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Exploring the Versatility of the Autotransporter BrkA for the Presentation of Enterovirus 71 Vaccine Candidates at the Surface of Attenuated *Bordetella pertussis*.

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Abstract

Enterovirus 71 (EV71) is one of the most virulent agents responsible for Hand, Foot and Mouth Disease (HFMD) which has been consistently associated with severe, life-threatening complications in infants and children during outbreaks in the Asia Pacific region. Neither vaccine nor effective treatment is currently available.

We have been investigating the use of live attenuated *Bordetella pertussis*, the agent of whooping cough, to present EV71 vaccine candidates to the respiratory mucosa. We have previously shown that *B. pertussis* BrkA autotransporter could successfully present at the bacterial surface a 15 aminoacid (aa) neutralizing B cell epitope from the (EV71) VP1 capsid protein, and triggered in mice a strong neutralizing antibody response upon nasal administration of the recombinant bacteria. With the goal to induce an even stronger immune response against EV71, we have explored the feasibility to express larger VP1 sub-fragments in attenuated *B. pertussis* using BrkA as carrier. VP1A and VP1C fragments corresponding to the first N-terminal 62aa and the last C-terminal 98aa of VP1, respectively, were fused to the passenger domain of BrkA and were expressed in a highly attenuated strain of *B. pertussis* strain. Immunostaining approaches showed that both chimeras were efficiently exposed at the bacterial surface. Furthermore, lung colonization profiles in mouse displayed by both recombinant strains were comparable to that obtained with the parental strain. Our data thus demonstrated that BrkA is a versatile display system that allows surface exposure of large heterologous antigens in *B. pertussis* without major detrimental effects on the outer membrane integrity, host cell viability and lung colonization efficacy of the recombinant strains.

Keywords: Enterovirus 71, Bordetella pertussis, BPZE1, BrkA autotransporter, live mucosal vaccine

1. Introduction

Bordetella pertussis, the etiological agent of whooping cough, colonizes the human respiratory tract very efficiently, and triggers strong and protective, local and systemic immune responses upon a single nasal administration (1-4), inducing immunity even at distant mucosal sites (5). Recently, a highly attenuated strain of *B. pertussis,* namely BPZE1 was constructed (3), with an excellent safety profile in wild type and immuno-compromised mice (3, 6). Furthermore, BPZE1 retained the ability to colonize the respiratory tract very effectively, and was shown to induce protective immunity against pertussis even more effectively than current acellular pertussis vaccines (3). These features make the *B. pertussis* BPZE1 strain a highly promising live pertussis vaccine candidate, and will shortly enter phase I safety trial in humans (http://www.child-innovac.org). In addition, BPZE1 appears to be particularly well-adapted for the nasal delivery of heterologous vaccine antigens and represents an attractive mucosal vaccine delivery system. All the foreign antigens that have so far been expressed in *B. pertussis* were fused to the filamentous haemagglutinin (FHA), the main *B. pertussis* adhesin (2, 7-10). However, to be efficiently translocated across the outer membrane and secreted into the extracellular milieu, the FHA-based chimeras must remain in an unfolded conformation; specifically, the presence of cysteine (Cys) residues in the passenger antigen was shown to impair the chimera secretion due to the formation of intramolecular disulfide bonds in the periplasmic space (7, 11). This limitation makes FHA an unsuitable carrier for many Cys-containing proteins, calling therefore for the development of other antigen display systems in *B. pertussis*.

Autotransporters have been successfully used in a variety of microorganisms to present heterologous antigens at the bacterial surface (12-15). Autotransporters are large secreted, often virulence-associated proteins of Gram-negative

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bacteria (16). They perform various functions such as proteolysis, serum resistance, cytotoxicity and adhesion. Autotransporters share a common characteristic domain structure that includes: (a) a signal peptide at the N-terminus; (b) a passenger domain which encodes for the functional part of the protein; and (c) a C-terminal translocation unit, conserved across autotransporters, which encodes a β-barrel that inserts into the outer membrane. Translocation across the inner membrane, in all known cases, occurs via the Sec apparatus. Subsequently, the translocation unit forms a β-barrel structure in the outer membrane, through which the passenger domain is translocated to the cell surface (17). Most autotransporters 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 (18) and plays a role in *B. pertussis* adhesion to, and invasion of host cells (19). Consistent with the model of autotransporter secretion, BrkA is expressed as a 103-kDa precursor that is processed during secretion to yield a 73-kDa N-terminal passenger domain and a 30-kDa C-terminal translocation unit (20). Following translocation, the cleaved passenger domain remains tightly associated with the bacterial surface (21). Recently, we showed that BrkA can be successfully used as carrier to expose at the surface of BPZE1 bacteria an Enterovirus 71 (EV71) vaccine antigen candidate, namely SP70 (22). SP70 is a 15 amino acids (aa) peptide spanning aminoacids 208 to 222 from the viral VP1 capsid protein of EV71, and has been shown to contain a neutralizing (23) and protective B-cell epitope (24). Three copies of SP70 peptide were fused to the BrkA passenger domain and the resulting BrkA-$(SP70)_3$ chimera was successfully exposed at the bacterial surface of BPZE1 (22). Nasal administration of the live recombinant bacteria induced in mice strong and sustained systemic neutralizing antibody response against EV71, indicating that BrkA can be used as carrier to present heterologous antigens to the respiratory mucosa.

To further exploit the potential of BrkA as surface-display system, we investigated the capability of BrkA to accommodate large, conformational heterologous proteins. We reasoned that bigger portions of the (EV71) VP1 capsid protein contain a higher number of sequential and conformational epitopes and therefore should trigger a stronger protective immunity against EV71 than the one obtained with the 15 aa-SP70 peptide. Two sub-fragments of the (EV71) VP1 capsid protein- VP1A and VP1C, were thus fused to the passenger domain of BrkA, respectively, and expressed in attenuated *B. pertussis* BPZE1 strain. VP1A and VP1C fragments correspond to the first N-terminal 62aa and the last C-terminal 98aa of VP1, respectively, and are at least 4 times larger in size than SP70 peptide.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. All *B. pertussis* strains were grown as described previously on Bordet-Gengou (BG) agar or in Stainer-Scholte (SS) liquid medium (25).

Strain	Relevant feature (s)	Reference
BPZE1	Attenuated Sm^r BPSM derivative lacking the *dnt* gene and producing inactive pertussis toxin and reduced tracheal cytotoxin	3
BPVP1A2	BPZE1 derivative producing BrkA-VP1A	This study
BPVP1C2	BPZE1 derivative producing BrkA-VP1C	This study
BPSY1 (BrkA K/O)	*brkA* knockout BPZE1 derivative	22

Table 1: Bacterial strains used in this study.

2.2. Construction of recombinant B. pertussis *strains*

To circumvent any problems in protein translation due to poor codon usage (26), the original *VP1* DNA sequence of EV71 strain 41 (5865/SIN/00009), was optimized to *B. pertussis* codon usage preference. The optimized *VP1* (*opVP1*) gene was cloned into pBBR1MCS (27) yielding pBBR1MCS3. To generate the BrkA-VP1A construct, VP1A-encoding DNA fragment was first obtained by amplifying the 60-256 nucleotide (nt) region of *opVP1* from pBBR1MCS3 using primers 5'-TTGGATCCGACCCAGGCCCT-3' and 5' –TTGGATCCCGCTGTCCAG GGT-3' (*BamH*I sites are underlined). Similarly, to generate the BrkA-VP1C construct, the VP1C-encoding DNA fragment was obtained by amplifying the 484-787nt region of *opVP1* using primers 5'-TTGGATCCGGAGTCGCGCGA-3' and 5'-TTCGGGATCCAGGCGCGCA-3' (*BamH*I sites are underlined). Subsequently, *BamH*I-digested VP1A and VP1C gene fragments were inserted into *BamH*I-opened pUCSY2, a pUC18-derivative which harbors two DNA fragments

that are homologous to the *brkA* ORF (22). The resulting plasmids are named pUC-VP1A and pUC-VP1C, respectively. Finally, the 2,141-bp *Hind*III fragment from pUC-VP1A and 2,245-bp *Hind*III fragment from pUC-VP1C were cloned into *Hind*III-opened suicide plasmid pJQmp200rpsL18 (28), yielding pJQVP1A and pJQVP1C, respectively. BPZE1 was electroporated with pJQVP1A and pJQVP1C, respectively, allowing the BrkA-VP1A and BrkA-VP1C constructs to integrate into the chromosomal DNA by allelic exchange (29) at the *brkA* locus, yielding recombinant *B. pertussis* strains BPVP1A2 and BPVP1C2.

2.3. Immunodetection of BrkA-VP1A and BrkA-VP1C chimeras

Western blot, flow cytometry analysis and immunofluorescence microscopy were carried out using polyclonal rabbit anti-BrkA immune serum as previously described (22).

2.4. Mice infection

The mice were kept under specific-pathogen free conditions in individual ventilated cages, and all the experiments were carried out according to the guidelines of the National University of Singapore animal study board. Female 9-week-old Balb/c mice (CARE, NUS) were infected intranasally (in.) with $5x10^6$ colony forming unit (CFU) of the different *B. pertussis* strains in 20µl as described previously (22). At the indicated time points, four mice per group were euthanized, 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 incubation at 37°C.

3. Results

3.1 Production of BrkA-VP1A and BrkA-VP1C chimeras by B. pertussis

The BrkA passenger domain [A52-H263] portion was replaced by sub-fragments VP1A and VP1C which correspond to the first N-terminal 62aa and the last C-terminal 98aa from the capsid protein VP1 of EV71, respectively (Fig. 1A), and both constructs were expressed in BPZE1 as described in Math&Meth. section. Production of BrkA-VP1A and BrkA-VP1C chimeras by the recombinant strains was analyzed by immunoblotting using anti-BrkA antibodies. Wild type and *brkA*-knockout (BrkA K/O) BPZE1 strains were used as controls. Two bands of 103 kDa and 73 kDa molecular weight corresponding to full-length wild-type BrkA and its passenger domain, respectively, were detected in the whole-cell extract of BPZE1 (Fig. 1B, Lane 1). Similarly, two bands at 88kDa and 58kDa, corresponding to full-length BrkA-VP1A and its passenger domain, respectively, were detected in the cell lysate of BPVP1A2 (Fig. 1B, Lane 2). Two bands at 90kDa and 60kDa, corresponding to the predicted sizes of full-length BrkA-VP1C and its passenger domain, respectively, were also detected from the whole-cell extract of BPVP1C2 (Fig. 1B, Lane 3). Despite loading of similar total protein amounts, much weaker signals were obtained for both chimeras compared to the signal obtained with wild type BrkA, indicating a significantly lower production yield of the chimeras compared to wild type BrkA. No bands were detected in the BrkA K/O strain confirming the specificity of the anti-BrkA antibodies (Fig. 1B, Lane 4).

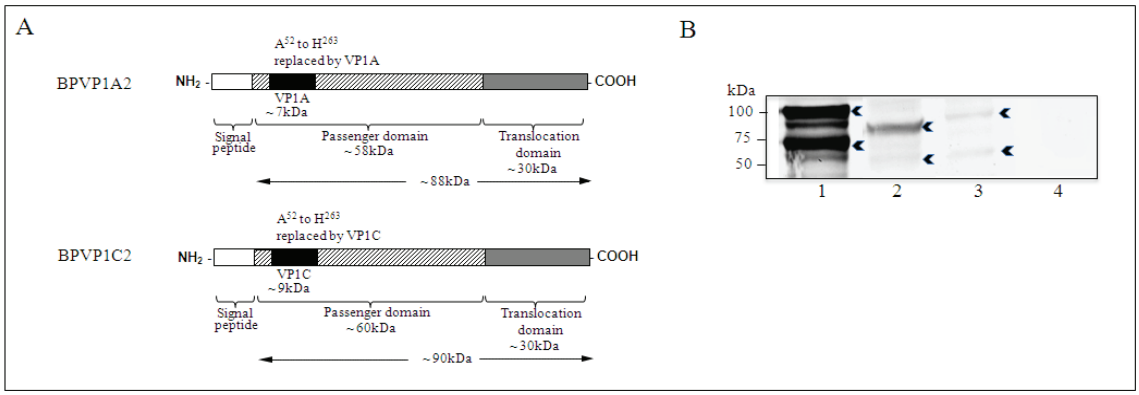

FIG. 1 Expression of BrkA-VP1A and BrkA-VP1C chimeras in live attenuated *B. pertussis* BPZE1 strain. (A) Schematic representation of BrkA-VP1A and BrkA-VP1C chimeras produced by recombinant *B. pertussis* strains, BPVP1A2 and BPVP1C2, respectively. (B) Western blot detection of BrkA-VP1A and BrkA-VP1C chimeras produced *by B. pertussis*. 20 µl of whole cell lysate from 1, BPZE1; 2, BPVP1A2; 3, BPVP1C2 and 4, BrkA K/O were analyzed by immunoblotting using polyclonal rabbit anti-BrkA antiserum. Arrows indicate the specific bands detected by the anti-BrkA antibodies.

Bacterial fitness of the recombinant BPVP1A2 and BPVP1C2 strains was studied *in vitro* by monitoring their growth kinetic profile and comparing to the parental BPZE1 and BrkA K/O strains. BPVP1A2 and BPVP1C2 strains clearly displayed reduced growth ability over the course of the *in vitro* culture (Fig. 2), whereby the cultures' OD_{600nm} were significantly lower than that obtained for the parental and BrkA K/O strains (Fig. 2). However, extensive clumping was noticed in the culture medium inoculated with BPVP1A2 and BPVP1C2 strains. It is thus possible that clumps formation, rather than genuine impaired growth ability, is responsible for the apparent reduced bacterial fitness of the BPVP1A2 and BPVP1C2 strains.

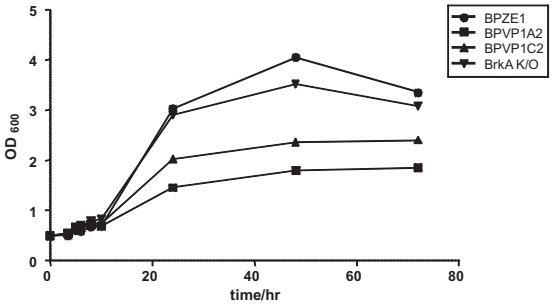

FIG. 2 Growth kinetic profiles of *B. pertussis* strains *in vitro*. *B. pertussis* BPZE1, BPVP1A2, BPVP1C2 and BrkA K/O strains were grown overnight in SS medium and fresh media were inoculated at initial OD_{600nm} of 0.15. The cultures were grown at 37°C with shaking (200 rpm). At indicated time points, OD_{600nm} were measured as an indication of the growth rate of *B. pertussis* strains *in vitro*.

3.3 Cell surface exposure of the BrkA-VP1A and BrkA-VP1C chimeras

Exposure of the BrkA-VP1A and BrkA-VP1C chimeras at the bacterial surface was investigated by flow cytometry using anti-BrkA antibodies on intact (non-permeabilized) BPVP1A2 and BPVP1C2 bacteria. Parental BPZE1 and BrkA K/O strains were used as positive and negative controls, respectively. Whereas background signal was obtained with BrkA K/O bacteria, the great majority of the parental BPZE1, and recombinant BPVP1A2 and BPVP1C2 cell populations were found positive, with differences not statistically significant (Fig. 3B). However, the fluorescence intensities obtained with BPVP1A2 and BPVP1C2 bacteria were significantly lower than that obtained for the parental strain BPZE1 (Fig. 3C). Consistent with the western blot data (Fig. 1B), FACS analysis thus indicated that the expression level of the BrkA chimeras was significantly lower than the wild type BrkA.

Surface exposure of the BrkA chimeras was further analyzed by immunofluorescence. BPZE1 and BPVP1A2 displayed strong and comparable fluorescence intensity signals (Fig. 4H & 4I, respectively), whereas a weaker signal was obtained with BPVP1C2 cells (Fig. 4J). No significant fluorescence emission was detected with the BrkA K/O strain (Fig. 4G).

Together, our data indicate that majority of the recombinant BPVP1A2 and BPVP1C2 bacteria are able to express the BrkA chimeras on their cell surface although at levels lower than the level obtained with the parental BPZE1 strain which expresses wild-type BrkA.

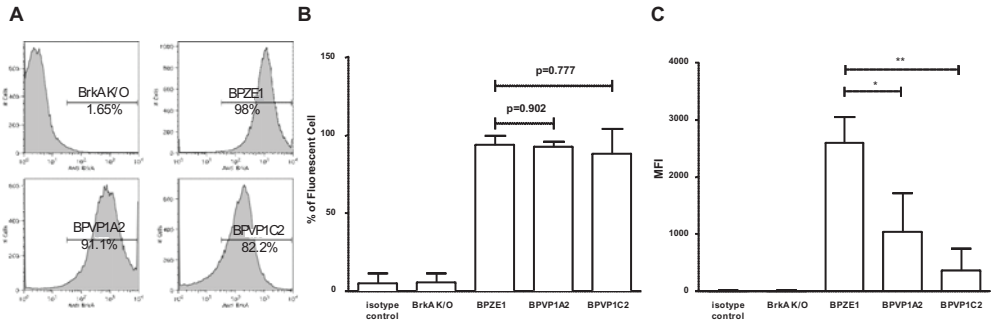

FIG. 3 Detection of the BrkA-VP1A and BrkA-VP1C chimeras by flow cytometry. Anti-BrkA polyclonal antibodies were co-incubated with intact *B. pertussis* strains as indicated. The parental BPZE1 and BrkA K/O strains were used as positive and negative controls, respectively. The isotype control consisted of BPZE1 bacteria stained with Cy2-conjugated secondary antibody. (A) Typical flow cytometry analysis histograms in a representative experiment. (B) Average values for the two independent experiments. (C) Mean Fluorescence Intensities of *B. pertussis* strains expressing BrkA protein. Data are expressed as the means ± standard deviation of two independent experiments. Significant statistical differences in relation to the BPZE1 group are represented by (*) $p < 0.05$ and (**) $p < 0.01$.

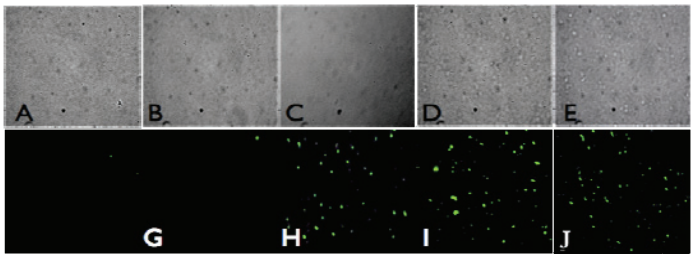

FIG. 4 Detection of the BrkA-VP1A and BrkA-VP1C chimeras by immunofluorescence. Anti-BrkA polyclonal antibodies were co-incubated with intact BrkA K/O (B and G), BPZE1 (C and H), BPVP1A2 (D and I) or BPVP1C2 (E and J) cells. The isotype control (A and F) consisted of BPZE1 bacteria stained with Cy2-conjugated secondary antibody. Panels A to E show corresponding phase-contrast images.

3.4 Lung colonization by BPVP1A2 and BPVP1C2

The ability of BPVP1A2 and BPVP1C2 strains to colonize the mouse respiratory tract was investigated and compared to the parental BPZE1 strain.

A significantly lower number of BPVP1A2 and BPVP1C2 bacteria were recovered from the mouse lungs at day 7 post-infection (p.i.) compared to the parental BPZE1 strain (Fig. 5). However, comparable numbers of CFU were obtained at the subsequent time points, i.e. 10 and 17 days p.i. These data demonstrated that recombinant BPVP1A2 and BPVP1C2 strains colonize efficiently the mouse respiratory tract, thereby suggesting that both strains should be able to prime effectively the host immune system at the nasal and respiratory mucosa.

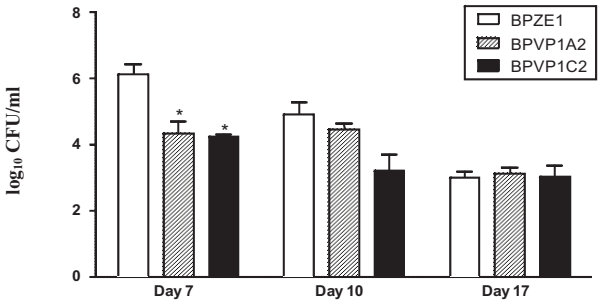

FIG. 5 Lung colonization by recombinant *B. pertussis* strains. Mice were infected intranasally with 2 x 10^6 CFU of BPZE1, BPVP1A2 and BPVP1C2 strains at 0 h. The lungs were harvested at the indicated time points, and the number of viable bacteria was determined after plating appropriate dilutions of the lung homogenates onto BG agar. Four mice per group per time point were assessed individually. Significant statistical differences in relation to the BPZE1 group are represented by (*) $p < 0.05$.

4. Discussion

Gram-negative bacteria have evolved various systems to transport proteins to the cell surface or secrete them into the extracellular milieu. Among these systems, autotransporters represent a solution of remarkable simplicity, as all information required for transport to the outer membrane and secretion is contained in the primary sequence itself (17).

Several autotransporters have been employed to express heterologous antigens at the bacterial surface (12-15). In addition to their inherent simplicity, autotransporters offer several advantages as a heterologous protein display system. Studies showed that autodisplay enables the recombinant expression of more than 10^5 proteins or peptide molecules on the surface of *E. coli* without reducing cell viability or integrity of the recombinant bacteria (15, 30-31), thus making autotransporters a high-valency display system of heterologous proteins (32). Furthermore, the mobility of the protein's β-barrel in the outer membrane enables passenger domains to form multimers at the cell surface (33-34). This special feature may be advantageous for the heterologous expression of proteins whose functionality requires multimerization. Altogether, these features make autotransporters obvious candidates as surface display systems for a wide range of biotechnological and biomedical applications (32).

Efficient surface expression of heterologous proteins using autotransporters as carrier appears to be multifactorial. Protein size and conformation are reported to greatly influence the success of the translocation process. Large proteins tightly folded or containing internal disulphide bonds are indeed in general more challenging to translocate through the β-barrel of the transport unit. However, although autotransporters share the same structural domain organization, their structural tolerance to translocate heterologous proteins across the outer membrane varies from one to another. The *Neisseria gonorrhoeae* IgA protease for example was reported unsuccessful to surface-expose the cholera toxin B

(CtxB) subunit which forms internal disulphide bonds (35). But the same autotransporter was reported to successfully transport single chain variable antibody domains in an active conformation with disulphide bonds preformed in the periplasm (36). Likewise the presence of cysteine bridges within the FimH lectin domain did not interfere with its surface display using the *E. coli* Ag43 autotransporter (37). Therefore, structural tolerance of autotransporters to translocate heterologous proteins across the outer membrane remains difficult to predict.

A previous study showed that deletion of amino acid residues A52 to P600 in the passenger domain of the *B. pertussis* BrkA autotransporter does not impair translocation and surface exposure of the protein (38). This finding thus prompted us to use BrkA as a carrier by replacing part of the A52-P600 region in the passenger domain with heterologous vaccine candidates. Here the [A52-H263] portion of the BrkA passenger domain was replaced by the N-terminal VP1A and C-terminal VP1C protein fragments of (EV71) VP1 capsid. Our data indicate that the BrkA chimeras were successfully produced and exposed at the bacterial surface of BPZE1, although with a lesser efficiency than the wild type BrkA molecules. The chimeras being smaller than wild type BrkA, size cannot be accounted for the lower production yield observed. Instead, the conformation of the recombinant passenger domains in the chimeras is likely to be different from the BrkA wild type conformation and may thus affect translocation efficacy across the outer membrane, thereby resulting in exposure of fewer molecules at the bacterial surface and degradation of the improperly folded chimeras. In addition, the presence of one Cys residue at position 63aa in VP1C fragment may also lead to the formation of intermolecular disulfide bonds in the perisplasmic space and impair the translocation efficacy of the dimerized recombinant proteins across the outer membrane. Consistently, production yield and surface-exposure of the BrkA-VP1C chimera were found to be the most impaired when compared to the BrkA-VP1A chimera and wild type BrkA.

In conclusion, this work shows that the *B. pertussis* BrkA autotransporter can support the surface exposure of large, conformational Cys-containing foreign inserts, although with lower efficacy compared to the wild type BrkA protein. Importantly, the BrkA chimeras can be displayed at the cell surface without significant detrimental effect on the bacterial colonization ability since both recombinant strains were able to persist for more than 14 days in the murine respiratory tract, which likely ensures efficient priming of the host immune system. The ability of the recombinant BPVP1A and BPVP1C2 strains at inducing specific cellular and humoral protective immune responses in mice upon nasal administration of the live recombinant bacteria is currently underway. The efficient presentation to the mucosal immune system of EV71 vaccine candidates expressed at the cell surface of an attenuated strain of *B. pertussis* provides an innovative approach to induce simultaneous protection against two childhood diseases, namely whooping cough and Hand, Foot and Mouth disease.

5. Acknowledgements

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