



Article

Capsule Regulation Shapes *Klebsiella pneumoniae* Pathogenesis by Balancing Adhesion, Biofilm Formation, and Intracellular Survival

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Abstract

Klebsiella pneumoniae is a major opportunistic pathogen, where the polysaccharide capsule is traditionally recognized as a critical virulence determinant. However, its role in surface interactions and intracellular adaptation remains incompletely understood. Here, we combined phenotypic assays with physicochemical analyses to dissect the contribution of the capsule. A *wza* knockout mutant displayed enhanced biofilm formation, adhesion, and invasion of epithelial cells compared to the encapsulated strain. Zeta potential and hydrodynamic size measurements revealed that capsule absence increased surface negativity and exposure of adhesion structures, thereby promoting host–cell interactions. In contrast, intracellular survival assays demonstrated that the capsule conferred a clear advantage for persistence and replication. Together, our results support a dynamic model in which capsule expression imposes a trade-off: restricting early adhesion and biofilm development but favoring long-term intracellular survival. This trade-off model expands the understanding of capsule biology and may inform novel strategies to disrupt colonization or persistence in antibiotic-resistant *K. pneumoniae*.

Keywords: bacterial capsule; biofilms; cell adhesion; *Klebsiella pneumoniae*; zeta potential

1. Introduction

Klebsiella pneumoniae is a Gram-negative, encapsulated and immobile rod-shaped bacterium that belongs to the family Enterobacteriaceae. It is a clinically important, opportunistic pathogen that colonizes human mucosal surfaces such as the nasopharynx, oropharynx and gastrointestinal tract of healthy people and has the ability to cause infections in immunocompromised people [1,2]. It is often associated with hospital-acquired infections such as pneumonia, urinary tract infections, post-operative wound infections, bacteremia, and septicemia [3]. The risk of infection with this bacterium increases considerably in patients who require breathing support equipment, urinary catheters, and neonatal catheters, due to the ability of the bacteria to form biofilm and remain viable on these devices for long periods of time [4]. This persistence reflects the combined contribution of several virulence factors, including fimbriae, siderophores, lipopolysaccharides (LPS), and the polysaccharide capsule.



Academic Editor: Pablo C. Baldi

Received: 17 November 2025

Revised: 15 December 2025

Accepted: 16 December 2025

Published: 25 February 2026

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A study based on data from Child Health and Mortality Prevention Surveillance (CHAMPS) in seven countries in sub-Saharan Africa and South Asia, showed that *K. pneumoniae* contributed considerably to high mortality in the first two years of life and was the underlying cause of death in 20% of these cases [5]. In 2019, *K. pneumoniae* antibiotic resistance was responsible for more than 600,000 deaths worldwide [6,7]. Recently, in 2024, the World Health Organization (WHO) published a list of bacterial priority pathogens with an alarming scenario in which, carbapenem-resistant *K. pneumoniae* is positioned in the top spot, while, *K. pneumoniae* resistant to third-generation cephalosporins ranks sixth [8].

K. pneumoniae employs a wide array of virulence determinants, including lipopolysaccharides, siderophores, and fimbriae, to establish infection [2]. Among these, the polysaccharide capsule remains a critical determinant of severe pathogenesis and resistance to host defenses [9]. Although well-characterized structurally, the functional plasticity of the capsule and its role in modulating the exposure of other surface factors during different stages of infection warrant further investigation.

The best described virulence factors for this pathogen are type 1 and 3 fimbriae, capsule, LPS and siderophores. Type 1 and 3 fimbriae promote adhesion to biotic surfaces (epithelial and immune cells) and facilitate invasion into the host, and also to abiotic surfaces, resulting in biofilm formation on medical devices [10,11]. The capsule, a polysaccharide structure that surrounds the bacterial cell, is classified into different serotypes (K antigens) based on variations in the sequence of its capsular polysaccharides [12]. *K. pneumoniae* produces more than 80 capsular serotypes that play a fundamental role in its protection and escape from the host's defenses, by e.g., promoting the inhibition of complement activation and masking structures and proteins involved in opsonization and subsequent bacterial phagocytosis [10,11]. The polysaccharide composition of these serotypes varies and determines different degrees of protection and efficiency in immune evasion [13].

The genes required to produce the capsule of this pathogen are in the *Cps* operon, which encodes a variety of enzymes and structural proteins essential for capsule assembly. The process begins with the action of glycosyltransferases (GTs), such as WcaJ, which transfers glucose, or WbaP, which transfers galactose to a lipid carrier on the inner membrane. Additional sugar residues—such as fucose and uronic acids—are sequentially added by other GTs. These oligosaccharide units are then flipped across the inner membrane by Wzx, polymerized by Wzy, and the length of the resulting polysaccharide chain is regulated by Wzc. The completed polysaccharide is exported to the extracellular space via Wza and anchored to the bacterial surface by Wzi, thereby finalizing capsule assembly [2,14]. In addition to protecting against immune recognition, the capsule has been suggested to influence surface interactions and biofilm development.

The capsule is thought to contribute to biofilm formation based both on its participation in initial surface adhesion, and through supporting the three-dimensional structure of mature biofilms [15]. Biofilms are structured communities of bacteria enclosed in a self-produced extracellular matrix composed of polysaccharides, proteins, and nucleic acids [3]. This matrix protects the bacteria from environmental stressors, such as temperature shifts and desiccation, and significantly increases their resistance to antimicrobial agents by limiting drug diffusion and enabling metabolic dormancy. Moreover, biofilm formation facilitates horizontal gene transfer, including the dissemination of antibiotic resistance plasmids, and impairs recognition and clearance by host immune defenses [3,16].

In this way, the biofilm acts as a reservoir of bacteria during infection, contributing to chronic and recurrent disease [17]. Despite this well-established role of biofilms in infection, it remains unclear how capsule expression modulates biofilm formation in *K. pneumoniae*. While the capsule has been proposed to participate in early adhesion and

to support mature biofilm structure [15], studies across different bacterial species have reported conflicting results. This knowledge gap raises the question of whether capsule loss promotes or impairs biofilm formation in *K. pneumoniae*.

Although capsule loss has been variably reported to increase or decrease biofilm formation, few studies have integrated host–cell assays with physicochemical measurements to propose a unified model. Here, we address this gap by demonstrating that capsule expression imposes a trade-off between early colonization and long-term persistence. Therefore, the central question of this study was: does the absence of the capsule affect the ability of *K. pneumoniae* to form biofilms, adhere to, and invade human epithelial cells? To address this, we compared encapsulated and non-encapsulated strains in in vitro models of biofilm formation on abiotic surfaces and host cells, as well as in adhesion, invasion, and intracellular survival assays.

2. Results

2.1. The Absence of the Capsule Interfered with Bacterial Growth

The strains compared in this work (Table 1) were first evaluated to confirm the absence of the capsule. For this, glucuronic acid quantification and negative staining were performed, as shown in the Supplementary Data (Supplementary Figures S1–S3). After this confirmation, growth curves were performed to evaluate the impact of capsule loss on bacterial replication dynamics (Figure 1) and to assess whether culture medium and growth conditions influenced bacterial fitness. Two growth conditions were tested: static and shaking. All growth curve experiments were performed in triplicate.

Table 1. Bacterial Strains.

Strains	Genotype/Phenotype
<i>K. pneumoniae</i> MKP103	Wild/capsule
<i>K. pneumoniae</i> KP07107	<i>wza</i> mutant/No capsule

In static cultivation (Figure 1A), growth was monitored for 8 h with OD600 nm measurements every 20 min. Both strains showed similar exponential growth in CDM and TSB; however, the capsule-deficient mutant (KP07107) entered stationary phase earlier, while the encapsulated strain (MKP103) continued to grow, reaching OD600 nm values of ~1.0 in CDM and ~0.7 in TSB.

In shaking cultivation (Figure 1B), growth was monitored for 5 h with OD600 nm readings every 20 min. Under these conditions, wild-type and mutant strains exhibited nearly identical growth patterns across both media. Overall, these results indicate that bacterial fitness was influenced primarily by the cultivation condition (static vs. shaking), rather than by the culture medium composition.

2.2. Capsule Deficiency Alters Surface Charge and Size

Bacterial adhesion to surfaces depends not only on the exposure of adhesion structures, such as fimbriae, but also on electrostatic, hydrophobic, van der Waals forces, and Brownian motion interactions [18]. In this way, zeta potential measurements were performed, providing important information to better understand the surface characteristics of the bacteria. Figure 2 shows that strain MKP103 has a larger hydrodynamic size compared with the non-encapsulated strain KP07107, which may be related to the presence of the capsular polysaccharide. However, the mutant strain exhibits a more negative zeta potential than the encapsulated wild-type strain.

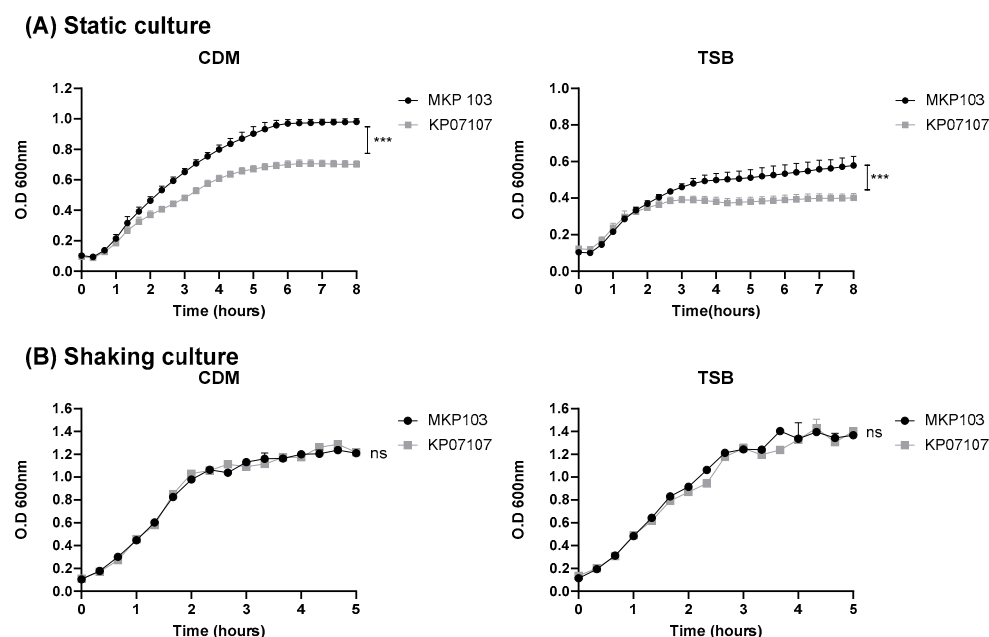


Figure 1. Bacterial Growth Curve. Strains MKP103 and KP07107 were inoculated into fresh culture medium (CDM or TSB) under two cultivation conditions: static (A), monitored for 8 h, and shaking at 200 rpm (B), monitored for 5 h. Growth was measured as optical density at 600 nm (OD₆₀₀). Statistical analysis was performed using a two-way ANOVA mixed-effects model (REML). Differences were considered significant when *** $p < 0.001$; ns: indicates no statistical significance.

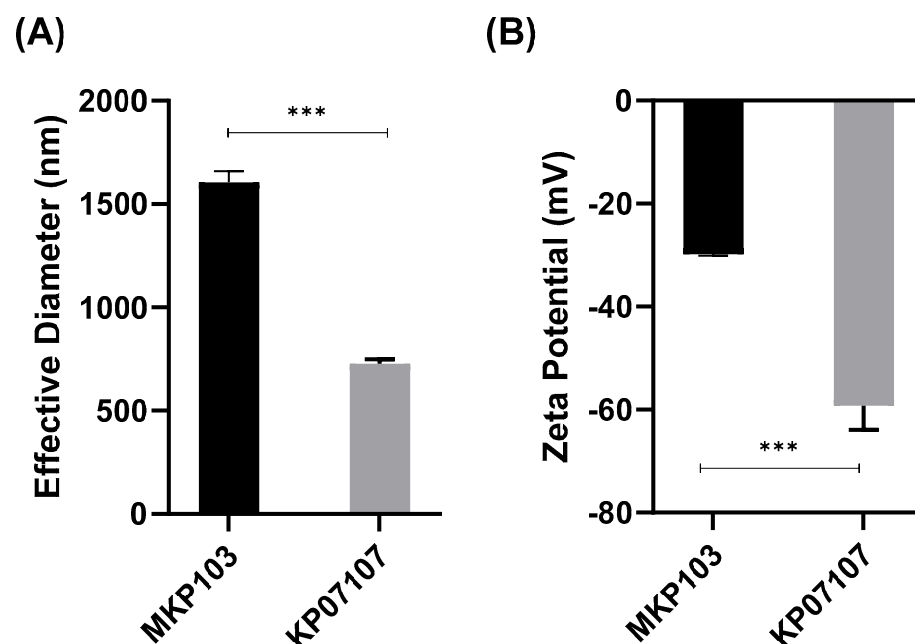


Figure 2. Comparison of the Effective Diameter (A) and zeta potential (B) between the wild-type and mutant strains. The graph shows the effective diameter and surface charge that was determined by calculating the zeta potential of the bacterium with capsule (MKP103) and without capsule (KP07107). Statistical analysis was performed using Student's *t*-test that *** = $p < 0.0001$.

2.3. Biofilm Formation on Abiotic Surface and Cell Substrate

Given the observed differences in growth patterns for planktonically grown bacteria, we next investigated whether this disparity would influence biofilm formation. The total cellular density in each well was determined, as well as the fraction of cells forming the biofilm, this allowed us to estimate the proportion of adherent (biofilm) cells relative to the

total bacterial population at 24, 48, and 72 h (Figure 3). The total cellular and biofilm formation were quantified using a plate reader at 600 nm and 590 nm, respectively, with biofilms being stained using crystal violet. The MKP103 strain consistently exhibited significantly higher total population than KP07107. However, crystal violet staining revealed an inverse pattern in biofilm formation: the capsule-deficient KP07107 strain produced greater biofilm biomass than MKP103 at all time points.

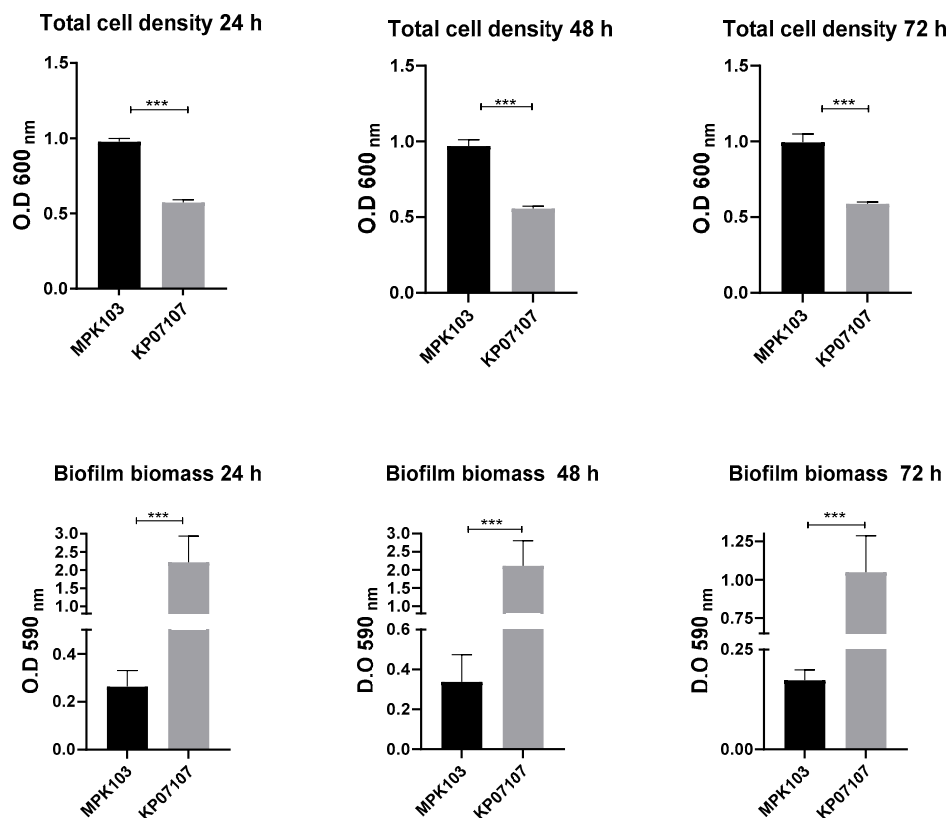


Figure 3. Biofilm formation on an abiotic surface. Biofilm formation was evaluated in 96-well plates after 24, 48, and 72 h. Subsequently, the absorbance of the total cell density was measured at 600 nm, and biofilm biomass was evaluated using crystal violet at 590 nm. Statistical analysis was performed using Student's *t*-test, where *** = $p < 0.0001$.

Despite the MKP103 strain exhibiting a higher total cell density in the well, only a fraction of the cells adhered. This demonstrates that greater planktonic growth does not necessarily translate into stronger surface attachment. Instead, the absence of the capsule seems to favor this process, suggesting that the capsule may play an inhibitory role.

A similar pattern was observed when measuring viable organisms (Figure 4). Viable plate counts were performed from both the attached biofilm growth and the planktonic supernatant in each well. After 24 h, the wild-type strain MKP103 exhibited higher cell density in the planktonic supernatant than the mutant strain. However, in the biofilm, this trend was opposite, with significantly more cells recovered for the capsule-deficient strain. At 48 h and 72 h, no significant differences were observed in planktonic cells between the strains, but biofilm-associated bacteria continued to be higher in the capsule-deficient strain. These results reinforce the findings of the crystal violet assay, confirming the enhanced biofilm-forming capacity of the capsule-deficient strain.

Given the greater ability of the capsule-deficient strain to form biofilms on abiotic surfaces, the experiment was repeated in a model that more closely resembles infection of host tissue, using respiratory (H292) and bladder (T24) epithelial cell lines as substrates for biofilm formation. As shown in Figure 5, the number of colony-forming units (CFU)

from biofilms formed on H292 cells by the capsule-deficient strain was 1.6-fold higher than with the encapsulated strain. On T24 cells, the difference was even more pronounced, with a 3-fold increase in CFUs measured from the capsule-deficient strain. It is worth noting that biofilm formation on abiotic surfaces and T24 cells were essentially similar, whereas a considerably higher CFU recovery was observed from biofilms formed on H292 cells. These results demonstrate a negative correlation between capsule expression and biofilm formation. Furthermore, the findings obtained with cellular substrates corroborate those observed on abiotic surfaces, indicating that the absence of the capsule enhances biofilm development in both contexts.

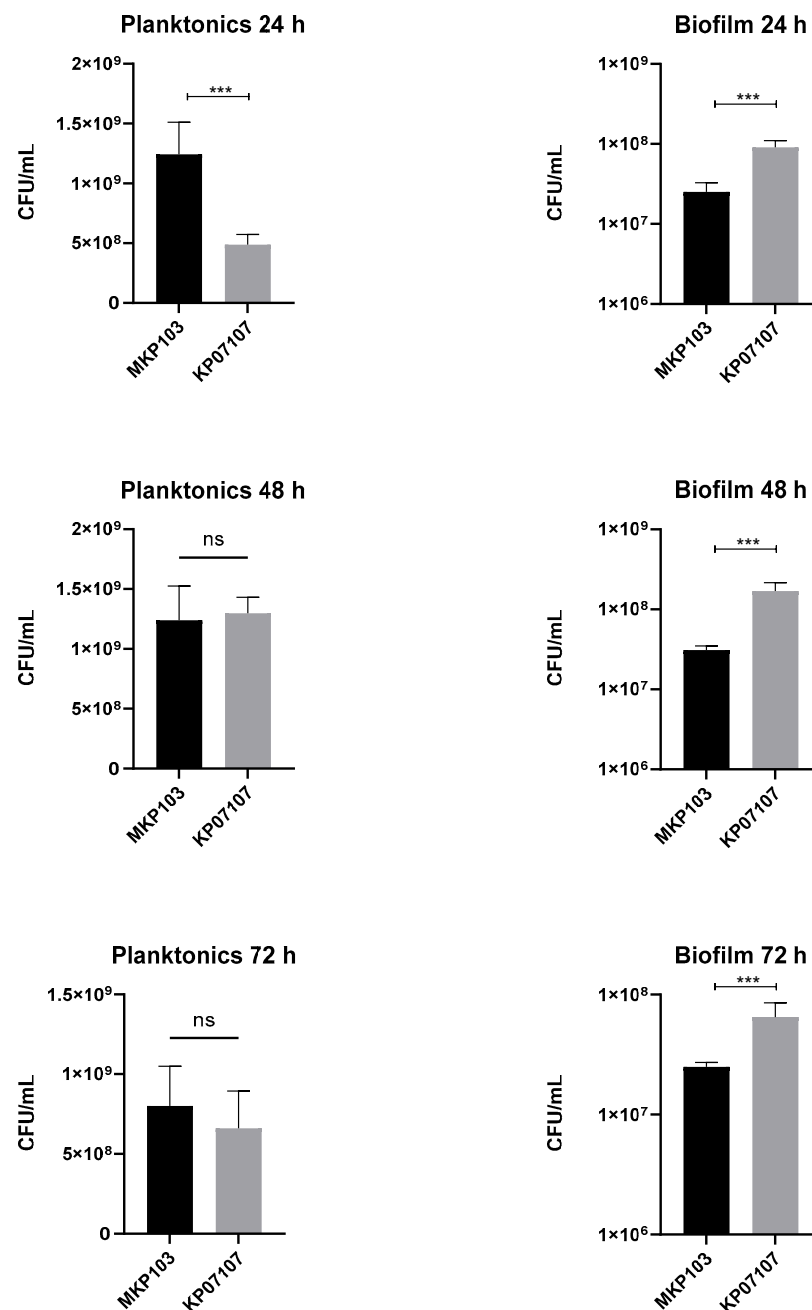


Figure 4. Colony recovery from planktonic cells and biofilm formed on abiotic surfaces. MKP103 and the *wza* mutant were grown in CDM medium in 24-well plates for 24, 48, and 72 h. Both planktonic and biofilm-associated cells were quantified by counting recovered colonies. Statistical analysis was performed with Student's *t*-test, in which *** = $p < 0.0001$ and ns = not significant.

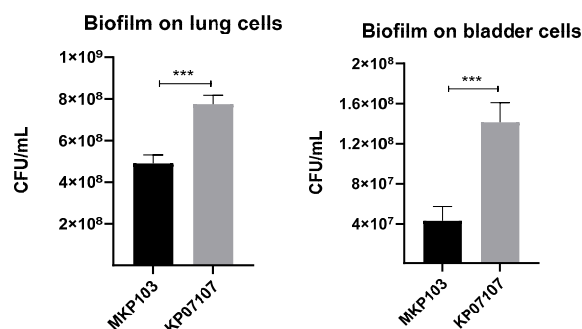
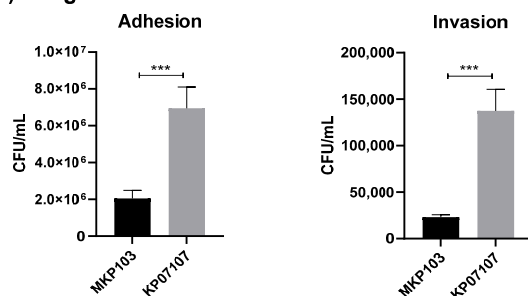


Figure 5. Biofilm formation on cell substrate. Wild-type and mutant strains were added to 24-well plates containing previously fixed lung cell line (H292) or bladder cell line (T24). The graph shows the number of colony-forming units recovered from the formed biofilm. Statistical analysis was performed with Student's *t*-test, in which *** = $p < 0.0001$.

2.4. The Lack of Capsular Polysaccharide Benefits Cell Adhesion and Invasion

To investigate the role of the polysaccharide capsule in bacterial colonization, specifically adhesion and cellular invasion, host–pathogen interaction assays were conducted using the same epithelial cell lines previously employed in the *in vitro* biofilm model. Consistent with the biofilm assays, the capsule-deficient strain exhibited a significantly greater ability to adhere to and invade both cell lines compared to the encapsulated strain (Figure 6). These findings support the notion that the absence of the capsule not only promotes adhesion and biofilm formation but also enhances cellular invasion. This phenomenon may be attributed to the fact that the capsule masks surface structures such as fimbriae, thereby reducing bacterial adhesion efficiency [17].

(A) Lung cells



(B) Bladder cells

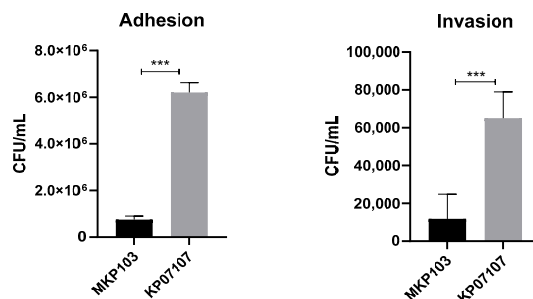


Figure 6. Colony-forming units of wild and mutant strains after adhesion and invasion assay in cells. Confluent lung and bladder cells were added to 12-well plates, and adhesion and invasion assays were performed using both cell lines. (A) The graphs show colonies recovered from lung cell adhesion and invasion. (B) The graphs show colonies recovered from bladder cell adhesion and invasion. Statistical analysis was performed using Student's *t*-test, in which *** = $p < 0.0001$.

2.5. The Presence of the Capsule Contributes to the Formation of Intracellular Bacterial Communities

Following cellular invasion, bacteria may form intracellular bacterial communities (IBCs), structures in which they replicate within host cells until the cytoplasm is filled. Upon maturation, these communities disperse to colonize new niches. This process is particularly relevant in urinary tract infections; therefore, T24 bladder epithelial cells were used in this experiment [19]. Figure 7 presents the results of the intracellular replication assay. For better comparison, the data on bacterial internalization previously shown in Figure 6B were included here as Figure 7A, allowing direct evaluation of bacterial uptake at 4 h (Figure 7A) and intracellular replication at 24 h (Figure 7B). In addition to assessing internalization, this experiment evaluated the bacteria's ability to persist and replicate within host cells over time. The capsule-deficient strain showed approximately a 4-fold increase relative to the initial number of bacteria after 24 h, whereas the encapsulated wild-type strain exhibited an approximately ~33.6 increase. These data indicate that both strains can replicate intracellularly, but at different rates.

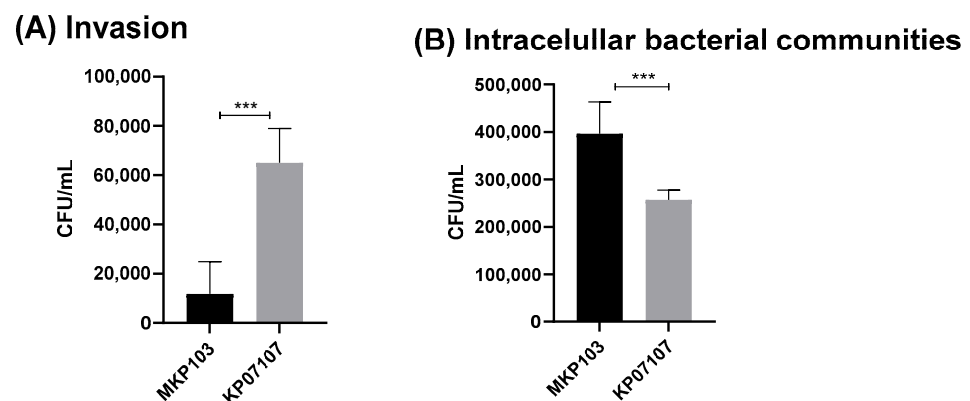


Figure 7. Colony-forming units of wild-type and mutant strains after intracellular replication assay in T24 cells. **(A)** Bacterial internalization after 4 h of infection in T24 cells. **(B)** Assays were conducted on T24 cells to evaluate the formation of intracellular bacterial communities (IBC) by wild-type and mutant *K. pneumoniae*. After 4 h of infection, the cells were treated with gentamicin (10 µg/mL) for 24 h, lysed with Triton X-100 and the lysate was plated for bacterial counting. Statistical analysis was performed using Student's *t*-test that *** = $p < 0.0001$.

Interestingly, the results contrasted with those observed for adhesion, biofilm formation, and invasion: after 24 h, a higher number of viable intracellular bacteria were recovered from the encapsulated strain compared to the capsule-deficient strain. This finding suggests that, while the absence of the capsule enhances initial interactions with host cells, while the capsule may confer an advantage for intracellular survival and replication.

3. Discussion

The emergence of *K. pneumoniae* strains that have become hypervirulent or acquired antibiotic resistance due to genetic modifications has raised serious public health concerns [2]. This scenario highlights the urgent need to better understand the pathogenic mechanisms of *K. pneumoniae*. In this study, we focused on one of its most important virulence factors, the polysaccharide capsule. Assays for glucuronic acid quantification (the main component of the capsule) and negative staining were performed to confirm the capsule-deficient phenotype. The results, presented in the Supplementary Material, confirmed the disruption of capsule expression in the mutant.

Next, two different culture media (CDM and TSB) and different growth conditions were analyzed to better understand bacterial fitness. The results showed that capsule loss

affected bacterial growth only under static conditions, in which the mutant strain entered the stationary phase earlier than the wild type. Under shaking conditions, however, the absence of the capsule did not influence growth, indicating that the cultivation method, rather than the medium, is the main determinant of bacterial fitness. These findings help to clarify controversies in the literature regarding the variable effects of the capsule on *K. pneumoniae* growth. Similarly, Zierke et al. [9], analyzed bacterial cell morphology and showed that deletion of *wza* leads to capsule loss and affects growth in TSB. Previous studies have shown that different mutations in capsule biosynthesis can reduce growth under static conditions [20], in nutrient-poor media, capsulated strains have a growth advantage while in rich media this difference disappears or may even reverse [21], and that under shaking conditions, the capsule impacts bacterial aggregate formation [22]. Therefore, our data reinforces that multiple experimental factors can influence the impact of the capsule, which exerts a modulatory effect depending on the cultivation conditions. This behavior may explain the discrepancies observed in the literature regarding its contribution to bacterial growth and organization in biofilms.

In the literature, studies have described the role of the capsule in biofilm formation, reporting that *wza* mutants produce less biofilm than wild-type strains, suggesting a positive correlation between capsule presence and biofilm formation [23,24]. Interestingly, our results differ from some previous reports, as the Δwza exhibited more pronounced biofilm formation than the encapsulated wild-type strain, both on abiotic surfaces and epithelial cells. This consistent phenotype indicates that it is a biologically relevant effect. Although the encapsulated strain MKP103 showed higher planktonic cell density, this did not translate into increased biofilm formation. On the contrary, the absence of the capsule promoted adhesion and biomass accumulation, as evidenced by the crystal violet assay and by the higher recovery of viable bacteria from biofilms on both abiotic surfaces and cellular substrates, confirming that the capsule acts as a negative modulator of biofilm formation, even in physiologically contexts. These results align with studies in *K. pneumoniae* and *A. baumannii*, in which acapsular mutants also showed increased biofilm formation. In *K. pneumoniae*, this effect has been associated with the exposure of type 3 fimbriae, which is normally masked by the capsule [25].

Consistently, the capsule-deficient strain KP07107 exhibited higher adhesion and invasion of epithelial cells compared to the encapsulated MKP103, corroborating the biofilm findings and indicating that capsule absence promotes not only biofilm formation but also direct interaction with host cells. This effect is likely due to capsule masking adhesion structures, such as fimbriae, thereby reducing adhesion and invasion efficiency. Our results agree with those of Tan et al., who observed increased adhesion in *wcaJ* and *wza* mutants, which was reversed upon complementation of the deleted genes [26]. Similarly, Zierke et al. reported increased internalization of the Δwza in epithelial cells compared to the wild-type strain [9].

Studies in pathogens, such as uropathogenic *E. coli* and *A. baumannii*, have shown that acapsular mutants adhere more to host cells but are more susceptible to phagocytosis and exhibit reduced intracellular replication capacity [27,28]. These findings highlight the dual role of the capsule: while it limits adhesion and biofilm formation, it protects against host clearance. In our model, we observed that both the encapsulated strain and the *wza* mutant were able to persist and replicate intracellularly, forming intracellular bacterial communities (IBCs), to different extents. The encapsulated strain MKP103 exhibited superior intracellular replication, suggesting that although capsule absence favors initial interactions with host cells (adhesion, biofilm formation, and invasion), capsule presence provides an advantage for intracellular persistence and replication, possibly protecting bacteria within the intracellular environment. This adaptation may facilitate colonization of new niches,

indicating that capsule loss does not prevent persistence but can modulate intracellular adaptation strategies.

In addition to the phenotypic effects, we performed further physicochemical characterizations to investigate the impact of the capsule on bacterial surface properties. Our results showed that the capsulated strain MKP103 has a larger hydrodynamic size, likely due to the presence of the polysaccharide capsule, whereas the acapsular mutant KP07107 exhibits a more negative zeta potential. These findings indicate that the capsule modulates both the apparent size and surface charge of the bacteria, suggesting a direct role in regulating adhesion to different substrates. These data, not previously reported in the literature, can be interpreted based on classical DLVO theory (Derjaguin, Landau, Verwey, Overbeek) [29], which describes physicochemical interactions at the bacteria–surface interface. Although similar charges promote initial repulsion, bacterial adhesion occurs through the reduction in the system’s total free energy, overcoming this barrier and allowing specific interactions, such as those mediated by fimbriae. By coupling infection assays with surface physicochemistry, we provide mechanistic evidence that the capsule alters electrostatic interactions at the bacteria–host interface, thereby modulating accessibility of fimbriae and other adhesins.

Regarding the limitations of this study, one notable constraint is the absence of a complemented mutant strain, which would have strengthened the interpretation of the phenotypic differences observed. Additionally, the adhesion, invasion, and intracellular bacterial community (IBC) formation assays conducted in T24 and H292 cells were performed under static conditions, with direct contact between bacteria and host cells. While this setup offers a controlled environment to study host–pathogen interactions, it does not fully replicate the complexity of *in vivo* settings, where hydrodynamic forces, immune components, and tissue architecture influence bacterial behavior. Therefore, *in vivo* experiments are essential to determine whether the *in vitro* observations translate into physiologically relevant outcomes; however, the consistency across multiple independent assays, including physicochemical characterization, mitigates this limitation.

4. Methods and Materials

4.1. Bacterial Strains

The bacterial strains used in this study were acquired from the Manoil Laboratory at the University of Washington and are listed in Table 1. The MKP103 strain is derived from the strain, KPNIH1, a clinical ST258 isolate from a hospital outbreak (<https://www.ncbi.nlm.nih.gov/bioproject/73191>, accessed on 1 November 2025). Mutagenesis was performed with a transposon resulting in the deletion of the carbapenemase gene and generating resistance to chloramphenicol (transposon T30). The mutant was generated through transposon-transposase [30]. Both strains were grown in Chemically Defined culture Medium (CDM) [31].

4.2. Quantitative Capsule Processing Through Glucuronic Acid Extraction and Measurement

Both strains were grown in CDM culture medium (chemically defined medium) [31] up to an optical density (OD) of 0.4–0.6. After reaching OD, 2 mL of the culture was centrifuged and resuspended in 500 µL of fresh CDM, followed by 250 µL of detergent *Zwittergent* 1% prepared in 100 mM citric acid (pH 2.0) was mixed and incubated at 50 °C for 20 min. After incubation, the tube was centrifuged at 14,000 rpm for 5 min, and then, 400 µL of the supernatant was transferred to a new tube and 1 mL of absolute ethanol was added and incubated at 4 °C for 20 min. Afterwards, the tube was again centrifuged at 14,000 rpm for 5 min, the supernatant was discarded, and the pellet was left to dry at room temperature for approximately 15 min. Then, the dry pellet was resuspended in 100 µL of *Milliq* sterile water, followed by addition of 600 µL of tetraborate at 12.5 mM diluted

in sulfuric acid (H_2SO_4). Next, the tube was vortexed and incubated in a water bath at 95°C for 5 min and left to cool in room temperature for 10 min. Finally, 20 μL of a solution containing 0.15% 3-hydroxydiphenyl prepared in sodium hydroxide (NaOH) 0.5% was added and absorbance was measured in spectrophotometer at 540 nm to determine the concentration of glucuronic acid from a standard curve.

4.3. Qualitative Assay of Polysaccharide Capsule—Negative Staining

To observe the capsule in the bacterium, the negative staining technique was performed. In this protocol, the dye is acidic and has a negative charge, as well as the capsule, so repulsion occurs with the bacterial surface and does not penetrate the cell, which generates the formation of a white halo around the cell, evidencing the capsular polysaccharide [32]. The MKP103 (wild-type) and KP07107 (*wza* mutant) strains were cultured in CDM medium up to $\text{OD}_{600\text{ nm}}$ 0.4–0.6 at 37°C and 200 rpm, 5 μL of the culture was added with 5 μL of Indian ink (*v/v*) on a microscopy slide, homogenized, after homogenization, the mixture was visualized under an optical microscope.

4.4. Growth Curves

Bacteria stored at -80°C were thawed, inoculated on LB agar medium, and incubated at 37°C under static conditions overnight. Bacterial growth curves were then produced from both static and shaking cultures. For static cultivation, colonies from overnight culture on plates were first cultured in fresh CDM or Tryptic Soy Broth (TSB) at 37°C and 200 rpm until reaching an optical density (OD_{600}) of 0.4–0.6. Cultures were then diluted in fresh medium in 96-well polystyrene plates to an initial $\text{OD}_{600\text{ nm}}$ of 0.1. Bacterial growth was monitored for 8 h at 37°C under static conditions, with $\text{OD}_{600\text{ nm}}$ measurements taken every 20 min using an automated spectrophotometer reader (Glomax®-Multi+ Microplate Multimode Reader (Promega Corporation, Madison, WI, USA). (GeneQuant™; Amersham Biosciences, Amersham, UK)

For shaking cultivation, colonies from overnight LB plates were inoculated into fresh medium at a low initial OD_{600} and incubated at 37°C with shaking (200 rpm). OD_{600} measurements were manually recorded every 20 min for 5 h, using a spectrophotometer reader (GeneQuant™; Amersham Biosciences, Amersham, UK). Measurements were continued up to 5 h to confirm growth stabilization under shaking conditions.

4.5. Physicochemical Characterization

Zeta potential analysis was performed to evaluate the surface charge of *K. pneumoniae* strains as described in [33], the experiment was conducted using a Zeta Plus Potential Analyzer (Brookhaven Instruments Corporation, Holtsville, NY, USA). The strains were grown on LB agar overnight and a bacterial suspension containing 1.5×10^8 bacteria/mL was prepared in milli-Q water, followed by centrifugation at 8000 rpm for 10 min and resuspension of the pellet in 2 mL of 1 mM NaCl. Particle size was first measured using the Particle Sizing Software 4.0 (Brookhaven Instruments Corporation, Holtsville, NY, USA). Following this, a 500 μL aliquot of the bacterial suspension was used for zeta potential analysis with the Zeta Plus Software version 4.0 (Brookhaven Instruments Corporation, Holtsville, NY, USA). The bacterial hydrodynamic size and the zeta potential were reported as the mean of 10 independent measurements.

4.6. Biofilm on Abiotic Surface Crystal Violet and Colony Recovery

Biofilm formation was quantified by a 96-well microplate biofilm formation assay. The formation of biofilm by the bacteria was evaluated using the protocol described by O'Toole and Kolter [34]. Briefly, both strains were cultured until *mid-log* phase and diluted to an initial $\text{OD}_{600\text{ nm}}$ of 0.1 in fresh CDM medium. Next, 200 μL of this dilution was inoculated

in flat-bottomed polystyrene microplates and incubated at 37 °C for 24, 48 and 72 h. The wells were washed with phosphate-buffered saline (PBS) solution, and the adhered bacteria were stained with 0.1% crystal violet for 15 min. The dye was discarded, the biofilm washed three times, and crystal violet that adhered to the biofilm was dissolved in 200 µL of 30% acetic acid. Finally, the absorbance was read in a spectrophotometer at 590 nm.

To assess viable bacteria from the biofilm, 24-well plates were used to grow biofilms as described above. After 24, 48, and 72 h, the adhered bacteria were dispersed using a 200 µL tip and subjected to an ultrasonic bath for 20 s. The contents of each well, including the planktonic fraction, were transferred to a microtube and vortexed for 10 s before serial dilution. Ten microliters of each dilution were plated on LB agar. After overnight incubation, colony-forming units (CFUs) were counted.

4.7. Biofilm on Cell Substrate

For this analysis, the methodology described by Marks, Parameswaran and Hakansson was used [31], which allows mimicking the conditions found by the bacterium in its natural niche for biofilm formation.

Pulmonary mucoepidermoid carcinoma cells (NCL-H292) and transitional cell carcinoma of the bladder (T24) were both thawed and transferred to ice and then incubated in a water bath at 37 °C. The cells were then transferred to a cell culture bottle containing RPMI-1640 culture medium supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin and amphotericin (antibiotic and fungicide), 1% sodium pyruvate, and 1% sodium bicarbonate (for H292 cells) or McCoy's 5A medium supplemented with 10% FBS, 1% sodium pyruvate, and 1% antibiotic and fungicide (for T24 cells). The cells were incubated in an incubator with 5% CO₂ at 37 °C, detached when confluent and used to seed 24-well polystyrene plates. After reaching confluency, the cells were washed twice with PBS and fixed with 4% paraformaldehyde (prepared in PBS, pH 7.2) and stored with PBS until use.

Before using the cell plate as a substrate in the co-culture, the wells were washed twice with PBS. Bacteria were adjusted to an OD_{600 nm} of 0.1 in a final volume of 1 mL per well and incubated for 60 h at the nasopharyngeal temperature 34 °C in 5% CO₂, with culture medium replaced every 24 h. After 60 h, the biofilm supernatant was removed, and the plate was washed to remove the planktonic bacteria, while the bacteria incorporated in the biofilm (bound to the human cells in the wells) were dispersed using a 200 µL tip and subjected to an ultrasonic bath for 20 s. Then, serial dilution and plating were performed to count the recovered colony-forming units (CFU). The number of CFU recovered was compared between the two strains to assess the biofilm-forming capacity.

4.8. Adhesion and Invasion Assay in H292 and T24 Cells

To explore other stages of the infectious process, adhesion and invasion assays were conducted in bladder (T24) and respiratory epithelial cells (H292), using the method described by [35]. Initially, the cells were seeded and grown in 12-well plates, as previously described above (point 4.7), until they reached confluency (approximately 4×10^5 cells per well).

Bacteria were cultured to *mid-log* phase (OD_{600 nm} of 0.4), washed three times with PBS (centrifuged at 10,000 rpm for 3 min), concentrated 10 times using RPMI medium or 5A McCoy medium without antibiotics for H292 and T24, respectively. Bacteria were added in a 200:1 multiplicity of infection (MOI) to plates with H292 or T24 cells and incubated as follows: For the adhesion assay, the infection period was 30 min at 37 °C, and the cells were then washed three times with saline Dulbecco phosphate buffer (DPBS) and lysed for 10 min at room temperature after addition of Triton X-100 (0.1% in PBS; Sigma, St. Louis,

MI, USA). To obtain the CFU count, serial dilutions were performed with lysate and plated on LB agar medium.

For the invasion assay, the initial infection period was 4 h, the cell monolayer was then washed once with DPBS buffer, incubated again for 1 h with pre-warmed culture medium containing the antibiotic gentamicin (25 µg/mL) to eliminate extracellular bacteria. The cells were then washed three times with DPBS and lysed with Triton X-100 0.1%, diluted and plated on LB agar medium to count the recovered colonies of bacteria present in the intracellular environment.

4.9. Intracellular Survival and Replication in Epithelial Cells

To analyze the ability of *K. pneumoniae* to replicate and form an intracellular bacterial community (IBC) in T24 cells [35], the invasion assay described above was performed, with some modifications. After the 4 h infection period, the cells were washed with DPBS buffer and incubated for 24 h in 5A McCoy medium containing 10 µg/mL of the antibiotic gentamicin. After this incubation, the cells were washed with DPBS, lysed with 0.1% Triton X-100 and serial dilutions of the lysate were plated on solid LB medium for colony counting.

4.10. Statistical Analysis

The Prism GraphPad software (version 8.01) was used to prepare the graphs and perform statistical analysis. The comparison between wild type and mutant bacteria was made by Student's *t*-test or a two-way ANOVA mixed-effects model (REML). A *p*-value ≤ 0.05 is considered statistically significant.

5. Conclusions

Our findings contribute to reconciling apparently conflicting reports regarding the role of the capsule in *K. pneumoniae* pathogenesis. By integrating host–cell assays with surface physicochemistry, we provide evidence for a context-dependent, dual role of the capsule. In early stages of infection, capsule absence exposes adhesive structures and favors electrostatic interactions, thereby enhancing adhesion, biofilm formation, and epithelial invasion. However, in later stages, capsule presence becomes advantageous by protecting bacteria during intracellular persistence. This trade-off model emphasizes that capsule expression is not simply beneficial or detrimental but dynamically modulates virulence depending on the infection stage. Importantly, this conceptual framework highlights capsule regulation as a potential therapeutic target, whereby modulating capsule expression could selectively impair either colonization or persistence.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27052169/s1>.

Author Contributions: M.E.S.G., G.D., R.V.C., I.C., L.F.C.F., A.P.H., R.G., M.D. and T.R.C. have drafted the manuscript. M.E.S.G., G.D. and R.V.C. conducted experiments. M.E.S.G. and T.R.C. produced the figures; M.E.S.G., G.D., R.V.C., I.C., L.F.C.F., A.P.H., M.D. and T.R.C. have analyzed the data. A.P.H., R.G., M.D. and T.R.C. have revised the text. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by FAPESP by the grants: 2019/23566-6, 2022/15111-1, and 2024/02517-5. Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq): 400099/2022-5 and 139133/2025-9. Vetenskapsrådet (Swedish Research Council): grant numbers: 2021-06050 and 2022-01296. Österlund Foundation, research grant. Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) 88887.994588/2024-00. We would like to thank Casa Nossa Senhora da Paz for financial support with the publication fee.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study and its Supplementary Materials are available in the Open Science Framework (OSF) repository at <https://doi.org/10.17605/OSF.IO/GZ37T>, accessed on 21 October 2025 [36].

Conflicts of Interest: 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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