

# Characterization of YvcJ, a Conserved P-Loop-Containing Protein, and Its Implication in Competence in *Bacillus subtilis*<sup>∇†</sup>

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**The uncharacterized protein family UPF0042 of the Swiss-Prot database is predicted to be a member of the conserved group of bacterium-specific P-loop-containing proteins. Here we show that two of its members, YvcJ from *Bacillus subtilis* and YhbJ, its homologue from *Escherichia coli*, indeed bind and hydrolyze nucleotides. The cellular function of *yvcJ* was then addressed. In contrast to results recently obtained for *E. coli*, which indicated that *yhbJ* mutants strongly overproduced glucosamine-6-phosphate synthase (GlmS), comparison of the wild type with the *yvcJ* mutant of *B. subtilis* showed that GlmS expression was quite similar in the two strains. However, in mutants defective in *yvcJ*, the transformation efficiency and the fraction of cells that expressed competence were reduced. Furthermore, our data show that YvcJ positively controls the expression of late competence genes. The overexpression of *comK* or *comS* compensates for the decrease in competence of the *yvcJ* mutant. Our results show that even if YvcJ and YhbJ belong to the same family of P-loop-containing proteins, the deletion of corresponding genes has different consequences in *B. subtilis* and in *E. coli*.**

Functional annotation of genomes is a major challenge of the postgenomic era, especially for eukaryotes, which have many more genes than prokaryotes. Nevertheless, prokaryotic genomes have revealed an unexpected complexity, since about one-third of their genes code for proteins of unknown functions (13). Among these proteins, some contain a specific signature in their sequence known as the Walker A motif (39). This signature [(G/A)X<sub>4</sub>GK(T/S)] is often used as a fingerprint to detect new putative ATP- or GTP-binding proteins. Indeed, the 3-dimensional structures show that proteins bearing this motif invariably form a loop that wraps around the polyphosphate moiety of the bound nucleotide, and hence it is referred to as the P-loop motif (35). However, the presence of this motif is not enough to clarify the physiological role of these proteins. Furthermore, the sequencing of genomes has revealed that genes encoding P-loop proteins are numerous in both prokaryotic and eukaryotic genomes (20), and many of them have evaded characterization.

In this study, we have investigated a bacterial P-loop-containing protein that belongs to the uncharacterized protein family UPF0042 of the Swiss-Prot database. This protein is conserved in several bacteria whose genomes have been sequenced. This broad conservation would suggest a central role in bacterial physiology. In *Bacillus subtilis* and *Escherichia coli*, the paradigmatic gram-positive and gram-negative bacteria, the designations of the corresponding genes are *yvcJ* and *yhbJ*, respectively. Nevertheless, neither *yvcJ* nor *yhbJ* is an essential gene in these two bacteria. In several proteobacteria, *yhbJ* is

found with genes related to the phosphoenolpyruvate:carbohydrate phosphotransferase system (4), whereas in most gram-positive bacteria, *yvcJ* is clustered with *yvcK*. The latter gene is required for the growth of *B. subtilis* on substrates of the pentose phosphate pathway and Krebs cycle intermediates, and its deletion has been shown to have a pleiotropic effect, since it affects the cell envelope and cell shape (14). A previous study of the *yvcIJKL-crh-yvcN* operon in *B. subtilis* showed that the deletion of *yvcJ* does not affect bacterial growth in any of the carbon sources tested (14). This observation would suggest a broader role of this operon in cell metabolism apart from its role in carbon source utilization.

Recently, it was shown that *E. coli* YhbJ is involved in glucosamine-6-phosphate synthase (GlmS) expression, by affecting the processing and stability of a small RNA, GlmZ (24, 32). The molecular mechanism that regulates the processing of GlmZ in *E. coli* is still unknown, but the authors suggest a pleiotropic role for YhbJ in regulating the activities of genes or proteins involved in RNA turnover control. In the gram-positive bacterium *B. subtilis*, the genetic mechanism for the control of *glmS* expression is well characterized (2, 42). Briefly, the 5' untranslated mRNA of the *glmS* gene contains a riboswitch, a highly structured domain sensitive to glucosamine-6-phosphate, which acts as a ribozyme. Indeed, direct activation of this ribozyme in the presence of glucosamine-6-phosphate leads to the processing of the *glmS* transcript by a site-specific self-cleavage, thereby repressing the expression of *glmS*. The participation of YvcJ in this mechanism has never been mentioned.

In this paper, we have biochemically characterized YvcJ and its homologue YhbJ, and we have investigated the cellular function of YvcJ in *B. subtilis*.

## MATERIALS AND METHODS

**Plasmids, bacterial strains, and general growth conditions.** The strains used in this study are listed in Table 1. DNA manipulations were performed by standard

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TABLE 1. *B. subtilis* strains used in this study

Strain	Genotype or description	Source or reference
168	<i>trpC2</i>	Laboratory stock
SG91	<i>trpC2 ΔyvcJ::cat</i>	This work
SG106	<i>trpC2 ΔyvcJ::cat amyE::yvcI kan</i>	This work
SG107	<i>trpC2 ΔyvcJ::cat amyE::yvcIJ kan</i>	This work
BD3836	<i>hisB2 leu-8 metB5 amyE::P<sub>his</sub>-comK spec</i>	27
SG119	<i>trpC2 amyE::P<sub>his</sub>-comK spec</i>	BD3836→168
SG120	<i>trpC2 ΔyvcJ::cat amyE::P<sub>his</sub>-comK spec</i>	SG119→SG91
BD2528	<i>hisB2 leu-8 metB5 pUB110 comS kan</i>	15
SG121	<i>trpC2 pUB110 comS kan</i>	BD2528→168
SG122	<i>trpC2 ΔyvcJ::cat pUB110 comS kan</i>	SG121→SG91
SG131	<i>trpC2 ΔyvcJ::tet</i>	This work
BD3196	<i>hisB2 leu-8 metB5 Δrok::kan</i>	1
BD2711	<i>hisB2 leu-8 metB5 comK-gfp cat</i>	37
SG147	<i>hisB2 leu-8 metB5 Δrok::kan comK-gfp cat</i>	BD2711→BD3196
SG152	<i>hisB2 leu-8 metB5 ΔyvcJ::tet Δrok::kan</i>	SG131→SG147
BD1243	<i>PcomK-gfp cat</i> <i>hisAl leuA8 metB5 comA124::Tn9171acZ</i>	33

procedures. Luria-Bertani (LB) broth was routinely used for bacterial growth. CSK-citrate contains 70 mM K<sub>2</sub>HPO<sub>4</sub>, 30 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 mM MgSO<sub>4</sub>, 10 μM MnSO<sub>4</sub>, 22 mg/liter ferric ammonium citrate, 20 mg/liter L-Trp, 8 g/liter potassium glutamate, 1 mM MgCl<sub>2</sub>, and 0.5% (wt/vol) citrate. Competence experiments were carried out in MD medium, containing 10.7 g/liter K<sub>2</sub>HPO<sub>4</sub>, 6 g/liter KH<sub>2</sub>PO<sub>4</sub>, 1 g/liter trisodium citrate · 5H<sub>2</sub>O, 2% (wt/vol) glucose, 50 mg/liter L-Trp, 11 mg/liter ferric ammonium citrate, 2.5 g/liter potassium aspartate, and 3 mM MgSO<sub>4</sub>. When necessary, the media were supplemented with the appropriate antibiotics (ampicillin at 100 μg/ml for *E. coli*; chloramphenicol at 5 μg/ml, spectinomycin at 150 μg/ml, and kanamycin at 10 μg/ml for *B. subtilis*). *E. coli* and *B. subtilis* cells were transformed with DNA by standard procedures (25, 34). Sequencing of PCR-derived DNA fragments in the final plasmid constructs was carried out by Genome Express (Meylan, France).

**Deletion of the *yvcJ* gene.** For the deletion of the *B. subtilis yvcJ* gene, its flanking sequences were amplified by PCR with two pairs of primers (see the list of primers in Table S1 in the supplemental material): BG1 (*yvcJ* [-20 to -3]) and BG49 (*yvcJ* [+477 to +459]), containing a BamHI site, and EF1 (*yvcK* [-83 to -61]) and BG56 (*yvcK* [+954 to +936]), containing an EcoRI site. These two PCR products were digested by BamHI or EcoRI. In parallel, a chloramphenicol or tetracycline resistance cassette without promoter and transcriptional terminators was amplified from pAC5 (29) or pDG1515 (23), respectively, using primers EF2 (*cat* [-19 to +6]) and EF3 (*cat* [+651 to +630]) or 5'-tet and 3'-tet, and was then digested at the BamHI and EcoRI sites within the primers. After ligation of the three fragments, the resulting DNA was reamplified using primers BG1 and BG56 before transformation of *B. subtilis* strain 168 by a double-crossover event and selection for either chloramphenicol or tetracycline resistance. In the resulting strain SG91 or SG131, the *cat* gene or the *tet* gene, which replaces the *yvcJ* gene, is expressed from the promoter located in front of *yvcI*, like the other genes of the operon, *yvcK*, *yvcL*, *crh*, and *yvcN*, located downstream (14).

**Strains for complementation.** Two PCR fragments containing the promoter region with either the *yvcI* gene or the *yvcIJ* genes were amplified with two pairs of primers, BG38 (promoter region [-100 to -81])–BG49 and BG38–BG67 (*yvcJ* [+888 to +870]), and were digested at the BamHI and EcoRI sites within the primers. These fragments were then ligated into the BamHI–EcoRI sites of pAC7 (41), so as to place these fragments between the arms of *amyE*, and were transformed into SG91 to create strains SG106 and SG107.

**Plasmid construction for protein purification.** Fragments containing the *yvcJ* gene or the *yhbJ* gene were amplified by PCR using chromosomal DNA of *B. subtilis* strain 168 or *E. coli* strain K-12 and two specific primers for each amplification; one containing a BamHI or EcoRI site and another containing an XhoI site. The BamHI–XhoI fragment containing the *yvcJ* gene or the EcoRI–XhoI fragment containing the *yhbJ* gene was cloned into the expression vector pET21a(+) (Novagen). A modified YvcJ or YhbJ protein, carrying a T7 tag fused to the N terminus and a polyhistidine tag fused to the C terminus, was expressed from the resulting plasmid pJL1 or pJL2 after transformation in the *E. coli* strain C41(DE3) (30). Then YvcJ or YhbJ was affinity purified on nickel-nitrilotriacetic acid resin (Qiagen) as previously described (12).

**Limited proteolysis.** Six micrograms of YvcJ was mixed with 40 mM NaCl, 10 mM Tris-HCl (pH 8.0) in the absence or presence of 1 mM ATP or GTP. After addition of 0.6 μg of endoproteinase Glu-C (Promega), the reaction mixture was

incubated for 0, 5, 15, or 30 min at 37°C. Digestion was stopped by addition of an equal volume of electrophoresis loading buffer to the assay mixtures and by heating for 5 min at 100°C before the samples were subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Fluorescence measurements.** All experiments were performed at 25 ± 0.1°C using a SAFAS flx-Xenius 5117 spectrofluorimeter. All spectra were corrected for buffer fluorescence. Fluorescence measurements were routinely carried out after dilution of YvcJ or YhbJ (final concentration, 0.5 μM) and equilibration for 5 min in 2 ml of a buffer containing 25 mM HEPES-KOH (pH 8) and 1 mM MgCl<sub>2</sub>.

Increasing concentrations of *N*-methylanthraniloyl (Mant) derivatives (Molecular Probes) were then added, and the emission fluorescence was scanned in the range of 300 to 500 nm, upon excitation at 282 nm. Fluorescent resonance energy transfer (FRET) between tryptophan residues of purified protein and bound Mant nucleotide derivatives was monitored by the appearance of a fluorescence emission peak between 400 and 500 nm, characteristic of bound nucleotide analogues. Peak integration was carried out for each ligand concentration, and the observed FRET was used for the calculation of ligand affinity. Each measure was determined in triplicate, and the curve fitting of the data was performed by using Graphit (version 4.0) software as previously described (11).

**Enzymatic activity measurements.** Enzymatic activities were measured at 37°C. For nucleoside triphosphatase (NTPase) activity, the buffer contained 25 mM morpholinepropanesulfonic acid (MOPS)-HCl (pH 7.5), 5 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 100 mM NaCl, 2 mM dithiothreitol (DTT), and 5 mM ATP or GTP, in the presence of an auxiliary regenerating system coupled to the oxidation of 0.3 mM NADH (4 mM phosphoenolpyruvate, 30 μg of pyruvate kinase, and 16 μg lactate dehydrogenase). The addition of 30 μg of purified protein (YvcJ or YhbJ) in a total volume of 1 ml initiated the reaction, and then NADH oxidation was recorded at 340 nm for 5 min. For *p*-nitrophenyl phosphatase (pNPPase) activity, the buffer contained 50 mM Tris-HCl (pH 7.4), 1 mM DTT, and 5 mM pNPP in a total volume of 100 μl. The addition of 2 μg of YvcJ or YhbJ initiated the reaction, and pNPP hydrolysis was recorded at 405 nm for 10 min. The kinetic parameters (*K<sub>m</sub>* and *V<sub>max</sub>*) were determined by double-reciprocal plots.

**mRNA isolation.** Bacteria were cultivated in competence medium until the transition phase was reached. Then 1 ml of the cell suspension was harvested, and RNA was prepared using the standard protocol with the High Pure RNA isolation kit (Roche, Basel, Switzerland) except that two DNase treatments were performed to avoid any DNA contamination. All the isolations were performed at least in triplicate from independent cultures.

**q-RT-PCR.** One microgram of RNA was reverse transcribed using the standard protocol with Superscript II reverse transcriptase (Invitrogen) and 100 ng of random primers. The resulting cDNA was diluted (1/16), and 5 μl was used for quantitative reverse transcription-PCR (q-RT-PCR). This step was performed on a Mastercycler ep realplex instrument (Eppendorf) by using the SYBR Premix Ex Taq (Perfect Real Time) PCR kit (Takara Bio Group, Japan) according to the manufacturer's instructions in a final volume of 20 μl. Specific primers are described in Table S1 in the supplemental material. Melting curves were analyzed to control for the specificity of the PCRs. Data from three independent experiments were analyzed and normalized with the software supplied with the Mastercycler. The relative units were calculated from a standard curve plotting four different dilutions (1/80, 1/400, 1/2,000, and 1/10,000) against the PCR cycle number at which the measured fluorescence intensity reached the threshold (*C<sub>T</sub>*), specified so that it is significantly above the noise band of the baseline (10 times the standard deviation).

**Global transcriptional analysis.** Experiments were done using DNA microarrays from Eurogentec (Seraing, Belgium). The Pronto Plus system (Promega, Madison, WI) was used, by following the protocol described by the manufacturer, to label cDNA by direct incorporation of Cy3- and Cy5-dCTP. Hybridization was performed at 42°C for 16 h with DIG Easy hybridization solution (Roche, Basel, Switzerland) and herring sperm DNA (10 mg/ml; 1 μl added to 30 μl hybridization solution). After hybridization, the glass array was washed using 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS for 5 min, 0.2× SSC for 2 min, and 0.1× SSC for 2 min, dried by centrifugation (1,000 rpm for 1 min), and then scanned with a Packard BioChip ScanArray, model 5000 (Perkin-Elmer), at a resolution of 10 μm per pixel. Image and data analyses were performed with GenePix Pro (version 6.0) and Acuity (version 4.0) software (Axon Instruments, Inc.) using locally weighted scatterplot smoothing (LOWESS) normalization (3). Data from spots with diameters of <90 μm and with signal intensities lower than twice the background were removed. Microarray analysis was done for three independent experiments.

**Measurement of transformation frequencies.** Strains were grown in MD medium at 37°C with shaking. Cells were transformed 2 h after the onset of stationary phase (*T*<sub>2</sub>), as described in reference 25, with chromosomal DNA

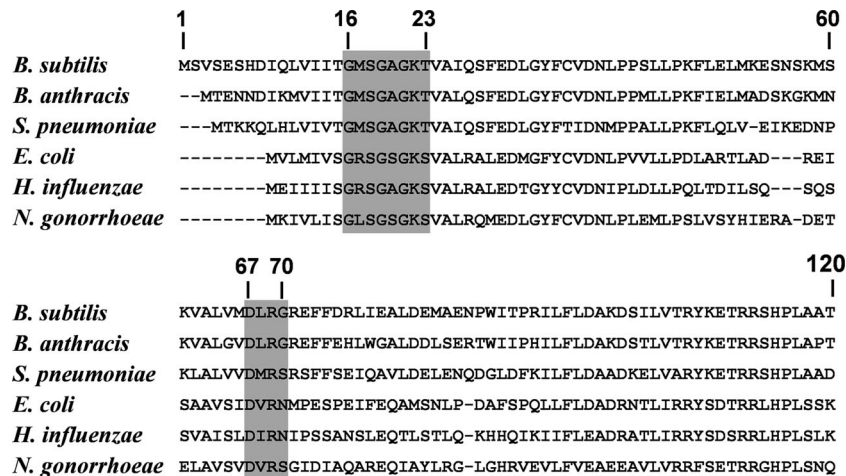


FIG. 1. Partial sequence alignment of YvcJ homologues in various bacteria, including *Bacillus anthracis*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria gonorrhoeae*, as well as *B. subtilis* and *E. coli*. The putative Walker A and B motifs are indicated by the two shaded boxes. Walker A is normally quoted in the literature by the consensus sequence (A/G)<sub>4</sub>GK(T/S), which is centered at a loop between a  $\beta$ -strand and an  $\alpha$ -helix (35, 39). The Walker B motif, much less conserved, can be represented by the consensus sequence DX<sub>2</sub>G (38), located at the end of a hydrophobic  $\beta$ -sheet.

carrying either a spectinomycin or a kanamycin marker, and were then plated on LB medium in the presence and absence of antibiotic. The transformation frequency was expressed as the ratio between the number of transformants per milliliter and the number of cells per milliliter.

**Phosphorylation assays.** Bacteria were grown at 37°C in 400 ml MD medium and were collected at the onset of stationary phase. They were then harvested by low-speed centrifugation, resuspended in 4 ml of lysis buffer containing 100 mM Tris-HCl (pH 7.4), 10.5 mM  $\beta$ -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 5 mg/ml lysozyme, and 10 U/ml of benzonase (Merck), and disrupted by sonication. After centrifugation, the supernatant was dialyzed against 100 mM Tris-HCl (pH 7.4) and 140  $\mu$ M  $\beta$ -mercaptoethanol. Phosphorylation assays were carried out in a total volume of 20  $\mu$ l. Three micrograms of YvcJ was incubated for 15 min at 37°C with 100  $\mu$ g of crude extract in 50 mM Tris · HCl (pH 7.4), 5 mM MgCl<sub>2</sub>, 1 mM DTT, and 50  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (2  $\mu$ Ci). The phosphorylation reaction was quenched by addition of an equal volume of SDS sample buffer to the reaction mixtures before SDS-PAGE analysis. Gels were then dried and exposed to autoradiography.

**Microarray data accession number.** All DNA microarray data, including the detailed protocol, the slide images, and the raw data, obtained in this study are available online at Array Express (<http://www.ebi.ac.uk/arrayexpress>; accession number: E-MEXP-1471).

## RESULTS

**ATP and GTP bind to *B. subtilis* YvcJ and induce conformational changes.** The presence of Walker A and B motifs in the N-terminal part of YvcJ (Swiss-Prot accession no. O06973) suggests that it is able to bind and hydrolyze ATP or GTP (Fig. 1) (39). In order to analyze its ability to bind nucleotides, the protein was overexpressed, purified, and assessed for its sensitivity to limited protease digestion. As shown in Fig. 2A, in the absence of nucleotides, YvcJ is sensitive to the endoprotease Glu-C. Indeed, after 30 min of digestion, the band at around 30 kDa, corresponding to YvcJ, is degraded, and only bands below 14 kDa are still detectable. In contrast, in the presence of ATP or GTP, YvcJ is more resistant to proteolysis, since bands around 30 kDa were still visible after 30 min of exposure to the endoprotease Glu-C. This indicates direct binding of both ATP and GTP to YvcJ, and this binding probably induces conformational changes in YvcJ that result in protection from endoprotease Glu-C cleavage.

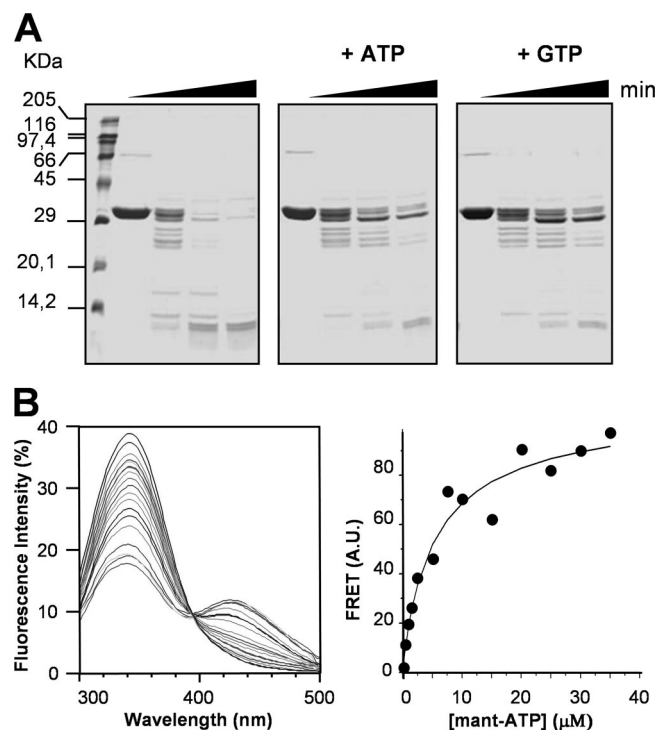


FIG. 2. YvcJ is a nucleotide-binding protein. (A) Coomassie blue-stained SDS-PAGE gel showing a partial proteolysis profile of YvcJ. YvcJ was incubated with endoprotease Glu-C (Promega) in the absence or presence of ATP or GTP for 0, 5, 15, or 30 min at 37°C. The digestion profiles were assessed by 15% SDS-PAGE. (B) Effect of Mant-ATP on the fluorescence of YvcJ. Increasing concentrations of Mant-ATP (from 0  $\mu$ M to 40  $\mu$ M) were added to a 2-ml assay medium containing 25 mM HEPES-KOH (pH 8.0), 1 mM MgCl<sub>2</sub>, and 0.5  $\mu$ M YvcJ, and the fluorescence intensity was recorded after each addition. The FRET, taken as the increase in fluorescence between 400 and 500 nm, was plotted against the concentration of Mant-ATP. A.U., arbitrary units.

Since YvcJ contains two tryptophans in its sequence (Trp-89 and Trp-223), we investigated the nucleotide binding by fluorescence measurements. The binding of the fluorescent nucleotide analogues Mant-ATP and Mant-GTP was measured. The addition of increasing concentrations of Mant nucleotides to YvcJ progressively quenched the Trp fluorescence emission peak (centered at around 340 nm) and gave rise concomitantly to a new peak of fluorescence (centered at around 430 nm). This new peak corresponds to a FRET between Trp residues and the Mant moiety (Fig. 2B). Fitted  $K_D$  (equilibrium dissociation constant) values were obtained from the plot of the FRET intensity against the Mant-ATP or Mant-GTP concentrations; they indicate that YvcJ possesses a better affinity for Mant-GTP than for Mant-ATP (apparent  $K_D$  values,  $0.46 \pm 0.04 \mu\text{M}$  and  $3 \pm 0.7 \mu\text{M}$ , respectively).

**YvcJ has GTPase and ATPase activities.** Since YvcJ binds nucleotides and possesses a Walker B motif often found in GTPases (38), we tested if YvcJ exhibited an intrinsic ATPase or GTPase activity. The NTPase activities were studied by a coupled enzymatic assay following NADH oxidation at 340 nm. Our results indicate that YvcJ is able to hydrolyze  $14.1 \text{ mol of ATP} \cdot \text{min}^{-1} \cdot \text{mol of enzyme}^{-1}$  and  $28.3 \text{ mol of GTP} \cdot \text{min}^{-1} \cdot \text{mol of enzyme}^{-1}$  in the presence of magnesium and, to a lesser extent, manganese (data not shown). Thus, GTP is hydrolyzed with a higher efficiency than ATP. In order to test if YvcJ is able to hydrolyze other substrates apart from nucleotides, we tested pNPP, which is often used to measure protein phosphatase activity but has also been found to be hydrolyzed by the  $\text{Na}^+/\text{K}^+$ -ATPase (22). Our results indicate that YvcJ is able to hydrolyze  $20.9 \text{ mol of pNPP} \cdot \text{min}^{-1} \cdot \text{mol of enzyme}^{-1}$ . To check if pNPPase and NTPase activities are interconnected, we carried out competition experiments. For this purpose, we measured pNPPase activities in a range of 2 to 10 mM pNPP in the presence of a constant concentration (4 mM) of ATP. We observed that the  $V_{\text{max}}$  of the enzyme did not change, but the  $K_m$  for pNPP increased (from 14.7 mM in the absence of ATP to 84.5 mM in the presence of 4 mM ATP), suggesting competitive inhibition between pNPP and ATP for YvcJ.

**YhbJ from *E. coli* is also a nucleotide-binding protein with NTPase activity.** YvcJ and YhbJ (Swiss-Prot accession no. P33995) possess 38% identity. Since YhbJ also contains two tryptophans (Trp-190 and Trp-228) in its sequence, we tested its ability to bind to nucleotides by fluorescence measurements as described previously. The binding of Mant-ATP and Mant-GTP was observed and measured. The  $K_D$  values obtained with YhbJ are similar to those obtained with YvcJ and indicate that YhbJ also has a better affinity for Mant-GTP than for Mant-ATP (apparent  $K_D$  values,  $0.65 \pm 0.03 \mu\text{M}$  and  $1.45 \pm 0.54 \mu\text{M}$ , respectively). We also investigated the enzymatic properties of YhbJ. We observed that YhbJ also displays NTPase and pNPPase activities with  $K_m$  values of the same magnitude but with a maximum velocity about 10-fold lower than that of YvcJ. Indeed, YhbJ was able to hydrolyze  $1.75 \text{ mol of pNPP} \cdot \text{min}^{-1} \cdot \text{mol of enzyme}^{-1}$ , with a  $K_m$  of 8.65 mM.

**Deletion of *yvcJ* affects competence efficiency.** In order to examine the role of YvcJ in *B. subtilis*, we first constructed a nonpolar mutant strain, SG91 (*yvcJ::cat*), in which *yvcJ* was replaced by a *cat* cassette, since the *yvcK* gene, located just downstream of *yvcJ*, was crucial for growth on some poor carbon sources (14). We then analyzed the crude extract from

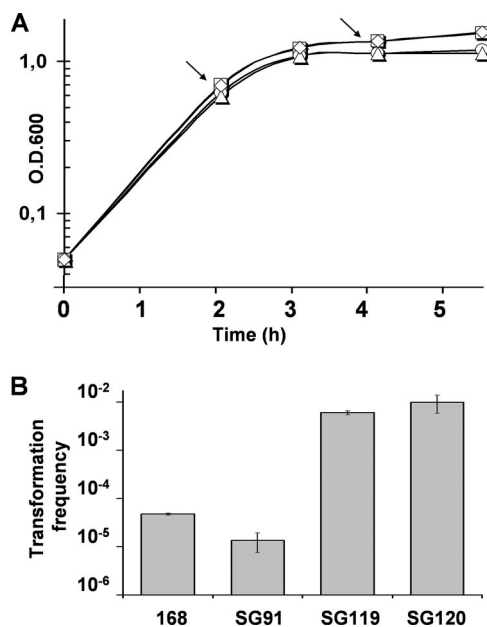


FIG. 3. Growth curves (A) and transformation frequencies (B) of wild-type and *yvcJ* mutant strains in which ComK was overexpressed. Wild-type strain 168 (squares) and mutant strains SG91 (diamonds), SG119 (circles), and SG120 (triangles) were grown on MD medium. *comK* expression was induced by 1 mM IPTG at an optical density at 600 nm of 0.7 (first arrow), and cells were transformed 2 h afterwards (second arrow) by using 0.5  $\mu\text{g}$  of a chromosomal DNA carrying a kanamycin marker. Transformation frequencies were determined by selection for  $\text{Km}^r$ . The transformation frequency corresponds to the ratio between the number of transformants per milliliter and the number of cells per milliliter.

*yvcJ* mutant cells after growth on LB medium by SDS-PAGE and staining with Coomassie blue. In contrast to recent results obtained with *E. coli* (strong overexpression of GlmS in a *yhbJ* mutant strain [24]), no obvious accumulation of proteins was detected (data not shown). We also monitored growth in other media, but no difference in growth curves was observed between the wild-type and mutant strains (Fig. 3A) (reference 14 and data not shown). We then undertook a phenotype screening. In particular, we tested if motility, biofilm formation, the secretion of degradative enzymes, competence, and sporulation were affected in the *yvcJ* mutant strain compared to the wild-type strain (data not shown). No obvious differences were detected between the two strains except for their competence efficiencies. Indeed, we observed that the transformation frequency of the mutant strain was about eightfold lower than that of the wild-type strain ( $0.6 \times 10^{-5}$  for SG91 versus  $4.6 \times 10^{-5}$  for the wild-type strain). To determine if the competence phenotype observed for strain SG91 was due only to the disruption of the *yvcJ* gene, we carried out complementation experiments by expressing *yvcJ* from the *amyE* locus under the control of its own promoter. When the frequency of transformation of SG106 (*yvcJ::cat amyE::yvcJ*) was compared to that of the isogenic strain SG107 (*yvcJ::cat amyE::yvcJII*), strain SG106 showed no complementation of the *yvcJ* mutation by *yvcI*. Strain SG107 grew more slowly than the wild-type strain, but the expression of the *yvcJII* genes from their own promoter at the *amyE* locus almost fully restored (about 90%) the wild-

TABLE 2. Relative expression levels of *glmS* and of genes involved in the competence pathway in a *yvcJ* mutant strain from transcriptome analysis

Gene	Expression level	Description
<i>glmS</i>	1.39	Glucosamine-6-phosphate synthase
Genes missing in the microarray		
<i>addA</i>		ATP-dependent DNase
<i>comEC</i>		Late competence operon required for DNA binding and uptake
<i>comP</i>		Two-component sensor histidine kinase involved in early competence
Genes with unchanged expression		
<i>comA</i>	0.84	Two-component response regulator of late competence genes and surfactin production
<i>comX</i>	1.00	Competence pheromone precursor
<i>comQ</i>	1.16	Transcriptional regulator of late competence operon and surfactin expression
<i>comS</i>	0.71	Assembly link between regulatory components of the competence pathway
<i>srfAA</i>	0.85	Surfactin synthetase/competence
<i>pnpA</i>	0.85	Polynucleotide phosphorylase (PNPase)
<i>ylbF</i>	0.73	Unknown; positively controls ComK at a posttranscriptional level
<i>mecA</i>	1.27	Negative regulator of competence
<i>clpX</i>	1.13	ATP-dependent Clp protease
<i>clpC</i>	0.84	Class III stress response-related ATPase
<i>clpP</i>	0.80	ATP-dependent Clp protease proteolytic subunit
<i>ypbH</i>	1.18	Unknown; similar to negative regulator of competence; MecA homologue
<i>codY</i>	0.88	Transcriptional pleiotropic repressor
<i>degU</i>	0.92	Two-component response regulator involved in degradative enzyme and competence
<i>abrB</i>	0.81	Transcriptional pleiotropic regulator of transition state genes
<i>rapC</i>	1.01	Response regulator, aspartate phosphatase
<i>addB</i>	0.97	ATP-dependent DNase
<i>rok</i>	1.29	Repressor of <i>comK</i>
<i>recA</i>	0.67	Multifunctional protein involved in homologous recombination and DNA repair
<i>spx</i>	1.70	Negative effector of competence
<i>spo0A</i>	1.35	Two-component response regulator central for the initiation of sporulation
Genes repressed at least twofold in the <i>yvcJ</i> mutant strain		
<i>comK</i>	0.42	Competence transcription factor
<i>comGA</i>	0.23	Late competence gene
<i>comGB</i>	0.24	DNA transport machinery
<i>comGC</i>	0.27	Exogenous DNA binding
<i>comGD</i>	0.26	DNA transport machinery
<i>comGE</i>	0.27	DNA transport machinery
<i>comGF</i>	0.29	DNA transport machinery
<i>comGG</i>	0.29	DNA transport machinery
<i>comER</i>	0.29	Nonessential gene for competence
<i>comEA</i>	0.20	Exogenous DNA-binding protein
<i>comEB</i>	0.39	Nonessential gene for competence
<i>comFA</i>	0.21	Late competence protein required for DNA uptake
<i>comFB</i>	0.26	Late competence gene
<i>comFC</i>	0.28	Late competence gene
<i>ywpH</i>	0.26	Unknown; similar to single-strand DNA-binding protein
<i>nin</i>	0.34	Inhibitor of the DNA-degrading activity of NucA
<i>comC</i>	0.41	Late competence protein
<i>yvyF</i>	0.36	Unknown; similar to flagellar protein
<i>nucA</i>	0.33	Membrane-associated nuclease

type efficiency of competence. In contrast, the frequency of transformation of strain SG106, in which only the *yvcJ* gene is expressed at the *amyE* locus, was the same as that of the SG91 mutant strain. This result indicates that the competence phenotype observed in strain SG91 is due to the disruption of the *yvcJ* gene.

**Transcriptome analysis of a *yvcJ* mutant.** In *B. subtilis*, competence develops postexponentially and only in some media (9). The effect of the deletion of the *yvcJ* gene on whole-genome expression was assayed by microarray analysis of DNA

from cells grown on MD medium and collected at the beginning of the stationary phase. cDNAs were generated from total RNA and were then hybridized to DNA arrays as described in Materials and Methods. Comparison of the gene expression profiles of the mutant strains versus the wild-type strain yields a total number of about 84 genes with  $\geq 2$ -fold changes in transcription levels (about 76 downregulated and 8 upregulated genes), many of which have unknown functions and a few of which are implicated in the competence pathway (Table 2). In particular, we observe that the transcription level of *comK*,

TABLE 3. ComS overexpression bypasses *yvcJ* deletion for competence and gene expression<sup>a</sup>

Strain	Relevant genotype	Transformation frequency (10 <sup>6</sup> )	Relative gene expression determined by q-RT-PCR		
			<i>comS</i>	<i>comK</i>	<i>comGA</i>
168	Wild type	46.0 ± 7.1	1	1	1
SG91	<i>yvcJ</i>	6.6 ± 1.6	0.7 ± 0.3	0.60 ± 0.18	0.60 ± 0.21
SG121	<i>pcomS</i>	64.5 ± 10.6	3.1 ± 0.4	1.06 ± 0.03	1.27 ± 0.06
SG122	<i>yvcJ pcomS</i>	31.5 ± 7.8	4.9 ± 1.8	1.60 ± 0.39	1.65 ± 0.44

<sup>a</sup> Cells of the wild-type strain and of *yvcJ* mutant strains in which ComS was overexpressed were transformed with 250 ng of a chromosomal DNA containing a spectinomycin cassette. Transformation frequencies were determined as described in Materials and Methods. The relative expression of the *comS*, *comK*, and *comGA* genes in the wild-type strain and in mutant strains in which ComS was overexpressed was determined by q-RT-PCR. All the values are averages of data from at least three independent experiments.

the gene encoding the main regulator of competence that activates the transcription of more than 100 genes (19), and of its regulon was repressed in the *yvcJ* mutant (Table 2). In contrast, the expression of the genes encoding proteins known to regulate *comK* expression either was not modified or was only slightly modified. We can also note that *glmS* expression was barely enhanced (1.4-fold) in the *yvcJ* mutant strain (Table 2), whereas a chromosomal transcriptional fusion of *lacZ* to *glmS* had a  $\beta$ -galactosidase activity 28 times higher in the *E. coli* homologue of the *yvcJ* mutant, the *yhbJ* mutant, than in the wild-type strain (24). Apart from the genes involved in the competence pathway, we could not obtain any clue as to the role of YvcJ; that is why we compared the expression profile of the *yvcJ* mutant strain with the wild-type strain under other growth conditions. We thus carried out transcriptome analyses under different conditions, such as growth of cells on CK-citrate or on LB medium and collection in mid-log phase, but they did not yield any new information on the cellular role of YvcJ (data not shown). We then focused our attention on genes involved in the competence pathway. The expression-profiling data were confirmed by q-RT-PCR performed with primers corresponding to genes involved in competence development (see Table S2 in the supplemental material). The estimated variations of *comK* expression are quite weak. This is because our result is obtained from the whole population, in which only about 10 to 20% of cells of a culture produce ComK and develop competence, even when all conditions are optimal (8).

**ComS or ComK overexpression bypasses *yvcJ* control of competence.** The effect of ComK overexpression on competence in wild-type or *yvcJ* backgrounds was tested by measuring the transformation levels in the presence or absence of overexpression of *comK*. We did not observe any difference in growth (Fig. 3A). However, in both strains SG119 and SG120, strong overexpression of ComK induced very high transformation efficiencies of >150-fold (Fig. 3B). This result clearly shows that ComK overexpression rescues the mutant phenotype of *yvcJ* inactivation for competence. Then we tested if constitutive expression of *comS* was able to bypass the mutant *yvcJ* phenotype for competence. It has been shown previously that ComK, targeted by MecA, is degraded by the ClpC-ClpP complex (36). This proteolysis is inhibited by ComS, an anti-adaptor protein that binds to MecA, thus preventing the degradation of the competence transcription factor, ComK (31).

As expected, when ComS was overexpressed, it rescued the *yvcJ* mutant phenotype for competence (Table 3) and brought the transformation efficiency back to the level of the wild-type strain, 168, or strain SG122. We also compared the expression of *comS*, *comK*, and *comGA* genes in these genetic backgrounds by q-RT-PCR. As shown in Table 3, the overexpression of ComS bypasses *yvcJ* for *comK* expression. This result confirms that the decrease in competence efficiency is correlated with lower expression of *comK* and consequently of the late competence genes.

**Deletion of *yvcJ* results in a decrease in the number of cells that express competence.** ComK expression shows a bistable pattern and occurs only in about 10% of the cells in a stationary-phase culture (27). Thus, a *yvcJ* mutation may result either in inhibition of *comK* expression within the competent cells and/or in a reduction in the number of cells that express competence. To investigate the second hypothesis, we used strain BD2711 (17), which contains a green fluorescent protein (GFP)-tagged ComK fusion protein expressed from its native promoter and in which the *yvcJ* gene is deleted. To enhance GFP expression, a *rok* mutation was also introduced to increase the fraction of cells that expressed competence (21). At 90 min after the end of exponential growth, the observation of *comK-gfp* expression by fluorescence microscopy indicated that strain SG152, in which *yvcJ* was deleted, showed a significantly lower fraction of fluorescent cells than strain SG147, containing the native *yvcJ* gene (Fig. 4): only 5% of cells from strain SG152 were fluorescent versus 16% for strain SG147 when about 2,000 cells from each strain were observed.

**YvcJ is involved in the phosphorylation of a cellular component.** We tried to determine how YvcJ affects the compe-

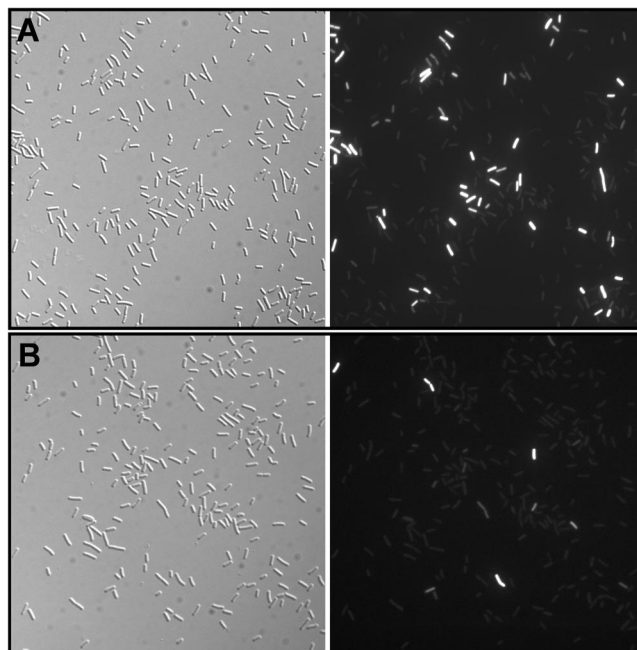


FIG. 4. Microscopy of cells harboring *comK-gfp* in strains SG147 (A) and SG152 (with *yvcJ* deleted) (B). Shown are images of cells from the cultures of strains SG147 and SG152 90 min after the transition between the exponential and the stationary-growth phase. (Left) Phase-contrast microscopy; (right) fluorescence microscopy.

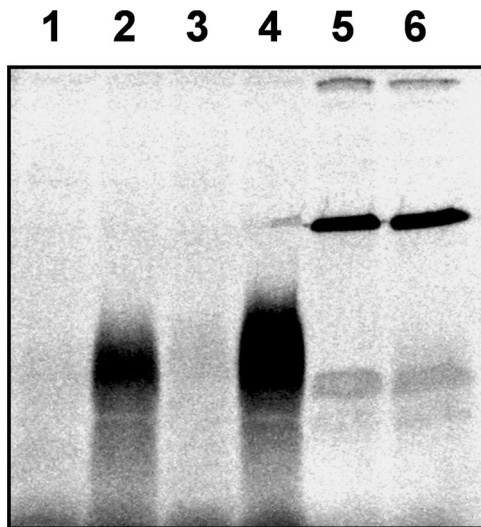


FIG. 5. ATP-dependent phosphorylation in the presence of YvcJ. Wild-type strain 168 (lanes 1 and 2) and strains BD1243 (lanes 3 and 4) and BD3836 (lanes 5 and 6) were grown on MD medium, and cells were collected at the onset of the stationary phase. For strain BD3836, *comK* overexpression was induced by addition of 1 mM IPTG to the growth medium. Crude extracts were prepared and then phosphorylated using [ $\gamma$ - $^{33}$ P]ATP in the absence (lanes 1, 3, and 5) or the presence (lanes 2, 4, and 6) of purified YvcJ. Phosphorylation assays were analyzed by 15% SDS-PAGE. Gels were then dried and exposed to autoradiography.

tence pathway. The large structural family of the P-loop-containing proteins is composed of NTPases but also of kinases. Therefore, we tested if YvcJ also possessed a kinase activity. We observed that when a crude extract of the *B. subtilis* wild-type strain, 168, was incubated with purified YvcJ and [ $\gamma$ - $^{33}$ P]ATP, a phosphorylated band of around 20 kDa could be detected (Fig. 5, lane 2), whereas no phosphorylation of the 20-kDa component could be observed in the absence of purified YvcJ (Fig. 5, lane 1). We tried to identify this substrate by various approaches, including 1- or 2 dimensional SDS gels and mass spectrometry analysis, but the signal was unstable and was thus impossible to isolate and identify by these biochemical techniques. We then tested if this radioactive signal was involved in the competence pathway by using two strains: BD1243, with a mutation in the *comA* gene and weakened competence, and BD3836, overexpressing *comK* and highly competent. All strains were grown on MD medium, and crude extracts were prepared and tested in phosphorylation assays. We observed that the intensity of the YvcJ-dependent radioactive signal increased over that for the wild type when a crude extract from strain BD1243 was incubated with purified YvcJ and [ $\gamma$ - $^{33}$ P]ATP (Fig. 5, lane 4). In contrast, no YvcJ-dependent phosphorylation was detected in a crude extract from strain BD3836 (Fig. 5, lane 6). These results suggest that YvcJ may act on the competence pathway via the phosphorylation of a cellular component.

## DISCUSSION

In this work we provide evidence that two members of the UPF0042 P-loop-containing proteins, the YvcJ protein from *B.*

*subtilis* and its homologue in *E. coli*, YhbJ, are both able to bind and hydrolyze ATP as well as GTP. Furthermore, the nucleotide binding induces a change of conformation in both proteins. In addition, YvcJ and YhbJ have fairly similar affinities for Mant-ATP and Mant-GTP, and both of them have better affinity for GTP. Interestingly, under the experimental conditions described here, YvcJ possesses a maximum velocity of hydrolysis 10-fold higher than that of YhbJ, implying that our in vitro conditions probably were not optimal for measuring nucleotide and pNPP hydrolysis by YhbJ and suggesting that some cofactor would be necessary to achieve a better enzymatic velocity.

Conserved P-loop ATPases and GTPases of unknown function constitute a significant group of proteins in bacteria (5). Among these, most studies have been carried out on essential proteins that seem to play a role in ribosome biogenesis. The YvcJ and YhbJ proteins do not belong to this group of conserved GTPases, since they are dispensable for the cell. For *E. coli*, it was shown that YhbJ was involved in the control of GlmS expression by affecting the processing and stability of a small RNA, GlmZ (24). In *B. subtilis*, we observed that YvcJ was not involved in GlmS expression but that the deletion of *yvcJ* decreased the efficiency of competence and weakly repressed the expression of *comK* and that of its downstream genes, thereby decreasing the proportion of cells that express competence.

*yvcJ* is a highly conserved gene (4), found in naturally competent bacteria but also in several noncompetent bacteria, and its role in the competence pathway, a phenomenon resulting from several cellular events, could be indirect. Indeed, the expression of *comK* is highly regulated, and it depends on the presence of several proteins and regulators (19). For example, the *sfA* operon, containing *comS*, is also involved in the synthesis of the lipopeptide surfactant surfactin, which is required for swarming motility (6). Furthermore, two phosphorelay proteins, Spo0A, involved in sporulation, and DegU, involved in degradative enzyme secretion, have been shown to regulate *comK* expression (16, 18). In the *yvcJ* mutant strain, motility, biofilm formation, secretion of degradative enzymes, and sporulation seem not to be affected, and no significant change in the expression of *sfA*, *spo0A*, or *degU* was detected by microarrays or q-RT-PCR. Our epistatic study showed that either ComK or ComS overexpression bypasses *yvcJ* deletion. Our transcriptome data indicate that *comS* transcription and the expression of the genes encoding proteins known to regulate *comK* expression either were not modified or were only slightly modified, highlighting the fact that only *comK* transcription is significantly repressed in the *yvcJ* mutant. Furthermore, our observation of the *comK-gfp* fusion indicates that *yvcJ* deletion is responsible for a threefold decrease in the number of cells that express competence. All these data suggest that the specific decrease in *comK* expression, due to the *yvcJ* deletion, is responsible for the decrease in the number of cells that express competence, thus causing a decrease in competence efficiency.

In order to obtain some clues about the mechanism by which YvcJ affects the expression of competence, we tested its potential kinase activity. We observed that when bacteria were grown on MD medium, YvcJ was involved in the phosphorylation of a 20-kDa cellular component. Despite our efforts, we

could not characterize this labile radioactive signal, which, however, seems correlated with the competence pathway. Indeed, in a *comA* mutant strain, affected in competence, the intensity of the YvcJ-dependent radioactive signal is higher than that for the wild-type strain. In contrast, no YvcJ-dependent phosphorylation was detected in a crude extract from a highly competent strain overexpressing *comK*. We also observed that under these conditions, the deletion of the *yvcJ* gene has no effect on competence efficiency (see the transformation frequencies of strains SG119 and SG120 [Fig. 3B]). These results suggest that YvcJ affects the expression of competence by phosphorylation of a cellular component.

In *E. coli*, YhbJ is proposed to have a pleiotropic effect by regulating the activities of genes or proteins involved in RNA turnover control (24). Hence, the *yvcJ* gene was expected to have a similar pleiotropic function in *B. subtilis*. However, our global transcriptional studies of cells grown under various conditions and our phenotypic screening yielded a role for YvcJ only in the competence of cells. Yet it has previously been reported that disruption of RNA stability affects competence (10, 28). In particular, PnpA, a polynucleotide phosphorylase involved in RNA stability (10), was shown to be necessary for competence and for the expression of late competence genes in *B. subtilis* (26). It may modify and stabilize the *srfA* transcript, since it stimulates the synthesis of surfactin synthetase and ComS (26). But while the whole-genome transcript analysis of a *pnpA* mutant strain showed high overexpression of *trp* operon genes when the strain was grown in the presence of tryptophan (7), the expression levels of two *trp* genes highly induced in a *pnpA* mutant, *trpB* and *trpE*, were not affected in the *yvcJ* mutant strain (data not shown). Furthermore, the *yvcJ* strain is able to swarm like the wild-type strain and does not have a cold-sensitive growth phenotype (data not shown), in contrast to the *pnpA* mutant (40). All these observations suggest that, in the *yvcJ* mutant strain, the activity of PnpA is not affected.

In conclusion, the deletion of the *yvcJ* and *yhbJ* genes, encoding two members of the P-loop protein family UPF0042, has different consequences in *B. subtilis* and *E. coli*. However, from our data, we cannot exclude the possibility that YvcJ, like its *E. coli* counterpart, can be involved, directly or indirectly, via a phosphorylation event, in the stability of some transcripts and thus can modulate the synthesis of ComS or ComK. All these hypotheses are now being tested in the laboratory to elucidate the molecular mechanism involving YvcJ and how this P-loop-containing protein is connected to the competence pathway.

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