CHARACTERIZATION OF THE *LACTOCOCCUS LACTIS* LACTOSE GENES AND REGULATION OF THEIR EXPRESSION

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Proefschrift
ter verkrijging van de graad van
doctor in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
dr. H. C. van der Plas,
in het openbaar te verdedigen
op woensdag 19 mei 1993
des namiddags te 16.00 uur in de Aula
van de Landbouwuniversiteit Wageningen.

Was ist denn Tat? was ist Nichttun?-
Das ists, was Weise oft verwirrt.
Denn achten muss man auf die Tat,
achten auf unerlaubtes Tun.
Muss achten auf das Nichttun auch-
der Tat Wesen ist abgrundtief.
Bhagavadgita, vierter Gesang

Stefan Zweig, "Die Augen des ewigen Bruders- Eine Legende"

Voor Willy en Robert
Voor mijn ouders

STELLINGEN

1. Om de netto speeltijd van een tenniswedstrijd te doen laten toenemen verdient het aanbeveling om de afmetingen van het servicevak te correleren aan de stijging van de gemiddelde lengte van de mens.

2. Het naleven van de maximumsnelheid binnen de bebouwde kom kan worden afgedwongen door de aanleg van meer en hogere verkeersdrempels, waarbij de onderlinge afstand is aangepast aan de gemiddelde acceleratie van een auto.

3. De "politieke" bijdrage aan een wetenschappelijk onderzoek wordt vaak overgewaardeerd in de vorm van een co-auteurschap.

4. Op basis van combinatie van de gegevens van de *hutR* mutant en de *hutP-lacZ* studies kan het aantal mogelijke regulatie mechanismen voor het *B.subtilis hut* operon beperkt worden tot twee.

   Chasin and Magasanik (1968) *J. Biol. Chem.* **243,** 5165-5178.
   Oda *et al.* (1992) *Mol. Microbiol.* **6,** 2573-2582.

5. Het nalaten van een goede statistische analyse bij het vergelijken van DNA sequenties bevordert 'wishful thinking'.

6. De handhaving van de DNA bindingsactiviteit van de TetR en LacI repressors na substitutie van het evolutionair sterk geconserveerde glycine residue in de turn van het α-helix-turn-α-helix motief met een (bijna) willekeurig ander aminozuur is een illustratie van de relatieve waarde van homologiestudies.

   Baumeister *et al.* (1992) *Proteins: Structure, Function, and Genetics* **14,** 168-177.
   Kleina and Miller (1990) *J. Mol. Biol.* **212,** 295-318.

7. Bij de optimalisatie van een produktieproces wordt door genetici het belang van het genotype en door procestechnologen het belang van het fenotype vaak overgewaardeerd.

9. De absolute geleverde prestatie bij het bereiken van de top in een bepaalde tak van sport is recht evenredig met het aantal serieuze beoefenaren.

10. De smaakvorming in kaas is hooguit indirect gecorreleerd aan het vrije aminozuurgehalte.

11. Het relatief grote aantal linkshandige topspelers bij racketsporten suggereert dat een relatief hoog percentage van de aanleg voor deze sporten is gelokaliseerd in de rechterhersenhelft.

Stellingen behorende bij het proefschrift:
Characterization of the Lactococcus lactis lactose genes and regulation of their expression

Rutger van Rooijen, Wageningen 19 mei 1993

VOORWOORD

Vanaf de start van je promotie tot aan de uiteindelijke afronding van het proefschrift zijn er een groot aantal mensen die op wat-voor-manier-dan-ook een steentje of steen hebben bijgedragen aan het uiteindelijke resultaat. Een zeer grote steen werd bijgedragen door mijn begeleider/promotor Prof. dr. W.M. de Vos. Beste Willem, bedankt voor je enthousiasme en stimulerende begeleiding. Je energie en werklust zijn voor mij steeds een voorbeeld geweest. Speciaal wil ik de stagiaires vermelden, in chronologische volgorde: Saskia van Schalkwijk, Wendy Dam, Theo Willems, Niek Wilmink en Koen Dechering, die ieder op hun eigen unieke wijze een bijdrage aan het onderzoek hebben geleverd. Verder waren daar natuurlijk de mede-promovendi, Martien van Asseldonk, Ronald Baankreis, Silke David, Nicolette Kleijn, Christ Platteeuw en Peter Rauch. We zaten allemaal in hetzelfde schuitje: bedankt voor jullie gezelschap (met name in de weekends en s'avonds), tips, en gezelligheid. De post-docs, Paul Bruinenberg, Oscar Kuipers, Jan Roelof van der Meer, John Mulders, Guus Simons en Pieter Vos bedank ik voor de inbreng van hun wetenschappelijke ervaring. Als ik eens wat nieuws wilde proberen, een idee had, of als experimenten technisch mislukten was er altijd wel een van jullie bij wie ik terecht kon voor suggesties of 'brainstorming'. Roland Siezen wil ik speciaal bedanken voor zijn immer kritische blik in het laatste stadium van een publicatie, waardoor de manuscripten in leesbaarheid toenamen. De technische (ex-)medewerkers van de Moleculaire Genetica groep Ingrid van Alen, Marke Beerthuyzen, Paul Doesburg, Miranda Hornes, Monique Nijhuis en Ger Rutten wil ik bedanken voor hun gezelligheid en de uitstekende verzorging van de "infrastructuur" van het lab. Van de mensen van 'boven' wil ik speciaal Harry Rollema, Charles Slangen, Arno Alting en Peter van Rooijen ("Pa") bedanken voor hun assistentie bij de verschillende biochemische experimenten.

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Tenslotte bedank ik jou Willy, om bij mij af en toe eens flink op de rem te trappen en me van voldoende geestelijke frisheid te voorzien.

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CONTENTS

" Ik heb een speelse wereld aangetroffen waar verbeeldingskracht, bezetenheid en ideé fixes een belangrijke rol spelen. Tot mijn verrassing waren degenen die het onverwachte bereikten en het onmogelijke bedachten, niet gewoon maar geleerde en methodische mensen. Het waren vooral mensen met een oorspronkelijke geest die moeilijkheden niet uit de weg gingen en die er een buitenissige visie op na hielden. Degenen die een vooraanstaande plaats inamen spreidden vaak een vreemde mengeling van onverschilligheid en gedrevenheid, van starheid en grilligheid, van Streberei en argeloosheid ten toon"

François Jacob, "La statue intérieure"

CHAPTER 1

GENERAL INTRODUCTION

Lactic acid bacteria that are used worldwide in industrial dairy fermentations include the four main genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, and *Streptococcus*. The main objective of their use in these fermentations is the rapid production of lactic acid, which inhibits growth of spoilage and pathogenic bacteria. In addition, strains that are used as a starter culture in these fermentations also determine the texture, flavor, and aroma of the resulting dairy products. The generation of the flavor and aroma of the fermented product is mediated by the degradation of milk caseins into small peptides and amino acids by the proteolytic system, that is present in some of the starter bacteria. Another important flavor component is diacetyl that is formed from citrate or lactose.

In the last decade many biochemical and genetic studies have been carried out concerning the various traits described above and additional properties, and have resulted in the molecular cloning and characterization of a large number of genes involved in e.g the utilization of sugar, citrate, and casein, phage resistance, and bacteriocin production (for reviews see David, 1992; De Vos, 1990; De Vos *et al.* 1992; Kok, 1990; Klaenhammer, 1988; Klaenhammer, 1987). However, although a considerable amount of promoters have been identified (for a review see van der Guchte *et al.*, 1992), only very limited data have emerged concerning the regulation of gene expression in lactic acid bacteria. The identification and characterization of homologous, strong, and controllable promoters is essential for the development of strains that have the ability to express homologous or heterologous genes of interest at a defined point in the fermentation. In order to identify such a promoter and to study gene regulation in lactic acid bacteria, control of expression of the genes involved in the lactose catabolism of *Lactococcus lactis* subsp. *lactis* was analyzed and is described in this thesis. In the following sections of this introduction some background information is given on lactose metabolism and gene regulation, both in model systems and lactic acid bacteria.

LACTOSE CATABOLISM IN LACTIC ACID BACTERIA

When lactic acid bacteria grow on milk, energy is provided by the conversion of lactose into lactate. The catabolic pathways involved in lactose uptake and degradation have been well established (Fig. 1). The conversion of lactose to lactate can either be homo- or heterofermentative. Whereas during homofermentative lactose degradation solely lactate is produced, heterofermentative degradation leads to the production of lactate, acetate, carbondioxide, and ethanol. In lactic acid bacteria two distinct systems for lactose uptake have been found: (i) the phospho*enol*pyruvate-dependent lactose phosphotransferase system (PEP-PTS^{lac}) and (ii) the lactose permease system (Fig. 1).

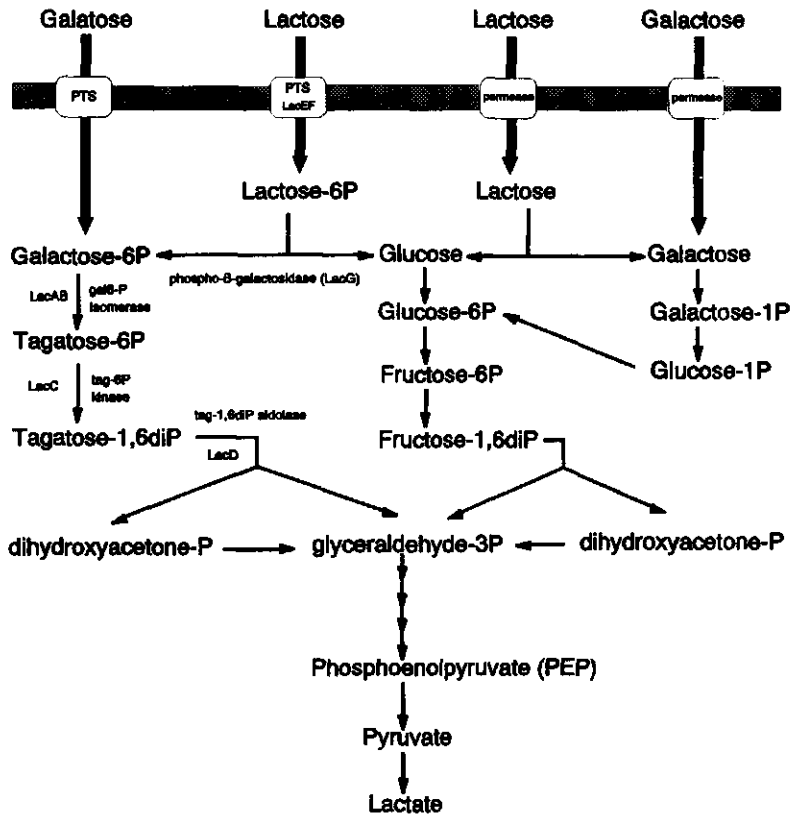

Fig. 1. Catabolic pathways of lactose and galactose that have been reported to operate in *Lactococci*. The enzymes involved in the breakdown of lactose via the phosphotransferase system are indicated. The tagatose-6-phosphate (Tag-6P), Embden-Meyerhof-Parnas (glycolysis), and LeLoir pathways are shown.

The phospho*enol*pyruvate-dependent lactose phosphotransferase system (PEP-PTS^{lac}). The PEP-PTS was first discovered by Kundig *et al.* (1964) in crude *E.coli* extracts as a glucose and mannose phosphorylating system with the following features: PEP was the phosphoryl donor, and three protein fractions were required for the overall reaction; these were designated Enzyme I (EI), HPr, and Enzyme II (EII). Four years later another soluble sugar-specific factor was found in extracts of *Staphylococcus aureus* designated Factor III (Simoni, 1968), now called Enzyme III (EIII). Enzyme I catalyzes the transfer of phosphate from PEP to a His residue in HPr, and EII/EIII catalyze the next step, the transfer to the sugar. In summary, there are two classes of PTS components: (i) the general PTS proteins HPr and EI, which are required for the phosphorylation of *all* PTS sugars, and (ii) the sugar-specific proteins EII and EIII. The properties, mechanisms and regulation of the sugar-PTS have been extensively studied

in the last decades both in gram-negative and Gram-positive bacteria (Meadow *et al.*, 1990; Saier, 1989; Roseman and Meadow, 1990).

In *L.lactis* strains used in industrial dairy fermentations (McKay, 1983) and some *Lactobacilli* (Chassy and Thompson, 1983) lactose is metabolized exclusively through the PEP-PTS^{lac}, that has only been found in gram-positive bacteria. In this system, Enzyme II^{lac} (LacE) and Enzyme III^{lac} (LacF) are the lactose-specific transport proteins. During transport lactose is phosphorylated to yield lactose-6-phosphate that is subsequently cleaved by phospho-ß-galactosidase into glucose and galactose-6-phosphate. Glucose is phosphorylated by the action of glucokinase to yield glucose-6-phosphate that is further converted into lactate in the glycolytic (Embden-Meyerhof-Parnas) pathway. The galactose-6-phosphate moiety is further metabolized in the tagatose-6-phosphate pathway into the glycolytic intermediates glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate by the enzymes galactose-6-phosphate isomerase, tagatose-6-phosphate kinase, and tagatose-1,6-diphosphate aldolase, respectively (Fig. 1). This pathway was first described in *Staphylococcus aureus* by Bisset and Anderson (1973). The *S.aureus* and *L.lactis* PEP-PTS^{lac} components appear to be strongly related, as was shown by McKay *et al.* (1970) who demonstrated that diluted cell-free extracts from *L.lactis* C2 could complement *S.aureus* HPr, EI, EII and EIII mutants. The genes encoding the tagatose-6-phosphate pathway, PEP-PTS^{lac}, and phospho-ß-galactosidase enzymes in *Lactococci* are located on conjugable plasmids (Crow *et al.* 1983; Gasson, 1990; Petzel and McKay, 1992). In *L.lactis* subsp. *lactis* strain NCDO 712, a 56.5 kb plasmid, pLP712, has been identified which harbors the lactose-PTS and proteinase genes (Gasson, 1983; Gasson, 1990). A 23.7-kb lactose mini-plasmid, pMG820, was constructed from pLP712 by transductional shortening and deletion of the proteinase genes (Maeda and Gasson, 1986). This plasmid was used as a starting point for the analysis of the *lac* genes. The first *lac* gene to be cloned was the *lacG* gene, encoding the phospho-ß-galactosidase enzyme (Maeda and Gasson, 1986). Since then, the nucleotide sequences of the *lacG* genes of *L.lactis* strains Z268 (Boizet *et al.*, 1988) and NCDO712 (De Vos and Gasson, 1989) have been reported. Also for *Lactobacillus casei* the nucleotide sequence of the plasmid-located *lacG* gene has been determined (Porter and Chassy, 1988). The deduced amino acid sequences of the *L.lactis* and *Lactobacillus casei* phospho-ß-galactosidases were found to be homologous to that of *S.aureus* and belong to the superfamily of ß-glycohydrolases (Henrissat, 1991; Hassouni *et al.*, 1992).

Regulation of the PEP-PTS^{lac}. Enzyme activities of the tagatose-6-phosphate pathway, PEP-PTS^{lac}, and phospho-ß-galactosidase enzymes in various *L.lactis* strains and *Lactobacillus casei* have been shown to be induced during growth on lactose or galactose (Bisset and Anderson, 1974; Le Blanc *et al.*, 1979; Molskness *et al.*, 1973; Chassy and Thompson, 1983). Growth diauxie was observed with cells of *L.lactis* 25Sp/R, that is a partial lactose-fermenting revertant of a *lac*$^-$ strain of *L.lactis* C2 (Cords and McKay,

1974), on media containing combinations of glucose and lactose (glucose/lactose) and glucose/galactose. Whereas in the wild-type strain C2 diauxie was observed on glucose/galactose, diauxie was not observed on glucose/lactose and is probably masked by the high basel levels of *lac* gene expression in glucose-grown cells in comparison with the apparent tight repression of the galactose transport system (Cord and McKay, 1974). From these results it can be concluded that (i) glucose, that is metabolized via the PEP-PTS^{man} (Thompson, 1978; Thompson and Chassy, 1985), is the preferred substrate of *L. lactis*, and (ii) repression of PEP-PTS^{lac} and phospho-ß-galactosidase activities during growth on glucose/lactose probably occurs through inducer exclusion. Mechanisms for the apparent hierarchical order of sugar utilization by the PEP-PTS have been proposed and will be briefly discussed. First of all, the affinities of the various sugar-specific EIIIs for P-HPr^{His} are different. For instance, exclusion of the lactose analog TMG in the presence of glucose has been associated with the preferential utilization of P-HPr^{His} by the PEP-PTS^{man} (Reizer and Peterkofsky, 1987). Secondly, phosphorylation of a serine residue in HPr by an ATP-dependent HPr^{Ser} kinase, that is stimulated by fructose-1,6-diphosphate (and other metabolites) and inhibited by inorganic phosphate, modulates the rate and order of sugar uptake (Deutscher and Saier, 1983; Deutscher *et al.*, 1984). The inactive P-HPr^{Ser}, that can no longer function as a substrate for phosphorylation by P-EI, can be reactivated by the action of a phosphoprotein phosphatase. Alternatively, a complex between P-HPr^{Ser} and various EIIIs can be formed resulting in the phosphorylation of P-HPr^{Ser} by EI to yield P-HPr^{Ser,His}, which functions as a phosphoryl donor for sugar transport (Deutscher *et al.*, 1985). Although low levels of cyclic AMP have been observed in lactic acid bacteria and other Gram positive bacteria (Ratliff and Stinson, 1980; Ratliff and Talburt, 1981), no second messenger system has been identified yet that, like the cAMP/CAP system in *E.coli* (De Crombrugghe, 1984; see below), gives a general control of expression of metabolic genes.

The lactose permease system. In strains of *Lactobacillus, Streptococcus* and *Leuconostoc*, lactose is taken up as a free sugar by the lactose permease. Subsequently, lactose is hydrolyzed by ß-galactosidase into glucose and galactose. Galactose is converted in the Leloir pathway into glucose-6-phosphate by the enzymes galactokinase, galacto-1-phosphate uridylyltransferase, uridine diphosphogalactose-4 epimerase, uridinediphosphoglucose synthase and phosphoglucomutase (Fig. 1; Adhya, 1987). Nucleotide sequences and characterization of genes from the Leloir pathway have been reported for *Lactobacillus helveticus* (*galK*, *galT* and *galM*, Mollet and Pilloud, 1991) and *Streptococcus thermophilus* (*galM* and *galE*, Poolman *et al.*, 1990). The Leloir pathway is blocked in *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, resulting in the stoichiometrical excretion of galactose into the medium via the lactose permease that acts as a lactose/galactose antiporter system (Poolman, 1990). The lactose permease

genes of *Streptococcus thermophilus* (Poolman *et al.*, 1989), and *Lactobacillus bulgaricus* (Leong-Morgenthaler *et al.*, 1991) have been cloned and sequenced and are organized in an operon-like structure together with the ß-galactosidase genes. Recently, a *Leuconostoc lactis* gene was cloned that could complement an *E.coli* LacY mutant. However, its deduced amino acid sequence did not show any similarity with those of the lactose permeases described above, but appeared to be strongly related to the membrane components of the *E.coli* GlnP and *Salmonella typhimurium* HisQ/HisM amino acid transport systems (David and De Vos, 1992). The nucleotide sequences of ß-galactosidase genes from several lactic acid bacteria have been reported and include those from *Streptococcus thermophilus* (Schroeder *et al.*, 1990), *Lactobacillus bulgaricus* (Schmidt *et al.*, 1989), *Lactobacillus casei* (Chassy, 1992) and *Leuconostoc lactis* (David *et al*, 1992) the latter two of which are encoded by two translationally coupled genes. The deduced amino acid sequences of the mentioned ß-galactosidase genes share a high degree of identity and, in addition, show also homology to those of *Clostridium acetobutylicum* and *E.coli* (David *et al.*, 1992).

REGULATION OF GENE EXPRESSION IN BACTERIA

In bacterial cells only a small portion of the available genetic information, consisting of approximately 2500-3000 genes, is expresssed at any given moment during its lifecycle. The fluctuations in the environment provide the cell with the signals that finally lead to the modulation of gene expression. Thereby, the cell responds to the extracellular signal and generates an intracellular signal (second messenger; for reviews see Roseman and Meadow, 1990; Botsford and Harman, 1992) that influences expression of its target gene(s). Some of the mechanisms involved in the translation of the second messenger to gene expression are summarized here. Gene expression can be regulated at the transcriptional (Fig. 2, sites 1 to 4) or translational level (Fig. 2, site 5) and include:

1. Transcription initiation by RNA polymerase modulated by the availability of RNA polymerase and appropriate sigma factor (for review see Losik and Perot, 1981).
2. Inhibition of transcription initiation by binding of repressor at operator. Discussed below.
3. Enhancement of transcription initiation by binding of activator. Discussed below.
4. Control of transcription elongation. Two main regulatory systems have been described: a) transcriptional attenuation and b) anti-termination (for reviews see Yanofsky and Crawford, 1987 and Reznikoff, 1984). Many biosynthetic operons (e.g. *trp*, *thr* and *leu*) are regulated by transcriptional attenuation (Gardner, 1979; Gemmill *et al.* 1979). A well studied anti-termination system is the *bglGFB* operon from *E.coli*, that is involved in the utilization of aromatic ß-glucosides (Houman *et al.*, 1990; Schnetz and Rak, 1990).
5. Control of translation initiation. The level of translation has been described to be mediated by a) a negatively acting protein (for examples see Yates and Nomura, 1981;

Zengel *et al.*, 1980 and Zaman *et al.*, 1990), b) antisense mRNA (for reviews see Inouye, 1988 and Simons, 1988) and c) translational attenuation (Lovett, 1990).

Since this thesis deals with repression and activation systems (Fig. 2, sites 2 and 3) these will be discussed in more detail below. In addition, initial experiments (Jacob and Monod, 1961) showed already that gene regulation mainly occurs at the level of transcription initiation, which seems plausible, since in this way no energy is wasted.

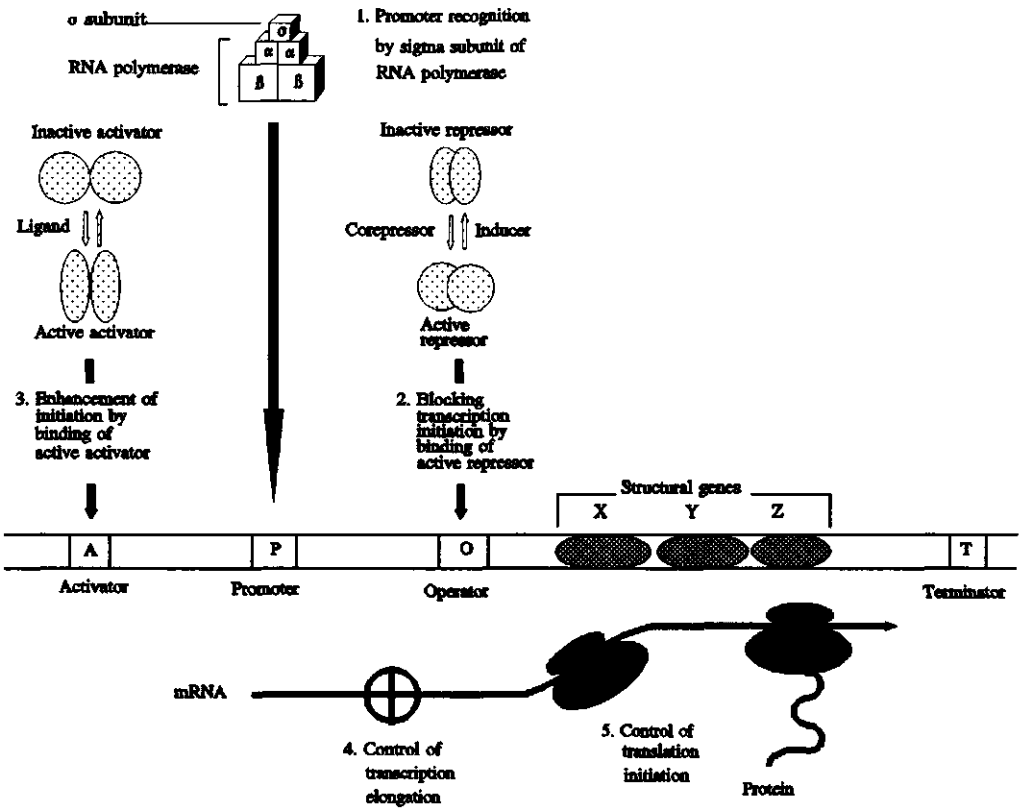

Fig. 2. The major sites of control of operon expression in bacteria. For details see text.

Regulation of transcription initiation.

The rate of transcription initiation has been reported to be controlled by positively and negatively acting regulatory proteins.

a. Negatively acting regulatory proteins (repressors). Repressors are called those protein regulators that bind to a DNA sequence (operator) and decrease the frequency of transcription initiation of the adjacent promoter (Reznikoff, 1984). The operators of repressors are found at a variety of locations relative to the promoters that they control. The the majority of repressor binding sites are located between -40 and +1 relative to the transcription initiation site (Collado-vides *et al.*, 1991). This seems plausible since the repressor has to compete for binding with RNA polymerase. To be physiologically useful, repressor activity must be capable of being modulated. Many repressors are allosteric proteins that in the absence and presence of an inducing molecule have a conformation that provides a high and low affinity for their operator(s), respectively (Fig. 2). Regulators might also be covalently modified. Members of the PhoB/OmpR response regulator superfamily, that includes repressors and activators (see below), are converted from an active to inactive form by reversible phosphorylation (Stock *et al.*, 1989). An even more drastic modification undergoes the *lexA* repressor that is irreversible proteolytically cleaved (Brent and Pthasne, 1981). A large number of DNA binding proteins (repressors and activators) show amino acid sequence homology in the domain that recognizes DNA (Brennan and Matthews, 1989; Harrison and Aggarwal, 1990). The DNA recognition of many DNA-binding proteins is mediated by a structural arrangement, termed the helix-turn-helix motif. Families of repressor proteins have been described that show homology in their amino acid sequences extending beyond the helix-turn-helix DNA-binding domains and include the LacI/GalR and DeoR family (Weickert and Adhya, 1992; Chapters 5 and 9, this thesis).

b. Positively acting regulatory proteins (activators). Positive regulation of transcription initiation involves the binding of an activator near the -35 region of the promoter. The majority of activators bind between -40 and -70 (Collado-vides *et al.*, 1991). Promoters that are regulated by activators have usually a very low basal level of activity. Activators increase the rate or extent of open complex formation, possibly by bending of the DNA molecule (Schultz *et al.*, 1991). The detailed molecular mechanisms concerning this activation have still to be elucidated. One of the best characterized activators is the cAMP-binding protein (CAP), a general activator, that is involved in e.g. *E.coli lacZYA* operon control (see below). Families of activator, that also contain repressors, have been described and include the LysR and the OmpR/PhoB family (Henikoff *et al.*, 1988; Stock *et al.*, 1989).

Control of lactose metabolism in *E.coli*.

The LacI repressor. Control of the *E.coli lacZYA* operon, encoding ß-galactosidase, lactose permease, and transacetylase, respectively, has been a paradigm for control of gene expression and was first described by Jacob and Monod (1961). The components involved in the regulation of the *lac* operon include the *lacI* repressor, the three *lac* operators, and the inducer (Reznikoff, 1992). During growth on glucose, in the absence of the natural inducer allolactose, that is a side-product of the cleavage of lactose by ß-galactosidase, the tetrameric LacI repressor (360 aa per subunit) binds to the *lac* operator, thereby inhibiting transcription initiation of the *lac* promoter. For almost two decades it was believed that LacI repressor acts by excluding the promoter from RNA polymerase (RNAP) binding. Recently it has been shown that inhibition is not due to direct steric hindrance of binding of RNAP, but that RNAP is engaged in a joint complex with LacI repressor at the *lac* promoter/operator. In this complex, RNAP cannot proceed with elongation but generates abortive RNA oligomers (Straney and Crothers, 1987; Lee and Goldfarb, 1991). In addition to the operator *lacO1*, that is located near the *lac* transcription initiation site and contains dyad symmetry, two LacI repressor binding sites (*lacO2* and *lacO3*) with different affinities have been identified, that are located 401 and 92 basepairs downstream and upstream of *lacO1*, respectively (Reznikoff *et al.*, 1974; Fried and Crothers, 1981). Deletion of either *lacO2* or *lacO3* resulted in a significant decrease of repression level. The three *lac* operators have been shown to cooperate in repression by the formation of a DNA loop mediated by the tetrameric LacI repressor (Oehler *et al.*, 1990; Chakerian and Matthews, 1992). Also for the *E.coli deo*, *gal*, and *ara* operons the involvement of DNA looping in transcriptional control has been reported (Matthews, 1992). The domains in the LacI repressor that are involved in DNA binding, inducer binding and multimerization have been identified (Chakerian and Matthews, 1992). Kaptein and coworkers (Kaptein *et al.*, 1985; Zuiderweg *et al.*, 1983) have resolved the solution structure of the first N-terminal 51 amino acids ("headpiece") in complex with the *lac* operator and proposed a folded secondary structure that is similar to other helix-turn-helix DNA binding proteins (Brennan and Matthews, 1989; Dodd and Egan, 1990). No X-ray structure of the entire LacI protein has yet been reported. Genetic studies with operator variants in combination with LacI repressor mutants correspond to the NMR structure and have established the contacts between the amino acid residues of the helix-turn-helix motif and operator base pairs (Sartorius *et al.*, 1990, 1991; Kleina and Miller, 1990). Based on the substantial amount of physical and genetic data obtained in the last decade, a model of the LacI repressor-operator complex was proposed by the Müller-Hill group (Kisters-Woike *et al.*, 1991).

Relatively little efforts have been made to elucidate the mechanism and nature of inducer response. In the presence of inducer, LacI repressor undergoes a conformational change that lowers its affinity for operator DNA without affecting non-specific DNA-binding properties (Lin and Riggs, 1975). A model for the sugar binding pocket of the

LacI repressor has been postulated (Sams *et al.*, 1984) based on homology with that of the arabinose-binding protein, of which the X-ray structure has been solved (Quiocho and Vyas, 1984). This model has been confirmed by genetic and biochemical studies (Kleina and Miller, 1990; Spotts *et al.*, 1991).

The catabolite gene activating protein (CAP). A central role in the control of catabolic activity in gram-negative bacteria is performed by cAMP. The intracellular concentration of cAMP is modulated primarily by the carbohydrate on which the cells grow, and is strongly decreased during growth on glucose (Botsford and Harman, 1992). When the intracellular level of cAMP increases, transcription initiation of a series of genes, including the *lac* operon, is activated by the binding of the catabolite gene activating protein (CAP) near the consensus promoter sequences. CAP only activates transcription initiation when complexed with cAMP. Genes that are activated in response to an increase in cAMP include those encoding the enzymes for the catabolism of lactose, maltose, arabinose, and other sugars (De Crombrugghe *et al.*, 1984). Recently, strong evidence has been obtained in support of the model that CAP activates *lac* transcription initiation through a protein-protein contact with RNA polymerase (Reznikoff, 1992). CAP mutants have been isolated that showed normal DNA binding properties but were defective in transcription activation. All of these mutants had residue changes within the same region, between amino acids 156 and 162 (Bell *et al.*, 1990; Eschenlauer and Reznikoff, 1991). With the elucidation of the molecular structure of the CAP-DNA complex (Schultz *et al.*, 1991) it could be shown that residues 156 to 162 coincide with a surface-exposed loop. It is proposed that this surface-exposed loop is the contact domain for RNA polymerase, which is supported by the inability of these mutants to interact with RNA polymerase *in vitro* (Reznikoff, 1992). Binding of CAP to its DNA target leads in a 90° bending (Schultz *et al.*, 1991) and it has been postulated that this feature might be important for the actual activation of transcription activation. However, the exact mechanism of activation of transcription initiation remains to be solved.

CONTROL OF GENE EXPRESSION IN LACTIC ACID BACTERIA

Various promoters from lactic acid bacteria have been isolated either by shotgun cloning upstream of promoterless CAT genes (Achen *et al.*, 1986; Van der Vossen *et al.*, 1987) or by characterization of the expression signals adjacent to cloned genes. The lactococcal -35 and -10 consensus sequences, TTGACA and TATAAT, respectively, are virtually identical to those of *E.coli*, and are usually spaced by 17 nucleotides. In addition, the three nucleotides just upstream of the -10 sequence seem to have a evolutionary preference for the sequence TGA (for reviews see De Vos, 1987 and Van de Guchte *et al*, 1992). Only during the last few years data have emerged concerning the regulation of expression of genes from lactic acid bacteria and are summarized in Table 1. For the *L.lactis* subsp. *cremoris* temperate bacteriophage BK5-T it has been reported that the *bpi*

gene repressed the activity of promoters that were isolated from the phage DNA (Laksmidevi *et al.*, 1990). Activation of the malolactic fermentation system in *L.lactis* is mediated by the *mleR* activator, that belongs to the *E.coli* LysR family of activators (Renault *et al.*, 1989). Expression of the sucrose uptake and metabolizing enzymes, encoded by the *sac* operon that is part of the conjugative transposon Tn5276 (Rauch and De Vos, 1992a; Rauch and De Vos, 1992b), is induced during growth on sucrose (Thompson and Chassy, 1981). In addition, the *srkI* gene, that encodes fructosekinase I and is closely linked to the *sac* operon, is also induced on sucrose (Thompson *et al.*, 1991; Rauch and De Vos, 1992b). The levels of *sac* mRNA in sucrose-grown cells were significantly higher than those on glucose, indicating that regulation of the *suc* operon occurs the transcriptional level (Rauch and De Vos, 1992b). Downstream of the *sacA* gene, encoding the sucrose-6-phosphate hydrolase, a gene, designated *sacR*, was identified that showed homology to the *E.coli* LacI/GalR family (Rauch and De Vos, 1992b). For the *S.thermophilus lacSZ* and *galME* genes it has been shown that transcription is strongly repressed during growth on glucose (Poolman *et al.*, 1990). The *Lactobacillus pentosus xylAB* genes, encoding xylose isomerase and xylulose kinase, have been shown to be induced during growth on xylose. This regulation occurs at the level of transcription and is mediated by the *xylR* repressor (Lokman *et al.*, 1991). Recently, the *L.lactis leu/ilv*, *trp*, and *his* operons encoding the enzymes involved in the biosynthesis of the branched chain amino acids (Godon *et al.*, 1992), tryptophane (Delorme *et al.*, 1992) and histidine (Bardowski *et al.*, 1992), respectively, have been sequenced and characterized. Various regulatory mechanisms have been proposed to be involved in the control of expression of these operons, including attenuation, anti-termination, and repression or induction (Godon and Renault, 1992). Some results concerning regulation of proteinase production, nisine expression and heat-shock response have emerged very recently. From expression and transcriptional fusion studies it has been deduced that transcription of the *L.lactis prtP* gene, encoding the proteinase enzyme, is induced during growth on milk-based media (De Vos, 1991; P. Bruinenberg; 1992). The regulatory protein mediating this regulation has not yet been identified. Expression of the *L.lactis nisA* gene appeared to be dependent of (precursor)nisin, as was evident from the absence of a *nisA* transcript in a strain in which a frame-shift was introduced in the chromosomally-located *nisA* gene. In addition, introduction of a plasmid containing the intact *nisA* gene restored transcription of the inactivated chromosomal copy of *nisA* (Kuipers, 1992). At the 3'-end of the *nis* operon a gene, designated *nisR*, has been identified that is essential for production of nisin(precursor) and encodes a protein with homology to the *E.coli* PhoB/OmpR family of regulators (Van der Meer *et al.*, 1992). Heat-shock proteins have been identified in *L.lactis* that are immunologically related to the *E.coli* GroEL proteins (Whitaker and Batt, 1991). Recently, a gene from *L.lactis* has been cloned, designated *dnaJ*, that is induced upon heat-shock and its deduced amino acid sequence sequence shows homology to those of *E.coli* and *B.subtilis*.

Upstream of the -35 and -10 consensus promoter sequences of *dnaJ* an inverted repeat was identified that is a possible target for a positive regulator (Van Asseldonk *et al.*, 1992). Although an increasing number of inducible systems from lactic acid bacteria have been identified, no data concerning the molecular mechanisms underlying this regulation have yet been described.

Species	Trait	Genes	Type of Regulation	Regulator	References
L.lactis	malolactic fermentation	*mle* locus	repression on glucose	*mleR*	Renault *et al.*, 1991
L.lactis	sucrose catabolism	*sacAB*	repression on glucose	*sacR*	Thompson and Chassy, 1981 Rauch and De Vos, 1992
L.lactis	sucrose metabolism	*srkI*	repression on glucose	*sacR* ?	Thompson *et al.*, 1991
S.thermo philus	lactose catabolism	*lacSZ/galME*	repression on glucose	unknown	Poolman *et al.*, 1990
L.lactis	lactose catabolism	*lac* operon	repression on glucose	*lacR*	This thesis
L.pentosus	xylose catabolism	*xylAB*	repression on glucose	*xylR*	Lokman *et al.*, 1991
L.cremoris	bacterio-phage BK5-T	unknown	unknown	*bpi*	Lakshmidevi *et al.*, 1990
L.lactis	proteinase production	*prtP*	induction on milk media	unknown	De Vos *et al.*, 1991 Bruinenberg, 1992
L.lactis	nisin production	*nisA*	induction by nisin?	*nisR*	Kuipers, 1992 Van der Meer *et al.*, 1992
L.lactis	heat-shock response	*dnaJ*	induction upon heat-shock	unknown	Van Asseldonk *et al.*, 1992
L.lactis	heat-shock response	unknown	induction of *E.coli* GroEL-like proteins	unknown	Whitaker and Batt, 1991
L.lactis	branched-chain amino acid synthesis	*leu/ilvP1P2* *leuP1* *aldP3* rbs *aldB*	repression by Ile attenuation Leu/Ile acetolactate induction translation	unknown leaderpeptide *aldR* tRNA synthetase	Godon *et al.*, 1992 Godon and Renault, 1992
L.lactis	tryptophane synthesis	*trpEGDCFBA*	induction upon Trp starvation	*B.subtilis* Mtr-like antiterminator?	Bardowski *et al.*, 1992
L.lactis	histidine synthesis	*hisCGDBHAF*	induction upon His starvation	unknown	Delorme *et al.*, 1992

Table 1. Properties of lactic acid bacteria that have reported to be subject to regulation.

Scope of this research

Following the General Introduction described in **Chapter 1,** Chapters 2 to 4 are concerned with the genetic organization and characterization of the structural genes of the *L. lactis lac* operon, while chapters 5 to 10, focus on the regulation of its expression.

Chapters 2 and 3 present the molecular cloning, nucleotide sequence, characterization, and transcriptional analysis of *L. lactis lacABCDFEGX* operon that consists of eight genes encoding the enzymes involved in the tagatose-6-phosphate pathway (LacABCD) and PEP-PTS^{lac} (LacEF), the phospho-ß-galactosidase enzyme (LacG), and a protein of yet unknown function (LacX). In addition, these chapters describe the homologies between the derived amino acid sequences of the proteins that are encoded by the *lac* operon and those of related proteins from other organisms. The nucleotide sequence and putative function of the *iso*-IS*S1* element that is flanking the 3'-end of the *L. lactis lac* operon is described in **Chapter 4.**

Chapter 5 describes the characterization and nucleotide sequence of the *lacR* gene encoding the LacR repressor. This chapter also shows a homology study between the LacR repressor and members of the *E. coli* DeoR family of repressors from which putative DNA-binding and inducer binding sites are postulated.

Chapter 6 presents the characterization of the promoter of the *L. lactis lac* operon. The contribution of LacR repressor and flanking DNA sequences to promoter activity was studied in *E. coli* and *L. lactis* by constructing transcriptional fusions between DNA fragments carrying the *L. lactis lac* promoter and the *cat*-86 reporter gene.

Chapter 7 presents the purification of the *L. lactis* LacR repressor and the characterization of the operators *lacO1* and *lacO2*. This chapter also describes the *in vitro* identification of the inducer tagatose-6-phosphate and a model for the action of LacR repressor in the regulation of *lac* operon expression.

Chapter 8 describes the construction of a *L. lactis* strain in which the *lacR* gene has been deleted by replacement recombination. The regulation of expression of the *lac* operon in the absence of *lacR* was studied and the data suggest the presence of a second control circuit.

Chapter 9 describes the identification of amino acid residues in the *L. lactis* LacR repressor that are involved in the binding of the inducer tagatose-6-phosphate.

In **Chapter 10,** amino acid residues in the putative DNA recognition helix of the LacR repressor were identified that are involved in DNA binding. A summary together with concluding remarks is presented in **Chapter 11.**

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CHAPTER 2

CHARACTERIZATION OF THE LACTOSE-SPECIFIC ENZYMES OF THE PHOSPHOTRANSFERASE SYSTEM IN *LACTOCOCCUS LACTIS*

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ABSTRACT

The plasmid-encoded lactose genes of the *Lactococcus lactis* phosphotransferase system encoding Enzyme III^{lac} (*lacF*) and Enzyme II^{lac} (*lacE*) have been identified and cloned in *Escherichia coli* and *L. lactis*. Nucleotide sequence and transcription analysis showed that these genes are organized into a lactose-inducible operon with the gene order *lacF-lacE-lacG-lacX*, the latter two genes encoding phospho-β-galactosidase and a 34-kD protein with an unknown function, respectively. The *lac*-operon is immediately followed by an IS element that is homologous to ISS1. Enzyme III^{lac} was purified from *L. lactis* and determination of its N-terminal sequence demonstrated that the *lacF* gene starts with a TTG codon and encodes a 105 amino acid protein (Mr = 11416). Cross-linking studies with the purified enzyme showed that Enzyme III^{lac} is active as a trimer. A mutant *lacF* gene was identified in strain YP2-5 and appeared to encode Enzyme III^{lac} containing the missense mutation G18E. The *lacF* gene could be expressed under control of vector-located promoter sequences resulting in overproduction of Enzyme III^{lac} in *E. coli* and complementation of the *L. lactis lacF* mutant YP2-5. The deduced amino acid sequence of Enzyme II^{lac} consists of 586 amino acids (Mr = 61562) and shows the characteristics of a hydrophobic, integral membrane protein. The deduced primary structures of the *L. lactis* Enzyme III^{lac} and Enzyme II^{lac} are homologous to those of *Staphylococcus aureus* (72 and 71 % identity, respectively) and *Lactobacillus casei* (48 and 47 % identity, respectively). In contrast, the organization of the lactose genes differs significantly between those Gram-positive bacteria. Heterogramic homology in specific domains was observed between the derived amino acid sequences of the lactose-specific enzymes and that of *E. coli* Enzyme III^{cel} and Enzyme II^{cel}, which suggests a common function in the transport and phosphorylation of these structurally related β-glucosides.

INTRODUCTION

The disaccharide lactose has been utilized extensively as model substrate for analyzing transport across biological membranes (Kaback, 1988; Franco *et al.* 1989). Most of the organisms used in these studies include bacteria such as *Escherichia coli*, that do not contain selective and efficient systems for the transport and degradation of lactose. In contrast, *Lactococcus lactis*, a Gram-positive lactic acid bacterium, is used for industrial milk fermentations mainly because of its ability to rapidly ferment lactose. Enzymatic complementation studies have shown that *L. lactis* contains a high-affinity (K_m=15 μM) lactose PTS consisting of two lactose-specific components, a membrane-located Enzyme II^{lac} and a soluble Enzyme III^{lac} (Thompson 1979; McKay *et al.* 1970). The intracellular lactose-6-phosphate thus generated is hydrolysed by a phospho-β-galactosidase that is unique for the lactose PTS (Hengstenberg *et al.*, 1970 ; de Vos and Simons, 1988). So far, no biochemical data have been reported for the *L. lactis* lactose PTS enzymes, that appear to be plasmid-encoded (Gasson, 1983; McKay, 1982). In contrast, Enzyme II^{lac} and Enzyme III^{lac} of *Staphylococcus aureus* have been purified and used to study the phosphorylgroup transfer by this PTS which has a K_m of 60 μM for lactose (Hengstenberg *et al.*, 1987; Hays *et al.* 1973). Although Enzyme II^{lac} was found to be inactive after purification (Schaefer *et al.*, 1981), Enzyme III^{lac} could be isolated in an active form that appeared to be a trimer (Hays *et al.* 1973). The primary structure of the 103 amino acid *S. aureus* Enzyme III^{lac} was resolved and its phosphorylation site determined (Stueber *et al.*, 1984). The cloning and nucleotide sequence determination of the *lacFEG* genes for the *S. aureus* lactose-specific PTS components confirmed the amino acid sequence of Enzyme III^{lac} (LacF) and provided the primary structures for Enzyme II^{lac} (LacE) and phospho-β-galactosidase (LacG; Breidt *et al.*, 1987).

Recently, the nucleotide sequences of the *lacG* genes from two *L. lactis* strains have been reported (Boizet *et al.*, 1988, de Vos and Gasson, 1989). The deduced amino acid sequences of the *L. lactis* phospho-β-galactosidase were found to be closely related to those of *S. aureus* and *Lactobacillus casei* (Porter and Chassy, 1988). In addition, we reported significant homology with an *E. coli* phospho-β-glucosidase and an *Agrobacterium* β-glucosidase, suggesting that those PTS-related phospho-β-glycosidases share a common ancestor with a non-PTS enzyme (De Vos and Gasson, 1989). To provide more information on the structure and function of the lactose PTS enzymes by comparing their biochemical properties and analysing interspecies sequence differences, we analysed the transcriptional organization and

nucleotide sequence of the *L.lactis* operon encoding the lactose PTS enzymes. In addition, the wild-type *L.lactis* Enzyme III^{lac} was overproduced, purified and analysed for its subunit composition, and a defective Enzyme III^{lac} was characterized.

MATERIALS AND METHODS

Bacterial strains, plasmids and media. *E. coli* strains MC1061 (Casadaban *et al.*, 1980), JM83 (Vieira and Messing, 1982), JM103 (Messing, 1983) and H1 trp (Remaut *et al.*, 1981) were used as hosts for cloning. *L.lactis* subsp. *lactis* strains used were MG1363 (Gasson, 1983), MG1820 (Maeda and Gasson, 1986), YP2-5 (Park and McKay, 1982). Plasmids used as vector included pUC18, pUC19 and pUC7 (Vieira and Messing, 1982), pAT153 (Twigg and Sherrat, 1981), pPLc28 (Remaut *et al.*, 1981), pNZ12 (De Vos, 1987) and pIL305 (Simon and Chopin, 1988). Media based on L-broth and M17 (Difco) were used for *E. coli* and *L.lactis*, respectively. For the selection of *L.lactis* Lac+ transformants use was made of Lactose Indicator Agar containing the pH indicator bromocresol purple as described (Park and McKay, 1982). Ampicillin was used at a final concentration of 50 μg ml^{-1} in *E. coli* and chloramphenicol and erythromycin were used in *L.lactis* at final concentrations of 5 and 10 μg per ml, respectively.

DNA manipulations and transfer. Isolation of plasmid DNA from *E. coli* was performed by the alkaline lysis method (Birnboim and Doly, 1979). Plasmid DNA was isolated from *L.lactis* by a modification of this procedure as described (De Vos and Gasson, 1989). All subsequent manipulations *in vitro* and in *E. coli* were performed as described by Maniatis *et al.* (1982). DNA was introduced into *L.lactis* by electroporation using a Genepulser (Bio-Rad) following a previously described protocol (De Vos *et al.* 1989). Restriction enzymes, Klenow polymerase and T4 DNA ligase were purchased from Bethesda Research Laboratories or Boehringer Mannheim and used as recommended by the suppliers.

DNA sequence analysis. A detailed physical map of the pMG820 regions flanking the *lacG* gene (De Vos and Gasson, 1989) was constructed with the use of three overlapping fragments that were cloned in *E. coli* strains MC1061 or JM83 (see Fig. 1): a central 4.3 kb *Xho*I fragment which was inserted into *Sal*I site of pNZ12 resulting in pNZ34 (De Vos and Gasson, 1989), a 2.6 kb *BstE*II fragment extending to the left side, that was made blunt using Klenow polymerase and cloned into *Hin*dII

linearized pUC7 resulting in pNZ301, and a 4.5 kb *Cla*I fragment extending to the right side, that was inserted into the *Cla*I site of pAT153 resulting in pNZ311. DNA fragments were subcloned in the single-stranded phage vectors Mp8, Mp9, Mp18 and Mp19 using JM103 as a host (Messing, 1983). Nucleotide sequences were determined of both strands by the dideoxy chain termination method as described by Sanger *et al.* (1977). The sequencing strategy used is outlined in Fig. 1. Sequence primers were synthesized on a Cyclone DNA Synthesizer (Biosearch). Sequence data were assembled using the PC/Gene program version 5.01 (Genofit, Geneva). The facilities of the Netherlands CAOS/CAMM Center (University of Nijmegen) were used to screen the protein data bases SWISS-PROT and NBRF/NEW, releases 12.0 and 23.0, respectively.

RNA isolation and analysis. *L.lactis* MG1820 was grown in M17 broth (100 ml) containing either 0.5% lactose or 0.5% glucose to an optical density (600 nm) of 0.6-0.8. Total RNA was isolated from protoplasts prepared by incubating washed cells in 10 ml buffer containing 50 mM Tris hydrochloride pH 7.4, 3 mM MgCl2, 25% (w/v) sucrose and 1 mg/ml lysozyme at 4 °C for 10 min. Subsequently, the protoplasts were collected by centrifugation, resuspended in 500 μl buffer containing 20 mM sodium acetate pH 5.5, 1 mM EDTA and 25% sucrose and lysed by the addition of 2 ml of the same buffer in which sucrose had been replaced by 0.5% SDS. RNA was extracted from the lysate by repeated acidic phenol and chloroform extractions as described (Aiba *et al.*, 1981), followed by precipitation by ethanol. RNA was glyoxylated, size-fractionated on a 1% agarose gel and either stained with ethidium bromide or blotted to a Gene Screen membrane by capillary transfer as recommended by the supplier (New England Nuclear). RNA size markers were obtained from Bethesda Research Laboratories. Hybridization and washing conditions were according to the protocols of New England Nuclear. Gel-purified restriction fragments that had been labeled by nick translation with α-32P dATP were used as hybridization probes. These included a *lacF*-specific probe isolated as a *Bam*HI fragment of pNZ302 (see below) and a *lacG*-specific probe obtained by digesting pNZ32 DNA (De Vos and Gasson, 1989) by *Eco*RI and *Hin*dIII (probes a and b, respectively, see Fig. 1).

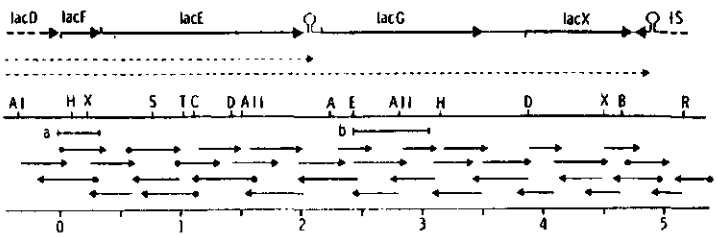

Fig. 1. Restriction and transcription map of the *L. lactis lac* genes and sequencing strategy. The locations of the *lac* genes and the IS element reported in this study are shown at the top together with the putative transcription terminators. The dashed lines indicate the transcriptional organization deduced from experiments with fragments a and b as hybridization probes. The lower part of the figure shows the sequencing strategy. Dotted arrows indicate sequences determined with the use of synthetic primers specific for the insert DNA. Other sequences were determined using an M13 universal primer. The size of the DNA (in kb) is indicated by the lower bar. Restriction sites are *Apa*I (A), *Hpa*I (AI), *Hpa*II (AII), *Aha*III (B), *Cla*I (C), *Eco*RI (E), *Hha*I (H), *Dra*I (D), *Eco*RV (R), *Stu*I (S), *Bst*EII (T), *Xho*I (X). The stars mark restriction sites used for cloning the flanking regions of the *lacG* gene.

Cloning and overexpression of the pMG820 *lacF* gene. The pMG820-located *lacF* gene is flanked by *Nco*I and *Xmn*I sites at positions 13 and 418, respectively (Fig. 2). It was isolated as a 0.4-kb *Nco*I-*Xmn*I fragment from pNZ301, provided by blunt ends using T7 Polymerase and cloned into *E. coli* JM83 using *Hin*dII-linearized pUC7, resulting in pNZ302. The *lacF* gene was isolated as a *Bam*HI fragment from pNZ302 and provided with useful flanking restriction sites by subcloning in *Bam*HI-linearized pUC18. The resulting plasmids, pNZ303 and pNZ304, differ by the orientation of the *lacF* gene, which is under control of the lac promoter in pNZ303. Subsequently, the *lacF* gene was isolated from either plasmid as a *Xba*I-*Eco*RI fragment and cloned into *L. lactis* MG1363 using *Xba*I-*Eco*RI-digested vector pIL253, resulting in pNZ305 and pNZ306. In pNZ305 the *lacF* gene is under control of a counterclockwise lactococcal-specific promoter located in the replicon part of the vector pIL253, whereas in pNZ306 the *lacF* gene is in the opposite orientation.

For the overexpression of the *lacF* gene, it was isolated from pNZ303 as an *Eco*RI-*Hin*dIII fragment and cloned under control of the lambda PL promoter in

pPL28c digested with *Eco*RI and *Hin*dIII. Cells of *E. coli* H1 *trp* carrying the resulting plasmid pNZ301 were induced at 42 °C to inactivate the thermosensitive cI857 repressor or non-induced as described previously (De Vos and Gasson, 1989). Samples were taken after 3 h, lysed and applied to a 0.2% SDS-15% polyacrylamide gel according to Laemmli (1970).

Isolation and analysis of the lacF gene from *L.lactis* YP2-5. Chromosomal DNA of *L.lactis* YP2-5 was isolated from protoplasts prepared as described (De Vos and Gasson, 1989) that were lysed by resuspension in 10 x TE buffer (100 mM Tris hydrochloride pH 7.4, 10 mM EDTA). The lysate was deproteinized by repeated neutral phenol extractions and finally dialysed against TE buffer. 50 μg chromosomal DNA was digested with *Xmn*I and *Nco*I and treated with T4 polymerase to generate blunt-ended fragments that were separated on a 1.0% agarose gel. Fragments with a size of approximately 0.4 kb were recovered and cloned into *Hin*dII-linearized Mp18. White plaques in *E. coli* JM103 were obtained, transferred to Colony Screen (New England Nuclear) and screened with a *lacF*-specific probe isolated from pNZ302 and labeled as described above (probe a in Fig. 1). Phage DNA was isolated from a positive reacting plaque and its insert was subcloned in Mp19 and sequenced.

Purification and analysis of Enzyme III^{lac} of *L.lactis*. Cells (330g) of *L.lactis* subsp. *lactis* 133 were disrupted in a Dynomill (Fa. Bachofen). The crude extract was centrifuged for 1 h at 22000 g and the supernatant was applied to a DEAE-cellulose column (DE-23, 12 x 30 cm, Whatman). The column was first washed with standard buffer (0.05 M Tris-HCl, 0.1 mM DTT, 0.1 mM PMSF and 0.1 mM EDTA) and the soluble proteins were eluted in as two-step gradient (6 liter each) of firstly 0-0.4 M NaCl and, secondly, 0.35-0.9 M NaCl in standard buffer. Enzyme III^{lac}-containing fractions were pooled and concentrated by a 45% ammonium sulphate precipitation. The Enzyme III^{lac}-containing pellet was dissolved in 140 ml of standard buffer, adjusted to 25% saturation with ammonium sulphate and applied to a Butyl-TSK column (2.2 x 15 cm, Merck, Darmstadt, West Germany) equilibrated with 30% ammonium sulphate in standard buffer. The column was eluted with a gradient of 30-0% ammonium sulphate in standard buffer. Enzyme III^{lac} fractions were pooled and concentrated by pressure dialysis (Amicon, UM-2 membrane, 76 mm) and then applied to a Sephadex G-75 column (5-90 cm). This column was eluted with standard buffer, and the resulting Enzyme III^{lac} pool was desalted on a Sephadex G-25 column (4x25 cm) and lyophilized. The described purification procedure resulted in 12 mg of

electrophoretically pure protein. The NH_2-terminal sequence was determined on a gas-phase sequenator according to Hewick *et al.* (1981).

Cross-linking experiments. Ten μg of purified Enzyme III^{lac} protein were mixed with DTBB or DMS (0-100 mM) dissolved in 0.2 M triethanolamine hydrochloride (pH 8.5) and incubated for 1 h at 37 °C. The samples were subsequently mixed with sample buffer containing 0 or 2% mercaptoethanol, incubated for 10 min at 37 °C and finally applied to a 0.2% SDS 10% polyacrylamide gel according to Schaegger and Von Jagow (1987).

RESULTS AND DISCUSSION

Nucleotide sequence analysis and location of the *lacFEGX* genes. Figure 2 shows the nucleotide sequence of a 5 kb DNA fragment that includes the *L. lactis* pMG820 *lacG* gene (located between position 2193 and 3599; De Vos and Gasson, 1989) and its surrounding regions. Three additional, complete open reading frames are present that all show the same orientation as the *lacG* gene.

The first reading frame, designated *lacF*, contains a GTG initiation codon at position 61 and spans 318 bp. It encodes Enzyme III^{lac} since the first 15 amino acids of its translation product (calculated molecular weight 11,416) are identical to that determined from purified *L. lactis* Enzyme III^{lac} (see below; underlined in Fig. 2). This also indicates that the GTG codon is translated into a methionine that is contained in the active *L. lactis* Enzyme III^{lac}. This is in contrast to *Lactobacillus casei* Enzyme III^{lac} where the N-terminal methionine is removed (Alpert and Chassy, 1988). *LacF* is preceded by a region of 4 kb DNA containing four other open reading frames (Van Rooijen and De Vos, unpublished results) from which the last one (*lacD*, Fig. 1), terminates at the TGA stop codon at position 31 that is separated from the *lacF* initiation codon by 30 bp.

Three bp downstream from *lacF* a second open reading frame initiates with an ATG codon at position 382 and stops at two adjacent ochre termination codons at position 2086 to 2091. This reading frame is designated *lacE* since its putative translation product is a highly hydrophobic, 568-amino-acid protein with a calculated molecular weight of 61,526, that is similar to Enzyme II^{lac} from *S. aureus* and *Lactobacillus casei* (see below).

The *lacE* and *lacG* genes are separated by a 102-bp region that contains an inverted repeated sequence (Fig. 2; De Vos and Gasson, 1989). Downstream from the

lacG gene there is an intergenic region of 292 bp extending to the last open reading frame that could encode a 299-amino-acid protein if the GTG codon at position 3891 is used for initiation. This reading frame is designated *lacX* since its location and the molecular weight of its deduced translation product (34,487) are in close agreement with that of the previously identified gene X, which in *E. coli* minicells results in the synthesis of the 37-kD protein X with an unknown function (Maeda and Gasson, 1986).

Seven bp downstream of *lacX* starts the right 18-bp inverted repeat of a 0.8 kb IS element with high homology to ISS1 (Polzin and Shimuza-Kadota, 1987). Only part of its sequence is shown here, including the 3' end of the transposase gene that terminates at position 4853. Within this transposase gene and approximately 100 bp downstream of *lacX*, two inverted repeats are located. The first and longest one resembles rho-independent transcription termination signals (Platt, 1986) since it could form a stable stem-loop structure and is followed by a long stretch of T-residues around position 4930.

Transcription analysis of the *lacFEGX* genes. The arrangement of the *lac* genes as deduced from the nucleotide sequence (Fig. 1) suggests an operon organization, in spite of the presence of intergenic regions flanking the *lacG* gene. To verify this, we analysed the transcriptional organization of the *lacFEGX* genes. Since it has been shown that the activity of the lactose PTS enzymes is induced approximately ten-fold during growth of *L.lactis* on lactose (LeBlanc *et al.*, 1979; Van Rooijen and De Vos, 1990), total RNA was isolated from cultures grown on lactose and glucose. The results (Fig. 3) show that two lactose-inducible transcripts of 6 and 8.5 kb hybridize with probes specific for *lacF* (Fig. 3A) and *lacE* (not shown). Interestingly, only the 8.5-kb transcript is found to hybridize with a probe for *lacG* (Fig 3C). Similar data were found with a *lacX*-specific probe (not shown). These results confirm the operon organization of the *lac* genes and indicate that the *lacFEGX* genes are transcribed into a single 8.5-kb mRNA that is predominantly present in lactose-grown cells. In addition, it shows that the *lac* operon gene expression is regulated at the transcriptional level. We recently presented evidence for the transcriptional control of the *L.lactis lac* operon by a repressor that is encoded by a further upstream located gene, *lacR* (Van Rooijen and De Vos, 1990). A similar type of transcriptional regulation has recently been reported for the lactose PTS genes of *S. aureus* (Oskouian and Stewart, 1990).

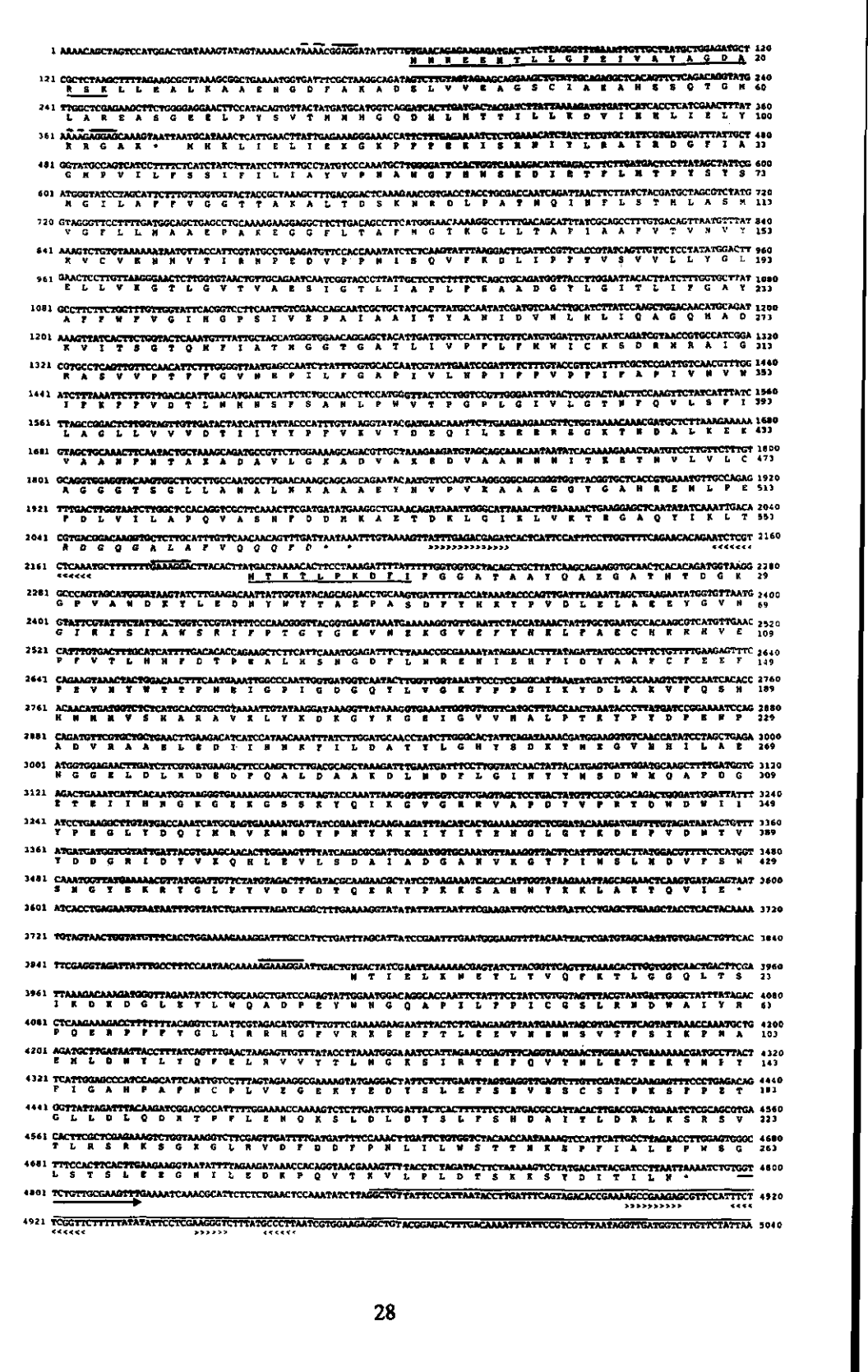

Fig. 2. Nucleotide sequence of the *L.lactis lac* genes and the predicted primary amino acid sequences of Enzyme III^{lac}, Enzyme II^{lac}, phospho-β-galactosidase and LacX. Amino acyl numbering starts with the initial methionine of each enzyme. Potential *L.lactis* ribosome binding sites (De Vos, 1987) are overlined. NH_2-terminal amino acid sequences that have been confirmed by protein sequencing are underlined. Dyad symmetries are indicated by broken arrows below the sequence. The IS-element is indicated by the arrow that marks the 18-bp terminal repeat and the overlined sequence that represents part of the coding strand for the transposase gene. The nucleotide sequence of *lacG* from position 2102 to 3626 has previously been reported (De Vos and Gasson, 1989) and is included for completeness.

The detection of a second, 6-kb lactose-inducible transcript specific for the *lacF* and *lacE* genes implies an unusual transcriptional organization of the *lac* genes (Fig. 1). The fact that both 8.5- and 6-kb transcripts are lactose-inducible and partially overlapping suggests that they initiate at the same position. This is compatible with the finding that a single, lactose-inducible promoter is present approximately 4 kb upstream from *lacF* (Van Rooijen and De Vos, unpublished results). We have previously suggested that the inverted repeat which is located in between the *lacE* and *lacG* gene and extends to position 2170, could function as a transcription terminator (De Vos and Gasson, 1989). If so, this may well be the termination site for the 6-kb transcript. Partial readthrough could then explain the 8.5 kb mRNA specific for *lacG* and *lacX* that is likely to terminate approximately 2.5 kb further downstream at the possible terminator around position 4930.

Overproduction, purification and subunit analysis of Enzyme III^{lac}. The nucleotide sequence analysis allowed the exact dissection of the *lacF* gene as a 0.4-kb DNA fragment that was cloned under control of the P_L promoter in *E. coli* containing a thermosensitive lambda repressor. After induction at 42 °C, a single protein was overproduced that was absent in uninduced cells (Fig. 4) and cross-reacted with antibodies raised against purified Enzyme III^{lac} from *S. aureus* (results not shown). The apparent molecular weight of the *lacF* gene product was estimated to be approximately 8 kD which is substantially smaller than that of 11.4 kD predicted from the gene sequence. This is not due to phosphorylation, since a similar mobility was observed with unphosphorylated Enzyme III^{lac} purified from *L.lactis* (Fig. 5).

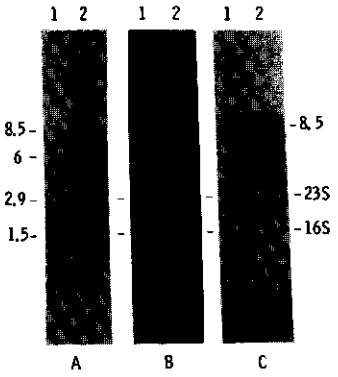

Fig. 3. Northern blot analysis of *lac* gene expression in *L.lactis*. Equal amounts of RNA (approximately 10 μg) were isolated from lactose- or glucose-grown cells (lanes 1 and 2, respectively) separated on agarose gels, that were either stained (B) or after blotting, hybridized with a *lacF*-specific probe (A) or a *lacG*-specific probe (C) and autoradiographed. The position of 23S and 16S rRNA, the presence of which results in a reduced hybridization signal, is indicated as is the estimated size (in kb) of the main *lac*-specific transcripts.

In addition, N-terminal cleavage of the protein can be ruled out since the sequence of the first 15 amino acids of purified Enzyme III^{lac} were determined and appeared to match that of the sequence deduced from the gene structure (Fig. 2). An anomalous mobility during SDS gel electrophoresis has been reported previously for the homologous (see below) *S. aureus* Enzyme III^{lac} (Hays *et al.*, 1973).

To determine the subunit composition of Enzyme III^{lac}, cross-linking studies were performed with the purified enzyme. After cross-linking with DTBB or DMS, two additional bands were detected on SDS-polyacrylamide gels with apparent molecular weights of 24 kD and 35 kD, respectively (Fig. 5). Mercaptoethanol fully inhibited cross-linking with DTBB when added to the samples before gel electrophoresis. This indicates that Enzyme III^{lac} of *L.lactis* consists of three identical subunits with a molecular weight of approximately 12 kD.

Fig. 4. Overproduction of *L.lactis* Enzyme III^{lac} in *E. coli*. Lysates of non-induced (lane 1) and induced (lane 2) *E. coli* H trp containing pNZ301 were separated and stained with Coomassie blue. The size (in kD) of parallel-run molecular-weight markers is indicated.

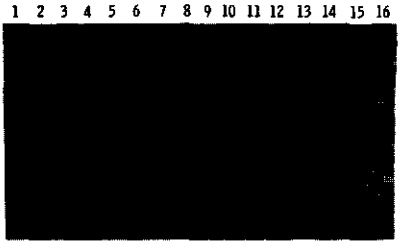

Fig. 5. Subunit composition of *L.lactis* Enzyme III^{lac}. Purified Enzyme III^{lac}, treated with DTBB or DMS in the absence or presence of mercaptoethanol, and marker proteins (5-10 μg) were separated by SDS 10% polyacrylamide gel electrophoresis (Schaegger and Von Jagow, 1987) and stained with Coomassie blue. Lanes 1-4: Enzyme III^{lac} treated with 0, 20, 50 and 100 mM of DTBB, respectively. Lanes 5-7, Enzyme III^{lac} treated with 0, 50 and 100 mM DTBB, respectively, in the presence of mercaptoethanol. Lanes 8-9, Enzyme III^{lac} treated with 50 mM DMS, with and without mercaptoethanol, respectively. Lane 10-16 molecular weight size markers, HPr from *Enterococcus faecalis* (9 kD), cytochrome c (13 kD), myoglobin (17 kD), chymotrypsinogen (24.5 kD), aldolase subunit (40 kD), ovalbumin (45 kD) and bovine serum albumin (68 kD), respectively.

Complementation of a *L. lactis lacF* mutant and analysis of its defect. The 0.4-kb *lacF* gene was cloned into the Enzyme III^{lac}-deficient *L.lactis* strain YP2-5' using the Gram-positive vector pIL253. The two resulting plasmids, pNZ305 and pNZ306, differ in orientation of the *lacF* gene, that in pNZ305 is under control of a vector-located promoter. Only pNZ305 could complement the *lacF* deficiency of the used host as concluded from the production of acid from lactose. In addition, strain YP2-5 harboring pNZ305 showed a readily detectable growth on lactose-containing media, although its generation time (90 min) appeared to be somewhat reduced in comparison with that of the wild-type strain MG1820 (48 min). These results confirm the identity of the *L.lactis lacF* gene and the necessity for a strong, exogenous promoter to drive its expression.

In order to analyse the structure and function of the *L.lactis* Enzyme III^{lac}, we isolated the mutant *lacF* gene from strain YP2-5 and determined its nucleotide sequence. Comparison of this sequence with that of the wild-type, pMG820-encoded *lacF* gene showed one nucleotide substitution, an A instead of G residue at position 113 (Fig. 2), resulting in a missense mutation Gly18Glu in the Enzyme III^{lac} of strain YP2-5. It is remarkable that this amino acid substitution is identical to that present in the defective Enzyme III^{lac} from a *S. aureus* mutant (Sobek *et al.*, 1984). Although no nucleotide sequence is available for the latter mutated *lacF* gene, this suggests that position 113 and/or 114 represent hot-spot mutation site(s) in the *lacF* genes of those two organisms that are highly identical (70%) at the nucleotide level.

Homogramic and heterogramic homology of the *L.lactis* lacPTS enzymes. The deduced amino acid sequences of Enzyme III^{lac} and Enzyme II^{lac} of *L.lactis* were compared pairwise with those of the similar-sized counterparts of *S. aureus* (Breidt *et al.*, 1987) and *Lactobacillus casei* (Alpert and Chassy 1988, 1990). The results (Table 1) demonstrate that the lactose-specific PTS enzymes of these unrelated Gram-positive bacteria are highly homologous (up to 72% identity) and that the *L.lactis* and *S. aureus* sequences show the greatest percentage of identical residues. A similar high degree of similarity has also been reported for the phospho-β-galactosidase (LacG) sequences (Porter *et al.*, 1988; De Vos and Gasson, 1989) that in general show more identity (up to 82%) than the PTS enzymes. An unexpected high degree of homology (35 identical residues) was found between the derived *L.lactis* Enzyme III^{lac} sequence and the COOH-terminal 102 amino acids of the putative *E. coli* Enzyme III^{cel} sequence. The latter enzyme has a deduced size of 116 amino acids and is required for the transport of both cellobiose and arbutin. It is encoded by the *celC* gene of the

cryptic *E. coli* cellobiose operon, that also contains the *celB* gene for a Enzyme II^{cel} (Parker and Hall, 1990). A similar degree of homology could be calculated for the *E. coli* Enzyme III^{cel} and the reported Enzyme III^{lac} sequences of *S. aureus* (as reported by Parker and Hall, 1990) and *Lactobacillus casei* (34 and 36 identical residues, respectively). A complete comparison of the deduced Enzyme III sequences is presented in Fig. 6; it shows that the only two His residues (at positions 54 and 78 in the *L.lactis* sequence) that are present in all three lactose-specific proteins are also present in Enzyme III^{cel}. His78 is located in a highly conserved segment comprising residues 76-92 and has been proposed as the phosphorylation site in *Lactobacillus casei* (Alpert and Chassy, 1988). Further support for this localization has been obtained by the isolation and characterization of peptides from ^{32}P-phosphoenolpyruvate-labeled *Lactobacillus casei* Enzyme III^{lac} (Hengstenberg *et al.*, 1989). It is conceivable that the conserved His78 is the main phosphorylation site in all these proteins. This possibility is currently being investigated using site-directed mutagenesis of this residue and His82 that has previously been identified as the phosphorylation site in *S. aureus* Enzyme III^{lac} (Stuber *et al.*, 1985) but is absent in the *Lactobacillus casei* sequence (Alpert and Chassy, 1988).

Table 1. Sequence identities between Enzymes III^{lac} (LacF), Enzymes II^{lac} and phospho-β-galactosidase from *L.lactis*, *S.aureus* and *Lactobacillus casei*.

protein	percentage identity		
	S. lactis *L. casei*	*S. lactis* *S. aureus*	*L. casei* *S. aureus*
LacF	45	79	44
LacE	47	71	44
LacG	54	82	54

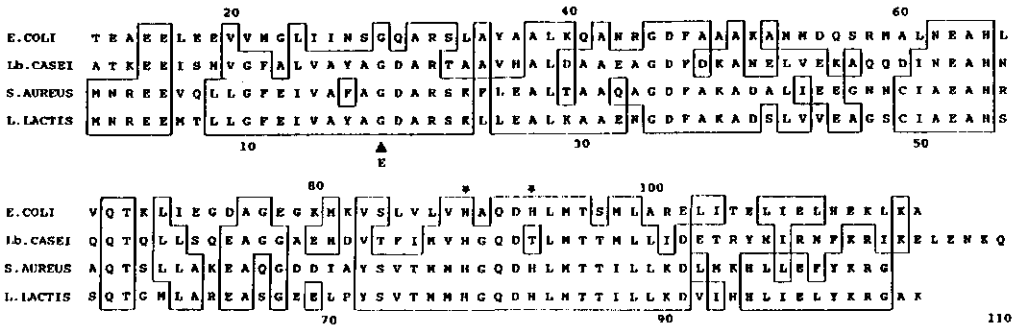

Fig. 6. Sequence homology between Enzyme III^{lac} from *L.lactis*, *S. aureus* and *Lactobacillus casei* and Enzyme III^{cel} from *E. coli*. The first 11 amino acid residues of Enzyme III^{cel} are not included in this comparison. The G18E mutation present in the defective Enzyme III^{lac} from *L.lactis* YP2-5 is indicated. Potential phosphorylation sites are marked by stars.

Another segment that is highly conserved includes the NH_2-terminal 21 amino acids of the lactose-specific proteins. Although the amino acid identity with *E. coli* Enzyme III^{cel} is less apparent, this sequence shows the same high hydrophobicity as that of the lactose-specific Enzymes III (not shown). A conserved Gly residue is present within this segment at position 18 that is changed into a charged Glu residue in the *S. aureus* and *L.lactis* defective Enzyme III^{lac} sequences (Sobek *et al.*, 1984; this paper). It is therefore very likely that, in analogy with *S. aureus*, this N-terminal part of Enzyme III provides the binding domain for the corresponding Enzyme II.

Detailed analysis of the *S. aureus* Enzyme III^{lac} sequence has shown that it contains an unusual COOH-terminal amphipathic α-helical segment (Saier *et al.* 1988). Helical wheel analysis (not shown) suggests that a similar amphipathic helix is present in all other lactose-specific Enzymes III and also in Enzyme III^{cel}. It is conceivable that this amphipathic sequence is involved in the interaction with the corresponding Enzymes II or, alternatively, participates in the formation of Enzyme III multimers. Support for the latter possibility is the presence of a possible amphipathic helix in the 16 COOH-terminal residues of the mannitol-specific *S. carnosus* Enzyme III, that is also found to be active as a trimer (Hengstenberg *et al.*, 1989).

The deduced *L. lactis* Enzyme II^{lac} sequence shows the characteristics of an integral membrane protein and contains an NH_2-terminal, highly charged and amphipathic sequence of 12 amino acid residues, that may have a function in its topogenesis (Saier *et al.*, 1989). The deduced *L. lactis* Enzyme II^{lac} sequence is even more hydrophobic than the reported Enzyme II^{lac} sequences from *S. aureus* and *Lactobacillus casei*, that all show similarly located hydrophobic domains (Fig. 7). Comparison of the deduced Enzyme II^{lac} primary sequences (results not shown) reveals that only three amino acid residues, viz. one His and two Cys residues (see Fig. 7) that are known to be phosphorylated in PTS enzymes, are conserved in all three proteins. Recent studies based on site-directed mutagenesis have shown that in *Lactobacillus casei* Cys483 is essential for the phosphoryl group transfer reaction (Alpert and Chassy, 1990). It is therefore very likely that the Cys473 residue that is located in a segment with high sequence identity within the deduced lactose-specific Enzyme II sequences (see Alpert and Chassy, 1990 for a detailed comparison) is the phosphorylation site in the *L. lactis* Enzyme II^{lac}.

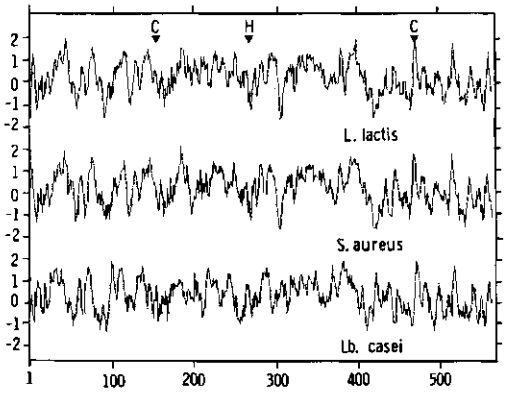

Fig. 7. Hydropathy analysis of the amino acid sequences of Enzyme II*lac* from *L.lactis*, *S. aureus* and *Lactobacillus casei*. The position of potential phosphorylation sites conserved in all three proteins are indicated.

In contrast to the lactose- and cellobiose-specific Enzyme III proteins that show a similar size and high sequence identity (approximately 30%; Fig. 6), the deduced sequences of Enzymes II^{lac} and that of *E. coli* Enzyme II^{cel} (Parker and Hall, 1990) differ in size (around 570 and 418 amino acid residues, respectively) and show only a

low degree of overall similarity (approximately 20% identity). Unexpectedly, the main homology is limited to three segments, one at the NH_2-terminus and two adjacent segments near the COOH-terminus of the Enzyme II sequences. The last segment is highly hydrophobic and contains unusual repeats of Pro residues (Fig. 8). No His or Cys residues are contained within these segments nor is the putative phosphorylation site of the lactose-specific Enzymes II. This indicates that a different phosphorylation site is present in Enzyme II^{cel} and suggests that the homologous segments are involved in the interaction with Enzyme III and/or the β-glucoside.

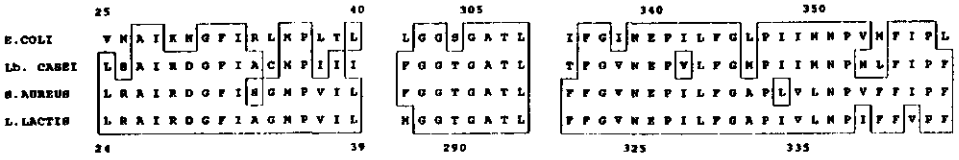

Fig. 8. Sequence conservation in Enzyme II^{lac} from *L. lactis*, *S. aureus* and *Lactobacillus casei* and Enzyme II^{cel} from *E. coli*.

Organization and evolution of the Gram-positive lactose PTS genes. In analogy with the deduced primary and predicted secondary structures of the lactose PTS enzymes, there exists a significant degree of identity between the genes encoding those enzymes of *L.lactis*, *S. aureus* and *Lactobacillus casei* (results not shown). However, the organization of the *lac*-genes differs considerably, as is illustrated in Fig. 9. The differences include the order of the *lac*-genes, the location of putative terminators, and the length of the intercistronic regions. In addition, an additional gene, *lacX*, appears to be part of the *L.lactis lac*-operon. The function of its 37-kD translation product is not known (Maeda and Gasson, 1986) but in analogy with *E. coli* PTS operons (Yamada and Saier, 1987; Davis *et al.*, 1988; Parker and Hall, 1990) it may participate in regulation or, alternatively, it may be involved in lactose catabolism, for instance as a glucokinase.

Although the transcriptional organization of the *lac*-genes has only been studied in *L.lactis* (Fig. 3), it is likely that also the *lac*-genes of *S. aureus* and *Lactobacillus casei* are organized into an operon. The 3-bp distance or overlap between the *lacF* and *lacE* genes in *L.lactis* (Fig. 2) and *S. aureus* (Breidt *et al.*, 1987), respectively, suggests translational coupling between those genes.

The characteristic modular organization of the *lac*-genes in the Gram-positive bacteria (Fig. 9) suggests that the individual *lacF*, *lacE* and *lacG* genes, and possibly other components of the *lac*-operon, have been acquired independently and/or have been reshuffled after acquisition, possibly in order to allow optimal gene expression and regulation. The observed heterogramic homologies of Enzyme III^{lac} with *E. coli* Enzyme III^{cel} (Parker and Hall, 1990; Fig. 6), and phospho-β-galactosidase with *E. coli* phospho-β-glucosidase and *Agrobacterium* β-glucosidase (Porter and Chassy, 1988; De Vos and Gasson, 1989) support the modular evolution of the lactose PTS genes and indicates that horizontal gene transfer has been involved in this process. The presence of an IS element immediately downstream of the *lac*-operon in *L. lactis* suggests that transposition is one of the possible mechanisms for the transfer of the *lac*-genes.

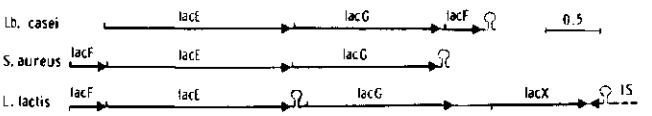

Fig. 9. Organization of the *L.lactis lac*-operon compared with that of the *lac*-genes in *S.aureus* and *Lb. casei*. ATG and GTG initiation codons are indicated by the upward or downward orientated *bars*, respectively.

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CHAPTER 3

MOLECULAR CLONING, CHARACTERIZATION, AND NUCLEOTIDE SEQUENCE OF THE TAGATOSE 6-PHOSPHATE PATHWAY GENE CLUSTER OF THE LACTOSE OPERON OF *LACTOCOCCUS LACTIS*

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ABSTRACT

The tagatose 6-phosphate pathway gene cluster (*lacAB*CD) encoding galactose 6-phosphate isomerase, tagatose 6-phosphate kinase, and tagatose 1,6-diphosphate aldolase of *Lactococcus lactis* subsp. *lactis* MG1820 has been characterized by cloning, nucleotide sequence analysis, and enzyme assays. Transcription studies showed that the four tagatose 6-phosphate pathway genes are the first genes of the lactose inducible lactose-phosphotransferase operon consisting of the *lacAB*CDFEGX genes. Using a T7-expression system, it could be shown that the *lacA*, *lacB*, *lacC* and *LacD* genes code for proteins with apparent molecular weights of 15, 19, 33, and 36 kDa, respectively. Cell-free extracts of induced and non-induced *E. coli* cells expressing the *lacAB*CD genes were used to determine the functions of the encoded proteins. Expression of both *lacA* and *lacB* was required to obtain galactose 6-phosphate isomerase activity. The *lacC* gene codes for tagatose 6-phosphate kinase, the deduced amino sequence of which is similar to that of *Escherichia coli* Pfk-2 phosphofructokinase, and *Staphylococcus aureus* LacC protein. The tagatose 1,6-diphosphate aldolase is encoded by the *lacD* gene and its deduced primary sequence, which is homologous to that of the *S. aureus LacD* protein, predicts an amino acid composition which is virtually identical to that of the previously purified *L. lactis* E8 tagatose 1,6-diphosphate aldolase.

INTRODUCTION

Lactose catabolism in lactic acid bacteria is initiated by either a lactose permease system (lac-PS; Thompson, 1987) or a phosphoenolpyruvate (PEP) dependent lactose phosphotransferase system (lac-PTS[2]; Hengstenberg et al., 1989; McKay, 1970). In the lac-PS the intracellular lactose is hydrolyzed by the enzyme ß-galactosidase into galactose and glucose, which are utilized in the Leloir (Maxwell et al., 1962) and Embden-Meyerhof-Parnas (Kandler, 1983) pathways, respectively. Lactococci that are used in industrial dairy fermentations, transport lactose exclusively via the lac-PTS, resulting in a rapid homolactic fermentation (de Vos and Simons, 1988). In this system, EnzymeII^{lac} (LacE) and EnzymeIII^{lac} (LacF) are the lactose-specific transport proteins. The resulting lactose 6-phosphate is hydrolyzed by phospho-ß-galactosidase (LacG) yielding glucose and galactose 6-phosphate. Galactose 6-phosphate is further metabolized in the tagatose 6-phosphate pathway by the enzymes galactose 6-phosphate isomerase, tagatose 6-phosphate kinase, and tagatose 1,6-diphosphate aldolase, respectively, as first described in *Staphylococcus aureus* by Bisset and Anderson (1973). The *S. aureus* tagatose 6-phosphate pathway enzymes have been partially purified and characterized (Bisset et al., 1980; Bisset and Anderson, 1980). Enzyme activities of the tagatose 6-phosphate pathway enzymes in various *Lactococcus lactis* strains have been determined and appeared to be induced during growth on lactose or galactose (Bisset and Anderson, 1974). The tagatose 1,6-diphosphate aldolase enzyme of *L. lactis* E8 has been purified and characterized (Crow and Thomas, 1982). In *L. lactis* H1 the genetic information for the tagatose 6-phosphate pathway enzymes is plasmid-encoded (Crow et al., 1983). The tagatose 1,6-diphosphate aldolase gene from this strain has been localized on plasmid pDI-1 and, subsequently, cloned and expressed in *E. coli* (Limsowtin et al.,1986; Yu et al., 1988). In *L. lactis* MG1820 the lactose-PTS genes have been characterized and are located on the plasmid pMG820, where they are organized in an operon structure (designated lac-PTS operon) with the gene order: *lacFEGX* (de Vos et al., 1990; de Vos and Gasson, 1989; Maeda and Gasson, 1986). The *lacFEG* gene order is also found in the *S. aureus* lac operon and these genes appear to be highly homologous to their *L. lactis* counterparts, although differences in the intercistronic regions have been described (de Vos et al., 1990; de Vos and Gasson, 1989). The *L. lactis lacX* gene, encoding a 34 kDa protein with unknown function, is not present in the *S. aureus* lac-operon. The *L. lactis* lac-PTS genes are transcribed as 6 and 8.5 kb polycistronic messengers and are induced 5 to 10-fold during growth on lactose as a sole energy source (de Vos et al., 1990). Regulation occurs at the transcriptional level, and is mediated by the LacR repressor, the product of the divergently transcribed *lacR* gene (van Rooijen and de Vos, 1990). Transcription of the *S. aureus lac*-operon also appears to be mediated by a repressor (LacR; Oskouian and Stewart, 1990), which shows high homology (44% identity) to the *L. lactis* LacR (Van Rooijen and De Vos, 1990). The main difference

is that the *S. aureus lacR* gene has the same orientation as the structural genes of the *lac*-operon (Oskouian and Stewart, 1990).

In the present study, we describe the molecular cloning, nucleotide sequence, and characterization of the tagatose 6-phosphate pathway gene cluster (*lacABCD*) of the lactose-PTS operon of *L. lactis* MG1820. The *lacAB*, *lacC*, and *lacD* genes appear to encode for the galactose 6-phosphate isomerase, tagatose 6-phosphate kinase, and tagatose 1,6-diphosphate aldolase, respectively.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. *E. coli* strains TG1 (Gibson, 1984), MC1061 (Casabadan and Cohen, 1980), JM83 (Vieira and Messing, 1982), and HMS174 (Campell et al., 1978) were used as recipients in the cloning experiments. For overproduction of the Lac proteins, *E. coli* K12 lysogen BL21(DE3)*lysS* was used. The *L. lactis* subsp. lactis strain used was MG1820, containing the lactose miniplasmid pMG820 (Maeda and Gasson, 1986). Media based on M17 broth (Difco) containing 0.5 % (w/v) glucose or lactose, and L-broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl), were used for the growth of *L. lactis* and *E. coli*, respectively. Ampicillin (Amp) and chloramphenicol (Cm) were purchased from Sigma and were used in *E. coli* at a final concentration of 50 μg/ml and 10 μg/ml, respectively. Plasmids used in the cloning experiments were pMG820, pUC18 (Yanisch-Perron et al., 1985), and pET8c (Studier et al., 1989).

Molecular cloning, reagents, and enzymes. Isolation of DNA from *E. coli* and *L. lactis* was performed by the alkaline lysis method (Birnboim and Doly, 1979) and a modified alkaline lysis method (de Vos and Gasson, 1989), respectively. All manipulations *in vitro* and in *E. coli* were performed as described by Maniatis et al (1989). All enzymes, IPTG, and rifampicin were purchased from Bethesda Research Laboratories, Biolabs, or Boehringer, and used according to the instructions of the manufacturers. Sequenase was purchased from Sophar Biochem., and [α-^{32}P]dATP and ^{35}S-methionine from Amersham. Oligonucleotides were synthesized on a Biosearch Cyclone DNA synthesizer.

Construction of plasmids. Plasmids pNZ390 and pNZ391 contain the 3.0 kb *Hpa*I-*Bst*EII fragment (filled in with Klenow DNA polymerase) and 2.9 kb *Eco*RI-*Kpn*I restriction fragment of pMG820 cloned into the *Sma*I and *Eco*RI-*Kpn*I of pUC18, respectively (Fig. 1). Plasmid pNZ390 contains the *lacR* (van Rooijen and de Vos, 1990), *lacA*, and *lacB* genes, and plasmid pNZ391 contains the *lacC* and *lacD* genes. Plasmid pNZ301 consists of the 2.6 kb *Bst*EII fragment (filled in with Klenow DNA polymerase) of pMG820 cloned into the *Hin*cII site of pUC7, and contains the *lacD* gene and parts of the *lacC* and *lacF* genes[2] (Fig. 1). In plasmid pNZ392 the 1.8 kb *Eco*RV restriction fragment of pMG820 (see Fig. 1) was cloned into the *Sma*I site of pUC18,

and contains the *lacA*, *lacB*, and (part of) *lacC* genes (Fig. 1).

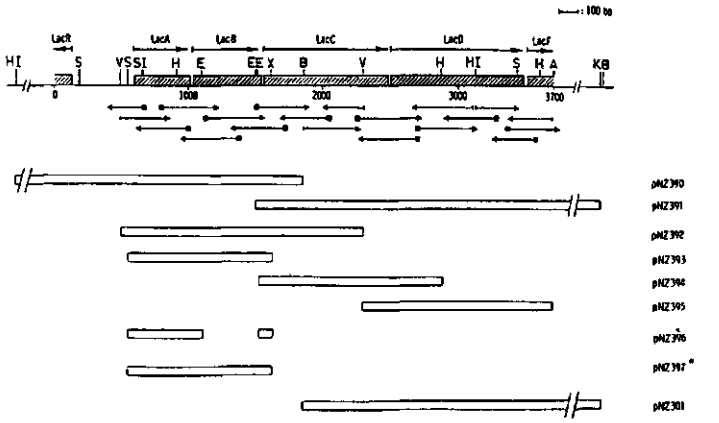

Fig. 1. Physical map and sequencing strategy of the region containing the *lacABCD* genes of the *L. lactis* MG1820 plasmid pMG820. The hatched bars and the arrows above indicate the coding regions and the direction of the *lacABCD* genes, respectively. Parts of the coding regions of the *lacR* and *lacF* genes are also presented[3] (Van Rooijen and De Vos, 1990). The positions of the restriction enzyme cleavage sites used in DNA-sequencing and cloning experiments are indicated: A, *Ava*I; B, *Bst*EII; E, *Eco*RI; H, *Hae*II; HI, *Hpa*I; K, *Kpn*I; S, *Ssp*I; SI, *Sty*I; V, *Eco*RV; X, *Xba*I. The arrows indicate the origin, direction, and extent of the individual sequencing reactions. A black box indicates the use of a sequence derived oligonucleotide primer. Open bars represent the DNA fragments used in the constructions of the various plasmids.
* A frameshift in the *lacA* coding region has been introduced by filling in the *Sty*I restriction site of plasmid pNZ393 with Klenow polymerase (∇).

Nucleotide sequence analysis. DNA fragments were cloned into the multiple cloning site of M13mp18 and M13mp19 (Yanisch-Perron et al., 1985). Nucleotide sequences of both strands were determined by the dideoxy chain termination method (Sanger et al., 1977) using either M13 universal primer or synthesized primer. Samples were electrophoresed on a 6% polyacrylamide, 7.5 M urea sequencing gel. The sequencing strategy is presented in Fig. 1. Sequence data were assembled and analyzed using the PC/GENE program version 5.01 (Genofit, Geneva). The facilities of the Netherlands CAOS/CAMM Center (University of Nijmegen) were used to screen the protein databases SWISS-PROT and NBRF/NEW, releases 14.0 and 25.0, respectively.

RNA isolation and northern blot analysis. *L. lactis* MG1820 cells growing on glucose or lactose (100 ml) were harvested and total RNA was isolated as previously described (van Rooijen and de Vos, 1990). RNA (50 μg) was glyoxylated, size fractionated, and

blotted to a membrane (Gene Screen; New England Nuclear). Prehybridization and hybridization were performed as described (van Rooijen and de Vos, 1990). A 1.8 kb *Eco*RI-*Bam*HI restriction fragment of pNZ392 was labelled by nicktranslation (Maniatis et al., 1989) and used as a hybridization probe.

Expression of *lacA*, *lacB*, *lacC*, and *lacD* in *E. coli*. A 1.1 kb *Ssp*I-*Xba*I fragment of pNZ390, containing the *lacA* and *lacB* genes (see Fig. 1), and a 1.35 kb *Eco*RI-*Hae*II (both sites made blunt with T4 DNA polymerase) and 1.5 kb *Eco*RV-*Ava*I (filled in with Klenow DNA polymerase) restriction fragment of pNZ391, containing the *lacC* and *lacD* genes, respectively, were cloned in the *Nco*I-*Bam*HI site (both sites filled in with Klenow DNA polymerase) of the T7 expression vector pET8c and used to transform *E. coli* HMS174. This resulted in the isolation of clones containing plasmids (see Fig. 1) designated as pNZ393 (*lacAB*), pNZ394 (*lacC*), and pNZ395 (*lacD*). In order to analyze the functions of the proteins encoded by the *lacA* and *lacB* genes, the latter were inactivated by manipulation of plasmid pNZ393. The *Sty*I restriction site located 128 bp downstream of the putative *lacA* ATG-startcodon was filled in by Klenow DNA polymerase (after partial digestion with *Sty*I) followed by ligation. As a consequence of this manipulation a frameshift in the *lacA* gene is introduced. The *lacB* gene of pNZ393 was partially deleted by an *Eco*RI digestion followed by ligation. As a consequence of this procedure, the ϕ_{10} terminator, located upstream of the pET8c-located *bla* gene, has been deleted. The ligation mixtures were used to transform *E. coli* HMS174 and the plasmids obtained from the isolated clones were designated pNZ396(*lacA*) and pNZ397(*lacB*). Since only pNZ397 was stably maintained in *E. coli* BL21(DE3), *E. coli* BL21(DE3)*lysS* was used for expression studies. This strain contains the T7 polymerase gene under control of the lac-promoter (*E. coli*) and LacI repressor and contains a pACYC184 derived plasmid containing a constitutively expressed T4 lysozyme, a natural inhibitor of T7 RNA polymerase, which reduces the basal activity of T7 RNA polymerase (Studier et al., 1989). Plasmids pNZ393, pNZ394, pNZ395, pNZ396, and pNZ397 were used to transform *E. coli* BL21(DE3)*lysS*, and the resulting strains were designated BL21-LacAB, BL21-LacC, BL21-LacD, BL21-LacA, and BL21-LacB, respectively. Expression of the various genes can be triggered by the addition of IPTG to the growth medium, resulting in the derepression of the T7 RNA polymerase gene. Proteins of induced and non-induced cells were labelled with ^{35}S-methionine as described (Tabor and Richardson, 1985; Studier et al., 1989) and separated on a SDS/polyacrylamide gel according to Laemmli (1970). After electrophoresis the gel was dried and the protein bands were visualized by autoradiography. As a negative control, cell-free extracts of *E. coli* BL21(DE3)*lysS*, carrying plasmid pET8c, were used.

Enzyme assays. *E. coli* cells were grown to an optical density (A600) of 0.5, and were divided in two parts. To one part 0.4 mM in IPTG was added (resulting in the

derepression of the T7 RNA polymerase), whereas no addition was made to the other part, and growth was continued for three hours at 37 °C. Cells were washed with one volume of assay-buffer (AB) consisting of 20 mM sodium-potassium phosphate buffer pH 6.5, containing 50 mM NaCl and 10 mM $MgCl_2$ (Crow et al., 1983). Subsequently, the cells were resuspended in 0.5 volume AB containing 1 mM dithiothreitol, and disrupted by sonification. Galactose 6-phosphate isomerase was assayed by a three-step procedure (Crow et al., 1983). The first step involved incubating extracts (2.5 μl) in an assay mixture (12.5 μl) containing 100 mM triethanolamine-HCl buffer (pH 7.8) and 10 mM D-tagatose 6-phosphate for 0, 3, 6, and 12 min, followed by terminating the reaction through a 5 min incubation at 100 °C. In step 2, 3.6 μl of 1 M glycine/NaOH buffer (pH 10,5), 1 μl alkaline phosphatase (1 U), and 7.4 μl H_2O, were added to the heat-treated reaction mixture, followed by incubation for 60 min at 25 °C. The final step involved the enzymatic determination of galactose as described by Kurz and Wallenfels (1974). The tagatose 6-phosphate kinase assays were performed as described by Bisset and Anderson (1980), the reaction mixture (0.25 ml) contained cell-free extract (1-5 μl), 67 mM glycylglycine-NaOH buffer (pH 8.5), 6.7 mM $MgCl_2$, 3.3 mM ATP, 3.3 mM phosphoenolpyruvate, 0.33 mM NADH, 0.33 mM D-tagatose 6-phosphate, and non-limiting amounts of pyruvate kinase (1.6 U) and lactate dehydrogenase (NH_4^+-salt, 10 U). Tagatose 1,6-diphosphate aldolase assays (0.25 ml) were performed as described (Crow and Thomas, 1982), and contained cell-free extract, 50 mM triethanolamine-HCl buffer (pH 7.8), 0.25 mM NADH, non-limiting amounts of the coupling enzymes α-glycerolphosphate dehydrogenase (1.5 U) and triose phosphate isomerase (4.5 U), and 0.16 mM tagatose 1,6-diphosphate. The reactions were monitored at 340 nm with a CARY 219 (Varian) absorbance-recording spectrofotometer thermostated at 25 °C. A correction for NADH oxidase and ATPase activities was obtained from a control reaction minus substrate. One unit of isomerase, kinase, and aldolase activity was defined as the amount of enzyme that catalyzed the formation of galactose 6-phosphate from tagatose 6-phosphate, the phosphorylation of D-tagatose 6-phosphate, and the cleavage of D-tagatose 1,6-diphosphate, respectively, at an initial rate of 1 μmol/min. Protein concentrations were measured according to Bradford (Bradford, 1976) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Nucleotide sequence and transcriptional analysis of the *L. lactis lacABCD* genes. Fig. 2 shows the nucleotide sequence of the 3.2 kb DNA region between the *lacR* and *lacF* genes of pMG820. Four large open reading frames (designated *lacABCD*) are present that all show the same orientation as the *lacFEG* genes. All open reading frames contain an ATG start codon (position 508, 950, 1476, and 2211, respectively) and are preceded by potential lactococcal ribosome-binding sites (ΔG^o values of complementarity to the *L.*

lactis 16S rRNA sequence: -16.6, -14.0, -8.4, and -9.8 kcal mol^{-1}, respectively (Tinoco et al., 1973)) at a distance that falls within the range (5-12) observed for *L. lactis* genes (de Vos, 1987). The deduced sizes of the proteins encoded by the *lacABCD* genes are 141, 171, 310, and 326 amino acids with calculated molecular sizes of 15,236, 18,926, 33,249, and 36,476 Da, respectively. Twenty-seven bp downstream of the *lacD* gene the lacF gene is initiated with an GTG start codon as described (de Vos et al., 1990). The *lacABCD* genes are preceded by a non-translated region containing the promoter, and a large amount of direct and inverted repeats involved in regulation of the lac-PTS operon[2].

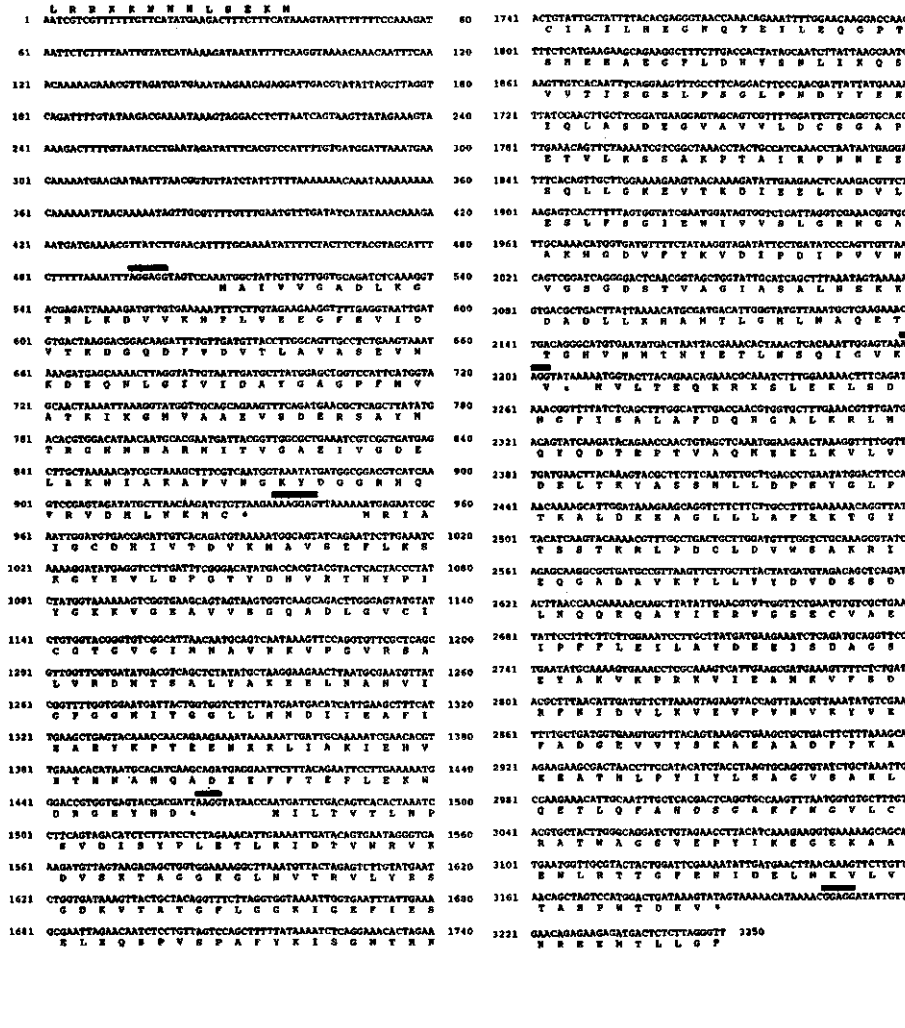

Fig. 2. Nucleotide sequence of the *L. lactis lacABCD* genes and deduced amino acid sequences of the encoded proteins. Translational stops (*) and putative ribosome binding sites ( ) are indicated. The N-terminal amino acid sequences of the LacR and LacF proteins are also shown, position 1-38, and 3219-3250, respectively (van Rooijen and de Vos, 1990; de Vos et al., 1990).

In order to investigate the transcriptional organization of the *lacABCD* genes, the 1.8 kb pMG820 insert of pNZ392 (containing the *lacABC* genes; see Fig. 1) was used as a probe. Fig. 3 shows the presence of 6 and 8.5 kb transcripts, the synthesis of which is induced during growth on lactose (lane 2 vs lane 1). These transcripts have the same sizes as those obtained with the *lacFEGX* genes as a probe and are a consequence of the presence of an intercistronic terminator between the *lacE* and *lacG* genes (de Vos et al., 1990). Therefore, we conclude that the tagatose 6-phosphate pathway gene cluster and the *lacFEGX* genes of *L. lactis* are part of the same lac-PTS operon. In addition, since we have previously observed (Van Rooijen and De Vos, 1990) that a *lacR* specific probe, upstream of the *Eco*RV site, did not hybridize with the *lac*-specific mRNA species, we conclude that the promoter of the lac-PTS operon must be located near the *Eco*RV (Fig. 1, position 500) site[2].

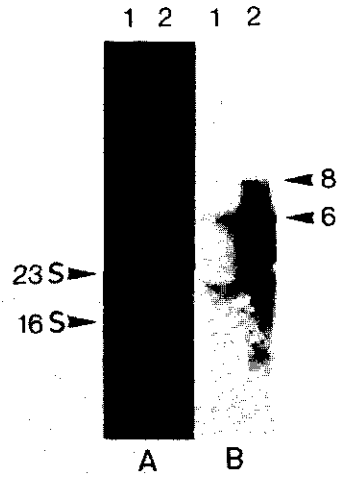

Fig. 3. Northern blot analysis of *lacABCD* gene expression in *L. lactis* MG1820. Cells were grown on glucose (lane 1) or lactose (lane 2) and 50 μg of isolated RNA was separated on a 1% denaturing agarose gel, that was either stained with ethidiumbromide (A) or, after blotting, hybridized with a *lacABC*-specific probe (B). The positions of the 23S (2.9 kb) and 16S (1.5 kb) rRNA's are indicated, as is the estimated size (kb) of the *lacABCD*-specific transcripts.

Expression of the *L. lactis lacABCD* genes in *E. coli*. For expression of the *lacABCD* genes in *E. coli* the expression vector pET8c was used, containing the ϕ_{10} T7 RNA polymerase promoter and its translation signals (Studier et al., 1989). Expression of the *lacAB*, *lacA*, and *lacB* genes is presented in Fig. 4 and resulted in the synthesis of 15 and 19 kDa (lane 12), 15 kDa (lane 6), and 19 kDa (lane 9) proteins, respectively. The 30 kDa protein in lanes 5 and 6 is the product of the bla gene of plasmid pNZ396, in which the ϕ_{10} terminator has been deleted (see Materials and Methods). Fig. 5 shows the expression of the *lacC* and *lacD* genes (lane 1 and lane 4, respectively) into 35 kDa proteins. In lane 4 the presence of an additional, strongly labelled protein of 7 kDa is visible. This is the gene product of a small open reading frame (201 nucleotides) that during the cloning procedure has been generated in pNZ395 and is preceded by the efficient ϕ_{10} ribosome binding and initiation site of the pET8c expression vector. The inefficient labelling of the LacD protein suggests that it is poorly expressed, although it shows significant enzyme specific activity in E.coli (see below). However, the abundant labelling of the 7 kDa protein (predicted to contain 6 methionine residues) may explain the less efficient incorporation of [^{35}S]methionine in the LacD protein.

The molecular weights of all induced Lac proteins correspond closely to that predicted from the deduced amino acid sequences of LacA, LacB, LacC, and LacD.

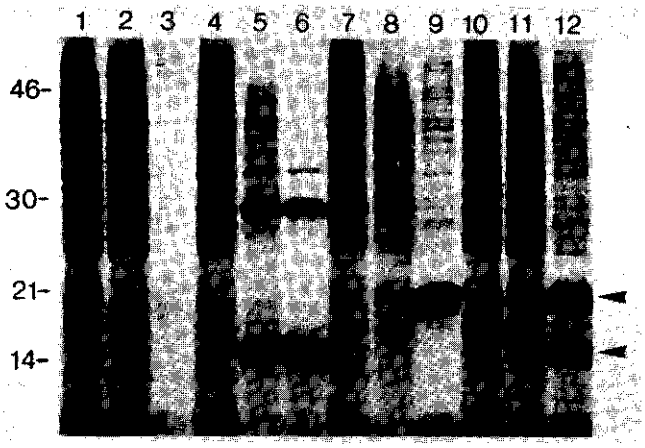

Fig. 4. Expression of the *L. lactis lacAB* genes in *E. coli*. Lane 1-3, 4-6, 7-9, and 10-12, contain extracts from non-induced, induced, and induced + rifampicin, *E. coli* BL21-pET8c (control), BL21-LacA, BL21-LacB, and BL21-LacAB cells, respectively. Proteins were labelled and separated on a 12.5% polyacrylamide/SDS gel. Molecular size markers (in kilodaltons) are indicated. Arrows indicate position of induced proteins.

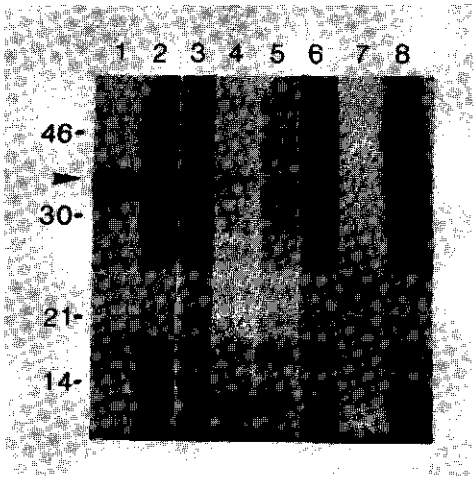

Fig. 5. Expression of the *L.lactis lacCD* genes in *E.coli*. Lane 1-3, *E.coli* BL21-LacC, induced + rifampicin, induced, and non-induced, respectively. Lane 4-6, *E. coli* BL21-LacD, induced + rifampicin, induced, and non-induced, respectively. Lane 7 and 8, control; *E. coli* BL21(DE3)*lysS* containing plasmid pET8c, induced + rifampicin, non-induced, respectively. Proteins were labelled and separated on a 12.5% polyacrylamide/SDS gel. Molecular size markers (in kilodaltons) are indicated. Arrow indicates position of induced proteins.

***LacAB* encodes galactose 6-phosphate isomerase.** In order to determine the functions of *lacA* and *lacB*, cell-free extracts were prepared from induced and non-induced cells of *E. coli* BL21-LacAB, BL21-LacA, BL21-LacB, respectively, followed by enzyme assays for galactose 6-phosphate isomerase (Table I). Induction of *E. coli* BL21-LacAB resulted in a 7-fold increase of the specific galactose 6-phosphate isomerase activity. The slight activity in extracts of non-induced *E. coli* BL21-LacAB cells (3.5 times higher than background activity in BL21-pET8c cells) could be attributed to the incomplete repression of the T7-polymerase dependent gene expression, that we occasionally observe. No galactose 6-phosphate isomerase activity was detected in extracts of induced or non-induced *E. coli* BL21-LacA or BL21-LacB. Therefore, we conclude that the galactose 6-phosphate isomerase activity is mediated by *lacA* and *lacB*. The galactose 6-phosphate isomerase of *S. aureus* has been partially purified and its native molecular weight has been estimated at 100 kDa (Bisset et al., 1980). Since the deduced amino acid sequences of the *L. lactis lacA* and *lacB* proteins are highly homologous to the *S. aureus lacA* and *lacB* proteins (Fig. 6), we assume that the *L. lactis* native galactose 6-phosphate isomerase is a multimer consisting of two subunits (*lacA* and *lacB*; 15 and 19 kDa, respectively). The nature of the interactions between these subunits awaits further investigation. Attempts to vizualize a native enzyme on a SDS-polyacrylamide gel by

omitting ß-mercaptoethanol during the preparation of the protein samples were unsuccessful (not shown). Combined extracts prepared from induced *E. coli* BL21-LacA and BL21-LacB cells (by incubating equal amounts of protein on ice for 30 min) did not result in detectable quantities of galactose 6-phosphate isomerase activity (results not shown). This could be due to an inefficient formation of the multimer from its subunits LacA and LacB. Alternatively, the presence of truncated LacA (49 aa) and LacB (32 aa) proteins in extracts of *E. coli* BL21-LacB and BL21-LacA could interfere with an efficient multimer formation.

TABLE I

Specific activities (activities expressed as nanomoles·min^{-1}·mg^{-1}) of enzymes in cell-free extracts prepared from induced or noninduced E. coli strains carrying the L. lactis MG1820 lacABCD genes

Strain	Galactose-6-P isomerase	Tagatose-6-P kinase	Tagatose-1,6-diP aldolase
BL21-LacAB			
+IPTG	330	—[a]	—
−IPTG	48	—	—
BL21-LacA			
+IPTG	10	—	—
−IPTG	8	—	—
BL21-LacB			
+IPTG	11	—	—
−IPTG	13	—	—
BL21-LacC			
+IPTG	—	92	—
−IPTG	—	10	—
BL21-LacD			
+IPTG	—	—	110
−IPTG	—	—	25
BL21-pET8c			
+IPTG	13	13	30
−IPTG	11	12	27

[a] Not determined.

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LacA (L. lactis) MAIVVGADLKGTRLKDVVKNFLVEEGFEVIDVTKDGQ-DFVDVTLAVASEVNKDEQNLGIVID
LacA (S. aureus) MAIIIGSDEAGKRLKEVIKSYLLDNKYDVVDVTEGQEVDFVDATLAVAKDVQSQEGNLGIVID
                 ***..* *  * ***.*.* .*... ..*.*** . . **** ***** .*  .* *******

LacB (L. lactis) EFLEKWDRGEYHD
LacB (S. aureus) EFLEKWDRGEYHD
                 *************
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Fig. 6. Homology between the deduced amino acid sequences of the N-terminal parts *L. lactis* LacA and *S. aureus* LacA (55%), and C-terminal parts of *L. lactis* LacB and *S. aureus* LacB (100%). The deduced partial amino acid sequences of the *S. aureus* LacA and LacB proteins have been published by Oskouian and Stewart (1990), and Rosey and Stewart (1989), respectively. In the LacA comparison one gap has been introduced to maximize identity. Identical and functionally related amino acids are indicated by an asterisk (*) and dot (.), respectively (Higgins and Sharp, 1988).

***LacC* and *lacD* encode tagatose 6-phosphate kinase, and tagatose 1,6-diphosphate aldolase, respectively.** The functions of *lacC* and *lacD* were determined by testing cell-free extracts, prepared from induced and non-induced cells of *E. coli* BL21-LacC and BL21-LacD, for tagatose 6-phosphate kinase and tagatose 1,6-diphosphate aldolase activity, respectively (Table I). Induction of *E. coli* BL21-LacC resulted in a 8-fold increase of the specific activity of tagatose 6-phosphate kinase. Therefore, we conclude that *lacC* encodes tagatose 6-phosphate kinase. It is conceivable that the observed *E. coli* background activity is due to the *E. coli* Pfk-2 protein that acts as a type II phosphofructokinase catalyzing the phosphorylation of tagatose 6-phosphate into tagatose 1,6-diphosphate in the galactitol metabolism (Lengeler, 1977). In a protein database search significant homology was found between the *L. lactis* LacC, *E. coli* Pfk-2 (Daldal, 1984) and *S. aureus* LacC (Rosey and Stewart, 1989) proteins (Fig. 7). The function of the *S. aureus* LacC protein has not yet been reported. The homology between the *L. lactis* LacC and *E. coli* Pfk-2 enzymes, both catalyzing the same reaction (i.e. phosphorylation of tagatose 6-phosphate), indicates that these enzymes have evolved from an common ancestor.

Induction of *E. coli* BL21-LacD resulted in the 4-fold increase of the specific tagatose 1,6-diphosphate aldolase activity. The tagatose 1,6-diphosphate aldolase gene of *L. lactis* H1 has been previously located on a 2.2 kb *Eco*RI-*Ava*I restriction fragment of plasmid pDI-1 (Yu et al., 1988). The *lacD* gene of *L. lactis* MG1820 is located on a similar sized *Eco*RI-*Ava*I plasmid-fragment (extending from position 1425 to 3700, Fig. 1). A comparison between the derived amino acid composition of the *L. lactis* LacD protein and that of the purified tagatose 1,6-diphosphate aldolase from *L. lactis* E8 (Crow and Thomas, 1982) showed that these proteins have an almost identical amino acid composition (Table II). From these data, the *E. coli* expression studies and enzyme assay, and the homology at the restriction map level between the *L. lactis* MG1820 *lacD* gene and the tagatose 1,6-diphosphate gene of *L. lactis* H1, we conclude that *lacD* encodes tagatose 1,6-diphosphate aldolase. Very strong homology was found between the *L. lactis* and *S. aureus* LacD proteins (73% identity, Fig. 8). No biological function for the *S. aureus* LacD protein has yet been published. The high degree of homology between the deduced amino acid sequences of the *L. lactis* and *S. aureus* LacABCD proteins strongly suggests that the *lacABCD* genes of *S. aureus* also code for the tagatose 6-phosphate pathway enzymes.

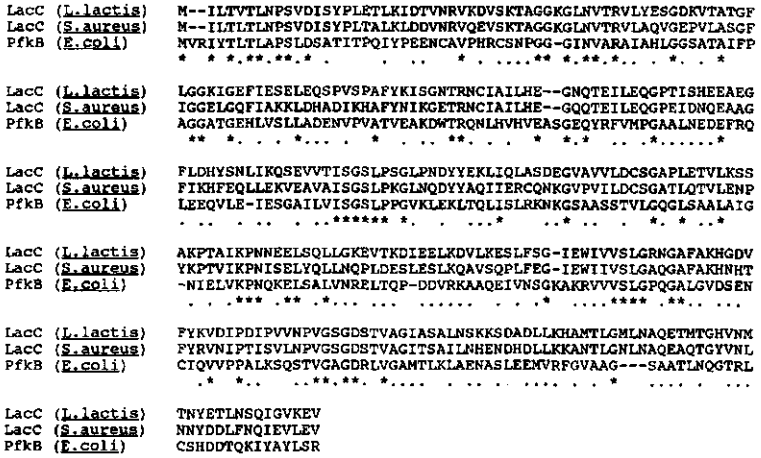

Fig. 7. Homology between the deduced amino acid sequences of *L. lactis* LacC, *S. aureus* LacC, and *E. coli* Pfk-2. The amino acid sequences have been aligned by introducing gaps to maximize identity. Percentage identity for pairwise comparisons are 61%, 26%, and 25% for *L. lactis* LacC and *S. aureus* LacC, *L. lactis* LacC and *E. coli* Pfk-2, and *S. aureus* LacC and *E. coli* Pfk-2, respectively. Identical and functionally related amino acids, present in all 3 proteins, are indicated by an asterisk star (*) and dot (.), respectively (Higgins and Sharp, 1988).

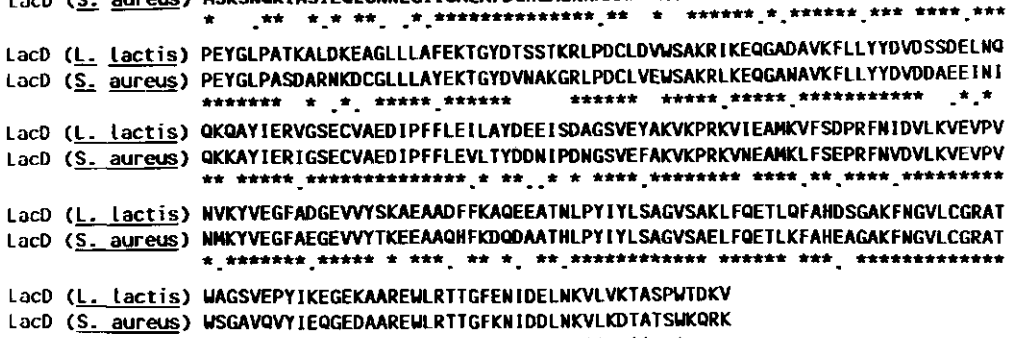

Fig. 8. Homology between the deduced amino acid sequences of *L. lactis* LacD and *S. aureus* LacD. Identical (*) and functionally related (.) amino acids are indicated (Higgins and Sharp, 1988). Percentage identity is 73%.

TABLE II

Comparison between the amino acid composition of purified tagtose-1,6-diphosphate aldolase (TDP-A) from L. lactis subsp. cremoris E8 (Crow and Thomas, 1982) and the deduced amino acid composition of L. lactis subsp. lactis MG1820 LacD

Amino acid	TDP-A (*L. cremoris*)	LacD (*L. lactis*)
Asp + Asn	30	31
Thr	17	15
Ser	25	20
Glu + Gln	46	44
Pro	13	11
Gly	19	17
Ala	34	35
Cys	ND[a]	3
Val	25	27
Met	4	5
Ile	9	11
Leu	30	32
Tyr	11	14
Phe	11	15
His	3	1
Lys	28	30
Arg	11	11
Trp	4	4
	320 + Cys	326

[a] ND, not determined.

CONCLUSIONS

In this paper, we present the nucleotide sequence of the genes (*lacABCD*) encoding the enzymes involved in the tagatose 6-phosphate pathway. The *lacAB*, *lacC*, and *lacD* genes code for the multimeric galactose 6-phosphate isomerase (15 and 19 kDa subunits), tagatose 6-phosphate kinase (33 kDa), and tagatose 1,6-diphosphate aldolase (36 kDa), respectively, and are located in between the *L. lactis lacR* and *lacFEGX* genes. Transcription studies showed that the *lacABCD* genes are transcribed as 6 and 8.5 kb polycistronic messengers together with the *lacFEGX* genes, and therefore, are part of the lac-PTS operon. To our knowledge this is the first molecular analysis of the tagatose 6-phosphate pathway, which has a pivotal role in the lactose and galactose metabolism in several Gram-positive bacteria.

Since it has been shown that a distinct galactose-PTS exists in *L. lactis* (Park and McKay, 1982; Crow et al., 1983; LeBlanc et al., 1979), it remains to be determined what the location is of the gal-PTS genes and whether they are coupled to the tagatose 6-phosphate pathway genes.

From this study and earlier studies (de Vos and Gasson, 1986; Van Rooijen and De Vos, 1990; de Vos et al., 1990), we conclude that the *L. lactis* lac-PTS regulon includes two distinct transcriptional units with the following gene order: *lacR-lacABCDFEGX*, which are followed by a *iso*-IS*S1* element.

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FOOTNOTES

[1] The abbreviations used are: IPTG, isopropyl-ß-D-galactopyranoside; kb, kilobases; PTS, phosphotransferase system; SDS, sodium dodecylsulphate.

[2] R.J. van Rooijen et al., manuscript in preparation

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CHAPTER 4

NUCLEOTIDE SEQUENCE OF AN *ISO*-IS*SI* ELEMENT FLANKING THE 3' END OF THE *LACTOCOCCUS LACTIS LAC* OPERON

Rutger J. van Rooijen and Willem M. de Vos

We have determined the nucleotide sequence of the 1.2 kb *Cla*I-*Pac*I DNA restriction fragment (Fig. 1) of the lactose miniplasmid pMG820 from *Lactococcus lactis* subsp. *lactis* strain MG1820 (1). The DNA fragment is located downstream of the *lac* operon, which encodes the lactose phosphotransferase and tagatose 6-phosphate pathway enzymes (*lacABCDFEGX*; 2,3), and appeared to contain an *iso*-IS*S1* element. The presence of this *iso*-IS*S1* element downstream of the *lac* operon has been reported previously (2). Analogous to IS*S1* (4) and other *iso*-IS*S1* elements (5-7) it contains an open reading frame (ORF1) that is flanked by 16-bp imperfect inverted repeats (Fig. 1). This ORF1 is orientated antisense with respect to the *lac* operon. The deduced amino acid sequence (226 residues) of ORF1 shows high identity (between 60 and 96%) to that of the putative transposases of other *iso*-IS*S1* elements (4-7). In addition, the inverted repeats that flank ORF1 are identical, with the exception of one base (right repeat), to those observed in other *iso*-IS*S1* elements (GGTTCTGTTGCAAAGTTT; (4)). Sixty-seven base pairs downstream of ORF1 the 3' end of the distal *lacX* gene of the *lac* operon is located (Fig. 1). ORF1 is preceded by a putative ribosome-binding site and promoter sequence (Fig. 1) that both correspond closely to those observed upstream of other lactococcal genes (8). The *iso*-IS*S1* copy described here is virtually identical to the IS-elements IS*S1*-N1 and IS*S1*-N2 that flank the *L.lactis* subsp. *cremoris* SK11 *prtM* and *prtP* proteinase genes (5,7). Interestingly, in many lactococcal strains both the proteinase and lactose genes are located on one large plasmid (9). This *iso*-IS*S1* element could be involved in the IS-promoted intermolecular rearrangements that have been described to occur during conjugal transfer of pMG820 or related lactose plasmids (10).

Fig. 1. Nucleotide sequence of the pMG820-located *iso*-IS*S1* element and deduced amino acid sequence of putative transposase. Imperfect inverted repeats (arrows), putative promoter sequence (underlined) and ribosome-binding site (###) are indicated. The C-terminal residues of LacX, that is encoded by the distal *lacX* gene of the *lac* operon (2), are shown.

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CHAPTER 5

MOLECULAR CLONING, TRANSCRIPTIONAL ANALYSIS AND NUCLEOTIDE SEQUENCE OF *LACR*, A GENE ENCODING THE REPRESSOR OF THE LACTOSE PHOSPHOTRANSFERASE SYSTEM OF *LACTOCOCCUS LACTIS*

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ABSTRACT

The repressor gene (*lacR*) of the lactose phosphotransferase system of *Lactococcus lactis* subsp. *lactis* strain MG1820 has been cloned and characterized. Transcription of *lacR*, into a 1.2 kb monocistronic messenger, is repressed approximately five-fold during growth on lactose. Nucleotide sequence analysis of the *lacR* gene showed the presence of an open reading frame of 861 bp. The deduced amino acid sequence of *lacR* is homologous to three *Escherichia coli* regulatory proteins (DeoR, FucR, and GutR) and includes a N-terminal domain (helix-turn-helix) involved in DNA-binding and a C-terminal domain that may be responsible for inducer binding. The *in vivo* function of *lacR* has been determined by introducing multiple copies of *lacR* into *L.lactis*, under control of its own or the unrelated *prtP* promoter. Growth rates and lactose PTS enzyme activities were measured during growth on lactose and glucose. The presence of *lacR* on a multicopy plasmid resulted in the decrease of lactose PTS activity, whereas only on lactose a decrease (25%) of growth rate was observed. No significant difference in growth rate was observed on glucose, indicating that *lacR* specifically represses the lactose genes of *L.lactis*.

INTRODUCTION

Regulation of gene expression in prokaryotes has been extensively studied in the last few decades (1,2,3). The lactose catabolic genes of *Escherichia coli* have been studied in great detail, both for historical reasons and because of the relatively easy enzyme assay of the gene products. The genes *lacZ* and *lacY*, encoding ß-galactosidase and lactose-permease, respectively, appear to be organized in an operon structure and are negatively controlled by a *lacI* encoded repressor protein (4,5). In contrast to *E. coli*, little is known about the origin and regulation of expression of the *lac* genes in Gram-positive bacteria. Metabolism of lactose by *Lactococcus lactis* is initiated by a phosphoenolpyruvate (PEP)[1] dependent phosphotransferase system (PTS). The lactose-specific components of PEP:PTS are a membrane-associated enzyme, EII^{lac}, and a soluble protein, $EIII^{lac}$ (6,7). Lactose appears intracellulary as lactose 6-phosphate, which is cleaved by phospho-ß-galactosidase to yield glucose and galactose 6-phosphate (8). The galactose 6-phosphate thus formed is metabolized via the tagatose 6-phosphate pathway as described for Staphylococcus aureus by Bisset and Anderson (9,10). Glucose is metabolized via the Embden-Meyerhof-Parnas pathway. The genes for phospho-ß-galactosidase (*lacG*), $enzymeII^{lac}$ (*lacE*) and $enzymeIII^{lac}$ (*lacF*) have been cloned and characterized[2] (11,12). The genetic arrangement of these genes in *L. lactis* is: *lacF-lacE-lacG*. The expression of these lac genes has been shown to be induced in *L. lactis*, *Streptococcus mutans*, Lactobacillus casei and *S. aureus* during growth on lactose or galactose (11,13,14,15). In *S. aureus*, a repressor gene has been characterized, located 2 kb upstream of *lacFEG* (16).

The present study describes the location, molecular cloning, and sequence and transcriptional analysis of the repressor gene (*lacR*) of the lactococcal *lac* operon. The *lacR* gene has been overexpressed in *E. coli* resulting in the synthesis of a 29-kDa protein. The deduced amino acid sequence of the *lacR* gene product was found to be homologous to that of various *E. coli* regulatory proteins. Furthermore, support for the functionality of *lacR* has been obtained by introducing into *L. lactis* a multicopy plasmid carrying the *lacR* gene.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* strains MC1061 (17), JM83 (18), TG1 (19) and HMS174 (20) were used as recipients in the cloning experiments. For overproduction of *lacR*, *E. coli* K12 lysogen BL21(DE3)*lysS* (21) was used. The *L. lactis* subsp. *lactis* strains used were MG1363 (plasmid-free strain, lac^- (22)), and its derivatives MG1820 (Lac^+), containing the lactose miniplasmid pMG820 (11), and MG5267 (Lac^+), containing a single chromosomally integrated copy of the *lac* operon (M.J. Gasson & W.M. de Vos, unpublished results). Plasmids used in the cloning experiments were pMG820, pNZ337

(23), pNZ18, derivative of pNZ12 (24), pUC18 (25), pET8c (21) and pKK232-8 (26).

Media, reagents and enzymes. Media based on M17 broth (Difco) containing 0,5% (wt/vol) glucose or lactose (27) and L-broth (1% tryptone, 0,5% yeast extract, 0,5% NaCl) were used for the growth of *L.lactis* and *E. coli*, respectively. Ampicillin (Ap), chloramphenicol (Cm), o-nitrophenyl-ß-D-galactopyranoside 6-phosphate (ONPG-P) and lysozyme were purchased from Sigma. Ampicillin and chloramphenicol were used in *E. coli* at a final concentration of 50 $\mu g\ ml^{-1}$ and 10 $\mu g\ ml^{-1}$, respectively. In *L.lactis*, chloramphenicol was used at a final concentration of 5 $\mu g\ ml^{-1}$. Restriction enzymes, T4 DNA ligase, RNAsin, DNAse, M-MLV reverse transcriptase, T4 DNA polymerase, T4 polynucleotide kinase, calf intestinal phosphatase, and IPTG were obtained from Promega, Bethesda Research Laboratories (BRL) or Biolabs and used under conditions as suggested by the suppliers. Sequenase and [α-^{32}P]dATP were purchased from Sopar Biochem. and Amersham, respectively.

DNA manipulations and transfer. Isolation of plasmid DNA from *E.coli* was performed by the alkaline lysis method (28). Isolation of plasmid DNA from *L.lactis* was performed by a modified alkaline lysis method using partially protoplasted cells, which were obtained by incubating cells in protoplast buffer (50 mM Tris hydrochloride pH 7.4, containing 25% (w/v) sucrose, 3 mM $MgCl_2$) and 1 mg lysozym per ml for 30 min at 37 °C. All subsequent manipulations of DNA *in vitro* and in *E.coli* , were performed as described by Maniatis *et al* (30). DNA was transformed into *L.lactis* by electroporation, using a Gene Pulser (Bio-Rad Laboratories). *L.lactis* MG1820 or MG5267 were grown to a optical density (600 nm) of 0.6-0.8. Cells were collected by centrifugation and washed three times with 1, 0.5 and 0.2 volumes of ice-cold 10% glycerol. Cells were resuspended in 1/50 volume of 10% glycerol and kept on ice for 10 min. DNA was added, and an electric pulse of 6.250 V/cm and 25 μF (1000 Ω resistance) was applied. Subsequently, ten volumes of M17-broth containing 0,5% (w/v) lactose were added and cells were incubated for 90 min at 30 °C before plating on selective media. Oligonucleotides were synthesized on a Biosearch Cyclone DNA synthesizer (New Brunswick Scientific).

RNA isolation. *L.lactis* MG1820 cells growing on glucose or lactose (100 ml) were harvested at an optical density (600 nm) of 0.6-0.8 and protoplasted by incubating in protoplastbuffer and 1 mg lysozym per ml for 10 min at 4 °C. Cells were pelleted, resuspended in 500 μl RNA-buffer I (20 mM Sodium acetate pH 5.5, 0.5 M sucrose, 1 mM EDTA) and lysed, by adding 2 ml of RNA-buffer II (20 mM Sodium acetate pH 5.5, 0.5 % SDS, 1 mM EDTA). As soon as lysis occurred 2.5 ml of hot (65 °C) acidic phenol (equilibrated with 20 mM Sodium acetate pH 5.5) was added followed by incubation for 10 min at 65 °C. After a acidic phenol/chloroform and chloroform

extraction, RNA was precipitated two times with 2.5 volumes ethanol and stored at -20 °C until use.

Northern blot analysis. RNA was glyoxylated, size fractionated on a 1.0 % agarose gel. One part of the gel was cut and the 16S and 23S rRNA's were visualized by staining in ethidium bromide and used as markers. The other part was blotted to a nylon membrane (Gene Screen; New England Nuclear) as recommended by the manufacturer. Prehybridization (2h) and hybridization (16h) were performed at 42 °C in 6xSSC (1xSSC is 0.15 M NaCl and 0.15 M sodium citrate), 50% formamide, 5x Denhart's solution (1x Denhart's is 0.2% Ficoll, 0.2% polyvinylpyrrolidone and 0.2% bovine serum albumin (29)), 0.1% sodium pyrophosphate, 0.1% SDS and 100 μg/ml sonicated denatured herring testis DNA. After hybridization, the membrane was washed twice with 2xSSC at room temperature for 5 min, and twice with 2xSSC, 0.1% sodium dodecyl sulphate at 65 °C for 30 min. Nicktranslated (29) restriction fragments were used as hybridization probes.

DNA sequence analysis. DNA fragments were inserted into the polylinker of M13mp18 and M13mp19 (25). Nucleotide sequences of both strands were determined by the dideoxy chain termination method as described by Sanger *et al* (30) using either M13 universal primer or synthesized primer. Samples were electrophoresed on a 6% polyacrylamide, 7.5 M urea sequencing gel. The sequencing strategy is presented in Fig. 1. Sequence data were assembled and analyzed using the PC/Gene program version 5.01 (Genofit, Geneva). The facilities of the Netherlands CAOS/CAMM Center (University of Nijmegen) were used to screen the protein databases SWISS-PROT and NBRF/NEW, releases 12.0 and 23.0, respectively.

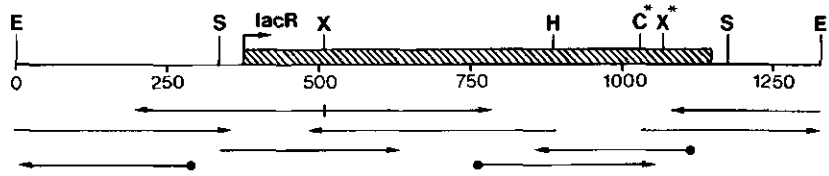

Fig. 1. Physical map and sequencing strategy of the region containing the *lacR* gene of the *L.lactis* MG1820 plasmid pMG820. The hatched bar indicates the coding region of the *lacR* gene, and the arrow shows the direction of the *lacR* gene. The positions of the restriction enzyme cleavage sites used in the DNA-sequencing and cloning experiments are indicated: C, *Cla*I; E, *Eco*RV; H, *Hin*dIII; S, *Ssp*I; X, *Xba*I; a star (*) above the restriction cleavage site indicates a *E.coli dam* methylation site. The arrows indicate the origin, direction, and extent of the individual sequencing reactions. A black dot preceding the arrow indicates the use of a sequence derived oligonucleotide primer.

Primer extension mapping. Primer extension was performed by annealing 1 pmol of oligonucleotide (5'-CATAAAGTAATTTTTTTCCA-3'; complementary to the mRNA from nucleotide 352-372) to 15 μg *L.lactis* MG1820 RNA (isolated during growth on glucose) in 70 mM Tris hydrochloride (pH 8.3), 10 mM $MgCl_2$, 15 mM dithiothreitol and 33 units RNAsin ribonuclease inhibitor in a total volume of 14 μl for 5 min at 65 °C. The mixture was allowed to cool to room temperature and adjusted to a final volume of 20 μl by the addition of dCTP, dGTP, dTTP (100 μM final concentration, each), dATP (10 μM final concentration), 15 μCi [α^{32}P]dATP and 20 units of M-MLV reverse transcriptase, followed by incubation for 30 min at 42 °C. Samples were deproteinized and ethanol precipitated. Pellets were dissolved in 3 μl H_2O and 3 μl sequence loading buffer, boiled for 3 min, and electrophoresed on a sequencing gel together with a M13-sequencing reaction obtained using the same oligonucleotide primer.

Phospho-ß-galactosidase and phosphotransferase assays. Cell-free extracts of 5 ml exponentially growing cultures were prepared by the glass bead method of Ranhand (31) with modifications. The cells were washed with 50 mM sodium phosphate buffer (pH 7.0), resuspended in 1 ml 50 mM sodium phosphate buffer (pH 7.0), 1 mM dithiothreitol and approximately 1.0 mg of glass beads (Zirconium Beads, 0.1 mm, Biospec) was added. The cells were broken by multiple cycles of high-speed vortexing (1 min) in the Biospec Mini BeadBeater, followed by cooling on ice (1 min). After disruption, the glassbeads and cell debris were removed by centrifugation. Phospho-ß-galactosidase activities were determined at 37 °C using the chromogenic substrate ONPG-P (11). Protein concentrations were measured according to Bradford (32) with bovine serum albumin as a standard. Phosphotransferase activities were measured as described by LeBlanc (33).

Overproduction and analysis of the *lacR* gene product. A *XmnI* site is located 11 bp downstream of the first ATG startcodon of the *lacR* gene. A double-stranded adaptor sequence was synthesized encoding amino acids 1-3 and part of amino acid 4, ending at the left arm of the *XmnI* site. This adaptor fragment was ligated together with a 1.1 kb *XmnI-Bam*HI-fragment of pNZ380 in the *Nco*I/*Bam*HI site of the T7 expression vector pET8c. The ligation mixture was used to transform *E.coli* HMS174. This resulted in the isolation of a clone containing a plasmid designated as pNZ387. For expression studies, pNZ387 was used to transform *E. coli* BL21(DE3)*lysS*, which contains the T7 RNA polymerase gene under control of *lacI* repressor, and the resulting *E.coli* strain was designated BL21-*lacR*. For overexpression of *lacR*, *E.coli* BL21-*lacR* was grown to an optical density of 0.8 at 37 °C and divided in two parts. To one part 0.4 mM in IPTG was added (resulting in the derepression of the T7 RNA polymerase), and growth was continued for another three hours at 37 °C. Samples (100 μl) were taken and the cells were collected by centrifugation. Pellets were resuspended in 30 μl lysis buffer [50 mM

Tris hydrochloride (pH 6.8), 0.5 % (wt/vol) SDS, 1 mM EDTA, 0.5% (vol/vol) ß-mercaptoethanol, 4% (vol/vol) glycerol and 0.001% bromophenol blue] and boiled for 3 min. Subsequently, the samples were electrophoresed on a 12.5% polyacrylamide/SDS gel and protein bands were visualized by staining with Coomassie blue (34). As a control, the same procedure was performed with *E.coli* BL21(DE3)*lysS* cells carrying plasmid pET8c.

Construction of plasmids. Plasmids pNZ380 and pNZ381 contain the 1.3 *Eco*RV DNA-fragment from pMG820 cloned into the *Sma*I-site of pUC18. pNZ380 contains the *lacR* gene in the same orientation as the *lacZ* gene, whereas in pNZ381 *lacR* has the reversed orientation. Plasmid pNZ382 contains a 1.3 kb *Pst*I-*Sst*I fragment from pNZ380 cloned into the *Pst*I-*Sst*I site of the lactococcal cloning vector pNZ18. Therefore, plasmid pNZ382 contains the *lacR* gene under control of its own promoter. For the construction of plasmid pNZ386 three subclones were constructed as followed: (1) a 0.35 kb *Hpa*I-*Kpn*I fragment from pNZ337, containing the proteinase (*prtP*) promoter (23), was cloned into the *Sma*I-*Kpn*I site of pUC18 to yield pNZ383, (2) a 0.9 kb SspI fragment from pNZ380, containing lacR without promoter sequence, was cloned into the *Sma*I site of pUC18 to yield pNZ384, and (3) a 0.9 kb *Bam*HI (filled in with Klenow DNA polymerase)-*Eco*RI fragment from pNZ384 was cloned into the Asp718 (filled in with Klenow DNA polymerase)-*Eco*RI site of pNZ383 to yield pNZ385. Finally, pNZ386 was constructed by cloning a 1.25 kb *Pst*I-*Sst*I fragment from pNZ385 into the *Pst*I-*Sst*I site of pNZ18. As a consequence of these manipulations, pNZ386 contains the *lacR* gene under control of the proteinase (*prtP*) promoter.
In order to test the functionality of the *lacR* promoter *in vivo*, a 0.3 kb SspI-*Kpn*I restriction fragment of pNZ380 was subcloned into the *Sma*I-*Kpn*I site of pUC18 to yield pNZ388, followed by cloning a 0.3 kb *Eco*RI (filled in with Klenow DNA polymerase)-*Bam*HI fragment of this plasmid into the *Sma*I-*Bam*HI site of the *E. coli* promoter/probe vector pKK232-8 (26). This plasmid was designated pNZ389.

RESULTS AND DISCUSSION

Transcriptional analysis of the *lac* genes of *L.lactis* MG1820. In *L.lactis* the activity of the lactose PTS enzymes is induced approximately ten-fold during growth on lactose (11,33). The *lac* genes of *L.lactis* MG1820 are localized on a 23.7 kb plasmid, pMG820 (Fig. 2). In order to investigate the transcriptional organization and nature of induction of the *lac* genes, Northern-blots were made using RNA isolated from *L.lactis* MG1820 grown on lactose or glucose. As DNA-probes, several pMG820 restriction-fragments in and upstream of the *lac*tose genes were used. The results obtained with the most upstream probe, the 1.3 kb *Eco*RV DNA fragment (Fig. 2, hatched bar), are presented in this paper. The results obtained with the other probes will be presented elsewhere[2,3].

The Northern-blot in Fig. 3 shows the presence of a 1.2 kb transcript, the synthesis of which is repressed (five-fold) during growth on lactose (lane 2). The direction of this transcript appeared to be opposite to that of the *lac* genes, as obtained from the sequence data and the primer extension experiment (see below). Thus, while the expression of the *lac* genes is induced during growth on lactose, the expression of a divergently transcribed gene, designated *lacR*, is repressed. A possible explanation for this could be that *lacR* encodes a protein that represses the transcription of the *lac* genes.

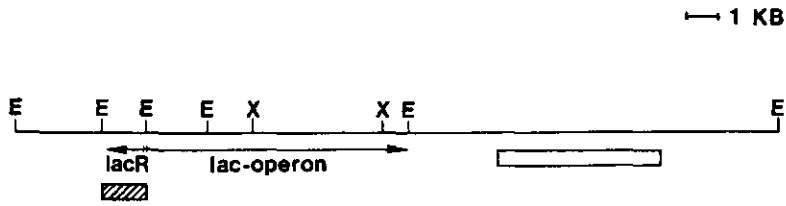

Fig. 2. Linear map of the *L.lactis* MG1820 plasmid pMG820. The *lac* operon and the *lacR* gene are indicated by arrows. The hatched bar represents the DNA probe used in the Northern blot experiment. The positions of some restriction enzyme cleavage sites are indicated: E, *Eco*RV; X, XhoI. The open bar represents the replication region (11).

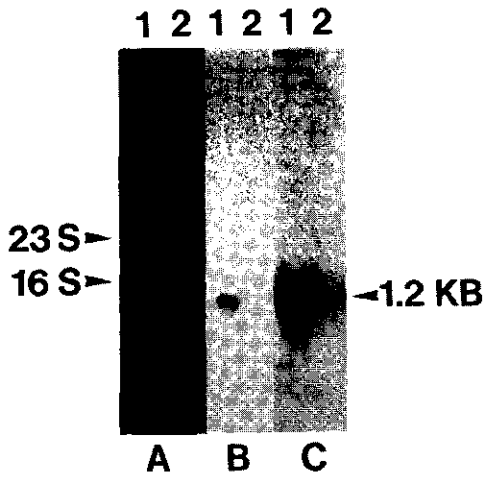

Fig. 3. Northern blot analysis of total RNA of *L.lactis*. *L.lactis* MG1820 was grown on glucose (lane 1) or lactose (lane 2) and as a hybridization probe a 1.3 kb *Eco*RV DNA fragment was used. As an internal control and molecular weight markers, part of the gel (A), containing the same samples, was stained with ethidium bromide. B: overnight exposure, C: 5 days exposure.

Nucleotide sequence of the *L.lactis lacR* gene. The nucleotide sequence of the 1.3 kb *Eco*RV DNA-fragment was determined (Fig. 1) and revealed an open reading frame of 861 base pairs, which is orientated antisense with respect to the *lac* genes (Fig. 4). Two ATG start codons in the open reading frame (at position 370, and 388, respectively) are preceded by a potential lactococcal ribosome-binding site (ΔG values of complementarity to the *L.lactis* 3' 16S rRNA sequence: -7.2 and -6.6 kcal mol^{-1} (35), respectively) at a distance that falls within the range (5-12) observed for *L.lactis* genes (24). The two open reading frames could encode proteins of 261 and 255 amino acids, having calculated molecular sizes of 29,342 and 28,617 Da, respectively. Although N-terminal sequence analysis of *lacR* must be performed to discriminate between these two possibilities, the homology comparison described below (Fig. 6), suggests that the second ATG start codon is used. An interesting feature of the nucleotide sequence preceding *lacR* is the presence of various long repeats and the high content (84%) of T-residues from position 31 to 68. Possibly, this region is involved in the regulation of expression of *lacR*. The sequence of the *lacR* gene is followed by an inverted repeat and T-stretch (Fig. 4), indicating a rho-independent transcription terminator with a ΔG value of -8.6 kcal mol^{-1} (35). Additional evidence for the functionality of this transcription terminator in *E.coli* and *L.lactis* was obtained by cloning this terminator between the vector-localized promoter and the *lacG* gene of the *L.lactis* promoter-probe vector pNZ336 (23). In both *E. coli* and *L.lactis* the phospho-ß-galactosidase activity was reduced five-fold (data not shown).

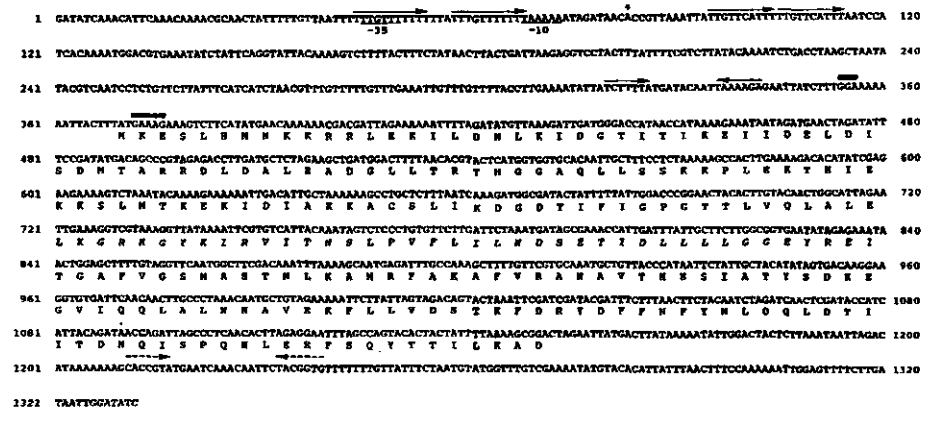

Fig. 4. Nucleotide sequence of the *L.lactis lacR* gene and derived amino acid sequence of the encoded protein. The transcription start site (*), promoter (underlined), and repeats (arrows) are indicated; ▬,putative ribosome binding site. Dotted arrows indicate the stem structure of the putative rho-independent terminator; ΔG value: -8.6 kcal mol^{-1}

Mapping and characterization of the *lacR* promoter. The exact start of the *lacR* transcript was determined by primer extension analysis, using an oligonucleotide primer, complementary to the coding strand from position 352 to 372, (see Fig. 4). One and the same primer-extension product was observed using RNA isolated from glucose and lactose grown cells (not shown). Using the sequencing ladder obtained with the same primer, the transcription start site has been mapped at the A-residue at position 83 (see Fig. 4). Therefore, the promoter sequence of the *lacR* gene is TTGTTT (-35) and TAAAAA (-10), separated by 17 base pairs and located 287 basepairs upstream of the first putative *lacR* startcodon (Fig. 4). This promoter sequence corresponds to the consensus promoter sequences found for other *L.lactis* genes (24). No extension product was found using a primer complementairy to the coding strand (not shown), confirming the orientation of *lacR* (Fig. 2). Since *lacR* is transcribed as a monocistronic messenger, with an estimated molecular size of 1.2 kb, these results are additional proof for the functionality of the trancriptional terminator, described above.

In order to test the functionality of the promoter *in vivo*, the promoter was cloned upstream the promoterless chloramphenicol transferase (CAT*) gene of the E.coli* promoter-probe vector pKK232-8 (26), to yield pNZ389. *E. coli* MC1061 clones harboring pNZ389 were chloramphenicol-resistant up to a concentration of 50 μg/ml, whereas *E.coli* cells containing pKK232-8 were sensitive to less than 1 μg/ml chloramphenicol.

***LacR* encodes a 29-kDa protein.** For overexpression of *lacR* in *E.coli* the expression vector pET8c was used, containing both the ϕ_{10} T7 RNA polymerase promoter and its translation initiation signals (21). The *E.coli* strain BL21-*lacR* contains a chromosomally localized T7 RNA polymerase under control of the *lacI* repressor, and harbors two plasmids: (1) a pACYC184 derived plasmid containing a constitutively expressed T4 lysozyme, a natural inhibitor of T7 RNA polymerase, which reduces the basal activity of T7 RNA polymerase (21), and (2) pNZ387, a pET8c derivative, containing the entire *lacR* gene. Expression of the *lacR* gene can be triggered by the addition of IPTG to the growth medium, resulting in the derepression of the T7 RNA polymerase gene. The total cellular protein shows the appearance of a 29-kD protein after 3 hours induction (Fig. 5, lane 4). The molecular weight of this induced protein corresponds closely to that predicted from the deduced amino acid sequence of *lacR*. This protein was not present in lysates of non-induced BL21-*lacR* (Fig. 5, lane 3) or in induced and non-induced cells containing plasmid pET8c (Fig. 5, lanes 2 and 1, respectively).

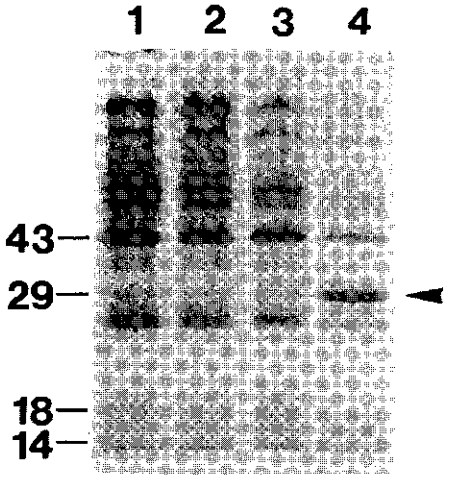

Fig. 5. Overexpression of *L.lactis* LacR in *E.coli* BL21-*lacR*. Lane 1 and 2, *E.coli* BL21(DE3)*lysS* containing plasmid pET8c, non-induced and induced, respectively. Lane 3 and 4, *E.coli* BL21-*lacR*, non-induced and induced, respectively. Samples were electrophoresed on a 12.5% polyacrylamide/SDS gel. Molecular size markers (in kilodaltons) are indicated. Arrow indicates position of the induced protein.

***LacR* represses the *lac* genes of *L.lactis*.** In order to determine the function of *lacR* in *L.lactis*, plasmids pNZ382, pNZ386, and pNZ18 were used to transform *L.lactis* MG5267, containing chromosomally located lactose genes. The growth rates of the transformants were measured on media containing glucose or lactose as a sole carbohydrate present (see Table I). The presence of *lacR* on a multicopy plasmid, under control of either its own or the *prtP* promoter (pNZ382 or pNZ386, respectively), reduces the growth rate of the *L.lactis* cells on lactose by 25%, as compared to that of strain MG5267 containing the vector pNZ18. No difference in growth rate was observed when the strains were grown on glucose. In order to verify this at the enzymatic level, phospho-ß-galactosidase and lactose-PTS activities were determined (see Table I). Both on lactose or glucose, the phospho-ß-galactosidase and lactose PTS activity were reduced as compared with the control strain (MG5267 harboring pNZ18). Apparently, this reduction results in a decreased growth rate only when cells are grown on lactose. Therefore, we conclude that *lacR* specifically represses the lactose catabolic genes of *L.lactis* The question arises why the presence of pNZ382 or pNZ386 does not result in a non-inducible phenotype, with phospho-ß-galactosidase and lactose-PTS activities comparable with activities obtained during growth on glucose. This could be attributed to the limited strength of the *lacR* or *prtP* promoters (23), resulting in synthesis of insufficient amounts of *lacR* to occupy all *lacR* binding sites. Alternatively, *lacR* may

control its own synthesis, as is the case for lambda repressor (2). Evidence for the presence of a control circuit for *lacR* expression has been obtained from the transcription data, as described above.

Table I. Effects of multiple copies of *lacR* on lac-PTS activity and growth rate. Growth rates, phospho-ß-galactosidase (P-ß-Gal), and lactose PEP-dependent phosphotransferase (PEP/PTS_{lac}) activities of *L. lactis* MG5267 carrying various plasmids, grown on glucose- or lactose-containing media.

Carbohydrate	Plasmid	Growth rate[a]	P-ß-gal activity[b]	PEP/PTS_{lac} activity[c]
lactose	pNZ382	67	1097	21
	pNZ386	65	503	29
	pNZ18	52	1287	53
glucose	pNZ382	41	93	2
	pNZ386	40	30	3
	pNZ18	40	103	6

[a] Growth rates expressed as generation time (min)

[b] Activities expressed as nanomoles o-nitrophenol released per minute per mg protein.

[c] Activities expressed as nanomoles per minute per milligram of cell dry weight.

LacR is homologous to other regulatory proteins. A protein data-base search was performed and significant homology was found with the repressor proteins of the deoxyribose (deo), fucose (*fuc*), and glucitol (*gut*) and galactitol (*gat*) operons of *E. coli* (36,37,38,39): DeoR (25% identity), FucR (29% identity), and GutR (33% identity), respectively (Fig. 6). Glucitol and galactitol catabolism in *E. coli* are initiated by a PEP-dependent phosphotransferase system (PTS), comparable to lactose catabolism in *L. lactis*. Intermediates of the galactitol catabolic pathway (*gat*) include tagatose 6-phosphate and tagatose 1,6-diphosphate, which are also intermediates in the lactose catabolic pathway of *L. lactis*. The highest homology was found in the region where DeoR is presumed to bind the operator of the *deo* operon (5,40). Computer-assisted analysis predicted the protein secondary structure of this region (position 25 to 45 for LacR) to have a helix-turn-helix motif, for all 4 proteins. This type of protein secondary structure is a common feature for DNA-binding proteins and is involved in the binding of operator-DNA (41). In addition, when the amino acid sequence of the putative helix-turn-helix motif was

compared with that of other DNA binding proteins (41), homology (27-36% identity) was found with TrpR, FnR, LacI, GalR, CAP, LysR, and MuB (data not shown). LacR, DeoR, GutR, and FucR showed a high overall homology, whereas in the other regulatory proteins homology was found only in the DNA-binding region. Since LacR, DeoR, GutR, and FucR are all regulatory proteins of a catabolic pathway, this could be an indication that they share a common ancestor. Another region of high homology is localized between amino acids 212 and 222 (see Fig. 6). When this amino acid sequence was used in a protein data-base search, homology was found with one *E.coli* regulatory protein, and several enzymes from prokaryotic and eukaryotic organisms involved in sugar or nucleoside metabolism (see Fig. 7). Because there are no common catalytic functions between these proteins, this region might be involved in binding of the various substrates. Since it has been postulated that the inducers of the *deo*, *fuc*, and *gut* operons of *E.coli*, and the *lac* genes of *L.lactis* are deoxyribose 5-phosphate, fuculose 1-phosphate, glucitol or glucitol 6-phosphate, and galactose 6-phosphate, respectively (42,39,43,44), this highly conserved region may be the inducer-binding site of these repressors.

Fig. 6. Homology between the deduced amino acid sequences of *L.lactis* LacR and other regulatory proteins. DeoR, GutR, and FucR are the repressor proteins of the deoxyribonucleoside, glucitol and galactitol, and fucose operons of *E.coli* (36,38,39,37). The amino acids sequences are given in the one-letter code and have been aligned by introducing gaps to maximize identity. Percentage identity for pairwise comparisons were

33%, 29%, and 25% for *L.lactis* LacR and *E.coli* GutR, FucR, and DeoR, respectively. Comparisons between *E.coli* GutR and *E.coli* FucR and DeoR, and between FucR and DeoR were, 28 %, 24%, and 22%, respectively. Functionally related and identical amino acids, present in all 4 proteins, are indicated by a black dot (.) and a star (*), respectively (45). The 'helix-turn-helix' motif and putative inducer-binding site are indicated by an "*open bar-line-open bar*" and *hatched* bar, respectively.

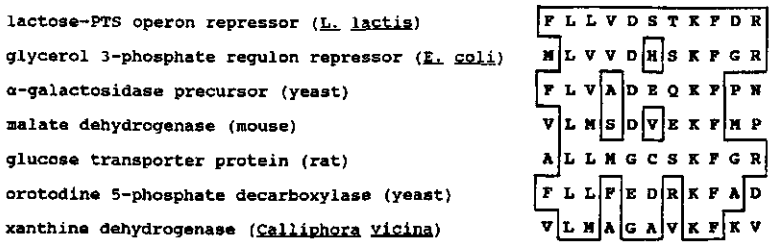

Fig. 7. Homology of the putative inducer-binding site of LacR. The amino acid sequence from a fragment of LacR (position 212-222) is aligned with homologous sequences of: glycerol 3-phosphate regulon repressor (*E.coli*), α-galactosidase precursor (yeast), malate dehydrogenase (mouse), glucose transporter protein (rat), orotodine 5-phosphate decarboxylase (yeast), xanthine dehydrogenase (Calliphora vicina); the codes of these proteins in the SWISS or NBRF protein databases are GLPR$ECOLI, AGAL$YEAST, MAOX$MOUSE, GTR2$RAT, DEBYOP, and XDH$CALVI, respectively. Functionally related and identical amino acids are boxed.

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FOOTNOTES

1 The abbreviations used are: IPTG, isopropyl-ß-D-thiogalactopyranoside; kb, kilobases; SDS, sodium dodecyl sulphate

2 W.M. de Vos, in preparation

3 R.J. van Rooijen, in preparation

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CHAPTER 6

CHARACTERIZATION OF THE *LACTOCOCCUS LACTIS* LACTOSE OPERON PROMOTER: CONTRIBUTION OF FLANKING SEQUENCES AND LACR REPRESSOR TO PROMOTER ACTIVITY

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ABSTRACT

The location, activity, and regulation were determined of the promoter of the *Lactococcus lactis* 8-kb lactose operon (*lacABCDFEGX*), encoding the enzymes of the lactose phosphotransferase system and the tagatose-6-phosphate pathway. The *lac* promoter sequence corresponds closely to the consensus promoter described for gram-positive bacteria and is located in a back-to-back configuration with the promoter of the divergently transcribed *lacR* gene, encoding the LacR repressor. The transcription start sites used under induced (lactose) and non-induced (glucose) conditions were determined. The minimal promoter region that could be isolated on a single restriction fragment included sequences ranging from -75 to +42. The effect of the presence of flanking sequences and the *lacR* gene on promoter activity and regulation was studied in *Escherichia coli* and *L.lactis* strains using transcriptional fusions with promoterless chloramphenicol acetyl transferase reporter genes. The results show that transcriptional regulation of the *lac* operon is mediated by the interaction between LacR repressor, *lac* promoter, and sequences in the non-coding region between the *lacR* and *lacA* genes. Sequences flanking the minimal promoter region appeared to enhance *lac* promoter activity much more in *L.lactis* (5- to 38-fold) than in *E.coli* (1.3- to 5-fold).

INTRODUCTION

The last decade has shown a considerable progress in the molecular genetics of lactic acid bacteria. Several genes that encode the key enzymes of catabolic pathways have been cloned and characterized, including those involved in sugar (16,17,37,49), citrate (12) and casein utilization (25,52). Relatively little is known about the regulation of expression of these and other genes in lactic acid bacteria. A consensus for *Lactococcus lactis* promoter sequences was postulated (15) that conformed to the consensus for Bacillus *subtilis* (22). However, significant differences in activities of identical promoter DNA sequences were observed between these two gram-positive species (47), indicating that different cellular factors are involved in determining promoter activity and/or messenger RNA stability. Until now two regulatory proteins both from *L. lactis*, have been identified: the MleR activator, that is homologous to the LysR family of positive regulators from gram-negative bacteria (38); and the LacR repressor, that belongs to the DeoR family of repressors (48). However, the molecular targets of these regulatory proteins have not yet been identified.

Regulation of expression of the *Escherichia coli* lactose catabolic genes (*lacZYA*) has been studied in great detail (27,34) and has been a paradigm for studying gene regulation in other bacteria. In *L. lactis* strains used in industrial dairy fermentations lactose is metabolized via a phospho*enol*pyruvate-dependent phosphotransferase system (PEP-PTS^{lac}; 17). The resulting intracellular lactose 6-phosphate is cleaved into galactose 6-phosphate and glucose that are subsequently metabolized via the tagatose-6-phosphate (4) and glycolytic pathways, respectively. The genes encoding the PEP-PTS^{lac} and tagatose 6-phosphate pathway enzymes are organized in the 8-kb *lac* operon comprising the *lacABCDFEGX* genes (Fig. 1; 17,49). Expression of the *lac* operon is repressed ten-fold during growth on glucose and is regulated at the transcriptional level (17,48) by the LacR repressor, the product of the divergently transcribed *lacR* gene (48). The *L. lactis* and *Staphylococcus aureus lac* operons and *lacR* genes appear to be highly homologous (17,36,48,49). The main differences in their genetic organizations is that the *S. aureus lacR* gene has the same orientation as the structural genes and that the distal *L. lactis lacX* gene is not present in the *S. aureus lac* operon (17,35). In this paper we present the molecular characterization of the promoter of the *L. lactis lac* operon. DNA sequences flanking the *lac* promoter appear to be involved in transcription activity, regulation, and/or stability of the produced transcript. Furthermore, the presence of the *lacR* gene results in a decreased activity of the *lac* promoter.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. *E.coli* strains MC1061 (8), HB101 (39), and JM83 (51) were used as recipients in the cloning experiments. The *L.lactis* subsp. *lactis* strains used were MG1363 (plasmid-free strain, Lac^-; 19) and its Lac^+ derivatives MG1820, containing the lactose miniplasmid pMG820 (29), and MG5267, containing a single chromosomally integrated copy of the *lac* operon (20). Media based on M17 broth (Difco Laboratories, Detroit, Mich.) containing 0.5 % (w/v) glucose or lactose (43), and L-broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl), were used for the growth of *L.lactis* and *E.coli*, respectively. If appropriate, media were supplemented with ampicillin (50 μg/ml), erythromycin (5 μg/ml), and chloramphenicol (Cm; 10 μg/ml for *E.coli* and 5 μg/ml for *L.lactis*). Plasmid vectors used in the cloning experiments were pACYC184 (9), pKK232-8 (7), and pUC18 (54) for *E.coli* and pGKV210 (47) for *E.coli* and *L.lactis*. The plasmids pNZ337 (41), pNZ390 (49), and pNZ380 (48) were used as a source for the *prtP* promoter (52), *lacRABC* genes, and the *lacR* gene including its expression signals, respectively. Plasmid copy numbers in *E.coli* were determined using part of plasmid pBR327::*recA* (14) as a probe for the *chromosomal RecA* gene (22).

Molecular cloning, reagents, and enzymes. Isolation of DNA from *E.coli* and *L.lactis* was performed by the alkaline lysis method (3) and a modified alkaline lysis method (16), respectively. All manipulations *in vitro* and in *E.coli* were performed as described (39). DNA was transformed into *L.lactis* by electroporation as described (52). All enzymes and butyryl-Coenzyme A were purchased from Bethesda Research Laboratories (Gaitersburg, Md.), New England Biolabs Inc. (Beverly, Mass.), or Promega Corporation (Madison, Wisconsin) and used according to the instructions of the manufacturers. Sequenase and o-nitrophenyl-ß-D-galactopyranoside 6-phosphate (ONPG-P) were purchased from U.S. Biochemical Corp. (Cleveland, Ohio) and Sigma (Chemical Company, St. Louis, U.S.A.), respectively. ^{14}C-Cm, [α-^{32}P]dATP, and [γ-^{32}P]ATP were supplied by Amersham International plc (UK). Oligonucleotides were synthesized on a Cyclone DNA synthesizer (Biosearch, San Rafael, Calif.).

RNA isolation and primer extension analysis. Total RNA was isolated from protoplasted glucose- or lactose-grown *L.lactis* MG1820 cells as described previously (48). Primer extension was performed by annealing 1 pmol of oligonucleotide (5'-GCCATTTGGACTACCT-3'; complementary to the *lac* operon mRNA, position 83-99) to 15 μg of RNA followed by cDNA synthesis as described (48). Primer extended products were separated on a 6% polyacrylamide/8 M urea sequencing gel together with the products of a double-stranded sequence reaction (10) obtained with the same primer and pMG820 DNA.

Enzyme assays. *L.lactis* and *E.coli* cultures were grown to late exponential phase (OD_{600}=0.7) and induced with 5 μg/ml Cm for 30 min. prior to harvesting (28). Cells were washed and resuspended in 0.25 M Tris-hydrochloride (pH 8.0) and, in the case of *L.lactis*, supplemented with 0.5 g/ml of Zirconium glass beads (0.1 mm, Biospec Products, Bartlessville, Oklahoma). *L.lactis* and *E.coli* cells were disrupted by high speed vortexing (2 min, 3 cycles; Biospec Mini BeadBeater) and sonification (15 s, 2 cycles; Heat Systems Inc. Sonicator), respectively. After disruption, cell-free extracts were isolated by centrifugation. Cm acetyl transferase (CAT) activities were determined at 37 °C by measuring the ^{14}C-labelled butyryl-CoA in the phase-extraction assay (40). Phospho-ß-galactosidase (P-ß-gal) activities were determined at 37 °C using the chromogenic substrate ONPG-P (29). Protein concentrations were measured according to Bradford (5) with bovine serum albumin as a standard.

Determination of plasmid copy numbers. Cells were grown and harvested as described in the previous section, and total DNA was isolated after lysis of protoplasted cells as described (25,39). Total DNA isolated from plasmid-containing *L.lactis* MG5267, MG1363, or *E.coli* MC1061 cells was digested with *Eco*RV, *Hind*III, and *Hin*fI, respectively, subsequently electrophoresed on a 1% agarosegel (39) and transferred to a GeneScreen Plus (New England Nuclear) membrane following the procedure recommended by the manufacturer. Since a single chromosomal copy of the *lac* promoter is present in MG5267, the membrane containing DNA from MG5267 cells was hybridized only with a γ[^{32}P]ATP end-labelled (39) primer specific for the *lac* promoter region (position -40 to +5). This resulted in hybridization of linearized plasmid DNA (4.6 to 5.9 kb) and a 1.3-kb chromosomal restriction fragment. The membrane containing DNA from MG1363 cells was hybridized simultaneously with two primers, the *lac* specific probe, and a probe for the chromosomally located single copy of the *usp* gene (45). This resulted in hybridization of linearized plasmids (*lac*-specific probe; 4.6 to 5.9 kb) and a 2.9-kb chromosomal restriction fragment (*usp*-specific probe). The membrane containing DNA from *E.coli* MC1061 cells was hybridized with the *lac*-specific probe and a nicktranslated (39) 0.45 kb *Eco*RV-*Eco*RI restriction fragment from pBR327::*rec*A, that contains part of the *E.coli rec*A gene (14,23). This resulted in hybridization of a 1.0-kb fragment (*rec*A-specific probe) and plasmid-derived fragments that varied in size between 0.5 and 1.5 kb (*lac*-specific probe). Following autoradiography, the hybridizing restriction fragments were cut out and total radioactivity was determined using a liquid scintillation counter (Beckman LS7500). The number of plasmid copies per chromosome in MG5267 was calculated based on the ratio between the plasmid- and chromosome-derived hybridization signals. Since the specific activities of the used *lac and usp* probes appeared to be identical, the ratio between the signals of the plasmid-located *lac* and chromosomal *usp* genes was used to calculate the plasmid copy number per chromosome in MG1363. We did not determine whether the specific activity of the *rec*A probe was

identical to that of the *lac* probe. Therefore, in *E.coli* MC1061 only the relative plasmid copy numbers were calculated.

Construction of plasmids. Plasmids pNZ398 and pNZ399 contain the 0.5-kb *Xmn*I-*Bgl*II and 0.35-kb *Ssp*I restriction fragments of pNZ390 cloned into the *Sma*I-*Bam*HI and *Sma*I sites of pUC18, respectively. Both plasmids contain the *L.lactis lac* promoter in the same orientation as the vector-localized *E.coli lacZα* gene. Plasmids pNZ3000 and pNZ3003 contain the 0.12-kb *Dra*I-*Bam*HI and 0.35-kb *Eco*RI-*Bam*HI fragments of pNZ399 cloned into the *Sma*I-*Bam*HI and *Eco*RI-*Bam*HI sites of the pGKV210, respectively. Plasmids pNZ3001, pNZ3002, and pNZ3004 contain the 0.2-kb *Dra*I-*Sal*I, 0.32-kb *Ava*II (filled in with Klenow DNA polymerase)-*Sal*I, and 0.5-kb *Eco*RI-*Sal*I fragments of pNZ398 cloned into the *Sma*I-*Sal*I, *Sma*I-*Sal*I, and *Eco*RI-*Sal*I sites of pGKV210, respectively. Plasmid pNZ3005 contains the 1.45-kb *Eco*RV-*Bgl*II fragment of pNZ390 cloned into the *Sma*I-*Bam*HI site of pGKV210. Plasmids pNZ3006, pNZ3007, and pNZ3008 contain the 0.5-kb *Xmn*I-*Bgl*II and 0.4-kb *Xmn*I-*Eco*RV fragments of pNZ390, and the 0.35-kb *Eco*RI (filled in with Klenow DNA polymerase)-*Bam*HI fragment of pNZ399 cloned into the *Sma*I-*Bam*HI, *Sma*I, and *Sma*I-*Bam*HI sites of pKK232-8, respectively. Plasmid pNZ3009 and pNZ3010 were obtained by cloning the blunt-end 1.3-kb *Eco*RI-*Bam*HI (*lacR* gene) and 0.3-kb *Hpa*I-*Bam*HI (*prtP* promoter) fragments of pNZ380 and pNZ337 into the *Eco*RI (filled in with Klenow DNA polymerase) and *Sma*I-*Bam*HI sites of pACYC184 and pKK232-8, respectively. As a control in the *E.coli* complementation studies, *Eco*RI-linearized pACYC184 was made blunt-end, ligated, and transformed to *E.coli* HB101 harboring either pNZ3006 or pNZ3010. The resulting pACYC184-derived plasmid, designated pACYC184Cm^s, contains a mutated *cat* gene. As a consequence, Cm resistance in the obtained multiplasmid strains is solely derived from pNZ3006 and pNZ3010.

RESULTS

Location of the *lac* operon promoter. The *L.lactis lac* operon that is located on the lactose miniplasmid pMG820 (29) encodes the *L.lactis* PEP-PTS^{lac} and tagatose 6-phosphate pathway enzymes (16,17,49), that are essential for rapid lactose fermentation (Fig. 1). In initial attempts to localize the *lac* promoter, pMG820 DNA was digested with *Bgl*II, *Xho*II, and *Bcl*I/*Bgl*II and the resulting restriction fragments were shotgun cloned upstream of the promoterless *cat* gene of the *E.coli* promoter-probe vector pKK232-8 (7) that was digested with *Bam*HI. Resistance to more than 200 μg/ml Cm was obtained in E.coli MC1061 with only one orientation of the 3.5-kb *Xho*II (6 clones analyzed), 3.8-kb *Bcl*I/*Bgl*II (1 clone analyzed), or 5.8-kb *Bgl*II (9 clones analyzed) fragments as shown in Figure 1. In all plasmids the *Bgl*II/*Xho*II site at map position 4.8

kb ([28], Fig. 1) appeared to be located immediately preceding the *cat* gene, indicating that the orientation of the promoter is towards the *lac* genes. To further locate the presumed *lac* promoter, the 2.0 kb *Eco*RI fragment that includes this site, was made blunt-end and cloned in *Sma*I-linearized pKK232-8. Since the resulting plasmid containing the expected orientation of the *Eco*RI fragment yielded high Cm resistance in *E.coli* we assumed that the *lac* promoter was located in the intercistronic region in between the *lac* operon and the divergently transcribed *lacR* gene (Fig. 1). Further subcloning and deletion analysis allowed the construction of plasmids containing small inserts of this region (Fig. 2) that were tested for *cat*-gene expression. *E.coli* MC1061 cells harboring plasmids pNZ3006 or pNZ3008 were resistant up to a concentration of 700 μg/ml Cm, whereas *E.coli* cells harboring vector pKK232-8 were sensitive to less than 1 μg/ml Cm. However, deletion of DNA sequences downstream of the *Eco*RV restriction site (pNZ3007, Fig. 2) dramatically decreased Cm-resistance to less than 10 μg/ml. Inspection of the nucleotide sequence of this region (48,49) showed the presence of a putative promoter that contains an *Eco*RV site in between the -35 and -10 sequences (Fig. 2). Since further analysis showed that these sequences constitute the *lac* promoter (see below) these results indicate that the *L.lactis lac* promoter is efficiently utilized in *E.coli*.

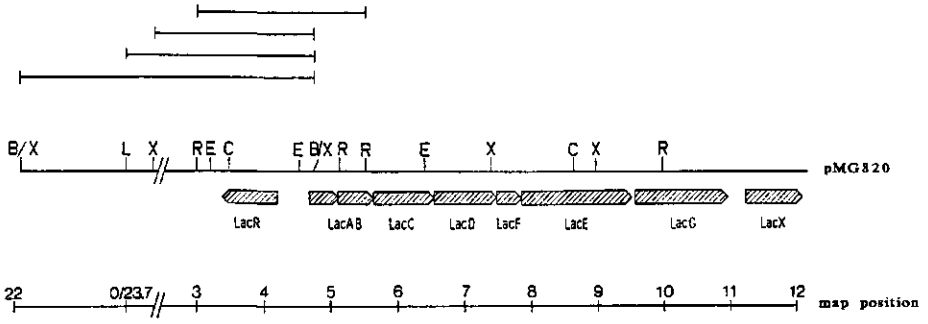

Fig. 1. Physical and genetic map of the *L.lactis* pMG820 *lac* regulon. The location is shown of the recognition sites for the restriction endonucleases *Bcl*I (L), *Bgl*II (B), *Cla*I (C), *Eco*RI (R), *Eco*RV (E), and *Xho*II (X) as determined by physical mapping (29) and deduced from sequence analysis (48). The location and orientation of the *lac* genes is shown; *lacR*, *lacAB*, *lacC*, *lacD*, *lacF*, *lacE*, *lacG* and *lacX* genes encode for LacR repressor, galactose 6-phosphate isomerase, tagatose 6-phosphate kinase, tagatose 1,6-diphosphate aldolase, enzyme III^{lac}, enzyme II^{lac}, P-ß-gal, and a protein with unknown function, respectively. The map positions (in kilobases) of the pMG820 DNA (29) and the DNA-fragments used in the localization of the *lac* promoter (├──────┤) are indicated.

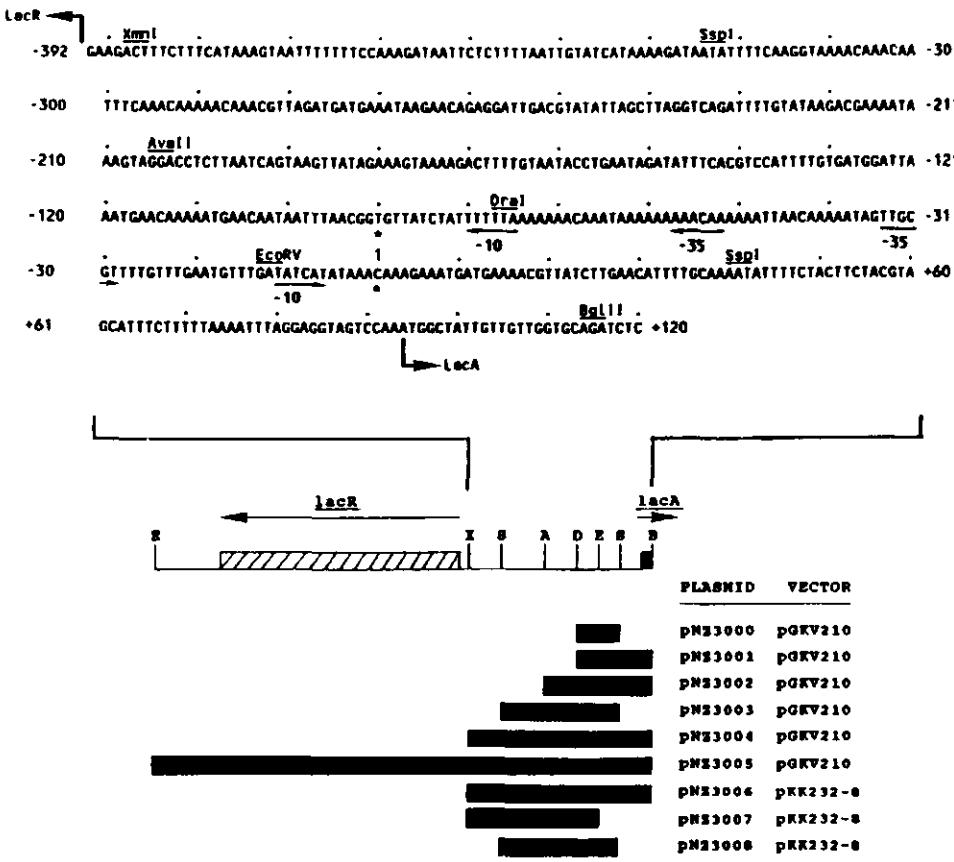

Fig. 2. Cloning, nucleotide sequence and location of the *lac* operon and *lacR* promoters. A physical map and part of its nucleotide sequence (48,49) is shown. Solid bars represent the DNA fragments used in the construction of the plasmids using the indicated vectors. The hatched and black bars indicate the coding regions of the *lacR* gene and part of *lacA* gene, respectively. The positions of the restriction enzyme cleavage sites used in the cloning experiments are indicated on the physical map and in the sequence: A, *Ava*II; B, *Bgl*II; D, *Dra*I; E, *Eco*RV; S, *Ssp*I; X, *Xmn*I. Transcription start sites (*), and the location and direction of the canonical sequences of the *lac* promoter (this study) and *lacR* promoter, that is located at the opposite strand (48), are indicated. The hooked arrows in the sequence indicate the putative translational start sites of the *lacR* and *lacA* genes.

Primer extension mapping of the *lac operon promoter*. To determine the transcription initiation site of the *L.lactis lac* operon, total RNA was isolated from glucose- and lactose-grown *L.lactis* MG1820 cells, and primer extension was performed using an oligonucleotide primer complementary to the coding strand of the *lacA* gene. In both cultures three similarly sized primer extended products were detected (Fig. 3) that were most abundant (5-10 times) in the lactose-grown cells. These results confirm that the *lac* operon is regulated at the transcriptional level as was shown previously (17,49). Assuming that the middle, most intense, band is the main primer extension product, transcription of the *lac* operon during growth on lactose initiates at the G residue at position 1 (Fig. 3). As a consequence, there is a 94 bp non-coding region upstream of the startcodon of *lacA*, the first gene of the *lac* operon (Fig. 2). In addition to the three primer extension products around +1, two minor, similary labelled, products were found on glucose and lactose (Fig. 3). Therefore, we cannot exclude the possibility that besides the inducible transcription initiation at position +1, minor constitutive transcription initiation occurs at positions +5 and +8.

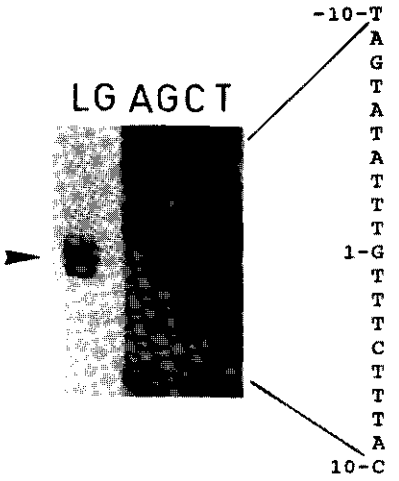

Fig. 3. Primer extension mapping of the *L.lactis lac* operon transcript. At the left site the plasmid pMG820 sequencing ladder obtained with the same primer is shown. L, lactose grown cells; G, glucose grown cells. The arrow indicates major transcription initiation site.

Flanking regions enhance *lac* promoter activity much more in *L.lactis* than in *E.coli*. Fragments containing the promoter and flanking DNA sequences were fused to the promoterless *cat*-86 gene of the *L.lactis* promoter-probe vector pGKV210, which is also capable of replication in *E.coli* (47). Constructs pNZ3000-pNZ3004 contain different fragments of the non-coding region between the *lacR* and *lacA* genes (Fig. 2). To determine the activity of the promoter in a Lac$^-$ host, these constructs were used to

transform *L.lactis* MG1363 and subsequently CAT-activities were measured (Table 1). The plasmid copy numbers of the constructs were determined in all strains and varied between 2.2 and 6.2 copies per chromosome (Table 1). Cells harboring plasmid pNZ3000 showed very low CAT-activity and were not able to grow on media containing more than 3 μg/ml Cm. Cells of MG1363 harboring pNZ3001, pNZ3002, pNZ3003, or pNZ3004 showed considerably higher (5-16 fold) CAT-activities. These results indicate that sequences downstream position +43 to +114; pNZ3001) and upstream (position -76 to -322; pNZ3003) of the *lac* promoter contribute significantly to the promoter activity.

Table I. CAT activities and plasmid copy numbers of *L.lactis* strains MG5267 and MG1363 harboring the indicated plasmids. Average CAT-activity values of two independent determinations are given s.d. less than 15%). Plasmid copy numbers were determined in the same cultures that were used for the determination of CAT and P-ß-gal activities. Energy sources used in the growth medium are indicated.

Plasmid	CAT-activity[1]			Plasmid copy number[2]	
	MG1363 glucose	MG5267 lactose	MG5267 glucose	MG1363 glucose	MG5267 glu-lac
pNZ3000	0.1 (2.8)[3]	0.1 (2.6)	0.1 (2.6)	2.8	3.0
pNZ3001	0.7 (14)	1.0 (31)	0.9 (28)	4.0	2.5
pNZ3002	1.0 (23)	2.7 (100)	1.7 (63)	3.4	2.1
pNZ3003	1.6 (28)	2.6 (23)	2.2 (19)	4.4	8.9
pNZ3004	3.7 (46)	5.5 (80)	1.4 (21)	6.2	5.3
pNZ3005	0.2 (7.1)	1.7 (73)	0.3 (13)	2.2	1.8
pGKV210	<0.01	<0.01	<0.01	ND	ND

[1] CAT-activity expressed as $U.mg^{-1}$

[2] Expressed as plasmid copies per chromosome; ND denotes not determined

[3] The numbers between brackets indicate relative CAT activities % of maximal value) that have been corrected for plasmid copy number.

To investigate whether the role of these flanking regions was host-specific, CAT-activities were determined in *E.coli* (Table 2) and corrected for the copy number of the *lac* promoter plasmids. The presence of sequences from position +43 to +114 (pNZ3001) or -322 to -76 (pNZ3003) flanking the minimal promoter fragment (pNZ3000) resulted in a 2.5- and 4-fold increase of CAT-activity, respectively. The highest increase (5-fold) was observed when sequences from position -387 to -76 and +43 to +114 (pNZ3004) were present. These results indicate that activity of the *L.lactis* *lac* promoter in *E.coli* is also enhanced by its upstream and downstream regions, but to a much lesser extent than in *L.lactis*.

Table 2. CAT-activities of *E.coli* MC1061 strains containing various constructs.

Plasmid	CAT-activity[1]	Plasmid copy number[2]
pNZ3000	4.5 (20)[3]	9.2
pNZ3001	3.1 (52)	2.4
pNZ3002	3.5 (47)	3.0
pNZ3003	6.8 (78)	3.5
pNZ3004	6.0 (100)	2.4
pNZ3005	1.1 (19)	2.3
pGKV210	<0.1	ND

[1] CAT-activity expressed as $U.mg^{-1}$

[2] Relative plasmid copy numbers are shown. ND denotes not determined.

[3] The numbers between brackets indicate relative CAT activities (% of maximal value) that have been corrected for plasmid copy number.

The presence of the *lacR* gene represses *lac* promoter activity in *L.lactis* and *E.coli*. To examine the role of LacR repressor in determining *lac* promoter activity, pNZ3005 was used to transform *E.coli* MC1061 and *L.lactis* MG1363. Plasmid pNZ3005 contains the complete *lacR* gene and the *lac* promoter fused to the *cat*-86 gene (Fig. 2). Both *E.coli* and *L.lactis* cells harboring pNZ3005 showed a decreased CAT-activity compared to pNZ3004-containing cells (Tables 1 and 2). Therefore, we conclude that the presence of the *lacR* gene decreases the activity of the *lac* promoter. Previously we found that in *L.lactis* LacR represses *lac* promoter activity *in trans* (48). To study the effect of *lacR* on *lac* promoter activity in *E.coli*, the pACYC184-derivative pNZ3009, containing the

lacR gene under control of its own expression signals, was used to transform *E.coli* HB101 carrying pNZ3006 (Fig. 2). Whereas HB101 harboring pNZ3006 and control plasmid pACYC184Cm^s was resistant up to 700 μg/ml, cells of HB101 harboring pNZ3006 and pNZ3009 were only resistant up to 200 μg/ml Cm. The specificity of the *lacR* gene product in inhibiting only *lac* promoter activity was examined by introducing pNZ3009 into strain HB101 harboring pNZ3010, that contains the unrelated *prtP* promoter upstream of the pKK232-8 cat gene. Similar Cm resistances (180 μg/ml) were observed in pNZ3010-containing HB101 cells harboring pNZ3009 or pACYC184Cm^s. From these and previous results (48) we conclude that the *lacR* gene product represses *lac* promoter activity *in trans* both in *E.coli* and *L.lactis*.

Regulation of the *lac* operon is mediated by the interaction between LacR and the *lac* promoter region. To study the influence of flanking DNA sequences and LacR repressor on regulation of *lac* promoter activity, constructs pNZ3000 to pNZ3005 were used to transform *L.lactis* strain MG5267 that contains a single chromosomal copy of the *lac* operon (20). The use of strain MG5267 has the additional advantage that the effect of the extrachromosomal *lac* promoter fragments on the *lac* operon expression can be determined via measurement of the activity of P-ß-gal (LacG, Fig. 1). Like *L.lactis* MG1820, which harbors pMG820, MG5267 shows a 5-10 fold induction of *lac* operon expression during growth on lactose (48). CAT and P-ß-gal activities were measured in order to determine plasmid and chromosomal derived *lac* promoter activities, respectively. The plasmid copy numbers were determined (Fig. 4, Table 1) and found to be identical in glucose- and lactose-grown cultures (not shown). The results (Tables 1 and 3) indicate that MG5267 cells harboring pNZ3000 show only very low CAT-activity both under induced and non-induced conditions, similar to MG1363 cells harboring this plasmid. In contrast, pNZ3004- and pNZ3005-containing MG5267 cells showed a lactose-inducible CAT-activity. Cells of MG5267 harboring pNZ3001, 3002, or 3003 showed no significant induction of CAT-activity during growth on lactose. The increased P-ß-gal activities of MG5267 cells harboring pNZ3003 or pNZ3004 (Table 3) when grown on glucose suggest that chromosomally encoded LacR repressor is titrated by the plasmid-derived copies of the *lac* promoter, resulting in a derepressed *lac* operon. Cells harboring pNZ3005 show super-repressed and lower P-ß-gal activities during growth on glucose and lactose, respectively. This may be attributed to the excess of plasmid-encoded LacR, resulting in an additional repression of the chromosomal copy of the *lac* promoter.

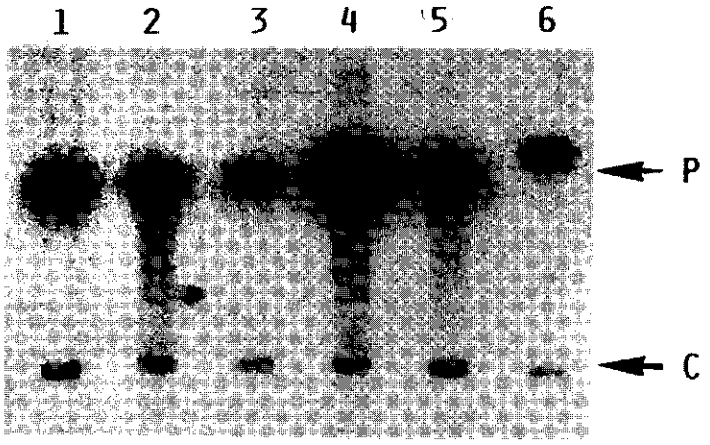

Fig. 4. Determination of plasmid copy number in lactose-grown *L.lactis* MG5267 cells. *Eco*RV-digested total DNA was separated on a 1% agarose gel, blotted to a GeneScreen Plus membrane and subsequently hybridized with an end-labelled *lac* promoter-specific probe. Lanes 1 to 6; MG5267 harboring pNZ3000, pNZ3001, pNZ3002, pNZ3003, pNZ3004, and pNZ3005, respectively. Arrows indicate positions of plasmid- (P) and chromosomal-derived (C) copies of the *lac* promoter.

Table 3. Phospho-ß-galactosidase activities (μmol.min^{-1}.mg^{-1}) of *L.lactis* MG5267 cells containing the indicated plasmids. Energy sources used are indicated.

Plasmid	P-ß-gal activity	
	lactose	glucose
pNZ3000	1.8	0.4
pNZ3001	1.8	0.4
pNZ3002	1.9	0.4
pNZ3003	2.0	1.0
pNZ3004	1.9	1.0
pNZ3005	1.3	0.2
pGKV210	1.9	0.4

DISCUSSION

Function of flanking sequences in determining *lac* promoter activity. We have determined the transcription initiation site of the promoter of the *L. lactis lac* operon (Fig. 3). The canonical -35 and -10 sequences and their spacing correspond closely to the extended promoter consensus sequence for gram-positive bacteria postulated by Graves and Rabinowitz (22), in which the TG dinucleotide at position -13 and an AT-rich stretch upstream of -35 are also conserved. The smallest restriction fragment (position -75 to +42) that contains these consensus sequences (minimal promoter fragment) was fused to a promoterless *cat*-86 gene in pNZ3000. Unexpectedly, the presence of pNZ3000 in *L. lactis* and *E. coli* resulted in relatively low CAT-activities, that were decreased 38-fold and 5-fold, respectively, compared with the highest activities (Table 1; pNZ3002 in MG5267 grown on lactose, Table 2; pNZ3005). The presence of DNA sequences downstream (position +43 to +114) of the minimal promoter fragment increased CAT-activities 5- (MG1363) to 11-fold (MG5267) in *L. lactis* (Table 1) and 2.5-fold in *E. coli* (Table 2). Sequences at position +43 to +114 (pNZ3001) could be involved in the stability of the *lac* operon transcript by participating in the formation of a stem-loop structure (Fig. 5). In the absence of this stem-loop structure (pNZ3000) the transcript might be less stable, resulting in significantly decreased CAT-activities. It has been shown that the 5'-leader sequences, that may contain stable stem-loop structures, of the *E. coli ompA* and *bla* (2), bacteriophage T4 gene 32 (21), and *B. subtilis sdh* transcripts (32) contribute to mRNA stabilization and protection against degradation. The observation that cells harboring pNZ3003 show considerably higher CAT activity than cells harboring pNZ3000 indicates that the presence of the sequences +43 and +114 is not the only factor involved in the efficiency of the *lac* promoter. Since it is unlikely that the upstream sequences -322 to -76 present in pNZ3003 affect the stability of the produced transcript, the 10-fold increase in CAT-activity of cells harboring this plasmid compared with cells harboring pNZ3000, may be attributed to enhanced *lac* promoter activity. In *E. coli*, upstream activating sequences have been shown to activate the expression of some genes in part by intrinsic bending (24); for other genes, these sequences are targets for activator proteins (33). In *B. subtilis*, DNA curvature of upstream regions appears to stimulate gene expression as has been shown for the Alu156 bacteriophage SP82 promoters (30,31). A common feature of these sequences is the high level of AT-residues. Since the -322 to -76 region of the *L. lactis lac* promoter is highly (74%) AT-rich, it is tempting to speculate that activity of the *lac* promoter could be stimulated similarly. Alternatively, this region could be a target for a protein that stimulates transcription, comparable to the catabolite activating protein of *E. coli* (13). In *E. coli*, the absence of sequences from +43 to +114 or -322 to -76 resulted in a less severe decrease of CAT-activity than in *L. lactis*, indicating that different mechanisms or cellular

factors might be involved in messenger RNA decay and enhancement of transcription initiation in *L.lactis* and *E.coli*.

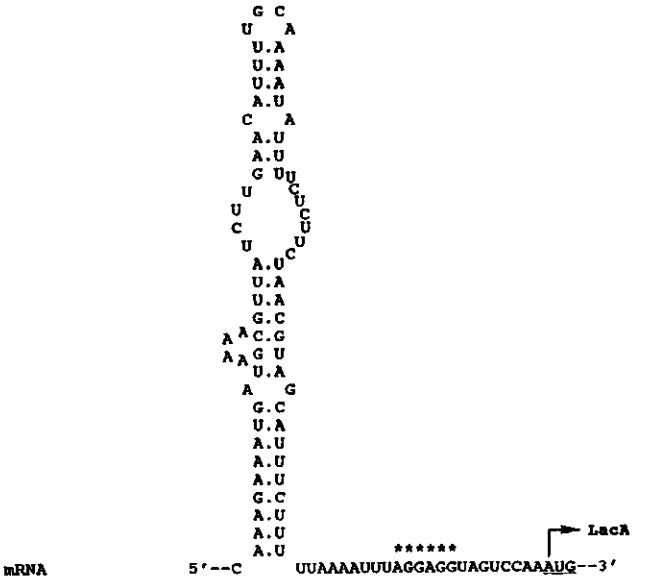

Fig. 5. Possible secondary structure of the 5' non-coding region of the *lac* operon transcript. Free energy was calculated according to Tinoco et al. (44) to be -5.8 kcal.mol^{-1}. Positions of LacA translation start (underlined) and putative ribosomal binding site (*****) are indicated.

Transcriptional regulation of the *L.lactis lac* operon. Expression of the *L.lactis lac* operon is repressed during growth on glucose and is mediated by the LacR repressor (48). Expression of the *lacR* gene, that is divergently transcribed from the *lac* operon (Fig. 1), is repressed during growth on lactose (48). Overproduction of LacR in lactose-grown cells resulted in a substantial decrease of growth rate and PEP-PTS^{lac} enzyme activity (48). In this paper we provide evidence that regulation is effected by the interaction between LacR repressor and the *lac* promoter region. The involvement of *lacR* in repressing promoter activity is evident from the low CAT-activities in *L.lactis* MG1363 and *E.coli* MC1061 cells harboring pNZ3005 (Table 1), whereas deletion of *lacR* resulted in a significant increase of activities (Tables 1 and 2, pNZ3004). In *E.coli*, the presence of the *lacR* gene *in trans* resulted in a decreased Cm-resistance of pNZ3006-containing cells, in which the *cat* gene is under control of the *lac* promoter. No decrease was observed when the *cat* gene is under control of the unrelated *prtP* promoter. These and earlier (48) results indicate that the promoter region in pNZ3006, which includes positions -387 to +114, is a target for the LacR repressor. As a

consequence, introduction of pNZ3004 (containing the same promoter region) into the Lac^+ *L.lactis* strain MG5267, that contains a chromosomal copy of all *lac* genes, results in an inducible Cm-resistance with CAT-activities that are 4-fold higher on lactose than on glucose (Table 1). The presence of additional copies of the *lacR* gene (pNZ3005) even results in a higher (6-fold) CAT induction in MG5267. Comparison of the P-ß-gal (LacG) activities, that reflect the activity of the chromosomal copy of the *lac* promoter, may register the distribution of LacR between the chromosomal and plasmid-derived copies of the *lac* promoter region. Cells of MG5267 harboring pNZ3004 show an increased P-ß-gal activity when grown on glucose, indicating that the chromosomally-encoded LacR molecules are titrated by the excess of plasmid-located *lac*-promoter regions. The presence of additional plasmid-encoded LacR as in MG5267 harboring pNZ3005 results in a relatively lower activity of the chromosomally located *lac* promoter, as is shown by the decreased P-ß-gal activities on glucose and lactose (Table 3). The ability of pNZ3003 to titrate LacR, as is indicated by the increased P-ß-gal activities of MG5267 cells harboring this plasmid, did not result in induced CAT-activities on lactose in contrast to cells of MG5267 harboring pNZ3004. This may be a consequence of the absence in pNZ3003 of the DNA-regions -387 to -322 and +43 to +114, which would suggest that additional LacR binding sites are located within this region. Alternatively, as a consequence of the absence of the putative stem-loop structure in pNZ3003, the higher promoter activity on lactose could be diminished by a higher turnover of messenger RNA. Although the mechanism of catabolite repression in gram-positive bacteria is poorly understood, there are reports of catabolite repression of amylase production and aconitase synthesis in *B. subtilis* (18,53). Therefore, we cannot exclude the possibility that a similar control system is operating in *L.lactis*, in addition to the LacR control circuit.

Previously we have shown that LacR repressor is homologous to *E.coli* DeoR, FucR, and GutR and *S. aureus* LacR (48) and contains a helix-turn-helix motif which is characteristic for DNA-binding proteins (6). Because the homology between these proteins is most significant in the helix-turn-helix motif, the DNA-regions that are involved in binding of these proteins might also be homologous. In order to identify such a sequence we have searched for homologies between the characterized *deo* operator (11) and the *L.lactis lac* promoter region. Operators involved in binding of the regulatory proteins of the *E.coli fuc* and *gut*, and *S. aureus lac* operons have not yet been characterized. Fig. 6 shows the homology between the $deoO_1$ operator and an imperfect inverted repeat at position -18 to +2 of the *L.lactis* promoter region. In analogy with the *deo* operon, LacR could bind to this sequence, resulting in the inhibition of transcription initiation of the *lac* promoter. The sole presence of this putative operator on multicopy plasmids does not affect the P-ß-gal activities of MG5267 cells harboring pNZ3000, pNZ3001, pNZ3002 or pGKV210 (control), indicating that no titration of the chromosomal encoded LacR occurs. Apparently, the excess of plasmid-located operators

do not compete efficiently with those present at the chromosome. Cooperative LacR binding may take place since *lac* promoter fragments containing region -322 to -205 (such as present in pNZ3003 and pNZ3004) are able to compete with the chromosomal copy of the *lac* promoter. This is supported by preliminary footprinting studies that have showed the presence of multiple operators in this region (50).

Fig. 6. Homology between the *E.coli deoO1* and putative *L.lactis lacO* operators. Identical residues (*), axis of symmetry (.), inverted repeats arrows), and transcription initiation sites are indicated.

Comparison of the specific CAT-activities obtained with the various *lac* promoter plasmids in *L.lactis* (Table 1) shows that the highest CAT-activities are found in MG5267 when grown on lactose, and not in MG1363. This was not to be expected since MG1363 lacks the *lacR* gene, and hence, no repression of the *lac* promoter by LacR occurs. The most likely explanation for the lower than expected CAT expression in MG1363 is the absence of a lactose-inducible activating factor that is encoded by the *lac* operon. It is tempting to speculate that the product of the *lacX* gene could be involved in this activation as has previously been suggested (17).

The results described here and in previous work (48) indicate that the promoters of the *lac* operon and the *lacR* regulator gene are organized in a back-to-back arrangement with a Regulator-Structural (R-S) type of regulation, as has been described for a variety of organisms (1). In this type of regulation, the regulatory molecule acts within the divergent transcription unit to control transcription of the structural genes, and often it also regulates its own synthesis (1). To the highly mobile *L.lactis lac* operon (42,46) this has the advantage that it may be translocated to new locations without loss of autonomous regulation.

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CHAPTER 7

PURIFICATION OF THE *LACTOCOCCUS LACTIS* LACR REPRESSOR AND CHARACTERIZATION OF ITS DNA BINDING SITES *LACO1* AND *LACO2*.

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ABSTRACT

Expression of the *Lactococcus lactis* lactose operon (*lacABCDFEGX*), encoding the tagatose-6-phosphate pathway and lactose phosphotransferase enzymes, is repressed during growth on glucose and is regulated at the transcriptional level by the product of the divergently transcribed *lacR* gene. The *lacR* gene was cloned in the T7 expression vector pET3b and overexpressed in *Escherichia coli*. Subsequently, the LacR repressor was purified and its interaction with the regulatory region of the *lac* operon was studied by gel mobility shift assays and DNase I footprinting. Two regions (*lacO1* and *lacO2*) were protected against cleavage by DNase I. *LacO1* is located at positions -31 to +6 and -96 to -59 relative to the transcription initiation sites of the *lac* operon and *lacR* gene, respectively. The affinity of the LacR repressor was three-fold higher for *lacO1* than *lacO2*, that is located at positions -313 to -279 and +189 to +223 relative to the transcription initiation sites of the *lac* operon and *lacR* gene, respectively. Cross-linking studies showed the ability of LacR to generate dimers. The formation of the complex between LacR repressor and the *lac* operator regions was studied in the presence of intermediates of the tagatose-6-phosphate and glycolytic pathways and was shown to be inhibited by tagatose-6-phosphate.

INTRODUCTION

Regulation of lactose catabolism has been extensively studied in *E.coli* and has been a paradigm for studying gene regulation in other bacteria. Expression of the *E.coli lacZYA* operon encoding ß-galactosidase, lactose permease and transacetylase, is under negative and positive control by the *lacI* repressor and CAP[1], respectively (Chakerian and Matthews, 1992, Lee and Goldfarb, 1991, Oehler *et al.*, 1990, Reznikof, 1992). In various gram-positive bacteria, including *Lactococcus lactis*, lactose catabolism has evolved differently and is initiated by uptake via the lactose-specific phospho*enol*pyruvate-dependent phosphotransferase system (PEP-PTS^{lac}), which includes Enzyme II^{lac} (LacE) and Enzyme III^{lac} (LacF). Subsequently, lactose-6-phosphate is cleaved by phospho-ß-galactosidase (LacG) into glucose and galactose-6-phosphate. Galactose-6-phosphate is converted into the glycolytic intermediates glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate by the tagatose-6-phosphate pathway enzymes (LacABCD). The specific activities of these enzymes in *L.lactis*, which are encoded by the *lacABCDFEGX* operon, are repressed when glucose is used as an energy source (De Vos *et al.*, 1990, Van Rooijen *et al.*, 1991). Expression of the *L. lactis lac* operon is regulated at the level of transcription through negative control by the product of the divergently transcribed *lacR* gene (Van Rooijen and De Vos, 1990; Van Rooijen *et al.*, 1992). Overexpression of *lacR* in *L. lactis* MG5267, that contains a single chromosomal copy of the *lac* regulon, significantly repressed *lac* operon activities resulting in a decreased growth rate on lactose (Van Rooijen and De Vos, 1990). The *lac* promoter region has been characterized and shown to be organized in a back-to-back configuration with the *lacR* promoter. Transcriptional fusions between various *lac* promoter DNA fragments and a *cat*-86 reporter gene have shown that the DNA region -387 to +114 relative to the *lac* transcription initiation site is required for repression of *lac* promoter activity by LacR in *L. lactis* (Van Rooijen *et al.*, 1992)

The *L. lactis* LacR repressor belongs to the DeoR family of repressors, that includes the *E. coli* DeoR, GutR, and FucR, *Staphylococcus aureus* LacR, *Streptococcus mutans* LacR and *Agrobacterium tumefaciens* AccR and contains a putative helix-turn-helix motif near the N-terminus (Van Rooijen and De Vos, 1990; Beck von Bodman *et al.*, 1992). The well-characterized DeoR repressor binds to three operators in the regulatory region of the *deo* operon thereby inhibiting transcription initiation from the *deoP1* promoter (Valentin-Hansen *et al.*, 1986; Mortensen *et al.*, 1989, Dandanell and Hammer, 1991).

In this report we describe studies of the physical interaction between the purified LacR repressor and the promoter/operator regions of the *lac* operon and *lacR* gene with gel mobility shift assays and DNase I footprinting. In addition, the effects of phosphorylated intermediates of the tagatose-6-phosphate and glycolytic pathways on the interaction between LacR repressor and the *lac* promoter/operator region were studied. Two operators were identified in the *lac* promoter region. The binding of LacR repressor

to those operators was negatively affected by the presence of tagatose-6-phosphate.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. *E. coli* strains HMS174 and BL21(DE3)plysE (Studier *et al.*, 1990) were used as recipients in the initial cloning and overexpression of *lacR*, respectively. *E. coli* MC1061 (Casabadan *et al.*, 1980) was used in routine cloning experiments. Media based on L-broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) were used for the growth of *E. coli*. Ampicillin and chloramphenicol were used at final concentrations of 50 μg ml^{-1} and 25 μg ml^{-1}, respectively. Plasmids used in the cloning experiments were pNZ381 (Van Rooijen and De Vos, 1990), pUC18 (Yanisch-Perron *et al.*, 1985), and pET3b (Studier *et al.*, 1990). Plasmid pNZ399 (Van Rooijen and De Vos, 1992) was used as a source for the *lac* promoter region.

Construction of *lacR* expression plasmid pNZ3011. Previously, we described the molecular cloning and DNA sequence of the *lacR* gene (Van Rooijen and De Vos, 1990). In this study two putative ATG start codons could be identified, that are separated by six codons and are both preceded by a relatively weak ribosome binding site. Overexpression of *lacR* initiated from the first ATG start codon has been presented (Van Rooijen and De Vos, 1990). To determine whether the first ATG start codon is utilized in *L. lactis* we used plasmid pNZ3005, that contains the *cat*-86 gene under control of the *lacR-lac* promoter regulon (Van Rooijen and De Vos, 1992). Introduction of plasmid pNZ3005 into the Lac$^+$ strain *L. lactis* MG5267 resulted in chloramphenicolacetyl transferase (CAT) activities that were repressed on glucose and induced on lactose (Van Rooijen and De Vos, 1992). A frameshift was introduced in the *lacR* open reading frame derived from the first ATG start codon by Klenow treatment of the *Nde*I restriction site. This manipulation does not affect the integrity of the open reading frame derived from the second ATG start codon and therefore a polypeptide of 255 amino acids can be generated. No differences in CAT-activities were observed between *L. lactis* MG5267 cells harboring pNZ3005 and the constructed plasmid (not shown). Therefore, we concluded that the first ATG start codon is not used in *L. lactis*, and we used the second ATG codon in the construction of the T7-expression vector pNZ3011. Plasmid pNZ3011 contains the 1.0 kb *Nde*I-*Eco*RI (filled in with Klenow DNA polymerase) restriction fragment from pNZ381 (*lacR* gene) cloned into the *Nde*I-*Bam*HI (filled in with Klenow DNA polymerase) site of the ATG vector pET3b. Therefore, in pNZ3011 the *lacR* gene is under control of the ϕ_{10} promoter, ribosome binding site, and terminator.

Purification of the LacR repressor. Plasmid pNZ3011 was used to transform *E. coli* BL21(DE3)lysE, which contains a chromosomal copy of the T7 RNA polymerase gene under control of the *lacUV5* promoter and plasmid pLysE that contains the T7 lysozyme

gene. T7 lysozyme represses the basal activity of T7 RNA polymerase, that appeared to interfere with the maintenance of pNZ3011 in *E. coli* BL21(DE3)(not shown). For LacR-overproduction, cells were grown at 37 °C and induced at an optical density at 600 nm of 0.6-0.7 by adding IPTG to an final concentration 0.4 mM. Incubation was continued for 3 hours and cells were harvested and resuspended in buffer A containing 50 mM Tris.HCl pH 8.0, 0.1 mM EDTA, 200 mM NaCl, 5 mM ß-mercaptoethanol and 10% glycerol. Extracts were prepared by sonification and cell-free extract was isolated after high speed centrifugation. Low molecular weight components that could disturb the resolution and performance of the first column, were removed by the following procedure. To cell-free extract 50 ml Q-Sepharose (Fast Flow, Pharmacia) was added and the mixture was gently stirred for 16 h at 4 °C. The Q-Sepharose was isolated by low speed centrifugation and repeatedly washed with buffer A containing 600 mM NaCl until an optical density at 280 nm of < 0.1. Supernatant was isolated and the NaCl concentration was adjusted to 200 mM (Fraction I). Fraction I was loaded on an anion exchange column (Q-Sepharose) and elution was performed with a 200-600 mM NaCl gradient using the Pharmacia FPLC system. The *lacR* protein eluted at $\approx$ 400mM NaCl. Fractions containing LacR were pooled and adjusted to 300 mM NaCl concentration. Final purification was achieved on a heparin-agarose column (Pharmacia). Elution was performed with a 300-600 mM NaCl gradient and the LacR protein eluted at $\approx$ 450 mM NaCl. After purification, the purified LacR protein was dialyzed three times against 50 volumes of 5 mM acetic acid pH 3.5, lyophilized, dissolved in 50 mM Tris.HCl pH 8, 100 mM NaCl, 0.1 mM EDTA, 1 mM ß-mercaptoethanol, 10% glycerol and stored at -80 °C. Fractions were analyzed by SDS-polyacrylamide (12.5%) gel electrophoresis. Protein concentration was determined spectrophotometrically according to Bradford (Bradford, 1976).

Amino acid analysis of LacR. Pure LacR repressor was hydrolysed for 24 and 96 hours in 6 M HCl at 110 °C; the 96 h hydrolysis was used for the complete hydrolysis of the Val-Ile bonds. The hydrolysates were concentrated by lyophilisation, dissolved in 0.2 M sodium citrate, pH2.2 and analyzed on an amino acid analyzer (LKB, type 4151).

Glutaraldehyde cross-linking studies. Cross-linking studies with glutaraldehyde were performed as described by Landschulz *et al.* (1989). Purified LacR repressor was incubated in a 10 μl volume with 0.01 % glutaraldehyde at a protein concentration of 5 μM in the presence or absence of 5 mg/ml bacterial protein. Incubations were carried out at room temperature for either 1, 3, or 10 minutes. Cross-linking was stopped by the addition of 10 μl SDS/TRIS-HCl sample buffer, and samples were loaded on a 11 % SDS/PAGE gel. Subsequently, proteins were transferred to nitrocellulose filters and probed with antibodies specific to LacR.

Preparation of operator DNA fragments. Plasmid pNZ399 contains the *lac* promoter region (position -322 to +42, relative to transcription initiation site of the *lacABCDFEGX* genes)(Van Rooijen and De Vos, 1992). A 419 bp *Eco*RI-*Hin*dIII fragment was excised from pNZ399 and labelled by filling in the 3' recessed ends of either the *Eco*RI or *Hin*dIII site with the Klenow fragment of DNA polymerase in the presence of [α-^{32}P]dATP (3000 Ci/mmol, Amersham Corp.), dCTP, dGTP, and dTTP. Since in the DNase footprinting studies the 419 bp *Eco*RI-*Hin*dIII fragment from pNZ399 is too large for mapping both DNA strands of *lacO1* and *lacO2*, the 124 bp *Dra*I-*Bam*HI (*lacO1*) and 139 bp *Eco*RI-*Ava*II (filled in with Klenow)(*lacO2*) fragments of pNZ399 were subcloned into the *Bam*HI-*Sma*I and *Eco*RI-*Sma*I sites of pUC18 and the resulting plasmids were designated pNZ3012 and pNZ3013, respectively. The *lacO1* and *lacO2*-specific probes were obtained by end-labelling the *Eco*RI-*Hin*dIII restriction fragments of pNZ3012 and pNZ3013, respectively. End-labelled probes were purified from a 5% non-denaturing polyacrylamide gel (Sambrook *et al.*, 1989).

Gel mobility shift assay. Binding of LacR with end-labelled probe was performed in 20 μl assay mixture as described by Garner and Revsin (1981), and contains: 10 mM Tris.HCl pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 50 μg/ml poly d(I-C), 10% glycerol, 5 fmol end-labelled fragment, and LacR. In the inducer binding studies, galactose 6-phosphate, tagatose-6-phosphate, glucose 6-phosphate, fructose 6-phosphate, tagatose-1,6-diphosphate, or fructose 1,6-diphosphate were added to the assay mixture to a final concentration of 4 mM. After 30 min of incubation at 4 °C, 1 μl of 20x sample buffer (200 mM Tris pH 8.0, 0.8 % bromo-phenol blue) was added and reaction mixtures were loaded on a 5% polyacrylamide gel (acrylamide:bisacrylamide, 60:1) in 50 mM Tris-borate, 1 mM EDTA (pH 8.3). The gel was pre-run for 30 min. at 15 V/cm at room temperature. Electrophoresis was performed under the same conditions. After drying the gel was autoradiographed.

DNase I footprinting. For DNase I footprinting, binding was conducted as described in the previous section with the modifications that a 50 μl reaction volume and 20 μg/ml poly d(I-C) was used. After the binding reaction, 2.5 mM $MgCl_2$ and 20 U/ml DNase I (Promega) were added and the reaction mixture was incubated at 25 °C for 90 or 180 sec. The DNase digestion was stopped by the addition of 20 mM EDTA, 0.2 % SDS. Samples were extracted with phenol/chloroform and then precipitated with ethanol. Pellets were resuspended in 3 μl of water and 3 μl of formamide dye mixture and 2 μl was electrophoresed on a 8% polyacrylamide-urea sequencing gel. The regions in the *Eco*RI- and *Hin*dIII-labelled probes that were protected by LacR against DNase I attack were identified by simultaneously electrophorese double-stranded sequence reactions obtained with primers 5'-AATTCGAGCTCGGTACCC-3'or 5'-AGCTTGCATGCCTGC-3', respectively, and the plasmid that the probe was isolated from (Sanger *et al.*, 1977).

RESULTS

Purification of the *L.lactis* LacR repressor from overproducing *E.coli* cells. In order to study its interaction with the promoter region of the *lac* operon *in vitro*, the LacR repressor was purified from an overproducing *E. coli* strain. To this purpose, the *L. lactis lacR* gene was cloned into the T7-expression vector pET3b and the resulting plasmid pNZ3011 was introduced into *E. coli* BL21(DE3) harboring plasmid pLysE (Studier *et al.*, 1990). Fig. 1 shows the overproduction (lane 2) and subsequent purification of LacR by a three-step procedure, that includes a Q-Sepharose batch treatment followed by Q-Sepharose ionexchange (lane 3) and heparin-agarose affinity chromatography (lane 4). This resulted in a single band band on SDS/PAGE with an apparent molecular mass of 28.5 kDa, which corresponds closely to the calculated value of 28,617 Da deduced from the nucleotide sequence of the *lacR* gene (Van Rooijen and De Vos, 1990). Analysis of the purified protein on a reversed-phase HPLC column showed that the protein was approximately 88% pure (data not shown). The amino acid composition of the purified protein was determined and was in good agreement with that derived from the *lacR* DNA sequence (Table 1).

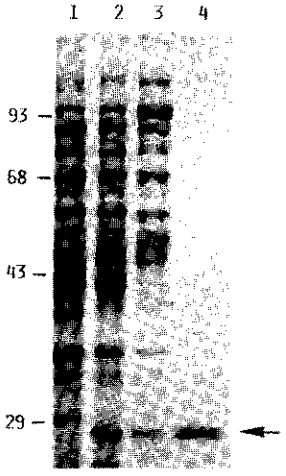

Fig. 1. Overexpression in *E.coli* and purification of the *L. lactis* LacR repressor. The *lacR* gene was cloned in the expression vector pET3b to yield plasmid pNZ3011. Expression of pNZ3011 was studied in *E. coli* BL21(DE3)lysE. After induction of the T7 promoter by 0.4 mM IPTG the LacR repressor was purified from cell-free extracts by Q-Sepharose and heparin-agarose chromatography. Lane 1, pET3b + 0.4 mM IPTG; Lane 2, pNZ3011 + 0.4 mM IPTG; Lane 3, purification of LacR, pool after the Q-Sepharose ion exchange chromatography; Lane 4, 5 μg of purified LacR after heparin-agarose chromatography. Protein samples were separated by SDS-PAGE (11%) and stained with Coomassie blue. The arrow indicates the position of the 28 kDa LacR repressor protein. Molecular weight markers (kDa) are indicated.

Molecular weight of the LacR repressor protein. Attempts to determine the native molecular weight by gel permeation chromatography were unsuccessful due to the excessive binding of LacR repressor to various column materials. Therefore, analysis of multimer formation of the LacR repressor in solution was carried out by glutaraldehyde-mediated cross-linking experiments followed by analysis on SDS/PAGE and Western blotting with anti-LacR antibodies. Fig. 2 shows the time-dependent formation of a cross-linked product with an apparent molecular mass twice (57 kDa) that of the LacR-monomer starting material. We assume this molecule to represent a covalently cross-linked dimer for the following reasons: (i) cross-linking occurred at low concentrations of LacR; (ii) the products of the cross-linking reaction were almost exclusively

Amino acid	LacR	*lacR*
Ala	19	18
Arg	10	10
Asx	30	32
Cys	1	1
Gly	16	14
Glx	23	23
His	4	4
Ile	22	24
Leu	33	33
Lys	24	24
Met	5	5
Phe	11	11
Pro	5	4
Ser	15	15
Thr	21	21
Tyr	6	6
Val	9	9

Table 1. Comparison beween the amino acid composition of the purified *L.lactis* LacR (LacR) repressor and that decuded from the *lacR* gene (*lacR*). Amino acids are given in the three-letter code.

monomeric and dimeric, virtually no other multimeric forms were generated, while in the case of a random collision of polypeptide chains all types of multimers might have been generated; (iii) cross-linking was restricted to LacR even in the presence of an excess of bacterial proteins (Fig. 2) or bovine serum albumin (not shown).

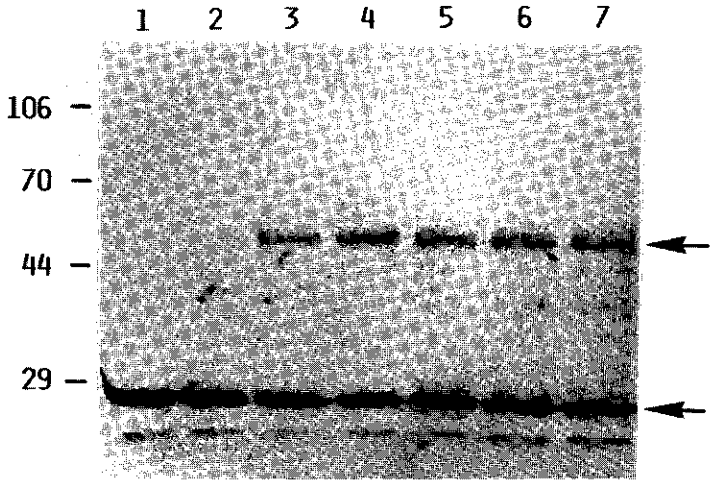

Fig. 2. Dimer formation of LacR repressor protein. LacR repressor (5 μM) was treated for 1, 3, and 10 min with 0.01 % glutaraldehyde in the presence (lanes 2-4) or absence (lanes 5-7) of an excess of E.coli protein (5 mg/ml). Lane 1, untreated sample. Proteins samples were separated on a 11% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with antibodies specific for LacR. Monomer and dimer positions (arrows) and molecular weight markers are indicated.

The *lac* promoter region contains two LacR-binding sites with different affinities. In order to investigate whether purified LacR could bind to the promoter region of the *lac* operon, gel mobility shift assays were carried out. An *Eco*RI-*Hin*dIII fragment of 419 bp was excised from pNZ399 (Fig. 3) and labelled with [α-^{32}P]dATP and Klenow polymerase. This fragment contains the intercistronic non-coding region between the *L. lactis lac* operon and the divergently transcribed *lacR* gene (Fig. 3; Van Rooijen *et al.*, 1992). The labelled fragment was incubated with increasing amounts of purified LacR repressor and then electrophoresed in a non-denaturing polyacrylamide gel. Fig. 4 shows that with increasing amounts of LacR the amount of free DNA is reduced and a new band with a slower migration rate appears (lanes 4 to 12). Since a large excess of non-specific DNA (poly d(I-C)) is present and the gel mobility shift is LacR-dependent, this slower moving band represents a specific LacR-DNA complex. The appearance of an additional LacR-DNA complex with a slower mobility was observed (Fig. 4, lanes 7 to 12, *lacO1O2*), but was more pronounced after an extended electrophoresis (not shown), suggesting that two LacR-binding sites are located on the 419-bp DNA-fragment. To obtain a higher resolution of the LacR-binding sites in the *lac* promoter region, three labelled restriction fragments, representing DNA sequences from -322 to -203 (fragment A), -202 to -76 (fragment B), and -75 to +42 (fragment C) relative to the *lac*

transcription initiation site, were tested for their ability to bind the to LacR repressor (Fig. 3). Fragments A and C contain LacR-binding sites as is indicated by the disappearance of free DNA and the appearance of a slower migrating band upon increasing amounts of LacR repressor. No gel mobility shift was observed with fragment B (data not shown), indicating that no LacR-binding site is located at this fragment. The LacR-binding site at fragment C, designated *lacO1*, has a higher affinity for LacR than that of fragment A, designated *lacO2*, as is indicated by the relatively slower disappearance of free DNA fragment A (Fig. 4). From these experiments we conclude that the promoter region of the *lac* operon contains at least two LacR binding sites with different affinities. After plotting the fraction of free DNA as a function of the LacR concentration, the following apparent dissociation constants (K_D) were determined: K_D^{lacO1}, 1.9×10^{-7} M; K_D^{lacO2}, 6.2×10^{-7} M; $K_D^{lacO1O2}$, 2.3×10^{-7} M (Fig. 5).

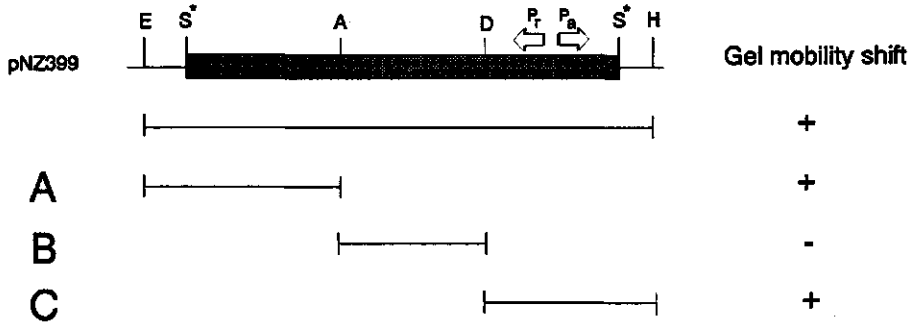

Fig. 3. Linear map of the *lac* promoter region in plasmid pNZ399 and summary of the gel mobility shift experiments. The bar represents DNA sequences originating from the intercistronic region between the *lacR* and *lacABCDFEGX* genes, located on a *Ssp*I restriction fragment (Van Rooijen *et al.*, 1992). As a result of the cloning procedure the *Ssp*I site was inactivated (S^*). DNA restriction fragments that were used in the gel mobility shift assays are indicated by the lines below the map. Arrows indicate positions and directions of the *lac* (P_a) and *lacR* (P_r) promoters. Restriction enzyme cleavage sites that were used in plasmid constructions and isolation of DNA probes are indicated: A, *Ava*II; D, *Dra*I; E, *Eco*RI; H, *Hin*dIII.

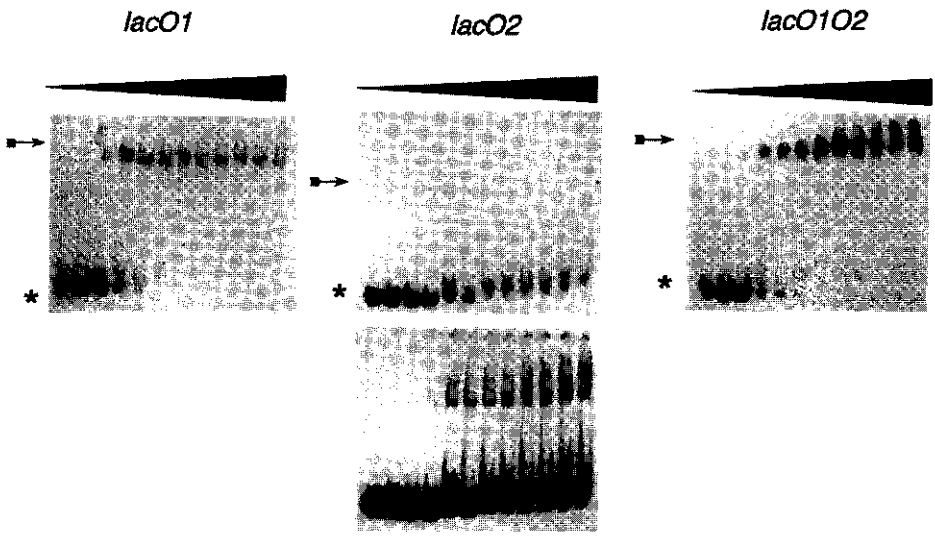

Fig. 4. Binding of LacR repressor to DNA fragments containing *lac* operators *lacO1O2*, *lacO1*, or *lacO2*. The DNA fragments (5 fmol) presented in Fig. 3 containing the *lac* promoter/operator region were radioactively labelled with [α-^{32}P]dATP and Klenow and incubated with an increasing amounts (<) , no, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 μM of LacR repressor. After incubation for 30 min at 4°C samples were separated onto a vertical 5 % polyacrylamide gel. After electrophoresis the gel was dried and free and complexed DNA were detected by autoradiography. Arrows and stars indicate positions of DNA-LacR complexes and free DNA, respectively. To visualize the *lacO2*-LacR complex, the result of an extended autoradiography is presented.

DNase I footprinting of the LacR binding sites *lacO1* and *lacO2*. To precisely determine the DNA sequences in the *lac* promoter/operator region that are involved in the binding of LacR repressor, DNase I footprinting experiments were carried out with the top and bottom DNA strands of the LacR binding sites *lacO1* or *lacO2* (Fig. 6). The labelled fragments were incubated with LacR repressor and partially digested with DNase I. After separation on a denaturing polyacrylamide gel, the products were visualized by autoradiography. In the absence of LacR, DNase I digestion results in a distinct pattern of bands (Fig. 6). Upon addition of LacR repressor protected regions were detected in the fragments that covered positions -31 to +6 (*lacO1*), and -313 to -279 (*lacO2*)

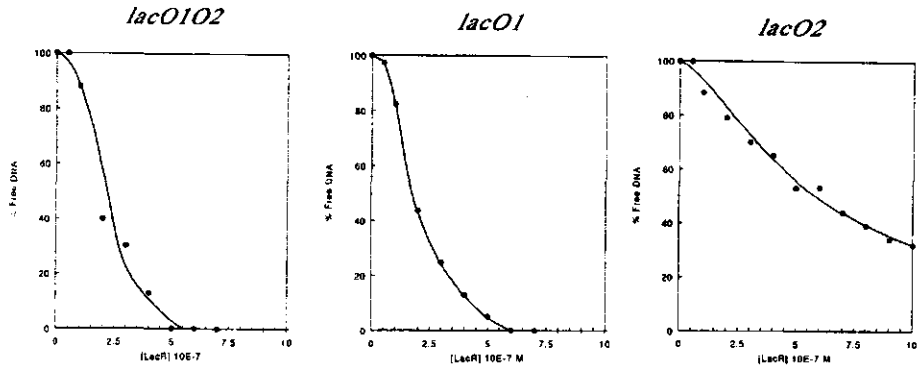

Fig. 5. Titration curves of LacR binding to DNA fragments containing *lacO1*, *lacO2*, or both (*lacO1O2*). The fraction of free DNA was quantitatively analyzed from the autoradiograms presented in Figure 3 using a two-dimensional scanner (Biorad). The fraction of free DNA (%) was plotted as a function of the LacR concentration in the reaction mixture. The apparent dissociation constants K_d (LacR concentration at half saturation point) were determined: K_D^{lacO1}, 1.9×10^{-7} M; K_D^{lacO2}, 6.2×10^{-7} M; $K_D^{lacO1O2}$, 2.3×10^{-7} M.

relative to the *lac* operon transcription initiation site. With respect to the *lacR* transcription initiation site, *lacO1* and *lacO2* were located at positions -96 to -59 and +189 to +223, respectively. The binding of LacR resulted in the appearance of DNAse I sensitive sites or the increase of sensitivity of some nucleotides to DNase I (Fig. 6), suggesting that DNA bending might occur upon LacR binding. No significant changes were found in the sensitivity of the bases between the two operators (not shown).

The LacR-operator complex dissociates in the presence of tagatose-6-phosphate. To identify the inducer of *lac* operon expression, binding between LacR and *lac* operator was studied in the presence of phosphorylated monosaccharides that are formed during growth on lactose and galactose. Derepression of *lac* operon expression is also obtained during growth on galactose, that is transported by the galactose-PTS (LeBlanc *et al.*, 1979; Park and McKay, 1982). Subsequently, the resulting galactose-6-phosphate, like that formed in the lactose metabolism, is further degraded via the tagatose-6-phosphate pathway (Bisset and Anderson, 1974; Crow *et al.*, 1983). The gel mobility shift assay presented in Fig. 7 shows that the LacR-operator complex is absent in the presence of tagatose-6-phosphate (lane 3). The other metabolites, galactose-6-phosphate, tagatose-1,6-diphosphate, glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6,diphosphate, did not affect the formation of the LacR-operator complex. From these data we conclude that tagatose-6-phosphate inhibits formation of the LacR-operator complex and hence, may play a pivotal role in the regulation of *L. lactis lac* operon expression.

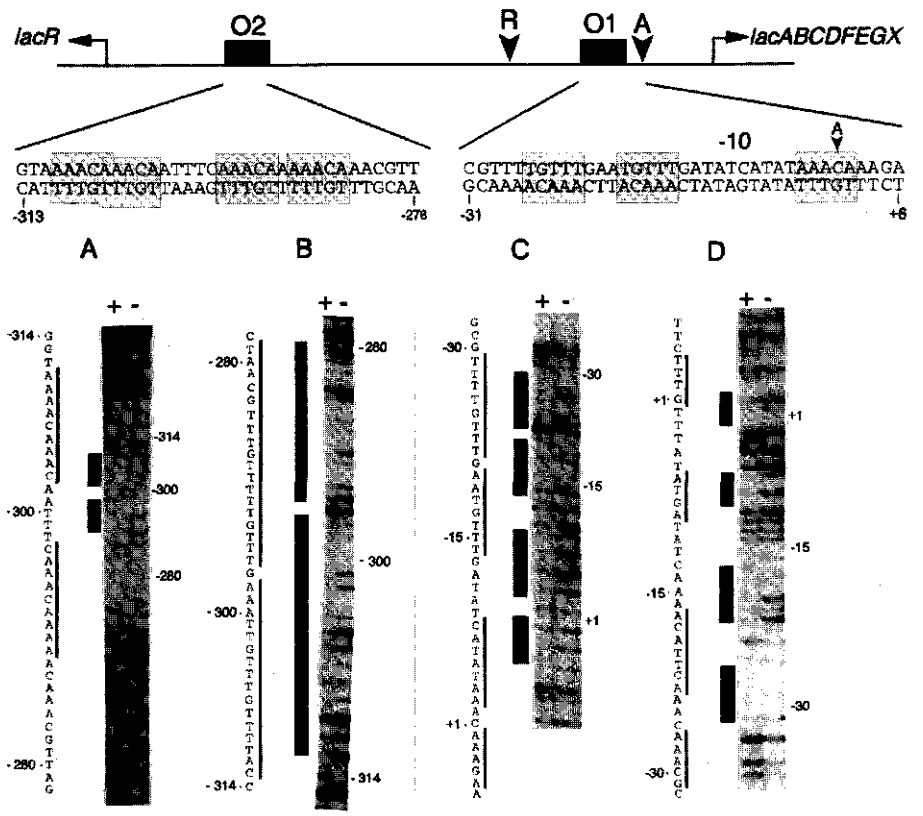

Fig. 6. Organization of the regulatory region of the L. lactis *lac* operon (top) and DNase I footprinting of the operators *lacO1* and *lacO2* (bottom). Transcription of the eight structural genes of *lac* operon, *lacABCDFEGX*, is initiated at the *lac* promoter (vertical arrowhead A). The divergently orientated *lacR* repressor gene is initiated at the *lacR* promoter (vertical arrowhead R), that is organized in a back-to-back configuration with the *lac* promoter. Operators *lacO1* (O1) and *lacO2* (O2) are indicated by black bars. Below, a summary of the DNase I footprinting experiments is presented, in which only the bases that are protected by LacR repressor against DNase I cleavage are shown. The positions relative to the transcription initiation site of the *lac* operon are indicated. The inverted and direct TGTTT repeats that coincide with the protected regions are boxed. DNase I footprints of top and bottom strands of the LacR binding sites *lacO2* (panels A and B) and *lacO1* (panels C and D) are shown at the bottom. End-labelled fragments containing *lacO1*, *lacO2* or both were digested with DNase I under conditions of limited digestion in the absence (-) of presence (+) of 1.0 μM of LacR repressor. The nucleotides in the *lacO1* and *lacO2* top and bottom strands that are protected against DNase I cleavage are indicated by black bars and lines.

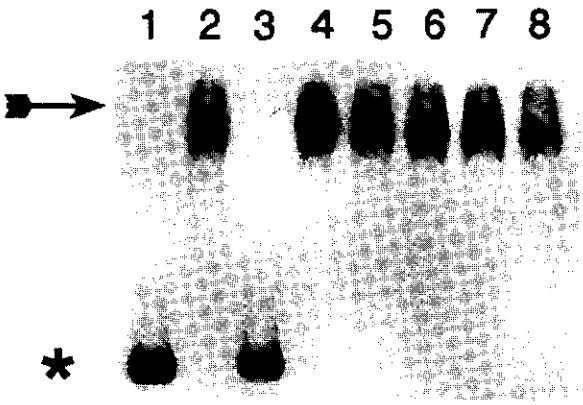

Fig. 7. Effect of phosphorylated metabolites on the LacR-operator complex. A 419-bp radioactively labeled DNA fragment containing operators *lacO1* and *lacO2* of the *lac* operon was incubated with the LacR repressor (5×10^{-7} M endconcentration) in the presence or absence of various phosphorylated monosaccharides (4 mM each). Free and complexed DNA were detected by autoradiography. Lane 1, DNA alone; Lane 2, incubation with LacR; Lanes 3-8, incubation with LacR in the presence of tagatose-6-phosphate, galactose-6-phosphate, tagatose-1,6-diphosphate, glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-diphosphate, respectively. Symbols as described in the legend of Fig. 4.

DISCUSSION

The *L. lactis* LacR repressor regulates expression of the *lac* operon by repressing *lac* promoter activity during growth on glucose. In *L. lactis* derepressed *lac* operon activities were observed during growth on glucose in the presence of multiple copies of the DNA region -387 to +114 relative to the transcription initiation site of the *lac* operon (Van Rooijen *et al.*, 1992). In this paper we have studied the *in vitro* interaction between the LacR repressor and the promoter/operator regions of the *lac* operon and the divergently orientated *lacR* gene. For this purpose the LacR repressor was purified from overproducing *E. coli* cells by a three-step procedure (Fig. 1).

It has been well established that the mode of action of repressors is the prevention of transcription initiation. Thereby, the repressor molecule binds to a region of dyad symmetry near the -35 and -10 promoter DNA consensus sequences (Collado-Vides *et al.*, 1991). The ability of the LacR repressor to bind to the *lac* promoter region is evident from the gel mobility shift assay experiments (Fig. 3 and 4). The operator sites *lacO1* and *lacO2* were localized by DNase I footprinting and found to cover the regions

-31 to +6 and +189 to +223 relative to the transcription initiation sites of the *lac* operon and *lacR* gene, respectively. An inverted repeat, $TGTTTN_{10}AAACA$, from position -18 to +2 coincides with the region that covers *lacO1* (Fig. 6). No such inverted repeat is present in the second LacR bindingsite *lacO2*. However, a direct repeat, TGTTT, that is part of the left arm of the *lacO1* inverted repeat, is also present in *lacO2*. Therefore, it is tempting to speculate that the TGTTT motif comprises the LacR recognition sequence. A similar pentanucleotide, TGTTA, is present in the operator half site of the *E.coli* LacI repressor variant 44 (Lehming *et al.*, 1988). Recently, we observed that the recognition helix of the *E.coli* LacI repressor variant 44 is homologous to that of *L.lactis* LacR[2]. Site-directed mutagenesis of the LacR recognition helix showed that residues Met-34 and Arg-38 are involved in DNA-binding[2]. Corresponding residues in the LacI repressor variant 44 have been shown to contact the TGTTA motif of the corresponding operator halfsite (Lehming *et al.*, 1988).

DNA fragments containing *lacO1* and *lacO1O2* showed comparable affinities for LacR repressor, whereas the affinity was approximately three-fold lower for the DNA fragment containing solely *lacO2* (Figs. 4 and 5). These results indicate that (i) the intrinsic affinity for LacR repressor is higher for *lacO1* than *lacO2*, and (ii) the presence of *lacO2 in cis* does not enhance binding of LacR to *lacO1*.

The subunit composition of LacR repressor was studied in the glutaraldehyde cross-linking studies (Fig. 2) and showed the ability of free LacR repressor to generate dimers in solution. The protected regions in *lacO1* and *lacO2* cover approximately four helical turns suggesting that two LacR dimers are bound. The exact nature and stoichiometry of binding between LacR dimers and *lac* operators remains to be determined. Recently, we have found additional evidence for the multimeric nature of LacR *in vivo*. LacR proteins that were mutated in their DNA-binding site were able to titrate the wild-type LacR protein in the wild-type *L.lactis* strain MG5267, resulting in a derepressed *lac* operon activity during growth on glucose[2].

It has been recently shown that during growth of *L. lactis* on glucose, *lac* operon expression was derepressed in the presence of multicopy plasmids containing both *lacO1* and *lacO2*. No derepression was observed in the presence of multicopy plasmids (3-8 copies) carrying solely *lacO1* (Van Rooijen *et al.*, 1992). In addition, transcriptional fusions between the *cat*-86 reporter gene and various *lac* promoter fragments demonstrated that in *L. lactis* the presence of both *lacO1* and *lacO2* was required to obtain inducible CAT activities (Van Rooijen *et al.*, 1992). This strongly suggests that during growth on glucose, repression of transcription initiation of the *lac* operon is accomplished by binding of the LacR repressor to both *lacO1* and *lacO2*. The contribution of *lacO2* to the *in vivo* repression might be the involvement in the formation of a repression loop. It has been proposed that DNA loops are important factors in the transcriptional control and efficient repression of the *E. coli lac*, *ara*, *gal*, and *deo* operons (Matthews, 1992). In gram-positive bacteria, the involvement of DNA looping

still has only been suggested for the transcriptional control of the *B. subtilis argC* operon by AhrC (Czaplewski *et al.*, 1992).

Expression of the *L. lactis lacR* gene is repressed during growth on lactose, in contrast to the divergently transcribed *lac* operon which is derepressed under these conditions (Van Rooijen and De Vos, 1990). The location of the *lacO1* operator from positions -96 to -59 relative to the transcription initiation site of *lacR* (Fig. 6) coincides with the distance that is frequently found in activation of transcription in *E.coli* (Collado-Vides *et al.*, 1991) and strongly suggests that *lacR* is autoregulated. Therefore, binding of LacR to *lacO1* might activate transcription of *lacR* during growth on glucose. At increasing LacR concentrations, LacR binds to *lacO2* thereby repressing its own synthesis. This would agree with the *in vitro* experiments showing that higher concentrations of LacR repressor are required to bind *lacO2* (Figs. 4 and 5). The organization of the operators of the *lac* operon strongly resembles that of the *E. coli* lambda cI repressor and lambda cro operators. The *cI* and *cro* promoters are organized divergently in a back-to-back configuration. Binding of cI repressor to O_R1 and O_R2 simultaneously represses transcription initiation of *cro* and activates its own transcription initiation. At high concentrations, cI repressor binds to O_R3 and represses *cI* transcription (see Ptashne, 1987). Also for other *E. coli* regulons it has been reported that the divergently transcribed regulator gene is autoregulated (Beck and Warren, 1988).

The nature of the signal that provides derepression of the *L. lactis lac* operon was studied. For this purpose we determined the effect of phosphorylated monosaccharides that are formed in the tagatose-6-phosphate and glycolytic pathways on the complex formation between the LacR repressor and the *lac* promoter/operator region. It was shown that the presence of tagatose-6-phosphate negatively affects the complex between LacR repressor and *lac* promoter/operator (Fig. 6). The presence of other phosphorylated sugars did not significantly affect formation of the LacR-DNA complex. Therefore, tagatose-6-phosphate, and not galactose-6-phosphate as has been previously suggested (Cords and McKay, 1974), is most probably the inducer of *L. lactis lac* operon transcription. For the DeoR repressor it has been shown *in vitro* that the presence of inducer of the *deo* operon, deoxyribose-5-phosphate, negatively affects formation of the *deoO1*-DeoR complex (Mortensen *et al.*, 1989). Also for the catabolic operons that are controlled by the other members of the DeoR repressor family it has been postulated that the physiological inducer is one of the phosphorylated intermediates that are formed in the metabolic routes they encode (Van Rooijen and De Vos, 1990). Based on data described in this paper and previous data (Van Rooijen and De Vos, 1990; Van Rooijen *et al.*, 1992) we propose the following model for the action of LacR repressor in the regulation of the *L. lactis lac* operon. During growth on glucose, first the LacR repressor binds to the *lacO1* operator and activates transcription of the *lacR* gene. No or only slight repression of transcription initiation of the *lac* promoter yet occurs. At increasing LacR concentrations, LacR repressor binds to *lacO2* thereby repressing both

transcription of the *lac* operon and that of its own gene. During growth on lactose, the inducer tagatose-6-phosphate that is generated in the tagatose-6-phosphate pathway binds to LacR. The tagatose-6-phosphate/LacR complex cannot bind to the *lac* promoter region resulting in a derepression of the *lac* operon.

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FOOTNOTES

[1] The abbreviations used are: CAP, catabolite activating protein; CAT, chloramphenicolacetyltransferase; IPTG, isopropyl-ß-D-galactopyranoside; kb, kilobases; PAGE, polyacrylamide gelelectrophoresis; PEP-PTS^{lac}, phospho*enol*pyruvate-dependente lactose phosphotransferase system; SDS, sodium dodecylsulphate.

[2] R.J. van Rooijen *et al.*, submitted for publication

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CHAPTER 8

DELETION OF THE *LACTOCOCCUS LACTIS LACR* REPRESSOR GENE AND ITS EFFECT ON THE REGULATION OF LACTOSE OPERON EXPRESSION

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ABSTRACT

The *lacR* gene, encoding the repressor of the chromosomally located *lacABCDFEGX* operon in *Lactococcus lactis* MG5267, was deleted by replacement recombination, resulting in strain NZ3015. Lactose phosphotransferase (LacEF) and phospho-ß-galactosidase (LacG) activities were determined in strain NZ3015, the wild-type strain MG5267, and NZ3015 containing the *lacR* gene on a multicopy plasmid. Introduction of multiple copies of *lacR* in NZ3015 resulted in LacEF and LacG enzyme activities on glucose and lactose that were lower than those of wild-type MG5267. Partial derepressed LacEF and LacG activities were observed in strain NZ3015 grown on glucose, confirming the contribution of the LacR repressor in the regulation of the lactose operon. However, the LacEF and LacG enzyme activities and *lac* messenger RNA levels in strain NZ3015 remained lower on glucose than on lactose and approximately one fifth of the wild-type regulation level was retained in NZ3015. These results indicate that expression of the *L.lactis lac* operon is not only controlled by the LacR repressor but is also subject to glucose (catabolite) repression. The *L.lactis lac* promoter region contains a DNA sequence that is conserved in the corresponding region of the *Staphylococcus aureus lac* operon, and shows homology to a postulated consensus sequence involved in catabolite repression in *Bacilli*.

INTRODUCTION

During growth of *Lactococcus lactis* in dairy fermentations, energy is obtained by the rapid conversion of lactose into lactic acid. The initial step in this conversion is the uptake and phosphorylation of lactose via the phospho*enol*pyruvate-dependent phosphotransferase system (PEP-PTS^{lac}), that includes the lactose-specific Enzyme II (LacE) and Enzyme III (LacF) and has only been found in gram-positive bacteria (7). Lactose-6-phosphate is subsequently cleaved by phospho-ß-galactosidase (LacG) into glucose and galactose-6-phosphate (6). The galactose-6-phosphate moiety is further metabolized in the tagatose-6-phosphate pathway, that includes three enzymatic steps (1). The plasmid-located genes encoding the PEP-PTS^{lac} and tagatose-6-phosphate pathway enzymes (LacABCD) have been cloned and characterized and found to be organized in the *lac* operon, with the order *lacABCDFEGX* (7, 29). Expression of the *lac* operon is repressed during growth on glucose via the product of the divergently transcribed *lacR* gene, encoding the LacR repressor (28, 30). The *L. lactis* LacR repressor, which belongs to the *Escherichia coli* DeoR family of repressors (24, 28), has been shown with footprinting studies to bind to two operators in the *lac* promoter region, and is assumed to inhibit transcription initiation from the *lac* promoter (31).

For the *Staphylococcus aureus lacABCDFEG* operon, that is homologous to the *L. lactis lac* operon (7, 19, 29), it has been observed that regulation of transcription is mediated by both catabolite (glucose) repression and the action of the LacR repressor (17). However, the relative contributions of each of those control systems have not yet been determined. The *S. aureus* LacR shares a high degree of amino acid similarity (44% identity) with the *L. lactis* LacR (28). In contrast to *L. lactis* (28) its *lacR* gene is transcribed in the same orientation as the *lac* operon (17).

Catabolite (glucose) repression has also been found to contribute to the regulation of several catabolic operons in other gram-positive bacteria, mainly *Bacilli* (for a review see 8). *Cis*-acting sequences have been proposed to be involved in this glucose repression (9, 15, 33). However, no general metabolite has yet been identified that, like *E. coli* cyclic AMP (5), senses the metabolic state of the cell and controls the expression of catabolic operons in these bacteria. Therefore, the mechanism of this global control mechanism remains to be established in gram-positive bacteria.

To investigate whether additional control elements are involved in the regulation of the *lac* operon, we have deleted by replacement recombination the *lacR* gene of *L. lactis* MG5267, which contains a single chromosomal copy of the *lac* regulon. This allowed us to study the expression of the *lac* operon in strains containing no, a single, or multiple copies of the lacR gene. The results described here confirm the essential role of the LacR repressor in controlling the expression of the *L. lactis lac* operon and provide evidence for the presence of a second control circuit.

MATERIALS AND METHODS

Bacterial strains, media and enzymes. *E.coli* strain MC1061 (4) was used as a recipient in the cloning experiments. *L.lactis* subsp. *lactis* strains used included MG5267, which contains a chromosomally integrated single copy of the *lac* operon (30), and its LacR-deficient derivative NZ3015 (see below). Growth of *L.lactis* and *E.coli* was performed in media based on M17 broth (Difco Laboratories, Detroit, Mich.) containing 0.5 % (w/v) glucose or lactose, and L-broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl), respectively. When appropriate, media were supplemented with ampicillin (50 μg/ml), erythromycin (5 μg/ml), and chloramphenicol (Cm; 10 μg/ml for *E.coli* and 5 μg/ml for *L.lactis*). Enzymes were purchased from Bethesda Research Laboratories (Gaitersburg, Md.), New England Biolabs Inc. (Beverly, Mass.), or Promega Corporation (Madison, Wisconsin) and used according to the instructions of the manufacturers. O-nitrophenyl-ß-D-galactopyranoside 6-phosphate (ONPG-P) was purchased from Sigma (Chemical Compagy, St. Louis, U.S.A.) and [α-^{32}P]dATP, [α-^{32}P]CTP and [γ-^{32}P]ATP were supplied by Amersham International plc (UK). Oligonucleotides were synthesized on a Cyclone DNA synthesizer (Biosearch, San Rafael, Calif.).

DNA procedures and construction of plasmids. DNA manipulations and transformation of *E.coli* were performed by standard procedures (21). A modified alkaline lysis method (6) was used for the isolation of plasmid DNA from *L.lactis*. Isolation of total DNA from *L.lactis* was performed after lysis of protoplasted cells as described (11). DNA was transformed into *L.lactis* by electroporation following the procedure of Holo and Nes (10). Prior to the construction of the *lacR* integration plasmid pNZ3015 (Fig. 1), a plasmid was constructed that contained DNA sequences downstream of the *lacR* coding sequences. For this purpose, a 1.7-kb *Cla*I (treated with Klenow DNA polymerase)-*Eco*RV restriction fragment of pMG820 (14), located downstream of *lacR* (Fig. 1), was cloned into the *Sma*I site of plasmid pKK232-8 (3) resulting in plasmid pNZ3014. Subsequently, a 1.5-kb *Eco*RI restriction fragment including the pMG820 *Cla*I-*Eco*RI fragment (Fig. 1, hatched box) was excised from plasmid pNZ3014 and cloned into the *Eco*RI-site of plasmid pUC19E (13), downstream of the erythromycin resistance (Em^R) gene, resulting in plasmid pNZ3018. Finally, a 1.1-kb *Nde*I (treated with Klenow DNA polymerase) restriction fragment from pNZ390 (29) was cloned in the unique *Bam*HI (site treated with Klenow DNA polymerase) of pNZ3018, upstream of the Em^R gene resulting in the 6.2-kb plasmid pNZ3015 (Fig. 1). Plasmid pNZ3016 contains the *lacR* gene and was constructed by cloning the 1.3-kb *Eco*RI-*Bam*HI restriction fragment of pNZ380 (28) in the *Eco*RI-*Bam*HI site of pNZ3017, a derivative of pGKV210 (27) containing the *prtP* promoter (32) upstream of the *cat*-86 gene. The copy number of pNZ3016 in *L.lactis* was determined following the previously described procedure (30)

and found to be 3.2 copies per chromosome. The *lacB* and *usp45* transcription plasmids pNZ3020 and pNZ3021 contain the 0.35-kb *Eco*RI and 0.6-kb *Pst*I restriction fragments of pNZ392 (29) and pNZ1011 (25) cloned in the *Eco*RI and *Pst*I sites of pGEM1 (Promega), respectively. The cloned DNA fragments in pNZ3020 and pNZ3021 were orientated in such a way that antisense RNA probes for the *lacB* and *usp45* genes were generated from the SP6 RNA polymerase promoter.
Sense RNA probes for for *lacB* and *usp45* were generated from the T7 RNA polymerase promoter.

Enzyme assays. PEP-PTS^{lac} (LacEF) activities were determined using permeabilized cells as described by LeBlanc *et al.* (12) with the modification that cells were resuspended in 0.1 M sodium-potassium phosphate buffer, 5 mM $MgCl_2$ pH 7.2 prior to the acetone/toluene treatment. Phospho-ß-galactosidase (LacG) activities were assayed with the chromogenic substrate ONPG-P as described (14). Protein concentrations were measured according to Bradford (2) with bovine serum albumin as a standard.

RNA analysis. Exponentially growing cells (25 ml) of *L.lactis* were pelleted and resuspended in 0.5 ml of cold 10 mM Tris.HCl pH 7.5, 1 mM EDTA. Subsequently, 0.6 g of Zirconium glass beads (0.1-mm diameter; Biospec Products, Bartlesville, Okla.), 0.17 ml 4% Macaloid clay suspension (a generous gift of KRONOS S.A/N.V., Rotterdam, The Netherlands), 0.5 ml phenol pH 7.5, and 50 µl 10% sodiumdodecyl sulphate were added and cells were disrupted by high-speed vortexing (2 min, 3 cycles; Biospec Mini BeadBeater) as described (18). Total RNA was separated from DNA, protein, and cell debris by centrifugation. The supernatant contains the RNA and the pellet, consisting of glass beads, phenol, macaloid and cell debris, contains DNA and protein (18). Finally, the sample was treated with phenol/chloroform and the RNA was precipitated with ethanol. RNA was denatured with glyoxal (21), adjusted to a final volume of 250 µl with sterile water, and dotted on a GeneScreen Plus membrane (New England Nuclear) with a Schleicher and Schuell dot blot apparatus. Generation of antisense *lacB* and *usp* RNA probes from plasmids pNZ3020 and pNZ3021 by a transcription reaction from the SP6 RNA polymerase promoter in the presence of [α-^{32}P]CTP followed by hybridization at 42°C was performed as described by the supplier (Promega). Following autoradiography, dots were cut out and total radioactivity was determined using a liquid scintillation counter (Beckman LKS 7500).

Blotting procedures. Chromosomal DNA fragments were separated by electrophoresis in a 1% agarose gel and transferred to a GeneScreen Plus membrane (New England Nuclear) with 1 M NaOH as a transfer buffer following the procedure recommended by the manufacturer. Oligonucleotide probes 5'-GCCATTTGGACTACC-3' (*lacA*, position 498-512)(29) and 5'-GTCATAATTCTAGTCCGC-3' (*lacR*, position 1147-1164)(28), and

plasmid pUC18 were radioactively labeled by standard procedures (21), and used as probes in the Southern analysis of the transformants. For Western blotting, total cellular protein was separated on a 12.5% polyacrylamide-SDS gel and transferred to a nitrocellulose membrane (BA85; Schleicher & Schuell). The membrane was treated with rabbit polyclonal LacR antibodies, obtained by repeated immunization with partially purified LacR repressor (31), and then incubated with peroxidase-labeled goat anti-rabbit antibodies as described by the supplier (Bio-Rad Laboratories, Richmond, Calif.).

RESULTS

Deletion of the *L.lactis* MG5267 *lacR* gene by replacement recombination. To study the contribution of the *lacR* gene in the control of *lac* operon expression, we deleted the *lacR* gene of the lactose-fermenting strain MG5267. For this purpose, the integration vector pNZ3015 was constructed, that allows replacement recombination to occur between the *lacR* gene and the vector part of pNZ3015 (Fig. 1). This strategy is an improvement of the previously described methods in *L.lactis* (13) since the resulting *L.lactis* strain NZ3015 is devoid of antibiotic resistance genes that are functional in this host. As a consequence, strain NZ3015 is a suitable host for further studies with the standard *L.lactis* cloning vectors.

Following transformation of *L.lactis* MG5267 with the 6.2-kb pNZ3015 DNA, two Em^R transformants (designated NZ3015-1 and NZ3015-2) were further analyzed. *Eco*RV-digested total DNA from both transformants was blotted, and hybridized with a labeled oligonucleotide from the *lacA* gene, that represents the right border sequence (Fig. 1). Two DNA fragments of 6.2 and 1.8 kb hybridized (Fig. 2B, lane 2; only one transformant is shown) as expected after a single cross-over event, since pNZ3015 contains a single *Eco*RV site (Fig. 1). No low molecular weight bands (< 20 kb) were detected in undigested total DNA (not shown), indicating that no autonomously replicating plasmid was present. Equally intense hybridization signals were observed from the 6.2- and 1.8-kb fragments (Fig. 2B, lane 2), derived from the integrated pNZ3015 and the endogenous *lacA* copy, respectively. Therefore, we conclude that the transformants carry a single copy of pNZ3015 integrated into the chromosome via a single cross-over event. Restriction mapping of NZ3015-1 chromosomal DNA demonstrated (not shown) that recombination had occurred at the *lacA* fragment (site 1, Fig. 1).

To obtain a strain that lacks the *lacR* gene, a second recombination event has to occur in strain NZ3015-1 between the two copies of the left border (site 2, Fig. 1). If a recombination occurs between the right border fragments, NZ3015-1 reverts back to wild-type and the *lacR-lac* operon (Fig. 1) is reconstituted. To allow this recombination to occur, *L.lactis* NZ3015-1 was grown on lactose for 100 generations in the absence of erythromycin. One hundred colonies were streaked out on agar plates that contained

either 0 or 5 μg/ml of erythromycin. Seven colonies were isolated that showed sensitivity to erythromycin. Western blot analysis with LacR antibodies showed that four did not produce LacR, whereas the remaining three still produced the 28-kDa LacR protein (Fig. 3, only one transformant of each type is shown). The chromosomal DNA of both types of transformants was further studied with vector (pUC19)-, *lacA*-, and *lacR*-specific probes (Fig. 2, lanes 3 and 4). With the vector-specific probe, a 5.2-kb hybridizing *Eco*RV fragment was observed in the transformants that did not produce LacR (Fig. 2A, lane 3). The size of this fragment corresponds to the size expected after a second recombination event between the left borders and also demonstrates that the vector part of pNZ3015 is present in the chromosome (Fig. 1). No such signals were detected in the LacR-producing cells (Fig. 2A, lane 4), indicating that the vector part was excised during the recombination event. With the *lacR* probe, a 1.3-kb hybridizing *Eco*RV fragment could be detected in the LacR-producing transformants (Fig. 2C, lane 4). The size of this fragment corresponds to that expected from the wild-type DNA sequence of the *lacR* gene (28), confirming the reversion event. In contrast, no signal was detected in the non-producers (lane 2C, lane 3). From these results we conclude that the replacement recombination was successful in constructing a *L. lactis* strain, designated NZ3015, in which the *lacR* gene has been replaced by the vector part of the integration plasmid.

Deletion of the *lacR* gene results in partially derepressed *lac* operon activities on glucose. To study the effect of the *lacR* deletion on the regulation of the *lac* operon, phospho-ß-galactosidase (LacG) and lactose phosphotransferase (LacEF) activities were determined (Table 1) in lactose- and glucose-grown cells of *L. lactis* strains NZ3015 and MG5267. Comparable enzyme activities were observed in strain NZ3015 grown on glucose and strain MG5267 grown on lactose, indicating that the absence of *lacR* in NZ3015 results in a derepressed *lac* operon expression. However, LacG and LacEF activities in strain NZ3015 grown on lactose were 1.3 and 2 times higher, respectively, than those observed during growth on glucose, suggesting additional regulation. In order to study whether this regulation occurs at the level of transcription, *lac* messenger RNA levels were determined in glucose- and lactose-grown NZ3015 and MG5267 cells (Fig. 4). As an internal control, the mRNA levels of the constitutively expressed *usp45* gene (26) were determined. The reduction of messenger RNA levels (Fig. 4) during growth on glucose as compared to lactose of strain MG5267 (6.8-fold) and strain NZ3015 (1.5-fold) corresponds to the observed reductions of LacEF and LacG activities (Table 1). Between 18 and 24 % of the wild-type induction levels remained after deletion of the *lacR* gene, indicating that, in addition to the LacR control circuit, a second regulatory circuit is involved in the transcriptional control of *lac* operon expression. In order to determine which sequences in the *lac* promoter region are involved in this additional control system, we introduced in strain NZ3015 multicopy plasmid pNZ3000, that

contains the minimal *lac* promoter fragment (positions -75 to +42)(30). This resulted in constitutive phospho-ß-galactosidase activities during growth on glucose and lactose, whereas in lactose-growing cells harboring control plasmid pNZ3017, phospho-ß-galactosidase activities were still 1.3-fold higher (data not shown). This suggests that a *trans*-acting factor is titrated by sequences in the *lac* minimal promoter fragment.

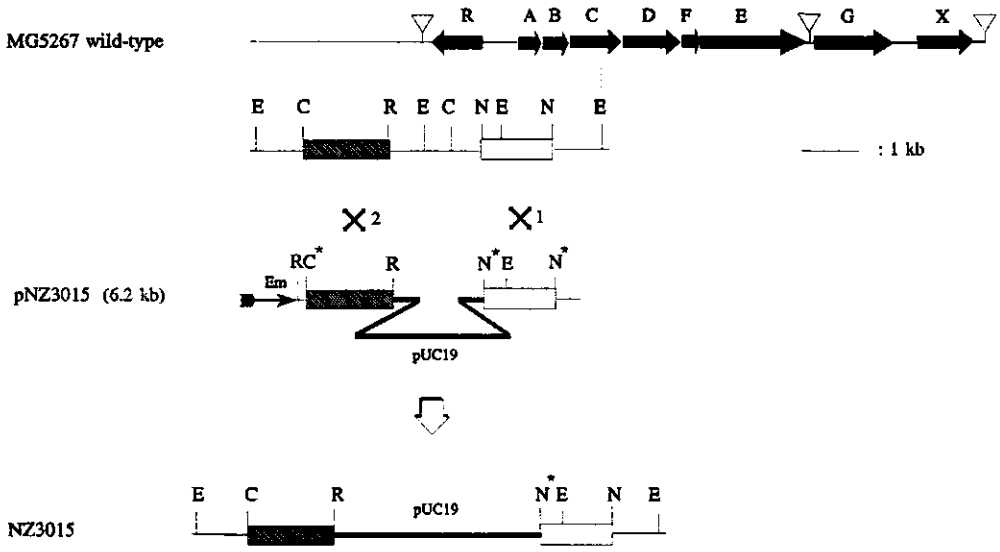

Fig. 1. Organization of the *L.lactis lacR-lac* operon in MG5267 and construction of the *L.lactis lacR*-deficient strain NZ3015. The location and orientation of the *lacABCDFEGX* genes and the divergently transcribed *lacR* repressor gene including transcriptional terminators are shown at the top. The homologous regions that flank the *lacR* gene and are also present in the integration plasmid pNZ3015 are represented by hatched and open boxes. The Em^R gene and pUC19 vector part of pNZ3015 are indicated. Following integration of one copy of pNZ3015 in the *lac* promoter region (recombination 1) resulting in strain NZ3015-1, strain NZ3015 was obtained after a second recombination-event (recombination 2). The restriction enzyme cleavage sites used in the cloning experiments and Southern-blot analysis are indicated: C, *Cla*I; E, *Eco*RV; R, *Eco*RI (only relevant sites are shown); N, *Nde*I. The *Cla*I and *Nde*I sites that were inactivated during the manipulations are indicated by C^* and N^*, respectively.

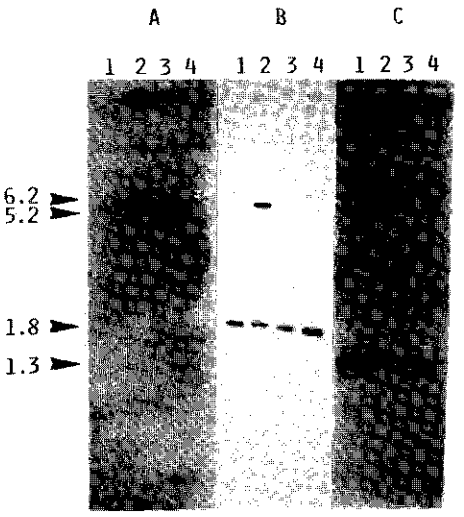

Fig. 2. Southern-blot analysis of transformants obtained after single and double cross-over events with the *lacR*-integration plasmid pNZ3015. Total DNA was digested with *Eco*RV, separated on a 1% agarose gel, blotted, and hybridized with the following radioactively labeled probes: pUC18 (A), and the *lacA* (B) and *lacR* (C) genes. Lane 1, MG5267; lane 2, NZ3015-1; lane 3, NZ3015; lane 4, MG5267 revertant. The molecular sizes (kb) of the hybridizing fragments are indicated by arrows.

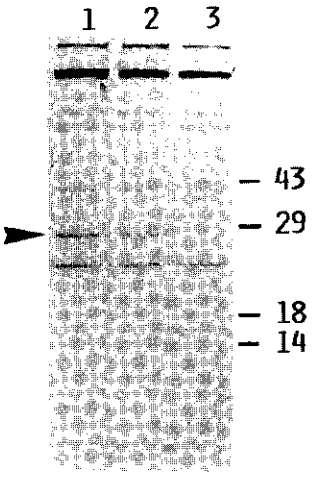

Fig. 3. Western-blot analysis of *L.lactis* MG5267 (lane 1), the wild-type revertant (lane 2), and NZ3015 (lane 3). Total cellular protein was separated according to size, blotted to a nitrocellulose membrane, and subsequently treated with polyclonal LacR-antibodies that react with LacR and some unrelated proteins in the lysate. Protein reacting with anti-LacR antibodies were visualized by staining with peroxidase-labeled goat anti-rabbit antibodies. The arrow indicates the position of the 28-kDa LacR protein. Molecular weight markers (kDa) are indicated.

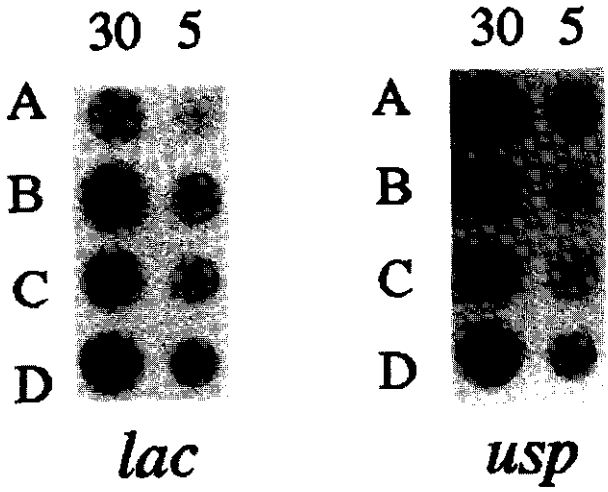

Fig. 4. Determination of *lac* messenger RNA levels in *L.lactis* MG5267 and NZ3015. Total RNA was isolated from glucose- and lactose-grown cells of *L.lactis* MG5267 (A and B, respectively) and NZ3015 (C and D, respectively). Subsequently, 30- and 5 μg of RNA was spotted on a GeneScreen Plus filter and hybridized with radioactively labelled antisense *lacB (lac)* and *usp45 (usp)* RNA probes. The *usp45* gene is expressed constitutively and serves as an internal control (26). In addition, hybridization with sense *lacB* and *usp45* RNA probes revealed no significant differences between RNA isolated from glucose- or lactose-grown cells (not shown). Following autoradiography, total radioactivity of each hybridizing dot was determined. Based on the ratio between the *lac* and *usp* derived signals, relative *lac* mRNA levels were calculated (relative values in %, variations between 30- and 5 μg derived signals are given): NZ3015 lactose, 100 $\pm$ 8; NZ3015 glucose, 67 $\pm$ 4; MG5267 lactose, 81 $\pm$ 10; MG5267 glucose, 12 $\pm$ 3. Ratios between relative mRNA levels on lactose and glucose induction factor) were 6.8 $\pm$ 1.9 and 1.5 $\pm$ 0.2 for strains MG5267 and NZ3015, respectively.

Table 1. Phospho-ß-galactosidase and lactose phosphotransferase activities, and generation time of *L.lactis* strains MG5267, NZ3015, and NZ3016. Average values of two independent determinations including the error are given. Energy sources used in the growth medium are indicated.

Strain	phospho-ß-galactosidase[a]		induction[b]	lactose phosphotransferase[c]		induction	generation time[d]	
	glucose	lactose		glucose	lactose		glucose	lactose
MG5267	0.48 ± 0.02	2.45 ± 0.06	5.1 ± 0.3	8.9 ± 1.0	100 ± 6.1	11.2 ± 1.7	37 ± 0.5	43 ± 0.5
NZ3015	2.35 ± 0.10	2.99 ± 0.10	1.3 ± 0.1	84 ± 3	166 ± 20	2.0 ± 0.3	43 ± 0.5	43 ± 0.5
NZ3016[e]	0.30 ± 0.01	1.92 ± 0.11	6.4 ± 0.6	4.8 ± 0.7	41 ± 1	8.5 ± 1.2	39 ± 0.5	47 ± 0.5

[a] Expressed as μmol.min^{-1} per milligram of protein
[b] Ratio between activities on lactose and glucose
[c] Expressed as nmol.min^{-1} per milligram of cell dry weight
[d] Expressed in min
[e] *L.lactis* NZ3015 harboring pNZ3016

Introduction of multiple copies of *lacR* in *L.lactis* NZ3015 results in the restoration of repressed *lac* operon activities on glucose. To study the effects of multiple copies of the *lacR* gene on regulation of *lac* operon expression, the multicopy plasmid pNZ3016 that contains the *lacR* gene was introduced in *L.lactis* NZ3015. Subsequently, LacEF and LacG activities were determined during growth on glucose and lactose (Table 1). Regulation of *lac* operon expression was restored in the resulting strain, as is indicated by the 6.0 and 8.5-fold decreased LacG and LacEF activities during growth on glucose, respectively. Both on glucose and lactose, the LacG and LacEF activities were significantly lower in strain NZ3015 harboring pNZ3016 than in strains NZ3015 and MG5267. The recovery of wild-type induction levels in strain NZ3015 harboring pNZ3016 confirms the central role of the *lacR* gene in the regulation of *lac* operon expression. In addition, the decreased *lac* operon expression (compared to wild-type) in the presence of multiple copies of the *lacR* gene during growth lactose suggests that the amount of inducer is limited.

Growth properties of *L.lactis* NZ3015. To determine the effect of the *lac* operon expression on the growth, we compared the maximal growth rates on glucose and lactose of *L.lactis* strain NZ3015 with those of strain MG5267 (Table 1). On lactose, no differences in growth rates were observed between strains MG5267 and NZ3015. However, while strain NZ3015 showed identical growth rates on either of both sugars, strain MG5267 grows faster on glucose than on lactose (increase of 6 min in generation time; Table 1). The presence of the *lacR*-encoding pNZ3016 in strain NZ3015 increases the growth rate on glucose to nearly wild-type level (Tabel 1). The slightly lower growth rate of the strain NZ3015 harboring pNZ3016 on glucose (a 2 min decrease in generation time) is most probably a consequence of the presence of plasmid DNA since MG5267 harboring the vector pNZ3017 exhibited a similar decreased growth rate on glucose (not shown). These results indicate that the high expression of the *lac* genes in NZ3015 accounts for the lower growth rate on glucose compared to that of the wild-type strain MG5267. This suggests that the high expression of the *lac* genes in strain MG5267 and other wild-type *L.lactis* strains (22) during growth on lactose is one of the factors contributing to the decreased growth rate on this sugar.

DISCUSSION

We have studied the role of the *lacR* repressor gene in the control of expression of the *L.lactis lac* operon. For this purpose, the *lacR* gene of *L.lactis* MG5267 was deleted by replacement recombination with the vector part of the integration plasmid pNZ3015 (Fig. 1). The effects of this deletion and the introduction of multiple copies of the *lacR* gene on the control of *lac* operon expression was studied by determining *lac* operon expression during growth on glucose and lactose.

Two partially overlapping transcripts have been detected from the *L.lactis lac* operon that are initiated at the same site (30). The largest transcript (8 kb) comprises the *lacABCDFEGX* genes and the smallest (6 kb) the *lacABCDFE* genes (7, 29). The size difference between those transcripts has been attributed to the presence of a putative transcription terminator or RNAse-resistant stem-loop structure in between the *lacE* and *lacG* genes (see Fig. 1)(6, 7). To study the effects of the *lacR* deletion on the regulation of synthesis of both transcripts, phospho-ß-galactosidase (LacG) and lactose phosphotransferase (LacEF) activities were determined (Table 1). In addition, total *lac* mRNA levels were determined using the *lacB* probe (Fig. 4). During growth on glucose, the absence of *lacR* in *L.lactis* NZ3015 resulted in derepressed LacEF and LacG activities and *lac* mRNA levels, that were comparable with those observed in wild-type MG5267 grown on lactose (Table 1, Fig.4), confirming the central role of *lacR* in the regulation of the *L.lactis lac* operon. However, the absence of the LacR control circuit in NZ3015 did not result in a complete constitutive *lac* operon expression on glucose. This is evident from the observation that in NZ3015, LacEF and LacG activities and *lac* messenger RNA levels were higher during growth on lactose than on glucose (2.0-, 1.3-, and 1.5-fold, respectively). The remaining regulation of *lac* operon expression in *L.lactis* NZ3015 equals approximately one fifth of that of the wild-type strain MG5267. Therefore, we conclude that in addition to the LacR regulatory circuit another control circuit, *L.lactis lac* operon expression is also subject to glucose (catabolite) repression.

For the *S.aureus lac* operon, that is homologous to that of *L.lactis*, it has been shown that, in addition to individual control by the LacR repressor, catabolite repression also contributes to regulation of expression (17). Also for *Bacilli* it has been reported that besides individual control, a distinct catabolite (glucose) repression system is operating (8). Two glucose-responsive elements (GREs) have been postulated that might be involved in catabolite repression in *Bacilli*: (i) TGWAANCGNTNWCA (GRE1; 33), and (ii) ATTGAAAG (GRE2; 15). Recently, it has been shown that GRE1 is involved in catabolite repression of the xylose genes of *B.megaterium* (20) and *S.xylosus* (22). In addition, in the *B.subtilis hut* operon a *cis*-acting site associated with catabolite repression has recently been identified (16). Careful inspection of its sequence showed that it also includes a sequence homologous to GRE1 (positions 203-216; one mismatch)(16). We searched for sequences in the promoter region of the *L.lactis lac* operon (30) that are

homologous to the GRE1 consensus sequence (Fig. 5). One DNA sequence, from position +12 to +25 was identified that showed strong homology to GRE1 (Fig. 5). This sequence is located five bp downstream of the *lacO1* operator, the binding site for the LacR repressor (31). Introduction of the multicopy plasmid pNZ3000 (30), that contains DNA sequences -75 to +42 relative to the *lac* transcription initiation site and includes the GRE1 sequence, in strain NZ3015 resulted in constitutive phospho-ß-galactosidase activities suggesting that a *trans*-acting factor that binds to the GRE1 sequence is titrated. Comparison of the nucleotide sequences of the *L. lactis* and *S. aureus lac* promoter regions (Fig. 5) showed that a sequence homologous to GRE1 is located at a comparable position in the *S. aureus lac* promoter region, although the transcription initiation site of the *S. aureus lac* operon has not been determined. In addition, a high degree of similarity was observed with the *L. lactis* -35 and -10 promoter consensus sequence and LacR binding site *lacO1* (Fig. 5). No significant homology was observed between the other parts of the non-coding regions between the *L. lactis* and *S. aureus lacR* and *lacA* genes. It has been postulated that DNA sequences around position -60 and -80 in the promoter region are involved in catabolite repression of the *S. aureus lac* operon (17). However, deletion of these sequences from a promoter-containing fragment cloned in a multicopy plasmid did not completely abolish titration of the catabolite repressor during growth on a glucose/galactose mixture (17), indicating that the -80 and -60 regions are not the only *cis* determinants in the apparent glucose repression.

The effects of multiple copies of *lacR* on the regulation of the *lac* operon were studied by introduction of the multicopy plasmid pNZ3016 into strain NZ3015. This restored the regulation of *lac* operon expression as is indicated by the 8.5 and 6-fold repressed LacEF and LacG activities, respectively, during growth on glucose. The repression levels were comparable to those observed in wild-type MG5267. The absolute LacEF and LacG activities in strain NZ3015 containing pNZ3016 were lower than those in MG5267 during growth on both glucose and lactose. It has been shown *in vitro* that tagatose-6-phosphate, formed during growth on lactose, inhibits binding of LacR to the *lac* promoter/operator region (31). In our current model for the derepression of the *L. lactis lac* operon during growth on lactose, the inducer tagatose-6-phosphate binds to LacR, that as a consequence does not bind to the *lac* operators, resulting in transcription initation of the *lac* operon. In the presence of multiple copies of *lacR*, some LacR molecules might escape from binding by the inducer, resulting in an additional repression of *lac* operon activities. This suggests that the amount of inducer that is formed in lactose-growing cells is limited. Since the LacEF and LacG activities in strain NZ3015 harboring pNZ3016 were lower than that of strain MG5267 during growth on glucose we conclude that in strain MG5267 repression of the *lac* operon is not maximal. The relative inefficient repression of the *L. lactis lac* operon on glucose may be regarded as genetic adaptation to the continuous availability of lactose during growth in milk, resulting in a decreased requirement for an efficient regulation system.

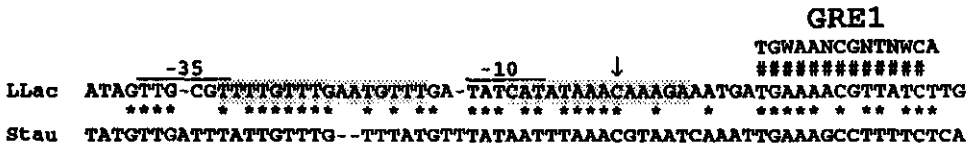

Fig. 5. Homology between DNA sequences in the promoter regions of the *L.lactis* (Llac) and *S.aureus* (Stau) *lac* operons (17, 30) and comparison with the consensus sequence of the putative glucose-responsive element GRE1 from *Bacillus* (33). Conserved residues are indicated (*) and gaps were introduced to maximize identity. Shadowed residues represent LacR binding site *lacO1* (31). *L.lactis lac* promoter -35 and -10 sequences and transcription initiation site (arrow) are indicated (30). Sequences in the *L.lactis lac* promoter region that are homologous to GRE1 are indicated (#): W, A or T nucleotide; N, any nucleotide. The probability of the occurence of GRE1 with one (*L.lactis*) or two mismatches (*S.aureus*) was calculated to be 5.9×10^{-5} (see example) and 1.6×10^{-3}, respectively.

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CHAPTER 9

LYSINES 72, 80, 213 AND ASPARTIC ACID 210 OF THE *LACTOCOCCUS LACTIS* LACR REPRESSOR ARE INVOLVED IN THE RESPONSE TO THE INDUCER TAGATOSE-6-PHOSPHATE LEADING TO INDUCTION OF *LAC* OPERON EXPRESSION

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ABSTRACT

Site-directed mutagenesis of the *Lactococcus lactis lacR* gene was performed to identify residues in the LacR repressor that are involved in the induction of *lacABCDFEGX* operon expression by tagatose-6-phosphate. A putative inducer binding domain located near the C-terminus was previously postulated based on homology studies with the *Escherichia coli* DeoR family of repressors, which all have a phosphorylated sugar as inducer. Residues within this domain and lysine residues that are charge conserved in the DeoR family were changed into alanine or arginine. The production of the *LacR* mutants K72A, K80A, K80R, D210A, K213A, and K213R in the LacR-deficient *L. lactis* strain NZ3015 resulted in repressed phospho-ß-galactosidase (LacG) activities and decreased growth rates on lactose. Gel mobility shift assays showed that the complex between a DNA fragment carrying the *lac* operators and LacR mutants K72A, K80A, K213A and D210A did not dissociate in the presence of tagatose-6-phosphate, in contrast to wild-type LacR. Other mutations (K62A/K63A, K72R, K73A, K73R, T212A, F214A, R216A, R216K) exhibited no gross effects on inducer response. The results strongly suggest that the lysines at positions 72, 80 and 213 and aspartic acid at position 210 are involved in the induction of *lac* operon expression by tagatose-6-phosphate.

INTRODUCTION

Expression of the *Lactococcus lactis lacABCDFEGX* operon, encoding the lactose phosphotransferase, phospho-ß-galactosidase and tagatose-6-phosphate pathway enzymes, is repressed during growth on glucose (De Vos *et al.*, 1990; Van Rooijen *et al.*, 1991). *In vivo* and *in vitro* studies have shown that repression is mediated by the binding of the *lacR* repressor to the *lac* operators, thereby inhibiting transcription initiation from the *lac* promoter (Van Rooijen and De Vos, in preparation; Van Rooijen et al., 1992). Since *in vitro* studies have shown that the LacR-operator complex dissociates in the presence of tagatose-6-phosphate (Van Rooijen and De Vos, in preparation), it is likely that this intermediate, which is formed during growth on lactose, is the inducer of *lac* operon expression.

The *L. lactis* LacR repressor (255 residues) belongs to the *Escherichia coli* DeoR family of repressors, which includes the *E. coli* DeoR, GutR, FucR, *Staphylococcus aureus* LacR and *Agrobacterium tumefaciens* AccR (Van Rooijen and De Vos, 1990; Beck von Bodman *et al.*, 1992). A common feature of the catabolic operons that are regulated by the members of this family is that their expression is induced by a phosphorylated sugar which is generated in the metabolic pathway they encode (Van Rooijen and De Vos, 1990). Based on homology studies, we have previously postulated that residues in the C-terminal part of the LacR repressor might be involved in binding of the inducer (Van Rooijen and De Vos, 1990).

In this paper we describe the identification of amino acid residues in the *L. lactis* LacR repressor that are involved in the response to the inducer tagatose-6-phosphate. For this purpose, we substituted conserved charged residues and residues that are part of the putative inducer-binding site in LacR by arginine or alanine. Mutant LacR proteins that resulted in constitutively repressed phospho-ß-galactosidase activities in the LacR-deficient *L. lactis* strain NZ3015 were purified. It was shown with gel mobility shift assays that their binding to the *lac* operators was not inhibited by tagatose-6-phosphate.

MATERIALS AND METHODS

Bacterial strains, media and plasmids. *E. coli* strain MC1061 (Casabadan *et al.*, 1980) was used as a host for the construction of mutations in the *lacR* gene. *L. lactis* strain NZ3015 is a Lac^+ derivative of MG5267, containing a single chromosomal copy of the *lac* operon (Van Rooijen *et al.*, 1992), in which the *lacR* gene has been deleted by replacement recombination. This strain was used as an expression host for the mutated *lacR* genes. Plasmid pNZ3016 contains the *lacR* gene (Fig. 1) and is based on the replicon pWV01 of pGKV210 (Van der Vossen *et al.*, 1985), that allows replication in *E. coli* and *L. lactis*. The construction of NZ3015 and pNZ3016 will be described elsewhere (Van Rooijen, unpublished results). Expression of the pNZ3016 *lacR* gene is

constitutive (see below), in contrast to the wild-type *L.lactis* MG1820 gene that is repressed on lactose (Van Rooijen and De Vos, 1990). This is most probably a consequence of the absence in the pNZ3016 *lacR* gene of one of the two identified *lac* operators, *lacO1*, that has been postulated to be involved in *lacR* autoregulation (Van Rooijen and De Vos, submitted). Plasmid pNZ3019 was constructed by filling in the *Eco*O1O9 site of pNZ3016 with Klenow polymerase followed by self-ligation. As a consequence, the *Eco*O1O9 (also *Ava*II) site, that is located upstream of the *lacR* ATG start codon, is inactivated and two unique *Ava*II sites flank the *lacR* codons 18 to 101 (Fig. 1). Plasmid pNZ399 containing the *lac* promoter/operator region (Van Rooijen *et al.*, 1992) was used as a source for the preparation of radioactively labelled operator fragments in the gel mobility shift assays. *E.coli* cells harboring the pNZ3016 derivatives were grown in media based on L-broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing chloramphenicol at a final concentration of 10 $\mu g.ml^{-1}$. Media based on M17 broth (Difco) containing 0.5% (wt/vol) glucose or lactose and erythromycin at a final concentration of 5 $\mu g.ml^{-1}$ were used for the growth of *L.lactis* harboring the pNZ3016 derivatives.

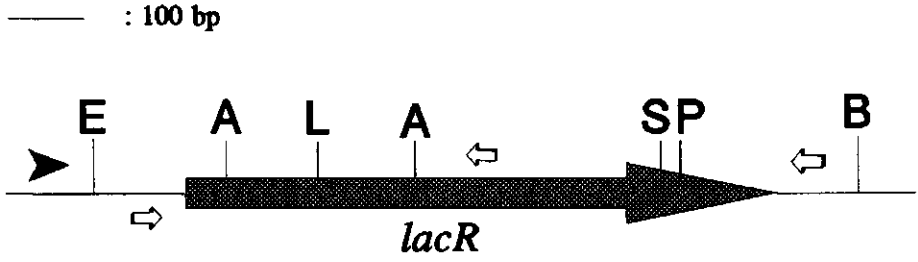

Fig. 1. Physical map of the *lacR* gene of plasmid pNZ3016. The *lacR* coding region and *lacR* promoter are indicated by a closed arrow and arrowhead, respectively. The positions of the restriction enzyme cleavage sites that are used in the cloning experiments are indicated; A, *Ava*II; B, *Bam*HI, E, *Eco*O1O9 (also *Ava*II); L, *Apa*LI; P, *Pvu*I; S, *Sca*I. In plasmid pNZ3019 the *Eco*O1O9 restriction site was inactivated by Klenow treatment. Direction and positions of general PCR primers used in the construction of the mutated *lacR* genes are indicated (open arrows).

Mutagenesis of the *lacR* gene. Mutations T212A, K213A, K213R, K213del, and F214A were constructed by cloning mutagenic synthetic linkers (Table 1) into the *Sca*I-*Pvu*I site of plasmid pNZ3016 (Fig. 1). Routine cloning procedures (Sambrook *et al.*, 1989) were used throughout. Mutations K62A/K63A, K72A, K72R, K73A, K73R, K80A, K80R, and K85A/K86A were constructed with the polymerase chain reaction (PCR) "megaprimer-method" as described (Landt *et al.*, 1990; Sarkar and Sommers, 1990) and modified by Kuipers *et al.* (1991). For this purpose two general PCR primers A and B were used

(Table 1). Mutagenic oligonucleotides (Table 1) were designed in such a way that they were preceded at their 5' end by a T-residue in the template (pNZ3016) strand. The 200-bp fragment that was generated in the first PCR reaction (primer A and mutagenic primer) was purified and used as a primer in the second PCR reaction with primer B. PCR was performed on a BioMed Thermocycler 60. The 0.5-kb PCR products were isolated, digested with *Ava*II (flanking codons 18 to 101) and subsequently cloned in *Ava*II-digested pNZ3019. Mutation D210A was obtained by cloning a 0.5-kb *Sca*I-*Apa*LI digested PCR fragment, that was generated with the D210A primer (Table 1) and primer A, into the *Sca*I-*Apa*LI sites of pNZ3016 (Fig. 1). For mutations R216A and R216K, PCR was carried out in the presence of the mutagenic primers and primer C (Table 1). Subsequently, the 0.3-kb PCR fragments were purified, digested with *Sca*I-*Bam*HI and cloned into the *Sca*I-*Bam*HI sites of pNZ3016. Plasmid DNA was isolated from all mutants and the nucleotide sequence of DNA that originated from the PCR or the DNA synthesizer was determined (Sanger *et al.*, 1977).

Primer	DNA sequence (5'-->3')	Remarks
A	TTTGAAATTGTTTGTTTTACCTTG	General PCR primer; positions -78 to -54 to *lacR* ATG startcodon
B	CTCTATATTCACCGCCAAGAAG	General PCR primer; positions +447 to +426 to *lacR* ATG startcodon
C	CTAGAGGATCCCCATCCAA	General PCR primer; 200 bp downstream of 3' end of *lacR* gene
K62A/K63A	AAGCTTTCCTCTGCAGCGCCACTTGAAAAGAC	Double mutation of lysines 62 and 63 to alanines
K72A	GAAAAGACACATATCGAGGCGAAAAGTCTAAATACAAAG	Lysine 72 to alanine
K72R	GAAAAGACACATATCGAGAGGAAAAGTCTAAATACAAAG	Lysine 72 to arginine
K73A	GAAAAGACACATATCGAGAAGGCAAGTCTAAATACAAAG	Lysine 73 to alanine
K73R	GAAAAGACACATATCGAGAAGAGAAGTCTAAATACAAAG	Lysine 73 to arginine
K80A	AGTCTAAATACAAAAGAAGCAATTGACATTGCTAAAAAAG	Lysine 80 to alanine
K80R	AGTCTAAATACAAAAGAAAGAATTGACATTGCTAAAAAAG	Lysine 80 to arginine
K85A/K86A	ATGACATTGCTGCAGCAGCCTGCTCTTTAATC	Double mutation of lysines 85 and 86 to alanines; *Pst*I site created
D210A	CGATCGAATTTAGTACTGGCTACTAATAAGAATTTTTCT	Aspartic acid 210 to alanine
T212A	GCTAAATTCGAT and CGAATTTAGC	Threonine 212 to alanine; *Sca*I site disappears after cloning
K213A	ACTGCATTCGAT and CGAATGCAGT	Lysine 213 to alanine
K213R	ACTCGATTCGAT and CaAATCGAGT	Lysine 213 to arginine; *Pvu*I site disappears after cloning
F214A	ACTAAAGCaGAT and CtGCTTTAGT	Phenylalanine to alanine; *Pvu*I site disappears after cloning
R216A	GTAGACAGTACTAAATTCGATGCATACGATTTC	Arginine 216 to alanine; *Nsi*I site is created
R216K	GTAGACAGTACTAAATTCGATAAATACGATTTC	Arginine 216 to lysine

Table 1. Primers and linkers used for the mutagenesis of the *L.lactis lacR* gene. Underlined and lower case bases represent the specific and silent mutations, respectively.

Phospho-ß-galactosidase activities and Western-blot analysis. Total cellular protein was isolated after the disruption of logarithmically growing cells by high-speed vortexing in the presence of zirconium glass beads using the Biospec Mini BeadBeater (Biospec Products, Bartlessville, Oklahoma) as described (Van Rooijen and De Vos, 1990). Phospho-ß-galactosidase (LacG) activities were assayed at 37 °C with the chromogenic substrate ortho-nitrophenyl-ß-D-galactopyranoside 6-phosphate (ONPG-P; Sigma) as described (Maeda and Gasson, 1986). For Western blotting, equal amounts of cells were treated with lysozyme as described (Maeda and Gasson, 1986), and boiled (5 min) in the presence of SDS/PAGE sample buffer. Subsequently, total cellular protein was separated

on a 12.5% polyacrylamide-SDS gel and transferred to a nitrocellulose membrane (BA85; Schleicher & Schuell). The membrane was treated with rabbit polyclonal LacR antibodies and then incubated with peroxidase-labeled goat anti-rabbit antibodies. Protein concentrations were measured according to Bradford (1976) with bovine serum albumin as a standard.

Purification of mutant LacR proteins. Mutant LacR proteins that resulted in constitutive repression of LacG activities in *L.lactis* were purified from their respective *E.coli* hosts. For this purpose, *E.coli* cells were grown overnight at 37 °C and mutant LacR protein was isolated by a Q-Sepharose batch treatment followed by heparin-agarose chromatography as described (Van Rooijen and De Vos, submitted). After purification, the purified mutant LacR protein was dialyzed three times against 50 volumes of 5 mM acetic acid pH 3.5, lyophilized, dissolved in 50 mM Tris.HCl pH 8, 100 mM NaCl, 0.1 mM EDTA, 1 mM ß-mercaptoethanol, 10% glycerol and stored at -80 °C.

Gel mobility shift assays. The 419 bp *Eco*RI-*Hin*dIII restriction fragment from pNZ399 containing the *lac* promoter/operator region was labelled with [α-^{32}P]dATP and isolated from a 5% non-denaturing polyacrylamide gel as described (Sambrook *et al.*, 1989). Binding of LacR with labelled probe was performed as described by Garner and Revsin (1981) in 20 μl of a mixture containing 10 mM Tris.HCl pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 50 μg/ml poly d(I-C), 10% glycerol, 5 fmol end-labelled fragment, and LacR. Incubations (30 min, 4 °C) were carried out in the presence or absence of 4 mM tagatose-6-phosphate. Sample buffer (200 mM Tris pH 8.0, 0.8 % bromo-phenol blue) was added and reaction mixtures were loaded on a 5% polyacrylamide gel (acrylamide:bisacrylamide, 60:1) in 50 mM Tris-borate, 1 mM EDTA (pH 8.3). Electrophoresis was performed at room temperature at 15 V/cm for 2 h, after a prerun of 30 min. After drying the gel was autoradiographed.

RESULTS AND DISCUSSION

Description of the expression system and mutagenesis strategy. Our aim in this study was to identify residues in the *L.lactis* LacR repressor that are involved in the response to the inducer tagatose-6-phosphate. Since the *L.lactis* LacR repressor belongs to the *E.coli* DeoR family of repressors (Van Rooijen and De Vos; 1990 Beck von Bodman, 1992), in which all members have in common that their inducer is a phosphorylated sugar, we reasoned that within this family there will probably be conserved residues that are involved in inducer response. Based on these considerations we previously postulated an inducer binding site that is located near the C-terminus from positions 207 to 216 (Van Rooijen and De Vos, 1990). The putative DNA-binding domain is located near the N-terminus and includes positions 19 to 42. A multiple sequence alignment of the DeoR

repressor family with the primary sequences of the *Staphylococcus aureus* and *Streptococcus mutans* LacR repressors is presented in Fig. 2. No crystal structure of the *L.lactis* LacR repressor or any of the other members of the DeoR family is available yet. Therefore, it is impossible to predict the effects of individual mutations on the overall structure and hence, biological activity of the LacR repressor.

An extensive study has been carried out by Kleina and Miller (1990) who identified twenty amino acids in the *E.coli lacI* repressor that, upon replacement by another amino acid, show a strong decrease of responsivity to inducer IPTG *in vivo* (I^S mutants). Six of these residues, located outside the DNA-binding domain, were charged (K84, D88, R195, R197, E248, D274). Lysine-84 and Arg-195 could be replaced by arginine and lysine, respectively, without a significant loss of response to inducer. No replacements were tolerated in the other charged residues (except E248Q). Based on homology with amino acid residues of the known sugar-binding site of the arabinose-binding protein, an inducer-binding site for the *lacI* repressor has been postulated (Sams *et al.*, 1984). The role of Arg-197 of the *E.coli lacI* repressor in inducer binding has recently been established *in vitro* by Spotts *et al.* (1991). Since charged amino acid residues in a protein are mainly exposed at the surface (Wells, 1991), we reasoned that changing these residues into alanine would least interfere with the folding into an active LacR repressor. The effects of the mutations on the activity of the LacR repressor were to be tested in *L.lactis* NZ3015, which contains a chromosomal copy of the *lac* regulon in which the *lacR* gene has been deleted by replacement recombination. Introduction of plasmid pNZ3016, containing the wild-type *lacR* gene, in NZ3015 leads to a repressed *lac* operon expression during growth on glucose as is reflected by a low phospho-ß-galactosidase (LacG) activity (Table 2). The first property that we tested was the ability of the mutant LacR proteins to repress phospho-ß-galactosidase activities in strain NZ3015 during growth on glucose. When the ratio between phospho-ß-galactosidase activities in the presence of mutant and wild-type LacR did not exceed 1.5, we assumed that the overall structure of the mutant LacR protein was not significantly affected by the introduced mutation. The effects of the mutations on the binding of the inducer tagatose-6-phosphate was initially studied indirectly by determining phospho-ß-galactosidase activities during growth on lactose. When mutant LacR repressor had lost its ability to bind the inducer tagatose-6-phosphate it was anticipated that during growth on lactose such a mutant LacR protein would not dissociate from its *lac* operators and hence, phospho-ß-galactosidase activities on this substrate would remain repressed.

Alanine scanning of residues in *L.lactis* LacR repressor that are conserved within the *E.coli* DeoR family of repressors. Five functionally conserved amino acid residues of the putative inducer binding site of the LacR repressor (D210, T212, K213, F214 and R216, Fig. 2) and lysines at positions 72, 73 and 80 that are charge conserved within the DeoR family were changed into alanine. Both lysines at positions 72 and 73 were

studied since in the multiple sequence alignment the gap preceding these residues is flexible between residues 72 and 80. Therefore, no discrimination between lysines 72 and 73 can be made concerning their position in the multiple sequence alignment (Fig. 2). In addition, alanine substitutions were made for the lysine residues at positions 62, 63 and 85, 86 (K62A/K63A and K85A/K86A) that are not conserved but are part of a lysine-rich segment (9 lysines in 25 residues) from positions 62 to 86.

After transformation of the plasmids carrying the mutated *lacR* genes to the LacR-deficient strain NZ3015, the amount of mutant LacR protein was estimated on Western blots using a polyclonal antibody against purified LacR. The results (Fig. 3) showed that the amounts of LacR repressor produced in all but one (K85A/K86A) of the mutants were comparable to that in the wild-type strain grown on lactose or glucose (lanes 1 and 2), indicating that the introduced mutations did not affect *lacR* expression or lead to increased sensitivity to proteolysis of the expressed mutant proteins. Therefore, the phospho-ß-galactosidase activities in these *lacR* mutants reflect the effects of the introduced mutation on LacR function. An exception is LacR K85A/K86A that showed significantly lower levels of mutant protein (Fig. 3, lane 14), possibly due to an increased sensitivity to proteolysis.

Phospho-ß-galactosidase activities were determined in all mutants during growth on glucose and lactose (Table 2). In all mutants, except for that containing LacR K85A/K86A, repressed phospho-ß-galactosidase activities were found during growth on glucose, comparable to those in NZ3015 containing wild-type LacR. From this result we conclude that the introduced mutations did not significantly affect the functionality of the LacR mutant proteins. The presence of LacR K85A/K86A did not result in repressed phospho-ß-galactosidase activities during growth on glucose indicating that one or both of these residues are important for DNA-binding, multimerization or folding of the protein. Alternatively, the low level of intact mutant LacR protein in this strain might result in an inefficient repression. Therefore, from this mutant no conclusions concerning the inducer binding site can be drawn. In contrast to cells containing the other mutant LacR proteins, phospho-ß-galactosidase activities were not derepressed (induction level < 1.7) in lactose-grown NZ3015 cells containing LacR K72A, K80A and K213A, whereas in NZ3015 containing LacR D210A a decreased induction level (3.1 versus 5.8 with the wild-type LacR) was observed. In addition, growth rates on lactose of cells containing these *lacR* mutations were significantly decreased compared to that of wild-type (Table 2). On glucose, growth rates of all mutant *L. lactis* strains were comparable to that of wild-type (data not shown), indicating that the presence of the LacR K72A, K80A, D210A and K213A mutants specifically affects lactose catabolism and is not a consequence of a general decrease in growth rate. To exclude the possibility that these observations were due to a mutation in the *lac* promoter region or in one of the genes of the *lacABCDFEGX* operon (De Vos *et al.*, 1991; Van Rooijen *et al.*, 1990), strains were cured of the plasmids containing the mutated *lacR* gene.

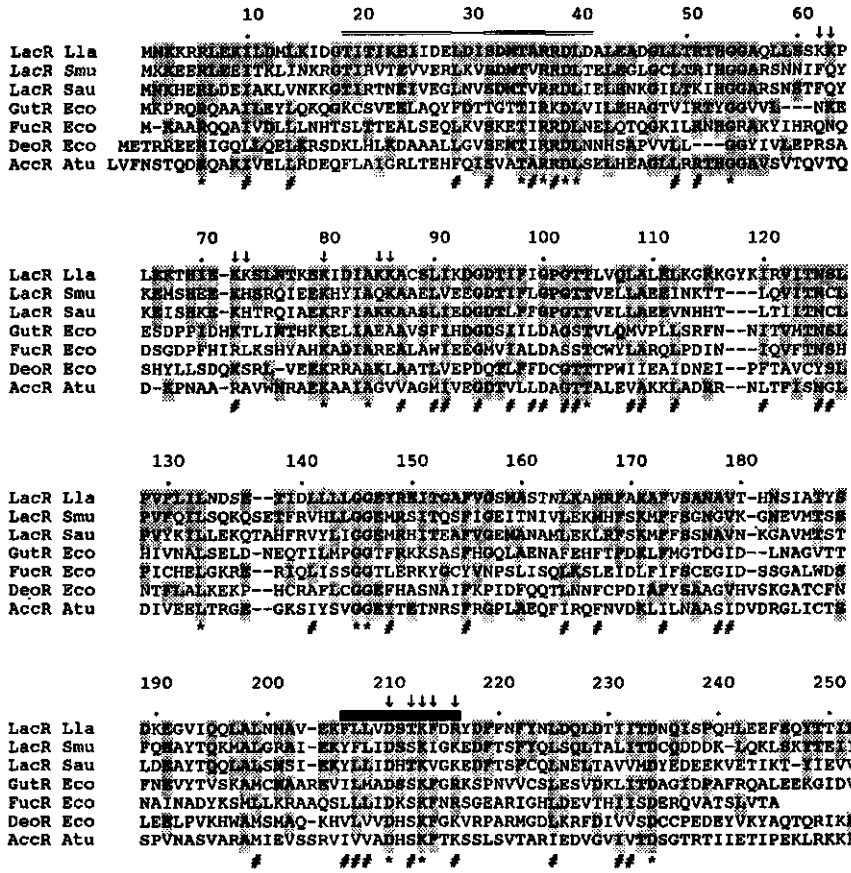

Fig. 2. Multiple sequence alignment between the members of the *E.coli* DeoR family of repressors. LacR Smu, LacR Sau, GutR Eco, FucR Eco, DeoR Eco and AccR Atu are proteins involved in the regulation of the *Streptococcus mutans* and *Staphylococcus aureus* lactose operons (Rosey and Stewart, 1992; Oskouian and Stewart, 1990), *E.coli* glucitol and galactitol, fucose, and deoxyribonucleoside operons (Yamada and Saier, 1988; Lu and Lin, 1989; Lin, 1987; Valentin-Hansen *et al.*, 1985), and *A.tumefaciens Acc* and *Tra* genes (Beck von Bodman, 1992), respectively. Amino acid sequences are given in the one-letter code. Multiple sequence alignment was performed with the CLUSTAL program (Higgins and Sharp, 1988) and gaps were introduced to maximize identity. Percentage identity for pairwise comparisons between *L.lactis* LacR and the other members were between 24 and 44 %. Functionally related (#), identical (*) and positions of amino acid residues are indicated. Amino acid residues that are identical to the *L.lactis* LacR repressor are shadowed and shown in boldface. The putative helix-turn-helix motif (double line-line-double line) and inducer binding site (black bar) in the N- and C-termini, respectively, are indicated. Amino acid residues in the *L.lactis* LacR repressor that were subject to site-directed mutagenesis are indicated with an arrow.

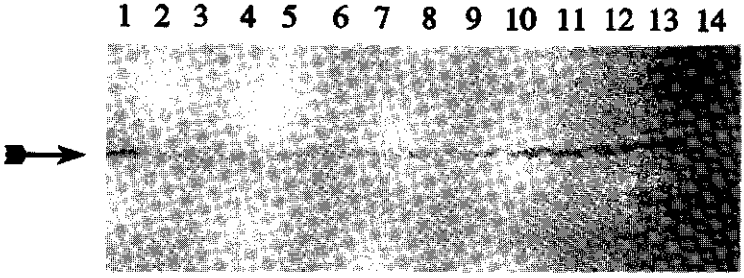

Fig. 3. Western blot analysis of mutant LacR proteins. Equal amounts of *L. lactis* cells harboring the various mutated *lacR* genes were lysed and total cellular protein was separated on a 12.5% SDS-PAGE gel, transferred to nitrocellulose and probed with polyclonal antibodies specific for LacR. The position of LacR is indicated by an arrow. Lane 1 and 2, pNZ3016 (wild-type *lacR* gene), after growth on lactose and glucose, respectively. Lanes 3-14, LacR mutants K72A, K73A, K80A, D210A, T212A, K213A, F214A, R216A, K72R, K80R, K213R, K85A/K86A, respectively, after growth on glucose. Comparable levels of LacR K62A/K63A, K73R and R216K were produced (not shown).

After curing, phospho-ß-galactosidase activities and growth rates on glucose and lactose were identical to those of the expression host NZ3015 (not shown). From these experiments we can deduce the following contributions of the mutated amino acid residues to inducer response: (i) Lysines at positions 62, 63, and 73, Thr-212, Phe-214 and Arg-216 do not seem to play an important role, although we cannot exclude the possibility that their function can be taken over by alanine. Residues Thr-212 and Arg-216 are functionally conserved within the DeoR family (Fig. 2). (ii) Replacement of the lysines at positions 72, 80 and 213 and Asp-210 by alanine leads to repressed phospho-ß-galactosidase activities in NZ3015 during growth on lactose, suggesting that these amino acids are involved in inducer response.

Lysines at positions 80 and 213 cannot be replaced by arginine. From the residues that are involved in inducer response, Lys-80 and 213 and Asp-210 are identical within the members of the DeoR family, whereas Lys-72 is charge conserved (Fig. 2). In order to study whether a positive charge at positions 72, 80, and 213 is essential for inducer response, residues at these positions were changed into arginine. In addition, Lys-73 and Arg-216, which were shown not to be involved in inducer binding (Table 2), were

replaced by arginine and lysine, respectively. All mutated *lacR* genes expressed wild-type amounts of LacR repressor in NZ3015 (Fig. 3). Phospho-ß-galactosidase activities were determined of *L.lactis* NZ3015 harboring the mutated *lacR* genes during growth on glucose and lactose (Table 2). All mutants showed repressed phospho-ß-galactosidase activities during growth on glucose, indicating that the mutations did not affect the functionality of LacR. Analogous to the alanine mutations, the presence of LacR K80R and K213R did not result in derepressed phospho-ß-galactosidase activities during growth on lactose (induction level < 1.3). Derepressed LacG activities were observed in the presence of LacR K72R, K73R and R216K, indicating that these mutations did not affect inducer binding.

Table 2. Phospho-ß-galactosidase activities and growth rates on glucose and lactose of *L.lactis* NZ3015 harboring the indicated *lacR* mutants.

	phospho-ß-galactosidase activities[a]			
lacR mutant	glucose	lactose	induction[b]	growth rate[c]
wild-type[d]	0.28 ± 0.02	1.61 ± 0.11	5.8	58
K62A/K63A	0.41 ± 0.05	1.63 ± 0.12	4.0	58
K72A	0.33 ± 0.01	0.54 ± 0.01	1.6	110
K72R	0.25 ± 0.01	0.97 ± 0.04	3.9	80
K73A	0.43 ± 0.01	1.74 ± 0.08	4.0	58
K73R	0.25 ± 0.02	1.25 ± 0.05	5.0	58
K80A	0.23 ± 0.04	0.21 ± 0.03	1.0	160
K80R	0.27 ± 0.04	0.24 ± 0.01	0.9	160
K85A/K86A	1.83 ± 0.12	1.81 ± 0.07	1.0	58
D210A	0.26 ± 0.01	0.80 ± 0.10	3.1	95
T212A	0.32 ± 0.02	1.51 ± 0.07	4.7	65
K213A	0.26 ± 0.03	0.29 ± 0.01	1.1	150
K213R	0.25 ± 0.02	0.29 ± 0.03	1.2	150
F214A	0.31 ± 0.04	1.79 ± 0.10	5.8	65
R216A	0.31 ± 0.01	1.36 ± 0.08	4.4	58
R216K	0.29 ± 0.02	1.33 ± 0.09	4.6	58

[a] Expressed as $U.mg^{-1}$; Average values and deviations of two independent determinations are given

[b] Ratio between phospho-ß-galactosidase activities on lactose and glucose

[c] Expressed as generation time (min). During growth on glucose, generation times of all mutants were comparable to that of the wild-type strain.

[d] Expression host NZ3015 harboring plasmid pNZ3016. Phospho-ß-galactosidase activities and growth rates of pNZ3019 harboring cells were similar to those harboring pNZ3016.

From these experiments we conclude that the lysines at positions 80 and 213 cannot be replaced by arginine without affecting the response to inducer. Therefore, the specific steric and electrostatic characteristics of the lysines at positions 80 and 213 of the wild-type LacR repressor seem to be important for inducer response. Since replacement of Lys-72 by arginine partially restored inducer response (induction factor 3.9), compared to the replacement by alanine (induction factor 1.6), we conclude that the positive charge and/or the size of the residue at position 72 significantly contributes to inducer response. The results show that for the positively charged residues that have been shown to be involved in inducer response of the *L.lactis* LacR repressor, replacement by another positive residue is only (partially) tolerated for Lys-72, and not for Lys-80 and Lys-213.

Binding of K72A, K80A, D210A and K213A LacR to the *L.lactis lac* operators is not inhibited by the inducer tagatose-6-phosphate. The *in vivo* results described above strongly suggest that the inducer of *lac* operon expression does not negatively affect the formation of the complex between the *lac* operators and LacR mutated at positions 72, 80, 210 or 213. Recently, we have shown that the LacR-operator complex dissociates *in vitro* in the presence of tagatose-6-phosphate (Van Rooijen and De Vos, submitted). In order to confirm the binding of the mutant LacR repressors to the *lac* operators *in vivo* and to study the effect of tagatose-6-phosphate on this interaction, the mutant LacR proteins K72A, K80A, D210A, and K213A were partially purified and used in gel mobility shift assays. First we determined the minimal amount of purified mutant LacR protein to give retention of a 419-bp DNA fragment carrying *lac* operators $lacO_1$ and $lacO_2$ (not shown). No gross differences were observed in the required amounts between the studied mutant LacR proteins, confirming the similar *in vivo* repression on glucose by the mutant and wild-type LacR proteins (Table 2). Subsequently, a gel mobility shift assay was carried out with this minimal amount of mutant LacR in the presence and absence of tagatose-6-phosphate as is shown in Fig. 4. The complex between wild-type LacR and the *lac* operators dissociates in the presence of tagatose-6-phosphate as is demonstrated by the appearance and disappearance of DNA with higher and lower mobility, respectively (Fig. 4, lane 3). In contrast, no dissociation was observed of the complexes between mutant LacR proteins and *lac* operators in the presence of the tagatose-6-phosphate. The congruence of these results and those obtained *in vivo*, as described above, provide additional support for the conclusion that tagatose-6-phosphate is the inducer of *L.lactis lac* operon expression. In addition, these data demonstrate the involvement of lysines 72, 80, and 213 and aspartic acid 210 of the *L.lactis* LacR repressor in the response to the inducer tagatose-6-phosphate.

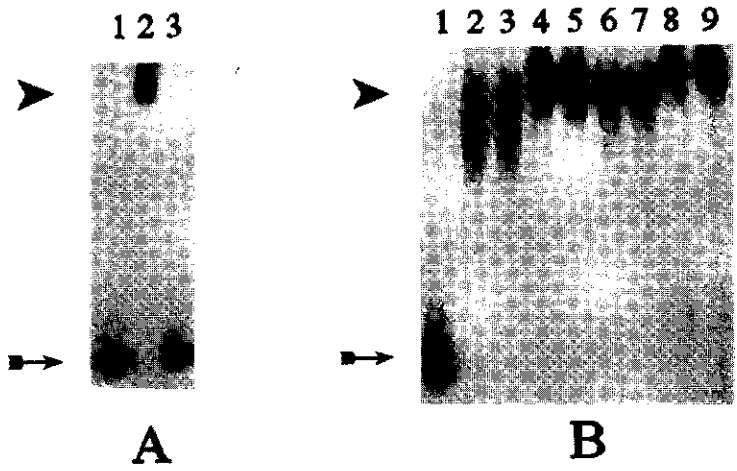

Fig. 4. Gel mobility shift assays with mutant LacR proteins. A 419-bp radioactively labelled DNA fragment containing operators $lacO_1$ and $lacO_2$ (5 fmol) was incubated with wild-type (panel A) or mutant LacR repressor (Panel B) in the presence or absence of the inducer tagatose-6-phosphate. The minimal amount of mutant LacR protein was determined (not shown) that resulted in the formation of the LacR-DNA complex and was used in this experiment. Free and complexed DNA are indicated by an arrow and arrowhead, respectively. Panel A; lane 1, free DNA, lanes 2 and 3, incubation of operator DNA with 20 ng/μl of purified LacR repressor in the absence and presence of 4 mM tagatose-6-phosphate, respectively. Panel B; lane 1, free DNA, lanes 2-9, incubation of operator DNA with 80 ng/μl of partially purified LacR K72A, LacR K80A, LacR D210A, LacR K213A in the absence, lanes 2, 4, 6 and 8, respectively, or presence, lanes 3, 5, 7 and 9, repectively, of 4 mM tagatose-6-phosphate.

Concluding remarks. In this paper we describe the identification of amino acid residues in the *L.lactis* LacR repressor that are involved in the inductive response which comprises the dissociation of the LacR repressor-operator complex resulting in transcription initiation of the *lacABCDFEGX* operon during growth on lactose. The presence of LacR mutants K72A, K80A, D210A or K213A in *L.lactis* NZ3015 leads to a repressed *lac* operon expression and decreased growth rates on lactose. In addition, the complex between purified LacR K72A, K80A, D210A and K213A and the *lac* operators did not dissociate in the presence of tagatose-6-phosphate, in contrast to wild-type LacR. Therefore, we conclude that the residues at positions 72, 80, 210 and 213 significantly contribute to the response to the inducer tagatose-6-phosphate. It remains to be determined whether the lack of response has to be attributed to a decreased affinity for

tagatose-6-phosphate or to the inability to generate a conformational change as a result of tagatose-6-phosphate binding. No significant effects were observed in the presence of LacR mutants K62A/K63A, K73A, K73R, T212A, F214A and R216A, R216K, indicating that the residues at these positions are not involved in inducer binding. In contrast to Lys-72, replacement of lysines 80 and 213 by arginine did not result in a partial reappearance of inducer sensitivity, indicating that the side chain of those lysines at positions 80 and 213 is important for inducer response, rather than their charge. It has been postulated that charged residues in the hypothetical sugar binding site of the *E.coli lacI* repressor can form hydrogen bonds with the inducing sugar (Sams *et al.*, 1984). Recently, this has been confirmed experimentally for Arg-197 (Spotts *et al.*, 1991). Although the *E.coli* LacI and the *L.lactis* LacR repressors share no homology, it is tempting to speculate that the identified charged residues at positions 72, 80, 210 and 213 of the *L.lactis* LacR repressor bind in a similar way to the sugar-part of tagatose-6-phosphate. The phosphate group of tagatose-6-phosphate might be contacted by one or more of the essential lysine residues.

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CHAPTER 10

CHARACTERIZATION OF THE DNA-BINDING HELIX OF THE *LACTOCOCCUS LACTIS* LACR REPRESSOR BY SITE-DIRECTED MUTAGENESIS.

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ABSTRACT

Site-directed mutagenesis of the *Lactococcus lactis lacR* gene, that controls expression of the *lacABCDFEGX* operon, was carried out to identify amino acid residues in the LacR repressor that are involved in binding to the *lac* operators. A putative DNA-binding domain, consisting of a helix-turn-helix motif, was postulated to be present between LacR residues 19 to 41. Mutations were made in residues 30 to 38, that are conserved in the other members of the *Escherichia coli* DeoR family of repressors. M34A-LacR and R38A-LacR were unable to repress phospho-ß-galactosidase (LacG) activities during growth on glucose in the LacR-deficient *L.lactis* strain NZ3015 and were *trans*-dominant over wild-type LacR in strain MG5267. This indicates that Met-34 and Arg-38 are directly involved in binding to the *lac* operators. S32A-LacR showed a 4-fold reduction of the *in vivo* DNA-binding constant, which might be attributed to the loss of a hydrogen bond with the DNA. D30A-, D33A-, and R37A-LacR showed a 3- to 4-fold increase in DNA binding. A model will be discussed for the binding of residues in the LacR recognition helix to the *lac* operators, based on a combination of the present data and the similarities between the recognition helices of *L.lactis* LacR, *E.coli* DeoR and *E.coli* LacI repressor variant 44.

INTRODUCTION

The enzymes involved in the lactose metabolism of *Lactococcus lactis* are encoded by the *lacABCDFEGX* operon and include the lactose phosphotransferase (LacEF), phospho-ß-galactosidase (LacG), and tagatose-6-phosphate pathway (LacABCD) enzymes (De Vos *et al.* 1990; Van Rooijen *et al.*, 1991). During growth on glucose, expression of the *lac* operon is repressed by the LacR repressor that is encoded by the divergently transcribed *lacR* gene (Van Rooijen and De Vos, 1990; de Vos *et al.*, 1990). Promoter-probe and DNase I footprinting studies have shown that repression is mediated by the interaction between the LacR repressor and the operators *lacO1* and *lacO2* that cover positions -31 to +6 and -313 to -278 relative to the *lac* operon transcription initiation site (Van Rooijen *et al.*, 1992; Van Rooijen and De Vos, 1993). The affinity of LacR for *lacO1 in vitro* is three times higher than for *lacO2* (van Rooijen and de Vos, 1993). The LacR repressor dissociates from both promoters in the presence of tagatose-6-phosphate. No dissociation was observed in the presence of other hexose phosphates, indicating that tagatose-6-phosphate is the inducer of the *lac* operon (Van Rooijen and De Vos, 1993). Amino acid residues of LacR that are involved in the response to the inducer tagatose-6-phosphate were identified previously, by using a site-directed mutagenesis approach (Van Rooijen *et al.*, 1993).

Bacterial regulators can be grouped into at least three families: the LysR family (Henikoff *et al.*, 1988), the GalR-LacI family (Weickert and Adhya, 1992) and the DeoR family (Van Rooijen and De Vos, 1990). The *L. lactis* LacR repressor belongs to the latter group. Following the rules described by Brennan and Matthews (1989b) and Dodd and Egan (1990) for the detection of DNA-binding helix-turn-helix (HTH) motifs, a putative DNA binding domain in LacR was postulated that is located between residues 19 to 41. In DNA binding of proteins containing a HTH motif one helix functions as the stabilization helix whereas the other helix is directly involved in the recognition and contact of the DNA target region (Brennan and Matthews, 1989a, Takeda *et al.*, 1983). DNA binding of the recognition helix is thought to be favoured by electrostatic interactions, whilst specificity is obtained through hydrogen-bonding with the bases of the DNA (Freemont, 1991, Mossing and Record, 1985, Seeman *et al.*, 1976).

To gain experimental evidence for the possibility that the HTH motif of the *L. lactis* LacR repressor is involved in DNA binding, several amino acid residues in the turn and the putative recognition helix were mutated. The effects of these substitutions were studied in the LacR-deficient strain *L. lactis* NZ3015 and wild-type strain MG5267 and showed that Met-34 and Arg-38, and possibly also Ser-32, are involved in DNA binding.

MATERIALS AND METHODS

Bacterial strains, media and plasmids. *Escherichia coli* strain MC1061 (Casabadan *et al.*, 1980) was used as a recipient in cloning procedures. *L. lactis* strains NZ3015 and MG5267 were used as hosts for the mutant *lacR* genes. Strain NZ3015 was constructed previously and is a *lacR*-deletion derivative of MG5267, that contains a single chromosomal copy of the *lac* operon (Van Rooijen *et al.*, 1993). *E. coli* cells were grown in media based on L-broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl). *L. lactis* cells harboring pNZ3019 and derivatives were grown in media based on M17 broth (Difco) containing 0.5% (w/v) glucose or lactose. When appropiate, media contained erythromycin or chloramphenicol to a final concentration of 10 μg/ml. Plasmid pNZ3019 is a derivative of plasmid pNZ3017 and contains a *lacR* gene in which codons 18 to 101 are flanked by unique *Ava*II restriction sites (Van Rooijen *et al.*, 1993). Expression from the *lacR* promoter is constitutive in pNZ3017, possibly due to partial deletion of operator *lacO1* as a result of the cloning procedure (Van Rooijen *et al.*, 1993).

DNA procedures, reagents and enzymes. Plasmid DNA from *E. coli* and *L. lactis* was isolated by an alkaline lysis (Birnboim and Doly, 1979) and a modified alkaline lysis method (De Vos and Gasson, 1989), respectively. All standard recombinant DNA procedures were performed according to Sambrook *et al.* (1989). DNA was transformed into *L. lactis* as described previously (Van Rooijen and De Vos, 1990). Enzymes were purchased from Bethesda Research Laboratories, New England Biolabs, Promega Corporation or Boehringer and used according to instructions of the manufacturer. O-nitrophenyl-ß-D-galactopyranoside-6-phosphate was purchased from Sigma Chemical Company. [α-^{32}P] was supplied by Amersham International plc. Oligonucleotides were synthesized on a Cyclone DNA synthesizer.

Mutagenesis of the *lacR* gene. Mutations in the *lacR* gene were constructed with the polymerase chain reaction (PCR) "megaprimer" method as described (Sarkar and Sommer, 1990; Landt *et al.* 1990) and modified by Kuipers *et al.* (1991). Table 1 lists the universal and mutagenic primers used in the PCR. Plasmid pNZ3019 was used as a template and PCR was performed in 30 cycles (1 min 93 °C, 1.5 min 45 °C, 2.5 min 72 °C) on a Biomed Thermocycler 60. The DNA fragments were purified by agarose gel electrophoresis and recovered using a Mermaid kit (Bio101 Inc.). The product of the second PCR reaction was digested with *Ava*II, heat inactivated and ligated in an *Ava*II-digested pNZ3019 vector. The ligation mix was transformed to *E. coli* strain MC1061 and transformants that were found by Western blotting to produce the full-length LacR were subsequently screened for the presence of the desired mutation in the *lacR* gene by DNA sequence analysis (Sanger, 1977).

Plasmids containing the mutated *lacR* genes were transformed to *L. lactis* strains NZ3015 and MG5267 and expression of the mutated *lacR* genes in strain NZ3015 was checked by Western blotting. In strain MG5267, a discrimination can be made between LacR mutants incapable of binding operator DNA as a result of the inability to form functional dimers, and LacR mutants which are unable to bind operator due to a loss of specific contacts. It is expected that only the latter group of mutant LacR proteins will titrate wild-type LacR by forming non-functional heterodimers resulting in derepressed activities on glucose.

Primer	Description	DNA sequence (5'-->3')
D30A	Asp30 to Ala	GCTTCTAGAGCATCAAGgTCTCTACGGGCTGTCATATCGGAAATAgCTAGTTCATCTATTATTTC
S32A	Ser32 to Ala	GCTTCTAGAGCATCAAGgTCTCTACGGGCTGTCATATCGGcAATATCTAGTTCATCTAT
D33A	Asp33 to Ala	GCTTCTAGAGCATCAAGgTCTCTACGGGCTGTCATAgCGGAAATATCTAGTTC
M34A	Met34 to Ala	GCTTCTAGAGCATCAAGgTCTCTACGGGCTGTCgcATCGGAAATATCTAG
A36I	Ala36 to Ile	AGCTTCTAGAGCATCAAGATCTCTACGGatTGTCATATCGG
R37A	Arg37 to Ala	GCTTCTAGAGCATCAAGgTCTCTAgcGGCTGTCATATCGGAAAT
R38A	Arg38 to Ala	GCTTCTAGAGCATCAAGgTCTgcACGGGCTGTCATATCGG
A	general primer	TTTGAAATTGTTTGTTTTACCTTG
B	general primer	CTCTATATTCACCGCCAAGAAG
C	general primer	GAGCAGGCTTTTTTAGC

Table 1. Primers used in the PCR site-directed mutagenesis of the *L.lactis lacR* gene. Bases in lower case indicate the specific mutation. In some cases a silent mutation (underlined) was introduced to create a *Bgl*II site to facilite the selection of mutants.

Western blot analysis. Equal amounts of cells were incubated for 15 min at 37°C in THMS (30 mM Tris.HCl pH 8.0, 3 mM $MgCl_2$, 25% sucrose) containing 2 mg/ml lysozyme, and boiled for 10 minutes in the presence of an equal volume of 2x SDS/PAGE sample buffer (4% SDS, 12% glycerol, 50 mM Tris.HCl pH 6.8, 2% ß-mercapto-ethanol, 0.1% bromophenolblue). Subsequently, total cellular protein was separated on a 12.5% polyacrylamide-SDS gel and transferred to a nitrocellulose membrane (BA85, Schleicher & Schuell). The membrane was treated with rabbit LacR antibodies and then incubated with peroxidase-labelled goat anti-rabbit antibodies and stained as described by the supplier (Bio-Rad Laboratories).

Phospho-ß-galactosidase assays. Cell-free extracts were isolated after the disruption of exponentially growing cells in a buffer consisting of 50 mM sodium phosphate buffer pH 7.0, 1 mM dithiotreitol, by vigorously shaking in the presence of 0.5 g of zirconium glass beads using a Mini Beadbeater (Biospec Products). The suspension was centrifuged and the supernatant was used in a phospho-ß-galactosidase (LacG) assay at 37 °C with ONPG-P (Sigma) as a chromogenic substrate as described previously (Maeda and Gasson, 1986). Protein concentrations were determined according to Bradford (1976) with bovine serum albumin as a standard.

Determination of relative DNA binding constants.
The relative binding constants were calculated according to the following equation (Betz and Sadler, 1976, Oertel-Bucheit *et al.*, 1992):

$$\frac{K_M}{K_{WT}} = \frac{\theta_M(1-\theta_{WT})}{\theta_{WT}(1-\theta_M)} = \frac{(1-Z_M/Z_D)Z_{WT}/Z_D}{(1-Z_{WT}/Z_D)Z_M/Z_D}$$

where the fractional occupancy of the operator *in vivo* (Θ) is given by ($1-Z/Z_D$), with Z being the phospho-ß-galactosidase activity during growth on glucose in presence of wild-type (Z_{WT}), mutant (Z_M) or no LacR repressor (Z_D).

RESULTS AND DISCUSSION

Experimental design. The *L.lactis* LacR repressor belongs to the *E.coli* DeoR family of repressors. The presence of a N-terminal HTH motif in this family, in which the second helix is highly conserved between its members (Fig. 1), has led to the hypothesis that the N-terminal part of these proteins is involved in DNA binding (Van Rooijen and De Vos, 1990). However, no experimental evidence has been reported that this conserved segment is actually involved in DNA binding in any of the members of the DeoR family. In order to elucidate whether the HTH motif of the *L.lactis* LacR repressor is involved in DNA binding, seven amino acid residues in the turn and part of putative recognition helix were altered using the alanine-scanning procedure (Wells, 1990). This approach is based on the assumption that removal of the side chains of amino acid residues involved in DNA recognition or binding would lead to a modification of operator binding. Since the residue at position 36 is already an alanine, this residue was replaced by isoleucine, which has been found to occur frequently at this position (Brennan and Matthews, 1989b).

The mutant LacR proteins were tested in the *L.lactis lacR*-deletion strain NZ3015 for their ability to repress *lac* operon expression measured as phospho-ß-galactosidase (LacG) activities during growth on glucose. Inducer response of the mutant LacR proteins was tested by growth on a lactose-containing medium. Since no 3D-structure of the *L.lactis* LacR repressor or one of the other members of the DeoR family has yet been determined it is difficult to predict the effect of individual mutations on the overall structure. Therefore, the mutant LacR proteins were tested for *trans*-dominance in the wild-type *L. lactis* strain MG5267 that contains a chromosomal copy of the *lac* operon, including the *lacR* gene. In this strain, a differentiation can be made between mutant LacR proteins that have lost their affinity for the *lac* operators as a result of a disruption of the overall (secundairy or tertiary) structure, and those due to the loss of a specific DNA contact. Since it has been shown that the LacR repressor is able to dimerize *in vitro* (Van Rooijen and De Vos, 1993), it was expected that only the latter mutant proteins will be able to dimerize with the wild-type LacR protein. It was anticipated that these heterodimers will have a decreased affinity for the *lac* operators resulting in phospho-ß-galactosidase activities during growth on glucose that were derepressed relative to those in the wild-type strain MG5267 harboring the control plasmid pNZ3017, which lacks the *lacR* gene. However, the presence of additional copies of the *lacR* gene in strain MG5267 after introduction of pNZ3019 or derivatives leads to an increase in repression level (Table 2). The presence of mutant LacR proteins with a decrease in affinity for the *lac* operator might therefore result in phospho-ß-galactosidase activities that equal those in MG5267 harboring pNZ3017. Therefore, a LacR mutant protein was called *trans*-dominant when in its presence the phospho-ß-galactosidase activity in strain MG5267 during growth on glucose exceeded or equaled those in the presence of plasmid pNZ3017 (i.e. 0.34 μmol.min^{-1}.mg^{-1}, Table 2). When during growth on glucose, a mutation in the *lacR* gene in pNZ3019 resulted in phospho-ß-galactosidase activities between 0.09 and 0.34 μmol.min^{-1}.mg^{-1} in MG5267, and exceeded 1.0 μmol.min^{-1}.mg^{-1} in NZ3015 (Table 2), it was concluded that the overall structure of the mutant protein was significantly disrupted.

Construction of mutated *lacR* genes and their expression in *L.lactis*. One residue in the turn and five residues in the putative recognition helix of the LacR repressor were replaced by an alanine, resulting in *lacR* mutants D30A, S32A, D33A, M34A, R37A, and R38A. The alanine residue at position 36 was changed into isoleucine, and designated A36I. From these residues Asp-30, Asp-33, Met-34, and Ala-36 are not conserved within the DeoR family, whereas the arginine residues 37 and 38 are functionally conserved (Fig. 1). After transformation of the plasmids carrying the mutated *lacR* genes to the LacR-deficient and wild-type *L.lactis* strains NZ3015 and MG5267, respectively, the amount of mutant LacR protein in NZ3015 was estimated

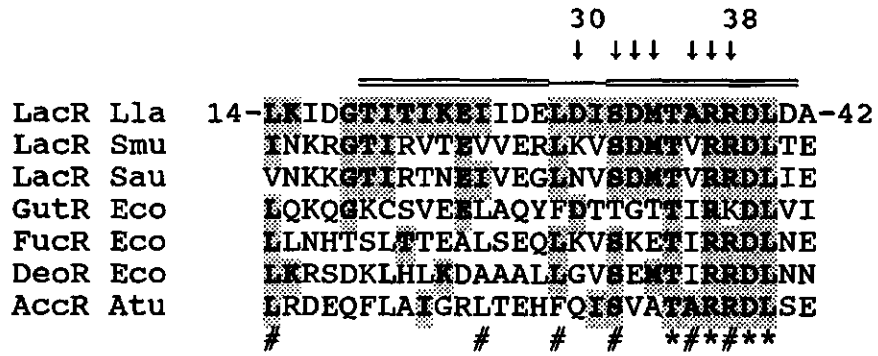

Figure 1. Multiple sequence alignment of the putative DNA-binding domains of the members of the DeoR family of repressors, including the repressors of the lactose operons of *L. lactis* (LacR Lla), *Streptococcus mutans* (LacR Smu) and *Staphylococcus aureus* (LacR Sau), the *E. coli* glucitol, galactitol (GutR Eco), fucose (FucR Eco) and deoxyribonucleoside (DeoR Eco) operons, and the *Agrobacterium tumefaciens acc* and *tra* genes (AccR Atu). Functionally related (#) and identical (*) residues are indicated. Amino acids that are identical to the *L. lactis* LacR repressor are shadowed and shown in boldface. The putative HTH motif and positions of the amino acid residues in the *L. lactis* LacR repressor are indicated. Residues that were subject to site-directed mutagenesis are indicated by arrows.

on Western blots using a polyclonal antibody against purified LacR. The results (Fig. 2) showed that the amounts of LacR repressor produced in all mutants were comparable to that in the wild-type strain grown on glucose (lane 1), indicating that the introduced mutations did not significantly affect *lacR* expression or lead to increased sensitivity to proteolysis of the expressed mutant proteins. Therefore, the phospho-ß-galactosidase activities of NZ3015 cells harboring the mutated *lacR* genes are considered to reflect the effects of the introduced mutations on the LacR function. As a consequence of the presence of the wild-type LacR repressor in MG5267, the expression and stability of the mutant LacR proteins could not be determined in this host. Since NZ3015 is a derivative of strain MG5267 and only differs in the absence of the *lacR* gene, we presumed that the expression and stability of the mutant LacR proteins in MG5267 were comparable to that in NZ3015. However, we cannot exclude possible effects of the mutant LacR proteins on the (auto)regulation of the wild-type *lacR* gene (Van Rooijen and De Vos, 1990). Table 2 shows the phospho-ß-galactosidase levels for the *L. lactis* MG5267 and NZ3015 strains harboring the different mutant *lacR* genes. In addition, the relative *in vivo* LacR-operator binding constants were calculated.

LacR D30A, D33A and R37A have increased affinities for operator DNA. LacR mutants D30A, D33A and R37A have a significantly higher affinity for the operator site than wild-type LacR, as is indicated by the lower phospho-ß-galactosidase levels in strain NZ3015 during growth on glucose. The relative *in vivo* binding constants for D30A-LacR, D33A-LacR, and R37A-LacR increased approximately 4-, 4-, and 3-fold, respectively. The higher DNA binding affinities of D30A-LacR and D33A-LacR, compared to that of wild-type LacR, were confirmed in strain MG5267 since during growth on glucose in the presence of these mutant LacR proteins lower phospho-ß-galactosidase activities were observed. The increased affinities of D30A-LacR and D33A-LacR mutants might be a result of the elimination of the negative charge of the aspartic acid residue and its lack of interference with the negative charge of the phosphate backbone of the DNA. Oerthel-Bucheit *et al.* (1992) have described *E.coli* LexA mutant repressors with enhanced DNA binding affinities in which a negative charge (glutamic acid) was replaced by a positive charge (lysine).

This replacement resulted in the formation of additional salt-bridges with operator DNA. For the lambda repressor it has been reported that operator affinity could be increased by replacing a negative charge for a positive charge in the recognition helix (Benson *et al.*, 1992, Nelson and Sauer, 1985).

During growth on lactose, *L. lactis* NZ3015 cells harboring D30A-LacR and D33A-LacR showed also decreased phospho-ß-galactosidase levels, in comparison with that observed in the presence of wild-type LacR. The decreased phospho-ß-galactosidase activities during growth on lactose in strain MG5267 harboring pNZ3019, that contains the wild-type *lacR* gene, compared to strain NZ3015 harboring pNZ3019 are most probably a consequence of the limited amount of the inducer tagatose-6-phosphate as has been proposed recently (Van Rooijen *et al.*, submitted). Therefore, the low phospho-ß-galactosidase activities of lactose-grown NZ3015 cells containing D30A-LacR and D33A-LacR might be attributed to a shift of the equilibrium between inducer and LacR-operator complex in the direction of the latter. In other words, the negative charges at positions 30 and 33 of the LacR repressor allow a high expression of the *lac* operon during growth on lactose. For rapid growth of *L. lactis* on lactose, full expression of the *lac* operon is required. Therefore, the negative charges found at positions 30 and 33 in the wild-type LacR repressor are, from an evolutionary point-of-view, probably of more benefit than neutral or positively charged residues.

Unexpectedly, the presence of R37A-LacR, which possesses an increased affinity for the operator (K_M/K_{WT} = 2.66; Table 2), in strain MG5267 resulted in a *trans*-dominant phenotype i.e derepressed phospho-ß-galactosidase activities during growth on glucose. Although the wild-type and R37A-LacR proteins each individually are able to give repression of phospho-ß-galactosidase activity in strain NZ3015, their simultaneous presence in strain MG5267, which may result in the formation of heterodimers, does not result in proper repression of the *lac* operon. It remains to be

determined whether the positioning of the different recognition helices of the wild-type and R37A-LacR subunits in the heterodimer with respect to the *lac* operators interferes with the DNA binding or the actual repression mechanism.

Table 2. Phospho-ß-galactosidase activities of cells of *L.lactis* strains NZ3015 (carrying the chromosomal *lacABCDFEGX* but no *lacR* gene) and MG5267 (carrying chromosomal *lacR* and *lacABCDFEGX* genes) harboring the different mutant LacR repressors and relative *in vivo* binding constants (K_M/K_{WT}) of the mutant LacR proteins compared to wild-type LacR. Activities are expressed in μmol.mg^{-1}.min^{-1} and are presented as mean values ± standard deviations as determined in 3 independent experiments.

[a] Control experiment with plasmid pNZ3017 (Van Rooijen *et al.*, 1993) which lacks the *lacR* gene.

[b] Control experiment with plasmid pNZ3019 which contains the wild-type *lacR* gene.

	NZ3015		MG5267		
lacR variant	lactose	glucose	lactose	glucose	K_m/K_{wt}
none[a]	2.00 ± 0.21	1.87 ± 0.11	1.38 ± 0.07	0.34 ± 0.04	-
wildtype[b]	1.61 ± 0.12	0.29 ± 0.05	0.79 ± 0.04	0.09 ± 0.00	1
D30A	0.83 ± 0.00	0.08 ± 0.00	0.88 ± 0.05	0.05 ± 0.00	4.10
S32A	1.48 ± 0.14	0.78 ± 0.04	1.21 ± 0.02	0.35 ± 0.04	0.27
D33A	0.43 ± 0.00	0.08 ± 0.00	0.34 ± 0.04	0.07 ± 0.00	4.10
M34A	1.39 ± 0.21	1.28 ± 0.05	1.21 ± 0.09	0.62 ± 0.04	0.10
A36I	1.96 ± 0.15	2.00 ± 0.10	1.59 ± 0.4	0.64 ± 0.02	0.00
R37A	0.82 ± 0.00	0.12 ± 0.02	1.51 ± 0.08	1.43 ± 0.28	2.66
R38A	1.97 ± 0.05	1.62 ± 0.03	1.53 ± 0.11	1.59 ± 0.25	0.04

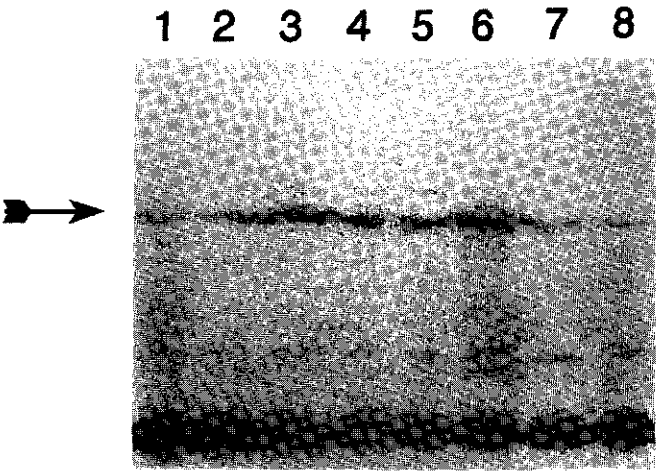

Figure 2. Western blot analysis of mutant LacR proteins. Equal amounts of *L.lactis* NZ3015 cells harboring the different mutant *lacR* genes were lysed and total cellular protein was separated on a 12.5% SDS-PAGE gel, transferred to nitrocellulose and probed with polyclonal antibodies specific for LacR. The position of LacR is indicated by an arrow. Lane 1, pNZ3019 (wild-type *lacR* gene), after growth on glucose. Lanes 2-8: LacR mutants D30A, S32A, D33A, M34A, A36I, R37A, and R38A, respectively.

M34A and R38A are 'loss-of-contact' mutants. The presence of LacR mutants M34A and R38A in strain NZ3015 resulted in derepressed phospho-ß-galactosidase activities comprising a 10- and 25-fold decrease *in vivo* binding constant, respectively (Table 2). The *trans*-dominant phenotype of these mutants in strain MG5267 indicates that their overall structure was not significantly affected. Therefore, we conclude that Met-34 and Arg-38 are involved in DNA binding. In addition, the *trans*-dominancy of these loss-of contact mutants in MG5267 confirms *in vivo* that LacR is probably active as a dimer or mutimer, as recently has been shown *in vitro* with cross-linking studies (Van Rooijen and De Vos, 1993).

Mutation A36I probably disrupts the recognition helix. Substituting the alanine residue at position 36 for isoleucine results in a complete loss-of-contact to operator DNA as is indicated by the inability of A36I-LacR to repress phospho-ß-galactosidase activities in strain NZ3015 during growth on glucose. A36I-LacR shows a weak *trans*-dominant phenotype in strain MG5267, compared to that of the loss-of-contact mutant R38A-LacR (see above), indicating that the overall structure of the A36I-LacR

protein might be slightly affected resulting in a decreased dimerization with the wild-type LacR. Computer-assisted predictions of secondary structures with the PC/GENE software package (Release 6.0; IntelliGenetics Inc.) following the method of Garnier *et al.* (1978) revealed a disruption of predicted α-helix when isoleucine occupies position 36. For the other mutations shown in Table 1 the secondary structure was predicted to be maintained. Combination of the computer predictions with the experimental results strongly suggests that the loss of affinity of A36I-LacR to operator DNA is a result of a disruption of the putative recognition helix.

The decreased affinity of S32A-LacR for operator DNA might be attributed to the loss of a hydrogen bond. Substitution of the serine residue at position 32 by an alanine leads to a 4-fold decrease of the *in vivo* DNA binding constant but still results in repressed phospho-ß-galactosidase activities in strain NZ3015 during growth on glucose (Table 2). Phospho-ß-galactosidase activities in the presence of S32A-LacR are fully inducible, indicating that this mutant LacR correctly responds to inducer. In addition, S32A-LacR shows *trans*-dominance in MG5267. Therefore, we conclude that the overall structure is not significantly affected. Since the only difference between a serine and an alanine is the presence of a hydroxyl group in the serine residue, it is tempting to speculate that the decreased affinity of the S32A mutant for the operator is a consequence of the loss of a hydrogen bond. Support for this assumption may be derived from calculating the difference in free energy between the S32A-LacR/operator and wild-type LacR/operator complexes since this should equal the energy required for a hydrogen bond between a protein and DNA. When the formation of an operator-LacR complex is supposed to be a simple first order process, the Gibbs free energy resulting from installing the equilibrium is given by:

$$\Delta G_0 = -RTlnK$$

From this equation, it can be deduced that for two reactions with different K values, the differential free energy is given by:

$$RTln\frac{K_1}{K_2} = \Delta G_2^O - \Delta G_1^O$$

As the ratio between K_1 and K_2 is known (Table 2), the difference in free energy between the S32A-operator complex and the wild-type operator complex can be calculated and amounts 3.4 kJ/mol (with R=8.31 J/mol.K, equilibrium at 308 K).

Calculating the energy required for a hydrogen bond between a protein and the DNA in an aqueous solutions is rather complex and little is known about the energies involved (Cantor and Schimmel, 1980). It has been shown that such binding energies are not additive (Lehming *et al.*, 1987, Mossing and Record, 1985). Furthermore, both the hydrogen donor and the acceptor may be hydrated in the free state, which may result in a small differential free energy (Freemont *et al.*, 1991). However, Fersht and coworkers have introduced an elegant approach to calculate hydrogen bonding energies. By specific deletion of side chains in the tyrosyl-tRNA synthetase and subsequent analysis of its kinetic properties, they were able to calculate the differential free energies. Their results showed that deletion of a side chain removing a hydrogen bond acceptor or donor weakens binding energy by 2.1 - 6.3 $kJ.mol^{-1}$ (Fersht *et al.*, 1985). The calculated differential energy between S32A and wild-type LacR is in good agreement with their calculations and support the possibility that deletion of the hydroxylgroup of Ser-32 results in the elimination of a hydrogen bond.

Concluding remarks. This paper presents evidence that amino acid residues in the N-terminal domain of the *L.lactis* LacR repressor are involved in binding to the *lac* operators. This domain includes a HTH motif that is often observed in bacterial regulators. To the best of our knowledge this is the first report of a member of the *E.coli* DeoR family in which experimental evidence has been provided for the involvement in DNA-binding of the highly conserved N-terminal HTH motif (Fig. 1). In the master set of DNA-binding HTH proteins the glycine residue located in the centre of the turn is highly conserved (Brennan and Matthews, 1989; Dodd and Egan, 1990). The apparent evolutionary preference for a glycine at this position is remarkable since for the *E.coli lac* and *tet* repressor it has been shown that this residue can be replaced by almost any amino acid residue except for proline (Baumeister *et al.*, 1992; Kleina and Miller, 1990). Also in the putative turn at position 30 of the *L.lactis* LacR repressor, replacement was allowed of the aspartic acid residue by an alanine. In the putative turns of other members of the *E.coli* DeoR family, a glycine residue is only observed in the DeoR repressor (Fig. 1).

Based on similarity with the binding of the LacI repressor variant 44 to its corresponding operator, a model has been proposed for the binding of the DeoR repressor to the *deoO1* operator (Lehming *et al.*, 1988). Using this model and the experimental data described in this paper, we propose a model for binding of the *L.lactis* LacR repressor to the *lacO1* (Fig. 3). The role of the aspartate residue at position 33, that is not conserved in the DeoR repressor and LacI repressor variant 44, in DNA binding is unclear. The model is supported by the following: (i) the experimental data clearly show the involvement of Met-34 and Arg-38 in DNA binding, (ii) these residues are conserved in the DeoR repressor and the LacI repressor variant 44, and in addition, (iii) the nucleotides

of the *E.coli lac* operator variant, that have been shown to be contacted by the LacI repressor variant 44 (Lehming *et al.*, 1988), are conserved in the *L.lactis lac* operators *lacO1* and *lacO2*.

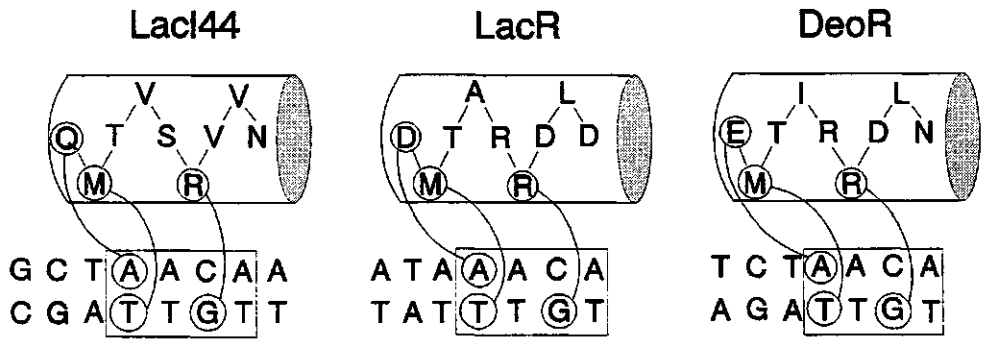

Fig. 3. Model for the binding of the *L.lactis* LacR recognition helix to the *lacO1* operator. The model is based on a combination between the experimental data presented in this paper and the proposed model for the *E.coli* DeoR repressor, that was based on similarity with that experimentally determined for the *E.coli* LacI repressor variant 44 (Lehming *et al.*, 1988). Amino acid residues are given in the one-letter code. Circles are drawn around amino acid residues and bases that contact each other. Bases that are conserved in the operators half-sites are boxed.

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CHAPTER 11

SUMMARY
AND
CONCLUDING REMARKS

An important trait of the lactic acid bacterium *Lactococcus lactis*, that is used in industrial dairy fermentations, is the conversion of lactose into lactic acid. The enzymatic steps involved in the breakdown of lactose, that is transported into the cell via a phospho*enol*pyruvate-dependent lactose phosphotransferase system (PEP-PTS^{lac}), have been well established (Fig. 1). However, except for the molecular cloning and characterization of the plasmid-located phospho-ß-galactosidase gene (Boizet *et al.*, 1988; De Vos and Gasson, 1989), relatively little data have emerged concerning the genetic information for the lactose catabolic enzymes. A solid genetic basis of this key metabolic route is essential for the development of food-grade selection markers and pathway engineering strategies for *L.lactis*. In addition, since high lactose-specific enzyme activities are observed during growth on lactose, which are repressed during growth on glucose, expression of the *lac* genes is probably under control of a strong and inducible promoter. Such a promoter would be applicable as a 'genetic switch' in the controlled overexpression of homologous and heterologous genes in *Lactococci*. Isolation and elucidation of the mechanism of control of the *lac* promoter would be beneficial for the development of such strains. This thesis describes the characterization and organization of the genes involved in the lactose metabolism of *L.lactis* subsp. *lactis*. In addition, several *cis*- and *trans*-acting factors that are involved in the regulation of their expression were identified.

In **Chapter 1** some background information is given about the enzymology and genetics of lactose metabolism in lactic acid bacteria. In addition, this Chapter provides a brief overview of the various mechanisms that may be involved in the regulation of gene expression in bacteria, and presents the state-of-the-art concerning gene regulation in lactic acid bacteria.

The characterization of the genetic determinants for lactose metabolism, including the PEP-PTS^{lac} (LacEF), phospho-ß-galactosidase (LacG) and tagatose-6-phosphate pathway enzymes (LacABCD), is presented in **Chapters 2 and 3**. The *lac* genes of the *L.lactis* subsp. *lactis* strain MG1820, that are located on the 23.7-kb plasmid pMG820, appeared to be organized in a 7.8-kb operon-structure with the gene order *lacABCDFEGX* (Fig. 1). The *lacE* and *lacF* genes encode the PEP-PTS^{lac} proteins Enzyme II^{lac} (62 kDa) and Enzyme III^{lac} (11 kDa), that are involved in the translocation across the cell membrane and subsequent phosphorylation of lactose (Chapter 2). Cross-linking studies with purified enzyme showed that Enzyme III^{lac} is active as a trimer. The identity of the *lacF* gene was confirmed by complementation of *lacF* deficiency in *L.lactis* strain YP2-5, that appeared to contain a G18E mutation in the deduced LacF protein. Homology was observed between the deduced amino acid sequences of the *L.lactis lacE* and *lacF* genes and those of *Lactobacillus casei* and *Staphylococcus aureus*. In addition, the deduced *L.lactis* LacE and LacF amino acid sequences were homologous to those of CelA, CelB and CelC that are involved in the cellobiose PTS of *Escherichia*

coli (Reizer *et al.*, 1990). The *lacG* gene codes for the phospho-ß-galactosidase enzyme (54 kDa) that catalyzes the hydrolysis of lactose-6-phosphate into galactose-6-phosphate and glucose (De Vos and Gasson, 1989). The *L.lactis* phospho-ß-galactosidase has been purified from an overexpressing *E.coli* strain (De Vos and Simons, 1988) and belongs to the superfamily of ß-glycohydrolases (Hassouni *et al.* 1992). The tagatose-6-phosphate pathway enzymes were shown to be encoded by the *lacABCD* genes (Chapter 3). The first enzyme of the tagatose-6-phosphate pathway, the galactose-6-phosphate isomerase (LacAB), is encoded by the first two genes of the *lac* operon, the *lacAB* genes. Galactose-6-phosphate activities were only observed in *E.coli* cells overexpressing both the *lacA* and *lacB* genes, whereas no activity was found in cells expressing solely LacA (15 kDa) or LacB (19 kDa). The *lacC* and *lacD* genes encode the tagatose-6-phosphate kinase (33 kDa) and tagatose-1,6-diphosphate aldolase (36 kDa), respectively, as was evident form their enzyme activities in overexpressing *E.coli* cells. The deduced amino acid sequences of the *lacABCD* genes appeared to be strongly homologous to those of *S.aureus* and *S.mutans* (Jagusztyn-Krynicka *et al.* 1992). In addition, the *L.lactis* LacC sequence is homologous to the *E.coli* enzyme phosphofructosekinase B, that catalyzes the phosphorylation of tagatose-6-phosphate in the galactitol catabolic pathway. The function of the distal *lacX* gene, encoding a 34-kDa protein, is still unclear. No significant homology was found with other sequences in DNA or protein databases. However, the *lacX* gene seems not to be essential for lactose catabolism, since *L.lactis* strains in which transcription of *lacX* was prevented did not show significantly altered growth characteristics or phospho-ß-galactosidase activities during growth on lactose (Simons *et al.*, 1993). Northern-blot analysis showed that the *lac* genes are transcribed as two 6.0- and 8.0-kb polycistronic transcripts, of the *lacABCDFE* and *lacABCDFEGX* genes, respectively. An inverted repeat which is located between the *lacE* and *lacG* genes could function as the transcription termination site for the 6.0-kb transcript. In cells shifted from glucose to lactose, *lac* operon transcription was induced similarly as lactose enzyme activities (approximately 5-10 fold), indicating that the expression of the *lac* operon is regulated at the transcriptional level. The 3' end of the *lacABCDFEGX* operon appeared to be followed by an *iso*-IS*S1* element (**Chapter 4**). This element is flanked by 16-bp inverted repeats and contains a divergently transcribed gene (*orf1*) encoding a putative transposase that is highly homologous to that of other *iso*-IS*S1* elements. It remains to be determined whether this IS-element, or one of the other IS-elements that have been located on pMG820 (Fig. 1; Van Rooijen, unpublished results), are involved in the conjugal transfer of this or related lactose plasmids.

Transcription of the *lacABCDFEGX* operon was found to be regulated by the product of the divergently transcribed 0.8-kb *lacR* gene (**Chapter 5**). The *lacR* gene was characterized by overexpression in *E.coli* and DNA sequencing and found to encode a 28-kDa protein. Northern-blot analysis showed that, in contrast to the *lacABCDFEGX* genes, the *lacR* gene is induced during growth on glucose. The deduced amino acid

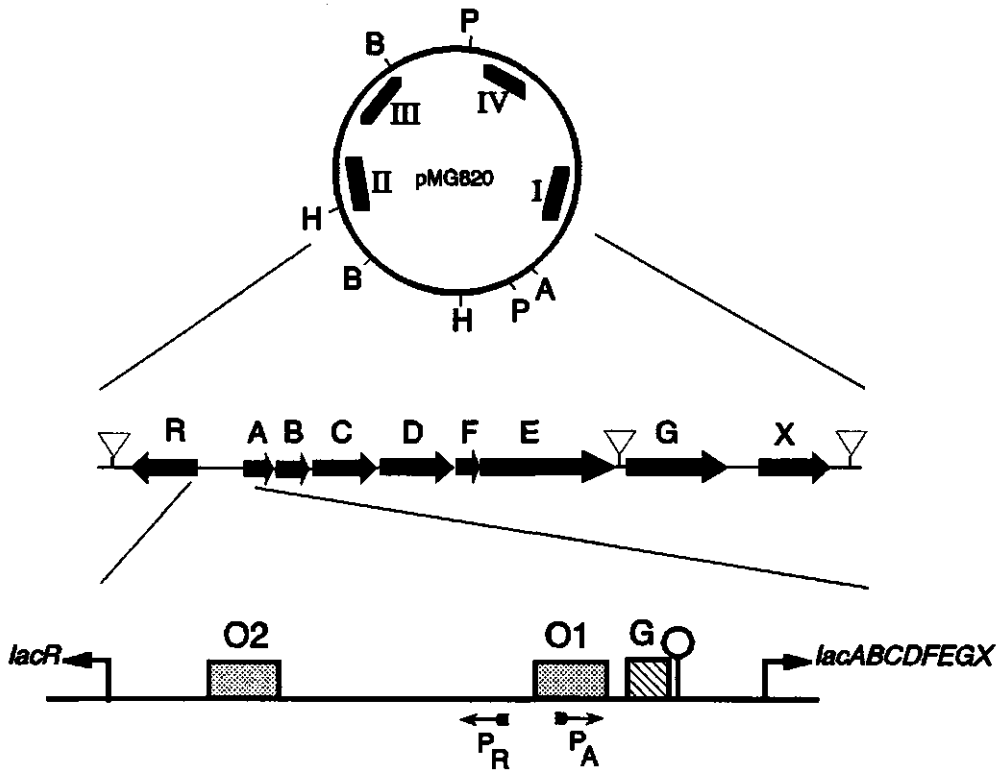

Fig. 1. Organization of the *L.lactis lac* regulon and positions of regulatory sites. The *lac* regulon is located on the 23.7-kb plasmid pMG820. Positions of restriction cleavage sites are indicated: A, *Apa*I; B, *Bgl*II; H, *Hpa*I; P, *Pst*I. Black bars indicate the positions of open reading frames that could encode proteins with homology to transposases of IS-elements (Van Rooijen, unpublished results): I, *iso*-IS*S1* homologue (Chapter 4); II, IS9 (TRA$MYCTU, EMBL entry); III, *S.aureus* Tn552 (SATN552, GenBank entry); IV, IS600 (YIS2&SHISO, EMBL entry). The sizes, location, and orientation of the *lacR* and *lacABCDFEGX* genes are indicated by black arrows. Positions of the putative transcription terminators are indicated by open triangles. The *lacR* (P_R) and *lacABCDFEGX* (P_A) promoters (arrows), operators *lacO1* (O1) and *lacO2* (O2), putative glucose-responsive element (G, hatched square), and stem-loop structure (open circle) in the *lac* regulatory region are indicated.

sequence of LacR appeared to be homologous to those of the *E.coli* DeoR, GutR, and FucR, *S.aureus* and *S.mutans* LacR, and *Agrobacterium tumefaciens* AccR repressors. None of these repressors belongs to one of the known LacI/GalR or LysR repressor

families. Since the DeoR repressor was the first repressor to be identified, this group of repressors was designated the *E.coli* DeoR family of repressors. Common characteristics of the members of the DeoR family are the presence of a helix-turn-helix motif near their N-termini and a conserved region near their C-termini, that for the *L.lactis* LacR repressor appeared to be involved in DNA and inducer binding, respectively (see below). In addition, all members have in common that expression of the catabolic operon they control is induced by a phosphorylated sugar, or a derivative thereof. The functionality of the *lacR* gene product as a repressor was demonstrated after introduction of multiple copies of the *lacR* gene in *L.lactis* strain MG5267, that contains a single chromosomal copy of the pMG820 *lac* operon. Whereas no effects were observed during growth on glucose, significant decreased growth rates and *lac* operon activities were observed during growth on lactose, indicating that *lacR* specifically represses expression of the *lac* operon.

Characterization of the *lac* promoter and modulation of promoter activity by the *lacR* gene product is presented in **Chapter 6.** The transcription initiation site of the *lac* promoter was determined by primer extension mapping. The *lac* promoter canonical -35 and -10 sequences correspond closely to those described for gram-positive bacteria and are located in a back-to-back configuration with those of the divergently orientated *lacR* promoter (Fig. 1). The effects on *lac* promoter activity of flanking sequences and the *lacR* gene were studied in *L.lactis* and *E.coli* by using transcriptional fusions with a promoterless chloramphenicol acetyltransferase (*cat-86*) gene. In the presence of the *lacR* gene both in *L.lactis* and *E.coli*, significantly decreased CAT activities were observed, indicating that the *lacR* gene product represses *lac* promoter activity. In addition, to obtain inducible CAT-activities a *lac* promoter fragment of at least 0.5 kb was required, suggesting that regions flanking the promoter are involved in regulation. These studies also showed that sequences flanking the *lac* promoter significantly contribute to the promoter efficiency in *L.lactis*. Enhancement of promoter activity in *L.lactis* of up to 38-fold was observed.

The interaction between the LacR repressor and the *lac* promoter region is described in **Chapter 7.** For this purpose, LacR was overexpressed in *E.coli* and purified in a three-step procedure. Cross-linking studies with glutaraldehyde showed the ability of LacR to generate dimers. Gel-mobility shift assays and DNase I footprinting studies demonstrated the presence of two LacR-binding sites, *lacO1* and *lacO2*, in the intercistronic region between the *lacA* and *lacR* genes (Fig. 1). The *lacO1* operator is located at positions -31 to +6 and -96 to -59 relative to the transcription initiation sites of the *lac* operon and *lacR* gene, respectively. The distances between *lacO1* and transcription initiation sites of the *lac* operon and *lacR* gene are comparable to those often observed for repressor and activator binding sites, respectively, as is illustrated in Fig. 2. The *lacO2* operator is located at positions -313 to -278 and +188 to +223 relative to the transcription initiation sites of the *lac* operon and the *lacR* gene,

respectively. Since a TGTTT sequence is present as an inverted repeat in *lacO1* and as a direct repeat in *lacO2*, we proposed that the TGTTT box comprises the LacR recognition sequence. Titration experiments with purified LacR and DNA-fragments containing *lacO1*, *lacO2*, or both (*lacO1O2*) showed that *lacO1* and *lacO1O2* have a three-fold higher affinity than *lacO2* for LacR binding. This indicated that the presence of *lacO2 in cis* does not significantly enhance binding of LacR to *lacO1*. To identify the metabolite that induces *lac* operon expression during growth on lactose, gel mobility shift assays were carried out with the LacR repressor and *lacO1O2* in the presence of various phosphorylated monosaccharide intermediates from the tagatose-6-phosphate and glycolytic pathways. Dissociation of the LacR-*lacO1O2* complex was observed only in the presence of tagatose-6-phosphate, which is an intermediate of the tagatose-6-phosphate pathway. No dissociation was observed with galactose-6-phosphate, tagatose-1,6-diphosphate, glucose-6-phosphate, fructose-6-phosphate and fructose-1,6-diphosphate. Therefore, it was concluded that tagatose-6-phosphate is the physiological inducer of *lac* operon expression. This is supported by the observation that *lac* operon expression is also induced during growth on galactose, that is transported via a galactose-PTS and is metabolized through the tagatose-6-phosphate pathway.

In order to study whether the LacR repressor is the only determinant in the control of *lac* operon expression and to develop an expression system in *L.lactis* that allowed screening of mutated *lacR* genes, the chromosomally located *lacR* gene of strain MG5267 was deleted by replacement recombination (**Chapter 8**). The resulting strain was designated *L.lactis* NZ3015. As expected, determination of phospho-ß-galactosidase (LacG) and lactose phosphotransferase (LacEF) activities, and *lac* mRNA levels of lactose- and glucose-grown NZ3015 cells showed that expression of the *lac* operon was significantly derepressed in the glucose-grown cells. However, approximately one fifth of the wild-type regulation level remained, as was demonstrated by the 1.6-fold (average) higher *lac* operon activities during growth on lactose than on glucose. This indicates that an additional control circuit is involved in the regulation of the *lac* operon. Since the RNA-studies demonstrated that this regulatory circuit mediates *lac* operon expression at the transcriptional level, we searched for DNA sequences in the *lac* promoter region that were homologous to a putative glucose-responsive-element (GRE) from *Bacillus*. Five basepairs downstream of the *lacO1* operator a sequence was detected that showed strong homology to the *Bacillus* GRE sequence. The *L.lactis* GRE sequence was also found in the promoter region of the *S.aureus lac* operon, that is strongly homologous to that of *L.lactis*.

The last two **Chapters 9** and **10** present the identification of amino acids in the *L.lactis* LacR repressor that are involved in the inducer response and binding to DNA, respectively. This was realized by studying the effects on the regulation of *lac* operon expression in the LacR-deficient strain NZ3015 and wild-type strain MG5267, after introduction of mutated *lacR* genes. Since LacR belongs to the *E.coli* DeoR family of

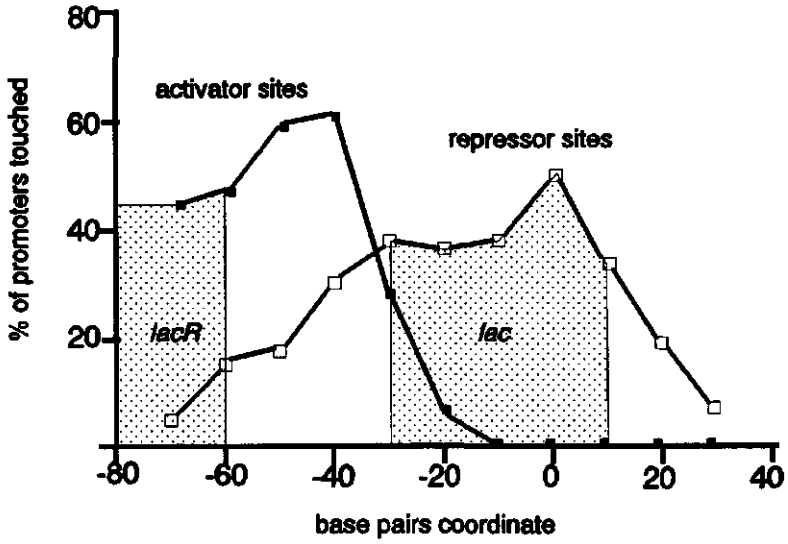

Fig. 2. Plot demonstrating the fraction of promoters with regulatory sites that touch the indicated promoter positions. The binding sites of 28 *E.coli* regulatory proteins relative to the transcription initiation sites are included. Filled and open squares represent activators and repressors, respectively. Thus, for example, 60% of activatible promoters have sites overlapping -40, and 49% of repressible promoters have operators overlapping the transcription initiation site +1 (data taken from Collado-Vides *et al.*, 1991). The grey boxes indicate the position of the *L.lactis lacO1* operator with respect to the transcription initiation sites of the *lac* operon (*lac*) and the *lacR* gene (*lacR*).

repressors, in which all members have in common that their inducer is a phosphorylated sugar, it was anticipated that within this family there will be conserved amino acid residues that are involved the response to the inducer tagatose-6-phosphate. Various amino acid residues in LacR that are conserved in other DeoR family members and located outside the DNA-binding motif, were replaced by alanine or arginine. Cells of strain NZ3015 containing K72A-, K80A-, D210A-, or K213A-LacR, were unable to derepress phospho-ß-galactosidase activities during growth on lactose. These low phospho-ß-galactosidase activities resulted in significantly decreased growth rates on lactose, and strongly suggested that these LacR mutant proteins had lost their ability to respond to inducer. This hypothesis was verified by carrying out gel mobility shift assays with *lacO1O2* operator and purified K72A-, K80A-, D210A-, and K213A-LacR proteins in the presence or absence of the inducer tagatose-6-phosphate. None of the complexes between the *lacO1O2* and the mutant proteins was affected by tagatose-6-phosphate,

whereas the complex between *lacO1O2* and wild-type LacR dissociated in the presence of tagatose-6-phosphate. From these experiments it was concluded that Lys-72, Lys-80, Asp-210, and Lys-213 are involved in the inducer response of the LacR repressor. It is not yet clear whether these residues are involved in the actual binding of tagatose-6-phosphate or, upon binding, the allosteric transition of LacR into a molecule with a decreased affinity for *lacO1O2*. In addition, these results confirm that *in vivo* tagatose-6-phosphate is the inducer of the *L.lactis lac* operon.

To identify the residues in LacR involved in DNA-binding, amino acid residues in the putative N-terminal DNA-binding domain, that contains a helix-turn-helix motif, were replaced by alanine. The LacR mutants M34A and R38A showed a 10- and 25-fold decrease of the *in vivo* DNA-binding constant, indicating that Met-34 and Arg-38 are involved in DNA-binding. Two LacR mutants, D30A and D33A, were constructed with a 4-fold increased DNA-binding constant, indicating that it is possible to improve the relatively weak binding of LacR to its operator. Based on the similarities between the LacR repressor and the *lacO1* operator and the *E.coli* LacI repressor variant 44 and its corresponding operator, a model for the binding of LacR to the *lacO1* operator was presented.

Based on the studies presented in this thesis a model for the action of the LacR repressor in the regulation of the *L.lactis lac* operon is proposed. Below, three stages of the model will be discussed.

1. Binding of LacR repressor to operator *lacO1* during growth on glucose results in autoactivation of *lacR* gene expression. The induction of *lacR* on glucose and the high affinity of the LacR repressor for *lacO1* are evident from the Northern-studies (Chapter 5) and gel mobility shift titration experiments (Chapter 7), respectively. The distance between location of *lacO1* and the *lacR* transcription initiation site coincides with the distance that is commonly observed for an activator (Fig. 2). The involvement of *lacO1* in the regulation of *lacR* is supported by the observation that partial deletion of *lacO1* resulted in the loss of *lacR* regulation (Chapter 9, Fig. 3). However, no experimental data have been generated to establish the direct involvement of LacR in activating expression of its own gene. Since the transcriptional fusion studies (Chapter 6) showed that *lacO1* alone is incapable of regulating CAT-expression, we presume that no repression of *lac* operon expression occurs at this stage.

2. Binding of LacR repressor to *lacO2* at increasing LacR concentrations during growth on glucose results in repression of *lacR* gene and *lac* operon expression. Since it has been shown that *lacO2* has a lower affinity for LacR than *lacO1* (Chapter 7), *lacO2* will only be bound at increasing LacR concentrations. The CAT-reporter studies showed that both *lacO1* and *lacO2* are required for repression of CAT-activity during growth on glucose (Chapter 6). Therefore, repression of transcription initiation of *lac* operon occurs when LacR is bound to both *lacO1* and *lacO2*. The exact repression mechanism has not been elucidated, but might include the formation of a

DNA loop between *lacO1* and *lacO2*, as has been described for other regulatory systems (Matthews, 1992). The postulated repression of *lacR* expression upon binding of LacR to *lacO2* would prevent the cell from overproduction of LacR due to continuous activation by *lacO1*, and results in a certain steady state concentration of LacR. However, no experiments have been carried out to establish the role of *lacO2* in the putative autoregulation of *lacR*.

3. Binding of LacR repressor to tagatose-6-phosphate during growth on lactose results in dissociation of the LacR-operator complex concomitant with the induction of *lac* operon expression. From the gel mobility shift studies in Chapter 7 it is evident that the LacR-*lacO1O2* complex dissociates in the presence of tagatose-6-phosphate, that is an intermediate of the tagatose-6-phosphate pathway. In addition, LacR mutants were constructed, the presence of which in *L.lactis* resulted in an inability to induce *lac* operon activity on lactose, that had lost their sensitivity to tagatose-6-phosphate. Therefore, the complex between LacR and tagatose-6-phosphate that is formed during growth on lactose does not bind to the *lac* operators, resulting in the restoration of transcription initiation from the *lac* promoter. As a result of the absence of LacR bound to *lacO1*, the *lacR* gene is probably no longer (auto)activated resulting in a decreased level of *lacR* expression. The presence of multiple copies of constitutively expressed *lacR* results in an additional repression of *lac* promoter activity during growth on both glucose and lactose (Chapters 5 and 6), suggesting that due to the overproduction of LacR relatively more *lacO2* is bound by LacR. Due to the limited amount of inducer (Chapter 8), it would under these conditions then be theoretically possible that, in contrast to the situation in wild-type cells, *lacR* expression is induced during growth on lactose. This might be a consequence of the dissociation of only the *lacO2*-LacR complex in these cells during growth on lactose. In contrast, in wild-type cells, where the LacR concentration is lower, LacR dissociates from both operators during growth on lactose.

The studies described in this thesis have provided more insight in the genetic basis and regulation of lactose catabolism in *L.lactis*. Parts of this knowledge have already been used for the development of a food-grade selection system for *L.lactis* based on the *lacF* gene (De Vos, 1988). In addition, the *lac* promoter has already been successfully used for the expression of mutated *nisZ* genes in *L.lactis* (Kuipers *et al.*, 1992). Since *L.lactis* preferentially metabolizes glucose it should be possible, by the starting the fermentation with a certain amount of glucose, to overexpress genes of interest under control of the *lac* promoter at a defined stage in a dairy fermentation.

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CHAPTER 12

SAMENVATTING

WAAR GAAT DIT PROEFSCHRIFT OVER?

Waar gaat dit proefschrift over?

Deze samenvatting geeft een vereenvoudigd overzicht van de inhoud van dit proefschrift. Voor een wetenschappelijke en gedetailleerde samenvatting wordt verwezen naar de Engelstalige samenvatting in hoofdstuk 11.

Melkzuurbacteriën

Bij de bereiding van zuivelprodukten als yoghurt, karnemelk en kaas worden melkzuurbacteriën bij de start van het bereidingsproces (de fermentatie) aan de melk toegevoegd. De combinatie van de gebruikte melkzuurbacteriën, de zogenaamde "starterculture", bepaalt de uiteindelijke smaak, textuur en aroma van het eindprodukt. De vorming van smaak en aroma wordt verzorgd door het vermogen van sommige bacteriën in de starterculture bepaalde typen melkeiwitten, de caseïnes, af te breken. De brokstukken van deze melkeiwitten, peptiden en aminozuren, geven een bepaalde smaak of zijn de voorlopers hiervan. Een andere belangrijke eigenschap van melkzuurbacteriën, hun naam geeft dit al aan, is de vorming van melkzuur uit melksuiker (lactose). De vorming van melkzuur geeft een daling van de zuurtegraad (pH), wat de groei van eventueel aanwezige ongewenste bacteriën (bederf) remt. De vorming van melkzuur tijdens een zuivelproces geeft dus een 'natuurlijke' conservering van het eindprodukt. Het volgende gedeelte behandelt de vraag hoe de bacteriecel lactose omzet in melkzuur.

De vorming van melkzuur uit lactose (melksuiker)

Tijdens het groeien heeft een melkzuurbacterie energie nodig voor de aanmaak van verschillende celmaterialen. Daartoe bezit de cel een systeem om stoffen uit zijn omgeving te gebruiken voor het maken van energie. Een belangrijke energiebron zijn suikers, zoals bv. druivesuiker (glucose), rietsuiker (sucrose) en melksuiker (lactose). De verschillende stappen in de afbraak van lactose (de metabole route) door de melkzuurbacterie *Lactococcus lactis* staan weergegeven in Fig. 1. Deze verschillende stappen vinden niet spontaan plaats maar worden vergemakkelijkt (gekatalyseerd) door bepaalde eiwitten (enzymen). Op specifieke punten in deze afbraakroute worden energierijke verbindingen (ATP) gemaakt. In hoofdstuk 1 worden de achtergronden van het lactose metabolisme besproken.

De genetische informatie voor de lactose afbraak

Enzymen betrokken bij de afbraak van lactose kunnen ook in andere organismen aanwezig zijn. Echter, de volgorde van de bouwstenen (aminozuren) waaruit zij zijn opgebouwd, waarvan er 20 in de natuur aanwezig zijn, is meestal uniek voor elke

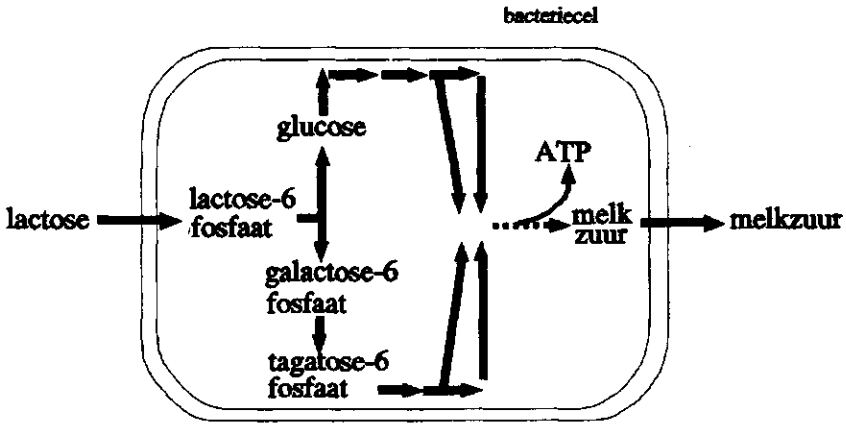

Fig. 1. Afbraakroute van lactose in *Lactococcus lactis*. De bacteriecel en de verschillende afbraakstappen staan schematisch weergegeven. Lactose wordt opgenomen en binnen in de cel omgezet tot melkzuur. Tijdens deze omzetting worden energie-rijke verbindingen (ATP) gegenereerd. Melkzuur gaat vervolgens weer naar buiten en zorgt voor verzuring van de omgeving.

bacteriesoort. Deze verschillen vinden hun oorsprong in verschillen in het genetisch materiaal van bacteriën. De blauwdruk voor de produktie en aminozuurvolgorde van eiwitten ligt opgesloten in het DNA, de drager van de erfelijke informatie. DNA (desoxy*ribo*nucleïnezuur) is opgebouwd uit vier verschillende bouwstenen, de nucleotiden A, G, C en T, en de volgorde van deze bouwstenen bepaalt tevens de volgorde van de aminozuren in een eiwit. Het stukje DNA dat "codeert" voor een eiwit noemen we een gen. Het DNA van de meeste bacteriën is opgebouwd uit zo'n 1-2 miljoen van deze bouwstenen. De lengte van een gen kan varieren van ongeveer 90 tot 12000 nucleotiden, coderend voor eiwitten van, respectievelijk, 30 tot 4000 aminozuren. De omzetting van een gen naar de aminozuurvolgorde van een eiwit verloopt via een genetisch tussenprodukt, het boodschapper ribonucleïnezuur (mRNA). Dit mRNA is een exacte copie van het gen dat moet worden vertaald. Vervolgens wordt het mRNA gebruikt als matrijs voor de koppeling van aminozuren in een volgorde zoals vastgelegd in het DNA. De vertaling van een gen (DNA) in een eiwit kan als volgt worden samengevat (Fig. 2):

Fig. 2. Het centrale dogma van de moleculaire biologie. De vertaling van een gen in een eiwit (enzym). Soms kan RNA ook worden omgezet in DNA (gestippelde pijl).

In hoofdstukken 2 en 3 van dit proefschrift staan de isolatie, nucleotide volgorde en karakterisatie van de genen beschreven die coderen voor de enzymen betrokken bij de afbraak van lactose in *Lactococcus lactis*. Het bleek dat deze genen, *lacABCDFEGX*, bijeen liggen in een zgn. operonstructuur. Dat wil zeggen dat bij de vertaling van deze genen in eiwitten slechts één mRNA molekuul wordt gemaakt. Verder is er een genetisch element gevonden, een *iso*-IS*S1* element (hoofstuk 4), dat mogelijk betrokken is bij de verplaatsing van de lactose genen naar melkzuurbacteriën die geen lactose als energiebron kunnen gebruiken (Fig. 3).

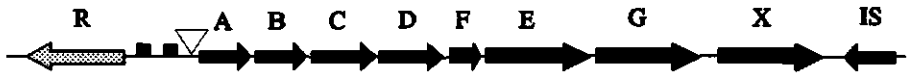

Fig. 3. De genetische informatie voor de lactose afbraak in *Lactococcus lactis*. De *lacABCDFEGX* genen die coderen voor de enzymen betrokken bij de afbraak van lactose en het *iso*-IS*S1* element (IS) worden voorgesteld door middel van zwarte pijlen. Onderdelen van de genetische schakelaar worden als volgt weergegeven: *lacR* gen, gestippelde pijl; *lac* promoter, open driehoek; LacR-bindingsplaatsen O1 en O2, zwarte vierkanten.

Suikerafbraak; een belangenconflict gedurende de evolutie

Gedurende zijn ontwikkeling in de loop van de tijd (de evolutie), is de melkzuurbacterie *Lactococcus lactis* vele suikers tegengekomen die als energiebron konden worden gebruikt. Het vermogen om zoveel mogelijk verschillende suikers te kunnen gebruiken was aantrekkelijk. Immers, wanneer in barre tijden slechts één suiker aanwezig was en deze niet kon worden benut, betekende dit de dood van de bacterie. Door natuurlijke selectie (alleen degenen die veel suikers konden benutten zijn overgebleven) kwam *Lactococcus lactis* in het bezit van een groot aantal enzymen die betrokken zijn bij de

afbraak van verschillende suikers, zoals bijvoorbeeld glucose, lactose, maltose en galactose. De bacterie had nu een grotere overlevingskans bij een regelmatig wisselend aanbod van suikers. Er zat echter ook een nadeel aan het bezit van een groot aantal verschillende suiker-afbrekende enzymsystemen. Het aanmaken van deze enzymsystemen kost namelijk ook energie. De cel verkeerde dus *in dubio*. Aan de ene kant moesten zo veel mogelijk enzymsystemen beschikbaar zijn om te anticiperen op de beschikbaarheid van verschillende suikers. Aan de andere kant zo min mogelijk omdat dit energie bespaarde. De cel heeft dit 'belangenconflict' zeer elegant opgelost door ervoor te zorgen dat de genetische informatie voor een bepaald (suiker-afbraak) enzymsysteem wel aanwezig is, maar dat er geen enzymen worden gemaakt wanneer daartoe geen aanleiding is. Wanneer een bepaalde suiker in de omgeving aanwezig is, worden de genen die coderen voor de afbraak-enzymen van het betreffende suiker 'aangeschakeld'.

De *lacABCDFEGX* genen van *Lactococcus lactis*; een genetische schakelaar

De *lacABCDFEGX* genen van *Lactococcus lactis* blijken ook te kunnen worden aan- en uitgeschakeld (gereguleerd). In hoofstukken 2 en 3 staat beschreven dat *Lactococcus lactis* bij groei op glucose ongeveer 10 keer minder van de lactose-afbrekende enzymen maakt dan bij groei op lactose. Zoals boven reeds besproken is dit economisch gezien gunstig voor de cel omdat nu geen energie wordt verspild voor de aanmaak van deze enzymen. Tevens bleek dat er bij groei op glucose een factor 10 minder *lacABCDFEGX* mRNA aanwezig was. Minder mRNA betekent tevens minder enzym (zie Fig. 2). Hieruit bleek dat de genetische schakelaar werkt op het niveau van de aanmaak van mRNA. Naast de *lacABCDFEGX* genen is er ook een stukje DNA (de promoter) van ongeveer 50 nucleotiden lang, wat samen met het RNA polymerase (een algemeen eiwit wat in de cel voorkomt) betrokken is bij de aanmaak van mRNA. Het RNA polymerase 'herkent' de promoter en begint vervolgens met de aanmaak van mRNA.

Voordat de genetische schakelaar kon worden onderzocht werd eerst de promoter van de *lacABCDFEGX* genen geïdentificeerd (de *lac* promoter), zoals staat beschreven in hoofdstuk 6. De ontdekking van een belangrijk onderdeel van de genetische schakelaar voor de *lacABCDFEGX* genen staat beschreven in hoofdstuk 5. Juist voor (stroomopwaarts van) de *lacABCDFEGX* genen werd een gen geïdentificeerd, het *lacR* gen (Fig. 3), waarvan het eiwitprodukt, de LacR repressor, betrokken bleek te zijn bij de remming van de *lac* promoter. Wanneer bijvoorbeeld een overdosis van het *lacR* gen in de cel werd gebracht, werden bij groei op lactose aanzienlijk minder lactose-afbrekende enzymen gemaakt en daalde de activiteit van de *lac* promoter. Dit vertaalde zich in een aanzienlijke afname van de groeisnelheid. Omdat bij groei op glucose geen afname van de groeisnelheid werd gevonden werd geconcludeerd dat de LacR repressor specifiek de aanmaak van de lactose-afbrekende enzymen remt en niet die van glucose.

Werkingsmechanisme van de *lacABCDFEGX* schakelaar

Zoals boven uiteen gezet bleek dus dat de LacR repressor op de een-of-andere manier de activeit van de *lac* promoter beinvloedt. Hoe doet de LacR repressor dit nu? Regulatie van genen in andere bacteriën, zoals bijvoorbeeld in de goed bestudeerde *Escherichia coli*, vindt vaak plaats door binding van een repressor nabij de promoter van het gereguleerde gen. Doordat de repressor in de buurt van de promoter bindt kan het RNA polymerase minder goed aan de promoter binden, waardoor er minder mRNA wordt gemaakt. Om te onderzoeken of de LacR repressor in staat is aan het *lac* promotergebied te binden werd LacR gezuiverd en werden DNA-eiwit binding studies uitgevoerd (hoofdstuk 7). Uit deze studies bleek dat de LacR repressor in staat is tot binding aan het *lac* promotergebied. Er werden twee LacR-bindingsplaatsen (operators) geïdentificeerd (Fig. 3). De hamvraag was vervolgens: Hoe weet de cel dat er lactose aanwezig is, en dat de binding van LacR aan de *lac* promoter moet worden opgeheven? Daartoe werd gekeken of tussenprodukten (intermediairen) van de afbraakroute van lactose de binding van LacR aan de *lac* promoter kunnen beinvloeden (hoofdstuk 7). Tagatose-6-fosfaat, een intermediair van de lactose-afbraak (Fig. 1), bleek in staat de binding van LacR aan de *lac* promoter te verhinderen, terwijl in aanwezigheid van andere intermediairen geen verlies van binding werd geconstateerd.

Een (in deze samenvatting sterk gesimplificeerd) model voor de regulatie van de *lacABCDFEGX* genen van *Lactococcus lactis* kan nu worden afgeleid. Bij groei op glucose bindt de LacR repressor aan het *lac* promoter gebied, en remt daarmee de mRNA synthese van de *lacABCDFEGX* genen. De *lacABCDFEGX* genen staan nu 'uit' geschakeld. Bij groei op lactose wordt op een zeker moment in de afbraakroute het intermediair tagatose-6-fosfaat gevormd. Dit bindt aan de LacR repressor, met als gevolg dat LacR niet meer in staat is tot binding aan het *lac* promoter gebied. Het RNA polymerase is nu weer in staat aan de *lac* promoter te binden, en de mRNA synthese van de *lacABCDGEFX* genen start. De *lacABCDFEGX* genen staan nu 'aan' geschakeld.

De onderdelen van de *lacABCDFEGX* schakelaar

In het voorafgaande is beschreven waar (aan de operators), en wanneer (als er geen tagatose-6-fosfaat aanwezig is), de LacR repressor aan het *lac* promoter gebied bindt. Op de vraag: "Hoe bindt LacR aan het DNA en aan tagatose-6-fosfaat?" is nog niet ingegaan. In hoofdstukken 8, 9 en 10 wordt op deze vraag gedeeltelijk ingegaan door eerst een *Lactococcus lactis* stam te construeren die het *lacR* gen mist (hoofdstuk 8), en vervolgens door middel van gerichte veranderingen in de LacR repressor een aantal aminozuren te identificeren die betrokken zijn bij binding van tagatose-6-fosfaat (hoofdstuk 9) en binding aan het DNA (hoofdstuk 10).

Tenslotte

Dit proefschrift beschrijft de karakterisering en regulatie van de genen die coderen voor enzymen die betrokken zijn bij een belangrijke eigenschap van *Lactococcus lactis*: de vergisting van lactose tot melkzuur. Deze kennis kan gebruikt worden bij de ontwikkeling van voedselveilige selectie markers in de verbetering van startercultures. Gebaseerd op het *lacF* gen is een dergelijke marker reeds ontwikkeld. De geïdentificeerde genetische schakelaar kan worden gebruikt in de gecontroleerde produktie van eiwitten in *Lactococcus*. Omdat *Lactococcus* glucose als suikerbron prefereert boven lactose moet het mogelijk zijn, door middel van een gedoseerde toevoeging van glucose bij aanvang van de fermentatie, een gen van interesse onder controle van de *lac* schakelaar op elk gewenst moment van de fermentatie tot expressie te brengen.

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 28 mei 1963 te Ede. Na het behalen van het VWO-B diploma aan het Christelijk Streeklyceum te Ede op 4 juni 1982, werd in september datzelfde jaar gestart met de studie Molekulaire Wetenschappen aan de Landbouwuniversiteit te Wageningen. Op 22 januari 1988 werd het doctoraalexamen behaald met als afstudeervakken Moleculaire Biologie (bij het NIZO te Ede; Prof. dr. van Kammen, Prof. dr. W.M de Vos) en Erfelijkheidsleer (Dr. J. Visser). Een stage werd uitgevoerd op de afdeling Biochemie van de Katholieke Universiteit Nijmegen (Dr. W. van de Ven). Van 1 januari 1988 tot 1 januari 1989 volgde een tijdelijke aanstelling als wetenschappelijk medewerker bij de afdeling MRDL (Recombinant DNA laboratorium) van Organon Int. te Oss, waar onder leiding van Dr. J. Meyerink en Prof. dr. W. Olijve onderzoek werd verricht aan receptoren. Hierop volgde per 15 januari 1989 een tijdelijke aanstelling als wetenschappelijk medewerker bij de afdeling Biofysische Chemie van het Nederlands Instituut voor Zuivel Onderzoek te Ede, waar onder leiding van Prof. dr. W.M de Vos het in dit proefschrift beschreven promotieonderzoek werd uitgevoerd. Sinds 1 oktober 1992 is hij werkzaam als wetenschappelijk medewerker binnen de afdeling Molecular and General Genetics (Genetics of Eukaryotic Microorganisms) van Gist-Brocades bv te Delft.

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