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Lactobacillus crispatus and its Nonaggregating Mutant in Human Colonization Trials

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ABSTRACT

A wild-type *Lactobacillus crispatus*, showing a cell aggregation phenotype and its spontaneous nonaggregating mutant were compared for their in vitro adhesion properties to human ileal mucus and to a cultured human colonic cell line (Caco2) and for their in vivo colonization and adhesion potential with colonoscopy patients as volunteers in feeding trials. The wild-type strain adhered better to mucus or to Caco2 cells than did the mutant. Altogether, three human trials with the wild type and two with the mutant strain were performed. In two of the trials, the wild type could be recovered from either fecal samples or biopsies taken from the colon, while the mutant strain could not be demonstrated in either of the trials where it was used. The *L. crispatus* colonies recovered from the trials were often mixed, and several enterococci and lactobacillus strains coaggregating with *L. crispatus* wild type could be isolated. The results indicate that the surface-mediated properties, such as aggregation, of lactobacilli can have a role in adhesion and colonization.
(**Key words:** probiotics, aggregation, colonization, *Lactobacillus*)

Abbreviation key: HH = HEPES-Hanks buffer, **PFGE** = pulsed field gel electrophoresis, **RAPD** = randomly amplified polymorphic DNA.

INTRODUCTION

Orally consumed viable bacteria with proposed beneficial health effects, the so-called probiotics, are increasingly used to treat disorders like lactose malabsorption, viral, bacterial, and radiotherapy-induced diarrhea, constipation, inflammatory bowel disease, and food allergy (Gilliland, 1990; Salimen et al., 1998). While the clinical efficacy of certain well-characterized probiotic strains has been documented (Salimen et al., 1998; Saxelin, 1997), little is known about the actual mechanisms of probiotic action. However, adhesion to human intestinal mucosa has been considered essential for efficient colonization of the gut, and it is used as an important criterion to select new probiotic strains (Huis in't Veld, 1996). The adhesion properties of the strains have traditionally been assessed with different in vitro assays with cultured human intestinal cell lines, while the in vivo colonization rate has been deduced from fecal samples. Colonization studies have, however, been hampered by the difficulties in the exact identification of the strains among the multitude of strains present in the intestinal microflora.

In addition to fecal samples, rectal mucosa and biopsies obtained from different parts of the large intestine (Alander et al., 1997, 1999; Johansson et al., 1993) have also been used in colonization studies. With this approach, a more exact picture of the in vivo adhesion properties of the probiotic strains can be obtained. By combining the use of colonic biopsies and molecular biological identification methods, the persistence of a probiotic strain in human colonic mucosa for a considerable time after the actual probiotic administration stops and the counts of the strain disappear below the detection limit in fecal samples (Alander et al., 1999).

However, the strain-specific properties responsible for colonization and adhesion are poorly known. Lipoteichoic acids have been suggested to have a role in the adhesion of certain lactobacilli to the squamous epithelium of the pig stomach (Tannock, 1990). A mannose-specific adherence mechanism was shown to mediate adherence of some *Lactobacillus plantarum* strains to colonic cell line HT29 (Aderberth et al., 1996). The authors suggested that a protein structure, located on the bacterial cell surface could be the mannose-sensitive adhesin of these strains. However, little is known

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about how surface structures are used by lactobacilli so that they remain in the human gut; a panel held in 1993 to promulgate the effect of probiotic food additives suggested the use of isogenic mutants, altered in some surface-linked properties, to elucidate colonization mechanisms (Sanders, 1993).

One well-studied mechanism of bacterial adhesion is the formation of dental plaque, in which Gibbons and coworkers postulated an important role for bacterial aggregation in the early 1970s (Gibbons and van Houte, 1975). Since that time, the relevant role of coaggregation in plaque formation and accretion has been proven by a large number of experimental works, [for a review, see Kolebrander (1991)], and it is now well established that nearly all human oral bacteria are able to aggregate with other bacteria.

In contrast, nothing is known about the role of aggregation on the persistence of *Lactobacillus* strains in the human gut, even if aggregation is a phenotype frequently detected in enteric lactobacilli.

Based on these premises, we tried to learn more about the role of aggregation by comparing the in vitro and in vivo behavior of a pair of isogenic lactobacilli.

The objective of this study was to compare a wild-type and a spontaneous mutant of a human isolate of *Lactobacillus crispatus* using in vitro tests and human colonization trials with volunteer colonoscopy patients as test subjects. The wild-type strain had a characteristic aggregating phenotype, mediated by a secreted aggregation promoting factor, similar to that already described in *Lactobacillus* 4B2 (Reiniero et al., 1992).

MATERIALS AND METHODS

Lactobacillus crispatus Strains M247 and MU5

Strain M247 was isolated from a fecal sample taken from a weaning baby. It was taxonomically identified by means of a positive hybridization reaction with the *L. crispatus* specific 23S rRNA-targeted probe (Ehrmann et al., 1994). Cells grown in MRS medium appeared as clumps discernible to the naked eye, which sedimented at the bottom of the tube, leaving the upper part of the medium clear.

A spontaneous nonclumping mutant of M247, named MU5, was isolated from the lower aqueous phase during an hydrophobicity assessment based on water-hexane partition assay. Fermentation patterns were determined with API 50CH galleries, while drug resistance was assayed by a disk diffusion test, and plasmid content was determined by means of an alkaline lysis method (Vescovo et al., 1982). Both strains were grown in MRS (Difco, Detroit, MI) broth or agar, at 37°C. Anaerobic conditions provided by the Gas Pack System (BBL Microbiology Systems, Cockeysville, MD) were used when required. Cultures are maintained in the Bacterial Collection of the Istituto di Microbiologia UCSC, Piacenza, and are also included in the Culture Collection of the European Union PROBDEMO project EU-FAIR CT96-1028.

Confirmation of the Isogenic Nature of M247 and MU5

The isogenic nature of the two *L. crispatus* strains was checked by four different genetic methods: plasmid profiling, RAPD-PCR, ribotyping, and pulsed field gel electrophoresis (**PFGE**). The randomly amplified polymorphic DNA (RAPD)-PCR procedure has been described (Cocconcelli et al., 1995). Briefly, the reaction mixture consisted of 2.5 U *Taq* polymerase (Boehringer GmbH, Mannheim, Germany), 2.5 μl of 10 × PCR buffer, 200 μM of each deoxyribonucleotide triphosphate (Boehringer), 3.5 mM of $MgCC_2$, 1 μM of a single random primer with an arbitrary nucleotide sequence (5′ AGCAGCGTGG 3′), and 10 ng of bacterial DNA in a final volume of 25 μl overlaid with 20 μl of mineral oil. Amplification reactions were carried out in a Perkin-Elmer Gene Amp 8700 thermocycler (Perkin-Elmer, Norwalk, CT). A 10-min preliminary cycle at 94°C was followed by 40 cycles each consisting of a denaturation step at 94°C for 1 min, a 1-min annealing step at 29°C for 1 min, ramp of 1.30 min to the extension temperature 72°C (2 min). The extension period in the final cycle was continued by additional 7 min at 72°C to complete partial polymerizations.

For ribotyping, the chromosomal DNA was obtained by the alkaline lysis method in which the denaturation step had been omitted (Vescovo et al., 1982). The *Sma*I and *Hind*III digestion products were electrophoresed for 4 h in 0.8% agarose gel at a constant voltage of 80 V. The gel was blotted on a nylon membrane (Boehringer) according to the instructions of the manufacturer. Hybridization was performed using *L. crispatus*-specific 23 S rRNA probe (Ehrmann et al., 1994) labeled with a DIG Oligonucleotide 3′-End Labeling Kit (Boehringer).

For PFGE, 1.5-ml samples of overnight cultures were successively washed with 50 mM EDTA, followed by 0.5% SDS, sterile ultrapure water (milliQ), and finally 1 M NaCl-10 mM Tris-HCl (pH 7.6). Finally, the cells were suspended in 300 μl of the last washing solution and mixed with an equal amount of 2% low melting point agarose (Sigma, St. Louis, MO). After the agar had solidified in molds, the cells were lysed in situ by treatment with 6 mM Tris-HCl (pH 7.6) containing 1% sarcosyl, 10 mg ml^{-1} of lysozyme, 50 U ml^{-1} of mutanolysin, and 28 μg ml^{-1} of RNAse for 18 h at 37°C, followed by a proteinase K treatment (1 mg ml^{-1}) (Ferrero et al., 1966). The DNA restriction was performed by using 40

Table 1. Universal and strain-specific primers used to identify *Lactobacillus crispatus* M27/MU5.

Primer	Length	Position	Reference	Sequence
		574-592		GTT GTC CGG ATT TAT TGG G
		821-802		GGG TAT CTA ATC CTG TTC GC
		137-155		TTA ATG CTG ATG GTA CCG C
		478-460		CAT TGC TGT TCT TTG AGC C

units of the following enzymes: *Not*I, *Apa*I, and *Sfi*I, all from Boehringer. The temperature was 37°C except for *Sfi*I, for which 50°C was applied. DNA fragments were separated in a 0.8% agarose gel using 0.5 × TBE buffer and a CHEF MAPPER apparatus (Bio-Rad, Richmond, CA) at a constant voltage of 6 V cm^{-1} with a pulse time of 2 to 30 s for a total running time of 18 h. The PFGE marker (Boehringer) was used as a molecular size standard.

Coaggregation Assay

The coaggregation of bacterial strains was assayed according to the procedure of Reniero et al. (Reniero et al., 1992). Briefly, equal amounts of overnight cultures of the two strains to be assayed were mixed together and then left at room temperature. Coaggregation was scored positive when clumps of coaggregated cells sedimented to the bottom of the tube, leaving a clear supernatant within 2 h.

Strain-Specific PCR Amplification for *L. crispatus* M247/MU5

Colony-forming units belonging to the strains used were identified by means of strain-specific multiplex PCR, using whole cells taken from colonies on agar plates. Bacterial colonies replicated on MRS agar were dispersed in 10 μl of sterile distilled water, heated by means of a microwave oven (10 min adjusted at the maximum power) then added to PCR reaction tubes. The reaction mixture (final volume 25 μl) contained 1× *Taq* buffer, 5 U of *Taq* polymerase, 1.5 m*M* $MgCl_2$, 200 μM each dNTP, and each of the primers at 0.5 μM. Primer design was carried out with the help of a commercial computer program (Vector NTI Deluxe, Informax, North Bethesda, MD). Strain-specific primers SL3 and SL4 were designed, starting from the sequence of the 1356 bp encoding for the surface layer (Slp247) of the M247/MU5 strains (EMBL Accession number AJ007839). The forward primer spanned from bp 137 to 155 and the reverse from 478 to 460 with an expected amplification product of 342 bp (Table 1). Lack of amplification might occur in some samples, due either to the absence of the specific target or to a failure of the amplification reaction caused by the variable conditions of PCR on whole cells taken from colonies on agar plates. To discriminate between these events, we used primers designed to recognize consensus 16S rRNA sequences of several *Lactobacillus* species obtained from GenBank (Lucchini et al, 1998). Forward primer LARNA5 and reverse primer LARNA6 generate a product of 248 bp in PCR amplification of every *Lactobacillus* tested to date. This primer pair does not give any taxonomic information, as the genomic region identified is conserved in many different bacteria, but it is a useful indication of positive amplification reaction. The multiplex PCR conditions were as follows: a start step of 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C. The final elongation step was performed at 72°C for 5 min. Amplification reactions were carried out in a Perkin-Elmer Gene Amp 8700 thermocycler. After cycling, 10 μl of each reaction was analyzed by electrophoresis on 2.0% agarose gel at 7 V/cm.

The In Vitro Acid, Bile, and Pancreatin Tolerances of M247 and MU5

Acid resistance was assayed in a Na-citrate buffer solution (pH 3.0) following the procedure outlined by Conway et al. (1987). Briefly, 0.1-ml portions of ten times concentrated overnight cultures were added to a series of 2.0 ml Na-citrate buffer tubes. Mixtures were incubated at 37°C, and the viable organisms were enumerated by plating after 90 min.

The bile tolerance was assayed by separate assays with two commercially available preparations, one consisting of essentially conjugated (Sigma B8381) and the other completely deconjugated bile salts (bile no. 3. L56, Oxoid Ltd, Basingstoke, Hampshire, UK). Resistance was assayed either in liquid cultures or on agar plates. In the former case, MRS broth was prepared with and without 0.5% (wt/vol) bile preparation and dispensed in 10-ml volumes and sterilized. For each strain to be tested, one tube was inoculated with 0.1% of an overnight culture. After a 24-h incubation at 37°C, the cultures were diluted and plated onto MRS agar. In the plate assays, an overnight culture of the tested strain was diluted and used to seed MRS agar medium containing 0.5% of the bile preparation. Solidified agar plates were then incubated at 37°C for 48 h.

To determine the pancreatin resistance, we grew the bacteria overnight in MRS broth. Cells were collected from 5 ml of the growth medium by centrifugation and washed once with sterile 150 m*M* $NaHCO_3$ solution containing 1.9 mg of pancreatin (pancreatin from porcine pancreas, Sigma). The cells were resuspended in 5 ml of pancreatin solution, and this suspension was used to inoculate 100 ml of the pancreatic solution, which was further incubated in a 37°C water bath for 3 h. The viable counts were determined both before and after the pancreatin treatment by plating on MRS agar plates.

In Vitro Adhesion Studies

Two test systems were applied to get an indication of the ability of the strains to adhere either to the small intestine or to the surface of the colon. Human ileostomy glycoproteins were used as a model for small intestinal mucus and Caco2 cell line as a model for colonic epithelium.

Lyophilized preparation of human ileostomy glycoproteins was a generous gift from J.G.H. Ruseler-van Embden (Erasmus University, Rotterdam, The Netherlands). In short, human ileostomy effluent was dissolved and homogenized in distilled water and centrifuged for 30 min at 10,000 × *g*. Clear supernatants were lyophilized and stored at −18°C (Ruseler-van Embden, 1991). The same preparation has been characterized by Ouwehand et al. (1995) and found to contain 55.4% carbohydrate, 12.6% protein, and 14.0% extractable lipids (by weight).

The adhesion of bacterial cells labeled with tritiated thymidine was quantitated according to a method described by Cohen and Laux (1995). Briefly, lyophilized human ileostomy glycoproteins were dissolved (0.5 mg/ml) in HEPES-Hanks buffer (**HH**; 10 m*M* HEPES; pH 7.4), and 100 μl of the solution was immobilized in polystyrene microtiter plate wells (Nunc, Roskilde, Denmark) by overnight incubation at 4°C. The wells were washed twice with 250 μl of HH. Suspension of labeled bacteria was prepared in HH and the optical density at 600 nm of each bacterial suspension was adjusted to 0.25 ± 0.01. Radiolabeled ([methyl-1′,2′-^{3}H] thymidine, 113Ci/mmol, Nycomed Amersham Life Sciences, Little Chalfont, Buckinghamshire, UK) bacteria (100 μl) were added to the wells. After incubation at 37°C for 1.5 h, the wells were washed three times with 200 μl of HH to remove unattached bacteria. To release and lyse the adherent bacteria, 1% SDS-0.1 *M* NaOH was added to wells and incubated at 60°C for 1 h. The radioactivity of the suspension containing the lysed bacteria was measured by liquid scintillation. The adhesion ratio (%) was calculated by comparing the radioactivity of the bacteria added (triplicate 100-μl samples) to the radioactivity of the bacteria bound.

The human colonic cancer cell line Caco2 was obtained from the American Type Culture Collection (ATCC HTB-37) (Manassas, VA). The cell culture medium was RPMI-HEPES (RPMI1640, GIBCO, Madison, WI) supplemented with 20% fetal calf serum (PAA Laboratories, Linz, Austria), 2 m*M* L-glutamate (GIBCO), 1% nonessential amino acids (GIBCO), and 100 IU ml^{-1} penicillin-streptomycin solution (GIBCO). The cells were seeded at a concentration of 3.2×10^4 cells ml^{-1} and incubated at 37°C in 5% CO_2 atmosphere for 14 days in Chamber Slide (Nunc) to obtain a monolayer. The culture medium was replaced every second day. Before the adhesion test, the cells were gently washed with 300 μl of PBS, pH 7.1, and overlaid with a 300-μl bacterial cell suspension in RPMI-HEPES-medium without supplements. Both M247 and MU5 were tested in different dilutions, the cell counts varying from 5×10^5 to 1×10^8 cfu ml^{-1}. After an incubation of 1 h at 37°C, the cells were washed gently six times with 300 μl of PBS. Adhesion was verified either visually from Gram-stained monolayer or by using bacterial cells labeled with tritiated thymidine. In tests with labeled bacteria, the cells were fixed with methanol for 10 min and subjected to liquid scintillation counting. The label associated with washed Caco2 cells was compared to that present in the original bacterial suspension.

Preparation of Fermented Whey Drinks for Human Colonization Trials

A commercial fruit-flavored drink based on lactose-hydrolyzed whey was used as a base for the experimental products. The drink was inoculated (1%) with overnight MRS cultures of either of the *L. crispatus* strains, and incubated for 24 h at 37 °C. The final bacterial counts at this time point (determined by plating on MRS agar) ranged from 5.0×10^7 up to 1×10^8 cfu ml^{-1}. The pH of the product decreased only slightly from the original value of 4.13 to pH 4.07 to 4.09 at the end of the fermentation. The drinks were refrigerated and sent immediately to volunteers, who started consumption the following day. The test persons were instructed to keep the drink refrigerated during the trial. A sample of the respective drinks was kept in the laboratory, and the bacterial count was determined at the end of the 8-d administration period.

The Feeding Trials

The following general scheme of the feeding trials, reviewed by the local ethical committee, was applied. The volunteers were patients coming to routine diag-

nostic colonoscopy, and, with the exception of various gastrointestinal symptoms, they reported themselves healthy and had no dietary restrictions. Patients with antibiotic therapy or diagnosed ulcerative colitis were excluded. All volunteers had given their informed consent to the study, and they were free to discontinue the trial whenever they wished. Taking of biopsies was a part of the standard diagnostic procedure, and thus the experimental setting did not involve any extra pain or discomfort for the patients. The volunteers took two 100-ml doses of fermented juice daily for 8 d before the colonoscopy. No other modification to their diet or general lifestyle was required.

Fecal and Biopsy Samples

Fecal samples were obtained from the volunteers immediately before the administration and again before the colonoscopy. The samples were stored in the home freezers of the patients, then transferred as frozen to the laboratory and stored there at −20°C until cultivation (within 2 wk of sampling). Before the colonoscopy, the evacuation of the colon was induced by three doses of a laxative (Pico-salax, Enska, Helsinki, Finland) consumed within 36 h. The instrument used for the colonoscopy and sampling of the biopsies was a Pentax ES-380IL. Three parallel biopsies were taken from the descending colon choosing the location from the apparently healthy part of the gut. The diameter of the biopsies was about 3 mm. Biopsies were immediately transferred into thioglycollate medium (Difco) and stored at 4°C until cultivation (within a day).

Bacteriological Examination of the Samples

Both fecal and biopsy samples were homogenized for 30 s in a stomacher (Stomacher 400, Seward) before dilution and cultivation on MRS agar. The plates were cultivated under anaerobic conditions (Anaerocult A, Merck, Darmstadt, Germany) for 3 d at 37°C. Colonies were then quantitatively picked from plates containing a sufficient number (approximately 100) of clearly separate isolates and subjected to *L. crispatus*-specific PCR.

The bacterial isolates from the mixed *L. crispatus* colonies recovered from the human samples were checked for cell morphology, sugar fermentation tests, isomers of lactic acid produced, and finally by hybridization to species-specific probes. The absence of detectable plasmids, RAPD-PCR profile, and ribotyping were used to confirm that the isolates positively recognised by strain-specific multiplex PCR belonged to M247 strain.

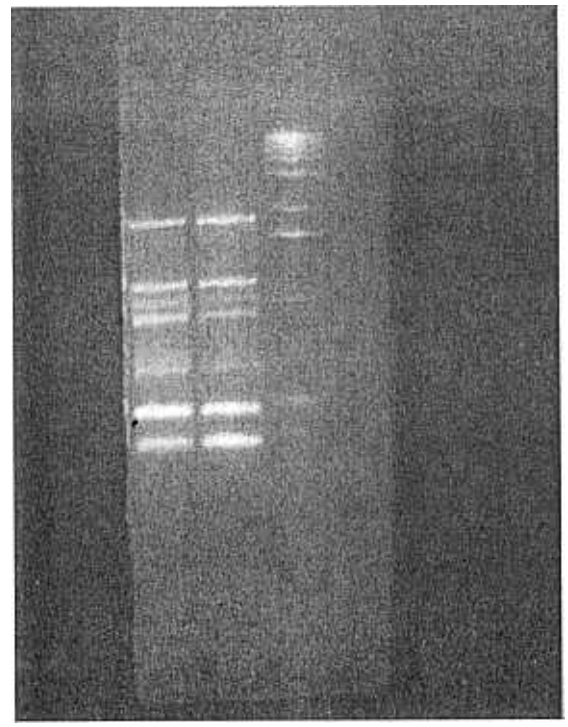

Figure 1. Randomly amplified polymorphic DNA patterns of the wild strain and its mutant: lane 1: *Lactobacillus crispatus* M247, lane 2: *L. crispatus* MU5, lane 3: DNA-molecular weight marker.

RESULTS

Characterization of M247 and MU5

Both strains were found to lack any detectable plasmid. In RAPD-PCR, both M247 and MU5 produced an identical pattern consisting of seven clearly distinguishable amplicons (Figure 1). Regardless of the enzyme used, ribotyping also resulted in similar profiles with both the strains (Figure 2). No differences between the strains could be detected in PFGE either (Figure 3). With the history of MU5 (isolation from a M247 culture), these findings, together with identical phenotypes in fermentation (API patterns) and drug resistance tests (data not shown), are all compatible with the assumption that MU5 is a spontaneous clonal variant of M247.

Acid, Bile, and Pancreatin Tolerances of M247 and MU5

The trials were performed three times, except for pancreatin tolerance, which was tested twice. In acid challenge tests, the viable counts of both the strains decreased approximately three log cycles from the starting level of 2 to 8 × 10^8 cfu ml^{-1}. With conjugated bile acids, the corresponding drop in viable counts was two

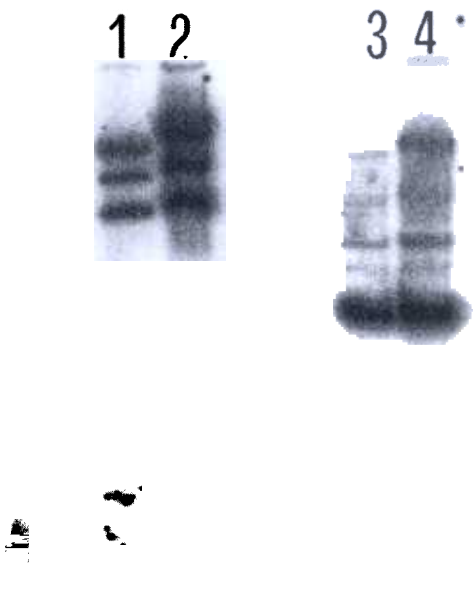

Figure 2. Ribotypes of M247 and MU5. Lanes 1 and 2: M247 and MU5, respectively, digested by *Sma*I; lanes 3 and 4, M247 and MU5 digested by *Hind*III.

log cycles (broth assays) or one log cycle (plate incorporation tests). Nonconjugated bile salts turned out to be considerably more toxic, resulting in a decrease of more than 5 log cycles. In pancreatin tolerance tests, an actual increase in cfu from 7 to 8 × 10^7 to 5 × 10^8 was

Figure 3. Pulsed field gel electrophoresis patterns of *Not*I, *Sfi*I, and *Apa*I digested genomic DNA of *Lactobacillus crispatus* M247 and MU5. Lanes 1 and 2, *Not*I digests of M247, and MU5, lane 3, molecular weight marker, lanes 4 and 5, *Sfi*I digests of M247 and MU5, lanes 6 and 7, *Apa*I digests of M247 and MU5.

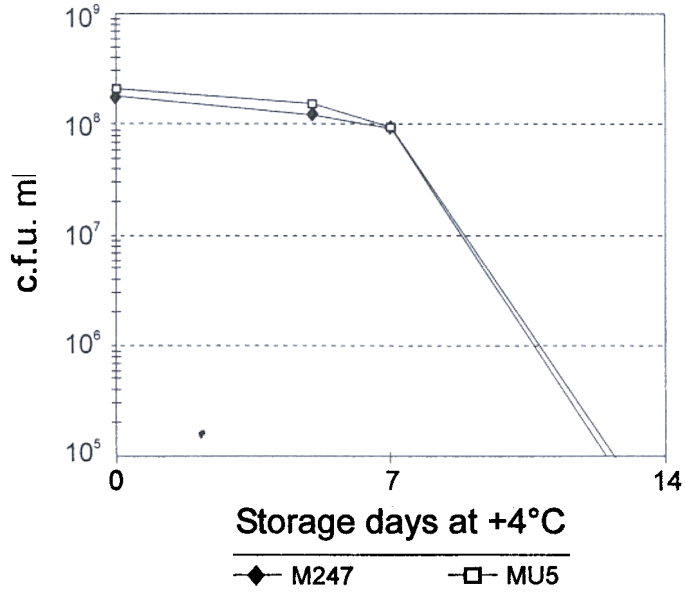

Figure 4. The stabilities of *Lactobacillus crispatus* M247 and MU5 strains in fermented juice products.

observed. No signs of differential resistance or sensitivity between the strains were detected in any of the challenge tests.

The In Vitro Adhesion Properties of the Strains

In the tested cell concentrations (approximately 10^7 cfu per well), M247 adhered significantly ($P < 0.001$) better to ileostomy glycoproteins than did MU5. The adhesion ratios (mean ± SD, n = 4) were 16.5 ± 6.0 and 1.9 ± 0.45, respectively.

In microscopic examination, M247 appeared to adhere better to Caco2 cells than did MU5. Because this was difficult to quantify because of the aggregation phenotype, a series of experiments were performed using tritium-labeled bacteria. The results of these trials were clearly dependent on the bacterial cell density. The difference between the strains were most prominent, when the bacterial cell counts in the assay were less than 10^8. The adhesion percentages (mean ± SD, n = 4) in the concentration range of 1.0 to 2.1 × 10^7 for M247 and 1.3 to 2.6 × 10^7 for MU5 were 13.5 ± 0.7 and 7.1 ± 0.3, respectively.

The Stability of *L. crispatus* in Experimental Drinks

The results of a representative stability trial are presented in Figure 4. Both M247 and MU5 were relatively stable for a week after the fermentation, when the drinks were stored refrigerated. However, after that period, the viable cell counts of both strains decreased

rapidly, which was one of the practical reasons to limit the human trials to 8 d.

Recovery of *L. crispatus* Strains from Human Volunteers

The results of the three human trials are summarized in Table 2. The first trial was a double-blind experiment with plates cultivated from the fecal and biopsy samples having been sent coded for the PCR examination of the colonies. The PCR results were also confirmed by RAPD-PCR and ribotyping. Only after the *L. crispatus* identifications had been performed were the codes broken, and the code corresponding each patient and sample matched with the outcome. The result was striking. While M247 could be recovered from all the fecal samples and from two of the biopsy samples, no positive isolates were detected in MU5 group (trial I, Table 3). The individual counts of total lactic acid bacteria and *L. crispatus* in the samples of the first trial are shown in Table 3. However, in a repeated experiment (trial II in Table 2), no positive isolates were recovered in either of the test groups. Therefore, a third confirmatory trial using only M247 with two additional volunteers was performed (trial III in Table 2), and this time the strain could be detected from the fecal sample of one patient and from the biopsy of the other. The amounts were comparable to those obtained in the first trial.

The Bacteriological Composition of the Positive Colonies of the First Trial

It was observed that some PCR amplification products obtained from colonies identified as M247 were just barely visible in the gel, while some were as distinct as those obtained from pure control cultures. It was therefore decided to replicate 100 randomly selected colonies from three subjects on MRS plates. Sixty of these colonies proved to contain M247 according to PCR amplification. However, 19 of these showed only faint specific bands in the gel. Microscopic examination of these isolates revealed mixed bacterial populations. Twelve isolates from two subjects contained enterococci, while the remaining seven, all isolated from one subject were vancomycin-resistant, ribose fermenting lactobacilli identified on the basis of a species-specific probe (Heertel et al., 1993) as *Lactobacillus paracasei*. The enterococcal isolates originated from mixed M247 colonies cultivated from both fecal and biopsy samples, while the *L. paracasei* isolates were recovered from a fecal sample of a single individual (patient no 3, in Table 3).

All the 19 *Enterococcus* and *Lactobacillus* isolates were assayed for their coaggregation capacities both with M247 and MU5. All the isolates aggregated with M247 but not with the mutant strain MU5.

DISCUSSION

This paper describes, to our knowledge for the first time, a human colonization experiment comparing isogenic variants of a *Lactobacillus* species. This approach has been recommended, among others, by a consensus panel (Sanders, 1993).

The mutant strain MU5 was selected from the wild-type *L. crispatus* M247 cultures without any chemical or physical mutagenic treatments. The three methods used to test the relatedness of the strains all confirmed that the mutant was indistinguishable from the parent strain. The presence of nonhydrophobic derivatives of hydrophobic strains in the lower aqueous phase of the hexadecane assay is well known (Rosenberg and Doyle, 1990), and it has been exploited to isolate spontaneous mutants with reduced hydrophobicity of *Acinetotobacter calcoaceticus* (Rosenberg and Kljellberg, 1981), *Serratia marcescens* (Rosenberg, 1984), and *Streptococ-*

Table 2. Summarized results of the human colonization trials with *Lactobacillus crispatus* strains M247 and MU5.

	Number of subjects in test groups		Daily dose cfu $\times 10^{10}$ [a]		Subjects with *L. crispatus* identified at the end of the 8-d administration			
					in feces		in biopsies	
Trial	M247	MU5	M247	MU5	M247	MU5	M247	MU5
	5	5	2.6		5		2	
	3	3	1.0		ND		ND	
	2		1.0		1[c]		1	

[a]Based on the colony-forming unit count of the experimental product in the beginning of the administration period.

[b]A double blind trial.

[c]A different individual from the one with a positive *L. crispatus* identification in the biopsies.

ND = None detected.

Table 3. The counts of total lactic acid bacteria and identified *Lactobacillus crispatus* in fecal and biopsy samples in the trial I.

Patient	Test strain	Sample[1]	Bacterial counts[2]	
			Total lactic acid bacteria	*L. crispatus*
			9.4×10^4	
			4.1×10^5	
			2.9×10^3	
2	M247		2.1×10^4	
			2.5×10^5	
			5.9×10^5	
3	M247		5.9×10^5	
			2.6×10^9	
			3.7×10^4	
4	M247		$<10^4$ •	
			6.5×10^4	
			8.3×10^5	
5	M247		1.6×10^6	
			4.7×10^7	
			7.9×10^6	
6	MU5		8.6×10^6	
			1.1×10^5	
			2.9×10^3	
7	MU5		3.9×10^6	
			2.0×10^7	
			4.8×10^5	
8	MU5		4.7×10^7	
			8.2×10^7	
			1.8×10^5	
9	MU5		4.0×10^7	
			1.5×10^7	
			1.8×10^4	
	MU5		1.2×10^6	
			3.5×10^7	
			3.8×10^5	

[1]Day 0 represents fecal samples obtained before the administration period, and d 8 the samples obtained immediately before the sigmoidoscopy.

[2]The counts are either cfu g^{-1} wet weight (fecal samples) or per biopsy.

ND = none detected.

cus sanguis (Jenkinson and Carter, 1988) and to compare their adhesion capacity.

The lack of the receptor of the coaggregation promoting factor in MU5 did not affect the performance of the strain in tests mimicking either the gastrointestinal stress factors or the growth parameters of the strain. Thus M247 and MU5 form an ideal strain pair for an experiment aiming to elucidate the effects of a single well-defined mutation and the consequent phenotype on the in vivo survival of the organism in the intestinal tract.

The in vitro tests M247, in the conditions reported here, consistently showed a better adhesion to the ileal mucus or to Caco2 cells than the mutant. The result obtained with the ileal mucus was similar to that obtained earlier with mucus extracted from the feces of infants and adults (Kirjavainen et al., 1998). This can be taken as an indication of the in vivo behavior of the strains. However, as pointed out by Greene and Klaenhammer (Hertel et al., 1993), in vitro test systems have their limitations. Especially in this case, in which the tendency of the wild type to aggregate may affect the result, it is necessary to obtain an in vivo confirmation of the ability of the strains to colonize the intestinal tract and to adhere to the colonic mucosa.

The results show that M247 can frequently be recovered in substantial amounts after an 8 d of administration from either fecal or biopsy samples of human volunteers. The pattern observed suggests that the doses applied are near the threshold level of successful permanency (apparently $\geq 10^{10}$ cfu/d), and minor fluctuations in the cell counts could affect the result. A "dose response" effect could be the real explanation of the impossibility to detect M247 during the second feeding trial, in which the beverage provided to volunteers contained a reduced amount of viable bacterial cells. The

mutant strain was consistently absent, or at least below the detection limit of the plate count method. The daily doses of the mutant were, if anything, slightly higher than those of the wild type and therefore insufficient dosing can be ruled out as an explanation for the results.

As can be seen in Table 3, the background levels of lactic acid bacteria were often lower in the group receiving M247 than in those being administered with MU5. There might have been less colonization resistance in M247-treated volunteers. However, *L. crispatus* could also be recovered from the individuals in M247-treated groups who had a background level of lactic acid bacteria comparable to the average of the MU5 group.

Unfortunately, the design of the study did not include a postfeeding examination of the volunteers for the determination of the presence of the strains after the continuous feeding period. This type of observation could be included in future trials to obtain data about the permanency of an aggregating strains into the human gut.

An unexpected outcome of the experiment was the frequent recovery of mixed colonies of M247 from the samples. This was all the more remarkable as the majority of accompanying isolates turned out to be enterococci, for which the plating medium used (MRS) was not specially suitable. The fact that in subsequent laboratory trials these isolates coaggregated strongly with M247 but not with the mutant, provokes the question, whether in vivo coaggregation plays a role in the survival and colonization of M247 in the human gastrointestinal tract.

The effect of coaggregation on the dental plaque formation is well known (Kolebrander, 1991). Yeasts coaggregate with bacteria, apparently to survive in the human gut (Millsap et al., 1998). To our knowledge, the role of coaggregation among different intestinal lactic acid bacterial species and genera has not even been speculated. However, lactobacilli able to aggregate with bacteria belonging to different genera have been shown to occur in the human urogenital tract (Reid et al., 1990).

The aim of this study was to investigate the effects of the aggregation phenotype on the behavior of *L. crispatus* in the human gastrointestinal tract. The results indicate that the ability to aggregate could have a definite role in the survival of the strain in the gut. Whether this is due to autoaggregation, Coaggregation, or some unknown factors associated with the phenotype and protecting the strain in the hostile environment, remains to be solved. The results further demonstrate the value of isogenic mutants in the study of essential colonization mechanisms, which have practical implications in the development of human or animal probiotics. The role of aggregation in intestinal microbial ecology is a fundamental question, and further research should be directed to its elucidation.

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