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The Fnr Regulon of *Bacillus subtilis*†

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The *Bacillus subtilis* transcriptional regulator Fnr is an integral part of the regulatory cascade required for the adaptation of the bacterium to low oxygen tension. The *B. subtilis* Fnr regulon was defined via transcriptomic analysis in combination with bioinformatic-based binding site prediction. Four distinct groups of Fnr-dependent genes were observed. Group 1 genes (*narKfnr*, *narGHJI*, and *arfM*) are generally induced by Fnr under anaerobic conditions. All corresponding promoters contain an essential Fnr-binding site centered −41.5/−40.5 bp upstream of the transcriptional start point, suggesting their induction by direct Fnr interaction. Group 2 genes (*alsSD*, *ldh lctP*, *ywcJ*, and *cydABCD*) are characterized by anaerobic repression in the presence of nitrate. Mutational analysis of the Fnr-binding sites found in three of the corresponding promoters excluded their function in Fnr-mediated repression. Genetic evidence showing that group 2 genes are anaerobically repressed by nitrate reductase formation was accumulated. A possible role of the redox regulator YdiH in the regulation of group 2 genes was initially investigated. Group 3 genes are characterized by their Fnr-dependent activation in the presence of nitrate and the lack of an Fnr-binding site in their promoters. The analysis of Group 3 gene transcription (*ykuNOP* and *ydbN*) indicated that Fnr induces nitrate reductase production, which leads to the formation of the regulatory compound nitrite from nitrate. Finally, the group 4 operon *acoABCL*, lacking an Fnr-binding site, requires Fnr-dependent nitrate reductase formation for its general anaerobic induction. A regulatory model for the observed complex Fnr-mediated gene expression was deduced.

The gram-positive model organism *Bacillus subtilis* adapts to an anaerobic environment by changing its metabolic activity (26). Under anaerobic conditions, *B. subtilis* performs a mixed-acid fermentation with lactate, acetate, and acetoin as the major products (7, 24). In the presence of nitrate, *B. subtilis* performs the respiratory process of ammonification (8, 13, 14, 22).

The regulatory network underlying anaerobic adaptation has been extensively studied during the last decade. A regulatory cascade describing the coordinated regulation of genes involved in anaerobiosis was established (7). One major regulatory switch in the adaptation to anaerobiosis is the two-component system ResDE (32, 35). While the mechanism of signal perception by ResDE is still unknown, the downstream regulatory network is elucidated to a significant depth. Activated ResD binds to promoter regions of *nasDE*, encoding the nitrite reductase, the flavohemoglobin gene *hmp*, and the gene encoding the redox regulator Fnr (23, 25). Fnr in turn is responsible for the induction of the *narGHJI* operon and *narK*, encoding the respiratory nitrate reductase and a potential nitrite extrusion protein, respectively (6, 24). Mutation of *fnr* strongly affects anaerobic growth of *B. subtilis* on nitrate (6, 24). Furthermore, Fnr activates the expression of the *arfM* gene encoding an anaerobic respiration and fermentation modulator protein by direct interaction with the *arfM* promoter region (16). The promoter regions of all three Fnr-regulated genes carry the highly conserved potential *B. subtilis* Fnr-binding site (TGTGA-N_6-TCACA) centered 41.5/40.5 bp from the transcriptional start point. Complementation experiments using an *Escherichia coli crp* mutant revealed that the DNA-binding domain of Fnr of *B. subtilis* is similar to that of Crp from *E. coli*, the well-studied cyclic AMP receptor protein (6). In *B. subtilis*, additional potential Fnr-binding sites were found in the promoter regions of a second potential nitrite transporter gene, *ywcJ*, as well as the fermentation operons *ldh-lctP* and *alsSD* (6, 7, 34). The latter operons encode lactate dehydrogenase, lactate permease, and acetolactate synthase and acetolactate decarboxylase, respectively. Transcription of *alsSD* and *ldh-lctP* was found to be anaerobically induced and repressed by the presence of nitrate (7). Nitrate repression was related to nitrate reductase activity (7).

Global transcriptional profiling was used to analyze changes in the mRNA population after adaptation to anaerobic growth conditions (35). Several hundred genes were observed to be induced or repressed under anaerobic conditions. Additionally, changes in gene expression patterns were measured for an anaerobically grown *resDE* mutant strain. About 50 genes, including 15 operons, were found to be ResDE dependent (35). However, these results did not distinguish between genes that are directly regulated by ResDE and indirect effects of ResDE resulting from the induction of *fnr* transcription.

In this study, the Fnr regulon was intensively characterized by an experimental approach combining DNA macroarrays and a bioinformatic investigation based on transcription factor binding site predictions. Identified promoters of interest were further investigated using genetic and molecular biology methods. Finally, a model of the Fnr regulon was proposed.

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

TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristics	Source or reference
JH642	*trpC2 pheA1*	BGSC[a]
THB1	*trpC2 pheA1 narGH::tet*	14
THB2	*trpC2 pheA1 fnr::spec*	16
LUW48	*trpC2 amyE::cydA′-lacZ*	33
LUW273	*ydiH*Ω pYDIH1 *spec*	15
QB700	*trpC2 amyE::acoA-lacZ cat*	1
TDB2	*trpC2 pheA1 amyE::narG lacZ cat*	This work
HRB5	*trpC2 pheA1 fnr::spec amyE::narG lacZ cat*	This work
HRB6	*trpC2 pheA1 narG::tet amyE::narG lacZ cat*	This work
TDB8	*trpC2 pheA1 amyE::*Δ*fnr*(−49/−48 TG→CC) *narG lacZ cat*	This work
HRB7	*trpC2 pheA1 amyE::alsS lacZ cat*	This work
HRB8	*trpC2 pheA1 fnr::spec amyE::alsS lacZ cat*	This work
HRB9	*trpC2 pheA1 narGH::tet amyE::alsS lacZ cat*	This work
HRB10	*trpC2 pheA1 amyE::*Δ*fnr*(−4/−5/−6 ACA→CGG) *alsS lacZ cat*	This work
HRB11	*trpC2 pheA1 fnr::spec amyE::*Δ*fnr*(−4/−5/−6 ACA→CGG) *alsS lacZ cat*	This work
HRB19	*trpC2 pheA1 amyE::ywcJ lacZ cat*	This work
HRB20	*trpC2 pheA1 fnr::spec amyE::ywcJ lacZ cat*	This work
HRB21	*trpC2 pheA1 amyE::*Δ*fnr*(−37/−36 TG→CC) *ywcJ lacZ cat*	This work
HRB22	*trpC2 pheA1 narGH::tet amyE::ywcJ lacZ cat*	This work
HRB23	*trpC2 pheA1 amyE::cydA′-lacZ*	This work
HRB24	*trpC2 pheA1 fnr::spec amyE::cydA′-lacZ*	This work
HRB25	*trpC2 pheA1 narGH::tet amyE::cydA′-lacZ*	This work
HRB26	*trpC2 pheA1 amyE::acoA-lacZ*	This work
HRB27	*trpC2 pheA1 narGH::tet amyE::acoA-lacZ*	This work
HRB28	*trpC2 pheA1 narGH::tet amyE::acoA-lacZ*	This work
HRB29	*ydiH*Ω pYDIH1 *spec amyE::ywcJ lacZ cat*	This work

[a] BGSC, Bacillus Genetic Stock Center.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All *B. subtilis* strains used in this study are listed in Table 1. *B. subtilis* strains were grown in Spizizen's minimal medium (10) supplemented with 10 mM sodium nitrate or 10 mM sodium nitrite where indicated. For aerobic growth, bacteria were inoculated with a culture grown overnight to a final optical density at 578 nm of 0.05 and grown in shake flasks with vigorous agitation (250 rpm) for the indicated times. For anaerobic growth, bacteria were inoculated with a culture grown overnight to a final optical density at 578 nm of 0.25 and incubated in transfusion flasks filled up to the top under conditions detailed above. For RNA preparations, bacteria were harvested at mid-log phase. For β-galactosidase assays, bacteria were harvested at late mid-log phase.

Preparation of RNA and Northern blot analysis. Preparation of RNA and Northern blot analysis were performed as described elsewhere previously (11).

DNA macroarray analysis. For the synthesis of labeled cDNA, 2 μg of total cellular RNA prepared from *B. subtilis* strains JH642 (wild type) and THB2 (*fnr* mutant) was mixed with 4 μl of primer labeling mix (Sigma-Genosys Ltd., The Woodlands, Tex.) and adjusted with water to a final volume of 12 μl. The samples were heated to 95°C for 10 min and subsequently incubated for 30 min at 42°C to allow annealing of the primers. For reverse transcription, a solution containing 10 μl first-strand buffer (supplied with the reverse transcriptase), 10 mM dithiothreitol, 400 mM dATP, 400 mM dGTP, 400 mM dTTP, 45 μCi [α-^{33}P]dCTP (Amersham Biosciences, Freiburg, Germany), and 300 U SuperScript II reverse transcriptase (Invitrogen Life Technologies GmbH, Karlsruhe, Germany) was mixed, and cDNA was synthesized for 1.5 h at 42°C in a total volume of 50 μl. Further steps were performed as described elsewhere previously (31).

Macroarray data analysis. Hybridized DNA arrays were read out using a phosphorimager (Molecular Imager FX; Bio-Rad Laboratories, Hercules, Calif.), and obtained data were processed with ArrayVision software (version 6.0; Imaging Research, St. Catherine's, Ontario, Canada). Background levels were deduced from the area surrounding all spots. A quality score was calculated from these values and used to distinguish absent, median, and present signals. Data normalization was done using GeneSpring software (version 4.2; Silicon Genetics, Redwood City, Calif.). Three independent measurements for each analyzed condition and mutant were performed. Results obtained were averaged accordingly. Finally, data were exported to Microsoft Excel, and expression ratios were calculated (raw data are presented in the supplemental material).

β-Galactosidase assays. Crude cell extracts were prepared as described previously (11). β-Galactosidase assays were performed as described elsewhere previously (17).

Construction of the reporter gene fusions and site-directed mutagenesis of potential Fnr-binding sites. A transcriptional fusion between the *E. coli lacZ* gene and the *alsS* upstream region was constructed. An 894-bp PCR fragment spanning the region from positions −341 to +540 relative to the translational start point of *alsS* was amplified with the primers EH46 (5′-AGTTGAATTCCT TGTCCGATTTG-3′) and EH47 (5′-GTGGATCCTGCCCTGCTGACGCTA T-3′). Using the restriction sites for EcoRI and BamHI created by the primers (underlined), we cloned the promoter region of *alsS* into plasmid pDIA5322 (6), resulting in plasmid p*alsS-lacZ*. This plasmid was transformed into *B. subtilis* JH642 (wild type), THB2 (*fnr* mutant), and THB1 (*narG* mutant) strains. Transformants were screened for double-crossover integration at the *amyE* locus, resulting in strains HRB7, HRB8, and HRB9, respectively.

A similar cloning strategy was used to create *narG-lacZ* and *ywcJ-lacZ* transcriptional fusions. A 456-bp PCR fragment spanning the region from positions −243 to +197 relative to the translational start point of *narG* was amplified with the primers EH18 (5′-GCGGATCCAATATTCCAGCTGCAAGA-3′) and EH19 (5′-CGGAATTCGGTATCTGCATACATCAC-3′) (restriction sites are underlined). Cloning of the PCR fragment into pDIA5322 resulted in plasmid p*narG-lacZ*. Transformation into *B. subtilis* strains JH642 (wild type), THB2 (*fnr* mutant), and THB1 (*narG* mutant) resulted in strains TDB2, HRB5, and HRB6, respectively. A 395-bp PCR fragment spanning the region from positions −203 to +176 relative to the translational start point of *ywcJ* was amplified with the primers EH20 (5′-CGGAATTCGCCTGCTTTACCAGTCAC-3′) and EH21 (5′-GCGGATCCAACGGAGAATCAGCCATA-3′). Cloning of the PCR fragment into pDIA5322 resulted in plasmid p*ywcJ-lacZ*. Transformation into *B. subtilis* JH642 (wild type), THB2 (*fnr* mutant), and THB1 (*narG* mutant) strains resulted in strains HRB19, HRB20, and HRB22, respectively.

To analyze *cydA-lacZ*, genomic DNA from strain LUW48 (33) was transformed into *B. subtilis* JH642 (wild type), THB2 (*fnr* mutant), and THB1 (*narG* mutant) strains. After selection for homologous recombination at the *amyE* locus, strains HRB23, HRB24, and HRB25 were obtained. The *acoA-lacZ* fusion of *B. subtilis* strain QB700 (1) was transformed into the *amyE* locus of *B. subtilis* JH642 (wild type), THB2 (*fnr* mutant), and THB1 (*narG* mutant), which resulted in strains HRB26, HRB27, and HRB28, respectively. To obtain strain HRB29, we transformed strain LUW273 (*ydiH* mutant) with plasmid p*ywcJ-lacZ* (15).

The potential Fnr-binding sites in the promoter regions of *alsS*, *narG*, and *ywcJ* were mutated using primers for crossover PCR (12). The potential Fnr-binding site of *alsS* was changed from AGTGA-CT-TCACA to AGTGA-CT-ATC**CGG** (exchanged bases are shown in boldface type). Crossover PCRs were performed with the following two primers containing the desired base exchanges (in boldface type): EH66 (5′-AGAGTGTATAGTGAAACTTATC**CGG**AGATA-3′) and EH67 (5′-TATCT**CCG**GATAAGTTTCCGGATACACTCT-3′). Two PCR products were generated with primer pairs EH46-EH67 (356 bp) and EH66-EH47 (568 bp). In a second PCR, we used the first two PCR products as templates and amplified the whole promoter region with the primer pair EH46-EH47. The complete promoter fragments were cloned into the plasmid pDIA5322 as described above for the wild-type sequence, resulting in the plasmid p*alsS*(Δ*fnr*)*-lacZ*. After transformation into *B. subtilis* strain JH642 and selection for double-crossover integration at the *amyE* locus, strain HRB10 was obtained.

The potential Fnr-binding site of *narG* was changed from 5′-TGTGA-TA-TCACA-3′ to **CC**TGA-TA-TCACA (exchanged bases are shown in boldface type). Crossover PCRs were performed with the following two primers containing the desired base exchanges (in boldface type): EH108 (5′-AAAAGCAGA GTG**CC**TGACATAGTT-3′) and EH109 (5′-ACTATGTCA**GG**CACTCTG CTTTT-3′). Two PCR products were generated with primer pairs EH19-EH109 (241 bp) and EH18-EH108 (291 bp). In a second PCR, we used the first two PCR products as templates, amplified the whole *narG* promoter region with the primer pair EH18-EH19, and cloned it into the plasmid pDIA5322 to create plasmid p*narG*(Δ*fnr*)*-lacZ*. Transformation of *B. subtilis* strain JH642 yielded strain TDB8.

The potential Fnr-binding site of *ywcJ* was changed from TGTGA-TA-TCACA to **CC**TGA-TA-TCACA (exchanged bases are shown in boldface type). Crossover PCRs were performed with the following two primers containing the desired base exchanges (in boldface type): EH24 (5′-TATTTCA**GG**ATTAAT TTTTACGAAT-3′) and EH25 (5′-ATTCGTAAAAATTAAT**CC**TGAAATA-3′). Two PCR products were generated with primer pairs EH20-EH24 (156 bp) and

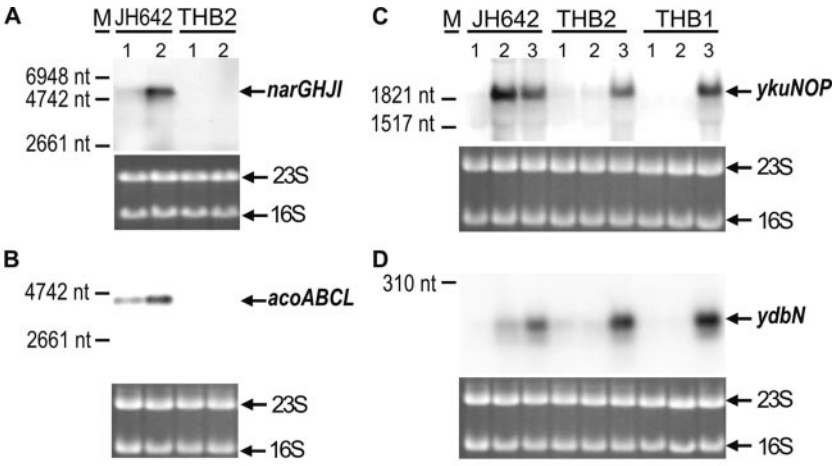

FIG. 1. Influence of anaerobiosis on transcription of *narGHJI*, *acoABCL*, *ykuNOP*, and *ydbN* in *B. subtilis* wild-type (JH642), *narG* mutant (THB1), and *fnr* mutant (THB2) strains. Northern blot hybridizations were performed with RNA extracted from cells grown under the following anaerobic conditions: fermentative (1), nitrate respiratory (2), and with the addition of nitrite (3). A *narG*-specific RNA probe detected a single transcript of 6.4 kb (A), and an *acoA*-specific probe detected a single transcript of 4,637 nt (B). Both transcripts were only found in wild-type cells. A threefold nitrate-dependent induction of both transcripts is visible when lanes 1 and 2 are compared. A single transcript of 1,900 nt was detected with the *ykuNOP*-specific probe (C), while the *ydbN*-specific probe detected an approximately 200-nt transcript, which corresponds to a single *ydbN* transcript (D). No obvious transcripts were detected for conditions of anaerobic fermentation in the wild-type strain and for fermentative and nitrate-respiratory conditions in the mutant strains. Ethidium bromide staining of the gels showed that equal amounts of RNA were analyzed. The size standard was RNA molecular weight marker no. 1 (Roche Diagnostics GmbH, Mannheim, Germany) and the 16S and 23S rRNA species.

EH21-EH25 (264 bp). The whole *ywcJ* promoter region was generated in a second PCR with the primer pair EH20-EH21 using the two PCR products as templates and cloned into the plasmid pDIA5322 to create plasmid p*ywcJ*(Δ*fnr*)-*lacZ*. Transformation of *B. subtilis* strain JH642 resulted in strain HRB21. In general, all transformants were tested for amylase activity to ensure integration of the promoter-*lacZ* construct at the *amyE* locus. In addition, two independently obtained clones of each newly constructed strain were used for β-galactosidase assays. All cloned fragments made by PCR were sequenced to check for PCR-induced errors.

Primer extension. For each primer extension analysis, 50 μg of total cellular RNA was used. Reverse transcription was initiated from the γ-^{32}P-end-labeled primer EH41 (5′-GGCCAAAATGGACCGAAGCACATAACG-3′) for *ywcJ* and primer EH113 (5′-TTATTTGAATGGTGTTCGATAGGAGAG-3′) for *narG* according to a standard procedure (2). The sequencing reactions were performed with the same primers used for reverse transcription. The primer extension products and the sequencing reactions were analyzed on a 6% denaturing polyacrylamide gel in Tris-borate buffer. The dried gel was analyzed by phosphorimaging.

Prediction of Fnr-binding sites. A position weight matrix (PWM) model of the Fnr-binding site was created by use of an aligned training set consisting of three sequences from the PRODORIC database (19). The PWM was computed using the widely accepted information theoretical approach, with some modifications (28). At first, the information vector $R_{\text{sequence}}(l)$ was generated from the alignment where $f(b,l)$ is the frequency of the base b at position l and was calculated as follows: $R_{\text{sequence}}(l) = 2 + \sum_{b=A}^{T} f(b,l) \log_2 f(b,l)$. We additionally considered the nucleotide bias in genomes by using a linear correction of noise (30). The position weight matrix $m(b,l)$ values were calculated afterwards as follows: $m(b,l) = f(b,l) \cdot R_{\text{sequence}}(l)$. For the case where $f(b,l) = 0$, we introduced a penalty function in dependence of the sample size n as follows: $f(b,l) = \frac{1-n}{n+2}$. Using the PWM as a scoring function, a genome-wide search for potential binding sites was performed using the program "Virtual Footprint" (20). The program is interconnected with the PRODORIC database (accessible at http://www.prodoric.de/vfp). The sequence logo was created using WebLogo software (http://weblogo.berkeley.edu) (29).

RESULTS AND DISCUSSION

Expression profiling using DNA macroarrays for definition of the Fnr regulon in *B. subtilis*. The global transcription profiles of *fnr* mutant strain THB2 and *B. subtilis* wild-type strain JH642 grown under fermentative conditions or nitrate-respiratory conditions were compared. To establish appropriate anaerobic growth conditions for RNA preparation, we monitored the anaerobic and Fnr-dependent expression of the *narGHJI* operon by Northern blot analysis. A single transcript of about 6,400 nucleotides (nt), which corresponds to the size of the *narGHJI* operon, was detected using a *narG*-specific RNA probe in total cellular RNA isolated from fermentatively grown cells. The amount of *narGHJI* transcript was found to be fourfold higher when nitrate was present in the growth medium (Fig. 1A). The *narGHJI* transcript was not present in RNA prepared from *B. subtilis fnr* mutant strain THB2. These results indicated that proper growth conditions were used for the RNA preparation.

Fnr-dependent transcription of *B. subtilis* genes was subsequently investigated by hybridization of labeled cDNAs prepared from mRNA isolated from the anaerobically grown *B. subtilis* wild type and a corresponding *fnr* mutant to DNA macroarrays representing about 4,100 of the genes of the organism. In total, we found the expression of 37 genes, including 10 operons, affected by the *fnr* mutation (Table 2).

The Fnr regulon consists of four groups of differentially expressed genes. Four different groups of Fnr-dependent genes were distinguished by their expression patterns. The first group consists of genes that were induced by Fnr under conditions of fermentative growth and anaerobic nitrate respiration. This group included *arfM*, *narKfnr*, and *narGHJI*. The *arfM* gene encodes an anaerobic modulator protein (16), and *narGHJI* encodes the respiratory nitrate reductase (13). The *narK* gene for a potential nitrate/nitrite transporter protein forms an operon with the *fnr* gene, and Fnr is required for transcription from the *narK* promoter (6). Next, we analyzed the promoter

TABLE 2. Transcriptome analysis for definition of the Fnr regulon[a]

Gene	Description	Putative Fnr box	Induction/repression (fold)	
			Fermentation	Nitrate respiration
Group 1				
narG	Nitrate reductase (alpha subunit)	++	1.3	1.0
narH	Nitrate reductase (beta subunit)		2.0	1.8
narI	Assembling factor		2.1	5.6
narJ	Nitrate reductase (gamma subunit)		3.1	1.9
narK	Nitrite extrusion protein	++	1.6	1.4
fnr	Anaerobic redox regulator		1.6	4.1
arfM	Anaerobic modulator	++	1.7	2.2
Group 2				
alsS	Alpha-acetolactate decarboxylase	+	(1.3)	(1.9)
alsD	Alpha-acetolactate synthase		(2.8)	(2.2)
cydA	Cytochrome *bd* ubiquinol oxidase (I)	−	(1.8)	(1.3)
cydB	Cytochrome *bd* ubiquinol oxidase (II)		(1.4)	(3.3)
cydC	ABC transporter		(1.3)	(2.5)
cydD	ABC transporter		(1.4)	(2.0)
ldh	L-Lactate dehydrogenase	+	(1.4)	(17.6)
lctP	L-Lactate permease		(0.9)	(4.0)
ywcJ	Similar to formate/nitrite transporter	+	(1.2)	(1.4)
Group 3				
dhbA	2,3-Dihydro-2,3-DHB dehydrogenase	−	1.6	1.8
dhbB	Isochorismatase		1.3	5.5
dhbC	Isochorismate synthase		1.5	5.1
dhbE	2,3-DHB-AMP ligase		1.1	2.7
dhbF	Similar to *E. coli* EntF		1.1	4.5
hmp	Flavohemoglobin	−	1.2	33.3
nasD	Nitrite reductase; subunit	−	1.0	12.4
nasE	Nitrite reductase; subunit		1.1	4.6
nasF	Uropophyrin-III *C*-methyltransferase		0.9	6.9
ycgT	Similar to thioredoxin reductase	−	1.2	2.5
ydbN	Unknown	−	1.3	11.4
ykjA	Unknown	−	0.9	3.5
ykuN	Unknown; similar to flavodoxin	−	1.5	5.5
ykuO	Unknown		1.9	19.5
ykuP	Unknown; similar to flavodoxin		1.3	29.8
Group 4				
acoA	Acetoin dehydrogenase; E1 subunit	−	7.1	8.6
acoB	Acetoin dehydrogenase; E1 subunit		2.5	2.8
acoC	Acetoin dehydrogenase; E2 component		5.3	3.4
acoL	Acetoin dehydrogenase; E3 component		4.1	4.5

[a] Total cellular RNA was prepared from wild-type *B. subtilis* and a corresponding *fnr* mutant strain grown anaerobically under the indicated conditions. Numbers give the severalfold induction and, when shown in parentheses, the severalfold repression mediated by Fnr. ++, Fnr boxes in the promoter region of the indicated genes responsible for a direct Fnr effect; +, found Fnr boxes without direct function; −, no Fnr box found.

regions of these genes for potential Fnr-binding sites using a bioinformatic approach. For this purpose, a position weight matrix for the Fnr-binding site was created. Two lines of evidence are currently available for functional *B. subtilis* Fnr-binding sites. First, the role of a proposed Fnr-binding site was proven indirectly by an in vivo complementation analysis. Here, a *narK* promoter-*lacZ* reporter gene fusion from *B. subtilis* was activated by the catabolite regulatory protein Crp of *E. coli* (6). Protein sequence alignments revealed that the amino acid residue RE-R motif within the helix-turn-helix DNA-binding domain of *E. coli* Crp is conserved in the potential DNA-binding domain of *B. subtilis* Fnr. Consequently, Fnr of *B. subtilis* was supposed to bind to DNA sequence motifs similar to those of Crp from *E. coli* (TGTGA-TA-TCACA).

Second, Marino et al. (16) identified such a motif (TGTGAA ATACATCACT) upstream of the Fnr-dependent gene coding for the anaerobic modulator ArfM (restriction sites are underlined). Mutations of this potential Fnr box centered −40.5 bp upstream of the transcriptional start site of *arfM* resulted in a complete loss of transcriptional activation under anaerobic growth conditions (16). Moreover, it was previously shown by Nakano et al. (25) that the transcription of *narGHJI* and *narK* is completely Fnr dependent. Consequently, within the promoter regions of both genes, a potential Fnr-binding sequence representing the *E. coli* Crp binding site motif was identified (6). Primer extension experiments performed previously by Cruz Ramos et al. revealed that an Fnr box was centered −41.5 bp from the transcriptional start point of *narK* (6). To prove whether the position of the Fnr-binding site upstream of the *narGHJI* operon also belongs to the same type of promoter, we determined the transcriptional start point of the *narGHJI* transcript by primer extension analysis (Fig. 2B). We found the Fnr-binding site located −40.5 bp upstream of the 5′ end of the *narGHJI* transcript (Fig. 2D). Therefore, a position weight matrix model for the *B. subtilis* Fnr-binding site was created, representing the three Fnr boxes found in the *narG*, *narK*, and *arfM* promoters and visualized as a sequence logo in Fig. 2A (29). Using the Virtual Footprint tool of the PRODORIC database and the corresponding *B. subtilis* Fnr position weight matrix, we screened the *B. subtilis* genome for potential Fnr boxes (20). All genes and operons found in group 1 of the macroarray analysis possess an Fnr-binding site. These promoters resemble class II catabolite activator protein-dependent promoters (5).

The second group of Fnr-dependent genes was characterized by the unaffected expression under anaerobic fermentative conditions but with Fnr-dependent repression under nitrate-respiring conditions. The genes *ldh-lctP* for lactate utilization, *alsSD* for acetoin formation, *cydABCD* for a potential high-affinity oxygen-dependent terminal cytochrome *bd* oxidase, and *ywcJ*, encoding a second potential nitrate/nitrite transport protein, belong to this group. The expression of group 2 genes is repressed in an Fnr-dependent manner. Putative Fnr-binding sites were found by Virtual Footprint centered at positions −16.5 and +6.5 relative to the transcriptional start point of the *ldh* promoter and at position −2.5 of the *alsS* promoter (Fig. 2D) (7). Obviously, the position of these Fnr boxes is different from those in Fnr-activated promoters. Comparable results were found for *E. coli* Fnr, where repression of transcription is mediated by Fnr binding to sites differently located compared to activating sites (9). To determine the position of the putative Fnr-binding site upstream of the *ywcJ* gene relative to the transcriptional start site, we performed a primer extension analysis (Fig. 2C). The putative Fnr-binding site was then mapped to a position centered at position −29.5 upstream of the transcriptional start point in the *ywcJ* promoter region (Fig. 2D). All genes of the Fnr-repressed group 2, with the exception of the *cydABCD* operon, possess Fnr-binding sites that are located closer to the transcriptional start point compared to the Fnr boxes of group 1 genes.

The third class of Fnr-dependent genes whose expression is not altered under fermentative conditions but strongly induced under nitrate-respiring conditions contains the *dhbABCEF*

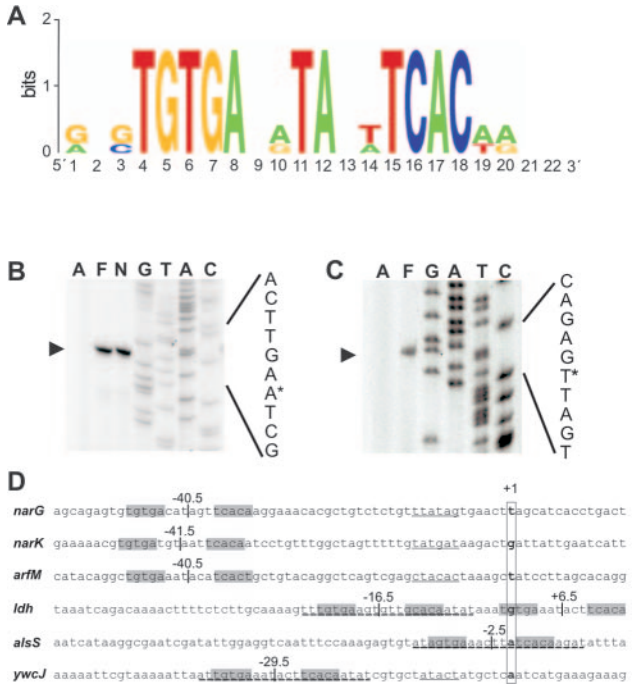

FIG. 2. Promoter analysis of group 1 and group 2 Fnr-dependent promoters. (A) Sequence logo of the Fnr-binding site of *B. subtilis* based on the information weight matrix model. The height of each stack of letters represents the degree of sequence conservation measured in bits of information according to the equation given in Materials and Methods. The height of each letter within a stack is proportional to its frequency at that position in the binding site. The letters are sorted with the most frequent on top. (B, C) Determination of transcription start points of *narG* and *ywcJ* by primer extension analysis. Total cellular RNA was extracted from wild-type strain JH642 grown aerobically (A) and anaerobically under fermentative (F) and nitrate-respiratory (N) conditions. The same primer used for the primer extension analysis was used for sequencing reactions (lanes G, A, T, and C). Arrows indicate the primer extension products, and asterisks mark the 5′ end of the *narG* (B) and *ywcJ* (C) mRNA in the sequence. (D) Localization of putative Fnr-binding sites in promoter regions of group 1 and group 2 Fnr-dependent genes. Putative Fnr-binding sites are marked by gray boxes, and their positions with respect to the transcriptional start points are given. Potential YdiH-binding sites are marked by dashed lines. The −10 promoter regions are underlined. Transcriptional start sites are boxed and indicated by boldface type.

operon for synthesis of 2,3-dihydroxybenzoate (DHB), its modification and esterification to the iron siderophore corynebactin, the *hmp* gene for the flavohemoglobin involved in nitric oxide (NO)/nitrite detoxification, and the *nasDEF* operon coding for nitrite reductase. Furthermore, open reading frames of unknown function, *ycgT*, *ydbN*, *ykjA*, and *ykuNOP*, belong to this group (Table 2). Interestingly, this cluster included genes of the Fur (*dhbABCEF*, *ydbN*, and *ykuNOP*) and ResDE (*hmp* and *nasDEF*) regulons (3, 35). Promoters of genes of the Fnr-dependent expression group 3 do not contain Fnr-binding sites at all. For those genes, we postulate an indirect effect of Fnr.

The fourth class of Fnr-dependent genes consists only of the *acoABCL* operon involved in acetoin utilization (1). While its anaerobic expression was completely dependent on the presence of the *fnr* gene, no obvious Fnr-binding site was detected in the corresponding promoter region (Table 2).

TABLE 3. Influence of anaerobiosis on transcription of *narG-lacZ*, *alsS-lacZ*, *ywcJ-lacZ*, *cydA-lacZ*, and *acoA-lacZ* in *B. subtilis* wild-type, *narG* mutant, *fnr* mutant, and *ydiH* mutant strains[a]

Strain	Genotype	Fusion	β-Galactosidase activity (Miller units)			
			Aerobic	Anaerobic		
				Fermentative	With nitrate	With nitrite
TDB2	Wild type	*narG-lacZ*	<5	19	81	ND[b]
HRB5	*fnr* mutant	*narG-lacZ*	<5	<5	<5	ND
TDB8	Wild type	*narG* (ΔFnr box)-*lacZ*	<5	<5	<5	ND
HRB6	*narG* mutant	*narG-lacZ*	<5	22	103	ND
HRB7	Wild type	*alsS-lacZ*	20	689	266	835
HRB8	*fnr* mutant	*alsS-lacZ*	31	720	718	ND
HRB10	wild type	*alsS* (ΔFnr box)-*lacZ*	30	714	304	ND
HRB11	*fnr* mutant	*alsS* (ΔFnr box)-*lacZ*	37	618	608	ND
HRB9	*narG* mutant	*alsS-lacZ*	30	567	597	ND
HRB19	Wild type	*ywcJ-lacZ*	<5	76	23	66
HRB20	*fnr* mutant	*ywcJ-lacZ*	<5	71	63	ND
HRB21	wild type	*ywcJ* (ΔFnr-box)-*lacZ*	10	248	248	ND
HRB22	*narG* mutant	*ywcJ-lacZ*	<5	73	79	ND
HRB29	*ydiH* mutant	*ywcJ-lacZ*	33	237	240	ND
HRB23	Wild type	*cydA-lacZ*	<5	519	262	570
HRB24	*fnr* mutant	*cydA-lacZ*	<5	580	596	ND
HRB25	*narG* mutant	*cydA-lacZ*	<5	619	655	ND
HRB26	Wild type	*acoA-lacZ*	93	1,289	1,703	2,663
HRB27	*fnr* mutant	*acoA-lacZ*	28	39	38	ND
HRB28	*narG* mutant	*acoA-lacZ*	35	26	30	ND

[a] Strains were grown anaerobically using 50 mM glucose as a carbon source and ammonia as the nitrogen source, with the indicated additions (10 mM nitrate or nitrite) to the mid-exponential phase of growth. Results represent the average of at least three independent experiments performed in duplicate, with a standard error of less than 10%.

[b] ND, not determined.

Furthermore, four genes, *ydbL*, *yceB*, *yceC*, and *ywiC*, containing a putative Fnr box in their promoter region were identified, but their expression was not influenced by Fnr under the growth conditions we tested.

Functional analysis of group 1 Fnr-dependent genes. The group 1 Fnr-dependent genes (*narKfnr*, *narGHJI*, and *arfM*) are characterized by their Fnr-dependent induction under all anaerobic conditions tested and by the presence of a conserved Fnr box in their upstream region. However, only the functionality of the Fnr box of the *arfM* promoter was characterized by a mutagenesis approach (16). Fnr-dependent regulation of the *narK* promoter via the Fnr box found −41.5 bp upstream of the transcriptional start point was concluded by its glucose-dependent induction via Crp in *E. coli* (6). Finally, the *narGHJI* promoter was only characterized via reporter gene fusion and *fnr* regulator mutants (25). In order to elucidate the detailed nature of the Fnr-dependent *narGHJI* promoter, we constructed new *lacZ* reporter gene fusions and integrated them into the *amyE* locus of *B. subtilis*. All previously published investigations of the *narGHJI* promoter were performed using a *lacZ* fusion located at the *nar* locus. Testing of *narG-lacZ* integrated at the *amyE* locus allowed the mutation of the Fnr-binding site via single base exchanges and then subsequent chromosomal integration and selection of appropriate strains. Furthermore, using this approach, it was also possible to test the expression of a *narG-lacZ* fusion in *narG* mutant strain THB1.

The expression of the newly constructed reporter gene fusion was analyzed in the wild type and an *fnr* mutant strain (THB2). Expression of *narG-lacZ* in wild-type cells was found to be induced fourfold under anaerobic growth conditions. Anaerobic expression was totally abolished in the *fnr* mutant strain (HRB5) (Table 3). To connect the observed regulation with the Fnr box of the *narG* promoter, we mutated the corresponding sequence upstream of *narG*. The mutation was performed according to the strategy used as described above for the *arfM* promoter region, where the 5′-TGTGA-3′ half-site was changed to 5′**CC**TGA-3′ (exchanged bases are shown in boldface type), destroying the originally palindromic sequence (16). The mutation of the putative Fnr box (strain TDB8) resulted in a total loss of anaerobic *narG-lacZ* induction, indicating the essential role of the Fnr box for Fnr-dependent gene activation. Surprisingly, in the presence of nitrate under anaerobic conditions, *narG* promoter expression was further increased by a factor of 4, indicating a further nitrate-dependent induction of the *narG* promoter. Very similar results were obtained during Northern blot analysis of the transcript derived from the *nar* operon (Fig. 1A). This nitrate-dependent induction was also observed for the *narJ* gene; however, it was not clearly visible for *narGH* and *narI* during the array analysis. Since the detected amount for all transcripts of the *narGHJI* operon was weak compared to the RNA amounts measured via Northern blot analysis, one can only speculate whether the missing induction of *narG* and *narH* is due to failure in reverse transcription during macroarray testing.

To prove whether nitrate itself or a product of the ammonification process is responsible for the observed additional induction, *narG-lacZ* was analyzed in a *narG* mutant strain (HRB6). Nitrate induction of *narG-lacZ* was still observed in

the *narG* mutant, indicating its independence of the metabolization of nitrate. Nitrate induction was only observed when analyzed cells were harvested in the exponential phase and when nitrate was still present in the medium. After 6 h of cultivation, when the cells entered the stationary phase, we no longer detected the nitrate-dependent *narG-lacZ* induction. Determination of the nitrate concentration in the medium revealed that nitrate is almost completely utilized and therefore can no longer act as an inducer. This observation might explain previously published results where no obvious nitrate induction of *narG* expression was observed (16).

Functional analysis of group 2 Fnr-dependent genes. The genes of group 2 (*alsSD*, *ldh-lctP*, *ywcJ*, and *cydABCD*) were characterized by their Fnr-dependent anaerobic repression in the presence of nitrate. However, only *alsSD*, *ldh-lctP*, and *ywcJ* carry an Fnr box-like sequence upstream of their coding region. The *cydABCD* promoter did not contain an obvious Fnr box. The potential Fnr boxes of the *alsS* and *ldh* promoters were centered at positions −16.5 and +6.5 for *ldh* and at position −2.5 for *alsS* with respect to the transcriptional start point. Primer extension analysis revealed that the 5′ end of the *ywcJ* mRNA is localized 27 bp upstream of the translational start, −29.5 bp downstream of the center of the Fnr box palindrome (Fig. 2D). In order to test general promoter and Fnr box function in vivo, *alsS-lacZ*, *ywcJ-lacZ*, and *cydA-lacZ* were integrated into the *amyE* locus and analyzed for their anaerobic expression. Due to the previously determined almost-identical expression behavior of the *alsS* and *ldh* promoters, we refrained from a detailed *ldh* promoter analysis (16).

First, the expression of *alsS-lacZ*, *ywcJ-lacZ*, and *cydA-lacZ* was found to be induced under fermentative conditions. But when nitrate was present, a consistent two- to threefold repression occurred for all three tested gene fusions (Table 3). Similar observations were previously made for *alsSD* and *ldh-lctP* expression (6). These findings demonstrate that essential genes for fermentation and for a high-affinity cytochrome oxidase required for microaerophilic growth are repressed during anaerobic nitrate-respiratory conditions. Clearly, *B. subtilis* adapts this way to the most efficient energy-generating pathway under anaerobic growth conditions. Interestingly, for the *fnr* mutant strain, we consistently found that the fermentative expression of *alsS-lacZ*, *ywcJ-lacZ*, and *cydA-lacZ* remained unaffected, indicating that *fnr* is not required for strong anaerobic expression (Table 3). However, nitrate-dependent repression of *alsS-lacZ*, *ywcJ-lacZ*, and *cydA-lacZ* gene expression was abolished in the *fnr* mutant strain, pointing towards a role of Fnr in mediating nitrate-dependent repression. These findings are in agreement with previous findings for the *alsS* and *ldh* promoters tested at their original chromosomal location (6). Next, the question arose whether Fnr directly interacts with the putative Fnr-binding sites found in the promoter regions of *alsS* and *ywcJ* to mediate the observed repression. To test this hypothesis, we mutated the corresponding putative Fnr-binding sites in both promoters. The putative Fnr box in the *alsS* promoter was changed from AGTGA-CT-TCACA to AGTGA-CT-TC**CGG**, while the *ywcJ* promoter-localized Fnr box was changed from TGTGA-TA-TCACA to **CC**TGA-TA-TCACA (exchanged bases are shown in boldface type). The mutation in the palindromic sequence did not result in a nitrate-dependent derepression of *alsS-lacZ* in the wild-type strain (Table 3). When the mutated promoter was analyzed in the *fnr* mutant strain (HRB11), derepression was still visible (Table 3). As previously demonstrated for *ldh* expression by Cruz Ramos et al. (7), these results indicate that the nitrate-dependent repression of *alsS-lacZ* is not mediated by the putative Fnr boxes in its upstream region.

In contrast, the expression of *ywcJ-lacZ* was found to be derepressed by about 3-fold under fermentative conditions and even 10-fold under nitrate-respiratory conditions, resulting in comparable levels of *ywcJ* expression under both conditions. However, this behavior was not observed in the *fnr* mutant strain with the *ywcJ* wild-type promoter, demonstrating that Fnr is not responsible for the dramatic derepression of *ywcJ* expression when the putative Fnr-binding site is mutated. These results suggest that *ywcJ* expression might be regulated by another yet-unknown transcription factor that recognizes the same or an overlapping consensus sequence like Fnr. In agreement with previous findings for *ldh-lctP* (7), these results demonstrate that expression of *alsSD* and *ywcJ* is not directly influenced by the *trans*-acting factor Fnr via a *cis*-acting element. The expression behavior mediated by the Fnr box-free *cydA* promoter perfectly fits the Fnr- and Fnr box-independent expression of the other group 2 genes.

Nevertheless, Fnr influences the transcription of *alsSD*, *ldh-lctP*, *ywcJ*, and *cydABCD* during anaerobic nitrate respiration. Next, we investigated the possible indirect Fnr effect on the nitrate-dependent repression of group 2 genes via the regulation of *narGHJI* expression. For this reason, we analyzed the expression of *alsS-lacZ*, *ywcJ-lacZ*, and *cydA-lacZ* reporter gene fusions in nitrate reductase mutant strains (HRB9, HRB22, and HRB25) (Table 3). Interestingly, for all three reporter gene fusions tested, a derepression similar to that of the *fnr* mutant strains was detected. Since Fnr is the major regulator of the nitrate reductase operon under anaerobic growth conditions, these results indicate that Fnr influences the transcription of *alsSD*, *ywcJ*, and *cydABCD* indirectly by controlling nitrate reductase function. The dissimilatory nitrate reductase converts nitrate to nitrite under anaerobic conditions. To test whether the product of the nitrate reductase causes the derepression, we analyzed gene expression in the wild-type strains in the presence of nitrite. Interestingly, nitrite showed no inhibitory effect on the transcription of *alsS-lacZ*, *ywcJ-lacZ*, or *cydA-lacZ* (Table 3). Similar results were previously observed for *ldh* expression (7).

These results demonstrate that Fnr mediates the nitrate-dependent repression of *alsSD*, *cydABCD*, *ldh lctP*, and *ywcJ* by anaerobic induction of nitrate reductase production. The observed effect is not mediated by the substrate and the product, nitrate and nitrite, of the nitrate reductase reaction. Nevertheless, active nitrate reductase is required for the observed regulation (Fig. 3). Possibly, parts of the electron transport system or the NADH-to-NAD^+ ratio is involved in the repression of *alsSD*, *cydABCD*, *ldh lctP*, and *ywcJ*. In *Streptomyces coelicolor*, expression of *cydABCD* is regulated by Rex, a novel redox-sensing repressor. The DNA-binding activity of Rex appears to be controlled by the redox poise of the NADH/NAD^+ pool (4). Rex homologues exist in most gram-positive bacteria. In *B. subtilis*, a Rex homolog, YdiH, encoded by the *ydiH* gene acts as a repressor for *cydABCD* transcription under aerobic growth conditions (27). Very recently, it was demonstrated that YdiH

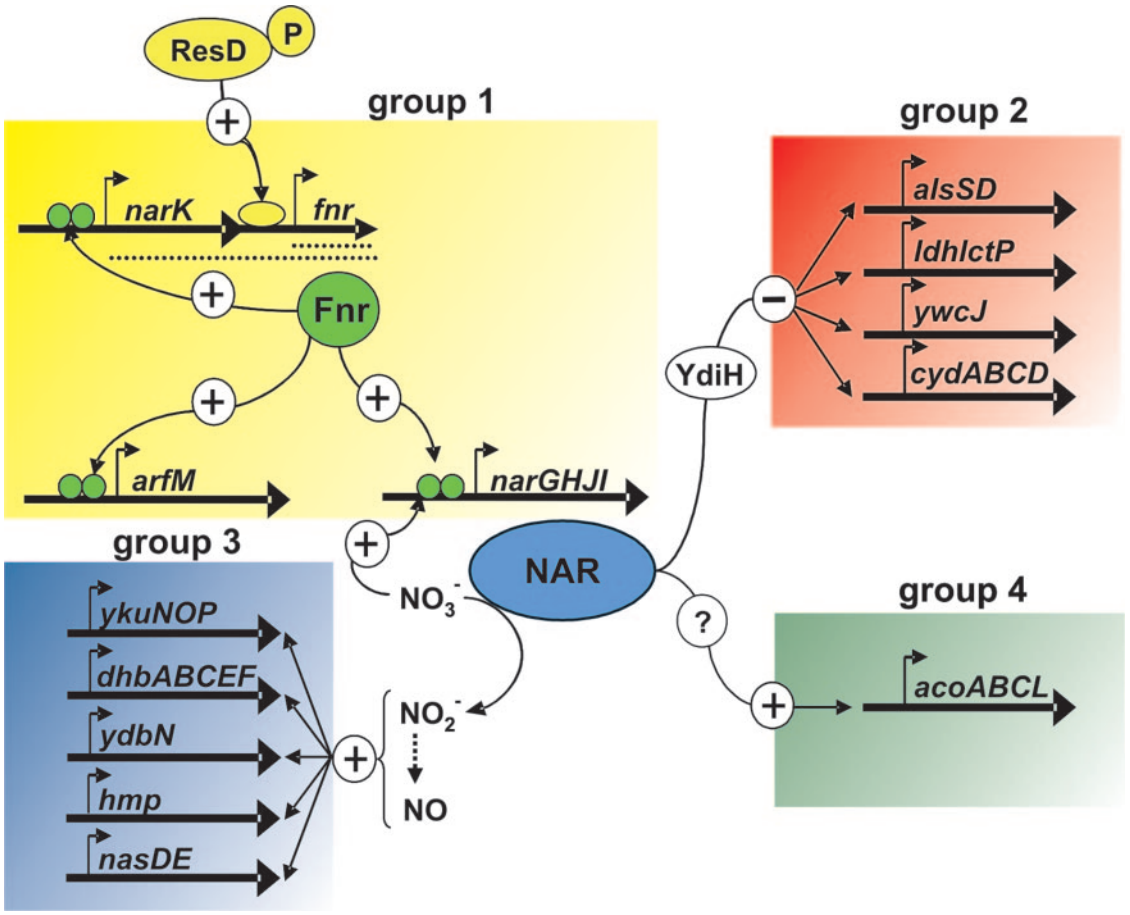

FIG. 3. Regulatory model for Fnr function during the transition to anaerobic growth conditions. Under anaerobic conditions, Fnr directly induces transcription of *narGHJI*, *arfM*, and *narKfnr* via a *cis*-acting Fnr box in the corresponding promoter regions (group 1 genes). By regulation of nitrate reductase formation, Fnr most likely mediates the repression of *alsSD*, *ldh lctP*, *ywcJ*, and *cydABCD* via YdiH (group 2 genes). Nitrite-derived nitric oxide serves as a second messenger and induces transcription of a subset of genes that overlap with the Fur, ResDE, and other unknown regulons (group 3 genes). Finally, *acoABCL* is the only member of expression group 4. Anaerobic expression of the operon is completely dependent on Fnr-dependent nitrate reductase formation.

of *B. subtilis* acts as a negative regulator of *cydABCD*, *ldh-lctP*, and *ywcJ* and coordinates the expression of these genes during the transition from aerobic to anaerobic growth (15). DNase I footprinting analysis revealed three binding sites of YdiH in the *cydA* promoter region, and a consensus sequence was proposed. Interestingly, the YdiH-binding sequence is also present in the *ywcJ* and *ldh* promoters overlapping the potential, nonfunctional Fnr-binding site. We already postulated the binding of a repressor at the *ywcJ* promoter, which is abolished by mutating the potential Fnr-binding site. To test whether YdiH functionally represses *ywcJ* expression under nitrate-respiratory conditions, we analyzed *ywcJ-lacZ* expression in a *ydiH* mutant strain (HRB29). A derepression of *ywcJ-lacZ* expression comparable to those when the potential Fnr-binding site of the *ywcJ* promoter was mutated was measured (Table 3). These results suggest that repression of *ywcJ* expression is mediated by YdiH and that the binding site of the repressor overlaps the deduced nonfunctional Fnr-binding site. The results previously reported by Larsson et al. (15) in combination with our findings provide the first insight into the redox regulatory network of *B. subtilis*. In the presence of nitrate under anaerobic growth conditions, this alternative electron acceptor is used to reoxidize NADH to NAD^+. With this NADH-to-NAD^+ ratio, the YdiH repressor is active and represses expression of the group2 genes *cydABCD*, *ldh-lctP*, *alsSD*, and *ywcJ*. After nitrate is used up, NADH accumulates and YdiH gets inactivated, which in turn leads to the derepression of group 2 genes. Thus, YdiH fills the missing link in how nitrate respiration and fermentation are coordinated at the transcriptional level (Fig. 3).

Functional analysis of group 3 Fnr-dependent genes. The third group of genes, *ydbN*, *ykuNOP*, *dhbABCEF*, *ykjA*, *ycgT*, *hmp*, and *nasDE*, identified by transcriptional profiling were found to be anaerobically induced in the presence of nitrate, and all lack Fnr-binding sites. To test how Fnr is involved in group 3 gene regulation, we analyzed the expression of *ykuNOP* and *ydbN*, encoding proteins of unknown functions, using the Northern blot technique. Using the *ykuN*-specific probe, a single transcript of about 1,900 nt, which corresponded to the size of the *ykuNOP* operon, was detected in equal amounts of RNA isolated from anaerobically grown cells in the presence of nitrate and nitrite (Fig. 1C). The transcript

was not detected in RNA from fermentatively grown cells. By analyzing an *fnr* mutant strain, we found the *ykuNOP* transcript only in RNA prepared from cells that were grown anaerobically in the presence of nitrite (Fig. 1C). A specific *ykuNOP* transcript was only detected in RNA prepared from a *narG* mutant strain (strain THB1) anaerobically grown in the presence of nitrite (Fig. 1C). Since this expression pattern was identical to those observed for the *fnr* mutant strain, an indirect Fnr function was obvious. The anaerobic expression of *ykuNOP* was only observed when nitrate was reduced to nitrite or when nitrite was directly added to the medium. Therefore, we conclude that *ykuNOP* expression is solely dependent on nitrite in the medium. The almost-identical expression pattern was found for *ydbN*, where we detected a specific transcript of about 200 nt in the Northern blot analysis (Fig. 1D). This size corresponds nicely to a single transcript of the predicted *ydbN* open reading frame of 177 bp. Consequently, Fnr anaerobically induces *narGHJI* transcription and formed nitrate reductase converts nitrate to nitrite, which in turn acts as an inducer for a yet-unknown *N*-oxide regulatory system (Fig. 3).

Some of the genes found in group 3 of our transcriptional profiling analysis have previously been described to be members of the Fur regulon, i.e., *dhbABCEF*, *ykuNOP*, and *ydbN* (4). NO can lead to nitrosylation of the ion center of Fur and thereby trigger derepression of the Fur regulon (18). Furthermore, members of the Fur regulon were found to be induced under nitrate-respiratory conditions (35). Moreover, *hmp* and *nasDE* belong to the ResDE regulon (35). Recent studies showed that the expression of most genes of this cluster (*hmp*, *nasD*, *dhbA*, *ykuN*, and *ycgT*) is dependent on the presence of NO (18, 21). Under cellular conditions, nitrite is spontaneously converted to nitric oxide (21). Thus, it is possible that the observed induction of group 3 genes upon the addition of nitrite is actually mediated by nitric oxide instead of nitrite.

Functional analysis of group 4 Fnr-dependent genes. The group 4 Fnr-dependent operon *acoABCL* was characterized by its strict Fnr-dependent expression under all anaerobic conditions tested and by the absence of an obvious Fnr box in its upstream region. Northern blot analysis revealed the strict dependence of *acoABCL* expression under fermentative and nitrate-respiratory conditions on the presence of an intact *fnr* gene (Fig. 1B). A slight nitrate induction was visible. Similar observations were made for the analysis of *acoA-lacZ* reporter gene fusions (Table 3). Again, a slight nitrate-dependent induction was observed. The total loss of reporter gene activity of the same fusion tested under anaerobic and nitrate-respiratory conditions in a *narG* mutant clearly demonstrated the indirect effect of Fnr via *narGHJI* induction. Therefore, the anaerobic expression of the *acoABCL* operon is, even under fermentative conditions, strictly dependent on the production of nitrate reductase mediated by Fnr.

Finally, a summary of the complex Fnr regulation pattern during the onset of anaerobic metabolism in *B. subtilis* is given in Fig. 3.

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