Encapsulation of the septal cell wall protects *Streptococcus pneumoniae* from its major peptidoglycan hydrolase and host defenses

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**Abstract**

Synthesis of the capsular polysaccharide, a major virulence factor for many pathogenic bacteria, is required for bacterial survival within the infected host. In *Streptococcus pneumoniae*, Wze, an autophosphorylating tyrosine kinase, and Wzd, a membrane protein required for Wze autophosphorylation, co-localize at the division septum and guarantee the presence of capsule at this subcellular location. To determine how bacteria regulate capsule synthesis, we studied pneumococcal proteins that interact with Wzd and Wze using bacterial two hybrid assays and fluorescence microscopy. We found that Wzd interacts with Wzg, the putative ligase that attaches capsule to the bacterial cell wall, and recruits it to the septal area. This interaction required residue V56 of Wzd and both the transmembrane regions and DNA-PPF domain of Wzg. When compared to the wild type, Wzd null pneumococci lack capsule at midcell, bind the peptidoglycan hydrolase LytA better and are more susceptible to LytA-induced lysis, and are less virulent in a zebrafish embryo infection model. In this manuscript, we propose that the Wzd/Wze pair guarantees full encapsulation of pneumococcal bacteria by recruiting Wzg to the division septum, ensuring that capsule attachment is coordinated with peptidoglycan synthesis. Impairing the encapsulation process, at localized subcellular sites, may facilitate elimination of bacteria by strategies that target the pneumococcal peptidoglycan.
During their cell cycle, Gram-positive bacteria can be frequently found surrounded by a cellular envelope, which includes glycopolymers such as peptidoglycan, wall teichoic acids or capsular polysaccharides, whose composition is adjusted to the external insults they may find. The clarification of how bacteria tune synthesis of these three macromolecules, which act as defensive layers, ensuring efficient enclosing of bacteria and their protection from the surrounding medium, will permit a better understanding of how bacteria propagate and may pave the way to the intelligent design of anti-infective strategies. In this manuscript, we have determined how Wzg, a candidate for the Streptococcus pneumoniae ligase that attaches capsular polysaccharides to the bacterial cell wall, is directed to the division septum to ensure the full encapsulation of bacteria. Perturbation of this process result in bacteria with reduced protection from external peptidoglycan binding receptors (such as the pneumococcal major hydrolase) and impaired in their ability to survive within the infected host.

Introduction

Streptococcus pneumoniae is an important Gram-positive bacterial pathogen, which can colonize asymptptomatically the nasopharynx of healthy individuals [1] or spread to other sites of the human host causing disease such as otitis media, pneumonia, or meningitis [2]. The capsule, a polysaccharide that, together with wall teichoic acids, decorates the peptidoglycan (PGN) macromolecule at the bacterial cell surface [3], is crucial for the ability of pneumococcus to cause invasive disease, since less encapsulated or non-encapsulated strains are less virulent or avirulent [4–6]. The capsule surrounds the surface of bacteria and acts as a shield that confers protection against the host's immune system [7–9]. A tight control of the amount of capsular polysaccharide (CPS) expressed or linked to the cell surface is expected to occur during the infection process, as a thick capsule may be disadvantageous during the colonization stage of infection, by preventing the exposure of bacterial molecules required for surface adhesion [2].

To date, more than one hundred capsular serotypes have been identified in pneumococcal bacteria [10–13]. The genes encoding proteins involved in CPS synthesis and regulation are, in most cases, located in the same region of the S. pneumoniae chromosome: the cps operon (S1 Fig, data adapted from Bentley et al. [14]). Most proteins encoded within the cps operon are serotype specific including those involved in the assembly of the repeating CPS unit, responsible for the transport of the CPS repeating unit from the inner to the outer face of the plasma membrane, involved in the polymerization of different repeating units and in the assembly of a mature CPS. However, the proteins encoded by the first four genes at the 5' end of the cps operon are highly conserved between serotypes and are proposed to be involved in the regulation of CPS synthesis [14]. The first gene in the cps operon encodes Wzg, also known as CpsA, an enzyme that belongs to the LCP (LytR–Cps2A–Psr) protein family. The initial observation that LytR protein produced by B. subtilis was involved in transcription regulation, has led to the proposal that Wzg (named Cps19A) had a similar role in serotype 19A encapsulated pneumococci [15,16]. However, a more recent study from Kawai and colleagues provided strong evidence that LCP proteins are in fact phosphotransferase enzymes implicated in anchoring anionic cell wall polymers, such as teichoic acids and capsular polysaccharides, to peptidoglycan [17]. Several reports suggest that Wzg may play a role in the attachment of CPS to the bacterial cell surface. Crystallography studies by Eberhardt and colleagues support the proposed
phosphotransferase activity for the serotype 2 Wzg protein [18]. Pneumococcal wzg mutant strains produce less total and cell wall associated CPS [19]. In addition, a pneumococcal wzg-lytR double mutant, which has a strongly distorted morphology, releases more capsule material to the supernatant when compared to the single wzg mutant and the parental strain [18]. Recently, a lytR single mutant was shown to be impaired in the retention of both CPS and teichoic acids [20]. However, the observed reduction in cell wall CPS does not directly imply a specific defect in attachment dependent on the activity of Wzg. Furthermore the three LCP homologs may have semi-redundant roles in the attachment of CPS and teichoic acids precursor chains to peptidoglycan [18].

Besides wzg, the conserved region of the cps operon contains wzh, wzd, and wze (S1 Fig), which encode three proteins that are considered to be part of a phosphoregulatory system required to control the synthesis of the capsule and its attachment to the cell wall [19]. Wze, also known as CpsD, is an autophosphorylating tyrosine kinase that belongs to the Bacterial Tyrosine Kinase family [21]. It has the conserved motifs characteristic of this protein family: a Walker A ATP binding motif and a C-terminal tyrosine cluster [22]. Wzd, also known as CpsC, is a membrane protein required for the autophosphorylation of Wze [23]. Wzh, also known as CpsB, is a phosphotyrosine protein phosphatase of the PHP family that dephosphorylates Wze [24]. For years, the role of tyrosine phosphorylation in capsular polysaccharide synthesis has been the subject of debate, with different reports presenting conflicting results, either supporting that Wze phosphorylation reduces capsule synthesis [22] or that it positively regulates CPS synthesis [25,26]. More recently, it has been reported that neither Wze phosphorylation, nor Wzh phosphatase activity, are determinants of capsule levels and factors outside the capsule locus have been suggested to play a role in the production of capsule [27].

Previously, we identified a novel role for Wzd and Wze as spatial regulators of capsule synthesis [28]. Wzd and Wze localize at the division septum in a manner that is dependent on the presence of both proteins, but independent of all other proteins encoded in the cps operon. In the absence of Wzd or Wze, capsule is synthesized, and covalently attached to the cell wall through phosphodiester bonds, given that it can only be removed through treatment with hydrofluoric acid [28], but it is absent from the division septum, the site where cell wall synthesis occurs during division. Therefore, we proposed that Wzd and Wze function as spatial regulators of capsule synthesis, ensuring that it occurs at the division septum, possibly to conceal newly synthesized cell wall [28]. A second role for the Wzd/Wze complex is to recruit the polysaccharide polymerase Wzy to the division site [29]. This suggests that CPS is produced only at the division septum by a single machinery [29]. In a wzd or wze null mutant, capsule is absent from the septum, but present in the rest of the cell surface, probably due to the observed delocalization of the CPS synthesis machinery [29]. This shows that these mutants are still able to export and attach polysaccharides to the cell surface.

Currently, the model for regulation of S. pneumoniae capsular polysaccharide synthesis proposes that when non-phosphorylated Wze interacts with Wzd, both proteins localize to the division septum [28,29]. Upon interaction with Wzd, Wze undergoes a conformational switch, promoting ATP binding and allowing autophosphorylation of its C-terminal tyrosine cluster [22,23]. Wze can then be dephosphorylated by the phosphatase Wzh and the cyclic phosphorylation/dephosphorylation of Wze could regulate the activity of the capsule assembly machinery [23,30]. Likely, Wzd and Wze capture the CPS synthesis machinery at the division site and trigger CPS export by the flippase Wzx, followed by polymerization by Wzy and anchoring to the peptidoglycan mesh by the phosphotransferase Wzg [14,17,18,29].

In this work, we examined the mechanism by which septal Wzd/Wze ensure CPS attachment to the septal cell wall. We demonstrate that this process is mediated by the recruitment of the Wzg ligase. When Wzg is not recruited to the division septum, there is an absence of
septal CPS that results in bacteria with septal cell wall more exposed to external PGN hydrolases or host PGN receptors.

**Results**

**The CPS-cell wall ligase Wzg interacts with Wzd**

We have previously found that Wzd and Wze act as spatial regulators of the *S. pneumoniae* capsule synthesis, to ensure that capsule is present at midcell, at the newly produced cell surface [28]. However, the mechanism mediating this regulation has remained unclear. We hypothesized that the Wzd/Wze complex either activates or recruits particular proteins of the CPS machinery synthesis to the division site [28].

In order to screen for possible interactions between Wzd or Wze proteins from *S. pneumoniae* ATCC6314 strain and other proteins involved in the synthesis of CPS, we have used a Bacterial Two-Hybrid (BTH) assay [31], which is based on the expression, in an *Escherichia coli* strain deficient in endogenous adenylate cyclase, of two inactive fragments of the catalytic domain of *Bordetella pertussis* adenylate cyclase, T25 and T18. When fused to interacting polypeptides, these fragments are brought together, activate the synthesis of cAMP and consequently restore the ability of the *E. coli* mutant strain to ferment lactose or maltose.

We screened for possible interactions between Wzd or Wze proteins from *S. pneumoniae* ATCC6314 strain, which produces a serotype 14 capsular polysaccharide, and two candidate proteins that could have a role in the control of the total amount of capsule present at the bacterial surface: WchA, the glycosyl transferase that links the first glucose residue of the capsule repeating unit to the lipid anchor present in the membrane of pneumococci [32]; and Wzg, the putative CPS-cell wall ligase that has been proposed to be involved in the linkage of assembled capsule molecules [18], which have been exported across the bacterial membrane but are still linked to a lipid anchor, to the peptidoglycan macromolecule, preventing the release of capsule to the surrounding medium [17]. An interaction was found only between Wzd and Wzg (Fig 1, column 18), which was dependent on the T25/T18 tag that was linked to the

![Fig 1. Wzg, a putative CPS-cell wall ligase, interacts with Wzd and with itself. Interactions between Wze, Wzd, Wzg and WchA were tested using Bacterial Two-Hybrid *E. coli* system [31]. β-galactosidase activity of cells expressing putative interaction partners was measured in cell extracts in at least three independent replicates. Black circles indicate median values and brackets show the 25% and 75% percentiles. Positive control (+): *E. coli* expressing T18 and T25 fragments linked to leucine zipper domains (zip) that can dimerize; Negative control (-): *E. coli* expressing untagged T18 and T25 fragments. Interactions were detected between T25-Wzd/T18 and T25-Wzg/T18-Wzg. ****P ≤ 0.0001. As previously described [28] combinations of plasmids expressing Wzd and Wze indicate that these proteins interact.](https://doi.org/10.1371/journal.ppat.1010516.g001)
interacting proteins (Fig 1, column 12), while no interactions were found between Wzd/Wze and WchA. Wzg interactions were observed when the T25-tag was fused to the N-terminal of the protein (Fig 1), but not when it was fused to the C-terminal (S2 Fig). Wzg was also found to interact with itself (Fig 1, column 23). In accordance with what has been previously reported [28,29], we were also able to detect an interaction between Wzd and Wze (Fig 1, columns 3–4 and 10–11).

**Wzg septal localization is dependent on the presence of Wzd/Wze proteins**

Given that Wzg interacted with Wzd in the BTH assay, and that Wzd has been shown to localize at the septum of encapsulated *S. pneumoniae* cells [28], we determined whether the septal localization of Wzg in dividing pneumococcal bacteria was dependent on the expression, or localization, of Wzd. For that, we constructed pneumococcal strains expressing a N-terminal fusion of Wzg linked to iGFP, an improved version of GFP for in vivo localization of proteins in *S. pneumoniae* [33]. The iGFP-Wzg fusion is functional as it can produce CPS at the surface of bacteria with the chromosomal wzg copy deleted from the cps locus (S3A Fig).

A pneumococcal plasmid expressing iGFP-Wzg was transformed into the wild-type ATCC6314 encapsulated strain (generating strain BCSJF005), into its unencapsulated isogenic strain that lacks the entire cps operon (generating strain BCSJF006), into the ATCC6314 wze null mutant in which Wzd is unable to localize at the septum [28] (generating strain BCSJF007), and into the ATCC6314 wzd null mutant (generating strain BCSJF008). When the resulting strains were observed by fluorescence microscopy, the fluorescent signal from iGFP-Wzg was found to be more enriched at the septal region of encapsulated BCSJF005 cells, than in cells from the unencapsulated strain that lack the cps operon or from the wze and wzd null mutant strains (Fig 2). In these, iGFP-Wzg was spread throughout the cell surface, presumably dispersed in the cell membrane, in accordance with the presence of three predicted transmembrane spanning domains in Wzg protein [34]. A quantitative analysis of these results was done by determining the ratio of the fluorescence signal at the septum versus the peripheral membrane (FR), as described in the Materials and Methods section. Notice that the septum contains two membranes, so only FR values higher than ~2 indicate preferential septal localization [35,36]. The encapsulated strain had a median FR value of 2.4, while strains lacking the cps operon, or the wze or wzd genes, had median FR values below 2 (1.6, 1.5 and 1.4, respectively, Fig 2), suggesting that Wzd is required for septal localization of Wzg. In agreement, complementation of the wzd null mutant with plasmid encoded Wzd (strain BCSJF010) caused an increase in recruitment of Wzg to the septum (median FR value of 2.0, Fig 2). It is possible that full complementation was not achieved because Wzd in the plasmid is under the control of a constitutive promoter, instead of the cps promoter. Septal enrichment of Wzg was not recovered upon complementation of the unencapsulated strain BCSJF009 with plasmid encoded Wzd (median FR value of 1.5, Fig 2). In this strain Wzd is present but it is delocalized due to the absence of Wze [28]. Therefore, septal Wzd appears to be required for the recruitment of Wzg to the septum, as expected if a physical interaction between the two proteins was responsible for Wzg localization. Similar data was obtained using a CFP fusion to Wzg (S3 Fig) showing that septal Wzg enrichment is independent of the fluorescent tag used.

In contrast to Wzg, WchA localization does not show septal enrichment. Expression of a functional derivative of WchA-CFP, which supports capsule production (S4 Fig, panel A), in an encapsulated or in a cps null mutant strain resulted in bacteria with a fluorescence signal dispersed throughout the membrane (S4B Fig).

Together, these results indicate that Wzg localizes at the division septum of pneumococcal cells, in a process that requires the correct expression and septal localization of Wzd/Wze.
The transmembrane and DNA-PPF domains of Wzg are required for its interaction with the membrane protein Wzd and with itself

Wzg is constituted by a small intracellular domain at its N-terminus, followed by three membrane spanning domains and a large extracellular C-terminal domain (Fig 3A). This large extracellular protein region contains an accessory domain, named DNA-PPF, present in some proteins of the LCP family, and the LytR-CpsA-PsR domain (Fig 3A), considered to be the core catalytic domain, present in all proteins of this family [34].

To determine which regions are involved in Wzg interactions, we used again a BTH assay. For this purpose, 6 different fragments of Wzg, shown in Fig 3A, were tested, showing that the three transmembrane domains and the first 120 amino acids of the extracellular domain (containing the accessory DNA-PPF domain), are required for self-interaction of Wzg monomers (Fig 3B, see columns 8/9 and 14/15) and for the interaction between Wzg and Wzd (Fig 3B, see columns 21 and 22). These results suggest that Wzd recruits Wzg to the pneumococcal
division septum through interaction with the membrane anchored accessory DNA-PPF domain.

Wzd V56 residue is critical for interaction with, and septal recruitment of, Wzg

Having determined the region of the Wzg ligase required for interaction with Wzd, we then enquired which region of the Wzd regulator was involved in this interaction.

Morona and colleagues have previously described that specific point mutations in Wzd, when in the presence of a mutated Wzh phosphatase, are associated with deficient attachment of capsular polysaccharide (CPS) to the cell wall [19]. Mutants bearing these alterations present a mucoid phenotype, in which total CPS levels are similar to the wild-type strain, but levels of CPS attached to the cell wall are much lower [19]. These observations indicate that the specific residues that were identified may be important for the ligation of CPS to the cell wall, but how...
this takes place has remained elusive. We hypothesized that these residues could be involved in Wzd interaction with the Wzg ligase. To test this hypothesis, and as the reported mucoid mutants carried other mutations beside those reported in Wzd, we first tested whether four of the reported mutations, namely Y39C, V56A, Y82F and V116A [19], impaired expression, or septal localization, of Wzd. All these residues are located in the extracellular loop of Wzd (Fig 4A), which could be responsible for its interaction with and recruitment of Wzg. We therefore expressed fluorescent derivatives of the four Wzd mutant alleles in a \textit{wzd} null mutant strain and determined their septal localization by fluorescence microscopy (Fig 4B). The ability of a mutated Wzd to localize at the division septum indicates that the protein is able to interact with Wze and may still regulate the synthesis of the pneumococcal CPS. The Y39C mutation may affect expression or stability of the Wzd protein, as no fluorescent cells were observed in the strain expressing WzdY39C. The V116A mutation impaired correct localization of Wzd at the septum, which became spread throughout the entire cell membrane. This mislocalization could be due to lack of interaction with Wze, which is required for septal localization of Wzd, or due to misfolding, or other alterations, that prevented the presence of an active Wzd. In contrast, mutations V56A and Y82F did not impair correct septal Wzd localization (Fig 4B). We then asked whether WzdV56A and WzdY82F mutants were altered in their ability to interact with Wzg and to recruit the capsule ligase to the division septum of pneumococci.

In the BTH assay WzdV56A, but not WzdY82F, lost the ability to interact with Wzg (Fig 5A, columns 66 and 67) indicating that V56 is critical for Wzd/Wzg interaction. We next tested whether WzdV56A was also unable to interact with Wzg in pneumococcal cells, leading to Wzg mis-localization. For this purpose, we co-expressed, in a \textit{wzd} null mutant, the fusion protein iGFP-Wzg with either Wzd (strain BCSJF010), WzdY82F (strain BCSJF012) or WzdV56A (strain BCSJF011). As described above, when Wzd was constitutively expressed
from a plasmid in a strain lacking wzd, iGFP-Wzg was able to localize at the division septum (median FR of 2.0). Similarly, WzdY82F was able to recruit iGFP-Wzg to the septum (median FR of 2.2) (Fig 5B). On the contrary, complementation with WzdV56A, did not recruit iGFP-Wzg to the division septum, as the fluorescent signal was distributed over the cell membrane (median FR of 1.3), similarly to the non-complemented strain BCSJF008 (median FR of 1.4) (Fig 5B).

Wzg recruitment to the septum by Wzd is required for the presence of capsular polysaccharide at midcell

Given the proposed role of Wzg in the attachment of capsule polysaccharide to the peptidoglycan, we questioned if the WzdV56A mutant, which can localize at the division septum, but is unable to recruit the Wzg ligase, was affected in capsule distribution at the bacterial surface. In encapsulated pneumococcal cells, the capsule is distributed throughout the surface, while in a
strain lacking Wzd, the capsule is detected at the cell surface, but it is absent from the division septum [28] (Fig 6). This is not due to an increased growth rate induced by the lack of capsule as the wild type ATCC6314 and its wzd null mutant (BCSMH001) strains have identical duplication times (33 min for both strains).

We transformed the wzd null mutant with plasmids encoding (i) iGFP-Wzd alone (strain BCSJF008); (ii) iGFP-Wzd and Wzd (strain BCSJF010); (iii) iGFP-Wzd and one of the variants WzdY82F (strain BCSJF012) or WzdV56A (strain BCSJF011) and visualized the presence of the capsule by immunofluorescence microscopy (Fig 6). Complementation of the wzd null mutant with a plasmid co-expressing iGFP-Wzd and Wzd, or WzdY82F, resulted in bacteria encapsulated, including at the septum. However, complementation with a plasmid co-expressing iGFP-Wzd with WzdV56A, resulted in bacteria that produce total capsule at similar levels as the parental strain but lower levels of CPS linked to the cell wall (S5 Fig). However, the CPS in cells expressing WzdV56A have a completely different pattern, as it was no longer homogeneously detected around the cells (Fig 6). Instead, CPS was absent from most regions of the cell and accumulated in dots near the division septum or at the cell poles. These CPS foci may reflect the subcellular localization of the machinery responsible for the polymerization and translocation of the CPS.

**Capsule absence at midcell results in increased exposure of bacterial septal cell wall**

The coordination of the Wzd/Wze complex with the Wzg ligase may ensure that newly synthesized cell wall is simultaneously produced and masked by CPS, so that bacteria permanently present a fully concealed cell wall. Absence of CPS at the division septum, caused by the lack of Wzd, may result in exposure of particular cell surface components, such as PGN or wall teichoic acids, to receptors produced by the host immune system.

To determine if wall teichoic acids are differently exposed at the bacterial cell surface in the absence of CPS, we used a recombinant and purified fluorescent derivative of LytA, a peptidoglycan hydrolase produced by S. pneumoniae that binds phosphorylcholine residues present in pneumococcal wall teichoic acids [37]. We hypothesized that localized or complete absence of capsule, in the wzd or in the cps null mutants respectively, could lead to an increased number of LytA-GFP molecules bound to the surface of bacteria. Addition of LytA-GFP to growing cultures of the wild-type ATCC6314 strain, its wzd null mutant (BCSMH001) and the capsule null mutant (BCSMC001), followed by epifluorescence microscopy to quantify levels of bound protein, showed that the fluorescent signal of bound LytA-GFP was ~1.5 times higher in the wzd null mutant than in the parental encapsulated strain. As expected, this increase was more pronounced in the unencapsulated strain (BCSMC001), where the fluorescent signal from bound LytA-GFP was ~6 times higher than for the parental strain (Fig 7A and 7B). The observed increased levels of LytA-GFP were not a consequence of an increased cell volume, as wzd mutant cells were significantly smaller than parental and non-encapsulated bacteria (S6 Fig and S1 Table).

Increased exposure of the cell wall of wzd mutant to LytA-GFP could be deleterious for bacteria, as it could increase susceptibility to lysis by external PGN hydrolases. To test this hypothesis, purified LytA-GFP was added to previously boiled bacterial cultures (required to inactivate native PGN hydrolases). Both the wzd null mutant and cps null mutant were more susceptible to lysis than the parental encapsulated ATCC6314 strain (Fig 7C). Interestingly, the wzd null mutant, with partial exposure of the bacterial cell surface, was as susceptible to LytA-GFP induced lysis as cps null mutant, with complete absence of capsule. This suggests that even a small breach in the concealment provided by CPS to the bacterial cell wall is
Expression of WzdV56A impairs the Wzg ability to produce a pneumococcal cell surface fully surrounded by capsule. A) Immunofluorescence microscopy images using a serotype-14 specific serum to detect the presence of the capsular polysaccharide at the cell surface. Wild-type encapsulated ATCC6314 (n = 115) expressed capsule all over the surface, while wzd null mutant strain BCSMH001 lacked capsule at midcell (n = 115). Cells of wzd null mutant strain were transformed with plasmids expressing (i) iGFP-Wzg alone (BCSJF008, n = 108), resulting in cells where the capsule is absent from the division septum; (ii) iGFP-Wzg and Wzd (BCSJF010, n = 104), or with WzdY82F (BCSJF012, n = 117), resulting in cells with homogeneous distribution of CPS at their surface or (iii) iGFP-Wzg and WzdV56A (strain BCSJF011, n = 105), resulting in cells where CPS accumulated in spots and was absent from most of the bacterial cell surface. Representative phase contrast (top panels, for visualization of bacteria), fluorescence microscopy (middle panels, for detection of the capsule associated with the bacterial cell surface) and overlay (bottom panels) images of each strain are shown. Arrows highlight bacteria that lack CPS at midcell. Scale bar, 2 μm. B) Graph shows the percentage of cells, for each strain, with different CPS patterns (grouped in 5 different classes). Numbers represent the percentage of cells fully covered with CPS. C) Classes of CPS patterns: cells fully covered with homogeneous CPS (dark red); cells fully covered with CPS whose staining is heterogeneous and has brighter regions (light red); cells partially covered with interruptions at the division septum (green); cells with CPS only in spots in particular regions (grey) or lacking CPS (black).

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Septal Wzg ensures correct encapsulation of pneumococci

A

Phase

LyA-GFP

WT

Δwzd

Δcps

B

Fluorescence (A.U.)

WT  Δwzd  Δcps

WT  Δwzd  Δcps

WT  Δwzd  Δcps

C

% of initial OD-600nm

0  50  100  150

Time (min)

- ATCC6314
- ATCC6314+LyA-GFP
- ATCC6314Δwzd
- ATCC6314Δwzd+LyA-GFP
- ATCC6314Δcps
- ATCC6314Δcps+LyA-GFP
sufficient to expose bacteria to cell wall binding molecules present in the growth medium, such as PGN hydrolases or host immune receptors.

**Absence of capsule at midcell impairs virulence**

To test the consequences of the absence of capsule at midcell on virulence, we used a zebrafish infection model. Due to its small size and rapid generation time, zebrafish (*Danio rerio*) is a powerful vertebrate model for studying host-pathogen interactions [38–41]. In particular, zebrafish embryos have been used as a model for host immune responses in systemic *S. pneumoniae* infections [42].

We performed survival assays by infecting zebrafish embryos, 3 days post-fertilization, with *S. pneumoniae* strains ATCC6314 (encapsulated), BCSMC001 (unencapsulated) and BCSMH001 (*wzd* null mutant). As observed in Fig 8, less than 30h post-infection, 0% of zebrafish embryos infected with the wild-type strain survived. However, 33% of embryos infected with the *wzd* null mutant strain (BCSMH001) were able to survive, even 72h after infection, close to the 47% of embryos that survive when infected with unencapsulated strain (BCSMC001). These results demonstrate that absence of capsule at the septum results in bacteria severely impaired in the ability to kill zebrafish embryos.

**Discussion**

We have previously proposed that Wzd and Wze act as spatial regulators of capsule synthesis, to ensure that it occurs at the division septum, possibly to conceal the newly synthesized cell wall [28]. Therefore, Wzd and Wze guarantee that the *S. pneumoniae* cell wall is completely surrounded and protected by the capsular polysaccharide during the entire cell cycle. However, how Wzd and Wze perform this role was not known. Two hypotheses could be envisioned: Wzd/Wze could recruit other members of the CPS synthesis machinery to the division septum or, alternatively, Wzd/Wze could interact and activate other members of the CPS synthesis machinery already present at that site. To understand the role of Wzd/Wze, we used a BTH assay and tested whether WchA, the first glycosyltransferase in the synthesis of the CPS repeating unit, or Wzg, the CPS-cell wall ligase, interacted with Wzd or Wze. While no interactions were detected between Wzd/Wze and WchA, we found that Wzg interacts with Wzd, as well as with itself. LCP enzymes such as Wzg are generally thought to work as monomers [43], although LcpA from *Corynebacterium glutamicum* can dimerize under particular conditions [44], and that may also be the case in *S. pneumoniae*.

Importantly, this work showed that Wzd/Wzg interaction is critical for correct localization of Wzg. As expected for a protein with three transmembrane domains, Wzg was found to be present at the membrane of pneumococcal cells. A Wzg fluorescent derivative was enriched at the septum of wild-type encapsulated ATCC6314 (serotype 14) cells, as previously described.
for the *S. pneumoniae* encapsulated D39 (serotype 2) and unencapsulated R6 strains [18]. However, contrary to what was previously reported, we observed that the localization of Wzg was dependent on the presence of the *cps* operon, which is required for the ability of bacteria to produce capsule. Wzg lost its septal enrichment in a mutant strain lacking the *cps* operon and, specifically, in the absence of the Wzd or Wze proteins. It is possible that the role of Wzd on Wzg localization varies for different serotypes, explaining the discrepancies in data obtained by different groups.

Morona and colleagues have previously proposed that alterations in Wzd/Wze expression could influence the levels of capsule associated with the cell wall or released into the growth medium [19]. These authors found that the change of a valine to an alanine at position 56 of Wzd, or of a tyrosine to a phenylalanine at position 82, in bacteria that lack the Wzh phosphatase, causes a mucoid phenotype in pneumococcal serotype 2 colonies [19]. These mutants produce total CPS levels that are similar to the parental strain, but the levels of CPS actually attached to the cell wall are lower, suggesting a decreased activity of the CPS ligase. As we showed that Wzd and Wzg interact, leading to Wzg septal recruitment, we hypothesized that
the V56A and Y82F mutations in Wzd might interfere with its interaction with and recruitment of Wzg. Indeed, WzdV56A did not interact with Wzg in a BTH assay and did not recruit Wzg to the division septum of pneumococcal cells. This was not the case for WzdY82F, which interacted with and recruited Wzg, indicating that the mucoid phenotype of this mutant arises via a different mechanism [19]. As substitution of Wzd tyrosine 82 by a cysteine was reported to cause a decrease in the phosphorylation of Wze [45], it is possible that this residue has a major role in the interaction between these two proteins or in the promotion of Wze phosphorylation by Wzd.

Wzg was initially thought to regulate transcription of the \textit{cps} operon [16]. This was based on the homology of its C-terminal domain with LytR, a \textit{B. subtilis} transcriptional regulator [15], and it was in accordance with a later report that showed that in \textit{Streptococcus agalactiae}, the homologue protein CpsIA functions as a transcriptional regulator [46]. Moreover, Wzg homologues from \textit{Streptococcus iniae} and \textit{S. agalactiae} bind specifically to DNA containing the capsule operon promoter region [47,48]. It seems therefore plausible that Wzg has two roles in the regulation of the CPS assembly: (i) to attach the capsular polysaccharide to PGN, through its extracellular domain, although it has been reported that \textit{wzg} insertion or deletion mutants can still attach CPS to the cell envelope [18,19,20], presumably through the activity of other LCP proteins; (ii) to control the transcription of the \textit{cps} operon, through its short cytoplasmic N-terminal end. The fact that in a BTH assay the transmembrane domain and the first part of the extracellular domain of Wzg are required for an interaction with Wzd, and that a point mutation in the extracellular domain of Wzd prevents this interaction, highlights the importance of the extracellular regions of both proteins in \textit{S. pneumoniae} CPS synthesis.

The role of Wzg, or of its successful interaction with Wzd, in the full encapsulation of the pneumococcal cell envelope may be specific to some serotypes, such as serotype 14, as it has been recently reported that CPS from serotypes 2, 8 and 31 is linked to peptidoglycan via a direct glycosidic bond, which would not require the role of this LCP protein [49], while serotype 14 CPS is connected through a phosphodiester bond to peptidoglycan which is labile to hydrofluoric acid [28]. We have further confirmed these results through sugar analysis of the cell envelope of serotype 14 cells. Hydrofluoric acid treatment released galactose, a sugar that is present in serotype 14 CPS, indicating that in these cells CPS is linked to peptidoglycan through a direct or indirect phosphodiester linkage.

More recently, the polymerase Wzy and the flippase Wzx were shown to localize exclusively at septum, suggesting that CPS synthesis occurs only at that place [29]. Interestingly, delocalization of Wzy was seen in a \textit{wzy} null mutant, which led to a sequential model where Wzd/Wze complex localize at the septum and then Wze captures Wzy resulting in its subsequent septal localization [29]. Considering these results, together with the data presented in this work, it is tempting to speculate that Wzd/Wze could control the elongation of the CPS chain (through recruitment of Wzy), as well as its ligation to the peptidoglycan (through recruitment of Wzg). Septal enrichment of Wzg may be crucial for CPS attachment to occur at a rate which is synchronized with the rate of PGN synthesis at the division septum. When this synchronization is lost, as it happens in the \textit{wzd} mutant, where septal enrichment of Wzg is lost, \textit{S. pneumoniae} cells synthesize PGN at the septum which is not immediately concealed by CPS. This results in cells where the cell wall at midcell is exposed to external WTA- or PGN-binding proteins. This model is line with the proposal that factor H binding proteins can protect the division septa of encapsulated bacteria against the host complement system [50]. In addition, in agreement with the idea that the septal cell surface requires a specific process of concealment, the \textit{wzd} null mutant bound higher amounts of the PGN hydrolase LytA to its surface and was more susceptible to lysis by LytA than the fully encapsulated parental strain. More importantly, a \textit{wzd} null mutant was dramatically impaired in virulence, in a zebrafish embryo infection...
model, a powerful vertebrate model for studying host-pathogen interactions due to its small size and rapid generation time [38–41], showing that full encapsulation of bacterial cells, covering the entire cell wall, is crucial for virulence. The observed small breaches in the concealment of bacteria, together with the reduced levels of CPS associated with bacteria, may explain why these mutants are unable to survive in the host.

**Materials and methods**

**Ethics statement**

Zebrafish larvae used in this study were 3 days post-fertilization and therefore are not subjected to any form of ethical regulation (European Union Directive 2010/63/EU). All the experiments were performed in strict compliance with national (DL 113/2013) and EU (Directive 2010/63/EU) regulations, in the i3S animal facility, with Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accreditation, that ensures high-quality care, providing animals with environmental enrichment, regular cleaning and daily checks. All personnel involved in animal handling and experimentation have individual training and authorization as per National/EU regulations.

**Bacterial strains and growth conditions**

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* bacteria were routinely grown in Luria Broth (LB) medium at 37°C, unless otherwise indicated. When appropriate, ampicillin (100 μg/ml) or kanamycin (50 μg/ml) were added to the growth media. For bacterial two-hybrid assays, MacConkey agar medium (Difco) and Maltose 1% (w/v) (Difco) were used.

*Streptococcus pneumoniae* bacteria were grown in liquid semi-defined C + Y medium [51] at 37°C, without aeration, or on tryptic soy agar (TSA, Difco) plates supplemented with 5% (v/v) sheep blood (ThermoScientific). When needed, tetracycline (1 μg/ml) was added to the growth media.

**DNA manipulation procedures**

*E. coli* and *S. pneumoniae* competent cells preparation and transformation were performed as previously described [52,53]. PCR products and plasmid DNA were purified with Wizard SV Gel and PCR Clean-up System and Wizard Plus SV Minipreps, respectively (Promega). PCR fragments were amplified with Phusion High-Fidelity DNA polymerase (Finnzymes). Restriction enzymes used were acquired from New England Biolabs or from Fermentas. Primers used in this work are listed in Table 2.

**Construction of plasmids for Bacterial Two-Hybrid assays**

Plasmids to test Wzd and Wze interactions with other capsule proteins, were constructed using inserts amplified from ATCC6314 chromosomal DNA, unless otherwise indicated.

Plasmids pBCSMC005, pBCSMH040 and pBCSMC007, encoding fusion proteins T18-Wzd, Wzd-T18 and Wzd-T25, were constructed by amplification of wzd with primers 1/2, 3/4 and 5/6, respectively, followed by restriction and ligation into plasmids pUT18C, pUT18 and pKNT25.

Plasmids pBCSMC006 and pBCSMC009, encoding fusions Wze-T18 and Wze-T25 were constructed by amplification of wze with primers 7/8, followed by restriction and ligation into pUT18 and pKNT25, respectively. Plasmid pBCSMC008, encoding fusion T25-Wze, was obtained by amplification of wze with primers 9/10 and cloning into pKT25.
Table 1. Bacterial strains and plasmids.

| Name | Relevant characteristics | Source/Reference |
|------|--------------------------|-----------------|
| **Strains** | | |
| *E. coli* DH5α | *recA endA1 gyrA96 thi-1 hsrR17 supE44 relA1* φ80 dlacZ M15 | Gibco-BRL |
| BL21(DE3) | F-ompT gal dcm lon hsdSB(rB-mB-) λ(DE3 lacI lacUV5-17 gene 1 ind1 sam7 nin5) | Lab Stock |
| BTH101 | Reporter strain for BTH system, F′ cya-99, araD139, galE15, galK16, rpsL1 (Str′), hsdR, mcrA1, mcrB1 | [31] |
| BCSRNN01 | DH5α transformed with pBCSRNN01 (pET21aLytA-GFP) | This work |
| **S. pneumoniae** ATCC6314 | Encapsulated strain, serotype 14 | American Type Culture Collection. |
| R36A | Non-encapsulated laboratory strain | [63] |
| BCSMC001 | ATCC6314Acps | [28] |
| BCSMH001 | ATCC6314Awze | [28] |
| BCSMH002 | BCSMH001 transformed with pBCSMH007 (wzd-citrine) | This work. |
| BCSMH036 | R36A transformed with pBCSMH020 (sfGFP) | [28] |
| BCSMH061 | BCSMH001 transformed with pBCSMH064 (wzdY39C-citrine) | This work. |
| BCSMH062 | BCSMH001 transformed with pBCSMH065 (wzdV56A-citrine) | This work. |
| BCSMH064 | BCSMH001 transformed with pBCSMH066 (wzdY82F-citrine) | This work. |
| BCSMH065 | BCSMH001 transformed with pBCSMH067 (wzdV116A-citrine) | This work. |
| BCSMH070 | ATCC6314 transformed with pBCSMH070 (wchA-CFP) | This work. |
| BCSMH071 | ATCC6314Acps transformed with pBCSMH070 (wchA-CFP) | This work. |
| BCSMH072 | ATCC6314 transformed with pBCSMH071 (iCFP-wzg) | This work. |
| BCSMH073 | ATCC6314Acps transformed with pBCSMH071 (iCFP-wzg) | This work. |
| BCSMH074 | ATCC6314Awze transformed with pBCSMH071 (iCFP-wzg) | This work. |
| BCSMH075 | ATCC6314Awze transformed with pBCSMH071 (iCFP-wzg) | This work. |
| BCSMH076 | ATCC6314Acps transformed with pBCSMH072 (iCFP-wzg) | This work. |
| BCSMH077 | ATCC6314Awze transformed with pBCSMH072 (iCFP-wzg) | This work. |
| BCSHF005 | ATCC6314 transformed with pBCSFH005 (iGFp-wzg) | This work. |
| BCSFH006 | BCSMC001 transformed with pBCSFH005 (iGFp-wzg) | This work. |
| BCSFH007 | BCSMH002 transformed with pBCSFH005 (iGFp-wzg) | This work. |
| BCSFH008 | BCSMH001 transformed with pBCSFH005 (iGFp-wzg) | This work. |
| BCSFH009 | BCSMC001 transformed with pBCSFH006 (iGFp-wzg) | This work. |
| BCSFH010 | BCSMH001 transformed with pBCSFH006 (iGFp-wzg) | This work. |
| BCSFH011 | BCSMH001 transformed with pBCSFH007 (iGFp-wzg) | This work. |
| BCSFH012 | BCSMH001 transformed with pBCSFH008 (iGFp-wzg) | This work. |
| BCSHP001 | ATCC6314Awzg | This work. |
| BCSHP002 | BCSP001 transformed with pBCSMH073 (wzg-GFP) | This work. |
| BCSHP003 | ATCC6314AwchA | This work. |
| BCSHP004 | BCSP003 transformed with pBCSMH070 (wchA-CFP) | This work. |
| BCSJC048 | BCSMH036 transformed with pBCSJ044 (lytA-GFP) | This work. |
| **Plasmids** | | |
| pET21a | T7p, T7t, His-Tag, lacI, Amp’ | Lab Stock |
| pBCSRN001 | pET21a containing lytA-GFP, Amp’ | This work. |
| **Plasmids for protein purification** | | |
| pUT18C | BTH plasmid, N-terminal cyaAT18 fusion, Amp’ | [31] |
| pUT18 | BTH plasmid, C-terminal cyaAT18 fusion, Amp’ | [31] |
| pKT25 | BTH plasmid, N-terminal cyaAT25 fusion, Kan’ | [31] |

(Continued)
Plasmids pBCSMH049 and pBCSMH051, encoding T18-Wzg and T25-Wzg, were constructed by amplification of wzg with primers 11/12, restriction and ligation into pUT18C and pKT25. Amplification of wzg with primers 11/13, restriction and ligation into pUT18 and pKT25.

| Name         | Relevant characteristics                                      | Source/Reference |
|--------------|--------------------------------------------------------------|------------------|
| pKNT25      | BTH plasmid, C-terminal cyaAT25 fusion, Kan’.                | [31]             |
| pKT25zip    | BTH control plasmid, Kan’.                                   | [31]             |
| pUT18Czip   | BTH control plasmid, Amp’.                                   | [31]             |
| pBCSMC001   | BTH plasmid containing cyaAT25-wzd fusion.                   | [28]             |
| pBCSMC002   | BTH plasmid containing cyaAT18-wze fusion.                   | [28]             |
| pBCSMC005   | BTH plasmid containing cyaAT18-wzd fusion.                   | This work.       |
| pBCSMC006   | BTH plasmid containing wze- cyaAT18 fusion.                  | This work.       |
| pBCSMC007   | BTH plasmid containing wzd- cyaAT25 fusion.                  | This work.       |
| pBCSMC008   | BTH plasmid containing cyaAT25-wze fusion.                   | This work.       |
| pBCSMC009   | BTH plasmid containing wze- cyaAT25 fusion.                  | This work.       |
| pBCSMH040   | BTH plasmid containing wzd- cyaAT18 fusion.                  | This work.       |
| pBCSMH049   | BTH plasmid containing cyaAT18-wzd fusion.                   | This work.       |
| pBCSMH050   | BTH plasmid containing wzg- cyaAT18 fusion.                  | This work.       |
| pBCSMH051   | BTH plasmid containing cyaAT25-wzd fusion.                   | This work.       |
| pBCSMH052   | BTH plasmid containing wzg- cyaAT25 fusion.                  | This work.       |
| pBCSMH053   | BTH plasmid containing cyaAT18-wchAfusion.                  | This work.       |
| pBCSMH054   | BTH plasmid containing wchA- cyaAT18 fusion.                 | This work.       |
| pBCSMH062   | BTH plasmid containing wzdV56A- cyaAT18 fusion.              | This work.       |
| pBCSMH063   | BTH plasmid containing wzdY82F- cyaAT18 fusion.              | This work.       |
| pBCSIF009   | BTH plasmid containing cyaAT25-wchA fusion.                  | This work.       |
| pBCSIF010   | BTH plasmid containing wchA-cyaAT25 fusion.                  | This work.       |

Replicative plasmids in *S. pneumoniae*

| Name         | Relevant characteristics                                      | Source/Reference |
|--------------|--------------------------------------------------------------|------------------|
| pBCSLF001   | High-copy-number vector, contains the -10 constitutive promoter of SigA from *S. pneumoniae*, Tet’. | [28]             |
| pBCSMH002   | Allows expression of Citrine fusion proteins, Tet’.         | [28]             |
| pBCSMH007   | pBCSMH002 containing wzd-citrine, Tet’.                     | [28]             |
| pBCSMH018   | Allows expression of CFP fusion proteins, Tet’.              | [28]             |
| pBCSMH020   | pBCSIF001 derivative, allows expression of sfGFP fusion proteins, Tet’. | [33]             |
| pBCSMH031   | Allows expression of CFP containing the first 10 aa of Wze at its N-terminus, Tet’. | [33]             |
| pBCSMH032   | Expression of sfGFP containing the first 10 aa of Wze at its N-terminus, Tet’. | [33]             |
| pBCSMH064   | pBCSMH002 containing wzdY39C-citrine, Tet’.                 | This work.       |
| pBCSMH065   | pBCSMH002 containing wzdV56A-citrine, Tet’.                 | This work.       |
| pBCSMH066   | pBCSMH002 containing wzdY82F-citrine, Tet’.                 | This work.       |
| pBCSMH067   | pBCSMH002 containing wzdV116A-citrine, Tet’.                | This work.       |
| pBCSMH070   | pBCSMH018 containing wchA-CFP, Tet’.                        | This work.       |
| pBCSMH071   | pBCSMH031 containing iCFP-wzg, Tet’.                        | This work.       |
| pBCSMH072   | pBCSMH031 containing iCFP-wzd, Tet’.                        | This work.       |
| pBCSMH073   | pBCSMH020 containing wzg-GFP, Tet’.                         | This work.       |
| pBCSIF005   | pBCSMH032 containing iGFP-wzg, Tet’.                        | This work.       |
| pBCSIF006   | pBCSMH032 containing iGFP-wzd, Tet’.                        | This work.       |
| pBCSIF007   | pBCSMH032 containing iGFP-wzgwzdV56A, Tet’.                 | This work.       |
| pBCSIF008   | pBCSMH032 containing iGFP-wzgwzdY82F, Tet’.                 | This work.       |
| pBCSIF044   | pBCSMH020 containing lytA-GFP, Tet’.                        | This work.       |

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### Table 2. Primers used in this work.

| Primers | Sequence 5’➔3’ | Features/ Restriction sites |
|---------|----------------|-----------------------------|
| 1       | CGGGATCCCGGAGGAAATGAATGAGG | BamHI |
| 2       | GGAATTCCCTCAAGTTGATCGAATTG  | EcoRI |
| 3       | CTAGCTAGATCTAGAGGAAAATGAGG | XbaI |
| 4       | CGCGGATCCCTCAAGTTGATCGAATTG | BamHI |
| 5       | GGAATTCCCTCAAGTTGATCGAATTG  | EcoRI |
| 6       | CGCGGATCCCTCAAGTTGATCGAATTG | BamHI |
| 7       | GGAATTCCCTCAAGTTGATCGAATTG  | EcoRI |
| 8       | CGCGGATCCCTCAAGTTGATCGAATTG | BamHI |
| 9       | GGAATTCCCTCAAGTTGATCGAATTG  | EcoRI |
| 10      | CGCGGATCCCTCAAGTTGATCGAATTG | BamHI |
| 11      | GGAATTCCCTCAAGTTGATCGAATTG  | EcoRI |
| 12      | CGCGGATCCCTCAAGTTGATCGAATTG | BamHI |
| 13      | GGAATTCCCTCAAGTTGATCGAATTG  | EcoRI |
| 14      | GGAATTCCCTCAAGTTGATCGAATTG  | PstI |
| 15      | CGCGGATCCCTCAAGTTGATCGAATTG | BamHI |
| 16      | GGAATTCCCTCAAGTTGATCGAATTG  | EcoRI |
| 17      | GGAATTCCCTCAAGTTGATCGAATTG  | NotI |
| 18      | GGAATTCCCTCAAGTTGATCGAATTG  | BglII; RBS |
| 19      | GGAATTCCCTCAAGTTGATCGAATTG  | BglII; RBS |
| 20      | GGAATTCCCTCAAGTTGATCGAATTG  | XhoI |
| 21      | GGAATTCCCTCAAGTTGATCGAATTG  | BamHI |
| 22      | GGAATTCCCTCAAGTTGATCGAATTG  | PstI |
| 23      | GGAATTCCCTCAAGTTGATCGAATTG  | KpnI |
| 24      | GGAATTCCCTCAAGTTGATCGAATTG  | NotI |
| 25      | GGAATTCCCTCAAGTTGATCGAATTG  | BglII |
| 26      | GGAATTCCCTCAAGTTGATCGAATTG  | XhoI |
| 27      | GGAATTCCCTCAAGTTGATCGAATTG  | BamHI |
| 28      | GGAATTCCCTCAAGTTGATCGAATTG  | PstI |
| 29      | GGAATTCCCTCAAGTTGATCGAATTG  | KpnI |
| 30      | GGAATTCCCTCAAGTTGATCGAATTG  | NotI |
| 31      | GGAATTCCCTCAAGTTGATCGAATTG  | BglII |
| 32      | GGAATTCCCTCAAGTTGATCGAATTG  | XhoI |
| 33      | GGAATTCCCTCAAGTTGATCGAATTG  | BamHI |
| 34      | GGAATTCCCTCAAGTTGATCGAATTG  | PstI |
| 35      | GGAATTCCCTCAAGTTGATCGAATTG  | KpnI |
| 36      | GGAATTCCCTCAAGTTGATCGAATTG  | NotI |
| 37      | GGAATTCCCTCAAGTTGATCGAATTG  | BglII |
| 38      | GGAATTCCCTCAAGTTGATCGAATTG  | XhoI |
| 39      | GGAATTCCCTCAAGTTGATCGAATTG  | BamHI |
| 40      | GGAATTCCCTCAAGTTGATCGAATTG  | PstI |
| 41      | GGAATTCCCTCAAGTTGATCGAATTG  | KpnI |
| 42      | GGAATTCCCTCAAGTTGATCGAATTG  | NotI |
| 43      | GGAATTCCCTCAAGTTGATCGAATTG  | BglII |
| 44      | GGAATTCCCTCAAGTTGATCGAATTG  | XhoI |
| 45      | GGAATTCCCTCAAGTTGATCGAATTG  | BamHI |
| 46      | GGAATTCCCTCAAGTTGATCGAATTG  | PstI |

(Continued)
pKNT25 produced plasmids pBCSMH050 and pBCSMH052, encoding the fusions Wzg-T18 and Wzg-T25, respectively.

Plasmids pBCSMH053 and pBCSMH054, encoding T18-WchA and WchA-T18, were constructed by amplification of \textit{wchA} with primer pairs 14/15 and 14/16, followed by restriction and ligation into pUT18C and pUT18, respectively.

Plasmids pBCSJF009 and pBCSJF010, encoding fusions T25-WchA and WchA-T25 were constructed by amplification of \textit{wchA} with primer pairs 17/18 and 19/20, respectively, restriction and ligation into pKT25 and pKNT25.

Plasmids pBCSMH062 and pBCSMH063, encoding fusions WzdV56A-T18 and WzdY82F-T18, were constructed using primers 3/4 to amplify the mutated \textit{wzd} genes from plasmids pBCSMH064 and pBCSMH066, respectively, followed by restriction and ligation into pUT18.

The nucleotide sequences of the inserts of the constructed plasmids were confirmed by sequencing.

**Bacterial two-hybrid assays**

Bacterial Two-hybrid assays were done in \textit{E. coli} strain BTH101 [31]. Transformants with plasmids mentioned above were plated in MacConkey media, supplemented with ampicillin, kanamycin and maltose. Plates were incubated at 30°C and screened for pink/white colonies, in which pink indicated a positive interaction. Single colonies were grown at 30°C in the presence of 0.5 mM of IPTG and the interactions confirmed by β-galactosidase activity measurements as described [54]. Part of these cultures (3 μl) were also used to spot fresh plates to visualize differences in the color of the bacterial lawn.

**Construction of plasmids for protein expression in \textit{S. pneumoniae}**

To determine the localization of Wzd proteins containing the point mutations Y39C, V56A, Y82F and V116A, plasmids expressing Citrine C-terminal fusions of the mutated proteins were constructed. The mutated \textit{wzd} genes were amplified in two different fragments using primers 21/22 and 23/24 for \textit{wzdY39C}; 21/25 and 26/24 for \textit{wzdV56A}; 21/27 and 28/24 for \textit{wzdY82F}; 21/29 and 30/24 for \textit{wzdV116A}. In each case, the two fragments were joined by overlap extension PCR using primers 21/24 and cloned in pBCSMH002, producing plasmids pBCSMH064-067.

Plasmid pBCSJF005, encoding a fluorescent derivative of Wzg, was constructed by amplification of \textit{wzg} from the ATCC6314 chromosomal DNA with primers 31/32, restriction and ligation into pBCSMH032.

Plasmids pBCSJF006-008 were constructed by amplification of \textit{wzd}, \textit{wzdV56A} and \textit{wzdY82F}, respectively, with primers 33/34 using plasmids pBCSMH007, pBCSMH065 and pBCSMH066 as templates, restriction and ligation into pBCSJF005. In the resulting plasmids, the protein fusion iGFP-Wzg is expressed in the presence of Wzd, WzdV56A or WzdY82F.

CFP fluorescent derivatives of Wzg were expressed through the construction of plasmid pBCSMH071 by amplification of \textit{wzg} from the ATCC6314 chromosomal DNA with primers 31/32, restriction and ligation into pBCSMH031. Amplification of \textit{wzd} with primers 33/34...
using plasmid pBCSMH007 as template, restriction, and ligation into pBCSMH071 produced plasmids pBCSMH072. In this plasmid, the protein fusion iCFP-Wzg is expressed in the presence of Wzd.

Plasmid pBCSMH070, was constructed by amplification of \textit{wchA} from the ATCC6314 chromosomal DNA with primers 35/36, restriction and ligation into pBCSMH018.

Plasmid pBCSJ044, encoding a LytA fluorescent derivatives, was constructed by amplification of \textit{lytA} with primers 37/38, restriction and ligation into pBCSMH020.

The nucleotide sequences of the inserts of the constructed plasmids were confirmed by sequencing.

**Construction of plasmids for protein expression in \textit{E. coli}**

To express in \textit{E. coli} and purify the protein LytA-GFP, amplification of \textit{lytAGFP} was done using primers 39 and 40 and the plasmid pBCSJ044 as template. Restriction and ligation into pET21a, produced plasmid pBCSRN001.

**Construction of null mutants of \textit{S. pneumoniae}**

Strains ATCC6314Δwzg and ATCC6314ΔwchA were constructed using the method described by Dalia and colleagues [55]. The upstream and downstream regions of target genes were amplified by overlap extension PCR to produce a single fragment. The \textit{rpsL1} gene cassette carrying streptomycin resistance was used for co-transformation experiments, using the corresponding overlap extension PCR amplicon, into the target \textit{S. pneumoniae} strain. Selection of transformants was made in the presence of streptomycin (100 μg/mL). For the construction of the ATCC6314Δwzg null mutants we used the primers 41–44. For the construction of the ATCC6314ΔwchA null mutants we used the primers 45–48.

**Protein purification**

The protocol for the purification of \textit{S. pneumoniae} LytA-GFP was adapted from [56]. Briefly, \textit{E. coli} BL21(DE3) cells were transformed with the plasmid pBCSRN001 and incubated overnight at 37˚C, with vigorous shaking, in LB supplemented with 100 μg/ml ampicillin and 2% (m/v) lactose. Cells were harvested by centrifugation, resuspended in 20 mM sodium phosphate buffer, pH 6.9, and broken by sonication. Clarified lysate was applied to DEAE-cellulose resin (DEAE Sephacel, GE Healthcare) and incubated at 4˚C for 1 h with stirring. Bound protein was washed five times with 20 mM sodium phosphate buffer containing 1.5 M NaCl, then eluted in the same buffer containing 2% (m/v) choline. Protein was dialyzed against 20 mM sodium phosphate buffer, pH 6.9 to remove the salts and aliquots were stored at 4˚C.

**Lysis assay**

For lysis assays, encapsulated ATCC6314, its capsule null mutant (BCSMC001) and the \textit{wzd} null mutant (BCSMH001) strains were grown O/N in 50ml of C+Y until OD~0.9, centrifuged, washed once with fresh C+Y medium, resuspended in 50 ml, and placed for 20 minutes in an ice bath. Cell suspensions were added to boiling water, at a flow that did not stop boiling, and further boiled for 40 minutes. Cell suspensions were then diluted to OD~1 and divided in two tubes, one of which received purified LytA-GFP at a final concentration of 6,75 μg/ml. OD600nm was followed in a Cary 100 UV-Vis Spectrophotometer (Agilent) using 10x10 mm cuvettes (Sarstedt) containing 3ml of culture, with a sterile gauze lid and a cuvette-adapted stirring bar (VWR) to allow continuous slow stirring. Lysis was followed by the decrease in OD600nm measured every 5 minutes for 150 minutes.
Growth assays

*S. pneumoniae* cells were grown O/N at 37°C without aeration in liquid semi-defined C + Y medium. When stationary phase was reached, at an OD≈0.8, cultures were diluted to OD≈0.05. The OD600nm was measured every 30 minutes until OD≈0.2 and then every 15 minutes. Absorbance values obtained during exponential growth were selected and used to determine duplication times (presented as a median value and n = 10).

Microscopy

*S. pneumoniae* strains were grown until early exponential phase and observed by fluorescence microscopy on a thin layer of 1% (m/v) agarose in PreC medium [51]. Images were acquired in a Zeiss Axio Observer microscope, equipped with a Photometrics CoolSNAP HQ2 camera (Roper Scientific), with appropriate exposure times (500–1000 ms for GFP; 5000 ms for Citrine and CFP; 100 ms for AlexaFluor secondary antibody), and analyzed using FIJI software [57] as well as eHooke software [58], which was developed in-house and is available at https://github.com/BacterialCellBiologyLab/eHooke.

Determination of the fluorescence ratio (FR) was performed as previously described [35]. Briefly, the intensity of the fluorescent signal at the division septum was divided by the fluorescent signal at the peripheral membrane. Average background fluorescence was subtracted from every value. An FR value higher than 2 is indicative of septal enrichment. Quantification was performed for at least 100 cells of each strain.

In vivo detection of the capsule produced at the surface of *S. pneumoniae* cells was performed as previously described [28], but Anti-rabbit Alexa Fluor 594 antibody (Invitrogen) was used as a secondary antibody. When necessary LytA-GFP purified protein was added to the media at 5 μg/ml concentration and incubated for 10 min at 37°C.

Determination of the CPS associated with pneumococcal cells

Cells were harvested at exponential growth-phase (OD_{600nm} ≈ 0.5) by centrifugation, washed with one volume of fresh C+Y medium and resuspended in water. After adjusting samples to same cell density, cells were lysed with deoxycholate (0.25 mg/ml for 30 min at 37°C) and boiled for 3 min before use. Samples of purified cell walls were prepared as previously described [59]. Briefly, cells were boiled into sodium dodecyl sulfate (SDS, final concentration, 4% (m/v)) for 30 min to inactivate any enzyme that could modify the bacteria cell wall. After removal of SDS, cell walls were mechanically broken by shaking with an equal volume of acid-washed glass beads with a FastPrepFP120 apparatus. Cell walls were digested with Dnase and Rnase (for 3 h at 37°C), and trypsin (overnight at 37°C), which were inactivated by boiling in 1% (m/v) (final concentration) SDS. Cell walls were washed twice with water, once with 8 M LiCl and then with 100 mM EDTA. Before lyophilization, broken cell walls (CW) were washed three times with water. In order to load in the dot-blot similar amounts of cell walls, the content of muramic acid was determined in each purified sample using HPAEC-PAD (High Performance Anion Exchange Chromatography coupled with Pulsed Amperometric Detection at the Chemical Analysis Laboratory at REQUIMTE-LAQV as previously described [60]. Samples were loaded onto Nitrocellulose (Amersham) membranes, which had been pre-equilibrated in PBS and placed on top of PBS soaked Hybond Blotting Paper. A volume of 3μl of each sample was loaded in triplicates, membranes were allowed to air-dry for 30 min and then blocked during 1 h in Blocking Buffer (5% (m/v) non-fat dried milk in PBS). Membranes were washed 3 times in PBS-T (PBS + 0.05% (v/v) Tween 20) and incubated overnight at 4°C with primary Anti-CPS14 antibody diluted 3/1000 in PBS-T, purified as previously described [28,61]. After washing with PBS-T, membranes were incubated during 1 h at room
temperature with the secondary antibody Anti-Rabbit StarBright Blue 700 (BioRad) diluted 1/5000 in PBS-T. Membranes were again washed 3 times with PBS-T and detected using the iBright FL1500 Imaging System.

Images were analyzed using FIJI software [57] to determine the average intensity present in each dot-blot spot. Average background fluorescence was subtracted from every value. Quantification was performed in membranes prepared in three independent days, each with 3–6 replicate spots from each sample.

**Infection assays using zebrafish embryos**

Wild-type AB zebrafish were obtained from the Zebrafish International Resource Center (Eugene). Embryos were raised at 28˚C in E3 medium. Larvae were anaesthetized with 200 µg/ml tricaine (Sigma) for the injection procedure. For injection of zebrafish larvae, cells were grown at 37˚C without aeration in liquid semi-defined C + Y medium, harvested (1 ml) at exponential growth (OD600nm=0.6), washed with fresh C+Y medium and resuspended in 100 µl of C+Y medium. A 70 kDa rhodamine dextran (Invitrogen/Molecular Probes) tracer was added in a proportion of 1:1 just before injection. Anaesthetized zebrafish larvae, 3 days post-fertilization (dpf), were microinjected in the hindbrain ventricle with 0.5–2 nl of bacterial suspension [62], so that a group of 12 embryos for each bacterial strain were injected with the same glass microcapillary needle filled with bacterial suspension. This experiment was repeated four times. Bacterial load upon infection was determined by plating at least three alive larvae onto TSA 5% (v/v) blood agar before and 2 hours post-injection, which confirmed that the number of bacteria injected for each strain were very similar. Each larvae was collected into an Eppendorf with 200 µl of lysis solution and then were mechanically smashed dilutions in C+Y were plated. Infected larvae were transferred into individual wells (containing 1 ml of E3 in 24 well plates), incubated at 30˚C and regularly observed under a stereomicroscope [42].

**Statistical analysis**

Statistical analyses of data presented in the figures were done using GraphPad Prism 8 software (GraphPad Software).

Analysis of data of the beta-galactosidase activity presented in Figs 1, 3, 5 and S2; of the Fluorescence ratios (FR) presented in Figs 2, 5, 7 and S3 and S4; and of the Area of bacteria in S6 Fig was done using a Kruskal-Wallis Test, and a Mann-Whitney Test. Results are available in S1 Table. P values ≤ 0.05 were considered as significant for all analysis performed and are indicated with asterisks: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 and ****P ≤ 0.0001.

Survival rates of the zebrafish embryos in the experiments reported in Fig 8, were also analyzed with the Graph Pad Prism 8 software. The statistical significance in these assay was measured using the Log-rank (Mantel-Cox) test. P values of <0.05 were considered significant.

**Supporting information**

S1 Fig. Schematic representation of the cps operon of serotype 14 ATCC6314 strain. Represented in figure are serotype specific genes that encode several glycosyltransferases that link or modify the different sugars present in the capsule repeating unit (light blue); that attach the first sugar of the repeating unit to the lipid anchor (WchA, light orange); the wzx gene (purple), which encodes the transporter of the CPS repeating unit from the inner to the outer face of the bacterial membrane; the wzy gene (orange), which encodes the polymerase involved in the polymerization of different repeating units and in the assembly of a mature CPS. The first four genes at the 5’ end of the cps operon are highly conserved between serotypes and are proposed to be involved in the regulation of the synthesis of CPS. The first gene is wzg (dark blue),
which encodes a ligase capable of attaching the capsule to the peptidoglycan macromolecule. The other three regulatory genes (yellow) encode Wze, an autophosphorylating tyrosine kinase; Wzd, a membrane protein required for the autophosphorylation of Wze and Wzh, a phosphotyrosine protein phosphatase that dephosphorylates Wze. Data adapted from Bentley et al. (14).

S2 Fig. Introduction of the T25 tag at the C-terminal of different capsule synthesis proteins may prevent their interaction with partner proteins. Wze, Wzd, Wzg and WchA interactions were tested using Bacterial Two-Hybrid E. coli system. β-galactosidase activity of cells expressing putative interaction partners was measured in cell extracts in at least three independent replicates. Black circles indicate median values and brackets show the 25% and 75% percentiles. Positive control (+): E. coli expressing T18 and T25 fragments linked to leucine zipper domains (zip) that can dimerize; Negative control (-): E. coli expressing untagged T18 and T25 fragments. No interactions were detected between tested proteins.

S3 Fig. A) Expression of iGFPWzg complements the ability of ATCC6314 wzg null to produce capsule. Immunofluorescence microscopy images using a serotype-14 specific serum to detect the presence of the capsular polysaccharide at the cell surface show that all cells of wild-type encapsulated ATCC6314 strain are surrounded by the capsule over the entire surface and that this number is reduced to 45% in its wzg null mutant strain (BCSSP001). Expression of iGFPWzg encoded in a replicative plasmid in BCSSP002 strain allows the expression of capsule in 95% of bacteria. Representative phase contrast (top panels, for visualization of bacteria) and fluorescence microscopy (middle panels, for detection of the capsule associated with the bacterial cell surface) images of each strain are shown. Scale bar, 2 μm. B) Septal localization of Wzg is dependent on the expression of Wzd/Wze. Graph shows the ratio of iCFP-Wzg fluorescence measured at the septum versus the peripheral wall in the S. pneumoniae wild-type encapsulated strain (BCSMH072, n = 132), the capsule null mutant (BCSMH073, n = 129), the wze null mutant (BCSMH074, n = 130), the wzd null mutant (BCSMH075, n = 112) and in the cps and wzd null mutants expressing Wzd from a constitutive promoter (BCSMH076, n = 135, and BCSMH077, n = 154, respectively). Enrichment of Wzg at the septum is only observed when Wzd is expressed and is localized at the division septum. Solid lines indicate median, and dashed lines indicate 25% and 75% percentiles. Representative phase contrast and fluorescence microscopy images of each strain are shown below the graph. Scale bar, 2 μm.

S4 Fig. A) Membrane localization of WchA is independent of the presence of the cps operon. Graph shows the ratio of WchA-CFP fluorescence measured at the septum versus the peripheral wall in the S. pneumoniae wild-type encapsulated strain (BCSMH070, n = 101) and in the capsule null mutant (BCSMH071, n = 101). No difference in the localization of WchA was observed between encapsulated and non-encapsulated bacteria. Solid lines indicate median, and dashed lines indicate 25% and 75% percentiles. Representative phase contrast and fluorescence microscopy images of each strain are shown below the graph. Scale bar, 2 μm. B) Expression of WchACFP complements the ability of ATCC6314 wchA null to produce capsule. Immunofluorescence microscopy images using a serotype-14 specific serum to detect the presence of the capsular polysaccharide at the cell surface show that all cells of wild-type encapsulated ATCC6314 strain are surrounded by the capsule over the entire surface and that no cells expressing capsule can be observed in its wchA null mutant strain (BCSSP003). Expression of WchACFP encoded in a replicative plasmid in BCSSP004 strain allows the expression...
of capsule in most bacteria. Representative phase contrast (top panels, for visualization of bacteria) and fluorescence microscopy (middle panels, for detection of the capsule associated with the bacterial cell surface) images of each strain are shown. Scale bar, 2 μm.

**S5 Fig. Expression of capsular polysaccharide in the presence of different Wzd proteins.** A) Cells from exponentially growing cultures of ATCC6314 (encapsulated parental strain, WT), BCSMC001 (non-encapsulated Δcps mutant strain), BCSMH001 (Δwzd mutant strain) and its derivatives strains that carry a plasmid expressing iGFPWzg in the presence of Wzd (BCSJF010 strain); of the mutated WzdV56A protein (BCSJF011 strain) and of the mutated WzdY82F protein (BCSJF012 strain) were analysed by dotblot using serotype-14 specific serum. Graph shows the intensity of the fluorescence signal measured in dot-blot assays in three independent experiments. Expression of WzdY82F and WzdV56A in the wzd null mutant strain allows expression of capsule at levels similar to those observed with the parental strain. B) Dot-blot assays performed with cell wall purified from the same strains.

**S6 Fig. Absence of Wzd results in smaller cell size.** Phase contrast microscopy images of wild-type ATCC6314 strain (WT), its capsule null mutant (BCSMC001; Δcps) and the wzd null mutant (BCSMH001; Δwzd) were used to determine cell size. Bacteria were grouped in three different classes depending on their cell cycle stage: (I) recently divided cells; (II) cells initiating division as seen from invagination of cell surface; (III) cells at the final steps of division, with deep invagination at division septum. Lack of Wzd, but not of CPS, results in smaller cells.

**S1 Table. Results obtained with the statistical analyses of data presented.** Analysis of data of the beta-galactosidase activity presented in the different figures was done using a Kruskal-Wallis Test, and a Mann-Whitney Test. P values ≤ 0.05 were considered as significant for all analysis performed.

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