Rational Engineering of a Pseudaminic Acid Synthase Enzyme Enables Access to a 3-Fluoro Sugar with Motility Inhibition in Bacterial Pathogens

James M. Jeffries,[a] Abigail J. Walklett,[a] Natasha E. Hatton,[a] Bartosz Kowalski,[a] Tessa Keenan,[a] Lickson Munjoma,[b] Richard Haigh,[b] Christopher D. Bayliss,[b] Gavin H. Thomas*[c] and Martin A. Fascione*[a]

Abstract: We report the rational engineering of a pseudaminic acid synthase (PseI) which enables the first synthesis of a 3-fluorinated pseudaminic acid sugar (3-(*eq*)-*F*-Pse5Ac7Ac), potentially establishing a new class of metabolic inhibitors targeting bacterial glycosylation. Pseudaminic acids are α-keto acid sugars essential for *O*-glycosylation of flagellin in pathogens such as *Campylobacter jejuni*, where they are critical for motility and virulence. By introducing rational mutations in the PseI active site, we achieve enhanced turnover with unnatural 3-fluoro-phosphoenolpyruvate, facilitating a scalable chemoenzymatic synthesis of the fluorinated sugar. Subsequent treatment of *C. jejuni* with 3-(*eq*)-*F*-Pse5Ac7Ac resulted in a significant, time-dependent reduction in motility, and *in vitro* studies demonstrated bacterial CMP-pseudaminic acid synthetase enzymes (PseF) can process the fluoro sugar to afford CMP-3-(*eq*)-*F*-Pse5Ac7Ac, potentially implicating the fluorinated pseudaminic acid or its glycosyltransferase CMP-donor as an anti-motilin *in vivo*. This study demonstrates, for the first time, that fluorinated pseudaminic acids can impair bacterial motility, paving the way for anti-virulence strategies in pathogenic bacteria.

This anti-motilin approach offers a promising alternative to traditional antibiotics, addressing the urgent need for novel strategies to combat antimicrobial resistance, and could be extended to other bacterial α-keto acid sugars.

[a] J. M. Jeffries, A. J. Walklett, Dr. N. E. Hatton, B. Kowalski, Dr. T. Keenan, Dr. M. A. Fascione
York Structural Biology Lab, Department of Chemistry
University of York, York, YO10 5DD, UK
E-mail: martin.fascione@york.ac.uk

[b] Dr. L. Munjoma, Dr. R. Haigh, Prof C. D. Bayliss
Division of Microbiology and Infection, College of Life Sciences,
University of Leicester, Leicester, LE1 7RH, UK

[c] Prof G. H. Thomas
Department of Biology,
University of York, York, YO10 5DD, UK
E-mail: gavin.thomas@york.ac.uk

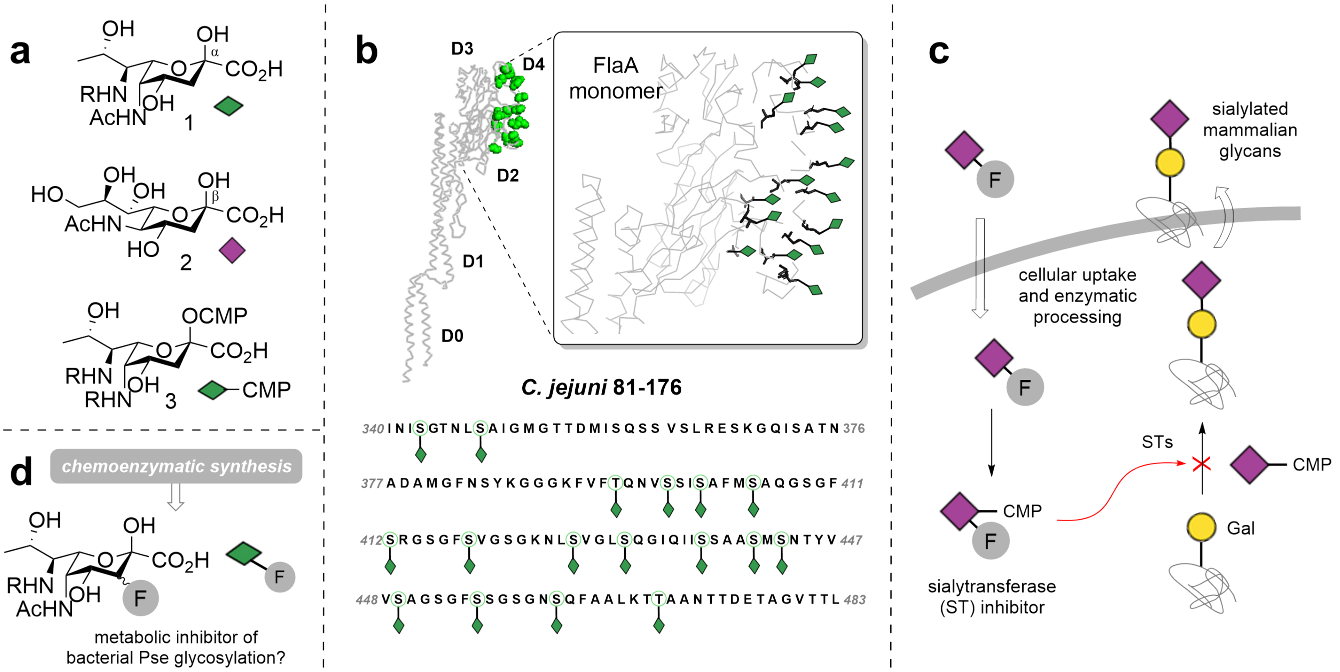

Figure 1. a. Structure of bacterial Pse sugars (R = Ac **1**, derivatives also include but not limited to; R = amidino, formyl, 3-(*R*)-hydroxybutyl, depicted in the α-anomeric configuration,) Neu5Ac **2** (depicted in the β-anomeric configuration), and nucleotide activated glycosyl donor CMP-Pse5Ac7Ac **3**. **b.** Representation of a single *C. jejuni* FlaA G508A subunit (PDB: 6X80) of an 11 protofilament complex with Pse5Ac7Ac **1** glycosylation sites in outer domain (D4).[23] **c.** Schematic of 3-*F*-Neu5Ac derivatives entering mammalian cells and competitively inhibiting SiaT activity following intracellular processing by esterases and CMP-sialic acid synthetase enzymes. **d.** Structure of proposed bacterial 3-*F*-Pse 'anti-motilin' scaffold.

5,7-di-*N*-acetyl pseudaminic acid (Pse5Ac7Ac) **1** (Figure 1a) and its derivatives (Pse)[1] are widely occurring α-keto acid sugars[2] analogous in structure to the ubiquitous "sialic acid" *N*-acetyl neuraminic acid (Neu5Ac) **2**,[3] which is central to cellular recognition in humans. In comparison, Pse sugars are non-mammalian and key components of lipopolysaccharides,[4–7] capsular polysaccharides[8–13] and other glycoconjugates in numerous bacteria, including WHO priority pathogens *Pseudomonas aeruginosa*[14] and *Acinetobacter baumannii*.[15,16] Notably, Pse sugars also adorn the flagella of motile bacteria that are highly prevalent in the human gut and capable of causing gastrointestinal infections, including *Campylobacter jejuni*,[17,18] the main cause of foodborne disease in the developed world, and *Helicobacter pylori*,[19,20] a risk factor for gastric cancer. The motility of these bacteria in the gut is important for colonization[21] and mediated by polar flagella which propel the helical pathogens through viscous gastrointestinal milieu.[22] Importantly, the major flagellin protein FlaA[23] (Figure 1b) is extensively *O*-glycosylated with Pse sugars at specific serine and threonine residues in both bacteria, and genetic knockout of *O*-pseudaminylation leads to aflagellated cells, reducing motility[18,20,24] and thus virulence. As such, the Pse biosynthetic pathway represents an 'Achilles heel' in these bacteria which could be targeted through the development of novel 'anti-motilins' which attenuate virulence by compromising flagella filament assembly,[25] facilitating more judicious use of antibiotics in the treatment of gut pathogens in which resistance is increasingly common.[26,27] Taking inspiration from the pioneering use of 3-*F*-Neu5Ac as metabolic inhibitors of sialylation in mammals (Figure 1c),[28–32] herein we use rational engineering of a synthase enzyme in the Pse biosynthetic pathway to chemoenzymatically synthesise 3-*F*-Pse and explore its potential as an *in vivo* metabolic inhibitor of *C. jejuni* motility (Figure 1d).

The biosynthesis of pseudaminic acids in *C. jejuni* and *H. pylori* (Figure 2a) has been well characterised *in vitro*,[33–35] with both pathogens synthesizing CMP-Pse5Ac7Ac **3**, the nucleotide donor required for glycosylation of FlaA, from UDP-GlcNAc **4** in a six- enzyme pathway (PseB, PseC, PseH, PseG, PseI and PseF). In contrast, the glycosyltransferase enzymes that catalyse the final *O*-pseudaminylation of FlaA proteins have yet to be unequivocally characterised *in vitro*, but elegant *in vivo* studies have identified glycosyltransferases genes termed motility associated factors (Mafs)[36–39] present with flagellin glycosylation islands comprising Pse5Ac7Ac biosynthesis and flagellin subunits genes, that are essential for flagellin glycosylation and motility. Wherein Maf dependent Pse glycosylation is hypothesised to facilitate the interaction of unfolded flagellin with its chaperone (FlaJ) prior to export via the T3SS secretion system.[38] Therefore, Maf pseudaminyltransferases (PseT) and enzyme inhibitors represent an attractive target for 'anti-motilin' therapeutic development. Although no PseT inhibitors have been identified to date, there is a significant precedent for inhibition of Neu5Ac sialyltransferases (SiaT) using 3-*F*-CMP-Neu5Ac, which can block mammalian cell sialylation through supply of metabolic inhibitor 3-*F*-Neu5Ac which is subsequently converted *in vivo* to the fluorinated CMP-donor (Figure 1c),[29,30] in which the electron withdrawing 3-fluoro group inductively destabilizes the formation of the SiaT oxocarbenium ion transition state.[40] Sialyltransferases Cst-I and Cst-II were previously shown to share structural homology to the central MAF_flag10 domain from the *Magnetospirillum magneticum* AMB-1 Maf protein, a domain that is common to all putative Maf sequences.[37] Docking studies suggested that CMP-Pse5Ac7Ac adopts a similar position to 3-F-CMP-Neu5Ac in the Cst-II inhibitor-bound complex. Therefore, we hypothesised that treating *C. jejuni* with a fluorinated Pse sugar could represent an analogous strategy to impair bacterial motility. Theorizing that following cellular uptake, 3-*F*-Pse **10** could be converted intracellularly by the bacterial CMP-pseudaminic acid synthetase (PseF) into the more hydrophilic 3-*F*-CMP-Pse5Ac7Ac **3** with subsequent potential inhibition of Maf PseT activity (Figure 2b), and/or build-up of the CMP-sugar, which is a feedback inhibitor of the first enzyme in the biosynthetic pathway (PseB).[41] We therefore set out to access the 3-fluorinated Pse scaffold through leveraging our understanding and expertise in the enzymatic synthesis of α-keto acid sugars.[34,42,43]

Although 3-*F*-Neu5Ac and other 'sialic acid' derivatives have routinely been synthesised utilising reversible sialic acid aldolase enzymes and fluoropyruvate,[44,45] to date no aldolases

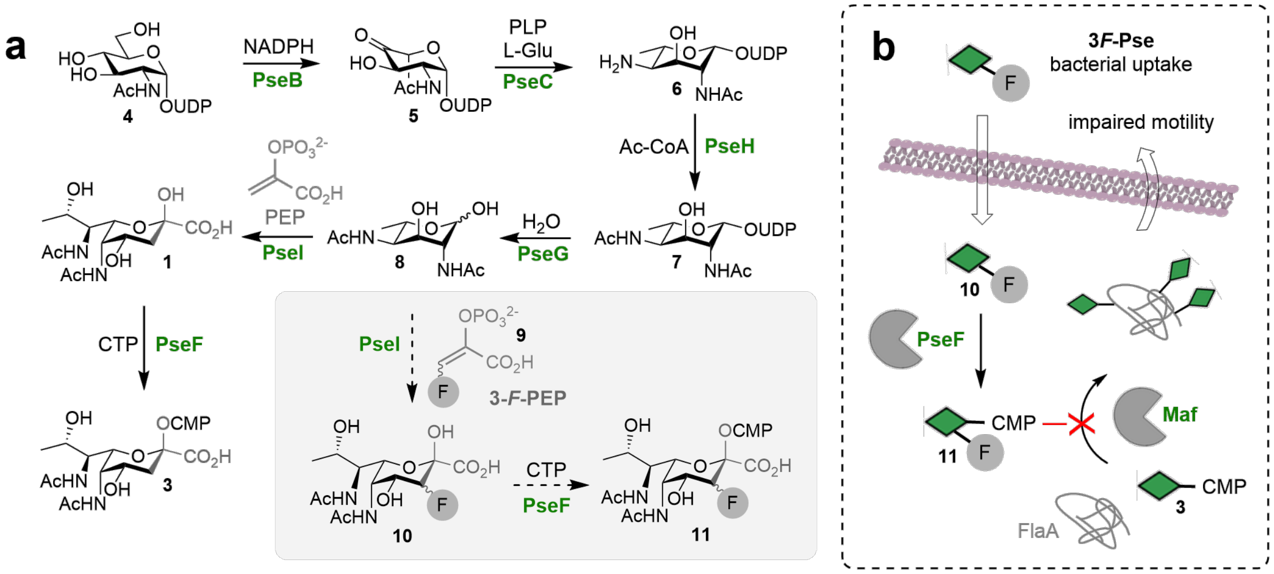

Figure 2. a. A proposed chemoenzymatic approach to CMP-3-*F*-Pse5Ac7Ac **11** utilising the biosynthetic pathway of Pse5Ac7Ac **1**, starting from UDP-GlcNAc **4** and proceeding through biosynthetic intermediates **5**, **6**, and **7**. Abbreviations: PLP= pyridoxal 5'-phosphate, L-Glu= L-glutamic acid, PEP= phosphoenolpyruvate, 3-*F*-PEP= 3-fluoro-phosphoenolpyruvate, CTP= cytidine triphosphate. **b.** Schematic of proposed bacterial uptake of 3-*F*-Pse5Ac7Ac **10** preceding enzymatic processing to CMP-3-*F*-Pse5Ac7Ac **11**, a potential inhibitor of Maf PseTs leading to impaired motility.

have been identified that are active in the reversible synthesis/degradation of the Pse scaffold. We therefore sought to determine if the *cj*PseI synthase enzyme, which catalyses the condensation between 6-deoxy-AltdiNAc **8** and phosphoenolpyruvate (PEP) in the biosynthesis of Pse5Ac7Ac **1** (Figure 2a), could be hijacked to facilitate fluorine incorporation into the Pse scaffold using the unnatural co-factor **9**. With notable precedent existing for activity of 3-deoxy-D-manno-2-octulosonate-8-phosphate (KDO8P) synthase and 3-deoxy-D-arabino-2-heptulosonate-7-phosphate (DAHP) synthase enzymes with the fluorinated co-factor.[46] Following chemical synthesis and purification of 3-*F*-PEP **9** as the (*Z*)-isomer, as previously described,[47] we setup a small-scale 5-enzyme one-pot synthesis[34,43] of the Pse5Ac7Ac sugar starting from UDP-GlcNAc **4**, using the wild type *C. jejuni* biosynthetic enzymes PseB, PseC, PseH, PseG, alongside their requisite natural cofactors, and PseI[48] with 3-*F*-PEP **9** as a substitute for PEP, and confirmed the putative formation of trace 3-*F*-Pse5Ac7Ac **10** by LC-MS analysis. Buoyed by this potential turnover, we chemically synthesised the 6-deoxy-AltdiNAc **8** PseG product[42] for scale-up and more detailed interrogation of the PseI enzyme. Subsequent ^{19}F NMR studies using wild-type PseI did indeed corroborate formation of 3-*F*-Pse5Ac7Ac **10** using the unnatural co-factor (Figure 3a, blue spectra) albeit in low conversion, evidenced by the decrease in signal intensity of (*Z*)-3-*F*-PEP **9** (-141 ppm) and increase in a new signal at -206 ppm over several days which we assigned as 3-*F*-Pse5Ac7Ac **10**, based on comparison with 3-*F*-Neu5Ac.[45] Cognisant the electron withdrawing nature of the fluorine atom may impede attack on the ring opened form of 6-deoxy-AltdiNAc **8** we sought to improve enzyme activity by introducing interactions with the fluorine through rational engineering of the PseI active site.[49] Utilising the crystal structure of PseI with PEP as a model, we built in the 6-deoxy-AltdiNAc substrate and (*Z*)-3-*F*-PEP and identified alanine 127 situated below the co-factor as a potential residue for mutagenesis, envisioning that mutation to a serine (A127S, Figure 3b) or glycine (A127G) might recoup nucleophilicity of the unnatural co-factor through H-F hydrogen bonding to the serine alcohol or a water molecule accommodated by the smaller glycine residue, respectively. Site-directed mutagenesis was therefore performed to afford the PseI A127S and A127G mutants which were then expressed and purified in high yields. Gratifyingly, ^{19}F NMR time-course (Figure 3a, red = A127G, pink = A127S) studies showed that after 16 hours both mutants afforded increased relative product formation compared to wild-type PseI with complete consumption of (*Z*)-3-*F*-PEP observed by 96 hours using 0.5 mg mL^{-1} enzyme and 4 mM substrate. Importantly, characteristic coupling constants of 12.3 and 49.9 Hz revealed the gauche orientation between the C3-fluorine and C-4 axial proton

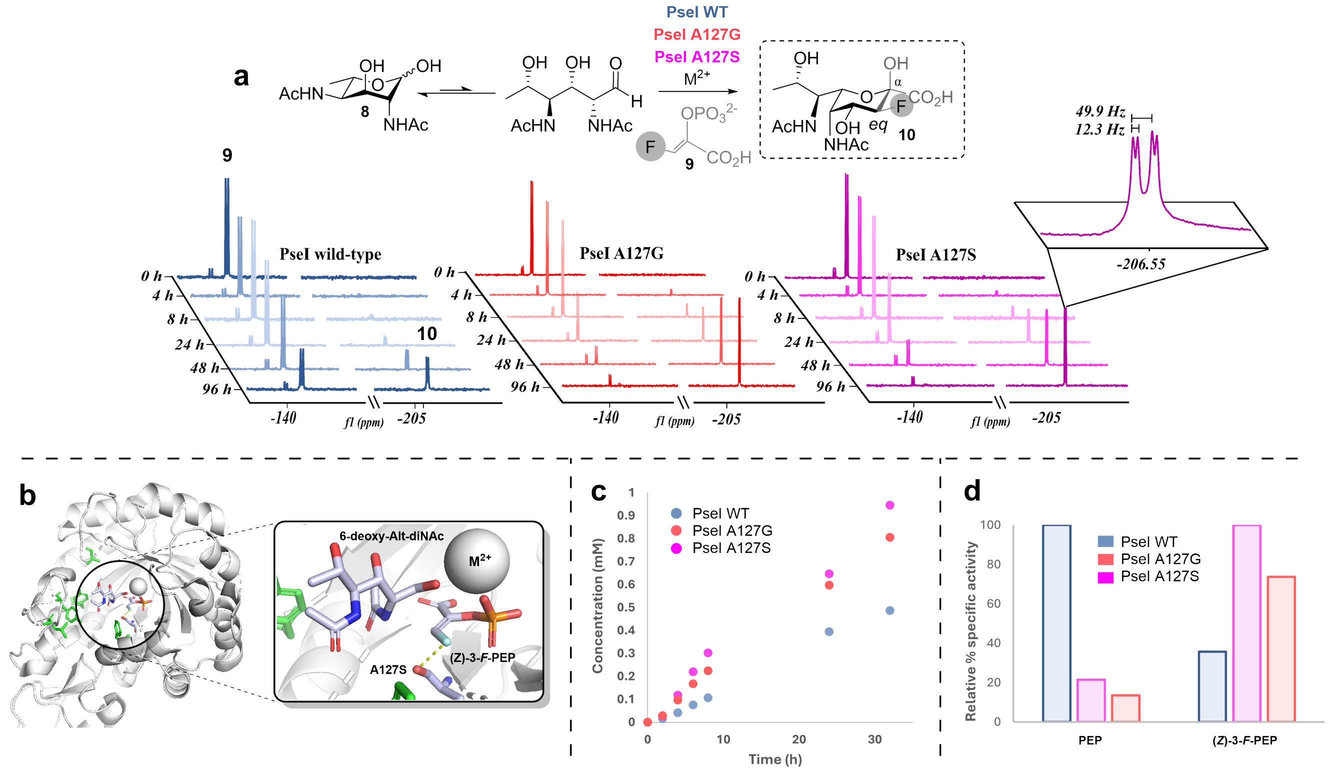

Figure 3. a. ^{19}F NMR time course showing formation of 3-(*eq*)-*F*-Pse5Ac7Ac **10** (-206.5 ppm) with wild-type PseI (blue), PseI A127G (red) and PseI A127S (pink) (0.5 mg mL^{-1}) with (*Z*)-3-*F*-PEP **9** (-141.3 ppm) (4 mM), 6-deoxy-Alt-di-NAc **8** (4 mM) and MgCl$_2$ (5 mM) in deuterated sodium phoshate buffer (pD 7.4, 50 mM). Reactions were monitored over 96 hours (400 MHz, D$_2$O, 256 scans). **b.** *cj*PseI A127S structure in complex with metal ion, modelled 6-deoxy-AltdiNAc **8** and (*Z*)-3-*F*-PEP **9,** highlighting conserved binding residues of **8** (A20, Y49, L75, Y79 and F129) **c.** LC-MS assay depicting quantitative formation of 3-(*eq*)-*F*-Pse5Ac7Ac **11** using wild-type PseI (blue), PseI A129G (red) and PseI A129S (pink) (0.1 mg mL $^{-1}$) over 32 hours when incubated (30 °C, 200 rpm) with (*Z*)-3-*F*-PEP **9** (4 mM) and 6-deoxy-Alt-diNAc **8** (4 mM) and MgCl$_2$ (5 mM) in sodium phosphate buffer (pH 7.4, 50 mM). **d.** Relative specific activities of PseI, PseI A127S and A127G with natural substrate PEP and unnatural substrate 3-*F*-PEP **9**.

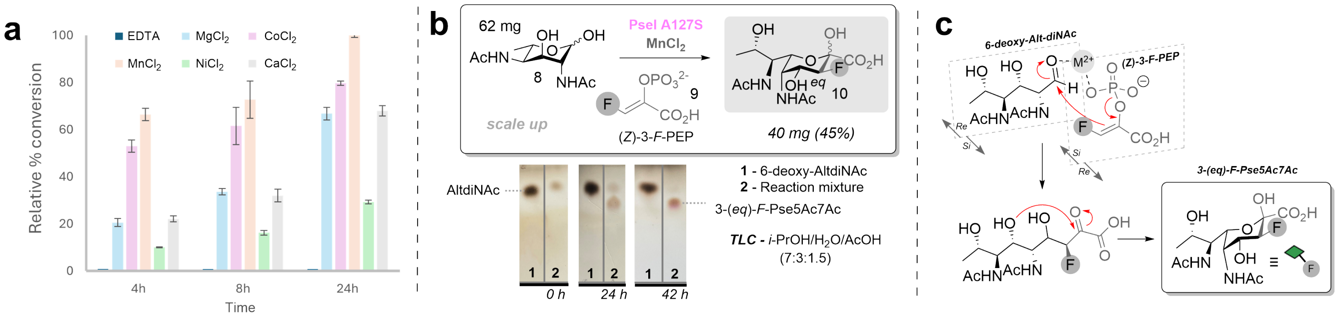

Figure 4. a. Screening of PseI A127S with 3-*F*-PEP **9** in the presence of different divalent metal ions **b.** Scale up of 3-(*eq*)-*F*-Pse5Ac7Ac **10** from 6-deoxy-AltdiNAc **8** and (*Z*)-3-*F*-PEP **9** using PseI A127S with MnCl$_2$. Reaction progress was monitored via TLC (*i*-PrOH/H$_2$O/AcOH 7:3:1.5) **c.** PseI shows stereospecificity towards (*Z*)-3-*F*-PEP **9** with the *si* face of 6-deoxy-AltdiNAc **8** attacked by the *si* face of unnatural substrate **9** to exclusively afford 3-(*eq*)-*F*-Pse5Ac7Ac **10**.

indicating formation of the equatorial isomer of 3-*F*-Pse5Ac7Ac **10** by both wild-type and mutant enzymes.

Cognisant that relative peak intensities in ^{19}F-NMR may be skewed by line broadening and unable to report on activity with the natural substrate, we also quantitatively characterised enzyme activity through LC-MS analysis (Figure 3c) by generating standard curves with purified products (Figures S10 and S11), and subsequently determining specific activities of PseI, PseI A127S and PseI A127G with both PEP and (*Z*)-3-*F*-PEP **9** (Figure 3d). Unsurprisingly, the specific activity of wild-type PseI with the natural co-factor PEP (5113 nmol min^{-1} mg^{-1}) was much greater than with the unnatural co-factor **9** (0.45) and ~5-fold greater in comparison to mutant PseI enzymes with PEP. However, the relative specific activity of both mutants with (*Z*)-3-*F*-PEP was 2-3-fold higher than that of wild-type enzyme (A127S: 1.26, A127G: 0.93), reinforcing the hypothesis that although A127 facilitates optimal binding of PEP, mutagenesis of this residue can increase the activity of the enzyme with unnatural fluorinated PEP, whilst reducing activity with PEP. This is potentially a result of a gain in advantageous interactions between the fluorine and the residue 127 on the *re*-face of the (*Z*)-3-*F*-PEP (see Figure 4c) in the active site, with previous structural studies on Neu5Ac synthases observing a notable lack of contacts between the enzyme and the *re*-face of the natural PEP co-factor.[50] However, in the absence of PseI structural data with a mutated active site and 3F-PEP bound, a more complex scenario involving other non-covalent and/or steric interactions cannot be discounted.

To further optimise this reaction, we subsequently performed metal screening with the PseI A127S mutant (Figure 4a). The metal dependence of the enzyme was confirmed with no activity in the presence of EDTA and highest activity observed in the presence of Mn^{2+} and Co^{2+} divalent cations, following a similar trend observed in previous characterisation of synthase enzymes,[48,50] With optimised conditions in hand, we next aimed to scale up the synthesis of 3-(*eq*)-*F*-Pse5Ac7Ac **10** *in vitro* (Figure 4b). To this end we reacted 6-deoxy Alt-diNAc **8** (62 mg) with (*Z*)-3-*F*-PEP **9**, MnCl$_2$ and PseI A127S, and monitored reaction progression by TLC until completion after 42 hours. The product 3-(*eq*)-*F*-Pse5Ac7Ac **10** was isolated (40 mg, 45%) from the reaction mixture via ion exchange chromatography, with no evidence of the 3-*F*-axial isomer. This is unsurprising considering enzyme mediated attack by the *si* face of the (*Z*)-3-*F*-PEP major isomer on the *si* face of 6-deoxy Alt-diNAc **8** aldehyde (Figure 4c), as previously observed for the *cj*Neu5Ac synthase,[51] would exclusively afford the 3-*F*-equatorial product. To determine if the minor (*E*)-3-*F*-PEP isomer was also a substrate for the enzyme we used a 5:1 *Z*:*E* 3-*F*-PEP mixture for ^{19}F-NMR studies, but observed no formation of 3-*F*-axial product (Figure S9), suggesting that akin to the *cj*Neu5Ac synthase,[51] the (*E*)-3-*F*-PEP isomer is not accepted as a substrate by *cj*PseI.

With 3-(*eq*)-*F*-Pse5Ac7Ac **10** synthesised on scale we set out to test its potential as an 'anti-motilin' in *C. jejuni* using plate-based motility assays (Figure 5a), using the Pse presenting 81116[52] laboratory strain. Bacteria were incubated with 100 µM 3-(*eq*)-*F*-Pse5Ac7Ac **10** for 1, 3 and 6 hours at 42 °C before inoculation onto motility agar plates and the halo diameter measured every 24 hours over the course 96 hours. Following incubation with the fluorinated sugar we observed a significant reduction in motility, with the effect more pronounced at longer incubation times, with cell viability studies (CFU) confirming treated *C. jejuni* were otherwise healthy. Notably, the greatest reduction in motility was observed 24 hours after plating, with an ~84% relative decrease in motility (3 hour incubation), recovering to ~51%, ~38%, and ~25% relative decreases in motility at 48, 72 and 96 hours respectively, potentially indicative of accumulating biosynthesis of new uninhibited Maf PseT, or degradation of the anticipated metabolic inhibitor CMP-3-(*eq*)-*F*-Pse5Ac7Ac **11**. Pre-incubation with 2 mM 3-(*eq*)-*F*-Pse5Ac7Ac **10** also led to a significant decrease in motility compared to untreated bacteria (Figure S17), but the lack of a concentration dependant response may allude to a saturation of a nonulosonic acid uptake pathway at the lower concentration.

To establish if the fluorinated CMP-donor **11** had accumulated *in vivo* we aimed to determine if recombinant PseF pseudaminic acid synthetase[42,53] could turnover the 3-(*eq*)-*F*-Pse5Ac7Ac **10** *in vitro*, whilst also providing a standard for metabolite analysis. LC-MS analysis of an initial PseF reaction provided preliminary evidence that a reaction between the fluorinated Pse **10** and co-factor CTP could indeed take place. We therefore opted to perform a scaled up chemoenzymatic synthesis of CMP-3-(*eq*)-*F*-Pse5Ac7Ac **11** in a one-pot two-enzyme reaction directly from 6-deoxy-AltdiNAc **8**, using PseI A127S and PseF in the presence of MnCl$_2$ and co-factors (*Z*)-3-*F*-PEP **9** and CTP (Figure 5b), affording the product **11** on a preparative scale (45 mg, 56%). Subsequent LC-MS analysis of metabolite extracts of *C. jejuni* cells treated with the fluorinated sugar **10** did reveal the intracellular presence of 3-(*eq*)-*F*-Pse5Ac7Ac **10** in treated samples (Figure S19), unequivocally demonstrating bacterial uptake of the unprotected pseudaminic acid sugar, but only trace evidence of CMP-3-(*eq*)-*F*-Pse5Ac7Ac **11** as a metabolite by comparison to our synthetic standard (Figure S20). We speculated that CMP-3-(*eq*)-*F*-Pse5Ac7Ac **11** may only be at low LC-MS detectable levels *in vivo* due to intracellular competition for PseF turnover between the endogenous substrate Pse5Ac7Ac **1** and fluorinated **10**. To explore this hypothesis, and assess the potential for *in vivo* metabolic conversion to the fluorinated CMP-donor in the presence of endogenous Pse5Ac7Ac **1**, we attempted to recapitulate this competition *in vitro* in an experiment using a 2:1 mixture of 3-(*eq*)-*F*-Pse5Ac7Ac **10** to Pse5Ac7Ac **1** and PseF and

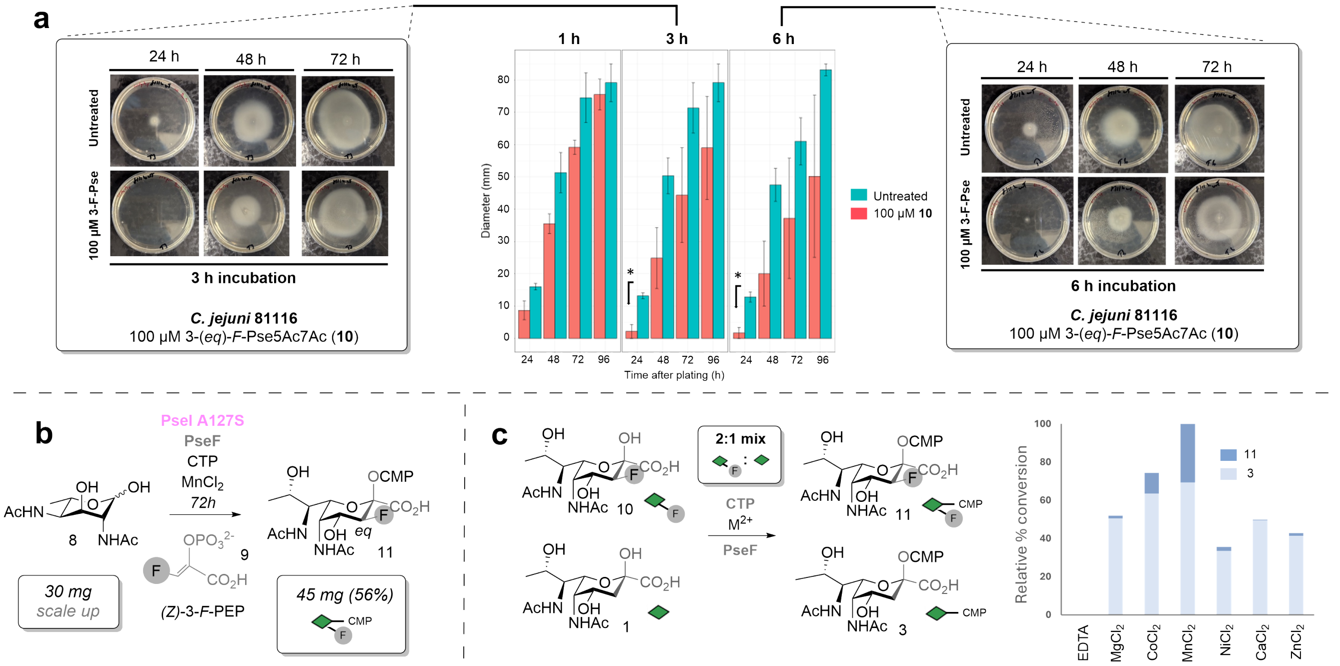

Figure 5. a. Motility-based assay of *C. jejuni* 81116 comparing treatment of 3-(*eq*)-*F*-Pse5Ac7Ac **10** (100 µM) to an untreated control. 81116 was grown overnight and adjusted to 0.05 OD_{600} nm. Bacteria were treated with 3-(*eq*)-*F*-Pse5Ac7Ac **10**, incubated at 42 °C under microaerobic conditions with shaking and sampled after 1, 3 and 6 hours. At each time point, 0.4% (w/v) MHA plates were inoculated in the centre with 10 µL of bacteria and incubated under microaerobic conditions (85% N_2, 10% CO_2 and 5% O_2). Halo diameter (mm) and photos taken over 96 hours. Data represents mean distance for 3 biological replicates ± SEM, each with 3 technical replicates. *Significant difference between groups determined by unpaired T-test. *, $p \leq 0.05$. **b.** One-pot two-enzyme scale up of CMP-3-(*eq*)-*F*-Pse5Ac7Ac **11** from 6-deoxy-AltdiNAc **8** and (*Z*)-3-*F*-PEP **9** using PseI A127S, PseF, CTP and $MnCl_2$. **c.** Screening of PseF with a 2:1 mix of 3-(*eq*)-*F*-Pse5Ac7Ac **10** and Pse5Ac7Ac **1** in the presence of different divalent metal ions.

limiting CTP for 6 h in the presence of various divalent metals that would be available to the enzyme intracellularly (Figure 5c). Intriguingly, little to no formation of the fluorinated product **11** was observed with most metal ions despite an excess of the fluorinated substrate **10**, with only incubation in the presence of Mn^{2+} affording appreciable CMP-3-(*eq*)-*F*-Pse5Ac7Ac **11** relative product formation (31%) albeit with CMP-Pse5Ac7Ac **3** still the dominant product (69%). This suggests that a biased contest *in vivo* between the endogenous and unnatural substrate in the final step of the CMP-donor biosynthesis could well constitute a bottleneck in accumulation of effective concentrations of CMP-3-(*eq*)-*F*-Pse5Ac7Ac **11** for Maf PseT inhibition. Thus highlighting potential future approaches for increasing the efficacy of this 'anti-motilin' strategy including bypass of PseF through direct use of a latently protected fluorinated CMP-donor, or exploration of 3-*F*-Pse analogues in search of better synthetase substrates, as has been recently observed in the case 3-*F*-Neu sugars.[30] Notably, we also demonstrated *in vitro* that, like the native nucleotide sugar,[41] CMP-3-*F*-Pse5Ac7Ac **11** is also able to act as a feedback inhibitor of the Pse biosynthetic pathway (Figure S22).

In conclusion, we have shown that the PseI pseudaminic acid synthase can be utilised in the chemoenzymatic synthesis of 3-fluorinated pseudaminic acid sugars and exclusively turns over the (*Z*)-isomer of 3-*F*-PEP to afford 3-(*eq*)-*F*-Pse5Ac7Ac **10**. Rational mutation of the A127 residue in the PseI active site increased the activity of the enzyme with the fluorinated cofactor ~3-fold compared to the wild type enzyme, whilst reducing activity -5-fold with the natural PEP cofactor. Subsequent treatment of *C. jejuni* 81116 with 3-(*eq*)-*F*-Pse5Ac7Ac as a metabolic 'anti-motilin' resulted in significant reduction in bacterial motility over a 96-hour period, potentially implicating *in vivo* inhibition of *O*-pseudaminylation by a CMP-3-*F*-Pse5Ac7Ac glycosyl donor, formation of which we demonstrated by PseF synthetase turnover *in vitro* even in competition with the natural substrate. Although direct inhibition by 3-(*eq*)-*F*-Pse5Ac7Ac cannot be discounted. This work thus showcases for the first time the potential utility of fluorinated pseudaminic acid sugars as chemical tools for dissecting and impairing bacterial motility, opening up the field for extension to other bacterial α-keto acid sugars.

Keywords: pseudaminic acid • fluorinated sugars • bacterial anti-motilin • chemoenzymatic synthesis • α-keto acid sugars • nonulosonic acids • rational mutagenesis • glycosyltransferases • sialic acid sugars

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