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The Rok Protein of *Bacillus subtilis* Represses Genes for Cell Surface and Extracellular Functions

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Rok is a repressor of the transcriptional activator ComK and is therefore an important regulator of competence in *Bacillus subtilis* (T. T. Hoa, P. Tortosa, M. Albano, and D. Dubnau, Mol. Microbiol. 43:15–26, 2002). To address the wider role of Rok in the physiology of *B. subtilis*, we have used a combination of transcriptional profiling, gel shift experiments, and the analysis of *lacZ* fusions. We demonstrate that Rok is a repressor of a family of genes that specify membrane-localized and secreted proteins, including a number of genes that encode products with antibiotic activity. We present evidence for the recent introduction of *rok* into the *B. subtilis-Bacillus licheniformis-Bacilllus amyloliquefaciens* group by horizontal transmission.

ComK is the major transcription factor driving the development of competence for transformation in *Bacillus subtilis* (37). *rok* encodes a direct repressor of *comK* transcription (18) and thereby plays an important, but incompletely understood, role in the complex regulation that takes place at the promoter of *comK*. Five proteins are known to bind to *PcomK* (reviewed in reference 9): ComK itself, DegU, Rok, AbrB, and CodY, the last three of which exert negative effects.

The use of a *rok-lacZ* fusion construct revealed no major change in *rok* transcription during growth in the population as a whole (18). Given this, it is surprising that the regulation of *rok* transcription is complex; *rok* is repressed by Rok itself, by ComK, and by the transition state regulators SinR and AbrB, although only ComK and Rok have been shown to bind directly to *Prok* (18). As cultures approach the end of exponential growth, the concentrations of active AbrB and SinR decrease (reviewed in references 31 and 32), leading to the expectation that *rok* transcription would increase. However, since ComK acts negatively on *Prok*, the increased synthesis of ComK in competent cells after the cessation of exponential growth would tend to place a limit on increased *rok* transcription. In addition, negative autoregulation at *Prok* would be expected to maintain a constant time-averaged level of *rok* expression. Transient changes in the concentration of Rok may play a role in the timing of competence expression, in the selection of which cells will develop competence, and in limiting the final level of *comK* expression in the competent subpopulation. Although transient changes have not been detected, it may be that our experiments lacked sufficient time resolution to detect these fluctuations. A major unanswered question concerns the possibility that the activity of Rok is somehow regulated, perhaps responding to the presence of an unknown corepressor.

The origin of *rok* is also interesting, since it has orthologs among sequenced organisms only in *Bacillus licheniformis* (68% identical [http://63.198.8.200/]) and *Bacillus amyloliquefaciens* (83% identical [R. Borriss, personal communication]). No orthologs are detected using either BLAST or Psi-BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), even in other bacilli, such as *B. anthracis*, *B. halodurans*, and *B. cereus* (reference 18 and unpublished data). Both *rok* and its convergently transcribed immediate downstream neighbor *yknT* are missing from these related species.

To extend our understanding of the role of Rok, we identified additional genes regulated by this protein and asked whether Rok was capable of acting positively or only as a repressor. By transcriptional profiling and the use of *lacZ* fusions, we have identified at least seven gene clusters that are negatively regulated by *rok*, including several involved in the production of bacteriocin-like antibiotics, and we have shown that this repression is caused by direct binding of Rok to target DNA sequences.

MATERIALS AND METHODS

Strains and general procedures. The *B. subtilis* strains used in this study are derived from strain 168 and most are isogenic with BD630 (*his leu met*). Selective and growth media, the growth of strains to competence, and transformation were described or referenced by Albano et al. (1) and by Tortosa et al. (35). Strains are listed in Table 1. Molecular biological methods were essentially as described by Sambrook et al. (30). β-Galactosidase assays were carried out as described previously (35) by using cultures grown in competence medium (1), which is a minimal salts medium supplemented with glucose, casein hydrolysate, and yeast extract.

Construction of *lacZ* fusions. To generate fusion constructs of *lacZ* to *sunT*, *yxaJ*, *yybM*, *yydI*, *yjcN*, and *sdpA*, internal fragments of these genes were isolated by PCR by using the primers listed in Table 2. The fragments were cleaved with HindIII and BamHI and inserted into pMUTIN2 (36), which was also cleaved with these two enzymes. The resulting recombinant plasmids were used to transform BD630 and BD2955 with selection for erythromycin resistance and were thereby integrated in the chromosomes of these strains by single reciprocal recombination (Table 1).

DNA microarrays and transcriptional profiling. We purchased a *B. subtilis* oligonucleotide library, manufactured by Sigma Genosys and designed by Compugen. The library consisted of a collection of 4,128 oligonucleotides (65-mers) representing 4,106 *B. subtilis* genes, 10 control oligonucleotides (from *Esche-*

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TABLE 1. Strains used in this study

Strain	Genotype	Source
BD630	*his leu met*	Laboratory stock
BD2955	*his leu met rok*::*miniTn10* (Spc^r)	18
BD3196	*his leu met rok* (Kan^r)	This work
ORB3147	*trpC2 pheA1 alb*::*pMUE1* (Erm^r)	40
ORB3162	*trpC2 pheA1* SPβc2del2::Tn*917*::pTK-*sboΔEB* (Erm^r Cm^r)	40
BD2121	*his leu met comK* (Kan^r)	38
BD3128	*his leu met comK rok*::*mini*Tn*10* (Kan^r Spc^r)	This work
BD3702[a]	*his leu met albA-lacZ*	This work
BD3703[a]	*his leu met albA-lacZ rok*::*mini*Tn*10* (Kan^r Spc^r)	This work
BD3704[b]	*his leu met sboA-lacZ* (Erm^r Cm^r)	This work
BD3705[b]	*his leu met sboA-lacZ rok*::*mini*Tn*10* (Erm^r Cm^r Spc^r)	This work
BD3761	*his leu met yxaJ-lacZ*	This work
BD3762	*his leu met yxaJ-lacZ rok*::*mini*Tn*10* (Erm^r Spc^r)	This work
BD3763	*his leu met yybM-lacZ* (Erm^r)	This work
BD3764	*his leu met yybM-lacZ rok* (Erm^r Kan^r)	This work
BD3767	*his leu met sunT-lacZ*	This work
BD3768	*his leu met sunT-lacZ rok*::*mini*Tn*10* (Erm^r Spc^r)	This work
BD3814	*his leu met sdpA-lacZ*	This work
BD3815	*his leu met sdpA-lacZ rok* (Erm^r Kan^r)	This work
BD3821	*his leu met yjcN-lacZ*	This work
BD3822	*his leu met yjcN-lacZ rok* (Erm^r Kan^r)	This work
BD3765	*his leu met yydl-lacZ*	This work
BD3766	*his leu met yydl-lacZ rok* (Erm^r Kan^r)	This work

[a] The *alb-lacZ* construct was obtained from ORB3147.
[b] The *sboA-lacZ* construct was obtained from ORB3162.

richia coli and Brome mosaic virus), and 12 random oligonucleotides. The oligonucleotides were designed to represent the *B. subtilis* genes as found in the genome data release R16.1 (26 April 2001) at the SubtiList website (http://genolist.pasteur.fr//SubtiList/). A single oligonucleotide was made for each gene. The oligonucleotides were spotted onto poly-L-lysine-coated glass slides at a concentration of 25 μM, and approximately 0.7 nl, containing ~17.5 fmol, was delivered per spot. Each gene was represented once per slide. The oligonucleotides were spotted, and the hybridized arrays were scanned in the Center for Applied Genomics facility maintained at the Public Health Research Institute.

BD2121 (*comK rok*$^+$) and BD3128 (*comK rok*::*mini*Tn*10*) were grown in competence medium (1), and samples were harvested for RNA isolation during exponential growth (corresponding to a reading in a Klett-Summerson colorimeter of about 70) and at T_2, defined as 2 h after the cultures departed from exponential growth. Samples were chilled rapidly by diluting them into an ice slurry of 0.3 M NaCl and 0.03 M Na citrate and collected by centrifugation at 4°C. Total cell RNA was isolated as described previously (5), from at least four independently grown cultures of the two strains. The RNA samples were analyzed on agarose gels to assess quality before being used in the preparation of cDNA.

cDNA was prepared from the RNA samples as previously described (5), by reverse transcription of 25 μg of total RNA to incorporate aminoallyl-dUTP into first-strand cDNA. The cDNA products were subsequently labeled by direct coupling either to Alexa Fluor 555 or Alexa Fluor 647 amine-reactive dyes (Molecular Probes), as described by the manufacturer. Labeled probes were mixed and purified using Qiaquick PCR spin columns; reciprocal labeling (dye swapping) of cDNA was done for all experiments.

Probes were purified for hybridization to microarrays essentially as described elsewhere (5) without filtration and were then boiled for 2 min, cooled to room temperature, and applied to the microarrays under coverglasses. The microarrays were placed in HybChambers (GeneMachines, San Carlos, Calif.) and incubated at 68°C overnight. The arrays were washed and dried as described previously (5). The slides were scanned, and images were produced with a GenePix 4000A microarray scanner (Axon Instruments).

Raw data files produced by GenePix (GPR files) were exported to Excel and prepared for analysis with CyberT software (24), as suggested by the program's authors (http://visitor.ics.uci.edu/genex/cybert/). The data were normalized by global scaling as described by Baldi and Hatfield (4). Each intensity value for each gene for a given wavelength was divided by the sum of the intensities for all genes at that wavelength. The resulting values were used to calculate red/green ratios, and the ratios were natural log transformed, as required by the software. A Bayesian paired expression value estimate was calculated for each gene according to the methods described in the CyberT website. Parameters for Bayesian standard deviation estimation were as suggested by the authors (sliding window size, 101; confidence value, 10).

Expression and purification of Rok-His$_6$. An overnight culture of *E. coli* strain ED428 (18) was diluted 1:50 into fresh TY medium supplemented with 100 μg of ampicillin/ml and 25 μg of kanamycin/ml and grown at 37°C with vigorous shaking. At an A_{600} of 0.6, expression of the recombinant protein was induced with 1 mM isopropyl thio-β-D-galactoside. Growth was continued for 4 h, and cells from 1 liter of culture were collected by centrifugation (10 min, 8,000 rpm, 4°C) in an Avanti J-20 XP centrifuge (Beckmann Coulter). The pellet was washed with 50 ml of buffer A (50 mM $NaHPO_4$, 300 mM NaCl, 10 mM imidazole, 3.5% glycerol, 1 mM β-mercaptoethanol; pH 8.0) and stored at −80°C for future use. The pellet was resuspended in 5 ml of buffer A and supplemented with Complete Mini protease inhibitor (Roche), and cells were disrupted by sonication. Cellular debris was removed by centrifugation (30 min, 25,000 rpm, 4°C), and the supernatant fraction was loaded onto a Superflow Ni-nitrilotriacetic acid resin column (QIAGEN) equilibrated with buffer A. The column was washed with buffer B (identical to buffer A, but with 20 mM imidazole). The protein was eluted from the column by using a linear gradient of 20 to 500 mM imidazole, and 0.5-ml fractions were collected. Fractions were checked for protein content and purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and for DNA content by ethidium bromide staining of agarose gels. Fractions containing the protein were pooled and loaded onto a MonoQ column (Amersham Pharmacia) and equilibrated with buffer C (20 mM Tris, 1 mM EDTA; pH 8.0) to remove DNA. Elution was carried out using a linear gradient of 0 to 2 M KCl. Fractions containing protein, but negligible amounts of DNA, were used in subsequent experiments. Protein was quantified using the RC/DC protein determination kit (Bio-Rad).

Gel retardation. Gel retardations were performed essentially as described previously (14). Fragments for use as probes were amplified with Expand (Roche) or *Pwo* (Roche) DNA polymerase using the primers from Table 2. The PCR products were end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. Protein and probe were mixed on ice and subsequently incubated for 20 min at 37°C. Samples were loaded onto a 6% nondenaturing polyacrylamide gel prepared with 1× TAE (40 mM Tris acetate [pH 8.0], 2 mM EDTA) and run in a 0.5-to-2.0× gradient of TAE at 100 V for 45 to 60 min in a MiniProtean electrophoresis system (Bio-Rad). Gels were dried in a vacuum dryer (model 583; Bio-Rad), and signals were recorded using phosphoscreens and a Cyclone PhosphorImager (Packard).

TABLE 2. Primers used in this study

Primer	Target fragment	Sequence	Reference
psboA1	*PsboA*	5′-CCTCATAAAAAGCATTTCCT-3′	This study
psboA2	*PsboA*	5′-AATTGAATCCTCCCTTTTTT-3′	This study
psunA1	*PsunA*	5′-ATCCATAATAGTCAATTTTA-3′	This study
psunA2	*PsunA*	5′-TTGTAAAACCTCCCCATTTG-3′	This study
pyjcN-F	*PyjcN*	5′-CTTTATTATGTAGTGGCTGCA-3′	This study
pyjcN-R	*PyjcN*	5′-TCATTTTTTCCTCCGATACCTT-3′	This study
pK-F	*PcomK*	5′-AATCTATCGACATATCCTGCAA-3′	This study
pKplusR-R	*PcomK*	5′-TTATACTAATAATCTATCATCTGTTT-3′	This study
rokint1	*rok* CS[a]	5′-CGGGATCCCGCTTGCGGTTAGAA-3′	This study
rokint2	*rok* CS[a]	5′-GGAATTCCGAAGCTCCGAGCTCT-3′	This study
psdpF	*PsdpA*	5′-CCCAAGCTTAGTTCCCAAATTCAATTCTG-3′	This study
psdpR	*PsdpA*	5′-GGAATTCTAATAGGAAACATATAGTCATTAC-3′	This study
pyydF-F	*PyydF*	5′-ATATTGATGTTTTACTTTTCAT-3′	This study
pyydF-R	*PyydF*	5′-CATATTATCCCTCCTCCT-3′	This study
pyybN-FL	*PyybN*	5′-CATGCAATTTAGTGATCCAA-3′	This study
pyybN-R	*PyybN*	5′-CATAATTTTACAATCCTTTCA-3′	This study
pKOH-R	*PcomK*	5′-TTCTGACTCATATTATGGCCT-3′	This study
pykuWpa	*Prok*	5′-CGGGATCCGCTTCTCTTTCATTAAACAT-3′	18
prokps	*Prok*	5′-CGGAATTCGATGTTTTTCCTCAATTTTAG-3′	18
pcomG1	*PcomG*	5′-CCGGAATTCATGGTGACCATGTCTGCT-3′	33
pcomG2	*PcomG*	5′-CGCGGATCCCTCTCCTTTCAACGC-3′	33
pskfA-F	*PskfA*	5′-CAAGCCGTACGAACGGACTG-3′	This study
pskfA-R	*PskfA*	5′-TGGATACGACCTCTTTGCCC-3′	This study
pyydH-F	*PyydH*	5′-GAAAAGTTAAATTCCAATTTGC-3′	This study
pyydH-R	*PyydH*	5′-TCATATTTTCCATCTTCATACTT-3′	This study
pbhlA-F	*PbhlA*	5′-GGCACAGTTAAGCTTGG-3′	This study
pbhlA-R	*PbhlA*	5′-GTTCACCACCTTAGTACC-3′	This study
pykuJ-F	*PykuJ*	5′-TAATTATGTGCATGACATTCAAAAAAAG-3′	This study
pykuJ-R	*PykuJ*	5′-TGCAGCCGTGTGATGATACC-3′	This study
pyxaJ-F	*PyxaJ*	5′-TACATGCAATATGGTATGGTG-3′	This study
pyxaJ-R	*PyxaJ*	5′-GTCATGATCGCCACGCTATT-3′	This study
sunT-F	*sunT*[b]	5′-GCCAAGCTTTGGGGATAAGGAAGGCT-3′	This study
sunT-R	*sunT*[b]	5′-CGGGATCCACCCACAACGAACAAGGA-3′	This study
yxaJ-F	*yxaJ*[b]	5′-GCCAAGCTTAGCTGGCATGTGGTCA-3′	This study
yxaJ-R	*yxa*[b]	5′-CGGGATCCGCCACAAACCAAATGACG-3′	This study
yybM-F	*yybM*[b]	5′-GCCAAGCTTTCTGGCACTTCCGT-3′	This study
yybM-R	*yybM*[b]	5′-CGGGATCCAAGGCTGTTATCAGGGA-3′	This study
yydl-F	*yydl*[b]	5′-GCCAAGCTTAGCAAAAGAATCGGCAG-3′	This study
yydl-R	*yydl*[b]	5′-CGGGATCCATCTTCTCGGGGTTCTC-3′	This study
yjcN-F	*yjcN*[b]	5′-CCCAAGCTTCTCCTGTTGTCTTTACAGCTTCTTCGG-3′	This study
yjcN-R	*yjcN*[b]	5′-CGCGGATCCCCCTGTGCCACAACTTACTTCGTATTC-3′	This study
sdpA-F	*sdpA*[b]	5′-CCCAAGCTTGAGGTTGAGCAGGACTACTATC-3′	This study
sdpA-R	*sdpA*[b]	5′-CGCGGATCCCTATTGCCTGAACTCTTCCTTC-3′	This study

[a] Coding sequence.
[b] The target sequences from this genes was inserted into pMUTIN2 to create a fusion to *lacZ*.

RESULTS

Transcriptional profiling. In order to further define the set of genes regulated by *rok*, we used transcriptional profiling by microarray, with a chip containing synthetic oligonucleotides representing all of the annotated open reading frames in the sequenced *B. subtilis* genome. RNA was isolated from a strain with an inactivated *rok* gene and from an isogenic strain with intact *rok*. Since Rok is known to be a repressor of *comK*, which in turn regulates many genes (5, 13, 27), both strains used as sources of RNA for this experiment also carried a *comK* null mutation, to simplify analysis of the results. RNA was isolated at two stages during growth in competence medium (1): from exponentially growing cultures, and 2 h after the departure from exponential growth (defined as T_2). Results of these transcriptional profiling experiments can be viewed at the website http://www.phri.org/research/res_pidubnau.asp and are summarized in Table 3 and in Fig. 1. The genes selected for inclusion in Table 3 satisfied three criteria: at least a 1.8-fold difference between the *comK rok* and *comK* RNA samples, Bayesian *P* values less than 0.01 calculated using the program Cyber T (24), and at least six successful independent measurements. If either the T_2 or exponential samples satisfied these criteria, the gene was listed in Table 3. Twenty of 39 genes listed in Table 3 satisfied these criteria at both growth stages. Four satisfied the criteria only during exponential growth, while 15 genes satisfied the criteria only at T_2, since they exhibited less than a 1.8-fold difference during exponential growth. This bias suggests that *rok* may be more active in stationary phase or, more likely, that the genes it controls may tend to be those that are expressed after the cessation of exponential growth, even in the absence of Rok. Two additional genes (*sdpA* and *ykuK*), which fall short of the criteria, are also listed in Table 3 because they are adjacent to *sdpB* and *sdpC* on the one hand and to *ykuJ* and *ykzF* on the other.

TABLE 3. Transcriptional profiling of *rok* mutants[a]

Gene	Ratio (T_2)[b]	P value (T_2)[c]	Ratio (Exp.)[b]	P value (Exp.)[c]
albA	3.5	5.6×10^{-5}	3.6	1.2×10^{-5}
albB	4.5	1.2×10^{-6}	3.6	9.1×10^{-6}
albC	4.2	1.1×10^{-6}	3.7	5.5×10^{-6}
albD	4.6	1.8×10^{-6}	4.3	8.0×10^{-7}
albE	3.8	4.4×10^{-5}	4.1	6.6×10^{-6}
albF	4.3	4.4×10^{-6}	4.4	4.8×10^{-6}
albG	2.8	6.2×10^{-5}	2.6	3.3×10^{-4}
argG	2.1	7.7×10^{-4}	1.2	0.2
bdbA	2.0	7.1×10^{-3}	4.8	3.4×10^{-6}
bdbB	2.1	1.6×10^{-3}	1.8	5.0×10^{-3}
bhlA	6.9	1.1×10^{-7}	5.2	8.4×10^{-7}
fur	1.9	9.7×10^{-3}	1.2	0.1
hom	2.4	4.8×10^{-3}	1.2	0.2
rapF	2.0	2.9×10^{-3}	1.2	0.2
rpsL	2.3	4.6×10^{-3}	1.3	0.3
sboA	13.3	1.4×10^{-9}	5.2	3.3×10^{-5}
sboX	12.2	3.3×10^{-8}	7.2	2.8×10^{-6}
sdpB	1.9	1.8×10^{-3}	1.2	0.3
sdpC	1.8	3.9×10^{-3}	1.1	0.8
serA	2.2	6.5×10^{-3}	1.3	0.1
sunA	9.7	2.1×10^{-8}	5.8	1.5×10^{-6}
sunT	3.1	1.0×10^{-4}	8.5	1.4×10^{-8}
thrC	2.4	6.7×10^{-3}	1.2	0.2
yjcN	2.3	1.4×10^{-4}	1.4	1.14×10^{-2}
ykuJ	2.0	2.9×10^{-3}	1.3	0.1
ykzF	2.0	4.1×10^{-3}	1.3	3.8×10^{-2}
ykzG	2.0	0.3×10^{-3}	1.3	0.2
ylbN	1.9	8.1×10^{-3}	1.1	0.7
yolJ	2.7	4.6×10^{-4}	5.3	1.5×10^{-6}
ypiF	2.0	7.0×10^{-3}	1.9	3.9×10^{-3}
yxaJ	1.7	9.3×10^{-3}	3.3	2.5×10^{-6}
yxaL	1.4	8.9×10^{-2}	3.5	1.9×10^{-6}
yxbC	1.7	4.0×10^{-2}	2.0	3.4×10^{-3}
yybK	2.3	1.9×10^{-4}	2.3	6.4×10^{-4}
yybL	2.1	5.5×10^{-3}	1.9	2.8×10^{-3}
yybM	2.9	1.4×10^{-4}	2.5	2.6×10^{-4}
yydH	1.7	3.4×10^{-2}	1.9	2.6×10^{-3}
yydI	2.0	1.8×10^{-3}	1.4	1.7×10^{-2}
yydJ	3.1	1.9×10^{-5}	2.6	2.8×10^{-5}
sdpA[d]	1.4	2.4×10^{-2}	1.0	0.7
ykuK[d]	1.4	0.1	1.2	0.2

[a] RNA was isolated from a *comK* strain and from an isogenic *comK rok* strain growing in competence medium at the indicated times. cDNA was prepared and hybridized to microarrays prepared from oligonucleotides designed from the genome sequence of *B. subtilis.*

[b] The ratios are normalized average values from the *rok* RNA divided by values from the *rok*$^+$ RNA. T_2 refers to a time 2 h after the departure from exponential growth. Exp. refers to a time during exponential growth. All of the T_2 values were derived from 8 successful measurements and all of the exponential sample values from 10, except that there were 9 measurements in the case of *ykuJ.*

[c] The P values are the Bayesian P values described in reference 24.

[d] This gene was not initially called by the criteria described in the text, but it is included because it is adjacent to genes that were called and because its values fall just below these criteria.

Although the listed genes were candidates for negative regulation by *rok*, no positively regulated genes were detected.

Many of the candidate *rok*-regulated genes may comprise operons, since they are adjacent to one another. These are illustrated in Fig. 1. In addition to *comK* and *rok* itself, 41 genes in 20 putative transcription units met our criteria for potential direct or indirect regulation by Rok.

Validation of transcriptional profiling results. In order to validate the results of the transcriptional profiling experiments, *lacZ* fusions of selected candidate *rok*-regulated genes were tested for β-galactosidase expression in wild-type and *rok* backgrounds (Fig. 2) grown in competence medium (1). In all cases, the fusion data served to validate the transcriptional profiling results. The expression patterns observed fell into three categories.

Among genes in the first category (*yydI*, *albA*, and *sboA*), the β-galactosidase specific activities remained constant or decreased slightly during growth, reaching a constant level after T_0, the end of exponential growth. At all points tested, higher expression was observed in the *rok* background, and a similar differential was observed between the wild-type and *rok* strains throughout growth. This is consistent with the transcriptional profiling data for all nine genes in the *sbo-alb* operon and with the genes in the *yydHIJ* operon, since approximately similar ratios were observed for them during exponential growth and at T_2 (Table 3). The relatively constant difference between the *rok* and *rok*$^+$ strains indicates that Rok is active as a repressor throughout growth, consistent with the aforementioned constant expression of *rok-lacZ* and the similar levels of Rok detected by Western blotting during growth (18).

The second group of genes (*yybM*, *yjcN*, and *sdpA*) exhibited a different pattern, with a gradual increase in β-galactosidase specific activity during growth, and again the *rok* strain expressed higher levels, but with a similar growth dependency. These β-galactosidase expression patterns suggested that the genes in question are regulated by an additional pathway, conferring growth-dependent expression, as well as being Rok repressed.

The patterns obtained with the third group of genes (*sunT* and *yxaJ*) were again distinct, in that differing patterns of growth dependence were observed in the *rok* and wild-type strains. In the cases of *sunT*, *yxaJ*, and *yxaL*, transcriptional profiling revealed a greater differential between the *rok* and wild-type strains during exponential growth, consistent with the data in Fig. 1.

In all cases, the data presented in Fig. 1 confirm the transcriptional profiling results for the genes tested, demonstrating repression by Rok and suggesting that many of the genes listed in Table 3, which were not studied with *lacZ* fusions, are probably also regulated by *rok*. The *lacZ* fusion constructs were all studied in *comK*$^+$ backgrounds, whereas the RNA samples for transcriptional profiling were isolated from isogenic *rok* and *rok*$^+$ strains in *comK* backgrounds to simplify interpretation of the data. Since the two sets of results were consistent, it appears that *rok* repression of the genes we have studied neither requires nor is prevented by ComK. Since the fusions were made using internal gene fragments, their Campbell-like integration resulted in gene knockouts. It follows that regulation by *rok* in these cases does not require the cooperation of the individual gene products, although it is possible that the magnitude of the regulatory effects was affected.

Rok binds directly to the promoter regions of *comK*, *rok*, *sunA*, *sboA*, *sdpA*, *yjcN*, *yybN*, *yxaJ*, and *yydH*. Although Rok apparently represses these genes, it may do so either directly, by binding to the various promoter regions, or indirectly. To distinguish between these possibilities, we performed gel electrophoresis mobility shift assays using His-tagged Rok protein (Rok-His$_6$) (Fig. 3) and ^{32}P-end-labeled, PCR-amplified promoter-probe fragments. For *comK* and *sboA*, the primers were

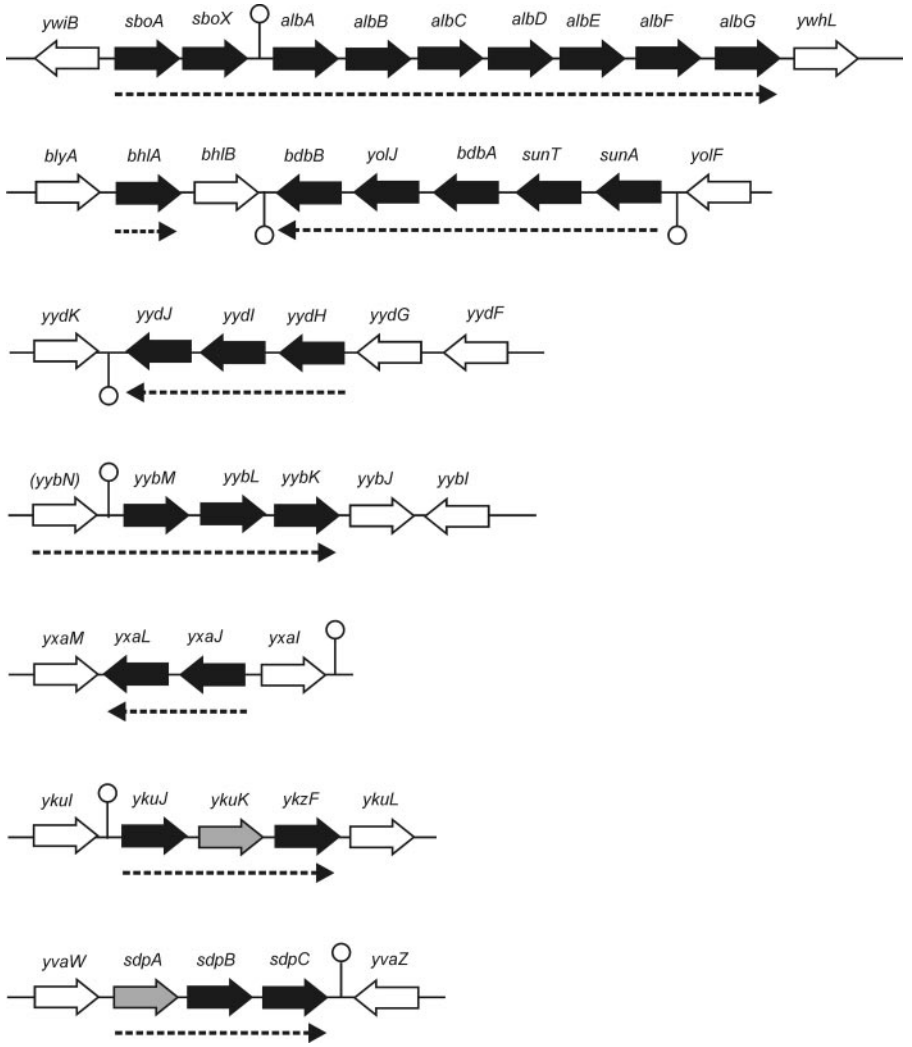

FIG. 1. Genetic maps of *rok*-regulated gene clusters. Genes are indicated with arrows, indicating the direction of transcription. Black arrows indicate genes that meet the criteria for *rok* regulation, as described in the text; grey arrows indicate genes that nearly met these criteria but are classified as *rok* regulated, based on their proximity to regulated genes; white arrows indicate genes that are judged as not *rok* regulated, based on the transcription profiling experiments. *yybN* is in brackets, since the transcription profiling for this gene was inconclusive (see the text). Dotted arrows indicate putative *rok*-regulated transcription units. Hairpins indicate terminators as derived from the Subtilist database (http://genolist.pasteur.fr/SubtiList/). The sizes in the figure are not proportional to the lengths of the open reading frames or intergenic regions.

designed based on published identification of the start sites of the transcription units (38, 40). For the remaining genes, we amplified fragments including 200 to 230 bp upstream from the apparent start codons of the first genes in each transcription unit.

In all cases, a shift was noted, suggesting that Rok was capable of direct binding to the probe fragments. The shifts observed with *PcomK* (Fig. 3A) and *Prok* (Fig. 3B) fragments, included as positive controls, yielded apparent K_d values of 26 to 56 nM, corresponding well with the previously reported estimate of 50 nM for both promoters (18).

We regard the binding observed with the newly identified Rok target fragments as specific, with the probable exceptions of *PykuJ* (Fig. 3K) and *PbhlA* (Fig. 3N). Several arguments serve to justify this conclusion. First, the gel shifts were carried out in the presence of a molar excess of poly(dI-dC). Second, several negative controls were used. No specific binding was observed to the promoter region of the ComK-regulated promoter *comG* (Fig. 3D), to a fragment amplified from within the coding region of *rok* (Fig. 3C), or to the promoter region of the arbitrarily selected gene *skfA* (Fig. 3E), except for some smearing of the probe at the highest concentration of Rok. Finally, the concentrations of Rok required to give half-maximal shifts were in the range from 30 to 100 nM for all but *PbhlA* (Fig. 3N) and *PykuJ* (Fig. 3K). This high-affinity binding, as well as the results obtained with the control fragments, is most consistent with the conclusion that Rok acts directly on the transcription units in question, repressing transcription.

The case of the *yybN-yybM-yybL-yybK* cluster (Fig. 1) deserves special mention. A 200-bp fragment derived from the sequence upstream from the start of *yybM* did not shift in the presence of Rok-His_6 (data not shown), although this gene is clearly repressed by Rok. A probe fragment was generated containing sequences preceding *yybN*, which lies immediately

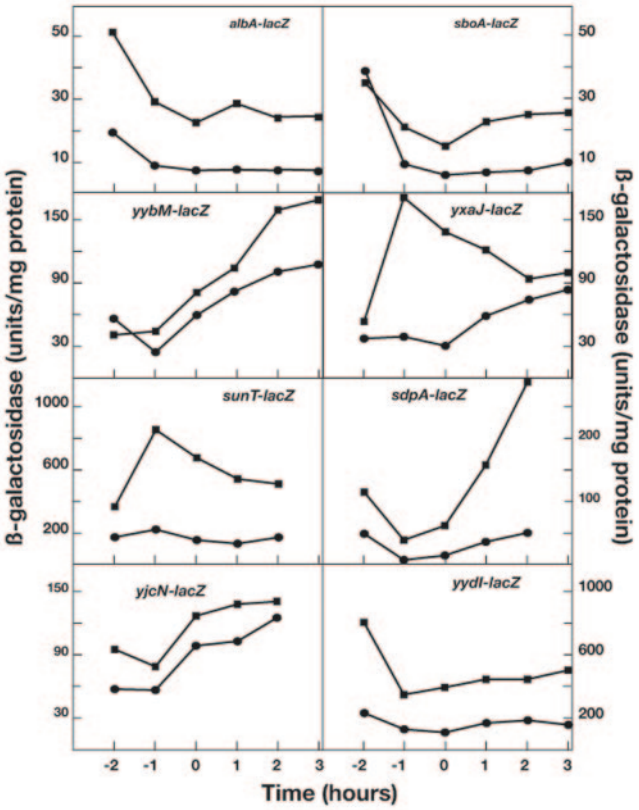

FIG. 2. Validation of transcriptional profiling results using *lacZ* fusions. Strains carrying fusions of *lacZ* to candidate *rok*-regulated genes were grown in competence medium, and samples were withdrawn at the indicated times for measurement of β-galactosidase specific activities. T_0 refers to the time of departure from exponential growth. Each fusion was placed in both *rok* (■) and *rok*$^+$ (●) backgrounds for determination of β-galactosidase.

upstream from *yybM*, and a half-maximal shift was observed at a Rok-His$_6$ concentration below 100 nM (Fig. 3H). Evidence for the regulation of *yybN* by *rok* could not be obtained from the transcriptional profiling data, since few reliable data points were obtained for this gene (5 out of 10 attempts with the exponential sample and 2 out of 8 for the T_2 sample). In spite of this failure, which was most likely due to a problem with the *yybN* oligonucleotide, we tentatively conclude that *yybN* is most likely the first gene in a Rok-repressed operon that also contains *yybM*, *yybL*, and *yybK* (Fig. 1).

Another feature worthy of mention, and evident in Fig. 3, is that Rok binding in all cases yields more than one shifted band, as noted previously for *PcomK* and *Prok* (18). This is most likely due to the association of more than one protein molecule with each regulatory sequence.

DISCUSSION

Nature of the *rok*-regulated genes. Transcription profiling, gel shift experiments, and the use of fusions to *lacZ* have established a set of genes that are repressed by Rok, most likely by direct binding. In addition to *comK*, the master regulator of competence development, and *rok* itself, we have confirmed nine Rok-repressed transcription units, seven of which appear to be operons with two or more open reading frames. These transcription units apparently include 30 new Rok-repressed genes. It is interesting that aside from *rok* and *comK*, none of the confirmed Rok-repressed genes appear to encode regulatory proteins. This is consistent with our conclusion, based on gel shift experiments, that nearly all of the genes tested appear to be directly regulated by Rok. In addition to the confirmed genes, Table 3 lists several that remain candidates for regulation by Rok, although they were not further validated. Several additional genes may well be Rok regulated, since their transcriptional profiling data fell just below our criteria for inclusion in Table 3 (see http://www.phri.org/research/res_pidubnau.asp).

Several of the newly identified genes encode proteins needed for the production of confirmed or probable extracellular molecules, including several bacteriocin-like antibiotics. Antibiotic-like exported molecules may play important roles in development and cell-cell signaling, as demonstrated by a recent report concerning the *Streptomyces coelicolor* product SapB (20), by the role of surfactin in fruiting body formation in *B. subtilis* (7), by the activity of secreted pheromones in the development of genetic competence (16, 25), and by the effects of subinhibitory concentrations of antibiotics on transcription (10). It is also possible that antibiotics induce the lysis of neighboring bacteria and the release of DNA, suggesting a possible link between competence and antibiotic synthesis.

The *sbo-alb* operon encodes the genes required for the synthesis and export of the peptide antibiotic subtilosin. The precursor of subtilosin is encoded by *sboA*, while the *albA-G* genes are needed to modify and transport this molecule, as well as to confer immunity to subtilosin (39, 40). An additional bacteriocin-like precursor peptide is apparently encoded by *sboX*, which partially overlaps *sboA*, but is not required for the production of subtilosin (39). All of the genes in this operon are repressed by the *rok* gene product, and the flanking genes, *ywiB* and *ywhL*, are not.

The Rok-repressed gene cluster, consisting of *sunA*, *sunT*, *bdbA*, *yolJ*, and *bdbB*, is involved in the production and secretion of sublancin, a lantibiotic peptide antibiotic (8, 28), and is part of the SPβ prophage. *sunA* encodes presublancin, and *sunT* encodes an apparent ABC transporter, surmised to export the antibiotic (28). *sunT* is a dual function protein, since it also includes a novel domain which is most likely responsible for the proteolytic processing of presublancin (28). *bdbA* and *bdbB* are thiol-disulfide oxido-reductases (6), and at least *bdbB* is needed for the production of active sublancin, which contains two disulfide bonds (8). YolJ is similar to a large family of glycosyl transferases, including one, PlnO (accession number NC_004567.1), which is annotated as a biosynthetic protein for the production of the *Lactobacillus plantarum* lantibiotic plantaricin.

BhlA, also encoded by the lysogenic phage SPβ, is a holin-like protein (29) and is similar to proteins annotated as involved in the production and release of bacteriocin-like molecules. If this is the role of BhlA, its substrates have not been identified.

The *sdp* operon has been shown to encode an extracellular factor, derived from SdpC, that results in a delay in sporulation (11). It appears likely that this signaling molecule stimulates increased energy production, thereby delaying the onset of

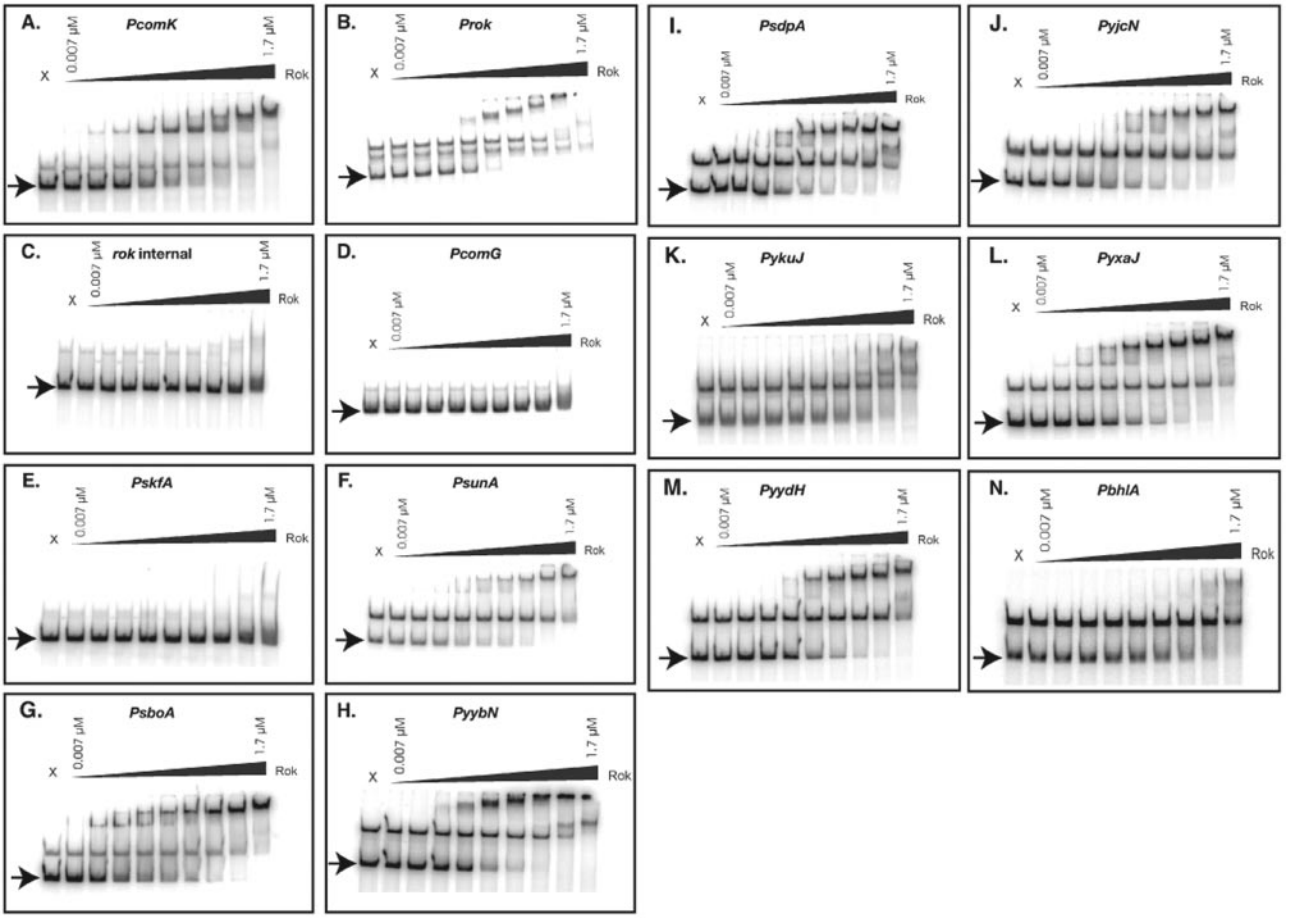

FIG. 3. Gel retardations. DNA fragments from the indicated genes were prepared by PCR and end labeled with ^{32}P. In each panel the lane marked with an x corresponds to probe alone. The remaining lanes correspond to probe samples incubated with Rok-His_6 at concentrations ranging from 7 to 1,792 nM. Each successive lane, from left to right, corresponds to a doubling in the concentration of Rok-His_6. The arrows indicate the positions of the unshifted probe fragments. In each case, the unshifted band that migrated slower than the probe probably corresponds to single-stranded DNA, due to slightly unequal concentrations of PCR primers. This material is not shifted by Rok-His_6.

sporulation. Although the SdpC-derived product is apparently not an antibiotic, it may be regarded as an extracellular signaling molecule.

The putative operon consisting of *yydJ*, *yydI*, and *yydH* encodes two polytopic membrane proteins (YydJ and YydH), predicted by TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) to contain six and five membrane-spanning sequences, respectively. YydI is not predicted to be a membrane protein but is similar to the family of ABC transporter, ATP binding proteins. It appears likely that this operon is involved in transport, probably in export, since it does not encode an obvious substrate binding protein.

The *yybN*, *yybM*, *yybL*, *yybK* cluster encodes one predicted protein with a likely N-terminal signal sequence (YybN) and three permease-like predicted polytopic membrane proteins, each with six likely membrane-spanning sequences. Again, it is plausible to suggest that this operon has a transport function.

The putative operon containing *yxaJ* and *yxaL* encodes two proteins, the first of which, YxaJ, is predicted to contain four transmembrane sequences and the second of which contains motifs that are typically found in bacterial dehydrogenases. YxaL has been shown to be a secreted protein, and the YxaL preprotein contains a signal sequence (34). The open reading frames in the *ykuJ-ykuK-ykzF* putative operon do not encode proteins with suggestive similarities, and all are predicted to be cytosolic gene products. Finally, *yjcN* encodes a protein with a single predicted transmembrane segment.

It appears, then, that nearly all of the newly identified genes that we have confirmed to be repressed by Rok encode proteins that are involved in secretion or transport, or are themselves secreted products. The competence proteins, which are indirectly regulated by Rok, fall into this category, since they are located in the membrane or associated with the cell wall and mediate the transport of DNA. In particular, several of the Rok-regulated proteins are involved in the production of secreted antibiotics or signaling molecules.

Although we have reported that the overproduction of Rok is lethal (18), none of the genes listed in Table 3 was shown to be essential for viability (19). We may consider several possibilities for this discrepancy. Most obviously, an essential gene may simply have failed to meet our criteria for inclusion in Table 3. Alternatively, an essential gene may be repressed by Rok, which binds to its promoter with low affinity. If so, we would not have identified this gene in our transcriptional profiling experiments, but overproduction of Rok would prevent

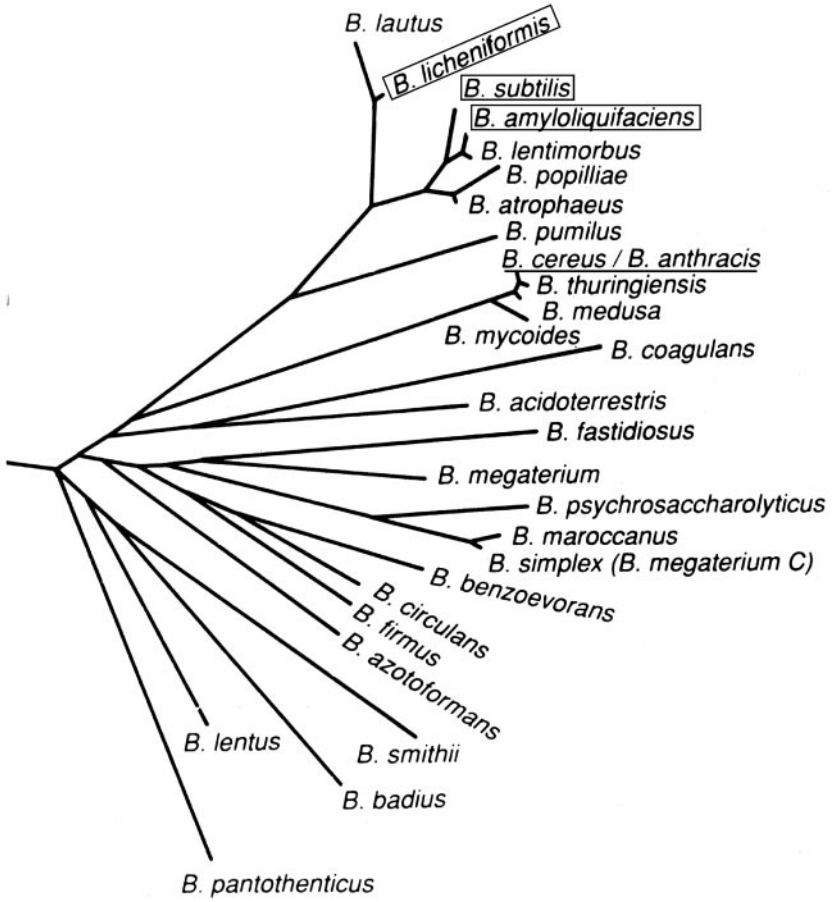

FIG. 4. Relationships among group 1 bacilli (adapted from reference 2). Species in which a *rok* ortholog have been identified are boxed, and those in which it is known to be absent are underlined.

adequate expression. It is also possible that a Rok-regulated gene may be essential under our growth conditions (competence medium) (18), but not in the rich medium used for the systematic search for essential genes (19). Finally, it is possible that the lethality we detected when Rok was overexpressed was due to some combination of Rok-repressed genes.

Regulation of Rok-repressed genes. The *sbo-alb* operon is known to be under complex control (26, 40). This operon is repressed by AbrB and also is induced by oxygen limitation via a mechanism that requires the two-component regulators ResD and ResE. Consistent with this is our observation that the expression of this operon is low in the *rok*$^+$ background, under aerobic conditions (Fig. 2). The *albA-lacZ* construct was tested under anaerobic conditions in complex medium (2xYT), and a dramatic induction was observed (data not shown), as expected. Under anaerobic conditions, an even greater induction was observed in the *rok* background, suggesting that Rok is not required for the anaerobiosis signaling pathway and that repression by Rok is still manifest under anaerobic conditions. Although the expression of *albA-lacZ* in competence medium was low and apparently not growth regulated, the expression of this construct in complex medium, whether anaerobic or aerobic, was clearly growth regulated, increasing markedly at the end of exponential growth (data not shown).

Several of the Rok-repressed genes listed in Table 1 (*sdpABC* [15], *comK* [12], *rok* [18], *hom* [15], *yxbC* [15], and the *sboA-alb* operon [40]) are also repressed by AbrB. There appears to be a significant overlap between the *rok* and *abrB* regulons.

Possible binding site for Rok. We have used a bioinformatics approach to search for a consensus Rok binding sequence. In particular, we have used BioProspector together with BioOptimizer (22, 23), as well as MEME (3). BioProspector finds candidate motifs with an improved Gibbs sampler search algorithm that allows for the introduction of gaps in the search parameters. BioOptimizer is an algorithm designed to take files containing candidate motifs identified by BioProspector as input. BioOptimizer then optimizes the candidate motif, if possible, providing a measure of confidence that the motif found by BioProspector is significant.

We have applied these programs, systematically varying the input data sets used (identities of the genes included as well as the lengths of the upstream sequences) and adjusting these data sets using information obtained from DNase protection footprinting experiments (data not shown). In each case we failed to obtain an optimized sequence, perhaps due to the subtle nature of the Rok binding motif and the inadequate size of our data set.

In spite of this failure to obtain an optimizable sequence, the

motif 5′-GATAG-3′ produced a relatively high score with both BioProspector and MEME and was found in repeated attempts with varied approaches. This motif is located in the DNase-protected regions for *comK*, *sboA*, and *sunA*, as well as in the upstream regions of the other six genes in our core data set. The significance of this motif awaits further experimental work.

The effect of *rok* on sporulation is not mediated solely by repression of *sdp*. Mutational inactivation of *rok* results in an oligosporogenic phenotype (18). Since Rok appears to be a direct repressor of the *sdp* operon, the possibility was considered that the oligosporogenic phenotype is explained by overexpression of *sdp*, which would be expected to delay spore formation (11). To test this, we determined whether an *sdp* null mutation would suppress the oligosporogenic phenotype of a *rok* knockout (data not shown). In fact, the sporulation frequency, measured by colony formation and by microscopic examination, was no higher in an *sdp rok* double mutant than in a *rok* single mutant. It appears that the oligosporogenic phenotype of the *rok* knockout mutation cannot be explained only by overexpression of the *sdp* operon.

Evolution of Rok. As noted above, Rok has an ortholog among sequenced genomes in *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens*, but not in other bacilli and spore-forming gram-positive bacteria, such as *B. halodurans*, *B. anthracis*, *B. cereus*, or *Oceanobacillus iyensis*. In fact *yknT*, the convergently transcribed gene immediately downstream from *rok*, is also absent from these organisms, whereas flanking genes (*ykuV* and *mobA*) are present. It is interesting that *yknT* (also called *cse15*) is dependent on sigma factor E for its expression in the spore mother cell, but no sporulation phenotype was observed in a gene knockout (17). It has been suggested that the *yknT* gene product plays a subtle role in spore coat formation (17), consistent with its absence from related spore-forming bacilli. *rok* and *yknT* may have been introduced into the *B. subtilis-B. licheniformis* lineage by horizontal transmission, or they may have been lost together from some common ancestor of *B. halodurans*, *B.cereus*, and *B. anthracis*. The combined average base composition of the *rok* and *yknT* coding sequences is 40.2%, which is less than the average for the entire *B. subtilis* genome (43.5%) (21). However, *rok* and *yknT* are located in a large region of lower-than-average percent GC (21), and the base composition differences therefore neither support nor refute the hypothesis of horizontal transmission.

Attention to the phylogenetic tree (Fig. 4) is more revealing. The *rok* determinant is present in the closely related *B. subtilis-B. licheniformis-B. amyloliquefaciens* group, but absent from the *B. cereus-B. anthracis* group. This permits two possibilities, if we assume a single relevant event. *rok* may have been introduced just before the branch point that led to the *B. subtilis* group and would therefore not be present in other more distantly related gram-positive spore formers. Alternatively, it may have been lost specifically from the *B. cereus* branch, in which case it would be present in the more distantly related spore-forming gram positives. Since *rok* is also missing from the outliers *B. halodurans* and *O. iyensis*, as well as from the gram-positive nonsporulating *Listeria monocytogenes*, the most parsimonious hypothesis is that *rok* was introduced by horizontal transfer into an ancestor of the branch that contains *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens*.

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