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Wzy-dependent Bacterial Capsules as Potential Drug Targets

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Abstract

The bacterial capsule is a recognized virulence factor in pathogenic bacteria. It likely works as an anti-phagocytic barrier by minimizing complement deposition on the bacterial surface. With the continual rise of bacterial pathogens resistant to multiple antibiotics, there is an increasing need for novel drugs. In the Wzy-dependent pathway, the biosynthesis of capsular polysaccharide (CPS) is regulated by a phosphoregulatory system, whose main components consist of bacterial-tyrosine kinases (BY-kinases) and their cognate phosphatases. The ability to regulate capsule biosynthesis has been shown to be vital for pathogenicity, because different stages of infection require a shift in capsule thickness, making the phosphoregulatory proteins suitable as drug targets. Here, we review the role of regulatory proteins focusing on *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Escherichia coli* and discuss their suitability as targets in structure-based drug design.

Keywords

antibiotic resistance, bacterial pathogens, capsule, drug discovery, protein tyrosine kinase, protein tyrosine phosphatase, Wzy.

Introduction

The continual rise of multiply antibiotic resistant bacterial pathogens is resulting in infections which are almost untreatable. The further lack of novel anti-microbials in the drug development pipeline may result in us retreating to a pre-antibiotic era [1]. Thus, the search for novel targets for anti-infectious agents is of utmost importance.

Polysaccharide capsules are critical determinants for the ability of a wide range of bacterial pathogens to cause invasive disease. They form the outermost layer of the cells, and are thought vital due to their ability to act as an anti-phagocytic factor. Our work focuses on understanding the mechanisms behind the biosynthesis of Wzy-dependent capsules, with a focus on the major human pathogen *Streptococcus pneumoniae.* However, other pathogens also possess Wzy-dependent capsules important for their virulence, including *Staphylococcus aureus, Escherichia coli* and *Klebsiella pneumoniae*.

The capsule of *S. pneumoniae* has been used as a relatively successful target for the development of vaccines, resulting in significant decreases in carriage and invasive disease. However, to date no drugs have been developed that target the biosynthesis and regulation of this crucially important virulence factor, even though unencapsulated bacteria are essentially avirulent.

The intent of this review is to provide an outline of how the mechanisms governing capsule synthesis and regulation can be approached as drug targets, as applied to major human pathogens. We will discuss the importance to virulence of the capsule, as well as the mechanisms involved in its synthesis and regulation, with a particular focus on *S. pneumoniae*. Finally, we will discuss some proteins which we feel could be targeted in an effort to produce novel antimicrobials to help us in the continuing fight against infectious disease.

Capsule as a virulence factor

Polysaccharide capsules are produced by an array of both Gram-positive and -negative bacteria, for all of whom they are important for virulence. In recent times, much work has been completed on furthering our understanding of the mechanisms involved in the contribution it makes to pathogenicity. We are focused on the synthesis and regulation of the capsule in *S. pneumoniae*, as for this pathogen it is widely recognised as its major virulence factor.

S. pneumoniae

Pneumonia is the single biggest killer of children under the age of five. As the major causative agent, *S. pneumoniae* alone accounts for 700,000 to 1 million deaths annually among children aged 1-59 months [2,3]. This includes deaths mainly due to pneumonia, meningitis, and febrile bacteremia, but not the compounding effects of co-infection with HIV. According to the World Health Organisation (WHO), the biggest disease burden is in the WHO African Region and WHO South-East Asia Region. With the majority of cases occurring in developing countries lacking proper estimates, however, the true scope of disease burden is unknown. In 2000 alone, 14.5 million cases of serious pneumococcal infection is estimated to have occurred in children aged 1-59 months, resulting in approximately 826,000 deaths [4].

As can be expected, HIV and other immune deficiencies greatly increase the risk of contracting pneumococcal disease, consequently mortality among HIV positive individuals is substantially higher.

Among conditions with a high morbidity but lower mortality, *S. pneumoniae* is the most common cause of otitis media, making it the single largest reason for prescribing antibiotic treatment [5]. This makes it responsible for massive impacts on health budgets in developed nations, with estimates in the US alone > 2 billion USD annually.

The pathogenesis of *S. pneumoniae* begins with asymptomatic colonisation of the nasopharynx, its natural reservoir responsible for the spread of the bacteria from person to person. The pathogen can then undergo a switch to invasive disease, resulting in upper or lower respiratory tract infections; give the bacteria the opportunity to cross over into the blood stream; or even cross the blood-brain barrier, with pneumonia, bacteremia, and meningitis, respectively, as possible outcomes. Furthermore, the increasing development of multi-antibiotic resistant strains and the inefficiencies associated with the current vaccines, means that the need for new treatments is vital. Traditional problems with compliance with antibiotic treatment regimens [6,7] have inevitably been a compounding factor in the rise of penicillin resistant *S. pneumoniae*. Treatment is further complicated by the fact that penicillin resistant strains tend to be multi-drug resistant as well. Many virulence factors are implicated in the ability of the pathogen to cause disease, and these have been reviewed recently [8]. However, the capsule is widely recognized as the one critical factor and as such has been a major focus of research into the understanding of this pathogen.

The polysaccharide capsule is the serotype-determining factor, and all but two of the 93 serotypes of *S. pneumoniae* found to date have the capsule synthesised by a Wzy-dependent mechanism [9]. Thus, this represents a promising target for drug development. The capsule is the target of the currently used vaccines. However, these provide serotype-specific protection, and as such protection is limited. Indeed, the very successful heptavalent pneumococcal conjugate vaccine (PCV7) targets only 7 serotypes. While it has provided protection in many populations since its introduction, and a recently introduced 13-valent vaccine shows promise, continual studies are illustrating that serotype replacement is occurring, resulting in a rise in the carriage prevalence of uncovered serotypes [10].

The importance of the capsule is evident in a number of different ways. All clinical isolates of *S. pneumoniae* causing invasive disease are encapsulated. Indeed, unencapsulated bacteria have only been associated with superficial infections such as conjunctivitis, and the capsule has been shown to enhance such infections [11]. Numerous studies have shown significant correlations of serotype with nasopharyngeal carriage prevalence, invasiveness, disease incidence, and even risk of death due to pneumococcal pneumonia [12]. This need for capsule has also been illustrated in numerous pneumococcal animal models of infection. One of the earliest studies by MacLeod & Krauss [13] showed the importance of capsule by correlating virulence in mice with the levels of capsule produced by different strains *in vitro*. In more recent times, defined mutations have produced similar results. Morona *et al.* [14,15] used defined mutations in various capsule regulatory genes (discussed below), which leads to various defects in the ability to produce capsule, and these strains all showed significant defects in the ability to colonise and cause invasive disease. The importance of capsule to

asymptomatic colonisation has also been illustrated using defined mutations in multiple strain backgrounds [16,17].

Thus, while the importance of the capsule to pneumococcal virulence is unquestioned, the obvious question is what precise role does it play? This role has long been postulated to involve its ability to act as an anti-phagocytic barrier. However, until recently there have been few studies investigating the exact mechanisms behind this. A recent study by Hyams *et al*. [18] examined this in more detail. The complement system is recognised as a crucial component of the host's ability to fight off infection by the pneumococcus, with neutrophil opsonophagocytosis of *S. pneumoniae* known to be largely dependent on complement [19,20]. Using defined unencapsulated mutants in multiple strain backgrounds, the authors illustrated the capsule is able to inhibit the classical complement pathway by preventing binding of both IgG and C-reactive protein, as well as reducing the activation of the alternative complement pathway [18]. Unencapsulated bacteria were more heavily opsonised by C3b/iC3b, as had been seen previously [21,22], which resulted in increased phagocytosis by neutrophils. Even in the absence of complement and antibody, unencapsulated bacteria were phagocytosed to a higher degree and killed. Thus, this clearly illustrated that the capsule employs multiple mechanisms to protect the pneumococcus from phagocytosis.

While numerous virulence factors undoubtedly contribute to the pathogen's ability to resist complement deposition and phagocytosis [23–25], two recent studies have illustrated the central dominance of the capsule in this strategy [24,26]. Both of these studies showed the fundamental importance of capsule by leaving the genetic background the same and only changing the capsule type. Using these strains they showed capsule serotype changes resulted in marked differences in C3 deposition, neutrophil phagocytosis and infection using *in vivo* models. These data correlate closely with the knowledge that capsule types differ in their epidemiological properties. Interestingly, these differences were unrelated to any significant difference in capsule thickness between the strains [27].

Additional recent studies have investigated the size and structure of the capsular polysaccharides of *S. pneumoniae* as a critical feature, testing if thicker capsule is more advantageous for infection [13]. Weinberger *et al.* [28] showed a correlation between the thickness of capsule, increased resistance to *in vitro* killing by neutrophils, and increased prevalence in nasopharyngeal colonisation in the community. Interestingly, they further identified a link between the more prevalent serotypes and their polysaccharide structure, suggesting that serotypes that produce less metabolically costly polysaccharides produce thicker capsules, and are thus more prevalent. A recent study has implicated another reason for the selection of particular serotypes. Lysenko *et al*. [29] used mathematical modelling as well as *in vivo* animal models to suggest the intraspecies competition, such as between *S. pneumoniae* and *H. influenzae* in the nasopharynx, will lead to the selection of particular capsular serotypes within the host.

However, the story of capsule is not all that simple. Indeed, while the capsule is critical for the ability of cells to cause disease, it also hinders the ability of the pathogen to attach to host epithelial cells [30,31], likely due to its ability to shield adherence factors [32]. The capsule has also been shown to inhibit the formation of biofilms of *S. pneumoniae in vitro* [33]. Additionally, the pneumococcus is known to undergo spontaneous phase variation between two phenotypes, opaque and transparent [34].

While the exact mechanism behind this is unknown, capsule is one factor that varies between the two. In the opaque phase, which is prevalent in invasive disease, capsule levels are significantly higher than those seen in the transparent phenotype, which are favoured in the asymptomatic colonisation of the nasopharynx [35]. The capsule is also known to be recognized by the host immune system, via C-type lectin expressed by macrophages in the spleen (SIGN-R1). With the spleen playing an important role in pneumococcal clearance [36], it is not surprising that mice with this receptor knocked out showed reduced survival to infection [37].

Thus, these observations suggest that in all situations a thicker capsule may not be optimal, and that regulation will be critical. Mechanisms other than resistance to opsonophagocytosis have also been implicated in the importance of the capsule. For instance, the capsule has been shown to interfere with the association of *S. pneumoniae* with neutrophil extracellular traps [38], and it can increase tolerance to antibiotics through an inhibition of lysis [39]. Additionally, Nelson *et al.* [16] suggested that rather than being due to protection from opsonophagocytosis, the capsule was required during colonisation as a result of the capsule enabling the pathogen to avoid association with the luminal mucus in the nasopharynx. This was hypothesized to be a result of the negative charge of the majority of pneumococcal capsule serotypes.

The charge of the capsule contributes to other interactions with the host as well. Human neutrophil defensins are cationic antimicrobial peptides that have been shown to be critical for the ability of neutrophils to kill the pneumococcus [40]. Beiter *et al*. [41] suggested that the negative charge of capsule makes pneumococci more susceptible to these antimicrobial peptides. Indeed, it may be interesting if this is the case with other antimicrobial peptides, as the majority are positively charged. However, this interaction may actually be beneficial for the bacteria. A recent publication has suggested that anionic capsular polysaccharide from *S. pneumoniae*, as well as *K. pneumoniae* and *P. aeruginosa*, can act as a decoy for cationic antimicrobial peptides, including human neutrophil α-defensin 1 [42]. This study suggests that bacteria can secrete varying levels of capsule proteins, which would then, as a protective mechanism, bind these important host defence molecules and result in resistance.

Other bacterial pathogens

Staphylococcus aureus is another Gram-positive pathogen for which capsule is critical. Approximately 20% of all humans have their nasal cavities colonised by *S. aureus* without displaying any symptoms [43]. Although the bacteria can be considered normal flora in these people, it is still considered to be an important source in the spread and pathogenesis of more serious infection. When it becomes invasive, *S. aureus* is a major cause of skin, soft-tissue, respiratory, bone, joint, and endovascular disorders [44]. Bacteremia from *S. aureus*, particularly when leading to sepsis, can cause multi-organ dysfunction in severe cases. It also a common causative agent of toxic shock syndrome due to the numerous toxins it produces [45]. These include serotoxins, such as protein-alpha toxin, which can cause proinflammatory changes in mammalian cells by pore-formation. Additionally, *S. aureus* produces a host of enterotoxins, all part of a family of staphylococcal and streptococcal pyrogenic exotoxins. These are all powerful

superantigens, in that they stimulate non-specific proliferation of T-cells [46]. While being one of the first bacteria to be treated with penicillin in the 1940's, antibiotic resistance was a clinical reality within the same decade. Today, methicillin-resistant *S. aureus* (MRSA) in particular is a global health concern [43,47,48]. Although more clonal complexes are known, 88% of all *S. aureus* that are isolated can be assigned to one of 11 previously known serotypes. Serotyping studies have shown that the majority of clinical isolates possess type 5 or type 8 capsules. Indeed, in recent studies in the United States and the Netherlands 92% and 82% of isolates were either type 5 or type 8 respectively [49,50]. As for pneumococcus, a capsule produced by a Wzy-dependent system is a critical factor [51,52]. It plays an important role in virulence, with unencapsulated mutants showing defects in animal models [53], while vaccination with capsule has also been shown to be protective [54]. An additional study, searching for *S. aureus* virulence genes using mariner-based mutagenesis, identified a number of genes likely involved in capsule synthesis to be important for virulence [55]. As with *S. pneumoniae*, the capsule likely acts as a protective barrier and helps the bacteria resist against opsonophagocytic uptake and death [53].

The discovery of new targets for virulence seems even more important in Gram-negative pathogens, which are traditionally more difficult to treat due to the presence of the outer membrane. Capsule in Gram-negative pathogens are also crucial virulence determinants as has been discussed recently for urinary tract infections caused by *E. coli* [56], through similar mechanisms to Gram-positive bacteria, via evasion of complement and inhibition of opsonophagocytosis, as well as their contributions to biofilm formation. In recent times, the capsule of *K. pneumoniae* has also shown to be critical, through its ability to act as an anti-phagocytic factor [57], while also promoting resistance to complement mediated lysis [58]. The emerging pathogen *Acinebacter baumanii* [59] also produces a polysaccharide capsule that has been implicated in its ability to cause infection. Thus, capsule of Gram-negative bacteria, some synthesized via alternative routes not including the Wzy-dependent pathway, are also valid drug targets, with studies already published looking for inhibitors to disrupt *E. coli* Group 2 capsule synthesis [60].

Capsule genes and synthesis

The capsule is comprised of a diverse array of polysaccharides on the bacterial cell surface, and the many chemically distinct sugar moieties available for the synthesis of polysaccharides result in high variability. These moieties can in turn be covalently linked by several different glycosidic bonds, leading to an even greater variation of both branched and unbranched polysaccharides. Three distinct capsular biosynthetic pathways have been identified: the Wzy-dependent, the synthase-dependent, and the ABC transporter-dependent pathways. The former two of these were recently reviewed by Yother in 2011 [9]. The ABC transporter-dependent pathway and the capsule of Gram-negative bacteria was recently reviewed by Cuthbertson *et al.* [61]. The ABC transporter-dependent pathway is not known to play any significant role in Gram-positive capsular biosynthesis, while the Wzy- and synthase-dependent pathways are well described in multiple Gram-positive bacteria.

Wzy pathway

The Wzy-dependent pathway is determined by the CPS biosynthesis (*cps*) locus situated between the *dexB* and *aliA* genes on the *S. pneumoniae* chromosome. The size of the locus across the > 90 serotypes studied varies from 10.3 to 30.3 kb with an average of 20.7 kb with flanking sequences showing evidence of mobile genetic elements. Despite size differences and transposable elements, the organisation of the *cps* locus is conserved across all serotypes. Starting at the 5' end, there is a cluster of 4 regulatory genes; *cpsA, cpsB, cpsC,* and *cpsD* (also known as *wzg, wzh, wzd, wze*) discussed in detail below. While *cpsA* is well conserved, *cpsB, cpsC* and *cpsD* are divided into two groups, with one set associated with carriage, and the other with invasive disease [62]. These regulatory genes are in most cases followed by *cpsE* (or *wchA*), the initial glucose phosphate transferase, and then *wzy*, the polysaccharide polymerase from which the pathway takes its name. After the succeeding flippase gene (*wzx*), the otherwise conserved order breaks up and is considered serotype specific, with glycosyl and acetyl transferases, genes for nucleotide diphosphate sugar biosynthesis and modification. Interestingly, while the majority of the locus has a G+C content consistent with the rest of the genome, the polymerase and the flippase (*wzy* and *wzx*) are more A+T rich [63].

Although a great deal of work remains before a complete model for Wzy-dependent synthesis of CPS can be postulated, much is known about the process. In *S. pneumoniae*, CPS synthesis begins in the cytoplasm by transfer of a sugar phosphate to undecaprenol-phosphate (Und-P); this forms a monosaccharide-PP-Und, the precursor molecule for oligosaccharide repeat unit synthesis. This first step is catalysed by an initiating glycosyl transferase, by transferring a sugar moiety such as glucose, N-acetyl-D-galactosamine or N-acetyl-D-glucosamine, depending on serotype. Several more sugar moieties, either identical or different, get added by glycosyl transferases until an oligosaccharide-PP-Und has been synthesised. This constitutes a repeat unit, and is subsequently flipped over by the Wzx flippase to the external side of the cytoplasmic membrane [64]. On this side, polysaccharides are extended by adding oligosaccharide-PP-Und repeat units to the reducing end of the chains. This is done in a non-processive manner by the Wzy polymerase and, once complete, the chain can be attached to an anchor. In capsule-forming Gram-positive bacteria, most polysaccharides are covalently attached to the cell, either to peptidoglycan or to other components in the cell membrane [9,65]. Some notable properties of CPS generated by the Wzy-dependent pathway is the variation in sugars making up the repeat units, the existence of branch points, and the degree of variation in number of repeat units making up the polymer.

Regulation

Regulation of capsular biosynthesis in a number of both Gram-positive and Gram-negative bacteria is mediated via a phosphoregulatory system, consisting of bacterial-tyrosine kinases (BY-kinases), and their cognate phosphatases. While tyrosine kinases were long thought to be present only in eukaryotes, in the past decade much work has shown the importance of BY-kinases with recent reviews highlighting these enzymes [66,67]. In *S. pneumoniae*, regulation of capsule production is mediated by

the manganese-dependent phosphotyrosine protein-phosphatase CpsB; the autophosphorylating protein-tyrosine kinase CpsD; and the membrane-bound CpsC (a polysaccharide co-polymerase (PCP) 2b protein) [68]), which is required for CpsD kinase activity. These regulatory genes are all located towards the 5'–end of the *cps* locus, along with CpsA, a member of the LytR-Cps2A-Psr (LCP) protein family. Deletion of all four of these genes have been shown to result in the production of significantly less capsule, highlighting their importance in capsule regulation [15].

While CpsA has been proposed to act as regulatory factor with evidence that is binds to the promoter region of the *cps* locus [69], a recent study has questioned this. Eberhart *et al.* (2012) have provided evidence that CpsA is responsible for the covalent attachment of the capsule to the cell wall [70]. CpsA is thought likely to bind the undecaprenol-pyrophosphoryl-linked capsule precursor to transfer the capsule to the cell wall. Interestingly, however, mutants in *cpsA* are just as virulent as the wt in *in vivo* models of pneumococcal infection [15], which may be due to the presence of other LCP family members (LytR and Psr) which may function to attach capsule in the absence of CpsA.

The remaining elements, CpsB, CpsC, and CpsD, are likely involved in a phosphoregulatory cycle, although the exact mechanism is still not understood. While we have seen a positive correlation between the phosphorylation of CpsD and capsule production, others have seen the opposite. However the system functions, it is clear that a cycle between phosphorylated and non-phosphorylated is important, not only in the pneumococcus, but other bacterial pathogens as well (e.g. *S. aureus* and *E. coli*) [14,15,71].

The organisation of the homologous BY-kinase differs between Gram-positive and -negative bacteria, although the function remains essentially identical. Both groups have an intracellular domain containing catalytic sequences called Walker A and B motifs, as well as an additional A' motif positioned between A and B [66]. Initially, the presence of the Walker motifs made it seem likely that these proteins were ATPases, since Walker A and B motifs predominantly occur in the P-loop of ATP/GTP binding proteins. Soon after their discovery though, they were shown to autophosphorylate and their role as BY-kinases established [72–74]. The BY-kinase Walker motifs are different from the canonical sequences though [75]. Of the [G/A]X(4)GK[S/T] Walker A motif, only the GK[S/T] amino acids are well conserved. Meanwhile, the Walker B motif is extended to [ILVFM](3)DX(2)P, following the additional Walker A' motif with sequence [ILVFM](3)DXDXR. Finally, following the Walker motifs, a tyrosine-rich C-terminus serves as the main site for phosphorylation, with up to 7 phosphorylatable tyrosine residues.

In Gram-negative bacteria, the BY-kinase is expressed as a single membrane-spanning polypeptide, also known as a PCP 2a protein [68], with the C-terminal kinase domain on the cytoplasmic side. Examples of this are Etk and Wzc in *E. coli* [76,77], but homologous proteins are also evident in other Gram-negative pathogens such as *K. pneumoniae* [78]. In Gram-positive bacteria, this protein is split into two such as in *S. pneumoniae* with the membrane spanning CpsC and cytoplasmic CpsD. The C-terminal region of CpsD contains the catalytic Walker motifs, as well as the phosphorylation sites of the tyrosine-rich cluster [79].

This is also the case in *S. aureus*, where the kinase consists of the membrane-bound CapA, whose 29 most C-terminal residues tightly interact with the soluble CapB protein. The determination of the CapAB structure has shown that the interface between CapB and CapA describes a shallow indentation of ~1350 $Å^2$ on the CapB surface (PISA server [80,81]). The C-terminal chain of CapA complements the central CapB β-sheet by adding a terminal strand to it; the interaction is otherwise predominantly hydrophobic in nature (Figure 2). Despite this organisational difference, both *E. coli* BY-kinases share a ~30% sequence identity with the corresponding *S. aureus* structure along with a high degree of structural similarity (Table 1). There is currently no BY-kinase structure available from *S. pneumoniae*. The closest homologous protein to CpsD is CapB from *S. aureus* with a sequence identity of 34%. By using the available structures from *E. coli* and *S. aureus* for a structure-based alignment, CpsD can be shown to have several features in common with those BY-kinases (Figure 1). This includes three aspartic acid residues that are part of or located near the conserved Walker A and B motifs. These residues are involved in the coordination of the β-phosphate of the ATP substrate, along with a conserved aspartic acid interacting with the ribose. All of these align perfectly in four sequences shown in Figure 1. While the role of CpsC in this cycle was at first less obvious, early work established its necessity for the proper function of CpsD [71,82,83]. Additionally, we recently showed that a number of defined amino acid mutations in CpsC can affect capsule polymerisation, CpsD phosphorylation and ligation of capsule to the cell wall [84]. A recent study showed that while not affecting the attachment of capsule to the cell wall, CpsC and CpsD played critical roles in the synthesis of the capsule at the division septum [85]. This did not seem to require phosphorylation of CpsD, as the C-terminal cluster of tyrosines was not required. Thus, this has led to the suggestion that there may be more than one method for controlling regulation of capsule in the pneumococcus – septal and non-septal.

The structures of bacterial protein tyrosine phosphatases were solved from both Gram-positive and Gram–negative organisms; CpsB from *S. pneumoniae*, and Wzb from *E. coli* [86]. These proteins share neither any significant sequence identity nor any structural similarity (Table 1). The structure of CpsB showed that it is a member of the polymerase and histidinol phosphatase (PHP) domain family of protein folds, as had been suggested by an earlier study via sequence comparisons [87]. On the other hand Wzb is associated with the low molecular weight phosphotyrosine protein phosphatases family [88]. The catalytic mechanism of Wzb could be inferred from previously studied phosphatases, but CpsB presented a novel metal-dependent mode of action. As a phosphatase, CpsB is not specific for its cognate autokinase CpsD, but has shown *in vitro* activity towards Wzc as well [86]. Thus, this would suggest that while these two phosphatases are relatively divergent, they share some similarity in their active sites. Recently, the structure of YwqE, a phosphatase from the Gram-positive bacterium *Bacillus subtilis* was solved [89].

While it is beyond doubt that CpsB is a major component in regulating CPS, the specific effects on the Wzy-dependent pathway has proven hard to conclusively show. We have shown in a variety of strains that defined mutations in CpsB lead to a reduction in capsule [15,71,90], while Bender *et al.* [91] saw a slight increase in the *S. pneumoniae* strain D39. This has led to confusion as to whether phosphorylation of CpsD is associated with a positive or negative effect on capsule expression.

In Gram-negative bacteria, evidence has suggested that phosphorylation is essential. In *E. coli* K-12 and K30, deletion of the gene encoding the protein tyrosine phosphatase Wzb results in no synthesis of colanic acid [1] (an exopolysaccharide produced by all *E. coli* isolates under stress conditions) and CPS [12]. In other words, this PTP is thought to be essential for CPS synthesis in Gram-negative bacteria. Whichever the case, CpsB and other bacterial tyrosine phosphatases are tightly bound to the regulation of CPS. Furthermore, defined mutations in CpsB have been shown to produce profound defects in a number of *in vivo* animal models [14,15], showing the importance of this protein tyrosine phosphatase to the pathogenicity of this organism.

Capsule has also been shown to be regulated by other mechanisms in *S. pneumoniae*. This includes the so far undefined phase variation [35], with some suggestion that part of this selection for phenotype may be caused by levels of oxygen. Indeed, CpsD phosphorylation levels vary in multiple strains with oxygen levels [92], while the specific activity of both CpsC and CpsB are affected by oxygen availability [9]. The catabolite control protein A (CcpA; also known as RegM), was previously shown to affect regulation of the *cps* regulon and to have an effect on the level of capsule in *S. pneumoniae* [93], while a recent study has suggested that this important regulator plays a role in the attachment of capsule to the cell wall at least in certain circumstances [94]. While this factor is undoubtedly important for virulence [93], further study is required to determine its effect on capsule regulation.

Scope for drug design

The Wzy-dependent pathway involves several key events which could potentially be targeted by rational drug design. The phosphoregulatory system in particular seems to be well suited for this, given the many studies that have shown its central role in CPS production, and its presence across a wide range of bacterial pathogens [72–74,95]. Of the two processes, phosphorylation and dephosphorylation, the effects of BY-kinases are the better studied and have been the focus of recent reviews [66,77]. The success of the last decade in targeting human kinases has made bacterial kinases tractable as drug targets as well. Although most work so far has been focused on bacterial histidine kinases [96], recent developments in the study of BY-kinases may generate more interest in them as drug targets. There are BY-kinase crystal structures from both Gram-positive and Gram–negative organisms available. The structures from *E. coli* and *S. aureus* are very similar; the proteins all share sequence identities ≥ 30% (Table 1). At the same time, these potential drug targets have very little in common with any known structure or protein sequence found in humans. This is a necessary, rather than sufficient, attribute in a drug target to minimize the potential drug from having adverse effects on human pathways. This marked difference from eukaryotic proteins is backed up by experimental evidence. BY-kinases have been shown to be insensitive to compounds known to have an effect on human kinases, and are unable to modify substrates easily phosphorylated by eukaryotic enzymes [97,98]. High resolution is advantageous for structure-based drug design approaches, and the highest resolution available crystal structure of a BY kinase is the *S. aureus* CapAB at 1.8 Å resolution [82]. The obvious site to focus on, the ATP-binding pocket, may not alone provide a sufficiently specific target. The nucleotide-binding

side of this pocket, however, is located in close proximity to the binding groove occupied by the C-terminal end of the interacting CapA (CapA-Ct; Figure 2). It is conceivable that a drug lead could garner enough specificity by interacting with features from both these sites on the CapB surface. Given the near-linear stretch of CapA-Ct leading up to the binding pocket, this could potentially be approached by using peptide-inhibitors as a lead towards peptidomimetic drugs. This is potentially the case in CpsC-Ct/CpsD as well; the majority of residues in the linear part of the CpsD binding groove are conserved between *S. pneumoniae* and *S. aureus* (Figure 1). The CapA-Ct/CapB interface is otherwise shallow and predominantly hydrophobic in nature, with few pockets that could potentially be exploited. Another feature of potential interest is the fact that BY-kinases have been shown to form large oligomeric complexes. In the *S. aureus* CapAB K55M mutant, a circular octamer is observed in the crystal structure, formed via two ~1000 $Å^2$ interfaces on each monomer. This interface, consisting mainly of four charged amino acid residues, also involves the tyrosine-rich C-terminal of one protomer interacting with the active site of the neighbouring chain. It is not known how well these findings reflect the *in vivo* situation. Although not observed in solution, it is conceivable that the octameric state is still present *in vivo* when stabilized by the transmembrane domain of the full length CapAB complex. Mutating these charged interface residues, all highly conserved in many BY-kinases (except *S. pneumoniae*), has no significant effect on the autokinase activity of the proteins [82]. However, since these were mutations to alanine residues, they would not be a sterical hinder for the transient interactions required for autophosphorylation. It seems feasible that this activity could be inhibited by disrupting the required protein-protein interaction. Although targeting active site cavities have a much longer history in drug design, and targeting protein interfaces presents its own set of challenges, the use of small molecules as drug leads by inhibiting protein-protein interactions have met with some recent success [99,100].

The *S. pneumoniae* protein tyrosine phosphatase CpsB shares very low sequence identity to any human proteins, with a putative deoxribonuclease TATP-domain protein as the most closely related structure. Although similar in overall structure, both belong to the TIM-barrel class of folds, their respective active sites are dissimilar enough that a prospective inhibitor could likely be made specific for the bacterial enzyme. On the other hand, the *E. coli* counterpart Wzb is clearly related in structure to human low molecular weight phosphatases, with sequence identities at ~30%. These are very similar in overall structure, and nearly identical in their phosphate-binding site, which renders some doubt over whether Wzb will be a useful drug target, while CpsB is more attractive. Of the bacterial phosphatases for which there is a structure, only YwqE from *B. subtilis* is utterly unlike any human structure or sequence to date. Likewise *S. aureus* phosphatases, for which there is currently no crystal structure, have little sequence identity with human proteins and could be considered a viable target on that basis (Figure 3). Although targeting phosphatases is a more recent development than using kinases as drug targets, dozens of new PTP inhibitors are reported annually [101]. These are mainly developed for the treatment of non-pathogenic diseases such as diabetes mellitus, cancer, and neural disorders. As potential drugs against pathogens, there has been some success in targeting PTPs in both Gram-positive and –negative organisms, for example *Mycobacterium tuberculosis* [102–104]

and *Yersinia pestis* [105,106]. Although demonstrably viable as drug targets, and increasingly specific compounds are under development, there are as of yet no clinically approved PTP inhibitors available.

Conclusions

Given the importance of proteins associated with the bacterial capsule as virulence factors, compromising the capsule synthesis during infections should sway the balance towards the innate immune defence of the host and allow control of bacterial numbers. This strategy would not directly lead to bacterial cell death, and as such inflammatory cell wall products should not be released into the hosts circulation on a large scale, and resistance selection pressure should also be reduced as the drug targets are not essential for bacterial viability and growth. In reality, this concept can only be investigated by using novel modulators that are currently being identified and developed (Add reference **Standish, A.J., A.A. Salim, H. Zhang, R.J. Capon, and R. Morona.** 2012. Chemical inhibition of bacterial protein tyrosine phosphatase suppresses capsule production. PLoS ONE.).

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Figure legends

Figure 1

Structure-based sequence alignment of BY-kinases from *S. aureus*, *S. pneumoniae*, and two from *E. coli*. Of the four, structures are available for all but *S. pneumoniae* and used for structure-based alignment. The split between the C-terminus of CapA and the N-terminus of CapB is indicated above the alignment, and the split between CpsC and CpsD is indicated below the alignment. CapB catalytic residues K55, S56, D77, D79, and D157 are labelled with "*". CapB residues directly interacting with the CapA linear C-terminus are labelled with "#". Sequence numbering is according to Uniprot records. Aligned by the T-Coffee (Expresso) web service [107].

Figure 2

Structure of the CapAB monomer. Non-carbon atom are in CPK colours. A) Ribbon representation of the overall structure; CapA C-terminus in grey, CapB in rainbow colouring from blue N-terminus to red C-terminus. Stick representation of ADP bound to the active site. B) Surface representation of the CapB binding groove interacting with the linear C-terminal stretch of CapA in pink. CapB surface collared by electrostatic potential from -0.5 V in red to 0.5 V in blue. CapA residues suffixed fixed by lower case ‘a’, CapB by lower case ‘b’. Interactions that a putative inhibitor could make use of include the hydrophobic pocket filled by L215a, and the pi stacking interaction between F221a and the nucleotide moiety of ADP. Figures produced using *CCP*4*mg* [108].

Figure 3

Structure-based sequence alignment of protein tyrosine phosphatases from *S. pneumoniae*, *B. subtilis*, and *S. aureus*. Of the three, structures are available for all but *S. aureus* and used for structure-based alignment. The CpsB residue R139, identified as the key residue for its catalytic activity, is labelled by double "*". CpsB residues interacting with the manganese cluster are labelled with single "*". Sequence numbering is according to Uniprot records. Aligned by the T-Coffee (Expresso) web service [107].

Table 1

PTKs				Protein		
Protein	**Organism**	**PDB**	**Ref**	**CapAB**	**Wzc**	**Etk**
CapAB	*S. aureus*	3bfv	[82]	1.8Å	31% seq id	29% seq id
Wzc	*E. coli*	3la6	[77]	Z: 15.2	2.5Å	57% seq id
Etk	*E. coli*	3cio	[76]	Z: 15.7	Z: 17.3	3.2Å

PTPs				Protein		
Protein	**Organism**	**PDB**	**Ref**	**CpsB**	**Wzb**	**YwqE**
CpsB	*S. pneumoniae*	2wjd	[86]	2.8Å	13% seq id	30% seq id
Wzb	*E. coli*	2wja	[86]	Z: 3.6	2.5Å	5% seq id
YwqE	*B. subtilis*	3qy6	[89]	Z: 12.8	Z: 3.4	1.8Å

Table 1 - text

Structures of representative phosphoregulatory proteins PTKs: protein tyrosine kinases; PTPs: protein tyrosine phosphatases; Å: highest resolution of structure. Z: Z-score; measures the statistical significance of a match in terms of Gaussian statistics. The higher Z-score, the higher the statistical significance of the match is. % seq id: fraction of pairs of identical residues in alignment. Data from PDBe-fold [109,110].

Figures

Figure 1

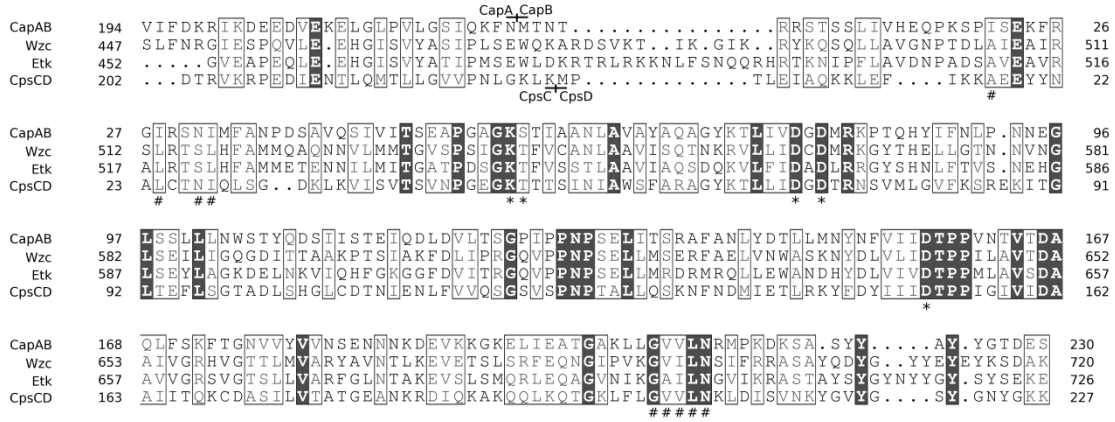


Figure 2

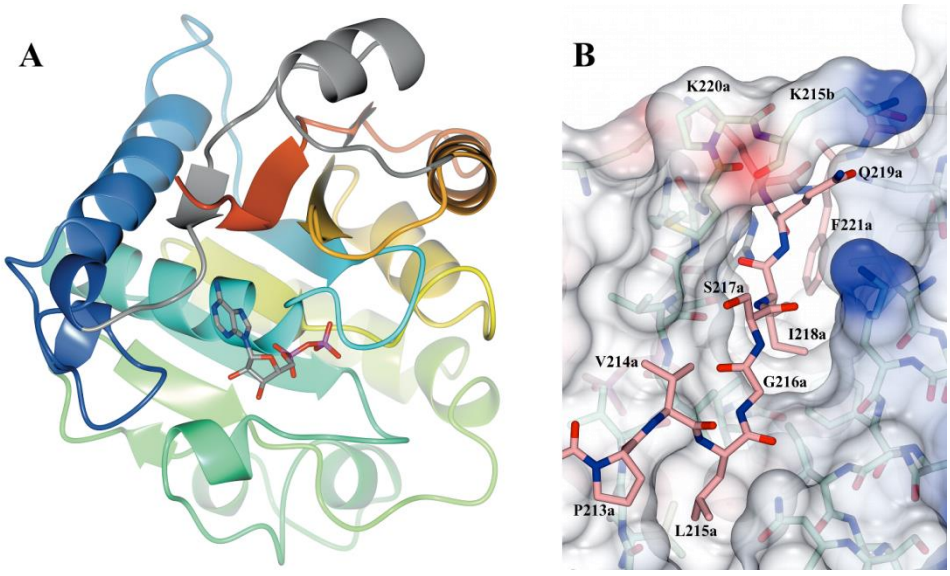
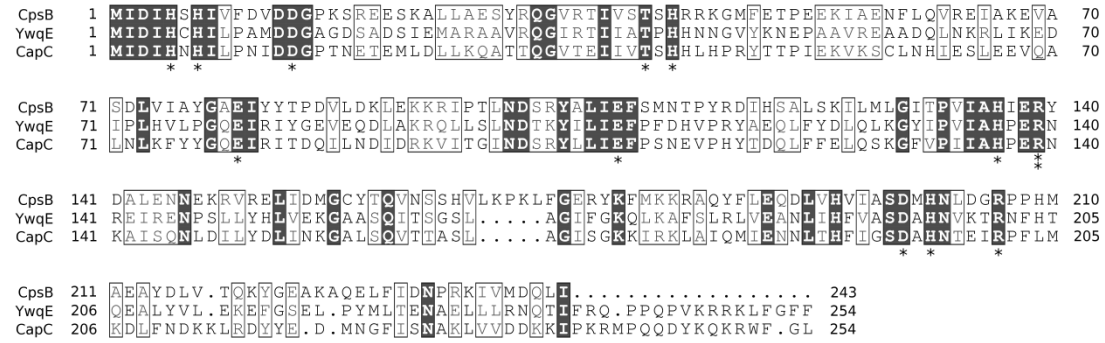

A
B
K220a
K215b
Q219a
F221a
S217a
I218a
V214a
G216a
P213a
L215a

Figure 3


CpsB 1 MIDIHSHIVFDVDDGPKSREESKALLAESYRQGVRTIVSTSHRRKGMFETPEEKIAENFLQVREIAKEVA 70
YwqE 1 MIDIHCHILPAMDDGAGDSADSIEMARAAVRQGIRTIIATPHHNNGVYKNEPAAVREAADQLNKRLIKED 70
CapC 1 MIDIHNHILPNIDDGPTNETEMLDLLKQATTQGVTEIIVTSHHLHPRYTTPIEKVKSCLNHIESLEEVQA 70
CpsB 71 SDLVIAYGAEIYYTPDVLDKLEKKRIPTLNDSRYALIEFSMNTPYRDIHSALSKILMLGITPVIAHIERY 140
YwqE 71 IPLHVLPGQEIRIYGEVEQDLAKRQLLSLNDTKYILIEFPFDHVPRYAEQLFYDLQLKGYIPVIAHPERN 140
CapC 71 LNLKFYYGQEIRITDQILNDIDRKVITGINDSRYLLIEFPSNEVPHYTDQLFFELQSKGFVPIIAHPERN 140
CpsB 141 DALENNEKRVRELIDMGCYTQVNSSHVLKPKLFGERYKFMKKRAQYFLEQDLVHVIASDMHNLDGRPPHM 210
YwqE 141 REIRENPSLLYHLVEKGAASQITSGSL.....AGIFGKQLKAFSLRLVEANLIHFVASDAHNVKTRNFHT 205
CapC 141 KAISQNLDILYDLINKGALSQVTTASL.....AGISGKKIRKLAIQMIENNLTHFIGSDAHNTEIRPFLM 205
CpsB 211 AEAYDLV.TQKYGEAKAQELFIDNPRKIVMDQLI..... 243
YwqE 206 QEALYVL.EKEFGSEL.PYMLTENAELLLRNQTIFRQ.PPQPVKRRKLFGFF 254
CapC 206 KDLFNDKKLRDYYE.D.MNGFISNAKLVVDDKKIPKRMPQQDYKQKRWF.GL 254