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Streptococcus pneumoniae galU gene mutation has a direct effect on biofilm growth, adherence and phagocytosis *in vitro* and pathogenicity *in vivo*.

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ONE SENTENCE SUMMARY: Mutation of the *galU* gene has an influence on *in vitro* interactions with immune cells and *in vivo* streptococcal pathogenicity using a *Galleria mellonella* infection model.

KEYWORDS: *Streptococcus pneumoniae*, *galU*, biofilm, cellular interactions, *Galleria mellonella*

DECLARATIONS OF INTEREST: none.

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ABSTRACT

Streptococcus pneumoniae, the most common cause of bacterial pneumonia, has developed a wide range of virulence factors to evade the immune system of which the polysaccharide capsule is the most important one. Formation of this capsule is dependent on the *cps* gene locus, but also involves other genes – like *galU*. The pyrophosphorylase encoded by *galU* plays a role in the UDP-glucose metabolism of prokaryotes and is required for the biosynthesis of capsular polysaccharides. In this paper, the effect of a *galU* mutation leading to a dysfunctional UDP-glucose pyrophosphorylase (UDPG:PP) on *in vitro* biofilm biomass, adherence to lung epithelial cells and macrophage phagocytosis is studied. Lastly, *in vivo* virulence using a *Galleria mellonella* model has been studied. We show that the mutation improves streptococcal adherence to epithelial cells and macrophage phagocytosis *in vitro*, while there is no definitive correlation on biofilm formation between parent and mutant strains. Moreover, *in vivo* virulence is attenuated for all mutated strains. Together, these results demonstrate that a *galU* mutation in *S. pneumoniae* influences host cell interactions *in vitro* and *in vivo* and can strongly influence the outcome of a streptococcal infection. As such, UDPG:PP is worth investigating further as a potential drug target.

INTRODUCTION

Streptococcus pneumoniae, also known as the pneumococcus, is the most common cause of bacterial pneumonia, and can cause otitis media and meningitis in children, the elderly and immunocompromised patients (Lundbo and Benfield 2017). Systemic streptococcal infections, such as invasive pneumonia, sepsis and meningitis are also known as inflammatory pneumococcal disease (IPD). From 2009 to 2011, 16% of diagnosed and hospitalized IPD patients in Belgium died during hospitalization (Verhaegen *et al.* 2014). In case of comorbidities, such as heart failure or renal insufficiency, a mortality rate of 20% was reported. These high mortality rates are partially due to ineffective treatments. Bacterial resistance towards first line antibiotics makes treating *S. pneumoniae* infections difficult (World Health Organisation (WHO) 2014). Moreover, vaccination does not have a positive outcome on pneumococcal pneumonia and it is only effective for other IPDs for the vaccinated serotypes and some crossovers (Huss *et al.* 2009). It has been observed that less common serotypes are replacing the ones used in vaccination (Vestjens *et al.* 2017).

The pneumococcus has developed a wide range of virulence factors to evade the immune system. The most important virulence factor is the polysaccharide capsule (Kadioglu *et al.* 2008). This capsule inhibits opsonization and subsequent macrophage phagocytosis through limitation of C3b/iC3b deposition (Hyams *et al.* 2010). In addition, the presence of the surrounding capsule restricts the serotype specific availability of antigens required for T-cell activation (Dockrell, Whyte and Mitchell 2012). In the pathogenesis of pneumonia, alveolar macrophages form the first line of defense to pneumococcal invasion (Dockrell *et al.* 2003; Dockrell, Whyte and Mitchell 2012). Deletion of the capsule has been shown to increase the phagocytosis rate *in vitro* and decrease virulence *in vivo* (Preston and Dockrell 2008). In contrast, non-encapsulated pneumococci show better adherence properties and an increase in biofilm formation, which is considered to be a part of the pneumococcal immune evasion strategy during pneumonia and otitis media (Domenech *et al.* 2013; Chao *et al.* 2015). Additionally, during nasopharyngeal colonization a decrease in capsule production is also observed. This could be due to its need for attachment. However, when the change from a commensal to a pathogenic lifestyle occurs, streptococci upregulate the formation of capsular polysacharides, which is likely due to the importance of the capsule in evading the immune system (Gilley and Orihuela 2014).

The main gene *locus* designated for capsule production is the *cps locus*, a highly variable region which gives rise to over 90 different pneumococcal serotypes (Geno *et al.* 2015). However, it is known that other genes are also involved in the regulation of capsule production (Llull *et al.* 1999). Regardless of the serotype, uridine diphosphate glucose (UDP-Glc) is a key component in the formation of a capsule. UDP-Glc is part of the glucose (Glc) and galactose (Gal) metabolism, where uridine diphosphate glucose pyrophosphorylase (UDPG:PP, EC 2.7.7.9) reversibly converts UDP-Glc to glucose-1-phosphate (Glc-1-P). UDPG:PP is encoded by the *galU* gene *locus* and is highly conserved between different bacterial

species. Mutants lacking a functional *galU* gene do not form any detectable amounts of capsular polysaccharides, due to their inability to interconvert Gal and Glc (Mollerach, López and García 1998).

As UDPG:PP is present in presumably all pneumococcal serotypes and deletion of the underlying gene deprives them of their most important virulence factor, this enzyme could present a potential effective drug target for the treatment of pneumococcal diseases. Furthermore, prokaryotic UDPG:PPs are structurally unrelated to their eukaryotic counterparts (Flores-Díaz *et al.* 1997; Berbís *et al.* 2015). This suggests that inhibition of bacterial UDPG:PP would not be harmful to the host, such as humans. UDPG:PP and UDP-Glc do not only play a role in the capsular polysaccharide pathway. In other bacteria, UDP-Glc is also involved in other functional pathways, such as those involving sugar metabolism and stress responses (Berbís *et al.* 2015). In this study, we investigated the role of a mutation in the *galU locus* in pneumococcal virulence. We determined the impact of a mutation of *galU* on *in vitro* adherence to lung epithelial cells and macrophages and phagocytosis in macrophages. As we found an effect on adherence, and this is the first step towards chronic biofilm infections, we also investigated biofilm formation. Additionally, to assess the effect of a *galU* mutation on *in vivo* virulence, a *Galleria mellonella* larvae model was also used. Our data demonstrate that a *galU* mutation impacts streptococcal interactions with host cells and overall virulence. These characteristics make this gene an interesting and potentially effective target for novel antimicrobial drug discovery.

MATERIALS AND METHODS

Bacterial strains and cell cultures. *S. pneumoniae* strains listed in **Table 1** were a kind gift from Prof. Mollerach, Universidad de Buenos Aires, Argentina. Briefly, mutants were obtained through an interruption in the last 102 nucleotides of the gene by mariner mutagenesis as described previously (Martin *et al.* 2000). All streptococcal strains are encapsulated as wild type strains, except for strain R6, which is an non-encapsulated strain of serotype 2. Reference strain TIGR4 (serotype 4) was obtained from ATCC® (ATCC® BAA-334™). Bacteria were cultured in brain-heart infusion (BHI) broth (LabM) or on 5% sheep blood agar plates (Tryptic Soy Agar, LabM, Oxoid) at 37°C and 5% CO_2. Human lung alveolar epithelial cells were obtained from ATCC® (A549, ATCC CCI-185™) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% pyruvate and 10% inactivated fetal calf serum (iFCS) (all from Sigma) under the same conditions. Murine macrophage cells were obtained from ATCC® (RAW 264.7, ATCC® TIB-71™) and grown in RPMI-1640 medium supplemented with 10% iFCS (all from Sigma) under the same conditions.

Table 1: *S. pneumoniae* strains used in this study.

Strain	Serotype
***S. pneumoniae* TIGR4**	Serotype 4
***S. pneumoniae* 85**	Serotype 14

***S. pneumoniae* 85 mutant**	Serotype 14, *galU* mutated
***S. pneumoniae* 88**	Serotype 5
***S. pneumoniae* 88 mutant**	Serotype 5, *galU* mutated
***S. pneumoniae* M23**	Serotype 3
***S. pneumoniae* M23 mutant**	Serotype 3, *galU* mutated
***S. pneumoniae* R6**	Serotype 2^-
***S. pneumoniae* R6 mutant**	Serotype 2^-, *galU* mutated

Planktonic growth. Planktonic growth curves were obtained over a 10 hour period. All strains were grown in BHI broth at 37°C and 5% CO_2 as advised by ATCC®. At 2-hour intervals, the concentration was determined by viable plate count and the optical density was measured using a cell density meter at 600 nm with 10 mm path length (Biochrom WPA CO8000).

Biofilm growth. Biofilm growth was set up in polystyrene 96-well plates (Greiner Bio-One). In each well, 150 µl of a bacterial culture with a concentration of 10^6 CFU mL^{-1} in Mueller-Hinton broth (MHB) (Sigma-Aldrich) supplemented with 5% lysed horse blood (Oxoid) was added (Vandevelde, Tulkens and Van Bambeke 2014) . Plates were wrapped in parafilm to counteract evaporation and incubated at 37°C and 5% CO_2 for 24 or 48 hours. After incubation, the medium was discarded and wells were washed with phosphate-buffered saline (PBS) (Life Technologies). Then biofilms were fixed with methanol (Merck), stained with 200 µl 0.01% crystal violet (Merck) and eluted with 33% acetic acid (Merck) as described previously (Toté *et al.* 2009). Absorbance was measured using a spectrophotometer at 570 nm (Labsystems Multiskan® MCC/340). For this assay, strain TIGR4 was used as a reference strain for biofilm formation (Lizcano *et al.* 2010).

Biofilm attachment. To assess the first step of biofilm formation, suspensions were made as described previously (see section biofilm growth). After 4 hours of incubation, wells were washed twice with PBS. Subsequently, attached bacteria were detached using 0.5% saponin (Riedel-de Haën). The number of attached bacteria was determined by viable plate count. For this assay, strain TIGR4 was used as a reference strain for biofilm formation (Lizcano *et al.* 2010).

Antibiotic activity. Minimal inhibitory concentrations (MIC) of standard antibiotics were determined using a resazurin assay as described previously (Torfs *et al.* 2018). The antibiotics that were used were: erythromycin (Sigma-Aldrich), amoxicillin (Sigma-Aldrich), doxycycline (Sigma-Aldrich), cefuroxime (CRS) and moxifloxacin (Fluka). Briefly, 100 µl of a 1:2 serial dilution series of antibiotics in BHI broth was added to 96-well plates, after which bacteria were added to final concentration of 2,5 * 10^5 CFU mL^{-1} in 200 µl. After 20 hours of incubation at 37°C and 5% CO_2, 20 µl of 0.005% resazurin was added. After an additional incubation of 90 min, fluorescence was measured at $\lambda_{emission}$ = 590 nm, $\lambda_{excitation}$ = 550 nm using a spectrophotometer (Promega Discover).

Epithelial adherence assay. Epithelial cells were grown to confluency for 24h at 37°C and 5% CO_2 in polystyrene 24-well plates. Pneumococcal cells were grown to mid log phase in BHI broth and diluted to obtain a multiplicity of infection (MOI) of 20 in DMEM supplemented with 1% glutamine and 10% iFCS (Letourneau *et al.* 2011). After 90 min of co-incubation, cells were washed three times with pre-warmed PBS to remove unbound bacteria. Adherent bacteria were detached using 0.5% saponin. The number of adherent bacteria was determined by viable plate count.

Macrophage assay. RAW264.7 cells were seeded into polystyrene 24-well plates at 2 * 10^5 cells per well and incubated at 37°C and 5% CO_2, 24 hours prior to infection. Bacteria were grown as described earlier for 4 hours prior to infection. Bacteria were added to cells at an MOI of 10 in DMEM + 10% iFCS as described previously (Domon *et al.* 2016). Plates were incubated for 90 min at 37°C and 5% CO_2. Cells were washed twice with PBS/Ca^+Mg^+ to wash away all loose bacteria. For determination of intracellular bacteria, 50 mg mL^{-1} gentamicin (Life Technologies) was added at 200 µl mL^{-1} in DMEM + 10% iFCS. Cells were incubated for 60 min at 37°C and 5% CO_2 to kill all extracellular bacteria. Afterwards, cells were lysed using 200 µl 0.1% Triton X-100 (Sigma-Aldrich) for 10 minutes at room temperature and the concentration of internal bacteria was determined using the viable plate count method. To determine the total amount of intracellular and adhered bacteria, 200 µl 0.1% Triton X-100 was added directly after washing the cells and the concentration was determined by viable plate count.

***Galleria mellonella* killing assay.** Larvae were purchased from a local vendor (Anaconda Reptiles, Kontich, Belgium) and stored in wood chips at 15°C before use. Four hours before use, larvae were put at 4°C. A sterile 20 µl Hamilton syringe was used to inject 10 µl aliquots of bacterial suspensions into the hindmost left proleg of *G. mellonella*. Larvae were infected with 10^4, 10^5 or 10^6 CFU per larvae, with a minimum of 15 larvae per group. The control group was injected with 10 µl PBS. Following the injections, larvae were incubated at 37°C in the dark for several days to allow the progression of the pneumococcal infection. Every 24 hours, larvae were scored as dead or alive. Larvae were determined dead when no signs of movement could be observed in response to external stimuli and when the larvae showed dark pigmentation due to melanization. For each strain, 5 independent repeats were carried out.

Statistical analyses. Data were analyzed for statistical significance using Graphpad Prism Version 6. Continuous variables were compared by two-way Anova, t-test or survival analyses. Statistical significance was defined as $p < 0.05$.

RESULTS

In vitro characterization of galU mutational strains: Planktonic growth and susceptibility to standard antibiotics

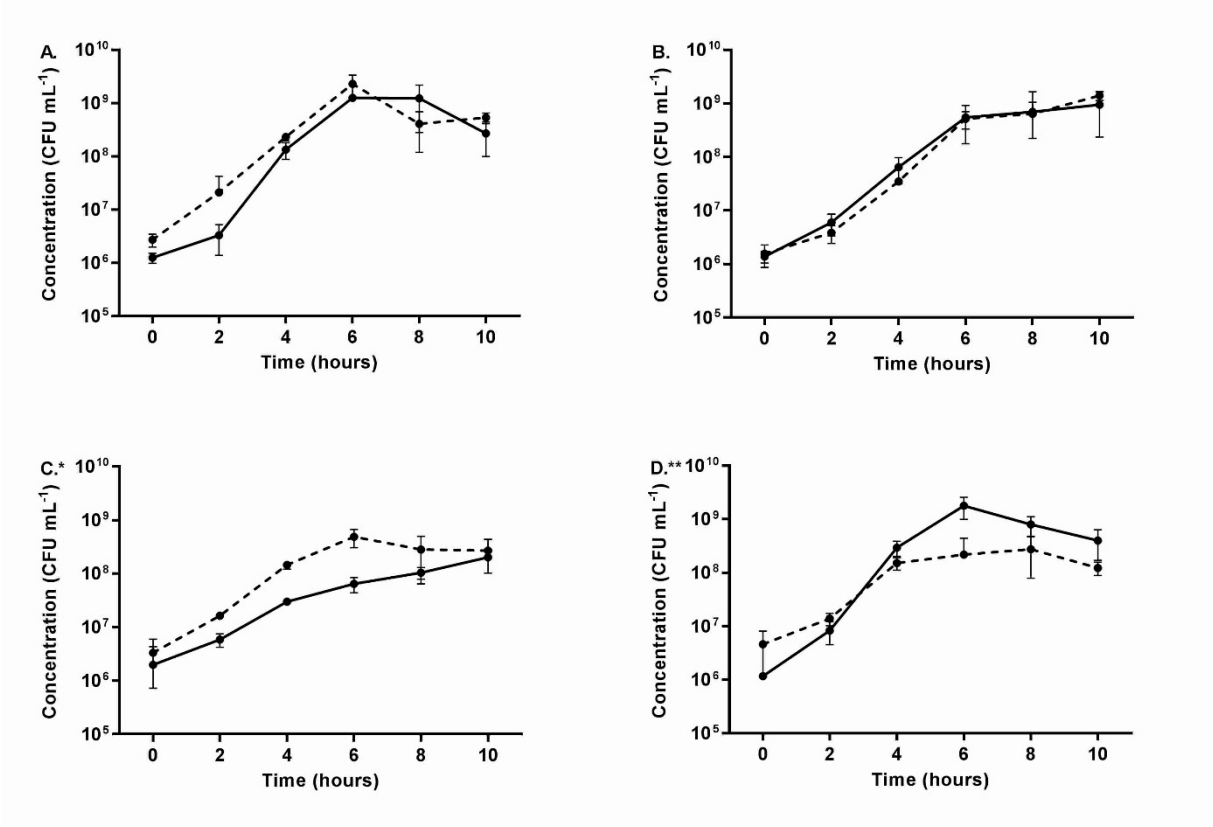

Figure 1: Planktonic growth curves for *S. pneumoniae galU* wild type strains (full line) and mutant strains (dotted line). A. Strain 85, B. Strain 88, C. Strain M23, D. Strain R6. Bacterial concentrations were determined using the viable plate count (VPC) method. Error bars represent SD. Asterisks represent statistical differences between wild type and mutant strains (two-way Anova, $p < 0.05$). (n = 3)

As UDPG:PP is part of the glucose and galactose pathway, a conformational mutation of this enzyme could lead to an overall change in metabolic state. To test this hypothesis, planktonic growth and antimicrobial susceptibility were investigated. No difference in planktonic growth between streptococcal strains with the *galU* mutation and their non-mutated wild type strains was observed in strain 85 and 88 (**Figure 1**). For strain M23, an increase in planktonic growth was observed for the mutant strain, while strain R6 was slightly growth impaired when the mutation was present. Also, the susceptibility to standard antibiotics remained the same in most wild type and mutant strains (**Table 2**). However, for strain R6 the susceptibility towards β-lactam antibiotics amoxicillin and cefuroxime increased over a 100-fold when the *galU* mutation was present. Taken together, these results suggest that no changes in metabolic state occur due to an effect of the mutation in encapsulated strains, but *galU* mutation has an influence on already non-encapsulated strains.

Table 2: Minimal inhibitory concentrations of 5 standard antibiotics (µg mL^{-1}) ± SD. MICs outside of tested range are indicated by < or >. (n = 4 x 3)

	MIC$_{90}$ (µg mL^{-1})							
	Strain 85	**Strain 85 mutant**	**Strain 88**	**Strain 88 mutant**	**Strain M23**	**Strain M23 mutant**	**Strain R6**	**Strain R6 mutant**
Erythromycin	0.111 ± 0.009	0.063 ± 0.004	0.081 ± 0.007	0.059 ± 0.001	> 2	> 2	0.056 ± 0.003	0.101 ± 0.011
Amoxicillin	0.977 ± 0.083	0.975 ± 0.030	< 0.0625	< 0.0625	< 0.0625	< 0.0625	1.861 ± 0.047	< 0.0625
Doxycycline	0.083 ± 0.007	0.112 ± 0.005	0.229 ± 0.007	0.118 ± 0.004	> 2	> 2	0.116 ± 0.010	0.238 ± 0.007
Cefuroxime	7.576 ± 0.182	6.074 ± 0.393	0.118 ± 0.002	0.119 ± 0.001	< 0.03125	< 0.03125	7.256 ± 0.421	0.057 ± 0.005
Moxifloxacin	0.117 ± 0.005	0.116 ± 0.008	0.225 ± 0.006	0.116 ± 0.002	0.121 ± 0.003	0.204 ± 0.010	0.117 ± 0.006	0.233 ± 0.012

Biofilm formation in vitro

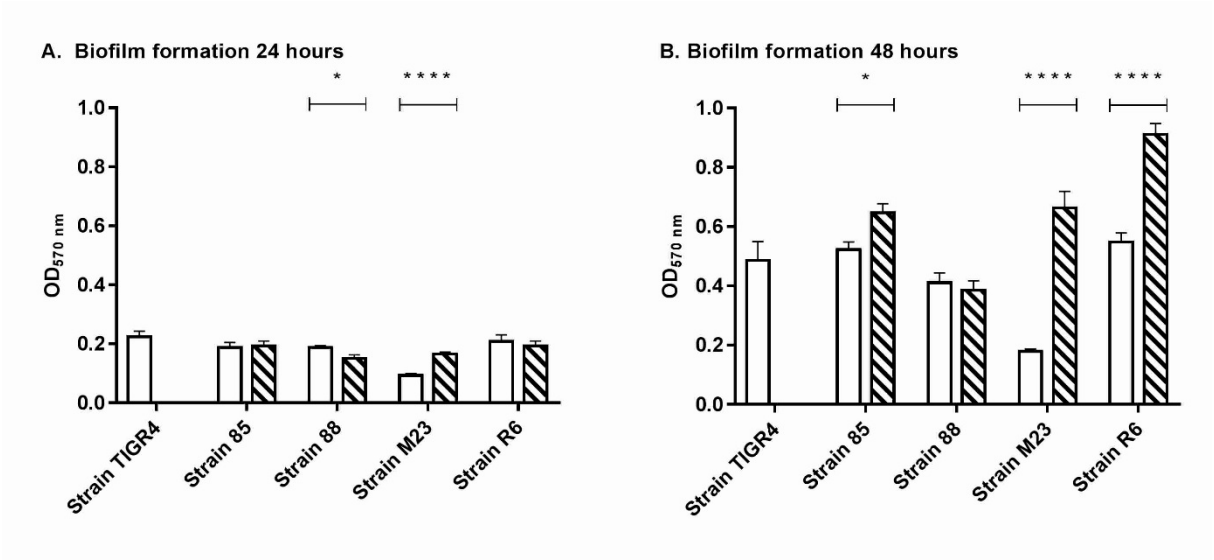

Figure 2: Biofilm formation of streptococcal strains. A. Biomass after 24 hours of incubation, B. Biomass after 48 hours of incubation. White bars represent wild type strains, striped bars represent mutants. There is no mutant of strain TIGR4 available. Asterisks represent statistical differences between wild type and mutant strains (Unpaired t-test, $p < 0.05$). Error bars represent SEM. (n = 5 x 12)

The biofilm formation was dependent on incubation time and serotype (**Figure 2**). Strain 85 and strain R6 showed no differences in biomass between wild type and mutant strain at 24 hours, but the biomass of the mutant strains significantly increased at 48 hours. For strain 88, no difference in biomass was observed after 48 hours ($p = 0.5977$). For strain M23, an increase was observed at 24 hours and 48 hours of incubation ($p < 0.0001$ at both time points). Compared to the reference strain TIGR4, which is a strain with good biofilm-forming capacities, strain M23 was a non-biofilm forming strain but it changed into a good biofilm former when the mutation was in place (Lizcano *et al.* 2010). Strain 85, which also is a

biofilm former compared to the reference strain TIGR4, became an even better biofilm former at 48 hours when mutated ($p = 0.0150$). The same effect was observed for strain R6 ($p < 0.0001$). Since planktonic growth was unaltered in almost the strains (**Figure 1**), the increase in biomass is hypothesized to be due to a difference in initial attachment rather than a difference in metabolism. However, we showed that the mutation leads to an increase in attachment for strains 88 and M23 but not for strain 85 and R6 (**Figure 3**), thus there is no clear correlation between primary attachment and biomass.

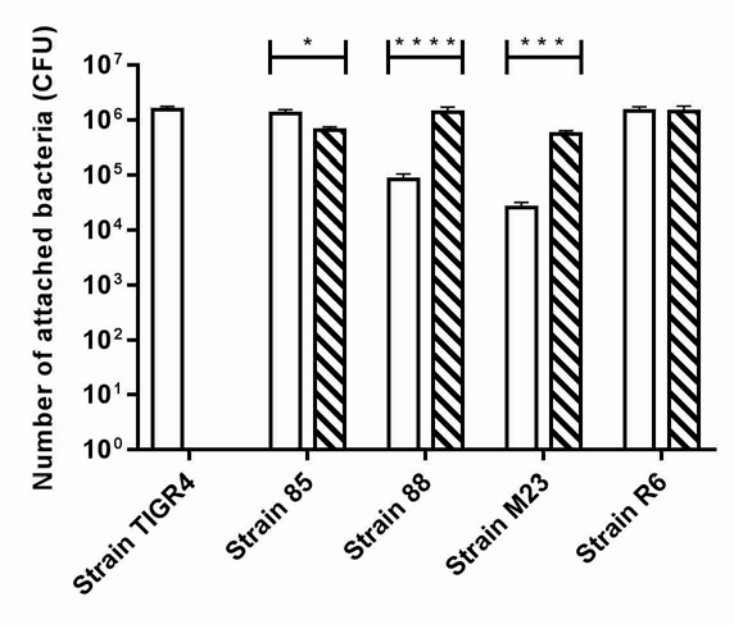

Figure 3: Number of attached bacteria to a 96-well plate after 4 hours of incubation. White bars represent wild type strains, striped bars represent mutants. There is no mutant of strain TIGR4 available. Asterisks represent statistical differences between wild type and mutant strains (Unpaired t-test, $p < 0.05$). Error bars represent SD. (n = 3 x 3)

In vitro adherence to epithelial cells and macrophage phagocytosis

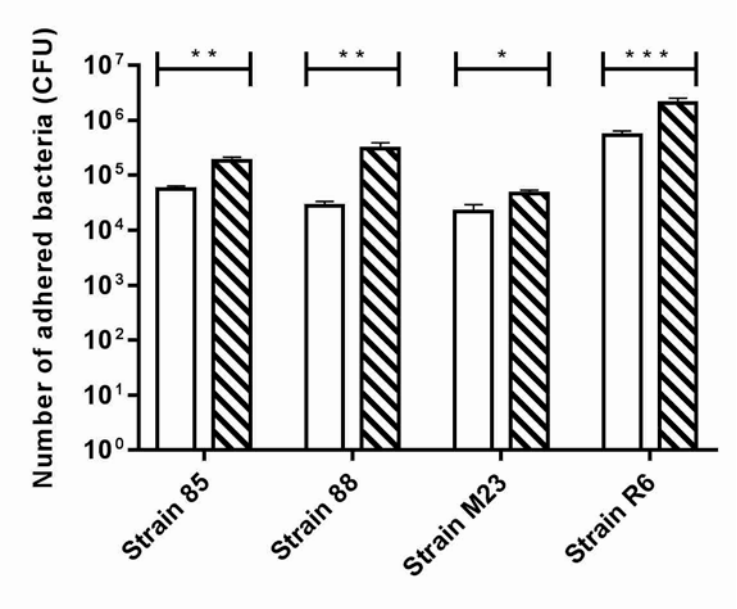

Figure 4: Number of adhered bacteria to A549 epithelial cells. White bars represent wild type strains, striped bars represent mutants. Asterisks represent statistical differences between wild type and mutant strains (Unpaired t-test, $p < 0.05$). Error bars represent SD. (n = 3 x 3)

The polysaccharide capsule is known to prevent bacterial adherence and phagocytosis of immune cells. Loss of this capsule makes bacteria more prone to interactions with the host. This is confirmed in our experiments, where non-encapsulated wild type strain R6 adheres significantly better compared to wild type strain 85 ($p = 0.0048$), strain 88 ($p = 0.0032$) and strain M23 ($p = 0.0004$). Furthermore, a significant increase in adhesion of bacteria with the *galU* mutation to A549 epithelial cells was observed for all strains (**Figure 4**). On RAW264.7 macrophage-like cells, the adherence and phagocytosis of wild type strains was even more serotype specific, showing a significant difference between almost all wild type strains (85 vs. 88 $p = 0.0006$, 85 vs. M23 $p < 0.0001$, 88 vs. M23 $p < 0.0001$, 85 vs. R6 $p = 0.2996$ (ns), 88 vs. R6 $p = 0.0012$, M23 vs. R6 $p < 0.0001$) (**Figure 5**). In accordance with the previous results using an epithelial cell line, all mutants showed an increased adherence and phagocytosis compared to their wild type strains. After gentamicin treatment, all mutants showed an increased phagocytosis compared to their wild type strains.

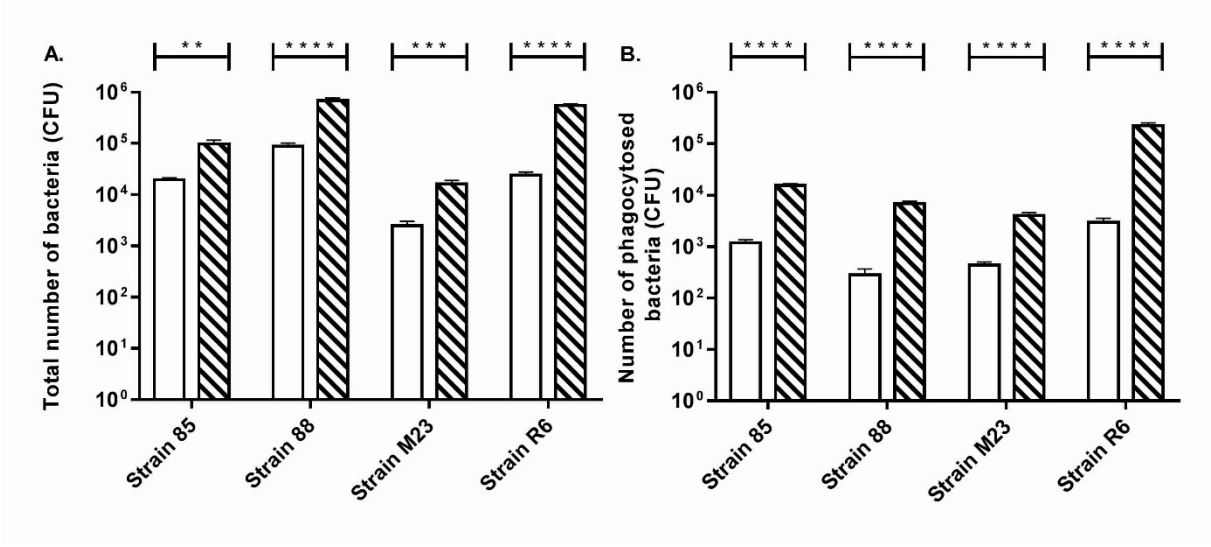

Figure 5: Number of adhered and phagocytosed bacteria with RAW264.7 macrophage cells. A: Total number of adhered and phagocytosed bacteria, obtained with Triton X-100 treatment B: Number of phagocytosed bacteria, obtained after removal of extracellular bacteria by gentamicin treatment. White bars represent wild type strains, striped bars represent mutants. Asterisks represent statistical differences between wild type and mutant strains (Unpaired t-test, $p < 0.05$). Error bars represent SD. (n = 3 x 3)

In vivo pathogenicity

The *G. mellonella* infection model was used to investigate pathogenicity of the mutant strains (**Figure 6**). Survival of *G. mellonella* larvae infected with mutant strains was significantly lower than those infected with mutant strains, independent of the serotype. Furthermore, there was a difference between the different serotypes. Strain M23 was found to be more virulent than strains 85 and 88. Only for avirulent strain R6, there was no difference between the wild type and the mutated strain.

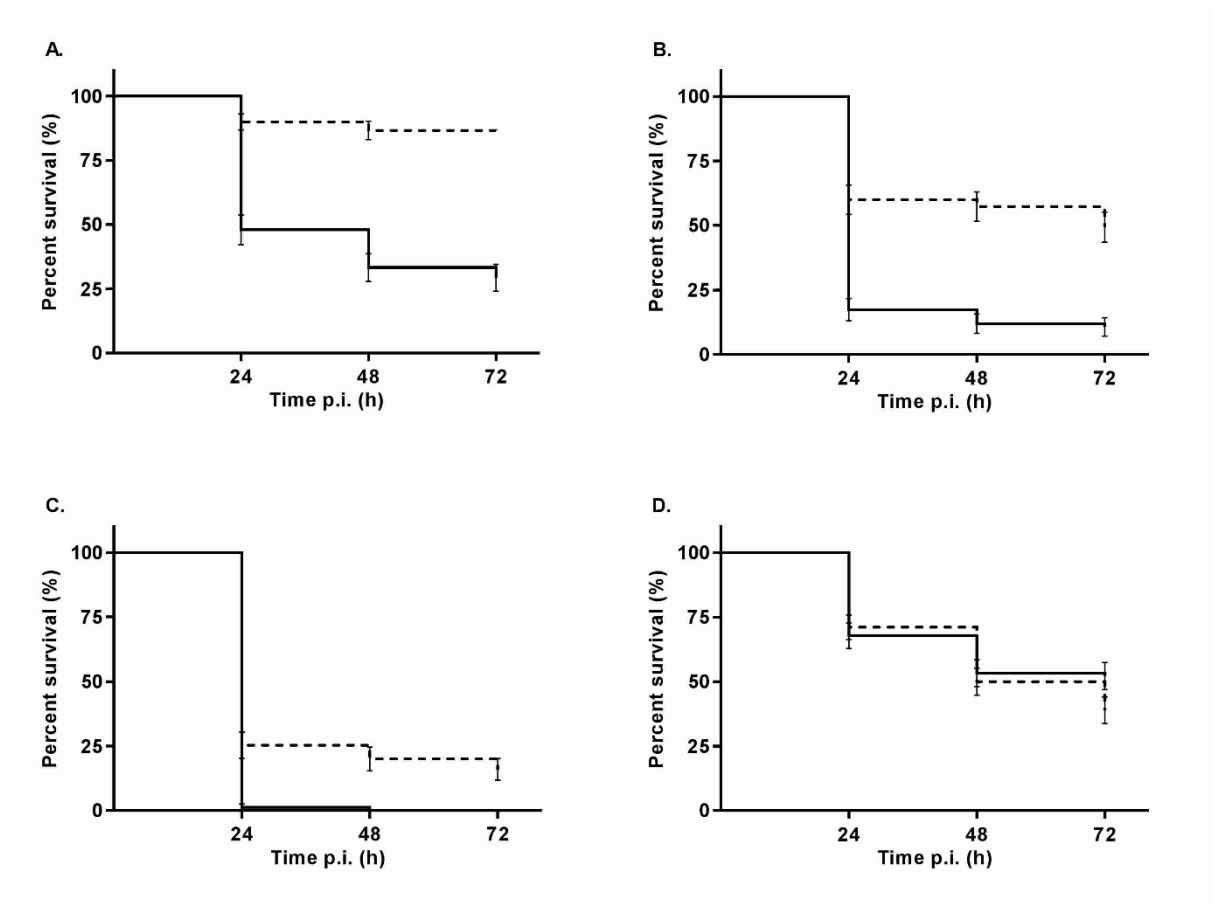

Figure 6: Kaplan-Meier survival curves of *G. mellonella* after infection with *S. pneumoniae* wild type strains (full line) and mutated strains (dotted line). A. Strain 85, B. Strain 88, C. Strain M23, D. Strain R6. Larvae were considered dead when they displayed no movement in response to shaking of the petri dish or touch with a pipette and were melanized. Error bars represent SE. Larval groups infected with mutant strains of strains 85, 88 and M23 show a significant difference in survival compared to groups infected with the respective wild type strain (Survival analysis, $p < 0.05$). There is no significant difference in survival between wild type strain 6 and its mutant strain (Survival analysis, $p = 0.5683$). ($n = 5 \times 15$)

DISCUSSION AND CONCLUSION

The *galU* gene codes for a UDPG:PP which is known to be involved in the UDP-glucose metabolism of prokaryotes and is needed for the biosynthesis of lipopolysaccharides, capsular polysaccharides and membrane derived oligosaccharides in other bacteria, such as *E. coli* and *P. aeruginosa* (Hyams *et al.* 2010; Dockrell, Whyte and Mitchell 2012). In *S. thermophiles*, the *galU* gene has been identified as an important gene in exopolysaccharide (EPS) production (Levander, Svensson and Rådström 2002). For other streptococcal species, such as *S. mitis* and *S. oralis*, a homologous gene has been identified (Mollerach and García 2000). In humans and other mammals, *galU* is also present. However, these UDPG:PPs are evolutionarily unrelated to their prokaryotic counterparts, while they do show homology with other eukaryotic UPDG:PPs (Flores-Díaz *et al.* 1997; Berbís *et al.* 2015). In *S. pneumoniae*, UDPG:PPs are found both in the capsule gene *locus* and outside of this *locus*. In contrast to UPDG:PP

inside the capsule gene *locus*, the gene outside the capsule *locus* has been shown to be irreplaceable for polysaccharide capsule production (Dockrell *et al.* 2003; Preston and Dockrell 2008). To date, apart from the effect on the streptococcal capsule, no other facets of a *galU* mutation have been investigated. Prokaryotic UDPG:PPs are rapidly gaining interest as target candidates for novel antimicrobial therapies. The determination of the effects of a *galU* mutation on the interactions with the host will provide some much needed insight and could aid the search for novel antimicrobial targets (Berbís *et al.* 2015; Zavala *et al.* 2017).

In this study, the effect of a *galU* mutation on host cell interactions was investigated using *in vitro* cell lines and an *in vivo G. mellonella* model. This mutation leads to a better phagocytosis of bacteria by macrophages, an increased adherence on different cell lines and a decreased virulence when compared to the effects of encapsulated parent strains. In a previous study, the *galU* mutation was shown to result in non-encapsulated streptococci (Mollerach, López and García 1998). However, thus far no further research has been conducted on this mutation. Deletion of the capsule in this manner will most likely highly influence the adherence to epithelial cells and macrophages, since this results in exposure of adhesive surface molecules (Hammerschmidt *et al.* 2005; Hyams *et al.* 2010). In this study, we showed that all UDPG:PP mutated strains indeed showed a significant increase in adhesion to epithelial cells. In macrophages, the number of phagocytosed bacteria was also significantly increased. Non-encapsulated parent strain R6 showed the highest adhesion and phagocytosis rates compared to other parent strains. All these *in vitro* data are in line with previous studies, where loss of capsule due to other, non-related, gene mutations in *S. pneumoniae* was investigated (Hammerschmidt *et al.* 2005; Gupta, Shah and Swiatlo 2009; Schaffner *et al.* 2014). However, the increase in adhesion and phagocytosis of the mutated R6 strain indicates an additional effect of the mutation, apart from the decrease in polysaccharide capsule. For other bacterial species, the effect of the *galU* gene product on virulence has already been investigated. In *E. coli*, the *galU* gene has been shown to play an important role in uropathogenesis. Mutants lacking a functional *galU* gene triggered a more intense cytokine response of macrophages *in vitro*, without affecting the bacterial growth or cytotoxicity towards macrophages (Meyer *et al.* 2015). *In vivo*, it has been demonstrated that streptococci lacking a capsule are significantly less virulent than encapsulated bacteria (Wessels *et al.* 1991). Additionally, a *galU* mutation in non-streptococcal bacterial species has been shown to decrease virulence in both animal-pathogenic and plant-pathogenic bacteria (Priebe *et al.* 2004; Liao *et al.* 2014). In this study, the *G. mellonella* model complies with these previous findings. Furthermore, this model confirms the results of the *in vitro* experiments, where mutants are more readily adhered and phagocytosed by immune cells than their wild type strains. For avirulent strain R6, no difference in *in vivo* pathogenicity is observed.

In previous studies, the absence of a polysaccharide capsule, unrelated to the *galU* gene, has been linked to an increase in streptococcal biofilm formation (Moscoso, Garcia and Lopez 2006; Domenech, García and Moscoso 2012). In this study, an effect of the *galU* mutation on biofilm formation was observed for

the first time. Interestingly, this trend was not apparent for all streptococcal strains. These findings suggest that there are likely also other factors, associated with the functionality of the *galU* gene product, which play a role in biofilm formation (Moscoso, Garcia and Lopez 2006). The number of attached bacteria after 4 hours of incubation was elevated in all mutant strains, as it was in host cell-associated assays. However, we found no correlation between this increase and the differences in biofilm biomass. In *E. coli*, the opposite effect has been observed previously. For this bacterium, a mutation in the *galU* gene led to a decrease of adherent abilities to a polystyrene surface (Genevaux *et al.* 1999). Since the composition of extracellular matrix in biofilms differs between streptococcal serotypes, it is possible that the effect of the *galU* mutation on this matrix differs between serotypes (Allegrucci *et al.* 2006). Another explanation could be that the overall metabolism of mutant strains differs from that of their respective wild type strains, giving rise to differences in biofilm biomass. However, no direct correlation between planktonic growth and biomass could be observed. Only strain M23 showed an increase in planktonic growth when the *galU* mutation was present. This elevated metabolism could partially explain the intense increase in biomass for this strain. Interestingly, there is an effect of the *galU* mutation in the non-encapsulated strain R6 on antimicrobial susceptibility towards β-lactam antibiotics. β-lactam antibiotics interfere with the peptidoglycan layer of the cell wall. Therefore, the increase in antimicrobial susceptibility towards this group of antibiotics in an already non-encapsulated strain indicates a broader involvement of UDPG:PP in the bacterial metabolism besides capsular polysaccharide production. In *E. coli*, UDP-glucose is used as an intracellular proxy for nutrient-dependent size control. An imbalance in the amount of intracellular UDP-glucose will impact cell size and other metabolic processes (Hill *et al.* 2013). These underlying processes could also impact biofilm formation and biomass.

The use of the *G. mellonella* model is currently proposed as an alternative model to the time-consuming, expensive and ethically challenging mouse models (López Hernández *et al.* 2015). As described previously, it can be used to investigate virulence factors such as the streptococcal capsule (Evans and Rozen 2012). However, this model does also have some limitations. There is only one company that provides standardized larvae for research purposes. Most research is conducted with larvae that are bred to feed reptiles and birds. More standardized larvae will provide statistically more reproducible results (Champion, Wagley and Titball 2016). The immune system of wax moths is similar to the innate immune system of vertebrates, but it lacks an adaptive immune response. Therefore, this model can be used to investigate the initial steps of infection, but cannot be used to investigate chronic infections containing an adaptive immune response (Jia *et al.* 2016). For streptococcal infections, previous studies have reported a decrease in virulence using non-encapsulated serotypes or serotypes lacking LytA, another important virulence factor (Evans and Rozen 2012). This highlights the applicability of this model to investigate and compare virulence. For several other microorganisms, such as *C. albicans*, *S. aureus* and bovine herpes simplex virus-1 it has been shown to be an adequate tool for virulence and/or

drug screening studies (Champion, Wagley and Titball 2016; Jia *et al.* 2016). Moreover, its ability to be incubated at several different temperatures, including 37°C, is a great benefit compared to other non-mammalian models. Also, the larvae are easy to maintain and to inoculate through injection in the last left proleg (Fuchs and Mylonakis 2006).

Recently, the binding capacities of several streptococcal UPDG:PP inhibitors to purified UDPG:PP enzyme have been tested, indicating the possibility to inhibit this enzyme (Zavala *et al.* 2017). As *galU* mutated strains are more readily phagocytosed, the effect of UDPG:PP inhibitors, possibly in combination with immunomodulatory compounds, should be evaluated in future studies. This could lead to a novel treatment strategy, where immunomodulatory drugs boosting the immune response are used in conjunction with drugs interfering with a basic bacterial enzyme, instead of direct bactericidal or bacteriostatic drugs. The effects of the *galU* mutation on biofilm formation are not yet fully understood and further studies (using techniques monitoring streptococcal viability and gene expression inside the biofilm and biomass composition) are needed to fully characterize this interaction. As biofilms are the primary growth state of all bacteria, it is important to determine the effects that an interference of the *galU* gene may have on biofilm formation, to ensure that bacteria are not more prone to forming hard to treat biofilms (Donlan 2001).

UDPG:PP is involved in a plethora of biosynthetical processes, including capsular polysaccharide production. Interfering with the underlying gene, *galU*, affects host cell interactions both *in vitro* and *in vivo*. As a *galU* mutation lowers *in vivo* virulence and increases phagocytosis by macrophages, dysfunctional bacterial UDPG:PP can strongly affect the outcome of streptococcal infections. Taken together, our results demonstrate that UDPG:PP is an enzyme that influences streptococcal virulence and further research into its potential role as a novel antimicrobial drug target is warranted.

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