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Expression of a pilin subunit BfpA of the bundle-forming pilus of enteropathogenic *Escherichia coli* in an *aroA* live salmonella vaccine strain

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Abstract

Enteropathogenic *Escherichia coli* (EPEC) is a major cause of childhood diarrhea in developing countries and is a leading cause of severe diarrheal illness among Brazilian infants. As one approach to constructing a vaccine candidate against diarrhea caused by EPEC, we evaluated whether the pilin subunit (BfpA) of the bundle-forming pilus (BFP) could be expressed by a live *Salmonella* vaccine strain. Several copies of the coding region of BfpA (*bfpA*) were amplified by PCR from a preparation of the EAF plasmid of EPEC strain B171 and cloned into plasmid vectors. An intact copy of *bfpA* was subcloned into the heat inducible prokaryotic expression vector pCYTEXP1, and the resulting pBfpA was used to transform the *aroA* *S. typhimurium* strain SL3261, generating SL3261(pBfpA). The recombinant vaccine strain was able to express, but not to process, rBfpA as evidenced by a prominent 21 kDa protein that crossreacted with anti-BFP antiserum found only in extracts of heat-treated SL3261(pBfpA), but not in strains of untreated SL3261(pBfpA) or SL3261 not carrying the plasmid. Furthermore, rBfpA accumulation was not toxic to the *Salmonella* host, as evidenced by similar plating efficiencies between induced and uninduced strains of SL3261(pBfpA). Finally, SL3261(pBfpA) orally administered to BALB/c mice was capable of eliciting a sustained and vigorous humoral immune response to BfpA, achievable even with a single oral dose of approximately 10^9 organisms. Therefore, this pilin product may serve as a potential immunogen as part of a live combined vaccine strategy to prevent two of the major public health problems in Brazil – salmonellosis and EPEC childhood diarrrhea. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: EPEC; BFP; Vaccine; Oral

1. Introduction

Enteropathogenic *Escherichia coli* (EPEC) is a major cause of diarrhea among infants of developing countries [1–6]. Diarrhea caused by EPEC is often more severe than that caused by other pathogens [7, 8] and is frequently associated with concurrent nutritional complications [9]. Furthermore, mortality rates due to EPEC diarrhea are high, exceeding 30% reported in some outbreaks [8]. In Brazil, it is esti-

mated that over 200 000 infants die every year from diarrheal diseases. These reported figures probably represent a small fraction of the actual number of cases, many of which are also associated with complications such as malnutrition. In Brazil, EPEC is one of the leading causes of childhood diarrhea in major urban centers [4–6] that require visits to emergency rooms; the incidence is double that due to rotavirus [4]. Nevertheless, despite the severity of illness among infants, most epidemiological data indicate that the disease due to EPEC in older children and adults living in endemic areas is less severe and uncommon [7]. This suggests that exposure to EPEC early in life may lead to long term protection against severe symptomatic infection. Serum levels of antibodies to this

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enterpathogen increase with age, and are seldom detectable among infants [10]. Therefore, the development of a vaccine against this pathogen may be feasible and may be of great benefit for infants in Brazil and other developing countries.

The initial phase of an EPEC infection is characterized by colonization of intestinal cells by clusters of bacteria [11,12]. This pattern of colonization, called localized adherence (LA) [13–15], is associated with symptomatic disease and is dependent on the presence of a 50–70 MDa plasmid, referred to as the EAF plasmid (EPEC adherence factor) [16,17]. Strains cured of the EAF plasmid no longer display LA in vitro [18] and are less virulent in human volunteers [17]. Recently, a colonization factor responsible for LA, called bundle-forming pilus (BFP) was identified [19,20] and the gene encoding its pilin subunit (*bfpA*) was cloned [21,22]. *bfpA* is located on the EAF plasmid and transposon mediated disruption of this gene abrogates LA [21]. Most importantly, mature, native BFP is expressed on the outer membrane of EPEC [20] and antiserum to it markedly inhibits LA [19], lending support to the hypothesis that an immune response to the colonization factor, or its pilin, may interfere with the normal pathogenic process of EPEC infection and possibly prevent or attenuate disease.

Vaccines administered by the oral route have many practical advantages. In this respect, the use of laboratory attenuated strains of *Salmonella* as a live vehicle for oral immunization has gained attention in the last decade [23–26]. These bacterial strains characteristically retain their ability to induce immunity against themselves, while eliciting an immune response against heterologous antigens expressed by them. Taking into account that *Salmonella typhi* and *Salmonella typhimurium* also represent relevant public health problems in Brazil, these vaccine strains also have the additional advantage of serving as basis for live combination vaccines. To test whether the pilin of the bundle-forming pilus of EPEC may be used as an immunogen in a *Salmonella* vector, we developed a *bfpA* recombinant *aroA* *Salmonella typhimurium* strain and evaluated its ability to elicit BfpA specific antibody in BALB/c mice.

2. Materials and methods

2.1. Plasmids and bacterial hosts for cloning and immunization

pCRII (Invitrogen, San Diego, CA, USA) was used to clone *bfpA* that was amplified by PCR. The heat-inducible prokaryotic expression plasmid pCYTEXP1 (GBF, Germany) [28] was used for the expression of

bfpA in *Salmonella typhimurium* SL3261 (Dr. Bruce Stocker, Stanford University), in laboratory *E. coli* strains DH5 α and HB101, and in EPEC B171-4. *E. coli* DH5 α was used as a cloning host and the *aroA* *S. typhimurium* strain SL3261 was used as a vaccine delivery vehicle. *S. typhimurium* strain LB5000 (Dr. Bruce Stocker, Stanford University) was used to modify the DNA constructs prior to transformation of SL3261. B171 is an EAF + , O111:NM, strain of EPEC, and B171-4 is an EAF-cured derivative of B171. All bacterial strains were maintained at -70°C or in stabs and slants of Luria Bertrani (LB) agar for long term and short term storage, respectively, and propagated in liquid LB broth supplemented with appropriate antibiotics when required. Bacterial chemical transformations were carried out by the CaCl_2 method described elsewhere [27].

2.2. DNA preparation, modification and sequencing

EAF plasmid DNA was extracted from an overnight suspension of B171, using the QIAGEN Plasmid Kit (QIAGEN, Germany) following the manufacturer's instructions. Plasmids used as cloning and expression vectors were extracted from their respective hosts by the alkaline lysis method described elsewhere [27]. Restriction enzyme digestion, PCR amplification, DNA ligation and dideoxy chain termination nucleic acid sequencing methods employed enzymes and buffers obtained from Boehringer Mannheim (Boehringer Mannheim Biochemicals, Indianapolis, IN) and Pharmacia (Pharmacia Biotech, Uppsala, Sweden) [27].

2.3. Protein extraction, electrophoresis and Western blotting

Whole cell protein extracts were prepared from bacterial suspensions grown in LB medium supplemented with the appropriate antibiotics and shaken at 180 rpm at 28°C until the optical density (OD_{600}) reached 0.4–0.6. The bacteria were harvested by centrifugation, resuspended in fresh medium and incubated at 42°C with low agitation and aeration for up to 3 h, followed by centrifugation and resuspension in distilled water to 1/40 of the original culture volume. It was then heated at 95°C in an equal volume of $2\times$ Laemmli's sample buffer for 5 min. These extracts were kept at -20°C until used. Wildtype EPEC B171 and its derivative B171-4 strains were grown overnight in DMEM to induce *bfpA* expression and then treated as above.

Sodium dodecyl sulfate polyacrylamide gel electrophoreses (SDS-PAGE) and Western blotting were performed as described elsewhere [27]. BFP and BfpA were detected by Western blots with a rabbit polyclonal antiserum against BFP kindly provided by J.A. Girón. An anti-rabbit polyclonal antiserum conjugated

to alkaline phosphatase was used (Bio-Rad Laboratories, Hercules, CA) as the secondary antibody and BCIP was used as a substrate for color development.

2.4. Immunization of mice

The *bfpA* recombinant SL3261 strain, referred to as SL3261(pBfpA), and the parent SL3261 strain were inoculated from frozen glycerol stocks (-70°C) into 5 ml of LB broth (supplemented with $100\ \mu\text{g ml}^{-1}$ of carbenicillin for the SL3261(pBfpA) suspension) and shaken at 75 rpm at 28°C overnight. The following day 50 μl of each culture were transferred into 50 ml of fresh medium and the bacterial suspensions were shaken at 180 rpm at 28°C until the OD_{600} reached 0.4. Then the suspensions were chilled on ice, pelleted at $8000 \times g$ at 4°C for 10 min, resuspended in 50 ml of fresh media, incubated at 42°C with low agitation and aeration for 2 h, chilled again, pelleted at $8000 \times g$ at 4°C for 10 min, resuspended in 2.5 ml of 1.5% sodium bicarbonate and kept on ice until the immunization of mice was performed.

To assess the immunogenicity of BfpA expressed by SL3261, we administered three groups of 7–9 weeks old female BALB/c mice (3 animals per group) one of the following preparations on days 0 and 30: (1) 100 μl of 1.5% sodium bicarbonate; (2) 10^9 cfu of SL 3261 in 100 μl of 1.5% sodium bicarbonate; or (3) 10^9 cfu of SL3261(pBfpA) in 100 μl of 1.5% sodium bicarbonate. On day 60, the animals were bled and the presence of anti-BfpA antibody was evaluated. In another experiment designed to evaluate the persistence of antibody levels in immunized animals, we performed the following experiments on three groups of 7–9 weeks old female BALB/c mice: (1) 12 animals received 100 μl of 1.5% sodium bicarbonate on days 0 and 30; (2) 12 animals received 10^9 cfu of SL3261 in 100 μl of 1.5% sodium bicarbonate on days 0 and 30; and (3) 21 animals received 10^9 cfu of SL3261(pBfpA) in 100 μl of 1.5% sodium bicarbonate on day 0; of the latter, 9 received booster immunizations on day 30. Sets of three animals from each of these groups were bled on days 30, 60, 90 and 120 after the initial immunization. Each set of three mice was bled only once in the study. Animals of group 3 boosted on day 30 were bled on days 60, 90 and 120 only. On day 0, three non-immunized animals maintained in the same cage as the immunized ones were bled and their sera served as the baseline nonimmune antibody. All oral administrations were carried out without sedation and with the inocula delivered via the oropharynx, using a 1 in. 21 gauge feeding needle. Food and water were removed from each cage 4 h prior to the oral inoculation and returned 30 min after inoculation.

2.5. Collection of blood and detection of anti-BfpA antibody in mouse serum samples

Blood was collected from the retroorbital venous plexus and allowed to clot. Serum samples were removed and stored frozen at -20°C until the analysis for anti-BFP antibody was performed. Whole cell extracts of SL3261(pBfpA) resolved by SDS-PAGE and transferred onto nitrocellulose paper were used for the detection of anti-BfpA antibody in serum samples. Alternatively, in one experiment, extracts of SL3261 were used to check whether the reactivity detected in the SL3261(pBfpA) extracts was specific to BfpA and not to another comigrating protein. Briefly, nitrocellulose strips containing SDS-PAGE-resolved whole cell extracts of SL3261(pBfpA) (or SL3261) were incubated with 3% gelatin in Tris buffered saline for one hour; after a cycle of washes with Tween containing Tris buffered saline, the strips were incubated overnight at room temperature with the serum samples to be tested diluted to 1:1000 and previously adsorbed with a specially prepared extract of SL3261. The membranes were washed again and incubated for two hours with alkaline phosphatase conjugated anti-mouse IgG (Sigma Chemical, St. Louis, MO) at a dilution of 1:1000; after a last cycle of washes, the strips were incubated for up to 45 min with alkaline phosphatase substrate BCIP (Bio-Rad Laboratories, Hercules, CA), rinsed extensively with water and allowed to air dry.

The immunoabsorption reagent was prepared as follows. A suspension of SL3261 expanded and heat treated as already described was pelleted, resuspended to 1/10 of the original volume in TBS (Tris-buffered saline), and then subjected to 5 freeze-and-thaw cycles, followed by five more cycles of sonication in an ice bath after which the resulting suspension was centrifuged at $8000 \times g$ at 4°C for 10 min. The supernatant was then collected and stored at -70°C until used. Prior to the immunodetection experiments, the aliquots of the pools of sera were incubated overnight at 4°C with slow shaking in 200 μl of the immunoabsorption reagent, and then centrifuged at $1200 \times g$ at 4°C for 15 min. The supernatant of this preparation was used for the Western blot analyses.

3. Results

3.1. *bfpA* cloning and expression

In order to clone the structural gene *bfpA*, we first amplified its coding region by PCR from a preparation of EAF plasmid of EPEC strain B171, using the following pair of oligonucleotide primers: 5':GCGC-CATATGTTTCTAAAATCATGAATA and 5':GCGCGCTCGACGATTACTTCATAAAAATATG (underlined areas are the target sites for the *Nde* I and *Sal* I

restriction enzymes added to the 5' ends of the forward and reverse primers, respectively; boldfaced nucleotides are the translational initiation and termination codons). The amplified PCR product had the expected size of approximately 600 bp and it was ligated into pCRII vectors. The identity of the amplicon was confirmed as *bfpA* by sequencing their 5' ends. After sequencing the entire insert DNA of several pCRII:*bfpA* constructs, we identified an intact copy of *bfpA* and subcloned it into the *Nde* I/*Sal* I fragment of the prokaryotic expression plasmid pCYTEXP1. In pCYTEXP1, the expression of cloned genes mediated by bacteriophage lambda P_R and P_L promoters is tightly controlled by a temperature sensitive form of the bacteriophage lambda CI repressor (CI^{ts}). Thus in transformed hosts, a heterologous gene expression can be tightly repressed at 28°C and fully de-repressed at 42°C.

The ability of pBfpA to drive *bfpA* expression was assessed in *E. coli* K12 derivatives HB101 and DH-5 α , and in the EAF minus EPEC strain B171-4 transformed with the recombinant plasmid. A suspension of each of these recombinant bacteria was expanded to mid-logarithmic stage at 28°C and then incubated at 42°C for up to 3 h. Then, whole cell protein extracts from samples of these suspensions were resolved by SDS-PAGE, transferred onto a piece of nitrocellulose membrane and the resulting blot was analyzed with a rabbit polyclonal antiserum raised against mature BFP (Fig. 1). A band representing a 21 kDa protein was recognized by the anti-serum in the extracts of all transformant strains (Fig. 1, lanes 3 to 6 and 8) but not in non-transformants run in parallel (Fig. 1, lanes 2 and 7), showing that *bfpA* could be successfully expressed by pBfpA in each of these *E. coli* hosts. Nevertheless, since the native BfpA has a molecular weight around 17 kDa, these recombinant *E. coli* hosts were able to produce, but not process rBfpA. This observation was further supported by the result that the B171-4 pBfpA transformants were unable to exhibit localized adherence on HeLa cell monolayers (data not shown).

3.2. *bfpA* expression and rBfpA stability in a *Salmonella*-based live vaccine strain transformed with pBfpA

A suspension of competent *aroA S. typhimurium* SL3261 was transformed with pBfpA previously modified by passage in *S. typhimurium* LB5000, and a resulting clone, referred to as SL3261(pBfpA), was evaluated for its ability to express *bfpA*. We analyzed the kinetics of *bfpA* expression in SL3261(pBfpA) by growing the suspension to mid-logarithmic stage at 28°C and incubating it for 3 h at 42°C; aliquots of the suspension were examined at different time points for the expression of the recombinant protein. A suspension of SL3261 treated similarly was used as the BfpA negative control. A protein of approximately 21 kDa was observed in samples of SL3261(pBfpA) as early as 1 h after the initiation of heat treatment, and the protein expression progressively increased in samples collected at 2 and 3 h time points (Fig. 2(a), lanes 2 to 4). The protein expression was not observable in samples of SL3261(pBfpA) collected before heat treatment (Fig. 2(a), lane 1) and was absent in all SL3261 samples (Fig. 2(a), lanes 5 to 8). The identity of this protein as rBfpA was confirmed by the Western blot analysis of the samples described above (Fig. 2(b)). The antiserum recognized the 21 kDa band, representing unprocessed rBfpA, only in SL3261(pBfpA) samples induced for 1–3 h, but not in samples of uninduced SL3261(pBfpA) or SL3261. In these previous experiments, a second heat inducible protein, migrating very close to the 21 kDa rBfpA band, was observed in SDS-PAGE gels of both SL 3261 and SL3261(pBfpA) samples (Fig. 2(a), lanes 2 to 4 and 6 to 8). Its presence in recombinant and non-recombinant *S. typhimurium* and the fact that it was not detectable in the Western blot (Fig. 2(b), lanes 2 to 8), suggest that it is a native bacterial protein probably expressed by heat stress, rather than a processed or degraded form of rBfpA, or another plasmid-encoded protein.

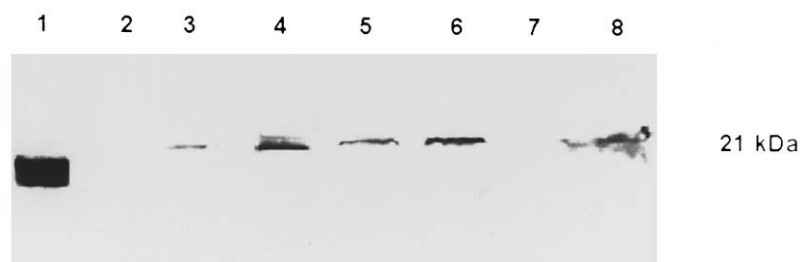


Fig. 1. *bfpA* expression in different bacterial hosts transformed with pBfpA. A Western blot of whole cell extracts from suspensions of different *E. coli* hosts: lane 1, EAF⁺ EPEC B171; lane 2, EAF⁻ EPEC B171-4; lane 3, EAF⁻ EPEC B171-4 transformed with pBfpA and incubated at 42°C for 2 h; lane 4, EAF⁻ EPEC B171-4 transformed with pBfpA and incubated at 42°C for 3 h; lane 5, *E. coli* HB101 transformed with pBfpA and incubated at 42°C for 2 h; lane 6, *E. coli* HB101 transformed with pBfpA and incubated at 42°C for 3 h; lane 7, *E. coli* DH5 α not transformed with pBfpA; lane 8, *E. coli* DH5 α transformed with pBfpA and incubated at 42°C for 3 h.

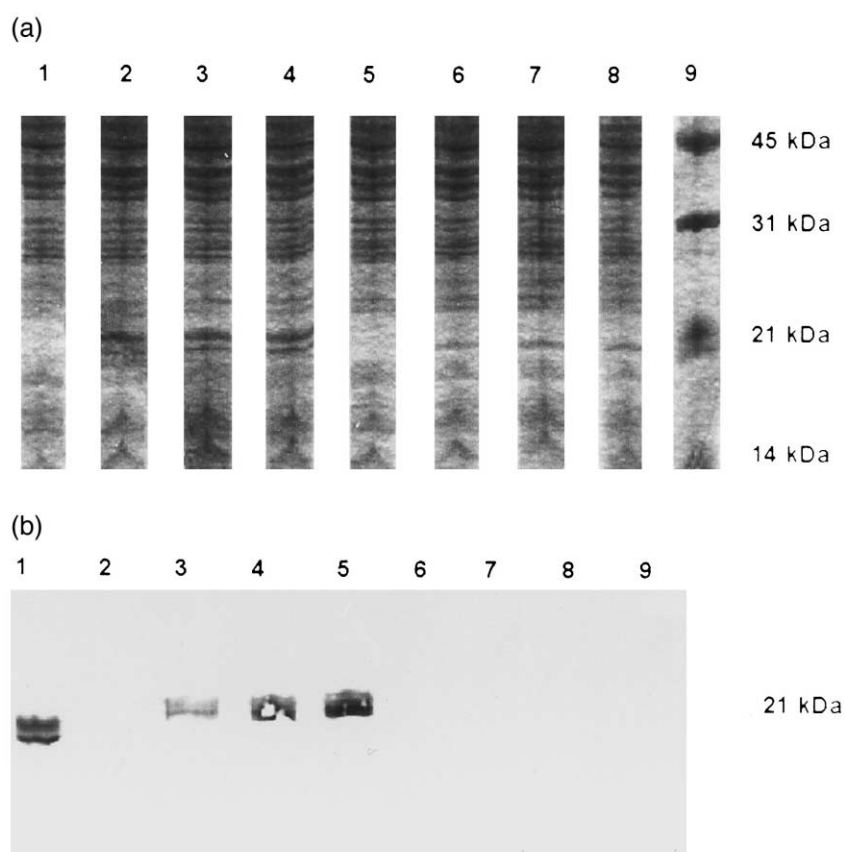


Fig. 2. Kinetics of *bfpA* expression in SL3261(pBfpA) and reactivity of rBfpA expressed in SL3261(pBfpA) to rabbit anti-BFP polyclonal antiserum. (a) SDS-PAGE of whole cell extracts of suspensions of SL3261(pBfpA) and SL3261, grown to mid-logarithmic stage at 28°C and incubated for up to 3 h at 42°C: lanes 1 to 4, SL3261(pBfpA) incubated 0, 1, 2 and 3 h at 42°C, respectively; lanes 5 to 8, SL3261 incubated 0, 1, 2 and 3 h at 42°C respectively; lane 9, molecular weight. (b) Western blot of samples from previous experiment, treated with rabbit anti-BFP polyclonal antiserum: lane 1, whole cell extract of EAF⁺ EPEC B171; lanes 2 to 5, whole cell extracts of SL3261(pBfpA) incubated 0, 1, 2 and 3 h at 42°C respectively; lanes 6 to 9, whole cell extracts of SL3261 incubated 0, 1, 2 and 3 h at 42°C respectively.

Finally, heat treatment and rBfpA accumulation were not detrimental to the *Salmonella* host as evidenced by similar numbers of colony forming units in heat treated suspensions of SL3261(pBfpA) and SL3261 (Table 1). Also, protein degradation was not detected as judged by a distinct single 21 kDa band in SDS-PAGE and Western blot (Fig. 2), indicating that rBfpA is quite stable in SL3261(pBfpA).

3.3. SL3261(pBfpA) induces a humoral immune response against the BFP pilin in BALB/c mice orally administered with suspensions of the recombinant vaccine strain

In order to evaluate whether BfpA presented to the immune system by SL3261(pBfpA) was immunogenic, we orally administered three groups of BALB/c mice

Table 1
Viability of SL3261 and SL3261(pBfpA) after the indicated durations of heat induction^a

Induction time (h)	SL3261			SL3261(pBfpA)		
	OD ₆₀₀ ^b	cols/plate ^c	cfu/ml ^d	OD ₆₀₀	cols/plate	cfu/ml
0	0.417	370	7.4	0.413	386	7.7
1	0.451	137	2.7	0.446	171	3.4
2	0.454	105	2.1	0.446	121	2.4
3	0.465	118	2.4	0.451	115	2.3

^aRepresentative experiment performed with the suspensions used for the first dose of immunization in the experiments depicted in Fig. 3(b) and (c). Results are expressed as means of two different determinations. ^bOptical densities of suspensions were determined at the end of the corresponding incubation period. ^cColony counts per LB plate. ^dCalculated colony forming units per ml of bacterial suspension.

with either 10^9 cfu of SL3261(pBfpA), 10^9 cfu of SL3261, or 100 μ l of 1.5% sodium bicarbonate twice (days 0 and 30) and collected their sera on day 60, pooled for each group, and tested them for the presence of anti-BfpA antibody. The antibody detection was performed with whole cell extracts of SL3261(pBfpA) resolved by SDS-PAGE, transferred onto strips of nitrocellulose membrane. The sera were pre-adsorbed with extracts of SL3261 in order to minimize background reactions. In this experiment, the serum samples collected from animals administered SL3261 or sodium bicarbonate showed no reactivity with the BfpA 21 kDa band on the blots, as shown in Fig. 3(a), lanes 1 and 2. On the other hand, the sera pooled from animals receiving SL3261(pBfpA) showed a strong reaction with the BfpA band even when tested at a dilution of 1:1000 (Fig. 3(a), lane 3), indicating that the recombinant vaccine strain was very effective in inducing a humoral immune response to the carried immunogen.

In another experiment designed to analyze the effect of immunization schedule on the persistence of the anti-BfpA response, we used four groups of animals: three groups were treated as in groups 1, 2 and 3 above, while a fourth group received 10^9 cfu of SL3261(pBfpA) on day 0 only. The presence of anti-BfpA in the serum of tested animals was evaluated on days 0, 60, 90 and 120 after the initial immunization, using the same schedule described above. The sera of three animals in each group were pooled and tested at each time point, making sure that the animals used at one time point were never retested. Once again, the animals receiving sodium bicarbonate or SL3261 did not show reactivity to the 21 kDa band in the blots at any of the time points. In contrast and still in accordance with the previous experiment, sera of animals that received SL3261(pBfpA) twice, displayed reactivity to the 21 kDa band as early as day 60 of the experiment (Fig. 3(b), lane 2). The sequential evaluation of humoral response in these animals showed that the reactivity remained as strong at least up to day 120 after the initial immunization when they were last analyzed (Fig. 3(b), lanes 3 and 4). On the other hand, animals receiving only one dose of SL3261(pBfpA) showed a delayed antibody response to the 21 kDa band, where it was not detectable at time points before day 90 after the initial immunization. However, as in animals that received two doses of SL3261(pBfpA), the antibody was detected on day 120 of the experiment (Fig. 3(c), lanes 3 and 4). Finally, to confirm that the anti-BfpA antibody was responsible for the reactivity of the tested sera to the 21 kDa band, we tested in parallel the serum samples obtained at day 90 from all four groups against blots containing extracts of either SL3261(pBfpA) or SL3261 resolved simultaneously by SDS-PAGE. The results in Fig. 4 show that only sera

of animals that received the test strains in one or two doses reacted with the 21 kDa band and that this reaction occurred only in blots of SL3261(pBfpA), proving that BfpA was indeed the target for the detected antibody.

Finally, none of the animals receiving the oral inocula showed any signs of toxicity (i.e. lethargy, fur alterations or death), indicating that there was no reversion of the attenuated phenotype of the carrier strain by the introduction of rBfpA and that the delivery of the vaccine strain in the oropharynx was not complicated by inhalation and subsequent pneumonia or sepsis.

4. Discussion

In this study we demonstrate that oral immunization of BALB/c mice with the pilin subunit of enteropathogenic *E. coli* bundle-forming pilus, expressed by an *aroA* *Salmonella typhimurium* strain, was capable of eliciting a strong and sustained anti-BfpA-specific antibody response. A single oral dose of the recombinant SL3261(pBfpA) strain was sufficient to elicit high titers of the anti-BfpA antibody. Of note was that this response became detectable only 12 weeks after immunization, whereas the typical pattern of a primary immune response has a peak within 14–21 days and decreases over time. The addition of a second dose to our immunization schedule elicited a response that was detectable as early as 8 weeks after the initial immunization (or 4 weeks after boosting). It is possible that these observations were due to the low sensitivity of the detection method we used. We believe that only a secondary immune response to BfpA, comprised of higher titers of the antibody of higher affinity, was detected and not the earlier, and probably weaker, primary immune response. The attenuated strains of *Salmonella* employed as vaccine vehicles are usually detectable in large numbers in lymphoid organs of immunized animals up to 30 days after oral administration, but they can still be observed in much lower numbers long thereafter. These remaining bacteria can potentially provide a continuous boost of rBfpA. The earlier detection of the anti-BfpA antibody in mice receiving two SL3261(pBfpA) doses was probably due to a prompt secondary response triggered by the large booster dose. Nonetheless, in spite of the immunization schedule used, high titers of the anti-BfpA antibody could still be detected in the serum of mice when last evaluated 16 weeks after initial SL3261(pBfpA) administration. This sustained response encompassed approximately 20% of the animal's lifespan. Such a response following a single vaccination dose, if only partially reproducible in human beings, would probably be sufficient to protect the vaccinated children for

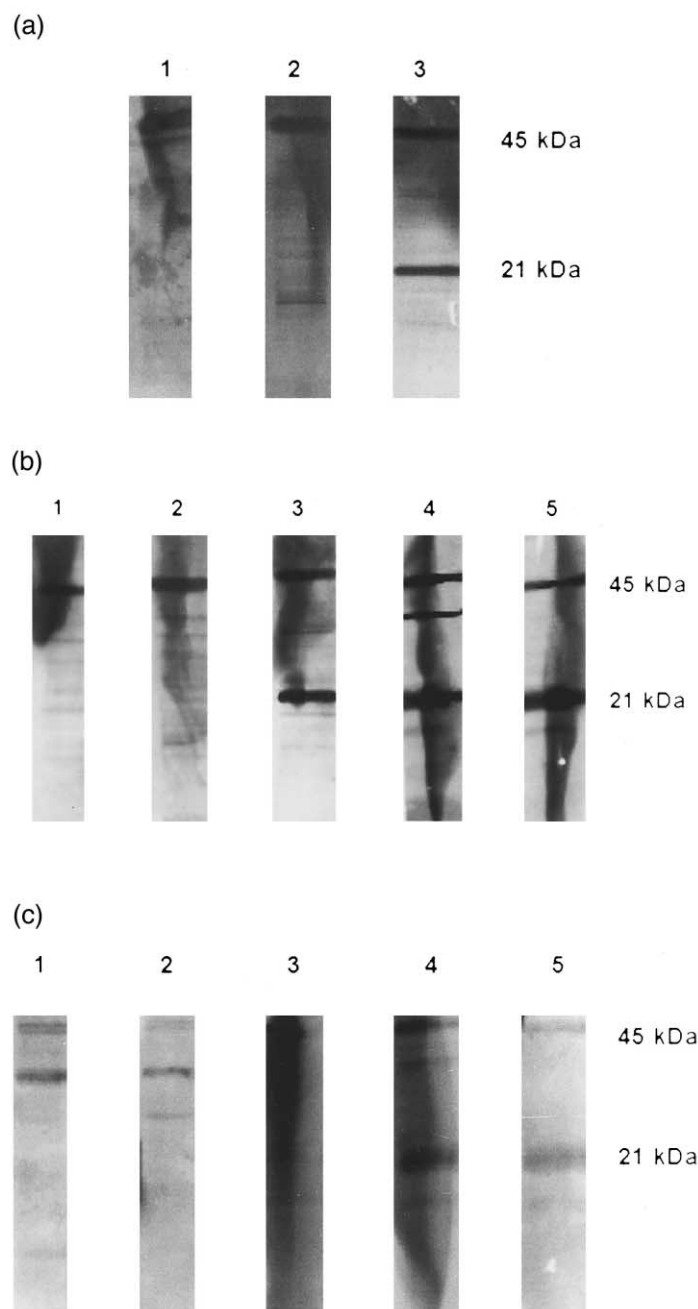


Fig. 3. Detection of anti-BfpA antibody in the sera of BALB/c mice orally administered with either SL3261(pBfpA), SL3261 or sodium bicarbonate in two different experiments, on strips containing extracts of SL3261(pBfpA) resolved by SDS-PAGE. (a) Immunization experiment 1: Three groups of three mice per group were administered either 10^9 cfu of SL3261(pBfpA), 10^9 cfu of SL3261 or 1.5% sodium bicarbonate on days 0 and 30 and the presence of anti-BfpA antibody was determined in the pools of sera from each group taken on day 60: Lane 1, sera of animals administered sodium bicarbonate; lane 2, sera of animals administered SL3261; lane 3, sera of animals administered SL3261(pBfpA). (b) Immunization experiment 2: A group of mice was administered 10^9 cfu of SL3261(pBfpA) on days 0 and 30 and the presence of anti-BfpA antibody was sequentially evaluated in pools of serum of three animals per time point: lane 1, pool from three pre-immune animals; lane 2, pool from animals bled on day 60; lane 3, pool from animals bled on day 90; and lane 4, pool from animals bled on day 120. (c) Immunization experiment 2: A group of mice was administered 10^9 cfu of SL3261(pBfpA) on day 0 only and the presence of anti-BfpA antibody was sequentially evaluated in pools of serum of three animals per time point: lane 1, pool from three pre-immune animals; lane 2, pool from animals bled on day 30; lane 3, pool from animals bled on day 60; lane 4, pool from animals bled on day 90; and lane 5, pool from animals bled on day 120.

the time period when they are at their highest risk for severe EPEC infection.

In expressing *bfpA* in SL3261 we opted for an inducible expression construct. Heterologous protein pro-

duction in prokaryotes often results in recombinant plasmid loss during the host's logarithmic stage of growth. Inducible expression allows for the full repression of the heterologous protein production during

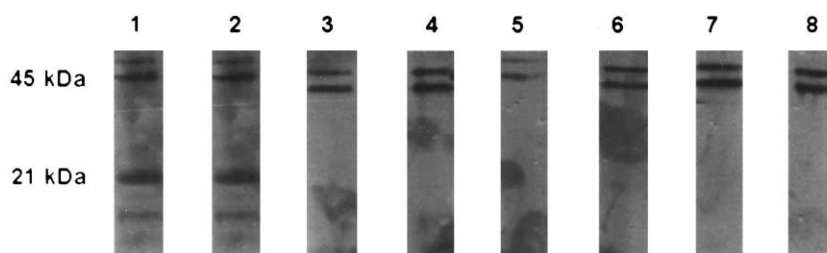


Fig. 4. Comparison of the reactivity of pools of sera drawn on day 90 from BALB/c mice administered either 10^9 cfu of SL3261(pBfpA) on days 0 and 30 (lanes 1 and 5), 10^9 cfu of SL3261(pBfpA) on day 0 only (lanes 2 and 6), 10^9 cfu of SL3261 on days 0 and 30 (lanes 3 and 7), or 1.5% sodium bicarbonate on days 0 and 30 (lanes 4 and 8), against nitrocellulose membrane strips containing extracts of SL3261(pBfpA) (lanes 1 to 4) or SL3261 (lanes 5 to 8) resolved by SDS-PAGE.

logarithmic growth, minimizing plasmid loss and selection of host strains that are unable to express the cloned antigen. Also, in such systems the expression of a heterologous protein can be maximized, thus providing vaccine inocula enriched in the immunogen. The densitometric analysis of SDS-PAGE protein bands of samples from our vaccine inocula showed that approximately 10% of the total SL3261(pBfpA) protein was comprised of rBfpA; it was the single most predominant polypeptide present in these bacterial extracts. We believe heat inducible constructs like pCYTEXP1 [28] are very promising for expression of immunogens in live vaccine strains for three major reasons: (1) they make scaling up cheaper and easier, thus obviating the use of expensive inducers; (2) all expression/regulation elements can be constructed into a small plasmid, thus not having to modify the host's chromosome; and (3) they offer the potential of in vivo expression at body temperature.

It is noteworthy that a *bfpA*-like gene has been detected in several *Salmonella* species and serotypes under low and medium stringency hybridization conditions, using *bfpA*-derived probes. In this study, the *aroA* *S. typhimurium* strain used as our delivery vehicle was reported to be *bfpA*-positive in one such study [22]. Nevertheless, at least with our protocol, only pBfpA transformed SL3261 displayed a protein cross-reactive with the anti-BFP antiserum (Fig. 2(b)), indicating that the gene detected using the *bfpA* probe either was not expressed in our experimental conditions or its product does not immunologically cross-react with EPEC BfpA. This last point was further reinforced by the observation that pre-adsorption of mice sera with SL3261 extracts did not affect the reactivity of the anti-BfpA antibody in samples taken from mice administered SL3261(pBfpA) (Figs. 3 and 4). With respect to the finding that rBfpA was not processed by SL3261(pBfpA), it was not surprising since it is in accordance with previous reports showing that other genes in the EAF plasmid are necessary for the proper biosynthesis of the native BFP [29, 30].

The *aroA* *S. typhimurium* we used tolerated well the large rBfpA load, corresponding to approximately

10% of its protein mass, as well as the prolonged heat stress to which it was subjected. Furthermore, the modifications imposed by *bfpA* expression and heat stress did not modify the characteristic attenuation of this particular *S. typhimurium* strain, as indicated by absence of deaths or disease in any of the vaccinated animals.

Over 200 000 Brazilian infants die of diarrhea every year. We have successfully expressed the pilin of the bundle-forming pilus of a leading bacterial pathogen in Brazil in a live vaccine strain. Such vaccine strains have been successfully used previously as live vehicles for oral vaccination in laboratory animals and in the field, and may ultimately serve as vehicles for combination vaccines, taking advantage of their polyvalency and ease of administration. Despite the fact that an attenuated *S. typhi* strain (Ty21a) is already approved for human use, we chose an auxotrophic *S. typhimurium* strain as the vehicle in the current study for two reasons: (1) it has a well established animal model that might be used for immunogenicity evaluation; and (2) it has been reported as the second major bacterial cause of diarrhea among Brazilian infants [5]. However, we realize that typhoid fever is also a major public health problem in our country, making Ty21a a good candidate as another vaccine vehicle for BfpA and/or other EPEC immunogens.

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