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The Tra Domain of the Lactococcal CluA Surface Protein Is a Unique Domain That Contributes to Sex Factor DNA Transfer

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CluA is a cell surface-presented protein that causes cell aggregation and is essential for a high-efficiency conjugation process in *Lactococcus lactis*. We know from previous work that in addition to promoting cell-to-cell contact, CluA is involved in sex factor DNA transfer. To define the CluA domains involved in aggregation and in transfer, we first performed random mutagenesis of the *cluA* gene using a modified mini-Tn*7* element which generated five amino acid insertions located throughout the encoded protein. Thirty independent *cluA* insertion mutants expressing modified CluA proteins at the cell surface were isolated and characterized further. The level of aggregation of each mutant was determined. The cell binding capacity of CluA was affected strongly when the protein had a mutation in its N-terminal region, which defined an aggregation domain extending from amino acid 153 to amino acid 483. Of the *cluA* mutants that still exhibited aggregation, eight showed an attenuated ability to conjugate, and six mutations were located in a 300-amino-acid C-terminal region of the protein defining a transfer domain (Tra). This result was confirmed by a phenotypic analysis of an additional five mutants obtained using site-directed mutagenesis in which charged amino acids of the Tra domain were replaced by alanine residues. Two distinct functional domains of the CluA protein were defined in this work; the first domain is involved in cell binding specificity, and the Tra domain is probably involved in the formation of the DNA transport machinery. This is the first report of a protein involved in conjugation that actively contributes to DNA transfer and mediates contact between donor and recipient strains.

CluA is a 136-kDa surface-bound protein encoded by the chromosomally located sex factor of *Lactococcus lactis* MG1363 (16); it is associated with cell aggregation linked to high-frequency transfer of the sex factor (13, 14). The amino terminus of CluA has a signal sequence typical of secreted proteins, and the carboxy terminus has a cell wall binding motif that includes a conserved LPXTGE motif (11). CluA exhibits significant homology with two groups of cell surface proteins found in other gram-positive bacteria (16): two enterococcal proteins involved in plasmid conjugation, Asa1 (12) and Asc10 (22), and two streptococcal proteins involved in binding to salivary glycoproteins, SpaP (23) and Ssp5 (8). A comparison of the amino acid sequences of CluA with the sequences of these four other proteins led to identification of six distinct domains in CluA (16), including four conserved domains (domains I, II, IV, and V), a variable domain (domain III), and an additional domain (designated domain IVB in this study) that is specific to CluA and is located at the C-terminal end between domains IV and V. In previous work, we investigated the role of CluA in aggregation and conjugation by controlling the level of protein expression using the lactococcal *nisA* promoter (29). In a sex factor-negative MG1363 derivative, overexpression of CluA resulted in aggregation of the cells, indicating that CluA is the only sex factor component required for aggregation. We clearly showed that CluA enhances sex factor transfer when it is expressed in a donor strain having a *cluA* gene deletion on the chromosome. However, expression of CluA in the recipient strain could not complement the lack of CluA in a *cluA* donor strain. This led us to the conclusion that CluA, in addition to its capacity to trigger cell-to-cell contact, has a direct role in DNA transport. This was surprising since the *Enterococcus faecalis* pheromone-inducible aggregation substance Asc10 provides efficient conjugation when the protein is expressed in either the donor or recipient cells (26). The functional domains of Asc10 were characterized using the phage-based Tn*lacZ*/in and Tn*phoA*/in transposition systems to construct a library of 22 in-frame insertions encoding 31 amino acids located throughout Asc10 (36). The plasmid conjugation efficiencies of the different insertion mutants correlated with aggregation in the different mutants, as expected. In our further characterization of CluA we used the well-developed pentapeptide scanning mutagenesis (PSM) technique (17) to insert randomly a variable 5-amino-acid cassette throughout CluA in order to determine the functional domains of the protein involved in aggregation and also the domain(s) that plays a role in DNA transfer.

PSM is a transposon-based strategy that generates a small fingerprint (15 bp) in the gene of interest, leading to a random 5-amino-acid insertion into the encoded protein. PSM can be performed after in vivo transposition using the Tn*4430* transposon originating from *Bacillus thuringiensis* that transposes efficiently in *Escherichia coli* (17, 18) or by using an in vitro transposition method based on the use of a Tn*7* derivative (2). The Tn*7*-based technique uses the TnsAB transposase associated with the ATP-dependent DNA binding protein $TnsC^{A225V}$ (28) to insert in vitro a mini-Tn*7* element containing the Tn*7* terminal *cis*-acting transposition sequences flanking an antibiotic resistance gene at random locations in the target DNA (for a review see reference 6). The mini-Tn*7* PmeI element is altered at both ends to generate PmeI restriction endonuclease sites (2). Removal of

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the mini-Tn*7* element using the PmeI restriction endonuclease sites leads to a 15-bp insertion that includes a unique PmeI site. An in-frame stop codon is generated in two of the six reading frames due to the UAA stop codon included in the PmeI sequence. The generation of these truncated proteins is the major limitation of this system for use in analyses of surface proteins anchored to the cell wall. Out-of-frame insertions occurring in the N-terminal region of the LPXTGE motif lead to mutated proteins that lack the membrane-associated domain and the cell wall anchor motif and could cause missorting or release of the proteins into the culture medium. Another limitation of this system is the use of the mini-Tn*7* PmeI selection markers, which are suitable only in gram-negative bacteria.

In this study, we developed a mutagenesis system suitable for gram-positive bacteria that produces only full-length proteins. This system was used successfully to generate a collection of derivatives with pentapeptide insertions located throughout CluA. These derivatives were employed for identification of regions of the protein associated with stability, aggregation, and conjugal transfer. Individual amino acid substitutions were created to further confirm the definition of the transfer domain.

MATERIALS AND METHODS

Media, growth conditions, and transformations. *E. coli* was grown at 37°C in Luria-Bertani medium (27) supplemented with 15 μg/ml of chloramphenicol or 150 μg/ml of erythromycin if necessary. *L. lactis* strains were grown at 30°C in M17 medium (30) with 0.5% glucose (GM17) or 0.5% sucrose (SM17) for selection of the nisin-sucrose transposon. Antibiotic resistance markers in *L. lactis* were selected using 5 μg/ml chloramphenicol or 5 μg/ml erythromycin. *E. coli* and *L. lactis* electrocompetent cells were prepared and transformed by the methods of Dower et al. (10) and Holo and Nes (19), respectively.

Bacterial strains. *E. coli* strain TG1 (15) was used for cloning experiments and plasmid propagation. *L. lactis* rifampin-resistant strain FI9979 (29) is a sex factor-negative strain that has a chromosomal copy of the complete nisin operon with a 20-bp insertion into *nisA* (9). This strain was used as a *cluA* background strain to monitor the aggregation of the different CluA mutants. Strains FI9980 and FI9981 (29) are FI9979 derivatives that harbor pFI2209, an expression vector containing the P_{nisA} promoter, and pFI2213, the same expression vector containing the wild-type *cluA* gene, respectively. These two strains were used as negative and positive controls to assess aggregation. FI9980 was also used as a recipient strain in conjugation experiments. *L. lactis* strain FI9983 (29) is a *cluA* derivative of FI8164 (containing a complete sex factor with the *tetM* gene from Tn*1545* [4] inserted into the tellurium resistance genes [C. J Pillidge, personal communication]). FI9983 also carries a chromosomal copy of the complete nisin operon with a 20-bp insertion into *nisA*. This strain was used in a complementation study to monitor the contributions of the different variants of CluA to conjugation. FI9984 (FI9983 containing the pFI2209 vector) and FI9985 (FI9983 carrying pFI2213) (29) were used as negative and positive controls, respectively, to assess conjugation efficiency.

Plasmid constructs. Phagemid pBluescript SK(−) (Stratagene) and plasmid pBR322 (3) were used for cloning experiments with *E. coli* TG1 (15). Plasmid pFI2453 was used as a target for insertion mutagenesis. This plasmid is a derivative of pFI2213 (expression vector pFI2209 containing the wild-type *cluA* gene under control of the P_{nisA} promoter) (29) in which the unique BsrGI site was removed by blunt ending the 5′ overhangs using the T4 DNA polymerase (Promega) under the conditions described by the manufacturer. All the relevant plasmids resulting from *cluA* insertional mutagenesis are listed in Table 1. Donor plasmid pFI2452 carrying the Ery^r mini-Tn*7* BsrGI element was constructed as follows. A 1,432-bp fragment of the pGPS4 plasmid (New England Biolabs) containing the Cm^r mini-Tn*7* PmeI element was isolated after digestion with KpnI and SacI and cloned into the SacI/KpnI restriction endonuclease sites of pBSK$^-$, generating pFI2449. The chloramphenicol resistance gene (903 bp) was then removed after BamHI digestion and religation of the vector, generating pFI2450. To amplify the Tn*7* recombination sequences, PCR was performed using primers LmaB and RmaB (see below) and pFI2450 as the template. The 508-bp PCR product was then digested with EcoRI and AvaI and inserted into the EcoRI/AvaI restriction endonuclease sites of pBR322, yielding pFI2451. The erythromycin resistance gene (*ermC*) of plasmid pE194 (20) was excised from pUC7E (Claire Shearman, personal communication) after digestion with BamHI and inserted into the BamHI restriction endonuclease site of pFI2451, which yielded pFI2452.

TABLE 1. *cluA* mutation plasmids generated during this study (plasmids resulting from *cluA* insertional mutations in pFI2453)

Plasmid	Nucleotide preceding the insertion	CluA amino acid[a]	Pentapeptide sequence[b]
pFI2454	A6	K2	M **K C V H M** K
pFI2455	C28	L10	L **L C T Q L** L
pFI2456	T357	Y119	D **Y C V H N** Y
pFI2457	C374	T125	S **T V Y T S** T
pFI2458	G457	D153	D **V C T Q D** D
pFI2459	G798	M266	Q **M C V H K** M
pFI2460	A841	M281	I **M C T Q T** M
pFI2461	G979	V327	T **V C T Q T** V
pFI2462	A1080	Q360	N **Q C V H N** Q
pFI2463	C1081	H361	H **L C T H L** H
pFI2464	T1200	S400	W **S C V H R** S
pFI2465	G1229	W410	T **V V Y T T** W
pFI2466	T1448	I483	P **I V Y T P** I
pFI2467	C1453	P485	P **P V Y T P** P
pFI2468	A1557	L519	A **L C V H T** L
pFI2469	C1748	T583	L **T V Y T L** T
pFI2470	A1778	N593	M **N V Y T M** N
pFI2471	A1814	E605	K **D V Y T K** E
pFI2472	G2092	A698	E **V C T Q D** A
pFI2473	G2200	A734	D **V C T Q D** A
pFI2474	T2342	M781	A **I V Y T P** M
pFI2475	A2513	D838	P **I V Y T P** M
pFI2476	C2573	T858	Y **T V Y T Y** T
pFI2477	A2735	K914	G **K C V H S** K
pFI2478	G2879	G960	T **G V Y T T** G
pFI2479	A3125	K1042	S **N V Y T S** K
pFI2480	A3247	T1083	D **M C T H D** T
pFI2481	T3370	L1124	T **L C T Q T** L
pFI2482	A3423	T1141	S **T C V H T** T
pFI2483	C3470	A1157	E **A V Y T E** A
pFI2484	C3650	A1217	A **A V Y T A** A

[a] The designation of an insertional mutation indicates the amino acid residue encoded by the codon that is disrupted by the insertion or that precedes it.
[b] Amino acid sequence of the inserted pentapeptide deduced from the DNA sequence of the mutated *cluA* gene. Pentapeptide sequences are in bold.

DNA manipulation, PCR, and sequencing. General molecular cloning techniques and electrophoresis of DNA on agarose gels were carried out essentially as described by Sambrook et al. (27). The PCRs were performed using the BIO-X-ACT proofreading polymerase as described by the manufacturer (Bioline). Primers LmaB (GATGAATTCTTTAGTGTGTACACACAATAAAGT TGGGAACTG) having an EcoRI site at its 5′ end and RmaB (GATCTCGGG CTAAATGTGTACACACAATAAAGTCTTAAACTG) having an AvaI site at its 5′ end were used to replace the PmeI sites of pGSP4 by PCR-directed mutagenesis (BsrGI restriction endonuclease sites are underlined in both primers). Nucleotide sequencing was performed by the dideoxy chain termination method, using fluorescent dye terminators and cycle sequencing reactions. Samples were analyzed with an Applied Biosystems 373A DNA sequencer.

Site-directed mutagenesis of *cluA*. PCR mutagenesis was performed with the *cluA* gene by using the BIO-X-ACT proofreading polymerase (Bioline) to replace charged amino acids with alanine residues in CluA. Two sets of primers were used to amplify two *cluA* fragments from MG1363 chromosomal DNA. A primer upstream of the charged amino acid cluster was paired with a mutagenic oligonucleotide to hybridize at the region within *cluA* at which the mutation occurred. The mutagenic primer contained the sequence specifying the bases for the alanine substitutions. A second PCR was performed by using a mutagenic oligonucleotide that was complementary to the oligonucleotide used in the first amplification paired with a primer downstream from the desired substitution. The two PCR products were mixed and used as a template in crossover PCR (24), using the two external primers to generate a recombined PCR fragment

TABLE 2. Sex factor DNA transfer abilities after induced expression of the *cluA* variants[a]

Plasmid with *cluA* variant	*cluA* allele[b]	CluA amino acid substitutions	Conjugation efficiency (10^{-4} transconjugant/donor)	
			Uninduced	Induced
pFI2213	Wild type		1.3 ± 0.2	35.4 ± 3.6
pFI2560	Tra1	K784A, E785A	1.7 ± 0.3	12.4 ± 2.9
pFI2561	Tra2	D838A, D840A	1.0 ± 0.2	2.7 ± 0.5
pFI2562	Tra3	K915A, K916A, E917A	1.0 ± 0.2	6.0 ± 1.3
pFI2563	Tra4	D868A, K869A, E870A	1.3 ± 0.1	12.1 ± 2.7
pFI2564	Tra5	K1053A, D1054A, E1055A, K1056A	1.1 ± 0.4	6.2 ± 0.7

[a] The different *L. lactis* donor strains containing a tetracycline resistance marker integrated into the *cluA* deleted sex factor were grown in the presence or absence of a nisin inducer. The cells were then mixed with a recipient strain, and the frequency of conjugation was assessed as described in Materials and Methods. The values are averages ± standard deviations for three independent assays.
[b] Different alleles of *cluA* carried by an expression vector in the *L. lactis* donor strains.

containing the alanine substitutions. The resulting PCR fragment was then digested using restriction enzymes whose sites were incorporated into the external primers. The fragment was cloned into the *cluA* gene carried by the pFI956 plasmid, a pBluescript SK(−) vector containing the *cluA* gene (29), to replace the wild-type *cluA* internal fragment. The fragments were sequenced in order to verify that no misincorporation had occurred in the DNA sequence during PCR amplification. The *cluA* gene containing the point mutations was excised with SmaI and XhoI and cloned into the pFI2209 expression vector (29). The five resulting plasmids are listed in Table 2.

Transposition reactions. The in vitro transposition reaction was performed with pFI2452 using the conditions described for the GPS-LS linker-scanning system (New England Biolabs) by the manufacturer. One microliter of the transposition reaction was used to transform *E. coli* TG1. The pBR322-based pFI2452 donor plasmid was digested prior to the transposition reaction with both the PvuI and PvuII restriction enzymes, each of which cut once in the plasmid outside the mini-Tn*7* BsrGI site to prevent replication in *E. coli*. A 0.02-μg portion of the pFI2452 digested donor DNA and 0.08 μg of the pFI2453 target DNA were used in the transposition reaction. After transformation of *E. coli* with the transposition reaction mixture DNA, transposition events were selected on plates containing erythromycin. A pool of plasmids extracted from 400 colonies was transformed into *L. lactis* FI9979, and the transformants were selected with erythromycin.

Nisin induction and quantification of the aggregation level. Appropriate dilutions of nisin (Aplin & Barret, Ltd.) were prepared for induction of the bacterial strains. One milliliter of an overnight cell culture grown in the presence of 50 ng/ml nisin was loaded into a 48-well microtitration plate to screen for *L. lactis* transformants containing mutated variants of pFI2213 that did not exhibit aggregation. The plate was then inverted several times until aggregation was visible. For a quantitative comparison of aggregation by the 30 different insertion mutants, a 1.5-ml overnight culture of the strains in SM17 containing 50 ng/ml of nisin was vortexed for 1 min to trigger aggregation and left at room temperature for 1 h, which allowed the aggregates to sediment. A 100-μl sample was then taken from the top (containing culture media with nonaggregated cells) and transferred to a 96-well microtitration plate. A 100-μl sample of the same culture that had not been vortexed was transferred to the same plate. The optical density at 600 nm (OD_{600}) of each sample was determined simultaneously using a THERMOmax microplate reader (Molecular Devices). The optical density of each mutant in both conditions was determined three times independently. The level of aggregation was determined using the following formula: $A\% = 100 - (OD_v/OD_{nv} \times 100)$, where A% is the percentage of aggregation, OD_v is the OD_{600} of the culture that had been vortexed, and OD_{nv} is the OD_{600} of the same culture that had not been vortexed.

Surface extraction and Western immunoblotting. A lysozyme surface extract of each strain was obtained as previously described (29). Briefly, the pellet from a 10-ml culture in SM17 was resuspended in a solution containing 50 mM Tris-HCl (pH 8), 0.1× protease inhibitor cocktail set II (Calbiochem), 10% sucrose, and 25 mg/ml lysozyme. The OD_{600} of the suspension was adjusted to 10, and the suspension was shaken gently at room temperature for 1 h and centrifuged at 3,500 rpm. Twenty microliters of the supernatant was loaded on a NuPage (Invitrogen) sodium dodecyl sulfate-polyacrylamide gel for electrophoresis. The amount of protein for each sample was checked by quantifying the signals using the TotalLab software (Nonlinear Dynamics) after staining of the gel with colloidal blue stain (Invitrogen). Western blot analyses were performed using a Western Breeze chromogenic kit by following the manufacturer's instructions (Invitrogen). Polyclonal antibodies raised against CluA (29) were used as primary antibodies. Quantification of the signals detected was performed using the TotalLab software.

Sex factor transfer. The FI9983 donor strain (tetracycline resistant) harboring plasmids carrying wild-type *cluA*, the different *cluA* insertion mutations, or the *cluA* point mutations or the strain carrying only the vector was grown overnight and diluted 100-fold in SM17 containing 5 μg/ml of chloramphenicol and 50 ng/ml of nisin. Recipient strain FI9980 (rifampin resistant) was grown under the same conditions but in the absence of nisin. Cell mating was triggered by centrifugation of a mixture of donor and recipient strains grown to the same optical density and not by vortexing as previously described (29). The conjugation efficiencies of the different strains were determined for donor and recipient cells grown to an OD_{600} of 0.8. Then 0.1 ml of donor cells and 0.9 ml of recipient cells were mixed, and the mixture was centrifuged at 5,000 rpm for 5 min. The pellet was resuspended in 1 ml of GM17 for all donor strains. Each mixture was incubated for 1 h at 30°C. Cells were then diluted and plated on GM17 agar containing rifampin (for recipient and transconjugant selection) or tetracycline (for donor and transconjugant selection) or both antibiotics (for transconjugant selection). The conjugation frequency for each donor was determined at least three times independently.

Statistical analysis. Statistical significance was calculated by determining confidence intervals for the differences of two population means when population variances were known. Cluster analysis was carried out to classify the strains on the basis of sex factor transfer level and aggregation. Hierarchical clustering (35) was used to determine the optimum number of clusters. The mean values for these clusters were used as seeds for a nonhierarchical cluster analysis (k-means) in order to optimize the classification of the strains. Statistical differences ($P < 0.05$) in the sex factor transfer level and/or level of aggregation between the three clusters were assessed by the Student-Newman-Keuls test for comparison of multiple means.

RESULTS

Construction of a CluA insertion library using an in vitro transposon mutagenesis system producing only full-length proteins. A transposon-based technique was used to define the regions of the CluA protein that are important for aggregation and the regions that may contribute to DNA transfer. To do this, we first engineered the mini-Tn*7* PmeI element included in the GPS-LS linker-scanning system (New England Biolabs) to make it suitable for the study of CluA. This minitransposon is flanked by two PmeI restriction endonuclease sites that result in one-third of the proteins being truncated after mutagenesis. We therefore decided to replace this restriction endonuclease site with a BsrGI site using primers LmaB and RmaB (see Materials and Methods). Introduction of a BsrGI site involved changing the R4 and R7 residues of the terminal inverted repeats of the mini-Tn*7* element, and these nucleotides could be altered without affecting the transposition activity (2). Furthermore, the resistance genes used to select the transposition events in the mini-Tn*7* PmeI system are suitable for use only in gram-negative bacteria. Consequently, we used a gram-positive erythromycin resistance gene that was also functional in *E. coli* to replace the chloramphenicol resistance cassette flanked by the Tn*7* terminal recombination sequences in mini-Tn*7* PmeI. Construction of the Ery^r mini-Tn*7* BsrGI system carried by donor plasmid pFI2452 is described in detail in Materials and Methods. The new Ery^r mini-Tn*7* BsrGI system led, after excision of the mini-Tn*7* element using BsrGI, to

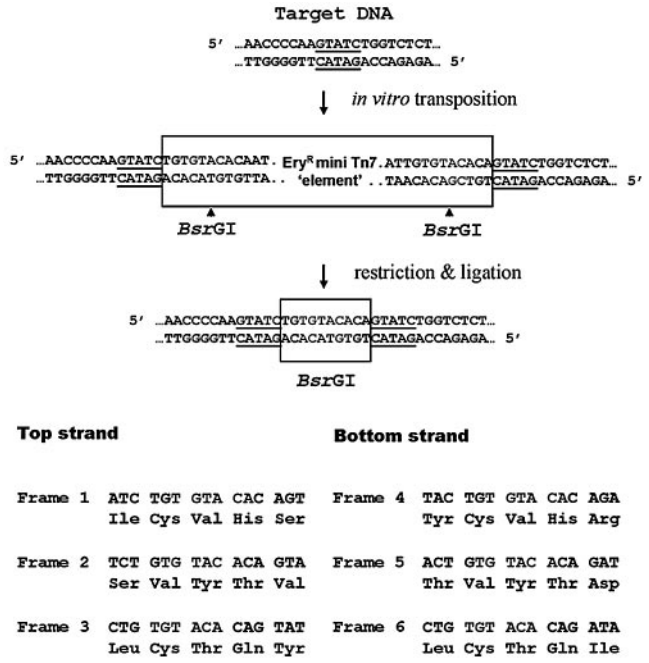

FIG. 1. Use of the newly developed mini-Tn*7* element in the PSM procedure. In vitro transposition is performed using the TnsABC^{225V} transposase and the Ery^r mini-Tn*7* BsrGI element. The sequence of the target DNA indicated is arbitrary. The mini-Tn*7* element inserts at random locations into the target DNA. The repeated sequence of the target DNA is underlined. The gray letters indicate BsrGI restriction endonuclease sites. BsrGI digestion removes all but 10 bp of the mini-Tn*7* element. Ligation creates a 15-bp insertion which includes a unique BsrGI site. The six possible reading frames resulting from target DNA translation are shown.

insertion of five amino acids in all six frames (Fig. 1), thus avoiding production of truncated proteins.

Independent 15-bp in-frame insertions located throughout *cluA* were obtained after use of the Ery^r mini-Tn*7* BsrGI element in a PSM experiment. For this, the new donor plasmid pFI2452 was used in an in vitro transposition reaction with the target plasmid pFI2453, a derivative of the pFI2213 plasmid carrying the wild-type *cluA* gene under control of the P_{nisA} nisin promoter (29) in which the unique BsrGI site was removed. The transposition events were selected in *E. coli* using erythromycin, and a pool of plasmids extracted from 400 colonies was transformed into *L. lactis* FI9979, a suitable strain for nisin induction and CluA aggregation assessment. To select for transposon insertions into the *cluA* gene, the aggregation abilities of 400 independent clones were tested, and 152 transformants were unable to clump. The mini-Tn*7* element was excised from a pool of the 152 corresponding plasmids using BsrGI, and the resulting plasmids were transformed back into FI9979. Of 130 nonaggregating transformants, 58 mutants carried a smaller plasmid with at least the 3′ half of *cluA* missing, resulting from DNA rearrangement events. In previous work, it has been observed that the full-length *cluA* gene is unstable in *E. coli* (29). The plasmids of the other 72 transformants were analyzed by restriction mapping and nucleotide sequencing in the vicinity of each insertion. Mapping of each plasmid revealed that 10 of the insertions had occurred between the nisin promoter and the ATG start codon of *cluA*. Surprisingly, seven insertions were located downstream and close to the *cluA* terminator. Fifty-five insertions were located in the *cluA* gene coding sequence, and there were 31 independent insertions. The designations of these insertional mutations indicate the amino acid residue encoded by the codon that was disrupted by the insertion or that preceded this codon (Table 1). These insertions were equally distributed along the *cluA* open reading frame (Fig. 2A), showing clearly that the mini-Tn*7* BsrGI element constructed in this work provided random insertions located throughout a target gene.

Consequences of insertions for the stability of CluA and determination of an N-terminal region responsible for cell aggregation. Previous to this study no structural data about CluA were available. We performed a Consensus Secondary Structure Prediction analysis of CluA using the Network Protein Sequence Analysis server (http://npsa-pbil.ibcp.fr) (5), and the results for the predicted alpha-helices are shown in Fig. 2B. In predicted domain I, the 14-amino-acid hydrophobic region (T4 to L17) is likely to be the region of the signal peptide that organizes into an alpha-helical conformation when it is in contact with the membrane lipid phase (34). Domain II consists mainly of alpha-helical structures, and a major structure is located between S75 and I154. Another important helical region is located in domain IV (T583 to Y600), and a final predicted alpha-helix is located downstream from the LP KTGE motif in domain V (A1207 to R1227), corresponding to the membrane-anchoring domain typical of cell wall-anchored proteins (11). Some pentapeptide insertions that occurred in

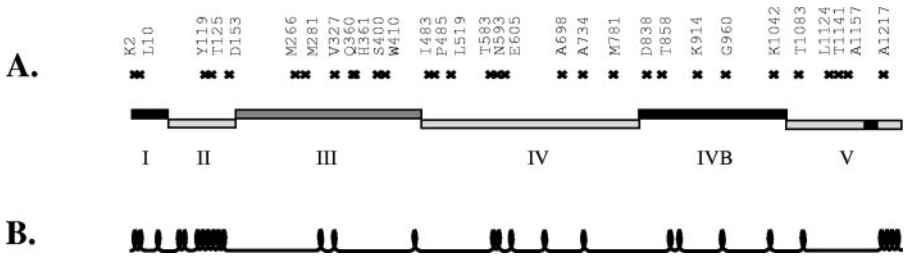

FIG. 2. Map of the 31 individual pentapeptide insertions generated in CluA. (A) Distribution of the 31 insertions in the different domains along the length of CluA. (B) CluA alpha-helical structures were predicted using the Consensus Secondary Structure Prediction software from the Network Protein Sequence Analysis server (http://npsa-pbil.ibcp.fr). Depending on the size, helices are represented by one loop or several successive loops. The black rectangle in domain V represents the LPKTGE motif of CluA, which is specific to cell wall-anchored proteins in gram-positive bacteria.

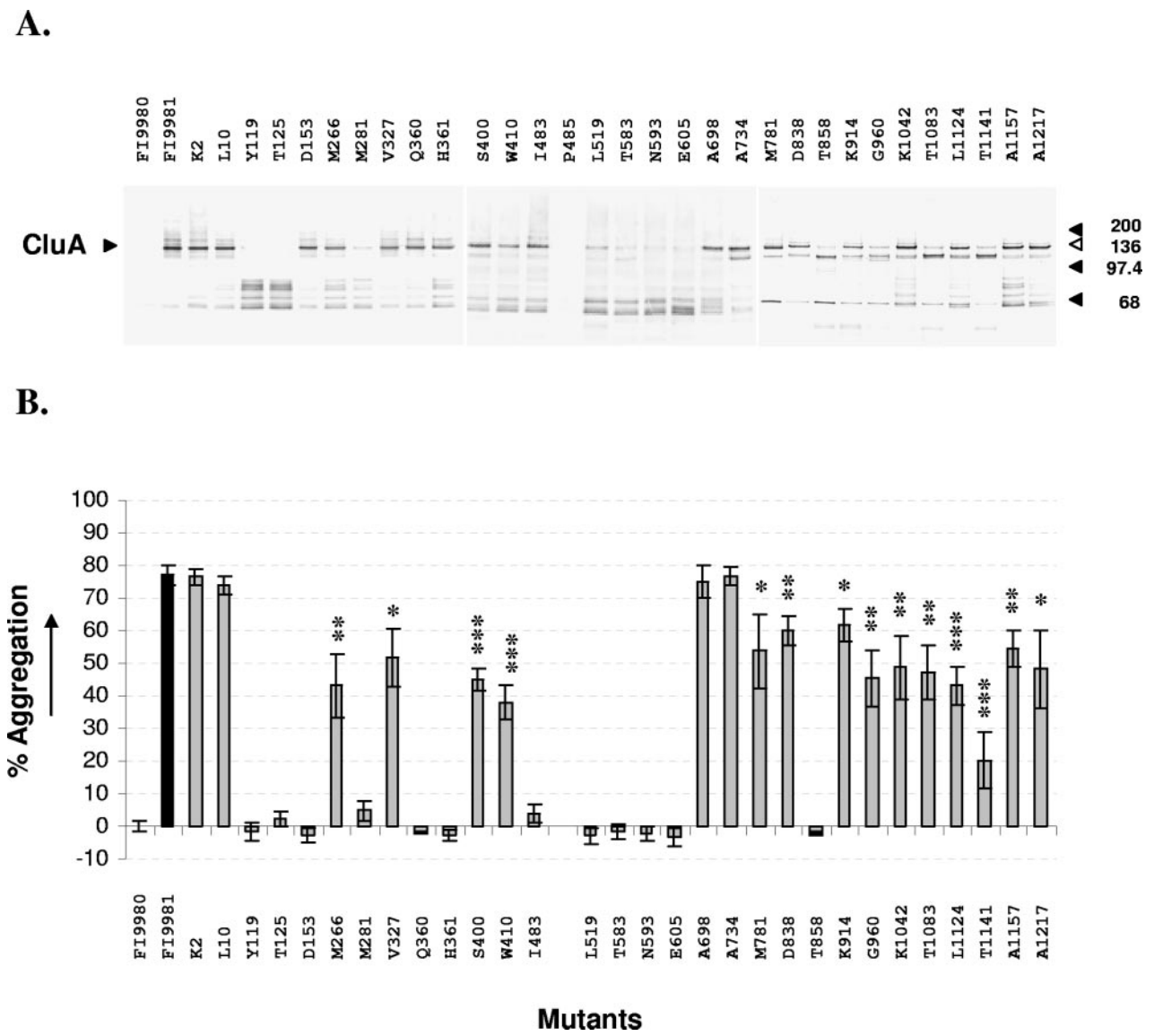

FIG. 3. (A) Immunodetection of CluA expressed at the cell surface of the 31 insertion mutants. Equivalent amounts of protein were loaded in the lanes. FI9980 is the strain containing the vector control; FI9981 expresses CluA$^+$. The position of full-length 136-kDa CluA is indicated on the left. The positions of molecular mass standard marker proteins are indicated on the right. (B) Levels of aggregation of 30 insertion mutants. The absorbance values for the culture media before and after sedimentation of the aggregate were determined (see Materials and Methods for details). The percentage of aggregation was defined as the proportion of cells involved in aggregate formation. FI9980 is the strain containing the vector control; FI9981 expresses CluA$^+$. Three asterisks, $P < 0.01$ for a comparison with FI9981; two asterisks, $P < 0.05$ for a comparison with FI9981; one asterisk, $P < 0.1$ for a comparison with FI9981.

predicted alpha-helical domains of CluA and led to protein instability are described below.

The presence of CluA on the cell surface of the 31 mutants was assessed by immunodetection of CluA following cell surface extraction, as shown in Fig. 3A. The controls were the negative control FI9980, carrying the pFI2209 empty vector, and FI9981, carrying pFI2213 that contained the wild-type *cluA* gene expressed under control of the P_{nisA} nisin promoter. As expected, the cell surface extract of FI9980 contained no reactive CluA protein, whereas wild-type CluA expressed in FI9981 was detected. For most of the insertion mutants, CluA was detected on the Western blot (Fig. 3A). The mutant carrying the P485 mutation contained no CluA and was not used in further experiments. A 136-kDa signal corresponding to the size of CluA was observed for all mutants except the strains carrying the Y119 and T125 mutations. For most of the mutants the total amount of detected signals comprising full-size CluA and its degradation products was similar to the amount of full-size CluA detected in FI9981 (data not shown). Since the extraction protocol released C-terminal cell wall-anchored CluA variants, it is likely that the 30 mutants expressed a full-size protein on the cell surface at a level comparable to that of wild-type CluA in FI9981. The effects of the 30 different insertions on CluA aggregation were quantified (see Materials and Methods), and the results are shown in Fig. 3B.

Aggregation was completely prevented for Y119 and T125 insertions, and this result agrees with the strong protein degradation observed after cell surface protein extraction (Fig. 3A). No signal above 70 kDa was detected for Y119 and T125, and a smear of strong bands migrating between 50 and 70 kDa corresponding to degraded CluA products was observed. This indicates that insertions located at positions Y119 and T125 affected the stability of CluA either in vivo or during the extraction process. According to the secondary structure prediction, these two insertions are located within the major predicted alpha-helical structure of predicted domain II (Fig. 2B), suggesting that this structure is important for protein stability.

For the different CluA mutants in which the 136-kDa product was observed, the insertions affecting aggregation were located mainly in the N-terminal half of the protein in a region bordered by the amino acids D153 and I483. Although the D153, Q360, H361, and I483 mutants showed little or no protein degradation after surface protein extraction (Fig. 3A), they were unable to aggregate (Fig. 3B). Likewise, no protein degradation was observed for the S400 and W410 mutants, whereas their aggregation abilities were significantly reduced ($P < 0.01$). This indicates that the insertions in these six mutants occurred in regions that are important for aggregation and likely to be involved in the control of CluA binding specificity. Although the M281 mutant showed low aggregation ability, the faint signals observed for the 136-kDa product and the degradation products suggest that an insertion at this location led to lower expression of the protein on the cell surface and/or decreased its stability.

The low levels of aggregation observed for the four mutants carrying the insertions located at positions L519 to E605 can be explained by the detection of a very weak signal at 136 kDa, whereas strong degradation products were observed around 60 kDa. The three insertions other than L519 are located within (T583 and N583) or close to (E605) the T583-Y600 predicted alpha-helical structure, suggesting that this region is also important for CluA stability. Although the mutants with mutations located in the D838-to-A1157 region exhibited reduced aggregation, all the corresponding proteins exhibited some degradation. In particular, the major product observed for the T858, T1083, and T1141 insertions was a 120-kDa CluA degradation product (Fig. 3A). This suggests that part of the C-terminal region is involved in aggregation and/or that the stability of the protein is affected by these insertions. In summary, the analysis of the insertions revealed a CluA domain that is responsible for cell-to-cell binding, including amino acids D153 to I483.

Definition of a CluA domain involved in transfer of the sex factor. In *L. lactis*, expression of CluA in the recipient strain was unable to complement DNA transfer of the sex factor from a donor strain lacking a *cluA* functional gene, whereas complementation took place when CluA was expressed in a *cluA* donor strain (29). This observation led to the conclusion that in addition to promoting cell-to-cell contact, CluA is involved in the sex factor DNA transfer machinery and that an association of CluA with one component or several components is necessary to enable DNA transfer.

It was anticipated that the region of the CluA protein associated with DNA transfer was different from the region involved directly in aggregation. The conjugation efficiencies of the mutants that were still able to aggregate were assessed to discriminate between potential insertions that affect this functional domain. The 17 plasmids carrying CluA mutations that were still able to promote aggregation (aggregation, >35%) were transformed into the FI9983 donor background strain containing a chromosomal *cluA* deletion. The sex factor transfer efficiencies were determined for the strain containing the empty expression vector (FI9984), the expression vector harboring the wild-type *cluA* gene (FI9985), and the 17 mutated plasmids. Since enhanced DNA transfer seems to be dependent on aggregation ability (12, 13, 29, 33) (see above) (i.e., on the ability of the donor strain to promote cell-to-cell contact), transfer efficiency data are presented in relation to aggregation efficiency in Fig. 4. We performed a cluster analysis using Ward's method (35) and identified three clusters of insertion mutants (Fig. 4). The first cluster (cluster 1) comprised the insertion mutants in which there was little effect on aggregation or sex factor transfer and which acted like the FI9985 CluA control. Mutants in this cluster exhibited the highest level of aggregation (76%) and the highest sex factor transfer value (80.4×10^{-4} transconjugant/donor cell on average), and both values were significantly different from the values for clusters 2 and 3. The second cluster (cluster 2) contained the CluA variants that exhibited a lower level of aggregation, which led to a lower sex factor transfer value. This was the result predicted if sex factor transfer relies solely on the level of aggregation. The third cluster (cluster 3) contained the insertion mutants that had intermediate levels of aggregation but sex factor transfer values that were lower than those that were predicted. There were no significant differences in the aggregation values between clusters 2 and 3 (ca. 48 to 49%),

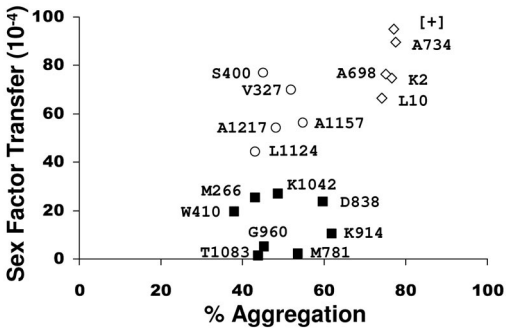

FIG. 4. Cluster analysis of DNA transfer efficiency in relation to aggregation: sex factor transfer of different insertion mutants in relation to aggregation ability. The sex factor transfer efficiency of strain FI9985 containing wild-type *cluA* ([+]) and of each mutant was expressed as the number of transconjugants per donor. The conjugation frequency for each donor was determined three times independently, and the standard deviation for each donor was less than 25% of the mean. The *P* values were as follows: $P < 0.001$ for a comparison with FI9985 for mutants M781 and T1083; $P = 0.001$ for a comparison with FI9985 for mutants K914 and G960; and $P < 0.01$ for a comparison with FI9985 for mutants M266, W410, D838, and K1042. A cluster analysis using Ward's method allowed the mutants to be classified in three clusters. Cluster 1 (◇) contained the strains that exhibited high transfer efficiencies and high levels of aggregation; cluster 2 (○) contained the strains that exhibited intermediate transfer efficiencies and intermediate levels of aggregation; and cluster 3 (■) contained the strains that exhibited low transfer efficiencies and intermediate levels of aggregation.

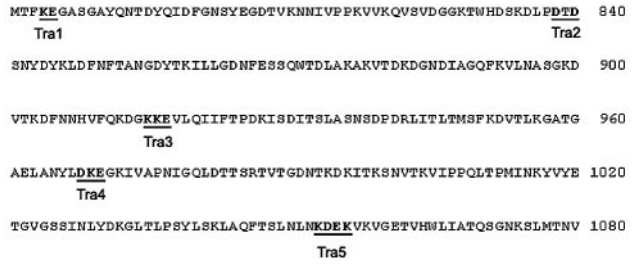

FIG. 5. CluA mutations: amino acid sequence of the M780-V1080 CluA region. Selected amino acids (boldface type) were replaced with alanine as described in Materials and Methods.

whereas the strains belonging to cluster 3 exhibited the lowest sex factor transfer efficiency, 14×10^{-4} transconjugant/donor cell and this value was significantly different from the transfer efficiency detected in cluster 2 (60.2×10^{-4} transconjugant/donor cell). The low transfer frequency observed in cluster 3 strains shows that these insertion mutants were affected in regions of CluA that play an important role in CluA's DNA transfer capacity. In this cluster, six of eight mutations affecting CluA were located in a region covering the 40 amino acids of the C-terminal end of predicted domain IV and in domain IVB (Fig. 2) specific for CluA. The M266 and W410 mutants had mutations that were located in the predicted variable domain III, and these two areas of CluA are also likely to be involved in DNA transfer.

Site-directed mutagenesis of the CluA Tra domain. To investigate further and refine the region of CluA involved in DNA transfer, we introduced point mutations in this part of the protein. In their study of RbsT's activity, a protein involved in stress response in *Bacillus subtilis* (38), Woodbury and coworkers replaced clusters of charged amino acids located throughout the length of the protein with alanine residues. Charged amino acids were chosen because they are residues that are likely to be involved in protein-ligand interactions (39) with a high probability that they are surface exposed and, consequently, less likely to affect the protein's general stability (38).

An analysis of charged amino acids groups in the entire CluA protein showed that these residues are the residues that are most frequently found in the transfer domain. Five clusters of charged amino acid residues located along the length of the CluA transfer region were found (Fig. 5). The charged amino acids at the sites were replaced by the uncharged amino acid alanine by using a PCR-based mutagenesis technique (see Materials and Methods for details). The five plasmids containing the five different sets of mutations are listed in Table 2. The alanine clusters in the transfer region of the five CluA mutants were designated Tra1 to Tra5 according to the orientation from the N terminus to the C terminus. The plasmids were used to transform FI9979, and the aggregation abilities of the resulting strains were assessed. All the strains exhibited levels of aggregation similar to that of the strain carrying the wild-type *cluA* plasmid (data not shown). The conjugation efficiency of each *cluA* variant expressed in the FI9983 donor strain was determined (Table 2), and in parallel, Western blotting was performed with cell surface extracts of the different donors (data not shown). The signals detected showed that the protein abundance and apparent size were similar to those of the wild type. Compared to the donor strain expressing wild-type CluA, the donor strains containing the Tra alleles were all affected in the DNA transfer capacity. The strain containing the mutant allele Tra2 had a 13-fold-lower DNA efficiency than the wild type, strains containing Tra3 and Tra5 had a 6-fold-lower DNA efficiency, and strains containing Tra1 and Tra4 had a 3-fold-lower transfer efficiency. The results for noninduced cells are also shown in Table 2, and the conjugation frequencies were similar to the conjugation frequency obtained for the same donor strain lacking *cluA*. Overall, the results showed clearly that charged amino acids belonging to the newly defined CluA transfer (Tra) domain bordered by amino acids K784 and K1056 are involved in the ability of CluA to transfer DNA, in addition to triggering cell-to-cell contact during conjugation.

DISCUSSION

The functional analysis of 30 *cluA* insertion mutants generated in this study identified two regions of the CluA protein with distinct functions: the D153-I483 region of CluA that mediates bacterial aggregation and the K784-K1056 Tra domain that is involved in DNA transfer and is responsible for a high conjugation frequency.

To define the regions of CluA important in promoting cell-to-cell binding, we determined and compared the levels of aggregation of the 30 *cluA* insertion mutants. Six of the CluA variants showed complete or partial inhibition of aggregation compared to the wild-type CluA strain (Fig. 3). These mutants defined a region extending from amino acid D153 to amino acid I483. This D153-I483 region includes predicted variable domain III of CluA, and this is in agreement with the two aggregation domains identified in Asc10 of *E. faecalis*, the K156-A358 region (37) that covers the N-terminal half of the Asc10 variable domain and the Q473-L683 region (36) that includes its C-terminal third. Furthermore, a 92-amino-acid region in the variable domain of the *E. faecalis* Asa1 aggregation substance has also been shown to play a dominant role in cell aggregation (25).

The CluA D153-I483 aggregation domain might extend farther into predicted domain IV as downstream from I483, four insertions (L519, T583, N593, and E605) were also affected in aggregation. However, the variant CluA proteins exhibited a degraded protein pattern, and it is not clear whether this occurred during extraction or in vivo, leaving the question of whether these insertions have a direct effect on aggregation open.

Five insertions in the C-terminal part of CluA (D838 to A1157) partially impaired CluA's aggregation capacity, and this region is likely to play a role in aggregation. In a similar way, the C-terminal domain of Asc10 has been shown to play an essential role in aggregation, and specific regions that bring the N-terminal functional domain into the correct conformation to mediate aggregation have been identified (36).

It is not known how the CluA protein interacts with the lactococcal cell surface to cause aggregation. In *E. faecalis* aggregation is mediated by a carbohydrate recognition process in which the lipoteichoic acid, a surface molecule that contains sugar residues, is the potential bacterial receptor (32, 37). Interestingly, the L238-V486 region of CluA exhibits signifi-

cant (E-value, 2×10^{-4}) homology (24% sequence identity) with the L554-I750 region of the central domain (21) of the SspB protein (7), a member of the streptococcal antigen I/II family of protein adhesins that recognize salivary agglutinin glycoprotein. The structure of this domain in *Streptococcus mutans* antigen I/II has been determined, and the results revealed its potential to bind sugars (31). Overall, these results suggest that CluA might also recognize and bind specific sugar motifs presented on the surface of some *L. lactis* strains (33), including MG1363.

Aggregating CluA mutants were tested for the ability to transfer the sex factor. All insertions located in the Tra domain, unique to CluA compared to other gram-positive cell surface protein homologues (16), were affected in sex factor transfer. The importance of this region was confirmed by replacement of charged amino acids located along the length of the Tra domain, which showed that these amino acids are essential for optimized DNA transfer. It is known that electrostatic interactions provided by charged amino acids at the binding interface play an important role in protein-ligand interactions (39). Sex factor transfer could be the result of a protein-ligand association involving the Tra domain of CluA and another component of the conjugation machinery, the nature of which still needs to be determined. A sequence database search with BLAST (1) using the Tra domain as a probe gave no significant homologies with other proteins.

Additional areas of CluA are essential for sex factor transfer. A decreased level of sex factor transfer was observed with the M266 and W410 insertions. These two positions in the CluA N terminus might play a role in DNA transfer by interacting with a component of the DNA transfer machinery or by directly or indirectly interacting with the CluA Tra domain to optimize DNA transfer.

Surprisingly, in mutants exhibiting similar levels of aggregation (Fig. 3), the sex factor transfer can vary, as observed for the S400 mutant, whose sex factor transfer was four times higher than that of the W410 mutant. The difference occurred despite the closeness of the two insertions, as S400 and W410 are only 9 amino acids apart. It is likely that the amino acid sequences SCVHR inserted into S400 and VVYTT inserted into W410 (Table 1) disrupted the protein differently, thus leading to noticeable differences in the capacity of the mutated CluA to transfer DNA.

This is the first report of a protein involved in conjugation that actively contributes to DNA transfer and also mediates contact between donor and recipient strains. To achieve this, we developed a mutagenesis tool adapted to the structural and functional analysis of CluA. The newly developed miniTn*7* BsrGI transposon has potential for use in diverse protein analyses in which full-length mutated proteins are required. Furthermore, the use of the erythromycin resistance gene to select miniTn*7* insertions makes it an appropriate tool for protein studies in diverse species of gram-positive bacteria.

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REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Biery, M. C., F. J. Stewart, A. E. Stellwagen, E. A. Raleigh, and N. L. Craig.** 2000. A simple in vitro Tn*7*-based transposition system with low target site selectivity for genome and gene analysis. Nucleic Acids Res. **28:**1067–1077.
3. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:**93–113.
4. **Caillot, F., C. Carlier, and P. Courvalin.** 1987. Physical analysis of the conjugative shuttle transposon Tn*1545*. Plasmid **17:**58–60.
5. **Combet, C., C. Blanchet, C. Geourjon, and G. Deléage.** 2000. NPS@: Network Protein Sequence Analysis. Trends Biochem. Sci. **25:**147–150.
6. **Craig, N. L.** 1996. Transposon Tn*7*. Curr. Top. Microbiol. Immunol. **204:** 27–48.
7. **Demuth, D. R., Y. Duan, W. Brooks, A. R. Holmes, R. McNab, and H. F. Jenkinson.** 1996. Tandem genes encode cell-surface polypeptides SspA and SspB which mediate adhesion of the oral bacterium *Streptococcus gordonii* to human and bacterial receptors. Mol. Microbiol. **20:**403–413.
8. **Demuth, D. R., E. E. Golug, and D. Malamud.** 1990. Streptococcal-host interactions. Structural and functional analysis of a *Streptococcus sanguis* receptor for a human salivary glycoprotein. J. Biol. Chem. **265:**7120–7126.
9. **Dodd, H. M., N. Horn, W. C. Chan, C. J. Giffard., B. W. Bycroft, G. C. Roberts, and M. J. Gasson.** 1996. Molecular analysis of the regulation of nisin immunity. Microbiology **142:**2385–2392.
10. **Dower, W. J., J. F. Miller, and C. W. Ragsdale.** 1988. High efficiency transformation of *Escherichia coli* by high voltage electroporation. Nucleic Acids Res. **16:**6127–6145.
11. **Fischetti, V. A., V. Pancholi, and O. Schneewind.** 1990. Conservation of a hexapeptide sequence in the anchor region surface proteins of Gram-positive cocci. Mol. Microbiol. **4:**1603–1605.
12. **Galli, D., F. Lottspeich, and R. Wirth.** 1990. Sequence analysis of *Enterococcus faecalis* aggregation substance encoded by the sex-pheromone plasmid pAD1. Mol. Microbiol. **4:**895–904.
13. **Gasson, M. J., and F. L. Davies.** 1980. High-frequency conjugation associated with *Streptococcus lactis* donor cell aggregation. J. Bacteriol. **143:**1260–1264.
14. **Gasson, M. J., S. Swindell, S. Maeda, and H. M. Dodd.** 1992. Molecular rearrangement of lactose plasmid DNA associated with high-frequency transfer and cell aggregation in *Lactococcus lactis* 712. Mol. Microbiol. **6:**3213–3223.
15. **Gibson, T. J.** 1984. Studies on the Epstein-Barr virus genome. Ph.D. thesis. Cambridge University, Cambridge, United Kingdom.
16. **Godon, J. J., K. Jury, C. A. Shearman, and M. J. Gasson.** 1994. The *Lactococcus lactis* sex-factor aggregation gene *cluA*. Mol. Microbiol. **12:**655–663.
17. **Hallet, B., D. J. Sherratt, and F. Hayes.** 1997. Pentapeptide scanning mutagenesis: random insertion of a variable five amino acid cassette in a target protein. Nucleic Acids Res. **25:**1866–1867.
18. **Hayes, F., and B. Hallet.** 2000. Pentapeptide scanning mutagenesis: encouraging old proteins to execute unusual tricks. Trends Microbiol. **8:**571–577.
19. **Holo, H., and I. F. Nes.** 1995. Transformation of *Lactococcus* by electroporation. Methods Mol. Biol. **47:**195–199.
20. **Horinouchi, S., and B. Weisblum.** 1984. Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibiotics. J. Bacteriol. **150:**804–814.
21. **Jenkinson, H. F., and D. R. Demuth.** 1997. Structure, function and immunogenicity of streptococcal antigen I/II polypeptides. Mol. Microbiol. **23:** 183–190.
22. **Kao, S. M., S. B. Olmsted, A. S. Vilksnins, J. C. Gallo, and G. M. Dunny.** 1991. Molecular and genetic analysis of a region of plasmid pCF10 containing positive control genes encoding surface proteins involved in pheromone-inducible conjugation in *Enterococcus faecalis*. J. Bacteriol. **173:**7650–7664.
23. **Kelly, C., P. Evans, L. Bermeier, S. F. Lee, A. Progulske-Fox, A. C. Harris, A. Aitken, A. S. Bleiweis, and T. Lehner.** 1989. Sequence analysis of the cloned streptococcal cell surface antigen I/II. FEBS Lett. **1:**127–132.
24. **Link, A. J., D. Phillips, and G. M. Church.** 1997. Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. J. Bacteriol. **179:**6228–6237.
25. **Muscholl-Silberhorn, A.** 1998. Analysis of the clumping-mediating domain(s) of sex pheromone plasmid pAD1-encoded aggregation substance. Eur. J. Biochem. **258:**515–520.
26. **Olmsted, S. B., S. M. Kao, L. J. van Putte, J. C. Gallo, and G. M. Dunny.** 1991. Role of the pheromone inducible surface protein Asc10 in mating aggregate formation and conjugal transfer of the *Enterococcus faecalis* plasmid pCF10. J. Bacteriol. **173:**7665–7672.
27. **Sambrook, J., and D. W. Russel.** 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

28. **Stellwagen, A. E., and N. L. Craig.** 1997. Gain-of-function mutations in TnsC, an ATP-dependent transposition protein that activates the bacterial transposon Tn*7*. Genetics **145:**573–585.
29. **Stentz, R., K. Jury, T. Eaton, M. Parker, A. Narbad, M. Gasson, and C. Shearman.** 2004. Controlled expression of CluA in *Lactococcus lactis* and its role in conjugation. Microbiology **150:**2503–2512.
30. **Terzaghi, B. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci. Curr. Microbiol. **7:**245–250.
31. **Troffer-Charlier, N., J. Ogier, D. Moras, and J. Cavarelli.** 2002. Crystal structure of the V-region of *Streptococcus mutans* antigen I/II at 2.4 Å resolution suggests a sugar preformed binding site. J. Mol. Biol. **318:**179–188.
32. **Trotter, K. M., and G. M. Dunny.** 1990. Mutants of *Enterococcus faecalis* deficient as recipients in mating with donors carrying pheromone-inducible plasmids. Plasmid **24:**57–67.
33. **van der Lelie, D., F. Chavarri, G. Venema, and M. J. Gasson.** 1991. Identification of a new genetic determinant for cell aggregation associated with lactose plasmid transfer in *Lactococcus lactis*. Appl. Environ. Microbiol. **57:**201–206.
34. **van Wely, K. H., J. Swaving, R. Freudl, and A. J. Driessen.** 2001. Translocation of proteins across the cell envelope of Gram-positive bacteria. FEMS Microbiol. Rev. **25:**437–454.
35. **Ward, J. H.** 1963. Hierarchical grouping to optimize an objective function. J. Am. Stat. Assoc. **58:**236–244.
36. **Waters, C. M., and G. M. Dunny.** 2001. Analysis of functional domains of the *Enterococcus faecalis* pheromone-induced surface protein aggregation substance. J. Bacteriol. **183:**5659–5667.
37. **Waters, C. M., H. Hirt, J. K. McCormick, P. M. Schlievert, C. L. Wells, and G. M. Dunny.** 2004. An amino-terminal domain of *Enterococcus faecalis* aggregation substance is required for aggregation, bacterial internalization by epithelial cells and binding to lipoteichoic acid. Mol. Microbiol. **52:**1159–1171.
38. **Woodbury, R. L., T. Luo, L. Grant, and W. G. Haldenwang.** 2004. Mutational analysis of RbsT, an activator of the *Bacillus subtilis* stress response transcription factor σ^B. J. Bacteriol. **186:**2789–2797.
39. **Xu, D., S. L. Lin, and R. Nussinov.** 1997. Protein binding versus protein folding: the role of hydrophilic bridges in protein associations. J. Mol. Biol. **265:**68–84.