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Integrative Expression System for Delivery of Antibody Fragments by Lactobacilli▽†

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A series of expression cassettes which mediate secretion or surface display of antibody fragments was stably integrated in the chromosome of *Lactobacillus paracasei*. *L. paracasei* producing surface-anchored variable domain of llama heavy chain (VHH) (ARP1) directed against rotavirus showed efficient binding to rotavirus and protection in the mouse model of rotavirus infection.

Lactobacilli are Gram-positive bacteria that are currently used in food fermentation and preservation. Lactobacilli are also normal constituents of the human microbiota and are generally regarded as safe for humans (GRAS). There is an increased interest in developing engineered lactobacilli as a system for delivery of therapeutic and prophylactic biomolecules (4, 19), and we have previously shown the potential of lactobacilli to deliver single-chain variable fragment (scFv) and variable domain of llama heavy chain (VHH) antibody fragments to reduce oral and gastrointestinal infections in animal models (7, 11, 15).

Stability and safety of the genetically engineered lactobacilli used for medical application are of the utmost importance. Various systems have previously been developed to stably integrate a heterologous gene into the chromosome, generating food-grade expression systems devoid of antibiotic selection genes (4). The recombinant proteins can be secreted into the environment or displayed on the cell surface with covalent (7, 14, 19) or noncovalent (2, 17) binding.

In this article, we describe a chromosomally integrated expression system based on the aggregation-promoting factor gene (*apf*) of *Lactobacillus crispatus* (9) to direct the expression and secretion of antibody fragments combined with the site-specific integration apparatus of the temperate bacteriophage A2 to mediate chromosomal integration (12). The APF protein has been identified in homofermentative lactobacilli, but proteins with a homologous C-terminal part are also present in heterofermentative lactobacilli and other Gram-positive bacteria (9, 17). This cell surface protein was originally hypothesized to be involved in autoaggregation, but more recent results suggest rather an involvement in the maintenance of cell shape (6, 18). The APF protein was selected as a vector molecule to deliver antibody fragments due to its high secretion level in the supernatant and noncovalent cell wall anchoring system (6, 9, 18).

Construction and selection of *apf* expression cassettes for production of antibody fragments in *Lactobacillus paracasei*. Our original publication described lactobacilli producing an scFv antibody fragment against the SAI/II adhesin of *Streptococcus mutans*, protecting against caries (7, 8). In order to optimize the level of expression, the secretion, and the localization of the antibody fragment, different translational fusions were made between the gene encoding scFv and the *apf* gene of *L. crispatus* M247 (see detailed materials and methods, Fig. S1, and Table S1 in the supplemental material). The *apf* gene of *L. crispatus* M247 encodes a 223-amino-acid protein containing a signal peptide (33 amino acids), an N-terminal domain (75 amino acids), a central region rich in asparagine, glutamine, threonine, and alanine (37 amino acids), and a C-terminal domain (the last 78 amino acids) (GenBank accession no. AF492458) (9) (Fig. 1A). Using different fragments of the *apf* gene and in some case the *prtP* gene region encoding the last 231 amino acids of proteinase P for cell wall covalent anchoring (7), a total of 11 expression cassettes were generated (Fig. 1B). In each cassette, the antibody fragment was also fused to an E tag for detection with an anti-E-tag antibody in Western blotting and enzyme-linked immunosorbent assay (ELISA). The pAF plasmid series (pAF100 to pAF1100) containing the 11 expression cassettes was introduced into *L. paracasei* (previously known as *L. casei* or *L. zeae* ATCC 393 pLZ15^{-}) (7, 15) by electroporation as previously described (7, 10), generating *L. paracasei* pAF100 to pAF1100. The most important bacterial strains and plasmids used in this study are listed in Table 1.

As shown by Western blot analysis of the supernatant and cell extract of *L. paracasei* transformants (pAF100 to pAF1100), fusion to different regions of the APF protein can affect the level of secretion and the localization of the antibody fragments (Fig. 1B). Cassettes lacking the middle region and C-terminal domain of APF generate scFv in the supernatant only (*L. paracasei* pAF100). The highest level of antibody frag-

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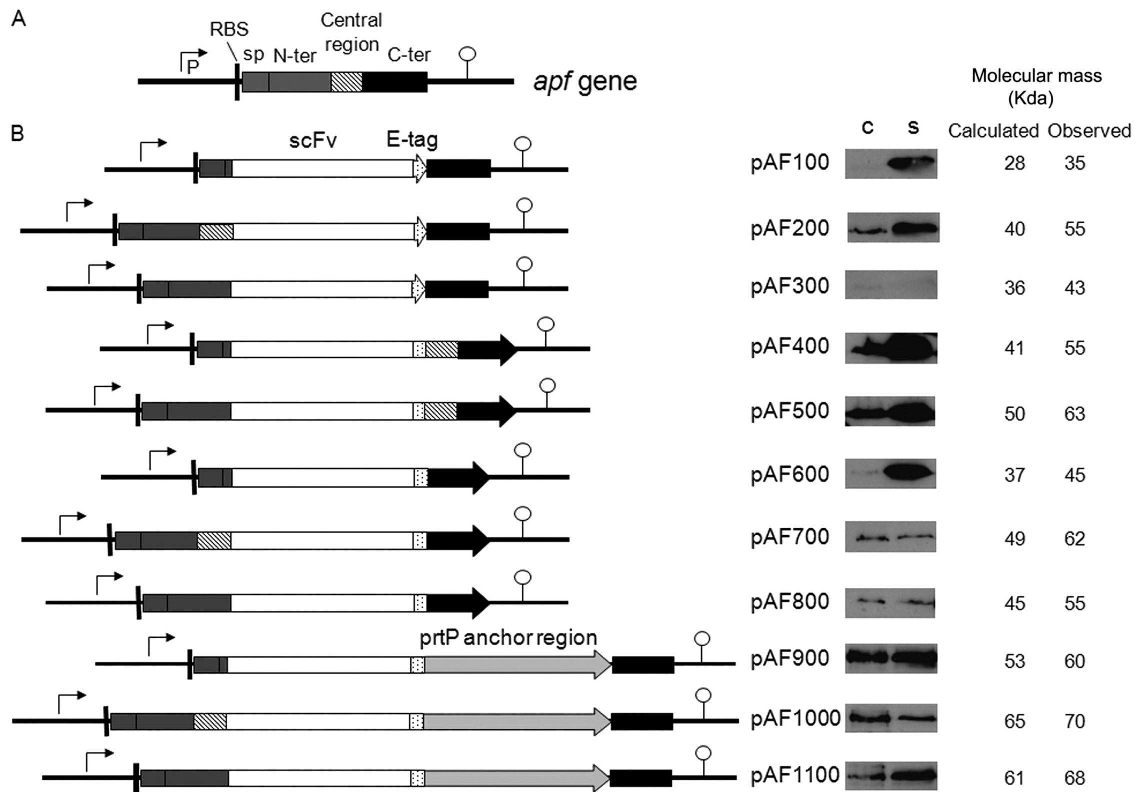

FIG. 1. Production of scFv anti-SAI/II by *L. paracasei* transformed with plasmids containing different expression cassettes. (A) The APF protein can be divided in three domains, N-terminal (N-ter), C-terminal (C-ter), and a central region which is rich in asparagine, glutamine, threonine, and alanine. The promoter (P), the ribosomal binding sites (RBS), the signal peptide (sp), the translational stop codon (arrowhead), and the transcription terminator (lollipop) are indicated. (B) scFv production was analyzed in cell extract (c) and supernatant (s) of *L. paracasei* transformed with the plasmids pAF100 to pAF1100. The experiment was repeated twice, and all transformants were analyzed at the same time. An equivalent of 125 μl supernatant and extract from 1×10^8 cells was loaded in each well.

ments in the supernatant was obtained when the scFv antibody fragment was fused to both the middle region and the C-terminal part of APF (*L. paracasei* pAF400 and pAF500). Furthermore, the highest level of scFv antibody in the cell extract was detected when scFv was fused either to the middle region and the C-terminal part of APF (*L. paracasei* pAF400 and pAF500) or to the C-terminal domain of PrtP (*L. paracasei* pAF900, pAF1000, and pAF1100).

It has previously been shown that the conserved APF carboxy termini might be involved, at least in part, in cell surface attachment of the protein and that the APF could be stripped from the surface of lactobacilli by LiCl treatment (18). The Western blot results suggest that both the central and C-terminal regions of the APF protein are important for cell wall attachment since scFv could be significantly detected in the cell extract only when fused to these two domains (pAF400 and pAF500) (Fig. 1B). The central region might be either directly involved in cell binding or indirectly involved by allowing the correct folding of the C-terminal domain, needed for binding. Treatment of the cell pellet of *L. paracasei* pAF400 with LiCl was shown to remove 75% of the scFv fusion protein from the cell pellet extract (Fig. 2Ai). In addition, a 55-kDa band, corresponding to the scFv fusion protein, was observed in the cell extract of nontransformed *L. paracasei* preincubated for 2 h with the culture supernatant of *L. paracasei* pAF400 (Fig. 2Aii). No band could be detected when *L. paracasei* had been previously incubated with the supernatant of *L. paracasei* pAF100 or nontransformed *L. paracasei*. These results confirm that scFv can attach to the cell wall through the central and C-terminal domains of APF. However, flow cytometry analysis using a mouse anti-E-tag antibody and Cy-2-conjugated goat anti-mouse immunoglobulin (5, 15) showed the presence of scFv fragments on the cell surface of *L. paracasei* transformed with the plasmids pAF900, pAF1000, and pAF1100 but not with the plasmid pAF400 (Fig. 2B).

Three expression cassettes were selected for future applications based on the amount of scFv produced and the localization of scFv. Expression cassettes producing fusion proteins with short APF N termini were preferred, since it could otherwise interfere with the folding of the antibody fragment. The three selected plasmids were pAF100, generating secreted scFv only, pAF400, generating both secreted and cell wall-attached scFv, and pAF900, generating surface-anchored scFv.

Validation of expression cassettes by cloning of other antibody fragment-encoding genes. Since chromosomal integration is cumbersome and we wanted to develop a universal expression system for production of a broad range of antibody fragments, we first verified that the selected cassettes contained in the plasmids pAF100, pAF400, and pAF900 were suitable for expression of other antibody fragments. scFv directed against human intercel-

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TABLE 1. Most important strains and plasmids used in this study

Strain or plasmid	Relevant properties	Reference or source
Strains		
E. coli DH5α		
L. paracasei	Previously considered a plasmid-free *L. casei* 393	15
L. paracasei pAF100	*L. paracasei* with pAF100 plasmid, secreted scFv anti-SAI/II	This work
L. paracasei pAF400	*L. paracasei* with pAF400 plasmid, secreted and attached scFv anti-SAI/II	This work
L. paracasei pAF900	*L. paracasei* with pAF900 plasmid, surface-anchored scFv anti-SAI/II	This work
L. paracasei pAF900-ARP1	*L. paracasei* with pAF900-ARP1 plasmid, surface-anchored ARP1	This work
L. paracasei EM171	*L. paracasei* with integrated pAF400 cassette, secreted and attached scFv anti-SAI/II	This work
L. paracasei EM181	*L. paracasei* with integrated pAF900 cassette, surface-anchored scFv anti-SAI/II	This work
L. paracasei EM182	*L. paracasei* with integrated pAF100 cassette, secreted scFv anti-SAI/II	This work
L. paracasei EM233	*L. paracasei* with integrated pAF900-ARP1 cassette, surface-anchored ARP1	This work
Plasmids		
pIAV7	Broad-range vector, Er^r, *lacZ*, pWV01 replication origin	16
pAF100 to pAF1100 series	pIAV7 with 11 expression cassettes	This work
pEM76	Integrative vector containing *six1*, A2 *int*, *attP*, and *six2*	12
pEM94	Containing the β-recombinase gene in order to delete the non-food-grade DNA present in the integrated plasmids by site-specific recombination	13
pEM171	pEM76 with expression cassette of pAF400	This work
pEM181	pEM76 with expression cassette of pAF900	This work
pEM182	pEM76 with expression cassette of pAF100	This work
pEM233	pEM76 with expression cassette of pAF900-ARP1	This work

lular adhesion molecule 1 (ICAM-1) (3) and VHH antibody fragments against SAI/II (S36) (8) and rotavirus (ARP1 or VHH1) (15) were selected since they were previously shown to be functional and to have a therapeutic effect *in vitro* and in animal models when produced by lactobacilli. Derivative pAF100, pAF400, and pAF900 plasmids were constructed for the expression of scFv anti-ICAM-1 (pAF100-ICAM, pAF400-ICAM, and pAF900 ICAM), S36 (pAF100-S36, pAF400-S36, and pAF900-S36), and ARP1 (pAF100-ARP1, pAF400-ARP1, and pAF900-ARP1) and introduced into *L. paracasei*.

Successful production of the antibody fragments by the modified *L. paracasei* bacteria was demonstrated using Western blotting (see Fig. S2A in the supplemental material) and was subsequently estimated by Western blot densitometry using purified scFv fused to an E tag as a standard. The amount of antibody fragments produced by *L. paracasei* transformed with the pAF100 derivative plasmids was lower for scFv (anti-SAI/II and anti-ICAM-1) (0.1 to 0.15 μg/ml) than for VHH (ARP1 and S36) (0.5 to 0.7 μg/ml). The largest amount of antibody fragments was measured in the supernatant of *L. paracasei* transformed with the pAF400 derivative plasmids (0.9 to 1 μg/ml of scFv and 3 to 5 μg/ml of VHH). The amounts of antibody fragments present in the cell extract of *L. paracasei* transformed with the pAF400 plasmids were similar for scFv and VHH (10^3 molecules/bacterium), but a larger amount of VHH than scFv molecules was observed in the cell extract of *L. paracasei* transformed with pAF900 derivative plasmids (3×10^3 to 6×10^3 versus 0.7×10^3 to 1×10^3 molecules/bacterium). As observed above, flow cytometry analysis showed the presence of scFv (anti-ICAM-1) and VHH (ARP1 and S36) fragments on the cell surface of *L. paracasei* transformed with the pAF900 plasmids (see Fig. S2B in the supplemental material) but not with the pAF400 plasmids.

In ELISA, binding activity toward ICAM-1, SAI/II, and rotavirus was observed in the supernatant of *L. paracasei* transformed with the pAF100 and pAF400 derivative plasmids and whole bacterial cells of *L. paracasei* transformed with the pAF900 derivative plasmids (see Fig. S3 in the supplemental material). However, no binding activity was observed using whole *L. paracasei* transformed with the derivative pAF400 plasmids. The latter could be due to the E-tag being poorly detected by the conjugated antibody when the fragment is attached to the cell through the central and C-terminal domain of APF as observed above. However, using *Bac*Light Green-stained bacteria in a spectrofluorometric microassay, we could observe binding to antigen-coated plates by *L. paracasei* transformed with pAF900 but not pAF400 plasmids (see Table S2). Furthermore, binding to rotavirus by *L. paracasei* pAF900-ARP1 but not pAF400-ARP1 was shown by flow cytometry using rabbit anti-rotavirus VP6 and donkey anti-rabbit phycoerythrin (PE)-conjugated antibodies (15).

The antibody fragments attached to the surface of *L. paracasei* via the central and C-terminal domain of APF may not protrude sufficiently outside the bacterial surface to bind to antigens or to be recognized by the anti-E tag in flow cytometry. We have previously shown that the length of the anchoring sequence is important to obtain a functional surface-displayed antibody (7). Insertion of a sequence between the middle region of APF and the antibody fragment might thus be necessary to elongate the fusion protein in order to improve the display of antibody fragments.

Our results confirm the possibility of producing different functional secreted and surface antibody fragments using the described expression system. Although the central and C-terminal part of APF does not allow the display of the antibody in a functional way, it favors the secretion and/or stability of the scFv antibodies in the supernatant. Furthermore, antibody fragments bound to antigen could potentially reattach to the *Lactobacillus* cells as suggested above (Fig. 2Aii). Most impor-

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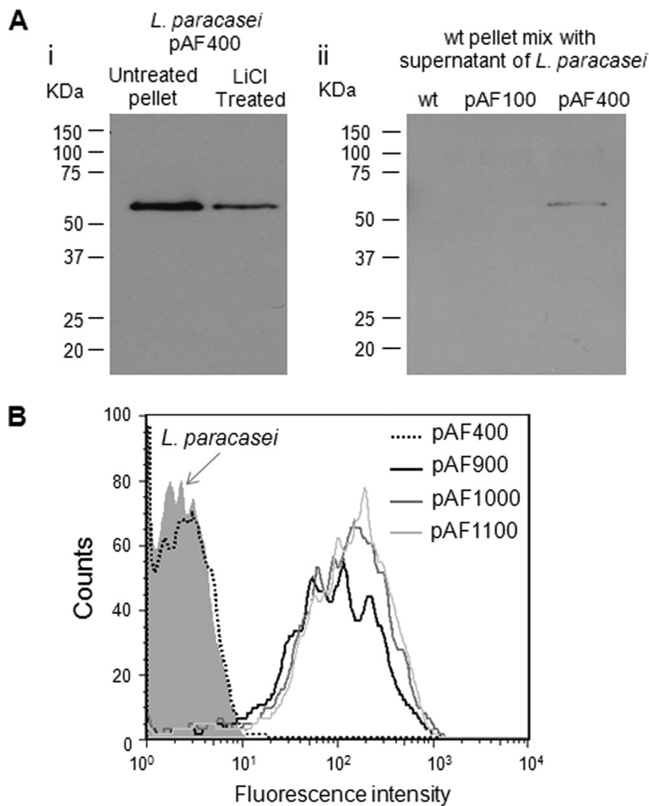

FIG. 2. Evaluation of surface display of scFv anti-SAI/II in modified *L. paracasei.* (A) Demonstration of noncovalent attachment of scFv to the surface of *L. paracasei* pAF400 by Western blotting. (i) The bacterial pellet was treated with LiCl (5 M) to strip surface proteins, and Western blotting of the cell extract was performed. (ii) Cell extract from wild-type *L. paracasei* incubated with the culture supernatant of wild-type *L. paracasei* (wt), *L. paracasei* pAF100, or *L. paracasei* pAF400 to evaluate reattachment of scFv. (B) Flow cytometry analysis showing the display of antibody fragments by *L. paracasei* transformants displaying surface-anchored scFv anti-SAI/II antibody fragments.

tant, display of antibody fragments is efficiently obtained using the PrtP anchor C-terminal domain.

Chromosomal integration of the gene encoding scFv anti-SAI/II and ARP1. The pEM76 integration vector was previously described and contains the phage A2 integrase gene (A2-*int*), which catalyzes the insertion of vector DNA containing the A2-*attP* site into an *attB* site present in the genome of all lactic acid bacteria tested so far (1). The pEM76 vector was used for integration of three selected cassettes fused to the gene encoding scFv anti-SAI/II and one cassette mediating the surface covalent anchoring of ARP1 into the chromosome of *L. paracasei*. The expression cassettes from the plasmids pAF100, pAF400, pAF900, and pAP900-ARP1 were cloned into the integrative plasmid pEM76, generating pEM182, pEM171, pEM181, and pEM233, respectively. The integrative plasmids were introduced by electroporation into *L. paracasei*. The resulting strains were subsequently electrotransformed with pEM94, a replicative plasmid that carries the β-recombinase gene, in order to delete, by site-specific recombination, the non-"food grade" DNA (antibiotic resistance gene, *Escherichia coli* DNA) located between two *six* sites (13). After this depuration step, the strains were cultured at 37°C to eliminate pEM94 (which carries a temperature-sensitive origin of replication). The obtained strains were designated *L. paracasei* EM171 (secreted and attached scFv), *L. paracasei* EM181 (covalently anchored scFv), *L. paracasei* EM182 (secreted scFv), and *L. paracasei* EM233 (covalently anchored ARP1), respectively. Each step (integration, depuration, and plasmid curing) was confirmed by PCR analysis and Southern blotting (data not shown).

When integrated in the chromosome, the amount of scFv in the supernatant of *L. paracasei* EM182 (secreted scFv) and in the cell extract of *L. paracasei* EM181 (surface-anchored scFv) was about 10-fold lower than that when the corresponding plasmid-based construct was used (approximately 10 ng/ml and 100 molecules/bacterium, respectively) as determined by Western blot densitometry (see Fig. S4A in the supplemental material). A 10-fold decrease in surface antibody display was also observed in *L. paracasei* EM181 as shown by flow cytometry (see Fig. S4B). However, the amount of scFv detected in the supernatant and in the cell extract of EM171 (secreted and attached scFv) was shown to be only 2-fold lower (approximately 500 ng/ml and 500 molecules/bacterium) than that in the plasmid-based system. Using ELISA, binding activity toward the SAI/II antigen was observed using the supernatant and whole bacterial cells of integrated constructs but to a reduced level in comparison to that with the equivalent plasmid constructs, which corresponds to the amount of antibody produced (see Fig. S4C).

We have previously shown using a plasmid expression system that lactobacilli producing surface-anchored ARP1 (but not secreted ARP1) are protective in a mouse model of rotavirus infection (15). Here we compared the activity of *L. paracasei* producing surface-anchored ARP1 using the plasmid (*L. paracasei* pAF900-ARP1) and integration system (*L. paracasei* EM233) *in vitro* and *in vivo*. *L. paracasei* EM233 also showed an intensity of the bands that was 10 times lower than that for *L. paracasei* pAF900-ARP1, which corresponds to approximately 600 molecules/bacterium (Fig. 3A). When the display of the ARP1 fragment on the surface of bacteria was evaluated by flow cytometry, the fluorescence intensity was shown to be 6 times lower for *L. paracasei* EM233 than for the corresponding plasmid construct, *L. paracasei* pAF900-ARP1 (Fig. 3B). The reason for the discrepancies between the two methods is probably that only a fraction of the molecules detected in the cell extract by Western blotting are displayed on the surface.

The binding of whole cells of *L. paracasei* EM233 and *L. paracasei* pAF900-ARP1 to rotavirus was shown to be similar using flow cytometry (Fig. 3C) and immunofluorescence microscopy (see Fig. S5 in the supplemental material), while with use of ELISA, whole cells of *L. paracasei* EM233 were shown to bind to rotavirus particles at a level about 3 times lower than that for the corresponding plasmid construct (Fig. 3D).

To evaluate construct stability, *L. paracasei* EM233 was also grown for 50 generations and fluorescence intensity was measured at generations 10, 20, 30, 40, and 50. No difference was observed in the fluorescence intensity between the different generations, showing that the integrated gene is stable.

The prophylactic effect of modified *L. paracasei* was subsequently tested in the mouse pup model of rotavirus infection

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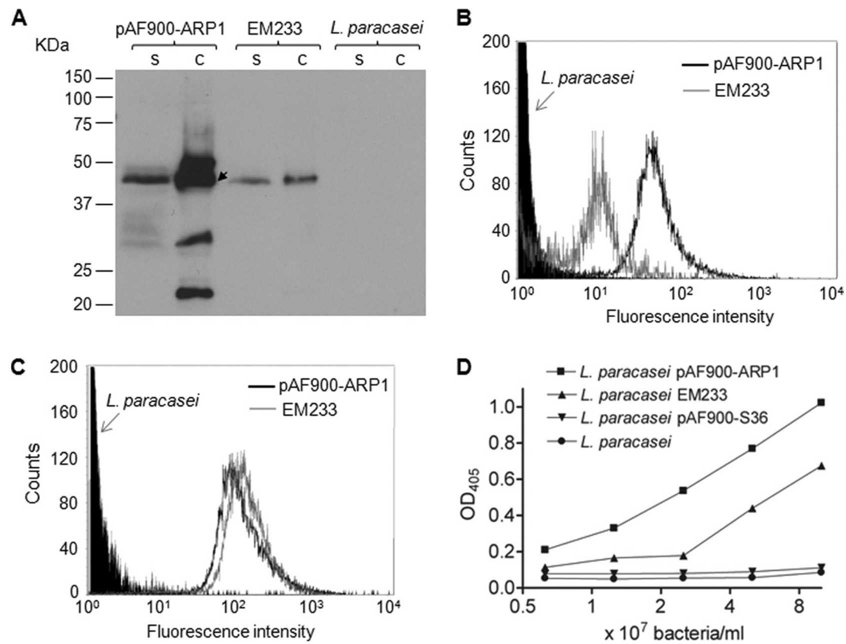

FIG. 3. (A) Production and binding activity of *L. paracasei* producing surface-anchored ARP1 using a plasmid-based (*L. paracasei* pAF900-ARP1) and chromosomally integrated (*L. paracasei* EM233) expression system. (A) Production of ARP1, determined by Western blot analysis of supernatant (s) and cell extract (c). An equivalent of 40 μl supernatant and extract from 3.5×10^7 cells was loaded in each well. For *L. paracasei* pAF900-ARP1, an arrowhead indicates the band corresponding to intact ARP1 fused to the C-terminal domain of PrtP. (B) Flow cytometry analysis showing the display of ARP1 on the bacterial surface. (C) Flow cytometry analysis showing binding activity of modified *L. paracasei* for rotavirus. (D) ELISA analysis showing binding activity of modified *L. paracasei* for rotavirus. Nontransformed *L. paracasei* and *L. paracasei* pAF900-S36 were used as negative controls. The coefficient of variation between triplicates is less than 10%.

using single oral inoculums of 20 50% diarrhea-inducing doses (DD_{50}) of rhesus rotavirus (RRV) on day 0 (15). *L. paracasei* pAF900-ARP1, *L. paracasei* EM233, and nontransformed *L. paracasei* were orally administered once daily starting on day −1 and continuing until day 3, and the diarrhea score was recorded daily until day 4. All mouse experiments were approved by the local ethics committee of the Karolinska Institutet at Karolinska University Hospital, Huddinge, Sweden. *L. paracasei* pAF900-ARP1 and *L. paracasei* EM233 were shown to reduce the duration and severity of diarrhea to similar levels (Table 2).

The pEM76 delivery system was shown to create functional and stable integration of expression cassettes mediating secretion, secretion and attachment, and surface covalent anchoring of antibody fragments in the model strain *L. paracasei*. The reduction in antibody production is acceptable considering that each bacterium contains one copy of the chromosome but multiple copies of the plasmid ($n = 162$) (16). Most important, the level of ARP1 antibody fragment displayed in *L. paracasei* EM233 was sufficient for the modified lactobacilli to reduce infection when tested in an animal model of rotavirus infection. Conforming to previous data in our laboratory (15), our present results showed that VHHs were produced at a higher level when secreted in the supernatant or covalently anchored on the surface than scFv antibody fragments. VHHs are smaller and, since they are formed by a single polypeptide, easier to produce in a recombinant form with an intact spatial structure. They are without a doubt the antibodies of choice for efficient production of antibody fragment using the integrated system.

The integration and expression system described in this article may potentially apply to a range of *Lactobacillus* species. In this study, we showed that the *apf* promoter originally from *L. crispatus* was active in *L. paracasei* in which the *apf* gene is absent. Furthermore, preliminary results also show a high expression in *Lactobacillus rhamnosus* GG (unpublished data). The delivery system can be integrated in various lactic acid bacteria since the integration machinery has some degree of flexibility with regard to the sequence of the *attB* site (1). This method eliminates the need to know the genome sequence since the DNA always integrates at the *attB* site. Since the temperate phage A2 is 44 kb in size, the integration of two or more expression cassettes encoding antibodies of different

TABLE 2. Duration and severity of rotavirus-induced diarrhea in the different treatment groups

Group	No. of mice	Duration[a] (mean ± SE, days)	Severity[b] (mean ± SE)
L. paracasei pAF900-ARP1	7	1.00 ± 0.22	1.00 ± 0.22[c]
L. paracasei EM233	7	1.14 ± 0.14	1.14 ± 0.14[c]
L. paracasei	7	1.43 ± 0.20	2.29 ± 0.36

[a] The duration was defined as the total sum of days with diarrhea.

[b] No stool or normal stool was given a score of 0, loose stool a score of 1, and watery diarrhea a score of 2. Severity was defined as the sum of diarrhea scores for each pup during the course of the experiment (severity = Σ diarrhea score [day 1 + day 2 + day 3 + day 4]).

[c] Statistically significant difference from results for the *L. paracasei* group by Kruskal-Wallis test ($P = 0.007$) and Dunn test ($P < 0.05$).

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specificities can probably be achieved. Taken together, this work represents the first expression system that can be applied to different lactobacilli and represents an important step forward in the development of modified live lactobacilli producing antibody fragments for the food industry and for health applications.

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