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Phosphorylation of the RitR DNA-binding domain by a Ser–Thr phosphokinase: implications for global gene regulation in the streptococci

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Summary

We report selective phosphorylation of the DNA-binding domain of the *Streptococcus pneumoniae* transcriptional regulator RitR. RitR is annotated as a two-component response regulator, but lacks a cognate His kinase as a neighbouring locus in the genome. In addition, Asn replaces Asp at the expected acceptor site. By the use of combinatorial phage display, we identified PhpP, a *S. pneumoniae* Ser–Thr eukaryotic-like PP2C phosphatase as an interacting partner of RitR. RitR interacts with the phage-displayed peptide VADGMGGR which forms a part of the active-site sequence of PhpP. RitR is phosphorylated *in vitro* by StkP, the presumed cognate kinase of PhpP, and the site on RitR that is phosphorylated has been localized to the RitR DNA-binding domain. PhpP together with its cognate kinase StkP appear to be necessary for Piu haem transporter expression. *In vitro* studies suggest that PhpP and StkP interact competitively with RitR in that RitR–PhpP–*piu* promoter ternary complexes are disrupted by StkP. Our findings indicate a regulatory link between RitR and Ser–Thr kinase–phosphatase-based bacterial signal transduction.

Introduction

Two-component signal transduction (TCST) in prokaryotes is based on the interaction between a His kinase and its cognate response regulator. In this interaction, commonly known as a His–Asp relay, a His kinase, upon activation, autophosphorylates and, in turn, transfers the phosphoryl moiety to its cognate response regulator at a canonical Asp residue located in the regulatory domain (RD). Phosphorylation of the RD alters the affinity of the response regulator for DNA, mediated through its C-terminal DNA-binding domain (DBD), which modulates expression at cognate promoter(s). RitR, however, contains an Asn residue at the predicted phosphorylation site in the N-terminal RD of RitR, leading to a question as to whether RitR is phosphorylated at all, and if so, which is the amino acid that is phosphorylated and where is it located? The absence of the expected phosphorylatable Asp residue that is characteristic of canonical response regulators suggests that if RitR is phosphorylated it would be at an alternate site.

The *Streptococcus pneumoniae* genome encodes 27 TCST components organized as 13 TCST His kinase–response regulator pairs plus a single unpaired orphan response regulator, RitR, which is not associated with a cognate kinase locus as an immediate neighbour in the genome. Throup *et al*. (2000) identified and inactivated each of the 27 TCST elements in the *S. pneumoniae* genome using allele replacement mutagenesis and noted markedly reduced virulence in several of the resultant knockout strains in an infected mouse lung model. One of the most pronounced reductions in virulence, 10^6-fold decrease in viable titre of colony forming units per lung, was seen by Throup *et al*. in response to inactivation of response regulator RR489. DNA microarray analysis showed that RR489 strongly repressed Piu transporter synthesis (Ulijasz *et al*., 2004), and it was subsequently renamed 'RitR' – repressor of iron transport. The functional role of TCST in adaptation and virulence of *S. pneumoniae* more broadly has been reviewed by Lange *et al*. (1999), Throup *et al*. (2000) and Paterson *et al*. (2006).

Although regulation by His–Asp relay systems dominates signal transduction studies in prokaryotes, the importance of other phosphorylation systems is emerging. Eukaryotic-like Ser–Thr protein kinase–phosphatase pairs in bacteria have been implicated in regulating diverse cellular activities such as virulence (for reviews see Archambaud *et al*., 2005; Cozzone, 2005) and

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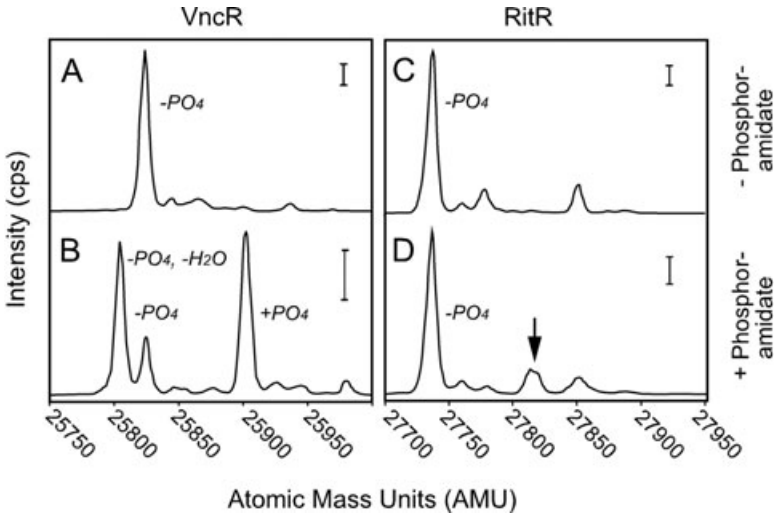

Fig. 1. Direct phosphorylation of response regulators *in vitro* with phosphoramidate. VncR and RitR, both purified to near homogeneity, were incubated with ammonium phosphoramidate, and analysed by HPLC-mass spectrometry. VncR alone (A), VncR plus phosphoramidate (B), RitR alone (C), RitR plus phosphoramidate (D). A minor peak indicated with an arrow in (D) is located at 80 amu above the mass peak seen for unmodified RitR. The vertical bar shown in the right upper corner of each panel corresponds to a peak intensity of 20 000 counts per second indicated on the ordinate.

secondary metabolism (for review see Zhang *et al*., 2005). Furthermore, Ser–Thr kinase signalling cascades have also been shown to interact with His–Asp relay systems of non-pathogens (for reviews see Gaidenko *et al*., 2002; Lux and Shi, 2005).

The Ser–Thr kinase–phosphatase systems in the streptococci are required for regulation of growth, cell division and virulence (Rajagopal *et al*., 2003; 2006; Echenique *et al*., 2004; Jin and Pancholi, 2006). In *S. pneumoniae*, a Ser–Thr kinase controls competence gene expression (Echenique *et al*., 2004) and also acts as a global regulator of genes associated with DNA repair, iron uptake and oxidative stress (Sasková *et al*., 2007). The regulation of the three latter systems has also been reported as a possible role for RitR (Ulijasz *et al*., 2004). Moreover, Novakova *et al*. (2005) identified phosphoglucosamine mutase GlmM, an enzyme in the cell wall synthesis pathway, as a substrate for the *S. pneumoniae* StkP/PhpP kinase–phosphatase system. Interestingly, and most relevant to the present studies, Rajagopal *et al*. (2006) have reported that Stk1, the *Streptococcus agalactiae* Ser–Thr kinase orthologue of StkP, phosphorylates CsrR(CovR), a response regulator that like RitR has 229 amino acids, 45% of which can be aligned identically.

In the present studies we report that RitR functionally interacts with the *S. pneumoniae* Ser–Thr kinase–phosphatase pair StkP and PhpP. As a part of this relationship, RitR is phosphorylated by StkP at the C-terminal RitR–DBD. RitR, PhpP and StkP also jointly participate in complex formation at the *piu* promoter *in vitro*. In addition, we demonstrate that RitR *vis-à-vis* StkP or PhpP shows reciprocal effects on *piu* transcription *in vivo*. These observations suggest that RitR utilizes a mechanism to regulate gene expression that differs from that used by most other response regulators.

Results

Phosphoramidate does not phosphorylate RitR

In the absence of an apparent His kinase that can phosphorylate RitR or of a phosphorylatable Asp residue at the expected site, we attempted to phosphorylate RitR directly by incubation with phosphoramidate (Lukat *et al*., 1992; Buckler and Stock, 2000). A shift of 80 atomic mass units (amu) was found when the positive control response regulator VncR was treated with phosphoramidate, as shown in Fig. 1A and B. Under similar conditions, phosphoramidate treatment of RitR showed only a minor peak at the expected 80 amu location (Fig. 1C and D). The minor peak seen at the 80 amu location could reflect a low level efficiency of specific RitR phosphorylation. Thus the absence of a phosphorylatable Asp and the absence of a cognate His kinase, together with our inability to phosphorylate RitR directly, suggested that RitR is not phosphorylated at all.

Identification of RitR-binding phage

We showed previously that combinatorial phage display can be used to discover an interacting partner of a TCST response regulator (Ulijasz *et al*., 2000). Specifically, in studies of the VanRS system, phages obtained by panning against VanR recapitulated the sequence around the active centre of VanS. To apply the same method here, microtitre plate wells coated with purified RitR were used to pan for phage from the combinatorial peptide library X10C. After three rounds of panning and amplification, phage was plaque-purified, amplified and tested for their ability to bind to wells coated with RitR and with BSA respectively. From a group of

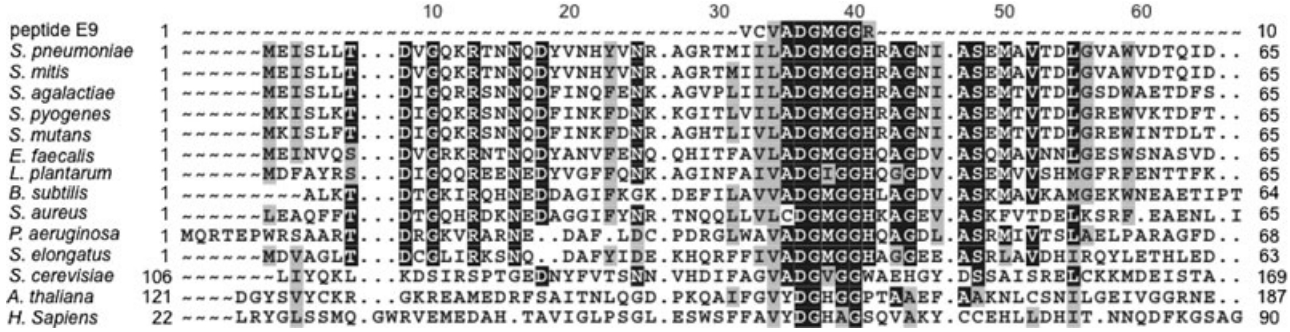

Fig. 2. Alignment of peptide E9 with PP2C phosphatases. Alignment of the N-terminal sequences PhpP from several streptococci , along with orthologues from other sources. Black boxes indicate identical residues. Grey boxes indicate chemically similar residues. For each sequence entry, source organism, NCBI protein database accession number and locus number are shown, respectively, as follows:

Streptococcus pneumoniae R6	AE007317	spr_1528
Streptococcus mitis	Unfinished genome	SMT_1070
Streptococcus agalactiae 18RS21	AAJO00000000	SAJ_0410
Streptococcus pyogenes MGAS10270	CP000260	spy_1452
Streptococcus mutans UA159	AE014133	SMU_483
Enterococcus faecalis V583	AE016830	EF_3121
Lactobacillus plantarum	AY096005	lp_1618
Bacillus subtilis 168	AL009126	BSU_1577
Staphylococcus aureus MW2	BA000033	MW_1102
Pseudomonas aeruginosa PA01	NC_002516	NP_250361
Synechococcus elongatus	NC_004113.1	tlr_2243
Saccharomyces cerevisiae	NC_001140	AAB_68888
Arabidopsis thaliana	NC_003070.5	NP_172196
Homo sapiens	AC_000146	NP_066283

phage samples that showed binding to RitR ≥ 3-fold over the BSA background, seven were randomly selected and sequenced. Six of these encoded the amino acid motif VCVADGMGGRC, designated E9. The E9 phage sequence was compared with the *S. pneumoniae* R6 genome using the TIGR Comprehensive Microbial Resource (CMR)-based protein–protein BLAST search program (http://blast.jcvi.org/cmr-blast), and PhpP was subsequently identified as a possible interacting partner of RitR. As shown in Fig. 2, the E9 amino acid sequence can be aligned with the conserved N-terminal sequences of PhpP and other PP2C-type Ser–Thr phosphatases. More extensive sequence information about this group of Ser–Thr kinases and phosphatases can be found at the Microbial Signal Transduction (MiST) database website (Ulrich and Zhulin, 2007).

Phage E9 binds selectively to the RitR DNA-binding domain

Phage ELISA was used to localize the domain of RitR which interacts with the E9 phage motif. Microtitre plate wells coated with Glutathione-S-transferase (GST)–RitR(DBD), GST–RitR(RD) or GST–RitR (full length) were probed with purified phage E9. Bound phage were detected with anti-M13 horseradish peroxidase (HRP) conjugate monoclonal antibody. Results, shown in Fig. 3, indicate that phage E9 bound both to full-length GST–RitR (lane 1) and to GST–RitR(DBD) (lane 3), but not detectably to GST–RitR(RD) (lane 2).

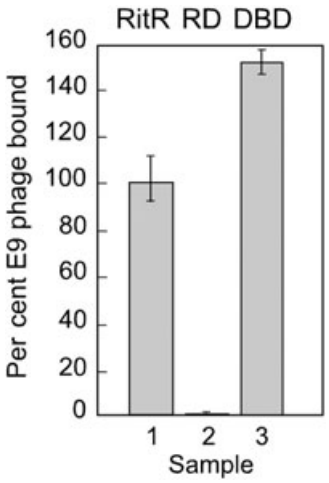

Fig. 3. RitR domain specificity of E9 phage binding. Microtitre plate wells were coated with solutions containing equimolar amounts of test protein as indicated, and probed with phage carrying the E9 sequence. Proteins used to coat the wells were: GST–RitR (lane 1); GST–RitR(RD) (lane 2); and GST–RitR(DBD) (lane 3). E9 phage binding to the protein samples was measured by addition of horseradish peroxidase-conjugated monoclonal rabbit anti-phage-epitope antibody.

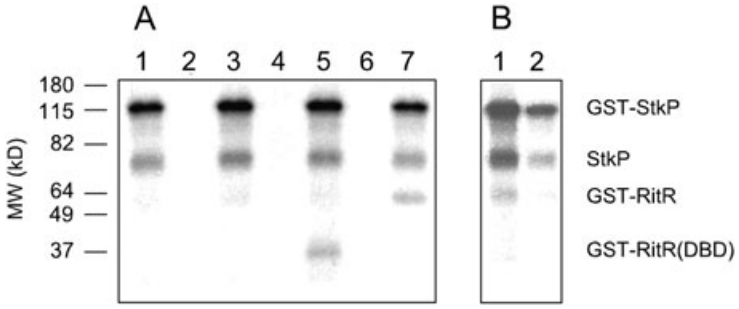

Fig. 4. *In vitro* phosphorylation of RitR by StkP.
A. Domain specificity of StkP phosphorylation of RitR: autophosphorylation of GST–StkP (lane 1), GST–RitR(RD) + GST–StkP (lane 3), GST–RitR(DBD) + GST–StkP (lane 5), GST–RitR full length + GST–StkP (lane 7), GST–RitR(RD) alone (lane 2), GST–RitR(DBD) alone (lane 4), RitR full length alone (lane 6).
B. Dephosphorylation of phosphoryl RitR by PhpP: GST–StkP + phospho RitR prior to addition of PhpP (lane 1), dephosphorylation of phospho RitR by addition of PhpP (lane 2).
Corresponding bands are labelled to the right of the figure.

StkP phosphorylates the DBD of RitR

The putative interaction between PhpP and RitR suggested that RitR might also interact with StkP, the genome neighbour and cognate kinase of PhpP. Autophosphorylated GST–StkP was incubated with GST–RitR, fractionated by PAGE and analysed by autoradiography. The larger of the two bands shown in Fig. 4A (lane 1) was identified as autophosphorylated GST–StkP, molecular weight approximately 100 kDa, in the non-linear part of the gel, and StkP with molecular weight 72 kDa, respectively, the latter presumed to have been formed by spurious proteolysis of GST–StkP. No intrinsic phosphorylation activity was seen with RitR(RD), RitR(DBD) or full-length RitR (lanes 2, 4 and 6 respectively). Results shown for the remaining lanes indicate that full-length GST–RitR, 53.7 kDa (lane 7) and GST–RitR(DBD), 39.8 kDa (lane 5), are phosphorylated. In contrast, StkP shows no phosphorylation activity on GST–RitR(RD), with an expected mobility equal to that of GST–RitR(DBD) (lane 3). Addition of PhpP to GST–phosphoryl RitR resulted in the removal of the label, as shown in Fig. 4B, GST–phosphoryl RitR (lane 1) and GST–phosphoryl RitR plus PhpP (lane 2).

In vitro *reconstitution of the* piu *promoter with RitR, PhpP and StkP*

A 217 nt DNA sequence containing the ^{32}P-labelled *piu* promoter, P*piu*, was incubated with RitR–DBD and complex formation was monitored by electrophoretic mobility shift analysis (EMSA). Results shown in Fig. 5A indicate that DNA binding was half saturated at 250 nM GST–RitR(DBD) and completely saturated at 500 nM. P*piu* incubated with PhpP alone showed no discernible shift (Fig. 5A, lane 2). P*piu*–RitR(DBD) complexes incubated with PhpP resulted in the formation of supershifted complexes, shown in Fig. 5B, suggesting PhpP binds to the RitR(DBD), and not directly to the *piu* promoter. The addition of GST–StkP to the supershifted complexes resulted in reversion to P*piu*–RitR(DBD) complexes, as shown in Fig. 5C, suggesting StkP is capable of competing with RitR–DBD for PhpP binding. These observations indicate that the RitR–P*piu* promoter interaction may be part of a more complex regulatory process at the *piu* promoter involving input from a Ser–Thr signal transduction system.

RitR, PhpP and StkP affect piu *expression*

We previously showed that RitR produced footprints at the *piu* promoter and repressed *piu* expression (Ulijasz *et al*., 2004). Northern blot analysis was used to determine whether PhpP and its cognate kinase StkP also contribute to *piu* expression. Quantification of Northern blot intensities is included in the Fig. 6 legend. The effect of StkP on *piu* expression is consistent with previous microarray studies on *S. pneumoniae* Δ*stkP* (Sasková *et al*., 2007). From these data, we infer that both PhpP and StkP participate in positively promoting *piu* expression; however, since PhpP and StkP have opposite effects on RitR phosphorylation, it is surprising that they affect *piu* expression in a similar manner.

Discussion

RitR and regulation of Fe-haem transport

Genomic studies of *S. pneumoniae* by Lange *et al*. (1999) and Throup *et al*. (2000) defined the set of 27 signal transduction elements (13 kinase–response regulator pairs plus one orphan response regulator) present in this organism. By systematically inactivating each of these elements and measuring virulence of the resultant strain in a mouse lung model, Throup *et al*. determined

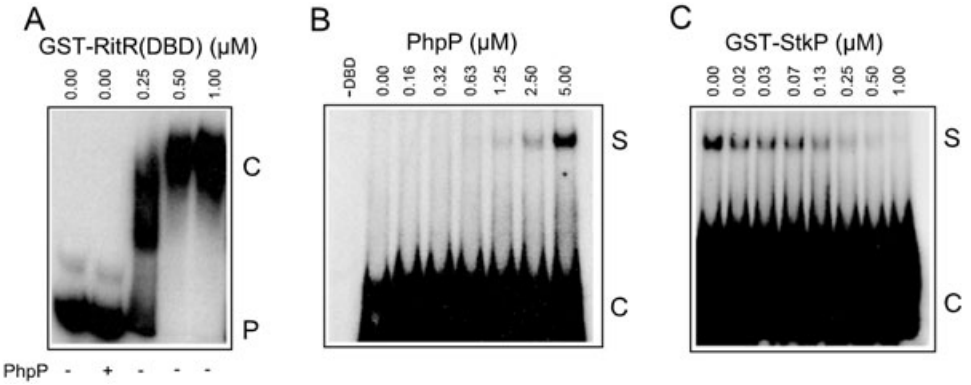

Fig. 5. EMSA of stepwise reconstituted *piu* promoter, P*piu*. P*piu* DNA was incubated with GST–RitR, PhpP and GST–StkP, and resultant complexes were characterized by EMSA. Annotations on the right side of each panel: 'P' denotes ^{32}P-labelled P*piu* DNA; 'C' denotes the shifted complex formed by the interaction between 'P' and GST–RitR(DBD); 'S' denotes the supershifted complex formed by the interaction of C with PhpP-RitR(DBD).
A. EMSA of ^{32}P-P*piu* DNA as a function of added GST–RitR(DBD). Complexes formed by adding increasing concentrations of GST–RitR(DBD) are shown in lanes 1, 3, 4 and 5. The absence of a shift if PhpP alone is added (lane 2).
B. Supershift of ^{32}P-P*piu*-GST–RitR(DBD) binary complex as a function of added PhpP. The supershifted complex produced by adding increasing concentrations of PhpP to the binary complex analysed in (A) is shown in lanes 2–8. Gels used in (B) were run twice as long as those used in (A) to facilitate demonstration of supershifted complexes. As a result, the radioactive sample shown in lane 1 in (A), labelled 'P', has run off the gel and cannot be seen in (B). The fastest moving material in (B) at the bottom of the gel corresponds to component C shown in (A).
C. Break-down of supershifted complexes as a function of added GST–StkP. The break-down of supershifted complexes formed in (B) upon addition of increasing concentrations of GST–StkP is shown in lanes 1–8.

the extent to which individual elements contribute to virulence. One strain in which RR489 had been inactivated showed a substantial reduction in virulence. In our hands, this strain, Ulijasz *et al*. (2004), showed a marked increase in Fe-haem uptake, suggesting its role as a repressor of haem-Fe transporter synthesis. RR489 was therefore renamed RitR (for Represor of iron transport). Brown and Holden (2002) and Tai *et al.* (2003) reviewed phenotypic aspects of the haem-Fe transporters of *S. pneumoniae* and the role that they play in virulence. Our complementary studies have focused on RitR as a genetic regulatory factor that controls expression of haem-Fe uptake transporter genes.

Regulation of RitR by a Ser–Thr kinase–phosphatase pair

The present studies extend our understanding of regulation of the *piu* promoter by identifying partners with which RitR interacts. The *piu* promoter exhibits three binding sites for RitR, thus acting as a scaffold for interaction with transcription factors and providing part of the physical basis for the observed regulation. In the absence of a cognate His kinase for RitR and the replacement of Asp by Asn at the expected response regulator receiver site, our initial studies left a question as to whether phosphorylation of RitR was a likely means for modulating its regulatory activity. The interaction of other proteins with RitR and with the *piu* promoter provides a new direction for studying regulation by RitR. Newer aspects of bacterial signalling that includes variations on the classical His–Asp phosphorelay model have been reviewed by Galperin (2004; 2006).

One would expect that StkP and PhpP exert opposing effects on regulation at the *piu* promoter. Instead, as shown in Fig. 6, a similar dependence of Piu on both was seen. Since StkP and PhpP are translationally linked adjacent loci, inactivation of one could affect the expression of the other, so that inactivation of either resulted in inactivation of both. Northern blot analysis of these mutants showed expression of StkP in the PhpP knockout and expression of PhpP on the StkP knockout,

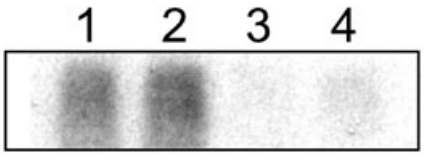

Fig. 6. PhpP positively regulates *piu* expression. The dependence of *piu* expression on RitR, PhpP and StkP was determined by Northern blot analysis. Equal amounts of total RNA were fractionated by formaldehyde agarose gel electrophoresis, transferred to nitrocellulose and probed with a ^{32}P-labelled *piuA*-specific RNA transcript. Wild type (lane 1), Δ*ritR* (lane 2), Δ*phpP* (lane 3), Δ*stkP* (lane 4). The autoradiogram from one of five experiments is shown. The blots were scanned and relative densities normalized to wild type, set at 100, were: 138.8 ± 21.2 for Δ*ritR* (lane 2), 30.0 ± 10.8 for Δ*phpP* (lane 3) and 35.5 ± 5.6 for Δ*stkP* (lane 4).

with both levels of expression apparently higher in the knockout than in the wild type (data not shown).

The use of combinatorial phage display provided a specific affinity reagent for RitR, namely, phage bearing the sequence VCVADGMGGRC (referenced as E9 in the present studies) that allowed us to identify PhpP as an interacting partner. Interestingly, this sequence corresponds to the conserved active centre of the Ser–Thr phosphatase PhpP, previously proposed to act as a global regulator of gene expression in *S. pneumoniae* by Novakova *et al*. (2005) and by Sasková *et al*. (2007). An ELISA based on phages carrying the E9 sequence surprisingly showed selective binding to the RitR–DBD rather than the RitR–DBD, suggesting a new regulatory paradigm part of which involves the interaction between the DBD of a response regulator and a phosphatase.

The association of RitR with the active site of PhpP suggests a possible catalytic interaction between PhpP and RitR. A likely candidate to test for phosphorylation of RitR would be StkP, the immediate genome neighbour and presumed cognate kinase of PhpP. *In vitro* phosphorylation studies suggest that RitR is capable of being phosphorylated, despite the lack of an Asp at the canonical phosphorylatable site in the RD. In these studies (Fig. 4A), StkP was shown capable of phosphorylating RitR selectively in the RitR(DBD). Additionally, PhpP was shown capable of enzymatically removing the phosphate from phosphoryl-RitR (Fig. 4B).

DNA microarray analyses of *S. pneumoniae* transcripts from an StkP knockout strain by Sasková *et al*. (2007) noted dependence of the Piu and Pia haem-Fe transporter expression on StkP. Our studies, Fig. 6, showed that *piu* expression is dependent on both PhpP and StkP individually. Since deletion of PhpP or StpK individually would be expected to have opposing effects, not seen in Fig. 6C and D, there may be other factors involved in regulation at the *piu* promoter.

PhpP is capable of interacting with *piu* promoter–RitR(DBD) complexes (Fig. 5B); however, PhpP does not bind directly to the *piu* promoter DNA (Fig. 5A) and does not compete with *piu* promoter DNA for RitR binding. StkP, however, is capable of breaking down the promoter DNA–RitR–PhpP ternary complex (Fig. 5C), possibly by displacing PhpP. The precise way in which *piu* expression is regulated by altered phosphorylation states of RitR remains unclear. Alternative explanations include the possibility that more definitive changes in *piu* expression may require host conditions absent in cells cultivated *in vitro*.

Previous studies in *S. pneumoniae* by Echenique *et al*. (2004) suggest that StkP belongs to a signalling network that more broadly regulates competence as well as pathogenesis in both lung and blood-borne infection models. Novakova *et al*. (2005) identified phosphoglucosamine mutase (GlmM) and RNA polymerase subunit alpha (αRNAP) as phosphorylatable substrates of StkP. They suggested a role for phosphorylation of GlmM by StkP in the regulation of cell wall biosynthesis. Thus the Ser–Thr kinases that phosphorylate RitR and related CovR(CsrR) response regulators receive environmental input from four C-terminal extracellular PASTA (penicillin-binding protein and serine/threonine kinase associated) domains. This type of sensor consists of a series of four PASTA subdomains, reported to be responsive to beta lactam antibiotics, cell wall peptidoglycan synthetic precursors or cell wall peptidoglycan-containing autolytic degradation products. This would suggest that StkP responds to physiological conditions under which the infecting streptococci grow, which in turn is reflected in the variety of peptidoglycans shed into the medium. PASTA domain physiology and genomics have been reviewed by Yeats *et al*. (2002).

The RitR amino acid sequence resembles that of the CovR(CsrR) family of global gene regulators

Reasons for classifying RitR as a member of the CovR (CsrR) family of gene regulators include: (i) RitR aligned with *Streptococcus pyogenes* CovR shares 102/229 identical amino acids; the similarity is even greater if one scores chemical properties of amino acid side-chains, and (ii) both RitR and CovR recognize a promoter DNA binding site that has an invariant ATTA core sequence which, in *S. pyogenes*, has been called the 'ATTARA' sequence (Gusa and Scott, 2005). Moreover, RitR and CovR play a major role in the regulation of virulence. Consistent with these data is the observation that the identity between the two sets of sequences is higher in the C-terminal half containing the DBD than in the N-terminal half containing the RD.

The combinatorial complexity of interactions at CovR(CsrR)-responsive promoters can be ascribed to: (i) the number of promoter ATTA-type binding sites and their relative orientations, (ii) Asp phosphorylation and its effect on the affinity between CovR and DNA, and (iii) Asp phosphorylation and its effect on cooperativity of protein–protein interactions at the promoter (Churchward, 2007). Compounding these factors with Ser–Thr phosphorylation of the DBD described in this work potentially adds an additional degree of complexity to the regulation of virulence in the streptococci. Genetic regulation of *S. pyogenes* virulence by CovR(CsrR) has been reviewed by Churchward (2007) and by Graham *et al*. (2002).

There are also strong similarities between *S. pneumoniae* RitR and the CovR(CsrR) virulence regulators in other streptococci . Rajagopal *et al*. (2003) studied the role of the Ser–Thr kinase phosphatase pair Stk1 and Stp1, respectively, in cell growth and virulence of *S. agalactiae* and identified an inorganic pyrophosphatase as a substrate of

this enzyme pair. Recently Rajagopal *et al*. (2006) linked phosphorylation of CovR by a Ser–Thr kinase to regulation of cytotoxin expression in *S. agalactiae*. A broad range of other studies of the role of the CovR(CsrR)-type regulators have been reported (Miller *et al*., 2001; Lamy *et al*., 2004; Orihuela *et al*., 2004; Gao *et al*., 2005; Jiang *et al*., 2008).

Results described above underscore the importance of the report of Rajagopal *et al*. (2006) that CovR(CsrR) of *S. agalactiae* is phosphorylated by Stk1, the Ser–Thr kinase orthologue of StkP. Under the assumption that phosphorylation of full-length *S. agalactiae* CovR by Stk1 (Rajagopal *et al*., 2006) is analogous to RitR phosphorylation shown above, we postulate a new paradigm for gene regulation in the streptococci. Under this model, response regulators may respond to two classes of external signals – one class linked a His kinase that phosphorylates the RD, and the other linked to a Ser–Thr kinase that phosphorylates the DBD. In streptococci with both Asp and Ser phosphorylation, this model leads to four possible response regulator phosphorylation states – phosphorylation of the RD alone, of the DBD alone, of both RD and DBD, and of neither.

The functional significance of DBD phosphorylation is not yet known. A sufficiently high degree of RitR labelling by StkP might make it possible to detect differences in the affinity with which RitR binds to promoter DNA, interacts cooperatively with RitR molecules bound to other sites in the promoter, or forms some higher-order complex with other proteins at a RitR-regulated promoter. In addition to *S. pneumoniae*, phosphorylation may be studied in *S. agalactiae* and *S. pyogenes* where CovR possesses the canonical Asp and can therefore be regulated by either a cognate His kinase or Ser-Thr kinase.

It will be interesting to see how the multiple phosphorylation sites in the CovR(CsrR) family combined with a multiplicity of binding sites and degrees of binding cooperativity at promoters regulated by this family contribute to the complexity of virulence regulation in the streptococci.

Experimental procedures

Bacterial strains and growth conditions

Streptococcus pneumoniae TIGR4 and derived strains were grown in THY medium (Todd Hewitt Broth + 0.5% yeast extract) supplemented with kanamycin (200 μg ml^{-1}) or chloramphenicol (2 μg ml^{-1}), as required. Cultures were incubated without shaking in 5% CO_2 at 37°C. *S. pneumoniae* TIGR4 was transformed as described by Bricker and Camilli (1999) using competence-stimulating peptide CSP2, a gift of D.A. Morrison (Pestova *et al*., 1996). *S. pneumoniae* TIGR4 Δ*ritR* was constructed by insertional displacement as described previously (Ulijasz *et al*., 2004).

Allele replacement mutagenesis

Streptococcus pneumoniae TIGR4 strains deficient in StkP, or PhpP alone, were constructed using allele replacement mutagenesis as described by Song *et al*. (2005). The knockout construct consisted of a *kan* cassette flanked by 300–500 nt sequences from the 5′ and 3′ regions, respectively, of the target open reading frame to be replaced. *S. pneumoniae* TIGR4 cells were transformed with the resultant construct followed by selection on Trypticase-Soy agar supplemented with 5% sheep's blood and kanamycin (200 μg ml^{-1}). The kanamycin gene was kindly provided by D.A. Morrison (Sung *et al*., 2001). Since *phpP* and *stkP* comprise an operon in which the two genes are translationally linked, gene disruption constructs were tested by Northern blot analysis to ensure the absence of expression of the replaced open reading frame as well as expression of the neighbouring open reading frame that was not disrupted.

Construction of RitR, StkP and PhpP expression vectors

Plasmids for preparative protein expression were constructed as described previously (Ulijasz *et al*., 2004). GST fusion proteins: GST–RitR, GST–PhpP and GST–StkP, were purified using a glutathione-Sepharose 4B affinity column. PhpP was liberated from GST by PreScission Protease (GE Healthcare). To construct the required plasmids, full-length *ritR*, *phpP* or *stkP* were PCR-amplified and inserted into plasmid pGEX-6P-1 DNA at the *EcoRI* and *BamHI* sites.

Phage display

Phage display was performed as described by Sparks *et al*. (1996). Microtitre plate wells coated with RitR were used to select binding phage from the combinatorial phage peptide display library 'X10C', a gift of B.K. Kay (Gee *et al*., 1998). Phage from the third round of selection were plaque purified and the level of binding to RitR-coated wells was measured by phage ELISA using a monoclonal anti-M13 phage HRP antibody conjugate (GE Healthcare). Phage binding to RitR was compared with background binding to BSA. Phage clones that bound RitR at a level more than threefold over the BSA background were sequenced.

Electrophoretic mobility shift analysis (EMSA)

EMSAs were performed as described by Ulijasz *et al*. (2004) with modifications as listed herein. P*piu*, the 217 bp promoter-regulatory region of the *piu* operon, from −107 to +110 relative to the transcriptional start site of *piuBCDA*, was amplified by PCR, 5′ end-labelled using [γ-^{32}P]-ATP and phage T4 polynucleotide kinase, and purified. GST–RitR(DBD), i.e. the DBD of RitR, PhpP and GST–StkP were added to 10 μl of binding reaction mixtures containing ^{32}P-P*piu* DNA, and incubated on ice for 10 min. The resultant incubation mix was fractionated by electrophoresis using 4% non-denaturing polyacrylamide gels cast in 1× TAE buffer: 0.05 M Tris-acetate (pH 8.0), 1 mM EDTA, followed by autoradiography.

Mass spectrometry analysis of RitR phosphorylation

Ammonium phosphoramidate was synthesized as described by Buckler and Stock (2000) and used to phosphorylate response regulators *in vitro*. The reaction mixture (total volume of 100 μl) contained: 50 mM HEPES (pH 7.2), 5 mM $MgCl_2$, 10 mM ammonium phosphoramidate and 9 μM response regulator protein. After incubation for 1 h at 37°C, part of the reaction mixture was used directly in analytical studies. A sample of the reaction mixture was analysed by an ABI 365 Electrospray Ionization Liquid Chromatography Mass Spectrometer (ESI-LC/MS) equipped with a C4 reverse-phase column. The column was eluted at 30 μl per minute with a 0–95% gradient of acetonitrile in 0.05% v/v TFA/H_2O. Samples were injected directly into the mass spectrometer for fragment analysis as they emerged from the column.

In vitro *enzymatic phosphorylation-dephosphorylation*

StkP was autophosphorylated in a total volume of 20 μl containing: 50 mM Tris-HCl (pH 8.0), 1 mM DTT, 5 mM $MgCl_2$, 100 μM ATP, 1 μCi of [γ-^{32}P]-ATP (Specific activity, > 6000 Ci mmole^{-1}) and 100 ng GST–StkP, followed by incubation at 30°C for 20 min. To phosphorylate RitR, GST–RitR, GST–RD or GST–DBD, equimolar amounts (approximately 1 μg) were added to the above reaction and incubated at 30°C for an additional 20 min. The reaction was stopped by addition of SDS loading buffer and the samples were fractionated by SDS-PAGE, mounted in cellophane, dried and analysed by autoradiography. Dephosphorylation of phosphorylated RitR was performed by adding 200 ng of GST–StkP and 1 μg of GST–RitR that had been previously phosphorylated, as described above, to a 20 μl reaction containing: 50 mM TrisHCl (pH 8.0), 5 mM $MnCl_2$ and 1 μg of purified PhpP. Reactions were incubated for 40 min at 30°C prior to fractionation by SDS-PAGE and autoradiography.

RNA purification and Northern blot analysis

Streptococcus pneumoniae cells were grown in THY medium to mid-log phase. RNA from cell pellets was extracted twice with hot acid phenol, ethanol precipitated and purified using the RNeasy (Qiagen) kit following the manufacturer's specifications. For Northern blot analysis, 5 μg of total RNA was separated by formaldehyde-agarose gel electrophoresis followed by transfer of the fractionated RNA to nitrocellulose. ^{32}P-labelled 300 bp RNA specific to the *piuA* open reading frame was synthesized with the RiboScribe kit (Epicentre) as specified by the manufacturer. The labelled RNA was used to probe the nitrocellulose membrane sample, which following autoradiography was analysed by densitometry.

Phage ELISA

Interaction between RitR and E9 phage was performed following established phage ELISA protocols (Sparks *et al*., 1996). All washes were performed with PBS + 0.1% Tween-20. Equimolar amounts of GST–RitR, GST–RitR(RD) or GST–RitR(DBD) were bound to Costar (Corning) protein binding plates. For phage ELISA, 5×10^9 plaque-forming units (pfu) of purified E9 phage was used per well, followed by quantification of bound phage with monoclonal anti-M13 phage HRP conjugate antibody at 1/20 000 dilution. Binding of antibody to phage was determined by the amount of tetramethylbenzidine (TMB) substrate converted by peroxidase measured by absorbance at 650 nm.

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