



HAL
open science

Lactate Dehydrogenase A Promotes Communication between Carbohydrate Catabolism and Virulence in *Bacillus cereus*.

Sabrina Laouami, Kahina Messaoudi, François Alberto, Thierry Clavel,
Catherine Duport

► **To cite this version:**

Sabrina Laouami, Kahina Messaoudi, François Alberto, Thierry Clavel, Catherine Duport. Lactate Dehydrogenase A Promotes Communication between Carbohydrate Catabolism and Virulence in *Bacillus cereus*. International Journal of Systematic Bacteriology, 2011, 193 (7), pp.1757-1766. 10.1128/JB.00024-11 . hal-01330279

HAL Id: hal-01330279

<https://hal.science/hal-01330279v1>

Submitted on 28 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Lactate dehydrogenase A promotes communication between carbohydrate**
2 **catabolism and virulence in *Bacillus cereus***

3
4 Sabrina Laouami^{1, 2, ±}, Kahina Messaoudi^{1, 2, ±}, François Alberto^{1, 2, §}, Thierry Clavel^{1, 2} and
5 Catherine Duport^{1, 2, *}

6
7
8
9 ¹ Université d'Avignon et des Pays de Vaucluse, UMR408, Sécurité et Qualité des Produits
10 d'Origine Végétale, F-84000 Avignon, France.

11
12 ² INRA, UMR408, Sécurité et Qualité des Produits d'Origine Végétale, F-84914 Avignon,
13 France.

14
15 [±]Both authors contributed equally

16 [§] Present address: Laboratoire de Chimie Bactérienne, UPR9043, F-13402 Marseille, France

17 * Author to whom correspondence should be addressed. Tel.: 334-322-507; Fax: 334-322-492;

18 E-mail: catherine.duport@univ-avignon.fr.

19
20
21
22 Running title: LdhA as a multifaceted fermentative enzyme

23 ABSTRACT

24

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

44

45 INTRODUCTION

46 *Bacillus cereus* strains express various putative virulence genes including hemolysins,
47 various enterotoxins, phospholipases, metalloproteases, collagenases and other proteins (7,

48

48 38). The contribution of these putative virulence factors to the pathogenicity of *B. cereus*
49 strains has not been fully elucidated. However, the amount of secreted non hemolytic
50 enterotoxin (Nhe) appeared to essentially dictate *B. cereus*-associated cytotoxic activity (29).
51 Nhe is a three-component enterotoxin encoded by the PlcR-regulated *nheABC* operon (*nhe*,
52 (1, 16, 26)). In *in vitro* culture, *nhe* transcription is responsive to a number of cues linked to
53 carbohydrate catabolism, including carbon source concentration, carbon source type,
54 extracellular oxidoreduction potential and growth phase (10, 28, 31, 42), which suggests there
55 are mechanisms to promote communication between carbohydrate catabolism and the *nhe*
56 regulatory network. Carbohydrates such as glucose are catabolized primarily through the
57 glycolytic pathway and to a lesser extent through the pentose phosphate pathway in growing
58 *B. cereus* cells (42). Glycolysis produces two molecules of pyruvate per molecule of glucose
59 consumed, and in the process reduces two molecules of NAD⁺ to NADH. The catabolic fate
60 of pyruvate is determined by growth conditions, specifically oxygen availability. During
61 fermentative anaerobic growth, pyruvate or acetyl-CoA derived from pyruvate serve as
62 electron acceptors for maintaining redox balance and as the substrate for generating ATP and
63 acetate. The enzymes responsible for redox balance in anoxic *B. cereus* cells are lactate
64 dehydrogenase (Ldh), malate dehydrogenase (Mdh), succinate dehydrogenase (Sdh), pyruvate
65 formate lyase (Pfl), aldehyde dehydrogenase (AdhE) and alcohol dehydrogenase (AdhA). The
66 production of acetate from acetyl-CoA is dependent on the phosphotransacetylase-acetate
67 kinase (Pta-Ack) pathway (Fig. 1A). The main fermentative by-products of *B. cereus* are a
68 mixture of organic acids, such as lactate, succinate, acetate and formate, and ethanol. Lactate
69 is the major product of fermentation and normally accounts for more than 65% of the total
70 produced from glucose by *B. cereus* F4430/73 culture. Acetate and formate are produced in
71 similar amounts (each accounting for 15% of total production), while ethanol and succinate
72 are minor fermentation products (less than 3% and 2% of total by-products produced from

73 glucose, respectively (11, 34). Producing this mixture of by-products in the appropriate ratio
74 helps maintain redox balance under anoxic conditions while also maximizing ATP yield per
75 glucose molecule to support high growth rates and cell yields. During aerobic growth, the
76 pyruvate dehydrogenase complex (Pdh) connects the glycolytic reactions to the tricarboxylic
77 cycle (TCA cycle) by catalyzing the production of acetyl-CoA from pyruvate. The NADH
78 generated during the complete oxidation of glucose is reoxidized to NAD⁺ by O₂ through the
79 aerobic respiratory chain with accompanying ATP production. Acetate excretion can occur
80 aerobically when carbon flux into the cells exceeds TCA cycle capacity (11, 34). In *B. cereus*,
81 the expression of most of the genes encoding metabolic enzymes is modulated by the two-
82 component ResDE system (11), the one-component Fnr system (43) and the catabolite control
83 protein CcpA (39). These three systems also contribute to virulence. ResDE and Fnr may act
84 as components of transcriptional regulatory complexes (13). Understanding how these
85 regulatory complexes detect metabolic cues is an important step towards understanding how
86 gene regulation contributes to the pathogenic potential of a *B. cereus* strain.

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

97

98 MATERIAL AND METHODS

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

123 described above for *ldhA* and *pfo*, with the following modifications. The *ldhB* gene was PCR-
124 amplified using oligonucleotide pair *ldhBmutF* and *ldhBmutR* (Table S1), then cloned into
125 pMAD using *BamHI* and *SalI*. The recombinant vector was then digested with *Csp45I* and
126 ligated with the 1.5-kb *spc*-containing fragment to obtain the vector used for *ldhB*
127 replacement. The *ldhC* gene was PCR-amplified using oligonucleotide pair *ldhCmutF* and
128 *ldhCmutR* (Table S1), and then cloned into pUC18 using *PstI* and *BamHI*. The pUC18*ldhC*
129 plasmid was then digested with *SnaBI* and ligated with the 1.5-kb *spc*-containing *SmaI*
130 fragment. The *NcoI*-*BglIII* fragment containing the disrupted *ldhC* gene was then isolated and
131 cloned into pMAD.

132 Wild-type strains F4430/73, F4810/72, F837/76 and PA and *ldhA* and *pfo* mutants
133 were grown in the synthetic MOD medium supplemented with 30 mM glucose as carbon
134 source (34, 42). A 2-L bioreactor (Discovery 100, Inceltech, Toulouse, France) was used for
135 all cultivations, and working volume was maintained at 1.3 L. Temperature was maintained at
136 37°C and pH was kept at a controlled 7.2 by automatic addition of 5M KOH. The regulated
137 batch was equipped with a Mettler Toledo polarographic oxygen electrode coupled with a
138 feedback regulation to maintain the setpoint dissolved oxygen tension value (pO_2) using air
139 sparging and agitation speed (10). Sparging the bioreactor with air alone set a pO_2 value of
140 100%. A pO_2 of 0% was obtained by continuously flushing the medium at $20 \text{ ml}\cdot\text{h}^{-1}$ with pure
141 N_2 gas previously passed through a Hungate column. Each bioreactor was inoculated with a
142 subculture grown overnight under anaerobiosis in such a way that initial optical density at 560
143 nm (OD_{560}) was equal to 0.02. For *ldhA* mutant complementation assays and analyses of
144 mutant F4430/73 strains *ldhB* and *ldhC*, uncontrolled aerobic and anaerobic cultures were
145 performed as previously described (34).

146

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

161
162 **Enzyme assay.** Lactate dehydrogenase activity was assayed with pyruvate on purified
163 enzymes. The assay mixture (1 ml) contained phosphate buffer at pH 6.6, NADH 0.3 mM,
164 fructose 1,6 diphosphate 3 mM, MgCl_2 5 mM and 5 to 10 μl of purified protein (10 $\mu\text{g}/\mu\text{l}$).
165 Sodium pyruvate 20 mM was added to start the reaction. NADH oxidation was followed by
166 the decrease in absorbance at 340 nm. One unit of lactate dehydrogenase activity is 1 A_{340}
167 unit per min.

168
169 **In vitro lactate dehydrogenase synthesis.** Proteins were synthesized by using the rapid-
170 translation system 500 *E. coli* HY kit (Roche Diagnostics), according to the manufacturer's
171 instructions. Template DNA fragments were prepared using a two-step PCR protocol. PCR

172 reaction 1 was used to obtain specific fragments (primer pairs are listed in Table 1). PCR
173 reaction 2 was used to obtain the products with regulatory elements and His-tag at the C-
174 terminus. The PCR products were purified by PCR purification kit (Roche). The purified
175 products were sequenced; after the sequence was confirmed to be correct, these products were
176 used for expressing corresponding proteins. The protein products were rapidly purified using
177 a Ni-NTA column. The expressed protein was confirmed by Western blotting using anti-His
178 antibody as first antibody.

179
180 **DNA sequencing and bioinformatics tools.** Sequencing was performed by Beckman Coulter
181 Genomics (UK). Homology and conserved domain searches were performed in the NCBI
182 database using the BLAST algorithm. Pairwise alignment was carried out using ClustalW
183 software. The presence of transmembrane helices in proteins was predicted using TMpred and
184 TMHMM version 2.0 software via the ExPASy proteomics server.

185
186 **RNA isolation, operon mapping and expression analysis.** To investigate the transcriptional
187 organization and expression of the *ldhA-pfo* locus, reverse transcription-PCR (RT-PCR) was
188 performed with total RNA isolated from *B. cereus* F4430/73 as previously described (11).
189 Portions of *ldhA*, *pfo* and the *ldhA-pfo* intergenic region were amplified by RT-PCR using the
190 Titan one Tube RT-PCR system following the manufacturer's protocol (Roche). The primer
191 pairs used for *ldhA*, *pfo* and the *ldhA-pfo* intergenic region are listed in Table S1 (see the
192 supplemental material). To check whether contaminant genomic DNA was present, each
193 sample was tested in a control reaction that did not contain reverse transcriptase.

194 The 5' end of *ldhA-pfo* mRNA was mapped from a 5' RACE PCR product obtained
195 with the 3'/5' RACE kit (Rapid amplification of cDNA ends, Roche). Briefly, the first-strand
196 cDNA was synthesized from total RNA with *ldhA-pfo*-specific SP1 primer (Table S1), avian

197 myeloblastosis virus reverse transcriptase, and the deoxynucleotide mixture of the 3'/5' RACE
198 kit following the manufacturer's instructions. After purifying and dA-tailing the cDNA, PCR
199 with the (dT)-anchor oligonucleotide primer and the *ldhA-pfo*-specific SP2 primer (Table S1)
200 followed by a nested PCR with SP3 primer (Table S1) led to a PCR product of approximately
201 140 bp, as revealed by 2% agarose gel electrophoresis. This PCR product was purified and
202 sequenced.

203 Real-time RT-PCR was performed using SYBR Green staining on a LightCycler
204 Instrument (Roche) as previously described (11). Sequences specifically used in the present
205 study are given in Table S1.

206
207 **Nucleotide sequence accession number.** Given that the *B. cereus* F4430/73 strain belongs to
208 the same genetic group as the ATCC 14579 strain (21), we designed primers (Table S1)
209 corresponding to the ATCC 14579 locus to clone and sequence its homolog from strain
210 F4430/73. The DNA sequence of the 2,748-bp cloned fragment corresponding to the *ldhA-pfo*
211 locus was deposited in GenBank under accession no HQ336744.

212 213 RESULTS

214 **Fermentative and respiratory capacities of strains F4810/72, F837/76 and PA versus** 215 **strain F4430/73**

216 The *B. cereus* strains F4430/73, F4810/72, F837/76 and PA were grown in glucose-
217 limited and pH-regulated-batch culture (pH 7.2) under anaerobiosis (pO₂ = 0%; generated
218 under an N₂ atmosphere) and aerobiosis (pO₂ = 100%). Table 1 summarizes the growth
219 features of the four strains. Under anaerobiosis, strains F4810/72, F837/76 and PA grew
220 significantly more slowly and reached the stationary growth phase at a lower final biomass
221 compared to strain F4430/73. This probably results from both lower levels and lower specific

222 rates of glucose uptake. Interestingly, strain F4810/72 exhibited the highest biomass yield on
223 glucose. Under aerobiosis, growth rate values were similar across the four strains, although
224 slightly lower in strains F837/76 and PA than in the two other strains. However, final biomass
225 and biomass yields were significantly higher for strains F4810/72, F837/76 and PA than for
226 strain F4430/73, while glucose uptake rates were lower. Taken together, these results indicate
227 that glucose supports lower glycolytic fluxes (or glucose uptake rate) in strains F4810/72,
228 F837/76 and PA compared to strain F4430/73 under both anaerobiosis and aerobiosis, leading
229 to lower fermentative growth and more efficient respiratory growth, respectively.

230 Figure 1A depicts the main catabolic pathways used by *B. cereus* strains. Glucose was
231 converted predominantly into lactate in all four strains (Fig. 1B). However, F4810/72,
232 F837/76 and PA cells produced lower amounts of lactate and concomitantly higher amounts
233 of formate, acetate (except for PA) and ethanol than F4430/73 cells. This suggests that the
234 carbon flux from pyruvate to lactate was lower in strains F4810/72, F837/76 and PA than in
235 strain F4430/73 under anaerobic fermentative conditions and that the carbon flux through
236 pyruvate formate lyase (Pfl) at the pyruvate node and then through the NADH recycling
237 alcohol dehydrogenase (AdhE-AdhA) pathway at the acetyl-CoA node was higher (Fig. 1A).
238 When oxygen was present, no detectable ethanol, formate or succinate was produced in all
239 four strains. Amount of secreted lactate was similar in all four strains, while strains F4810/72,
240 F837/76 and PA secreted lower amounts of acetate than strain F4430/73 (Fig. 1B). Since
241 aerobic acetate generation results from an overflow metabolism in *B. cereus* (34), glucose
242 could support a lower overflow metabolism in strains F4810/72, F837/76 and PA than in
243 strain F4430/73 under aerobic respiratory conditions.

244 Table 2 shows the catabolic gene expression profiles in strains F4810/72, F837/76 and
245 PA *versus* strain F4430/73 from cells cultured under fermentative anaerobic and respiratory
246 aerobic growth conditions. The data revealed that strains F4810/72, F837/76 and PA exhibited

247 a very different pattern of catabolic gene expression than strain F4430/73. Interestingly, we
248 observed that the mRNA profile of the three genes predicted to encode L-lactate
249 dehydrogenase (*ldhA*, *ldhB* and *ldhC*) differed significantly between strains F4810/72,
250 F837/76 and PA and strain F4430/73 in absence and presence of oxygen: (i) *ldhA* expression
251 was moderately reduced in strain F4810/72 and severely reduced in strains F837/76 and PA
252 under both anaerobiosis and aerobiosis; (ii) *ldhB* expression differed between strains and
253 between condition sets; (iii) *ldhC* expression was inversely correlated to *ldhA* expression ($r =$
254 -0.63 , $p < 0.05$) in all the three strains F4810/72, F837/76 and PA whatever the growth
255 conditions. In addition, concomitantly to *ldhA*, *adhE*, which encodes a key enzyme of the
256 ethanol generating pathway, and *citZ*, which encodes an enzyme that governs carbon entry
257 into the TCA cycle, were significantly down-regulated in strains F4810/72, F837/76 and PA
258 compared to strain F4430/73 under both anaerobiosis and aerobiosis.

260 **Nhe secretion capacity of strains F4810/72, F837/76 and PA versus strain F4430/73**

261 Strains F4810/72, F837/76 and PA secreted lower amounts of Nhe than strain
262 F4430/73 under both anaerobiosis and aerobiosis (Fig. 1C). Among strains F4810/72,
263 F837/76 and PA, F4810/72 was clearly the largest producer of Nhe (1.1 and $0.4 \text{ U} \cdot \text{g}^{-1}$ of dry
264 cells under anaerobiosis and aerobiosis, respectively, compared to 0.2 and 0.002 for strain
265 F837/76 and 0.5 and 0.01 for strain PA).

266 Table 2 shows that *nhe* expression was strongly reduced in strains F4810/72, F837/76
267 and PA compared to strain F4430/73 under both anaerobiosis and aerobiosis. This may
268 explain why Nhe was less secreted in these strains. Transcript levels of *nhe*, regulatory genes
269 and genes encoding catabolic enzymes that were ≥ 2 -fold up- or down- regulated under both
270 anaerobiosis and aerobiosis were subjected to Pearson's r correlation analysis. The results
271 indicated that only the *ldhA*, *adhE* and *citZ* mRNA levels were significantly correlated

272 (p<0.05) with *nhe* levels ($r = +0.62$, $+0.91$ and $+0.77$, respectively). This suggests a
273 regulatory link between these catabolic genes and *nhe*. To validate such a link, we studied the
274 *ldhA* gene in relation to *nhe* in strain F4430/73.

276 Analysis of the *ldhA* locus in *B. cereus* F4430/73

277 As in reference strain ATCC 14579 (GenBank accession no. AE016877), the stop
278 codon of *B. cereus* F4430/73 *ldhA* is located 14-bp upstream of the start codon of an ORF,
279 annotated *pfo* by Ivanova *et al.* (24), suggesting these genes are co-operonic (Fig. 2A). RT-
280 PCR experiments demonstrated that *ldhA* and *pfo* were effectively co-transcribed under both
281 aerobiosis and anaerobiosis (Fig. 2B). A transcriptional start site (G) located 48 bp from the
282 *ldhA* start codon was identified by 5' RACE PCR (Fig. 2C). Upstream of this start site, we
283 identified a potential σ_A -type -10 sequence, TATAAT, that is not preceded by a typical -35
284 sequence (TTGACA, (22)). A review of the *ldhA* promoter regions of the other *B. cereus*
285 strains confirmed the absence of the typical -35 region. Inspection of the 5' UTR sequence
286 revealed the presence of a palindromic sequence spanning positions -64 to -41. Each
287 symmetrical arm contains highly conserved potential binding sites for the redox regulators
288 Fnr and ResD (8, 11, 23, 43). No CcpA binding site was found (39). Furthermore, the *ldhA*-
289 *pfo* locus appeared to be followed by an inverted repeat ($\Delta G = - 23.1$ kcal/mol) that may be a
290 transcriptional terminator, confirming that *ldhA* and *pfo* could form a transcriptional unit.

292 Expression of *B. cereus ldhA* gene in *E. coli*

293 To demonstrate that *B. cereus ldhA* encodes lactate dehydrogenase, *ldhA* was
294 expressed as a His-tagged protein using a cell-free *E. coli* translation system in the presence
295 of GroES and GroEL chaperone proteins. Figure 3 shows that LdhA peptide (Fig.3A), like
296 LdhB and LdhC peptides (Fig.3B), was successfully expressed *in vitro*. The purified LdhA

297 protein has a specific Ldh activity of $40 \pm 4 \text{ U.mg}^{-1}$, using pyruvate as substrate and NADH as
298 the co-substrate. Comparatively, LdhB and LdhC exhibited specific Ldh activities of 50 ± 6
299 U.mg^{-1} and $83 \pm 10 \text{ U.mg}^{-1}$, respectively. Taken together, these results demonstrate that *B.*
300 *cereus ldhA*, like *ldhB* and *ldhC*, encodes functional lactate dehydrogenase.

302 Primary structure analysis of LdhA and PfoR

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

314 Physiological characteristics of *B. cereus* F4430/73 *ldhA* mutant

315 A *B. cereus* F4430/73 *ldhA* mutant was created by introducing a spectinomycin
316 resistance gene deprived of its transcription initiation and termination sequence, as described
317 in the Material and Methods. To prove the non-polar effect of *ldhA* disruption, we also
318 constructed a *pfo* mutant and compared the growth features of the *ldhA* and *pfo* mutants to the
319 parental F4430/73 strain under anaerobiosis and aerobiosis. Table 3 showed that both *ldhA*
320 and *pfo* disruption did not significantly affect the parameters of *B. cereus* F4430/73 growth
321 under aerobiosis. However, *ldhA* disruption severely impaired the growth rate of fermentative

322 F4430/73 cells under anaerobiosis, probably due to a lower specific rate of glucose
323 consumption. Analysis of glucose by-product spectra at the end of growth only showed a
324 significant decrease in formate secretion in the *ldhA* mutant strain compared to the parental
325 strain. This lower formate generation is also observed in the *pfo* mutant concomitantly with
326 higher acetate generation. More importantly, the ethanol-to-acetate-ratio was higher in the
327 *ldhA* mutant strain compared to the parental strain and the *pfo* mutant while NADH recovery
328 is similar. This indicates an effective NAD⁺ regeneration in spite of higher NADH availability
329 in *ldhA* mutant cells (35). To determine if this higher NADH availability could be due to a
330 defect of lactate secretion at the beginning of the exponential growth phase ($\mu = \mu_{\max}$), we
331 compared the amounts of lactate produced by the *ldhA* mutant and the parental strain. The
332 results showed that the *ldhA* mutant strain produced lower lactate than the parental strain
333 (compared 0.15 to 0.23 mol/mol of glucose, respectively), indicating a defect in NADH
334 recycling lactate pathway. Table 4 shows that *ldhB* expression was up-regulated by the
335 disruption of *ldhA* under both anaerobiosis and aerobiosis, although more strongly under
336 anaerobiosis. Based on these data, we hypothesized that overexpression of *ldhB* would be
337 sufficient for maintain redox balance (NADH/NAD⁺ ratio) and that higher NADH availability
338 rather than ineffective NAD⁺ regeneration is limiting glycolytic flow and subsequently
339 fermentative growth.

340 Table 3 shows that *ldhA* disruption but not *pfo* disruption significantly reduced Nhe
341 secretion under both aerobiosis and anaerobiosis. Table 4 shows that transcription of *nhe* was
342 strongly down-regulated under these two conditions. Taken together, these data indicate that
343 the main control of Nhe production in *ldhA* mutant was oxygen-independent and occurs
344 mainly at transcriptional level. Interestingly, no significant correlation could be highlighted
345 between the variations in *nhe* transcripts levels and those of other tested gene.

346 Table 4 also shows that *pfo* mRNA levels were not affected in aerobic *ldhA* cells while
347 *pfo* transcripts were significantly decreased in fermentative anaerobic cells when *ldhA* was
348 disrupted. The same results were obtained with *ldhA-pfo* intergenic region mRNA (data not
349 shown). This highlights the non-polar effect of spectinomycin cassette insertion and suggests
350 a possible role of *ldhA* in *ldhA-pfo* transcription, i.e. in its self-regulation. Finally, to
351 definitively confirm that the defect in fermentative growth and enterotoxin production was
352 specifically ascribable to the disruption of *ldhA*, wild-type copies of *ldhA* and *ldhA-pfo* loci
353 were cloned into the pHT304 vector (see Material and Methods) and each resulting vector
354 was used to complement the *ldhA* mutant strain. Whatever the vector used, *trans*
355 complementation only partially restored the fermentative growth of the *ldhA* mutant and
356 enabled the mutant to produce 2-fold higher Nhe levels (data not shown).

357 358 **Validation of *ldhA* as a specific lactate dehydrogenase gene of pathogenic relevance**

359 To compare the role played by LdhA with the roles of LdhB and LdhC in glucose
360 catabolism and Nhe secretion, we disrupted the *ldhB* and *ldhC* genes in *B. cereus* strain
361 F4430/73 as described in the Material and Methods section. The ability of these mutants to
362 grow and produce Nhe under both anaerobiosis and aerobiosis was assessed in uncontrolled
363 batch cultures. Unlike *ldhA* disruption, *ldhB* and *ldhC* disruptions did not induce fermentative
364 growth deficiency or lower Nhe secretion (Table 5). This suggests that LdhA, unlike LdhB
365 and LdhC could play a major role in both physiology and Nhe production in *B. cereus*
366 F4430/73. Because *ldhA* disruption caused the same effects in strain ATCC 14579 and
367 F837/76 than in strain F4430/73 (data not shown), we cannot not rule out the possibility that
368 LdhA could play a major role in several species of *B. cereus* group.

369 In addition to *nhe*, *B. cereus* expresses several other toxins or putative toxins (7). We
370 determined the effect of *ldhA* disruption on the mRNA levels of these enterotoxin genes.

371 Table 6 reports that all these genes were down-regulated in *ldhA* mutant cells, suggesting that
372 *ldhA* should participate in the regulatory network of toxinogenesis in *B. cereus* F4430/73.

373

374 DISCUSSION

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

380 *B. cereus* group species contains three high-conserved genes proposed to encode L-
381 lactate dehydrogenase (www.ncbi.nlm.nih.gov). The results obtained in the current study
382 showed that these three genes (*ldhA*, *ldhB* and *ldhC*) were responsible for active fermentative
383 lactate dehydrogenase. *LdhA* expression, like *ldhB* and *ldhC* expression, was detectable in
384 both fermenting and respiring *B. cereus* cells according to their ability to secrete lactate in
385 these two conditions, which is in line with our previous studies (28). Low expression of *ldhA*
386 was coupled with high expression of *ldhC* in strains F4810/72, F837/76 and PA whereas
387 disruption of *ldhA* induced *ldhB* overexpression in strain F4430/73 under both anaerobiosis
388 and aerobiosis. This suggests that the regulation network that coordinates the differential
389 expression of *ldhA*, *ldhB* and *ldhC* in *B. cereus* could function independently of the presence
390 or absence of oxygen in a strain-dependent manner. The existence of strain-dependent *ldh*
391 gene regulation in aerobic respiring cells where lactate dehydrogenase activity is not
392 necessary to maintain redox homeostasis indicates that fine regulation of lactate
393 dehydrogenase synthesis could confer *B. cereus* a metabolic advantage when grown under
394 aerobiosis, as is the case in *Staphylococcus aureus* (33). Expression of *ldh* genes is affected by
395 the redox sensors ResDE and Fnr, although direct binding of the regulators and their

396 regulatory involved have not been elucidated (11, 43). ResD and Fnr were showed to regulate
397 each other and their own synthesis at the transcriptional level (13-14). Since no correlation
398 was observed between the mRNA levels of these two regulators and those of *ldhA*, we
399 conclude that strain-dependent regulation of *ldh* genes cannot be simply related to ResDE and
400 Fnr.

401 The importance of LdhA, unlike LdhB and LdhC, to maintain redox homeostasis in
402 fermentative cells was demonstrated by (i) the inability of low-*ldhA* expressing F4810/72,
403 F837/76 and PA cells to sustain rapid anaerobic growth compared to high-*ldhA* expressing
404 F4430/73 cells and by (ii) the fermentative anaerobic growth defect of F4430/73 *ldhA* mutant.
405 The importance of LdhA on enterotoxin gene expressions in fermentative anaerobic and
406 respiring aerobic *B. cereus* cells was highlighted by (i) the lower capacity of low-*ldhA*
407 expressing F4810/72, F837/76 and PA cells to express *nhe* mRNA levels compared to high-
408 *ldhA* expressing F4430/73 cells and by (ii) the down-regulation of *nhe* and other putative
409 enterotoxin gene in strain F4430/73 lacking LdhA. A key question is how LdhA impacts on
410 the enterotoxin gene expression? A detailed analysis of this impact was beyond the scope of
411 this study. However, we speculate that under fermentative conditions, the defect in LdhA
412 synthesis may indirectly down-regulated enterotoxin gene expression through its impact on
413 NADH availability and subsequently on redox potential of the cytoplasm and on growth rate
414 (31). Activation of *nhe* expression in fermentative anaerobiosis has been shown to involve
415 ResDE and Fnr regulators (11, 43). These redox regulators activate their own transcription in
416 response to redox potential changes (13-14). Unlike *nhe* mRNA levels, *resD* and *fnr* mRNA
417 levels did not change significantly in *ldhA* mutant strain compared to the parental strain. This
418 suggests that ResDE and Fnr are not the key regulators of *nhe* expression in *ldhA* mutant
419 cells. This is probably also the case for auto-regulatory PlcR transcriptional regulator (16). In
420 *Bacillus subtilis* (25), as in other gram positive bacteria (36), changes in NADH availability is

421 sensed by the transcriptional regulator Rex. Rex homologue is encoded in the genome of *B.*
422 *cereus* (www.ncbi.nlm.nih.gov and unpublished data). Interestingly, we found Rex binding
423 motifs (32), overlapping ResD and Fnr binding motifs in *ldhA*, *ldhB* (but not *ldhC*) and *nhe*
424 (data not shown). It remains to be examined whether these sequences are really targets for this
425 redox regulator that could thus regulate directly and simultaneously *nhe* and *ldhA*.

426 Previous results showed that ResDE, Fnr and PlcR also activate *nhe* expression in
427 aerobic respiring cells (11, 17, 43). Our transcriptomic data suggest that down-regulation of
428 *nhe* expression in *ldhA* mutant cells could be not simply controlled by these three regulators
429 under aerobiosis like under anaerobiosis. Under aerobiosis, involvement of Rex seems more
430 questionable since no NADH-dependent physiological change was observed in the *ldhA*
431 mutant strain compared to the parental strain. We thus speculate that LdhA could directly
432 control *nhe* expression under aerobiosis and may be under anaerobiosis in addition to its
433 probable indirect control. According with this latter hypothesis, we observed that lack of
434 LdhA led to a higher downregulation of *nhe* expression under anaerobiosis compared to
435 aerobiosis (Table 4). That LdhA has a regulatory role is not unfounded because (i) our results
436 indicate that LdhA regulates its own expression, (ii) LdhA is structurally very different to
437 other *B. cereus* classical fermentative lactate dehydrogenase (2, 15) and (iii) lactate
438 dehydrogenases have been identified as single-stranded-DNA (ssDNA) binding proteins in
439 eukaryotic cells, and speculated to be components of transcriptional co-activator complexes
440 (6, 19). Lactate dehydrogenase binding to ssDNA involved a tyrosine residue located near the
441 coenzyme binding site that was regulated by NADH/NAD⁺ ratio (40). LdhA possesses this
442 Tyr residue; its position and structural environment (2, 15) differ from those encountered in
443 LdhB and LdhC (Fig. 4). It is thus tempting to speculate that LdhA could be a partner
444 regulator of a complex involving at least ResD and Fnr (and possibly Rex) to regulate its own
445 transcription as well as *nhe* transcription. Such a complex could also activate the expression

446 of other toxin genes, since we demonstrated that a lack of LdhA down-regulated their
447 expression (Table 6). Finally, LdhA could function as both a main fermentative enzyme and a
448 virulence-associated transcriptional regulator, although further analyses are necessary to
449 confirm and further define its non-enzymatic role.

450 In conclusion, this study exploring the catabolism and *nhe* expression of different *B.*
451 *cereus* strains was a successful approach to discover a fermentative enzyme of pathogenic
452 relevance. In addition, this study indicates that links between metabolic sensors and virulence
453 factor transcription could be established directly through enzymes that participate in
454 carbohydrate metabolism in *B. cereus* like in other bacteria (41). These roles further
455 underscore the need to consider the non-enzymatic functions of central metabolism enzymes
456 in studies aiming to determine the virulence potential of a *B. cereus* strain, and more widely
457 of any other pathogenic bacterial strain. Finally, LdhA, like adapted glycolytic enzymes in
458 eukaryotes, may act as a link between environmental cues and regulatory circuits, ensuring *B.*
459 *cereus* adaptation and propagation in the anoxic *versus* oxic conditions such as found in the
460 human intestine (27).

463 **ACKNOWLEDGMENTS**

464 S.L. received a fellowship from the Provence Alpes Côte d'Azur region and INRA and K.M.
465 from the Franco-Algerian intergovernmental program. We thank Tefisoa Randrianarivony and
466 Chahla Louldji for technical assistance.

467 **FIGURE LEGENDS**

468

469 **Figure 1:** Metabolic response of *B. cereus* strains F4430/73, F4810/72, F837/76 and PA. **A.**
470 Pathways for the excretion of partially-oxidized metabolites. The reactions are represented by
471 the names of the corresponding genes: *ldhA*, *ldhB*, *ldhC*, L-lactate dehydrogenase; *pfl*,
472 pyruvate formate lyase; *pdh*, pyruvate dehydrogenase; *pta*, phosphotransacetylase; *ack*,
473 acetate kinase; *adhE*, acetaldehyde dehydrogenase; *adhA*, alcohol dehydrogenase; *mdh*,
474 malate dehydrogenase; *sdh*, succinate dehydrogenase; *citB*, aconitase hydratase; *citZ*, citrate
475 synthase. Reactions that normally function during aerobiosis are represented by gray arrows,
476 and reactions that normally function during anaerobiosis by black arrows. **B.** Extracellular
477 metabolite analyses. **C.** Specific Nhe production. Bars indicate the standard error of the mean.
478 CIT, citrate; ICT, isocitrate; AKG, α -ketoglutarate; SUC, succinate; FUM, fumarate; MAL,
479 malate; OAA, oxaloacetate; TCA, tricarboxylic acid cycle.

480

481 **Figure 2:** Operon mapping and expression of *B. cereus* F4430/73 *ldhA-pfo* operon. **A.** Solid
482 arrows represent the *ldhA* and *pfo* ORFs. The small arrows flag the positions and directions of
483 primers used in RT-PCR. Lengths of expected PCR products are also shown for every primer
484 pair. **B.** RT-PCR analyses for detecting *ldhA*, *pfo* and the intergenic (IG) region between *ldhA*
485 and *pfo* in strain F4430/73. **C.** The nucleotide sequence of the promoter region of F4430/73
486 *ldhA* is shown. The transcriptional start site (+1) and the putative -10 are in bold and
487 underlined. Putative regulatory sequences are highlighted. The start codon is boxed.

488

489 **Figure 3:** Cell-free expression of *ldhA*, *ldhB* and *ldhC*. Lactate dehydrogenases were
490 expressed as His-tagged proteins using the RTS 500 *E. coli* HY kit in the presence of GroE
491 supplement. Purified LdhA, LdhB and LdhC were visualized by Western blotting using anti-

492 His antibodies. **A.** Purified LdhA (34.6 kDa). **B.** Lane 1, purified LdhB (34.5 kDa). Lane 2,
493 purified LdhC (34.7 kDa). The sizes of the molecular mass markers (in thousands) are
494 indicated on the left.

495
496 **Figure 4:** Amino acid sequence alignment of the three *B. cereus* L-lactate dehydrogenases.
497 Black and gray boxes correspond to identical and similar amino acid residues, respectively.
498 The tyrosine residue reported as acting in both dehydrogenase activity and ssDNA binding in
499 eukaryotic cells is indicated with * (40).

500

501 REFERENCES

502

- 503 1. **Agaisse, H., M. Gominet, O. A. Okstad, A. B. Kolsto, and D. Lereclus.** 1999. PlcR
504 is a pleiotropic regulator of extracellular virulence factor gene expression in *Bacillus*
505 *thuringiensis*. Mol Microbiol **32**:1043-1053.
- 506 2. **Arai, K., T. Ishimitsu, S. Fushinobu, H. Uchikoba, H. Matsuzawa, and H.**
507 **Taguchi.** 2010. Active and inactive state structures of unliganded *Lactobacillus casei*
508 allosteric L-lactate dehydrogenase. Proteins **78**:681-694.
- 509 3. **Arantes, O., and D. Lereclus.** 1991. Construction of cloning vectors for *Bacillus*
510 *thuringiensis*. Gene **108**:115-119.
- 511 4. **Arnaud, M., A. Chastanet, and M. Debarbouille.** 2004. New vector for efficient
512 allelic replacement in naturally nontransformable, low-GC-content, gram-positive
513 bacteria. Appl Environ Microbiol **70**:6887-6891.
- 514 5. **Awad, M. M., and J. I. Rood.** 2002. Perfringolysin O expression in *Clostridium*
515 *perfringens* is independent of the upstream *pfoR* gene. J Bacteriol **184**:2034-2038.

- 516 6. **Cattaneo, A., S. Biocca, N. Corvaja, and P. Calissano.** 1985. Nuclear localization of
517 a lactic dehydrogenase with single-stranded DNA-binding properties. *Exp Cell Res*
518 **161**:130-140.
- 519 7. **Clair, G., S. Roussi, J. Armengaud, and C. Duport.** 2010. Expanding the known
520 repertoire of virulence factors produced by *Bacillus cereus* through early secretome
521 profiling in three redox conditions. *Mol Cell Proteomics* **9**:1486-1498.
- 522 8. **Cruz Ramos, H., T. Hoffmann, M. Marino, H. Nedjari, E. Presecan-Siedel, O.**
523 **Dreesen, P. Glaser, and D. Jahn.** 2000. Fermentative metabolism of *Bacillus*
524 *subtilis*: physiology and regulation of gene expression. *J Bacteriol* **182**:3072-3080.
- 525 9. **Dong, J. M., J. S. Taylor, D. J. Latour, S. Iuchi, and E. C. Lin.** 1993. Three
526 overlapping *lct* genes involved in L-lactate utilization by *Escherichia coli*. *J Bacteriol*
527 **175**:6671-6678.
- 528 10. **Duport, C., S. Thomassin, G. Bourel, and P. Schmitt.** 2004. Anaerobiosis and low
529 specific growth rates enhance hemolysin BL production by *Bacillus cereus* F4430/73.
530 *Arch Microbiol* **182**:90-95.
- 531 11. **Duport, C., A. Zigha, E. Rosenfeld, and P. Schmitt.** 2006. Control of enterotoxin
532 gene expression in *Bacillus cereus* F4430/73 involves the redox-sensitive ResDE
533 signal transduction system. *J Bacteriol* **188**:6640-6651.
- 534 12. **Ehling-Schulz, M., B. Svensson, M. H. Guinebretiere, T. Lindback, M.**
535 **Andersson, A. Schulz, M. Fricker, A. Christiansson, P. E. Granum, E.**
536 **Martlbauer, C. Nguyen-The, M. Salkinoja-Salonen, and S. Scherer.** 2005. Emetic
537 toxin formation of *Bacillus cereus* is restricted to a single evolutionary lineage of
538 closely related strains. *Microbiology* **151**:183-197.

- 539 13. **Eselin, J., J. Armengaud, A. Zigha, and C. Duport.** 2009. ResDE-dependent
540 regulation of enterotoxin gene expression in *Bacillus cereus*: evidence for multiple
541 modes of binding for ResD and interaction with Fnr. J Bacteriol **191**:4419-4426.
- 542 14. **Eselin, J., Y. Jouanneau, J. Armengaud, and C. Duport.** 2008. ApoFnr binds as a
543 monomer to promoters regulating the expression of enterotoxin genes of *Bacillus*
544 *cereus*. J Bacteriol **190**:4242-4251.
- 545 15. **Eventoff, W., M. G. Rossmann, S. S. Taylor, H. J. Torff, H. Meyer, W. Keil, and**
546 **H. H. Kiltz.** 1977. Structural adaptations of lactate dehydrogenase isozymes. Proc
547 Natl Acad Sci U S A **74**:2677-2681.
- 548 16. **Gohar, M., K. Faegri, S. Perchat, S. Ravnum, O. A. Okstad, M. Gominet, A. B.**
549 **Kolsto, and D. Lereclus.** 2008. The PlcR virulence regulon of *Bacillus cereus*. PLoS
550 One **3**:e2793.
- 551 17. **Gohar, M., N. Gilois, R. Graveline, C. Garreau, V. Sanchis, and D. Lereclus.**
552 2005. A comparative study of *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus*
553 *anthracis* extracellular proteomes. Proteomics. **5**:3696-3711.
- 554 18. **Gompertz, B.** 1925. On the nature of the function expressive of the law of human
555 mortality, and on a new mode of determining the value of life contingencies. Philos.
556 Trans. R. Soc. **115**:513-585.
- 557 19. **Grosse, F., H. P. Nasheuer, S. Scholtissek, and U. Schomburg.** 1986. Lactate
558 dehydrogenase and glyceraldehyde-phosphate dehydrogenase are single-stranded
559 DNA-binding proteins that affect the DNA-polymerase-alpha-primase complex. Eur J
560 Biochem **160**:459-467.
- 561 20. **Guinebretiere, M. H., V. Broussolle, and C. Nguyen-The.** 2002. Enterotoxigenic
562 profiles of food-poisoning and food-borne *Bacillus cereus* strains. J Clin Microbiol
563 **40**:3053-3056.

- 564 21. **Guinebretiere, M. H., F. L. Thompson, A. Sorokin, P. Normand, P. Dawyndt, M.**
565 **Ehling-Schulz, B. Svensson, V. Sanchis, C. Nguyen-The, M. Heyndrickx, and P.**
566 **De Vos.** 2008. Ecological diversification in the *Bacillus cereus* Group. Environ
567 Microbiol **10**:851-865.
- 568 22. **Haldenwang, W. G.** 1995. The sigma factors of *Bacillus subtilis*. Microbiol Rev
569 **59**:1-30.
- 570 23. **Hartig, E., H. Geng, A. Hartmann, A. Hubacek, R. Munch, R. W. Ye, D. Jahn,**
571 **and M. M. Nakano.** 2004. *Bacillus subtilis* ResD induces expression of the potential
572 regulatory genes yclJK upon oxygen limitation. J Bacteriol. **186**:6477-6484.
- 573 24. **Ivanova, N., A. Sorokin, I. Anderson, N. Galleron, B. Candelon, V. Kapatral, A.**
574 **Bhattacharyya, G. Reznik, N. Mikhailova, A. Lapidus, L. Chu, M. Mazur, E.**
575 **Goltsman, N. Larsen, M. D'Souza, T. Walunas, Y. Grechkin, G. Pusch, R.**
576 **Haselkorn, M. Fonstein, S. D. Ehrlich, R. Overbeek, and N. Kyrpides.** 2003.
577 Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus*
578 *anthracis*. Nature **423**:87-91.
- 579 25. **Larsson, J. T., A. Rogstam, and C. von Wachenfeldt.** 2005. Coordinated patterns of
580 cytochrome bd and lactate dehydrogenase expression in *Bacillus subtilis*.
581 Microbiology **151**:3323-3335.
- 582 26. **Lund, T., and P. E. Granum.** 1996. Characterisation of a non-haemolytic enterotoxin
583 complex from *Bacillus cereus* isolated after a foodborne outbreak. FEMS Microbiol
584 Lett **141**:151-156.
- 585 27. **Marteyn, B., F. B. Scorza, P. J. Sansonetti, and C. Tang.** 2010. Breathing life into
586 pathogens: the influence of oxygen on bacterial virulence and host responses in the
587 gastrointestinal tract. Cell Microbiol. doi:10.1111/j.

- 588 28. **Messaoudi, K., T. Clavel, P. Schmitt, and C. Duport.** 2010. Fnr mediates
589 carbohydrate-dependent regulation of catabolic and enterotoxin genes in *Bacillus*
590 *cereus* F4430/73. Res Microbiol **161**:30-39.
- 591 29. **Moravek, M., R. Dietrich, C. Buerk, V. Broussolle, M. H. Guinebretiere, P. E.**
592 **Granum, C. Nguyen-The, and E. Martlbauer.** 2006. Determination of the toxic
593 potential of *Bacillus cereus* isolates by quantitative enterotoxin analyses. FEMS
594 Microbiol Lett **257**:293-298.
- 595 30. **Murphy, E.** 1985. Nucleotide sequence of a spectinomycin adenylyltransferase AAD(9)
596 determinant from *Staphylococcus aureus* and its relationship to AAD(3") (9). Mol Gen
597 Genet **200**:33-39.
- 598 31. **Ouhib, O., T. Clavel, and P. Schmitt.** 2006. The production of *Bacillus cereus*
599 enterotoxins is influenced by carbohydrate and growth rate. Curr Microbiol **53**:222-
600 226.
- 601 32. **Pagels, M., S. Fuchs, J. Pane-Farre, C. Kohler, L. Menschner, M. Hecker, P. J.**
602 **McNamarra, M. C. Bauer, C. von Wachenfeldt, M. Liebeke, M. Lalk, G. Sander,**
603 **C. von Eiff, R. A. Proctor, and S. Engelmann.** 2010. Redox sensing by a Rex-family
604 repressor is involved in the regulation of anaerobic gene expression in *Staphylococcus*
605 *aureus*. Mol Microbiol **76**:1142-1161.
- 606 33. **Richardson, A. R., S. J. Libby, and F. C. Fang.** 2008. A nitric oxide-inducible
607 lactate dehydrogenase enables *Staphylococcus aureus* to resist innate immunity.
608 Science **319**:1672-1676.
- 609 34. **Rosenfeld, E., C. Duport, A. Zigha, and P. Schmitt.** 2005. Characterization of
610 aerobic and anaerobic vegetative growth of the food-borne pathogen *Bacillus cereus*
611 F4430/73 strain. Can J Microbiol **51**:149-158.

- 612 35. **Sanchez, A. M., G. N. Bennett, and K. Y. San.** 2005. Effect of different levels of
 613 NADH availability on metabolic fluxes of *Escherichia coli* chemostat cultures in
 614 defined medium. J Biotechnol. **117**:395-405. Epub 2005 Apr 2007.
- 615 36. **Somerville, G. A., and R. A. Proctor.** 2009. At the crossroads of bacterial
 616 metabolism and virulence factor synthesis in Staphylococci. Microbiol Mol Biol Rev
 617 **73**:233-248.
- 618 37. **Spira, W. M., and J. M. Goepfert.** 1975. Biological characteristics of an enterotoxin
 619 produced by *Bacillus cereus*. Can J Microbiol **21**:1236-1246.
- 620 38. **Stenfors Arnesen, L. P., A. Fagerlund, and P. E. Granum.** 2008. From soil to gut:
 621 *Bacillus cereus* and its food poisoning toxins. FEMS Microbiol Rev **32**:579-606.
- 622 39. **van der Voort, M., O. P. Kuipers, G. Buist, W. M. de Vos, and T. Abee.** 2008.
 623 Assessment of CcpA-mediated catabolite control of gene expression in *Bacillus cereus*
 624 ATCC 14579. BMC Microbiol **8**:62.
- 625 40. **Williams, K. R., S. Reddigari, and G. L. Patel.** 1985. Identification of a nucleic acid
 626 helix-destabilizing protein from rat liver as lactate dehydrogenase-5. Proc Natl Acad
 627 Sci U S A **82**:5260-5264.
- 628 41. **Yesilkaya, H., F. Spissu, S. M. Carvalho, V. S. Terra, K. A. Homer, R. Benisty, N.**
 629 **Porat, A. R. Neves, and P. W. Andrew.** 2009. Pyruvate formate lyase is required for
 630 pneumococcal fermentative metabolism and virulence. Infect Immun **77**:5418-5427.
- 631 42. **Zigha, A., E. Rosenfeld, P. Schmitt, and C. Duport.** 2006. Anaerobic cells of
 632 *Bacillus cereus* F4430/73 respond to low oxidoreduction potential by metabolic
 633 readjustments and activation of enterotoxin expression. Arch Microbiol **185**:222-233.
- 634 43. **Zigha, A., E. Rosenfeld, P. Schmitt, and C. Duport.** 2007. The redox regulator Fnr
 635 is required for fermentative growth and enterotoxin synthesis in *Bacillus cereus*
 636 F4430/73. J Bacteriol **189**:2813-2824.

Postprint

Version définitive du manuscrit publié dans / Final version of the manuscript published in : Journal of Bacteriology, 2011, vol.193, no.7, DOI: :10.1128/JB.00024-11

- 637 44. **Zwietering, M., I. Jongenburger, F. Rombouts, and K. Van't Riet.** 1990. Modeling
638 of the bacterial growth curve. Appl. Environ. Microbiol. **56**:1875-1881.

639

640

Manuscrit d'auteur / Author manuscript

Manuscrit d'auteur / Author manuscript

Manuscrit d'auteur / Author manuscript

Postprint

Version définitive du manuscrit publié dans / Final version of the manuscript published in : Journal of Bacteriology, 2011, vol.193, no.7, DOI: 10.1128/JB.00024-11

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

	Anaerobic growth				Aerobic growth			
	F4430/73	F4810/72	F837/76	PA	F4430/73	F4810/72	F837/76	PA
Maximal specific growth rate (μ , h ⁻¹)	0.9	0.5	0.5	0.5	1.4	1.3	1.1	1.1
Final biomass (g.l ⁻¹) ^a	0.8	0.3	0.3	0.3	2.6	2.9	3.7	3.0
Glucose consumption (%) ^a	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 Final biomass and glucose consumption were determined at the end of growth, i.e. 1.5 h after OD₅₆₀ reached its maximal value.

Comment citer ce document :

Laouami, S., Messaoudi, K., Alberto, F., Clavel, T., Duport, C. (2011). Lactate dehydrogenase A promotes communication between carbohydrate catabolism and virulence in *Bacillus cereus*. Journal of Bacteriology, 193 (7), 1757-1766. DOI : 10.1128/JB.00024-11

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 strain F4430/73.^a

Gene	Difference in mRNA level (<i>n</i> -fold) ^b under the indicated conditions					
	Anaerobic growth			Aerobic growth		
	F4810/72	F837/76	PA	F4810/72	F837/76	PA
Glucose catabolism						
<i>ldhA</i>	-2.5	-100	-100	-2.0	-100	-100
<i>ldhB</i>	+2.5	+2.0	-5.0	-50	-100	-5.0
<i>ldhC</i>	+3.2	+6.2	+6.5	+2.2	+12.3	+5.4
<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	+2.0
<i>adhE</i>	-7.7	-4.8	-7.7	-4.5	-33.3	-3.7
<i>adhA</i>	-1.1	+1.7	+1.7	+2.3	-1.8	+1.1
<i>sdhA</i>	-2.2	+1.0	-2.5	+3.0	+2.9	+2.4
<i>citZ</i>	-100	-100	-16.0	-50.0	-33.0	-2.7
<i>citB</i>	+1.1	-12.5	-10.0	+2.0	+1.5	-2.3
<i>mdh</i>	+1.6	+4.8	+4.9	-1.1	-1.1	+1.2
Enterotoxin						
<i>nhe</i>	-10	-100	-100	-3.1	-500	-14.3
Regulators						
<i>plcR</i>	+3.0	+1.4	+4.2	+2.9	+1.1	-1.1
<i>resD</i>	+4.0	+3.2	+5.6	+2.2	+1.9	+2.3
<i>fnr</i>	+6.7	+6.2	+7.5	-1.8	+2.4	+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 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 measures from two independent RNA samples taken from the mid-exponential growth phase ($\mu = \mu_{max}$) of the same culture were analyzed in parallel. Each data point is an average of the results of the combined experiments. Only ratios ≤ -2 and $\geq +2$ were considered significant (i.e. $p \leq 0.05$) according to the precision of the method. + and - indicate up- and down-regulation of genes and are boxed in gray and black, respectively.

TABLE 3. Results of controlled batch cultures (pH=7.2) of *ldhA* and *pfo* mutants and their parent strain *B. cereus* F4430/73 (wt). 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. Nz, yield was below 0.01. Nd, not determined.

	Anaerobic growth			Aerobic growth		
	wt	<i>ldhA</i>	<i>pfo</i>	wt	<i>ldhA</i>	<i>pfo</i>
<u>Growth parameters</u>						
Maximal specific growth rate (μ , h ⁻¹)	0.9	0.5	1.0	1.4	1.4	1.1
Final biomass (g.l ⁻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
<u>Yields of end-products (mol.mol glucose⁻¹)</u>						
Lactate	1.50	1.60	1.70	0.03	0.04	nz
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	nd	nd
NADH recovered ^a	1.1	1.1	1.2	nd	nd	nd
Total Nhe levels (U.g ⁻¹ of dry cells)	5.30	1.25	4.35	1.41	0.09	1.29

^aNADH recovery was calculated as the ratio of pathways producing NADH *versus* those consuming NADH

Comment citer ce document :

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

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

^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 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 measures from two independent RNA samples taken from the mid-exponential growth phase ($\mu = \mu_{max}$) of the same culture were analyzed in parallel. Each datapoint is an average of the results of the combined experiments. Only ratios ≤ -2 and ≥ 2 were considered significant (i.e. $p \leq 0.05$) according to the precision of the method. + and - indicate up- and down-regulation of genes and are boxed in gray and black, respectively. ND, not detectable.

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

	Anaerobic growth					Aerobic growth				
	wt	<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>
Maximal specific growth rate (μ , 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

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

Toxin genes	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 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 ($\mu = \mu_{\max}$) of the same culture were analyzed in parallel. Each datapoint 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 down-regulation of genes.

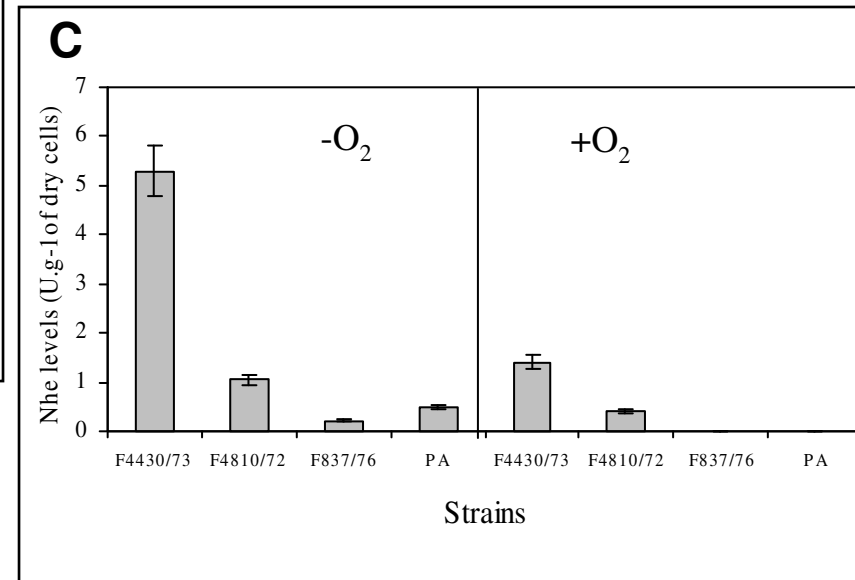
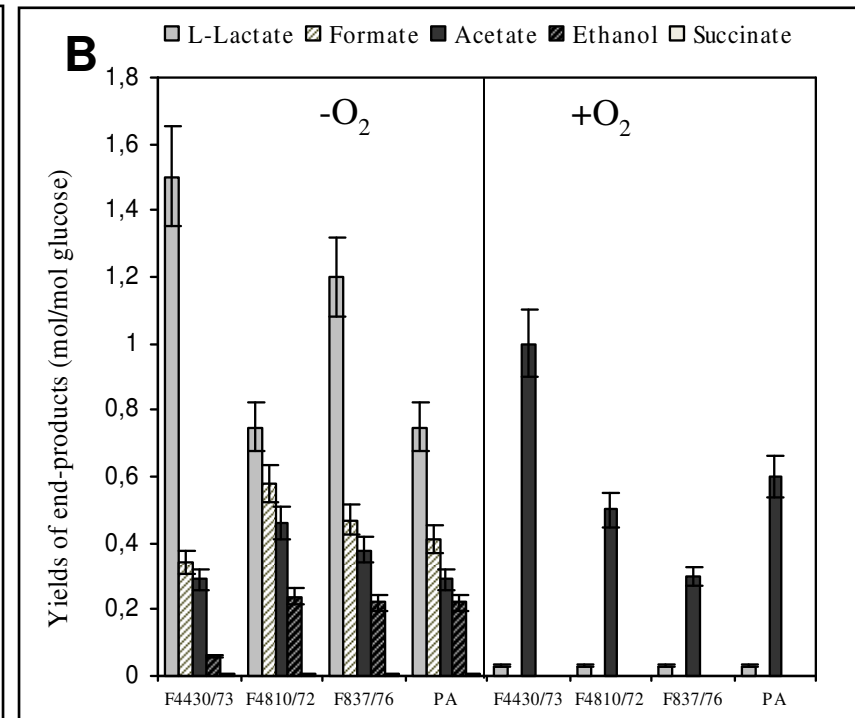
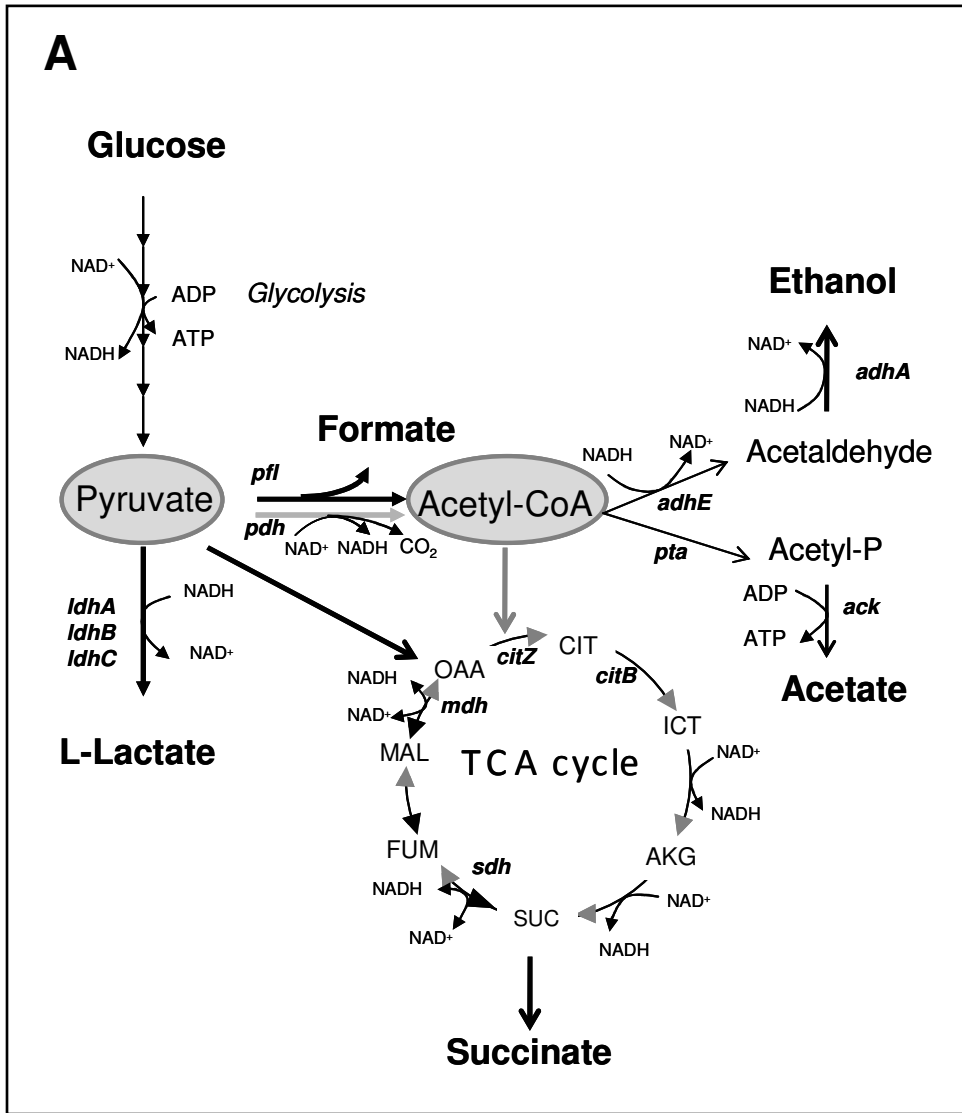


Figure 1

Postprint

Version définitive du manuscrit publié dans / Final version of the manuscript published in : Journal of Bacteriology, 2011, vol.193, no.7, DOI: 10.1128/JB.00024-11

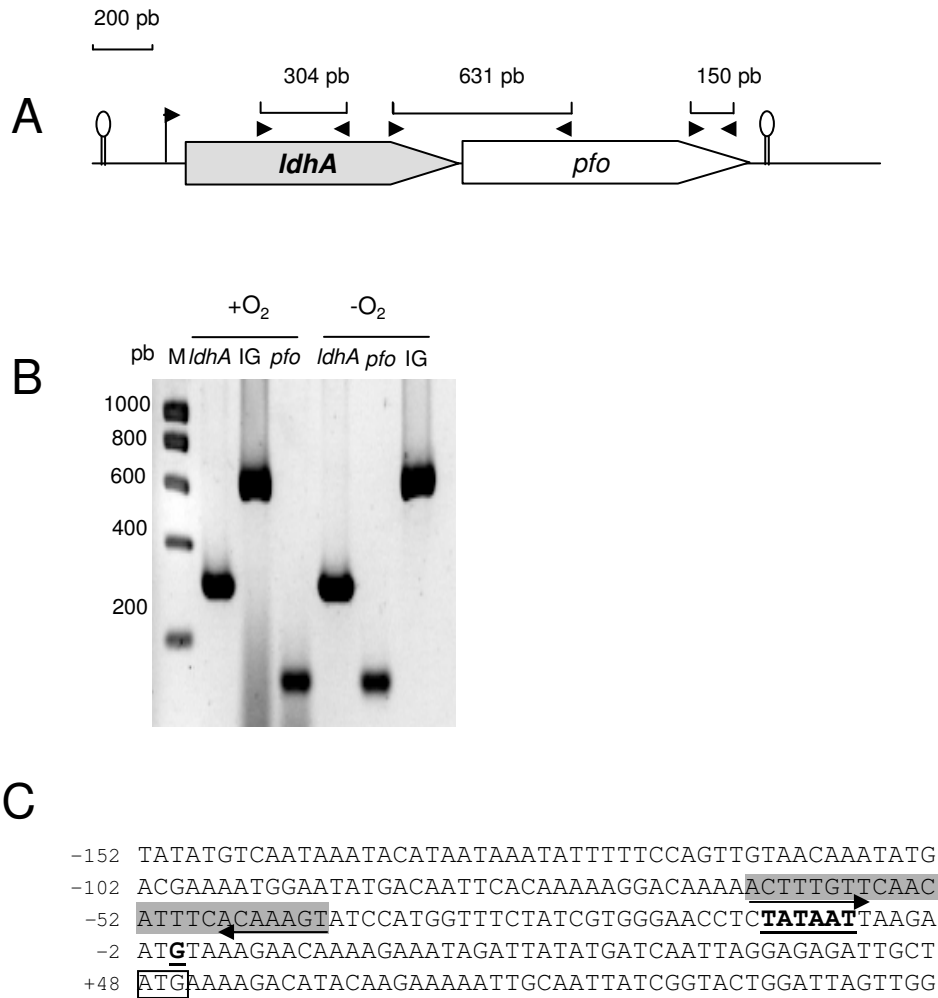


Figure 2

Comment citer ce document :

Laouami, S., Messaoudi, K., Alberto, F., Clavel, T., Duport, C. (2011). Lactate dehydrogenase A promotes communication between carbohydrate catabolism and virulence in *Bacillus cereus*. Journal of Bacteriology, 193 (7), 1757-1766. DOI : 10.1128/JB.00024-11

Postprint

Version définitive du manuscrit publié dans / Final version of the manuscript published in : Journal of Bacteriology, 2011, vol.193, no.7, DOI: 10.1128/JB.00024-11

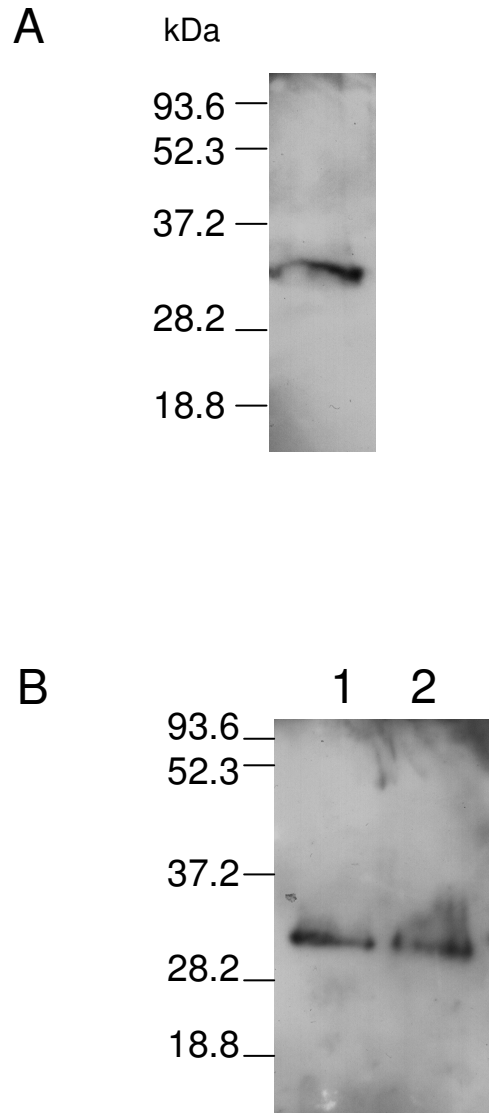


Figure 3

Comment citer ce document :

Laouami, S., Messaoudi, K., Alberto, F., Clavel, T., Duport, C. (2011). Lactate dehydrogenase A promotes communication between carbohydrate catabolism and virulence in *Bacillus cereus*. Journal of Bacteriology, 193 (7), 1757-1766. DOI : 10.1128/JB.00024-11

Postprint

Version définitive du manuscrit publié dans / Final version of the manuscript published in : Journal of Bacteriology, 2011, vol.193, no.7, DOI: 10.1128/JB.00024-11

```
LdhB MKKGINRVVLVGTGAVGCSYAYCMINQAVAEFFVLVDVNEAKAEGEAMD 50
LdhC MKKGINRVVLVGTGAVGCSYAYSMINQGVAEFFVLVDVNEAKAEGEAMD 50
LdhA MKRHTRKIATIGTGLVGSSCAYSIVNOGICEELLLIDINHERAVGEAMD 50

LdhB SHAVPFAPAPTRVVKGSYEDCKDADLVVITAGLPQKPGETRLDI.VEKNAK 100
LdhC SHAVPFSPSPTKVWSGSYADCKDADLVVITAGLPQKPGETRLDLVEKNTK 100
LdhA SHCINFTNRTTKVYAGSYEDCKMDIVTITAGPAPKPGQSRDLTLGASAK 100

LdhB IFKQIVRSIMDSGFDGIFLIATNPVDILTYYVTWKESGLPKERVIGSGTTL 150
LdhC IFKQIVRGIMDSGFDGIFLIATNPVDILTYYVTWKESGLPKERVIGSGTTL 150
LdhA IMESVVGVMASGFDGIFLLASNPVDITTYQVWKL SGLP RN RVIGITGTS L 150

LdhB DSARFRYMLGEYFDIGPHNIHAYII GEHGDELPVWSHVSVG-IQKLOTL 199
LdhC DSARFRYMLGDYLDVDP RN VHAYIV GEHGDELPVWSHATIG-VOKLETI 199
LdhA DSSRLRTILSEM LHVDP RSIHGYSI GEHGDSQMVAVWSHVTVGGKPILOIL 200

LdhB LEKDNTYNQEDLDKIFINVRDAAYHI IERKGATYYGIGMSLLRVTKAILN 249
LdhC LANNEQYKQEDLDKIFENVRDAAYQVIERKGATYYGIGMSLLRVTKAILN 249
LdhA EEQKEREGEIDLDEIVEKTAKAGWEIYKRKGTYYGIGNSLAYLASSIFN 250
* *

LdhB DENSVLTVSAYLEGOYGQKDVYI GVPVAVLN RGGVREI LVELSEDEELKF 299
LdhC NENSVLTVSAYLEGOYGEKDAYV GVPVAVINREGVREI VLELNEDEKAKF 299
LdhA DDHRVLA VSA TL DGEYGEVDICTGVPATITRDGIREVVELNLTEDEESRF 300

LdhB DHSVQVLKETMAPVL- 314
LdhC AHSVQVLKETMAPVL- 314
LdhA AKSNDILRDYMKTI GY 316
```

Figure 4

Comment citer ce document :

Laouami, S., Messaoudi, K., Alberto, F., Clavel, T., Duport, C. (2011). Lactate dehydrogenase A promotes communication between carbohydrate catabolism and virulence in *Bacillus cereus*. Journal of Bacteriology, 193 (7), 1757-1766. DOI : 10.1128/JB.00024-11