestly reduced in the Δ*clpYQ* mutant, but probably significantly enough to alter biofilm regulation due to the ultrasensitivity of the expression of biofilm genes to SinR protein levels. Western immunoblotting also showed that the abundance of CodY, whose gene is clustered with *clpYQ* in the same operon, was not impacted on by Δ*clpYQ*. Lastly, our results suggested that, unlike in *Escherichia coli*, ClpYQ does not play an essential role in heat-shock response in both *B. subtilis* and *Bacillus cereus*. In conclusion, we propose that the ClpYQ protease is primarily involved in multicellular development in *B. subtilis*.

INTRODUCTION

ATP-dependent proteases are ubiquitous in bacteria. They play essential roles in protein quality control and serve various regulatory functions [1, 2]. Multiple ATP-dependent proteases have been characterized in bacteria and shown to either degrade non-native proteins (e.g. misfolded proteins during heat shock) as a protein quality-control mechanism, or alter the activity of native proteins through proteolysis as a regulatory function, or both [1]. Among these proteases, Lon and FtsH are composed of multiple functional subdomains in a single polypeptide, whereas Clp proteases such as ClpAP, ClpXP and ClpYQ consist of two separate subunits, one as the ATPase substrate-binding subunit (unfoldase) and the other as the catalytic subunit (peptidase) [1]. Further, ClpYQ best resembles the 26S proteasome in the eukaryotic cells [3].

In the bacterium *Bacillus subtilis*, there are five characterized ATP-dependent proteases: ClpCP, ClpEP, ClpXP, Lon and FtsH. These five proteases have been shown to be collectively involved in heat-shock response, protein quality control, the regulation of native proteins and the control of cell development [4–8]. For example, ClpCP was shown to regulate the stability of ComK, a master regulator for genetic competence [6], and SlrR, an important regulatory protein for both motility and biofilm formation [9]. ClpXP was shown to be involved in regulating general stress tolerance [10] and the activity of Sda, a small checkpoint protein for controlling the entry into sporulation in *B. subtilis* [7]. FtsH is an ATP-dependent cytoplasmic membrane protease involved in the control of membrane protein quality, cell division heat-shock response and biofilm formation [5, 11]. More recently, Mukherjee *et al.* reported that the Lon protease plays an important role in the switch of the *B. subtilis* cells from swimming in the aqueous environment to swarming on a surface by regulating SwrA, a key activator for swarming motility in *B. subtilis* [8]. Several studies also demonstrated that the Lon and Clp proteases were localized

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Abbreviations: iTRAQ, isobaric tags for relative and absolute quantification; LacZ, beta-galactosidase; γ-PGA, γ-poly-DL-glutamic acid.

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Six supplementary figures and two supplementary tables are available with the online version of this article.

in distinct cellular compartments, although in some cases, the biological importance for such localization patterns was still not clear [12–14]. Overall, many of those ATP-dependent proteases seem to play critical regulatory functions in cell physiology and development in *B. subtilis*.

ClpYQ, on the other hand, is perhaps the least studied ATP-dependent protease in *B. subtilis* and other Gram-positive bacteria. ClpYQ contains a 19 kDa protein (ClpQ) that harbours the peptidase activity, and a 50 kDa protein (ClpY) that provides essential ATPase activities. The biological function and regulation of ClpYQ are poorly understood in *B. subtilis*, with the exception that some biochemical and structural studies have suggested ClpYQ as a serine protease, instead of a threonine protease in the case of the ClpYQ counterpart in *Escherichia coli* [15]. In one recent study, a defect in swarming motility was observed in a *B. subtilis* transposon insertion mutant of the *clpY* gene [8]. In another Gram-positive bacterium, *Staphylococcus aureus*, it was suggested that ClpYQ might play a minor role in virulence and act as an accessory ATP-dependent protease in the heat-shock response [16]. We recently showed that in the soil bacterium *Bacillus cereus*, ClpYQ played a role in biofilm formation, although the molecular mechanism was not further explored in that study [17]. In *E. coli*, ClpYQ is also known as HslVU and is encoded by the *hslVU* operon [3, 18]. The *hslVU* operon is strongly induced under heat shock in *E. coli* [3]. Hence, one of the primary functions of ClpYQ (or HslVU) is to degrade non-native (misfolded) proteins during the heat-shock response.

B. subtilis is capable of forming biofilms on solid surfaces, at the air–liquid interface, or submerged in liquid [19–21]. *B. subtilis* has served as a model system for studies of biofilm formation [22, 23]. In *B. subtilis*, cells adopt alternative cell fates in response to various environmental signals [22, 24, 25]. For example, it has been well characterized that *B. subtilis* cells switch between free living and biofilm formation, two mutually exclusive cell fates [26, 27]. Under nutrient-limited conditions, planktonic cells may settle down by producing adhesins for surface attachment and the polymeric biofilm matrix, which allows individual cells to stick to each other and form multicellular aggregates [21, 28]. At the molecular level, the switch involves the down-regulation of a number of genes for free living, including those involved in motility, chemotaxis and cell separation [22, 26]. The switch also involves antagonization of the biofilm master repressor SinR, which in turn results in the derepression of SinR-controlled matrix genes for the production of the TasA fibres and exopolysaccharides of the biofilm matrix [29–32]. *B. subtilis* is also known to produce other polymeric substances, such as γ-poly-DL-glutamic acid (γ-PGA) [33, 34]. The biological function of γ-PGA is not entirely clear. Previous studies have indicated its importance in biofilm formation, surface colonization and host interactions [33, 35].

In this study, we constructed a *clpYQ* in-frame deletion mutant in *B. subtilis* and showed that this mutant formed early and robust biofilms, while the mutant was also simultaneously impaired for swarming and swimming motility. We further observed that the *clpYQ* deletion mutant of *B. subtilis* lost colony mucoidy on the agar plate, indicating a significant reduction in the production of γ-PGA. Our evidence also suggested that ClpYQ has a dispensable role in heat-shock response in both *B. subtilis* and *B. cereus*, which differs from what has been seen in Gram-negative bacteria. We propose that the primary function of the ATP-dependent protease ClpYQ in *B. subtilis* is to regulate multicellular development.

METHODS

Strains, media and growth conditions

B. subtilis and *B. cereus* strains were routinely cultured in lysogenic broth (LB) (10 g tryptone, 5 g yeast extract and 5 g NaCl l^{-1} broth) or on LB plates solidified with 1.5 % agar at 37 °C. For biofilm formation assays, the biofilm-inducing medium, LBGM, was used [24]. LBGM is composed of LB broth (or solidified LB agar) supplemented with 1 % glycerol and 100 μM $MnSO_4$. All of the strains, plasmids and primers used in this study are listed in Table 1. Antibiotics were added at the following final concentrations: 100 μg ml^{-1} of spectinomycin, 5 μg ml^{-1} of chloramphenicol, 5 μg ml^{-1} of tetracycline and 1 μg ml^{-1} of erythromycin plus 2.5 μg ml^{-1} of lincomycin (for selection of MLS resistance). Chemicals were purchased from Sigma. Restriction enzymes and other enzymes for molecular cloning were obtained from New England Biolabs. All primers were ordered from IDT DNA technology. DNA sequencing was performed at Genewiz.

Strain construction

To construct the non-polar in-frame deletion mutation in *clpYQ* in *B. subtilis* 3610 (FY170), the temperature-sensitive suicide vector pMAD was used [36]. Briefly, approximately 1 kb regions both upstream and downstream of the *clpYQ* genes were amplified by PCR using primers clpYQ(Bs)-P1 and -P2, and clpYQ(Bs)-P3 and -P4, respectively. The two PCR products were cloned sequentially into the pMAD plasmid, resulting in the recombinant plasmid pMAD-ΔclpYQ (Bs). The resulting plasmid was then introduced into PY79 by transformation. Transformants with the plasmid integrated into the chromosomal locus via Campbell integration were selected at non-permissive temperature (45 °C) on LB agar plates (+Mls, +X gal). The integrated plasmid was then transferred from the PY79 background into 3610 by SPP1 phage-mediated transduction [37]. MLS-resistant blue colonies were picked and grown at permissive temperature (25 °C) in LB broth to the stationary phase to allow the integrated plasmid to excise from the chromosome. The cells were then diluted 1000-fold to fresh LB broth and grown at non-permissive temperature (45 °C) for 4 h. They were then diluted again and plated on LB agar plates (+X gal). The next day white colonies were picked from the plates and checked for loss of MLS drug resistance. The presence of the in-frame deletion in *clpYQ* was confirmed by PCR amplification of the *clpYQ* locus and DNA sequencing of the PCR product.

Table 1. Strains, plasmids and primers used in this study

	Description	Reference
Strains		
PY79	A laboratory strain of *B. subtilis* used for genetic manipulation	[55]
3610	An undomesticated strain of *B. subtilis*, capable of forming biofilms	[20]
AR156	An environmental isolate of *B. cereus*, capable of forming robust biofilms	[56]
DH5α	*E. coli* strain for molecular cloning	Invitrogen
FY6	Δ*pgsBCAD* in *B. subtilis* 3610, Sp^R	[33]
FY170	*clpYQ* in-frame deletion mutant in *B. subtilis* 3610	This study
FY171	The complementation strain for Δ*clpYQ* in *B. subtilis* 3610, Spc^R	This study
FY173	*codY* insertional deletion mutant in *B. subtilis,* Mls^R	This study
FY175	*clpYQ* in-frame deletion mutant in *B. cereus* AR156	This study
FY184	*amyE*::P_{hag}-*lacZ* in 3610, Cm^R	This study
FY185	*amyE*::P_{hag}-*lacZ* in the Δ*clpYQ* mutant of *B. subtilis*, Cm^R	This study
RL3854	Δ*sinR* in *B. subtilis* 3610, Sp^R	[31]
YC844	Synonymous substitutions at ser16, 18 and 33(TCA>TCG) in *sinR*, Kan^R	[52]
YC1000	*amyE*::P_{epsA}-*lacZ* in 3610, Cm^R	[52]
YC1275	*amyE*::P_{pgsB}-*lacZ* in 3610, $Spec^R$	[47]
YC1276	*amyE*::P_{pgsB}-*lacZ* in the Δ*clpYQ* mutant of *B. subtilis*, $Spec^R$	This study
YY64	*clpYQ* in-frame deletion, *amyE*::P_{epsA}-*lacZ* in 3610, Cm^R	This study
YY100	*clpYQ* in-frame deletion, ser32(AGC>TCA) and ser76(AGT>TCA) in *sinR*, Kan^R	This study
YY145	*clpQ* in-frame deletion mutant in *B. subtilis* 3610	This study
YY146	*clpY* in-frame deletion mutant in *B. subtilis* 3610	This study
YY265	*clpYQ* in-frame deletion and *codY*::*mls* in *B. subtilis* 3610, Mls^R	This study
Plasmids		
pIC333	A suicide vector with the mini-Tn10 transposon element, Amp^R, Spc^R, Mls^R	[38]
pMAD	A suicide vector for effecting deletion mutation, Mls^R	[36]
pDR111	An *amyE* integration vector with *hyspank* promoter, Spc^R, Amp^R	[24]
Primers		
Tn10(113-98)	5′- GCCGCGTTGGCCGATTC-3	
Tn10(2235-2249)	5′- GATATTCACGGTTTA-3′	
clpYQ(Bs)-P1	5′- GGAAGATCTTCGAGACTTTCCTGCGCGTTC-3′	
clpYQ(Bs)-P2	5′- CTTTATGTCATCTTTTCATGCGGGAATTCCGC-3′	
clpYQ(Bs)-P3	5′- GCGGAATTCCTTAAGTCAATTTATATTGTGA-3′	
clpYQ(Bs)-P4	5′- CAAAAAGTTTCAAAAATGCCGGGATCCCGCG-3′	
clpY-IDT-F	5′-GAGCCGCGTTAGAAACAGC-3′	
clpY-IDT-R	5′-CGCAGCTTGCAGCATGGAG-3′	
clpQ-IDT-F	5′-CAAGAACTGCTCGGGCATT-3′	
clpQ-IDT-R	5′-TTAATGCCACGGCGACAGC-3	
clpYQ-F1	5′- GCATGTCGACTAAAGGAGGCCCTTTATGTCATCTTTTCATGCGACC-3′	
clpYQ-R1	5′ -GCGCGCTAGCTCACAATATAAATTGACTTAAATCTTTG-3′	

To construct the complementation strain of Δ*clpYQ* in *B. subtilis* (FY171), we first amplified the entire coding region of *clpYQ* by PCR and using primers *clpYQ*-F1 and *clpYQ*-R1. The PCR product was then cloned into the plasmid pDR111, which contains an IPTG-inducible promoter *hyper-spank* [24]. The resulting recombinant plasmid was introduced into the strain FY170 to generate the complementary strain FY171.

To construct the individual non-polar in-frame deletion mutants for *clpQ* and *clpY* in the *B. subtilis* 3610 background, the insertional deletion strains of *clpY* (Δ*clpY*::erm^R) and *clpQ* (Δ*clpQ*::erm^R) in the *B. subtilis* 168 background were obtained from the Bacillus Genetic Stock Center (BGSC, www.bgsc.org). The mutation cassettes (Δ*clpY*::erm^R and Δ*clpQ*::erm^R) were then introduced into 3610 by transformation. In order to remove the associated antibiotic cassettes and generate non-polar deletion mutations of the genes, we followed the protocol provided with the purchased strains. Briefly, pDR244(*cre+ts*), a plasmid with a temperature-sensitive origin and the *cre* gene encoding the Cre recombinase that recognizes the flanking sequences of the antibiotic cassettes, was transformed into the insertional mutation strains bearing those antibiotic cassettes. The transformants were selected at 30 °C for spectinomycin resistance for introduction

of the plasmid. The colonies were then streaked out at 42 °C without antibiotic selection for excision of the antibiotic cassette and simultaneously the loss of the pDR244(*cre +ts*) plasmid. The non-polar deletion mutation strains were confirmed (YY145 for Δ*clpQ* and YY146 for Δ*clpY*) by both loss of erm^R and PCR verification with gene-specific primers (clpY-IDT-F: 5′-GAGCCGCGTTAGAAACAGC-3′, clpY-IDT-R: 5′-CGCAGCTTGCAGCATGGAG-3′, clpQ-IDT-F: 5′-CAAGAACTGCTCGGGCATT-3′, clpQ-IDT-R: 5′-TTAATGCCACGGCGACAGC-3′).

To construct the *codY* insertional deletion mutation in *B. subtilis* 3610, the insertional deletion mutant of *codY* in the *B. subtilis* 168 strain background (Δ*codY*::erm^R) was obtained from the *Bacillus* Genetic Stock Center (BGSC). Genomic DNA was prepared from the above strain by using the genomic DNA Prep kit (Promega, Madison, WI, USA) and introduced into 3610 by genetic transformation. The transformants were selected on LB supplemented with the antibiotic MLS, resulting in strain FY173. For the construction of various reporter strains of *B. subtilis*, the genomic DNA containing the reporter fusions was obtained from previously constructed strains and introduced into *B. subtilis* 3610 derivatives by either genetic transformation or phage-mediated general transduction.

To construct the Δ*clpQ*Δ*codY* double mutant, the DNA containing the Δ*codY*::erm^R insertional mutation was prepared from the strain FY173 and introduced into strain YY145, which contains a non-polar deletion mutation in *clpQ* (Δ*clpQ*). The resulting doubly mutated strain (YY265, Δ*clpQ*Δ*codY*) was verified for the presence of the erm^R antibiotic marker (associated with Δ*codY*) on the LB plate supplemented with MLS and the presence of the in-frame deletion in *clpQ* by PCR amplification of the corresponding genetic locus and DNA sequencing using the primers clpQ-IDT-F and clpQ-IDT-R.

Pellicle and colony biofilm formation assays

To analyse pellicle biofilm formation, cells were first grown in 3 ml LB broth to the late-exponential growth phase (OD_{600}=1). Then 3 µl culture was added to 3 ml LBGM medium (a 1000-fold dilution) in 12-well polyvinyl plates (VWR). The plates were incubated statically at 30 °C for the indicated period of time (usually 24 or 48 h). To analyse the formation of colony biofilms, 3 µl of log-phase growing cells was spotted onto the LBGM plates solidified with 1.5 % agar. The plates were incubated at 30 °C for the indicated period of time (usually 48 or 72 h). Images were taken using either a Nikon CoolPix camera or a Leica DMC2900 dissecting microscope.

Swarming motility assays

Swarming motility assays were performed for the wild-type or mutant strains by following a published protocol [38] with some modifications. LB plates solidified with 0.75 % agar were poured and dried overnight (~12 h) at room temperature. One millilitre of mid-late log-phase cells were collected, washed twice with PBS buffer and resuspended in 100 µl PBS buffer. Then 5 µl samples were spotted on the centre of the swarming soft agar plates. The plates were dried for 10 min in a laminar hood and then incubated at 37 °C. The swarming plates were removed from incubation at indicated time points, and then dried for an hour in a laminar hood. The plates were then left on the bench at room temperature overnight. The diameter of the swarming zone was measured the next day. For swarming by *B. subtilis* strains, the incubation time at 37 °C is about 8 h.

β-galactosidase activity assays

Cells were cultured in LB or LBGM medium at 37 °C in a shaking water bath. One millilitre of culture was collected at various time points and cells were spun down. Cell pellets were resuspended in 1 ml of Z buffer (40 mM NaH_2PO_4, 60 mM Na_2HPO_4, 1 mM $MgSO_4$, 10 mM KCl and 38 mM 2-mercaptoethanol) supplemented with 10 µl of 20 mg ml^{-1} freshly made lysozyme. All cell samples were incubated at 37 °C for 30 min. Then 200 µl ONPG (O-nitrophenyl-β-D-galactopyranoside) dissolved in Z buffer was added to the solution to start the reactions. The reactions were stopped by adding 500 µl of 1 M Na_2CO_3 after the solutions turned yellow. The samples were vortexed vigorously, briefly spun down and applied for measurement of the OD_{420} using the Bio-Rad Smartspec 3000. The activity was calculated according to the following equation: $OD_{420}\times1000/(\Delta T_{min}\times OD_{600})$.

Western immunoblotting

Western immunoblotting was performed to examine whether there was an alteration in the abundance of the CodY and SinR proteins in different strain backgrounds. Cell lysates were prepared as follows. Cells were grown in LB broth to indicated growth stages as measured by optical cell density at OD_{600}. Five millilitres of cell culture was harvested and washed with 2 ml of cold PBS buffer (pH 7.2). For CodY immunoblotting assays, cells were collected after being grown to different stages, namely OD_{600} at 0.5, 1, 2, 3 and 4.5, respectively, from both the wild-type cells and the *clpYQ* deletion mutant. Cell pellets were then resuspended in 500 µl PBS buffer supplemented with 200 µg per ml of freshly prepared lysozyme and incubated on ice for 30 min. The mixture was then sonicated on ice (3–5 times, 15–20 pulses each time). The cell lysates were centrifuged at 15000 ***g*** for 20 min at 4 °C to remove cell debris. Supernatants were transferred to new cold tubes. The supernatant samples were analysed using 12 % SDS-PAGE. Then 15 µl of supernatant was mixed with the SDS-PAGE loading dye and loaded per lane. After size fractionation, the proteins were transferred onto a nitrocellulose membrane (Bio-Rad) by electro-transfer for 2 h at 150 mA. After the proteins were transferred, the membrane was briefly washed with TBS buffer (20 mM Tris pH 8.0 and 200 mM NaCl, with 0.1 % Tween 20), and blocked for about 1 hour at room temperature in the TBS buffer supplemented with 0.1 % Tween 20 and 5% skim milk. After blocking, the membrane was incubated in 20 ml TBS buffer with 0.1 % Tween 20 and 5% skim milk. The primary CodY or SinR antibody was then added to the incubation in a 1 : 5000 dilution, and the

membrane was incubated for 4 hours at room temperature with gentle shaking. After being washed briefly with TBS buffer supplemented with 0.1 % Tween 20 and 5% skim milk three times, the secondary antibody (goat-anti-rabbit, Bio-Rad) was added at a 1 : 5000 dilution, and the membrane was incubated for an additional hour at room temperature with gentle shaking. The membrane was then washed three times with TBS buffer plus 0.1 % Tween 20 and 5% skim milk, and developed with the application of the chemiluminescence detection kit (Thermo). The membrane was scanned by a Typhoon Imager (GE Healthcare) for image development and result analysis. For purification of the SinR antibody, the SinR antiserum was mixed with TBS buffer (1 : 500) and the mixture was then incubated with a nitrocellulose membrane pre-transferred with the cleared protein lysate from the Δ*sinR* mutant cells. Pre-absorption of the antibodies to the proteins on the membrane was allowed under gentle shaking (50 r.p.m.) at 4 °C for 3 h prior to use of the SinR antibody.

Isobaric tags for relative and absolute quantitation (iTRAQ)

Cell sample preparation

The wild-type strain and the *clpYQ* in-frame deletion mutant of *B. subtilis* were grown in LBGM to the mid-log phase (OD_{600}=1). Fifty millilitres of cells were spun down, and the cell pellets were flash frozen in dry ice. Total protein preparation, trypsin digestion, peptide labelling, MS analysis and bioinformatics analysis were all performed at BGI Americas (http://bgiamericas.com) following the established protocols.

Protein preparation

The cells were suspended in lysis buffer (7 M urea, 2 M thiourea, 4 % CHAPS, 40 mM Tris-HCl, pH 8.5, 1 mM PMSF, 2 mM EDTA) and sonicated on ice. The proteins were reduced with 10 mM DTT (final concentration) at 56 °C for 1 h and then alkylated by 55 mM iodoacetamide (IAM; final concentration) in the darkroom for 1 h. The reduced and alkylated protein mixtures were precipitated by adding 4× volume of chilled acetone at −20 °C overnight. After centrifugation at 4 °C, 30 000 ***g***, the pellet was dissolved in 0.5 M TEAB (Applied Biosystems, Milan, Italy) and sonicated on ice. An aliquot of the supernatant was taken for determination of protein concentration. The proteins in the supernatant were kept at −80 °C for further analysis.

iTRAQ labelling

Total protein (100 µg) was taken out from each sample solution and then the protein was digested with Trypsin Gold (Promega, Madison, WI, USA) with the ratio of protein: trypsin=30 : 1 at 37 °C for 16 h. After trypsin digestion, the peptides were dried by vacuum centrifugation. The peptides were reconstituted in 0.5 M TEAB and processed according to the manufacture's protocol for 8-plex iTRAQ reagent (Applied Biosystems). Briefly, one unit of iTRAQ reagent was thawed and reconstituted in 24 µl isopropanol. Samples were labelled with the iTRAQ tags. The peptides were labelled with the isobaric tags and incubated at room temperature for 2 h. The labelled peptide mixtures were then pooled and dried by vacuum centrifugation.

SCX fractionation

SCX chromatography was performed with an LC-20AB HPLC pump system (Shimadzu, Kyoto, Japan). The iTRAQ-labelled peptide mixtures were reconstituted with 4 ml buffer A (25 mM NaH2PO4 in 25 % acetonitrile (ACN), pH 2.7) and loaded onto a 4.6×250 mm Ultremex SCX column containing 5-µm particles (Phenomenex, Torrance, CA, USA). The peptides were eluted at a flow rate of 1 ml min^{-1} with a gradient of buffer A (5 % ACN, 0.1 % formic acid, FA) for 10 min, 5–60 % buffer B (25 mM NaH2PO4, 1 M KCl in 25 % ACN, pH 2.7) for 27 min and 60–100 % buffer B for 1 min. The system was then maintained with 100 % buffer B for 1 min before equilibration with buffer A for 10 min prior to the next injection. Elution was monitored by measuring the absorbance at 214 nm, and fractions were collected every 1 min. The eluted peptides were pooled into 20 fractions, desalted with a Strata X C18 column (Phenomenex, Torrance, CA, USA) and vacuum-dried.

Liquid chromatography/electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) analysis

Each fraction was resuspended in buffer A and centrifuged at 20 000 ***g*** for 10 min. The final concentration of peptide was about 0.5 µg $µl^{-1}$ on average. Ten microlitres of supernatant were loaded by the autosampler onto a 2 cm C18 trap column in a LC-20AD nanoHPLC (Shimadzu, Kyoto, Japan). Then, the peptides were eluted onto a 10 cm analytical C18 column (inner diameter 75 µm) that was packed in-house. The samples were loaded at 8 µl min^{-1} for 4 min, and then the 35 min gradient was run at 300 nl min^{-1} starting from 2 to 35 % B (95 % ACN, 0.1 % FA), followed by a 5 min linear gradient to 60 %, with this being followed by a 2 min linear gradient to 80 %, and maintenance at 80 % B for 4 min, before finally returning to 5 % in 1 min.

Data acquisition

Data acquisition was performed with a TripleTOF 5600 System (AB SCIEX, Concord, ON, Canada) fitted with a Nanospray III source (AB SCIEX, Concord, ON, Canada) and with a pulled quartz tip as the emitter (New Objectives, Woburn, MA, USA). Data were acquired using an ion spray voltage of 2.5 kV, curtain gas of 30 p.s.i., nebulizer gas of 15 p.s.i. and an interface heater temperature of 150 °C. The mass spectrometer was operated with an RP of greater than or equal to 30 000 FWHM for TOF MS scans. For IDA, survey scans were acquired in 250 ms [as many as 30 product ion scans were collected if a threshold of 120 counts per second (counts s^{-1}) was exceeded] and with a 2+ to 5+ charge state. The total cycle time was fixed at 3.3 s. The Q2 transmission window was 100 Da for 100 %. Four time bins were summed for each scan at a pulser frequency value of 11 kHz through monitoring of the 40 GHz multichannel TDC detector with a four-anode channel detection ion. A sweeping collision energy setting of 35±5 eV coupled with iTRAQ adjust rolling collision energy was applied to all precursor ions for collision-induced dissociation. Dynamic exclusion

was set at 1/2 of peak width (15 s), and then the precursor was refreshed off the exclusion list.

Data analysis

Raw data files acquired from the Orbitrap were converted into MGF files using Proteome Discoverer 1.2 (PD 1.2, Thermo) and the MGF files were searched. Protein identification was performed by using the Mascot search engine (Matrix Science, London, UK; version 2.3.02) and the NCBI database. For protein identification, a small mass tolerance was permitted for intact peptide masses and for fragmented ions, with allowance for one missed cleavage in the trypsin digests. Gln->pyro Glu (N-term Q), oxidation (M) and deamidated (NQ) were the potential variable modifications, while carbamidomethyl (C), iTRAQ8plex (N-term) and iTRAQ8plex (K) were the fixed modifications. The charge states of the peptides were set to +2 and +3. Specifically, an automatic decoy database search was performed in Mascot by choosing the decoy checkbox in which a random sequence of the database is generated and tested for raw spectra as well as the real database. To reduce the probability of false peptide identification, only peptides at or greater than the 95 % confidence level measured by a Mascot probability analysis were counted as identified, and each confident protein identification involved at least one unique peptide. For protein quantitation, it was required that a protein contained at least two unique spectra. The quantitative protein ratios were weighted and normalized by the median ratio in Mascot. Only ratios with *P*-values <0.05 were used, and only fold changes of >1.2 were considered to be significant.

Heat-shock experiment

The wild-type strains or the *clpYQ* deletion mutants of *B. subtilis* or *B. cereus* were streaked out on LB agar plates. The plates were then incubated at various temperatures (30, 37, 45 and 50 °C) for 24 h. Images of the plates were taken using a Nikon Coolpix Camera.

Measurement of survival rate

The *B. subtilis* wild-type strain, and single and double mutants of *clpYQ* were grown in shaking LB broth at 37 °C to the early log phase. The incubation temperature was then shifted from 37 to 50 °C and the cultures were incubated for an additional 30 min or 120 min. The cells were serial-diluted and colony-forming units (c.f.u.s) were calculated by the plating method. The survival rate was calculated as the ratio of c.f.u.s before and after 50 °C heat treatment. The c.f.u. assays were performed in triplicate.

Colony mucoidy assays

The wild-type strain or the *clpYQ* deletion mutant of *B. subtilis* was streaked out on LB agar plates. The plates were then incubated at 37 °C for 12 h. Images were taken using either a Nikon CoolPix camera or a Leica DMC2900 dissecting microscope (for zoom-in images).

RESULTS

Loss of *clpYQ* results in early and robust biofilms in *B. subtilis*

Little is known about the biological function of the ClpYQ protease in Gram-positive bacteria. In our previous work, we observed that the *clpYQ* deletion mutant of *B. cereus* had an early and robust biofilm phenotype, suggesting that ClpYQ has a role in biofilm formation in *B. cereus* [17]. We were interested in finding out whether the *clpYQ* genes might also be involved in biofilm formation in *B. subtilis*. In *B. subtilis*, *B. cereus* and other related Gram-positive bacteria, the *clpY* and *clpQ* genes are clustered in an operon with two other genes, *codY* and *xerC* (Fig. 1a) [39]. Of the other two genes, *codY* encodes a global regulator for stationary-phase metabolism and growth in *B. subtilis* as well as virulence in some Gram-positive bacteria [39–42]. *xerC* encodes a site-specific recombinase that may be involved in cell division [39]. The genetic organization of these genes is highly conserved even in *Staphylococcus aureus* and *Listeria monocytogenes* (Fig. 1a). To avoid a potential polar effect on the downstream *codY* gene, we constructed a non-polar in-frame deletion of the *clpYQ* genes by removing about 95 % of the *clpYQ* coding sequences in the *B. subtilis* strain 3610 genome (Fig. 1a). The resulting deletion mutant was tested for biofilm formation. As shown in Fig. 2(a, b), the mutant showed early induction of pellicle biofilm formation; after 24 h of incubation at 30 °C in the biofilm medium LBGM, the mutant had already formed robust floating pellicles with highly wrinkled structures, whereas the wild-type cells just started to form a thin layer of featureless pellicles (Fig. 2a, 24 h and Fig. 2b). This suggested that ClpYQ might have a role in biofilm formation in *B. subtilis*, similar to what was seen in *B. cereus* [17]. The mutant also showed a mild phenotype in colony biofilm formation, being modestly more robust than the wild-type strain (Fig. 2c). In colony biofilm formation, the difference in the timing of biofilm induction between the wild-type and the mutant was less obvious (data not shown).

To further test whether the observed biofilm phenotype in the *clpYQ* mutant was due to alteration in the expression of biofilm matrix genes, we compared the activities of the *epsA-O* operon, which encodes proteins involved in biosynthesis of exopolysaccharides of the biofilm matrix in *B. subtilis* [22]. The wild-type strain and the *clpYQ* deletion mutant bearing a transcriptional fusion between the promoter of *epsA* and the *lacZ* gene (P_{epsA}–*lacZ*) were constructed. The strains were cultured in LBGM and the activities of the P_{epsA}–*lacZ* reporter were assayed for cells grown to different stages (OD_{600}=1, 2 and 3). We observed early induction of the *epsA* operon in the *clpYQ* deletion mutant (Fig. 3a). This suggested that ClpYQ plays a role in biofilm formation by regulating the transcription of the biofilm genes. Lastly, we also complemented the Δ*clpYQ* deletion mutation by providing an IPTG-inducible copy of the wild-type *clpYQ* genes at the ectopic *amyE* locus on the chromosome. The complementation strain was also tested

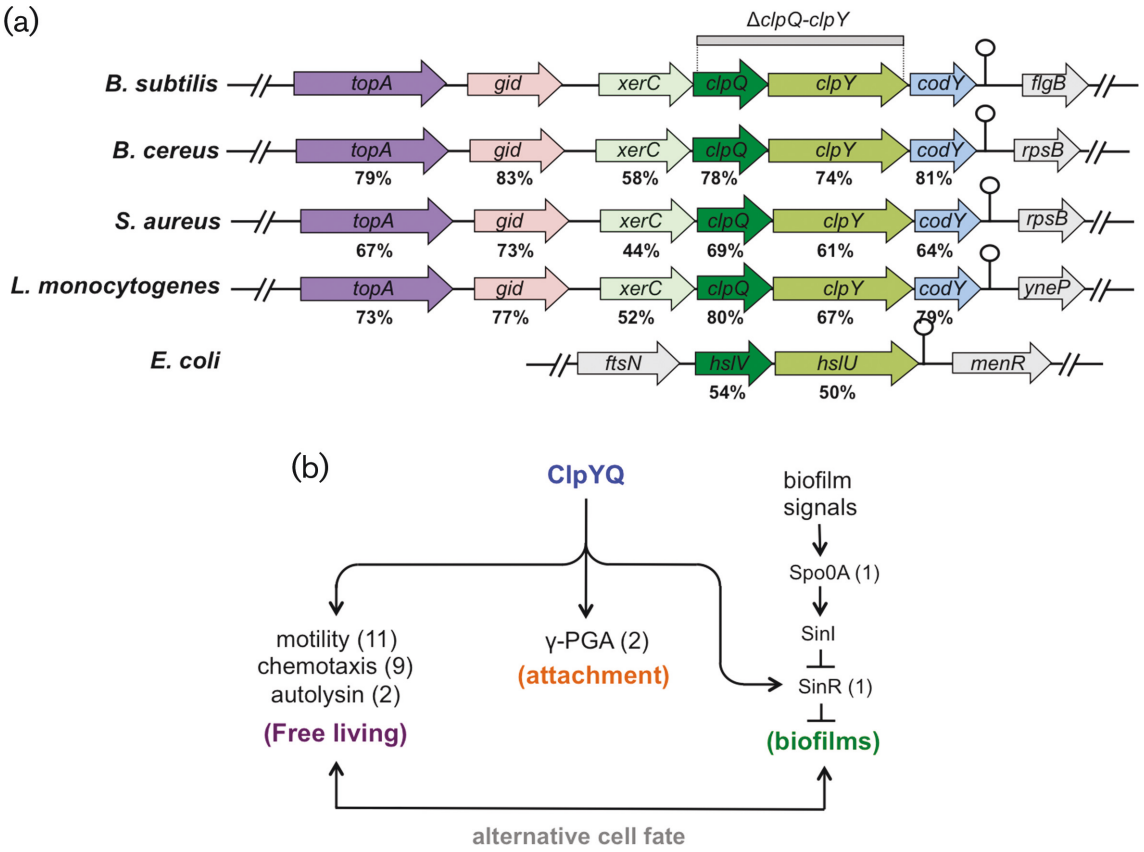


Fig. 1. (a) Schematic drawing of the *xerC-clpQ-clpY-codY* operons and flanking regions on the chromosomes of five different bacteria. The amino acid sequence identity between the encoded proteins in *B. subtilis* and the homologous proteins in other bacteria is noted underneath the genes. *topA* encodes a DNA topoisomerase I and *gid* encodes a putative glucose-inhibited cell division protein. The product of *xerC* resembles a DNA recombinase. The transposon insertion site in the *clpY* gene on the *B. cereus* AR156 chromosome is indicated by a triangle. The regions within the *clpYQ* coding sequences that were removed during construction of the non-polar deletion are highlighted. (b) A proposed model for how the ATP-dependent protease ClpYQ may be involved in controlling the decision-making process during the switch between the free living state and formation of multicellular communities in *B. subtilis*. The numbers in parentheses represent the number of proteins whose accumulation was significantly decreased in the *clpYQ* mutant based on global proteomic analysis (Table S1).

for biofilm formation. Upon the addition of IPTG (10 μM), the complementation strain formed pellicle biofilms that were similar to those of the wild-type, but were clearly produced more slowly and were less robust than those of the *clpYQ* deletion mutant after 24 h of incubation (Fig. 2a, 24 h) After 48 h of incubation, the wild-type strain, the deletion mutant and the complementation strain all formed similarly robust pellicle biofilms (Fig. 2a, 48 h), suggesting that ClpYQ likely played a role in regulating the timing of biofilm induction.

ClpQ, but not ClpY, is primarily responsible for biofilm regulation

The ClpYQ protease consists of the ATPase substrate-binding subunit (ClpY) and the catalytic subunit (ClpQ) [15]. To test which subunit (or both) of the protease is more important for biofilm regulation in *B. subtilis*, we constructed individual non-polar in-frame deletion mutants for *clpY* and *clpQ*, respectively. We then tested the biofilm phenotype of the two single-deletion mutants. Surprisingly, only the Δ*clpQ* deletion mutant showed a robust phenotype in both colony and pellicle biofilm formation, similar to what was seen in the Δ*clpYQ* double mutant, while the Δ*clpY* mutant behaved almost identically to the wild-type strain (Fig. 2d). This result suggested that only the catalytic subunit ClpQ is involved in regulating biofilm formation in *B. subtilis*, while the role of the ATPase substrate-binding subunit ClpY in biofilm formation is dispensable. Distinct phenotypes of the individual subunit mutants in the Clp proteases are not uncommon, not least because the catalytic subunit ClpP is shared among several ATPase substrate-binding subunits in both *E. coli* and *B. subtilis* [1, 2]. In the case of ClpYQ in *B. subtilis*, one possible explanation for this somewhat surprising result is the fact that ClpQ may be able to work with substrate-binding subunits from other Clp proteases, such as ClpX and ClpC, in proteolysis.

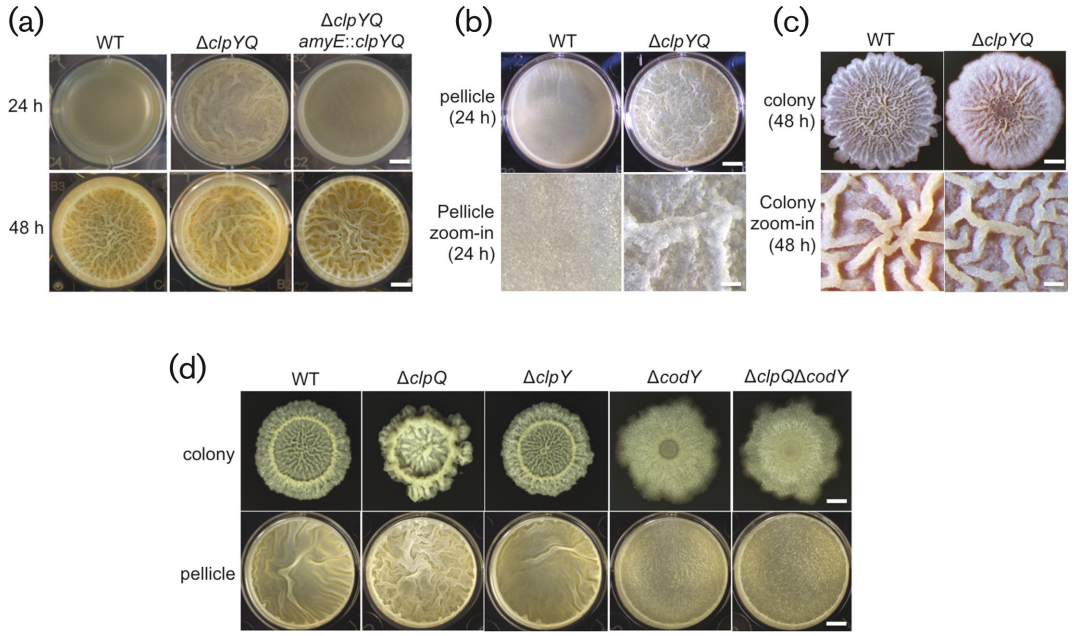


Fig. 2. Alteration in biofilm formation in various *clpYQ* mutants of *B. subtilis*. (a) Pellicle biofilms formed by the wild-type (WT) strain (3610), the *clpYQ* deletion mutant (FY170) and the complementation strain of Δ*clpYQ* (FY171). Cells were inoculated in LBGM broth in 12-well polyvinyl plates (VWR) and incubated at 30 °C. Pictures were taken after 24 or 48 h of inoculation. The scale bars represent 5 mm in length. (b) Morphology of pellicle biofilms formed in LBGM at 24 h by the wild-type (3610) and the *clpYQ* deletion mutant (FY170), with features highlighted in zoom-in images (lower panels). Scale bars in the upper panels, 5 mm; scale bars in the lower panels, 200 µm. (c) Morphology of colony biofilms formed in LBGM at 48 h by the wild-type (3610) and the *clpYQ* deletion mutant (FY170), with features highlighted in zoom-in images (lower panels). Scale bars in the upper panels, 2 mm; scale bars in the lower panels, 100 µm. (d) Colony and pellicle biofilms formed by the wild-type strain (3610), the Δ*clpQ* deletion mutant (YY145), the *clpY* deletion mutant (YY146), the Δ*codY* insertional deletion mutant (FY173) and the double mutant of Δ*clpQ*Δ*codY* (YY265). Cells were spotted on LBGM agar plates or inoculated in LBGM broth in 12-well polyvinyl plates (VWR) and incubated at 30 °C. Pictures were taken after 48 h of inoculation. Scale bars in the upper panel, 2 mm; scale bars in the lower panel, 5 mm.

codY is the last gene in the operon, implying a possible functional relatedness between CodY and ClpYQ (Fig. 1a). We thus constructed an insertional deletion mutant of *codY*. Interestingly, the Δ*codY* mutant demonstrated a weaker biofilm phenotype; both the colony and pellicle biofilms of the mutant lacked robust structural features (Fig. 2d). This was in contrast to the case of Δ*clpQ* (Fig. 2d). More importantly, we found that Δ*codY* was epistatic to Δ*clpQ*, since the Δ*codY*Δ*clpQ* double mutant showed a similar biofilm phenotype to that of the Δ*codY* single mutant (Fig. 2d). Our genetic evidence seems to suggest a possibility that *codY* lies downstream of *clpQ* in the same pathway that is involved in biofilm regulation. Alternatively, the above result can be simply explained as Δ*codY* having a stronger influence on the biofilm phenotype than Δ*clpQ*.

The *clpYQ* genes are important for swarming motility in *B. subtilis*

We also had evidence that the *clpYQ* genes may be involved in swarming motility (Fig. 3c), another multicellular behaviour in *B. subtilis*. We performed swarming assays for the wild-type strain and various deletion mutants on LB plates solidified with 0.75 % agar. Interestingly, the Δ*clpYQ* mutant exhibited the most severe swarming defect (Fig. 3c). The defect was rescued to a great extent in the complementation strain (Fig. 3c). For the single-deletion mutants, Δ*clpQ* had a slight but consistent defect in swarming motility, while no defect was observed in the Δ*clpY* mutant (Fig. 3c). On the other hand, the Δ*codY* mutant also showed a modest defect in swarming motility (Fig. 3c). Based on these results, we conclude that ClpYQ is also important for swarming motility in *B. subtilis*. Lastly, we briefly tested the potential impact of ClpYQ on swimming motility. To do so, we measured the motility of the wild-type and the single and double mutants of *clpYQ* on the surface of LB plates semi-solidified with 0.4 % agar. We found that, very similarly to the results of the swarming motility assay, the *clpYQ* double mutant, but not the single mutants, demonstrated a severe defect in swimming motility (Fig. S1, available in the online version of this article). The regulation of swarming and swimming motility are likely contributed to by both ClpY and ClpQ, since the double mutant showed a much stronger defect than the single mutants (Figs 3c, S1), whereas we previously showed that for the regulation of biofilm development, ClpQ, but not ClpY, played a primary role (Fig. 2d). We do not know yet why there is such a disparity between the regulation of swarming motility and that of biofilm development by ClpYQ.

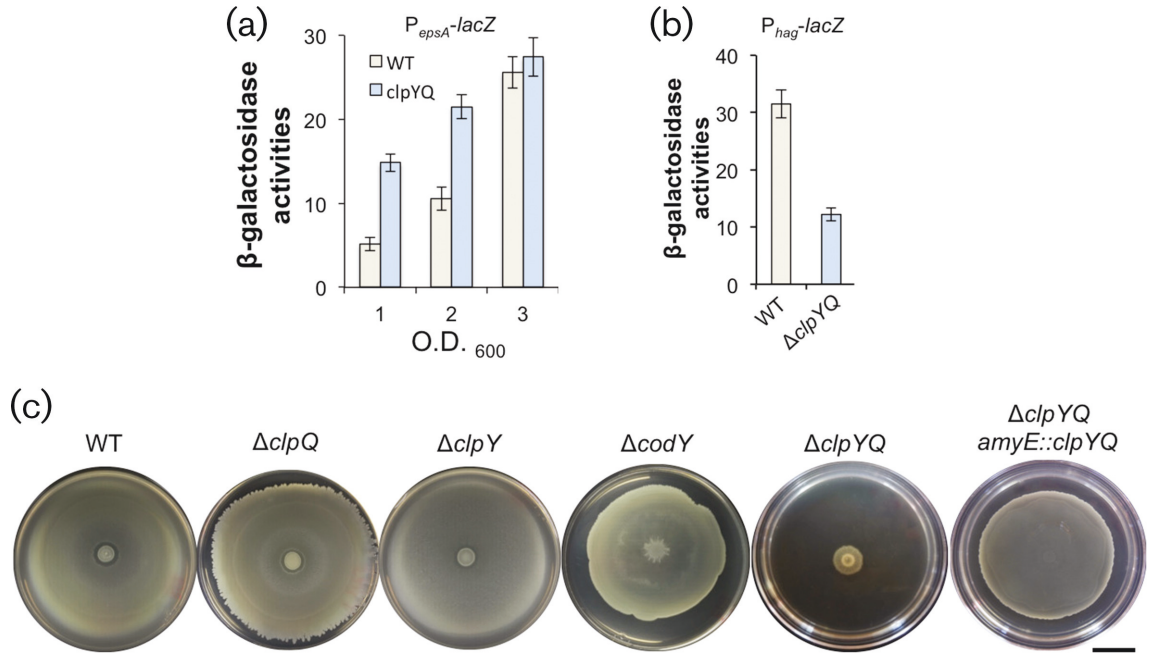


Fig. 3. The Δ*clpYQ* deletion impacted on the swarming motility in *B. subtilis*. (a) β-galactosidase activities of the wild-type (WT) strain (YC1000) and the *clpYQ* deletion mutant (YY64) harbouring the P_{epsA}–*lacZ* reporter fusion at the chromosomal *amyE* locus. Cells were grown in LBGM to different cell densities (OD_{600}=1, 2 and 3, respectively), harvested and assayed for β-galactosidase activities. The error bars represent standard deviations. Assays were performed in triplicate. (b) β-galactosidase activities of the wild-type strain (FY184) and the *clpYQ* deletion mutant (FY185) harbouring the P_{hag}–*lacZ* reporter fusion at the chromosomal *amyE* locus. Cells were grown in LB to OD_{600}=0.5, harvested and assayed for β-galactosidase activities. The error bars represent standard deviations. Assays were performed in triplicate. (c) Swarming motility assays for the wild-type strain (3610), the *clpQ* deletion mutant (YY145), the *clpY* deletion mutant (YY146), the Δ*codY* insertional deletion mutant (FY173), the *clpYQ* double deletion mutant (FY170) and the complementation strain of Δ*clpYQ* (FY171) on LB plates solidified with 0.75 % agar. The plates were incubated at 37 °C for about 8 h. The plates were air-dried for 20 min and then left overnight at room temperature before the images were taken. Scale bar, 2 cm.

We next asked whether the motility defect in the Δ*clpYQ* double mutant was due to alteration in the expression of genes involved in swarming motility. We similarly applied a transcriptional reporter (P_{hag}–*lacZ*) that measures the activity of the gene encoding flagellin, the protein subunit of flagella [43]. The reporter fusion was introduced into the chromosomal *amyE* locus in the wild-type and the *clpYQ* deletion mutant, respectively. Assays of the β-galactosidase activities were conducted in shaking cultures. As shown in Fig. 3(b), expression of the P_{hag}–*lacZ* reporter was significantly reduced in the *clpYQ* deletion mutant. This suggested that ClpYQ down-regulated the expression of the *hag* gene in *B. subtilis*. In the quantitative proteomic analysis (which we discuss later in this study), we identified 11 motility proteins (including Hag) whose amounts were significantly reduced in the *clpYQ* mutant, which further supported the role of ClpYQ in swarming and swimming motility.

Production of γ-PGA is likely reduced in the Δ*clpYQ* mutant of *B. subtilis*

Wild strains of *B. subtilis* are capable of producing γ-poly-DL-glutamic acids (γ-PGA) [44, 45], so that the colonies of *B. subtilis* on the agar surface look somewhat mucoid during early stages of growth (Fig. 4a) (although, after prolonged incubation at 37 °C, colonies of 3610 growing on the LB plate gradually lose mucoidy). The biosynthesis of γ-PGA relies on the protein products of the *pgsBCAD* operon [46]. The exact biological function of γ-PGA in the cell physiology of *B. subtilis* is not fully understood. In this study, we observed that the *clpYQ* deletion mutant of *B. subtilis* lost colony mucoidy during early growth when the cells were streaked out on LB agar plates and grown at 37 °C for about 12 h (Fig. 4a). The phenotype was clearly different from that of the wild-type cells on the same plate, but similar to that of the Δ*pgsBCAD* mutant (Fig. 4a). The complementation strain of Δ*clpYQ* looked very similar to the wild-type strain in colony mucoidy on the same plate (Fig. 4a).

We suspected that the lack of mucoidy observed for the *clpYQ* deletion mutant was due to reduced production of γ-PGA. We further suspected that expression of the *pgsBCAD* operon was altered in the Δ*clpYQ* mutant. To test this, a transcriptional fusion with the regulatory region of the *pgsBCAD* operon fused to *lacZ* (P_{pgsB}–*lacZ* [47]) was introduced into the wild-type and the *clpYQ* mutant. Cells were grown in LB shaking culture to OD_{600}=1.0 and assayed for β-galactosidase activities. Our results showed that the

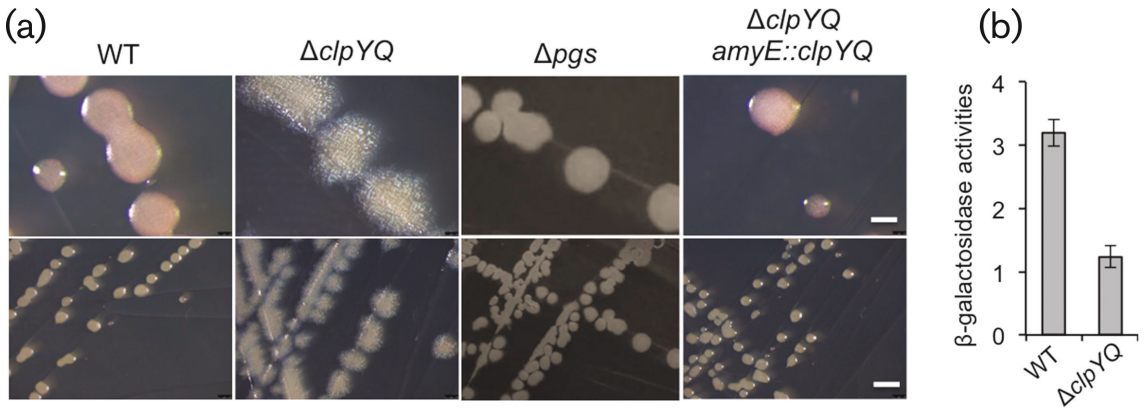


Fig. 4. The Δ*clpYQ* deletion impacted on colony mucoidy and expression of γ-PGA biosynthesis genes. (a) Colony morphology of the wild-type (WT) strain (3610), the *clpYQ* deletion mutant (FY170), the *pgs* operon deletion mutant and the complementation strain of Δ*clpYQ* (FY171) on LB plates after 10 h of inoculation at 37 °C. Upper panels show zoom-in images for the corresponding panels below. Scale bar in the upper panel, 20 µm; scale bar in the lower panel, 4 µm. (b) β-galactosidase activities of the wild-type strain (YC1275) and the *clpYQ* deletion mutant (YC1276) harbouring the P_{pgsB}–*lacZ* reporter fusion. In both (a) and (b), assays were performed on harvested cells grown in LB broth to the late log phase (OD_{600}=1).

activity of the reporter fusion was significantly down-regulated in the *clpYQ* deletion mutant (Fig. 4b). This was further confirmed in the iTRAQ analysis (which we discuss later in this study), which showed that the levels of the two enzymes directly involved in γ-PGA biosynthesis were significantly reduced (Fig. 5c). In conclusion, our results suggest that ClpYQ also plays a positive role in regulating γ-PGA production in *B. subtilis*.

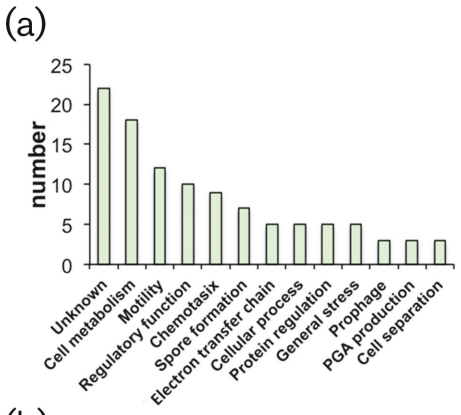
ClpYQ is not important in heat-stress response in either *B. subtilis* or *B. cereus*

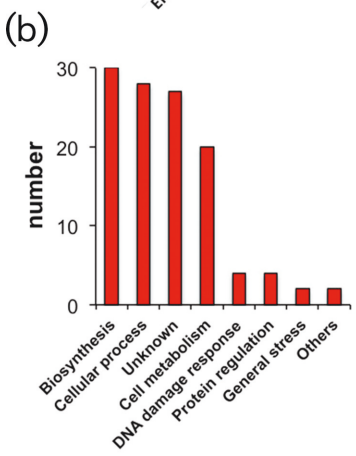
So far, our results suggest that ClpYQ is an ATP-dependent protease involved in multicellular development in *B. subtilis*. In *E. coli* however, there is strong evidence that *clpYQ* (*hslVU*) is induced by heat stress, and one of the primary functions for ClpYQ is to degrade non-native (misfolded) proteins generated during heat stress [3]. In Gram-positive bacteria, it is unclear whether ClpYQ is an ATP-dependent protease in response to heat stress, in part because very few studies have been published on this subject. To test whether ClpYQ plays an important role in the heat-stress response in *B. subtilis*, we first compared the colony-forming ability of the wild-type strain and the *clpYQ* deletion mutant of *B. subtilis* incubated at various temperatures (30, 37, 45 and 50 °C). Our results showed that after 16 h of incubation, both the wild-type and the *clpYQ* mutant grew well under all tested temperatures and both formed colonies of comparable size (Fig. S2a). The only difference was a moderate decrease in the size of the colonies formed by the *clpYQ* mutant when they were grown at 50 °C, as compared to those of the wild-type strain under the same conditions (Figs S2a and 50 °C). We also tested the potential importance of ClpYQ in heat stress in the related bacterium *B. cereus* by similarly comparing the colony-forming ability of the wild-type strain and the *clpYQ* deletion mutant that we constructed in our previous study [17] on LB agar plates under the same temperatures (30 37 45 and 50 °C). The results were very similar to those for *B. subtilis* (Fig. S2b). To further investigate the possible role of ClpYQ in heat-stress response in *B. subtilis*, we compared the growth profile of the wild-type strain and those of the *clpYQ* deletion mutant and the single-deletion mutants at both 37 and 50 °C in shaking conditions (Fig. S3a, b). The results showed that at 37 °C no significant difference in growth profile was seen among the tested strains, while at 50 °C, after about 7 h of shaking incubation, the *B. subtilis* cultures experienced a decrease in cell density, possibly due to cell lysis after prolonged heat stress. Interestingly, the *clpYQ* double mutant managed to perform slightly better in maintaining cultural density (Fig. S3b). Next, we also measured the survival rate of the wild-type and the single and double mutants of *clpYQ* after heat shock treatment at 50 °C for either 30 or 120 min. Our results showed no decrease (if not a slight increase) in the survival rate after heat-shock treatment in both the single and double mutants when compared to that of the wild-type (Fig. S3c).

Lastly, we briefly compared the expression of the *clpYQ* genes in the wild-type *B. subtilis* and *B. cereus* cells grown under two different temperatures (37 vs 45 °C) by performing real-time quantitative PCR. No statistically significant difference was seen (data not shown), indicating that the *clpYQ* genes were not induced by heat shock in either *B. subtilis* or *B. cereus*. These results indicate that ClpYQ is not essential in the heat-stress response in either *B. subtilis* or *B. cereus*. This is quite different from the roles of their counterparts in Gram-negative bacteria [1].

Search for candidate protein targets of ClpYQ in *B. subtilis* using iTRAQ

The *clpYQ* deletion mutants showed multiple phenotypes, as we demonstrated above. We were thus very interested in





(c)

	ratio(clpYQ/WT)	Protein	Desription	Coverage	COG E-Value
motility	0.362	FliG	Flagellar motor switch protein FliG	24.6	0
	0.372	FliF	Flagellar M-ring protein	21.1	0
	0.401	FlgE	Flagellar basal-body rod protein FlgG	26.9	3.00E-147
	0.493	MotA	Motility protein MotA	14.1	2.00E-153
	0.496	MotB	Motility protein MotB	38.7	4.00E-150
	0.5	Hag	Flagellin	44.7	7.00E-173
	0.525	FliH	flagellar assembly protein FliH	26	1.00E-116
	0.554	BS_fliL	Flagellar protein FliL	29.3	4.00E-72
	0.589	FliM	Flagellar motor switch protein FliM	22.6	0
	0.622	FliY	Flagellar motor switch phosphatase FliY	36.5	5.00E-150
	0.644	YviF	Flagellar assembly factor FliW	21.7	2.00E-79
chemotaxis	0.339	McpB	Methyl-accepting chemotaxis protein McpB	29.2	0
	0.365	TlpA	Methyl-accepting chemotaxis protein TlpA	12.4	0
	0.375	CheV	Chemotaxis protein CheV	48.5	2.00E-90
	0.384	McpA	Methyl-accepting chemotaxis protein McpA	18	0
	0.468	CheW	Chemotaxis protein CheW	51.9	2.00E-85
	0.614	CheA	Chemotaxis protein CheA	21.7	0
	0.625	TlpC	Methyl-accepting chemotaxis protein TlpC	6.8	0
	0.643	CheC	CheY-P phosphatase CheC	16.3	7.00E-117
	0.647	CheB	Chemotaxis response regulator protein	20.7	0
cell separation	0.606	LytB	Amidase enhancer	26.5	0
	0.482	YhdD	hypothetical protein, similar to major autolysin	12.9	8.00E-142
biofilm sporulation	0.681	Spo0A	Stage 0 sporulation protein A, Spo0A	22.5	8.00E-66
	0.754	SigF	RNA polymerase sigma-F factor	12.5	2.00E-146
	0.761	SpoIIAA	Anti-sigma F factor antagonist	21.4	7.00E-63
	0.767	SinR	HTH-type transcriptional regulator SinR	55	1.00E-59
PGA biosynthesis	0.227	CapC	PGA biosynthesis protein CapC	10.7	0
	0.431	YwtB	PGA biosynthesis protein CapA	18.4	0
insertase	0.738	MisCA	Membrane protein insertase MisCA	8.4	4.00E-151
oxidative respiration	0.643	CydA	Cytochrome bd ubiquinol oxidase subunit 1	2.6	0
	0.722	CtaD	cytochrome c oxidase subunit I, aa3 type	3.7	0
	0.724	YocJ	FMN-dependent NADH-azoreductase 1	37.5	4.00E-120
	0.724	CtaC	Cytochrome c oxidase subunit 2	16.9	6.00E-147
	0.796	QcrA	Menaquinol-cytochrome c reductase	52.7	2.00E-97
	0.796	HemH	Ferrochelatase	36.5	0
	0.798	ResB	Cytochrome c biogenesis protein ResB	16.2	0

Fig. 5. A global search for candidate protein targets of ClpYQ in *B. subtilis* by iTRAQ. (a, b) Distribution of 229 out of 2025 proteins based on differential accumulation in the wild-type (WT) and the *clpYQ* mutant. A protein whose abundance differs by a factor of 1.2-fold with a *P*-value of less than 0.05 is considered to be differentially accumulated in the two different strains. A total of 107 proteins show down-regulation [green bars in (a)], and a total of 122 proteins show up-regulation [red bars in (b)] in the *clpYQ* mutant when compared to the wild-type strain. Different functional categories are also assigned to those candidate proteins based on known or predicted functions of the proteins. (c) A partial list of proteins with reduced accumulation in the *clpYQ* mutant and different categories of those proteins in motility, chemotaxis, cell separation, sporulation and biofilm formation, oxidative respiration, etc. The ratio of protein accumulation in the wild-type vs the *clpYQ* mutant is shown; the numbers in the coverage column refer to percentages (%).

characterizing putative protein target(s) whose activities are regulated by ClpYQ in *B. subtilis*. We took both a global approach by performing isobaric tags for relative and absolute quantification (iTRAQ), a mass spectrometry-based technique allowing quantitative proteomic analysis [48], and a targeted approach by applying Western immunoblotting to selected proteins. For iTRAQ, total proteins were prepared from the wild-type cells and the *clpYQ* deletion mutant of *B. subtilis* grown in the biofilm medium LBGM to OD_{600}=1. Quantitative proteomic analysis and bioinformatics analyses were performed as described in the Methods section. A total of 10 630 unique peptides and 2025 distinct protein species were detected in the iTRAQ analysis (Fig. S4a). This is several-fold higher than the number of proteins that could be identified by traditional two-dimensional gel electrophoresis [49]. The coverage of the proteins ranged from ~5 % to~90 % and averaged around 20 % (Fig. S4b). A protein whose abundance differs by a factor of 1.2-fold between the wild-type and the mutant (up- or down-regulation) with a *P*-value of less than 0.05 was considered to be a valid candidate for differential accumulation in the bioinformatics analysis. Thus, 229 proteins met the criteria and were considered to be differentially accumulated between the wild-type and the *clpYQ* mutant (represented by red and green triangles in Figs 5a, b and S4c). Among the 229 candidate proteins, 107 proteins showed decreased abundance in the *clpYQ* mutant (Figs 5a and S4d, Table S1), while 122 proteins showed increased abundance in the mutant (Figs 5b and S4d, Table S2). These candidate proteins fall into different functional categories for cell development, metabolism, stress response, etc. (Fig. 5a, b).

To highlight some of the notable results, dozens of proteins involved in motility and chemotaxis were found to have decreased abundance in the *clpYQ* mutant (Fig. 5c and Table S1), providing molecular evidence for the impaired motility observed in the *B. subtilis clpYQ* mutant (Figs 3c

and S1). The proteins involved in γ-PGA production, PgsC (also named CapC) and PgsA (also named CapA or YwtB), showed approximately 4.4- and 2.3-fold decreases in abundance, respectively, in the *clpYQ* mutant (Fig. 5c and Table S1). This result was also consistent with the reduced expression of the *pgs* operon in the *clpYQ* mutant shown earlier (Fig. 4b), and may explain the lack of colony mucoidy in the *clpYQ* mutant (Fig. 4a). Lastly, several proteins involved in the regulation of sporulation and biofilm formation were also in the list of those whose abundance decreased in the *clpYQ* mutant, including Spo0A, SigF, SpoIIAA and SinR (Fig. 5c), although we did not observe a significant change in the sporulation efficiency in the heat–kill experiment (data not shown). The biofilm master regulator SinR showed a decrease in protein abundance of about 25 % (Fig. 5c).

Finally, one striking feature we noticed in the list of 107 proteins with decreased abundance in the *clpYQ* mutant is that many of those proteins are (verified or putative) membrane-associated. The reason for this is currently not clear to us. We did observe a moderate decrease in the abundance of a membrane protein insertase (MisCA, Fig. 5c). With respect to the proteins whose abundance increased in the *clpYQ* mutant, many are metabolic proteins or enzymes, as well as proteins with hypothetical functions. A few ofare involved in DNA damage and general stress responses (Fig. 5b).

CodY abundance is not influenced by Δ*clpYQ* in *B. subtilis*

In addition to iTRAQ, we also applied a targeted approach. Since in other Gram-positive bacteria such as *S. aureus* and *L. monocytogenes, clpYQ* genes are in the operon with *codY*, which encodes a well-studied global regulator (Fig. 1a) [42, 50], it was tempting for us to speculate that CodY might be a potential target of ClpYQ. In addition, our genetic evidence from the epistasis assay (Fig. 2d) suggested at least a possibility that *clpYQ* could function upstream of *codY* in the shared pathway. Nevertheless, CodY was not in the list of proteins that showed differential accumulation in the *clpYQ* mutant in the iTRAQ analysis (Tables S1 and S2).

To test our hypothesis, we performed immunoblot assays with the total protein lysates prepared from the wild-type strain, the Δ*clpYQ* deletion mutant and the complementation strain of Δ*clpYQ* by using antibodies against *B. subtilis* CodY (a gift from Linc Sonenshein, Tufts University). The *codY* mutant of *B. subtilis* was used as a control. However, we did not observe a significant difference in the abundance of CodY among the three strains, suggesting that under the tested conditions (cells were grown to the early stationary phase in LB), CodY abundance was not significantly influenced by Δ*clpYQ* (Fig. 6a). In a previous study, Slack *et al.* also tested whether CodY was regulated by ClpYQ by using genetic approaches. No change in CodY activities was found in the *clpYQ* deletion mutant in that study either [39]. It is possible that varied accumulation of CodY in the *clpYQ* mutant is growth-stage dependent, since CodY primarily functions during stationary-phase growth. To test this, we harvested cells of the wild-type and the mutant grown to different stages (from OD_{600} of 0.5 to 4.5) and repeated the immunoblotting assay. Again, no clear difference in CodY abundance was observed between the wild-type and the mutant (Figs 6c and S5). In conclusion, our results do not support the idea that CodY protein abundance is regulated by ClpYQ, even though the corresponding genes are located in the same operon.

SinR abundance is moderately reduced in the Δ*clpYQ* mutant

SinR is a biofilm master regulator in *B. subtilis*. SinR protein abundance showed a moderate decrease (~25 %) in the *clpYQ* mutant in the iTRAQ experiment (Fig. 5c). Such a moderate change in the SinR protein level may still be significant enough to alter the biofilm regulation, as we showed in previous studies that expression of the biofilm genes and therefore biofilm induction were extremely sensitive to changes in the SinR protein level [51, 52]. We hoped to confirm the moderate decrease in SinR levels shown in the iTRAQ by Western immunoblotting. The total protein lysates were prepared from different strains and SinR proteins were detected by using SinR antibodies. Although no substantial difference was seen in the SinR levels among the different samples, we did notice a moderate decrease in the level of the SinR proteins in the *clpYQ* deletion mutant, and the moderate decrease disappeared in the complementation strain (Fig. 6b). To further confirm this, we semi-purified the SinR antibody (see the Methods section) and repeated the Western immunoblot with the serial-diluted protein lysates. Our results again showed that there was a moderate decrease in the SinR levels in the *clpYQ* mutant when compared to the wild-type (Fig. 6d). We estimated that the decrease was about 40 % by quantifying the pixel density using the imaging software MicrobeJ [53]. A similar decrease in the SinR levels was also seen in the *clpQ* single-deletion mutant, but not in the *clpY* deletion mutant (data not shown), which was consistent with the observed enhanced biofilm robustness of the *clpQ* single-deletion mutant (Fig. 2d).

SinR-mediated repression on the biofilm matrix genes is ultrasensitive to the SinR protein levels. A modest change in the protein level of SinR may cause a much bigger shift in the matrix gene expression and result in a strong biofilm phenotype [51, 52]. In a previous study [52], we showed that in a *sinR* synonymous mutant ($sinR^S$ in Fig. S6), synonymous substitutions of the serine codon [at the amino acid positions 16, 18, 33 (TCA >TCG) in *sinR*] caused a decrease of about 50 % in the SinR protein levels as measured by Western immunoblotting. This is likely due to ribosome pausing on specific serine codons during *sinR* translation, as revealed by ribosome profiling [52]. Interestingly, this *sinR* synonymous mutant had robust colony and pellicle biofilm phenotypes, similar to what was seen in the *clpQ* deletion mutant (Fig. S6). Therefore, we argued that the observed moderate decrease in the protein level of SinR in the *clpYQ* deletion mutant could be significant enough to explain the early and robust biofilm phenotype of the mutant.

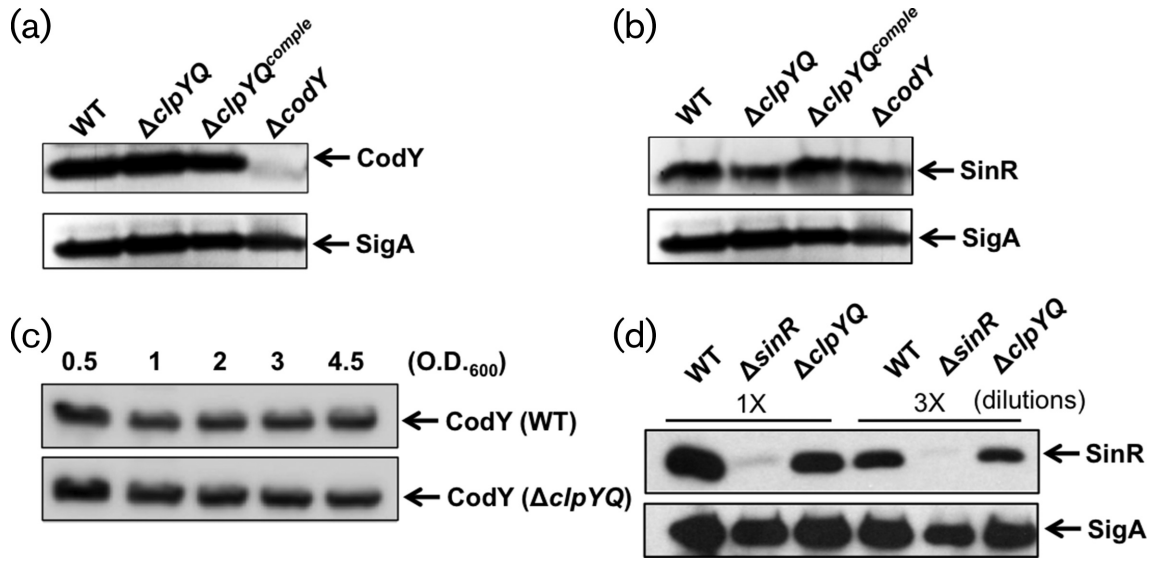


Fig. 6. A moderate decrease in SinR protein abundance in the *clpYQ* mutant of *B. subtilis*. (a) Western immunoblot to compare the protein abundance of CodY (a) and SinR (b) in the total protein lysates prepared from the wild-type (WT) strain (3160), the *clpYQ* deletion mutant (FY170), the complementation strain of Δ*clpYQ* (FY171) and the *codY* insertional deletion mutant (FY173). SigA was used as a control. (c) Western immunoblot to compare the protein abundance of CodY in the total protein lysates prepared from the wild-type strain and the *clpYQ* deletion mutant grown to different growth stages (from OD_{600}=0.5 to 4.5). In this assay, the amount of total proteins was used to normalize the sample load for each lane. (d) Western immunoblot to assay for SinR abundance in the protein lysates prepared from wild-type and the *clpYQ* deletion mutant. The loaded total lysates were either from undiluted samples or diluted threefold. In this assay, the anti-SinR antibodies were purified prior to use by pre-absorption with the total protein lysate from Δ*sinR*. The total protein lysate from Δ*sinR* was also included as a control.

DISCUSSION

ClpYQ is one of several major ATP-dependent proteases that are highly conserved in both Gram-positive and Gram-negative bacteria [1]. However, the biological function of ClpYQ is poorly understood in Gram-positive bacteria. In this study, we showed that the *clpYQ* deletion mutants of *B. subtilis* had multiple motility and multicellularity phenotypes. First, the mutant formed early and robust biofilms (Fig. 2). Second, the deletion mutant had a defect in swarming motility (Fig. 3c). The mutant also lost colony mucoidy on the agar plate (Fig. 4a), suggesting a defect in the production of secreted polymeric γ-PGA. Although we did not compare the amounts of γ-PGA produced by the wild-type and the *clpYQ* deletion mutant directly, the expression of the biosynthesis genes for γ-PGA was down-regulated significantly and the abundance of the biosynthetic proteins for γ-PGA was greatly reduced in the *clpYQ* deletion mutant (Figs 4b, 5c). Combined with our previous phenotypic characterization of the *clpYQ* deletion mutant of *B. cereus*, we propose that in *Bacillus* species, ClpYQ may function primarily in bacterial multicellularity.

Our results from the global proteomic analysis further showed that the accumulation of a number of proteins whose genes are under the positive control of the sigma factor D (SigD) is reduced in the *clpYQ* deletion mutant of *B. subtilis* (Fig. 5c). This suggests that the observed swarming and swimming defects are possibly due to reduced activities of SigD, which controls the expression of many motility-related genes [27]. Unfortunately, the SigD protein itself did not show up in the list of proteins with altered abundance in the *clpYQ* mutant. One possibility could be that the SigD levels changed slightly in the *clpYQ* deletion mutant, but the change was below the cutoff (20 % difference) that we used in the analysis. The second possibility we could think of is that *sigD* activities were shown to be bistable in the *B. subtilis* population [27], meaning that changes in SigD activities or levels might only occur in a subpopulation. Our proteomic approach was unable to address such population heterogeneity and characterize the differences within the subpopulation.

The regulation of PGA production and swarming motility is shown to overlap in *B. subtilis* [45, 54]. For example, the DegS–DegU two-component system positively regulates both PGA production (by activating the *pgsBCAD* operon directly) and motility (by activating the *fla/che* operon, whose protein products are involved in motility and chemotaxis) [45, 54]. It is possible that ClpYQ directly or indirectly regulates such a shared protein target that is involved in the regulation of both PGA production and swarming motility.

Early and robust biofilm formation is observed in the *clpYQ* mutant in both *B. subtilis* and *B. cereus*. Our results from both the immunoblotting assay and iTRAQ showed that the protein level of the biofilm master repressor SinR reduced to some extent (by about 40 %) in the *clpYQ* mutant, a

difference that is possibly sufficient to result in a significant biofilm phenotype due to the ultrasensitivity of SinR-mediated repression [51, 52]. Indeed, there was evidence that a previously studied *sinR* synonymous mutant (*sinR*^{S}, Fig. S6), which had a decrease of about 50 % in the SinR protein level as compared to the wild-type strain, had an early and robust biofilm phenotype that was similar to what was seen in Δ*clpYQ* (Fig. S6). How ClpYQ might regulate the protein abundance of SinR is not clear, but it likely acts indirectly, since we saw a decrease in the protein levels of SinR in the protease mutant, as opposed to the increase that one would expect for a direct target of a protease. Lastly, we briefly looked at sporulation by the *clpYQ* mutant in both *B. subtilis* and *B. cereus*. No significant difference was observed when compared to that of the wild-type strains (data not shown).

It is worth emphasizing that our investigations suggest that ClpYQ is probably not a first-line heat-shock-responsive protease in either *B. subtilis* or *B. cereus*, in contrast to what was shown in *E. coli* and other Gram-negative bacteria [1, 18]. One previous study in another Gram-positive bacterium, *S. aureus*, showed that ClpYQ is dispensable under modestly elevated temperatures (up to 45 °C). It was only when the temperatures were shifted to above 45 °C that a difference in colony-forming ability started to appear between the wild-type and the *clpYQ* deletion mutant [16]. Unlike in *E. coli*, but similar to what we have seen, expression of the *clpYQ* genes was only moderately increased in response to heat stress in *S. aureus* [16]. Overall, the evidence from our work and previous studies suggests that in Gram-positive bacteria the primary role of ClpYQ may have shifted from coping with heat-stress response to cell developmental control, such as biofilm formation.

In summary, we propose that in *B. subtilis*, ClpYQ is an ATP-dependent protease that is primarily involved in bacterial multicellularity, and the decision-making during the switch between the free-living state and formation of multicellular communities in particular (Fig. 1b). Under planktonic growth, ClpYQ plays a positive role in the accumulation of the proteins involved in motility, chemotaxis and cell separation. ClpYQ also seems to positively regulate the production of γ-PGA, a secreted polymer that may function as an adhesin for surface attachment and host interaction. Lastly, ClpYQ acts as a checkpoint-like regulator for biofilm formation by maintaining an appropriate level of SinR for the repression of genes involved in making the biofilm matrix. Upon biofilm induction, altered levels or activities of ClpYQ result in a decrease in the abundance of the proteins involved in motility and chemotaxis, as well as a mild decrease in the biofilm master repressor SinR. Cells thus shut off motility and switch to becoming a matrix producer and forming multicellular communities.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Gottesman S, Wickner S, Maurizi MR. Protein quality control: triage by chaperones and proteases. *Genes Dev* 1997;11:815–823.
2. Sauer RT, Baker TA. AAA+ proteases: ATP-fueled machines of protein destruction. *Annu Rev Biochem* 2011;80:587–612.
3. Rohrwild M, Coux O, Huang HC, Moerschell RP, Yoo SJ *et al.* HslV-HslU: A novel ATP-dependent protease complex in *Escherichia coli* related to the eukaryotic proteasome. *Proc Natl Acad Sci USA* 1996;93:5808–5813.
4. Frees D, Savijoki K, Varmanen P, Ingmer H. Clp ATPases and ClpP proteolytic complexes regulate vital biological processes in low GC, Gram-positive bacteria. *Mol Microbiol* 2007;63:1285–1295.
5. Yepes A, Schneider J, Mielich B, Koch G, García-Betancur JC *et al.* The biofilm formation defect of a *Bacillus subtilis* flotillin-defective mutant involves the protease FtsH. *Mol Microbiol* 2012; 86:457–471.
6. Turgay K, Hahn J, Burghoorn J, Dubnau D. Competence in *Bacillus subtilis* is controlled by regulated proteolysis of a transcription factor. *Embo J* 1998;17:6730–6738.
7. Ruvolo MV, Mach KE, Burkholder WF. Proteolysis of the replication checkpoint protein Sda is necessary for the efficient initiation of sporulation after transient replication stress in *Bacillus subtilis*. *Mol Microbiol* 2006;60:1490–1508.
8. Mukherjee S, Bree AC, Liu J, Patrick JE, Chien P *et al.* Adaptor-mediated Lon proteolysis restricts *Bacillus subtilis* hyperflagellation. *Proc Natl Acad Sci USA* 2015;112:250–255.
9. Chai Y, Kolter R, Losick R. Reversal of an epigenetic switch governing cell chaining in *Bacillus subtilis* by protein instability. *Mol Microbiol* 2010;78:218–229.
10. Gerth U, Krüger E, Derré I, Msadek T, Hecker M. Stress induction of the *Bacillus subtilis* clpP gene encoding a homologue of the proteolytic component of the Clp protease and the involvement of ClpP and ClpX in stress tolerance. *Mol Microbiol* 1998;28:787–802.
11. Deuerling E, Mogk A, Richter C, Purucker M, Schumann W. The ftsH gene of *Bacillus subtilis* is involved in major cellular processes such as sporulation, stress adaptation and secretion. *Mol Microbiol* 1997;23:921–933.
12. Kirstein J, Strahl H, Molière N, Hamoen LW, Turgay K. Localization of general and regulatory proteolysis in *Bacillus subtilis* cells. *Mol Microbiol* 2008;70:682–694.
13. Kain J, He GG, Losick R. Polar localization and compartmentalization of ClpP proteases during growth and sporulation in *Bacillus subtilis*. *J Bacteriol* 2008;190:6749–6757.
14. Simmons LA, Grossman AD, Walker GC. Clp and Lon proteases occupy distinct subcellular positions in *Bacillus subtilis*. *J Bacteriol* 2008;190:6758–6768.
15. Kang MS, Lim BK, Seong IS, Seol JH, Tanahashi N *et al.* The ATP-dependent CodWX (HslVU) protease in *Bacillus subtilis* is an N-terminal serine protease. *Embo J* 2001;20:734–742.
16. Frees D, Thomsen LE, Ingmer H. *Staphylococcus aureus* ClpYQ plays a minor role in stress survival. *Arch Microbiol* 2005;183: 286–291.
17. Yan F, Yu Y, Gozzi K, Chen Y, Guo JH *et al.* Genome-wide investigation of biofilm formation in *Bacillus cereus*. *Appl Environ Microbiol* 2017;83:e00561-17.

18. **Bochtler M, Hartmann C, Song HK, Bourenkov GP, Bartunik HD *et al.*** The structures of HslU and the ATP-dependent protease HslU-HslV. *Nature* 2000;403:800–805.
19. **Hamon MA, Lazazzera BA.** The sporulation transcription factor Spo0A is required for biofilm development in *Bacillus subtilis*. *Mol Microbiol* 2001;42:1199–1209.
20. **Branda SS, González-Pastor JE, Ben-Yehuda S, Losick R, Kolter R.** Fruiting body formation by *Bacillus subtilis*. *Proc Natl Acad Sci USA* 2001;98:11621–11626.
21. **Stanley NR, Britton RA, Grossman AD, Lazazzera BA.** Identification of catabolite repression as a physiological regulator of biofilm formation by *Bacillus subtilis* by use of DNA microarrays. *J Bacteriol* 2003;185: 1951–1957.
22. **Vlamakis H, Chai Y, Beauregard P, Losick R, Kolter R.** Sticking together: building a biofilm the *Bacillus subtilis* way. *Nat Rev Microbiol* 2013;11:157–168.
23. **Aguilar C, Vlamakis H, Losick R, Kolter R.** Thinking about *Bacillus subtilis* as a multicellular organism. *Curr Opin Microbiol* 2007;10: 638–643.
24. **Shemesh M, Chai Y.** A combination of glycerol and manganese promotes biofilm formation in *Bacillus subtilis* via histidine kinase KinD signaling. *J Bacteriol* 2013;195:2747–2754.
25. **Gozzi K, Ching C, Paruthiyil S, Zhao Y, Godoy-Carter V *et al.*** *Bacillus subtilis*utilizes the DNA damage response to manage multicellular development. *NPJ Biofilms Microbiomes* 2017;3:8.
26. **Chai Y, Norman T, Kolter R, Losick R.** An epigenetic switch governing daughter cell separation in *Bacillus subtilis*. *Genes Dev* 2010;24:754–765.
27. **Kearns DB, Losick R.** Cell population heterogeneity during growth of *Bacillus subtilis*. *Genes Dev* 2005;19:3083–3094.
28. **Kobayashi K.** Gradual activation of the response regulator DegU controls serial expression of genes for flagellum formation and biofilm formation in *Bacillus subtilis*. *Mol Microbiol* 2007;66:395–409.
29. **Diehl A, Roske Y, Ball L, Chowdhury A, Hiller M *et al.*** Structural changes of TasA in biofilm formation of*Bacillus subtilis*. *Proc Natl Acad Sci USA* 2018;115:3237–3242.
30. **Erskine E, Morris R, Schor M, Earl C, Gillespie RMC *et al.*** Formation of functional non-amyloidogenic fibres by recombinant *Bacillus subtilis* TasA. *bioRxiv* 2017.
31. **Kearns DB, Chu F, Branda SS, Kolter R, Losick R.** A master regulator for biofilm formation by *Bacillus subtilis*. *Mol Microbiol* 2005; 55:739–749.
32. **Branda SS, Chu F, Kearns DB, Losick R, Kolter R.** A major protein component of the *Bacillus subtilis* biofilm matrix. *Mol Microbiol* 2006;59:1229–1238.
33. **Yu Y, Yan F, Chen Y, Jin C, Guo J-H *et al.*** Poly-γ-glutamic acids contribute to biofilm formation and plant root colonization in selected environmental isolates of *Bacillus subtilis*. *Frontiers in Microbiology* 1811;2016:7.
34. **Stanley NR, Lazazzera BA.** Defining the genetic differences between wild and domestic strains of *Bacillus subtilis* that affect poly-γ-dl-glutamic acid production and biofilm formation. *Mol Microbiol* 2005;57:1143–1158.
35. **Morikawa M, Kagihiro S, Haruki M, Takano K, Branda S *et al.*** Biofilm formation by a *Bacillus subtilis* strain that produces γ-polyglutamate. *Microbiology* 2006;152:2801–2807.
36. **Arnaud M, Chastanet A, Débarbouillé M.** New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. *Appl Environ Microbiol* 2004;70:6887–6891.
37. **Yasbin RE, Young FE.** Transduction in *Bacillus subtilis* by bacteriophage SPP1. *J Virol* 1974;14:1343–1348.
38. **Kearns DB, Chu F, Rudner R, Losick R.** Genes governing swarming in *Bacillus subtilis* and evidence for a phase variation mechanism controlling surface motility. *Mol Microbiol* 2004;52:357–369.
39. **Slack FJ, Serror P, Joyce E, Sonenshein AL.** A gene required for nutritional repression of the *Bacillus subtilis* dipeptide permease operon. *Mol Microbiol* 1995;15:689–702.
40. **Pohl K, Francois P, Stenz L, Schlink F, Geiger T *et al.*** CodY in *Staphylococcus aureus*: a regulatory link between metabolism and virulence gene expression. *J Bacteriol* 2009;191:2953–2963.
41. **Roux A, Todd DA, Velázquez JV, Cech NB, Sonenshein AL.** CodY-mediated regulation of the *Staphylococcus aureus* Agr system integrates nutritional and population density signals. *J Bacteriol* 2014;196:1184–1196.
42. **Belitsky BR, Sonenshein AL.** Genome-wide identification of *Bacillus subtilis* CodY-binding sites at single-nucleotide resolution. *Proc Natl Acad Sci USA* 2013;110:7026–7031.
43. **Mirel DB, Chamberlin MJ.** The *Bacillus subtilis* flagellin gene (hag) is transcribed by the sigma 28 form of RNA polymerase. *J Bacteriol* 1989;171:3095–3101.
44. **Ashiuchi M, Misono H.** Biochemistry and molecular genetics of poly-γ-glutamate synthesis. *Appl Microbiol Biotechnol* 2002;59:9–14.
45. **Stanley NR, Lazazzera BA.** Defining the genetic differences between wild and domestic strains of *Bacillus subtilis* that affect poly-γ-dl-glutamic acid production and biofilm formation. *Mol Microbiol* 2005;57:1143–1158.
46. **Ashiuchi M, Soda K, Misono H.** A poly-γ-glutamate synthetic system of *Bacillus subtilis* IFO 3336: gene cloning and biochemical analysis of poly-γ-glutamate produced by *Escherichia coli* clone cells. *Biochem Biophys Res Commun* 1999;263:6–12.
47. **Gao T, Greenwich J, Li Y, Wang Q, Chai Y.** The bacterial tyrosine kinase activator TkmA contributes to biofilm formation largely independent of the cognate kinase PtkA in *Bacillus subtilis*. *J Bac* 2015;197:3421–3432.
48. **Ross PL, Huang YN, Marchese JN, Williamson B, Parker K *et al.*** Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol Cell Proteomics* 2004;3:1154–1169.
49. **Bernhardt J, Völker U, Völker A, Antelmann H, Schmid R *et al.*** Specific and general stress proteins in *Bacillus subtilis* - a two-deimensional protein electrophoresis study. *Microbiology* 1997;143: 999–1017.
50. **Brinsmade SR, Alexander EL, Livny J, Stettner AI, Segrè D *et al.*** Hierarchical expression of genes controlled by the *Bacillus subtilis* global regulatory protein CodY. *Proc Natl Acad Sci USA* 2014;111: 8227–8232.
51. **Chai Y, Norman T, Kolter R, Losick R.** Evidence that metabolism and chromosome copy number control mutually exclusive cell fates in *Bacillus subtilis*. *Embo J* 2011;30:1402–1413.
52. **Subramaniam AR, Deloughery A, Bradshaw N, Chen Y, O'Shea E *et al.*** A serine sensor for multicellularity in a bacterium. *Elife* 2013;2:e01501.
53. **Ducret A, Quardokus EM, Brun YV.** MicrobeJ, a tool for high throughput bacterial cell detection and quantitative analysis. *Nat Microbiol* 2016;1:16077.
54. **Mordini S, Osera C, Marini S, Scavone F, Bellazzi R *et al.*** The role of SwrA, DegU and P(D3) in fla/che expression in B. subtilis. *PLoS One* 2013;8:e85065.
55. **Youngman P, Perkins JB, Losick R.** Construction of a cloning site near one end of Tn917 into which foreign DNA may be inserted without affecting transposition in *Bacillus subtilis* or expression of the transposon-borne *erm* gene. *Plasmid* 1984;12:1–9.
56. **Niu DD, Liu HX, Jiang CH, Wang YP, Wang QY *et al.*** The plant growth-promoting rhizobacterium *Bacillus cereus* AR156 induces systemic resistance in *Arabidopsis thaliana* by simultaneously activating salicylate- and jasmonate/ethylene-dependent signaling pathways. *Mol Plant Microbe Interact* 2011;24:533–542.

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