Surface display of functional fibronectin-binding domains on *Staphylococcus carnosus*

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Abstract The surface expression in *Staphylococcus carnosus* of three different fibronectin binding domains (FNBDs), derived from fibronectin binding proteins of *Streptococcus dysgalactiae* and *Staphylococcus aureus*, has been investigated. Surface localization of the chimeric proteins containing the FNBDs was demonstrated. All three surface-displayed FNBDs were demonstrated to bind fibronectin in whole-cell enzyme-linked binding assays. Furthermore, for one of the constructs, intranasal immunizations with the recombinant bacteria resulted in improved antibody responses to a model immunogen present within the chimeric surface proteins. The implications of the results for the design of live bacterial vaccine delivery systems are discussed.

Key words: Surface display; Fibronectin binding; Staphylococcal protein A; Albumin binding protein; Mucosal immunization; *Staphylococcus carnosus*

1. Introduction

The display of heterologous proteins on bacterial surfaces has become an important tool in bioscience, with a variety of research applications, such as live recombinant bacterial vaccines, systems for display and screening of peptide and antibody libraries, whole-cell adsorbents, recombinant whole-cell biocatalysts, and cell-based solid-phase diagnostic reagents [1,2]. Surface display on Gram-negative bacteria, predominantly represented by *Escherichia coli* and *Salmonella* spp., has been achieved by inserting target gene fragments into genes encoding outer membrane proteins, e.g. the genes encoding OmpA [3–5], PhoE [6], and LamB [7], of *E. coli*. Genetic fusions to proteins of filamentous structures, such as fimbriae, flagella and pili proteins, have also resulted in the successful surface display of heterologous proteins on Gram-negative bacteria [8,9], and several other systems for surface display have been described (for reviews see [1,2]). The most common application of bacterial surface display is the development of live bacterial vaccine delivery systems, and for this purpose, several non-pathogenic Gram-positive bacteria have recently been investigated, with the major advantage of lack of invasive properties, and thereby no risk of reversion to pathogenicity [2,10]. Both commensal bacteria, such as *Streptococcus gordonii* [10,11], and food-grade bacteria, such as staphylococcal and lactococcal species [12], are being investigated in this context. Two interesting candidates which are being investigated for such applications are *Staphylococcus xylosus* and *Staphylococcus carnosus*, which are both used as starter cultures in meat fermentation applications [13,14]. Of the two staphylococcal species, it was recently shown that *S. carnosus* displayed a higher number of heterologous surface proteins [15]. Surface display of antigens on *S. xylosus* [16] has been achieved using fusions to the gene encoding the signal sequence and cell surface anchoring parts, of *Staphylococcus aureus* protein A (SPA). When using *S. carnosus* as a host for surface expression, the signal sequence and propeptide of the *Staphylococcus hyicus* lipase gene were used together with the SPA cell surface anchoring sequences [17]. Surface-expressed foreign antigens have been anchored to the *S. gordonii* cell wall using fusions to a fragment of the streptococcal protein M gene [18], a gene which has also been successfully expressed in *Lactobacillus lactis* [19]. Although antibody responses to surface-displayed antigens have been evoked by oral immunization of mice [20–22], prolonged persistence of recombinant bacteria would potentially enhance the immune responses. To achieve increased persistence of the recombinant live bacterial vectors at the mucosal surfaces, an interesting strategy would be the surface expression of adherence molecules. We have recently shown that the surface expression of the cholera toxin B subunit (CTB) from *Vibrio cholerae* on staphylococci resulted in recombinant staphylococci with the capacity to bind the ganglioside GM1 [23], present on all epithelial cells. This strategy, however, depends on the assembly of functional pentamers of CTB, which is not obviously achievable when having N- and C-terminal extensions to CTB [24]. More efficient adhesion to mucosal epithelial cells by the recombinant bacteria might possibly be achieved by surface display of a protein domain with the capacity to bind in a monomeric form to its ligand. Fibronectin, present in extracellular matrices and on epithelial cells [25], is known to bind various bacteria, which carry surface proteins with fibronectin binding properties [26]. Hanski and coworkers reported increased capacity of *Streptococcus pyogenes* to adhere to respiratory epithelial cells by the expression of the fibronectin binding protein F in a non-fibronectin binding strain [27]. Fibronectin binding domains (FNBDs) have previously been expressed in a functional form on the surface of *E. coli* as fusions to flagellin [28], and as fusions to the outer membrane protein OmpS on the surface of *V. cholerae* [29]. It would thus be of interest to investigate whether *S. carnosus*, which normally lacks fibronectin binding properties, would become able

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Abbreviations: ABP, albumin binding protein; CTB, cholera toxin B subunit; FACS, fluorescence-activated cell sorting; FNBD, fibronectin binding domain; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with Tween; SPA, staphylococcal protein A

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to bind fibronectin by heterologous display of FNBDs. Here, the surface expression of three different fibronectin binding domains on *S. carnosus*, using the surface display system previously described [17], is investigated. The FNBDs included in the study are: (i) A2, a 43 amino acid domain from *Streptococcus dysgalactiae* fibronectin binding protein A [30], (ii) B3, a 36 amino acid domain, from *S. dysgalactiae* fibronectin binding protein B [30], and (iii) D3, a 37 amino acid domain, from *S. aureus* fibronectin binding protein A [31]. Surface localization and functionality of the recombinant fibronectin binding surface proteins were analyzed by a variety of assays.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli strain RRIDM15 [32] was used as bacterial host during the plasmid constructions. The *S. carnosus* strain TM300 [33] was used for surface expression of heterologous fibronectin binding proteins. The general surface display vector pSPPmABPXM [17] was used for the construction of the expression vectors encoding the fibronectin binding proteins.

2.2. Preparation and transformation of protoplasts

The preparation and transformation of protoplasts from *S. carnosus* cells were performed as described by Götz and co-workers [34,35].

2.3. Assembly of the gene fragments encoding fibronectin binding domains

The oligonucleotides (Table 1) were synthesized by phosphoramidite chemistry on an Expedite nucleic acid synthesis instrument (PerSeptive Biosystems, Framingham, MA, USA), and desalted using NAP-10 columns (Amersham Pharmacia Biotech, Uppsala, Sweden). After lyophilization, the oligonucleotides were dissolved in water to a final concentration of 0.5 nmol/ml. The oligos were annealed pairwise by heating to 70°C followed by slow cooling to 37°C. The oligonucleotide pairs were 5′-phosphorylated according to Maniatis et al. [36] prior to the assembly of the gene fragments by mixing the phosphorylated pairs, and slowly cooling the mixture from 37°C to room temperature. The assembled double-stranded gene fragments were ligated into *Bam*HI/*Sal*I-digested plasmid pSPPmABPXM, yielding the different expression vectors pScA2, pScB3, and pScD3. The nucleotide sequences of the introduced gene fragments, encoding the different fibronectin binding domains, were verified by cycle sequencing on the plasmids with one-dye labelled terminators [37] (DuPont, Wilmington, DE, USA) using the sequencing primer SAPA1 (Table 1). The Sanger fragments were analyzed on an ALF DNA sequencer (Amersham Pharmacia Biotech). The expression vectors pScA2, pScB3, and pScD3 were used for transforming *S. carnosus* protoplasts.

2.4. Monoclonal antibodies

Monoclonal antibody FN-3E2, binding to human cellular fibronectin and used in the detection of fibronectin binding capacity of surface displayed FNBDs on *S. carnosus*, was purchased from Sigma (St. Louis, MO, USA). An albumin binding protein (ABP)-reacting mouse monoclonal antibody 13F10 [38] was used in the FACScan assay.

2.5. FACScan analysis of the staphylococcal surface display

This assay was performed essentially as described earlier by Robert and coworkers [15]. Briefly, wild-type and recombinant *S. carnosus* cells were grown at 37°C to $A_{580nm} \approx 7–8$. The bacteria were resuspended in 0.1% sodium azide in PBS to a final concentration of $A_{580nm} = 1$. Aliquots of 25 μl of the stock suspension were added to each conical well of a 96 well microtiter plate and sedimented by centrifugation for 10 min at 550×*g* at 4°C. After removing the supernatant, the bacterial suspension was incubated for 30 min with 100 μl of an ABP-reacting mouse monoclonal antibody 13F10 [38] diluted 1:1000 in PBS. The cells were resuspended and subsequently incubated 30 min at 4°C with 100 μl of fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG (Silenius Laboratories, Melbourne, Australia) at a dilution of 1:200 in PBS. The cells were washed twice, resuspended in 1 ml of PBS and analyzed on the basis of fluorescence intensity on a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA, USA), using 488 nm as excitation wavelength at 15 mW for the argon ion laser, with saline as sheath buffer. Data for 5000–10 000 cells were collected in List Mode by a LYSYS II software (Becton Dickinson). The fluorescent light was collected through a band pass interference filter, with emission detection between 515 and 545 nm. The cell suspension fluorescence distribution is represented by fluorescence histograms and mean fluorescence is given for each histogram.

2.6. Detection of surface displayed fibronectin binding domains

Samples from overnight cultures of recombinant or wild-type *S. carnosus* cells were diluted 1:100, grown at 37°C to $A_{578nm} \approx 1$, and harvested. In the following assay, unless otherwise indicated, the incubations were performed for 30 min at room temperature, and the cells were washed twice in PBST (PBS with 0.05% Tween 20) between the incubation steps. The cells were resuspended in PBST and incubated with fibronectin (Sigma) at a concentration of 13 μg/ml. After incubation with the monoclonal anti-fibronectin antibody FN-3E2 (Sigma), diluted 1:500 in PBST, the cells were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG antibodies (Dako), diluted 1:1000 in PBST. The cells were washed once with PBST and once with substrate buffer (1 M diethanolamine-HCl, pH 9.8, 0.5 mM

Table 1
Nucleotide sequences of the oligonucleotides used in this study

Name	Nucleotide sequence (5′-3′)
SAPA-1	CCGAATTCTCGAGGCTCCTAAAGAAAATAC
A2-1	GATCGGCGGCCGCTGCTAGCAGAAGATTCTAAACCAAGTCAAGAAGATGA
A2-2	CCACCAATAATTACTTCATCTTCTTGACTTGGTTTAGAATCTTCTGCTAGCAGCGGCCGCC
A2-3	AGTAATTATTGGTGGTCAAGGTCAAGTAATTGATTTTACAGAAGATACTCAATCT
A2-4	CACCAGACATACCAGATTGAGTATCTTCTGTAAAATCAATTACTTGACCTTGA
A2-5	GGTATGTCTGGTGATAATAGTCATACAGATGGTACAGTTTTAGAAGAGGATCCTGAG
A2-6	TCGACTCAGGATCCTCTTCTAAAACTGTACCATCTGTATGACTATTAT
B3-1	GATCGGCGGCCGCTGCTAGCAGAAGAAAGTTTACCAACTGAACAAGGTCAAT
B3-2	GTTGTACTACCAGATTGACCTTGTTCAGTTGGTAAACTTTCTTCTGCTAGCAGCGGCCGCC
B3-3	CTGGTAGTACAACTGAAGTAGAAGATAGTAAACCTAAATTATCTATTCATTTT
B3-4	GGCCATTCATTATCAAAATGAATAGATAATTTAGGTTTACTATCTTCTACTTCA
B3-5	GATAATGAATGGCCTAAAGAAGATGAGGATCCTGAG
B3-6	TCGACTCAGGATCCTCATCTTCTTTA
D3-1	GATCGGCGGCCGCTGCTAGCATTTAATAAACATACTGAAATTATTGAAGAAGATACA
D3-2	AATAATTTCAGTATGTTTATTAAATGCTAGCAGCGGCCGCC
D3-3	AATAAAGATAAACCTAGTTATCAATTTGGTGGACATAATAG
D3-4	AAATTGATAACTAGGTTTATCTTTATTTGTATCTTCTTC
D3-5	TGTTGATTTTGAAGAAGATACATTACCAAAAGTATCGGATCCTGAG
D3-6	TCGACTCAGGATCCGATACTTTTGGTAATGTATCTTCTTCAAAATCAACACTATTATGTCCACC

$MgCl_2$), before they were resuspended in substrate buffer to $A_{578nm} \approx 0.4$. After the addition of *p*-nitrophenyl phosphate, the change in A_{405nm} was measured in an ELISA reader (SLT EAR 340AT; SLT-Labinstruments, Grödig, Austria).

Surface-displayed fibronectin binding domains were also detected employing a colorimetric assay previously used for detection of surface displayed albumin binding domains, as earlier described in detail [15,17]. Briefly, the recombinant bacteria were incubated with biotinylated fibronectin (Sigma), biotinylated with D-biotinoyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester (Boehringer Mannheim, Mannheim, Germany) according to the supplier's recommendations at a final concentration of 2 μg/ml for 30 min at 30°C. Subsequently, the bacteria were incubated with streptavidin-alkaline phosphatase, and fibronectin binding was monitored by measuring the change in A_{405nm} after addition of the substrate *p*-nitrophenyl phosphate.

2.7. Intranasal immunization of mice

Groups of five BALB/c mice (5 weeks old) were immunized intranasally on days 0, 10 and 20 with 10^9 wild-type or recombinant *S. carnosus* cells in 40 μl of PBS (20 μl per nostril) without any adjuvant. Serum samples were taken on day 0 (preimmune) and day 30, and ABP-reactive IgG antibodies were measured in an ELISA, essentially as previously described [39]. Briefly, microtiter plates were coated overnight at 4°C with 100 μl of recombinant ABP (1 μg/ml) and blocked with 0.5% gelatin in PBS. All sera were diluted 1:100 in dilution buffer (PBS containing 0.1% gelatin and 0.05% Tween 20). Plates were washed with PBS and incubated with 100 μl horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce, Rockford, IL, USA) (1:5000). The reaction was developed with 50 μl tetramethyl benzidine (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD, USA). A_{450nm} was monitored using an integrated EIA Management System (Labsystems). ELISA titers were expressed as the reciprocal of the last dilution with $A_{450nm} > 0.2$.

2.8. Nucleotide sequence accession number

The GenBank accession number for the expression vector pSPPmABPXM is U15516.

3. Results and discussion

3.1. Background

The non-pathogenic bacterium *S. carnosus*, which is widely used in food fermentation applications [14], has been extensively investigated as a delivery system for subunit vaccines [22]. Immunogens of viral, bacterial, or parasitic origin has been expressed in a surface-exposed form, and immunizations have been made using the bacteria as whole-cell immunogens. Moderate antibody responses to the surface-displayed immunogens have been reported when using mucosal route administration of the bacteria [22]. The short persistence of the bacteria, estimated to be approximately 70 h [22], could probably account for the somewhat low antibody responses. One strategy to increase the immune responses to the surface-displayed immunogens could be the co-display of protein domains which potentially could have the capacity to bind to molecules present on the mucosal epithelium. It could thus be of relevance to investigate whether fibronectin binding protein domains could be expressed in a functional form on *S. carnosus*, which is devoid of inherent fibronectin binding activity [33]. For the purpose of surface expression on *S. carnosus*, we selected three different FNBDs: (i) a 43 amino acid domain, denoted A2, from *S. dysgalactiae* fibronectin binding protein A [30], (ii) a 36 amino acid domain B3, from *S. dysgalactiae* fibronectin binding protein B [30], and (iii) D3, a 37 amino acid domain, from *S. aureus* fibronectin binding protein A [31]. The three FNBDs were chosen since they have previously been shown to inhibit the binding of fibronectin to bacterial cells [30,31,40].

3.2. Assembly of gene constructs encoding fibronectin binding domains

Gene constructs encoding three different fibronectin binding domains were assembled from synthetic oligonucleotides, using a method similar to the solid-phase gene assembly method described by Ståhl and co-workers [41]. The oligonucleotides (Table 1), used in the gene assembly, were designed to be pairwise complementary, except for single-stranded 5′ protrusions of 4–15 nucleotides. The assembled gene fragments had 5′ protrusions of four nucleotides, identical with the cohesive ends given by restriction with endonucleases *Bam*HI and *Sal*I, respectively. The gene fragments could therefore successfully be ligated with the *Bam*HI/*Sal*I-restricted general expression vector pSPPmABPXM yielding the three expression vectors pScA2, pScB3, and pScD3 (Fig. 1).

3.3. Expression vectors for surface display of fibronectin binding domains on S. carnosus

Three novel *E. coli*-staphylococci shuttle vectors were constructed, designed for surface display of hybrid fibronectin binding domains on *S. carnosus*. The constructed shuttle vectors, designated pScA2, pScB3 and pScD3 (Fig. 1), encode the recombinant receptors PP-A2-ABP-XM′, PP-B3-ABP-XM′, and PP-D3-ABP-XM′, respectively (Fig. 1), anchored in the cell wall of *S. carnosus*. The expression vectors utilize the promoter, signal sequence, and propeptide sequence (PP) from a *S. hyicus* lipase gene construct, optimized for expres-

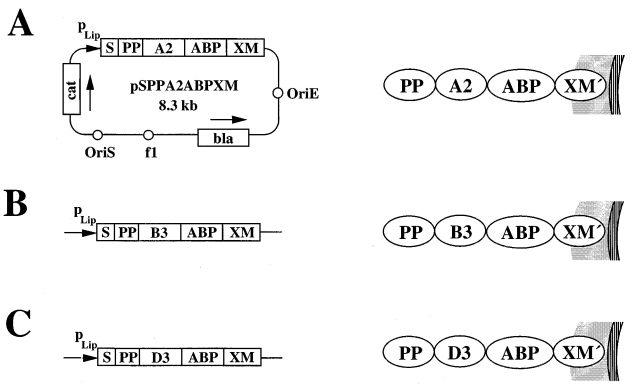

Fig. 1. Expression vectors with encoded gene products. Abbreviations: bla, β-lactamase encoding gene; cat, chloramphenicol acetyltransferase encoding gene; f1, origin of replication for phage f1; OriE, origin of replication from *E. coli*; OriS, origin of replication from *S. aureus*; p_{Lip}, *S. hyicus* promoter region designed for *S. hyicus* lipase production in *S. carnosus*. The expression vectors, suitable for surface display in *S. carnosus*, are shown with their encoded fusion products illustrated as anchored to the cell surface. Note that the propeptide (PP) from the *S. hyicus* lipase is not processed in *S. carnosus* [33], while it is processed in its homologous host, *S. hyicus* [47]. This propeptide has been shown to be essential for secretion of heterologous gene fusion products from *S. carnosus* [41,48] when using the lipase signal peptide for the secretion. M′ represents the processed and covalently anchored form [44,45] of the M sequence of SpA. A: Surface expression vector pScA2 encoding fibronectin binding domain A2 from *S. dysgalactiae* FnBA, and the processed gene fusion product PP-A2-ABP-XM′. B: Expression cassette of the surface expression vector pScB3 encoding fibronectin binding domain B3 from *S. dysgalactiae* FnBB, and the processed gene fusion product PP-B3-ABP-XM′. C: Expression cassette of the surface expression vector pScD3 encoding fibronectin binding domain D3 from *S. aureus* fibronectin binding protein A, FnBPA, and the processed gene fusion product PP-D3-ABP-XM′.

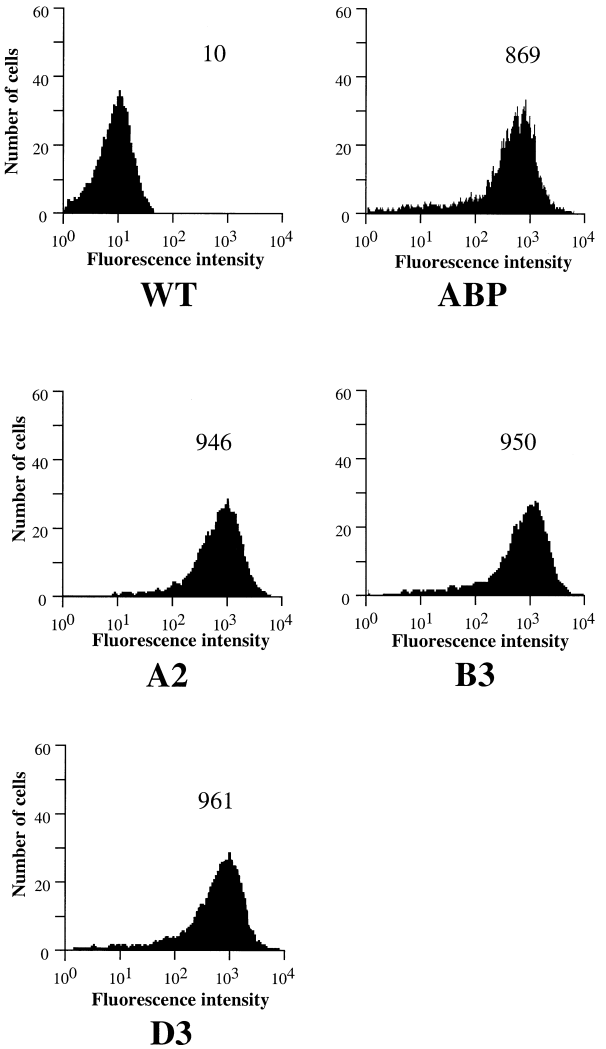

Fig. 2. Flow cytometry analysis (FACScan) of wild-type and recombinant *S. carnosus* cells probed with a monoclonal antibody 13F10 [38], reactive with the ABP part of the recombinant receptors, and stained with FITC-labelled secondary antibody. The histograms show non-stained bacterial cells displayed to the left in the histograms and cells which expose ABP-containing surface proteins on their surfaces shifted to the right. The type of analyzed bacteria and mean fluorecense reactivity is given for each histogram. Wild-type (WT) and recombinant *S. carnosus* cells; pSPPmABPXM-transformed (ABP), pScA2-transformed (A2), pScB3-transformed (B3), pScD3-transformed (D3)

sion in *S. carnosus* [42]. The vector system contains gene fragments from *S. aureus* protein A (SPA); comprising X, a charged repetitive region postulated to interact with the peptidoglycan cell wall [43], and M, a region, common for Gram-positive cell surface bound receptors, required for cell surface anchoring [44,45]. The gene encoding an ABP, derived from streptococcal protein G [17,46], is also present in all expression vectors. The ABP region is expressed as the part of the recombinant receptors closest to the cell wall anchoring motifs [17]. It has been demonstrated to be useful as a reporter peptide in a colorimetric assay to analyze surface accessibility of the hybrid surface proteins [15,17], and has previously also been shown to act as a spacer protein to increase surface accessibility [22].

3.4. Detection of recombinant surface-displayed chimeric proteins

To investigate if the encoded chimeric surface proteins were expressed and surface-exposed, wild-type and recombinant *S. carnosus* cells were analyzed in a fluorescence-activated cell sorting (FACScan) assay [15,17]. Since the ABP region was present in the various chimeric surface proteins, and since the surface exposure of this region has proven to be indicative of a successful surface display [15,17,38,39], the presence of the ABP region was monitored by FACScan analysis using an ABP-reactive mouse monoclonal antibody 13F10 [38]. The bacterial cells were probed with the monoclonal antibody, and thereafter fluorescently stained using a FITC-labelled secondary antibody, to allow FACS analysis. Positive responses in the FACScan assay, shown as an increase in fluorescence intensity (Fig. 2), were obtained for *S. carnosus* cells transformed with the parental vector pSPPmABPXM, carrying surface-exposed PP-ABP-XM′ fusion proteins, and *S. carnosus* cells transformed with plasmids pScA2, pScB3 and pScD3, while wild-type *S. carnosus* cells, as expected, remained negative in this assay (Fig. 2). The significant and homogeneous increase in fluorescence intensity observed for the recombinant staphylococci, as compared to wild-type *S. carnosus* (Fig. 2), indicate that close to 100% of the recombinant bacteria expose numerous copies of the chimeric surface proteins [39]. In a previous study, it was demonstrated that *S. carnosus* cells transformed with the parental vector pSPPmABPXM carried approximately 10^4 surface-exposed chimeric proteins [38]. Since the fluorescence histograms for the pSPPmABPXM-transformed *S. carnosus* and the staphylococci carrying the FNBDs have similar intensity distributions and mean fluorescence reactivities (Fig. 2), it is likely that these

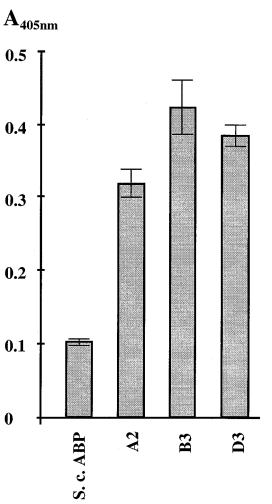

Fig. 3. Functional analysis of fibronectin binding domains displayed by whole cells. Histogram of whole-cell binding capacity of the recombinant staphylococci to human cellular fibronectin. Intact staphylococcal cells were incubated with fibronectin, followed by incubation with a monoclonal anti-fibronectin antibody. After incubation with an alkaline-phosphatase conjugate and the addition of a chromogenic substrate, the fibronectin binding capacity of the recombinant staphylococci was monitored by a color shift. Bars indicate the A_{405nm} response for *S. carnosus* cells: pSPPmABPXM-transformed (bar 1), pScA2-transformed (bar 2), pScB3-transformed (bar 3), pScD3-transformed (bar 4). The background response of the *S. carnosus* wild-type cells is subtracted from the responses of the recombinant *S. carnosus* cells. All samples were run in triplicate.

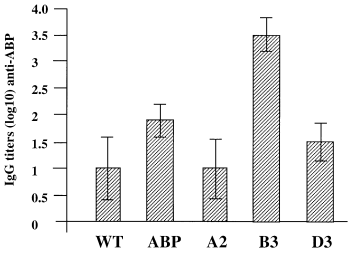

Fig. 4. Serum IgG responses to ABP after intranasal immunization of mice with wild-type (bar 1) and recombinant *S. carnosus* cells: pSPPmABPXM-transformed (bar 2), pScA2-transformed (bar 3), pScB3-transformed (bar 4), pScD3-transformed (bar 5).

recombinant staphylococci also display surface proteins in numbers of the same order of magnitude.

Recombinant and wild-type *S. carnosus* cells were further analyzed in a previously described colorimetric assay [15,17], for the presence of ABP-containing surface-accessible receptors. Recombinant and wild-type *S. carnosus* cells were incubated with biotinylated human serum albumin, prior to the addition of a streptavidin-alkaline phosphatase conjugate, and the presence of ABP-containing surface-accessible receptors was analyzed by the addition of chromogenic substrate and the color shift was monitored. This assay gave very similar results as the FACScan assay (data not shown). These results demonstrate that hybrid receptors with serum albumin binding capacity were accessible on the surfaces of the recombinant *S. carnosus* cells.

3.5. Functional characterization of the fibronectin binding receptors

The fibronectin binding capacity of the surface-displayed hybrid receptors was characterized in an assay based on the binding of human fibronectin to the recombinant *S. carnosus* cells. Fibronectin bound to the cells was detected by an enzyme-linked immunoassay (Fig. 3). The staphylococcal cells were incubated with fibronectin, and a monoclonal anti-fibronectin antibody was allowed to react with the cells. After incubation with enzyme-labelled goat anti-mouse IgG antibodies, the presence of cell-bound fibronectin was detected with a chromogenic substrate. Significant binding of fibronectin was observed for the recombinant staphylococci harbouring plasmids pScA2, pScB3 and pScD3 (Fig. 3, bars 2, 3, and 4), while only background binding was demonstrated for *S. carnosus* cells transformed with the parental vector carrying the PP-ABP-XM′ chimeric protein (Fig. 3, bar 1). Furthermore, wild-type *S. carnosus* cells showed only background reactivity in this assay (data not shown).

The recombinant *S. carnosus* cells expressing hybrid receptors were further investigated in a modified assay for their fibronectin binding capacity. The binding of biotinylated fibronectin to the cells was analyzed by the addition of streptavidin-conjugated alkaline phosphatase, and subsequently its substrate, and again, the *S. carnosus* cells harboring plasmids pScA2, pScB3, and pScD3 showed significantly higher binding to biotinylated fibronectin than did the wild-type cells and the cells harboring the parental plasmid pSPPmABPXM (data not shown). These results clearly suggest that the recombinant *S. carnosus* cells have gained fibronectin binding capacity by the expression of FNBDs as parts of their chimeric surface receptors.

3.6. Immunization experiments

Since the goal of the study was to improve the antibody responses to surface-displayed proteins on the staphylococcal cells, mice were immunized intranasally with the wild-type and recombinant *S. carnosus* cells in order to evaluate whether the fibronectin binding capacity would influence the antibody responses to the ABP region, seen as a model immunogen in this assay. Serum IgG responses to ABP were monitored, and interestingly, *S. carnosus* cells harboring the pScB3 plasmid did evoke significantly higher serum IgG responses to ABP than did the other constructs (Fig. 4). All preimmune sera were negative in this assay (data not shown). The reason why the B3 FNBD-displaying staphylococci induced higher immune responses to ABP, while the two other constructs did not, remains to be elucidated.

3.7. Concluding remarks

We have in this study assembled gene fragments encoding three different fibronectin binding domains, and expressed these as parts of chimeric surface proteins on the food-grade Gram-positive bacterium *S. carnosus*. It was found that the FNBD-containing surface proteins were successfully exposed on the surface of the staphylococci, as analyzed by a FACScan assay. Furthermore, it could be concluded that the recombinant *S. carnosus* cells, carrying surface-exposed FNBDs, had obtained fibronectin binding capacity. This was demonstrated using a monoclonal antibody reactive to fibronectin. In addition, recombinant *S. carnosus* cells, carrying the B3 domain from *S. dysgalactiae* fibronectin binding protein B, demonstrated improved antibody responses to the ABP region, present in the chimeric protein, thus suggesting that this construct might have gained improved properties as a mucosal route vaccine delivery system. Before stating that this is a general phenomenon, several questions need to be further addressed. For example, it would be of interest to evaluate whether the recombinant staphylococci show improved persistence in the gastrointestinal or in the nasal tract after oral or intranasal administration. Nevertheless, we have demonstrated that an adherence molecule, as exemplified by the bacterial FNBDs, could be expressed in a functional form on the surface of recombinant staphylococci intended for subunit vaccine delivery, and that the surface display of B3 FNBD demonstrated positive effects on the antibody responses to a co-displayed model antigen. We believe that such strategies will be of importance to improve mucosal route vaccine delivery systems.

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