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The Engineering of Bacteria Bearing Azido-Pseudaminic Acid-Modified Flagella

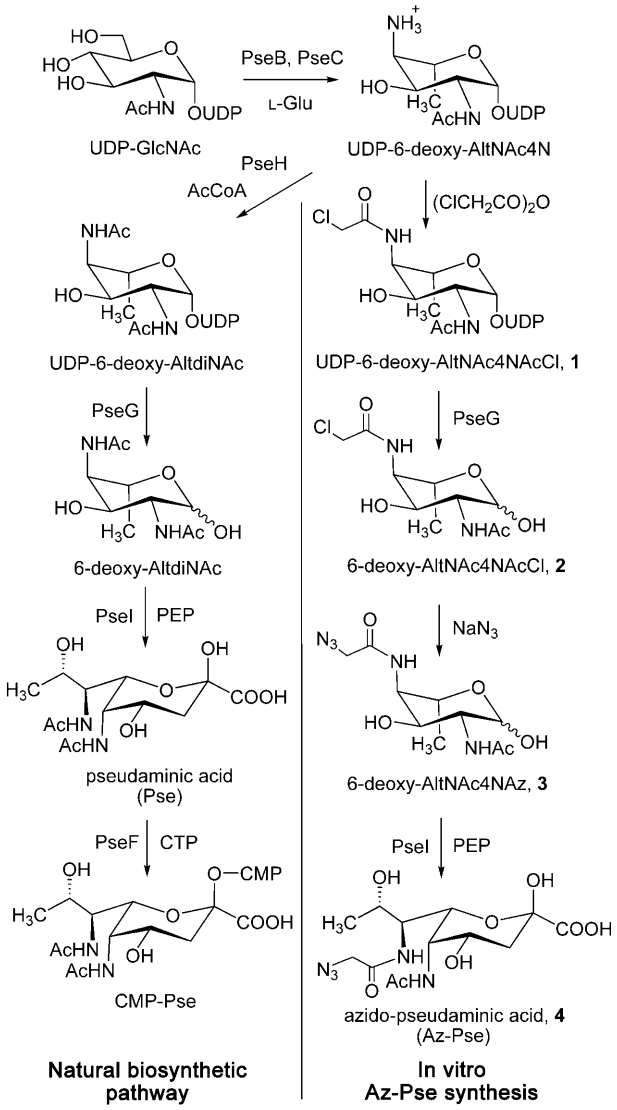
Feng Liu,[a] Annie J. Aubry,[b] Ian C. Schoenhofen,[b] Susan M. Logan,*[b] and Martin E. Tanner*[a]

Pseudaminic acid (Pse) is a nine-carbon α-keto acid that is related in structure to the sialic acids (Scheme 1).[1] Pse is found as an *O*-linked post-translational modification of the flagellin proteins in the pathogenic bacteria *Helicobacter pylori* (the causative agent of human ulcers) and *Campylobacter jejuni* (a major cause of food poisoning in humans).[2–4] The flagellins of these organisms are heavily glycosylated with as many as 19 sites of glycosylation on *C. jejuni* 81–176 flagellin and ten sites on *H. pylori* 1061 flagellin. Because glycosylation of the flagellin is required for the assembly of functional flagella, Pse biosynthesis is required for both motility and pathogenicity of these bacteria.

The display of Pse on bacterial flagella presents an exciting opportunity for cell surface engineering.[5–7] Pioneering studies from the Bertozzi group have shown that feeding azide-modified precursors to eukaryotic cells can result in the display of azide-modified sialic acids on their surface.[8,9] The azide group might then act as a bio-orthogonal chemical reporter, as these azido-sugars can be selectively modified by using reactions such as the Staudinger ligation.[10] This manuscript describes the feeding of an azido-labelled Pse precursor to mutant *C. jejuni* cells that are deficient in the Pse pathway. This results in the production of bacteria bearing flagella decorated with the bio-orthogonal chemical tag, azido-pseudaminic acid (Az-Pse, **4**, Scheme 1).

The biosynthetic pathway of CMP-pseudaminic acid (CMP-Pse) has recently been elucidated and begins with UDP-GlcNAc (Scheme 1).[11] The action of an inverting dehydratase, PseB, and an aminotransferase, PseC, serve to generate UDP-4-amino-4,6-dideoxy-β-L-AltNAc (UDP-6-deoxy-AltNAc4N).[12–16] An acetyltransferase, PseH, acetylates the free amino functionality and the hydrolase, PseG, cleaves the UDP–glycosidic linkage to give 2,4-diacetamido-2,4,6-trideoxy-L-altrose (6-deoxy-AltdiNAc).[17] This compound is condensed with phosphoenolpyruvate by pseudaminic acid synthase, PseI, to give Pse.[18] Finally, Pse is activated for protein glycosylation as a CMP-sugar by the cytidylyltransferase, PseF.[11]

The first goal of this project was to prepare a neutral precursor that might be taken up by *C. jejuni* cells and converted into an azido-labelled Pse derivative in vivo. The only uncharged compound in the Pse biosynthetic pathway is 6-deoxy-AltdiNAc and therefore 2-acetamido-4-azidoacetamido-2,4,6-trideoxy-L-Alt (6-deoxy-AltNAc4NAz; **3**), became the synthetic target (Scheme 1, in vitro Az-Pse synthesis). UDP-GlcNAc was treated with PseB and PseC to give UDP-6-deoxy-AltNAc4N, and this compound was chemically acetylated with chloroacetic anhydride to give UDP-2-acetamido-4-chloroacetamido-2,4,6-trideoxy-β-L-Alt (UDP-6-deoxy-AltNAc4NAcCl; **1**). Subsequent hydrolysis of the UDP-glycosidic linkage to give 2-acetamido-4-chloroacetamido-2,4,6-trideoxy-L-Alt (6-deoxy-AltNAc4NAcCl), **2**, was then achieved under very mild conditions



Scheme 1. The biosynthesis of CMP-pseudaminic acid (left) and the synthesis of azido-pseudaminic acid (right).

[a] *F. Liu, Prof. Dr. M. E. Tanner*
Department of Chemistry, The University of British Columbia
2036 Main Mall, Vancouver, B.C. V6T 1Z1 (Canada)
E-mail: mtanner@chem.ubc.ca

[b] *A. J. Aubry, Dr. I. C. Schoenhofen, Dr. S. M. Logan*
Institute for Biological Sciences, National Research Council
Ottawa, Ontario K1A 0R6 (Canada)
E-mail: susan.logan@nrc-cnrc.gc.ca

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by using the enzyme PseG. Treatment of compound **2** with sodium azide generated the desired precursor 6-deoxy-AltNAc4NAz, **3**. For compound **3** to be useful in generating azido-labelled Pse, it must serve as an alternate substrate for PseI. This was examined by incubating compound **3** with phosphoenolpyruvate (PEP), PseI, and Mn^{II} under in vitro conditions (Scheme 1, in vitro Az-Pse synthesis). This led to the clean production of Az-Pse **4**, which was fully characterized by ^{1}H NMR spectroscopy and mass spectrometry.

The generation of azido-labelled bacteria requires that Pse precursors be taken up from the media by *C. jejuni* cells. This was first tested with the natural precursor, 6-deoxy-AltdiNAc. *C. jejuni* 81–176 is motile, and produces a diffuse spreading pattern when grown in semisolid motility agar (Figure 1 A). In contrast, a mutant strain *C. jejuni* 81–176 *pseG::cat*, which is unable to produce the UDP-6-deoxy-AltdiNAc hydrolase PseG, lacks flagella and is completely nonmotile as evidenced by the small, sharply delineated colony that is restricted to the centre of inoculation within the motility agar plate (Figure 1 B).[19] When 6-deoxy-AltdiNAc is included in the motility agar (8 mM final concentration), the motility of *C. jejuni* 81–176 *pseG::cat* is restored as evidenced by a spreading diffuse growth pattern, but not to wild-type levels (Figure 1 C). This indicates that the Pse precursor is taken up from the medium at sufficient levels to generate functional flagella. To test the feasibility of generating azido-labelled bacteria in this manner, the same motility assay was performed by using the azido-labelled precursor, compound **3**. *C. jejuni* 81–176 *pseG::cat* cells grown in the presence of compound **3** also displayed the spreading diffuse growth pattern typical of motile bacteria (Figure 1 D). This clearly demonstrates that 6-deoxy-AltNAc4NAz **3** is also taken up by *C. jejuni* cells and is able to restore motility. Additionally, it indicates that the compound is accepted by all of the enzymes required to synthesize (PseI), activate (PseF), and transfer Pse to the flagellin monomer, as well as by the machinery required to assemble the flagellin monomers into functional flagella.

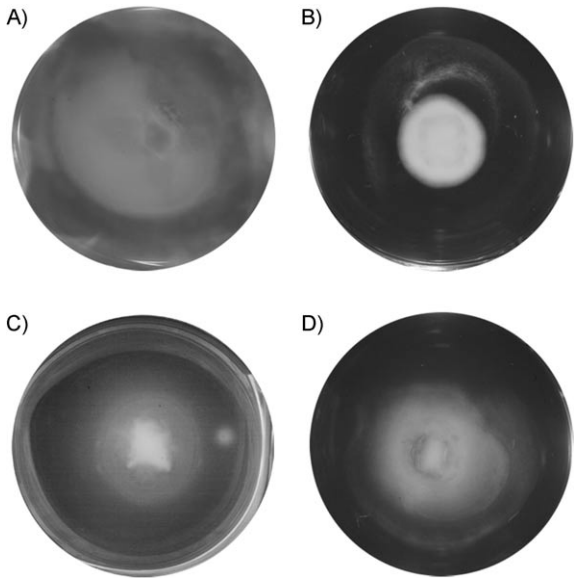
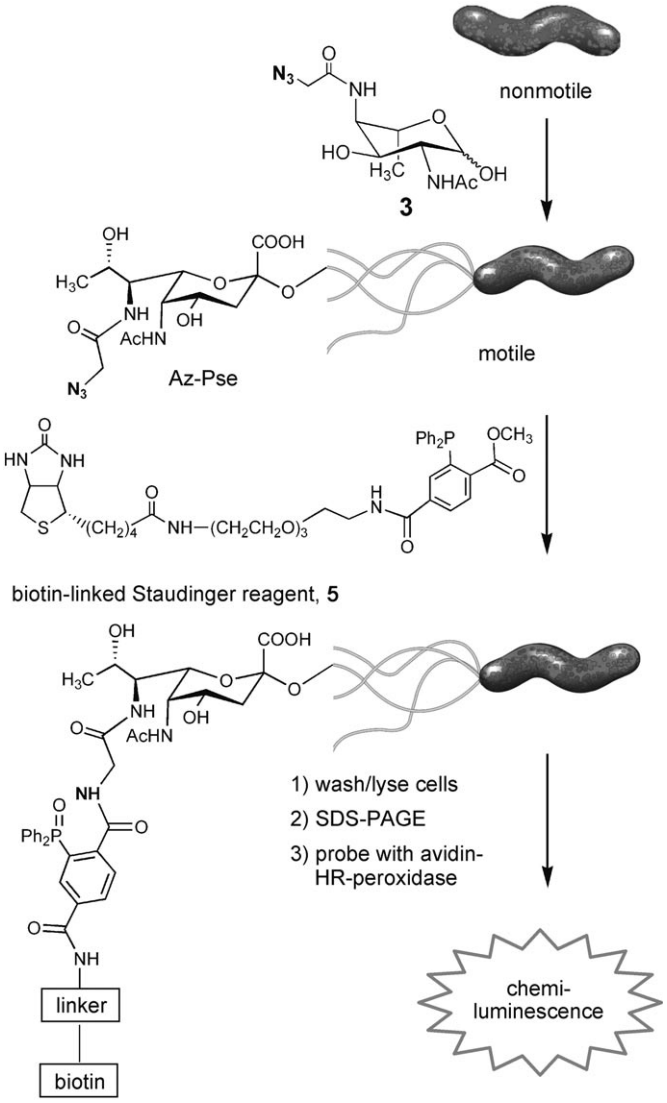


Figure 1. Motility of *C. jejuni* 81–176 and *C. jejuni* 81–176 *pseG::cat*. A) *C. jejuni* 81–176 in MH agar. B) *C. jejuni* 81–176 *pseG::cat* in MH agar. C) *C. jejuni* 81–176 *pseG::cat* in MH agar containing 6-deoxy-AltdiNAc. D) *C. jejuni* 81–176 *pseG::cat* in MH agar containing 6-deoxy-AltNAc4NAz.

To demonstrate that the azido functionality survived the in vivo conditions, and that Az-Pse was still amenable to chemical modification, the bacterial cell surface was probed with a biotin-linked Staudinger reagent, **5** (Scheme 2).[9,20–22] Following



Scheme 2. Generation of azido-pseudaminic-acid-bearing bacteria and probing with biotin-linked Staudinger reagent **5**.

overnight growth on MH agar containing 6-deoxy-AltNAc4NAz **3**, (8 mM final concentration), *C. jejuni* 81–176 *pseG::cat* and *C. jejuni* 81–176 cells were harvested into PBS containing Staudinger reagent **5** (2 mM final concentration) and reacted for 2 h at room temperature. Bacterial cells were collected by centrifugation and biotinylated proteins were detected by using Streptavidin-HRP on western blots of whole-cell lysates. A biotinylated protein was detected in both *C. jejuni* 81–176 *pseG::*

cat and *C. jejuni* 81–176 cells grown in the presence of **3** (Figure 2A, lanes 1 and 4). No streptavidin-reactive bands were present in either strain when grown in the absence of 6-deoxy-AltNAc4NAz (lanes 2 and 3). To confirm that this streptavidin-reactive band was flagellin, the whole-cell lysates were probed with an antiflagellin antibody (Figure 2B lanes 1–3) and the reactive bands in each strain were shown to have an identical migration pattern to the streptavidin-reactive band. This experiment demonstrates that the azido functionality was still intact and was displayed on the surface of the bacterial flagella. The absence of any other streptavidin-reactive proteins in whole-cell lysates of both *C. jejuni* 81–176 and *C. jejuni* 81–176 *pseG::cat* grown with **3** indicates that the flagellin proteins are the only cell-surface-associated proteins that are glycosylated with Pse. The incorporation of 6-deoxy-AltNAc4NAz in wild-type *C. jejuni* 81–176 flagellin demonstrates clearly that this substrate competes readily with the natural substrate and is well tolerated by the pathway biosynthetic enzymes.

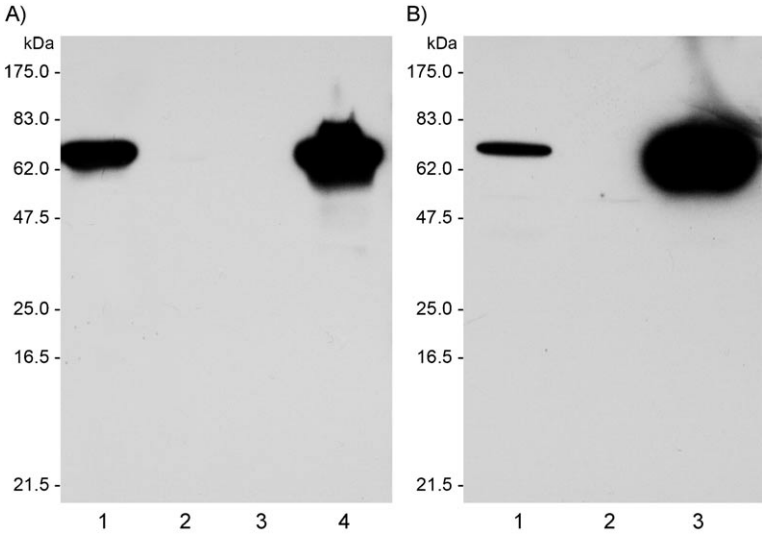


Figure 2. Incorporation of Az-Pse into the flagellin glycoprotein. A) Streptavidin-HRP detection of biotinylated flagellin in whole-cell lysates. Lane 1) *C. jejuni* 81–176 *pseG::cat* on MH medium containing 6-deoxy-AltNAc4NAz **3**, lane 2) *C. jejuni* 81–176 *pseG::cat* on MH medium, lane 3) *C. jejuni* 81–176 on MH medium, Lane 4) *C. jejuni* 81–176 on MH medium containing 6-deoxy-AltNAc4NAz **3**. B) Flagellin detection in whole-cell lysates by using antiflagellin single-domain antibody. Lane 1) *C. jejuni* 81–176 *pseG::cat* on MH medium containing 6-deoxy-AltNAc4NAz **3**, lane 2) *C. jejuni* 81–176 *pseG::cat* on MH medium, lane 3) *C. jejuni* 81–176 on MH medium.

The ability to incorporate reactive chemical handles selectively onto the flagella of pathogenic bacteria provides a useful tool for further studies. Such tags could be used for labeling of cells to be used in in vivo animal models of infection, biophysical studies of bacterial motility, or in studies probing flagella assembly and surface accessibility of glycan modifications on flagellin proteins. More exotic uses could include using labelled bacteria as drug delivery vehicles or taking advantage of the immunogenicity of the flagellin proteins in the development of novel conjugate vaccines. Current efforts are underway to visualize Az-Pse on the flagella of intact bacterial cells.

Experimental Section

Chemical synthesis: Experimental details describing the synthesis of compounds **1**–**5** can be found in the Supporting Information.

Motility assays: Strains were grown overnight on MH agar at 37 °C under microaerophilic conditions. Motility stab assays were performed in four-well tissue culture plates (Nunc, Fisher Scientific, Ottawa, Canada). Test wells contained 0.4% semi-solid MH agar (1 mL) or 0.4% semi-solid MH agar (1 mL) containing either 6-deoxy-AltdiNAc or 6-deoxy-AltNAc4NAz, **3** (8 mM final concentration). A loopful of an overnight culture was stab inoculated into the centre of the agar in each well and then incubated for 24 h at 37 °C under microaerophilic conditions. The growth pattern was observed and recorded.

Cell surface tagging of Az-Pse with Staudinger reagent, 5: *C. jejuni* 81–176 and *C. jejuni* 81–176 *pseG::cat* were inoculated onto MH agar or agar containing 6-deoxy-AltNAc4NAz (2 mL), **3** at 8 mM final concentration in 5 cm Petri plates (Nunc, Fisher Scientific). Following overnight growth at 37 °C, cells were harvested from the surface of the agar by using a loop and resuspended in PBS that contained Staudinger reagent **5** (2 mM final concentration) and reacted for 2 h at RT. Bacterial cells were then collected by centrifugation, washed twice in PBS and lysed in SDS-PAGE solubilisation buffer by heating to 100 °C for 10 min.

Streptavidin HRP detection of biotinylated Az-Pse: Following SDS-PAGE of whole-cell lysates on 12.5% acrylamide gels as described by Laemmli,[23] proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Biorad, Mississauga, ON, Canada) as described by Towbin et al.[24] Western blotting was performed by using Streptavidin-HRP (Cedar Lane, Hornby, ON, Canada) and developed with a chemiluminescence kit (Pierce, Rockford, IL, USA) according to the manufacturer's directions. The theoretical masses of the unglycosylated proteins FlaA and FlaB are 59543 and 59704 Da, respectively. A Coomassie-blue-stained SDS-PAGE gel of these samples is shown in Figure S5.

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Keywords: azides · *C. jejuni* · cell surfaces · flagella · metabolism · Staudinger reaction

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