Rewiring *Lactococcus lactis* for Ethanol Production

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Lactic acid bacteria (LAB) are known for their high tolerance toward organic acids and alcohols (R. S. Gold, M. M. Meagher, R. Hutkins, and T. Conway, J. Ind. Microbiol. 10:45–54, 1992) and could potentially serve as platform organisms for production of these compounds. In this study, we attempted to redirect the metabolism of LAB model organism *Lactococcus lactis* toward ethanol production. Codon-optimized *Zymomonas mobilis* pyruvate decarboxylase (PDC) was introduced and expressed from synthetic promoters in different strain backgrounds. In the wild-type *L. lactis* strain MG1363 growing on glucose, only small amounts of ethanol were obtained after introducing PDC, probably due to a low native alcohol dehydrogenase activity. When the same strains were grown on maltose, ethanol was the major product and lesser amounts of lactate, formate, and acetate were formed. Inactivating the lactate dehydrogenase genes *ldhX*, *ldhB*, and *ldh* and introducing codon-optimized *Z. mobilis* alcohol dehydrogenase (ADHB) in addition to PDC resulted in high-yield ethanol formation when strains were grown on glucose, with only minor amounts of by-products formed. Finally, a strain with ethanol as the sole observed fermentation product was obtained by further inactivating the phosphotransacetylase (PTA) and the native alcohol dehydrogenase (ADHE).

More than 85 billion liters of bioethanol is currently being produced globally each year (Renewable Fuels Association, 2012 [<http://www.ethanolrfa.org/>]), and ethanol is the main fermentation-derived biofuel on the market, with a well-established technology behind the process. Yeast has been and still is the major production organism, partly because of its robustness but also because it is able to grow on cheap industrial media (1, 2). But there are drawbacks to yeast. Being a eukaryote, it grows and ferments slowly, is capable of fermenting only a few sugars naturally, and tolerates high temperatures poorly (3). The desire to use lignocellulosic biomass (LCB) as a raw material for ethanol production also points to production organisms other than yeast. LCB is readily available (4, 5) and contains large amounts of carbohydrates (6), but the pretreatment process required releases or generates compounds, such as acetate, that are inhibitory to growth of microorganisms (7, 8). Yeast is no exception, and especially acetate affects growth negatively (9). A huge effort has been put into developing the process, and novel genetically engineered microorganisms for ethanol fermentation have also been constructed (4). Common approaches have been to improve existing ethanol-producing microorganisms, e.g., by increasing the capability to metabolize various sugars (10) or the tolerance toward inhibitors (11–13). The ethanol pathway has also been introduced into hosts with advantageous properties such as ease of genetic manipulation (14), high ethanol tolerance (15), or the ability to degrade LCB-derived sugars (16). Lactic acid bacteria (LAB) as a group share many of these useful properties. Many LAB naturally ferment both hexoses and pentoses, tolerate high concentrations of organic acids, and tolerate harsh conditions (17). LAB have an innate high tolerance to ethanol and are frequently found in bioethanol plants as contaminants (18). They are able to grow at low pH, and some also thrive at elevated temperatures (19), both properties that are important for avoiding contamination (18), and in addition they meet the requirements for simultaneous saccharification and fermentation (SSF), as the cellulases work optimally at around pH ~5 and 50°C.

It has previously been attempted to turn LAB into ethanol producers, but these efforts have met with only limited success (15, 17, 20–22). In this study, the target organism is *Lactococcus lactis*,

which is the best-characterized LAB to date and one of the Gram-positive model organisms. We introduce codon-optimized genes encoding *Z. mobilis* pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADHB), either in the wild-type strain or in strains with combinations of competing pathways eliminated. The strains are characterized in growth experiments, and the best-performing strains produce exclusively ethanol.

MATERIALS AND METHODS

Strains and plasmids. The plasmid-free strain *Lactococcus lactis* subsp. *cremoris* MG1363 (23) or derivatives thereof were used for the studies described in this article. LB436, a derivative of MG1363 containing the plasmid pLB65, expressing phage TP901-1 integrase (24), was used for integrating genes into the TP901-1 attachment site (*attB*) on the chromosome. *Escherichia coli* strain ABLE-C, *E. coli* C *lac*(*lacZ*[−]) [Kan^r McrA[−] McrCB[−] McrF[−] Mrr[−] HsdR (r_K[−] m_K[−])] [F' *proAB lacI*^qZΔM15 Tn10 (Tet^r)] (Stratagene) was used for cloning purposes. The plasmid pCS1966 (25) was used for the purpose of deleting various genes in *L. lactis*. The plasmid pLB85 (24) was used for integrating genes into the chromosome of *L. lactis*. The plasmid pTD6 (this work) was used for expressing the pyruvate decarboxylase and alcohol dehydrogenase genes.

Growth media and growth conditions. *E. coli* strains were grown aerobically at 30°C in Luria-Bertani broth (26). For growth experiments, *L. lactis* was grown in batch cultures (250-ml flasks) with slow stirring and no aeration in defined SA medium (27) devoid of acetate but supplemented with 2 μg/ml of lipoic acid and nucleosides (20 μg/ml of adenosine, guanosine, cytidine, thymidine, uridine, and inosine) (SALN). The cultures were supplemented with 2.0 g/liter of glucose or maltose and incubated at 30°C. For other purposes, rich M17 broth (28) supplemented with 10 g/liter glucose was used. When required, antibiotics were added at the following concentrations: erythromycin, 200 μg/ml for *E. coli* and 5 μg/ml for *L. lactis*; tetracycline, 8 μg/ml for *E. coli* and 5 μg/ml for *L. lactis*.

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HPLC analysis. Regular measurements of optical density at 600 nm (OD_{600}) were made, and high-pressure liquid chromatography (HPLC) samples were taken for measuring the product formation and glycolytic flux. HPLC analysis of the fermentation broth was carried out on an Ultimate 3000 high-pressure liquid chromatography system (Dionex, Sunnyvale, CA) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA) and a Shodex RI-101 detector (Showa Denko K.K., Tokyo, Japan). The column oven temperature was set at 60°C, the mobile phase was at 5 mM H_2SO_4 , and the flow rate was 0.5 ml/min.

DNA techniques. All manipulations were performed according to Sambrook et al., 1989 (26). A description of the PCR primers used can be seen in Table 1. PfuX7 polymerase (29) was used for PCR applications. Chromosomal DNA from *L. lactis* was isolated using the method described for *E. coli* with the modification that cells were treated with 20 μ g of lysozyme per ml for 2 h before lysis. Cells of *E. coli* were transformed using electroporation. Cells of *L. lactis* were made electrocompetent by growth in GM17 medium containing 1% glycine and transformed by electroporation as previously described by Holo and Nes (30). The plasmid vector pCS1966 (25) was used for deleting genes in *L. lactis*. The primers used are indicated in Table 1. Generally, when deleting chromosomal genes, 800-bp regions upstream and downstream of the deleted region were PCR amplified and inserted into pCS1966. The resulting plasmids were used as described previously (25). The primers used for amplifying the upstream and downstream regions are indicated in Table 1 as “geneX ups” and “geneX dwn,” respectively.

Construction of *L. lactis* strains with pyruvate decarboxylase activity. A PCR product was generated using primers 690/691 and digested with XbaI/PstI and ligated to plasmid pLB85 digested with the same enzymes. The ligation was transformed into *L. lactis* LB436, in which an integrase mediates site-specific integration into the chromosome. Transformants were selected on GM17 with erythromycin and 200 μ g/ml 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-Gluc). The strains with the highest expression of pyruvate decarboxylase were CS3720, CS3723, CS3756, and CS3754.

Deleting the LDH genes. Derivatives of pCS1966 for deleting lactate dehydrogenase (LDH) genes *ldhX*, *ldhB*, and *ldh* were constructed as described above. The plasmids used for deleting *ldhX*, *ldhB*, and *ldh* are pCS4026, pCS4020, and pCS4104, respectively. Deletion was verified using primers 774/777 (*ldhX*), 769/771 (*ldhB*), and 788/789 (*ldh*). The strain containing all three deletions was named CS4099 or MG1363 Δ^3ldh .

Construction of the first series of strains with pyruvate decarboxylase and alcohol dehydrogenase activity based on MG1363 Δ^3ldh . The pyruvate decarboxylase gene (*pdh*) was amplified using primers 690/829, and the alcohol dehydrogenase gene *adhB* was amplified using primers 830/791. In both cases, the templates were synthetic codon-optimized genes based on *Zymomonas mobilis* *pdh* and *adhB* genes (GenScript). These fragments were digested with XbaI/EcoRI and EcoRI/PstI, respectively, and ligated to pLB85 digested with XbaI/PstI. The ligation was transformed into CS4099 (MG1363 Δ^3ldh) containing the integrase plasmid pLB65. The strains CS4113 to CS4118 were the best ethanol producers, and the integrity of *pdh* and *adhB* was verified by sequencing.

Construction of the second series of strains with pyruvate decarboxylase and alcohol dehydrogenase activity based on MG1363 Δ^3ldh . SP (synthetic promoter)-*pdh-adhB* PCR fragments were generated using primers 845/263, 846/263, 847/263, and 848/263 and CS4116 chromosomal DNA as the template. These PCR products were digested with XbaI/PstI and ligated to PCR-amplified (using primers 702/703) pLB85 digested with the same enzymes. Each digested SP-*pdh-adhB* fragment was ligated to the digested vector and transformed into CS4099 (MG1363 Δ^3ldh) containing the integrase plasmid pLB65. The resulting strains are CS4137 (promoter from CS3720), CS4138 (promoter from CS3723), CS4139 (promoter from CS3756), and CS4157 (promoter from CS3754).

Introduction of ethanol genes in an MG1363 Δ^3ldh Δpfl strain. The plasmid pCS4122 was used for deleting *pfl* as described above. The MG1363 Δ^3ldh Δpfl strain was transformed with the integrase plasmid

pLB65, resulting in strain CS4197. The ligations used for constructing the second series of strains with pyruvate decarboxylase and alcohol dehydrogenase were transformed into *E. coli* ABLE-C, and clones containing plasmids with correct-size inserts were isolated (CS4177 to CS4193). Strain CS4210, the result of transforming CS4197 (MG1363 Δ^3ldh Δpfl) with pCS4184 (plasmid from CS4184), was characterized further in this study.

Constructing an MG1363 Δ^3ldh Δpta $\Delta adhE$ strain expressing the *pdh-adhE* operon from plasmid pTD6. The *pta* and *adhE* genes were deleted using pCS4230 and pCS4273, respectively, as described above. The *L. lactis* strain with deleted *ldh* genes and *pta* is CS4234, and CS4363 is MG1363 Δ^3ldh Δpta $\Delta adhE$. An SP-*pdh-adhB* fragment was amplified from CS4116 chromosomal DNA using primers 947/894, phosphorylated using T4 polynucleotide kinase, and ligated to pTD6 amplified using primers 891/892. The ligation was transformed directly into CS4363 (MG1363 Δ^3ldh Δpta $\Delta adhE$). CS4435 is a strain expressing the *pdh-adhB* genes and was characterized in this study. The plasmid pTD6 is a derivative of pAK80 (31) containing a *gusA* reporter gene. A PCR fragment was amplified from pAK80 using primers ptd45/ptd46. The *gusA* gene was amplified from *E. coli* MG1655 (32) using primers ptd1/ptd2. After digesting both fragments with BglII and AvrII, the fragments were ligated and transformed into *E. coli* strain MC1000 (33). The resulting plasmid was named pTD5. The erythromycin marker of pTD5 was replaced by a tetracycline marker by PCR amplifying pTD5 using primers ptd49/ptd50, digesting the product with StuI/ApaI, and ligating it with a StuI/ApaI fragment containing the tetracycline resistance genes amplified from pG⁺host8 (34) using primers ptd51/ptd52. The ligation was introduced into *E. coli* MC1000, and the result was plasmid pTD6.

RESULTS

Ethanol tolerance of *L. lactis* MG1363. When choosing an organism for production of ethanol, it is reasonable to select one that has a high tolerance toward ethanol. In this case, it is known that LAB generally are quite tolerant, and the main reason for choosing *L. lactis* is because of its status as the model organism of LAB and the well-developed genetic toolbox available for it and not for its ethanol tolerance. Nonetheless, in this work we have determined the tolerance of the wild-type *L. lactis* strain MG1363 and a mutant derivative selected by growing MG1363 in the presence of increasing ethanol concentrations over 2 months. Table 2 shows that the wild-type strain grows, although slowly, in the presence of 6% ethanol. The mutant, however, is capable of growth in the presence of 8% ethanol.

Introduction of pyruvate decarboxylase activity in *L. lactis*. Previous attempts to divert the product flux toward ethanol in LAB have not been completely successful (15, 17, 20–22). In the current study, a codon-optimized version of the *Z. mobilis* gene encoding PDC (*pdh*) was introduced and expressed from synthetic promoters as described in Materials and Methods. The synthetic promoter approach used for this study is described in Solem and Jensen, 2002 (35). It involves fusing genes to a range of synthetic promoters in a single step using PCR. The synthetic promoters are incorporated in a PCR primer and consist of consensus sequences flanked by degenerate regions, which in principle should generate all promoter strengths possible. A transcriptional fusion between *pdh* and the *E. coli* *gusA* gene encoding β -glucuronidase was created to facilitate subsequent screening. Four strains with high expression levels were characterized in growth experiments, and the product composition was determined. *L. lactis* naturally produces ethanol, but usually in small amounts, and the major fermentation product on fast-fermentable sugars is lactate. An overview of the glucose/maltose catabolism can be seen in Fig. 1, in which the most common products are indicated. During growth on glucose,

TABLE 1 Primers used in this study^a

Name	Use	Sequence (5' → 3')
43 (T3)	Verify insert in pCS1966	AATTAACCCCTCACTAAAGGG
263	5' end of <i>gusA</i> , rev	CGCGATCCAGACTGAATG
603	Verify insert in pCS1966	ATCAACCTTTTGATACAAGGTTG
690	Modulate <i>pdh</i> expression, XbaI	CTAGTCTAGACTGAACCCCTACCGNNNNNAGTTTATTCTTGACANNNNNNNNNNNNNNTGRTAT AATNNNNNAAGTAATAAAATATTCGGAGGAATTTGAAATGAGTTATACAGTAGGAAC
691	Used with 690, PstI	TCGGACTGCAGTTACAACAATTTATTTACAGGTTTTT
702	pLB85	GAGTCGACCTGCAGAATTC
703	pLB85, XbaI	TCGACTCTAGAGGATCTAC
768	<i>ldhB</i> ups, PstI	AATTCCTGCAGCATATTAAATATGAACCAAGTCATTG
769	<i>ldhB</i> ups, BamHI	TAGTGGATCCTGGTAAATCCAAACACAACAAC
770	<i>ldhB</i> dwn, PstI	AATTCCTGCAGTAATTTCCAGCTCTTACAATAAC
771	<i>ldhB</i> dwn, XhoI	GACCTCGAGTCAGAAACTTTCTTTACCCAGAG
772	pCS1966, BamHI	GCGGGGATCCACTAGTTCTAG
773	pCS1966, XhoI	ATACCGTCGACCTCGAG
774	<i>ldhX</i> ups, BamHI	TAGTGGATCCCTGTTTCAGGTCTTGATAG
775	<i>ldhX</i> ups, EcoRI	CCGATGAATTCTCATTAGCACGTTTAACAAGAG
776	<i>ldhX</i> dwn, EcoRI	CCGATGAATTCATCAGCGTAGTCTGCTGC
777	<i>ldhX</i> dwn, KpnI	CGGGGTACCATTTAATCCTAAAGTCGTTATTAC
785	<i>Ldh</i> ups, EcoRI	CCGATGAATTCTTAAGTCAAGACAACAGAGGTC
786	<i>Ldh</i> dwn, EcoRI	CCGATGAATTCGACCTTGTGAAAAAATCTTC
787	<i>Ldh</i> ups, BamHI	TAGTGGATCCGTACAATGGCTACTGTAAAC
788	<i>Ldh</i> dwn, XhoI	GACCTCGAGGATGAACAGACTTTTTTATTATAG
789	Verify <i>ldh</i> deletion	AAAACCAGGTGAACTCGTC
791	<i>adhB</i> rev, PstI	TCGGACTGCAGTTAAATGCTGATAAAAAACAATCTTC
827	pCS1966, BamHI	ATACCGTCGACCTCGAG
828	pCS1966, XhoI	CGATAAGCTTGATATCGAATTC
829	Used with 690, EcoRI	CCGATGAATTCCTTACAACAATTTATTACAGGTTTTT
830	<i>adhB</i> fwd, EcoRI	CCGATGAATTCCTTAAGGAGAATTAGAATGGCAAGTAGTACATTTT ATATTC
834	<i>pfl</i> ups, XbaI	CTAGTCTAGACAAGTGATGTACCAATGAC
835	<i>pfl</i> ups, BamHI	CGCGGATCCTTTGAAATCTCCTTTGTTCT
836	<i>pfl</i> dwn, BamHI	CGCGGATCCTTCTTAGTATTAATAAATATAAAG
837	<i>pfl</i> dwn, XhoI	GGTACTCGAGTGTGATTCACCCCTATTCT
845	SPP, <i>pdh</i> , XbaI	CTAGTCTAGACTGAACCCCTACCGTGGGGAGTTTATTCTTGACAAGTTCTCTTGAGTTGA TATAATCAAGAAGTAATAAAATATTCGGAGGAATTTTG
846	SPP, <i>pdh</i> , XbaI	CTAGTCTAGACTGAACCCCTACCGTGCAGAGTTTATTCTTGACATCGTTACCGTAGGTTGGT ATAATCTTGAAGTAATAAAATATTCGGAGGAATTTTG
847	SPP, <i>pdh</i> , XbaI	CTAGTCTAGACTGAACCCCTACCGCAAGAGTTTATTCTTGACAATTACCTGTTTCGCTTGATAT AATAAGGAAGTAATAAAATATTCGGAGGAATTTTG
848	SPP, <i>pdh</i> , XbaI	CTAGTCTAGACTGAACCCCTACCGTGCAGAGTTTATTCTTGACAATCTGTGAGATCAGTGATA TAATACAGAAGTAATAAAATATTCGGAGGAATTTTG
878	<i>pta</i> ups, USER	ATCCCTCGGTTACAAGTTTCU
879	<i>pta</i> dwn, USER	AGAAACTTGTAAACCGAGGGAUATAATAGATTGAAATTCTGTCAG
880	<i>pta</i> ups, USER	ATTCGATATCAAGCTTATCGAUCAAAAATGTGGTAGAATATATAG
881	<i>pta</i> dwn, USER	AGGTGCGAGGTATCGATAUUCCTAGTTCAATTGATGTGAC
882	pCS1966, USER	ATCGATAAGCTTGATATCGAAU
883	pCS1966, USER	ATTATCGATACCGTCGACCU
891	pTD6, USER	ACAGATTAAAGGTTGACCAAGTAU
892	pTD6, USER	ACCAATTCTGTGTGCGCAU
894	<i>adhB</i> rev, USER	ATACTGGTCAACCTTTAATCTGUTTAATAATGCTGATAAAAAACAATTCTT
920	pCS1966, USER	ATAAGCTUGATATCGAATTCCT
921	pCS1966, USER	ATTCCTTUAGTGAGGGTTAAT
927	<i>adhE</i> ups, USER	ATGTGTACGUTCTCCTTTGTG
928	<i>adhE</i> dwn, USER	ACGTACACAUATTATAGTATTTGGAACCGAAC
929	<i>adhE</i> ups, USER	AAGCTTAUGGTGCTCTTGTACTTGTG
930	<i>adhE</i> dwn, USER	AAAGGGAUTCTGCGGAGCTATATATG
947	Modulate <i>pdh</i> expression, USER	ATGCGCAACACAGAATTGGUGGCCNNNNNAGTTTATTCYRAMANNNNNNNNNNNNNT GRYAYAAAYNNNNNAAGTAATAAAATATTCGGAGGAAT
ptd1	<i>gusA</i> fwd	GACTGAAGTCTAGTATCTGCAGAGCTTGTGACCCGGGTACCTCGAGCTCCATGGC ATATGCGGCCGCATGCGCAACACAGAATTGGTTAAAC
ptd2	<i>gusA</i> rev	ACCTCTCCTAGGATTAAAAAATAAAAAAGAACCCACTCGGGTT CTTTTTTTTACTAGCTAGCTAATGGTGCGCCAGGAGAGTTG
ptd45	pAK80	TTCTGCAGATCTTCAGTCAAGTCAAGAGGTTTGATGACTTTGAC
ptd46	pAK80	TTAATCCTAGGATTGTGGGAAATTTAGGCG
ptd49	pTD5, StuI	ATGCTAGGGCCACGGGGAATTTGTATCGATG
ptd50	pTD5, ApaI	TGATAAAGGCCTTACTGCACTATCAACACACT
ptd51	pG ⁺ host8, StuI	TTAAGAAGGCCTAAGAAATTTGCCAGTCG
Ptd52	pG ⁺ host8, ApaI	GCGCCTGGGCCGCCACTCATAGTTCTAAAC

^a Abbreviations: fwd, forward; rev, reverse; ups, upstream; dwn, downstream; SPP, synthetic promoter primer; USER, uracil-containing primers for USER cloning.

the strains expressing PDC did produce ethanol, but lactate was the major fermentation product (Fig. 2A), probably reflecting that on a rapidly metabolizable sugar such as glucose, the native alcohol dehydrogenase (ADHE) is expressed only to a low level. Other

sugars, including galactose and maltose, that are metabolized more slowly induce a change in the fermentation mode leading to substantial amounts of formate, acetate, and ethanol (mixed-acid mode) (36), and it is therefore expected that the alcohol dehydro-

FIG 2 Fluxes and specific growth rate as a function of pyruvate decarboxylase expression. Strains containing a transcriptional fusion of *pdc* and *gusA* were generated as described in Materials and Methods. The x axis carries Miller units for quantification of GusA activity. The left y axis corresponds to the fluxes, whereas the right y axis corresponds to the specific growth rate. (A) Growth on glucose; (B) growth on maltose. The curves are included as a visual aid and have no physiological relevance. The data shown are averages of three or more experiments, and standard deviations are indicated as error bars (GusA error bars shown for glucose/maltose data only).

used codon-optimized genes. In addition, by using synthetic promoters, we were able to achieve high expression levels. Synthetic promoters, in an easy way, deliver all the possible expression levels in one simple step (35). The strains constructed here have not yet been fully optimized, and further improvements are possible, but still ethanol productivities comparable to what has been reported previously for even optimized *Saccharomyces cerevisiae* strains, i.e., 29 to 31 mmol/(gdw · h) (37, 38), were obtained. Besides productivity, tolerance is an important issue. In this study, we chose *L. lactis* mainly because of its model organism status and because it is easy to engineer. Still, we found it to be more ethanol tolerant than *E. coli*, the first prokaryote engineered into producing ethanol (14), which despite its limitations continues to be optimized for ethanol production (12, 39, 40). Wild-type *E. coli*

strains are completely inhibited at 35 g/liter (4.4%, vol/vol) (41). Evolution experiments have resulted in strains able to grow at 50 g/liter (6.3%, vol/vol) (41), but this is still far from the 20% (vol/vol) (158 g/liter) that has been reported for some LAB strains (42). We did a short time evolution experiment and were able to increase tolerance from 6 to 8% in just 2 months, which is not far from the 10 to 12% tolerated by the commonly used *S. cerevisiae* and *Z. mobilis* (43). There are many other LAB that definitely would be much better suited for industrial ethanol production than the dairy strain MG1363, and the only direct application of the strain developed here probably would be production of ethanol from whey. Other LAB able to grow in simple or industrial-type media, with higher ethanol tolerance and the ability to grow at higher temperatures, would be a better starting point.

In this study, we deleted several genes, including three lactate dehydrogenase genes, *pta*, and *adhE*. It is probably necessary to delete only two genes, i.e., the main lactate dehydrogenase gene, *ldh*, and *pta*. The other lactate dehydrogenase genes are not expressed significantly and were deleted only to increase genetic stability. After introducing PDC and ADHB activities, the growth rate was, however, not drastically reduced compared to the wild-type strain rate, and the strains should therefore be relatively stable. When PDC activity was introduced into a wild-type background, we observed a decrease in specific growth rate of approximately 20% on glucose, whereas on maltose the effect was a 30% reduction. The stronger effect observed on maltose can be explained by the reduction of flux toward acetate, which will result in less ATP being produced (Fig. 1). In contrast, in the Δ^3ldh background a stimulation of growth was observed, indicating that the decline in acetate-coupled ATP production is compensated for by an increased glycolytic flux. Deleting *adhE* is probably not necessary, since formate and acetoin would be produced only in the absence of PDC and ADHE activities. When the latter two enzyme activities are present, the internal pyruvate concentration is expected to be low, and the flux through acetolactate synthase, which has a high K_m , should be minimal (36). We first attempted to avoid formate and acetate formation by deleting *pfl*. This, however, led to slow growth, probably because this interfered with acetyl-CoA formation, which is essential for lipid biosynthesis.

FIG 3 Fluxes and specific growth rate as a function of pyruvate decarboxylase and alcohol dehydrogenase expression in the Δ^3ldh strain. Strains containing a transcriptional fusion of *pdc-adhB* and *gusA* were generated as described in Materials and Methods. The x axis carries Miller units for quantification of GusA activity. The left y axis corresponds to the fluxes, whereas the right y axis corresponds to the specific growth rate. The curves are included as a visual aid and have no physiological relevance. The data shown are averages of three or more experiments, and standard deviations are indicated as error bars. The GusA activities shown are the same as the ones in Fig. 2A.

TABLE 3 Comparison of the $\Delta^3ldh \Delta pta$ and the $\Delta^3ldh \Delta pta \Delta adhE$ (expressing *pdC* + *adhB*) derivatives of MG1363^a

Strain	Relevant genotype	μ (h^{-1})	Flux [mmol/(gdw · h)]						
			Glc	EtOH	Lac	Ace	For	Act	Pyr
CS4234	$\Delta^3ldh \Delta pta$	0.63 (0.00)	16.6 (0.1)	13.7 (0.2)	ND	ND	14.8	8.2 (3.4)	2.6 (0.3)
CS4435	$\Delta^3ldh \Delta pta \Delta adhE$ + pET	0.76 (0.04)	17.9 (1.1)	31.1 (2.7)	ND	ND	0.7 (0.2)	ND	ND

^a Symbols and abbreviations: μ , specific growth rate; Glc, glucose; EtOH, ethanol; Lac, lactate; Ace, acetate; For, formate; Act, acetoin; Pyr, pyruvate; ND, not determined. Values are averages of three independent experiments, and standard deviations are indicated in parentheses.

PFL is expected to be the main generator of acetyl-CoA, since under anaerobic conditions the NADH/NAD⁺ ratio is high, which inhibits the pyruvate dehydrogenase complex (PDHC) (44). Formation of acetyl-CoA was not a problem in the $\Delta^3ldh \Delta pta \Delta adhE$ strain, in which acetyl-CoA can still be formed via PFL. It should be mentioned that in *L. lactis* more than 90% of the metabolized sugar ends up as various fermentation products, and the small amounts of acetyl-CoA generated would result only in insignificant amounts of formate. As expected, when the native alcohol dehydrogenase, ADHE, was inactivated, the resulting strain (MG1363 $\Delta^3ldh \Delta pta \Delta adhE$) was unable to grow anaerobically due to its inability to regenerate NAD⁺. In the presence of oxygen, under which condition the NADH oxidase (*noxE*) regenerates NAD⁺, growth was possible. Introducing PDC and ADHB activity restored capacity for anaerobic growth. Whereas the $\Delta^3ldh \Delta pta$ strain formed a mixture of ethanol, formate, acetoin, and a small amount of pyruvate, the sole measurable fermentation product of the $\Delta^3ldh \Delta pta \Delta adhE$ strain expressing PDC and ADHB was ethanol (Table 3). As with the Δ^3ldh strain, introduction of the ethanol genes also resulted in faster growth than in the $\Delta^3ldh \Delta pta$ strain, and also higher glucose and ethanol fluxes were observed (Table 3).

To sum up, *L. lactis*, the model organism of LAB, was engineered into producing ethanol in relatively few steps. The work demonstrated the potential in using LAB for ethanol production and justifies further work.

REFERENCES

- Kuyper M, Winkler AA, Van Dijken JP, Pronk JT. 2004. Minimal metabolic engineering of *Saccharomyces cerevisiae* for efficient anaerobic xylose fermentation: a proof of principle. *FEMS Yeast Res.* 4:655–664.
- van Maris AJ, Abbott DA, Bellissimi E, van den Brink J, Kuyper M, Luttik MA, Wisselink HW, Scheffers WA, van Dijken JP, Pronk JT. 2006. Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. *Antonie Van Leeuwenhoek* 90:391–418.
- la Grange DC, den Haan R, van Zyl WH. 2010. Engineering cellulolytic ability into bioprocessing organisms. *Appl. Microbiol. Biotechnol.* 87:1195–1208.
- Geddes CC, Nieves IU, Ingram LO. 2011. Advances in ethanol production. *Curr. Opin. Biotechnol.* 22:312–319.
- Schubert C. 2006. Can biofuels finally take center stage? *Nat. Biotechnol.* 24:777–784.
- Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresour. Technol.* 96:673–686.
- Chung IS, Lee YY. 1985. Ethanol fermentation of crude acid hydrolyzate of cellulose using high-level yeast inocula. *Biotechnol. Bioeng.* 27:308–315.
- Palmqvist E, Hahn-Hägerdal B. 2000. Fermentation of lignocellulosic hydrolysates. II. Inhibitors and mechanisms of inhibition. *Bioresour. Technol.* 74:25–33.
- Huang H, Guo X, Li D, Liu M, Wu J, Ren H. 2011. Identification of crucial yeast inhibitors in bio-ethanol and improvement of fermentation at high pH and high total solids. *Bioresour. Technol.* 102:7486–7493.
- Bettiga M, Bengtsson O, Hahn-Hägerdal B, Gorwa-Grauslund MF. 2009. Arabinose and xylose fermentation by recombinant *Saccharomyces cerevisiae* expressing a fungal pentose utilization pathway. *Microb. Cell Fact.* 8:40.
- Heer D, Heine D, Sauer U. 2009. Resistance of *Saccharomyces cerevisiae* to high concentrations of furfural is based on NADPH-dependent reduction by at least two oxidoreductases. *Appl. Environ. Microbiol.* 75:7631–7638.
- Miller EN, Jarboe LR, Yomano LP, York SW, Shanmugam KT, Ingram LO. 2009. Silencing of NADPH-dependent oxidoreductase genes (*yqhD* and *dkgA*) in furfural-resistant ethanologenic *Escherichia coli*. *Appl. Environ. Microbiol.* 75:4315–4323.
- Wang X, Miller EN, Yomano LP, Shanmugam KT, Ingram LO. 2012. Increased furan tolerance in *Escherichia coli* due to a cryptic *ucpA* gene. *Appl. Environ. Microbiol.* 78:2452–2455.
- Ingram LO, Conway T, Clark DP, Sewell GW, Preston JF. 1987. Genetic engineering of ethanol production in *Escherichia coli*. *Appl. Environ. Microbiol.* 53:2420–2425.
- Rana NF, Gente S, Rincé A, Auffray Y, Laplace J. 2012. Genetic modifications and introduction of heterologous *pdC* genes in *Enterococcus faecalis* for its use in production of bioethanol. *Biotechnol. Lett.* doi:10.1007/s10529-012-0964-x.
- Bi C, Rice JD, Preston JF. 2009. Complete fermentation of xylose and methylglucuronoxylase derived from methylglucuronoxylan by *Enterobacter asburiae* strain JDR-1. *Appl. Environ. Microbiol.* 75:395–404.
- Nichols NN, Dien BS, Bothast RJ. 2003. Engineering lactic acid bacteria with pyruvate decarboxylase and alcohol dehydrogenase genes for ethanol production from *Zymomonas mobilis*. *J. Ind. Microbiol. Biotechnol.* 30:315–321.
- Lucena BTL, dos Santos BM, Moreira JLS, Moreira APB, Nunes AC, Azevedo V, Miyoshi A, Thompson FL, de Moraes MA, Jr. 2010. Diversity of lactic acid bacteria of the bioethanol process. *BMC Microbiol.* 10:298–308.
- Tashiro Y, Kaneko W, Sun Y, Shibata K, Inokuma K, Zendo T, Sonomoto K. 2011. Continuous D-lactic acid production by a novel thermotolerant *Lactobacillus delbrueckii* subsp. *lactis* QU 41. *Appl. Microbiol. Biotechnol.* 89:1741–1750.
- Gold RS, Meagher MM, Tong S, Hutkins RW, Conway T. 1996. Cloning and expression of the *Zymomonas mobilis* “production of ethanol” genes in *Lactobacillus casei*. *Curr. Microbiol.* 33:256–260.
- Liu S, Dien BS, Cotta MA. 2005. Functional expression of bacterial *Zymobacter palmae* pyruvate decarboxylase gene in *Lactococcus lactis*. *Curr. Microbiol.* 50:324–328.
- Liu S, Nichols NN, Dien BS, Cotta MA. 2006. Metabolic engineering of a *Lactobacillus plantarum* double *ldh* knockout strain for enhanced ethanol production. *J. Ind. Microbiol. Biotechnol.* 33:1–7.
- Gasson MJ. 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J. Bacteriol.* 154:1–9.
- Brøndsted L, Hammer K. 1999. Use of the integration elements encoded by the temperate lactococcal bacteriophage TP901-1 to obtain chromosomal single-copy transcriptional fusions in *Lactococcus lactis*. *Appl. Environ. Microbiol.* 65:752–758.
- Solem C, Defoor E, Jensen PR, Martinussen J. 2008. Plasmid pCS1966, a new selection/counterselection tool for lactic acid bacterium strain construction based on the *oroP* gene, encoding an orotate transporter from *Lactococcus lactis*. *Appl. Environ. Microbiol.* 74:4772–4775.
- Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

27. Jensen PR, Hammer K. 1993. Minimal requirements for exponential growth of *Lactococcus lactis*. *Appl. Environ. Microbiol.* 59:4363–4366.
28. Terzaghi BE, Sandine WE. 1975. Improved medium for lactic streptococci and their bacteriophages. *Appl. Microbiol.* 29:807–813.
29. Nørholm MHH. 2010. A mutant Pfu DNA polymerase designed for advanced uracil-excision DNA engineering. *BMC Biotechnol.* 10:21. doi:10.1186/1472-6750-10-21.
30. Holo H, Nes IF. 1989. High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. *Appl. Environ. Microbiol.* 55:3119–3123.
31. Israelsen H, Madsen SM, Vrang A, Hansen EB, Johansen E. 1995. Cloning and partial characterization of regulated promoters from *Lactococcus lactis* Tn917-*lacZ* integrants with the new promoter probe vector, pAK80. *Appl. Environ. Microbiol.* 61:2540–2547.
32. Bachmann BJ. 1987. Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12, p 1191–1219. In Ingraham JL, Low KB, Magasanik B, Schaechter M, Umberger HE (ed), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, DC.
33. Casadaban MJ, Cohen SN. 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. *J. Mol. Biol.* 138:179–207.
34. Maguin E, Prévost H, Ehrlich SD, Gruss A. 1996. Efficient insertional mutagenesis in lactococci and other gram-positive bacteria. *J. Bacteriol.* 178:931–935.
35. Solem C, Jensen PR. 2002. Modulation of gene expression made easy. *Appl. Environ. Microbiol.* 68:2397–2403.
36. Coccain-Bousquet M, Garrigues C, Loubiere P, Lindley ND. 1996. Physiology of pyruvate metabolism in *Lactococcus lactis*. *Antonie Van Leeuwenhoek* 70:253–267.
37. Alper H, Moxley J, Nevoigt E, Fink G, Stephanopoulos G. 2006. Engineering yeast transcription machinery for improved ethanol tolerance and production. *Science* 314:1565–1568.
38. Bakker BM, Bro C, Kötter P, Luttik MAH, van Dijken JP, Pronk JT. 2000. The mitochondrial alcohol dehydrogenase Adh3p is involved in a redox shuttle in *Saccharomyces cerevisiae*. *J. Bacteriol.* 182:4730–4737.
39. Kim Y, Ingram LO, Shanmugan KT. 2007. Construction of an *E. coli* K-12 mutant for homoethanologenic fermentation of glucose or xylose without foreign genes. *Appl. Environ. Microbiol.* 73:1766–1771.
40. Turner PC, Miller EN, Jarboe LR, Baggett CL, Shanmugan KT, Ingram LO. 2011. YqhC regulates transcription of the adjacent *Escherichia coli* genes *yqhD* and *dkgA* that are involved in furfural tolerance. *J. Ind. Microbiol. Biotechnol.* 38:431–439.
41. Jarboe LR, Grabar TB, Yomano LP, Shanmugan KT, Ingram LO. 2007. Development of ethanologenic bacteria. *Adv. Biochem. Eng. Biotechnol.* 108:237–261.
42. Gold RS, Meagher MM, Hutkins R, Conway T. 1992. Ethanol tolerance and carbohydrate metabolism in lactobacilli. *J. Ind. Microbiol.* 10:45–54.
43. Bai FW, Anderson WA, Moo-Young M. 2008. Ethanol fermentation technologies from sugar and starch feedstocks. *Biotechnol. Adv.* 26:89–105.
44. Snoep JL, de Graef MR, Westphal AH, de Kok A, de Mattos MJT, Neijssel OM. 1993. Differences in sensitivity to NADH of purified pyruvate dehydrogenase complexes of *Enterococcus faecalis*, *Lactococcus lactis*, *Azotobacter vinelandii* and *Escherichia coli*: implications for their activity in vivo. *FEMS Microbiol. Lett.* 114:279–284.