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Metabolic Engineering of *Lactococcus lactis*: Influence of the Overproduction of α-Acetolactate Synthase in Strains Deficient in Lactate Dehydrogenase as a Function of Culture Conditions

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The *als* gene for α-acetolactate synthase of *Lactococcus lactis* MG1363 was cloned on a multicopy plasmid under the control of the inducible *L. lactis lacA* promoter. More than a hundredfold overproduction of α-acetolactate synthase was obtained in *L. lactis* under inducing conditions as compared with that of the host strain, which contained a single chromosomal copy of the *als* gene. The effect of α-acetolactate synthase overproduction on the formation of end products in various *L. lactis* strains was studied under different fermentation conditions. Under aerobic conditions and with an initial pH of 6.0, overexpression of the *als* gene resulted in significant acetoin production that amounted to more than one-third of the pyruvate converted. However, the effect of the α-acetolactate synthase overproduction was even more pronounced in the lactate dehydrogenase-deficient strain *L. lactis* NZ2700. Anaerobic cultivation of this strain resulted in a doubling of the butanediol formation of up to 40% of the converted pyruvate. When cultivated aerobically at an initial pH of 6.8, overexpression of the *als* gene in *L. lactis* NZ2700 resulted in the conversion of more than 60% of the pyruvate into acetoin, while no butanediol was formed. Moreover, at an initial pH of 6.0, similar amounts of acetoin were obtained, but in addition, approximately 20% of the pyruvate was converted into butanediol. These metabolic engineering studies indicate that more than 80% of the lactose can be converted via the activity of the overproduced α-acetolactate synthase in *L. lactis*.

Diacetyl is an essential flavor compound in dairy products such as butter, buttermilk, and cheese and in many nondairy products where a butter-like taste is desired. It is formed during milk fermentations by citrate-utilizing lactic acid bacteria, including *Leuconostoc*, *Lactobacillus*, and *Lactococcus* species. Although several mechanisms have been proposed for the biosynthesis of diacetyl in citrate-utilizing lactococci, recent studies on citrate metabolism have established that diacetyl is formed by an oxidative decarboxylation of the metabolic intermediate α-acetolactate (13, 25, 28). α-Acetolactate is produced from two molecules of pyruvate by the activity of α-acetolactate synthase, which has a low affinity for pyruvate (K_m, 50 mM) and therefore will be most active under conditions of pyruvate excess (19, 24). α-Acetolactate is further decarboxylated into acetoin by the action of α-acetolactate decarboxylase. Strains lacking α-acetolactate decarboxylase activity internally accumulate α-acetolactate, which can subsequently be oxidized to diacetyl under aerobic conditions (25).

The production of diacetyl or its precursor α-acetolactate by lactic acid bacteria has been studied as a function of various fermentation conditions and usually is associated with citrate utilization. This seriously limits the production of diacetyl, since citrate is a minor component in most foodstuffs. Diacetyl could be generated more efficiently from sugars such as lactose or glucose. Redirection of the sugar metabolism in lactic acid bacteria has recently been realized by constructing lactate dehydrogenase-deficient strains of *Lactococcus lactis* by site-directed mutagenesis (8). In these strains, pyruvate cannot be converted into lactate, and a mixed acid fermentation is obtained with formate and ethanol as major products. Under aerobic conditions, pyruvate is accumulated, leading to the formation of high levels of acetoin and butanediol.

Recently, the gene for α-acetolactate synthase of *L. lactis* subsp. *lactis* biovar *diacetylactis* has been cloned and further characterized. Expression of this *als* gene in *Escherichia coli* resulted in only low yields of acetoin (9, 18). Here we describe the cloning of the *als* gene from the non-citrate-utilizing *L. lactis* MG1363 and its inducible overexpression in lactococcal strains under various physiological conditions. The production of acetoin and other fermentation products was studied in *als*-overexpressing *L. lactis* strains with an active lactate dehydrogenase or strains that were made deficient for this enzyme by inactivation of the *ldh* gene. Under the appropriate conditions, it was found that more than 80% of the pyruvate was converted via the activity of the overproduced α-acetolactate synthase.

MATERIALS AND METHODS

Bacterial strains and media. *L. lactis* subsp. *lactis* biovar *diacetylactis* RU4 (13) and the plasmid-free *L. lactis* strains MG1363 (6) and MG5267, carrying a single chromosomal copy of the *lac* operon (7, 27), were cultivated at 30°C in M17 medium (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% lactose or 0.5% glucose (26). The fermentation experiments were performed with 50-ml cultures grown in M17 medium containing 0.5% lactose set with H_2SO_4 at an initial pH of 6.0 or 6.8 that were incubated at 30°C in 250-ml serum bottles with shaking in a G76 waterbath (New Brunswick Scientific, Edison, N.J.) at 200 rpm (aerobic conditions) or in 50-ml serum bottles that were not shaken (anaerobic conditions). *E. coli* MC1061 (4) was grown in Luria broth at 37°C (23). If

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TABLE 1. Product formation and lactose consumption by *L. lactis* MG5267 harboring pNZ2500[a]

Fermentation conditions		Product formed (mM)						Lactose utilized (mM)
Initial pH	Aeration	Lactate	Formate	Acetate	Ethanol	Acetoin	Butanediol	
6.8	−	54.6 (100)[b]	ND[c]	ND	ND	ND	ND	18.1
6.0	−	41.2 (99.7)	ND	0.1 (0.2)	ND	ND	ND	9.7
6.8	+	52.0 (70.8)	ND	2.3 (3.1)	ND	9.6 (26.1)	ND	19.2
6.0	+	42.5 (55.4)	ND	2.0 (2.6)	ND	16.1 (42.0)	ND	18.7

[a] Data represent the average of at least two experiments.
[b] Values in parentheses are percentages of pyruvate converted into the product.
[c] ND, not detected.

appropriate, media were supplemented with erythromycin (10 μg/ml), ampicillin (100 μg/ml), and tetracycline (1 μg/ml for *L. lactis* or 10 μg/ml for *E. coli*).

Molecular cloning, reagents, and enzymes. Plasmid DNA was isolated from *E. coli* cells essentially by established protocols (23). Lactococcal plasmid (30) and chromosomal (17) DNA was isolated as described previously. *L. lactis* was electroporated as described before (31). All other DNA manipulations were performed by established procedures (23). Restriction enzymes and T4 ligase were purchased from Bethesda Research Laboratories (Gaithersburg, Md.), New England BioLabs, Inc. (Beverly, Mass.), or Boehringer GmbH (Mannheim, Germany) and used as described in the instructions of the manufacturers. Oligonucleotides were synthesized on a Cyclone DNA synthesizer (Biosearch, San Rafael, Calif.).

Construction of plasmids and strains. The *als* gene was isolated from *L. lactis* RU4 by the PCR (22). The primers used for the PCR were based on the nucleotide sequence of *L. lactis* subsp. *lactis* biovar *diacetylactis* DSM20384 and had the following sequences: 5′-ATACGTCGACGTCAAAATAATTGTAAAAGG-3′ and 5′-TAATCTGCAGTTATGCCACAAATTGCTTG-3′, positions 1129 to 1149 and 2900 to 2923, respectively, on the basis of the numbering of Marugg et al. (18) (restriction sites are underlined). PCR amplification resulted in a single DNA fragment with the expected size of 1.8 kb, which was digested with *Sal*I and *Pst*I and purified by agarose gel electrophoresis. Subsequently, this fragment was cloned in pUC19, and the resulting plasmid was designated pNZ-ALS. pNZ2500 was constructed by cloning the 1.8-kb *Sal*I-*Pst*I fragment from pNZ-ALS in pNZ3004 (27), digested with the same enzymes. Plasmid pNZ2007 was constructed by isolating the 5′ and 3′ truncated MG1363 *ldh* gene from pNZ2006 (8) as a 0.7-kb *Hin*dIII-*Pst*I fragment. This fragment has been made blunt with Klenow polymerase and was cloned into pCI182 digested with *Pvu*II. pCI182 is a pBR322 derivative carrying the Tn*919 tetM* gene and incapable of replicating in *L. lactis* (10). The lactate dehydrogenase-deficient strain *L. lactis* NZ2007 was constructed by single crossover chromosomal integration of plasmid pNZ2007 in *L. lactis* MG5267 and selection for resistance to tetracycline.

Enzyme assays. Cell extracts were prepared as described previously (13). L-Lactate dehydrogenase activity was determined by the method of Hillier and Jago (11). α-Acetolactate synthase measurements were performed as described by Hugenholtz and Starrenburg (13). Protein was assayed as described by Bradford (3), with bovine serum albumin as a standard.

Products of fermentation. Acetate, formate, lactate, ethanol, and butanediol were determined by high-performance liquid chromatography as described previously (25). α-Acetolactate, diacetyl, and acetoin were determined chemically as described by Veringa et al. (29). In all cases, the carbon balances, when corrected for the produced CO_2, were close to unity. In addition, under anaerobic conditions, the redox balances were also close to unity.

RESULTS

Cloning and expression of the *L. lactis* MG1363 *als* gene. *L. lactis* MG5276 harboring a chromosomal copy of the *lac* operon contains a low α-acetolactate synthase activity when grown in M17 medium containing lactose (0.44 U/mg), which is similar to that observed during growth in glucose-containing M17 medium (0.37 U/mg). To obtain high production of α-acetolactate in *L. lactis*, the lactococcal *als* gene was isolated and cloned under the control of the strong lactose-inducible *lacA* promoter, resulting in pNZ2500. When grown in glucose-containing M17 medium, strain MG5267 harboring pNZ2500 contained high α-acetolactate synthase activity (23.08 U/mg). This activity increased approximately twofold when grown under inducing conditions in lactose-containing media (54.80 U/mg). This represents a hundredfold increase of specific α-acetolactate activity as compared with that of strain MG5267, which contains only a single chromosomal copy of the *als* gene.

Metabolites produced by overexpression of the *als* gene. To investigate the effect of α-acetolactate synthase overproduction in *L. lactis*, the metabolites were determined in batch cultures of MG5267 harboring pNZ2500 grown at different initial pHs and with or without aeration (Table 1). When cultivated anaerobically at an initial pH of 6.8, *L. lactis* MG5267 harboring pNZ2500 showed a homofermentative fermentation, converting all pyruvate into lactate. The same fermentation products were obtained under these conditions with the host strain MG5267 (data not shown). It has been shown that α-acetolactate synthase production is inversely related to the pH (13, 19) and that the internal pH of *L. lactis* can be affected by the external pH (16). Therefore, the product formation of MG5267 harboring pNZ2500 was also studied in medium set at an initial pH of 6.0 under anaerobic conditions. However, no change in the metabolic end products was observed, and all pyruvate was converted into lactate (Table 1).

It is well known that aeration affects the product formation of metabolites from lactose in lactococci (2, 5, 20). *L. lactis* is known to contain an active NADH oxidase activity that under aerobic conditions reduces the NADH pool in the cell and thereby affects the rate of pyruvate reduction into lactate by the lactate dehydrogenase activity. Therefore, we studied the influence of aeration on the product formation in strains that overexpressed the *als* gene. When cultivated aerobically, *L. lactis* MG5267 harboring pNZ2500 was found to produce acetoin in addition to lactate. Under anaerobic conditions, no acetoin was found, and the fermentation products were not significantly affected by the culture pH. However, at an initial pH of 6.8, 26% of the pyruvate appeared to be converted to acetoin, while at an initial pH of 6.0, the acetoin production was increased almost twice. Under the latter conditions, 42% of the pyruvate was converted into acetoin via the α-acetolactate synthase activity (Table 1).

Metabolites produced by overexpression of the *als* gene in a lactate dehydrogenase-deficient strain. To investigate whether the amount of acetoin in an α-acetolactate synthase-overproducing strain could be increased further, a lactate dehydrogenase-deficient derivative was constructed from the lactose-fermenting strain MG5267, designated *L. lactis* NZ2007. Strain NZ2007 showed no lactate dehydrogenase activity (results not shown), and this resulted in a mixed acid fermentation under anaerobic conditions (Table 2) (8). Under aerobic conditions and an initial pH of 6.0, approximately half of the pyruvate was converted into acetoin, and one-third was further converted into butanediol (Table 2).

The combination of both increasing the pyruvate pool in the cell by disrupting the *ldh* gene and overexpression of the *als* gene could result in a higher yield of α-acetolactate and derivatives. Therefore, pNZ2500 was introduced in strain NZ2007. Under anaerobic conditions, the lactate dehydrogenase-deficient strain *L. lactis* NZ2007 harboring pNZ2500 produced a

TABLE 2. Product formation and lactose consumption by lactate dehydrogenase-deficient strain *L. lactis* NZ2007[a]

Fermentation conditions		Product formed (mM)						Lactose utilized (mM)
pH	Aeration	Lactate	Formate	Acetate	Ethanol	Acetoin	Butanediol	
6.8	−	5.5 (5.1)[b]	36.4 (33.8)	0.8 (0.7)	30.2 (28.0)	5.7 (10.6)	11.7 (21.7)	19.5
6.0	−	4.7 (5.4)	29.6 (33.8)	1.6 (1.8)	22.8 (26.1)	4.7 (10.7)	9.7 (22.2)	13.9
6.8	+	2.2 (2.0)	19.9 (18.2)	17.5 (16)	12.5 (11.4)	28.6 (52.3)	ND[c]	23.7
6.0	+	4.7 (4.7)	ND	0.7 (0.7)	17.3 (17.2)	23.2 (46.0)	15.9 (31.5)	23.7

[a] Data represent the average of at least two experiments.
[b] Values in parentheses are percentages of pyruvate converted into the product.
[c] ND, not detected.

range of metabolites. These products differed from those obtained from strain NZ2700 without pNZ2500. Approximately half of the pyruvate was converted into formate and ethanol (Table 3). The other half of the pyruvate was converted into α-acetolactate and its derivatives. This is an increase of 17% compared with that obtained with strain NZ2700 without pNZ2500. The major part of the acetoin produced was further reduced to butanediol (39.4% of the converted pyruvate). Lowering of the initial pH to 6.0 showed no significant effect on the distribution of the end products.

Under aerobic conditions, the variation in product formation at different pHs was much more pronounced (Tables 2 and 3). At an initial pH of 6.8, α-acetolactate and acetoin appeared to be produced at high concentrations in a lactate dehydrogenase-deficient strain overexpressing *als*. Approximately 64% of the pyruvate was converted into these products via the α-acetolactate synthase. However, no significant amounts of diacetyl were found. The other products resulted from pyruvate conversion via the pyruvate formate lyase. Furthermore, no butanediol is formed, which was also the case with the lactate dehydrogenase-deficient *L. lactis* strain NZ2007 under aerobic conditions at an initial pH of 6.8 (Table 2). Apparently, under these conditions, all reducing equivalents are efficiently removed by the NADH-oxidizing activities or are used for reduction of pyruvate to ethanol. At a lower initial pH of 6.0, no formate was formed, as a result of an efficient inhibition of the pyruvate formate lyase, and the vast majority (84.8%) of the pyruvate was converted into products from the α-acetolactate synthase pathway. The formed α-acetolactate was not accumulated but was immediately converted into acetoin under these conditions. One-fourth of the acetoin formed was further reduced to butanediol, which constituted 18.9% of the metabolized pyruvate.

DISCUSSION

The glycolytic intermediate pyruvate is a central metabolite during sugar fermentation of *L. lactis*. Four enzyme activities are known to convert pyruvate under different physiological conditions (Fig. 1): (i) α-acetolactate synthase, which is active at high pyruvate concentrations and low pH (13, 19, 24); (ii) L-Lactate dehydrogenase, with maximal activities at high sugar concentrations and high intracellular NADH levels; (14); (iii) pyruvate-formate lyase, which is active at a relatively high pH (pH above 6) and under anaerobic conditions (1); and (iv) pyruvate dehydrogenase, which is active under aerobic conditions and low pH (24). In this paper, we describe the product formation of the pyruvate conversion from lactose in strains which overproduce α-acetolactate synthase under different physiological conditions, including pH and aeration. Furthermore, the overproduction of α-acetolactate synthase was studied in both a wild-type strain and in a strain deficient in lactate dehydrogenase.

The lactose-utilizing strain *L. lactis* MG5267 harboring pNZ2500 produced high α-acetolactate synthase activity. Since the *als* gene in pNZ2500 is preceded by the *L. lactis lacA* promoter, both strong and regulated expression could be observed. In *L. lactis* MG5267 harboring the expression vector pNZ3004, which was used to construct pNZ2500, the regulation of the *lacA* promoter was shown to be fourfold when grown on glucose or lactose, based on the expression of the promoterless *cat* gene (27), while only a twofold difference in α-acetolactate synthase activity encoded by pNZ2500 was found. Since the *als* gene cloned in pNZ2500 is preceded by its own promoter, the higher activity of α-acetolactate synthase in MG5267 harboring pNZ2500 when grown on glucose may be the result of the high constitutive expression of the *als* promoter.

In the wild-type strain *L. lactis* MG5267 harboring pNZ2500, no effect has been observed in the product formation by the overproduction of α-acetolactate synthase under anaerobic conditions (Table 1). The strain showed a homolactic lactose fermentation. This can be explained by the high K_m value of α-acetolactate synthase for pyruvate (50 mM) (13, 19) in comparison with the low K_m value of lactate dehydrogenase for

TABLE 3. Product formation by lactate dehydrogenase-deficient strain *L. lactis* NZ2007 harboring pNZ2500[a]

Fermentation conditions		Product formed (mM)							Lactose utilized (mM)
Initial pH	Aeration	Lactate	Formate	Acetate	Ethanol	Acetoin	α-AL[b]	Butanediol	
6.8	−	3.5 (3.1)[c]	29.1 (25.7)	0.8 (0.7)	24.1 (21.3)	5.5 (9.7)	0.1 (0.2)	22.3 (39.3)	16.6
6.0	−	2.3 (2.8)	21.9 (26.6)	0.1 (0.1)	17.8 (21.6)	5.5 (13.3)	0.3 (0.7)	14.4 (34.9)	7.0
6.8	+	1.2 (1.1)	17.6 (15.5)	11.8 (10.4)	10.5 (9.4)	35.0 (61.7)	1.2 (2.1)	ND[d]	20.2
6.0	+	1.1 (1.1)	ND	5.9 (5.3)	8.7 (7.8)	36.6 (65.7)	0.6 (1.1)	10.5 (18.9)	20.2

[a] Data represent the average of at least two experiments.
[b] α-AL, α-acetolactate.
[c] Values in parentheses are percentages of pyruvate converted into the product.
[d] ND, not detected.

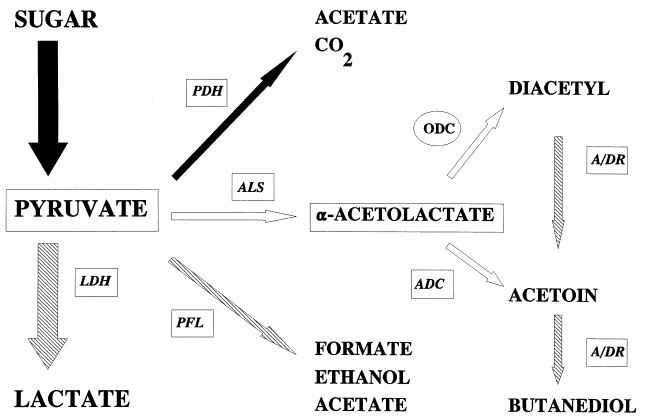

FIG. 1. Pyruvate pathway in *L. lactis*. Black and shaded arrows indicate conversions that generate and consume NADH, respectively. Enzymatic and chemical conversions are indicated by boxes and circles, respectively. Abbreviations: ALS, α-acetolactate synthase; LDH, lactate dehydrogenase; PFL, pyruvate formate lyase; PDH, pyruvate dehydrogenase; ADC, α-acetolactate decarboxylase; A/DR, acetoin and diacetyl reductase; ODC, oxidative decarboxylation.

pyruvate (≤1 mM) (12). Apparently, all pyruvate formed from lactose degradation was converted directly to lactate. Under aerobic conditions, the metabolic flexibility of strain MG5267 becomes apparent, and the α-acetolactate formed by the overproduced α-acetolactate synthase is directly converted into acetoin by the α-acetolactate decarboxylase activity. A lower initial pH of the culture leads to a lower intracellular pH, which is known to increase the α-acetolactate synthase activity (13, 16). This may explain why higher acetoin production was observed under these conditions (Table 1). To further increase the variability of the metabolic end products, a lactate dehydrogenase-deficient derivative of MG5267 was constructed, extending previous work with a lactate dehydrogenase-deficient derivative of MG1363 that could utilize only glucose (Table 2) (8).

As a consequence of the inactivation of the *ldh* gene in *L. lactis* NZ2007 harboring pNZ2500, the intracellular pyruvate concentration is presumably higher than it is in the wild-type strain. Therefore, the influence of the high α-acetolactate synthase activity was even more pronounced. Under anaerobic conditions, relatively high concentrations of butanediol, formate, and ethanol were formed. Since NADH cannot be used for the reduction of pyruvate to lactate, the reduction equivalents should be used in the production of ethanol and butanediol. As observed in lactate dehydrogenase-deficient or α-acetolactate synthase-overexpressing strains, lowering the initial pH had only a minimal effect on the product balance under anaerobic conditions. Aeration of *L. lactis* NZ2007 harboring pNZ2500 resulted in the production of high concentrations of acetoin. At a high initial pH of 6.8, no butanediol was formed and equal amounts of acetate and ethanol were formed. Lowering the initial pH resulted in higher concentrations of acetoin and butanediol, but no formate was formed. The reduction of acetoin to butanediol and the relatively high amount of ethanol that was formed by the alcohol dehydrogenase indicate the presence of an excess of NADH, especially at low pH. This has also been observed in a lactate dehydrogenase-deficient derivative of *L. lactis* MG1363 grown on glucose (8). In the lactate dehydrogenase-deficient strains NZ2007 and NZ2007 harboring pNZ2500, more reduced metabolites were formed under aerobic conditions at low pH. This could indicate that the dehydrogenase enzymes utilizing NADH are more active at pH 6.0 or that the NADH oxidases of *L. lactis* are less active at low pH. Overproduction of α-acetolactate synthase in *L. lactis* NZ2007 harboring pNZ2500 as compared with that in *L. lactis* NZ2007 resulted in a 5 to 10% rerouting from acetate or ethanol to α-acetolactate.

In this paper, we show that metabolic engineering of *L. lactis* is possible by altering fermentation conditions (pH and aeration) together with the modulation of the activity of key enzymes, such as lactate dehydrogenase and α-acetolactate synthase. Under optimal fermentation conditions and with appropriately constructed strains, up to 85% of the pyruvate and hence the carbon source lactose was converted via the α-acetolactate synthase pathway to acetoin and butanediol. To produce high yields of diacetyl, the conversion of α-acetolactate into acetoin by the activity of α-acetolactate decarboxylase should be prevented. It may be anticipated that the vast majority of the end products could then consist of α-acetolactate and diacetyl. The level of diacetyl produced in such a strain would be dependent on the efficient reduction of the NADH pool, the activity of diacetyl reductase, and the possible toxicity of a high diacetyl concentration (15). Strains deficient for α-acetolactate decarboxylase, obtained either via screening or by inactivating its recently discovered gene (21), will allow the testing of this hypothesis and further extend the potential of metabolic engineering of *L. lactis.*

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REFERENCES

1. **Abbe, K., S. Takahashi, and T. Yamada.** 1982. Involvement of oxygen-sensitive pyruvate formate-lyase in mixed-acid fermentation by *Streptococcus mutans* under strictly anaerobic conditions. J. Bacteriol. **152:**175–182.
2. **Bassit, N., C.-Y. Boquien, D. Picques, and G. Corrieu.** 1993. Effects of initial oxygen on diacetyl and acetoin production by *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*. Appl. Environ. Microbiol. **59:**1893–1897.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
4. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138:**179–207.
5. **Condon, S.** 1987. Responses of lactic acid bacteria to oxygen. FEMS Microbiol. Rev. **46:**269–280.
6. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154:**1–9.
7. **Gasson, M. J.** Personal communication.
8. **Gasson, M. J., S. Swindell, H. Griffin, C. Platteeuw, J. Hugenholtz, and W. M. de Vos.** Unpublished data.
9. **Goelling, D., and U. Stahl.** 1988. Cloning and expression of an α-acetolactate decarboxylase gene from *Streptococcus lactis* subsp. *diacetylactis* in *Escherichia coli*. Appl. Environ. Microbiol. **54:**1889–1891.
10. **Hill, C., G. Venema, C. Daly, and G. Fitzgerald.** 1988. Cloning and characterization of the tetracycline resistance determinant of and several promoters from within the conjugative transposon Tn*919*. Appl. Environ. Microbiol. **54:**1230–1236.
11. **Hillier, A. J., and G. R. Jago.** 1982. L-Lactate dehydrogenase, FDP-activated, from *Streptococcus cremoris*. Methods Enzymol. **89:**362–367.
12. **Hugenholtz, J.** Unpublished results.
13. **Hugenholtz, J., and M. J. Starrenburg.** 1992. Diacetyl production by different strains of *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* and *Leuconostoc* spp. Appl. Microbiol. Biotechnol. **38:**17–22.
14. **Kandler, O.** 1983. Carbohydrate metabolism in lactic acid bacteria. Antonie van Leeuwenhoek **49:**209–224.
15. **Kaneko, T., Y. Watanabe, and H. Suzuki.** 1990. Enhancement of diacetyl production by a diacetyl-resistant mutant of citrate-positive *Lactococcus lactis* subsp. *lactis* 3022 and by aerobic conditions of growth. J. Dairy Sci. **73:**291–298.
16. **Kashket, E. R.** 1987. Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance. FEMS Microbiol. Rev. **46:**233–244.
17. **Leenhouts, K. J., J. Kok, and G. Venema.** 1989. Campbell-like integration of heterologous plasmid DNA into the chromosome of *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. **55:**394–400.
18. **Marugg, J. D., D. Goelling, U. Stahl, A. M. Ledeboer, M. Y. Toonen, W. M.**

Verhue, and C. T. Verrips. 1994. Identification and characterization of the α-acetolactate synthase gene from *Lactococcus lactis* subsp. *lactis* biovar diacetylactis. Appl. Environ. Microbiol. **60:**1390–1394.
19. **Monnet, C., V. Phalip, P. Schmitt, and C. Diviès.** 1994. Comparison of α-acetolactate synthase and α-acetolactate decarboxylase in *Lactococcus* spp. and *Leuconostoc* spp. Biotechnol. Lett. **16:**257–262.
20. **Petit, C., F. Vilchez, and R. Marczak.** 1989. Formation and stabilization of diacetyl and acetoin concentrations in fully grown cultures of *Streptococcus lactis* subsp. *diacetylactis*. Biotechnol. Lett. **11:**53–56.
21. **Phalip, V., C. Monnet, P. Schmitt, P. Renault, J.-J. Godon, and C. Divies.** 1994. Purification and properties of the α-acetolactate decarboxylase from *Lactococcus lactis* subsp. *lactis* NCDO 2118. FEBS Lett. **351:**95–99.
22. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. G. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Snoep, J. L., M. J. Teixeira de Mattos, M. J. C. Starrenburg, and J. Hugenholtz.** 1992. Isolation, characterization, and physiological role of the pyruvate dehydrogenase complex and α-acetolactate synthase of *Lactococcus lactis* subsp. *lactis* bv. diacetylactis. J. Bacteriol. **174:**4838–4841.
25. **Starrenburg, M. J., and J. Hugenholtz.** 1991. Citrate fermentation of *Lactococcus* and *Leuconostoc* spp. Appl. Environ. Microbiol. **57:**3535–3540.
26. **Terzaghi, P. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophage. Appl. Environ. Microbiol. **29:**807–813.
27. **van Rooijen, R. J., M. J. Gasson, and W. M. de Vos.** 1992. Characterization of the *Lactococcus lactis* lactose operon promoter: contributions of flanking sequences and LacR repressor to promoter activity. J. Bacteriol. **174:**2273–2280.
28. **Verhue, W. M., and F. S. Tjan.** 1991. Study of the citrate metabolism of *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* by means of ^{13}C nuclear magnetic resonance. Appl. Environ. Microbiol. **57:**3371–3377.
29. **Veringa, H. A., E. H. Verburg, and J. Stadhouders.** 1984. Determination of diacetyl in dairy products containing α-acetolactic acid. Neth. Milk Dairy J. **38:**251–263.
30. **Vos, P., M. van Asseldonk, F. van Jeveren, R. Siezen, G. Simons, and W. M. de Vos.** 1989. A maturation protein essential for the production of active forms of *Lactococcus lactis* SK11 serine proteinase located in or secreted from the cell envelope. J. Bacteriol. **171:**2795–2802.
31. **Wells, J. M., P. W. Wilson, and R. W. F. Le Page.** 1993. Improved cloning vectors and transformation procedure for *Lactococcus lactis*. J. Appl. Bacteriol. **74:**629–636.