Immunogenicity and protective potential of *Bordetella pertussis* biofilm and its associated antigens in a murine model

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Abstract

The resurgence of whooping cough reflects novel genetic variants of *Bordetella pertussis* and inadequate protection conferred by current acellular vaccines (aP). Biofilm is a source of novel vaccine candidates, including membrane protein assembly factor (BamB) and lipopolysaccharide assembly protein (LptD). Responses of BALB/c mice to candidate vaccines included IFN-γ and IL-17a production by spleen and lymph node cells, and serum IgG1 and IgG2a reactive with whole bacteria or aP. Protection was determined using bacterial cultured from lungs of vaccinated mice challenged with virulent *B. pertussis*. Mice vaccinated with biofilm produced efficient IFN-γ responses and more IL-17a and IgG2a than mice vaccinated with planktonic cells, aP or adjuvant alone. Vaccination with aP produced abundant IgG1 with little IgG2a. Mice vaccinated with aP plus BamB and LptD retained lower bacterial loads than mice vaccinated with aP alone. Whooping cough vaccines formulated with biofilm antigens, including BamB and LptD, may have clinical value.

Key words: *Bordetella pertussis* vaccine, biofilm, biofilm-associated proteins, whooping cough

1. Introduction

An ideal whooping cough vaccine would induce Th1 and Th17 immune responses, as well as antibodies [1]. The first-generation whole-cell pertussis (wP) vaccines did this, but were associated with adverse reactions including neurological disorders in some children [2]. Safer, second generation acellular pertussis (aP) vaccines induce potent IgG1 antibody in children, consistent with a Th2 response, with immunity waning after 5 years [3] and poor induction of memory B-cells [4, 5]. T-cell responses are not increased by aP boosters in aP-primed children, while responses of wP-primed children are boosted by aP or natural infections [6, 7]. Suboptimal responses to aP vaccination may favour the emergence of genetically distinct variants of *B. pertussis* [8]. Strains with polymorphisms in genes encoding pertactin (Prn) and pertussis toxin (Ptx) were described in 1998 [9] and constitute a novel "P3" lineage that is now common in most countries. Alarmingly, the "P3" lineage is antigenically distinct from vaccine strains and produces higher levels of toxin [10, 11]. Strains that do not produce Prn are also found in most developed countries [12-14], including Australia [15]. These findings illustrate the need for improved whooping cough vaccines that can stimulate potent T-cell responses and confer long lasting immunity.

All currently available pertussis vaccines are formulated with antigens derived from planktonic bacterial cells. However, new variants of circulating *B. pertussis* isolates form biofilms more readily than the common vaccine strain *B. pertussis* Tohama I [16-18]. Biofilm formation may enhance the virulence and persistence of *B. pertussis* in the human nasopharynx, facilitating transmission to susceptible infants [18-20]. *Bordetella* intermediate protein A (BipA) was the only *B. pertussis* biofilm-derived membrane protein shown to protect mice against virulent *B. pertussis* infection [21].

Our study explored responses to pertussis biofilm vaccination and novel candidate whooping cough vaccine antigens. Production of cytokines (IFN-γ, IL-17a) and antibodies (IgG1, IgG2a) was followed in mice immunized with biofilm, planktonic cells, a current aP vaccine or the adjuvant alone. We also tested two proteins upregulated in biofilm, outer membrane protein assembly factor (BamB) and lipopolysaccharide assembly protein (LptD). Expression of BamB was upregulated in the biofilm of the clinical isolate ID20, while LptD was upregulated in the biofilm of Tohama I and ID20, relative to Tohama I planktonic cells [16]. The ability of our vaccine candidates to protect mice against infection with a new variant of *B. pertussis* carrying *ptxP3* allele was investigated.

2. Materials and Methods

2.1 Mice

Male BALB/c mice were obtained from Animal Resources Centre (Murdoch University, Western Australia) at 6-8 weeks of age and maintained under pathogen-free conditions. All animal experiments were approved by Curtin University's Animal Ethics Committee and conducted in accordance with the Australian Animal Welfare Act 2002.

2.2 Bacterial strains and growth conditions

Bordetella pertussis strains were grown on Bordet-Gengou (BG) agar (Becton Dickinson, Sparks, MD) supplemented with 15% sheep blood, charcoal agar (Thermo Scientific, Waltham, MA) or Stainer-Scholte (SS) broth. Mice were challenged with live *B. pertussis* Tohama I or clinical isolate ID20, the strongest biofilm producer in our previous study [16].

Escherichia coli DH5α (New England Biolabs, Ipswich, MA), *Escherichia coli* BL21-DE3 and electro-competent *E. coli* with the pETM-11 vector for expression of recombinant proteins were grown on Luria Bertani agar containing 1% tryptone, 0.5% yeast extract (Oxoid, Hampshire, UK), 1% NaCl and 1.5% bacteriological agar with or without 50 μg/mL kanamycin (Biochemicals, Taren Point, Australia) and 100 μg/mL chloramphenicol (Sigma, St. Louis, MO).

2.3 Cloning and purification of recombinant BamB (rBamB) and LptD (rLptD)

A 685 nucleotide fragment of *bamB* (bases 396-1080) and a 2166 nucleotide fragment of *lptD* (bases 61-2166) were amplified using primers designed using NEBuilder® Assembly tool v1.12.15 (New England Biolabs, Ipswich, MA). PCR amplification was carried out using Q5® Hi-Fidelity DNA polymerase (New England Biolabs). Amplicons were excised from 1% agarose gels, purified (Bioline, Alexandria, Australia) and cloned into the *Bam*HI and *Nco*I sites of pETM-11 using the NEBuilder® HiFi DNA Assembly cloning kit (New England Biolabs), to generate N-terminal His_6-tagged rBamB and rLptD. *E. coli* BL21-DE3 expression hosts were transformed and plated on to agar containing 50 μg/mL and 30 μg/mL kanamycin and chloramphenicol, respectively. Carriage of the insert was verified by DNA restriction analysis (*Sph*I restriction enzyme for pETM11-BamB, and *Xba*I and *Bsa*I for pETM11-LptD) and sequencing. Expression of recombinant proteins was induced by overnight culture at 18°C with 0.4mM isopropyl-β-D-1-thiogalactopyranoside (IPTG; Sigma). Cells were harvested by centrifugation and resuspended in wash buffer containing 20 mM sodium phosphate, 500 mM

NaCl and 20mM imidazole with 1 mM PMSF (Sigma). The cells were disrupted using a high pressure instrument (Constant System Ltd, Northants, UK) at 30kPa. Lysates were centrifuged (30,000g, 30 min, 4°C) and supernatant protein was purified using Ni-NTA sepharose® (Qiagen, Nutley, NJ), followed by size exclusion chromatography (Sepharose, 200 on an AKTApurified™ 10 FPLC system, GE Healthcare, Uppsala, Sweden). Protein was identified at A_{280}, positive fractions were pooled and purity was determined by 1D-PAGE using gradient 4-12% Novex® NuPAGE® Bis-Tris protein gels (Invitrogen, Carlsbad, CA). Identity was confirmed by mass spectroscopy (Proteomics International, Western Australia). Results are shown in Supplementary Figures 1 and 2.

2.4 Preparation of vaccines

Planktonic cells and biofilms were prepared as described previously [16, 21]. Briefly, bacterial cells were cultured for 18 h in SS broth at 37°C with shaking at 180 rpm, washed 3 times with PBS and planktonic cells harvested by centrifugation (8,000 g, 20 min). To prepare biofilm, ~200 ml of planktonic cultures were placed in a tissue culture flask and allowed to form biofilm at 37°C for 96 h. The supernatants were gently removed and the attached biofilm was washed three times with PBS. It was then harvested by scraping from the surface of the flasks into sterile PBS and collected by centrifugation (10,000g, 10 min). Both biofilm and planktonic preparations were adjusted to A600=0.1. Colony forming counts (CFU) were determined using 10 fold dilutions in triplicate.

2.5 Experimental design

To investigate immunogenicity, groups of 6 mice were immunised subcutaneously on days 0 and 14 with 10^7 and 10^9 CFU/mL (respectively) *B. pertussis* planktonic bacteria or biofilm, that had been heat (56°C) inactivated and mixed 1:1 with alum adjuvant (Imject™; ThermoFisher Scientific)]. A third group was immunised with 1/50th of the human dose of the commercially available hexavalent acellular vaccine Infanrix® hexa (DTaP; GlaxoSmithKline) [21]. Control mice received Imject alone. On day 24 and 35, three mice from each group were sacrificed to collect blood, spleens and lymph nodes (LN). The days were based on an optimization study where day 24 represents the peak response and day 35 assesses persistence.

To investigate protection, groups of 7 mice were immunized (as described above) and challenged on day 24 with 2x10^7 CFU of virulent *B. pertussis* Tohama I or the ID20 resurgent strain of *B. pertussis* which carries the *ptxP3* allele [16]. Mice were lightly sedated with Isoflurane (Parimal

Critical Care, Bethlehem, PA) and 40 μL of bacterial suspension was instilled slowly into their nostrils [21]. Mice were weighed and health monitored twice daily by a single observer. Seven days after challenge, lungs were harvested into sterile SS broth. Ten-fold serial dilutions of lung homogenates were inoculated on to charcoal agar plates to determine bacterial loads. Blood was collected and serum was stored at -80°C until required.

2.6 Quantitation of pertussis-reactive IgG1 and IgG2a

The enzyme-linked immunosorbent assay (ELISA) was adapted from Raymonds et al [22]. Briefly, 96-well plates were coated overnight with A_{600} = 0.1 of heat-inactivated *B. pertussis*. Plates were washed with phosphate buffered saline (PBS) containing 0.05% Tween-20 (BDH Prolabo, Fontenay-sous-Bois, France) and blocked with 5% bovine serum albumin (BSA; Bovogen Biologicals, Keilor, Australia). Four serial dilutions of mouse sera were added for 2h at room temperature, followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1 or IgG2a (Abcam, Melbourne, Australia) for 1h. After washing, 3,3',5,5'–tetramethylbenzidine (TMB; Sigma) containing 1 μL H_2O_2 (Sigma) was dispensed into each well (15 or 30 min for IgG1 and IgG2a, respectively). Reactions were stopped with 25 μL 1M H_2SO_4 and absorbance was measured at 450 nm. To assess antibodies reactive with DTaP antigens, plates were coated with 5 μg/mL DTaP vaccine (overnight, 4°C) and developed as described above. Standards and quality control samples were separate pools of high titre sera assigned values as arbitrary units per mL (AU/mL). Antibody concentrations were determined from standard curves using least-squares curve-fitting.

2.7 Quantitation of spleen and lymph node cells producing IFN-γ or IL-17a

Spleen and LN cells were separated using a mesh, washed and resuspended in 3 mL RPMI-1640 with 250 mM ammonium chloride (Sigma) (on ice, 2 min). Cells were washed twice, resuspended in 1 mL culture medium (RPMI-1640 with 10% foetal calf serum, 100 μg/mL streptomycin, 100 U penicillin/mL) and viability was determined by Trypan blue exclusion. For enzyme linked immunosorbent spot (ELISPOT) assays, 100 μL cells were transferred to an equivalent volume of medium containing antigens to obtain $2.5x10^5$ cells/well. Production of IFN-γ and IL-17a was assessed using Mouse IFN-γ and IL-17a ELISPOT Ready-SET-GO!® kits (eBioscience, San Diego, CA). Following stimulation with heat-inactivated biofilm or planktonic bacteria, DTaP, rBamB or rLptD, spots were counted using an automated ELISPOT plate reader and data analysed using EliSpot2.9 software (Autoimmun Diagnostica, Strassberg, Germany). Images were checked and results are reported as spot-forming units (SFU) / $2x10^5$ cells.

2.8 Statistical analyses

Two-tailed Mann-Whitney *U* tests were used to compare bacterial loads in the lungs of mice (n=7/group). Unpaired Student's t-tests were used to compare immunological data. All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). $P \leq 0.05$ was accepted as a significant difference.

3. Results

3.1 Vaccination with *Bordetella pertussis* biofilm generates optimal IFN-γ and IL-17a responses to whole bacteria

Groups of 6 mice were vaccinated with inactivated *B. pertussis* planktonic bacteria, biofilm, or DTaP. Control mice received Imject (alum adjuvant) alone and responses to vaccines were assessed relative to this group. Spleen and LN cultures were stimulated with planktonic cells, biofilm, DTaP, rBamB or rLptD. Cytokine profiles were determined using ELISPOT assays applied to 3 mice / group on days 24 and 35. rBamB did not induce IFN-γ or IL-17a in cells from any vaccinated mice (<3 SFU/2x10^5 cells, data not shown). However efficient IFN-γ and IL17a production was evident when splenocytes from vaccinated mice were stimulated with planktonic cells or biofilm (Figures 1A, 1B, 2A, 2B).

When stimulated with planktonic bacteria, splenocytes of mice vaccinated with biofilm displayed more IFN-γ SFU on day 24 than mice vaccinated with planktonic bacteria (P=0.006), DTaP (P =0.013) or Imject (P <0.001). Biofilm vaccination remained superior to DTaP at day 35 (P=0.004; Figure 1A). Similarly following stimulation with biofilm, splenocytes of mice vaccinated with biofilm displayed more IFN-γ SFU on day 24 than mice vaccinated with planktonic bacteria (P=0.04), DTaP (P=0.006) or Imject (P <0.001). Biofilm vaccination remained superior to DTaP (P =0.007) and Imject (P=0.02) at day 35; Figure 1B).

When cultures were stimulated with DTaP, the differences were less clear but mice vaccinated with biofilm produced more IFN-γ than control mice on day 24 (P=0.001; Figure 1C). Whilst rBamB did not induce significant production of IFN-γ in splenocytes from any vaccinated mice (data not shown), rLptD stimulated IFN-γ production by splenocytes of mice vaccinated with planktonic bacteria (P=0.005), biofilm (P=0.001) or DTaP (P=0.01) compared to control mice, with no significant differences between the vaccines (Figure 1D).

When stimulated with planktonic bacteria, splenocytes of mice vaccinated with biofilm also displayed more IL-17a SFU on day 24 than mice vaccinated with planktonic bacteria (P =0.012), DTaP (P =0.01) or Imject (P <0.001). Biofilm vaccination remained superior to DTaP (P=0.028) and Imject (P=0.032) at day 35 (Figure 2A). Similarly following stimulation with biofilm (Figure 2B), splenocytes of mice vaccinated with biofilm displayed more IL-17a SFU on day 24 than mice vaccinated with planktonic bacteria (P=0.001), DTaP (P=0.001) or Imject (P=0.002). Biofilm vaccination again remained superior to DTaP (P=0.001) and Imject (P=0.001) at day 35.

As with IFN-γ, IL-17a production was low and similar in all cultures stimulated with DTaP (Figure 2C). rLptD induced significant IL-17a production in splenocytes of mice vaccinated with biofilm (P=0.01, Figure 2D), but low values precluded other analyses.

LN cells from each group of mice were pooled to obtain enough cells to test all antigens, so no statistical analyses were possible. However on day 35, LN cells from biofilm-vaccinated mice produced several fold more IFN-γ and IL-17a SFU than cells from mice vaccinated with planktonic bacteria, DTaP or Imject (Table 1).

3.2 A predominance of IgG2a links biofilm vaccination with an efficient Th1 response.

In mice, IgG1 reflects a Th2 response, while IgG2a is associated with Th1 [23]. When assayed in ELISA plates coated with heat-killed planktonic bacteria, mice vaccinated with planktonic bacteria or biofilm produced higher IgG1 responses at day 24 and 35, than mice vaccinated with DTaP or Imject (Figure 3A). IgG1 responses to planktonic bacteria and biofilm were similar, but biofilm-vaccinated mice produced markedly higher levels of IgG2a than other groups (Figures 3B). An ELISA based on DTaP antigen confirmed that mice vaccinated with DTaP produced higher levels of IgG1 antibody than mice immunized with planktonic bacteria or biofilm (Figure 3C, P<0.001). No IgG2a was detected with this assay (data not shown).

3.3 *Bordetella pertussis* biofilm and associated proteins confers protection.

Given the strong Th1, Th17 and IgG2a responses to *B. pertussis* biofilms, groups of 7 mice were vaccinated twice over 2 weeks with biofilm, planktonic cells, DTaP or Imject and challenged with virulent *B. pertussis* Tohama I. Mice vaccinated with biofilm had lower bacterial loads in

their lungs than mice vaccinated with DTaP (P=0.02) or Imject (P=0.002) (Figure 4A). There was no difference between mice given biofilm or planktonic bacteria.

The recent clinical isolate ID20 carries the *ptxP3* allele and Prn variant, distinct from Tohama I [16]. Mice were vaccinated with planktonic cells or biofilm (from Tohama I), DTaP, rBamB or rLptD (alone or with DTaP), and challenged with ID20. Vaccination with planktonic bacteria or biofilm reduced bacterial loads in the lungs compared to Imject-vaccinated mice (P<0.05; Figure 4B).

3.4 Vaccination with rBamB or rLptD protects mice against ID20.

Vaccination with rBamB or rLptD reduced bacterial loads in the lungs compared to Imject control mice (P=0.003, P<0.001, respectively; Figure 4B). Moreover, a combined formulation of rBamB-rLptD-DTaP was a more potent vaccine against ID20, with lower bacterial loads than achieved with DTaP (P=0.02). Six days after challenge with ID20, all signs of illness had resolved in mice vaccinated with planktonic bacteria, biofilm or rBamB-rLptD-DTaP, but symptoms persisted in mice given DTaP, rLptD, rBamB or Imject alone (Table 2). Hence, both bacterial loads and the health of the mice indicate that rBamB and rLptD enhance the protective efficacy of DTaP.

4. Discussion

Current whooping cough vaccines are formulated with antigens expressed in planktonic bacteria, so biofilm may offer novel protective antigens. This may be attributed to different constituents of biofilm matrix. For example, extracellular DNA has been identified as one of the major components of *B. pertussis* biofilm [24]. Protection against *B. pertussis* biofilm may interrupt the persistence and spread of infections *in vivo* [19]], but we are aware of only one *B. pertussis* biofilm vaccine trial [21]. This is critical because 5% of proteins were upregulated and 3.5% are downregulated in biofilm cultures of clinical isolate ID20 [16]. Though other antigens warrant similar studies, we selected BamB and LptD as candidate antigens in our study since they were upregulated in biofilm of Tohama I and ID20.

The data support evidence from mice [1] and humans [25] that current aP vaccines induce predominantly Th2 responses. *Bordetella pertussis* biofilm induced higher IgG1 levels and up to 50-fold more IgG2a compared to vaccination with DTaP or Imject alone (Figure 3A and B). An ELISA detecting DTaP-specific antibody responses confirmed that mice vaccinated with

DTaP produce IgG1 but not IgG2a (Figure 3C). Splenocytes from biofilm-vaccinated mice produced more IFN-γ and IL-17a than planktonic-, DTaP- or Imject-vaccinated mice (Figures 1 and 2). Biofilm vaccination also induced higher responses by LN cells on Day 35 (Table 2). Induction of IFN-γ by biofilm vaccination was also demonstrated by high serum levels of the chemokine CXCL10 in a pilot study of mice challenged with live bacteria (data not shown).

Having demonstrated the immunogenicity of the biofilm and associated proteins, we assessed protection. Here biofilm vaccination reduced bacterial loads more effectively than DTaP. Mice vaccinated with rBamB or rLptD alone had significantly lower bacterial loads than control mice ($P<0.05$, Figure 4B). However, a rBamB-rLptD-DTaP formulation enhanced the protective efficacy over DTaP alone and had an acceptable safety profile (Figure 4B, Table 2). This is a novel and important finding. de-Gouw et al [21] showed that vaccination of mice with the biofilm protein, BipA, reduced colonisation of the lungs following virulent *B. pertussis* challenge. However the authors did not address the effect of BipA on the protective potential of DTaP.

In summary, a vaccine comprising inactivated *B. pertussis* biofilm may induce a protective immune response against resurgent *B. pertussis* strains, as assessed by IFN-γ, IL-17a or IgG2a. Our planktonic cell vaccine induced lower IFN-γ and IgG2a responses than biofilm. The current aP vaccine, Infanrix® hexa (DTaP), induces predominantly IgG1 antibody response with little or no detectable IgG2a, IFN-γ or IL-17a. Importantly, DTaP may be sub-optimal in protecting against infection with a resurgent strain, but formulation of DTaP with BamB and LptD may enhance the protective potential of DTaP against the resurgent strains of *B. pertussis*.

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Figure legends

Figure 1. IFN-γ production in mice vaccinated with B. pertussis vaccine candidates. Splenocytes of vaccinated mice were stimulated with planktonic cells (A), biofilm (B), DTaP (C) or rLptD (D) and IFN-γ production was assessed by ELISpot. Each bar represents n=3 mice. Results are presented as spot forming units (SFU) per $2x10^5$ cells (median ± range). D24, D35: day 24, day 35. Vxn: vaccination. Horizontal lines represent significant differences between groups (Unpaired Student's t-tests, $P<0.05$).

Figure 2. IL-17a production in mice vaccinated with *B. pertussis* vaccine candidates. Splenocytes of vaccinated mice were stimulated with planktonic cells (A), biofilm (B), DTaP (C) or rLptD (D) and IL-17a production was assessed by ELISpot. Each bar represents n=3 mice. Results are presented as spot forming units (SFU) per $2x10^5$ cells (median ± range). D24, D35: day 24, day 35. Vxn: vaccination. Horizontal lines represent significant differences between groups (Unpaired Student's t-tests, $P<0.05$).

Figure 3. Humoral responses to *Bordetella pertussis* in vaccinated mice.
Sera from vaccinated mice assessed using ELISA plates coated with heat-killed planktonic bacteria to determine levels of IgG1 (A) and IgG2a (B) antibody, and using plates coated with DTaP to assess IgG1 (C) and IgG2a (not shown). Columns represent median ± range for 3 mice/group. D24, D35: day 24, day 35. Vxn: vaccination. Horizontal lines represent significant differences between groups (Mann-Whitney, $P<0.05$).

Figure 4. Protective potential of *Bordetella pertussis* biofilm and associated proteins.
Mice were immunised on days 0 and 14 with vaccine candidates and challenged with Tohama I (A) or clinical isolate ID20 (B) on day 24. Bacterial loads were determined from lung homogenates collected 7 days post-challenge. Each symbol represents the bacterial load of one mouse. Median values indicated. Horizontal lines represent significant differences between groups (Mann-Whitney, $P<0.05$).

Supplementary figure legends

Supplementary Figure 1. Cloning and purification of rBamB.

The *bamB* gene was cloned into the pETM-11 vector and confirmed by PCR using *bamB* or pETM-11 primers in DE3-BL21 *E. coli* cells. Carriage of the *bamB* insert was verified by *Sph*I restriction (A) and nucleotide sequencing of the insert. AKTA-purified rBamB was separated on SDS-gel (B) and protein identification of purified rBamB was performed with MS using MSPnr100 database search (C) at Proteomics International, Western Australia.

Supplementary Figure 2. Cloning and purification of rLpTD

The *lptD* gene was cloned into the pETM-11 vector and confirmed by PCR using *lptD* or pETM-11 specific PCR in DE3-BL21 *E. coli* cells (A). Carriage of the insert was verified by restriction with *Xba*I and *Bsa*I (A) and nucleotide sequencing of the insert. AKTA-purified rLptD was separated on SDS-gel (B) and protein identification of purified rLptD was performed with MS using MSPnr100 database search (C) at Proteomics International, Western Australia.

The resurgence of whooping cough reflects inadequate protection conferred by current acellular vaccines (aP). Biofilm has been suggested as a source of immunogenic proteins with potential as vaccines, but few have been tested. We have identified two candidates. This work establishes their potential as components of a more effective vaccine.

- Mice vaccinated with biofilm produced efficient IFN-γ responses and more IL-17a and IgG2a than mice vaccinated with planktonic cells, aP or adjuvant alone.
- Vaccination with aP produced abundant IgG1 with little IgG2a confirming a Th2 bias
- Mice vaccinated with aP plus BamB and LptD retained lower bacterial loads than mice vaccinated with aP alone, and displayed better health.

Immunogenicity and protective potential of *Bordetella pertussis* biofilm and its associated antigens in a murine model

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Abstract

The resurgence of whooping cough reflects novel genetic variants of *Bordetella pertussis* and inadequate protection conferred by current acellular vaccines (aP). Biofilm is a source of novel vaccine candidates, including membrane protein assembly factor (BamB) and lipopolysaccharide assembly protein (LptD). Responses of BALB/c mice to candidate vaccines included IFN-γ and IL-17a production by spleen and lymph node cells, and serum IgG1 and IgG2a reactive with whole bacteria or aP. Protection was determined using bacterial cultured from lungs of vaccinated mice challenged with virulent *B. pertussis*. Mice vaccinated with biofilm produced efficient IFN-γ responses and more IL-17a and IgG2a than mice vaccinated with planktonic cells, aP or adjuvant alone. Vaccination with aP produced abundant IgG1 with little IgG2a. Mice vaccinated with aP plus BamB and LptD retained lower bacterial loads than mice vaccinated with aP alone. Whooping cough vaccines formulated with biofilm antigens, including BamB and LptD, may have clinical value.

Key words: *Bordetella pertussis* vaccine, biofilm, biofilm-associated proteins, whooping cough

1. Introduction

An ideal whooping cough vaccine would induce Th1 and Th17 immune responses, as well as antibodies [1]. The first-generation whole-cell pertussis (wP) vaccines did this, but were associated with adverse reactions including neurological disorders in some children [2]. Safer, second generation acellular pertussis (aP) vaccines induce potent IgG1 antibody in children, consistent with a Th2 response, with immunity waning after 5 years [3] and poor induction of memory B-cells [4, 5]. T-cell responses are not increased by aP boosters in aP-primed children, while responses of wP-primed children are boosted by aP or natural infections [6, 7]. Suboptimal responses to aP vaccination may favour the emergence of genetically distinct variants of *B. pertussis* [8]. Strains with polymorphisms in genes encoding pertactin (Prn) and pertussis toxin (Ptx) were described in 1998 [9] and constitute a novel "P3" lineage that is now common in most countries. Alarmingly, the "P3" lineage is antigenically distinct from vaccine strains and produces higher levels of toxin [10, 11]. Strains that do not produce Prn are also found in most developed countries [12-14], including Australia [15]. These findings illustrate the need for improved whooping cough vaccines that can stimulate potent T-cell responses and confer long lasting immunity.

All currently available pertussis vaccines are formulated with antigens derived from planktonic bacterial cells. However, new variants of circulating *B. pertussis* isolates form biofilms more readily than the common vaccine strain *B. pertussis* Tohama I [16-18]. Biofilm formation may enhance the virulence and persistence of *B. pertussis* in the human nasopharynx, facilitating transmission to susceptible infants [18-20]. *Bordetella* intermediate protein A (BipA) was the only *B. pertussis* biofilm-derived membrane protein shown to protect mice against virulent *B. pertussis* infection [21].

Our study explored responses to pertussis biofilm vaccination and novel candidate whooping cough vaccine antigens. Production of cytokines (IFN-γ, IL-17a) and antibodies (IgG1, IgG2a) was followed in mice immunized with biofilm, planktonic cells, a current aP vaccine or the adjuvant alone. We also tested two proteins upregulated in biofilm, outer membrane protein assembly factor (BamB) and lipopolysaccharide assembly protein (LptD). Expression of BamB was upregulated in the biofilm of the clinical isolate ID20, while LptD was upregulated in the biofilm of Tohama I and ID20, relative to Tohama I planktonic cells [16]. The ability of our vaccine candidates to protect mice against infection with a new variant of *B. pertussis* carrying *ptxP3* allele was investigated.

2. Materials and Methods

2.1 Mice

Male BALB/c mice were obtained from Animal Resources Centre (Murdoch University, Western Australia) at 6-8 weeks of age and maintained under pathogen-free conditions. All animal experiments were approved by Curtin University's Animal Ethics Committee and conducted in accordance with the Australian Animal Welfare Act 2002.

2.2 Bacterial strains and growth conditions

Bordetella pertussis strains were grown on Bordet-Gengou (BG) agar (Becton Dickinson, Sparks, MD) supplemented with 15% sheep blood, charcoal agar (Thermo Scientific, Waltham, MA) or Stainer-Scholte (SS) broth. Mice were challenged with live *B. pertussis* Tohama I or clinical isolate ID20, the strongest biofilm producer in our previous study [16].

Escherichia coli DH5α (New England Biolabs, Ipswich, MA), *Escherichia coli* BL21-DE3 and electro-competent *E. coli* with the pETM-11 vector for expression of recombinant proteins were grown on Luria Bertani agar containing 1% tryptone, 0.5% yeast extract (Oxoid, Hampshire, UK), 1% NaCl and 1.5% bacteriological agar with or without 50 μg/mL kanamycin (Biochemicals, Taren Point, Australia) and 100 μg/mL chloramphenicol (Sigma, St. Louis, MO).

2.3 Cloning and purification of recombinant BamB (rBamB) and LptD (rLptD)

A 685 nucleotide fragment of *bamB* (bases 396-1080) and a 2166 nucleotide fragment of *lptD* (bases 61-2166) were amplified using primers designed using NEBuilder® Assembly tool v1.12.15 (New England Biolabs, Ipswich, MA). PCR amplification was carried out using Q5® Hi-Fidelity DNA polymerase (New England Biolabs). Amplicons were excised from 1% agarose gels, purified (Bioline, Alexandria, Australia) and cloned into the *Bam*HI and *Nco*I sites of pETM-11 using the NEBuilder® HiFi DNA Assembly cloning kit (New England Biolabs), to generate N-terminal His_6-tagged rBamB and rLptD. *E. coli* BL21-DE3 expression hosts were transformed and plated on to agar containing 50 μg/mL and 30 μg/mL kanamycin and chloramphenicol, respectively. Carriage of the insert was verified by DNA restriction analysis (*Sph*I restriction enzyme for pETM11-BamB, and *Xba*I and *Bsa*I for pETM11-LptD) and sequencing. Expression of recombinant proteins was induced by overnight culture at 18°C with 0.4mM isopropyl-β-D-1-thiogalactopyranoside (IPTG; Sigma). Cells were harvested by centrifugation and resuspended in wash buffer containing 20 mM sodium phosphate, 500 mM

NaCl and 20mM imidazole with 1 mM PMSF (Sigma). The cells were disrupted using a high pressure instrument (Constant System Ltd, Northants, UK) at 30kPa. Lysates were centrifuged (30,000g, 30 min, 4°C) and supernatant protein was purified using Ni-NTA sepharose® (Qiagen, Nutley, NJ), followed by size exclusion chromatography (Sepharose, 200 on an AKTApurified™ 10 FPLC system, GE Healthcare, Uppsala, Sweden). Protein was identified at A_{280}, positive fractions were pooled and purity was determined by 1D-PAGE using gradient 4-12% Novex® NuPAGE® Bis-Tris protein gels (Invitrogen, Carlsbad, CA). Identity was confirmed by mass spectroscopy (Proteomics International, Western Australia). Results are shown in Supplementary Figures 1 and 2.

2.4 Preparation of vaccines

Planktonic cells and biofilms were prepared as described previously [16, 21]. Briefly, bacterial cells were cultured for 18 h in SS broth at 37°C with shaking at 180 rpm, washed 3 times with PBS and planktonic cells harvested by centrifugation (8,000 g, 20 min). To prepare biofilm, ~200 ml of planktonic cultures were placed in a tissue culture flask and allowed to form biofilm at 37°C for 96 h. The supernatants were gently removed and the attached biofilm was washed three times with PBS. It was then harvested by scraping from the surface of the flasks into sterile PBS and collected by centrifugation (10,000g, 10 min). Both biofilm and planktonic preparations were adjusted to A600=0.1. Colony forming counts (CFU) were determined using 10 fold dilutions in triplicate.

2.5 Experimental design

To investigate immunogenicity, groups of 6 mice were immunised subcutaneously on days 0 and 14 with 10^7 and 10^9 CFU/mL (respectively) *B. pertussis* planktonic bacteria or biofilm, that had been heat (56°C) inactivated and mixed 1:1 with alum adjuvant (Imject™; ThermoFisher Scientific)]. A third group was immunised with 1/50th of the human dose of the commercially available hexavalent acellular vaccine Infanrix® hexa (DTaP; GlaxoSmithKline) [21]. Control mice received Imject alone. On day 24 and 35, three mice from each group were sacrificed to collect blood, spleens and lymph nodes (LN). The days were based on an optimization study where day 24 represents the peak response and day 35 assesses persistence.

To investigate protection, groups of 7 mice were immunized (as described above) and challenged on day 24 with $2x10^7$ CFU of virulent *B. pertussis* Tohama I or the ID20 resurgent strain of *B. pertussis* which carries the *ptxP3* allele [16]. Mice were lightly sedated with Isoflurane (Parimal

Critical Care, Bethlehem, PA) and 40 μL of bacterial suspension was instilled slowly into their nostrils [21]. Mice were weighed and health monitored twice daily by a single observer. Seven days after challenge, lungs were harvested into sterile SS broth. Ten-fold serial dilutions of lung homogenates were inoculated on to charcoal agar plates to determine bacterial loads. Blood was collected and serum was stored at -80°C until required.

2.6 Quantitation of pertussis-reactive IgG1 and IgG2a

The enzyme-linked immunosorbent assay (ELISA) was adapted from Raymonds et al [22]. Briefly, 96-well plates were coated overnight with A_{600} = 0.1 of heat-inactivated *B. pertussis*. Plates were washed with phosphate buffered saline (PBS) containing 0.05% Tween-20 (BDH Prolabo, Fontenay-sous-Bois, France) and blocked with 5% bovine serum albumin (BSA; Bovogen Biologicals, Keilor, Australia). Four serial dilutions of mouse sera were added for 2h at room temperature, followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1 or IgG2a (Abcam, Melbourne, Australia) for 1h. After washing, 3,3',5,5'–tetramethylbenzidine (TMB; Sigma) containing 1 μL H_2O_2 (Sigma) was dispensed into each well (15 or 30 min for IgG1 and IgG2a, respectively). Reactions were stopped with 25 μL 1M H_2SO_4 and absorbance was measured at 450 nm. To assess antibodies reactive with DTaP antigens, plates were coated with 5 μg/mL DTaP vaccine (overnight, 4°C) and developed as described above. Standards and quality control samples were separate pools of high titre sera assigned values as arbitrary units per mL (AU/mL). Antibody concentrations were determined from standard curves using least-squares curve-fitting.

2.7 Quantitation of spleen and lymph node cells producing IFN-γ or IL-17a

Spleen and LN cells were separated using a mesh, washed and resuspended in 3 mL RPMI-1640 with 250 mM ammonium chloride (Sigma) (on ice, 2 min). Cells were washed twice, resuspended in 1 mL culture medium (RPMI-1640 with 10% foetal calf serum, 100 μg/mL streptomycin, 100 U penicillin/mL) and viability was determined by Trypan blue exclusion. For enzyme linked immunosorbent spot (ELISPOT) assays, 100 μL cells were transferred to an equivalent volume of medium containing antigens to obtain $2.5x10^5$ cells/well. Production of IFN-γ and IL-17a was assessed using Mouse IFN-γ and IL-17a ELISPOT Ready-SET-GO!® kits (eBioscience, San Diego, CA). Following stimulation with heat-inactivated biofilm or planktonic bacteria, DTaP, rBamB or rLptD, spots were counted using an automated ELISPOT plate reader and data analysed using EliSpot2.9 software (Autoimmun Diagnostica, Strassberg, Germany). Images were checked and results are reported as spot-forming units (SFU) / $2x10^5$ cells.

2.8 Statistical analyses

Two-tailed Mann-Whitney *U* tests were used to compare bacterial loads in the lungs of mice (n=7/group). Unpaired Student's t-tests were used to compare immunological data. All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). $P \leq 0.05$ was accepted as a significant difference.

3. Results

3.1 Vaccination with *Bordetella pertussis* biofilm generates optimal IFN-γ and IL-17a responses to whole bacteria

Groups of 6 mice were vaccinated with inactivated *B. pertussis* planktonic bacteria, biofilm, or DTaP. Control mice received Imject (alum adjuvant) alone and responses to vaccines were assessed relative to this group. Spleen and LN cultures were stimulated with planktonic cells, biofilm, DTaP, rBamB or rLptD. Cytokine profiles were determined using ELISPOT assays applied to 3 mice / group on days 24 and 35. rBamB did not induce IFN-γ or IL-17a in cells from any vaccinated mice (<3 SFU/2x10^5 cells, data not shown). However efficient IFN-γ and IL17a production was evident when splenocytes from vaccinated mice were stimulated with planktonic cells or biofilm (Figures 1A, 1B, 2A, 2B).

When stimulated with planktonic bacteria, splenocytes of mice vaccinated with biofilm displayed more IFN-γ SFU on day 24 than mice vaccinated with planktonic bacteria (P=0.006), DTaP (P =0.013) or Imject (P <0.001). Biofilm vaccination remained superior to DTaP at day 35 (P=0.004; Figure 1A). Similarly following stimulation with biofilm, splenocytes of mice vaccinated with biofilm displayed more IFN-γ SFU on day 24 than mice vaccinated with planktonic bacteria (P=0.04), DTaP (P=0.006) or Imject (P <0.001). Biofilm vaccination remained superior to DTaP (P =0.007) and Imject (P=0.02) at day 35; Figure 1B).

When cultures were stimulated with DTaP, the differences were less clear but mice vaccinated with biofilm produced more IFN-γ than control mice on day 24 (P=0.001; Figure 1C). Whilst rBamB did not induce significant production of IFN-γ in splenocytes from any vaccinated mice (data not shown), rLptD stimulated IFN-γ production by splenocytes of mice vaccinated with planktonic bacteria (P=0.005), biofilm (P=0.001) or DTaP (P=0.01) compared to control mice, with no significant differences between the vaccines (Figure 1D).

When stimulated with planktonic bacteria, splenocytes of mice vaccinated with biofilm also displayed more IL-17a SFU on day 24 than mice vaccinated with planktonic bacteria (P =0.012), DTaP (P =0.01) or Imject (P <0.001). Biofilm vaccination remained superior to DTaP (P=0.028) and Imject (P=0.032) at day 35 (Figure 2A). Similarly following stimulation with biofilm (Figure 2B), splenocytes of mice vaccinated with biofilm displayed more IL-17a SFU on day 24 than mice vaccinated with planktonic bacteria (P=0.001), DTaP (P=0.001) or Imject (P=0.002). Biofilm vaccination again remained superior to DTaP (P=0.001) and Imject (P=0.001) at day 35.

As with IFN-γ, IL-17a production was low and similar in all cultures stimulated with DTaP (Figure 2C). rLptD induced significant IL-17a production in splenocytes of mice vaccinated with biofilm (P=0.01, Figure 2D), but low values precluded other analyses.

LN cells from each group of mice were pooled to obtain enough cells to test all antigens, so no statistical analyses were possible. However on day 35, LN cells from biofilm-vaccinated mice produced several fold more IFN-γ and IL-17a SFU than cells from mice vaccinated with planktonic bacteria, DTaP or Imject (Table 1).

3.2 A predominance of IgG2a links biofilm vaccination with an efficient Th1 response.

In mice, IgG1 reflects a Th2 response, while IgG2a is associated with Th1 [23]. When assayed in ELISA plates coated with heat-killed planktonic bacteria, mice vaccinated with planktonic bacteria or biofilm produced higher IgG1 responses at day 24 and 35, than mice vaccinated with DTaP or Imject (Figure 3A). IgG1 responses to planktonic bacteria and biofilm were similar, but biofilm-vaccinated mice produced markedly higher levels of IgG2a than other groups (Figures 3B). An ELISA based on DTaP antigen confirmed that mice vaccinated with DTaP produced higher levels of IgG1 antibody than mice immunized with planktonic bacteria or biofilm (Figure 3C, P<0.001). No IgG2a was detected with this assay (data not shown).

3.3 *Bordetella pertussis* biofilm and associated proteins confers protection.

Given the strong Th1, Th17 and IgG2a responses to *B. pertussis* biofilms, groups of 7 mice were vaccinated twice over 2 weeks with biofilm, planktonic cells, DTaP or Imject and challenged with virulent *B. pertussis* Tohama I. Mice vaccinated with biofilm had lower bacterial loads in

their lungs than mice vaccinated with DTaP (P=0.02) or Imject (P=0.002) (Figure 4A). There was no difference between mice given biofilm or planktonic bacteria.

The recent clinical isolate ID20 carries the *ptxP3* allele and Prn variant, distinct from Tohama I [16]. Mice were vaccinated with planktonic cells or biofilm (from Tohama I), DTaP, rBamB or rLptD (alone or with DTaP), and challenged with ID20. Vaccination with planktonic bacteria or biofilm reduced bacterial loads in the lungs compared to Imject-vaccinated mice (P<0.05; Figure 4B).

3.4 Vaccination with rBamB or rLptD protects mice against ID20.

Vaccination with rBamB or rLptD reduced bacterial loads in the lungs compared to Imject control mice (P=0.003, P<0.001, respectively; Figure 4B). Moreover, a combined formulation of rBamB-rLptD-DTaP was a more potent vaccine against ID20, with lower bacterial loads than achieved with DTaP (P=0.02). Six days after challenge with ID20, all signs of illness had resolved in mice vaccinated with planktonic bacteria, biofilm or rBamB-rLptD-DTaP, but symptoms persisted in mice given DTaP, rLptD, rBamB or Imject alone (Table 2). Hence, both bacterial loads and the health of the mice indicate that rBamB and rLptD enhance the protective efficacy of DTaP.

4. Discussion

Current whooping cough vaccines are formulated with antigens expressed in planktonic bacteria, so biofilm may offer novel protective antigens. This may be attributed to different constituents of biofilm matrix. For example, extracellular DNA has been identified as one of the major components of *B. pertussis* biofilm [24]. Protection against *B. pertussis* biofilm may interrupt the persistence and spread of infections *in vivo* [19]], but we are aware of only one *B. pertussis* biofilm vaccine trial [21]. This is critical because 5% of proteins were upregulated and 3.5% are downregulated in biofilm cultures of clinical isolate ID20 [16]. Though other antigens warrant similar studies, we selected BamB and LptD as candidate antigens in our study since they were upregulated in biofilm of Tohama I and ID20.

The data support evidence from mice [1] and humans [25] that current aP vaccines induce predominantly Th2 responses. *Bordetella pertussis* biofilm induced higher IgG1 levels and up to 50-fold more IgG2a compared to vaccination with DTaP or Imject alone (Figure 3A and B). An ELISA detecting DTaP-specific antibody responses confirmed that mice vaccinated with

DTaP produce IgG1 but not IgG2a (Figure 3C). Splenocytes from biofilm-vaccinated mice produced more IFN-γ and IL-17a than planktonic-, DTaP- or Imject-vaccinated mice (Figures 1 and 2). Biofilm vaccination also induced higher responses by LN cells on Day 35 (Table 2). Induction of IFN-γ by biofilm vaccination was also demonstrated by high serum levels of the chemokine CXCL10 in a pilot study of mice challenged with live bacteria (data not shown).

Having demonstrated the immunogenicity of the biofilm and associated proteins, we assessed protection. Here biofilm vaccination reduced bacterial loads more effectively than DTaP. Mice vaccinated with rBamB or rLptD alone had significantly lower bacterial loads than control mice ($P<0.05$, Figure 4B). However, a rBamB-rLptD-DTaP formulation enhanced the protective efficacy over DTaP alone and had an acceptable safety profile (Figure 4B, Table 2). This is a novel and important finding. de-Gouw et al [21] showed that vaccination of mice with the biofilm protein, BipA, reduced colonisation of the lungs following virulent *B. pertussis* challenge. However the authors did not address the effect of BipA on the protective potential of DTaP.

In summary, a vaccine comprising inactivated *B. pertussis* biofilm may induce a protective immune response against resurgent *B. pertussis* strains, as assessed by IFN-γ, IL-17a or IgG2a. Our planktonic cell vaccine induced lower IFN-γ and IgG2a responses than biofilm. The current aP vaccine, Infanrix® hexa (DTaP), induces predominantly IgG1 antibody response with little or no detectable IgG2a, IFN-γ or IL-17a. Importantly, DTaP may be sub-optimal in protecting against infection with a resurgent strain, but formulation of DTaP with BamB and LptD may enhance the protective potential of DTaP against the resurgent strains of *B. pertussis*.

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Figure legends

Figure 1. IFN-γ production in mice vaccinated with B. pertussis vaccine candidates. Splenocytes of vaccinated mice were stimulated with planktonic cells (A), biofilm (B), DTaP (C) or rLptD (D) and IFN-γ production was assessed by ELISpot. Each bar represents n=3 mice. Results are presented as spot forming units (SFU) per $2x10^5$ cells (median ± range). D24, D35: day 24, day 35. Vxn: vaccination. Horizontal lines represent significant differences between groups (Unpaired Student's t-tests, $P<0.05$).

Figure 2. IL-17a production in mice vaccinated with *B. pertussis* vaccine candidates. Splenocytes of vaccinated mice were stimulated with planktonic cells (A), biofilm (B), DTaP (C) or rLptD (D) and IL-17a production was assessed by ELISpot. Each bar represents n=3 mice. Results are presented as spot forming units (SFU) per $2x10^5$ cells (median ± range). D24, D35: day 24, day 35. Vxn: vaccination. Horizontal lines represent significant differences between groups (Unpaired Student's t-tests, $P<0.05$).

Figure 3. Humoral responses to *Bordetella pertussis* in vaccinated mice.
Sera from vaccinated mice assessed using ELISA plates coated with heat-killed planktonic bacteria to determine levels of IgG1 (A) and IgG2a (B) antibody, and using plates coated with DTaP to assess IgG1 (C) and IgG2a (not shown). Columns represent median ± range for 3 mice/group. D24, D35: day 24, day 35. Vxn: vaccination. Horizontal lines represent significant differences between groups (Mann-Whitney, $P<0.05$).

Figure 4. Protective potential of *Bordetella pertussis* biofilm and associated proteins.
Mice were immunised on days 0 and 14 with vaccine candidates and challenged with Tohama I (A) or clinical isolate ID20 (B) on day 24. Bacterial loads were determined from lung homogenates collected 7 days post-challenge. Each symbol represents the bacterial load of one mouse. Median values indicated. Horizontal lines represent significant differences between groups (Mann-Whitney, $P<0.05$).

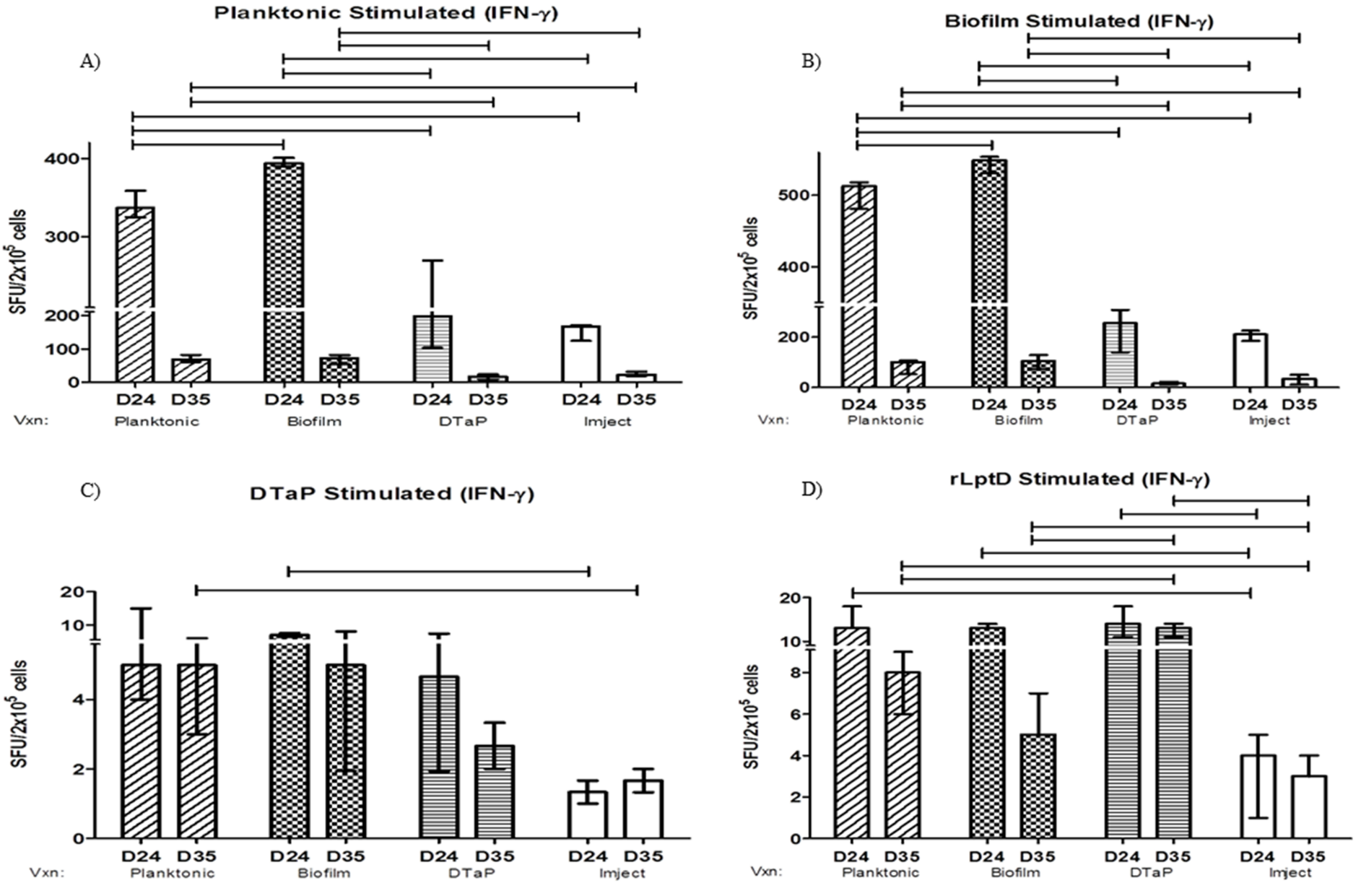
Supplementary figure legends

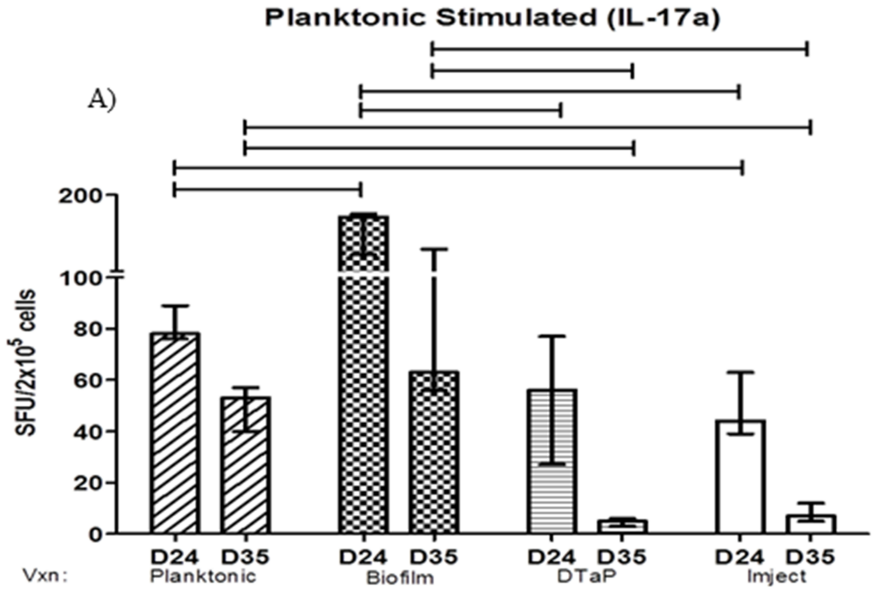
Supplementary Figure 1. Cloning and purification of rBamB.

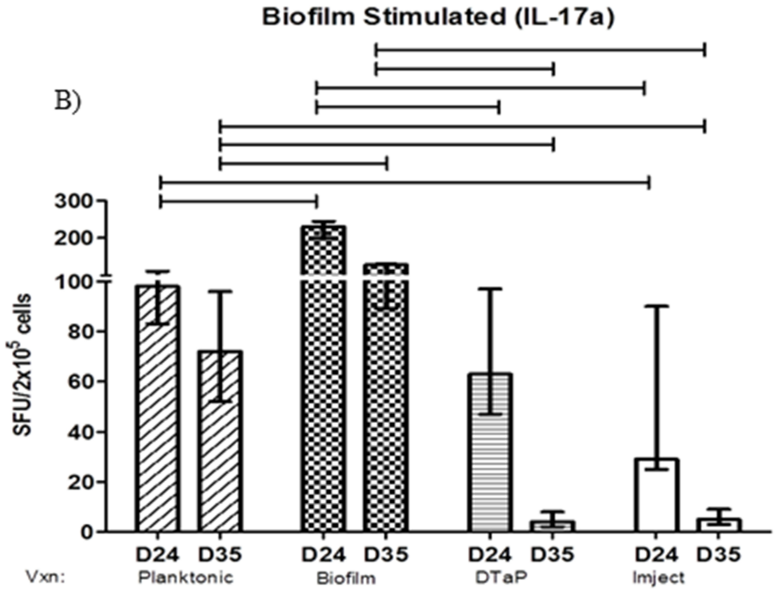
The *bamB* gene was cloned into the pETM-11 vector and confirmed by PCR using *bamB* or pETM-11 primers in DE3-BL21 *E. coli* cells. Carriage of the *bamB* insert was verified by *Sph*I restriction (A) and nucleotide sequencing of the insert. AKTA-purified rBamB was separated on SDS-gel (B) and protein identification of purified rBamB was performed with MS using MSPnr100 database search (C) at Proteomics International, Western Australia.

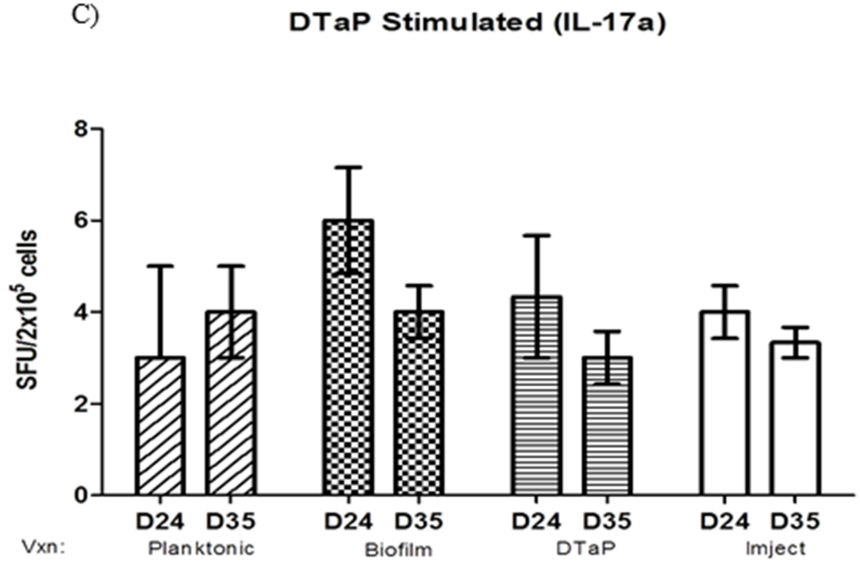
Supplementary Figure 2. Cloning and purification of rLpTD

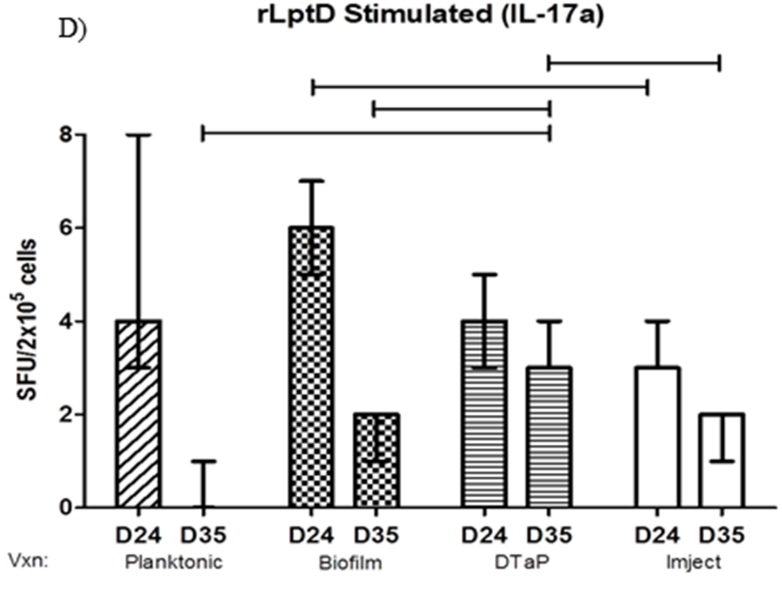
The *lptD* gene was cloned into the pETM-11 vector and confirmed by PCR using *lptD* or pETM-11 specific PCR in DE3-BL21 *E. coli* cells (A). Carriage of the insert was verified by restriction with *Xba*I and *Bsa*I (A) and nucleotide sequencing of the insert. AKTA-purified rLptD was separated on SDS-gel (B) and protein identification of purified rLptD was performed with MS using MSPnr100 database search (C) at Proteomics International, Western Australia.

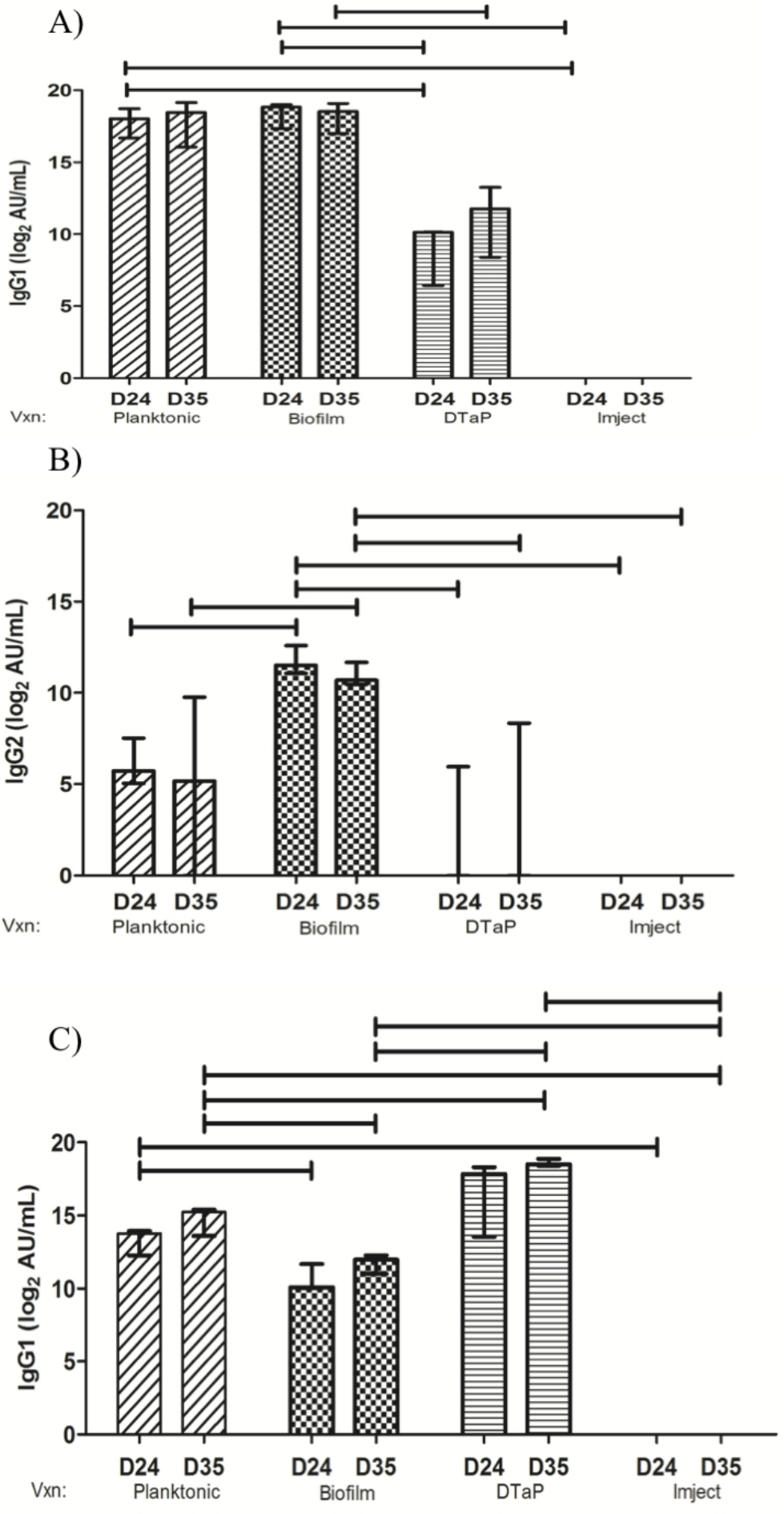

A)
Planktonic Stimulated (IFN-γ)
SFU/2x10^5 cells
400
300
200
100
0
D24 D35 D24 D35 D24 D35 D24 D35
Vxn: Planktonic Biofilm DTaP Imject
B)
Biofilm Stimulated (IFN-γ)
SFU/2x10^5 cells
500
400
200
0
D24 D35 D24 D35 D24 D35 D24 D35
Vxn: Planktonic Biofilm DTaP Imject
C)
DTaP Stimulated (IFN-γ)
SFU/2x10^5 cells
20
10
4
2
0
D24 D35 D24 D35 D24 D35 D24 D35
Vxn: Planktonic Biofilm DTaP Imject
D)
rLptD Stimulated (IFN-γ)
SFU/2x10^5 cells
20
10
8
6
4
2
0
D24 D35 D24 D35 D24 D35 D24 D35
Vxn: Planktonic Biofilm DTaP Imject

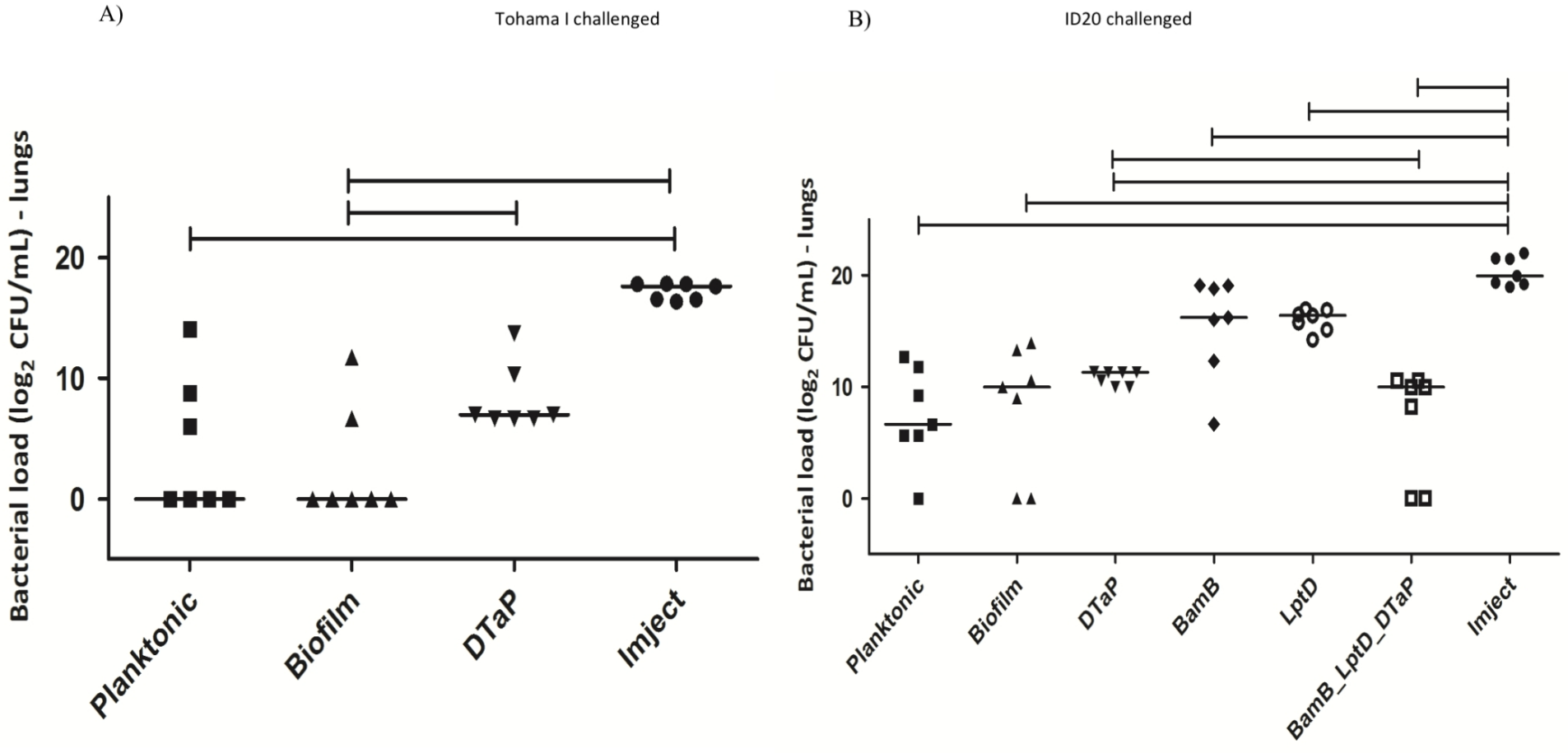

Planktonic Stimulated (IL-17a)
A)
SFU/2x10^5 cells
200
100
80
60
40
20
0
D24 D35 D24 D35 D24 D35 D24 D35
Vxn: Planktonic Biofilm DTaP Imject


Biofilm Stimulated (IL-17a)
B)
SFU/2x10^5 cells
300
200
100
80
60
40
20
0
D24 D35 D24 D35 D24 D35 D24 D35
Vxn: Planktonic Biofilm DTaP Imject

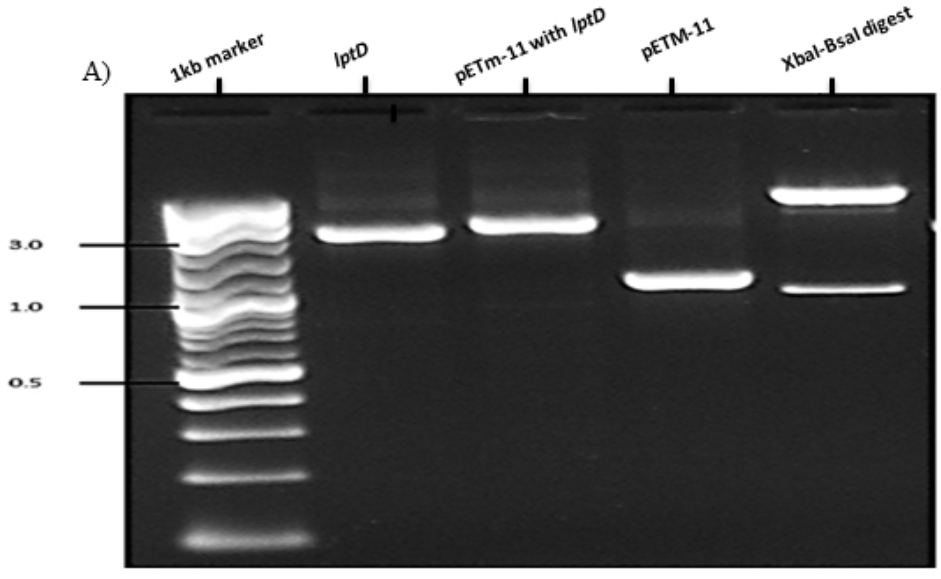

C)
DTaP Stimulated (IL-17a)
SFU/2x10^5 cells
8
6
4
2
0
D24 D35 D24 D35 D24 D35 D24 D35
Vxn: Planktonic Biofilm DTaP Imject


D)
rLptD Stimulated (IL-17a)
SFU/2x10^5 cells
8
6
4
2
0
D24 D35 D24 D35 D24 D35 D24 D35
Vxn: Planktonic Biofilm DTaP Imject


A)
IgG1 (log2 AU/mL)
20
15
10
5
0
D24 D35 D24 D35 D24 D35 D24 D35
Vxn:
Planktonic
Biofilm
DTaP
Imject
B)
IgG2 (log2 AU/mL)
20
15
10
5
0
D24 D35 D24 D35 D24 D35 D24 D35
Vxn:
Planktonic
Biofilm
DTaP
Imject
C)
IgG1 (log2 AU/mL)
20
15
10
5
0
D24 D35 D24 D35 D24 D35 D24 D35
Vxn:
Planktonic
Biofilm
DTaP
Imject


A)
Tohama I challenged
Bacterial load (log2 CFU/mL) - lungs
20
10
0
Planktonic
Biofilm
DTaP
Imject
B)
ID20 challenged
Bacterial load (log2 CFU/mL) - lungs
20
10
0
Planktonic
Biofilm
DTaP
BamB
LptD
BamB_LptD_DTaP
Imject

A)



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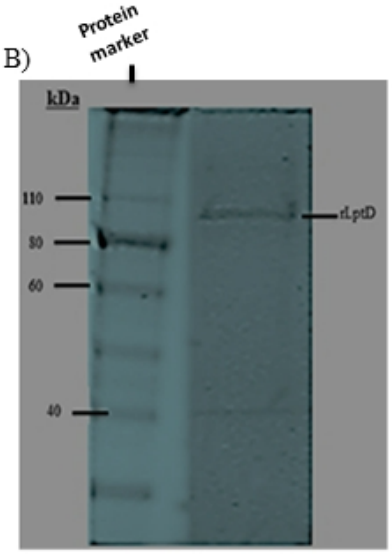

c)

{MATRIX SCIENCE} **MASCOT Search Results**

Protein View: Q7VU13

sp|Q7VU13|lptD LPTD_BORPE LPS-assembly protein LptD n=6 Tax_Id=257313 [Bordetella pertussis (strain Tohama I / ATCC BAA-589 / NCTC 13251)]

Database:	MSPnr100
Score:	1538
Nominal mass (M$_r$):	88533
Calculated pI:	8.32
Taxonomy:	Unknown species

Sequence similarity is available as an NCBI BLAST search of Q7VU13 against nr.

Search parameters

MS data file:	C:\Documents and Settings\Proteomics\Desktop\3975B-20160927T020048Z\3975B\160923 3975B.mgf
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Variable modifications:	Oxidation (M)

Protein sequence coverage: 40%

Matched peptides shown in ***bold red***.

```
   1 MRMLRWLILS AFSVAGAVQA QGNQDSAAAS APSASIGAPV LRTSPGLRVH
  51 RLPDEKIPAF MEADQISGDP DSEVILTGNA QVRRVDGIIK GDRINYRRDT
 101 GDVDVQGSAR MLRDGTLITG PSARLNVDTY SGEIQEPNFW IGASGGTAQA
 151 RHADIFSKSQ MRLSQVTYSG CPCPKPSWYI KADTVDLDFD ENEGVARNGV
 201 LYFKDVPILA SPYLTFPVKK ERKSGFLMPT YGTTSNSGFD ISLPYYFNLA
 251 PNYDLTLVPR YLSKRGAQLG GEFRYLGSGY RGVAIGTYLP DDNETGRDRW
 301 MYRTYHRQLL GNGFYTDWDI AGASDDNYFR DISELGLNTA STTYLPRRGR
 351 VGWSSTYWQT YAQVYKYQTL QDFDAPLAPF YDKVPELWLK GARYDWGGFD
 401 AEWVSTAVRF QRSLLNGRRL GPDGDRLQTY PTVSYPIGRP GWFLVPKVGV
 451 HYTQYRIDWY NRDWNRIGLS NYKRTESRTV PIMSLDAGMI FERDASLFGK
 501 AATQTLEPRL YYLRVPYRDQ SALPVYDTTL ADFSFDQAFQ ENIYTGGWDR
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 601 VRSDFLVGAT AALTDTLITD VAAQYNFYDN KWSRGMVSAR WSPQRLTTVA
 651 VAYRYQRDPL PGISYQPQGQ NQVSLAVQWP IHRRWYGVGR VDYSLRSEPA
 701 IAAAAEQSPR VTQAIAGLEY KGDCCWVGRV VYQRYAVSAA DINTALFFQL
 751 ELTGLGALGT DPISLLNRSI PGYQSVVPPT PTGTTFERYE
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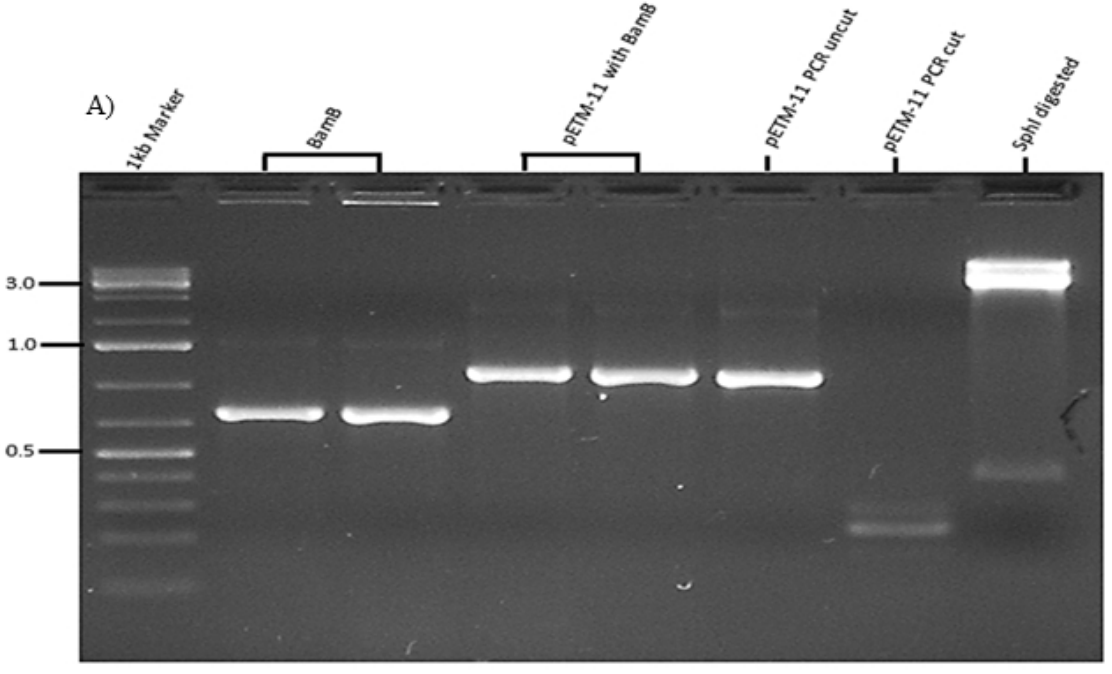

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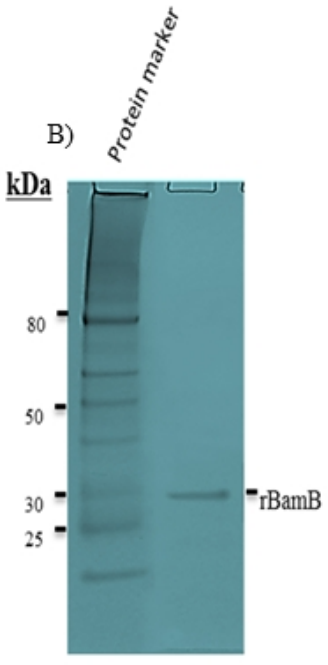

C)

MASCOT Search Results

User :
E-mail :
MS data file : C:\Documents and Settings\Proteomics\Desktop\3945\160715 3945A.mgf
Database : MSPnr100 (75,925,788 sequences; 27,045,014,025 residues)
Taxonomy : Bacteria (Eubacteria) (51,492,816 sequences)
Timestamp : 20 Jul 2016 at 09:19:47 GMT

Protein Family Summary

Significance threshold p< 0.05 Max. number of families AUTO
Ions score or expect cut-off 0 Dendrograms cut at 0
Preferred taxonomy All entries

Protein families 1–6 (out of 6)

A0A058YGG7 101 tr|A0A058YGG7|bamB A0A058YGG7_BORBO Outer membrane protein assembly factor BamB n=1 Tax_Id=1331202...

	Score	Mass	Matches	Sequences	emPAI
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tr|A0A058YGG7|bamB A0A058YGG7_BORBO Outer membrane protein assembly factor BamB n=1 Tax_Id=1331202 [Bordetella bronchiseptica 99-R-0433]

▼11 samesets of A0A058YGG7

A0A0E1QT54	101	40066	18 (3)	4 (2)	

tr|A0A0E1QT54|bamB A0A0E1QT54_BORBO Outer membrane protein assembly factor BamB n=3 Tax_Id=1208657 [Bordetella bronchiseptica 1289]

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A0A0E8DJ16	101	39935	18 (3)	4 (2)	

tr|A0A0E8DJ16|yfgL A0A0E8DJ16_BORPT Quinoprotein n=1 Tax_Id=520 [Bordetella pertussis]

Table 1. IFN-γ and IL17a production by lymph node cells was more persistent after biofilm vaccination

***In vitro* stimulants**	**Days**	**Vaccination**			
		Planktonic bacteria	**Biofilm**	**DTaP**	**Imject**
IFN-γ production					
Planktonic bacteria	24	390	446	270	33
	35	38	**212**	25	21
Biofilm	24	471	506	368	56
	35	70	**177**	17	40
IL-17a production					
Planktonic bacteria	24	138	159	16	1
	35	23	**87**	0	3
Biofilm	24	225	263	26	8
	35	29	**143**	7	12

Data are presented as spot forming units/$2x10^5$ LN cells (mean value from 3 wells per pooled preparation). Figures in bold indicate >2-fold higher SFU following biofilm vaccination.

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Table 2. Clinical features of mice challenged with ID20 after *Bordetella pertussis* vaccination.

Vaccination	**Clinical score 1 to 7-days post-challenge**						
	D1	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**
Planktonic bacteria	2 (1-2)	3 (2-3)	3 (3-3)	2 (2-2)	1 (1-1)	0 (0-0)	0 (0-0)
Biofilm	2 (1-3)	3 (3-3)	3 (3-3)	2 (1-3)	1 (1-2)	0 (0-0)	0 (0-0)
DTaP	3 (1-3)	3 (1-3)	3 (1-3)	3 (1-3)	2 (1-3)	2 (1-2)	1 (1-1)
rBamB	3 (3-3)	3 (3-3)	3 (3-3)	3 (3-3)	2 (1-3)	2 (1-3)	2 (1-3)
rLptD	3 (3-3)	3 (3-3)	3 (3-3)	2 (2-2)	1 (1-3)	1 (0-2)	1 (0-2)
rBamB-rLptD-DTaP	2 (1-3)	3 (3-3)	3 (3-3)	2 (1-3)	1 (0-2)	0 (0-1)	0 (0-1)
Imject control	3 (3-3)	3 (3-3)	3 (3-3)	3 (3-3)	3 (3-3)	2 (1-3)	2 (1-3)

Clinical features were scored by monitoring their coats (ruffled-smooth), grooming activity and/or movement post-challenge. 0 - no macroscopic changes, 1- low grade, 2- medium grade, 3- severe grade. Median (range) values are presented. Observations were made at a fixed time of day by a single observer (DD). D=days post-challenge.