A _Klebsiella pneumoniae_ Regulatory Mutant Has Reduced Capsule Expression but Retains Hypermucoviscosity

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**ABSTRACT** The polysaccharide capsule is an essential virulence factor for _Klebsiella pneumoniae_ in both community-acquired hypervirulent strains as well as health care-associated classical strains that are posing significant challenges due to multidrug resistance. Capsule production is known to be transcriptionally regulated by a number of proteins, but very little is known about how these proteins collectively control capsule production. RmpA and RcsB are two known regulators of capsule gene expression, and RmpA is required for the hypermucoviscous (HMV) phenotype in hypervirulent _K. pneumoniae_ strains. In this report, we confirmed that these regulators performed their anticipated functions in the ATCC 43816 derivative, KPPR1S: _rcsB_ and _rmpA_ mutants are HMV negative and have reduced capsule gene expression. We also identified a novel transcriptional regulator, _RmpC_, encoded by a gene near _rmpA_. The Δ_rmpC_ strain has reduced capsule gene expression but retains the HMV phenotype. We further showed that a regulatory cascade exists in which _KvrA_ and _KvrB_, the recently characterized MarR-like regulators, and _RcsB_ contribute to capsule regulation through regulation of the _rmpA_ promoter and through additional mechanisms. In a murine pneumonia model, the regulator mutants have a range of colonization defects, suggesting that they regulate virulence factors in addition to capsule. Further testing of the _rmpC_ and _rmpA_ mutants revealed that they have distinct and overlapping functions and provide evidence that HMV is not dependent on overproduction of capsule. This distinction will facilitate a better understanding of HMV and how it contributes to enhanced virulence of hypervirulent strains.

**IMPORTANCE** _Klebsiella pneumoniae_ continues to be a substantial public health threat due to its ability to cause health care-associated and community-acquired infections combined with its ability to acquire antibiotic resistance. Novel therapeutics are needed to combat this pathogen, and a greater understanding of its virulence factors is required for the development of new drugs. A key virulence factor for _K. pneumoniae_ is the capsule, and community-acquired hypervirulent strains produce a capsule that causes hypermucoidy. We report here a novel capsule regulator, _RmpC_, and provide evidence that capsule production and the hypermucoviscosity phenotype are distinct processes. Infection studies showing that this and other capsule regulator mutants have a range of phenotypes indicate that additional virulence factors are in their regulons. These results shed new light on the mechanisms controlling capsule production and introduce targets that may prove useful for the development of novel therapeutics for the treatment of this increasingly problematic pathogen.

**KEYWORDS** HMV, RmpA, RmpC, capsular polysaccharide, hypervirulent

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acquired infections, *K. pneumoniae* is more commonly associated with nosocomial infections, as individuals in health care settings tend to be more susceptible to opportunistic pathogens. *K. pneumoniae* is also a member of the carbapenem-resistant *Enterobacteriaceae* (CRE), a group of Gram-negative pathogens that have acquired resistance to this important therapeutic drug. This problem of resistance prompted the CDC to classify CRE bacteria as an urgent threat; at the time of that report, *K. pneumoniae* was responsible for nearly 90% of CRE infections (3). *K. pneumoniae* isolates have also been found to be resistant to colistin, and they frequently produce extended-spectrum β-lactamases (ESBLs), making them resistant to most β-lactams currently in use (5).

Despite decades of research on *K. pneumoniae*, its repertoire of known virulence factors remains small compared to other pathogens. This organism generally possesses type 1 and type 3 fimbriae, lipopolysaccharide, siderophore iron acquisition systems, and a polysaccharide capsule that are necessary for virulence in animal models (1). More recently, *K. pneumoniae* isolates have been broadly classified as classical or *hv* (1, 4). The classical strains are commonly found circulating through health care facilities and tend to possess resistance to numerous antibiotics. *hv* strains are less commonly associated with antibiotic resistance, but they are considered community acquired as they can infect healthy hosts, often manifesting as pyogenic liver abscesses (reviewed in reference 4). Unlike *hv* strains, classical strains typically do not cause lethal disease in mouse models (11, 13). Two features that are known to distinguish classical from *hv* strains are the number of siderophore systems and the abundance of capsule (4). Classical strains typically have one or two siderophore systems, whereas *hv* strains have three or four, and *hv* strains are known to produce a very thick capsule associated with a hypermucoid phenotype called hypermucoviscosity (HMV). Although more than 130 capsule types have been identified (18), *hv* strains most frequently are type K1 or K2 (2; reviewed in references 1 and 4). Of particular concern is the recent emergence of strains that are both multidrug resistant and *hv* (6). The genes encoding resistance and genes associated with hypervirulence are often found on plasmids, suggesting that the frequency of strains carrying both virulence traits is likely to increase due to the ease with which these DNA elements are acquired.

The production of capsule appears to be a carefully orchestrated process. The genes encoding the proteins responsible for sugar synthesis, polymerization, and transport through the inner and outer membrane are transcriptionally regulated by several proteins. The Rcs phosphorelay system, first identified as a regulator of colonic acid synthesis in *Escherichia coli* (7), regulates capsule gene (*cps*) expression in a number of organisms, including *Klebsiella* (8, 9). Recent studies implicate H-NS (10), CRP (12), the iron-responsive regulator IcrR (14), and the response regulators KvgA, KvhA, and KvhR (16). RmpA was first reported in 1989 as a regulator of the mucoidy phenotype (17) and has since been linked to *hv* strains possessing the HMV phenotype, but its exact role is not understood (19). RmpA is encoded either on a virulence plasmid, the chromosome (on the ICEKp genomic island), or both (17, 20–22). We recently reported two new regulators of *cps* expression, KvrA and KvrB (23). The *kvrA* and *kvrB* genes are found in both classical and *hv* strains, but they appear to regulate capsule synthesis only in *hv* strains. This work sparked our interest in capsule regulation and in examining this process in more detail. From these endeavors, we identified RmpC as a novel protein that contributes to capsule regulation in KPPR1S, a derivative of the hypervirulent strain ATCC 43816. In addition, we confirmed the roles of RcsB and RmpA in capsule gene expression and the HMV phenotype in this strain. Through a series of epistasis experiments, we found that *rmpl* expression is dependent on RcsB, KvrA, and KvrB, but the roles of these proteins in capsule production are not limited to controlling *rmpl* expression. Furthermore, we found that loss of the *rmpl* gene results in decreased capsule gene expression, but curiously, it retains the HMV phenotype. This study provides evidence that capsule production is a distinct process from the process leading to the HMV phenotype. Understanding that these are separate processes will
allow for specific probing of the HMV phenotype and how it contributes to enhanced virulence of hv strains.

RESULTS

rmpA, rcsB, and rmpC are required for normal capsule production. Our identification of KvrA and KvrB as regulators of capsule (cps) gene expression (23) combined with the importance of capsule for virulence prompted us to explore cps regulation in more detail. Due to the heterogeneity of K. pneumoniae genomes, we examined the roles of the regulators RmpA and RcsB in cps expression in K. pneumoniae KPPR1S to determine whether their predicted roles were maintained in this strain. In closely examining the region around the rmpA gene (VK055_5097), we noted a neighboring gene with a predicted LuxR-type DNA binding domain (VK055_5099; designated rmpC). Its proximity to rmpA hinted at a related function, so it was targeted for deletion, and the effect of this mutation on capsule was examined along with strains carrying deletions of the rmpA and rcsB genes. Strains carrying gene deletions of the recently reported regulators KvrA and KvrB (23) were included for comparison. Because each of these genes encodes a putative DNA binding domain (DBD), they are collectively referred to as DBD mutants. These mutants were first assessed for HMV using the string test, where a colony is touched with a loop and lifted upward; colonies that stretch at least 5mm are string test positive (24). Unlike the mutations in rmpA and rcsB (and kvrA and kvrB), the ΔrmpC strain was still mucoid and showed a positive string test result; like ΔkvrA and ΔkvBR mutant strains, ΔrmpA and ΔrcsB mutants were string test negative. As the string test is purely qualitative, we next performed two quantitative assays for capsule. Uronic acid (UA) is a key component of many capsules and has historically been used as an indicator of capsule content. UA was measured in KPPR1S and mutant derivatives from late-log cultures grown in M9 with glucose and Casamino Acids (referred to hereafter as M9-CAA). KPPR1S produces about 7 μg UA/OD600, whereas the ΔmanC capsule mutant produces about 1 μg/OD600. The DBD mutants produced about 20 to 30% less UA than KPPR1S (5 to 6 μg/OD600) (Fig. 1A). This reduction is slight and did not reflect the striking differences we observed in colony morphology, so we turned to the mucoviscosity assay. HMV strains do not sediment well during centrifugation, and the supernatant remains turbid. Measurement of the turbidity after centrifugation can therefore serve as a quantitative indicator of HMV. The strains were grown as described above, diluted to an optical density at 600 nm (OD600) of 1, and then subjected to low-speed centrifugation. The supernatant OD600 of KPPR1S was 0.3, and, consistent with the string test result, a similar value was obtained for the ΔrmpC mutant (Fig. 1B). The remaining mutants all formed tight pellets with nearly cleared superna-
tants (OD$_{600}$ < 0.08). These results indicate that all of these DBD mutants have decreased UA content but that UA levels do not necessarily correlate with mucoviscosity.

**Mucoviscosity impacts host cell associations.** As capsule is known to have a number of protective roles during infection, we wanted to assess the phenotypes of the DBD mutants using *in vitro* models. Capsule is known to be an antiphagocytic factor and to block adherence to mammalian cells (reference 1 and references therein). Using bone marrow-derived macrophages (BMDM), we assayed the adherence and internalization of the DBD mutants. KPPR1S was barely adherent, with 1.5% of the inoculum recovered. The Δ$\text{manC}$ strain was recovered at 55% of the inoculum, an ~35-fold increase over the KPPR1S strain (Fig. 2A). The Δ$\text{rmpC}$ strain exhibited a wild-type-like phenotype with very low adherence at 3%, whereas the other strains had intermediate adherence levels (8 to 15%). An identical trend in phenotypes was observed for internalized bacteria. Only 0.2% of the inoculum of KPPR1S was intracellular, but nearly 7% of Δ$\text{manC}$ was intracellular (Fig. 2B). The DBD mutants each measured about ~1%, with the exception of the Δ$\text{rmpC}$ mutant at 0.3%. In each assay, the Δ$\text{kvrA}$, Δ$\text{kvrB}$, Δ$\text{rmpA}$, and Δ$\text{rcsB}$ strains showed between 5- to 8-fold increases in host-cell associations compared to KPPR1S. All five of the DBD mutants produced similar levels of capsule, but only the Δ$\text{rmpC}$ strain retained the HMV phenotype. Thus, it appears that HMV, rather than UA content, is a more critical determinant in avoiding host cell associations.

**Attenuation of the DBD mutants reveals different roles in virulence.** A mouse model of pneumonia was used to assess the *in vivo* impact of these loss-of-function mutations. C57BL/6j mice were intranasally inoculated with 2 × 10$^4$ CFU and sacrificed 24 and 72 h postinoculation (hpi) to determine bacterial burdens in the lungs and spleens. Given the defects in capsule, we anticipated that each mutant would be attenuated and this was indeed observed. In the lungs at 24 hpi, the Δ$\text{rcsB}$ and Δ$\text{rmpA}$ strains had colonization levels about 4 logs lower than that of strain KPPR1S (Fig. 3A). The Δ$\text{rmpC}$ strain was also attenuated, with nearly 2 logs less bacteria recovered from the lungs than KPPR1S. At 72 hpi, the lungs of mice inoculated with the Δ$\text{rcsB}$ or Δ$\text{rmpA}$ strains were nearly cleared, and the burden from those inoculated with the Δ$\text{rmpC}$ mutant was very low. In the spleens at 24 hpi, the levels of strain KPPR1S are typically very low. Although not significantly different, the median burden of the Δ$\text{rcsB}$ mutant was about 1,500 CFU/g, while that of KPPR1S was 165 CFU/g (Fig. 3B). Nearly all mice inoculated with the Δ$\text{rmpC}$ mutant had undetectable CFU in the spleens, and this was a significant reduction compared to KPPR1S. By 72 hpi, each DBD mutant showed a significant decrease in bacterial burden in the spleens compared to KPPR1S. Although the CFU recovered from mice infected with the Δ$\text{rcsB}$ mutant was
higher than KPPR1S at 24 hpi, it fell to barely detected levels at 72 hpi. Chromosomal complementation of the \( rmpA \) and \( rcsB \) genes restored the bacterial burdens to wild-type levels at each time point (Fig. 3C). For reasons we do not understand, we were unable to generate the plasmid needed for chromosomal complementation of the \( rmpC \) gene. We therefore tested a strain carrying a plasmid-borne copy of \( rmpC \), which showed complementation in the lungs at 24 hpi (Fig. 3D); no bacteria were recovered at 72 hpi, indicating that the plasmid likely had been lost during the infection (data not shown). Complementation was not observed in the spleen; this could be due to loss of the plasmid during dissemination or to potential consequences from multicopy expression.

**Gene expression at the \( cps \) locus is altered in DBD mutants.** Because \( rcsB \), \( rmpA \), \( rmpC \), \( kvrA \), and \( kvrB \) are known or predicted to encode proteins with DNA binding domains and mutations in these genes affected capsule-associated phenotypes, we sought to examine whether these mutations impacted \( cps \) expression. The \( cps \) locus contains three characterized promoters, located upstream of \( galF, wzi \), and \( manC \) (Fig. 4A). The regions upstream of these genes that should contain promoters were cloned into a \( gfp \) reporter plasmid that was then transformed into strain KPPR1S and each DBD mutant to assay expression levels. Expression from the \( wzi \) promoter was not altered in the mutants, suggesting that it is not regulated by any of these proteins.
**A. The capsule locus**

![Schematic of the capsule locus containing genes for sugar precursor biosynthesis, polymerization, and export.](image)

**B. galF-gfp**

![Graph showing relative fluorescence units (RFU) for galF-gfp in WT and mutant strains](image)

**C. wzi-gfp**

![Graph showing relative fluorescence units (RFU) for wzi-gfp in WT and mutant strains](image)

**D. manC-gfp**

![Graph showing relative fluorescence units (RFU) for manC-gfp in WT and mutant strains](image)

**E. manC-gfp**

![Graph showing relative fluorescence units (RFU) for manC-gfp in WT and mutant strains](image)

**FIG 4** Capsule gene expression is affected by loss of DBD genes. (A) Schematic of the capsule locus containing genes for sugar precursor biosynthesis, polymerization, and export. (B to E) Saturated cultures of WT and mutant strains carrying plasmids with transcriptional *gfp* fusions were subcultured and grown as described in the legend to Fig. 1. Relative fluorescence units (RFU) were measured, normalized first to the culture OD$_{600}$, and then to WT (set at 100). The three characterized promoters tested were *galF-gfp* (pPROBE_galF) (B), *wzi-gfp* (pPROBE_wzi) (C), *manC-gfp* (pPROBE_manC) (D). (E) Complementation assays were performed using strains transformed with *manC-gfp* and individual complementation plasmids (pRcsB [pKW173], pRmpA [pKW184], pRmpC [pKW185], pKvrA [pTM006], and pKvrB [pTM007]). The one-way ANOVA test was performed to determine statistically significant differences between each mutant and WT. ***, P < 0.001; ****, P < 0.0001.

(Fig. 4C). The *galF* and *manC* promoters were both affected by all the mutants, including the Δ*mpc* mutant (Fig. 4B and D). Both promoters had decreased expression compared to WT, ranging from a 2- to 7-fold reduction in *galF* expression (Fig. 4B) and a 4- to 39-fold reduction in *manC* expression (Fig. 4D). Plasmids containing the individual regulator genes were transformed into the respective DBD mutants with the *manC-gfp* reporter, and restored (or enhanced) expression was observed when the gene is expressed in trans (Fig. 4E). Thus, the capsule alterations described above may be a consequence of reduced expression of the biosynthetic genes.

*KvrA, KvrB, and RcsB activate the rmpA promoter.* When multiple regulators impact transcription, questions arise as to whether each individually impacts transcription or whether some act indirectly by regulating the direct regulator. To begin to address these questions, we cloned the putative promoter regions upstream of each DBD gene into our *gfp* reporter plasmid and transformed them into KPPR1S and mutant strains. We were unable to detect expression from the region upstream of *rpmC* and tested to determine whether it was in an operon with *rpmA*. Using primers that flank the intergenic region between *rpmA* and *rpmC*, a product was obtained when cDNA or
RmpC affects cps expression but not hypermucoviscosity

genomic DNA was provided as a template, but not in the negative controls (Fig. 5A), thus indicating that rmpC is in an operon with rmpA. RmpA and KvrB autoregulate their own expression by about 2-10-fold, respectively (Fig. 5B and C), while expression of kvrA and rcsB was largely unregulated (Fig. 5D and E). Expression of rmpA was dependent on KvrA, KvrB, and RcsB; loss of KvrA resulted in a 9-fold reduction in rmpA levels, and loss of KvrB or RcsB resulted in 2- and 2.5-fold reductions, respectively (Fig. 5B).

This prompted us to determine whether the decreased expression from the galf and manC promoters in ΔkvrA, ΔkvrB, and ΔrcsB strains were due to loss of expression from the rmpA promoter. We therefore transformed pRmpA into these strains carrying the manC-gfp reporter to determine whether an extrachromosomal copy of rmpA could compensate for loss of kvrA, kvrB, or rcsB. No changes in manC expression were observed in the ΔkvrA and ΔrcsB strains, but pRmpA did restore manC expression in the ΔkvrB mutant (Fig. 5F and G). These data suggest that KvrA and RcsB have an impact on the manC promoter independent of their impact on rmpA expression but that the impact of KvrB on expression may solely be indirect, through regulation of the rmpA promoter.

To further dissect this network, we constructed a strain lacking kvrA, kvrB, rmpA, and rmpC, referred to as Δquad. We transformed pKvrA, pKvrB, pRmpA, or pRmpC into the Δquad strain containing the manC-gfp reporter and assayed manC expression. We attempted to address the role of RcsB using a strain lacking all five regulator genes, but this strain displayed some pleotropic defects and the data were unreliable, so these same plasmids along with pΔrcsB were transformed into the ΔrcsB strain. Ectopic expression revealed that rmpC, but not rmpA, kvrA, or kvrB, restored manC expression in the absence of the other three regulators, and this is observed only if rcsB is present (Fig. 5G). Very high levels of manC-gfp were detected from the Δquad strain with pRmpC, but no expression was detected from ΔrcsB with pRmpC. Consistent with the data from the Δquad strain, neither pKvrA, pKvrB, nor pRmpA could restore expression of manC-gfp when transformed into the ΔrcsB mutant. Collectively, these data suggest that RcsB is required for any level of manC transcription and that plasmid-encoded RmpC (thus likely produced at higher levels) leads to high levels of manC expression, even in the absence of rmpA, kvrA, and kvrB.

RmpA and RmpC have overlapping and independent roles. Through the course of the experiments that included plasmids for expression of rmpA or rmpC, we noted some intriguing phenotypes. First, strains carrying pRmpC have exceptionally high expression of manC-gfp at levels 4- to 5-fold higher than that of the WT (Fig. 4E and 5G). Second, broth cultures of strains carrying pRmpA become viscous after several hours of growth with the inducer anhydrous tetracycline (aTc). We decided to examine this more closely by transforming KPPR1S, ΔrmpA, and ΔrmpC strains with either pRmpA, pRmpC, or pRmpAC and measuring mucoviscosity and manC-gfp expression. As before, introduction of pRmpC resulted in very high levels of manC-gfp expression in each mutant (Fig. 6A). Introduction of pRmpA only restores expression in ΔrmpA, indicating that RmpC is essential for maximal manC expression. Curiously, introduction of pRmpAC, presumably overexpressing both genes, resulted in manC-gfp levels similar to that of the WT in all three mutant strains. Thus, it appears that the stoichiometry of RmpA and RmpC is important for normal expression from the manC promoter. Although pRmpC led to high manC-gfp expression, it also led to a striking reduction in mucoviscosity in all strains, including WT (Fig. 6B). Strains with pRmpA showed a significant increase in mucoviscosity in all strains, consistent with its role in the HMV phenotype. Introduction of pRmpAC into the WT, ΔrmpA, and ΔrmpC strains led to increased mucoviscosity similar to that seen with these strains containing pRmpA, even though strains with pRmpAC have normal levels of manC-gfp expression. Collectively, these data show that RmpA is necessary for “normal” expression of manC and for the HMV phenotype. RmpC is necessary for full manC expression, and overexpression leads to elevated manC expression but loss of HMV. Thus, it appears that the relative amounts of RmpA and RmpC are critical for the HMV phenotype as well as cps expression. Importantly, these
The data indicate that while there is some cooperativity between RmpA and RmpC, they do not perform identical functions. Furthermore, expression levels of capsule biosynthetic genes do not necessarily correlate with HMV, hinting that the composition of the extracellular material that produces this phenotype may not be identical to the polysaccharides that comprise the capsule.

**FIG 5** RcsB, KvrA, and KvrB control rmpA expression. (A) rmpC is in an operon with rmpA. Standard PCR was performed with primers CB472 and CB498 (black arrows) using wild-type genomic DNA (gDNA), samples from cDNA synthesis reactions with reverse transcriptase (RT +) and without reverse transcriptase (RT −), or with no template (nt). The RNA used to generate the cDNA was isolated from strain KPPR1S grown in LB from a previously published data set (23). Promoter-gfp fusions for rmpA (rmpA-gfp, pKW174) (B), kvrB (kvrB-gfp, pPROBE_kvrB) (C), kvrA (kvrA-gfp, pPROBE_kvrA) (D), and rcsDB (rcsDB-gfp, pKW170) (E) were transformed into the indicated strains and grown as described in the legend to Fig. 1. (F and G) Strains containing manC-gfp were transformed with the indicated complementing plasmids; plasmid names are as given in the legend to Fig. 4. The one-way ANOVA test was performed to determine statistically significant differences between each mutant and WT. ***, P ≤ 0.001; ****, P ≤ 0.0001.
DISCUSSION

In this work, we introduce a new capsule regulator, RmpC, and we have shown that RcsB and RmpA contribute to capsule gene expression and production of the HMV phenotype in the hypervirulent ATCC 43186 derivative KPPR1S. The roles of RcsB and RmpA were also identified as contributing to capsule production in this strain using methodology that specifically selected for mutants with altered hypermucoidy (25).

The fact that RcsB and RmpA regulate capsule gene synthesis in strain KPPR1S is not surprising, as they have had this role in every other Klebsiella strain examined. However, new information indicates there is a regulatory cascade in place by which several regulators encoded by the K. pneumoniae core genome (KvrA, KvrB, and RcsB) control rmpA expression in addition to cps expression. RmpA in turn auto-regulates its expression, and that of rmpC as well, since they are coexpressed and no promoter could be detected upstream of rmpC. Furthermore, experiments with rmpA and rmpC mutants suggest that RmpA and RmpC have overlapping and separate functions that contribute to cps expression and the HMV phenotype. Last, although each of these regulators is required for cps expression, they have different virulence phenotypes in a mouse pneumonia model, suggesting that their roles extend beyond capsule regulation.

![Graph A: manC-gfp](image)

**FIG 6** RmpA and RmpC have overlapping and independent functions. The WT, ΔrmpA, and ΔrmpC strains were transformed with manC-gfp (pPROBE_manC) and pRmpA (pKW184), pRmpC (pKW185), or pRmpAC (pKW186), grown as described in the legend to Fig. 1 and assayed for manC expression (A) or mucoviscosity (B). The one-way ANOVA test was performed to determine statistically significant differences between each mutant and WT. **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001.
As the polysaccharide capsule is a critical virulence factor in *K. pneumoniae*, its production is tightly controlled. Numerous transcriptional regulators have been identified as contributing to capsule gene expression in various *K. pneumoniae* strains. In addition to the Rcs phosphorelay system and RmpA, these include CRP, H-NS, IscR, the response regulators KvhA, KvgA, and KvhR, and the newly reported regulators KvrA and KvrB (10, 12, 14, 16, 17, 23). A considerable amount of work has been dedicated to the Rcs phosphorelay system, and many of the mechanisms involved in regulation of and by this system have been elucidated in *E. coli* (9), and identification of the RcsAB box upstream of *galF* in *K. pneumoniae* suggests that its function is likely conserved (8). RmpA was identified in the late 1980s as contributing to the HMV phenotype (17) and appears to be primarily limited to the Klebsiellae; however, its mechanism of action remains largely unknown. In the HMV+/K2 type strain GC43, RmpA was shown to interact with RcsB, and both proteins were required for activation of capsule gene expression (26). The HMV+/K2 type strain NTUH-K2044 contains both chromosome- and plasmid-encoded copies of *rmpA* that are nearly identical (>90% amino acid identity). One study reported that only the plasmid-borne gene, and not the chromosomal copy, impacted capsule production *in vitro*, but neither gene appeared to contribute to virulence in mice inoculated intragastrically or intraperitoneally (19). This contradicts results obtained from two different HMV+/K2 type strains that showed significant increases in the 50% lethal dose (LD$_{50}$) for *rmpA* mutants compared to the isogenic WT in intraperitoneal models (17, 26). One conundrum of this body of research is that most of these regulators were studied for their independent contributions using only single deletion strains. Further complicating the interpretation is that these studies have been conducted using a variety of different *K. pneumoniae* strains that may or may not have the same complement of regulators, and therefore have potentially varying mechanisms of regulation of *cps* and other virulence genes.

The *rmpA* and *rmpC* genes in strain KPPR1S are nearly identical to those on the chromosome of NTUH-K2044 (Fig. 7), and the protein sequences show only one amino acid change in RmpA right near the C terminus and complete identity in RmpC. Comparing strain KPPR1S to the NTUH-K2044 plasmid copies, there are 17 different...
residues in the RmpA protein and 15 differences in RmpC. The other two strains in which RmpA has been studied each contain only one copy of rmpA on large virulence plasmids (17, 20, 22), and both more closely match that of the NTUH-K2044 plasmid. However, these changes may have only subtle or no effects, as all the RmpA proteins appear to be functional in some context, except perhaps the NTUH-K2044 chromosomal copy. Expression from the NTUH-K2044 manC promoter is reduced when expressed in the KPPR1S ΔrmpA mutant compared to the WT KPPR1S strain, and expression is restored to WT levels when either NTUH-K2044 or KPPR1S rmpA is expressed in trans (K. A. Walker and V. L. Miller, unpublished results). This suggests that the NTUH-K2044 chromosomal copy has the potential for functionality even though this was not evident when examined (19). It is possible that the plasmid and chromosomal promoters are differentially regulated in response to different signals, such that only the plasmid copy is produced or active in broth (where cps expression was tested). Infection data with a mutant lacking both copies of rmpA was not reported (19); thus, the lack of attenuation observed from the single mutants could be due to functional redundancy in vivo. Another key difficulty in interpreting the prior work with RmpA is that rmpC had not been identified, and thus, the impact of rmpA mutations on rmpC expression and function were not taken into account.

In strain KPPR1S, there is severe attenuation of the ΔrmpA strain in our pneumonia model, and this phenotype is nearly identical to the phenotypes of the ΔrcsB and ΔkvrA strains (23). Curiously, the level of attenuation observed for the ΔrmpC strain is not as severe, despite the similar decreases in galF and manC expression and UA levels in these mutants. The ΔkvrB mutant also has an intermediate phenotype, with lung colonization levels higher than those of the ΔrmpC strain at 72 hpi (23). Therefore, the changes in cps expression observed in vitro cannot fully account for the virulence defects in these DBD mutants. Consistent with this notion, the ΔrmpC strain behaves like the WT in adherence and phagocytosis assays. The capacity to avoid host cell contact and uptake appears to be driven more by HMV rather than by cps expression levels, and the retention of HMV by ΔrmpC may be why it is not as severely attenuated in the pneumonia model.

Although a regulatory cascade exists in which RcsB, KvrA, and KvrB control rmpA expression, RcsB and KvrA also appear to have impacts on manC expression beyond regulating rmpA. This is evidenced by the lack of complementation of manC expression in the ΔrcsB, ΔkvrB, or ΔkvrA strains when rmpA was expressed in trans. However, overexpressing rmpC does complement for loss of all regulators except RcsB. Because no other regulator tested could compensate for the loss of RcsB and because manC expression is barely (and often not at all) detected in the ΔrcsB mutant, it appears that RcsB is necessary for basal level expression from the manC promoter. One function of the other regulators may be to elevate cps expression above this basal level, perhaps in response to different signals.

The differences in virulence assays between the ΔrmpA and ΔrmpC mutants are particularly intriguing. That these genes are chromosomal neighbors suggests they have overlapping functions. However, the distinct mucoviscosity phenotypes of these mutants indicate that their functions are separate. The ΔrmpA mutant lost the HMV phenotype and is string test negative, while the ΔrmpC mutant is string test positive and HMV positive, yet both mutants display similar decreases in manC expression and UA levels. It had been speculated that the HMV phenotype is due to an overabundance of capsule (27), but the investigators that identified RmpA concluded HMV was not due to overproduction of capsule because their nonmucoid rmpA mutant strain produced an equivalent amount of capsule as the parent strain (17). The phenotypes of the ΔrmpC strain support the notion that HMV is not simply due to an overabundant capsule. When the ΔrmpA or ΔrmpC mutation is complemented in trans, the defects in expression and virulence are restored, but overexpression leads to some other interesting phenotypes. First, overexpression of rmpA leads to increased HMV such that the broth cultures become viscous. In the ΔrmpC strain with pRmpA, HMV increases but manC expression is not restored. This is a key indicator that HMV is not necessarily
dependent on capsule production. Second, overexpression of rmpC leads to very high levels of manC expression (even in the absence of other regulators) but a complete loss of HMV, even in WT. Thus, it appears that RmpA is primarily responsible for the HMV phenotype and that RmpC is primarily responsible for cps expression. Expression of rmpC will be reduced in a rmpA mutant due to the loss of positive autoregulation by RmpA. We speculate that the association of rmpA as a regulator of cps transcription may actually be due to effects on rmpC expression, as most strains we examined that have the rmpA gene also have rmpC. Using tBLASTn and selecting only complete sequences (whole chromosome or plasmid), we identified about 40 strains that encode both RmpA and RmpC; from the available sequences, only three were found to encode RmpA alone. Further supporting the notion that RmpA is an indirect regulator of cps transcription is that overexpression of rmpA does not restore manC expression in the ΔrmpC strain. Despite these distinct roles, these proteins do appear to have some coordinated function. Overexpressing both genes in either the ΔrmpA or ΔrmpC mutant restores WT level of manC expression, although HMV is still elevated. Thus, the ratio of RmpA to RmpC seems to be an important aspect of cps expression. Proteins containing LuxR-type DNA binding domains are known to dimerize. Several models can be envisaged, assuming dimerization occurs. The simplest model proposes that RmpA homodimers regulate HMV and RmpC homodimers regulate cps expression. In this scenario, RmpA–RmpC heterodimer could serve to sequester RmpA and RmpC, thus controlling appropriate levels of both HMV and cps transcription by affecting the concentration of the homodimers. When the balance is shifted by overexpression of one regulator, the ratio of homodimers is skewed to favor increased HMV or cps expression. It is also possible that the RmpA–RmpC heterodimer binds DNA and thus directly impacts HMV and cps expression. RmpA was shown to interact with RcsB (26), and it is plausible that the RmpA–RcsB heterodimer regulates HMV. An abundance of RmpC could thus block HMV by preventing the formation of the RmpA–RcsB dimer through sequestration of RmpA. These models are clearly overly simplistic, as they ignore the contributions of the other regulators, but they provide a framework for future experimentation. Ongoing experiments will test these models and probe the individual regulons to identify other virulence-related genes as well as those that contribute to HMV. A greater understanding of how each regulator functions within the context of other regulators and within a single strain will provide much needed information about the production of capsule and HMV and may promote the development of new therapeutics against this problematic pathogen.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Table 1 lists the bacterial strains and plasmids used in this work. E. coli strains were grown in LB medium at 37°C. K. pneumoniae strains were grown in LB at 37°C for mouse and BMDM infections or M9 medium supplemented with 0.4% glucose and 0.2% Casamino Acids for capsule regulation and production assays. Where appropriate, antibiotics were added at the following concentrations: kanamycin (Kan), 50 μg/ml; rifampin (Rif), 30 μg/ml, spectinomycin (Sp), 50 μg/ml. For expression of genes cloned into pMWO-078, 50 or 100 ng/ml anhydrous tetracycline (aTc) was added to the media at the time of subculture.

Plasmid and strain construction. The primers used for each construct are listed in Table 2. Plasmids for making in-frame deletions were generated using pKAS46 as described previously (23) with minor modifications. Briefly, fragments of 500 to 800 bp upstream and downstream of the targeted gene were amplified, digested, ligated into pKAS46, and electroporated into E. coli S17-1 λpir. The resulting plasmids (pCB43, pCB058, pCB096, pCB109, and pKW189) were verified by sequencing and introduced into K. pneumoniae via conjugation. Transconjugants were selected by growing on LB agar containing Rif (30 μg/ml) and Kan (50 μg/ml). Several Rif/Kan colonies were streaked onto LB agar with 2.5 mg/ml streptomycin (Str) to select for clones that had undergone the second recombination step (28). Following another isolation streak on LB-Str, Str/Kan colonies were subjected to PCR to determine whether the second recombination event yielded the wild-type or mutant genotype. Strains with the resulting in-frame deletions in rcsB, rmpA, rmpC, and manC were named VK248, VK352, VK487, and VK506, respectively. Strain VK429 (Δquad) was generated by sequential deletion of kvrA, kvrB, then rmpAC.

Plasmids for gfp transcriptional fusions were generated by amplifying a 500- to 700-bp region upstream of the predicted start codon of the target gene, digesting the product, ligating it into pPROBE-tagless (29), and electroporating into E. coli DH5α. The resulting plasmids, pKW170 (rcsB-gfp)
and pKW174 (rmpA-gfp), were verified by sequencing and then introduced into K. pneumoniae by electroporation.

Plasmids for complementation in trans were similarly constructed; PCR-amplified inserts were ligated into pMWO-078 (30). These plasmids, pKW173 (rcsB), pKW184 (rmpA), pKW185 (rmpC), pTM006 (pKvrA), and pTM007 (pKvrB), were verified by sequencing and then introduced into K. pneumoniae by electroporation.

Chromosomal complementation was done by allelic exchange. The plasmids for chromosomal complementation were generated by amplifying a single fragment that spanned the same upstream and downstream regions as the deletion constructs using the outermost primers. This insert was cloned into pKAS46 and verified by sequencing. These plasmids, pKW190 (rcsB) and pCB112 (rmpA), were introduced into the desired K. pneumoniae strain via conjugation, and the same protocol was followed as for generating in-frame deletions.

Electroporation of plasmids into Klebsiella. For efficient electroporation of plasmids into Klebsiella, saturated overnight cultures grown in LB at 26°C (to minimize capsule production) were subcultured into fresh LB containing 0.7 mM EDTA and grown for 2 h at 37°C (31). One milliliter of culture was washed twice with 10% glycerol, and the pellet was resuspended in 100 μl of 10% glycerol. Plasmid DNA was added to 50 μl cell suspension and subjected to electroporation. Cells were allowed to recover for 1 h in SOC medium prior to plating on LB agar with appropriate antibiotics.

Uronic acid measurement. The uronic acid (UA) content was measured using a modified protocol (32, 33) essentially as described previously (34); UA was extracted from a 300-ml culture with zwittergent, precipitated with ethanol, and resuspended in tetraborate/sulfuric acid. Following addition of phenylphenol, UA was detected by absorbance at 520 nm. A standard curve was generated with glucuronic acid.

Mucoviscosity assay. Mucoviscosity of the capsule can be assessed by low-speed centrifugation of liquid cultures (35). Various strains of K. pneumoniae were grown as in the UA assay. After the 6-h

### TABLE 1 Bacterial strains and plasmids used in this work

| Strain or plasmid | Relevant genotype or phenotype | Source or reference |
|-------------------|--------------------------------|---------------------|
| **E. coli**       |                                |                     |
| DH5α             | F′ p80ΔlacZM15 Δ(lacZYA-argF)U169 deoP recA1 endA1 hsdR17 (rK− mK−) | Invitrogen          |
| S17-1pir         | Tp′ Str′ thi pro hsdR hsdM+ RP4::Tc::Mu::Km Tn7 & pir lysogen | 38                  |
| **K. pneumoniae**|                                |                     |
| KPPR15           | ATCC 43816, RifR Str′         | 39                  |
| VK248            | KPPR15, ΔrcsB                 | This work           |
| VK332            | KPPR15, rcsB reconstituted    | This work           |
| VK277            | KPPR15, ΔkvrA                 | 23                  |
| VK352            | KPPR15, ΔrmpA                 | This work           |
| VK379            | KPPR15, rmpA reconstituted    | This work           |
| VK410            | KPPR15, ΔkvrB                 | 23                  |
| VK429            | KPPR15, ΔkvrA ΔkvrB ΔrmpAC (Δquad) | This work         |
| VK487            | KPPR15, ΔrmpC                 | This work           |
| VK506            | KPPR15, ΔmanC                 | This work           |
| **Plasmids**     |                                |                     |
| pPROBE-E         | KanR; gfp transcriptional reporter vector | 29                  |
| pKAS46           | Kan′; MobRP4 oriR6K, cloning vector | 28                  |
| pMWO-078         | Sp′; p15A ori cloning vector, tetO | 30                  |
| pCB096           | rmpA in-frame deletion in pKAS46 | This work          |
| pCB109           | rmpAC in-frame deletion in pKAS46 | This work          |
| pKW189           | rmpC in-frame deletion in pKAS46 | This work          |
| pCB058           | rcsB in-frame deletion in pKAS46 | This work          |
| pCB043           | manC in-frame deletion in pKAS46 | This work          |
| pCB112           | rmpA region in pKAS46         | This work          |
| pKW190           | rcsB region in pKAS46         | This work          |
| pKW173           | rcsB in pMWO-078              | This work          |
| pKW184           | rmpA in pMWO-078              | This work          |
| pKW185           | rmpC in pMWO-078              | This work          |
| pKW186           | rmpAC in pMWO-078             | This work          |
| pTM006           | kvrA in pMWO-078              | This work          |
| pTM007           | kvrB in pMWO-078              | This work          |
| pPROBE-manC      | manC promoter region in pPROBE | 23                  |
| pPROBE-galF      | galF promoter region in pPROBE | 23                  |
| pPROBE-wzi       | wzi promoter region in pPROBE  | 23                  |
| pPROBE-kvrA      | kvrA promoter region in pPROBE | This work          |
| pPROBE-kvrB      | kvrB promoter region in pPROBE | This work          |
| pKW174           | rmpA promoter region in pPROBE | This work          |
| pKW170           | rcsDB promoter region in pPROBE | This work          |
incubation, cultures were normalized to 1 OD600/ml and centrifuged at 1,000 × g for 5 min. The OD600 values of the supernatant were determined and plotted. HMV strains do not form tight pellets, and the supernatants therefore have higher absorbance readings.

Transcriptional gfp reporter assays. Plasmids containing various promoter-gfp fusions were transformed into the desired K. pneumoniae strains. The resulting strains were grown overnight in M9-CAA, subcultured in fresh medium, and grown for 6 h. Relative fluorescence units (RFU) were measured from bacterial cultures diluted 1:10 using a Synergy H1 plate reader (Bio-Tek, Winooski, WI). The OD600 of each culture was measured to calculate RFU/OD600 and then normalized to the activity from the WT strain in each assay.

Host cell attachment and internalization assays. BMDM were harvested from the femurs of 8-week-old female C57BL/6j mice (Jackson Laboratories, Bar Harbor, ME) and used without further passage. Nonadherent macrophages were used for these studies. BMDM were plated at 1 × 10^5 cells per well in a 24-well plate, and incubated overnight in DMEM with 10% FBS. BMDM were then inoculated with bacteria that were grown overnight at 37°C in LB with Rif (30 μg/ml) at a multiplicity of infection (MOI) of 50 and allowed to incubate for 1 h. Plasmid-containing BMDM were then incubated with 2 μM cytochalasin D (Sigma-Aldrich, St. Louis, MO) to halt internalization of bacteria 1 h prior to next steps. Following incubation, all wells were gently washed three times with 1× PBS, lysed with 0.5% saponin, and assayed for fluorescence. For internalization assays, after a 1-h incubation with bacteria, the cells were rinsed three times with 1× PBS and then incubated in fresh medium containing 200 μM gentamicin to kill extracellular bacteria. After 30 min in gentamicin, the cells were rinsed, lysed, and assayed for fluorescence.

Murine pneumonia model. All animal studies were approved by the Institutional Animal Care and Use Committee of UNC-Chapel Hill (protocols 14-110 and 17-033). Prior to inoculation, mice had unlimited access to food and water. Inoculated mice were monitored daily, and mice were euthanized upon showing signs of moribundity. Five- to 8-week-old female C57BL/6j mice (Jackson Laboratories, Bar Harbor, ME) were anesthetized by intraperitoneal injection of ketamine/xylazine and inoculated with 2 × 10^5 CFU as described previously (23, 34). At the indicated time points, mice were euthanized by
i.p. injection with sodium pentobarbital. Lungs and spleens were removed, macerated in 1× PBS, serially diluted, and plated for bacterial enumeration. Organ weights were recorded, and the data are presented as CFU/gram tissue.

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