Characterization of Anaerobic Fermentative Growth of *Bacillus subtilis*: Identification of Fermentation End Products and Genes Required for Growth

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Bacillus subtilis can grow anaerobically by respiration with nitrate as a terminal electron acceptor. In the absence of external electron acceptors, it grows by fermentation. Identification of fermentation products by using *in vivo* nuclear magnetic resonance scans of whole cultures indicated that *B. subtilis* grows by mixed acid-butanediol fermentation but that no formate is produced. An *ace* mutant that lacks pyruvate dehydrogenase (PDH) activity was unable to grow anaerobically and produced hardly any fermentation product. These results suggest that PDH is involved in most or all acetyl coenzyme A production in *B. subtilis* under anaerobic conditions, unlike *Escherichia coli*, which uses pyruvate formate lyase. Nitrate respiration was previously shown to require the ResDE two-component signal transduction system and an anaerobic gene regulator, FNR. Also required are respiratory nitrate reductase, encoded by the *narGHJI* operon, and *moaA*, involved in biosynthesis of a molybdopterin cofactor of nitrate reductase. The *resD* and *resDE* mutations were shown to moderately affect fermentation, but nitrate reductase activity and *fnr* are dispensable for fermentative growth. A search for genes involved in fermentation indicated that *ftsH* is required, and is also needed to a lesser extent for nitrate respiration. These results show that nitrate respiration and fermentation of *B. subtilis* are governed by divergent regulatory pathways.

Recent studies have shown that *Bacillus subtilis*, which had been widely believed to be a strict aerobe, can grow anaerobically in the presence of nitrate (3, 8, 13, 15, 20, 25, 27, 29, 34). Respiratory nitrate reductase encoded by the *narGHJI* operon (3) was shown to be responsible for nitrate respiration (13, 15). Mutations in *moaA* (formerly *narA*), the product of which shows homology to the *Escherichia coli moaA* gene product (26), impair nitrate respiration, probably by conferring a defect in the biosynthesis of the nitrate reductase cofactor (8). Transcription of *narGHJI* and *narK* (required for nitrite extrusion [3]) is induced by oxygen limitation, and the induction is completely abolished by mutations in *fnr*, the second gene of the *narK-fnr* operon (3, 15). FNR is known to be a global anaerobic gene regulator in *E. coli* and has amino acid sequence similarity to the catabolite activator protein (30). In *E. coli*, the activity of FNR, but not the expression of *fnr*, was shown to be stimulated by anaerobiosis. This is believed to be due to the cluster of cysteine residues in the amino terminus of the protein that may play a role in modulating FNR activity by a mechanism involving bound iron (9, 10, 37). Unlike *E. coli*, in which *fnr* expression is weakly repressed by anaerobiosis, *fnr* expression in *B. subtilis* is strongly induced by oxygen limitation (3, 20). Anaerobic induction of *fnr* transcription is controlled at two levels. First, *fnr* transcription at an intergenic *fnr*-specific promoter is activated by oxygen limitation and requires phosphorylated ResD, the production of which depends on a cognate histidine sensor kinase, ResE (20, 34). FNR, thus produced, activates transcription of the *narK-fnr* and *narGHJI* operons probably by binding to the putative FNR binding sites at the *narK* and *narG* operon promoters (3, 20).

In the absence of external electron acceptors, i.e., oxygen (aerobic respiration) or nitrate or nitrite (anaerobic respira-

tion), some facultative organisms such as *E. coli* can grow anaerobically by fermenting sugars. In fermentation, NADH generated by glycolysis cannot be reoxidized by electron transport systems. Instead, NAD⁺ is generated with endogenous electron acceptors produced during metabolism of pyruvate, while ATP is generated by substrate-level phosphorylation, unlike the case of respiration, in which proton potential is used to drive ATP synthesis (2). It has been shown that *B. subtilis* lacks or has a very inefficient glucose fermentation pathway (27, 29). In this paper, however, we report that *B. subtilis* grows anaerobically by fermentation either when both glucose and pyruvate are provided or when glucose and mixtures of amino acids are present. We also show that expression of genes involved in nitrate respiration and fermentation is regulated by distinct regulatory pathways.

MATERIALS AND METHODS

Strains and growth conditions. All *B. subtilis* strains used in this study are derivatives of JH642(*trpC2 pheA1*) (Table 1). Defined medium for anaerobic growth was Spizizen's minimal medium (33) supplemented with 1% glucose or glycerol (the latter was used to examine anaerobic growth by either nitrate or fumarate respiration). Also added were trace element solutions at final concentrations per liter as follows: CaCl₂, 5.5 mg; FeCl₂ · 6H₂O, 13.5 mg; MnCl₂ · 4H₂O, 1.0 mg; ZnCl₂, 1.7 mg; CuCl₂ · 2H₂O, 0.43 mg; CoCl₂ · 6H₂O, 0.6 mg; Na₂MoO₄ · 2H₂O, 0.6 mg; Na₂SeO₄, 0.47 mg. For growth by nitrate respiration, 0.2% KNO₃, 0.2% glutamate, and 50 µg of tryptophan and phenylalanine per ml were added to the medium, or glutamate, tryptophan, and phenylalanine were replaced with a mixture of 20 amino acids (50 µg/ml). For fermentative growth, 0.2% glutamate, 1% sodium pyruvate, tryptophan, and phenylalanine were added, or alternatively, these were replaced with amino acid mixtures. Agar plates were incubated in an anaerobic jar under an H₂-CO₂ atmosphere with a gas-generating system (Becton Dickinson, Cockeysville, Md.). Inocula for liquid anaerobic cultures were prepared by growing cells overnight aerobically in Spizizen's medium supplemented with glucose, glutamate, and auxotrophic requirements. To inoculate media for growth experiments, a sample of the overnight culture was diluted 100-fold in fresh medium containing the various supplements described above (starting absorbance of the culture was between 0.04 and 0.06). Anaerobic growth conditions were maintained by static incubation as described previously (20) after the medium was flushed with N₂ for 1 min. Antibiotics were added

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TABLE 1. *B. subtilis* strains used in this study

Strain	Relevant genotype	Source and/or reference(s)
JH642	<i>trpC2 pheA1</i>	J. A. Hoch
MH5418	<i>trpC2 pheA1 ΔresE::cat</i>	F. M. Hulett; 34
SJB4 (61442)	<i>trp met ace</i>	A. L. Sonenshein; 7
LAB1955	<i>trpC2 pheA1 nar-15</i>	15
LAB2135	<i>trpC2 pheA1 ΔresDE::tet</i>	20, 34
LAB2136	<i>trpC2 pheA1 fnr::spc</i>	3, 20
LAB2234	<i>trpC2 pheA1 ΔresE::spc</i>	This study
LAB2339	<i>trpC2 pheA1 fisH erm</i>	This study
LAB2341	<i>trpC2 pheA1 fisH erm</i>	This study
LAB2408	<i>trpC2 pheA1 ΔnarGH::phleo</i>	This study
LAB2439	<i>trpC2 ace</i>	This study
LAB2506	<i>trpC2 pheA1 resD::cat</i>	This study; 34

when necessary at the following concentrations: 25 µg of ampicillin per ml, 5 µg of chloramphenicol per ml, 75 µg of spectinomycin per ml, 1 µg of erythromycin per ml and 25 µg of lincomycin per ml, 10 µg of tetracycline per ml, and 0.5 µg of phleomycin per ml.

Construction of *fnr* (3, 20), *ΔresE* (34), *resD* (34), and *ΔresDE* (20, 34) mutants was previously described. Plasmid pJL62 (spectinomycin resistant) (16) was used to replace a chloramphenicol resistance (*Cm*^r) gene inserted in *resE* in the MH5418 strain with a spectinomycin resistance (*Spc*^r) gene as follows. MH5418 (*ΔresE*) cells were transformed with linearized pJL62, and the LAB2234 strain was obtained among *Spc*^r *Cm*^r transformants. A *ΔnarGH1* (LAB2408) strain was constructed by the following procedure. Strain LAB1955, which has a plasmid (erythromycin resistant) integration mutation in *narG* was previously isolated (15). Chromosomal DNA prepared from LAB1955 cells was digested with *Bam*HI or *Hind*III, the recognition sites of which flank the *narG* insert. The digests were ligated at a low DNA concentration to allow self-ligation and were used to transform *E. coli* AG1574. pMMN248 was generated from the *Hind*III-cleaved LAB1955 DNA and carries the *B. subtilis* DNA containing a part of *narG* and a downstream region including the entire *narHII* genes. Plasmid pMMN249 was created from the *Bam*HI-digested DNA and contains a part of *narG*, the entire *fnr* gene, and most of the *narK* region located upstream of the *narGHII* operon. By ligating the larger *Hind*III-*Hpa*I fragment of pMMN248 and the smaller *Hind*III-*Hpa*I fragment of pMMN249, we constructed pMMN293, in which 4,066 bp of DNA containing most of *narG* and *narH* was deleted. A phleomycin resistance (*Phleo*^r) marker was inserted in the *Hpa*I site of pMMN293, resulting in the replacement of the *narGH* fragment with the *Phleo*^r marker. The resultant plasmid, pMMN295, was used to transform JH642 with selection for phleomycin resistance followed by a screen for erythromycin sensitivity (the latter ensured that the double-crossover recombination event occurred after transformation). The resulting *ΔnarGH* transformant was designated LAB2408. LAB2439 carrying the *ace* (acetate requirement) mutation was constructed by conjugation. JH642 was transformed with chromosomal DNA prepared from SJB4 with selection for Phe⁺. An acetate-requiring congressant was selected and designated *ace* mutant strain LAB2439. Potassium acetate (0.2%) was added to culture media for growth of LAB2439.

Isolation of *fisH* mutants. *fisH* mutants were identified as being defective in anaerobic growth by fermentation. These were isolated by transformation of *B. subtilis* JH642 cells with two plasmid libraries (28) carrying *B. subtilis* chromosomal DNA as described previously (15, 31). The libraries contain *Alu*I or *Hpa*I fragments (around 0.45 kb) inserted into the *Eco*RV site of pPS34 (erythromycin resistance plasmid; a derivative of pBluescript SK⁺ [Stratagene, La Jolla, Calif.]). Transformants were replica plated to duplicate Luria-Bertani glucose plates supplemented with erythromycin-lincomycin and 0.5% sodium pyruvate. One set of the duplicate plates was incubated aerobically, and the other set was incubated anaerobically. Colonies that did not grow anaerobically were chosen as the candidates defective in fermentative growth. In order to confirm that the defect in anaerobic growth was due to the plasmid integration, JH642 was retransformed with the chromosomal DNA of the candidates. Chromosomal DNA isolated from the mutants obtained by the retransformation was digested with restriction enzymes (*Eco*RI, *Bam*HI, *Hind*III, or *Pst*I), whose recognition sites were located on either side of the original cloned inserts, to isolate flanking chromosomal DNA sequences. The restriction enzyme digests were ligated at a low DNA concentration to allow self-ligation, and the ligation mixtures were used to transform *E. coli* AG1574 to isolate plasmids carrying the *B. subtilis* DNA. These plasmids were used for double-stranded DNA sequence analysis by using reverse or T7 primers that hybridize to DNA located adjacent to the inserts.

Observation of fermentation products by NMR. We monitored the synthesis of fermentation products by performing *in vivo* nuclear magnetic resonance (NMR) scans of whole cultures (1, 22, 23). *B. subtilis* cells were grown aerobically in E medium (per liter; 0.2 g of MgSO₄ · 7H₂O, 2 g of citric acid, 13.1 g of K₂HPO₄, 3.5 g of NaNH₄PO₄ · 4H₂O, pH 7.0) containing 1% glucose, 1% sodium pyru-

vate, 0.2% glutamate, trace element solutions, and the auxotrophic requirement. The *ace* mutant was grown in the medium supplemented with 0.2% potassium acetate. When the cell density reached 5 × 10⁸/ml, the cells were collected by centrifugation at 5,000 × g for 2 min at 4°C. The cell pellets were resuspended in M9 salts (per liter; 12.8 g of Na₂HPO₄ · 7H₂O, 3.0 g of KH₂PO₄, 0.5 g of NaCl, 1.0 g of NH₄Cl) supplemented with 1% glucose and 1% sodium pyruvate. A 0.9-ml cell suspension was placed in a 5-mm-diameter NMR tube with 0.1 ml of D₂O, and the suspension was then bubbled with argon gas to remove oxygen. The cell suspension was incubated at 37°C for 4 h, after which proton NMR spectra were measured with a Varian VXR-500 spectrometer operating at 500 MHz. The parameters used were the following: temperature, 37°C; pulse width, 6 ms (30°); delay time, 3 s; 16 acquisitions per spectrum. The water peak was suppressed by irradiation at high power during the preacquisition delay time. The field was locked onto the solvent D₂O, and internal H₂O was used as a reference peak (4.8 ppm). Authentic fermentation product samples (acetate, acetoin, 2,3-butanediol, ethanol, lactate, pyruvate, and succinate) were also used to assign the NMR signals of the metabolites.

RESULTS

***B. subtilis* grows anaerobically under two different culture conditions.** Recent studies showed that *B. subtilis* grows anaerobically with nitrate as a terminal electron acceptor (3, 8, 13, 15, 20, 25, 27, 29, 34). In previous studies, six-carbon sources such as glucose (3, 29), sorbitol (29), or glucuronate (29) were used to examine anaerobic growth of *B. subtilis* in minimal media in the presence of nitrate. In order to determine whether nitrate utilization in *B. subtilis* is truly respiratory and is coupled to energy production, we examined anaerobic growth on a nonfermentable carbon source. JH642 cells were anaerobically grown in Spizizen's minimal medium supplemented with trace element solutions, 1% glycerol, 0.2% glutamate, 0.2% nitrate, and auxotrophic requirements (tryptophan and phenylalanine) (Fig. 1A). When the cells were grown in the minimal medium with glycerol in the absence of nitrate, no anaerobic growth was observed after the *A*₆₀₀ doubled and the cells began to lyse. The cells grew well when the glycerol medium was supplemented with nitrate but not when it was supplemented with fumarate. The doubling time (3.8 h) in glycerol-nitrate medium was longer than that (2.2 h) observed with the glucose-nitrate medium. Therefore, we concluded that *B. subtilis* is able to grow by nitrate respiration but not by fumarate respiration.

In the absence of external electron acceptors, some bacteria such as *E. coli* can grow anaerobically by fermentation. *B. subtilis* was reported not to ferment glucose under anaerobic conditions (27, 29). As shown in Fig. 1A, JH642 cells grew at a reduced rate in the minimal medium with glucose until *A*₆₀₀ = 0.2 to 0.3 and no increase in absorbance was observed thereafter, confirming that *B. subtilis* has a very inefficient, if any, glucose fermentation system. However, *B. subtilis* did grow anaerobically with a doubling time of 2.8 h when both glucose and pyruvate were supplied (Fig. 1A). The maximal cell yield in fermentation (*A*₆₀₀ = 0.7 to 0.8) was attained only upon a longer incubation than that needed in nitrate respiration.

Figure 1B shows the anaerobic growth curve of JH642 grown in Spizizen's medium supplemented with trace element solutions and a mixture of 20 amino acids. The growth curves were generally similar to those in Fig. 1A. However, in contrast to the results shown in Fig. 1A, glucose alone supported anaerobic growth and addition of pyruvate showed no significant effect, indicating that amino acids can substitute for pyruvate in a medium supporting fermentative growth. The fermentative growth rate (doubling time, 1.6 h) in this medium was higher than the rate in glucose-pyruvate (2.8 h). However, the maximum yields of the two cultures were almost identical. These results demonstrate that *B. subtilis* grows anaerobically, first by nitrate respiration and second by fermentation, which requires both glucose and pyruvate (or amino acid mixtures).

FIG. 1. Anaerobic growth of *B. subtilis* JH642 cultured in Spizizen's minimal medium (33) with trace element solutions, 0.2% glutamate, and 50 μ g of tryptophan and phenylalanine per ml (A) or with trace element solutions and 50 μ g of 20-amino-acid mixtures per ml (B). Each medium was supplemented with 1% glucose (○), 1% glycerol (□), 1% glucose and 0.2% KNO_3 (△), 1% glycerol and 0.2% KNO_3 (●), 1% glucose and 1% pyruvate (■), or 1% glycerol and 0.2% fumarate (▲). OD600, optical density at 600 nm.

NMR analysis of fermentation products. In an attempt to elucidate the fermentation pathway utilized by *B. subtilis*, fermentation end products were analyzed by *in vivo* NMR. The wild-type strain, JH642, was incubated anaerobically in M9 salts with glucose and pyruvate in an NMR tube and scanned for the presence of fermentation products as described in Materials and Methods. Fermentation products produced in the wild-type strain include acetate, ethanol, and lactate, as shown in Fig. 2A. The two peaks at 2.43 and 1.55 ppm were from pyruvate which was present in the medium; its net change before and after fermentation was unknown. Since control samples of pyruvate and succinate gave signals which were very close in chemical shift (within 0.03 ppm), we cannot exclude

the possibility that the peak at 2.43 ppm may contain succinate as well as pyruvate. A peak at 2.28 ppm and a doublet at 1.45 ppm are those of acetoin, although another possibility is that the latter peak was derived from alanine. Since the two peaks increase (or decrease) in parallel, it is more probable that the two peaks were due to one product, i.e., acetoin. In some experiments, a small peak of 2,3-butanediol at 1.15 ppm was also observed (data not shown). The pattern of the fermentative end products of *B. subtilis* is similar to that observed for *E. coli* (1) or *Bacillus licheniformis* (29), although 2,3-butanediol and acetoin are not major products of *E. coli* fermentation and no formate peak of around 8 ppm was detected for *B. subtilis* (data not shown). The possibility that formate is converted quickly to CO_2 and H_2 is unlikely since no gas production was observed. These results indicate that *B. subtilis* performs mixed acid-butanediol fermentation as shown in Fig. 3 and that pyruvate is probably not metabolized by pyruvate formate lyase (PFL), which produces acetyl coenzyme A (CoA) and formate, but oxidatively decarboxylated by pyruvate dehydrogenase (PDH) under anaerobic conditions. Involvement of PDH in pyruvate metabolism was shown in an anaerobic chemostat culture of *Enterococcus faecalis* at low pH (32). In an attempt to determine if pyruvate is a substrate for PDH during fermentation in *B. subtilis*, fermentation products were analyzed in an *ace* mutant (7). The *ace* mutant was shown to lack PDH activity due to an inactive E1 component having reduced affinity for the E2E3 subcomplex (12). The *ace* mutant (LAB2439) was precultured aerobically in the presence of 0.2% potassium acetate, and the cells were resuspended in M9 salts with glucose and pyruvate and incubated in an NMR tube to detect fermentation products. The *ace* mutant produced hardly any fermentation products as only the pyruvate originally present in the medium was detected (Fig. 2B). This result showed that pyruvate is converted to acetyl-CoA mostly (if not exclusively) by PDH in the fermentative growth of *B. subtilis*.

Effect of mutations on nitrate respiration and fermentation.

Previous studies have identified various genes required for nitrate respiration. These include genes for respiratory nitrate reductase activity (*narGHJ* for the enzyme and *moaA* for the cofactor) and regulatory genes, *fnr* and *resD-resE*, which are indispensable for expression of some anaerobically induced genes. Since we showed above that *B. subtilis* has an alternative mode of anaerobic metabolism, fermentation, we were interested to see if the genes required for nitrate respiration also have an essential role in fermentation. Figure 4A shows that *fnr*, *resD*, *resE*, and *narGH* are required for growth by nitrate respiration, although *resE* showed a slightly leaky phenotype with respect to anaerobic growth, as reported previously (34). Among these mutations, only *resD* and *resDE* mutants showed moderate defects in fermentative growth (Fig. 5A). Cells of strain LAB2439, bearing the *ace* mutation, showed poor growth in either the presence or the absence of acetate, both by nitrate respiration (Fig. 4B) and by fermentation (Fig. 5B), suggesting that PDH is essential in both respiratory and fermentative anaerobic growth.

The effect of the various mutations on anaerobiosis was also determined by examining growth on agar plates. The number of CFU of JH642 on a glucose-nitrate or glucose-pyruvate plate was approximately 80% of that under aerobic conditions. *resD*, *resE*, *resDE*, *fnr*, and *narGH* mutants were not able to form colonies under anaerobic conditions on plates supplemented with nitrate as previously shown. Although the *resE* mutant was unable to grow anaerobically from a single cell to form a colony, as in the case of the *resD* and *resDE* mutants, it exhibited slight growth when it was streaked on the agar plate, unlike the *resD* and *resDE* mutants, which did not grow at all.

FIG. 2. NMR of fermentation products. *B. subtilis* JH642 (wild type) (A) and LAB2439 (*ace*) (B) were incubated anaerobically in M9 salts with glucose and pyruvate. NMR scans of fermentation products were run as described in Materials and Methods. Abbreviations: A, acetate; An, acetoin; E, ethanol; L, lactate; P, pyruvate.

This is in good agreement with the result shown in Fig. 4A, in which the *resE* mutation had a less drastic effect on anaerobic liquid growth than did the *resD* mutation. The *fnr* and *narGH* mutants exhibited slightly higher values for CFU when grown anaerobically on plates containing glucose and pyruvate than when grown on the corresponding aerobic plates. CFU of anaerobically grown *resE*, *resD*, and *resDE* mutants on the glucose-pyruvate plates were around 0.4, 0.04, and 0.01% of the corresponding aerobic cultures, respectively. These results confirm that *fnr* and nitrate reductase activity are not required

for fermentation and that the ResD-ResE two-component regulatory system is required for full expression of the fermentation pathway.

Isolation of *ftsH* mutants which are defective in nitrate respiration and fermentation. The results described in the preceding section indicate that there are distinct regulatory pathways for gene expression required for nitrate respiration and fermentation, although both of them require the ResD-ResE two-component signal transduction system. In hopes of elucidating the regulatory pathway controlling fermentation, we sought to isolate additional mutants which are defective in fermentation, as described in Materials and Methods. Two such mutants were found to have a plasmid integration in the *ftsH* gene, as determined by DNA sequence analysis. One of the *ftsH* mutants (LAB2339) had undergone an insertion mutation caused by a Campbell-type integration of a plasmid carrying a 720-bp *AluI* fragment from the *ftsH* gene (nucleotides 1013 to 1734 from the beginning of the coding sequence), and the other (LAB2341) bears an integration of a plasmid carrying a 255-bp *HpaI* fragment (nucleotides 956 to 1210). As a result, LAB2339 and LAB2341 potentially produce truncated FtsH proteins of 578 and 403 amino acids, respectively (the intact *ftsH* gene encodes a protein of 637 amino acids). Both LAB2339 and LAB2341 did not grow anaerobically by nitrate respiration (Fig. 4B) or by fermentation (Fig. 5B). However, the defect in nitrate respiration is less severe than in fermentation as judged by growth phenotype, particularly on agar plates. The *ftsH* mutants could not form colonies anaerobically on the glucose-pyruvate plates, but they showed a leaky phe-

FIG. 3. Fermentation pathways of *B. subtilis*. The pathways are deduced from identification of the end products (shown by boldface). Production of succinate could not be confirmed with certainty as described in the text. Enzyme abbreviations: ACK, acetate kinase; ADH, alcohol dehydrogenase; ALDC, acetolactate decarboxylase; ALDH, aldehyde dehydrogenase; ALS, acetolactate synthase; AR, acetoin reductase; FRD, fumarate reductase; FUM, fumarase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; PDH, pyruvate dehydrogenase; PTA, phosphotransacetylase; PYC, pyruvate carboxylase.

FIG. 4. Anaerobic growth of wild-type and mutant strains by nitrate respiration. Cells were grown anaerobically in Spizizen's medium supplemented with trace element solutions, 1% glucose, 0.2% glutamate, 0.2% KNO_3 , and 50 μg of tryptophan and phenylalanine per ml. (A) $\circ$, JH642 (wild type); $\blacksquare$, LAB2135 (ΔresDE); $\square$, LAB2136 (*fnr*); $\bullet$, LAB2234 (ΔresE); $\triangle$, LAB2408 (ΔnarGH); $\blacktriangle$, LAB2506 (*resD*). (B) $\circ$, JH642 (wild type); $\bullet$, LAB2339 (*ftsH*); $\square$, LAB2341 (*ftsH*); $\triangle$, LAB2439 (*ace*). OD600, optical density at 600 nm.

notype when grown anaerobically on glucose-nitrate plates (number of CFU under anaerobic conditions was around 1% of that in aerobic culture).

DISCUSSION

Previous studies showed that *B. subtilis* grows anaerobically in the presence of nitrate (3, 8, 13, 15, 20, 25, 27, 29, 34). The work presented in this paper has shown that the anaerobic growth with nitrate is truly nitrate respiration. We also showed that *B. subtilis* is able to grow anaerobically by fermentation of glucose, which is stimulated by pyruvate, in contrast to *E. coli*, which can ferment either glucose or pyruvate (24). Since pyruvate is produced by fermentation of glucose, the stimulatory effect of pyruvate is puzzling. In the presence of glucose but not of pyruvate, *B. subtilis* cells were unable to grow well, but

FIG. 5. Anaerobic growth of wild-type and mutant strains by fermentation. Cells were grown anaerobically in Spizizen's medium supplemented with trace element solutions, 1% glucose, 1% pyruvate, 0.2% glutamate, and 50 μg of tryptophan and phenylalanine per ml. (A) $\circ$, JH642 (wild type); $\blacksquare$, LAB2135 (ΔresDE); $\square$, LAB2136 (*fnr*); $\bullet$, LAB2234 (ΔresE); $\triangle$, LAB2408 (ΔnarGH); $\blacktriangle$, LAB2506 (*resD*). (B) $\circ$, JH642 (wild type); $\bullet$, LAB2339 (*ftsH*); $\square$, LAB2341 (*ftsH*); ∇ , LAB2439 (*ace*). OD600, optical density at 600 nm.

the same fermentation products identified in the presence of glucose and pyruvate were observed in much lower amounts (data not shown). One possibility is that the amount of pyruvate accumulated by glycolysis is not sufficient to induce expression of genes involved in pyruvate catabolism (substrate induction) or to activate some enzyme activity. In this case, amino acids can also stimulate fermentation since some amino acids generate pyruvate. Conversely, excess pyruvate could function as a source of certain amino acids, the synthesis of which may be inefficient in fermentation due to the higher demand of pyruvate.

In an attempt to characterize the fermentation pathway in *B. subtilis*, fermentation end products were analyzed by NMR. A

previous study identified acetate as the major product, with small amounts of lactate and succinate from the culture supernatant of *B. subtilis* grown by nitrate respiration (29). Our result indicates that *B. subtilis* undergoes mixed acid-butenediol fermentation; the pathway is shown in Fig. 3. In the fermentation, metabolism of pyruvate to fermentative end products regenerates NAD^+ from NADH. Pyruvate is converted to lactate by lactate dehydrogenase and to oxaloacetate by pyruvate carboxylase (6), which is converted successively to malate, fumarate, and succinate, although production of succinate in *B. subtilis* was not clearly evident from the result of the NMR scan (Fig. 2). Pyruvate is also metabolized to acetoin and 2,3-butanediol by the steps shown in Fig. 3. Each conversion described above contributes to reoxidation of NADH. Finally, pyruvate is converted to acetyl-CoA, which is further metabolized to acetate and ethanol; the former reaction is accompanied by the synthesis of ATP, and the latter involves recycling NADH. The conversion of pyruvate to acetyl-CoA is catalyzed in aerobic cultures by the PDH complex, which produces NADH. Studies with *E. coli* showed that synthesis of the PDH complex is repressed under anaerobic conditions, and residual PDH activity is assumed to be inhibited by NADH (11). Instead, this reaction is replaced by that which is catalyzed by PFL (2, 17). The shift from PDH to PFL is favorable for cells grown under fermentation conditions, in which NADH generated during glycolysis is not reoxidized by functional respiratory chains linked to oxygen and alternative electron acceptors. However, in *Enterococcus faecalis*, PDH was shown to be active at low pH when pyruvate was used as the energy source during anaerobiosis (32). Two observations presented here show that PDH is likely to catalyze the conversion of pyruvate to acetyl-CoA in fermentation. First, the *ace* mutant defective in PDH activity was impaired in fermentative growth. Second, almost no acetate and ethanol were produced by fermentation of the *ace* mutant.

Evidence indicating a role for PDH in nitrate respiration in *B. subtilis* was also provided by the observed defect of the *ace* mutant in nitrate-dependent anaerobic growth. In *E. coli*, either PFL or PDH can be used to catabolize pyruvate in nitrate respiration, since growth of either mutant which lacks PFL or PDH activity was indistinguishable from that of the wild type under nitrate respiratory conditions (14). Further studies of gene expression of PDH as well as examination of the enzyme activity present under fermentative conditions will shed light on the role of pyruvate in anaerobiosis as it occurs in *B. subtilis*.

As has been shown in studies of *E. coli*, bacteria sense environmental changes such as the availability of oxygen and alternative electron acceptors and then respond by switching their regulatory mechanisms to ensure that the most energetically favorable processes are active under a given environmental condition. For example, oxygen is preferred to nitrate and nitrate is favored over fumarate, which is preferred to endogenously generated electron acceptors. The results presented in this paper indicate that genes required for fermentation in *B. subtilis* are controlled by a regulatory pathway distinct from that governing nitrate respiration.

The search for genes involved in fermentation identified *ftsH* as a gene required for both fermentation and nitrate respiration. The *ftsH* gene, essential for cell viability in *E. coli*, encodes an integral membrane protein with a putative ATP binding domain and an amino acid sequence resembling an active-site motif of zinc metalloproteases (35, 36). The *B. subtilis ftsH* homolog was first identified by the genome sequencing project (21), and the gene was later shown to be essential for survival under high osmolarity (5). Recent reports also showed that *ftsH* is required for survival after heat treatment, for entry

into the sporulation pathway, and for secretion of exoproteins (4, 18). We also found that our *ftsH* mutants showed defects in sporulation (the sporulation frequency was 10^{-5} of that of the wild-type strain) and competence development (the transformation frequency is 10^{-3} of that of the wild-type parent). Lysenko et al. reported that their *ftsH* mutant did not grow in glucose minimal medium at all (18). Our mutants grow aerobically in Spizizen's minimal medium with glucose and glutamate, although the doubling time (1.4 h) is slightly longer than that (1.1 h) of the parent strain. Reasons for the discrepancy in the aerobic growth observed for the two mutants are unknown at present. The truncated protein likely to be produced in LAB2339 has a zinc binding motif, but this sequence is absent in the FtsH protein produced by cells of LAB2341. However, both mutants are viable and exhibit the same mutant phenotype, including the defect in anaerobiosis. This may suggest that the defect in diverse cellular functions of these mutants is due to loss of function other than the proteolytic activity of FtsH. Alternatively, LAB2339 may lack FtsH-catalyzed proteolytic activity due to structural changes within the protein. Suppressor mutations of *ftsH* have been frequently isolated, and one of the suppressor mutants restored all of the defects caused by the *ftsH* mutation (19). Characterization of the suppressor mutants may provide insight as to how *ftsH* plays a role in multiple cellular functions, including anaerobiosis in *B. subtilis*.

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