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Novel Surface Display System for Proteins on Non-Genetically Modified Gram-Positive Bacteria

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A novel display system is described that allows highly efficient immobilization of heterologous proteins on bacterial surfaces in applications for which the use of genetically modified bacteria is less desirable. This system is based on nonliving and non-genetically modified gram-positive bacterial cells, designated gram-positive enhancer matrix (GEM) particles, which are used as substrates to bind externally added heterologous proteins by means of a high-affinity binding domain. This binding domain, the protein anchor (PA), was derived from the *Lactococcus lactis* peptidoglycan hydrolase AcmA. GEM particles were typically prepared from the innocuous bacterium *L. lactis*, and various parameters for the optimal preparation of GEM particles and binding of PA fusion proteins were determined. The versatility and flexibility of the display and delivery technology were demonstrated by investigating enzyme immobilization and nasal vaccine applications.

Heterologous display of proteins or peptides on the surface of bacteria is a useful research tool and has been associated with a broad range of interesting applications, including development of live vaccine delivery systems, diagnostics, whole-cell absorbents, and novel biocatalysts (16). For gram-positive bacteria, Hansson et al. (11) were the first to describe anchoring of heterologous proteins on the surface of *Staphylococcus xylosus* by translational fusion of the recombinant proteins to the LPXTG cell wall-anchoring motif of staphylococcal protein A (SpA). This anchoring mechanism requires processing by a sortase for covalent anchoring of the protein to the peptidoglycan of the bacterial cell wall (37). Various other anchoring proteins have also been exploited for surface display, but in all cases expressed proteins were anchored to the producer cells, thus making the host strain for surface display a genetically modified organism (GMO) (10, 36). However, the use of GMOs in applications that involve uncontrolled release into the environment (e.g., in foods and vaccines) is less desirable or at least still being debated.

Noncovalent binding domains have the potential to be used in *trans* for the binding of chimeric proteins to non-GMOs. An example of such a domain is the C-terminal cell wall-binding domain of lysostaphin produced by *Staphylococcus simulans*. However, most if not all of this type of binding domains seem to be species specific in their targeting and therefore have limited use (27). Here, we report on the development of a novel surface display tool suitable for a broad range of gram-positive bacteria that avoids the use of GMOs in the final application. This tool is based on the peptidoglycan-binding domain of the major autolysin AcmA of *Lactococcus lactis* (5) that enables functional display of heterologous proteins on the surface of genetically unmodified gram-positive bacteria (4). The cell wall-binding domain was designated the protein anchor (PA). The PA comprises three LysM motifs consisting of about 45 amino acids separated by spacer sequences. The LysM motif is a common module found in many cell wall-degrading enzymes and proteins involved in bacterial pathogenesis. The LysM motif has also been found in a number of eukaryotic proteins (2). Furthermore, it was recently demonstrated that LysM domains are involved in plant recognition of symbiotic bacteria (22, 24, 30). It has been proposed that the LysM-type cell wall-binding domain binds noncovalently to peptidoglycans of various gram-positive bacteria (39). Following secretion of the lactococcal AcmA, the PA directs the protein to the cell wall. Hybrid PA fusions exhibited similar properties, as has been demonstrated for MSA2, β-lactamase, α-amylase, viral capsid proteins, and FedF-PA fusions (4, 17, 23, 31). It was observed in all cases that only a small amount of the secreted PA fusion bound to the *L. lactis* producer cells. Most of the secreted PA-fusion protein remained in the culture supernatant. In some cases it was demonstrated that the PA fusions in the growth medium could be used in *trans* to bind to nonproducer *L. lactis* cells, albeit again in small amounts (31, 39). Steen et al. (39) discovered that boiling of *L. lactis* whole cells in trichloroacetic acid (TCA) followed by washing and neutralization prior to binding dramatically increased the amount of MSA2-PA that can be bound to the cells.

Here, we found that several acids can be used for pretreatment of *L. lactis* cells in order to enhance the binding of PA fusions. The acid-pretreated nonliving lactococcal cells were designated "gram-positive enhancer matrix (GEM) particles," and they may provide a suitable non-GMO support for PA-mediated cell surface display of all kinds of heterologous proteins. Therefore, we constructed a generally applicable surface display system based on lactococcal GEM particles and the PA. We optimized the conditions for preparation of GEM particles, analyzed their DNA and protein contents, and determined the maximum loading capacity for PA fusions. In addition, the influence of the number of PA LysM motifs in the PA on binding efficiency was determined, and LysM-containing

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motifs of other proteins were tested for the ability to bind to lactococcal GEM particles.

Moreover, we constructed a versatile lactococcal PA display vector that allows efficient inducible expression and secretion of PA fusions into the culture medium. The addition of cell-free culture medium containing a PA fusion to lactococcal GEM particles results in efficient, strong, and selective surface binding of the chimeric anchor fusion protein without a need for additional purification steps. To demonstrate the applicability of this display system, two biocatalysts, α-amylase and β-lactamase, were functionally displayed in different ratios on the surface of *L. lactis* GEM particles. Furthermore, we found that PA-mediated surface display of epitopes of the *Plasmodium berghei* malaria circumsporozoite protein antigen on *L. lactis* GEM particles induces high levels of immunoglobulin G (IgG)-specific serum antibodies in nasally immunized mice. Altogether, this system provides a cheap, flexible, effective, easy-to-handle alternative for surface display of proteins and peptides on non-genetically modified bacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. *L. lactis* strains were grown at 30°C in M17 broth (Oxoid) containing 0.5% (wt/vol) glucose (GM17) and, when necessary, supplemented with chloramphenicol (5 μg/ml) or erythromycin (5 μg/ml) for plasmid selection. Induction for P_{nisA}-driven gene expression was done with the culture supernatant of the nisin-producing organism *L. lactis* strain NZ9700 as described previously (14). *Lactobacillus* spp. were grown in MRS broth (Oxoid) in standing cultures at 30°C. *Bacillus subtilis* was grown aerobically in TY broth at 37°C. *Mycobacterium smegmatis* was grown aerobically in Middlebrook medium (Oxoid) at 37°C. *Escherichia coli* strains were grown in Luria-Bertani liquid medium or on agar plates at 37°C; both media were supplemented with 100 μg/ml ampicillin.

General molecular biology. Unless specified otherwise, all protein analysis and standard recombinant DNA techniques were performed as described previously (35) or as specified by the manufacturers. Enzymes and buffers were purchased from New England Biolabs or Roche. Electrotransformation of *L. lactis* was carried out as described previously (12) using a Bio-Rad Gene Pulser (Bio-Rad). Nucleotide sequence analyses were performed by BaseClear (Leiden, The Netherlands).

The PA6 concatemer constructs were made as follows. The first LysM repeat of the AcmA protein anchor (nucleotides 819 to 1038) was amplified by PCR using oligonucleotides cA1repeat1.fw (5′-CCGTCTCCAATTGGAGCTTCTTC AGCTGGAAATAC) and cA1repeat1.rev (TAATAAGCTTAAAGGTCTCCA ATTCCTGTCAGTACAAGTTTTTGACCAATG). The resulting amplification product had at the 5′ end an Esp3I site that generated EcoRI sticky ends. The 3′ end contained an Eco31I site that generated EcoRI sticky ends and 3′ of that a HindIII site. The amplicon was digested with Esp3I and HindIII and ligated into pPA3 that was digested with EcoRI and HindIII, yielding plasmid pPA6. Successive PA6 modules were added by ligating the Esp3I (which generated an EcoRI sticky end)/HindIII-digested amplification product into pPA6 digested with Eco31I (which generated a EcoRI sticky end) and HindIII. After ligation, the Eco31I recognition site in the vector was lost, but a new one was introduced with insertion of the PCR fragment, which allowed insertion of the following LysM PCR fragment.

RTQ-PCR. An *E. coli* plasmid containing the lactococcal *htrA* gene (pTOPO-htrA) was used as a standard. The number of *htrA* copies was derived from the DNA concentration of the isolated pTOPO-htrA, which was measured spectrophotometrically at 260 nm. A standard real-time quantitative PCR (RTQ-PCR) was performed with 10^2 to 10^8 *htrA* copies per PCR mixture.

Primers HtrA.5 (5′-GAGGATATCATAATCATCCTCAG) and HtrA.6 (5′-GTCGGCTCACCTTTAGGTAG) (Sigma-Genosys) were used to target the *htrA* gene. In a conventional PCR these primers yielded a single specific 0.9-kb amplicon with total DNA of *L. lactis* or plasmid pTOPO-htrA as the template.

RTQ-PCR was performed with 1 μl of total DNA isolated from 2.5×10^9 nontreated *L. lactis* cells or lactococcal GEM particles using an iCycler IQ real-time PCR detection system (Bio-Rad). Total DNA was isolated as described by Leenhouts et al. (19), with one modification: cell pellets were lysed in 0.5% sodium dodecyl sulfate (SDS) after incubation with lysozyme (2 mg/ml) and mutanolysin (15 U) at 55°C for 15 min. The reaction mixtures (total volume, 25 μl) contained IQSYBR Green Supermix (Bio-Rad) and 2 μM of each primer. The temperature program consisted of one cycle of 95°C for 60 s, followed by 40 cycles of 60 s at 95°C, 60 s at 50°C, and 60 s at 72°C for data acquisition. To evaluate the specificity of the amplification, a melting curve was determined over the range from 60°C to 95°C with increments of 0.5°C for 10 s. Standard samples were included in each run to establish a standard curve. The iCycler IQ software (Bio-Rad) was used for data analysis.

***L. lactis* pretreatments and binding conditions.** Chemical pretreatment of *L. lactis* NZ9000(Δ*acmA*) was routinely done with 10% trichloroacetic acid (TCA) (0.6 M) as follows. Cells of stationary-phase cultures were collected by centrifugation and washed once with 0.5 volume of phosphate-buffered saline (PBS) (58 mM $Na_2HPO_4 \cdot 2H_2O$, 17 mM $Na_2H_2PO_4 \cdot H_2O$, 68 mM NaCl; pH 7.2). Cells were resuspended in 0.2 volume of a 10% TCA solution and boiled for 30 min. Subsequently, the GEM particles formed in this way were washed three times with PBS and resuspended in PBS so that the average concentration was 2.5×10^{10} GEM particles/ml, as determined with a Burker-Turk hemocytometer. GEM particles were either used immediately for binding experiments or stored in 0.5-ml aliquots at −80°C until use. The following chemicals and conditions were used to examine the effect of pretreatment on the binding capacity of *L. lactis* cells for PA fusion proteins: 0.2 M TCA, 0.01 M hydrochloric acid (HCl), 5.6 M acetic acid (HAc), 0.72 M lactic acid, 10% SDS, and 4 M guanidine hydrochloride. An alternative pretreatment was performed with Tris-buffered phenol for 15 min at 55°C. The effects of the pretreatments on viability were analyzed by recovery and plating of the cells under the conditions used after electroporation of *L. lactis* cells (12).

In a typical binding experiment 2.5×10^9 GEM particles were incubated for 30 to 60 min at room temperature in an end-over-end rotator with 0.5 to 50 ml of cell-free culture medium containing a PA fusion protein. The fusions used in this study, listed in Table 1, all contained the PA at the C terminus of the protein. When appropriate, culture medium containing PA fusions was concentrated prior to binding using a Vivaflow 200 (Vivascience, Germany). After binding, GEM particles were collected by centrifugation, washed twice with PBS, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), immunoblotting, or enzymatic activity analysis.

Protein purification and analysis. PA3 was purified essentially as described previously (39). SDS-PAGE was carried out by using standard procedures (15). After separation, proteins were either stained with Coomassie brilliant blue or electroblotted on polyvinylidene difluoride membranes (Roche). In immunoblots, PA fusions were detected with horseradish peroxidase-conjugated anti-*c-myc* antibody (Roche). The membrane was developed with a 3-amino-g-ethylcarbazole (AEC)/H_2O_2 substrate solution (Sigma) in 20 ml 100 mM HAc-NaOH (pH 5.0), as specified by the manufacturer.

The amount of PA6 concatemers was determined densitometrically by analysis of scans of Coomassie brilliant blue-stained SDS–12.5% polyacrylamide (PAA) gels with the Total Lab image analysis software, version 1.0 (Non Linear Dynamics, United Kingdom). A calibration curve was generated using bovine serum albumin (BSA) protein standards on the same PAA gel.

For His tag isolation of CSP[2xB]-His, *E. coli* BL21DE3(pPA193) was cultured in 1 liter growth medium and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an optical density at 600 nm of ~0.5. Cells were harvested 2 h after induction, resuspended in lysis buffer (50 mM Tris-HCl, 50 mM NaCl; pH 8.0), and disrupted by two passages through a One Shot cell disruptor (Constant Systems, United Kingdom) at $2{,}500 \times 10^5$ Pa. Cleared lysate was applied to Ni-nitrilotriacetic acid resin prewashed with binding buffer (300 mM NaCl, 50 mM NaH_2PO_4, 10 mM imidazole; pH 8.0), and His-tagged protein was eluted in elution buffer (binding buffer with 100 mM NaCl and 240 mM imidazole). Fractions containing CSP[2xB]-His were pooled and subjected to buffer exchange against water with a HiPrep 26/10 column (Pharmacia). A total of 13 mg purified CSP[2xB]-His was obtained in this way.

Enzyme assays. The enzyme activities of bound α-amylase– and β-lactamase–PA fusions (α-PA and β-PA, respectively) were measured colorimetrically. GEM particles loaded with both enzymes were spun down and washed twice with PBS. α-Amylase activity was determined by incubating the loaded GEM particles in 1 ml of an amylose azure (Sigma) substrate solution (0.6 mg/ml amylose azure in 20 mM K_2HPO_4/KH_2PO_4 buffer containing 50 mM NaCl [pH 7.5]) at 37°C and 200 rpm. After 60 min, GEM particles and insoluble amylose azure were spun down, and the absorbance at 595 nm was determined.

β-Lactamase activity was measured by adding 40 μl nitrocefin (CalBiochem) to GEM particles loaded with β-PA in 1 ml (final volume) of PBS. After 30 min the absorbance at 486 nm was determined.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype(s) or genotype(s)[a]	Source or reference
Strains		
E. coli BL21DE3	Allows IPTG-inducible expression	Novagen
E. coli TOP10	Cloning host	Invitrogen
L. lactis MG1363	Plasmid-free derivative of NCDO712	9
L. lactis NZ9700	Nisin producer	14
L. lactis NZ9000	MG1363 *pepN*::*nisRK*, allows nisin-inducible expression	14
L. lactis NZ9000(Δ*acmA*)	Derivative of NZ9000 lacking *acmA*	4
L. lactis PA1001	Derivative of NZ9000 lacking *acmA* and *htrA*	This study
Lactobacillus casei	Wild type	ATCC 393
Lactobacillus sake	Wild type	NCFB 2714
Lactobacillus salivarius	Wild type	NCFB 2747
Lactobacillus curvatus	Wild type	NCFB 2739
Bacillus subtilis	Wild type	Laboratory collection
Mycobacterium smegmatis	Wild type	ATCC 700084
Plasmids		
pNG400	Cm^r, pNZ8048 derivative containing P_{nisA}, $usp45_{ss}$, and a transcription terminator	Laboratory collection
pNG3041	Cm^r, pNZ8048 derivative containing P_{nisA}, *prtP* preprosequence, and *msa2-acmA* PA (MSA2-PA)	39
pNG3042	Cm^r, pNZ8048 derivative containing P_{nisA}, *prtP* preprosequence, and *msa2-acmD* PA (nucleotides 1286 to 2371)[b]	This study
pPA1	Cm^r, pNZ8048 derivative containing c-*myc, B. licheniformis* α-amylase (*amyL*) under control of P_{nisA}, and $usp45_{ss}$	This study
pPA3	Cm^r, pNZ8048 derivative containing c-*myc*, the *acmA* PA (nucleotides 835 to 1492)[c] under control of P_{nisA}, and $usp45_{ss}$ (PA3)	This study
pPA5	pPA3 derivative, *B. licheniformis* α-amylase (*amyL*) upstream of the *acmA* PA sequence (α-PA)	This study
pPA6 (1–6)	pPA3 derivative containing one to six copies of first LysM repeat of *acmA* PA (nucleotides 819 to 1038)[c]	This study
pPA95	Cm^r, pNZ8048 derivative containing c-*myc*, the *E. coli mltD* LysM repeats (nucleotides 927 to 1313)[d] under control of P_{nisA}, and $usp45_{ss}$	This study
pPA165	Cm^r, pNZ8048 derivative containing c-*myc*, human LysM repeat *erap140* (amino acids 116 to 158 [accession no. AAM27392]; in duplicate) under control of P_{nisA}, and $usp45_{ss}$	This study
pPA171	Cm^r, pNZ8048 derivative containing *P. berghei* CSP B-cell epitope sequences upstream of the *acmA* PA sequence under control of P_{nisA} and $usp45_{ss}$; the epitopes PPPPNPND and NANDPAPP were duplicated and fused to the PA, $[PPPPNPND]_2$-$[NANDPAPP]_2$-PA (CSP[2×B]-PA)	This study
pPA193	Ap^r, pET32c (Novagen) derivative producing CSP[2×B]-His	This study
pGBL1	Em^r, pGB14 derivative, β-lactamase secretion vector	28
pGBLR	Em^r, pGBL1 derivative producing secreted β-lactamase fused to the AcmA PA (β-PA)	4
pINT-HtrA	Em^r, pORI280 derivative containing a 900-bp chromosomal DNA fragment 5′ of *htrA* and a 1,200-bp fragment 3′ of *htrA*; primers used to generate the chromosomal fragments were HtrA.1 (5′-CGGTCTCACTAGCGCATTTTCTAAATTTTATTTTTGCGG), HtrA.2 (5′-CCCCCCGCGGCCGCCCTTTCAATTATTACTCTTTGTAGC), HtrA.3 (5′-GGGGGGGCGGCCGCCATACTAAAGTCTGAAATTTTG), and HtrA.4 (5′-CGGTCTCAAATTACGTTTATATTTCTGTAAAGTCTATC)[e]	This study
pTOPO-htrA	Ap^r Km^r, derivative of pCR4Blunt-TOPO (Invitrogen) containing the *L. lactis htrA* gene	This study

[a] P_{nisA}, nisin-inducible promoter; $usp45_{ss}$, signal sequence of *usp45*.
[b] See reference 3.
[c] See reference 5.
[d] See reference 8.
[e] See reference 18. Sequences homologous to the MG1363 chromosome sequence are underlined.

Microscopy. For immunofluorescence microscopy, suspensions containing 100 μl of GEM particles incubated with a PA fusion were washed twice with demineralized water and resuspended in an equal volume of PBS containing 1% BSA and mouse anti-c-myc antibody that was diluted 1:50. After incubation for 1 h at room temperature, the particles were washed three times with 1 ml PBS. Subsequently, they were incubated with 1 volume of PBS containing 1% BSA and Oregon Green-labeled goat anti-mouse immunoglobulin (Molecular Probes) diluted 1:100. After one wash with 2 volumes of PBS and two washes with 2 volumes of demineralized water, the GEM particles were resuspended in 100 μl demineralized water. A 10-μl aliquot of the suspension was spread onto a polysin microslide (Menzel-Gläser) and air dried. After application of 8 μl Vectashield (Vector, Burlingame, CA) and mounting of a coverslip, the samples were examined by fluorescence microscopy using a Zeiss microscope (Carls Zeiss, Thornwood, CA) and an Axion Vision camera (Axion Technologies, Houston, TX). MSA2-PA was detected by fluorescence microscopy using anti-MSA2 polyclonal rabbit serum (32) and Oregon Green-conjugated goat anti-rabbit immunoglobulin with H+L specificity (Molecular Probes) as the secondary antibody.

For transmission electron microscopy, samples were fixed by adding glutaraldehyde (Fluka) to a final concentration of 0.1%. After 3 h of fixation at 0°C the material was washed with 0.1 M sodium cacodylate (CAC buffer) (pH 7.2) and postfixed for 2 h in a solution containing 1% osmium tetroxide and 5% sodium bichromate in CAC buffer. Subsequently, the specimens were stained overnight

in 0.5% uranyl acetate. After dehydration with a graded ethanol series the specimens were embedded in Epon (Serva). Ultrathin sections were cut with an LKB ultramicrotome and examined with a Philips CM10 transmission electron microscope.

For scanning electron microscopy, bacteria were fixed in 2% glutaraldehyde in 0.1 M CAC buffer (pH 7.4) for 12 h at 4°C, and this was followed by rinsing with 0.1 M CAC buffer at room temperature. Cell suspensions were spotted on clean poly(L-lysine)-coated (0.1 to 0.2%) mica slides. After dehydration with a graded ethanol series, samples were dried under CO_2 using a critical point drier (Balzers CPD20; Bal-Tec, Liechtenstein). Samples were Au/Pd sputter coated (Balzers 07120B; Bal-Tec), and images were obtained with a JEOL 6301-F scanning electron microscope (JEOL Europe, Belgium) equipped with the Soft Imaging system (PAES, Netherlands).

Vaccine preparation. Cultures of strain *L. lactis*(pPA171) were grown and induced as described above to express CSP[2xB]-PA. The cell-free supernatant was used as a source of secreted CSP[2xB]-PA. Unconcentrated cell-free culture supernatant contained approximately 0.75 mg CSP[2xB]-PA per liter. For each vaccine dose, 2.5×10^9 *L. lactis* GEM particles were incubated for 30 min at room temperature with 0.8 ml of 33-fold-concentrated cell-free culture supernatant containing CSP[2xB]-PA. GEM particles with the bound CSP[2xB]-PA were collected by centrifugation and washed twice in PBS. Finally, the GEM particles were resuspended in 20 μl PBS for nasal immunizations and in 100 μl PBS for subcutaneous immunizations and stored at −80°C until use. The amount of bound CSP[2xB]-PA was estimated by using Coomassie brilliant blue-stained gels and by comparison to BSA protein standards. Each vaccine dose contained approximately 20 μg CSP[2xB]-PA (about 1.75×10^5 molecules/cell).

Immunization of mice. Six-week-old female BALB/c mice (Harlan, The Netherlands) were used for immunization. Groups consisted of 10 mice. Mice that were vaccinated nasally were lightly anesthetized with 2% (vol/vol) isoflurane over oxygen (1.2 liters/min) and NO_2 (0.6 liter/min), which was administered with a calibrated vaporizer. Anesthesia was confirmed by observation that there was no pinch reflex reaction. Once anesthetized, the animals were scruffed, held in the supine position, and vaccinated intranasally by placing a series of small droplets (20 μl) of the inoculum into the nostrils for the mice to involuntarily inhale. After vaccination the mice were laid on their backs until recovery. Nasally immunized mice received either GEM particles with CSP[2xB]-PA, GEM particles, or PBS alone. Subcutaneous immunization with GEM particles with CSP[2xB]-PA was performed by injecting 50 μl of the inoculum into each flank under light inhalation anesthesia.

The mice were immunized on days 1, 22, and 43, and sera were collected on days 9, 30, and 55 by orbital puncture under light anesthesia. The sera were stored at −20°C until use.

Measurement of the serum antibody response. To determine the titer of the antibodies against CSP[2xB], an enzyme-linked immunosorbent assay was used. Briefly, high-binding-capacity microtiter plates (Greiner) were coated with CSP[2xB]-His (0.2 μg/well) in 0.05 M carbonate buffer (pH 9.6) overnight at 4°C. The plates were washed with PBS (pH 7.4) with 0.02% Tween 20 and then incubated for 1 h with 5% BSA in PBS-Tween. Sera were diluted appropriately, threefold dilutions were added to the plates, and the plates were incubated for 2 h at room temperature. After washing, the alkaline phosphatase secondary antibody directed to mouse IgG-Fc (Sigma) was incubated for 1.5 h at a dilution of 1:5,000. A colorimetric reaction was obtained by addition of *p*-nitrophenyl phosphate substrate (Sigma) diluted in 0.05 M carbonate buffer (pH 9.6) containing 0.001 mM $MgCl_2$. The enzymatic reaction was stopped with NaOH, and the results were read at 405 nm. Concentrations were calculated from a calibration curve prepared with purified mouse IgG (Sigma).

RESULTS AND DISCUSSION

PA-mediated surface display. The three LysM motifs in the AcmA PA are responsible for binding to the cell wall peptidoglycans of various gram-positive bacteria (39). The N-terminal part of the protein is thus exposed to the outer surface of the cell wall. It was found that exogenous addition of a hybrid protein containing PA (i.e., one PA C-terminally fused to MSA2 [MSA2-PA], a malaria parasite merozoite-stage surface antigen) caused specific binding to *L. lactis* cells. MSA2-PA bound to the cell poles of living cells, whereas it was able to bind to the entire cell surface when the cells were first boiled in TCA and washed with PBS prior to addition of the fusion protein (39). The resulting TCA-pretreated *L. lactis* cells, designated GEM particles, exhibited considerably increased binding capacity for PA fusions (see below). The method described here for preparation of GEM particles is generally applicable to gram-positive bacteria. We obtained similar results for *L. lactis* and *Lactobacillus casei* using MSA2-PA (39) and also using other PA fusion proteins, like PA3 (see below), with various other gram-positive bacteria, including *Lactobacillus sake*, *Lactobacillus salivarius*, *Lactobacillus curvatus*, *B. subtilis*, and *M. smegmatis* (data not shown).

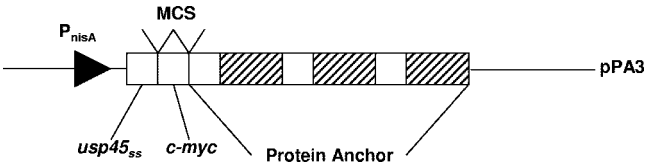
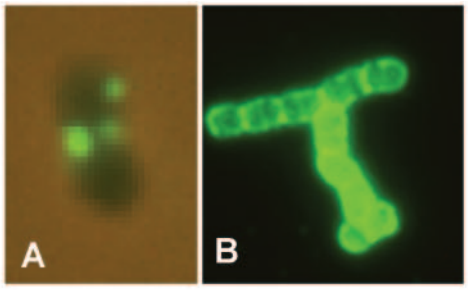
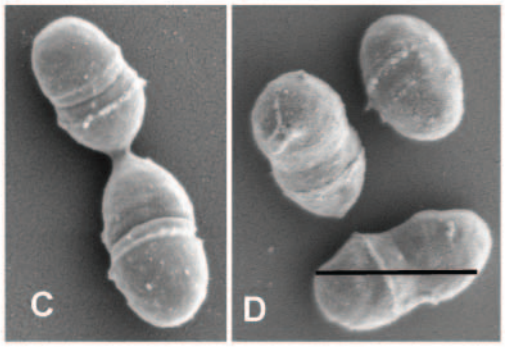


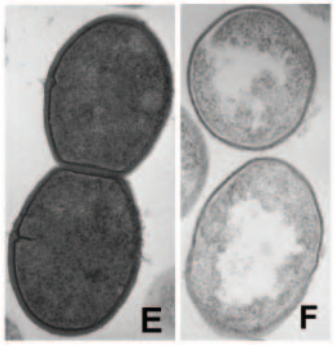
FIG. 1. Schematic diagram of the organization of the general display vector pPA3. Abbreviations: P_{NisA}, inducible *nisA* promoter; $usp45_{ss}$, *usp45* signal sequence; c-*myc*, region encoding an epitope for immunodetection; MCS, multiple cloning site. Cross-hatched boxes indicate the LysM repeat sequences.

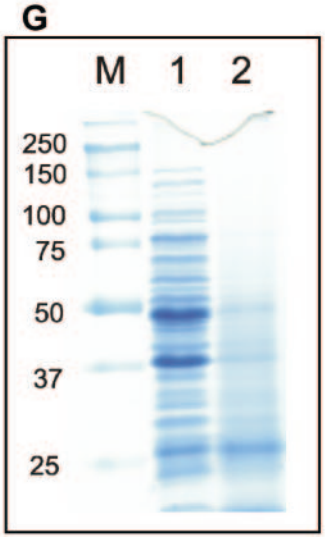
In addition to the AcmA PA, a number of alternative LysM-type PAs were produced as c-Myc fusions and tested for their ability to bind to lactococcal GEM particles. The LysM domains included are the peptidoglycan-binding motifs of (i) the membrane-bound lytic transglycosylase MltD from *E. coli* (8) (two LysM sequences), (ii) the putative peptidoglycan hydrolase AcmD of *L. lactis* (3) (three LysM sequences), and (iii) a human LysM domain present in the estrogen receptor-associated protein ERAP140 (38) (one LysM sequence, but it is expressed here as a duplicate). Lactococcal GEM particles were incubated with *L. lactis* PA1001 cell-free culture supernatant containing these LysM-type PAs fused to the c-Myc epitope. Western blot analysis of the GEM particles and supernatant fractions after binding revealed that all the non-AcmA PA domains exhibited no or only poor binding, whereas all of the AcmA PA was completely bound to the GEM particles (data not shown). Thus, under the conditions used even the lactococcal AcmD PA homolog exhibited poor binding. Since AcmA PA can be bound to various gram-positive bacteria, it seems to have relaxed peptidoglycan binding specificity, while non-AcmA PAs exhibit a higher degree of specificity or require more specific binding conditions.

On the basis of these results we developed a broadly applicable surface display system which takes advantage of the ability of AcmA PA-containing proteins to bind noncovalently to lactococcal GEM particles. PA-mediated surface display differs in a number of aspects from bacterial surface display systems that have been described previously (36). First, the strain for production of PA-containing hybrid proteins and the host strain for surface display can be different, allowing the use of a non-GMO display strain. In contrast, previously described bacterial display systems rely on recombinant host strains to which the anchoring domain is covalently attached to the cell surface during translocation (16). Second, the AcmA PA has a high affinity for gram-positive cell walls, and *L. lactis* growth medium contains only a few other proteins (3). Therefore, the cell-free culture medium is directly suitable for addition to lactococcal GEM particles, and further purification steps are









not necessary. Finally, AcmA PA fusions can be bound to living as well as nonliving gram-positive bacteria. Moreover, PA fusions bound to GEM particles were found to be very stable. No loss or degradation of the PA fusion was observed during storage of the loaded GEM particles in PBS at room temperature, 4°C, and −80°C (41).

PA display vector. In order to create a generally applicable PA fusion system, we constructed the versatile surface display vector pPA3. This vector has the following parts (Fig. 1): the lactococcal pNZ212 origin of replication; a chloramphenicol acetyltransferase gene for selection; the P_{nisA} promoter to direct inducible expression of PA fusions (7); the lactococcal Usp45 signal sequence to drive secretion (40); the c-Myc epitope, which was included for detection of secreted protein; a multiple cloning site to allow insertion of recombinant genes; and the gene fragment encoding the PA (C-terminal 218-amino-acid cell wall-binding domain of AcmA of *L. lactis*, as described by Buist et al. [5]), including its stop codon and transcriptional terminator. *L. lactis* strains carrying pPA3 secrete the c-Myc–PA fusion (PA3) into the growth medium upon induction of the promoter.

L. lactis NZ9000 and NZ9000(Δ*acmA*) appeared not to be efficient hosts for the production of full-length PA3 since PA degradation products were observed in the growth medium (data not shown). These degradation products were most likely due to the activity of the surface-located housekeeping protease HtrA, which has been shown to degrade the AcmA cell wall-binding domain (4, 5, 26, 29). Efficient production of full-length PA3 was obtained by using an *htrA*-deficient *L. lactis* strain. To this end, a deletion in the chromosomal copy of *htrA* of *L. lactis* NZ9000(Δ*acmA*) was made using the pORI280 replacement recombination system (18). The resulting strain, *L. lactis* PA1001, was used for production of PA derivatives throughout this study. *L. lactis* PA1001(pPA3) produced and secreted only full-length PA3, only a small part of which was bound to the *L. lactis* production cells. Most of the PA3 was secreted as soluble protein into the culture supernatant. *L. lactis* PA1001(pPA3) produced about 10 mg PA3 per liter GM17, as estimated from Coomassie brilliant blue-stained SDS-PAA gels (data not shown).

Maximum binding capacity of lactococcal GEM particles. Purified PA3 was used to determine the maximum binding capacity of lactococcal GEM particles. Different amounts of purified PA3 (20 to 160 μg) were added to 2.5×10^9 lactococcal GEM particles in 1 ml PBS. Standard binding conditions were used as described in Materials and Methods. After binding, the GEM particles were separated from the supernatant by centrifugation and washed twice with 1 ml PBS. Subsequently, the amount of PA3 on the GEM particles and the

FIG. 2. Fluorescence microscopic (A and B), scanning electron microscopic (C and D), and transmission electron microscopic (E and F) images of *L. lactis* cells and *L. lactis* GEM particles. (A, C, and E) Untreated cells; (B, D, and F) GEM particles. Typical examples are shown. The samples in panels A and B contained bound PA3. The bar in panel D represents 1 μm. (G) Coomassie brilliant blue-stained SDS-PAA gel of total protein extracts of 5×10^8 *L. lactis* cells (lane 1) and an equal number of *L. lactis* GEM particles (lane 2). The sizes of the marker proteins (lane M) (in kDa) are indicated on the left.

amount of the PA3 remaining in the supernatant were analyzed using Coomassie brilliant blue-stained SDS-PAA gels. Control samples containing nontreated *L. lactis* cells (which bound very little PA3 [Fig. 2A]) or containing no cell particles were included in the analyses to exclude nonspecific entrapment of PA3 between particles and nonspecific binding of PA3 to the reaction vials, respectively. These analyses showed that 140 to 150 μg PA3 bound specifically to the GEM particles; with these amounts no PA3 was found in the supernatant, whereas most PA3 (> 90%) and all PA3 remained in the supernatants of the nontreated *L. lactis* cells and the no-particle control samples, respectively. After addition of more than 150 μg PA3 some PA3 remained in the supernatant after binding. These results indicated that under the conditions used at most 150 μg PA3 can bind to 2.5×10^9 lactococcal GEM particles. Thus, GEM particles saturated with PA3 (molecular mass, 28 kDa) contained about 10^6 PA3 molecules per cell. The amount may be smaller for PA fused to more bulky polypeptides. Nevertheless, the surface density of PA3 on GEM particles compares favorably with the surface densities for other surface display systems, which rely on expression of proteins on the surface of bacterial cells or spores. For example, surface densities of 10^4 and 1.5×10^3 molecules per cell have been reported for *Staphylococcus carnosus* (1) and *B. subtilis* spore (13) surface display systems, respectively.

Influence of the number of LysM motifs in the PA on binding. The AcmA PA consists of three LysM motifs (5). In the LysM protein family the number of LysM repeat motifs varies between 1 and 12. Proteins containing multiple LysM motifs are considered multivalent with the potential to bind to their substrates with high affinity. PA, for example, binds very strongly to its natural substrate, the *L. lactis* cell wall. Bound PA was only partially removed from GEM particles after treatment with 8 M LiCl (not shown). This compound is commonly used to remove noncovalently bound proteins from bacterial cell walls. Thus, although PA binds to bacterial cell walls in a noncovalent manner, the binding interactions appear to be very strong.

In order to test the effect of the number of LysM repeats on cell wall binding, we first expressed and secreted a single binding module, PA6, comprising the first LysM motif and spacer of the PA (amino acid residues 215 to 287; AcmA numbering as described by Buist et al. [5]). This single binding module was able to bind to GEM particles. However, binding was poor since a considerable amount of PA6 remained in the supernatant after binding (data not shown). Subsequently, PA6 modules were added one by one in chimeric constructs, yielding concatemers consisting of up to six PA6 modules (*n*PA6, with $n = 1$ to 6). All the *n*PA6 chimeras were secreted by *L. lactis* PA1001, and supernatants containing the different concatemers were used to analyze binding to *L. lactis* GEM particles. SDS-PAGE analysis of PA6 concatemers showed that proteins containing up to four PA6 modules were produced at comparable levels (data not shown). Production of 5PA6 and 6PA6 was considerably lower. The lower protein yields could be related to proteolytic degradation of the proteins, as well as to plasmid instability (data not shown).

Binding of PA6 concatemers to GEM particles was monitored colorimetrically (Fig. 3). In this case, binding was detected using the c-Myc tag as a reporter. Different amounts of

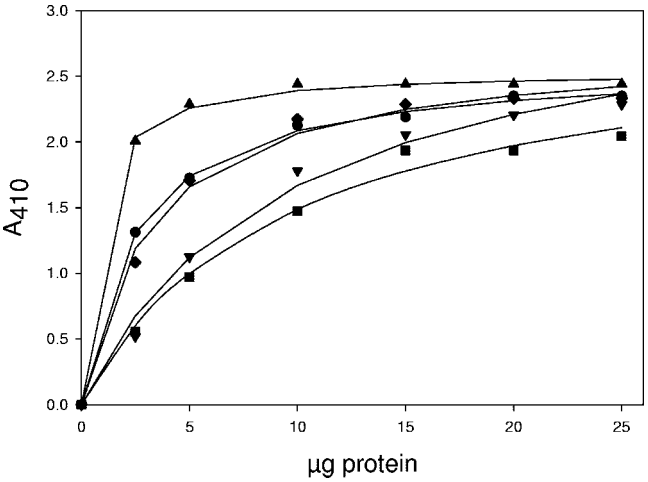


FIG. 3. Colorimetric assay for detection of surface-displayed PA6 concatemers on lactococcal GEM particles. Different amounts of PA6 concatemers were added to 2.5×10^9 GEM particles. After binding, particles were collected by centrifugation and washed twice with PBS buffer. Surface-displayed PA6 concatemers were detected with horseradish peroxidase-conjugated anti-c-myc antibody (Roche). GEM particles were washed three times with PBS and developed with 1 ml of the horseradish peroxidase substrate 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid (ABTS) (Fluka)–H_2O_2. Absorbance at 405 nm was determined after a suitable time. ■, PA6; ▲, 2PA6; ●, 3PA6; ▼, 4PA6; ◆, PA3.

PA6 concatemers carrying an N-terminally exposed c-Myc tag were allowed to bind to lactococcal GEM particles. As expected, the lowest level of binding was found for 1PA6. In other experiments we determined that 1PA6 without a spacer domain (amino acid residues 243 to 286; AcmA numbering as described by Buist et al. [6]) still bound to GEM particles, albeit with lower affinity. The spacer domain itself (amino acid residues 215 to 242; AcmA numbering as described by Buist et al. [5]) did not show binding to GEM particles (data not shown). A steep increase in binding affinity was obtained by addition of a second PA6 domain, and the level was even somewhat higher than that for wild-type PA3 (Fig. 3). Three PA6 modules exhibited binding affinity similar to that of the wild-type PA3, whereas addition of a fourth domain had a negative effect on the binding affinity.

In summary, strong binding to lactococcal GEM particles was already obtained with two PA6 modules (i.e., two identical AcmA LysM domains).

Lactococcal GEM particles. In the method to generate lactococcal GEM particles that was initially described 10% TCA is used. This acid is commonly used for purification of peptidoglycan from cell wall extracts. TCA is known to specifically remove (lipo)teichoic acids from cell walls. It has been proposed that these cell wall components prevent PA from binding to the cell surface (39).

For some applications TCA may be a less desirable chemical for use in large-scale production of GEM particles. A number of alternative acids were examined, including HCl, acetic acid, lactic acid, and some other chemicals generally used for purification of cell wall peptidoglycan. The acid concentration was optimized to prevent cellular lysis. The effect of various chemical treatments on the loading capacity of GEM particles was

investigated using a fusion protein consisting of α-amylase from *Bacillus licheniformis* and PA (α-PA). α-PA was functionally displayed on the surface of lactococcal GEM particles (see below). Culture medium containing α-PA was added to chemically pretreated lactococcal cells. α-PA bound to GEM particles was visualized by incubating loaded GEM particles with the chromogenic amylase substrate amylase azure. The absorbance at 595 nm was a measure of the amount of bound α-PA. The results, summarized in Fig. 4, clearly show that all of the acids tested were equally effective at the concentrations indicated in improving the loading capacity of *L. lactis* cells. Compared to nonpretreated *L. lactis* cells, 10- to 15-fold increases in absorbance were obtained with GEM particles, illustrating the greater loading capacity of the GEM particles. Pretreatment of lactococcal cells with a number of chemicals generally used for purification of cell wall peptidoglycan, such as phenol, guanidine hydrochloride, and SDS, did not affect the loading capacity of the cells (not shown). All the treatments with the acids and other chemicals tested resulted in complete killing of the lactococcal cells (plating efficiency, 0).

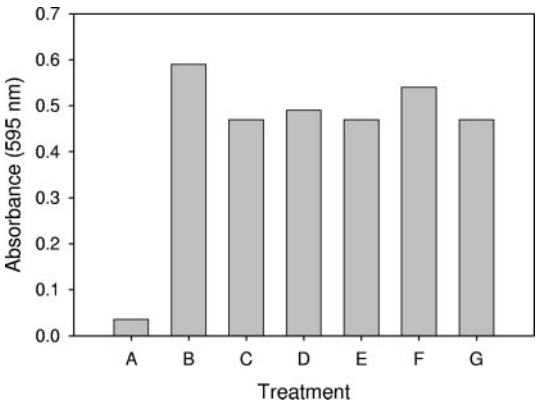


FIG. 4. Effect of different acid pretreatments on the binding capacity of *L. lactis* particles. α-PA was added to an equal number of GEM particles. Bound α-PA was determined with the chromogenic substrate amylose azure. Bar A, no treatment; bar B, 10% TCA; bar C, 0.2% TCA; bar D, 5.6 M HAc; bar E, 0.01 M HCl; bar F, 0.72 M lactic acid; bar G, 0.56 M formic acid. The means for three determinations (<15% difference) are shown.

Fluorescence and electron microscopic analyses of *L. lactis* cells pretreated with 10% TCA showed that the harsh treatments left the cellular morphology largely intact (Fig. 2A to D). Similar results were obtained with cells treated with HCl, H_2SO_4, and HAc (data not shown). Due to the acid pretreatment, binding of PA3 occurred on the entire cell surface, whereas in nonpretreated cells PA3 bound only to the cell poles (Fig. 2A and B). This clearly illustrated the increased binding capacity of lactococcal GEM particles.

The harsh acid pretreatment of the lactococcal cells most likely affected the protein and DNA contents of the GEM particles. The intracellular contents of the GEM particles seemed to be partially released or degraded (Fig. 2E and F). Similar results were obtained for all acids tested; however, the optimal acid concentrations could differ for each acid (41). Figure 2G shows a typical example of an SDS-PAGE analysis of proteins from lysed acid-pretreated cells. Substantial amounts of proteins were removed from the GEM particles and/or degraded. The DNA content of lactococcal GEM particles was determined by RTQ-PCR. Total DNA isolated from approximately 2.5×10^9 nontreated *L. lactis* cells or lactococcal GEM particles was used as the template. Three separate runs of RTQ-PCR were performed to determine the DNA content of lactococcal cells. For nontreated *L. lactis* cells an average of one to two copies of the *htrA* gene per cell was calculated, which is close to the theoretical value (one copy). For GEM particles we calculated that for each particle there was about 10^{-6} copy of the *htrA* gene. In other words, in only 1 cell out of 10^6 GEM particles was a copy of the *htrA* gene present. This indicated that acid pretreatment of *L. lactis* cells degrades or removes almost all DNA from the cells.

In conclusion, lactococcal GEM particles are nonviable spherical peptidoglycan microparticles with a diameter of approximately 1 μm. They have a lower protein content, contain virtually no DNA, can be generated by boiling in various acids, and exhibit enhanced binding capacity for PA fusions.

Applications of the GEM particle-PA display system. (i) Immobilizing enzymes. Due to the enhanced binding capacity of *L. lactis* GEM particles and their nonproteolytic character, the GEM particle-PA system could provide an attractive alternative method for immobilizing enzymes. Enzymes can be attached to GEM particles by fusion to the PA. To test this, we used *B. licheniformis* α-amylase (AmyL) as a reporter enzyme. The α-PA fusion was bound to GEM particles. PA3 and wild-type AmyL incubated with GEM particles were used as controls. Amylase activity assays clearly demonstrated that α-PA was functionally displayed on the surface of GEM particles (Fig. 5a). As expected, no significant amylase activity was observed with GEM particles loaded with PA3 or with GEM particles incubated with supernatant containing AmyL (Fig. 5a).

Exogenous addition of PA fusions to lactococcal GEM particles allows controlled surface display of multiple proteins, in contrast to previously described surface display systems, which are based on expression of proteins on the cell surface. In these cases display of multiple proteins would require large plasmid constructs encoding multiple proteins or multiple plasmids in a single host organism. Moreover, the display level is largely dependent on the efficiency of expression and translocation. With PA-mediated surface display these problems are circumvented since display of multiple proteins can be achieved easily by incubating GEM particles with culture medium containing different PA fusions. This was illustrated by displaying two enzymes, *B. licheniformis* α-amylase (α-PA) and *E. coli* β-lactamase (β-PA), together on GEM particles. Different volumes of cell-free culture supernatant containing α-PA or β-PA were added, sequentially or premixed, to the GEM particles. Surface display of both α-PA and β-PA was visualized by assaying enzyme activity using the chromogenic substrates amylase azure and nitrocefin, respectively. The results are summarized in Fig. 5b and show that both enzymes were active when they were immobilized individually on lactococcal GEM particles (groups A and B). The cell-free culture supernatant volumes with α-PA used in groups C to E were the same as the volume used in group B, whereas the cell-free culture supernatant volumes used with β-PA varied; in group C the volume was one-third the volume in group A, in group D the volume was two-thirds the volume in group A, and in group E the volume

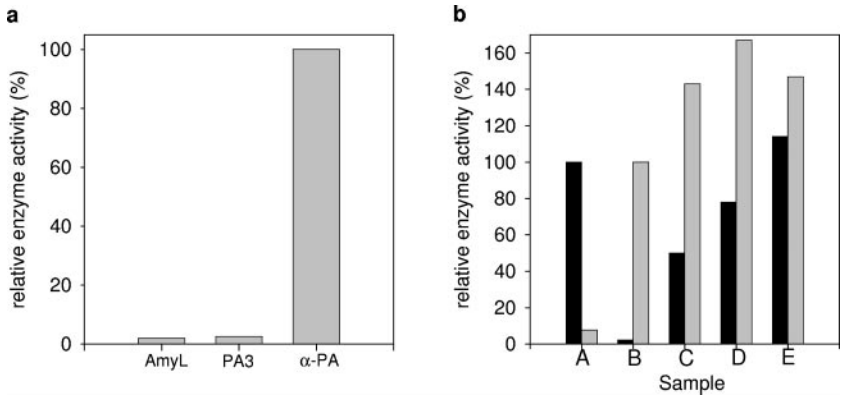


FIG. 5. Immobilization of enzymes on the surface of lactococcal GEM particles. (a) Relative α-amylase activities on GEM particles incubated with culture medium containing soluble AmyL, PA (PA3), or α-PA. (b) Relative α-amylase and β-lactamase activities on GEM particles carrying α-PA and β-PA at different ratios. Sample A, incubation with 1 volume of cell-free culture supernatant containing β-PA; sample B, incubation with 1 volume of cell-free culture supernatant containing α-PA; sample C, incubation with 1 volume of cell-free culture supernatant containing α-PA and 1/3 volume of β-PA; sample D, incubation with 1 volume of cell-free culture supernatant containing α-PA and 2/3 volume of β-PA; sample E, incubation with 1 volume of cell-free culture supernatant containing α-PA and 1 volume of β-PA. The mean value for three determinations (<15% difference) was normalized to the activities for samples A and B, which were defined as 100%. Solid bars, nitrocefin as the substrate (specific for β-lactamase activity); gray bars, amylose azure as the substrate (specific for α-amylase activity).

was the same as the volume in group A. The increase in β-PA activity correlated nicely with the added volume of culture supernatant, indicating that there were increased amounts of β-PA on the GEM particles. Strikingly, the amylase activities in groups C to E were higher than expected since the same amounts of α-PA were bound in these groups as in group B. The reason for this observation is not clear at present, and investigating it in more detail was beyond the scope of this study.

The results demonstrated that externally added enzymes can be functionally immobilized on lactococcal GEM particles by fusing them to the PA. In addition, combinations of enzymes can be immobilized in controlled ratios. Immobilization of PA fusions is fast and easy with no need for chemical treatments or extensive purification steps.

(ii) Mucosal vaccine delivery. The use of bacterial surface display systems for the development of vaccines is an emerging research area (16). Heterologous antigens have been displayed on bacterial cells in order to elicit antigen-specific antibody responses. Recently, mucosal vaccination using bacterial display systems has received a great deal of attention (6, 10, 20, 21). However, current systems for bacterial surface display are based on attenuated pathogenic strains and/or recombinant bacteria (25). The potential abilities of these (attenuated or recombinant) bacterial strains to flourish on or invade mucosal surfaces may cause problems, such as inflammation and disease or possibly the induction of immune tolerance. Furthermore, the presence of recombinant DNA in such vaccines may be considered less desirable because of shedding of the recombinant DNA into the environment and the possible risk of transmission to other organisms. Vaccines based on the GEM particle-PA display system eliminate some of the drawbacks associated with the use of genetically modified bacterial delivery systems.

In order to evaluate the efficacy of the GEM particle-PA display system as a mucosal delivery tool, a nasal vaccine was constructed on the basis of the circumsporozoite surface antigen (CSP) of the *Plasmodium berghei* malaria parasite (33). The CSP epitopes PPPPNPND and NANDPAPP, both fused as duplicates in one construct to the PA (CSP[2xB]-PA), were bound to GEM particles. This vaccine was nasally administered to mice without the use of an additional adjuvant. The mice were immunized three times at 3-week intervals. For comparison, another group of mice was subcutaneously immunized with the same vaccine and dose using the same immunization schedule. The mice received a total of 60 μg CSP[2xB]-PA in the three immunizations. Control groups consisted of groups of mice that received GEM particles or only PBS nasally. Sera collected 8 to 12 days after each immunization were used to detect CSP[2xB]-specific IgGs with an enzyme-linked immunosorbent assay. CSP[2xB]-specific serum IgGs were detected only in the mice that received GEM particles loaded with CSP[2xB]-PA (Fig. 6). In the subcutaneously immunized mice the CSP[2xB]-specific IgG levels reached approximately 4, 24, and 35 μg/ml after the first, second, and

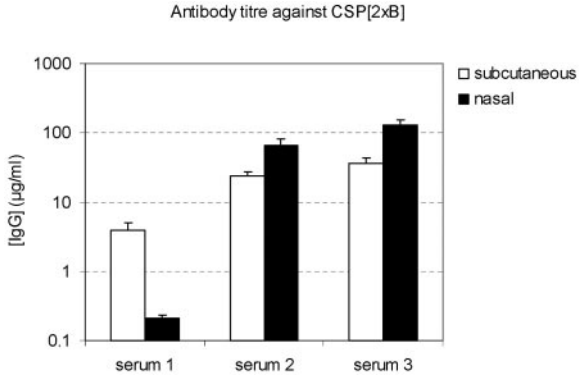


FIG. 6. IgG-specific serum antibody response to the CSP[2xB] epitopes. Groups of 10 mice were immunized nasally or subcutaneously with GEM particles (CSP[2xB]-PA). The sera were obtained as described in Materials and Methods.

third immunizations, respectively. The nasal GEM particle (CSP[2xB]-PA) vaccine induced a weak but significant CSP[2xB]-specific IgG response after the first immunization (0.2 μg/ml). However, a steep increase in the CSP[2xB]-specific IgG level was observed after the second immunization (65 μg/ml), which increased to approximately 120 μg/ml after the third immunization.

These results clearly show the potential of the GEM particle-PA display system as a delivery vehicle for mucosal vaccines and that this system can be used to elicit antigen-specific systemic antibodies. In addition, the results demonstrate that an antigen produced at a relatively low level (0.75 mg/liter) can be efficiently concentrated on GEM particles in a simple process using the PA binding domain and thus create an effective immunogen.

Conclusions. Lactococcal GEM particles are a suitable nonliving, non-GMO support for the display of proteins for various applications. *L. lactis* is an organism that is generally recognized as safe because it has a long history of safe use in foods (34), and large quantities can be easily cultivated. Acid pretreatment can be done with various acids, including acids that are compatible with the use of GEM particles in humans. Several applications can be envisaged. Immobilized enzymes can be used in industrial processes, diagnostic kits, or foods or to clean up environmental spills. The ability to bind multiple different proteins or peptides at a controlled ratio on a single GEM particle can be an additional advantage compared to existing particle systems. Also, the high loading capacity and high binding affinity are advantageous. The GEM particle-PA system might also be used for purification purposes, although a proper elution buffer has not been identified yet. So far, complete elution has been obtained only with SDS-containing solutions, which makes downstream processing difficult. This difficulty could be circumvented by incorporation of a protease recognition site between the PA and the desired protein, as has been done for similar affinity-based purification systems. For applications in mucosal vaccines the GEM particle-PA system combines the advantage of recombinant live bacterial delivery systems (i.e., presentation of antigens by a bacterial particle) with the advantage of inert microparticle delivery systems (i.e., safety).

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