Metabolic Engineering of *Bacillus subtilis* for Ethanol Production: Lactate Dehydrogenase Plays a Key Role in Fermentative Metabolism[▽]

Susana Romero,[1] Enrique Merino,[2] Francisco Bolívar,[1] Guillermo Gosset,[1] and Alfredo Martinez[1]*

*Departamento de Ingeniería Celular y Biocatálisis[1] and Departamento de Microbiología Molecular,[2]
Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México*

Wild-type *Bacillus subtilis* ferments 20 g/liter glucose in 48 h, producing lactate and butanediol, but not ethanol or acetate. To construct an ethanologenic *B. subtilis* strain, homologous recombination was used to disrupt the native lactate dehydrogenase (LDH) gene (*ldh*) by chromosomal insertion of the *Zymomonas mobilis* pyruvate decarboxylase gene (*pdc*) and alcohol dehydrogenase II gene (*adhB*) under the control of the *ldh* native promoter. The values of the intracellular PDC and ADHII enzymatic activities of the engineered *B. subtilis* BS35 strain were similar to those found in an ethanologenic *Escherichia coli* strain. BS35 produced ethanol and butanediol; however, the cell growth and glucose consumption rates were reduced by 70 and 65%, respectively, in comparison to those in the progenitor strain. To eliminate butanediol production, the acetolactate synthase gene (*alsS*) was inactivated. In the BS36 strain (BS35 Δ*alsS*), ethanol production was enhanced, with a high yield (89% of the theoretical); however, the cell growth and glucose consumption rates remained low. Interestingly, kinetic characterization of LDH from *B. subtilis* showed that it is able to oxidize NADH and NADPH. The expression of the transhydrogenase encoded by *udhA* from *E. coli* allowed a partial recovery of the cell growth rate and an early onset of ethanol production. Beyond pyruvate-to-lactate conversion and NADH oxidation, an additional key physiological role of LDH for glucose consumption under fermentative conditions is suggested. Long-term cultivation showed that 8.9 g/liter of ethanol can be obtained using strain BS37 (BS35 Δ*alsS udhA*+). As far as we know, this is the highest ethanol titer and yield reported with a *B. subtilis* strain.

Ethanol production from renewable resources, such as biomass, is a promising alternative to compete with and eventually replace nonrenewable fossil fuels; it has considerable advantages in terms of sustainability, lower greenhouse gas emissions, and cost reduction (25). Currently, fuel ethanol is produced by using *Saccharomyces cerevisiae* that ferments sucrose from sugarcane or glucose from hydrolyzed cornstarch. Nonetheless, large amounts of sugars are available in plant biomass. This biomass is a complex mixture of carbohydrate polymers, mainly cellulose and hemicellulose. Cellulose utilization requires a previous enzymatic breakdown to release glucose; however, the use of cellulases raises the cost of ethanol production (18, 22, 25).

Gram-positive bacteria have several traits, such as the capacity to withstand relatively low pH, high temperature, high sugar, salt and ethanol concentrations, and various other harsh conditions, which could be used to develop an advanced biocatalyst and improve the commercial competitiveness of fuel ethanol production (9, 17). Several groups have tried to develop ethanologenic gram-positive bacteria, but with limited success (2, 15, 19, 21, 27, 32).

Different chimerical *pet* operons have been constructed by employing *pdc* and *adhB* genes from *Zymomonas mobilis* (gram-negative bacterium) or by using the *pdc* gene from *Sarcina ventriculy* (gram-positive bacterium) for expression in monocopy or multicopy vectors in gram-positive bacteria. In some of these cases, functional pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) enzymes have been detected, although the ethanol yields have been low or nonexistent (19, 21, 27, 32).

Bacillus subtilis has a generally-recognized-as-safe status (29), metabolizes an extensive range of sugars (31), and can efficiently synthesize a wide variety of proteinases and transport them out of the cell using secretion systems. This capacity can potentially be used to export cellulases, which could break down plant wastes, releasing cellobiose and glucose. Hence, ethanologenic *B. subtilis* strains engineered in the future might consume both carbohydrates to produce ethanol. This design could help in reducing fuel ethanol production cost. However, under fermentative conditions, *B. subtilis* produces lactate, acetate, butanediol, and traces of ethanol from glucose, amino acids, and pyruvate (Fig. 1) (7). Lactate is produced by the reduction of pyruvate, a reaction catalyzed by lactate dehydrogenase (LDH), with the simultaneous oxidation of one molecule of NADH per molecule of pyruvate reduced (7). In this study, the heterofermentative metabolism of *B. subtilis* was modified to obtain a gram-positive strain that produces ethanol as the main fermentation product. By inactivating the lactate dehydrogenase gene (*ldh*) via chromosomal integration of the *Z. mobilis pdc* and *adhB* genes, also eliminating butanediol synthesis, and by inserting the *E. coli udhA* transhydrogenase in the *alsS* locus, the resulting recombinant *B. subtilis* strain produced ethanol as the sole fermentation product.

* Corresponding author. Mailing address: Departamento de Ingeniería Celular y Biocatálisis, Instituto de Biotecnología, Universidad Nacional Autónoma de México, A.P. 510-3 Cuernavaca, Mor. 62250, México. Phone: (52-777) 3291601. Fax: (52-777) 3172388. E-mail: alfredo@ibt.unam.mx.
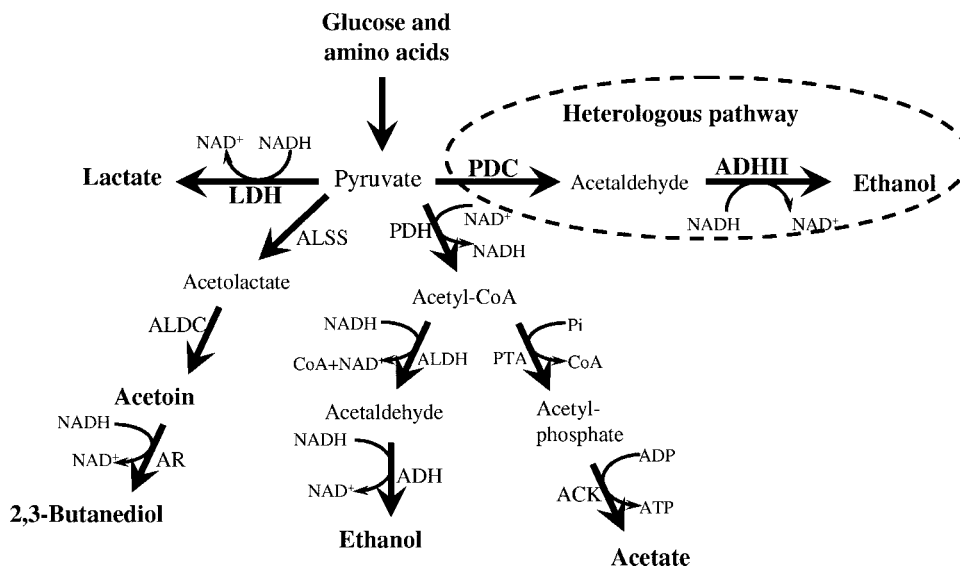
FIG. 1. *Bacillus subtilis* fermentation pathways. The heterologous pathway used in this study is highlighted with a dashed-line ellipse. PDC and ADHII are from *Z. mobilis*. ALDC, acetolactate decarboxylase; AR, acetoin reductase; PDH, pyruvate dehydrogenase; CoA, coenzyme A; ALDH, acetaldehyde dehydrogenase; PTA, phosphotransacetylase; ACK, acetate kinase. (Modified from references 7 and 26 with permission.)

LDH inactivation caused a *B. subtilis* growth defect in spite of complementation with a stoichiometrically equivalent redox pathway (PDC-ADHII). We found that LDH oxidizes NADH to NAD$^+$ and NADPH to NADP$^+$. The apparent K_m value for both cofactors is comparable to K_m values of LDH for NADH of other gram-positive bacteria (14). Hence, an additional key physiological role of LDH for glucose consumption under fermentative conditions is suggested. The results presented in this work clearly indicate that this key physiological role of LDH for glucose consumption under fermentative conditions should be considered in the development of new ethanologenic *B. subtilis* strains.

MATERIALS AND METHODS

Strains and plasmids. The *B. subtilis* strains and plasmids used in this study are listed in Table 1, and the oligonucleotides in Table 2. To avoid the Trp requirement, competent *B. subtilis* WB700 cells (35) were transformed by using chromosomal DNA from a Trp$^+$ *B. subtilis* BSR1 strain (20), recombinants were

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Strains		
B. subtilis WB700	*B. subtilis* 168 *trpC2* Δ*nprE* Δ*aprE* Δ*epr* Δ*bpf* Δ*mpr* Δ*nprB* Δ*vrpE* Ery^r Lin^r	35
B. subtilis CH1	WB700 prototroph	This study
B. subtilis CH1 Δ*alsS* mutant	CH1 *als*::Spt	This study
B. subtilis BS35	CH1 *ldh*::*pdc-adhB* Cm^r	This study
B. subtilis BS36	CH1 *ldh*::*pdc-adhB* Cm^r *als*::Spt	This study
B. subtilis BS37	CH1 *ldh*::*pdc-adhB* Cm^r *als*::*udhA*_Spt	This study
Plasmids		
pUC19	pMB1 *ori* Ap^r	BioLabs
pCR-Blunt II-TOPO	pBR322 *ori* Km^r *ccdB* selection	Invitrogen
pLOI276	pUC19 carrying *pdc*$_{Zm}$	5
pLOI284	pUC19 carrying *adhB*$_{Zm}$	6
IpTOPO-*pdc*	pCR carrying *pdc*$_{Zm}$	This study
pTOPO-*adh-term*	pCR carrying *adhB*$_{Zm}$-*cryIIIAt*	This study
pTOPO-*pdc-adh-term*	pCR carrying *pdc*$_{Zm}$, *adhB*$_{Zm}$, and Cm^r	This study
pSG-PLK	pBR322 *ori* Ap^r	20
pSG-operon	pSG-PLK carrying *pdc*$_{Zm}$, *adhB*$_{Zm}$, and Cm^r	This study
pUC-*ldh*	pUC19 carrying *ldh*$_{Bs}$	This study
pUC-*ldh*::operonCm	pUC19 carrying *ldh*$_{Bs}$::(*pdc*$_{Zm}$ *adhB*$_{Zm}$)	This study
pTOPO-*alsS*	pCR carrying *alsS*	This study
pCm::Spt	pIC177 carrying Cm^r::Spt^r	30
pTOPO-*alsS*::Spt	pCR carrying *alsS*::Spt^r	This study
pTOPO-*alsS*::*udhA*_Spt	pCR carrying *alsS*::(*udhA*$_{Ec}$ Spt^r)	This study

[a] Zm, *Z. mobilis*; Bs, *B. subtilis*; Ec, *E. coli*.

TABLE 2. Oligonucleotides used in this study

Primer	Target gene	Sequence (5′→3′)
1	Z. mobilis pdc	GTC GGC CGG CCA AGG AGG AGT AAG CAA TGA GTT ATA CTG TCG G
2	Z. mobilis pdc	CTT GGC GCG CCT TAC GGC TGT TGG CGG GCA GC
3	Z. mobilis adhB	TAA GGC GCG CCA AGG AGG GTA TAG CTA TGG CTT CTT CAA CTT TTT ATA TTC
4	Z. mobilis adhB	TCC GGT CAA TTG GAG TGA TGT CCG TTT TCC TGT TTT GAA ATT AG
5	B. thuringiensis cryIIIAt	AAA AGA ATT CAA AAA AAA ACG GAC ATC ACT CCG GTC AAT TGG AGT G
6	B. subtilis ldh	CCG CGG ATC CAA GGA GGG ATG ATT AAT GAT GAA ACA ACA TGT A
7	B. subtilis ldh	GCC CGA ATT CAC TCT AAA GTT GCG GTT AGT TGA C
8	B. subtilis alsS	GTG TCA CAC ATG TAA TTG GCA TTC C
9	B. subtilis alsS	GGC TGA GCA CTT AAA TGT TGC TTT C
10	E. coli udhA	GCC GGT CGA CAA GGA GGA CCC TAC CAT GCC ACA TTC C
11	E. coli udhA	GCC GGT CGA CTT AAA ACA GGC GGT TTA AAC CG

selected in mineral medium containing glucose, and the resulting prototrophic strain was designated CH1 (Table 1). *B. subtilis* CH1 was used to construct ethanologenic *B. subtillis* strains. During strain and plasmid construction, strains were grown in Luria-Bertani (LB) agar (23) containing appropriate antibiotics. The final antibiotic concentrations were as follows: 5 µg/ml chloramphenicol (Cm), 5 µg/ml erythromycin (Ery), 5 µg/ml lincomycin (Lin), 100 µg/ml spectinomycin (Spt), 10 µg/ml tetracycline (Tc), 50 µg/ml kanamycin (Km), and 100 µg/ml ampicillin (Ap).

Construction of ethanologenic *B. subtilis* strains. (i) Genetic procedures. Standard procedures were used for plasmid preparation, restriction enzyme digestions, ligations, transformations, and agarose gel electrophoresis (23). *E. coli* XL1-Blue was used as the host for plasmid constructions, and cells were grown in LB medium. *B. subtilis* was transformed by using the natural competence method (8). Chromosomal integrations and deletions were confirmed by using appropriate antibiotic markers and PCR analysis and by the examination of fermentation products.

(ii) Inactivation of *ldh* and chromosomal integration of *Z. mobilis pdc* and *adhB* genes. Specific primers were designed to amplify *Z. mobilis pdc* and *adhB* genes (Table 2). The primer design included the *B. subtilis* Shine-Dalgarno

consensus sequence and also the conserved distance between this motif and the start codon of each gene. The *Z. mobilis pdc* gene was amplified from vector pLOI276, using primers 1 and 2. The 1.7-kb PCR-amplified segment was cloned into pCR-TOPO, obtaining the vector IpTOPO-*pdc*.

The *Z. mobilis adhB* gene, followed by the terminator of the *Bacillus thuringiensis cryIIIA* gene (*cryIIIAt*), was amplified (34). In the first PCR, using primers 3 and 4, the *adhB* gene was amplified from vector pLOI284, including half of *cryIIIAt*; in the second PCR, using primers 3 and 5, the *adhB* gene, followed by the complete terminator, was amplified; this PCR segment was cloned into pCR-TOPO, generating pTOPO-*adh-term*.

The *pdc* gene was purified as a 1.7-kbp EcoRV-AscI fragment from vector IpTOPO-*pdc*. This fragment was ligated into the blunt-treated BamHI and AscI sites upstream from the *adhB* gene in pTOPO-*adh-term*, obtaining the vector pTOPO-*pdc-adh-term*. The *pdc* and *adhB* genes were purified as a 2.88-kbp EcoRI-NaeI fragment from vector pTOPO-*pdc-adh-term*. This fragment was ligated into the EcoRI-HincII sites of vector pSG-PLK (20), obtaining the vector pSG-operon. This plasmid contained the *pdc* and *adhB* genes followed by the transcription terminator *cryIIIAt* and the chloramphenicol-resistant cassette (*cat*).
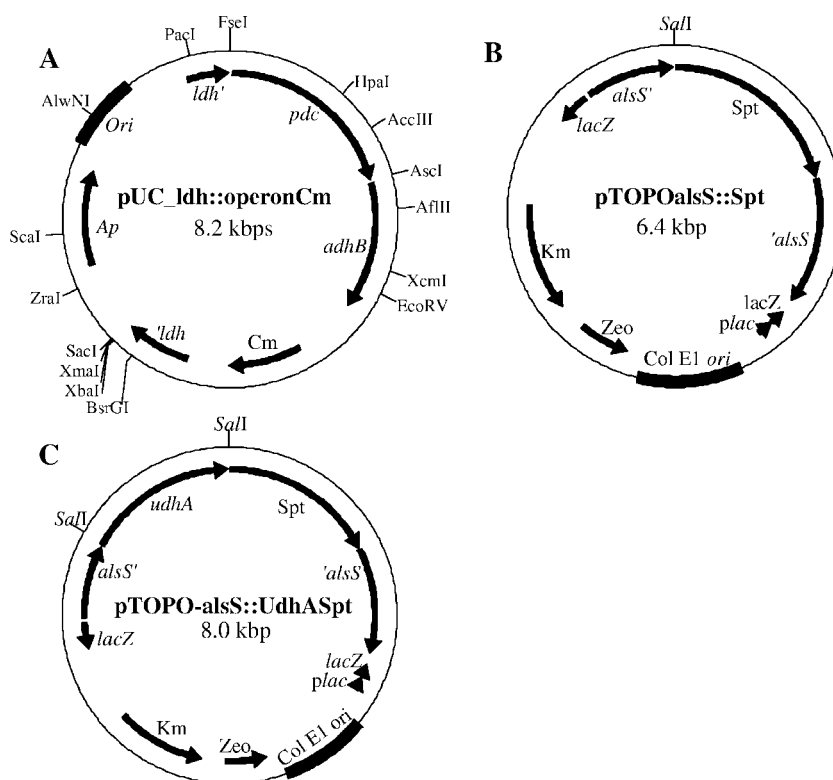

FIG. 2. Vectors constructed to inactivate (A) the *B. subtilis ldh* gene (*ldh*$_{Bs}$) by chromosomal integration of the *Z. mobilis pdc* gene (*pdc*$_{Zm}$) and *adhB*$_{Zm}$, (B) *alsS*$_{Bs}$ by chromosomal integration of the Spt cassette, and (C) *alsS*$_{Bs}$ by chromosomal integration of *E. coli udhA* and the Spt cassette.

The *ldh* gene of *B. subtilis* was amplified by PCR, using primers 6 and 7, and cloned into the HincII site of plasmid pUC19 to construct pUC-*ldh*.

The promoterless *pdc* and *adhB* genes, *cryIIIAt*, and the *cat* gene were purified as a 4.5-kbp blunt-treated AccI fragment from vector pSG-operon and ligated into the EcoRV site of the *ldh* gene in pUC-*ldh*; the resulting plasmid was pUC-*ldh*::operonCm (Fig. 2A).

Competent *B. subtilis* CH1 cells were transformed by using the suicide vector pUC-*ldh*::operonCm; thereby, *pdc* and *adhB* genes were integrated by double-crossover homologous recombination in the *ldh* locus into the *B. subtilis* CH1 genome (8) under the control of the *ldh* promoter. *B. subtilis* recombinants were selected by Cm^r and identified as being defective in lactate production under fermentative conditions. The resulting strain was named BS35.

(iii) *alsS* cloning and inactivation. The *alsS* gene was amplified by PCR from the *B. subtilis* genome using primers 8 and 9. This segment was cloned into vector pCR-TOPO in order to construct pTOPO-*alsS*. A 1.2-kbp segment containing the Spt cassette was cut out by double digestion with BamHI-HindIII from vector pCm::Spt (30). After the ends were blunt-treated, the Spt cassette was ligated into the SspI site of the *alsS* gene of pTOPO-*alsS*, generating the pTOPO-*alsS*::Spt plasmid (Fig. 2B). The inactivation of the *alsS* gene, by the insertion of the Spt cassette contained in this plasmid, was transferred to the *alsS* locus of *B. subtilis* BS35 by a double homologous recombination event. *alsS* mutants were selected by Spt^r and identified for the absence of 2,3-butanediol production under fermentative conditions. This new strain was designated BS36.

The CH1 *alsS* mutant strain was constructed to evaluate the apparent K_m of LDH in the cellular extracts of the *B. subtilis* strain. Competent cells of strain CH1 were transformed with plasmid pTOPO-*alsS*::Spt. *alsS* mutants were selected by Spt^r and identified for the absence of 2,3-butanediol production under fermentative conditions.

(iv) Inactivation of *alsS* by chromosomal integration of *E. coli* transhydrogenase gene (*udhA*). The primer design to amplify the *E. coli udhA* gene included the same translational elements used in the amplification of the *pdc* and *adhB* genes. The PCR product containing the *udhA* gene, amplified from the *E. coli* JM101 genome using primers 10 and 11, was ligated into the unique SalI site of pTOPO-*alsS*::Spt. The resulting plasmid was pTOPO-*alsS*::UdhA_Spt (Fig. 2C). Competent *B. subtilis* BS35 cells were transformed with this plasmid; the *udhA* gene was thereby integrated by double homologous recombination into the *alsS* locus into the *B. subtilis* BS35 chromosome (8), allowing the expression of *udhA* under the control of the *alsS* promoter. Transformants were selected on LB-Spt agar plates. *B. subtilis* BS37 (BS35 Δ*alsS* *udhA*$^+$) was confirmed by PCR amplification of the *udhA* gene from the *alsS* mutant's genome and identified as being defective in lactate and butanediol production under fermentative conditions.

Inoculum preparation and fermentation conditions. LB-glucose 0.2% agar plates were inoculated with cells stored at $-70°$C in glycerol and incubated overnight at 37°C. These cells were used to inoculate 500-ml flasks containing 150 ml of LB broth with 20 g/liter glucose. The flasks were incubated overnight at 37°C and 100 rpm. Cells from the flasks were centrifuged and used as inocula at an initial optical density at 600 nm (OD$_{600}$) of 0.1. Cultures were performed by duplication in minifleakers (3) containing 200 ml of LB supplemented with 20 g/liter glucose. The working conditions were 35°C, 100 rpm, and pH 7 (controlled by the automatic addition of 2 N KOH). The growth was monitored by measuring the OD$_{600}$ with a spectrophotometer (Lambda 11; Perkin Elmer, Pomona, CA). Samples were periodically taken and centrifuged; supernatants were used for analytical determinations. Cells with an OD$_{600}$ adjusted to 1 were collected at 48 h elapsed time of fermentation, centrifuged, and utilized for enzymatic activity assays.

Analytical methods. Biomass measured by the OD$_{600}$ was converted to dry cellular weight (DCW) using a standard curve (1 OD$_{600}$ = 0.35 g DCW/liter). The PDC and ADHII enzymatic activities were assayed at 30°C following protocols previously described (5, 6). The LDH activity was assayed using pyruvate, NADH, and the buffers used for the PDC assay. The apparent K_m was determined in cellular extracts of *B. subtilis* CH1 Δ*alsS*, from cells collected at 48 h elapsed time of fermentation. *B. subtilis* BS35 and BS36 were included as negative controls. For the apparent K_m determination, different concentrations of NADH (0 to 0.15 mM) and NADPH (0 to 1.3 mM) were tested, with a constant pyruvate concentration (5.0 mM), employing the same buffers used for the PDC assay, at 30°C.

The protein concentration was determined by using a commercial Bradford reagent according to the manufacturer's instructions (Bio-Rad Laboratories, Inc., United States). The glucose, pyruvate, lactate, and butanediol levels were measured by high-pressure liquid chromatography and the ethanol levels by gas chromatography as previously reported (13).
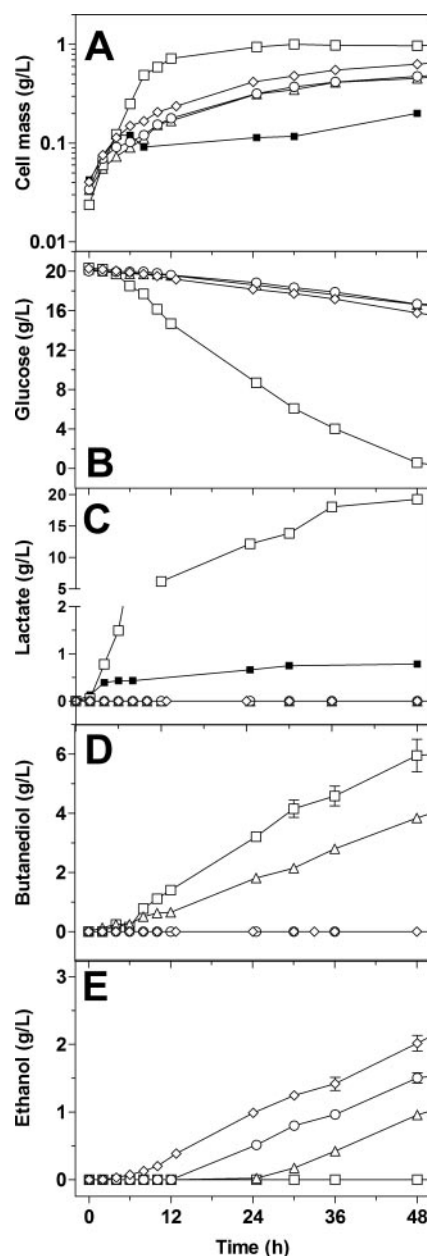

FIG. 3. Characterization of *B. subtilis* strains under fermentative conditions in LB medium containing glucose (20 g/liter). Cell mass formation (A), glucose consumption (B), lactate (C), butanediol (D), and ethanol (E) production of *B. subtilis* strains CH1 (□), BS35 (△), BS36 (○), and BS37 (◇). *B. subtilis* CH1 (■) was included as a control, using LB medium without glucose. The error bars represent the variations of duplicated experiments.

RESULTS

***B. subtilis* produces lactate and butanediol under fermentative conditions.** *B. subtilis* under anaerobic conditions metabolizes glucose to different products, depending on the culture conditions, culture media, and terminal electron acceptor (7, 10, 26). The baseline performance of the prototrophic strain CH1 was characterized under nonaerated conditions, using LB supplemented with 20 g/liter glucose (Fig. 3). During the first

TABLE 3. Kinetic parameters of *B. subtilis* CH1 and its transformants under fermentative growth in LB broth with 20 g glucose/liter[a]

Strain	μ (h^{-1} ± SD)[b]	q glucose[c] (g glucose/g DCW/h ± SD)		q ethanol (g ethanol/g DCW/h ± SD)[d]	q lactate (g lactate/g DCW/h ± SD)[d]	q butanediol (g butanediol/g DCW/h ± SD)[d]
		Exponential phase	Stationary phase			
CH1	0.35 ± 0.014	1.86 ± 0.03	0.44 ± 0.002	0	0.43 ± 0.005	0.15 ± 0.000
BS35	0.11 ± 0.015	0.66 ± 0.01	0.26 ± 0.010	0.08 ± 0.002	0	0.27 ± 0.016
BS36	0.09 ± 0.007	0.44 ± 0.02	0.23 ± 0.006	0.12 ± 0.002	0	0
BS37	0.11 ± 0.014	0.70 ± 0.03	0.23 ± 0.010	0.11 ± 0.009	0	0

[a] q, specific rate.
[b] Specific growth rate.
[c] Specific glucose consumption rate.
[d] Specific rates of ethanol, lactate, and butanediol production during the stationary phase.

10 h of the culture, *B. subtilis* CH1 grew exponentially, generating 0.6 g/liter of cell mass, producing mainly lactate (4.7 g/liter) and 2,3-butanediol (1 g/liter). Table 3 shows a summary of the kinetic parameters determined for these cultures.

During the stationary phase, a large amount of lactate (14.5 g/liter) was accumulated (Fig. 3C and Table 3). The specific glucose consumption rate diminished 75% in comparison with the rate in the exponential phase, and glucose was depleted at 48 h. A total of 5 g/liter of 2,3-butanediol was formed. Ethanol, acetate, succinate, formate, and acetoin were not detected during the culture.

B. subtilis CH1 was included as a control, using LB medium without glucose. The CH1 cells grew exponentially, generating only 0.11 g/liter of cell mass and 0.8 g/liter of lactate (Fig. 3).

B. subtilis containing *pdc* and *adhB* genes produced ethanol, but *ldh* knockout reduced growth rate and glucose consumption. Based on the high lactate productivity observed during the exponential and stationary growth phases, the *ldh* promoter was considered a good candidate to be used for driving heterologous gene expression under nonaerated conditions. Therefore, this promoter was selected to drive *pdc* and *adhB* expression in the *B. subtilis* chromosome.

Characterization of strain BS35 (CH1 *ldh::pdc-adhB*) under nonaerated conditions showed that the heterologous genes *pdc* and *adhB* are expressed, leading to the synthesis of functional enzymes. Table 4 shows a summary of the PDC, ADHII, and LDH enzymatic activities determined in strains CH1 and BS35. Interestingly, interruption of the *ldh* gene impaired the growth capacity of strain BS35 (Fig. 3A), diminishing its growth rate by 70% in comparison with that of strain CH1, possibly as a consequence of a 65% decrease in the specific glucose consumption rate (Fig. 3B and Table 3). In comparison with the titer and specific rate of butanediol production in strain CH1, the final titer and the specific rate of butanediol production were slightly reduced in strain BS35, but lactate production

was completely abolished (Table 3 and Fig. 3C and D). Even though the growth and glucose consumption rates in strain BS35 were impaired, it produced ethanol during the stationary phase (Fig. 3E), with a specific productivity of 0.08 g ethanol/g DCW/h (Table 3). Interestingly, ethanol accumulation in the culture broth was detected until 24 h elapsed time of fermentation, and 0.95 g/liter was obtained at 48 h.

Butanediol synthesis suppression increased ethanol production capacity. Acetoin synthesis from pyruvate involves two steps, catalyzed by acetolactate synthase (ALSS) and acetolactate decarboxylase (Fig. 1). Butanediol is formed from acetoin reduction (7). Thus, ALSS directly competes with PDC for pyruvate in strain BS35. Hence, to increase pyruvate availability, the *alsS* gene was interrupted as described in Materials and Methods. Insertional inactivation of *alsS* totally abolished butanediol production (Fig. 3D) and allowed the onset of ethanol production at 12 h of culture time (Fig. 3E). The specific ethanol production rate in strain BS36 increased 50% in comparison with the rate in strain BS35 (Table 3). Analysis of glucose consumption and ethanol production in the stationary phase (from 12 to 48 h) revealed that 1.5 g/liter of ethanol was produced from 3 g/liter of glucose consumed. This value corresponds to 98% of the maximum theoretical yield (0.51 g ethanol/g glucose). In comparison with the rates in strain CH1, the growth and glucose consumption rates in BS36 remained impaired.

B. subtilis L-LDH can use both NADH and NADPH as cofactors. The heterologous ethanol pathway in strain BS35 is equivalent to the native lactate pathway in strain CH1 in terms of NADH reoxidation (Fig. 1 and Table 4). Nevertheless, as shown above, the growth and glucose consumption rates were impaired when the homologous lactate pathway was replaced with the heterologous ethanol pathway.

To determine the LDH cofactor specificity, we evaluated if NADPH could be a substrate of *B. subtilis* LDH. Enzymatic assays clearly showed that both NADH and NADPH are substrates for LDH (Table 5). A control test was also performed using BS35 and BS36 cellular extracts, showing that in the absence of LDH, no NADPH reduction can be detected (Table 5).

Furthermore, to verify that only the LDH activity was responsible for NADPH oxidation, the apparent K_m (Michaelis-Menten affinity constant) was determined in cellular extracts from a *B. subtilis ldh$^+$ ∆alsS* strain (CH1 *alsS* mutant strain)

TABLE 4. Specific enzymatic activities of PDC, ADH, and LDH in *B. subtilis* strains[a]

Strain	LDH (IU/mg protein ± SD)	PDC (IU/mg protein ± SD)	ADHII (IU/mg protein ± SD)
B. subtilis CH1	7.36 ± 0.02	0.00	0.03 ± 0.02
B. subtilis BS35	0.02 ± 0.01	4.21 ± 0.03	3.19 ± 0.04

[a] Samples were collected at 48 h of nonaerated culture. Activities were replicated four times.

TABLE 5. Determination of specific enzymatic activities of LDH of *B. subtilis* strains towards two different cofactors[a]

Strain	Cofactor	Sp act (IU/mg protein ± SD)
BS35	NADPH	0.01 ± 0.01
BS36	NADPH	0.02 ± 0.005
CH1	NADPH	0.68 ± 0.04
CH1	NADH	7.36 ± 0.02

[a] Activities were replicated four times. Samples were collected at 48 h of nonaerated culture.

(Table 1). The apparent K_m values of LDH were 0.013 mM for NADH and 0.288 mM for NADPH.

The expression of a transhydrogenase improved growth and reduced the onset time for ethanol production. The *E. coli udhA* gene encodes a soluble pyridine nucleotide transhydrogenase (4). This enzyme catalyzes the reversible transfer of reducing equivalents between $NADP^+$ and NAD^+ pools according to the following equation: $NADPH + NAD^+ \Leftrightarrow NAP^+ + NADH$.

Neither any putative transhydrogenase-encoding gene nor any enzymatic system(s) that could fulfill a similar function has been identified in the *B. subtilis* genome. To evaluate if LDH could play a role in maintaining NADPH/NADP$^+$ equilibrium, the *udhA* gene from *E. coli* was integrated into the *B. subtilis* BS35 chromosome to disrupt the native *alsS* gene and, at the same time, be under the control of the *alsS* promoter (see Materials and Methods for details). Under anaerobic conditions, the *alsS* promoter is induced during the exponential phase and its expression is maintained during the stationary phase (7). A 22% improvement in the growth rate and a 59% increase in the glucose consumption rate during the exponential phase were observed (Fig. 3 and Table 3); as expected, butanediol production was abolished. Ethanol production started at 6 h of culture, and 2 g/liter of ethanol was produced at 48 h; 4.1 g/liter of glucose was consumed during this time, indicating a high conversion yield of glucose into ethanol (96% of the theoretical maximum).

***B. subtilis* can produce lactate and butanediol from rich medium.** The results with the progenitor strain CH1 indicated that higher yields were obtained in comparison with the amount of glucose consumed, considering that the theoretical maximum yields of lactate and butanediol from glucose are 1.0 g lactate/g glucose and 0.5 g butanediol/g glucose (Fig. 3). In addition, some lactate was produced by strain CH1 in glucose-free LB medium (Fig. 3). These results indicated that components from the rich medium were used to build up cell mass and synthesize fermentation products. Furthermore, LB medium supplemented with glucose allowed the generation of reducing power by strain CH1, to produce large amounts of lactate and butanediol. On one hand, BS35 produced 0.95 g ethanol/liter and 3.8 g butanediol/liter. After the inactivation of ALSS in strain BS36, no butanediol but only 1.5 g/liter of ethanol was produced at 48 h; only 0.65 g/liter more of ethanol was produced than in strain BS35, and a larger amount could be expected. Based on carbon, 2 mol of pyruvate is used to produce 1 mol of butanediol, but just 1 mol of NADH is needed. In comparison, 2 mol of pyruvate and NADH is required to form 2 mol of ethanol. Thereby, in terms of reducing

power, although a greater availability of carbon precursors to produce ethanol probably exists in BS36, the reducing power must be limiting. These results suggest that BS35 produced butanediol from carbon sources obtained from rich medium components with a lesser reducing power than glucose.

In addition, *ldh* inactivation reduced cell mass formation, but small amounts of the glucose consumed were efficiently converted into ethanol in the BS36 and BS37 strains. In these cases, it is suggested that components from the LB were only used for cell mass formation. BS37 was also cultivated in LB without glucose, and biomass formation was only 0.19 g/liter (similar to that found for the first 12 h of culture with glucose); no ethanol was produced in this condition (data not shown). These results confirm that components from rich medium were used to build up cell mass, and that actual ethanol obtained with the BS36 and BS37 strains was synthesized from the glucose consumed.

Exogenous pyruvate was channeled to ethanol, improving the growth rate. *B. subtilis* has a poor capacity to grow anaerobically in glucose-mineral medium (26). However, when glucose-mineral medium is supplemented with pyruvate or amino acids, *B. subtilis* can grow anaerobically, producing lactate, acetate, and butanediol (7, 26). Consequently, to test if pyruvate availability or the PDC- and ADHII-specific activities limit BS37's anaerobic growth in rich medium (LB), LB-glucose medium was supplemented with 2 g/liter of pyruvate (Fig. 4). It was observed that glucose and pyruvate were cometabolized during the exponential growth phase. During this phase, pyruvate and glucose were consumed at the same specific rate. The growth rate of BS37 increased from 0.11 h^{-1} (Table 3) to 0.27 h^{-1} when pyruvate was added (Fig. 3 and 4). Pyruvate was exhausted at 12 h, coincidental with the end of the exponential growth phase.

The onset of ethanol production was at 6 h of culture time; the ethanol production rate was 0.9 g ethanol/g DCW/h when glucose and pyruvate were consumed simultaneously. This rate is similar to the ethanol production rates of ethanologenic organisms like *E. coli* KO11 and yeast (13, 33). In molar terms, the sum of the glucose and pyruvate consumption rates was similar to the ethanol production rate (19 versus 22 mmol ethanol/g DCW/h), indicating that the PDC and ADHII levels in BS37 do not limit ethanol production; therefore, the expres-
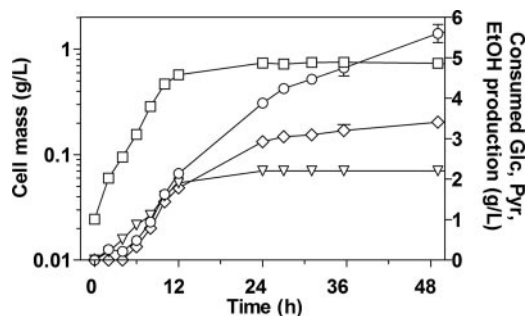

FIG. 4. Characterization of *B. subtilis* BS37 in LB supplemented with glucose (Glc; 20 g/liter) and pyruvate (Pyr; 2 g/liter) under fermentative conditions. Cell mass (□), consumed glucose (○), consumed pyruvate (▽), and ethanol (EtOH) production (◇). The error bars represent the variations of duplicated experiments.
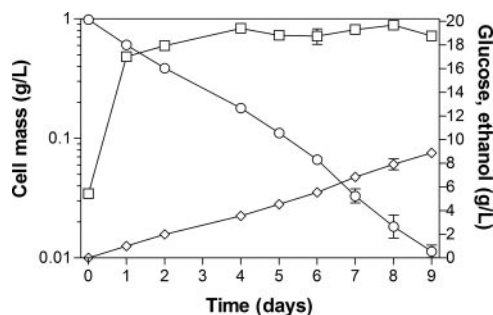
FIG. 5. Long-term fermentation of *B. subtilis* BS37 in LB supplemented with glucose (20 g/liter). Cell mass (□), glucose consumption (○), and ethanol production (◇). The error bars represent the variations of duplicated experiments.

sion level of these enzymes was not the reason for glucose consumption and growth impairment. After pyruvate depletion, 3.5 g/liter of glucose was consumed and 1.6 g/liter of ethanol was produced (i.e., 90% of the theoretical yield).

In long-term cultivation, *B. subtilis* BS37 produced 8.9 g/liter ethanol from glucose. The growth and formation of products from *B. subtilis* BS37 (*ldh::pdc_adhA ΔalsS udhA*⁺) were characterized in LB broth with 20 g/liter glucose under nonaerated conditions in long-term cultivation (Fig. 5). During the first 4 days, strain BS37 produced ethanol (3.6 g/liter) from glucose (7.5 g/liter) with a high ethanol yield (95% of the theoretical). Although the specific rate of ethanol production was low, it was constant during the 9 days of batch culture. Hence, *B. subtilis* BS37 produced 8.9 g/liter of ethanol from 20.13 g/liter of glucose, despite the ethanol yield diminishing (87% of the theoretical) during the last days, which could be an effect of ethanol evaporation. The ethanol formation during the 9 days indicates that PDC and ADHII were functional during this long period.

DISCUSSION

Several groups have tried to develop ethanologenic gram-positive bacteria by engineering lactic acid bacteria, *Corynebacterium*, and *Bacillus* species. Nevertheless, little or no ethanol has been produced by these recombinant microorganisms (2, 15, 19, 21, 27, 32).

Barbosa and Ingram (2) tried to express the *Z. mobilis pdc* and *adhB* genes by using a multicopy vector in *B. subtilis*. Using Western blot analysis, they observed that both proteins were synthesized in *B. subtilis*, although no enzymatic activity or ethanol production was reported (2). Talarico et al. (32) constructed different chimeric operons using *pdc* genes from gram-positive and gram-negative bacteria and from *Saccharomyces cerevisiae*, to be expressed in multicopy vectors in *Bacillus megaterium*. They reported different PDC activity levels for each source, indicating the importance of differences in codon usage between gram-positive and gram-negative bacteria. Despite high levels of PDC and ADH in the recombinant *B. megaterium* strain, in vivo ethanol production was null, and an in vitro assay was necessary to prove ethanol production from pyruvate (32).

LDH plays a complex role in *B. subtilis* fermentative metabolism. It has been reported that *ldh* inactivation reduced the

rate of growth of *B. subtilis* under fermentative conditions (7). In the present study, with the substitution of the combined PDC and ADH activities for LDH activity, we tried to maintain the NADH/NAD⁺ redox balance; however, the glucose consumption and growth rates of the *B. subtilis ldh* mutant were impaired. Two hypotheses could explain this phenomenon.

(i) The PDC and ADHII activity levels might not be sufficient to substitute for the previous LDH activity; thus, the NADH/NAD⁺ redox balance in this strain might be limited. Nevertheless, the *Z. mobilis* PDC and ADHII enzymes were found to be functional in *B. subtilis*, with enzymatic activity levels similar to those observed in ethanologenic *E. coli* (28). The addition of pyruvate led to an increase of 32% in the glucose consumption rate. Furthermore, it was also shown that the activities of these enzymes were sufficient to produce ethanol at a rate similar to that reported for ethanologenic *E. coli* KO11 and yeast (0.9 g ethanol/g DCW/h) (13, 33). This fact demonstrates the potential of ethanol production in BS37 and eliminates the possibility that the PDC and ADHII levels were not sufficient to support the anaerobic growth of *B. subtilis*.

(ii) *B. subtilis* LDH might contribute to maintaining NADPH/NADP⁺ equilibrium under fermentative conditions. It has been reported that *B. subtilis* LDH oxidizes NADH to NAD⁺ (14, 36). Our data proved that *B. subtilis* LDH can use both NADH and NADPH as cofactors, although the apparent K_m of LDH showed a preferential binding of NADH over NADPH. The K_m value for NADH was similar to that reported by Yoshida (36). The K_m values of LDH for NADH from different gram-positive bacteria range from 0.001 to 0.22 mM. LDH from *B. subtilis* has one of the largest reported activities and highest affinities for NADH (14). The K_m value for NADPH of *B. subtilis* LDH was similar to the K_m values for NADH of LDH from *Lactobacillus acidophilus* and *Lactobacillus jensenii* (14). Thus, the LDH from *B. subtilis* has a significant affinity for NADPH, whose influence in the NADPH/NADP⁺ equilibrium must be decisive in the fermentative metabolism of *B. subtilis*.

The transhydrogenase's enzymatic activity (4) partially restored the observed diminished growth rate, supporting the hypothesis that LDH might have an important physiological and regulatory role related to the NADPH/NADP⁺ balance. In this sense, the cofactor-level imbalance provoked by LDH inactivation might affect the metabolism of *B. subtilis*, diminishing glucose consumption in the ethanologenic *B. subtilis*—a role that the ethanologenic pathway might not fulfill.

LDH inactivation in *Lactobacillus* has also been reported to have negative effects on the glucose consumption rate and cellular growth (1, 12, 21) and on peptidoglycan precursor synthesis (11). Based on the results of these previous works and those presented in this study, it can be speculated that the LDH role in some gram-positive bacteria is as complex as it is in *B. subtilis*; it sustains not only the pyruvate transformation to lactate and NADH⁺ oxidation but also, apparently, the NADPH oxidation. Hence, the development of an ethanologenic microorganism derived from a gram-positive bacteria, such as *B. subtilis*, might require the use of other enzymatic activities, such as those of a transhydrogenase, or the directed evolution of protein to obtain new ADHs that might fulfill the complex role of LDH in the metabolism of gram-positive bac-

teria, or metabolic strategies to reduce the generation of NADPH in glucose catabolism.

High ethanol production from glucose in *B. subtilis*. This study reports the metabolic engineering of *B. subtilis* for the production of ethanol as the main fermentation product. The strategy was based on the replacement of the LDH activity by the PDC and ADHII activities. The resulting strain produced ethanol, while the parental strain did not produce this compound under the same growth conditions. To ensure the efficient expression of these genes to transcriptional and translational levels in this study, the use of a strong promoter (*ldhp*) and the *B. subtilis* Shine-Dalgarno consensus sequence (AAG GAAG) of *B. subtilis* placed at an optimum distance from the ATG start codon were included (16). In addition, to avoid metabolic burden and to have a genetically stable strain, the heterologous *pet* operon was integrated by disrupting the native *ldh* gene. At the same time, the *ldh* promoter (*ldhp*) would drive *pdc* and *adhB* expression. The combination of all these factors allowed the generation of a *B. subtilis* strain with relatively high enzymatic activities for PDC and ADHII. The enzymatic activity levels were comparable to those of the patented ethanologenic *E. coli* KO11 (24, 28), indicating adequate transcription, translation, and protein folding of PDC and ADHII in *B. subtilis* under nonaerated conditions.

When *alsS* was inactivated, the resulting ethanologenic *B. subtilis* strain reached an ethanol yield of 88.8% of the maximum theoretical value from the glucose consumed in 48 h of batch cultures, although the ethanologenic *B. subtilis* consumed only 3 g/liter during this time; this is because the specific rate of glucose consumption was low. Interestingly, besides the partial restoration of the growth rate with *udhA* expression, the onset of ethanol production was shortened, similar to the onset of lactate production in the parental CH1 strain. With the aforementioned strategy, the ethanologenic *B. subtilis* BS37 produced 9 g/liter of ethanol with an ethanol yield on glucose close to 90% during 9 days of fermentation culture. These results clearly demonstrated that it is possible to engineer *B. subtilis* to produce ethanol as a single fermentation product. The specific rate of ethanol production was relatively low and was very closely related to the specific rate of glucose consumption. In conclusion, this is the first time that a recombinant strain of *B. subtilis* could produce ethanol in vivo as a sole fermentation product with a high ethanol yield from glucose. These results open new possibilities to develop a process that takes advantage of the *B. subtilis* secretion system by using complex substrates for ethanol production.

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