Studies of the assembly of the *Streptococcus pneumoniae* capsular polysaccharide

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Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras, December, 2012

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Knowledge Creation

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First edition, December 2012

Cover image

Left, upper panel: Localization of Wze-Citrine, expressed from the natural chromosomal locus of the *wze* gene, in encapsulated *Streptococcus pneumoniae* cells.

Right, lower panel: Detection of the capsular polysaccharide, by immunofluorescence, on the Wze null mutant.

Back image: Detection of the capsular polysaccharide, by immunofluorescence, on encapsulated wild-type *S. pneumoniae* cells.

Aos meus pilares,

Mãe, Pai, Ivo

From left to right: Professor Mário Ramirez, Professor Leonilde Moreira, Professor Carlos Romão, Doctor Rut Carballido-López, Mafalda X. Henriques, Doctor Sérgio Filipe, Professor Adriano Henriques

December, the 17th of 2012

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Acknowledgments

The development of a PhD thesis is never an individual process. Many people contribute to its construction, either by suggesting experiments, discussing ideas, keeping us focused on the problem at hand or simply by listening to our complaints after a bad day of work. To all the people that accompanied me in this journey, I would like to declare my acknowledgments.

I will start by thanking my supervisor, Dr. Sérgio Filipe. When I joined the Bacterial Cell Surfaces and Pathogenesis laboratory I was not sure I wanted, or had the necessary characteristics, to enroll in a PhD. I was lucky to be given a very interesting and exciting project, and soon I was completely involved with it. For that opportunity and for all his guidance and ideas throughout these years, I would like to thank him.

I would like to thank the members of my Thesis Committee, Dr. Mariana Pinho and Dr. Ana Rute Neves. Dr. Mariana Pinho has accompanied my work very closely and her suggestions have no doubt been extremely helpful, as well as the different perspective and suggestions of Dr. Ana Rute Neves.

I deeply thank my lab colleagues at the Bacterial Cell Surfaces and Pathogenesis laboratory. To Magda Atilano, for her sincere friendship, advices and help in the lab. A month after we met I was going to be away from the lab for a couple of days and you said you would miss having me around. That meant a lot to me and I still remember it. I miss you already! I thank Tatiana Rodrigues for all her help in the lab but mostly for her friendship, her joy and the good moments and laughs we shared. I truly think that without you I would not been able to endure part of this thesis. Thank you very much. The lab is not the same since you left. I thank James Yates for his ideas and his willingness and availability

to help every time. I also thank all other members of the lab, past and present, for providing a good work environment.

I also thank my colleagues from the Bacterial Cell Biology laboratory. Ana Jorge, thank you for your friendship, for sharing your knowledge with me and for teaching me new protocols. I think we hit it of very quickly and I truly miss having you around. I thank Pedro Matos for his ideas, willingness to help and sense of humor. I thank Patricia Reed, for sharing all her knowledge and tips with me when I was lost in unexpected results. I also thank all other members of the Bacterial Cell Biology group, past and present.

I thank all my friends and colleagues from other labs. It was a pleasure and an honor to share this time with you.

I greatly thank Teresa Baptista da Silva for all her help in the lab. You have made our life so much easy, thank you very much!

I thank Instituto Tecnologia Química e Biológica for the ooportunity to work and develop my project in an institute with such excellent conditions. ITQB is truly a good place to work. To Fundação para a Ciência e Tecnologia I thank for providing the funding for my PhD.

Finalmente, quero agradecer às pessoas mais importantes para o desenrolar deste percurso. Aos meus pais, por toda a formação, educação, amizade, carinho e amor que sempre me deram. A vocês devo tudo o que sou e agradeço-vos profundamente. Não há palavras para descrever o quanto vos adoro. Ao Ivo, por me acompanhar há mais de um terço da minha vida. Obrigada pelo teu carinho, companheirismo e compreensão. E pela tua paciência para os meus longos e frequentes desabafos... agradeço-te do fundo do meu coração. Considero-me uma pessoa priveligiada por vos ter aos três na minha vida. Não poderia desejar algo melhor.

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Abbreviations and Acronyms

Amp	Ampicillin
ATP	Adenosine triphosphate
BY-kinase	Bacterial tyrosine kinase
CFP	Cyan fluorescent protein
CPS	Capsular polysaccharide
CW	Cell wall
CWPS	Cell wall polysaccharide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
Em	Erythromycin
Gal	Galactose
GFP	Green fluorescent protein
Glc	Glucose
GlcNAc	*N*-acetylglucosamine
GlcUA	Glucoronic acid
HMW	High molecular weight
HPLC	High pressure liquid chromatography
IPD	Invasive pneumococcal disease
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kan	Kanamycin
LB	Luria-Bertani broth
LMW	Low molecular weight
NDP	Nucleotide diphosphate
NETs	Neutrophil extracellular traps
PCP	Polysaccharide co-polymerase
PGN	Peptidoglycan
PHP	Polymerase and histidinol phosphatase

PMSF	Phenylmethylsulfonylfluoride
PBP	Peptidoglycan binding protein
Rha	Rhamnose
Rib	Ribitol
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sylfate polyacrylamide gel electrophoresis
Tet	Tetracycline
TSA	Tryptic soy agar
UDP	Uracil diphosphate
Und-P	Undecaprenyl phosphate
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Abstract

The capsular polysaccharide (CPS), or capsule, is one of the main virulence factors of *Streptococcus pneumoniae*. This gram-positive bacterium can asymptomatically colonize the nasopharynx of healthy individuals, but it also has the capacity to spread to other parts of the host's body and cause disease. The ability of pneumococcal bacteria to cause invasive disease is dependent upon the presence of the CPS, which surrounds the entire cell surface, protecting it from the host immune system.

There are more than 90 different types of capsule currently known. Importantly, in almost all serotypes the regulation of polymerization and cell wall attachment of the CPS seems to be dependent upon two proteins belonging to the Bacterial Tyrosine Kinase family. These proteins, Wzd and Wze, are proposed to function as main regulators of CPS synthesis in *S. pneumoniae*. In this work, the study of the dependence of CPS synthesis upon these two proteins was performed. Investigation of how capsule synthesis proceeds and its coordination with other essential cellular processes, such as cell division and cell wall synthesis, is described in this thesis.

Chapter II of this dissertation describes the construction and improvement of tools for *S. pneumoniae* cell biology studies. The developed tools allow the *in vivo* localization of proteins fused, either at the N- or C- terminus, to fluorescent proteins mCherry, Citrine, CFP and sfGFP. These new cell biology tools were subsequently used to study the cellular localization of Wzd and Wze. As described in chapter III, Wzd, a membrane protein, and Wze, a cytoplasmic protein, localized at the division septum when in the presence of each other. This process did not require any additional proteins encoded in the *cps* operon. Using a

Bacterial Two-Hybrid assay, Wzd and Wze were found to interact and the roles of the two conserved motifs of Wze, a Walker A ATP-binding motif and a C-terminal tyrosine cluster, in this interaction were determined. Mutation of conserved residues in the Wze Walker A ATP-binding motif, which prevents ATP binding to Wze, was shown to eliminate the interaction with Wzd, while substitution of the C-terminal tyrosines for phenylalanines had no effect. Moreover, Wze containing a mutated Walker A ATP-binding motif no longer localized to the septum of encapsulated *S. pneumoniae* cells, but became dispersed throughout the cytoplasm. In the absence of Wzd and Wze, capsular polysaccharide was produced and attached to the cell wall, but it was found to be absent from the division septum, the site of cell wall synthesis. These results suggest that Wzd and Wze are spatial regulators of capsule synthesis, ensuring that it occurs at the correct cellular localization.

In chapter IV the investigation of how Wzd and Wze spatially regulate capsule synthesis is described. Using a Bacterial Two-Hybrid assay, screens for interactions between Wzd and Wze, and other components of the CPS synthesis machinery, were performed. Interestingly, a positive interaction between Wzd and the CPS-cell wall ligase, Wzg, was detected. Fluorescent derivatives of Wzg were found to localize at the division septum of encapsulated bacteria in the presence of Wzd and Wze. This suggests that one role of the Wzd and Wze proteins may be to activate the attachment of the CPS to the cell wall at the division septum, ensuring that the whole surface is protected by the capsule.

The work described in this thesis has contributed to the elucidation of the mechanism of capsule synthesis in *S. pneumoniae*. A new role for the main regulators of this process, Wzd and Wze, was identified and interactions with other members of the CPS synthesis

machinery were found. The findings described here may have important implications in the design of new strategies against pneumococcal infection.

Resumo

O polissacárido capsular (CPS), ou cápsula, é um dos principais factores de virulência de *Streptococcus pneumoniae*. Esta bactéria Gram-positiva pode colonizar assintomaticamente a nasofaringe de indivíduos saudáveis, possuindo também a capacidade de se espalhar para outras partes do corpo do hospedeiro e causar doença. A aptidão das bactérias pneumocócicas de causar doença invasiva é dependente da presença de CPS, que rodeia toda a superfície celular, protegendo-a do sistema imunitário do hospedeiro.

Existem mais de 90 tipos diferentes de cápsula actualmente conhecidos. No entano, em quase todos os serótipos, a regulação da polimerização e ligação da cápsula à parede celular parece ser dependente de duas proteínas que pertencem à família das Tirosinas Cinases Bacterianas. Estas proteínas, Wzd e Wze, são propostas funcionar como os principais reguladores da síntese de CPS em *S. pneumoniae*. Neste trabalho, estudou-se a dependência da síntese de CPS destas proteínas. A investigação de como decorre a síntese da cápsula e de como esta é coordenada com outros processos celulares essenciais, como a divisão celular e a síntese de parede celular, é descrita nesta tese.

O capítulo II desta dissertação descreve a construção e optimização de ferramentas para estudos de biologia celular de *S. pneumoniae*. As ferramentas desenvolvidas permitem a localização *in vivo* de proteínas fundidas, quer no seu N- ou C-terminal, às proteínas fluorescentes mCherry, Citrine, CFP e sfGFP. Estas novas ferramentas de biologia celular foram subsequentemente utilizadas no estudo da localização celular do Wzd e do Wze. Como descrito no capítulo III, o Wzd, uma proteína de membrana, e o Wze, uma proteína citoplasmática, localizaram-se no septo de divisão, quando na presença uma da outra. Este

processo não requereu nenhuma proteína adicional codificada no operão *cps*. Usando um ensaio de Bacterial Two-Hybrid, demonstrou-se que o Wzd e o Wze interagem e determinou-se o papel dos dois motivos conservados do Wze, um motivo Walker A de ligação de ATP e um grupo de tirosinas no seu C-terminal, nesta interacção. A mutação dos resíduos conservados no motivo Walker A de ligação de ATP do Wze, impedindo a ligação de ATP, eliminou a interacção com o Wzd, enquanto que a substituição das tirosinas do C-terminal por fenilalaninas não teve qualquer efeito. Além disso, o Wze contendo um motivo Walker A de ligação de ATP mutado deixou de se localizar no septo de células encapsuladas de *S. pneumoniae*, encontrando-se disperso pelo citoplasma. Na ausência do Wzd ou do Wze, o polissacárido da cápsula era produzido e ligado à parede celular, mas encontrava-se ausente do septo de divisão, o local da síntese de parede. Estes resultados sugerem que o Wzd e o Wze são reguladores espaciais da síntese da cápsula, garantindo que esta ocorre na sua correcta localização celular.

No capítulo IV descreve-se a investigação de como o Wzd e o Wze regulam espacialmente a síntese de cápsula. Usando um ensaio de Bacterial Two-Hybrid, testaram-se interacções entre o Wzd e o Wze e outros componentes da maquinaria de síntese de CPS. Curiosamente, detectou-se uma interacção positiva entre o Wzd e o Wzg, a proteína que liga o CPS à parede celular. Derivados fluorescentes do Wzg localizaram-se no septo de divisão de bactérias encapsuladas na presença do Wzd e do Wze. Isto sugere que um dos papéis das proteínas Wzd e Wze poderá ser a activação da ligação do CPS à parede celular no septo de divisão, assegurando que toda a superfície celular é protegida pela cápsula.

O trabalho descrito nesta tese contribuiu para a elucidação do mecanismo da síntese de cápsula em *S. pneumoniae*. Um novo papel para os principais reguladores deste processo, o Wzd e o Wze, foi identificado

e interacções com outros membros da maquinaria de síntese do CPS foram encontradas. As descobertas aqui descritas poderão ter importantes implicações no desenvolvimento de novas estratégias contra a infecção por pneumococos.

Chapter I

General Introduction

When, in 1880, Pasteur described the isolation of *Streptococcus pneumoniae*, he noted: "Each of these little particles is surrounded at a certain focus with a sort of aureole which corresponds perhaps to a material substance." [5]. This structure, observed by Pasteur more than a century ago, was in fact the capsule or capsular polysaccharide (CPS) that surrounds pneumococcal bacteria. Since then, the CPS of *S. pneumoniae* has been at the center of major scientific observations in the fields of genetics, immunology and pathogenesis. Its study elucidated the role of capsular polysaccharides in bacterial virulence [6] and in protective immunity observed in the infected host [56], but perhaps the more important contribution to biology was the discovery of the capsule transformation reaction [34], which ultimately led to the identification of DNA as the genetic material [7]. Today, the study of pneumococcal capsule remains an area of active research, particularly with the aim of developing strategies to fight *S. pneumoniae* infections.

Structure of the *S. pneumoniae* capsular polysaccharide

The capsular polysaccharide forms the outermost layer of all *S. pneumoniae* fresh clinical isolates. The CPS is approximately 200 – 400 nm thick [96] and is usually found covalently attached to the outer surface of the cell wall peptidoglycan [95]. Currently, a total of 94 capsular serotypes have been reported in *S. pneumoniae* [14, 16, 82], and it is expected that additional serotypes will probably be identified in the future [114].

Capsular polysaccharides differ in their sugar composition and in the linkages between each of their components. They are complex structures containing different sugars, linkages and sometimes side chains (Figure I-1). Some capsular polysaccharides, like teichoic acids, contain ribitol or phosphorylcholine, such as types 6 and 15, while others possess

a simpler structure, being composed of only one or two sugars, such as types 3 and 37 [112].

Type	Repeating Unit
2	-3)-α-L-Rha-(1,3)-α-L-Rha-(1,3)-β-L-Rha-(1,4)-β-D-Glc-(1- 2 1 α-D-GlcUA-(1,6)-α-D-Glc
3	-3)-β-D-GlcUA-(1,4)-β-D-Glc-(1-
6A	-3)-α-L-Rha-(1,3)-D-Rib-(5-PO_4^-, 2)-α-D-Gal-(1,3)-α-Glc-(1-
14	-6)-β-D-GlcNAc-(1,3)-β-D-Gal-(1,4)-β-D-Glc-(1- 4 1 β-D-Gal

Figure I-1: *S. pneumoniae* capsular polysaccharide repeating units. The capsule repeating units of specific *S. pneumoniae* serotypes are shown to demonstrate their diversity. Glc, glucose; Gal, galactose; GlcNAc, N-acetylglucosamine; Rha, rhamnose; GlcUA, glucuronic acid; Rib, ribitol. Adapted from references [83, 112].

Role of the *S. pneumoniae* capsular polysaccharide

The essential role of the capsule in pneumococcal virulence was found by Avery and co-workers, when they discovered that enzymatic removal of the capsule protected mice from a pneumococcal infection [6]. Since then, the essential role of capsule in *S. pneumoniae* virulence has been demonstrated with unencapsulated mutants or with mutants that produce reduced amounts of capsule. These strains contained mutations in essential capsule genes, which render them avirulent in mice models [11-12, 36, 66], or mutations that impaired the activity of cellular enzymes that are required for capsule synthesis [36]. Importantly, pneumococcal

virulence requires not only the expression of capsule, but also its attachment to the cell wall [70].

Cell surface protection

Although structurally diverse, capsules perform the same major role: to confer resistance to opsonophagocytosis by reducing access of phagocytic receptors to complement bound to the cell wall of *S. pneumoniae*.

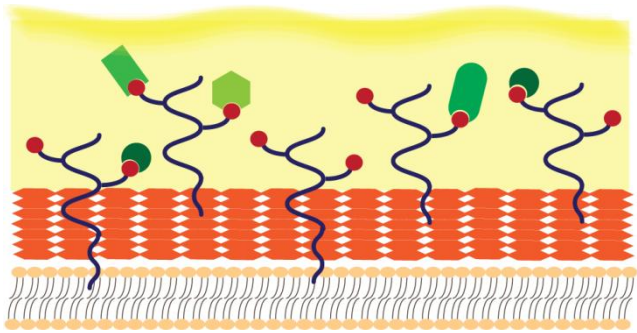

Figure I-2: Scheme of the *S. pneumoniae* cell surface. The membrane (light orange) is surrounded by the cell wall (dark orange). Teichoic acids and lipoteichoic acids (blue lines), choline (red circles) and Choline-binding proteins (green shapes) are shown. The capsule surrounds the entire surface of the cell (yellow). Modified from reference [81].

The *S. pneumoniae* cell wall can activate the complement system. In the absence of capsule, antibodies and C3b molecules, bound to the surface of the cells, are easily recognized by specific receptors produced by the host's phagocytes. In encapsulated bacteria, the CPS acts as a shield or mechanical barrier (Figure I-2) that prevents a successful interaction. This renders the pneumococcal cells resistant to phagocytosis [73, 106]. More recently, capsule was also shown to reduce the trapping

of *S. pneumoniae* by neutrophil extracellular traps (NETs), but not to be required for resistance to NET-mediated killing [103].

Contribution to the colonization of the host

Although a thick capsule prevents the exposure of surface molecules that might be important for the establishment of the colonization stage, the total absence of capsular polysaccharide is nevertheless deleterious for pneumococcal bacteria even at this stage. In fact, unencapsulated mutants are not able to colonize mouse models, requiring at least ~20% of the amount of capsular polysaccharide found in the parental encapsulated strains to be able to colonize at parental levels [58]. The reason for this might be the reduced clearance of the pneumococcus from the mucus, in the absence of CPS. Unencapsulated strains remain agglutinated in the luminal mucus and are less likely to migrate to the epithelial surface, where stable colonization occurs. The capsule inhibits mucus binding, in a process possibly mediated by electrostatic repulsion [74].

Importance of capsule type

The capsular type that is expressed by a particular pneumococcal strain has a direct role in the virulence and in the accessibility of surface adhesins and antigens to antibodies, although CPS structure does not seem to be the only factor involved in these mechanisms [1, 44, 87]. Also, isolates of the serotypes more commonly found in invasive disease resist better to complement deposition [62].

Importantly, the occurrence of spontaneous capsular transformation events, i. e. the exchange of genes that determine capsule type, have been identified *in vivo* [75]. This might be a mechanism by

which *S. pneumoniae* avoids opsonophagocytosis by serotype-specific antibodies during infection. Most importantly, it can lead to the spreading of a multiresistant phenotype into new serotypes, and to the increase in blood virulence and invasive phenotype of multiresistant strains [75].

Despite the differences between capsular types, the virulence observed with different strains within the same serotype is correlated with the amount of capsular polysaccharide that is expressed at the surface of the cells [57].

Why certain serotypes are more prevalent than others is a question that remains to be answered. It has been proposed that the serotypes producing a less metabolically costly CPS will be more heavily encapsulated, and hence be able to resist phagocytosis and persist in carriage. However, many serotypes with low metabolic cost remain rare, indicating that this is not sufficient for high prevalence [104].

Genetic organization of the *S. pneumoniae* capsular polysaccharide locus

In all the *S. pneumoniae* serotypes, with the exception of serotype 37, the genes that encode the enzymes responsible for the capsular polysaccharide synthesis are located in the same chromosomal region, the *cps* operon, located between the *dexB* and *aliA* genes [13, 113]. Although some of the nucleotide diphospho-sugars necessary for CPS production that are common to other metabolic pathways may be obtained from cellular pools [37, 113], most of the enzymes required for capsule synthesis are encoded in this region.

The 5' region of the *cps* operon is highly conserved between serotypes and comprises four genes: *wzg*, *wzh*, *wzd* and *wze* (also known has *cpsABCD*) (Figure I-3). The fifth gene of the operon is also conserved

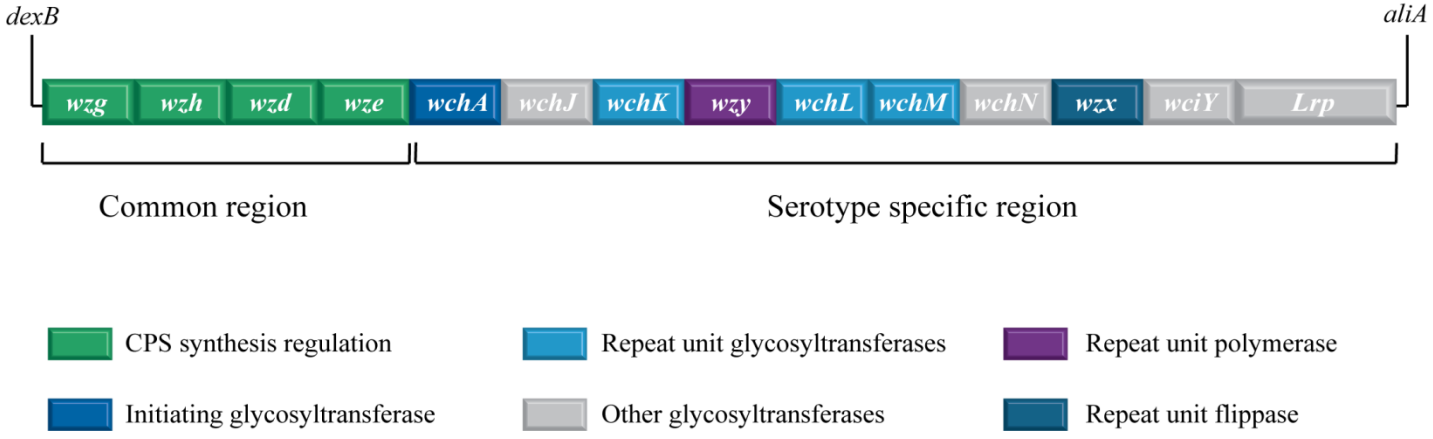

Figure I- 3: Organization of the *cps* capsule locus from *S. pneumoniae* serotype 14. The conserved region between serotypes and the serotype specific region are indicated. The color of the boxes indicates the function of the protein encoded by the gene. Adapted from reference [13].

and encodes the initiating glycosyltransferase. This is constituted by WchA (CpsE) in the 68 serotypes in which glucose (Glc) is the first sugar of the capsule repeat unit [13, 113]. The sequence of gene *wzg* is the most conserved. It falls into only one sequence cluster, whereas the sequences of *wzh* through *wze* or *wchA* can be divided into two different clusters [61, 69, 101]. Interestingly, these two clusters have been associated with ancestral forms of the capsular regulatory genes or with sequences probably acquired by lateral gene transfer, which in turn correlated with a predominance of carriage serotypes or invasive serotypes, respectively [101].

The downstream region of the *cps* operon is considered to be serotype specific (Figure I-3). This sequence is not conserved as the 5' region and it encodes the enzymes responsible for the assembly of the specific sugar repeating unit of each serotype [13, 48]. This includes genes encoding enzymes responsible for the synthesis of NDP-sugars that are unique to the CPS, glycosyltransferases and sugar modification enzymes. Also in this region is encoded the enzyme responsible for the transport of the repeat unit across the membrane, the Wzx flippase, as well as the enzyme required for its polymerization, the Wzy polymerase [13, 113].

The *cps* locus of the *S. pneumoniae* serotype 3 is also located between genes *dexB* and *aliA* and it includes the common region described above. However, this is not transcribed and all the genes except *wzd* are mutated. The 3' end of the type 3 locus is also constituted by truncated sequences and only two genes, *ugd* (*cps3D*) and *wchE* (*cps3S*) are required for serotype 3 capsular polysaccharide synthesis [4, 23, 113].

As mentioned above, serotype 37 is the only exception to this chromosomal location. Strains belonging to this serotype contain a *cps* locus correspondent to serotype 33, between the *dexB* and *aliA* genes, but

this is mutated. A single gene, *tts*, located elsewhere in the pneumococcal genome, is responsible for the synthesis of the type 37 capsule [55].

S. pneumoniae capsular polysaccharide synthesis

The majority of the pneumococcal capsular polysaccharide types are synthesized through the Wzy-dependent mechanism [13]. In this mode of synthesis, which proceeds in a similar way as the synthesis of peptidoglycan, the repeating units of the polymer are assembled in the inner face of the cytoplasmic membrane, transported across it to the outer face by a Wzx flippase and then polymerized in a nonprocessive manner by a Wzy polymerase. The defining enzymes of this pathway are the Wzx flippase and the Wzy polymerase [113]. Only in serotypes 3 and 37 the CPS synthesis occurs by a different mechanism, the synthase-dependent mechanism [13]. In this case, a single enzyme is responsible for the initiation, polymerization and transport of the capsular polysaccharide polymer [113]. The polysaccharides synthesized through this mechanism are simpler than those synthesized by the Wzy-dependent manner, consisting of only one or two repeating sugars. Also, defined repeating units are not formed, instead single sugars are added directly to the growing chain while it is being synthesized and transported across the cytoplasmic membrane.

Synthase dependent synthesis

Synthesis of the capsular type 3 is the paradigm for the synthase dependent capsule mode of synthesis in *S. pneumoniae*. It requires only one membrane associated enzyme, WchE (Cps3S), to transport and link the two sugar residues, from UDP-glucose (UDP-Glc) and UDP-glucuronic acid (UDP-GlcUA), that are part of the type 3 CPS [19].

Synthesis initiates with the addition of one of the sugar residues, the majority of the times from UDP-Glc, to phosphatidylglycerol [20]. Subsequently, alternating UDP-Glc and UDP-GlcUA units are used until the oligosaccharide reaches a critical length of ~8 monosaccharides [31]. This causes the oligosaccharylphosphatidylglycerol to become tightly associated with the synthase and shifts the enzyme to a highly processive phase, leading to the production of polymer of high molecular weight [31].

The rate of capsule synthesis in serotype 3 is regulated by the concentration of the UDP-sugars. In the absence or presence of insufficient levels of one of the UDP-sugars, synthesis is stopped and the polysaccharide is ejected from the enzyme in an abortive translocation reaction [30]. The presence of the second UDP-sugar prevents the release of the polysaccharide and, in a concentration-dependent manner, leads to the processive addition of sugars to the polymer [18]. Since cellular levels of UDP-Glc are usually sufficient to inhibit the ejection reaction, UDP-GlcUA levels are the limiting factor in this process [31]. Because capsule production correlates with capsule chain length, the concentration of UDP-GlcUA also modulates this property [102].

Wzy-dependent synthesis

Synthesis of the *S. pneumoniae* capsule by the Wzy-dependent mechanism (Figure I-4) begins with reversible transfer of an activated sugar, the majority of the times glucose (Glc) [13], to the C55 undecaprenyl-phosphate (Und-P) lipid carrier, leading to the formation of Und-PP-Glc [17]. This transfer was shown to be catalyzed by WchA, the first glycosyltransferase encoded in the *cps* locus, in serotypes 2, 8, 9V and 14 [17, 47, 84, 91], and it is assumed that it catalyzes this reaction in all the serotypes in which glucose is the first sugar of the repeating unit.

Once the formation of the repeating unit is initiated, subsequent sugars are added by additional glycosyltransferases. These are usually identified by homology from the genes encoded in the *cps* locus, but for serotype 14 the activities of the putative enzymes have been confirmed [46, 49]. WchK is a β-1, 4-galactosyltransferase that transfers galactose to lipid-linked glucose, the second step in the assembly of the type 14 repeating unit. WchL is a β-1, 3-N-acetylglucosaminyltransferase that adds the third sugar of the repeating unit, N-acetylglucosamine. Finally, WchM, a β-1, 4-galactosyltransferase, adds the last sugar to the repeating unit (Figure I-4).

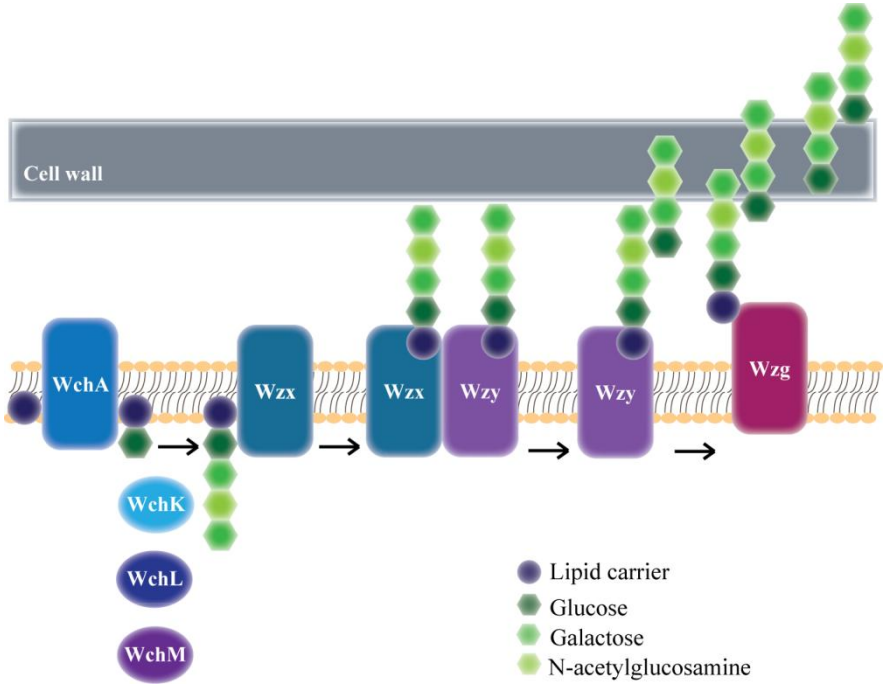

Figure I-4: CPS Wzy-dependent synthesis pathway. The steps in CPS synthesis of serotype 14 are shown as example. Protein WchA adds the first glucose of the CPS14 repeating unit to the lipid carrier. Proteins WchK, WchL and WchM successively add the next sugars of the repeating unit, which is then transported across the membrane by the flippase Wzx. At the outer surface, repeating units are added by the Wzy polymerase and high-molecular weight capsular polysaccharide is formed. The final step in CPS synthesis is the covalent

linkage of the capsule to the cell wall peptidoglycan, a reaction catalyzed by protein Wzg. See text for more details.

Following the assembly of the repeating unit in the inner face of the cytoplasmic membrane, the polymer is "flipped" to the external side by protein Wzx. At the outer face of the cytoplasmic membrane, repeating units are added and high molecular weight capsular polysaccharide is formed in a reaction catalyzed by the Wzy polymerase [113] (Figure I-4). The Wzy polymerases are generally predicted by their putative membrane topologies. Only for the *Escherichia coli* O-polysaccharide biosynthetic pathway (in an *in vitro* system) has unambiguous biochemical evidence that Wzy acts as a sugar polymerase been presented [107].

The final step in capsular polysaccharide synthesis is the covalent linkage of the capsule to the cell wall peptidoglycan [95]. For a long time the enzyme responsible for this step has remained unknown. Recently, Kawai and colleagues [43] have identified the Wzg protein, the product of the first gene of the *cps* operon, as the long searched capsule-cell wall ligase. Wzg belongs to a family of proteins, named LCP (**L**ytR – **C**ps2A – **P**sr), that carry out the transfer of anionic cell wall polymers, such has teichoic acids and acidic capsular polysaccharides, from their lipid-linked precursors to peptidoglycan. Crystallographic studies have demonstrated the phosphotransferase activity of the serotype 2 Wzg protein, supporting the proposed enzymatic function of the protein [25]. Importantly, the LCP proteins apparently do not recognize the repeating chain of the anionic polymer, recognizing instead the lipid and pyrophosphorylated part [25]. Therefore, these proteins are not specific and have somewhat redundant roles. The precise linkage of the CPS to peptidoglycan is not known.

In the last years, protein WchA has gained importance has a central player in the control of polymer assembly. Knockout mutants of serotype 2 *wzx*, *wzy* and *ugd* (encoding Ugd, a UDP-glucose

dehydrogenase necessary for side chain synthesis in this serotype) were only obtained in the presence of suppressor mutations [111]. In the majority of the cases, the suppressor mutations were mapped in *wchA*. It was proposed that the lethality of the *wzx*, *wzy* and *ugd* mutations was due to sequestration of Und-P in the capsule synthesis pathway, preventing its turnover for utilization in other essential cellular pathways, like peptidoglycan synthesis, or desestabilization of the membrane due to the accumulation of lipid-linked intermediates. The suppressors led to a reduction in the amount of capsule, presumably to a level that did not seriously affect the levels of available Und-P. The mutations in WchA occurred in the non-conserved extracytoplasmic and in the conserved cytoplasmic glyosyltransferase domains of the protein, indicating both have important roles [111]. It is possible that these mutations disrupt interactions with other proteins of the pathway, causing a reduction of its global activity.

Regulation of capsular polysaccharide synthesis in the Wzy-dependent mode occurs through a complex phosphoregulatory system, which is discussed in the following section.

Regulation of the *S. pneumoniae* capsular polysaccharide synthesis

The production of capsule and the length of the CPS chain in the Wzy-dependent synthesis are regulated by proteins from the Bacterial Tyrosine Kinase (BY-kinase) family. The members of this family are often involved in the regulation of the synthesis of polysaccharides, such as exopolysaccharides and capsular polysaccharides [33].

The BY-kinase regulatory systems consist of a membrane sensor domain, a catalytic cytoplasmic domain and a phosphatase that

dephosphorylates the latter. Two transmembrane domains and an extracytoplasmic loop between them, which is proposed to participate in the sensing of signals from the outside, constitute the membrane sensor domain. The catalytic cytoplasmic domain is the kinase part of the system. It possesses conserved Walker A and B ATP binding motifs and a tyrosine cluster at its C-terminal end. In Gram-negative bacteria and in Actinobacteria, these two domains are present in the same protein. On the other hand, in the firmicutes, such as *S. pneumoniae*, the two domains exist as two separate, interacting proteins [33] (Figure I-5).

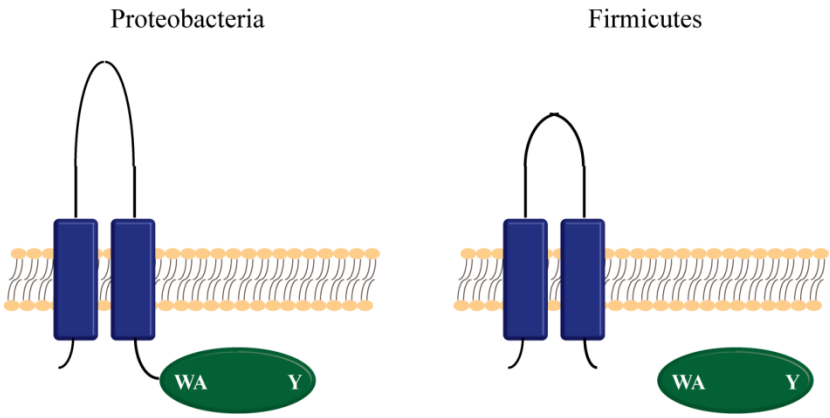

Figure I-5: Organization of Bacterial Tyrosine Kinases. BY-kinases are constituted by a transmembrane "sensory" domain (blue) and an intracellular catalytic domain (green). The cytoplasmic catalytic domain possesses conserved motifs: a Walker A ATP binding (WA) and a C-terminal tyrosine cluster (Y). In proteobacteria, the BY-kinases exist as a single protein, whereas in firmicutes they exist as two separate interacting polypeptides. The loop between the two transmembrane α-helices is shorter in firmicutes than in proteobacteria.

Protein Wzd is the sensor transmembrane domain in *S. pneumoniae* CPS synthesis. It belongs to the Polysaccharide Co-Polymerase (PCP) family [72] and it was shown to be required for the autophosphorylation of Wze [12]. In the absence of Wzd, pneumococcal colonies exhibit a rough phenotype, indicating capsule is not produced

[71]. However, immunoblot analysis revealed the production of short-chain CPS polymers in these mutants [11]. Also, immunofluorescence experiments detected the presence of low levels of CPS-related material at the cell poles of some of the cells [71].

Protein Wze is the catalytic cytoplasmic domain part of the BY-kinase system involved in *S. pneumoniae* CPS synthesis. As mentioned above for these types of proteins, it possesses the conserved motifs characteristic of the group: the Walker A and B ATP binding motif and the C-terminal tyrosine cluster [71]. Wze autophosphorylates at this cluster and this requires the binding of ATP and the presence of protein Wzd [12, 71]. Null mutants of *wze* produced the same phenotype as described above for *wzd* [11, 66, 71]. Interestingly, mutation of the two conserved motifs of Wze had different effects on capsule synthesis. Mutation of the Walker A ATP binding motif eliminated capsule production and prevented tyrosine phosphorylation, whereas mutation of the C-terminal tyrosines to phenylalanines still allowed capsule production [71]. However, deletion of the whole tyrosine cluster produces a phenotype indistinguishable from a *wze* null mutant and at least two functional tyrosine repeats are required for the normal function of the protein [67], indicating that this motif is involved in CPS production.

As mentioned above, the third member of the BY-kinase regulatory system is a phosphatase, responsible for dephosphorylating the cytoplasmic kinase. In *S. pneumoniae* CPS synthesis, the phosphatase is protein Wzh, a manganese-dependent phosphotyrosine-protein phosphatase that belongs to the Polymerase and Histidinol Phosphatase (PHP) family [68]. Wzh was shown to bind and dephosphorylate Wze, but also to inhibit its phosphorylation [12]. Pneumococcal *wzh* null mutant colonies have a rough phenotype, but immunobloting and immunofluorescence experiments have shown that, although at lower levels, capsule is produced and expressed all around the surface of the

cells [66, 71]. Also, the capsule produced has a normal chain size, as seen by the CPS ladder pattern observed in immunoblots, which is identical to the WT [11]. Point mutations in conserved aspartate and histidine residues of *wzh*, predicted to be involved in catalysis and metal binding, respectively, inactivate the protein. This leads to an increase in the levels of phosphorylated Wze and produces the same phenotype as a *wzh* null mutant [68].

For a long time, the role of the tyrosine phosphorylation in capsular polysaccharide synthesis has been the subject of debate, since apparently conflicting results were obtained by different authors. Weiser *et al*. observed that changes in the availability of environmental oxygen had an effect in the amount of capsule produced by transparent (T variants, producing less CPS) and opaque (O variants, producing more CPS) pneumococci [105]. Oxygen had an inhibitory effect in capsule production and this correlated with a decrease in the levels of tyrosine phosphorylation of Wze. Therefore, it seemed that tyrosine phosphorylation positively regulated capsule synthesis. This was also observed by Bender and colleagues, who found that the amounts of capsule and tyrosine-phosphorylated Wze correlated directly [11]. However, Morona and colleagues observed the opposite trend: tyrosine phosphorylation appeared to reduce capsule synthesis [67, 71]. It was only when the distinction between capsule synthesized and capsule attached to the cell wall was made that this question was elucidated. Mutants in which capsule was synthesized at normal levels but was defectively attached to the cell wall expressed mutated Wzd proteins, whose specific point mutations allowed the protein to function independently of Wze tyrosine phosphorylation [70]. Also, *wzh* mutants produced less CPS than the WT strain, but the proportion of attached CPS versus total amount produced was higher in these strains [70]. These data indicated that high cellular levels of the phosphorylated form of protein Wze reduce the overall rate

of CPS biosynthesis and increase the rate of cell wall-CPS attachment [70].

Currently, the model for *S. pneumoniae* capsular polysaccharide synthesis regulation is the following [70] (Figure I-6): nonphosphorylated Wze interacts with Wzd, and ATP binds to the Walker A motif of Wze. This promotes the interaction with other proteins of the machinery such that CPS biosynthesis/polymerization proceeds at a maximal level (Figure I-6A). At some point, due to an as yet unknown signal, Wze becomes phosphorylated. This changes the interactions between the proteins in the assembly machinery, slowing down CPS polymerization and promoting instead the transfer of the polymer to the cell wall ligase and consequent linkage to peptidoglycan. When Wzh dephosphorylates Wze, the cycle can be repeated (Figure I-6B).

Structural studies of the Wzd and Wze *Staphylococcus aureus* homologues, CapA and CapB, have provided a model for the molecular mechanism of polysaccharide synthesis regulation. Olivares-Illana *et al.* solved the structure of the *S.aureus* tyrosine kinase CapB fused at its N-terminus with the carboxyl-terminal cytoplasmic part of its cognate transmembrane modulator, CapA [80]. The unphosphorylated form of the protein was found to associate in a ring-shaped octamer and this led to the proposal of a structural-mechanical cycling process. Phosphorylation/dephosphorylation of the tyrosine kinase would induce cyclic dissociation-association of the octamer. These conformational changes would probably be transmitted to the other members of the polysaccharide synthesis machinery, affecting their interactions, this way modulating the capsule synthesis process [80].

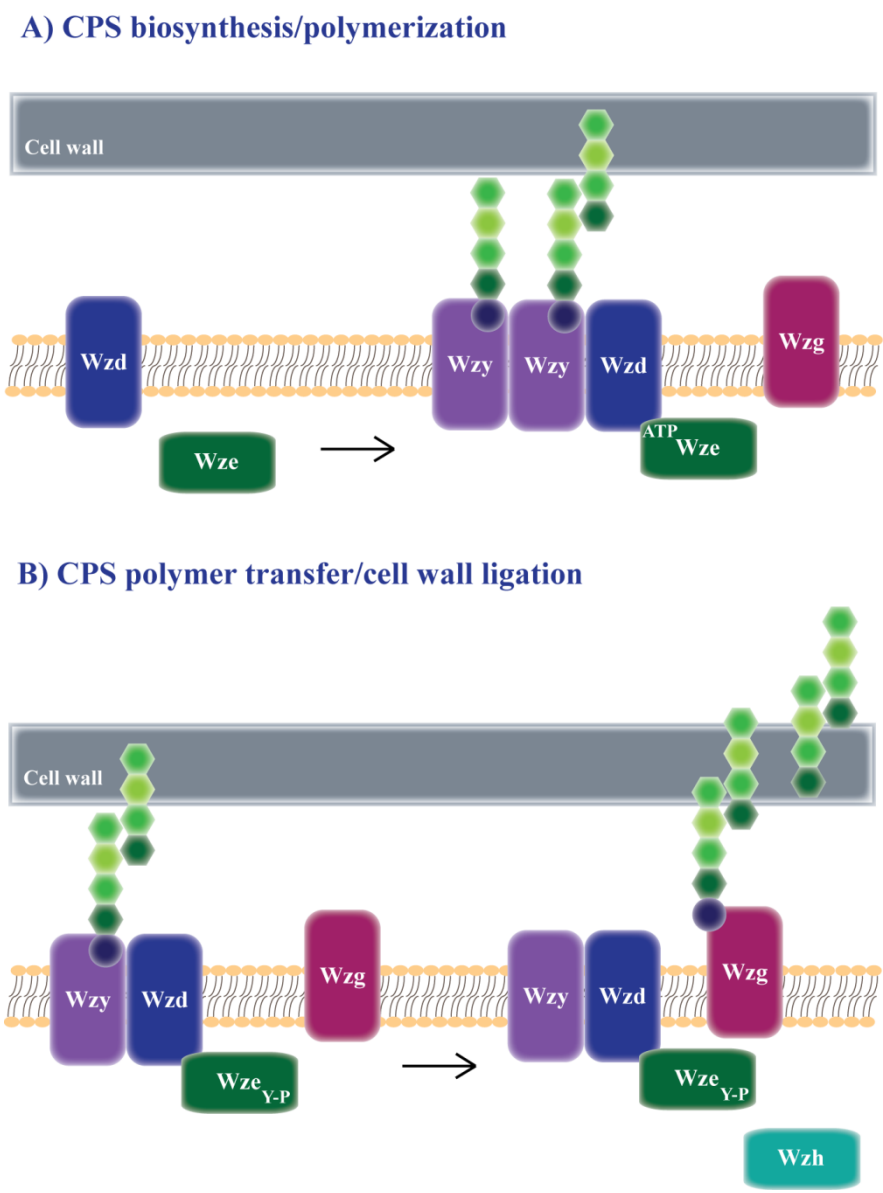

Figure I-6: Current model for the regulation of *S. pneumoniae* CPS synthesis. A) Wzd and Wze interact allowing ATP to bind to the Walker A motif of Wze. At this stage, the interactions between the proteins of the CPS machinery occur in a way that promotes CPS polymerization by the Wzy polymerase and leads to the formation of high-molecular weight CPS. B) Wze autophosphorylates at its C-terminal tyrosine cluster, due to an as yet unknown signal. This changes the interaction with Wzd, and with the other proteins in the complex. CPS synthesis is slowed down and the transfer of the capsular polysaccharide to the Wzg ligase and subsequent cell wall ligation is promoted. When the Wzh phosphatase dephosphorylates Wze, the cycle can be repeated.

S. pneumoniae capsule synthesis as a potential drug target

Importance of *S. pneumoniae* as a human pathogen

S. pneumoniae is the leading cause of otitis media, community-acquired pneumonia and bacterial meningitis. The pneumococcus spreads from person-to-person by aerosols and nasopharynx colonization is generally asymptomatic, with carriage rates ranging from 5 – 10% of healthy adults to 20 – 40% of healthy children. When the bacteria present at the nasopharynx migrates to other parts of the human body, spreading into the inner ear, lungs, bloodstream or brain, invasive pneumococcal disease (IPD) develops. IPD risk groups include infants, the elderly and immunocompromised individuals [81].

Although the rates of colonized individuals which develop IPD are low, the morbidity associated with *S. pneumoniae* is very high, due to its high colonization levels. In 2005, it was estimated that pneumococcus was the cause of 1.6 million deaths annually, including 0.7 – 1 million deaths of children under the age of 5 [110]. The situation is of most concern in developing countries, in which the majority of the latter occur. In 2000, 19% of the deaths of children under the age of five, which were HIV negative, due to pneumococcal disease, occurred solely in India and more than half occurred in only six countries (India, Nigeria, Ethiopia, Democratic Republic of the Congo, Afghanistan and China) [108]. In Europe and the United States, *S. pneumoniae* is the most common cause of community-acquired bacterial pneumonia in adults. In these economically developed regions, IPD is also responsible for high mortality levels. The mortality rates average 10 – 20% for adults with pneumococcal pneumonia, reaching as much as 50% in high-risk groups [109]. Furthermore, pneumococcal disease is also responsible for a high economical burden. In 2004 in the United States, it was estimated that

direct medical costs reached as much as 3.5 billion US dollars [41]. When one considers the work loss and productivity, the value is even higher. Pneumococcal disease still remains a substantial cause of morbidity and mortality in developed countries nowadays.

Current strategies to fight *S. pneumoniae* infections

Given the figures presented above, it is clear that effective strategies against *S. pneumoniae* disease are required. In the search for strategies to fight pneumococcal infections, the capsule has been a subject of interest for a long time.

Despite the existence of several *S. pneumoniae* serotypes, the majority of the disease is caused by only a small subset [8]. Therefore, polysaccharide vaccines (containing capsular polysaccharide) and conjugated vaccines (containing capsular polysaccharides conjugated with a highly immunogenic protein) against these CPS types have been developed [86]. Unfortunately, they only confer protection to the particular serotypes contained in their formulation. This has led to a phenomenon of replacement, in the population, of the vaccine serotypes by other non-vaccine serotypes [3, 38, 42]. New approaches, preferably directed at several serotypes simultaneously, are therefore required and would be a major breakthrough in the field. Vaccines against non-variable pneumococcal surface structures are being developed and will possibly be of great use in the future [59].

BY-kinases as novel drug targets

Since proteins Wzd, Wze and Wzh, as seen above, are key enzymes involved in CPS regulation in all but two of the pneumococcal serotypes, they present themselves as natural candidates for new strategies

designed against pneumococcal infections. Moreover, the fact that they belong to the BY-kinase family is another important factor, since this family of proteins has been raising attention as novel drug targets.

Several features of BY-kinases meet the criteria for an antibacterial target [21]. These enzymes control the metabolism of polysaccharides that are present at the surface of the bacterial cells, but not of the host cells, and they are embedded in the membrane, allowing relatively easy access. Also, through the phosphorylation of other cellular targets, they are involved in a broad spectrum of essential metabolic pathways. Most importantly, the structures of two BY-kinases, Etk from the Gram-negative *E. coli* [54] and CapB from the Gram-positive *S. aureus* [80], have been solved and they revealed some important features. Besides the absence of sequence similarity with tyrosine kinases from eukaryotes, the fold of BY-kinases is also entirely different from their eukaryotic counterparts, as well as their mechanism of action [53].

Not only BY-kinases serve as good candidates for drug development, their cognate phosphatases are also promising targets. The first structure of a tyrosine phosphatase from a Gram-positive, Wzh from *S. pneumoniae* serotype 4, was solved and shown to have a completely different structure from its *E. coli* functional homologue Wzb [35]. Recently, a screening for inhibitors of the phosphatase Wzh led to the finding of a metabolite produced by a marine sponge that inhibited the activity of the enzyme, causing a decrease in capsule synthesis [97]. Interestingly, despite their different structures, this metabolite also inhibited the phosphatase activity of the *E. coli* Wzb, demonstrating the value of these enzymes as drug development targets.

State-of-the-art of *S. pneumoniae* cell biology

In order to fully comprehend how important and essential mechanisms for the survival of bacteria occur and how they are regulated, it is mandatory to determine where the proteins involved in those mechanisms are localized in the cell and what factors control that localization. In *S. pneumoniae*, fundamental processes such as cell wall synthesis [64-65] and cell division [27, 32, 51, 78] have been studied through the use of immunofluorescence techniques. However, these types of techniques present several disadvantages. They require the fixation of the cells and their lysis in order to allow access of antibodies, therefore they are prone to the production of artifacts. Importantly, they also do not allow the visualization of live cells, making it impossible to follow the dynamics of a particular protein throughout the cell cycle of the bacteria [90].

The discovery of the GFP protein and its application to the construction of protein fusions that allow their *in vivo* visualization has revolutionized the cell biology field. Several characteristics of GFP make it an attractive reporter, in particular the fact that no external co-factors are necessary for its fluorescence and its self-contained domain structure. This reduces the chance of perturbations of the fluorescence by the protein to which GFP is fused, while simultaneously allowing that protein to retain its function [60, 100]. However, GFP maturation requires post-translational oxidation of the protein, which has hindered the application of this technology in microaerophiles such as *S. pneumoniae* [100].

In 2000, Acebo and colleagues provided the first report on the expression of GFP in *S. pneumoniae* [2]. They were able to measure directly the fluorescence of GFP in cells expressing single or multiple copies of the gene, opening the way to the possibility of following the fate of the pneumococcal cells during colonization. Shortly after, other

strategies in the same direction and allowing tighter control of GFP expression followed [76-77].

It was only in 2009 that the first study of protein localization through GFP fusions, in live pneumococcal cells, was reported [26]. Eberhardt and colleagues successfully localized proteins involved in choline decoration of teichoic acids, and since then several proteins involved in processes such as chromosome segregation [63], cell division [10, 29] or capsule synthesis [25] have been studied.

The use of GFP protein fusions in *S. pneumoniae* has no doubt been an important breakthrough in the cell biology of this important human pathogen, and the constant laboratory evolution and improvement of the GFP proteins [28, 94] is constantly increasing its range of applications [24]. Despite this, pneumococcal processes are still being studied through immunofluorescence techniques [9, 50, 92]. This indicates that the field of pneumococcal cell biology is still in need of more and better tools for the *in vivo* study of protein localization.

S. pneumoniae cell division

S. pneumoniae cells have the shape of an elongated ellipsoid, similar to a rugby ball. Cocci bacteria which have this shape have been designated as ovococci, to distinguish from the truly spherical cocci such as *Staphylococcus aureus*. Pneumococcal cells can be visualized as isolated cells, as diplococcic or as small chains, a characteristic which is the result of their mode of division which proceeds in successive parallel planes, perpendicular to the long axis of the cells [115].

S. pneumoniae possesses two modes of cell wall synthesis

In rod-shaped cells, cell wall growth occurs in two phases, elongation and division. On the contrary, cocci do not possess a cell wall elongation machinery, so that all cell wall growth is associated with division. Ovococcal cells possess an annular outgrowth of peptidoglycan which encircles the cells at their middle [39, 98], termed the equatorial ring (Figure I-7A), and this is the only site where new cell wall material is incorporated [15, 99]. In these bacteria, peptidoglycan is synthesized in two ways: peripheral synthesis and septal synthesis [40].

In ovococci, division initiates with the appearance of an ingrowth below the equatorial ring (Figure I-7B). When the equatorial ring splits at its middle, peripheral cell wall synthesis takes place as new material is incorporated between the two separating equatorial rings (Figure I-7C). Septal cell wall synthesis begins when the ingrowth, which has remained equidistant from the two rings, starts to grow centripetally, leading to the formation of the cross-wall, perpendicular to the main axis of the cell (Figure I-7D-E) [40, 115]. It is important to note that both types of cell wall synthesis in ovococci are division specific, since peripheral synthesis is different from the elongation, or lateral wall synthesis, that occurs in rod-shaped bacteria.

PBPs (Penicillin-binding proteins) are enzymes responsible for the last steps of peptidoglycan synthesis: the transglycosylation of disaccharide units that form the glycan strands; and the transpeptidation that cross-links the glycans through peptide bridges [89]. These proteins can be classified into high-molecular-weight (HMW) and low-molecular-weight (LMW) PBPs. HMW PBPs can be further divided into class A, which includes bifunctional proteins with both transglycosylase and transpeptidase activities, and class B, which includes proteins only with transpeptidase activity. LMW PBPs are carboxypeptidases or

endopeptidases [89]. *S. pneumoniae* has 3 class A HMW PBPs (PBP1a, PBP1b and PBP2a), two class B HMW PBPs (PBP2b and PBP2x) and one LMW PBP (PBP3) [115].

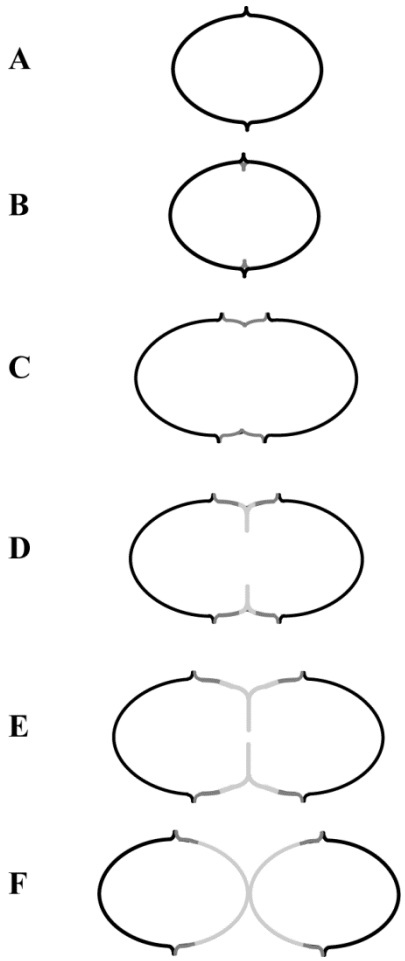

Figure I-7: Schematic representation of the ovococci mode of division. Ovococci cells possess an outgrowth at their middle, termed the equatorial ring (A). Division starts with the appearance of an ingrowth below the equatorial ring (B). The equatorial ring is split and the two resulting rings spread from each other as new cell wall material is incorporated during peripheral cell wall synthesis (C). Septal cell wall synthesis occurs when the ingrowth, which has remained equidistant from the two departing rings, starts to grow centripetally (D). Division proceeds with the addition of cell wall material at midcell (E) until the septum is closed and the two daughter cells separated (F). Cell wall material of peripheral and septal synthesis origin is represented in dark and light gray, respectively.

Initial immunofluorescence studies on the localization of the pneumococcal HMW PBPs led to the identification of two distinct localization patterns, septal and equatorial, which seemed to relate with the organization of the PBPs in two cell wall synthesis machineries, septal and peripheral [65]. Importantly, subsequent studies have shown that this patterns were an artifact and that all PBPs have the same localization at the middle of the cells [115]. However this does not rule out the existence of two cell wall synthesis machineries in ovococci, which is supported by recent data.

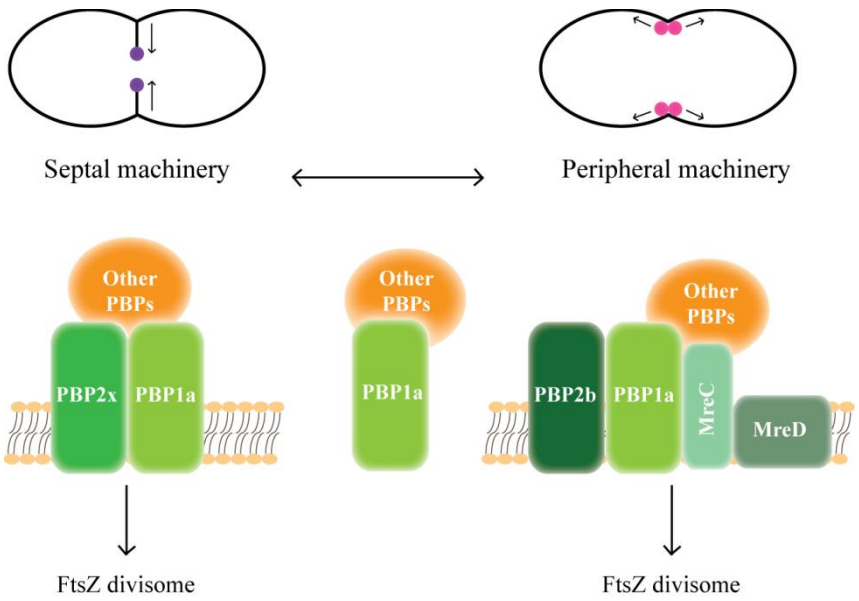

Figure I-8: Model for the composition of the peripheral and septal peptidoglycan synthesis machineries in *S. pneumoniae*. Both septal and peripheral peptidoglycan synthesis machineries are dependent on the division protein FtsZ. In *S. pneumoniae*, PBP2x is proposed to be part of the septal machinery, whereas PBP2b is proposed to be part of the peripheral machinery. The peripheral machinery also includes proteins MreC and MreD, as well as PBP1a. Based on studies with *B. subtilis*, PBP1a is proposed to shuttle between the two peptidoglycan synthesis machineries. The location of the other pneumococcal HMW PBPs has not been yet determined. Adapted from reference [93].

First, *Lactococcus lactis*, an ovococcus, was found to have the ability to become rod-shaped. Studies on the mechanism that allows this morphological transition led to the identification of PBPs associated with septal growth (PBP2x) and peripheral growth (PBP2b), which function independently of each other. This allows elongation to occur during inhibition of division and vice versa [85]. Second, proteins MreC and MreD, which are required to maintain the shape of rod-shaped bacteria, were found to direct peripheral peptidoglycan synthesis in *S. pneumoniae*. MreCD achieved this through the control of PBP1a localization or activity [50]. Based on these results and on other studies from *Bacillus subtilis*, which showed that PBP1 shuttles between peripheral and septal peptidoglycan synthesis, a model for the composition of the septal and peripheral peptidoglycan synthesis machineries has been proposed (Figure I-8) [93].

Interestingly, dual labeling of PBPs showed that different populations of PBPs are differentially localized within the same region of a cell. The observation that the activities of PBPs are not uniform in the midcell regions is in accordance with the model that two cell wall synthesis machineries, septal and peripheral, exist and operate at that site [45].

Coordination of cell wall synthesis with cell division

The first event in cell division is the recruitment of the tubulin-like protein FtsZ to the division site, where it assembles into a ring structure at the inner face of the cytoplasmic membrane. Following the formation of the Z-ring other division proteins are recruited to the division site, including the PBPs [115]. These were initially thought to localize through protein-protein interactions, but increasing evidence has been accumulating that supports the idea that substrate recognition plays a part

in this process. The *S. pneumoniae* LMW PBP3, a carboxypeptidase that hydrolyses the D-Ala-D-Ala terminus of the peptidoglycan precursor, was found to localize all over the cell surface of the pneumococcal cells, but absent from the future division site. This implies that pentapetides are only available at the equator, making the localization of the HMW PBPs at that site dependent on the availability of their substrate. In the absence of PBP3, the HMW PBPs rings were no longer co-localized with the FtsZ ring [64]. Also, in *L. lactis* the substitution of the terminal D-Ala of the peptidoglycan precursors for D-Lac led to the production of spherical cells, instead of ovococcal, that remained attached in chains [22]. The changes in the structure of the peptidoglycan precursor were postulated to affect the activity of the carboxypeptidase peptidoglycan processing enzymes, which in turn affected the recruitment of other PBPs.

Other proteins seem to be important in *S. pneumoniae* cell division. DivIVA, a protein that in *B. subtilis* is involved in division site selection, localizes as a ring at the division septum and as dots at the poles of *S. pneumoniae* cells. In the absence of DivIVA, the percentage of cells with constricted FtsZ rings is reduced, indicating a possible role in Z-ring progression. Using a Bacterial Two-Hybrid assay, DivIVA was found to interact with itself and with FtsZ, as well as with several other division proteins [27]. Protein DivIB seems to have a role in the early and late stages of the division process, since in its absence cells form chains and a small fraction are enlarged and have defective septa [52]. DivIB forms a trimeric complex with proteins DivIC and FtsL, the function of which has been proposed to be related to septal peptidoglycan synthesis [78].

Recently, protein StkP has emerged as a main regulator of pneumococcal cell division. StkP is the only eukaryotic-type Ser/Thr protein kinase in *S. pneumoniae* and it was shown to affect the transcription of several genes encoding proteins involved in several processes, including cell wall metabolism [88]. StkP mutant cells are

round or elongated and form chains, with non-functional septa and with delocalized sites of peptidoglycan synthesis [10, 29]. Importantly, the cell division defects observed in the absence of StkP are the result of the lack of phosphorylation of its targets [10], among which is DivIVA [79]. StkP localizes to the division site [10, 32], and this localization occurs through the sensing of uncross-linked peptidoglycan, since the protein is delocalized in the presence of antibiotics that target the late stages of peptidoglycan synthesis, as well as in cells that stopped dividing [10]. It seems that cell wall synthesis and uncross-linked peptidoglycan are sensed by StkP, leading to its recruitment to the cell division sites and to the stimulation of its autophosphorylation activity. StkP, trough the phosphorylation of key division substrates, would in this way regulate cell division and control the shift from peripheral to septal cell wall synthesis [10].

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Chapter II

Construction of improved tools for protein localization in *Streptococcus pneumoniae*

The work presented in this chapter was submitted for publication:
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Abstract

We have constructed a set of plasmids that allow efficient expression of both N- and C-terminal fusions of proteins of interest to fluorescent proteins mCherry, Citrine, CFP and sfGFP in the Gram-positive pathogen *Streptococcus pneumoniae*.

In order to improve expression of the fluorescent fusions to levels that allow their detection by fluorescence microscopy, we have introduced a 10 amino acid tag at the N-terminal end of the fluorescent proteins. This caused increased expression due to improved translation efficiency.

The availability of the new plasmids described in this work should greatly facilitate studies of protein localization in an important clinical pathogen.

Introduction

Studies on the sub-cellular localization of bacterial proteins have changed our view on the organization of bacterial cells. Initially, these studies were essentially restricted to the model organisms *Escherichia coli* and *Bacillus subtilis*. Despite their importance, these organisms do not eliminate the need for specific studies using different clinically important bacterial species, given the diversity in terms of morphology, physiology and metabolism among bacteria. Therefore, we have recently witnessed the development of new tools to allow cell biology studies in different pathogenic bacteria [6, 24].

Streptococcus pneumoniae is a major cause of morbidity and mortality worldwide. This Gram-positive bacterium is associated with a range of infections, which can vary from simple otitis media to more complicated ones, such as pneumonia or meningitis. Infection by this important human pathogen is of particular concern in developing countries, in which pneumococcal septicemia causes 25% of all preventable deaths in children under the age of five [11].

In order to design new and more efficient strategies to fight pneumococcal infections it is essential to understand how these bacteria divide or perform specific tasks important for their survival inside the host, such as the synthesis of peptidoglycan, the target of β-lactam antibiotics which are widely used against *S. pneumoniae*, or the synthesis of the capsular polysaccharide, the target of several successful anti-pneumococcal vaccines. An important step to accomplish this goal is the study of the localization of proteins involved in these processes. However, for a long time, cell biology studies in *S. pneumoniae* were limited by the lack of appropriate tools. Localization of pneumococcal proteins involved in cell wall synthesis [20-21] and cell division [7, 16, 22] was initially

performed using immunofluorescence techniques, which require cell fixation and lysis to allow access of the antibodies to the target proteins. Therefore, immunofluorescence cannot be used with live cells and is prone to the generation of artifacts [25]. It was only recently that the first studies on the localization of proteins in live pneumococcal cells, using fluorescent protein fusions tagged to the Green Fluorescent Protein (GFP), was reported [6]. Since then, other proteins involved in processes such as cell division [3, 19], cell wall synthesis [18] and capsular polysaccharide synthesis [5, 10] have been localized in live pneumococcal cells. However, the variety of tools available for these studies is still limited.

In this chapter, we report the construction of new plasmids that expand the tools available for *S. pneumoniae* cell biology studies by allowing the expression of N- or C- terminal protein fusions to different fluorescent reporters, namely mCherry, Citrine, CFP and sfGFP. For this purpose, we have improved the expression of the various fluorescent proteins in *S. pneumoniae*, by introducing an upstream tag, named “i-tag”, which increases protein translation. The availability of these plasmids should greatly facilitate studies of protein localization in this important clinical pathogen.

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Tables SII-1 and SII-2, respectively, in the Supplementary Information. *S. pneumoniae* was grown in C + Y liquid medium [14] at 37ºC, without aeration, or in tryptic soy agar (TSA, Difco) plates supplemented with 5% sheep blood (Probiologica). Tetracycline was added to the media at 1 μg/ml final concentration.

DNA manipulation procedures

S. pneumoniae competent cells preparation and transformation was executed as previously described [17]. PCR products and plasmid DNA were purified with kits 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 were from New England Biolabs. Primers used in this study are listed in Table SII-3 in Supplementary Information.

Construction of plasmids for protein expression in *S. pneumoniae*

For expression of CFP and sfGFP, not fused to any protein, we constructed plasmids pBCSMH018 and pBCSMH020 by amplification of the CFP and sfGFP coding sequences from plasmids pMUTIN-CFP and pTrc99A-sfGFP, respectively, with primers 1/2 and primers 7/8, followed by restriction and ligation to the vector backbone obtained by amplification of pBCSMH001 with primers 3 and 4.

For expression of Wze-CFP and Wze-sfGFP, the wze gene was amplified with primers 5 and 6 and cloned in pBCSMH018 and

pBCSMH020, upstream of the fluorescent protein gene, producing plasmids pBCSMH019 and pBCSMH021, respectively.

Construction of the plasmids encoding truncated mCherry, in which successive parts of the N-terminus of the encoded protein were removed, was obtained by ligation of the PCR products resulting from the amplification of plasmid pBCSMH001 with primer 12 combined with primers 15 – 18. The plasmids obtained in this way were named pBCSMH024 – 027.

In order to express different truncated forms of Wze-Citrine we constructed plasmids pBCSJC003, pBCSJC004 and pBCSJC005 that lacked the DNA encoding the N-terminus, central and C-terminus regions of Wze fused to Citrine, respectively. This was done by restriction and ligation of the PCR products resulting from amplification of plasmid pBCSMH004 using primer pairs 19/20, 21/22, and 23/9, respectively.

Construction of the plasmids that allowed the expression of Citrine in fusion with the first 3, 5 and 7 aminoacids of Wze at its N-terminus was carried out by ligation of the PCR products resulting from the amplification of plasmid pBCSMH004 with primer 20 combined with primers 28 – 30, respectively. The plasmids obtained in this way were named pBCSMJ008 – 010. Construction of the plasmid pBCSJC002, which allowed the expression of Citrine in fusion with the first 50 aminoacids of Wze at its N-terminus, was carried out by ligation of the PCR products resulting from the amplification of plasmid pBCSMH004 with primer 9 combined with primer 11. Construction of the plasmid pBCSJC007, which allowed the expression of Citrine in fusion, at its N-terminus, with the peptide sequence that in Wze is located between positions 11 to 50 was carried out by ligation of the PCR product resulting from the amplification of plasmid pBCSJC002 with primer pair 20/33.

Construction of plasmids pBCSMH030, pBCSJC001, pBCSMH031 and pBCSMH032, in which the fluorescent proteins mCherry, Citrine, CFP and sfGFP, respectively, are expressed in fusion with the first 10 aa of Wze, the "i-tag", at their N-terminus, was carried out by amplification of plasmids pBCSMH003, pBCSMH004, pBCSMH019 and pBCSMH021, respectively, with primers 9 and 10, followed by restriction and auto-ligation.

In order to express Citrine in fusion, at its N-terminus, with an "i-tag" whose *wze* nucleotide sequence was modified so that it carried a silent mutation we constructed plasmid pBCSJC006. This plasmid was obtained by restriction and ligation of the PCR product resulting from amplification of plasmid pBCSJC001 using primer pair 31/32.

For expression of iCitrine-Wze and Citrine-Wze, *wze* was amplified with primers 24 and 25 and cloned into pBCSJC001 and pBCSMH002, to produce plasmids pBCSJF001 and pBCSJF002, respectively. Plasmids pBCSJF003 and pBCSJF004, which allowed the expression of iCFP-Wzd and CFP-Wzd, respectively, were constructed through amplification of *wzd* with primers 42 and 43 and cloning in plasmids pBCSMH031 and pBCSMH018, respectively. For expression of iCFP-FtsZ and CFP-FtsZ, amplification of *ftsZ* was carried out with primers 26 and 27. The resulting PCR product was cloned into pBCSMH018 and pBCSMH031 to produced plasmids pBCSMH035 and pBCSMH036, respectively. The nucleotide sequences of the modified regions of the constructed plasmids were confirmed by sequencing.

Microscopy

S. pneumoniae strains were grown until early exponential phase (O. D. (600nm) = 0.2 – 0.3) and observed by fluorescence microscopy on a thin layer of 1% agarose in PreC medium [14]. Images were obtained

using a Zeiss Axio Observer Z1 microscope equipped with a Plan-Apochromat objective (100×/1.4 Oil Ph3; Zeiss) and a Photometrics CoolSNAP HQ2 camera (Roper Scientific). Semrock GFP, CFP, YFP and Texas Red filters were used. After acquisition, images were analyzed and cropped using Metamorph software (Meta Imaging series 7.5) and Image J software [1]. Fluorescence quantification was done using the Metamorph software by measuring the integrated fluorescence intensity in a defined region of 2 by 2 pixels and subtracting the average background fluorescence from every value. The obtained values were then normalized to the higher value. Quantification was performed for at least 100 cells of each strain.

Protein analysis

Bacterial cell aliquots of 1ml of culture were harvested at mid-exponential growth phase. Cells were incubated at 37ºC during 30 minutes in deoxicholate (0.25 mg/ml), RNase (10 mg/ml), DNase (10 mg/ml) and PMSF (1mM). For fluorescent protein analysis, proteins were incubated with solubilization buffer (200 mM Tris-HCl pH 8.8, 20% glycerol, 5 mM EDTA pH 8.0, 0.02% bromophenol blue, 4% SDS, 0.05M DDT) [4] at 37ºC during 5 minutes and separated on SDS-PAGE. Gel images were acquired on a FUJI FLA 5100 laser scanner (Fuji Photo Film Co.) with 635 nm excitation and > 665 nm band pass emission filter for protein molecular weight marker detection and 473 nm excitation and > 510 nm band pass emission filter for Citrine detection.

For western-blot analysis, cells extracts were boiled during 5 minutes before being separated on SDS-PAGE. Proteins were transferred into a Hybond PVDF Membrane (Amersham) and probed with Living Colors ® Av. Peptide Antibody (Clontech) for the detection of Citrine, used at 1:500, followed by 1:100000 of goat anti-rabbit conjugated to

horseradish peroxidase. Detection was done with ECL Plus™ Western Blotting Detection Reagents (Amersham).

RNA isolation and reverse transcriptase PCR (RT-PCR)

S. pneumoniae strains were grown in C+Y until early-exponential phase for RNA extraction. Prior to harvesting, RNAprotect Bacteria Reagent (twice the culture volume, QIAGEN) was added to the culture and the mixture was immediately vortexed for 10 sec. The cells were harvested, the pellet was frozen in liquid N_2 and stored at -80º C overnight. The next day, the pellet was resuspended with 200µl of sodium deoxycholate 0,25mg/ml during 30 min at 37ºC. RNA was extracted with RNeasy Mini kit (QIAGEN) and resuspended in milli-Q water. Total RNA was quantified using a Nanodrop Spectrophotometer ND-100. For RT-PCR, purified RNA was treated with Turbo DNase (Ambion) and screened for absence of contaminating DNA by PCR. 100 ng of DNase-treated RNA was subjected to reverse transcription using the OneStep RT-PCR Kit (QIAGEN). To amplify the fluorescent genes, the following nucleotides were used: 40/41 for *citrine* and 18/40 for *mCherry*. As a negative control, RNA isolated from strain BCSMH031 was used.

Quantitative Real-Time PCR

cDNA was generated from 250 ng of each RNA sample using TaqMan RT Reagents (Applied Biosystems, Branchburg, NJ, USA). The reaction mixture included 5.5 mM $MgCl_2$, 500 µM dNTPs, 2.5 µM random hexamers, 1 × RT Buffer, 0.8 U/µl RNase Inhibitor and 1.25 U/µl MultiScribe RT in a final volume of 50 µl. The Reverse Transcription conditions were 10 min at 25ºC, 15 min at 42ºC and 5 min at 99ºC. Quantification of Citrine and mCherry expression was achieved using the ABI7000SDS (Applied Biosystems), SYBR Green chemistry, and the

standard curve method for relative quantification. The PCR reagents consisted of: 1 × SYBR Green PCR Master Mix (Applied Biosystems), 400 nM of each primer, and 5 µl of sample cDNA, in a final volume of 25 µl. The thermocycling profile was: 10 min at 95ºC followed by 40 cycles of 15 s at 95ºC and 1 min at 60ºC. qPCR primers for *mCherry* (34 and 35) *citrine* (36 and 37) and *tetracycline* (38 and 39) were designed using ABI7000SDS – specific software, Primer Express (Applied Biosystems).

Optical plates included plasmid standard curves for Citrine and mCherry, and duplicates of each cDNA sample. "No template" and "no RT" controls were also included in every qPCR assays. For each sample, the expression of Citrine or mCherry was determined from the respective standard curve by conversion of the mean threshold cycle values, and normalization was obtained by dividing the quantity of Citrine (or mCherry) cDNAs by the quantity of cDNA amplified within the gene encoding for the tetracycline resistance protein (used as the endogenous control), which is cloned in the same plasmid. The specificity of the amplified products was verified by analysis of the dissociation curves generated by the ABI 7000 software based on the specific melting temperature for each amplicon. The final qPCR results were based on two independent experiments.

Results and Discussion

Expression of mCherry, Citrine, CFP and sfGFP in *S. pneumoniae*

S. pneumoniae is a microaerophile organism and therefore can only grow in the presence of low levels of oxygen, which may impair the correct folding of GFP-like proteins, known to require the presence of oxygen [27]. We have expressed fusions of Wze, a protein required for the regulation of the synthesis of the capsule polysaccharide [11], to four different fluorescent proteins, mCherry [26], Citrine [9], CFP [12] and sfGFP [8] (a robustly folded variant of the GFP protein [23]), two of which (CFP and sfGFP) had not been previously used in *S. pneumoniae*. The protein fusions Wze-CFP (BCSMH029) and Wze–sfGFP (BCSMH030), expressed in the encapsulated strain ATCC6314, allowed the visualization of Wze protein at the septum (Figure II-1A), in accordance to what we have previously described for Wze-mCherry (BCSMH015) and Wze-Citrine (BCSMH016) [10]. This indicates that all fluorescent proteins tested were able to fold correctly in the growth conditions used.

In order to test which of the four fluorescent proteins could potentially be used together for applications that require co-localization studies in *S. pneumoniae*, we mixed and analyzed various combinations of strains expressing the fluorescent proteins (data not shown). Fluorescence microscopy analysis of a mixture of three different cultures of the unencapsulated laboratory R36A strain expressing Wze-mCherry (BCSMH006), Wze-Citrine (BCSMH007) and Wze-CFP (BCSMH035) in the cytoplasm (Figure II-1B) showed that the fluorescent signals of these three proteins did not overlap.

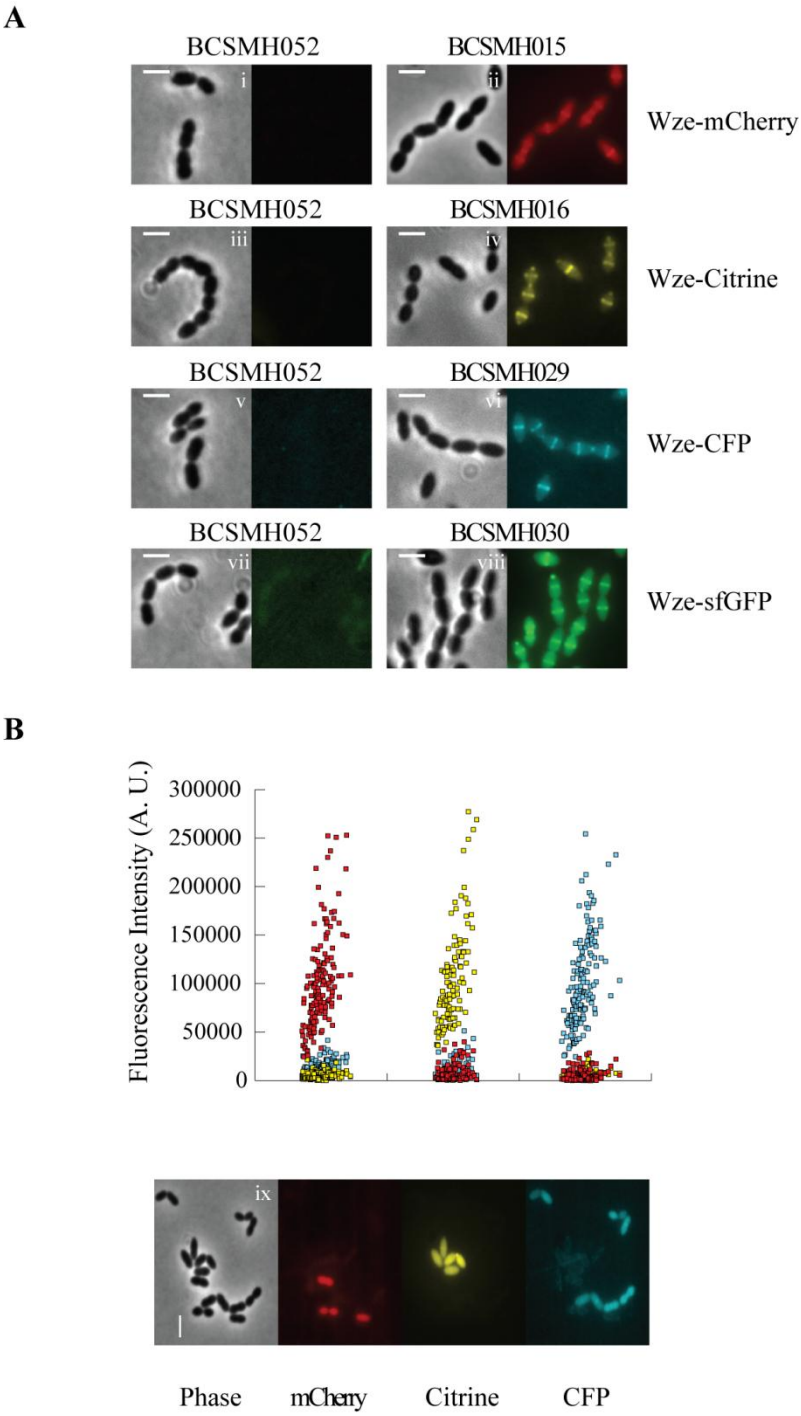

Figure II-1: Fluorescent signals emitted by mCherry, Citrine, CFP and sfGFP protein fusions are detectable in live *S. pneumoniae* bacteria. (A) The

septal localization of Wze protein fusions expressed in the encapsulated ATCC6314 strain is shown for Wze-mCherry (strain BCSMH015), Wze-Citrine (strain BCSMH016), Wze-CFP (strain BCSMH029) and Wze-sfGFP (strain BCSMH030). No comparable signal was detected in cells containing an empty vector (strain BCSMH052) visualized using appropriate filters for each fluorescent protein. (B) Three cultures of unencapsulated R36A strain expressing Wze-mCherry (strain BCSMH006, red dots), Wze-Citrine (strain BCSMH007, yellow dots) and Wze-CFP (strain BCSMH035, blue dots) were prepared for fluorescence microscopy observation as described in the Materials and Methods section, mixed on the same slide, and visualized using appropriate filters for each fluorescent protein. Fluorescence intensity was plotted, showing that the signals from each protein do not overlap. A representative image is shown at the bottom of the figure. Exposure times were 5000 msec. Scale bar: 2 µm.

We then constructed new vectors for cell biology studies in *S. pneumoniae* (plasmids pBCSMH001, pBCSMH002, pBCSMH018 and pBCSMH020) expressing the four fluorescent proteins, not fused to any protein. Surprisingly, all strains expressing solely the untagged fluorescent proteins mCherry (BCSMH032), Citrine (BCSMH033), CFP (BCSMH034) and sfGFP (BCSMH036) showed extremely low levels of fluorescence (Figure II-2).

Optimization of expression of fluorescent proteins in *S. pneumoniae*

The four fluorescent proteins used in our studies all included, at their N-terminal, a linker region, which introduced a spacer between the fluorescent protein and the protein of interest, and a GFP-like terminus region that had been proposed to stabilize the fluorescent signal [26] (Figure II-3). We therefore asked whether this N-terminal linker was responsible for the lack of fluorescence in *S. pneumoniae*. To answer this question we constructed a series of plasmids, encoding mCherry or Citrine, in which successive fragments of the linker were removed,

leaving intact the original promoter region and starting codon (Figure II-3).

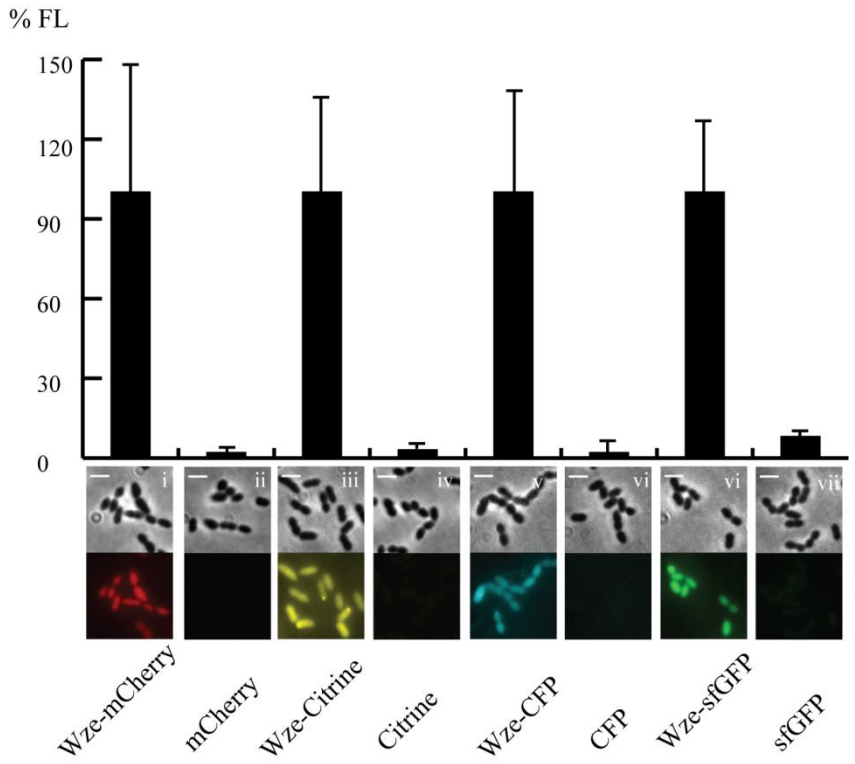

Figure II-2: Fluorescent signals emitted by untagged mCherry, Citrine, CFP and sfGFP proteins are not detectable. The intensity of the fluorescence signal in unencapsulated bacteria expressing mCherry (strain BCSMH032), Citrine (strain BCSMH033), CFP (strain BCSMH034) and sfGFP (strain BCSMH036), is plotted as a percentage relative to the signal obtained with expression of the same fluorescent protein fused to the C-terminus of Wze (strains BCSMH006, -007, -033 and -035, respectively). Representative images of each strain are shown at the bottom. Scale bar: 2 µm.

Quantification of the fluorescent signal expressed by pneumococcal strains encoding truncated mCherry proteins (strains BCSMH040 to BCSMH043) showed that removal of the linker caused only a small increase in the observed fluorescence (Figure II-4), which

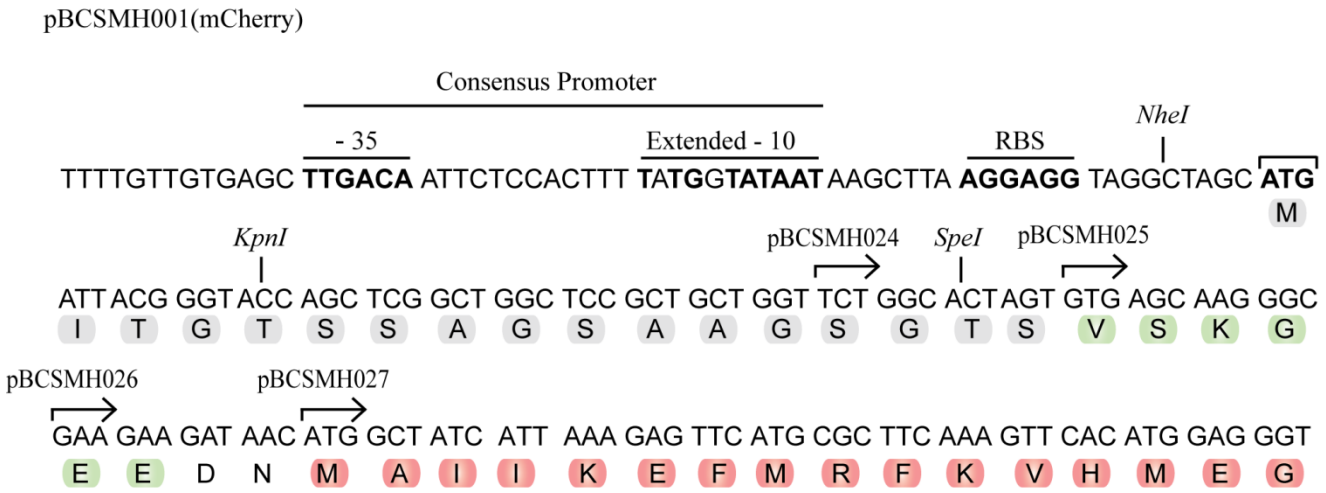

Figure II-3: The plasmids from which the fluorescent proteins are expressed include a linker region upstream of the protein coding sequence. Partial sequence of plasmid pBCSMH001 highlights the linker region (grey), the GFP-like termini region (green) and the encoded mCherry (red). Amino acids between the first methionine and the aminoacids indicated by the arrows were removed to generate plasmids pBCSMH024-027 encoding truncated forms of mCherry.

was still 80% lower than the signal obtained for strain BCSMH006, expressing Wze-mCherry [10]. No increase in the fluorescence signal was observed when the linker region was removed from the Citrine protein (data not shown) indicating that this region was not impairing fluorescence in pneumococcal bacteria.

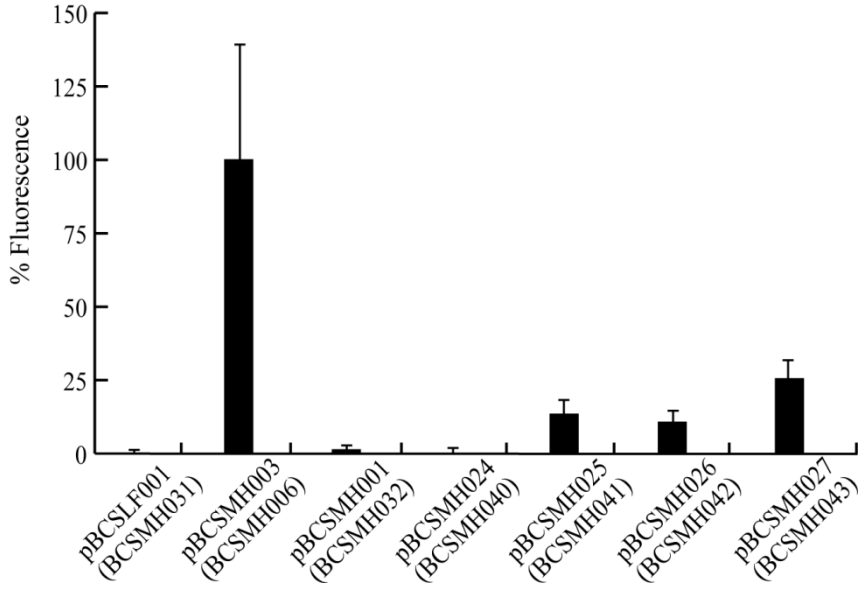

Figure II- 4: The linker region present at the N-terminal of fluorescent proteins does not impair expression of fluorescence. The fluorescence signal obtained from strains expressing truncated mCherry forms (strains BCSMH040 – 043) and mCherry alone (strain BCSMH032) is plotted as percentage relative to the signal from the strain expressing Wze-mCherry (strain BCSMH006).

As a second hypothesis, we asked whether there was a specific nucleotide sequence present in the coding sequence of Wze that increased protein expression in the Wze-Citrine fusion. This was based on the fact that the 5´ coding region of the mRNA, immediately after the start codon, has been proposed to have an important role in ensuring protein translation in bacteria. This effect seems to be more dependent on the predicted mRNA secondary structure of this region than on codon usage

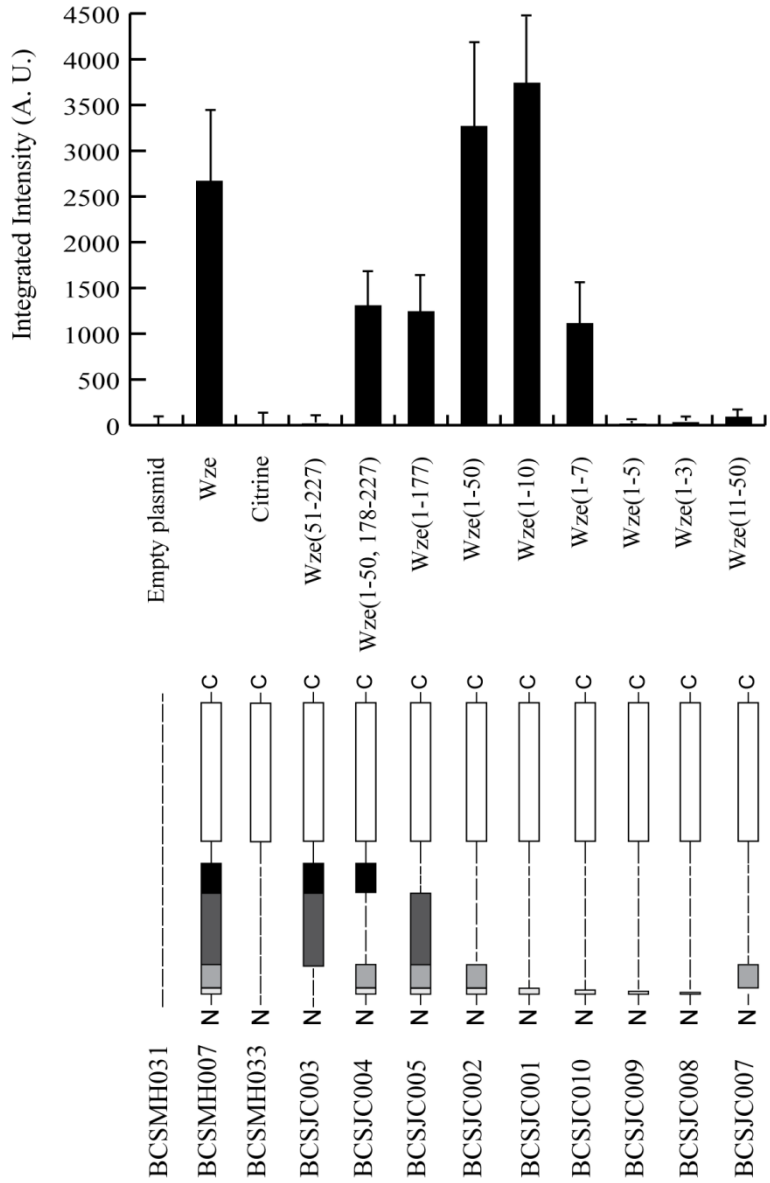

Figure II- 5: The first ten amino acids of Wze are required to obtain high levels of fluorescence. (Upper panel) Fluorescence (in arbitrary units) emitted by Citrine fused to specific aminoacid sequences of Wze, as indicated below the graphic, in *S. pneumoniae* R36A strain. Strain BCSMH031 was used as a negative control. Exposure time was 5000 msec. (Bottom panel) Schematic representation of each Wze construct. The white boxes represent the Citrine protein, while the gray boxes represent different regions of Wze protein: lighter grey - aminoacids 1 to 10, light grey - remaining N-terminus region between

aminoacids 11 to 50, dark grey - central region between aminoacids 51-176, black box - C-terminus region between aminoacids 178-227. Strain names are indicated below.

bias or GC content [13]. In order to identify that putative nucleotide sequence, pneumococcal bacteria were transformed with plasmids encoding different truncated forms of Wze-Citrine fusion (Figure II-5). Deletion of the central region (amino acids 51-177, strain BCSJC004) or the C-terminal region (amino acids 178-227, strain BCSJC005) of Wze resulted in proteins that were still fluorescent, although a decrease of about 40% in the intensity of the fluorescence signal was observed (Figure II-5). However, when the first 50 amino acids of the N-terminal region of Wze were deleted, in strain BCSJC003, the expression of fluorescence was completely lost (Figure II-5).

In order to determine the minimum size of the N-terminal region of Wze required for expression of fluorescence, *S. pneumoniae* R36A strain was transformed with plasmids encoding the first 3, 5, 7, 10 or 50 aminoacids of Wze linked to the N-terminal of Citrine, and analyzed by fluorescence microscopy. Strains BCSJC008 and BCSJC009, expressing the Citrine protein fused to the first 3 or 5 amino acids of Wze, respectively, showed no significant fluorescence (Figure II-5). Strain BCSJC010, which expresses the Citrine protein fused to the first 7 amino acids of Wze, showed a decreased fluorescence relative to the strain expressing Wze-Citrine. However, strains BCSJC001 and BCSJC002, expressing the Citrine protein fused to the first 10 or 50 amino acids of Wze, respectively, showed fluorescence levels similar to, or even higher than, that obtained for strain BCSMH007, expressing the original Wze-Citrine fusion (Figure II-5). Expression of Wze aminoacids 11 to 50 fused to Citrine did not result in a fluorescent protein (Figure II-5), confirming that the first 10 amino acids of Wze were necessary and sufficient for

expression of Citrine in *S. pneumoniae*. We have named this 10 amino acid tag, which improved protein expression in pneumococcal bacteria, "i-tag".

The increased fluorescence due to the presence of the i-tag fused to Citrine could be the result of higher mRNA or higher protein levels. We therefore quantified, by real-time PCR, the levels of mRNA encoding untagged Citrine, Wze-Citrine fusion and the various truncated forms of this fusion, in exponentially growing bacteria, relatively to the mRNA for the tetracycline resistance marker, encoded in the plasmid backbone. Figure II-6 shows that the levels of the different mRNAs were not sufficiently different to explain the observed variability in fluorescence expression.

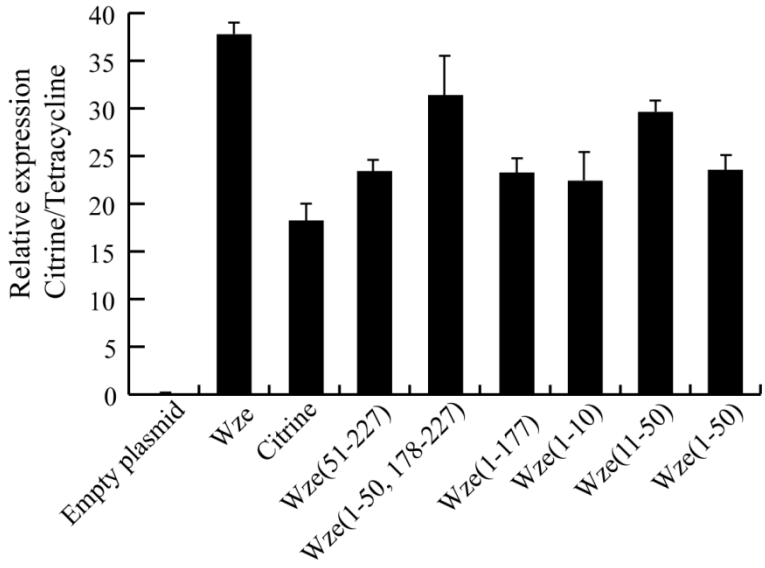

Figure II- 6: mRNA levels are identical in the presence or absence of the "i-tag". mRNA encoding Citrine was quantified by Real-time PCR in strains expressing specific aminoacid sequences from Wze fused to Citrine, relatively to the mRNA for the tetracycline resistance protein which is encoded in the plasmid backbone. Strains analyzed were BCSMH031 (transformed with an empty vector), BCSMH007 (expressing full Wze-Citrine), BCSMH033 (expressing Citrine), BCSJC003 (expressing $Wze_{(51-227)}$-Citrine), BCSJC004 (expressing

$Wze_{(1\text{-}50,\ 178\text{-}227)}$-Citrine), BCSJC005 (expressing $Wze_{(1\text{-}177)}$-Citrine), BCSJC001 (expressing $Wze_{(1\text{-}10)}$-Citrine), BCSJC007 (expressing $Wze_{(11\text{-}50)}$-Citrine) and BCSJC002 (expressing $Wze_{(1\text{-}50)}$-Citrine).

However, analysis of Citrine protein levels in the same strains (Figure II-7) showed a direct correlation between strains in which Citrine protein could be detected and strains which were fluorescent, namely those encoding for Wze-Citrine (strain BCSMH007) and all that included the first 10 amino acids of the Wze fused to Citrine (strains BCSJC001, BCSJC002, BCSJC005 and BCSJC004). Taken together these results

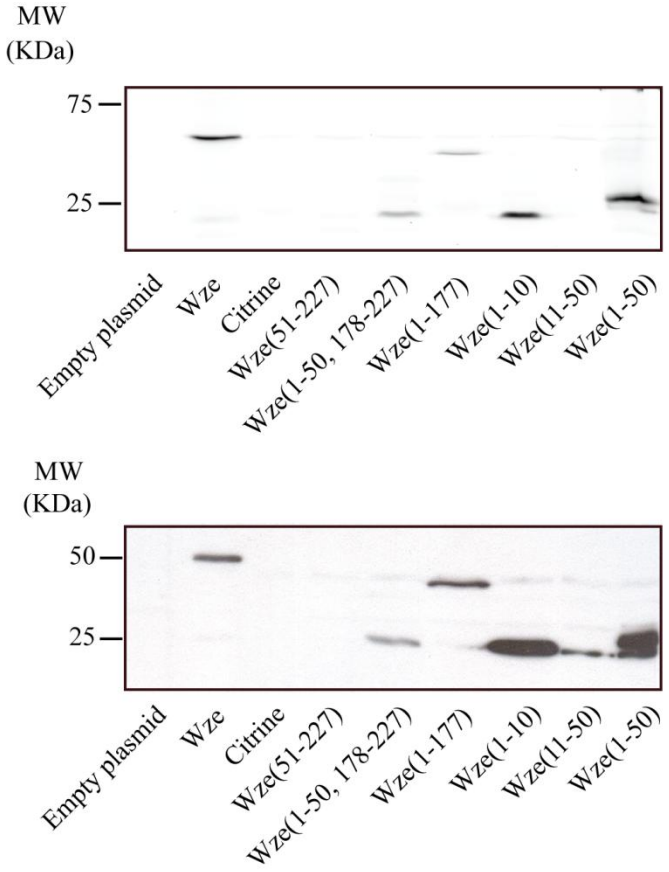

Figure II- 7: Increased fluorescence resulting from the presence of the "i-tag" is due to increased protein levels. Cell extracts from strains expressing Citrine fused to the C-terminus of the indicated Wze fragments were separated by SDS-PAGE and analyzed using a Fluorescent Image Analyzer (Upper panel).

Western-blot using an antibody that recognizes the Citrine protein was performed (Lower panel), and showed that fluorescence in strains containing the i-tag is due to increased protein levels.

show that fusion of the i-tag to Citrine increased fluorescence levels due to increased protein levels and not increased mRNA levels.

To determine if increased protein levels resulted from higher translation rates or increased protein stability, we generated silent mutations in the sequence encoding the i-tag, fused to Citrine, and analyzed the fluorescence of the resulting constructs. We were able to identify mutations that did not alter the amino acid sequence of the tag, but resulted in loss of fluorescence, namely the substitution of the UUA leucine codon by CUC (Figure II-8). Given that the protein sequence was

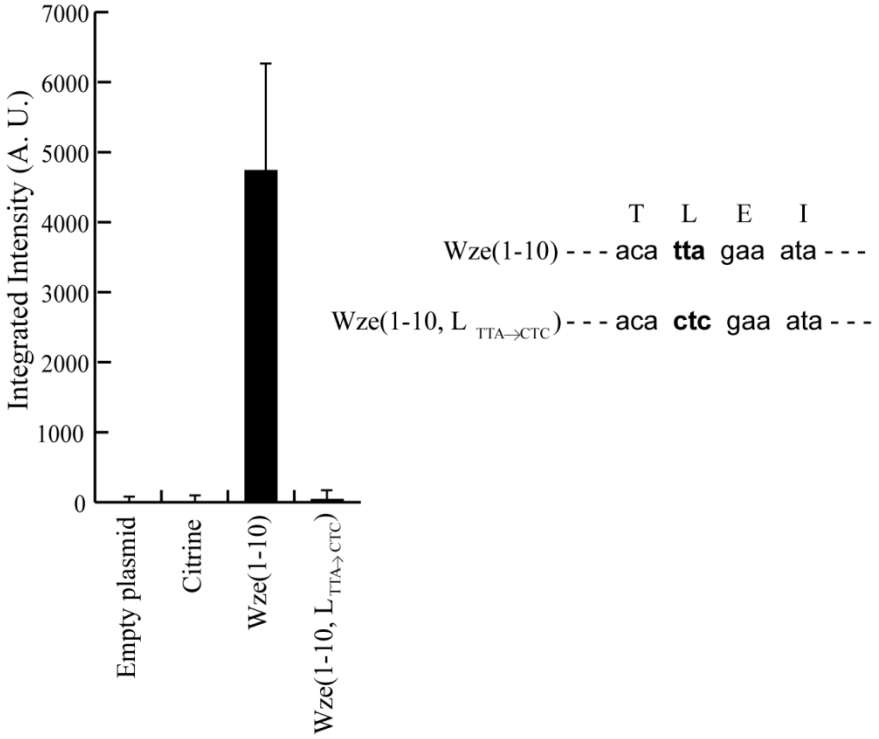

Figure II- 8: The nucleotide sequence of the i-tag determines the expression of fluorescence. (Left panel) Fluorescence (in arbitrary units) emitted by Citrine

from the following strains: BCSMH031 (empty plasmid), BCSMH033 (expressing Citrine), BCSJC001 (expressing $Wze_{(1-10)}$-Citrine), BCSJC006 (expressing $Wze_{(1-10)*}$-Citrine in which the sequence encoding for Leucine was changed from TTA to CTC). (Right panel) Sequence encoding aminoacids 3-6 of Wze present in $Wze_{(1-10)}$-Citrine and $Wze_{(1-10)*}$-Citrine.

not altered, we can rule out the hypothesis that the i-tag acted by increasing the stability of the fusion proteins. Therefore, we favor the hypothesis that the presence of the nucleotide sequence encoding the i-tag results in increased translation rates, possibly by destabilizing the mRNA structure of this region and thus facilitating ribosome binding to the mRNA molecule.

Construction of plasmids for the expression of fluorescent protein fusions in *S. pneumoniae*

We have redesigned plasmids pBCSMH001, pBCSMH002, pBCSMH018 and pBCSMH020, expressing untagged fluorescent proteins

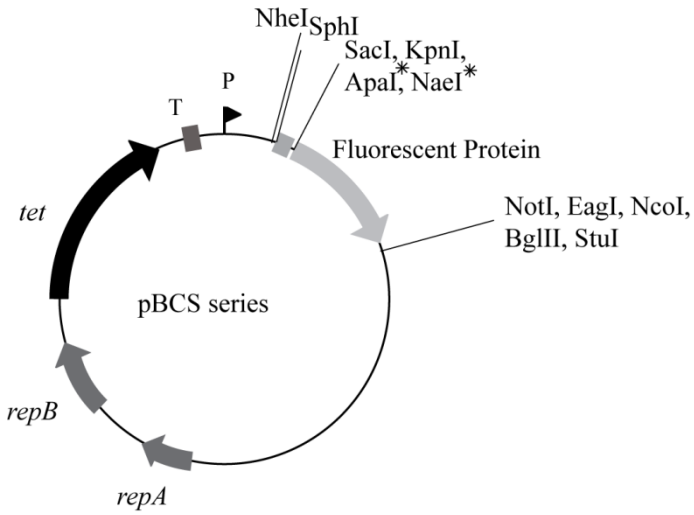

Figure II- 9: **New plasmids for *S. pneumoniae* cell biology studies.** Map of the pBCS plasmids. Fluorescent protein refers to mCherry, Citrine, CFP or sfGFP,

encoded by plasmids pBCSMH030, pBCSJC001, pBCSMH031 and pBCSMH032, respectively. ApaI and NaeI restriction sites, highlighted with an asterisk, are not available in plasmid pBCSMH030. *repA*, *repB*, plasmid replication genes. *tet*, tetracycline resistance marker. T, transcription terminator. P, promoter.

(Figure II-2) to improve expression of mCherry (pBCSMH030), Citrine (pBCSJC001), CFP (pBCSMH031) and sfGFP (pBCSMH032) by including the "i-tag" upstream of the fluorescent proteins (Figure II-9). Additionally, we have introduced unique restriction sites flanking the genes encoding the fluorescent proteins, so that the resulting plasmids can be used to express both N- and C-terminal fluorescent fusions of *S. pneumoniae* proteins, under the control of a constitutive consensus *S. pneumoniae SigA* promoter [15].

Analysis of strains containing these new plasmids showed that expression of i-tagged fluorescent proteins generates cells with fluorescence levels comparable to the ones expressing the Wze-fusions (Figure II-10). Importantly, the i-tagged fluorescent proteins were dispersed throughout the cytoplasm in the encapsulated pneumococcal cells (data not shown) indicating that the i-tag did not interact with Wzd or Wze proteins, normally localized at the dividing septum of encapsulated strains [10].

The developed tools were used to localize different proteins in *S. pneumoniae*, namely FtsZ, a central protein for bacterial division, Wzd, a membrane protein, and Wze, a cytoplasmic tyrosine kinase, both of which localize at the bacterial division septum when expressed together in pneumococcal cells. Figure II-11 shows that fusions of these proteins to the C-terminal of CFP or Citrine only produced a fluorescent signal when the improved versions, containing the "i-tag", of the fluorescent proteins were used, and not with the normal untagged versions.

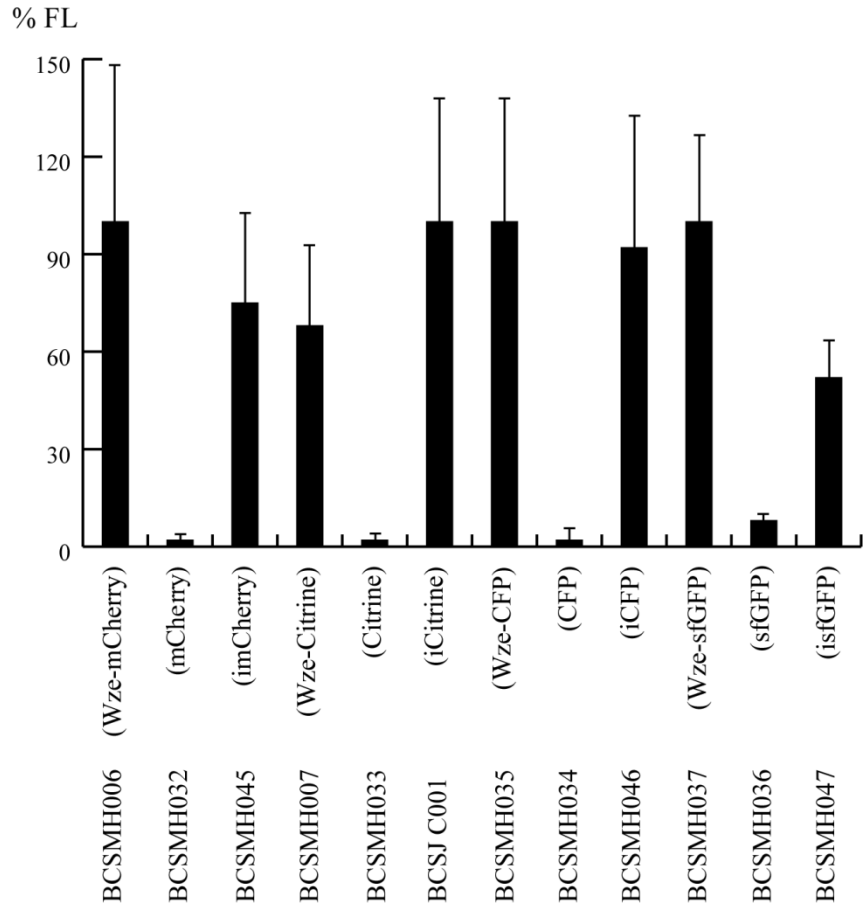

Figure II- 10: New plasmids for *S. pneumoniae* cell biology studies produce high levels of fluorescence. Comparison of fluorescence emitted by strains expressing mCherry, Citrine, CFP and sfGFP alone, their improved i-tag versions and their Wze fusions. Data is presented as a percentage relative to the strain with highest signal for each fluorescent protein. Strain names are indicated at the bottom.

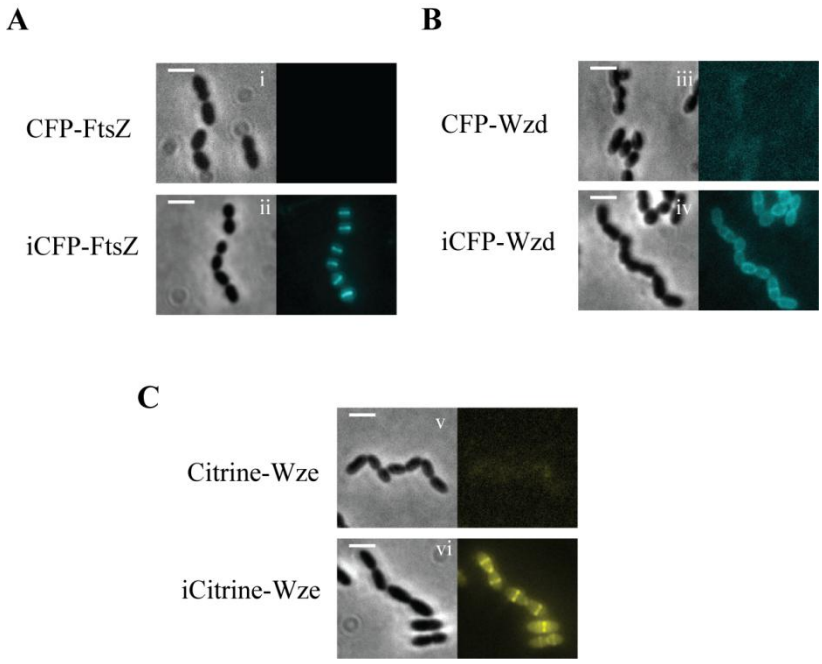

Figure II- 11: Applications of the developed tools for localization of *S. pneumoniae* proteins. (A) Localization of the cell division protein FtsZ as an N-terminal fusion to CFP (CFP-FtsZ, strain BCSMH050) and to i-tagged CFP, (iCFP-FtsZ, strain BCSMH051). (B) Localization of the membrane protein Wzd as an N-terminal fusion to CFP (CFP-Wzd, strain BCSJF004) and to improved i-tagged CFP, (iCFP-Wzd, strain BCSJF003). (C) Localization of the Wze tyrosine kinase as an N-terminal fusion to Citrine (Citrine-Wze, strain BCSJF002) and to improved i-tag Citrine (iCitrine-Wze, BCSJF001). The i-tagged versions of the fluorescent reporters allowed the visualization of each protein at the expected sub-cellular region of bacteria, the division septum. Exposure times: 5000 msec. Scale bar: 2 μm.

Final Remarks

We were able to significantly improve the expression of four fluorescent proteins, mCherry, Citrine, CFP and sfGFP in the Gram-positive bacteria *S. pneumoniae*, by designing a tag that increases translation efficiency of heterologous proteins. The set of plasmids encoding improved versions of these fluorescent proteins allows the expression of both N- and C-terminal fluorescent fusions of pneumococcal proteins and should greatly facilitate cell biology studies in this important pathogen.

Acknowledgements

This study was funded by Fundação para a Ciência e Tecnologia (FCT), Lisbon, Portugal, through research grants PTDC/SAU-MII/75696/2006 and PTDC/BIA- MIC/100747/2008 awarded to S. R. F. The work performed at Instituto de Tecnologia Química e Biológica was supported additionally by FCT through grant # PEst-OE/EQB/LA0004/2011. MXH and MJC were supported by FCT fellowships SFRH/BD/ 43797/2008 and SFRH/BPD/77758/2011, respectively. MXH designed and performed a great part of the experiments described in this chapter and wrote the manuscript.

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Supplementary Information

Table SII-1: Bacterial Strains.

Name	Relevant Characteristics	Reference
ATCC6314	Encapsulated strain, serotype 14.	American Type Culture Collection.
BCSJC001	R36ApBCSJC001, Tet^r.	This work.
BCSJC002	R36ApBCSJC002, Tet^r.	This work.
BCSJC003	R36ApBCSJC003, Tet^r.	This work.
BCSJC004	R36ApBCSJC004, Tet^r.	This work.
BCSJC005	R36ApBCSJC005, Tet^r.	This work.
BCSJC006	R36ApBCSJC006, Tet^r.	This work.
BCSJC007	R36ApBCSJC007, Tet^r.	This work.
BCSJC008	R36ApBCSJC008, Tet^r.	This work.
BCSJC009	R36ApBCSJC009, Tet^r.	This work.
BCSJC010	R36ApBCSJC010, Tet^r.	This work.
BCSJF001	R36ApBCSJF001, Tet^r.	This work.
BCSJF002	R36ApBCSJF002, Tet^r.	This work.
BCSJF003	R36ApBCSJF003, Tet^r.	This work.
BCSJF004	R36ApBCSJF004, Tet^r.	This work.
BCSMC001	ATCC6314Δ*cps*	[1]
BCSMH006	R36ApBCSMH003, Tet^r.	This work.
BCSMH007	R36ApBCSMH004, Tet^r.	This work.
BCSMH015	ATCC6314pBCSMH003, Tet^r.	[10]
BCSMH016	ATCC6314pBCSMH004, Tet^r.	[10]
BCSMH029	ATCC6314pBCSMH019, Tet^r.	This work.
BCSMH030	ATCC6314pBCSMH021, Tet^r.	This work.
BCSMH031	R36ApBCSLF001, Tet^r.	This work.
BCSMH032	R36ApBCSMH001, Tet^r.	This work.

Table SII-1(Cont.): Bacterial Strains.

Name	Relevant Characteristics	Reference
BCSMH033	R36ApBCSMH002, Tet^r.	This work.
BCSMH034	R36ApBCSMH018, Tet^r.	This work.
BCSMH035	R36ApBCSMH019, Tet^r.	This work.
BCSMH036	R36ApBCSMH020, Tet^r.	This work.
BCSMH037	R36ApBCSMH021, Tet^r.	This work.
BCSMH040	R36ApBCSMH024, Tet^r.	This work.
BCSMH041	R36ApBCSMH025, Tet^r.	This work.
BCSMH042	R36ApBCSMH026, Tet^r.	This work.
BCSMH043	R36ApBCSMH027, Tet^r.	This work.
BCSMH045	R36ApBCSMH030, Tet^r.	This work.
BCSMH046	R36ApBCSMH031, Tet^r.	This work.
BCSMH047	R36ApBCSMH032, Tet^r.	This work.
BCSMH050	R36ApBCSMH035, Tet^r.	This work.
BCSMH051	R36ApBCSMH036, Tet^r.	This work.
BCSMH052	BCSMC001pBCSLF001, Tet^r.	This work.
R36A	Non-encapsulated laboratory strain.	[2]

Table SII-2: Plasmids used in this work.

Name	Relevant Characteristics	Reference
S. pneumoniae		
pBCSJC001	pBCSMH004 derivative, allowing expression of Citrine containing the first 10 aa of Wze at its N-terminus, Tet^r.	This work.
pBCSJC002	pBCSMH004 derivative, allowing expression of Citrine containing the first 50 aa of Wze at its N-terminus, Tet^r	This work.

Table SII-2 (Cont.): Plasmids used in this work.

Name	Relevant Characteristics	Reference
pBCSJC003	pBCSMH004 derivative, lacking the N-terminal 147 nucleotides of *wze*, Tet^r.	This work.
pBCSJC004	pBCSMH004 derivative, lacking the central 381 nucleotides of *wze*, Tet^r.	This work.
pBCSJC005	pBCSMH004 derivative, lacking the C-terminal 147 nucleotides of *wze*, Tet^r.	This work.
pBCSJC006	pBCSJC001 derivative, TTA→CTC change in codon 4 of Wze, Tet^r.	This work.
pBCSJC007	pBCSJC002 derivative, lacking the N-terminal 33 nucleotides of Wze, Tet^r.	This work.
pBCSJC008	pBCSJC001 derivative, allowing expression of Citrine containing the first 3 aa of Wze at its N-terminus, Tet^r.	This work.
pBCSJC009	pBCSJC001 derivative, allowing expression of Citrine containing the first 5 aa of Wze at its N-terminus, Tet^r.	This work.
pBCSJC010	pBCSJC001 derivative, allowing expression of Citrine containing the first 7 aa of Wze at its N-terminus, Tet^r.	This work.
pBCSJF001	pBCSJC001 containing i*Citrine-wze*, Tet^r.	This work.
pBCSJF002	pBCSMH002 containing *Citrine-wze*, Tet^r.	This work.

Table SII-2 (Cont.): Plasmids used in this work.

Name	Relevant Characteristics	Reference
pBCSJF003	pBCSMH031, containing i*CFP-wzd*, Tet^r.	This work.
pBCSJF004	pBCSMH018 containing *CFP-wzd*, Tet^r.	This work.
pBCSLF001	High-copy-number vector, contains the -10 constitutive promoter of *SigA* from *S. pneumoniae*, Tet^r.	[10]
pBCSMH001	pBCSLF001 derivative, allows expression of mCherry fusion proteins, Tet^r.	[10]
pBCSMH002	pBCSLF001 derivative, allows expression of Citrine fusion proteins, Tet^r.	[10]
pBCSMH003	pBCSMH001 containing *wze-mCherry*, Tet^r.	[10]
pBCSMH004	pBCSMH002 containing *wze-Citrine*, Tet^r.	[10]
pBCSMH018	pBCSLF001 derivative, allows expression of CFP fusion proteins, Tet^r.	This work.
pBCSMH019	pBCSMH018 containing *wze-cfp*, Tet^r.	This work.
pBCSMH020	pBCSLF001 derivative, allows expression of sfGFP fusion proteins, Tet^r.	This work.
pBCSMH021	pBCSMH018 containing *wze-sfgfp*, Tet^r.	This work.
pBCSMH024	pBCSMH001 derivative, removal of the first ten codons between the ATG and the mCherry sequence Tet^r.	This work.

Table SII-2 (Cont.): Plasmids used in this work.

Name	Relevant Characteristics	Reference
pBCSMH025	pBCSMH001 derivative, removal of the first fourteen codons between the ATG and the mCherry sequence Tet^r.	This work.
pBCSMH026	pBCSMH001 derivative, removal of the first eighteen codons between the ATG and the mCherry sequence Tet^r.	This work.
pBCSMH027	pBCSMH001 derivative, removal of the twenty two codons between the ATG and the mCherry sequence, Tet^r.	This work.
pBCSMH030	pBCSMH001 derivative, expression of mCherry containing the first 10 aa of Wze at its N-terminus, Tet^r.	This work.
pBCSMH031	pBCSMH018 derivative, allowing expression of CFP containing the first 10 aa of Wze at its N-terminus, Tet^r.	This work.
pBCSMH032	pBCSMH020 derivative, allowing expression of sfGFP containing the first 10 aa of Wze at its N-terminus, Tet^r.	This work.
pBCSMH035	pBCSMH018 containing *cfp-ftsz*, Tet^r.	This work.
pBCSMH036	pBCSMH031 containing i*cfp-ftsz*, Tet^r.	This work.
E. coli		
pMUTIN-CFP	Plasmid containing the CFP coding sequence.	[12]
pTrc99A-sfGFP	Plasmid containing the sfGFP coding sequence.	[8]

Table SII-3: Primers used in this work.

Primer	Sequence 5' → 3'	Features/ Restriction sites
1	GGACTAGTGGGCCCGCCGGCATGGTGAGCAAGGGCG	SpeI, ApaI, NaeI
2	GAAGATCTAATCCATGGCATATGAGCGGCCGCCTTGTACAGCTCGTCC	BglII
3	GGATTAGATCTCAGGAATTG	BglII
4	CCCTTGCTCACACTAGTGCC	SpeI
5	CTAGCTAGCATGCCGACATTAGAAATAGCAC	NheI
6	CGGGGTACCTTTTTTACCATAATTTCCATAGGAAGC	KpnI
7	GGACTAGTGGGCCCGCCGGCATGAGTAAAGGAGAAG	SpeI
8	GAAGATCTAATCCATGGCATATGAGCGGCCGCGTCGACTTTGTATAGTTCATCC	BglII
9	GCGGAGCTCTGTTGCTGTTACCAAG	SacI
10	GCGGAGCTCCAGTTTTTTTTGTGCTATTTC	SacI
11	GCGGAGCTCAGTTGTTTTTCCTTCCCCAGG	SacI
12	GGGGTACCCATGCTAGCCTACCTCCTTAAGC	KpnI
13	GGGGTACCAGCTCGGCTGGCTCCGCTGC	KpnI
14	GGGGTACCTCCGCTGCTGGTTCTGGC	KpnI
15	GGGGTACCTCTGGCACTAGTGTGAGCAAGG	KpnI
16	GGGGTACCGTGAGCAAGGGCGAAGAAGATAAC	KpnI
17	GGGGTACCGAAGAAGATAACATGGCTATC	KpnI
18	GGGGTACCATGGCTATCATTAAAGAGTTC	KpnI
19	GCGGAGCTCACTACTTCCGTAAATATAG	SacI
20	GTAGAGCTCCATGCTAGCCTACCTCCTTAAG	SacI
21	GATGAGCTCGGTGAGGCGAATAAACGTGATG	SacI
22	GATGAGCTCTGTTTTTCCTTCCCCAGGG	SacI
23	GATGAGCTCGGTACCAGCTCGGCTG	SacI
24	ATAAGAATGCGGCCGCAATGCCGACATTAGAAATAG	NotI
25	CATGAGATCTTTATTTTTTACCATAATTTCC	BglII
26	CATGCCATGGATGACATTTTCATTTGATAC	NcoI
27	GAAGGCCTTTAACGATTTTTGAAAAATGGAGG	StuI
28	GCGGAGCTCTGTCGGCATGCTAGCCTACCTCC	SacI
29	GCGGAGCTCTTCTAATGTCGGCATGCTAGCC	SacI
30	GCGGAGCTCTGCTATTTCTAATGTCGGCATGC	SacI
31	GCGGCTAGCATGCCGACACTCGAAATAGCAC	NheI

Table SII-3 (Cont.): Primers used in this work.

Primer	Sequence 5' → 3'	Features/ Restriction sites
32	GCGGCTAGCCTACCTCCTTAAGCTTATTATACC	NheI
33	GCGGAGCTCGAGTTCATTAAGAAGGCAG	SacI
34	GCCATTATGACGCTGAAGTGAA	
35	TGCACAGGTTTCTTGGCTTTG	
36	GAGCTGAAGGGCATCGACTT	
37	CTTGTGCCCCAGGATGTTG	
38	AATGGTTGTAGTTGCGCGCTAT	
39	AATGCTTTACCCCTATTTTCCTTTG	
40	CCGGAATTCTTACTTGTACAGCTCGTCCATGC	
41	GGGGTACCATGGTGAGCAAGGGCGAGGAGC	
42	ATTTGCGGCCGCTCATATGATGAAGGAACAAAACAC	NotI
43	CATGCCATGGCTATTTCAACTTACTCAAG	NcoI

Chapter III

Synthesis of capsular polysaccharide at the division septum of *Streptococcus pneumoniae* is dependent on a bacterial tyrosine kinase

The work presented in this chapter was published in:
Henriques, M. X., Rodrigues, T. Carido, M., Ferreira, L. and Filipe, S. R. (2011). "Synthesis of capsular polysaccharide at the division septum of *Streptococcus pneumoniae* is dependent on a bacterial tyrosine kinase." *Mol. Microbiol* 82(2): 515 -534.

Abstract

One of the main virulence factors of the pathogenic bacterium *Streptococcus pneumoniae* is the capsule, present at the bacterial surface, surrounding the entire cell. Virtually all the 90 different capsular serotypes of *S. pneumoniae*, which vary in their chemical composition, express two conserved proteins, Wzd and Wze, which regulate the rate of the synthesis of capsule.

In this work, we show that Wzd, a membrane protein, and Wze, a cytoplasmic tyrosine kinase, localize at the bacterial division septum, when expressed together in pneumococcal cells, without requiring the presence of additional proteins encoded in the capsule operon. The interaction between the two proteins and their consequent septal localization was dependent on a functional ATP binding domain of Wze.

In the absence of either Wzd or Wze, capsule was still produced, linked to the cell surface, but it was absent from the division septum. We propose that Wzd and Wze are spatial regulators of capsular polysaccharide synthesis and, in the presence of ATP, localize at the division site, ensuring that capsule is produced in coordination with cell wall synthesis, resulting in full encapsulation of the pneumococcal cells.

Introduction

Streptococcus pneumoniae is associated with a variety of infections that can range in severity from otitis media to pneumonia or meningitis [22]. This Gram-positive bacterium is a common respiratory pathogen and a frequent cause of community-acquired pneumonia in developed countries. Despite the increased availability of new antibiotics, the mortality rate of patients presenting pneumococcal pneumonia and concurrent septicemia has remained unchanged, with values higher than 20%. In developing countries pneumococcal septicemia is a major cause of infant mortality, causing 25% of all preventable deaths in children under the age of 5 [22].

A capsular polysaccharide (capsule or CPS), surrounds pneumococcal bacteria, forming a thick layer of 200-400 nm [46]. CPS may be linked to bacterial peptidoglycan [45], an heterogeneous polymer, that surrounds and protects bacterial cells, made of glycan chains of β-(1-4)-linked N-acetylglucosamine and N-acetylmuramic acid, and crosslinked by short peptides [47].

The pneumococcal CPS plays a major role in the colonization and persistence of *S. pneumoniae* in the infected host as non-encapsulated mutants of clinical pneumococcal isolates are non-virulent [22]. This is probably due to the ability of the CPS to form a shield that prevents antibodies and complement components, which bind to deeper cell structures such as teichoic-acids and cell-surface proteins, from interacting with their receptors on the host phagocytic cells [37, 49]. The capsule also seems important to reduce the deposition of complement on the bacterial cell surface [1] and the trapping of bacteria in neutrophil extracellular traps [48].

Although more than 90 different pneumococcal capsule serotypes are known, only a limited number seems to be associated with invasive pneumococcal disease [7] and these have been specifically targeted by the pneumococcal capsular polysaccharide conjugate vaccines recently developed [40]. The vaccination programs in different countries seem to have led to a reduction of the total incidence of pneumococcal disease caused by the vaccine serotypes [20]. However, an increase of pneumococcal disease caused by serotypes that are not covered by the different vaccines, has been observed [3], as well as the alteration of the serotypes most frequently found in asymptomatic human carriers [16, 21]. This worrying trend indicates that vaccines effective against most, if not all, serotypes would be of great importance.

The capsular biosynthetic loci of 90 different pneumococcal serotypes have been recently sequenced [10]. In virtually all serotypes, the *cps* operon includes serotype-specific genes, namely those that encode enzymes required for the synthesis of the specific sugar components of each capsular polysaccharide, as well as genes that are conserved among different serotypes (absent only in serotypes 3 and 37). These proteins are involved in the translocation of the capsular polysaccharide repeat unit to the outer surface of the plasma membrane (Wzx), in the polymerization of the capsule (Wzy), leading to the formation of high-molecular weight capsular polysaccharide, or in the regulation of CPS synthesis (Wzg, Wzh, Wzd and Wze, the products of the first four genes of the *cps* operon) [10]. In this work, we focused on the role of two of these proteins widely conserved in the different *S. pneumoniae* serotypes: Wzd and Wze.

Wze (also known as CpsD) is an autophosphorylating tyrosine kinase [35]. Some proteins that belong to the bacterial tyrosine kinase family function as co-polymerases in the biosynthesis of capsular and extracellular polysaccharides [17-18]. These proteins usually possess a

transmembrane domain and an intracellular catalytic domain that, in Gram-negative bacteria, form a single polypeptide. On the contrary, in Gram-positive bacteria, the two domains occur in the form of two distinct polypeptide chains [17-18]. In the case of *S. pneumoniae*, Wzd (also known as CpsC), a membrane protein that belongs to the polysaccharide co-polymerase family [36], constitutes the external domain, and Wze constitutes the catalytic domain. Because members of the bacterial tyrosine kinase family regulate the production and export of bacterial polysaccharides which play essential roles in the initial stages of infection by pathogenic bacteria, these proteins have raised attention as potential novel antibacterial targets [12]. Most important, structural studies have shown that these bacterial tyrosine kinases possess unique features with no equivalent in eukaryotic kinases [38].

As a bacterial tyrosine kinase, Wze possesses the conserved Walker A and B ATP binding motifs and the C-terminal tyrosine rich region, whose phosphorylation seems to regulate the rate of the synthesis of the capsular polysaccharide [35]. The binding of ATP to Wze is necessary for capsular polysaccharide synthesis, as Wze mutants carrying an inactive ATP-binding site ($G_{48}A$, $K_{49}A$) seem to be unable to produce CPS, similarly to a *wze* null mutant [35]. On the other hand, tyrosine phosphorylation of Wze does not seem necessary for capsular polysaccharide production. In fact, *S. pneumoniae* expressing mutant forms of Wze, where the C-terminal tyrosines have been replaced by phenylalanines, making the protein unable to be phosphorylated, can still produce CPS [35]. More recently, an additional role of the phosphorylated Wze in the attachment of CPS to the pneumococcal cell wall, required for pneumococcal invasive disease, has been proposed [34].

Wzd is essential for the synthesis of CPS as no significant amounts of capsule, determined by colony phenotype or reaction with

specific polyclonal antiserum, were found in *wzd* deletion mutants [8, 35]. Wzd is thought to be involved in the regulation of the synthesis of the bacterial capsule through the promotion of the phosphorylation of the cytoplasmic Wze [9].

The current model for capsule synthesis states that Wzd and Wze interact in the presence of ATP to promote CPS synthesis. When Wzd induces Wze autophosphorylation at its C-terminal tyrosines, a reduction of the rate of CPS synthesis occurs, probably due to changes in interactions with Wzy (the capsule polymerase). This allows the CPS to be linked to the peptidoglycan by an unknown ligase. Finally, dephosphorylation of Wze enables the cycle to be repeated [22].

In this chapter we report a previously uncharacterized role of Wzd and Wze in the coordination of the synthesis of the CPS with pneumococcal cell division, by showing that these two proteins localize at the division septum and that this localization is required for the presence of CPS at the septum. Pneumococcal Wzd and Wze null mutants were able to produce a capsule attached to the cell surface but this polysaccharide was absent from the division septum. We therefore propose that Wzd/Wze proteins may act as spatial regulators of capsule metabolism, ensuring the CPS is produced synchronously with the synthesis of peptidoglycan at the division septum.

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Tables SIII-1 and SIII-2, respectively, in Supplementary Information. *E. coli* was routinely grown in LB medium at 37ºC unless otherwise indicated. When needed, antibiotics were used at the following concentrations: ampicillin 100 μg/ml, kanamycin 50 μg/ml and erythromycin 100 μg/ml. Isopropyl-β-D-thiogalactopyranoside (IPTG, Apollo Scientific Ltd) was used at 0.5 mM and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, Apollo Scientific Ltd) at 40 μg/ml. *S. pneumoniae* was grown in C + Y liquid medium [26] at 37ºC, without aeration, or in tryptic soy agar (TSA, Difco) plates supplemented with 5% sheep blood. Tetracycline (Sigma-Aldrich) and erythromycin (Apollo Scientific Ltd) were added to the media when appropriate to make a final concentration of 1 and 0.25 μg/ml, respectively. For white/blue colony selection, X-Gal was used at 120 μg/ml. *Lactococcus lactis* was grown in M17 broth (Difco), supplemented with glucose (0.5% w/v), at 30ºC. Erythromycin was used at 100 μg/ml.

DNA manipulation procedures

E. coli competent cells were prepared and transformed as described [41]. *S. pneumoniae* competent cells preparation and transformation was executed as previously described [32]. PCR products and plasmid DNA were purified with kits Wizard® SV Gel and PCR Clean-up System and Wizard® Plus SV Minipreps, respectively (Promega). PCR fragments were amplified using Phusion High-Fidelity DNA polymerase (Finnzymes). Restriction enzymes were from New

England Biolabs. Primers used in this study are listed in Table SIII-3 in Supplementary Information.

Construction of null mutants and substitution of capsule wild-type genes for fluorescent derivatives

In order to delete the entire *cps* gene cluster from the chromosome of *S. pneumoniae* ATCC6314 strain, plasmid pORI280 was used [25]. Fragments of DNA corresponding to the upstream and downstream regions of the *cps* operon were amplified with primers 1 and 2 and primers 3 and 4, respectively. Primers 2 and 3 have a 23 base pair overlapping region. After this first amplification, the two fragments were joined by overlapping PCR using primers 1 and 4. The product of this reaction was restricted with *Bam*HI and *Pst*I, cloned into pORI280, resulting plasmid pBCSMC005, and transformed to *E. coli* strain EC101. The encapsulated ATCC6314 *S. pneumoniae* strain (serotype 14) was transformed with pBCSMC005. The *cps* null-mutant, strain BCSMC001, was obtained by excision of the plasmid as previously described [25].

Construction of *wzd* and *wze* null-mutants (strains BCSMH001 and BCSMH002, respectively) was done in the same way, using primers 5 to 8 and 9 to 12, respectively. These primer pairs were used to clone the upstream and downstream regions of *wzd* and *wze*, resulting in plasmids pBCSMH012 and pBCSMH013, respectively. In the construction of these mutants ~50 bp of the 3' end of the deleted gene were left intact, to guarantee that the expression of the following gene on the operon was not disturbed.

Plasmid pORI280 was also used to substitute *wzd* and *wze* in the *S. pneumoniae* genome by genes encoding the correspondent Citrine fluorescent derivatives. For the construction of strain BCSMH003 (ATCC6314*wzd*::*wzd_citrine*), primers 13 and 14 were used to amplify

the 3'-part of *wzd* and the entire *citrine* coding sequence, using plasmid pBCSMH007 as template DNA. Primers 15 and 16 were used to amplify the downstream region of *wzd*, using ATCC6314 chromosomal DNA as template. Primers 14 and 15 possess an overlapping region, allowing the above fragments to be joined by overlap PCR. The resulting fragment was restricted with enzymes *BamHI* and *EcoRI* and cloned in pORI280, resulting in pBCSMH010. For the construction of strain BCSMH004 (ATCC6314*wze*::*wze_citrine*), a fragment comprising the 3'-part of *wze* and the entire *citrine* sequence was amplified with primers 17 and 18 from plasmid pBCSMH004. The downstream region of *wze* was amplified with primers 11 and 19, from the chromosomal DNA of strain ATCC6314. Primers 11 and 18 contain an overlapping region so that the two fragments could be joined by overlap PCR. The resulting fragment was restricted with *BamHI* and *EcoRI* and cloned in pORI280, giving pBCSMH011. As for the construction of the null mutants, a duplicated fragment of ~50 bp of the 3' end of each gene was left on the chromosome, after the *citrine* sequence, so that the expression of the following gene on the operon was maintained at normal levels.

Plasmids were routinely propagated in *E. coli* EC101 or *Lactococcus lactis* LL108 and purified before being transformed into *S. pneumoniae* bacteria.

Construction of plasmids for protein expression in *S. pneumoniae*

Plasmid pBCSLF001 was constructed by amplification of plasmid pLS578 with primers 20 and 21, and subsequent auto ligation. This eliminated the CAT reporter gene, which encodes a chloramphenicol acetyltransferase, from pLS578 and added *Nhe*I, *Nco*I and *Bgl*II restriction sites.

The mCherry coding sequence was amplified from plasmid pROD17 with primers 22 and 23 and cloned in plasmid pBCSLF001. In

this process, *Kpn*I and *Not*I restriction sites were added upstream and downstream of the mCherry sequence, respectively, and plasmid pBCSLF002 was obtained. This plasmid was again amplified with primers 24 and 25, restricted with *Spe*I and ligated. This procedure mutated two guanines nucleotides to adenine, eliminating an internal ribosome binding site present on the 5' end of the mCherry sequence. The resulting plasmid was named pBCSMH001. The Citrine gene was amplified from plasmid pcDNA3_Citrine with primers 28 and 29 and ligated to the product of amplification of pBCSMH001 with primers 26 and 27, producing plasmid pBCMH002. The coding sequence for Wze was amplified with primers 30 and 31 and cloned in pBCSMH001, upstream of the mCherry sequence, giving plasmid pBCSMH003. Cloning of the same DNA in pBCSMH002 produced pBCSMH004. In order to mutate the Walker A ATP binding motif of *wze*, the gene was amplified in two different fragments with primer pairs 30 and 32 and 33 and 31. The two fragments were joined by overlapping PCR using primers 30 and 31 and the resulting product was cloned in pBCSMH001, producing plasmid pBCSMH005. Primers 32 and 33 encode for the following mutations: $G_{48}\rightarrow A$; $K_{49}\rightarrow A$. A second mutation of the Walker A ATP motif was done by the same procedure, using primers 46 and 47 instead of primers 32 and 33, respectively. The resulting plasmid is pBCSMH015, in which Wze carries a $K_{49}M$ mutation. Substitution of the four tyrosines (residues 218, 221, 224 and 227) for phenylalanines on the C-terminal tyrosine cluster of Wze was achieved through a single PCR step with primers 30 and 34. Cloning of this DNA fragment in pBCSMH001 produced pBCSMH006.

The *wzd* gene was amplified with primers 35 and 36, and cloned on plasmid pBCSMH002 generating the plasmid pBCSMH007.

In order to co-express the Wze and Wzd fluorescent derivatives in the same cell, plasmid pBCSMH008 was constructed. This

was done by amplification of the *wzd_citrine* sequence from plasmid pBCSMH007 with primers 37 and 38, and cloning on pBCSMH003. Plasmid pBCSMH009 was also constructed, through amplification of *wzd* with primers 37 and 39, restriction and ligation into pBCSMH003, to allow co-expression of Wze-mCherry and untagged Wzd.

Plasmids for the constitutive expression of untagged Wzd and Wze were also constructed. For this purpose, plasmids pBCSMH007 and pBCSMH004 were used as templates in a PCR reaction with primers 26 and 48 and 26 and 49, respectively. The resulting DNA fragments were restricted with BglII and autoligated, giving plasmids pBCSMH016 (Wzd expression) and pBCSMH017 (Wze expression).

In order to determine the localization of Wzd linked to Wze, fragments corresponding to both genes were amplified with primers 35 and 40 and 41 and 31, respectively, and joined by overlap PCR with primers 35 and 31. The resulting fragment that encode for Wzd linked to Wze through the linker NH_2-AERGSVKTIKG-COOH was cloned into pBCSMH002 and originating the plasmid pBCSTR001. The same procedure was done for the Wze(WA) mutant, giving plasmid pBCSTR002.

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

Bacterial Two-Hybrid

To test *in vitro* the interaction between proteins Wzd and Wze, a Bacterial Two-Hybrid activity assay was employed. Plasmid pBCSMC001, encoding the fusion protein T25-Wzd, was constructed by cloning the DNA fragment amplified with primers 42 and 43 from ATCC6314 chromosomal DNA into plasmid pKT25. Plasmid pBCSMC002, encoding the fusion protein T18-Wze, was constructed by

cloning the DNA fragment amplified with primers 44 and 45 from ATCC6314 chromosomal DNA into plasmid pUT18C. Cloning of the *wze* mutated forms in plasmid pUT18C was done using primers 44 and 45 and plasmids pBCSMH005, pBCSMH015 and pBCSMH006 as templates. The resulting plasmids are pBCSMC003 (encoding T18-Wze(WA)), pBCSMH014 (encoding T18-Wze(WA2)) and pBCSMC004 (encoding T18-Wze(Y)). The nucleotide sequences of the modified regions of the constructed plasmids were confirmed by sequencing.

Interactions between proteins Wzd and Wze were first tested by transformation of BTH101 cells with the constructed bacterial two-hybrid plasmids. Plates were incubated at 30ºC and screened for blue/white colonies, in which blue indicated a positive interaction. Single colonies were grown at 30ºC in the presence of 0.5 mM of IPTG and the interactions confirmed by β-galactosidase activity measurements, as described [23].

Microscopy

S. pneumoniae strains were grown until early exponential phase and observed by fluorescence microscopy on a thin layer of 1% agarose in PreC medium [26]. Membranes were labeled with Nile Red (10 μg/ml, Molecular Probes) and DNA with Hoechst 33342 (0.2 μg/ml, Molecular Probes). To determine the site of cell wall synthesis, cells were labeled with a 1:1 mixture of vancomycin (Sigma) and the fluorescent BODIPY FL conjugate of vancomycin (VanFL; Molecular Probes) at a final concentration of 0.6 μg/ml, during 5 minutes on ice. Images were obtained using a Zeiss Axio Observer. Z1 microscope equipped with a Photometrics CoolSNAP HQ2 camera (Roper Scientific). After acquisition, these images were analyzed, adjusted and cropped using Metamorph software (Meta Imaging series 7.5) and Image J software [2].

Quellung reactions were performed using 1 ml aliquots of liquid cultures (OD_{600}~0.5). Cells were washed 3 × with fresh C + Y medium at 37ºC and resuspended in a final volume of 50 µl of C + Y medium. A volume of 2 µl of this suspension was mixed with 2 µl of CPS14 pneumococcal antisera (SSI Diagnostica) and the resulting reaction was observed under the microscope.

In vivo detection of the capsule present at the surface of *S. pneumoniae* cells was achieved using an antibody specific for the capsular polysaccharide of serotype 14 (SSI Diagnostica), purified against the nonencapsulated strain BCSMC001, as previously described [50]. Briefly, 300 ml of a culture of the null-mutant strain at OD ~0.3 was heat-killed during 45 min at 56ºC, followed by centrifugation and washing with PBS. Cells were suspended in 300 µl of Anti-CPS14 diluted 1/100 in PBS and incubated o/n at 4ºC with gentle agitation. On the next day, the sample was centrifuged, the supernatant filtered (0.2 µm pore, Millipore) and stored at 4ºC until further use. Immunofluorescence of live pneumo cells was done by incubating, on ice during 5 min, 1 ml aliquots of early exponential phase cultures with 50 µl of purified antibody. Samples were washed 2 × with C + Y at 37ºC, and incubated with 0.4 µg/ml of Anti-rabbit Alexa Fluor 488 (Invitrogen), on ice during 5 min. Cells were washed twice with fresh C + Y medium, suspended in 50 µl and 2 – 3 µl loaded on PreC + 1% agarose slides.

Fluorescence ratio (FR) calculation was done similarly to a previously described method [5, 39]. FR was determined by quantifying the fluorescence at the septum of cells initiating division (cells that belong to class III as defined in figure III-2) divided by the average of the fluorescence at the lateral cell wall of the two future daughter cells. Average background fluorescence was subtracted from every value. Quantification was performed for at least 100 cells of each strain.

Fluorescent protein analysis

For the preparation of *S. pneumoniae* total cell extracts, cells were harvested from mid-exponential phase cultures, ressuspended in PBS and disrupted in a FastPrep FP120 bead beater (Thermo Electron Corporation). The cell breakage program used was 3 cycles of 90 seconds at 6.0 rpm, followed by 5 min intervals of incubation on ice. Total cell extracts were obtained after centrifugation at 14 000 rpm, during 20 min at 4ºC (Eppendorf 5430R), to remove unbroken cells and isoluble material. The BCA Protein Assay Kit from Pierce (Thermo Scientific) was used to quantify the total amount of protein.

For the separation of the membrane and cytoplasmic fractions, total cell extracts of uncapsulated bacteria constitutively expressing Wzd-Citrine (strain BCSMH008) and Wze-Citrine (strain BCSMH007) were prepared as described above. These total cell extracts were then ultracentrifuged for 20 min at 20 000 rpm (Beckman TL 100). The supernatants resulting from this centrifugation were ultracentrifuged again, this time at 80 000 rpm during 45 min. The pellet obtained in this step contained membrane bound proteins, such as Wzd-Citrine, while the supernatant contained Wze-Citrine and other cytoplasmic proteins.

Protein samples were incubated with solubilization buffer (200 mM Tris_HCl pH8.8, 20% glycerol, 5 mM EDTA pH 8.0, 0.02% bromophenol blue, 4% SDS, 0.05 M DTT) [14] at 37ºC during 5 minutes and separated on SDS-PAGE gels. Gel images were acquired on a Fuji FLA 5100 laser scanner (Fuji Photo Film Co.) with 635 nm excitation and >665 nm band pass emission filter for protein molecular weight marker detection, 532 nm excitation and > 575 nm band pass emission filter for mCherry fusions detection and 473 nm excitation and > 510 nm band pass emission filter for Citrine fusions detection.

Western-Blot

Bacterial cell lysates of 1 ml of culture (OD_{600}~ 0.3) were harvested and boiled during 3 minutes before being separated on a 10% SDS-PAGE gel. Proteins were transferred into a Hybond PVDF Membrane (Amersham) and probed with mouse anti-phosphotyrosine monoclonal antibody cocktail clone 4G10® Platinum (Millipore) used at 1:2000, followed by 1:5000 of goat anti-mouse antibody conjugated to horseradish peroxidase (Amersham). Detection was done with ECL Plus™ Western Blotting Detection Reagents (Amersham). For the detection of capsule proteins fluorescent derivatives, the cultures were harvested at OD_{600}~0.5. Proteins were transferred and detected as above. Probing was done with Living Colors® Av. Peptide Antibody (Clontech) used at 1:500, followed by 1:100000 of goat anti-rabbit antibody conjugated to horseradish peroxidase.

Dot-blot

Cell samples were prepared by harvesting cells at early exponential growth-phase (OD_{600}~0.3), and resuspending them in water. After adjusting the samples to the same cell density, cells were lysed with deoxycholate and boiled for 3 minutes before use. Samples of purified cell walls and peptidoglycan were prepared as previously described [43]. Briefly, cells were boiled into sodium dodecyl sulfate (SDS, final concentration, 4%) 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 FastPrep FP120 apparatus. Cell walls were digested with Dnase and Rnase (for 3h at 37ºC), and trypsin (overnight at 37ºC), which were inactivated by boiling in 1% (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. For purification of peptidoglycan, the cell walls (5 mg) were treated with 2 ml of 49% hydrofluoric acid (HF) for 48h at 4°C, which is known to cleave phosphodiester bonds and thus remove teichoic acids and polysaccharides covalently bound to the bacterial peptidoglycan. The peptidoglycan (PGN) was recovered and washed as previously described [42].

In order to load in the dot-bot similar amounts of cell walls and peptidoglycan, the content of muramic acid was determined in each purified sample, by fluorescence HPLC analysis after an hydrolysis process, as described [4]. 20 µl of peptidoglycan (10 mg/ml) and 50 µl of cell wall (20 mg/ml) were mixed with 37.25 µl of 12 M HCL and incubated at 95ºC for 2 hours. The mixture was lyophilized, washed with 0.5 ml of water, lyophilized again and the final pellet ressuspended in 100 µl of water. 20 µl of sample were mixed with 80 µl of OPA reagent, which was prepared by dissolving 68 mg of o-phtaldialdehyde (Fluka) in 50 µl of 2-mercaptoethanol and 1.4 ml methanol, followed by completion of the volume to 10 ml with borate buffer (0.4 M H_3BO_3, pH 10.4). Samples were injected onto Hypersil ODS column C18 (Thermo Electron Corporation). The flow rate rate of the mobile phase (0.05 M sodium citrate, 0.05 M sodium acetate, methanol and tetrahydrofuran at a ratio of 90: 8.5: 0.75: 0.75 in vol %) was 1.5 ml/min at 35ºC for 80 min, followed by a 20 min gradient from 0 to 80% of a cleaning buffer (water and methanol at a ratio of 50: 50 in vol %) and 20 min mobile phase again for re-conditioning of the column. Fluorometric emission of the amino sugar derivatives was measured at a wavelength of 445 nm with 340 nm as the excitation wavelength (Shimadzu RF-10Axl).

Samples were loaded into Hybond PVDF (Amersham) membranes, pre-equilibrated in PBS and placed on top of PBS-soaked

Hybond Blotting Paper. The membranes were allowed to air-dry for 30 minutes and then blocked during 1 hour in Blocking Buffer (5% non-fat dried milk in PBS). Membranes were washed in PBS-T (PBS + 0.05% Tween 20) and incubated overnight at 4ºC with primary antibodies Anti-CPS14, purified as described above, and Anti-CWPS (SSI Diagnostica) diluted 1/1000 in PBS-T. The latter serum, Anti-CWPS, was used to verify that the samples were retained in the membrane of the dot-blot, as it recognizes the phosphorylcholine residues of the Cell Wall Polysaccharide, or pneumococcal teichoic acids. After washing with PBS-T, membranes were incubated during 1 hour at room temperature with secondary antibody Anti-Rabbit IgG peroxidase linked diluted 1/100000 in PBS-T. Membranes were again washed with PBS-T and detected using the ECL Plus™ Western Blotting Detection Reagents (Amersham).

Results

Membrane-linked Wzd and cytoplasmic Wze are found at the division septa of encapsulated *Streptococcus pneumoniae* bacteria

The localization of the capsule synthetic machinery in *S. pneumoniae* has not been previously studied. In order to determine the localization of Wzd and Wze in the pneumococcal cell we have constructed fluorescent derivatives of these proteins in which their C-terminal end was fused to the fluorescent protein Citrine. Citrine is a variant of the Green Fluorescent Protein that fluoresces in the yellow range of the light spectrum and that has increased photostability and resistance to fluctuations of the environmental pH [19]. Genes encoding for Wzd-Citrine and Wze-Citrine proteins were individually introduced into the *cps* operon of the encapsulated ATCC6314 strain, substituting the native *wzd* and *wze* genes, in strains BCSMH003 and BCSMH004, respectively.

Wzd-Citrine and Wze-Citrine proteins were found at the division septa of pneumococcal bacteria and had similar localization patterns at different stages of the cell cycle (Figure III-1), suggesting that the recruitment of Wzd and Wze to the division septum occurs in a similar manner.

To determine the temporal window of the pneumococcal cell cycle during which the localization of Wze takes place at the septum, we labeled an exponentially growing culture of strain BCSMH004, expressing Wze-Citrine, with DAPI, a fluorescent stain that labels DNA, and with Nile-Red, a dye that becomes fluorescent when incorporated into bacterial membranes. Using these dyes we were able to localize bacterial

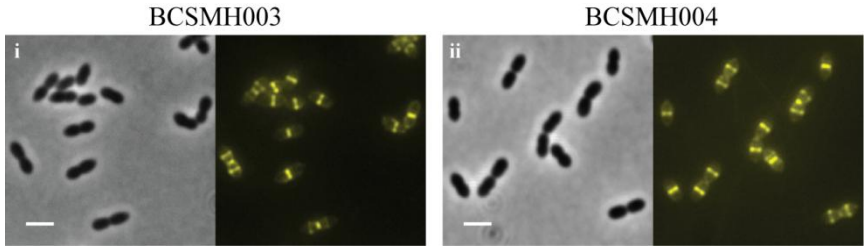

Figure III-1: Localization of proteins Wzd and Wze in encapsulated pneumococcal cells. Fluorescence microscopy images of strains i) BCSMH003 and ii) BCSMH004, expressing Wzd and Wze Citrine fluorescent derivatives, respectively, from their native promoter. Scale bar, 2 µm.

chromosomes and evaluate the stage of membrane invagination during the formation of the division septum. We then assigned labeled bacteria to five different classes, according to the progression of the cell cycle, from newborn bacteria with only one chromosome and no membrane at the division septum (class I, Figure III-2) to bacteria that have nearly completed division, with fully segregated chromosomes and with a clear invagination of the division septum (class V, Figure III-2). The same cells were also classified according to the localization of fluorescent Wze-Citrine protein (classes A to E, Figure III-2). This analysis showed that in newborn bacteria, even before the onset of septum invagination (classes I and II), Wze is already localized at mid-cell, seen as two spots that indicate the formation of a ring (classes A and B). Later on the pneumococcal cell cycle, when a membrane septum is being formed and chromosome segregation has proceeded (class III), Wze-Citrine is observed as a line consistent with septal localization (class C). In a small percentage of cells (1.6 %, n=1254), we could observe that fluorescence was spread between the old and the new septa (class D). This is consistent with a migration of the Wze protein between the old and new septa along the membrane. Additionally, in a small percentage of the cells (2.1 %,

n=1254), Wze is already found at the mid-cell of the newly born cells while still present at the old division septum (class E).

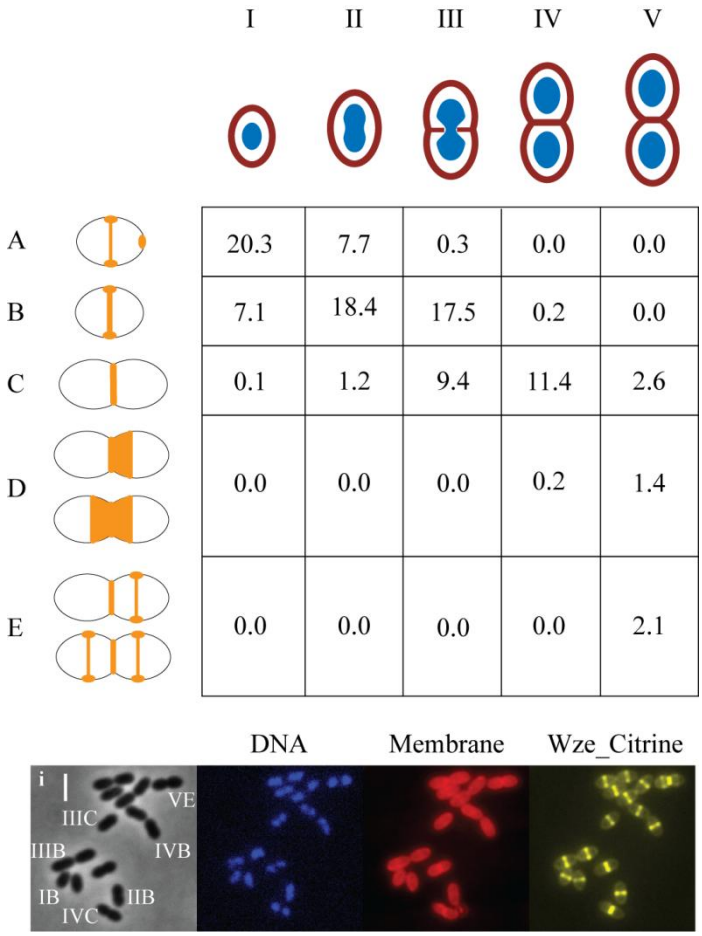

Figure III-2: Classification of encapsulated pneumococcal cells expressing Wze-Citrine according to cell division stage. Classification of BCSMH004 pneumococcal cells (n=1254) according to cell division stage (I – V) and Wze-Citrine localization (A – E). Representatives of the classes are shown in i). DNA was labeled with DAPI and membranes with Nile Red. Scale bar 2 µm.

To test for the functionality of Wzd and Wze fluorescent derivatives expressed by BCSMH003 and BCSMH004, respectively, we performed a Quellung reaction. For that purpose, these strains were incubated with rabbit-serum raised against pneumococcal capsular

polysaccharide serotype 14, which resulted in a positive reaction of agglutination. Agglutination reactions were also observed with the parental encapsulated ATCC6314 strain, with the Wzd null mutant expressing constitutively untagged Wzd (strain BCSMH025) and with the Wze null mutant expressing constitutively untagged Wze (strain BCSMH026) (Supplementary Figure SIII-1). This indicated that both Wzd-Citrine and Wze-Citrine protein fusions were functional, as Wzd and Wze null mutants (strains BCSMH001 and BCSMH002 respectively) seemed impaired in their ability to produce capsule as they failed to agglutinate (Supplementary Figure SIII-1).

We further confirmed that the fluorescent derivatives of Wzd and Wze were functional by looking for phosphorylated Wze. It was previously shown that Wzd is required for Wze autophosphorylation at its C-terminal tyrosines [9]. Therefore, if the Wze fluorescent derivative retained its ability to autophosphorylate, and the Wzd fluorescent derivative its capacity to induce Wze autophosphorylation, this would further indicate the protein fusions were functional. We checked for the presence of the phosphorylated Wze fluorescence derivatives in unencapsulated strains BCSMH006 and BCSMH011, expressing only Wze-mCherry, and in strains BCSMH009 and BCSMH014, expressing Wze-mCherry together with Wzd-Citrine, by western-blot using an antibody against phosphorylated tyrosines. Wze-mCherry was phosphorylated when Wzd-Citrine was also expressed but not in its absence (Supplementary Figure SIII-2A), indicating that both fusions were functional. We did not detect additional bands that could result from phosphorylated untagged Wze, originated from cleavage of the fusion proteins. The phosphorylated Wze protein was only observed in the encapsulated ATCC6314 control strain (Supplementary Figure SIII-2A).

Importantly for experiments described below, we observed that expressing Wzd-Citrine or Wze-Citrine in the encapsulated strains BCSMH019 and BCSMH016, respectively, under the control of a constitutive promoter, also resulted in a fluorescent signal localized at the division septum (see below). Expression of Wze-Citrine fluorescent protein from the replicative plasmid pBCSMH004 in strain BCSMH016 occurred at similar levels as when this protein was produced from the coding sequence inserted into the chromosome, under the control of the chromosomal *cps* promoter, in strain BCSMH004 (Supplementary Figure SIII-3A). The possibility that Wzd and Wze fluorescent derivatives could be being cleaved, which would result in functional and untagged Wzd, and Wze proteins was ruled out because BCSMH004 and BCSMH003 strains produced fluorescent proteins of the expected molecular weight of the fusion proteins. This was determined by western blotting with a specific antibody against the fluorescent domain of the protein fusions (Supplementary Figure SIII-3B).

Co-expression of Wzd and Wze is sufficient for septal localization of both proteins

Having determined that fluorescent derivatives of Wzd and Wze localized at the division septum of encapsulated pneumococcal bacteria, we then asked whether this localization was dependent on the ability of bacteria to produce a capsule. To answer this question, plasmids pBCSMH007 and pBCSMH004 were transformed into unencapsulated strains R36A (unencapsulated laboratory strain) and BCSMC001 (unencapsulated strain derived from ATCC6314, in which the entire *cps* operon was deleted). Constitutive expression of either Wzd-Citrine in unencapsulated BCSMH008 and BCSMH013 strains or Wze-Citrine in

unencapsulated BCSMH007 and BCSMH012 strains, did not result in localization of the fluorescent signal at the division septum (Figure III-3): Wzd-Citrine was distributed all over the membrane and Wze-Citrine was dispersed throughout the entire cytoplasm.

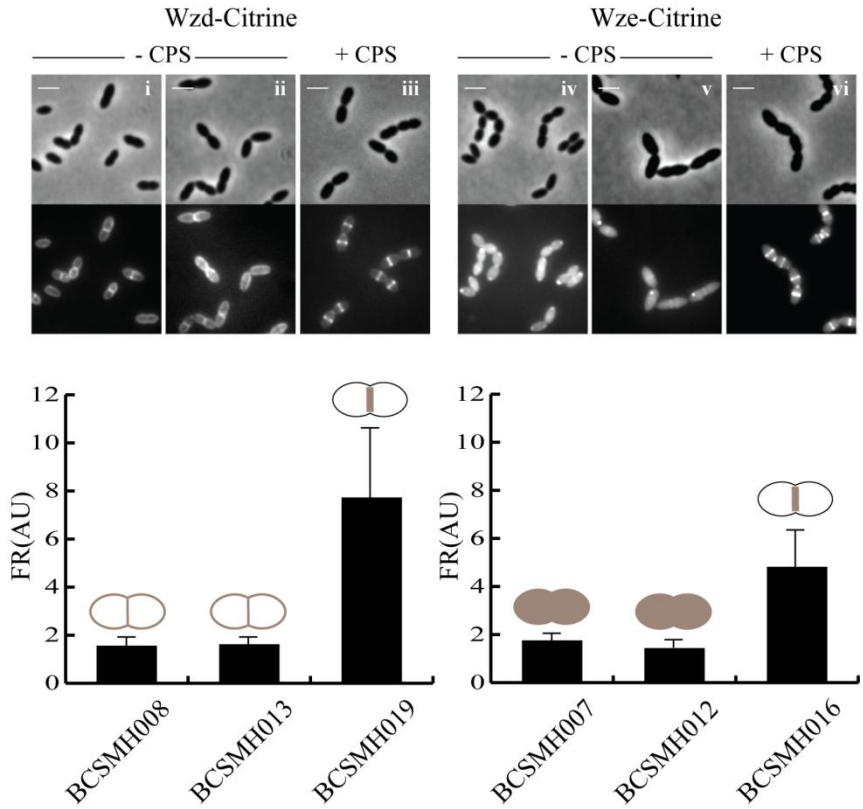

Figure III-3: Localization of Wzd and Wze fluorescent derivatives in different pneumococcal cells. Microscopy images of *S. pneumoniae* strains expressing constitutively Wzd-Citrine (strains i) BCSMH008, ii) BCSMH013 and iii) BCSMH019) or Wze-Citrine (strains iv) BCSMH007, v) BCSMH012 and vi) BCSMH016) are shown in the upper panel. Scale bar 2 µm. Quantification of septum versus lateral membrane fluorescence (fluorescence ratio, FR) of the same strains is also shown. FR values over 2 indicate preferential septal localization, whereas FR values equal to or lower than 2 indicate that a protein is dispersed over the cell surface.

We further confirmed the cellular localization of Wzd-Citrine and Wze-Citrine by cell fractionation analysis, which showed that Wzd-

Citrine was found at the membrane while Wze-Citrine was found in the cytoplasm (Supplementary Figure SIII-4). These results are in accordance with the fact that Wzd carries two hydrophobic regions, responsible for its insertion in the membrane, while Wze is predicted to be a cytoplasmic protein.

In order to confirm that the localizations observed in the unencapsulated strains are indeed different from the ones observed in the encapsulated strains, we calculated the fluorescence ratio (FR) between the intensity of the fluorescent signal at the division septum, of cells that had initiated division (class III), and the fluorescent signal measured at the lateral cell wall of the same cells. When a fluorescent protein is evenly distributed all over the membrane, the intensity of the fluorescence at the septum, that contains two membranes, should be approximately twice the fluorescence at the lateral wall. On the other hand, when a protein is localized at the septum, the fluorescence ratio should be higher than two [5, 39]. Determination of the FR in encapsulated strains BCSMH019 and BCSMH016 clearly indicated that Wzd and Wze, respectively, were localized at the septum (Figure III-3). This is different from the result obtained for the unencapsulated strains expressing Wzd (BCSMH008 and BCSMH013) and Wze (BCSMH007 and BCSMH012) fluorescent derivatives. The FR determined for these strains was consistent with an even distribution of the proteins over the cells (Figure III-3).

We ruled out the hypothesis that the fluorescent domain of the protein fusions was preferably cleaved in the unencapsulated strains, which would result in the presence of the fluorescent signal throughout the cytoplasm of the cells, because expression of Wzd fluorescent derivative in both encapsulated BCSMH019 and in the unencapsulated BCSMH008 and BCSMH013 strains produced protein products of similar molecular weight and at similar levels (Figure III-4, left panels). Similar

results were obtained with expression of Wze fluorescent derivative in both encapsulated BCSMH016 and in the unencapsulated BCSMH007 and BCSMH012 strains (Figure III-4, right panels). Wzd and Wze fluorescent derivatives were not cleaved as shown by western blotting with a specific antibody that recognizes Citrine protein (Figure III-4, lower panels).

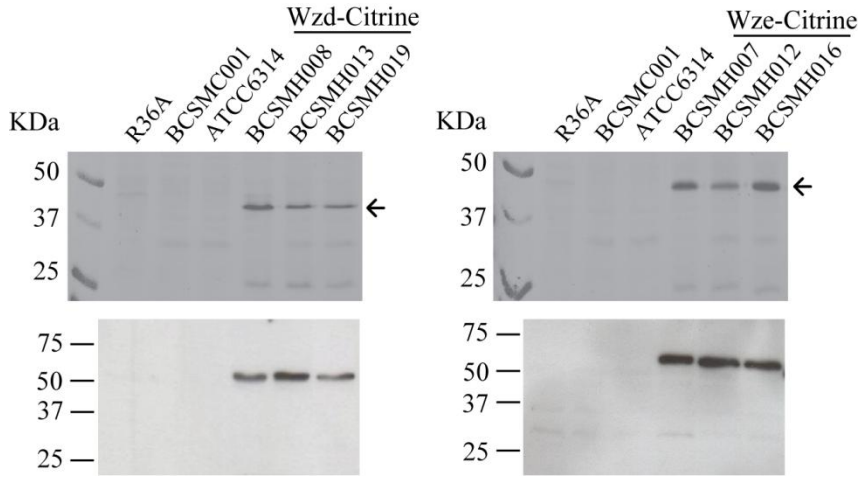

Figure III-4: Cell extracts of *S. pneumoniae* strains expressing Wzd and Wze fluorescent derivatives. Analysis of total cell extracts of *S. pneumoniae* separated by SDS-PAGE showing the absence of cleavage of the Wzd and Wze fluorescent derivatives. Fluorescence images of the SDS-PAGE gels are shown in the upper panels and a Western-Blot analysis, using an antibody that recognizes protein Citrine is shown in the lower panels. Unencapsulated strains, R36A and BCSMC001 (ATCC6314Δ*cps*), and the encapsulated ATCC6314 parental strain are shown as the controls. Strains BCSMH008, BCSMH013 and BCSMH019, which express Wzd-Citrine (indicated by and arrow), are shown on the left. Strains BCSMH007, BCSMH012 and BCSMH016, which express Wze-Citrine (indicated by and arrow) are shown on the right. The size of the detected bands in SDS-PAGE gels and Western-Blot was different as protein extracts were boiled prior loading into gels for Western-Blot analysis.

The observation that fluorescent derivatives of Wzd and Wze could localize at the division septum in bacteria capable of producing a capsular polysaccharide, but not in unencapsulated strains, suggested that

there was an additional protein, encoded by the *cps* operon, involved in recruitment of Wzd and Wze to the septa. Alternatively, a protein complex containing Wzd and Wze could be able to find the division septum on its own, or by interacting with a topological marker present at the pneumococcal division septum. We therefore decided to co-express, in unencapsulated pneumococcal bacteria, Wze, fused to the fluorescent mCherry protein, and Wzd-Citrine. Expression of Wze-mCherry alone, in strains BCSMH006 and BCSMH011, resulted in a dispersed fluorescent signal throughout the entire cytoplasm. Expression of Wzd-Citrine alone, in strains BCSMH008 and BCSMH013, resulted in dispersal of the fluorescent signal all over the membrane (Figure III-5 and Supplementary Figure SIII-5), similarly to what was described in Figure III-3. However, when Wze-mCherry and Wzd-Citrine were co-expressed in the unencapsulated strains BCSMH009 and BCSMH014 both proteins localized at the septum (Figure III-5 and Supplementary Figure SIII-5).

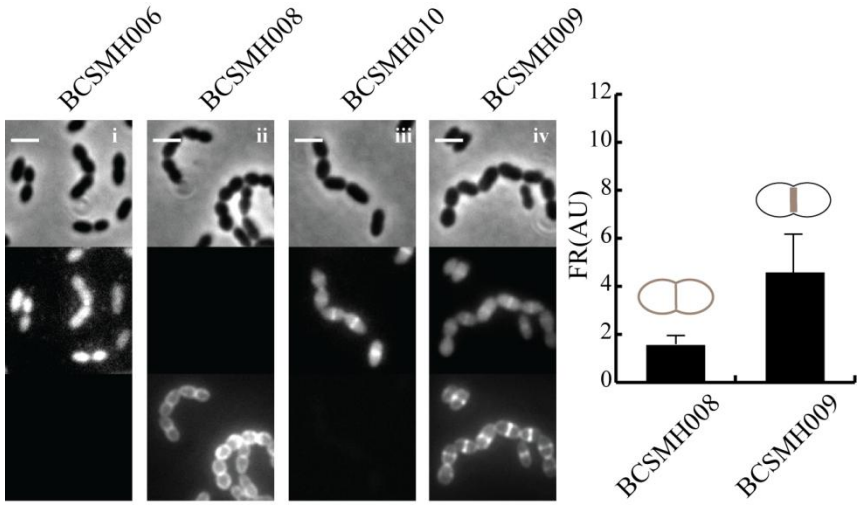

Figure III-5: Co-localization of Wzd-Citrine and Wze-mCherry in the unencapsulated strain R36A. Fluorescent microscopy images of representative fields of strains i) BCSMH006 (expressing Wze-mCherry), ii) BCSMH008 (expressing Wzd-Citrine), iii) BCSMH009 (expressing Wze-mCherry and Wzd)

and iv) BCSMH010 (expressing Wze-mCherry and Wzd-Citrine) are shown. Scale bar, 2 μm. Fluorescent ratios (FR) determined for the Wzd-Citrine localization in strains BCSMH008 and BCSMH009 are shown on the right.

Similar results were obtained when Wze-mCherry was co-expressed with Wzd in the uncapsulated BCSMH010 strain. We confirmed that in all strains there was no cleavage of the fluorescent domains of the Wzd and Wze derivatives, and that the proteins were being produced at similar levels (Supplementary Figure SIII-6). Furthermore, we confirmed that, as expected, untagged mCherry (expressed in the unencapsulated BCSLF001 strain and in the encapsulated BCSLF002 strain) was found dispersed throughout the entire cytoplasm (Supplementary Figure SIII-7).

These results show that the pair Wzd/Wze is recruited to the division septa in the absence of any additional protein encoded by the *cps* operon.

Wzd and Wze interact and this interaction requires the Wze ATP binding domain.

As co-expression of proteins Wzd and Wze is necessary and sufficient for their localization at the division septum, it seemed likely that these two proteins interacted. In order to test this hypothesis, we used a bacterial two-hybrid system [24], based on the expression, in an *E. coli* strain deficient in endogenous adenylate cyclase, of two inactive fragments of the catalytic domain of *Bordetella pertussis* adenylate cyclase, T25 and T18, that when fused to interacting polypeptides can activate the synthesis of cAMP and consequently restore the ability of the *E. coli* mutant strain to ferment lactose or maltose. This approach showed that Wzd was indeed capable of interacting with Wze, as extracts from *E.*

coli cells expressing the proteins T25-Wzd and T18-Wze had a β-galactosidase activity 30 times higher than that resulting from the sole expression of the untagged protein fragments T25 and T18 (Figure III-6).

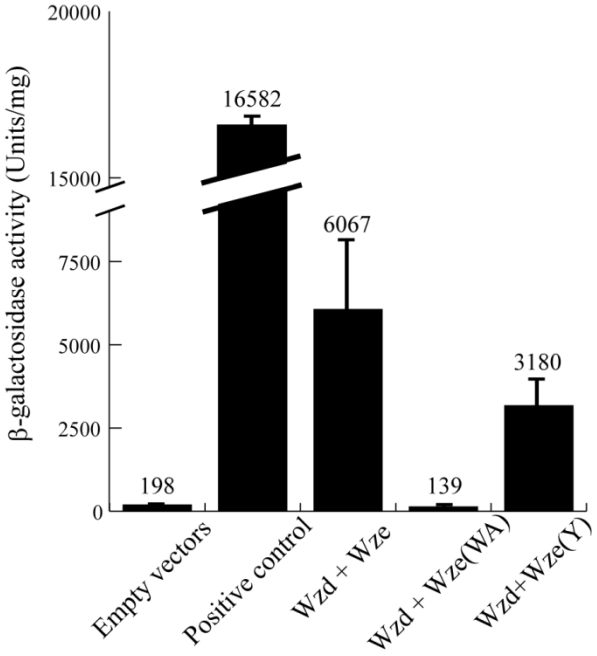

Figure III-6: Requirement of a functional Wze ATP binding domain for its interaction with Wzd. Interaction between Wzd and Wze was tested by a Bacterial Two-Hybrid assay. The averages and standard deviations of three β-galactosidase activity measurement experiments are shown. Empty vectors: expression of untagged T18 and T25 fragments; Positive control: expression of T28 and T25 fragments linked to two leucine zipper domains, Wzd+Wze: interactions between T25-Wzd and T18-Wze; Wzd+Wze(WA): interaction between T25-Wzd and the Walker A ATP binding motif mutant form of T18-Wze (Wze(WA)); Wzd+Wze(Y): interaction between T25Wzd and T18-Wze with a mutated C-terminal tyrosine cluster (Wze(Y)).

A functional Walker A motif and the enriched Tyr cluster in Wze were previously shown to be important for the regulation of the synthesis of the capsule in *S. pneumoniae* [35]. We therefore asked if these motifs were also important for the ability of Wze to interact directly with Wzd, and consequently for the localization of the pair Wzd/Wze at

the division septum. We used again the bacterial two-hybrid system to test the interaction between Wzd and Wze(WA), a previously described mutated form of Wze in which the Walker A motif is inactivated by the mutations $G_{48}A$ and $K_{49}A$ [35]. In this case, the β-galactosidase activity was similar to the negative control (Figure III-6). Similar results were obtained with a mutated form of Wze, named Wze(WA2), in which the Walker A motif was inactivated by the mutation $K_{49}M$ (data not shown). This mutation results in an inactive P-loop in the Wze homologue of *S. aureus*, CapB, without significant alterations of the protein structure [38]. On the contrary, when the mutant Wze(Y) protein, with the tyrosines of the C-terminus cluster mutated to phenylalanines, was tested in the bacterial two-hybrid system, it resulted in β-galactosidase activity 16 times higher than the negative control (Figure III-6).

These results show that a functional Walker A motif is essential for Wze to interact with Wzd, while the ability of Wze to phosphorylate its C-terminal tyrosines is not.

The ATP binding domain of Wze is required for recruitment of the pair Wzd/Wze at the division septum

Since mutations in the conserved Walker A and C-terminal tyrosine cluster motifs of Wze had different effects on its interaction with Wzd, we tested the effect of those mutations on the localization of Wze. In order to do that, we transformed the encapsulated ATCC6314 strain with plasmids pBCSMH005 and pBSMH006, encoding the Walker A, Wze(WA), and the tyrosine cluster, Wze(Y), mutant forms of Wze, respectively, fused to fluorescent protein mCherry, obtaining strains BCSMH017 and BCSMH018.

Microscope visualization of the encapsulated BCSMH017 strain, expressing Wze(WA)-mCherry showed that the protein did not localize at the division septa (Figure III-7A). Similar results were obtained with encapsulated BCSMH024 strain, expressing Wze(WA2)-mCherry (data not shown). On the other hand, expression of Wze(Y)-mCherry in the encapsulated BCSMH018 strain, resulted in septal localization (Figure III-7A). The three forms of Wze fluorescent derivatives had similar sizes and were produced at similar levels (Figure III-7B).

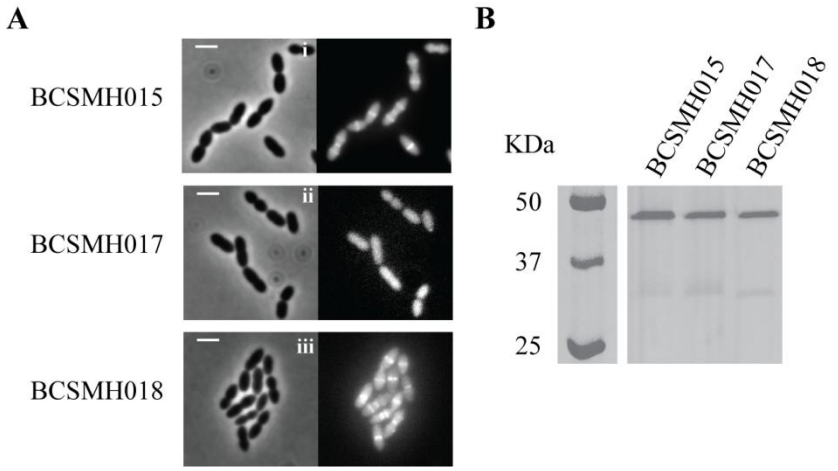

Figure III-7: Requirement of a functional Wze ATP binding domain for the septal localization of the protein. A) Fluorescent microscopy images of encapsulated strains expressing Wze-mCherry fluorescent derivatives from a constitutive promoter. i) BCSMH015, wild-type Wze; ii) BCSMH017, Wze Walker A ATP binding motif mutant (Wze(WA)); iii) BCSMH018, Wze C-terminal tyrosine cluster mutant (Wze(Y)). Scale bar, 2 μm. B) Analysis of total cell extracts of strains in A, separated by SDS-PAGE, showing that all fluorescent Wze-mCherry proteins are expressed at the same levels.

These observations indicated that the ability of Wze to bind ATP, but not to autophophorylate its C-terminal tyrosines, is necessary for its interaction with Wzd and consequent recruitment to the division septa.

In order to determine if ATP binding was required only for the interaction of Wze with Wzd or also for the actual recruitment of the Wzd/Wze complex to the division septum, we linked both proteins through an 11 aminoacid flexible peptide linker (L11), which should overcome the requirement of ATP binding for Wze to interact with Wzd and allow us to evaluate its role solely on the localization of the pair of proteins.

Strain BCSTR001, expressing the chimera protein Wzd-L11-Wze-Citrine in the background of unencapsulated R36A, showed a fluorescent signal localized at the division septum, similarly to what was observed in strain BCSMH009, when Wzd-Citrine was co-expressed together with Wze-mCherry (Figure III-8). On the contrary, strain BCSTR002, unencapsulated R36A strain expressing the protein Wzd-L11-Wze(WA)-Citrine, which has the inactivated Walker A motif, presented a fluorescent signal which was no longer localized at the septum (Figure III-8). The results obtained here showed that ATP binding is required not only for the ability of Wze to interact with Wzd but also for the correct localization of these two proteins at the division septa.

The chimera protein Wzd-L11-Wze-Citrine seems functional, because when this protein was expressed in strain BCSTR003, lacking Wzd, or in strain BCSTR004, lacking Wze it could restore capsule production as determined by Quellung reaction (Supplementary Figure SIII-1). Furthermore, detection of phosphorylated tyrosines in the unencapsulated R36A expressing Wzd-L11-Wze-Citrine (strain BCSTR001), in a protein with the correct size for the fusion protein, indicated that this was not cleaved and that both Wzd and Wze domains were functional (Supplementary Figure SIII-2B), as it has been shown that Wze only phosphorylates its C-terminal tyrosines in the presence of Wzd [9].

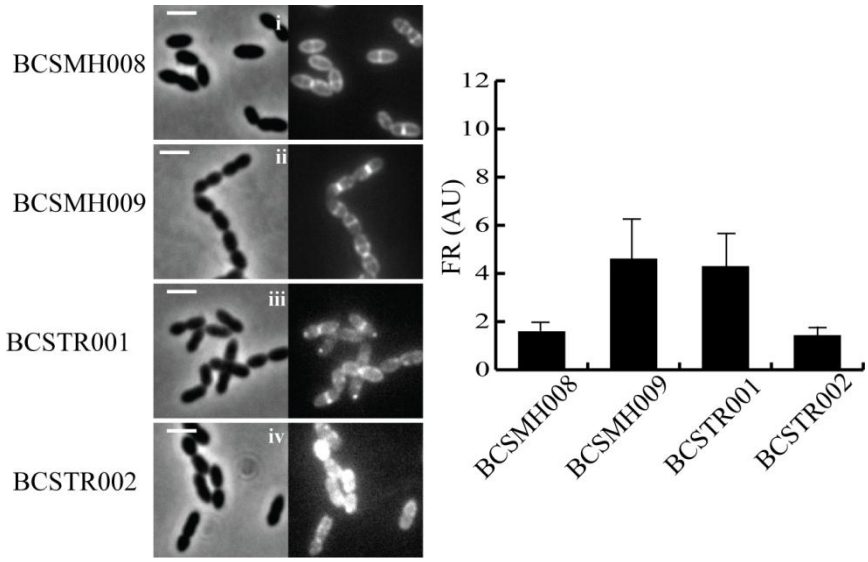

Figure III-8: Localization of the Wzd-L11-Wze chimera fluorescent derivatives. Fluorescence microscopy images of representative fields of unencapsulated strains i) BCSMH008 (Wzd-Citrine), ii) BCSMH009 (Wzd-Citrine co-expressed with Wze-mCherry), iii) BCSTR001 (Wzd-L11-Wze-Citrine) and iv) BCSTR002 (Wzd-L11-Wze(WA)-Citrine). The Wzd-L1-Wze-Citrine chimera protein can localize at the division septum of pneumococcal cells when a functional Wze ATP binding domain is present. Scale bar, 2 μm. The fluorescence ratios (FR) determined for these strains are shown at the bottom.

Capsular polysaccharide is produced in *S. pneumoniae* Wzd or Wze null mutants, but it is absent from the division septa

In order to study the effect of lack of Wzd or Wze on CPS production we deleted the *wzd* and *wze* genes from the chromosome of the encapsulated ATCC6314 strain, obtaining the BCSMH001 and BCSMH002 strains, respectively. No resistance marker was left in the CPS operon to minimize polar effects in downstream genes. The last 50 nt of each deleted gene were kept in the chromosome of the mutant strains because we found that they were required for the expression of the protein encoded by the gene located downstream in the *cps* operon (data not

shown). Both BCSMH001 and BCSMH002 strains seemed to have an impaired ability to synthesize the capsular polysaccharide, compared to the parental ATCC6314 strain, as assayed by Quellung reaction, in which the mutant strains showed a decreased ability to agglutinate in the presence of serum against the type 14 capsular polysaccharide (Figure III-9 and Supplementary Figure SIII-1).

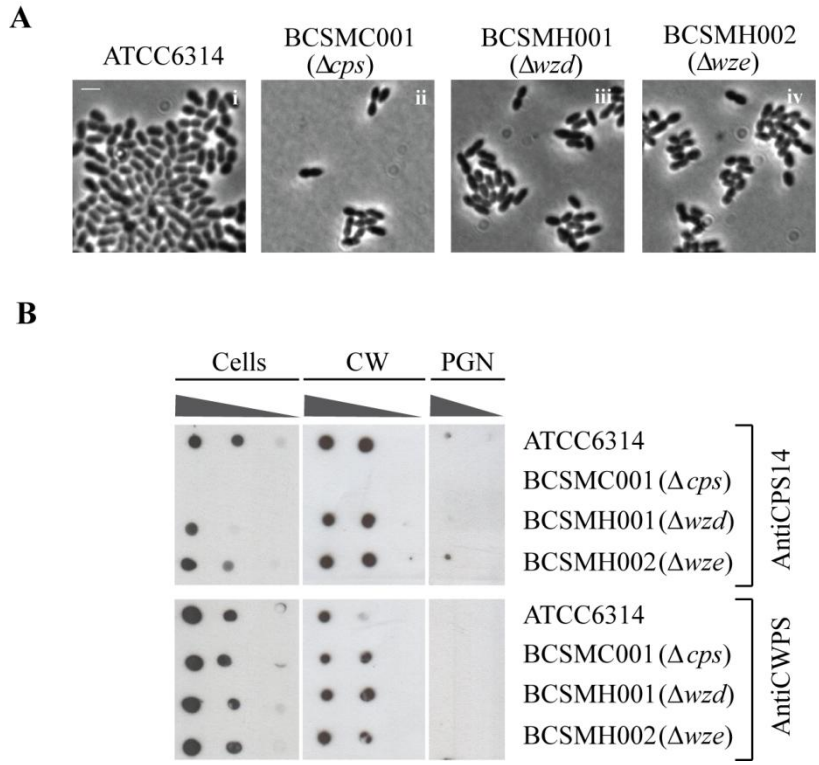

Figure III-9: Expression of capsular polysaccharide in the absence of Wzd and Wze conserved proteins. A) Brightfield images of the agglutination resulting from Quellung reaction. i) ATCC6314, encapsulated parental strain; ii) BCSMC001, unencapsulated ATCC6314Δ*cps* strain; iii) BCSMH001, ATCC6314Δ*wzd* mutant strain; iv) BCSMH002, ATCC6314Δ*wze* mutant strain. Scale bar, 2 μm. B) Dot-blot assays performed with whole cells, cell wall (CW) and peptidoglycan (PGN) samples from strains as in A. Detection of capsular polysaccharide was done with a purified serotype-14 specific serum (AntiCPS14, upper panel). As a control, detection of phosphorylcholine with a cell wall polysaccharide serum was performed (AntiCWPS, lower panel).

We performed a dot-blot with purified commercially available serum, which recognizes capsular polysaccharide 14, to determine if capsule was present in *wzd* and *wze* null mutant cells, in their purified cell walls and in samples of purified peptidoglycan. As seen in figure III-9, we found that capsule was being produced at lower levels in the *wzd* and *wze* null mutant cells than in the wild-type encapsulated ATCC6314 cells and it was absent from the unencapsulated BCSMC001 cells. Moreover, the capsule was present in samples of purified cell walls from the *wzd* and *wze* null mutants, similarly to the parental encapsulated strain, and absent in samples of the purified peptidoglycan (Figure III-9). These results suggest that the capsule was covalently attached to the peptidoglycan in both *wzd* and *wze* null mutants, identical to what was observed in the parental strain, and it was absent when hydrofluoric acid was used to remove material linked to peptidoglycan through phosphodiester bounds, a step used in the purification of bacterial peptidoglycan. Similar amounts of cells walls and peptidoglycan were present in the dot-blot (see methods and Figure III-9).

In order to determine not only if, but also where, capsule was present, we labeled the capsular polysaccharide using again the purified rabbit-serum raised against pneumococcal capsular polysaccharide serotype 14 and showed by immunofluorescence microscopy that in BCSMH001 and BCSMH002, the ATCC6314 *wzd* and *wze* null mutants strains, respectively, the capsular polysaccharide was absent from the division septum (Figure III-10). This is the site where Wzd and Wze proteins can localize if co-expressed, and where new cell wall is synthesized (see below). However, the capsule was still present in the mature cell wall, as judged by the observation of an immunofluorescence signal. This is in accordance with what Morona and colleagues have reported previously [35]. As expected, the capsule was present over the

entire surface of the pneumococci cells in the parental ATCC6314 strain, and no signal was detected in BCSMC001, the *cps* null mutant of ATCC6314 (Figure III-10).

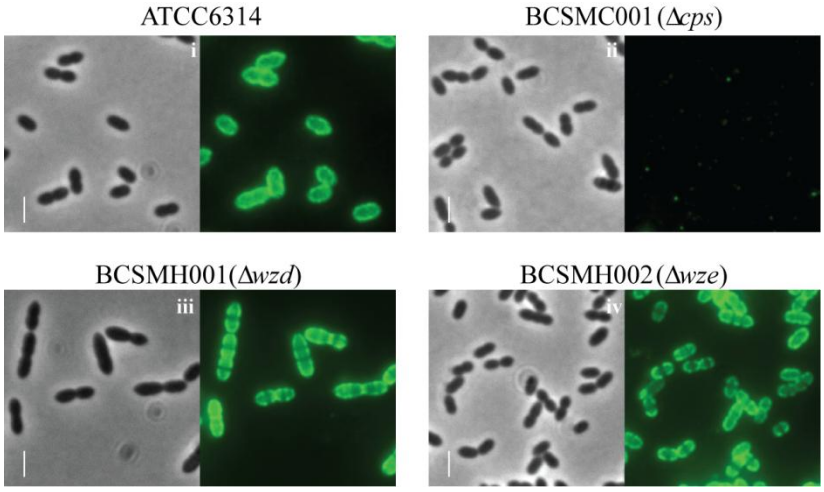

Figure III-10: Distribution of capsular polysaccharide at the cell surface of different pneumococcal cells. Capsular polysaccharide immunofluorescence. i) ATCC6314, encapsulated parental strain; ii) BCSMC001, unencapsulated ATCC6314Δ*cps* strain; iii) BCSMH001, ATCC6314Δ*wzd* mutant strain; iv) BCSMH002, ATCC6314Δ*wze* mutant strain. Capsular polysaccharide is absent from the division septum of cells lacking Wzd (BCSMH001) or Wze (BCSMH002). Scale bar, 2 μm.

To determine where the new cell wall was being synthesized and if it corresponded to the same place where Wze and Wzd localized, we labeled encapsulated bacteria BCSMH015, expressing Wze-mCherry, with Van-FL, a fluorescent derivative of vancomycin, an antibiotic that binds to the recently translocated peptidoglycan precursors that still carry an intact D-Ala-D-Ala carboxyl terminus [13]. Fluorescent signals of Van-FL and Wze-mCherry co-localized at the division septum (Figure III-11), indicating that Wze localizes where the new peptidoglycan is being synthesized.

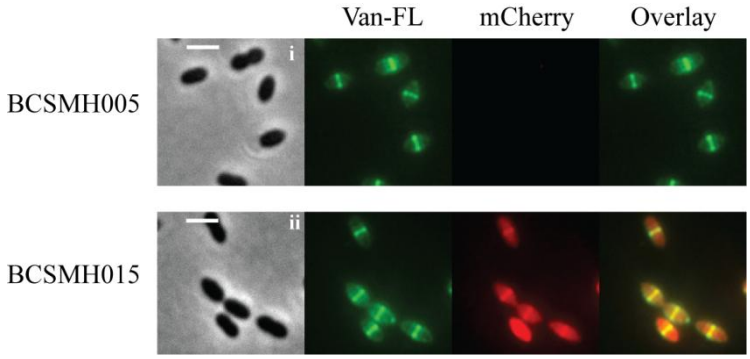

Figure III-11: Co-localization of Wze-mCherry and new cell wall synthesis. Van-FL staining of strain BCSMH015, expressing Wze-mCherry. Strain BCSMH005, transformed with the empty vector, is shown for comparison. Scale bar, 2 μm.

The observation that Wzd and Wze were required for the presence of the capsular polysaccharide at the division septum suggests that these proteins are involved in the spatial-temporal regulation of the capsule metabolism, ensuring the capsule is present at the division septum, where the new cell wall is being synthesized.

Discussion

Protein localization studies in *S. pneumoniae* have been done mainly by immunofluorescence, probably due to the fact that these bacteria are microaerophiles. Low levels of oxygen could prevent post-translational oxidation of GFP fusion proteins with the consequent absence of fluorescent signal, when expressed in pneumococci. However, Eberhardt and colleagues have recently described the localization in live cells of fluorescent derivatives of proteins involved in the essential pathway of choline metabolism in *S. pneumoniae* [15].

In this work we have studied the localization of the regulators of capsule synthesis in *S. pneumoniae*, Wzd and Wze. We substituted *wzd* and *wze* in the *cps* operon by genes encoding the fluorescent derivatives of Wzd and Wze. This strategy increases the likelihood that fluorescent proteins are produced at the same level as the native unlabeled proteins, with minimal alterations in the expression of the other proteins encoded in the *cps* operon.

Wzd and Wze localized at the septum of live and dividing encapsulated pneumococcal cells. Both proteins were recruited to the septum early in the cell cycle, as the corresponding fluorescent signal was observed at mid-cell in newborn bacteria, before the onset of invagination.

Surprisingly, we found that when the localization of the fluorescent derivatives of Wzd and Wze was determined in unencapsulated bacteria both Wzd and Wze proteins were no longer localized at the septum, but had a dispersed localization consistent with their aminoacid composition: Wzd was found all over the cell membrane, while Wze was dispersed throughout the cell cytoplasm.

The observation that Wzd and Wze localized at the division septum only in encapsulated bacteria suggested that one or more proteins,

encoded in the *cps* operon, could recruit Wzd and Wze to the division septum. An alternative hypothesis was that Wzd and Wze, assembled in a protein complex and were able to find the septum on their own, or through the interaction with a topological marker present at the pneumococcal division septum. The second scenario seems more likely, as we observed that co-expression of fluorescent derivatives of both Wzd and Wze in unencapsulated strains resulted in a clear septal localization. We have not identified the topological marker that determines Wzd/Wze septal localization. Interestingly, Wze has homology with proteins from the large P-loop NTPase superfamily that include members such as the MinD ATPase, a constituent of the MinCDE complex that contributes to the determination of the place where the division septum is formed in different bacteria [44]. In Gram-positive bacteria, such as *Bacillus subtilis*, MinD can interact with the bacterial membrane and accumulate at cell division sites and cell poles [31]. This results in the localization of MinC, an inhibitor of the polymerization of FtsZ, at these sites, which ensures that bacterial division is temporally and spatially regulated [31].

Bacterial two-hybrid assays showed that Wzd and Wze interact and that a functional ATP binding domain in Wze is required for their interactions. This is in agreement with recent data obtained with Wzd and Wze homologues from *Streptococcus thermophilus* using a yeast two-hybrid assay [11]. Wze contains a divergent Walker A motif (A/G-X-X-X-X-G-K-S/T, where X can be any amino acid); and a Walker B ATP binding motif (h-h-h-h-D, where h represents a hydrophobic amino acid) [30]. Besides these two motifs, Wze also has an additional motif, termed Walker A' (D-X-D), generally found in cell division regulatory proteins of the MinD/Mrp family and present in different bacterial tyrosine kinases involved with the synthesis of capsular polysaccharides [12]. Morona and colleagues have described that a mutation of the Walker A motif, which

inactivates the ATP binding ability of the protein, resulted in the elimination of capsule synthesis [35]. On the contrary, mutation of the tyrosines at the C-terminus of the protein had no apparent effect on capsule production. We now found that a functional Walker A motif in Wze is also essential for its interaction with Wzd, while the tyrosines at the C-terminus of Wze are not. These results suggest that the reason why an ATP binding Wze mutant is impaired in its ability to produce capsule, may be a consequence of its inability to interact with Wzd and properly localize.

Wze belongs to a bacterial tyrosine kinase protein family which includes members reported to act as co-polymerases in the biosynthesis of capsular and extracellular polysaccharides [17-18]. In Gram-negative bacteria, the tyrosine kinase domain is linked to a transmembrane domain, functional homologous to Wzd, forming a single polypeptide. We have reproduced the Gram-negative organization of bacterial tyrosine kinases in *S. pneumoniae* by constructing a fluorescent derivative of the chimera Wzd-L11-Wze protein, in which Wzd was linked to Wze through an 11-aminoacid flexible linker. This strain allowed us to test if ATP binding activity was required only for Wzd/Wze interactions (as showed in the bacterial two-hybrid assays), or also for the septal localization of these proteins, as the need of ATP binding for the interaction between Wzd and Wze would be by-passed by the physical linkage of the two proteins. The fact that expression of Wzd-L11-Wze-Citrine, but not Wzd-L11-Wze(WA)-Citrine (containing a Walker A mutant form of Wze), in the unencapsulated strain R36A resulted in localization at the division septum, showed that the ability of Wze to bind ATP is required not only for its interaction with Wzd, but also for the correct localization of the proteins. However, at this moment, we cannot rule out the possibility that the absence of a functional ATP-binding domain may alter the structure of

Wze(WA) in such a way that Wzd and Wze(WA) domains cannot interact properly in the Wzd-L11-Wze(WA)-Citrine protein. If this were the case, it would also result in the inability of the fusion protein to localize at the division septum.

In order to clarify the role of Wzd and Wze in the production of capsular polysaccharide, we constructed mutants of the encapsulated strain ATCC6314 where the genes encoding these proteins were deleted. The synthesis of the capsule in these *wzd* and *wze* null mutants seemed to be affected, as seen by their reduced ability to agglutinate when incubated with serotype specific serum in a Quellung reaction. These results are in accordance with previous reports describing that Wzd or Wze mutants were not able to produce capsule, as judged by Quellung reactions and ELISA experiments [9, 33, 35].

However, we have now determined that *wzd* and *wze* mutants still produce capsule but in a different manner from the parental ATCC6314 strain. In the encapsulated parental strain the capsule was distributed all around the surface of the cells, as expected. However, in the *wzd* and *wze* null mutants, capsule was produced at lower levels than those found in the encapsulated ATCC6314 parental strain, as determined by quantitative detection of capsule bound to cells in a dot-blot assay. More interestingly, the capsular polysaccharide was absent from the division septum of the *wzd* and *wze* null mutant cells, but was still present in the mature cell wall as determined by a immunofluorescence microscopy assay using a purified serum against serotype 14 capsular polysaccharide. A similar observation has been made by Morona and colleagues [35], with deletion mutants constructed in an encapsulated serotype 19 *S. pneumoniae* strain. The authors explained this observation as resulting from impaired synthesis of capsule, where this polysaccharide was synthesized, exported and attached to the cell wall at very low basal rate [35].

In theory, absence of the capsule from the division septum of *wzd* and *wze* null mutants could result in the inability of these mutants to attach capsule to the newly assembled peptidoglycan. In fact, phosphorylated Wze has been previously proposed to have a role in the attachment of capsule to the cell wall [34]. However, we do not favor this hypothesis, as we observed that the capsule produced by the *wzd* and *wze* null mutants was as tightly associated to peptidoglycan as in the parental strain (Figure III-9B). The capsular polysaccharide was removed from the peptidoglycan only after incubation with hydrofluoric acid, which is known to cleave phosphodiester bonds and thus remove teichoic acids and polysaccharides covalently bound to the bacterial peptidoglycan, similarly to what has been previously described [45]. It should be emphasized that the proposed role for Wzd/Wze in attaching the capsule to the cell wall has been made based on the characterization of the amount of capsule produced by mutants strains constructed in the background of the encapsulated serotype 2 *S. pneumoniae* D39 strain. As different serotypes are known to produce variable amounts of capsule polysaccharide, we cannot exclude that the absence of Wzd/Wze may affect differently the synthesis of the capsule in different serotypes.

We propose that Wzd/Wze function as spatial regulators of the capsular polysaccharide synthesis, ensuring that it occurs at the proper place, the division septum, and at the proper time, possibly to ensure the concealment of the newly synthesized cell wall. According to this model, wild-type Wzd and Wze interact and migrate to the middle of the dividing cells, events that are dependent on a functional ATP binding Walker A motif of Wze (Figure III-12). At the septum, the complex Wzd/Wze is probably subjected to cycles of phosphorylation/dephosphorylation events at the C-terminal tyrosine cluster of Wze, which may affect other components of the polysaccharide synthesis and assembly complex

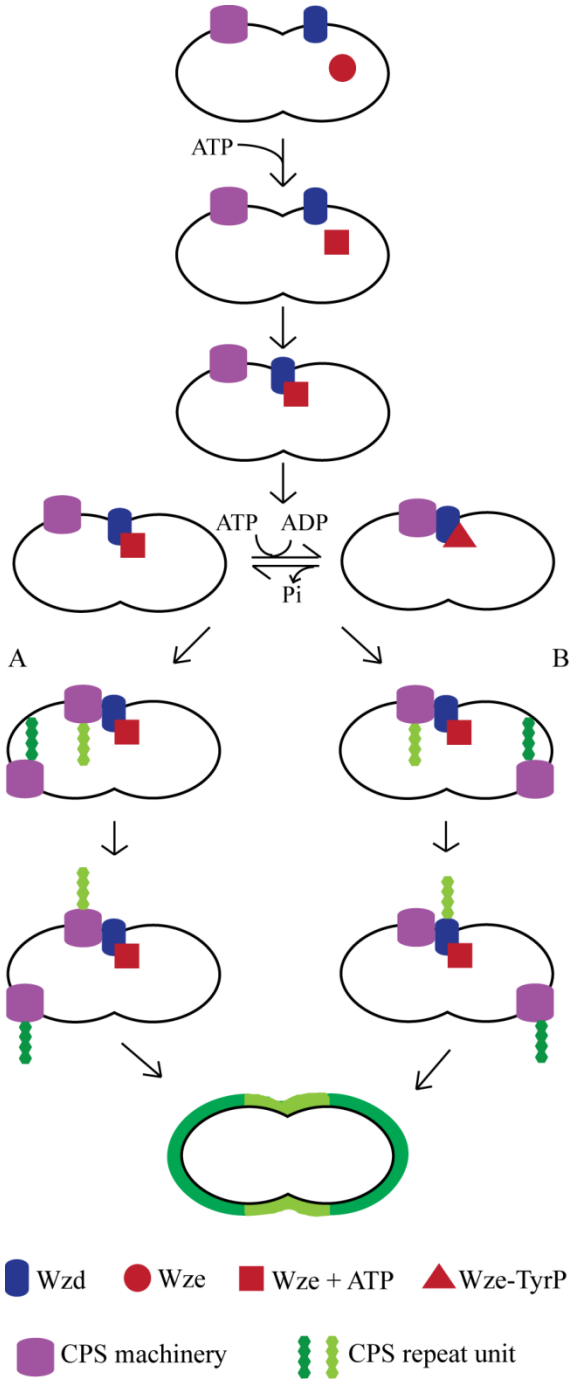

Figure III-12: Model for the regulation of capsule synthesis by Wzd and Wze. Upon ATP binding to the Walker A motif of Wze, the protein is able to interact with Wzd and the complex migrates to the cell septum. At the septum, the

complex is subjected to cycling phosphorylation/dephosphorylation events at the C-terminal tyrosine cluster of Wze. Two hypothesis are proposed for capsular polysaccharide synthesis and export at the division site: A) Wzd/Wze function as activators of CPS export at the septum, ensuring that it occurs at the correct place; or B) Wzd/Wze function themselves as the exporter at the septum, interacting with the synthetic machinery and transferring the CPS repeat unit to the outer surface of the plasma membrane at this site. Both hypothesis result in the expression of capsule all around the surface of the pneumococcal cells.

through conformational changes as previously proposed from the studies of the *S. aureus* Wzd/Wze homologues, CapA and CapB [38].

The formation of capsule at the division septum of the pneumococcal cells seems to be regulated by Wzd/Wze proteins. If ATP is not able to bind to Wze, or in the absence of Wzd or Wze, capsule is absent from the septum, where the new cell wall is synthesized, resulting in the presence of capsule at the surface of cells only in regions of mature cell wall. This raises the hypothesis that there are two modes to synthesize the capsular polysaccharide in pneumococcal bacteria (septal and non-septal), similarly to what has been proposed for peptidoglycan synthesis [51]. The absence of capsule from the septum could then be explained by the existence of two capsular polysaccharide synthetic machineries or alternatively by the activation of the synthetic machinery at the lateral cell wall or at the division septum, the latter dependent on the pair Wzd/Wze.

Why bacteria require such fine-tuned regulation of the synthesis of the capsular polysaccharide is unknown. One possibility is that the newly synthesized cell wall, located at the septum, has to be concealed or covered by the capsular polysaccharide to avoid bacteria recognition by the host complement system, or prevent detection by the host innate immune system. The pair Wzd/Wze would therefore assure that the capsule polysaccharide is produced or exported where it is most needed: the division septum of bacteria.

Acknowledgments

We thank Dr. Mariana Pinho for critical reading of the manuscript and Magda Atilano for helping us in performing the analysis of the different samples by HPLC. This work was funded by "Fundação para a Ciência e Tecnologia" through research Grants PTDC/SAU-MII/75696/2006 and PTDC/BIA-MIC/100747/2008 (to S. R. F.) and fellowships SFRH/BD/43797/2008 (to M. X. H.). M. X. H. designed and performed the experiments and wrote the manuscript.

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Supplementary Information

Table SIII-1: Bacterial strains.

Name	Relevant characteristics	Comments/Source/ Reference
E. coli		
DH5α	*recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1* Φ80 d*lac*Z ΔM15	Gibco-BRL
EC101	*supE thi* (*lacproAB*) (F' *traD36 proAB lacI*^{q} ZΔM15), *repA* from pWV01 integrated in the chromosome	[28]
BTH101	Reporter strain for BTH system, F$^-$ *cya*-99, *ara*D139, *gal*E15, *gal*K16, *rps*L1 (Str^r), *hsd*R2, *mcr*A1, *mcr*B1	[24]
Lactococcus lactis		
LL108	RepA$^+$ MG1363, Cm^r	[29]
S. pneumoniae		
R36A	Non encapsulated laboratory strain	[6]
ATCC6314	Encapsulated strain, serotype 14	American Type Culture Collection
BCSLF001	R36ApBCSLF002, Tet^r	R36A expressing constitutively mCherry, this work
BCSLF002	ATCC6314pBCSLF002, Tet$_r$	ATCC6314 expressing constitutively mCherry, this work
BCSMC001	ATCC6314Δ*cps*	This work
BCSMH001	ATCC6314Δ*wzd*	This work
BCSMH002	ATCC6314Δ*wze*	This work
BCSMH003	ATCC6314*wzd::wzd_citrine*	This work
BCSMH004	ATCC6314*wze::wze_citrine*	This work
BCSMH005	ATCC6314pBCSLF001, Tet^r	ATCC6314 transformed with pBCSLF001, this work

Table SIII-1 (Cont.): Bacterial strains.

Name	Relevant characteristics	Comments/Source/ Reference
BCSMH006	R36ApBCSMH003, Tet^r	R36A expressing constitutively Wze-mCherry, this work
BCSMH007	R36ApBCSMH004, Tet^r	R36A expressing constitutively Wze-Citrine, this work
BCSMH008	R36ApBCSMH007, Tet^r	R36A expressing constitutively Wzd-Citrine, this work
BCSMH009	R36ApBCSMH008, Tet^r	R36A expressing constitutively Wze-mCherry and Wzd-Citrine, this work
BCSMH010	R36ApBCSMH009, Tet^r	R36A expressing constitutively Wze-mCherry and Wzd, this work
BCSMH011	BCSMC001pBCSMH003, Tet^r	ATCC6314Δ*cps* expressing constitutively Wze-mCherry, this work
BCSMH012	BCSMC001pBCSMH004, Tet^r	ATCC6314Δ*cps* expressing constitutively Wze-Citrine, this work
BCSMH013	BCSMC001pBCSMH007, Tet^r	ATCC6314Δ*cps* expressing constitutively Wzd-Citrine, this work
BCSMH014	BCSMC001pBCSMH008, Tet^r	ATCC6314Δ*cps* expressing constitutively Wze-mCherry and Wzd-Citrine, this work
BCSMH015	ATCC6314pBCSMH003, Tet^r	ATCC6314 expressing constitutively Wze-mCherry, this work
BCSMH016	ATCC6314pBCSMH004, Tet^r	ATCC6314 expressing constitutively Wze-Citrine, this work
BCSMH017	ATCC6314pBCSMH005, Tet^r	ATCC6314 expressing constitutively Wze(WA)-mCherry, this work
BCSMH018	ATCC6314pBCSMH006, Tet^r	ATCC6314 expressing constitutively Wze(Y)-mCherry, this work

Table SIII-1 (Cont.): Bacterial strains.

Name	Relevant characteristics	Comments/Source/ Reference
BCSMH019	ATCC6314pBCSMH007, Tet^r	ATCC6314 expressing constitutively Wzd-Citrine, this work
BCSMH020	R36ApBCSLF001, Tet^r	R36A transformed with pBCSLF001, this work
BCSMH021	BCSMC001pBCSLF001, Tet^r	BCSMC001 transformed with pBCSLF001, this work
BCSMH022	BCSMH001pBCSMH007, Tet^r	ATCC6314Δ*wzd* expressing constitutively Wzd-Citrine, this work
BCSMH023	BCSMH002pBCSMH004, Tet^r	ATCC6314Δ*wze* expressing constitutively Wze-Citrine, this work
BCSMH024	ATCC6314pBCSMH015, Tet^r	ATCC6314 expressing constitutively Wze(WA2)-mCherry, this work
BCSMH025	BCSMH001pBCSMH016, Tet^r	ATCC6314Δ*wzd* expressing constitutively Wzd, this work
BCSMH026	BCSMH002pBCSMH017, Tet^r	ATCC6314Δ*wze* expressing constitutively Wze, this work
BCSTR001	R36ApBCSTR001, Tet^r	R36A expressing constitutively Wzd–L11-Wze-Citrine, this work
BCSTR002	R36ApBCSTR002, Tet^r	R36A expressing constitutively Wzd–L11-Wze(WA)-Citrine, this work
BCSTR003	BCSMH001pBCSTR001, Tet^r	ATCC6314Δ*wzd* expressing constitutively Wzd-L11-Wze-Citrine, this work
BCSTR004	BCSMH002pBCSTR001, Tet^r	ATCC6314Δ*wze* expressing constitutively Wzd-L11-Wze-Citrine, this work

Table SIII-2: Plasmids.

Name	**Relevant characteristics**	**Comments/Source/ Reference**
Replicative plasmids in *E. coli*		
pRod17	Plasmid containing the mCherry coding sequence	David Sherrat
pcDNA3_Citrine	Plasmid containing the Citrine coding sequence	[19]
Plasmids for Bacterial Two Hybrid Assay		
pKT25	BTH plasmid, N-terminal *cyaA*T25 fusion, Kan^r	[24]
pUT18C	BTH plasmid, N-terminal *cyaA*T18 fusion, Amp^r	[24]
pKT25zip	BTH control plasmid, Kan^r	[24]
pUT18Czip	BTH control plasmid, Amp^r	[24]
pBCSMC001	BTH plasmid containing *cyaA*T25-*wzd* fusion	This work
pBCSMC002	BTH plasmid containing *cyaA*T18-*wze* fusion	This work
pBCSMC003	BTH plasmid containing *cyaA*T18-*wze(WA)* fusion	This work
pBCSMC004	BTH plasmid containing *cyaA*T18-*wze(Y)* fusion	This work
pBCSMH014	BTH plasmid containing *cyaA*T18-*wze(WA2)* fusion	This work
Replicative plasmids in *S. pneumoniae*		
pLS578	Contains the extended -10 constitutive promoter of *SigA* from *S. pneumoniae*, Tet^r	[27]
pBCSLF001	High-copy-number vector, contains the -10 constitutive promoter of *SigA* from *S. pneumoniae*, Tet^r	This work
pBCSLF002	pBCSLF001 derivative, mCherry constitutive expression, Tet^r	This work
pBCSMH001	pBCSLF002 derivative, allows expression of mCherry fusion proteins, Tet^r	This work

Table SIII-2 (Cont.): Plasmids.

Name	Relevant characteristics	Comments/Source/ Reference
pBCSMH002	pBCSLF001 derivative, allows expression of Citrine fusion proteins, Tet^r	This work
pBCSMH003	pBCSMH001 containing *wze-mCherry*, Tet^r	This work
pBCSMH004	pBCSMH002 containing *wze-citrine*, Tet^r	This work
pBCSMH005	pBCSMH001 containing *wze(WA)-mCherry*, Tet^r	This work
pBCSMH006	pBCSMH001 containing *wze(Y)-mCherry*, Tet^r	This work
pBCSMH007	pBCSMH002 containing *wzd-citrine*, Tet^r	This work
pBCSMH008	pBCSMH001 containing *wze-mCherry*, *wzd-citrine*, Tet^r	This work
pBCSMH009	pBCSMH001 containing *wze-mCherry*, *wzd*, Tet^r	This work
pBCSMH015	pBCSMH001 containing *wze(WA2)-mCherry*, Tet^r	This work
pBCSMH016	Derivative of pBCSMH007 in which *citrine* was removed, Tet^r	This work
pBCSMH017	Derivative of pBCSMH004 in which *citrine* was removed, Tet^r	This work
pBCSTR001	pBCSMH002 containing *wzd-L11-wze-citrine*, Tet^r	This work
pBCSTR002	pBCSMH002 containing *wzd-L11-wze(WA)-citrine*, Tet^r	This work
Non replicative plasmids in *S. pneumoniae*		
pORI280	Em^r, $LacZ^+$, ori^+ of pWV01	[29]
pBCSMC005	pORI280 containing up- and downstream regions of *cps* operon, Em^r, $LacZ^+$	This work
pBCSMH010	pORI280 containing the 3' of *wzd*, the entire *citrine* sequence, and the downstream region of *wzd*, Em^r, $LacZ^+$	This work

Table SIII-2 (Cont.): Plasmids.

Name	Relevant characteristics	Comments/Source/ Reference
pBCSMH011	pORI280 containing the 3' of *wze*, the entire *citrine* sequence, and the downstream region of *wze*, Em^r, $LacZ^+$	This work
pBCSMH012	pORI280 containing up- and downstream regions of *wzd*, Em^r, $LacZ^+$	This work
pBCSMH013	pORI280 containing up- and downstream regions of *wze*, Em^r, $LacZ^+$	This work

Table SIII-3: Primers used in this work.

Primer	Sequence 5'→3'	Features/ Restriction sites
1	CGGGATCCCGGCTGCTTGCGGCCAATCAGG	BamHI
2	AATATCATCACTGTTCTTGAAGGCACCTATAC CTTGAACATCG	Region overlapping primer 3
3	CCTTCAAGAACAGTGATGATATTGAGATTGT TAAAATCTTGCG	Region overlapping primer 2
4	GAACTGCAGCCTTTAAAACTTTCTCTCCA	PstI
5	CGCGGATCCGGCGCAGAGATATACTATAC	BamHI
6	CTCCCAAAAGTGTCATCTGCACTAAATTAATT GATCCATTATAA	Region overlapping primer 7
7	GCAGATGACACTTTTGGGAGTTGTACC	Region overlapping primer 6
8	CCGGAATTCGTGATAATTACCGCATCAATAA C	EcoRI
9	CGCGGATCCATCAAGACTTGCAGGCAGG	BamHI
10	GCGTAAACTCCATACTTATCAACCGACTATTT CAACTTACTC	Region overlapping primer 11

Table SIII-3 (Cont.): Primers used in this work.

Primer	Sequence 5'→3'	Features/ Restriction sites
11	GGTTGATAAGTATGGAGTTTACGCTTCCTATGG	Region overlapping primer 10
12	CCGGAATTCCTGCTACCAGTTTCCATGAAAG	EcoRI
13	CGCGGATCCGAAGTCTGGTTTAACCAATC	BamHI
14	CTCCCAAAAGTGTCATCTGCTTACTTGTACAGCTCGTCCATGCCGAGAGTG	Region overlapping primer 15
15	GGACGAGCTGTACAAGTAAGCAGATGACACTTTTGGGAGTTGTACC	Region overlapping primer 14
16	CCGGAATTCCCGCATCAATAACAATTCCAACAGG	EcoRI
17	CGCGGATCCGCAAGGTCGTTTGCGCGTGC	BamHI
18	GCGTAAACTCCATACTTATCAACCTTACTTGTACAGCTCGTCCATGCCG	Region overlapping primer 11
19	CCGGAATTCCACTTACTGCTACCAGTTTCC	EcoRI
20	AGCTAGCAACCATGGATTAGATCTCAGAATTGCAGATAGGC	NheI, NcoI, BglII
21	AGCTAGCCTACCTCCTTAAGC	NheI
22	AGCTAGCATGATTACGGGTACCAGCTCGGC	NheI, KpnI
23	AACCATGGCCTACACCCGGGTTACATAGAGCGGCCGCCTTGTACAGCTCGTCCATGC	NcoI, NotI
24	GGACTAGTGTGAGCAAGGGCGAAGAAGATAACATGGCTATC	SpeI
25	GGACTAGTGCCAGAACCAGCAGCGGAGCC	SpeI
26	GGATTAGATCTCAGGAATTG	BglII
27	CCCTTGCTCACACTAGTGCC	SpeI
28	GGACTAGTGGGCCCGCCGGCATGGTGAGCAAGGGCG	SpeI, ApaI, NaeI
29	GAAGATCTAATCCATGGCATATGAGCGGCCGCCTTGTACAGCTCGTCC	BglII, NotI
30	CTAGCTAGCATGCCGACATTAGAAATAGCAC	NheI
31	CGGGGTACCTTTTTTACCATAATTTCCATAGGAAGC	KpnI

Table SIII-3 (Cont.): Primers used in this work.

Primer	Sequence 5'→3'	Features/ Restriction sites
32	CCCTGGGGAAG**C**A**GC**AACAACTACTTCCG	Mutation of Walker A motif ($G_{48}A$; $K_{49}A$); region overlapping primer 33
33	CGGAAGTAGTTGTT**GC**T**G**CTTCCCCAGGG	Mutation of Walker A motif ($G_{48}A$; $K_{49}A$); region overlapping primer 32
34	CGGGGTACCTTTTTTACCA**A**AATTTCCA**A**AG GAAGCG**A**AAACTCCA**A**ACTTATCAACCG	Mutation of the C-terminal tyrosines of Wze; KpnI
35	CTAGCTAGCATGAAGGAACAAAACACTTTGG	NheI
36	GGGGTACCCTATTTCAACTTACTCAAG	KpnI
37	CATGCCATGG**TAAGGAGGT**GCTGCGGCCAT GAAGGAACAAAACAC	RBS; NcoI
38	GGAAGATCTCTTGTACAGCTCGTCCATGCC	BglII
39	GAAGATCTCTATTTCAACTTACTCAAG-	BglII
40	TCCTTTAATAGTTTTAACAGATCCTCTCTCCG CTTTCAAC	Region overlapping primer 41
41	TCTGTTAAAACTATTAAAGGAATGCCGACAT TAGAAATAGC	Region overlapping primer 40
42	GGGGATCCCGGAGAAAATATGAAGG	BamHI
43	GAATTCCCCTCTCTCCTATTTC	EcoRI
44	GGATCCAATGCCGACATTAGA	BamHI
45	GAATTCCCCTAAGTTATTTTTTACC	EcoRI
46	CCCTGGGGAAGGAATGACAACTACTTCCG	Mutation of Walker A motif ($K_{49}A$); region overlapping primer 53

Table SIII-3 (Cont.): Primers used in this work.

Primer	**Sequence 5'→3'**	**Features/ Restriction sites**
47	CGGAAGTAGTTGTCATTCCTTCCCCAGGG	Mutation of Walker A motif ($K_{49}A$); region overlapping primer 52
48	GGATTAGATCTCGGAGCCAGCCGAGCTGGTACCCTATTTCAACTTACTCAAG	BglII
49	GGATTAGATCTCGGAGCCAGCCGAGCTGGTACCCTATTTTTTACCATAATTTCC	BglII

Supplementary figures

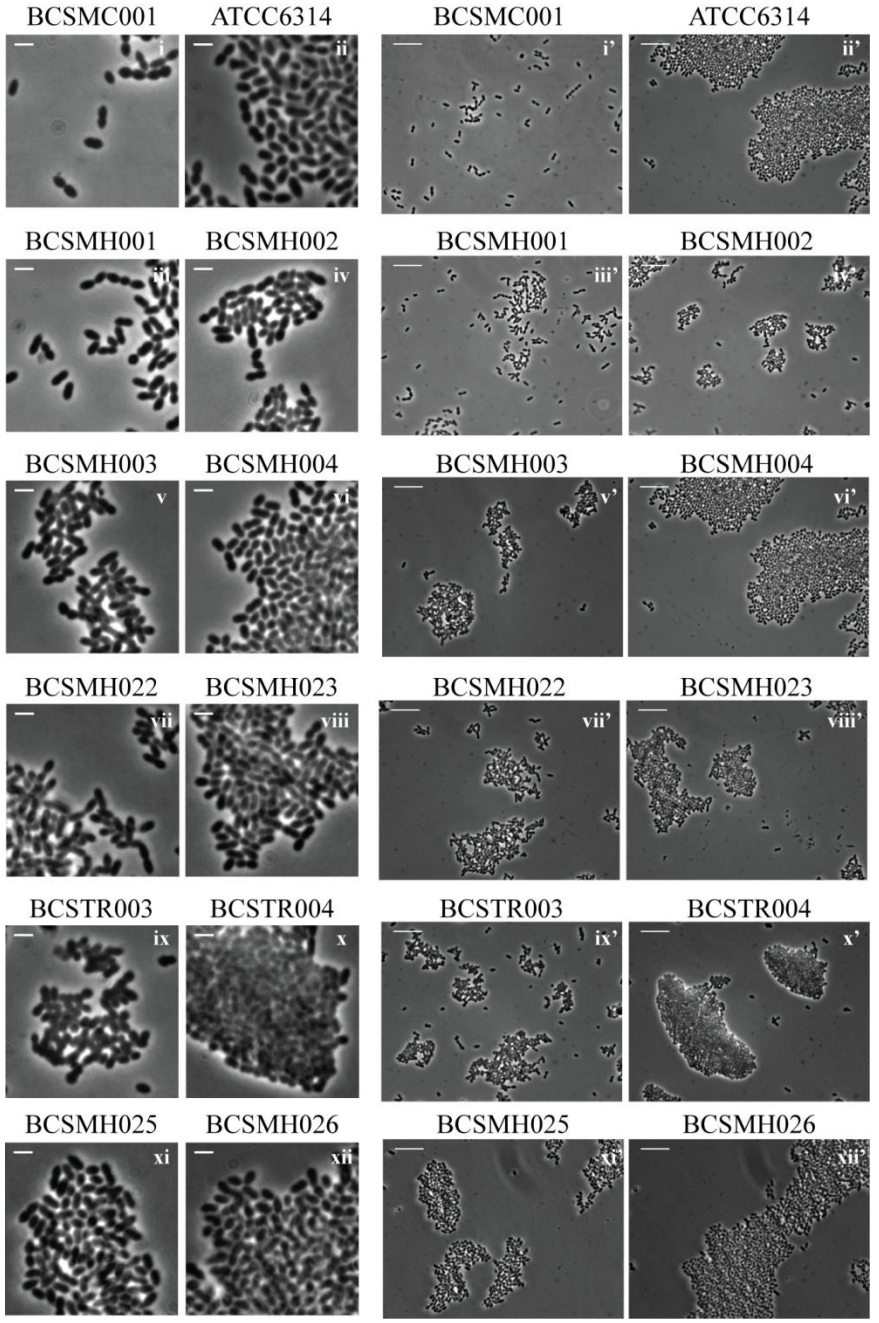

Figure SIII-1: Functionality of the different Wzd and Wze fluorescent derivatives. Fluorescent derivatives of Wzd and Wze are functional as they complement the production of capsule in Wzd and Wze null mutants.

Representative brightfield images of the agglutination resulting from Quellung reactions, with a purified serotype 14 specific serum, are shown: whole fields on the right hand side of the figure (scale bar, 10 µm) and zoomed view of chosen sections on the left hand side of figure (scale bar, 2 µm). The strains were the following: i and i') BCSMC001 (Δ*cps*); ii and ii') ATCC6314 (WT); iii and iii') BCSMH001 (Δ*wzd*); iv and iv') BCSMH002 (Δ*wze*); v and v') BCSMH003 (ATCC6314*wzd::wzd_citrine*); vi and vi') BCSMH004 (ATCC6314*wze::wze_citrine*); vii and vii') BCSMH022 (Δ*wzd* expressing Wzd-Citrine constitutively); viii and viii') BCSMH023 (Δ*wze* expressing Wze-Citrine constitutively); ix and ix') BCSTR003 (Δ*wzd* expressing Wzd-L11-Wze-Citrine constitutively); x and x') BCSTR004 (Δ*wze* expressing Wzd-L11-Wze constitutively); xi and xi') BCSMH025 (Δ*wzd* expressing Wzd constitutively); xii and xii') BCSMH026 (Δ*wze* expressing Wze constitutively).

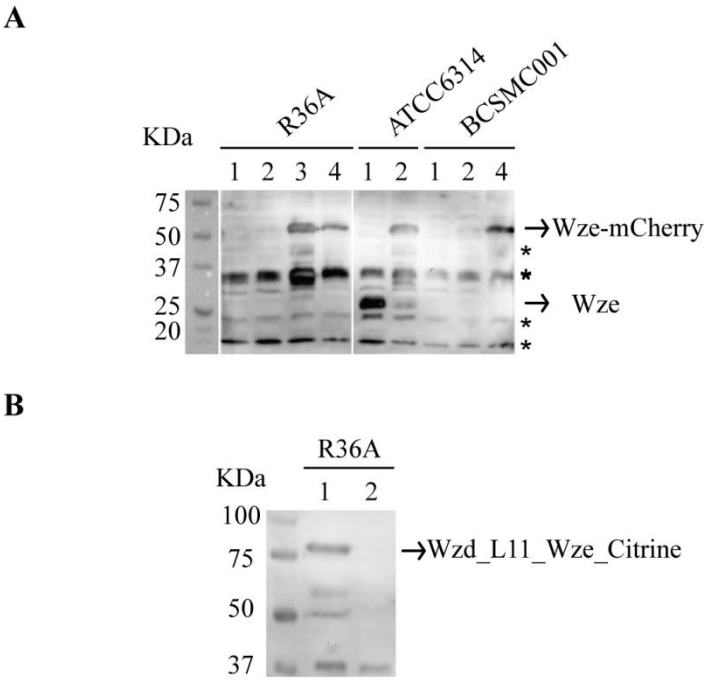

Figure SIII-2: Detection of tyrosine phosphorylated Wze derivatives. Analysis by western-blotting with a primary antibody against phosphorylated tyrosines. A) Strains R36A, ATCC6314 (parental encapsulated strain) and BCSMC001 (ATCC6314Δ*cps*) expressing constitutively the following proteins: 1) control, empty plasmid (strains BCSMH020 and BCSMH021; 2) Wze-mCherry (strains BCSMH006, BCSMH015 and BCSMH011); 3) Wze-mCherry and Wzd (strain BCSMH010); 4) Wze-mCherry and Wzd-Citrine (strains BCSMH009 and BCSMH014). The bands correspondent to Wze-mCherry and to Wze alone are indicated. Wze-mCherry is only detected, as it is phosphorylated,

in the presence of Wzd. Non-specific bands, present in all strains, are labeled with "*". B) the Wzd-L11-Wze chimera is phosphorylated. Strains analysed: BCSTR001 (R36A expressing constitutively Wzd-L11-Wze) in lane (1); BCSTR002 (R36A expressing constitutively Wzd-L11-Wze(WA) in lane (2). The band correspondent to the phosphorylated form of Wzd-L11-Wze is indicated.

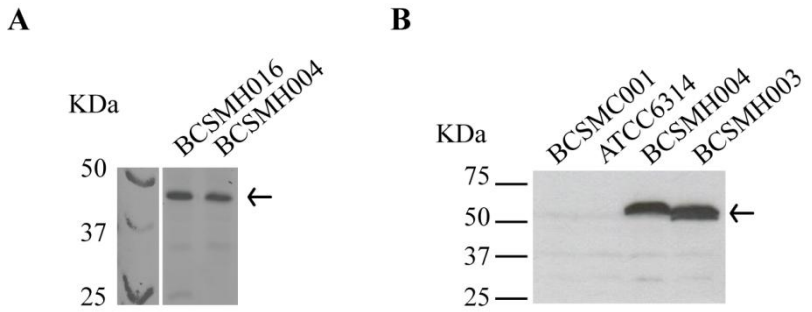

Figure SIII-3: Analysis of Citrine fluorescent derivatives. A) Fluorescent protein analysis by SDS-PAGE. Total cell extracts were loaded in each lane showing that the Wze-Citrine fluorescent derivative is expressed at similar levels from the constitutive promoter in plasmid pBCSMH004 (lane 1, strain BCSMH016) and from its native promoter (lane 2, strain BCSMH004). B) Western-blot analysis, with a primary antibody against the Citrine protein, showing that Wze-Citrine and Wzd-Citrine are expressed at similar levels from their native promoter and do not suffer proteolytic cleavage. Strains BCSMC001 (ATCC6314Δ*cps*), ATCC6314 (encapsulates parental strain), BCSMH004 (ATCC6314*wze::wze-citrine*) and BCSMH003 (ATCC6314*wzd::wzd-citrine*) were analysed.

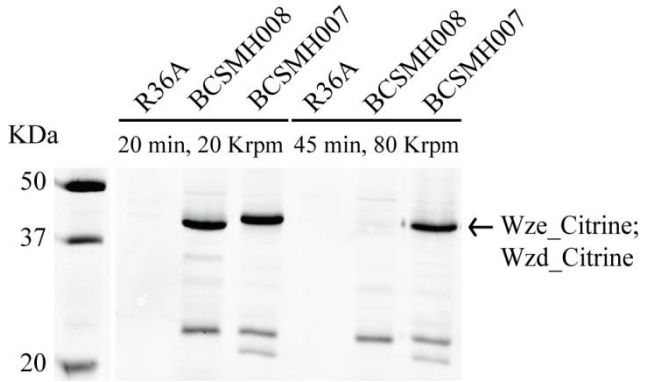

Figure SIII-4: SDS-PAGE fluorescent protein analysis after cell fractionation. Strains analysed: R36A, BCSMH008 (R36A expressing Wzd-

Citrine constitutively) and BCSMH007 (R36A expressing Wze-Citrine constitutively). Total cell extracts ultracentrifuged during 20 minutes at 20 Krpm had similar amounts of Wzd-Citrine and Wze-Citrine. However, when these samples were ultracentrifuged during 45 minutes at 80 Krpm, the membrane Wzd-Citrine protein was pulled down into the pellet fraction, while the cytoplasmic Wze-Citrine protein remained in the supernatant.

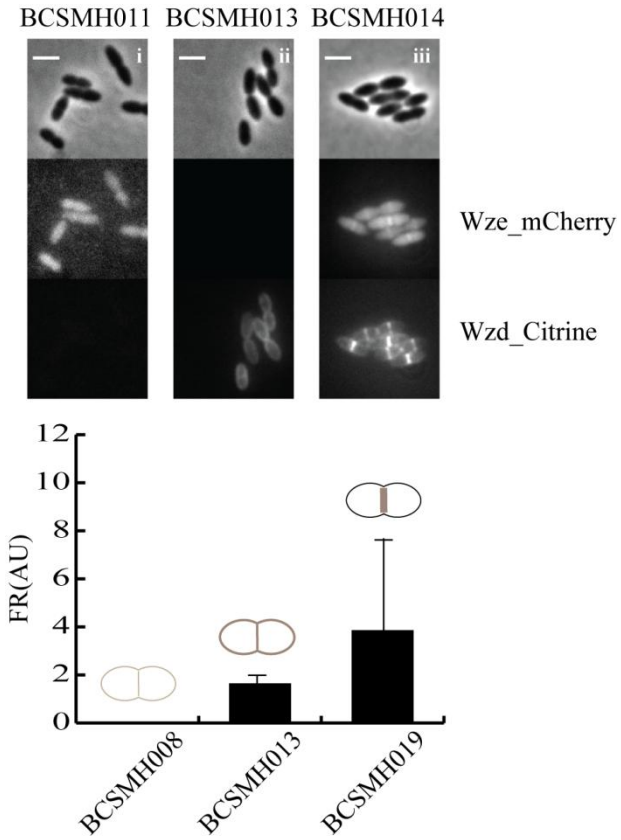

Figure SIII-5: Co-localization of Wzd-Citrine and Wze-mCherry on the capsule null mutant strain BCSMC001. Fluorescence microscopy images of representative fields are shown. Scale bar 2 μm. i) BCSMH011, expressing constitutively Wze-mCherry; ii) BCSMH013, expressing constitutively Wzd-Citrine; iii) BCSMH014, expressing constitutively Wze-mCherry and Wzd-Citrine. Fluorescence ratios determined for the Wzd-Citrine localization in the strains above are shown in the lower panel.

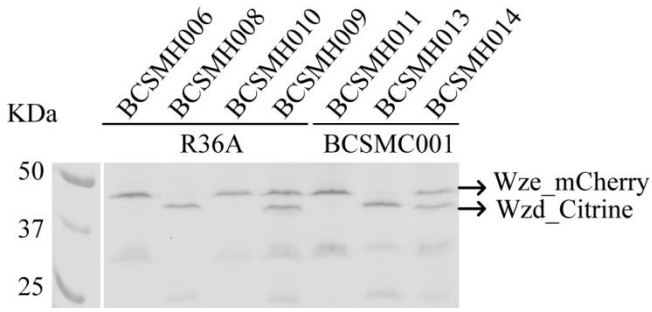

Figure SIII-6: SDS-PAGE analysis of total cell extracts of unencapsulated strains R36A and BCSMC001 (ATCC6314Δ*cps*) co-expressing Wzd and Wze fluorescent derivatives. R36A strains: BCSMH006, expressing Wze-mCherry; BCSMH008, expressing Wzd-Citrine; BCSMH010, expressing Wze-mCherry and Wzd; and BCSMH009, expressing Wze-mCherry and Wzd-Citrine. BCSMC001 strains: BCSMH011, expressing Wze-mCherry; BCSMH013, expressing Wzd-Citrine; and BCSMH014, expressing Wze-mCherry and Wzd-Citrine. Proteins Wze-mCherry and Wzd-Citrine are indicated by arrows.

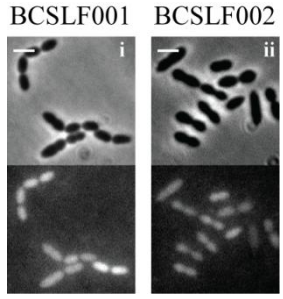

Figure SIII-7: Fluorescence microscopy images of strains expressing mCherry alone. i) strain BCSLF001, R36A transformed with pBCSLF002; ii) strain BCSLF002, ATCC6314 transformed with pBCSLF002. Scale bar 2 μm.

Chapter IV

Wzd, a spatial regulator of capsule synthesis, interacts with and recruits to the septum the capsular polysaccharide ligase Wzg of *Streptococcus pneumoniae*

The results presented in this chapter are preliminary, unpublished data.

Abstract

The capsule or capsular polysaccharide (CPS) is a major virulence factor of *Streptococcus pneumoniae* and its production, at the surface of the cells at different stages of infection, is tightly regulated. Wze, an autophosphorylating tyrosine kinase, and Wzd, a membrane protein required for Wze autophosphorylation, are the main regulators of this process. Wzd and Wze were shown to regulate the polymerization of the CPS and its attachment to the cell wall. More recently, we have proposed that Wzd/Wze act as spatial regulators of capsule synthesis and ensure full encapsulation of pneumococcal bacteria. However, how the Wzd/Wze complex carries out this function remains unknown.

In the present work, we performed a Bacterial Two-Hybrid screening (BTH) for interactions between components of the Wzd/Wze complex and other proteins involved in the assembly and regulation of the synthesis of capsule. No interactions were found between Wzd, or Wze, and the following proteins: Wzh (a phosphotyrosine phosphatase that dephosphorylates Wze), Wzx (the flippase that translocates the CPS repeating unit across the membrane) and Wzy (the polymerase that assembles the CPS). Interestingly, we found that Wzd interacts with Wzg, the CPS-cell wall ligase, in the BTH assay. In accordance, fluorescent derivatives of Wzg accumulated at the septum of the pneumococcal cells only when Wzg fusions were produced in the presence of functional Wzd/Wze. We hypothesize that the Wzd/Wze complex recruits Wzg to the septum to ensure the efficient attachment of the CPS to the cell wall at that particular site. In this way, the *S. pneumoniae* cell surface is fully encapsulated and protected by the capsular polysaccharide.

Introduction

Streptococcus pneumoniae is an important Gram-positive bacterial pathogen, which can colonize asymptomatically the nasopharynx of healthy individuals. Having the ability to spread to other sites of the human host, it is also capable of causing diseases such as otitis media, pneumonia or even meningitis [14]. The expression of capsule or capsular polysaccharide (CPS) is central to the ability of pneumococcus to cause invasive disease. This structure surrounds the surface of the bacteria, acting as a shield that confers protection against the host's immune system. However, a thick capsule may be disadvantageous during the colonization stage of infection, by preventing the exposure of bacterial molecules required for surface adhesion [14]. Therefore, a tight control of the amount of CPS expressed is expected to occur during the infection process.

To date, more than 90 types of capsular polysaccharide have been identified [4]. In the majority of them, the genes that encode proteins involved in CPS synthesis and regulation are located at the same region of the *S. pneumoniae* chromosome: the *cps* operon [3]. The 5' end of this operon is highly conserved between serotypes and is constituted by four genes, the products of which are proposed to be involved in CPS synthesis regulation [3], as discussed below. The 3' end of the *cps* operon is the serotype specific region of the locus, comprising genes that encode the enzymes necessary for the formation of the specific capsule repeating unit of each serotype [3]. This region also includes the genes that encode the proteins responsible for the transfer of the CPS repeating units from the inner to the outer face of the plasma membrane, Wzx, and for the polymerization of the CPS, Wzy.

The first gene in the *cps* operon, *wzg*, encodes an enzyme whose function was discovered only recently. Wzg was initially identified as a transcriptional regulator, based on its homology with LytR from *Bacillus subtilis* [9], but more recently Kawai and colleagues have determined that Wzg is in fact the long searched CPS-cell wall ligase [16].

The other genes of the conserved region of the *cps* operon, *wzh*, *wzd*, and *wze*, encode three proteins that are part of a phosphoregulatory system required to control the synthesis of the capsule and its attachment to the cell-wall. Wze is an autophosphorylating tyrosine kinase that belongs to the Bacterial Tyrosine Kinase family [8]. It possesses the conserved motifs characteristic of this protein family: a Walker A ATP binding motif and a C-terminal tyrosine cluster [26]. Wzd is a membrane protein that is required for the autophosphorylation of Wze [2]. Wzh is a phosphotyrosine protein phosphatase that dephosphorylates Wze [24]. These three proteins are proposed to regulate the capsule synthesis process in the following way [25]: Wze, upon ATP binding to its Walker A motif, interacts with Wzd in such a way that CPS synthesis proceeds at its maximal level until an unknown signal causes Wze to autophosphorylate its C-terminal tyrosines. This probably leads to conformational changes that alter the interaction with Wzd and with other members of the CPS synthesis machinery, causing a decrease in the CPS synthesis rate. At the same time the transfer of the CPS to the CPS-cell wall ligase Wzg is favored. When Wzh dephosphorylates Wze, the cycle can be repeated.

Recently, we have identified a novel role for Wzd and Wze as spatial regulators of capsule synthesis [13]. These two proteins were found to localize at the septum, in the presence of each other, with no additional factor encoded in the *cps* operon being required for that localization. Moreover, in the absence of Wzd or Wze, capsule was synthesized and attached to the cell wall, but it was absent from the

division septum of the cells, the site where the two proteins localize and where cell wall synthesis occurs. 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 [13].

In this work, we wanted to determine how Wzd and Wze can spatially regulate the CPS synthesis. We hypothesized that Wzd/Wze could recruit other proteins from the CPS synthesis machinery to a septal location, or alternatively, that they could activate other members of the CPS synthesis machinery at that site. This chapter describes the preliminary results obtained in our attempt to test these hypotheses. Using a Bacterial Two-Hybrid system, we asked whether proteins Wzd and Wze could interact with other proteins of the CPS synthesis machinery. We identified a putative interaction between protein Wzd and Wzg, the CPS-cell wall ligase, which was also found to be capable of interacting with itself. Moreover, analysis of the *in vivo* localization of Wzg revealed that it is associated with the membrane and enriched at the septal region. In accordance with a possible interaction of Wzd with Wzg, the ability of Wzg to localize at the division septum was dependent on the presence of Wzd/Wze.

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Tables SIV-1 and SIV-2, respectively, in Supplementary Information. *E. coli* was routinely grown in LB medium at 37ºC, unless otherwise indicated. When appropriate, ampicillin (100 μg/ml), kanamycin (50 μg/ml) and erythromycin (100 μg/ml) were added to the growth media. Isopropyl-β-thiogalactopyranoside (IPTG, Apollo Scientific Ltd.) was used at 0.5 mM and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, Apollo Scientific Ltd.) at 40 μg/ml.

S. pneumoniae was grown in C + Y liquid medium [18] at 37ºC, without aeration, or in tryptic soy agar (TSA, Difco) plates supplemented with 5% sheep blood (Probiológica). When needed, tetracycline (1 μg/ml) and erythromycin (0.25 μg/ml) were added to the growth media. For white/blue colony selection, X-Gal was used at 120 μg/ml. *Lactococcus lactis* was grown in M17 broth (Difco), supplemented with glucose (0.5% w/v), at 30ºC. Erythromycin was used at 100 μg/ml.

DNA manipulation procedures

E. coli and *S. pneumoniae* competent cells preparation and transformation were executed as previously described [22, 28]. PCR products and plasmid DNA were purified with kits 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. The primers used in this work are listed in Table SIV-3 in Supplementary Information.

Construction of plasmids for Bacterial Two-Hybrid assays

In order to test *in vitro* whether Wzd and Wze could interact with other capsule proteins, several plasmids were constructed. All genes were amplified using 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.

Amplification of *wze* with primers 7/8, followed by restriction and ligation into pUT18 and pKNT25 produced plasmids pBCSMC006 and pBCSMC009, encoding fusions Wze-T18 and Wze-T25, respectively. Plasmid pBCSMC008, encoding fusion T25-Wze, was obtained by amplification of *wze* with primers 9/10 and cloning into pKT25.

Construction of plasmid pBCSMH041, which allows the co-expression of Wzd with T18-Wze was achieved by amplification of *wzd* with primers 11/12, restriction and ligation into pBCSMC002. The same approach was followed for the construction of pBCSMH042, in which Wze is co-expressed with Wzd-T18. In this case, *wze* was amplified with primers 13/14 and cloned into pBCSMH040. Plasmids pBCSMH045 and pBCSMH046, in which Wzd is co-expressed with T25-Wze and Wze is co-expressed with T25-Wzd, respectively, were obtained by amplification of *wzd* with primers 11/12 and *wze* with primers 8/16, restriction and ligation into pBCSMC008 and pBCSMC001.

Cloning of the fusion protein Wzd-L11-Wze into the Bacterial Two-Hybrid plasmids was executed using plasmid pBCSTR001 as DNA template for the amplification reaction. For the construction of pBCSMH043 and pBCSMH047, encoding fusions T18-Wzd-L11-Wze

and T25-Wzd-L11-Wze, respectively, primers 10/15 were used, followed by restriction and ligation into pUT18C and pKT25. Construction of pBCSMH044 and pBCSMH048, encoding fusions Wzd-L11-Wze-T18 and Wzd-L11-Wze-T25, respectively, was done using primers 8/15, restriction and ligation into pUT18 and pKNT25.

Plasmids pBCSMH049 and pBCSMH051, encoding T18-Wzg and T25-Wzg, was achieved by amplification of *wzg* with primers 17/18, restriction and ligation into pUT18C and pKT25. Amplification of *wzg* with primers 17/19, restriction and ligation into pUT18 and pKNT25 produced plasmids pBCSMH050 and pBCSMH052, encoding the fusions Wzg-T18 and Wzg-T25, respectively.

The amplification of *wchA* with primer pairs 20/21 and 20/22, followed by restriction and ligation into pUT18C and pUT18, respectively, produced plasmids pBCSMH053 and pBCSMH054. These encode the fusions T18-WchA and WchA-T18. Construction of plasmids encoding the fusions T18-Wzx (pBCSMH055), Wzx-T18 (pBCSMH056), T18-Wzy (pBCSMH057) and Wzy-T18 (pBCSMH058) was done by amplification of *wzx* with primer pairs 23/24 and 23/25 and of *wzy* with primer pairs 26/27 and 26/28, respectively, restriction and ligation into pUT18C and pUT18.

Plasmids pBCSMC010 and pBCSMC012, encoding fusions T18-Wzh and T25-Wzh were done by amplification of *wzh* with primers 29/30, restriction and ligation into pUT18C and pKT25. Amplification of *wzh* with primers 31/32, restriction and ligation into pUT18 and pKNT25 produced plasmids pBCSMC011 and pBCSMC013, encoding the fusions Wzh-T18 and Wzh-T25, respectively.

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

Construction of plasmids for protein expression in *S. pneumoniae*

In order to express fluorescent derivatives of Wzg, plasmid pBCSMH039 was constructed by amplification of *wzg* from the ATCC6314 chromosomal DNA with primers 33/34, restriction and ligation into pBCSMH031.

Construction of the *wzg* null mutant

In order to construct strains BCSMH057-059, derivatives of the encapsulated ATCC6314 strain in which the *wzg* gene is deleted, plasmid pORI280 was used. Fragments of DNA corresponding to the upstream and downstream regions of the *wzg* gene were amplified with primers 35/36 and 37/38, respectively. Primers 36 and 37 possess an overlapping region. After this first amplification, the two fragments were joined by overlapping PCR using primers 35 and 38. The resulting fragment was restricted with *BamH*I and *Ecor*I and cloned into pORI280, producing plasmid pBCSMH059. This plasmid was transformed into strain ATCC6314 and transformants were selected by their ability to grow in the presence of erythromycin. Excision of pBCSMH059 was executed as previously described [17] and produced strains BCSMH057-059. In the construction of these mutants ~50 bp of the 3' region of the *wzg* gene were left intact, to guarantee that the expression of the downstream gene on the operon was not disturbed.

Plasmids were routinely propagated in *E. coli* EC101 or *L. lactis* LL180 and purified before being transformed into *S. pneumoniae* bacteria.

Bacterial Two-Hybrid assays

Interactions were tested by transformation of *E. coli* strain BTH101 with plasmids of interest. Transformants were plated in LA media, supplemented with ampicillin, kanamycin, IPTG and X-Gal, at the

concentrations indicated above. Plates were incubated at 30ºC and screened for blue/white colonies, in which blue indicated a positive interaction.

Microscopy

S. pneumoniae strains were grown until early exponential phase and observed by fluorescence microscopy on a thin layer of 1% agarose in PreC medium [18]. Images were acquired in a Zeiss Axio Observer microscope, equipped with a Photometrics CoolSNAP HQ2 camera (Roper Scientific) and analyzed and cropped using Metamorph (Meta Imaging series 7.5) and Image J software [1].

Determination of the Fluorescence ratio (FR) was performed as previously described [27]. The intensity of the fluorescent signal by quantifying the fluorescence at the septum was divided by the fluorescent signal at the poles. Average background fluorescence was subtracted from every value. An FR value higher than 2 is indicative of a septal localization. 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 [13].

Results

Screening for CPS proteins capable of interacting with Wzd/Wze

Following the finding that Wzd and Wze act as spatial regulators of capsule synthesis in *S. pneumoniae* [13], it was our goal to discover how these two proteins were capable of performing that role. Using a Bacterial Two-Hybrid assay, we previously detected a positive interaction between proteins Wzd and Wze [13]. Therefore, we decided to test whether Wzd/Wze could interact with other capsule synthesis proteins using the same method. We chose Wzh, Wzg, WchA, Wzx, and Wzy as the first targets of our screening and tested them for interactions with Wzd, Wze and Wzd co-expressed with Wze. The results obtained are summarized in figure IV-1. No interactions were found between Wzd or Wze and proteins WchA, Wzx and Wzy. Surprisingly, interactions were also not detected between Wze and Wzh, the cognate phosphatase of the Wze tyrosine kinase [24]. However, qualitative analysis of the color of the colonies transformed with plasmids encoding the fusions Wzd-T18 and T25-Wzg revealed that they were blue, albeit less intense than the color observed with the positive control (data not shown), suggesting a possible interaction between Wzd and Wzg (Figure IV-1).

	T18-**Wzd**	**Wzd**-T18	T18-**Wze**	**Wze**-T18	T25-**Wzd**	**Wzd**-T25	T25-**Wze**	**Wze**-T25	T25-**Wzd** + **Wze**
T18-**Wzh**	n.d.	n.d.	n.d.	n.d.	–	–	–	–	–
Wzh-T18	n.d.	n.d.	n.d.	n.d.	–	–	–	–	–
T25-**Wzh**	–	–	–	–	n.d.	n.d.	n.d.	n.d.	n.d.
Wzh-T25	–	–	–	–	n.d.	n.d.	n.d.	n.d.	n.d.
T18-**Wzg**	n.d.	n.d.	n.d.	n.d.	–	–	–	–	–
Wzg-T18	n.d.	n.d.	n.d.	n.d.	–	–	–	–	–
T25-**Wzg**	–	+	–	–	n.d.	n.d.	n.d.	n.d.	n.d.
Wzg-T25	–	–	–	–	n.d.	n.d.	n.d.	n.d.	n.d.
T18-**WchA**	n.d.	n.d.	n.d.	n.d.	–	–	–	–	–
WchA-T18	n.d.	n.d.	n.d.	n.d.	–	–	–	–	–
T18-**Wzx**	n.d.	n.d.	n.d.	n.d.	–	–	–	–	–
Wzx-T18	n.d.	n.d.	n.d.	n.d.	–	–	–	–	–
T18-**Wzy**	n.d.	n.d.	n.d.	n.d.	–	–	–	–	–
Wzy-T18	n.d.	n.d.	n.d.	n.d.	–	–	–	–	–

Figure IV-1: Summary of the interactions tested between Wzd and Wze and other capsule proteins. Interactions between Wzd and Wze and capsule proteins Wzh, Wzg, WchA, Wzx and Wzy were tested using a bacterial two-hybrid system. A positive interaction was detected only when Wzd-T18 and T25-Wzg were co-expressed (n.d. – not determined).

Wzg, the CPS-cell wall ligase, interacts with Wzd and with itself in a BTH assay

The weak signal observed when Wzd-T18 and T25-Wzg were expressed could be explained if Wzg can interact better with the pair Wzd/Wze than with Wzd alone. We extended our screening to include additional plasmids that allowed the co-expression of Wzd and Wze in different combinations and the expression of a chimera protein of Wzd

linked to Wze through an 11 aminoacid linker (Figure IV-2). A fluorescent derivative of the latter, designated Wzd-L11-Wze, was shown to localize at the septum of *S. pneumoniae* encapsulated and unencapsulated cells [13].

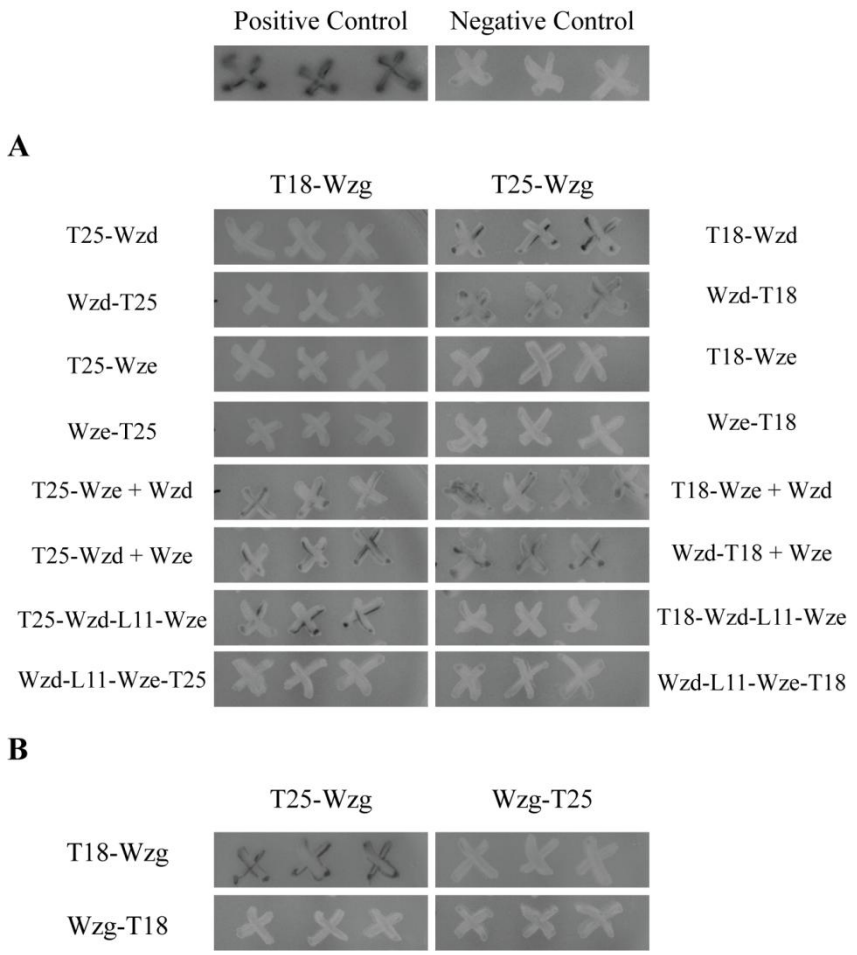

Figure IV-2: Wzg may interact with Wzd and with itself. Three colonies of each bacterial two-hybrid transformation with plasmids encoding the indicated fusions were re-streaked to show the blue/white color. Positive and negative controls, constituted by transformation with plasmid pairs pUT18C-zip/pKT25-zip and pUT18C/pKT25, respectively, are shown at the top. A) Interactions between protein Wzg and proteins Wzd, Wze, Wzd and Wze co-expressed in different combinations and Wzd-L11-Wze. B) Interactions of Wzg with itself.

In this extended screening, we confirmed that the fusion protein T25-Wzg may interact with the fusion Wzd-T18, as expression of these proteins again produced light blue colonies. The same result was obtained when T25-Wzg was expressed with T18-Wzd and with Wzd-T18 in the presence of Wze, as well as with T18-Wze in the presence of Wzd (Figure IV-2A). No interactions were observed between T18-Wzg and Wzd or Wze. Only a weak signal was observed when T18-Wzg was co-expressed with T25-Wze in the presence of Wzd, with T25-Wzd in the presence of Wze and with T25-Wzd-L11-Wze (Figure IV-2A). Linking T18 or T25 to the C-terminus of Wzg seems to abolish its ability to interact with Wzd or Wze. Interactions between fusions Wzg-T18 and Wzg-T25 and the different Wzd and Wze fusions described above produced only white colonies (data not shown).

Furthermore, we also asked whether Wzg could interact with itself. The expression of fusions T18-Wzg and T25-Wzg produced blue colonies on X-Gal plates, indicating that the CPS-cell wall ligase Wzg may interact with itself (Figure IV-2B). No interactions were detected for fusions Wzg-T18 and Wzg-T25.

Wzg septal localization is dependent on the expression of Wzd/Wze

Following the indication that Wzg may interact with Wzd, we decided to determine whether fluorescent derivatives of Wzg would also localize to where Wzd is normally found, the division septum of encapsulated bacteria. For that purpose, we constructed an N-terminal fusion of Wzg to iCFP, an improved version of CFP for *in vivo* localization of proteins in *S. pneumoniae*, recently developed by us [12]. The plasmid expressing iCFP-Wzg was transformed into the wild-type

ATCC6314 encapsulated strain (producing strain BCSMH053), into an unencapsulated derivative that lacks the *cps* operon (producing strain BCSMH054), into the ATCC6314 *wzd* null mutant (producing strain BCSMH055) and into the ATCC6314 *wze* null mutant (producing strain BCSMH056). The resulting strains were observed by fluorescence microscopy (Figure IV-3A).

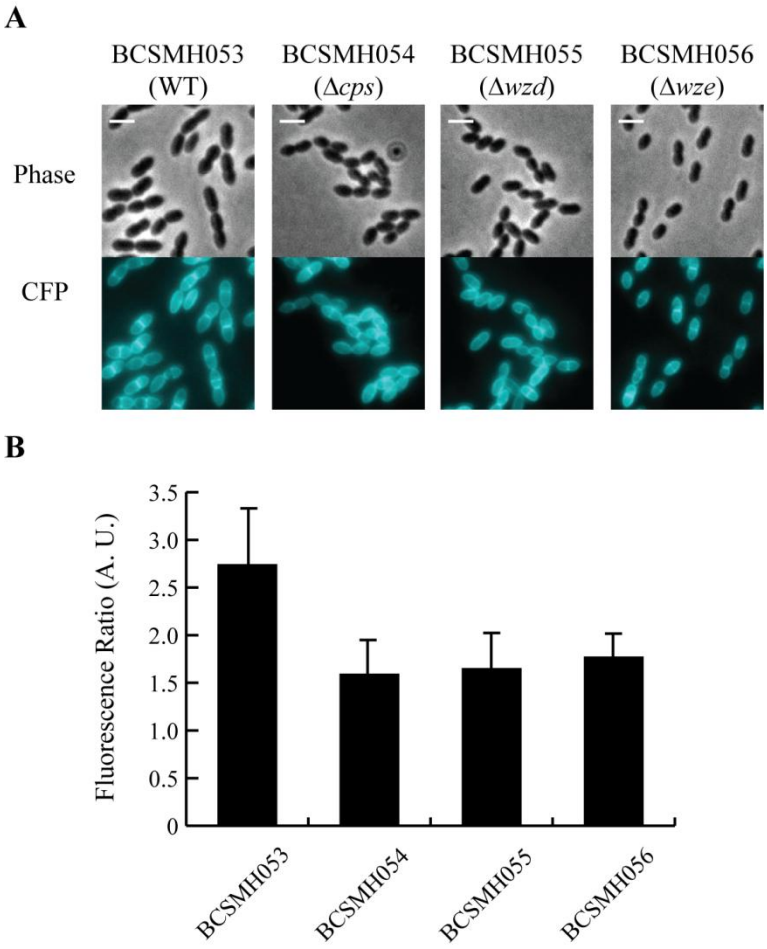

Figure IV-3: A fluorescent derivative of Wzg localizes to the division septum of *S. pneumoniae* cells, in a Wzd/Wze dependent manner. A) Localization of iCFP-Wzg fusion in the *S. pneumoniae* encapsulated strain (BCSMH053), the capsule null mutant strain (BCSMH054), the *wzd* null mutant strain (BCSMH055) and the *wze* null mutant strain (BCSMH056). Representative images are shown. Exposure times: Phase 100 msec, CFP 5000 msec. Scale bar, 2

μm. B) Fluorescence ratios determined for the strains above: BCSMH053, n= 117; BCSMH054, n= 108; BCSMH055, n= 105; BCSMH056, n= 112.

The iCFP-Wzg fusion was found to be localized at the membrane of the cells (Figure IV-3A), in accordance with the prediction of its three transmembrane spanning regions. Interestingly, in the encapsulated strain (BCSMH053) there seemed to be an enrichment of the fluorescent signal at the septal region of the cells. This enrichment was not observed in the unencapsulated strain, or in the *wzd* and *wze* null mutants (Figure IV-3A).

In order to confirm the patterns of localization observed when iCFP-Wzg was expressed in the different strains, we determined the fluorescence ratio for each strain, as described in the Materials and Methods section. FR values higher than 2 indicate that a protein is localized at the septum of the cells and not spread throughout the entire membrane. This quantification confirmed that indeed the septal region of the encapsulated strain was enriched in iCFP-Wzg. This was not observed in strains lacking the *cps* operon, the *wzd* or the *wze* genes (Figure IV-3B).

Lack of Wzg results in pneumococcal cells that produce various levels of capsule at their surface

We next decided to determine the distribution of the capsular polysaccharide at the surface of the pneumococcal cells, in the absence of Wzg. For that purpose, we constructed *wzg* null mutants as previously described [13]. Several mutants were obtained in two independent experiments. These were analyzed by immunofluorescence microscopy using a purified antibody against the serotype 14 capsular polysaccharide.

Three phenotypes of CPS distribution at the surface of the cells were obtained (Figure IV-4). In phenotype 1, represented by strain BCSMH057, the capsule was distributed all around the surface of the cells

as in the wild-type cells of the encapsulated ATCC6314 strain. However, some cells presented a fluorescence signal stronger than the wild-type cells, while others presented a weaker signal. In phenotype 2, represented by strain BCSMH058, cells were not labeled by the anti-capsular antibody, identically to the capsule null mutant BCSMC001 strain. Finally, in phenotype 3, represented by strain BCSMH059, cells presented a strong fluorescence signal, but this was not homogeneously distributed around the entire surface of the cells (Figure IV-4).

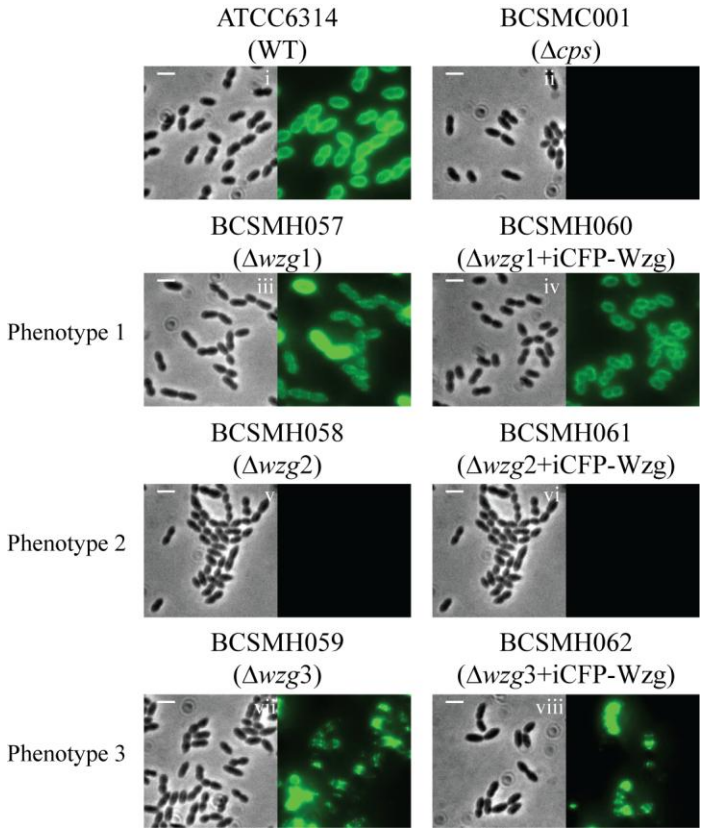

Figure IV-4: Lack of Wzg results in pneumococcal cells that produce various levels of capsule at their surface. Detection of the presence of the capsular polysaccharide at the surface of pneumococcal bacteria was carried out by immunofluorescence as described in Material and Methods. i) ATCC6314, encapsulated parental strain; ii) BCSMC001, unencapsulated ATCC6314Δ*cps*

strain; iii) BCSMH057, ATCC6314Δ*wzg* strain representative of phenotype 1; iv) BCSMH060, strain BCSMH057 expressing constitutively iCFP-Wzg; v) BCSMH058, ATCC6314Δ*wzg* strain representative of phenotype 2; vi) BCSMH061, strain BCSMH058 expressing constitutively iCFP-Wzg; vii) BCSMH059, ATCC6314Δ*wzg* strain representative of phenotype 3; viii) BCSMH062, strain BCSMH059 expressing constitutively iCFP-Wzg. Scale bar, 2 μm.

The great majority of the mutants obtained (95%, n= 19) presented a CPS distribution correspondent to phenotypes 2 (58%, n= 11) or 3 (37%, n= 7). On the other hand, only one mutant displaying the phenotype 1 of CPS distribution at the surface of the cells was obtained. Despite this low frequency, we believe that the phenotype 1 corresponds to the real phenotype caused by the absence of *wzg*, since this was the only phenotype that was complemented upon expression of the protein fusion iCFP-Wzg (Figure IV-4): strain BCSMH060, which results from transformation of strain BCSMH057 with plasmid pBCSMH039, no longer presents cells with different fluorescence intensities when capsular polysaccharide is detected by immunofluorescence. On the other hand, strains BCSMH061 and 062, expressing iCFP-Wzg constitutively from plasmid pBCSMH039, presented the same phenotype as their parental null mutant strains, BCSMH058 and 059, respectively. The phenotypes of these mutants are probably the result of suppressor mutations in other capsule genes.

Discussion

The regulation of capsule synthesis in *S. pneumoniae* has been the subject of several studies. These led to the proposal that Wzd and Wze act as the main regulators of this process, through the control of the capsule synthesis and cell wall attachment rates. Recently, we have proposed a new role for these two proteins as spatial regulators of capsule synthesis, which ensure that it occurs at the division septum, possibly to conceal the newly synthesized peptidoglycan [13]. Therefore, Wzd and Wze guarantee that the *S. pneumoniae* cell surface is completely surrounded, and protected, by the capsular polysaccharide. However, how proteins Wzd and Wze perform this role is still not known. Two hypotheses can be envisioned: Wzd/Wze may recruit other members of the CPS synthesis machinery to the division septum or, alternatively, Wzd/Wze can interact and activate other members of the CPS synthesis machinery already present at that site. In order to better understand the role of Wzd/Wze, we decided to use a Bacterial Two-Hybrid assay and test whether different proteins involved in CPS synthesis could interact with Wzd, Wze or Wzd co-expressed with Wze.

No interactions were detected between Wzd, or Wze, and WchA, the first glycosyltransferase in the synthesis of the CPS repeating unit, Wzx, the CPS flippase, and Wzy, and the CPS polymerase. Surprisingly, also no interactions were detected between Wzh, the cognate phosphatase of Wze, and Wze [24]. It is possible that this interaction is transient and therefore undetectable with the Bacterial Two-Hybrid system. In fact, a weak interaction has been found between the Wzh and Wze homologues from *Streptococcus thermophilus* using a Yeast Two-Hybrid system [5]. Alternatively, Wzh, since it dephosphoylates Wze, may only interact with the phosphorylated form of the protein. If this is the case, and because

Wzd is required for the autophosphorylation of Wze [2], we would expect that an interaction would be obtained only when Wzh is co-expressed with Wze in the presence of Wzd. However, we failed to observe an interaction between the fusions T18-Wzh and Wzh-T18 with the fusion T25-Wzd co-expressed with Wze. It is possible that co-expression of Wzh with tagged Wze in the presence of Wzd or with the tagged Wzd-L11-Wze chimeric protein will produce a positive result. A direct fusion of the *S. thermophilus* Wzd and Wze homologues did not interact with Wzh probably due to conformational problems [5]. This may not occur in the Wzd-L11-Wze chimera, as this protein seems to be functional [13]. This chimera construct has a 11 amino acids linker connecting Wzd and Wze that should allow the mobility of both proteins.

In this work, we found that the CPS-cell wall ligase Wzg, may interact with Wzd in a bacterial two-hybrid assay. *E. coli* colonies expressing both proteins, linked to different fragments of the catalytic domain of *Bordetella pertussis* adenylate cyclase, were light blue. We hypothesized that the weak signal could be due to the fact that Wzg interacts with the pair Wzd/Wze, and not with Wzd alone. Therefore we tested the interactions of Wzg with several combinations of Wzd and Wze co-expressed, as well as with the chimera protein Wzd-L11-Wze. However, an increase in the intensity of the color of the colonies on indicative plates did not occur. The light blue color obtained for the colonies expressing the pair of fusions T18-Wzg + T25-Wze, co-expressed with Wzd, and T25-Wzg + T18-Wze, co-expressed with Wzd, may result not from a direct interaction of Wzg with Wze, but from an interaction via Wzd, as Wze is known to interact with Wzd [13]. This would generate a complex Tagged-Wzg/Wzd/Tagged-Wze where the T18 and T25 tags could be brought close enough to produce blue colonies. Protein Wzg was also found to interact with itself.

Since one of our initial hypotheses was that Wzd/Wze could spatially regulate the capsule synthesis by recruiting other capsule proteins to the division septum, we decided to determine if the presence of Wzd/Wze was required for the correct localization of Wzg. We therefore determined the localization of Wzg, fused at its N-terminus end to iCFP. As expected due to its three transmembrane domains, Wzg was found to be present at the membrane of the pneumococcal cells. Interestingly, the Wzg fluorescent derivative was enriched at the septum of the wild-type encapsulated cells, as recently described by other authors for the *S. pneumoniae* encapsulated D39 and unencapsulated R6 strains [7]. However, we did not observe the septal enrichment of Wzg in the *cps* operon null mutant strain and, more importantly, in the absence of proteins Wzd or Wze. Our results are in disagreement with those obtained by Eberhardt and colleagues [7], and the reason for this discrepancy remains to be determined.

In the absence of Wzg, the capsular polysaccharide is produced and present all around the surface of the pneumococcal cells. However, the levels of CPS seem to differ between cells, as seen by the different fluorescent intensity signals observed. This phenotype is identical to the one reported for serotype 2 strain D39 [23]. The fact that capsule is still produced and attached to peptidoglycan in the absence of Wzg, the CPS-cell wall ligase is in accordance with previous studies [7]. These showed that proteins from the LCP protein family to which Wzg belongs have (semi)redundant roles. Therefore, in the absence of Wzg other ligases may fulfill its role and attach the capsular polysaccharide to the peptidoglycan.

The determination of the function of Wzg in the attachment of the capsular polysaccharide to peptidoglycan has been reported very recently [16]. Based on the homology of its C-terminal domain with LytR [9], a *B. subtilis* transcriptional regulator [20], it was believed for a long time that

Wzg regulated the transcription of the *cps* operon. In fact, in *Streptococcus agalactiae*, recent evidence that the homologue protein CpsIA functions as a transcriptional regulator has been provided [6]. Moreover, the latest data regarding the Wzg homologues from *Streptococcus iniae* and *S. agalactiae* shows that these proteins bind specifically to DNA containing the capsule promoter region [10-11]. Interestingly, the extracellular domain of Wzg, the one whose structure has been solved and which is involved in the attachment of the capsule to the cell wall, is not involved in DNA binding [10-11]. DNA binding occurs through the less than 30 amino acids of Wzg present in the cytoplasm, but the sequential truncation of the *S. agalactiae* Wzg extracellular domain from its C-terminus led to a decrease in the specificity of the DNA binding [11]. It seems therefore plausible that Wzg has two roles in the regulation of the CPS assembly. It may attach the capsular polysaccharide and also control the transcription from the *cps* operon. The binding to DNA, or to the CPS polymer, could be modulated by conformational changes in Wzg. It is tempting to speculate that the conformational changes proposed to occur during CPS synthesis, and to regulate this process through the cycles of phosphorylation and dephosphorylation at the C-terminal tyrosines of Wze, are transmitted to Wzg by its interaction with Wzd, possibly through their extracellular domains. Wzg could be recruited to the division septum by Wzd and attach the capsular polysaccharide at that site. A second function would be the regulation of the transcriptional levels of the operon mediated by structural changes in Wzg that modulate its binding to DNA. These hypotheses remain to be tested.

In this work we aimed to elucidate how *S. pneumoniae* capsule synthesis is spatially regulated by proteins Wzd and Wze. We found that the CPS-cell wall ligase weakly interacts with Wzd. Wzd seems to recruit

Wzg to the division septum, since in its absence or when it is delocalized from the septum due to the absence of Wze, the septal enrichment of Wzg is no longer observed. Therefore, Wzd guarantees the attachment of the capsular polysaccharide at the septum by recruiting the Wzg ligase.

Acknowledgments

This study was funded by Fundação para a Ciência e Tecnologia (FCT), Lisbon, Portugal, through research grants PTDC/SAU-MII/75696/2006 and PTDC/BIA- MIC/100747/2008 awarded to S. R. F. The work performed at Instituto de Tecnologia Química e Biológica was supported additionally by FCT through grant # PEst-OE/EQB/LA0004/2011. MXH was supported by FCT fellowship SFRH/BD/ 43797/2008. MXH designed and performed the experiments described in this chapter.

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Supplementary Information

Table SIV-1: Bacterial strains.

Name	Relevant characteristics	Comments/Source/ Reference
E. coli		
DH5α	*recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1* Φ80 d*lac*Z ΔM15	Gibco-BRL
BTH101	Reporter strain for BTH system, F^- *cya*-99, *ara*D139, *gal*E15, *gal*K16, *rps*L1 (Str^r), *hsd*R2, *mcr*A1, *mcr*B1	[15]
EC101	*supE thi* (*lacproAB*) (F' *traD36 proAB lacI*^{q} ZΔM15), *repA* from pWV01 integrated in the chromosome	[19]
L. lactis		
LL108	$RepA^+$ MG1363, Cm^r	[21]
S. pneumoniae		
ATCC6314	Encapsulated strain, serotype 14	American Type Culture Collection
BCSMC001	ATCC6314Δ*cps*	[13]
BCSMH001	ATCC6314Δ*wzd*	[13]
BCSMH002	ATCC6314Δ*wze*	[13]
BCSMH053	ATCC6314 transformed with pBCSMH039.	This work.
BCSMH054	ATCC6314Δ*cps* transformed with pBCSMH039.	This work.
BCSMH055	ATCC6314Δ*wzd* transformed with pBCSMH039.	This work.
BCSMH056	ATCC6314Δ*wze* transformed with pBCSMH039.	This work.
BCSMH057	ATCC6314Δ*wzg*1	This work.
BCSMH058	ATCC6314Δ*wzg*2	This work.
BCSMH059	ATCC6314Δ*wzg*3	This work.
BCSMH060	BCSMH057 transformed with pBCSMH039.	This work.

Table SIV-1 (Cont.): Bacterial strains.

Name	**Relevant characteristics**	**Comments/Source/ Reference**
BCSMH061	BCSMH058 transformed with pBCSMH039.	This work.
BCSMH062	BCSMH059 transformed with pBCSMH039.	This work.

Table SIV-2: Plasmids.

Name	**Relevant characteristics**	**Comments/Source/ Reference**
Plasmids for Bacterial Two Hybrid Assay		
pUT18C	BTH plasmid, N-terminal *cya*AT18 fusion, Amp^r.	[15]
pUT18	BTH plasmid, C-terminal *cya*AT18 fusion, Amp^r.	[15]
pKT25	BTH plasmid, N-terminal *cya*AT25 fusion, Kan^r.	[15]
pKNT25	BTH plasmid, C-terminal *cya*AT25 fusion, Kan^r.	[15]
pKT25zip	BTH control plasmid, Kan^r.	[15]
pUT18Czip	BTH control plasmid, Amp^r.	[15]
pBCSMC001	BTH plasmid containing *cya*AT25-*wzd* fusion.	[13]
pBCSMC002	BTH plasmid containing *cya*AT18-*wze* fusion.	[13]
pBCSMC003	BTH plasmid containing *cya*AT18-*wze(WA)* fusion.	[13]
pBCSMC004	BTH plasmid containing *cya*AT18-*wze(Y)* fusion.	[13]
pBCSMH014	BTH plasmid containing *cya*AT18-*wze(WA2)* fusion.	[13]
pBCSMC005	BTH plasmid containing *cya*AT18-*wzd* fusion.	This work.
pBCSMH040	BTH plasmid containing *wzd*-*cya*AT18 fusion.	This work.

Table SIV-2 (Cont.): Plasmids.

Name	Relevant characteristics	Comments/Source/ Reference
pBCSMC007	BTH plasmid containing *wzd-cyaA*T25 fusion.	This work.
pBCSMC006	BTH plasmid containing *wze-cyaA*T18 fusion.	This work.
pBCSMC008	BTH plasmid containing *cyaA*T25-*wze* fusion.	This work.
pBCSMC009	BTH plasmid containing *wze-cyaA*T25 fusion.	This work.
pBCSMH041	BTH plasmid containing *cyaA*T18-*wze* fusion and *wzd*.	This work.
pBCSMH042	BTH plasmid containing *wzd-cyaA*T18 fusion and *wze*.	This work.
pBCSMH043	BTH plasmid containing *cyaA*T18-*wzd-L11-wze* fusion.	This work.
pBCSMH044	BTH plasmid containing *wzd-L11-wze- cyaA*T18 fusion.	This work.
pBCSMH045	BTH plasmid containing *cyaA*T25-*wze* fusion and *wzd*.	This work.
pBCSMH046	BTH plasmid containing *cyaA*T25-*wzd* fusion and *wze*.	This work.
pBCSMH047	BTH plasmid containing *cyaA*T25-*wzd-L11-wze* fusion.	This work.
pBCSMH048	BTH plasmid containing *wzd-L11-wze- cyaA*T25 fusion.	This work.
pBCSMH049	BTH plasmid containing *cyaA*T18-*wzg* fusion.	This work.
pBCSMH050	BTH plasmid containing *wzg-cyaA*T18 fusion.	This work.
pBCSMH051	BTH plasmid containing *cyaA*T25-*wzg* fusion.	This work.
pBCSMH052	BTH plasmid containing *wzg-cyaA*T25 fusion.	This work.
pBCSMH053	BTH plasmid containing *cyaA*T18-*wchA*fusion.	This work.
pBCSMH054	BTH plasmid containing *wchA-cyaA*T18 fusion.	This work.

Table SIV-2 (Cont.): Plasmids.

Name	Relevant characteristics	Comments/Source/ Reference
pBCSMH055	BTH plasmid containing *cyaA*T18-*wzx* fusion.	This work.
pBCSMH056	BTH plasmid containing *wzx*-*cyaA*T18 fusion.	This work.
pBCSMH057	BTH plasmid containing *cyaA*T18-*wzy* fusion.	This work.
pBCSMH058	BTH plasmid containing *wzy*-*cyaA*T18 fusion.	This work.
pBCSMC010	BTH plasmid containing *cyaA*T18-*wzh* fusion.	This work.
pBCSMC011	BTH plasmid containing *wzh*-*cyaA*T18 fusion.	This work.
pBCSMC012	BTH plasmid containing *cyaA*T25-*wzh* fusion.	This work.
pBCSMC013	BTH plasmid containing *wzh*-*cyaA*T25 fusion.	This work.
Replicative plasmids in *S. pneumoniae*		
pBCSMH031	Allows expression of iCFP fusion proteins, Tet^r.	[12]
pBCSMH039	pBCSMH031 containing *iCFP*-*wzg*, Tet^r.	This work.
pBCSTR001	Contains *wzd-L11-wze-citrine*, Tet^r.	[13]
Non replicative plasmids in *S. pneumoniae*		
pORI280	Em^r, $LacZ^+$, ori^+ of pWV01	[21]
pBCSMH059	pORI280 containing up- and downstream regions of *wzg*, Em^r, $LacZ^+$	This work.

Table SIV-3: Primers used in this work.

Primer	Sequence 5'→3'	Features/ Restriction sites
1	CGGGATCCCGGAGAAAATATGAAGG	BamHI
2	GGAATTCCCCTCTCTCCTATTTC	EcoRI
3	CTAGTCTAGAGATGAAGGAACAAAACACTTTGG	XbaI
4	CGGGGATCCCCTTTCAACTTACTCAAGTTTGG	BamHI
5	CGGGATCCTAAGGAGGAAATATGA	BamHI
6	GGAATTCCATTTCAACTTACTCAAG	EcoRI
7	CGGGGATCCTAGGAGGGAGGAATGCCG	BamHI
8	GGAATTCCATTTTTTACCATAATTTCC	EcoRI
9	CGGGGATCCAATGCCGACATTAGA	BamHI
10	GGAATTCCCCTAAGTTATTTTTTACC	EcoRI
11	CGGAATTC**TAAGGAGGT**GCTGCGGCCATGAAGGAACAAAACACTTTGG	EcoRI, **RBS**
12	CGGAATTCCTATTTCAACTTACTCAAGTTTGG	EcoRI
13	CCCAAGCTT**TAAGGAGGT**GCTGCGGCCATGCCGACATTAGAAATAGC	HindIII, **RBS**
14	GCTCTAGATTATTTTTTACCATAATTTCC	XbaI
15	CTAGTCTAGAGATGAAGGAACAAAACACTTTGG	XbaI
16	CGGGGATCC**TAAGGAGGT**GCTGCGGCCATGCCGACATTAGAAATAGC	BamHI, **RBS**
17	CGCGGATCCAATGAGTAGACGTTTTAAAAAATCACG	BamHI
18	CCGGAATTCTCATCTACCCTCCATCACATCC	EcoRI
19	CCGGAATTCTTTCTACCCTCCATCACATCC	EcoRI
20	AACTGCAGAATGGATAAAAAAGGATTGG	PstI
21	CGCGGATCCTTACTTCGCTCCATTTCTC	BamHI
22	CGCGGATCCTTCTTCGCTCCATTTCTC	BamHI
23	AACTGCAGAATGAGTAATAAAATCAG	PstI
24	CGCGGATCCTTATAATATATTTTCATAACC	BamHI
25	CGCGGATCCTTTAATATATTTTCATAACC	BamHI
26	AACTGCAGAATGAAAATTAGAATAG	PstI
27	CGCGGATCCTCATCCAACTATTTCC	BamHI
28	CGCGGATCCTTTCCAACTATTTCC	BamHI
29	CGGGATCCCGAAATGATAGACATCC	BamHI

Table SIV-3 (Cont.): Primers used in this work.

Primer	**Sequence 5'→3'**	**Features/ Restriction sites**
30	GGAATTCCCTCCTAAATTAATTGATCC	EcoRI
31	CGGGATCCTGAAGGAGGGTAGATGAAATG	BamHI
32	GGAATTCCAAATTAATTGATCCATTAT	EcoRI
33	ATAAGAATGCGGCCGCAATGAGTAGACGTTT TAAAAAATCACG	NotI
34	GAAGATCTTCATCTACCCTCCATCACATCC	BglII
35	CGCGGATCCGGTAAGAAAAACTTCTG	BamHI
36	CTACTATCATCGATTAACACCTATACCTTGAA CATCGTAC	Region overlapping primer 37
37	GGTGTTAATCGATGATAGTAGTTTAGCTGTA GTTAAAGC	Region overlapping primer 36
38	CCGGAATTCGGTTTTGAAACATGATAAC	EcoRI

Chapter V

General Discussion and

Future Perspectives

The cell wall structure, which surrounds the surface of bacteria, constitutes the frontier, and therefore the first contact, between a pathogen and the infected host. The capsular polysaccharide, a major component of the cell wall that surrounds *S. pneumoniae* cells, has been extensively investigated throughout the years.

In this thesis, we contributed to the elucidation of the regulation of capsular polysaccharide synthesis in *S. pneumoniae*. We unraveled a new role for the proteins Wzd and Wze, which form a complex that localizes at the division septum and act as spatial regulators. This ensures CPS is expressed around the entire surface of the cells [3]. We shed some light on how Wzd/Wze fulfill this function, since our preliminary results indicate that Wzg, the CPS-cell wall ligase, is recruited to the septum when Wzd is produced in the presence of Wze. Importantly, our results suggest the existence of two CPS synthesis machineries, similar to what has been proposed for peptidoglycan synthesis [8, 15]. In order to perform our studies we have developed novel tools for *in vivo* protein localization in *S. pneumoniae*. These tools constitute an important help to researchers working in the field of pneumococcal cell biology and should be of great use in the future.

Despite the important contributions described in this thesis towards the understanding of *S. pneumoniae* CPS synthesis and regulation, many questions still remain and are currently being addressed.

How does the Wzd/Wze complex localize to the septum?

We have shown that Wzd and Wze, the well accepted main regulators of capsule synthesis in *S. pneumoniae*, interact when the Walker A ATP binding motif of Wze is intact. This complex is then able to localize to the division septum of the pneumococcal cells, without

requiring any other protein encoded in the *cps* operon. However, the factors that determine the septal localization of proteins Wzd and Wze are still unknown.

The localization of the Wzd/Wze complex to the septal region of *S. pneumoniae* may require its recruitment by another protein, not involved in capsule synthesis. This hypothesis implies that proteins Wzd and Wze, as a complex, should interact with that unknown protein. Also, the recruiting protein should be distributed in the pneumococcal cell with an identical pattern as Wzd/Wze, but independent on the ability of the bacteria to produce CPS. PBPs are attractive candidates to fulfill this role. First, these proteins were shown to localize at the midcell region [7, 12]. Second, the *S. pneumoniae* capsular polysaccharide is covalently attached to the peptidoglycan [17], indicating that proteins involved in the assembly of the two macromolecules may interact, or probably share a similar localization, during a specific window of the bacterial cell cycle. Third, we showed that Wzd/Wze co-localized with the signal from fluorescent vancomycin labeling, i. e., co-localized with the sites where synthesis of peptidoglycan is taking place. In order to enquire whether Wzd/Wze can be recruited to the division septum by PBPs the localization of Wzd/Wze should be determined in the absence of the PBPs, when possible, and/or in conditions that affect their localization, such as incubation with specific antibiotics that target different steps of peptidoglycan synthesis. Moreover, we could also test their ability to interact directly using Bacterial Two-Hybrid and/or pull-down assays.

Proteins MreC and MreD are also distributed in the pneumococcal cell in a very identical pattern as Wzd/Wze [8]. Furthermore, their role in peripheral peptidoglycan synthesis makes them also possible candidates for the recruitment of the complex. StkP shows the same apparent localization pattern as Wzd/Wze [1-2] and interestingly it delocalizes in

the presence of antibiotics that affect the latest stages of peptidoglycan synthesis [1]. Other proteins, which seem to distribute identically to Wzd/Wze, like FtsA, DivIB and FtsL [9, 14], also constitute possible candidates for the recruitment of Wzd/Wze and would be interesting to test.

Another hypothesis for the localization of the complex Wzd/Wze to the division septum is the recognition of a specific motif present at the septal region. Membrane curvature has been recently shown to act as a cue for the localization of two proteins, SpoVM and DivIVA, in *B. subtilis* [16]. We have shown that Wzd/Wze localize to the division region early in the cell cycle, so the hypothesis of a membrane curvature dependent Wzd/Wze localization should not be ruled out.

Alternatively, the enrichment of certain phospholipids in discrete regions of the membrane may be required for the localization of Wzd/Wze to the division septum. In *E. coli* and *B. subtilis*, enrichment of cardiolipin at the septum and poles has also been identified as a protein targeting effector [11]. MinD interacts with the membrane through an amphipathic helix that has a preference for anionic phospholipids such as cardiolipin [11]. Interestingly, Wze belongs to the same superfamiliy of P-loop NTPases as MinD, the MinD/Mrp-Etk superfamily [10]. One can speculate that an identical mechanism of localization might be at place for the localization of Wzd/Wze. To our knowledge, no studies on the existence of lipid rich domains in the *S. pneumoniae* membrane have been reported.

How does the Wzd/Wze complex control CPS synthesis at midcell?

In this thesis, we have shown that the complex Wzd/Wze is required for the expression of capsular polysaccharide all around the surface of the *S. pneumoniae* cells. In the absence of one of the proteins, or in conditions in which the complex cannot be formed, such as prevention of ATP binding to Wze, we observed that CPS is produced and attached to peptidoglycan, but it is absent from the new, septal wall. Therefore, we have proposed that proteins Wzd and Wze are spatial regulators of capsule synthesis that guarantee that this process occurs at the division septum. How do proteins Wzd/Wze perform this regulation is an aspect that we are currently trying to elucidate.

Two hypotheses can be envisioned for the control of CPS expression at midcell by Wzd and Wze: i) the Wzd/Wze complex recruits to the septum one or more proteins involved in CPS synthesis; ii) the Wzd/Wze complex activates one or more proteins involved in CPS synthesis that are already localized at the division septum. In either hypotheses, the CPS proteins that, based on their function and the current model for CPS synthesis [5, 13], are more likely to be under the regulation of Wzd/Wze are Wzx, the capsular polysaccharide flippase; Wzy, the capsular polysaccharide polymerase; and Wzg, the capsule-cell wall ligase.

Interestingly, we found that Wzg can interact with Wzd in a Bacterial Two-Hybrid assay. More importantly, we observed that Wzg could localize at the septum of encapsulated pneumococcal cells. This enrichment was not present in the unencapsulated strain or in the absence of Wzd or Wze. This suggests that the complex Wzd/Wze can recruit the Wzg ligase to the septum and that the lack of capsular polysaccharide at

that site in the *wzd* and *wze* null mutants might be due to its inefficient attachment at that location. These results are still preliminary and pull-down experiments to confirm the interaction between Wzg and Wzd are needed. It would also be interesting to test if the defect in capsule-cell wall attachment observed previously in *S. pneumoniae* expressing Wzd single residue mutants [13] is due to a deficient interaction of Wzd with Wzg. If this is the case, Wzg should be delocalized when these single residue Wzd mutants are expressed, identically to the *wzd* null mutant. Interestingly, the referred mutations are in aminoacids localized in the extracellular region of Wzd. Therefore, it is tempting to speculate that Wzd and Wzg interact through their extracellular domains.

Although no interactions have been found between Wzd/Wze and proteins Wzx and Wzy using the Bacterial Two-hybrid assay, we cannot rule out their existence. More experiments are needed to ascertain if besides Wzg other capsule proteins are under the control of Wzd/Wze. This will further elucidate the manner in which CPS synthesis proceeds.

Are there two CPS synthesis modes in *S. pneumoniae*?

Perhaps one of the most striking results obtained in this thesis is the indication that, similarly to the synthesis of peptidoglycan, there are two modes to synthesize CPS. The concept of two cell wall synthesis machineries, one peripheral and the other one septal, as emerged early in ovococcus cell division studies [4] and has recently gained strong support [8, 15]. Our capsular polysaccharide immunofluorescence results point to an identical phenomenon regarding CPS synthesis. The observed distribution of capsule on the surface of the pneumococcal cells that lack Wzd or Wze can be explained by the existence of two modes of synthesis,

one associated with peripheral cell wall and the other with septal cell wall (Figure V-I), which can be discriminated by fluorescence microscopy.

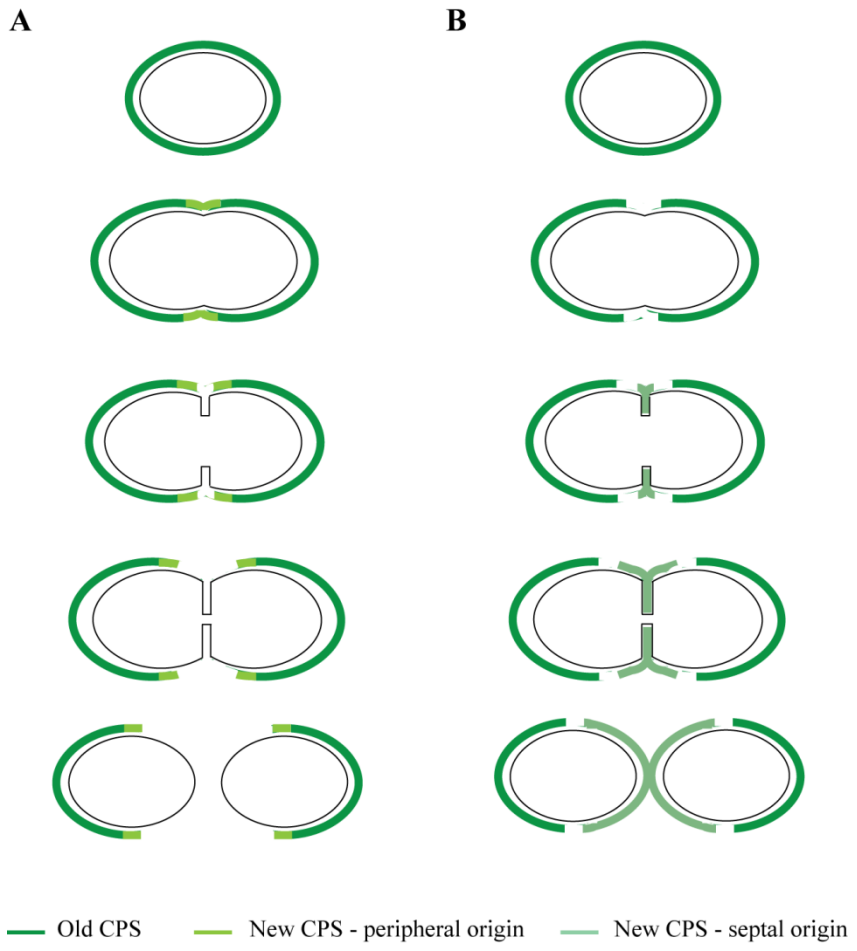

Figure V-1: Production of capsular polysaccharide associated with the septal and peripheral cell wall. Schematic representation of the expected patterns of CPS expression at the surface of *S. pneumoniae* cells when one mode of synthesis, septal (A) or peripheral (B), is not-functional.

Careful analysis of *wzd* and *wze* null mutant cells and integration with the current model for *S. pneumoniae* cell wall synthesis allows us to propose the type of cell wall with which Wzd/Wze are associated. The

association of Wzd/Wze with septal growth should lead to the appearance of half-stained cells, as represented in Figure V-IA, and cells stained only at the middle region. These were not observed. On the other hand, association of Wzd/Wze with peripheral growth would lead to the lack of fluorescence signal only at the middle region of the cells (Figure V-IB), the pattern observed in the *wzd* and *wze* null mutants [3]. Therefore, we favor the hypothesis that the complex Wzd/Wze regulates the synthesis of CPS, and its attachment to peptidoglycan, at the peripheral cell wall.

In order to test this model, the expression of capsule at the surface of cells in conditions of peripheral or septal growth inhibition should be examined. If Wzd and Wze are indeed associated with the production of CPS attached to the peripheral cell wall, and not necessary for the synthesis of CPS linked to the septal cell wall, then the inhibition of the peripheral cell wall growth in the *wzd* and *wze* null mutants would lead to round cells expressing capsule all around their surface. On the other hand, inhibition of septal growth would lead to elongated cells lacking capsular polysaccharide at multiple sites.

Since the proteins known to be specifically involved in the different types of growth, peripheral (MreCD, PBP2b) and septal (PBP2x) [8, 15] are essential [6, 8], this model cannot be tested through the construction of knockout mutants. Instead, inhibition of peripheral and septal growth can be achieved through the use of β-lactam antibiotics from different classes that differentially target the PBPs. One hypothesis is the use of piperacillin and cefotaxime, which displayed preferential affinities for PBP2b and PBP2x [18], respectively, allowing us to distinguish between the peripheral and septal modes of growth.

Concluding remarks

The *S. pneumoniae* capsular polysaccharide is one of the main virulence factors of this major pathogen. It is essential for the bacterium during the different stages of interaction with its host and the amount of CPS expressed at the surface of the cells in these different environments is highly regulated. For these reasons, the *S. pneumoniae* capsular polysaccharide has been the subject of several studies, many with the aim of developing new and more efficient strategies against pneumococcal infection. In order to achieve this goal, a complete understanding of the manner in which CPS synthesis occurs and is regulated is of central importance. The results obtained in this thesis have no doubt contributed to the elucidation of the capsular synthesis process in *S. pneumoniae*. However, many questions still remain, some raised by the results described in this manuscript. The answer to those questions will have important implications in the development of new strategies to fight *S. pneumoniae* infections.

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