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Fermentative Metabolism of *Bacillus subtilis*: Physiology and Regulation of Gene Expression

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Bacillus subtilis **grows in the absence of oxygen using nitrate ammonification and various fermentation processes. Lactate, acetate, and 2,3-butanediol were identified in the growth medium as the major anaerobic fermentation products by using high-performance liquid chromatography. Lactate formation was found to be dependent on the** ***lctEP*** **locus, encoding lactate dehydrogenase and a putative lactate permease. Mutation of** ***lctE*** **results in drastically reduced anaerobic growth independent of the presence of alternative electron acceptors, indicating the importance of NADH reoxidation by lactate dehydrogenase for the overall anaerobic energy metabolism. Anaerobic formation of 2,3-butanediol via acetoin involves acetolactate synthase and decarboxylase encoded by the** ***alsSD*** **operon. Mutation of** ***alsSD*** **has no significant effect on anaerobic growth. Anaerobic acetate synthesis from acetyl coenzyme A requires phosphotransacetylase encoded by** ***pta*****. Similar to the case for** ***lctEP*****, mutation of** ***pta*** **significantly reduces anaerobic fermentative and respiratory growth. The expression of both** ***lctEP*** **and** ***alsSD*** **is strongly induced under anaerobic conditions. Anaerobic** ***lctEP*** **and** ***alsSD*** **induction was found to be partially dependent on the gene encoding the redox regulator Fnr. The observed** ***fnr*** **dependence might be the result of Fnr-induced** ***arfM*** **(*****ywiD*****) transcription and subsequent** ***lctEP*** **and** ***alsSD*** **activation by the regulator ArfM (YwiD). The two-component regulatory system encoded by** ***resDE*** **is also involved in anaerobic** ***lctEP*** **induction. No direct** ***resDE*** **influence on the redox regulation of** ***alsSD*** **was observed. The alternative electron acceptor nitrate represses anaerobic** ***lctEP*** **and** ***alsSD*** **transcription. Nitrate repression requires** ***resDE*****- and** ***fnr*****-dependent expression of** ***narGHJI*****, encoding respiratory nitrate reductase. The gene** ***alsR*****, encoding a regulator potentially responding to changes of the intracellular pH and to acetate, is essential for anaerobic** ***lctEP*** **and** ***alsSD*** **expression. In agreement with its known aerobic function, no obvious oxygen- or nitrate-dependent** ***pta*** **regulation was observed. A model for the regulation of the anaerobic fermentation genes in** ***B. subtilis*** **is proposed.**

Bacillus subtilis was long considered to be unable to grow in the absence of molecular oxygen as a terminal electron acceptor. However, as in the case of other members of the genus *Bacillus*, the ability of *B. subtilis* to utilize nitrate as an alternative electron acceptor has been described by several groups (5, 9, 14, 31). During the process of anaerobic nitrate ammonification, nitrate is reduced by a respiratory nitrate reductase (NarGHI) to nitrite, which is subsequently reduced further to ammonia by a general cellular nitrite reductase (NasDE) (4, 8, 9, 22). The *nar* locus, consisting of the *narGHJI* operon (encoding respiratory nitrate reductase), *narK* (for a potential nitrite extrusion protein), and the open reading frames *ywiC* and *ywiD* (of unknown function) also contains the regulatory gene *fnr* (4). The nitrite reductase genes *nasDE* were found downstream of the *nasAB* operon, which encodes assimilatory nitrate reductase (24). Two regulatory systems for the transition from aerobic to anaerobic nitrate respiratory conditions have already been identified (4, 21). First, Fnr, a member of *Escherichia coli* Crp-Fnr regulatory protein family, acts directly on the expression of several anaerobic transcriptional units (i.e., *narK* and *narGHJI*), most likely via interaction with a conserved DNA binding site similar to the *E. coli* Crp binding site, which is usually centered 41.5 nucleotides (nt) upstream of the transcriptional start point (4). Second, ResD-ResE, the pleiotropic two-component response regulator system encoded by the last two genes of the *resABCDE* operon, regulates, directly or indirectly, aerobic and anaerobic respiration (23, 33). The binding site for the phosphorylated form of ResD, the active form of the regulator, is still unknown. *B. subtilis fnr* is strongly induced in the absence of oxygen in a Fnr-independent manner (4). This induction is abolished in a *resDE* background (23). It is not known, however, if ResD~P acts directly to activate *fnr* transcription or if an unknown intermediary regulator is required. This contrasts with mainly autonomous *fnr* function in *E. coli* (34, 35).

Several groups demonstrated that *B. subtilis* is able to grow anaerobically on minimal media in the absence of terminal electron acceptors (8, 19). *E. coli* and other bacteria use a mixed acid fermentation for glucose metabolism to form the end products ethanol, succinate, lactate, acetate, formate, hydrogen, and carbon dioxide (2). Typical indicators of this process arise from the activity of its key enzyme, pyruvate formatelyase (Pfl), which leads to massive excretion of formate and

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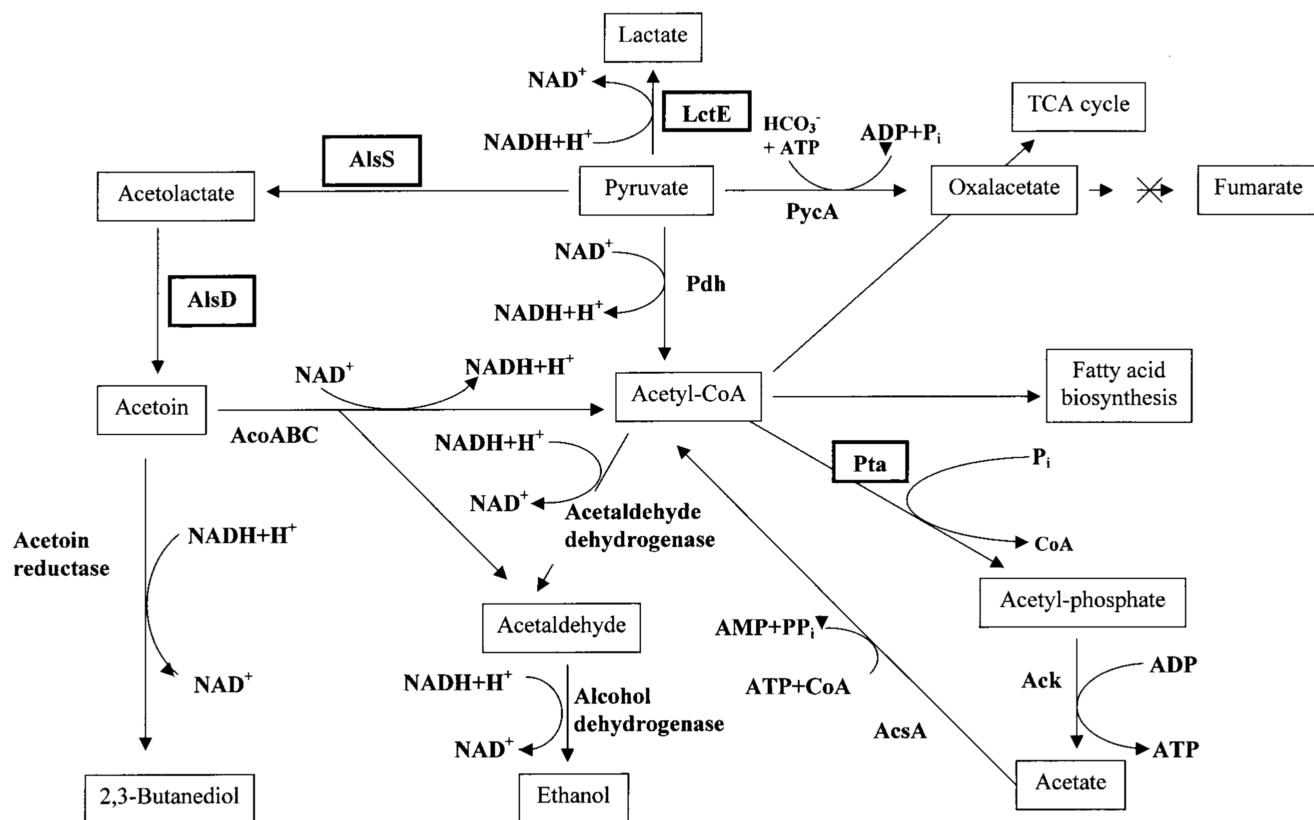

FIG. 1. Proposed pathways for anaerobic fermentation and related catabolism in *B. subtilis* (modified from references 11 and 19). Enzymes with known coding genes are as follows: LctE, lactate dehydrogenase; AlsS, acetolactate synthase; AlsD, acetolactate decarboxylase; Pta, phosphotransacetylase; Ack, acetate kinase; AcoABC, acetoin dehydrogenase; Pdh, pyruvate dehydrogenase; PycA, pyruvate carboxylase; AcsA, acetyl-CoA synthetase. TCA, tricarboxylic acid.

acetate as fermentative by-products. For *B. subtilis*, Nakano and coworkers (19) have identified, via nuclear magnetic resonance analysis, lactate, acetate, acetoin, ethanol, and succinate as main fermentation products (Fig. 1). No significant amounts of formate were detected (19), although the gas phase was not investigated. This observation is supported by the absence of any obvious counterpart to *E. coli* Pfl among the protein sequences deduced from the complete *B. subtilis* genome sequence (12).

To study the molecular basis for the coordinated induction for anaerobic respiration and fermentation, the completely sequenced *B. subtilis* genome was analyzed for loci potentially involved in fermentation. Three loci on the *B. subtilis* chromosome were investigated for their potential involvement in fermentative metabolism and corresponding regulation: *lctEP*, *alsSD*, and *pta* (Fig. 1). The *lctE* gene, encoding a protein with similarity to known dissimilatory lactate dehydrogenases, was identified during the systematic sequencing of the *B. subtilis* genome but has not yet been further studied (36). However, 20 years ago, the aerobic regulation of lactate dehydrogenase formation in *B. subtilis* was investigated using biochemical methods (37). The *alsSD* operon, which has been shown to encode an acetolactate synthase and an acetolactate decarboxylase, is responsible for acetoin production. Its aerobic catabolite-sensitive and growth phase regulation has been studied (29). Aerobic *alsSD* transcription is activated in late exponential growth phase. The *alsR* gene, located upstream of the *alsSD* operon and transcribed in the opposite direction, encodes a regulator involved in this activation process. As observed for *alsSD* expression, lactate dehydrogenase synthesis is induced upon the onset of the stationary phase (37). Finally, acetate formation from acetyl coenzyme A (acetyl-CoA) is catalyzed in a two-step reaction by phosphotransacetylase (*pta*) and acetate kinase (*ack*). Two-dimensional gel electrophoresis showed that Pta formation is aerobically regulated by various stress conditions (1). The second gene, *ack*, located in a different position of the genome was found to be subject to catabolite regulation mediated by the catabolite regulator CcpA (6, 27, 32). Here we describe the investigation of the roles played by *lctEP*, *alsSD*, and *pta* in anaerobic metabolism. Their oxygen tension- and nitrate-dependent expression dependent on the regulatory loci *resDE* and *fnr* was investigated. An initial regulatory model is proposed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All *B. subtilis* and *E. coli* strains used throughout this work are listed in Table 1. Luria-Bertani medium was used for standard cultures of *B. subtilis* and *E. coli* if not indicated otherwise (16). 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 L-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. The bacteria were incubated in completely filled flasks with rubber stoppers and with shaking at 100 rpm in an incubation shaker to minimize aggregation of the bacteria. Inoculation was performed aerobically at a 1:100 ratio of aerobically grown overnight culture and prewarmed medium. Anaerobic conditions were achieved after a short time through consumption of residual oxygen by the inoculated bacteria. After five doubling times, in the middle of the exponential growth phase, samples for β-galactosidase were taken. The minimal medium for the high-performance

TABLE 1. Strains

B. subtilis strain	Relevant genotype	Source or reference
168	*trpC2*	BGSC[a]
BSIP1104	*trpC2 pta-lacZ cat*	27
BSIP1171	*trpC2 pta*::*aphA3*	27
BSIP1173	*trpC2 alsS*::*alsS-lacZ2 cat*	This study
BSIP1174	*trpC2 alsS*::*alsS-lacZ cat pta*::*aphA3*	This study
BSIP1185	*trpC2 lctE-lacZ1 cat*	This study
BSIP1186	*trpC2 alsR*::*spc*	This study
BSIP1187	*trpC2 amyE*::*alsS-lacZΔfnr cat*	This study
BSIP1188	*trpC2 lctE-lacZ1 cat alsR*::*spc*	This study
BSIP1189	*trpC2 lctE-lacZΔFnr cat*	This study
BSIP1190	*trpC2 lctE*::*lctE-lacZ2 cat*	This study
BSIP1191	*trpC2 lctE*::*lctP-lacZ cat*	This study
BSIP1192	*trpC2 alsS-lacZ1 cat*	This study
BSIP1194	*trpC2 alsS-lacZ1 cat alsR*::*spc*	This study
MH5081	*trpC2 pheA1 ΔresDE*::*tet*	32
MMB100	*trpC2 pta-lacZ cat ΔresDE*::*tet*	This study
MMB101	*trpC2 pta-lacZ cat fnr*::*spc*	This study
MMB110	*trpC2 lctE-lacZ1 cat ΔnarGH*::*tet fnr*::*spc*	This study
MMB111	*trpC2 lctE-lacZ1 cat ΔresDE*::*tet fnr*::*spc*	This study
MMB112	*trpC2 alsS-lacZ1 cat ΔnarGH*::*tet fnr*::*spc*	This study
MMB113	*trpC2 alsS-lacZ1 cat ΔresDE*::*tet fnr*::*spc*	This study
MMB114	*trpC2 lctE-lacZΔFnr cat fnr*::*spc*	This study
MMB115	*trpC2 lctE-lacZΔFnr cat ΔresDE*::*tet*	This study
MMB116	*trpC2 lctE-lacZΔFnr cat ΔnarGH*::*tet*	This study
THB1	*trpC2 pheA1 ΔnarGH*::*tet*	9
THB2	*trpC2 pheA1 fnr*::*spc*	20
THB357	*trpC2 alsS-lacZ1 cat ΔresDE*::*tet*	This study
THB361	*trpC2 lctE-lacZ1 cat ΔresDE*::*tet*	This study
THB457	*trpC2 alsS-lacZ1 cat fnr*::*spc*	This study
THB461	*trpC2 lctE-lacZ1 cat fnr*::*spc*	This study
THB557	*trpC2 alsS-lacZ1 cat ΔnarGH*::*tet*	This study
THB561	*trpC2 lctE-lacZ1 cat ΔnarGH*::*tet*	This study

[a] BGSC, *Bacillus* Genetic Stock Center.

liquid chromatography (HPLC) determination of the fermentation products produced by the various investigated strains consisted of 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 $Na_2\ MoO_4 \cdot 2H_2O$, 50 mM glucose, 50 mM pyruvate, and where indicated, 10 mM nitrate or 10 mM nitrite. Anaerobic growth was performed as described above (4, 8, 20).

DNA methods and genetic techniques. *E. coli* was transformed as described by Chung and Miller (3). *B. subtilis* cells were transformed as described by Kunst and Rapoport (13). RNA extractions were performed as described by Hagen and Young (7). Southern blotting and Northern blotting were performed as described by Sambrook et al. (30). Membranes were further hybridized with nonradioactively digoxigenin-labeled probes (Boehringer, Mannheim, Germany). The *lctE*- and *lctP*-specific DNA fragments used as probes in the Northern blotting experiments were amplified by PCR using the following pairs of oligonucleotides: 5′-GCGGAATTCTTTAATCGGAGCGGGT-3′ and 5′-ACCTGCGATCCCTCCGC-3′ for *lctE* and 5′-TCTCGAATTCCTTTTGGCTTTAACTGT-3′ and 5′-CTCCCGTGACAACCTGC-3′ for *lctP*. Primer extensions experiments using reverse transcriptase were performed as described by Pikielny and Rosbash (26). The two oligonucleotides used as primers for mapping the *lctE* promoter were 5′-CGCAAATGCATAACTGCTTCCAAC-3′ and 5′-TGACCACAAGCTCATCTGTGATCCC-3′. The SubtiList database was used to search for sequence patterns in the *B. subtilis* genome (17).

Construction of fusion and mutant strains. *B. subtilis* strains containing transcriptional fusions between the *E. coli lacZ* gene and the *lctE*, *lctP*, *pta*, and *alsS* upstream regions were constructed using PCR-amplified chromosomal fragments and the integrative plasmid pJM783 (25) or the pAC5 derivative pDIA5322 (integration at the *amy* locus) (Table 1) (15). In pDIA5322, the pAC5 *Eco*RI-*Sac*I DNA fragment encompassing the 3′ part of the *lacZ* gene was replaced by the equivalent fragment including the *spoVG* initiation codon from pJM783. The oligonucleotides pairs used and their relative positions with respect to each start codon are described below. During the amplification, *Eco*RI and *Bam*HI restriction sites were created at opposite ends of the amplified fragment (underlined). After digestion using these two enzymes, the amplified fragment was inserted into pJM783 digested with the same enzymes. The plasmid pDIA5373, containing the *lacZ* fusion P*lctE-lacZ1*, was constructed by the integration of the region −206 to +434 (with respect to the start codon of *lctE*), amplified using the primers 5′-CCGGAATTCTCGGGCTTAAGCGGTTC-3′ and 5′-CGCGGATCCAATCACCCGCTCT-3′, into pJM783. The plasmid pDIA5374 harbors the fusion P*lctE-lacZ2*, consisting of the region +29 to +434 relative to *lctE* initiation codon amplified using the primers 5′-GCGGAATTCTTTAATCGGAGCGGGT-3′ and 5′-CGCGGATCCAATCACCCGCTCT-3′, inserted into pJM783. The plasmid pDIA5375, with the fusion P*lctE-lacZΔfnr*, was constructed using region −383 to −54 of *lctE* amplified using the primers 5′-GTAATTGAATTCACCGGATCTTGGCCTGGA-3′ and 5′-CCCGGATCCTTTCACATTTATATTGTGCAACACTTCACAAACTTTTGC-3′ and inserted into pJM783 (see Fig. 4). The vector pDIA5376 with P*lctP-lacZ* contains region +78 to +405 of *lctP* amplified using the primers 5′-TCTCGAATTCCTTTTGGCTTTAACTGT-3′ and 5′-ATCGGGATCCGAACACCAAAACCGGCC-3′ and integrated into pJM783. Plasmid pDIA5377, containing P*alsS-lacZ1* with region −481 to +394 with respect to the start codon of *alsS* amplified using the primers 5′-AGTTGAATTCCTTGTCCGATTTG-3′ and 5′-CCGTGGATCCTGCCCTGCTGACGCTAT-3′, was constructed using pJM783. Plasmid pDIA5378 contains the fusion P*alsS-lacZ2* spanning the region +105 to +394 of *alsS*, which was amplified using the primers 5′-GCAGGAATTCATGGCCCAAGCAGTC-3′ and 5′-CCGTGGATCCTGCCCTGCTGACGCTAT-3′. Plasmid pDIA5379, harboring P*alsS-lacZΔfnr*, was constructed using region −481 to −143 of *alsS* amplified using the primers 5′-AGTTGAATTCCTTGTCCGATTTG-3′ and 5′-GCGAGGATCCGATAAGTTTCACTATACACTC-3′ and integrated into pDIA5322. P*als-lacZΔfnr* was inserted at the *amyE* locus of *B. subtilis* 168 after a double crossover event to generate strain BSIP1187. P*lctE-lacZ1*, P*lctE-lacZ2*, P*lctE-lacZΔfnr*, P*lctP-lacZ*, P*alsS-lacZ1*, and P*alsS-lacZ2* were integrated into the corresponding genes of *B. subtilis* 168, leading in some cases to the inactivation of the gene (P*lctE-lacZ2*, P*lctP-lacZ*, and P*alsS-lacZ2*). The resulting strains were named BSIP1185, BSIP1190, BSIP1189, BSIP1191, BSIP1192, and BSIP1173, respectively. The description of the employed *pta-lacZ* fusion was given previously (27). *B. subtilis* strains in which *alsR* was disrupted by a spectinomycin resistance gene (18) were constructed by homologous recombination using plasmid pDIA5372. pDIA5372 was constructed by the insertion of a spectinomycin cassette into the unique *Stu*I site of the pUC18 derivative pDIA5370, previously obtained by a shotgun cloning experiment (28). In the resulting pDIA5372, the *alsR* gene was disrupted 270 bp downstream of the translational start codon. Linearized pDIA5372 was used to transform *B. subtilis* 168 to generate BSIP1186. P*lctE-lacZ1* and P*alsS-lacZ1* were transferred into BSIP1186 to generate BSIP1188 and BSIP1194, respectively. The strain THB361 was constructed by the transfer of Δ*resDE*::*tet* from MH5081 into BSIP1185 via transformation of BSIP1185 using genomic DNA from MH5081 and appropriate antibiotic selection. All newly created strains were checked using PCR and hybridization experiments. THB461 and THB561 resulted from the transfer of *fnr*::*spc* (THB2) and Δ*narGH*::*tet* (THB1), respectively, to BSIP1185. BSIP1188 contains *alsR*::*spec* from BSIP1186 in BSIP1185. THB357, THB457, THB557, and BSIP1194 were formed by the transfer of Δ*resDE*::*tet*, *fnr*::*spc*, Δ*narGH*::*tet*, and *alsR*::*spc* from MH5081, THB1, THB2, and BSIP1186, into BSIP1192, respectively. *B. subtilis* MMB100 and MMB101 were created by the transformation of BSIP1104 with genomic DNA prepared from MH5081 and THB2, respectively. BSIP1174 was formed by the transfer of *pta*::*aphA3* to BSIP1173. MMB110, MMB111, MMB112, and MMB113 were created by the transfer of Δ*narGH*::*tet* or Δ*resDE*::*tet* into THB461 and THB457. MMB114, MMB115, and MMB116 resulted from the transformation of BSIP1189 with genomic DNA prepared from THB2, MH5081, and THB1, respectively.

HPLC analysis of fermentation products. Fermentation products were separated and quantified by HPLC using an LKB-Pharmacia liquid chromatograph (Amersham Pharmacia Biotech, Freiburg, Germany). Metabolites were separated on a Eurocat H 300- by 8-mm, 10-μm-pore-size cation-exchange resin (Knauer, Berlin, Germany). Chromatography was performed at 60°C, with a flow rate of 0.6 ml/min in 0.01N H_2SO_4. Eluted compounds were registered and quantified by a refractive index detector equipped with a computer-powered integrator. Soluble fermentation products were identified by comparison with retention times and peak areas of corresponding standards.

β-Galactosidase assays. β-Galactosidase activity was assayed as previously described (16, 20).

RESULTS AND DISCUSSION

Analysis of *B. subtilis* fermentation products using HPLC. Previously, Nakano and coworkers demonstrated by nuclear magnetic resonance the formation of acetate, ethanol, lactate, acetoin, and, under certain conditions, small amounts of 2,3-butanediol from the carbon source glucose in combination with pyruvate (19). To monitor more precisely the flow of carbon from glucose and pyruvate into the various fermentation products, a semiquantitative HPLC analysis was established. A cation-exchange column was calibrated with glucose (retention time, 12.1 min), pyruvate (12.8 min), succinate (16.7 min), lactate (18.3 min), acetate (22.2 min), 2,3-butanediol (25.8 min), and ethanol (28.1 min). *B. subtilis* was grown anaerobi-

TABLE 2. Fermentation product formation in wild-type *B. subtilis* and various fermentation gene mutants[a]

B. subtilis strain (relevant genotype)	Electron acceptor (10 mM)	Lactate (mM)	Acetate (mM)	2,3-Butanediol (mM)	Total product concn (mM)	Concn of cells (g [wet wt]/liter)
168 (wild type)	Nitrate	23.3	16.4	16.7	56.4	8.0
BSIP1190 (*lct*)	Nitrate	1.1	14.3	13.4	28.8	2.0
BSIP1171 (*pta*)	Nitrate	9.0	3.7	4.2	16.9	4.3
BSIP1173 (*als*)	Nitrate	38.3	18.1	ND[b]	56.4	7.8
BSIP1174 (*pta als*)	Nitrate	2.7	1.7	ND	4.4	2.4
168 (wild type)	Nitrite	25.9	16.0	11.1	53.0	7.3
BSIP1190 (*lct*)	Nitrite	0.9	14.4	13.1	28.4	2.7
BSIP1171 (*pta*)	Nitrite	12.3	3.0	1.1	16.4	3.0
BSIP1173 (*als*)	Nitrite	38.0	15.5	ND	53.5	7.5
BSIP1174 (*pta als*)	Nitrite	2.0	1.0	ND	3.0	2.1
168 (wild type)		38.4	13.3	9.5	61.2	7.3
BSIP1190 (*lct*)		3.0	7.0	7.5	16.5	1.8
BSIP1171 (*pta*)		28.8	2.5	18.2	49.5	3.2
BSIP1173 (*als*)		39.4	12.5	ND	51.9	7.4
BSIP1174 (*pta als*)		15.0	1.1	ND	16.1	2.0

[a] The indicated *B. subtilis* strains were incubated anaerobically for 10 h at 37°C in defined minimal media using 50 mM glucose and 50 mM pyruvate as carbon sources. Fermentation products were isolated from the growth media and quantified using HPLC as described in Materials and Methods.

[b] ND, not detectable.

cally in minimal medium containing 50 mM glucose and 50 mM pyruvate as carbon sources. Ammonia served as a nitrogen source. Where indicated, 10 mM nitrate or nitrite was added. Since fermentation products are excreted by *B. subtilis*, metabolites present in the growth medium before and after anaerobic growth were quantified. Fermentative growth in the presence of 50 mM glucose and 50 mM pyruvate led to the detection of lactate (38.4 mM), 2,3-butanediol (9.5 mM), and acetate (13.3 mM). Surprisingly, the addition of the electron acceptors nitrate and nitrite did not drastically change the overall formation of fermentation products (Table 2). In both cases lactate formation was found to be slightly reduced, while the formation of acetate and 2,3-butanediol was slightly increased. These observations indicate the presence of the various fermentation processes during anaerobic respiration in *B. subtilis*. In all cases only a minor part of the initial added glucose was consumed, while the pyruvate was no longer detectable at the end of the growth (data not shown). At a higher pyruvate concentration (80 mM), excretion of acetoin was observed (data not shown). Ethanol was not detected in the growth media under any of the employed conditions using the HPLC method.

Functional identification of genes involved in anaerobic lactate, 2,3-butanediol, and acetate formation. Predicted metabolic pathways for the synthesis of lactate, acetate, and 2,3-butanediol are shown in Fig. 1 (11, 19). To identify the genetic loci involved in anaerobic fermentation product formation, we investigated mutants with mutations in potential fermentation genes previously identified by the sequencing project or other approaches.

First, the compounds excreted by those mutants when grown under anaerobic fermentative and respiratory conditions were compared using HPLC. Lactate is usually produced by reduction of pyruvate in a single step (Fig. 1). This reaction is catalyzed by lactate dehydrogenase, with the simultaneous oxidation of one molecule of NADH per molecule of pyruvate reduced. The *lctE* gene, potentially encoding lactate dehydrogenase from *B. subtilis*, was identified through systematic sequencing (36). In a *lctE* mutant (strain BSIP1190), almost no lactate accumulation was observed under any tested anaerobic condition in minimal (Table 2) or rich (data not shown) medium. The lack of lactate dehydrogenase led to a severe defect in anaerobic growth in rich and minimal media, although the *lct* mutant still accumulates significant amounts of acetate and 2,3-butanediol (Fig. 2; Table 2). In agreement with the observed lactate accumulation pattern for the wild-type strain, the observed reduction in anaerobic growth of the *lctE* mutant was independent of the presence of alternative electron acceptors, such as nitrate and nitrite (Fig. 2). These observations indicate that the lactate dehydrogenase encoded by *lctE* is generally important for anaerobic growth. Its function in the reoxidation of reducing equivalents is not limited to fermentation but is also important for respiratory growth with nitrate and nitrite.

Aerobic acetoin synthesis from pyruvate in *B. subtilis* was studied before (29). It involves two steps catalyzed by acetolactate synthase and acetolactate decarboxylase, which are encoded by *alsS* and *alsD*, respectively (Fig. 1). The two genes are organized in an operon (29). An additional step catalyzed by acetoin reductase converts acetoin to 2,3-butanediol. The corresponding *B. subtilis* gene is presently unknown. The insertional inactivation of the *als* operon (strain BSIP1173) totally abolished 2,3-butanediol production under all tested conditions (Table 2). However, the mutation had only a small effect on the growth behavior of *B. subtilis* under the tested anaerobic conditions (Fig. 2).

The energetically most efficient fermentative pathway is acetate production. After conversion of pyruvate to acetyl-CoA, the phosphotransacetylase (Pta) and acetate kinase (Ack) form acetate in a two-step reaction (Fig. 1). Usually, one ATP molecule per molecule of acetate is produced. Significantly reduced amounts of acetate were produced in a *pta* background (BSIP1171) under all tested conditions (Table 2). A significant reduction of growth was observed under all tested anaerobic conditions independent of the presence of alternative electron acceptors. Similar to the conclusions drawn from the growth behavior of the *lctE* mutant, *pta* plays a general role in the anaerobic energy metabolism. Recent investigation of the aerobic function of *pta* revealed its contribution to the growth in the presence of oxygen (27, 32). The *pta* gene is subject to a complex catabolite regulation involving *ccpA*, *hprK*, *ptsH1*, and *crh* (27).

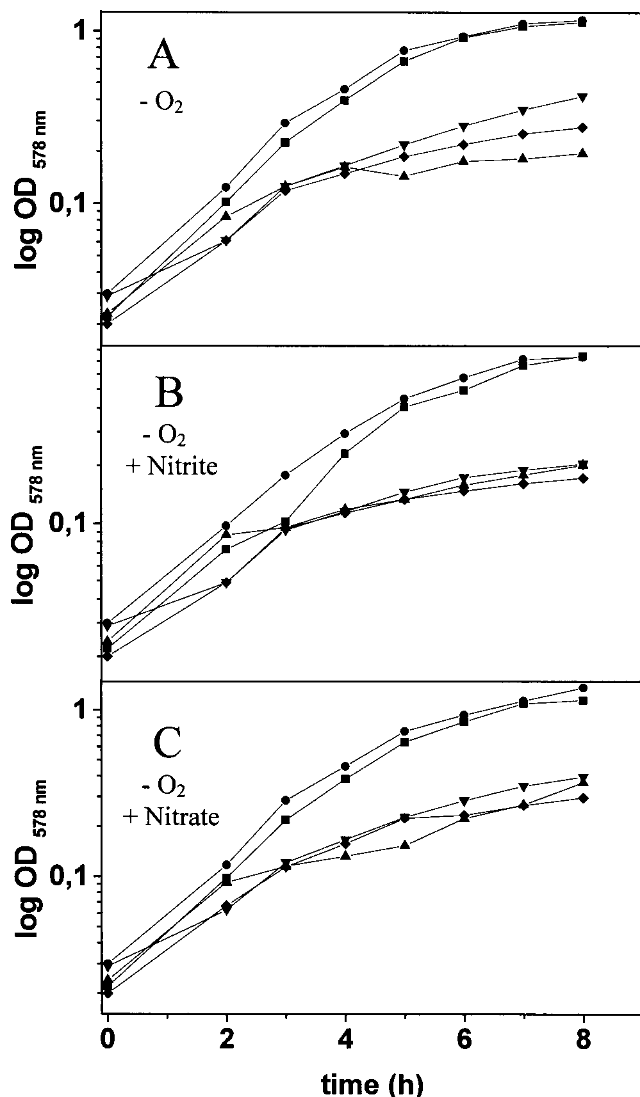

FIG. 2. Growth of wild-type *B. subtilis* (■); mutants with mutations in the *alsS* (●), *lctE* (▲), and *pta* (▼) genes; and a *pta alsS* double mutant (◆) under fermentative conditions (A) and nitrite (B) and nitrate (C) respiratory conditions. Growth was monitored by determination of the optical density at 578 nm (OD_{578} nm) at the indicated time points. Values reported are the averages from at least five independent experiments performed in triplicate.

We have also investigated the effect of a *pta als* double mutation (BSIP1174). In agreement with the results of the investigation of the strains with single mutations, the double mutant was unable to produce acetate or 2,3-butanediol and its growth was severely reduced compared to that of the wild type strains (Table 2; Fig. 2).

Organization of the *lctEP* operon. Initial inspection of the DNA sequence of *lctE*, encoding lactate dehydrogenase, and *lctP*, encoding a putative lactate permease, suggested the presence of a single transcriptional unit. Northern blot experiments confirmed this expectation (Fig. 3A). Total cellular RNA was prepared from wild-type *B. subtilis* grown under aerobic conditions (Fig. 3A, lanes 1) and under anaerobic conditions in the presence (Fig. 3A, lanes 2) and absence of nitrate (Fig. 3A, lanes 3). The addition of fumarate reflects fermentative conditions (Fig. 3A, lanes 4). Probes specific to *lctE* and *lctP* were employed for the detection of specific mRNA. The *lctE* probe detected three bands of approximately 2,700, 1,050, and 650 nt. The *lctP* probe revealed only the single band of higher molecular weight of approximately 2,700 nt, which was also seen with the *lctE* probe. First, the observed pattern indicated that the two genes are indeed organized in an operon and that no internal promoter upstream of *lctP* was active under the conditions tested. The large transcript of approximately 2,700 nt has exactly enough coding capacity for LctE and LctP. The observed transcriptional polarity may contribute to a lower synthesis of the potential lactate permease compared to lactate dehydrogenase. Second, comparison of band intensities for the RNAs prepared from *B. subtilis* grown under various growth conditions showed that the *lctEP* operon is significantly induced on the transcriptional level under anaerobic conditions in the absence of nitrate (Fig. 3A, lanes 3 and 4). The presence of nitrate significantly reduced anaerobic *lctEP* expression. However, the utilization of increased amounts of total cellular RNA for the Northern blot and primer extension experiments revealed the presence of *lctEP* mRNA even in the presence of nitrate in the growth medium (data not shown). The molecular basis of the observed regulatory phenomena was further investigated as outlined below.

Promoter structure of the *lctEP* operon. The 5′ end of the *lctEP* mRNA was investigated using the primer extension technique. As shown in Fig. 3B, the mRNA appeared to start at a guanosine residue 62 nt upstream of the translational start codon (Fig. 2B and 3). Potential −10 (CACAAT) and −35 (TTGCAA) regions for a σ^A-dependent promoter, separated by 17 bp, were deduced (Fig. 4). No other mRNA 5′ ends were detected by these mapping experiments, indicating the absence of other active promoters under the conditions tested. In

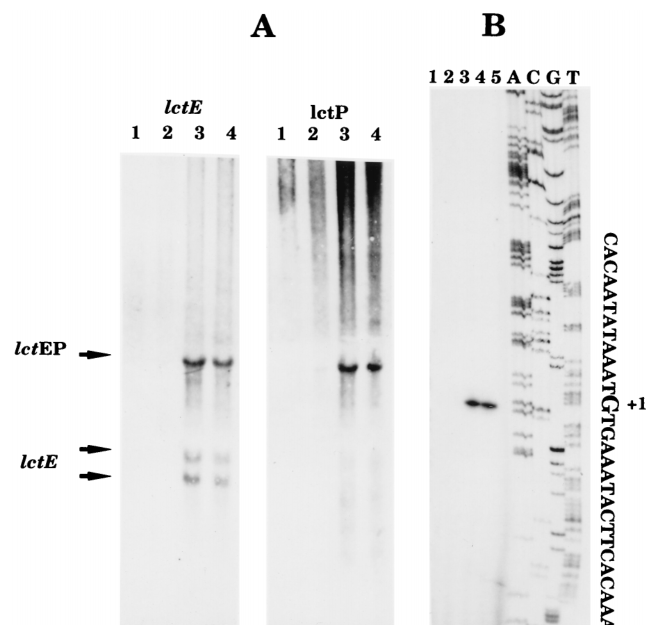

FIG. 3. mRNA analysis of the *lctEP* operon. (A) Northern blot analysis. The *lctE*- and *lctP*-specific probes were generated and the blotting was performed as outlined in Materials and Methods. (B) Primer extension mapping. The location of the 5′ end of the *lctEP* mRNA was deduced from the lengths of the cDNA bands. The length was obtained by comparison with the sequencing reaction products (in the order A, C, G, and T) performed with the primer used for the extension reaction. For both panels, the RNA used in each experiment was extracted from *B. subtilis* grown under the following conditions: lane 1, exponential phase in complex medium in the presence of oxygen; lane 2, without oxygen and with 10 mM nitrate; 3, without oxygen or further additions; 4, without oxygen and with 5 mM fumarate; 5, control without RNA.

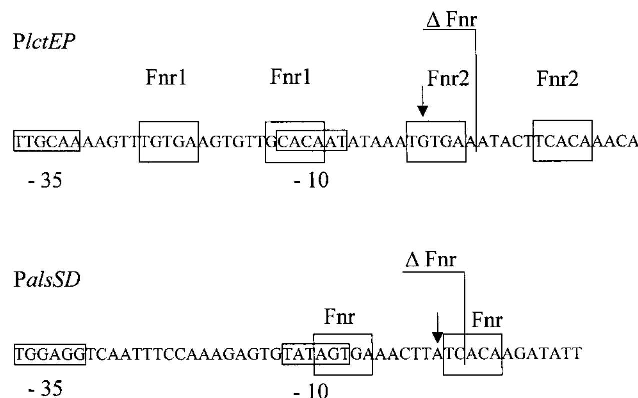

FIG. 4. DNA sequences of the *B. subtilis lctEP* and *alsSD* promoter regions. The mapped mRNA ends are indicated by arrows. Predicted −10 and −35 regions are boxed. Predicted Fnr binding sites are also boxed. The 3′ ends of the *lctE* and *alsS* promoter regions in the P*lct-lacZ*ΔFnr and P*als-lacZ*ΔFnr fusions are shown. The *alsSR* start point was described by Renna et al. (29).

agreement with the Northern experiment results and reporter gene fusion experiments (see below), the strongest primer extension signal was observed using RNA prepared from fermentatively grown *B. subtilis*.

Analysis of the DNA sequence upstream of the *lctEP* operon revealed a palindromic sequence spanning positions 89 to 44 bp upstream of the translational start codon and overlapping the determined transcriptional start site. Each symmetrical arm contains a highly conserved potential binding site for the redox regulator Fnr (Fnr box 1, 5′-TGTGA-AGTGTT-GCA CA-3′; Fnr box 2, 5′-TGTGA-AATACT-TCACA-3′) (4). The deduced promoter region is partly within the inverted repeat sequence (Fig. 4). The predicted −10 sequence is relatively poorly conserved and coincides with the second half of the first potential Fnr site. An initial characterization of this promoter region is described.

Redox- and nitrate-regulated expression of the *lctEP* operon. In order to further substantiate the transcriptional regulation highlighted by the mRNA analysis, we have studied the expression of the *lctEP* and *alsSD* operons by using transcriptional fusions with the *E. coli lacZ* gene as a reporter gene. The *lacZ* fusions were constructed as described in Materials and Methods. In the strain carrying P*lctE-lacZ1* or P*alsS-lacZ1*, the wild-type copy of the corresponding gene was maintained. Integration of P*lctE-lacZ2*, P*lctP-lacZ*, and P*alsS-lacZ2* resulted in the disruption of *lctE*, *lctP*, and *alsS*, respectively. Expression of these fusions was analyzed during aerobiosis and after a shift to anaerobiosis in the presence or absence of nitrate or nitrite.

The β-galactosidase activity of the PlctE-*lacZ1* fusion was rapidly induced (more than 70-fold) after a shift to anaerobic conditions (Table 3). Anaerobic induction of the P*lctE-lacZ1* fusion was suppressed fivefold if nitrate was present in the medium. The presence of nitrite had no inhibitory effect on anaerobic P*lctE-lacZ1* expression. The P*lctP-lacZ* fusion induction profile was similar to that of P*lct-lacZ1*. Inactivation of *lctE* did not significantly change the observed anaerobic induction of *lctE*. However, the nitrate repression of *lctE* transcription was reduced. Changes in *lctE-lacZ* expression due to structural differences between the employed fusions cannot be excluded.

Oxygen tension- and nitrate-regulated expression of the *alsSD* operon. Similar to the case for *lctE* expression, an approximately 60-fold anaerobic induction of β-galactosidase activity was observed for the P*alsS-lacZ1* fusion in the *B. subtilis* wild-type strain grown under fermentative conditions and in the presence of nitrite (Table 4). Again, nitrate reduced *alsS* expression threefold. Mutation of *alsS* did not influence anaerobic P*alsS-lacZ2* expression (Table 4). To investigate the participation of various known redox regulators and of AlsR in redox- and nitrate-dependent *alsSD* and *lctEP* expression, *lacZ* fusions were tested in *B. subtilis* strains carrying mutations in the *fnr*, *resDE*, and *alsR* loci.

Participation of *fnr*, *resDE*, and *alsR* in *lctEP* and *alsSD* expression. Mutation of *fnr* reduced anaerobic P*lctE-lacZ1* and

TABLE 3. Regulation of *lctEP* expression by oxygen tension and nitrate, the regulatory loci *resDE*, *fnr*, and *alsR*, and the nitrate reductase genes *narGHJI*[a]

Strain (relevant genotype)	Fusion	β-Galactosidase activity (Miller units)[b]			
		Aerobic	Anaerobic		
			Fermentative	With nitrate	With nitrite
BSIP1185 (wild type)	P*lctE-lacZ1*	<10	730	140	630
THB361 (Δ*resDE*)	P*lctE-lacZ1*	15	180	130	170
THB461 (*fnr*)	P*lctE-lacZ1*	<10	330	320	320
THB561 (Δ*narGH*)	P*lctE-lacZ1*	<10	630	620	610
BSIP1188 (*alsR*)	P*lctE-lacZ1*	<10	60	30	60
BSIP1190 (*lctE*)	P*lctE-lacZ2*	<10	780	510	600
MMB110 (*fnr* Δ*narGH*)	P*lctE-lacZ1*	<10	480	470	320
MMB110 (*fnr* Δ*resDE*)	P*lctE-lacZ1*	20	220	240	170
BSIP1191 (*lctP*)	P*lctP-lacZ*	<10	380	40	390
BSIP1189 (wild type)	P*lctE-lacZ*Δ*fnr*	<10	1,200	520	1,430
MMB114 (*fnr*)	P*lctE-lacZ*Δ*fnr*	<10	1,315	1,230	1,020
MMB115 (*resDE*)	P*lctE-lacZ*Δ*fnr*	30	450	320	460
MMB116 (Δ*narGH*)	P*lctE-lacZ*Δ*fnr*	<10	1,220	1,210	1,480

[a] Construction of the various *B. subtilis* strains carrying the indicated *lacZ* fusions is described in Materials and Methods. The indicated strains were grown either aerobically or anaerobically with 50 mM glucose as a carbon source and ammonia as a nitrogen source, with the indicated additions (10 mM nitrate or nitrite), to the mid-exponential growth phase as outlined in detail before (8, 20).
[b] β-Galactosidase activities were assayed as described before (16, 20). The results represent averages from at least five independent experiments performed in triplicate, with a standard error of less than 10%.

TABLE 4. Regulation of *als* expression by oxygen tension and nitrate, the regulatory loci *resDE*, *fnr*, and *alsR*, and the nitrate reductase genes *narGHJI*[a]

Strain (relevant genotype)	Reporter gene fusion	β-Galactosidase activity (Miller units)[b]			
		Aerobic	Anaerobic		
			Fermentative	With nitrate	With nitrite
BSIP1192 (wild type)	P*alsS-lacZ1*	<10	610	190	600
THB357 (Δ*resDE*)	P*alsS-lacZ1*	<10	740	880	940
THB457 (*fnr*)	P*alsS-lacZ1*	<10	390	380	330
THB557 (Δ*narGH*)	P*alsS-lacZ1*	<10	530	630	790
MMB112 (*fnr* Δ*narGH*)	P*alsS-lacZ1*	<10	270	280	250
MMB113 (*fnr* Δ*resDE*)	P*alsS-lacZ1*	<10	410	390	440
BSIP1194 (*alsR*)	P*alsS-lacZ1*	<10	40	20	30
BSIP1173 (*alsS*)	P*alsS-lacZ2*	<10	610	200	610
BSIP1187 (wild type)	P*alsS-lacZ*Δ*fnr*	<10	<10	<10	<10

[a] Construction of the various *B. subtilis* strains carrying the indicated *lacZ* fusions is described in Materials and Methods. The indicated strains were grown either aerobically or anaerobically with 50 mM glucose as a carbon source and ammonia as a nitrogen source, with the indicated additions (10 mM nitrate or nitrite), to the mid-exponential growth phase as outlined in detail before (8, 20).
[b] β-Galactosidase activities were assayed as described before (16, 20). The results represent averages from at least five independent experiments performed in triplicate, with a standard error of less than 10%.

P*alsS-lacZ1* expression by half (Tables 3 and 4). Expression of *lctEP* and *alsSD* is subject to *arfM* (*ywiD*) regulation (10; M. Marino, H. Cruz Ramos, T. Hoffman, P. Glaser, and D. Jahn, unpublished observations). Transcription of *arfM* (*ywiD*) was found to be completely *fnr* dependent. Due to the similar degrees of *arfM* and *fnr* regulation, it was concluded that there is an indirect participation of *fnr* in *lctEP* and *alsSD* expression via *arfM* induction (10; M. Marino et al., unpublished observations). Moreover, the location and initial analysis of the potential Fnr binding sites (see below) made their participation in a direct Fnr-mediated anaerobic induction of both operons very unlikely.

The second consequence of *fnr* mutation was the complete loss of nitrate repression on both operons (Tables 3 and 4). Interestingly, mutation of *resDE* also led to the loss of nitrate repression. These findings prompted us to investigate the participation of *narGHJI*, encoding respiratory nitrate reductase, in nitrate repression, since *narGHJI* expression is strictly dependent on *fnr* and *resDE*. As shown in Tables 3 and 4, *narGH* deletion also completely abolished nitrate repression of both operons. These results suggest that the observed nitrate regulation depends on the enzymatic nitrate reductase activity. Consequently, only an indirect participation of *fnr* and *resDE* via *narGHJI* induction in nitrate repression of anaerobic *lctEP* and *alsSD* transcription is suggested.

A clear difference was observed for the consequences of a *resDE* mutation on the overall anaerobic *lctEP* and *alsSD* expression. While P*lctE-lacZ1* expression was reduced three- to fourfold in a *resDE* mutant, P*alsS-lacZ1* expression remained mainly unchanged. This observation indicated an additional direct or indirect participation of *resDE* in anaerobic *lctEP* induction.

In agreement with the results obtained for the investigation of *lctEP* expression in single *fnr* and *resDE* mutants, analysis of a P*lctE-lacZ1* fusion in an *fnr resDE* double mutant revealed significantly reduced anaerobic induction with the complete loss of nitrate repression (Table 3). An *fnr resDE* double mutant led to the reduction of *alsSD* expression slightly below the level determined for the *fnr* mutant alone and to the complete loss of nitrate repression. This is again in agreement with the analysis of single regulatory mutants, where the redox regulation of *alsSD* did not significantly respond to *resDE* mutation (Table 4). In agreement with the observed effects of single *fnr* and *narGH* mutation on *lctEP* and *alsSD* expression, an *fnr narGH* double mutation reduced anaerobic *lctEP* and *alsSD* induction and completely abolished nitrate repression (Table 3).

The regulatory gene *alsR*, located upstream of the *alsSD* operon, was found to be essential for both P*lctE-lacZ* and P*alsS-lacZ* expression under all tested anaerobic conditions. Previous investigations suggested the intracellular pH as well as the acetate concentration in the growth medium as possible signals for AlsR-dependent regulation (29).

Initial analysis of the potential Fnr binding site in the *lctEP* and *alsSD* promoters. The analysis of the *lct* promoter region revealed a complex structure. In order to assess the role of the apparent Fnr binding sites in regulation, a truncated fusion was constructed (P*lctE-lacZ*Δ*fnr* [Fig. 4]). In this fusion only the first five bases after the transcription start point were kept, while the downstream half of the second putative Fnr site was removed as shown in Fig. 4. The expression of this P*lctE-lacZ*Δ*fnr* fusion was monitored under aerobic and various anaerobic growth conditions (Table 3). The loss of the second half site led to a higher anaerobic induction of *lctE* expression and a significant decrease of nitrate repression. These results indicate that this region might be involved in a mechanism of repression the *lct* transcription. In agreement with the data obtained for the analysis of the wild-type *lctE* promoter, *resDE* mutation significantly reduced anaerobic *lctEP* induction via the mutated promoter. However, the values obtained for the *resDE* mutant still documented the same degree of derepression observed for the wild-type strain (Table 3). Moreover, similar to the wild-type *lctEP* promoter, the mutated promoter revealed significantly reduced nitrate repression in a *resDE* mutant. In complete agreement with the case for the wild-type *lctEP* promoter, the mutated promoter responded to a *narGH* mutation with complete loss of nitrate repression. The degree of anaerobic depression of the mutated promoter in the *narGH* mutant was identical to that of the wild-type strain. Surprisingly, mutation of *fnr* did not decrease transcription from the mutated *lctEP* promoter, while the native promoter partially lost its anaerobic induction capacity. This observation indicated the importance of the mutated promoter sequence for direct or indirect influence of *fnr* on *lctEP* expression.

TABLE 5. Investigation of dependence of *pta* expression on changing oxygen tension and nitrate as well as nitrite[a]

Strain (relevant genotype)	Reporter gene fusion	β-Galactosidase activity (Miller units)[b]			
		Aerobic	Anaerobic		
			Fermentative	With nitrate	With nitrite
BSIP1104 (wild type)	P*pta-lacZ*	210	185	200	200
MMB100 (Δ*resDE*)	P*pta-lacZ*	180	175	170	170
MMB101 (*fnr*)	P*pta-lacZ*	215	185	170	170

[a] Construction of the various *B. subtilis* strains carrying the indicated *lacZ* fusions is described in Materials and Methods. The indicated strains were grown either aerobically or anaerobically with 50 mM glucose as a carbon source and ammonia as a nitrogen source, with the indicated additions (10 mM with nitrate or nitrite), to the mid-exponential growth phase as outlined in detail before (8, 20).

[b] β-Galactosidase activities were assayed as described before (16, 20). The results represent averages from of at least five independent experiments performed in triplicate, with a standard error of less than 10%.

Interestingly, a similar Fnr-like binding site is found in the vicinity of the *als* operon transcription starting point (Fig. 4). In order to assess if this sequence is involved in *als* regulation, the P*als-lacZ*Δ*fnr* fusion was constructed. In this fusion only the first three bases downstream of the transcription starting point were kept (Fig. 4). In the resulting strain, BSIP1187, the transcription of the P*als-lacZ*Δ*fnr* fusion was abolished during both aerobiosis and anaerobiosis (Table 4). These results could be interpreted in two ways: either the binding of an essential activator has been abolished or the integrity of the overall promoter structure has been disturbed in this construct. In both cases the effect of an *fnr* gene mutation on *lctEP* and *alsSD* expression contrasts with the results of the deleted-promoter study. In the case of *lctEP*, an *fnr* mutation decreased *lctEP* transcription, while the mutation of the putative Fnr box derepressed *lctEP* transcription. The location of the putative Fnr box with respect to the transcriptional start site of *alsSD* suggested that a protein binding to this site would act as a repressor. However, an *fnr* mutation again led to decreased transcription, and deletion of the putative Fnr box totally abolished transcription.

These results indicate that the Fnr box-like elements of the *lctEP* and *alsSD* promoters do not serve known Fnr-dependent regulatory functions.

Expression of *pta* under anaerobic conditions. The third fermentative locus, *pta*, was investigated for its potential oxy-

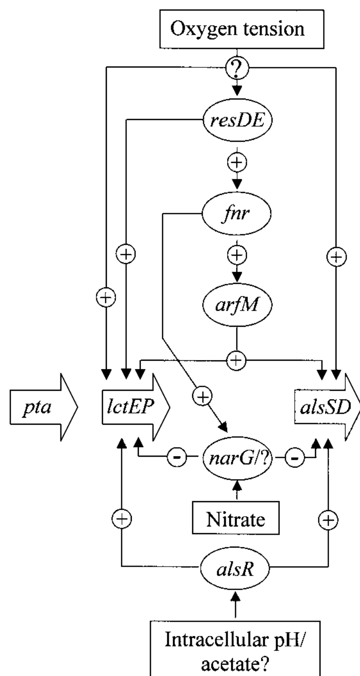

FIG. 5. Regulatory model for the anaerobic expression of the *lctEP*, *alsSD*, and *pta* loci.

gen- or nitrate-dependent control of transcription (Table 5). In agreement with its general aerobic and anaerobic function, no obvious regulation by oxygen tension, nitrate, or nitrite was observed. Mutation of *resDE* and *fnr* did not change *pta* transcription. These data in combination with the observed *pta* growth phenotypes and a recent analysis of anaerobic *pta* function (27) demonstrated the overall importance of acetate formation for aerobic and anaerobic growth.

A model for the regulation of anaerobic fermentation genes in *B. subtilis*. The *lctEP* and *alsSD* operons are an integral part of the anaerobic modulon of *B. subtilis*. Under anaerobic conditions, both are induced via a regulatory cascade from an unknown sensor via ResDE, Fnr, and ArfM (Fig. 5). However, ArfM activation is responsible for only a part of the observed degree of anaerobic *lctEP* and *alsSD* induction. Additional, yet-unknown, redox regulatory components are required for full anaerobic gene expression. A similar regulatory cascade was recently determined for *B. subtilis hemN* and *hemZ* transcription (10).

Nitrate only partially represses *lctEP* and *alsSD* expression. The nitrate regulatory system involved is still unknown. Intact nitrate reductase and lactate dehydrogenase are required to allow nitrate regulation. One could speculate that the redox status of the cell (NAD-to-NADH ratio) or nitrate-dependent membrane-localized electron flow is a signal for this unknown system. Alternatively, an already-known regulatory system responding to the outlined changes in intracellular parameters could indirectly mediate nitrate regulation in *B. subtilis*.

Lactate and 2,3-butanediol formation was clearly detectable in the presence of nitrate and nitrite, indicating the importance of NADH reoxidation even for anaerobic respiratory growth. The obvious absence of a proton translocating NADH dehydrogenase of the Nuo type from the *B. subtilis* genome and the observed strictly aerobic expression of the non-proton-pumping NADH dehydrogenase of the Ndh type explain the observed general anaerobic *lctEP* and *alsSD* expression (13, 17; Marino et al., unpublished observations).

Both operons are additionally subject to AlsR regulation. The postulated signal(s) for AlsR activity is the intracellular pH and/or acetate or derived metabolites (29). The observed rapid anaerobic induction of *alsSD* and *lctEP* could result from the accumulation of acidic compounds like pyruvate and acetate. Further experiments should shed light on the complex regulatory systems involved in the control of anaerobic metabolism of *B. subtilis*.

Finally, acetate formation is a general part of the aerobic and anaerobic *B. subtilis* metabolism (27). In agreement with this observation, *pta* expression is not subject to redox regulation.

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