

## High frequency of conjugation in *Lactobacillus* mediated by an aggregation-promoting factor

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*Lactobacillus plantarum* strain 4B2, which exhibits a strong autoaggregating phenotype, receives the broad-host-range plasmid pAM $\beta$ 1 with conjugation efficiencies as high as  $10^{-2}$  transconjugants per donor using solid matings; broth matings also occur, but at low transfer frequencies. Filter-sterilized spent supernatant of this strain contains a 32 kDa protein that promotes aggregation, and consequently a high frequency of conjugation, in lactic acid bacteria containing  $\alpha$ -1,2-glucose-substituted lipoteichoic or teichoic acids. It appears, therefore, that the substituted lipoteichoic or teichoic acids act as receptors for the aggregation-promoting protein.

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### Introduction

Since the earliest report of conjugal transfer of a drug resistance plasmid into *Lactobacillus casei* (Gibson *et al.*, 1979), there have been several further reports of plasmid transfer mediated by bacterial mating in lactobacilli. Most of the reports deal with the introduction of pAM $\beta$ 1, a broad-host-range conjugative plasmid conferring macrolide resistance (Clewell *et al.*, 1974), into lactobacilli using as donors *Enterococcus faecalis* or *Lactococcus lactis* subsp. *lactis* (Fitzgerald & Gasson, 1988). Successful conjugal transfer of this plasmid into *Lb. casei* (Gibson *et al.*, 1979), *Lb. reuteri* (Vescovo *et al.*, 1983; Tannock, 1987), *Lb. acidophilus* (Vescovo *et al.*, 1983; Luchansky *et al.*, 1989), *Lb. plantarum* (West & Warner, 1985; Shrago *et al.*, 1986) and *Lactobacillus* sp. (Romero & McKay, 1985) has also been described using agar or filter mating. Some difficulties in using lactobacilli as pAM $\beta$ 1 donors have been reported (Gibson *et al.*, 1979; West & Warner, 1985), and deleted forms of pAM $\beta$ 1, which were not able to promote their own transfer, were found in *Lb. casei* transconjugants (Iwata, 1989). Other workers, however, have shown that lactobacilli can act as donors in inter- and intragenic matings on solid media (Cocconcelli *et al.*, 1985; Tannock, 1987; Luchansky *et al.*, 1989), and in the intestinal tract of gnotoxenic mice (Morelli *et al.*, 1988).

Plasmid conjugation in *Lactobacillus* is a low-efficiency process: reported frequencies using agar or filter matings generally range from  $10^{-7}$  to  $10^{-5}$  transconjugants per donor or recipient. A notable exception is *Lb. acidophilus* strain ADH, which acts as a pAM $\beta$ 1 recipient at a frequency of  $10^{-4}$ – $10^{-2}$  (Luchansky *et al.*, 1989). It was also reported, however, that it was not possible to use ADH transconjugants as donors in a second round of matings. Attempts to achieve conjugal transfer in broth were always unsuccessful, a phenomenon typical of the majority of Gram-positive organisms, which mate only on solid surfaces. Two exceptions to this general rule are known: *E. faecalis* (for reviews see Clewell & Weaver, 1989; Dunny, 1990) and *Lactococcus* (reviewed by Gasson, 1990).

Donor strains of *E. faecalis* are induced by small peptides (the so-called sex pheromones), secreted by putative recipient strains, to produce new surface proteins which form an aggregation substance that mediates the visible autoaggregation ('clumping') of the donor cells, or their coaggregation with the recipient strain. Aggregation reactions also require a binding substance, present on the outermost envelopes of both the donor and the recipient, which was tentatively identified as lipoteichoic acid (LTA) anchored to the cell membrane and protruding outside the murein complex (Ehrenfeld *et al.*, 1986).

In *Lactococcus* the high frequency of conjugation is the ultimate result of complex, and not yet completely understood events, starting from an intermolecular rearrangement, taking place during matings, in which plasmid or chromosomal DNA is inserted into the

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Abbreviations: APF, aggregation-promoting factor; EPE, electroendosmosis preparative electrophoresis; LTA, lipoteichoic acid; TA, teichoic acid.

conjugative lactose plasmid. The resulting transconjugant progeny have an increased donor ability and a constitutive cell aggregation phenotype (Gasson, 1990; van der Lelie *et al.*, 1991).

Thus, in Gram-positive bacteria, high-frequency conjugation and cell aggregation are closely associated phenotypes. Auto- and coaggregating lactobacilli have been described (Wadstrom *et al.*, 1987; Reid *et al.*, 1988; Reniero *et al.*, 1990) but no information is available on the conjugation efficiencies of these strains. This report shows that an autoaggregating *Lb. plantarum* strain was able to act as a donor or recipient to pAM $\beta$ 1, with a high efficiency of plasmid transfer, when mated on solid surfaces, and at a low rate when conjugations took place in broth. Our results suggest that cell aggregation and high frequency of conjugation are associated with a secreted protein of 32 kDa, which recognizes, and specifically binds to, kojibiose [*O*- $\alpha$ -D-glucopyranosyl-(1,2)-D-glucose] substitutions on LTAs or teichoic acids (TAs).

## Methods

**Bacterial strains and cultural conditions.** Autoaggregating *Lb. plantarum* 4B2 and *Lb. casei* 5WR were isolated by the authors from the faeces of human newborns, and then classified and maintained in the Bacterial Collection of the Istituto di Microbiologia UCSC, Piacenza, following routine procedures. Other strains used in conjugation experiments were the previously described *Lb. reuteri* DSM 20016 and its pAM $\beta$ 1-carrying derivative (Vescovo *et al.*, 1983), *Lb. plantarum* NCFB 1988 (isolated as C106; characters relevant for this study were described by Adams *et al.*, 1969; Archibald *et al.*, 1969; Knox & Wicken, 1973; Sharpe *et al.*, 1973), *Lb. plantarum* NCFB 343 and *Lb. acidophilus* ATCC 4356. The lactose-negative, pAM $\beta$ 1-containing *Lc. lactis* subsp. *lactis* SH4174 (Gasson & Davies, 1980), the erythromycin-sensitive, spectinomycin-resistant *Lc. lactis* subsp. *lactis* SH4160 (Gasson & Davies, 1980; Cocconcelli *et al.*, 1985) and the plasmid-free *E. faecalis* JH2-2 were also used.

Lactobacilli were grown in MRS (Oxoid) broth or agar, while enterococci were propagated on Brain Heart Infusion (Oxoid); the incubation temperature for both was 37 °C. Anaerobic conditions provided by the Gas Pack System (BBL) were used when required. Lactococci were cultivated on glucose M17 (Terzaghi & Sandine, 1975) at 30 °C.

**Isolation of the aggregation-promoting factor.** The aggregation-promoting factor (APF) was isolated from culture supernatants of *Lb. plantarum* 4B2 using a modification of the method of Tang *et al.* (1989). Briefly, a 100 ml overnight culture of *Lb. plantarum* 4B2 was centrifuged at 3000 g for 15 min and the supernatant filtered through a 0.45  $\mu$ m membrane filter. A concentration step was then performed by dialysis at room temperature for 4 h through a semipermeable membrane tubing, with a 12000 *M*<sub>r</sub> cut-off, against polyethylene glycol (*M*<sub>r</sub> 20000). The concentrated preparation was then precipitated with ammonium sulphate at 70% saturation, resuspended in 5 mM-Tris/HCl buffer pH 6.5, dialysed against the same buffer and reprecipitated with ammonium sulphate at 50% saturation. A further dialysis against

10 mM-Tris/HCl buffer pH 7 was performed as a desalting step. These preparations were analysed by SDS-PAGE according to Laemmli (1970).

Recovery and purification of active APF molecules were achieved by electroendosmosis preparative electrophoresis (EPE) (Curioni *et al.*, 1988, 1989). The electroendosmotic preparative electrophoresis unit ELFE (Genofit, Grand-Lancy, Switzerland) was connected to a detector (model 440; Waters) set at 280 nm and a fraction collector (FC 203; Gilson). The method of Laemmli (1970) was used for the preparation of both the running gel (*T* = 12%) and the stacking gel (*T* = 4%) for the ELFE unit. The sample was loaded on the top of the stacking gel and the fraction collector device was set to obtain one fraction every 10 drops (0.19–0.20 ml). The pooled fractions were then dialysed against 10 mM-potassium phosphate buffer pH 5.8 with several changes for at least 10 hours at 4 °C, to remove SDS. Protein concentration was determined by means of BCA Protein Assay Reagent (Pierce).

**Aggregation tests.** Overnight cultures of appropriate strains were washed with distilled water at least three times, resuspended in the same initial volume of one-quarter-strength (N/4) Ringer solution and incubated at room temperature in the presence of at least 1% (v/v) of freshly prepared sterile culture supernatant or 2–5  $\mu$ g APF ml<sup>-1</sup>. Aggregation was scored positive when clearly visible sand-like particles, formed by the aggregated cells, gravitated to the bottom of the tubes, leaving a clear supernatant, within 2 h.

The role of LTAs in cell aggregation (Ehrenfeld *et al.*, 1986) was evaluated by adding to the aggregation tubes variable amounts of commercially available LTAs (Sigma) isolated from *Streptococcus mutans*, *E. faecalis*, *Bacillus subtilis* and *Staphylococcus aureus*.

To test for a possible pheromone-like aggregation induction effect, 1–10% (v/v) of sterilized culture supernatant or 2–5  $\mu$ g APF ml<sup>-1</sup> was added to the water-washed cells and left in contact for periods from 10 min to 3 h. Cells were then washed again in order to remove the added APF and finally resuspended in N/4 Ringer solution to score the aggregation reaction.

**Mating experiments.** Procedures outlined by Morelli *et al.* (1988) were followed. In brief, mating mixtures were prepared by using equal amounts, of overnight donor and recipient cells, as inferred by optical density values. A 0.2 ml portion of each mixture was spread on a modified MRS agar medium, free of sodium acetate and adjusted to pH 7.4, and incubated at 37 °C in anaerobic conditions for 24 h. For broth matings the mixture was diluted 10-fold in the modified MRS broth and then incubated anaerobically at 37 °C for 24 h. In another set of mating experiments 1–10% (v/v) of filter-sterilized *Lb. plantarum* 4B2 supernatant or 5–10  $\mu$ g APF ml<sup>-1</sup> was added to mating mixtures before plating. At the end of the incubation period, the agar-plated cells were collected in 1 ml one-quarter-strength (N/4) Ringer solution, while for broth matings, cells were collected by centrifugation and resuspended in 1 ml of the same Ringer solution. Dilutions were then plated to select donors, recipients and drug-resistant putative transconjugants, using phenotypes already described (Gasson & Davies, 1980; Vescovo *et al.*, 1983). Lactobacilli were enumerated on Rogosa selective medium (Oxoid), while enterococci were selected on Slanetz & Bartley medium (Oxoid) and lactococci on glucose M17 (GM17). Erythromycin was added, when required, at a concentration of 50  $\mu$ g ml<sup>-1</sup>. To test for the presence of a possible pheromone-like mating substance, cells were treated as described in the aggregation test and then spread on mating plates.

**Identification of the putative transconjugants.** Colonies of the drug-resistant putative transconjugants obtained during the different matings were randomly selected for further characterization. Plasmid profiles were also examined using the alkaline lysis method and gel conditions routinely used in our laboratory (Vescovo *et al.*, 1983).

## Results

### Isolation of the aggregation-promoting factor

Among all the strains studied, only *Lb. plantarum* 4B2 showed a strong autoaggregating phenotype. However, after centrifugation and several washings of the cells with distilled water, the autoaggregation properties were lost and the cells remained evenly dispersed when resuspended in Ringer solution. The observation that the addition of 1–10% (v/v) of freshly prepared filter-sterilized supernatant restored the aggregated phenotype prompted us to investigate the factor(s), present in the supernatant, which were able to promote cell aggregation (Fig. 1).

Filtered supernatant of *Lb. plantarum* 4B2 lost its aggregation-promoting ability when incubated with Pronase E or Proteinase K (both 1 mg ml<sup>-1</sup> final concentration for 30 min at 37 °C), suggesting that APF is a protein or polypeptide. To identify and isolate this substance, supernatants of *Lb. plantarum* 4B2 were concentrated by ammonium sulphate precipitation and dialysed as described in Methods. These preparations retained aggregation-promoting ability. SDS-PAGE of the material revealed two distinct bands with apparent molecular masses of 32 kDa and 29 kDa (Fig. 2). These two proteinaceous bands were separated by means of an EPE system, and the results shown in Fig. 3 demonstrated that it was possible to recover fractions containing pure forms of the two proteins. These samples were dialysed to remove SDS and then tested for their aggregation-promoting properties; only the 32 kDa protein was able to promote aggregation of water-washed cells of *Lb. plantarum* 4B2, *Lb. reuteri* DSM 20016, *Lb. plantarum* NCFB 1988 and *E. faecalis* JH2-2.

### Aggregation properties

The addition of filtered *Lb. plantarum* supernatant or APF to cultures of *Lb. reuteri* DSM 20016, *Lb. plantarum* NCFB 1988 and *E. faecalis* JH2-2 led to a clear aggregation reaction. Lactococci showed a weak aggregation when 10% (v/v) of supernatant was added, while *Lb. casei* 5WR, *Lb. acidophilus* ATCC 4356 and *Lb. plantarum* NCFB 343 did not aggregate when filtered supernatant or APF was added. The presence of pAMβ1 did not influence the aggregation reactions.

Induction tests, in which the supernatant or the APF were removed from the cell suspension after variable contact times led to the conclusion that the presence of these substances was necessary for aggregation to occur. APF was thus unable to promote the formation of new aggregation substance(s), but was itself the clumping agent.

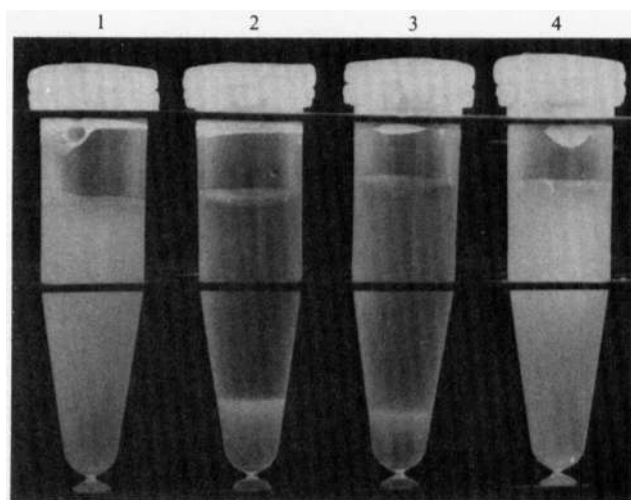


Fig. 1. Aggregation tests with *Lb. plantarum* 4B2. In tube 1, cells were washed three times with water and resuspended in one-quarter-strength Ringer solution. In tube 2, cells treated as in (1) were added to their own filter-sterilized supernatant. In tube (3) *B. subtilis* LTAs (1 mg ml<sup>-1</sup>) were added to cells treated as in (2). In tube 4, *E. faecalis* LTAs (50 µg ml<sup>-1</sup>) were added to cells treated as in (2).

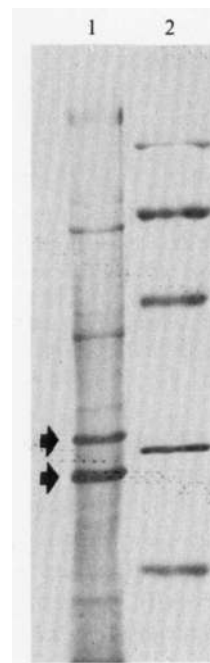


Fig. 2. SDS-PAGE of proteins present in *Lb. plantarum* 4B2 culture supernatants. Lane 1, partially purified preparation; arrows indicate the 32 kDa and 29 kDa proteins. Lane 2, molecular mass markers (rabbit muscle phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 42.7 kDa; bovine carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa. Separating gel, T = 12%; stacking gel, T = 3%.

Table 1. Frequencies of conjugal transfer using solid-surface or broth matings

Donor	Recipient	Frequency* using	
		Solid mating	Broth mating
<i>Lc. lactis</i> subsp. <i>lactis</i> SH 4174 (pAM $\beta$ 1)	<i>Lb. plantarum</i> 4B2	$1.0 \times 10^{-2}$	$3.0 \times 10^{-7}$
	<i>Lb. casei</i> 5WR	$1.5 \times 10^{-7}$	ND
	<i>Lb. reuteri</i> DSM 20016	$5.1 \times 10^{-6}$	ND
	<i>Lb. plantarum</i> NCFB 343	$2.2 \times 10^{-6}$	ND
	<i>Lb. plantarum</i> NCFB 1988	$8.5 \times 10^{-7}$	ND
	<i>Lb. acidophilus</i> ATCC 4356	$2.0 \times 10^{-8}$	ND
<i>Lb. reuteri</i> DSM 20016 (pAM $\beta$ 1)	<i>Lc. lactis</i> subsp. <i>lactis</i> SH 4160	$3.9 \times 10^{-7}$	ND
	<i>Lb. plantarum</i> 4B2	$3.0 \times 10^{-2}$	$5.0 \times 10^{-7}$
	<i>Lb. plantarum</i> NCFB 343	$2.0 \times 10^{-6}$	ND
	<i>Lb. plantarum</i> NCFB 1988	$3.2 \times 10^{-7}$	ND
	<i>E. faecalis</i> JH2-2	$8.2 \times 10^{-6}$	ND
<i>Lb. plantarum</i> 4B2 (pAM $\beta$ 1)	<i>Lc. lactis</i> subsp. <i>lactis</i> SH 4160	$3.0 \times 10^{-5}$	ND
	<i>Lb. reuteri</i> DSM 20016	$5.0 \times 10^{-2}$	$1.2 \times 10^{-7}$
	<i>E. faecalis</i> JH2-2	$4.2 \times 10^{-3}$	$8.1 \times 10^{-7}$
	<i>Lb. plantarum</i> NCFB 343	$8.3 \times 10^{-6}$	ND
	<i>Lb. plantarum</i> NCFB 1988	$1.2 \times 10^{-2}$	$7.2 \times 10^{-5}$

\* Mean frequencies expressed as transconjugants per donor, of at least five different experiments.  
ND, Not detected.

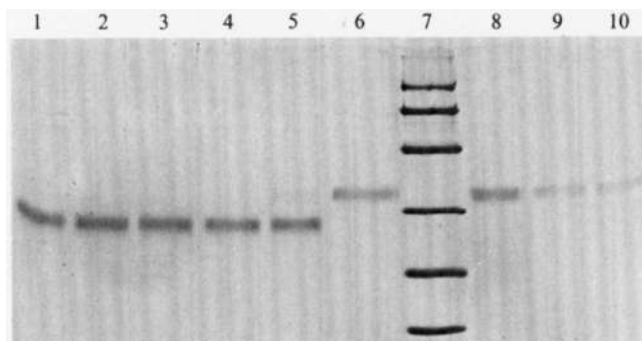


Fig. 3. Purification and separation by EPE (see Methods) of the two major proteins present in *Lb. plantarum* 4B2 supernatant. In lanes 1–4 the 29 kDa protein recovered using EPE is shown, while lanes 6, 8, 9 and 10 demonstrate the recovery of the 32 kDa protein. Lane 5 shows the original preparation containing the two bands. Lane 7 contains molecular mass markers (as in Fig. 2, plus hen egg white lysozyme, 14.4 kDa). Separating gel, T = 15%; stacking gel, T = 3%.

To test the possible role of LTAs in cell aggregation, we added variable amounts of commercially available LTAs to tubes in which aggregation reactions were tested. When  $50 \mu\text{g ml}^{-1}$  (final concentration) of *E. faecalis* LTA was added, together with the sterile spent supernatant or the APF, clumping of cells was not detected in *Lb. plantarum* 4B2, *Lb. reuteri* DSM 20016, *Lb. plantarum* NCFB 1988 or *E. faecalis* JH2-2; in contrast  $1 \text{ mg ml}^{-1}$  of the three other LTAs did not affect the aggregation properties in these strains (see Fig. 1).

#### Mating experiments

Table 1 summarizes mating experiments performed on solid surfaces or in broth cultures. Frequencies higher

than those generally reported were obtained when *Lb. plantarum* 4B2 was the recipient, whatever the donor used. Transfer of pAM $\beta$ 1 was also obtained in the liquid matings, but at low frequencies.

When one *Lb. plantarum* 4B2 transconjugant was used as the pAM $\beta$ 1 donor, high-frequency conjugal transfer was obtained only when recipients sensitive to the APF were used.

The addition of filtered *Lb. plantarum* 4B2 supernatants or the APF to the aggregation-sensitive strains increased the efficiency of conjugation (Table 2).

Induction tests did not influence the frequency of conjugation (data not shown).

Table 2. Frequencies of plasmid transfer in solid-surface matings in the presence of filtered *Lb. plantarum* 4B2 supernatants or APF

Donor	Recipient	Frequency* with	
		Supernatant	APF
<i>Lc. lactis</i> subsp. <i>lactis</i> SH 4174 (pAM $\beta$ 1)	<i>Lb. reuteri</i> DSM 20016	$4.1 \times 10^{-4}$	$5.0 \times 10^{-2}$
	<i>Lb. plantarum</i> NCFB 1988	$5.0 \times 10^{-3}$	$4.2 \times 10^{-2}$
<i>Lb. reuteri</i> DSM 20016 (pAM $\beta$ 1)	<i>E. faecalis</i> JH2-2	$2.1 \times 10^{-3}$	$3.0 \times 10^{-2}$
	<i>Lb. plantarum</i> NCFB 1988	$2.0 \times 10^{-4}$	$4.2 \times 10^{-3}$

\* Mean frequencies, expressed as transconjugants per donor, of at least five different experiments. Culture supernatant was added at 10% (v/v), and APF at  $5 \mu\text{g ml}^{-1}$ .

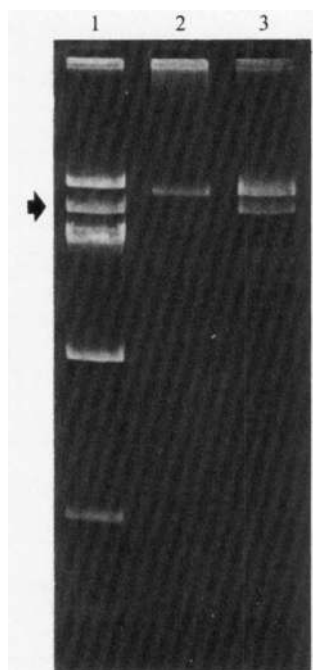


Fig. 4. Agarose gel electrophoresis of plasmid profiles. Lane 1, *Lc. lactis* subsp. *lactis* SH 4174 (pAM $\beta$ 1 donor). Lane 2, *Lb. plantarum* 4B2 (containing one native plasmid). Lane 3, a *Lb. plantarum* 4B2 transconjugant containing pAM $\beta$ 1. Arrow indicates pAM $\beta$ 1.

#### Identification of the putative transconjugants

The presence of pAM $\beta$ 1 in randomly selected Ery<sup>R</sup> transconjugants was confirmed by isolation of plasmid DNA and visualization on agarose gel electrophoresis (Fig. 4).

## Discussion

In this study we have shown that high-frequency pAM $\beta$ 1 conjugal transfer can be obtained in lactobacilli. Among Gram-positive bacteria only some strains of enterococci and lactococci are able to transfer plasmid DNA at a high rate; in both organisms this capacity is linked to the appearance of a 'clumping' phenotype (reviewed by Clewell & Weaver, 1989; Gasson, 1990), which causes the aggregation of donor and recipient cells. These observations prompted us to investigate the effect on pAM $\beta$ 1 conjugal transfer efficiency when autoaggregating bacteria were used.

A preliminary screening allowed us to select *Lb. plantarum* 4B2 for study. Its coaggregation capacity was lost when cells were washed and resuspended in water, but could be restored by adding its own supernatant. The proteinaceous nature of the APF was then determined and the substance was isolated and shown to be a 32 kDa protein. This protein, as well as the filter-sterilized

supernatant, was able to induce aggregation not only in the producer strain, but also in some strains of *E. faecalis*, *Lb. reuteri*, *Lb. plantarum* and, to a lesser extent, *Lc. lactis*. The APF was not active on *Lb. casei*, *Lb. acidophilus* and another strain of *Lb. plantarum*. These results suggest that a specific binding substance present on the outermost envelopes of positively reacting strains is required for aggregation to occur.

The nature of the receptor for the aggregating protein is indicated by comparison of the composition of the cell walls of the aggregating and non-aggregating strains. *Lb. plantarum* NCFB 1988 lacks the precipitinogen-specific group of *Lb. plantarum* (Adams *et al.*, 1969), which is a glucosylribitol TA (Sharpe *et al.*, 1973). Instead, walls of this strain contain a glycerol TA, with glucosyl substituents attached to the primary hydroxyl group of glycerol (Adams *et al.*, 1969). Moreover, these substituents occur as kojibiose [*O*- $\alpha$ -D-glucopyranosyl-(1,2)-D-glucose], a complex sugar characterized by the formation of a 'pocket' in its structure (Knox & Wicken, 1973). The observation that *E. faecalis* LTAs also have the same substituents (Knox & Wicken, 1973), supports the identification of the receptor as kojibiose-containing LTAs. *Lc. lactis* strains also have  $\alpha$ -1,2-diglucosyl substituents on their LTAs, but only as terminal components. No information is available, to our knowledge, on the LTA composition of *Lb. reuteri* strains; *Lb. casei* strains do not have glucosylated LTAs, while LTAs of *Lb. acidophilus* strains do not contain the  $\alpha$ -1,2 linkage in their glycosyl substituents (Wicken, 1980). Inhibition of aggregation reactions was obtained only when *E. faecalis* LTAs were used, confirming the identification of the binding substance.

This report shows that the presence of an aggregation phenotype in *Lactobacillus* is mediated by a protein able to establish a bridge between two bacterial cells containing, as binding substance, the kojibiose-substituted LTAs or TAs. Coaggregated bacteria are able to transfer plasmid DNA at a very high rate when matings occur on solid surfaces, and at a low frequency in broth mating. No induction reaction resembling those described for the sex pheromone of *E. faecalis* was ever detected.

The highest conjugation frequencies were detected when *Lb. plantarum* strain 4B2 was used as recipient; a possible explanation of this might be that intra-recipient conjugal transfer took place during the mating, in which the first transconjugants acted as donors during the subsequent rounds of cell replication.

The aggregation mechanism described here strongly resembles the protein-mediated adhesion of a *Lb. fermentum* strain to mouse stomach squamous epithelium described by Conway & Kjelleberg (1989). Moreover, *Lb. acidophilus* ADH, the only *Lactobacillus* strain for which

high-frequency plasmid transfer, by agar matings, has been reported, is able to adhere to intestinal tissue (Luchansky *et al.*, 1989).

The observation that secreted proteins in *Lactobacillus* can play a central role in a number of relevant phenotypes clearly encourages further research in this area.

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