A bacterial prophage small peptide counteracts DnaA activities in *B. subtilis*.

Magali Ventroux and Marie-Francoise Noirot-Gros*

Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, 78350, Jouy-en-Josas, France.

*** Correspondence:**
marie-francoise.noirot-gros@inrae.fr

Keywords: small ORF, SEP, skin element, DnaA, Spo0A, *B. subtilis*

Abstract

Bacteriophages are able to hijack host essential machineries to benefit their fitness and assemble their own progeny. Phage proteins targeting major bacterial pathways can be powerful tools to understand cell functions and have possible applications in human health and industry. Bacterial genomes also harbor cryptic prophages carrying genes that may contribute to their host fitness and properties. The cryptic prophages are mostly transcriptionally silent and most of the functions they encode are not annotated. In *B. subtilis*, the 48 kb-long *skin* element is a prophage carrying the *yqaF-yqaN* operon, which is tightly regulated by the Xre-like repressor *sknR*. The small *yqaH* gene potentially encodes the protein *YqaH* in absence of SknR. It was previously reported that YqaH interacts with the replication initiator DnaA in yeast two-hybrid assay and its expression in *B. subtilis* causes defects in the chromosomal cycle. In this study, we report that, in addition to DnaA, YqaH interacts with Spo0A, a master regulator of sporulation. To decipher *yqaH* mode of action, we used the yeast two-hybrid to isolate single mutations in *yqaH* that separate interactions with DnaA and Spo0A. We isolated mutations that caused loss-of-interaction (LOI) with DnaA but not Spo0A. However, all mutations disrupting the interaction with Spo0A were also DnaA-LOI functions, suggesting that these functions could not be separated. We found that expression YqaH carrying DnaA-LOI mutations affects both chromosome integrity and DnaA-mediated transcription, leading to growth inhibition as well as preventing bacterial development such as sporulation and biofilm formation. These results show that YqaH acts as an antimicrobial peptide in *B. subtilis* and pave the way for the structural design of mutants with improved antibacterial action.

1 Introduction

Small proteins encoded by short open reading frames (smORFs) has emerged as a new class of micropeptides widespread in both in eukaryotic and prokaryotic genomes (Albuquerque et al., 2015; Couso and Patraquim, 2017; Hellens et al., 2016; Samayoa et al., 2011; Storz et al., 2014). Due to their small size, smORFs have been overlooked for a long time. However, with advanced computation and ribosome profiling-based biochemical methods, small proteins are being now more

widely identified and some has been functionally characterized (Chu et al., 2015; He et al., 2018; Samayoa et al., 2011; Straub and Wenkel, 2017; VanOrsdel et al., 2018). Growing evidence indicates they often encode bioactive peptides (Chu et al., 2015; Saghatelian and Couso, 2015). However, their contribution to cellular functions remains largely unexplored.

Micropeptides (for the smallest <30aa or microProteins up to 100 aa) were identified to act as regulators of diverse cellular processes in eukaryotes (Staudt and Wenkel, 2011). In plants, characterized micro-proteins were found to regulate transcription factors by sequestering them into nonfunctional states, preventing DNA binding or transcriptional activation (Dolde et al., 2018; Graeff and Wenkel, 2012). In Drosophila melanogaster, smORF-encoded peptides (SEPs) represent about 5% of the transcriptome and play an important role in controlling drosophila development by triggering post-translational processing of transcriptional regulators (Albuquerque et al., 2015; Zanet et al., 2015). In human, SEPs has been discovered with specific subcellular localization, suggesting they can fulfill biological functions (Slavoff et al., 2013). As example, a 69 aa long SEP, MRI-2, has been described to play a role in stimulating DNA repair through binding to the DNA end-binding protein complex Ku (Slavoff et al., 2014). SEPs represent about 2% of the genome of *S. cerevisiae* (Erpf and Fraser, 2018). Their function remains largely elusive but few has been identified to play regulatory roles in diverse physiological processes such as iron homeostasis (An et al., 2015) or DNA synthetis (Chabes et al., 1999; Erpf and Fraser, 2018; Lee et al., 2008). Genomic analysis of the *S. cerevisiae* sORFs revealed that a significant part are conserved in other eukaryotes even as phylogenetically distantly related such as humans, thus emphasizing their biological significance (Kastenmayer et al., 2006).

In Prokaryotes, small proteins are encoded by 10 to 20% of sRNA in average, and are often species-specific (Friedman et al., 2017; Miravet-Verde et al., 2019; VanOrsdel et al., 2018; Yang et al., 2016; Zuber, 2001). SEPs with characterized functions were found involved in various cellular processes (Storz et al., 2014). In *P. aeruginosa*, PtrA (63-aa long) and PtrB (59-aa long) repress the type III secretion system in response to DNA stress (Ha et al., 2004; Wu and Jin, 2005). In *E. coli*, the 43-aa long peptide SrgT interferes with the PTS glucose transport system allowing cells to utilize alternative non-PTS carbon sources to rapidly adapt to environmental changes in nutrient availability (Lloyd et al., 2017). In *B. subtilis*, SEPs have been found to participate in regulating cell division and stress responses (Ebmeier et al., 2012; Handler et al., 2008; Schmalisch et al., 2010). A compelling example is the recently characterized developmental regulator MciZ (mother cell inhibitor of FtsZ), a 40-aa long peptide which prevents cytokinesis in the mother cell during sporulation (Araujo-Bazan et al., 2019; Bisson-Filho et al., 2015). In this bacteria, about 20% of the total core protein of the mature spores is composed by the small acid-soluble spore proteins (SASPs) playing an important role in protecting DNA in the dormant spores (Moeller et al., 2008; Setlow, 2007). Notably, several smORFs identified in intergenic regions were reported to be expressed during sporulation (Schmalisch et al., 2010). The sporulation inhibitor *sda* encodes a 52aa long protein, which acts as a checkpoint system coordinating DNA replication with sporulation initiation (Burkholder et al., 2001; Cunningham and Burkholder, 2009; Rowland et al., 2004). Its mode of action has been extensively characterized at molecular level. Sda binds to the primary sporulation kinase KinA, preventing its activation and subsequent phosphorelay-mediated activation of the master sporulation regulator

Spo0A (Cunningham and Burkholder, 2009; Veening et al., 2009). By linking DNA replication to a phosphorylation-dependent signaling cascade that triggers cellular development, this system illustrates an important biological role played by a SEP in blocking sporulation in response to DNA stress in *bacillus* (Veening et al., 2009).

Small proteins encoded by phage or by prophage-like regions of bacterial genomes have been identified to hijack the host cellular machineries, as part of a strategy to shift host resources toward the production of viral progeny (Liu et al., 2014; Duval and Cossart, 2017). The bacteriophage T7 gene 2 encodes the 64 aa-long gp2 protein essential for infection of *E. coli*. Studies of it biological role revealed that gp2 inhibits transcription by binding to RNA polymerase (RNAP), promoting a host-to-viral RNAP switch (Nechaev and Severinov, 2003; Savalia et al., 2010). Another illustration is the 52-aa long protein ORF104 of phage 77 infecting *S. aureus*. ORF104 is able to interfere with the host chromosome replication by binding to the ATPase domain of the helicase loader protein DnaI, thus preventing the loading of the DNA helicase DnaC (Liu et al., 2004; Hood and Berger, 2016).

DNA replication is an essential process in all living organisms. Owing to their essentiality, proteins that compose the orisome and the replisome machineries are potential targets for the development of antimicrobials. In bacteria, DNA replication is initiated by the conserved initiator protein DnaA that assembles to the chromosomal replication origin to elicit local DNA strand opening (Hwang and Kornberg, 1992; Leonard and Grimwade, 2011; Mott and Berger, 2007; Ozaki and Katayama, 2009). This step triggers the coordinated assembly of the proteins that will further built a functional replication fork, from the DNA helicase, unwinding the DNA duplex, to the many components of the replisome that form the replication machinery (Messer, 2002). In addition to its initiator activity, DnaA acts as a transcription factor repressing or activating genes (Messer and Weigel, 2003). The activity of the initiator DnaA is tightly controlled to coordinate chromosomal replication initiation with other cellular processes during the bacterial cell cycle (Katayama et al., 2010; Scholefield and Murray, 2013). Part of this control is mediated by protein-protein interactions and involves various protein regulators that bind DnaA and affect his activity (Felicori et al., 2016; Jameson and Wilkinson, 2017; Katayama et al., 2017; Riber et al., 2016; Skarstad and Katayama, 2013). In *B. subtilis*, four proteins SirA, Soj, DnaD and YabA have been identified to regulate DnaA activity or its assembly at OriC through direct interaction (Bonilla and Grossman, 2012; Felicori et al., 2016; Martin et al., 2019; Murray and Errington, 2008).

Phage SEPs targeting important functions in bacteria are regarded as promising antimicrobial peptides, and ignited a strong interest in their identification and characterization (Hood and Berger, 2016; Liu et al., 2004). The phage-like element skin of *B. subtilis* encodes about 60 proteins. The skin element is repressed under most physiological conditions (Nicolas et al., 2012), silenced by the skin repressor SknR (Figure 1A) (Kimura et al., 2010). Excision of skin from the genome restore the integrity of sigK gene encoding the late sporulation σK factor (Kunkel et al., 1990). Among the skin ORFs, *yqaH* encodes the 85 aa-long polypeptide YqaH that has been previously identified to bind to the replication initiator protein DnaA in a yeast two-hybrid genomic screen (Noirot-Gros et al., 2002; Marchadier et al., 2011). When *yqaH* is overexpressed, bacillus cells exhibits aberrant

nucleoid morphological defects suggestive of replication deficiency (Kimura et al., 2010). In this study, we further characterized the function of *yqaH* in antagonizing DnaA. In addition, we found that YqaH also interacts with the master regulator Spo0A involved in developmental transitions to sporulation and biofilm formation (Dubnau et al., 2016). As DnaA, Spo0A is a DNA-binding protein, which, under its activated phosphorylated form (Spo0A-P) controls the expression of numerous genes during the early stages of sporulation (Molle et al., 2003). To further understand the biological role of this sORF in *B. subtilis* we performed the functional dissection of YqaH. Using a reverse yeast two-hybrid system, we selected *yqaH* alleles that selectively disrupted the YqaH/DnaA complex. This approach allowed us to link specific DnaA loss-of-interaction with loss-of-function phenotypes

2 Material and Methods

2.1 Strains, plasmids and primers

Experiments were performed in strains 168 trp+ or TF8A a phage-cured strain lacking the prophage-like element 'skin' element as well as the prophages SPβ and PBSX (Nicolas et al., 2012; Westers et al., 2003) (Table S1A). Saccharomyces cerevisiae PJ69-4a or α strains used for yeast-two-hybrid experiments (James et al., 1996). Escherichia coli strain DH10B (Durfee et al., 2008) was used as a cloning host. Plasmids constructs are listed in Table S1B. Primers are listed in Table S2. Sequences of interest cloned or mutated in this study were verified by DNA sequencing.

2.2 Bacterial culture conditions

Bacteria strains were grown at 37°C in LB medium containing ampicillin 100 µg/ml for plasmid selection (in *E. coli*), spectinomycin 60 µg/ml, kanamycin 5 µg/ml or chloramphenicol 5 µg/ml (for *B. subtilis*), and inducer molecules IPTG 0.5 mM, or D-xylose 0.5%, when necessary. *B. subtilis* strains containing pDG148 and pDG148-*yqaH* constructions were grown overnight in LB complemented with kanamycin. ON cultures were then diluted at OD600 0.01on fresh media and grown to mid-exponential phase (OD600 0.3-0.4) prior to addition of IPTG. The effect of *yqaH* expressing on growth has been performed in 96 microwells plates in a final volume of 200 µL.

2.3 Strains constructions

yqaH expression:

The *yqah* wild type and mutated gene derivatives, were PCR-amplified using the *yqaH-HindIII-RBS-F/pYqaH-R* primer pair and inserted in plasmid pDG148 between HindIII and SalI restriction sites to be placed under control of the IPTG-inducible *Pspac* promoter (Stragier et al., 1988). The plasmid constructs were extracted from *E.coli* and transformed in *B. subtilis*. Expression of YqaH wild-type and mutated proteins were assessed using 3xflag-fusions.

Construction of yqaH-gfp fusion:

yqaH WT and mutated gene were PCR-amplified from the pGAD-*yqaH* yeast vector derivatives carrying either the WT or LOI mutations (see below) using *yqaH-apa* and *pYqaH-R* primer pair. DNA fragments were digested by ApaI and SalI and inserted in between ApaI and XhoI restriction sites of pSG1154m, a version of the pSG1154 vector (Lewis and Marston, 1999) encoding a monomeric version of the GFP (GFPm). Final constructions, consisting of translational fusions of the *gfpm* gene to the C-terminal region encoded *yqaH* under the control of the P*xyl* promoter, were transferred at the *amyE* locus of the *B. subtilis* chromosome by transforming competent *B. subtilis* cells with plasmid DNA. The transfer of single mutations in the *spo0A* chromosomal locus of *B. subtilis* were performed by gene replacement using the "pop-in pop-out" gene replacement method (Fabret et al., 2002; Tanaka et al., 2013).

Yeast two-hybrid plasmid constructs:

Genes encoding for full size YqaH, Spo0A, YabA and DnaA, as well as truncated DnaA (boundaries as illustrated figure 1D) were translationally fused to the activating domain (AD) or the binding domain (BD) of the transcriptional factor Gal4 by cloning into pGAD and pGBDU vectors, respectively (James et al., 1996). DNA fragments were amplified by PCR using appropriated primer sets (Table S2), double digested by EcoR1 and SalI and ligated to corresponding pGAD or pGBDU linearized vector to generate a translational fusion with Gal4-AD or BD domains. Plasmid constructs were first transformed into *E. coli* prior to be introduced pJ69-4α (pGAD-derivatives) or pJ69-4a (pGBDU-derivatives) haploid strain. pGAD- and pGBDU- plasmid derivatives were selected onto SD media lacking leucine (SD-L) or Uracyl (SD-U), respectively (James et al., 1996).

2.4 Sporulation conditions

Sporulation of *B. subtilis* was induced by nutrient limitation by the re-suspension in Sterlini-Mandelstam medium (SM) (Sterlini and Mandelstam, 1969). The beginning of sporulation (t0) is arbitrarily defined as the moment of re-suspension of the cells in SM. To study the effects of *yqaH* overexpression on *B. subtilis* sporulation, ON cultures of strains containing the pDG148 constructions were then diluted in CH medium at starting OD600 of 0.05. When cultures reached an OD600=1, cells were re-suspended in an equivalent volume of SM medium complemented with 0.5M IPTG (t0).To monitored sporulation efficiency, cells were collected at different times after sporulation induction (t0) until t6 (6 hours after induction, defined as a stage of production of mature spores) and t18. Asymmetric septa and spores were enumerated by microscopic observations from all samples.

2.5 Spores survival assay

Cells were spread and grown ON at 30°C on DSM agar medium (Schaeffer et al., 1965), prior to be inoculated in preheated liquid DSM at OD600 0.1 and incubated at 37°C until reaching OD600 1.5. From this point (taken as T0) cultures were grown for 20 h to produce matured resistant spores. Samples were collected and half of the material was heated at 80°C (10 min) before plating to kill potential vegetative cells prior to be plated on LB. Colony counts were performed after 36 hours at

37°C. The percentage of spores was calculated as the ratio of colonies forming units (cfu) from heated and unheated samples.

2.6 Yeast-two-hybrid assay

The yeast-two-hybrid assays were performed as described (Marchadier et al., 2011; Noirot-Gros et al., 2002; Noirot-Gros et al., 2006). PJ69-4A and α haploid yeast strains transformed by pGAD- and pGBDU- plasmid derivatives were mixed onto YPD-rich media plates to allow formation of diploids. Diploids containing both pGAD and pGBDU type of plasmids were then selected on SD-UL and interacting phenotypes were monitored by the ability of diploids to grow on SD-LUHA medium further lacking histidine (H) or adenine (A).

2.7 Generation of Loss of Interaction (LOI) mutation

YqaH_LOI mutants were identified using a yeast two-hybrid-based assay as described elsewhere (Natrajan et al., 2009; Noirot-Gros et al., 2006; Quevillon-Cheruel et al., 2012). Random mutagenesis of the targeted genes were achieved by PCR amplification under mutagenic conditions that promotes less that one miss incorporation per amplification cycle (Noirot-Gros et al., 2006). For *yqaH*, a library of mutated pGAD-*yqaH** were constructed by gap-repair recombination into yeast (PJ69–4α strain). About 1000 individual transformants were organized in 96-well format on plates containing a defined medium lacking leucine (-L) to form an arrayed collection of AD-*yqaH** gene fusion mutants. This organized library was then mated with PJ69–4a strains containing either pGBDU-*dnaA* or pGBDU-*spo0A*, or an empty pGBDU plasmid as a negative control. Selective pressure for interacting phenotypes was then applied on media lacking –LUH or –LUA. Diploids that failed to grow on interaction selective media are considered as potentially expressing a loss-of-interaction (LOI) mutant of *yqaH*. Importantly, any particular AD-YqaH*_LOI proteins unable to trigger interacting phenotypes in the presence of BD-DnaA while still producing interacting phenotypes when expressed in the presence of BD-Spo0A is defined as a DnaA-specific LOI mutant. The corresponding *yqaH_LOI* genes were retrieved from the initially organized haploid library and the mutations identified by DNA sequencing. Only mutations resulting from single substitutions were considered.

2.8 Ori/Ter ratios measurements by qPCR

To determine the ratio of amounts of origin-proximal and terminus-proximal DNA sequences, ON cultures of *B. subtilis* strains containing pDG148 or pDG148-*yqaH* plasmids were freshly diluted (OD600=0.01) in LB supplemented with kanamycin 5 µg/mL in the presence of IPTG 0.5 mM, and grown at 37°C in up to mid-exponential phase (OD600 = 0.3 to 0.4). A determined volume of culture was taken, mixed with similar volume of sodium azide solution (1%, 0.5% final) to stop all metabolic activities, and subjected to total lysis. Total genomic DNA extracts were kept at -80°C and thawed aliquots were used only once. Quantitative real time PCR were performed on a Mastercycler® ep realplex (Eppendorf) thermocycler device using ABsolute™ Blue QPCR SYBR® Green ROX Mix (ABgene), to amplify specific origin or terminus proximal sequences. Primers used for sequence amplification were chosen using Primer3Plus program (http://www.bioinformatics.nl/cgi-

bin/primer3plus/primer3plus.cgi/). Amplification using the ORI pair of primers (oriL3F and oriL3R, Table S2) targeting the 4212889-4211510 region of the *B. subtilis* chromosome, yields a 128 bp size product corresponding to sequence at the left side of the origin. The terminus sequence is a 122 bp long fragment obtained from the TER pair of primers (terR3F and terR3R, Table S2) amplifying the region 2016845-2017711 at the right side of the terminus. The two primer pairs ORI and TER exhibited ≥ 95% of amplification efficiency. Data analysis was performed using the software Realplex (Eppendorf) and the quantification with the ΔΔCt method. The formula reflects the efficacy (Eff) of the primers (Rate = (1+Eff)^(-ΔΔCt)).

2.9 Gene expression quantifications

To quantify the expression rates of genes regulated by DnaA, sample of cells (as collected for Ori/Ter ratios measurements) were also harvested in exponential growth at OD600=0.3-0.4, and RNA extractions were performed. To quantify the expression rates of genes regulated by Spo0A, sporulating cells were harvested when reaching stages T2-T3 of sporulation ($t_{2,5}$) in SM medium. In both case, a determined volume of culture was mix with equivalent volume of centrifuged of sodium azide solution, and subjected to lysis followed by total RNA extraction as described (Nicolas et al., 2012). Total RNA was reversely transcribed and quantitative real time PCR were performed on cDNA. Primers used for sequence amplification were chosen using Primer3Plus program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) and are detailed in Table S2. The couples of primers exhibited ≥ 95% amplification efficiency. Data analysis was performed using the software Realplex (Eppendorf) and the quantification with the ΔΔCt method. The formula reflects the efficacity (Eff) of the primers (Rate = (1+Eff)^(-ΔΔCt)).

2.10 Fluorescence microscopy

The cytolocalization of proteins fused to GFP were observed by epifluorescence microscopy on vegetative or sporulating cells. The expression of *yqaH-gfp* gene fusions from the *B. subtilis amyE* chromosomal locus was trigered by supplementing exponential growth or sporulation cultures with xylose 0.5%. Sample of cells were harvested and rinsed in a minimal transparent media to avoid LB auto-fluorescence prior to be mounted onto 1.2% agarose pads. When necessary, bacterial membranes were stained with FM4-64 dye and nucleoids with DAPI. Fluorescence microscopy was performed on a Leica® DMR2A (100X UplanAPO objective with an aperture of 1.35.) coupled with CoolSnap HQ camera (Roper Scientific). System control and image processing were achieved using Metamorph software (Molecular Devices, Sunnyvale, CA, USA). Counts of cells, spores, foci or nucleoids were determined with the ImageJ® software, from at least 500 cells.

2.11 Biofilm assay

Production and analysis of air-to-liquid biofilm pellicles were performed as already described (Garcia Garcia et al., 2018). Briefly, strains expressing yqaH, wild type or K17E mutant derivative as well as control strain were grown in LB to OD600 of 1.0 and inoculated in 12-well culture plates containing

3.5 ml of MSgg media at starting OD600 = 0.1. Cultures were maintained at 28°C and 70% humidity, with no agitation. After 48 hours, wells were filled out with MSgg media (slowly added at the edge) to lift the biofilm pellicles up to the top of the wells. The pellicles were then peeled-off onto a 2.5 cm diameter circular cover slide. The cover slides with intact biofilm pellicles were mounted onto an Attofluor Cell Chamber and stained with the Film Tracer FM 1-43 Green Biofilm dye (Thermo Fisher Scientific). Stained biofilms were observed using a spinning disk confocal microscope [Nikon Eclipse Ti-E coupled with CREST X-LightTM confocal imager; objectives Nikon CFI Plan Fluor 10X, DIC, 10x/0.3 NA (WD = 16 mm); excitation was performed at 470 nm and emission recorded at 505 nm]. Images were processed using IMARIS software (Bitplane, South Windsor, CT, United States). Biofilm images were quantified using the surface function in IMARIS (XTension biofilm). Biovolumes were calculated based on total volume (µm3) per area (µm2) from n ≥ 4 samples.

2.12 Protein immunodectection

Production of 3FLAG-YqaH mutated derivatives was determined from total protein extract of *B. subtilis* by immuno-detection using monoclonal antibody anti-FLAG® M2 (Sigma). An IgG goat secondary antibody (Sigma) peroxidase conjugated was used at 1/10000e to detect the anti-FLAG® M2. Proteins immuno-detections were performed using the Clarity Western ECL kit (Biorad) according to the supplier's indications followed by chemiluminescence detection (ChemiDoc imager, Biorad). Images were analysed with the software Image Lab™.

2.13 Structure predictions

Secondary structures predictions were performed with the computer server Jpred 3 (http://www.compbio.dundee.ac.uk/www-jpred/) and 3D structures predictions with the server Phyre[2] (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index).

3 Results

3.1 The Skin element SPORF YqaH interacts with DnaA and Spo0A

YqaH was originally identified as interacting with the replication initiator DnaA in a yeast two-hybrid screen of a *B. subtilis* genomic library (Noirot-Gros et al., 2002). Interestingly, we also identified YqaH as binding partner to Spo0A in a subsequent screen targeting the sporulation transcriptional factor (Figure 1B). Other DnaA-binding proteins SirA, Soj, DnaD and YabA also exert regulatory functions by interacting with different functional domains of DnaA (Figure 1B). To gain insights into YqaH mode of action, we characterized the minimal interacting domain of DnaA. We defined the C-terminal domain IV as necessary and sufficient to trigger the interaction phenotypes in a yeast two-hybrid binary assay (Figure 1C). This 118 AA long fragment spans residue 328 to 446, and carries the signature motif for binding to double stranded DNA. This result distinguishes YqaH from other regulators, found to inhibit DnaA binding to *oriC* through interacting with domains I, responsible for self-oligomerization (SirA), and/or the central AAA+ ATPase domain III, (SirA, Soj, YabA and DnaD).

3.2 YqaH expression triggers *dnaA*-related mutant phenotypes

The interaction of YqaH with DnaA hints at a potential role in replication initiation control. We first investigated the effect of *yqaH* expression on cell growth and viability. *B. subtilis* strains carrying a plasmid expressing *yqaH* under control of an IPTG-inducible promoter or carrying a control plasmid with no expressed gene were treated under identical conditions Addition of IPTG at early exponential stage specifically halted cell growth after two hours in cells expressing *yqaH* (Figure 2A). We found that cell viability was affected almost instantly in the presence of *yqaH*, leading to about 30x decrease after three hours (Figure 2A). Expression of *yqaH* also affects cell morphology leading to significant filamentation (Figure 2B). Closer examination of nucleoids revealed aberrant segregation of chromosomes with both diffused and compacted nucleoids unequally distributed within filaments, as well as a large portion of cells with no DNA. Most importantly, septum-entrapped nucleoids were also observed indicative of impaired nucleoid occlusion (NO) (Wu and Errington, 2011). These observations highlighted that the expression of YqaH triggered a large panel of chromosomal disorders suggestive of replication and segregation stress, and compatible with replication initiation deficiency, in agreement to earlier described (Kimura et al., 2010). We further investigate a role of YqaH by investigating DnaA-dependant transcriptional regulation. Among genes of the DnaA-regulon, active DnaA-ATP negatively regulate its own expression by binding to *dnaA* promoter region (Goranov et al., 2005). We monitored the *dnaA* mRNA levels in the presence or absence of YqaH and shown that expression of *yqaH* led to a 2 fold increase in *dnaA* mRNA, indicating a regulatory defect. Taking all together, these results are in agreement with a direct role of YqaH in counteracting DnaA activity.

3.3 YqaH expression impairs sporulation

We investigated the possible role of YqaH in Spo0A functions, by examining the effect of *yqaH* expression on sporulation. Cells carrying either the control or the *yqaH*-inducible plasmids were put to sporulate by the re-suspension method. To prevent an inhibitory effect of YqaH during vegetative growth, the *yqaH* gene expression was induced only at the onset of sporulation (T0). The formation of spores was monitored over time. Appearance of asymetric septa at early stage (T2,5) was imaged by fluorescent microscopy after staining by a red-fluorescent membrane-dye, while the engulfed forespore (T6 and T18) was revealed using bright field (Figure. 3A). We observed that the counts of spore forming bacteria was drastically affected in the presence of YqaH (Figure 3B). We also examined the effect of *yqaH* expression on Spo0A-mediated transcriptional regulation. Among the genes under the direct control of Spo0A are *spoIIE*, encoding the protein serine phosphatase SpoIIE and *spoIIGA*, encoding a pro- σE processing protease (Molle et al., 2003). These two genes are involved in the activation of the alternative sporulation sigma factors σF and σE in the forespore and mother cell compartments, respectively (Baldus et al., 1994; Bradshaw et al., 2017; Errington and Wu, 2017; Fujita et al., 2005). Examination of their expression at the onset of sporulation revealed a significant downregulation (about 7 and 8 fold, respectively) in the presence of *yqaH* (Figure 3C). Altogether, these results pointed to a negative effect of YqaH in Spo0A-mediated function.

3.4 Functional dissection of YqaH

To further decipher YqaH mechanism, we looked for *yqaH* separation of function mutations that selectively affect its ability to interact to one protein partner while preserving its interaction with the other. Using a yeast two-hybrid based assay, we screened a *yqaH* mutant library specific loss-of-interaction (LOI) phenotypes (Figure S1A). This approached is based on the selection of single amino acid changes in the protein of interest that selectively disrupt the interaction with one partner without affecting the interaction with the other partner. Such screening favors the substitution of residues located at the interacting surface, leading to a LOI phenotype with the targeted partner, while preserving the overall 3D-structure of the protein that remains proficient for interaction with another partner (Natrajan et al., 2009; Noirot-Gros et al., 2006; Quevillon-Cheruel et al., 2012). We identify three single residue substitutions K17E, E38K and K48E that elicited a complete loss-of-interaction phenotype with DnaA without affecting interaction with Spo0A (Figure S1B, Table 1). Six additional substitutions affecting residues R16G, M27T, Y37H, E38V A44V and R56W were only partially abolishing interaction phenotypes with DnaA (Figure S1B, Table 1). Two substitutions, S25T and A40T were found to partially affect both interaction with DnaA and Spo0A, and 4 substitutions, D10G, S20L, L28P, L43P and L57P were totally abolishing interaction phenotypes with both DnaA and Spo0A (Figure S1B, Table1). In the latter case, these substitutions could have affected the overall YqaH 3D-structure integrity. Is it worthy of note that, while several specific DnaA-LOI mutants were obtained, no substitution that specifically prevent interaction with Spo0A were identified in our screen. The YqaH protein is predicted to fold into three successive alpha helix (Figure S1B). The residues important for interaction with DnaA mapped within the two main helix presumably involved in a coiled-coil structure.

3.5 DnaA-LOI mutants of yqaH restore replication and transcriptional regulation defects caused by YqaH expression

We investigated the effect of two DnaA-LOI mutants carrying substitutions K17E and R56W, exhibiting total (K17E) or partial (R56W) loss of interaction phenotypes with YqaH in our yeast two-hybride assay. (Table 1). Both substitutions abolished the nucleoid segregation defects resulting from *yqaH* expression (Figure 4A). To rule out that this phenotypic compensation could potentially result from expression of the gene copy from the silent SknR operon, we confirmed that the only source of YqaH in the cell arisen from plasmidic expression (Figure S3). No YqaH protein was detected in cells carrying a control plasmid devoid of yqaH coding sequence, indicating that the chromosomal copy remained completely silent during our experimental conditions.

We further examined the effect of YqaH on replication initiation by measuring the ratio between chromosomal origin-proximal and terminus-proximal sequences (Figure 4B). The average of origin-to-terminus (*ori:ter*) ratio was determined by qPCR on genomic DNA harvested from exponentially growing cells as previously described (Soufo et al., 2008). The *ori:ter* ratio was about 4 in control cells that do not expressed *yqaH*, indicating that two events of replication initiation has taken place in most cells, in agreement to already observed in similar experimental conditions (Murray and Koh, 2014). In the presence of *yqaH*, the *ori:ter* ratio dropped to 2, indicating that only one replication

initiation event per cell has taken place in average (Figure 4B). This observation suggests that the YqaH protein could exerts a negative effect on replication initiation by counteracting DnaA activity. We observed that this effect was abolished upon expression of the *yqaH*_DnaA LOI variants carrying either the K17E or the R56W substitutions (Figure 4B). In cells, expressing YqaH-R56W the replication initiation rate was similar to control cells while expression of YqaH-K17E restored about 75% of the initiation rate indicating a significant but partial complementation. In another assay, we analyzed the number of chromosomal origin in individual cells using the *lacO*/LacI-GFP system. Cells carrying a *lacO* repeat array near the replication origin and expressing the LacI-GFP fusion were observed by fluorescence microscopy, in the presence of absence of YqaH (Figure 4C). In the absence of YqaH, the number of lacI foci per nucleoid was found to be 3.6 in average, in agreement with the *ori-to-ter* ratio (Figure 4D). This number dropped to 1 upon expression of YqaH, suggestive of replication deficiency. As observed earlier, cell were also exhibiting aberrant nucleoids and segregation defects. Conversely, expression of YqaH-K17E mutant fully restored nucleoid and cell morphology and partially restored replication defects with an average of LacI-GFP foci about 2.3. Together these observations indicate that YqaH binds to DnaA and antagonized its activity in replication initiation.

To substantiate the role of YqaH/ DnaA interaction, we also examined the effect of the DnaA-LOI mutant on *dnaA* transcription (Figure 4E). We found that the loss of DnaA-mediated control of *dnaA* expression in the presence of wild type YqaH was restored by the introduction of both K17E and R56W substitutions. This result further supports the conclusion that the binding of YqaH to DnaA antagonizes DnaA functions.

3.6 The YqaH-mediated defects in sporulation and biofilm formation requires interaction with DnaA.

Although we did not identified any YqaH_SpoA-LOI mutant in our screen, we thought that the YqaH_K17E DnaA-LOI mutant provided for a convenient separation-of-function for a more reliable investigation of the potential role of *yqaH* in *spo0A*-mediated phenotypes. Indeed, this K17E variant remains fully proficient for interacting with Spo0A in our yeast 2HB assays, allowing to investigate the role of *yqaH* during sporulation while circumventing DnaA-related defects during vegetative cycle. We compared the sporulation efficiencies of strains expressing the wild-type YqaH or the YqaH_K17E variant in both 168 and TF8A (devoid of Skin element) backgrounds. The percent of cells ongoing sporulation was determined at t6 (i.e stage VI, refers as the spore maturation stage, Figure 5A). Surprisingly, we found that the K17E-LOI substitution abolished the defect elicited by the wild type YqaH (Figure 5). This observation indicates that the sporulation deficiency mediated by YqaH requires its ability to interact with DnaA. To further investigate the potential role of YqaH in Spo0A-related processes, we also examined its effect on biofilm formation (Figure 5B, Figure S4). We observed that the production of biofilm pellicles at the air-medium interface was impaired in strains expressing YqaH, leading to a loss of biomass and cohesion. Damaged biofilm pellicles were not observed in cells expressing the YqaH_K17E DnaA-LOI mutant (Figure 5B, Figure S4B,C) thus pointing to a role of DnaA during biofilm formation.

4 Discussion

Our study sheds light on the mechanism of action of YqaH, a small protein with antimicrobial activities encoded by the *B. subtilis* skin element. By physically interacting with two master regulators DnaA and Spo0A, the YqaH protein holds the promise to inhibit two essential cellular processes under two different life styles of the bacteria. Upon nutrient stress and other environmental conditions, B. subtilis cells switch from vegetative growth to sporulation or biofilm formation. DnaA is essential during vegetative growth, acting as both replication initiator and transcriptional factor, while the response regulator Spo0A controls developmental transitions.

In Bacillus, several regulatory proteins inhibit DnaA by targeting different functional domains of the protein. In cells committed to sporulation, the protein SirA prevents DnaA from binding to the replication origin OriC by interacting with its structural domains I and III (Jameson et al., 2014). During vegetative growth, the regulatory proteins YabA interacts with DnaA during most of the cell cycle (Felicori et al., 2016; Noirot-Gros et al., 2006; Soufo et al., 2008). YabA as well as the primosomal protein DnaD affect DnaA cooperative binding to OriC by interacting with DnaA structural domain III (Merrikh and Grossman, 2011; Scholefield and Murray, 2013). Finally, the ATPase protein Soj negatively regulates DnaA by also interacting with the structural domain III, preventing oligomerization (Scholefield et al., 2011). Our results indicate that YqaH controls DnaA activity by a mode distinct from the other known regulators, specifically by contacting the structural domain IV of DnaA that is responsible for DnaA-box sequence recognition (Fujikawa et al., 2003).

B. subtilis cells expressing YqaH exhibited various DnaA-related phenotypes that spanned from a general growth defect, aberrant nucleoid morphology, impaired replication initiation and loss of transcriptional control (Figure 2 and 4). These cells also exhibited Spo0A-related phenotypes such as a dramatic reduction of sporulation efficiency (Figure 3A, B), an inhibition of the expression of Spo0A-dependent genes (Figure 3C) and a strong impairment of biofilm formation (Figure 5). These observations are in agreement with a role of YqaH in counteracting both DnaA and Spo0A activities during vegetative growth and sporulation. However, it is well documented that initiation of sporulation is closely coupled to the cell cycle and DNA replication in order to ensure that sporulation occurs only in cells containing two fully replicated chromosomes (Veening et al., 2009). Because of this intricate relationship between DNA replication and sporulation, it is difficult to separate the phenotypes of YqaH related to its interactions with DnaA on one hand and Spo0A on the other hand. By using YqaH single point mutants unable to interact with DnaA while remaining proficient for interaction with Spo0A, we identified mutational pattern on YqaH (Figure S2) and demonstrated their direct involvement in DnaA-related a loss-of-function phenotype. In our screens, we did not obtain YqaH mutants that affected only the interaction with Spo0A. However, the YqaH DnaA_LOI mutants revealed that sporulation and biofilm formation phenotypes of YqaH expression required interaction with DnaA. Thus, we could not establish whether interaction with Spo0A plays a role in these phenotypes. Our results could be explained by the role of DnaA as a pleiotropic transcriptional regulator controlling the expression of several sporulation genes, including the checkpoint histidine kinase Sda (Washington et al., 2017). Sda is known to bind to KinA, inhibiting its phosphorylation activity required to activate Spo0A (Burkholder et al., 2001). A recent study

highlighted that a large part of the role of DnaA into gene expression is indirect and mediated by sda (Washington et al., 2017). Sda is positively regulated by both DnaA and Spo0A, and a coupling between expression of Sda and the replication cycle results in cyclic bursts of Spo0A activation (Narula et al., 2015). Many genes that appear regulated by DnaA are involved in sporulation and biofilm formation, illustrating the complexity of regulatory circuits that control transitions in lifestyle. In conclusion, our study characterizes the role of small peptide YqaH that negatively interfers with a broad range of cellular processes by counteracting the activity of DnaA. Controlling YqaH expression could be part of a strategy to prevent the dissemination of engineered B. subtilis strains in a controlled environment.

5 Acknowledgements

6 Author contributions

MV and MFNG designed the experiments. MV performed all experiments. MV and MFNG interpreted the results. MFNG wrote the manuscript. Both authors contributed to data interpretation and reviewed the final version of the manuscript.

7 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

8 Supplementary material

Figure S1: Yeast 2HB screening of a yqaH gene mutant library for specific LOI phenotypes.

Figure S2: Mapping of the YqaH single LOI mutations with DnaA

Figure S3: Immunodetection of YqaH in cell extracts after induction

Figure S4: DnaA-dependant YqaH-mediated defects in biofilm formation

Figure S5: ORF conservation within bacillus species

Table S1: Strains and Plasmids

Table S2: Primers list

9 References

Albuquerque, J.P., Tobias-Santos, V., Rodrigues, A.C., Mury, F.B., and da Fonseca, R.N. (2015). small ORFs: A new class of essential genes for development. Genetics and molecular biology *38*, 278-283.

An, X., Zhang, C., Sclafani, R.A., Seligman, P., and Huang, M. (2015). The late-annotated small ORF LSO1 is a target gene of the iron regulon of Saccharomyces cerevisiae. MicrobiologyOpen *4*, 941-951.

Araujo-Bazan, L., Huecas, S., Valle, J., Andreu, D., and Andreu, J.M. (2019). Synthetic developmental regulator MciZ targets FtsZ across Bacillus species and inhibits bacterial division. Mol Microbiol *111*, 965-980.

Baldus, J.M., Green, B.D., Youngman, P., and Moran, C.P., Jr. (1994). Phosphorylation of Bacillus subtilis transcription factor Spo0A stimulates transcription from the spoIIG promoter by enhancing binding to weak 0A boxes. J Bacteriol *176*, 296-306.

Bisson-Filho, A.W., Discola, K.F., Castellen, P., Blasios, V., Martins, A., Sforca, M.L., Garcia, W., Zeri, A.C., Erickson, H.P., Dessen, A., *et al.* (2015). FtsZ filament capping by MciZ, a developmental regulator of bacterial division. Proc Natl Acad Sci U S A *112*, E2130-2138.

Bonilla, C.Y., and Grossman, A.D. (2012). The primosomal protein DnaD inhibits cooperative DNA binding by the replication initiator DnaA in Bacillus subtilis. J Bacteriol *194*, 5110-5117.

Bradshaw, N., Levdikov, V.M., Zimanyi, C.M., Gaudet, R., Wilkinson, A.J., and Losick, R. (2017). A widespread family of serine/threonine protein phosphatases shares a common regulatory switch with proteasomal proteases. Elife *6*.

Burkholder, W.F., Kurtser, I., and Grossman, A.D. (2001). Replication initiation proteins regulate a developmental checkpoint in Bacillus subtilis. Cell *104*, 269-279.

Chabes, A., Domkin, V., and Thelander, L. (1999). Yeast Sml1, a protein inhibitor of ribonucleotide reductase. J Biol Chem *274*, 36679-36683.

Chu, Q., Ma, J., and Saghatelian, A. (2015). Identification and characterization of sORF-encoded polypeptides. Crit Rev Biochem Mol Biol *50*, 134-141.

Couso, J.P., and Patraquim, P. (2017). Classification and function of small open reading frames. Nature reviews Molecular cell biology *18*, 575-589.

Cunningham, K.A., and Burkholder, W.F. (2009). The histidine kinase inhibitor Sda binds near the site of autophosphorylation and may sterically hinder autophosphorylation and phosphotransfer to Spo0F. Mol Microbiol *71*, 659-677.

Dolde, U., Rodrigues, V., Straub, D., Bhati, K.K., Choi, S., Yang, S.W., and Wenkel, S. (2018). Synthetic MicroProteins: Versatile Tools for Posttranslational Regulation of Target Proteins. Plant physiology *176*, 3136-3145.

Dubnau, E.J., Carabetta, V.J., Tanner, A.W., Miras, M., Diethmaier, C., and Dubnau, D. (2016). A protein complex supports the production of Spo0A-P and plays additional roles for biofilms and the K-state in Bacillus subtilis. Mol Microbiol *101*, 606-624.

Durfee, T., Nelson, R., Baldwin, S., Plunkett, G., Burland, V., Mau, B., Petrosino, J.F., Qin, X., Muzny, D.M., Ayele, M., *et al.* (2008). The complete genome sequence of Escherichia coli DH10B: Insights into the biology of a laboratory workhorse. Journal of Bacteriology *190*, 2597-2606.

Ebmeier, S.E., Tan, I.S., Clapham, K.R., and Ramamurthi, K.S. (2012). Small proteins link coat and cortex assembly during sporulation in Bacillus subtilis. Mol Microbiol *84*, 682-696.

Erpf, P.E., and Fraser, J.A. (2018). The Long History of the Diverse Roles of Short ORFs: sPEPs in Fungi. Proteomics *18*, e1700219.

Errington, J., and Wu, L.J. (2017). Cell Cycle Machinery in Bacillus subtilis. Sub-cellular biochemistry *84*, 67-101.

Fabret, C., Ehrlich, S.D., and Noirot, P. (2002). A new mutation delivery system for genome-scale approaches in Bacillus subtilis. Mol Microbiol *46*, 25-36.

Felicori, L., Jameson, K.H., Roblin, P., Fogg, M.J., Garcia-Garcia, T., Ventroux, M., Cherrier, M.V., Bazin, A., Noirot, P., Wilkinson, A.J., *et al.* (2016). Tetramerization and interdomain flexibility of the replication initiation controller YabA enables simultaneous binding to multiple partners. Nucleic Acids Res *44*, 449-463.

Friedman, R.C., Kalkhof, S., Doppelt-Azeroual, O., Mueller, S.A., Chovancova, M., von Bergen, M., and Schwikowski, B. (2017). Common and phylogenetically widespread coding for peptides by bacterial small RNAs. BMC genomics *18*, 553.

Fujita, M., Gonzalez-Pastor, J.E., and Losick, R. (2005). High- and low-threshold genes in the Spo0A regulon of Bacillus subtilis. J Bacteriol *187*, 1357-1368.

Graeff, M., and Wenkel, S. (2012). Regulation of protein function by interfering protein species. Biomolecular concepts *3*, 71-78.

Ha, U.H., Kim, J., Badrane, H., Jia, J., Baker, H.V., Wu, D., and Jin, S. (2004). An in vivo inducible gene of Pseudomonas aeruginosa encodes an anti-ExsA to suppress the type III secretion system. Mol Microbiol *54*, 307-320.

Handler, A.A., Lim, J.E., and Losick, R. (2008). Peptide inhibitor of cytokinesis during sporulation in Bacillus subtilis. Mol Microbiol *68*, 588-599.

He, C., Jia, C., Zhang, Y., and Xu, P. (2018). Enrichment-Based Proteogenomics Identifies Microproteins, Missing Proteins, and Novel smORFs in Saccharomyces cerevisiae. J Proteome Res *17*, 2335-2344.

Hellens, R.P., Brown, C.M., Chisnall, M.A.W., Waterhouse, P.M., and Macknight, R.C. (2016). The Emerging World of Small ORFs. Trends in plant science *21*, 317-328.

Hood, I.V., and Berger, J.M. (2016). Viral hijacking of a replicative helicase loader and its implications for helicase loading control and phage replication. Elife *5*.

Hwang, D.S., and Kornberg, A. (1992). Opening of the replication origin of Escherichia coli by DnaA protein with protein HU or IHF. J Biol Chem *267*, 23083-23086.

James, P., Halladay, J., and Craig, E.A. (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics *144*, 1425-1436.

Jameson, K.H., and Wilkinson, A.J. (2017). Control of Initiation of DNA Replication in Bacillus subtilis and Escherichia coli. Genes *8*.

Kastenmayer, J.P., Ni, L., Chu, A., Kitchen, L.E., Au, W.C., Yang, H., Carter, C.D., Wheeler, D., Davis, R.W., Boeke, J.D., *et al.* (2006). Functional genomics of genes with small open reading frames (sORFs) in S. cerevisiae. Genome research *16*, 365-373.

Katayama, T., Kasho, K., and Kawakami, H. (2017). The DnaA Cycle in Escherichia coli: Activation, Function and Inactivation of the Initiator Protein. Front Microbiol *8*, 2496.

Katayama, T., Ozaki, S., Keyamura, K., and Fujimitsu, K. (2010). Regulation of the replication cycle: conserved and diverse regulatory systems for DnaA and oriC. Nat Rev Microbiol *8*, 163-170.

Kimura, T., Amaya, Y., Kobayashi, K., Ogasawara, N., and Sato, T. (2010). Repression of sigK intervening (skin) element gene expression by the CI-like protein SknR and effect of SknR depletion on growth of Bacillus subtilis cells. J Bacteriol *192*, 6209-6216.

Lee, Y.D., Wang, J., Stubbe, J., and Elledge, S.J. (2008). Dif1 is a DNA-damage-regulated facilitator of nuclear import for ribonucleotide reductase. Molecular cell *32*, 70-80.

Leonard, A.C., and Grimwade, J.E. (2011). Regulation of DnaA assembly and activity: taking directions from the genome. Annual review of microbiology *65*, 19-35.

Lewis, P.J., and Marston, A.L. (1999). GFP vectors for controlled expression and dual labelling of protein fusions in Bacillus subtilis. Gene *227*, 101-110.

Liu, J., Dehbi, M., Moeck, G., Arhin, F., Bauda, P., Bergeron, D., Callejo, M., Ferretti, V., Ha, N., Kwan, T., *et al.* (2004). Antimicrobial drug discovery through bacteriophage genomics. Nature biotechnology *22*, 185-191.

Lloyd, C.R., Park, S., Fei, J., and Vanderpool, C.K. (2017). The Small Protein SgrT Controls Transport Activity of the Glucose-Specific Phosphotransferase System. J Bacteriol *199*.

Marchadier, E., Carballido-Lopez, R., Brinster, S., Fabret, C., Mervelet, P., Bessieres, P., Noirot-Gros, M.F., Fromion, V., and Noirot, P. (2011). An expanded protein-protein interaction network in Bacillus subtilis reveals a group of hubs: Exploration by an integrative approach. Proteomics *11*, 2981-2991.

Martin, E., Williams, H.E.L., Pitoulias, M., Stevens, D., Winterhalter, C., Craggs, T.D., Murray, H., Searle, M.S., and Soultanas, P. (2019). DNA replication initiation in Bacillus subtilis: structural and functional characterization of the essential DnaA-DnaD interaction. Nucleic Acids Res *47*, 2101-2112.

Messer, W. (2002). The bacterial replication initiator DnaA. DnaA and oriC, the bacterial mode to initiate DNA replication. FEMS microbiology reviews *26*, 355-374.

Messer, W., and Weigel, C. (2003). DnaA as a transcription regulator. Methods in enzymology *370*, 338-349.

Miravet-Verde, S., Ferrar, T., Espadas-Garcia, G., Mazzolini, R., Gharrab, A., Sabido, E., Serrano, L., and Lluch-Senar, M. (2019). Unraveling the hidden universe of small proteins in bacterial genomes. Molecular systems biology *15*, e8290.

Moeller, R., Setlow, P., Horneck, G., Berger, T., Reitz, G., Rettberg, P., Doherty, A.J., Okayasu, R., and Nicholson, W.L. (2008). Roles of the major, small, acid-soluble spore proteins and spore-specific and universal DNA repair mechanisms in resistance of Bacillus subtilis spores to ionizing radiation from X rays and high-energy charged-particle bombardment. J Bacteriol *190*, 1134-1140.

Molle, V., Fujita, M., Jensen, S.T., Eichenberger, P., Gonzalez-Pastor, J.E., Liu, J.S., and Losick, R. (2003). The Spo0A regulon of Bacillus subtilis. Mol Microbiol *50*, 1683-1701.

Mott, M.L., and Berger, J.M. (2007). DNA replication initiation: mechanisms and regulation in bacteria. Nat Rev Microbiol *5*, 343-354.

Murray, H., and Errington, J. (2008). Dynamic control of the DNA replication initiation protein DnaA by Soj/ParA. Cell *135*, 74-84.

Murray, H., and Koh, A. (2014). Multiple regulatory systems coordinate DNA replication with cell growth in Bacillus subtilis. PLoS Genet *10*, e1004731.

Natrajan, G., Noirot-Gros, M.F., Zawilak-Pawlik, A., Kapp, U., and Terradot, L. (2009). The structure of a DnaA/HobA complex from Helicobacter pylori provides insight into regulation of DNA replication in bacteria. Proc Natl Acad Sci U S A *106*, 21115-21120.

Nicolas, P., Mader, U., Dervyn, E., Rochat, T., Leduc, A., Pigeonneau, N., Bidnenko, E., Marchadier, E., Hoebeke, M., Aymerich, S., *et al.* (2012). Condition-dependent transcriptome reveals high-level regulatory architecture in Bacillus subtilis. Science *335*, 1103-1106.

Noirot-Gros, M.F., Dervyn, E., Wu, L.J., Mervelet, P., Errington, J., Ehrlich, S.D., and Noirot, P. (2002). An expanded view of bacterial DNA replication. Proc Natl Acad Sci U S A *99*, 8342-8347.

Noirot-Gros, M.F., Velten, M., Yoshimura, M., McGovern, S., Morimoto, T., Ehrlich, S.D., Ogasawara, N., Polard, P., and Noirot, P. (2006). Functional dissection of YabA, a negative regulator of DNA replication initiation in Bacillus subtilis. Proc Natl Acad Sci U S A *103*, 2368-2373.

Ozaki, S., and Katayama, T. (2009). DnaA structure, function, and dynamics in the initiation at the chromosomal origin. Plasmid *62*, 71-82.

Quevillon-Cheruel, S., Campo, N., Mirouze, N., Mortier-Barriere, I., Brooks, M.A., Boudes, M., Durand, D., Soulet, A.L., Lisboa, J., Noirot, P., *et al.* (2012). Structure-function analysis of pneumococcal DprA protein reveals that dimerization is crucial for loading RecA recombinase onto DNA during transformation. Proc Natl Acad Sci U S A *109*, E2466-2475.

Riber, L., Frimodt-Moller, J., Charbon, G., and Lobner-Olesen, A. (2016). Multiple DNA Binding Proteins Contribute to Timing of Chromosome Replication in E. coli. Frontiers in molecular biosciences *3*, 29.

Rowland, S.L., Burkholder, W.F., Cunningham, K.A., Maciejewski, M.W., Grossman, A.D., and King, G.F. (2004). Structure and mechanism of action of Sda, an inhibitor of the histidine kinases that regulate initiation of sporulation in Bacillus subtilis. Molecular cell *13*, 689-701.

Saghatelian, A., and Couso, J.P. (2015). Discovery and characterization of smORF-encoded bioactive polypeptides. Nat Chem Biol *11*, 909-916.

Samayoa, J., Yildiz, F.H., and Karplus, K. (2011). Identification of prokaryotic small proteins using a comparative genomic approach. Bioinformatics *27*, 1765-1771.

Schaeffer, P., Millet, J., and Aubert, J.P. (1965). Catabolic repression of bacterial sporulation. Proc Natl Acad Sci U S A *54*, 704-711.

Schmalisch, M., Maiques, E., Nikolov, L., Camp, A.H., Chevreux, B., Muffler, A., Rodriguez, S., Perkins, J., and Losick, R. (2010). Small genes under sporulation control in the Bacillus subtilis genome. J Bacteriol *192*, 5402-5412.

Scholefield, G., and Murray, H. (2013). YabA and DnaD inhibit helix assembly of the DNA replication initiation protein DnaA. Mol Microbiol *90*, 147-159.

Setlow, P. (2007). I will survive: DNA protection in bacterial spores. Trends in microbiology *15*, 172-180.

Skarstad, K., and Katayama, T. (2013). Regulating DNA replication in bacteria. Cold Spring Harbor perspectives in biology *5*, a012922.

Slavoff, S.A., Heo, J., Budnik, B.A., Hanakahi, L.A., and Saghatelian, A. (2014). A human short open reading frame (sORF)-encoded polypeptide that stimulates DNA end joining. J Biol Chem *289*, 10950-10957.

Slavoff, S.A., Mitchell, A.J., Schwaid, A.G., Cabili, M.N., Ma, J., Levin, J.Z., Karger, A.D., Budnik, B.A., Rinn, J.L., and Saghatelian, A. (2013). Peptidomic discovery of short open reading frame-encoded peptides in human cells. Nat Chem Biol *9*, 59-64.

Soufo, C.D., Soufo, H.J., Noirot-Gros, M.F., Steindorf, A., Noirot, P., and Graumann, P.L. (2008). Cell-cycle-dependent spatial sequestration of the DnaA replication initiator protein in Bacillus subtilis. Dev Cell *15*, 935-941.

Staudt, A.C., and Wenkel, S. (2011). Regulation of protein function by 'microProteins'. EMBO reports *12*, 35-42.

Sterlini, J.M., and Mandelstam, J. (1969). Commitment to sporulation in Bacillus subtilis and its relationship to development of actinomycin resistance. The Biochemical journal *113*, 29-37.

Storz, G., Wolf, Y.I., and Ramamurthi, K.S. (2014). Small proteins can no longer be ignored. Annu Rev Biochem *83*, 753-777.

Stragier, P., Bonamy, C., and Karmazyn-Campelli, C. (1988). Processing of a sporulation sigma factor in Bacillus subtilis: how morphological structure could control gene expression. Cell *52*, 697-704.

Straub, D., and Wenkel, S. (2017). Cross-Species Genome-Wide Identification of Evolutionary Conserved MicroProteins. Genome biology and evolution *9*, 777-789.

Tanaka, K., Henry, C.S., Zinner, J.F., Jolivet, E., Cohoon, M.P., Xia, F., Bidnenko, V., Ehrlich, S.D., Stevens, R.L., and Noirot, P. (2013). Building the repertoire of dispensable chromosome regions in Bacillus subtilis entails major refinement of cognate large-scale metabolic model. Nucleic Acids Res *41*, 687-699.

VanOrsdel, C.E., Kelly, J.P., Burke, B.N., Lein, C.D., Oufiero, C.E., Sanchez, J.F., Wimmers, L.E., Hearn, D.J., Abuikhdair, F.J., Barnhart, K.R., *et al.* (2018). Identifying New Small Proteins in Escherichia coli. Proteomics *18*, e1700064.

Veening, J.W., Murray, H., and Errington, J. (2009). A mechanism for cell cycle regulation of sporulation initiation in Bacillus subtilis. Genes & development *23*, 1959-1970.

Westers, H., Dorenbos, R., van Dijl, J.M., Kabel, J., Flanagan, T., Devine, K.M., Jude, F., Seror, S.J., Beekman, A.C., Darmon, E., *et al.* (2003). Genome engineering reveals large dispensable regions in Bacillus subtilis. Mol Biol Evol *20*, 2076-2090.

Wu, L.J., and Errington, J. (2011). Nucleoid occlusion and bacterial cell division. Nat Rev Microbiol *10*, 8-12.

Wu, W., and Jin, S. (2005). PtrB of Pseudomonas aeruginosa suppresses the type III secretion system under the stress of DNA damage. J Bacteriol *187*, 6058-6068.

Yang, X., Jensen, S.I., Wulff, T., Harrison, S.J., and Long, K.S. (2016). Identification and validation of novel small proteins in Pseudomonas putida. Environmental microbiology reports *8*, 966-974.

Zanet, J., Benrabah, E., Li, T., Pelissier-Monier, A., Chanut-Delalande, H., Ronsin, B., Bellen, H.J., Payre, F., and Plaza, S. (2015). Pri sORF peptides induce selective proteasome-mediated protein processing. Science *349*, 1356-1358.

Zuber, P. (2001). A peptide profile of the Bacillus subtilis genome. Peptides *22*, 1555-1577.

10 Legends to figures

Figure 1

A) SknR transcriptionally repress the yqaF-yqaN operon of the skin element**. B-C) Yeast two hybrid interaction assay.** Haploid yeast strains expressing yqaH, dnaA and yabA infusion with the BD and AD domains of gal4 are separately introduced into haploid yeast strains. Binary interactions are tested by the ability of diploids to grow onto selective media. **D) DnaA interaction with YqaH and regulators.** Numbers refer to amino acid boundaries (see also Figure S1**).** Schematic representation of the architecture of the four functional domains of DnaA with associated functions as illustrated by colors. The binding of negative regulators to their targeted DnaA functional domain is illustrated accordingly. The YqaH interacting domains of DnaA with associated yeast 2HB interacting phenotypes (IP) are indicated. (+ and -) refers growth or absence of growth on selective media, reflecting interacting or loss of interaction phenotype, respectively.

Figure 2:

***YqaH* triggers *dnaA*-related phenotypes. A) Effect of *yqaH* expression on growth.** Cells carrying plasmids pDG148 (control, plain lines) or pDG148-yqaH (dashed lines) were examined in the presence of IPTG (0.5 mM) over 5 hours. Growth was monitored either by OD600 (left x-axe, black) or by cells viability, measured as the number of colony forming units per ml, normalized by OD600 (right x-axe, red). **B) Nucleoid morphological defects**. Samples of living cells (OD or time?) were examined by fluorescent microscopy after staining with of FM4-64 (membrane dye false-colored in red) and DAPI (DNA dye, false-colored in blue). White and yellow arrows indicate guillotined chromosomes resulting from septal closing over nucleoids and aberrant nucleoid segregation patterns, respectively and typical example of chromosomal segregation defects is magnified. Scale bares are 5 µm. **C) YqaH affects dnaA expression**. Cells harboring either the pDG148 or pDG148-yqaH were grown in LB in the presence of IPTG. RNAs from exponentially grown cells (OD600~0.3) were extracted and expression levels of the dnaA gene were monitored by qPCR in the presence (+) or absence (-) of YqaH (*** $P<0.001$).

Figure 3:

YqaH triggers sporulation and Spo0A-related phenotypes. A-B) YqaH expression inhibits sporulation. Cells carrying plasmids pDG148 (control) or pDG148-*yqaH* were grown in DSM media in the presence of IPTG (0.5 mM) added at the onset (t_0) of sporulation. Sporulant cells were observed at different time after initiation of sporulation. **A)** Snapshot captures of light and fluorescence microscopy at indicated t_{hrs} time. Cells were stained with fluorescent membrane dye FM4-64 (left panel). **B)** Sporulating cells were quantified by monitoring asymmetric septa, engulfed forespore and free spores, in the presence (+) or absence (-) of YqaH. Ratio were determined from observation of > 500 cells over 2 independent experiments and 3 biological replicates per experiment. **C) YqaH affects expression Spo0A-regulated genes**. Cells harboring either the

pDG148 or pDG148-yqaH were grown in DSM. IPTG was. Expression levels of *spoIIE* and *spoIIGA* genes from the Spo0A regulon were monitored by real-time qPCR in the presence (+) or absence (-) of YqaH and expressed as relative expression ratio compared to control (-) (*** P <0.001, n≥6).

Figure 4:

Restoration of replication initiation defects by YqaH_DnaA LOI mutation. Exponentially growing *B. subtilis* cells carrying either pDG148 (-), pDG148-*yqaH* (WT), pDG148-*yqaH* LOI mutant derivatives K17E or R56W were grown in the presence of IPTG and harvested at similar OD600~0.3 and assessed for various *dnaA*-related phenotypes: **(A) Nucleoid segregation and morphology**. Cells were treated with DAPI to reveal nucleoids (false-colored blue) and with the membrane dye FM4-64 (false colored red). **B-C-D) Analysis of DnaA-dependent replication initiation phenotypes. B**) Ori:ter ratio; Origin-proximal and Terminus proximal DNA sequences were quantified by qPCR. **C)** Visualization of origins foci in living cells. Origins are tagged through binding of the GFP-lacI repressor to LacO operator sequences inserted at proximal location from OriC. **D**) Averaged number of replication origins per cell determined as the number of LacI-GFP foci upon induced condition in control (-), WT and K17E- mutant derivative of YqaH. Statistical significance are illustrated by stars (t-test; . Ori:ter: n=6; *dnaA* mRNA: n=12; P< 0.01 *; P<0.001**). **E**) **Effect of YqaH LOI mutant on dnaA expression**. Cells harboring either the pDG148, pDG148-yqaHWT or K17E and R56W mutated derivatives were grown in LB in the presence of IPTG. RNAs from exponentially grown cells (OD600~0.3) were extracted and expression levels of the dnaA gene were monitored by qPCR in the presence (+) or absence (-) of YqaH (*** P<0.001).

Figure 5:

Role of K17E DnaA_LOI substitution in sporulation and biofilm formation. A) 168 and Tf8 strains carrying plasmids pDG148 (control) or pDG148-*yqaH* and K17E mutant were grown in DSM media in the presence of IPTG (0.5 mM) added at the onset (t_0) of sporulation. Sporulant cells were observed - hours after initiation of sporulation (as described in figure 3). **B**) Surface rendering of biofilms pellicles of 168 strain expressing yqaH or yqaH-K17E mutant after 48h in Mgss media. Biomass was averaged from 8 samples. Pairwise comparisons were performed using the Tukey Method (*p <= 0.05 **p <= 0.01 ***p <= 0.001).

Table 1:

YqaH_LOI mutational screen. Aminoacid substitution affecting interaction with DnaA and/or DnaN are indicated with their associated interaction phenotype. (-) refers as a total loss of interaction phnotype leading to absence of growth on both –LUH and –LUA media. (+/-) refers as a partial loss of interaction leading to some growth on the –LUH but not –LUA; see also Figure S1 for additional explanation.

Figure 1

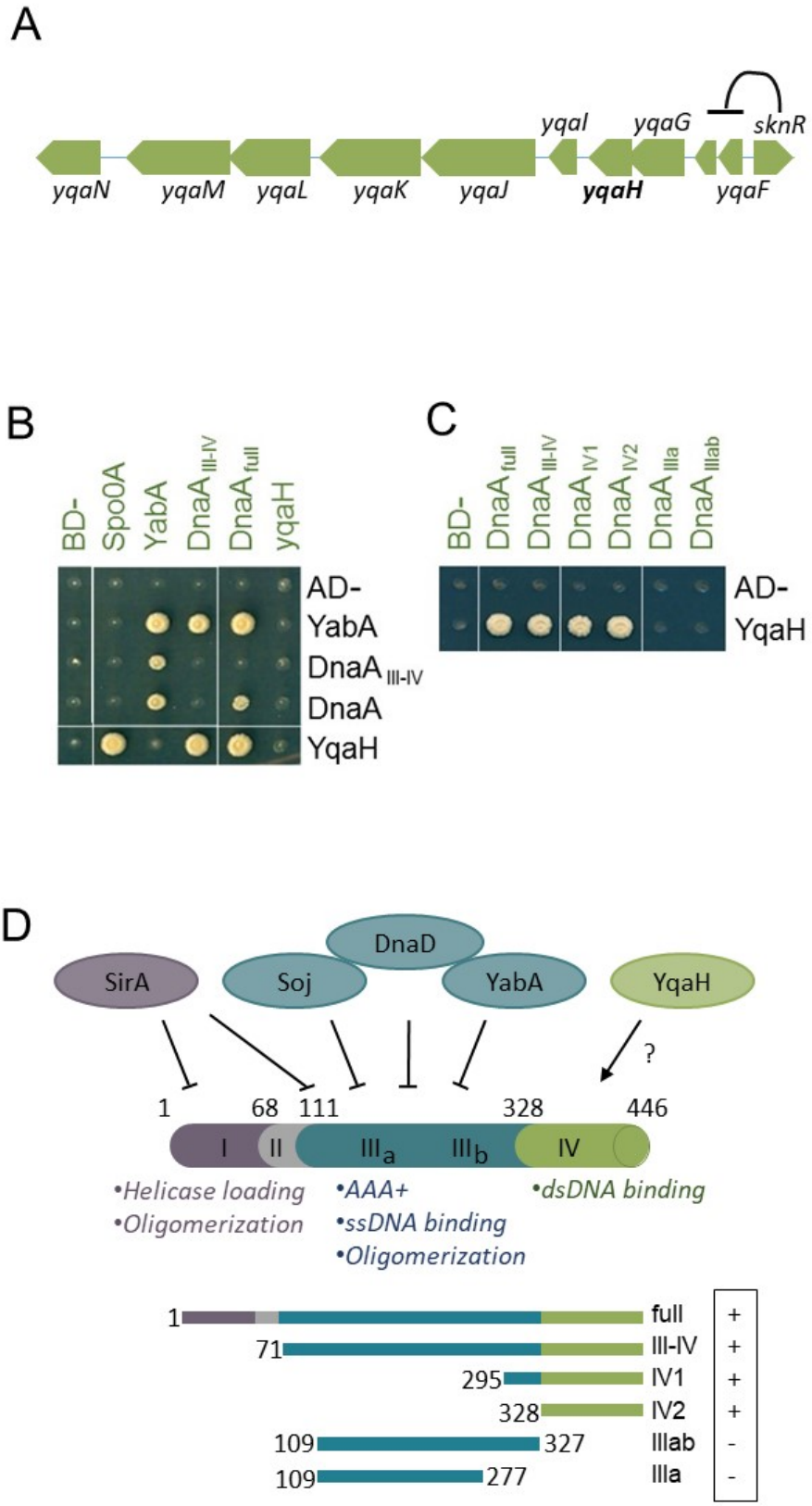
A
yqaI
yqaG
sknR
yqaN
yqaM
yqaL
yqaK
yqaJ
yqaH
yqaF
B
BD-
Spo0A
YabA
DnaA III-IV
DnaA full
yqaH
AD-
YabA
DnaA III-IV
DnaA
YqaH
C
BD-
DnaA full
DnaA III-IV
DnaA IV1
DnaA IV2
DnaA IIIa
DnaA IIIab
AD-
YqaH
D
DnaD
SirA
Soj
YabA
YqaH
?
1
68
111
328
446
I
II
IIIa
IIIb
IV
•Helicase loading
•Oligomerization
•AAA+
•ssDNA binding
•Oligomerization
•dsDNA binding
1
full
+
71
III-IV
+
295
IV1
+
328
IV2
+
109
327
IIIab
-
109
277
IIIa
-

Figure 2

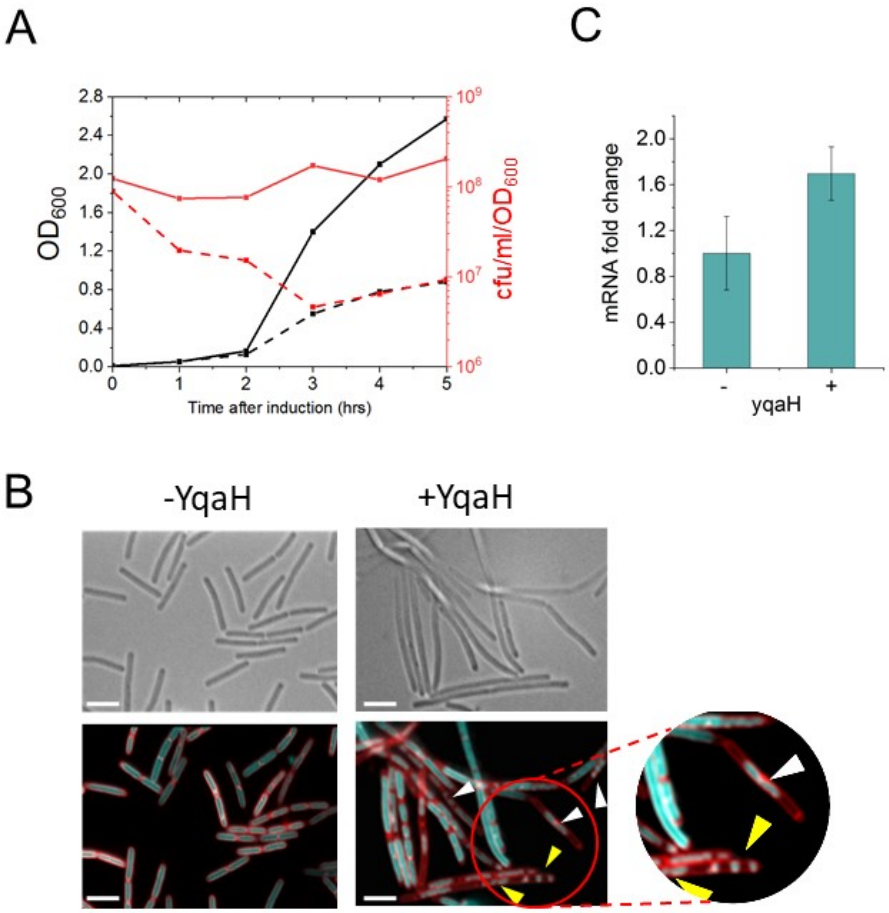
A
C
B
-YqaH
+YqaH
OD600
cfu/ml/OD600
Time after induction (hrs)
mRNA fold change
yqaH
-
+

Figure 3

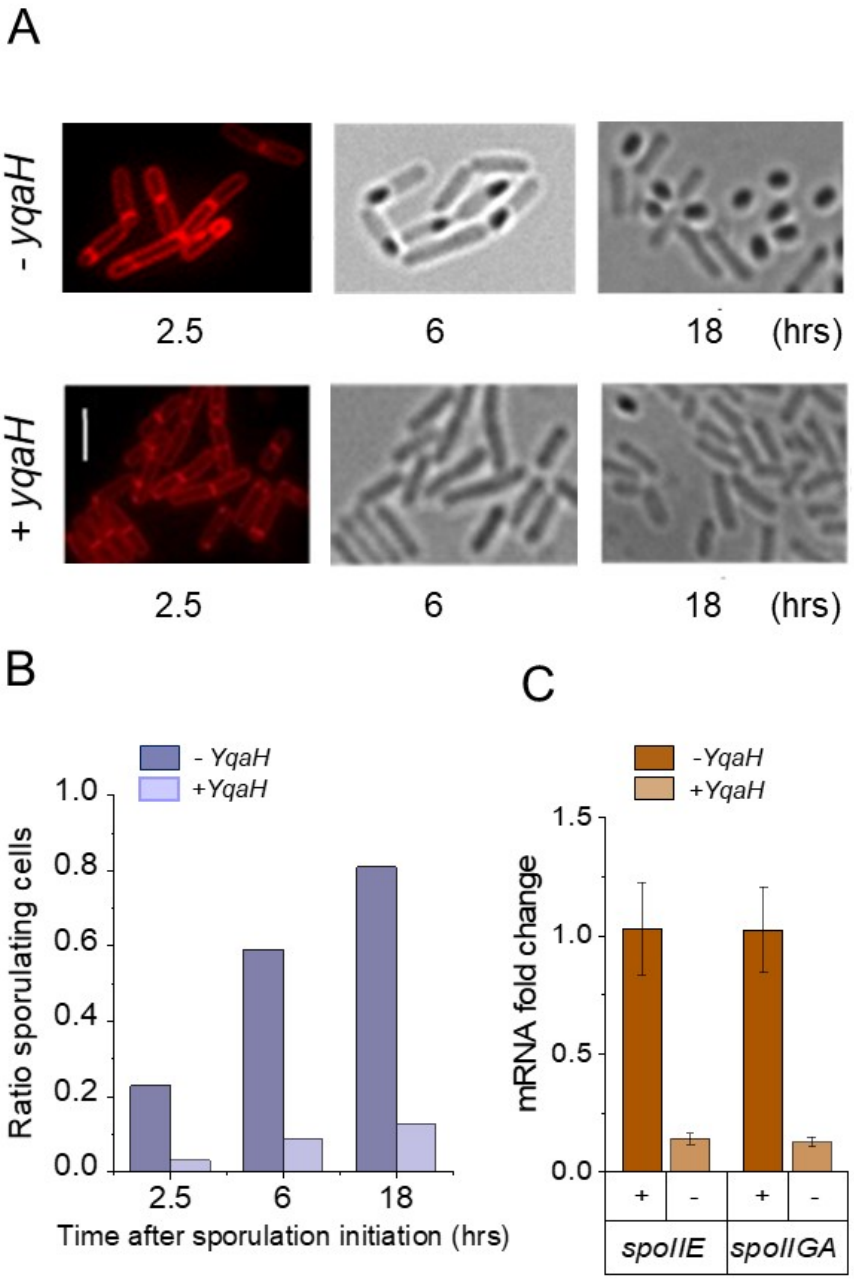
A
- yqaH
2.5
6
18
(hrs)
+ yqaH
2.5
6
18
(hrs)
B
- YqaH
+YqaH
Ratio sporulating cells
1.0
0.8
0.6
0.4
0.2
0.0
2.5
6
18
Time after sporulation initiation (hrs)
C
-YqaH
+YqaH
mRNA fold change
1.5
1.0
0.5
0.0
+
-
+
-
spoIIE
spoIIGA

Figure 4

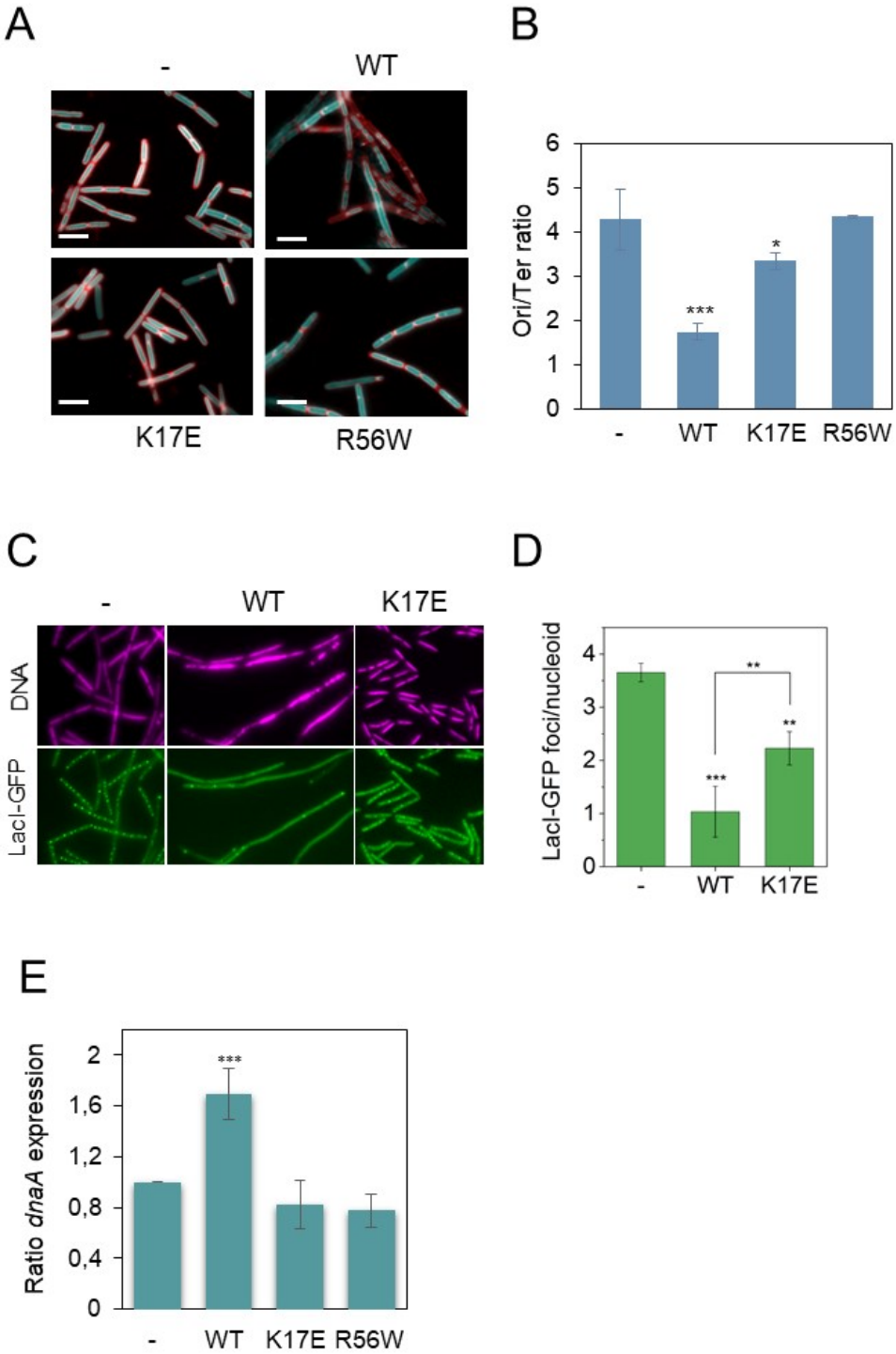
A
-
WT
K17E
R56W
B
Ori/Ter ratio
6
5
4
3
2
1
0
***
*
-
WT
K17E
R56W
C
-
WT
K17E
DNA
LacI-GFP
D
LacI-GFP foci/nucleoid
4
3
2
1
0
**
***
**
-
WT
K17E
E
Ratio dnaA expression
2
1,6
1,2
0,8
0,4
0
***
-
WT
K17E
R56W

Figure 5

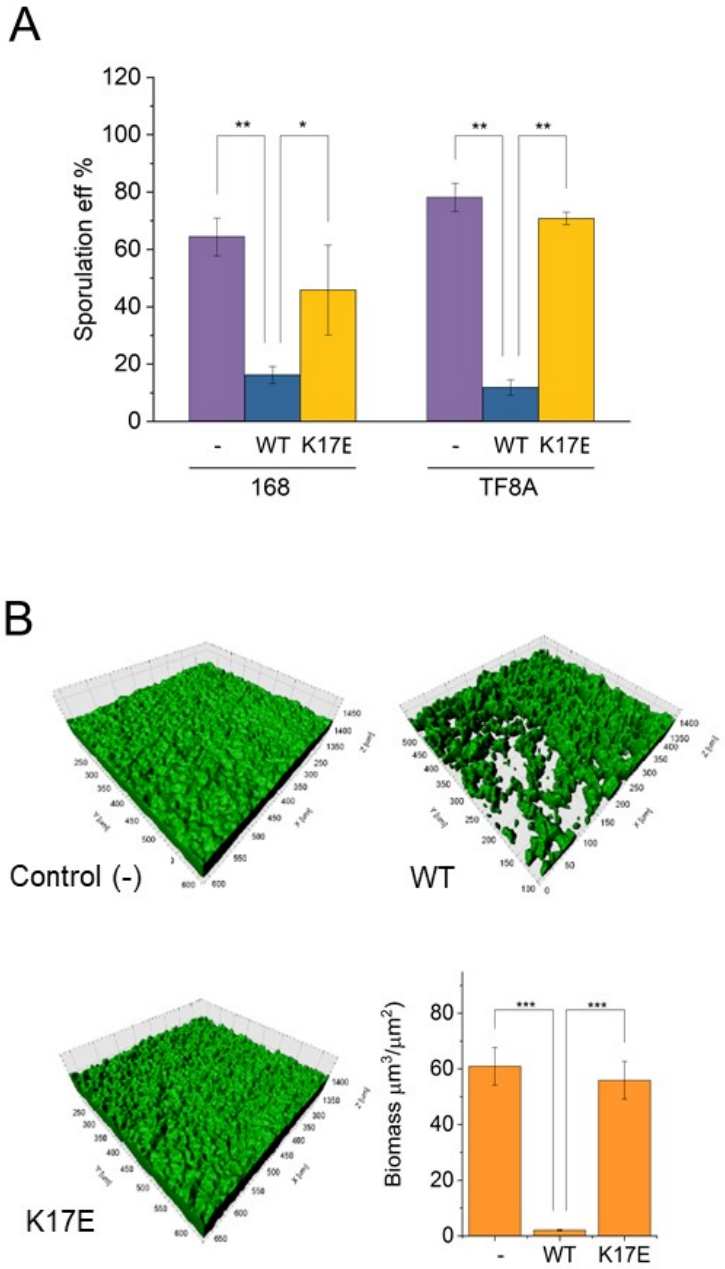
A
Sporulation eff %
120
100
80
60
40
20
0
**
*
**
**
-
WT
K17E
-
WT
K17E
168
TF8A
B
Control (-)
WT
K17E
Biomass µm³/µm²
80
60
40
20
0
***
***
-
WT
K17E

Table 1

		DnaA	Spo0A
YqaH_LOI	D10G	-	-
	R16G	+/-	+
	K17E	-	+
	S20L	-	-
	S25T	+/-	+/-
	M27T	+/-	+
	L28P	-	-
	Y37H	+/-	+
	E38K	-	+
	E38V	+/-	+
	A40T	+/-	+/-
	L43P	-	-
	A44V	+/-	+
	K48E	-	+
	R56W	-	+
	L57P	-	-