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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 chromatography, 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 *ykrK* 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 P*ykrL-gfp* reporter strain showed that both proteins are repressors of *ykrL* expression. In addition, conditions which activated P*ykrL* (membrane protein overproduction, dissipation of the membrane potential, and salt and phenol stress) point to the involvement of YkrL in membrane protein quality control.

The quality of bacterial membrane proteins, essential for the viability of the cell, may be challenged during environmental stresses that can eventually lead to accumulation of misfolded proteins in the membrane. Therefore, the membrane protein folding state must be constantly monitored and damaged proteins must be repaired or eliminated. This is facilitated by membrane protein quality control systems. In *Escherichia coli*, two structurally unrelated membrane-integrated metalloproteases, HtpX and the ATP-dependent protein FtsH, are involved in membrane protein quality control by facilitating proteolytic degradation of proteins (reviewed in reference 1). Involvement of HtpX in membrane quality control is supported by the observation that disruption of both *htpX* and *ftsH* in a strain with the *ftsH* suppressor mutation *sfhC21* results in thermosensitivity, while single disruptions are silent (32). The *htpX* gene of *E. coli* is under the control of the CpxR/CpxA extracytoplasmic stress response system (32), which also regulates expression of a protein involved in secretion stress, DegP (HtrA) (8), and has a homologue in the Gram-positive model organism *Bacillus subtilis*, i.e., CssRS (9). Little is known about how the quality of membrane proteins is monitored in *B. subtilis* and how cells respond to membrane stress. In another study (B. C. Marciniak, H. Trip, P. J. van der Veek, and O.P. Kuipers, submitted for publication), we found that membrane stress caused by membrane protein (*Lactobacillus pentosus* xyloside transporter XylP and *Lactococcus lactis* multidrug transporter LmrA) overproduction in *B. subtilis* led to an increase of *sigW* and *ykrL* expression. The *B. subtilis* SigW regulon consists of ~60 genes (5) and is induced in response to cell envelope stress (6, 40). *ykrL* codes for a homologue of *E. coli* HtpX and is also predicted to be a membrane protein itself. In this study, the regulation of *ykrL* expression in *B. subtilis* was investigated and turned out to be very different from that of *htpX* in *E. coli*. We show that *ykrL* expression is repressed by Rok and by a novel type of DNA binding protein, YkrK, encoded by a gene adjacent to *ykrL* but divergently transcribed, and that expression of *ykrL* is stimulated under conditions potentially influencing membrane protein structure.

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* NZ9000 (20) was used as an intermediate cloning host for pNZ-xylP 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 xyloside transporter XylP 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 NICE system (10). Cells were grown in GM17 medium to mid-exponential phase (optical density at 600 nm [OD_{600}] = 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

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Supplemental material for this article may be found at http://jb.asm.org/.

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
Bacterial strains		
E. coli		
DH5α	F$^-$ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, *hsdR17*(r_K^- m_K^+), λ$^-$	13
ED428	*rok-his*$_6$	17
L. lactis		
NZ9000	*pepN*::*nisRK*	20
B. subtilis		
168	*trpC2*	21
NZ8900	*trpC2 amyE*::*spaRK* (Kan^r)	4
JW8900	*trpC2 thrC*::*spaRK* (Erm^r)	J. W. Veening, unpublished data
JW8940	*trpC2 thrC*::*spaRK ykrL* (Erm^r Spc^r)	This study
RH100	*trpC2 thrC*::*spaRK* (Spc^r)	R. Nijland, unpublished data
KB100	*trpC2 thrC*::*spaRK sigW* (Spc^r Kan^r)	This study
BC300	*trpC2 thrC*::*spaRK* (Spc^r) P*ykrL-gfp* (Cm^r)	This study
BC400	*trpC2* P*ykrL-gfp* (Cm^r)	This study
BC401	*trpC2* P*ykrL-gfp rok* (Cm^r Kan^r)	This study
BC402	*trpC2* P*ykrL-gfp ykrK* (Cm^r Tet^r)	This study
BC403	*trpC2* P*ykrL-gfp rok ykrK* (Cm^r Kan^r Tet^r)	This study
BC404	*trpC2* P*ykrL-gfp amyE*::*spaRK ykrK* (Cm^r Kan^r Tet^r)	This study
HT400	*trpC2 amyE*::P*ykrL-lacZ* (Cm^r)	This study
Plasmids		
pNZ8902	P*spaSpn* (Erm^r)	4
pNZ-xylP	P*spaSpn-xylP* (Erm^r)	This study
pNZ-ykrK-strep	P*spaSpn-ykrK-strep* (Erm^r)	This study
pNZ-ykrK	P*spaSpn-ykrK* (Erm^r)	This study

Products). Cell debris was removed by centrifugation, and the supernatant was incubated with Strep-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-His$_6$ was expressed in *E. coli* ED428 and purified using a Superflow Ni-nitrilotriacetic 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 *ykrK-ykrL* intergenic region was amplified using primers PykrL-oligo4 and PykrL-oligo5, of which PykrL-oligo4 was provided in a biotinylated form on the 5′ end (Biolegio). The amplified fragment covered a region from −412 to + 70 with respect to the translational start site of *ykrL*. The PCR product labeled with biotin was immobilized on SiMag-streptavidin magnetic beads (Bio-Nobile). For cytoplasmic fraction isolation, *B. subtilis* NZ8900 was grown in 600 ml LB medium. Cells were collected at late exponential phase, washed with TGED 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/in^2. 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 destained 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 P*ykrL-gfp* fusion were diluted to an OD_{600} 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 Na_2HPO_4, 17 mM NaH_2PO_4, 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-^{33}P]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 8], 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.05 mg ml^{-1} poly[d(I-C)], and 0.05 mg ml^{-1} bovine serum albumin[BSA]} and subsequently incubated for 30 min at room temperature. Glycerol was added to a final concentration of 10%, and the samples were loaded on a nondenaturing 6% polyacrylamide gel. Gels were run in 1× TAE buffer (40 mM Tris-acetate [pH 8.0], 2 mM EDTA) at 100 V for 45 or 55 min, depending on the probe size, and dried in a vacuum gel dryer (Bio-Rad). Radioactivity was visualized using phosphor screens and a Cyclone phosphorimager (Packard). In all the EMSA experiments, *secA* promoter (P*secA*) amplified with primers SQ_PsecA_o1 and SQ_PsecA_o2 was used as a negative control.

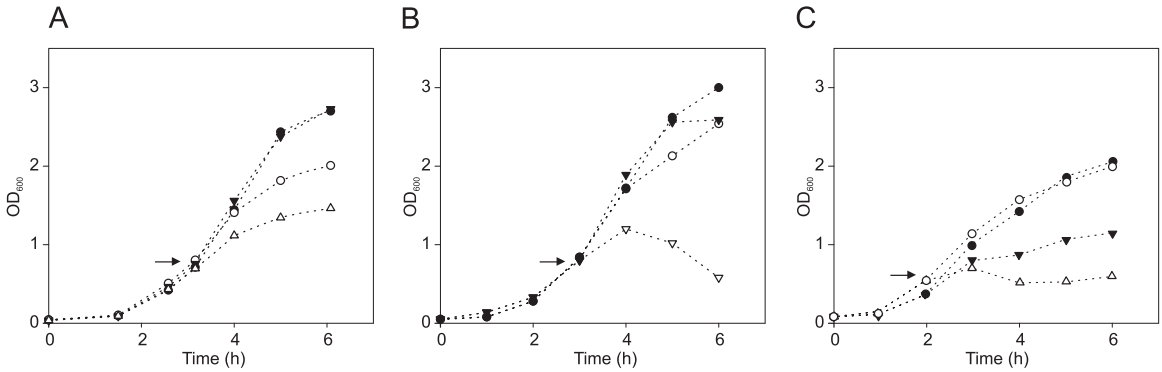

FIG 1 Sensitivity of *ykrL* (A and C) and *sigW* (B) deletion mutants to membrane protein (XylP) overproduction (A and B) or to membrane potential (ΔΨ) dissipation (C). (A and B) Growth of wild type (circles) and Δ*ykrL* (JW8940 [A]) or Δ*sigW* (KB100 [B]) strains (triangles) overexpressing *xylP* (open symbols) or harboring the empty vector pNZ8902 (closed symbols). Expression of *xylP* was induced at an OD_{600} of 0.8 (arrow) with 0.1% subtilin. (C) Growth of wild type (JW8900 [closed symbols]) and Δ*ykrL* (JW8940 [open symbols]) in LB medium with 200 mM KCl with (triangles) or without (circles) addition of 0.5 μM valinomycin. Lower growth rates are due to addition of KCl. Arrow indicates time point of valinomycin addition. All growth curves are representative of at least three independent experiments.

Protein-DNA binding affinities (dissociation constants [K_d]) were determined by fitting binding kinetics to the data derived from radiographs. Bound and unbound fractions were determined by measuring band intensities using ImageJ software (version 1.44i; National Institutes of Health, United States [http://rsb.info.nih.gov/nih-image/]).

YkrK binding motif search. In order to find a YkrK binding motif, the intergenic sequences between *ykrK* and *ykrL* homologue genes in *B. subtilis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus halodurans*, and *Bacillus pumilus* were compared using Motif Sampler tool (37). The motif weight matrix was generated using Genome2D (3).

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* (encoding a close homologue of HtpX of *E. coli*) (44% identity), *sigW*, and most of the SigW regulon members (Marciniak et al., submitted). Knockout mutants of *ykrL* and *sigW* were constructed, yielding strains JW8940 and KB100, respectively, which were tested for sensitivity to membrane stress (Fig. 1A and B) caused by overexpression of *xylP* from pNZ-xylP. Overproduction of XylP resulted in a significantly higher inhibition of growth for both the Δ*ykrL* and Δ*sigW* mutants than for the control cells harboring the empty vector pNZ8902. The Δ*sigW* mutant was most sensitive, showing cell lysis after 1 h of induction of expression of *xylP*. Overproduction of LmrA was also 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 Δ*ykrL* 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 Δ*ykrL* 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 Δ*ykrL* 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* (P*ykrL-gfp*) integrated at the native locus. As *ykrL* was induced by membrane protein overproduction in the transcriptome analyses, activation of P*ykrL* by overexpression of *xylP* in the P*ykrL-gfp* reporter strain (BC300) was tested using flow cytometry (Fig. 2A). P*ykrL* 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 P*ykrL-gfp* reporter strain (BC400) growing in LB medium supplied with 200 mM KCl resulted in a strong induction of P*ykrL* (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 P*ykrL* activity, not even after prolonged incubation (Fig. 2C). This, as well as the sensitivity of the Δ*ykrL* 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 P*ykrL* activity and membrane energetics was suggested by results from a

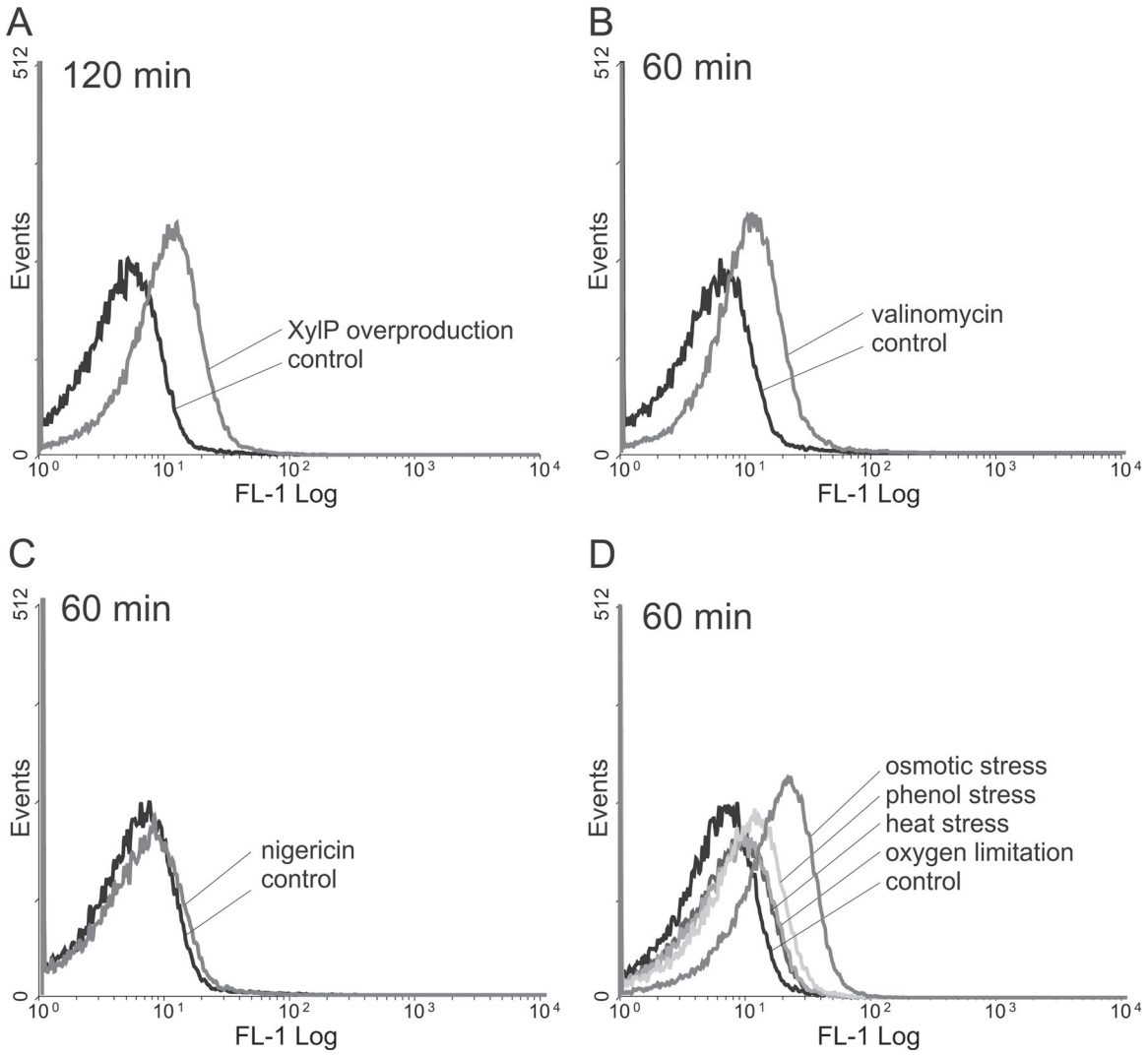

FIG 2 Flow cytometry histograms of the P*ykrL-gfp* reporter strains BC300 (A) and BC400 (B to D) showing P*ykrL* activity under different stress conditions. (A) *xylP* overexpression from pNZ-xylP induced by subtilin; control, strain carrying empty pNZ8902. (B) Membrane potential ($\Delta\Psi$) dissipation using 0.5 μM valinomycin. (C) ΔpH 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 OD_{600} 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 P*ykrL-gfp* activity.

random transposon mutagenesis screening in a P*ykrL-lacZ* reporter strain (HT400), which showed that P*ykrL* 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 P*ykrL* (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 P*ykrL* either directly or indirectly.

In addition, P*ykrL* 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 P*ykrL*, while phenol, heat, and anaerobic stress resulted in milder activation of P*ykrL*.

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 spectrom-

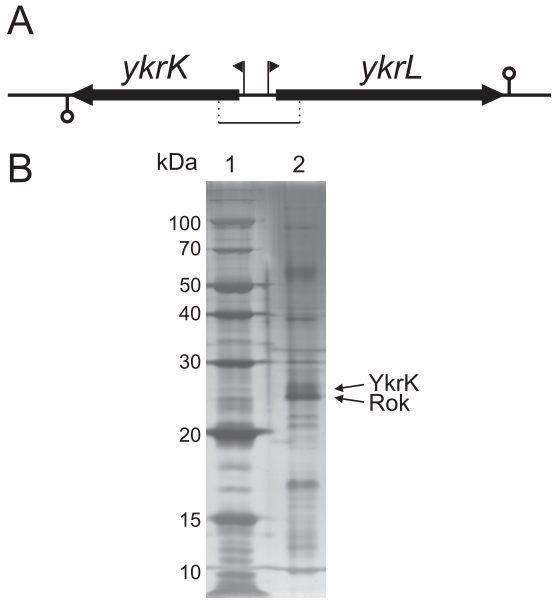

FIG 3 Identification of proteins binding to the *ykrK-ykrL* intergenic region using DNA affinity chromatography. (A) Schematic organization of the *ykrK-ykrL* locus in *B. subtilis*. Lollipops indicate terminator structures. Flags indicate predicted promoters. The line below indicates the fragment used for the affinity chromatography. (B) SDS-PAGE and silver stain analysis of the proteins enriched from the cytoplasmic fraction of a *B. subtilis* NZ8900 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*.

etry. 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, dihydrolipoamide acetyltransferase (*acoC*), lipoamide acyltransferase (*bkdB*), and dihydrolipoyl dehydrogenase (*lpdV*). Since these proteins were not (potential) DNA binding proteins, their role in *ykrL* regulation 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 P*ykrL-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, P*ykrL* activity was clearly higher in the Δ*ykrK* and Δ*rok* Δ*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 P*ykrL* activity also increased in the Δ*rok* mutant at late stationary growth phase (Fig. 4B). Overexpression of *ykrK* from pNZ-ykrK using the SURE system (4) in the Δ*ykrK* mutant (BC404) resulted in full repression of P*ykrL-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 *ykrK* promoter (P*ykrK*) and the predicted *ykrL* promoter (P*ykrL*), respectively. Fragment C covered the region between these two promoters, including predicted −35 boxes, and fragment D covered the whole region. The Rok and YkrK proteins were expressed in *E. coli* and *L. lactis* and purified using a C-terminal His_6 tag and Strep tag, respectively. Binding of Rok-His_6 and YkrK-Strep to these fragments was tested by electrophoretic mobility shift assays (EMSAs) (Fig. 5B). Binding affinities were estimated from the decrease in signal of the unbound DNA using binding kinetics curve fitting. Both proteins bound to fragment B (P*ykrL*), although YkrK with much higher affinity than Rok (apparent K_d [K_d app] of 0.1 μM and 1.2 μM, respectively). YkrK also showed significant binding to fragments A (K_d app = 3.4 μM) and C (K_d app = 2.7 μM), albeit with much lower affinity than to fragment B, indicating that next to *ykrL*, YkrK might regulate its own expression. The mobility shift patterns of fragment D were consis-

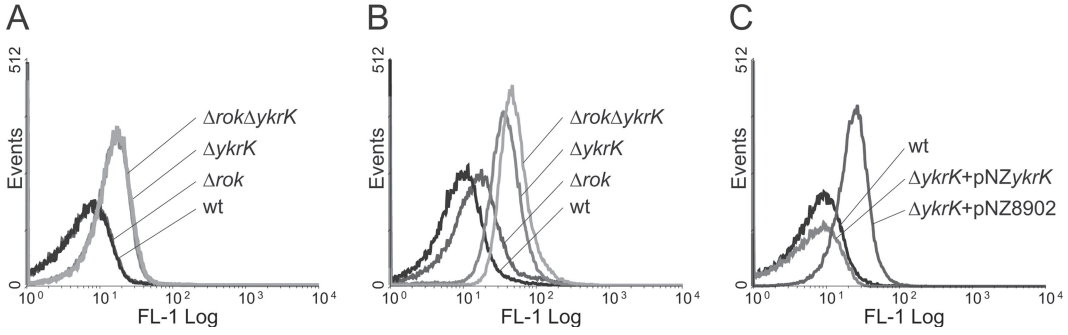

FIG 4 Flow cytometry histograms showing P*ykrL* activity in P*ykrL-gfp* reporter strains: wild type (BC400), Δ*rok* (BC401), Δ*ykrK* (BC402), Δ*rok*Δ*ykrK* (BC403), Δ*ykrK*+pNZ-ykrK (BC404 carrying pNZ-ykrK), and Δ*ykrK*+pNZ8902 (BC404 carrying pNZ8902). (A) GFP signal in cells growing exponentially; (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 P*ykrL-gfp* activity.

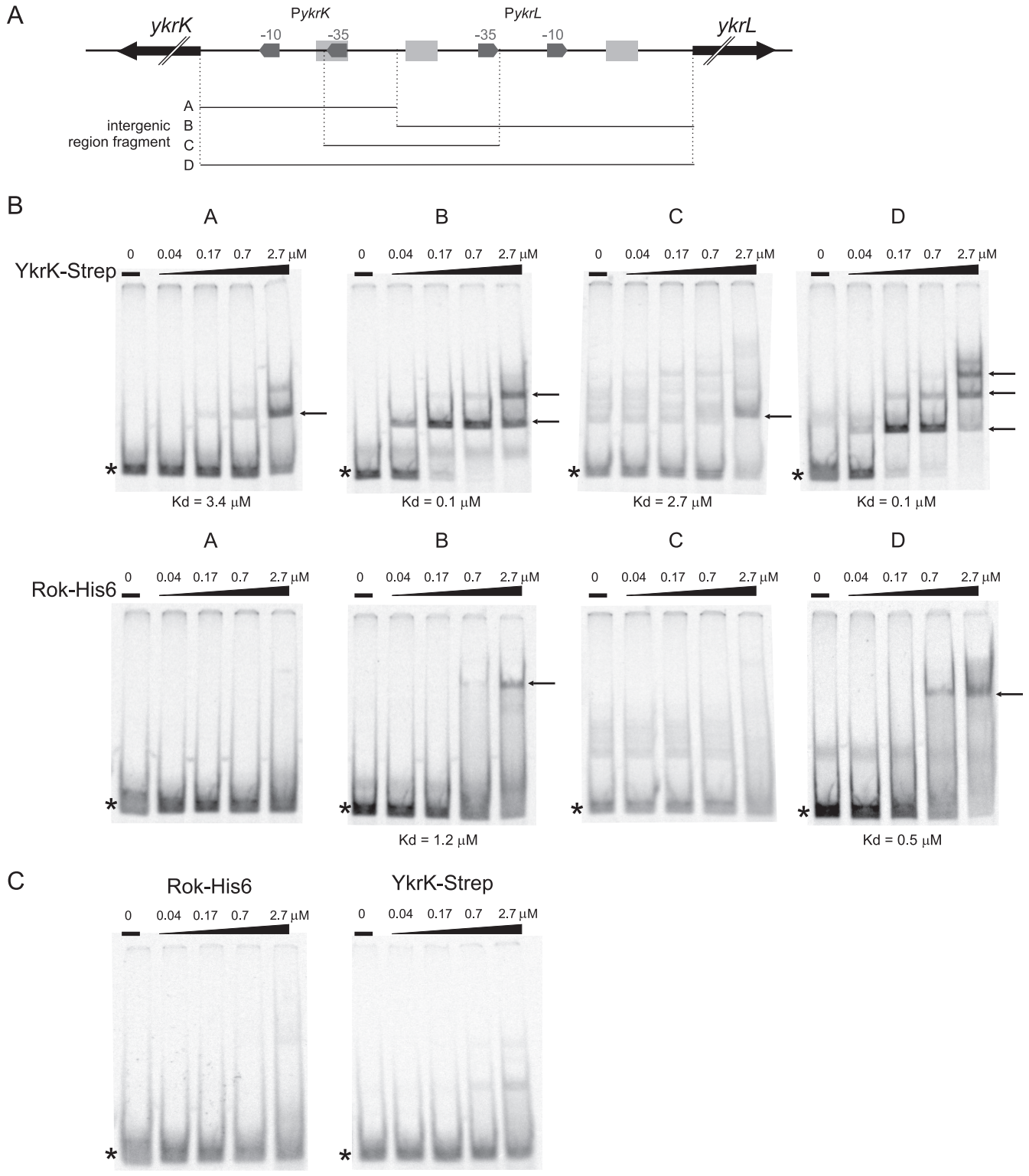

FIG 5 *In vitro* binding of YkrK-Strep and Rok-His$_6$ to fragments of the *ykrK-ykrL* intergenic region. (A) Schematic representation of the *ykrK-ykrL* intergenic region and fragments A (P*ykrK*), B (P*ykrL*), C, and D used for EMSA. Predicted −35 and −10 boxes of P*ykrK* and P*ykrL* are shown as boxed 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 P*secA* DNA. Asterisks indicate free ^{33}P-DNA probes, and arrows indicate the position of the shifted probe. The calculated K_d values are indicated below each binding assay graph.

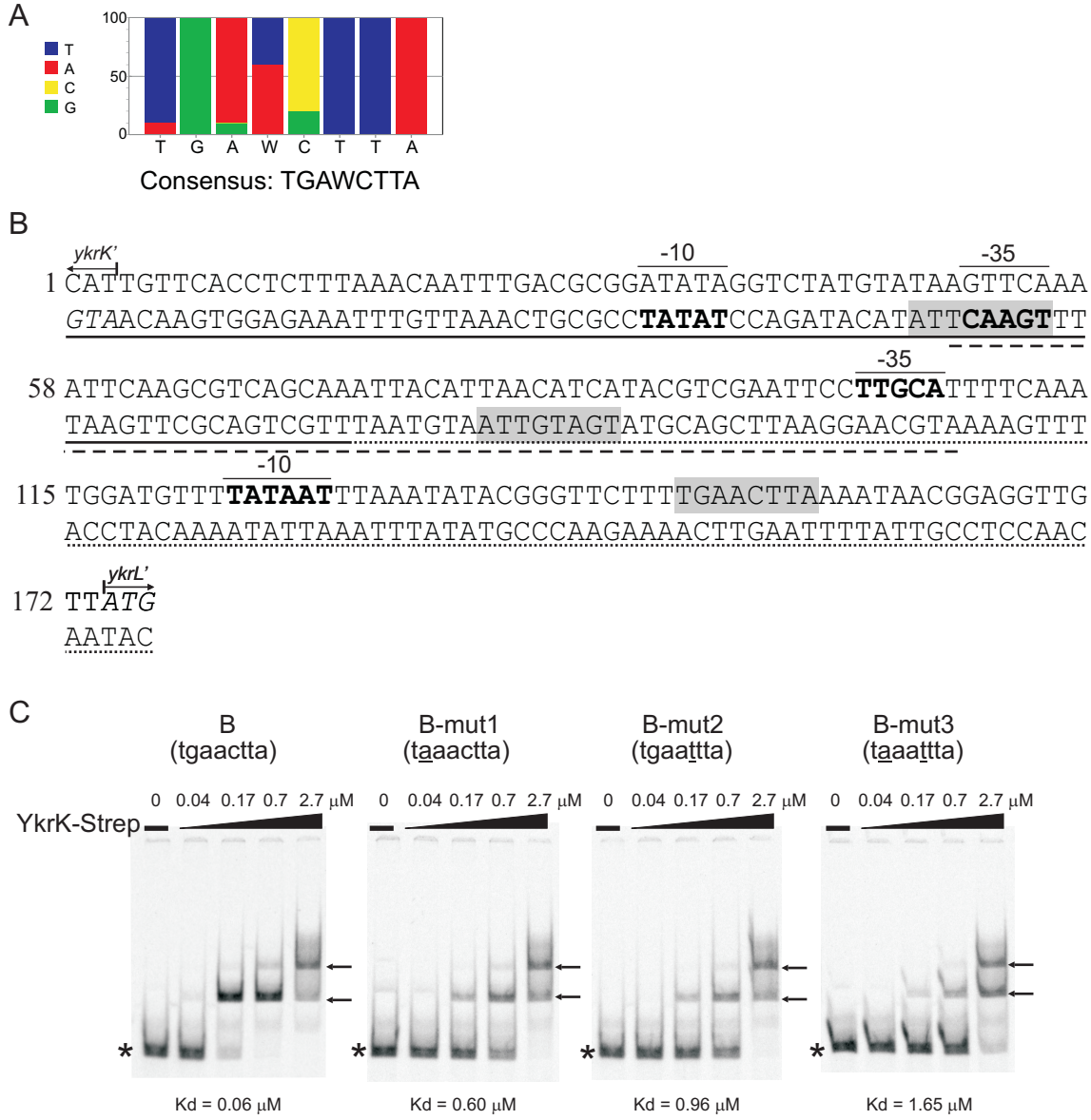

FIG 6 YkrK binding motif search and validation. (A) Weight matrix of consensus sequence present within the *ykrK-ykrL* intergenic region in different *Bacillus* species. (B) Sequence of the *ykrK-ykrL* intergenic region of *B. subtilis*, with putative YkrK binding sites in gray shading; start codons of *ykrL* and *ykrK* are in italics and predicted −10 and −35 boxes in bold. The fragments used for EMSA experiments in Fig. 5 are underlined: solid line, fragment A (P*ykrK*); dotted line, fragment B (P*ykrL*); dashed line, fragment C. (C) EMSA of YkrK-Strep binding to fragment B containing the consensus motif, and with derivatives containing point mutations in the motif (B-mut1, B-mut2, and B-mut3). Point mutations are underlined. Asterisks indicate free ^{33}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 P*ykrK*, within P*ykrL*, 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 P*ykrK*, downstream of the −10 box of P*ykrL*, 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 P*ykrL*, 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, P*ykrL*. In contrast, the *ykrL* mutation did not increase sensitivity to dissipation of the chemical proton gradient, ΔpH, which together with the $\Delta\Psi$ constitutes the proton motive force. Nor did this condition induce P*ykrL*. 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 P*ykrL* 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 P*ykrL* 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*, CssRS, 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 P*ykrL* (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 P*ykrL*-controlled expression, together with other studies discussed above, indicate YkrL as a quality control system for membrane proteins.

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