

## Contribution of a Response Regulator to the Virulence of *Streptococcus pneumoniae* Is Strain Dependent

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**Bacterial two-component signal transduction systems (TCS) enable bacteria to respond to environmental changes and regulate a range of genes accordingly. They have a crucial role in regulating many cellular responses and have excellent potential as antibacterial-drug targets. We have constructed mutations in a TCS response regulator gene for two different strains of the human pathogen *Streptococcus pneumoniae*. These mutants have been analyzed in our murine model of infection. Data suggest that in a D39 background the response regulator gene is essential for virulence; an isogenic mutant is avirulent via intraperitoneal, intranasal, and intravenous routes of infection. This mutant, which does not show impaired growth in vitro, is unable to grow in the lung tissue or in blood. Mutation of the response regulator in a 0100993 background results in a strain that is fully virulent intraperitoneally and intravenously but shows decreased levels of bacteremia and increased murine survival following intranasal infection. The ability to grow in the lung tissue is not impaired in this mutant, suggesting that it has an impaired ability to disseminate from the lungs to the systemic circulation. Our data highlight the importance of assessing the contribution of putative virulence factors to the infection process at different sites of infection and provide evidence that virulence determinants can behave very differently based on the genetic background of the bacterial strain. These important findings may be relevant to other bacterial pathogens.**

*Streptococcus pneumoniae* remains an important human pathogen responsible for much morbidity and mortality worldwide. The disease burden from pneumococcal disease is increasing, even in developed countries, and resistance to commonly used antibiotics is becoming more prevalent (18). Evidence also exists linking penicillin-resistant invasive isolates to increased morbidity and mortality (9). The polysaccharide vaccine currently in use fails to protect those most susceptible to invasive disease, namely, infants and the elderly. Recently licensed conjugate vaccines have helped overcome such problems, but the serotype coverage of these vaccines is greatly restricted. Furthermore, there are concerns over the high cost of such vaccines, potential capsular switching, and serotype replacement (36). The need to identify novel therapies and vaccine candidates for successful management of this pathogen is evident from the recent efforts of researchers using genome-wide strategies to search for virulence factors (11, 20, 22, 24, 25, 34). Such techniques have been fundamental in identifying a multitude of potential drug and vaccine candidates but have limited capacity to analyze individual genes in detail for their contributions to virulence.

Among the genes identified in genomewide searches for pneumococcal virulence factors are those encoding proteins of bacterial two-component signal transduction systems (TCS). These systems generally comprise a transmembrane receptor protein, or histidine kinase (HK), and its cognate intracellular response regulator (RR), involved in the regulation of one or

several bacterial genes. TCS become active when an external signal, such as temperature, pH, ion concentration, or nutrient concentration, is detected by the HK, which undergoes autophosphorylation on a histidine residue. The phosphoryl group is transferred onto the N-terminal region of the cognate response regulator, resulting in a conformational change and subsequent gene regulation. Response regulator proteins are thought to act as transcription factors and may alter the expression of several genes or subsets of genes. TCS are widespread among prokaryotes and are fundamental to the way in which bacteria sense their external environment and regulate genes accordingly. Genes essential for viability and important virulence factors have been found to be under the regulation of TCS (reviewed in reference 8). Furthermore, bacterial TCS homologues have not been identified in vertebrates, making these systems excellent targets for antibacterial therapies (2).

Thirteen TCS have been identified in the genome of *S. pneumoniae*, together with a single, unpaired response regulator (19, 31). All TCS are present in all three genomes sequenced to date (7, 14, 30). Mutations in individual genes of all pneumococcal TCS have been made and evaluated in murine models of infection. In one study, inactivation of all response regulator genes in serotype 22 and serotype 3 backgrounds did not result in any significant attenuation in virulence compared to that of isogenic parental strains when bacteria were given intraperitoneally (19). A second study, however, mutated TCS genes in a serotype 3 background and found that many mutants showed a significantly attenuated ability to colonize lung tissue following intranasal infection (31). These findings may highlight important differences in the contributions of certain bacterial virulence factors at various sites of infection, as demonstrated by the recent findings of Hava and Camilli (11).

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Furthermore, the genetic background of the murine model and/or of the bacterial strains chosen, together with differences in techniques used to create mutants, may result in the discrepancies observed (10).

We have analyzed null mutations in the gene encoding RR09 in two different pneumococcal strains, D39 (serotype 2) and 0100993 (serotype 3). Together with *hk09*, this response regulator forms *tcs09* (the nomenclature used is that of Lange et al. [19]). The gene name for this response regulator is *sp0661* (TIGR4 sequence) or *spr0578* (R6 sequence) (<http://www.tigr.org/>). The whole *rr09* gene in strain 0100993 was mutated by allelic replacement. This null mutation was subsequently transformed into D39, resulting in two otherwise isogenic mutants of two different parental strains. All strains were subjected to comprehensive in vivo analysis to determine their virulence in a murine model of infection. Such detailed characterization has allowed us to identify a role for this response regulator in virulence in both pneumococcal strains and highlights the importance of utilizing different routes of infection when analyzing bacterial virulence factors. Our work also demonstrates that genes make different contributions to virulence depending on the genetic background of the parental strain, a property that must be investigated fully before any bacterial protein can be considered as a vaccine candidate or drug target.

#### MATERIALS AND METHODS

**Pneumococcal strains.** Wild-type *S. pneumoniae* strains D39 (serotype 2; NCTC 7466) and 0100993 (serotype 3; NCIMB 40794; kindly provided by M. K. Burnham, GlaxoSmithKline), both encapsulated, virulent strains, were used for all studies. The isogenic  $\Delta rr09$  (from parent strain D39) and  $\Delta 488$  (from parent strain 0100993; provided by M. K. Burnham, GlaxoSmithKline) mutants were created as described below. *S. pneumoniae* strains were maintained in brain heart infusion (BHI) broth (Oxoid Ltd., Basingstoke, United Kingdom) or on blood agar base no. 2 (BAB; Oxoid Ltd.) supplemented with 5% (vol/vol) defibrinated horse blood (E&O Laboratories, Bonnybridge, United Kingdom). For culture of all mutants, the medium or agar was supplemented with 1.0  $\mu\text{g}$  of erythromycin  $\text{ml}^{-1}$ . All strains were confirmed as being *S. pneumoniae* by Gram staining, optochin sensitivity, the presence of alpha-hemolysis on BAB plates, and multilocus sequence typing. The Quellung reaction was used to confirm the capsular serotype.

**Construction of mutants.** Mutants of strain 0100993 with null mutations of the response regulator gene ( $\Delta 488$ ) were provided by M. K. Burnham (GlaxoSmithKline). These were created by allelic replacement as described previously (31). PCR primers R9F (GCGGAGCCGAGTAGGAGATTCTCACC) and R9R (TGTAGAAATGACCTGACCAG) were used to amplify the mutated response regulator gene from the  $\Delta 488$  mutant. PCR products were transformed into the virulent strain D39 by using the protocol described below. Transformants were selected on erythromycin. Chromosomal DNA was prepared as described below, and mutants were confirmed by PCR and subsequent sequencing of the product. Sequencing was performed at the Sequencing Service, University of Glasgow. Two independent mutants were created and analyzed.

**Confirmation of the TCS09 locus and surrounding genes in different strains.** Chromosomal DNA was prepared from bacterial strains as described below. PCR was performed using primers designed at various regions within, upstream of, and downstream of the TCS09 locus (see Fig. 1). Primer sequences were as follows: for primer 1, CAGTTAGCCAAGGAGGAA; for primer 2, CCTCAA TATCTGACGAG; for primer 3, AGTCTTACCCAAGCTGGTCC; and for primer 4, ACAGATGCTTCTGTCCT.

**Transformation of *S. pneumoniae*.** Cultures were grown to an optical density at 600 nm of 0.1 in BHI supplemented with 1 mM  $\text{CaCl}_2$  (final concentration). One milliliter of culture was incubated with a competence-stimulating peptide (CSP-1 [12]), to a final concentration of 100  $\text{ng ml}^{-1}$ , at 37°C for 15 min. Sample DNA (8 to 10  $\mu\text{g}$ ) was added, and cultures were incubated for 75 min at 37°C. Potential transformants were selected by their ability to grow on BAB supplemented with erythromycin. As a control, D39 was transformed with plasmid pVA838, which is able to replicate in the pneumococcus and confers erythromycin resistance on transformants (21).

**Preparation of *S. pneumoniae* chromosomal DNA.** Cultures at mid-log phase were centrifuged for 15 min at 4,000  $\times g$ . The pellet was resuspended in 1 ml of extraction buffer (10 mM Tris [pH 8.0], 100 mM EDTA [pH 8.0], 0.5% sodium dodecyl sulfate) and incubated for 1 h at 37°C. Proteinase K was added to a final concentration of 100  $\mu\text{g ml}^{-1}$ , and the mixture was incubated for 3 h at 50°C. RNase was added to a final concentration of 20  $\mu\text{g ml}^{-1}$ , and the mixture was incubated at 37°C for 30 min. The resultant suspension was mixed with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol) and centrifuged at 13,000 rpm for 3 min. The aqueous layer was added to 0.2 volume of 10 M ammonium acetate. Ethanol (100%) was added to precipitate the DNA, and the mixture was centrifuged at 18,000  $\times g$  for 30 min. The DNA pellet was left to air dry for 10 min and then resuspended in DNase-free Tris-EDTA buffer (pH 7.4).

**In vitro growth and stability.** BHI was inoculated with  $1.0 \times 10^6$  CFU of bacterial strains  $\text{ml}^{-1}$  and incubated at 37°C. Samples were removed every 2 h for 20 h. The optical density at 600 nm was determined, and viable cells were enumerated as described previously (6). To assess the ability of mutant strains to retain erythromycin resistance, cultures were grown in the absence of erythromycin. At predetermined time points, viability in the presence and absence of erythromycin was determined. Experiments were performed in duplicate and repeated on at least two separate occasions.

**In vivo analysis.** Female outbred MF1 mice (Harlan Olac, Bicester, United Kingdom) aged 9 to 13 weeks and weighing 30 to 35 g were used for all studies. All mice were provided with sterile pelleted food (B&K Universal, North Humberside, United Kingdom) and water ad libitum. All animal work was carried out under appropriate licensing and with approval from the Home Office and the University of Glasgow.

Prior to use in mice, strain 0100993 and the corresponding isogenic  $\Delta 488$  mutant were passaged in mice as described previously (6). D39 and the corresponding isogenic  $\Delta rr09$  mutant were used without prior passaging due to the inability to induce bacteremia following intraperitoneal injection with the mutant. Aliquots of bacteria were stored at  $-70^\circ\text{C}$ . When required, aliquots were thawed rapidly, harvested by centrifugation, and resuspended in sterile phosphate-buffered saline (PBS).

In order to determine experimental end points, mice were monitored frequently for symptoms of infection and were culled prior to reaching, or upon reaching, a moribund state. When animals were actively culled, the time was recorded and used as a measure of "mortality" for analysis of survival data. Mice that survived the course of infection (7 days, unless stated otherwise) were assigned an arbitrary survival time of 168 h for statistical analysis.

For intraperitoneal infection, mice were each given  $1.0 \times 10^5$  CFU resuspended in sterile PBS, administered intraperitoneally. Symptoms were monitored for 168 h postinfection, and mice were culled prior to reaching, or upon reaching, a moribund state.

For intranasal challenge, mice were lightly anesthetized with 2.5% (vol/vol) halothane (Zeneca Pharmaceuticals, Adlery Edge, United Kingdom), and  $1.0 \times 10^7$  CFU resuspended in sterile PBS was administered into the nares in a total volume of 50  $\mu\text{l}$ . The inoculum dose was confirmed by plating on BAB as described above. At predetermined time points, a small volume of blood was removed from a tail vein by using a 1-ml insulin syringe (Micro-fine, 12.7 mm; Becton Dickinson), and bacteremia was determined by viable-cell enumeration (6). Symptoms were monitored for 168 h postinfection, and mice were culled prior to reaching, or upon reaching, a moribund state.

To determine the bacterial load in the lung tissue, mice were challenged intranasally as described above. Subsequently, mice were sacrificed at predetermined time points by cervical dislocation. Lungs were removed, weighed, and homogenized in 5 ml of sterile PBS by using an electric tissue homogenizer. Bacterial counts in lung tissue were determined by viable-cell enumeration (6).

For intravenous infection, mice were each administered  $1.0 \times 10^6$  CFU, resuspended in sterile PBS, directly into a tail vein. At predetermined time points, a small volume of blood was removed from a tail vein by using a 1-ml insulin syringe (Micro-fine; 12.7 mm; Becton Dickinson), and bacteremia was determined by viable-cell enumeration (6). Symptoms were monitored for 168 h postinfection, and mice were culled prior to reaching, or upon reaching, a moribund state.

**Statistical analysis.** All results are given as geometric means  $\pm 1$  standard error of the mean (SEM). Virulence studies were analyzed using Mann-Whitney U tests. All other data were analyzed using Student's *t* test (these data were normally distributed). A *P* value of  $<0.05$  was considered statistically significant. For all animal work, a minimum of five animals were evaluated per strain for each time point.

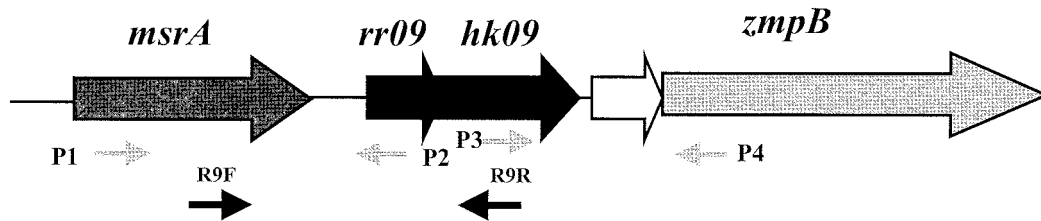


FIG. 1. Gene organization around the TCS09 locus. The *rr* and *hk* genes make up *S. pneumoniae tcs09*. An erythromycin resistance cassette was inserted to replace the *rr09* gene. R9F and R9R, primers used to create and confirm mutations within the *rr09* gene. Downstream of the *tcs09* locus is a conserved hypothetical protein (open arrow) and a gene, *zmpB*, encoding a putative zinc metalloprotease (3, 23). Immediately upstream lies *msrA*, encoding a methionine sulfoxide reductase, also important in virulence (35). Primer pairs P1–P2, P3–P4, and P1–P4 were used to confirm that the organization of genes surrounding *tcs09* was conserved in the two strains used in this study. The diagram is not drawn to scale.

**RESULTS**

**Construction of mutants.** A knockout mutation in *rr09* in strain 0100993 of *S. pneumoniae* was transferred into strain D39 by transformation, creating the isogenic  $\Delta 488$  and  $\Delta rr09$  mutants, respectively. PCR, using primers flanking the mutated gene, was used to confirm the replacement of the target gene with a cassette encoding erythromycin resistance (data not shown). The PCR fragments were subsequently sequenced to ensure that the entire gene was deleted and to ensure that the downstream *hk* gene was not disrupted. Preliminary microarray analysis has also confirmed the expression of *hk09* in the  $\Delta rr09$  mutant, demonstrating that no polar effects have occurred (C. E. Blue and T. J. Mitchell, unpublished data). Genomic analysis suggests that *tcs09* comprises a single transcript, so polar effects on other downstream genes will be

eliminated. Two independent mutants were analyzed for virulence and were found to demonstrate the same phenotype (data not shown).

**Confirmation of the TCS09 locus and surrounding genes in different serotypes.** PCR, with several primer pairs, was used to determine whether the *tcs09* locus and surrounding genes identified in the available pneumococcal genomes sequenced to date (14, 30) were present in the bacterial strains used for this study. Figure 1 shows the gene organization in strains TIGR4 and R6, together with the relative positions of primers used. The results showed that the gene organization of D39 and 0100993 is the same as that of the sequenced strains (data not shown).

**In vitro growth and stability.** Isogenic mutants were analyzed for their abilities to grow in culture. No growth defects

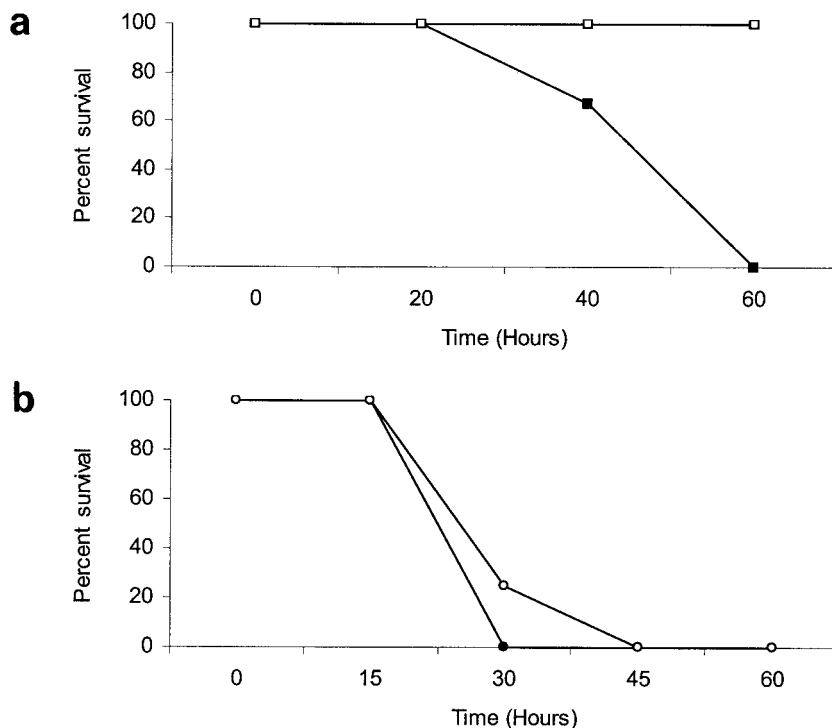


FIG. 2. Survival of mice following intraperitoneal infection with  $10^5$  CFU of wild-type or mutant bacteria. (a) Strains on a D39 background. ■, wild-type strain D39; □, the  $\Delta rr09$  mutant.  $P < 0.05$  for survival time with the  $\Delta rr09$  mutant compared to that with the D39 parental strain. (b) Strains on a 0100993 background. ●, wild-type strain 0100993; ○, the  $\Delta 488$  mutant. No difference in survival was seen between animals challenged with the  $\Delta 488$  mutant and the 0100993 parental strain.

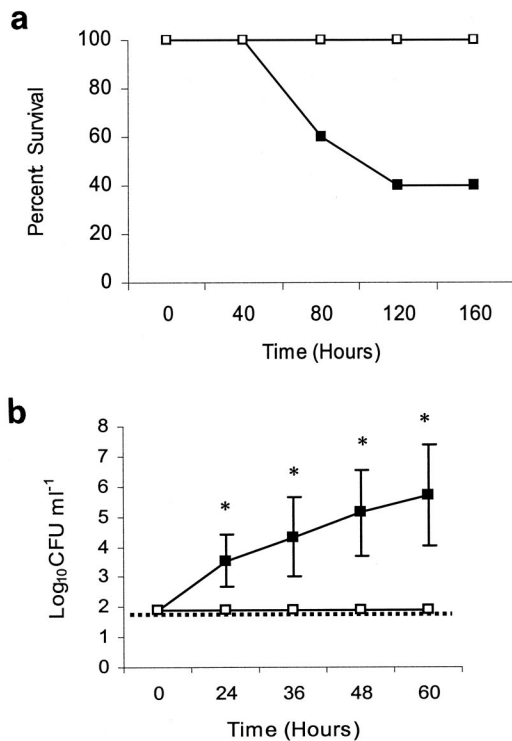


FIG. 3. Intranasal challenge with  $10^7$  CFU on a D39 background. (a) Survival; (b) bacteremia. ■, wild-type strain D39; □, the  $\Delta r709$  mutant.  $P < 0.05$  for survival and for bacteremia at all time points after 0 h. In panel b, error bars represent SEMs; dashed line represents the limit of detection ( $\log_{10} 1.92 \text{ CFU ml}^{-1}$ ).

were identified for either mutant in comparison to the parental strains (data not shown), a finding which is in agreement with previous studies (31). Mutants also retained their erythromycin resistance phenotype during in vitro culture (data not shown).

**Virulence of transformed D39 compared to that of wild-type D39.** To confirm that transformation of D39 does not select for avirulent or reduced-virulence mutants, the virulence of D39 transformed with the control plasmid pVA838 (21) was compared with that of nontransformed wild-type D39. No difference was detected between transformed D39 and wild-type D39 following either intraperitoneal or intranasal challenge (data not shown). Thus, the mutant phenotypes discussed in this work are due to the relevant mutation and not to the transformation procedure used to create the mutants. Transformation efficiency was also compared between the mutants and their isogenic parental strains. No difference was observed (data not shown).

**Intraperitoneal infection.** Prior to commencing any in vivo work, our laboratory routinely passages pneumococcal strains in mice by intraperitoneal injection. This enhances the virulence of bacteria and ensures that differences in virulence are not due to loss of virulence with in vitro culture. High levels of bacteremia are seen 24 h postinjection, and bacteria recovered from the blood are isolated and used to produce stocks for subsequent analysis of virulence. Infection of mice by this route resulted in the rapid appearance of symptoms with strains D39 and 0100993 and with the  $\Delta 488$  mutant. Such mice all succumbed to infection within 3 days, and median survival times were 24, 28, and 28 h, respectively. Intraperitoneal in-

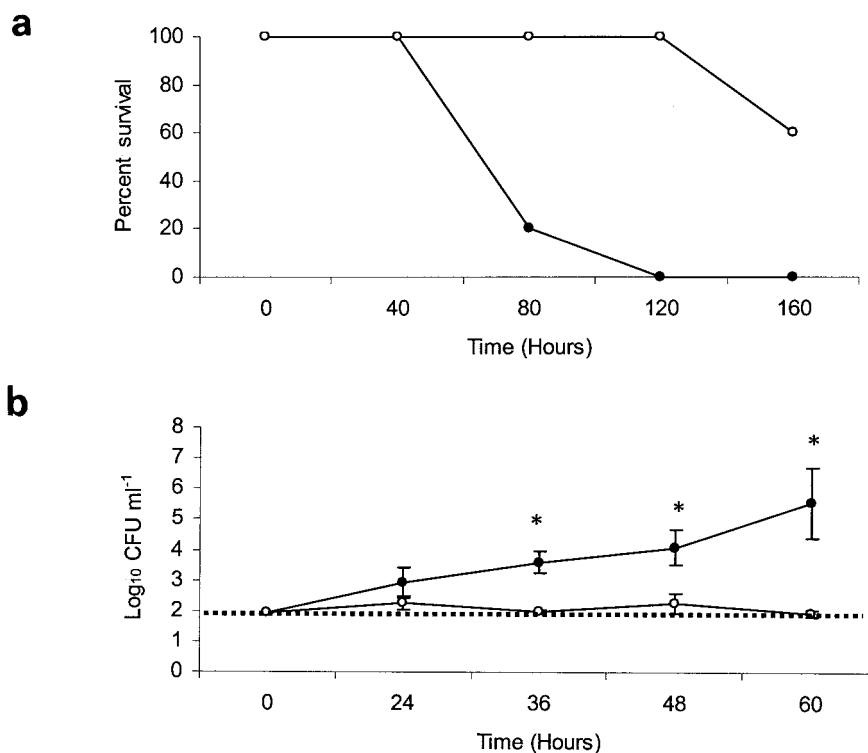


FIG. 4. Intranasal challenge with  $10^7$  CFU on a 0100993 background. (a) Survival; (b) bacteremia. ●, wild-type 0100993; ○, the  $\Delta 488$  mutant.  $P < 0.05$  for survival and for bacteremia at all time points after 24 h. In panel b, error bars represent SEMs; dashed line represents the limit of detection ( $\log_{10} 1.92 \text{ CFU ml}^{-1}$ ).

jection of the  $\Delta rrr09$  mutant, however, did not result in bacteremia at any time point following infection, and all mice survived for more than 5 days without showing any symptoms of disease (Fig. 2). Increasing the dose of the  $\Delta rrr09$  mutant administered as much as 1,000-fold still did not result in bacteremia or cause murine mortality (data not shown).

For all subsequent in vivo analysis, passaged bacteria were used for strain 0100993 and its  $\Delta 488$  mutant only. Non-animal-passaged glycerol stocks were used for all subsequent challenges with D39 and the  $\Delta rrr09$  mutant.

**Intranasal challenge.** Strains were analyzed for their abilities to cause disease in a pneumonia model of infection. Mice were given  $1.0 \times 10^7$  CFU intranasally and monitored for survival. Following challenge with D39, 60% of mice succumbed to infection by 120 h (median survival time, 96 h), but all mice infected with the  $\Delta rrr09$  mutant survived the entire course of the experiment (Fig. 3a). These observations were matched by the levels of bacteremia at predetermined time points following infection. Levels of bacteria in the blood increased over time for D39-infected mice, but no bacteremia was detected in mice infected with the  $\Delta rrr09$  mutant at any of the time points examined (Fig. 3b). The 100% mortality usually seen with wild-type D39 infection was not obtained in this study; this can be attributed to the fact that bacteria were not passaged prior to infection (our laboratory, unpublished data).

Infection of mice with strains on a 0100993 background resulted in 100% mortality with the wild-type strain and 40% mortality with the isogenic  $\Delta 488$  mutant (Fig. 4a). Median survival times of mice were 66 h and 168 h, respectively. Blood counts were similar to those seen for wild-type D39 infection, where a steady increase in counts for strain 0100993 was observed but levels of the  $\Delta 488$  mutant remained very low up to 60 h postchallenge (Fig. 4b). However, some mice did succumb to infection with the  $\Delta 488$  mutant, indicating that bacterial levels had increased later in the infection (when moribund, these mice had counts of  $10^7$  to  $10^9$  CFU ml<sup>-1</sup> in their blood).

**Lung counts.** Bacterial loads within lung tissue were determined 24 and 48 h after intranasal infection with  $10^7$  CFU. Challenge with strains on a D39 background resulted in low numbers of bacteria within lung tissue at both 24 and 48 h postinfection; median counts of the  $\Delta rrr09$  mutant were below the detection limit (Fig. 5a). In contrast, counts in lung tissue following challenge with strains on a 0100993 background were high at both time points, showing an increase between 24 and 48 h postinfection (Fig. 5b). No significant difference was observed between counts of the wild-type strain 0100993 and its  $\Delta 488$  mutant at either time point studied.

**Intravenous challenge.** Bacterial strains were inoculated directly into the veins of mice in order to determine their abilities to grow in the blood. Counts at 0 h confirmed the bacterial dose administered, and subsequent levels of bacteremia were examined at predetermined time points. Mice were also monitored for survival. Forty percent of mice given D39 survived the infection, while all mice injected with the  $\Delta rrr09$  mutant survived (Fig. 6a). The median survival time for mice given D39 was 84 h. The experiment was allowed to proceed for 7 days. By 8 h postinfection, counts in the blood of mice administered D39 or the  $\Delta rrr09$  mutant had fallen significantly, although the exact time postinfection at which this decrease occurred was not determined. Levels of D39 began to increase

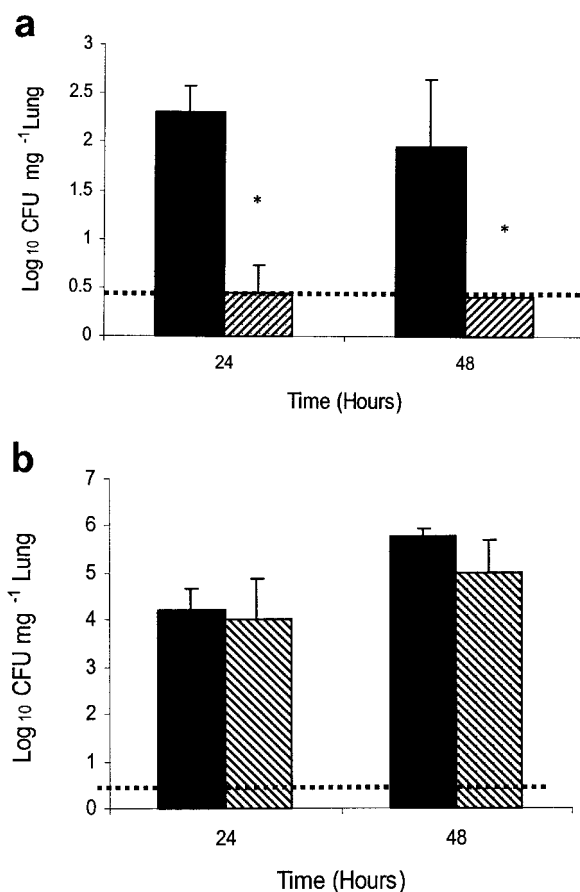


FIG. 5. Bacterial loads in the lung tissue 24 and 48 h following intranasal infection with  $10^7$  CFU. (a) Strains on a D39 background. Filled bars, wild-type D39; hatched bars, the  $\Delta rrr09$  mutant.  $P < 0.05$  for wild-type versus mutant lung counts at both time points. (b) Strains on a 0100993 background. Filled bars, wild-type 0100993; hatched bars, the  $\Delta 488$  mutant. No differences were found between wild-type and mutant lung counts at either time point studied. Values are geometric means  $\pm$  SEMs. The dashed line represents the limit of detection.

after this time point, albeit slowly, while counts of the  $\Delta rrr09$  mutant remained below the detection limit at all time points studied (Fig. 6b). Counts of D39 presumably increased within the blood at time points later than those examined, as 60% of mice eventually succumbed to infection, and when moribund, these mice had counts of  $10^7$  to  $10^9$  CFU ml<sup>-1</sup> in their blood.

All mice infected with strain 0100993 or the  $\Delta 488$  mutant succumbed to infection very quickly, and in both cases, mice had median survival times of 36 h (Fig. 7a). Bacterial counts in the blood of mice administered 0100993 or the  $\Delta 488$  mutant were very similar at all time points examined (Fig. 7b). After 36 h, no further time points were studied, as many of the mice had become moribund by this stage and had to be culled.

## DISCUSSION

Genomewide approaches have identified several genes comprising pneumococcal TCS as being important in virulence, including TCS01, TCS04, TCS05, TCS07, TCS09, TCS12, and TCS13 (11, 20, 22, 25; annotations are based on those assigned

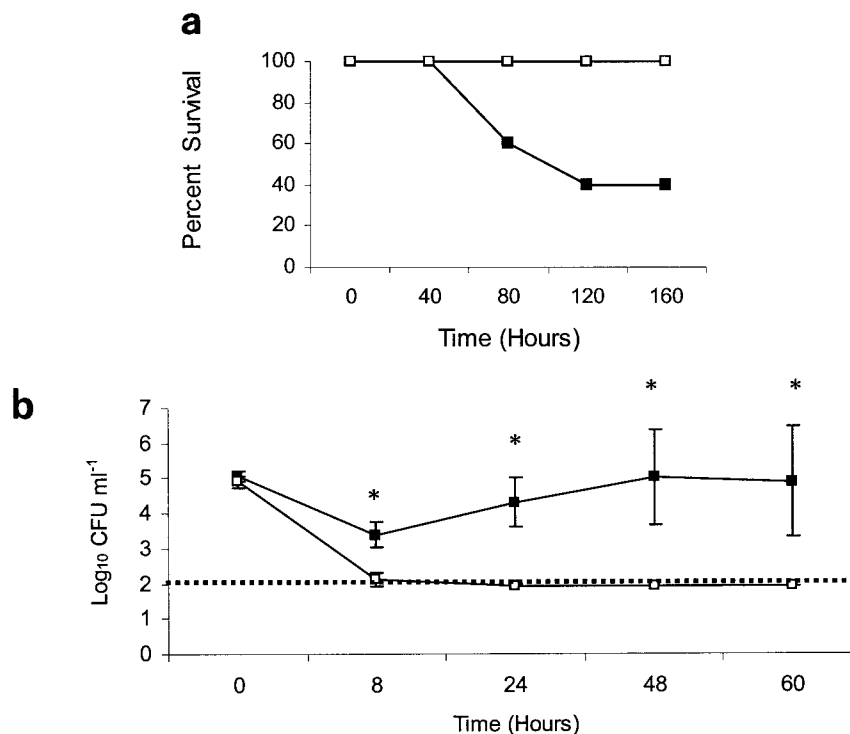


FIG. 6. Intravenous challenge with  $10^6$  CFU on a D39 background. (a) Survival; (b) bacteremia. ■, wild-type D39; □, the  $\Delta rr09$  mutant.  $P < 0.05$  for survival and for bacteremia at all time points after 0 h. In panel b, error bars represent SEMs; dashed line represents the limit of detection ( $\log_{10} 1.92$  CFU ml<sup>-1</sup>).

by Lange and colleagues [19]). Microbial TCS are ideal targets for antimicrobial therapy for a number of reasons. First, they have been found to be essential for viability (1, 19, 33) and virulence (8, 19, 26, 27, 31; our laboratory, unpublished data). Homology exists between TCS of different bacteria, which could facilitate the design of broad-spectrum drugs. Similarly, regions unique to individual TCS could be targeted to produce drugs specific for a given organism. Furthermore, homologous systems have not been identified in vertebrates, so any drugs developed could have low toxicity to host cells (13). Inhibitors of bacterial TCS have been identified and are currently being characterized for use as therapeutic agents (28, 29).

This study presents data on one particular system, TCS09, that is important in virulence in two different strains of the pneumococcus, D39 and 0100993. Control studies using D39 transformed with plasmid pVA838, which does not integrate into the chromosome, have shown that the virulence of this strain is not compromised by the transformation procedure used to create the mutants, confirming observations made by Kadioglu et al. (15). Administration of *S. pneumoniae* via the intraperitoneal route generally results in high levels of bacteremia within 24 h of injection together with the display of moderate to severe symptoms (hunched stance, piloerection, lethargy) which progress until the mice become moribund. This pattern of infection was observed when mice were given strain D39 or 0100993 or the  $\Delta 488$  mutant, but intraperitoneal challenge with the  $\Delta rr09$  mutant did not result in any detectable bacteremia and mice displayed no signs of infection. This finding indicated that *rr09* is essential for virulence in a D39 background but not in a 0100993 background when pneumo-

cocci are given to MF1 mice via intraperitoneal injection. Lange and colleagues (19) created knockout mutations in the *rr09* genes of a type 22 and a type 3 pneumococcus and found no difference in virulence between the mutants and the wild type strains when they were given to C57/BL mice via the intraperitoneal route of infection. These data are in agreement with the results we report with the  $\Delta 488$  mutant of 0100993.

Mice were subsequently challenged with mutants and their parental strains via an intranasal route of infection. The D39 bacteria used and the isogenic  $\Delta rr09$  mutant were not animal passaged prior to challenge. Mice given  $10^7$  CFU of D39 showed significantly higher levels of bacteremia at all time points examined and had significantly shorter survival times than those challenged with the isogenic  $\Delta rr09$  mutant. Challenge with the wild-type 0100993 resulted in levels of bacteremia comparable to those with D39 infection. Infection with the  $\Delta 488$  mutant did not result in detectable levels of bacteremia at any time point, although 40% of mice did eventually succumb to infection. These data indicate that the *rr09* gene is important in virulence via an intranasal route of infection in both strains studied.

Bacterial loads in the lung tissue following intranasal challenge were determined in order to assess if the abilities of mutant strains to grow in the lungs were impaired. Significant differences were seen between the counts of D39 and the  $\Delta rr09$  mutant at 24 and 48 h postchallenge, when the lung tissue of mice challenged with the  $\Delta rr09$  mutant failed to yield detectable bacterial counts. No significant difference was seen between the bacterial counts of mice challenged with strain 0100993 and those with the  $\Delta 488$  mutant. This suggests that

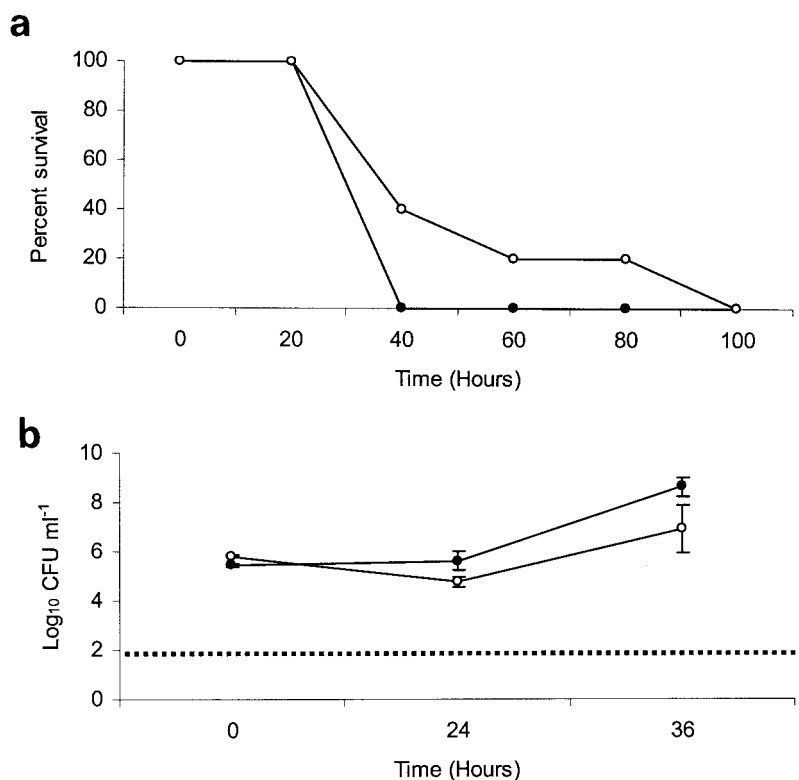


FIG. 7. Intravenous challenge with  $10^6$  CFU on a 0100993 background. (a) Survival; (b) bacteremia. ●, wild-type 0100993; ○, the  $\Delta 488$  mutant. No differences were seen in survival or level of bacteremia between mice infected with 0100993 or the  $\Delta 488$  mutant at any of the time points studied. In panel b, error bars represent SEMs; dashed line represents the limit of detection ( $\log_{10}$  1.92 CFU  $\text{ml}^{-1}$ ).

*rr09* null mutants on a D39 background are unable to colonize the lung tissues of MF1 mice, while the ability of mutants on a 0100993 background to grow in the lungs is not impaired. The data obtained for the type 3 pneumococcus are in agreement with the findings of Throup and colleagues (31).

Intravenous administration suggested that the  $\Delta rr09$  mutant was unable to sustain itself in the blood, and all mice given the  $\Delta rr09$  mutant via this route survived the infection. Mice challenged with the  $\Delta 488$  mutant showed no statistical difference in survival or bacteremia from those infected with the isogenic wild-type strain 0100993.

Overall, the *in vivo* infections suggest that in a murine model of infection, RR09 is important for virulence in both strains examined, but its contribution to virulence differs between strains. In the D39 background, RR09 is essential for virulence, and null mutants are unable to survive any of the routes of infection examined. Since the ability of these mutants to grow in culture media *in vitro* is not impaired (data not shown), it appears that the loss of the RR09 protein is preventing survival in the blood and the lungs. The data indicate that the  $\Delta 488$  mutant on a 0100993 background is impaired in its ability to disseminate from the lung tissue into the bloodstream or that transition from the lungs to the blood results in the mutants being less well adapted for systemic survival. This could result from an inability to detect certain environmental signals that are required for the regulation of genes or sets of genes required for systemic virulence and survival following transition from the lung tissue into the blood. Certain aspects of the

host immune system may be activated during this transition, via tissue damage or the inflammatory response, and the ability of the mutants to resist such defense mechanisms may be impaired relative to that of wild-type bacteria.

In a recently published signature-tagged mutagenesis screen, Hava and Camilli (11) identified an *rr09* mutant on a serotype 4 background as being highly attenuated in a pneumonia model based on counts within lung tissue 44 h after challenge with  $10^7$  CFU. The same mutant was not highly attenuated in a model of bacteremia on the basis of bacterial counts in the blood 20 h post-intraperitoneal challenge ( $5 \times 10^5$  CFU). The challenge doses given in this study were representative of the doses used in our work, although Hava and Camilli used a different strain of mice (Swiss Webster). Their data are in agreement with our findings that RR09 is important in the virulence of the pneumococcus but that its exact role in virulence depends on the genetic background of the parental strain.

Although it has been generally accepted that the virulence of pneumococcal strains in both humans and mice is strongly influenced by certain capsular serotypes (5, 17), the reasons for this are largely unknown. However, several recent studies, using capsular switch experiments, have demonstrated that capsule type is not the sole determinant of the virulence of any given serotype and that the genetic background of the bacterial strain also plays an important role (16, 17). This finding highlights the fact that the interaction of the capsule with other factors dictates virulence.

Very little has been published highlighting differences in the role of pneumococcal virulence determinants in different strains or serotypes. Tomasz and colleagues (32) created mutations in the major autolysin gene, *lytA*, in serotypes 3 and 6 and found no difference in virulence between either mutant and its parental strain (intraperitoneal challenge with three different challenge doses). In contrast, Berry and colleagues also created a *lytA* mutation in a serotype 3 strain and showed a significant decrease in virulence following intraperitoneal challenge (4). The strains of type 3 pneumococcus used in these studies were different, which could suggest that strain differences could exist alongside serotype differences, but because the groups used different mutagenesis techniques and different strains of mice, the reasons for the differences in their findings cannot be accurately assessed. Thus, our study is the first we are aware of where the same mutation introduced into the same gene of two pneumococcal strains has resulted in large differences in virulence.

Our data, combined with those of Hava and Camilli (11), strongly suggest differences in the way RR09 behaves in different pneumococcal strains and serotypes. We have no data that allow us to explain the differences seen or to extrapolate the significance of our findings to other pneumococcal genes. The function of TCS09 in *S. pneumoniae* remains unknown, although Lange and colleagues predicted a putative role in nutrient perception based on homology with chemotaxis-associated sensors found in *Bacillus subtilis* (19). This is in agreement with some preliminary microarray studies carried out in our laboratory with D39 and the  $\Delta rr09$  mutant (Blue and Mitchell, unpublished). It is thus tempting to speculate that different serotypes have different nutritional requirements. Experiments to investigate this hypothesis are under way. Preliminary microarray analysis has not identified any obvious candidates (such as known pneumococcal virulence factors) to suggest why the  $\Delta rr09$  mutants used in this study have reduced virulence *in vivo* (data not shown). However, more in-depth analysis is currently under way in our laboratory to further characterize the role of this pneumococcal TCS and to help explain the differences observed in the *in vivo* role of RR09 in different strains. This analysis will provide useful insight into the genes regulated by this system.

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