Recombinant *Staphylococcus* Strains as Live Vectors for the Induction of Neutralizing Anti-Diphtheria Toxin Antisera

CÉCILE FROMEN-ROMANO, 1 PASCAL DREVET, 1 ALAIN ROBERT, 2 ANDRÉ MÉNÉZ, 1 AND MICHEL LÉONETTI* 1

CEA, Département d’Ingénierie et d’Études des Protéines (DIEP), Centre d’Etude de Saclay, 91191 Gif-Sur-Yvette Cedex, 1 and Centre d’Immunologie Pierre Fabre (CIPF), 74164 Saint-Julien en Genevois, 2 France

Received 8 March 1999/Returned for modification 20 May 1999/Accepted 7 July 1999

We have investigated whether the nonpathogenic gram-positive bacteria *Staphylococcus xylosus* and *S. carnosus* can display a whole domain of a toxic protein on their surface and if such vectors are suitable for immunization of BALB/c mice. The nucleotide sequence encoding the receptor-binding domain (DTR; amino acids 382 to 535) of diphtheria toxin (DT) was inserted into plasmids pSE mp18ABPXM and pSPPmABPXM, which were designed to display heterologous proteins on *S. xylosus* and *S. carnosus* cell surfaces, respectively. Western blot analysis of the resulting bacterial lysates indicates that DTR is produced by each expression system. However, analysis of rabbit anti-DTR antisera binding to the transformed live bacteria shows that DTR is not displayed on the surface of *S. xylosus* cells whereas it is efficiently exposed on *S. carnosus*. A significant anti-DT antibody response was raised in BALB/c mice immunized intraperitoneally with *S. carnosus* displaying DTR, and the antisera abolished DT cytotoxicity on Vero cells. Thus, only *S. carnosus* can display a whole domain of a toxic protein and represents a potential vector for humoral vaccination.

The advent of genetic manipulation has allowed the development of nonpathogenic live bacteria as vehicles for antigens (31). The interest in these vectors resides in their potential ability to induce a durable immune response (27), to bypass the use of adjuvants, and to induce a mucosal immune response following oral or nasal administration (16). For safety reasons, the live vector must be nonpathogenic or at least of greatly attenuated pathogenicity. In this context, several types of gram-negative and gram-positive bacteria, such as *Salmonella* (29, 30), *Mycobacterium* (29, 30), and *Staphylococcus* (10, 18, 21), have been previously engineered to express foreign antigens.

Among these bacterial strains, *Staphylococcus xylosus* and *S. carnosus* represent particularly safe and potentially interesting vectors for immunization. These two nonpathogenic strains possess a low level of DNA homology to the pathogenic strain *S. aureus* and are currently used for applications in meat fermentation (26). Furthermore, they do not produce toxins, hemolysins, protein A, coagulase, or clumping factors (7). Also, two expression systems have recently been developed for the surface display of heterologous proteins on *S. xylosus* (17, 18) and *S. carnosus* (25) cells, and the two live vectors were shown to be efficient for protein or protein fragment expression (8, 14, 21).

In the present work, we investigated whether a structurally well-defined domain of a toxic protein could be expressed on the surface of *S. xylosus* or *S. carnosus* and if the resulting live vector could trigger, in mice, antitoxin antibodies with neutralizing potency. We focused our work on the diphtheria toxin (DT) fragment from amino acids 382 to 535, called receptor-binding domain (DTR), which mediates the targeting of DT to a cell surface receptor (22). DTR was selected because (i) it is structurally organized as a whole domain in DT (1–3), (ii) it is devoid of any cytotoxicity per se (15), (iii) a large proportion of antibodies able to neutralize DT cytotoxicity are directed against the DTR region (11, 33), and (iv) DTR expressed as a soluble fusion protein is capable of eliciting neutralizing anti-DT antibodies in rabbits (15).

In this report, we describe the insertion of the nucleotide sequence encoding amino acids 382 to 535 of DT in plasmids pSE mp18ABPXM and pSPPmABPXM, which were developed for surface display of heterologous proteins on *S. xylosus* and *S. carnosus* cells, respectively. We examined DTR cell surface expression and investigated the immunogenic properties of *S. carnosus* displaying DTR in BALB/c mice and the capacity of the resulting antisera to neutralize DT cytotoxicity in vitro.

MATERIALS AND METHODS

Bacterial strains, plasmids, and DNA manipulation. *Escherichia coli* MC1061 was used as a host in subcloning the DTR fragment in the *S. carnosus* expression vector. *S. carnosus* TM300 and *S. xylosus* SJ21 were provided by the Centre d’Immunologie Pierre Fabre (CIPF) (Saint-Julien en Genevois, France). The expression vectors pSE mp18ABPXM (17, 18) and pSPPmABPXM (25) were also provided by CIPF.

All DNA manipulations were performed as described by Sambrook et al. (24). Bacteria were grown acrobically in basic broth medium (Dilco, Detroit, Mich.). Culture medium was supplemented with ampicillin (200 µg/ml) for selection of pSE mp18ABPXM or pSPPmABPXM in *E. coli* or chloramphenicol (10 µg/ml) for selection in *Staphylococcus* species.

The nucleotide sequence coding for amino acids 382 to 535 of DT, corresponding to the receptor domain of the toxin (DTR), was excised from pCP-DTR (15) by *SacI* and *HindIII* restriction enzymatic restriction. The DNA fragment was then inserted in the mp18 multichroming site of pSE mp18ABPXM by using the *SacI* and *HindIII* restriction sites, and the resulting plasmid was called pSE-DTR. From pSE-DTR, a BamHI-Xhol DNA fragment containing DTR was extracted and ligated to a BamHI-Xhol-restricted pSPPmABPXM plasmid, leading to pSPPDTR.

Preparation and transformation of the protoplasts from *Staphylococcus* cells were carried out by a method adapted from that of Götze (7).

Western blot analysis. Overnight cultures of *Staphylococcus* cells were diluted in basic broth medium to give an absorbance of 1 at 600 nm. Diluted cultures (2-ml fractions) were centrifuged for 5 min at 3,900 × g. The cells were then suspended in 150 µl of lysis buffer (50 mM Tris-HCl [pH 8], 10 mM EDTA, 5 µg of lysisostaphin per ml, 500 µg of lysozyme per ml). After 1 h of incubation at 37°C, bacterial lysates were diluted twofold in Laemmli buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on an 8% acrylamide gel. After migration, proteins were electrotransferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membrane was
first saturated with a phosphate-buffered saline (PBS)–2% bovine serum albumin solution and then incubated in PBS–0.1% Tween 20 (PBST) containing a mouse polyclonal anti-albumin-binding protein (ABP) antiserum (CIPF) diluted 1/80,000. After being washed with PBST, the membrane was incubated with a goat anti-rabbit immunoglobulin G (IgG) conjugated to peroxidase (Jackson Immunoresearch, West Grove, Pa.) diluted 1/5,000 in PBST. After the membrane was washed, labeling was assessed by using 20 ml of 100 mN Tris-HCl (pH 7.6) containing 10 mg of diaminobenzidine (Sigma) and 100 mN of 3% H2O2.

Detection of DT fragments on the surface of S. carnosus. Overnight cultures of S. xylosus containing pSE-DTR and S. carnosus containing pSPPPDTR were diluted in culture medium to 2.6 × 108 CFU/ml. Samples were added to a 96-well microtiter plate (MADV N65; Millipore), at 50 µl per well and incubated in the presence of 50 µl of either an anti-aa-DTR rabbit serum or an anti-aa-DTR rabbit antiserum (15) (final dilution, 1/150). After 2 h at 4°C, the contents of the plates were filtered with the Millipore multiscreen assay system and the cells were washed five times with PBS. Goat anti-rabbit IgG conjugated to peroxidase was then added at a dilution of 1/5,000, and the mixture was incubated for 30 min at room temperature. After extensive washing of the mixture with PBS, 250 µl of a 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate solution was added per well. The mixtures were transferred (100 µl per well) to an enzyme-linked immunosorbent assay microtiter plate (Nunc) 30 min later, and the plate was read at 414 nm.

Immunization of mice. Three groups of four BALB/c mice (IFFA CREDO) were injected intraperitoneally with 3 × 108 CFU of S. carnosus containing pSPPPDTR. The mice were immunized every 3 or 4 days (nine injections in total [group C]), every 7 days (five injections in total [group B]), or every 14 days (three injections in total [group A]). As a control, four BALB/c mice were injected intraperitoneally at 14-day intervals with 5 × 106 CFU of S. carnosus containing pSPPMABPXM (three doses in total [group D]). Blood samples were collected 14 days after the last injection.

Determination of anti-DT titer. Microtiter ELISA plates were coated overnight at 4°C with DT (Calbiochem, La Jolla, Calif.) (0.1 µg/well) diluted in 50 mM phosphate buffer (pH 7.4) and subsequently saturated with 200 µl of 0.1 M phosphate buffer (pH 7.4)–0.3% bovine serum albumin per well. The DT-coated plates were washed, and dilutions of the different antisera were added (100 µl/well), and the mixtures were incubated overnight at 4°C. After extensive washing, a goat anti-mouse IgG antibody conjugated to peroxidase was added (100 µl/well; dilution, 1/5,000), and the mixture was incubated for 30 min at room temperature. After extensive washing, 200 µl of ABTS substrate solution was added per well. The plates were read at 414 nm after 60 min. The titer was defined as the highest serum dilution giving an absorbance of 0.6 above the negative control. As a control, we used mouse preimmune sera.

In vitro neutralization assay. Vero cells were grown in 250-ml culture flasks (Falcon) at 37°C in Dulbecco modified Eagle medium (Biological Industries, Beit Haemek, Israel) supplemented with 10% fetal calf serum without penicillin or a preimmune serum were diluted 1/10 in a synthetic culture medium supplemented with 10% fetal calf serum without penicillin or a preimmune serum were diluted 1/10 in a synthetic culture medium supplemented with 10% fetal calf serum. The cells were grown to confluence and detached from the flasks with 0.02% trypsin–0.05% EDTA solution and then plated to a final density of 2.6 × 108 CFU/ml. Samples were added to a 24-well plate (Falcon) at 100 µl per well and centrifuged for 15 min at 1,000 × g. After extensive washing, a solution containing 3 × 108 CFU of S. carnosus was added per well. After 3.5 h at 37°C, the medium was removed and the cells were washed with cold Hank's balanced salt solution (Biological Industries). The cells were then incubated in Leu-deficient minimal essential medium (Sigma, St. Louis, Mo.) for 1 h at 37°C. The medium was then removed and the cells were washed twice with cold Hank's balanced salt solution. The cells were then sylvanized with 0.4 M KOH for 10 min. Proteins were precipitated with 10% trichloroacetic acid (TCA) and collected on filters by using a TOMTEC apparatus (Wallac, Turku, Finland). The filters were dried, and [14C]Leu incorporation was measured by liquid scintillation with a 1450 Microbeta counter (Wallac).

RESULTS

Insertion of the sequence coding for the receptor-binding domain of DT in two expression systems. Two expression systems, called pSE’mp18ABPX and pSPPMABPX, have been developed for surface display of heterologous proteins by S. xylosus and S. carnosus, respectively (17, 25). The two vectors contain (i) the origin of replication from E. coli and the β-lactamase gene conferring ampicillin resistance (13), (ii) the origin of replication from S. aureus and the chloramphenicol acetyltransferase gene for Staphylococcus expression, and (iv) a multicloning site. In both systems, the heterologous polypeptide was inserted in the N-terminal part of a serum albumin-binding region (ABP) of protein G from Streptococcus sp. strain G148 followed by the cell surface-anchoring regions of protein A from S. aureus. In pSE’mp18ABPX, the recombinant polypeptide was preceded by the promoter region, the signal peptide, and fragment E (6 residues) of protein A; PP, 207-residue propeptide of a lipase from S. hyicus.

We selected the DTR region for insertion in each expression system. This choice was supported by the previous observation that a soluble fusion protein containing DTR is able to abolish the cytotoxicity of DT for Vero cells and to elicit neutralizing antibodies in rabbits, suggesting that this region, which is organized as a whole domain in DT (1–3), can fold independently in a type of native structure (15).

The sequence coding for DTR was isolated from plasmid pCP-DTR (15) and transferred to pSE’mp18ABPX. The insertion of this sequence in the resulting pSE’DTR plasmid was then assessed by restriction analysis. S. xylosus was subsequently transformed with the plasmid. To transfer the sequences coding for DTR in the S. carnosus expression vector, a BamHI-XhoI fragment was isolated from pSE’DTR and inserted in pSPPmABPX. S. carnosus was transformed with the resulting plasmid, called pSPPPDTR.

Western blot analysis. The synthesis of the recombinant proteins was checked by Western blot analysis. Extracts of overnight cultures of Staphylococcus strains containing pSE’mp18ABPX, pSPPMABPX, pSE’DTR, pSPPPDTR, or no plasmids were subjected to SDS-PAGE and Western blot
analysis with ABP-reactive antibodies (Fig. 2). An immunoreactive product was detected in the lysates of S. xylosus and S. carnosus containing either pSE\textsuperscript{mp18ABPXM} derivative plasmids (Fig. 2A, lanes 2 and 3) or pSPPmABPXM derivative plasmids (Fig. 2B, lanes 1 and 2) but not in the lysates of the untransformed staphylococci (Fig. 2A, lane 1, and Fig. 2B, lane 3). The estimated sizes of the proteins produced by cells containing pSPPmABPXM and pSE\textsuperscript{mp18ABPXM} were 89 ± 3 and 50 ± 3 kDa, respectively. For the hybrid proteins, the estimated sizes were 108 ± 3 kDa for pSPPDTR and 66 ± 4 kDa for pSE\textsuperscript{DTR}. These values indicate that the sequence of DTR (17 kDa) is expressed by the two transformed bacteria.

**Immunological characterization of the surface display of DTR by S. carnosus and S. xylosus cells.** The display of heterologous proteins on the surface of transformed bacteria can be estimated by investigating the binding of antibodies specific to the inserted proteins (21). We therefore assessed the surface display of DTR by using a rabbit antiserum raised against the soluble fusion protein ZZ-DTR and a second rabbit antiserum raised against the region of DT from residues 168 to 220 \((ZZ-DT_{168-220})\) (15). The rabbit anti ZZ-DT\textsubscript{168–220} antiserum was used as a negative control since it does not recognize DTR (data not shown). The two antisera were incubated with the recombinant bacteria in microfilter plates for 3 h. After extensive washing and filtering, the antibodies still bound to the transformed staphylococci were detected by using a goat anti-rabbit IgG covalently coupled to peroxidase and with ABTS as the substrate.

As shown in Fig. 3, S. carnosus containing pSPPDTR was efficiently recognized by the ZZ-DTR antiserum but was only weakly bound by the ZZ-DT\textsubscript{168–220} antiserum. In contrast, the two antisera did not differ significantly in their binding to S. xylosus containing pSE\textsuperscript{DTR}. Hence, DTR is efficiently displayed on the surface of recombinant S. carnosus but weakly exposed on S. xylosus.

**Immunogenicity of S. carnosus displaying DTR in BALB/c mice.** We investigated the ability of S. carnosus displaying DTR to elicit a humoral immune response in BALB/c mice. Four BALB/c mice were injected intraperitoneally with \(3 \times 10^8\) CFU of the live recombinant bacteria every 3 or 4 days (group C), every 7 days (group B), or every 14 days (group A). As a control, a fourth group of BALB/c mice was injected with \(5 \times 10^8\) CFU of S. carnosus containing pSPPmABPXM every 2 weeks. Blood samples were collected 2 weeks after the last injection, and antisera were subsequently tested for their ability to bind to DT on microtiter enzyme-linked immunosorbent assay plates. The anti-DT titers measured on pooled antisera from group C, or a fixed dilution of preimmune serum. Titers were distributed over a range of 10% around the value measured for the pooled sera (data not shown), indicating that surface display of DTR on S. carnosus cells can trigger a homogeneous immune response in BALB/c mice.

**Neutralization of DT cytotoxicity by immune sera.** The neutralizing potency of the antiseras raised against recombinant S. carnosus displaying DTR was tested in vitro with toxin-sensitive Vero cells. Various dilutions of DT were preincubated overnight at 4°C with culture medium, a fixed dilution of antisera from group C, or a fixed dilution of preimmune serum. Vero cells were subsequently added to these mixtures, and DT cytotoxicity was estimated by measuring \([^{14}C]\text{Leu incorporation into TCA-precipitable material.}\)

As shown in Fig. 4, the preimmune serum did not alter the ability of DT to inhibit the protein synthesis of the Vero cells. In contrast, in the presence of the pooled immune sera from group C mice, approximately 10 times more DT was required to reach a level of inhibition similar to that observed with culture medium only. Therefore, nine intraperitoneal injections of BALB/c mice with \(3 \times 10^8\) CFU of S. carnosus displaying DTR elicited anti-DT antibodies with in vitro neutralizing potency.

**DISCUSSION**

Vaccination with a nonpathogenic bacterium expressing a fragment of a heterologous protein is a promising concept. Numerous heterologous antigens have been tentatively expressed in the cytoplasm (5, 31), in the periplasm (9, 12), or on the cell surface (6, 29) of or secreted by (23) different bacterial strains. In principle, bacterial surface display is particularly suitable for eliciting a humoral immune response because Igs expressing B lymphocytes can bind the heterologous antigen.
directly. However, the development of this approach is partly limited by the need to display a protein fragment in a structure that resembles the conformation adopted by the same fragment in the cognate protein. In this context, we previously observed that a soluble fusion protein containing the DTR domain is able to abolish efficiently the cytotoxicity of DT for Vero cells and to elicit neutralizing antibodies in rabbits, suggesting that DTR may fold independently in a native type of structure (15). These observations prompted us to investigate whether such a structurally well-defined domain (1–3) may be expressed on the surface of S. xylosus and S. carnosus and if the resulting vectors can trigger, in mice, anti-toxin antibodies with neutralizing potency.

Our results show that the two recombinant bacteria differ markedly in the expression of the DTR domain. DTR was not displayed on the surface of S. xylosus, but it was efficiently produced on S. carnosus. There are various possible explanations for these differences. First, DTR may be translated efficiently by S. carnosus but only weakly by S. xylosus. This hypothesis is supported by a recent report showing that the expression system developed for S. carnosus is more efficient in its ability to translocate heterologous proteins on the cell surface than is the system developed for S. xylosus (21). Second, the heterologous protein can be degraded by the extracellular protease activity exhibited on the surface of S. xylosus; whereas S. carnosus has been shown to be devoid of such extracellular activity (26). Third, the folding and/or degradation of the DTR domain can be favored or affected by the N-terminal region of the hybrid, which differs in the two expression systems. In S. carnosus, the DTR fragment is preceded by a 209-residue propeptide from lipase, whereas in S. xylosus, this extension is replaced by a 10-residue propeptide from protein A of S. aureus. At present, we cannot determine which of these possibilities applies. However, the weak capacity of S. xylosus to display DTR prompted us to exclude this recombinant vector from our immunization experiments.

The anti-DT antibody titer measured after intraperitoneal injections of BALB/c mice with S. carnosus displaying DTR and the “in vitro” neutralizing capacities of the resulting antisera indicate that this live vector efficiently presents the DTR domain to the immune system. Although these results are promising, it cannot yet be concluded that the live vector is appropriate for heterologous immunizations, because nine injections of high doses of recombinant live bacteria (3 × 10⁸ CFU) were required to raise an efficient anti-DT antibody response. Furthermore, the intraperitoneal route was selected since preliminary experiments (results not shown) we observed a weak antibody response after subcutaneous injections of BALB/c mice. These observations raised the question of how to increase the immunogenicity of the recombinant bacterium. Since the antibody response is related to the amount of heterologous protein expressed by the live vector (4), one way might be to increase the proportion of DTR displayed on the surface of S. carnosus. Another approach would consist of targeting the recombinant bacterium to appropriate cells of the immune system by using specific antibodies. The latter seems particularly well suited to S. carnosus, since this bacterium has been successfully used for surface display of the single-chain variable fragment of immunoglobulin (ScFv) (8).

ACKNOWLEDGMENTS

We gratefully acknowledge T. N. Nguyen for his advice on the expression of the DT fragments on the surface of staphylococci. This work was supported by grant B102CT-CT920089 from the European Biotechnology Programme, “New approaches for oral vaccination against infectious diseases and autoimmune disorders.”

REFERENCES