Supporting Information

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SI Materials and Methods

Bacterial Strains and Plasmids. Bacterial strains, plasmids, and primers used in this study are listed in Table S1. *Bacillus coagulans* strain P4-102B was used as the wild type strain. *Escherichia coli* strain Top10 (Invitrogen) and *Bacillus subtilis* strain HB1000 (1) were used as hosts during construction of various plasmids used in this study. Plasmid pGK12 carries chloramphenicol and erythromycin-resistance genes and replicates in several Gram-positive bacteria and *E. coli* (2, 3). Although this plasmid has a broad host-range, its replication is naturally restricted to temperatures ≤42 °C. This temperature sensitive nature of plasmid pGK12 replication at 50 °C provides an opportunity to select for chromosomal DNA integrants of *B. coagulans* that can grow at 50–55 °C. Plasmid pGK12 and its derivatives were maintained in *B. subtilis* strain HB1000 at 37 °C. When transformed into *B. coagulans*, the transformants were selected and maintained at 37 °C. Various plasmids used in the construction of mutants are listed in Table S1.

Medium and Growth Condition. L-broth (LB) (4) was used as the rich medium for culture of bacteria at pH 5.0 or 7.0, as needed. Glucose was sterilized separately and added to the medium at the indicated concentration, before inoculation. Chloramphenicol, erythromycin, and ampicillin were added to LB medium at 7.5 $mg\,L^{-1}$, 5 $mg\,L^{-1}$, and 100 $mg\,L^{-1}$, respectively, when needed. Calcium carbonate medium was prepared by overlaying glucose-supplemented (2%, wt/vol) LB-agar medium with 2.5 mL of $CaCO_3$ agar [solid $CaCO_3$ suspended in water (0.1 M) with 1.5% agar] as described previously (5).

Aerobic cultures were grown in a shaker at 200 rpm. Fermentations were carried out either in small custom fermenters (200 mL medium in 500 mL vessels) (6) or in 2.5 L fermenters (New Brunswick Scientific Bioflo 110) with 1.2 L medium (7). Culture pH was maintained at the set value by automatic addition of 2N or 6N KOH. Solid $CaCO_3$ (Fisher Scientific, Pittsburgh, PA) was added at the beginning of fermentation, as needed, at a concentration of 0.2 M unless specified otherwise. Inoculum for these cultures was grown in the same medium aerobically at 50 °C overnight and fermentations were started with 1% (vol/vol) inoculum. Medium in small fermenters was stirred at 200 rpm using a magnetic stirrer with air in the gas phase. Bacterial growth started aerobically due to low starting cell density. Dissolved O_2 concentration was <0.2% when the culture density reached an optical density (OD) of about 0.6 at 420 nm. Dissolved O_2 was not detectable by O_2 electrode when the OD of the culture reached about 1.5 at 420 nm. Samples were removed periodically for determination of fermentation products and residual sugar concentration. Simultaneous saccharification and fermentation (SSF) of crystalline cellulose was as described previously (8). Solka floc (International Fiber Corp., North Tonawanda, NY) was used as the crystalline cellulose at 40 $g\,L^{-1}$ and the fungal cellulase was Biocellulase W from Kerry Biosciences (Cork, Ireland).

Metabolic Evolution. Metabolic evolution was carried out by sequentially subculturing under indicated conditions using small pH-controlled fermenters. Transfer conditions were adjusted to different time and inoculum amount. On an average, transfers were after every 2 or 3 d of growth with 2% (vol/vol) inoculum during metabolic evolution.

Transformation of *E. coli*, *B. subtilis*, and *B. coagulans*. *E. coli* transformation was based on standard techniques as described previously (9). For transformation of *B. subtilis*, cells from an overnight culture in stationary phase of growth in LB medium was inoculated (10% vol/vol) into 10 mL of freshly prepared modified competence medium (10), which contained 100 mM phosphate buffer (pH 7.0), 3 mM trisodium citrate, 3 mM magnesium sulfate, 2% glucose, 22 $\mu g\,mL^{-1}$ ferric ammonium citrate, 0.1% casein hydrolysate and 0.2% potassium glutamate, in a 125 mL Erlenmeyer flask and incubated at 37 °C with shaking for 3 h. When the $OD_{600\,nm}$ reached around 0.6, 0.6 mL of the culture was removed to a 13×100 mm test tube and DNA was added to the cells. This culture with DNA was incubated in a rotator at 37 °C for 2.5 h. Cells were collected by centrifugation at room temperature and resuspended in 0.1 mL of LB and plated on LB-agar with appropriate antibiotics. Plates were incubated at 37 °C and transformants were selected next day.

A modification of a procedure described previously was used for transformation of wild type *B. coagulans* P4-102B (11). Cells growing in 10 mL of LB in a 125 mL flask at 50 °C ($OD_{420\,nm}$ 0.3) was inoculated (10% vol/vol) into 100 mL of LB medium in a 1 liter flask. Cells were incubated at 50 °C with shaking (200 rpm) for about 3–4 h until the OD at 420 nm reached about 0.3–0.5. Cells were collected by centrifugation (4 °C; 4,300 × *g*; 10 min) and washed three times with 30, 25, and 15 mL of ice-cold SG medium (sucrose, 0.5 M, glycerol, 10%). These electro-competent cells were used immediately. The cell suspension (75 µL) was mixed with 0.1 µg of plasmid DNA and transferred to chilled electroporation cuvette (1 mm gap). The electroporation condition was set as square wave for 5 ms at 1.75 KV (BioRad electroporator; BioRad Laboratories, Hercules, CA). After electroporation, cells were transferred to 2 mL of prewarmed (37 °C or 50 °C) RG medium (LB medium with 0.5 M sucrose, 55.6 mM glucose and 20 mM $MgCl_2$). These cells were transferred to a 13×100 mm screw cap tube and incubated in a tube rotator for 3 h at 50 °C before plating on selective antibiotic medium. For transformation of temperature sensitive plasmids, the regeneration temperature was 37 °C and the cultures were incubated overnight. For transformation of the mutant strain QZ4, the DNA concentration was increased to 1 µg plasmid DNA, and the electroporation condition was altered to a time constant of 10 ms at 1.5 KV, 25 µF and 600 ohms.

Development of Genetic Tools for Deletion of L(+)-Lactate Dehydrogenase Gene (*ldh*) in *B. coagulans* Strain P4-102B. Several methods for constructing gene deletions in bacteria are available and many of these utilize appropriate linear DNA with a positive selection gene such as an antibiotic resistance gene flanked by short DNA sequence corresponding to the target gene (12–14). However, attempts to construct Δ*ldh* mutants using linear DNA were unsuccessful in *B. coagulans*. This failure to obtain desired mutants could be a result of low transformation efficiency of *B. coagulans* combined with the need for the incoming DNA to recombine to generate selectable transformants. In order to overcome this limitation, an alternate method that has proven useful in gene deletions was used (15). A temperature sensitive plasmid with appropriate target *ldh* gene sequence and erythromycin-resistance gene was constructed using plasmid PGK12 as plasmid vector (plasmid pQZ44). After transformation of *B. coagulans* strain P4-102B by electroporation, plasmid pQZ44-containing transformants were selected at 37 °C that supported stable maintenance of the plasmid. Presence of plasmid in each cell in a population (10^9 CFU mL^{-1}) at 37 °C helps to overcome the low transformation efficiency of plasmid DNA into this bacterium

and chromosomal integrants in the population were readily identified when the plasmid was eliminated by growth at 50–55 °C. Continued culturing of this plasmid-containing derivative is expected to mobilize the plasmid into the chromosome in a fraction of the population by a single homologous recombination event at the *ldh* gene. During growth at 50 °C, due to the inability of the plasmid to replicate at this temperature, plasmids will be cured off the cells and erythromycin-resistant colonies that appear at 50 °C are expected to have the plasmid DNA at the chromosomal *ldh* gene. Further cultivation of these derivatives is projected to lead to DNA rearrangements and deletion of the target gene *ldh* as indicated in Hamilton, et al. (15).

Construction of Δ*ldh* Mutant of *B. coagulans* (Strain QZ4). For construction of *ldh* deletion derivative of *B. coagulans* strain P4-102B lacking L-LDH, two sets of primers were used [primers 9 (BsaAI) and 10 (EcoRI); primers 11 (EcoRI) and 12 (StuI); Table S1] to amplify the 5′ and 3′-ends of the *ldh* gene separately using the genomic DNA from *B. coagulans* strain P4-102B. The genome sequence of *B. coagulans* strain 36D1 (GeneBank Accession number CP003056) was used to guide the design of primers for *B. coagulans* strain P4-102B genes. These primers have the indicated (in parenthesis) endonuclease recognition sequences at the 5′-end. The two amplified fragments were digested with EcoRI and ligated together. The ligation product was used as template (primers 9 and 12) to produce a promoterless *ldh* gene fragment that is lacking a 100 bp region in the middle of the *ldh* coding region starting at 431 bp from the "A" in the "ATG" of the *ldh* gene. This fragment was digested with BsaAI and StuI and ligated to similarly digested plasmid pGK12 (plasmid pQZ44). Insert in this plasmid was confirmed by sequencing. Plasmid pQZ44 was transformed into strain P4-102B and erythromycin-resistant colonies were selected at 37 °C. One of the transformants was cultured at 50 °C and an erythromycin-resistant derivative that was also L-LDH-minus (about 1%) was selected (strain QZ3). Presence of the plasmid DNA in the *ldh* gene in the chromosome of strain QZ3 was confirmed by PCR amplification of the genomic DNA with appropriate primers and sequencing the amplified product. During subcultures in medium without erythromycin, the *ldh*-minus property of strain QZ3 was found to be unstable and ldh^+ revertants were readily isolated. Strain QZ3 was serially transferred into fresh medium (1% vol/vol inoculum) everyday at 55 °C without erythromycin for 10 d. The final culture was diluted and plated on LB-agar medium. After overnight growth at 50 °C, the colonies were transferred by replica plating to LB-agar, LB-agar + erythromycin and LB-agar + calcium carbonate medium. Colonies that grew on LB-agar, but not on LB-agar + erythromycin and also did not produce lactic acid based on calcium carbonate medium (5) were picked and tested further in liquid cultures for lactate production. The second recombination is expected to yield erythromycin-sensitive derivatives lacking L-LDH activity because of the 100 bp deletion in the *ldh* gene. In these experiments, the frequency of Δ*ldh* was 1 in about 5,000 erythromycin-sensitive colonies. This method yielded *ldh* mutant strain QZ4 that was identified by the loss of erythromycin-resistance and absence of lactate as a fermentation product. The deletion within the chromosomal *ldh* gene was confirmed by PCR (primers 9 and 12; Table S1). Strain QZ4, a Δ*ldh* mutant, was selected for further study.

Using similar methods, a Δ*ldh* mutant could not be isolated using chloramphenicol as a selective marker during several attempts. Irrespective of the plasmid backbone, presence of chloramphenicol resistance gene was found to target plasmid DNA insertion to a unique location in the chromosome that is unrelated to the *B. coagulans* chromosomal DNA in the plasmid. These results indicate that chloramphenicol resistance gene is unsuitable for mutant construction in this *B. coagulans* strain.

Construction of a Δ*alsS* Mutant of *B. coagulans*. A mutant derivative of strain QZ4 lacking acetolactate synthase activity is not expected to produce acetoin and 2,3-butanediol, fermentation products produced by *ldh* mutant of *B. coagulans* (5). As a first step towards construction of this double mutant (Δ*ldh* Δ*als*), the *alsD* (alpha-acetolactate decarboxylase) and *alsS* (acetolactate synthase) DNA sequences were amplified by PCR from the genomic DNA of strain P4-102B using primers 17 and 21 (Table S1) designed based on the genome sequence of *B. coagulans* strain 36D1. This PCR fragment was treated with T4 polynucleotide kinase and ligated to HincII digested plasmid vector pUC19 to form plasmid pQZ45. This *alsSD* DNA insert in plasmid pQZ45 was verified by sequencing with appropriate primers. Primers 18 and 22 were used to amplify by PCR a 2,380 bp DNA from plasmid pQZ45 containing only the *alsSD* coding regions (without the promoter). The amplified DNA was cloned into the HincII site of plasmid pUC19 generating plasmid pQZ45-1. A 596 bp region of the *alsS* was removed from plasmid pQZ45-1 after digestion by AfeI and HincII and an erythromycin-resistance gene cassette was inserted at that location. This new plasmid, pQZ54 served as template (primers 18 and 22) to amplify a fragment with the *alsSD* genes with a 596 bp deletion in *alsS* and the gene encoding erythromycin-resistance. The PCR product was phosphorylated with polynucleotide kinase and ligated to plasmid pGK12 digested with BsaAI and AfeI. The resulting plasmid, pQZ64 was transformed into *B. coagulans* strain QZ4 (Δ*ldh*) by electroporation and erythromycin-resistant colonies were selected at 37 °C. Using the procedures described above for construction of Δ*ldh*, Δ*alsS* mutation was introduced into strain QZ4. This method yielded several *alsS* mutants differing in their growth rates under both aerobic and anaerobic condition. One of the mutants with the highest aerobic growth rate (strain QZ5) was selected for further study.

DNA sequence of the *ldh*, *ldhA* and *alsSD* genes from *B. coagulans* strain P4-102B were deposited in GenBank and their accession numbers are HQ699676, HQ699678, and HQ699677, respectively.

Determination of mRNA Levels. For determination of mRNA levels in *B. coagulans*, cells were collected by centrifugation (16,000 × *g*; 30 sec, room temperature). RNA was isolated using the acid phenol extraction method described before (5). Total RNA concentration was determined from the absorbance at 260 nm (NanoVue, GE, Piscataway, NJ). The cDNA copy was prepared with Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) using primers specific for the gene of choice. The cDNA (mRNA) concentration was determined by PCR using gene-specific primers and SYBR-green containing PCR reaction mix (BioRad Laboratories). The threshold cycle for each of the PCR reaction with different concentrations of cDNA was determined and compared against a standard DNA template that was also run at the same time (16). From these results, a ratio of the concentration of gene-specific mRNA in the sample was calculated. Reported results are the average of at least three experiments with variation of less than 15%.

The primers used for RT-PCR are listed in Table S1; *ldh* primers (L-LDH)—primers 23 and 24, *ldhA* primers (D-LDH)—primers 29 and 30, *polA* primers used as internal control—primers 33 and 34, *gldA* primers—primers GDH11-F and GDH10-R.

Cloning *gldA101* from *B. coagulans* Strain QZ19. Plasmid pQZ115 that contained the *gldA101* allele encoding mutated form of glycerol dehydrogenase (GlyDH*) from *B. coagulans* strain QZ19 was constructed using plasmid pUC19 as vector and a PCR product derived from strain QZ19 genomic DNA as template using primers D-LDH-3 and GDH-6 (Table S1). Appropriate primer design for PCR was based on the *gldA* sequence derived from the genome sequence of a related *B. coagulans* strain 36D1. The

PCR product was treated with T4-polynucleotide kinase and ligated to plasmid pUC19 linearized with HinCII. After transformation of *E. coli* strain Top10, plasmid-containing derivatives were selected as white colonies on LB-agar medium with ampicillin and X-gal. One of the plasmids, pQZ115, contained a 3,422 bp insert DNA that was confirmed by DNA sequencing. The same selection also yielded plasmid pQZ109 that contained a 3,185 bp insert, also verified by DNA sequencing. Insert in plasmid pQZ109 lacks 237 bp of DNA at the 3′-end of the insert that removed the last 10 amino acids of the GlyDH* protein in addition to the 3′-noncoding region of *gldA101*. Plasmid pQZ113, used for expression and purification of His-tagged GlyDH* from *E. coli*, was constructed by inserting a PCR product (only the *gldA101* coding region) derived from strain QZ19 genomic DNA (primers GDH-7 and GDH-12) into plasmid vector pET15b linerized with XhoI and BamHI. After confirming the sequence of the insert, plasmid pQZ113 was transformed into *E. coli* strain Rosetta 2 and the His-tagged GlyDH* was expressed and purified as described before for other His-tagged proteins (16).

Purification of the Enzyme with D-LDH Activity in Strain QZ19. Strain QZ19 was cultured in a pH-controlled fermenter to midexponential phase of growth in LB + glucose (0.16 M) and the cells were harvested at 4 °C after centrifugation at 4,000 × *g* for 10 min. Cells were washed once in 50 mM phosphate buffer, pH 7.5 (phosphate buffer) and resuspended in the same buffer. Cells were broken by passage through a French pressure cell operating at 20,000 PSI. Cell extract was collected after centrifugation at 20,000 × *g*. Proteins were differentially precipitated with ammonium sulfate and the D-LDH activity remained in solution after precipitation of proteins with ammonium sulfate at 60% saturation. After dialysis, the protein fraction with D-LDH activity was loaded on a Q-sepharose column equilibrated with phosphate buffer and the proteins were eluted by a NaCl gradient (0.1 to 0.5 M in phosphate buffer). The D-LDH activity eluted at about 0.3 M NaCl. Fractions with D-LDH activity were pooled and dialyzed. The next purification step, hydrophobic chromatography, utilized HiTrap Butyl-sepharose (GE-life sciences). Starting buffer was phosphate buffer with 1 M ammonium sulfate. Proteins were eluted with a decreasing concentration of ammonium sulfate from 1 to 0 M. Fractions with D-LDH activity were identified and tested for purity by SDS-PAGE (17). Several fractions contained a single protein as determined by SDS-PAGE. One of the fractions was also tested for purity by 2-D-PAGE. The 2-D-PAGE (pH range 3–10) was performed by the Protein core at the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida. The protein present in the gel was identified using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) after trypsin digestion, also by ICBR. LC-MS/MS analysis was carried out on a hybrid quadrapole-TOF mass spectrometer (QSTAR elite, Applied Biosystems). Tandem mass spectra were extracted by ABI Analyst Version 2.0 and the samples were analyzed using Mascot (Matrix Science, London, United Kingdom). Mascot was set up to search NCBI database including *B. coagulans* strain 36D1 draft genome sequence. Scaffold (Proteome software, Inc. Portland, OR) was used to validate MS/MS based peptide and protein identifications (18, 19).

Enzyme Assays. To determine the level of various enzyme activities in crude extracts, cells were cultured in LB-glucose medium in a fermenter until the culture reached mid- to late- exponential phase of growth. Cells were harvested by centrifugation (10,000 × *g*, 10 min; room temperature), washed once with 10 mL of phosphate buffer (50 mM, pH 7.0) and resuspended in 5.0 mL of same phosphate buffer. Cells were broken by passage through a French pressure cell (20,000 PSI). The cell extract was centrifuged at 12,000 × *g* for 30 min at 4 °C to remove the cell debris and the supernatant was used for enzyme assay. Protein concentration was determined by Bradford method with bovine serum albumin as standard (20).

LDH activity was assayed as described previously (21) as the oxidation of NADH in the presence of pyruvate. Each 1 mL reaction mixture contained potassium phosphate buffer (50 mM; pH 7.5), NADH (0.1 mM) and crude extract. Reaction was started by the addition of pyruvate (25 mM). Glycerol dehydrogenase activity was determined as described previously (22) in carbonate/bicarbonate buffer (125 mM; pH 10.0) with NAD^+ (3.3 mM) and ammonium sulfate (30 mM). Reaction was started with glycerol (100 mM). Enzyme activity is expressed as μmoles min^{-1} mg $protein^{-1}$.

Analytical Methods. Glucose and fermentation products were determined by HPLC with Aminex HPX-87H ion exclusion column (300 mm × 7.8 mm) as before (23). Optical isomers of D-(−)- and L-(+)- lactic acids were determined by HPLC with a Chirex 3126(D)-penicillamine column (150 × 4.6 mm, 5 micron) (Phenomenex) with 2 mM $CuSO_4$ as mobile phase. The D-(−)-lactate was also analyzed by enzyme-based method with D-lactate dehydrogenase (Sigma Chemical Co., St. Louis, MO).

Materials. Biochemicals were from Sigma Chemical Co. and organic and inorganic chemicals were from Fisher Scientific (Pittsburgh, PA). Molecular biology reagents and supplies were from New England Biolabs (Ipswich, MA), Invitrogen (Carlsbad, CA), or BioRadLaboratories (Hercules, CA).

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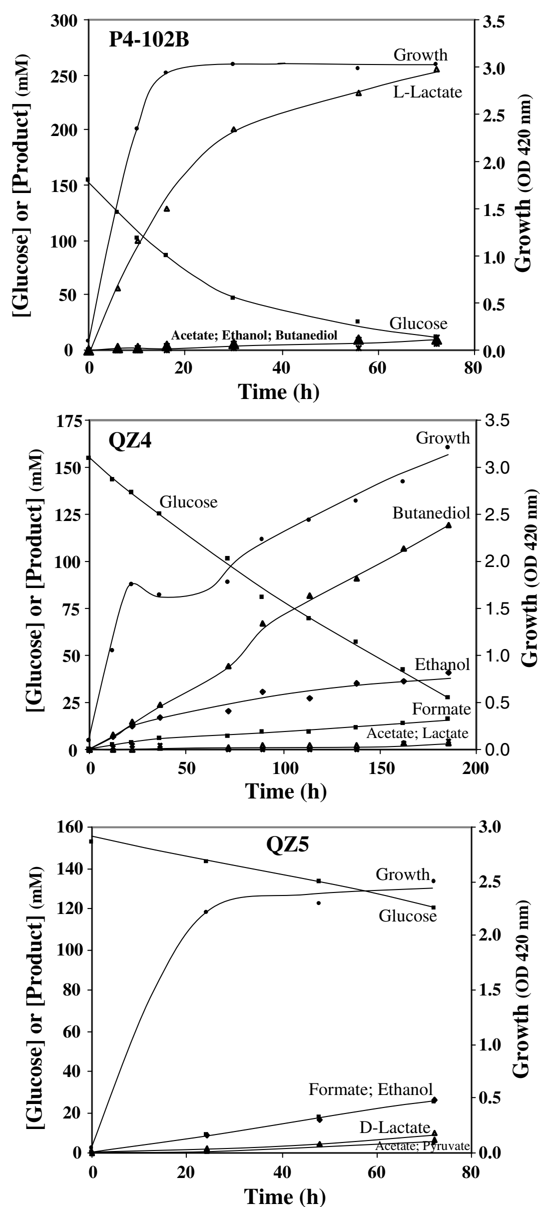

Fig. S1. Fermentation profile of *B. coagulans* wild type P4-102B and its *ldh* (strain QZ4), and *ldh*, *als* (strain QZ5) mutants. Fermentations were at 50 °C in small fermenters with pH control at 5.0 by automatic addition of KOH in LB + glucose (0.16 M).

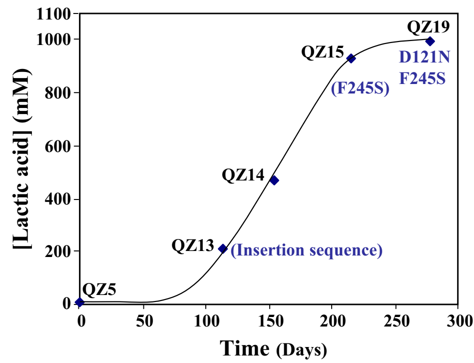

Fig. S2. Metabolic evolution of *B. coagulans* strain QZ5 to D-lactate producing strain QZ19 in small fermenters. Lactic acid titer during metabolic evolution was determined every three days. See the section on *Metabolic Evolution* in the text and also Fig. S3 legend for other details.

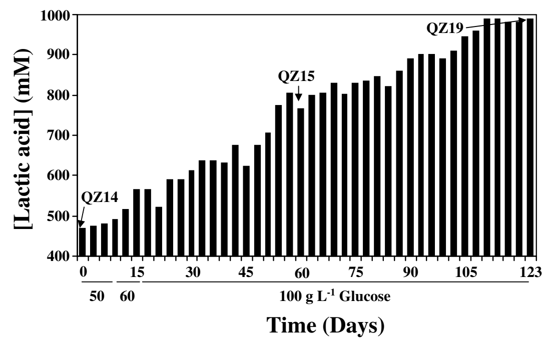

Fig. S3. Metabolic evolution of *B. coagulans* strain QZ14 in small fermenters in LB + glucose at pH 7.0 with increasing glucose concentration. Medium also contained 0.2 M $CaCO_3$. Starting glucose concentration was 50 g L^{-1} (0.28 M). After the third transfer glucose concentration was increased to 60 g L^{-1} (0.33 M). Glucose concentration of the medium was 100 g L^{-1} (0.56 M) after the fifth transfer. Lactic acid titer of the culture was determined after 3 d of each transfer. After 60 d of incubation, strain QZ15 that produced about 0.8 M lactic acid was isolated. Strain QZ19 was isolated after an additional 63 d of metabolic evolution.

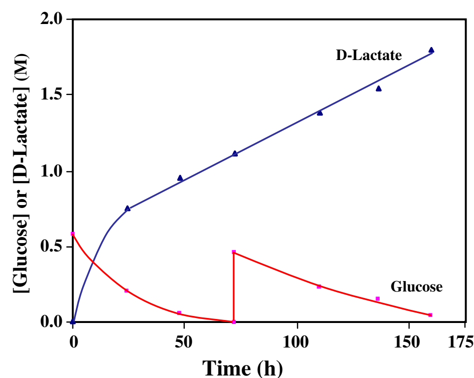

Fig. S4. Fed-batch fermentation of glucose to D-lactic acid by *B. coagulans* strain QZ19, at pH 5.0 and 50 °C. Fermentations with pH control in LB + glucose were started with 100 g L^{-1} (0.56 M) of glucose and at 72 hours, another 100 g L^{-1} (0.56 M) of glucose was added. Culture pH was maintained at 5.0 by $Ca(OH)_2$ addition. Results from a typical experiment are presented.

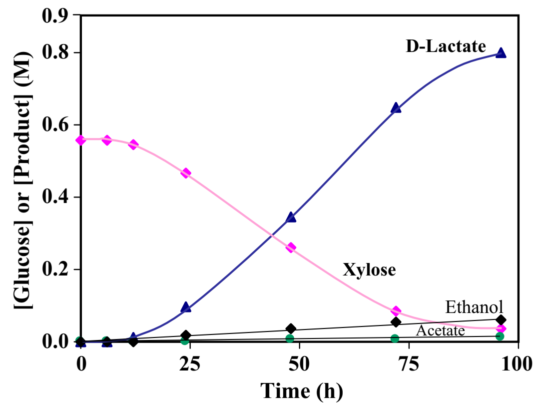

Fig. S5. Fermentation of xylose to D-lactic acid by *B. coagulans* strain QZ19 at 50 °C and pH 5.0. LB medium contained 80 g L^{-1} (0.53 M) of xylose and 0.2 M $CaCO_3$. Results from a typical experiment are presented.

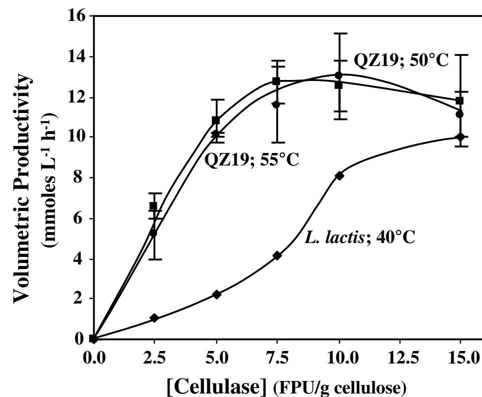

Fig. S6. Simultaneous saccharification and fermentation of crystalline cellulose to D-lactic acid by *B. coagulans* strain QZ19 at 50 °C, pH 5.0. *Lactococcus lactis* data was from Ou, et al. (8) and included for comparison. Initial cellulose (Solka Floc) concentration was 40 g L^{-1}.

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  1 mTKIITSPSK FIQGPDELSR LSAYTERlgk KAFIIADDFV TGLVGKTVEE SYAGKETGYQ
 61 mALFGGEcSK PEIERLcEMS KSEEADVVVG IGGGKtldta kavgyynnip vivaptiast
121 naptsalsvi ykengefeey lmlplnptfv imdtkviasa parllvsgmg dalatyfear
181 atkrankttm aggrvteaai alaklcydtq ileglkakLA AEKHLVTEAV EKiieantyl
241 sgigsesggl aaahaihngl tvleethhmy hgekVAFGTL AQLILEDAPK aeieevvsfc
301 lsvglpvtlg dlgvkelnee klrkvaelsc aegetiynmp fevtpdlvya aivtadsvgr
361 yykekwa
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Fig. S7. Amino acid sequence of glycerol dehydrogenase with D-lactate dehydrogenase activity from *B. coagulans* strain QZ19. The amino acids in upper case and red were identified after trypsin digestion and LC-MS/MS of fragments.

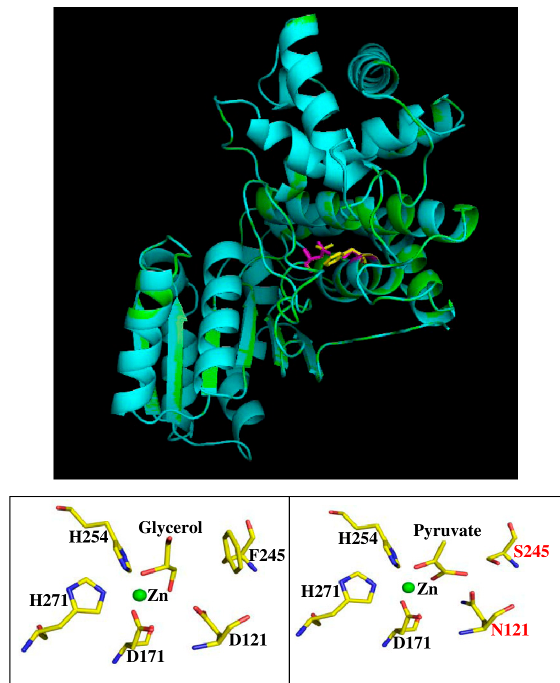

Fig. S8. Model of *B. coagulans* GlyDH based on *B. stearothermophilus* GlyDH (PDB 1JPU) constructed by Swiss-Model. Native enzyme is colored green and the enzyme from QZ19 (GlyDH*) is in cyan and are superimposed. Amino acids 121 and 245 are highlighted; native amino acid in yellow and the altered amino acid (strain QZ19) in magenta. Bottom: Only the amino acids at the active site are listed. Left, native GlyDH with glycerol at the active site; Right, GlyDH* (D121N, F245S) with pyruvate at the active site.

Table S1. Bacterial strains, plasmids and primers used in this study

Strain	Relevant genotype	Source or reference
B. coagulans P4-102B	wild type	(1)
E. coli Top10	*mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*Δ*M15* Δ*lacX74 nupG recA1 araD139* Δ(*ara-leu*)*7697 galE15 galK16 rpsL*(Str^R) *endA1*	Invitrogen
E. coli AH242	Δ*ldhA*, Δ(*focA-pflB*)	(2)
B. subtilis HB1000	SPβc2Δ2::Tn*917*::pSK106 *attSP*β	(3)
QZ3	P4-102B *ldh*::pQZ44, Em^R	this work
QZ4	QZ3 Δ*ldh*	this work
QZ5	QZ4 Δ*alsS*	this work
QZ13	QZ5 evolved at pH 5.0 for higher cell yield	this work
QZ14	QZ13 evolved at pH 7.0 for higher lactic acid titer	this work
QZ15	QZ14 evolved for higher sugar use	this work
QZ19	QZ16 further evolved for higher rate of lactate production.	this work
Plasmid		
pGK12	broad host-range, Cm^R, Em^R	(4)
pUC19	plasmid vector Ap^R	lab stock
pQZ44	pGK12 with promoterless *ldh* (P4-102B) with 100 bp deletion	this work
pQZ45	pUC19 with P4-102B *alsSD*	this work
pQZ45-1	pUC19 with 2,380 bp promoterless P4-102B *alsSD*	this work
pQZ54	pQZ45-1 with 596 bp *alsS* deletion with Em^R gene insertion	this work
pQZ64	pGK12 with 506 bp *alsS* deletion with Em^R gene insertion	this work
pQZ109	pUC19 with 3,185 bp fragment from QZ19 with truncated *gldA*	this work
pQZ113	pET15b with *gldA* from QZ19	this work
pQZ115	pUC19 with 3,422 bp fragment from QZ19 with *gldA*	this work
Primer		
Primer9	ccctacgtaTTGGAACGGGTGCAGTTGGT	this work
Primer10	cccgaattcCCGGGTTGCTGGCAACAAGA	this work
Primer11	cccgaattcTTTGAGCGCCCAATTTGGAA	this work
Primer12	cccaggcctCCGGAACGCCAACGTACACA	this work
Primer17	ACGAGCCGCTGACACTGGAT	this work
Primer18	GCCGTCTTCGCCTTCGTTCA	this work
Primer21	TGTCATAAGTCGCCGAACCG	this work
Primer22	TGATTGTATGCCGCCACGAA	this work
Primer23	GGTGTTGCAGAAGAGCTTGT	this work
Primer24	GTGCCGCAATCGGAATAATC	this work
Primer29	AGATCTTAAGCCGTGTGGAG	this work
Primer 30	CGCAACAATACTGCCGATTC	this work
Primer33	TTGGAGGCGAACAAAGAACA	this work
Primer 34	CGGCAATGGAAAAAGAAATG	this work
Primer GDH10-R	AGTCCGACACTCAGGCAGAA	this work
Primer GDH11-F	GGCTTACCGTGCTCGAAGAA	this work
D-LDH-3	GCGTTCTTCTGCTAACATCC	this work
GDH-6	GTGCTCGCTTCCTATATCGT	this work
GDH-7	gggctcgagATGACGAAAATCATTACCTC	this work
GDH-12	gggggatccGTGCTCGCTTCCTATATCGT	this work

Capital letters in the primers represent *B. coagulans* sequence. Lower case letters in the primers indicate the restriction enzyme recognition sequence and 5′extensions for optimum cleavage of the PCR-amplified product by the respective enzyme.

1 Patel MA, et al. (2006) Isolation and characterization of acid-tolerant, thermophilic bacteria for effective fermentation of biomass-derived sugars to lactic acid. *Appl Environ Microbiol* 72:3228–3235.
2 Kim Y, Ingram LO, Shanmugam KT (2007) Construction of an *Escherichia coli* K-12 mutant for homoethanologenic fermentation of glucose or xylose without foreign genes. *Appl Environ Microbiol* 73:1766–1771.
3 Fredrick K, Helmann JD (1996) FlgM is a primary regulator of sigmaD activity, and its absence restores motility to a *sinR* mutant. *J Bacteriol* 178:7010–7013.
4 Kok J, van der Vossen JM, Venema G (1984) Construction of plasmid cloning vectors for lactic streptococci which also replicate in *Bacillus subtilis* and *Escherichia coli*. *Appl Environ Microbiol* 48:726–731.