H-NS Nucleoid Protein Controls Virulence Features of *Klebsiella pneumoniae* by Regulating the Expression of Type 3 Pili and the Capsule Polysaccharide

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**INTRODUCTION**

*Klebsiella pneumoniae* is an opportunistic pathogen causing nosocomial infections. Main virulence determinants of *K. pneumoniae* are pili, capsular polysaccharide, lipopolysaccharide, and siderophores. The histone-like nucleoid-structuring protein (H-NS) is a pleiotropic regulator found in several gram-negative pathogens. It has functions both as an architectural component of the nucleoid and as a global regulator of gene expression. We generated a Δhns mutant and evaluated the role of the H-NS nucleoid protein on the virulence features of *K. pneumoniae*. A Δhns mutant down-regulated the mrkA pilin gene and biofilm formation was affected. In contrast, capsule expression was derepressed in the absence of H-NS conferring a hypermucoviscous phenotype. Moreover, H-NS deficiency affected the *K. pneumoniae* adherence to epithelial cells such as A549 and HeLa cells. In infection experiments using RAW264.7 and THP-1 differentiated macrophages, the Δhns mutant was less phagocytized than the wild-type strain. This phenotype was likely due to the low adherence to these phagocytic cells. Taken together, our data indicate that H-NS nucleoid protein is a crucial regulator of both T3P and CPS of *K. pneumoniae*.
been reported (Nordmann et al., 2009; Hirsch and Tam, 2010; Schwaber et al., 2011). The main virulence determinants of *K. pneumoniae* are: capsular polysaccharide (CPS), lipopolysaccharide, siderophores, and pili (Gerlach et al., 1989; Podschun and Ullmann, 1998; Brisse et al., 2009). The *K. pneumoniae* genome codes for different pili such as Type 1 pili (T1P), Type 3 pili (T3P), and *E. coli* common pilus (ECP; Allen et al., 1991; Schurtz et al., 1994; Struve et al., 2009; Alcántar-Curiel et al., 2013). In contrast to T1P, the T3P can cause mannose-resistant agglutination of tannic acid-treated human erythrocytes (Podschun and Ullmann, 1998). The biosynthesis of T3P is dependent on the *mrkABCDF* operon (Hornick et al., 1995; Huang et al., 2009). The filament is composed of the major pilus subunit MrkA and the tip adhesion protein MrkD (Gerlach et al., 1989). *K. pneumoniae* T3P mediate adherence to tracheal epithelial cells, renal tubular cells, basolateral surfaces of lung tissue and are crucial in biofilm formation (Tarkkanen et al., 1997; Sebghati et al., 1998; Langstraat et al., 2001; Jagnow and Clegg, 2003; Schroll et al., 2010). While the pili are required during the initial colonization of the host, the CPS impairs macrophage-mediated phagocytosis (Highsmith and Jarvis, 1985; Podschun and Ullmann, 1998; Alvarez et al., 2000). CPS is a complex layer of surface-associated polysaccharides which is important for the pathogenesis of *K. pneumoniae* in both, animal models as well as in infections of cultured cells (Cortés et al., 2002; Lawlor et al., 2005; Regueiro et al., 2006; March et al., 2013).

The histone-like nucleoid-structuring protein (H-NS) is a DNA-binding protein found in enteropathogens such as *Escherichia*, *Salmonella*, *Shigella*, *Vibrio*, and *Yersinia* (Tendeng and Bertin, 2003). It has functions as an architectural component of the nucleoid and as a global regulator of gene expression (Tendeng and Bertin, 2003; Dorman, 2004). It has been proposed that H-NS affects bacterial evolution by direct repression of AT-rich foreign DNA (i.e., pathogenicity islands) acquired by horizontal transfer events, to facilitate tolerance of the foreign genetic sequences and to integrate them into a pre-existing regulatory network (Navarre et al., 2006, 2007; Dorman, 2007). Mutations in *hns*-like genes have pleiotropic effects in several bacteria and may cause defects in their growth or even bacterial cell death (Zhang et al., 1996; Tendeng et al., 2000; Heroven et al., 2004; Ellison and Miller, 2006; Lucchini et al., 2006; Navarre et al., 2006; Baños et al., 2008; Castang and Dove, 2012). Similar to other enterobacteria, *K. pneumoniae* is known to possess regions of horizontally acquired genetic sequences. However, there are no reports about the role of H-NS in this pathogen.

In this work we describe the effect of H-NS protein on the expression of both T3P and CPS, two of the main virulence determinants of *K. pneumoniae*. The absence of H-NS down-regulated the T3P and affected biofilm formation. In contrast, expression of CPS was derepressed in a Δ*hns* mutant, conferring a hypermucoviscous phenotype. Finally, the absence of H-NS resulted in low adherence to epithelial cells and macrophages and in high resistance to macrophage phagocytosis.

### MATERIAL AND METHODS

#### Bacterial Strains and Culture Conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Bacterial cultures were routinely grown in Luria-Bertani (LB) broth with or without antibiotics [200 μg/ml (ampicillin), 50 μg/ml (kanamycin), 30 μg/ml (chloramphenicol), or 10 μg/ml (tetracycline)] after overnight growth with shaking at 37°C.

#### Construction of *K. pneumoniae* Mutants

*K. pneumoniae* 123/01 was isolated from a patient with pneumonia by bronchoalveolar washing. Capsular serotype K39 was determined by sequencing of *wzc* gene as previously described (Pan et al., 2013). *K. pneumoniae* was targeted for mutagenesis of *hns*, *mrkA* and *cps* following the procedure reported by Datsenko and Wanner (2000) with some modifications. Each purified PCR product was electroporated into competent *K. pneumoniae* carrying the lambda-Red recombinase helper plasmid pKD119, whose expression was induced by adding L-(+)-arabinose (Sigma) at a final concentration of 1.0%. For the Δ*cps* mutant, we deleted the

### Table 1 | Bacterial strains and plasmids used in this study.

| Strain or plasmid | Genotype or description | References or source |
|-------------------|-------------------------|----------------------|
| **K. pneumoniae STRAINS** |
| Kpn 123/01        | Wild-type, serotype K39 | Clinical isolate     |
| Kpn hns           | Δ*hns:*Km<sup>R</sup>    | This study           |
| Kpn mrkA          | Δ*mrkA:*Km<sup>R</sup> | This study           |
| Kpn cps           | Δ*cps:*Km<sup>R</sup> | This study           |
| Kpn hns cps       | Δ*hns:*Km<sup>R</sup> Δ*cps:*Cm<sup>R</sup> | This study           |
| **E. coli K12 STRAIN** |
| DH5α              | Laboratory strain       | Invitrogen           |

| PLASMIDS |
|----------|
| pMPM-T3  | p15A derivative low-copy-number cloning vector, lac promoter, Tc<sup>R</sup> | Mayer, 1995       |
| pT3-H-NS | pMPM-T3 derivative expressing H-NS from the lac promoter | This study         |
| pMPM-T6  | p15A derivative cloning vector, pBAD (ara) promoter, Tc<sup>R</sup> | Mayer, 1995       |
| pT6-MrkH | pMPM-T6 derivative expressing MrkH from the pBAD (ara) promoter | This study         |
| pKD119   | pINT-ts derivative containing the λ red recombinase system under an arabinose-inducible promoter, Tc<sup>R</sup> | Datsenko and Wanner, 2000 |
| pKD4     | pANT<sub>S</sub> derivative template plasmid containing the kanamycin cassette for λ Red recombination, Ap<sup>R</sup> | Datsenko and Wanner, 2000 |
| pKD3     | pANT<sub>S</sub> derivative template plasmid containing the chloramphenicol cassette for λ Red recombination, Ap<sup>R</sup> | Datsenko and Wanner, 2000 |

Ap<sup>R</sup>, ampicillin resistance; Km<sup>R</sup>, kanamycin resistance; Cm<sup>R</sup>, chloramphenicol resistance; Tc<sup>R</sup>, tetracycline resistance.
chromosomal region from galF to wzi [Δ(galF-orf2-wzi)]. PCR fragments containing hns, mrkA and cps sequences flanking a kanamycin cassette were generated using gene-specific primer pairs (Table 2), and DNA of the pKD4 plasmid was used as template. For the Δhns Δcps double mutant, we generated a PCR fragment containing cps sequence flanking a chloramphenicol cassette using the pKD3 plasmid as template. The respective mutations were confirmed by PCR and sequencing.

Construction of Plasmids

The pT3-H-NS plasmid was generated by cloning a PCR product containing the corresponding hns region of K. pneumoniae into the pMPM-T3 plasmid (see primers in Table 2). The PCR product was digested with Xhol and EcoRI and ligated into pMPM-T3 digested with the same enzymes. pT6-MrkH was constructed by cloning a PCR product containing the mrkH region, which was digested with Ncol and HindIII and ligated into pMPM-T6 (see Table 2). The identities of the inserts were confirmed by DNA sequencing.

Quantitative RT-PCR

Total RNA extraction was performed using the hot phenol method as previously described (Jahn et al., 2008). DNA was removed with TURBO DNA-free (Ambion, Inc.) and the quality of RNA was assessed using a NanoDrop (ND-1000; Thermo Scientific). Specific primers were designed with the Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and are listed in Table 2. For LightCycler reactions, a master mix of the following components was prepared: 3.0 µl PCR-grade water, 1.0 µl (10 µM) forward primer, 1.0 µl (10 µM) reverse primer, 10 µl 2x SYBR Green I Master Mix, and 5.0 µl cDNA (50–100 ng). A multwell plate was sealed with sealing foil, centrifuged at 1500 g for 3 min and loaded into the LightCycler 480 instrument (Roche). Amplification was performed in triplicate wells for each sample analyzed. Control reactions with no template (water) and minus-reverse transcriptase (RNA) were run with all reactions. Real-time PCR analysis was performed using the following conditions: denaturation (95°C for 10 min); amplification and quantification repeated for 45 cycles (95°C for 10 s, 57°C for 20 s, 72°C for 30 s with a single fluorescence measurement); melting curve (95°C for 10 s, 65°C for 1 min with continuous fluorescence measurement at 97°C); and finally a cooling step at 40°C for 10 s. Melting curve analysis was performed after each run to confirm specificity of the primers. 16S rRNA was used as a reference gene for normalization and the relative gene expression was calculated using the 2−ΔΔCt method (Livak and Schmittgen, 2001).

Mucoviscosity

The mucoviscosities of K. pneumoniae strains were determined by a string test and measured by centrifugation as described

### Table 2 | Primers used in this study.

| Primer | Sequence (5’-3’) | Target gene | RE |
|--------|-----------------|-------------|----|
| **FOR qPCR** | | | |
| mrkA-5’ | CCGTCGACCTGATTGATGATCTCA | mrkA | |
| mrkA-3’ | GCAGCGCTGCGCAGGAGAC | | |
| fimA-5’ | ACTGTTAACCCACCAAGCTGAC | fimA | |
| fimA-3’ | CTGCATACGGATGGCAGTAC | | |
| ecpA-5’ | ACCCTACGCTATATACGCAAA | ecpA | |
| ecpA-3’ | CGCTGATGATGAGAAGAGAG | | |
| mrkH-5’ | AAAATCAAAGGCTCTAGAGAC | mrkH | |
| mrkH-3’ | TGCGATGCTGCTGCAATAG | | |
| mrkI-5’ | CCAAGGCAAAAAAGAGATGC | mrkI | |
| mrkI-3’ | AATAATCCGCCCTGATTCCT | | |
| mrkJ-5’ | CGCTATCCGCGTTATCCACT | mrkJ | |
| mrkJ-3’ | TATGATGTTGCGCGTAGA | | |
| fimB-5’ | ATGGATATGCTGACCTGCT | fimB | |
| fimB-3’ | TAAAATCTTGGCGGAGAAG | | |
| fimE-5’ | AAAAGGGAGACGACCTGTT | fimE | |
| fimE-3’ | TGGCCTCTTTAACACCGATTT | | |
| ecpR-5’ | ATTTGGTTCTGCAGGATTCCT | ecpR | |
| ecpR-3’ | ATTTGGTTCTGCAGGATTCCT | | |
| rscA-5’ | ACGGATATGCTGCAATTAAAG | rscA | |
| rscA-3’ | AGGTGATGACGGTGCTGAC | | |
| galF-5’ | CAAAGGCAATTCCAAAGGAAG | galF | |
| galF-3’ | TGGCCTGACACAACAAATCT | | |
| wzi-5’ | CAGGGGTTTTGCGACACACA | wzi | |
| wzi-3’ | GTTTGACGTCGATGCATCTG | | |
| manC-5’ | AGGGGATCTGGTATTGGTCG | manC | |
| manC-3’ | AAATGATGCTGCGATGTGCT | | |
| msh-5’ | CAACCCAACTGGAATCTGAGA | msh | |
| msh-3’ | GTTACCCTCCATCTGGCTTTCCT | | |
| **FOR GENE CLONING** | | | |
| hns-Xhol-5’ | GGCGCTCGAGTTAGTTCAACAAAAA | hns | Xhol |
| hns-Xho | | | |
| hns-EcoRI-3’ | GGCGAGATTACGCCAAAAAAACCT | EcoRI | |
| mrkH-Ncol-5’ | AACCCATGAGATACAGAGGGAAGA | mrkH | Ncol |
| mrkH-HindIII-3’ | CCAAGGCTTTTCAAGATGATGATGATG | HindIII | |
| **FOR GENE DELETIONS** | | | |
| Kpn-hns-H1P1 | TATAAGGTTAATCTCTCAATCAG | hns | |
| Kpn-hns-H2P2 | TTTTATGGCGATCCAAACGGGATTG | | |
| Kpn-mrkA-H1P1 | CACCTTCCAGGAAGGATGGGAATCT | mrkA | |
| Kpn-mrkA-H2P2 | CAGTTTATGTTTCTGGCGG | | |

(Continued)
previously (Pan et al., 2011; Lin et al., 2012). The string test was performed stretching a colony that had been grown overnight on a blood agar plate, using a loop. To further measure the levels of mucoviscosity, a low speed centrifugation was performed. Briefly, equal numbers of exponential phase-cultured bacteria were centrifuged at 1000 g for 5 min. The supernatant was subjected to measurement of the absorbance at 600 nm.

**Glucuronic Acid Analysis**

Capsular polysaccharides were extracted and quantified using a colorimetric assay for glucuronic acid as previously described (Lin et al., 2009). Basically, 500 μl of bacterial cultures were mixed with 100 μl of 1% zwittergent 3–14 in 100 mM citric acid and then the mixtures were incubated at 50°C for 20 min. After centrifugation, 250 μl of supernatants were transferred into new tubes, and 1 ml of absolute ethanol was added to precipitate the CPS. The pellets were dissolved in 200 μl of distilled water, and then 1200 μl of 12.5 mM borax in concentrated H₂SO₄ were added. The mixtures were vigorously vortexed, boiled for 5 min, and then cooled. 20 μl of 0.15% 3-hydroxydiphenol in 0.5% NaOH were added to the mixture and the absorbance was measured at 520 nm. The glucuronic acid concentration in each sample was determined from a standard curve of glucuronic acid measured at 520 nm. The glucuronic acid concentration in each sample was determined from a standard curve of glucuronic acid and expressed in micrograms/10⁶ CFU.

**Adherence Assays to Cultured Eukaryotic Cells**

Monolayers of HeLa (ATCC CCL-2) human cervix epithelial and A549 (ATCC CCL-185) human lung epithelial cell lines (7 × 10⁵) were infected with the indicated strains of an LB broth overnight culture at a multiplicity of infection (MOI) of 100. Epithelial cells were grown in DMEM (Invitrogen) with 10% fetal bovine serum (FBS). After infection, eukaryotic cells were incubated in DMEM with no FBS for 2 h at 37°C under an atmosphere of 5% CO₂. After the 2 h incubation period, cells were rinsed three times with PBS to remove unbound bacteria. For quantification of adherence, the cells were lysed with a solution of 0.1% Triton X-100. After homogenization, 10-fold serial dilutions were plated onto LB agar plates to determine total CFUs. The results shown are the mean of at least three experiments performed in triplicate on different days.

**Phagocytosis of Bacteria by Macrophages**

THP-1 (ATCC TIB-202) human monocites (differentially to macrophages with 200 nM of phorbol 12-myristate 13-acetate for 24 h) and RAW264.7 (ATCC TIB-71) murine macrophages (6 × 10⁵) were seeded into 24-well tissue culture plates. Bacteria were grown in 5 ml of LB broth to the exponential phase. Macrophages were infected with a MOI of 100 in a final volume of 1 ml RPMI 1640 tissue culture medium supplemented with 10% heat-inactivated FBS. To synchronize infection, plates were centrifuged at 200 g for 5 min. Plates were incubated at 37°C under an humidified 5% CO₂ atmosphere. After 2 h, cells were rinsed three times with PBS and incubated for an additional 60 min with 1 ml of RPMI 1640 containing 10% FBS and gentamicin (100 μg/ml) to eliminate extracellular bacteria. Cells were then rinsed again three times with PBS and lysed with 0.1% Triton X-100. After homogenization, 10-fold serial dilutions were plated onto LB agar plates to determine total CFUs. Adherence of *K. pneumoniae* strains (grown to the exponential phase) to macrophages was performed as previously described (Rosales-Reyes et al., 2012), incubating 1 h at 4°C to inhibit phagocytosis.

**Biofilm Formation Assay on Abiotic Surface**

Adhesion to abiotic surface (polystyrene) was analyzed using 96-well plates as described previously (Saldaña et al., 2014). Overnight cultures of bacteria grown in LB broth (10 μl) were added to 1 ml of LB. This volume was distributed in quintuples of 1 ml RPMI 1640 tissue culture medium supplemented with 10% heat-inactivated FBS and 10% heat-inactivated FBS. To synchronize infection, plates were centrifuged at 200 g for 5 min. Plates were incubated at 37°C under an humidified 5% CO₂ atmosphere. After 2 h, cells were rinsed three times with PBS and lysed with 0.1% Triton X-100. After homogenization, 10-fold serial dilutions were plated onto LB agar plates to determine total CFUs. Adherence of *K. pneumoniae* strains (grown to the exponential phase) to macrophages was performed as previously described (Rosales-Reyes et al., 2012), incubating 1 h at 4°C to inhibit phagocytosis.

**Statistical Analysis**

For statistical differences, one-way ANOVA followed by the Tukey’s comparison test was performed using Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). *P* ≤ 0.05 was considered statistically significant.

**RESULTS**

**Generation of an hns Mutant of *K. pneumoniae***

H-NS amino acid sequences of *K. pneumoniae* strains were homologous to H-NS proteins of enteric bacteria such as *Salmonella, Yersinia, Shigella*, and *E. coli* (Figure 1A). The hns gene of the strain 123/01 used in this study was completely identical to all *K. pneumoniae* sequenced strains (data not shown). By using the λ-red homolog recombinase (Datsenko and Wanner, 2000), we were able to replace the hns gene of the *K. pneumoniae* genome with a kanamycin resistance cassette. The hns gene of *K. pneumoniae* was cloned into a
A

K. pneumoniae-MGH  
K. pneumoniae-342  
K. pneumoniae-NTUH-K2044  
S. dysenteriae  
S. boydii  
S. flexneri  
EHEC  
EPEC  
E. coli K-12  
S. Typhimurium  
S. Typhi  
Y. pestis  
Y. pseudotuberculosis  
Y. enterocolitica  

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K. pneumoniae-342  
K. pneumoniae-NTUH-K2044  
S. dysenteriae  
S. boydii  
S. flexneri  
EHEC  
EPEC  
E. coli K-12  
S. Typhimurium  
S. Typhi  
Y. pestis  
Y. pseudotuberculosis  
Y. enterocolitica  

B

FIGURE 1 | H-NS protein in K. pneumoniae. (A) Alignment of amino acid sequences of H-NS proteins from several enterobacteria: K. pneumoniae (MGH, 342 and NTUH-K2044), Shigella dysenteriae (Sd197), Shigella boydii (Sb227), Shigella flexneri (2a str. 2457T), Enterohemorrhagic E. coli (EDL933), Enteropathogenic E. coli (E2348/69), E. coli K-12 (MG1655), Salmonella enterica serovar Typhimurium (LT2), Salmonella enterica serovar Typhi (CT18), Yersinia pestis (KIM 10), Yersinia pseudotuberculosis (IP 32953) and Yersinia enterocolitica (8081). Analysis was performed using the ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Growth kinetics of wild-type K. pneumoniae, and the isogenic mutants at 37°C (B) and 25°C (C). Bacterial cultures were grown for 8 h in LB medium.
plasmid yielding pT3-H-NS to complement the Δhns mutant. In terms of resistance to antibiotics, the Δhns mutant did not differ with respect to the wild type strain (data not shown). The growth in LB broth of K. pneumoniae strains was followed over a period of 8 h at 37 and 25°C. Growth of the Δhns mutant was slightly attenuated at 37°C, mainly in the exponential phase but reaching the stationary phase like the wild-type strain (Figure 1B). In contrast, at 25°C the Δhns mutant did not reach the growth of the wild-type strain in the stationary phase (Figure 1C). Growth of the complemented strain harboring pT3-H-NS was restored to wild-type levels at both temperatures. This observation indicates that H-NS is required for optimal growth of K. pneumoniae as has been shown for other enterobacteria (Zhang et al., 1996; Tendeng et al., 2000; Heroven et al., 2004; Ellison and Miller, 2006; Lucchini et al., 2006; Navarre et al., 2006; Baños et al., 2008; Castang and Dove, 2012).

**H-NS Differentially Regulates the Fimbrial Repertoire of K. pneumoniae**

We reported previously that ecpA, mrkA and fimA fimbrial genes are highly prevalent in K. pneumoniae strains (Alcántar-Curiel et al., 2013). To demonstrate the role of H-NS in regulation of fimbrial genes, we determined transcriptional expression levels of ecpA, mrkA and fimA by qRT-PCR. Both, ecpA and fimA were derepressed in the absence of H-NS. In contrast to ecpA and fimA, mrkA was repressed in the absence of H-NS (Figure 2A). In addition to pilin genes themselves, we evaluated the expression of genes regulating expression of three pili types. Regulatory genes for the pilins analyzed were up-regulated in the absence of H-NS. The mrkJ gene codes for a phosphodiesterase (Johnson and Clegg, 2010) and negatively regulates mrkA expression by degrading c-di-GMP, thereby inhibiting MrkH activity, which is the central activator of T3P (Wilksch et al., 2011). To corroborate decreased expression of the mrkA pilin gene in absence of H-NS, we performed biofilm formation assays. Biofilm formation in K. pneumoniae is MrkA-dependent (Langstraat et al., 2001; Wilksch et al., 2011). Indeed, the absence of MrkA reduced K. pneumoniae biofilm formation by 10-fold (Figure 2B). The Δhns mutant was impaired in biofilm formation (5-fold) similar to the ΔmrkA mutant, while this phenotype was counteracted by complementing the Δhns mutant with the pT3-H-NS plasmid (Figure 2B). In addition to T3P, the capsule polysaccharide (CPS) is a crucial virulence determinant in K. pneumoniae. To evaluate the role of CPS in biofilm formation, we assayed the Δcps and Δhns Δcps mutants. The Δcps mutant was not affected in...
biofilm formation. However, the Δhns Δcps double mutant was impaired in biofilm formation similar to the Δhns single mutant (Figure 2B). These observations suggest that CPS has no role in biofilm formation and that downregulation of T3P expression is the main reason for the biofilm phenotypes observed in the Δhns mutant. To corroborate that the effect of H-NS on biofilm formation is due to the transcriptional repression of mrkA and not due to overexpression of the capsule possibly resulting in steric hindrance of T3P, we generated wild type and Δhns mutant strains overexpressing the MrkH activator protein. Indeed, MrkH overexpression positively affected biofilm formation (∼2-fold) in the wild-type strain (Figure 2C). Interestingly, overexpression of the MrkH protein counteracted the decrease in biofilm formation observed in the Δhns mutant (∼9-fold) regardless of the excess production of CPS (Figure 2C). These data corroborated H-NS as a positive regulator of TP3 gene expression.

The Absence of H-NS Results in a Hypermucoviscous Phenotype of K. pneumoniae

The Δhns mutant colony morphology was hypermucoviscous compared to wild-type bacteria on agar plates while the complemented strain was similar to the wild-type strain (data not shown). To determine the levels of mucoviscosity, we measured the supernatant of suspensions of wild-type, Δhns mutant and complemented Δhns mutant bacteria centrifuged at low speed. Indeed, the absence of H-NS resulted in higher mucoviscosity levels compared to the wild-type strain (∼3-fold), while complementation restored the wild-type phenotype (Figures 3A,B). To determine the amount of CPS quantitatively, we performed a biochemical assay, which measured the capsular glucuronic acid. As shown in Figure 3C, the CPS level increased in the absence of H-NS (∼3-fold), while the complemented Δhns strain had similar amounts of CPS compared to the wild-type strain. The absence of MrkA did not alter the CPS production; however in both the Δcps and Δhns Δcps mutants the CPS amount was considerably diminished (∼4-fold), indicating that increasing of the capsule in the hns background is dependent on capsular genes. Genetically, capsule-generating genes of K. pneumoniae are encoded in the cps cluster, including three transcriptional units, being galF, wzi, and manC the first genes for each promoter (Chou et al., 2004; Chuang et al., 2006; Pan et al., 2011). K. pneumoniae strains belonging to serotype K39 contain the three capsule-generating genes described above (Pan et al., 2013, 2015). In addition, cps genes are activated by the RcsA regulatory protein (Wehland and Bernhard, 2000; Lin et al., 2011, 2013). Using qRT-PCR we monitored the expression of

![FIGURE 3](image-url)
**Fimbrial and Capsular Genes of* K. pneumoniae* are Thermoregulated by H-NS**

H-NS nucleoid protein has been reported to be an essential component in thermoregulation of virulence factors in several pathogenic bacteria (Falconi et al., 1998; Umanski et al., 2002; Ono et al., 2005; Duong et al., 2007). To analyze if the repressor effect of H-NS was temperature-dependent, we performed qRT-PCR experiments determining the transcriptional expression of both fimbrial (mrkA, fimA, ecpA) and capsule-generating (galF, wzi, manC) genes in the wild-type and Δhns mutant at 37 and 25°C. At 25°C, the repressor effect of H-NS was higher than at 37°C, specifically 3.33-, 8.72-, and 9.66-fold for mrkA, fimA, and ecpA, respectively (Figure 4A). For capsular genes, H-NS-mediated repression was about 4-fold higher at 25°C compared to 37°C (Figure 4B). These data clearly indicate that at low temperatures (25°C), H-NS efficiently represses both fimbrial and capsular genes as compared to 37°C by maintaining down-regulation of these virulence genes.

**H-NS is Involved in* K. pneumoniae* Adherence to and Phagocytosis by Eukaryotic Cells**

*K. pneumoniae* is able to adhere to different epithelial cell lines such as A549 and HeLa (Moranta et al., 2010; Alcántar-Curiel et al., 2013). We evaluated the adherence of the *K. pneumoniae* wild-type bacteria, the Δhns mutant, the complemented Δhns mutant, the ΔmrkA mutant, the Δcaps mutant and the Δhns Δcaps double mutant to A549 or HeLa cells (≈4500-fold), while the complemented Δhns mutant showed an adherence that was similar to that of the wild-type strain (Figure 5A). Interestingly, the ΔmrkA mutant was less adherent than the wild-type strain (≈9-fold) but not comparable to the Δhns mutant, suggesting that the phenotype observed with the Δhns mutant cannot be explained solely by the decrease of MrkA expression (Figure 5A). Similar to the biofilm assay, we overexpressed the MrkH activator protein to exclude possible effects of capsule-mediated steric hindrance on T3P activity. Overexpression of MrkH enhanced the adherence of *K. pneumoniae* to HeLa cells for both, wild-type bacteria (≈14-fold) and Δhns mutant (≈263-fold) (Figure 5B), supporting the notion that H-NS affects the adherence by transcriptional control of mrkA pilin. The absence of CPS resulted in a slight increase in adherence of *K. pneumoniae* to both epithelial cell types (≈5-fold). Bacteria deficient in both hns and cps genes adhered in higher numbers to epithelial cells compared to those deficient in hns alone (≈15-fold). However, the levels of adherence of the Δhns Δcps double mutant did not reach the numbers of the wild-type strain, indicating that CPS is partially involved in the low adherence observed with the Δhns single mutant (Figure 5A). To discard that biofilm formation on plastic surfaces did not affect the adherence assays during 2 h of incubation, we quantified the CFU/ml of adhered bacteria on plastic wells at this time with no eukaryotic cells. We were unable to find adhered bacteria on the plastic surface during 2h. Furthermore, biofilm formation assays were performed and all strains examined did not form biofilm at 2 h of incubation in the conditions examined for eukaryotic cells (DMEM or RPMI, 37°C, 5% CO₂). A crucial event in the pathogenesis of *K. pneumoniae* is the evasion of macrophage phagocytosis. We used THP-1 differentiated macrophages and RAW264.7 cells to evaluate the effect of H-NS deletion on phagocytosis. A Δhns mutant was considerably less phagocytized by macrophages (≈55-fold) and the complemented strain was recovered in numbers similar to the wild-type strain (Figure 5C). Bacterial growth rates during adherence and phagocytosis were similar in the different strains analyzed (data not shown), indicating that the low levels of phagocytosis observed with the Δhns mutant were not due to growth defects. The absence of MrkA did not alter the phagocytosis of *K. pneumoniae* by macrophages,
FIGURE 5 | Adherence and phagocytosis of the K. pneumoniae Δhns mutant. (A) Comparison of adherence levels of K. pneumoniae wild-type, Δhns mutant, complemented Δhns mutant, ΔmrkA mutant, Δcps mutant, and Δhns Δcps mutant to HeLa and A549 cells. (B) Adherence levels of wild-type strain and isogenic Δhns mutant to HeLa cells, overexpressing the MrkH activator protein (0.1% arabinose). (C) Comparison of phagocytic uptake of indicated K. pneumoniae strains by RAW264.7 and THP-1 macrophages. (D) Adherence levels of K. pneumoniae wild-type strain and isogenic mutants to RAW264.7 and THP-1 macrophages. Results represent means and standard deviations of the results obtained from the 3 experiments performed in triplicates. ns, not significant; statistically significant with respect to the wild-type strain ***p < 0.001; **p < 0.01; *p < 0.05.

while the absence of CPS increased the phagocytosis by both cell lines (~67-fold). In contrast, a Δhns Δcps double mutant was phagocytosed in similar bacterial numbers as the Δhns single mutant. To determine if the low level of phagocytosis showed by the Δhns Δcps double mutant was due to the initial stage of adherence, we performed adherence assays using both macrophages cell lines. The Δhns and the Δhns Δcps mutants presented the same low levels of adherence to macrophages, indicating that the evasion of phagocytosis is mainly due to impaired adherence (Figure 5D). These observations suggest that the H-NS nucleoid protein in K. pneumoniae is relevant for both, adherence to and phagocytosis by eukaryotic cells.

DISCUSSION

H-NS is a pleiotropic regulator, which modulates expression of virulence determinants of several enteropathogens (Dorman, 2004). This study describes for the first time the role of H-NS in expression of K. pneumoniae virulence features. The K. pneumoniae H-NS protein is homologous to other H-NS-like proteins of different enteropathogens such as E. coli, Salmonella, Yersinia, and Shigella. In terms of bacterial fitness, the absence of H-NS resulted in a slight defect in K. pneumoniae growth in LB broth at 37°C, but more evident at low temperature such as 25°C. As previously reported in other hns mutants, this could be due to dysregulated expression of non-related genes (Navarre et al., 2007). Pili are relevant for the adherence to host cells as well as in biofilm formation (Podschun and Ullmann, 1998; Langstraat et al., 2001; Wilksch et al., 2011). Interestingly, a Δhns mutant differentially regulated fimbrial genes, as we observed an increase of both fimA and ecpA and a repression of mrkA expression. FimA and EcpA pilin subunits were previously reported to be repressed by H-NS in E. coli, showing similar regulation for pilin genes as for regulatory genes in both bacteria (Dorman and Ni
The mrkA expression was down-regulated in the absence of H-NS, likely by up-regulation of mrkJ, which is a negative regulator of T3P (Johnson and Clegg, 2010), albeit MrkH and MrkJ transcriptional regulators were also derepressed. In the absence of H-NS, high levels of MrkJ may deplete c-di-GMP affecting the MrkH activity as transcriptional activator of the mrkA gene (Wilksch et al., 2011). The positive effect of H-NS on mrkA suggests a post-transcriptional process indirectly affecting MrkA expression, as has been previously described for other genes regulated by H-NS (Bertin et al., 1994; Suzuki et al., 1996; Johansson et al., 1998; Park et al., 2010). The positive role of H-NS on mrkA expression was corroborated by the fact that the Δhns mutant was impaired in biofilm formation similarly to the mutant deficient in mrkA. Since pilus are important in the early stage of infection, we analyzed the contribution of H-NS and Mrk to the adherence to human epithelial cells. A Δhns mutant was dramatically affected in the adherence to both, A549 and HeLa cells, while the absence of MrkA led to a significant but comparatively mild decrease in adherence. T3P have been expressed in an E. coli background (Tarkkanen et al., 1997), yet this is the first report about the contribution of MrkA pilus to the adherence to A549 and HeLa epithelial cells using a ΔmrkA mutant. The low adherence levels of the Δhns mutant could not be observed with the ΔmrkA mutant, suggesting that this decrease in adherence to human epithelial cells may be caused by different factors. In addition to transcriptional repression, hypermucoviscosity observed in the Δhns mutant could block the aggregation of Mrk pili and therefore interfere with their attachment to the abiotic surface. Previous studies showed that T1P function can be inhibited by the presence of the capsule by steric overcrowding and it has been suggested that this would also affect aggregation of Mrk pili (Schembri et al., 2005; Wilksch et al., 2011). Overexpression of MrkH activator protein showed, however, that T3P are not affected by capsule-mediated steric hindrance in the hns background neither with respect to biofilm formation, nor during adherence to epithelial cells. Hypervirulent K. pneumoniae strains produce large amounts of CPS, which confer both, a mucoviscous phenotype and resistance to phagocytosis (Lin et al., 2004; Regueiro et al., 2006). We found that the Δhns mutant was hypermucoviscous with respect to the wild-type strain. This increase in CPS correlated with the derepression of capsular genes (Chuang et al., 2006; Ho et al., 2011; Lin et al., 2013). Controversial results regarding the involvement of capsule on biofilm formation in K. pneumoniae have been reported (Schembri et al., 2005; Boddicker et al., 2006; Balestrino et al., 2008; Wu et al., 2011; Wang et al., 2015). However, our data support observations stating that CPS is not related with biofilm formation.

As to CPS regulation by H-NS, a mucoid morphology in an E. coli hns background has been shown in previous reports (Jbel and Trempy, 1999). This phenotype was due to up-regulation of rcsA, which activates the cps locus (Sledjeski and Gottesman, 1995). Interestingly, we observed the same phenomenon in K. pneumoniae, since capsular structural genes (galF, wzi, and manC) and a capsular regulator (rcsA) were derepressed in the absence of H-NS. Moreover, the K. pneumoniae Δhns mutant was phagocytized in lower numbers as the wild-type strain, likely related to its high mucoviscosity which confers resistance to phagocytic uptake by macrophages (Williams et al., 1983; Cortés et al., 2002). Surprisingly, uptake of bacteria deficient in both, hns and cps was similar to that of mutants deficient in hns alone. This decrease was likely due to impaired adherence to macrophages, indicating that H-NS regulates the initial stages of K. pneumoniae recognition by phagocytic cells. Our data show that there are probably others factors involved in macrophage adherence in addition to CPS. Pili of gram-negative bacteria such as E. coli T1P, Porphyromonas gingivalis FimA (also called T2P), Gram-positive Lactobacillus rhamnosus ScaCBA pilin and Streptococcus pneumoniae RrgA pili have been described to be required for the phagocytic uptake by macrophages (Baorto et al., 1997; Wang et al., 2007; Orrskog et al., 2012; Vargas García et al., 2015). The absence of MrkA, however, did not affect macrophage phagocytosis, indicating that T3P are not required for this phenomenon. LPS and outer membrane proteins (OMPs) of K. pneumoniae have been reported to be involved in resistance to phagocytosis (March et al., 2013). Our group currently studies the effect of H-NS on other K. pneumoniae virulence factors such as LPS, OMPs, and siderophores.

Change of temperature is an environmental condition that affects the oligomerization state of H-NS and subsequently its DNA-binding properties, being crucial for control of transcriptional regulation (Ono et al., 2005; Stella et al., 2005, 2006). In bacteria such as E. coli, Shigella and Salmonella, virulence genes are thermoregulated by H-NS: down-regulated at low temperature and expressed at mammalian body temperature (37°C) (Maurelli and Sansonetti, 1988; Falconi et al., 1998; Umanski et al., 2002; Ono et al., 2005; Duong et al., 2007). In agreement with the thermoregulatory functions of H-NS on various virulence factors, fimbrial and capsular genes of K. pneumoniae were differentially repressed at 37 and 25°C, showing that H-NS-mediated repression is higher at the lower temperatures likely encountered outside of the host.

In summary, we have described for the first time the important role of H-NS in K. pneumoniae. Our data show that similar to other pathogens, K. pneumoniae H-NS is a master regulator assuring optimal expression of both T3P and CPS whose uncontrolled expression may severely impact bacterial fitness.

**AUTHOR CONTRIBUTIONS**

Conceived and designed the experiments: MA, JF RR, MJ, MA, MD. Performed the experiments: MA, JF RR, MJ, MA, MD. Analyzed the data: MA, JT, JG, MA, MD. Wrote the paper: MA, KV, MA, MD.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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