

SCIENTIFIC REPORTS

OPEN

PilVax – a novel peptide delivery platform for the development of mucosal vaccines

Dasun Wagachchi^{1,2}, Jia-Yun C. Tsai^{1,2}, Callum Chalmers¹, Sam Blanchett¹, Jacelyn M. S. Loh^{1,2} & Thomas Proft^{1,2}

Peptide vaccines are an attractive strategy to engineer the induction of highly targeted immune responses and avoid potentially allergenic and/or reactogenic protein regions. However, peptides by themselves are often unstable and poorly immunogenic, necessitating the need for an adjuvant and a specialised delivery system. We have developed a novel peptide delivery platform (PilVax) that allows the presentation of a stabilised and highly amplified peptide as part of the group A streptococcus serotype M1 pilus structure (PilM1) on the surface of the non-pathogenic bacterium *Lactococcus lactis*. To show proof of concept, we have successfully inserted the model peptide Ova_{324–339} into 3 different loop regions of the backbone protein Spy0128, which resulted in the assembly of the pilus containing large numbers of peptide on the surface of *L. lactis*. Intranasal immunisation of mice with *L. lactis* PilM1-Ova generated measurable Ova-specific systemic and mucosal responses (IgA and IgG). Furthermore, we show that multiple peptides can be inserted into the PilVax platform and that peptides can also be incorporated into structurally similar, but antigenically different pilus structures. PilVax may be useful as a cost-effective platform for the development of peptide vaccines against a variety of important human pathogens.

Vaccines remain the most cost-effective and feasible means of infectious disease control in the community. They have resulted in the eradication of small pox and polio, and a substantial reduction in the incidence of other serious diseases such as measles, tetanus and diphtheria¹. Vaccine development has progressed from live-attenuated or inactivated forms of microbial pathogens to subunit vaccines using one or a few selected proteins to elicit a protective immune response^{2,3}. These formulations are generally regarded as the safer options, as many proteins within a whole organism are not only unnecessary for protective immunity, but often also carry the risk of triggering allergic or autoreactive responses⁴. More recently, peptide vaccines have been introduced as an alternative strategy. Short peptides can be synthesised that carry only the necessary epitopes for highly specific T cell and/or B cell responses. Unfortunately, peptides by themselves are often not very immunogenic and prone to proteolytic digestion^{5,6}. Consequently, peptide vaccines require time-consuming and expensive chemical coupling to carriers for delivery and adjuvancy^{4,7,8}.

Here we present a novel peptide delivery platform (PilVax) that uses a *Streptococcus pyogenes* (Group A Streptococcus, GAS) pilus expressed on the surface of *Lactococcus lactis*. Pili (*sing.* pilus) are hair-like peritrichous protrusions from the cell surface of a variety of Gram-positive and Gram-negative bacteria, with a main, but not exclusive function in host tissue adhesion^{9,10}. In Gram-positive bacteria, such as *S. pyogenes*, pili usually consist of 3 structural proteins that are covalently linked to each other by a specialised pilus-assembly sortase. During assembly, a single tip adhesin is linked to a backbone pilin monomer, followed by integration of >100 additional backbone monomers to form the elongated fibre. Pilus extension is terminated after insertion of an adaptor protein that is then recognised by the house-keeping sortase A and bound to the cell wall peptidoglycan¹¹.

The pilus encoded in the Fibronectin- and Collagen-binding T antigen-2 (FCT-2) region of serotype M1 strains¹² is thus far the best-characterised GAS pilus. Collagen-binding protein (Cpa, Spy0125) is the tip adhesin that confers the adhesive properties of the pilus structure^{13,14}. Spy0128 (FctA, T-antigen) is the backbone pilin that generates the pilus fibre¹⁵, and Spy0130 (FctB) is the anchor protein that links the FCT-2 pilus to the cell wall¹⁶.

¹Department of Molecular Medicine & Pathology, School of Medical Sciences, The University of Auckland, Auckland, 1023, New Zealand. ²Maurice Wilkins Centre for Molecular Biodiscovery, Auckland, 1023, New Zealand. Correspondence and requests for materials should be addressed to J.M.S.L. (email: mj.loh@auckland.ac.nz) or T.P. (email: t.proft@auckland.ac.nz)

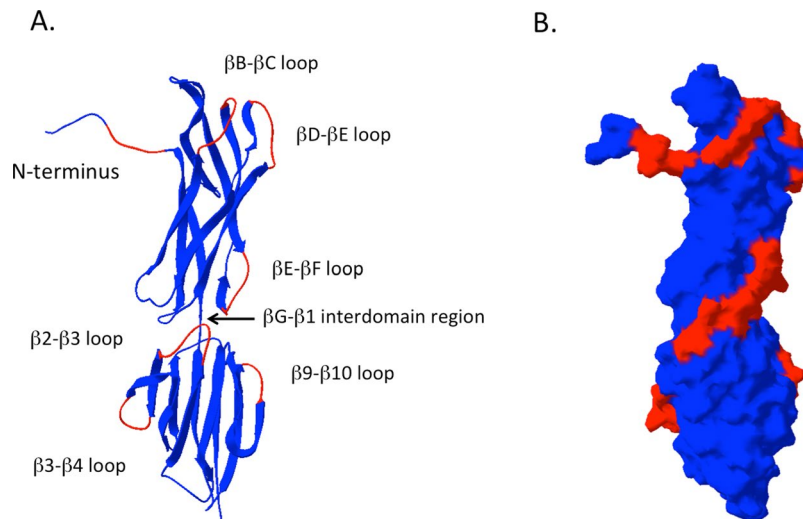


Figure 1. Protein structure of the backbone pilin Spy0128 with selected peptide insertion sites. The protein structure was generated with the PDB Swiss Viewer version 4.1.0.⁵⁴ using coordinates downloaded from the Brookhaven database (3B2M). The selected peptide insertion regions are shown in red. **(A)** Ribbon diagram structure. **(B)** Accessible surface structure.

The crystal structure of the FCT-2 type pilus backbone protein (Spy0128) revealed an immunoglobulin-like fold with intramolecular isopeptide bonds resulting in a protease-resistant and very stable protein conformation¹⁵. Linkage between Spy0128 monomers is achieved by a transpeptidase (sortase C, Spy0129) reaction between the carboxyl group of a C-terminal threonine and the ϵ -amino group of an internal lysine, leaving the N-terminus protruding along the pilus fibre¹⁵. The pili are distributed over the entire GAS cell surface¹⁷.

Mucosal routes for vaccine delivery have gained increased interest in modern vaccinology. More recently, non-pathogenic food grade bacteria, such as *L. lactis* were shown to work as safe and efficacious live antigen carriers that elicit specific and protective immune responses against the target protein^{18–22}. *L. lactis* secreting human IL-10 has also been used in a phase I clinical trial to treat patients with Crohn's disease²³.

It was shown previously that complete *S. pyogenes* pilus structures can be expressed on the surface of *L. lactis*^{24–28}. Furthermore, it was reported that a model protein, maltose-binding protein (MBP), fused to the tip pilin of a GAS serotype M3 strain was able to induce mucosal MBP-specific antibody responses, showing proof-of-concept for a potential vaccine strategy²⁷.

The aim of our study was to demonstrate that *L. lactis* bacteria expressing a complete GAS pilus structure can be used as a platform to carry stabilised peptide antigens for the generation of mucosal vaccines against selected human pathogens. We show that the model peptide Ova_{324–339}, a well characterised B cell epitope^{29,30} inserted at suitable regions of the protease-resistant backbone pilus protein can be stably expressed in large numbers as part of the multimeric GAS pilus structure on the surface of *L. lactis* (Supplementary Figure 1) and triggers peptide-specific mucosal immune responses after intranasal immunisation of mice.

Results

Insertion of a model peptide into selected regions within Spy0128. The GAS serotype M1 pilus (PilM1) encoded in the FCT-2 region has been extensively characterised^{13,14,16,17} and the crystal structure of the pilus backbone protein Spy0128 has been solved¹⁵. In order to identify potential integration sites for peptide epitopes, the protein structure of Spy0128 was analysed for surface accessible non-structured loop regions (Fig. 1). Based on this analysis, we selected 6 different loop regions for peptide integration. These include the β B- β C loop, the β D- β E loop and the β E- β F loop in the N-terminal domain and the β 2- β 3 loop, the β 3- β 4 loop and the β 9- β 10 loop in the C-terminal domain (Fig. 1). Furthermore, the free Spy0128 N-terminus and the region between the two Spy0128 domains (β G- β 1 interdomain region) were also selected (Fig. 1). As the interdomain region is not well exposed we also inserted a peptide linker at this position.

As Spy0128 is expressed as a precursor with a N-terminal signal peptidase domain and a predicted cleavage site between Gly23 and Glu24, the N-terminus peptide integration site was introduced between Asn28 and Gly29. Table 1 shows the amino acid sequences of all the targeted regions before and after the introduction of the model peptide. The complete pilus structure was expressed in the food-grade bacterium *L. lactis* under the control of the constitutive P23 lactococcal promoter. To evaluate the successful assembly of the modified pili, *L. lactis* cell wall extracts were analysed in Western blots using specific anti-M1_Spy0128 antibodies (Fig. 2). Correctly assembled pili appear as a high molecular weight (HMW) ladder pattern due to the covalent linkage of variable numbers of individual pilin monomers, as seen for *L. lactis* expressing the unmodified M1 pilus (Fig. 2A). Unfortunately, insertion of the Ova_{324–339} peptide into the N-terminal region or the β G- β 1 interdomain region of Spy0128 did not result in expression of a pilus structure (Supplementary Figure 2). The addition of a GSGSG linker region on both ends of the Ova_{324–339} peptide in the β G- β 1 interdomain region also failed to produce pili (data not shown). Introduction of the Ova_{324–339} peptide into the β B- β C, the β D- β E, and the β 2- β 3 loops also affected pilus assembly

Site	Original sequence	Sequence after peptide insertion
a) M1_Spy0128		
N-terminus	...TVVNGAK...	...TVVNLESQAVHAAHAEINEAGRVEGAK...
β G- β 1_Ova	...SLDSTTLT...	...SLDLESQAVHAAHAEINEAGRVEVETTL...
β G- β 1-L	...SLDSTTLT...	...SLDLDGSGSGLEGSQVHAAHAEINEAGRVEVETTL...
β G- β 1-L_Ova	...SLDSTTLT...	...SLDLDGSGSGLESQVHAAHAEINEAGRVEGSQVHAAHAEINEAGRVEVETTL...
β B- β C_Ova	...PDTTVNEDGNK...	...PDTLESQAVHAAHAEINEAGRVEGNK...
β D- β E_Ova	...FDFSEVTFEKPVGYY...	...FDFLESQAVHAAHAEINEAGRVEGVYY...
β E- β F_Ova	...TEEKIDKVPVGS...	...TEELESQAVHAAHAEINEAGRVEGVGS...
β 2- β 3_Ova	...LKANQYKASEK...	...LKALESQAVHAAHAEINEAGRVESEK...
β 3- β 4_Ova	...KTTKGGQAPVQ...	...KTTLESQAVHAAHAEINEAGRVEPVQ...
β 9- β 10_Ova	...SPQDGAVKNI...	...SPQLESQAVHAAHAEINEAGRVEKNI...
β E- β F_ova-J14	...TEEKIDKVPVGS...	...TEELESQAVHAAHAEINEAGRVEKQAEKDVKASREAKKQVEKALEQLEDKVVQVEGVGS...
b) M18_Spy0128		
β E- β F_Ova	...SEVNGNKAGIAY...	...SEVLESQAVHAAHAEINEAGRVEIAY...

Table 1. Amino acid sequences of the peptide insertion sites in Spy0128. The Ova_{324–339} and J14 peptide sequences are shown in bold underlined. Amino acids deriving from restriction enzyme recognition sites are shown in bold. Note that cloning of a *Sall* site (GTCGAC) into a *XhoI* site (CTCGAG) results in the hybrid sequence GTCGAG encoding amino acids VE. Peptide linker regions are underlined.

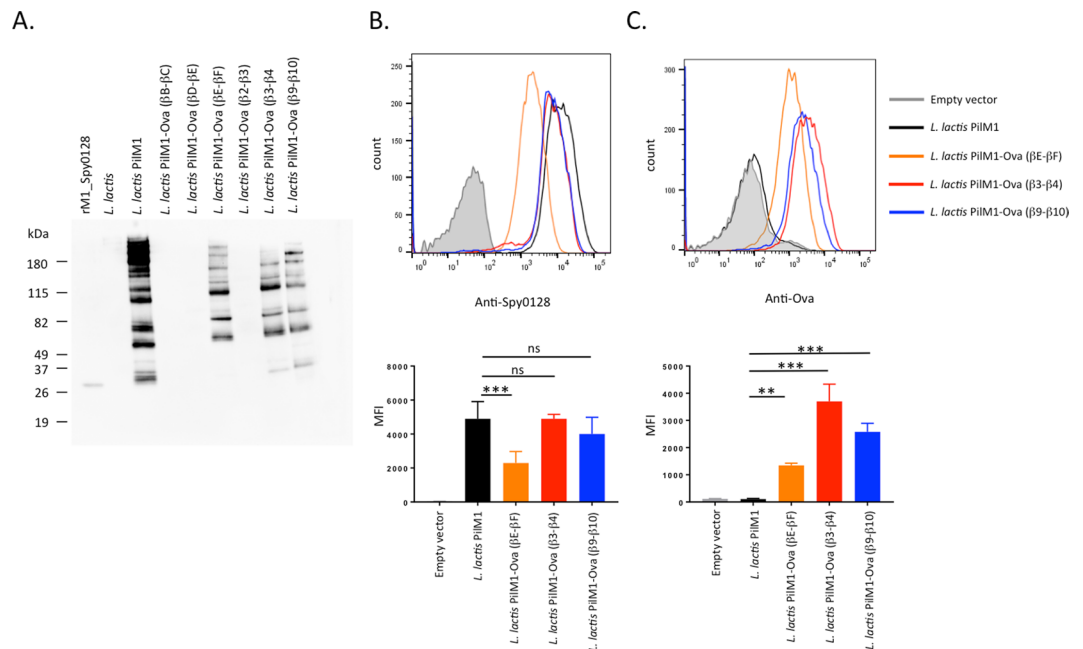


Figure 2. Expression of the PilM1 structure on the surface of *L. lactis* with the model peptide Ova_{324–339} inserted at selected sites within the Spy0128 backbone pilus protein. (A) Western blot analysis of *L. lactis* cell wall extracts (CWE) with antiserum specific for M1_Spy0128 (pilus backbone protein). The high molecular band patterns are indicative of pilus assembly. For *L. lactis* strains that showed pilus expression after peptide insertion, flow cytometry was used to compare the expression levels of M1_Spy0128 (B) and Ova_{324–339} peptide (C). Error bars show the standard deviation from 3 independent experiments. ** $p < 0.005$; *** $p < 0.0005$; one-way ANOVA followed by a Holm-Sidak test.

(Fig. 2A). Western-blot analysis of the individual cell fractions revealed that *L. lactis* with Ova-peptide engineered into the β C- β D, β 2- β 3 loops or the interdomain region (β G- β 1) expressed monomeric Spy0128 protein in the protoplast fraction with a slightly higher molecular weight than rSpy0128. In contrast, *L. lactis* with Ova-peptide at the β D- β E or the N-terminal region did not show Spy0128 protein in any cell fraction (Supplementary Figure 2). However, insertion of the Ova_{324–339} peptide into the β E- β F, the β 3- β 4 or β 9- β 10 loop regions resulted in assembled pili, indicated by a high-molecular weight banding pattern on Western blots with anti-M1_Spy0128 antibodies (Fig. 2A). Flow cytometry was used to investigate whether or not peptide integration in the β E- β F loop, the β 3- β 4 loop or the β 9- β 10 loop had an effect on the amount of Spy0128 pilus proteins expressed on

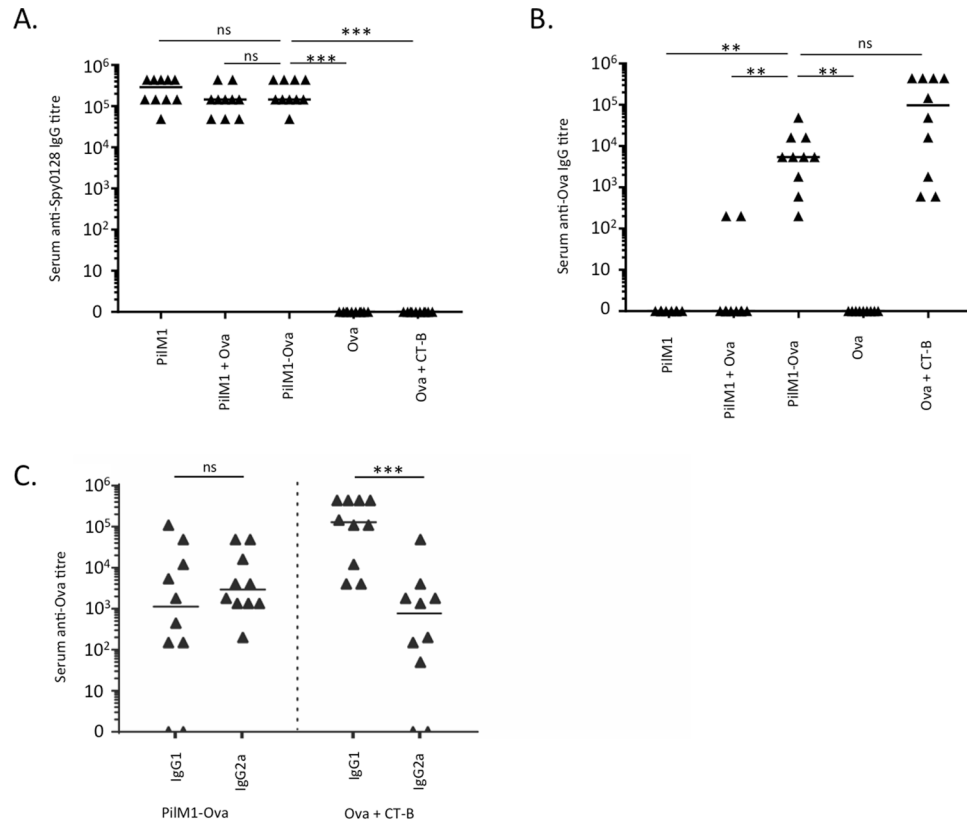


Figure 3. Intranasal immunisation of mice with *L. lactis* that expresses the M1 pilus with the model peptide Ova₃₂₄₋₃₃₉ inserted at the β E- β F loop region (PilM1-Ova) induces IgG responses. Groups of Balb/c mice ($n = 5$) were immunised intranasally with 1×10^8 CFU live recombinant *L. lactis* PilM1-Ova. The data from two independent experiments were combined. *L. lactis* without inserted peptide (PilM1) or mixed with synthetic Ova₃₂₄₋₃₃₉ (PilM1 + Ova) were used as controls. Synthetic Ova₃₂₄₋₃₃₉ peptide alone (Ova) or mixed with Cholera Toxin B subunit adjuvant (Ova + CT-B) were used as additional controls. Total serum IgG titres against (A) immobilised recombinant Spy0128 or (B) commercial ovalbumin were measured by ELISA. ** $p < 0.005$; *** $p < 0.0005$; One-way ANOVA with Dunn's multiple comparisons test. (C) Ova-specific responses by IgG subclasses. *** $p < 0.0005$; Mann-Whitney test.

the cell surface of *L. lactis*. As shown in Fig. 2B, the choice of peptide insertion site had a notable effect on pilus expression. Insertion of Ova₃₂₄₋₃₃₉ peptide into the β 3- β 4 loop or the β 9- β 10 loop only slightly reduced Spy0128 expression, whereas the *L. lactis* strain with Ova₃₂₄₋₃₃₉ peptide at the β E- β F loop expressed considerably less Spy0128 compared to wild-type. This was further confirmed by flow cytometry analysis using antibodies against the Ova₃₂₄₋₃₃₉ peptide (Fig. 2C).

Intranasal immunisation generates mucosal and systemic antibody responses in mice. After successful integration of the Ova₃₂₄₋₃₃₉ peptide into the Spy0128 pilus backbone protein and confirmed assembly of the modified pilus structure on the surface of the food-grade bacterium *L. lactis*, we were able to test our hypothesis that this peptide delivery platform could be used as a strategy to generate mucosal immune responses. Mice were immunised intranasally with live *L. lactis* bacteria that expressed the M1 pilus with Ova₃₂₄₋₃₃₉ peptide inserted into the β E- β F loop region of Spy0128 (*L. lactis* PilM1-Ova). *L. lactis* expressing the unmodified M1 pilus without the peptide (*L. lactis* PilM1), *L. lactis* PilM1 mixed with synthetic Ova₃₂₄₋₃₃₉ peptide (*L. lactis* PilM1 + Ova) and synthetic Ova₃₂₄₋₃₃₉ peptide (with or without CT-B as an adjuvant) were used as additional controls. After 3 additional booster immunisations at weeks 2, 4, and 6, serum, saliva and bronchoalveolar lavage (BAL) fluid samples were obtained and analysed for Spy0128 and Ova-specific antibodies. Mice immunised with *L. lactis* PilM1, *L. lactis* PilM1 + Ova or *L. lactis* PilM1-Ova showed evenly high Spy0128-specific IgG titres (5×10^4 to 5×10^6) in serum samples, confirming reliable and consistent administration of the bacterial suspensions (Fig. 3A). Immunisation with *L. lactis* PilM1-Ova or synthetic Ova₃₂₄₋₃₃₉ administered with the mucosal adjuvant CT-B, resulted in measurable and significant Ova-specific serum IgG responses (titres of 5×10^3 to 1×10^4) compared to mice immunised with the *L. lactis* PilM1 or *L. lactis* PilM1 + Ova controls ($p < 0.005$), which did not generate significant anti-Ova responses (Fig. 3B). Administration of synthetic Ova₃₂₄₋₃₃₉ without CT-B also failed to generate Ova-specific IgG ($p < 0.005$). Notably, strong IgG responses were also seen with synthetic Ova₃₂₄₋₃₃₉ when administered with CT-B. However, it should be mentioned that the amount of synthetic Ova₃₂₄₋₃₃₉ was approximately 36,000 times higher than the amount of Ova₃₂₄₋₃₃₉ peptide displayed on the surface of *L. lactis* PilM1-Ova.

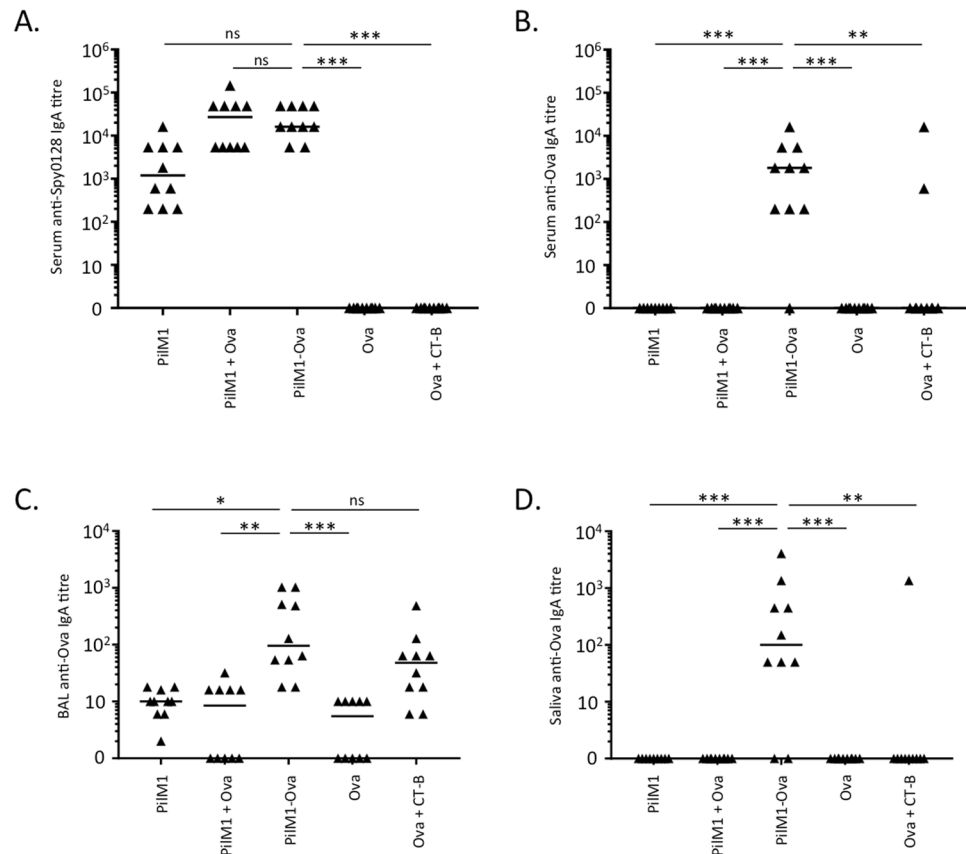


Figure 4. Intranasal immunisation of mice with *L. lactis* PilM1-Ova induces systemic and mucosal IgA responses. Groups of Balb/c mice ($n = 5$) were immunised intranasally with 1×10^8 CFU live recombinant *L. lactis* PilM1-Ova. The data from two independent experiments were combined. *L. lactis* without inserted peptide (PilM1) or mixed with synthetic Ova_{324–339} (PilM1 + Ova) were used as controls. Synthetic Ova_{324–339} peptide alone (Ova) or mixed with Cholera Toxin B subunit adjuvant (Ova + CT-B) were used as additional controls. (A) Specific IgA responses against recombinant Spy0128 were measured by ELISA. ELISAs were also performed to measure specific anti-ovalbumin IgA responses in serum (B), BAL fluid (C) and saliva (D). * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; ns = not significant; One-way ANOVA with Dunn's multiple comparisons test.

To investigate if *L. lactis* PilM1-Ova preferentially targets specific IgG subclasses, we tested IgG1 and IgG2a responses in serum from vaccinated mice (Fig. 3C). There was no significant difference between IgG1 and IgG2a responses when mice were vaccinated with *L. lactis* PilM1-Ova. However, mice vaccinated with synthetic Ova_{324–339} plus CT-B generated significantly more IgG1 than IgG2a ($p < 0.05$).

As intranasal immunisation is expected to also generate mucosal responses, IgA titres were measured in serum (Fig. 4A and B), BAL fluid (Fig. 4C) and saliva (Fig. 4D). As seen for IgG, consistent IgA responses in serum of *L. lactis* PilM1, *L. lactis* PilM1 + Ova and *L. lactis* PilM1-Ova groups were detected against Spy0128, with titres of approximately 10^3 to 5×10^4 (Fig. 4A). Importantly, significant Ova-specific serum IgA responses were found in most mice immunised with *L. lactis* PilM1-Ova (titres $\geq 10^3$, $p < 0.0005$). In contrast, mice immunised with *L. lactis* PilM1, *L. lactis* PilM1 + Ova or synthetic Ova_{324–339} peptide (even when mixed with CT-B) all failed to generate detectable Ova-specific serum IgA (Fig. 4B). Similar responses were also seen in BAL fluid and saliva samples, although IgA titres were generally lower (Fig. 4C and D). The only difference was that Ova_{324–339} peptide plus CT-B induced Ova-specific IgA responses in BAL fluid, at a similar level to mice immunised with *L. lactis* PilM1-Ova.

Expression of multiple peptides in the PilM1 pilus. Our cloning strategy (peptides with flanking XhoI and Sall sites into the XhoI site of the *spy0128* gene) allows the insertion of multiple peptides in succession. XhoI and Sall digestions generate compatible restriction site overhangs, which result in a hybrid site after ligation that is not recognised by either XhoI or Sall. Therefore, cloning of the first peptide into the pilin gene neutralises one XhoI site and leaves the remaining XhoI site for cloning of a second peptide in frame with the first peptide. Using this strategy, we have inserted a second model peptide (J14, derived from the *S. pyogenes* M protein³¹), downstream of the Ova_{324–339} peptide in the β E- β F loop (Table 1a). This peptide fusion consists of a total of 47 amino acids and was successfully expressed within the PilM1 pilus on the surface of *L. lactis* as shown by the formation of high-molecular weight bands on Western blots (Fig. 5A). However, flow cytometry revealed that the expression

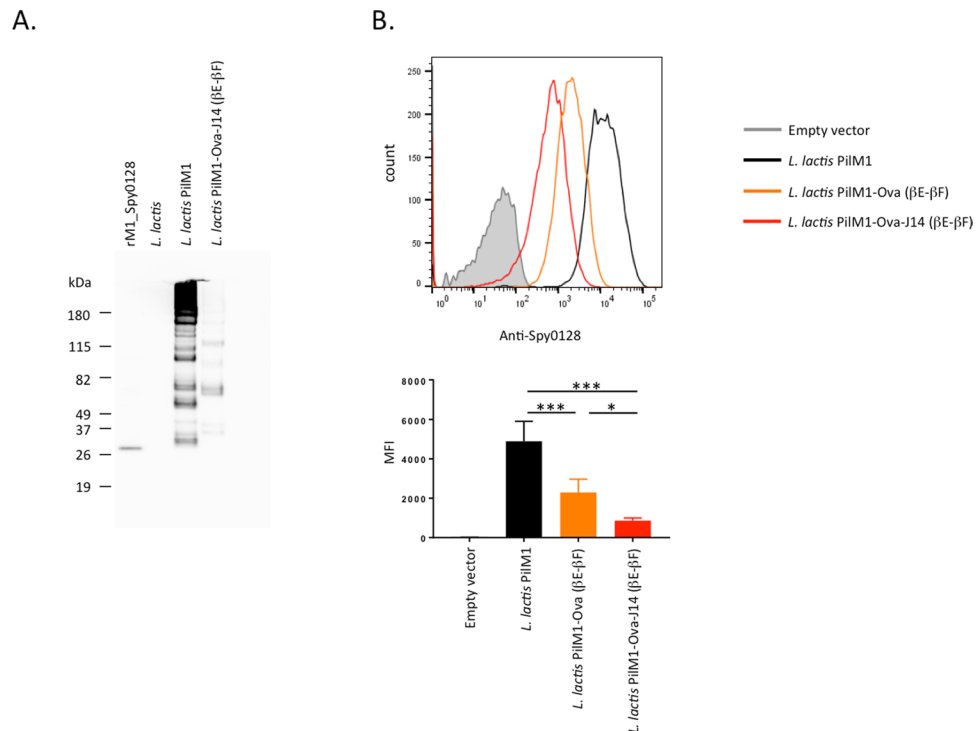


Figure 5. More than one peptide can be inserted in frame within a Spy0128 loop region. Cloning of a *Xho*I-*Sal*I fragment into a *Xho*I site generates one non-cleavable hybrid site and one intact *Xho*I site downstream of the inserted DNA region. This allows the addition of consecutive DNA fragments (peptide sequences). A second peptide (J14) was added to the Ova_{324–339} peptide within the PilM1 β E- β F loop region (PilM1-Ova-J14). **(A)** Western blot analysis of *L. lactis* cell wall extracts (CWE) with antiserum specific for M1_Spy0128. The high molecular band patterns are indicative of pilus assembly. **(B)** Flow cytometry analysis shows expression of M1_Spy0128 on the surface of *L. lactis*, but with notably reduced expression after insertion of peptides into the β E- β F loop region. Error bars show the standard deviation from 3 independent experiments. * $p < 0.05$; *** $p < 0.0005$; one-way ANOVA followed by a Holm-Sidak test.

level of Spy0128 was even lower (about 3 times) compared to the *L. lactis* PilM1-Ova construct with a single peptide in the β E- β F loop (Fig. 5B).

Expression of peptides in the backbone pilins of other *S. pyogenes* pilus types. Several antigenically different pilus types have been found in *S. pyogenes*¹⁷. The FCT-3 and FCT-4 pili each possess a backbone pilin with a predicted two-domain structure similar to the FCT-2 backbone pilin (Spy0128), despite rather low amino acid identities (38% and 40%, respectively). We used the SWISS PDB modeller to generate protein structure models of both the FCT-3 type (from *S. pyogenes* serotype M18) and the FCT-4 type (from *S. pyogenes* serotype M28) backbone pilins (Fig. 6A). This enabled us to identify the loop regions equivalent to the β E- β F, β 3- β 4 and β 9- β 10 loops in the M1_Spy0128. Insertion of the Ova_{324–339} peptide into the β E- β F loop region of M18_Spy0128 (Table 1b) resulted in expression and assembly of the M18 pilus (PilM18) on the surface of *L. lactis* (Fig. 6B). Similar to Spy0128 β E- β F-Ova, the insertion of the Ova_{324–339} peptide into the β E- β F loop region of M18_Spy0128 resulted in decreased expression of the backbone pilin (Fig. 6C).

Discussion

Mucosal routes for vaccine delivery have gained an increased interest in modern vaccinology. Mucosal surfaces represent a major entry site of many pathogens. Hence, the ability of a vaccine to induce a local mucosal immune response is an efficient prophylactic strategy. Mucosal vaccination also has an advantage over parenterally administered vaccines in that it can elicit both mucosal and systemic immune responses, generally with reduced secondary effects³². Additionally, mucosal vaccines can be administered without needles and syringes, eliminating the need for trained personnel³³.

There is growing emphasis on the use of peptide vaccines due to their specificity, thereby reducing unnecessary antigenic load and potentially allergenic and/or reactogenic responses⁴. However, the foremost challenge is that peptides are often poorly immunogenic on their own and undergo enzymatic degradation more quickly than folded proteins³⁴. These features necessitate chemical coupling to a stabilising carrier, and/or administration with potent immunostimulatory adjuvants³⁵, posing greater costs and potential safety concerns. To overcome these problems, we have developed a novel peptide carrier platform for the presentation of peptides as an integral part of the GAS serotype M1 pilus on the surface of *L. lactis*. *L. lactis* is a non-pathogenic bacterium which has a 'generally recognised as safe' (GRAS) status³⁶ and has been thoroughly investigated as a delivery vehicle and

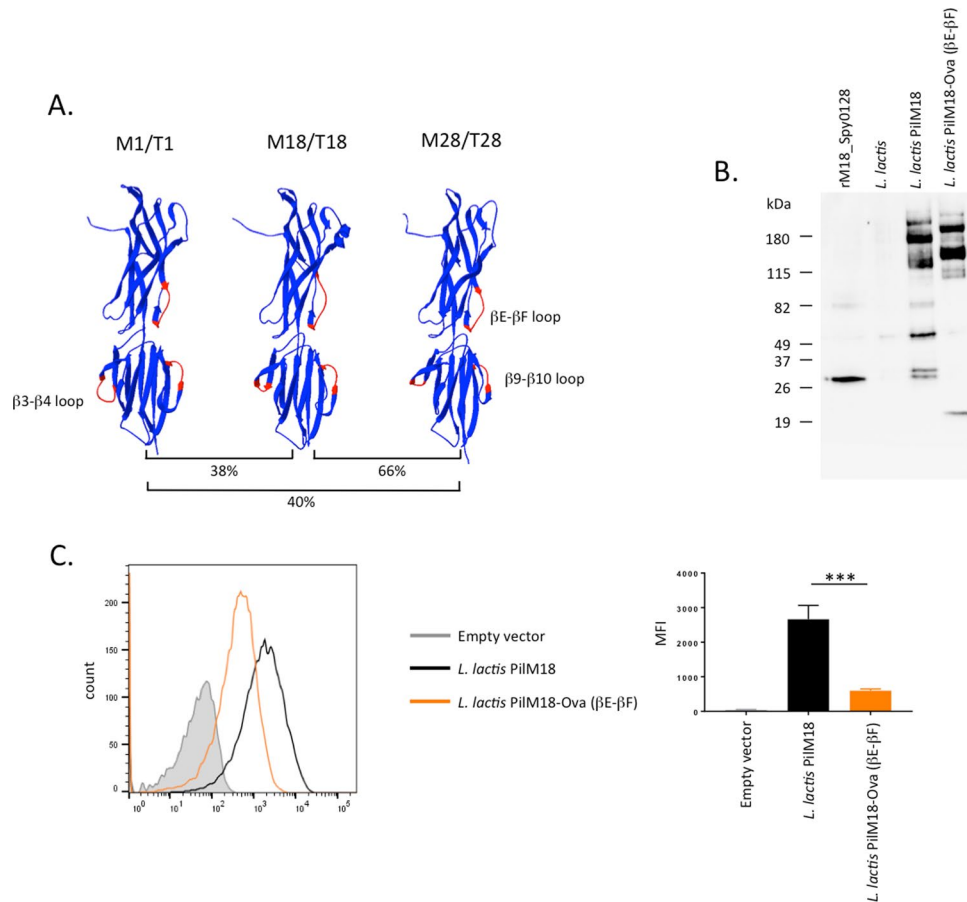


Figure 6. Peptides can be inserted into loop regions from structurally related backbone pilus proteins. **(A)** The structures of the backbone pilus proteins found in serotype M18/T18 and serotype M28/T28 were modelled onto the Spy0128 (serotype M1/T1) crystal structure (3B2M) using the Swiss PDB modeling server⁵³. Despite low amino acid sequence identities, the models predicted conserved structures, which allowed the identification of loop regions equivalent to those analysed in Spy0128 (M1/T1). The model peptide Ova_{324–339} was inserted into the β E- β F loop region of M18_Spy0128 using the same cloning strategy as for M1_Spy0128. **(B)** Western blot analysis of *L. lactis* cell wall extracts (CWE) with antiserum specific for M18_Spy0128. **(C)** Flow cytometry analysis with anti-M18_Spy0128 antiserum shows expression of M18_Spy0128 on the surface of *L. lactis*, but with notably reduced expression after insertion of peptides into the β E- β F loop region. Error bars show the standard deviation from 3 independent experiments. ***p < 0.0005; one-way ANOVA followed by a Holm-Sidak test.

adjuvant for mucosal vaccines^{18,22,37,38}. However, *L. lactis* produces a cell envelope structural component known as a polysaccharide pellicle, similar to a bacterial capsule, which might restrict accessibility of the heterologous protein³⁹. It has previously been proposed that foreign proteins expressed on the tip of a pilus might overcome this problem by exposing the protein at a distance from the cell envelope²⁷. The pilus structure of GAS has been shown to be highly immunogenic, and *L. lactis* possesses natural adjuvancy that induces IL-2 and IFN- γ cytokines⁴⁰. The combination of these two elements provides the basis of promising efficacy for the PilVax platform as a vaccine strategy.

The GAS serotype M1 pilus consists of 3 structural proteins that are covalently connected by a pilus assembly sortase on the cell surface to form a long protruding peritrichous structure. The length of the pilus is determined by the number of backbone pilins (Spy0128) incorporated into the pilus fibre, which is estimated to consist of around 50–100 Spy0128 monomers^{10,15,17}. Our strategy was to increase peptide stability and immunogenicity by inserting the model peptide Ova_{324–339} into the compact (trypsin-resistant) and highly immunogenic backbone pilin, resulting in multimerisation of the peptide when the pilus structure is assembled (Supplementary Figure 1). Of the 8 potential integration sites within Spy0128 we analysed, 3 loop regions were found to be suitable for peptide expression. These include the β E- β F loop region in the N-terminal domain and the β 3- β 4 loop and β 9- β 10 loop regions in the C-terminal domain. Insertion of the model peptide Ova_{324–339} into the β B- β C, β 2- β 3 loop regions or the β G- β 1 interdomain regions resulted in expression of monomeric Spy0128 in the protoplast fraction indicating a lack of pilus assembly. Notably, the Spy0128 proteins were slightly larger than the rSpy0128, which lacks the N-terminal secretion sequence and the C-terminal sortase C assembly domain. This suggests that lack of proper protein maturation is responsible for the failed pilus assembly. In contrast, insertion of the

Ova peptide into the β D- β E loop or at the free N-terminal region between Asn28 and Gly29 did not result in detectable Spy0128 in any of the cell fractions or the cell culture supernatant. This suggests that the proteins either failed to express or were rapidly degraded after expression. The exact reason for the failed expression of pili was not further analysed as this is irrelevant to this study. In the successful constructs, pilus expression was generally lower than in *L. lactis* expressing unmodified PilM1 without peptide. The reduced pilus expression level could be explained by shorter pili being assembled, or lower amounts of pili being produced on the surface. As only the overall amount of Ova_{324–339}-carrying Spy0128 pilins is important for our study, not the length of the pili, we have not investigated this any further.

One of the successful constructs, *L. lactis* expressing PilM1 with Ova_{324–339} at the β E- β F loop region (*L. lactis* PilM1-Ova) was used to intranasally immunise groups of mice and to test for Ova-specific antibody responses. Our study shows that *L. lactis* PilM1-Ova induces detectable Ova-specific IgA responses at mucosal sites (measured in saliva and BAL fluid) and in serum after intranasal vaccination. Antibody responses were measurable against ovalbumin by ELISA, indicating that the antibodies recognised the Ova_{324–339} peptide as part of the complete protein. This suggests that PilVax might be a suitable peptide delivery platform to develop mucosal vaccines against respiratory pathogens. In addition, we found detectable Ova-specific IgG responses in serum samples of immunised mice. As systemic antibodies can reduce bacterial spreading from mucosal sites and prevent systemic disease⁴¹, this is an important finding demonstrating the suitability of developing the PilVax platform into effective vaccines. Furthermore, serum-derived IgG can also contribute significantly to immune defence in the lower respiratory tract and in the genitourinary mucosa⁴². Notably, Ova-specific IgG or IgA were not detected in serum or mucosal sites when synthetic Ova_{324–339} was mixed with *L. lactis* PilM1. This indicates that the adjuvant property of *L. lactis* alone is not sufficient to induce Ova-specific immunity and suggests that the physical integration of the peptide into the pilus structure is important, probably due to peptide stabilisation and prevention of enzymatic degradation. The Ova-specific IgA response in BAL fluid was similar between *L. lactis* PilM1-Ova and Ova + CT-B immunised mice, even though the amount of synthetic Ova peptide was approximately 36,000-times higher in the Ova + CT-B group. This might again be due to the increased stability of the peptide when incorporated into the rigid pilus structure. Interestingly, no significant Ova-specific IgA titers were found in the serum after immunisation with Ova + CT-B. However, the reason for this is unclear.

Further investigation into the type of immune response generated by *L. lactis* PilM1-Ova showed a mixed IgG1/IgG2a systemic response. This was in contrast to the control immunisation with synthetic Ova_{324–339} mixed with CT-B adjuvant, which generated a predominant IgG1 response. In mice, IgG1 is associated with a Th2-like response, while a Th1 response promotes the induction of IgG2a antibodies⁴³. Yanase *et al.* recently reported that ovalbumin conjugated to nanoparticles (Ova-NPs) also stimulated a mixed IgG1/IgG2a Ova-specific response, and only low levels of unwanted and potentially toxic IgE antibodies in mice. In contrast, immunisation with Ova in alum stimulated Ova-specific IgG1 and IgE, but only low levels of IgG2a/IgG2b antibodies⁴⁴. Notably, IgA was not detected in either of these schedules, likely due to the subcutaneous route of immunisation used in this study⁴⁴.

We have further shown that it is possible to insert more than one peptide into the same integration site, expanding the versatility of the PilVax platform. Introducing unique restriction enzyme recognition sites into each of the 3 accommodating loop regions within the same construct could also further increase the number of different peptides that could be presented. Finally, we have shown that peptide epitopes can be incorporated into structurally similar, but antigenically different pilus structures. However, PilM18 expression was much lower than observed with PilM1 and therefore the suitability of this pilus type for peptide-presentation requires further evaluation.

One drawback of the PilVax platform is that peptide insertion, at least for the model Ova_{324–339} peptide tested in this study, resulted in notable reduction in pilus expression level on the *L. lactis* surface. However, we have shown that significant Ova-specific IgA and IgG levels could be generated in mice immunised with the construct that had the lowest pilus expression (*L. lactis* PilM1-Ova β E- β F). Based on the assumption that higher pilus/peptide expression would lead to improved immune responses it would be advisable that for any future application with a vaccine peptide, all integration sites should be investigated in order to identify the construct that shows the highest pilus expression levels.

Although live *L. lactis* vectors have been investigated as vaccines against a variety of different antigens from various pathogens^{18–22,37,38,45,46}, the lack of a biological containment strategy has hindered progress into clinical trials. One solution is the use of an auxotroph mutant to prevent selection by a plasmid-encoded antibiotic resistance gene, e.g. the *L. lactis* NZ9130 strain, which carries an alanine racemase gene deletion preventing growth in the absence of alanine^{47,48}. More recently, a non-GMO alternative to live *L. lactis* has been introduced which is based on hot acid-treated nucleotide-free *L. lactis* known as bacterium-like particles (BLP)^{49,50}.

In conclusion, we have developed a novel peptide delivery platform that might be useful for mucosal vaccinations against a variety of pathogens that colonise mucosal sites, such as *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and *Staphylococcus aureus*. As proof of concept, we have shown that insertion of the model peptide Ova_{324–339} into the monomeric pilus backbone protein Spy0128 increases immunogenicity most likely due to peptide stabilisation and multimerisation. Other benefits of PilVax include low production costs, as no chemical coupling is required and the modified bacteria can easily be shipped in lyophilised form. Furthermore, no potentially toxic adjuvants are required and the vaccine can be delivered needle-free to a mucosal site. These advantages in safety, low production cost and ease of administration are highly desired in developing countries, where efficacious vaccines are most needed.

Methods

Bacterial strains and culture conditions. *S. pyogenes* strains SF370 (serotype M1) and MGAS8232 (serotype M18) were grown stationary in Brain Heart Infusion (BHI) broth (BD) at 37 °C, *L. lactis* MG1363 was grown stationary in M17 media (BD) supplemented with 0.5% glucose at 28 °C, and *E. coli* DH5 α was grown aerobically

Name	Sequence (5' to 3')
PilM1.fw	GCGGATCCGATATGATGTACATTGAGAG
PilM1.rev	GCGGATCCGTCGTGGGGCAATAAAAAATTC
PilM18.fw	GCGGATCCGACCATATACACGTTGCAGGA
PilM18.rev	CGGCTGCAGTCTTATATGGTACAAAAACCTATCAGC
PilM28.fw	GCGGATCCGATTTTATTCAGAAGGAGAGG
PilM28.rev	CGGCTGCAGTCTTATATGGTACAAAAACCTATCAGC
PilM1_N29.fw	CCCGCTCGAGGGAGCCAACTAACAGTTAC
PilM1_N29.rev	CCCGCTCGAGGTTTACAACAGTCTCCCC
PilM1_βG-β1.fw	GAGAGACTCGAGACATTAACGGTGAAGAAAAAG
PilM1_βG-β1.rev	GAGAGACTCGAGATCTAAGCTATTTTGAAGCTG
PilM1_βB-βC.fw	GAGAGAGACTCGAGGAAATAAGTTTAAAGGTGTAGC
PilM1_βB-βC.rev	GAGAGAGACTCGAGAGTATCAGGTTTCGATTTTAAATG
PilM1_βD-βE.fw	GAGAGAGACTCGAGGGTGTATTATTACAAAG
PilM1_βD-βE.rev	GAGAGAGACTCGAGAAAATCAAATTCGTCAG
PilM1_βE-βF.fw	GAGAGAGACTCGAGGGTGTTCCTTATGATACAAAC
PilM1_βE-βF.rev	GAGAGAGACTCGAGTCTCCTCAGTTACTTTG
PilM1_β2-β3.fw	GAGAGAGACTCGAGTCAGAAAAAGTCATGATTGAG
PilM1_β2-β3.rev	GAGAGAGACTCGAGTGTCTTTAAAGTCAGACC
PilM1_β3-β4.fw	GAGAGAGACTCGAGCCTGTTCAAACAGAGGCTAG
PilM1_β3-β4.rev	GAGAGAGACTCGAGAGTTGTCTTCTCAATCATGAC
PilM1_β9-β10.fw	GAGAGAGACTCGAGAAAAATATCGCAGGTAATTC
PilM1_β9-β10.rev	GAGAGAGACTCGAGTTGAGGACTAACTTCCACG
PilM18_βE-βF.fw	GAGAGAGACTCGAGATAGCTTATGATTCTCAAC
PilM18_βE-βF.rev	GAGAGAGACTCGAGTACTTCAGAAACCGTATAAC
PilM28_βE-βF.fw	GAGAGAGACTCGAGGGGATTAATACGATACC
PilM28_βE-βF.rev	GAGAGAGACTCGAGTATTTCTGAAACCGTATAAC
Ova ₃₂₄₋₃₃₉ .fw	CCCGCTCGAGTCACAAGCTGTTTCATGCTGCACATGCAGAAAT
Ova ₃₂₄₋₃₃₉ .rev	CCCGTTCGACTCTACCTGCTTCATTAATTTCTGCATGTGCAGC
J14.fw	CCCGTTCGAGAAACAAGCTGAAGATAAAGTTAAAGCTTCACGTGAAGCCAAAAAGCAAGTCG
J14.rev	CCCGTTCGACTTGAACCTTATCTTCAAGTTGTTCAAGAGCTTTTTCGACTTGCTTTTGGC
PilM1_linker.fw	ACGCGTTCGACGGCAGCGGTACGGC CTCGAG GGC
PilM1_linker.rev	ACGCGTTCGACGCCGCTACCGCTGCC CTCGAG GCCG

Table 2. List of oligos used in this study Primer sequences used in this study. Restriction sites are shown in bold letters.

at 37°C in LB broth (HiMedia). When required, antibiotics were added: ampicillin (50 µg/ml, *E. coli*), kanamycin (50 µg/ml, *E. coli*; 200 µg/ml, *L. lactis*).

Genetic manipulations. The complete pilus operon was amplified from *S. pyogenes* SF370 or MGAS8232 genomic DNA by PCR using iProof DNA polymerase (Roche) and primers shown in Table 2. The purified PCR products were digested with BamHI (PilM1) or BamHI/PstI (PilM18) and cloned into plasmid pLZ12km2_P23R^{51,52} to generate pLZ12km2_P23R: PilM1 and pLZ12km2_P23R: PilM18.

The unique XhoI in the vector's multiple cloning site was first deleted, then re-introduced by full circle PCR with specific primers shown in Table 2 followed by XhoI digestion and religation. This allowed the insertion of peptide-encoding DNA sequences with flanking XhoI or SalI restriction sites. The DNA encoding Ova₃₂₄₋₃₃₉ and J14³¹ peptides were generated by PCR with overlapping primers (see Table 2), digested with XhoI and SalI and cloned into selected XhoI sites previously introduced into the pilus backbone genes as described above.

DNA sequences were confirmed by Sanger sequencing using the DNA sequencing service at the School of Biological Sciences (The University of Auckland, New Zealand). *L. lactis* strain MG1363 was transformed by electroporation with the resulting plasmids at 2100 V, 25 µF, 200 Ω in a 2 mm cuvette using a Gene Pulser Xcell™ (Bio-Rad).

Bioinformatic analysis. The structural model of the FCT-3 backbone pilin was generated using the Swiss PDB modelling server at the Swiss Institute of Bioinformatics⁵³ with Spy0128 (3B2M) as a template. The structural images were created with the PDB Swiss Viewer version 4.1.0.⁵⁴

Cell wall extracts and Western blots. An overnight *L. lactis* culture was spun down at 4,500 rpm for 15 min at 4°C. The bacterial cell pellet was washed once with PBS, then resuspended at 0.1 g/ml in cold protoplast buffer (40% sucrose, 10 mM MgCl₂, 0.1 M KPO₄, 2 mg/ml lysozyme, 400 U mutanolysin, Roche EDTA-free protease inhibitor) and incubated for 3 h at 37°C with constant rotation. Cellular debris was removed by

centrifugation at $13,000 \times g$ for 15 min at 4°C , and the supernatant containing cell surface proteins were run on a NuPAGE™ Novex™ 4–12% gradient gel (Invitrogen). Separated proteins were transferred onto a nitrocellulose (N+) membrane (Biotrace NT, Pall Life Science, USA) in NuPAGE™ Transfer Buffer (Invitrogen) using a Hoefer TE77 Semi-phor semi-dry transfer unit. The membrane was blocked with 5% (w/v) non-fat milk in TBS, 0.5% (v/v) tween 20 (TBS-T), then probed with polyclonal rabbit antibodies against M1_Spy0128, M18_Spy0128²⁶ or serum from Ova + CT-B immunised mice. After intensive washing in TBS-T, the membrane was then probed with goat anti-rabbit or goat anti-mouse HRP-conjugated antibody. The membrane was washed again before being developed using Amersham ECL Prime Western blotting detection reagent (GE Healthcare). Chemiluminescence signals were captured and digitised into image using a ChemiDoc™ Imaging System (Bio-Rad).

Flow cytometry. *L. lactis* strains were grown overnight in GM17 medium, harvested by centrifugation ($5000 \times g$, 5 min), and resuspended in 1 ml blocking buffer (PBS/3% FBS/5 mM EDTA) at an OD_{600} of 0.4. Cells were dispersed in a water bath sonicator for 2 min, then incubated on ice for 30 min. Blocking buffer was removed by centrifugation, and bacteria were washed once with FACS buffer (PBS/1% FBS/5 mM EDTA) and incubated with anti-Spy0128 antibody in FACS buffer on ice for 30 min. Bacteria were washed once, then incubated with anti-rabbit IgG FITC (Abacus ALS Ltd.) on ice for 30 min. Flow cytometry analysis was performed on a LSRII Flow Cytometer and analysed using FlowJo software.

Immunisations. Frozen aliquots of *L. lactis* were prepared as previously described²⁶ and enumerated by plating. On immunisation days, an aliquot was thawed at RT, washed and resuspended in PBS. Five to six week old Balb/c mice ($n = 5$) were immunised intranasally, under isoflurane anaesthesia, with 1×10^8 CFU live recombinant *L. lactis* with or without 1.4 ng Ova peptide, 1.4 ng Ova peptide alone, or 50 μg Ova peptide + 4 μg CT-B, in a 10 μl volume. The bacterial suspensions were administered on 3 consecutive days (day 0, 1 and 2) as described previously²⁷. An additional 3 boosters were given, 1–2 weeks apart, for a total of 12 doses (days 0, 1, 2, 14, 15, 16, 28, 29, 30, 35, 36, 37). Pre-immune serum was collected on day 0 and immune serum was collected 1 week after final immunisation (day 44). Bronchoalveolar lavage (BAL) was performed with 1 ml PBS, and saliva samples collected by rinsing the oral cavity with 50 μl PBS on day 44.

ELISA. MaxiSorp plates (Nunc) were coated with 1 $\mu\text{g}/\text{ml}$ recombinant Spy0128 protein or commercial ovalbumin protein (Invivogen) in PBS overnight at 4°C , prior to incubation with titrated serum, BAL fluid or saliva. Detection was performed using goat anti-mouse IgG/IgG1/IgG2a-HRP (Life Technology) or goat anti-mouse IgA-HRP (Invitrogen) and 3,3',5,5'-tetramethylbenzidine (ThermoFisher). Absorbance was determined on an EnSpire multilabel plate reader (Perkin Elmer). Endpoint titres were determined as the minimum serum dilution above the control (absorbance of 1:200 dilution of pre-immune serum plus 3 times the standard deviation).

Animal Ethics. All animal experiments were performed in the Vernon Jansen Unit (University of Auckland, New Zealand) in accordance with relevant guidelines and regulations approved by the University of Auckland Animal Ethics Committee.

Data availability. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

References

- Ehreth, J. The global value of vaccination. *Vaccine* **21**, 596–600 (2003).
- Clark, T. G. & Cassidy-Hanley, D. Recombinant subunit vaccines: potentials and constraints. *Develop Biol* **121**, 153–163 (2005).
- Hansson, M., Nygren, P. A. & Stahl, S. Design and production of recombinant subunit vaccines. *Biotech Appl Biochem* **32**(Pt 2), 95–107 (2000).
- Li, W., Joshi, M. D., Singhanian, S., Ramsey, K. H. & Murthy, A. K. Peptide. *Vaccine: Progress and Challenges*. *Vaccines* **2**, 515–536 (2014).
- Lee, V. H. Enzymatic barriers to peptide and protein absorption. *Crit Rev Ther Drug Carrier Sys* **5**, 69–97 (1988).
- Purcell, A. W., McCluskey, J. & Rossjohn, J. More than one reason to rethink the use of peptides in vaccine design. *Nat Rev Drug Discov* **6**, 404–414 (2007).
- Bijker, M. S., Melief, C. J., Offringa, R. & van der Burg, S. H. Design and development of synthetic peptide vaccines: past, present and future. *Exp Rev Vaccines* **6**, 591–603 (2007).
- Vartak, A. & Sucheck, S. J. Recent Advances in Subunit Vaccine Carriers. *Vaccines* **4**, <https://doi.org/10.3390/vaccines4020012> (2016).
- Danne, C. & Dramsi, S. Pili of gram-positive bacteria: roles in host colonization. *Res Microbiol* **163**, 645–658 (2012).
- Proft, T. & Baker, E. N. Pili in Gram-negative and Gram-positive bacteria - structure, assembly and their role in disease. *Cell Mol Life Sci* **66**, 613–635 (2009).
- Hendrickx, A. P. A., Budzik, J. M., Oh, S.-Y. & Schneewind, O. Architects at the bacterial surface — sortases and the assembly of pili with isopeptide bonds. *Nat Rev Microbiol* **9**, 166–176 (2011).
- Bessen, D. E. & Kalia, A. Genomic localization of a T serotype locus to a recombinatorial zone encoding extracellular matrix-binding proteins in *Streptococcus pyogenes*. *Infect Immun* **70**, 1159–1167 (2002).
- Kreikemeyer, B. et al. *Streptococcus pyogenes* collagen type I-binding Cpa surface protein. Expression profile, binding characteristics, biological functions, and potential clinical impact. *J Biol Chem* **280**, 33228–33239 (2005).
- Linke-Winnebeck, C. et al. Structural model for covalent adhesion of the *Streptococcus pyogenes* pilus through a thioester bond. *J Biol Chem* **289**, 177–189 (2014).
- Kang, H. J., Coulibaly, E., Clow, F., Proft, T. & Baker, E. N. Stabilizing isopeptide bonds revealed in Gram-positive bacterial pilus structure. *Science* **318**, 1625–1628 (2007).
- Linke, C. et al. Crystal structure of the minor pilin FctB reveals determinants of Group A streptococcal pilus anchoring. *J Biol Chem* **285**, 20381–20389 (2010).
- Mora, M. et al. Group A *Streptococcus* produce pilus-like structures containing protective antigens and Lancefield T antigens. *Proc Natl Acad Sci USA* **102**, 15641–15646 (2005).
- Bahey-El-Din, M. *Lactococcus lactis*-based vaccines from laboratory bench to human use: an overview. *Vaccine* **30**, 685–690 (2012).

19. Bahey-El-Din, M., Gahan, C. G. & Griffin, B. T. *Lactococcus lactis* as a cell factory for delivery of therapeutic proteins. *Curr Gene Ther* **10**, 34–45 (2010).
20. Berlec, A., Ravnikar, M. & Strukelj, B. Lactic acid bacteria as oral delivery systems for biomolecules. *Die Pharmazie* **67**, 891–898 (2012).
21. Bermudez-Humaran, L. G., Kharrat, P., Chatel, J. M. & Langella, P. Lactococci and lactobacilli as mucosal delivery vectors for therapeutic proteins and DNA vaccines. *Microb Cell Fact* **10**(Suppl 1), S4, <https://doi.org/10.1186/1475-2859-10-s1-s4> (2011).
22. Mercenier, A., Muller-Alouf, H. & Grangette, C. Lactic acid bacteria as live vaccines. *Curr Iss Mol Biol* **2**, 17–25 (2000).
23. Braat, H. *et al.* A phase I trial with transgenic bacteria expressing interleukin-10 in Crohn's disease. *Clin Gastroenterol Hepatol* **4**, 754–759 (2006).
24. Becherelli, M. *et al.* The ancillary protein 1 of *Streptococcus pyogenes* FCT-1 pili mediates cell adhesion and biofilm formation through heterophilic as well as homophilic interactions. *Mol Microbiol* **83**, 1035–1047 (2012).
25. Edwards, A. M. *et al.* Scavenger receptor gp340 aggregates group A streptococci by binding pili. *Mol Microbiol* **68**, 1378–1394 (2008).
26. Loh, J. M. S., Lorenz, N., Tsai, C. J.-Y., Khemlani, A. & Proft, T. Mucosal vaccination with pili from Group A Streptococcus expressed on *Lactococcus lactis* generates protective immune responses. *Sci Rep* (2017).
27. Quigley, B. R. *et al.* A foreign protein incorporated on the Tip of T3 pili in *Lactococcus lactis* elicits systemic and mucosal immunity. *Infect Immun* **78**, 1294–1303 (2010).
28. Young, P. G., Proft, T., Harris, P. W., Brimble, M. A. & Baker, E. N. Structure and activity of *Streptococcus pyogenes* SipA: a signal peptidase-like protein essential for pilus polymerisation. *PLoS one* **9**, e99135, <https://doi.org/10.1371/journal.pone.0099135> (2014).
29. Johnsen, G. & Elsayed, S. Antigenic and allergenic determinants of ovalbumin-III. MHC Ia-binding peptide (OA 323–339) interacts with human and rabbit specific antibodies. *Mol Immunol* **27**, 821–827 (1990).
30. McFarland, B. J., Sant, A. J., Lybrand, T. P. & Beeson, C. Ovalbumin(323–339) peptide binds to the major histocompatibility complex class II I-A(d) protein using two functionally distinct registers. *Biochemistry* **38**, 16663–16670 (1999).
31. Olive, C., Clair, T., Yarwood, P. & Good, M. F. Protection of mice from group A streptococcal infection by intranasal immunisation with a peptide vaccine that contains a conserved M protein B cell epitope and lacks a T cell autoepitope. *Vaccine* **20**, 2816–2825 (2002).
32. Bermudez-Humaran, L. G. *et al.* A novel mucosal vaccine based on live Lactococci expressing E7 antigen and IL-12 induces systemic and mucosal immune responses and protects mice against human papillomavirus type 16-induced tumors. *J Immunol* **175**, 7297–7302 (2005).
33. Yuki, Y. & Kiyono, H. Mucosal vaccines: novel advances in technology and delivery. *Expert Rev Vaccines* **8**, 1083–1097 (2009).
34. Kaumaya, P. T. *et al.* Peptide vaccines incorporating a 'promiscuous' T-cell epitope bypass certain haplotype restricted immune responses and provide broad spectrum immunogenicity. *J Mol Recog: JMR* **6**, 81–94 (1993).
35. O'Hagan, D. T. & De Gregorio, E. The path to a successful vaccine adjuvant—'the long and winding road'. *Drug Discov Today* **14**, 541–551 (2009).
36. Song, A. A., In, L. L., Lim, S. H. & Rahim, R. A. A review on *Lactococcus lactis*: from food to factory. *Microb Cell Fact* **16**, 55 (2017).
37. Thole, J. E. *et al.* Live bacterial delivery systems for development of mucosal vaccines. *Curr Opin Mol Therap* **2**, 94–99 (2000).
38. Wyszynska, A., Kobińska, P., Bardowski, J. & Jagusztyn-Krynicka, E. K. Lactic acid bacteria—20 years exploring their potential as live vectors for mucosal vaccination. *Appl Microbiol Biotechnol* **99**, 2967–2977 (2015).
39. Chapot-Chartier, M. P. *et al.* Cell surface of *Lactococcus lactis* is covered by a protective polysaccharide pellicle. *J Biol Chem* **285**, 10464–10471 (2010).
40. Bermudez-Humaran, L. G. *et al.* An inducible surface presentation system improves cellular immunity against human papillomavirus type 16 E7 antigen in mice after nasal administration with recombinant lactococci. *J Med Microbiol* **53**, 427–433 (2004).
41. Lima, F. A. *et al.* Controlled inflammatory responses in the lungs are associated with protection elicited by a pneumococcal surface protein A-based vaccine against a lethal respiratory challenge with *Streptococcus pneumoniae* in mice. *Clin Vaccine Immunol: CVI* **19**, 1382–1392 (2012).
42. Holmgren, J. & Czerkinsky, C. Mucosal immunity and vaccines. *Nat Med* **11**, S45–53 (2005).
43. Germann, T. *et al.* Interleukin-12 profoundly up-regulates the synthesis of antigen-specific complement-fixing IgG2a, IgG2b and IgG3 antibody subclasses *in vivo*. *Eur J Immunol* **25**, 823–829 (1995).
44. Yanase, N. *et al.* OVA-bound nanoparticles induce OVA-specific IgG1, IgG2a, and IgG2b responses with low IgE synthesis. *Vaccine* **32**, 5918–5924 (2014).
45. Chamcha, V., Jones, A., Quigley, B. R., Scott, J. R. & Amara, R. R. Oral Immunization with a Recombinant *Lactococcus lactis*-Expressing HIV-1 Antigen on Group A Streptococcus Pilus Induces Strong Mucosal Immunity in the Gut. *J Immunol (Baltimore, Md.: 1950)* **195**, 5025–5034 (2015).
46. Trombert, A. Recombinant lactic acid bacteria as delivery vectors of heterologous antigens: the future of vaccination? *Beneficial Microbes* **6**, 313–324 (2015).
47. Bron, P. A. *et al.* Use of the *alr* gene as a food-grade selection marker in lactic acid bacteria. *Appl Environ Microbiol* **68**, 5663–5670 (2002).
48. Lu, W., Kong, J. & Kong, W. Construction and application of a food-grade expression system for *Lactococcus lactis*. *Mol Biotech* **54**, 170–176 (2013).
49. Lu, J. *et al.* Systemic and mucosal immune responses elicited by intranasal immunization with a pneumococcal bacterium-like particle-based vaccine displaying pneumolysin mutant Plym2. *Immunol Lett* **187**, 41–46 (2017).
50. van Roosmalen, M. L. *et al.* Mucosal vaccine delivery of antigens tightly bound to an adjuvant particle made from food-grade bacteria. *Methods (San Diego, Calif)* **38**, 144–149 (2006).
51. Loh, J. M. & Proft, T. Toxin-antitoxin-stabilized reporter plasmids for biophotonic imaging of Group A streptococcus. *Appl Microbiol Biotech* **97**, 9737–9745 (2013).
52. Okada, N., Tatsuno, I., Hanski, E., Caparon, M. & Sasakawa, C. *Streptococcus pyogenes* protein F promotes invasion of HeLa cells. *Microbiology (Reading, England)* **144**(Pt 11), 3079–3086 (1998).
53. Arnold, K., Bordoli, L., Kopp, J. & Schwede, T. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics (Oxford, England)* **22**, 195–201 (2006).
54. Johansson, M. U., Zoete, V., Michielin, O. & Guex, N. Defining and searching for structural motifs using DeepView/Swiss-PdbViewer. *BMC Bioinformatics* **13**, 173 (2012).

Acknowledgements

This work was supported by research grants from the Auckland Medical Research Foundation (AMRF, grant number 1111016) and Auckland UniServices (grant number 34543.002). JMSL is a New Zealand Heart Foundation Research Fellow. D.W. was the recipient of a University of Auckland doctoral scholarship.

Author Contributions

D.W. generated the majority of *L. lactis* PilM1 constructs and conducted the initial mouse experiments; J.-Y.C.T. generated the *L. lactis* PilM18 constructs and some of the PilM1 constructs and contributed to manuscript

preparation. C.C. and S.B. generated some of the *L. lactis* PilM1 constructs, performed the Western blots, and flow cytometry; J.M.S.L. jointly supervised the work, re-validated the animal experiments and contributed to manuscript preparation; T.P. secured the funding, developed the original concepts of the work, jointly supervised the work and wrote the first draft of the manuscript. All authors reviewed the manuscript.

Additional Information

Supplementary information accompanies this paper at <https://doi.org/10.1038/s41598-018-20863-7>.

Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2018