Regulation of ykrL (htpX) by Rok and YkrK, a Novel Type of Regulator in Bacillus subtilis

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Expression of ykrL of Bacillus subtilis, encoding a close homologue of the Escherichia coli membrane protein quality control protease HtpX, was shown to be upregulated under membrane protein overproduction stress. Using DNA affinity chromotography, two proteins were found to bind to the promoter region of ykrL: Rok, known as a repressor of competence and genes for extracytoplasmic functions, and YkrK, a novel type of regulator encoded by the gene adjacent to ykrL but divergently transcribed. Electrophoretic mobility shift assays showed Rok and YkrK binding to the ykrL promoter region as well as YkrK binding to the ykrL promoter region. Comparative bioinformatic analysis of the ykrL promoter regions in related Bacillus species revealed a consensus motif, which was demonstrated to be the binding site of YkrK. Deletion of rok and ykrK in a Pykrl-gfp reporter strain showed that both proteins are repressors of ykrL expression. In addition, conditions which activated Pykrl (membrane protein overproduction, dissipation of the membrane potential, and salt and phenol stress) point to the involvement of YkrK in membrane protein quality control.

MATERIALS AND METHODS

Bacterial strains and growth conditions. B. subtilis strains used in this study are isogenic derivatives of strain 168 (21) and are listed in Table 1. L. lactis NZ29000 (20) was used as an intermediate cloning host for pNZ-xyIP and pNZ-ykrK-strep and E. coli DH5α for pPykrL-gfp. B. subtilis and E. coli strains were grown in LB medium (26) at 37°C with shaking. L. lactis was grown at 30°C in M17 broth (Oxoid, Basingstone, England) supplemented with 0.5% (wt/vol) glucose. LB medium was supplemented with the following, when needed: 100 μg ml⁻¹ ampicillin and 25 μg ml⁻¹ kanamycin for E. coli; 5 μg ml⁻¹ kanamycin, 100 μg ml⁻¹ spectinomycin, 0.5 μg ml⁻¹ erythromycin, 5 μg ml⁻¹ chloramphenicol, or 9 μg ml⁻¹ tetracycline for B. subtilis; and 5 μg ml⁻¹ chloramphenicol or 5 μg ml⁻¹ erythromycin for L. lactis. Solid media were prepared by adding 1.5% agar.

The membrane located xylose transporter XylIP from Lb. pentosus was overexpressed in B. subtilis using the subtilin-regulated gene expression (SURE) system (4). Molecular techniques were carried out as described before (30). For plasmid and strain constructions, see the supplemental material. Primers used in this study are listed in Table S1 in the supplemental material.

Expression and purification of YkrK and Rok. YkrK-strep was expressed in L. lactis NZ9000 from pNZ-ykrK-strep, using the NICSE system (10). Cells were grown in GM17 medium to mid-exponential phase (optical density at 600 nm [OD₆₀₀] = 0.5) and induced with 5 ng ml⁻¹ nisin (Sigma). After 2 h, cells were harvested, washed, and resuspended in buffer W (100 mM Tris-HCl [pH 8.0], 150 mM NaCl). Lysozyme (5 mg ml⁻¹) was added, and after 10 min of incubation at 45°C, cells were disrupted by bead beating for 2 min at 4°C in a Mini-Beadbeater-16 (Biospec

Received 1 March 2012 Accepted 16 March 2012 Published ahead of print 23 March 2012 Address correspondence to Oscar P. Kuipers, o.p.kuipers@rug.nl * Present address: Molecular Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Groningen, The Netherlands. Supplemental material for this article may be found at http://jb.asm.org/ Copyright © 2012, American Society for Microbiology. All Rights Reserved. doi:10.1128/JB.00324-12
TABLE 1 Bacterial strains and plasmids used in this study

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Products). Cell debris was removed by centrifugation, and the supernatant was incubated with Strept-Tactin Sepharose (IBA) with gentle rotation for 2 h at 4°C. The mixture was loaded onto a Bio-Spin disposable chromatography column (Bio-Rad) and washed three times with buffer W. The protein was eluted from the column with a solution containing 100 mM Tris-HCl (pH 8.0), 150 mM EDTA, and 2.5 mM desthiobiotin.

The protein concentration was determined using an ND-1000 spectrophotometer (NanoDrop Technologies). Fractions containing pure YkrK were used in gel retardation analysis.

Rok-His6 was expressed in *E. coli* ED428 and purified using a Superflow Ni-nitrotriacetic acid resin column (Qiagen) as described before (2).

**DNA affinity chromatography.** DNA affinity chromatography was performed as described before (11, 12), with modifications. The 542-bp ykrL-ykrl intergenic region was amplified using primers Pykrl-oligo4 and Pykrl-oligo5, of which Pykrl-oligo4 was provided in a biotinylated form on the 3' end (Biolegio). The amplified fragment covered a region from -412 to +70 with respect to the translational start site of yklrL. The PCR product labeled with biotin was immobilized on Silmag-streptavidin magnetic beads (Bio-Noble). For cytoplasmic fraction isolation, *B. subtilis* NZ8900 was grown in 600 ml LB medium. Cells were collected at late exponential phase, washed and diluted again, and grown under the desired conditions. For flow cytometry, cells were washed and diluted in filter-sterilized PBS buffer (58 mM NaHPO4, 17 mM NaH2PO4, 68 mM NaCl [pH 7.3]) and analyzed using a BD FACSCanto (BD Biosciences) operating on an argon laser at 488 nm. The green fluorescent protein (GFP) signal (FL-1) was collected through a fluorescein isothiocyanate (FITC) filter. The photomultiplier tube voltage was set at 700 V. In each measurement, 50,000 events (cells) were counted. The data were then analyzed using WinMDI software (version 2.9; http://facs.scripps.edu/software.html).

**EMSAs.** Electrophoretic mobility shift assays (EMSAs) were performed essentially as described before (15). DNA probes were amplified using Phusion high-fidelity DNA polymerase (NEB) and primers listed in Table S1 in the supplemental material. The PCR fragments were end labeled with T4-polynucleotide kinase using [γ-32P]ATP. Various amounts of protein were mixed on ice with 5,000 cpm (corresponding to approximately 4 ng) of probe DNA in binding buffer (20 mM Tris-HCl [pH 7.5], 1 mM EDTA, 10% [vol/vol] glycerol, 0.01% [vol/vol] Triton X-100, 100 mM NaCl, 1 mM dithiothreitol [DTT]), resuspended in the same buffer supplemented with 60 mg lysozyme and protease inhibitor cocktail Complete (Roche), and incubated for 1 h at 37°C. Cells were broken by passage three times through a French press operated at 13,000 lb/in². Preparation of a cytoplasmic fraction, incubation with immobilized and biotinylated DNA, and subsequent washing and elution steps were performed as described before (11, 12). Elution fractions were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and detected by silver staining. Gel slices were excised from the gel and digested in a 1:1 solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate. Proteins were identified by in-gel trypsin digestion followed by liquid chromatography-mass spectrometry (LC-MS/MS) as described before (28). For protein identification, the MS data were submitted to Mascot (version 2.1; Matrix Science, London, United Kingdom) and searched against the *B. subtilis* proteome sequence.

**Flow cytometry.** Overnight cultures of the *B. subtilis* reporter strain carrying the Pykrl-gfp fusion were diluted to an OD600 of 0.05, grown to exponential phase, diluted again, and grown under the desired conditions. For flow cytometry, cells were washed and diluted in filter-sterilized PBS buffer (58 mM NaHPO4, 17 mM NaH2PO4, 68 mM NaCl [pH 7.3]) and analyzed using a BD FACSCanto (BD Biosciences) operating on an argon laser at 488 nm. The green fluorescent protein (GFP) signal (FL-1) was collected through a fluorescein isothiocyanate (FITC) filter. The photomultiplier tube voltage was set at 700 V. In each measurement, 50,000 events (cells) were counted. The data were then analyzed using WinMDI software (version 2.9; http://facs.scripps.edu/software.html).
RESULTS

Deletion of ykrL leads to a higher sensitivity to membrane protein overproduction and to dissipation of the membrane potential. Heterologous overproduction of the membrane proteins XylP, a xyloside transporter from Lb. pentosus, and LmrA, a multidrug transporter from L. lactis, in B. subtilis caused significant upregulation of ykrL. Overexpression of XylP from pNZ-xylP, resulted in significantly higher inhibition of growth for both the ΔykrL and ΔsigW mutants than for the control cells harboring the empty vector pNZ8902. The ΔsigW mutant was most sensitive, showing cell lysis after 1 h of induction of expression of xylP. Overproduction of LmrA was tested and showed highly similar effects (data not shown). The stronger effect of the sigW deletion is not surprising, as it regulates the expression of approximately 60 genes (5).

HtpX of E. coli has proteolytic activity on membrane proteins and has been suggested to be involved in degradation of misfolded membrane proteins (1). Since the transmembrane electrical potential (ΔΨ) has been shown to be important for correct insertion of membrane proteins into the membrane via the protein secretion machinery (34, 38), the effect of membrane potential dissipation using valinomycin was tested on the ykrL mutant (JW8940) (Fig. 1C). Valinomycin is an antibiotic that acts as a K⁺ carrier specifically dissipating ΔΨ in the presence of a sufficiently high extracellular concentration of K⁺ ions (31). When added at a final concentration of 0.5 μM (sublethal concentration for wild-type cells) to exponentially growing cells in LB medium supplied with 200 mM KCl, the ΔykrL strain showed a much higher sensitivity than wild-type cells. In a control experiment without addition of KCl, in which valinomycin leads to hyperpolarization due to K⁺ efflux rather than depolarization, the ΔykrL strain was not more sensitive than the wild type. After the addition of different concentrations of nigericin, which dissipates the proton gradient across the membrane (ΔpH) by H⁺/K⁺ exchange (16), no difference in sensitivity between the mutant and wild-type strains was observed. This indicates that the stress caused by membrane protein overproduction against which YkrL is acting is not proton leakage through the membrane via misfolded membrane proteins but possibly the accumulation of misfolded protein itself. In agreement, experiments in which the membrane potential was measured in ΔykrL and wild-type cells, with or without overexpression of xylP, did not show differences in the ability to maintain or build up membrane potential (data not shown).

Stress conditions activating the ykrL promoter. To investigate the regulation of ykrL expression, a reporter strain was constructed containing a fusion of the ykrL promoter region to gfp (PykrL-gfp) integrated at the native locus. As ykrL was induced by membrane protein overproduction in the transcriptome analyses, activation of PykrL by overexpression of xylP in the PykrL-gfp reporter strain (BC300) was tested using flow cytometry (Fig. 2A). PykrL clearly showed a higher activity upon XylP overproduction than in cells not producing XylP.

Dissipation of membrane potential (ΔΨ) by addition of a sublethal concentration of valinomycin to the PykrL-gfp reporter strain (BC400) growing in LB medium supplied with 200 mM KCl resulted in a strong induction of PykrL (Fig. 2B). In the absence of KCl, no induction was observed (data not shown). In contrast, dissipation of the proton concentration gradient (ΔpH) by addition of a sublethal concentration of nigericin did not affect PykrL activity, not even after prolonged incubation (Fig. 2C). This, as well as the sensitivity of the ΔykrL strain to valinomycin but not nigericin (see above), shows a relation of the membrane potential, but not the proton concentration gradient, with stress caused by membrane protein overproduction. A relation between the PykrL activity and membrane energetics was suggested by results from a
random transposon mutagenesis screening in a PykrL-lacZ reporter strain (HT400), which showed that PykrL is induced by mutations in resE, resB, sdhC, and sdhA (see Fig. S1 in the supplemental material). resE codes for a sensor histidine kinase of the ResDE two-component system, which regulates expression of genes involved in aerobic and anaerobic respiration (23), including its upstream genes resABC (37); resB is essential for cytochrome c synthesis (5), and sdhC and sdhA encode components of succinate dehydrogenase, which plays a role in the electron transport chain (17). Although the effects of these mutations are pleiotropic, they constituted almost two-thirds of the identified mutations that induced PykrL (data not shown), and the genes all play a role in generation of membrane potential. This, together with the above-mentioned results, suggests that the membrane potential component of the proton motive force could affect the activity of PykrL either directly or indirectly.

In addition, PykrL activity was tested under different stress conditions that can influence the membrane protein structure or were reported to induce ykrL expression previously (phenol stress [36] and salt stress [23]). The conditions tested were addition of 16 mM phenol, addition of 650 mM NaCl, submission to heat stress at 42°C and 50°C, and anaerobic growth (a closed vessel filled up to 80% with culture) (Fig. 2D). Salt stress had a strong effect on PykrL, while phenol, heat, and anaerobic stress resulted in milder activation of PykrL.

Rok and YkrK bind to the intergenic region of ykrK and ykrL. In order to find proteins binding to the promoter of ykrL, DNA affinity chromatography was performed. A 542-bp DNA fragment containing the ykrK-ykrL intergenic region with the predicted ykrK and ykrL promoters (Fig. 3A), was amplified, biotinylated, immobilized on magnetic streptavidin beads, and incubated with a cytoplasmic fraction of B. subtilis cells. Proteins bound to the DNA were eluted and analyzed on an SDS-PAGE gel (Fig. 3B) followed by in-gel digestion and identification by mass spectrometry.

FIG 2 Flow cytometry histograms of the PykrL-gfp reporter strains BC300 (A) and BC400 (B to D) showing PykrL activity under different stress conditions. (A) xylP overexpression from pNZ-xylP induced by subtilin; control, strain carrying empty pNZ8902. (B) Membrane potential (∆Ψ) dissipation using 0.5 µM valinomycin. (C) ∆pH dissipation with 7.5 nM nigericin. (D) Oxygen limitation (vessel containing 20% air), heat stress (42°C), phenol stress (16 mM), and salt stress (650 mM NaCl). Stress conditions were applied to cultures at an OD₆₀₀ of 0.5, and GFP fluorescence was monitored at time intervals. Shown are measurements 120 (A) and 60 (B to D) minutes after applying stress. The shift in fluorescent signal (FL-1) to the right indicates higher PykrL-gfp activity.
FIG 3 Identification of proteins binding to the ykrK-ykrL intergenic region using DNA affinity chromatography. (A) Schematic organization of the ykrK-ykrL locus in B. subtilis. Lollipops indicate terminator structures. Flags indicate predicted promoters. The line below indicates the fragment used for the affinity chromatography. (B) SDS-PAGE and silver stain analysis of the proteins enriched from the cytoplasmic fraction of a B. subtilis NZ8000 culture using the ykrK-ykrL intergenic region immobilized on streptavidin beads. Lane 1, protein ladder; lane 2, proteins enriched with the ykrK-ykrL intergenic region of B. subtilis.

ometry. Among the most abundant proteins that bound to the ykrK-ykrL intergenic DNA fragment were Rok and YkrK. Rok is known to act as a negative regulator of competence (17) and genes coding for membrane-localized and secreted proteins (2), and it was shown to bind to A+T-rich DNA (33). The other protein, YkrK, is an unknown 233-amino-acid protein encoded by ykrK, the gene adjacent to but divergently transcribed from ykrL (Fig. 3A). Apart from close homologues in closely related Bacillus species, no significant homology was found with any other known protein. It shows minor local similarity to MerR family regulators but can be considered a novel type of DNA binding protein. The Bacillus species that harbor YkrK homologues share the same genetic organization as the divergent ykrL and ykrK corresponding genes.

Other, less abundant proteins identified in the elution fraction were 30S and 50S ribosomal proteins, dihydriopioamidase acetyltransferase (acoC), lipoamide acetyltransferase (bkdB), and dihydriopioyl dehydrogenase (lpdV). Since these proteins were not (potential) DNA binding proteins, their role in ykrL expression was not further analyzed.

Rok and YkrK are repressors of ykrL expression. To test how YkrK and Rok regulate expression of ykrL, i.e., by activation or repression, strains containing the Pyrkl-gfp fusion combined with a deletion of rok (BC401), ykrK (BC402), or both rok and ykrK (BC403) were constructed. GFP fluorescence was measured every hour throughout growth (only chosen time points are shown in Fig. 4). During early exponential phase, Pyrkl activity was clearly higher in the ∆ykrK and ∆rok ∆ykrK mutants (Fig. 4A). Later in growth (transition from exponential to stationary phase and early stationary phase), the difference in GFP signal between the wild type and these two mutants became larger, and Pyrkl activity also increased in the ∆rok mutant at late stationary growth phase (Fig. 4B). Overexpression of ykrL from pNZ-ykrK using the SURE system (4) in the ∆ykrK mutant (BC404) resulted in full repression of Pyrkl-gfp (Fig. 4C). Altogether, we demonstrate that both Rok and YkrK are repressors of the ykrL promoter.

Rok binds to the ykrL promoter region; YkrK binds to both the ykrK and ykrL promoter regions. To determine more precisely the regions to which Rok and YkrK bind, four fragments, A, B, C, and D (Fig. 5A), covering the ykrK-ykrL intergenic region were amplified. DNA fragments A and B contained the predicted promoters, including predicted promoter regions. The line below indicates the fragment used for the affinity chromatography. (B) SDS-PAGE and silver stain analysis of the proteins enriched from the cytoplasmic fraction of a B. subtilis NZ8000 culture using the ykrK-ykrL intergenic region immobilized on streptavidin beads. Lane 1, protein ladder; lane 2, proteins enriched with the ykrK-ykrL intergenic region of B. subtilis.

FIG 4 Flow cytometry histograms showing Pyrkl activity in Pyrkl-gfp reporter strains: wild type (BC400), ∆rok (BC401), ∆ykrK (BC402), ∆rok∆ykrK (BC403), ∆ykrK+pNZ-ykrK (BC404 carrying pNZ-ykrK), and ∆ykrK+pNZ8902 (BC404 carrying pNZ8902). (A) GFP signal in cells growing exponentially; (B) GFP signal in cells in stationary phase; (C) GFP signal in cells growing exponentially 30 min after inducing ykrK expression with subtilin. The shift in fluorescent signal (FL-1) to the right indicates higher Pyrkl-gfp activity.
**FIG 5** *In vitro* binding of YkrK-Strep and Rok-His$_6$ to fragments of the *ykrK-ykrL* intergenic region. (A) Schematic representation of the *ykrK-ykrL* intergenic region and fragments A (PykrK), B (PykrL), C, and D used for EMSA. Predicted $-35$ and $-10$ boxes of PykrK and PykrL are shown as boxed arrows and putative YkrK binding sites as light gray rectangles. (B) EMSAs of YkrK-Strep (top panel) and Rok-His$_6$ (bottom panel) binding to fragments A, B, C, and D. (C) Negative control, Rok-His$_6$ and YkrK-Strep binding to *secA* DNA. Asterisks indicate free $^{32}$P-DNA probes, and arrows indicate the position of the shifted probe. The calculated $K_d$ values are indicated below each binding assay graph.
tent with one binding site for Rok in the intergenic region and three for YkrK, the latter appearing as a sum of the YkrK binding patterns of fragments A and B.

A YkrK binding motif is present within PykrK, within PykrL, and in between the two promoters. In order to identify a consensus sequence within the ykrK'-ykrL' intergenic region that may represent a YkrK binding motif, we compared the sequences of this region from five Bacillus species which contain a corresponding, divergent ykrK'-ykrL' pair—B. subtilis, B. amyloliquefaciens, B. licheniformis, B. halodurans, and B. pumilus—using the Motif Sampler tool (37). A conserved sequence TGAWCTTA (W = A/T) was found (Fig. 6A). This 8-nucleotide (nt) motif is present in the ykrK'-ykrL' intergenic region of B. subtilis in three places (Fig. 6B): overlapping with the predicted −35 box of PykrK, downstream of the −10 box of PykrL, and, with more deviation from the consensus, in between these two promoters. The locations of the motif correlates with the observed YkrK binding to the four DNA fragments in the EMSA experiment (compare Fig. 5).

To confirm experimentally the result obtained with the YkrK binding motif search, a YkrK binding assay was performed with DNA fragment B, covering the predicted PykrL, in its original sequence and with three variants carrying point mutations in the predicted binding motif: B-mut1 (G2A), B-mut2 (C5T), and B-mut3 (G2A/C5T) (Fig. 6C). YkrK bound to the wild-type DNA...
fragment B with an estimated $K_d$ of 60 nM. The single point mutations on the second (B-mut1) and fifth (B-mut2) positions of the predicted motif caused at least a 10-fold decrease in affinity of YkrK binding (apparent $K_d$ values of 600 nM and 960 nM, respectively). When the two mutations were present simultaneously (B-mut3), affinity decreased even more substantially ($K_d$ app = 1.65 μM). This result confirms that the conserved sequence TGAWCTTA ($W = A$ or $T$) is the YkrK binding motif, or at least constitutes the major part of the binding site. Up- and downstream base pairs may be involved as well, which could explain the difference of the YkrK binding affinity between the ykrL promoter and the other two binding sites, present in the ykrK promoter and in the region in between.

Since ykrL was possibly not the only target of YkrK regulation, ykrK was overexpressed in B. subtilis and the transcriptome was compared to control cells using DNA microarrays. The upstream regions of significantly regulated genes (see Table S2 in the supplemental material) were searched for the YkrK binding motif using the genome-scale DNA pattern tool (39) with default parameters. Next to ykrK and ykrL, only two genes were found to possess the putative YkrK binding motif (TGAWCTTA) in their promoter regions: yrkA, coding for a putative membrane protein of unknown function, and penP, encoding a secreted penicillinase. The motif of penP, however, is in the reverse orientation, while the expression of the adjacent but divergently transcribed gene yoaZ was not changed upon YkrK overexpression. It appears that YkrK regulates only a small number of genes, including ykrL and possibly ykrK, but this needs further research.

**DISCUSSION**

The regulation of ykrL of B. subtilis, encoding a close homologue of E. coli HtpX, is described. In addition, insight in the physiological role of YkrL was obtained from a ykrL knockout mutant as well as from stress conditions inducing ykrL expression. YkrL is a membrane protein with four predicted transmembrane segments and contains all the conserved residues present in the active site of the 44% identical HtpX of E. coli, a zinc-dependent metalloprotease involved in membrane protein quality control (1, 27). It was shown that a ykrL knockout mutant was more sensitive to membrane protein overproduction stress and dissipation of transmembrane electrical potential ($\Delta\Psi$) than the wild-type parent strain. These conditions also induced expression from the ykrL promoter, PykrL. In contrast, the ykrL mutation did not increase sensitivity to dissipation of the chemical proton gradient, $\Delta pH$, which together with the $\Delta\Psi$ constitutes the proton motive force. Nor did this condition induce PykrL. This suggests that the stress that is sensed and leads to induction of ykrL expression is the presence or accumulation of misfolded proteins in the membrane, rather than proton leakage as a result of disturbed membrane integrity. In this case, the observed activation of PykrL by membrane potential dissipation can be explained by the requirement of membrane potential for correct insertion of membrane proteins (34, 38).

We suggest that YkrL, similar to HtpX of E. coli, is involved in a membrane protein stress response, likely by proteolytic degradation of misfolded membrane proteins, and serves as a membrane protein quality control system. Supporting this hypothesis is the strongly increased amount of YkrL observed in the membrane proteome in a conditional double knockout of spoIIIJ and yqjG, encoding YidC homologues involved in membrane protein insertion (29). However, differences between the HtpX and YkrL functions may exist. The htpX gene is induced in E. coli by heat stress (32), whereas the effect of heat stress on ykrL expression in B. subtilis, albeit significant, was less strong than that of other tested conditions such as salt stress. The strong induction of PykrL by salt stress and phenol, observed before (23, 36), may be explained by misfolding of proteins induced by the hyperosmotic conditions and denaturation, respectively. Indeed, Hahne et al. (14) observed that salt stress induced cytosolic as well as membrane protein quality control proteins in B. subtilis, although ykrL was not indicated as significantly regulated in that study.

In E. coli, next to HtpX, the membrane-located ATP-dependent metalloprotease FtsH is involved in membrane protein quality control (1). The gene encoding FtsH of B. subtilis, sharing 47% amino acid sequence identity with E. coli FtsH, was, unlike ykrL, not induced by membrane stress caused by membrane protein overproduction (Marciniak et al., submitted). Although we cannot exclude a general role for FtsH in membrane protein quality control, its previously described functions (7, 22) may represent a more specific role in the physiology of B. subtilis.

YkrL has recently been shown to associate with the MreB cytoskeleton (19) forming a spiral structure immediately underneath the cell membrane and coordinating cell wall synthesis together with MreC and MreD (18). This spiral localization of MreB is disrupted under dissipation of the membrane potential (35). The site where YkrL is recruited may be close to the cell wall synthesis machinery (MreB complex), where, during rapid growth, cells could benefit from a membrane protein quality control system.

In E. coli, expression of htpX is regulated by the CpxR/CpxA two-component system (32), which regulates many genes with an important role in envelope protein folding (25), including the gene encoding the secretion stress protein DegP (Htra) (8). The corresponding system in B. subtilis, CspRS, regulating htrA and htrB expression (9), appeared not to be involved in ykrL expression. Instead, two proteins binding to the ykrL promoter region were identified by DNA affinity chromatography: the unknown protein YkrK, encoded by ykrK, located upstream of ykrL but divergently transcribed, and Rok, a known repressor of competence and of genes with extracytoplasmic function (2, 17). Both proteins were demonstrated to act as repressors of ykrL expression. Electrophoresis mobility shift assays showed that Rok binds to the ykrL promoter region, whereas YkrK binds at three positions in the ykrK-ykrL intergenic region: with high affinity to the ykrL promoter region, with low affinity to the ykrK promoter region, and with low affinity to a region in between these. A consensus motif for YkrK binding was identified by comparative bioinformatic analysis of homologous ykrK-ykrL intergenic regions of related Bacillus species. The motif, TGAWCTTA ($W = A$ or $T$), was confirmed by making point mutations, which led to a drastically lowered affinity for YkrK. The presence of the binding motif in the ykrK promoter region, overlapping with its predicted −35 sequence, and the weak but significant binding of YkrK to this region, suggests that YkrK may be involved in its own regulation, but this needs further research.

The exact binding site for Rok in the ykrK-ykrL intergenic region was not determined, but recent work demonstrated that Rok binding occurs at A+T-rich DNA (33). The fragment of the ykrK-ykrL intergenic region to which Rok bound contains an uninterrupted 18-bp stretch of A+T, overlapping with the predicted −10 box of PykrL (Fig. 6B). The fragments to which Rok did not bind
do not contain such uninterrupted stretches, although the average A+T content of the fragments did not significantly differ.

The functioning of YkrK is an interesting target for further research, as it may be either directly or indirectly involved in the sensing of the stress signal that leads to its dissociation from the ykrL promoter. As this stress is occurring and sensed at or in the membrane, interaction of YkrK with other membrane-associated or -embedded proteins can be expected. At the C terminus of YkrK, a 34-amino-acid sequence containing a large proportion of hydrophobic amino acids is predicted as a relatively hydrophobic α-helix by secondary-structure prediction tool POLYVIEW (24), which may play a role in such interaction.

In conclusion, this study addressed the so far relatively unexplored area of membrane protein stress in *B. subtilis*, in particular the regulation of the membrane stress-responsive gene *ykrL*. Two regulators were identified, Rok and the novel YkrK protein, for which a binding motif was revealed. Results on a *ykrL* knockout strain and data for *PykR*-controlled expression, together with other studies discussed above, indicate YkrK as a quality control system for membrane proteins.

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