Contribution of Aggregation-Promoting Factor to Maintenance of Cell Shape in *Lactobacillus gasseri* 4B2

Ivana Jankovic,^{1*} Marco Ventura,¹ Valerie Meylan,¹ Martine Rouvet,¹
Marina Elli,² and Ralf Zink¹

Nestlé Research Center, Vers-chez-les-Blanc, 1000 Lausanne 26, Switzerland,¹ and Università Cattolica del Sacro Cuore, Istituto di Microbiologia, 29100 Piacenza, Italy²

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Aggregation-promoting factor (APF) was originally described as a protein involved in the conjugation and autoaggregation of *Lactobacillus gasseri* 4B2, whose corresponding *apf* gene was cloned and sequenced. In this report, we identified and sequenced an additional *apf* gene located in the region upstream of the previously published one. Inactivation of both *apf* genes was unsuccessful, indicating that APF function may be essential for the cell. Overproduction of APF proteins caused drastic alteration in the cell shape of this strain. These cells were irregular, twisted, enlarged, and tightly bound in unbreakable clumps of chains. Down-regulation of APF synthesis was achieved by cloning of the *apf2* promoter region on a high-copy-number plasmid, which recruited a putative *apf* activator. As a consequence, the shape of the corresponding recombinant cells was elongated (filamentous) and cell division sites were no longer visible. None of the induced changes in APF production levels was clearly correlated with modifications of the aggregation phenotype. This report shows, for the first time, that APF proteins are mainly critical for *L. gasseri* 4B2 cell shape maintenance.

Lactic acid bacteria are normal inhabitants of the human oral cavity and gastrointestinal (GI) and urogenital tracts. Interest in the health-promoting effects of specific lactic acid bacterial strains has increased in recent years (10, 12). Survival and persistence of probiotic bacteria in the human GI tract is often reported to provide them a competitive advantage. One of the proposed mechanisms that could increase the potential of bacteria to survive and persist in the GI tract is their ability to aggregate (13). However, this hypothesis has never been confirmed.

To study the importance of aggregation, we chose an aggregating strain, *Lactobacillus gasseri* 4B2 (previously classified as *L. plantarum* 4B2), whose 32-kDa aggregation-promoting factor (APF) has already been described (25). This protein was purified from the culture supernatant as one of the most abundant proteins. The function of APF as a factor of aggregation was shown in vitro. After three subsequent washing steps, *L. gasseri* 4B2 lost its ability to aggregate. Addition of purified APF or of filtered supernatant reaggregated the washed cells. Furthermore, the role of APF in conjugation was also demonstrated (25). The frequency of pAM β 1 conjugal transfer (among *Lactobacillus* species whose aggregation is APF dependent) was increased upon addition of *L. gasseri* 4B2 filtered supernatant to the mating mixture. Since neither the *apf* gene nor any *apf* mutant was available, the exact physiological role of APF could not be demonstrated. Subsequent N-terminal sequencing of the *L. gasseri* 4B2 APF protein enabled the cloning and sequencing of the *apf* gene (accession no. Y08498; L. Morelli et al., direct submission).

Recently, we showed that four *L. johnsonii* and two *L. gasseri*

strains contain two *apf* genes in their genomes (34). Analysis of the gene organization, amino acid composition, and physical properties of APF supported certain similarities of APF to S-layer proteins. Although APF was originally isolated from the cell supernatant, significant amounts of APF were found in a lithium chloride preparation of the surface proteins, showing that APF is able to noncovalently attach to the cell surface.

In this report, we demonstrate the presence of a second *apf* gene in *L. gasseri* 4B2. We further show that an imbalance of the APF concentration in the cell results in dramatic alterations of *L. gasseri* 4B2 cell shape.

MATERIALS AND METHODS

Bacterial strains and media. *L. gasseri* strain 4B2 and its aggregation-negative mutant *L. gasseri* Agg[−], which were used in the present study, were kindly provided by L. Morelli (University of Piacenza, Piacenza, Italy). Cloning in *Escherichia coli* was performed with strain EC101 (17). *Lactobacillus* strains were grown in Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories, Detroit, Mich.) at 37°C for 24 h under static conditions or on MRS agar in an anaerobic atmosphere (with the AnaeroGen system [Oxoid, Basingstoke, United Kingdom]) supplemented with 10 μ g of chloramphenicol per ml or 5 μ g of erythromycin per ml where appropriate. *E. coli* was grown under agitation at 37°C in Luria-Bertani broth supplemented with 30 μ g of chloramphenicol per ml or 150 μ g of erythromycin per ml where appropriate.

Microbial techniques. The aggregation phenotype was scored positive if the overnight cultures were clear with cells clumped at the bottom of the tube (Fig. 1). The strains were considered nonaggregating if the overnight cultures were turbid.

The isogenicity of *L. gasseri* Agg[−] and 4B2 was confirmed by rapid amplified ribosomal DNA restriction analysis (33) with restriction enzymes *Sau*3AI, *Hin*II, and *Dra*I and by the random amplified polymorphic DNA method (8).

Sensitivity of strains to different cell wall-targeted antibiotics was tested with ready-to-use antibiotic discs (BioMérieux). Bacterial cultures were plated on MRS plates, and antibiotic discs were placed on the agar surface. After 16 h of incubation at 37°C, the zones of inhibition were measured and compared to each other.

For plasmid stability testing, cultures were subcultured five times with no antibiotic. Each subculture was diluted and plated on MRS agar. The grown colonies were replicated on chloramphenicol (10 μ g/ml)-containing MRS plates. The percentage of resistant colonies was calculated for each growth cycle.

* Corresponding author. Mailing address: Nestlé Research Center, P.O. Box 44, Vers-Chez-Les-Blanc, 1000 Lausanne 26, Switzerland. Phone: 41-21-785-8346. Fax: 41-21-785-8549. E-mail: ivana.jankovic@rdls.nestle.com.

FIG. 1. Aggregation and nonaggregation phenotypes. (A) Aggregating *L. gasseri* 4B2. (B) Nonaggregating *L. gasseri* Agg⁻.

DNA manipulations and transformation. DNA manipulations, plasmid isolation, and transformation of *E. coli* were performed in accordance with standard procedures (26). All plasmid constructions were performed with *E. coli*. Plasmid DNA was introduced into *Lactobacillus* strains by electroporation (24). PCR was carried out with *Taq* polymerase (Roche) and the Expand high-fidelity PCR system (Roche).

Inactivation of *apf* genes of *L. gasseri* 4B2. For double inactivation of the *apf1* and *apf2* genes, a derivative of plasmid pDP600 (R. D. Pridmore, unpublished data) was constructed. Plasmid pDP600 was constructed by exchanging the erythromycin resistance gene of pGh9 (19) with a chloramphenicol resistance gene. The erythromycin cassette (*eryR*) was reinserted back into the multiple cloning site, thereby creating multiple cloning sites upstream and downstream of the *eryR* gene. For double *apf* gene inactivation, the regions flanking *apf1* and *apf2* were cloned upstream and downstream of *eryR*, respectively. The upstream fragment was amplified with primers B10 and B12 (Table 1) and cloned into the *Xba*I and *Bam*HI restriction sites of plasmid pDP600, upstream of the *eryR* cassette (Fig. 2). For downstream fragment amplification, primers B7 and B8 (Table 1) were used. The fragment was then cloned into the *Hind*III and *Sal*I cloning sites downstream of the *eryR* cassette (Fig. 2). The resulting plasmid, pVM20, was transformed into *L. gasseri* 4B2, which was grown at a permissive

temperature (30°C) in the presence of 10 µg of chloramphenicol per ml or 5 µg of erythromycin per ml. After raising the temperature to a nonpermissive 40°C, we screened for erythromycin-resistant, chloramphenicol-sensitive colonies in which gene replacement occurred and which had lost the plasmid.

To inactivate the *apf1* gene of *L. gasseri* 4B2, a fragment proximal to the N terminus of the gene was amplified with primers B13 and B14 (Table 1; Fig. 2). The fragment was cloned into the *Spe*I and *Sal*I sites of the pGh9 (19) temperature-sensitive vector to produce pVM21. For *apf2* inactivation, primers B1 and B2 (Table 1; Fig. 2) were used. The amplified fragment was cloned into the *Eco*RI and *Sal*I sites of the pGh9 vector to produce pVM22. The resulting plasmids, pVM21 and pVM22, were transformed into *L. gasseri* 4B2, which was grown at a permissive temperature (30°C) in the presence of 5 µg of erythromycin per ml. After the temperature was raised to a nonpermissive 40°C, erythromycin-resistant colonies containing the plasmid integrated into either of the *apf* genes in the chromosome were selected. Disruption of the *apf1* gene was confirmed by PCR with primer B10 and pGh9-specific primer G1 (Table 1). Disruption of the *apf2* gene was confirmed by PCR with primers B3 and G1 (Table 1).

Cloning of *apf1* and *apf2* into a high-copy-number plasmid. The *apf1* and *apf2* genes of *L. gasseri* 4B2 were amplified from chromosomal DNA with the primer pairs B10/B15 and B3/B4, respectively (Table 1; Fig. 2). The *apf1* fragment was cloned into the *Xba*I and *Hind*III restriction sites of pNZ124, and the *apf2* fragment was cloned into the *Bam*HI and *Eco*RI restriction sites of pNZ124 (32). The resulting plasmids, pVM23 and pVM24, carried the *apf1* and *apf2* genes of *L. gasseri* 4B2 under the control of their own promoters, respectively. Correct cloning was confirmed by restriction analysis and DNA sequencing with pNZ124-specific primers N-L and N-R (Table 1).

Cloning of the *apf2* promoter region into a high-copy-number plasmid. The promoter region of the *apf2* gene of *L. gasseri* 4B2 was amplified from chromosomal DNA with primers B3 and L12 (Table 1; Fig. 2). The amplicon was cloned into the *Bam*HI and *Eco*RI restriction sites of pNZ124 (23) to produce pVM25. Correct cloning was confirmed by restriction analysis and DNA sequencing with pNZ124-specific primers N-L and N-R (Table 1).

DNA sequencing. Sequences upstream of *apf2* in *L. gasseri* 4B2 and the *apf* locus of *L. gasseri* Agg⁻ were obtained by primer walking on chromosomal DNA or PCR fragments. All sequencing reactions were performed by Microsynth GmbH (Balgach, Switzerland).

RNA preparation and primer extension analysis. For RNA isolation, the cells were harvested in the exponential growth phase. Bacterial cell pellets were disrupted with 106-µm glass beads in a Mini-Beadbeater-8 cell disrupter (Biospec Products). Total RNA was isolated with the RNeasy midi kit (Qiagen, Hilden, Germany). Primer extension analyses were performed as described previously (2), with avian myeloblastosis virus reverse transcriptase (Stratagene) and infrared dye (IRD800)-labeled primers. To map the transcription start sites of the *apf1* and *apf2* genes, primers A1 and A2 (Table 1), labeled with the infrared dye IRD800 at the 5' end were used, respectively. Sequencing reactions were performed by the chain-terminating sequencing method (27) with a fluorescence-

TABLE 1. Oligonucleotides used in this study

Primer	Sequence ^a	Modification
B1	5'-AATCAGAATTCAATCTATCTTAACTAAG-3'	<i>Eco</i> RI
B2	5'-AATCAGTCGACGTTGTACATTGTAGTTG-3'	<i>Sal</i> I
B3	5'-AATCAGGATCCCGCTACGGTTCATGGA-3'	<i>Bam</i> HI
B4	5'-AATCAGAATTCCACCAAAAGGTGATCTTCC-3'	<i>Eco</i> RI
B5	5'-CCTTGGTGCAAACCAATGGG	
B7	5'-AATCAAGCTTACAGCTACGCTTCAGC-3'	<i>Hind</i> III
B8	5'-AATCAGTCGACGAACCAGCGCATGATGG	<i>Sal</i> I
B10	5'-AATCATCTAGACTAAACGATGGGACGAGCG-3'	<i>Xba</i> I
B12	5'-AATCAGGATCCAAGCAGTTGCGTGTGG-3'	<i>Bam</i> HI
B13	5'-AATCAACTAGTGCTTAACAATAATTGAAATCAAC-3'	<i>Spe</i> I
B14	5'-AATCAGTCGACCTACTGAAGTCTTAGGAGCTG-3'	<i>Sal</i> I
B15	5'-AATCAAAGCTTAAAAGTGCCCTATGTTACAG-3'	<i>Hind</i> III
L12	5'-AATCAGAATTCTACCAAAACCAAGTTGCGG	<i>Eco</i> RI
G1	5'-GTAAAACGACGGCCAGT-3'	
N-L	5'-GTCATAACCTGCCCCG-3'	
N-R	5'-CTCATTGAGAAGATTGCCG-3'	
A1	5'-CTTGTGGCCAACAGTAACCTCTGC	IRD800
A2	5'-GCTACCAAACCAAGTTGCGG-3'	IRD800

^a Introduced restriction site is underlined.

FIG. 2. Genetic organization of the *apf* locus in *L. gasseri* 4B2. The nucleotide sequences of the *apf1* and *apf2* promoter regions are shown at the top. Putative RNA polymerase binding sites are underlined. Transcriptional start sites are shown in bold type and indicated by vertical arrows. Putative ribosomal binding sites (RBS) are underlined. Primers and their directions are represented by horizontal arrows. PCR amplicons are presented at the bottom, and cloning sites are indicated. The cloning strategy is outlined on the right. k.o., knockout; o.p., overproduction; d.r., down regulation.

labeled primer cycle sequencing kit (Amersham Biosciences Europe GmbH, Freiburg, Germany) and the same primers. Reverse transcripts, together with sequencing reactions, were run on 8% polyacrylamide-urea gels and detected in a Li-Cor DNA sequencer.

Anti-APF antibodies. Polyclonal anti-APF antibodies were raised in a rabbit (Eurogentec, Seraing, Belgium). Two polypeptides were selected from the APF sequence for a high probability of surface exposure and for a high antigenic index. The peptides EP011668 (H₂N-CADNYVKSRYGSGWTG-CONH₂) and EP011669 (H₂N-CGRESGGSYSARNGOY-CONH₂) were synthesized and coupled with keyhole limpet hemocyanin. Testing of APF specificity by Western blot analysis showed that the polyclonal antibodies against the first peptide (EP011668) were more specific, and they were subsequently used in all experiments.

Western blot analysis. Crude protein extracts were prepared from cultures grown overnight on MRS agar. The cells were scraped from the surface, washed in phosphate-buffered saline (PBS) (26), and disrupted in a Mini-Beadbeater-8 cell disrupter (Biospec Products). Protein concentrations were determined spectrophotometrically by the method of Bradford (5), and 20 µg of total protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. Western blot analysis was performed with polyclonal anti-APF antibodies and a chemiluminescent Western Breeze kit (Invitrogen).

Scanning electron microscopy. The cells grown overnight were scraped from MRS agar, washed twice in PBS, fixed overnight with 2.5% glutaraldehyde in PBS, and washed with PBS. Postfixation was done with 1% osmium tetroxide in PBS, followed by dehydration in increasing concentrations of ethanol. Samples were subsequently dried to the critical point and coated with 20 nm of gold in a scanning electron microscopy coating unit (ES100, Polaron LTT). All samples were examined with a Philips SEM 505 scanning electron microscope at an accelerating voltage of 30 kV.

Nucleotide sequence accession number. The DNA sequence reported here was submitted to the GenBank database and assigned accession no. AY245438.

RESULTS

***apf* sequence analysis.** In a previous study (25), only one APF protein of *L. gasseri* 4B2 was identified and the corresponding gene was sequenced later (accession no. Y08498). However, in all of the strains of *L. gasseri* and *L. johnsonii* analyzed so far, two adjacent *apf* genes have been found (34). To determine if another *apf* gene is present in the genome of *L. gasseri* 4B2, we sequenced the region upstream of the published *apf* gene, which, according to the sequence similarity, was a homologue of *apf2*. As expected, the presence of a second gene (*apf1*) upstream of *apf2* was confirmed (Fig. 2).

Both *apf* genes are preceded by a putative ribosomal binding site and are initiated by a TTG start codon. Putative rho-independent terminator structures with calculated ΔG values of -10.8 and -13.2 kcal follow *apf1* and *apf2*, respectively (Fig. 2). The corresponding APF1 and APF2 proteins are 261 and 297 amino acids long, with predicted molecular masses of 28.19 and 31.98 kDa, respectively. They exhibit 52% identity to each other on the amino acid sequence level. APF1 is preceded by a signal sequence with the predicted cleavage site VKA-AE between amino acids 38 and 39, while the predicted APF2

FIG. 3. (A) Domain architecture of proteins containing conserved C termini. Functionally distinct proteins containing a conserved C terminus were clustered into three groups: group I, containing APF proteins, group II, containing putative proteins with a LysM domain (peptidoglycan binding motif), and group III, containing putative proteins with an SLT domain (lytic transglycosylases). Signal peptides (sp) are indicated by black boxes. Conserved domains LysM and SLT are in gray. The homologous sequence region is indicated by striped boxes. (B) Alignment of conserved C termini. CLUSTAL software was used (11). Lagas1, APF1 protein from *L. gasseri* 4B2; Lagas2, APF2 protein from *L. gasseri* 4B2; Lajoh1, APF1 protein from *L. johnsonii* NCC533 (La1) (accession no. AAN63951); Lajoh2, APF2 protein from *L. johnsonii* NCC533 (La1) (accession no. AAN63952); Enfcm1, a protein of *Enterococcus faecium* (accession no. NZ_AAAK01000149); Enfcm2, a protein of *E. faecium* (accession no. NZ_AAAK01000213); Enfae, LysM domain protein of *Enterococcus faecalis* (EF0443, The Institute for Genomic Research, unfinished genome); Stpne1, LysM domain protein of *Streptococcus pneumoniae* (accession no. NC_003028); Stpne2, a protein of *S. pneumoniae* R6 (accession no. NC_003098); Stmut, LysM domain protein from *Streptococcus mutans* (accession no. NP_722430); Staga, LysM domain protein from *Streptococcus agalactiae* (accession no. NC_004116); Stcoe1, a protein from *Streptomyces coelicolor* (accession no. AL357591); Stcoe2, a protein from *S. coelicolor* (accession no. AL391754); Thfus, a protein from *Thermobifida fusca* (DOE_2021, Joint Genome Institute, unfinished genome).

cleavage site, AQA-AT, lies between amino acids 34 and 35 (22).

Similarity searches of databases identified several proteins sharing homology with the whole sequences of the APF proteins. The hypothetical proteins of the recently sequenced *L. gasseri* ATCC 33323 genome (accession no. ZP_00047488 and ZP_00047489) share 96 and 79% identity with *L. gasseri* 4B2 APF1 and APF2, respectively. The *L. acidophilus* APF protein (accession no. AJ438291) is 83% identical to *L. gasseri* 4B2 APF2. Moreover, proteins of five *L. gasseri* and *L. johnsonii* strains (34) show identities with *L. gasseri* 4B2 ranging from 48 to 99% for APF1 and from 81 to 99% for APF2. In addition, 10 hypothetical proteins sharing homology with the APF proteins only in the C-terminal part of the sequence were identified (Fig. 3). Closer sequence analysis revealed that these proteins, which are predicted to be secreted (22), could be classified into three different groups. The proteins of the first group consist of APF homologues for which surface localization has been demonstrated (34). The second group includes proteins containing LysM, a domain that, together with additional sites, probably mediates binding to the peptidoglycan (3). The proteins of the third group have a recognizable SLT motif, typical for lytic transglycosylases (16), which also need to be attached to the cell surface in order to reach their substrate. Thus, this C-terminal domain, shared by three different protein

groups of different microorganisms, is a good candidate for a novel cell surface anchoring region in gram-positive bacteria.

Analysis of *apf* gene transcription. To determine the transcriptional start points of *apf1* and *apf2*, primer extension reactions were carried out with *apf1*- and *apf2*-specific primers. The transcriptional start point of the *apf1* gene was determined to be at position 63 upstream of the *apf1* coding region (Fig. 2). For *apf2*, two equally intense primer extension products were obtained at positions 42 and 43 upstream of the coding region (Fig. 2). Upstream of the transcription start sites, a -10 box (TATTAT) was identified in both promoter regions. While a typical -35 box was identified in the promoter region of *apf2* at a suboptimal position only 11 bp upstream of the -10 region, no evident -35 box was found in the promoter region of *apf1*. The -10 hexamers of both *apf* promoters are extended by a TG motif at positions -13 and -14. The TG motif was shown to be responsible for the increased activity of the extended -10 boxes of some promoters with no recognizable -35 sequence (4, 6, 18). Thus, the *apf1* and *apf2* genes are independently transcribed from the promoters with TG extension of the -10 sequence and atypical -35 boxes.

Inactivation of *apf* genes. To analyze the functional role of the *L. gasseri* APF proteins, we first tried to inactivate both the *apf1* and *apf2* genes. For that purpose, a double-crossover knockout construct, pVM20, was made (Fig. 2) and trans-

FIG. 4. Western blot analysis of the APF proteins of *L. gasseri* Agg⁻ (lane 1), JA11 (*apf1* knockout; lane 2), JA12 (*apf2* knockout; lane 3), 4B2(pVM23) (APF1 overproducer; lane 4), 4B2(pVM24) (APF2 overproducer; lane 5), 4B2(pVM25) (with APF down-regulated; lane 6), 4B2(pVM25) (wild-type-looking revertant; lane 7), and 4B2(pNZ124) (control; lane 8). The crude extracts from *L. gasseri* 4B2 cells grown on agar plates were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. The APF proteins were recognized with polyclonal anti-APF antibodies. k.o., knockout; o.p., overproduction; d.r., down regulation; rev., revertants; wt, wild type.

formed into *L. gasseri* 4B2 and the inactivation procedure was performed as described in Materials and Methods. However, all attempts to obtain a double knockout were unsuccessful.

Inactivation of individual *apf* genes was achieved by a single plasmid integration. For inactivation of *apf1* and *apf2*, pGh9 derivatives pVM21 and pVM22 were constructed (Fig. 2) and gene disruption was performed as described in Materials and Methods. Plasmid integration in the *apf1* or *apf2* gene by single crossover resulted in *L. gasseri* strains JA11 and JA12, respectively. The interruption of *apf1* and *apf2* was verified by PCR. The absence of APF1 or APF2 in the corresponding strain was confirmed by Western blot assays of crude cell extracts with anti-APF antibodies (Fig. 4, lanes 2 and 3). Notably, the overnight cultures of *L. gasseri* JA11 and JA12 with disrupted *apf1* and *apf2*, respectively, were still clumping like the wild type, showing no changes in their ability to aggregate. Thus, single *apf* knockouts imply that coexistence of the two proteins is not required for cell aggregation and that only one protein is needed for survival.

Characterization of the *L. gasseri* 4B2 aggregation-negative spontaneous mutant. Since a double *apf* knockout seemed not to be viable in *L. gasseri* 4B2, we took the opportunity to analyze a spontaneous nonaggregating mutant of *L. gasseri* 4B2, *L. gasseri* Agg⁻ (Fig. 1). This mutant was isolated after plating of a lyophilized *L. gasseri* 4B2 culture on MRS agar as a colony that appeared smoother and shinier than the wild type. This colony turned out to be a mutant that had lost the ability to aggregate in liquid medium. The isogenicity of *L. gasseri* Agg⁻ and 4B2 was confirmed by rapid amplified ribosomal DNA restriction analysis and random amplified polymorphic DNA methods (data not shown).

We hypothesized that *L. gasseri* Agg⁻ might be mutated in one of the *apf* genes, thus producing truncated or eventually no APF1 or APF2. We analyzed the APF proteins of *L. gasseri* Agg⁻ by Western blotting. The amounts of APF proteins pro-

duced by the wild-type and mutant strain were similar (as estimated from several different blots [not shown]), and no truncation of APF1 or APF2 was observed in *L. gasseri* Agg⁻ (Fig. 4, lanes 1 and 8). To exclude the possibility of small in-frame deletions or point mutations, we amplified the *apf* genes of *L. gasseri* Agg⁻ with primers B10 and B4 and sequenced the amplified fragment. The sequence obtained from *L. gasseri* Agg⁻ was 100% identical to the corresponding sequence in the wild-type strain. Despite the normal production of intact APF proteins, *L. gasseri* Agg⁻ is not able to aggregate, suggesting that APF is not the sole component involved in aggregation and that another component(s) might be mutated in this strain.

APF overproduction in the wild-type and Agg⁻ strains. Since neither inactivation of the *apf* genes in the wild type nor complementation of the nonaggregating strain was possible, we analyzed the role of *apf* in cell metabolism by modulating the cellular concentrations of APF. To overproduce APF, the *apf1* and *apf2* genes were cloned with their own promoters into high-copy-number vector pNZ124 to produce pVM23 and pVM24, respectively (Fig. 2). The latter plasmids were transformed into wild-type *L. gasseri* 4B2, and overproduction of APF1 and APF2 in *L. gasseri* 4B2(pVM23) and 4B2(pVM24) was confirmed by Western blotting with anti-APF antibodies (Fig. 4, lanes 4 and 5). Upon overproduction of APF1, the amount of APF2 synthesized from the chromosomal gene copy was slightly reduced (Fig. 4, lanes 4 and 5). The same decrease in APF1 production was observed when APF2 was overproduced, suggesting the possibility of positive regulation of *apf1* and *apf2* gene expression. It should be noted that the 10-kDa band (Fig. 4) probably corresponds to the C-terminal part of the degraded APF2 protein and is recognized by C terminus-specific anti-APF antibodies.

Careful observation of colony morphology revealed that the wild-type and APF-overproducing strains looked different on agar plates (Fig. 5). While wild-type colonies were flat, both

FIG. 5. Scanning electron microscopic analysis of *L. gasseri* 4B2 strains. Cells grown on MRS agar were prepared as described in Materials and Methods. *L. gasseri* 4B2(pNZ124) (A) exhibits the wild-type morphology of rod-shaped cells; *L. gasseri* 4B2(pVM23) (B), overproducing APF1, and *L. gasseri* 4B2(pVM24) (C), overproducing APF2, exhibit twisted cells of increased diameter. *L. gasseri* 4B2(pVM25) (D), with downregulated APF production, exhibits elongated cells with no visible cell separation sites. Two different magnifications and the colony morphology are presented for each strain. WT, wild type; o.p., overproduction; d.r., down regulation.

APF-overproducing strains were convex. Microscopic analysis demonstrated that strains producing increased amounts of APF1 or APF2 had an altered cell shape (Fig. 5). They were twisted and exhibited an enlarged diameter compared to that of wild-type cells. Chains of the APF-overproducing strains were curled, producing tight clumps.

In selective liquid medium, both APF1- and APF2-overproducing *L. gasseri* strains JA11 and JA12 grew as a mixed population of wild-type-looking cells and cells with altered shape (data not shown). Repeated attempts to isolate single colonies with a uniform phenotype failed. In the absence of selective pressure, *L. gasseri* 4B2 carrying pVM23 or pVM24 rapidly lost the plasmid and consequently reverted to wild-type morphology. After three subcultures, 90% of the cells were cured of the plasmid. This suggests that increased amounts of APF represent a metabolic burden for *L. gasseri* 4B2 cells.

When plasmids carrying *apf1* or *apf2* (pVM23 or pVM24, respectively) were transformed into *L. gasseri* Agg⁻, APF overproduction similar to that of the wild type was observed in Western blot assays (data not shown). The resulting strains, overproducing APF1 or APF2, were still nonaggregating but showed the same cell shape alterations as the APF-overproducing wild-type strains. Hence, the mechanisms by which APF influences cell shape and aggregation seem to be distinct.

APF down-regulation in the wild type. While analyzing crude protein extracts of APF-overproducing strains *L. gasseri* 4B2(pVM23) and 4B2(pVM24), we noticed that overproduction of one APF protein led to a slight reduction in the level of the other APF protein (Fig. 4, lanes 4 and 5). This indicated that both *apf* genes may be regulated by the same positive regulator. In the APF1- and APF2-overproducing strains, this hypothetical activator may be recruited by the high copy number of *apf* from the plasmid (pVM23 or pVM24) and thereby be less available for activation of the chromosomal *apf* gene copies.

To test if *apf* transcription could be decreased by recruiting the hypothetical activator, we cloned the *apf2* promoter region into high-copy-number plasmid pNZ124 to produce pVM25 (Fig. 2). Transformants of the wild-type strain exhibited a filamentous morphology (Fig. 5). Analysis of crude extracts by Western blot assays demonstrated that these cells produce much less APF1 and APF2 than the wild type (Fig. 4, lane 6), demonstrating that both *apf* genes are regulated by the same transcriptional activator. Microscopic analysis of the filamentous colonies revealed that the recombinant cells of *L. gasseri* 4B2 with down-regulated APF production were extremely elongated (Fig. 5). Such elongated cells had no obvious cell separation sites. This result confirmed the involvement of APF in the determination of *L. gasseri* 4B2 cell shape.

When grown in liquid medium, *L. gasseri* 4B2(pVM25) was still aggregating. However, microscopic analysis showed that the elongated cell phenotype was unstable even in the presence of antibiotic pressure. When the liquid culture of *L. gasseri* 4B2(pVM25) was plated on selective agar plates, two types of colony morphology appeared: filamentous and wild type looking. The spontaneous revertants exhibiting the wild-type cell shape still contained the intact pVM25 plasmid, but their levels of APF1 and APF2 were close to those of wild-type *L. gasseri* strain 4B2 (Fig. 4, lanes 7 and 8). This suggests that a suppression mutation occurred elsewhere in the genome, enabling

optimal APF production. Thus, any imbalance of cellular APF concentrations definitely leads to cell shape alterations of *L. gasseri* 4B2.

DISCUSSION

In this study, we analyzed the physiological role of APF of *L. gasseri* 4B2. Of the six strains known to contain actively transcribed *apf* genes (34), three are nonaggregating (*L. johnsonii* NCC533 [La1] and ATCC 332 and *L. gasseri* ATCC 19992). Since the presence of *apf* genes was not obviously related to an aggregation phenotype, the role of APF in cell physiology deserved to be further studied. We first confirmed that *L. gasseri* 4B2 also contains two *apf* genes. All attempts to inactivate both *apf* genes failed. Single *apf* gene disruption was successful but did not change the ability of mutant *L. gasseri* strains JA11 and JA12 to aggregate. Moreover, sequencing of the spontaneous nonaggregating mutant form of *L. gasseri* 4B2, strain Agg⁻, revealed that its *apf* genes contained no mutations. The APF protein concentrations of *L. gasseri* Agg⁻ were similar to those of the wild-type strain, suggesting that regulation of APF production was also intact. Since neither of the *apf* genes nor the level of APF production in *L. gasseri* Agg⁻ was altered, it is possible that the mutation of *L. gasseri* Agg⁻ lies in a second component involved in aggregation, e.g., an APF receptor molecule. In a previous study, it was shown that aggregation promoted by APF occurs only in strains containing α -1,2-glucose-substituted lipoteichoic or teichoic acid (25), which were proposed as APF receptors on the *L. gasseri* 4B2 cell surface. Lack of knowledge about APF receptors prevented us from identifying a suitable strain containing an APF receptor but carrying no *apf* genes in which heterologous complementation of aggregation could be shown. Actually, the results obtained in this study do not seem to support the role of APF proteins as aggregation factors. If APF proteins are indeed involved in cell aggregation, they probably need an additional actor(s) to be functional.

However, we clearly demonstrated that APF proteins influence the shape of *L. gasseri* 4B2 cells. As a consequence of APF overproduction, the cells of *L. gasseri* 4B2(pVM23) and 4B2(pVM24) became twisted and enlarged in diameter. A similar twisted cell shape phenotype was observed with mutants of penicillin binding protein PBP2a, which is involved in peptidoglycan production in *Bacillus subtilis* (28). Also, other mutations that influence peptidoglycan production cause different changes in the shape of *B. subtilis* (28) and *E. coli* (20, 21, 28, 30, 35) cells. Altered cell wall composition may lead to changes in the affinity of antibiotics for their target or their susceptibility to their target and make the cells more or less sensitive to cell wall-targeted antibiotics. However, we found no significant differences in the growth inhibition of the wild-type strain and strains containing increased APF copy numbers by penicillin G, vancomycin, cefotaxime, cefixime, or cefuroxime (data not shown). Thus, it seems unlikely that overproduction of APF influences *L. gasseri* 4B2 cell shape by interfering with peptidoglycan synthesis or regulation.

As shown by Western blot analysis of the crude extracts, when one APF protein was overproduced, the production of the other was reduced. In addition, an increased copy number of the *apf2* promoter region caused the reduction of both APF

protein levels in the cell, suggesting that both *apf* genes are regulated by an unknown positive regulator. The previous observation that *apf* genes are expressed more strongly in the exponential than in the stationary phase also indicated transcriptional *apf* regulation (34). Their strong expression in the exponential growth phase, together with the fact that the *apf* genes seem to be essential for cell survival, points to a role for APF in cell structure determination.

As a confirmation that APF influences cell shape in *L. gasseri* 4B2, the opposite cell shape alteration was obtained when APF production was reduced: the cells were elongated, with no visible cell division sites. Different variations of similar elongated cell phenotypes were observed with temperature-sensitive *ftsZ*, *ftsQ*, *ftsA*, and *pbpB* cell division mutant strains of *E. coli* (1, 31). The phenotype of the *ftsZ* mutant especially resembles that of the elongated cells of *L. gasseri*(pVM25). FtsZ is a homologue of the eucaryotic cytoskeleton protein tubulin, which is responsible for the initial phase of septum formation.

A comparison of the sequences of APF and FtsZ and other cell shape-affecting proteins revealed no significant matches. However, with the fold recognition technique 3D-PSSM (15), a tentative similarity with an E value of 1.07 was found with eucaryotic profilins, which are sophisticated regulators of polymerization of the main cytoskeleton protein actin (7). Recently, genes encoding analogues of eucaryotic actin, *mreB* or *mlb*, whose mutation resulted in cell shape alterations, were discovered in *B. subtilis* (14). Actin-like and Fts cell division proteins are conserved among bacteria, and the corresponding genes are present in the recently sequenced genome of *L. gasseri* (http://www.jgi.doe.gov/JGI_microbial/html/index.html). It could be hypothesized that APF interacts with these proteins or interferes with their synthesis and thereby influences the cell shape of *L. gasseri* 4B2. In gram-negative archaeobacteria, it has been shown that the S-layer is involved in cell shape determination and in the cell division process (29). The amino acid composition, physical properties, and gene organization of APF indeed resemble those of S-layer proteins (34). However, whether APF can interact with cytoskeleton molecules and if the cell shape changes caused by APF are due to imbalance of cell division or actin-like molecules in *L. gasseri* 4B2 remains to be elucidated.

Genes affecting bacterial cell shape are typically essential for viability (9), which may explain our failure to obtain an *apf* double knockout. Double inactivation of *apf* genes was also unsuccessful in *L. johnsonii* NCC533 (La1) (34), suggesting that the physiological function of APF is essential. In addition, in the absence of selective pressure, both overexpressing (pVM23 and pVM24) and downregulating (pVM25) constructs were very unstable in *L. gasseri* 4B2. Moreover, the elongated cells of *L. gasseri* 4B2(pVM25) reverted spontaneously to the wild-type phenotype even in the presence of antibiotic pressure, which was accompanied by increased amounts of APF to wild-type levels. Thus, either by aborting the plasmid or by producing a suppression mutation, cells very quickly recover optimal cellular APF amounts.

Our data suggest, for the first time, that APF proteins are essential for *L. gasseri* 4B2, and they show that any changes in their cellular concentration lead to alteration of cell shape. Whether APF acts directly to control cell shape, indirectly interferes with processes like peptidoglycan, exopolysaccha-

ride, lipoteichoic acid, and teichoic acid synthesis, or interacts with other proteins involved in bacterial cell shape maintenance remains to be established.

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