Controlled expression of CluA in *Lactococcus lactis* and its role in conjugation

Régis Stentz, Karen Jury, Tracy Eaton, Mary Parker, Arjan Narbad, Mike Gasson and Claire Shearman

Institute of Food Research, Norwich Research Park, Norwich NR4 7UA, UK

CluA is a 136 kDa surface-bound protein encoded by the chromosomally located sex factor of *Lactococcus lactis* MG1363 and is associated with cell aggregation linked to high-frequency transfer of the sex factor. To further investigate the involvement of CluA in these phenomena, the *cluA* gene was cloned on a plasmid, downstream from the lactococcal *nisA* promoter. In a sex-factor-negative MG1363 derivative, nisin-controlled CluA expression resulted in aggregation, despite the absence of the other genes of the sex factor. Therefore, CluA is the only sex factor component responsible for aggregation. The direct involvement of CluA in the establishment of cell-to-cell contact for aggregate formation was observed by electron microscopy using immunogold-labelled CluA antibodies. Inactivation of *cluA* in an MG1363 background led to a dramatic decrease in sex factor conjugation frequency compared to the parental strain. Increasing levels of CluA expressed in trans in the *cluA*-inactivated donor strain facilitated a gradual restoration of conjugation frequency, reaching that of the parental strain. In conclusion, CluA is essential for efficient sex factor transfer in conjugation of *L. lactis*.

INTRODUCTION

Lactose plasmid conjugation in the related strains *Lactococcus lactis* 712, C2 and ML3 frequently involves plasmid cointegration with a sex factor; often this is associated with a cell aggregation phenotype and a very high-frequency transfer ability (Gasson & Davies, 1980; Anderson & McKay, 1984; Walsh & McKay, 1981). The lactococcal sex factor exists integrated in the chromosome but it can excise as a closed circular form and it can be lost from the cell (Gasson et al., 1992). In neither the integrated nor the excised closed circular state does the sex factor express a cell aggregation phenotype. Cell aggregation has only been observed following sex factor and lactose plasmid cointegration.

Surface proteins in the Gram-positive bacteria have a multitude of functions, including binding to host tissues or specific immune system components, protein processing, nutrient acquisition and interaction between bacteria during conjugation (for a review see Navarre & Schneewind, 1999). Recent reports have shown that many cell-surface proteins are involved in aggregation and adhesion processes, including the colonization of oral and commensal bacteria (Kolenbrander & London, 1993; Jenkinson, 1994; Jenkinson & Demuth, 1997) and the initiation of infection by pathogens (Kreft et al., 1992; Jett et al., 1998; Schlievert et al., 1999; Rakita et al., 1999; Süssmuth et al., 2000).

Cell aggregation provides the first cell-to-cell contact that is necessary for conjugal transfer. This association of conjugation with cell aggregation has been observed in *Enterococcus faecalis* (Clewell & Weaver, 1989; Clewell, 1993), *Bacillus thuringiensis* (Andrup et al., 1993; Jensen et al., 1995) and *Lactobacillus* (Reniero et al., 1992). In *Ent. faecalis* cell aggregation is dependent on a cell-surface protein in the donor cell that binds to the recipient cell surface. This protein is characteristically induced by a small peptide sex pheromone secreted by the recipient (Clewell, 1993). The genes encoding the surface proteins derived from several *Ent. faecalis* plasmids, including pAD1, pPD1 and pCF10, have been sequenced (Galli et al., 1990; Galli & Wirth, 1992; Kao et al., 1991). The cloning and sequencing of the *cluA* gene in *L. lactis* was reported previously (Godon et al., 1994). The molecular structure of the CluA protein resembles that of the general cell-surface proteins involved in conjugation in *Enterococcus* as well as several streptococcal proteins that have adhesion properties. The amino terminus of the CluA protein has the features of a typical hydrophobic signal sequence and the carboxy terminus has an LPXTG motif similar to those shown to be important in cell wall sorting in other Gram-positive bacteria, notably *Staphylococcus* (Fischetti et al., 1990; Schneewind et al., 1995; Ton-That et al., 1997; Navarre & Schneewind, 1999; Bolken et al., 2001; Osaki et al., 2002). It is likely that the CluA protein is attached to the cell wall through a covalent

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**Abbreviation:** BCP, bromocresol purple.
link to the peptidoglycan mediated by a sortase-type protein.

Two approaches are available to relate the clumping phenotype to the expression of the cluA gene: (i) creation of a clumping phenotype by direct expression of the cluA gene and (ii) the removal of the clumping capacity by gene disruption. Godon et al. (1994) reported that subcloning of the complete cluA gene and 400 bp upstream on a lactococcal plasmid vector did not produce a clumping phenotype, possibly because the immediate upstream region of the cluA gene does not contain a lactococcal sortase promoter. However, the integration of the dnaK promoter upstream of the cluA gene in situ in the chromosomally located sex factor did result in a constitutive cell aggregation phenotype. In this case, aggregation was weaker than that associated with a strain (MG1827) carrying the sex factor::lactose plasmid cointegrate. Heat-shock induction did not have a significant effect on the aggregation phenotype; this may reflect the small (threefold) induction observed for the DnaK protein (Eaton et al., 1993a). The effect of increased temperature on clumping is unknown; there is also the possibility that other proteins besides CluA were required for cell aggregation and these may be suboptimally expressed in the dnaK construct.

This study investigates both the native and heterologously controlled expression of CluA protein and its effect on aggregation and conjugation.

METHODS

Bacterial strains and plasmids. Escherichia coli strain TG1 (Gibson, 1984) was used for cloning experiments and plasmid propagation. TG1 was transformed with pFI2209 and pFI2213 (see below), leading to strains F9469 and F9509, respectively. All the L. lactis strains used in this study are held in the Institute of Food Research LAB strain collection and are listed in Table 1. F9012, a derivative of MG1827 was transformed with plasmid pFI973. FI9012::lacZ::nisA, with an additional SalI fragment from sex factor Godon et al. (1994), was used for the construction of the fusion.

Table 1. L. lactis subsp. cremoris strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics*</th>
<th>Source or reference</th>
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<tr>
<td>Strains</td>
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<tr>
<td>MG1363</td>
<td>Plasmid-free derivative of NCDO 712</td>
<td>Gasson (1983)</td>
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<td>MG1827</td>
<td>MG1363 with co-integrate lactose sex factor plasmid pMG827</td>
<td>Gasson et al. (1992)</td>
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<td>MG2567</td>
<td>MG1363 with a chromosomal copy of the lactose operon</td>
<td>MacCormick et al. (1995)</td>
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<td>FI7847</td>
<td>FI5876 derivative with a 20 bp insertion in nisA, suc+, StrR RifR</td>
<td>Dodd et al. (1996)</td>
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<td>FI8164</td>
<td>MG1363 with tetracycline-marked sex factor, TetR</td>
<td>C. Pillidge, pers. comm.</td>
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<td>FI9012</td>
<td>Spontaneous sf− MG1614 derivative, StrR RifR</td>
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<tr>
<td>FI9097</td>
<td>FI8164 with cluA inactivated using pFI1063, TetR</td>
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<td>FI9388</td>
<td>FI8164 with mobA inactivated, TetR</td>
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<td>FI7847/pFI2213, nisA− suc+, StrR RifR</td>
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<td>Plasmids</td>
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<td>pBS carrying 5.8 kb BclI–PstI fragment from sex factor</td>
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<td>pBS carrying cluA under the control of the lacR promoter</td>
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<td>pTRKH2</td>
<td>EryR, shuttle vector between E. coli and Lactococcus</td>
<td>O’Sullivan &amp; Klaenhammer (1993)</td>
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<td>pTRKH2 containing cluA under the control of the lacA promoter</td>
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<td>pFI1003 containing cluA from pFI973</td>
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<td>pFI2414</td>
<td>pFI2213 containing C-terminal His-tagged cluA gene</td>
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<td>EryR, ori+ of pWV01, replicates only in strains with repA in trans</td>
<td>Leenhouts et al. (1996)</td>
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<td>pFI1063</td>
<td>5′-end and 3′-end of cluA cloned into pOri280</td>
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*Amp, ampicillin; Ery, erythromycin; Cam, chloramphenicol; Tet, tetracycline; Str, streptomycin; Rif, rifampicin; sf−, sex factor negative; suc+, sucrose-positive.
spontaneous sex-factor-negative MG1614 derivative, was isolated following identification of a colony that did not hybridize with a cluA probe. The loss of the complete sex factor was confirmed by Southern blotting and PCR (data not shown). Five per cent of the colonies had spontaneously deleted the sex factor. All other strains constructed during this study are detailed in Results. All the plasmids used in this study are listed in Table 1. To construct the expression vector plasmid pFI2209, a multiple cloning site generated by the adaptor-duplex technique (Cravchik & Matus, 1993) using the oligonucleotides K240 (5'-GATCCTCGGGGGAGCTCGAGA- AGCTT-3') and K241 (5'-AGGGCCTTCTAGGAGCTCTTCGA- ACTTAA-3') was cloned in the BglII/EcoRI sites of pFI1003 (Karakaš Sen et al., 1999).

**Media, growth conditions and transformations.** *E. coli* was grown at 37 °C in L broth medium (Sambrook & Russell, 2001) supplemented with 15 μg chloramphenicol ml⁻¹, 150 μg erythromycin ml⁻¹ or 100 μg ampicillin ml⁻¹ as necessary. *L. lactis* strains were grown at 30 °C in M17 medium (Terzaghi & Sandline, 1975) with 0.5 % (w/v) glucose (GM17), lactose (LM17) or sucrose (SM17). Antibiotic-resistance markers in *L. lactis* were selected using 5 μg chloramphenicol ml⁻¹, 5 μg tetracycline ml⁻¹, 200 μg streptomycin ml⁻¹ or 200 μg rifampicin ml⁻¹. McKay’s indicator plates containing 0.5 % (w/v) sucrose and bromocresol purple (BCP) indicator (Kondo & McKay, 1982) with appropriate antibiotic selection were used to select conjugative transposition of the nisin transposon from FI7847. *E. coli* and *L. lactis* electrocompetent cells were prepared and transformed by the methods of Dower et al. (1988) and Holo & Nes (1995), respectively.

**DNA manipulation, PCR and sequencing.** General molecular cloning techniques and electrophoresis of DNA on agarose gels were carried out essentially as described by Sambrook & Russell (2001). Chromosomal DNA from *L. lactis* was prepared by the method of Lewington et al. (1987). The PCR reactions were performed using the BLO-X-ACT proof-reading polymerase in the conditions described by the manufacturer (Bioline). Nucleotide sequencing was performed by the dideoxy chain-termination method using fluorescedyce terminators and cycle-sequencing reactions using an ABI Big Dye Terminator Cycle Sequencing Ready Reaction Kit as described in the manufacturer’s manual. Sequencing samples were analysed on an Applied Biosystems 373A DNA sequencer.

**Oligonucleotides.** The two primer pairs used to amplify the His-tagged cluA gene by PCR were UCL1 (5'-TTGATGTTAACGCAACTAAC-3') and DCL2 (5'-CTAAATGATGATGATGATGATGATGATGAACCTCTTTGGGAAAGTAACCTGTAGTTTTTTTACACAGC-3') and DEXT2 (5'-CATCATCATCATTAGTGGGAAATCCTTTTGGAGGTG-3') and DEXT3 (5'-GACTCGAGATATCAATAAGGATATGGAGGTA-3'); HpaI and XhoI restriction sites are underlined.

**Nisin induction and subsequent aggregation.** A preparation containing 1 ng nisin ml⁻¹ (Aplin & Barret) was stored at −20 °C. Appropriate dilutions were made for induction of the bacterial strains. To test strains for aggregation, 1 ml of cell culture was grown overnight in a 24-well microtitre plate. The plate was then inverted several times until aggregation was visible.

**Purification of an His-tagged CluA and production of antibodies.** The overexpression of the His-tagged CluA was carried out using the F9982 strain. To first check the presence of the His-tagged CluA in the culture supernatant, 1 ml of an overnight culture induced with 100 ng nisin ml⁻¹ was filtered and concentrated fourfold by centrifugation using a Centricron YM-30 filter device (Millipore). For a larger scale, the strain was grown in 500 ml GM17 supplemented with 100 ng nisin ml⁻¹. After 24 h growth, the culture was centrifuged and the supernatant was concentrated using an Amicon (Millipore) concentrator (YM-30 membrane 63-5 mm, using a nitrogen cylinder at room temperature, for 6 h). The 10 ml retentate was loaded onto a column containing Ni-NTA agarose (Qiagen) and the His-tagged CluA protein was eluted by an imidazole gradient under the conditions recommended by the manufacturer. The imidazole was then removed by gel-filtration using a PD-10 desalting column (Pharmacia Amersham). To generate polyclonal rabbit serum, approximately 100 μg purified His-tagged CluA emulsified in Freund’s complete adjuvant was used to immunize two New Zealand White rabbits by subcutaneous injection. The rabbits were given a booster immunization with the same amount of protein emulsified with Freund’s incomplete adjuvant 21 days later. On day 42 after the first immunization, the rabbits were bled, and serum was prepared and stored at −20 °C.

**Transmission electron microscopy.** To purify the polyclonal antibodies raised against CluA, 100 μl rabbit antiserum was mixed with 900 μl FI9012 protein extract obtained after sonication and incubated for 1 h at 37 °C. The mixture was then centrifuged at 3500 r.p.m., and the supernatant was diluted 1:500 in PBS TWEEN containing 1 % BSA and used as a primary antibody solution. The bacterial cultures were sedimented at low speed with a bench-top centrifuge and the supernatants removed. The pellets were fixed for 2 h in 0.5 % glutaraldehyde, 3 % paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2, at room temperature. The bacteria were then washed in buffer and mixed with a small amount of 2 % low-gelling-temperature agarose (Sigma), which was solidified at 4 °C and chopped. These encapsulated samples were dehydrated in a graded ethanol series of increasing 10 % steps followed by three changes of 100 % ethanol. They were then infiltrated over a period of 3 days with LR White resin (London Resin Company) and polymerized overnight at 60 °C in gelatin capsules. Sections showing gold interference colours were cut with a diamond knife and collected on gold grids. All grids were pre-incubated for 15 min in 3 % bovine serum albumin (BSA Fraction V, Sigma) in PBS TWEEN at pH 7.4, then incubated in primary antibody. Grids were washed (3 x 5 min) in 1 % BSA in PBS TWEEN and immersed in secondary antibody, goat anti-rabbit 15 nm gold (British BioCell International), diluted 1:100 with PBS TWEEN containing 1 % BSA. Sections, unstained or stained with uranyl acetate and lead citrate, were examined and photographed in a JEOL1200EX/B transmission electron microscope. For all samples, control grids in which the primary antibody was omitted were also run.

**Surface extraction and Western immunoblotting.** A lysozyme surface extract of each strain was obtained as previously described for *Ent. faecalis* (Galli et al., 1990) following modification of the extraction buffer. Briefly, the cell pellet from a 10 ml culture in GM17 was resuspended in 50 mM Tris/HCl, pH 8, containing 1/10 diluted protease inhibitor Cocktail Set II (Calbiochem), 10 % (w/v) sucrose and 25 mg lysozyme ml⁻¹. The suspension was adjusted to an OD₆₀₀ of 10 and was shaken gently at room temperature for 1 h and centrifuged at 3500 r.p.m. Twenty microlitres of the supernatant was loaded on a NuPage (Invitrogen) SDS-polyacrylamide gel for electrophoresis. The success of extraction of protein samples used for the Western blots was checked by visualizing proteins after staining with Colloidal Blue Stain (Invitrogen). Western blot analyses were performed using the Western Blotting Chemogenic Kit as described by the manufacturer (Invitrogen). The quantification of detected signals was performed using the TotalLab software (Nonlinear Dynamics).

**Conjugation.** Conjugative transposition of the nisin transposon was performed using FI7847 (*nisA⁻ suc⁺*; see Table 1) as a donor strain. Transfer of the nisin transposon can be detected by the cotransfer of sucrose metabolism. Sucrose metabolism can be distinguished on McKay’s indicator plates containing sucrose and BCP (Kondo & McKay, 1982). Overnight cultures (0.3 ml) of FI7847 and the recipient strains were mixed together and 100 μl of the mixture
was then plated onto GM17 agar and incubated for 16 h at 30 °C. The cells were resuspended in 3 ml 0.9% (w/v) NaCl and serially diluted. One hundred microlitres of each dilution was spread on McKay’s indicator plates containing sucrose and BCP with appropriate antibiotic selection for the recipient strains. Sucrose-positive transconjugants could be detected as large yellow colonies on a thin white lawn formed by the recipient strains. The nisA+ transposon from FI7847 was introduced directly into the FI9097 cluA strain, giving the transconjugant strain FI9983 confirmed as suc+, tetracycline resistant, and streptomycin and rifampicin sensitive. Transfer of the nisA+ transposon from FI7847 into the FI9012 strain was via an intermediate cross with FI9388. FI9388 is equivalent to FI9097 except that the non-mobilizable sex factor is mobA+ instead of cluA-. The resulting transconjugant strain from the FI7847 and FI9388 mating was suc+, tetracycline resistant, and streptomycin and rifampicin sensitive; this strain was further crossed with FI9012 to give FI9979, suc+, tetracycline sensitive, and streptomycin and rifampicin resistant. The intermediate cross was to ensure that the nisuc-suc transposon moved into the FI9012 sex-factor-negative background without the sex factor. In this way the nisA+ transposon from FI7847 was introduced into the FI9097 cluA+ and FI9012 sex-factor-negative backgrounds for the cluA expression under nisin control (see Table 1).

The method used to measure sex factor transfer is an adaptation of the method previously described for plasmid transfer measurement in Ent. faecalis (Waters & Dunny, 2001). Briefly, overnight cultures of donor and recipient strains grown in the presence of tetracycline and rifampicin, respectively, were diluted to 1/100 in fresh GM17 medium. After 2 h growth, nisin was added to the medium at the same concentration for donor or recipient strains when induction of nisin promoter was required. After 4 h growth, donor and recipient strains were mixed at a ratio of 1:10. The mixture was vortexed for 30 s, left 1 h at 30 °C and serially diluted and plated on M17 agar containing rifampicin (recipient and transconjugant selection), tetracycline (donor and transconjugant selection) or both antibiotics (transconjugant selection). Transfer frequencies were calculated as transconjugants per donor.

RESULTS AND DISCUSSION

Expression of CluA using the lacA and lacR promoters

Initial attempts to overexpress the CluA protein focussed on expression under control of the inducible lactose promoters lacA and lacR (van Rooijen et al., 1992). The cluA gene (originating from pFI767; Godon et al., 1994) was first cloned on an E. coli pBluescript plasmid downstream from a lactococcal lactose promoter fragment. The pFI956 construct with cluA under the control of the lacA lactose operon promoter was stable in E. coli. In contrast, pFI975, a plasmid carrying cluA under the lacR lactose regulator promoter, was structurally unstable and the E. coli transformants grew poorly and had impaired viability. The lactose promoter–cluA fusions were subcloned onto the pTRKH2 lactococcal vector (O’Sullivan & Klaenhammer, 1993) and introduced into MG1363 or MG5267, which has the lactose operon and the lacR gene integrated on the chromosome (MacCormick et al., 1995). Lacococcal in the culture medium induces lacA and lacR promoters in MG5267. In both strains cell aggregation was observed in transconjugants where the cluA gene was under the control of the lactose repressor promoter (data not shown). No phenotypic change was observed when cluA expression was controlled by the lacA lactose operon promoter in pFI973. Eaton et al. (1993b) reported that transcription from the lactose repressor promoter gives higher expression than transcription from the lacA lactose operon promoter. This observation suggested that high levels of CluA expression were needed for visible clumping.

Expression of CluA using the nisin promoter

The positively regulated nisin expression system is tightly regulated, allowing complete ‘turn-off’ of genes under its control, but the requirement for additional control elements meant that new suitable lactococcal strain backgrounds had to be generated to study CluA expression. The expression level from the nisA promoter (PnisA) depends linearly on the concentration of the externally added nisin in a nisA+ background (De Ruyter et al., 1996). In addition, when fully induced PnisA is the most powerful promoter described in L. lactis. Constructions were carried out using pFI2209, an expression vector containing the PnisA promoter (see Methods). A Smal/XhoI fragment from pFI973 (see above) including the complete cluA gene and its transcription terminator was cloned in the Smal/XhoI sites of the pFI2209 polynucleotide to create pFI2213, a cluA expression vector under PnisA control.

Plasmids pFI2213 (cluA expression) and pFI2209 (negative control) were transformed into FI7847, an MG1363 derivative strain that carries a chromosomal copy of the complete nisin operon with an inactive nisA gene (Dodd et al., 1996). In this background, activation of the nisin promoter occurs only in the presence of exogenous nisin. The two resultant strains FI9523 (FI7847/pFI2213) and FI9518 (FI7847/ pFI2209) were grown for 16 h in the presence of 100 ng nisin ml–1, and after shaking of both culture flasks, visible aggregation was observed only for FI9523, the strain carrying pFI2213 (cluA gene under PnisA). To address the question whether products of the chromosomally located sex factor are also needed for cell aggregation, both plasmids were moved into a sex-factor-cured background (strain FI9979), leading to FI9981 (FI9979/pFI2213) and FI9980 (FI9979/pFI2209). FI9979 carries a nisA+ nisin transposon (see Table 1) introduced by conjugative transposition using FI7847 as a donor. After nisin induction and shaking, a clumping phenotype similar to the one observed for FI9523 was seen in FI9981, whereas no clumping was observed for the negative control strain FI9980. Thus, no additional components encoded by the sex factor are required for cell aggregation. In Ent. faecalis the cell surface aggregation protein Asc10 has been overexpressed in a strain lacking the sex pheromone plasmid which is still able to form aggregates (Hirt et al., 2000). Like CluA, Asc10 is the only sex pheromone protein required for aggregate formation. As shown in Fig. 1, between 1 and 2·5 ng ml–1 of nisin was required in the medium to induce a visible aggregation phenotype. The turbidity of the medium decreased when nisin concentration increased because more cells are
involved in cell aggregates under high CluA expression levels.

**Purification of a C-terminal His-tagged CluA protein**

Previous attempts to isolate CluA protein failed either because the amount of CluA present on the cell surface was limited or because the CluA protein was firmly attached and not released by the isolation methods used. Expression of the cluA gene under control of P_{nisA} ensures the production of a high level of CluA protein. The C-terminal end of the CluA protein was tagged by replacing the LPKTGE motif (LPKTGE in CluA) (Fischetti et al., 1990) with six successive histidine residues (Fig. 2a). Because no anchor is present, the overexpressed protein was expected to be secreted into the culture medium.

PFI2213 was used as the start point for construction of a plasmid expressing a C-terminal His-tagged CluA. The primer pairs UCL1/DCL2 and DEXT2/DEXT3 were used to amplify two fragments encoding the CluA C-terminal region corresponding to the regions up- and downstream of the LPKTGE motif of the protein. The downstream fragment contained the putative transcription terminator of cluA (Godon et al., 1994). The two resulting PCR fragments of 1649 bp and 271 bp, respectively, contained a 21 bp complementary extension encoding six successive histidine residues preceded by a thrombin cleavage site. The fragments were mixed and used as a template in cross-over PCR (Link et al., 1997) with the primers UCL1 and DEXT3. The resulting 1899 bp PCR fragment was digested with HpaI and XhoI and cloned into the HpaI/XhoI sites of pFI2213, replacing the original HpaI/XhoI fragment of the plasmid. Because of the stability problems already observed in E. coli the resultant plasmid pFI2414 was cloned directly into L. lactis FI9979 (sex factor negative), generating the strain FI9982 (FI9979/pFI2414).

Secretion of His-tagged CluA into the culture supernatant was investigated after overnight growth in GM17 containing 100 ng nisin ml^{-1}. Controls included strain FI9980 (FI9979/pFI2209) and FI9982 grown in the absence of nisin. The three cultures were centrifuged and the supernatants concentrated fourfold by filtration as described in Methods. Each supernatant was electrophoretically separated by SDS-PAGE and the stained gel is shown Fig. 2(b). A thick band with a size of about 136 kDa was present only in the case of FI9982 grown in the presence of nisin. The size corresponds to that expected for CluA (Godon et al., 1994). This result confirmed that FI9982 secreted His-tagged CluA when

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**Fig. 1.** Aggregation of cells expressing cluA under the control of the P_{nisA} nisin promoter. The FI9981 cells were grown for 16 h in a microtitre plate in the presence of different concentrations of nisin. The microtitre plate was then shaken until cell aggregation occurred.

**Fig. 2.** Purification of a C-terminal His-tagged CluA protein. (a). Schematic representation of CluA. Grey box, signal peptide sequence; white box, membrane anchor. The cluA gene was altered by PCR to encode an anchorless CluA protein where the LPKTGE motif has been replaced by six successive histidine residues (hatched box). (b) The altered cluA gene was expressed under the control of the nisA promoter (P_{nisA}). The His-tagged protein was secreted from the cells and the supernatant concentrated fourfold. The concentrated samples from three different cultures were loaded on an SDS-polyacrylamide gel (NuPAGE Bis-Tris 4–12%). Lanes: 1, FI9980 (carrying pFI2209) induced with 100 ng nisin ml^{-1}; 2, FI9982 (carrying pFI2414, encoding anchorless His-tagged CluA) non-induced; 3, FI9982 induced with 100 ng nisin ml^{-1}. The molecular masses (in kDa) of the standard proteins are indicated on the left. The size of the His-tagged CluA is indicated on the right.
induced with nisin. A large-scale overexpression of His-tagged CluA protein was performed, using a 500 ml culture. The supernatant was ultra-filtrated and the His-tagged CluA protein purified by affinity chromatography followed by gel-filtration. A total amount of 5 mg purified His-tagged CluA was obtained. The protein was used to raise polyclonal antibodies in rabbits.

Use of antibodies raised against CluA to monitor the expression of CluA at different stages of growth

The antiserum was used in a Western immunoblotting experiment using lysozyme extracts of the wild-type strain MG1363 and MG1827, which carries the sex factor::lactose plasmid cointegrate (Gasson et al., 1992). The sex-factor-deleted strain FI9012 was used as a negative control. The three strains were grown simultaneously and samples were collected when they reached an \( \text{OD}_{600} \) of 0.4 or 1.2, or after 16 h of culture (\( \text{OD}_{600} \sim 3.0 \)). Cell aggregation was tested (Fig. 3a) and CluA protein analysed by Western immunoblotting (Fig. 3b). Surface proteins were extracted using lysozyme and loadings standardized per cell. As expected, the immunodetection of His-tagged CluA showed a unique band located at 136 kDa. No CluA expression could be detected in the FI9012 extracts, whereas bands at about 136 kDa were observed in all other cases. This confirms that antibodies raised against His-tagged CluA are also specific for the natural protein. Neither aggregation nor CluA expression could be detected for FI9012. At the three different growth stages, no aggregation phenotype was observed for MG1363, even when the cells were concentrated twofold in PBS. A faint band corresponding to CluA could be observed at the mid-exponential phase (\( \text{OD}_{600} 0.4 \)) whereas the expression increased dramatically in the early stationary phase (\( \text{OD}_{600} 1.2 \)). Unlike MG1363, the aggregation phenotype in MG1827 could already be observed at an \( \text{OD}_{600} \) of 0.4 when the cells were concentrated in PBS, although the amount of detected CluA was equivalent to or lower than that observed for MG1363 at the early or late stationary phase. This suggests that MG1363 expresses additional factors that inhibit or reduce aggregation in spite of cluA expression. In MG1827, aggregation could also be observed at the early or late stationary phase, directly in the culture medium. As in MG1363, the expression of CluA in MG1827 increased at the early stationary phase. In all cases, the amount of CluA present on the cell surface was much higher for MG1827 than for MG1363, perhaps due to the higher copy number of the cluA gene in MG1827 or expression change due to the fusion point within the lactose plasmid cointegrate. These results of CluA detection at the MG1363 and MG1827 cell surface during growth suggest that the cluA gene expression is dependent on the growth stage and seems to be optimal only when cells have reached the early stationary phase.

Immunodetection of CluA on the cell surface

To check whether CluA is exposed on the cell surface and directly involved in the formation of cell to cell contact, an immunogold labelling experiment was performed on sections of MG1827 cells grown overnight. As a control, sections of FI9012 cells were treated simultaneously, under the same conditions. Electron microscopy revealed black spots corresponding to CluA protein located on the surface of the cells at the external side of the cell wall (Fig. 4). The majority of CluA detected was located precisely between two cells that were close to each other, confirming that CluA is directly involved in the establishment of cell-to-cell contact. No signal could be detected for the sex-factor-negative strain FI9012 (data not shown).
Comparison of conjugation frequencies in the presence or absence of CluA expression

A cluA<sup>-</sup> donor strain was constructed for use as a background strain in which to express the CluA protein. Fragments spanning domains I and II of CluA upstream and domain IV and part of domain V downstream (as defined by Godon <i>et al.</i>, 1994) were cloned contiguously into pOri280 to give pFI1063. This plasmid, pFI1063, was used in a gene replacement experiment involving double cross-over recombination (Leenhouts <i>et al.</i>, 1996) to introduce a deletion into the <i>cluA</i> gene on the chromosomally located sex factor of FI8164. The resulting strain, FI9097 (<i>cluA</i><sup>-</sup>), was used as a background for the controlled expression of CluA and to monitor its effect on conjugation.

The role of CluA in conjugation was assessed by first comparing the conjugation frequencies of the FI9097 donor containing a <i>cluA</i> deletion with the parental strain FI8164 (tetracycline-marked sex factor). FI9980 was used as a recipient (rifampicin selection) and FI8164 and FI9097 as donors. The conjugation frequency obtained for FI8164 was 61 times higher than for FI9097 (Fig. 5) after vortexing the donor/recipient mixture. Vortexing did not increase the conjugal transfer of the sex factor from FI9097. Gentle pipetting of the mixture up and down led to a conjugation frequency only seven times higher for FI8164 than for FI9097. Complementation of the <i>cluA</i> deletion was carried out to confirm that the impaired conjugation in FI9097 was due to the absence of CluA. For this, different expression levels of CluA were obtained using the donor strain FI9985 (i.e. FI9097 containing pFl2213 and a <i>nisA</i><sup>-</sup> nisin transposon) by the addition of increasing concentrations of nisin as shown in Fig. 6(a). FI9980 was used as a recipient strain. No significant difference of conjugation frequency could be observed between FI9984 (carrying pFl2209) in the presence of nisin and FI9985 (carrying pFl2213) in the absence of nisin. Immunoblotting confirmed that CluA was not expressed in either strain. The conjugation frequencies determined for FI9985 grown in the presence of nisin at 0.1, 1 and 10 ng ml<sup>-1</sup> were approximately 3 times, 22 times and 41 times (respectively) higher than those of cells grown in absence of nisin. In each case, the intensity of the signal obtained after immunodetection of CluA by Western blotting (Fig. 6a) was quantified. It emerged that 12 times more CluA was located on the surface of cells grown in presence of 1 ng nisin ml<sup>-1</sup> than of cells grown in presence of 0.1 ng nisin ml<sup>-1</sup> and 18 times more CluA on the surface of cells grown in presence of 10 ng nisin ml<sup>-1</sup>. These results showed that the amount of CluA exposed at the surface of the cell was correlated with conjugal transfer efficiency of the sex factor. In general, conjugation frequencies were approximately 3 times lower for cells harbouring the nisin transposon and the pFl2109 derivative plasmids compared to the parental strain. In contrast to <i>Ent. faecalis</i>, in which transfer of pCF10 is drastically improved when surface protein Asc10 is expressed in the recipient strain (Olmsted <i>et al.</i>, 1991), no difference in terms of conjugation frequency...

**Fig. 4.** Electron microscopy of MG1827 cells. A primary rabbit polyclonal antibody against CluA was used, followed by a secondary antibody labelled with 5 nm diameter gold particles (see Methods). The arrows indicate the black spots corresponding to the detected signals. Scale bar, approximately 0.5 μm.

**Fig. 5.** Conjugation frequencies and CluA expression in FI8164 and FI9097 used as donor strains. The FI9980 sex-factor-negative strain was used as a recipient. Each conjugation experiment was repeated three times. 1, Donor and recipient mixed by gentle pipetting; 2, Donor and recipient mixed by vortexing. A CluA immunodetection was performed on a lysozyme extract of each donor strain, prior to the mating mix. The conjugation frequencies are expressed as the number of trans-conjugants per donor cell. The position of CluA (approx. 136 kDa) is indicated.
could be observed when recipient strain FI9981 was induced with nisin (Fig. 6b).

This suggests that conjugation depends on an association between CluA and other sex-factor-encoded transfer proteins and that the CluA protein is involved in more than cell-to-cell contact mediated by aggregation.

Conclusions
In this work, cell aggregation and enhanced sex factor transfer were shown to be correlated with the presence of the CluA surface protein. It was shown that CluA can trigger aggregation in the absence of all other sex factor components. The expression of the cluA gene is dependent on the growth stage and seems to be optimal only when cells have reached the early stationary phase. A strain carrying an internal deletion of the sex factor cluA gene was impaired in its ability to transfer the sex factor. Complementation studies using this strain showed that CluA was able to restore the capacity of this strain to conjugate at frequencies similar to the parental strain. Complementation experiments were also conducted in which CluA was expressed in the recipient strain rather than the donor (CluA was induced in the recipient strain and the donor strain carried the sex factor cluA deletion). In this case, there was no restoration of conjugation frequency. This suggests that the CluA protein is involved in more than cell-to-cell contact in the conjugation process. This additional role of CluA is being further investigated.

A method to purify the protein in an anchorless form containing a C-terminal His-tag tail was developed. The His-tagged CluA protein was secreted from the cell and the protein was purified directly from the culture supernatant by nickel affinity chromatography. This method could be easily adapted to other surface-bound proteins in L. lactis or in other Gram-positive species. A polyclonal antibody to CluA was raised and used to visualize the involvement of CluA in cell-to-cell contact formation using immunogold electron microscopy.

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REFERENCES
The role of CluA in conjugation


