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The aggregation-promoting factor of *Lactobacillus crispatus* M247 and its genetic locus

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ABSTRACT

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Aims: Characterization of the aggregation-promoting factor (APF) of the human intestinal isolate *Lactobacillus crispatus* M247 and its homologous nonaggregating mutant Mu5.

Methods and Results: Western blot analysis revealed that the supernatant of both M247 and Mu5 contains a 28-kDa protein which cross reacts with the antiserum produced against the APF of *Lact. gasseri* 4B2. The *apf* genes of M247 and Mu5 strains were identical and were shown to be 672 nucleotides in length and encoding a protein of 223 amino acids with a predicted molecular weight of 24·0 kDa.

Conclusion: Our results shows that the lost of aggregation in Mu5 is not related to a defect in secretion of the APF protein or a mutation in the *apf* gene.

Significance and Impact of the Study: These results suggest that the mutation in Mu5 may be contained in another molecule involved in aggregation such as a possible receptor for APF.

Keywords: aggregation, aggregation-promoting factor, *apf* gene, colonization, lactobacilli, nonaggregating mutant.

INTRODUCTION

Lactobacilli are normal inhabitants of the oral cavity, the gastrointestinal tract and the urogenital tract (Ahrne *et al.* 1998; Hillier *et al.* 1993). The capacity of *Lactobacillus* to colonize the mucosa may be conferred by direct attachment to the host surface or by aggregation to other micro-organisms (Conway *et al.* 1987; Wadstrom *et al.* 1987; Conway and Kjelleberg 1989; Wilcox *et al.* 1993). Strains with a high autoaggregation and coaggregation capacity have been isolated from the oral, vaginal and intestinal tract of both humans and animals (Wilcox *et al.* 1993; Kmet *et al.* 1995; Boris *et al.* 1997; Kmet and Lucchini 1997, 1999; Cesena *et al.* 2001). Cell aggregation seems to involve the interaction between cell surface components such as lipoteichoic acid, proteins, glycoproteins, lipoproteins, carbohydrates and pheromones (Wilcox *et al.* 1993; Kmet *et al.* 1995; Boris *et al.* 1997; Kmet and Lucchini 1997, 1999; Granato *et al.* 1999).

The presence of the aggregation phenotype in *Lactobacillus gasseri* 4B2 (previously misclassified as *Lact. plantarum*), an enteric strain, was previously shown to be mediated by a secreted 32-kDa protein, consequently named aggregation-promoting factor (APF) (Reniero *et al.* 1992). This protein was also able to induce aggregation in some strains of *Enterococcus faecalis*, *Lact. reuteri* and *Lact. plantarum*. However, more recent results do not seem to support the role of APF as an aggregation protein but rather suggest an involvement in the maintenance of the cell shape (Ventura *et al.* 2002; Jankovic *et al.* 2003). The *apf* gene from six different *Lact. gasseri* and *Lact. johnsonii* human faecal isolates have recently been identified and sequenced. The amino acid composition, physical properties, and genetic organization were found to be quite similar to those of

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S-layer proteins. APF proteins were also shown to noncovalently attach to the cell surface (Ventura *et al.* 2002).

An autoaggregation positive phenotype strain, *Lact. crispatus* M247, has previously been isolated from newborn infant faecal samples. A nonaggregating isogenic spontaneous mutant, Mu5, has also been derived from M247 (Cesena *et al.* 2001). It has been shown that the strain M247 (aggregation phenotype) could colonize the intestinal tract while the Mu5 mutant could not, suggesting a role of aggregation in colonization of *Lactobacillus* in the intestinal tract (Cesena *et al.* 2001). In this study, we determined if the nonaggregation phenotype of the Mu5 mutant was due to a defect in APF expression. The genetic loci of *apf* of M247 and Mu5 were also sequenced.

MATERIALS AND METHODS

Strains and culture condition

Autoaggregating *Lact. crispatus* M247 and *Lact. gasseri* 4B2 were isolated from the faeces of human newborns (Cesena *et al.* 2001). The nonaggregating mutant strain Mu5 has been obtained from the *Lact. crispatus* M247 strain using absorption of the M247 culture to a hydrophobic phase (hexadecane) and successive reinoculation of an aliquot of acquous phase in fresh MRS medium (Cesena *et al.* 2001). The isogenic nature of the two *Lact. crispatus* strains has been confirmed by plasmid profiling, RAPD-PCR, ribotyping and pulsed-field gel electrophoresis (Cesena *et al.* 2001). Both strains showed identical profiles of fermentation and antibiotic sensitivity, and the same level of resistance to acid and bile (Cesena *et al.* 2001).

Hydrophobicity of M247 and Mu5

The level of hydrophobicity was evaluated by measuring the affinity of bacterial cells to a hydrophobic phase in a two phase system (water : hexadecane) (Watanabe *et al.* 1987). Cells were grown in MRS medium to stationary phase at 37°C, centrifuged (3000 *g*, 15 min) and washed twice with water. The pellet was resuspended in an appropriate volume of water to obtain an optical density of 560 nm ($O.D._{560}$) between 0·5 and 0·6. Equal volumes (4 ml) of bacterial suspension and hexadecane were mixed together and the suspension was then left 30 min at room temperature to allow the two phases to separate. The aqueous phase was removed carefully and the $O.D._{560}$ was measured. The percentage of hydrophobicity was evaluated as the percentage of reduction in the optical density of the aqueous phase.

Aggregation assay

The aggregation test was performed according to Reniero *et al.* (1992). Cells were grown in MRS broth for 18 h at 37°C, centrifuged (3000 *g*, 15 min), washed three times with sterile distilled water and resuspended in an equal volume of sterile water. The bacterial suspension was incubated with 1% (v/v) of filtered sterilized supernatant at room temperature. The reactions were scored positive when a clearly visible sand-like particle, gravitated to the bottom of the tube, leaving a clear supernatant, within 1 h. *Lact. gasseri* 4B2 was added as a positive control.

Western blotting

Bacteria were grown in MRS broth for 18 h at 37°C and centrifuged (3000 *g*, 15 min). The protein profile of M247 and Mu5 was compared by Western blot analysis using a rabbit antiserum prepared against the APF of *Lact. gasseri* 4B2. Ten millilitres of supernatants were precipitated by adding sodium deoxycholic acid 0·04% (w/v, 15 min at room temperature) followed by trichloroacetic acid (TCA) 10% (v/v). After centrifugation, the pellet was neutralized with 5 μl NaOH 1 mol l^{-1}, resuspended in 100 μl loading buffer [0·075 mol l^{-1} Tris–HCl pH 6·8, 10% (v/v) glycerol, 5% (v/v) mercaptoethanol, 2% (w/v) SDS, 0·1% (w/v) bromophenol blue] and boiled for 3 min. A quantity of 15 μl of samples were run on a 10% SDS-PAGE gel and was electroblotted on nitro-cellulose by semi-dry transfer. The membrane was blocked for 1 h with a 3% solution of dry milk in tris-buffered saline [0·1 mol l^{-1} Tris–HCl pH 7·5 + 0·9% (w/v) NaCl] containing 0·05% (v/v) Tween-20 (TTBS). The membrane was then incubated for 2 h at room temperature with an anti-APF rabbit antiserum, diluted (1/500) in 3% (w/v) milk/TTBS. After washing with TTBS, the membrane was incubated for 2 h with goat anti-rabbit antibodies (Sigma) (1/10000) in 3% milk/TTBS and the membrane was developed with 5-bromo-4-chloro-3-indolyl phosphate and *p*-nitro blue tetrazolium chloride (NBT) (Sigma).

Sequencing and sequence analysis

The nucleotide sequence of the DNA adjacent to the *apf* gene of *Lact. crispatus* M247 was determined by inverse PCR and by amplification of long PCR fragments. The inverse PCR was initiated using pairs of divergent primers that had been designed based on *apf*A (accession number Y08498) from *Lact. gasseri* 4B2 [APF17 anti-sense (5′-ATCATTT TGAACAATAGTAGCAGCT-3′)/APF7 sense (5′-AATG GATCATGGCTAAGTACACTGT-3′); APF10 anti-sense (5′-AGCTGCTGCAAGTGACTTAGTTAA-3′)/APF7 sense; APF21 anti-sense (5′-TTCTGGCAAACTAACG GTTGGTACT-3′) and APF2 sense (5′-GTATTGAC CATTTCTTGCTGAGTAT-3′)]. Finally, long PCRs were performed using primers designed based on the promotor region of the sequenced *apf* gene of M247 and

primers designed based on upstream and downstream regions obtained by inverse PCR. A 17·2-kb fragment upstream the *apf* was obtained using the primers HM65 (5′-TGCAGAACTATAACAGGAATATCAC-3′) and HM9 (5′-TGTCAGTATTATCGTCCATTTCAGC-3′) and a 6·7-kb fragment downstream was obtained using primers APF6 (5′-TTTACTTGTTGGCTCTATTTGAATG-3′) and HM66 (5′-GTGATATTCCTGTTATAGTTCTG CA-3′). An overlapping 4·3-kb PCR fragment was also obtained using the primers APF14 (5′-AGTACCA ACCGTTAGTTTGCCAGAA-3′) and HM32 (5′-ATCG CCATTAAGATACCACCACCAA-3′). In order to compare the *apf* locus between M247 and Mu5, the same fragments were PCR amplified from chromosomal DNA of the nonaggregating mutant Mu5 and a region of 4·4-kb, which include the *apf* gene, was sequenced. Direct automated sequencing of long PCR fragments was performed as previously described (Iannelli *et al.* 1998). Blast was used to conduct homology searches in the GeneBank database (http://www.ncbi.nlm.nih.gov/BLAST) and the microbial genome database (http://www.ncbi.nlm.nih.gov/sutils/genom_table_cgi) available at the National Center for Biotechnology Information website. RNA secondary structure was predicted by the program GeneBee (http://www.genebee.msu.su/services/rna2_reduced.html). The presence of a signal peptide in APF and the site of cleavage was predicted using SignalP V2·0 (http://www.cbs.dtu.dk/services/SignalP) (Nielsen *et al.* 1997).

RESULTS

Characterisation of M247 and Mu5

M247 and Mu5 were shown to be different in their aggregation phenotype and in hydrophobicity. When grown in MRS medium, *Lact. crispatus* M247 spontaneously aggregated, leaving a clear supernatant while Mu5 remained in suspension (Fig. 1). As expected, Mu5 showed a reduced hydrophobicity in comparison with M247 and the maximum difference was found in the exponential phase (90% hydrophobicity for M247 vs 50% for Mu5).

Nature of the aggregation factor

The Mu5 and M247 were compared for the production of an aggregation factor. *Lact. crispatus* M247 and *Lact. gasseri* 4B2 showed a strong autoaggregation phenotype in MRS broth. After centrifugation and washing with distilled water, the autoaggregation property was lost and cells remained in suspension. The addition of 1% supernatant, including the supernatant from Mu5, was able to restore aggregation in water-washed cells of M247 and 4B2 but not of Mu5. The presence of APF was

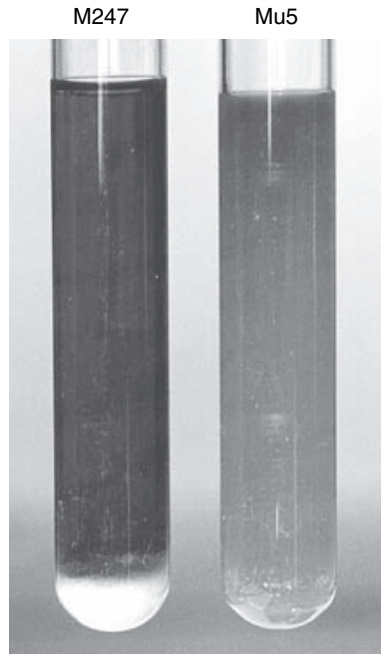

Fig. 1 Aggregation in *Lactobacillus crispatus* M247 and the isogenic nonaggregating mutant Mu5. Bacteria were grown overnight at 37°C in MRS medium

confirmed by Western blot analysis of M247 and Mu5 supernatants using a rabbit anti-APF serum (prepared against the APF of *Lact. gasseri* 4B2). A band of 28 kDa was observed both in M247 and Mu5 supernatants indicating secretion of the APF protein by both the aggregating and nonaggregating strains (Fig. 2). The amount of APF proteins produced by the wild-type and mutant strain was shown to be similar by the analysis of several different blots (data not shown).

Sequencing of the *apf* locus

Organization of the apf locus. A total of 24 582-bp were sequenced including 17 242-bp upstream and six 668-bp downstream of the M247 *apf* gene (GenBank accession number AF492458) (Fig. 3). The GC content was around 40% over the whole sequence and no major variation was observed. Homology of each open reading frame and proposed function are presented in Table 1. A 4·4-kb DNA region was also sequenced from PCR fragments amplified from chromosomal DNA of Mu5 (GenBank accession number AY583362) (Fig. 3). The sequence, which includes the *orf*14 (truncated cellobiose EIIC-like protein gene), the *orf*15 (*apf* gene) and the *orf*16 (transposase gene), was 100% identical between M247 and Mu5. This finding confirms the results obtain by Western blot showing that the Mu5 strain can secrete a normal APF.

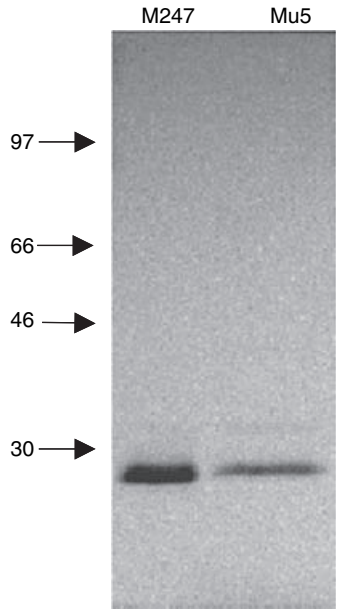

Fig. 2 Western blot analysis of proteins from supernatants of *Lactobacillus crispatus* M247 and Mu5. The proteins were detected using an antiserum against the APF of *Lactobacillus gasseri* 4B2. In both strains, a major band at 28 kDa is detected. Numbers on the left refer to molecular masses in kilodaltons.

The *apf* gene was found to be an open reading frame (ORF) of 672 nucleotides preceded by a consensus ribosome binding site (aggagag). The *apf* gene initiates with a TTG start codon and encodes a protein of 223 amino acids with a predicted molecular weight of 24·0 kDa. The protein also contains a putative signal peptide with a predicted cleavage site between the amino acids 33 and 34. The predicted leader has a mass of 3·3 kDa which means that a protein of 20·6 kDa would result after cleavage.

In the intergenic region upstream of the *apf* (367 bp), putative transcriptional start sites are located but no potential ORF. A putative transcription terminator sequence can also be found immediately after the gene upstream of *apf* (truncated cellobiose EIIC-like protein gene). A strong rho independent stem-loop structure with a free energy of $-16{\cdot}5$ kcal mol^{-1} was detected 24 bases downstream the *orf*14.

The spatial organization of the chromosomal region surrounding the *apf* gene was compared with the homologous regions of the complete genome of *Lact. johnsonii* NC 533 (accession number AE017198). Two APF genes are found in *Lact. johnsonii* NC 533 (accession number NC_005362). This comparison showed no conservation of the ORFs of the *Lact. crispatus* M247 genome region to the one surrounding the *apf* genes in *Lact. johnsonii*. Only the ORFs 3, 4, 5 and 9 were found to be present upstream the *apf* genes in *Lact. johnsonii* while the downstream region was different (Table 1).

Homology of APF of Lact. crispatus M247. The deduced amino acid sequence of APF was used for homology searches in the databases. The *Lact. crispatus* protein is highly homologous in its N-terminal (first 106 amino acids) and C-terminal (last 79 amino acids) part to the sequenced APF genes of *Lact. acidophilus*, *Lact. gasseri* and *Lact. johnsonii* (Fig. 4). In between these two regions of homology, a central part rich in asparagine, glutamine, threonine and alanine, varies in length between the different APF (Fig. 4). Contrary to the *Lact. gasseri* and *Lact. johnsonii* strains which contain two tandem APF genes, *Lact. crispatus* M247 contains only one copy of the *apf* gene. The *apf* gene was shown to be absent from *Lact. plantarum* WCFS1 genome (accession number NC_004567).

The C-terminus of *Lact. crispatus* APF (last 79 amino acids) also showed a strong homology with the C-terminus (last 73 to 79 amino acid) of the following proteins: three extracellular proteins (accession number NP_784118, NP_786208 and NP_786209) of *Lact. plantarum* WCFS1 (respectively 76, 74 and 78% identity), the APF1-like protein (accession number AAP94109) of *L. reuteri* DSM20016 (80% identity), a hypothetical protein (accession number ZP_00036556) of *Streptococcus agalactiae* NEM316 (80% identity), the LysM domain protein (accession number AE007327) of *Strep. pneumoniae* TIGR4 (78% identity), the LysM domain protein (accession number NP_814228) of *Ent. faecalis* V583 (78% identity), a hypothetical protein (accession number ZP_00036556) of *Ent. faecium* (75% identity), and a hypothetical protein (accession number AAN59736) of *Strep. mutans* UA159 (72% identity). All these proteins contain a LysM domain at the N-terminus. The LysM domain is absent in APF and no homology was found between the N-terminus of APF and the N-terminus of these LysM domain proteins.

DISCUSSION

In a previous study, the presence of an aggregation phenotype in *Lact. gasseri* 4B2, an enteric strain, was shown to be induced by a secreted 32-kDa protein, named APF (Reniero *et al.* 1992). In this study, we characterized the aggregation phenotype and the nature of the aggregation factor in *Lact. crispatus* M247, and the nonaggregating mutant Mu5. The *apf* loci of M247 and Mu5 strains were also sequenced.

The nonaggregating mutant strain was shown to be less hydrophobic than the parent strain. Relation between hydrophobicity and autoaggregation of *Lactobacillus* have been previously reported (Wadstrom *et al.* 1987; Reniero *et al.* 1992; Kmet and Lucchini 1997). The hydrophobicity is generally associated with proteins and lipoteichoic acid or a complex between the two molecules (Wadstrom *et al.* 1987).

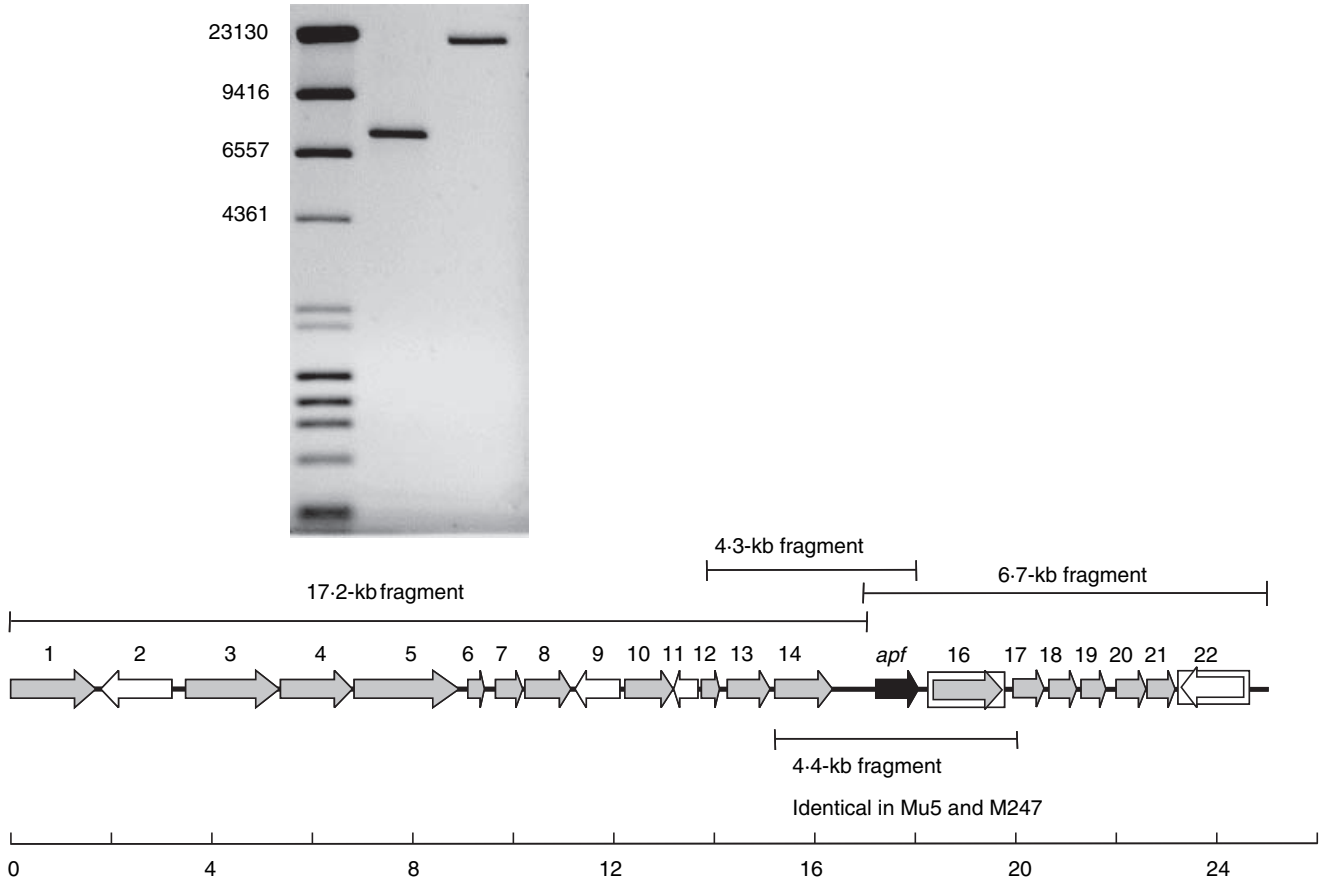

Fig. 3 Structure of the *apf* locus of *Lactobacillus crispatus* M247. Three PCR fragments were used as a template for sequencing. The agarose gel shows the 6·7- and 17·2-kb fragments that were sequenced. An overlapping 4·3-kb region was also sequenced (not shown on the gel). The ORFs and their direction are represented by arrows. Sequence data analysis showed the presence of 22 ORFs of which 18 are in the same direction. The ORF 14 is truncated. The insertion elements encoding for ORF 16 and ORF 22 are represented by squares. A 4·4-kb region (including the *apf* gene) of the isogenic nonaggregating mutant Mu5 was also sequenced and was found to be 100% homologous to M247. The scale is in kilobases

The supernatant of M247 and Mu5, contained a factor similar to that of 4B2, which is able to induce aggregation. Although the Mu5 mutant cannot aggregate, we showed that its supernatant can induce aggregation in both *Lact. crispatus* M247 and *Lact. gasseri* 4B2. Furthermore, the Mu5 mutant produced a similar level of APF in the supernatant as the aggregating wild type M247 strain. The APF locus has been sequenced and no difference was seen between the two strains. These results confirm previous results obtained by Jankovic *et al.* (2003), showing no difference in APF secretion between aggregating and nonaggregating mutant strains of *Lact. gasseri* 4B2. Aggregation is a complex phenomenon that may involve interactions between surfaces and molecules. The nonaggregation phenotype in Mu5 may be due to an anomaly in the expression or the synthesis of another molecule involved in aggregation, including an APF receptor. One of the candidate receptors for APF has been identified as α-1,2-glucose-substituted lipoteichoic acid or teichoic acid (Reniero *et al.* 1992). The APF-mediated aggregation of *Lact. gasseri* 4B2 was shown to be inhibited by lipoteichoic acid containing a glucosyl substituent (*O*-α-D-glucopyranosyl-(1,2)-D-glucose) attached to the primary hydroxyl group of glycerol (Reniero *et al.* 1992). A defect in glucosylation of teichoic acid could explain the nonaggregation phenotype in *Lact. crispatus* Mu5 but further experiment are necessary to determine the existence and nature of an APF receptor.

Sequence analysis of the *apf* locus showed no conservation of the ORFs of the *Lact. crispatus* M247 genome region in comparison with the one surrounding the *apf* gene in *Lact. johnsonii* and *Lact. gasseri* strains (Ventura *et al.* 2002; Pridmore *et al.* 2004). These results suggest that the *apf* is not part of an organized gene cluster including genes encoding the receptor or molecules involved in conjugation as reported for other aggregation proteins (Weidlich *et al.* 1992). Similarities in the gene organization around the *apf*

Table 1 Homologies of the ORFs with other microbial genes

ORF*	Position	Homologous gene†	Origin	Proposed function of gene product*	*E* value	Amino acid identity (%)	Amino acid similarity (%)
*orf*1 (>512)	<1–1540	LJ1296 (NP_965151)	*Lact. johnsonii* NCC 533	ABC transporter ATPase and permease components (539)	1e-104	207/510 (40)	319/510 (62)
*orf*2 (552)	Complement (1631–3289)	LJ0149 (NP_964165)	*Lact. johnsonii* NCC 533	Hypothetical protein LJ0149 (552)	0·0	497/551 (90)	518/551 (94)
		Lgas1113 (ZP_00046936)	*Lact. gasseri*	Phosphoenolpyruvate carboxykinase (413)	0·0	379/412 (91)	395/412 (95)
*orf*3 (616)	3473–5323	LJ1762 (NP_965568)	*Lact. johnsonii* NCC 533	Hypothetical protein LJ1762 (626)	1e-102	230/632 (36)	360/632 (56)
*orf*4 (442)	5328–6656	Lgas0524 (ZP_00046357)	*Lact. gasseri*	Hypothetical protein (444)	0·0	372/440 (84)	410/440 (93)
		LJ1761 (NP_965567)	*Lact. johnsonii* NCC 533	Hypothetical protein LJ1761 (444)	0·0	368/440 (83)	410/440 (93)
*orf*5 (743)	6668–8899	Lgas0525 (ZP_00046358)	*Lact. gasseri*	Lhr-like helicases (740)	0·0	575/739 (77)	647/739 (87)
		LJ1760 (ZP_00046357)	*Lact. johnsonii* NCC 533	Hypothetical protein LJ1760 (740)	0·0	574/739 (77)	645/739 (87)
*orf*6 (125)	8931–9308	None					
*orf*7 (416)	9532–10782	*amtB* (NP_784158)	*Lact. plantarum* WCFS1	Ammonium transporter (438)	1e-120	249/412 (60)	311/412 (75)
*orf*8 (254)	10828–11592	LJ0257 (NP_964274)	*Lact. johnsonii* NCC 533	Nitro/flavin reductase (252)	6e-32	85/246 (34)	135/246 (54)
*orf*9 (135)	Complement 11967–12374	LJ1747b (NP_965553)	*Lact. johnsonii* NCC 533	Hypothetical protein (134)	2e-10	38/67 (56)	53/67 (79)
*orf*10 (200)	12515–13117	lp_2761 (NP_786141)	*Lact. plantarum* WCFS1	O-acetyltransferase (200)	3e-68	127/192 (66)	144/192 (75)
*orf*11 (131)	Complement (13169–13564)	LJ1847 (NP_965826)	*Lact. johnsonii* NCC 533	Hypothetical protein LJ1847 (132)	8e-14	44/122 (36)	66/122 (54)
		Lgas1370 (ZP_00047190)	*Lact. gasseri*	Hypothetical protein (132)	4e-12	34/98 (34)	55/98 (56)
*orf*12 (76)	13661–13891	None					
*orf*13 (290)	14281–15153	TM0742 (AE001744)	*Thermotoga maritime*	Serine/threonine protein phosphatase (209)	4e-15	61/189 (32)	91/189 (47)
*orf*14 (248)‡	15538–16284	LJ0531 (NP_965724)	*Lact. johnsonii* NCC 533	PTS system EIIC, probable cellobiose specific (443)	0·0	354/440 (80)	400/440 (90)
apf (223)	17243–17914	apf1 (AY148189.1)	*Lact. johnsonii* ATCC 332	Aggregation-promoting factor (257)	9e-43	139/258 (53)	169/258 (65)
		LJ1746 (NP_965551)	*Lact. johnsonii* NCC 533	Aggregation-promoting factor (267)	1e-35	120/239 (50)	144/239 (60)
		apf1 (AY245438.1)	*Lact. gasseri* 4B2	Aggregation-promoting factor (261)	6e-37	127/255 (49)	157/255 (61)
*orf*16 (248)	18255–19481	*orfX* (L26311)	*Lact. helveticus*	Transposase for insertion sequence IS1201, mutator family (369)	0·0	333/368 (90)	352/368 (95)
*orf*17 (273)	19700–20521	*prgA* (AE006493)	*Streptococcus pyogenes* M1	Surface exclusion protein (873)	8e-07	71/288 (24)	120/288 (41)
*orf*18 (191)	20597–21172	lin1208 (AL596168)	*Listeria innocua*	Phosphoglycerate mutase (199)	1e-28	77/188 (40)	106/188 (55)
*orf*19 (196)	21179–21769	*Orf*18 (This study)	*Lact. crispatus* M247	ORF 18 (191)	8e-40	88/192 (45)	120/192 (61)
		lin1208 (AL596168)	*L. innocua*	Phosphoglycerate mutase (199)	2e-23	72/184 (39)	98/184 (53)
*orf*20 (219)	21838–22497	FN1347 (AE010639)	*Fusobacterium nucleatum*	Hypothetical cytosolic protein (218)	1e-9	48/197 (24)	92/197 (46)
*orf*21 (240)	22508–23230	glpQ1 (NP_784968)	*Lact. plantarum* WCFS1	Glycerophosphodiester phosphodiesterase (228)	1e-53	100/221 (45)	148/221 (66)
*orf*22 (344)	Complement (23361–24395)	ISLdl-3 (AJ316615)	*Lact. delbrueckii* subsp. *lactis*	Transposase for insertion sequence element, IS30 family (342)	6e-80	165/345 (47)	223/345 (63)

*The number of amino acids is in parentheses.

†The accession number is shown in parentheses.

‡The ORF is truncated at position 248. The stop (TAG) at position 248 of the protein sequence has been confirmed by sequencing of different PCR fragments.

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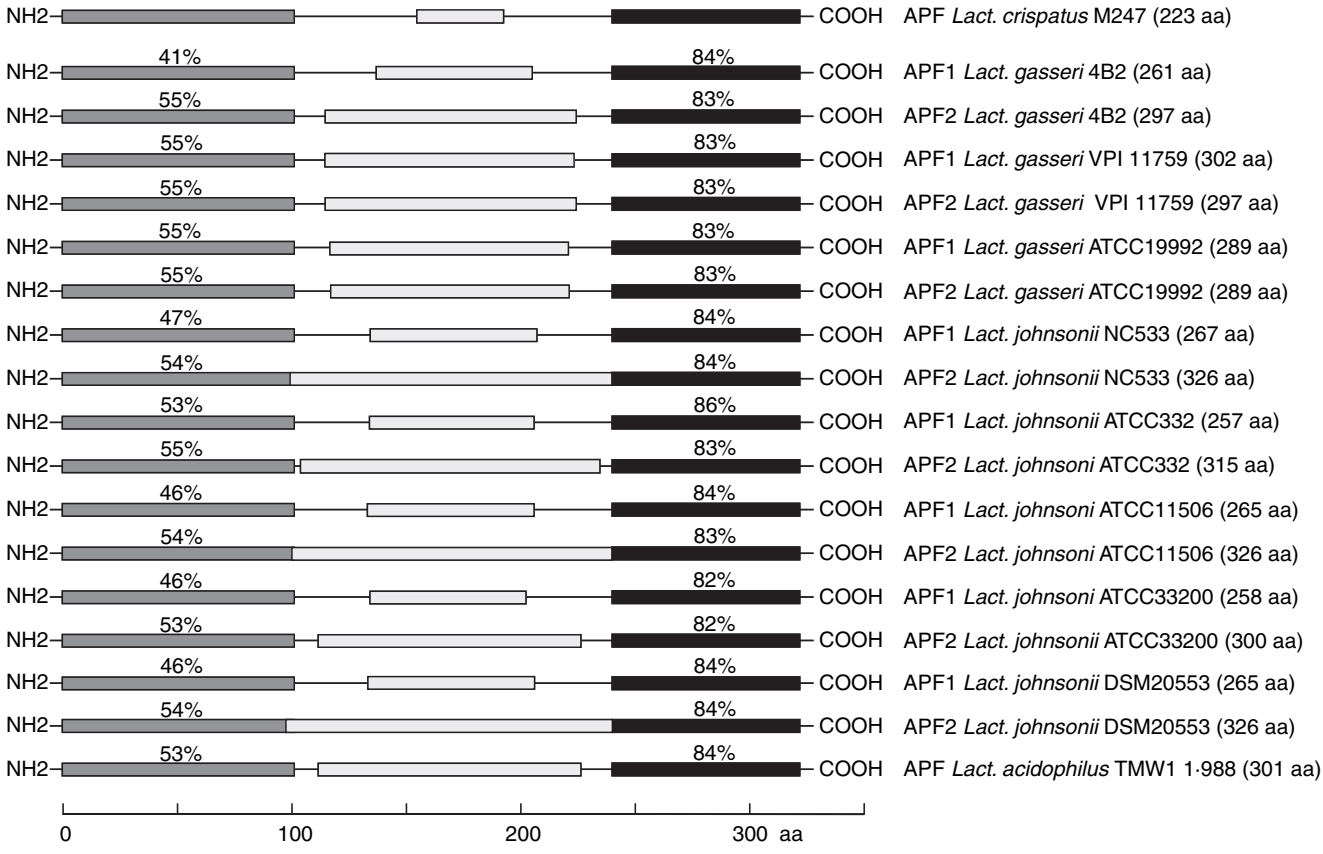

Fig. 4 Homology of *Lactobacillus crispatus* M247 APF with other *Lactobacillus* APF proteins. The APF can be divided in three domains. The homology was more pronounced at the C-terminus (78–86%) (black box) than at the N-terminus (40–55%) (dark grey box). The central region rich in asparagine, glutamine, threonine and alanine, varies in length between different APFs and is of lower homology (light grey box). The homology was found with the APF of *Lact. acidophilus* TMW1·988 (accession number AJ438291) and to the two APF genes (APF1 and APF2) of the following strains: *Lact. gasseri* 4B2 (accession number AY245438), *Lact. gasseri* VPI 11759 (accession number AY148188 and AF543464), *Lact. gasseri* ATCC 19992 (accession number AF543458 and AF543459), *Lact. johnsonii* ATCC332 (accession number AY148189 and AF543460), *Lact. johnsonii* NC533 (La1) (accession number NC_005362), *Lact. johnsonii* ATCC 33200 (accession number AY148190 and AF543461), *Lact. johnsonii* ATCC 11506 (accession number AY148192 and AF543463), and *Lact. johnsonii* DSM 20553 (accession number AY148191 and AF543462)

gene of seven *Lact. gasseri* and *Lact. johnsonii* strains have been reported but probably because these strains are more closely phylogenetically related (Ventura *et al.* 2002).

Until now, the *apf* gene has only been found in obligately homofermentative lactobacilli (*Lact. crispatus*, *Lact. acidophilus*, *Lact. gasseri* and *Lact. johnsonii*) and it is absent in *Lact. plantarum*, an heterofermentative species (Ventura *et al.* 2002; Kleerebezem *et al.* 2003, this study). Only one copy of the *apf* gene is present in *Lact. crispatus* M247 and *Lact. acidophilus* TMW1·988 while two copies are present in *Lact. gasseri* and *Lact. johnsonii* strains. The C-terminus of the APF protein was shown to be more conserved and also showed a strong homology with the C-terminus of LysM domain proteins from *Lact. plantarum*, *Lact. reuteri*, *Streptococcus* spp. and *Enterococcus* spp. The conserved C-termini among *Lactobacillus*, *Streptococcus* and *Enterococcus* must represent a new domain in Gram-positive bacteria. The function of this domain has still to be determined but it could be involved in the attachment of proteins (including APF proteins) to the cell wall of Gram-positive bacteria. The LysM domain proteins contain a LysM domain at their N-terminus, which is absent in APF. The available evidence suggests that the LysM domain has a general peptidoglycan-binding function (Bateman and Bycroft 2000).

A previous study has shown that the nonaggregating mutant Mu5 has a reduced capability to colonize the human intestinal tract in comparison with the wild-type M247, suggesting an importance of aggregation in colonization (Cesena *et al.* 2001). Our results showed that the lost aggregation capacity in Mu5 and consequently its reduced colonization ability is not related to a defect in secretion of

the APF protein or a mutation in the *apf* gene. Our results rather suggest that the mutation in Mu5 may be contained in another molecule involved in aggregation such as a possible receptor for APF.

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