# Conversion of *Lactococcus lactis* from homolactic to homoalanine fermentation through metabolic engineering

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**We report the engineering of *Lactococcus lactis* to produce the amino acid L-alanine. The primary end product of sugar metabolism in wild-type *L. lactis* is lactate (homolactic fermentation). The terminal enzymatic reaction (pyruvate + NADH→L-lactate + $NAD^+$) is performed by L-lactate dehydrogenase (L-LDH). We rerouted the carbon flux toward alanine by expressing the *Bacillus sphaericus* alanine dehydrogenase (L-AlaDH; pyruvate + NADH + $NH_4^+$→L-alanine + $NAD^+$ + $H_2O$). Expression of L-AlaDH in an L-LDH-deficient strain permitted production of alanine as the sole end product (homoalanine fermentation). Finally, stereospecific production (>99%) of L-alanine was achieved by disrupting the gene encoding alanine racemase, opening the door to the industrial production of this stereoisomer in food products or bioreactors.**

Keywords: alanine, alanine racemase, alanine dehydrogenase, *Lactococcus lactis*



The world market for amino acids amounts to about US$3 billion. More than half of the L-amino acids (Ala, Asn, Gln, His, Ile, Leu, Met, Pro, Ser, Tyr, and Val) are produced in the range of 1,000 tons/year with an average price of $50/kg[1]. With the sole exception of methionine, the L-form of amino acids is required for most food industry and pharmaceutical applications[1]. L-alanine is currently used as a food sweetener[1–3] and for pharmaceutical applications in which it is incorporated together with several other L-amino acids in standard infusions for parenteral administration in clinical pre- and postoperative nutrition therapy[1].

A number of microorganisms (i.e., *Corynebacterium gelatinosum, Arthrobacter oxydans, Brevibacterium lactofermentum, Clostridium* sp. P2, and *Pyrococcus furiosus*) are able to produce alanine from sugar fermentation[1,4–7]. However, the maximal sugar conversion rate is 50–60%, and a mixture of both isomers is generally obtained due to the presence of an alanine racemase, which is required for D-alanine production for cell wall biosynthesis[1]. An industrial process has been developed for the exclusive production of L-alanine through L-aspartate decarboxylation[1,8]. This process uses immobilized cells or suspensions of *Pseudomonas dacunhae* as biocatalysts with a yield >90%[1,8]. A metabolic engineering approach for L-alanine production was recently attempted in *Escherichia coli* by the coexpression of formate dehydrogenase and alanine dehydrogenase[9]. Alanine (80% L-isoform) was produced directly from pyruvate, but the yield decreased upon increasing pyruvate concentration and, moreover, the relative D-alanine isomer concentration increased with incubation time due to alanine racemase activity[9]. Metabolic engineering to produce alanine from glucose has been achieved in *Zymomonas mobilis* by expressing an alanine dehydrogenase, but the end products were a mixture of ethanol and alanine (presumably both isomers) with a glucose conversion rate into alanine of 85%. However, the maximal glucose consumption was low (30%), and the final alanine concentration did not exceed 80 mM[10,11].

*Lactococcus lactis* belongs to the lactic acid bacteria (LAB) group and is widely used as a starter for the production of fermented dairy products. The extensive knowledge of this species at the genetic and physiological levels has permitted the rerouting of various catabolic pathways in bioreactors or fermented food products[12]. The main advantage of LAB for metabolic engineering stems from the nearly complete uncoupling of the basic catabolic and cell biosynthetic pathways. *Lactococcus lactis* converts excess sugars primarily into L-lactate through glycolysis (homolactate fermentation)[12]. As with other LABs, *L. lactis* contains neither a functional electron transport chain nor a citric acid cycle. ATP is generated mainly from glycolysis by two substrate-level phosphorylation reactions coupled to the reduction of $NAD^+$ into NADH. The $NAD^+$ cofactor is regenerated through the action of L-lactate dehydrogenase (L-LDH), which converts pyruvate into L-lactate (Fig. 1).

This work reports homoalanine fermentation in an *L. lactis* L-LDH-deficient strain overexpressing L-alanine dehydrogenase (L-AlaDH). Exclusive production of L-alanine was achieved by disruption of the endogenous alanine racemase gene.

## Results and discussion

Overexpression of the *Bacillus sphaericus* alanine dehydrogenase gene (*alaD*). AlaDH and LDH both use pyruvate as substrate and NADH as cofactor (Fig. 1). Furthermore, the $K_M$ values of the *B. sphaericus* L-AlaDH[13] for pyruvate and NADH are very close to those of the *L. lactis* L-LDH[14] (Fig. 1). We expressed L-AlaDH from a nisin-inducible promoter because of the ability to fine-tune its activity by varying the levels of inducer and because expression at levels of up to 50% of total cell protein have been reported with this system[15,16].

The *B. sphaericus alaD* gene[17] was fused to the *nisA* promoter (pNZ2650) and introduced into *L. lactis* strain NZ3900 (ref. 15), which expressed the *nisRK* regulatory genes stably integrated at the *pepN* locus. As competition between L-AlaDH and L-LDH was expected in the wild-type strain, we created an isogenic L-LDH-deficient strain, NZ3950 by homologous recombination. NZ3950 dis-

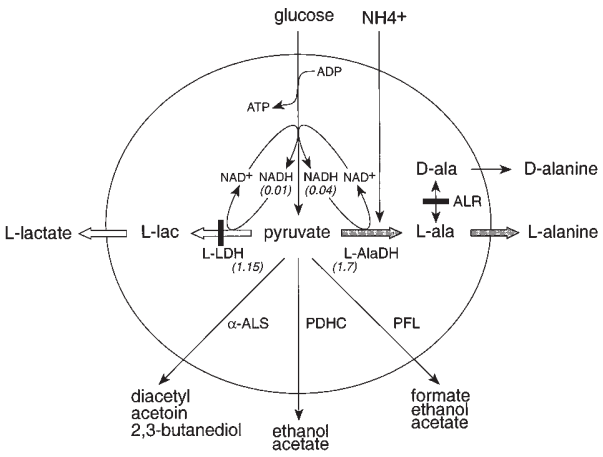


**Figure 1. Rerouting carbon flux toward L-alanine by overproduction of alanine dehydrogenase (L-AlaDH) in an L-lactate dehydrogenase (L-LDH) and alanine racemase (ALR)-deficient strain. The alternative pathways of pyruvate catabolism in the L-LDH–deficient strain[18] take place through α-acetolactate synthase (α-ALS), pyruvate dehydrogenase complex (PDHC), and pyruvate formate lyase (PFL). The $K_M$ values for pyruvate and NADH of L-LDH and L-AlaDH are indicated between brackets.**

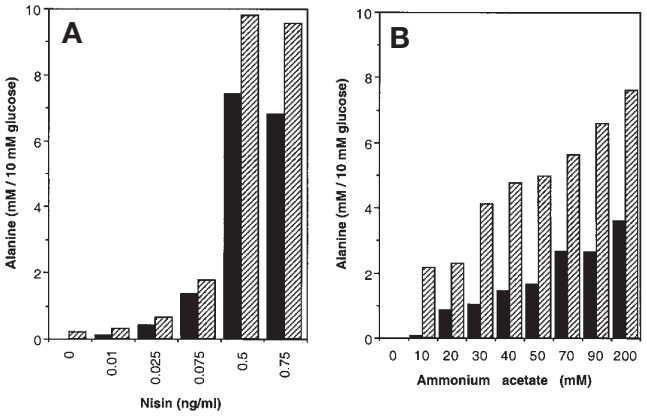


**Figure 3. Alanine production as a function of nisin concentration and ammonium supply. (A) Effect of nisin concentration on alanine production by cell suspensions of the wild-type NZ3900 strain (black bars) and the L-LDH–negative NZ3950 strain (hatched bars). (B) Effect of ammonium acetate supply on alanine production. (Experimental conditions were as in (A) except that the cells were induced at a fixed nisin concentration of 0.5 ng/ml). Symbols as in (A).**

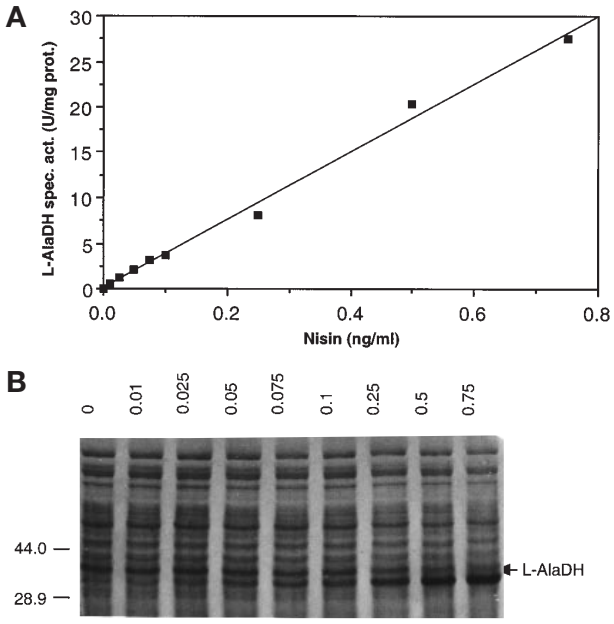


**Figure 2. Nisin-dependent overproduction of alanine dehydrogenase. (A) Effect of nisin concentration on L-AlaDH specific activity. (B) Coomasie brilliant blue-stained gel after SDS–PAGE of cell extracts from cultures induced with increasing concentrations of nisin (ng/ml) as indicated above each lane. Molecular mass markers (in kilodaltons) are indicated on the left, and the L-AlaDH band is indicated by arrow.**

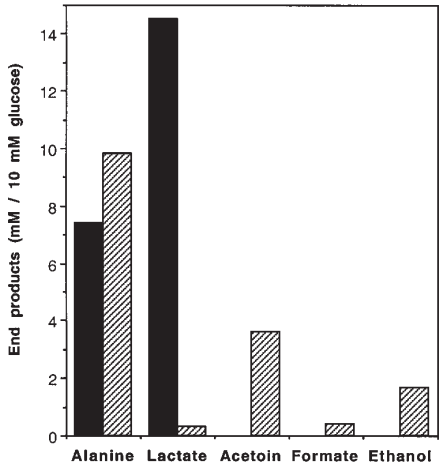


**Figure 4. End products formation by cell suspensions of the wild-type NZ3900 strain (black bars) and the L-LDH–deficient NZ3950 strain (hatched bars), each harboring pNZ2650. Cells were induced at a fixed nisin concentration of 0.5 ng/ml. Production of acetate, 2,3-butanediol, and pyruvate was not observed.**

played no L-LDH activity and produced only trace amounts of L-lactate (data not shown), as previously observed for a similar strain[18]. L-AlaDH was expressed in NZ3950 cells, and its specific activity was determined as a function of increasing concentrations of nisin in the medium (0–1 ng/ml). A linear relationship between nisin concentration and L-AlaDH-specific activity was observed (Fig. 2A), ranging from 0.05 U/mg protein to a maximum of 28 U/mg protein at 0.75 ng/ml nisin (550-fold induction). Increasing the nisin concentration to 1 ng/ml resulted in both decreased growth and L-AlaDH activity (data not shown). Since purified L-AlaDH from *B. sphaericus* had a specific activity of 157 U/mg protein[13], the expressed L-AlaDH was estimated to represent approximately 20% of cell proteins. Analysis of cell extracts by SDS–PAGE revealed a protein band with the predicted molecular mass (39.5 kDa; Fig. 2B). At maximal induction conditions, the L-AlaDH protein amounted to about 30–40% of total soluble protein. Similar results were obtained with wild-type NZ3900 cells expressing L-AlaDH (data not shown). The discrepancy in quantification compared with the 20% evaluation based on specific activities could be explained if only a fraction of the overexpressed protein is active or, alternatively, if some inhibitory compounds affect the enzymatic assay performed on crude extracts.

Use of *L. lactis* resting cells as biocatalyst for alanine production. In order to assay alanine production, L-AlaDH was induced in both wild-type and LDH-deficient cells, which were then washed and resuspended at a concentration of approximaetly 3.3 g dry weight/L in potassium sodium phosphate buffer (100 mM, pH 7.0) supplemented with glucose and ammonium acetate. An evaluation of various production parameters was performed with small-scale (4 ml) cell suspensions. Both strains produced alanine at a rate that was

dependent on the nisin concentration (Fig. 3A) and the ammonium supply (Fig. 3B). Alanine production was always higher and required lower nisin concentrations and ammonium amounts in the L-LDH-deficient strain relative to the wild-type strain (Fig. 3). This was probably due to the absence of competition between L-AlaDH and L-LDH in the L-LDH mutant. Carbon balances (corrected for $CO_2$ production) were calculated from the concentration of the different end products for each strain and were found to vary between 95 and 110%. At maximal L-AlaDH induction, the end products of glucose fermentation in the wild-type strain (Fig. 4) were a mixture of L-lactate (65% [percentage of total amount of carbon converted]) and alanine (35%). In the L-LDH mutant (Fig. 4), the end products were mainly a mixture of alanine (50%) and acetoin (35%) together with small amounts of ethanol (8%), formate (2%), and lactate (1.5%). These data show that L-AlaDH is able to compete with L-LDH in the wild-type strain and even more efficiently with the alternative pathways of pyruvate catabolism responsible for a mixed fermentation profile in the L-LDH-deficient strain (Fig. 1). Residual L-lactate production in the L-LDH mutant suggests the presence of alternative pathways for lactate production as reported with similar mutants obtained in LAB[18,19]. The influence of increasing the initial pH in the medium from 5 to 8 was also examined. Alanine production gradually increased up to a maximum at pH 8 (internal pH of 8.5, ref. 20) (data not shown), in agreement with the reported pH optimum (8.5–9) for amination[11].

As the pH played a critical role in alanine production, a number of fermentations were conducted using larger cell suspensions (400 ml) maintained at a fixed pH of 7.5. Figure 5 illustrates the time course of the most important products (glucose, alanine, and lactate) for fermentation performed with initial concentrations of glucose and $(NH_4)_2SO_4$ of 100 mM each. Ammonium acetate was replaced by $(NH_4)_2SO_4$ because consumption of acetate by the L-LDH mutant complicated the calculation of the carbon balances (data not shown). The wild-type strain (Fig. 5A) produced a mixture of lactate (104 mM), alanine (68 mM), and acetate (8 mM) with 35% rerouting toward alanine. Thus, controlling the pH in this case does not improve the rerouting, probably because L-LDH can still compete with L-AlaDH at an alkaline pH. Conversely, the L-LDH mutant (Fig. 5B) under similar conditions showed an improved rerouting toward alanine (140 mM, 75%) in comparison with uncontrolled pH conditions. The following additional end products were detected in low amounts: pyruvate, lactate, acetate, and formate, altogether accounting for <6 mM. A number of unidentified compounds were also observed on the high-pressure liquid chromatography (HPLC) profile, but acetoin and 2,3-butanediol, expected to arise from the α-acetolactate synthase (α-ALS) pathway (Fig. 1), were not detected. Acetoin is expected to be the major end product of pyruvate catabolism in an L-LDH mutant of *L. lactis*[18], but the α-ALS with an optimum pH of 6.0 could be inactive at an alkaline pH, as previously suggested[18,21]. Thus, maintaining the pH in the alkaline range for the L-LDH mutant has the advantage of favoring the L-AlaDH reaction (closer to the optimum pH) and limits alternative pathways of pyruvate catabolism.

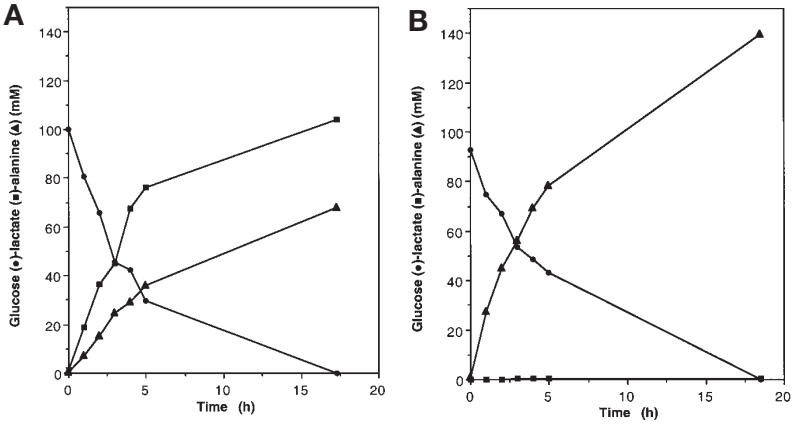


**Figure 5. Time course of glucose consumption and alanine and lactate production by (A) the wild-type NZ3900 strain and (B) the L-LDH–deficient NZ3950 strain, each containing pNZ2650. Cell suspensions were initially supplemented with 100 mM glucose and 100 mM $(NH4^+)_2SO_4$, and the pH was maintained at 7.5 with NaOH.**

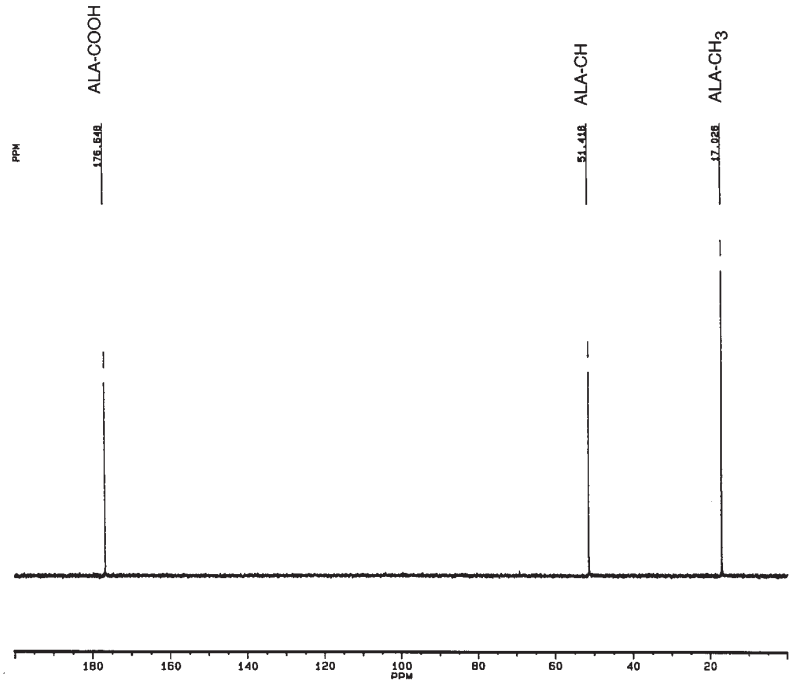


**Figure 6. $^{13}C$ NMR spectrum (0–200 p.p.m.) of end products from a cell suspension of the L-LDH–deficient NZ3950 strain containing pNZ2650 initially supplemented with 100 mM glucose and 150 mM $(NH4^+)_2SO_4$ after 17 h of fermentation at pH 7.5.**

Theoretically, a 100% conversion of 100 mM glucose into 200 mM alanine should require 200 mM ammonium (100 mM $[NH_4^+]_2SO_4$). We suspected that 100 mM $(NH_4^+)_2SO_4$ could be limiting for a complete rerouting toward alanine because the $K_m$ value of L-AlaDH for ammonium[13] is quite high (28.2 mM). Therefore, a sample from a 17 h fermentation with 150 mM $(NH_4^+)_2SO_4$ was subjected to $^{13}C$ nuclear magnetic resonance (NMR) analysis (Fig. 6). Alanine was detected as the sole product with a purity close to 99.5%. Thus, glucose (100 mM) was completely converted into alanine (200 mM) by controlling the pH in the presence of an excess of ammonium.

As autolysis of *L. lactis* occurs when cells are resuspended in buffer without any carbon source or when the efficiency of glycolysis is low[22,23], we assayed cell lysis by following the release of cellular proteins during fermentation. Fermentations (17 h) performed in the presence of 100 mM ammonium sulfate displayed 1% and 12% cell lysis in the LDH-positive and LDH-negative strains, respectively. Ten percent cell lysis of the

LDH-negative strain was observed within the first 4 h. This was reduced to 4% by increasing the initial amount of ammonium sulfate (150 mM) during the fermentation. The higher lysis level (12%) observed at low ammonium sulfate concentration is probably caused by ammonium limitation resulting in less efficient fermentation. Preliminary data obtained with alginate-immobilized cells are very encouraging in terms of cell stability and alanine production efficiency, which is maintained at the initial level for at least 24 h, indicating that the level of cell lysis must be very low (data not shown). Should autolysis become a problem for long-term cell stability in process development, the major autolysin of *L. lactis* could be inactivated, which should result in strongly reduced autolytic behavior without negatively affecting growth[24].

**Exclusive production of L-alanine isomer in an alanine racemase knockout mutant.** L-alanine is an important isomer for applications in the food, feed, and pharmaceutical industries[1]. The production of each isomer was determined from samples obtained from small- or large-scale fermentations using enzymatic assays. A mixture of both isomers was observed in all cases with the D-isomer amounting to 10–15% of the total, independent of fermentation time (data not shown). This proportion of D-alanine is rather low in comparison to the racemic mixture generally observed with strains that naturally produce alanine[1,2,4]. D-alanine is essential for peptidoglycan biosynthesis and alanine racemase is responsible for its conversion from L-alanine[25]. The racemization observed with the constructed recombinant strains is the result of an enzymatic conversion: incubation with D-chloroalanine (50 µg/ml), a specific inhibitor of alanine racemase[26], led to a significant reduction in the proportion of the D-isomer (<3% of total alanine).

In order to prevent racemization, the alanine racemase gene (*alr*) of *L. lactis* was disrupted by homologous recombination. The Alr$^-$ derivatives of the *L. lactis* strains NZ3900 and NZ3950, respectively named PH3900 and PH3950, were unable to grow without supplementation of D-alanine (2.25 mM). D-alanine auxotrophy was previously reported for Alr$^-$ strains of *E. coli*[27], *Bacillus subtilis*[28], and *Lactobacillus plantarum*[29]. L-AlaDH was expressed in both strains, which were then cultured in the presence of D-alanine to ensure growth during the nisin induction step. D-alanine was removed during the washing step, preceding the final fermentation. While the D-alanine isomer was produced at the expected levels in the control strains, it was not detected in the Alr$^-$ mutants (L-enantiomer excess >99%). In contrast to *E. coli* or *B. subtilis*, in which two alanine racemases were identified[27,28], *L. lactis* apparently contains a single racemase.

In conclusion, the use of a straightforward metabolic engineering strategy consisting of the controlled overproduction of L-AlaDH and disruption of *ldh* and *alr* genes led to a complete conversion of *L. lactis* from the naturally occurring homo-L-lactate fermentation into a homo-L-alanine fermentation. To our knowledge, this is the first example of a complete metabolic rerouting toward a metabolite that is not a normal end product in the host. Nearly complete rerouting toward an endogenous end product (ethanol) has previously been reported in *E. coli*[30].

These results confirm the suitability of *L. lactis*, which displays a simple and efficient fermentation metabolism both aerobically and anaerobically, as a host for metabolic engineering[12]. The use of resting cells of *L. lactis* as biocatalysts for the conversion of cheap sugar sources (i.e., sucrose, whey, and starch) into L-alanine opens interesting perspectives for the development of a new process for the production of this amino acid as a fine chemical. Such a process could use gel-immobilized steady-state cells (as was already implemented for L-lactate production)[31]. Another interesting perspective could be the development of alanine-producing dairy starters for in situ production of the natural sweetener alanine in dairy products such as cheese, buttermilk, or yogurt.

## Experimental protocol

**Bacterial strains and media.** *E. coli* MC1061 and TG1 were grown in Luria Broth medium with aeration at 37°C[32,33]. *L. lactis* NZ3900 (ref. 15) and its derivatives were grown at 30°C in M17 medium (Merck, Darmstadt, Germany) supplemented with 0.5% (wt/vol) glucose (GM17). D-alanine was added at a final concentration of 2.25 mM to ensure growth of the alanine racemase mutants. Chloramphenicol, erythromycin, and tetracycline were used at 20 µg/ml, 250 µg/ml, 12.5 µg/ml, respectively, for *E. coli*, and at 10 µg/ml (5 µg/ml when used in combination), 5 µg/ml, and 1 µg/ml, respectively, for *L. lactis*.

**DNA techniques and transformation.** *E. coli* MC1061 or TG1 strains were used as intermediate hosts for cloning. Lactococcal plasmid and chromosomal DNA were isolated as described previously[34,35]. DNA sequencing was performed with an Applied Biosystems model 377 DNA sequencer and a dye-labeled terminator sequencing kit (Applied Biosystems, Foster City, CA). PCR reactions were performed with the Expand High Fidelity PCR system (Boehringer, Mannheim, Germany) in a DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT) with the following regimen: denaturation at 95°C for 1 min, annealing at 40°C for 1 min, and extension at 72°C for 1 min for a total of 25 cycles. All other DNA manipulations were performed using established procedures[33]. *L. lactis* was electroporated as reported before[36].

**Construction of plasmids and mutant strains.** The promoterless *B. sphaericus alaD* gene was isolated as a 1.22 kb *Sma*I-*Hin*dIII fragment from plasmid pBM20*alaD* (a gift from G.A. Sprenger, Institut für Biotechnologie, Jülich, Germany) and cloned into *Hpa*I-*Hin*dIII–digested pGIT032 (ref. 37), to generate pGIT503. This intermediate plasmid positioned the strong *L. plantarum ldhL* terminator[37] downstream of the *alaD* open reading frame (ORF). The *alaD* ORF-*ldhL* terminator module was then cloned as a 1.64 kb *Bam*HI-*Xba*I fragment into similarly digested pNZ8020 (ref. 15). The resulting pNZ2650 expresses the *alaD* gene from the *nisA* promoter.

To create the *alr* gene knockout, partial sequence (5′ end region) of the *alr* gene from *L. lactis* IL1403 was kindly provided by P. Renault (INRA, Jouy en Josas, France). A 0.692 kb PCR product (corresponding to a 5′ and 3′ truncated *alr* gene) was amplified from NZ3900 chromosomal DNA using primers derived from the IL1403 DNA sequence (LLALR1: 5′-CGAGGATCCGGCTCGGTTGAGGTTTCTAAAGCGG-3′ containing a *Bam*HI site [underlined] and LLALR2: 5′-CGCGAGCTCACTTGTTTCATAAGGCACCGTAACC-3′ containing an *Sst*I site [underlined]). The DNA fragment was identified by sequencing and displayed 94% identity to the IL1403 DNA sequence (data not shown). The fragment was then restricted with *Bam*HI and *Sst*I and cloned into the corresponding sites of the suicide plasmid pJDC9 (ref. 38) to generate pGIP011.

The L-LDH–deficient derivative of NZ3900 (NZ3950) was obtained by single crossover chromosomal integration of plasmid pNZ2007 (5′ and 3′ truncated *ldh*, *tetM*) and validated as described previously[18]. The Alr$^-$ strains were constructed by transformation of pGIP011 DNA into strains NZ3900 and NZ3950. Transformants corresponding to single crossover chromosomal integration of pGIP011 were selected on GM17 containing D-alanine and erythromycin. The resulting Alr-deficient strain PH3900 and its L-LDH–deficient variant PH3950 were identified by PCR using primers located 5′ and 3′ to the fragment used for homologous recombination (data not shown). The Alr–deficient phenotype was validated on the basis of absolute dependence upon D-alanine for growth.

**Nisin induction and fermentation.** An overnight culture of *L. lactis* containing pNZ2650 was diluted (1:20) in GM17 supplemented with the appropriate antibiotics and grown until an $OD_{600nm}$ of 0.5. The cells were induced with different concentrations of nisin A (referred to as nisin) and incubated until an $OD_{600nm}$ of 2.0, at which point the cells were either harvested for extraction or used as cell suspensions. Small-scale cell suspensions made use of a 20 ml culture that had reached an $OD_{600nm}$ of 2.0. Cells were harvested by centrifugation (12,000 *g*, 10 min, 20°C), washed with an equal volume of 100 mM K-Na $PO_4$ (pH 7.0) and resuspended in 4 ml of the same buffer supplemented with 50 mM glucose and different concentrations of ammonium acetate. The cell suspensions were subsequently incubated under shaking for 1 h at 30°C followed by centrifugation and supernatant recovery for analysis of end products. Large-scale cell suspensions (400 ml) were obtained using the same procedure as described above, but nisin concentration was fixed at 0.75 ng/ml, the initial culture volume was increased to 2 L and ammonium acetate was replaced by ammonium sulfate (100 or 150 mM). The cell suspension was transferred to a 1-liter fermentor (Applikon Dependable Instruments, Schiedam, The Netherlands). The pH was kept at 7.5 by the addition of 1 M NaOH, the temperature was maintained at 30°C, and the stirrer speed was set at 120 r.p.m.

Cell extracts, enzyme assays, and protein analysis. Preparation of cell extracts and protein assays were performed as described[37]. L-LDH activity was determined by the method of Hillier and Jago[14]. L-AlaDH activity was measured according to the procedure of Ohashima and Soda[13]. Specific activities were expressed as units (U, µmol of NADH/min) per mg protein. SDS–PAGE was performed according to the protocol of Laemmli[39]. Samples (10 µg of total proteins) were heated for 1 min at 100°C and loaded on a 10% polyacrylamide gel. The gels were stained with Coomasie brilliant blue. The prestained molecular weight markers were obtained from Bio-Rad Laboratories (Richmond, CA). The protein fractions were quantified as a percentage of the total intracellular proteins as reported before[15].

Analysis of fermentation products. Glucose, lactate, acetate, formate, ethanol, pyruvate, acetoin, and 2,3-butanediol were analyzed by HPLC as described before[40]. Total alanine (L- and D-isomers) analysis was performed on a 4151 Alpha Plus amino acid analyzer (Pharmacia Biotech, Uppsala, Sweden) after appropriate dilutions of the samples. Enzymatic determination of L-alanine was perfomed by coupling the glutamate–pyruvate transaminase (pig heart, 10 mg/ml; Boehringer) reaction to the L-LDH (rabbit muscle, 5 mg/ml; Boehringer) reaction according to the enzyme manufacturer's instructions. The enzymatic measurement of D-alanine concentration made use of the same solutions as for L-alanine quantification, but the initial mixture contained 550 µl of triethanolamine HCl (750 mM)–EDTA (7.5 mM), 950 µl of $H_2O$, 50 µl of sample at appropriate dilution, and 30 µl of D-amino acid oxidase (hog kidney, 5 mg/ml, Boehringer). The mixture was incubated for 1 h at 37°C under strong shaking, 50 µl of NADH–$Na_2$ $2H_2O$ (6 mM)–$NaHCO_3$ (120 mM) were added and a first measurement at 340 nm was performed. The reaction was initiated with 10 µl of L-LDH (rabbit muscle, 5 mg/ml; Boehringer). The next steps were identical to L-alanine assays. $^{13}C$ NMR experiments were conducted as follows: 0.5 ml of $D_2O$ was added to a 2 ml sample in a 10 mm tube. $^{13}C$ spectra were recorded at 298 K using an AM 500 Bruker instrument operating at 125.7 MHz. Fourier transform parameters were: spectral width, 26 kHz; data points, 32 K; pulse angle, 90° (23 µs); recycle time, 9 s. After 5,000 transients were accumulated, a line broadening of 1.5 Hz was applied to the free induction decay before Fourier transformation. Chemical shifts were accurate at 0.05 p.p.m.

Nucleotide sequence accession number. The nucleotide sequence of the 5′ and 3′ truncated *alr* gene from *L. lactis* NZ3900 (MG1363 derivative) has been submitted to the EMBL Nucleotide Sequence Database under accession number Y18148.

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