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KpsS1 Mediates the Glycosylation of Pseudaminic Acid in *Acinetobacter Baumannii*

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Pseudaminic acid (Pse) is found in the polysaccharide structures of the cell surface of various Gram-negative pathogenic bacteria including *Acinetobacter baumannii* and considered as an important component of cell surface glycans including oligosaccharides and glycoproteins. However, the glycosyltransferase that is responsible for the Pse glycosylation in *A. baumannii* remains unknown yet. In this study, through comparative genomics analysis of Pse-positive and negative *A. baumannii* clinical isolates, we identified a potential glycosyltransferase, KpsS1, located right downstream of the Pse biosynthesis genetic locus. Deletion of this gene in an Pse-positive *A. baumannii* strain, Ab8, impaired the glycosylation of Pse to the surface CPS and proteins, while the gene knockout strain, Ab8Δ*kpsS1*, could still produce Pse with 2.86 folds higher amount than that of Ab8. Furthermore, impairment of Pse glycosylation affected the morphology and virulence potential of *A. baumannii*, suggesting the important role of this protein. This study will provide insights into the further understanding of Pse in bacterial physiology and pathogenesis.

Introduction

Pseudaminic acid (Pse), also known as 5,7-diacetamido-3,5,7,9-tetradeoxy-L-glycero-L-manno-nonulosonic acid,[1] is found in the polysaccharide structures of the cell surface of various Gram-negative pathogenic bacteria. Pse is an important component of cell surface glycans including oligosaccharides and glycoproteins, for example, LPS O-antigens, capsular polysaccharides, pili, and flagellin.[1] Flagella glycans in *Campylobacter jejuni* were found to be highly modified with Pse and legionaminic acid[2] and the study demonstrated that Pse significantly affected flagellum formation and function. Another study found that the flagella in *Helicobacter pylori* were post-translationally modified with Pse in FlaA and FlaB proteins. Insertion mutagenesis in genes related to motility, flagellar glycosylation, or polysaccharide biosynthesis resulted in a phenotype that was non-motile and had no structural flagella filament.[3] These findings demonstrated that Pse is related to bacterial virulence. The process and enzymes for biosynthesis of Pse have been well characterized starting from the substrate UDP-GlcNAc to the product cytidine-5′-monophosphate (CMP)-Pse5Ac7Ac.[4] However, there is little known about how Pse is glycosylated to proteins and polysaccharides. In this study, we performed the biological investigation of the KpsS1 protein to understand the glycosylation process of Pse. During the course of our study, a report showed that KpsS1 was able to glycosylate Pse in an *in vitro* enzymatic reaction.[5] Our study, however, provides directed evidence of the *in vivo* role of this enzyme in *A. baumannii*, which complements very well with the recent report.

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Materials and Methods

Detection of Pse in *kpsS1* Knockout Strains

Bacterial strains, including wild type and knockout, were cultured in LB broth at 37 °C overnight with shaking at 250 rpm. The strains were inoculated at 1 : 100 fraction into fresh LB broth and incubated at 37 °C with shaking at 250 rpm until OD_{600} reached 0.6. The bacterial cells were pelleted by centrifuge at 5,000 rpm for 3 min. On the other hand, 2.5 μg mouse anti-Pse antibody[6] was mixed with 4 μg donkey anti-mouse IgG secondary antibody conjugated with Alexa Fluor® 647 (Abcam) in 400 μL PBS and incubated at room temperature for 1 h. After that, the bacterial cell pellet was resuspended in 200 μL premixed antibody with an addition of SYTO 9, a fluorescent nucleic acid stain and incubated in the dark at room temper-

ature for 1 h. The bacterial cells were washed twice with PBS and resuspended in 50 μL PBS. The image was taken and visualized using the Nikon Eclipse Ti2-E Live-cell Fluorescence Imaging System.

Detection of Conjugated Pse in Bacterial Cell

The glycosylated Pse was detected by western blot. Bacterial strains, including wild type and knockout, were cultured in LB broth at 37 °C overnight with shaking at 250 rpm. 2 mL of bacterial culture was collected and centrifuged to separate culture medium supernatant and bacterial pellet. The pellet was resuspended in 300 μL of PBS, lysed using sonication and centrifuged. The culture medium supernatant, cell lysates and cell supernatant of the wild type and knockout strains were boiled with SDS running buffer for 10 min and were subsequently separated by SDS-PAGE. Proteins were transferred to a PVDF membrane followed by blocking by skimmed milk for 1 h and incubated with mouse anti-Pse antibody at 4 °C overnight. The goat anti-mouse antibody was used as the secondary antibody. The signal was generated by HRP substrate and detected by chemiluminator.

Detection of Intracellular Pse

Bacterial strains, including wild type and knockout, were cultured in LB broth at 37 °C overnight with shaking at 250 rpm. After incubation, 3 mL of bacterial culture was pelleted by centrifugation, resuspended in 30 μL of PBS, and lysed using sonication. The cell debris was removed by centrifugation at 21,000×g for 20 min. To remove the remaining protein, a Amicon Ultra-0.5 (nominal molecular weight limit [NMWL] = 3000) centrifugal filter device was used to filter out the remaining protein. The cell lysate was then analyzed using a mass spectrometer to detect the intracellular Pse.

Scanning Electron Microscopy

To perform the scanning electron microscopy (SEM), overnight cultured bacteria of the two strains were first prepared and inoculated into fresh 6 mL LB broth in 1% inoculation, incubated at 37 °C with shaking until OD_{600} reached 0.4. After that, the bacterial culture was transferred into a 6-well plate containing a sterile ITO glass slide and incubated for 2 h. To fix the bacteria on the slide, the slide was first washed with PBS and fixed with 4% Paraformaldehyde (PFA) overnight at 4 °C. After fixing, the bacteria sample was washed with PBS and dehydrated by washing with 50%, 70%, 90% and 100% ethanol for 15 min each. Then, the slide was gold sprayed and observed under SEM.

Determination of the Role of KpsS1 in Virulence Expression in *A. baumannii* Using a Mouse Sepsis Model

Female C57BL/6 J inbred strains of mice (six to eight weeks old, ~20 g) were obtained from the Laboratory Animal Research Unit (LARU), City University of Hong Kong. Animals were handled in strict accordance with the Animals (Control of Experiments) Ordinance (Cap. 340), Hong Kong. All animal experiments were approved by the Animal Research Ethics Sub-Committee (ARESC) of City University of Hong Kong. Animals were housed under specific pathogen-free conditions during the experiment. All efforts were made to minimize animal suffering.

A mouse sepsis model was adopted to assess the difference in virulence level between the wild-type and gene knockout strains. The test strains were cultured overnight in LB broth and then diluted 1 : 100 in fresh LB broth, followed by incubation at 37 °C with shaking until OD_{600} reached 0.6. The bacteria cells were then pelleted by centrifugation at 6,500×g for 3 min; the cell pellet was washed with PBS three times, resuspended in PBS, and adjusted to an appropriate concentration. A total volume of 200 μL bacterial suspension containing 5×10^7 CFU bacteria was then injected intraperitoneally into 6–8 weeks old inbred C57BL/6 J female mice; each strain was tested in 10 mice. The survival rate of the mice was observed and recorded for 3 days post-infection at 12 h intervals. The bacterial load in different organs of the test mice was examined to determine the difference between the virulence level and survival rate of the test strains. A total volume of 200 μL bacterial suspension containing 5×10^7 CFU bacteria was injected intraperitoneally into 6–8 weeks old inbred C57BL/6 J female mice; each strain was tested in 4 mice. The infected mice were anesthetized at 12 h after being inoculated with bacteria. Blood and different organs including the spleen, kidney, lung, liver, and heart were removed aseptically from the mouse and were homogenized by using a homogenizer. The samples were serially diluted in 96 well plates and spread on LB agar plates; the number of bacterial colonies that formed were counted for determination of the bacterial load in different samples.

Results and Discussion

Upon development of chemical synthesis of Pse in our lab,[7–9] we conjugated the Pse to keyhole limpet hemocyanin (KHL) to have developed a monoclonal antibody that specifically recognizes Pse in *A. baumannii* and other bacteria.[6] *A. baumannii* is one of the leading pathogens responsible for death associated with drug resistance.[10] Pse can be found in the capsule polysaccharide of the *A. baumannii*. We have screened over 100 clinical *A. baumannii* strains using the monoclonal antibody developed and found that over 50% of the strains carried Pse. Comparative genomics were performed on Pse-positive (Ab191) and Pse-negative (Ab11854) strains, which enabled us to identify the genetic locus of Pse, which contains *psb*1 to *psb*6, also named as *pse*BCFGHI, six enzymes involved in Pse biosynthesis (Figure 1). We also identified a gene named, *wagF*, also

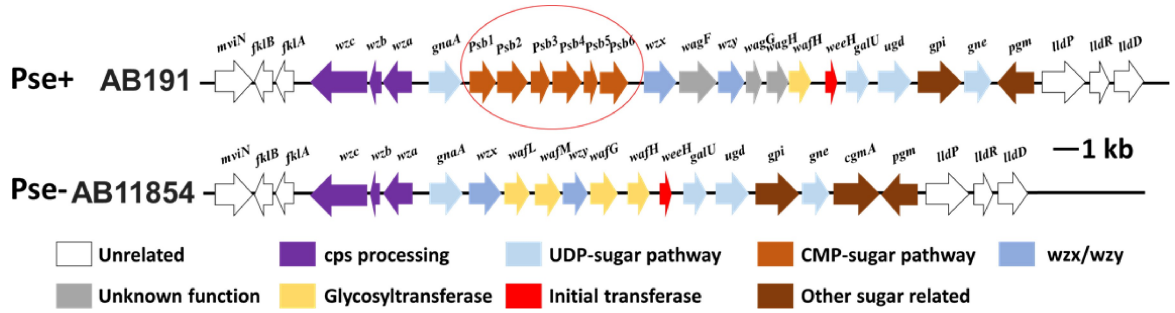

Figure 1. Genetic loci of Pse-positive and –negative A. *baumannii*. The *psb*1~6 region also named as *pse*BCFGHI, is responsible for Pse biosynthesis. WagF also named KpsS1 is the potential glycosyltransferase.

named *kpsS1*, which was located right downstream of the *pse* genetic locus. We hypothesized that this might be the glycosyltransferase that mediates the glycosylation of Pse to CPS and other proteins in *A. baumannii*.

To investigate the contribution of *kpsS1* to the Pse glycosylation, we utilized a CRISPR-Cas9/RecAb-based genome-editing platform to generated *kpsS1* gene knockout mutant in *A. baumannii* strain, Ab8, to obtain Ab8Δ*kpsS1*.[11] To check if the deletion of *KpsS1* affected the conjugation of Pse to the surface CPS of Ab8 strain, an anti-Pse antibody mentioned above was used to stain the CPS on the surface of bacteria. Our data showed that the wild-type strain, Ab8, could be stained by fluorescence signal by the anti-Pse antibody, whereas the knockout strain, Ab8Δ*kpsS1*, did not exhibited any fluorescence signal indicating that upon knock-out of *kpsS1*, no Pse could be conjugated to the CPS on the bacteria cell surface (Figure 2A).

To further confirm the loss of glycosylation of CPS and proteins in Ab8Δ*kpsS1*, we performed western blot to check the glycosylation of Pse in the bacteria cell. Our data showed that glycosylated CPS and proteins could be detected in Ab8 strain, with upper band representing CPS and lower band representing proteins, while there was no signal detected in the knock-out strain (Figure 2B). The result indicated that after knock-out of *kpsS1* gene, there was no Pse could be glycosylated to either CPS or proteins in Ab8.

To prove that KpsS1 is the glycosyltransferase, but not the enzyme involved in Pse biosynthesis, we set out to test the production of free Pse that could be produced by Ab8 and the knockout strains. The released Pse from *A. baumannii* was labeled with 1,2-diamino-4,5-methylenedioxybenzene.2HCl (DMB) for fluorophore detection by LC/MS. The MS result showed a corresponding peak as the DMB-Pse which could be detected in both Ab8 and Ab8Δ*kpsS1*, with higher density in the knockout strain. The data is consistent with the fact that after deletion of glycotransferase, the Pse could be produced and accumulated in Ab8Δ*kpsS1*, therefore leading to the high density of Pse in the knockout strain (Figure 3A&B). We further quantified the amount of Pse inside bacteria cell by LC/MS with results showing that the Pse intensity in the Ab8Δ*kpsS1* was 2.68 times higher than that of Ab8 further confirming the role of KpsS1 as the Pse glycosyltransferase (Figure 3C).

Next, we further investigated the effect of deletion of *kpsS1* gene on the morphological changes and virulence of *A. baumannii* in mice sepsis model. Scanning electron microscopy was used to investigate the morphology change after blocking the glycosylation of Pse to CPS. The knockout strain appeared flattened and sticked together when comparing to the wild type suggesting that glycosylation of Pse to CPS contributes to maintain the optimal morphology of *A. baumannii* (Figure 4A). To check if Pse contributed to the virulence of *A. baumannii*, a dose of $5x10^7$ CFU bacteria cells was injected into the C57BL/6 J mouse to initiate infection. The survival rate post-infection was observed and recorded at 12 h intervals. The survival rate of mice infected by wild-type *A. baumannii* was 80% after 12 h of infection but dropped to 0% 24 h post-infection (Figure 4B). However, all test mice infected by the *kpsS1* gene knockout strain remained viable throughout the experiment. The result indicates that glycosylation of Pse to CPS and proteins is essential for virulence expression in bacteria. To further investigate the effect of Pse glycosylation on bacterial invasion during the infection process, blood and various internal organs including the spleen, kidney, lung, liver, and heart were removed aseptically from the test mice. The bacterial count in each organ and the blood sample was recorded. Among the blood and internal organ samples tested, the bacterial load of the gene knock-out strain Ab8Δ*kpsS1* was much lower than that of the wildtype strain. Notably, the most significant difference in bacteria load was observed in blood, with that of the wild-type and gene knockout strain being ~10^9 and ~10^2 CFU/mL, respectively (Figure 4B).

Pse biosynthesis pathway has been well characterized,[4] however little is known about the transfer of Pse to other glycans to form capsule polysaccharide and Pse-glycosylated proteins. In this work, we identified a potential Pse glycosyl-transferase, KpsS1, and confirmed its role through *in vivo* biological characterization, which provides further evidence for the current *in vitro* functional study of this enzyme as a novel glycosyltransferase.

We discovered that the knockout strain has a significantly lower level of virulence in mice models. Both the survival rate and overall bacteria load in mice affected with Ab8Δ*kpsS1* decreased significantly compared to the wild type strain. A recent study showed that Pse on bacteria exopolysaccharide can bind to Siglec-10 receptor and suppress phagocytosis.[11] This results in the immune escape from the host. We determined that KpsS1 is crucial for the glycosylation of Pse on

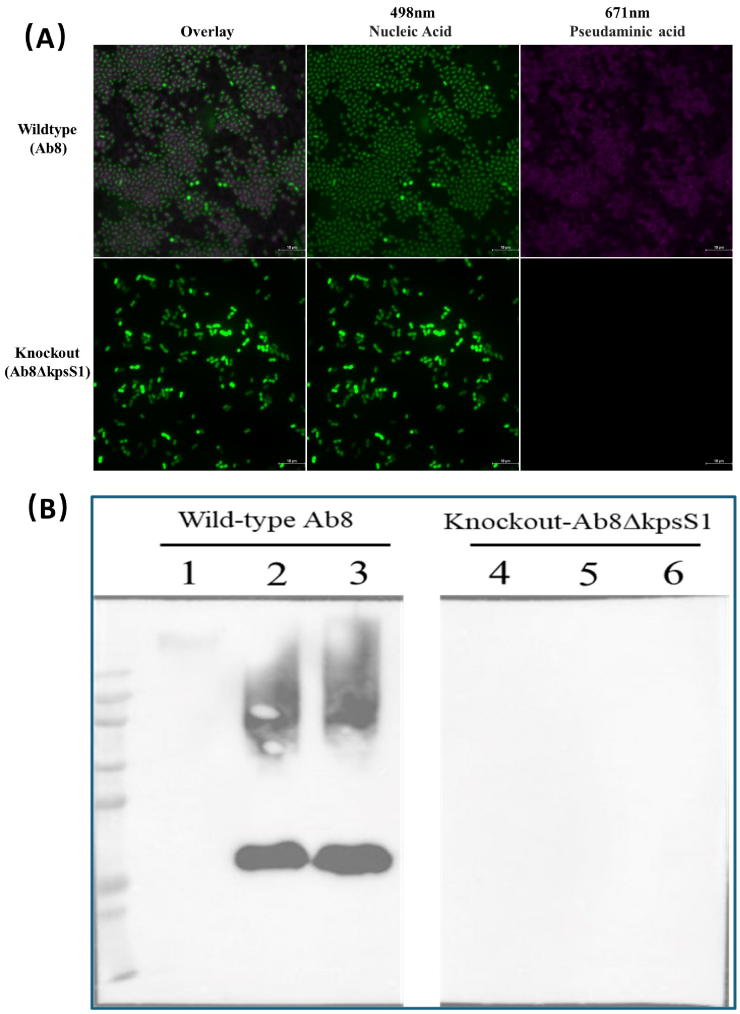

Figure 2. Detection of Pse on *A. baumannii* Ab8 strain and its *kpsS1* knockout mutant. (A) Fluorescence microscopy detection of Pse in live bacterial cells. (B) Western-blot detection of Pse on CPS and proteins. The upper band represents CPS and lower band represents glycosylated proteins. (Lane 1–3: The culture medium supernatant, cell lysates and cell supernatant of Ab8 strain. Lane 4–6: The culture medium supernatant, cell lysates and cell supernatant of Ab8Δ*kpsS1* strain.)

the exopolysaccharide known as CPS, the absence of Pse on CPS could potentially decrease its binding to the Siglec-10 receptor, consequently reducing the virulence level. Additionally, our study revealed that the glycosylation of Pse on CPS is responsible for maintaining its structural integrity, and its absence may result in morphological changes in bacterial cells. However, the precise mechanism by which Pse glycosylation affects virulence remains unclear, necessitating further investigation.

Conclusions

This study presents a pioneering investigation into the impact of Pse glycosylation in bacterial cells, a topic that has received limited attention thus far. Our research offers compelling evidence for the *in vivo* significance of the KpsS1 glycosyltransferase in *A. baumannii*. Through the use of a knockout strain, we unequivocally demonstrated that KpsS1 plays a critical role in the glycosylation of Pse within bacterial cells, leading to an elevated abundance of Pse within the cells. Furthermore, we established that the glycosylation of Pse influences the morphology of bacterial cells and that the presence of glycosylated Pse enhances virulence. This study significantly advances our understanding of the role of Pse in bacterial pathogenesis.

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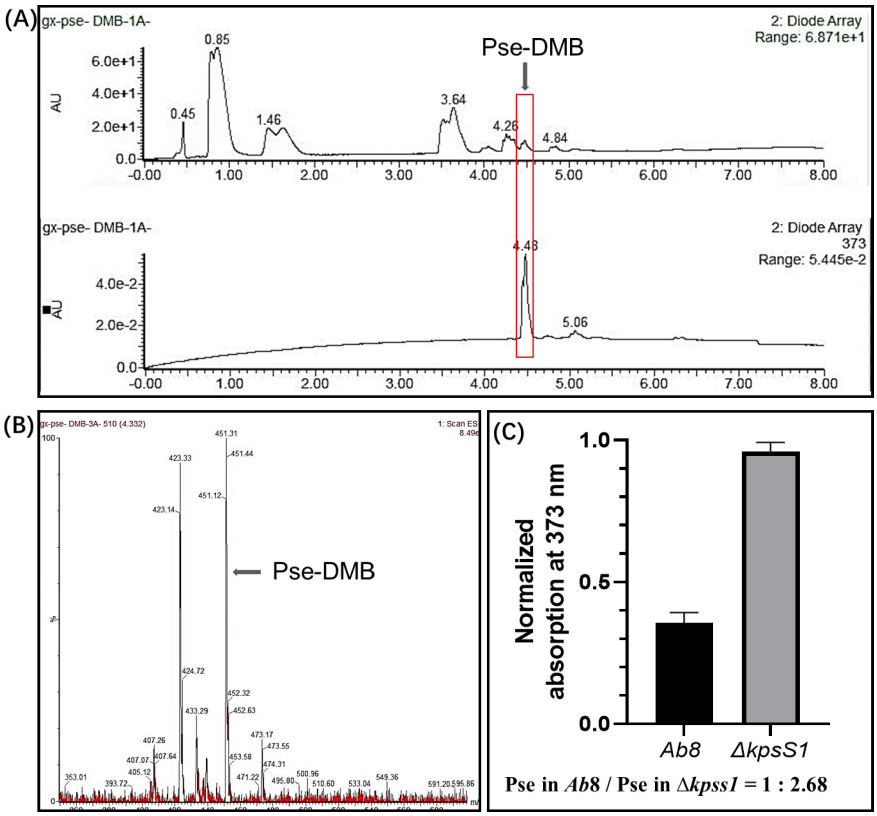

Figure 3. LC/MS analysis of intracellular Pse. (A) The UPLC spectrum of intracellular lysate. Upper panel is full spectrum at all wavelength; lower panel is full spectrum at 373 nm wavelength. Because 373 nm is the characteristic fluorescence excitation wavelength of the conjugated structure formed after derivatization of DMB, which is equivalent to the characteristic absorption wavelength. (B) The peak region of MS spectrum was shown. The peak is desired MS(M + H) of DMB-Pse as 451. (C) Pse intensity in Ab8 and Ab8Δ*kpsS1*.

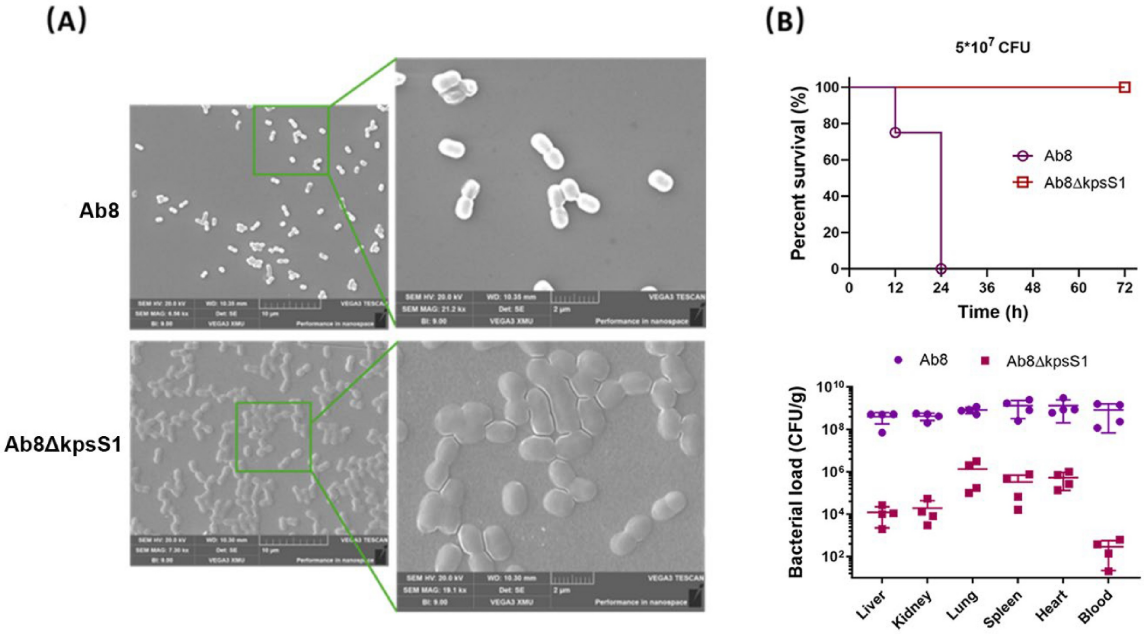

Figure 4. Contribution of Pse glycosylation to morphology and virulence of *A. baumannii*. (A) Scanning electron microscopy of wild type and knockout strain. The knockout strain shows morphology change comparing to the wildtype. (B) Survival rate of mouse sepsis model and bacterial load in blood and other organs. (Upper panel) Survival rate of mice infected with wild type and knockout strain in 72 h. (Lower panel) Bacterial load in blood and different organs in mice after 12 h of infection. A dose of 5×10^7 CFU bacteria cells was injected into the C57BL/6 J mouse to initiate infection. The survival rate post-infection was observed and recorded at 12 h intervals. Ab8, a clinical *A. baumannii* strain that produces Pse. Ab8Δ*kpsS1* is the gene deletion mutant of the Ab8 strain.

Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: *Acinetobacter baumannii* · Glycotransferase · KpsS1 · Pseudaminic acid · and Virulence

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