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Modulation of Anaerobic Energy Metabolism of *Bacillus subtilis* by *arfM* (*ywiD*)

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Bacillus subtilis **grows under anaerobic conditions utilizing nitrate ammonification and various fermentative processes. The two-component regulatory system ResDE and the redox regulator Fnr are the currently known parts of the regulatory system for anaerobic adaptation. Mutation of the open reading frame *ywiD* located upstream of the respiratory nitrate reductase operon *narGHJI* resulted in elimination of the contribution of nitrite dissimilation to anaerobic nitrate respiratory growth. Significantly reduced nitrite reductase (NasDE) activity was detected, while respiratory nitrate reductase activity was unchanged. Anaerobic induction of *nasDE* expression was found to be significantly dependent on intact *ywiD,* while anaerobic *narGHJI* expression was *ywiD* independent. Anaerobic transcription of *hmp,* encoding a flavohemoglobin-like protein, and of the fermentative operons *lctEP* and *alsSD,* responsible for lactate and acetoin formation, was partially dependent on *ywiD*. Expression of *pta,* encoding phosphotransacetylase involved in fermentative acetate formation, was not influenced by *ywiD*. Transcription of the *ywiD* gene was anaerobically induced by the redox regulator Fnr via the conserved Fnr-box (TGTGA-6N-TCACT) centered 40.5 bp upstream of the transcriptional start site. Anaerobic induction of *ywiD* by *resDE* was found to be indirect via *resDE*-dependent activation of *fnr*. The *ywiD* gene is subject to autorepression and nitrite repression. These results suggest a ResDE → Fnr → YwiD regulatory cascade for the modulation of genes involved in the anaerobic metabolism of *B. subtilis*. Therefore, *ywiD* was renamed *arfM* for anaerobic respiration and fermentation modulator.**

Under anaerobic growth conditions, *Bacillus subtilis* can generate ATP via nitrate ammonification or fermentation (2, 5, 14). During nitrate respiration, nitrate is reduced by the respiratory nitrate reductase (NarGHI) to nitrite (2, 5, 9). Nitrite is further reduced to ammonia by a general nitrite reductase (NasDE) (4, 13). The latter enzyme also contributes to the nitrite assimilation process (13). During anaerobic fermentation, carbon sources are transformed via pyruvate into the end products lactate, acetoin, 2,3-butanediol, ethanol, acetate, and succinate (3, 12). NAD^+ regeneration is primarily mediated by a cytoplasmic lactate dehydrogenase, encoded by *lctE*, that converts pyruvate to lactate (3). Acetoin is synthesized from pyruvate in a two-step reaction catalyzed by acetolactate synthase and acetolactate decarboxylase, encoded by the *alsSD* operon (3, 20). Subsequently, acetoin is converted to 2,3-butanediol by acetoin reductase (12). The third major fermentation product, acetate, is formed from acetyl-coenzyme A in a two-step reaction catalyzed by phosphotransacetylase and acetate kinase, encoded by *pta* and *ack*, respectively. The latter step usually leads to the formation of ATP.

Due to the drastically different ATP yields of respiratory and fermentative processes, bacteria usually use a fine-tuned regulatory system to maintain the most efficient mode of ATP generation. In *B. subtilis* only parts of the anaerobic redox regulatory system are known. The pleiotropic two-component regulating system ResDE, encoded by the *resABCDE* operon, is activated by an unknown redox-sensing system (21). Activated ResD binds directly to DNA elements (TTTGTGAAT) located within anaerobically induced promoter regions. Activator binding at this conserved promoter element and transcriptional activation were demonstrated for *nasDE*, the flavohemoglobin gene *hmp*, and the redox regulatory gene *fnr* (14, 16, 21). The redox regulator Fnr, possibly containing an iron sulfur cluster similar to its *Escherichia coli* counterpart, is subsequently responsible for the induction of the *narGHJI* operon and *narK*, encoding respiratory nitrate reductase and a potential nitrite extrusion protein, respectively (2).

All known Fnr-regulated genes have a highly conserved potential *B. subtilis* Fnr-binding site (TGTGA-N_6-TCACA) in their promoter regions. Additional potential Fnr-binding sites were found in the 5′ regions of a second potential nitrate/nitrite transporter gene, *ywcJ*, the fermentation operons *lctEP* and *alsSD*, and *ywiD*, encoding a protein of unknown function (2, 3).

The regulation of genes involved in fermentation was described recently (3). Transcription of *alsSD* and *lctEP* is induced anaerobically and repressed by the presence of nitrate (3). However, Fnr is only partially responsible for anaerobic *lctEP* and *alsSD* induction. These findings, in combination with

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the incompletely understood molecular basis for anaerobic *nasDE* and *hmp* induction, raise the possibility of additional redox regulatory components in *B. subtilis*. Here we provide evidence that *ywiD*, located in the 5′ region of the *narGHJI* operon, is an important part of the anaerobic regulatory system, responsible for the modulation of anaerobic gene expression. Since *ywiD* expression is Fnr dependent, a regulatory cascade from an unknown sensor proceeding via *resDE* through *fnr* and *ywiD* to multiple target genes is proposed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All *B. subtilis* strains used are listed in Table 1. For the investigation of the expression of the various *lacZ* fusions, the host strains were grown anaerobically at 37°C on Luria-Bertani medium supplemented with 20 mM K_3PO_4 (pH 7), 2 mM $(NH_4)_2SO_4$, 1 mM glutamic acid, 1 mM L-tryptophan, 0.8 mM L-phenylalanine, 0.005% (wt/vol) ammonium iron(III) citrate, 1 mM glucose, and, when indicated, 10 mM nitrite or nitrate (4, 13).

Inoculation of the test culture was performed under the described conditions and started in all experiments with identical amounts of cultured cells. For all strains tested, β-galactosidase activities were followed over the whole growth phase. Values obtained from comparable growth phases are listed. For β-galactosidase assays, cells were harvested at an appropriate optical density at 578 nm (OD_{578}) by centrifugation. The cell pellet was resuspended in 400 μl of Z-buffer (60 mM $Na_2HPO_4 \cdot 2H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 50 mM β-mercaptoethanol, pH 7.0). Lysozyme-DNase solution (2 μl; 8 mg of lysozyme in 950 μl of sterile water and 50 μl of DNase [2.5 mg/ml in 3 M sodium acetate]) was added and incubated for 15 min at 37°C. The crude cell extract was centrifuged for 5 min to remove cell debris. The supernatant was recovered. Protein concentration of the cell extract was determined using the Roti-Quant (Roth, Karlsruhe, Germany) protein detection assay. Then 600 μl of Z-buffer was added to 200 μl of crude cell extract.

The reaction was started by adding 200 μl of *ortho*-nitrophenylgalactopyranoside stock solution (4 mg/ml). To stop the reaction, 500 μl of 1 M Na_2CO_3 was added, the reaction time was noted, and the OD_{420} was determined versus the reference reaction. The specific activity was determined using the following equation: units per milligram of protein = 1,500/[test volume (milliliters) × time (minutes) × protein concentration (milligrams per milliliter)] × OD_{420}.

For the growth experiments, minimal medium containing 80 mM K_2HPO_4, 44 mM KH_2PO_4, 0.8 mM $MgSO_4 \cdot 7H_2O$, 1.5 mM thiamine, 40 μM $CaCl_2 \cdot 2H_2O$, 68 μM $FeCl_2 \cdot 4H_2O$, 5 μM $MnCl_2 \cdot 4H_2O$, 12.5 μM $ZnCl_2$, 24 μM $CuCl_2 \cdot 2H_2O$, 2.5 μM $CoCl_2 \cdot 6H_2O$, 2.5 μM $Na_2MoO_4 \cdot 2H_2O$, 50 mM glucose, and, where indicated, 10 mM nitrate or 10 mM nitrite was used. Antibiotics were added when necessary at the following concentrations (milligrams per liter): ampicillin, 100; chloramphenicol, 5; kanamycin, 10; and spectinomycin, 60. Bacteria were grown at 37°C in all experiments.

DNA manipulations and genetic techniques. *E. coli* was transformed as described by Chung and Miller (1). *B. subtilis* cells were transformed as described before (7). Transcriptional fusions with the *E. coli lacZ* gene were constructed using integrative plasmid pJM783 (16). A pUC18 derivative was obtained during the pDIA5348 shotgun cloning experiment described before (2), encompassing the complete intergenic region between the *ywiC* and *ywiD* genes.

To construct a transcriptional fusion with the *ywiD* gene, the chromosomal insert from the pUC18 derivative was excised as a ~0.34-kb *Hae*III-*Bcl*I fragment and inserted between the *Sma*I and *Bam*HI sites in pJM783, leading to plasmid pDIA5562. The *lacZ* gene in this construct was placed after the 38th codon of the *ywiD* gene. Both transcriptional fusions were introduced into the *B. subtilis* 168 chromosome by Campbell-type recombination events to generate *B. subtilis* BSIP1203 and BSIP1204, respectively. The *ywiD-lacZ* fusion was transferred from BSIP1204 into the *B. subtilis* JH642-based *fnr* mutant THB2, the *resDE* mutant MH5081, the *resDE* mutant LAB2313 carrying *fnr* under the control of the IPTG (isopropylthiogalactopyranoside)-inducible P*spac* promoter, and the *ywiD* mutant to generate *B. subtilis* MMB2, MMB4, MMB20, and MMB21, respectively.

A *B. subtilis* strain in which *ywiD* was interrupted by a kanamycin resistance gene (11) was constructed by homologous recombination using pDIA5564. Plasmid pDIA5564 was created from another pUC18 derivative (2), encompassing the 5′ end of the *ywiC* gene and the complete *ywiD* gene (pDG782). The plasmid was linearized by *Bcl*I and ligated to a *Bam*HI-*Bgl*II restriction site-flanked kanamycin cassette, interrupting the *ywiD* gene after the 38th codon. Plasmid pDIA5564 was linearized and used to transform *B. subtilis* JH642.

A strain in which the wild-type *ywiD* gene was replaced by the disrupted copy, *ywiD*::*kan* (THB110), was selected as a kanamycin-resistant transformant. The *ywiD*::*kan* mutation was transferred into LAB2854 (*nasD-lacZ*), LAB2143 (*narG-lacZ*), MMB61 (*lctE-lacZ*), MMB57 (*alsS-lacZ*), MMB101 (*pta-lacZ*), and

TABLE 1. Bacterial strains used in this study

B. subtilis strain	Relevant characteristics	Source or reference
168	*trpC2*	BGSC[a]
BSIP1104	*trpC2 pheA1pta-lacZ cat*	18
BSIP1204	*trpC2 arfM-lacZ cat*	This study
BSIP1185	*trpC2 lctE-lacZ cat*	3
BSIP1192	*trpC2 alsS-lacZ cat*	3
JH642	*trpC2 pheA1*	BGSC
LAB2000	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pML26 (*hmp-lacZ*) *cat*	This study
LAB2143	*trpC2 pheA1 amyE*::*narG-lacZ cat*	13
LAB2313	*trpC2 pheA1* Δ*resDE*::*tet fnr*::pMMN297 (P*spac-fnr*)	This study
LAB2854	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::*pMMN392* (*nasD-lacZ*) *cat*	13
MH5081	*trpC2 pheA1* Δ*resDE*::*tet*	This study
MMB2	*trpC2 pheA1 fnr*::*spc arfM-lacZ cat*	This study
MMB4	*trpC2 pheA1* Δ*resDE*::*tet arfM-lacZ cat*	This study
MMB8	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::*pML26* (*hmp-lacZ*) *cat arfM*::*kan*	This study
MMB9	*trpC2 pheA1 arfM*::*kan amyE*::*narG-lacZ cat*	This study
MMB10	*trpC2 pheA1 arfM*::*kan nasD-lacZ cat*	This study
MMB14	*trpC2 pheA1 arfM*::*kan alsS-lacZ cat*	This study
MMB15	*trpC2 pheA1 arfM*::*kan lctE-lacZ cat*	This study
MMB20	*trpC2 pheA1* Δ*resDE*::*tet cat fnr*::pMMN297 (P*spac-fnr*) *arfM-lacZ cat*	This study
MMB21	*trpC2 pheA1 arfM*::*kan arfM-lacZ cat*	This study
MMB25	*trpC2 pheA1 arfM*ΔFnr-*lacZ cat*	This study
MMB38	*trpC2 pheA1pta-lacZ cat arfM*::*kan*	This study
MMB40	*trpC2 pheA1 arfM*::*kan fnr*::*spc*	This study
MMB41	*trpC2 pheA1 arfM*::*kan* Δ*resDE*::*tet*	This study
MMB46	*trpC2 pheA1 arfM*::*kan fnr*::*spc lctE-lacZ cat*	This study
MMB47	*trpC2 pheA1 arfM*::*kan* Δ*resDE*::*tet lctE-lacZ cat*	This study
MMB48	*trpC2 pheA1 arfM*::*kan fnr*::*spc* SPβ*c2del2*::Tn*917*::pMMN392 (*nasD-lacZ*) *cat*	This study
MMB49	*trpC2 pheA1 arfM*::*kan fnr*::*spc* SPβ*c2del2*::Tn*917*::pML26(*hmp-lacZ*) *cat*	This study
MMB50	*trpC2 pheA1 arfM*::*kan* Δ*resDE*::*tet alsS-lacZ cat*	This study
MMB51	*trpC2 pheA1 arfM*::*kan fnr*::*spc amyE*::*narG-lacZ cat*	This study
MMB52	*trpC2 pheA1 arfM*::*kan fnr*::*spc arfM-lacZ cat*	This study
MMB54	*trpC2 pheA1 arfM*::*kan* Δ*resDE*::*tet arfM-lacZ cat*	This study
MMB55	*trpC2 pheA1 arfM*::*kan* Δ*resDE*::*tet amyE*::*narG-lacZ cat*	This study
MMB56	*trpC2 pheA1 arfM*::*kan* Δ*resDE*::*tet* SPβ*c2del2*::Tn*917*::pMMN392(*nasD-lacZ*) *cat*	This study
MMB57	*trpC2 pheA1 arfM*::*kan* Δ*resDE*::*tet* SPβ*c2del2*::Tn*917*::pML26(*hmp-lacZ*) *cat*	This study
MMB67	*trpC2 pheA1 lctE-lacZ cat fnr*::*spc*	This study
MMB68	*trpC2 pheA1 alsS-lacZ cat fnr*::*spc*	This study
MMB70	*trpC2 pheA1 fnr*::*spc* SPβ*c2del2*::Tn*917*::pMMN392(*nasD-lacZ*) *cat*	This study
MMB71	*trpC2 pheA1 amyE*::*narG-lacZ cat fnr*::*spc*	This study
MMB72	*trpC2 pheA1 fnr*::*spc* SPβ*c2del2*::Tn*917*::pML26 (*hmp-lacZ*) *cat*	This study
MMB74	*trpC2 pheA1 lctE-lacZ cat* Δ*resDE*::*tet*	This study
MMB75	*trpC2 pheA1 alsS-lacZ cat* Δ*resDE*::*tet*	This study
MMB77	*trpC2 pheA1* Δ*resDE*::*tet* SPβ*c2del2*::Tn*917*::pMMN392(*nasD-lacZ*) *cat*	This study
MMB78	*trpC2 pheA1 amyE*::*narG-lacZ cat* Δ*resDE*::*tet*	This study
MMB79	*trpC2 pheA1* Δ*resDE*::*tet* SPβ*c2del2*::Tn*917*::pML26(*hmp-lacZ*) *cat*	This study
MMB103	*trpC2 pheA1 ywiC*::*spc*	This study
MMB104	*trpC2 pheA1 arfM*::*kan*	This study
THB2	*trpC2 pheA1 fnr*::*spc*	This study
THB216	*trpC2 pheA1 arfM-lacZ cat*	This study

[a] BGSC, Bacillus Genetic Stock Center.

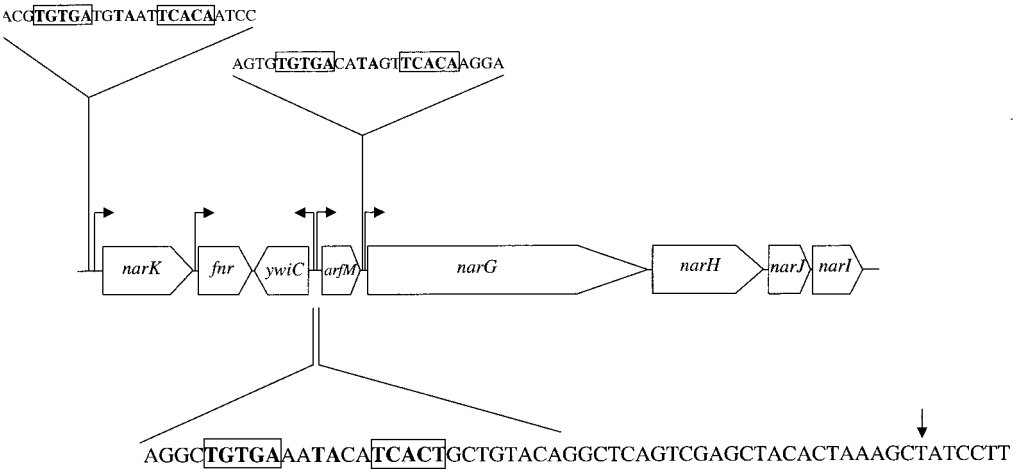

FIG. 1. *nar* locus of *B. subtilis*. The *fnr* gene encodes a redox regulatory protein, *narK* a putative nitrate/nitrite transporter protein, and *narGHJI* the respiratory nitrate reductase. The open reading frame *arfM* is the subject of this investigation. Potential Fnr binding sites are boxed. The 5′ end of the *arfM* mRNA is indicated by the arrow.

LAB2000 (*hmp-lacZ*) to generate MMB10, MMB9, MMB15, MMB14, MMB102, and MMB8, respectively. The *ywiD* mutation of THB110 was transferred into the *fnr* mutant THB2 and the *resDE* mutant MH5081 to generate the *ywiD fnr* and *ywiD resDE* double mutants MMB40 and MMB41, respectively. Finally, the *nasD-lacZ* (from LAB2854), *narG-lacZ* (LAB2143), *lctE-lacZ* (BSIP1185), *alsS-lacZ* (BSIP1192), *ywiD-lacZ* (BSIP1204), and *hmp-lacZ* (LAB2000) fusions were transferred to the double mutants, resulting in the strains listed in Table 1. We failed to obtain a strain carrying an *alsS-lacZ* fusion in a *ywiD fnr* mutant background.

Mutation of the *fnr* site upstream of *ywiD* from 5′-TGTGA-AATACA-TCA CT-3′ to 5′-*CC*TGA-AATACA-TCACT-3′ localized on the *ywiD-lacZ* fusion-carrying plasmid pDIA5562 was performed using the Quick Change site-directed mutagenesis kit (Stratagene, Heidelberg, Germany) according to the instructions of the manufacturer. The resulting plasmid, pDIA5562ΔFnr, was introduced into JH642 to generate MMB25 (*ywiD* Δ*fnr-lacZ*).

PCR and Southern blotting experiments were used to confirm the appropriate substitution of the wild-type gene by the mutated copy in mutant strains and to verify that only a single copy of the *lacZ* fusion was integrated.

Primer extension analysis of 5′ end of *ywiD* mRNA. Total cellular RNA was prepared from *B. subtilis* using the RnEasy minikit (Qiagen, Hilden, Germany), according to the instructions of the manufacturer. The 5′ ends of mRNAs encoded by the *ywiD* gene were mapped with an oligonucleotide that was complementary to positions 55 to 78 (5′-GTGCCTCTATCCATTGTCGAAACC-3′) of the *ywiD* gene and fluorescence labeled with 5′-indodicarbocyanine (5′-Cy5). For each experiment, 20 to 100 μg of RNA was incubated with 0.2 pmol of labeled primer for 3 min at 70°C in 34 mM Tris-HCl (pH 8.3)–50 mM NaCl–5 mM $MgCl_2$–5 mM dithiothreitol. The primer-RNA hybrids were extended with 10 U of avian myeloblastosis virus reverse transcriptase and 0.5 mM each nucleoside triphosphate, 12.5 mM dithiothreitol, 12.5 mM Tris-HCl (pH 8.3), and 7.5 mM $MgCl_2$ for 1 h at 42°C in the presence of 10 U of RNasin. Extension products were purified by phenol extraction, subjected to denaturing polyacrylamide gel electrophoresis (PAGE), and monitored by the ALF Express system (Amersham Pharmacia Biotech, Freiburg, Germany). A sequencing reaction performed with the same primer set was run in parallel on the same gel and allowed direct identification of the *ywiD* mRNA 5′ end.

HPLC analysis of *B. subtilis* fermentation products. Analysis of excreted fermentation product by high-pressure liquid chromatography was performed as outlined before (3).

RESULTS AND DISCUSSION

Reduced anaerobic growth of *B. subtilis ywiD* mutant. In the 5′ region of the *narGHJI* operon encoding respiratory nitrate reductase, an open reading frame of unknown function termed *ywiD* was found (Fig. 1). The open reading frame *ywiD* would encode a protein of 158 amino acid residues and a calculated molecular mass of 18,137 Da. The deduced protein showed no significant similarity to any other protein of known function in the database. To investigate its potential participation in anaerobic growth processes, a genomic knockout mutation of the gene was constructed and its growth behavior in minimal medium was compared to that of wild-type *B. subtilis* under aerobic and various anaerobic growth conditions.

Deletion of *ywiD* had no obvious influence on aerobic growth (Fig. 2). However, anaerobic growth in minimal medium was significantly reduced in the presence of nitrate or nitrite and under fermentative conditions (Fig. 2). The typical biphasic anaerobic growth curve for wild-type *B. subtilis* on minimal medium in the presence of nitrate was not observed

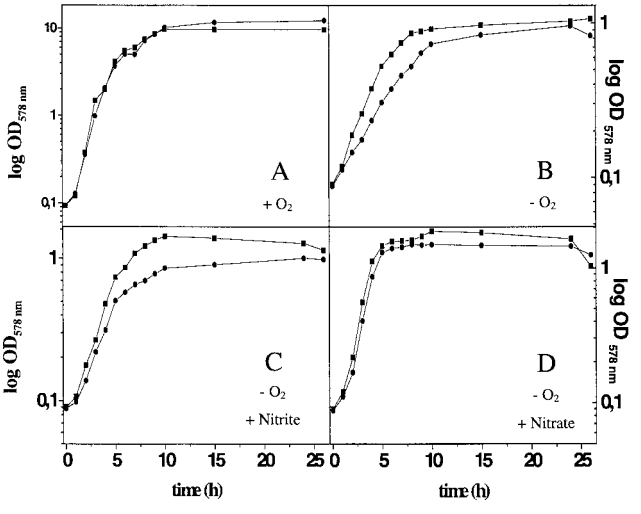

FIG. 2. Aerobic (A) and anaerobic fermentative (B) and nitrite (C) and nitrate (D) respiratory growth of *B. subtilis* wild-type JH642 (■) and the *arfM* mutant MMB104 (●). The minimal medium is described in Materials and Methods. Growth was monitored by determination of the OD_{578} at the indicated time points. Values reported are the averages from at least five independent experiments performed in triplicate.

TABLE 2. Respiratory nitrate and nitrite reductase activities in cell extracts prepared from wild-type *B. subtilis* and the *arfM* mutant[a]

B. subtilis strain	Relevant genotype	Activity ± SD (mU/mg)	
		Nitrite reductase activity	Nitrate reductase activity
JH642	Wild type	144 ± 10	250 ± 10
THB97	*arfM*::*kan*	29 ± 5	256 ± 10

[a] NADH-dependent nitrite reductase activities were measured using cell extracts prepared from *B. subtilis* strains grown anaerobically in minimal medium supplemented with 10 mM nitrite as described in the text. Benzyl viologen-dependent nitrate reductase activities were determined using cell extracts prepared from *B. subtilis* strains grown anaerobically in minimal medium with 10 mM nitrate as outlined before (4). Values reported are the averages of at least three independent experiments performed in triplicate.

for the *ywiD* mutant. The biphasic character of the curve for anaerobic nitrate respiratory growth results from the sequential utilization of first nitrate and then nitrite (4). The observed growth curve of the *ywiD* mutant mirrored the anaerobic growth behavior of a *nasD* mutant on nitrate-containing medium, missing the growth enhancement from nitrite dissimilation via *nasDE*-encoded nitrite reductase (13). In agreement with this observation, reduced anaerobic growth of the *ywiD* mutant on nitrite-containing medium was observed (Fig. 2). Moreover, a reduction in anaerobic fermentative growth by the *ywiD* mutant was detected. From these results, we conclude that *ywiD* is generally important for anaerobic metabolism of *B. subtilis*. From these results and others presented below, we have renamed *ywiD arfM* (anaerobic respiration and fermentation modulator).

Mutation of *arfM* leads to reduced nitrite reductase activity. To differentiate between the *arfM* influence on various anaerobic respiratory systems, cell extracts prepared from wild-type *B. subtilis* and the *arfM* mutant grown under various anaerobic growth conditions were compared for respiratory nitrate reductase and nitrite reductase activity. No significant influence of *arfM* on respiratory nitrate reductase activity was observed (Table 2). This is in agreement with the anaerobic growth behavior of the *arfM* mutant on nitrate-containing medium. Interestingly, reduced nitrate reductase activity was observed in the wild-type and the *arfM* mutant under nitrite dissimilatory conditions, indicating nitrite repression (data not shown). In contrast to the results of the nitrate reductase activity measurements, the nitrite reductase activity was reduced approximately fivefold in the *arfM* mutant, indicating the participation of *arfM* in *nasDE* expression or nitrite reductase formation or activity (Table 2).

HPLC analysis of fermentation products of *arfM* mutant. The amounts of the fermentation products lactate, acetate, and 2,3-butanediol excreted by wild-type *B. subtilis* and an *arfM* mutant were compared using HPLC analysis (3). The amount of accumulated lactate and 2,3-butanediol formed under all anaerobic growth conditions tested using glucose and pyruvate as carbon sources was found to be reduced by approximately 60% for the *arfM* mutant compared to the wild-type strain, taking the difference in growth yield between the two strains into account (data not shown). No obvious change in acetate production was observed. These results indicate an impact of *arfM* on lactate and 2,3-butanediol formation and provide an explanation for the *arfM* growth phenotype under anaerobic fermentative conditions.

***arfM* gene is involved in anaerobic *nasDE* but not *narGHJI* expression.** To test the influence of *arfM* on the transcription of various genes encoding anaerobic metabolism enzymes, the expression of reporter gene fusions of corresponding promoter regions with *lacZ* were tested. β-Galactosidase activities in wild-type *B. subtilis* and the *arfM* mutant grown under various anaerobic conditions were compared. In agreement with the growth phenotypes and enzymatic activities, no significant influence of *arfM* on *narGHJI* expression was found (Table 3). In agreement with the enzyme activity measurements, less *narGHJI* expression was observed in the presence of nitrite, again indicating nitrite inhibition (Table 3). The *arfM* mutation significantly reduced *nasDE* expression under all anaerobic growth conditions tested (Table 3). This is in agreement with the observed growth phenotype and reduced nitrite reductase activities of an *arfM* mutant.

***arfM* gene is involved in anaerobic *hmp* induction.** The *hmp* gene, encoding a flavohemoglobin-like protein, is another target of redox regulation in *B. subtilis*. The importance of *resDE* and nitrite for anaerobic induction of *hmp* was described previously (8). As outlined by LaCelle et al., the observed anaerobic induction by nitrate is of an indirect nature (8). Respiratory nitrate reductase reduces nitrate to nitrite. The nitrite formed subsequently induces *hmp* transcription via a still unknown regulatory system. Anaerobic *hmp* transcription was found to be significantly reduced in an *arfM* mutant under all growth conditions tested (Table 3).

TABLE 3. Influence of regulatory genes *resDE, fnr*, and *arfM* on transcription of the nitrate and nitrite dissimilatory loci *narGHJI* and *nasDE* and the flavohemoglobin gene *hmp*[a]

Fusion	Strain (relevant genotype)	β-Galactosidase activity (U/mg of protein)			
		Aerobic	Anaerobic		
			Fermentative	With nitrate	With nitrite
narG-lacZ	LAB2143 (wild type)	<10	811	852	455
	MMB71 (*fnr*)	<10	<10	<10	<10
	MMB78 (*resDE*)	<10	80	64	42
	MMB9 (*arfM*)	<10	822	840	468
	MMB51 (*arfM fnr*)	<10	<10	<10	<10
	MMB55 (*arfM resDE*)	<10	42	35	21
nasD-lacZ	LAB2854 (wild type)	<10	251	1,954	2,452
	MMB70 (*fnr*)	<10	35	33	1,230
	MMB77 (*resDE*)	<10	33	35	28
	MMB8 (*arfM*)	<10	73	771	955
	MMB48 (*arfM fnr*)	<10	25	35	872
	MMB56 (*arfM resDE*)	<10	<10	<10	12
hmp-lacZ	LAB2000 (wild type)	<10	376	7,715	14,833
	MMB72 (*fnr*)	<10	<10	<10	4,011
	MMB79 (*resDE*)	<10	49	24	35
	MMB8 (*arfM*)	<10	211	2,714	4,212
	MMB49 (*arfM fnr*)	<10	11	<10	4,126
	MMB57 (*arfM resDE*)	<10	25	15	16

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

TABLE 4. Influence of *resDE*, *fnr*, and *arfM* on transcription of the fermentative loci *lctEP*, *alsSD*, and *pla*[a]

Fusion	Strain (relevant genotype)	β-Galactosidase activity (U/mg of protein)			
		Aerobic	Anaerobic		
			Fermentative	With nitrate	With nitrite
lctE-lacZ	BSIP1185 (wild type)	<10	10,121	1,523	8,744
	MMB67 (*fnr*)	<10	3,125	2,560	2,746
	MMB74 (*resDE*)	<10	1,505	1,233	1,304
	MMB15 (*arfM*)	<10	3,237	782	2,536
	MMB46 (*arfM fnr*)	<10	2,924	1,828	2,127
	MMB47 (*arfM resDE*)	<10	275	314	199
alsS-lacZ	BSIP1192 (wild type)	<10	5,715	1,705	5,824
	MMB68 (*fnr*)	<10	2,513	2,127	1,827
	MMB75 (*resDE*)	<10	3,798	3,501	2,117
	MMB14 (*arfM*)	<10	2,525	759	1,714
	MMB50 (*arfM resDE*)	<10	1,874	1,253	1,752
pta-lacZ	BSIP1104 (wild type)	1,051	874	1,116	1,081
	MMB38 (*arfM*)	993	1,215	1,189	1,076

[a] See Table 3, footnote *a*.

***arfM* gene is involved in anaerobic induction of the fermentation loci *lctEP* and *alsSD*.** The involvement of *fnr*, *resDE*, *alsR*, and the respiratory nitrate reductase operon *narGHJI* in oxygen-, pH-, and nitrate-dependent *lctEP* and *alsSD* expression control was described previously (3). However, *fnr* was only partially responsible for anaerobic *lctEP* and *alsSD* expression. No obvious oxygen or nitrate regulation was observed for *pta* (3).

Reporter gene fusions of the promoter regions of the fermentation loci *lctEP*, *alsSD*, and *pta* were tested for the participation of *arfM* in their anaerobic expression. A significant reduction in anaerobic *lctE-lacZ* expression in an *arfM* mutant compared to wild-type *B. subtilis* was observed (Table 4). The previously observed nitrate repression of *lctE* transcription remained mainly unchanged (3). *alsS-lacZ* expression was also found to be reduced in the *arfM* mutant under conditions of fermentative as well as nitrate and nitrite dissimilatory growth. Again, nitrate repression of *alsS-lacZ* expression remained mainly unaffected (Table 4).

Consequently, *arfM* contributes to anaerobic *lctEP* and *alsSD* induction. No *arfM* involvement in *pta* transcription was observed (Table 4). This result was expected because *pta* transcription lacks obvious redox regulation (3). These results identify *arfM* as an important component of the redox regulatory system in *B. subtilis*. To investigate the relationship of *arfM* to the other known members of the redox system encoded by *resDE* and *fnr*, the regulation of *arfM* transcription was investigated.

***resDE-fnr*-dependent anaerobic *arfM* induction.** Redox-dependent *arfM* transcription was investigated using *arfM-lacZ* fusions. As shown in Table 5, *arfM* was exclusively expressed anaerobically. Comparable values of *arfM-lacZ* expression were obtained for fermentative and nitrate respiratory growth, while the presence of nitrite resulted in a 50% reduction in *arfM* expression. A similar reduction in anaerobic gene expression by the presence of nitrite was also observed for *narGHJI* transcription (see above).

Anaerobic *arfM* expression was completely dependent on the presence of intact *fnr* (Table 5). The involvement of *fnr* in *arfM* transcription was expected because the 5′ region of *arfM* harbors a conserved potential Fnr binding site (TGTGA-6N-TCACT). An *arfM* promoter analysis is described below. Anaerobic expression of *arfM* in a *resDE* mutant was found to be significantly reduced (Table 5). However, *resDE*-independent expression of *fnr* from an IPTG-inducible P*spac* promoter in a *resDE* mutant restored anaerobic *arfM* expression to almost the wild-type level (Table 5). These findings indicate that the observed role of *resDE* in *arfM* expression is indirect, via *resDE*-dependent *fnr* induction. As observed for various other regulatory genes, *arfM* represses its own expression (Table 5).

These results suggest that the anaerobic induction of various genes of anaerobic metabolism is mediated by a regulatory cascade consisting of an unknown signal, *resDE*, *fnr*, and *arfM*. Investigation of the contribution of this potential regulatory cascade to the induction of various anaerobic loci using regulatory double mutants in combination with reporter gene fusions is described below.

Analysis of *fnr*-dependent *arfM* promoter. Primer extension analysis revealed a single 5′ end for *arfM* mRNA (data not shown). A transcriptional start site located 25 bp from the translational start was determined (Fig. 1). Signal intensities during the primer extension experiments varied depending on the growth conditions used for the *B. subtilis* employed for RNA isolation. In agreement with the reporter gene fusions, primer extension signals were only observed with RNA prepared from anaerobically grown *B. subtilis*. Centered at 40.5 bp upstream of the transcriptional start, we found a DNA sequence (TGTGA-N_6-TCACT) with a high degree of sequence identity to potential *B. subtilis* Fnr binding sites.

However, no direct experimental proof was available for the function of this promoter element in *B. subtilis*. Therefore, the upstream half of the palindromic sequence was mutated from 5′-TGTGA-3′ to 5′-CCTGA-3′. As shown in Table 5, the mutations in the putative Fnr-box (strain MMB25) completely abolished anaerobic induction of *arfM*. These results confirmed the complete *fnr* dependence of anaerobic *arfM* induction and experimentally verified the 5′-TGTGA-N_6-TCACT-3′ sequence as the Fnr-box.

TABLE 5. Influence of *resDE*, *fnr*, and *arfM* on transcription of the *arfM* gene[a]

Strain (relevant genotype)[b]	β-Galactosidase activity (U/mg of protein)			
	Aerobic	Anaerobic		
		Fermentative	With nitrate	With nitrite
THB216 (wild type)	<10	1,027	1,579	612
MMB2 (*fnr*)	<10	<10	<10	<10
MMB25 (ΔFnr site)	<10	<10	<10	<10
MMB20 (*resDE pSpac-fnr*)				
Without IPTG	<10	182	225	85
With IPTG	<10	1,035	1,483	472
MMB4 (*resDE*)	<10	17	25	27
MMB21 (*arfM*)	<10	2,314	3,025	3,123
MMB52 (*arfM fnr*)	<10	<10	<10	<10
MMB54 (*arfM resDE*)	<10	28	24	22

[a] See Table 3, footnote *a*.
[b] All strains carried the *arfM-lacZ* fusion.

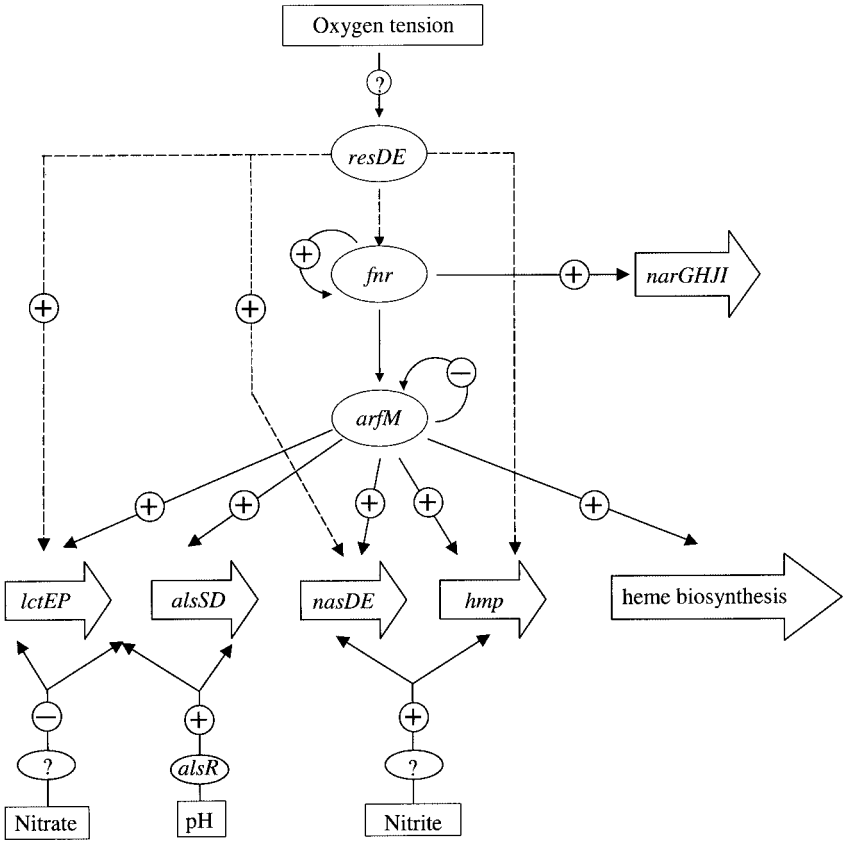

FIG. 3. Proposed regulatory cascade involved in the induction of gene expression under low oxygen tension conditions in *B. subtilis* (2, 3, 6, 8, 13, 13, 16, 20, 21). Various stimuli (oxygen tension, nitrate, nitrite, and pH) are transferred and integrated via various regulatory loci (*resDE*, *fnr*, *arfM*, and *alsR*) to differentially control the expression of various metabolic target genes encoding respiratory nitrate reductase (*narGHJI*), nitrite reductase (*nasDE*), flavohemoglobin-like protein (*hmp*), enzymes of lactate (*lctEP*) and acetoin (*alsSD*) fermentation and heme biosynthesis (*hemN* and *hemZ*).

Role of *resDE-fnr-arfM* regulatory cascade for differential anaerobic gene expression. The results from the experiments described above and from a previous study of *B. subtilis hemN* and *hemZ* expression led us to propose a model for *arfM* function (Fig. 3). The protein encoded by *arfM* is currently the last part of a regulatory chain in *B. subtilis* responsible for adaptation to anaerobic growth conditions. The signal of low oxygen tension is measured by a still unknown receptor and transferred directly or indirectly to the two-component regulatory system ResDE. Subsequently ResDE directly activates *fnr* transcription (14). Moreover, several other genes of anaerobic metabolism such as *hmp* and *nasDE* are influenced directly in their expression by *resDE*.

Fnr directly activates the most efficient anaerobic mode of ATP generation, nitrate respiration, via induction of the nitrate reductase operon *narGHJI* and nitrate/nitrite transporter genes. Fnr also activates *arfM* transcription. Finally, *arfM* modulates the expression of genes encoding proteins which further sustain nitrate respiration, such as heme biosynthesis genes (6). It also enhances alternative modes of ATP generation to nitrate respiration such as fermentation and nitrite dissimilation. This level of the molecular response to anaerobiosis is further fine tuned by additional environmental and cellular stimuli mediated by additional unknown redox regulatory components, by a pH-responding system, and by nitrate as well as nitrite regulatory systems (15).

In order to obtain further evidence for this regulatory model (Fig. 3), double mutant strains defective in *resDE*, *fnr*, or *arfM* were constructed, and the expression patterns of *lacZ* reporter gene fusions with all investigated genes were measured (Tables 3 and 4). Combination of the *arfM* mutation with the *fnr* or *resDE* mutation did not significantly change their already detrimental effects on *narG-lacZ* expression (Table 3). As expected, combining the *arfM* and *fnr* mutations resulted in similar β-galactosidase activity values resulting from *nasD-lacZ* and *hmp-lacZ* expression compared to expression in a simple *fnr* mutant (Table 3). Combination of the *arfM* and *resDE* mutations led to a complete loss of transcription from the *nasD* and *hmp* promoters. These additive effects were expected due to the significant direct role of *resDE* in *nasDE* and *hmp* transcription.

The comparable β-galactosidase activity values derived from the *lctE-lacZ* fusion in the *arfM* and *fnr* single mutants as well as the *arfM fnr* double mutant are in good agreement with our cascade regulatory model (Table 4). The lower values from the *resDE* mutants are in agreement with the previously determined independent role of *resDE* in *lctE* expression (3). This conclusion is further sustained by the significantly reduced *lctE*

expression in a *resDE arfM* double mutant. The similar values obtained for *alsS-lacZ* expression in *resDE*, *fnr*, and *arfM* mutants and the *arfM resDE* double mutant are in agreement with the proposed regulatory cascade. Surprisingly, no *arfM fnr* double mutant carrying an *alsS-lacZ* fusion was obtained. The reason remains unclear.

Previously, we described the nitrate repression of *lctE-lacZ* and *alsS-lacZ* mediated by the presence of intact *narGHJI*. In agreement with the lack of *arfM* influence on *narGHJI* transcription, the *arfM* mutation did not significantly affect nitrate repression. Combination of an *arfM* mutation with either an *fnr* or a *resDE* mutation resulted in the loss of nitrate repression due to decreased *narGHJI* expression. For similar reasons, anaerobic *nasD-lacZ* and *hmp-lacZ* expression in the presence of nitrate was reduced in an *arfM* mutant and totally abolished in an *fnr* mutant (Table 3). In the *fnr* mutant, *narGHJI* expression is greatly reduced. The synthesis of the strongly stimulatory nitrite from nitrate by nitrate reductase is missing. Since the *arfM* mutation has no effect on *narGHJI* expression, the combination of both regulatory mutations resulted in the expected *fnr* mutant-like expression pattern.

Similar to findings for *B. subtilis hemN* and *hemZ* transcription, *nasDE*, *hmp*, *lctEP*, and *alsSD* transcription is subject to *resDE-fnr-arfM* cascade regulation.

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