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GENETICS OF MESOPHILIC CITRATE METABOLIZING LACTIC ACID BACTERIA

Proefschrift
ter verkrijging van de graad van
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1. Gezien de recente publicatie van Janes et al. is het waarschijnlijk, dat alle koper bevattende amine oxidases een topaquinon als hun katalytische prosthetische groep bevatten in plaats van het tot nu toe gepostuleerde covalent gebonden PQQ.
Janes, S.M., et al. 1990. Science 248:981-987.

2. Het feit dat gemodificeerde aminozuren in de eiwitketen van enzymen bij kunnen dragen aan het katalytisch mechanisme, maakt het de moeite waard om in enzymen, waarvan het werkingsmechanisme nog onopgehelderd is, te zoeken naar dergelijke aminozuur modificaties.
McIntire, W.S., et al. 1991. Science 252:817-824.

3. Het gebruik door Citro et al. van polyklonale antilichamen ter identificatie van vrij en covalent gebonden PQQ sluit niet uit dat kruisreagerende moleculen gedetecteerd werden.
Citro, G. et al. 1989. FEBS Lett. 247:201-204.

4. Het volstaat niet, bacteriële species te identificiëren op basis van overeenkomsten in morfologische en fysiologische eigenschappen.
Dit proefschrift.

5. Het pH afhankelijke citraat gebruik van de aromabacteriën *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* en *Leuconostoc* spp. wordt in eerste instantie niet bepaald door de pH gevoeligheid van de bij het citraat metabolisme betrokken enzymen als wel door de pH afhankelijke opname van citraat.
Hugenholtz, J., and M.C.J. Starrenburg. 1991. Appl. Envirom. Microbiol. 57:3535-3540.
Dit proefschrift.

6. Vergeleken met de onderzoeksresultaten van Hugenholtz en Starrenburg is het opmerkelijk dat bij de door Lin et al. gebruikte pH waardes citraat gebruik waar te nemen is.
Hugenholtz, J., and M.C. J. Starrenburg. 1991. Appl. Environ. Microbiol. 57:3535-3540.
Lin. J. et al. 1991. Appl. Microbiol. Biotechnol. 34:628-631.

7. De nauwkeurigheid van de onderzoeks resultaten wordt bepaald door de exactheid waarmee de vraagstelling geformuleerd is.

8. Het besluit bij veel universiteiten om de verplichting tot het formuleren van stellingen achterwege te laten is zeker terecht met betrekking tot de toch al grote tijdsdruk die op de promovendus rust, maar maakt het voor een leek minder aantrekkelijk een proefschrift open te slaan.

9. De alsmaar stijgende prijzen van het openbaar vervoer alsmede vertragingen en gebrekkig materieel lijken bepaald geen goed uitgangspunt te zijn voor het stimuleren van verminderd autogebruik.

10. Wanneer de term gehanteerd wordt "de vervuiler betaalt" zou de hoogte van de hondenbelasting gerelateerd moeten worden aan de grootte van de hond.

11. Het is aan te bevelen om tijdens het "achterom" afdalen van een route evenveel aandacht aan het zekeren te besteden als tijdens het beklimmen ervan.

12. Een halve steur in de Merwede maakt nog geen (hele) schone Rijn.
NRC Handelsblad, 27 jan.1992.

13. Het gevoel dat een westerse tourist krijgt als hij voor het eerst op Sri Lankaanse wegen rijdt moet vergelijkbaar zijn met de sensatie die een spookrijder op een westerse autoweg beleeft.

Stellingen behorende bij het proefschrift van Silke David (1992).

You see things, and say why?
But I dream of things that never were,
and say, why not?

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CONTENTS

CHAPTER I

General introduction

Mesophilic lactic acid bacteria belonging to the genera *Lactococcus* and *Leuconostoc* are important components of starter cultures used for a variety of industrial food fermentations, including dairying, processing of meat and vegetables and in wine making (12, 15, 33, 41, 55).

During the manufacture of fermented dairy products, the most important function of these bacteria is the conversion of lactose into lactic acid which serves as a food preservative. In addition, the degradation of casein by the proteolytic system of *Lactococcus* strains is essential for rapid growth in milk and important for cheese ripening, texture formation and flavor development. The quality of many fermented dairy products is dependent on the ability of starter bacteria to produce flavor and aroma compounds. One such compound, diacetyl, is responsible for the characteristic aroma of products such as butter, sour cream, cottage cheese and buttermilk. Diacetyl is generated during the conversion of citrate, which is present in milk at concentrations up to 8 mM. Flavor producing lactic acid bacteria include *Leuconostoc* spp. and *Lactococcus lactis* subsp. *lactis* var. *diacetylactis*. The latter organism is the only *Lactococcus lactis* variant that is able to convert citrate. A summary of the citrate fermenting ability of the most commonly used lactic acid bacteria in dairy starters is given in Table 1.

Lactococcus lactis subsp. *lactis* var. *diacetylactis* and *Leuconostoc* spp. are included in starter cultures mainly for their ability to convert citrate into diacetyl and CO_2 (13, 10). The CO_2 generated during citrate fermentation, contributes to the formation of "eyes" in some cheese-types. Another important reason for having *Leuconostoc* spp. in mixed starter cultures is based on their ability to reduce the aroma component acetaldehyde (14), which may be produced by lactococci and is considered undesirable in some dairy products.

TABLE 1. Overview of the citrate fermenting ability of lactic acid bacteria (26).

Bacterial species	citrate fermentation[#]
Lactococcus lactis	-*
Leuconostoc spp.	+(-)
Lactobacillus spp.	-@
Streptococcus thermophilus	-

[#] +: fermentation of citrate; -: no citrate fermentation observed; +(-): occasionally strains are found that cannot ferment citrate. *: only *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* ferments citrate; @: only *Lactobacillus brevis* can ferment citrate.

Many properties of lactic acid bacteria that are of eminent importance for successful dairying are unstable. This may cause problems in large-scale fermentations and has stimulated the development of genetically stable strains. Features that are readily lost in lactococci include lactose utilization, protein degradation, citrate fermentation, bacteriocin production, phage resistance, resistance against UV-light and restriction/modification systems. In recent years it has been demonstrated by plasmid curing, conjugation, transduction, and transformation experiments that many of the above-mentioned properties are plasmid-linked in *Lactococcus* spp., and that loss of those plasmids accounts for the unstable character of these traits (5, 29, 31, 49, 50, 62, 64, 68, 71, 82, 83, 84). Only few data are available on the plasmid complement of industrially important leuconostoc strains. Certain characters, however, are unstable which suggests that they are plasmid-encoded. In *Leuconostoc lactis* and *Leuconostoc mesenteroides* a positive correlation could be demonstrated between the presence of certain plasmids and the ability of those strains to ferment lactose and citrate (70). Additionally, recently Fantuzzi et al. (24) reported the plasmid-linkage of these features in *Leuconostoc cremoris* and *Leuconostoc dextranicus*.

Two strategies can be used to stabilize plasmid-encoded properties. The first involves the integration of plasmid-encoded genes into the chromosome. In the second method an essential gene is inserted in the plasmid DNA, thus imposing a selection pressure for the presence of the plasmid. For both methods it is essential to know the genetic basis of the metabolic traits. Knowledge of gene organization and regulation is also required for the construction of expression systems for genetic, metabolic and protein engineering, offering a means of starter

improvement. Finally, the application of this knowledge allows the development of food-grade selection systems for lactic acid bacteria which are acceptable for applications in biotechnological processes in the food-industry.

LACTOSE METABOLISM IN LACTIC ACID BACTERIA

Lactose is the only sugar, which in industrial dairy fermentations is metabolized by lactic acid bacteria, leading to the acidification of dairy products.

The biochemical pathways involved in lactose transport and degradation have been well established and studied extensively (reviews: 17, 37, 44). Two basically different systems for lactose uptake have been found in lactic acid bacteria, which differ in the way lactose is transported across the cell membrane and in the key enzyme for hydrolysis of the ß-glycosidic bond (Fig.1). The bioenergetics and complexity of the two transport systems differs considerably (46, 53, 97) and they determine the fermentative route that is followed for further conversion of lactose into lactate.

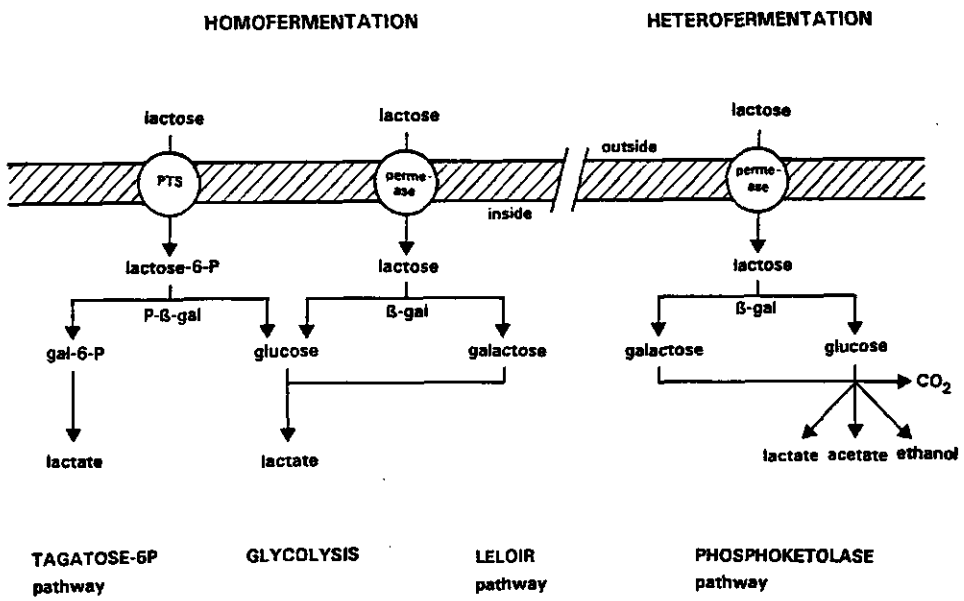

FIG. 1. Pathways of lactose transport in lactic acid bacteria. PTS indicates the phosphoenolpyruvate dependent phophotransferase system; P-ß-gal and ß-gal indicate the two different lactose hydrolyzing enzymes phospho-ß-galactosidase and ß-galactosidase, respectively.

In the first system, which is found in industrial *Lactococcus lactis* strains (59) and some lactobacilli such as *Lactobacillus casei* (4) lactose is transported via a phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS). In this system lactose is phosphorylated during transport into lactose-6-P, which is subsequently hydrolysed by phospho-ß-galactosidase into glucose and galactose-6-P. Galactose-6-P is further metabolized by the D-tagatose-6-P pathway resulting in the formation of intermediates (dihydroxyaceton phosphate and glyceraldehyde-3-P) of the glycolytic pathway.

Lactose can also enter the cell via a permease. The unmodified lactose is then hydrolyzed by a ß-galactosidase to glucose and galactose. In homofermentative lactose metabolism these compounds can subsequently be metabolized further by the glycolytic (Embden-Meyerhof-Parnas) or Leloir pathway, or, during heterofermentative lactose degradation, by the phosphoketolase pathway. Glycolysis is performed by the *Lactococcus* spp., and the *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, leading to homolactic fermentation with lactate as sole end product (Fig.1). The latter bacterial species do not metabolize the galactose moiety of lactose but excrete it stochiometrically into the medium. Heterofermentative lactic acid bacterial species like *Lactobacillus brevis*, *Lactobacillus bruckneri*, *Lactobacillus casei* and *Leuconostoc* spp. convert lactose via the phosphoketolase pathway. In this pathway glucose is converted into glucose-6P or fructose-6P and subsequent metabolism leads to the production of lactate, CO_2, acetate and ethanol (Fig.1). In contrast to other lactic acid bacteria, *Leuconostoc* spp. can metabolize the galactose moiety of lactose (8).

The genes of the lactose phosphotransferase system

The fact that lactose fermentation is a plasmid-encoded trait in most lactococcal strains has greatly facilitated the genetic studies of lactose metabolism in this species. The gene coding for the key enzyme, P-ß-galactosidase, was cloned from two lactose plasmids (2, 57) and characterized. Recently the molecular analysis of the entire *Lactococcus lactis* MG1820 lactose operon was completed (16, 100). This operon includes eight genes with the order *lacABCDFEGX*. The first four genes, *lacABCD*, code for enzymes involved in the tagatose-6-P pathway (101) and the following lactose-specific three genes, *lacFEG*, encode Enzyme III^{lac}, Enzyme II^{lac}, and P-ß-galactosidase, respectively. The function of the protein encoded by *lacX* remains unidentified until now. Upstream of the *lac*-operon a divergently

transcribed, glucose-inducible gene, *lacR*, is located that encodes a protein that functions as a repressor and regulates transcription of the *lac*-operon (16, 100). Immediately downstream of the *lac*-operon the presence of parts of an iso-ISS1 element have been reported (16), suggesting a possible mechanism for the transfer of the *lac*-genes by means of transposition.

The genes involved in the lactose permease system

The cloning of ß-galactosidase genes of lactic acid bacteria in *Escherichia coli* ß-galactosidase mutants has been facilitated by the relative simple detection of complementing clones using chromogenic substrates (66). In Table 2 the source and location of the cloned ß-galactosidase genes in lactic acid bacteria is listed. The genes for ß-galactosidase of most lactic acid bacteria are chromosomally located, although examples of plasmid-encoded ß-galactosidases have been reported in *Lactobacillus casei* (26) and *Leuconostoc lactis* (this thesis, Chapter IV), respectively.

TABLE 2. Summary of the cloned ß-galactosidase genes of lactic acid bacteria and their genetic location.

	Strain[•]	plasmid[*]	reference
Lb.bulg.	B131		(85)
	NCDO1489		(67)
Lb.casei	ATCC 393	pLZ15	(4)
S.therm.	A147		(77)
	A054		(87)
	ATCC 19258		(38)
Lc.lactis	NZ6009	pNZ63	(this thesis, Chapter IV)

[•] *Lb.bulg.*: *Lactobacillus bulgaricus*; *Lb.casei*: *Lactobacillus casei*; *S.therm*: *Streptococcus thermophilus*; *Lc.lactis*: *Leuconostoc lactis*. [*] only plasmid location is indicated, in the other cases the gene encoding for ß-galactosidase activity is located on the chromosome.

The complete DNA sequences of ß-galactosidases of several lactic acid bacteria have been described recently (56, 85, 87). The deduced amino acid sequences of ß-galactosidases from *Streptococcus thermophilus* (87) and *Lactobacillus bulgaricus* (85) are strongly identical (48%). In addition, the ß-galactosidases from Gram-positive bacteria show substantial

homology with ß-galactosidases from Gram-negative bacteria, including the *lacZ* and *ebgA* genes of *E.coli* (43, 95) and the ß-galactosidase gene of *Klebsiella pneumoniae* (3).

Recently, the lactose permease gene of *Streptococcus thermophilus* has been characterized (76). Only three bases separated the termination codon of the lactose permease (*lacS*) gene from the initiation codon for the ß-galactosidase gene, suggesting that the genes involved in lactose utilization are organized into an operon in these bacteria. The same structure of the lactose genes is observed in *Lactobacillus bulgaricus* (56, 87). As a consequence, the genetic organization of the lactose genes in these yoghurt bacteria, differs from the well-known genetic organization of the lactose genes in *E.coli*, that have the gene order *lacZ-lacY*. The deduced amino acid sequence of the lactose permeases of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* show no homology with the *lacY* gene of *Escherichia coli*. The *Streptococcus thermophilus* and *Lactobacillus bulgaricus* lactose permease were found to be hybrid proteins having homology in the N-terminal part with the *E.coli* melibiose carrier (MelB) and with Enzymes III of several PEP-dependent PTS systems in the C-terminal domain (56, 77). The lactose transport protein of *Streptococcus thermophilus* has been found to act as a lactose-galactose antiporter system (77; see below).

CITRATE METABOLISM

The mechanism of diacetyl production from citrate by the mesophilic lactic acid bacteria *Lactococcus lactis* ssp. *lactis* var. *diacetylactis* and *Leuconostoc lactis* has been studied to some extent (6, 7, 65, 90, 102, 103). The pathway for the metabolism of citrate is outlined in Fig. 2.

The first step in the metabolism of citrate is the uptake of citrate via a permease, which seems to be most efficient at or around pH 5.0 in *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* (35). The internalized citrate is subsequently hydrolyzed to acetate and oxaloacetate by citrate lyase (91). This is a constitutive enzyme in *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* (6) and an inducible one in *Leuconostoc* spp. (65). Oxaloacetate is further converted to pyruvate and acetaldehyde-thiamine pyrophosphate, which can condense with another molecule of pyruvate to form α-acetolactate. There are conflicting reports on the involvement of divalent cations such as Mg^{2+} and Mn^{2+} in the acetolactate synthase

activity (9, 12). However, recent studies indicate that no cofactors are required for the acitvity of the α-acetolactate synthetase (40).

There has been a strong controversy about the mechanism of diacetyl production *in vivo*. One mechanism, as proposed by Stadhouders (92), is the oxidative conversion of the unstable compound α-acetolactate, which can also non-oxidatively be decarboxylated to acetoin (Fig.2).

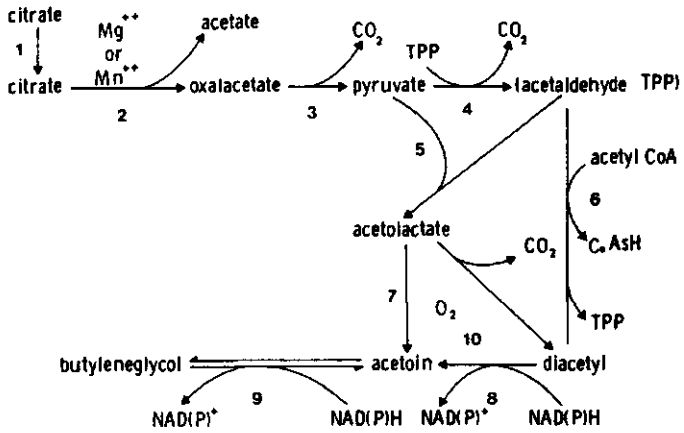

FIG. 2. Pathway of citrate metabolism in *Leuconostoc* spp. and *Lactococcus lactis* subsp. *lactis* var. *diacetylactis*. The reactions are catalyzed by the following enzymes: 1, citrate permease; 2, citrate lyase; 3, oxaloacetate synthase; 4, pyruvate decarboxylase; 5, acetolactate synthase; 6, diacetyl synthase; 7, acetolactate decarboxylase; 8, diacetyl reductase;9, acetoin reductase. Reaction 10 is the oxidative conversion of α-acetolactate to diacetyl (see text).

Another mechanism, proposed by Harvey and Collins (36) postulates that diacetyl-formation results from an enzymatic reaction of the condensation product of acetaldehyde-tyrosinepyrophophate and acetyl-CoA. However, none of the enzymes involved in this reaction have yet been identified or characterized. Recent studies (40) suggest that diacetyl production in *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* and *Leuconostoc* spp. is entirely due to chemical oxidation or decarboxylation of α-acetolactate, confirming the earlier observations (92).

In the heterofermentative *Leuconostoc* spp. citrate is metabolized over a pH range from pH 6.3 to 4.5, but diacetyl and acetoin are only produced at acidic pH (6, 7). Cogan et al. (9)

demonstrated that acetolactate synthase is inhibited by intermediates of the phosphoketolase pathway, and that this inhibition disappeares at acidic pH. In *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* production of acetoin and diacetyl is affected by pH and temperature in non-growing cells (73). A strong pH dependence of citrate fermentation was also observed by Hugenholtz et al. (40).

It has generally been assumed that citrate cannot be used as an energy source by the aroma-producing bacteria, although it has been demonstrated that growth of aroma-bacteria on lactose can be stimulated by citrate (65, 6). However, recent experiments (40, 93) showed that in chemostat cultures *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* strains is able to grow on citrate as sole energy source. The conditions that allow for growth are dependent on the pH of the growth medium. At pH values below 5.5 citrate becomes growth inhibitory, whereas at pH values above 6.0 no citrate is utilized. On the other hand, in *Leuconostoc* spp. in the presence of citrate only stimulation of growth was observed (93). This growth stimulation can be explained by the action of citrate as external electron donor which results in the production of more acetate instead of ethanol during heterofermentative lactose conversion, therefore resulting in the net gain of 1 ATP-equivalent per citrate molecule (86, 93).

The biotechnological importance of the citrate metabolism is widely appreciated and various physiological aspects have been documented, but the genetics of this metabolism has hardly been studied. For *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* it has been well established, that citrate uptake is linked to a 7.9-kb plasmid (48, 29, 88). Genes involved in the metabolism of citrate appeared to be unstable in *Leuconostoc* spp., suggesting plasmid linkage of (some) of those genes (24, 69, Chapter VII of this thesis).

SOLUTE TRANSPORT IN LACTIC ACID BACTERIA

Lactic acid bacteria, being Gram-positive organisms, are surrounded by a cytoplasmic membrane, which functions as a barrier to hydrophilic molecules. Therefore, for the transport of such molecules transmembrane proteins are required.

The biochemical and physical aspects of systems required for the transport of nutrients and maintenance of homeostasis in lactic acid bacteria has been studied extensively in lactococci

symport with a stoichiometry of 3 protons transported per molecule citrate. Lactose transport in *Streptococcus thermophilus* has been proposed recently to proceed via a lactose/galactose antiporter (77, 78). Antiport systems have been described first for the uptake of amino acids. In *Lactococcus lactis*, uptake of arginine has been shown to be linked to the excretion of ornithine by a very efficient antiport system. The driving forces for this process are supplied by the chemical gradients of arginine and ornithine alone (74, 21). Other examples of antiport mechanisms are proposed by Poolman (78), one of them explaining the metabolic energy production during malolactic fermentation in *Lactococci* and *Leuconostoc* spp.. In this model, divalent malate and monovalent lactate are transported via an electrogenic malate/lactate antiport mechanism.

GENE TRANSFER IN LACTOCOCCI

Genetic studies in lactococci have focussed on the transfer of natural and recombinant plasmids into these strains. A variety of different naturally occurring systems have been discovered and analyzed. The first system to be studied was the transductional transfer of genetic material by temperate bacteriophages, which erroneously packaged host DNA and transferred it to the recipient cell by bacteriophage infection (60, 61, 62). Apart from transfer of genetic material from one cell to another, transduction proved to be a means by which integration of plasmid-encoded traits into the chromosome could be achieved. For instance, transduction of lactose plasmids into Lac$^-$ variants of *Lactococcus lactis* strains C2 and 712 was achieved at low frequencies (63, 90). Since the resultant Lac$^+$ strains did not contain plasmid DNA it was concluded that the lactose genes had integrated into the chromosome.

Another mechanism of natural transfer of plasmid encoded information is conjugation, by means of which genetic material is transferred from a donor cell to a recipient cell during close contact of both cells. Plasmids involved in this form of transfer must encode their own transfer functions. However, plasmids lacking these functions may also be transferred in the presence of a conjugative plasmid (94). The enterococcal broad-host range plasmid pAMß1 was efficiently transferred to various lactococcal strains demonstrating the potential of this system (29). Additionally, high-frequency conjugal transfer of the transposable element Tn*919* has been demonstrated (39) and offers a means for chromosomal integration, since

transfer and transposition are combined.

Reintroduction of isolated plasmid DNA is vital in the application of recombinant DNA technology. Because natural competence is lacking in lactococci many efforts have been directed towards the development of a transformation system. For a long time protoplasts have been used as a method to obtain DNA transfer by transformation (30, 51, 89, 99) and transfection (32). However, protoplast production and regeneration are tedious and time consuming methods and the transformation frequencies are poorly reproducible and vary widely between different lactococcal strains (28). The introduction of the electroporation technique has greatly facilitated the transformation of many lactococcal strains (34, 79). It also allowed transformation of *Lactococcus lactis* subsp. *cremoris* strains, of which protoplasts were known to regenerate poorly. However, optimal conditions of this widely used method are still strain specific.

OUTLINE OF THIS THESIS

A prerequisite for the stabilization of important features, such as aroma production, in starter strains used in dairy fermentations, is an extensive knowledge of the genetic basis of these properties. In this thesis the genetic basis of citrate metabolism in *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* and *Leuconostoc* spp. are studied and genetic aspects of citrate-fermenting *Leuconostoc* strains are analyzed.

- Chapter II describes the development of a cloning system for *Leuconostoc* which was necessary due to the lack of natural transformation systems.
- Chapter III describes the use of polymerase chain reaction to classify *Leuconostoc* strains.
- Since lactose is the most important carbohydrate used by lactic acid bacteria, knowledge of the genetic basis of the genes involved in lactose metabolism can offer important data concerning gene regulation and expression. Chapters IV and V describe the cloning and expression of the lactose genes of *Leuconostoc* ssp.
- Cloning and functional expression in *Escherichia coli* of the the citrate permease gene of *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* are described in Chapter VI.
- A comparison of the lactococcal citrate permease with the *Leuconostoc lactis* citrate

permease gene is described in Chapter VII.

- A summary together with concluding remarks are given in Chapter VIII.

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CHAPTER II

Plasmid transformation by electroporation of *Leuconostoc paramesenteroides* and its use in molecular cloning.

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Plasmid Transformation by Electroporation of *Leuconostoc paramesenteroides* and Its Use in Molecular Cloning

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In this report, we demonstrate the utility of electroporation as an efficient method for genetic transformation of *Leuconostoc paramesenteroides*. We optimized several factors which determine the transformation frequency, resulting in transformation efficiencies of up to 4×10^3 transformants per μg of pNZ12 DNA, which contains the promiscuous *Lactococcus lactis* pSH71 replicon. Slightly lower efficiencies were obtained with a deletion derivative of the broad-host-range plasmid pAMβ1. These plasmids could be stably maintained in *L. paramesenteroides* NZ6009 for more than 100 generations, even in the absence of selective pressure. In order to show the use of the developed host-vector system, we cloned the *Lactococcus lactis* gene encoding phospho-β-galactosidase in *L. paramesenteroides*. Expression of this heterologous gene in *L. paramesenteroides* under control of *Lactococcus lactis* expression signals was evident from the presence, in transformants, of phospho-β-galactosidase activity and a specific phospho-β-galactosidase protein band on Western blots (immunoblots). In addition, we transformed a lactose-deficient derivative of *L. paramesenteroides* with a plasmid carrying a *Lactococcus lactis-Escherichia coli lacZ* gene fusion. The resulting transformants synthesized high levels of β-galactosidase, indicating the efficiency of heterologous gene expression signals in *L. paramesenteroides*.

Bacteria of the genus *Leuconostoc* are gram-positive cocci that are phenotypically and ecologically related to group N streptococci (13). *Leuconostoc* spp. are widely used in the food industry; they are involved in the fermentation of vegetables, in wine making, and in the manufacture of fermented milk products, such as butter, buttermilk, and cheese (12). Their wide application as components of starter cultures in the dairy industry relies on their ability to produce CO_2 and the aroma component diacetyl from citrate. Furthermore, the heterofermentative conversion of lactose contributes to the formation of additional amounts of CO_2.

During the last few years, an increasing number of reports on genetic studies of *Leuconostoc* spp. have been published. It is known that many *Leuconostoc* dairy strains contain plasmid DNA (27), and the conjugal transfer of plasmid DNA from *Lactococcus lactis* to *Leuconostoc* spp. has been reported (30, 34, 35). The plasmids used in these studies encode readily identifiable properties, such as lactose fermentation, nisin production, and resistance to erythromycin and chloramphenicol. In addition, the transposon Tn*919*, conferring tetracycline resistance, has been transferred to *Lactococcus lactis* (18).

Until now, no host-vector system has been described for *Leuconostoc* species. The development of a plasmid-mediated DNA transformation system for *Leuconostoc* spp. is, however, a prerequisite for the application of recombinant DNA technology for both the fundamental genetic analysis and the practical improvement of these bacteria.

Electroporation has been used to transfer DNA into a variety of cell types by production of high-intensity electric fields of short duration, during which the cell is reversibly made permeable. Many reports have described transformation by electroporation of mammalian cells (28) and of plant and yeast protoplasts (11, 17). In addition, electroporation has been reported for gram-negative bacteria, such as *Escherichia coli* (9, 10) and *Campylobacter jejuni* (24), as well as for gram-positive bacteria, including the lactic acid bacteria *Lactobacillus casei* (5), *Lactococcus lactis* (16, 29), and *Streptococcus thermophilus* (32). Transformation by electroporation is easily and rapidly performed compared with transformation systems described for various lactic acid bacteria that involve protoplast preparation and complex regeneration media (14, 19). In the present communication, we describe the optimization of an electroporation procedure for *Leuconostoc paramesenteroides* and the use of the procedure in molecular cloning.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Strain NZ6009 is a spontaneous mutant of *Leuconostoc* strain Lcm9 and lacks an endogenous cryptic plasmid with an approximate size of 3 kilobase pairs. Its sensitivity to the Lcm9-specific bacteriophage BA-Lcm9 is the same as the sensitivity of the parent strain. Both strain Lcm9 and its phage BA-Lcm9, which was used for strain identification in subsequent experiments, were obtained from B. Lébert, Nestec Research Laboratory, Vevey, Switzerland.

Strain NZ6091 is a lactose-deficient derivative of strain NZ6009 that lacks β-galactosidase activity and that was isolated after plasmid curing by electroporation (unpublished results).

Growth of bacteria. *E. coli* and *Bacillus subtilis* strains were propagated in L broth at 37°C; all further manipulations of these bacteria were as described for *E. coli* by Maniatis et al. (22).

L. paramesenteroides and *Lactococcus lactis* strains were grown in complete MRS broth (Difco Laboratories, Detroit, Mich.) or in MRS broth containing lactose as the sole carbon source (MRS-lactose). For plating, MRS broth was supplemented with 1.5% agar; cells were plated by using a top-agar

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant feature(s)[a]	Reference or source[b]
L. mesenteroides NCDO 523	Type strain	NCDO
L. paramesenteroides		
NZ6009	Lac$^+$, Cit$^+$	This study
NZ6091	Lac$^-$, Cit$^+$	Lactose-deficient derivative of NZ6009 (unpublished results)
Lactococcus lactis subsp. *diacetylactis* NCDO 176	Cit$^+$	NCDO
Lactococcus lactis		
MG1363	Plasmid-free, Lac$^-$	15
MG1820	Lac$^+$	22
E. coli MC1061	Plasmid-free	4
B. subtilis 168	Plasmid-free	1
ΔpAMβ1	10.6 kb, Em^r	36
pNZ12	4.3 kb, Cm^r	7
pNZ36	6.8 kb, Cm^r, pNZ12 with a 2.5-kb fragment containing the *Lactococcus lactis* phospho-β-galactosidase gene	de Vos and Gasson, J. Gen. Microbiol., in press
pNZ262	10.7 kb, Cm^r, pNZ12 with a 6.3-kb fragment containing a *Lactococcus lactis-E. coli lacZ* gene fusion	8

[a] kb, Kilobase pairs; Cm^r and Em^r, resistance to chloramphenicol and erythromycin, respectively. Cit$^+$ and Cit$^-$ and Lac$^+$ and Lac$^-$ indicate the ability and inability to dissimilate citrate and to ferment lactose, respectively.
[b] NCDO, National Collection of Dairy Organisms.

overlay (MRS broth containing 0.7% agar). When required, chloramphenicol or erythromycin (both purchased from Sigma Chemical Co., St. Louis, Mo.) were each added at 10 μg/ml. All lactic acid bacterium strains were cultivated at 28°C.

For the identification of strain NZ6009, the following characteristics were determined: (i) the ability to form CO_2 from glucose, (ii) the ability to utilize citrate (halo formation on citrate agar plates [12]), (iii) the formation of either D-(−)-lactate or L-(+)-lactate from glucose (26), and (iv) sensitivity to vancomycin (Sigma) (27). For differentiation between *Leuconostoc* species, the ability to form acid from various carbohydrates was analyzed by using API test strips (API-50 CHL; API systems S.A., La Balme les Grottes, France). The ability to form dextran from sucrose was tested on sucrose agar plates (12).

Plasmid DNA isolation. Plasmid DNA isolations from *Lactococcus lactis* and *L. paramesenteroides* NZ6009 were performed on a small scale by using the alkaline lysis method (2) with the following modifications. Five milliliters of exponentially growing cells (A_{600} of 0.5) was incubated in THMS buffer (30 mM Tris hydrochloride [pH 8.3], 3 mM $MgCl_2$, 25% sucrose) containing 2 mg of lysozyme per ml for 30 min at 37°C. The suspension was chilled on ice, 2 volumes of a solution containing 0.2 N NaOH and 1% (wt/vol) sodium dodecyl sulfate (SDS) were added, and the procedure was continued as described for *E. coli* (23). For use in electroporation, plasmid DNA was purified by CsCl/ethidium bromide density gradient centrifugation (23).

Restriction endonuclease digestions were performed according to the suppliers' recommendations (Bethesda Research Laboratories, Inc., Gaithersburg, Md., and Boehringer GmbH, Mannheim, Federal Republic of Germany).

Electroporation. *L. paramesenteroides* NZ6009 was grown overnight in MRS broth supplemented with 40 mM DL-threonine (MRS-T). This culture was diluted 1:20 in MRS-T, and exponentially growing cells were harvested by centrifugation (6,000 × *g*, 20°C), washed twice with ice-cold electroporation buffer (5 mM potassium phosphate buffer [pH 7.4], 2 mM $MgCl_2$ in 25% sucrose), and finally resuspended in ice-cold electroporation buffer to an A_{600} of 2.0.

Subsequently, 0.8 ml of the cell suspension was transferred into a sterile Gene Pulser cuvette (Bio-Rad Laboratories, Richmond, Calif.) with an inter-electrode distance of 0.4 cm. Plasmid DNA in 10 μl of TE buffer (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA) was added, and the mixture was left to stand on ice for 10 min prior to electroporation.

High-voltage pulses were delivered with a Gene Pulser apparatus (Bio-Rad) by using the 25-μF capacitor. After 10 min on ice, the cells were diluted into 8 ml of MRS broth and kept at 28°C for at least 1 h to allow for the expression of the antibiotic resistance marker. Dilutions of cells were plated on MRS agar plates containing the appropriate antibiotic. Colonies were visible after 24 to 36 h of incubation at 28°C.

Control experiments to determine the survival of the cells and the occurrence of spontaneous antibiotic-resistant mutants were performed by plating cells either that had received no electrical pulse or that were electroporated without plasmid DNA, on media with or without an antibiotic.

Enzymatic assays. To determine the phospho-β-galactosidase and β-galactosidase activities in *Lactococcus lactis* and *L. paramesenteroides*, exponentially growing cells were used that were protoplasted in THMS buffer containing 2 mg of lysozyme per ml, washed twice in THMS buffer, and lysed in 50 mM sodium phosphate buffer (pH 7.2) containing 1 mM dithiothreitol. To obtain complete lysis, the mixture was sonicated three times for 5 each time at 4°C in a model W-375 sonifier (Heatsystems Ultrasonic Inc., Plainview, N.Y.) at the maximal microtip setting. Aliquots were tested for phospho-β-galactosidase and β-galactosidase by using as substrates *o*-nitrophenyl-β-D-galactopyranoside-6-phosphate (ONPG-P) and *o*-nitrophenyl-β-D-galactopyranoside (ONPG), respectively(both purchased from Sigma Chemical Co.), as described by Okamoto and Morichi (25).

Protein content was determined by the method of Bradford (3) by using the Bio-Rad protein assay with bovine serum albumin as a standard.

Plasmid stability. The stability of plasmid DNA in *L. paramesenteroides* NZ6009 was determined by serially passaging the strains in MRS broth in the absence of an antibiotic. Cells were plated on MRS agar after 100 generations, and at least 200 individual colonies were picked and plated on selective and nonselective plates to determine the fraction of antibiotic-resistant colonies.

Southern blot hybridizations. Plasmid DNA was separated on a 0.8% agarose gel, and the DNA was transferred to Gene Screen Plus membranes and hybridized as described by the supplier (Du Pont, NEN Research Products, Boston, Mass.). Prehybridization was performed for at least 2 h in (pre-) hybridizing solution at 65°C.

Hybridization was performed overnight at 65°C by using 2

TABLE 2. Summary of characteristic features of *L. paramesenteroides* NZ6009 and comparison with those of *Streptococcus lactis* subsp. *diacetylactis* NCDO 176 and *L. mesenteroides* NCDO 523[a]

Strain	CO_2 from glucose	Type of lactic acid from glucose	Vancomycin (μg/ml)	Citrate dissimilation	Growth in litmus milk	Dextran formation	Growth at 37°C
NZ6009	+	D-(−)	>500	+	Slight	−	+
NCDO 176	−	L-(+)	<10	+	+	−	+
NCDO 523	+	ND	>500	−	Slight	+	−

[a] +, Reaction or growth observed; −, reaction or growth not observed; ND, not determined.

ml of hybridization solution and 0.3 μg of probe DNA, which was prepared by nick translation by using [α-^{32}P]dATP (Du Pont, NEN Research Products) by the method of Maniatis et al. (23).

After hybridization, the filter was washed three times for 30 min each time at 65°C in 0.1× SSC and 0.1% SDS and subsequently autoradiographed by using XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) and Du Pont Cronex Lightning-Plus intensifying screens at −80°C (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

Western blot (immunoblot) analysis. Whole-cell protein samples were prepared by incubating cells from 1.5-ml exponentially growing cultures in 500 μl of THMS buffer with 2 mg of lysozyme per ml for 1 h at 37°C. Cells were pelleted and resuspended in 100 μl of sample buffer (60 mM Tris hydrochloride [pH 6.8], 1% SDS, 1% β-mercaptoethanol, 10% glycerol, 0.01% bromphenol blue), and the lysates were heated at 100°C for 5 min. Samples (35 μl each) were applied to 12.5% polyacrylamide–SDS gels (20), which were run for 16 h at 35 V. The gels were blotted to nitrocellulose filters (BA85; Schleicher & Schuell, Inc., Keene, N.H.) in Tris-glycine buffer (25 mM Tris hydrochloride [pH 7.4], 192 mM glycine) containing 20% (vol/vol) methanol by using a blotting apparatus (Transblot; Bio-Rad) for 4 h at 300 mA.

Phospho-β-galactosidase was visualized by using rabbit antibodies against purified *Lactococcus lactis* phospho-β-galactosidase (8) and swine anti-rabbit peroxidase conjugate (Dakopatts A/S, Glostrup, Denmark), with 1-chloro-4-naphthol as a substrate.

RESULTS

Strain identification. Various essential properties of strain NZ6009 are summarized in Table 2.

Because of the ability of this strain to form acid from arabinose, xylose, and mannitol and its inability to form dextran from sucrose, strain NZ6009 was classified as *L. paramesenteroides*. As observed in other *Leuconostoc* species, *L. paramesenteroides* contains plasmid DNA (27). Strain NZ6009 harbors four endogenous plasmids with molecular weights ranging from 35 to 1.8 kilobase pairs, as is shown in Fig. 1.

Electroporation of various plasmids into NZ6009. In initial experiments, *L. paramesenteroides* NZ6009 was transformed with 1 μg of DNA from the broad-host-range vectors pNZ12 and ΔpAMβ1 by using a single pulse of 6.25 kV/cm. The percentage of cell death under these conditions was less than 10%.

Transformation efficiencies per microgram of plasmid DNA ranged from 1×10^3 to 4×10^3 for pNZ12 and were a factor of 10 lower (2×10^2) for ΔpAMβ1. Cells of strain NZ6009 that had been electroporated in the absence of DNA did not produce colonies resistant to either chloramphenicol or erythromycin.

The plasmid content of transformants was analyzed by agarose gel electrophoresis of plasmid DNA isolated on a small scale; the presence of the donor plasmid DNA could be demonstrated in all antibiotic-resistant transformants. Examples of plasmid profiles from *L. paramesenteroides* NZ6009 transformants are shown in Fig. 2. Comparison of the intensities of the undigested and linearized DNA bands (Fig. 2, lanes A and C and B and D, respectively) of pNZ12 and pNZ36 with those of the endogenous plasmids indicates that pNZ12 and its derivative, pNZ36, have a higher copy number in *L. paramesenteroides* than do the endogenous plasmids detected.

The presence of donor plasmids in the transformants was confirmed in hybridization experiments as shown in Fig. 3. In all experiments, the strain identity of the transformants was confirmed by testing their sensitivity to the NZ6009-specific phage BA-Lcm9.

Electroporation conditions. To determine the conditions required for optimal electroporation of *L. paramesenteroides* NZ6009, the strain was electroporated under several different conditions by using pNZ12 DNA. Experiments were performed that varied the initial electrical field strength, the amount of plasmid DNA, and the source of plasmid DNA. A strong effect of the electrical field strength on electroporation efficiency was observed.

No transformants could be detected at the lowest voltage applied. Between 3.75 and 6.25 kV/cm, the efficiency of transformation (i.e., the number of antibiotic-resistant colonies per microgram of DNA) increased almost exponentially

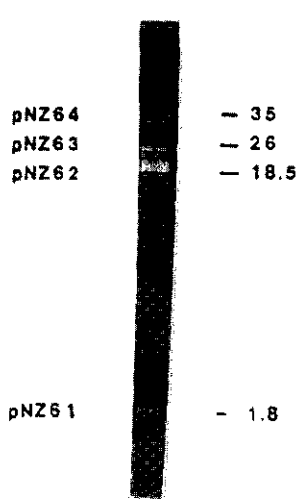

FIG. 1. Agarose gel electrophoresis of CsCl/ethidium bromide-purified plasmid DNA from *L. paramesenteroides* NZ6009. Numbers to the right indicate plasmid sizes (in kilobase pairs). On the left, plasmid designations are given.

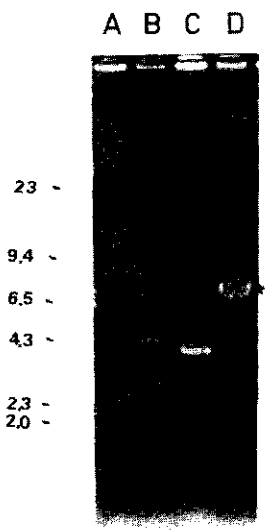

FIG. 2. Agarose gel electrophoresis of plasmid DNA isolated on a small scale from *L. paramesenteroides* NZ6009 transformants. Numbers on the left indicate sizes of molecular size standards (in kilobase pairs). Lanes: A and B, plasmid DNA from transformants harboring pNZ12, undigested and digested with *Sal*I, respectively; C and D, plasmid DNA from transformants harboring pNZ36, undigested and digested with *Eco*RI, respectively. Asterisks mark the positions of the linearized pNZ12 (4.3 kilobase pairs) and pNZ36 (6.8 kilobase pairs).

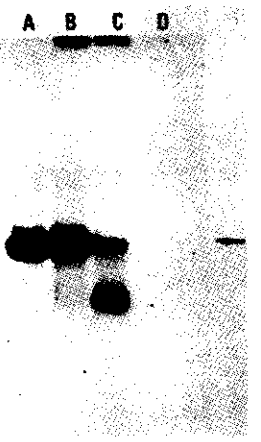

FIG. 3. Autoradiogram obtained from a Southern blot of plasmid DNA from *L. paramesenteroides* NZ6009 transformants harboring pNZ12 after hybridization with ^{32}P-labeled pNZ12 DNA. Lanes: A, pNZ12 DNA digested with *Sal*I; B and C, total plasmid DNA of NZ6009 harboring pNZ12, digested with *Sal*I and undigested, respectively; D, total plasmid DNA of NZ6009 (not transformed). The arrow indicates the linearized 4.3-kilobase-pair pNZ12 plasmid band.

with the field strength. At the highest voltage applied (6.25 kV/cm), efficiencies of 1×10^3 to 4×10^3 transformants per μg of DNA were observed. This corresponds to frequencies of about one transformant per 10^7 recipient cells.

The relationship between the amount of transforming plasmid DNA and the number of transformants obtained by electroporation was examined and is presented in Table 3. In the range of 0.3 to 3.0 μg of plasmid DNA, a linear dose response was observed between DNA concentration and transformation efficiency, indicating that one molecule is sufficient to transform one host cell. The values in Table 3 indicate that saturation occurs at higher DNA concentrations.

To determine whether the source of plasmid DNA had any effect on the efficiency of *Leuconostoc* transformation, we transformed *L. paramesenteroides* NZ6009 with pNZ12 DNA isolated from *Lactococcus lactis* MG1363, *E. coli* MC1061, or *L. paramesenteroides* NZ6009. Similar frequencies, approximately 10^3 transformants per μg of DNA, were obtained (data not shown), indicating that the source of DNA had no influence on the transformation efficiencies.

Stability of plasmid DNA in *L. paramesenteroides* NZ6009. After growth for 100 generations without selective pressure, no chloramphenicol- or erythromycin-sensitive colonies could be detected for strain NZ6009 containing either pNZ12 or ΔpAMβ1. All antibiotic-resistant colonies tested (24 of 24) appeared to contain the intact pNZ12 or ΔpAMβ1, as could be demonstrated by analysis of plasmid DNA content (data not shown). Growing the transformants mentioned above in the presence of the curing agent acriflavine (5 μg/ml) did not result in the curing of either pNZ12 or ΔpAMβ1 (data not shown), confirming the stability of these plasmids in *L. paramesenteroides*.

Expression of the *Lactococcus lactis* phospho-β-galactosidase gene in *L. paramesenteroides*. In order to test the utility of the established host-vector system for *L. paramesenteroides* NZ6009, the *Lactococcus lactis* phospho-β-galactosidase gene located on pNZ36 (Table 1) was introduced into this strain. The phospho-β-galactosidase activity of NZ6009 harboring pNZ36 (Table 4) was elevated only slightly as compared with the untransformed control (NZ6009). This is a consequence of relatively high background activity of strain NZ6009. However, the expression of the *Lactococcus lactis* phospho-β-galactosidase gene is evident in the lactose-deficient derivative NZ6091 containing pNZ36 (Table 4). The levels of phospho-β-galactosidase activity specified by pNZ36 were comparable in *Lactococcus lactis* and *L. paramesenteroides*.

Expression of phospho-β-galactosidase activity in transformants carrying pNZ36 was further demonstrated by Western blot analysis (Fig. 4). The protein extracts of NZ6009 harboring pNZ36 (Fig. 4, lane E) clearly show an additional band that migrates the same distance as the

TABLE 3. DNA dependence of electroporation (effect of the concentration of pNZ12 on the transformation efficiency of *L. paramesenteroides* NZ6009)

μg of DNA	No. of transformants[a]	Transformation efficiency[b]
0.3	2.5×10^2	8.3×10^2
0.5	4.2×10^2	8.4×10^2
1.0	6.5×10^2	6.5×10^2
3.0	2.1×10^3	7×10^2
10.0	3.6×10^3	3.6×10^2

[a] Total number of antibiotic-resistant colonies observed.
[b] Number of transformants per microgram of DNA.

TABLE 4. Specific phospho-β-galactosidase and β-galactosidase activities of *L. paramesenteroides* (NZ6009 and NZ6091) and *Lactococcus lactis* strains (MG1363 and MG1820) containing plasmids carrying the *Lactococcus lactis* phospho-β-galactosidase gene (pNZ36) or the *Lactococcus lactis-E. coli lacZ* gene fusion (pNZ262)

Strain	Sp act of[a]:	
	Phospho-β-galactosidase	β-Galactosidase
NZ6009	42	2.3×10^3
NZ6009(pNZ36)	75	2.4×10^3
NZ6091	<0.1	<0.1
NZ6091(pNZ36)	92	<0.1
NZ6091(pNZ262)	<0.1	0.7×10^3
MG1363	<0.1	<0.1
MG1363(pNZ36)	79	<0.1
MG1363(pNZ262)	<0.1	1.0×10^3
MG1820	2×10^3	<0.1

[a] Specific activities are expressed as nanomoles per minute per milligram of protein.

purified phospho-β-galactosidase (lane D). This band is also present in the phospho-β-galactosidase-positive *Lactococcus lactis* MG1820 (lane A) and in MG1363 harboring pNZ36 (lane C), whereas such a band could not be detected in the phospho-β-galactosidase-deficient *Lactococcus lactis* MG1363 or in *L. paramesenteroides* NZ6091 (lanes B and F, respectively).

Expression of the *E. coli* β-galactosidase gene in *L. paramesenteroides*. The plasmid pNZ262 is a pNZ12 derivative containing a gene fusion between the *Lactococcus lactis* SK11 proteinase transcription and translation initiation signals and the *E. coli lacZ* gene (8). The lactose-deficient *L. paramesenteroides* strain NZ6091 appeared to be devoid of β-galactosidase activity and was therefore chosen as the subject of transformation with pNZ262 DNA.

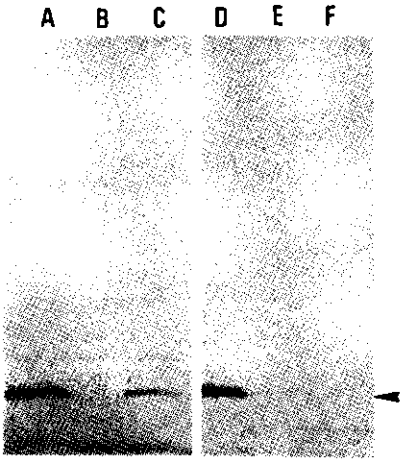

FIG. 4. Immunoblot analysis of cell lysates from various *Lactococcus lactis* and *L. paramesenteroides* strains after incubation with antiserum raised against *Lactococcus lactis* phospho-β-galactosidase. Lanes: A, *Lactococcus lactis* MG1820 (Lac^+); B, *Lactococcus lactis* MG1363 (Lac^-); C, *Lactococcus lactis* MG1363 harboring pNZ36; D, *Lactococcus lactis* NCDO 712 phospho-β-galactosidase purified from an overproducing *E. coli* strain (8); E, *L. paramesenteroides* NZ6009 harboring pNZ36; F, *L. paramesenteroides* NZ6009 (untransformed). The arrow indicates the specific phospho-β-galactosidase band.

The β-galactosidase activity of *L. paramesenteroides* transformants was comparable to the specific activity observed in *Lactococcus lactis* MG1363 carrying pNZ262 (Table 4) and only slightly lower than the activity in wild-type, lactose-proficient *L. paramesenteroides* NZ6009. However, the presence of the *lacZ* gene in NZ6091 did not allow strain NZ6091 to grow on MRS-lactose (data not shown).

DISCUSSION

The results presented here show that *L. paramesenteroides* NZ6009 can be transformed efficiently by means of electroporation with two unrelated broad-host-range plasmids. The conjugal transfer of pAMβ1 to *L. dextranicus* and *L. cremoris* was already demonstrated previously (30), indicating the functionality of its replicon in *Leuconostoc* species. The successful transformation of *L. paramesenteroides* by pNZ12 DNA exemplifies the wide host range of this vector. The replicon of pNZ12 is derived from the cryptic *Lactococcus lactis* NCDO 712 plasmid pSH71 and is functional in various streptococcal hosts, as well as in *B. subtilis* (7), *Lactobacillus casei* (5), *Staphylococcus aureus* (7), and *E. coli* (7).

Plasmid pNZ12 was chosen for initial experiments designed to establish optimal conditions for the electroporation of *L. paramesenteroides* NZ6009. The transformation efficiencies obtained in *L. paramesenteroides* NZ6009 amounted to 1×10^3 to 4×10^3 transformants per μg of pNZ12 DNA. A linear dose response was observed up to an amount of 3.0 μg of pNZ12 DNA per 2×10^9 cells. Saturation occurred when higher DNA concentrations were used. Minor variations between experiments were observed that may be attributed to differences in growth phases of the cell preparations (24). Our experimental outline was based on earlier data on electroporation of lactic acid bacteria (5, 29, 32), showing that the voltage applied is the most significant parameter.

Application of pulses up to 6.25 kV/cm, which represented the maximum voltage that could be reached, resulted in a linear increase in the number of transformants, indicating that higher voltages might give rise to an even larger number of transformants (9). However, preliminary experiments using cuvettes with a 0.2-cm inter-electrode distance, resulting in a higher electrical field strength, showed that transformation efficiencies do not exceed 4×10^3/μg of pNZ12 DNA. This indicates that the efficiencies obtained by using 6.25 kV/cm may be the maximal efficiencies obtainable with this strain under the given experimental conditions.

Efficiencies of transformation in *L. paramesenteroides* NZ6009 were lower than those achieved by transformation of *Lactococcus lactis* (29) and *Lactobacillus casei* (5). Results of the electroporation of *Streptococcus thermophilus* (32) and very recently published results on the electroporation of *L. lactis* and *L. dextranicus* (21) showed comparable transformation efficiencies. However, the system for the electroporation of these *Leuconostoc* strains has not been optimized or used to study the expression of new genes in this species. *L. paramesenteroides* NZ6009 cells were very resistant to high electrical pulses, with only a 10% decline in the number of CFU at 6.25 kV/cm. The apparent large number of surviving cells may not reflect the actual number of viable cells but could be due to electroporation-induced rupture of cell chains, giving rise to more CFU.

Leuconostoc spp. are heterofermentative and do not possess a lactose phosphotransferase system (6). Therefore, the phosphotransferase system-specific phospho-β-galactosidase activity, which hydrolyzes the intracellular phosphorylated lactose, is absent in this genus. Instead, *Leuconostoc* spp. hydrolyzes the internalized lactose by using a β-galactosidase. Little is known about the sugar uptake system in this organism, but it may be mediated by a proton motive force-dependent permease system, which might function in a manner analogous to that in other heterofermentative organisms (33). Since the galactose moiety of lactose is completely metabolized by *Leuconostoc* spp.(6), a permease acting as a lactose-galactose antiporter, as has recently been proposed for *Streptococcus thermophilus* (27a), is not likely to exist in these bacteria.

Transformants containing pNZ36 showed specific phospho-β-galactosidase activities comparable to the specific activity seen in *Lactococcus lactis* MG1363 (pNZ36). However, these activities could be assayed accurately only in a β-galactosidase-negative derivative (NZ6091). Recent experiments have shown that this strain does not contain the lactose plasmid detected in the wild-type strain (unpublished results). Background activity of NZ6009 might be due to phosphatase activity that converts ONPG-P to ONPG, which subsequently can be hydrolyzed by β-galactosidase (32).

The *E. coli lacZ* gene located on pNZ262 was introduced into NZ6091, and transformants could express the *E. coli* β-galactosidase by using the *Lactococcus lactis* SK11 proteinase transcription and translation initiation signals. Comparing the levels of β-galactosidase activity in both a lactose-deficient *Lactococcus lactis* strain and a *Leuconostoc* strain, we can conclude that lactococcal expression signals can be efficiently recognized by *L. paramesenteroides*. The fact that pNZ262 results in high levels of β-galactosidase synthesis in *L. paramesenteroides* NZ6091 but does not complement for its lactose-deficient phenotype suggests that this strain also lacks other components of the lactose pathway.

The results described here demonstrate the possibilities of heterologous gene expression in *L. paramesenteroides* by using established lactic acid bacterium cloning and expression vectors. Furthermore, the results provide opportunities for further genetic studies in *Leuconostoc* spp., including the analysis of genes encoding important dairy functions, such as lactose and citrate metabolism.

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CHAPTER III

Reclassification of two *Leuconostoc* strains using PCR-derived specific DNA-probes of 16S rRNA variable regions.

S. David and W. M. de Vos

SUMMARY

Two *Leuconostoc* strains that had previously been classified as *Leuconostoc paramesenteroides* based on phenotypic characteristics were reclassified. The method used was a hybridization of specific DNA probes with PCR-amplified highly variable regions of 16s rRNA. Based on the results of these studies the *Leuconostoc* strains were grouped as *Leuconostoc lactis*.

INTRODUCTION

Traditionally the classification of micro-organisms has been based to a great extent on similarities in their morphology and nutritional requirements. For the differentiation of species within the genus *Leuconostoc* phenotypic characterization based mainly on carbohydrate fermentation patterns have been used for a long time (9,10). However, classification of strains using this method often proves to be unreliable, since the fermentation patterns of lactic acid bacteria belonging to different genera often overlap and their morphology can vary according to growth conditions (9).

Nucleic acid hybridization studies have proved useful in distinguishing between closely related lactic acid bacteria such as the heterofermentative *Lactobacillus* spp. and *Leuconostoc* spp., with *Leuconostoc oenos* forming a distinct group (7,8). Other studies demonstrated that *Leuconostoc paramesenteroides* was more closely related to *Lactobacillus* spp. than to the non-acidophilic species *Leuconostoc mesenteroides* and *Leuconostoc lactis* (3,17,18,20).

The 16S and 23S rRNA genes reveal an unique organization in which highly conserved regions alternate with more variable regions (4). Comparative analysis of 16S rRNA sequences and the use of DNA probes directed to the variable regions have been successfully used for the identification and phylogenetic analysis of different bacterial species (1,2,6,20). This type of analysis has provided significant insight into the natural relationships among lactic acid bacteria and has led to important changes in their taxonomy and nomenclature. This has resulted to a natural grouping of *species of Leuconostoc* and *Lactobacillus*, in which the *Leuconostoc paramesenteroides* was clearly placed into a specific subgroup (Fig.1; 3,12,20).

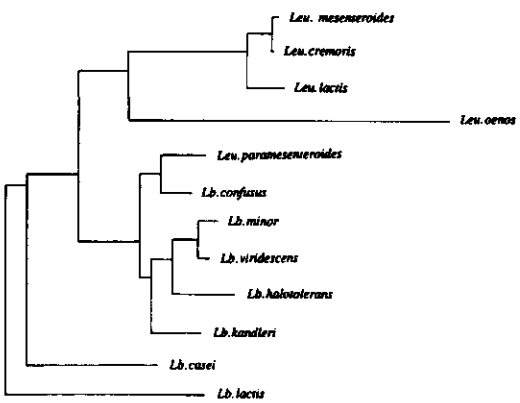

FIG. 1. Phylogenetic tree showing the relationship of the *Leuconostoc* branch of the genus *Lactobacillus* (reproduced from ref. 20). *Leu.* = *Leuconostoc*; *Lb.* = *Lactobacillus*.

More recently, the polymerase chain reaction (PCR; 15) has been applied to amplify rRNA variable sequences, using primers located in the flanking conserved regions. The amplified PCR-products could subsequently be used as targets in hybridization experiments with

specific probes (1). Using this approach, Klijn et al. (11) developed specific probes for the identification of mesophilic lactic acid bacteria, based on highly variable regions, derived from published rRNA sequences (3,12,16,19). PCR-amplified DNA of the variable V1 and V3 regions of 16S rRNA were hybridized with these probes, enabling discrimination between various *Lactococcus* strains and *Leuconostoc* species, respectively.

In this Chapter we describe the use of PCR and specific DNA probing in the reclassification of two *Leuconostoc* strains, which had previously been grouped on basis of their fermentation patterns.

MATERIALS AND METHODS

Two *Leuconostoc* strains NZ6070 (obtained as Lp7-1 from A. Harrington, University College, Cork) and NZ6009 (5) and their cured derivatives, NZ6071 (Cit^-) and NZ6091 (Lac^-), respectively were analyzed to study their previous classification as *Leuconostoc paramesenteroides*. *Leuconostoc* strains NCDO523, NCDO533 and NCDO803 as well as *Lactococcus* strain NCDO176 were obtained from the National Collection of Dairy Organisms. Strains were cultured and maintained in MRS medium (Difco) at 30°C.

The ability to form CO_2 from glucose was determined using Durham tubes in MRS broth. The formation of D(-)-lactate as opposed to L-(+)-lactate was determined by HPLC analysis (13).

Acid production from various carbohydrates was tested using API test strips (API-50 CHL; API systems S.A., La Balme Les Grottes, France), and the ability of the strains to form dextran from sucrose was tested on sucrose agar plates (9). Citrate utilization was tested on citrate agar plates (9) by determining the halo-formation around colonies.

DNA isolation of the strains and further manipulations were performed as described by Klijn et al. (11).

RESULTS AND DISCUSSION

The identifcation as *Leuconostoc* strains was based on the essential properties suggested by

Garvie (9) that are listed in Table 1. The heterofermentative character of the strains was evident from their ability to form CO_2 from glucose. Additionally, a high vancomycin resistance clearly distinguishes the strains from other lactic acid bacteria (14).

TABLE 1. Characteristic features of strains NZ6009 and NZ6070 in comparison with those of *Leuconostoc mesenteroides* NCDO523, *Leuconostoc lactis* NCDO533 and *Lactococcus lactis* NCDO176.@

Strain	CO_2 from glucose	Type of lactic acid from glucose	Vancomycin resistance (μg/ml)
NZ6009	+	D(-)	> 500
NZ6070	+	D(-)	> 500
NCDO523	+	D(-)	> 500
NCDO533	+	D(-)	n.t.
NCDO176	-	L(+)	< 10

@ +, Reaction or growth observed; -, reaction or growth not observed; n.t. not tested.

Further differentiation between *Leuconostoc* species was performed by assaying for carbohydrate fermentation. The results of those tests are summarized in Table 2. The inability of the strains to form dextran from sucrose in combination with the acid formation from xylose (only with strain NZ6009), lactose, melibiose and raffinose (only with strain NZ6070) resulted in the classification of these strains as *Leuconostoc paramesenteroides*.

Fermentation patterns are known to vary between experiments, due to minor differences of growth conditions. Additionally variations between strains of the same species are known to occur and similarity of the patterns are found with different species (Table 2; 9). Therefore, we decided to confirm the classification of strains NZ6009 and NZ6070 with a more reliable and sensitive method as soon as it became available. For this purpose we applied the method which was recently described by Klijn et al. (11) using specific DNA-probes based sequences within the variable regions V1 and V3 of 16S rRNA genes for hybridization experiments on DNA of these variable regions, amplified using PCR. Using primers P1 and P2 (Fig.2) for the amplification of the V1 region, it is possible to differentiate between *Leuconostoc paramesenteroides* and other *Leuconostoc* species, since the V1 region of *Leuconostoc paramesenteroides* is larger (V1 = 110 bp) than that of other

Leuconostoc species (V1 = 90 bp) (12). This difference in size of the amplified DNA fragments can be visualized on ethidium bromide stained agarose gels. Furthermore, a V1 region-specific DNA-probe for *Lactococcus*

TABLE 2. Summary of characteristic features used for the differentiation between species of Leuconostoc strains.

	Strain				
	NZ 6009	NZ 6070	NCDO 523	NCDO 533	NCDO 803
colonies on sucrose agar	-	-	+	-	-
Halo-formation on citrate plates	+	+	-	-	-
Fermentation of:					
Arabinose	-	-	+	+	-
Xylose	+	-	+	-	-
Lactose	+	+	-	+	+
Mannitol	-	-	+	-	-
Melibiose	+	+	+	-	+
Raffinose	-	+	+	-	+
Threhalose	-	-	+	-	+

+, growth or reaction observed; - no growth or reaction observed. Strains used for comparison are culture type strains *Leuconostoc mesenteroides* NCDO523; *Leuconostoc lactis* NCDO533, and *Leuconostoc paramesenteroides* NCDO803.

cremoris (PLc; 5'-TTCAAATTGGTGCAAGCACC-3')(Fig.2) was used it is possible to distinguish *Leuconostoc paramesenteroides* from *Lactococci* and several other *Leuconostoc* species, since the *Leuconostoc paramesenteroides* DNA sequence differs markedly in this region (Fig.2). Further distinction between *Leuconostoc mesenteroides* and *Leuconostoc lactis* is possible based on specific hybridization of PCR-amplified V3 regions of the 16S rRNA sequences of these species (see Fig.2), since this region shows marked sequence differences between *Leuconostoc mesenteroides* and *Leuconostoc lactis* allowing the design of specific probes.

In order to classify the *Leuconostoc* strains NZ6009 and NZ6070 amplification of the V3 regions of those strains together with that of two culture type strains, *Leuconostoc*

mesenteroides NCDO523 and *Leuconostoc lactis* NCDO533 was performed using primers P3 and P4 (Fig.2). Hybridization of the amplified DNA with a *Leuconostoc lactis* V3 region-specific probe (PLCl; 5'-ATGCTAGAATAGGGAATGAT-3') (Fig.2) showed hybridizing bands with the amplified DNA of all strains except that of strain *Leuconostoc mesenteroides* NCDO523 (Fig.3 panel B). The size of the amplified DNA, visible on the ethidium bromide stained gel (Fig.3, panel A) was the same for all DNAs analyzed, ruling out the possibility that they were *Leuconostoc paramesenteroides*.

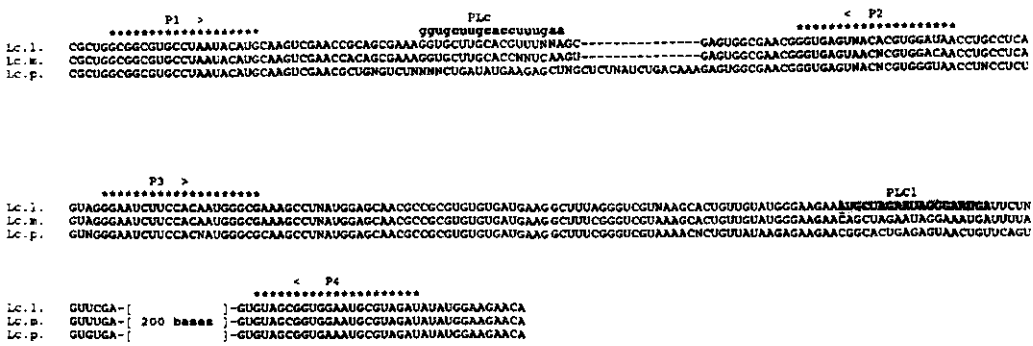

FIG. 2. Alignment of 16S rRNA sequences of *Leuconostoc lactis* NCDO533, *Leuconostoc mesenteroides* NCDO523 and *Leuconostoc paramesenteroides* NCDO803 (12). Alignment gaps are indicated by dashes, N designates an undetermined nucleotide. Regions marked by stars and designated P1, P2, P3 and P4 represent the location of the primers that were used to amplify the V1 and V3 variable regions, respectively (11). The direction of the primers are marked by arrows. Specific DNA probes for the V1 region and for the V3 region that were used in hybridizations are indicated in small, bold print above the corresponding sequence in *Leuconostoc* (PLc, V1 region-specific probe) or by highlightening the sequence (PLCl, V3 region-specific probe).

The above results clearly indicate that the strains NZ6009 and NZ6070 together with their cured derivatives, NZ6091 (results not shown) and NZ6071 respectively, should be reclassified as *Leuconostoc lactis*. Furthermore, this study shows that errors can easily be made using phenotypical properties of bacteria for their determination and that PCR-amplification of 16S rRNA in combination with specific hybridization offers a relatively simple and fast method to unambiguously classify bacteria.

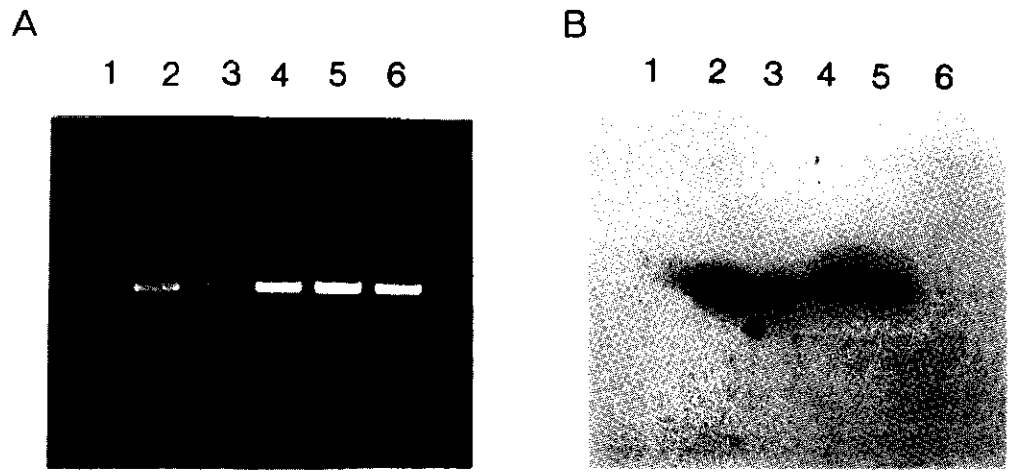

FIG. 3. Identification of *Leuconostoc* species. Panel A: Ethidiumbromide stained 2% agarose gel showing PCR-amplified DNA of the variable V3 region of 16S rRNA genes. Panel B: Southern blot hybridized with the *Leuconostoc lactis*-specific PLCl-probe located within the V3 region. Lane 1: no DNA; lane 2: NZ6070; lane 3: NZ6071; lane 4: NZ6009; lane 5: *Leuconostoc lactis* NCDO533; lane 6: *Leuconostoc mesenteroides* NCDO523.

Acknowledgements

The author would like to thank N. Klijn for helpful discussions and the practical assistance in performing the PCR experiments.

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CHAPTER IV

Leuconostoc lactis ß-Galactosidase Is Encoded by Two Translationally Coupled Genes

S. David, H. Stevens, M. van Riel, G. Simons, and W. M. de Vos

SUMMARY

We report the molecular characterization and overexpression in *Escherichia coli* of two translationally coupled genes, *lacL* and *lacM*, from *Leuconostoc lactis* NZ6009, that encode a functional ß-galactosidase. A 16-kilobase *Bam*HI fragment of the *L.lactis* lactose plasmid pNZ63 coding for ß-galactosidase activity was cloned in *E.coli* MC1061 using pACYC184. Further deletion and complementation analysis showed that the coding region for the ß-galactosidase was located on a 5.8-kilobase *Sal*I-*Bam*HI fragment. Its nucleotide sequence was determined and contained two partially overlapping genes, *lacL* and *lacM*, of 1878 and 963 base pairs, respectively, that could encode proteins with a calculated size of 72,113 and 35,389. The *L.lactis* ß-galactosidase was overproduced in *E.coli* using the lambda P_L promoter and resulted in the appearance of two main new proteins with a Mr of 75,000 and 36,000. The N-terminal sequences of these proteins were determined and corresponded to those deduced from the *lacL* and *lacM* gene sequences. Mutation and deletion analysis showed that the *lacL* and *lacM* genes are translationally coupled and both required for the production of a functional ß-galactosidase in *E.coli*. The deduced amino acid sequences of the LacL and LacM proteins showed considerable identity with that of the N- and C-terminal parts, respectively, of ß-galactosidases from other lactic acid bacteria or *E.coli*. DNA and

protein sequence alignments suggest that the *L.lactis lacL* and *lacM* genes have been generated by an internal deletion in an ancestral ß-galactosidase gene.

INTRODUCTION

Two systems for lactose transport and hydrolysis are known among bacteria. The first system has only been found in Gram-positive bacteria and involves a phospho*enol*pyruvate-dependent phosphotransferase system (PTS), by which lactose is phosphorylated during transport and subsequently hydrolysed by a phospho-ß-galactosidase (for reviews see references 13, 22). In the second, more widespread system lactose is transported across the cellular membrane by a galactoside permease and the unmodified internalized sugar is hydrolysed by a ß-galactosidase. Most research has focused on the lactose permease (*lacY*) and ß-galactosidase (*lacZ*) genes from *Escherichia coli* (for reviews see references 3, 24, 25) and its *lacZ* gene has been developed into a useful tool in molecular genetics. Similar *lac* genes located on chromosomal or plasmid DNA have been found in other Gram-negative bacteria (20) and in one instance a *lac* transposon (Tn*951*) has been reported (10).

Recently, *lac* genes have been characterized in lactic acid bacteria that are used as starter cultures in dairy fermentations and, therefore, are highly specialized lactose utilizers. Genetic studies have shown that the lactose-specific PTS enzymes are homologous and plasmid encoded in *Lactococcus lactis* (30, 13-15) and *Lactobacillus casei* (1,2,38). In contrast, the homologous *lac* genes of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* are chromosomally located, and found to encode unique lactose permeases (28, 37), and ß-galactosidases that share high similarity with those of Gram-negative bacteria (42,43). A plasmid-encoded ß-galactosidase has been reported in *Lactobacillus casei* ATCC 393 (9). In addition, we showed recently that *Leuconostoc lactis* strain NZ6009 also contains a lactose plasmid that codes for a ß-galactosidase (11). Bacteria of the genus *Leuconostoc* are heterofermentative, Gram-positive cocci that are used for industrial milk and wine fermentations. We recently started the genetic characterization of *Leuconostoc* spp. (11) and focused on the plasmid-located *lac* genes in *L.lactis* NZ6009 (12). Here we describe the molecular characterization of a DNA fragment from the lactose plasmid pNZ63 that encodes a functional ß-galactosidase. We demonstrate that the *L.lactis* ß-galactosidase consists of two

subunits that are encoded by the translationally coupled *lacL* and *lacM* genes. These *L.lactis lac* genes are highly homologous to the corresponding genes isolated from *Lactobacillus casei* ATCC 393 (17).

MATERIALS AND METHODS

Bacterial strains, plasmids and media. The sources and relevant properties of the bacterial strains and plasmids used are listed in Table 1. *Leuconostoc lactis* strain NZ6009, previously designated *Leuconostoc paramesenteroides* (11), has been reclassified based on the hybridization of the V3-region of its 16S rRNA with probes specific for *Leuconostoc* spp. (26). Comparison of the plasmid complements of *L.lactis* strain NZ6009 and its lactose-deficient derivative NZ6091, revealed the absence of the 23-kb plasmid pNZ63 in strain NZ6091 that lacked ß-galactosidase activity (11). *Leuconostoc* strains were grown at 30°C in MRS broth (Difco Laboratories, Detroit, Mich.). *E.coli* strains were grown at 37°C in L-broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl), if appropriate supplemented with chloramphenicol, 10 μg/ml, or ampicillin, 50 μg/ml. For selection of ß-galactosidase-positive *E.coli* strains L-broth agar plates were supplemented with 40 μg/ml 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal, Sigma Chemical Co., St. Louis).

Cloning procedures and sequence analysis. Plasmid DNA was isolated from *L.lactis* by a modified alkaline lysis procedure (5) as described previously (11). Further purification of plasmid DNA by CsCl/ethidium bromide density-gradient centrifugation, its cloning in *E.coli*, and subsequent manipulations were performed according to established procedures (41). Restriction endonucleases and other enzymes used for DNA manipulations were purchased from Bethesda Research Laboratories (Gaitersburg, Md.) or New England Biolabs Inc. (Beverly, Mass.) and used as specified by the suppliers.

Sequence analysis by the dideoxy chain method (40) was performed on two strands by cascade sequencing on single-stranded DNA by cloning in bacteriophage M13mp18 or M13mp19 (33) using standard M13 or oligonucleotide primers and sequenase (U.S. Biochemical Corp., Cleveland, Ohio.). All primers were synthesized on a Cyclone DNA synthesizer (Biosearch, San Rafael, Calif.). DNA sequence data were assembled using the PC/GENE programs (version 5.01 Genofit, Geneva). Amino acid comparisons were

performed screening the protein data bases SWISS-PROT and NBRF/NEW, releases 12.0 and 23.0 respectively, using the facilities of the CAOS/CAMM Center (University of Nijmegen, The Netherlands).

TABLE 1. Bacterial strains and plasmids

Strains and plasmids	relevant properties	source or reference
Escherichia coli		
MC1061	hsdR *rpsL ara*D139 (ara-leu) 7697 *lac*74 *gal*K	(7)
ΔH1Δ*trp*		(39)
TG1		(18)
Plasmids		
pACYC184	Cm^R	(8)
pAT153	Ap^R, TcR	(45)
pNZ28	derivative of pPLc28 (39) containing the *Eco*RI-*Hind*III fragment of the M13mp18 multiple cloning site (33), inserted behind the lambda p_L-promoter	(46)

Cloning of the ß-galactosidase gene. Total plasmid DNA of *Leuconostoc lactis* was digested with *Bam*HI and the 16-kb *Bam*HI fragment derived from the plasmid pNZ63 was isolated and ligated to *Bam*HI-linearized pACYC184. The ligation mixtures were used to transform *E.coli* MC1061. Subclones were made using the *E.coli* vectors pAT153 and pNZ28.

Overproduction and characterization of *L.lactis* ß-galactosidase. Restriction fragments of pNZ63 were cloned in *E.coli* ΔH1 Δ*trp* under control of the λP_L promoter using the expression vector pNZ28. Cells of ΔH1 Δ*trp* harboring the pNZ28- derivatives were grown at 28°C to an OD_{600} of 0.5, at which time the culture was divided into two equal parts. One

part was further incubated at 28°C and the other part at 42°C to inactivate the host-encoded thermosensitive *cI857* repressor. Analysis of the expression products was performed on sodium dodecyl sulphate-polyacrylamide gels (27). The N-terminal amino acid sequence of induced protein bands was determined with the aid of a gas-phase sequenator (Applied Biosystems, Foster City, Calif.) after transfer of the protein to Problott paper (Applied Biosystems) as described (47).

Assay of ß-gal activity. Cell-free extracts of exponentially growing cultures were prepared by ultrasonication and ß-galactosidase activities were determined at 37°C using the chromogenic substrate o-nitrophenyl ß-D-galactopyranoside (ONPG, Sigma) (34). One unit of ß-galactosidase activity produced 1 nmol of *o*-nitrophenol per min. Protein concentrations were determined as described (6).

RESULTS

Location and cloning of a *L.lactis* DNA fragment encoding ß-galactosidase. A partial restriction map of the 23-kb lactose plasmid pNZ63 of *L.lactis* strain NZ6009 was constructed and appeared to contain a single 16-kb *Bam*HI fragment (Fig. 1). Insertion of this 16-kb *Bam*HI fragment into the *E.coli* vector pAT153 resulted in two plasmids, pNZ620 and pNZ621, representing both orientations of the fragment in the vector. After introduction into the lac⁻ *E.coli* strain MC1061 both plasmids give rise to the formation of blue colonies on X-Gal plates. However, after prolonged propagation, white colonies appeared. Analysis of the plasmid complement of these white colonies revealed that the large insert of pNZ620 or pNZ621 had been deleted. Since this instability might be due to the high copy number of pAT153, we repeated the cloning of the 16-kb *Bam*HI fragment of pNZ63 using the low copy number vector pACYC184. Only one orientation of the cloned fragment could be stably maintained in pACYC184 and the resulting plasmid was designated pNZ601 (Fig.1). *E.coli* MC1061 transformed with pNZ601 showed production of active ß-galactosidase as could be demonstrated on plates containing X-Gal and by measuring enzyme activities (Fig. 1). Several subfragments of pNZ601 were cloned in *E.coli* and analyzed for ß-galactosidase production. The results (Fig. 1) showed that the coding region for the *L.lactis* ß-galactosidase was located on a 5.8-kb *Sal*I-*Bam*HI fragment, that is contained in pNZ623. A frame-shift

mutation was introduced in pNZ623 by filling in the unique *Nco*I site by Klenow polymerase. *E.coli* harboring the resulting plasmid, pNZ623ΔN, produced white colonies on X-Gal plates, indicating that the *Nco*I site was located within the region encoding ß-galactosidase production.

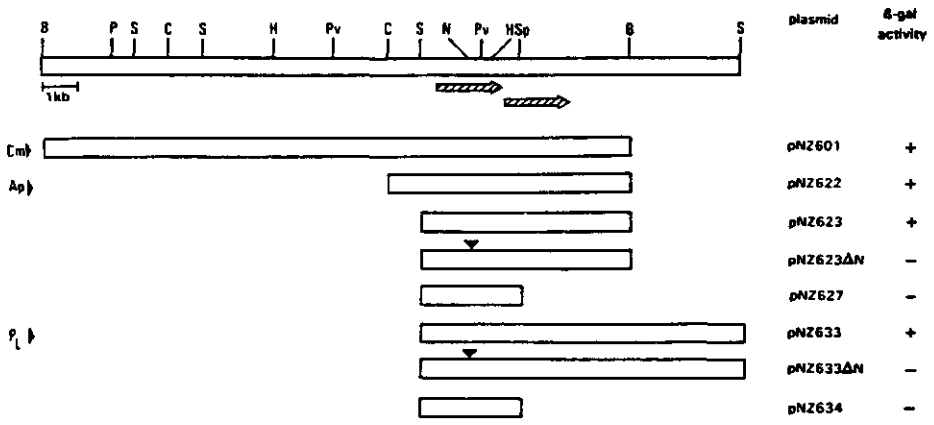


FIG.1. Restriction map of pNZ63 and its derivatives. pNZ63 fragments were cloned in pACYC184 (pNZ601), pAT153 (pNZ622, pNZ623, pNZ623ΔN, pNZ627) or pNZ28 (pNZ633, pNZ633ΔN, pNZ634). The orientation of the inserts with respect to vector-located promoters is illustrated by arrowheads. Phenotypes of *E.coli* MC1061 transformed with these plasmids are indicated on right part of the figure; +: blue colonies on X-gal plates and ß-galactosidase activity (more than 0.5 units/mg protein); -: white colonies on X-gal plates and no ß-galactosidase activity (less than 0.01 units/mg protein). Open boxes indicate insert DNA and the black triangles indicate a frame-shift mutation created by filling in the *Nco*I-site by Klenow polymerase. The location and orientation of the *lacL* and *lacM* genes is indicated.

Nucleotide sequence of the *L.lactis* ß-galactosidase coding region. The nucleotide sequence of a 3.2-kb region of pNZ623 comprising the *Nco*I site (Fig. 2) showed the presence of two large open reading frames. Two ATG initiation codons (positions 196 and 277) were found at the start of the first open reading frame that stops at position 2155. The N-terminal sequence of its expression product (described below) indicated that the second ATG (underlined in Fig. 2) is the start of a gene, designated *lacL*, encoding a protein of 626 amino acids with a calculated molecular weight of 72,113 Da.

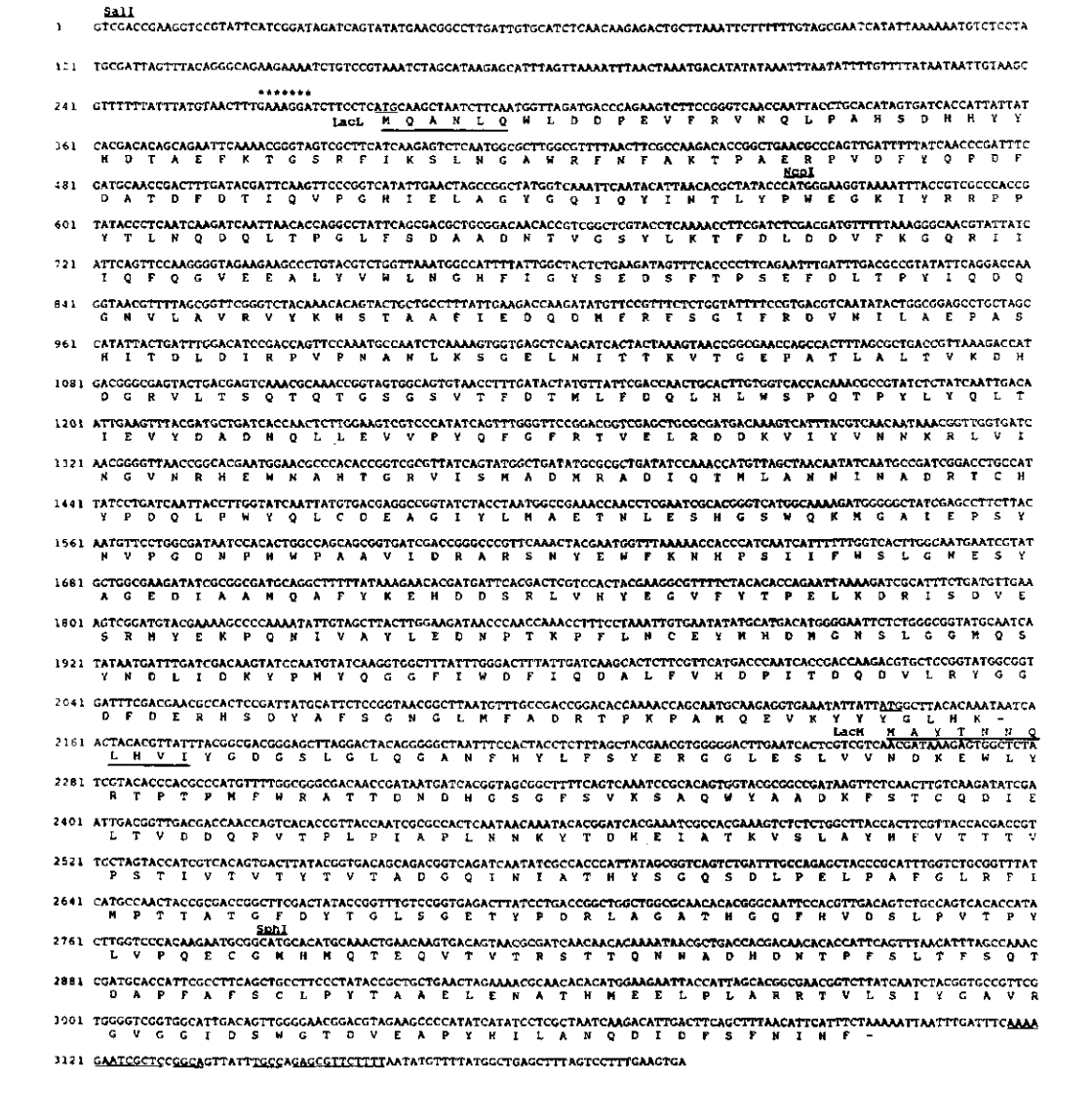


FIG. 2. Nucleotide sequence of the *L. lactis* NZ6009 ß-galactosidase coding region and flanking sequences. Numbering starts at the *SalI* site (see Fig. 1); relevant restriction sites are indicated. The start and stop codons of *lacL* and *lacM* are underlined. The putative ribosome binding site in front of *lacL* gene is marked by stars. A region of dyad symmetry, representing a putative terminator of transcription is indicated by arrows. The N-terminal amino acid residues that have been determined by amino acid sequencing are underlined.

The second open reading frame, that starts with an ATG at position 2141 and contains a termination codon at position 4005, partially overlaps with the 3' end of the *lacL* gene. The N-terminal sequence of its expression product (described below), indicated that the ATG at position 2141 (underlined in Fig. 2), is the start of a gene, designated *lacM*, encoding a protein of 321 amino acids with a calculated molecular weight of 35,389 Da.

Downstream from the termination codon of *lacM* a region of dyad symmetry followed by a T-rich region could be identified (Fig. 2) that shows the features of a rho-independent terminator of transcription (36). No other open reading frames were detected immediately upstream or downstream of the *lacLM* genes.

Overproduction of the *L.lactis* ß-galactosidase in *E.coli* and identification of the LacL and LacM proteins. In order to analyze the expression products of the *L.lactis lacL* and *lacM* genes, we cloned a 9.0-kb *Sal*I fragment comprising the ß-galactosidase coding region into the *Sal*I-linearized expression vector pNZ28. This resulted in two plasmids, pNZ632 and pNZ633, representing the two orientations of the fragment within the vector, which both gave rise to blue colonies on X-Gal plates after transformation to *E.coli* MC1061 (Fig. 1). Subsequently, both plasmids were introduced into *E.coli* ΔH1 Δ*trp* and the thermoinduction of ß-galactosidase activity was determined. Only the ß-galactosidase activity of *E.coli* ΔH1 Δ*trp* carrying pNZ633 was found to be inducible and increased from 1.8 U/mg protein (after 45 min at 28°C) to 8.9 U/mg protein (after 45 min at 42°C). The activity decreased several-fold after incubation at 42°C for 3 h and was absent after overnight incubations at 42°C. In contrast, no induction of ß-galactosidase activity was observed in ΔH1 Δ*trp* harboring pNZ632.

Analysis of the total cellular proteins of *E.coli* ΔH1 Δ*trp* cells harboring pNZ633, showed that after induction at 42°C, two major proteins were overproduced with sizes of 75 kDa and 36 kDa, that are not found in ΔH1 Δ*trp* harboring the vector pNZ28 alone (Fig. 3). In addition, a protein with a size of 58 kDa appeared to be induced to a lower extent. Since this 58-kDa protein was not found in induced lysates of strain ΔH1 Δ*trp* harboring pNZ633ΔN, containing a frameshift in the unique *Nco*I site within the *lacL* gene (Fig. 3), this protein most likely represents a degradation product of the 75 kDa-protein.

The N-terminal sequences of the major inducible proteins encoded by pNZ633 were determined using a gas-phase sequencer. The sequence of the first six residues obtained with the 75 kDa-protein was Met-Gln-Ala-Asn-Leu-Gln. This sequence is identical to the N-

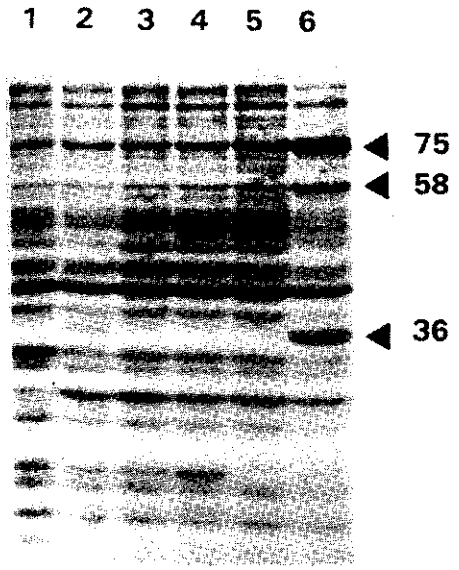


FIG. 3. Expression products of the *lacL* and *lacM* genes. SDS-polyacrylamide gelelectrophoresis of whole cellysates of *E.coli* ΔH1 Δtrp containing pNZ28 (lanes 1 and 4), pNZ633ΔN (lanes 2 and 5) or pNZ633 (lanes 3 and 6). Lanes 1-3 show the lysates of cells grown at 28°C; lanes 4-6 show lysates of cells after induction at 42°C during 45 min. Molecular weights (in kDa) of induced proteins are indicated at the right.

terminal sequence predicted for the *lacL* gene product (underlined in Fig. 2). With the smaller band of 36 kDa two sequences were obtained and ten residues were determined: the most prominent sequence was Ala-Tyr-Thr-Asn-Asn-Gln-Leu-His-Val-Ile and the less obvious Met-Gln-Ile-Tyr-Ser-Lys-Asp-Gly-Asn-Lys. With the exception of the N-terminal Met residue, the removal of which is not unusual in *E.coli* (4), the most prominent sequence was identical to the N-terminal sequence deduced from the *lacM* gene. These results demonstrate that the products of the *lacL* and *lacM* genes are the 75- and 36-kDa proteins, respectively.

The *lacL* and the translationally coupled *lacM* gene are both required for a functional ß-galactosidase. In order to determine whether the *lacL* gene alone or in combination with the *lacM* gene was encoding an active ß-galactosidase, derivatives of pNZ623 or pNZ633 (Fig. 1) were introduced in *E.coli* strains that either contained a frameshift mutation in the *lacL* gene (pNZ623ΔN or pNZ633ΔN) or in which the major part of the *lacM* gene had been deleted (pNZ627 or pNZ634). *E.coli* MC1016 harboring either of the mutated plasmids showed white colonies on X-gal plates and no detectable ß-galactosidase activity (Fig. 1),

indicating that both *lacL* and *lacM* genes are required for the formation of a functional ß-galactosidase.

To further analyze the expression of the *lacL* and *lacM* genes, proteins present in lysates from induced and non-induced cells of strain ΔH1 Δtrp harboring either pNZ635ΔN or pNZ634 were compared with that from cells harboring pNZ633. The results (Fig. 4) showed that deletion of the last 0.3 kb from the *lacM* coding region, as in pNZ634, results in the formation of a truncated LacM product of approximately 20 kDa, while the production of the LacL product remains unaffected. In contrast, introduction of the frameshift mutation into the *lacL* gene affects the production of both LacL and LacM indicating that the *lacM* gene is translationally coupled to the *lacL* gene.

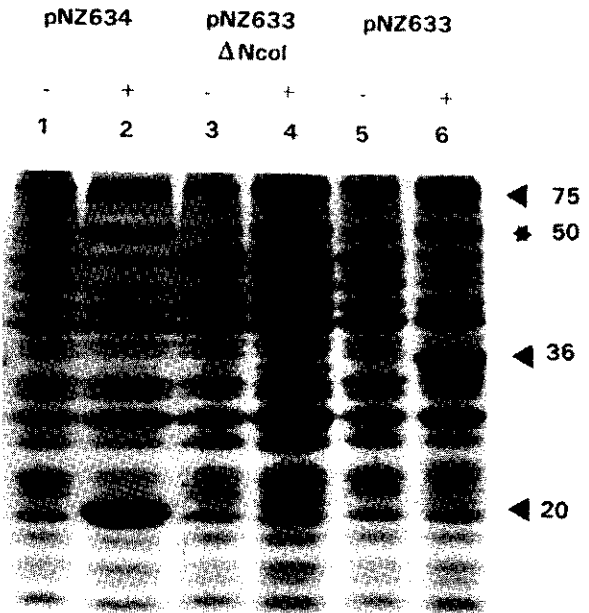


FIG. 4. Expression products of mutated *lacLM* genes. SDS-polyacrylamide gelelectrophoresis of whole cell lysates of *E.coli* ΔH1 Δtrp containing pNZ634 (lanes 1 and 2), pNZ633ΔN (lanes 3 and 4), or pNZ633 (lanes 5 and 6). Lanes 1, 3, and 5 show the lysates of cells grown at 28°C; lanes 2, 4, and 6 show lysates of cells after induction at 42°C during 45 min. Molecular weights (in kDa) of induced proteins are indicated at the right. An induced protein also found in the control of ΔH1Δ*trp* harboring pNZ28 (not shown) is indicated by a star.

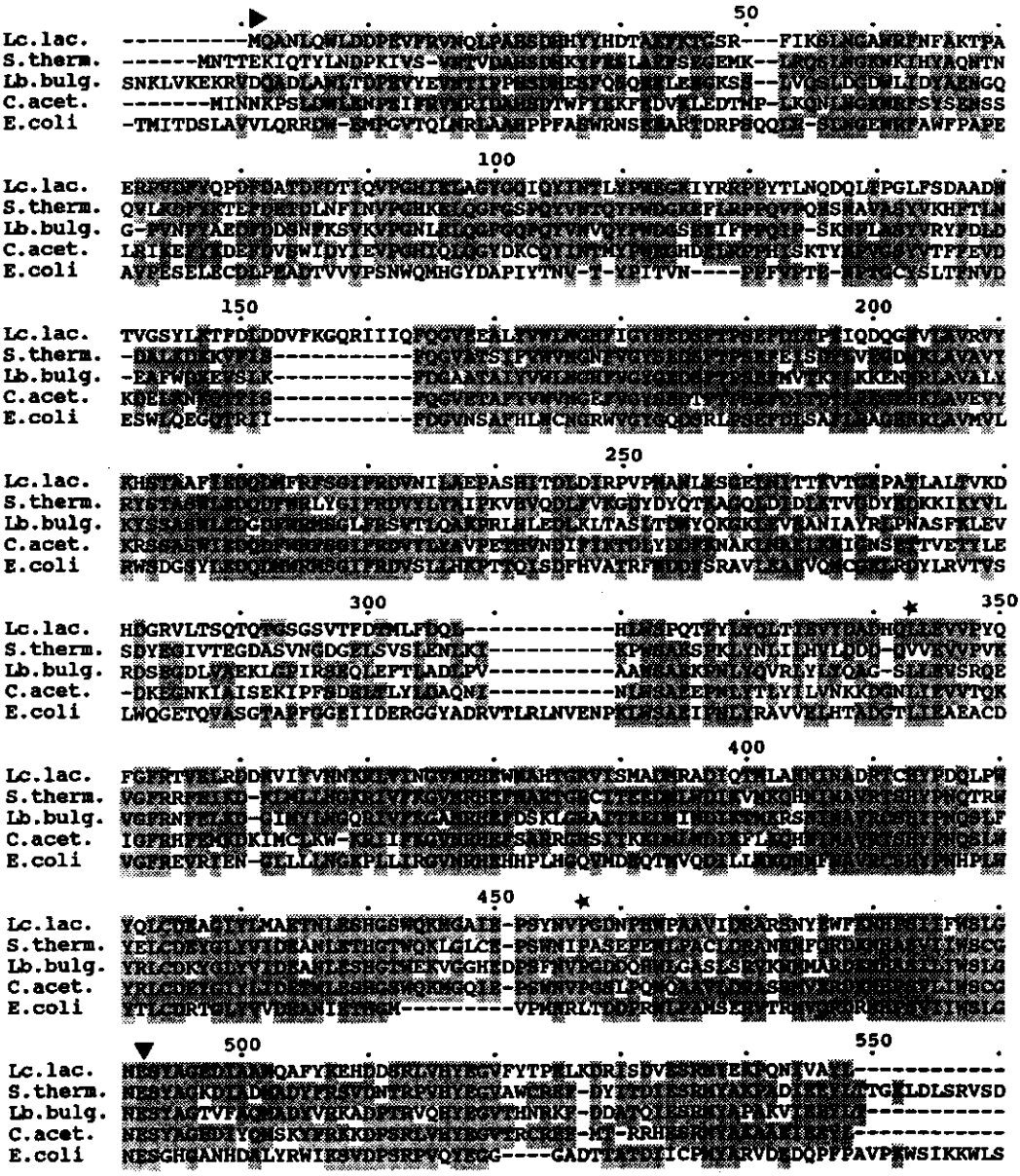
DISCUSSION

From expression, mutation and sequencing studies described here, we conclude that the plasmid-encoded ß-galactosidase of *L.lactis* NZ6009 is encoded by two overlapping genes *lacL* and *lacM*, that are both required for the production of a functional ß-galactosidase in *E.coli*. The N-terminal sequences of the 75-kDa LacL and 36-kDa LacM proteins produced at high levels in *E.coli* were identical with those predicted from the DNA sequences. Although transcomplementation of the *E.coli* M15 ß-galactosidase is well-known and that by the α-fragment exploited widely (3, 41, 48), this is to our knowledge the first example of a naturally occurring ß-galactosidase consisting of two non-identical subunits.

The introduction of a frameshift mutation in the *lacL* gene results in the loss of production both the LacL and LacM proteins (Fig. 3 and 4), indicating that the *lacM* gene is translationally coupled to the *lacL* gene (44). This, and the fact that the *lacM* gene partially overlaps the *lacL* gene, suggests that both genes are organized into an operon, the transcription of which could be initiated at the AT-rich region preceding the *lacL* gene. Further transcriptionstudies will be required to verify this possibility.

In spite of the phylogenetic analysis based on *Leuconostoc* 16S rRNA sequences (32), to date the sequence of the 3' end is not known. However, 8 bp preceding the start codon of the *lacL* gene (Fig. 2) a potential ribosome binding site was found with the sequence 5'-GAAAGGA-3' (position 263-269) that shows high complementarity to the 3' end of the 16S RNA from the phylogenetically related *Lactococcus lactis* (29) or *Bacillus subtilis* (19). The *lacM* gene is preceded by a stretch of ten pyrimidines, that are unlikely to act as a ribosome binding site as is not uncommon amongst translationally coupled genes (44). Translational coupling is an efficient and simple way to regulate gene expression. In this case, translational ccoupling could ensure a constant ratio between the amount of LacL and LacM protein (44). Preliminary estimations of the *Leuconstoc lactis* ß-galactosidase apparent molecular weight indicated that it is a heterodimer. This would suggest that the translational coupling should be highly efficient, resulting in the synthesis of equal amounts of LacL and LacM proteins.

The instability of the *L.lactis* ß-galactosidase that is apparent after prolonged incubations at 42°C, might be due to dissociation of LacL and LacM at elevated temperatures. Alternatively, it may be attributed to proteolysis. The generation of a 58-kDa protein that is encoded by the *lacL*gene (Fig. 3) is in favor of the latter possibility.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Lc.lac.
S.therm.
Lb.bulg.
C.acet.
E.coli

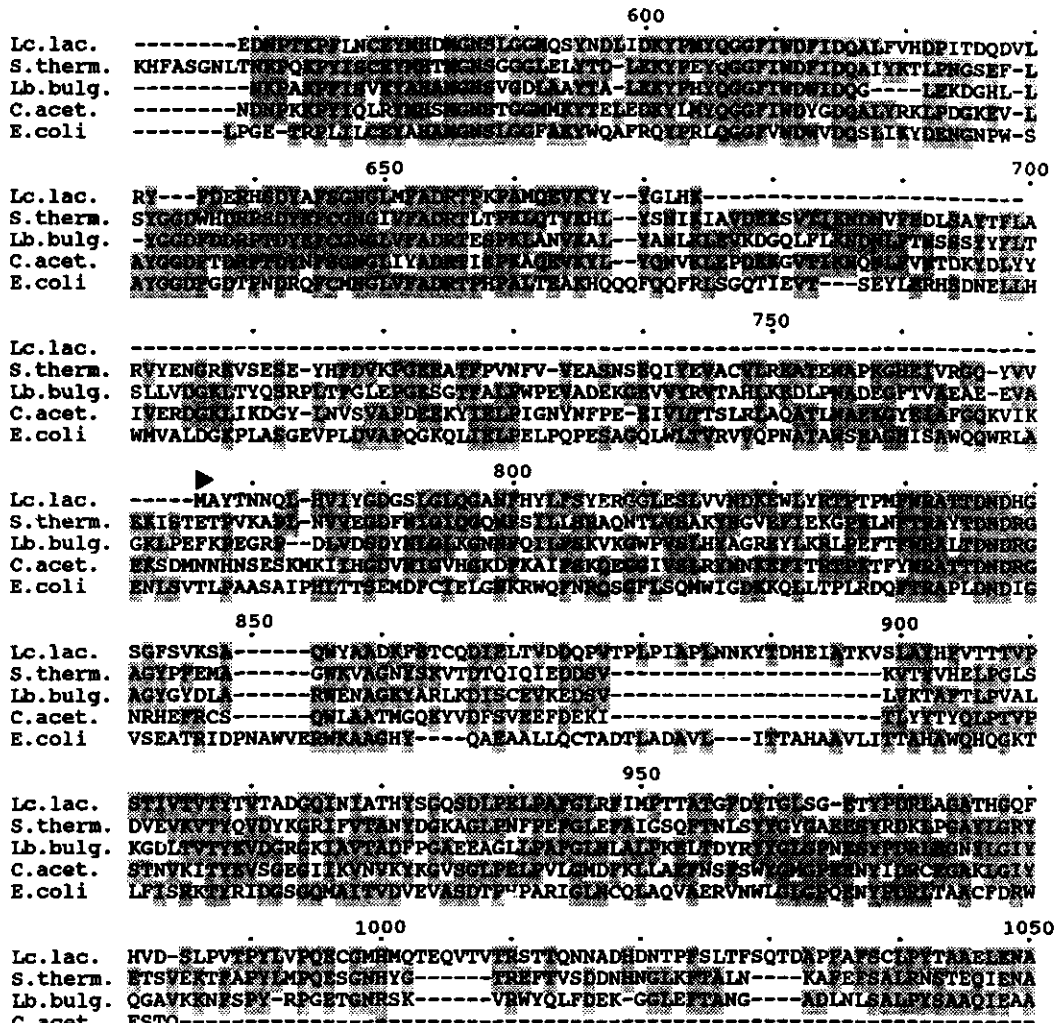

FIG. 5. Amino acid sequence alignment (from top to bottom) of the *Leuconostoc lactis* (Lc.lac.), *Lactobacillus bulgaricus* (Lb.bulg.), *Streptococcus thermophilus* (S.therm.), *Clostridium acetobutylicum* (C.acet.) and *Escherichia coli* (E.coli) ß-galactosidases. Identical amino acids in the sequences are indicated by shaded boxes. Residues possibly involved in the active site or affecting the thermostability are indicated by an arrowhead or stars, respectively.

The amino acid sequence deduced from the *L.lactis lacL* gene was compared with that of the ß-galactosidases from *E.coli* (25), *Streptococcus thermophilus* (43), *Lactobacillus bulgaricus* (42) and *Clostridium acetobutylicum* (21). A computer alignment of these sequences is given in Fig. 5 and shows that LacL has a large degree of sequence similarity with the N-terminal region of approximately 700 residues of the other ß-galactosidases. In addition, the deduced LacM amino acid sequence showed homology to the C-terminal part of approximately 300 residues of the ß-galactosidases of the other bacteria. Several regions of high similarity were detected and the average percentage identity between the ß-galactosidase sequences varied between 22 and 45 % (Table 2). The highest identity was obtained between the gram-positive bacteria and, unexpectedly, the *L.lactis* ß-galactosidase showed higher identity with the *C.acetobutylicum* sequence than with the ß-galactosidases from the other lactic acid bacteria. These results strongly suggest that these enzymes have evolved from a common ancestral ß-galactosidase gene. The putative active site residue (Glu-461) of the *E.coli* ß-galactosidase (23) and the two residues (Leu-316 and Pro-429), substitution of which increased the cold sensitivity of the *Lactobacillus bulgaricus* ß-galactosidase (31), were all conserved in the *L.lactis* ß-galactosidase (Fig. 5). The alignment shows that, in spite of the small insertions at the N-terminus of LacL and the C-terminus of LacM, the combined size of the *L.lactis* LacL and LacM proteins is smaller than the size of the other ß-galactosidases. A specific domain, located in a region of low homology (amino acid position 675-775; Fig. 5), is not found in either LacL or LacM, and hence does not seem to be required for activity of the *L.lactis* ß-galactosidase. In the *E.coli* ß-galactosidase there is a elastase cleavage site just a few residues C-terminal from this region (position 779-780; Fig. 5). It has been shown that cleavage of this site, generating a N- and C-terminal fragment that are not any longer covalently linked, does not affect the activity of the *E.coli* enzyme (23). The present results with the *L.lactis* ß-galactosidase support this finding and further indicate that the C-terminal fragment (LacM) is required for a functional enzyme. It is very likely that the *L.lactis lacL* and *lacM* genes have been generated by one or more deletion events within an ancestral ß-galactosidase gene that included the fragment coding for the dispensable domain. Spontaneous deletions within the ß-galactosidase gene of *Lactobacillus bulgaricus* strain NCDO1489 have been found to occur frequently and in one case (lac150) included the fragment lacking in the *L.lactis* ß-galactosidase coding region (35).

TABLE 2. Amino acid identity (in percentage) between pairs of ß-galactosidases from *Lactococcus lactis* (Lc.lac), *Streptococcus thermophilus* (S.therm), *Lactobacillus bulgaricus* (Lb.bulg.), *Clostridium acetobutylicum* (C.acet.), and *Escherichia coli* (E.coli).

	Amino acid Identity				
	Lc.lac	S.therm	Lb.bulg	C.acet	E.coli
Lc.lac	-				
S.therm	33.3				
Lb.bulg	28.2	45.5			
C.acet	36.2	42.0	40.1		
E.coli	22.7	27.4	27.2	28.3	-

In *Streptococcus thermophilus* and *Lactobacillus bulgaricus* the genes for the ß-galactosidase gene and the lactose permease are organized in an operon structure (28,37), in which the lactose permease gene is located immediately upstream of the ß-galactosidase, separated by only three base pairs. This organization is different from that of the *lac* genes in *E.coli*, that have the gene order *lacZY*. We could detect no open reading frames in the sequences immediately upstream or downstream of the *L.lactis lacLM* genes. Recent results have shown (12), that the lactose permease is also encoded by the lactose plasmid pNZ61 in *L.lactis* NZ6009 and its gene, *lacP*, has been located approximately 4 kb upstream of the *lacL* gene. The organization of the plasmid encoded genes involved in the lactose metabolism in *L.lactis* is therefore considerably different from that described for the *lac* genes of other bacteria.

The nucleotide and deduced amino acid sequence of the plasmid-encoded ß-galactosidase genes from *Lactobacillus casei* ATCC 393 have recently been reported (17). The *lactobacillus casei* ß-galactosidase has a heterodimeric subunit structure similar to that of *L.lactis*.

Comparison of these sequences reveals almost complete identity on the gene and protein level. Fifteen base substitutions were found in the *lacL* gene, resulting in 11 amino acid substitutions. Seven base substitutionswere observed in the *lacM* gene, which lead to only 2 amino acid substitutions. The near-perfect homology extends from 103 bp upstream of the *lacL* gene and 95 bp downstream of the *lacM* gene, and includes the ß-galactosidase coding region, the putative ribosome binding site and the transcription terminator. Outside this region of 3916 bp no significant sequence homology was observed, indicating that the lactose

plasmids of the two hosts are quite different. The nearly complete identity of the *L. lactis* and *Lactobacillus casei* ß-galactosidase genes indicate that they not only share a common ancestor but very recently have been acquired. Plausible routes for the dissemination of the *lacL* and *lacM* genes include horizontal plasmid transfer followed by transposition or recombination into endogenous plasmids.

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CHAPTER V

Characterization of the *lacP* Gene Encoding the Novel Lactose Carrier of *Leuconostoc lactis* NZ6009

S.David and W.M. de Vos

SUMMARY

The plasmid-encoded *lac*P gene for the *Leuconostoc lactis* NZ6009 lactose transport protein was cloned in *Escherichia coli* and found to complement the *lacY* mutation of different strains. Subcloning and mutation analysis indicated that the *lacP* gene was located on a 2.9-kb *SalI-EagI* fragment. Functional expression of the lactose transport activity was shown by determining the uptake of ^{14}C-labeled lactose or methyl-ß-D-thiogalactopyranoside in whole cells. Expression of the *lacP* gene under control of the T7 promoter in *E.coli* BL21 indicated that it coded for a protein with an apparent M_r of 26,000.

The nucleotide sequence of the *lacP* gene and its surrounding regions was determined and showed that it was not flanked by a ß-galactosidase gene. The *lacP* gene comprised 654 base pairs, coding for a hydrophobic protein of 218 amino acids, with a calculated molecular weight of 23,957. A second open reading frame of 256 amino acids was found downstream of the *lacP* gene, showing no homology to any known amino acid sequences. The deduced amino acid sequence of the *Leuconostoc lactis* lactose permease revealed no sequence homology to other known lactose transport proteins, but showed significant similarity to the *E.coli* glutamine transport protein GlnP (72%) and the HisQ (66%) and HisM (65%) proteins involved in histidine transport in *Salmonella typhimurium*, that are all components of binding-

protein dependent amino acid transport systems.

INTRODUCTION

Lactic acid bacteria used for the manufacture of fermented dairy products, depend on lactose as their primary source of energy and therefore contain efficient transport systems for the transport of this disaccharide across the membrane. These bacteria are known to transport lactose either via the phosphoenolpyruvate-dependent phosphotransferase system (PTS) (17, 38), like in lactococci (13, 27) and certain lactobacilli (1, 8), or by means of a permease system, like in *Streptococcus thermophilus* (29), *Lactobacillus bulgaricus* (24) and *Leuconostoc* spp. (9). In the first system lactose is phosphorylated during transport into the cell and the resulting lactose-6-phosphate is subsequently hydrolyzed by a phospho-ß-galactosidase. The lactose PTS enzymes are only found in gram-positive bacteria and the organization and expression of their genes have been studied in *Lactococcus lactis* (13,14) and *Lactobacillus casei* (1, 2, 31). In the permease system, lactose is not modified during transport and the internalized lactose is hydrolyzed to glucose and galactose by a ß-galactosidase. The *lac* genes of *Streptococcus thermophilus* (29), and *Lactobacillus bulgaricus* (24, 34) have been cloned and sequenced and were found to encode highly homologous ß-galactosidases and lactose permeases. In both lactic acid bacteria these *lac* genes are separated by 3 base pairs and their order is opposite to that of the *lac* operon in *E.coli*. The deduced sequence of their ß-galactosidases showed considerable similarity to that of *E.coli*. However, the lactose transport proteins of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* differed considerably from the *E.coli* lactose permease and appeared to be hybrid proteins with homology to the melibiose carrier of *E.coli* at the N-terminal end and similarity to PTS Enzyme III sequences at the C-terminal end (29). It has been proposed that *in vivo* this unusual lactose permease functions as a lactose-galactose antiporter although it also catalyzes galactose/H^+ symport (29, 30). This is in agreement with the observation that *Streptococcus thermophilus* excretes the galactose moiety of lactose stochiometrically into the medium in the presence of excess lactose (39). In contrast, *Leuconostoc* spp. are able to ferment lactose completely. Recently, we identified a lactose plasmid, pNZ63, in *Leuconostoc lactis* strain NZ6009 (10), and started the analysis of its *lac*-genes. Sequence

analysis of the pNZ63-encoded ß-galactosidase coding region did not reveal any significant flanking open reading frames, suggesting a different genetic organization of the *lac* genes in this lactic acid bacterium (12). In this study we describe the characterization and functional expression in *E.coli* of the lactose permease gene from *L.lactis* and show that it codes for a hydrophobic protein with homology to amino acid carriers of Gram-negative bacteria.

MATERIALS AND METHODS

Bacterial strains and plasmids. The following *E.coli* strains were used for selection of lactose permease positive transformants: strain HB101 [*hsd20* ($r^- m^-$) *recA13 ara-14 proA2 lacY1 galK2 rps* (Sm^r) *xyl-5 mtl-1 supE44* lambda$^-$ F^-] and strain BY1, a *lacY* derivative strain of JC5412 (40) obtained by N-ethyl-N'-nitro-N-nitrosoguanine mutagenesis (kindly provided by B. Poolman). *E.coli* strains MC1061 (6) and BL21 (DE3) (35) (obtained from F.Studier) were used as hosts for routine cloning or expression experiments, respectively. *E.coli* TG1 (15) was used for the isolation of M13 single-stranded DNA. *L.lactis* strain NZ6009 and its Lac$^-$ derivative NZ6091 that lacks the lactose plasmid pNZ63, have been described previously (10) as *Leuconostoc paramesenteroides* strains but have been reclassified based on rRNA typing (11). pNZ601 (12) contains a 16-kb *BamH*I fragment of pNZ63 cloned in the unique *Bam*HI site of the *E.coli* cloning vector pACYC184 (7) and was used as the source of the lactose transport gene of *L.lactis* NZ6009. Other *E.coli* vectors used in this work included pHSG576 (37), pT7-5 and pT7-6 (obtained from S.Tabor and C.C. Richardson)(36).

Media and growth conditions. *E.coli* strains were routinely propagated at 37°C in L-broth (33), supplemented with 10 µg/ml chloramphenicol or 50 µg/ml ampicillin, if appropriate.

For selection of transformants able to transport lactose, cells were spread on MacConkey agar (Difco Laboratories, Detroit, Mich.) plates containing 1% lactose (MacConkey-lac plates) and the appropriate antibiotic. *L.lactis* strains were grown on MRS based media (Difco Laboratories) as described (10).

Molecular cloning and nucleotide sequence analysis. Isolation and purification of plasmid DNA by CsCl/ethidium bromide density-gradient centrifugation, and subsequent routine DNA manipulations were performed in *E.coli* essentially as described (33). Restriction enzymes and other enzymes used for DNA manipulations were purchased from Bethesda Research

Laboratories (Gaitersburg, Md.) and New England Biolabs Inc. (Beverly, Mass.). Restriction fragments were separated on 0.8% agarose gels and recovered using Gene Clean (BIO 101, La Jolla, Calif.). Frame shift mutations were introduced by cutting with either *Cla*I or *Bgl*II, filling up the sites using Klenow polymerase and dNTP's, and religating the fragments.

Sequence analysis by the dideoxy chain method (32) was performed on single-stranded DNA obtained by cloning in M13mp18 and M13mp19 vectors (28) or double-stranded DNA, using primers synthesized on a Cyclone DNA synthesizer (Biosearch, San Rafael, Calif.) and sequenase (U.S. Biochemical Corp., Cleveland, Ohio). Sequence data were analyzed using the programs of PC/GENE (version 5.01; Genofit, Geneva, Switzerland), and protein comparisons were performed using the programs and computer facilities of the CAOS/CAMM Center, Nijmegen University, with the National Biomedical Research Foundation (NBRF/PIR) (release 23.0) and SWISS-PROT (release 13.0) data bases.

Transport assays. Transport of [D-glucose-1-^{14}C]lactose (57 mCi/mmol Radiochemical Centre, Amersham, England) and [^{14}C]methyl-ß-D-thiogalactopyranoside (TMG) (50 mCi/mmol, New England Nuclear Research Products, Boston, Mass.) was assayed in exponentially growing *E.coli* BY1 cells, carrying the appropriate plasmids, or *L.lactis* NZ6009 and NZ6091, essentially as described by Poolman et al. (29). Protein concentrations were determined by the method of Bradford (4) by using the Bio-Rad protein assay with bovine serum albumin as a standard.

Analysis of plasmid-encoded polypeptides. For radioisotope labeling of proteins encoded by derivatives of pNZ601, DNA fragments were cloned in *E.coli* BL21 using pT7-5 and pT7-6. The expression products were labeled with [^{35}S]methionine (Amersham) by the procedure of Tabor and Richardson (36) and analyzed on sodium dodecyl sulfate-12.5% polyacrylamide gels (23) that were subjected to autoradiography using Kodak X-AR-5 films.

RESULTS

Cloning and functional expression of the *L.lactis* lactose transport gene (*lacP*). The Lac$^-$ derivative NZ6091 of *L.lactis* strain NZ6009 has lost the lactose plasmid pNZ63, previously

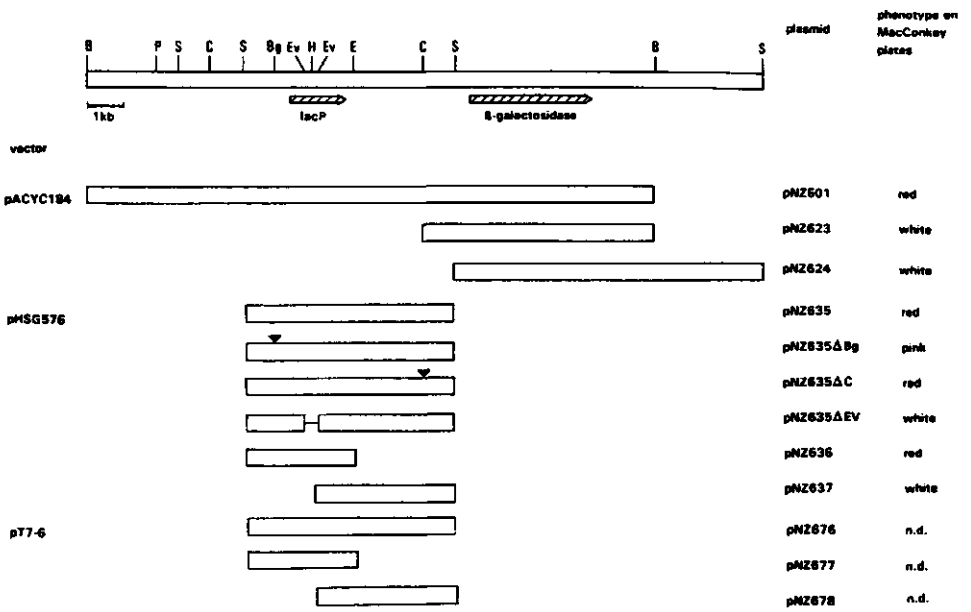

FIG. 1. Restriction map of a 20-kb region of the *Leuconostoc lactis* NZ6009 lactose plasmid pNZ63 and characterization of the subclones used in this study. The plasmids pNZ601 and pNZ635 were constructed using pACYC184 (7) and pHSG576 (37), respectively. Plasmids pNZ623 and pNZ624 have been constructed using pAT153 as described elsewhere (12). The 0.45-kb *Eco*RV fragment was deleted in pNZ635ΔEV. pNZ636 and pNZ637 and pNZ677 and pNZ678 were constructed by deleting the 5' and 3' part of the insert, using restriction sites *Bam*HI and *Hin*dIII, located within the multiple cloning site of the vector pHSG576 and the unique *Eag*I and *Hpa*I sites on the fragment, respectively. The vectors that were used for the constructions are indicated at the left. Designations of the recombinant plasmids are indicated at the right together with the ability of these plasmids to complement the *lacY* mutation of *E.coli* BY1. This was determined on MacConkey-lac agar plates, and in the case of complementation confirmed using transport studies with ^{14}C-labeled lactose or TMG. The arrows indicate the direction and localization of the ß-galactosidase (12) and the lactose permease (*lacP*) genes, respectively. The sites of the following restriction enzyme sites are indicated: B: *Bam*HI; Bg: *Bgl*II; C: *Cla*I; E: *Eag*I; EV: *Eco*RV; H: *Hpa*I; P: *Pvu*I; S: *Sal*I.

shown to encode a functional ß-galactosidase (10, 12). Transport assays showed that washed cells of strain NZ6091 in contrast to those of strain NZ6009, were unable to take up ^{14}C-labeled lactose (results not shown), suggesting that the ability to transport lactose was also encoded by pNZ63. A 16-kb *Bam*HI fragment of pNZ63 was cloned in *E.coli* MC1061 using pACYC184 and the resulting plasmid pNZ601, appeared to express a functional ß-galactosidase in *E.coli* (12). Upon transformation of pNZ601 into the lactose-permease

deficient *E.coli* strains HB101 and BY1, chloramphenicol-resistant red colonies could be detected on MacConkey-lac agar plates, indicating the complementation of the *lacY* mutation of these strains. However, as reported previously (29), a relatively high frequency of false positives (pink-red colonies) were observed using *E.coli* HB101 as a host, probably due to restoration of the point mutation within the *lacY* gene. The *lacY* mutation of *E.coli* strain BY1 appeared to be more stable, and therefore this strain was used for further studies

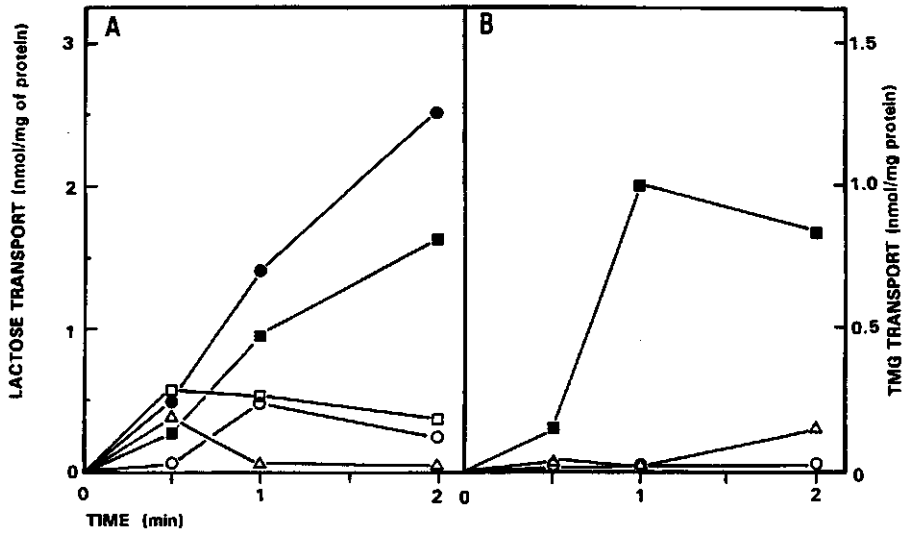

FIG. 2. Transport of [^{14}C]lactose and [^{14}C]TMG by washed cells of *E.coli* BY1 without plasmid (Δ) or cells harboring plasmids pNZ635 (▬; Lac$^+$), pNZ636 (●; Lac$^+$),pNZ635ΔEV (□;lac$^-$) or pNZ637 (○ ;lac$^-$). [D-glucose-1-^{14}C] lactose was added to a final concentration of 50 μM to cell suspensions containing about 0.5 mg/ml protein. Uptake was stopped at different time intervals by adding 2ml 0.5M $LiCl_2$.

In order to locate the lactose permease determinant on pNZ601, subclones were made in pHSG576 and analyzed in *E.coli* BY1 for the complementation of the *lacY* mutation (Fig.1). These experiments showed that only BY1 harboring pNZ635, consisting of a 6.0-kb *Sal*I fragment of pNZ601 cloned into the unique *Sal*I site of pHSG576, showed red colonies on MacConkey-lac plates. Further delineation of the lactose transport determinant was achieved by introducing frameshift mutations or deletions in the insert of *L.lactis* DNA contained in pNZ635 (Fig.1). Introduction of the deletion derivatives pNZ635ΔEV or pNZ637 in *E.coli* BY1 resulted in white colonies on MacConkey-lac agar plates, indicating that the 0.45-kb *Eco*RV fragment is essential for complementation of the *lacY* mutation of this host. *E.coli* BY1 harboring pNZ635ΔBg, containing a mutation at the *Bgl*II site, gave rise to pink

colonies on MacConkey-lac plates. In contrast, introduction of a frameshift mutation in the *Cla*I site or deletion of the 3.1-kb *Eag*I-*Bam*HI fragment did not affect the capacity of the resulting plasmids (pNZ625ΔC or pNZ636, respectively) to complement the *lacY* mutation of *E.coli* BY1. These results show that the coding region for the *L.lactis* lactose carrier is located on the 2.9-kb *Sal*I-*Eag*I fragment contained in pNZ636.

Further confirmation for the functional complementation of the *lacY* mutation in *E.coli* BY1 was obtained from transport studies with radiolabeled lactose or TMG. Cells harboring pNZ635 or pNZ636 were capable of transporting significant amounts of ^{14}C-lactose, with an initial rate of about 1.0 to 1.5 nmol/min x (mg of protein), whereas cells harboring pNZ635ΔEV or pNZ637 did not accumulate ^{14}C-lactose above background quantities (Fig. 2A). Similar results were found with ^{14}C-labeled TMG (Fig. 2B), that was taken up with a rate of 1.0 nmol/min x (mg of protein).

Nucleotide sequence analysis of the *lacP* gene and its flanking regions. The nucleotide sequence of a 1.9-kb DNA region comprising the *Bgl*II, *Eco*RV and *Eag*I sites was determined. Two large open reading frames were detected that partially overlapped. The nucleotide and deduced amino acid sequence of these open reading frames, designated ORF1 and ORF2 respectively are shown in Fig. 3.

The first open reading frame (ORF1) starts with an ATG at position 439, contains 654 base pairs (bp) and is preceded by many stop codons in all reading frames. It could encode a protein of 218 amino acids with a calculated molecular weight of 23,957. This ORF1 is preceded by a stretch of purines (5'-AGAAAGGA-3'), which shows high complementarity to the 3' end of 16S rRNA sequences of other gram positive bacteria like *Lactococcus lactis* (25) and *Bacillus subtilis* (16), and could function as a ribosome binding site (RBS). This putative RBS is spaced from the ATG initiation codon by 12 bp. The second initiation codon in ORF1 is located at position 493 but is not preceded by a consensus RBS.

The second open reading frame, from position 1077 to position 1844, could code for 256 amino acids with a calculated molecular weight of 28,777 and overlaps ORF1 with 6 amino acids. The second triplet of ORF2 is an ATG that is preceded by a putative RBS (5'-GAAAGGA) spaced by 7 base pairs. The results from the deletion analysis (Fig. 1) indicate that ORF2 is not involved in lactose uptake, since its major part (starting from the *Eag*I site at position 1381; Fig. 3) is deleted in pNZ636, that still shows lactose transport activity in *E.coli* BY1. In addition, pNZ637 that contains an intact ORF2 is not able to complement the

lacY mutation of *E.coli* BY1. We therefore conclude that the lactose transport activity is encoded by ORF1, that has been designated the *lacP* gene.

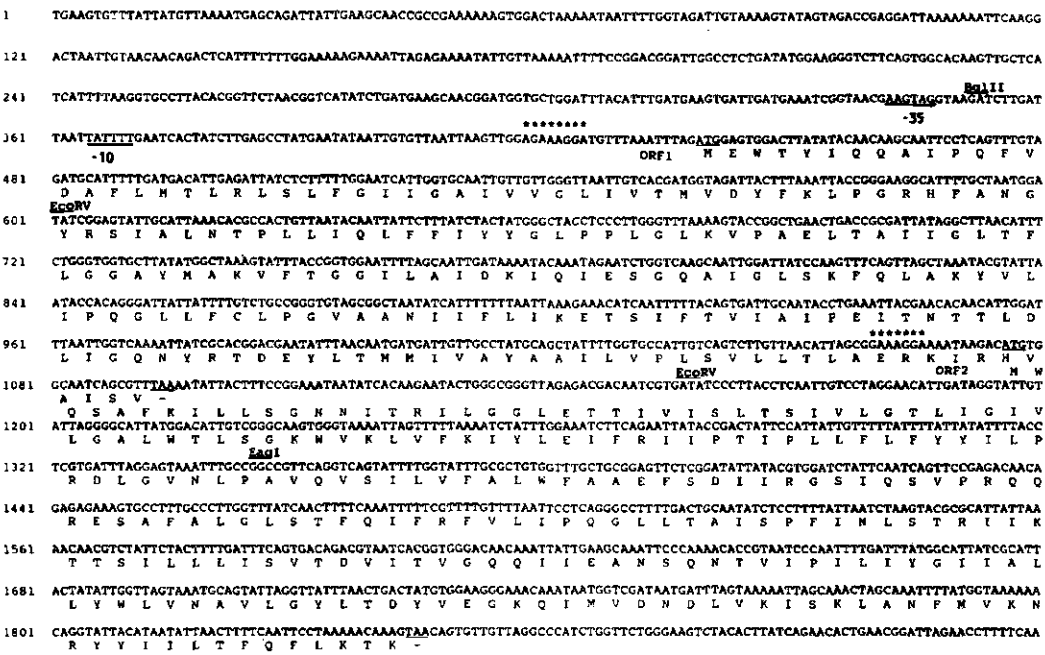

FIG. 3. Nucleotide sequence and deduced amino acid sequence of ORF1 and ORF2. The initiation and stop codons of the two open reading frames are underlined. Putative ribosome binding sites are marked with stars and consensus -10 and -35 regions of a putative promoter are underlined. The recognition sites for *Bgl*II, *Eag*I and *Eco*RV are indicated. Putative membrane spanning regions are overlined.

T7 expression studies. To analyze the expression products of the *lacP* gene and flanking regions, the 6.0-kb *Bam*HI-*Pst*I fragment of *L.lactis* DNA was isolated from pNZ635 and cloned into the expression vectors pT7-5 or pT7-6, that had been digested with *Bam*HI and *Pst*I. The resulting plasmids, pNZ576 and pNZ676, were introduced into *E.coli* BL21 and

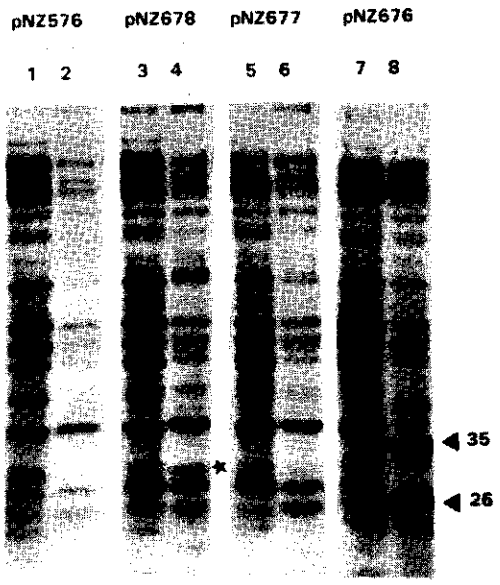

FIG. 4. Autoradiography of protein extracts derived from *E.coli* BL21 cells harboring the *lacP* gene under T7 promoter control. Extracts of cells harboring pNZ576 (lanes 1 and 2) , pNZ678 (lanes 3 and 4), pNZ677 (lanes 5 and 6) and pNZ676 (lanes 7 and 8) are shown. Lanes 1, 3, 5 and 7 represent non-induced cells; lanes 2, 4, 6 and 8 represent cells after induction with 400 μM IPTG and addition of 100 μg/ml rifampicin. Molecular weights of the relevant bands are indicated at the right. A star indicates the 30 kDa induced bands in pNZ678 (lane 4).

the [^{35}S] methionine-labeled proteins synthesized after IPTG induction of the T7 polymerase gene were analyzed (Fig. 4). Additional labeled proteins were only visible in cells harboring pNZ676, containing the *lacP* gene and flanking regions under control of the T7 promoter. The most strongly labeled proteins had an apparent molecular weight of 26 and 35 kilodalton (kDa), while three less intensively labeled bands of 24 kDa, 33 kDa and 38 kDa were also detected (Fig. 4, lane 8). Deletion of the *Eag*I-*Bam*HI fragment of pNZ676 resulted in pNZ677, with the same insert as pNZ636, that contains only the *lacP* gene under control of the T7 promoter. Following introduction of pNZ677 into *E.coli* BL21, labeled proteins of 26 and 30 kDa were obtained after IPTG induction. Since the 26-kDa protein is the only labeled protein present in BL21 strains harboring the *lacP* expressing plasmids pNZ676 or pNZ677, we assume that this protein is the *L.lactis* lactose permease.

E.coli BL21 cells harboring the deletion plasmid pNZ678 (Fig.1), containing the *Hpa*I/*Sal*I fragment under control of the T7 promoter, showed a labeled protein band of approximately 30 kDa after induction. This band could represent the protein encoded by the second ORF

that was found downstream of *lacP*.

Amino acid sequence analysis and homology. The deduced amino acid sequences of the products of *lacP* and ORF2 exhibited a large degree of hydrophobicity, showing a hydrophobicity index of 0.91 for LacP and 0.85 for the putative ORF2 gene product, suggesting that they are integral membrane proteins.

Upon comparison of the deduced amino acid sequences of LacP and the putative ORF2 gene product with the NBRF and Swiss protein data bases no homology was detected for the ORF2 gene product. In contrast, a significant amount of similarity was found for the deduced amino acid sequence of the *Leuconostoc lactis lacP* gene product with the *E.coli* glutamine transport protein (the product of the *glnP* gene; 26) and the *Salmonella typhimurium* histidine permease protein (the product of *hisQ* gene; 19). An alignment of the deduced amino acid sequences of *Leuconostoc lactis* LacP, the *E.coli* GlnP and *Salmonella typhimurium* HisQ is shown in Fig.5. The alignment shows that all three proteins have a similar size but that the *Leuconostoc lactis lacP* shows a higher degree of identity with GlnP (31%) than with HisQ (18%).

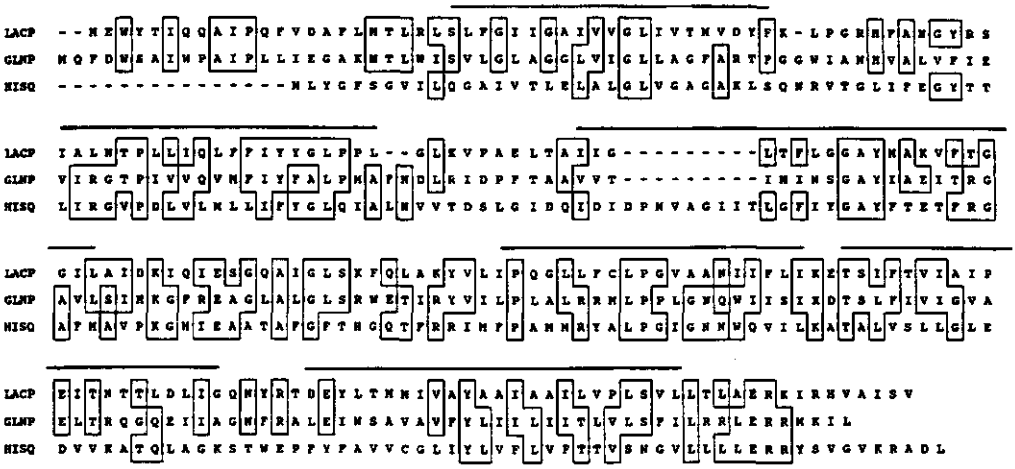

FIG. 5. Alignment of the deduced amino acid sequences of the *Leuconostoc lactis* lactose transport protein (LacP) with that of the glutamine transport protein of *E.coli* (GlnP) and the *S.typhimurium* histidine transport protein (HisQ). Identical residues are boxed. Putative membrane spanning domains are overlined.

DISCUSSION

We describe the cloning, expression and nucleotide sequence determination of the plasmid-encoded *lacP* gene of *L.lactis* NZ6009. The lactose permease-deficient *E.coli* strain HB101 and BY1 could be complemented with the *L.lactis lacP* gene and transport of ^{14}C-labeled lactose and TMG was demonstrated in cells of BY1 harboring the LacP expressing plasmids. The pink colonies that were observed on lactose-MacConkey agar plates of *E.coli* BY1 carrying pNZ635ΔBg could be due to a lower functional expression of the *lacP* gene. The frame shift mutation in the *Bgl*II site in pNZ635ΔBg may affect the *lacP* expression since it is located in a region that contains a putative promoter sequence (Fig.3).

Expression studies revealed that only one labeled protein of 26 kDa is found after IPTG induction in all *E.coli* BL21 strains harboring pNZ676 or pNZ677 that contain the *lacP* gene under control of the T7 promoter (Fig.4) and, therefore, presumably represents the *lacP* gene product. The less intensively labeled protein band of 30 kDa, visible after induction of cells harboring pNZ678 (Fig.4, lane 4) might represent the protein encoded by ORF2, since it is not found in BL21 harboring pNZ677. The absence of this protein band in induced cells harboring pNZ676 (which contains both *lacP* and ORF2), could be explained by the fact that due to the overlap of the *lacP* and ORF2 (Fig.3), the expression of the *lacP* gene prevents proper translation initiation of the ORF2 gene.

Nucleotide sequence analysis of the *L.lactis lacP* gene and its flanking regions showed that it encodes a highly hydrophobic (hydrophobicity index 0.91) protein with a calculated molecular weight of 23,957. Homology studies indicated that the *L.lactis lacP* gene encodes a third class of lactose carriers that differs from the previously described *E.coli* LacY (5) and *Streptococcus thermophilus* and *Lactobacillus bulgaricus* LacS (24, 29). In contrast, the deduced amino acid sequence of the *lacP* gene product of *L.lactis* shows considerable similarity with the *E.coli* GlnP (72%) (26) and *Salmonella typhimurium* HisQ (66%) (19) HisM (65%) (19) proteins (Fig. 5), all representing components of binding protein-dependent transport systems. The identity of the amino acid sequences of LacP to those of GlnP, HisQ and HisM is 32%, 24% and 20% respectively. The *E.coli glnP* gene product is thought to be the membrane component of the glutamine transport system involved in the permease activity (26). The *Salmonella typhimurium* HisQ protein may, in conjunction with the HisM protein, associate in the membrane to form a pore that with a functional HisP would allow

the substrate to enter the cell (19). Studies on the energetics of transport systems utilizing periplasmic binding proteins in *E.coli* and *Salmonella typhimurium* have shown that they function independently of a proton motive force but require intracellular ATP (3, 20). The homologies of the *L.lactis* lactose permease to the membrane components of those amino acid transport systems, suggest that the energy coupling of lactose transport in *L.lactis* might resemble that found in these transport systems. The results of the complementation studies in *E.coli lacY* mutants indicate that a single protein, LacP, is sufficient for lactose transport. It remains to be established, however, if LacP is sufficient for lactose uptake in *L.lactis*, and what role ORF2 has in its natural host.

The *lacP* gene of *L.lactis* NZ6009 is not flanked by a ß-galactosidase gene (Fig. 4), in contrast to the *lacS* genes for the lactose carriers of the other lactic acid bacteria *Lactobacillus bulgaricus* (24) and *Streptococcus thermophilus* (29), that are localized immediately upstream of the ß-galactosidase genes. Recent studies have shown that a DNA region encoding ß-galactosidase in *L.lactis* NZ6009 is located about 4.2-kb downstream of the *lacP* gene (11). This difference in organization of the *lac* genes and the fact that the *lacS* and *lacP* genes code for unrelated lactose carriers strongly suggest that the elements of the lactose metabolism have evolved in a different way in *L.lactis* as compared to *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, and may indicate that *L.lactis* has assembled the genes required for transport and hydrolysis of lactose from different sources.

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CHAPTER VI

Nucleotide sequence and expression in *Escherichia coli* of the *Lactococcus lactis* citrate permease gene.

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Nucleotide Sequence and Expression in *Escherichia coli* of the *Lactococcus lactis* Citrate Permease Gene

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The plasmid-encoded citrate determinant of the *Lactococcus lactis* subsp. *lactis* var. diacetylactis NCDO176 was cloned and functionally expressed in a Cit$^-$ *Escherichia coli* K-12 strain. From deletion derivative analysis, a 3.4-kilobase region was identified which encodes the ability to transport citrate. Analysis of proteins encoded by the cloned fragment in a T7 expression system revealed a 32,000-dalton protein band, which correlated with the ability of cells to transport citrate. Energy-dependent [1,5-^{14}C]citrate transport was found with membrane vesicles prepared from *E. coli* cells harboring the citrate permease-expressing plasmid. The gene encoding citrate transport activity, *citP*, was located on the cloned fragment by introducing a site-specific mutation that abolished citrate transport and resulted in a truncated form of the 32,000-dalton expression product. The nucleotide sequence for a 2.2-kilobase fragment that includes the *citP* gene contained an open reading frame of 1,325 base pairs coding for a very hydrophobic protein of 442 amino acids, which shows no sequence homology with known citrate carriers.

As in members of the family *Enterobacteriaceae* (25), the ability to utilize citrate is a useful metabolic characteristic for identifying *Lactococcus lactis* species (6, 34). The citrate-fermenting ability of these gram-negative bacteria appears to be linked to the presence of genetically unstable determinants such as plasmids (13, 14, 18, 32, 33, 37, 38) or transposons (15). The presence of plasmid- or transposon-encoded citrate transport systems enables members of the *Enterobacteriaceae* to utilize citrate as the sole carbon source. In contrast, the citrate-fermenting lactococcal strains, designated *L. lactis* subsp. *lactis* var. diacetylactis (7, 34), require an additional source of metabolic energy for the transport of citrate (5, 12). Although biochemical details of lactococcal citrate metabolism have been the subject of many studies (12, 36, 41), the energetics of citrate uptake are not yet understood. Kempler and McKay (19) demonstrated that the ability to transport citrate was linked to a 7.9-kilobase (kb) plasmid that appears to be present in all citrate-fermenting *L. lactis* strains analyzed. A detailed physical map of one of these citrate plasmids, pCT176, has been reported (10).

In the bacterial species described until now, the ability to grow on citrate is associated with cation-dependent transport systems. Na^+-dependent citrate utilization is found in *Enterobacter aerogenes* (16, 28) and *Salmonella typhimurium*, which also possess a K^+-dependent transport system (1, 18, 40). In *Bacillus subtilis* citrate transport is coupled to magnesium ion transport (2). Cit$^+$ *Escherichia coli* strains contain a citrate permease, which seems to be H^+ dependent (30), whereas two citrate transport systems are present in *Klebsiella pneumoniae*, one being dependent on H^+ (45) and the other being dependent on Na^+ (9). The genes for H^+-dependent citrate transport systems of *E. coli* and *K. pneumoniae* have been isolated, and sequence analysis has shown that they code for related citrate-transport proteins (van der Rest et al., in press).

To assess the characteristics of citrate transport in lactococci, we describe in this paper the cloning, functional expression, and sequencing of the citrate carrier of *L. lactis* NCDO176 in *E. coli*. Additionally, we present an initial characterization of the mechanism of citrate uptake mediated by the lactococcal citrate carrier.

MATERIALS AND METHODS

Bacterial strains and plasmids. *L. lactis* subsp. *lactis* var. diacetylactis NCDO176 was the source of the Cit$^+$ determinant in plasmid pCT176 (11). *E. coli* K-12 strain DH1 [F$^-$ *recA1 endA1 gyrA96 thi-1 hsdR17*(r$^-$ m$^-$) *supE44 relA1* lambda$^-$] was used for selection of Cit$^+$ transformants. *E. coli* DH1 harboring plasmid pES1 containing the citrate carrier of *K. pneumoniae* (35) was the Cit$^+$ positive control in these experiments. *E. coli* NZ1021 is a derivative of MC1061 (4) carrying plasmid pGP1 (43) and was used in T7 RNA polymerase expression experiments. *E. coli* BL21 (DE3) (42) (F$^-$ *hsdR gal*) (obtained from F. W. Studier) was used for membrane vesicle isolations. *E. coli* cloning vectors pBR328 (39) and pT75 (obtained from S. Tabor and C. C. Richardson) were used to clone the citrate determinant from strain NCDO176.

Media and growth conditions. *E. coli* strains were grown in L-broth (24) with vigorous shaking at 37°C. When appropriate, the medium was supplemented with carbenicillin (100 μg/ml), kanamycin (20 μg/ml), or tetracycline (12.5 μg/ml) or a combination of these antibiotics.

Citrate-positive recombinants of *E. coli* DH1 were selected after overnight incubation on Simmons citrate agar plates (Difco Laboratories).

Cloning of the *citP* gene. CsCl-ethidium bromide density gradient-purified plasmid DNA from *L. lactis* NCDO176 was prepared by the method of Maniatis et al. (24) with minor variations as described previously (6) and was digested to completion with *Eco*RI. The 7.9-kb linearized plasmid band of pCT176 was isolated, inserted into the unique *Eco*RI site of vector pBR328, and transformed to *E. coli* MC1061.

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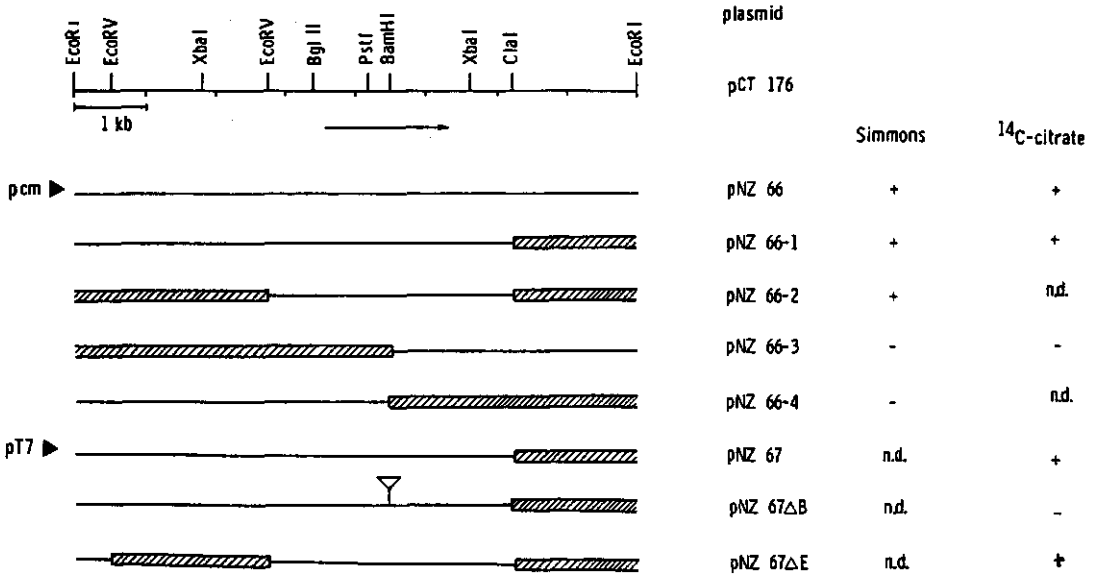

FIG. 1. Physical map and subcloning of pCT176. A partial restriction nuclease map of the citrate plasmid pCT176 and deletion derivatives of the fragment cloned in pBR328 and pT75 are shown. Symbols: →, location and orientation of the citrate permease (*citP*) gene on pCT176; ▨, regions that have been deleted in the cloned fragment. Plasmid pNZ66 contains pCT176 cloned as an *Eco*RI fragment (indicated by the line) into pBR328. The direction of the promoter of the chloramphenicol resistance gene located on pBR328 in front of the cloned fragments is indicated. Plasmid pNZ67 is a derivative of pT75. The 5-kb *Eco*RI-*Cla*I fragment of pCT176 was cloned behind the T7 promoter. The direction of the promoter transcription is indicated. In plasmid pNZ67ΔB the frameshift mutation within the *Bam*HI site of the cloned fragment is indicated. In pNZ67ΔE, an *Eco*RV fragment was deleted from the original *Eco*RI-*Cla*I fragment. The ability to transport citrate which is conferred by these plasmids on *E. coli* DH1 is shown in the right half of the figure and was determined by using Simmons citrate indicator agar plates and by [^{14}C]citrate uptake studies with intact cells. The amount of radioactivity found in citrate-positive cells ranged from 6,000 to 16,000 cpm, whereas in citrate-negative cells less than 3,000 cpm was detected. Symbols: +, citrate uptake or utilization; −, no citrate uptake or utilization; n.d., not determined.

Further subcloning and other DNA manipulations were performed as described by Maniatis et al. (24).

T7 expression experiments. For radioisotope labeling of proteins encoded by pCT176, DNA fragments were cloned in *E. coli* NZ1021 by using pT75 and the expression products were analyzed as described by Tabor and Richardson (43) on sodium dodecyl sulfate-12.5% polyacrylamide gels (21).

Membrane vesicle preparation. Membrane vesicles were prepared by the method of Kaback (17) from exponentially growing cells (A_{600}=0.8 to 1.0) of *E. coli* BL21, containing the appropriate plasmids, after induction of logarithmically growing cells with 400 μM isopropyl-β-D-thiogalactopyranoside for 90 min. Membrane vesicles were suspended in 50 mM potassium phosphate (pH 6.6) and stored in liquid nitrogen.

Transport assays (whole cells and membrane vesicles). We studied the transport of citrate in exponentially growing *E. coli* cells which were washed three times with 50 mM potassium phosphate (pH 5.5) containing 2 mM $MgSO_4$ and resuspended in the same buffer to 10 to 20 mg of total cellular protein per ml. Transport was assayed over 20 min with 9 μM [1,5-^{14}C]citric acid (110 mCi/mmol; The Radiochemical Centre, Amersham, England) at room temperature with samples containing 1 to 2 mg of protein per ml.

Incubation and processing were performed as described by Reynolds and Silver (30) for both whole cells and membrane vesicles, except that for transport studies in membrane vesicles we used 50 mM potassium piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (K-PIPES; pH 6.6). Controls were assayed for the transport of L-[U-^{14}C]proline (154.5 mCi/mmol). The energy for citrate transport was supplied by 10 mM ascorbate and 100 μM phenylmethosulfonate (PMS). Protein determinations were performed by the method of Lowry et al. (22).

DNA sequence analysis. The DNA sequence of a 2.2-kb *Bgl*II-*Xba*I fragment of plasmid pCT176 (Fig. 1) was determined by using the method of Sanger et al. (31). Sequence data were analyzed by using PC/Gene, version 5.01 (Genofit, Geneva, Switzerland), nucleic acid and protein analysis programs and the computer facilities of the CAOS/CAMM Center, Nijmegen University, with the National Biomedical Research Foundation (NBRF/PIR) (release 23.0) and SWISS-PROT (release 13.0) data bases.

RESULTS

Cloning and functional expression of the Cit$^+$ determinant in *E. coli*. Cells of a derivative of *L. lactis* NCDO176, lacking the 7.9-kb plasmid pCT176, were unable to take up radioactively labeled citrate, indicating that this plasmid encodes a citrate permease (results not shown). Tetracycline-resistant transformants of *E. coli* DH1(pNZ66) were tested on Simmons citrate agar plates, on which colonies with a Cit$^+$ phenotype have a blue halo around the colonies. As a positive control in these experiments, we used *E. coli* DH1 (pES1), a pBR325 derivative containing the *K. pneumoniae*

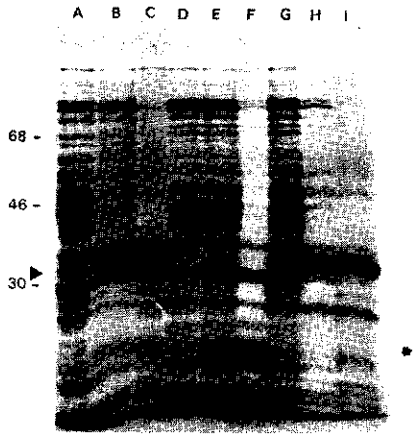

FIG. 2. Expression of the *citP* gene under T7-RNA polymerase control. *E. coli* NZ1012 cells containing plasmids pNZ67 (lanes A to C), pNZ67ΔB (lanes D to F), or pNZ67ΔE (lanes G to I) are shown. Lanes A, D, and G contain noninduced cells; lanes B, E, and H contain 42°C induced cells with no rifampin added; and lanes C, F, and I contain induced cells to which rifampin was added. The arrowhead indicates the position of the induced *citP* gene product. The asterisk indicates the position of the truncated *citP* gene product.

citrate transport gene (35). The pCT176 fragment was found to contain a functional citrate permease gene (*citP*), which was also demonstrated by the ability of transformants to take up citrate (Fig. 1). The Cit$^+$ phenotype was expressed in only one of the two possible orientations of the 7.9-kb *Eco*RI-cut plasmid DNA within the vector, suggesting that the promoter of the citrate permease gene either was not present on the cloned fragment or was not recognized in *E. coli*. The *Eco*RI fragment of pCT176 was further subcloned in pBR328 to narrow down the region encoding the *citP* gene (Fig. 1). Growth of these deletion derivatives in *E. coli* DH1 revealed that a 3.4-kb *Eco*RV-*Cla*I fragment in pNZ66-2 was still capable of conferring the Cit$^+$ phenotype.

T7 expression experiments. To analyze the proteins encoded by the cloned DNA fragments, we made additional constructs in the expression vector pT75. One of these recombinant plasmids, pNZ67 (Fig. 1), contained the 6.4-kb *Eco*RI-*Cla*I fragment of pCT176 under control of the T7 RNA polymerase promoter. One derivative of pNZ67, pNZ67ΔB, containing a frameshift mutation in the *Bam*HI site of the insert, was constructed by cutting with *Bam*HI, filling up the protruding ends with Klenow DNA polymerase, and religating the fragment. A second derivative, pNZ67ΔE, had the 2-kb *Eco*RV fragment deleted from the insert. [^{35}S]methionine-labeled proteins specified by the recombinant plasmids were analyzed. After temperature induction, the presence of a 32-kilodalton (kDa) protein band was visible in preparations of cells harboring pNZ67 or pNZ67ΔE but not pNZ67ΔB (Fig. 2). Also, the cells showing the 32-kDa protein band were able to take up radioactively labeled citrate. Cells containing plasmid pNZ67ΔB were unable to transport citrate (Fig. 1). This strain showed a band of approximately 20 kDa, which was absent in cells containing plasmid pNZ67ΔE or pNZ67ΔB. Strain NZ1021 harboring pNZ67 or pNZ67ΔB showed an additional protein band of approximately 30 kDa, which was absent in cells harboring pNZ67ΔE.

Transport studies in membrane vesicles. Membrane vesicles were prepared from *E. coli* cells carrying pNZ67 or pNZ67ΔB. IPTG induction proved to be a more reproducible and efficient method than temperature for induction of T7 polymerase-dependent citrate transport in vesicle preparations. Plasmids pNZ67 and pNZ67ΔB were transformed to *E. coli* BL21 containing a chromosomally linked T7 RNA polymerase gene under control of the IPTG-inducible *tac* promoter (42). Membrane vesicles of BL21 cells harboring pNZ67 or pNZ67ΔB accumulated proline in the presence of

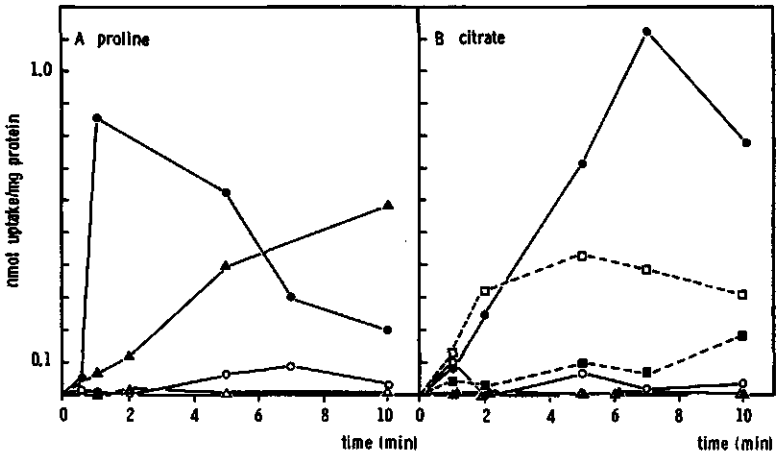

FIG. 3. Uptake studies of [^{14}C]proline (2 μM) (A) and [^{14}C]citrate (4.5 μM) (B) by membrane vesicles of *E. coli* BL21 with ascorbate-PMS as the electron donor (●, ▲, ■) and without ascorbate-PMS (○, △, □). Symbols: ●, ○, uptake of membrane vesicles of strain BL21(pNZ67); ▲, △, uptake of membrane vesicles of BL21(pNZ67ΔB); ■, □, [^{14}C]citrate uptake of ascorbate-PMS-energized membrane vesicles after the addition of valinomycin (□) or nigericin (■). The values are averages of at least two separate experiments.

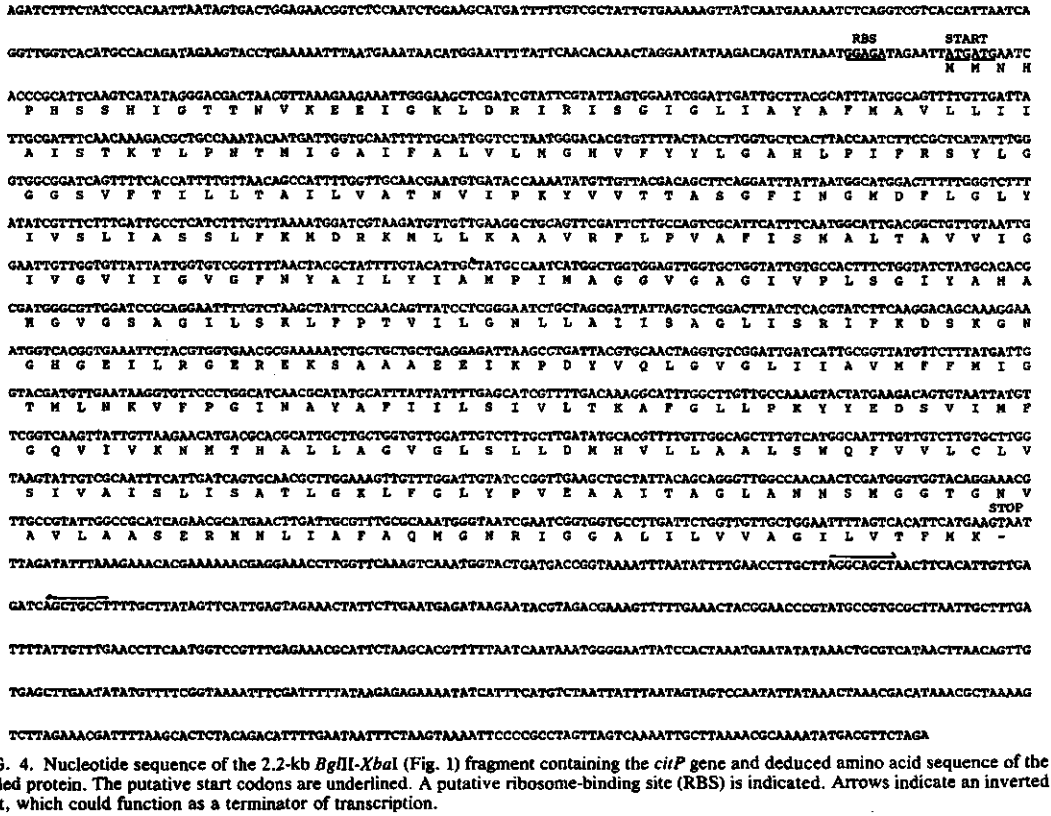

FIG. 4. Nucleotide sequence of the 2.2-kb *Bgl*II-*Xba*I (Fig. 1) fragment containing the *citP* gene and deduced amino acid sequence of the encoded protein. The putative start codons are underlined. A putative ribosome-binding site (RBS) is indicated. Arrows indicate an inverted repeat, which could function as a terminator of transcription.

ascorbate-PMS, as expected (Fig. 3A). The differences in proline uptake between the strains harboring either plasmid pNZ67 or pNZ67ΔB may be attributed to differences in the vesicle preparations. Similar differences were observed with different vesicle preparations of one strain (data not shown). Membrane vesicles from Cit^+ cells, containing plasmid pNZ67, were also able to take up citrate (Fig. 3B). Citrate transport in these vesicles appeared to be driven by the proton motive force (ΔP), since dissipation of the $\Delta\psi$ by valinomycin or of the ΔpH by nigericin inhibited the accumulation of citrate (Fig. 3B).

Nucleotide sequence of the *citP* gene and flanking regions. The nucleotide sequence of a 2.1-kb fragment containing the citrate transport gene (*citP*) was determined (Fig. 4). There was only one open reading frame, from positions 131 to 1557, of sufficient size to encode a 32-kDa protein. The DNA sequence for the open reading frame contained the single *Bam*HI site at position 852. Introduction of a frameshift mutation within this site introduces a stop codon at position 872 and results in the formation of a truncated, nonfunctional protein product (Fig. 2 and 3). The open reading frame for *citP* starts with two adjacent ATG triplets. If the first initiation codon was used (position 259), the gene would encode a protein of 442 amino acids with a calculated molecular weight of 46,645.

Analysis of the deduced amino acid composition of the presumed citrate carrier indicates that this protein is highly hydrophobic, with 12.4% polar and 87.5% nonpolar amino acid residues. The ratio of basic (lysine, arginine, and histidine) to acidic (glutamate and aspartate) residues is 2.1:1 and indicates that *citP* encodes a basic protein with a calculated isolectric point of 9.97. The deduced amino acid sequence of the citrate carrier contains segments of high hydrophobicity that alternate with short hydrophilic segments (data not shown, 20). Both N- and C-terminal ends of the amino acid sequence are more hydrophilic, as is the region between residues 240 and 280.

There was no homology between the *Lactococcus* citrate carrier protein and other known citrate carriers, such as those of *E. coli* (32) and *K. pneumoniae* (45). Also, a search of the NBRF and SWISS data bases with the predicted primary sequence of *citP* failed to detect significant homology to any of the published sequences.

DISCUSSION

We describe the cloning, expression in *E. coli*, and nucleotide sequence of the plasmid-encoded *citP* gene of *L. lactis* NCDO176. Transport of [^{14}C]citrate by whole cells and membrane vesicles of *E. coli* harboring *citP* expressing plasmids was demonstrated.

The *citP* gene product is a protein with a gel electrophoresis rate corresponding to an apparent molecular mass of 32 kDa (Fig. 2). The 32-kDa protein band is probably the *citP*

gene product, and the 20-kDa band visible in strains carrying pNZ67ΔB may represent a truncated derivative of the *citP* gene product. The 30-kDa protein band encoded by pNZ67ΔE may be a second protein encoded by the *Eco*RV fragment that has no apparent function in citrate transport. The results were supported by citrate uptake studies in membrane vesicles of *E. coli* cells carrying *citP*-expressing plasmids (Fig. 3). These studies show that citrate transport is driven by the ΔP. More extensive studies are needed to reveal the nature of the cations symported with citrate and the contribution of the components of the ΔP in the transport process.

The nucleotide sequence of *citP* (Fig. 4) was identified which starts with two ATG codons. At this stage we do not know which initiation codon is actually used. A putative ribosome-binding site (GGAG at position 247), complementary to the 16S rRNA of *E. coli* (ΔG° of −7.2 kcal/mol [ca. −30.1 kJ/mol], calculated by the method of Tinoco et al. [44]) is present 9 nucleotides preceding the first of the two possible initiation codons. However, regions that are similar to *E. coli* (26) or *L. lactis* (8) consensus promoter transcription initiation sequences were not found. An inverted repeat 97 base pairs downstream of the stop codon at position 1557 showed homology to typical ρ-independent terminators of transcription (29) (Fig. 4). It was also found that in other citrate carrier genes of *E. coli* (32) and *K. pneumoniae* (45), no promoter sequences were present in the region preceding the sequence encoding the citrate carrier. A second open reading frame, located 54 base pairs in front of the citrate carrier gene, is proposed to be necessary for undelayed citrate utilization in *E. coli* (32). In *K. pneumoniae* no such open reading frame has been detected, although the inability to obtain functional expression in some deletion derivatives has been interpreted as evidence for the presence of such an open reading frame (45). There are no indications of a similar structure in *L. lactis*, since deletion of a region upstream of the *citP* gene, such as in pNZ67ΔE, did not show any delayed growth or delayed uptake of labeled citrate into whole cells (Fig. 1 and results not shown).

The hydropathy profile of *citP* resembles those of other membrane-associated proteins. For instance, the citrate transport proteins of both *E. coli* and *K. pneumoniae* also contain a central hydrophilic region as well as hydrophilic N and C termini. The hydropathy profile of a hydrophobic protein may be a good description of the folding structure of the protein (27). The hydrophobic regions of the sequence may well represent membrane-spanning domains. These results strongly suggest that the *L. lactis* citrate permease is an integral membrane protein; this is in agreement with the location of the *citP* expression product in the cytoplasmic membrane.

The molecular mass calculated from the deduced primary sequence of the putative citrate carrier is 46.6 kDa, larger than the molecular mass of 32 kDa estimated from the mobility of the *citP* gene product on a sodium dodecyl sulfate-polyacrylamide gel. Such an aberrant migration on sodium dodecyl sulfate-polyacrylamide gels is well documented for a variety of hydrophobic proteins (3, 23).

The lack of homology between the citrate carriers of gram-negative bacteria and the lactococcal CitP suggests that the *L. lactis* citrate permease belongs to a different class of carriers. The observation that the *citP* gene can functionally complement *E. coli* suggests that all information for citrate transport is contained in its gene product.

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CHAPTER VII

Cloning and Nucleotide Sequence Analysis of the Plasmid Located *Leuconostoc lactis* NZ6070 Citrate Permease Gene.

S. David and W. M. de Vos

SUMMARY

In *Leuconostoc lactis* NZ6070 the gene for the citrate permease (*citP*) is plasmid located as could be demonstrated by hybridization studies of plasmid DNA from the wild-type strain and a cured citrate-deficient derivative strain NZ6071 with a DNA fragment containing the *citP* gene from *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* NCDO176. Two overlapping hybridizing NZ6070 plasmid fragments, a 5.0 kb *Pst*I fragment and a 4.5 kb *Bgl*II fragment, were identified and cloned into *Escherichia coli*. Using oligonucleotide primers based on the lactococcal *citP* gene, the nucleotide sequence of the *Leuconostoc lactis* NZ6070 citrate permease gene could be determined. Analysis of the nucleotide sequence of the *Leuconostoc lactis* NZ6070 *citP* gene revealed almost complete identity to that derived from the lactococcal strain NCDO176. A significant degree of sequence identity was found in the regions flanking the *citP* genes of both organisms, that included the putative ribosome binding site and terminator of transcription. The high identity of the *citP* genes of *Leuconostoc lactis* and *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* suggests recent acquisition of the *citP* gene by those lactic acid bacteria from a common ancestral gene.

INTRODUCTION

Leuconostoc species are an essential component of mesophilic starter cultures used in the manufacture of various fermented dairy products (3). The major function of *Leuconostoc* spp. in these fermentations are the heterofermentative conversion of lactose and the ability to produce CO_2 and the aroma component diacetyl from citrate (2, 9, 24). *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* is another important citrate-fermenting species, that is present in many mesophilic starter cultures. In all citrate fermenting *Lactococcus* strains that have been investigated sofar, the genetic information for citrate transport is located on a 7.9-kilobase (kb) plasmid species (11, 15, 23). Recently, we reported the nucleotide sequence of the plasmid-encoded structural gene for citrate permease (*citP*) of *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* NCDO176 (5).

Various *Leuconostoc* strains used in dairy fermentations have been reported to contain plasmids (6, 16, 17, 19). Although, most of these plasmids are cryptic, it has been suggested that genes involved in the utilization of lactose and citrate might be plasmid encoded in *Leuconostoc* spp., since these properties appeared to be unstable (17, 19) Recent results have confirmed that structural genes involved in lactose metabolism are plasmid encoded in *Leuconostoc lactis* NZ6009 (6, 8).

The loss of the ability to convert citrate is known to occur during prolonged propagations of *Leuconostoc* and *Lactococcus* strains in industrial fermentations, and may lead to the development of undesired flavor or texture defects in the final product. Studies on the citrate fermentation pathway in those lactic acid bacteria have shown that the citrate permease is a key enzyme in this process (4, 24).

In this report we demonstrate that the capacity to transport citrate are plasmid encoded in *Leuconostoc* strains. Additionally, we present the nucleotide sequence of the *Leuconostoc lactis citP* gene and a comparison with the *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* (5) and a *Klebsiella pneumoniae* (25) citrate permease genes.

MATERIALS AND METHODS

Bacterial strains and media. *Leuconostoc lactis* NZ6070 and its ethidiumbromide cured,

Cit$^-$, Lac$^-$ derivative strain NZ6071 (18) (kindly provided by A. Harrington, University College, Cork, Ireland) were grown in MRS medium (Difco Laboratories, Detroit), supplemented with glucose (0.5%), for the selection of Lac$^-$ strains, or glucose and lactose (both 0.5%), at 28°C without aeration. Whey serum agar with calcium citrate (10) was used for the selection of citrate-negative colonies. *Escherichia coli* strains MC1061 (1) and TG1 (12) were propagated in L-broth (20) at 37°C with vigorous shaking, and were supplemented, if appropriate, with 50 μg/ml ampicillin.

^{14}C-citrate transport assays. Transport of [1,5-^{14}C]citric acid (110 mCi/mmol; The Radiochemical Centre, Amersham, England) was assayed in exponentially growing *Leuconostoc* cells as described previously (5).

Cloning of the *citP* gene and its sequence analysis. Total plasmid DNA of *Leuconostoc lactis* strain NZ6070 was isolated following a procedure described previously (6) and digested to completion with restriction enzyme *Pst*I or *Bgl*II. The cleaved DNA was fractionated on a 0.8% agarose gel, after which appropriate fragments were eluted from the gel using Gene Clean (BIO 101, La Jolla, Calif.). The isolated *Pst*I fragments were cloned in the unique *Pst*I site of the vector pAT135. The *Bgl*II fragments were cloned in M13mp18 (New England Biolabs), that had been linearized with *Bam*HI. Both vectors had been dephosphorylated prior to ligation. *E.coli* MC1061 was used as a host for the pAT153 derivatives and *E.coli* TG1 was used to propagate the M13 clones. Plasmids containing the *Leuconostoc citP* gene were identified by hybridization with primer 1202 (5'-CATTAGGACCAATGC-'3). All DNA manipulations in *E.coli* were performed essentially as described by Sambrook et al. (20).

Nucleotide sequences of DNA fragments were determined by the dideoxy-chain termination method (21) on double-stranded or single-stranded templates, using synthetic oligonucleotides, synthesized on a Cyclone DNA synthesizer (Biosearch, San Rafael, Calif.). All enzymes were used according to the instructions of the suppliers (New England Biolabs Inc., Beverly, Mass.)

Hybridization techniques. DNA fragments were separated on 0.8% agarose gels that were blotted for 1 hour on a Gene Screen Plus membrane (Dupont, NEN Research products, Wilmington, Del.) using a blotting device (Vacugene, Pharmacia LKB Biotechnology) and with 0.4 N NaOH as transferbuffer. Colony or phage plaque screening was performed following the protocol provided by the manufacturer (Dupont, NEN). Oligonucleotides were labeled with [gamma-^{32}P]-ATP (Amersham) using polynucleotide kinase (Bethesda search

Laboratories, Gaitersburg, Md.) and restriction-fragments by nick-translation using DNAse/Polymerase (BRL) and [α-^{35}P]-dATP (Amersham) according to the methods described by Sambrook (20). Hybridization was performed at 65°C in a 0.5 M sodium phosphate buffer pH 7.2 and the blots were subsequently washed at 65°C in 1 x SSC (150 mM NaCl, 15 mM Na citrate).

RESULTS

Identification and cloning of the *citP* gene in *Leuconostoc lactis* NZ6070. *Leuconostoc lactis* strains NZ6070 and NZ6071 appeared to contain a complex plasmid complement, with plasmids ranging from 7 to 42 kb (Table 1 and Fig. 1, panel A).

TABLE 1. Plasmid content of *Leuconostoc lactis* strains NZ6070 and NZ6071.

Strain	plasmids (kilo base pairs)	phenotype[a,b]
NZ6070	7.0, 11.5, 23, 27, 32, 42	Cit$^+$,Lac$^+$
NZ6071	-, 11.5, -, 27, 32, 42	Cit$^-$,Lac$^-$

a: cit phenotype determined by growth on whey agar plates and ^{14}C-citrate uptake;
b: lac phenotype determined by growth in MRS-medium +/- lactose. For details see Materials and Methods section.

Comparison of the plasmid contents of the two strains, revealed the loss of at least two plasmids of 7.0 and 23 kb in the Cit$^-$ strain NZ6071 as compared to NZ6070 (Table 1 and Fig.1, panel A). The curing of these plasmids correlated with the inability of strain NZ6071 to form halos around colonies on whey agar plates and inability to transport ^{14}C-labeled citrate (Fig.2). Additionally, strain NZ6071 was unable to grow on medium containing lactose a sole carbon source.

Plasmid DNA of both strain NZ6070 and strain NZ6071 appeared to hybridize with pNZ66, which contains the citrate plasmid of *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* NCDO176 cloned in pBR328 (5) (Fig. 1, panel B). This hybridization showed one prominent band in the NZ6070 plasmid DNA, whereas chromosomal DNA from both strains

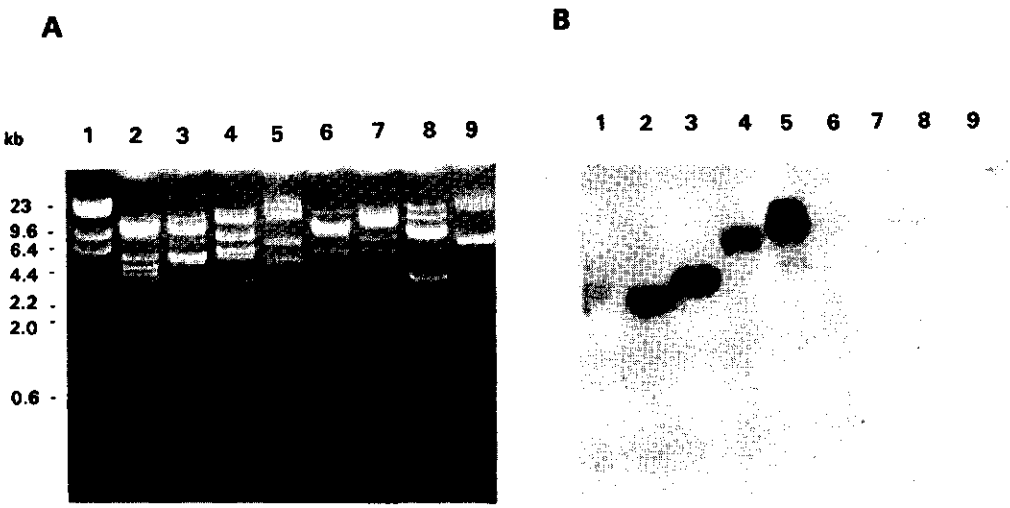

FIG. 1. Agarose gelelectrophoresis (panel A) and southern blot analysis (panel B) of plasmid DNA from *Leuconostoc lactis* strain NZ6070 (lanes 1 to 4) and its citrate-negative derivative strain NZ6071 (lanes 5 to 8). Lanes 1 and 5: *Pst*I digests, lanes 2 and 6: *BamH*I digests, lanes 3 and 7: *Sal*I digests, lanes 4 and 8: undigested plasmid DNA of NZ6070 and NZ6071 respectively. Lane 9: *Pst*I digest of plasmid DNA from *Lactococcus diacetylactis* NCDO176. The Southern blot was probed with a *PstI/BglII* fragment of pCT176. On the left the plasmid sizes are indicated in kilo base pairs (kb). Asterisks mark the plasmids that are absent in the cit' derivative strain NZ6071.

did not reveal any additional hybridizing bands (results not shown). These results suggested that the putative *citP* gene of *Leuconostoc lactis* was located on a single plasmid. In order to establish specific hybridization, a 1-kb *PstI-BglII* fragment of pCT176 containing the 5' part of the *citP* gene (5) was used to probe different restriction enzyme digests of plasmid DNA of strains NZ6070 and NZ6071. Only DNA from strain NZ6070 showed strong hybridization and contained single restriction fragments that hybridized with the probe. The same hybridization results were found (not shown) using the oligonucleotide primer 1202 (Fig. 3B) , that is complementary to the coding region of the lactococcal *citP* gene (5). The hybridizing fragments could be assigned to a 23-kb plasmid (pCT71), which is missing in strain NZ6071. A restriction enzyme map of a part of pCT71 was constructed (Fig.3A) and contained a 1.8-kb region that showed a comparable restriction site organization as the *BglII/AccI* fragment of pCT176 encoding the *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* NCDO176 *citP* gene. These results suggest that the common 1.8-kb *BglII/AccI*

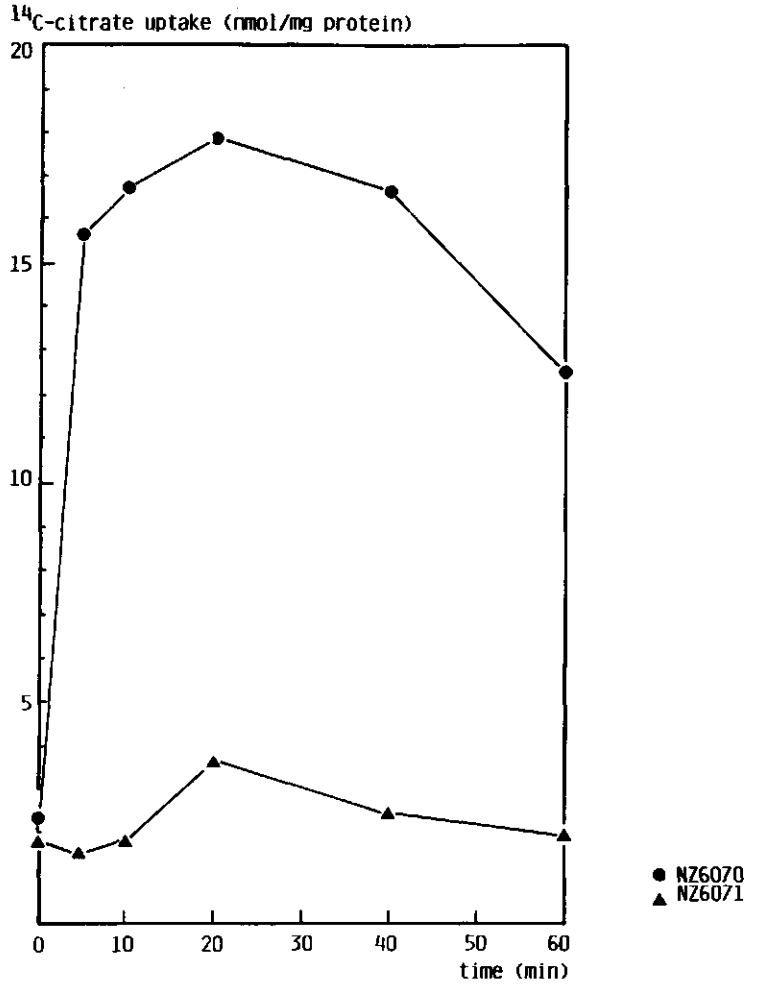

FIG. 2. ^{14}C-citrate uptake of whole cells of *Leuconostoc lactis* strains NZ6070 and NZ6071. Transport was assayed with washed cells in 50 mMpotassium phosphate (pH5.5) containing 2mM $MgSO_4$ with 9 μM [1,5-^{14}C]citric acid (110mCi/mmol), at room temperature with samples containing 1 to 2 mg of protein per ml.

fragment present in both plasmids contains the *Leuconostoc lactis citP* gene. Therefore, overlapping 5.0-kb *Pst*I and 4.5-kb *Bgl*II fragments, that hybridized to the oligonucleotide primer 1202 were cloned in *Pst*I- and *Bgl*II- linearized pAT135 and M13mp19, resulting in pNZ641 and pNZ642, respectively.

Sequence analysis of the putative *Leuconostoc citP* gene. The complete nucleotide sequence of the putative *Leuconostoc lactis* NZ6070 *citP* gene could be determined using the oligonucleotide sequence primers, that had previously been used to sequence the *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* NCDO176 *citP* gene (Fig.3B), suggesting strong conservation at the nucleotide level. Fig.4 shows the nucleotide sequence of the putative

Leuconostoc lactis NZ6070 *citP* gene and flanking sequences and a comparison with that of the *Lactococcus lactis* subsp. *lactis* var. *diacetylactis citP* gene. The sequence contained one open reading frame of 1423 base pairs, starting with two ajacent ATG triplets, that could encode a protein of 441 amino acids with a calculated molecular weight of 46,639, if the first initiation codon is used. The lactococcal and *Leuconostoc* sequences appeared to be nearly identical. The differences include 47 base substitutions, 13 of which are located within the coding region of the *citP* gene. The nucleotide substitutions resulted in the following amino acid substitutions in the the *Leucocnostoc* CitP as compared to the lactococcal: Val33Ile, Met103Ile and Asp257Lys (Fig.5). Additionally, two fragments of seven and three nucleotides (Fig.4) were deleted in the *Leuconostoc* sequence, the latter of which was

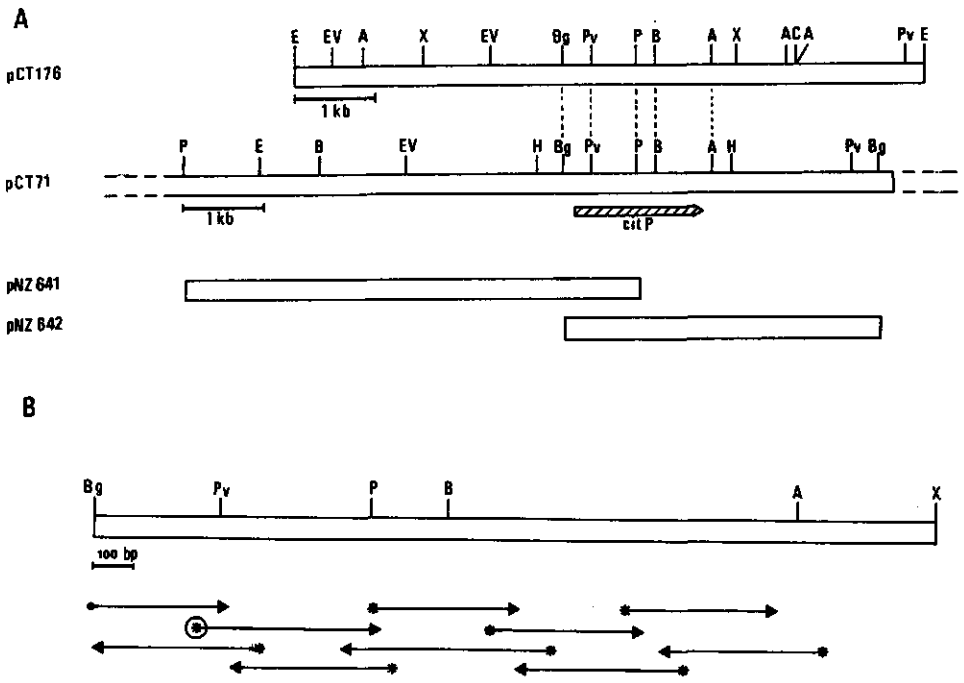

FIG. 3. A: Physical maps of the *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* citrate plasmid pCT176 and part of the *Leuconostoc lactis* NZ6070 citrate plasmid pCT71. The arrow indicates the location and direction of the *citP* gene of pCT176. The black bar indicates the region of pCT176 which has been sequenced.
Restriction enzyme abbreviations: A:*Acc*I; B:*Bam*HI; Bg:*Bgl*II;; C:*Cla*I; E:*Eco*RI; EV:*Eco*RV; H:*Hin*dIII; P:*Pst*I; Pv:*Pvu*I; X:*Xba*I
B: Sequence strategy of the putative *Leuconostoc lactis citP* gene and identification of *citP*-containing clones. Sequence reactions obtained using an universal primer are indicated with a dot, whereas the positions of the oligonucleotide primers are indicated by stars. The primer used as a probe in hybridization studies is marked with a circle.

contained within the *citP* gene and resulted in the deletion of a Ser-residue at position 258. The homology between the nucleotide sequences is not restricted to the coding region of the *citP* gene but continues to a large extent in the regions directly upstream and downstream of the coding region, including the putative ribosome binding site and terminator of transcription, that were identified in the lactococcal *citP* (Fig.4; 5).

DISCUSSION

We show here that similarly to *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* NCDO176 also *Leuconostoc lactis* NZ6070 contains a plasmid, pCT71, that encodes the ability to transport citrate and which is cured in strain NZ6071. An alignment of the restriction maps of the cloned fragments of pCT71 and plasmid pCT176 showed that the conservation of restriction sites is limited to the region encoding the *citP* genes. The putative *cit*P gene of *Leuconostoc lactis* exhibits extensive homology to the lactococcal *cit*P gene as could be demonstrated by hybridization studies and sequence analysis of fragments from pCT71. Recently Lin et al. (16) described the curing of a 22-kb plasmid and the concommitant loss of citrate utilizing ability in a *Leuconostoc mesenteroides* strain. In absence of data on the physical organization of this plasmid and the phenotype it encodes, we can only speculate about the possible identity between this plasmid and pCT71.

The putative amino acid sequence of the *Leuconostoc lactis* CitP is one amino acid smaller than that of *Lactococcus lactis* subsp. *lactis* var. *diacetylactis*, and contains three amino acid substitutions i.e. Val33Ile, Met103Ile and Asp257Lys. The deduced amino acid sequence of *Leuconostoc lactis* NZ6070 citrate permease was aligned to that of the sodium-dependent citrate carrier of *Klebsiella pneumoniae* (Fig.5; 25) The comparison shows that 132 out of 442 amino acid residues are identical (30% identity; 51% similarity). Some of those residues are also conserved in the H^+-dependent citrate carriers of *E.coli* (13, 22) and *Klebsiella pneumoniae* (26). Since there are no data available concerning the involvement of specific amino acid residues in the transport mechanism of either of the two classes of transport proteins, it is not yet possible to speculate about the significance of the conserved residues. This comparison and results of previous studies with *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* citrate carrier (5) suggest strongly that the Gram-positive carrier is probably also

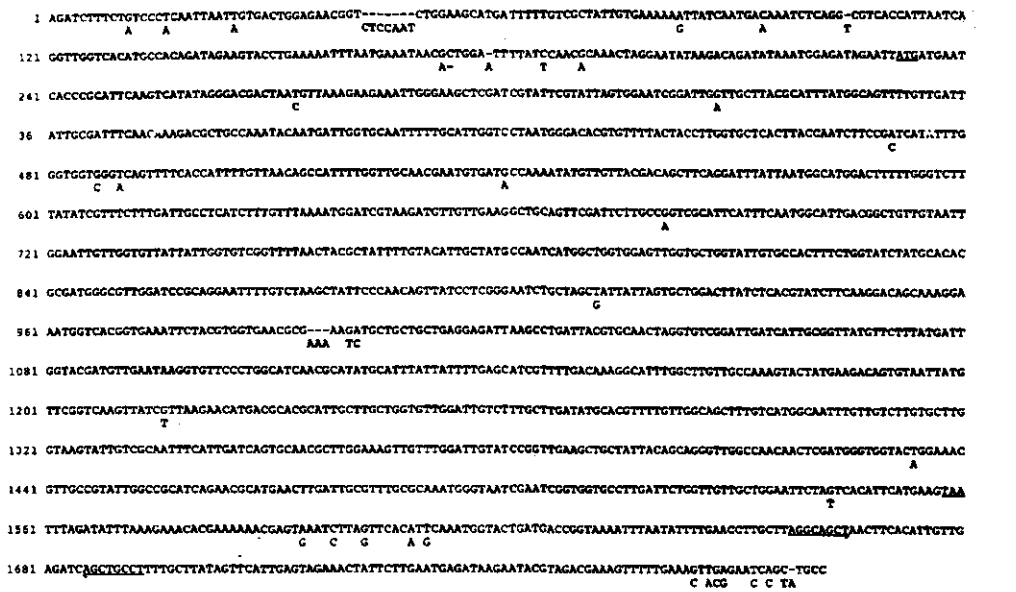

FIG. 4. Nucleotide sequence of the putative *Leuconostoc lactis* NZ6070 *citP* gene and its flanking sequence. Differences with the *Lactococcus lactis* subsp. *lactis* var. *diacetylactis citP* nucleotide sequence are indicated below the *Leuconostoc lactis* sequence. Start and stop codons of the *Leuconostoc lactis citP* sequence are underlined. The putative ribosome binding site is indicated with stars. The putative terminator of transcription is indicated with arrows below the inverted repeat.

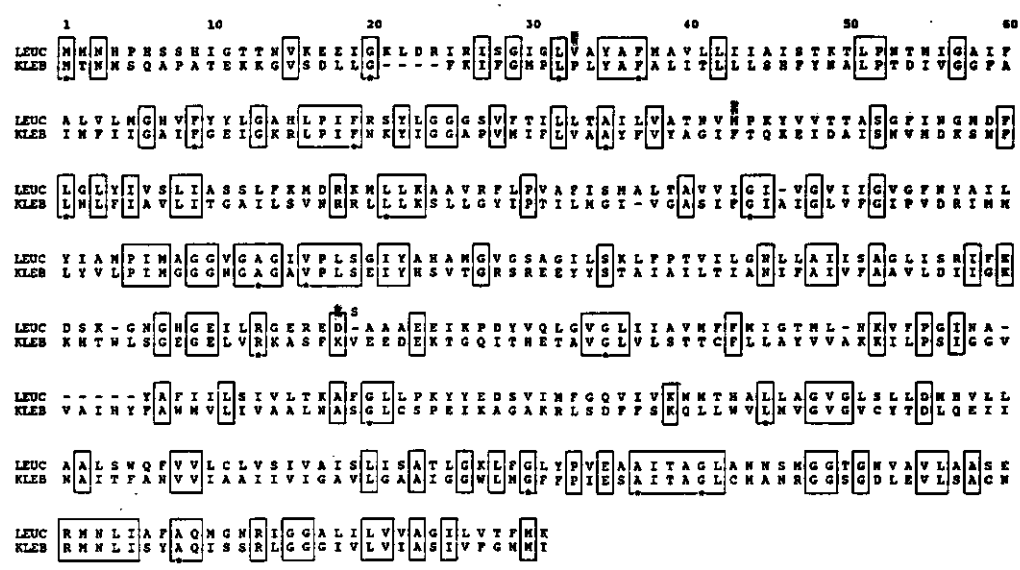

FIG. 5. Alignment of the putative deduced amino acid sequences of the *Leuconostoc lactis* NZ6070 and *Klebsiella pneumoniae* citrate permease genes (25,26). The conserved amino acid residues are boxed. Shaded residues are different amino acid residues in the *Lactococcus diacetylactis* NCDO176 CitP. Residues that are conserved in the H^+-dependent *E.coli* and *Klebsiella pneumoniae* citrate carriers are indicated by stars.

Na^+-dependent, and may function in a similar way. The Na^+-dependent citrate carrier in *Klebsiella pneumoniae* is believed to transporte citrate together with two protons and two Na^+ ions, providing the oxaloacetatedecarboxylase with the required energy in the form of Na^+ (8). The action of the *Klebsiella pneumoniae* citrate carrier is therefore dependent of a pH and Na^+ gradient across the membrane. Previous experiments with the *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* NCDO176 citrate carrier had already provided evidence suggesting the requirement of a pH for transport of citrate in *E.coli* membrane vesicles containing the lactococcal citrate carrier. Further experiments are needed to establish the role of positively charged ions in the transport process in *Lactococcus* and *Leuconostoc* spp.

The high amount of sequence identity between the *cit*P genes of *Lactococcus diacetylactis* NCDO176 and *Leuconostoc lactis* suggests a very recent evolution from a common ancestor. Recently, part of an iso-ISSI-element has been identified on a lactococcal citrate plasmid that is similar if not identical to the well-studied pCT176 (14). Therefore, it is tempting to speculate that the acquisition of the *cit*P genes in *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* and *Leuconostoc lactis* has occurred via IS-element mediated events, leading to the horizontal transfer of *cit*P genes in lactic acid bacteria.

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CHAPTER VIII

Summary and Concluding Remarks

Mesophilic lactic acid bacteria, belonging to the related genera *Lactococcus* and *Leuconostoc*, are important components of starter cultures used in the manufacture of cheese, butter and buttermilk. The most important function of these bacteria is the fermentative conversion of lactose into lactic acid. Furthermore, the application of both *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* and *Leuconostoc* spp. in starter cultures is based on their ability to produce CO_2 that is essential for the formation of eyes in cheese, and the important aroma component diacetyl during the conversion of citrate, which is present in milk. Although the biotechnological importance of the citrate metabolism is widely appreciated, little is known about its genetic basis. In addition, none of the used *Leuconostoc* spp. have been studied on the genetic level. A better knowledge of the genes involved in important metabolic functions and a detailed molecular analysis of signals regulating their expression is essential for constructing starter strains with controlable and desired properties. This thesis describes the molecular characterization of genes involved in lactose metabolism in *Leuconostoc lactis* and the genetic determinants involved in citrate metabolism in both *Leuconostoc lactis* and *Lactococcus lactis* subsp. *lactis* var. *diacetylactis*.

In **Chapter I** a brief overview is presented of the physiology and genetics of lactose metabolism in *Lactococci* and physiological aspects of the citrate metabolism in *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* and *Leuconostoc* spp.. Furthermore, several other general aspects relating to lactic acid bacterial genetics and physiology are summarized, providing background information for the following chapters.

Chapter II describes the development of a transformation system for *Leuconostoc* spp. which is a prerequisite for further genetic studies in this organism. It is shown that

electroporation can be used as an efficient method for genetic transformation with commonly used broad host range vectors. An optimized electroporation procedure was developed for *Leuconostoc lactis* NZ6009, resulting in transformation effieciencies of up to 4 x 10^3 transformants per μg of plasmid DNA. The use of the developed host-vector system was demonstrated by cloning and expression of the *Lactococcus lactis lacG* gene encoding the phospho-ß-galactosidase in *Leuconostoc lactis* NZ6009. Additionally, the efficiency of heterologous gene expression was demonstrated by transforming *Leuconostoc lactis* NZ6091, a lactose-deficient derivative of strain NZ6009, with a plasmid, pNZ36, carrying a *Lactococcus lactis-Escherichia coli lacZ* gene fusion, which subsequently synthesized high levels of ß-galactosidase. However, the resulting transformants were not complemented for growth on lactose, suggesting the absence of a functional transport system for lactose in the host strain. In *Leuconostoc lactis* NZ6091 the lactose deficiency was correlated with the absence of the endogenous plasmid pNZ63, and additionally to the inability of NZ6091 to grow on galactose, suggesting the involvement of pNZ63 in lactose and galactose metabolism in *Leuconostoc lactis* (see below).

Chapter III describes the reclassification of *Leuconostoc* strains. Those had previously been classified as *Leuconostoc paramesenteroides* based on phenotypic characteristics such as fermentation patterns. However, this method proved to be relatively undependable. A very reliable way to distinguish between bacterial species and subspecies is offered by using hybridization of specific DNA-probes to PCR-amplified, highly variable regions of 16S rRNA genes. Application of this method unambiguously showed that the two *Leuconostoc* strains used in this thesis should be classified as *Leuconostoc lactis*.

Chapters IV and V describe the cloning and expression in *E.coli* of the two important lactose genes of *Leuconostoc lactis* NZ6009, the ß-galactosidase and lactose permease genes. Both properties are located on the same plasmid, pNZ63, in *Leuconostoc lactis* NZ6009, as was apparent from studies described in **Chapter II**.

Chapter IV describes the cloning, localization, and expression of the ß-galactosidase gene in a lactose-deficient *E.coli* strain. Nucleotide sequence analysis of the DNA region, that showed complementation of a Lac$^-$ *E.coli* strain, revealed two partially overlapping genes, *lacL* and *lacM*, that could encode proteins of calculated molecular weights of 72,113 and 35,389. Those calculated sizes coincided well with those of protein bands of 75 kDa and 35 kDa, detected using SDS-PAGE after overexpression of the *lacLM* genes using the lambda

P_L promoter. The N-terminal sequences of the overexpressed proteins were determined and they corresponded to those of the deduced LacL and LacM protein sequences. Mutation and deletion analysis was used to demonstrate that the *lacL* and *lacM* genes are translationally coupled and that they are both required for the production of a functional ß-galactosidase in *E.coli*. Considerable amino acid sequence homology of the deduced sequences of LacL and LacM was found with the N- and C-terminal parts, respectively, of the *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, *Clostridium acetobutylicum* and *E.coli* ß-galactosidases which could implicate evolution of the ß-galactosidase genes from a common ancestral gene. Alignments of both DNA and amino acid sequences suggest that a deletion has occurred in the *Leuconostoc lactis* ß-galactosidase gene, which has generated the two genes, *lacL* and *lacM*. The same molecular organization and almost identical DNA sequence is observed for the plasmid encoded ß-galactosidase of *Lactobacillus casei* (2), suggesting horizontal gene transfer of these lactose genes between *Leuconostoc* and *Lactobacillus* species.

Chapter V describes the characterization of the *Leuconostoc lactis lacP* gene encoding lactose transport, that complemented the *lacY* mutation of different *E.coli* strains. Analysis of subclones and deletion mutants in *E.coli* allowed the localization of the *lacP* gene on a 2.9-kb *Sal*I-*Eag*I fragment. Functional expression of lactose transport activity was monitored on lactose-MacConkey agar plates, where Lac$^-$ *E.coli* strains, harboring *lacP* expressing plasmids, formed red colonies. Additionally, the ability of these transformants to transport lactose was determined by measuring the uptake of ^{14}C-lactose and the non-metabolizable lactose-analog, ^{14}C-methyl-ß-D-thiogalactopyranoside (TMG), in whole cells. In a T7-expression system the cloned fragment expressed a protein with an apparent M_r of 26,000, the presence of which correlated with the ability of the cells to transport lactose. Nucleotide sequence analysis showed that *lacP* comprised 654 base pairs which could encode a protein of 218 amino acids with a calculated molecular weight of 23,957. Homology searches showed no homology of LacP to other known lactose permeases, but revealed significant similarity to the permease components of binding-protein dependent amino acid transport systems in *E.coli* (GlnP, 72% homology) and in *Salmonella typhimurium* (HisQ and HisM, 66 and 65% homology respectively).

Although both genes, coding for proteins necessary for lactose metabolism, the ß-galactosidase and the lactose permease, are located on the same plasmid in *Leuconostoc lactis*

NZ6009, their genetic organization is different from other known lactose genes (Fig. 1; 7,8,10). This different organization of the lactose genes in *Leuconostoc lactis* compared to *lac* genes in other bacteria, suggests a cassette-like evolution of the elements of the lactose metabolism in *Leuconostoc lactis*, in which the genes necessary for lactose metabolism were assembled from different sources.

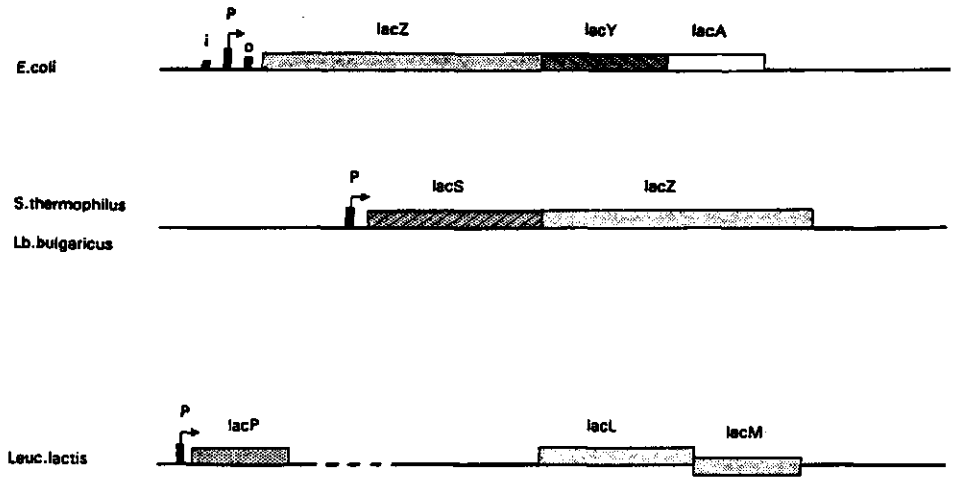

FIG. 1. Schematic representation of the organization of the lactose genes of *Escherichia coli* (E.coli), *Streptococcus thermophilus* (S.thermophilus), *Lactobacillus bulgaricus* (Lb.bulgaricus) and *Leuconostoc lactis* (Leuc.lactis). P: promoter; o: operator; i: inducer of the *E.coli lac* operon. The shading of the boxes indicates protein similarities and related functions; ß-galactosidase; , , lactose permease.

Previous observations had indicated that in *Leuconostoc lactis* NZ6091 the loss of pNZ63 was not only correlated with the loss of lactose fermenting ability, but also to the ability to utilize galactose. In many organisms the genes for galactose metabolism, galactokinase (*galK*), UDPglucose 4-epimerase (*galE*) and UDPglucose-hexose-1-phosphate uridylyltransferase (*galT*) are present in a single operon with the gene order *galETK* as shown for *E.coli* (1), *Salmonella typhimurium* and *S.typhi* (4). Recently, genes involved in galactose metabolism, *galM* and *galE*, coding for mutarotase and UDPglucose-4-epimerase, respectively were identified immediately upstream of the lactose operon in *Streptococcus thermophilus* (9). From our sequencing data of the upstream and downstream regions of *lacP* and *lacLM* it was already evident that these immediately flanking regions showed no

homology to any of the known *gal* genes. In order to determine whether other fragments of pNZ63 encoded genes involved in galactose metabolism, we looked for complementation of *E.coli* strains that lacked elements of the galactose metabolism. For this purpose *E.coli* strains MC1061 (*galK*), CGSC4467 (*galT*) and CGSC4498 (*galE*) (CGSC4467 and CGSC4498 were kindly provided by E.Vaugham, University College, Cork) were transformed with plasmids used for the cloning of the lactose genes, containing fragments of the *Leuconostoc* lactose plasmid pNZ63 (**Chapter IV and V**). The plasmids that were used for transformations are pNZ601, pNZ624 and pNZ635. Galactose fermentation of transformants was monitored on MacConkey agar plates containing galactose as sole carbon source. A red phenotype of the colonies, indicating complementation of galactose fermenting ability was only visible in *E.coli* MC1061 (*galK*) harboring pNZ601. This result suggests that a galactokinase is encoded by pNZ63 in a 3.5-kb region upstream of *lacP*, since neither pNZ624 nor pNZ635 could complement the *galK* mutation of *E.coli* MC1061. Colonies of *E.coli* CGSC4467 transformed with either pNZ601 or pNZ635 were pink after overnight culturing, turning red only after longer incubation times (> 36 hours). The longer incubation times necessary for a red phenotype of the colonies could reflect a lower level of expression of the *galT* gene. However, control cells harboring only the vector, which formed white colonies after overnight incubations, also turned red after prolonged incubation times, rendering the interpretation of the *galT* complementation was rendered less reliable. Transformants of *E.coli* CGSC4498 (*galE*) never showed a red phenotype on galactose MacConkey plates.

The results of the complementation analysis suggest that elements of the galactose metabolism are encoded by pNZ63. However, further experiments are needed to exactly localize the galactokinase gene on pNZ63. The constructs that were tested in these experiments do not contain all fragments of plasmid pNZ63. Therefore, cloning of the missing fragments and complementation analysis in other Gal$^-$ *E.coli* strains may allow the identification of other elements of the galactose metabolism in *Leuconostoc lactis*.

Chapters VI and VII describe the first examples of elucidating of the genetics of the citrate metabolism by analyzing the genetic determinants for citrate transport in *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* NCDO176 and *Leuconostoc lactis*. The studies were started in *Lactococcus lactis* NCDO176 (described in **Chapter VI**), since it was known that the citrate permease was plasmid-encoded in this organism, which therefore facilitated the

cloning of the gene. The citrate-deficient *E.coli* strain DH1 was transformed with cloned plasmid fragments and constructs were selected by their ability to complement the Cit⁻ phenotype on Simmons-citrate agar plates. Deletion analysis allowed the localization of the citrate transport gene (*citP*) on a 3.4-kb region. Transformants that showed complementation on Simmons-citrate agar plates were also able to transport ^{14}C-citrate. Cloning of a fragment containing the *citP* gene using a T7 expression vector (resulting in pNZ67) in *E.coli* BL21 resulted in the production of a 32 kDa protein band. Introduction of a site-specific mutation in pNZ67, resulting in pNZ67ΔB, which abolished citrate transport, showed a truncated form of the 32 kDa expression product. The nucleotide sequence of the *citP* gene was determined and its deduced 442 amino acids showed a high hydrophobicity, characteristic of an integral membrane protein. No homology of the deduced CitP sequence was found to other citrate carriers known at that time. However, the sequence of a Na^+-dependent *Klebsiella pneumoniae* citrate permease (11) shows significant similarities (**Chapter VII**; Fig.4), which may imply that these carriers have a similar mode of citrate transport. Membrane vesicles prepared from *E.coli* cells harboring pNZ67 showed energy-dependent ^{14}C-citrate transport. Preliminary studies were performed to establish the pH dependence of the ^{14}C-citrate transport in vesicles of transformed *E.coli* cells. Citrate uptake was measured at pH 5.5, 6.0, 6.5 and 7.0 and showed that the citrate transport is most efficient at low pH (pH 5.5) (1.2 nmol uptake per mg protein) and with hardly any uptake occurring at pH 7.0 (0.02 nmol/mg protein). This correlates well with earlier observations *in vivo*, that citrate fermentation occurrs most efficiently at low pH (3,5). The effect of the pH on citrate transport could be caused by a variation of the magnitude of the ΔpH, which has a higher value at pH 5.5 than at pH 7.0. A second possibility is that the effect of the pH on citrate transport reflects the availability of the citrate species, which can occur in various protonated forms, ranging from the fully protonated form at pH 2.0 to $citrate^{3-}$ that is predominantly present at at pH 8.0. Obviously, more detailed studies are required to determine the nature of the transported species of citrate and nature of the symported proton by the *citP*.

The lactococcal *citP* gene was subsequently used as a probe in hybridization studies with plasmid DNA of a *Leuconostoc lactis* strain (**Chapter VII**). This analysis showed strong hybridization with restriction fragments derived from one of the plasmids which was lacking in a Cit⁻ derivative strain. Two overlapping hybridizing fragments were cloned in *E.coli* and the large amount of homology with the lactococcal *citP* gene allowed us to sequence the

putative *Leuconostoc citP* gene using oligonucleotide primers based on the lactococcal *citP* nucleotide sequence. The comparison of the lactococcal and the *Leuconostoc citP* nucleotide sequences revealed only 13 single base substitutions in the coding region of *citP*, resulting in 3 amino acid substitutions, and two small deletions of 7 and 3 amino acids. A significant degree of sequence homology was also found in the *citP* flanking regions, including a putative ribosome binding site and terminator of transcription. This high degree of identity suggests a recent horizontal transfer of the citrate plasmids between those bacterial species. The presence of an *iso*-ISS*1* element on the citrate plasmid of *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* (6) suggest that acquisition of those genes may have occurred by transposition.

The studies of the here described location and organization of the genes involved in important metabolic traits, such as lactose and citrate metabolism in mesophilic lactic acid bacteria, suggest that their acquisition has taken place only recently in evolution. Additionally, these studies have demonstrated the versatility of this group of lactic acid bacteria with respect to their potential to exchange genetic material, which contributes to the genetical variability. Further analysis of the plasmids encoding the *Leuconostoc citP* and *lac* genes may provide insight in the mechanism of the dissemination of those genes to this bacterial species.

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SAMENVATTING

De mesofiele melkzuurbacterien behorende tot de genera *Lactococcus* en *Leuconostoc*, zijn belangrijke bestanddelen van starter kultures, die bij de bereiding van kaas, boter en karnemelk gebruikt worden. De belangrijkste functie van deze bacterien is de fermentatieve omzetting van lactose (melksuiker) in melkzuur. Daarnaast is het gebruik van *Lactococcus lactis* susp. *lactis* var. *diacetylactis* en *Leuconostoc* spp. in starter kultures gebaseerd op hun vermogen om CO_2 te produceren, dat belangrijk is bij de vorming van zgn. ogen in de kaas. Voorts zijn zij van belang door hun vermogen de aroma component diacetyl te vormen tijdens de omzetting van citroenzuur, dat in de melk aanwezig is. Ondanks het feit, dat het biotechnologische belang van het citroenzuur metabolisme al geruime tijd erkend wordt, is nog weinig bekend van de genetische basis van dit metabolisme. Bovendien zijn geen van de *Leuconostoc* spp. op genetisch niveau bestudeerd. Een betere kennis van de genetica van belangrijke metabole functies en een gedetaileerde moleculaire bestudering van signalen die de expressie reguleren, is essentieel voor de ontwikkeling van starter kultures, die controleerbare en gewenste eigenschappen bezitten. In dit proefschrift wordt een moleculaire karakterisering gegeven van de genen, die betrokken zijn bij het lactose metabolisme in *Leuconostoc lactis*, en de omzetting van citroenzuur in zowel *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* als *Leuconostoc lactis*.

In **Hoofstuk I** wordt een kort overzicht gepresenteerd van de fysiologie en de genetica van het lactose metabolisme in *Lactococcen* en de fysiologische aspecten van het citroenzuur metabolisme in *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* en *Leuconostoc* spp. Verder worden verschillende andere aspecten, die samenhangen met de genetica en fysiologie van melkzuurbacterien samengevat, om achtergrond informatie te geven voor de volgende hoofdstukken.

Hoodstuk II beschrijft de ontwikkeling van een transformatie systeem voor *Leuconostoc* spp., hetgeen een voorwaarde is voor verder genetisch onderzoek in dit organisme. Uit deze proeven blijkt, dat electroporatie gebruikt kan worden als een efficiente methode om *Leuconostoc lactis* NZ6009 genetisch te transformeren met algemeen gebruikte vectoren, die een breed gastheer bereik bezitten. Er is een geoptimaliseerd electroporatie protocol

ontwikkeld voor *Leuconostoc lactis* NZ6009. Het nut van het ontwikkelde gastheer-vector systeem kon aangetoond worden door de klonering en expresie in *Leuconostoc lactis* van het *Lactococcus lactis lacG* gen, dat voor fosfo-ß-galactosidase kodeert. Bovendien kon de efficientie van de heterologe genexpressie aangetoond worden door een lactose-deficiente stam van *Leuconostoc lactis* NZ6009 te transformeren met een plasmide, dat een *Lactococcus lactis-Escherichia coli lacZ* genfusie bevat en dat, na transformatie in de lactose-negatieve stam NZ6091, aanleiding gaf tot de vorming van grote hoeveelheden ß-galactosidase. Ondanks de functionele expressie van het *E.coli* ß-galactosidase in *Leuconostoc lactis* NZ6091 kunnen transformanten nog steeds niet groeien op lactose. Dit betekent, dat op het plasmide, pNZ63, dat in *Leuconostoc lactis* NZ6091 ontbreekt ten opzichte van de oorspronkelijke stam NZ6009, nog andere determinanten gelegen moeten zijn, die betrokken zijn bij het lactose metabolisme. Hierop wordt in latere hoofdstukken teruggekomen.

Hoofdstuk III beschrijft de herindeling van *Leuconostoc* stammen. Deze waren eerder geklassificieerd als *Leuconostoc paramesenteroides*, gebaseerd op fenotypische kenmerken, zoals fermentatie patronen. Deze indelingsmethode blijkt echter vaak niet eenduidige en wisselende resultaten te geven. Een zeer betrouwbare manier om een onderscheid te kunnen maken tussen bacteriele species en subspecies is mogelijk door gebruik te maken van de hybridisatie van specifieke DNA probes met PCR-geamplificeerde variabele gebieden van 16S rRNA genen. Toepassing van deze methode liet eenduidig zien, dat de twee *Leuconostoc* stammen, die in dit proefschrift gebruikt worden, *Leuconostoc lactis* zijn.

In **Hoofdstukken IV en V** wordt de klonering en expressie in *E.coli* beschreven van twee belangrijke lactose genen van *Leuconostoc lactis*, de genen coderend voor ß-galactosidase (lactose omzetting) en voor lactose opname (lactose permease). Beide eigenschappen zijn gelegen op het lactose plasmide, pNZ63, van *Leuconostoc lactis* NZ6009, hetgeen was gebleken uit studies beschreven in **Hoofdstuk II.**

Hoofdstuk IV beschrijft de klonering, lokalisering en expressie van het ß-galactosidase gen in een lactose-deficiente *E.coli* stam. De nucleotide sequentie van het DNA gebied, dat komplementatie gaf in een Lac$^-$ *E.coli* stam liet twee overlappende genen zien, *lacL* en *lacM*, die beide noodzakelijk zijn voor de productie van een functioneel ß-galactosidase in *E.coli*. Verdere genetische studies toonden aan dat de translatie van het *lacM* gen gekoppeld is aan die van het *lacL* gen. Tussen de afgeleide aminozuur sequenties van LacL en LacM en de respectievelijk N- en C-terminale gedeeltes van een aantal andere beschreven ß-

galactosidases werd een aanzienlijke aminozuur homologie gevonden. Deze gelijkenis zou, evolutionair gezien, op een gemeenschappelijke voorouder kunnen wijzen. Uit een dergelijke vergelijking blijkt eveneens dat in het *Leuconostoc* ß-galactosidase een deletie opgetreden is, ten opzichte van de andere ß-galactosidase genen, die vermoedelijk verantwoordelijk is voor de splitsing in *lacL* en *lacM*.

Het *lacP* gen uit *Leuconostoc lactis* NZ6009, koderend voor het lactose transport eiwit, kon gekloneerd worden door komplementatie van een*lacY E.coli* stam, die het lactose permease mist (dit wordt beschreven in **Hoofdstuk V**). De complementatie kon zowel op indicator platen als met behulp van opname van ^{14}C-gelabeld lactose en een niet- metaboliseerbaar analoog ^{14}C-TMG gemeten worden. Door middel van constructie van deletie plasmiden kon vervolgens een fragment van pNZ63 aangewezen worden, dat voldoende was om lactose opname in de *lacY E.coli* stam te geven. Nucleotide sequentie bepaling van *lacP* en vergelijking van de afgeleide aminozuur sequentie liet geen gelijkenis zien tussen LacP en andere lactose transport eiwitten. Wel kon er homologie aangetoond worden met de permeases van bindingseiwit-afhankelijke transport systemem voor aminozuren in *E.coli* (GlnP) en *Salmonella typhimurium* (HisQ en HisM).

Ondanks het feit, dat beide eiwitten, die nodig zijn voor lactose metabolisme op het zelfde plasmide gelegen zijn in *Leuconostoc lactis*, liggen zij niet vlak bij elkaar. Daardoor verschilt de moleculaire organisatie van de *Leuconostoc* lactose genen van die beschreven voor andere *lac* genen, die vlak bij elkaar in een operon gelegen zijn. Dit verschil suggereert, dat *Leuconostoc* de genen voor het lactose metabolisme wellicht vanuit verschillende bronnen verzameld heeft.

In **Hoofdstukken VI en VII** wordt de eerste aanzet beschreven om de genetica van het citroenzuur metabolisme op te helderen in *Lactococcus* spp. en *Leuconostoc* spp. Hiervoor is in eerste instantie het *citP* gen, de genetische determinant voor het citroenzuur transport van *Lactococcus lactis subsp.* lactis var. *diacetylactis* NCDO176, gekloneerd in *E.coli* en vervolgens functioneel tot expressie gebracht en geanalyseerd. De klonering werd vergemakkelijkt, omdat bekend was, dat het *citP* gen op een plasmide gelegen was in *Lactococcus lactis* stam NCDO176. Het fragment van het citraat plasmide dat codeerde voor *citP* kon, middels komplementatie analyse in een Cit$^-$ *E.coli* stam, geidentificeerd worden. Het transport van citroenzuur kon in *E.coli* cellen, die het gekloneerde *citP* gen op een plasmide bevatten, gemeten worden met ^{14}C-gemerkt citroenzuur. Bovendien kon in een T7

expressie systeem overproductie van het 32 kDa citroenzuur transport eiwit aangetoond worden. Door opname te meten van ^{14}C-gelabeld citroenzuur in membraan vesicles van *E.coli* cellen, die het komplementeernde plasmide bevatten, kon aangetoond worden, dat citroenzuur transport afhankelijk is van de proton motive force. Van de nucleotide sequentie van *citP* kon een kodeernde sequentie afgeleid worden voor een eiwit van 442 aminozuren , dat gezien zijn hydrofobiciteit waarschijnlijk geassocieerd is met de membraan.

Het *citP* gen van *Lactococcus lactis* NCDO176 is vervolgens gebruikt als probe in hybridisatie studies met plasmide DNA van *Leuconostoc lactis* stam NZ6070. Deze proeven lieten sterke hybridisatie zien met restriktie fragmenten afkomstig van een plasmide, dat in een Cit$^-$ stam miste. Twee overlappende hybridiserende fragmenten werden gekloneerd in *E.coli*, en de grote mate van homologie met het NCDO176 *citP* maakte het mogelijk om met behulp van de daarvan afgeleide oligonucleotide primers het gehele vermoedelijke *Leuconostoc citP* gen te sequencen. De vergelijking van de nucleotide sequentie van de twee *citP* genen liet slechts enkele (13) base substituties zien in de koderende sequentie. Een significante mate van homologie werd eveneens gevonden in de flankerende gebieden, die een mogelijke ribosoom bindingsplaats en een terminator van transcriptie bevatten. Deze grote mate van gelijkenis wijst op een zeer recente uitwisseling van de genetische determinant tussen deze twee bacteriele species.

De afgeleide aminozuur sequenties van het *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* en het *Leuconostoc lactis citP* vertoonden geen homologie met bekende sequenties. Recent bleek dat het melkzuurbacterie *citP* homoloog is met een Na-afhankelijke citroenzuur transport eiwit van *Klebsiella pneumoniae*. Dit suggereert dat ook het melkzuurbacterie citroenzuur transport Na-afhankelijk kan zijn.

In **Hoofdstuk VIII** wordt een samenvatting gegeven van de gevonden onderzoeksresultaten en worden enkele aanvullende gegevens besproken.

CURRICULUM

De schrijfster van dit proefschrift werd geboren op 10 september 1959 te Kassel (Duitsland). In 1978 werd het eindexamen diploma van het Gymnasium behaald aan de Deutsche Schule te Den Haag. In datzelfde jaar werd begonnen met de studie Biologie aan de Vrije Universiteit te Amsterdam. Het Kandidaats-examen B5' (medische Biologie) werd behaald op 17 november 1982. De studie werd voortgezet met als doctoraalvakken Moleculaire Microbiologie (prof. dr. F.K. de Graaf), gedeeltelijk bewerkt bij het Rijksinstituut voor Volksgezondheid en Milieuhygiene (RIVM) te Bilthoven onder begeleiding van dr. F.R.Mooi. Daarnaast werden twee bijvakken bewerkt, respectievelijk Experimentele Immunologie (prof. dr. T.Sminia) aan de Medische Faculteit van de Vrije Universiteit en Virologie (dr. R. Nusse) aan het Nederlands Kankerinstituut (AVL) te Amsterdam. Het doctoraal examen werd op 20 augustus 1986 behaald.

Van december 1986 tot december 1990 was zij als wetenschappelijk medewerkster, in tijdelijke dienst verbonden aan het Nederlands Instituut voor Zuivelonderzoek (NIZO) te Ede, bij de werkgroep Moleculaire Genetica. Gedurende deze periode werd het hier beschreven onderzoek verricht onder begeleiding van prof. dr. W.M. de Vos.

Sinds juni 1991 is zij als wetenschappelijk medewerkster in tijdelijke dienst verbonden aan het E.C. Slater Instituut bij de werkgroep energetica en regulatie van metabolisme, onder leiding van Dr. P. Postma, bij de vakgroep Biochemie van de Universiteit van Amsterdam.

LIJST VAN PUBLICATIES

Artikels

* **David, S., G. Simons, and W. M. de Vos.** 1989. Plasmid transformation by electroporation of *Leuconostoc paramesenteroides* and its use in molecular cloning. Appl. Environ. Microbiol. 55:1483-1489.
* **De Vos, W. M., P. Vos, G. Simons and S. David.** 1989. Gene organization and expression in mesophilic lactic acid bacteria. J. Dairy Sci. 72:3398-3405.
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* **David, S., H. Stevens, M. v. Riel, G. Simons, and W. M. de Vos.** 1991. *Leuconostoc lactis* ß-galactosidase is encoded by two translationally coupled genes. J. Bacteriol. submitted.
* **David, S., and W. M. de Vos.** 1991. Characterization of the *lacP* gene encoding the novel lactose carrier of *Leuconostoc lactis* NZ6009. J. Bacteriol. submitted.

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* **David, S. and W.M. de Vos.** 1987. Genetic characterization of *Leuconostoc* ssp. from dutch starter culture, FEMS Microbiol. Rev. 46:P-26, A44.
* **David, S., G. Simons, and W. M. de Vos.** 1989. Cloning and expression of the *Leuconostoc paramesenteroides* ß-galactosidase gene in *E.coli*. Proc. 46th Ann. Meeting of the Soc. for Ind. Microbiol. Seattle. G. A. Somkuti and S. A. Orndorff (eds.) P-61,p.92.
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NAWOORD

Net terug van vacantie en meteen weer in de stress van: het moet nu snel naar de drukker! - Planning is soms moeilijk! Maar met vers gewonnen energie kan ik het laatste stukje text van dit boekje schrijven. Ongetwijfeld is dit het meest gelezen gedeelte van elk proefschrift, en terecht, want vanaf deze laatste bladzijden wil ik iedereen bedanken, die direct of indirect, met of zonder medeweten bijgedragen heeft aan het tot stand komen van dit boekje. Het zijn teveel mensen om allemaal met naam genoemd te worden. Toch zijn er enkelen, die ik vanuit deze plek nog eens extra wil bedanken.

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