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

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Supplemental Materials and Methods

Plasmid and strain construction.

All primers used in this study are listed in Table S1 and strains are listed in Table 1. The *ykrL* deletion was constructed as follows. First, the *ykrL* locus including 735 bp upstream the start codon and 584 bp downstream the stop codon, was amplified using primers ykrLdel-fw and ykrLdel-rv. The PCR product was ligated into pCR2.1-TOPO (Invitrogen). The resulting vector, pTOPO-DykrL-1, was digested with *Swa*I and *Eco*47III enzymes. The spectinomycin resistance gene was amplified from pDG1726 (3) using primers specBsu-F and specBsu-R and ligated into pTOPO-DykrL-1. The resulting plasmid pTOPO-DykrL-2 carries *ykrL* flanking regions and the spectinomycin resistance gene replacing 874 bp of the *ykrL* gene. Strain JW8940 was constructed by transforming JW8900 with pTOPO-DykrL-2 resulting in replacement of the *ykrL* gene with the spectinomycin cassette by double recombination.

The *sigW* deletion mutant was constructed as follows. The *sigW* locus including 540 bp upstream the start codon and 376 bp downstream the stop codon was amplified using primers sigW-del-fw and sigW-del-rv. The PCR product was ligated into pCR2.1-TOPO (Invitrogen) resulting in pTOPO-DsigW-1. Next, the kanamycin resistance marker was amplified from pDG780 (3) using the primers Km-dsigW-fw and Km-dsigW-rv. After digestion with *Sac*I and *Acc*I, the product was ligated into pTOPO-DsigW-1, from which a 374 bp internal fragment of sigW was removed by digestion with the same enzymes. The resulting vector, pTOPO-DsigW-2 was transformed to the *B. subtilis* RH100, giving rise to strain KB100 which contains the kanamycin resistance cassette replacing the 374 bp internal *sigW* fragment.

To disrupt a *ykrK*, the deletion construct pDG1514-ykrK was made in two steps. An upstream flanking region of *ykrK* was amplified with primers ykrK::tc-o1 and ykrK::tc-o2, digested with *Bam*HI and *Pst*I and ligated to the corresponding sites of pDG1514 (3), upstream the tetracycline resistance cassette. Next,

a downstream flanking region of *ykrK* was amplified with primers ykrK::tc-o3 and ykrK::tc-o4, digested with *Hind*III and *Xho*I and ligated at the same sites downstream the resistance cassette, resulting in pDG1514-ykrK. The construct was then transformed to BC400 and BC401, which gave strains BC402 and BC403, respectively, with a disrupted *ykrK* gene.

The P*ykrL-gfp* reporter strains were obtained as follows. First, a DNA fragment of 510 bp upstream of *ykrL* containing the promoter region of *ykrL* was amplified using primers PykrL-fw and PykrL-rv. The PCR product was digested with *Kpn*I and *Pst*I and ligated into the same sites of pSG1151 (6), which resulted in pPykrL-gfp. pPykrL-gfp was transformed to the strains 168 and RH100 resulting, by means of Campbell-type integration, in strains BC400 and BC300, respectively.

The BC401 strain carrying the P*ykrL-gfp* fusion and *rok* deletion was made by transforming BC400 with chromosomal DNA from *B. subtilis* BD3196, carrying a *rok* mutation (1).

Strain HT400 was constructed as follows. A 463 bp *ykrL* promoter region of *B. subtilis* was amplified using primers PykrLpDL-fw and PykrLpDL-rv, digested with *BamH*I and *Kpn*I and ligated upstream of the β-galactosidase gene (*bgaB*) in pDL, an *amyE* integration vector (8), which was cut with corresponding enzymes. The resulting plasmid pDL-PykrL was transformed to *B. subtilis* 168, resulting in integration of the P*ykrL-bgaB* (P*ykrL-lacZ*) fusion in the *amyE* locus.

Plasmid pNZ-ykrK-strep was made by amplifying *ykrK* using primers ykrK-strep-F and ykrK-strep-R carrying the Strep-tag sequence for a C-terminal fusion. The product was digested with *Pag*I and *Kpn*I and ligated into pNZ8948 (4), cut beforehand with *Nco*I and *Kpn*I.

To construct pNZ-ykrK, *ykrK* gene was amplified using primers ykrK-fw and ykrK-rv. The product was digested with *Pag*I and *Hind*III and ligated into pNZ8902 digested with *Nco*I and *Hind*III.

To make pNZ-xylP, *xylP* of *Lactobacillus pentosus* was amplified using primers xylP-fw and xylP-rv. The PCR product was digested with *Pag*I and *Xba*I and ligated into pNZ8902 digested with *Nco*I and *Xba*I.

DNA Microarray analysis.

The transcriptional profile of *B. subtilis* NZ8900 carrying pNZ-ykrK was compared to the profile of strain NZ8900 strain carrying the empty vector pNZ8902. For *ykrK* induction, SURE expression system was used (2). Strains were grown in TY broth and at OD_{600} of 0.8, 0.1% subtilin was added to both cultures. After 30 minutes further incubation, 10 OD units (1 OD_{600} unit corresponds to 1 ml of a culture of an OD_{600} of 1.0) of each culture was collected for RNA isolation. The microarray experiment was performed in three biological replicates and one technical replicate.

RNA isolation, amino allyl-modified cDNA synthesis, cDNA labeling with Cy3 and Cy5 dyes, hybridization to oligonucleotide microarrays, washing, scanning, image analysis and normalization of data as well as statistical analysis were performed as described previously (7).

The complete microarray data is available at GEO data repository (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi) under accession number GSE33456.

Transposon mutagenesis

Detection of transposition events. Random mutagenesis of the *B. subtilis* strain HT400 was performed using the TnYLB-1 transposon following the protocol described before (5) with modifications. The HT400 strain was transformed with pMarA carrying the TnYLB-1 transposon. Transformants were selected on plates for erythromycin resistance at 30°C, permissive for plasmid replication. A positive clone was grown for three hours at 30°C, whereupon the temperature was shifted to 45°C (nonpermissive temperature) and grown for additional 4 hours. Proper dilutions were plated on TY agar containing 5 μg ml^{-1} kanamycin and 0.01 % X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside, Sigma) and

incubated at 45°C. To confirm that the transposition event was efficient, the same dilutions were plated on TY agar containing 1 μg ml^{-1} erythromycin.

Identification of transposon insertion sites. 1 μg of chromosomal DNA from transposon mutants was digested with *Taq*I or *Hind*III, purified and circularized in a ligation reaction using T4 DNA ligase. 100 ng of ligated DNA was used as a template for inverse-PCR (iPCR) using primers oIPCR1 and oIPCR2 (5). iPCR products were purified and sequenced with oIPCR3 primer (5) by ServiceXS (Leiden, The Netherlands).

Table S1. Primers used in this study.

Primer	**Sequence (5'- 3')**	**Application**
ykrLdel-fw	GCTCTGTCTCCTGTTCGGCG	Deletion mutants
ykrLdel-rv	GATCAGAAACAGTACCTGCTTC	
specBsu-F	GGGCTTGGATCCCAACGCTGTCGACGTTGTAAAACGACGG	
specBsu-R	CGCATAGCTTTCCGGTCGCCGCAGCTATGACCATGATTACGC	
sigW-del-fw	GCGAAAGGTACCTCTGCCTTACAAGCAGAGGG	
sigW-del-rv	GCGAAATCTAGAATTCGGCTGCTTGGACACGC	
Km-dsigW-fw	TAATACGACTCACTATAGGGC	
Km-dsigW-rv	ACCGGGCCCCCCCTCGAGGTATACGGTATCGATACAAATTCC	
ykrK::tc-o1	GCGAAAGGATCCCGATACTGCGACAAGTGAACG	
ykrK::tc-o2	GCGAAACTGCAGTGACATGTTCGCTGATTGGACG	
ykrK::tc-o3	GCGAAAAAGCTTAGATTATCGCCATTACAGTCC	
ykrK::tc-o4	GCGAAACTCGAGAAGAAGAGAAAGCGGATGACC	
PykrL-fw	GCGAAAGGTACCGTTCAATTGAGTATTTTTTGCCC	Transcriptional fusion P*ykrL-gfp*
PykrL-rv	GCGAAACTGCAGCGCCATAACAACCTCCGTTATTTT	
PykrLpDL-fw	GCGAAAGGATCCTTGCCCAGCTCAGGGTGTCCG	Transcriptional fusion P*ykrL-lacZ*
PykrLpDL-rv	GCGAAAGGTACCAAAAGAACCCGTATATTTAAATTA	
ykrK-strep-F	CGTAGTCATGATGAACATTTTTAAACTCTCTCGAACCGATATGG	YkrK-Strep fusion
ykrK-strep-R	CGTCCGGTACCTCATTTTTCAAATTGTGGATGGCTCCATAGAATGTCTGCTGTCATTTTATG	
ykrK-fw	GCGAAATCATGAACATTTTTAAACTCTCTCGAACCG	YkrK overexpression
ykrK-rv	GCGAAAAAGCTTTTATTTATGCTGGCAATTGTTGTGG	
xylP-fw	CGCATATCATGAGCGTTAGTATGCAGC	XylP overexpression

Table S1. Continued.

xylP-rv	GCGAAATCTAGATTAATGGTGATGGTGATGG TGCTTTTGATCGTCAGCAA	
PykrL-oligo4	ACAAAACGATCCCGATTGTTG	DNA affinity chromatography
PykrL-oligo5	TGTCCGATCAGATCTTTGATATCAC	
SQ_PsecA_o1	CAAATTCTTTGGAAATAACAAAAGGTATGAT ATGATAATGAGAGGTATACATGGACTAG	EMSA, P*secA*
SQ_PsecA-o2	CTAGTCCATGTATACCTCTCATTATCATATCA TACCTTTTGTTATTTCCAAAGAATTTG	
KL1-o1	ATGTTCATTGTTCACCTC	EMSA, forward primer for DNA fragment A and D
KL1-o2	TTGCTGACGCTTGAATTTTG	EMSA, revers primer for DNA fragment A
KL2-o1	ATTACATTAACATCATACGTCG	EMSA, forward primer for DNA fragment B
KL2-o2	TTCGCCATAACAACCTCC	EMSA, revers primer for DNA fragment B and D
KL3-f	AGTTCAAAATTCAAGCGTC	EMSA, forward primer for DNA fragment C
KL3-r	ATGCAAGGAATTCGACGTATG	EMSA, revers primer for DNA fragment C
KL2-o2-a	TTCGCCATAACAACCTCCGTTATTTTAAGTTT AAAAGAACCCGTATATTTAAATTATA	EMSA, B-mut1
KL2-o2-t	TTCGCCATAACAACCTCCGTTATTTTAAATTC AAAAGAACCCGTATATTTAAATTATA	EMSA, B-mut2
KL2-o2-at	TTCGCCATAACAACCTCCGTTATTTTAAATTT AAAAGAACCCGTATATTTAAATTATA	EMSA, B-mut3

Table S2. Genes with significantly changed expression ratio (0.4 > ratio > 2.5, Bayesian p < 0.01) upon YkrK overproduction.

Gene	Ratio	Product	YkrK binding motif		
			Sequence [a]	Start [b]	End [b]
ykrK	136.94	hypothetical protein	atttTGAACTTAtaca atttTGAACTTAtaca*	-52 -153	-45 -146
ykzE	6.75	hypothetical protein			
guaC	5.43	guanosine 5'-monophosphate oxidoreductase			
bmrU	5.05	diacylglycerol kinase			
yqjF	4.07	hypothetical protein			
purC	3.89	phosphoribosylaminoimidazole-succinocarboxamide synthase			
purS	3.59	phosphoribosylformylglycinamidine synthase subunit PurS			
purB	3.46	adenylosuccinate lyase			
amyC	2.96	maltose and multiple sugars ABC transporter permease			
amyD	2.91	carbohydrate ABC transporter permease			
ytlD	2.82	permease			
purQ	2.79	phosphoribosylformylglycinamidine synthase I			
ywjC	2.76	hypothetical protein			
purE	2.76	phosphoribosylaminoimidazole carboxylase I			
msmE	2.69	multiple sugar-binding lipoprotein			
purK	2.61	phosphoribosylaminoimidazole carboxylase ATPase subunit			
yfnF	0.40	glycosyltransferase			
yktB	0.38	hypothetical protein			
penP	0.36	beta-lactamase precursor	caatTGATCTTAtatt*	-106	-99
sspG	0.36	small acid-soluble spore protein			
ykrL	0.22	heat shock protein HtpX	ctttTGAACTTAaaat atttTGAACTTAtaca*	-25 -126	-18 -119
yrkA	0.21	membrane associated protein	ctttTGAACTTAtaat	-88	-81
ykoX	0.19	integral inner membrane protein			

[a] Predicted YkrK binding motif (in capitals) with flancing sequences.
[b] Distance calculated from the first nucleotide of the start codon.
* Motifs found in the dirction reverse to the gene.

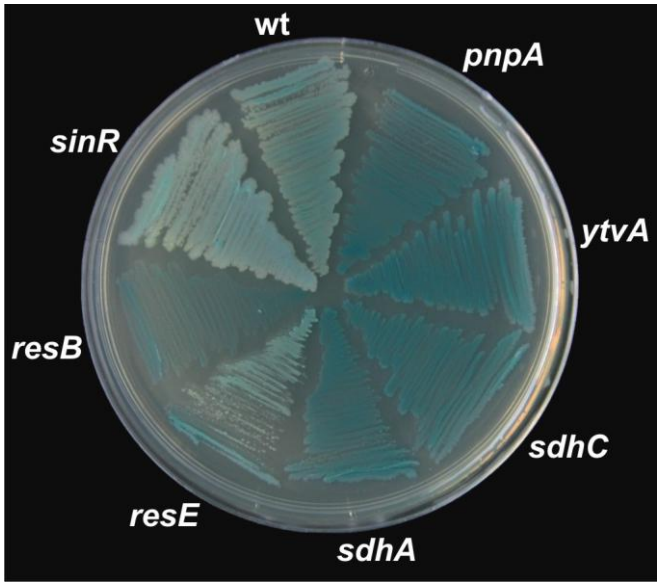

Fig. S1. Random mutagenesis of the P*ykrL-lacZ* reporter strain HT400. Strains carrying TnYLB-1 transposon mutations with positive (*pnpA*, *ytvA*, *sdhC*, *sdhA*, *resE*, *resB*) and negative (*sinR*) effects on P*ykrL* activity. WT, HT400; *pnpA*, polynucleotide phosphorylase; *ytvA*, positive regulator of sigma-B activity; *sdhC* and *sdhA*, succinate dehydrogenase subunits; *resE*, two-component sensor histidine kinase, global regulation of aerobic and anaerobic respiration; *resB*, cytochrome c biogenesis protein; *sinR*, master regulator of biofilm formation. Strains were plated on LB solid medium supplemented with 0.008% X-gal.

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