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The Retaining Pse5Ac7Ac Pseudaminyltransferase KpsS1 Defines a Previously Unreported glycosyltransferase family (GT118)

Abigail J. Walklett⁺, Emily K. P. Flack⁺, Harriet S. Chidwick, Natasha E. Hatton, Tessa Keenan, Darshita Budhadev, Julia Walton, Gavin H. Thomas,^{*} and Martin A. Fascione^{*}

In memory of Professor Paul A. Clarke

Abstract: Cell surface sugar 5,7-diacetyl pseudaminic acid (Pse5Ac7Ac) is a bacterial analogue of the ubiquitous sialic acid, Neu5Ac, and contributes to the virulence of a number of multidrug resistant bacteria, including ESKAPE pathogens *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. Despite its discovery in the surface glycans of bacteria over thirty years ago, to date no glycosyltransferase enzymes (GTs) dedicated to the synthesis of a pseudaminic acid glycosidic linkage have been unequivocally characterised in vitro. Herein we demonstrate that *A. baumannii* KpsS1 is a dedicated pseudaminyltransferase enzyme (PseT) which constructs a Pse5Ac7Ac- α (2,6)-Glc_p linkage, and proceeds with retention of anomeric configuration. We utilise this PseT activity in tandem with the biosynthetic enzymes required for CMP-Pse5Ac7Ac assembly, in a two-pot, seven enzyme synthesis of an α -linked Pse5Ac7Ac glycoside. Due to its unique activity and protein sequence, we also assign KpsS1 as the prototypical member of a previously unreported GT family (GT118).

acid (Pse5Ac7Ac) **1** (Figure 1a) and its analogues, is an underexplored area of glycoscience.

Pse sugars are bacterial analogues of the ubiquitous sialic acid sugar *N*-acetyl neuraminic acid (Neu5Ac) **2**,^[1] and are present within the lipo- (LPS) and capsular polysaccharides (CPS), and linked to the surface of flagella in a number of pathogenic bacteria.^[2] These include *Campylobacter jejuni*,^[3] *Helicobacter pylori*,^[4] *Shigella boydii* (a cause of dysentery),^[5] *Aeromonas caviae* (a causative agent of diarrhoea in children),^[6] and multidrug resistant ESKAPE pathogens *Pseudomonas aeruginosa*,^[5,7] and *Acinetobacter baumannii*,^[8] which are emerging causes of hospital acquired infections.^[9] Tantalisingly, disruption of Pse biosynthesis in a number of these bacteria results in drastically reduced

The biological roles of glycoconjugates containing the non-mammalian nonulosonic acid sugar 5,7-diacetyl pseudaminic

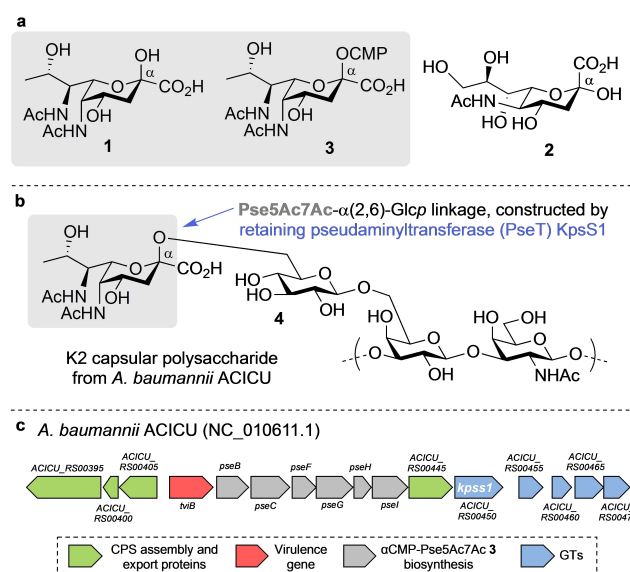


Figure 1. a. Structure of the bacterial sugar Pse5Ac7Ac **1** (depicted in α anomeric configuration), nucleotide activated glycosyl donor α -CMP-Pse5Ac7Ac **3**, and ubiquitous sialic acid sugar Neu5Ac **2** (depicted in α anomeric configuration). b. Structure of the K2 capsular polysaccharide from *A. baumannii* ACICU, containing a Pse5Ac7Ac- α (2,6)-Glc_p linkage. c. Genetic islands encoding *A. baumannii* ACICU KL2 capsular polysaccharide (CPS) biosynthesis and assembly genes. Islands contain genes for biosynthesis of **3** (grey), CPS repeat unit assembly and export (green), virulence genes (red) and glycosyltransferases (GTs, blue).

[*] A. J. Walklett,⁺ Dr. E. K. P. Flack,⁺ Dr. H. S. Chidwick, Dr. N. E. Hatton, Dr. T. Keenan, Dr. D. Budhadev, J. Walton, Dr. M. A. Fascione
 Department of Chemistry
 University of York
 York, YO10 5DD, UK
 E-mail: martin.fascione@york.ac.uk

Dr. E. K. P. Flack,⁺ Prof. G. H. Thomas
 Department of Biology
 University of York
 York, YO10 5DD, UK
 E-mail: gavin.thomas@york.ac.uk

[†] These authors contributed equally

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virulence through loss of flagella.^[6,10] In addition, in vivo experiments also suggest that Pse-glycosylated flagella can bind to Siglec-10, a cell surface sialic acid binding protein, and induce an anti-inflammatory response.^[11] These studies therefore validate Pse bacterium glycosylation as a *bona fide* target for therapeutic intervention in the ongoing battle to overcome antimicrobial resistance.

Although the enzymes required for the biosynthesis of cytidine-5'-monophosphate (CMP)-Pse5Ac7Ac **3** (Figure 1a), the glycosyl donor utilised by glycosyltransferase enzymes (GTs) in Pse glycosylation, have been well characterised.^[12,13] To date no GTs dedicated to the construction of the Pse glycosidic linkage have been unequivocally identified, despite elegant in vivo studies.^[14] Herein we present the first in vitro evidence of Pse glycosylation by a dedicated GT, the pseudaminyltransferase (PseT) KpsS1 from *A. baumannii*.

A. baumannii is a Gram-negative opportunistic bacteria that is listed as a critical-priority pathogen by the World Health Organisation due to the emergence of strains that are resistant to all commercially available antibiotics.^[9] The CPS of *A. baumannii* is a major virulence factor^[15] and a number of strains have been shown to incorporate Pse glycans into their capsule, including highly virulent *A. baumannii* strain ACICU, which bears a K2 type CPS repeat unit **4** (Figure 1b), reportedly encoded within >10% of deposited *A. baumannii* genomes.^[8b] NMR characterisation of **4** had previously identified the presence of a terminal Pse5Ac7Ac attached to a glucopyranoside through an $\alpha(2,6)$ -linkage.^[8b,c] This *axial* α -Pse5Ac7Ac glycosidic linkage is in contrast to the presentation of Neu5Ac **2** in nature, which exists exclusively linked to other glycans through an *equatorial* α -linkage^[1] (note for Pse5Ac7Ac glycosides: α = axial and β = equatorial linkages, whilst for Neu5Ac glycosides: α = equatorial and β = axial linkages). The enzymatic construction of this α -Pse5Ac7Ac linkage would therefore require a PseT that proceeds with a retention of anomeric configuration if using α -CMP-Pse5Ac7Ac **3** as a donor. Analysis of the *A. baumannii* ACICU KL2 biosynthetic locus reveals the presence of a cluster of six genes (*pseBCFGHI*) responsible for the biosynthesis of α -CMP-Pse5Ac7Ac **3** (Figure 1c). In addition, downstream from this cluster of genes, a number of GTs are encoded that are likely responsible for the enzymatic assembly of the CPS trisaccharide core in the repeat unit, and an as yet uncharacterised *kpsS1* gene.^[8b,c]

Although no retaining sialyltransferases that utilise a Neu5Ac substrate have been discovered to date, KpsS1 shares 30% sequence identity to *Escherichia coli* KpsS,^[16] which was recently characterised as the first retaining β -Kdo (3-deoxy-d-manno-oct-2-ulosonic acid) transferase, and a member of the GT107 family of carbohydrate active enzymes.^[17] It was therefore tentatively predicted that KpsS1 may be responsible for construction of the Pse5Ac7Ac- $\alpha(2,6)$ -GlcP linkage in **4**,^[8c] and act as a dedicated retaining PseT. We set out to test this proposed function through expression and in vitro characterisation of the enzyme.

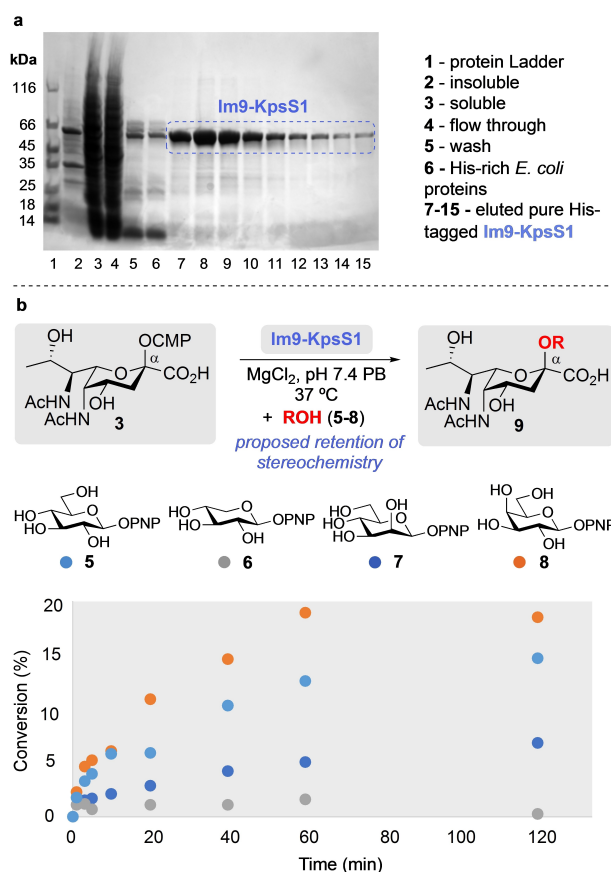
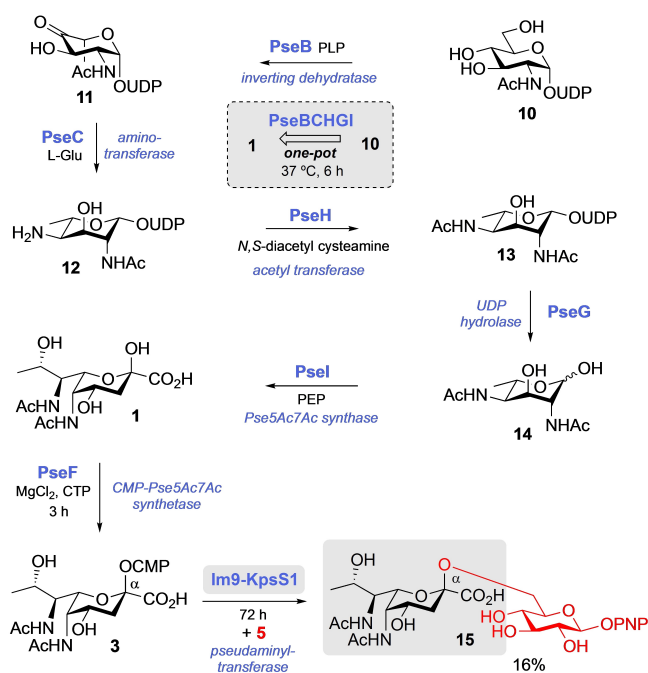


Figure 2. a. Optimised nickel affinity purification of Im9-KpsS1 following expression in *E. coli* BL21 (DE3). b. PseT activity assay. All reactions were performed over 120 min at 37 °C on a 20 μ L scale in 50 mM pH 7.4 sodium phosphate buffer (PB), containing 1 mM donor **3**, 10 mM acceptor **5–8**, 10 mM $MgCl_2$ and 50 μ M Im9-KpsS1. c. Plot of PseT time-course activity. Conversions measured using HILIC-LCMS, comparing UV 280 nm chromatogram peak area of remaining CMP-Pse5Ac7Ac donor and Pse5Ac7Ac-disaccharide product **9**.

The full length *kpsS1* gene was cloned initially into a pET-15b vector to encode a recombinant protein with an N-terminal hexahistidine tag, however expression trials performed in *E. coli* under a variety of conditions yielded only insoluble protein. We therefore opted to construct a fusion protein (Supporting Information Table S1–S3), a strategy which has previously been used successfully in the production of challenging GTs.^[16] We assembled a plasmid encoding the 86-amino acid colicin E9 immunity protein Im9,^[18] bearing an N-terminal His tag, fused to KpsS1, and this Im9-KpsS1 fusion was then taken forward for expression trials in *E. coli* BL21 (DE3) cells. Again, under many conditions the fusion was insoluble, however low levels of soluble Im9-KpsS1 (Mw: 68.9 kDa) was detected in cell lysate at 4 h post induction (Supplementary Figure S1).

With crude Im9-KpsS1 lysate in hand we undertook preliminary activity screens using the predicted α -CMP-Pse5Ac7Ac donor **3**, and a series of glycosyl acceptors (Supplementary Figure S6). Reaction samples were then subjected to negative ion ESI LC–MS analysis to determine if any Pse5Ac7Ac glycoside products had formed. Consider-



Scheme 1. Enzymatic synthesis of disaccharide **15** starting from UDP-GlcNAc **10**, using a tandem five enzymes one-pot (PseBCHGI) synthesis of **1** from **10**, and a two enzyme one-pot (PseF and Im9-KpsS1) synthesis of **15** from **1**. Abbreviations: PLP= pyridoxal 5'-phosphate, L-Glu=L-glutamic acid, PEP= phosphoenolpyruvate, CTP= cytidine triphosphate.

ing the structure of the *A. baumannii* CPS repeat unit **4**, we anticipated KpsS1 might utilise β -D-glucopyranoside structures as substrates, and therefore initially screened the simple Me- β -D-Glcp as an acceptor. However, under the reaction conditions screened no turnover of the donor **3** was observed. It has been reported that GTs in *A. baumannii* gene clusters are often distributed in inverse order to function.^[8c] As *kpsS1* is the first GT in the KL2 gene cluster, upstream from the remaining putative GTs, it therefore likely uses a di, tri, or tetrasaccharide acceptor in vivo. So next we screened para-nitrophenyl (PNP)- β -D-Glcp **5** as an acceptor, in the hope that the aryl aglycone would occupy the region of the enzyme active site reserved for binding to a larger acceptor. Pleasingly, after 18 h, all CMP-donor **3** was consumed and a peak for the expected disaccharide product was observed in LCMS. To test enzyme specificity towards the Glcp in the acceptor, PNP- β -D-Xylp **6** which lacks the nucleophilic 6-OH group present in Glcp was also screened and unsurprisingly no turnover was observed, reaffirming the hypothesis that KpsS1 is a PseT that forms a linkage to the 6-OH of Glcp in vivo. We also sought to determine whether the enzyme could utilise the alternative β -CMP glycosyl donor of Neu5Ac **2**. Under otherwise identical conditions using PNP- β -D-Glcp **6** as an acceptor and β -CMP-Neu5Ac as a donor, no turnover to product was catalysed by the enzyme (Supplementary Figure S7). An internal competition reaction between α -CMP-Pse5Ac7Ac **3** and β -CMP-Neu5Ac for the acceptor **6** under the same

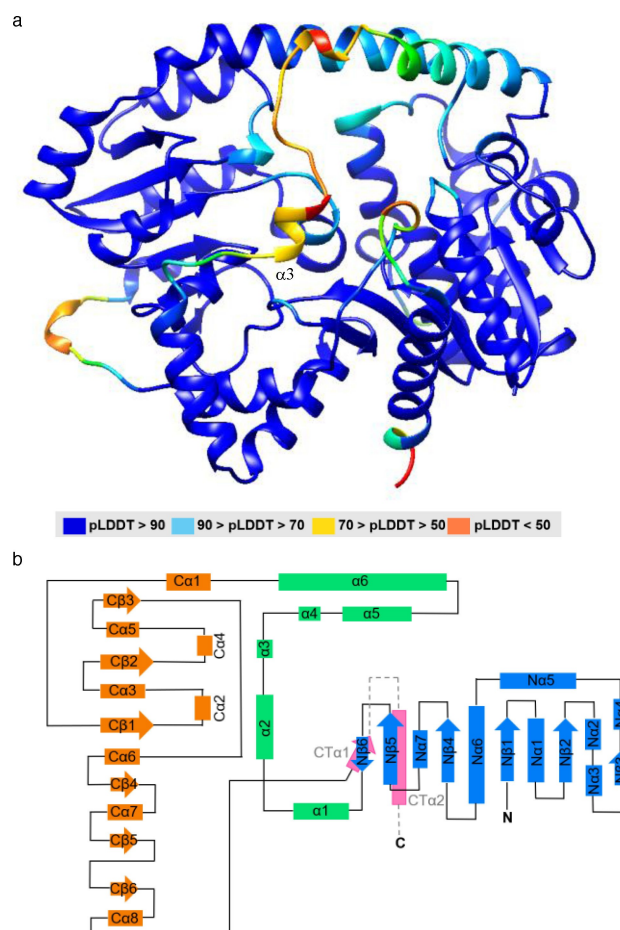


Figure 3. a. AlphaFold prediction for KpsS1 coloured by per-residue confidence score (pLDDT) with α 3 highlighted. b. Topology of KpsS1. α -helices are depicted as rectangles, β -strands as arrows and loops as lines.

conditions also yielded only Pse5Ac7Ac-Glcp disaccharide, with the β -CMP-Neu5Ac donor remaining.

We next set out to improve expression and purification conditions of Im9-KpsS1 to obtain enough pure enzyme for further study. Optimised production was achieved in *E. coli* BL21 (DE3) cells induced at OD₆₀₀ 0.8 and incubated at 30°C for 4 h post induction, followed by lysis using 0.5 % non-ionic detergent and nickel affinity purification using a two-step isocratic elution (Figure 2a). We subsequently performed PseT activity time-course assays using pure Im9-KpsS1 and a range of PNP-acceptors (Figure 2b).^[19] Over 120 min we again observed no turnover using PNP-Xylp **6**, lacking the 6-OH. But we did observe increasing product formation over the time-course using PNP-Manp **7**, albeit low level, and more rapid increased conversion to disaccharide product (approaching 20 % at 120 min) for both PNP-Galp **8** and PNP-Glcp **5** acceptors. These results demonstrate that the purified fusion enzyme is active in vitro and also shows some substrate promiscuity with respect to glycosyl acceptors, with both PNP-Glcp and Galp acceptors used by the enzyme with comparable efficiency. We subsequently used a CMP-Glo™ assay^[20] to measure kinetic

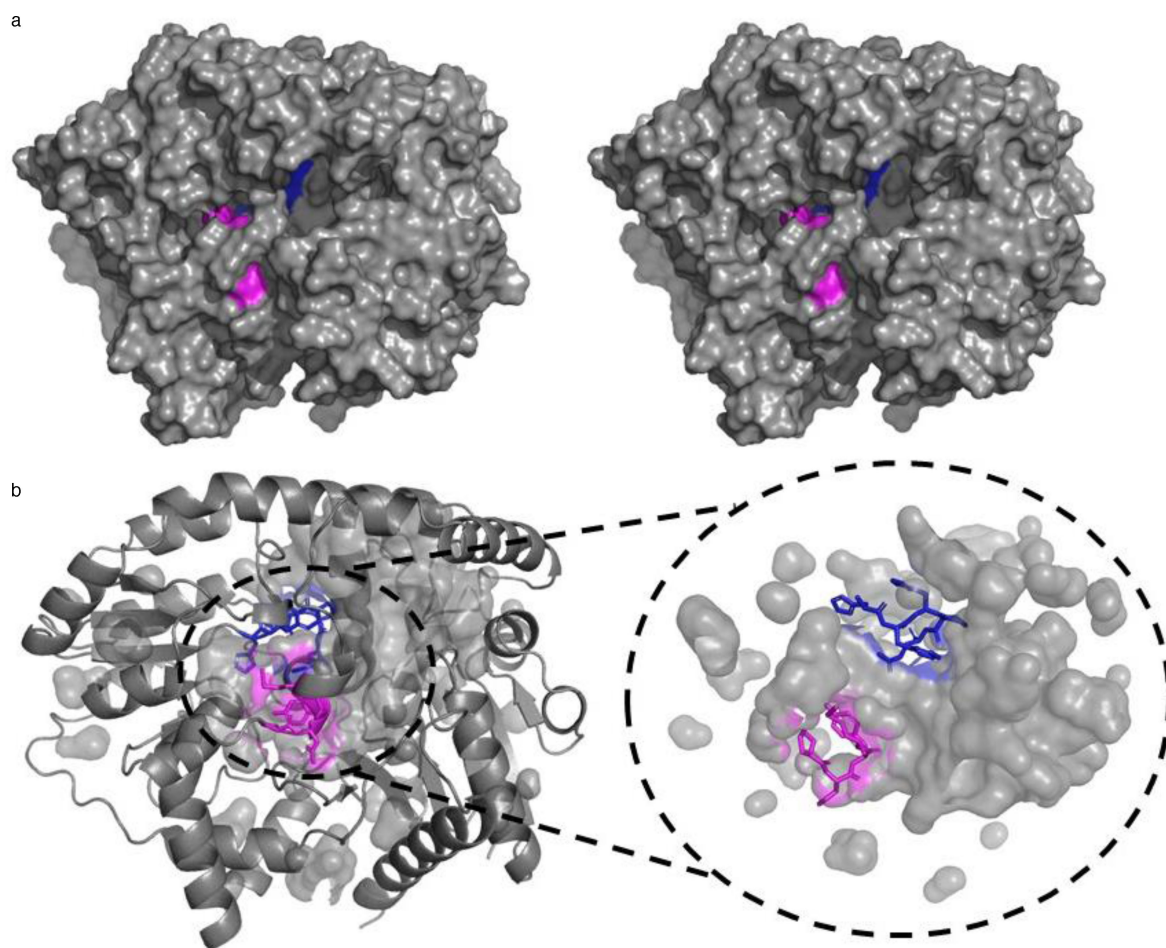


Figure 4. a. A stereoview representation of the exterior surface (light grey) of the KpsS1 AlphaFold model depicting a solvent-accessible large cavity (dark grey) with the H(F/Y) HPE (blue) and PDYM (pink) motifs highlighted. b. KpsS1 AlphaFold model with cavity depicted as the surface layer (light grey) and expansion showing positioning of the H(F/Y) HPE (blue) and PDYM (pink) motifs within the cavity.

parameters of Im9-KpsS1 (Supplementary Figures S9 and S11) and determined the enzyme bound CMP-Pse5Ac7Ac **3** with a K_m of approximately 105 μM , which is comparable to the donor affinity of other bacterial sialyltransferases.^[21]

Although these enzyme activity assays provided evidence of turnover, the limited availability of pure CMP-Pse5Ac7Ac **3** donor restricted the scale on which these reactions could be performed and precluded NMR characterisation of the products. This was required to unequivocally assign the stereochemistry of the glycosidic linkage formed and therefore whether KpsS1 was indeed a retaining glycosyltransferase enzyme. We therefore chose to scale up the enzymatic glycosylation using Glc acceptor **5** to yield enough disaccharide product for full characterisation, and comparison to the unambiguous assignment of the Pse5Ac7Ac- α 2,6-Glcp linkage in **4**.^[8c] To achieve this, we reconstituted the biosynthetic pathway for production of CMP-Pse5Ac7Ac **3** in vitro (Scheme 1).^[22] Starting from 30 mg of UDP-GlcNAc **10**, using the five biosynthetic enzymes from *C. jejuni*, PseB, C, H, G and I and required co-factors, we synthesised Pse5Ac7Ac **1** in one pot in 6 h, with negative ion ESI LCMS confirming product formation and no remaining biosynthetic intermediates **11–14**. To this reaction mixture

we then added the CMP-Pse5Ac7Ac synthetase PseF from *A. caviae*,^[23] CTP and MgCl_2 for 3 h until CMP-Pse5Ac7Ac **3** was observed. Finally, acceptor **6** and Im9-KpsS1 were then added to the reaction mixture before incubation for a further 72 h, when all donor was consumed. From this tandem seven enzyme two-pot synthesis the desired Pse5Ac7Ac- α 2,6-PNP- β -D-Glcp disaccharide **15** was synthesised in 16% yield. The stereochemistry of the newly formed glycosidic bond was unambiguously assigned as α based on the 0.54 ppm difference between the δ values of the Pse5Ac7Ac $\text{H}_{3_{\text{ax}}}$ and $\text{H}_{3_{\text{eq}}}$ protons in 700 MHz ^1H NMR analysis (reported as 0.55 ppm in the characterisation of repeating unit **4** at 600 MHz).^[8c,24] Thus providing unequivocal evidence that KpsS1 acts as a retaining pseudaminyltransferase.

Whilst the mechanism employed by inverting sialyltransferases has been well studied,^[25] the retaining mechanism utilised by KpsS1 is an intriguing open question that is difficult to determine without in depth structural characterisation of the active site. However, the overall high per-residue confidence (pLDDT) scores in KpsS1's AlphaFold model make it a suitable foundation from which to base preliminary mechanistic hypotheses. This model predicts

KpsS1 adopts a GT-B fold^[26] comprised of two $\beta/\alpha/\beta$ Rossmann domains linked by a flexible α -helix rich region (Figure 3a–b). A DALI^[27] search against the PDB using this model returned two retaining β -Kdo transferases, KpsC from *E. coli* (GT107 family),^[14] and WbbB from *Raoultella terrigena* (GT99 family).^[17] Structural studies of both KpsC and WbbB suggest that an internal return S_Ni mechanism,^[26] often proposed for retaining GTs, is unlikely for these enzymes due to narrow active sites.^[14,17] Moreover the presence of an indispensable Asp residue in a conserved QXXXD motif in the active site of both enzymes, raised the possibility of a double displacement mechanism,^[26] a hypothesis recently reinforced by trapping of mutant Kdo-WbbB/KpsC (QXXXD) covalent enzyme intermediates.^[28] Although analysis of the KpsS1 sequence revealed an equivalent QNGLD motif, it also confirmed the presence of an extended H(F/Y)HPE motif, similar to an invariant HP motif in the retaining β -Kdo-transferases,^[17a,29] a CMP-binding motif also highly conserved in inverting sialyltransferases.^[25] This H(F/Y)HPE motif, alongside a unique PDYM motif also not present in the GT99 and GT107 β -Kdo-transferases, were both found to be highly conserved in putative KpsS1 PseTs returned in a BLASTp^[30] search (Supplementary Figure S16), including from other *A. baumannii* strains (Figure S14). These include KpsS1_{KL23} which shares 84 % sequence identity to KpsS1,^[8c] KpsS2_{KL33} which is predicted to form the same $\alpha(2,6)$ -linkage to a β -D-Glcp as KpsS1, and KpsS2_{KL42} from the *A. baumannii* LUH5550K42 gene cluster,^[31] which likely utilises CMP-Pse5Ac7Hb as a donor, a Pse sugar modified with (*R*)-3-hydroxybutanoyl group at N7. Conversely, the CPS of *A. baumannii* RBH4K6 contains an equatorial $\beta(2,6)$ -Pse5Ac7Ac linkage, and notably *kpsS1* is replaced in this KL6 capsule locus by a sequence with similarity to characterised inverting sialyltransferases.^[32] A webFlaGs^[33] search, which identifies putative orthologues to inputted sequences based on shared sequence identity and genomic context, also returned a number of putative PseT sequences from more divergent bacterial species (Supplementary Figure S13) in which both the H(F/Y)HPE and PDYM motifs were highly conserved (Supplementary Figure S15). Notably, the KpsS1 AlphaFold model lacks confidence in a short, loop embedded $\alpha 3$ helix (Figure 3a), indicative of a flexible region, which covers a large cavity in the enzyme where these conserved motifs are clustered (Figure 4), potentially highlighting their positioning in a conformationally flexible active site.

In conclusion, we have unequivocally established that *A. baumannii* ACICU KpsS1 is a PseT enzyme. To the best of our knowledge, this is the first example of dedicated PseT activity in vitro. Additionally, as a result of the functional studies on the Im9-KpsS1 enzyme, a Pse5Ac7Ac- $\alpha 2,6$ -PNP- β -D-Glcp disaccharide **15** was synthesised in a tandem two-pot enzymatic process using seven enzymes, confirming that KpsS1 is also a retaining PseT. Although full elucidation of the nature of this retaining mechanism will likely require structural and biochemical studies using Pse-based probes, this study confirms that KpsS1 defines a previously unreported GT family (GT118) which includes homologous

proteins with conserved sequence motifs that are early candidates for mechanistic exploration.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: enzymes · pseudaminic acid · proteins · structure elucidation

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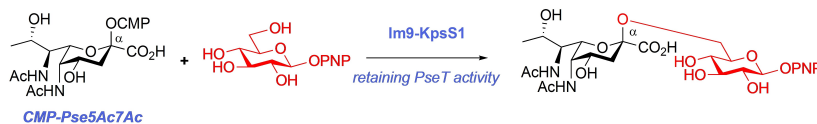
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Communications

Enzymes

A. J. Walklett, E. K. P. Flack, H. S. Chidwick,
N. E. Hatton, T. Keenan, D. Budhadev,
J. Walton, G. H. Thomas,*
M. A. Fascione* ————— e202318523

The Retaining Pse5Ac7Ac Pseudaminyl-
transferase KpsS1 Defines a Previously
Unreported glycosyltransferase family
(GT118)



Pse5Ac7Ac glycosyltransferase (GT) activity has been demonstrated in characterisation of the retaining pseudaminyl-transferase (PseT) KpsS1, the prototypical member of a previously unreported GT family (GT118). KpsS1 is

utilised in a two-pot, seven enzyme synthesis of a glycoside terminating in Pse5Ac7Ac, an important bacterial analogue of the ubiquitous sialic acid Neu5Ac.