CLONING AND EXPRESSION OF CITRATE PERMEASE GENE *LACTOCOCCUS LACTIS* SUBSP. *LACTIS* BIOVAR. *DIACETYLACTIS* MAD61

SİTRAT PERMEAZ GENİNİN *LACTOCOCCUS LACTIS* SUBSP. *LACTIS* BIOVAR. *DIACETYLACTIS* MAD61 SUŞUNDAN KLONLANMASI VE İFADESİ

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ABSTRACT: The citrate permease determinant (Cit-P) in *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* MAD61 *(L. diacetylactis)* was demonstrated to be encoded by a 8.4 kb plasmid, pMC8, by protoplast induced plasmid curing experiments. The 1.9 kb Cit-P gene fragment was cloned into the *HindIII / PstI* site of plasmid pNZ9019. Resulting recombinant plasmid, pNZ9020, enabled *Escherichia coli* E10-1 and citrate permease negative *(Cit-P⁻)* *L. diacetylactis* MAD61-22 to transport and utilise citrate, indicating expression of the citrate permease from strain MAD61 to interspecific and intraspecific hosts.

ÖZET: Protoplastlarda indüklenen plazmid giderme çalışmaları sonucu, *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* *(L. diacetylactis)* MAD61 suşunda sitrat permeaz (Cit-P) determinantının 8.4 kb büyüklükteki pMC8 plazmidi olduğu tanımlandı. 1.9 kb büyüklükteki Cit-P gen fragmenti pNZ9019 plazmidinin *HindIII / Pst* I bölgesi içerisine klonlandı. Oluşturulan rekombinant plazmid pNZ9020 *E. coli* E10-1 ve sitrat permeaz negatif *(Cit-P⁻)* *L. diacetylactis* MAD61-22 suşuna aktarıldı ve kullanımı sağlandı. Bu sonuçlar, MAD61 suşundan aktarılan sitrat permeaz geninin tür içi ve türler arası konakçılarda ifade edilebildiğini gösterdi.

INTRODUCTION

Lactococci are well known for their use as starter cultures in the manufacture of fermented dairy products (LEEWACHARAMAS et al., 1997). A number of traits of economic importance in lactococci such as lactose utilisation, proteinase activity, phage resistance and citrate metabolism were reported to be unstable because of the genes responsible for these traits are associated with plasmid DNA (HARLANDER et al., 1984; LEENTHOUS et al., 1991; AKÇELİK and TUNAİL, 1992; KOK, 1996). *L. lactis* subsp. *lactis* biovar. *diacetylactis* *(L. diacetylactis)* can utilise citrate to produce diacetyl, an aromatic constituent in fresh cheese, butter and some fermented milk (HADDAD et al., 1997). Citrate is transported inside the cell by citrate permease (Cit-P) and then to acetate and oxalacetate by citrate lyase (Cit-L). Oxalacetate is decarboxylated by oxalacetate decarboxylase, yielding pyruvate. α-Acetolactate lyase synthase transforms pyruvate to acetaldehyde-thiamine phosphate and condenses it with a second molecule of pyruvate to from α-acetolactate. Diacetyl is originated from the chemical oxidative decarboxylation of α-acetolactate (PLATTEUW et al., 1995; GASSON et al., 1996; BOUMERDASSI et al., 1997) and aceton is originated from the decarboxylation of α-acetolactate by α-acetolactate decarboxylase, by reduction of diacetyl reductase, or by nonoxidative chemical decarboxylation (BOUMERDASSI et al., 1997). In several cases it has been demonstrated that the citrate permease system associated by 8.0 kb plasmid DNA (KEMPLER and McKAY 1979; MAGNI et al., 1994) and genes cloned and expressed in intraspecific and interspecific host strains (DAVID et al., 1990; SESMA et al., 1990; MORITA et al., 1997; BANDELL et al., 1998).

In this study, cloning of the Cit-P$^+$ gene from the strain *L. diacetylactis* MAD61 into the *Hind*III / *PstI* site of the vector pNZ9019, designed as pNZ9020, allowed expression of the Cit-P$^+$ gene in *Escherichia coli* E10 and *L. diacetylactis* MAD61-22 was reported.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. *Lactococcal* strains were routinely grown in M17 medium (TERZAGHI and SANDINE 1975) containing 0.5 % glucose and lactose and incubated at 30 °C. *Escherichia coli* E10 was grown in L broth (SILHAVY et al., 1984) at 37 °C. Antibiotic chloramphenicol (Cm) was added to selective media at level of 10 µg/ml. Culture stocks were stored in broth containing 40 % glycerol at -40 °C.

Plasmid curing and characterisation of citrate positive (Cit^+) and citrate negative (Cit^-) phenotypes

Plasmid cured variants of *L. diacetylactis* MAD61 were obtained by using the protoplast induced curing method described by GASSON (1983). Citrate utilisation by *L. diacetylactis* was scored by using the medium of KEMPLER and McKAY (1980). Medium used for screening citrate utilisation by E. coli was Christensen agar (SESMA et al., 1990).

Isolation of plasmid DNA and gene cloning

The lysis procedure of ANDERSON and McKAY (1983) was used to isolate plasmid DNA from *L. diacetylactis*. *E. coli* plasmid DNA was extracted by using the method of ISH-HOROWICZ and BURKE (1981). Plasmid DNA samples were purified by cesium chloride-ethidium bromide density gradient and desalted as described by LAIBLE et al. (1987). Agarose gel electrophoresis was performed using 0.7 % agarose gels in tris-acetate buffer (pH 8.0) at 4 V/cm followed by staining in ethidium bromide (0.5 µg/ml) (COFFEY et al., 1993).

Table 1. Bacterial Strains and Plasmids

Strains and plasmids	Relevant characteristics	Molecular weights of plasmid(s) (kb)	Source or Reference
L. diacetylactis			
MAD61	Cit-P^+, Cit-L^+, Cm^s, wild type strain	42.4, 36.8, 32.5, 30.1, 22.3, 17.6, 8.4, 6.3, 1.8	This study
MAD61-9	Cit-P^+, Cit-L^+, Cm^s, One plasmid cured variant of MAD61	42.4, 32.5, 30.1, 22.3, 17.6, 8.4, 6.3, 1.8 30.1, 22.3	This study
MAD61-13	Cit-P^-, Cit-L^+, Cm^s, Multiple plasmid cured variant of MAD61		This study
MAD61-20	Cit-P^+, Cit-L^+, Cm^s, Multiple plasmid cured variant of MAD61	22.3, 8.4	This study
MAD61-22	Cit-P^-, Cit-L^+, Cm^s, Plasmid free variant of MAD61	-	This study
MAD61-22T1	Cit-P^-, Cit-L^+, Cm^r, pNZ9020 carrying transformant of MAD61-22	4.9	This study
E. coli			
E10-1	Cit-P^-, Cm^s, Plasmid free variant of E10	-	Demircan et al. (1995)
E10-1T17	Cit-P^+, Cm^r, PNZ9020 carrying transformant of E10-1	4.9	This study
Plasmids			
pMC8	Cit-P^+, Cm^s,	8.4	This study
pNZ9019	Cit-P^-, Cm^r,	3.3	Demircan et al. (1995)
pNZ9020	Cit-P^+, Cm^r,	4.9	This study

Cit-P^+: citrate permease positive, Cit-P^-: citrate permease negative, Cit-L^+: citrate lyase positive, Cit-L^-: citrate lyase negative, Cm^r: chloramphenicol resistance, Cm^S: chloramphenicol sensitivity, kb: kilobase

Restriction enzymes, T4 DNA ligase and other DNA modifying enzymes were purchased Gibco/BRL Life Technologies, New England Biolabs or Promega Corporation and used as recommended by manufacturers. Plasmid DNA transformation and standard recombinant DNA techniques were done as described by SAMBROOK et al., (1989). Transformation of *L. diacetylactis* was performed as described DAVID et al., (1990). Chloramphenicol resistant (Cm^r) transformants were selected on KEMPLER and McKAY (1980) agar and Christensen agar (SESMA et al., 1990) for *L. diacetylactis* and *E. coli*, respectively.

Citrate transporting activity and measurement of diacetyl/acetoin

The citrate-transporting activity was assessed by the method of REYNOLDS and SILVER (1983). Residual citrate content was determined by using citrate assay kit (Boehringer GmbH, Mannheim, Germany). Diacetyl/acetoin were determined as described by WALSH and COGAN (1974).

RESULTS AND DISCUSSION

After protoplast induced plasmid curing of *L. diacetylactis* MAD61, Cit^+ and Cit^- plasmid variants were selected on KEMPLER and McKAY (1980) agar plates. In order to determine the involvement of plasmid DNA in the citrate utilisation, strain MAD61 and its Cit^+ and Cit^- variants were examined for their plasmid contents. Strain MAD61 was found to be harbour nine distinct plasmid species of 42.4, 36.8, 32.5, 30.1, 22.3, 17.6, 8.4, 6.3 and 1.8 kb (Figure 1). The plasmid profiles of Cit^+ and Cit^- variants were also shown in Figure 1. Plasmid contents of Cit^+ variant MAD61-20 and Cit^- variant MAD61-13 differed in two plasmids that 8.4 kb present in MAD61-20 and 30.1 kb present in MAD61-13. These results pointed out that citrate utilisation linked by 8.4 kb plasmid, designed as pMC8, in *L. diacetylactis* MAD61.

To develop a cloning system for Cit-P gene, pCM8 preparation from *L. diacetylactis* MAD61 was digested with *Pst*I and *Hind*III and ligated with *Pst*I / *Hind*III cleaved pNZ9019. The resulting plasmid pNZ9020 (Figure 2) was used to transform $Cit\text{-}P^-$ / $Cit\text{-}L^+$ *L. diacetylactis* MAD61-22 and $Cit\text{-}P^-$ *E. coli* E10-1 strains. Cm^r / $Cit\text{-}P^+$ transformants were selected on KEMPLER and McKAY (1980) agar and Christensen agar (SESMA et al., 1990) including 10µg/ml chloramphenicol. Plasmid DNAs obtained from stable blue colonies of *L.diacetylactis* and red colour produced colonies in the streaked line of *E. coli* were analysed. Two transformants, MAD61-22T1 and E10-1T17 were found to be harbour pNZ9020 containing an insert of 1.9 kb. Since the 1.9 kb region present in pNZ9020 was a segment of citrate plasmid pMC8, it appeared that the gene for citrate permease system resided in this segment.

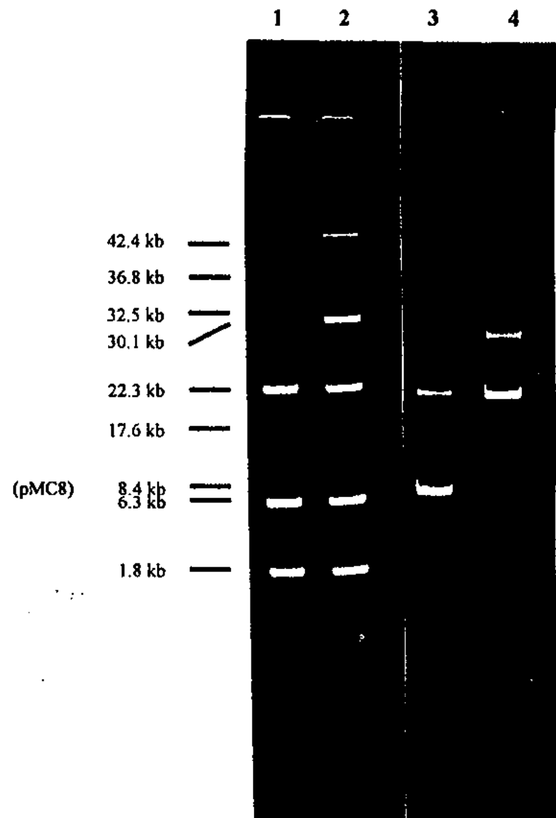

Figure 1. Plasmid contents of *L. lactis* subsp. *lactis* biovar. *diacetylactis* strain MAD61 and its citrate positive (Cit^+) and citrate negative (Cit^-) variants. Lanes; 1: MAD61-9 (Cit^+); 2: MAD61 (wild type strain); 3: MAD61-20 (Cit^+); 4: MAD61-13 (Cit^-).

As shown in Table 2, *L. diacetylactis* MAD61, its Cit+ variants (MAD61-9 and MAD61-20) and pNZ9020 carrying transformants (MAD61-22T1 and E10-1T17) were able to transport citrate into the cells, whereas Cit⁻ variant of MAD61 (MAD61-13), plasmid free *L. diacetylactis* MAD61-22 and *E. coli* could not transport. Citrate transporting activity and accumulation of diacetyl / acetoin in the Cit+ variants MAD61-9 and MAD61-20 and the transformant MAD61-22T1 were the same as wild type strain MAD61. But the citrate permease activity of the transformant strain *E. coli* E10-1T17 was weaker than *L. diacetylactis* MAD61 and accumulation of diacetyl/acetoin in the E10-1T17 cells was not detected. *E. coli* E10-1 is unable to utilise citrate as a sole source of carbon and energy. However, *E. coli* possesses all of the enzymes for citrate metabolism, since citrate is a substrate in the tricarboxylic acid cycle, the major metabolic pathway of aerobically growing cells. Therefore the inability to transport citrate is the major barrier to utilisation of citrate by *E. coli* (LARA and STOCK 1952; SESMA et al., 1990). Citrate transporters (CitPs) have been found in strain belonging to the genera *Lactococcus* and *Leuconostoc*, bacteria in which the mechanisms of citrate fermentation has been studied in detail (MARTY-TEYSSET et al., 1995; MARTIN et al., 2000). Cloning experiments in this study demonstrated that the 8.4 kb plasmid encoded citrate transport system of *L. diacetylactis* MAD61 is active in E. coli. The weaker Cit-P activity in the *E. coli* E10-1T17 cells than *L. diacetylactis* MAD61 may be was due to not its own promoter on recombinant plasmid pNZ9020.

Table 2. Citrate Permease Activity in the Cells and Diacetyl/Acetoin Content in the Presence of Ditrate

Strain	Citrate utilisation*	Citrate uptake (mmoles/ml/mg dry wt. cells)	Diacetyl/acetoin content (mM)
L. diacetylactis			
MAD61	Cit+	0.39	15
MAD61-9	Cit+	0.39	15
MAD61-13	Cit−	<0.00	<0.0
MAD61-20	Cit+	0.39	15
MAD61-22	Cit−	<0.00	<0.0
MAD61-22T1	Cit+	0.39	15
E. coli			
E10-1	Cit−	<0.00	<0.0
E10-1T17	Cit+	0.23	<0.0

* Detected by using KEMPLER and McKAY (1980) agar plate, Cit+: citrate utilisation, Cit−: no citrate utilisation

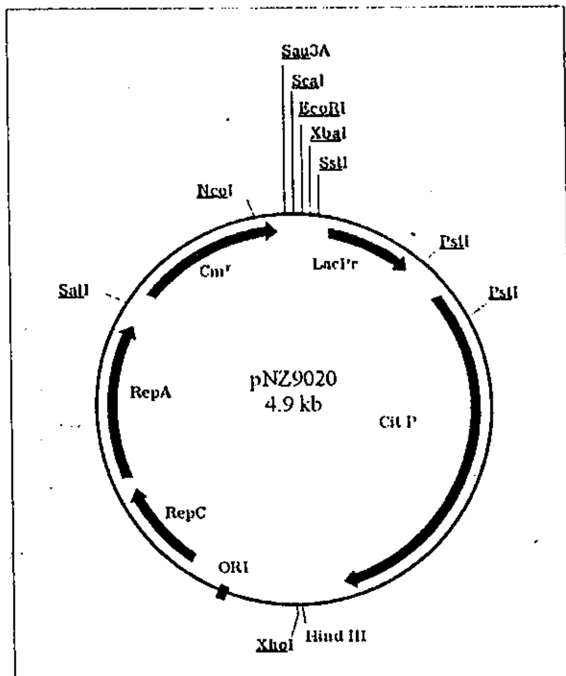

Figure 2. Schematic map of plasmid pNZ9020, showing the restriction sites used for construction. The lac promoter region, and the Cit-P gene. kb: kilobase

Genes coding for CitPs have been described for strains of *L. diacetylactis* and *Leuconostoc lactis* (DAVID et al., 1990; VAUGHAN et al., 1995; BANDELL et al., 1998). Clearly, although the transporters are almost identical, the genetic context of the *cit*P genes is different in *Lactococcus* and *Leuconostoc* strains, and it has been shown that the mechanism that controls expression of the genes are different (MAGNI et al., 1994; BANDELL et al.,

1997; MARTIN et al., 2000). The introduction of well characterised citrate genes into existing cloning vectors may result in food grade selection systems for lactococci which are acceptable for applications in the dairy and food industry.

Attemps are presently being made to sequence nucleotide of cloned citrate permease gene from *L. diacetylactis* MAD61.

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