Surface Display of the Cholera Toxin B Subunit on Staphylococcus xylosus and Staphylococcus carnosus

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The heterologous surface expression of the cholera toxin B subunit (CTB) from Vibrio cholerae in two staphylococcal species, Staphylococcus xylosus and Staphylococcus carnosus, has been investigated. The gene encoding native CTB (103 amino acids) was introduced into gene constructs encoding chimeric receptors designed to be translocated and anchored on the outer cell surface of the staphylococci. Since functionality of CTB is correlated with its ability to form pentamers and the capacity of the pentameric CTB to bind the GM1 ganglioside, both the surface accessibility and the functionality of the surface-displayed CTB receptors were evaluated. It could be concluded that the chimeric receptors were targeted to the cell wall of the staphylococci, since they could be released by lysostaphin treatment and, after subsequent affinity purification, identified as full-length products by immunoblotting. Surface accessibility of the chimeric receptors was demonstrated by a colorimetric assay and by immunofluorescence staining with a CTB-reactive rabbit antiserum. Pentamerization was investigated by using a monoclonal antibody described to be specific for pentameric CTB, and the functionality of the receptors was tested in a binding assay with digoxigenin-labelled GM1. It was concluded that functional CTB was present on both types of staphylococci, and for S. carnosus, the reactivity to the pentamer-specific monoclonal antibody and in the GM1 binding assay was indeed significant. The implications of the results for the design of live bacterial vaccine delivery systems intended for administration by the mucosal route are discussed.

The display of heterologous proteins on the outer surfaces of bacteria has become an emerging topic in different fields of research within applied bacteriology, biotechnology, and vaccinology (7, 12, 31). Genetic insertion of a target sequence into the genes for various outer membrane proteins has constituted the general strategy to enable secretion and subsequent surface anchoring of the recombinant target gene products. For gram-negative bacteria, represented by Escherichia coli and Salmonella spp., a number of different types of heterologous proteins have been surface displayed, including antigenic determinants for the purpose of vaccine development (4, 19, 37, 60), enzymes (9, 10), metal-chelating histidyl peptides (53) and single-chain antibodies (8, 11), as well as entire peptide libraries (33). Surface display on gram-positive bacteria also has recently started being considered in the context of vaccine development. Approaches based on attenuated mycobacteria (57), commensal streptococci (35, 42, 44, 45), and nonpathogenic food-fermenting staphylococci (22, 38, 39, 48, 55) and lactococci (40), as well as sporulating bacteria, e.g., Bacillus subtilis (1), are being conducted. Even active single-chain antibody fragments (17) and heterologous enzymes (58) have been surface displayed on recombinant staphylococci. Such recombinant bacteria could be envisioned as inexpensive tools in diagnostics and as novel types of microbial biocatalysts.

Bacteria used in food fermentation processes, such as Staphylococcus xylosus and Staphylococcus carnosus (14, 20, 30), are interesting vehicles for the delivery of subunit vaccines by mucosal routes since they are considered nonpathogenic and safe to eat (55). However, although significant serum antibody responses to surface-displayed antigenic determinants have been evoked by oral immunization of mice with such recombinant bacteria (39, 55), increased antibody titers would in many cases be desired. Prolonged persistence of bacteria at mucosal surfaces would potentially increase the capacity to induce immune responses. Along this line of research, Hanski and coworkers (21) reported that non-fibronectin-binding isolates of Streptococcus pyogenes could become fibronectin binding by heterologous expression of a fibronectin-binding protein. These recombinant bacteria thereby gained the capacity to adhere to respiratory epithelial cells (21).

Another interesting target molecule to utilize in order to achieve adherence to mucosal surfaces would be the monosialoganglioside GM1, which is present on all epithelial cells of mucosal surfaces (24). The nontoxic cholera toxin B subunit (CTB), which is present in a pentameric form in the cholera holotoxin of Vibrio cholerae, is responsible for the cell attachment via its GM1 binding capacity (34). CTB, which binds GM1 only as pentameric CTB (23), might thus be able to function as an adherence molecule to mucosal epithelial cells if it could be expressed in a functional form on the outer surface of bacteria. In addition, CTB is of general interest for mucosal vaccine development, since it is considered to carry immunopotentiating capacities (6, 24, 54). Although CTB epitopes (37, 43) and even the entire CTB (27, 28) have been surface displayed on recombinant E. coli, reports that demonstrate any functionality, i.e., pentamerization and/or GM1 binding, of surface-displayed CTB are lacking.

Here, the surface display of CTB on S. xylosus and S. carnosus, obtained by taking advantage of previously reported surface display systems (22, 38, 48), is described. To achieve surface anchoring of the chimeric receptors containing the CTB subunit, the cell surface attachment regions from Staphylococcus aureus protein A were utilized in both expression...
systems. Surface localization, accessibility on the outer surface of the recombinant staphylococci, and functionality of the CTB-containing chimeric receptors were analyzed by a variety of assays.

**MATERIALS AND METHODS**

**Bacterial strains and plasmid vectors.** The strains and plasmids used in this study are listed in Table 1.

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**TABLE 1. Strains and plasmids**

**Preparation and transformation of protoplasts.** The preparation and transformation of protoplasts from *S. xylosus* and *S. carnosus* cells were performed as described by Götz and coworkers (15, 16).

**DNA constructions.** The CTB gene was amplified by PCR with plasmid pJS752-3 (3) as a template and the CTB-specific primers pJS752-3, giving the plasmid pK3CTC3, which in turn was digested with the same enzymes. The resulting plasmid, pECTBmp18, was cleaved with the same enzymes. The polyclonal rabbit antiserum R1802 used in Western blotting and the monoclonal antibody (Ab) LT39 (13) used in the whole-cell detection assay were kindly provided by Jan Holmgren.

**Extraction and affinity purification of recombinant receptors.** The recombinant receptors were extracted from staphylococcal cell wall fractions and affinity purified essentially as described by Samuelson and coworkers (48). Recombinant *S. xylosus* cells, harboring the expression vector pSEmp18ABXPX or pSECTCABPX, and *S. carnosus* cells, harboring the expression vector pSPPmAABPX or pSPCTCABPX, were grown overnight at 37°C in tryptic soy broth (Difco, Detroit, Mich.) (30 g/liter) supplemented with yeast extract (Difco) (5 g/liter) and chloramphenicol (Boehringer, Mannheim, Germany) (10 mg/liter). Samples were diluted 1:50 and grown to an *A*₅₇₈ of ~1. An amount of cells corresponding to an optical density of 95 was harvested and washed twice in phosphate-buffered saline (PBS) (pH 7.3) and resuspended in 5 ml of a modified SMM medium (16) composed of 7.5 parts SMM (1 M sucrose, 0.04 M maleic acid, and 0.04 M MgCl₂, pH 6.5) and 2.5 parts of 7% Penassay broth (Lab Technologies, Paisley, United Kingdom). Lysozymin (Sigma) was added to a final concentration of 25 U/ml, and incubation followed for 1.5 h at 37°C. The released cell wall proteins were separated from the protoplast fraction by centrifugation twice at 7000 × *g* for 20 min at room temperature. The supernatant containing solubilized receptors was diluted 1:20 in Tris-buffered saline containing TWEEN (25 mM Tris-HCl [pH 8.0], 0.2 M NaCl, 1 mM EDTA, 0.05% TWEEN 20) and subjected to affinity chromatography on a human serum albumin (HSA)-Sepharose column for purification of the hybrid receptors (56). Relevant fractions, as determined by *A*₅₇₈ measurements, were pooled and lyophilized prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis using 12.5% gels under reducing conditions (Bio-Rad, Hercules, Calif.). The gels were either stained with Coomassie brilliant blue or subjected to immunoblot analysis with polyclonal rabbit antiserum reactive with CTB.

**Colorimetric assay for detection of surface-displayed ABR-containing protein (ABP)-containing receptors was performed essentially as described by Samuelson and coworkers (48). Briefly, samples of overnight cultures of wild-type or recombinant *S. xylosus* and *S. carnosus* cells were grown in broth supplemented with chloramphenicol (10 mg/liter) to an *A*₅₇₈ of ~1. The cells were harvested and washed twice in PBS with 0.05% TWEEN 20 (PBST). One milliliter of cell suspension, diluted in PBST to an *A*₅₇₈ of ~1, was incubated with biotinylated HSA (biotinylated with biotinyl-F-aminoethylamide) or biotinylated CTA (CTA hydroxysterol) according to the supplier’s recommendations at a final concentration of 2 μg/ml for 15 min at 30°C. The cells were washed twice in PBST before being resuspended in 1 ml of PBST containing 0.5 U of streptavidin-alkaline phosphatase (Boehringer) and incubated for 15 min at 30°C. After two washing steps, the first in PBST and the second in substrate buffer (1 M diethanolamine-HCl [pH 9.8], 0.5 mM MgCl₂), the cells were diluted in substrate buffer to an *A*₅₇₈ of 0.1, and three aliquots of 100 μl from each cell type were used in a microplate reader (SLT EAR 340AT; SLT-Labinstruments, Großig, Austria).

**Immunofluorescence assay for detection of CTB-containing receptors on the cell surface of staphylococci.** The immunofluorescence assay was performed essentially as described by Samuelson and coworkers (48). *S. xylosus* and *S. carnosus* cells harboring the different constructs were grown overnight in broth supplemented with chloramphenicol (10 mg/liter). The overnight cultures were diluted 1:50 and grown at 37°C to an *A*₅₇₈ of ~1. Before the preparation the immunofluorescence slides was carried out, the cells were washed three times in PBS. Fifty-well multiwell slides were incubated with coating buffer (15 mg sodium carbonate, 35 mM NaHCO₃, pH 9.6) in a humid chamber for 30 min at room temperature. The coating buffer was displaced by a 0.15 M PBS solution (final concentration, ~0.15 M), and the slides were incubated for 30 min before the fixation step, with 1% glutaraldehyde in PBS for 5 s. The slides were washed in distilled water and air dried. The cells were incubated with one drop of polyclonal rabbit anti-CTB serum at a dilution of 1:25 in PBS for 1 h at room temperature and the cells were washed twice in PBS between the following assay, unless otherwise indicated, the incubations were performed for 1 h at room temperature and the cells were washed twice in PBS between the incubation steps. The cells were washed twice in PBS, and an amount of cells equivalent to an *A*₅₇₈ of ~5 was incubated with a MAB, LT39, described to be specific for pentameric CTB (13), diluted 1:50 in PBS. After being washed, the cells were incubated with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) (15 μg/ml) (Vector, Burlingame, Calif.) for 30 min according to the supplier’s recommendation. After three washes, incubation with avidin-conjugated fluorescein isothiocyanate (50 μg/ml) (Vector), and a final wash, the slides were examined under a UV microscope. Photographs were taken with a 30× exposure time.

**Immunodetection of surface-displayed pentameric CTB.** Samples from overnight cultures of recombinant *S. xylosus* cells (harboring pSEmp18ABXPX or pSECTCABPX) and *S. carnosus* cells (harboring pSPPmAABPX or pSPCTCABPX) were diluted 1:100 in 50 ml of the medium described above, grown at 37°C to an *A*₅₇₈ of ~1, and harvested by centrifugation at 4000 × *g* in the following assay, unless otherwise indicated, the incubations were performed for 1 h at room temperature and the cells were washed twice in PBS between the incubation steps. The cells were washed twice in PBS, and an amount of cells equivalent to an *A*₅₇₈ of ~5 was incubated with a MAB, LT39, described to be specific for pentameric CTB (13), diluted 1:50 in PBS. After being washed, the cells were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG antibodies (Dako) diluted 1:100 in PBS (25 mM Tris-HCl [pH 8.0], 0.2 M NaCl, 1 mM EDTA). After being washed twice in PBS, the cells were washed once in substrate buffer (see above) and resuspended in substrate buffer to an *A*₅₇₈ of 0.1. Three aliquots of 100 μl of each cell type were loaded into wells of a microtitre plate before addition of 100 μl of p-nitrophenyl phosphate substrate solution. The change in *A*₅₇₈ after 30 min was measured in an ELISA reader.

**GM1-binding assay to characterize the cell surface-displayed CTB hybrid receptors.** The GM1 binding assay was carried out essentially like the immunodetection assay, described above, for analysis of pentameric CTB receptors with the MAB. Here, the surface-displayed CTB-containing receptors were probed on intact staphylococci by analyzing the binding of digoxigenin-labelled GM1 ganglioside. The GM1 (Sigma) was digoxigenin labelled with digoxigenin-3-O-suc-
Two novel E. coli-staphylococcal shuttle vectors, designed for surface display of hybrid receptors containing CTB on S. xylosus or S. carnosus, respectively, were constructed. The CTB-encoding gene was PCR amplified and introduced into two general surface expression vectors, pSEmp18ABPXM (38) and pSPPmABPXM (48), designed for S. xylosus and S. carnosus, respectively. The two shuttle vectors constructed, designated pSECTBABPXM (Fig. 1A) and pSPPCTBABPXM (Fig. 1B), encode the recombinant receptors CTB-ABP-XM (Fig. 1A) and PP-CTB-ABP-XM (Fig. 1B), anchored in the cell walls of S. xylosus (18), and a region common for gram-positive cell surface-bound receptors (M) which is required for cell surface anchoring (36, 50–52). Both encoded chimeric receptors also contain an ABP, derived from streptococcal protein G (41), which is expressed closest to the cell wall-anchoring domains (38, 48).

The ABP region (i) can be used as an affinity tag for purification of extracted chimeric receptors (38, 48), (ii) has been used as a reporter peptide in a colorimetric assay to analyze surface accessibility of the hybrid receptors (46, 48), and (iii) has been shown to act as a spacer protein to increase surface accessibility (55).

**RESULTS**

**Expression vectors for surface display of CTB on staphylococci.** Two novel E. coli-staphylococcal shuttle vectors, designed for surface display of hybrid receptors containing CTB on S. xylosus or S. carnosus, respectively, were constructed. The CTB-encoding gene was PCR amplified and introduced into two general surface expression vectors, pSEmp18ABPXM (38) and pSPPmABPXM (48), designed for S. xylosus and S. carnosus, respectively. The two shuttle vectors constructed, designated pSECTBABPXM (Fig. 1A) and pSPPCTBABPXM (Fig. 1B), encode the recombinant receptors CTB-ABP-XM (Fig. 1A) and PP-CTB-ABP-XM (Fig. 1B), anchored in the cell walls of S. xylosus and S. carnosus, respectively. The S. xylosus expression vector (Fig. 1A) takes advantage of the promoter and signal sequence from S. aureus protein A (SpA) (59), while the S. carnosus vector (Fig. 1B), utilizes the promoter, signal sequence, and propeptide sequence (PP) from a Staphylococcus hyicus lipase gene construct (48). Both vector systems contain gene fragments encoding the cell wall-anchor-
3 and 4, respectively). The proteins released from the staphylococcal cell walls migrated as proteins somewhat larger than expected (ABP-XM, 43 kDa; CTB-ABP-XM, 54 kDa; PP-ABP-XM, 65 kDa; and PP-CTB-ABP-XM, 76 kDa), a phenomenon observed earlier for gene products containing the cell surface-anchoring regions of gram-positive bacterial receptors (48, 58).

In an immunoblotting experiment (Fig. 2B), the affinity-purified fusion proteins extracted from S. xylosus transformed with pSECTBABPXM (Fig. 2B, lane 2) and from S. carnosus transformed with pSPPCTBABPXM (Fig. 2B, lane 4) were recognized with the CTB-reactive rabbit antiserum, whereas the fusion proteins extracted from the staphylococci harboring the respective parental vectors were not stained (Fig. 2B, lanes 1 and 3). These results demonstrate that the hybrid receptors were properly expressed and localized to the cell walls of the recombinant S. xylosus and S. carnosus cells and that CTB was present where expected, since the cell wall extracts containing these receptors reacted positively in CTB-specific immunoblotting (Fig. 2B).

Colorimetric assay for detection of recombinant surface-displayed receptors. The surface accessibility of the expressed hybrid receptors was investigated by a colorimetric enzymatic assay (48), taking advantage of the albumin-binding reporter molecule ABP. Recombinant and wild-type S. xylosus and S. carnosus cells were grown to early logarithmic phase and harvested. Whole cells were incubated with biotinylated HSA, and after incubation with a streptavidin-alkaline phosphatase conjugate and a chromogenic substrate allowed for monitoring of a color shift. Bars indicate the $A_{405}$ response for wild-type (bars 1 and 4), pSEmp18ABPXM-transformed (bar 2), pSECTBABPXM-transformed (bar 3), pSPPmABPXM-transformed (bar 5), and pSPCTBABPXM-transformed (bar 6) cells. OD, optical density unit. Error bars indicate standard deviations.

Immunofluorescence assay. In order to further investigate the nature of the surface-expressed hybrid receptors detected by the colorimetric assay described above, an immunofluorescence assay was performed with antibodies reactive with CTB. Recombinant S. xylosus and S. carnosus cells harboring the two constructs presented in Fig. 1 were assayed in the immunofluorescence assay, with S. xylosus and S. carnosus cells harboring the corresponding parental vectors as controls. The cells were stained with a CTB-reactive polyclonal antiserum (Fig. 4). It was demonstrated that S. xylosus and S. carnosus cells containing plasmid pSECTBABPXM or pSPPCTBABPXM were efficiently stained by antibodies reactive with CTB (Fig. 4, B and D, respectively). The CTB-reactive serum did not stain the staphylococcal cells harboring pSEmp18ABPXM or pSPPmABPXM (Fig. 4, A and C, respectively). In the examples with positive immunofluorescence, close to 100% of the recombinant bacteria were stained (Fig. 4), demonstrating an efficient surface display of CTB-containing recombinant receptors. These results show that CTB is present in an accessible form within the expected chimeric receptors displayed on the surface of recombinant staphylococci, but they do not give any
information about the nature or possible functionality of the CTB, since a polyclonal serum was used.

**Functional characterization of the CTB receptors.** To investigate whether the CTB molecules present within the cell surface-exposed hybrid receptors were functional, as indicated by pentamerization and GM1 binding (34), two whole-cell assays were performed in a microtiter plate format. The four recombinant staphylococci analyzed in the serum albumin binding assay (Fig. 3) and the immunofluorescence assay (Fig. 4) were analyzed (i) with a MAb, LT39, described to be specific for
pentameric CTB (13) (Fig. 5A) and (ii) for their binding to the ganglioside GM1 (Fig. 5B). The MAb was allowed to react with nonfixed intact staphylococcal cells, and after incubation with enzyme-labelled goat anti-mouse IgG, the presence of pentameric CTB was detected with a chromogenic substrate. Although a certain background binding to the _S. xylosus_ cells expressing the hybrid receptor without CTB (pSEmp18ABPXM transformed) was observed (Fig. 5A, bar 1), a significant increase in reactivity was observed for the recombinant _S. xylosus_ cells carrying hybrid CTB-containing receptors (pSECTBABPXM transformed) (Fig. 5A, bar 2). This type of background binding was not observed for the parental vector-transformed _S. carnosus_ cells (Fig. 5A, bar 3), and a significant reactivity was observed for the recombinant _S. carnosus_ cells carrying CTB-containing receptors (pSPPCTBABPXM transformed) (Fig. 5A, bar 4). The observed reactivity of the recombinant CTB-carrying staphylococci with the MAb indicates that pentameric forms of CTB receptors might be assembled on the bacterial surfaces.

In the GM1 binding assay, digoxigenin-labelled GM1 was allowed to react with intact staphylococcal cells, and after incubation with enzyme-labelled sheep anti-digoxigenin Fab fragments, the GM1 binding was detected by the addition of an alkaline phosphatase-conjugated digoxigenin-specific Fab fragments and the monitoring of a color shift after the addition of a chromogenic substrate. Hatched bars indicate the _A_405 response for _S. xylosus_ cells, pSEmp18ABPXM transformed (bar 1) or pSECTBABPXM transformed (bar 2), and open bars represent the _A_405 response for _S. carnosus_ cells, pSPPmABPXM transformed (bar 3) or pSPPCTBABPXM transformed (bar 4). OD, optical density unit. Error bars indicate standard deviations.

**DISCUSSION**

We have described how CTB, from the gram-negative bacterium _Vibrio cholerae_, has been expressed in a functional form on the surfaces of _S. xylosus_ and _S. carnosus_. Localization to the staphylococcal cell wall was demonstrated by lysostaphine extraction, followed by purification by HSA affinity chromatography and analysis by SDS-PAGE and immunoblotting to verify the presence of CTB within the chimeric receptors. Surface accessibility of chimeric receptors was demonstrated by a colorimetric assay based on ABP, present as one region within the composite receptors, and surface accessibility of CTB within the chimeric receptors was proven by an immunofluorescence assay with a CTB-reactive rabbit antiserum. Reactivity of intact recombinant staphylococci to a MAB specific for pentameric forms of CTB indicated that the chimeric CTB-containing receptors might assemble into pentameric structures on the
staphylococcal cell surfaces. This hypothesis was further supported by the fact that recombinant staphylococci with CTB-containing surface receptors bound the GM1 ganglioside, since it has been previously shown that only pentameric CTB exhibits this ability (23).

Since our observations were based on the reactivities of polyclonal antibodies and a MAB reactive to CTB, we reconfirmed the specificities of the antibodies by immunoblotting experiments. The noncovalent interactions responsible for pentamer formation are strong enough to allow analysis of intact CTB pentamers by nonreducing SDS-PAGE (23). We thus used the MAB LT39 in immunoblotting to both monomeric and pentameric commercial CTB, and it could be concluded that the reactivity of MAb LT39 to the pentameric form of CTB was completely dominating (data not shown), as expected (13). Interestingly, the two polyclonal rabbit antisera used in the different analyses also showed a stronger reactivity to pentameric CTB than to monomeric CTB, thus indicating that the immunodominant epitopes for CTB are conformational and are formed when pentamers assemble.

Although a number of antigenic determinants (22, 38, 39, 46, 48), as well as a functional single-chain Fv antibody fragment (17) previously have been surface displayed on S. xylosus and S. carnosus, it was not obvious that we would succeed in expressing CTB in a functional form on the staphylococcal cell surface. Considering that the translocation process is accompanied by a covalent linkage of the receptor to cell wall constituents (50), it was somewhat surprising that we could detect pentameric forms of CTB, as well as GM1 binding activity on the staphylococci carrying surface-displayed CTB. However, it has been estimated with the help of fluorescence-activated cell sorting technology that the recombinant staphylococci might carry up to 104 heterologous surface receptors (1a). This could mean that at least a fraction of the surface-displayed receptors would be able to form pentameric CTB structures. It has earlier been debated whether CTB with N- and/or C-terminal extensions was able to form functional pentamers (3, 6), and it was only recently demonstrated that carrying a 25-kDa fusion at both termini was able to form functional pentamers (30a). These results would thus indicate the potential to use CTB introduced within chimeric receptors to achieve binding to GM1-carrying epithelial cells.

It would be of great interest to test in vitro the recombinant CTB-displaying staphylococci for binding to epithelial cells (which express GM1 in abundance). It might also be possible to evaluate the binding to epithelial cells in vivo by testing the recombinant staphylococci for increased persistence in the mouse gut. Similar staphylococci which do not expose CTB on their surface show a persistence in the mouse gut of about 70 h (55).

To conclude, we have expressed CTB in a functional form on the surfaces of S. xylosus and S. carnosus. These results first demonstrate the possibility of surface expression of proteins, which have to assemble from subunits to become functional, and second could lead to an interesting way for targeting of bacteria, intended as subunit vaccine vehicles, to mucosal surfaces.

ACKNOWLEDGMENTS

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REFERENCES


