Effectiveness of engineering the nontypeable *Haemophilus influenzae* antigen Omp26 as an S-layer fusion in bacterial ghosts as a mucosal vaccine delivery

Eva M Riedmann[1], Werner Lubitz[1], John McGrath[2], Jennelle M Kyd[2] and Allan W Cripps[3*]

[1]BIRD-C GmbH&CoKG, Hauptstr. 88, 3420 Austria

[2] Central Queensland University, Rockhampton Qld 4702, Australia

[3] Griffith Health Institute, School of Medicine, Gold Coast campus, Griffith University, QLD 4222, Australia

Key words: Bacterial ghosts, NTHi, Omp26, mucosal immunization

[*] Corresponding author: Griffith Health Institute, School of Medicine, Gold Coast campus, Griffith University, QLD 4222, Australia

Tel.: +61 (07) 5678 0711; Fax: +61 (07) 5678 0795; E-mail: allan.cripps@griffith.edu.au

Abstract

The potential of empty bacterial cell envelopes (ghosts) as a delivery system for mucosal immunization was assessed in a rat model and different routes of immunization were evaluated. Animals were mucosally immunized targeting either gut only or gut and lung mucosal sites with *Escherichia coli* ghosts harbouring the nontypeable *Haemophilus influenzae* (NTHi) antigen Omp26. Omp26 was expressed as either a part of an S-layer fusion or as a soluble protein in the periplasm. In the gut/lung regime two initial gut targeted inoculations with the ghosts were followed by an intratracheal (IT) boost with purified Omp26. The gut only immunization regime showed a moderate enhancement of bacterial clearance following pulmonary challenge whereas the gut/lung immunization regime resulted in significantly enhanced pulmonary clearance of NTHi. Both immunization regimes induced high levels of Omp26 specific antibodies in the serum of immunized rats, with higher levels in the groups that received the IT boost with purified Omp26. Analysis of IgG isotypes present in serum suggest that the immune response was predominantly of a T-helper1 type. Additionally, immunization induced a significant cellular immune response with lymphocytes from animals vaccinated using the gut/lung regime responding significantly to Omp26 when compared to control groups. The results of this study show that mucosal immunization with recombinant Omp26 in *E. coli* ghosts followed by a boost with purified Omp26 can induce a specific and protective immune response in a rodent model of acute lung infection.

Introduction

Vaccines have traditionally been administered by intramuscular or subcutaneous injection, which often only elicits systemic immune responses. Since many diseases are caused by live microbes entering the host through mucosal membranes it seems reasonable to develop vaccine strategies that induce mucosal immunity. Thus, immunity is stimulated at the sites where the pathogen initially infects the host, which, for many diseases may lead to improved vaccine efficacy. There is considerable interest in the development of novel mucosal delivery systems, that can be used to package and deliver a variety of antigens.

The bacterial ghost platform technology has been proposed as an advanced delivery system for potential vaccine candidates. Bacterial ghosts are empty bacterial cell envelopes produced by controlled expression of cloned bacteriophage PhiX174 lysis gene *E* in Gram-negative bacteria.[1] Bacterial ghosts derived from pathogens, such as *Vibrio cholerae* or *Actinobacillus pleuropneumoniae*, have shown potential as non-living candidate vaccines in different animal models. In addition bacterial ghosts, especially ones derived from the model organism *E. coli* K12, have been extensively studied as carrier and delivery systems for a variety of heterologous protein antigens.[2,3]

The potential of bacterial ghosts as a mucosal vaccine delivery system has been demonstrated in several studies. Aerosol immunization of pigs with *A. pleuropneumoniae* ghosts induced protective immunity against a homologous aerosol challenge.[4] In another study, immunization with *A. pleuropneumoniae* ghosts and formalin-inactivated whole-cells has been compared. While both vaccination groups were protected against clinical disease, colonization of the lungs with *A. pleuropneumoniae* was only prevented in ghost-vaccinated pigs.[5] Mice orally immunized with *Salmonella typhimurium* ghosts show significantly prolonged survival after a lethal challenge when compared to control vaccination groups.[6] Prophylactic oral vaccination experiments using *Helicobacter pylori* ghosts in mice showed a

significant reduction of the bacterial load in the ghost group.[7] Intranasal and intramuscular immunization of mice with *Vibrio cholerae* ghosts expressing the major outer membrane protein Omp1 from *Chlamydia trachomatis* induced a strong Th1 type immune response in the genital mucosa, which provided protective immunity.[8,9] A study investigating intragastric immunization of mice with enterohemorrhagic *E. coli* (EHEC) ghosts without the addition of any adjuvant showed that immunization induced cellular and humoral immunity which resulted in 86% survival against lethal challenge with a heterologous EHEC strain after single-dose oral immunization and 93.3% after one booster at day 28.[10]

To use bacterial ghosts as a carrier and adjuvant for foreign proteins, different strategies for immobilizing the antigen have been developed.[9] Besides membrane anchoring the antigen, the sealed periplasmic space of a ghost can be filled with soluble proteins. Expression of recombinant S-layers in bacterial ghosts further extends their carrier capacity. In addition to immobilizing an antigen in bacterial ghosts, the ability of S-layers to assemble into large regular structures endows them with immune stimulating and intrinsic adjuvant properties.[11,12] The potential of S-layers as antigen carriers for vaccine preparations has previously been shown.[11,13,14]

Nontypeable *Haemophilus influenzae* (NTHi) is a common cause of respiratory tract infections and otitis media in humans.[15,16] At present, no vaccine is available for prevention of infection by this pathogen. Several outer membrane proteins of NTHi have been found to elicit immune responses in animal models, however, many exhibit significant ´between strain` sequence heterogeneity which limits their capacity to protect against infection with heterologous bacterial strains.[15,17,18,19] A NTHi outer membrane protein (Omp26) has been shown to be present on all NTHi strains tested and is highly conserved between strains.[20,21] Rats immunized mucosally with Omp26 enhanced the clearance of both homologous and heterologous strains of NTHi post pulmonary challenge and more recently, Omp26 was shown to be an effective immunogen against NTHi otitis media and nasopharyngeal carriage

in the chinchilla model.[20,21,22,23] Thus, these animal experiments provide evidence to support the potential of Omp26 as a vaccine candidate.

Previously we showed the construction and immunogenicity of bacterial ghosts harbouring the NTHi antigen Omp26 as part of different S-layer self assembly structures.[24] The current study investigated the feasibility of mucosally delivering Omp26 in bacterial ghosts to induce an effective immune response that enhances clearance of an acute pulmonary challenge with NTHi. Bacterial ghosts carrying Omp26 either as part of an S-layer self assembly structure in the cytoplasm or as soluble protein in the periplasm were administered to rats via different mucosal immunization routes, that either targeted the gut-associated lymphoid tissue alone or in combination with the bronchus-associated lymphoid tissue of the lung. The study has shown that the combined gut/lung immunization was the most effective in both the immune response induced and enhancing clearance of the NTHi from the lungs.

Materials and Methods

Construction of a MBP/Omp26 fusion protein

For the construction of a maltose binding protein (MBP) Omp26 fusion protein, the *omp26* gene was amplified by PCR from the vector pQE30-omp26[21] with oligonucleotides ER-Omp26B (5`-ggc gga tcc atg aaa aac atc gca aaa gt-3`) and ER-Omp26H (5`-ggc aag ctt tta ttt ttt ctc ttg agc ttt ttc tga agc-3`) containing *Bam*HI and *Hin*dIII restriction enzyme sites, respectively (restriction enzyme sites are underlined). PCR was performed as previously described.[24] After purification and restriction, the amplification product was cloned into the commercially available vector pMAL-p2X (New England BioLabs, Hitchin Hertfordshire, UK) resulting in the construct pREM-26. The vector pMAL-p2X is designed to produce maltose binding protein (MBP) fusions. The *malE* gene of this vector includes the sequence coding for the amino terminal signal peptide of MBP, which directs the fusion protein to the periplasm of *E. coli*.

Production of recombinant bacterial ghosts

Recombinant bacterial ghosts, expressing the Omp26 fusion proteins were produced from *Escherichia coli* pop2135 as described previously.[24] Briefly, bacteria harbouring a combination of expression and lysis plasmid were grown at 28°C in Luria Bertani (LB) medium supplemented with ampicillin (100µg ml^{-1}) and kanamycin (50µg ml^{-1}). The lysis plasmids pML1 and pDKL01 [6,25] containing the lysis gene *E* of the bacteriophage PhiX174 were used in combination with the expression plasmids pREM-26 (encoding for MBP/Omp26) and pREM-A4 (encoding for SbsA/Omp26), respectively. The previously constructed vector pREM-A4 [24] contains the *omp26* gene inserted in the S-layer gene *sbsA*

from *Bacillus stearothermophilus* PV72. 400 ml of medium was inoculated with an overnight culture descending from a single transformant colony and used as a preculture for a laboratory-scale stirring batch fermenter (Meredos, Bovenden, Germany). Bacteria were grown in the fermenter in a volume of 10 litres with aeration and agitation until the culture reached an OD_{600} of 0.3. At this point the expression of SbsA/Omp26 was induced by a temperature upshift from 28°C to 42°C, or the expression of MBP/Omp26 was induced by the addition of 3mM isopropyl ß-D-thiogalactopyranoside (IPTG) (Sigma). Thirty min later gene *E* mediated lysis was induced from the vectors pDKL01 or pML1 by the addition of 2.5mM of toluolic acid or a temperature upshift from 28°C to 42°C, respectively. The course of lysis was monitored by measuring the OD_{600} at intervals of 15 min and light microscopy was performed on samples of the lysing culture. The efficiency of lysis was determined by viable cell counts prior to and at the end of the lysis process. Samples for western blot experiments (1.5 ml) were collected before induction and at the end of lysis. After complete lysis, cells were harvested by centrifugation at 5000×g. The ghosts were washed twice in a large volume of 0.85% saline and once in distilled water, and subsequently lyophilized for long-term storage.

SDS-PAGE and western blot analysis

SDS-PAGE and western blot analysis was performed as described previously.[24] Briefly, after centrifugation of the collected samples the resulting pellets were resuspended in reducing buffer, boiled for 5 min and separated on 7.5% polyacrylamide. Western immunoblotting was performed using the following primary antibodies: rabbit anti-SbsA 1/10000, rat anti-Omp26 1/100 and rabbit anti-MBP 1/10000. All were monoclonal except for anti-Omp26, which was a polyclonal antiserum to Omp26 derived from immunization with the protein alone.[20] The

secondary antibodies used were: goat anti-rabbit IgG 1/3000 and goat anti-rat IgG 1/10000 which were coupled to alkaline phosphatase and obtained from Sigma.

Antigens used for immunization

Recombinant Omp26 (rOmp26) was heterologously expressed in *E. coli* XL1-blue with a His-tag and subsequently purified using a Ni-nitrilotriacetic acid resin (Qiagen Pty Ltd, Clifton Hill, Victoria, Australia) under conditions recommended by the manufacturer. For intratracheal (IT) immunization, 20µg of rOmp26 in PBS was administered to each animal.

The recombinant bacterial ghosts either harboured Omp26 as a soluble protein in the periplasmic space (Ghosts/MBP/Omp26) or as part of an S-layer self assembly structure in the cytoplasm (Ghosts/SbsA/Omp26). Ghosts lacking Omp26 were used for control immunizations (Ghosts/MBP and Ghosts/SbsA). Freeze dried bacterial ghosts were resuspended in PBS for immunization. Depending on the immunization regime, 10µg or 20µg of Omp26 protein in bacterial ghosts were administered to each animal.

Killed whole cell (KWC) preparations of NTHi-289 were used in immunization experiments as positive controls. Bacteria were killed by suspension in 1% (wt/vol) paraformaldehyde in PBS and incubated at 37°C for 2 h. The bacteria were washed four times in PBS, and the concentration was adjusted to a bacterial equivalent of $2x10^{10}$ ml^{-1}. Bacteria were then emulsified in a 1:1 ratio with IFA so that each animal received approximately $5x10^{8}$ bacteria.

Immunization regimes

Specific pathogen free male DA rats, aged 8 to 10 weeks, were used for all immunization experiments. In a gut immunization regime rats were immunized on day 0 by

inoculation of intestinal Peyer´s patches (IPP), followed by a second immunization on day 14 into the duodenum (ID). In the combined gut/lung immunization regime these initial inoculations were followed by an intratracheal (IT) boost on day 21. The procedures for immunization were essentially the same as previously described.[19] Briefly, animals were sedated with sodium pentobarbital and the small intestine exposed through an abdominal incision. Each animal received the antigen prepared in a 50µl volume. Bacterial ghosts were resuspended in PBS, whereas the KWC NTHi was emulsified in PBS and IFA in a 1:1 ration for IPP immunization. For IPP immunizations, antigen was injected sub-serosally to each Peyer´s patch located along the wall of the small intestine. For ID immunizations the antigen (in 50µl) was injected directly into the lumen of the duodenum. For the IT boost, the rats were sedated with halothane and the antigen in sterile PBS was administered in a 50µl volume via a cannula inserted orally into the trachea. The antigen was dispersed with two 5ml volumes of air. The different immunization groups are shown in Table 1.

Bacterial challenge

Animals were challenged with live NTHi 28 days after the first immunization. Bacteria were prepared by overnight culture on chocolate agar plates at 37°C in 5% CO_2, washed and resuspended in PBS. The concentration of the inoculum was estimated by optical density at 405 nm and confirmed by counting the CFU of the overnight plating of serial dilutions of the inoculum. Animals were sedated with halothane, and $5x10^8$ CFU of live NTHi-289 in 50µl of PBS was introduced into the lungs via an intratracheal cannula and dispersed with two 5ml volumes of air. Four hours after the challenge the animals were killed by an intraperitoneal injection of pentobarbital sodium. Blood was collected by heart puncture and aliquots of the serum were stored at –20°C for antibody analysis. Lungs were lavaged with five 2ml volumes of PBS and the pooled bronchoalveolar lavage fluid (BAL) was

assessed for clearance by plating several serial dilutions of the washings for CFU determination. Lungs were removed following lavage and homogenized in 10ml of PBS, and bacterial counts were determined. The bacteria recovered were expressed as a percentage of that recovered from animals that where immunised with control bacterial ghosts. The spleen was collected and lymphocytes prepared for proliferation studies.

Antigen specific enzyme-linked immunosorbent assay (ELISAs)

The Omp26 specific ELISAs were performed as reported previously.[20] Briefly, ELISA plates were coated with 1 mg/ml of purified Omp26. Serial dilutions of rat sera taken at day 28 were examined for Omp26 specific IgG, IgG1 and IgG2a. The BAL was examined for the presence of Omp26 specific IgA. Horseradish peroxidase conjugated goat-anti-rat IgG (1/2000), goat-anti-rat IgG1 (1/500), goat-anti-rat IgG2a (1/500) or goat-anti-rat IgA (1/1000) were used as the secondary antibody.

Antigen specific lymphocyte assay

Lymphocytes were obtained from the spleen by passing tissue through a stainless steel sieve and washing in cold sterile PCM buffer prepared with PBS containing calcium and magnesium supplemented with 5% (vol/vol) fetal calf serum (heat inactivated at 57°C for 30 min), penicillin (100U ml^{-1}), streptomycin (100µg ml^{-1}) and amphotericin B (Fungizone; 0.25µg ml^{-1}). Viable cells were counted by trypan blue exclusion with a hemocytometer and resuspended in culture medium [Multicel RPMI 1640 (Cytosystem, Castle Hill, NSW Australia) containing 0.01M HEPES (pH 7.2), 0.05mM ß-mercaptoethanol, 2mM L-glutamine (ICN, Costa Mesa, California), 5% fetal calf serum, and penicillin-streptomycin-amphotericin B (as described above)] to obtain a final concentration of 10^6 cells ml^{-1}. The

antigen rOmp26 was resuspended in culture medium and sterile filtered to give final assay concentrations of 1 and 10µg ml^{-1}. The cell suspension and antigen were added in triplicate to flat-bottomed multiwell microculture plates (Nunc) to give a final volume of 0.2ml $well^{-1}$. Lymphocyte proliferation was estimated by [^{3}H]thymidine (Amersham) incorporated for the last 8 h of a 4 day culture. Results were calculated by subtraction of background from the mean of triplicate wells and these data were then expressed as dosage related stimulation index (SI) = (cpm experimental for 10µg Omp26/ cpm experimental for 1µg Omp26). Values represent the mean of each group ± standard error of the mean (SEM).

Statistical analysis

The bacterial clearance and antibody data were assessed for statistical significance by an independent *t* test (Macintosh Systat), and the lymphocyte proliferation data were assessed by a factorial analysis of variance (Macintosh Systat).

Results

Construction and expression of a MBP/Omp26 fusion protein

Expression from the vector pREM-26, harbouring the *malE-omp26* fusion under the control of LacPO, led to strong expression of MBP/Omp26 upon induction with IPTG. Compared to samples taken before induction of MBP/Omp26 expression, samples taken 2h after induction showed an additional protein band on SDS-PAGE. This additional band had an apparent molecular mass of 66 kDa and could be detected with anti-Omp26 as well as anti-MBP sera (data not shown).

Production of recombinant bacterial ghosts

E. coli pop2135 cells harbouring a combination of lysis- and expression plasmids were utilized for the production of bacterial ghosts. The expression of the Omp26 fusion proteins was induced prior to gene *E* mediated lysis. Onset of lysis was observed 30 min after induction of gene *E* expression by a decrease of OD_{600} from 0.43 to 0.22 or 0.42 to 0.28 in bacterial cultures expressing SbsA/Omp26 and MBP/Omp26, respectively. A shift from intact to lysed cells was also observed under the light microscope, where ghosts could clearly be distinguished from their living counterparts by their more transparent appearance. Loss of viability of the ghost preparation was assessed by viable cell counts. Colony forming units of *E. coli* expressing SbsA/Omp26 in the cytoplasm decreased from 1.52×10^8 ml^{-1} before induction of lysis to 4.25×10^4 ml^{-1} at the end of the lysis process, representing a lysis efficiency of 99.97%. *E. coli* expressing MBP/Omp26 in the periplasm showed a decrease of colony forming units from 1.8×10^8 ml^{-1} before induction of lysis to 2.2×10^6 ml^{-1} at the end of the lysis process, representing a lysis efficiency of 98.78%. Following the subsequent lyophilization procedure no viable bacteria were detected by culture of 10-fold immunization dose on agar plates. Expression of SbsA/Omp26 from the vector pREM-A4 30 min prior to induction of gene *E* mediated lysis revealed a band of about 155 kDa on a polyacryamide gel (the expected size of the construct), which reacted strongly with both anti-SbsA and anti-Omp26 sera (Fig. 1, lanes 4 and 5) in western blot. Expression of MBP/Omp26 from the vector pREM-26 resulted in an additional protein band of 66 kDa on a polyacrylamide gel, the expected size for the MBP/Omp26 fusion and which could be detected with anti-Omp26 sera (Fig. 1, lane 2). The total amount of Omp26 protein in the bacterial ghost preparations was quantified by densitometry from a western blot and was shown to be 6.3µg Omp26 mg^{-1} and 8.7µg mg^{-1} freeze dried ghosts for SbsA/Omp26 and MBP/Omp26, respectively.

Immunization with ghost preparations containing no NTHI antigen

Clearance of NTHI from the lungs of animals immunized with bacterial ghosts not expressing the Omp26 was compared to nonimmunized animals (data not shown). There was no significant clearance of bacteria in the BAL and lung homogenate in animals that received the gut immunization regime with Ghost/SbsA or Ghost/MBP preparations. However, in animals that received the combined gut/lung immunization regime, there was enhanced clearance in the Ghosts/SbsA group when compared with the nonimmunized animals. Therefore the clearance of bacteria from the lungs for each experimental group was compared against the relevant bacterial ghost control.

Bacterial clearance and immune responses following a gut immunization regime

The immune responses to bacterial ghosts harbouring the Omp26 protein were studied in a rat model, and their effectiveness was assessed by pulmonary challenge. When compared with their control groups, animals immunized with Ghosts/SbsA/Omp26 cleared approximately 50% of the bacterial load in the first four hours of infection as measured in both the BAL and the lung homogenate (Fig. 2A). In contrast, animals immunized with the Ghosts/MBP/Omp26 preparation only reduced the bacterial load in the lung homogenate (Fig. 2B). While the group that received Ghosts/SbsA/Omp26 showed a significant difference to both its control group (Ghosts/SbsA) and untreated animals, this was not the case for the Ghosts/MBP/Omp26 group which was only statistically different to untreated rats and not its control group.

The total number of white blood cells in the BAL was not significantly different for any of the immunized groups (data not shown).

Antibody to Omp26 was measured in the serum and BAL samples of rats by ELISA. High Omp26 specific antibody titers for IgG, IgG1 and IgG2a were found in serum of immunized animals (Fig. 3A, B and C). The highest levels were observed in the group

receiving Ghosts/MBP/Omp26 (IgG: 4523µg ml^{-1}, IgG2a: 1443µg ml^{-1}, IgG1: 35µg ml^{-1}) followed by the group immunized with Ghosts/SbsA/Omp26 (IgG: 2993µg ml^{-1}, IgG2a: 926µg ml^{-1}, IgG1: 31µg ml^{-1}). Control groups immunized with ghosts lacking Omp26 showed significantly lower levels of antibodies. In all groups much higher levels of IgG2a than of IgG1 were detected. No Omp26 specific IgA was found in the BAL for any of the immunized groups.

Bacterial clearance and immune responses following a gut/lung immunization regime

Previous results had shown that a boost delivered to the lungs was important in enhancing the immune response to the immunogen that effected enhanced clearance in this rat model.[19],[26] Immunization with killed NTHi to the gut followed by an Omp26 IT boost was used as a positive control to gauge the level of clearance observed with the Ghost/SbsA/Omp26 regimes. These animals significantly enhanced clearance of NTHi from the lungs when compared to untreated controls ($p<0.005$) and the level of clearance was still greater than that measured for the Ghost/SbsA control group (Fig. 4). The immunogenicity of recombinant bacterial ghosts with an Omp26 boost was tested in the rat model, and the effectiveness of the immune response was assessed by pulmonary challenge. Groups that received a gut immunization with the Ghost/SbsA/Omp26 preparation followed by an IT boost with Omp26 cleared the bacteria significantly better from the lung and the BAL of either the Ghost/SbsA immunized (Fig. 4) or untreated animals (not shown). In addition, animals immunized with Ghosts/SbsA followed by an IT boost with purified Omp26 or animals that received only an Omp26 IT boost did not differ from animals gut immunized with Ghost/SbsA and receiving a PBS IT boost (Fig. 4). Whether animals were immunized IPP/ID or ID/ID with Ghosts/SbsA/Omp26 did not make any statistically significant difference (Fig. 4).

Compared to the nonimmune control ($0.84x10^7 \pm 1.11$), animals immunized IPP/ID with killed whole cell NTHi ($1.71x10^7 \pm 0.22$), Ghosts/SbsA/Omp26 ($2.21x10^7 \pm 0.38$) or ID/ID with Ghosts/SbsA/Omp26 ($2.13x10^7 \pm 0.15$) showed a significant increase in the total number of white blood cells in the BAL.

Antibody to Omp26 was measured in serum and BAL samples of rats by ELISA. Omp26 specific antibody titers for IgG, IgG1 and IgG2a were found in serum (Fig. 5A, B and C), with the highest levels observed for the groups receiving Ghosts/SbsA/Omp26 followed by an IT boost with purified Omp26 (IgG: 6583µg ml^{-1}, IgG2a: 3353µg ml^{-1} and IgG1: 80µg ml^{-1}). In all groups much higher levels of IgG2a than of IgG1 were detected. Some Omp26 specific IgA was found in the BAL, again the highest levels in the groups immunized with Ghosts/SbsA/Omp26 (Fig. 5D).

Lymphocytes isolated from the spleen of rats immunized using the gut/lung regime, were set up in culture against rOmp26 protein. Cells from the Ghosts/SbsA/Omp26 immunized groups responded significantly to Omp26 and a difference in the response could be observed for the different dosages of coating antigen (Fig. 6). Cells immunized with Ghosts/SbsA also showed some proliferation to Omp26, however the difference in response to the different dosages was not significant (Fig. 6).

4.5. Discussion

In the present study, *Escherichia coli* ghosts harbouring the NTHi antigen Omp26 either as part of an S-layer fusion protein in the cytoplasm or as soluble protein in the periplasm were used for mucosal immunization studies. Rats immunized via inoculation of the Peyer`s patches and the duodenum showed a significant increase in Omp26 specific IgG, IgG1 and IgG2a titers in serum. Following pulmonary challenge with the homologous NTHi strain, only limited bacterial clearance was observed. Animals that received an additional

intratracheal boost with purified Omp26 showed very high levels of Omp26 specific IgG, IgG1 and IgG2a in serum and low levels of IgA in the BAL. In rats that received the Omp26 boost, bacteria were cleared more efficiently from both the lung and the BAL. Lymphocytes isolated from the spleen of immunized rats responded significantly to Omp26 in culture.

The natural assembly of S-layer proteins into large two-dimensional arrays endows them with immune stimulating and intrinsic adjuvant properties.[11,12] T-cell immunity to often unresponsive oligosaccharide haptens can be induced in Balb/C mice when the haptens are immobilized within crystalline S-layers.[11] Further, the use of S-layer proteins as a carrier for the birch pollen allergen has been reported to direct the immunological bias from an allergic Th2 mediated response to a more favourable Th0/Th1 response.[14] In this study, the Omp26 antigen was either immobilized in the cytoplasm of bacterial ghosts as part of an S-layer self assembly structure or as a soluble protein entrapped in the sealed periplasmic space of the ghost.[24] The amount of ghosts used for immunization was adjusted to equate to 10µg of Omp26 per animal. Animals immunized IPP/ID with either of the two ghost preparations containing Omp26 showed bacterial clearance when compared to the nonimmune group, however, the difference between the two groups was not significant. The Omp26 specific antibody titers were significantly higher in animals immunized with ghosts harbouring the Omp26 as a soluble protein in the periplasm and the ratio of IgG1 to IgG2a was similar for animals immunized with either of the two ghost preparations. In this particular immunization the S-layer does not appear to have a beneficial effect on the immunogenicity of Omp26 delivered in bacterial ghosts. However it is an efficient way of immobilizing antigens in bacterial ghosts and might be especially useful in regard to the development of combination vaccines.[2]

Since most diseases are caused by live microbes entering the host through mucosal membranes, the development of vaccine strategies that induce mucosal immunity is of considerable interest. There is a great demand for novel mucosal delivery systems, which can

be used to package and deliver a variety of antigens. It is well established that particulate antigens are more effective for oral immunization than soluble antigens.[27] Polylactide-co-glycolide (PLG) microparticles have been extensively studied and show great potential for mucosal immunization.[28] The potency of microparticles may be improved by their use in combination with additional adjuvants.[29] This favourable composition is naturally found in bacterial ghosts. On one hand, bacterial ghosts represent a particulate delivery vehicle and on the other hand they contain many immune stimulating compounds, such as LPS, lipid A and peptidoglycan.[30] As such they are able to non-specifically enhance immunity. We observed this in developing the protocols for this study where gut administration of bacterial ghosts without NTHi antigen was able to enhance to some extent the clearance of lung challenge with NTHi. This was particularly so for Ghosts/SbsA. It is possible that non-specific as well as the induction of specific cross-reactive responses explain this observation. It is known that *E. coli* has a number of outer membrane homologs with NTHI, for example OmpA and P5.[31] The potential of bacterial ghosts for mucosal immunization has been demonstrated in several studies using cell envelope preparations from *A. pleuropneumoniae, S. typhimurium, H. pylori, E. coli* or *V. Cholerae.*[4,6,7,8,10] In all these immunization experiments bacterial ghosts were administered without the addition of extra adjuvant.

In the current study *E. coli* ghosts harbouring Omp26 have been evaluated as a candidate vaccine using different mucosal immunization regimes in rats. To mimic an oral immunization but overcome the problems of abundant proteolytic enzymes and low acidity in the stomach in the absence of enteric formulation, animals were immunized via inoculation of the Peyer`s patches and the duodenum. This immunization regime induced high levels of Omp26 specific antibodies, however only moderate bacterial clearance was observed following pulmonary challenge. Animals that received double the amount of bacterial ghosts and an additional intratracheal boost with purified Omp26 induced even higher levels of antibodies and very efficient bacterial clearance of the challenge strain from both the lung and

the BAL. These results are in accordance with previous findings showing a combination of IPP/IT to be very successful for immunization with purified Omp26.[20,21] Bacterial clearance achieved by delivering Omp26 in bacterial ghosts without the addition of adjuvants is comparable to that seen following IPP/IT immunization with purified Omp26 formulated in incomplete Freund`s adjuvant (IFA). IPP/IT immunization with recombinant bacterial ghosts elicited slightly lower levels of Omp26 specific antibodies than immunization with purified Omp26. This is in accordance with previous studies on NTHi infections showing that correlation of antibody titers with protection is not consistent.[32] IgG2a appears to be the dominant isotype for both mucosal immunization with purified Omp26 [20] and mucosal immunization with recombinant bacterial ghosts, suggesting a Th1 type response. The induction of secretory IgA in the BAL was only seen in animals immunized via a combined gut/lung route. In another study, intragastric immunization with cholera toxin has been reported to result in IgA antibody restricted to the small intestine.[33] In rats, orally primed with *Pseudomonas aeruginosa*, a booster immunization in the lung seemed to be essential for a high antibody response in the respiratory tract.[34] The authors suggested that after seeding of antigen-reactive cells to distant mucosal sites, reexposure to the antigen at those distant mucosal sites may be important for an enhanced immune response, possibly because of local proliferation of antigen-reactive cells in response to the booster exposure to the antigen.[33] Mucosal delivery of Omp26 in bacterial ghosts followed by a boost with purified Omp26 led to a strong and specific humoral response and very efficient bacterial clearance of the challenge strain from the lung and the BAL. These results demonstrate the potential of bacterial ghosts as mucosal delivery system for the NTHi antigen Omp26.

The results presented in the current study provide evidence of the potential of bacterial ghosts to induce a primary immune response to an NTHi antigen. Further studies will investigate the feasibility of optimizing an oral immunization regime with the ghost technology. This will require appropriate formulation for oral vaccination to ensure passage

through the stomach without antigen degradation by the stomach acid or inappropriate dilution of the antigen bolus by the stomach contents.

Acknowledgements

The authors thank Dr Adam Smith and Dr Sara Gomez-Gallego for there advice; and Ms Nancy Fisher and Ms Claire Batum for their technical assistance with the mouse experiments.

References

1. Szostak MP, Lubitz W. Recombinant bacterial ghosts as multivaccine vehicles. In: Chanock RM, ed. Modern approaches to new vaccines including prevention of AIDS Vaccines 91 New York, Cold Spring Harbor Laboratory Press, 1991:409-14.
2. Lubitz W. Bacterial ghosts as carrier and targeting systems. Expert Opin Biol Ther 2001; 1:765-71.
3. Lubitz W, Witte A, Eko FO, Kamal M, Jechlinger W, Brand E. Extended recombinant bacterial ghost system. J Biotechnol 1999; 73:261-73.
4. Katinger A, Lubitz W, Szostak MP, Stadler M, Klein R, Indra A. Pigs aerogenously immunized with genetically inactivated (ghosts) or irradiated *Actinobacillus pleuropneumoniae* are protected against a homologous aerosol challenge despite differing in pulmonary and antibody responses. J Biotechnol 1999; 73:251-60.
5. Huter V, Hensel A, Brand E, Lubitz W. Improved protection against lung colonization by *Actinobacillus pleuropneumoniae* ghosts: characterization of a genetically inactivated vaccine. J Biotechnol 2000; 83:161-72.
6. Szostak MP, Hensel A, Eko FO, Klein R, Auer T, Mader H. Bacterial ghosts: non living candidate vaccines. J Biotechnol 1996; 44:161-70.
7. Panthel K, Jechlinger W, Matis A, Rodhe M, Szostak MP, Lubitz W. Generation of *Helicobacter pylori* ghosts by PhiX protein E-mediated inactivation and their evaluation as vaccine candidates. Infect Immun 2003; 71:109-16.
8. Eko FO, Lubitz W, McMillan L, Ramey K, Moore TT, Ananaba GA. Recombinant *Vibrio cholerae* ghosts as a delivery vehicle for vaccinating against *Chlamydia trachomatis*. Vaccine 2003; 21:1694-703.
9. Jechlinger W, Haidinger W, Paukner S, Mayrhofer P, Riedmann E, Marchart J. Bacterial ghosts as carrier and targeting systems for antigen delivery. In: Goebel GAaW, ed. Vaccine Delivery Strategies. Wymondham: Horizon Scientific Press, 2002:163-84.
10. Mayr UB, Haller C, Haidinger W, Atrasheuskaya A, Bukin E, Lubitz W. Bacterial ghosts as an oral vaccine: a single dose of Escherichia coli O157:H7 bacterial ghosts protects mice against lethal challenge. Infect Immun 2005; 73:4810-7.
11. Smith RH, Messner P, Lamontage LR, Sleytr UB, Unger FM. Induction of T-cell immunity to oligosaccharide antigens immobilized on cyrstalline bacterial surface layers (S-layers). Vaccine 1993; 11:919-24.
12. Messner P, Unger FM, Sleytr UB. Vaccine Development based on S-layer technology. In: Sleytr UB, Messner P, Pum D, Sara M, eds. Crystalline Bacterial Cell surface Proteins. Austin: R.G. Landes Company, 1996:161-73.
13. Messner P, Mazid MA, Unger FM, Sleytr UB. Artificial antigens. Synthetic carbohydrate haptens immobilized on crystalline bacterial surface layer glycoproteins. Carbohydr Res 1992; 233:175-84.
14. Jahn-Schmid B, Messner P, Unger FM, Sleytr UB, Scheiner O, Kraft D. Toward selective elictation of T_H1-controlled vaccination responses: vaccine applications bacterial surface layer proteins. J Biotechnol 1996; 44:225-31.
15. Foxwell AR, Kyd JM, Cripps AW. Nontypeable *Haemophilus influenzae*: pathogenesis and prevention. Microbiol Mol Biol Rev 1998; 62:294-308.
16. St Geme III JW. The pathogenesis of nontypeable *Haemophilus influenzae* otitis media. Vaccine 2001; 19:S41-S50.
17. Bolduc GR, Bouchet V, Jiang R-Z, Geisselsoder J, Truong-Bolduc QC, Rice PA. Variability of outer membrane protein P1 and its evaluation as a vaccine candidate against experimental otitis media due to nontypeable *Haemophilus influenzae* : an unambiguous multifaceted approach. Infect Immun 2000; 68:4505-17.
18. Kyd JM, Cripps AW. Modulation of antigen-specific T and B cell responses influence bacterial clearance of nontypeable *Haemophilus influenzae* from the lung in a rat model. Vaccine 1996; 14:1471-8.
19. Kyd JM, Dunkley ML, Cripps AW. Enhanced respiratory clearance of nontypeable *Haemophilus influenzae* following mucosal immunization with P6 in a rat model. Infect Immun 1995; 63:2931-40.
20. Kyd JM, Cripps AW. Potential of a novel protein, OMP26, from nontypeable *Haemophilus influenzae* to enhance pulmonary clearance in a rat model. Infect Immun 1998; 66:2272-8.
21. El-Adhami W, Kyd JM, Bastin DA, Cripps AW. Characterisation of the gene encoding a 26-kilodalton protein (OMP26) from nontypeable *Haemophilus influenzae* and immune responses to the recombinant protein. Infect Immun 1999; 67:1935-42.
22. Poolman JT, Bakaletz L, Cripps A, Danoël P, Forsgren A, Kyd J. Developing a nontypeable *Haemophilus influenzae* (NTHi) vaccine. Vaccine 2000; 19:S108 - S15.

23. Kyd JM, Cripps AW, Novotny LA, Bakaletz LO. Efficacy of the 26-kilodalton outer membrane protein and two P5 fimbrin-derived immunogens to induce clearance of nontypeable *Haemophilus influenzae* from the rat middle ear and lungs as well as from the chinchilla middle ear and nasopharynx. Infect Immun 2003; 71:4691-9.
24. Riedmann EM, Kyd JM, Smith AM, Gomez-Gallego S, Jalava K, Cripps AW. Construction of recombinant S-layer proteins (rSbsA) and their expression in bacterial ghosts--a delivery system for the nontypeable *Haemophilus influenzae* antigen Omp26. FEMS Immunol Med Microbiol 2003; 37:185-92.
25. Kloos DU, Stratz M, Guttler A, Steffan RJ, Timmis KN. Inducible cell lysis system for the study of natural transformation and environmental fate of DNA released by cell death. J Bacteriol 1994; 176:7352-61.
26. Wallace FJ, Clancy RL, Cripps AW. An animal model demonstration of enhanced clearance of nontypeable *Haemophilus influenzae* from the respiratory tract after antigen stimulation of the gut-associated lymphoid tissue. Am Rev Respir Dis 1989; 140:311-6.
27. O'Hagan DT. Microparticles and polymers for the mucosal delivery of vaccines. Adv Drug Del Rev 1998; 34:305-20.
28. Challacombe SJ, Rahman D, O'Hagan DT. Salivary, gut, vaginal and nasal antibody responses after oral immunization with biodegradable microparticles. Vaccine 1997; 15:169-75.
29. O'Hagan DT, MacKichan ML, Manmohan S. Recent development on adjuvants for vaccines against infectious diseases. Biomol Eng 2001; 18:69-85.
30. Jalava K, Eko FO, Riedmann E, Lubitz W. Bacterial ghosts as carrier and targeting systems for mucosal antigen delivery. Expert Rev Vaccines 2003; 2:45-51.
31. Webb DC, Cripps AW. A method for the purification and refolding of a recombinant form of the nontypeable Haemophilus influenzae P5 outer membrane protein fused to polyhistidine. Protein Expr Purif 1999; 15:1-7.
32. Cripps AW, Kyd JM. A vaccine for nontypable *Haemophilus influenzae*. In: Ellis R, Brodeur B, eds. New Bacterial Vaccines. New York: Kluwer Academic/Plenum Publishers, 2003:244-59.
33. Ogra PL, Faden H, Welliver RC. Vaccination strategies for mucosal immune responses. Clin Microbiol Rev 2001; 14:430-45.
34. Freihorst CE, Merick JM, Ogra PL. Effect of oral immunization with *Pseudomonal aeruginosa* on the development of specific antibacterial immunity in the lungs. Infect Immun 1989; 57:235-8.

563

564

565 **Table 1. Different antigens and routes used for immunization**

566

Immunization Groups (Antigen)	Day 0	Day 14	Day 21	Day28
Gut immunization regime (10µg Omp26 in Ghosts)				
NI	-	-	-	live NTHi challenge
Ghosts/SbsA	IPP	ID	-	live NTHi challenge
Ghosts/SbsA/Omp26	IPP	ID	-	live NTHi challenge
Ghosts/MBP	IPP	ID	-	live NTHi challenge
Ghosts/MBP/Omp26	IPP	ID	-	live NTHi challenge
Gut/Lung immunization regime (20µg Omp26 in Ghosts + 20µg rOmp26)				
NI	-	-	-	live NTHi challenge
Boost only	-	-	IT rOmp26	live NTHi challenge
KWC	IPP	ID	IT rOmp26	live NTHi challenge
Ghosts/SbsA	IPP	ID	-	live NTHi challenge
Ghosts/SbsA	IPP	ID	IT rOmp26	live NTHi challenge
Ghosts/SbsA/Omp26	IPP	ID	IT rOmp26	live NTHi challenge
Ghosts/SbsA/Omp26	ID	ID	IT rOmp26	live NTHi challenge

567

Figure Legends

Figure 1

Western blot analysis of whole cell and bacterial ghost protein extracts of *E. coli* pop2135. Molecular weight marker in kDa **(M)**. Cells prior to induction of the fusion proteins MBP/Omp26 **(lane 1)** and SbsA/Omp26 **(lane 3)**. Bacterial ghosts harbouring the fusion proteins MBP/Omp26 **(lane 2)** or SbsA/Omp26 **(lanes 4 and 5)**. The presence of the Omp26 epitope was demonstrated with native Omp26-specific antiserum and anti-rat IgG-AP conjugates **(lanes 1-4)**. The carrier protein SbsA was detected using SbsA-specific antiserum and anti-rabbit IgG-AP conjugates **(lane 5)**.

Figure 2

Bacterial recovery in the BAL **(A)** and lung homogenate **(B)** at 4h following pulmonary challenge with NTHI-289 following gut immunisation. Rats were immunized with either Ghost/SbsA expressing Omp26 (Ghost/SbsAOmp26) or Ghost/MBP expressing Omp26 (GhostsMBPOmp26) or their respect control ghosts not expressing Omp26. Bacterial recovery is measured as a percentage of that recovered in the respective control ghost immunized group. Each bar represents the mean±the standard error of the mean for samples from 4 or 5 animals. *Significance of $P<0.05$.

Figure 3

Omp26 specific levels of IgG **(A)**, IgG1 **(B)** and IgG2a **(C)** in serum samples of an untreated control group **(I)** and animals immunized with Ghosts/SbsA **(II)**, Ghosts/SbsA/Omp26 **(III)**, Ghosts/MBP **(IV)** or Ghosts/MBP/Omp26 **(V)**. Values represent means ± SEM for serum from four to five rats per group. $* P < 0.05$, $** P < 0.005$ significant against nonimmune data.

Figure 4

Bacterial recovery in the BAL **(A)** and lung homogenate **(B)** at 4h following pulmonary challenge with NTHI-289 following gut/lung immunisation. Immunization antigens and route for the primary, secondary and tertiary immunizations are shown in the table under each bar (§ primary immunization was by ID). Bacterial recovery is measured as a percentage of that recovered in the GhostSbsA control group that received a PBS boost. Each bar represents the mean±the standard error of the mean for samples from 4 or 5 animals. *Significance of P<0.005 compared with untreated animals, # P<0.05 with the GhostSbsA control group.

Figure 5

Omp26 specific levels of IgG **(A)**, IgG1 **(B)** and IgG2a **(C)** in serum, or IgA **(D)** in BAL samples of an untreated control group **(I)** and animals immunized with Omp26 IT **(II)**, KWC NTHi-289 IPP/ID + Omp26 IT **(III)**, Ghosts/SbsA IPP/ID **(IV)**, Ghosts/SbsA IPP/ID + Omp26 IT **(V)**, Ghosts/SbsA/Omp26 IPP/ID + Omp26 IT **(VI)**, Ghosts/SbsA/$Omp26_{878}$ ID/ID + Omp26 IT **(VII)**. Values represent means ± SEM for serum from four to five rats per group. $* P < 0.05$, $** P < 0.005$ significant against nonimmune data.

Figure 6

Antigen specific proliferation of lymphocytes isolated from the spleen of gut/lung immunized rats. Shown are lymphocyte responses to culture with Omp26 of untreated animals **(A)** and animals immunized with rOmp26 IT **(B)**, KWC NTHi IPP/ID rOmp26 IT **(C)**, Ghosts/SbsA IPP/ID rOmp26 IT **(D)**, Ghosts/SbsA/Omp26 IPP/ID rOmp26 IT **(E)** or Ghosts/SbsA/Omp26 ID/ID rOmp26 IT **(F)**. Data are expressed and calculated as dosage related stimulation index (SI) = (cpm experimental for 10µg Omp26/ cpm experimental for 1µg Omp26). Values represent the mean of each group ± SEM. $* P < 0.05$, $** P < 0.005$ significant against nonimmune data.