Development of an electroporation procedure for gene disruption in *Lactobacillus helveticus* CNRZ 32

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An electroporation-mediated transformation method was developed and optimized for *Lactobacillus helveticus* CNRZ 32. The effects of electroporation buffers, growth conditions, cell-wall-weakening agents and field strength on transformation frequency were examined. Optimal conditions yielded a frequency of 2×10^4 transformants ($\mu\text{g pGK12}^{-1}$). Using this procedure, an integration plasmid was introduced into *L. helveticus* CNRZ 32 to inactivate the chromosomal X-prolyl dipeptidyl aminopeptidase gene (*pepXP*). The integration plasmid, designated pSUW200, consisted of pUC19, the pE194 erythromycin resistance gene and a 1.6 kb internal fragment of the *pepXP* gene. The erythromycin resistance transformants obtained were X-prolyl dipeptidyl aminopeptidase (X-PDAP) negative. Southern hybridization results indicated that pSUW200 had integrated into the *L. helveticus* CNRZ 32 chromosome via Campbell-type integration. After growth of the pSUW200-derived transformants for 112 generations under non-selective conditions, erythromycin-sensitive X-PDAP⁺ isolates were obtained. Southern hybridization results indicated that precise excision of pSUW200 had occurred via recombination between the 1.6 kb *pepXP*-derived nontandem repeats.

Introduction

Lactobacillus helveticus is an industrially important micro-organism which is used as a starter culture in the manufacture of a variety of fermented dairy products (Auclair & Accolas, 1983), as a flavour adjunct capable of reducing bitterness and accelerating cheese flavour development (Bartels *et al.*, 1987*a, b*), and is considered by some to be the organism of choice for the industrial production of lactate. However, understanding of the basic physiology and genetics of this organism is limited, due in part to the lack of modern molecular approaches for examining these organisms.

We have chosen to concentrate on *L. helveticus* CNRZ 32 because of its use as a cheese flavour adjunct (Bartels *et al.*, 1987*a, b*) and the availability of data concerning its proteolytic enzyme system (Khalid & Marth, 1990*a, b*, 1991). The proteolytic enzyme system is thought to be responsible for this strain's ability to reduce bitterness and accelerate cheese flavour development. To date, we have constructed a gene bank of this organism in *Escherichia coli* and identified clones expressing six distinct peptidases (Nowakowski *et al.*, 1993). The construction of isogenic derivatives of CNRZ 32, differing only in the activity of a single peptidase, would

allow us to determine the impact of individual peptidases on this strain's ability to function as a flavour adjunct. Therefore, the objectives of this study were to develop an efficient electroporation-mediated transformation procedure for *L. helveticus* CNRZ 32, and to use this procedure to develop an approach, based on homologous recombination, for constructing isogenic derivatives of CNRZ 32.

Methods

Bacterial strains, growth conditions and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. *L. helveticus* strains were grown in MRS broth or agar (De Man *et al.*, 1960) and *Escherichia coli* in LB broth or agar (Sambrook *et al.*, 1989) at 37 °C. Erythromycin (Em) and chloramphenicol (Cm) were added at 4 and 7.5 $\mu\text{g ml}^{-1}$ for *L. helveticus* and 1000 and 25 $\mu\text{g ml}^{-1}$ for *E. coli*, respectively. Stock cultures were maintained at –80 °C and working cultures were prepared from frozen stocks through two transfers in MRS or LB broth for *L. helveticus* and *E. coli*, respectively.

Plasmid and chromosomal DNA isolation. Plasmid DNA was isolated from *E. coli* using alkaline lysis (Sambrook *et al.*, 1989) and from lactobacilli using the procedure of Anderson & McKay (1983). Preparative plasmid purification was conducted by CsCl₂/EtBr density centrifugation (Sambrook *et al.*, 1989). Chromosomal DNA was isolated from *L. helveticus* strains using the lysis procedure described by Anderson & McKay (1983) and the extraction procedure described by Marmur (1961).

DNA manipulations. Restriction enzymes and T4 ligase were obtained from Bethesda Research Laboratory and used according to the

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Table 1. *Bacterial strains and plasmids*

Strain or plasmid	Characteristics	Source/Reference
<i>L. helveticus</i> CNRZ 32	X-PDAP ⁺ , parental strain	Khalid & Marth (1990)
<i>L. helveticus</i> JLS 100 to 102	Em ^r , X-PDAP ⁻ , pSUW200 integrated in the <i>pepXP</i> region of CNRZ 32	This study
<i>L. helveticus</i> JLS 100a	Em ^s , X-PDAP ⁺ , derivative of JLS100	This study
<i>E. coli</i> DH5 α	F ⁻ ϕ 80dlacZ Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (<i>r_k⁻</i> , <i>m_k⁺</i>) <i>supE44λ⁻ thi-1 gyrA96 relA1</i>	BRL
pGK12	Em ^R , Cm ^R	Kok <i>et al.</i> (1984)
pUC19E	pUC19 containing Em gene of pE194, Amp ^r , Em ^r	Leenhouts (1990)
pSUW200	pUC19E containing an 1.6 kb <i>pepXP</i> internal fragment, Em ^r , Amp ^r , X-PDAP ⁻	This study

manufacturer's instructions. Recombinant DNA techniques were carried out essentially as described by Sambrook *et al.* (1989).

Optimized electroporation procedure for *L. helveticus*. Electroporation was conducted using a Gene Pulser apparatus including a Pulse Controller unit (Bio Rad). The optimized procedure described below was developed by systematic analysis of the parameters described in this communication. A 1% inoculation from a working culture of *L. helveticus* CNRZ 32 was made into MRS broth containing 1% (w/v) glycine (MRSG) and incubated at 37 °C for 20 h. This culture was used to inoculate (1%, v/v) 250 ml MRSG broth, with incubation at 37 °C until OD₆₀₀ = 0.25–0.3. Cells were harvested by centrifugation at 7520 g for 10 min at 4 °C. Cells were washed four times with an equal volume of ice-cold electroporation buffer containing 0.1 mM-HEPES (Sigma) and 0.5 M-sucrose (Ultra pure grade, BRL), pH 7.0, and resuspended in 1.6 ml electroporation buffer. The cell suspension (800 μ l) was mixed with 10 μ l pGK12 (100 ng μ l⁻¹ dissolved in 10 mM-Tris/HCl, 1 mM-EDTA, pH 8.0) and kept on ice for 5 min. The mixture was then transferred into an electroporation cuvette (0.40 cm inter-electrode gap) and electroporated immediately using a single electric pulse. The electroporator settings were 2.5 kV, 25 μ F and 200 Ω . After electroporation, the DNA/cell mixture was kept on ice for an additional 5 min, diluted with 10 ml MRS broth containing 10 mM-CaCl₂, and incubated at 37 °C for 3 h. To determine the transformation frequency, cells were diluted, plated on MRS/CaCl₂ (10 mM) agar containing the appropriate antibiotic, and incubated anaerobically (Becton Dickinson) for 72 h at 37 °C. Electroporations without the addition of pGK12 served as the control.

Southern hybridization. Chromosomal DNA (4 μ g per lane) was digested with *Sph*I and electrophoresed in 0.7% agarose gels using Tris/acetate buffer (Sambrook *et al.*, 1989). After electrophoresis, the DNA was transferred onto nylon membranes (Micron Separations) as described by Southern (1975), except the acid treatment was omitted. Preparation of probes, hybridizations and detections were carried out according to the manufacturer's instructions using the non-radioactive Genius kit (Boehringer Mannheim).

Integrand stability. To determine the stability of the Em^r and X-prolyl dipeptidyl aminopeptidase negative (X-PDAP⁻) phenotypes, *L. helveticus* JLS100, JLS101, and JLS102 were grown in MRS broth without erythromycin. After 112 generations, cells were diluted and plated on media with and without Em. Additionally, 100 colonies were randomly picked from plates without Em to plates with and without Em. The X-PDAP activity of colonies was determined by flooding plates with 5 ml reaction mixture containing Gly-Pro- β -naphthylamide (2 mM final concentration) dissolved in *N,N* dimethyl formamide as described previously (Bhowmik & Marth, 1990). In this assay, X-

PDAP⁺ colonies stain red, while X-PDAP⁻ colonies remain white. Additionally, the chromosomal structure of a Em^s X-PDAP⁺ isolate was examined by Southern hybridization.

X-PDAP assay. The X-PDAP activity of broth cultures was assayed essentially as described previously (Bhowmik & Marth, 1990), except that the cell suspension (approximately 10⁷ c.f.u. in 5 ml 0.05 M-HEPES, pH 7.0) was permeabilized by mixing vigorously with 30 μ l chloroform for 1 min.

All buffers and chemicals were reagent grade and purchased from Sigma unless otherwise indicated.

Results

The effects of several parameters on the electrotransformation frequency were systematically examined and the results are summarized as follows.

Effect of electroporation buffers

Cells were grown in MRS broth to OD₆₀₀ = 0.7, harvested by centrifugation, washed four times with an equal volume of buffer, and electroporated using the parameters described earlier. The effect of several buffers on the transformation frequency of *L. helveticus* CNRZ 32 using pGK12 were examined (Table 2). Sucrose (0.5 M) was used as an osmostabilizer in 1 mM-HEPES buffer, pH 7.0. Use of 1 mM-HEPES buffer, pH 7.0, gave the highest transformation frequency (4 $\times$ 10³). Interestingly, the addition of MgCl₂, a commonly used divalent cation in electroporation buffers, completely inhibited electrotransformation of *L. helveticus* CNRZ 32.

Effect of growth media

To determine the effect of growth medium, cells were grown in APT broth (Difco), Selective Lactobacilli (SL) broth (Difco), MRS broth containing no phosphate salt (MRSNP) and MRS broth containing 1 mM-CaCl₂ (MRSC) to OD₆₀₀ $\approx$ 0.7. Cells grown in MRS broth

Table 2. Effect of different buffers on electroporation frequency

MRS-grown cells were used. Gene Pulser settings: 2.5 kV, 25 μ F and 200 Ω . Transformants were selected on MRS plates containing Cm (7.5 μ g ml⁻¹).

Buffers	Transformants per μ g pGK12
SMEB (3.5 $\times$)*	$< 1 \times 10^2$
EPB†	$< 1 \times 10^2$
HEPES‡	4×10^3
HEPES+EDTA (1 mM)	4×10^3
HEPES+CaCl ₂ (1 mM)	3×10^3
HEPES+MgCl ₂ (1 mM)	$< 1 \times 10^2$
HEPES+Lysozyme§	$< 1 \times 10^2$

* Sucrose magnesium electroporation buffer (Luchansky *et al.*, 1988).

† Electroporation buffer (Powell *et al.*, 1988).

‡ HEPES (1 mM) buffer, containing 0.5 M sucrose, pH 7.0.

§ Final concentration, 2 kU ml⁻¹ (Powell *et al.*, 1988).

served as a control. Cells were then washed four times with 1 mM-HEPES buffer, pH 7.0 and electroporated as above. Transformation frequencies with MRS, MRSC, SL, APT and MRSNP broth were 4×10^3 , 2.5×10^3 , 8×10^2 , 2×10^2 , and 1×10^2 , respectively. Therefore MRS broth was used throughout this study unless otherwise indicated.

Effect of cell-wall-weakening agents

The effect on transformation frequency of adding cell-wall-weakening agents to MRS broth was investigated. In this instance, cells were grown to OD₆₀₀ = 0.25–0.30. Cells were then harvested, washed and electroporated as described above. Cells were also grown in unsupplemented MRS as a control. The transformation frequencies observed with MRS, MRS supplemented with glycine (1%, w/v), MRS supplemented with DL-threonine (40 mM) and MRS supplemented with lysine (40 mM) were 4.3×10^3 , 6×10^3 , 4×10^3 and 1.2×10^3 , respectively. No transformants were observed using MRS broth supplemented with 200 mM-DL-threonine. Addition of glycine (1%, w/v) resulted in a slightly higher transformation frequency (6×10^3) compared to that of the control and other agents examined. Therefore MRS broth supplemented with 1% glycine (MRSG) was used routinely.

Effect of growth phase

To examine the effect of growth phase on transformation frequency, samples were taken at predetermined intervals from an MRSG broth culture, and were washed and electroporated as described above. The highest transformation frequency (6×10^3) was obtained in the early

Fig. 1. Effect of growth phase on transformation frequency of MRSG-grown *L. helveticus* CNRZ 32. ●, Transformants per μ g pGK12, selected on MRS plates containing Cm (7.5 μ g ml⁻¹); ○, OD₆₀₀.

exponential growth phase (OD₆₀₀ = 0.25), after which time electroporation frequency decreased (Fig. 1). A similar pattern was observed when unsupplemented MRS broth was used as the growth medium; however, the overall electroporation frequency was lower (data not shown).

Effect of pH of HEPES buffer

To determine the effect of buffer pH on transformation frequency, MRSG-grown cells were washed and electroporated with HEPES buffer adjusted in the range pH 4.0–8.0. No transformants were observed at pH 4.0. Transformation frequencies at pHs 5.0, 6.0, 7.0 and 8.0 were 1.5×10^3 , 4.1×10^3 , 7.5×10^3 and 3.7×10^3 , respectively. Therefore subsequent trials used HEPES buffer, pH 7.0.

Effect of buffer strength

To examine the effect of buffer strength, cells grown in MRSG broth were washed with, and resuspended in, buffer containing various HEPES concentrations (0.01 to 1.5 mM), pH 7.0. Transformation frequencies at 0.01, 0.1, 0.5, 1.0 and 1.5 mM were 3.8×10^3 , 2.0×10^4 , 6.9×10^3 , 7.4×10^3 and 4.0×10^3 , respectively. Therefore HEPES buffer was used at 0.10 mM in subsequent trials.

Effect of field strength

To determine the effect of field strength on transformation frequency, cells were grown in MRSG broth, washed with HEPES (0.10 mM) buffer, pH 7.0 and

Fig. 2. (a) Construction of pSUW200. (b) *pepXP* gene disruption by Campbell-type integration. (c) Recombination between the nontandem *pepXP* derived repeats, resulting in precise excision of pSUW200. ▨, Internal fragment of *pepXP* gene; ■, *pepXP* gene; ●, erythromycin resistance gene; —, CNRZ 32 chromosomal DNA; —, pUC19; E, K and S represent restriction sites for *EcoRI*, *KpnI*, and *SphI*, respectively. Figure not drawn to scale.

electroporated in the same buffer. Transformation frequencies at field strengths of 4000, 6250, 8000 and 12500 V cm⁻¹ were 1.0×10^3 , 2.0×10^4 , 4.3×10^3 and 1.8×10^3 , respectively. Therefore subsequent trials were done with a field strength of 6250 V cm⁻¹.

Inactivation of the *L. helveticus* CNRZ 32 *pepXP* gene

To obtain an X-PDAP⁻ derivative of *L. helveticus* CNRZ 32, an integration vector, designated pSUW200, was constructed (Fig. 2a). An internal 1.6 kb *EcoRI*–*KpnI* fragment of the *pepXP* gene (Ünlü & Steele; unpublished data) was cloned in pUC19E. This plasmid replicates in *E. coli*, but not in *L. helveticus* CNRZ 32, and expresses no detectable X-PDAP activity. The optimized electroporation procedure was used to introduce pSUW200 into *L. helveticus* CNRZ 32. In one experiment, three Em^r transformants were obtained from 10 µg of pSUW200 and designated JLS100–102. No transformants were obtained when electroporations were conducted using pUC19E. All three transformants were

Fig. 3. Analysis of chromosomal structure by Southern hybridization. (a) Agarose gel electrophoresis of DNA samples. Lanes: 1, λ/*HindIII* molecular mass standards; 2, *L. helveticus* CNRZ 32 chromosomal DNA digested with *SphI*; 3, *L. helveticus* JLS100 chromosomal DNA digested with *SphI*; 4, *L. helveticus* JLS100a chromosomal DNA digested with *SphI*. (b) Southern hybridization of the gel shown in (a) with a probe synthesized from a 1.6 kb *EcoRI*–*KpnI* internal fragment of *pepXP*.

determined to be X-PDAP⁻ by the flood-plate and chloroform-permeabilized cell assays.

Mechanism of chromosomal integration

To determine the mechanism of integration of pSUW200, chromosomal DNA from *L. helveticus* CNRZ 32 and JLS100 was isolated, and analysed by Southern hybridization. Chromosomal DNA from both strains was digested with *SphI* and hybridized with a probe synthesized from the 1.6 kb *EcoRI*–*KpnI* *pepXP* internal fragment (Fig. 3). The *pepXP* probe hybridized with a 15.5 kb *SphI* chromosomal fragment of *L. helveticus* CNRZ 32 (Fig. 3b, lane 2). Bands of 10.5, 8.8 and 5.3 kb were observed in the JLS100 lane (Fig. 3b, lane 3). The 5.3 kb band was significantly more intense and corresponds in size with pSUW200. Similar results were obtained for JLS101 and JLS102 (data not shown).

Stability of X-PDAP phenotype

The stability of the X-PDAP⁻ and Em^r phenotypes of JLS100, JLS101 and JLS102 were determined and the results are presented in Table 3. While 78–90% of

Table 3. Stability of the *Em^r* phenotype after 112 generations under non-selective growth conditions

Strain	<i>Em^r</i> c.f.u. (%)*	
	Direct plating	With tooth picks
CNRZ 32	0	0
JLS100	13	17
JLS101	97	78
JLS102	87	84

* Values were determined by direct plating or picking individual colonies with tooth picks to media with and without erythromycin ($4 \mu\text{g ml}^{-1}$).

JLS101 and JLS102 remained *Em^r* after 112 generations under non-selective conditions, only 13–17% of the population of JLS100 remained *Em^r*. Regardless of the strain, all the *Em^r* colonies examined were found to be X-PDAP⁻, while all the *Em^s* colonies were X-PDAP⁺. The chromosomal structure of a *Em^s* X-PDAP⁺ revertant of JLS100, designated JLS100a, was examined via Southern hybridization using a probe synthesized from the 1.6 kb *EcoRI*–*KpnI* *pepXP* internal fragment. A single hybridizing band of 15.5 kb, which was indistinguishable from the band detected in the CNRZ 32 lane, was detected in the JLS100a lane (Fig. 3b, lane 4).

Discussion

L. helveticus is an industrially important micro-organism which has received relatively little research attention. To apply modern molecular techniques to the study of this organism, an efficient genetic transformation system is required. Initially, we attempted to electrotransform *L. helveticus* CNRZ 32 with pGK12 using the procedures of Luchansky *et al.* (1988) and Powell *et al.* (1988); however, no transformants were obtained with either procedure. Therefore, the first objective of this study was the development of an efficient electrotransformation procedure for *L. helveticus* CNRZ 32.

The procedure optimized for CNRZ 32 yields 2×10^4 transformants per μg pGK12. The electroporation buffer system employed was determined to be the single most critical parameter examined. No transformants were obtained when buffer systems described by Luchansky *et al.* (1988) or Powell *et al.* (1988) were employed, whereas 4×10^3 transformants per μg pGK12 were obtained using a HEPES buffer system. Optimization of other parameters such as growth media, growth phase, buffer pH, buffer concentration, and field strength increased the frequency to 2×10^4 transformants per μg pGK12. Recently, two other groups have reported electrotransformation of *L. helveticus*. Gavilan-Reyes *et al.* (1990) reported a frequency of 1.2×10^2 transformants

per μg pGK12 with *L. helveticus* CNRZ 1340 using the procedure described by Luchansky *et al.* (1988). Recently the procedure developed by Hashiba *et al.* (1990) yielded 1.3×10^4 *L. helveticus* subsp. *jugurti* SBT2161C transformants per μg pLHR, a vector based on a cryptic plasmid from *L. helveticus* subsp. *jugurti* SBT2161. Because of the composition of the electroporation buffers employed in these procedures, it is unlikely that either of these approaches would yield transformants of *L. helveticus* CNRZ 32. Both electroporation buffers contain 1 mM-MgCl₂, which we have demonstrated completely inhibits electroporation of CNRZ 32; additionally the buffer strength (7 mM) of both procedures was much higher than the buffer strength (0.1 mM) found to be optimal for CNRZ 32. These results indicate that there is significant variation between strains in the electroporation buffers required for electrotransformation in *L. helveticus*.

L. helveticus CNRZ 32 has been reported to enhance cheese flavour development; however the roles of individual gene products have not been examined, due to the lack of a procedure for constructing isogenic strains. Campbell-type integration of a nonreplicating plasmid is an approach that has been widely employed to construct isogenic strains differing in a single gene product. If the nonreplicating vector contains an internal fragment of the target gene, then a single cross-over event between the plasmid construct and the chromosome will result in two incomplete copies of the target gene, yielding a mutant deficient in the target gene product. This approach has been successfully applied in the construction of isogenic strains in Gram-positive bacteria such as *Listeria monocytogenes* (Wuenschel *et al.*, 1991), *Bacillus subtilis* (Beall & Lutkenhaus, 1989), *Streptococcus mutans* (Barletta *et al.*, 1988; Hudson & Curtiss, 1990) and *Streptococcus pneumoniae* (Berry *et al.*, 1989). Since no homology was observed between pUC19E and chromosomal DNA of CNRZ 32 (data not shown), the pSUW200 *Em^r* transformants most likely resulted from recombination between the internal *pepXP* fragment of pSUW200 and the CNRZ 32 chromosomal *pepXP*. The X-PDAP⁻ phenotype of the *Em^r* transformants obtained, and the physical evidence provided by Southern hybridizations, strongly support this conclusion. Additionally, our results indicate that significant amplification of the integrated pSUW200 occurred. The expected product of Campbell-like integration, the vector flanked by regions of homology (Fig. 2b), is a structure known to be susceptible to amplification (Anderson & Roth, 1977), and amplification of such structures has been observed in several other Gram-positive bacteria (Duncan *et al.*, 1978; Niaudet *et al.*, 1982; Young, 1983; Vosman *et al.*, 1986; Chopin *et al.*, 1989; Leenhouts *et al.*, 1989, 1990). Therefore, the observed amplification of

pSUW200 also implies that Campbell-like integration has occurred. To our knowledge, this is the first report of the construction of isogenic strains in lactobacilli via homologous recombination.

The structure produced by integration of pSUW200 into the CNRZ 32 chromosome was unstable during growth in the absence of selection for the pSUW200 Em^r gene. Growth of two isolates without selection for Em^r resulted in 3–13 % of the population becoming Em^s after 112 generations; in the case of the third isolate, approximately 86 % of the population was Em^s after 112 generations. The variation in the stability of the Em^r phenotype may reflect a difference in the extent of amplification of pSUW200 between the isolates. The results obtained by the direct plating of the culture on media with and without Em were similar to the results observed when colonies were transferred to media with and without Em. This differs from the results of Leenhouts *et al.* (1989) who observed a wide variation of the calculated stability obtained with these two methods. These authors hypothesized that the discrepancy between the two methods was the result of widely different levels of amplification of the integration plasmid within a culture. Southern analysis of a CNRZ-32-derived isolate which had lost the Em^r phenotype, and the observation that Em^s colonies were X-PDAP⁺, suggests that precise excision of pSUW200 occurred via recombination between the nontandem *pepXP*-derived repeats (Fig. 2c). Precise excision via recombination between nontandem repeats produced via Campbell-like integration has been observed by other investigators (Chopin *et al.*, 1989; Leenhouts *et al.*, 1991).

In conclusion, an efficient method for electroporation-mediated transformation of *L. helveticus* CNRZ 32 has been developed and used to construct an X-PDAP⁻ derivative of CNRZ 32 via Campbell-type integration. This approach should be useful in determining the contributions of individual components of the CNRZ 32 proteolytic enzyme system in the hydrolysis of casein.

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