HHS Public Access

Author manuscript

Microbes Infect. Author manuscript; available in PMC 2016 March 01.

Published in final edited form as:

Microbes Infect. 2015 March ; 17(3): 237–242. doi:10.1016/j.micinf.2014.11.002.

Commensal *Streptococcus mitis* is a unique vector for oral mucosal vaccination

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Abstract

The development of vaccine approaches that induce mucosal and systemic immune responses is critical for the effective prevention of several infections. Here, we report on the use of the abundant human oral commensal bacterium *Streptococcus mitis* as a delivery vehicle for mucosal immunization. Using homologous recombination we generated a stable *rS. mitis* expressing a *Mycobacterium tuberculosis* protein (Ag85b). Oral administration of *rS. mitis* in gnotobiotic piglets resulted in efficient oral colonization and production of oral and systemic anti-Ag85b specific IgA and IgG antibodies. These results support that the commensal *S. mitis* is potentially a useful vector for mucosal vaccination.

Keywords

Streptococcus mitis; Ag85b; vaccine vector; oral immunization; mucosal vaccine; gnotobiotic piglets

1. INTRODUCTION

Most vaccines currently approved for humans are administered parenterally; therefore, mucosal, needle-free vaccination would not only improve compliance in immunization programs, particularly in developing countries, but would also generate a more relevant protective immune response at mucosal sites of the body. A successful example is the trivalent attenuated poliovirus oral vaccine, the keystone of the Global measures that led to the eradication of poliomyelitis [1]. Other currently available and FDA approved oral vaccines include rotavirus [2, 3], typhoid [4] and adenovirus [5].

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Over the years many attenuated strains of viruses and bacteria have been tested as live vectors of antigen delivery for mucosal immunization against a variety of diseases. Examples of these systems include *Salmonella enteric* serovar typhi (*S. typhi*) [6] and *S. enteric* serovar typhimurium (*S. typhimurium*) [7], *Shigella* [8], *Vibrio cholera* [9], *Listeria monocytogenes* [10], *Mycobacterium bovis* (Bacille Calmette-Guerin, BCG) [11], and *Yersinia enterocolitica* [12]. Although some preclinical success was achieved with these vectors, several issues on the downstream steps of vaccine development for human use have emerged including substandard safety performance and poor long-term colonization. A few commensal organisms, e.g., *Streptococcus gordonii* and *Lactobacillus* spp have also been tested as vectors of antigen delivery. However, these bacteria, similar to the attenuated strains of virulent organisms, are either poor colonizers (*Lactobacillus)* in humans [13] or less abundant (*S. gordonii)* than *S. mitis* [14].

S. mitis possesses unique features that make it an attractive vaccine vector for eliciting human mucosal immunity. It is the most abundant member of the normal human oral flora and an excellent colonizer of this mucosal site [13], inhabiting the human oral cavity as early as 1-3 days postpartum [15]. Microbiological studies have shown that during infancy and adult life, *S. mitis* can predominate, both in prevalence and proportion of oral streptococci recovered in the mouth [16, 17]. Mucosal immune responses to *S. mitis* are well-documented. Salivary IgA antibody to *S. mitis* is present soon after birth and persists into adulthood [18].

Because of this exceptional biological association with the human host, we explored *S. mitis* as a possible mucosal vaccine vector. Here we report the initial and successful validation of this unique system of oral immunization.

2. MATERIALS AND METHODS

2.1. Bacteria, vector plasmid, and growth conditions

Streptococcus mitis strain NCTC 12261 (ATCC) and plasmid pCR2.1TOPO (Invitrogen) were used in this study. *S. mitis* was grown in Todd Hewitt Broth (THB) medium and *S. mitis* expressing an *Mtb* antigen were grown in THB containing 50 μg/ml erythromycin at 37°C under anaerobic conditions.

2.2. Experimental animals

Gnotobiotic piglets were delivered by caesarian section and were maintained in specific-pathogen-free conditions. The protocol for pig experiments was conducted under the approval of the IACUC at the Cummings School of Veterinary Medicine, Tufts University, Grafton, MA.

2.3. Construction and characterization of recombinant S. mitis

The *Mycobacterium tuberculosis* (*Mtb*) Ag85b gene (which codes for the vaccine candidate Ag85b) was codon-optimized for optimal expression in *S. mitis* followed by synthesis, both steps were done at Blue Heron Biotech, Bothel, WA (www.blueheronbio.com) using a proprietary technology which allows 100% accuracy. The synthesized DNA, which included

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the restriction enzyme sequences *Sac*I and *Not*I (5' and 3' respectively), was cloned into pUCminusMCS (Blue Heron) followed by sequencing, which confirmed the designed optimized sequence. The gene was then cut and ligated in-frame with the 250bp 5' end of the pullulanase gene (*pulA*/Smt0163) encoding a signal peptide that allows processing and secretion of the vaccine antigens [19]. For stable expression, the *Mtb* gene was integrated into the *pulA* gene using a gene cassette consisting of the 250bp 5' end of the pullulanase gene (*pulA*/Smt0163) that is in-frame with the codon-optimized *Mtb* gene followed by the erythromycin resistance gene (*erm*R) amplified from the plasmid pVA838 [20] and the 250bp 3' end of the *pulA* gene (Fig. 1A). The gene cassette was inserted into the pCR2.1 Topo vector to create p5E3 which was transformed into *S. mitis* by electroporation to allow for integration of the *Mtb* gene via homologous recombination into the *pulA* gene of *S. mitis* (Fig. 1A). Erm-resistant *S. mitis* transformants were selected on THB agar plates containing 50 μg/ml erythromycin.

Transformation and integration was confirmed by PCR and sequencing. Genomic DNA was isolated from overnight cultures using the DNeasy Blood and Tissue kit (Qiagen, CA). To assess *Ag8b* gene integration, we used the sense primer 5'-AAT<u>GAGCTC</u>TTTTCTCGTCCTGGTTTG-3' and the antisense primer 5'-TAAT<u>GCGGCCGC</u>GCTCCTTGGAAGCTGTCAGT-3' (*Sac*I and *Not*I restriction sequences are underlined) which amplifies a 1868 bp fragment comprising *S. mitis pul*A signal peptide, the in frame Ag85b, and the erythromycin gene.

2.4. Western blot analysis

Protein samples were prepared from 10 ml of bacterial cultures (OD 600 ≊1). After centrifugation, culture supernatants were transferred to new tubes containing protease inhibitor cocktail. Supernatants were concentrated 20x using a 3 kDa Amicon centrifugation device (Millipore, Billerica, MA). The concentrated supernatants were separated on 4-20% gradient sodium dodecyl sulfate-gels (SDS-gels) and then transferred to polyvinylidene difluoride (PVDF) membranes. Antigen detection was performed with specific rabbit anti-Ag85b antiserum (Colorado State University, NIAID/NIH Tuberculosis Research Materials contract no. 1-A125174, Fort Collins, CO) and developed with horseradish peroxidase-conjugated anti-rabbit IgG (Abcam, Cambridge MA). Visualization was carried by incubation with ECL reagents (Invitrogen).

2.5. Mass spectrometry

Concentrated culture supernatant from the recombinant *S. mitis* construct (*rS. mitis*) was separated by SDS-PAGE. A band corresponding to the size of Ag85b protein (~35 kDa) was excised from the Coomassie-stained gel and was submitted for mass spectroscopy analysis. The sample was subjected to in-gel digestion and peptide mass determination was carried out using microcapillary LC/MS/MS techniques (Taplin Biological Mass Spectrometry Facility, Harvard Medical School).

2.6. Colonization of rS. mitis in the oral cavity of piglets

rS. mitis cultures were adjusted to 10^9 cell/ml in THB medium. A total of 3 pigs received 2 oral doses of 10^9 bacteria on 2 consecutive days. Three oral samples and one fecal sample

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were collected from each animal weekly for up to 5 weeks post inoculation. Two oral samples were collected by cotton swabs (saliva) and one sample was collected by brushing all oral surfaces (brush swab). For fecal samples, cotton swabs were used to collect anal samples. To definitively identify the colonizing bacteria, samples were grown anaerobically in THB medium. Genomic DNA was prepared from bacterial cultures as described above. Amplification reaction was carried out with 0.3 μg of genomic DNA and the sense and antisense primers mentioned above. Saliva samples collected from animals before inoculation were used as negative controls. The PCR products were analyzed on agarose gels.

For antibody evaluation saliva was centrifuged and transferred to fresh tubes and tested undiluted using ELISA. For systemic antibody evaluation, sera were collected at week 3, 4, and 5 post infection. To assess the basal antibody levels, serum samples were collected from littermate naïve piglets (week 0). Sera were kept at -20°C until assayed.

2.7. Antibody measurements by Enzyme Linked Immunosorbent Assay (ELISA)

Costar high binding 96-well flat-bottomed plates were coated overnight at 4°C with 2μg/ml of recombinant Ag85b protein (Colorado University). After blocking with 1% bovine serum albumin in PBS-Tween (PBS-T), saliva or serum samples were then added to the plates. Saliva was used undiluted while serum was added at 1:100 dilution and incubated for 1 h at room temperature. For IgA detection an HRP labeled rabbit anti-pig IgA isotype antibody (Bethyl laboratories, Montgomery, TX) was used. Anti-Ag85b IgG was detected with biotin-labeled protein A. Plates were developed using TMB (3, 3’, 5, 5’-tetramethylbenzidine) substrate (KPL). Absorbance was read at 450 nm using a microplate ELISA reader (Molecular Devices, Sunnyvale, CA) and SoftMax Pro5. Samples were tested in duplicate.

2.8. Statistics

Statistical analysis was performed using student *t*-test. *P* values of 0.05 or less were considered significant.

3. RESULTS

3.1. Construction of S. mitis expressing Ag85b protein

Our approach was to generate *rS. mitis* that express vaccine antigens as secreted proteins (like the native Ag85b) because such microbial antigens are in general highly immunogenic [21]. To facilitate expression/secretion of candidate vaccine antigens, the DNA sequence of the signal peptide of the pullulanase A (pulA) gene of *S. mitis* was fused with the *Ag85b* gene. The signal peptide has a putative amino-terminal region (N), a hydrophobic core (H), a signal peptidase cleavage site (C), and an accessory Sec transport motif (AST). The *pulA* gene was selected as the integration site because it exhibits putative signal sequence for gram-positive protein expression that are both associated with the cell as well as in secreted form [19]. The *Ag85b* gene was ligated in frame with the 250 bp 5’ end of the *pulA* gene encoding signal peptide. A DNA fragment containing the *Mtb* gene and *ermr* flanked by 250bp *pulA* 5’ and 3’ fragments was constructed for transformation and integration at the

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pulA locus (Fig. 1A). Genomic PCR analysis revealed that the transformation of *S. mitis* with the DNA cassette resulted in successful integration of the *Ag85b* gene into the *pulA* locus (Fig 1B).

3.2. Expression analysis of Ag85b produced by rS. mitis vaccine vector

To determine whether the *rS. mitis* expressed and secreted the *Mycobacterium* antigen, the engineered bacteria as well as the parental strain were grown in THB medium. The cultures' supernatants were concentrated 20x using amicon filters (3kDa cutoff) and the presence of Ag85b was then examined by Western blot probed with a specific rabbit anti-Ag85b antibody. Figure 1C clearly shows that Ag85b was present in the culture supernatant of the *rS. mitis* but absent in the culture supernatant of the WT bacteria. These results confirm that the *rS. mitis* expresses the Ag85b and as in *Mtb*, releases the protein into the extracellular culture environment.

To definitively confirm the identity of the protein band identified by the Western blot as the Ag85b, a second PAGE was run and Coomassie blue stained. A protein band corresponding to the same MW of the protein recognized in the Western blot analysis was cut from the gel and subjected to mass spectrometry for protein sequence. Analysis of the digested protein yielded 5 peptides with 100% identity to Ag85b (Fig 1D). Since multiple peptide sequences spanning at different segments of Ag85b were identified, these results are unequivocal indication that the *S.mitis*Ag85b expresses and releases Ag85b into the bacterial culture supernatant. To rule out that these peptide sequences could be in *S. mitis* proteins their sequences were individually examined using a BLAST analysis against *S. mitis* genome. The results clearly indicated that this microorganism does not have genes coding for the peptides' sequences detected by mass spectroscopy (Fig. 1E).

To confirm that the vaccine construct was stably integrated in the *S. mitis* genome the bacteria was successively cultured (every other day for 80 passages) in Todd medium (in the absence of selective antibiotic) and the presence of the insert was routinely monitored on these cultures (Fig. 2A). These results, in addition to confirming the stability of the mutant they also clearly indicate that *pul*A gene is not essential for the bacterial growth.

3.3. Colonization of engineered S. mitis in the oral cavity and gastrointestinal tract of gnotobiotic piglets

Unfortunately, small rodents (e.g., mice) are unsuitable to perform these experiments because most bacteria present in the human oral microbiome including *S. mitis* do not colonize in the oral cavity of these animals. To address this key issue we used the gnotobiotic piglet model for colonization of human microbial species [22]. r*S. mitis* was pipetted into the oral cavity of the animals on two consecutive days. Oral and fecal samples were collected and examined microscopically for the presence of r*S. mitis*. The presence of Ag85b in the colonizing bacteria was confirmed by PCR in all samples (Fig. 2B). These results confirm that r*S. mitis* is capable of colonizing gnotobiotic pigs.

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3.4. Detection of Ag85b-specific IgA and IgG in saliva and sera of colonized pigs

To determine whether colonizing *rS. mitis* in the gnotobiotic piglets induced mucosal and/or systemic immune response, we measured anti-Ag85b IgA and IgG in saliva and sera using ELISA. Colonized pigs showed anti-Ag85b-specific IgG and IgA in both fluids (Fig. 3A & 3B). Anti-Ag85b antibodies were not detected either in saliva or sera before immunization or from naïve litter-mate pigs. Saliva samples of colonized pigs exhibited detectable levels of Ag85b-specific IgA as early as day 9 after colonization (Fig. 3A). The level of anti-Ag85b IgA in sera was not examined on day 9 post inoculation; however on day 24 lower levels of this antibody were detected in sera compared to saliva (Fig. 3B). In addition to the IgA response, *S. mitis* colonized pigs exhibited increased Ag85b-specific IgG antibodies (Fig. 3A & 3B). Collectively these findings indicate that r*S.mitis*Ag85b is capable of persisting in the oral cavity of colonized animals and inducing local and systemic antibody responses.

4. DISCUSSION

An important innovative aspect of this study is utilization of the commensal Gram-positive bacteria *S. mitis* which is an excellent colonizer of the normal human oral mucosal throughout life. As a proof-of-concept, we engineered *S. mitis* to express the Ag85b, a well-known antigen used in TB vaccine development experiments. To assure the stable expression of vaccine antigen for many bacterial generations, the recipient *S. mitis* was engineered to integrate the *Mtb* gene at the pullulanase locus via homologous recombination. This allowed for expression of the *Mtb* gene under the stable control of the efficient resident promoter of the *pulA* gene without the need for selective pressure to maintain the construct. The stability and secretion of integrated vaccine candidates were confirmed by PCR even after 80 generations *in vitro*.

To assess the feasibility of using *rS. mitis* as a mucosal delivery vector, we used the gnotobiotic piglet model of colonization by human oral microbiome organisms. Colonization of *S. mitis* and most other commensals of the human oral cavity is difficult to achieve in mice and other rodents. In contrast, gnotobiotic piglets have been used successfully as a model to study the pathophysiologic role of the gastrointestinal human microbiome [23]. Therefore, we opted to use this well-established model of colonization of the human microbiome to investigate the ability of the *rS. mitisAg85b* to colonize the oral mucosa and to induce antigen-specific mucosal immunization. The results clearly showed that oral delivery of *rS. mitis* resulted in colonization of the oral cavity of piglets for up to the five week duration of the experiment. Importantly, persistence of the engineered bacteria was accompanied by the induction of local mucosal and systemic humoral immune responses to the vaccine antigen. These observations constitute a strong proof of concept for the use of *S. mitis* as a delivery system of mucosal immunization for a variety of diseases.

On the other hand, it is important to stress that colonization of the mutant *S. mitis* in gnotobiotic pigs is not an ideal model to study oral mucosal immunization for vaccine development purposes. However, the aim of these preliminary studies was centered on the validation of the *S. mitis* mutant and not on the validation of the gnotobiotic model for future vaccine studies. We chose the gnotobiotic pigs because they constitute a proven animal

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model to study the human microbiome and thus ideal for this preliminary study, vis-à-vis that *S. mitis* is a poor colonizer in most animal models. Therefore, we believe that colonization of the mutant in the presence of selective pressure of other microbial species is not necessarily an impediment to investigate if the mutant, once colonized in the oral mucosa, could induce an immune response to the target vaccine candidate.

In light that protection against tuberculosis is dependent of lung mucosal immunity [24] future studies are forthcoming to examine development of mucosal immunity in non-human primates (NHP) in response to Mtb antigens expressed by *rS. mitis.* NHP are the ultimate model used in pre-clinical vaccine development studies for tuberculosis vaccine. Because *S. mitis* is normally and abundantly present in the microbiome of the oral cavity of NHP [25], colonization of the recombinant bacteria in this animal model, even in the presence of an already established microbial flora as it happens in humans [26, 27], should not be a concern. Moreover, the present studies strongly suggest that *S. mitis* can be an excellent vaccine delivery system against diseases that primarily affect the gastrointestinal mucosa for which IgA mediates immunity, e.g. diarrheas caused by *Clostridium difficile*.

The approach, described herein using *S. mitis* as delivery vector has many advantages; (i) chromosomal integration of genes encoding vaccine candidate antigens assures stable expression by the recombinant bacteria; (ii) the ability of *S. mitis* to propagate and persist in the oral cavity allows ample time for immunostimulation to occur; and (iii) oral application induces oral and remote mucosal as well as systemic immune. In conclusion, the data presented here indicate the potential clinical utility of *S. mitis* as a vector for delivery of vaccine candidates to mucosal sites. Further studies will assess, in suitable animal models, the protective efficacy of this promising antigen delivery system of oral mucosal immunization not only to tuberculosis but other diseases like AIDS, and *C. difficile*-associated diarrhea.

Acknowledgments

This study was supported in part by NIH grants R03 DE022525 (M.C.), R01 DE015931 (M.D.), and R01 AI076425 (A.C-N.).

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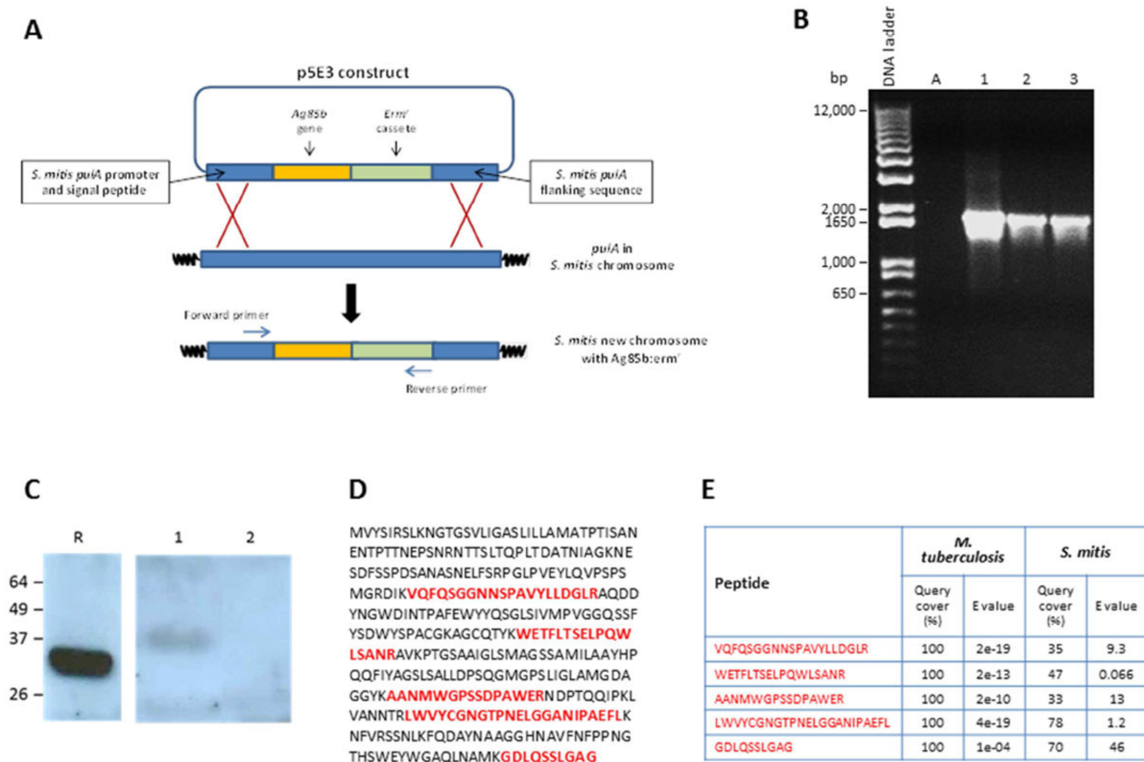

Peptide	*M. tuberculosis*		*S. mitis*	
	Query cover (%)	Evalue	Query cover (%)	Evalue
VQFQSGGNNSPAVYLLDGLR	100	2e-19	35	9.3
WETFLTSELPQWLSANR	100	2e-13	47	0.066
AANMWGPSSDPAWER	100	2e-10	33	13
LWVYCGNGTPNELGGANIPAEFL	100	4e-19	78	1.2
GDLQSSLGAG	100	1e-04	70	46

Figure 1. Homologous recombination and molecular characterization of recombinant Ag85b *S. mitis* construct

(A) Schematic representation of the homologous recombination. The integrating fragment into *S. mitis* genome contains 250 bp of *S. mitis* 5' flanking sequence that includes the *pulA* promoter and signal peptide sequences, the in frame antigen 85b, the erythromycin resistance (Erm^r) gene cassette, and 250 bp of *S. mitis* 3' *pulA* flanking gene sequence. The *S. mitis* flanking sequences permit integration of the fragment into the *S. mitis* chromosome by homologous recombination. (B) Amplification of a DNA fragment containing *Ag85b* from genomic DNA of erythromycin resistant *S. mitis* transformants. Lane A: genomic DNA from wild type *S. mitis.* DNA ladder is shown with molecular weights (bp). Lanes 1-3: genomic DNA preparations obtained from three different clones of the engineered *rS. mitis*. Forward and reverse primers were from the flanking regions of the *pulA* gene and *erm^r* genes respectively as illustrated in A. (C) Detection of Ag85b in culture supernatants of *rS. mitis*. Supernatants from *rS. mitis* and WT cultures in THB medium were analyzed for expression of Ag85b by Western blot probed with rabbit anti-Ag85b antiserum; Lane R: recombinant Ag85b, Lane 1: culture supernatant of *rS. mitis*, Lane 2: culture supernatant of WT. Note that the Ag85b produced by rS. mitis has a slightly higher MW than the rAg85b (produced in *E. coli*) because it contains the signal peptide sequence of *pul*A. The presence of Ag85b in culture supernatant of engineered *rS. mitis* was further confirmed by mass spectroscopy. (D) A protein band corresponding to the molecular weight of Ag85b, identified by Western blot, was cut from SDS-PAGE and submitted for mass spectrometry, which identified the highlighted peptides (red/bold) within the amino acid sequence of Ag85b. Note that because several different peptides spanning at different sequence positions of Ag85b were identified, this finding shows unambiguously that the engineered *rS. mitis*

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strain produces the *Mtb* antigen. (E) BLAST analyses of the identified peptides performed against both *M. tuberculosis* and *S. mitis* genomes. Note that none of five peptide sequences could be assigned to *S. mitis* because only partial sequences of the peptides had homologies with sequences present in the genome of this microorganism. In addition their E-values were highly insignificant. In contrast, the peptides were 100% homologous with *M. tuberculosis* sequences and the E-values were highly significant.

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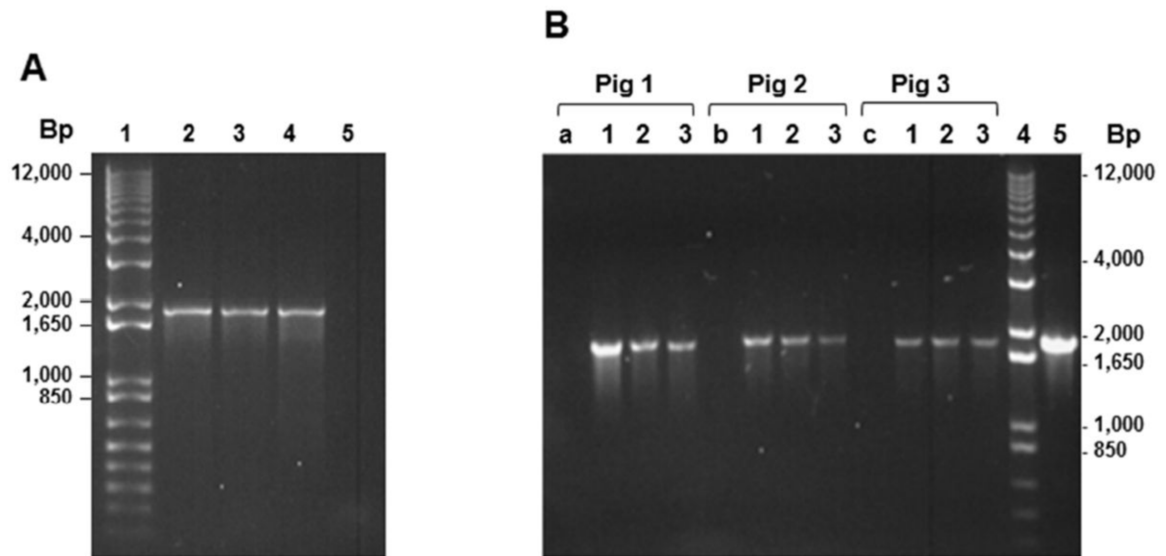

Figure 2. Insertion of Ag85b gene in *S. mitis* genome is stable and r*S.mitis*Ag85b efficiently colonizes piglets

(A). Ag85b PCR products from successive passages of *rS.mitisAg85b* in Todd medium. Lane 1 DNA ladder; Lane 2, passage #10; Lane 3, passage #50; Lane 4 passage # 80; Lane 5 no DNA. (B). Ag85b PCR products detected from biological samples obtained from piglets 9 days after oral inoculation with *rS. mitis* (passage # 10). Lanes a, b, and c are saliva samples collected before oral inoculation of the pigs; Lanes 1, 2, and 3, are saliva, swab of oral surfaces, swab of anal area collected nine days after oral inoculation; Lane 4, DNA ladder; Lane 5, positive control (DNA purified from r*S. mitis*).

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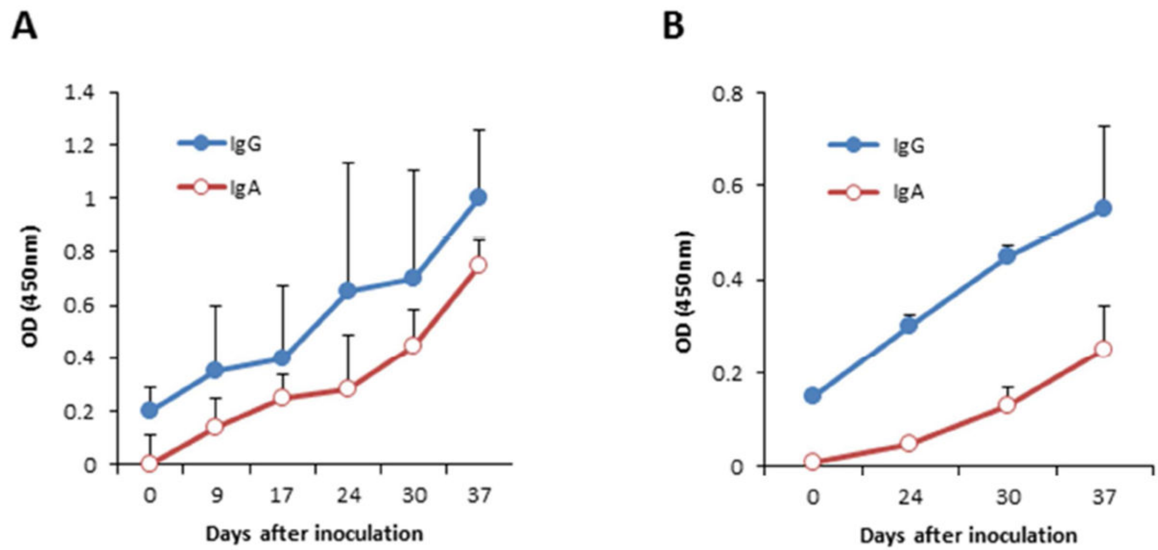

Figure 3. Ag85b-specific antibody response in *rS. mitis* colonized piglets

Gnotobiotic piglets (n= 3) were given two oral doses of *S.mitis*Ag85b on two consecutive days. The anti-Ag85b IgA and IgG antibody levels were measured by ELISA in (A) saliva samples (undiluted) collected on day 0, 9, 17, 24, 30, and 37 and (B) serum samples (1/100 dilution) collected on days 0, 24, 30, and 37 after colonization.