Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 321 (2004) 192–196
BBRC
www.elsevier.com/locate/ybbrc

Expression and characterization of cholera toxin B—pneumococcal surface adhesin A fusion protein in *Escherichia coli*: ability of CTB-PsaA to induce humoral immune response in mice

Ana Paula Mattos Arêas [a,b], Maria Leonor Sarno Oliveira [a], Eliane Namie Miyaji [a], Luciana Cezar Cerqueira Leite [a,b], Karina Araújo Aires [a], Waldely Oliveira Dias [a], Paulo Lee Ho [a,b,c,*]

[a] *Centro de Biotecnologia, Instituto Butantan, São Paulo, Brazil*
[b] *Instituto de Química, USP, São Paulo, Brazil*
[c] *Instituto de Biociências, USP, São Paulo, Brazil*

Received 14 June 2004

Abstract

Cholera toxin B subunit (CTB) is responsible for CT holotoxin binding to the cell and has been described as a mucosal adjuvant for vaccines. In this work, the ctxB gene was genetically fused to the psaA gene from *Streptococcus pneumoniae*, a surface protein involved in its colonization in the host that is also considered a vaccine antigen candidate against this pathogen. The CTB-PsaA fusion protein was expressed in *Escherichia coli*, and the purified protein was used for intranasal immunization experiments in Balb/C mice. CTB-PsaA was able to induce both systemic and mucosal antibodies evaluated in serum, saliva, and in nasal and bronchial wash samples, showing that CTB-PsaA is a promising molecule to be investigated as *S. pneumoniae* vaccine antigen candidate.

Keywords: CTB; Cholera toxin; PsaA; *Streptococcus pneumoniae*; Nasal immunization; Pneumococcus; Bacterial adhesin; Pneumonia; Mucosa

Cholera toxin B subunit (CTB) is the pentameric non-toxic portion of cholera toxin (CT), responsible for the holotoxin binding to the GM1 ganglioside receptor, a glycolipid that is constitutively expressed in most cells in the body, including leucocytes. CTB is also described as a potent mucosal adjuvant. Chemical conjugations with CTB have been performed using many different heterologous antigens, such as *Schistosoma mansoni* [1] and SIV virus [2] proteins, as well as polysaccharide antigens [3,4] from *Streptococcus pneumoniae*. Coadministered protein antigens from *S. pneumoniae* with CTB also provided protective immunity against *S. pneumoniae* challenge [5]. In addition, some works have reported genetic fusions of CTB with several antigens, such as epitopes from hepatitis C virus [6] and foot-and-mouth disease virus [7]. Both conjugation and genetic fusion to CTB can elicit strong mucosal antibody responses to the linked antigens. These strategies aimed the delivery of the covalently attached antigens to the mucosal cells, via GM1 ganglioside.

In many developing and developed countries, *S. pneumoniae* is the most important cause of bacterial pneumonia, meningitis, bacteremia, and otitis media [8]. Due to the ineffectiveness of the available polysaccharide vaccine (Pneumovax 23) in young children (<2 years), elderly, and patients with immunodeficiences,

* Corresponding author. Fax: +55-11-3726-1505.
E-mail address: hoplee@butantan.gov.br (P.L. Ho).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.06.118

such as AIDS [9], a number of protein vaccines have been investigated. In contrast to polysaccharide vaccines, conjugated and protein-based vaccines can elicit a T cell-dependent immune response. In fact, new conjugated vaccines, composed of 7–11 polysaccharides from different prevailing serotypes in USA conjugated with a bacterial toxoid, such as CRM-197, are efficient vaccines in the groups mentioned above [9]. However, besides the high cost of this vaccine, there is also a possibility that large-scale use of the conjugated vaccines may result in a shift in prevailing serotypes from those that are present in the vaccines to those that are not included in the formulation [9]. For these reasons, several pneumococcal surface proteins have been tested as candidate vaccines; one of them is PsaA (Pneumococcal surface antigen A), a highly conserved protein within all 90 pneumococcal serotypes [5]. PsaA appears to have a role in promoting bacterial adhesion to host cells since mutant PsaA$^-$ pneumococci are avirulent in a mouse model [10–12]. This effect may be due to the absence of some adhesins, such as the choline binding protein A (CbpA) modulated by the presence or absence (in PsaA$^-$ mutant pneumococci) of Mn^{2+} or Zn^{2+} [11]. In addition to this, anti-PsaA antibodies naturally developed in humans or when elicited by recombinant PsaA in animals can prevent the adherence of pneumococci to nasopharyngeal epithelial cells [13].

Colonization of the mucosal surface of the human nasopharynx is the crucial first step in the pathogenesis of all pneumococcal diseases, and in the most serious cases, the pathogen can reach the bloodstream [14]. Therefore, stimulation of the mucosal-associated lymphoid tissue (MALT), which releases pathogen-specific secretory immunoglobulin A (sIgA), can prevent both mucosal colonization and pathogen spread into the systemic circulation [3]. In this study, a CTB-PsaA fusion protein was expressed in *Escherichia coli* in an active pentameric form. Purified protein was used to evaluate the ability to induce the systemic and mucosal antibody production.

Materials and methods

Cloning steps and screening of the recombinant plasmids. The psaA gene (867 bp), from *S. pneumoniae* serotype 6B (strain 472/96 from the Instituto Adolfo Lutz, São Paulo, Brazil), was amplified from plasmid pCI-psaA [15] by PCR. The mixture was subjected to a program consisting of a DNA denaturation step at 94 °C for 5 min, 25 cycles at 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 1 min. The following oligonucleotides were used: For: 5′ CCC **ACG CGT** GCT AGC GGA AAA AAA GAT ACA ACT TCT GG 3′ and Rev: 5′ CC **AAG CTT** TTA TTT TGC CAA TCC TTC AGC 3′. In bold are the *Mlu*I and *Hin*dIII restriction sites, respectively.

After amplification, the psaA fragment was gel-purified using the kit "Concert Gel Purification" (Invitrogen), as described by the manufacturer. The PCR fragment was cloned into the *Mlu*I and *Hin*dIII sites of the pAE-ctxB, a construct previously obtained [16], in order to generate the pAE-ctxB-psaA plasmid. The pAE-psaA was obtained by the digestion of the construct previously described with *Nhe*I, present in the upstream regions of both ctxB and psaA genes, and further religation.

Expression and purification of the recombinant proteins. BL21(SI) *E. coli* strain was used to express CTB [16], PsaA, and CTB-PsaA. BL21(SI) *E. coli* competent cells (Invitrogen) were transformed with the pAE-ctxB, pAE-psaA or pAE-ctxB-psaA plasmids and grown overnight at 30 °C. Ampicillin-resistant colonies were inoculated in 5 mL on 2YTON-amp (2YT broth without NaCl) and grown overnight at 30 °C. In the following day, cultures were diluted 30-fold in 2YTON-amp and grown until A_{600} reached 0.8, when NaCl was added to the medium at a final concentration of 300 mM. After 3 h, cells were harvested by centrifugation and bacterial pellets were lysed by the addition of 1-fold SDS–PAGE sample buffer. Aliquots of total extracts were analyzed by 15% (for CTB) [16], 12% (for PsaA and CTB-PsaA) SDS–PAGE. The best clone was chosen for expression in a larger scale (1 L). Recombinant PsaA and CTB-PsaA were expressed in a soluble form, whereas CTB was recovered from the inclusion bodies [16]. Cells were collected by centrifugation, resuspended in 100 mL lysis buffer, pH 8.0 (100 mmol L^{-1} NaCl and 20 mmol L^{-1} Tris–Cl), and lysed by French pressure. Cellular lysates were centrifuged at 26,000*g* for 15 min.

All proteins were expressed with a 6× His tag at N-terminus to facilitate the purification through Ni^{2+}-charged column chromatography. After the adsorption of PsaA or CTB-PsaA proteins to Ni^{2+} Chelating Sepharose Fast Flow resins (Amersham Biosciences), washes with 10 volumes of binding buffer (100 mmol L^{-1} NaCl, 20 mmol L^{-1} Tris–Cl, pH 8.0) containing 5, 20, 40, and 60 mmol L^{-1} imidazole were performed. Proteins were eluted with 5 volumes of the same solution containing 1 mol L^{-1} imidazole. Fractions were analyzed by 12% SDS–PAGE. Proteins were dialyzed in one step. The equilibrium was established using 2 L of a 10 mmol L^{-1} Tris–Cl, pH 8.0, 20 mmol L^{-1} NaCl, 0.1% (m/V) glycine solution. The CTB purification was made as previously described [16].

Western blot. Nitrocellulose membranes, after protein transfer from 15% SDS–PAGE, were blocked with 5% (m/V) non-fat milk in 0.05% Tween 20/phosphate buffered saline (PBS-T). Membranes were washed three times for 10 min with PBS-T, and further incubated with a proper dilution of mouse polyclonal anti-CTB or anti-PsaA antibodies in 5% non-fat milk-PBS-T, for 1 h, followed by washing as described above. Membranes were incubated with a proper dilution of anti-mouse IgG peroxidase conjugate (Sigma) in 5% non-fat milk-PBS-T, washed with the same procedure, and revealed with ECL reagent (Amersham Biosciences).

SDS–PAGE of the CTB-PsaA and GM1-ELISA. The ability of the CTB-PsaA fusion protein to fold into pentamers was analyzed by a 6% SDS–PAGE. For this, samples were not boiled and a sample buffer that did not contain β-mercaptoethanol was used.

The ability of the pentamers to bind to its cellular receptor was assessed by GM1-ELISA. This protocol was adapted from a reference published elsewhere [17]. Briefly, microtiter 96-well plates were coated with 10 μg mL^{-1} GM1 ganglioside in PBS, pH 7.2, or BSA in 0.05 mol L^{-1} carbonate–bicarbonate buffer, pH 9.6, at 37 °C for 16 h. After washing three times with PBS-T, the plates were blocked by incubation with a 1% (m/V) BSA-PBS-T solution for 30 min at room temperature. The recombinant proteins were diluted, from 300 to 0.3 ηmol L^{-1}, in 1% BSA-PBS-T, added to the plates, and incubated for 2 h at room temperature. Unbound proteins were removed from the plate by washing three times with PBS-T, and then, a proper dilution of commercial anti-CT polyclonal antibody (Sigma) was added to the plates and incubated for 1 h at room temperature. After washing three times with PBS-T, a peroxidase-conjugated goat anti-rabbit IgG (Sigma), in a proper dilution, was added to the plate and incubated for 1 h at room temperature. After washing, plates were revealed by the addition of 8 mg *o*-phenylenediamine (OPD) in 20 mL of a 0.2 mol L^{-1} citrate-phosphate buffer, pH 5.0, in the presence of 10 μL H_2O_2.

The reaction was stopped by the addition of 4 mol L^{-1} H_2SO_4. The absorbance was measured at 492 nm.

Immunization of mice with CTB-PsaA fusion protein. Seven-week-old female Balb/C mice (six per condition) were immunized intranasally twice a week for three consecutive weeks. Ten microliters was inoculated in each nostril using a micropipette. The following conditions were used: saline, CTB (4 μg), PsaA (1 μg), PsaA (1 μg) + CTB (4 μg), PsaA (1 μg) + CT (4 μg), as a positive control, and CTB-PsaA fusion protein (1 and 5 μg). After 21 days, mice were bled by the retrorbital plexus. For the collection of saliva, a 0.01% pilocarpine solution was injected intraperitoneally. The animals were sacrificed, and nasal and bronchial washes were performed [18]. Anti-PsaA IgG from serum and IgA from saliva and nasal and bronchial washes were determined by ELISA as described below.

ELISA. Microtiter plates (Maxisorp-NUNC) were incubated at 4 °C for 16 h with 10 μg mL^{-1} PsaA in 0.05 mol L^{-1} carbonate–bicarbonate buffer, pH 9.6. After three washes with PBS-T, plates were blocked with 10% (m/V) non-fat milk-PBS-T for 1 h at 37 °C, and then, washed three times with PBS-T. Serum, saliva or nasal and bronchial wash dilutions were added to the plates in 1% (m/V) bovine serum albumin (BSA) and incubated at 37 °C for 1 h. After washing, proper dilutions of a peroxidase-conjugated goat anti-mouse IgG (Sigma) or a peroxidase-conjugated goat anti-mouse IgA (Sigma) were added to the plates and incubated for an additional hour at 37 °C. Plates were revealed by the addition of 8 mg *o*-phenylenediamine (OPD) in 20 mL of a 0.2 mol L^{-1} citrate-phosphate buffer, pH 5.0, in the presence of 10 μL H_2O_2. The reaction was stopped by adding 4 mol L^{-1} H_2SO_4. The absorbance was measured at 492 nm.

Results and discussion

Expression and purification of the recombinant proteins

Recombinant PsaA, CTB-PsaA, and CTB (as described in [16]) were expressed in BL21 (SI) *E. coli* strain and purified through a Ni^{2+}-charged column chromatography. Cellular extracts of induced BL21 (SI) and purified CTB-PsaA (45 kDa) and PsaA (32 kDa) are shown in Fig. 1. Quantification of the recovered proteins revealed that they reached a yield of about 40 mg of purified protein per liter of induced culture.

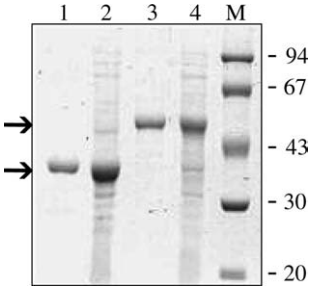

Fig. 1. Expression and purification of recombinant PsaA and CTB-PsaA fusion protein. Cellular extracts and purified proteins were loaded onto 12% SDS–PAGE. Lanes 2 and 4 show cellular extracts from *E. coli* BL21 (SI) expressing PsaA (32 kDa) and CTB-PsaA (45 kDa), respectively, whereas lanes 1 and 3 show their respective recombinant proteins in a purified form. Protein bands are indicated by arrows.

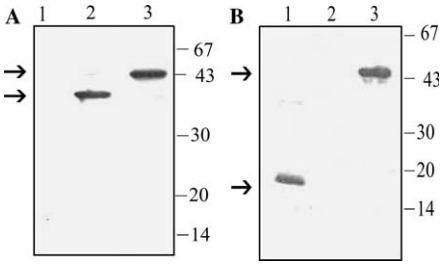

Fig. 2. Western blot analysis of recombinant CTB-PsaA fusion protein. Nitrocellulose membranes were revealed, after incubations with the antibodies anti-PsaA (A) and anti-CTB (B) antibodies and immunoconjugates. CTB-PsaA is in lane 3. Recombinant CTB (lane 1), PsaA (lane 2) were used as controls. Protein bands are indicated by arrows.

Western blot

The CTB-PsaA fusion protein was strongly and specifically recognized by polyclonal anti-PsaA and anti-CTB antibodies in an immunoblot assay (Figs. 2A and B, respectively). PsaA and CTB were used as positive controls. A single band corresponding to CTB-PsaA can be observed in the immunoblot, indicating that the fusion protein did not undergo degradation.

Pentamer formation

To analyze the ability of CTB-PsaA to form pentamers, the protein was resuspended in sample buffer without β-mercaptoethanol and was directly loaded onto a 6% SDS–PAGE (Fig. 3A). In the reduced and boiled samples, a single band of 45 kDa (Fig. 3A, lane 1) was observed, whereas, in the non-reduced and non-boiled samples, a band of about 225 kDa, the predicted size of the pentameric form of CTB-PsaA, was detected (Fig. 3A, lane 2).

To confirm the ability of the CTB-PsaA pentamers to bind GM1, a GM1-ELISA was performed (Fig. 3B). Recombinant CTB and CTB-PsaA were able to bind GM1 in a dose-dependent manner. In addition to this, their curves presented the same profile. As negative controls, we used PsaA that should not be able to bind GM1 and all the proteins were evaluated using BSA as the coating protein. Considering these results, we assumed that the fold of CTB-PsaA may be correct, at least for the CTB portion and, it seems that the presence of five molecules of PsaA in the structure does not abrogate the binding of the pentamer to its receptor.

Immunization of mice with CTB-PsaA fusion protein

Intranasal immunization was performed in Balb/C mice in order to evaluate the ability of CTB-PsaA fusion protein to induce antibody production. Results are shown in Table 1. Five micrograms of CTB-PsaA fusion protein induced the greatest levels of both anti-PsaA

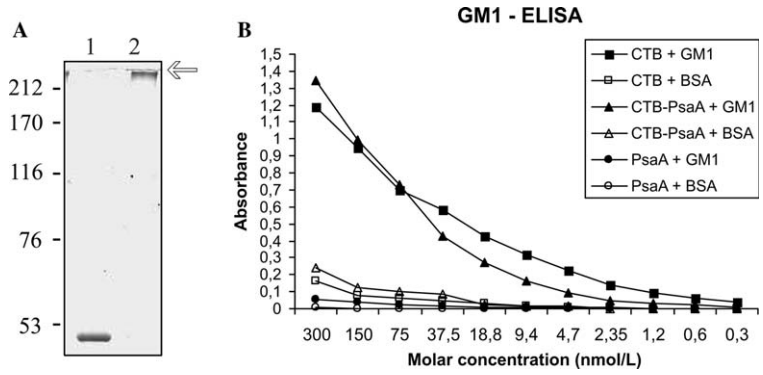

Fig. 3. Analysis of CTB-PsaA pentamer formation (A) and its ability to bind GM1 receptor (B). (A) To evaluate the CTB-PsaA pentamer formation, 4 μg of the protein was loaded onto a 6% SDS–PAGE. Lane 1 shows the sample boiled and in reducing conditions, whereas lane 2 shows the same protein not submitted to boiling in non-reducing conditions. A band of about 225 kDa, indicated by an arrow, can be observed in non-boiled and non-reduced samples. (B) In order to verify the pentamer ability to bind GM1 gangliosides, a GM1-ELISA, using recombinant CTB-PsaA, CTB, and PsaA, was performed. The ELISA was performed by coating a 96-well plate with GM1 or BSA.

Table 1
Induction of antibodies by CTB-PsaA after nasal immunization

	IgG	IgA		
	Serum	Saliva	Nasal wash	Bronchial wash
Saline	<20	<2	<2	<2
CTB	20	<2	<2	<2
PsaA	20	<2	<2	<2
PsaA + CTB	<20	<2	2	<2
PsaA + CT	2560	4	16	<2
CTB-PsaA 1 μg	320	8	4	<2
CTB-PsaA 5 μg	2560	128	32	4

Reciprocal IgG titers from pooled serum and IgA titers from pooled saliva, and nasal and bronchial washes are shown in this table. The titer was defined as the last dilution in which an absorbance of 0.1 can be observed. These results are representative of two experiments.

IgG in the serum and IgA in saliva, in nasal and bronchial washes, even better than PsaA + CT, the positive control. The effect of the fusion protein in the antibody production was dose-dependent, since a lower amount of CTB-PsaA (1 μg) elicited a lower titer of both systemic and mucosal antibodies. The other conditions in which the antigen was administered, such as PsaA, PsaA + CTB, were not able to induce detectable levels of IgG and IgA.

In conclusion, the use of CTB-PsaA fusion protein for vaccination has several advantages. In terms of manufacturing the final product, the antigen and the adjuvant constitute the same molecule. Thus, it requires only one fermentation step. Moreover, CTB-PsaA was expressed in high levels in a soluble form and it can be directly purified, whereas CTB is expressed in inclusion bodies [16], requiring denaturing–renaturing steps. This work shows that CTB-PsaA can be produced in large amounts in the active form, which is easily assessed by 6% SDS–PAGE and GM1-ELISA. In contrast to conjugation processes, in which the proportion between the proteins is much more difficult to control, the genetic fusion produces a hybrid molecule, with a determined number of antigen molecules. The CTB pentamer moiety is capable to bind the receptor and to deliver five molecules of the antigen. This may contribute to the higher immunogenicity observed for this protein than for coadministered CTB and PsaA, or PsaA alone. In terms of protective immune response, some experiments have to be performed in order to verify if the adjuvant effect evoked by the CTB portion is maintained by the CTB-PsaA fusion protein. A suitable model to assess this information is the nasopharyngeal colonization by pneumococci. In this case, CTB-PsaA can be evaluated in terms of effective immune response inducer as an immunogen as well as an adjuvant to other vaccine, such as non-capsulated pneumococci [19]. In the light of these results, CTB-PsaA may be a promising candidate to be included in a pneumococcal vaccine formulation.

Acknowledgments

This work was supported by FAPESP, CNPq, and Fundação Butantan. We thank Ingrid Rani Santos Pimenta and Erika Regazoli for the excellent technical assistance.

References

[1] J.-S. Sun, N. Mielcarek, M. Lakew, J.-M. Grzych, A. Capron, J. Holmgrren, C. Czerkinsky, Intranasal administration of a *Schistosoma mansoni* glutathione S–transferase-cholera toxoid conjugate vaccine evokes antiparasitic and antipathological immunity in mice, J. Immunol. 163 (1999) 1045–1052.

[2] S.-M. Kang, Q. Yao, L. Guo, R.W. Compans, Mucosal immunization with virus-like particles of simian immunodeficiency virus conjugated with cholera toxin subunit B, J. Virol. 77 (18) (2003) 9823–9830.

[3] S-Y. Seong, N-H. Cho, I.C. Kwon, S.Y. Jeong, Protective immunity of microsphere-based mucosal vaccines against lethal

intranasal challenge with *Streptococcus pneumoniae*, Infect. Immun. 67 (7) (1999) 3587–3592.
[4] C. Bergquist, T. Lagergard, J. Holmgren, Anticarrier immunity suppresses the antibody response to polysaccharide antigens after intranasal immunization with the polysaccharide–protein conjugate, Infect. Immun. 65 (5) (1997) 1579–1583.
[5] J-Y. Seo, S.Y. Seong, B-Y. Ahn, I.C. Kwon, H. Chung, S.Y. Jeong, Cross-protective immunity of mice induced by oral immunization with pneumococcal surface adhesin A encapsulated in microspheres, Infect. Immun. 70 (3) (2002) 1143–1149.
[6] L.G. Nemchinov, T.J. Liang, M.M. Rifaat, H.M. Mazyad, A. Hadidi, J.M. Keith, Development of a plant-derived subunit vaccine candidate against hepatitis C virus, Arch. Virol. 145 (12) (2000) 2557–2573.
[7] H. Song, L. Zhou, W. Fang, Y. Li, X. Wang, H. Fang, X. Li, M. Wu, B. Qiu, High-level expression of codon optimized foot-and-mouth disease virus complex epitopes and cholera toxin B subunit chimera in *Hansenula polymorpha*, Biochem. Biophys. Res. Commun. 315 (1) (2004) 235–239.
[8] S.E. Johnson, J.K. Dykes, D.L. Jue, J.S. Sampson, G.M. Carlone, E.W. Ades, Inhibition of Pneumococcal carriage in mice by subcutaneous immunization with peptides from the common surface protein pneumococcal surface adhesin A, J. Infect. Dis. 185 (2002) 489–496.
[9] Pneumococcal vaccines: WHO position paper, Weekly Epidemiological Record 74 (1999) 177–183.
[10] H-J. Tseng, A.G. McEwan, J.C. Paton, M.P. Jennings, Virulence of *Streptococcus pneumoniae*: PsaA mutants are hypersensitive to oxidative stress, Infect. Immun. 70 (3) (2002) 1635–1639.
[11] M.J. Jedrzejas, Pneumococcal virulence factors: structure and function, Microbiol. Mol. Biol. Rev. 65 (2) (2001) 187–207.
[12] D.J. Rigden, M.Y. Galperin, M.J. Jedrzejas, Analysis of structure and function of putative surface-exposed proteins encoded in the *Streptococcus pneumoniae* genome: a bioinformatics-based approach to vaccine and drug design, Crit. Rev. Biochem. Mol. Biol. 38 (2) (2003) 143–168.
[13] S. Romero-Steiner, T. Pilishvili, J.S. Sampson, S.E. Johnson, A. Stinson, G.M. Carlone, E.W. Ades, Inhibition of pneumococcal adherence to human nasopharyngeal epithelial cells by anti-PsaA antibodies, Clin. Diagn. Lab. Immunol. 10 (2) (2003) 246–251.
[14] T.L. McCool, T.R. Cate, G. Moy, J.N. Weiser, The immune response to pneumococcal proteins during experimental human carriage, J. Exp. Med. 195 (3) (2002) 359–365.
[15] E.N. Miyaji, W.O. Dias, M. Gamberini, V.C. Gebara, R.P. Schenkman, J. Wild, P. Rield, J. Reimann, R. Schirmbeck, L.C.C. Leite, PsaA (pneumococcal surface adhesin A) and PspA (pneumococcal surface protein A) DNA vaccines induce humoral and cellular immune responses against *Streptococcus pneumoniae*, Vaccine 20 (5–6) (2001) 805–812.
[16] A.P.M. Arêas, M.L.S. Oliveira, C.R.R. Ramos, M.E. Sbrogio-Almeida, I. Raw, P.L. Ho, Synthesis of cholera toxin B subunit gene: cloning and expression of a functional 6XHis-tagged protein in *Escherichia coli*, Protein Expr. Purif. 25 (2002) 481–487.
[17] C.A. Menezes, J. Amianti, H.S. Harayama, P.C.M. Koga, L.R. Trabulsi, R.M.F. Piazza, Inhibition of *Escherichia coli* heat-labile enterotoxin by neoglycoprotein and anti-lectin antibodies which mimic GM1 receptor, FEMS Microbiol. Lett. 216 (2002) 67–70.
[18] H-Y. Wu, M.H. Nahm, Y. Guo, M.W. Russell, D.E. Briles, Intranasal immunization of mice with PspA (Pneumococcal surface protein A) can prevent intranasal carriage, pulmonary infection, and sepsis with *Streptococcus pneumoniae*, J. Infect. Dis. 175 (1997) 839–846.
[19] R.Malley, S.C. Morse, L.C.C. Leite, A.P.M. Arêas, P.L. Ho, F.S. Kubrusly, I.C. Almeida, P. Anderson, Evaluation of adjuvants given intranasally with killed noncapsulated pneumococci in multi-serotype protection of mice against colonization and middle ear infection, Infect. Immun. 72 (2004) 4290–4292.