The impact of the ancillary pilus-1 protein RrgA of *Streptococcus pneumoniae* on colonization and disease

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**Abstract**
The Gram-positive bacterium *Streptococcus pneumoniae*, the pneumococcus, is an important commensal resident of the human nasopharynx. Carriage is usually asymptomatic, however, *S. pneumoniae* can become invasive and spread from the upper respiratory tract to the lungs causing pneumonia, and to other organs to cause severe diseases such as bacteremia and meningitis. Several pneumococcal proteins important for its disease-causing capability have been described and many are expressed on the bacterial surface. The surface located pneumococcal type-1 pilus has been associated with virulence and the inflammatory response, and it is present in 20%–30% of clinical isolates. Its tip protein RrgA has been shown to be a major adhesin to human cells and to promote invasion through the blood-brain barrier. In this review we discuss recent findings of the impact of RrgA on bacterial colonization of the upper respiratory tract and on pneumococcal virulence, and use epidemiological data and genome-mining to suggest trade-off mechanisms potentially explaining the rather low prevalence of pilus-1 expressing pneumococci in humans.

**Keywords**
colonization, meningitis, pili, pneumococci, RrgA adhesin, *Streptococcus pneumoniae*

**1 | INTRODUCTORY PARAGRAPh**
Pili are composed of several proteins in a long multi-subunit structure. Assembly of pili in Gram-positive bacteria is regulated by transpeptidases, so called sortases, that crosslink single pilus subunits (pilin monomers) and attach them covalently to the peptidoglycan cell wall (Mandlik, Das, & Ton-That, 2008). Pili of Gram-positive bacteria play important roles in bacterial colonization to host tissues, and in modulation of host immune responses and interaction with host cells that may lead to invasive disease. In this review, we focus on pilus-1 of *Streptococcus pneumoniae* (the pneumococcus), and in particular the role of the pilus-1 adhesin protein RrgA and its multirole in nasopharyngeal colonization, in interaction with host immune cells and human cell barriers. In addition, we provide a genomic analysis of pilus-1 and presence of RrgA among 8,351 publicly available *S. pneumoniae* genomes.

**1.1 | The pneumococcal pilus-1 and its adhesin RrgA**
Pilus-1 is expressed by about 20%–30% of *S. pneumoniae* strains (Moschioni et al., 2008), and its presence has been associated with...
the capacity of pneumococci to interact with different types of host cells, including both epithelial and endothelial cells. The multicomponent pilus-1 of \textit{S. pneumoniae} was first demonstrated in the strain TIGR4 and found to be encoded by the \textit{rlrA} pathogenicity islet (Barocchi et al., 2006). This islet includes a transcriptional regulator (\textit{RlrA}), three structural proteins \textit{RrgA}, \textit{RrgB} and \textit{RrgC}, and three sortases (SrtC-1, SrtC-2 and SrtC-3) (Barocchi et al., 2006; Hilleringmann et al., 2009; LeMieux, Hava, Bassett, & Camilli, 2006). Using a TIGR4 mutant lacking the \textit{rlrA} islet it was shown that pilus-1 is important for colonization, virulence in a pneumonia model, and for the inflammatory response in mice (Barocchi et al., 2006). Moreover, introduction of the \textit{rlrA} islet into the encapsulated \textit{rlrA}-negative isolate D39 allowed pilus expression and enhanced adherence to lung epithelial cells. Also, pilus-1 expression in D39 provided a competitive advantage in a mixed intranasal challenge of mice (Barocchi et al., 2006). Since the discovery of pilus-1, the role played by its three structural proteins, \textit{RrgA}, \textit{RrgB} and \textit{RrgC}, as well as the composition of the pilus, has been investigated. The stalk of the pilus was shown to be composed mainly of \textit{RrgB}, with the minor pilus proteins \textit{RrgA} at the tip, and \textit{RrgC} at the base (Hilleringmann et al., 2009), covalently linked to \textit{RrgB} through \textit{rlrA} encoded sortases. A reason for why \textit{RrgA} specific antibodies also bound along the pilus (Barocchi et al., 2006) may be that each pilus is composed of two or more protofilaments arranged in a coiled-coil superstructure (Hilleringmann et al., 2008), and tip location on a \textit{RrgB} polymer could also enhance surface exposure of \textit{RrgA} on encapsulated pneumococci (Pathak et al., 2018).

In 2007, Nelson et al showed that \textit{RrgA} of pilus-1 is a major adhesin to respiratory epithelial cells (Nelson et al., 2007). Pneumococci lacking \textit{RrgA} were significantly less capable to adhere to human nasopharyngeal epithelial A549 cells than wild-type (WT) bacteria. When \textit{RrgA} was overexpressed, bacterial adhesion was enhanced (Nelson et al., 2007). The adhesive phenotype was dependent exclusively on \textit{RrgA}, since bacteria expressing \textit{RrgB} and \textit{RrgC}, but lacking \textit{RrgA} were impaired in their adhesive capacity. In addition, when \textit{RrgB} and \textit{RrgC} were absent, and the bacteria expressed only \textit{RrgA}, and hence the bacteria did not form pili, their attachment to A549 cells was the same as observed for WT pneumococci (Nelson et al., 2007). Even though no clinical isolates have been reported to express \textit{RrgA} in the absence of \textit{RrgB}, it appears that not all \textit{RrgA} molecules produced become pilus-associated. Furthermore, in the absence of \textit{RrgB}, the bacterial cell surface is covered by \textit{RrgA} in a non-polymerized form (Fälker et al., 2008). It is therefore likely that non-pilus-associated \textit{RrgA} may also be covalently linked directly to the peptidoglycan like other LPxTG proteins. Apparently, cell wall associated \textit{RrgA} must be presented such that they can interact with host receptors despite capsular production.

However, the backbone proteins in Gram-positive pili may also provide a significant role in adherence under conditions of mechanical stress (Konto-Ghiorghi et al., 2009). This has been explained by the force promoted breakage of isopeptide bonds in the CNA A domain within these pilus backbone proteins, resulting in an extension of each subunit of the polymer causing dissipation of the mechanical stress (Echelman et al., 2016). Hence, the backbone \textit{RrgB} may contribute to adhesion, but primarily under conditions of mechanical force (Becke et al., 2019), that likely occurs in the upper respiratory tract with the upward beating of cilia for example. Such a force buffering role for the pilus backbone protein \textit{RrgB} is likely to stabilize pneumococcal adherence in the respiratory tract, with its constant outward flow of mucus, even though \textit{RrgA} is the major cell adhesive protein of pilus-1.

The adhesive role played by \textit{RrgA} was also confirmed in vivo using a mouse colonization model in which mice infected with piliated pneumococci, but lacking \textit{RrgA}, showed lower bacterial numbers in the upper respiratory tract as compared to mice challenged with WT bacteria. Also, non-piliated pneumococci expressing only \textit{RrgA} had similar bacterial numbers colonizing the nasopharynx as piliated WT bacteria, confirming that \textit{RrgA} is the major adhesin to the nasopharyngeal epithelium among the pilus-1 proteins (Nelson et al., 2007). Colonization of the upper respiratory tract involves biofilm formation (Marks, Davidson, Knight, & Hakansson, 2013). Interestingly, only mutations in \textit{rrgA} have been shown to cause an impaired bacterial capacity to form biofilm, while mutations in the \textit{rrgB} or \textit{rrgC} genes did not influence the biofilm forming capacity (Muñoz-Elias, Marcano, & Camilli, 2008). Thus \textit{RrgA} seems to be the pilus-1 component that affects biofilm formation.

### 1.2 The crystal structure and interaction partners of \textit{RrgA}

The crystal structure of \textit{RrgA} has been solved and reveals an elongated 190 Å long protein with four domains: D4 interacting with \textit{RrgB} followed by D2 and D1, and at the edge D3, with an integrin I domain that carries a MIDAS (metal ion-dependent adhesion site) motif (Izoré et al., 2010). It is likely that the integrin I domain mediates the established \textit{RrgA} interaction with collagen, laminin and fibronectin, that are common components of the eukaryotic extracellular matrix (ECM) (Hilleringmann et al., 2008). Single molecule force microscopy was recently used to demonstrate that not only domain D3 of \textit{RrgA}, but also D4 may simultaneously interact with fibronectin (Becke et al., 2018). Pre-incubation of pilus-1 expressing pneumococci with antibodies specific to either \textit{RrgB} or \textit{RrgA} decreased adhesion to human epithelial cells. Pre-incubating pneumococci with a monoclonal antibody directed against the D2 domain on \textit{RrgA} also reduced adhesion. Thus, even though domain 3 at the edge of \textit{RrgA} clearly is the major adhesive part of pilus-1, \textit{RrgB} and other domains on \textit{RrgA} may contribute to host cell interactions, leading to bacterial colonization of the upper respiratory tract (Amerighi et al., 2016).

Domain D3 of \textit{RrgA} was also shown to possess a large, cradle-shaped and highly basic surface that might interact with positively charged molecules on host cells such as glycosaminoglycans (Izoré et al., 2010). It was recently demonstrated that all three pilus-1 proteins act as lectins in glucan-array binding assays, with \textit{RrgA} having the broadest glycan recognition of the three. Furthermore, the blood group H trisaccharide could efficiently block adherence of TIGR4 to epithelial cells (Day et al., 2017). Therefore, the role of \textit{RrgA} as an adhesion promoting colonization factor of the nasopharynx...
may at least partly be due to its recognition of a variety of carbohydrate structures present on extracellular matrix proteins and on glycosylated host cell proteins.

Moschioni et al observed that the minor pilus-1 protein RrgA exists in two variants, clades I and II (Moschioni et al., 2010). Available X-ray data from a clade I variant showed that the sequence variability between the two clades exclusively occurred within the adhesive domain 3 at the edge of RrgA, while the other domains were largely conserved. They did not observe any differences between the two variants in binding to respiratory epithelial cells and to ECM proteins (Moschioni et al., 2010). It is possible that the sequence variation can add to the binding repertoire of RrgA. Another possibility is that it represents a mean of immune evasion.

### 1.3 The rrgA islet is present in 20%–30% of clinical isolates and is associated with certain clonal lineages

We examined 8,351 pneumococcal genomes available on PubMLST Pneumococcal Genome Library (Jolley, Bray, & Maiden, 2018) for the presence of rrgA, and therefore likely expressing pilus-1. The concatenated multi-locus sequence typing (MLST) alleles (aroE, gdh, gki, recP, spi, xpt, dll) of these genomes were aligned using MAFFT v.7.407 (Katoh & Standley, 2013), phylogenetic trees were constructed using FastTree v.2.1.10 (Price, Dehal, & Arkin, 2010) and annotated using iTOL (Letunic & Bork, 2016). The presence of rrgA in these genomes was queried using blastn (90% of query length and annotated using iTOL (Letunic & Bork, 2016). The presence of rrgA positive genomes are highlighted on the tree (light blue clades) (Figure 1a) (https://itol.embl.de/tree/13023 796136218811572352847#). The inner circle in Figure 1a shows the sequence type (ST) as determined using MLST, and the outermost circle presents the capsular serotype. The total number of rrgA positive hits are 1854, that is, 22%. The genome tree in Figure 1a shows that the presence of pilus-1 is specific to a subset of clonal lineages (STs) types (Figure 1c). Since one ST may express one or more serotypes, and one serotype belong to one or more clonal lineages. There is a lower association between serotype and presence of rrgA.

The tight association of pilus-1 to clonally related lineages of pneumococci has been observed previously (Moschioni et al., 2008). One of the major pilus-1 carrying lineages, ST4414, comprises a single clone of 19A isolates from the Mela collection (Chewapreecha et al., 2014), and interestingly this clone also carries a megacystis cell resistance (Croucher et al., 2014). The pilus-1 positive lineages ST558 (serotype 35B) and ST156 (9V, 35B, 9A and 19A) have been associated with decreased susceptibility to penicillin (Olarte et al., 2017; Sjöström et al., 2007), and ST802 and ST4413 are single locus variants and are of serotype 23A. Interestingly, the only exceptional lineage where only 15% of the strains carry the pilus-1 islet is ST199 (15/SB, 19A). ST199 has been shown to be pilus-negative and existed prior to the introduction of pneumococcal conjugated vaccines (PCVs) in the childhood vaccination program, and was reported to be involved in vaccine escape capsular switch events (Brueggemann, Pai, Crook, & Beall, 2007; Moschioni et al., 2008). Interestingly, it was reported that pilus-1 positive strains show a biphasic expression with two subpopulations, one expressing pilus and the other not expressing pilus, indicating a regulation on the transcriptional level (Bassett et al., 2011; De Angelis et al., 2011). This regulation can be seen independent of serotype and ST and is an intrinsic property of the pneumococcus.

### 1.4 Possible trade-off mechanisms of pilus-1 expression

Multiple colonization events with pneumococci occur during early childhood and available data suggest that recolonization is due to acquisition of new strains (Sime et al., 2019). The reason why colonizing strains come and go is not known, but is likely due to that pneumococcal colonization is an immunizing event. It has thus been demonstrated that sera from healthy adults without a previous pneumococcal disease contain antibodies to a number of pneumococcal surface proteins, including the pilus-1 stalk protein RrgB (Giefing et al., 2008). The high immunogenicity of pilus-1 proteins, and accessibility of pilus antigens to specific antibodies (Pathak et al., 2018) may act as a trade-off mechanism decreasing the likelihood by which a colonization event by a pilus-1 expressing strain is followed by recolonization with another likewise pilus-1 expressing strain.

Molecular epidemiological studies have shown that some important serotypes (such as serotypes 1, 7F and 3) are rarely found in carriage, but are common in invasive disease (Brueggemann et al., 2003; Sandgren et al., 2005; Sjöström et al., 2006). Interestingly, pilus-1 has not been detected in clonal types expressing these three capsular serotypes (Figure 1a). We argued that strains belonging to these serotypes have a high invasive disease potential (Lindström et al., 2016; Sjöström et al., 2006). Mechanisms for this finding are unknown but could depend on a high rate of spread within a population and a short or non-existent colonization phase. One support for this hypothesis is that the non-piliated serotype 1 clone ST306 increased endemically tenfold, as a cause of bacteremia, during a five-year period in Sweden (Henriques-Normark et al., 2001). In Sub-Saharan Africa, the endemically spreading serotype 1 clone ST217 (not related to ST306) has been a major cause of invasive disease including meningitis, but is rare in carriage, suggesting a low colonization ability but high transmissibility (Chaguza et al., 2016).

In the post-vaccination era some non-vaccine type strains (such as serotype 8) have expanded as causes of invasive disease, without expanding in the carrier population (Galantis et al., 2016). These strains do not belong to clonal types expressing pilus-1. Also interesting is that non-piliated serotype 3 strains (predominantly of ST180) have increased in the post-vaccination era as causes of invasive disease, but is low among child carriers (Lindström et al., 2016; Naule et al., 2017), even though PCV13 targets serotype 3. We hypothesize that pneumococcal strains having a short duration of colonization and/or are colonizing at low numbers may not lead to immunization and protection against recolonization/infection by the same strain lineage.

Transmission efficiency may be another trade-off mechanism for pilus-1 expressing strains. If pilus-1 mediated adherence is of
FIGURE 1  Prevalence of rrgA in Streptococcus pneumoniae. (a) Phylogenetic tree based on the concatenated MLST alleles of 8,351 pneumococcal genomes. The highlighted clades (light blue color) indicate the presence of rrgA in these genomes. The inner circle corresponds to the ST and the outermost circle indicates the corresponding serotype of these isolates. (https://itol.embl.de/tree/1302379613621881572352847#wileyonlinelibrary.com) (b) Distribution of the pilus-1 positive strains by serotype. (c) Distribution of the pilus-positive strains by ST.
advantage for duration of colonization of the nasopharynx, the lack of pilus-1 expression could be of advantage for transmission from one individual to another. Even though transmission experiments have been carried out in mice and ferrets, none of these studies have allowed piliated and non-piliated strains to be compared (Ammar Zafar, Wang, Hamaguchi, & Weiser, 2017; McCullers et al., 2010).

In summary experimental and epidemiological data together suggest that pneumococcal lineages expressing pilus-1 may have a competitive advantage in colonizing the human nasopharynx. However, pilus-1 mediated immunogenicity preventing re-colonization, and RrgA-mediated stickiness to the nasopharynx reducing transmissibility may be two trade-off mechanisms explaining the relatively low frequency of pilus-1 expressing strains among clinical isolates.

PCV-vaccination has eliminated efficient colonizers expressing vaccine type capsule and belonging to clonal lineages expressing pilus-1, but led to an expansion of non-vaccine serotypes belonging to pilus-1 expressing lineages. We suggest that the frequency of pilus-1 expressing strains remains low in the post-vaccination era probably due to the same trade-off mechanisms, high immunogenicity and low transmissibility.

### 1.5 The impact of RrgA in pneumococcal virulence

*Streptococcus pneumoniae* is unusual among members of the airway microbiota in that it is adapted to an inflammatory environment, an environment that may be promoted by the organism itself for example through its production of pneumolysin (Ammar Zafar, Wang, Hamaguchi, & Weiser, 2017). Also, pilus-1 expression has been demonstrated to increase the production of pro-inflammatory cytokines, such as IL-6 and TNF-α, after intraperitoneal challenge in mice (Barocchi et al., 2006). It was subsequently found that this pro-inflammatory role of pilus-1 was mediated by a surface exposed 49 amino-acid sequence present in domain 3 of RrgA that acted as a TLR2 agonist (Basset et al., 2013). TLR2 dependent sensing of pneumococci have been attributed to lipoproteins, and possibly lipoteichoic acids, but it has been argued that lipopeptides might have contaminated the lipoteichoic acid preparations. Also, cell wall anchored proteins such as the PfbA adhesin have been suggested to be recognized by TLR2 (Gisch et al., 2013; Travassos et al., 2004; Yamaguchi et al., 2019; Zähringer, Lindner, Inamura, Heine, & Alexander, 2008).

The onset of invasive pneumococcal disease is often dependent on the capacity of the bacterium to interact with human cell barriers and spread into tissues, and to defend itself from the clearance action of immune cells. Pneumococci use specific proteins or structures that have the capability to engage in physical contact with the plasma membrane of different type of human cells. Pilus-1, through the adhesive action of RrgA, has been described to confer to *S. pneumoniae* the ability to interact with both macrophages, and endothelial cells. Thus, it was shown that the uptake by murine and human macrophages of pneumococci that have pili expressing RrgA is increased as compared to pneumococci lacking RrgA (Orrskog et al., 2012). This uptake was abolished in complement receptor 3 (CR3)-deficient macrophages. It was further demonstrated that purified RrgA physically interacts with CR3. Also, a more rapid progression of invasive disease from the upper respiratory tract to the blood stream was observed in WT mice in comparison to CR3-deficient mice challenged with pneumococci (Orrskog et al., 2012).

A crucial step preceding the onset of infectious disease is the capacity of bacteria to cross human cell barriers and spread into the surrounding tissues. *S. pneumoniae* is the main etiological cause of bacterial meningitis, inflammation of the meninges, occurring as a consequence of a bacterial infection in the brain (Iovino, Seinen, Henriques-Normark, & van Dijl, 2016; van de Beek, Gans, Tunkel, & Wijdicks, 2006). Through high-resolution immunofluorescence microscopy using ex vivo brain tissues from infected mice, it was shown that pneumococci expressing pilus-1 are significantly more prone, than non-piliated pneumococci, to adhere to the vascular endothelium of the blood-brain barrier (BBB) (Iovino, Hammarlöf et al., 2016). Pneumococci expressing pilus-1, but lacking RrgA, showed a very poor adhesion to the BBB endothelium, in comparison to WT pneumococci, and pneumococci expressing RrgA, but lacking RgbB and Rgc (Iovino, Hammarlöf et al., 2016). This finding suggests an important role for pilus-1, and in particular the ancillary RrgA, as a virulence factor in pneumococcal meningitis. It was subsequently demonstrated that the polymeric immunoglobulin receptor (plgR) and the platelet endothelial cell adhesion molecule (PECAM-1), expressed on the surface of brain endothelial cells, are two host receptors that bind to RrgA (Iovino et al., 2017). Brain biopsies from patients with fatal pneumococcal meningitis, where the causative organism had been recovered, was further examined by immunofluorescence using serotype specific capsular antibodies, demonstrating co-localization between the pneumococcus and the endothelial receptors PECAM-1 and plgR. suggesting that this interaction between the two receptors and pneumococci also occurs in humans. Five of the six patients analyzed with fatal pneumococcal meningitis were infected with pilus-1 expressing strains (Iovino et al., 2017). Even though the total number of cases was low, the data suggest that pilus-1 expression is a contributing factor to lethal pneumococcal meningitis. Pneumococci expressing pilus-1 were also shown to significantly enhance activation of microglia, the resident macrophages of the brain, in comparison to pneumococci lacking pilus-1, suggesting that pilus-1 enhances inflammation of the brain (Iovino, Hammarlöf et al., 2016). These data are promising and opens for new possible therapeutic approaches. Thus, by blocking the endothelial receptors plgR and PECAM-1 or RrgA on the bacterial surface, it was shown that pneumococcal invasion into the brain could be reduced significantly with an increased survival of mice (Iovino et al., 2017; Iovino, Thorsdottir, & Henriques-Normark, 2018).

### 1.6 Pilus-1 and RrgA expression promotes formation of small round cocci that facilitate entry across the blood-brain barrier

*Streptococcus pneumoniae* is typified by its ellipsoid size, and most often appear as ellipsoid diplococci and chains of different lengths. This growth pattern is due to two modes (peripheral and septal) of
cell wall synthesis that is orchestrated by the serine/threonine kinase StkP, and its cognate phosphatase GpsB, and the division protein DivIVA. StkP is recruited to the mid cell after formation of the helical FtsZ ring, which requires recruitment and phosphorylation of DivIVA. Both GpsB and DivIVA play a crucial role in pneumococcal cell division and morphogenesis. While DivIVA is required for proper cell elongation, GpsB prevents cell elongation therefore acting as a negative regulator of DivIVA (Fleurie et al., 2014). In the absence of DivIVA, bacteria take on a rounded phenotype, whereas absence of GpsB hamper cell division and promote elongation (Fleurie et al., 2014; Iovino, Hammarlöf et al., 2016). StkP possesses four cell wall binding PASTA-domains that interconnects phosphorylation of its target proteins with septal wall remodeling, allowing cell division at midcell (Beilharz et al., 2012; Fleurie et al., 2014; Zucchini et al., 2018).

Clinical isolates of S. pneumoniae lacking pilus-1 found in the mouse brain in a meningitis model appeared as ellipsoid diplococci and chains. This was in contrast to pilus-1 expressing strains, including also a naturally non-piliated strain in which the rlrA islet was introduced, and a mutant lacking the backbone RrgB and the ancillary RrgC proteins, thereby only expressing RrgA, where all appeared as spherical single cocci. Interestingly, in the blood stream non-piliated pneumococci were found as diplococci or chains, while RrgA containing bacteria appeared as spherical single cocci in a small (2%–5%), but significant, fraction of bacteria. The single cocci from the brain contained a FtsZ ring, proving the cell division capability of these bacteria, however there was no focal recruitment of DivIVA, in contrast to normal ellipsoid diplococci and chains. Aside from the single cocci, pneumococci in the brain detected in the division process expressed DivIVA. Two stages of cell division were observed, at the early stage when bacteria displayed a round coccus shape, DivIVA was detected at the poles of the cells. At later stages of the cell division, the bacterial cell was more elongated and more close to a diplococcus shape rather than a single coccus, and at this stage the DivIVA signal detected on the two newly formed cocci of the diplococcus was severely impaired (Iovino, Hammarlöf et al., 2016). Furthermore, they expressed high levels of RrgA, appearing as one bright spot on each coccus (Iovino, Hammarlöf et al., 2016). Underlying mechanisms by which RrgA expression promotes formation of small round single cocci is not known, but is likely related to RrgA being, directly or indirectly, a cell wall anchored protein that is linked to the peptidoglycan that may affect the delicate cell wall sensing function of StkP, and hence recruitment and phosphorylation of DivIVA. It is believed that pneumococci pass the endothelium of the BBB through transcytosis (Surve et al., 2018), and it is likely that small bacterial size matters for this process, explaining the superior ability for small single cocci to enter the brain. The two other meningitis pathogens, Haemophilus influenzae and Neisseria meningitidis, form small short rods and diplococci respectively.

1.7 | RrgA as a pneumococcal vaccine candidate

Today there are still two main problems related to the pneumococcal conjugate vaccines (PCVs): (a) PCVs are based on polysaccharide capsules which are poorly immunogenic in humans and (b) PCVs confer protection only to a limited number of known serotypes circulating in the society (7 in PCV7, 10 in PCV10, 13 in PCV13), while no protection is conferred against most of the other pneumococcal serotypes (more than 97 capsular serotypes have been identified so far). In the post-vaccination era there has been an increase in invasive disease such as meningitis caused by serotypes that are not included in current PCVs, including also strains resistant to several antibiotics (Browall et al., 2014; Galanis et al., 2016; Weinberger, Malley, & Lipsitch, 2011). An alternative vaccine approach, to elicit a better immune response toward pneumococcal infections, is to develop a protein-based vaccine using pneumococcal antigens that show conserved sequences among different pneumococcal serotypes, and that are present in all pneumococcal strains, that is, found in the core genome. Moreover, it could be an advantage to also include protein antigens that are encoded by the accessory genome, such as the rlrA islet, and therefore achieve protection against specific clonal lineages, either by preventing their colonization, or by preventing their tissue invasive effects when they reach the circulation.

Serum and salivary antibodies to RrgA and RrgB in children and adults have been analyzed by ELISA and immunoblotting. Significant levels of anti-RrgA antibodies were observed to develop early in childhood. Notably, anti-RrgA IgG titers in both serum and saliva were significantly higher in culture-negative children in comparison to in patients with cultures positive for S. pneumoniae. These results show the strong ability of RrgA to elicit an antibody response, which can be important when building the natural immunity toward pneumococcal carriage in children (Ahmed et al., 2014). Notably, from a recent clinical trial of a novel vaccine candidate, PnuBioVax, performed in healthy young adults, it was observed that most subjects receiving the vaccine had ≥ 2-fold increase in antibodies against a group of pneumococcal proteins, including RrgA (Entwisle et al., 2017). Gianfaldoni et al investigated the immunogenicity of RrgA in vivo mice. Intraperitoneal immunization with RrgA, RrgB or RrgC, led to high levels of IgGs specific for the three pilus components in the sera (Gianfaldoni et al., 2007). The most abundant IgGs detected were those specific for RrgA upon RrgA (alone) immunization (Moschioni et al., 2010), pointing out the high immunogenicity of RrgA (Gianfaldoni et al., 2007). Importantly, immunization with RrgA alone, or RrgA together with RrgB and RrgC, conferred high protection in mice, preventing bacteremia upon intraperitoneal challenge (Gianfaldoni et al., 2007). In addition, Moschioni et al investigated the capacity of both RrgA variants, clades I and II, in conferring protection toward pneumococcal infection. Rabbit polyclonal antisera against RrgA clades I and II were used for intraperitoneal immunization of mice that were challenged intraperitoneally with pneumococci after immunization. A significant reduction of bacteremia levels was observed in mice immunized with RrgA clade I rabbit antisera compared to control mice that were immunized with normal rabbit serum (Moschioni et al., 2010). A similar scenario was observed in mice immunized with RrgA clade II rabbit antisera, which showed a significant reduction of bacteremia in comparison to the control group (Moschioni et al., 2010).

Nucleotide sequence analysis of 44 pneumococcal clinical isolates pointed out that the rlrA gene is subjected to a strong positive
selection (Muzzi, Moschioni, Covacci, Rappuoli, & Donati, 2008). As previously observed in Neisseria species, advantageous alleles that are under positive selection increase in prevalence (Smith, Maynard Smith, & Spratt, 1995). Such positive selection may be an explanation for the adaptation of RrgA in various types of interactions with the host. This broad involvement of RrgA in host-pathogen interaction may make RrgA a good vaccine candidate. Even though only a minority of the pneumococcal isolates found in healthy carriage express pilus-1 and RrgA, it should be beneficial to eliminate them by an anti-colonizing vaccine approach, as clonal lineages with decreased susceptibility to penicillin frequently express pilus-1 (Sjöström et al., 2007). Furthermore, their elimination from healthy carriers may decrease the incidence of severe meningitis among the compromised elderly.

2 | CONCLUDING REMARKS

In this review we aimed at shedding light on different features, resulting from biochemical, structural, in vitro and in vivo host-pathogen interaction studies, that make RrgA an important pneumococcal protein impacting S. pneumoniae colonization and pathogenicity. The pneumococcus is the main cause of community-acquired pneumonia (File, 2004). The ability of pneumococci to cause pneumonia is considered to be facilitated by the capacity of the bacteria to colonize the nasopharyngeal epithelium. Here, we have summarized recent findings describing that the ability of pneumococci to adhere to respiratory epithelial cells is largely due to the presence of pilus-1, and in particular RrgA. This adhesive capacity may also be a direct consequence of specific features that have been observed by many research groups analyzing the protein structure of pilus-1. The involvement of RrgA in invasive pneumococcal disease ranges from interactions with immune cells, like macrophages, to adhesion and interactions with vascular endothelial cells. Even though pilus-1, and RrgA, is only present in about 20%–30% of clinical pneumococcal strains, its strong impact on the pathogenesis of severe pneumococcal diseases make it a potential candidate in a protein-based vaccine, as shown from some in vivo studies in which RrgA was used to immunize mice prior to pneumococcal infection.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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