Lactate Dehydrogenase A Promotes Communication between Carbohydrate Catabolism and Virulence in *Bacillus cereus*^{∇†}

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The diarrheal potential of a *Bacillus cereus* strain is essentially dictated by the amount of secreted nonhemolytic enterotoxin (Nhe). Expression of genes encoding Nhe is regulated by several factors, including the metabolic state of the cells. To identify metabolic sensors that could promote communication between central metabolism and *nhe* expression, we compared four strains of the *B. cereus* group in terms of metabolic and *nhe* expression capacities. We performed growth performance measurements, metabolite analysis, and mRNA measurements of strains F4430/73, F4810/72, F837/76, and PA cultured under anoxic and fully oxic conditions. The results showed that expression levels of *nhe* and *ldhA*, which encodes lactate dehydrogenase A (LdhA), were correlated in both aerobically and anaerobically grown cells. We examined the role of LdhA in the F4430/73 strain by constructing an *ldhA* mutant. The *ldhA* mutation was more deleterious to anaerobically grown cells than to aerobically grown cells, causing growth limitation and strong deregulation of key fermentative genes. More importantly, the *ldhA* mutation downregulated enterotoxin gene expression under both anaerobiosis and aerobiosis, with a more pronounced effect under anaerobiosis. Therefore, LdhA was found to exert a major control on both fermentative growth and enterotoxin expression, and it is concluded that there is a direct link between fermentative metabolism and virulence in *B. cereus*. The data presented also provide evidence that LdhA-dependent regulation of enterotoxin gene expression is oxygen independent. This study is the first report to describe a role of a fermentative enzyme in virulence in *B. cereus*.

Bacillus cereus strains express various putative virulence genes, including genes encoding hemolysins, various enterotoxins, phospholipases, metalloproteases, collagenases, and other proteins (7, 38). The contribution of these putative virulence factors to the pathogenicity of *B. cereus* strains has not been fully elucidated. However, the amount of secreted non-hemolytic enterotoxin (Nhe) appeared to essentially dictate *B. cereus*-associated cytotoxic activity (29). Nhe is a three-component enterotoxin encoded by the PlcR-regulated *nheABC* operon (*nhe*) (1, 16, 26). In *in vitro* culture, *nhe* transcription is responsive to a number of cues linked to carbohydrate catabolism, including carbon source concentration, carbon source type, extracellular oxidation-reduction potential, and growth phase (10, 28, 31, 42), which suggests that there are mechanisms to promote communication between carbohydrate catabolism and the *nhe* regulatory network. Carbohydrates such as glucose are catabolized primarily through the glycolytic pathway and to a lesser extent through the pentose phosphate pathway in growing *B. cereus* cells (42). Glycolysis produces two molecules of pyruvate per molecule of glucose consumed and in the process reduces two molecules of NAD⁺ to NADH. The cat-

abolic fate of pyruvate is determined by growth conditions, specifically oxygen availability. During fermentative anaerobic growth, pyruvate or acetyl coenzyme A (acetyl-CoA) derived from pyruvate serves as an electron acceptor for the maintenance of redox balance and as the substrate for generating ATP and acetate.

The enzymes responsible for redox balance in anoxic *B. cereus* cells are lactate dehydrogenase (Ldh), malate dehydrogenase (Mdh), succinate dehydrogenase (Sdh), pyruvate formate lyase (Pfl), aldehyde dehydrogenase (AdhE), and alcohol dehydrogenase (AdhA). The production of acetate from acetyl-CoA is dependent on the phosphotransacetylase-acetate kinase (Pta-Ack) pathway (see Fig. 1A). The main fermentative by-products of *B. cereus* are a mixture of organic acids, such as lactate, succinate, acetate and formate, and ethanol. Lactate is the major product of fermentation and normally accounts for more than 65% of the total produced from glucose by *B. cereus* F4430/73 culture. Acetate and formate are produced in similar amounts (each accounting for 15% of total production), while ethanol and succinate are minor fermentation products (less than 3% and 2% of total by-products produced from glucose, respectively [11, 34]). Producing this mixture of by-products in the appropriate ratio helps maintain redox balance under anoxic conditions while also maximizing ATP yield per glucose molecule to support high growth rates and cell yields. During aerobic growth, the pyruvate dehydrogenase complex (Pdh) connects the glycolytic reactions to the tricarboxylic acid cycle (TCA cycle) by catalyzing the production of acetyl-CoA from pyruvate. The NADH generated during the complete oxidation of glucose is reoxidized to NAD⁺

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by O_2 through the aerobic respiratory chain with accompanying ATP production. Acetate excretion can occur aerobically when carbon flux into the cells exceeds TCA cycle capacity (11, 34). In *B. cereus*, the expression of most of the genes encoding metabolic enzymes is modulated by the two-component ResDE system (11), the one-component Fnr system (43), and the catabolite control protein CcpA (39). These three systems also contribute to virulence. ResDE and Fnr may act as components of transcriptional regulatory complexes (13). Understanding how these regulatory complexes detect metabolic cues is an important step toward understanding how gene regulation contributes to the pathogenic potential of a *B. cereus* strain.

In the present study, we first compared the catabolic capacities of four *B. cereus* strains (F4430/73, F4810/72, F837/76, and PA) grown under anoxic and oxic conditions in relation to their capacities for expressing *nhe*. This led us to study the role of lactate dehydrogenase A (LdhA) as a fermentative enzyme contributing to *nhe* expression regulation. We showed that disruption of *ldhA* affected the fermentative capacity of anaerobically grown F4430/73 cells and the *nhe* expression capacities of both anaerobically and aerobically grown F4430/73 cells. We demonstrated that *ldhA* plays a role in the transcription of several toxin or putative toxin genes under both aerobic and anaerobic growth conditions. These effects were not observed with other *B. cereus* L-lactate dehydrogenase genes (*ldhB* and *ldhC*). We discuss the possible role of LdhA in regulation of enterotoxin gene expression.

MATERIALS AND METHODS

Bacterial strains, mutant construction, and growth conditions. The four *B. cereus* strains used in this study were the food-poisoning strains F4430/73 (37), F4810/72 (AH187) (12), and F837/76 (DSM4222) (20) and the food-borne strain PA (20). *ldhA* and *pfo* deletion mutants were constructed as follows. Two SalI-BglII DNA fragments of 1,309 bp and 1,011 bp encompassing the *ldhA* and *pfo* open reading frames (ORFs), respectively, were amplified by PCR using chromosomal DNA as the template and the primer pairs *ldhA*mutF-*ldhA*mutR and *pfo*mutF-*pfo*mutR, respectively (see Table S1 in the supplemental material). The amplified DNA fragments were digested with the appropriate enzymes and inserted between the corresponding pMAD sites (4), producing pMAD*ldhA* and pMAD*pfo*, respectively. These plasmids were then digested by AflII and BamHI, respectively, and end filled with T4 DNA polymerase. A 1.5-kb SmaI fragment containing the entire spectinomycin resistance gene *spc* (30) was purified from pDIA (a generous gift from I. Martin-Verstraete). This purified DNA fragment was ligated into the digested pMAD*ldhA* and pMAD*pfo* plasmids. The resulting plasmids were introduced into *B. cereus* strains by electroporation, and the *ldhA* and *pfo* genes were deleted by a double-crossover event as described by Arnaud et al. (4). Chromosomal allele exchanges were confirmed by PCR with oligonucleotide primers located upstream and downstream of the DNA regions used for allelic exchange. To complement the *ldhA* gene in *trans*, the 1,767-bp *ldhA* locus and the 2,748-bp *ldhA-pfo* locus were first PCR amplified using the primer pairs *ldhA1*compF-*ldhA1*compR and *ldhA2*compF-*ldhA2*compR, respectively (see Table S1 in the supplemental material) and then cloned into the pCRXL-TOPO plasmid (Invitrogen). The PCR fragments were then cut with HindIII and PstI and ligated to similarly digested pHT304 (3). The integrity of the inserts of the recombinant vectors (pHT304*ldhA* and pHT304*ldhApfo*) was verified by sequencing, and the vectors were then used to transform the *B. cereus* mutant strain.

The *ldhB* and *ldhC* mutants were also constructed using pMAD as described above for *ldhA* and *pfo*, with the following modifications. The *ldhB* gene was PCR amplified using oligonucleotide pair *ldhB*mutF-*ldhB*mutR (Table S1) and then cloned into pMAD using BamHI and SalI. The recombinant vector was then digested with Csp45I and ligated with the 1.5-kb *spc*-containing fragment to obtain the vector used for *ldhB* replacement. The *ldhC* gene was PCR amplified using oligonucleotide pair *ldhC*mutF-*ldhC*mutR (Table S1) and then cloned into pUC18 by using PstI and BamHI. The pUC18*ldhC* plasmid was then digested

with SnaBI and ligated with the 1.5-kb *spc*-containing SmaI fragment. The NcoI-BglII fragment containing the disrupted *ldhC* gene was then isolated and cloned into pMAD.

Wild-type strains F4430/73, F4810/72, F837/76, and PA and *ldhA* and *pfo* mutants were grown in synthetic MOD medium supplemented with 30 mM glucose as a carbon source (34). A 2-liter bioreactor (Discovery 100; Incubatech, Toulouse, France) was used for all cultivations, and the working volume was maintained at 1.3 liters. The temperature was maintained at 37°C, and the pH was kept at a controlled 7.2 by automatic addition of 5 M KOH. The regulated batch was equipped with a Mettler Toledo polarographic oxygen electrode coupled with feedback regulation to maintain the set point dissolved oxygen tension value (pO_2) using air sparging and agitation speed (10). Sparging the bioreactor with air alone set a pO_2 value of 100%. A pO_2 of 0% was obtained by continuously flushing the medium at 20 ml h^{-1} with pure N_2 gas previously passed through a Hungate column. Each bioreactor was inoculated with a subculture grown overnight under anaerobiosis in such a way that the initial optical density at 560 nm (OD_{560}) was equal to 0.02. For *ldhA* mutant complementation assays and analyses of *ldhB* and *ldhC* mutant F4430/73 strains, uncontrolled aerobic and anaerobic cultures were performed as previously described (34).

Analytical procedures and physiological parameters. *B. cereus* growth was monitored spectrophotometrically at 560 nm and calibrated against cell dry weight measurements as previously described (10). The specific growth rate (μ) was determined using the modified Gompertz equation (18, 44). For substrate, by-product, and Nhe measurements, a 4-ml sample was centrifuged at 10,000 $\times g$ for 5 min at 4°C and the supernatants were frozen at -80°C until analysis. Glucose, lactate, ethanol, formate, acetate, and succinate concentrations were determined using commercial enzymatic kits (from Diffranch, Lyon, France, from R-Biopharm, Saint-Didier au Mont-d'Or, France, and from Roche, Meylan, France). The specific glucose consumption rate, defined as the differential change in glucose concentration with time, was calculated from the equation $q_{\text{glucose}} = \mu/Y_x$, where μ is the specific growth rate (h^{-1}) and Y_x is the biomass yield ($g \cdot mol \text{ carbon substrate}^{-1}$). Amounts of Nhe were estimated by measuring the optical density at 420 nm (one unit was defined as one OD unit at 420 nm) as previously described (42). Specific Nhe production was defined as the amount of Nhe produced per gram of cell dry mass ($U \cdot g^{-1}$) or per OD_{560} unit ($U \cdot OD^{-1}$).

Enzyme assay. Lactate dehydrogenase activity was assayed with pyruvate on purified enzymes. The assay mixture (1 ml) contained phosphate buffer at pH 6.6, 0.3 mM NADH, 3 mM fructose 1,6-diphosphate, 5 mM $MgCl_2$, and 5 to 10 μl of purified protein (10 $\mu g/\mu l$). Sodium pyruvate (20 mM) was added to start the reaction. NADH oxidation was followed by the decrease in absorbance at 340 nm. One unit of lactate dehydrogenase activity is 1 A_{340} unit per min.

In vitro lactate dehydrogenase synthesis. Proteins were synthesized by using the rapid-translation system RTS 500 *E. coli* HY kit (Roche Diagnostics) according to the manufacturer's instructions. Template DNA fragments were prepared using a two-step PCR protocol. The first step (PCR 1) was used to obtain specific fragments (primer pairs are listed in Table S1 in the supplemental material). The second step (PCR 2) was used to obtain the products with regulatory elements and His tag at the C terminus. The PCR products were purified by use of a PCR purification kit (Roche). The purified products were sequenced; after the sequence was confirmed to be correct, these products were used for expressing corresponding proteins. The protein products were rapidly purified using a Ni-nitrilotriacetic acid (NTA) column. The expressed protein was confirmed by Western blotting using anti-His antibody as the first antibody.

DNA sequencing and bioinformatics tools. Sequencing was performed by Beckman Coulter Genomics (United Kingdom). Homology and conserved domain searches were performed with the NCBI database by using the BLAST algorithm. Pairwise alignment was carried out using ClustalW software. The presence of transmembrane helices in proteins was predicted using TMpred and TMHMM version 2.0 software via the ExPASy proteomics server.

RNA isolation, operon mapping, and expression analysis. To investigate the transcriptional organization and expression of the *ldhA-pfo* locus, reverse transcription-PCR (RT-PCR) was performed with total RNA isolated from *B. cereus* F4430/73 as previously described (11). Portions of *ldhA*, *pfo*, and the *ldhA-pfo* intergenic region were amplified by RT-PCR using the Titan one-tube RT-PCR system following the manufacturer's protocol (Roche). The primer pairs used for *ldhA*, *pfo*, and the *ldhA-pfo* intergenic region are listed in Table S1 in the supplemental material. To check whether contaminant genomic DNA was present, each sample was tested in a control reaction that did not contain reverse transcriptase.

The 5' end of *ldhA-pfo* mRNA was mapped from a 5' rapid amplification of cDNA ends (RACE) PCR product obtained with the 3'/5' RACE kit (Roche). Briefly, the first-strand cDNA was synthesized from total RNA with the *ldhA*-

TABLE 1. Growth parameters determined from controlled batch cultures (pH 7.2) of strains F4430/73, F4810/72, F837/76, and PA^a

Parameter	Anaerobic growth				Aerobic growth			
	F4430/73	F4810/72	F837/76	PA	F4430/73	F4810/72	F837/76	PA
$\mu_{\max}$ (h ⁻¹)	0.9	0.5	0.5	0.5	1.4	1.3	1.1	1.1
Final biomass (g · liter ⁻¹) ^b	0.8	0.3	0.3	0.3	2.6	2.9	3.7	3.0
Glucose consumption (%) ^b	100	28	42	35	100	100	100	100
Y_{glucose} (g of cells · mol of glucose ⁻¹)	26	34	27	24	86	97	123	100
Maximal specific glucose consumption rate (mmol · mg ⁻¹ · h ⁻¹)	35	6	11	7	16	13	9	11

^a Cells were grown under N₂-generated anaerobiosis (pO₂ = 0%) and full aerobiosis (pO₂ = 100%). Data are the means of duplicate cultures. For clarity, standard deviations (below 10%) are not shown.

^b Final biomass and glucose consumption were determined at the end of growth, i.e., 1.5 h after the OD₅₆₀ reached its maximal value.

pfo-specific SP1 primer (Table S1), avian myeloblastosis virus reverse transcriptase, and the deoxynucleotide mixture of the 3'/5' RACE kit by following the manufacturer's instructions. After purifying and deoxyribosyladenine (dA) tailing the cDNA, PCR with the deoxyribosylthymine (dT) anchor oligonucleotide primer and the *ldhA-pfo*-specific SP2 primer (Table S1) followed by a nested PCR with the SP3 primer (Table S1) led to a PCR product of approximately 140 bp, as revealed by 2% agarose gel electrophoresis. This PCR product was purified and sequenced.

Real-time RT-PCR was performed using SYBR green staining on a Light-Cycler instrument (Roche) as previously described (11). Sequences specifically used in the present study are given in Table S1.

Nucleotide sequence accession number. Given that the *B. cereus* F4430/73 strain belongs to the same genetic group as the ATCC 14579 strain (21), we designed primers (Table S1) corresponding to the ATCC 14579 locus to clone and sequence its homolog from strain F4430/73. The DNA sequence of the 2,748-bp cloned fragment corresponding to the *ldhA-pfo* locus was deposited in GenBank under accession no. HQ336744.

RESULTS

Fermentative and respiratory capacities of strains F4810/72, F837/76, and PA relative to those of strain F4430/73. *B. cereus* strains F4430/73, F4810/72, F837/76, and PA were grown in glucose-limited and pH-regulated batch culture (pH 7.2) under anaerobiosis (pO₂ = 0%; generated under an N₂ atmosphere) and aerobiosis (pO₂ = 100%). Table 1 summarizes the growth features of the four strains. Under anaerobiosis, strains F4810/72, F837/76, and PA grew significantly more slowly and reached the stationary growth phase at a lower final biomass than strain F4430/73. This probably resulted from both lower levels and lower specific rates of glucose uptake. Interestingly, strain F4810/72 exhibited the highest biomass yield on glucose. Under aerobiosis, growth rates for the four strains were similar, although those for strains F837/76 and PA were slightly lower than those for the two other strains. However, final biomass and biomass yields of strains F4810/72, F837/76, and PA were significantly higher than those of strain F4430/73, while the glucose uptake rates for these three strains were lower. Taken together, these results indicate that glucose supports lower glycolytic fluxes (or glucose uptake rates) in strains F4810/72, F837/76, and PA than in strain F4430/73 under both anaerobiosis and aerobiosis, leading to lower fermentative growth and more efficient respiratory growth, respectively.

Figure 1A depicts the main catabolic pathways used by *B. cereus* strains. Glucose was converted predominantly into lactate in all four strains (Fig. 1B). However, F4810/72, F837/76, and PA cells produced smaller amounts of lactate and concomitantly larger amounts of formate, acetate (except for PA), and ethanol than F4430/73 cells. This suggests that the carbon flux from pyruvate to lactate was lower in strains F4810/72,

F837/76, and PA than in strain F4430/73 under anaerobic fermentative conditions and that the carbon flux through pyruvate formate lyase (Pfl) at the pyruvate node and then through the NADH-recycling alcohol dehydrogenase (AdhE-AdhA) pathway at the acetyl-CoA node was higher (Fig. 1A). When oxygen was present, no detectable ethanol, formate, or succinate was produced in any of the four strains. The amounts of secreted lactate in all four strains were similar, while strains F4810/72, F837/76, and PA secreted smaller amounts of acetate than strain F4430/73 (Fig. 1B). Since aerobic acetate generation results from an overflow metabolism in *B. cereus* (34), glucose could support a lower overflow metabolism in strains F4810/72, F837/76, and PA than in strain F4430/73 under aerobic respiratory conditions.

Table 2 shows the catabolic gene expression profiles in strains F4810/72, F837/76, and PA relative to those in strain F4430/73 from cells cultured under fermentative anaerobic and respiratory aerobic growth conditions. The data revealed that strains F4810/72, F837/76, and PA exhibited a pattern of catabolic gene expression very different from that of strain F4430/73. Interestingly, we observed that the mRNA profiles of the three genes predicted to encode L-lactate dehydrogenase (*ldhA*, *ldhB*, and *ldhC*) in strains F4810/72, F837/76, and PA differed significantly from those of these genes in strain F4430/73 in the absence and presence of oxygen. They differed in the following ways: (i) *ldhA* expression was moderately reduced in strain F4810/72 and severely reduced in strains F837/76 and PA under both anaerobiosis and aerobiosis, (ii) *ldhB* expression levels differed between strains and between condition sets, and (iii) *ldhC* expression was inversely correlated to *ldhA* expression ($r = -0.63$, $P < 0.05$) in the three strains F4810/72, F837/76, and PA, whatever the growth conditions. In addition, concomitantly with *ldhA*, *adhE*, which encodes a key enzyme of the ethanol-generating pathway, and *citZ*, which encodes an enzyme that governs carbon entry into the TCA cycle, were significantly downregulated in strains F4810/72, F837/76, and PA compared to strain F4430/73 under both aerobiosis and anaerobiosis.

Nhe secretion capacities of strains F4810/72, F837/76, and PA compared to that of strain F4430/73. Strains F4810/72, F837/76, and PA secreted smaller amounts of Nhe than strain F4430/73 under both anaerobiosis and aerobiosis (Fig. 1C). Among strains F4810/72, F837/76, and PA, F4810/72 was clearly the greatest producer of Nhe (1.1 and 0.4 U · g⁻¹ of dry cells under anaerobiosis and aerobiosis, respectively, com-

FIG. 1. Metabolic responses of *B. cereus* strains F4430/73, F4810/72, F837/76, and PA. (A) Pathways for the excretion of partially oxidized metabolites. The reactions are represented by the names of the corresponding genes, as follows: *ldhA*, *ldhB*, and *ldhC*, L-lactate dehydrogenase; *pfl*, pyruvate formate lyase; *pdh*, pyruvate dehydrogenase; *pta*, phosphotransacetylase; *ack*, acetate kinase; *adhE*, acetaldehyde dehydrogenase; *adhA*, alcohol dehydrogenase; *mdh*, malate dehydrogenase; *sdh*, succinate dehydrogenase; *citB*, aconitase hydratase; *citZ*, citrate synthase. Reactions that normally function during aerobiosis are represented by gray arrows, and reactions that normally function during anaerobiosis are represented by black arrows. CIT, citrate; ICT, isocitrate; AKG, α -ketoglutarate; SUC, succinate; FUM, fumarate; MAL, malate; OAA, oxaloacetate; TCA, tricarboxylic acid cycle. (B) Extracellular metabolite analyses. (C) Specific Nhe production. Error bars indicate the standard errors of the means.

pared to 0.2 and 0.002 for strain F837/76 and 0.5 and 0.01 for strain PA, respectively).

Table 2 shows that *nhe* expression was strongly reduced in strains F4810/72, F837/76, and PA compared to strain F4430/73 under both anaerobiosis and aerobiosis. This may explain why Nhe was secreted less in these strains. Transcript levels of *nhe*, regulatory genes, and genes encoding catabolic enzymes that were ≥ 2 -fold upregulated or downregulated under both anaerobiosis and aerobiosis were subjected to Pearson's *r* correlation analysis. The results indicated that only the *ldhA*, *adhE*, and *citZ* mRNA levels were significantly correlated ($P < 0.05$) with *nhe* levels (r values of +0.62, +0.91, and +0.77, respectively). This suggests a regulatory link between these catabolic genes and *nhe*. To validate such a link, we studied the *ldhA* gene in relation to *nhe* in strain F4430/73.

Analysis of the *ldhA* locus in *B. cereus* F4430/73. As in the case for reference strain ATCC 14579 (GenBank accession no. AE016877), the stop codon of *B. cereus* F4430/73 *ldhA* is located 14 bp upstream of the start codon of an ORF, annotated *pfo* by Ivanova et al. (24), suggesting that these genes are

cooperonic (Fig. 2A). RT-PCR experiments demonstrated that *ldhA* and *pfo* were effectively cotranscribed under both aerobiosis and anaerobiosis (Fig. 2B). A transcriptional start site (G) located 48 bp from the *ldhA* start codon was identified by 5' RACE PCR (Fig. 2C). We identified upstream of this start site a potential σ^A -type -10 sequence, TATAAT, that is not preceded by a typical -35 sequence (TTGACA) (22). A review of the *ldhA* promoter regions of the other *B. cereus* strains confirmed the absence of the typical -35 region. Inspection of the 5' untranslated region (UTR) sequence revealed the presence of a palindromic sequence spanning positions -64 to -41. Each symmetrical arm contains highly conserved potential binding sites for the redox regulators Fnr and ResD (8, 11, 23, 43). No CcpA binding site was found (39). Furthermore, the *ldhA-pfo* locus appeared to be followed by an inverted repeat (change in Gibbs free energy [ΔG], -23.1 kcal/mol) that may be a transcriptional terminator, confirming that *ldhA* and *pfo* could form a transcriptional unit.

Expression of the *B. cereus* *ldhA* gene in *E. coli*. To demonstrate that *B. cereus* *ldhA* encodes lactate dehydrogenase, *ldhA*

TABLE 2. mRNA levels of genes involved in glucose catabolism and enterotoxin synthesis in *B. cereus* strains F4810/72, F837/76, and PA relative to those of strain F4430/73^a

Gene	Difference in mRNA level (<i>n</i> -fold) ^b under the indicated condition					
	Anaerobic growth			Aerobic growth		
	F4810/72	F837/76	PA	F4810/72	F837/76	PA
Glucose catabolism gene						
<i>ldhA</i>	-2.5	-100	-100	-2.0	-100	-100
<i>ldhB</i>	<u>+2.5</u>	<u>+2.0</u>	-5.0	-50	-100	-5.0
<i>ldhC</i>	<u>+3.2</u>	<u>+6.2</u>	<u>+6.5</u>	<u>+2.2</u>	<u>+12.3</u>	<u>+5.4</u>
<i>pfl</i>	-4.0	-1.2	-2.2	-1.4	-3.7	-3.1
<i>pdhA</i>	+1.4	+1.9	+1.6	-1.1	+1.0	+1.0
<i>pta</i>	-5.0	-3.3	-3.8	-2.5	-2.0	+1.5
<i>ackA</i>	-3.3	-2.3	+1.6	-1.7	-1.5	<u>+2.0</u>
<i>adhE</i>	-7.7	-4.8	-7.7	-4.5	-33.3	-3.7
<i>adhA</i>	-1.1	+1.7	+1.7	<u>+2.3</u>	-1.8	+1.1
<i>sdhA</i>	-2.2	+1.0	-2.5	<u>+3.0</u>	<u>+2.9</u>	<u>+2.4</u>
<i>citZ</i>	-100	-100	-16.0	-50.0	-33.0	-2.7
<i>citB</i>	+1.1	-12.5	-10.0	<u>+2.0</u>	+1.5	-2.3
<i>mdh</i>	+1.6	<u>+4.8</u>	<u>+4.9</u>	-1.1	-1.1	+1.2
Enterotoxin gene <i>nhe</i>	-10	-100	-100	-3.1	-500	-14.3
Regulator genes						
<i>plcR</i>	<u>+3.0</u>	+1.4	<u>+4.2</u>	<u>+2.9</u>	+1.1	-1.1
<i>resD</i>	<u>+4.0</u>	<u>+3.2</u>	<u>+5.6</u>	<u>+2.2</u>	+1.9	<u>+2.3</u>
<i>fnr</i>	<u>+6.7</u>	<u>+6.2</u>	<u>+7.5</u>	-1.8	<u>+2.4</u>	+1.5
<i>ccpA</i>	-1.4	-1.1	-2.3	-3.6	-1.7	-1.2

^a *B. cereus* cells were grown under oxic and anoxic conditions in controlled batch cultures (pH 7.2) on MOD medium with 30 mM glucose as the carbon source.

^b Each change (*n*-fold) represents the mean value of the mRNA levels of one strain sample in relation to that of the F4430/73 sample. For each experiment, two measurements from two independent RNA samples taken from the mid-exponential growth phase ($\mu_{\max}$) of the same culture were analyzed in parallel. Each data point is an average of the results of the combined experiments. Only ratios of ≤ -2 and $\geq +2$ were considered significant (i.e., $P \leq 0.05$) according to the precision of the method. + and - indicate up- and downregulation of genes, and significant values for upregulation and downregulation are underlined and boldface, respectively.

was expressed as a His-tagged protein by using a cell-free *Escherichia coli* translation system in the presence of GroES and GroEL chaperone proteins. Figure 3 shows that the LdhA peptide (panel A), like LdhB and LdhC peptides (panel B), was successfully expressed *in vitro*. The purified LdhA protein has a specific Ldh activity of $40 \pm 4 \text{ U} \cdot \text{mg}^{-1}$, using pyruvate as the substrate and NADH as the cosubstrate. In comparison, LdhB and LdhC exhibited specific Ldh activities of $50 \pm 6 \text{ U} \cdot \text{mg}^{-1}$ and $83 \pm 10 \text{ U} \cdot \text{mg}^{-1}$, respectively. Taken together, these results demonstrate that *B. cereus* *ldhA*, like *ldhB* and *ldhC*, encodes functional lactate dehydrogenase.

Primary structure analysis of LdhA and PfoR. Although it displays all the characteristics of the lactate dehydrogenase/malate dehydrogenase (LDH/MDH) superfamily, LdhA shares no more than 50% identity over 316 amino acids with the two other *B. cereus* lactate dehydrogenases, LdhB and LdhC (Fig. 4). Unlike LdhB and LdhC, LdhA is produced from a bicistronic mRNA. The second coding sequence of this mRNA is predicted to produce a transmembrane protein. This protein was proposed to function as a perfringolysin O regulator in strain ATCC 14579 and named PfoR (24). Our analysis of *B. cereus* F4430/73 PfoR failed to detect any significant similarity to this regulator (5). However, we found that PfoR could display the hydrophobic characteristics of a transport protein, although no homology between this protein and known bacterial permeases was found (5, 9).

Physiological characteristics of the *B. cereus* F4430/73 *ldhA* mutant. A *B. cereus* F4430/73 *ldhA* mutant was created by introducing a spectinomycin resistance gene deprived of its

transcription initiation and termination sequence, as described in Materials and Methods. To prove the nonpolar effect of *ldhA* disruption, we also constructed a *pfo* mutant and compared the growth features of the *ldhA* and *pfo* mutants to those of the parental F4430/73 strain under anaerobiosis and aerobiosis. Table 3 shows that neither *ldhA* nor *pfo* disruption significantly affected the parameters of *B. cereus* F4430/73 growth under aerobiosis. However, *ldhA* disruption severely impaired the growth rate of fermentative F4430/73 cells under anaerobiosis, probably due to a lower specific rate of glucose consumption. Analysis of glucose by-product spectra at the end of growth showed only a significant decrease in formate secretion in the *ldhA* mutant strain compared to formate secretion of the parental strain. This lower level of formate generation was also observed for the *pfo* mutant concomitantly with higher-level acetate generation. More importantly, the ethanol-to-acetate ratio of the *ldhA* mutant strain was higher than those of the parental strain and the *pfo* mutant, while NADH recovery levels were similar. This indicates an effective NAD^+ regeneration in spite of greater NADH availability in *ldhA* mutant cells (35). To determine if this greater NADH availability could be due to a defect of lactate secretion at the beginning of the exponential growth phase (corresponding to the maximal specific growth rate [$\mu_{\max}$]), we compared the amounts of lactate produced by the *ldhA* mutant and the parental strain. The results showed that the *ldhA* mutant strain produced less lactate than the parental strain (0.15 versus 0.23 mol/mol of glucose, respectively), indicating a defect in the NADH-recycling lactate pathway. Table 4 shows that *ldhB* expression was

FIG. 2. Operon mapping and expression of the *B. cereus* F4430/73 *ldhA-pfo* operon. (A) Large arrows represent the *ldhA* and *pfo* ORFs. The small arrowheads flag the positions and directions of primers used in RT-PCR. Lengths of expected PCR products are also shown for every primer pair. (B) RT-PCR analyses for detecting *ldhA*, *pfo*, and the intergenic (IG) region between *ldhA* and *pfo* in strain F4430/73. M, molecular mass marker. (C) The nucleotide sequence of the promoter region of F4430/73 *ldhA* is shown. The transcriptional start site (+1) and the putative -10 sequence are in bold and underlined. Putative regulatory sequences are highlighted in gray, the start codon is boxed, and inverted repeat sequences are indicated by arrows.

upregulated by the disruption of *ldhA* under both anaerobiosis and aerobiosis, although upregulation occurred more strongly under anaerobiosis. Based on these data, we hypothesized that overexpression of *ldhB* would be sufficient for maintaining redox balance (NADH/NAD⁺ ratio) and that greater NADH availability rather than ineffective NAD⁺ regeneration limits glycolytic flow and subsequently fermentative growth.

Table 3 shows that *ldhA* disruption, but not *pfo* disruption, significantly reduced Nhe secretion under both aerobiosis and anaerobiosis. Table 4 shows that transcription of *nhe* was strongly downregulated under these two conditions. Taken together, these data indicate that the main control of Nhe production in the *ldhA* mutant was oxygen independent and occurred mainly at the transcriptional level. Interestingly, no significant correlation between the variations in *nhe* transcript levels and those of other tested genes could be highlighted.

Table 4 also shows that *pfo* mRNA levels were not affected in aerobic *ldhA* cells, while *pfo* transcripts were significantly decreased in fermentative anaerobic cells when *ldhA* was disrupted. The same results were obtained with *ldhA-pfo* intergenic region mRNA (data not shown). This highlights the nonpolar effect of spectinomycin resistance cassette insertion and suggests a possible role of *ldhA* in *ldhA-pfo* transcription, i.e., in its self-regulation. Finally, to definitively confirm that the defect in fermentative growth and enterotoxin production was specifically ascribable to the disruption of *ldhA*, wild-type

copies of *ldhA* and *ldhA-pfo* loci were cloned into the pHT304 vector (see Materials and Methods) and each resulting vector was used to complement the *ldhA* mutant strain. Whatever the vector used, *trans*-complementation only partially restored the fermentative growth of the *ldhA* mutant and enabled the mutant to produce 2-fold-higher Nhe levels (data not shown).

Validation of *ldhA* as a specific lactate dehydrogenase gene of pathogenic relevance. To compare the role played by LdhA with the roles of LdhB and LdhC in glucose catabolism and Nhe secretion, we disrupted the *ldhB* and *ldhC* genes in *B. cereus* strain F4430/73 as described in Materials and Methods. The abilities of the mutants to grow and produce Nhe under both anaerobiosis and aerobiosis in uncontrolled batch cultures were assessed. Unlike *ldhA* disruption, *ldhB* and *ldhC* disruptions did not induce fermentative growth deficiency or lower-level Nhe secretion (Table 5). This suggests that LdhA, unlike LdhB and LdhC, could play a major role in both physiology and Nhe production in *B. cereus* F4430/73. Because *ldhA* disruption caused the same effects in strains ATCC 14579 and F837/76 and in strain F4430/73 (data not shown), we cannot rule out the possibility that LdhA could play a major role in several species of the *B. cereus* group.

In addition to expressing the toxin encoded by *nhe*, *B. cereus* expresses several other toxins or putative toxins (7). We determined the effect of *ldhA* disruption on the mRNA levels of these enterotoxin genes. Table 6 reports that all these genes

FIG. 3. Cell-free expression of *ldhA*, *ldhB*, and *ldhC*. Lactate dehydrogenases were expressed as His-tagged proteins by using the RTS 500 *E. coli* HY kit in the presence of GroE supplement. Purified LdhA, LdhB, and LdhC were visualized by Western blotting using anti-His antibodies. (A) Purified LdhA (34.6 kDa). (B) Lane 1, purified LdhB (34.5 kDa); lane 2, purified LdhC (34.7 kDa). The sizes of the molecular mass markers (in kDa) are indicated on the left.

were downregulated in *ldhA* mutant cells, suggesting that *ldhA* should participate in the regulatory network of toxinogenesis in *B. cereus* F4430/73.

DISCUSSION

Our exploration of the physiology and *nhe* expression profile of different *B. cereus* strains revealed that the *ldhA* gene, encoding L-lactate dehydrogenase A (LdhA), is required for *B. cereus* fermentative metabolism and toxinogenesis under both aerobiosis and anaerobiosis. This makes LdhA the first classical catabolic enzyme of both physiological relevance and pathogenic relevance in *B. cereus*.

B. cereus group species contain three high-conserved genes proposed to encode L-lactate dehydrogenase (<http://www.ncbi.nlm.nih.gov/nuccore/29899096>). The results obtained in the current study showed that these three genes (*ldhA*, *ldhB*, and *ldhC*) were responsible for active fermentative lactate dehydrogenase. *ldhA* expression, like *ldhB* and *ldhC* expression, was detectable in both fermenting and respiring *B. cereus* cells according to their ability to secrete lactate under these two conditions, which is in line with the results of our previous

studies (28). Low-level expression of *ldhA* was coupled with high-level expression of *ldhC* in strains F4810/72, F837/76, and PA, whereas disruption of *ldhA* induced *ldhB* overexpression in strain F4430/73 under both anaerobiosis and aerobiosis. This suggests that the regulation network that coordinates the differential expression of *ldhA*, *ldhB*, and *ldhC* in *B. cereus* could function independently of the presence or absence of oxygen in a strain-dependent manner. The existence of strain-dependent *ldh* gene regulation in aerobic respiring cells where lactate dehydrogenase activity is not necessary to maintain redox homeostasis indicates that fine regulation of lactate dehydrogenase synthesis could confer upon *B. cereus* a metabolic advantage when grown under aerobiosis, as is the case for *Staphylococcus aureus* (33). Expression of *ldh* genes is affected by the redox sensors ResDE and Fnr, although direct binding of the regulators and their binding sites have not been elucidated (11, 43). ResD and Fnr were showed to regulate each other and their own synthesis at the transcriptional level (13, 14). Since no correlation between the mRNA levels of these two regulators and those of *ldhA* was observed, we conclude that strain-dependent regulation of *ldh* genes cannot be simply related to ResDE and Fnr.

The importance of LdhA, unlike LdhB and LdhC, in maintaining redox homeostasis in fermentative cells was demonstrated by (i) the inability of low-level *ldhA*-expressing F4810/72, F837/76, and PA cells to sustain rapid anaerobic growth compared to high-level *ldhA*-expressing F4430/73 cells and (ii) the fermentative anaerobic growth defect of the F4430/73 *ldhA* mutant. The importance of LdhA for enterotoxin gene expression in fermentative anaerobic and respiring aerobic *B. cereus* cells was highlighted by (i) the capacity for low-level *ldhA*-expressing F4810/72, F837/76, and PA cells to express *nhe* mRNA being lower than that for high-level *ldhA*-expressing F4430/73 cells and (ii) the downregulation of *nhe* and other putative enterotoxin genes in strain F4430/73 lacking LdhA. A key question is how LdhA impacts enterotoxin gene expression. A detailed analysis of this impact was beyond the scope of this study. However, we speculate that under fermentative conditions, the defect in LdhA synthesis may indirectly downregulate enterotoxin gene expression through its impact on NADH availability and subsequently on redox potential of the cytoplasm and on growth rate (31). Activation of *nhe* expression in fermentative anaerobiosis has been shown to involve ResDE and Fnr regulators (11, 43). These redox regulators activate their own transcription in response to redox potential changes (13, 14). Unlike *nhe* mRNA levels, *resD* and *fnr* mRNA levels did not differ significantly between the *ldhA* mutant strain and the parental strain. This suggests that ResDE and Fnr are not the key regulators of *nhe* expression in *ldhA* mutant cells. This is probably also the case for the autoregulatory PlcR transcriptional regulator (16). In *Bacillus subtilis* (25), as in other Gram-positive bacteria (36), changes in NADH availability are sensed by the transcriptional regulator Rex. A Rex homologue is encoded in the genome of *B. cereus* (www.ncbi.nlm.nih.gov and unpublished data). Interestingly, we found Rex binding motifs (32), overlapping ResD and Fnr binding motifs in *ldhA*, *ldhB* (but not *ldhC*), and *nhe* (data not shown). It remains to be examined whether these sequences are really targets for this redox regulator that could thus regulate directly and simultaneously *nhe* and *ldhA*.

LdhB	MKKGINRVVLVGTGAVGCSYAYCMINQAVAEFFVLVDVNEAKAEGEAMD	50
LdhC	MKKGINRVVLVGTGAVGCSYAYSMINQGVAEFFVLVDVNEAKAEGEAMD	50
LdhA	MKRHRKIALITGTGLVSSCAYSIVNOGICEELLIDINHERAVGEAMD	50
LdhB	SHAVPFAPAPTRVWKGSYEDCKDADLVVITAGLPQKPGETRLDLVEKNAK	100
LdhC	SHAVPFSPSPTKVWSGSYADCKDADLVVITAGLPQKPGETRLDLVEKNAK	100
LdhA	SHCTINFINTRTKVYAGSYEDCKMDITVITAGPAPKPGQSRDLTLGASAK	100
LdhB	IFKQIVRSIMDSGFDGIFLIATNPVDILTYYVTWKESGLPKERVIGSGTTL	150
LdhC	IFKQIVRGIMDSGFDGIFLIATNPVDILTYYVTWKESGLPKERVIGSGTTL	150
LdhA	IMESVVGVMASGFDGIFLLASNPVDITTYQVWKLISGLPRNRVIGTGTSL	150
LdhB	DSARFRYMLGEYFDIGPHNIHAYTIGEHGDTLPVWSHVSIG-IQKLTLL	199
LdhC	DSARFRYMLGIDYLDVDPNRVHAYTIVGEHGDTELPVWSHATIG-VQKLETL	199
LdhA	DSSRLRLTILSEMLHVDPRSIIHGYSIGEHGDSQMVAVSHVTVGKPILOTLL	200
LdhB	LEKDNTYNOEDLDKIFINVRDAAYHIIEKKGATYYGIGMSLLRVTKAILN	249
LdhC	LANNEQYKQEDLDKIFENVRDAAYQVIERKGATYYGIGMSLLRVTKAILN	249
LdhA	EEQKERFGEIDLDEIVEKTAAGWETIKRKGTITYYGIGNSLAYIASSTFN	250
	* *	
LdhB	DENSVLTVSAYLEGQYGOKDVIYIGVPAVLNRGGVREILEVELSEDEELKF	299
LdhC	NENSVLTVSAYLEGQYGEKDAYVGVPAVINREGVREIVELELNEDEKAKF	299
LdhA	DDHRVIAVSATILDGEYGEYDICTGVPATITRDGIREVVELNLTEDEESRF	300
LdhB	DHSVQVLKETMAPVL-	314
LdhC	AHSVKVLKETMAPVL-	314
LdhA	AKSNDILRDYMKTIIGY	316

FIG. 4. Amino acid sequence alignment of the three *B. cereus* L-lactate dehydrogenases. Black and gray boxes correspond to identical and similar amino acid residues, respectively. The tyrosine residue reported as acting in both dehydrogenase activity and ssDNA binding in eukaryotic cells is indicated with an asterisk (40). Dashes indicate gaps.

Previous results showed that ResDE, Fnr, and PlcR also activate *nhe* expression in aerobic respiring cells (11, 17, 43). Our transcriptomic data suggest that downregulation of *nhe* expression in *ldhA* mutant cells could not be simply controlled

by these three regulators under aerobiosis as they were under anaerobiosis. Under aerobiosis, involvement of Rex seems more questionable, since no NADH-dependent physiological change in the *ldhA* mutant strain relative to the parental strain

TABLE 3. Results from controlled batch cultures (pH 7.2) of *ldhA* and *pfo* mutants and their parent strain, *B. cereus* F4430/73^a

Growth parameter	Anaerobic growth			Aerobic growth		
	WT ^b	<i>ldhA</i>	<i>pfo</i>	WT	<i>ldhA</i>	<i>pfo</i>
$\mu_{\max}$ (h ⁻¹)	0.9	0.5	1.0	1.4	1.4	1.1
Final biomass (g · liter ⁻¹)	0.8	1.0	0.9	2.6	2.5	2.6
Glucose consumption (%)	100	100	100	100	100	100
Y_{glucose} (g of cells · mol of glucose ⁻¹)	26	33	30	86	83	86
Maximal specific glucose consumption rate (mmol · mg ⁻¹ · h ⁻¹)	35	15	34	16	17	13
Yields of end products (mol · mol glucose ⁻¹)						
Lactate	1.50	1.60	1.70	0.03	0.04	NZ ^c
Acetate	0.29	0.26	0.37	1.0	2.0	2.0
Formate	0.34	0.24	0.19	NZ	NZ	NZ
Ethanol	0.06	0.07	0.08	NZ	NZ	NZ
Succinate	0.01	0.01	0.01	NZ	NZ	NZ
Ethanol/acetate	0.21	0.27	0.21	ND ^d	ND	ND
NADH recovered ^e	1.1	1.1	1.2	ND	ND	ND
Total Nhe level (U · g ⁻¹ of dry cells)	5.30	1.25	4.35	1.41	0.09	1.29

^a Cells were grown under N₂ anaerobiosis (pO₂ = 0%) and full aerobiosis (pO₂ = 100%). Data are the means of triplicate measures obtained from two independent cultures. For clarity, standard deviations (below 10%) are not shown.

^b WT, wild-type parent strain *B. cereus* F4430/73.

^c NZ, yield was below 0.01 mol · mol glucose⁻¹.

^d ND, not determined.

^e NADH recovery was calculated as the ratio of pathways producing NADH versus those consuming NADH.

TABLE 4. Comparison of the mRNA levels of genes involved in glucose catabolism and enterotoxin synthesis in *ldhA* and *pfo* mutant strains^a

Gene	Difference in mRNA levels (<i>n</i> -fold) ^b under the indicated conditions			
	Anaerobic growth		Aerobic growth	
	<i>ldhA</i>	<i>pfo</i>	<i>ldhA</i>	<i>pfo</i>
Glucose catabolism genes				
<i>ldhA</i>	ND	+1.2	ND	+1.5
<i>pfo</i>	-4.0	ND	-1.1	ND
<i>ldhB</i>	<u>+32.0</u>	-3.4	<u>+2.4</u>	+1.1
<i>ldhC</i>	-1.4	+1.2	+1.0	-1.2
<i>pfl</i>	+1.4	-1.1	<u>+2.8</u>	<u>+3.5</u>
<i>pdhA</i>	-1.5	+1.5	+1.0	+1.2
<i>pta</i>	-2.0	+1.3	-2.4	+1.9
<i>ackA</i>	<u>+2.4</u>	<u>+5.8</u>	<u>+2.3</u>	<u>+10.8</u>
<i>adhE</i>	<u>+3.9</u>	-1.7	<u>+5.8</u>	<u>+2.6</u>
<i>adhA</i>	<u>+2.7</u>	-2.4	<u>+2.6</u>	+1.9
<i>sdhA</i>	+1.0	+1.5	<u>+2.1</u>	<u>+3.4</u>
<i>citZ</i>	-2.7	<u>+3.2</u>	+1.4	<u>+2.4</u>
<i>citB</i>	+1.4	+1.0	<u>+2.6</u>	+1.6
<i>mdh</i>	-1.3	+1.1	<u>+2.1</u>	+1.3
Enterotoxin gene <i>nhe</i>	-166	+1.8	-12.6	<u>+2.4</u>
Regulator genes				
<i>plcR</i>	-2.2	+1.2	-1.6	+1.6
<i>resD</i>	-1.3	+1.2	+1.2	<u>+2.6</u>
<i>fnr</i>	-1.2	-1.1	+1.4	+1.1
<i>ccpA</i>	-1.1	+1.1	+1.1	<u>+4.8</u>

^a *B. cereus* cells were grown under aerobic and anaerobic conditions in controlled batch cultures (pH 7.2) on MOD medium with 30 mM glucose as the carbon source.

^b Each change (*n*-fold) represents the mean value of the mRNA levels of one mutant strain sample in relation to that of the parent strain, F4430/73. For each experiment, two measurements from two independent RNA samples taken from the mid-exponential growth phase (μ_{max}) of the same culture were analyzed in parallel. Each data point is an average of the results of the combined experiments. Only ratios of ≤ -2 and ≥ 2 were considered significant (i.e., $P \leq 0.05$) according to the precision of the method. + and - indicate up- and downregulation of genes, and significant values for upregulation and downregulation are underlined and boldface, respectively. ND, not detectable.

was observed. We thus speculate that LdhA could directly control *nhe* expression under aerobiosis and maybe under anaerobiosis in addition to its probable indirect control. In accordance with the latter hypothesis, we observed that a lack of LdhA led to a greater downregulation of *nhe* expression under anaerobiosis than that seen under aerobiosis (Table 4). The idea that LdhA has a regulatory role is not unfounded, because (i) our results indicate that LdhA regulates its own expression, (ii) LdhA is structurally very different from other *B. cereus* classical fermentative lactate dehydrogenases (2, 15), and (iii)

TABLE 6. Differences in mRNA levels of toxin and putative toxin genes in the *ldhA* mutant compared to those of the parental F4430/73 strain^a

Toxin gene	Difference in mRNA level (<i>n</i> -fold) ^b under the indicated conditions	
	Anaerobic growth	Aerobic growth
<i>hbl</i>	-15.4	-4.1
<i>cytK</i>	-123	-18.5
<i>hlyI</i>	-3.7	-8.9
<i>entFM</i>	-2.0	-2.7
<i>entA</i>	-3.4	-3.4
<i>entB</i>	-8.1	-2.0
<i>entC</i>	-6.7	-4.3

^a *B. cereus* cells were grown under aerobic and anaerobic conditions in controlled batch cultures (pH 7.2) on MOD medium with 30 mM glucose as the carbon source.

^b Each change (*n*-fold) represents the mean value of the mRNA levels of the *ldhA* mutant strain sample in relation to that of the parent strain, F4430/73. For each experiment, two measures from two independent RNA samples taken from the mid-exponential growth phase (μ_{max}) of the same culture were analyzed in parallel. Each data point is an average of the results of the combined experiments. All ratios were ≤ -2 and thus considered significant (i.e., $P \leq 0.05$) according to the precision of the method. - indicates downregulation of genes.

lactate dehydrogenases have been identified as single-stranded DNA (ssDNA) binding proteins in eukaryotic cells and speculated to be components of transcriptional coactivator complexes (6, 19). Lactate dehydrogenase binding to ssDNA involved a tyrosine residue located near the coenzyme binding site that was regulated by the NADH/NAD⁺ ratio (40). LdhA possesses this Tyr residue; its position and structural environment (2, 15) differ from those encountered in LdhB and LdhC (Fig. 4). It is thus tempting to speculate that LdhA could be a partner regulator of a complex involving at least ResD and Fnr (and possibly Rex) to regulate its own transcription as well as *nhe* transcription. Such a complex could also activate the expression of other toxin genes, since we demonstrated that a lack of LdhA downregulated their expression (Table 6). Finally, LdhA could function as both a main fermentative enzyme and a virulence-associated transcriptional regulator, although further analyses are necessary to confirm and further define its nonenzymatic role.

In conclusion, this study exploring the catabolism and *nhe* expression of different *B. cereus* strains was a successful approach for the discovery of a fermentative enzyme of pathogenic relevance. In addition, this study indicates that links between metabolic sensors and virulence factor transcription could be established directly through enzymes that participate in carbohydrate metabolism in *B. cereus* like in other bacteria (41). These roles further underscore the need to consider the

TABLE 5. Growth parameters and Nhe production determined from uncontrolled batch cultures of *ldhA*, *pfo*, *ldhB*, and *ldhC* mutants and their parent strain, F4430/73^a

Parameter	Anaerobic growth					Aerobic growth				
	WT ^b	<i>ldhA</i>	<i>pfo</i>	<i>ldhB</i>	<i>ldhC</i>	WT	<i>ldhA</i>	<i>pfo</i>	<i>ldhB</i>	<i>ldhC</i>
μ_{max} (h ⁻¹)	0.90	0.45	0.82	0.94	0.96	1.40	1.33	1.43	1.48	1.35
Final OD ₅₆₀	0.42	0.17	0.42	0.37	0.42	1.49	1.56	1.51	1.51	1.39
Total Nhe level (U · OD ₅₆₀ ⁻¹)	12.90	0.13	10.10	12.40	13.90	6.10	0.52	5.20	5.00	5.10

^a Cells were grown under anaerobiosis and aerobiosis. Data are the means of duplicate cultures. For clarity, standard deviations (below 15%) are not shown.

^b WT, wild-type parent strain *B. cereus* F4430/73.

nonenzymatic functions of central metabolism enzymes in studies aiming to determine the virulence potentials of a *B. cereus* strain and, more widely, of any other pathogenic bacterial strain. Finally, LdhA, like adapted glycolytic enzymes in eukaryotes, may act as a link between environmental cues and regulatory circuits, ensuring *B. cereus* adaptation and propagation in anoxic and oxic conditions, such as those found in the human intestine (27).

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