Review

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Role of two-component systems in the virulence of *Streptococcus pneumoniae*

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Understanding of how the human pathogen *Streptococcus pneumoniae* perceives and responds to its environment in the host offers insight into the pathogenesis of disease caused by this important bacterium and the potential for improved interventions. A central role in this environmental response is played by two-component systems (TCSs), which both sense the environment and drive the cellular response. Molecular advances in the form of genome sequencing, signature-tagged mutagenesis, differential fluorescence induction and microarray analysis have yielded considerable progress in the study of these systems in *S. pneumoniae*. These recent advances are discussed here, focusing in particular on the role of TCSs in the virulence of *S. pneumoniae*.

Introduction

Integral to the survival of a bacterium is the ability to sense and respond to its environment. This can have huge significance in infection processes through the regulation of virulence factors, such as *Bacillus anthracis* spore germination and virulence gene expression in response to phagocytosis by alveolar macrophages (Guidi-Rontani, 2002).

Two-component systems (TCSs), also referred to as two-component signal transduction systems, are recognized as a key mechanism through which bacteria perceive and respond to their environment. Here we discuss the significant advances that have been made in the understanding of TCSs in *Streptococcus pneumoniae* (the pneumococcus), in particular with regards to the role of these systems in the virulence of this important human pathogen.

The pneumococcus

With the exception of atypical equine isolates (Whatmore *et al.*, 1999), *S. pneumoniae* is normally found as a harmless commensal of the human upper respiratory tract. However, depending on host and bacterial factors that are not fully understood, the pneumococcus is also a major cause of diseases such as pneumonia, meningitis, septicaemia, bronchitis and otitis media. An illustration of its significance as a pathogen comes from the estimate that *S. pneumoniae* causes the death of 1 million young children per year in developing countries (Mulholland, 1997). Its impact, however, is also significant in the developed world; for example, in the USA the pneumococcus is responsible for 50 000 cases of pneumonia, 3000 cases of meningitis and 7 million cases of otitis media annually (Obaro, 2000). Furthermore, inadequate diagnosis, especially in the developing world, is suspected to underestimate the true burden imposed by pneumococcal disease. Current vaccines based on the

pneumococcal polysaccharide capsule have significant disadvantages (Bogaert *et al.*, 2004). In the case of purified polysaccharides, these are poorly immunogenic in children under 2 years old, an age group that suffers a high incidence of pneumococcal disease. Conjugate vaccines with purified capsular polysaccharide conjugated to a protein carrier resolve this problem. However, conjugate vaccines are beset by high production costs and limited coverage of pneumococcal serotypes, of which 90 are known. Adding to concerns over pneumococcal disease is the increase and spread of antibiotic resistance (Tan, 2003). Renewed efforts are now being made to understand the pathogenesis of pneumococcal disease with TCSs receiving much attention of late.

Bacterial TCSs

Bacterial adaptation to external stimuli is often mediated by systems known as two-component systems or two-component signal transduction systems (Hoch, 2000; Stock *et al.*, 2000). The basic model TCS (Fig. 1) is composed of two proteins: a membrane-associated sensor histidine kinase (HK) and a cytoplasmic cognate response regulator (RR). Upon receipt of a specific external stimulus the kinase domain of the HK sensor protein is activated to autophosphorylate a conserved histidine residue. HK proteins tend to be found as homodimers that operate *in trans* with the kinase domain of one catalysing phosphorylation of the second (Dutta *et al.*, 1999). This phosphate group is then transferred by the HK to a conserved aspartate residue in its cognate RR. In turn, the RR undergoes a conformational change allowing it to regulate gene expression or protein function. In most cases this is through the activity of the RR as a DNA-binding transcriptional regulator. Such histidine-phosphorelay systems are widespread among bacteria and have been shown to modulate a variety of cellular responses including osmoregulation, chemotaxis, sporulation, photosynthesis

Fig. 1. Schematic of bacterial two-component signal transduction. Following detection of its environmental stimulus, the HK uses cellular ATP to autophosphorylate a conserved histidine residue (A). The phosphoryl group is transferred from the HK to an aspartate residue on a cognate RR protein, found in the cytoplasm (B). The phosphorylated RR protein can control gene regulation by binding to promoter regions upstream of its target genes (C).

and pathogenicity (Hoch, 2000; Stock *et al.*, 2000). Some systems are essential for bacterial viability (Fabret & Hoch, 1998; Lange *et al.*, 1999; Martin *et al.*, 1999; Throup *et al.*, 2000) and because TCSs are absent in vertebrates they have received attention as potential targets for antimicrobials (Barrett & Hoch, 1998).

Pneumococcal TCSs

Prior to genome sequencing only four pneumococcal TCSs had been identified (Guenzi *et al.*, 1994; Novak *et al.*, 1999a, b; Pestova *et al.*, 1996). Subsequent examination of the pneumococcal genome sequence allowed the full repertoire to become apparent (Lange *et al.*, 1999; Throup *et al.*, 2000). TCS features are sufficiently characterized and conserved to allow their identification from sequence data, and two independent groups, screening for pneumococcal TCSs, identified the same complement of 13 HK:RR pairs with an additional 'orphan' unpaired RR (Lange *et al.*, 1999; Throup *et al.*, 2000). Both groups investigated the role of these TCSs in virulence. In the case of Lange *et al.* (1999) analysis of *rr* mutants in two pneumococcal strains (serotypes 3 and 22)

yielded no attenuation in comparison with wild-type in an intraperitoneal infection of mice. In contrast, Throup *et al.* (2000) demonstrated a significant role in virulence for most tested TCSs in a mouse pneumonia model using a different serotype 3 strain, 0100993. The conflict between these results shows the complexity of these systems in pneumococcal virulence and likely reflects experimental differences such as the use of different bacterial and mouse strains and site of infection. Subsequent work has confirmed important bacterial strain and infection site dependent effects for some TCS mutants.

These investigations provided the foundation for analysis of these systems in pneumococcal biology. The better-studied pneumococcal TCSs with regards to virulence are discussed here, with Table 1 providing a summary of all known pneumococcal TCSs and their contribution to virulence.

TCS09

Demonstrating the significant contribution that a TCS can make to pneumococcal virulence, a *rr09* mutant in strain D39 was essentially avirulent in mouse models of pneumonia (intranasal infection) and bacteraemia (intraperitoneal and intravenous infection) (Blue & Mitchell, 2003). In contrast to wild-type-infected mice, all *rr09*-mutant-infected mice survived infection, with bacteria being rapidly cleared. Interestingly, the same mutant made in a second strain, 0100993, did not show the same pattern of attenuation as seen in the D39 background. Although attenuated (but not avirulent) compared with wild-type in the pneumonia infection, the 0100993 *rr09* mutant behaved similarly to wild-type in bacteraemia models. Together these results illustrate the considerable impact that a TCS may have on pneumococcal virulence as evidenced by the avirulent phenotype seen in D39 when this gene is deleted. Importantly, this contribution to virulence varies between strains and between infection sites. Interestingly, despite being attenuated in a pneumonia model of intranasal infection as seen by improved mouse survival and reduced bacteria blood counts, the 0100993 *rr09* mutant did not show reduced bacterial counts in the lung compared with the parental wild-type strain. This suggests that, in strain 0100993, *rr09* attenuation during pneumonia is not due to impaired bacterial survival in the lung. Instead, given that the mutant was not significantly attenuated following systemic infections, showing that the mutant is able to survive in the blood as well as the wild-type, the attenuation seen in the pneumonia infection probably reflects reduced dissemination from the lung to the blood. It is also possible that the mutant has reduced ability to adapt properly during transition between lung and blood. This feature of TCS09 in strain 0100993 was missed in the work of Throup *et al.* (2000) as only lung bacterial counts were measured in a pneumonia model and only a modest attenuation was seen. These data highlight the value of measuring multiple parameters of infection and using different routes of infection to gain improved insight into the action of bacterial virulence factors.

Table 1. Pneumococcal TCSs and their contribution to virulence

TCS	Alternative names*	TCS family†	Demonstrated role in virulence	References for role in virulence
01	480	Pho	Yes	Throup <i>et al.</i> (2000); Hava & Camilli (2002)
02	<i>vic</i> , <i>micAB</i> , <i>yycFG</i> , 492	Pho	Yes	Wagner <i>et al.</i> (2002); Kadioglu <i>et al.</i> (2003)
03	474	Nar	No	—
04	<i>pnpRS</i> , 481	Pho	Yes	McCluskey <i>et al.</i> (2004)
05	<i>ciaRH</i> , 494	Pho	Yes	Throup <i>et al.</i> (2000); Marra <i>et al.</i> (2002); Ibrahim <i>et al.</i> (2004a)
06	478	Pho	Yes	Throup <i>et al.</i> (2000); Standish <i>et al.</i> (2005)
07	539	Lyt	Yes	Throup <i>et al.</i> (2000); Hava & Camilli (2002)
08	484	Pho	Yes	Throup <i>et al.</i> (2000)
09	<i>zmpSR</i> , 488	Lyt	Yes	Lau <i>et al.</i> (2001); Throup <i>et al.</i> (2000); Hava & Camilli (2002); Blue & Mitchell (2003)
10	<i>vncRS</i> , 491	Pho	No	—
11	479	Nar	No	—
12	<i>comDE</i> , 498	Agr	Yes	Lau <i>et al.</i> (2001); Bartilson <i>et al.</i> (2001); Hava & Camilli (2002)
13	<i>blpRH</i> , 486	Agr	Yes	Throup <i>et al.</i> (2000)
Orphan RR	<i>ritR</i> , 489	Pho	Yes	Throup <i>et al.</i> (2000); Ulijasz <i>et al.</i> (2004)

*Number code refers to designation used by Throup *et al.* (2000).

†As assigned by Throup *et al.* (2000).

Further supporting its role as a pneumococcal virulence factor, *rr09* was identified in the signature-tagged mutagenesis (STM) screen of the sequenced strain TIGR4 (Hava & Camilli, 2002). Additional investigation by these workers showed that in TIGR4, *rr09* contributed to virulence in pneumonia but not to bacteraemia. This result is similar to that seen with strain 0100993 and again shows that the importance of genes to virulence varies with the site of infection.

RR09 (and presumably TCS09) therefore has the potential to contribute significantly to pneumococcal virulence but this contribution varies between pneumococcal strains and infection sites. The genes regulated by this system and the reason for these strain- and site-specific effects are yet to be characterized. Interestingly, a second pneumococcal RR, RR04, also has strain-specific effects on virulence, the basis of which is beginning to be elucidated (see below).

TCS04

TCS04 was first identified as a pneumococcal virulence factor by Throup *et al.* (2000), where they demonstrated that a 0100993 mutant in *rr04* was attenuated during murine pneumonia. This was expanded upon by the work of McCluskey *et al.* (2004) with the comparison of *rr04* mutants in three different strains, TIGR4, D39 and 0100993, using a similar pneumonia model. Only the TIGR4 *rr04* mutant was attenuated relative to its parental wild-type in this second study thereby expanding the finding of a strain-specific effect in virulence for *rr09* to include *rr04* also. Microarray analysis of the transcriptome of these mutants was used to investigate the genes regulated by RR04 and the reason for the strain-specific effects on virulence.

The strains showed considerable variation with regard to the genes regulated by RR04. In particular, the *psa* operon encoding a manganese ABC transporter system was down-regulated in the TIGR4 *rr04* mutant but not in D39 and 0100993 mutants. This operon, consisting of *psaB*, *C* and *A*, is known to contribute to pneumococcal virulence and resistance to oxidative stress (McAllister *et al.*, 2004). Based on those data, the down-regulation of this operon in TIGR4 was postulated to contribute, at least in part, to the specific attenuation of this strain. Indeed, in agreement with reduced *psaBCA* expression the TIGR4 *rr04* mutant was hypersensitive to killing by hydrogen peroxide (McCluskey *et al.*, 2004). The reason for these strain-specific differences in *rr04*-dependent transcription have yet to be elucidated but RR04 itself is highly conserved.

Interestingly, the results of McCluskey *et al.* (2004) using the 0100993 *rr04* mutant differ from the findings of Throup *et al.* (2000) using the same mutant and parent strain. Whereas the former found no attenuation in a pneumonia model based on survival and blood and lung counts, Throup *et al.* (2000) showed a significant reduction in lung counts for the mutant compared with wild-type (10^6 -fold reduction). The cause of this discrepancy is not known but may be caused by the use of different infection models, in particular the use of different mouse strains, adding further to the complexity of studying these systems.

Inter-strain variation, TCSs and virulence

The strain-to-strain variation in the role of *rr09* and *rr04* in virulence is a feature repeated in several pneumococcal virulence factor studies (Blue & Mitchell, 2003; Chapuy-Regaud *et al.*, 2003; McCluskey *et al.*, 2004; Orihuela *et al.*,

2004; Paterson & Mitchell, 2006). Such variation is perhaps to be expected given the diversity of the pneumococcus as a bacterial species. For example, microarray examination of genome content showed that 8–10 % of genes are divergent/absent in any one clinical strain relative to the reference strain TIGR4 with the pool of variable genes making up ~20 % of the TIGR4 genome (Hakenbeck *et al.*, 2001). Actual genomic diversity will likely be much greater, given this analysis would miss test strain-specific genes. Thus conflicting results with different strains likely reflect the diversity in natural pneumococcal populations. The growing data on strain variation and strain-specific effects show clearly the complexity of this bacterium and argue strongly for the analysis of multiple strains in future investigations. Likewise, the data highlight the dangers of extrapolation of results from one strain to another and the over-reliance on any single 'reference' or 'model' strain.

TCS12 competence and virulence

The pneumococcus is naturally competent, a well-studied phenomenon contributing to its genetic diversity (Claverys & Havarstein, 2002). It is through TCS12 that competence is activated (Fig. 2). TCS12 consists of the HK encoded by *comD* and the RR encoded by *comE*, which together respond to competence stimulating peptide, CSP, the product of

comC, secreted via the ComA/B ABC transporter. TCS12 mutants were not tested in the pneumonia model of Throup *et al.* (2000). However, a clear link between competence and virulence was demonstrated with the finding that a *comD* (*hk12*) mutant in D39 was attenuated in models of both pneumonia and bacteraemia (Bartilson *et al.*, 2001). This link was further supported by the serotype 3 STM of Lau *et al.* (2001), where ComB, the ATP-binding protein in the ComAB CSP transporter, was identified as a virulence factor in a bacteraemia model. However, it cannot be ruled out that ComAB transports additional substrates responsible for this effect. Further investigation showed *comD* (*hk12*) to contribute to pneumonia and bacteraemia models in competitive infections with wild-type (Lau *et al.*, 2001). Additionally, *comD* was identified as a virulence factor in pneumonia by the TIGR4 STM screen (Hava & Camilli, 2002). How competence is tied to virulence is not yet fully clear. The most comprehensive analysis of the effect of competence on gene expression comes from the use of microarray expression analysis following exposure to CSP (Dagkessamanskaia *et al.*, 2004; Peterson *et al.*, 2004). Together these groups identified a repertoire of ~240 CSP-responsive genes. The power of microarray technology is clearly demonstrated given that prior to these studies only ~40 CSP-responsive genes were known. CSP-responsive genes represented ~7–8 % of the

Fig. 2. Competence regulation in *S. pneumoniae*. Competence in *S. pneumoniae* involves a complex series of events that are required for the uptake of DNA from the external environment (transformation). It is mediated by competence stimulating peptide (CSP), which is encoded, as a precursor, by the *comC* gene. The peptide precursor is cleaved during export through the ATP-binding cassette, encoded by *comA/B*. Mature, secreted CSP accumulates in the external medium and, upon reaching a critical density, activates the ComD histidine kinase (HK). Phosphoryl transfer proceeds from the activated HK protein to its cognate intracellular response regulator (RR) protein, encoded by *comE*. Activated/phosphorylated RR can auto-regulate expression of the *comC/D/E* and *comA/B* operons and activates transcription of *comX*, which encodes an alternative sigma factor. ComX serves to activate genes involved in competence, including DNA uptake apparatus, together with genes not involved in competence. Phosphorylated ComE has also been shown to activate expression of *comW*, a component recently shown to have a role in the stabilization of ComX (Luo *et al.*, 2004; Sung & Morrison, 2005).

genome, illustrating the considerable global impact of competence on the cell. Of the responsive genes identified by Peterson *et al.* (2004), the majority were shown to be dispensable for transformation. Therefore competence has the ability to affect a large number of genes, many of which are apparently unrelated to transformation. With regards to the link between competence and virulence, 18 of the 124 (14.5 %) up-regulated genes seen by Peterson *et al.* (2004) were identified in the TIGR4 STM screen as virulence factors, thereby providing a mechanistic explanation for the role of competence in virulence (Hava & Camilli, 2002). These up-regulated virulence factors included the autolysin *lytA*, *htrA*, a stress response protein and a choline-binding protein gene (*cbpD*).

Many of these CSP-responsive genes will likely not be regulated directly by ComC but indirectly via induction of the alternative sigma factor ComX, which regulates expression of late competence genes (Luo *et al.*, 2003; Luo & Morrison, 2003).

Adding further to the complexity of competence and pneumococcal TCSs is the involvement of TCS02 and the TCS05, which act to repress competence development (Echenique *et al.*, 2000; Echenique & Trombe, 2001).

TCS02

RR02 is notable as being the only RR essential for pneumococcal viability (Lange *et al.*, 1999; Throup *et al.*, 2000). In agreement with this, TCS02 shows homology to the essential YycFG TCS of *Bacillus subtilis* and *Staphylococcus aureus* (Fabret & Hoch, 1998; Martin *et al.*, 1999). A difference of note, however, is that in these latter two systems, both the *rr* and *hk* are essential; in the case of the pneumococcus this appears true only for the *rr* gene. Presumably, the essential function of RR02 is phosphorylation-independent or possibly RR02 may be phosphorylated from an alternative donor in the absence of HK02 (Throup *et al.*, 2000). Recent work shows that TCS02 contributes to the regulation of cell wall and fatty acid biosynthesis as well as expression of the virulence factor *pspA* (Mohedano *et al.*, 2005; Ng *et al.*, 2003, 2005). The essential status of *rr02* can be suppressed by overexpression of the murein-hydrolase-encoding gene *pcsB*, showing that control of this gene is a key function of TCS02 (Ng *et al.*, 2003, 2004).

Overexpression and deletion of various components of the TCS02 operon, which includes a third ORF of unknown function, caused attenuated virulence in intraperitoneal infections (Wagner *et al.*, 2002). The finding that overexpression mutants had reduced virulence shows that controlled expression of TCS02, and presumably other TCSs, is vital to virulence. Furthermore, *hk02* mutants in two different strains showed attenuation in a pneumonia model (Kadioglu *et al.*, 2003). This latter work is in conflict with the findings of Throup *et al.* (2000), where a *hk02* knockout in 0100993 was not attenuated. The reason for this is unclear but may relate to strain and experimental differences.

TCS05/CiaRH

TCS05 or the CiaRH system was the first pneumococcal TCS identified (Guenzi *et al.*, 1994). The influences of this TCS on pneumococcal biology are diverse and complex, affecting virulence, competence and antibiotic resistance (Guenzi *et al.*, 1994; Guenzi & Hakenbeck, 1995; Ibrahim *et al.*, 2004a; Throup *et al.*, 2000; Zahner *et al.*, 2002). In addition, analysis of cells undergoing transformation suggests that CiaRH is important in protecting cells from the stress of competence development (Dagkessamanskaia *et al.*, 2004). In the absence of *ciaR*, cultures showed enhanced autolysis in response to competence induction. A role in virulence through the use of knockout mutants has been demonstrated by several groups (Ibrahim *et al.*, 2004a; Marra *et al.*, 2002; Throup *et al.*, 2000). Investigation of the CiaRH regulon identified the major virulence factor *htrA* as being down-regulated in *ciaRH* mutants (Ibrahim *et al.*, 2004a; Mascher *et al.*, 2003; Sebert *et al.*, 2002). Restoration of *htrA* expression in a *ciaRH* mutant restores the virulence defect of the TCS mutant, showing that up-regulation of *htrA* is a key component in the contribution of CiaRH to virulence (Ibrahim *et al.*, 2004a). The exact role of *htrA*, a stress response serine protease, in virulence is unclear (Hava & Camilli, 2002; Ibrahim *et al.*, 2004b). It should be noted, however, that several other known and putative virulence factors were regulated or potentially regulated by CiaRH and these too may contribute significantly to the role of CiaRH in virulence (Sebert *et al.*, 2002; Mascher *et al.*, 2003). These include the *dlt* and *pit2/pia* operons. Evidence suggests that the pneumococcal response to oxygen and calcium ions involves CiaRH (Echenique & Trombe, 2001; Giammarinaro *et al.*, 1999).

TCS13

TCS13 was identified as contributing to pneumococcal virulence in the TCS identification and characterization work of Throup *et al.* (2000). They found that a *rr13* mutant was significantly attenuated in their respiratory tract infection model with bacterial lung counts reduced by about 10 000-fold compared with wild-type 0100993. This system was subsequently named *blp* TCS for bacteriocin-like peptide when it was found by microarray expression analysis to control a 16-gene quorum-sensing regulon controlling the synthesis and export of bacteriocin-like peptides and immunity proteins (de Saizieu *et al.*, 2000). The system is paralogous to the competence system (TCS12) with the small peptide BlpC signalling via Blp TCS to up-regulate target genes including *blpC* itself. Further confirmation of a role in virulence for this system came from the TIGR4 STM screen, where *blpA*, purportedly involved in BlpC export, was identified as a virulence factor (Hava & Camilli, 2002). Interestingly, several strains, including the commonly studied strains TIGR4, R6 and D39, contain a reading frame shift mutation in the *blpA* gene resulting in premature termination of translation. Not all strains have this feature and its significance is as yet unclear (de Saizieu *et al.*, 2000). Bacteriocins are bacterial products that kill or inhibit the growth of related strains or species, with immunity proteins

protecting the producing organism. Although poorly characterized as yet, pneumococcal bacteriocin activity has been described previously and presumably provides a growth advantage in the microbial rich nasopharynx (Mindich, 1966). However, the bacteriocin activity of *blp* products and a role in colonization remains unstudied. Interestingly, given that the lung is sterile, the function of bacteriocins in virulence at this site presumably does not involve killing competing microbes at this site. Rather, it is proposed that *blp* bacteriocins may be acting via a cytotoxic affect on host cells (de Saizieu *et al.*, 2000).

RR489/RitR, the orphan response regulator

Unlike the other pneumococcal TCSs, *rr489* is not located in the genome next to a cognate *hk*. It appears, however, to have a key role in virulence given that a knockout mutant showed a $>10^4$ reduction in pulmonary bacterial counts compared with wild-type in a murine pneumonia model (Throup *et al.*, 2000). This contribution to virulence was confirmed by Ulijasz *et al.* (2004) and furthermore found to be tissue-specific whereby a mutant was attenuated for growth in the lung but not in the thigh following infection of these organs. The mechanism for this appears to be due, at least in part, to repression by RitR of the *piu* iron uptake system (Ulijasz *et al.*, 2004); hence the renaming of *rr489* as *ritR* (regulator of iron transport). This repression appeared to be a direct effect of RitR given it was shown to bind the *piu* promoter. While iron is essential for the growth of most bacteria it can also be deleterious through the Fenton reaction, where it catalyses the synthesis of reactive oxygen intermediates from hydrogen peroxide. It is therefore essential to regulate its levels. RitR appears to be one such system for the regulation of iron in the pneumococcus. In line with increased iron uptake in the absence of *ritR*, a *ritR* mutant was more sensitive to iron-dependent killing by streptonigrin. Resistance to oxidative stress was also altered, with the mutant showing increased susceptibility to hydrogen peroxide killing and this likely contributes to decreased virulence. While increased iron overload may itself contribute to this sensitivity, the *ritR* mutant also showed reduced expression of various genes implicated in resistance to oxidative stress. Presumably the importance of *ritR* varies with different sites of infection based on the levels of iron and/or oxidative stress. How RitR operates in the absence of a cognate HK remains unclear. Indeed the mechanism(s) by which the pneumococcus senses iron is unknown. Interestingly, even although RitA contains the conserved aspartate residue which is phosphorylated in other RRs, various phosphate donors did not alter RitR binding to the *piu* promoter, hinting at a possible phosphorylation-independent regulation (Ulijasz *et al.*, 2004).

Other pneumococcal TCSs and virulence

The remaining TCSs are relatively poorly characterized although most have been shown to contribute to virulence (Table 1). TCS10 has been implicated in tolerance to the antibiotic vancomycin, a finding now considered controversial

(Haas *et al.*, 2004; Robertson *et al.*, 2002), while TCS06 has recently been shown to regulate the important virulence factor *cbpA* (Standish *et al.*, 2005).

As yet only TCS03, 10 and 11 have not been associated with virulence in at least one study. In the pneumonia model of Throup *et al.* (2000) where most systems were examined, no attenuation was seen with mutants in *tsc03* and *rr11* while a *rr10* mutant showed only a trend towards mild attenuation that was not significant. However, Hava & Camilli (2002) speculate that the attenuation of STM strain STM237 may be due to polar effects on adjacent *tcs11*. TCS03 and 11 have recently been implicated in the pneumococcal stress response to antibiotic treatment (Haas *et al.*, 2005).

Pneumococcal TCSs as antimicrobial targets

The role of TCSs in the viability and virulence of a number of pathogenic bacteria, coupled with their absence in mammals, make them potential targets for novel antimicrobial drugs (Barrett & Hoch, 1998). In particular, pneumococcal RR02 being essential for viability makes it a potentially attractive target. Its biochemical characterization *in vitro* and structural studies may pave the way for rational drug design against this target (Bent *et al.*, 2003, 2004; Clausen *et al.*, 2003; Echenique & Trombe, 2001; Riboldi-Tunncliffe *et al.*, 2004; Wagner *et al.*, 2002). However, a TCS need not be essential for viability to be a useful target. Inhibition of those with a major role in virulence may also prove beneficial. However, strain- and infection-type-specific roles in virulence as described for *rr04* and *rr09* may limit the value of specific targeting of such systems in the pneumococcus.

Gene regulation *in vivo*

An important challenge in understanding genetic regulation by TCSs is to characterize and understand this in the context of *in vivo* infection. Knowledge of gene regulation *in vivo* offers insight into bacterial adaptation to the host environment, greatly enhancing basic understanding of the infection process, and is something that cannot be fully gained by *in vitro* studies alone. This significant advantage is, however, beset by the technical difficulties of recovering sufficient quantities of bacterial RNA of adequate quality from infected tissues. Several studies have shown that the expression of key pneumococcal virulence factors is up-regulated *in vivo* (Ogunniyi *et al.*, 2002; Orihuela *et al.*, 2000, 2001). For example, *pspC* mRNA has recently been shown to be up-regulated a massive 870-fold *in vivo* compared to growth *in vitro* (Quin *et al.*, 2005). However, such studies are limited by focusing on only a small subset of genes.

A more comprehensive investigation of the pneumococcus response to growth *in vivo* came following the development of differential fluorescence induction (DFI). First employed on *Salmonella typhimurium*, DFI allows the identification of promoters activated in response to a particular environment (Valdivia & Falkow, 1996). It works through the cloning of random genome fragments upstream of promoterless

green fluorescence protein and screening and sorting by flow cytometry to identify and isolate clones with up-regulated promoter activity following exposure to a specific environment, for example *in vivo*. The cloned DNA is then sequenced to identify the responsive promoter and ORF. DFI has the advantages of circumventing the requirement for recovering bacterial RNA and makes it easier to simultaneously examine many promoters. Using three infection models and five *in vitro* cultures mimicking various *in vivo* conditions a total of 73 *in vivo* responsive promoters were identified in strain D39 (Marra *et al.*, 2002). However, the fullest examination of pneumococcal transcription in the host has come through the use of microarrays, comparing the transcriptional profile of bacteria recovered from mouse blood, rabbit cerebral spinal fluid or after adherence to human epithelia cells *in vitro* with that of control cultures (Orihuela *et al.*, 2004). A distinct profile was seen for each 'infection' type showing a site-specific transcriptional response. Expression differences between the different infections show that the pneumococcus is responding not just to being in the host but also to specific *in vivo* environments. This is relevant to human infections with the pneumococcus able to cause infections at different sites such as during pneumonia, otitis media, meningitis and bacteraemia. Presumably these expression differences correspond to exposure to different conditions and stresses and reflect different adaptive strategies to survive in these conditions. While this study is not without important limitations it provides insight into the gene expression changes occurring *in vivo* and provides a foundation to understand how the pneumococcus responds to the host environment. The challenges ahead include identification of the stimuli, the pathways involved and understanding the biological significance of these transcriptional changes.

Conclusion/summary

Pneumococcal TCSs are important virulence factors of this significant human pathogen. Interestingly, their contribution to virulence can vary depending on pneumococcal strain and infection site. The reasons for this are largely unclear as yet. Microarray analysis is allowing insights into the genes regulated by these systems but important challenges remaining include understanding gene regulation *in vivo*, understanding of how TCSs interact with each other and investigation of their potential as novel antimicrobial targets.

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