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Highly Attenuated *Bordetella pertussis* Strain BPZE1 as a Potential Live Vehicle for Delivery of Heterologous Vaccine Candidates▽

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***Bordetella pertussis*, the causative agent of whooping cough, is a promising and attractive candidate for vaccine delivery via the nasal route, provided that suitable attenuation of this pathogen has been obtained. Recently, the highly attenuated *B. pertussis* BPZE1 strain has been described as a potential live pertussis vaccine for humans. We investigated here the use of BPZE1 as a live vehicle for heterologous vaccine candidates. Previous studies have reported the filamentous hemagglutinin (FHA), a major *B. pertussis* adhesin, as a carrier to express foreign antigens in *B. pertussis*. In this study, we also examined the BrkA autotransporter as a surface display system. Three copies of the neutralizing peptide SP70 from enterovirus 71 (EV71) were fused to FHA or in the passenger domain of BrkA, and each chimera was expressed in BPZE1. The FHA-$(SP70)_3$ and BrkA-$(SP70)_3$ chimeras were successfully secreted and exposed at the bacterial surface, respectively. Nasal administration of the live recombinant strains triggered a strong and sustained systemic anti-SP70 antibody response in mice, although the titers and neutralizing activities against EV71 were significantly higher in the sera of mice immunized with the BrkA-$(SP70)_3$-producing strain. These data indicate that the highly attenuated BPZE1 strain is a potential candidate for vaccine delivery via the nasal route with the BrkA autotransporter as an alternative to FHA for the presentation of the heterologous vaccine antigens.**

Live recombinant bacteria adapted to the respiratory tract appear to be attractive and promising vehicles for the presentation of vaccine antigens to the respiratory mucosa. *Bordetella pertussis*, the etiological agent of whooping cough, colonizes the human respiratory tract very efficiently and induces strong and protective local and systemic immune responses after a single nasal administration (39, 40, 49), with induction of immunity even at distant mucosal sites, such as the urogenital tract (41).

Consequently, *B. pertussis* has been successfully used as a live bacterial vector for the presentation of foreign antigens to the respiratory mucosa in mouse models (33, 38). However, suitable attenuation is mandatory in order to use *B. pertussis* as a live recombinant vector of vaccination. Recently, the highly attenuated *B. pertussis* BPZE1 strain was described (40). Mielcarek et al. reported markedly reduced lung inflammation in mice nasally infected with BPZE1, while the ability to colonize and induce protective immunity against pertussis infection was maintained. Furthermore, BPZE1 was found to induce protection in infant mice that was superior to the protection provided by the current acellular pertussis vaccines. These features make the *B. pertussis* BPZE1 strain an attractive live pertussis vaccine candidate and also a potential vehicle for vaccine delivery via the nasal route. The expression of heterologous antigens in BPZE1 and the ability of this strain to induce specific immune responses upon nasal administration of live recombinant bacteria have not been described previously.

Several heterologous antigens have been produced in recombinant *B. pertussis*, including the *Schistosoma mansoni* 28-kDa glutathione *S*-transferase (42), fragment C of tetanus toxin (50), transferrin-binding protein B (TbpB) from *Neisseiria meningitidis* (15), and HtrA from *Haemophilus influenzae* (3). These antigens have been fused to the filamentous hemagglutinin (FHA), a major adhesin of *B. pertussis* (34). FHA is a 220-kDa monomeric protein that is both surface exposed and secreted into the extracellular milieu (16, 29). It is highly immunogenic (2, 8, 52) and displays adjuvant properties (47), prompting its use as a carrier to present heterologous antigens to the respiratory mucosa. However, efficient secretion of FHA chimeras across the outer membrane requires a totally unfolded conformation of the passenger (24, 50), which limits the use of FHA as a carrier.

Autotransporters have been successfully used in *Salmonella* and *Escherichia coli* to present heterologous antigens at the bacterial surface (31, 32, 62), and they are able to translocate folded protein domains across the outer membrane (58). Autotransporters are large, secreted, often virulence-associated proteins of gram-negative bacteria (25). They display a characteristic domain structure that includes (i) a signal peptide at the N terminus; (i) a passenger domain, which encodes the functional part of the protein, and (iii) a C-terminal translocation unit, which is conserved in the autotransporter family. The latter domain consists of a beta barrel that is embedded in the outer membrane and through which the passenger domain is translocated to the cell surface (26). Most autotransporters

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TABLE 1. *B. pertussis* strains used in this study

Strain	Relevant feature(s)	Reference
BPZE1	Attenuated Sm^r BPSM derivative lacking the *dnt* gene and producing inactive pertussis toxin and reduced tracheal cytotoxin	40
BPSQ5	BPZE1 derivative producing BrkA-$(SP70)_3$	This study
BPSY13.1	BPZE1 derivative producing FHA-$(SP70)_3$	This study
BPSY1	*brkA* knockout BPZE1 derivative	This study

are proteolytically processed, releasing an α-domain which comprises most of the passenger domain.

The *B. pertussis* BrkA autotransporter confers serum resistance by inhibiting the classical pathway of complement activation (6, 20) and plays a role in *B. pertussis* adhesion to and invasion of the host cells (19, 20). It is expressed as a 103-kDa precursor and is processed during secretion, which yields a 73-kDa N-terminal passenger domain and a 30-kDa C-terminal translocation unit (53). Following translocation, the cleaved passenger domain remains tightly associated with the bacterial surface (44). A truncated version of BrkA with a large deletion within its passenger domain has been reported and shown to be efficiently translocated across the outer membrane (45). We therefore hypothesized that this domain may be permissive for replacement at least in part by heterologous antigens.

Here, we report the expression of the neutralizing SP70 peptide from enterovirus 71 (EV71) in the highly attenuated *B. pertussis* BPZE1 strain using FHA or BrkA as a carrier. EV71 is a major causative agent of hand, foot, and mouth disease and has a propensity to cause severe neurological complications leading to significant morbidity and mortality in infants and children (36, 46). Since 1997, several outbreaks of EV71 infection have been reported in East and Southeast Asia, including Singapore and Japan, and its epidemic activity has been on the rise in the Asia-Pacific region (10, 12, 27). Several reports have indicated that the EV71 VP1 capsid protein is protective in animal models (13, 14, 55, 59) and is highly immunogenic in humans (57). We have recently shown that the SP70 peptide, spanning amino acids 208 to 222 of VP1, contains a neutralizing (23) and protective (22) B-cell epitope and is highly conserved among the EV71 subgenogroups.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. BPSY13.1, BPSY1, and BPSQ5 were derived from *B. pertussis* BPZE1, a streptomycin-resistant Tohama I derivative producing inactivated pertussis toxin, no dermonecrotic toxin, and background levels of tracheal cytotoxin (40). All *B. pertussis* strains were grown at 37°C for 72 h on Bordet-Gengou (BG) agar (Difco, Detroit, MI) supplemented with 1% glycerol, 10% defibrinated sheep blood, and 100 μg/ml streptomycin (Sigma Chemical Co., St Louis, MO). Liquid cultures were grown as described previously (37) in Stainer-Scholte medium containing 1 g/liter heptakis(2,6-di-*o*-methyl)-β-cyclodextrin (Sigma).

All DNA manipulations were carried out with chemically competent *Escherichia coli* One-Shot TOP10 (Invitrogen). The bacteria were grown at 37°C overnight on Luria-Bertani agar or in Luria-Bertani broth with vigorous shaking. When appropriate, 100 μg/ml ampicillin, 50 μg/ml ampicillin, or 10 μg/ml gentamicin was added to select for antibiotic-resistant strains.

Virus growth and purification. EV71 strain 5865/SIN/00009 (GenBank accession no. AF316321) was propagated in rhabdomyosarcoma (RD) cells using minimum essential medium (Gibco, United States) supplemented with 5% fetal calf serum, 1% sodium pyruvate, and 1.5% sodium bicarbonate. The virus particles were purified as described previously (23). Briefly, infected cells were lysed by subjecting them to freeze-thaw cycles. The virus particles were precipitated in 7% polyethylene glycol 8000 by centrifugation on a 30% sucrose cushion at 25,000 × *g* for 4 h. The virus titer was expressed as the 50% tissue culture infective dose with RD cells based on typical cytopathic effects (CPE) produced by viral infection. Before injection into mice, the virus suspension was heat inactivated at 56°C for 30 min. The amount of virion protein was quantified by the Bradford method (Bio-Rad Laboratories, United States).

Oligonucleotides, peptides, and antibodies. To circumvent any problems in protein translation due to poor codon usage (28), the original *sp70* DNA sequence was optimized to *B. pertussis* codon usage preference. To generate the FHA-$(SP70)_3$ construct, the upper and lower DNA strands of optimized *sp70* (5′-GATCGGCTACCCGACCTTCGGCGAGCACAAGCAGGAGAAGGACCTGGAGTACGA-3′ and 5′-GATCTCGTACTCCAGGTCCTTCTCCTGCTTGTGCTCGCCGAAGGTCGGGTAGCC-3′) were chemically synthesized and annealed, generating cohesive BglII-compatible ends. To generate the BrkA-$(SP70)_3$ construct, the upper and lower DNA strands of optimized *sp70* (5′-GATCTGTACCCGACCTTCGGCGAGCACAAGCAGGAGAAGGACCTGGAGTACTG-3′ and 5′-GATCCAGTACTCCAGGTCCTTCTCCTGCTTGTGCTCGCCGAAGGTCGGGTACA-3′) were chemically synthesized and annealed, generating cohesive BamHI-compatible ends.

Unconjugated SP70 peptide (22) was chemically synthesized at Mimotopes Pty. Ltd. (Clayton Victoria, Australia).

Rabbit anti-BrkA polyclonal antibodies were a kind gift from Rachel Fernandez (University of British Columbia, Canada).

Construction of recombinant *B. pertussis* strains. To construct the recombinant *B. pertussis* BPSY13.1 strain producing the FHA-$(SP70)_3$ chimera, a 1,620-bp HindIII PCR fragment was amplified from the BPZE1 chromosomal DNA using primers 5′-TTAAGCTTGCGAACGCGCTGCTGTGGG-3′ and 5′-TTAAGCTTCGCATCGGCGCTGCCCAGC-3′ (HindIII sites are underlined) and cloned into HindIII-opened plasmid pBR322 (7), yielding pBRSY0. The PCR fragment corresponded to nucleotides (nt) 5221 to 6840 of the *fhaB* open reading frame (ORF) and contained its unique BglII site. Three copies of the *sp70* DNA sequence were inserted in tandem and sequentially into the unique BglII site of pBRSY0. Insertion of one copy of *sp70* DNA into BglII-digested pBRSY0 restored a BglII site only at the 3′ end of the *sp70* DNA fragment, allowing insertion of a second *sp70* copy and then a third *sp70* copy, finally yielding pBRSY3. The 1,755-bp HindIII fragment from pBRSY3 was then cloned into HindIII-opened suicide plasmid pJQmp200rpsL18 (48), yielding pJQSY3. BPZE1 was electroporated with pJQSY3, allowing the *fha*-$(sp70)_3$ construct to integrate into the chromosomal DNA by allelic exchange (56) at the *fhaB* locus.

To construct the recombinant *B. pertussis* BPSQ5 strain, which express the BrkA-$(SP70)_3$ chimera, a 1-kb SalI-BamHI PCR fragment and a 945-bp BamHI-HindIII PCR fragment were cloned into pUC19 (60) using the corresponding restriction sites, yielding pUCSY2. Both PCR fragments were amplified from BPZE1 chromosomal DNA. The 1-kb SalI-BamHI PCR fragment was amplified using primers 5′-TTGTCGACGTAGTATCCCTTGGCCGCGC-3′ and 5′-TTGGATCCTGCGCATGCGGCGCGCC-3′ (SalI and BamHI sites are underlined) and encompassed the 5′ end of the *brkA* ORF (nt 1 to 151), its promoter region, and the first 529 nt of the adjacent *brkB* ORF, which is transcribed in the opposite direction. A 945-bp BamHI-HindIII PCR fragment was obtained using primers 5′-TTGGATCCACGCCGGCCAGGACGGCAA-3′ and 5′-TTAAGCTTCACGACCCAGGTTCCGCCC-3′ (BamHI and HindIII sites are underlined) and corresponded to nt 789 to 1735 of the *brkA* ORF. Three copies of the *sp70* gene fragment were then inserted in tandem and sequentially into BamHI-digested pUCSY2. Insertion of one copy of *sp70* DNA into BamHI-digested pUCSY2 restored a BamHI site only at the 3′ end of the *sp70* DNA fragment, which allowed insertion of a second copy and a third copy of *sp70*, finally yielding pUCSQ2. The 2,125-bp HindIII fragment from pUCSQ2 was finally cloned into HindIII-opened pJQmp200rpsL18, yielding pJQSQ1. BPZE1 was electroporated with pJQSQ1, which allowed the *brkA*-$(sp70)_3$ construct to integrate into the chromosomal DNA by allelic exchange at the *brkA* locus.

To construct the *brkA* knockout strain BPSY1, the 1,963-bp HindIII fragment from pUCSY2, which contained both the 1-kb SalI-BamHI and 945-bp BamHI-HindIII PCR fragments described above, was inserted into HindIII-opened pJQmp200rpsL18, yielding pJQSY1. BPZE1 was then electroporated with

pJQSY1, which allowed allelic exchange at the *brkA* chromosomal locus and resulted in deletion of nt 151 to 789 in the *brkA* ORF. BPSY1 therefore produced a truncated BrkA protein consisting of 103 amino acids.

Whole-cell extract preparation and supernatant concentration. Ten milliliters of mid- to late-exponential-phase bacteria in SSAB medium was centrifuged at 7,000 rpm for 15 min at room temperature. The supernatant was concentrated 10-fold using a 30-kDa-cutoff Ultra-4 centrifugal filter device (Amicon) according to the manufacturer's protocol. The bacterial pellet was resuspended in 500 μl of ultrapure water. An equal volume of 2× loading buffer was added before the preparation was heated at 95°C for 10 min. The chromosomal DNA was sheared by passing the suspension 10 times through a 27-gauge needle; this was followed by heating at 95°C for 15 min before 30 μl was loaded onto a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel for Western blot analysis.

Immunodetection of FHA-$(SP70)_3$ and BrkA-$(SP70)_3$ chimeras. Concentrated (10×) culture supernatants or whole-cell extracts of the *B. pertussis* strains were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 8 or 12% polyacrylamide gels. The proteins were electrotransferred onto nitrocellulose membranes and incubated with mouse anti-SP70 polyclonal antibodies (23) diluted 1:100, mouse anti-FHA monoclonal antibodies diluted 1:250 (49), or rabbit anti-BrkA polyclonal antibodies diluted 1:30,000 (45) in Tris-buffered saline containing 0.1% Tween 20 and 2% bovine serum albumin. Alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit immunoglobulin G (IgG) secondary antibodies (Sigma), both diluted 1:3,000, were used for chromogenic detection of the proteins after addition of the alkaline phosphatase substrate (nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate reagents; Sigma).

FACS. *B. pertussis* strains grown on BG agar were washed three times with sterile phosphate-buffered saline (PBS) supplemented with 5% glycerol. Fluorescence-activated cell sorting (FACS) was then conducted with the intact *B. pertussis* cells using a Coulter Epics machine (Beckman Coulter, Palo Alto, CA). Intact bacteria were incubated with rabbit anti-BrkA polyclonal antibodies (45) diluted 1:200 and then with Cy2-conjugated goat anti-rabbit IgG (Jackson Laboratories, West Grove, PA) diluted 1:100. Samples were analyzed with laser excitation at 488 nm, and the data were acquired using the EXPO version 2.0 software (Applied Cytometry Systems, Sheffield, United Kingdom) and analyzed with the WinMDI-2.8 software. The assay was performed two times independently.

Immunofluorescence. Intact *B. pertussis* cells were prepared as described above for FACS and spotted onto glass slides pretreated with 100 μl of 0.1% poly-L-lysine. The samples were examined with blue light excitation (488 nm) using an epifluorescence microscope (BX40; Olympus, Japan) at a magnification of ×1,000.

i.n. infection. The mice were kept under specific-pathogen-free conditions in individual ventilated cages, and all the experiments were carried out using the guidelines of the National University of Singapore animal study board. For colonization studies, 9-week-old outbred CD1 mice (Biopolis Research Center, Singapore) were each infected intranasally (i.n.) with 5×10^6 CFU of the different *B. pertussis* strains in 20 μl as described previously (1). At the indicated time points, four mice per group were sacrificed, and their lungs were aseptically removed and homogenized in PBS. Serial dilutions from individual lung homogenates were plated onto BG agar, and the total numbers of CFU per lung were determined after 4 to 5 days of incubation at 37°C. For immunization studies, groups of six 5-week-old BALB/c mice (Biopolis Research Center, Singapore) were infected i.n. twice at a 4-week interval with 5×10^6 CFU of the different *B. pertussis* strains in 20 μl. An additional group of six mice was inoculated intraperitoneally (i.p.) twice at a 4-week interval with 10 μg of heat-inactivated EV71 in a 50% emulsion of complete and incomplete Freund's adjuvant. At the indicated time points, the mice were bled at the retroorbital sinus.

Antibody detection. The levels of antibodies to SP70 and *B. pertussis* were measured by an enzyme-linked immunosorbent assay (ELISA). The 96-well microtiter plates (COSTAR; Corning) were coated overnight at 4°C with 50 μl of 0.1 M carbonate buffer (pH 9.6) containing 10 μg/ml of unconjugated SP70 peptide or total *B. pertussis* BPZE1 cell lysate. After blocking with 2% bovine serum albumin in PBS containing 0.1% Tween 20, 50 μl of serum diluted 1:50 (for anti-SP70 detection) or 1:800 (for anti-*B. pertussis* detection) was added to the wells. The plates were incubated at 37°C for 1 h, rinsed in PBS-0.1% Tween 20, and incubated at 37°C for 1 h with 50 μl of horseradish peroxidase-conjugated goat anti-mouse IgG(H+L) secondary antibodies (Sigma) at a 1:3,000 dilution. To detect the various IgG subtypes, horseradish peroxidase-conjugated goat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 secondary antibodies (Jackson Laboratories) were used at a 1:5,000 dilution. The reaction was then developed using *o*-phenylenediamine dihydrochloride substrate (Sigma) at room temperature for 30 min in the dark and stopped by addition of 1 M sulfuric acid. The absorbance at 490 nm was determined with an ELISA plate reader (Tecan Sunrise, United States).

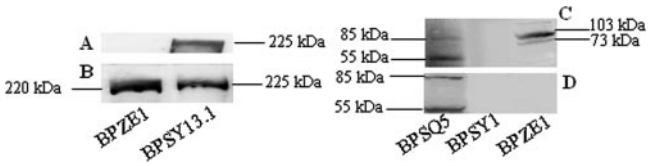

FIG. 1. Detection of FHA-$(SP70)_3$ and BrkA-$(SP70)_3$ chimeras by immunoblotting. Tenfold-concentrated culture supernatants from BPZE1 and BPSY13.1 cultures (A and B) or whole-cell extracts from BPZE1, BPSQ5, and BPSY1 cultures (C and D) were assayed by immunoblotting using anti-SP70 polyclonal antibodies (B and D), anti-FHA monoclonal antibodies (A), and anti-BrkA polyclonal antibodies (C). Fifty microliters of supernatant or 10 μl of cell extract was loaded.

EV71 neutralization assay. The presence of neutralizing antibodies against EV71 was determined by an in vitro microneutralization assay using RD cells, as described previously (23). Mouse serum samples were first incubated at 56°C for 30 min to inactivate complement activity. Briefly, 25 μl of twofold serial dilutions of heat-treated serum was coincubated with equal volumes containing 10^3 50% tissue culture infective doses of virus in a 96-well microtiter plate. Two hours later, 5×10^4 RD cells were added to each well and incubated at 37°C for 48 h. The cells were examined for CPE, and the neutralizing antibody titer was defined as the highest dilution of serum that inhibited virus growth by 100%, thereby preventing CPE. The assay was performed three times independently.

Statistical analysis. The results were analyzed using the unpaired Student *t* test. Differences were considered significant if the *P* value was <0.05.

RESULTS

Production of FHA-$(SP70)_3$ and BrkA-$(SP70)_3$ chimeras by *B. pertussis*. Up to 85% of the passenger domain of BrkA can be deleted without affecting the efficacy of translocation of the protein across the outer membrane (45). However, the passenger domain of BrkA has recently been found to possess adjuvant properties and immunogenic activities (9), which may be important when BrkA is used as a carrier for the display of vaccine candidates. We therefore decided to truncate the BrkA protein from amino acid A52 to H263, corresponding to a deletion of 32% of the passenger domain. Three copies of the 15-amino-acid SP70 neutralizing peptide from EV71 were then fused in tandem in the truncated passenger domain of BrkA. Three copies of SP70 were also inserted in tandem into full-length FHA. The chimeric proteins were designated BrkA-$(SP70)_3$ and FHA-$(SP70)_3$, respectively. The corresponding DNA constructs were introduced by allelic exchange into the *brkA* and *fhaB* chromosomal loci, respectively, of attenuated *B. pertussis* BPZE1, resulting in strains BPSQ5 and BPSY13.1, respectively.

The production of the FHA-$(SP70)_3$ and BrkA-$(SP70)_3$ chimeras by the recombinant strains was analyzed by immunoblotting using anti-SP70 and anti-FHA or anti-BrkA antibodies. A 225-kDa band corresponding to the predicted size of FHA-$(SP70)_3$ was detected in the culture supernatant of BPSY13.1 using anti-FHA and anti-SP70 antibodies (Fig. 1A and B, respectively). Two bands at 103 and 73 kDa, corresponding to full-length wild-type BrkA and its passenger domain, respectively, were detected in the whole-cell extract of BPZE1 using anti-BrkA antibodies (Fig. 1C). Similarly, two bands at 85 and 55 kDa, corresponding to the predicted sizes of full-length BrkA-$(SP70)_3$ and its passenger domain, respectively, were detected in the whole-cell extract of BPSQ5 using

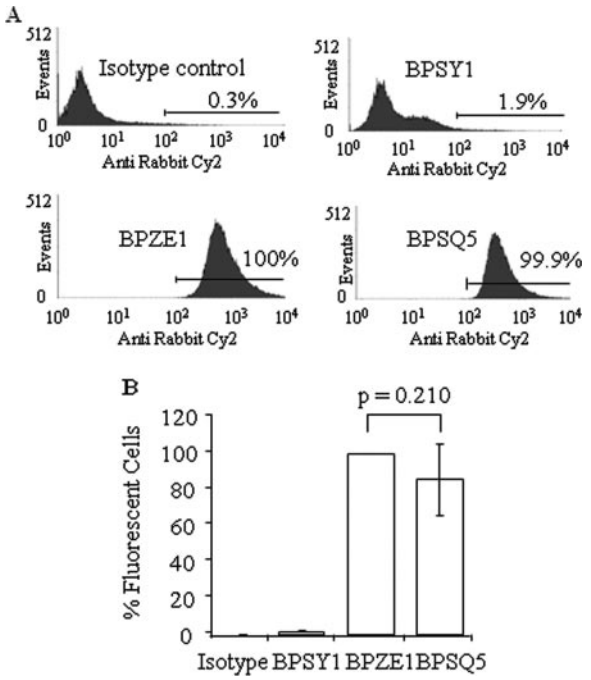

FIG. 2. Detection of the BrkA-$(SP70)_3$ chimera by FACS. Anti-BrkA polyclonal antibodies were coincubated with intact BPSY1, BPZE1, and BPSQ5 cells as indicated. The isotype control was BPZE1 bacteria stained with Cy2-conjugated secondary antibody. (A) Graphs representative of two independent experiments. (B) Average values for the two independent experiments. The results are expressed as means ± standard deviations.

anti-BrkA and anti-SP70 antibodies (Fig. 1C and D, respectively). No bands were detected in the *brkA* knockout BPSY1 strain using either antibody (Fig. 1C and D). FHA and BrkA were not detected by anti-SP70 antibodies (Fig. 1B and D, respectively).

These data demonstrate that FHA-$(SP70)_3$ and BrkA-$(SP70)_3$ were successfully produced by BPSY13.1 and BPSQ5, respectively. Moreover, they indicate that FHA-$(SP70)_3$ was efficiently secreted by BPSY13.1 into the extracellular milieu. In contrast and as expected, BrkA-$(SP70)_3$ was not secreted by BPSQ5 at an appreciable level (data not shown).

Cell surface exposure of the BrkA-$(SP70)_3$ chimera. To assess whether BrkA-$(SP70)_3$ was exposed at the bacterial surface of BPSQ5, FACS was performed with intact (nonpermeabilized) BPSQ5 cells using anti-BrkA antibodies. The parental BPZE1 and *brkA* knockout BPSY1 strains were used as positive and negative controls, respectively. As shown in Fig. 2, 100% of the parental BPZE1 cells exhibited surface exposure of BrkA. Similarly, the majority of BPSQ5 cells were found to be positive, and the difference from the parental strain was not statistically significant ($P = 0.21$) (Fig. 2B). As expected, the BPSY1 strain did not display any significant fluorescence.

The surface exposure of BrkA-$(SP70)_3$ was further confirmed by immunofluorescence analysis using anti-BrkA antibodies on intact BPSQ5, BPZE1, and BPSY1 cells. As shown in Fig. 3, BPZE1 and BPSQ5 cells displayed strong and comparable fluorescence signals (Fig. 3G and H, respectively), while no significant fluorescence emission was detected with the BPSY1 strain (Fig. 3F).

Due to a high background value, the anti-SP70 immune serum could not be used as the primary antibody in FACS and immunofluorescence studies to confirm the data obtained with the anti-BrkA immune serum (data not shown).

These results demonstrate that BrkA-$(SP70)_3$ is exposed at the bacterial surface of BPSQ5 at levels comparable to the levels of the wild-type BrkA protein in BPZE1.

Lung colonization by *B. pertussis* BPSY13.1 and BPSQ5. FHA and BrkA have been shown to play a role in the colonization efficiency of *B. pertussis* (1, 18). To study whether the recombinant BPSQ5 and BPSY13.1 strains retained the capacity to colonize the murine respiratory tract, mice were infected i.n. with either strain, and the colonization profiles were compared to those of the parental BPZE1 and *brkA* knockout BPSY1 strains.

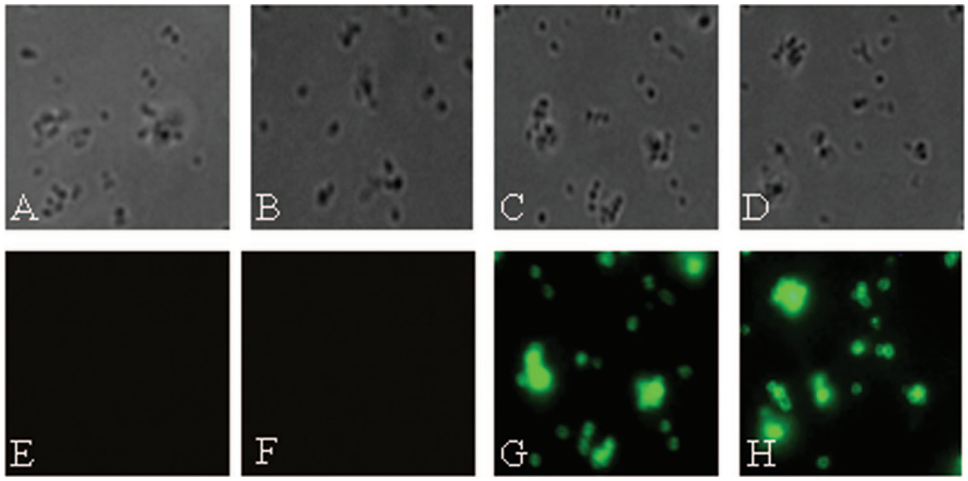

FIG. 3. Detection of the BrkA-$(SP70)_3$ chimera by immunofluorescence microscopy. Anti-BrkA polyclonal antibodies were coincubated with intact BPSY1 (B and F), BPZE1 (C and G), or BPSQ5 (D and H) cells. The isotype control (A and E) was BPZE1 bacteria stained with Cy2-conjugated secondary antibody. Panels A to D show corresponding phase-contrast images.

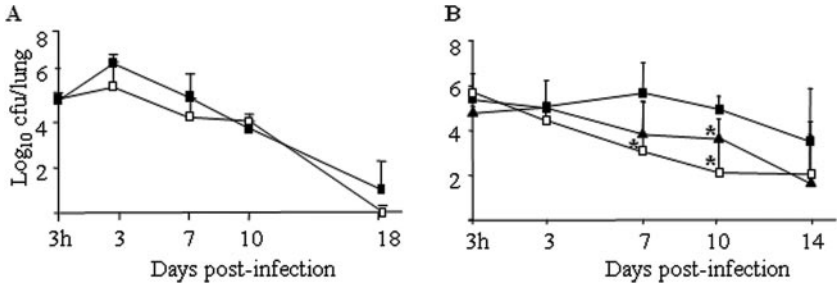

FIG. 4. Lung colonization by the recombinant *B. pertussis* strains. (A) CD1 mice were infected i.n. with 5×10^6 CFU of *B. pertussis* BPZE1 (solid squares in panels A and B), BPSY13.1 (open squares in panel A), BPSY1 (solid triangles in panel B), or BPSQ5 (open squares in panel B). The lungs of infected mice were harvested at the indicated time points, and appropriate dilutions of the lung homogenates were plated to determine the total number of CFU per lung. Four mice per group and per time point were assessed individually. Asterisk, $P < 0.05$ compared to BPZE1.

BPSY13.1 colonized the lungs as efficiently as the parental BPZE1 strain; a peak of multiplication was observed, followed by progressive clearance of the bacteria from the lungs (Fig. 4A), indicating that the insertion of three copies of SP70 into full-length FHA did not impair the adhesion function of the protein. However, similar to the colonization efficiency of the *brkA* knockout strain BPSY1, the colonization efficiency of BPSQ5 was found to be slightly but significantly ($P < 0.05$) reduced 7 and 10 days postinfection compared to that of BPZE1 (Fig. 4B). This observation suggests that the BrkA-$(SP70)_3$ chimera did not retain the full adhesion function of the wild-type BrkA protein and/or that other functions of BrkA, such as resistance to serum killing (20) and to antimicrobial peptides (21), were impaired in the BrkA-$(SP70)_3$ chimera, which might account for the reduced colonization ability observed with BPSQ5.

Systemic anti-SP70 and anti-*B. pertussis* antibody responses in mice. To examine the abilities of the two recombinant *B. pertussis* strains to trigger a systemic anti-SP70 antibody response upon nasal administration, groups of six BALB/c mice were infected i.n. twice at a 4-week interval with BPSY13.1, BPSQ5, or BPZE1. As a reference for anti-SP70 antibody production, an additional group of mice was inoculated i.p. with heat-inactivated EV71 using the same immunization schedule. The systemic anti-SP70 and anti-*B. pertussis* IgG responses were measured by ELISA 2 weeks after the boost.

Both BPSY13.1- and BPSQ5-infected mice developed a strong systemic anti-*B. pertussis* antibody response comparable to the response observed in the BPZE1-infected mice (Fig. 5A). As expected, no anti-*B. pertussis* antibody response was seen in the EV71-inoculated animals. However, the EV71-inoculated mice all showed high anti-SP70 antibody responses, while the naïve and BPZE1-infected mice displayed only background absorbance (Fig. 5B). Two of six BPSY13.1-infected mice (mice M5 and M6) produced significant anti-SP70 IgG antibody levels. In contrast, five of six BPSQ5-immunized mice produced significant anti-SP70 antibody levels, and the titers were significantly higher than the titers obtained for the BPSY13.1-immunized group ($P < 0.05$). However, the antibody titers measured for both groups of mice were found to be significantly lower than the titers measured for the EV71-inoculated group.

An anti-SP70 IgG subtype analysis was carried out for the immune sera from all the mouse groups and showed that there was production of significant levels of IgG2a/IgG2b antibodies in the BPSQ5- and BPSY13.1-immunized mice, indicative of a Th1-oriented immune response (Table 2).

The anti-*B. pertussis* and anti-SP70 antibody responses were monitored over a period of 8 weeks after the boost in the BPSY13.1- and BPSQ5-immunized groups and were found to be as high as the titers measured 2 weeks after the boost (data not shown), demonstrating that the antibody responses trig-

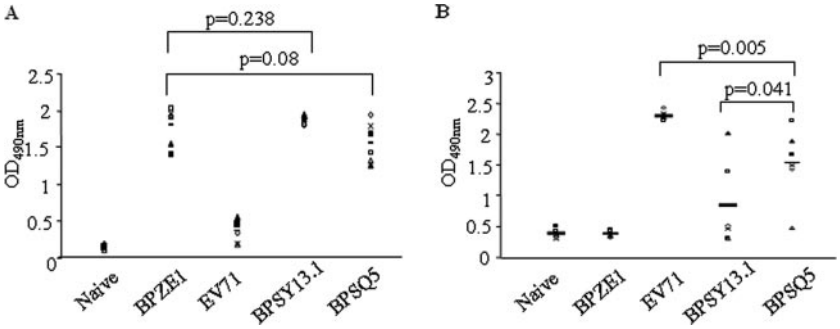

FIG. 5. Detection of specific antibody responses. Groups of six mice were infected i.n. twice at a 4-week interval with 5×10^6 CFU of BPZE1, BPSY13.1, or BPSQ5. The EV71 group was inoculated i.p. with 10 μg of inactivated virus using the same immunization schedule. The mice were bled 2 weeks after the boost, and the anti-*B. pertussis* (A) and anti-SP70 (B) IgG(H+L) titers were determined by ELISA with the individual sera diluted 1/800 and 1/50, respectively, using *B. pertussis* whole-cell lysate and SP70 peptide as coating antigens, respectively. ◇, mouse M1; ■, mouse M2; △, mouse M3; ×, mouse M4; ▲, mouse M5; □, mouse M6. The average is indicated by a horizontal line. OD_{490nm}, optical density at 490 nm.

TABLE 2. Isotype profiles of the immune sera

Immunogen	Mouse serum	Titer[a]			
		IgG1	IgG2a	IgG2b	IgG3
Naïve	Pooled	0.12	0.08	0.11	0.11
BPZE1	Pooled	0.10	0.10	0.12	0.11
EV71	Pooled	2.36	2.23	2.36	2.33
BPSQ5	M1	0.18	0.80	1.62	0.11
	M2	0.11	1.57	2.27	0.11
	M3	0.10	0.13	0.22	0.10
	M4	0.09	1.52	1.21	0.09
	M5	0.10	0.65	2.54	0.12
	M6	1.42	2.17	2.34	0.13
BPSY13.1	M1	0.22	0.25	0.78	0.07
	M2	0.08	0.08	0.08	0.09
	M3	0.09	0.07	0.07	0.07
	M4	0.27	0.71	0.08	0.08
	M5	2.16	1.42	1.33	0.17
	M6	0.39	2.00	2.03	0.19

[a] The anti-SP70 IgG1, IgG2a, IgG2b, and IgG3 titers were determined by ELISA using the sera diluted 1/50 and using SP70 peptide as the coating antigen. Sera from individual mice belonging to the naïve, BPZE1, and EV71 groups were pooled, while serum from each BPSY13.1- and BPSQ5-immunized mouse was tested individually.

TABLE 3. Neutralizing activities of the immune sera[a]

Immunogen	Mouse serum	Serum dilution[b]
Naïve	Pooled	NP
BPZE1	Pooled	NP
EV71	Pooled	1/128
BPSQ5	M1	1/32
	M2	1/2
	M3	NP
	M4	1/16
	M5	1/32
	M6	1/32
BPSY13.1	M1	NP
	M2	NP
	M3	NP
	M4	NP
	M5	1/16
	M6	NP

[a] Twofold serial dilutions of the sera from each group were incubated with EV71 before infection of RD cells, and the CPE were observed 48 h later. Sera from individual mice belonging to the naïve, BPZE1, and EV71 groups were pooled, while serum from each BPSY13.1- and BPSQ5-immunized mouse was tested individually.

[b] Highest dilution with which total protection was observed. NP, no protection.

gered by nasal administration of the recombinant strains was sustained.

Neutralizing activity of the immune sera against EV71. To evaluate the functional activities of the sera from the BPSY13.1- and BPSQ5-immunized mice, an in vitro EV71 neutralization assay was used. Serially diluted sera were coincubated with EV71 before infection of RD cells. CPE were determined 48 h later. The sera from the naïve, BPZE1-infected, and EV71-inoculated mice were pooled within groups, while the sera from the BPSY13.1- and BPSQ5-infected mice were analyzed individually.

In contrast to uninfected cells, which had a flattened and spindle-like shape, infected cells appeared to be rounded and swollen with microbodies, as described elsewhere (23; data not shown). As expected, the sera from the naïve and BPZE1-infected mice failed to protect RD cells from viral infection (Table 3). In contrast, the pooled serum from the EV71-inoculated mice provided complete protection to the cells up to a serum dilution of 1:128. For the six BPSY13.1-infected mice, only the serum from mouse M5, corresponding to the highest anti-SP70 IgG titer, displayed significant neutralizing activity against the virus. This serum conferred complete protection to the cells up to a dilution of 1:16. The sera from the five BPSQ5-infected mice, which were found to produce significant anti-SP70 antibodies, showed the ability to neutralize the virus, and complete protection was obtained with serum dilutions ranging from 1:2 to 1:32. Surprisingly, the highest neutralization titer did not correspond to the highest anti-SP70 antibody titer. For example, the sera from mice M1 and M5, which contained significantly different anti-SP70 antibody levels (Fig. 5B), were found to be equally able to neutralize EV71 in vitro.

These results show that nasal administration of BPSQ5 and, to a lesser extent, nasal administration of BPSY13.1 are able to trigger the production of systemic antibodies capable of neutralizing EV71 infection in vitro.

DISCUSSION

Despite high vaccination coverage, *B. pertussis* remains endemic in many areas, and reports of an increasing incidence of infection worldwide have been accumulating for the past 20 years (5, 11, 17). The resurgence of pertussis is believed to be due to waning vaccine-induced immunity in adults and to antigenic shift and adaptation of the circulating *B. pertussis* strains to the current acellular pertussis vaccines (43). Natural infection with *B. pertussis* has long been known to induce strong and long-lasting immunity that wanes later than vaccine-induced immunity (4). Furthermore, natural infection with *B. pertussis* induces measurable antigen-specific Th1 immune responses even in very young children (as young as 1 month of age) (35). However, the neonatal immune system is too immature for effective development of protective immunity upon administration of acellular vaccines (54). These observations suggest that live vaccines that can be administered by the nasal route in order to mimic as closely as possible natural infection may be attractive alternatives to the currently available subunit vaccines. Such a strategy would allow early immunization, possibly at birth, thereby reducing the incidence of pertussis in the very young and most vulnerable age groups. The highly attenuated *B. pertussis* BPZE1 strain has been described recently as a promising live pertussis vaccine (40). In this study we investigated the use of BPZE1 as a live vehicle to deliver a heterologous peptide vaccine candidate via the nasal route.

The heterologous antigens which have been produced in *B. pertussis* so far were fused to either full-length or truncated FHA (3, 15, 42, 50). To be efficiently secreted, an FHA chi-

mera must be in an unfolded conformation, precluding the fusion of any foreign antigens with cysteine residues susceptible to formation of disulfide bonds (24, 50). To further develop *B. pertussis* as a vehicle for vaccine delivery, we explored the use of the autotransporter BrkA as a surface display system. The efficient translocation across the outer membrane of folded protein domains containing disulfide bonds has been demonstrated for autotransporters in other bacterial species (58). We report here that insertion in tandem of three copies of the 15-amino-acid SP70 peptide within the full-length FHA and within the truncated passenger domain of BrkA does not impair the secretion and surface exposure, respectively, of the chimeras.

The colonization efficiency of BPSQ5, which produces the BrkA-$(SP70)_3$ chimera, was found to be slightly reduced compared to that of BPZE1, and the profile was comparable to that of a *B. pertussis brkA* knockout mutant. A previous study using a *B. pertussis brkA* knockout mutant showed that BrkA is involved in colonization efficacy in mice (18). Our findings further suggest that the A52-H263 region of the passenger domain of BrkA likely plays a role in this process. A role of BrkA in direct adherence to and invasion of the host cells has also been described (19, 20). However, whether the A52-H263 region of the BrkA passenger domain is part of the adhesion domain of the protein is not known. Other functions of BrkA, such as resistance to serum killing (20) and to antimicrobial peptides (21), might be impaired in the BrkA-$(SP70)_3$ chimera, which might account for the reduced colonization ability observed with BPSQ5.

Both BPSQ5- and BPSY13.1-immunized mice developed a strong anti-*B. pertussis* antibody response, and the titers were comparable to those obtained for mice immunized with parental strain BPZE1, demonstrating that fusion of three copies of SP70 to either FHA or BrkA did not alter the immunogenicity of the bacterial vector. The systemic anti-SP70 IgG response measured in the BPSQ5-immunized mice was found to be significantly higher than the response measured in the BPSY13.1-immunized mice. Several factors might account for the differential ability to trigger an anti-SP70 antibody response using BrkA or FHA as the carrier. These include the yield of each chimera produced by the recombinant strains and the size ratio between the carrier and the three copies of SP70 peptide, as well as the intrinsic immunogenicity of the carrier. Indeed, FHA is known to be highly immunogenic (2, 8, 52). However, this feature, if too prominent, may be a handicap as it may skew the immune response towards the carrier at the expense of the passenger antigen. Moreover, the subcellular localization of the chimera might also play a role in the presentation of the heterologous antigen. A recent study showed that *B. pertussis* BrkA, as well as autotransporters from other rod-shaped gram-negative bacteria, is localized at the bacterial pole, which may have profound implications for the nature and efficiency of the pathogen-host interactions (30). Thus, concentration of the BrkA-$(SP70)_3$ molecules at one pole of the bacteria might allow more efficient presentation to and processing by the host's antigen-presenting cells.

The lack of a suitable animal model to examine vaccine efficacy is a major obstacle to the development of EV71 vaccines. Mice are susceptible to EV71 infection in the first 4 days of life and then become completely resistant by 6 days of age (61, 51). EV71 infection has been found to be asymptomatic in all strains of adult mice tested, including BALB/c, C3H, ICR, CD28 knockout, and tumor necrosis factor alpha receptor knockout mice (59). Therefore, the efficacy of an EV71 vaccine candidate cannot be evaluated with actively immunized mice but can be addressed only using passive immunization, in which newborn (1-day-old) mice are challenged with EV71 and subsequently inoculated with the immune serum from actively immunized adult mice, as previously described (22, 61). We recently reported that anti-SP70 antisera with a neutralizing titer of 1:32 were able to confer up to 80% in vivo passive protection in suckling mice and that the survival rate correlated well with the neutralizing titers measured in vitro (22). Here, we found that the sera from the BPSQ5-immunized mice displayed significant neutralizing activities against EV71 in vitro and had titers of up to 1:32, demonstrating the presence of anti-SP70 neutralizing antibodies in the antisera. Moreover, the anti-SP70 serum antibody isotypes induced in the BSQ5-immunized mice were predominantly IgG2a and IgG2b, indicative of a Th1-oriented immune response. In contrast, parenteral administration of conjugated SP70 peptide induced a Th2 immune response with production of high levels of anti-SP70 IgG1 antibodies (23). This apparent discrepancy, very likely due to the vehicle used to deliver the SP70 peptide (live recombinant bacteria versus conjugated SP70 emulsified in Freund's adjuvant), suggests therefore that the neutralizing activity of the anti-SP70 antibodies is not restricted to one particular IgG isotype.

In conclusion, we describe here the feasibility of using the highly attenuated *B. pertussis* BPZE1 strain as a bacterial vector to deliver heterologous vaccine candidates, thereby allowing simultaneous protection against pertussis and the target disease. Since whooping cough and hand, foot, and mouth disease are two childhood diseases, combined immunization soon after birth would be highly desirable. Furthermore, we show that the BrkA autotransporter is a promising display system for foreign antigens in *B. pertussis*. This study opens up new avenues for the development of safe live attenuated *B. pertussis* as a vehicle for vaccine delivery via the nasal route.

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