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MOLECULAR PATHOGENESIS

High Levels of Cyclic Di-GMP in *Klebsiella pneumoniae* Attenuate Virulence in the Lung

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ABSTRACT The bacterial second messenger bis-(3′-5′)-cyclic dimeric GMP (c-di-GMP) has been shown to influence the expression of virulence factors in certain pathogenic bacteria, but little is known about its activity in the increasingly antibiotic-resistant pathogen *Klebsiella pneumoniae*. Here, the expression in *K. pneumoniae* of a heterologous diguanylate cyclase increased the bacterial c-di-GMP concentration and attenuated pathogenesis in murine pneumonia. This attenuation remained evident in mice lacking the c-di-GMP sensor STING, indicating that the high c-di-GMP concentration exerted its influence not on host responses but on bacterial physiology. While serum resistance and capsule expression were unaffected by the increased c-di-GMP concentration, both type 3 and type 1 pili were strongly upregulated. Importantly, attenuation of *K. pneumoniae* virulence by high c-di-GMP levels was abrogated when type 1 pilus expression was silenced. We conclude that increased type 1 piliation may hamper *K. pneumoniae* virulence in the respiratory tract and that c-di-GMP signaling represents a potential therapeutic target for antibiotic-resistant *K. pneumoniae* in this niche.

KEYWORDS *Klebsiella pneumoniae*, cyclic di-GMP, pneumonia, murine model, type 1 pili, type 3 pili, virulence

Klebsiella pneumoniae is a versatile pathogen that causes numerous human infections, including pneumonia, urinary tract infection (UTI), and sepsis (1). These infections are increasingly difficult to treat, given the accelerating antibiotic resistance profile of this organism, the genome of which frequently encodes extended-spectrum beta-lactamases (ESBLs), *K. pneumoniae* carbapenemases (KPCs), and other antibiotic resistance determinants (2–5). *K. pneumoniae* is the most prevalent of the carbapenem-resistant *Enterobacteriaceae* (CRE), an emerging worldwide problem which results in mortality rates of up to 50% (4, 6, 7). Given that all forms of nosocomial *K. pneumoniae* infection are on the rise, novel therapeutic targets are urgently needed to combat this organism (8, 9).

While the virulence repertoire of *K. pneumoniae* is incompletely defined, several factors have been studied in detail. *K. pneumoniae* produces at least 79 distinct polysaccharide capsules that are vital for infection of the respiratory tract (10–13). Conversely, type 1 pili, adhesive fimbriae encoded by the *fim* operon, are essential for the initiation of UTI, but deletion of these pili does not impair virulence in *K. pneumoniae* infections of the respiratory tract (14, 15). Another form of adhesive fimbriae, type 3 pili, is encoded by the *mrk* operon and promotes biofilm formation and binding to collagen or abiotic surfaces (16, 17). Expression of type 3 pili is regulated by MrkH in response to the bacterial second messenger bis-(3′-5′)-cyclic dimeric GMP (c-di-GMP) (18–21).

c-di-GMP is a ubiquitous bacterial signaling molecule produced by diguanylate

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TABLE 1 *K. pneumoniae* strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
TOP52	*K. pneumoniae* K6 isolate (wild-type parent strain)	46
TOP52 Δ*mrkH*	Deletion of *mrkH*, regulator of type 3 pili	This study
TOP52 Δ*mrkA*	Deletion of *mrkA*, type 3 pilus structural subunit	This study
TOP52 Δ*fimS-K*	Deletion of entire type 1 pilus operon from promoter *fimS* through *fimK*	This study
TOP52 Δ*rfaH*	Deletion of *rfaH*, transcription antitermination factor	This study
ATCC 43816	*K. pneumoniae* K2 isolate	47
Plasmids		
pKD46s	Bacteriophage lambda Red recombinase, *repA101*(Ts), spectinomycin resistant	13
pCP20	FLP recombinase, chloramphenicol resistant	48
pEVS143	Parent IPTG-inducible vector, kanamycin resistant	49
pCMW75	Vector containing IPTG-inducible, active QrgB	28
pCMW98	Vector containing IPTG-inducible, inactive QrgB*	28

cyclases (containing GGDEF domains) and broken down by phosphodiesterases (containing EAL or HD-GYP domains) (22, 23). The genomes of many bacterial species encode a large number of these proteins; *K. pneumoniae* strains generally harbor ~12 proteins with GGDEF domains, 9 with EAL domains, and 6 with both domains (19). c-di-GMP in various bacteria stimulates the biosynthesis of adhesins, inhibits motility, and can even modulate antibiotic resistance (22–24). Meanwhile, the mammalian host may sense c-di-GMP via the innate immune receptor STING (stimulator of interferon genes) and induce proinflammatory cytokine and chemokine production (25, 26).

Despite the myriad of c-di-GMP-modifying enzymes encoded by the *K. pneumoniae* genome, the effects of c-di-GMP on lung pathogenesis or expression of virulence factors (beyond type 3 pili) are unknown. In this study, we leveraged a plasmid system allowing the inducible expression of a c-di-GMP-synthesizing enzyme to demonstrate that high levels of c-di-GMP attenuate *K. pneumoniae* virulence in the lung and upregulate the expression of both type 3 and type 1 pili. This attenuation of infection of the respiratory tract was abrogated when *fim* expression was silenced, indicating that, in contrast to the critical role of type 1 pili in the urinary tract, type 1 pilus expression may be detrimental to *K. pneumoniae* pathogenesis in the lung.

RESULTS

pCMW75 increases the global c-di-GMP concentration in *K. pneumoniae* TOP52. The *K. pneumoniae* genome encodes as many as 27 distinct enzymes bearing GGDEF domains, EAL domains, or both that could synthesize or hydrolyze c-di-GMP (19, 27). As it would be infeasible to inactivate all of these genes, we used an inducible plasmid system to raise global c-di-GMP levels in *K. pneumoniae*. Plasmid pCMW75 encodes isopropyl-β-D-1-thiogalactopyranoside (IPTG)-inducible QrgB, a *Vibrio harveyi* GGDEF protein which synthesizes c-di-GMP and has no further homology to *K. pneumoniae* proteins (28). The comparator plasmid pCMW98 encodes QrgB*, an inactive AAEEF mutant that is unable to synthesize c-di-GMP. We transformed pCMW75, pCMW98, or the parent vector, pEVS143, into wild-type *K. pneumoniae* strain TOP52 and grew these transformants in the presence of kanamycin and IPTG (Table 1). We developed a liquid chromatography-mass spectrometry (LC-MS) assay, using a known concentration of xanthosine 3′,5′-cyclic monophosphate (c-XMP; not synthesized by bacteria) as an internal standard, to measure the normalized c-di-GMP concentration in *K. pneumoniae* pellets (Fig. 1). Wild-type TOP52 bearing the parent vector pEVS143 contained 0.77 ng c-di-GMP/mg bacterial pellet. In contrast, induction of active QrgB in TOP52/pCMW75 resulted in a greater than 30-fold increase in the c-di-GMP concentration relative to that in both TOP52/pEVS143 ($P = 0.0002$) and TOP52/pCMW98 (containing inactive QrgB*; $P = 0.0007$).

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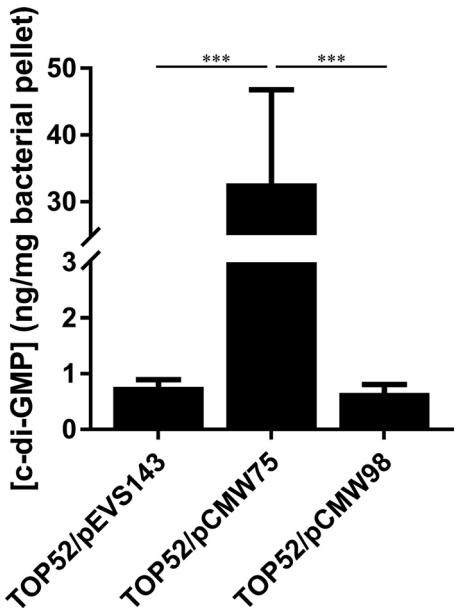

FIG 1 Expression of diguanylate cyclase QrgB increases the concentrations of c-di-GMP in *K. pneumoniae* TOP52. *K. pneumoniae* TOP52 was transformed with plasmid pEVS143 (the parent vector), pCMW75 (with active QrgB), or pCMW98 (with inactive QrgB*). The concentration of c-di-GMP in the bacterial pellets was measured by liquid chromatography-mass spectrometry. Data are shown as means ± SEMs and were combined from 4 independent experiments. ***, $P < 0.001$, Mann-Whitney U test.

A high c-di-GMP concentration impairs *K. pneumoniae* TOP52 virulence in the lungs. To assess how *K. pneumoniae* with a globally high c-di-GMP concentration performs *in vivo*, we utilized an established preclinical model of *K. pneumoniae* respiratory tract infection (29). Overnight, IPTG-induced cultures were grown statically at 37°C and then resuspended in phosphate-buffered saline (PBS), and no differences in bacterial growth or aggregation were observed (data not shown). Intratracheal inoculation with 10^7 CFU of TOP52/pEVS143, TOP52/pCMW75, or TOP52/pCMW98 into wild-type C57BL/6J mice was followed by lung and spleen harvest at 24 h postinfection (hpi). Mice infected with *K. pneumoniae* containing a high c-di-GMP concentration (TOP52/pCMW75) exhibited lower lung bacterial burdens than mice infected with basal levels of c-di-GMP (TOP52/pEVS143, $P < 0.0001$; TOP52/pCMW98, $P = 0.0002$) (Fig. 2A). While spleen bacterial titers (a marker for systemic dissemination) were low in all infected mice, they were significantly lower in mice infected with TOP52/pCMW75 than in mice infected with TOP52/pEVS143 and TOP52/pCMW98 ($P < 0.0001$ for both comparisons) (Fig. 2B). Lungs infected with TOP52/pCMW75 demonstrated less severe histopathology than lungs infected with TOP52/pEVS143 or TOP52/pCMW98, which contained dense, largely neutrophilic, inflammatory infiltrates (Fig. 2C). These data suggest that *K. pneumoniae* strains with high c-di-GMP levels are attenuated in murine pneumonia.

Attenuation of *K. pneumoniae* TOP52 virulence by high c-di-GMP levels is secondary to pathogen-specific changes. The mammalian host can sense and respond to c-di-GMP, along with other cyclic dinucleotides, and thus, it was not clear if the attenuation phenotype observed with TOP52/pCMW75 was a result of c-di-GMP effects on the bacteria or the host (25, 30). To investigate this, we intratracheally infected mice lacking STING (the host receptor for c-di-GMP) with TOP52/pEVS143 or TOP52/pCMW75. At 24 hpi in both the lungs and spleens, TOP52/pCMW75 was attenuated relative to TOP52/pEVS143 in the STING$^{-/-}$ mouse background ($P < 0.0001$) (Fig. 3), similar to its attenuation in wild-type C57BL/6J mice. These data suggest that the decrease in pulmonary virulence with *K. pneumoniae* containing high c-di-GMP levels reflects the effects of c-di-GMP on the bacteria and not augmented host sensing of the increased c-di-GMP levels.

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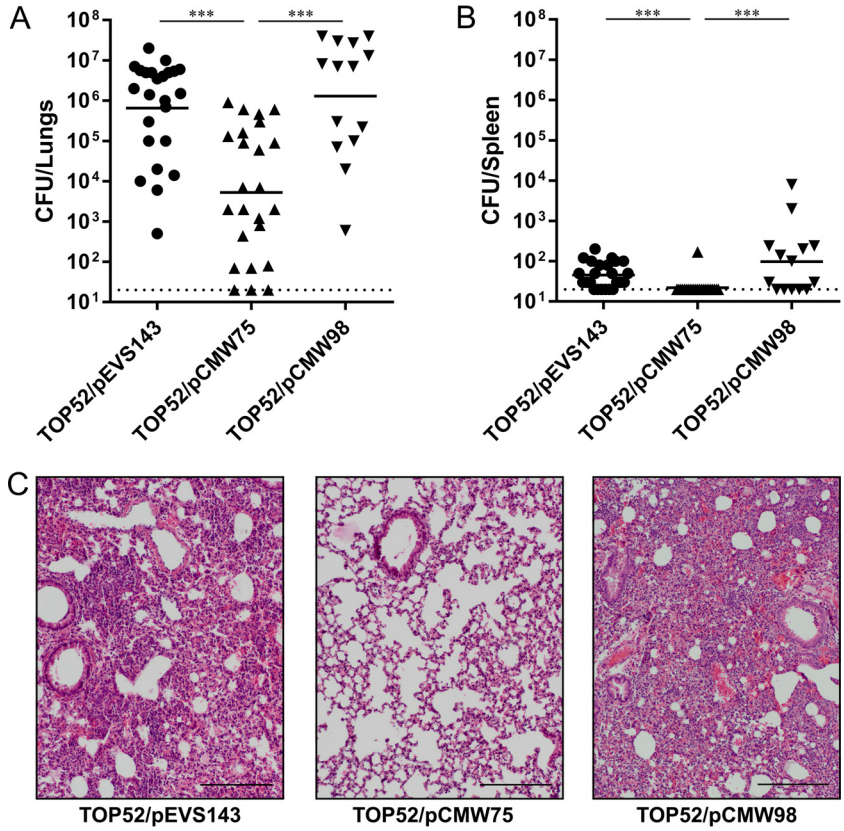

FIG 2 A high bacterial c-di-GMP concentration attenuates *K. pneumoniae* TOP52 virulence. Female C57BL/6J mice were infected with 10^7 CFU of TOP52/pEVS143, TOP52/pCMW75 (which has high levels of c-di-GMP), or TOP52/pCMW98 by intratracheal inoculation. Lung (A) and spleen (B) bacterial loads were quantified at 24 hpi. Data from at least 3 independent experiments were combined. Each symbol represents the result for one animal, short bars represent the geometric mean for each group, and full dotted horizontal lines represent the limits of detection. ***, $P < 0.001$, Mann-Whitney U test. (C) Murine lungs infected with *K. pneumoniae* were harvested at 24 hpi, and representative hematoxylin-and-eosin-stained sections are shown.

High c-di-GMP levels do not affect *K. pneumoniae* TOP52 serum resistance or capsule production. The abilities of *K. pneumoniae* to survive in serum and produce an exopolysaccharide capsule are important for infection of the lung and dissemination (10, 11, 31). To investigate if increased c-di-GMP levels affect the ability of *K. pneumoniae* to survive in serum, we performed serum resistance assays with TOP52/pEVS143, TOP52/pCMW75, and TOP52/pCMW98 along with two control strains. We constructed the negative-control strain TOP52 Δ*rfaH*, a mutant lacking the antiterminator regulatory factor RfaH, which is important in capsule production and serum resistance (13). We also used ATCC 43816, a murine-adapted *K. pneumoniae* strain known to produce abundant capsule and resist serum killing (11, 13). These assays demonstrated no significant differences in serum survival between TOP52/pCMW75 ($P = 0.7532$) or TOP52/pCMW98 ($P = 0.5344$) and TOP52/pEVS143 (Fig. 4A). As expected, the rate of survival in serum was lower for TOP52 Δ*rfaH* and higher for ATCC 43816 ($P < 0.0001$ for each strain compared to TOP52/pEVS143). To quantify capsule production, we measured the levels of uronic acid, a component of *K. pneumoniae* capsule, in extracts from the same five strains. Again, no significant differences in uronic acid levels were observed between TOP52/pCMW75 ($P = 0.4116$) or TOP52/pCMW98 ($P = 0.5414$) and TOP52/pEVS143 (Fig. 4B). As expected, TOP52 Δ*rfaH* ($P = 0.0024$) and ATCC 43816 ($P = 0.0008$) produced less and more uronic acid, respectively, than TOP52/pEVS143. Together, these data indicate that high c-di-GMP levels in TOP52/pCMW75 do not alter capsule production or serum resistance and that these factors do not explain TOP52/pCMW75 attenuation *in vivo*.

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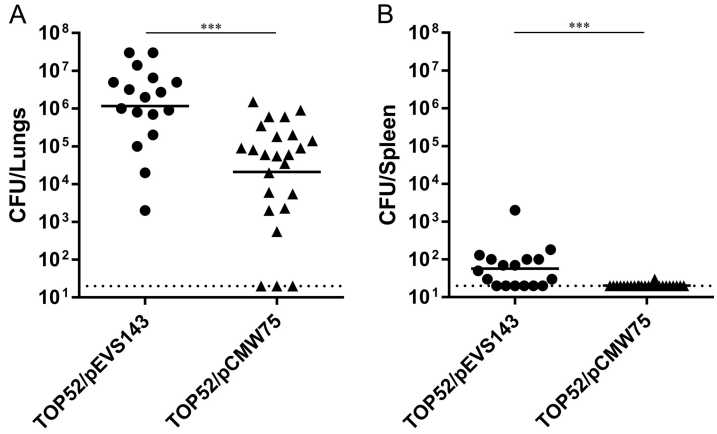

FIG 3 A high c-di-GMP concentration attenuates *K. pneumoniae* TOP52 virulence in STING$^{-/-}$ mice. Female STING$^{-/-}$ mice were infected with 10^7 CFU of TOP52/pEVS143 or TOP52/pCMW75 (which has high levels of c-di-GMP) by intratracheal inoculation. Lung (A) and spleen (B) bacterial loads were quantified at 24 hpi. Data were combined from 5 independent experiments. Each symbol represents the result for one animal, short bars represent the geometric mean for each group, and full dotted horizontal lines represent the limits of detection. ***, $P < 0.001$, Mann-Whitney U test.

High c-di-GMP levels promote type 1 piliation in *K. pneumoniae* TOP52. c-di-GMP is generally thought to promote the expression of surface adhesins by bacteria. Specifically, in *K. pneumoniae*, c-di-GMP has been shown to promote type 3 pilus expression via the sensor protein MrkH (18–20). Quantitative reverse transcription-PCR (qRT-PCR) for *mrkA*, the main type 3 pilus subunit, confirmed that type 3 pilus expression is upregulated in TOP52/pCMW75 relative to its expression in TOP52 harboring the vector control ($P < 0.0001$) (see Fig. S1A in the supplemental material).

It was unknown whether c-di-GMP influences the regulation of other pilus systems relevant to virulence. In *K. pneumoniae* (as in *Escherichia coli*), type 1 pili are transcriptionally regulated by an invertible phase switch, *fimS*, upstream of the gene encoding the main pilus subunit, *fimA*. A PCR-based phase assay was used to assess the proportions of *K. pneumoniae* populations in the phase-on versus phase-off promoter orientations. While TOP52/pEVS143 and TOP52/pCMW98 were almost exclusively phase off after growth in static culture, TOP52/pCMW75, which has high levels of c-di-GMP, displayed a significant phase-on population (Fig. 5A). As measured by qRT-PCR, TOP52/pCMW75 exhibited ~4-fold increased levels of the *fimA* transcript relative to those in TOP52/pEVS143 or TOP52/pCMW98 ($P < 0.0001$ and $P = 0.0003$, respectively) (Fig. 5B).

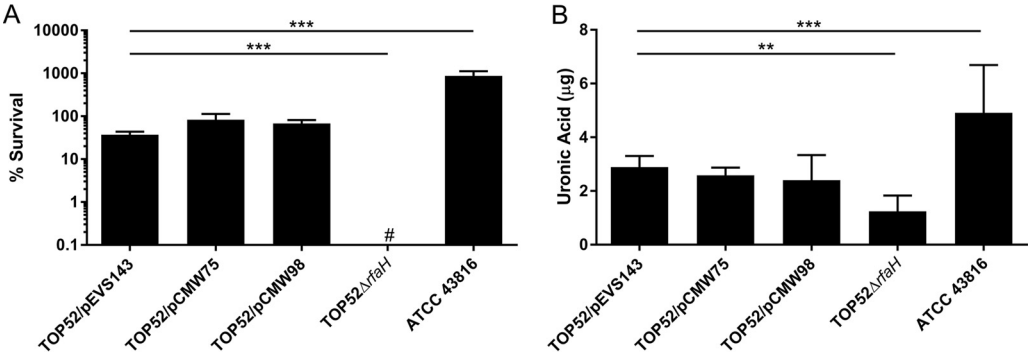

FIG 4 A high c-di-GMP concentration does not affect *K. pneumoniae* TOP52 serum resistance or capsule production. (A) In serum resistance assays, bacterial strains were incubated with pooled human active serum for 3 h; bacterial survival is expressed as a percentage of the input number of CFU. (B) Capsule extraction and quantification of the capsule component uronic acid were performed on the indicated strains. Data were combined from at least 3 independent experiments, and the results are shown as the means ± SEMs. #, survival was <0.01%. *P* values were determined by the Mann-Whitney U test. **, $P < 0.01$; ***, $P < 0.001$.

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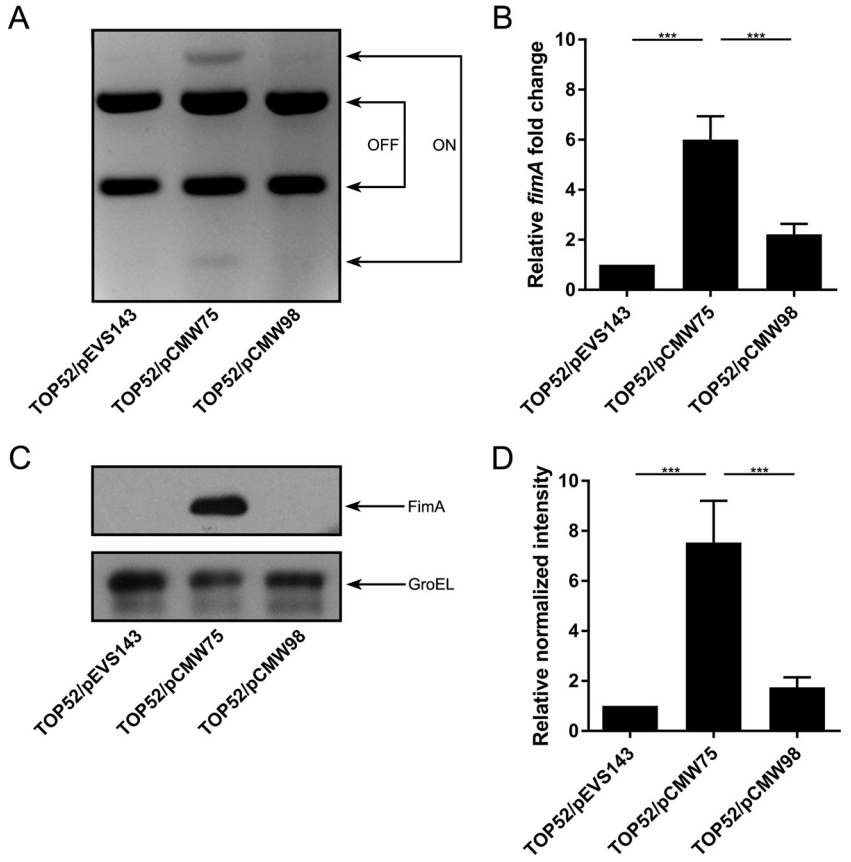

FIG 5 A high c-di-GMP concentration promotes type 1 piliation in *K. pneumoniae* TOP52. (A) Phase assays of the *fimS* type 1 pilus promoter switch were performed to assess the populations of phase-on versus phase-off bacteria in cultures. (B) qRT-PCR was performed to quantify the relative expression of *fimA*. (C) Immunoblots were performed using antibodies to FimA and the control protein GroEL. (D) Quantification of overexposed immunoblots was performed with ImageJ software. All data are representative of those from at least 3 independent experiments. The data in the graphs are shown as means ± SEMs. ***, $P < 0.001$, Mann-Whitney U test.

To confirm that high c-di-GMP levels influenced the actual production of type 1 pili in *K. pneumoniae*, we performed immunoblots for FimA. With the standard exposure, a significant FimA band was evident for TOP52/pCMW75, but the band was difficult to visualize for the TOP52/pEVS143 and TOP52/pCMW98 controls (Fig. 5C). Relative quantification of the band intensity after prolonged blot exposure demonstrated significantly increased FimA levels in TOP52/pCMW75 compared to TOP52/pEVS143 or TOP52/pCMW98 ($P < 0.0001$ and $P = 0.0015$, respectively) (Fig. 5D). In total, these data argue that, besides type 3 pili, a high c-di-GMP concentration also promotes the production of type 1 pili in *K. pneumoniae* TOP52.

c-di-GMP promotion of type 1 pilus production is not mediated by MrkH. MrkH features a PilZ domain which can sense c-di-GMP and, in turn, bind to the *mrkA* promoter, resulting in the transcriptional activation of type 3 pili (18, 19). Given the novel finding that c-di-GMP also promotes type 1 pilus production in *K. pneumoniae*, we asked whether this upregulation was also mediated via MrkH. We deleted *mrkH* in strain TOP52 and assessed type 1 pilus expression in the setting of high c-di-GMP concentrations. The c-di-GMP concentration was confirmed to be higher in TOP52 Δ*mrkH*/pCMW75 than in the control strains TOP52 Δ*mrkH*/pCMW98 and TOP52 Δ*mrkH*/pEVS143 when measured by LC-MS (data not shown). Of note, qRT-PCR did demonstrate the upregulation of *mrkA* in TOP52 Δ*mrkH*/pCMW75, although it was not nearly to the extent to which *mrkA* was upregulated in TOP52/pCMW75 (Fig. S1). These data indicate that while MrkH is the primary mediator of c-di-GMP signaling leading to

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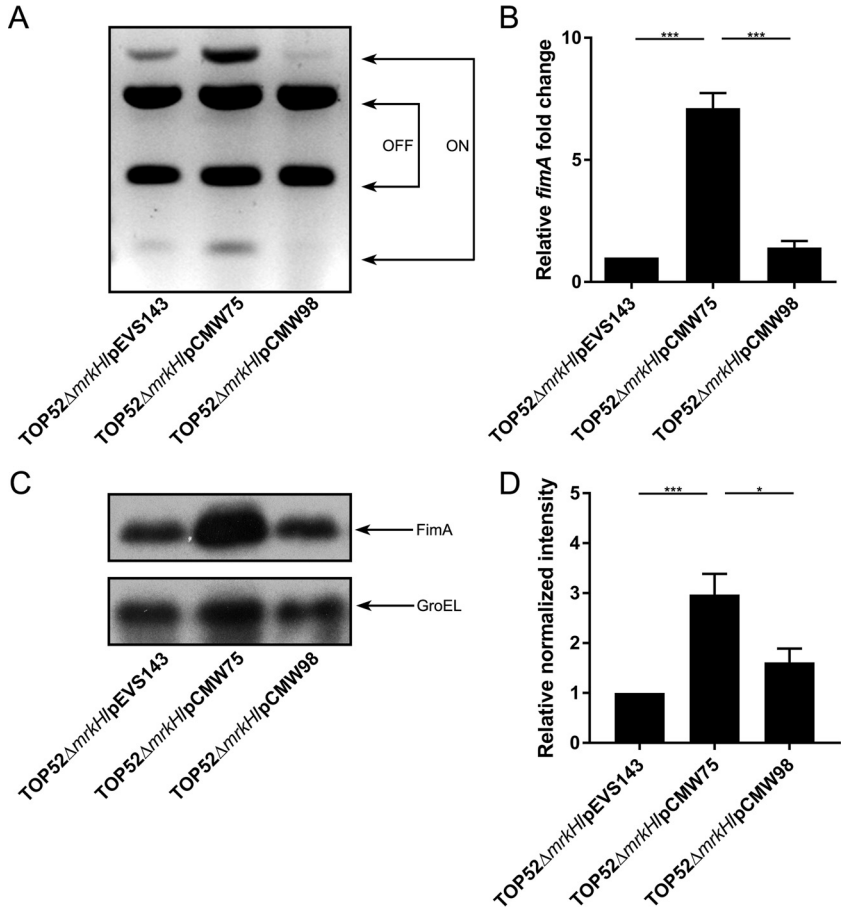

FIG 6 A high c-di-GMP concentration promotes type 1 piliation in *K. pneumoniae* TOP52 in the absence of *mrkH*. (A) Phase assays of the *fimS* type 1 pilus promoter switch were performed to assess the populations of phase-on versus phase-off bacteria in cultures. (B) qRT-PCR was performed to quantify the relative expression of *fimA*. (C) Immunoblots were performed using antibodies to FimA and the control protein GroEL. (D) Quantification of overexposed immunoblots was performed with ImageJ software. All data are representative of those from at least 3 independent experiments. The data in the graphs are shown as means ± SEMs. *P* values were determined by the Mann-Whitney U test. *, $P < 0.05$; ***, $P < 0.001$.

type 3 pilus production, type 3 pili are also regulated by c-di-GMP via MrkH-independent mechanisms.

Consistent with prior reports (32, 33), decreased type 3 pilus production in TOP52 Δ*mrkH* was associated with increased type 1 piliation relative to that in wild-type *K. pneumoniae*. While the sizes of the *fimS* phase-on populations were appreciable in all TOP52 Δ*mrkH* transformants, the size of the phase-on population of TOP52 Δ*mrkH*/pCMW75 was increased compared to that for the controls (Fig. 6A). In addition, qRT-PCR of TOP52 Δ*mrkH*/pCMW75 demonstrated increased amounts of the *fimA* transcript relative to those for TOP52 Δ*mrkH*/pEVS143 or TOP52 Δ*mrkH*/pCMW98 $P < 0.0001$ for both comparisons (Fig. 6B). While FimA bands were evident for all TOP52 Δ*mrkH* transformants, immunoblots demonstrated increased production of FimA in TOP52 Δ*mrkH*/pCMW75 compared to TOP52 Δ*mrkH*/pEVS143 or TOP52 Δ*mrkH*/pCMW98 ($P = 0.0002$ and $P = 0.0207$, respectively) (Fig. 6C and D). These data indicate that increases in type 1 pilus production triggered by high c-di-GMP concentrations are not mediated by MrkH.

Increased expression of type 1 pili, but not type 3 pili, is detrimental for *K. pneumoniae* TOP52 virulence in the lung. To determine if the increased production of type 1 or type 3 pili contributed to the attenuated virulence of *K. pneumoniae* strains with high-c-di-GMP levels in the lung, we constructed mutant strains unable to produce

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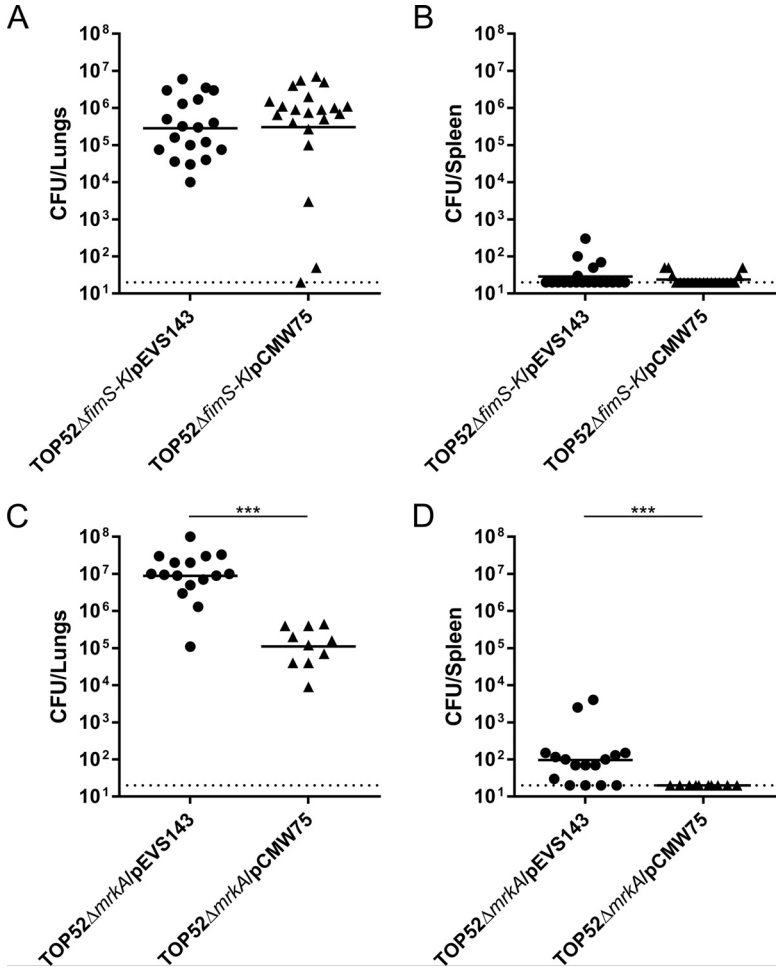

FIG 7 A high c-di-GMP concentration attenuates *K. pneumoniae* TOP52 virulence in the absence of type 3 pili but not in the absence of type 1 pili. Female C57BL/6J mice were infected with 10^7 CFU of TOP52 Δ*fimS-K*/pEVS143, TOP52 Δ*fimS-K*/pCMW75 (which has high levels of c-di-GMP), TOP52 Δ*mrkA*/pEVS143, or TOP52 Δ*mrkA*/pCMW75 (which has high levels of c-di-GMP) by intratracheal inoculation. Lung (A, C) and spleen (B, D) bacterial loads were quantified at 24 hpi. Data were combined from at least 3 independent experiments. Each symbol represents one animal, short bars represent the geometric mean for each group, and full dotted horizontal lines represent limits of detection. ***, $P < 0.001$, Mann-Whitney U test.

type 1 pili (TOP52 Δ*fimSAICDFGHK* [TOP52 Δ*fimS-K*]) or type 3 pili (TOP52 Δ*mrkA*) and intratracheally infected wild-type C57BL/6J mice with transformants confirmed to have basal or high levels of c-di-GMP. Infection with TOP52 Δ*fimS-K*/pCMW75 showed no attenuation in the lung or spleen bacterial burden at 24 hpi compared to that with infection with TOP52 Δ*fimS-K*/pEVS143 (Fig. 7A and B). In contrast, infection with TOP52 Δ*mrkA*/pCMW75 maintained significant reductions in lung and spleen bacterial burdens at 24 hpi relative to the burdens of TOP52 Δ*mrkA*/pEVS143 ($P < 0.0001$ and $P = 0.0004$, respectively) (Fig. 7C and D). The failure of high c-di-GMP levels to attenuate pneumonia in the absence of type 1 pili but not in the absence of type 3 pili indicates that the increased production of type 1 pili in the lung may hamper *K. pneumoniae* virulence in this niche.

DISCUSSION

In the face of accelerating antimicrobial resistance in *K. pneumoniae*, additional therapeutic targets are needed. The present work is the first to examine the influence of c-di-GMP on *K. pneumoniae* pulmonary virulence. These murine experiments suggest that *K. pneumoniae* in a state with high c-di-GMP levels is less virulent in the lung.

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Manipulation of c-di-GMP synthetic and degradative pathways in pathogenic bacteria may thus represent a promising means of targeting virulence. Efforts are under way to synthesize and develop small-molecule broad-EAL-domain inhibitors that could alter a pathogen's ability to cleave c-di-GMP (34, 35). It should be noted that while high c-di-GMP levels attenuated *K. pneumoniae* infection in the lung, this state may not be detrimental for pathogenesis in other niches. For example, one could postulate that increased type 1 piliation triggered by high c-di-GMP levels might augment *K. pneumoniae* virulence in the urinary tract; ongoing studies will address such questions.

Our experiments relied on the elevated c-di-GMP concentrations achieved via expression of a diguanylate cyclase enzyme in *trans*, and our controls (the QrgB* mutant) helped to ensure that the QrgB protein itself was not exerting off-target effects. This system may not, however, reflect the differential functions of apparently redundant c-di-GMP-modifying enzymes in *K. pneumoniae* or the potential temporal and spatial regulation of c-di-GMP synthesis within the cell (22, 36). Thus, natural perturbations in c-di-GMP levels may have different effects on virulence factors downstream. Additionally, while we suggest that high c-di-GMP levels result in a *K. pneumoniae* virulence profile that is attenuated in initial infection of the lung, the effects of the perpetual induction of high c-di-GMP levels over the course of a longer infection have not been investigated.

In our focused analyses, we demonstrated that both type 1 and type 3 pilus adhesins are upregulated in the setting of high c-di-GMP levels, whereas capsule expression did not seem to be significantly affected. However, c-di-GMP is a ubiquitous second messenger and, as such, could impart numerous downstream effects in *K. pneumoniae* and other bacteria; transcriptional profiling and other unbiased approaches may help to further define these in future work. Regardless of the mechanism, however, if the phenotypes observed in the present model extend to other *K. pneumoniae* strains or additional lung pathogens, then c-di-GMP pathways remain attractive therapeutic targets.

Of additional note, c-di-GMP has been shown to stimulate the innate immune system in mice and has even been proposed to be a vaccine adjuvant (30, 37). However, the levels of c-di-GMP used in studies showing host-protective innate immune effects were ~10,000-fold higher than the level contained in our intratracheal inocula. Additionally, the persistence of the attenuated *K. pneumoniae* phenotype with high c-di-GMP levels in STING$^{-/-}$ mice suggests that the effects on virulence in this study were not host mediated.

This work begins to illustrate the complexity of interactions between type 1 pili, type 3 pili, and c-di-GMP in *K. pneumoniae*. For example and as previously reported (33, 34), the loss of type 3 pili results in increased type 1 pilus production through regulatory mechanisms that remain unclear. Furthermore, while c-di-GMP promotes increased expression of each adhesin, for type 3 pili, this is mediated by both MrkH-dependent and MrkH-independent effects, while type 1 pili exhibit only MrkH-independent regulation. The specific mechanism by which c-di-GMP exerts its effects on these fimbrial operons in the absence of MrkH remains to be identified.

In conclusion, c-di-GMP promotes the expression of both type 1 and type 3 pili in *K. pneumoniae* while attenuating virulence in the lung. Further efforts addressing c-di-GMP pathways as a means of thwarting pathogenesis *in vivo* are warranted.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The *K. pneumoniae* strains and plasmids used in this study are shown in Table 1. Growth curves measuring the optical density at 600 nm (OD_{600}) over time, as well as confirmation by plating at multiple time points, demonstrated no significant differences in growth among the strains used (data not shown). For all experiments, including those involving murine infections, and unless otherwise specified, bacteria were prepared by growing them statically in 20-ml cultures at 37°C for 16 h in Luria-Bertani (LB) broth. When pEVS143, pCMW75, or pCMW98 was present, 50 μg/ml kanamycin and 0.5 mM IPTG were also added at culture onset. For murine experiments, bacteria were centrifuged at 8,000 $\times$ *g* for 10 min, resuspended in sterile PBS, and diluted to the desired inoculum concentration by measuring the OD_{600}. The inocula were verified by serial dilution and plating.

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Bacterial mutagenesis. Bacteriophage lambda Red recombinase mutagenesis was modified for use with *K. pneumoniae* as previously described (13). Briefly, bacteria containing pKD46s were grown with shaking overnight at 30°C with 50 μg/ml spectinomycin and 0.7% arabinose in LB broth, subcultured 1:100, and grown to an OD_{600} of 0.5 to 0.6. Cells were transformed with a kanamycin resistance determinant flanked by sequences with homology to the target gene (the primers are listed in Table 2) and recovered overnight in superoptimal broth containing 20 mM glucose (SOC medium). Colonies were selected on LB agar containing 50 μg/ml kanamycin. The kanamycin resistance gene was excised by transforming the cells with temperature-sensitive plasmid pCP20 carrying the FLP recombinase. Clones were verified by direct sequencing.

Quantification of c-di-GMP. Measurements were performed using an LC-MS method (38) with addition of an internal standard for the absolute quantification of the c-di-GMP concentration (expressed as the amount per milligram of bacterial pellet). Briefly, 4 ml of bacterial cultures was pelleted, and the pellet was weighed and homogenized in 500 μl water containing 100 ng of c-XMP (Sigma-Aldrich, St. Louis, MO) using an Omni Bead Ruptor 24 apparatus (Omni International, Kennesaw, GA). After centrifugation of the homogenized liquids, the supernatants were analyzed by LC-MS alongside calibration samples of cyclic di-GMP containing 100 ng of c-XMP. LC-MS analysis was performed with a Shimadzu 20AD high-performance liquid chromatography system and a Leap PAL autosampler coupled to a triple-quadrupole mass spectrometer (API 4000; Applied Biosystems, Foster City, CA) operated in the multiple-reaction-monitoring mode. The positive-ion electrospray ionization mode was used for detection of both c-di-GMP and c-XMP. Study samples were injected in duplicate in each independent experiment. Data were processed with Analyst (version 1.5.1) software (Applied Biosystems).

Mouse infections, organ titers, and histology. All animal procedures were approved by the Institutional Animal Care and Use Committee at Washington University School of Medicine. Female C57BL/6J and C57BL/6J-*Tmem173*^{gt}/J (STING$^{-/-}$) mice (The Jackson Laboratory, Bar Harbor, ME) aged 7 to 8 weeks were intratracheally inoculated as previously described (39). Briefly, each mouse was anesthetized with inhaled isoflurane, and the trachea was exposed via surgical dissection. The inoculum (20 μl containing 1×10^7 to 2×10^7 CFU) was injected intratracheally using a 30-gauge, caudally directed needle. The skin was closed using Vetbond tissue adhesive (3M Animal Care Products, St. Paul, MN). Mice received 1 mg/kg of body weight sustained-release buprenorphine subcutaneously for pain control. To determine the bacterial burden at 24 hpi, the animals were sacrificed and their organs (lungs, spleens) were homogenized in sterile PBS by use of a Bullet Blender homogenizer (Next Advance, Averill Park, NY) for 5 min; aliquots were serially diluted and plated. Organs that were removed for histology were washed in PBS, fixed in 10% neutral buffered formalin, dehydrated in ethanol, and paraffin embedded; 5-μm sections were stained with hematoxylin and eosin. Micrographs were obtained using an Olympus DP25 camera and a BX40 light microscope.

Serum resistance. Serum bactericidal assays were adapted from those previously described (40). Blood was drawn by venipuncture from healthy adult donors according to a protocol approved by the Human Research Protection Office at Washington University. Briefly, 25 μl of statically grown bacteria (10^6 CFU/ml in PBS) was mixed in 96-well microplates with 75 μl of pooled human serum, and the mixture was incubated for 3 h at 37°C. Samples were serially diluted and plated to calculate percent survival (ratio of the number of CFU at 3 h to the input number of CFU).

Capsule quantification. Capsule extraction and uronic acid quantification were performed using a modified protocol (41, 42). Five hundred microliters of overnight static LB cultures was mixed with 100 μl of 1% Zwittergent 3-14 (Sigma-Aldrich) in 100 mM citric acid, and the mixture was incubated at 50°C for 20 min. Following centrifugation, the supernatants were precipitated with 1 ml cold ethanol. After recentrifugation, the pellet was dissolved in 200 μl water and 1.2 ml of 12.5 mM tetraborate in concentrated H_2SO_4 was added. Samples were vortexed, boiled at 95°C for 5 min, and mixed with 20 μl of 0.15% 3-hydroxydiphenol (Sigma-Aldrich) in 0.5% NaOH. The absorbance at 520 nm was measured. The uronic acid concentration in each sample was determined from a standard curve of glucuronic acid (Sigma-Aldrich).

Phase assay for type 1 pili. To determine the orientation of the *fimS* phase switch in *K. pneumoniae*, a phase assay was adapted as previously described (15). PCR primers (Table 2) were used to amplify an 817-bp DNA region including *fimS*, and the PCR product was digested with HinfI (New England BioLabs, Ipswich, MA). A phase-on switch yields products of 605 and 212 bp, and a phase-off switch yields products of 496 and 321 bp.

Quantitative reverse transcription PCR. qRT-PCR was performed as previously described, and the amounts of the PCR products were normalized to those of a *K. pneumoniae* housekeeping gene, *rpoB* (43). The cultures were grown overnight with antibiotics, as appropriate, subcultured 1:100 with antibiotics and inducing agents, and grown to an OD_{600} of 0.4 to 0.6. mRNA was isolated using an RNeasy minikit (Qiagen, Germantown, MD). cDNA was synthesized with an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) using a C1000 Touch thermal cycler (Bio-Rad). qRT-PCR was performed with SsoAdvanced Universal SYBR green Supermix (Bio-Rad) and the primers listed in Table 2 using an ABI 7500 real-time PCR system (Applied Biosystems).

Immunoblots. Acid-treated whole-cell immunoblotting was performed as previously described (44) using 1:2,000 rabbit anti-type 1 pilus (45) and 1:500,000 rabbit anti-GroEL (Sigma-Aldrich) primary antibodies. Amersham ECL horseradish peroxidase-linked donkey anti-rabbit IgG (GE Healthcare, Buckinghamshire, UK) secondary antibody (1:2,000) was applied, followed by application of Clarity enhanced chemiluminescence (ECL) substrate (Bio-Rad), and the membrane was developed using GeneMate blue autoradiography film (BioExpress, Kaysville, UT). Relative band intensities were quantified using ImageJ software (http://rsb.info.nih.gov/ij/).

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TABLE 2 Primers used in this study

Primer purpose and primer	Description	Sequence[a]
Mutagenesis		
mrkH-P1-F	Amplifies kanamycin resistance cassette from pKD4 for insertion into *mrkH* (type 3 pilus regulatory gene)	TCAGAATGTTGCTATTGCTATAAGAAAAATCAAACGCCTCACGACAACTATTTACAAGGGGTGTAGGCTGGAGCTGCTTC
mrkH-P2-R	Amplifies kanamycin resistance cassette from pKD4 for insertion into *mrkH* (type 3 pilus regulatory gene)	AGATTGAGTGACCAATGAGATTGTCATTGGTGTACAGAAATATACTGTCCAAGGTTGTCACATATGAATATCCTCCTTAG
*mrkH*checkF	Verifies TOP52 Δ*mrkH* sequence	CCGGTCTCCAGCTTGGAGGC
*mrkH*checkR	Verifies TOP52 Δ*mrkH* sequence	GCACAGCCCGACAATGTCGC
mrkA-P1-F	Amplifies kanamycin resistance cassette from pKD4 for insertion into *mrkA* (type 3 pilus subunit gene)	GCTGACCTCAGAATAAAAATACAAGCGGCGGACATTGCCGCTTTATTATTGTTATTAACTGTGTAGGCTGGAGCTGCTTC
mrkA-P2-R	Amplifies kanamycin resistance cassette from pKD4 for insertion into *mrkA* (type 3 pilus subunit gene)	TTCTGGTTCTATGCGAATTCACAGTGTGCTCATTGATTCGTAATTCACTCTGACAAGGAACATATGAATATCCTCCTTAG
*mrkA*checkF	Verifies TOP52 Δ*mrkA* sequence	ATTGTTGGCATGGGCCGCA
*mrkA*checkR	Verifies TOP52 Δ*mrkA* sequence	CAGCATCGCCTGATGTTTCTGG
fimS-P1-F	Amplifies kanamycin resistance cassette from pKD4 for insertion into *fim* operon (type 1 pilus operon)	GAAAGAAATAATCTTTTAGAGGAAAAAGACCAGAAGAAGAAAAATGAATTAACAGATTGAGTGTAGGCTGGAGCTGCTTC
fimK-P2-R	Amplifies kanamycin resistance cassette from pKD4 for insertion into *fim* operon (type 1 pilus operon)	GATATTCGCGCATGACGTACCGGCACCGGTGCTAACCGGTGCGCTTTTCTCGCACCCTCA CATATGAATATCCTCCTTAG
*fimS*checkF	Verifies TOP52 Δ*fimS-K* sequence	CACGTTTCGCTGGCATCTGG
*fimK*checkR	Verifies TOP52 Δ*fimS-K* sequence	CAGCTGGTGGCGAAGGTAGTGG
rfaH-P1-F	Amplifies kanamycin resistance cassette from pKD4 for insertion into *rfaH* (transcription antitermination gene)	CGCGATTACACCAGCCATTTGTTATGCTTGCCGTTATTAGAAGTGGAATGAGTCATTATGGTGTAGGCTGGAGCTGCTTC
rfaH-P2-R	Amplifies kanamycin resistance cassette from pKD4 for insertion into *rfaH* (transcription antitermination gene)	GAGGCGCAAACGGACGCAAACGTCAGGGCGCTGTTTGGCGTCGACATTAACGGCGTTTAGCATATGAATATCCTCCTTAG
*rfaH*checkF	Verifies TOP52 Δ*rfaH* sequence	TACGGTGTTCGACGGCGCG
*rfaH*checkR	Verifies TOP52 Δ*rfaH* sequence	TGCCGCATATCCTGGCCAG
qRT-PCR		
q*rpoB*-F	Amplifies *rpoB* (housekeeping gene)	CTGATGCCTCAGGATATGATCAAC
q*rpoB*-R	Amplifies *rpoB* (housekeeping gene)	CTGGCTGGAACCAAAGAACTCT
q*fimA*-F	Amplifies *fimA* (type 1 pilus gene)	GGACCGTGCATTTTAAAGGA
q*fimA*-R	Amplifies *fimA* (type 1 pilus gene)	GGCCTAACTGAACGGTTTGA
q*mrkA*-F	Amplifies *mrkA* (type 3 pilus gene)	CCTGTTTAGTGCCATCAGCA
q*mrkA*-R	Amplifies *mrkA* (type 3 pilus gene)	CTCCGGTAACCCTGACTGAA
Phase assay		
*Kleb*phaseF	Amplifies *fimS* for orientation analysis	GGGACAGATACGCGTTTGAT
*Kleb*phaseR	Amplifies *fimS* for orientation analysis	GGCCTAACTGAACGGTTTGA

[a]All primers are listed in the 5′-3′ orientation.

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Statistical analysis. Comparisons between two groups of continuous variables were analyzed using the nonparametric Mann-Whitney U test. All tests were two-tailed, and P values of <0.05 were considered significant. Analyses were performed using GraphPad Prism (version 7.01) software.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00647-17.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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D.A.H. serves on the board of directors of BioVersys AG.

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