



Flagellin glycosylation with pseudaminic acid in *Campylobacter* and *Helicobacter*: prospects for development of novel therapeutics

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Abstract Many pathogenic bacteria require flagella-mediated motility to colonise and persist in their hosts. *Helicobacter pylori* and *Campylobacter jejuni* are flagellated epsilonproteobacteria associated with several human pathologies, including gastritis, acute diarrhea, gastric carcinoma and neurological disorders. In both species, glycosylation of flagellin with an unusual sugar pseudaminic acid (Pse) plays a crucial role in the biosynthesis of functional flagella, and thereby in bacterial motility and pathogenesis. Pse is found only in pathogenic bacteria. Its biosynthesis via six consecutive enzymatic steps has been extensively studied in *H. pylori* and *C. jejuni*. This review highlights the importance of flagella glycosylation and details structural insights into the enzymes in the Pse pathway obtained via a combination of biochemical, crystallographic, and mutagenesis studies of the enzyme–substrate and –inhibitor complexes. It is anticipated that understanding the underlying structural and molecular basis of the catalytic mechanisms of the Pse-synthesising enzymes will pave the way for the development of novel antimicrobials.

Keywords Biosynthetic pathway · Bacterial flagellum · O-linked glycosylation · Crystal structure · Small molecule inhibitor · Bacterial virulence · Bacterial motility

Introduction

Helicobacter pylori and *Campylobacter jejuni* are representative members of the epsilon (ϵ) Proteobacteria. *H. pylori* and *C. jejuni* infections cause a broad range of human and animal diseases. *H. pylori* is found primarily in humans, where it colonises gastric mucosa. *H. pylori* is the etiological agent of dyspepsia, gastritis, duodenal and gastric ulcers, mucosa-associated lymphoid tissue lymphoma and gastric carcinoma [1–6]. In addition, association between *H. pylori* infection and extra-gastric diseases including iron-deficiency anaemia [7, 8], idiopathic thrombocytopenic purpura [9, 10], cardiovascular diseases [11], chronic liver disorder [12], pancreatic cancer [13], chronic respiratory illness [14], skin diseases [15] and diabetes [16] has been reported. The presence of *H. pylori* significantly affects the natural microecology of the stomach [17]. It is the first bacterium to be classified as a group I (definite) carcinogen for human gastric cancer by the International Agency for Research on Cancer [18]. Around 75% of gastric cancer and 90% of duodenal ulcer patients had a previous history of *H. pylori* infection [19, 20]. The clinical outcome of *H. pylori* infection is determined by a complex interplay between the genetic properties of the bacteria and host genetic factors [21], and may be influenced by co-infection with other bacterial pathogens [22, 23].

The most common mode of transmission is thought to be by person-to-person contact, via gastro-oral, oral–faecal, and oral–oral routes [24, 25], although the full spectrum of *H. pylori* transmission routes is yet to be determined. Multiple socioeconomic, environmental and behavioural factors contribute to the acquisition and spread of infection. These factors include low socioeconomic status [26, 27], diet [28], use of tobacco [29], a higher number of siblings [30] and a lower educational status of the parents [31,

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32]. The prevalence of *H. pylori* infection increases with age [33] and is significantly higher in developing countries compared to developed countries. About 80% of individuals in developing countries harbour this bacterium, compared with 15–50% of the population in developed ones [34, 35]. For example, a 2011 nationwide study in Australia showed that the prevalence of *H. pylori* was 15% among adults [35]. The occurrence of *H. pylori* infection has been decreasing in developed countries [36, 37], largely due to improving hygiene practices and higher standards of living. However, the relative prevalence in developed countries remains significantly higher among people with lower socioeconomic status, such as indigenous and migrant populations [35, 38].

Currently, there is no effective vaccine or single drug available to treat *H. pylori* infection. The common therapy for the treatment of such infections is a combination of a proton pump inhibitor and two or three antibiotics [39, 40]. However, the effectiveness of this therapy has significantly declined, mainly due to the widespread increase in resistance to clarithromycin and metronidazole [41, 42].

Campylobacter jejuni is a zoonotic pathogen that usually colonises the gut of birds and mammals, but can also infect humans, with variable clinical outcomes including mild, self-limiting, non-inflammatory diarrhea, severe, inflammatory, bloody diarrhea with pyrexia, abdominal cramps, inflammatory bowel disease, Barrett's oesophagus and irritable bowel syndrome [43]. *C. jejuni* infections are also associated with acute cholecystitis and celiac disease [43]. The sequelae of the infection include neurological and autoimmune diseases such as Guillain-Barré syndrome [44], Reiter syndrome [45] and reactive arthritis [46].

The prevalence of *C. jejuni* varies significantly between countries. In developed countries, human infections occur sporadically and at a low frequency [47, 48]. In developing countries, campylobacteriosis is endemic, and asymptomatic infections are common [47, 49]. It is believed that contaminated poultry is the main source of *C. jejuni* infection [47, 50, 51].

Factors that contribute to the pathogenicity and virulence of *H. pylori* include the *cag* pathogenicity island [52], vacuolating cytotoxin A [53], adhesins (including blood-antigen binding protein A [54], sialic acid-binding adhesin [55], heat-shock protein 60 [56], adherence-associated proteins [57], outer membrane protein HopZ [58], *N,N'*-diacetyllactosamine-binding adhesin [59] and neutrophil-activating protein [60]), duodenal ulcer promoting gene [61], urease [62], and γ -glutamyl transpeptidase [63]. Furthermore, chemotaxis [64] and flagella-mediated motility [64, 65] have been shown to also play an important role in the development of a robust infection.

In *C. jejuni*, a diverse group of virulence factors including flagella-mediated motility, chemotaxis, iron acquisition, adhesion, invasion of epithelial cells and quorum sensing

contributes to the development of successful infection [64, 66, 67]. The role of motility and chemotaxis in *C. jejuni* pathogenesis has been the subject of intensive studies, particularly since it has been shown that flagellin biosynthesis and modification of genes are important for colonisation [68, 69].

Bacteria use flagella-driven motility to relocate toward favourable environments and away from toxic chemicals. Flagella-mediated motility allows *H. pylori* and *C. jejuni* to penetrate the gastric/intestinal mucus and reach the underlying layer of epithelial cells, where they colonise. In addition, these bacteria rely on their high motility in the viscous layer covering the gastric mucosa of the host to persist despite the natural flow of the gastrointestinal mucus [64]. *H. pylori* uses motility to colonise the stomach [70] and to establish robust infection [71]. Chemotaxis plays an important role in this, as it enables *H. pylori* to sense, and responds to, changes in various conditions, including pH, concentration of urea/ammonium and cellular energy status [64]. Deletion of motility-associated genes in *C. jejuni* and *H. pylori* was shown to result in attenuated growth in animal models [71, 72]. Furthermore, wild-type *H. pylori* was shown to have a significantly lower minimum infectious dose in animal models than its non-motile or nonchemotactic mutants [71, 73].

***Helicobacter* and *Campylobacter* flagella**

Helicobacter pylori and *C. jejuni* possess unipolar and bipolar flagella, respectively [66, 74, 75]. The flagellum is assembled from ~ 40 different proteins [76, 77] and has three components: a membrane-embedded basal body, a hook, and a long filament [78, 79]. Interestingly, each *H. pylori* flagellar filament is covered by a sheath (extension of the outer membrane) which is believed to serve as a protective shield against the low pH of the human stomach [80]. The basal body together with the stator proteins serves as motor that turns the extracellular helical-shaped filament at its base. Cryo-electron tomography studies revealed that the *H. pylori* basal body possesses a unique periplasmic cage-like structure [75]. This cage is not present in *C. jejuni*; however, *C. jejuni* possesses an additional, large periplasmic basal disk at a similar position [81]. It was proposed that these additional structures serve as a robust scaffold for recruitment of stator complexes to the motor, an important feature thought to be linked to the high motility of *H. pylori* and *C. jejuni* in viscous environments [75, 81].

The central rod component of the basal body is connected via a hook to a helical filament. The flagellar filament is composed of two proteins, the major flagellin FlaA and the minor flagellin FlaB [82]. The flagellins are exported via the type III secretion apparatus within the basal body following

translation and post-translational modification in the cytoplasm [83].

The role of FlaA and FlaB in the biosynthesis of fully functional flagella in *C. jejuni* and *H. pylori* has been investigated [84, 85]. In *H. pylori*, FlaA (53 kDa) and FlaB (54 kDa) show around 56% amino acid sequence identity [85]. A mutagenesis study revealed that isogenic *H. pylori* *flaA* mutants were aflagellated and non-motile, while the *flaB* mutants were flagellated but only partially motile [86, 87]. The *C. jejuni* FlaA (60 kDa) and FlaB (60 kDa) are more similar to each other (92–95% sequence identity) [82]. The *C. jejuni* *flaA* mutant had a truncated flagellum and was unable to colonise the host [88]. Although the *C. jejuni* *flaB* gene is not essential for motility [87], its product was reported to play an important role in the defence of *C. jejuni* against bacteriophage infection [89]. In 1999, it became evident that flagellins of *Helicobacter* and *Campylobacter* spp. are glycosylated [87, 90]. *C. jejuni* flagellins are among the most heavily glycosylated proteins identified to date, with the carbohydrate moieties contributing up to ~ 10% of the total molecular weight [91–93].

Protein glycosylation

Glycosylation (a covalent addition of sugar moieties) of proteins is a ubiquitous co-translational or post-translational modification occurring in all kingdoms of life. More than two-thirds of eukaryotic proteins are believed to be glycosylated [94]. Since the discovery of glycoproteins in archaea *Halobacterium* sp. and hypothermophilic bacteria *Clostridium* sp. [95, 96], protein glycosylation in microorganisms has been studied extensively, yielding insights into the structure of the oligosaccharide building blocks of the glycan moieties and the mechanisms of glycosylation [97].

Classification of glycosylation

The three most common types of protein glycosylation, classified according to the nature of the atom of the amino acid to which the sugar moiety is attached, are (1) N-linked, (2) C-linked, and (3) O-linked glycosylation.

N-linked glycosylation

N-linked glycosylation is a common type of post-translational modification of secreted or membrane-embedded proteins, where a sugar molecule is attached to a nitrogen atom (usually the N4 atom of asparagine residues) by oligosaccharyltransferase (OTase) [98]. N-glycosylation contributes to folding, stability, and function of a wide range of proteins that play a role in the regulation of cell

differentiation, cell signalling and pathogenesis [99–101]. In eukaryotes, the assembly of the building blocks of the glycan occurs at the endoplasmic reticulum (ER) membrane, while in prokaryotes, this process takes place at the plasma membrane. Preassembled blocks of 14 sugars, containing two *N*-acetylglucosamine, nine mannose, and three glucose residues, are transferred onto the lipid anchor to generate a lipid-linked oligosaccharide (LLO). The LLO is then flipped from the cytosolic side to the luminal face of the eukaryotic ER or to the outer layer of the plasma membrane in prokaryotes, where it serves as a glycosyl donor for the transfer reaction catalysed by OTase [102]. Proteins harbouring the conserved sequence Asn-X-Ser/Thr/Cys in eukaryotes [98, 103] and Asp/Glu-X-Asn-X-Ser/Thr in bacteria [104, 105] act as acceptors in the ER lumen or the bacterial extracytoplasmic space, respectively [102]. Following the transfer, the N-glycan can be modified via terminal glycosylation which gives rise to its structural diversity.

C-linked glycosylation/C-mannosylation

C-linked glycosylation (also known as C-mannosylation) is a relatively rare event that is defined as the covalent attachment of mannose by specific mannosyltransferase to the indole C2 carbon atom of a tryptophan residue on an acceptor protein via a C–C link [106, 107]. This type of glycosylation has been reviewed elsewhere [108, 109]. C-mannosylation is restricted to mammals and related species [110]. The consensus motif for C-linked glycosylation is Trp-X-X-Trp or Trp-X-X-Cys/Phe (where X can be any residue except proline), and the addition of the mannose sugar usually occurs at the first Trp residue [108, 111, 112], although in a motif (Trp-X-X-Trp-X-X-Trp) C-mannosylation can occur on all tryptophan residues [110, 113]. This type of post-translational modification plays a role in protein folding, stability and cell signalling mechanisms [109, 110].

O-linked glycosylation

O-glycosylation is a covalent linkage of a sugar to the side-chain hydroxyl oxygen of serine, threonine, tyrosine, hydroxylysine or hydroxyproline residue [114, 115]. Proline-rich sequences (Thr-Ala-Pro-Pro, Thr-Val-X-Pro, Ser/Thr-Pro-X-Pro and Thr-Ser-Ala-Pro, where X can be any amino acid) are usually preferred for O-glycosylation [114]. However, the consensus sequence of O-glycosylation is yet to be identified.

O-glycosylation in eukaryotes takes place in the Golgi apparatus, ER, and cytoplasm [116–118] and involves the sequential addition of nucleotide-activated monosaccharides to acceptor proteins [116]. Sugar units can be of a different chemical nature, which is the source of diversity of O-linked glycan chains. O-linked *N*-acetylgalactosamine glycans

(O-GalNAc) are the most abundant type. They are commonly found in, for example, mucins, fetuin, gonadotropins, and glycoporphins [114, 119]. O-linked glycans incorporating *N*-acetylglucosamine, fucose, xylose, galactose, mannose, glucose, and arabinose have also been found [119, 120].

In prokaryotes, O-glycosylation is believed to occur in the cytoplasm or inner membrane [116]. Two distinct mechanisms of bacterial O-glycosylation have been identified: OTase-dependent and OTase-independent [115]. As the first step of the OTase-dependent O-glycosylation, an initiating glycosyltransferase (GT) catalyses the attachment of the first nucleotide-activated monosaccharide to the membrane-embedded lipid carrier (undecaprenolphosphate) on the cytoplasmic side of the inner membrane of the cell [121]. Further individual monosaccharide groups are subsequently added one by one by different GTs. Then the undecaprenolphosphate-linked glycan is flipped onto the periplasmic side of the membrane and transferred onto the acceptor protein by OTase [121]. The OTase-dependent O-glycosylation pathway appears to exist only in Gram-negative bacteria [122]. A wide range of structurally and functionally diverse membrane-associated proteins, including the components of type IV pili, has been reported to be glycosylated through this mechanism [115, 123]. OTase-independent O-glycosylation, where nucleotide-activated monosaccharides are directly transferred onto acceptor proteins by cytoplasmic GTs, has been observed for proteins that are exported to the outer membrane (e.g. adhesins, autotransporters) or secreted by the basal body [115]. One important function of O-glycosylation is to help pathogenic bacteria to evade host defence mechanisms [92, 93, 115, 124, 125]. The remaining part of this review focuses on the OTase-independent O-glycosylation of flagellins in the representative members of ϵ -Proteobacteria *C. jejuni* and *H. pylori*.

Pseudaminic acid biosynthesis pathway

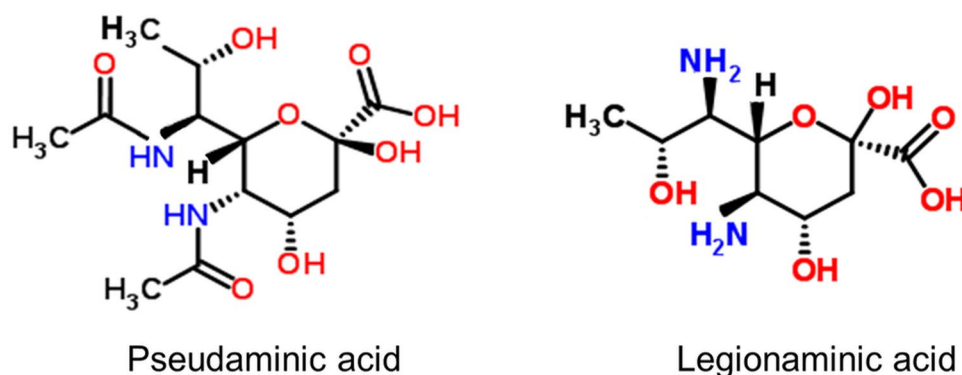
Flagellin modification via O-linked glycosylation has been characterised in many bacteria, including *H. pylori* and *C.*

jejuni [91, 115, 126]. *C. jejuni* FlaA is glycosylated at up to 19 sites [93]; *C. jejuni* FlaB is also glycosylated, but the exact number of sites remains to be established [127]. FlaA and FlaB in *H. pylori* are glycosylated at seven and ten sites, respectively [92]. *H. pylori* decorates its flagellins with glycans that contain only one type of sugar, the sialic acid-like nonulosonate pseudaminic acid (Pse, 5,7-diacetamido-3,5,7,9-tetra-deoxy-*L*-glycero- α -*L*-manno-2-nonulopyranosonic acid) [91]. In contrast, O-linked glycans attached to *C. jejuni* flagellins can contain more than one type of sugar, including Pse, legionaminic acid and related derivatives of nonulosonate [93] (Fig. 1). The Pse biosynthesis has been extensively studied in *H. pylori* and *C. jejuni*. Nucleotide-activated pseudaminic acid is synthesised via six consecutive enzymatic steps, illustrated in Fig. 2 [128, 129], and then transferred onto flagellins via O-linked glycosylation.

Step one

The first step of the Pse biosynthesis pathway is catalysed by the uridine-5'-diphosphate *N*-acetylglucosamine (UDP-GlcNAc) 5-inverting 4,6-dehydratase (also known as pseudaminic acid biosynthesis protein B, PseB, EC 4.2.1.115). PseB is a bifunctional enzyme that belongs to the short-chain dehydrogenase/reductase (SDR) superfamily and uses nicotinamide adenine dinucleotide phosphate (NADP⁺) as a cofactor [130]. PseB catalyses oxidation at the C-4'' atom of the nucleotide sugar UDP-GlcNAc by adding a ketone group, and a subsequent reduction at the C-6'' atom to generate UDP-2-acetamido-2,6-dideoxy- β -*L*-arabino-4-hexulose (UDP-6-deoxy-4-keto-HexNAc) (Fig. 2) [130–132]. PseB from *H. pylori* and *C. jejuni* share 63% amino acid sequence identity and follow the same catalytic mechanism [131]. In *H. pylori*, this enzyme is also involved in the alteration of the O-antigen composition in the lipopolysaccharide (LPS) and the modulation of the urease activity, in addition to its role in the flagellin glycosylation [133]. Inactivation of the *pseB* gene in *H. pylori* resulted in an aflagellated non-motile phenotype which suggested that

Fig. 1 Structures of pseudaminic acid and legionaminic acid



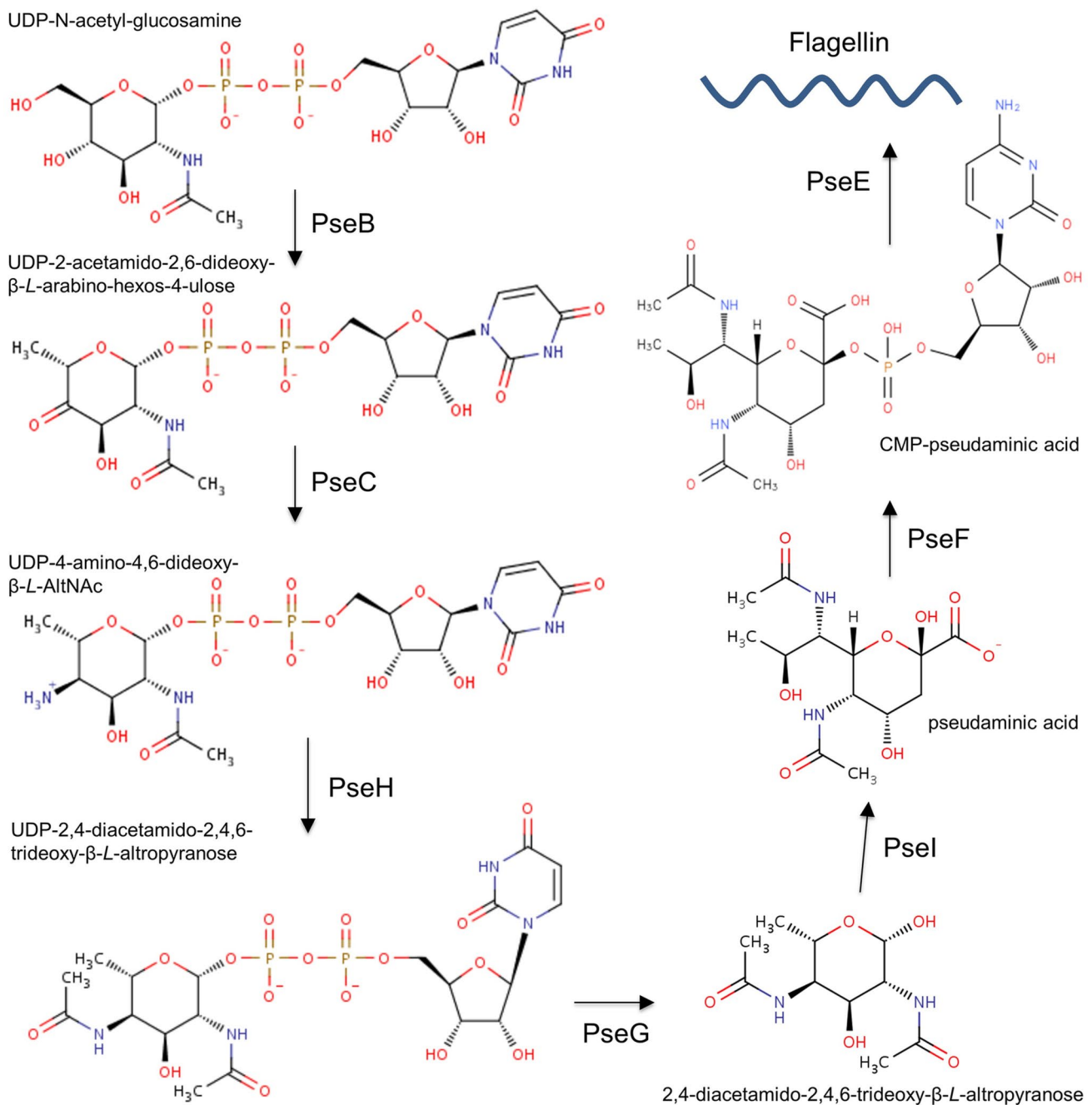


Fig. 2 The Pse biosynthesis pathway in *H. pylori* and *C. jejuni* [129]

PseB likely plays an important role in bacterial pathogenesis and colonisation [133].

Helicobacter pylori PseB (HpPseB, 333 aa) exists as a hexamer both in solution and in crystal [132]. Each HpPseB monomer consists of two lobes: an N-terminal large lobe (residues 1–174, 208–234, and 265–317) and C-terminal small lobe (residues 175–207, 235–264, and 318–333) (Fig. 3). The N-terminal lobe adopts a Rossmann fold with four additional β -strands, while the C-terminal

lobe harbors three α -helices and two β -strands. Detailed structural analysis of the HpPseB/NADP/UDP-N-acetyl-glucosamine complex revealed that the cofactor binds to the Rossmann fold part of the N-terminal lobe, whereas the sugar substrate binding site is located in the C-terminal lobe (Fig. 3). Biophysical and mutagenesis studies confirmed the presence of three catalytic residues (D132, K133, and Y141) in HpPseB, one of which (K133,

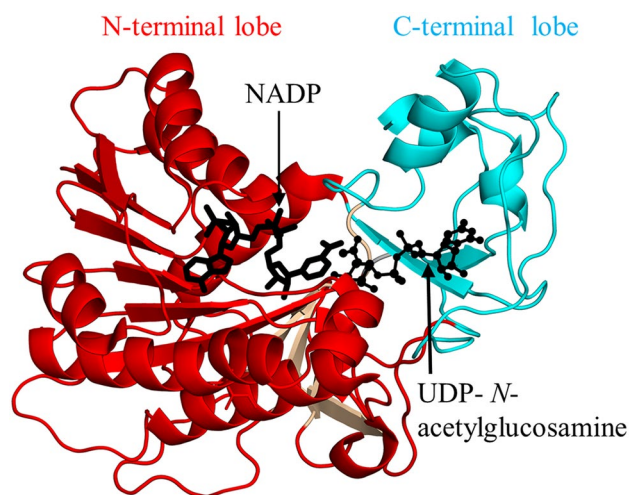


Fig. 3 Cartoon representation of the structure of *H. pylori* PseB in complex with nicotinamide adenine dinucleotide phosphate (NADP⁺) and uridine-diphosphate-*N*-acetylglucosamine (PDB ID: 2GN6 [132]). The part of the N-terminal domain that has the Rossmann fold is coloured cyan, and the additional four β -strands are coloured wheat. The NADP molecule is drawn as black sticks, the substrate is drawn using a ball-and-stick representation

equivalent to K127 in *C. jejuni* PseB) is believed to serve as both a catalytic acid and base during the reaction [131, 132].

Step two

The second step of the Pse biosynthetic pathway (Fig. 2), axial transfer of an amino group onto the C4 atom of UDP-6-deoxy-4-keto-HexNAc to produce UDP-4-amino-4,6-dideoxy- β -L-AltNAc, is catalysed by the UDP-4-amino-4,6-dideoxy-*N*-acetyl- β -L-altrosamine transaminase, also known as pseudaminic acid biosynthesis protein C, PseC (EC 2.6.1.92). This enzyme belongs to the pyridoxal 5'-phosphate (PLP)-dependent transferase superfamily [128, 134]. PseC uses PLP as a cofactor and L-glutamate as an amino group donor [134, 135]. Insertional inactivation of the *H. pylori* *pseC* gene (HP0366) produced a non-motile phenotype that did not produce flagella and lacked O-antigen [133]. In addition, an isogenic *H. pylori* *pseC* mutant showed reduced invasion of human gastric epithelial cells. *H. pylori* PseC (HpPseC) shares no homology with any of the genes for the biosynthesis of the related nine-carbon sugar, sialic acid, in humans and, therefore, it represents a potential target for the design of novel anti-*H. pylori* therapeutics [136].

Biochemical and structural analyses have shown that HpPseC exists as a homodimer both in solution and in crystal [136]. The HpPseC monomer comprises two domains (Fig. 4). The N-terminal domain (residues 13–245) has a central mixed seven-stranded β -sheet, in which the β -strands are arranged in the topological order

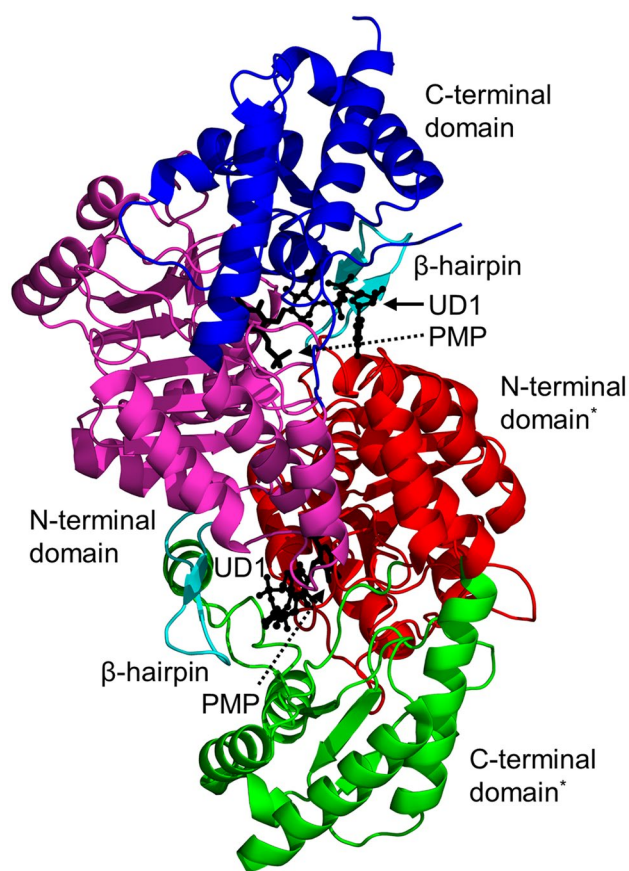


Fig. 4 Cartoon representation of the structure of the *H. pylori* PseC dimer (PDB ID: 2FNU [136]). The N- and C-terminal domains of one of the two monomers are coloured green and magenta, respectively. The β -hairpin involved in domain swapping is coloured cyan; the bound pyridoxamine-5'-phosphate (PMP) cofactor is drawn as black sticks; the product UDP-4-amino-4,6-dideoxy- β -L-AltNAc (UD1) is shown using a ball-and-stick representation. The star (*) indicates domains from the second monomer of the PseC homodimer

$\uparrow\beta 1-\downarrow\beta 7-\uparrow\beta 6-\uparrow\beta 5-\uparrow\beta 4-\uparrow\beta 2-\uparrow\beta 3$. The β -sheet is flanked on either side by several α -helices. This domain harbors a β -hairpin structure (residues 211–225 on $\uparrow\beta 8$ and $\downarrow\beta 9$) that is projected away from one monomer and interacts with the second monomer at the dimer interface (Fig. 4) [136]. The C-terminal domain (residues 1–12 and 246–374) contains an antiparallel β -sheet with the strands arranged in the order $\uparrow\beta 10-\downarrow\beta 11-\uparrow\beta 12$. This β -sheet is sandwiched between three α -helices on one side and the edge of the central β -sheet in the N-terminal domain on the other side. Two large and deep active site cavities are located at the dimer interface, and both monomers provide the residues that form the walls of these cavities (Fig. 4) [136]. Detailed crystallographic analysis of the HpPseC/PLP and HpPseC/PLP/UDP-4-amino-4,6-dideoxy- β -L-AltNAc (reaction product) complexes revealed that the cofactor PLP binding site is located at the bottom of the cavity near the C-terminal edge of the central β -sheet.

The UDP-sugar binds at a different part of the pocket, connected to the bottom of the PLP binding cavity, and interacts with residues from both monomers. Site-directed mutagenesis has confirmed the crucial role of K183 (equivalent to K181 in *C. jejuni* PseC) in the catalytic mechanism of HpPseC [135, 136].

Step three

The third step of the Pse biosynthesis pathway (Fig. 2) is catalysed by a nucleotide sugar-linked *N*-acetyltransferase, also known as pseudaminic acid biosynthesis protein H (PseH), or flagellin modification protein H (FlmH) (EC 2.3.1.202). It acetylates at the C4 of the UDP-sugar produced in the previous step, using acetyl-CoA (AcCoA) as an acetyl donor, and produces UDP-2,4-diacetamido-2,4,6-trideoxy- β -L-altrose (UDP-6-deoxy-AltDiNAc) [128]. Mutational inactivation of the *C. jejuni* *pseH* gene resulted in the inhibition of the flagellum assembly, thereby rendering bacteria non-motile. This finding suggests that PseH plays a crucial role in bacterial motility and, likely, virulence [124, 137]. PseH is a member of the general control non-repressible 5 (GCN5)-related *N*-acetyltransferases (GNAT) superfamily [138], representatives of which are present in all kingdoms of life. A common feature of the GNAT enzymes is a V-shaped active site cavity at the central β -sheet and a P-loop that interacts with the pyrophosphate arm of the acetyl-CoA (AcCoA) cofactor [138]. Most members of this superfamily follow the direct acetyl transfer mechanism that proceeds via formation of a tetrahedral intermediate [138].

Helicobacter pylori PseH (HpPseH) (21.4 kDa) is dimeric in solution and in crystal [129, 139]. X-ray crystallographic analysis of the PseH/AcCoA complex revealed that each monomer has a core β -sheet made up of eight β -strands which are arranged in the topological order $\uparrow\beta_0\text{--}\downarrow\beta_1\text{--}\uparrow\beta_2\text{--}\downarrow\beta_3\text{--}\uparrow\beta_4\text{--}\uparrow\beta_5\text{--}\downarrow\beta_7\text{--}\uparrow\beta_6$ (Fig. 5) [129]. The central β -sheet is flanked by three α -helices on each side. The cofactor AcCoA binds at the V-shaped cavity between strands β_4 and β_5 , so that its pyrophosphate arm interacts with the P-loop between the β_4 -strand and α_4 -helix. Analysis of the modelled structure of the PseH/substrate/cofactor complex suggested that the nucleotide- and 4-amino-4,6-dideoxy- β -L-AltNAc-binding pockets are the elements that contribute to the substrate specificity most. Furthermore, a hydrophobic pocket harbouring the 6'-methyl group of the altrose determines preference to the methyl over the hydroxyl group [129]. Examination of the conservation of the amino acid residues in the enzyme active site suggested that PseH follows the common GNAT catalytic mechanism that involves direct acetyl transfer from AcCoA without an acetylated enzyme intermediate, and that S78 and Y138 likely act as a general base and acid in the PseH-catalysed

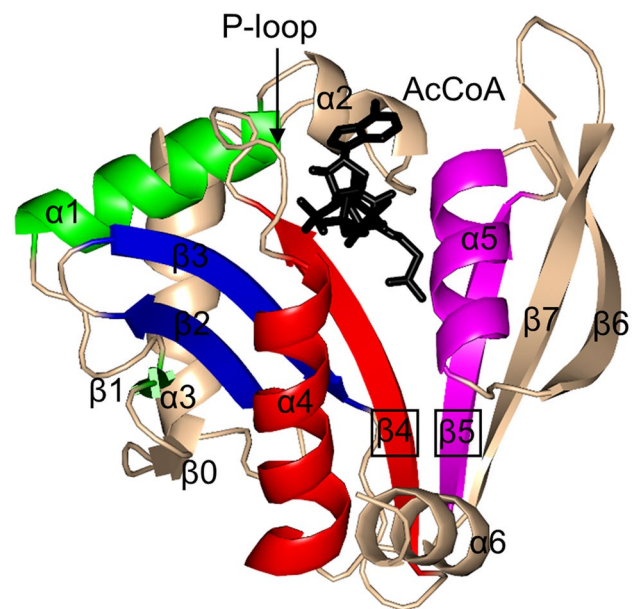


Fig. 5 Cartoon representation of the structure of *H. pylori* PseH in complex with AcCoA (PDB ID: 4RI1 [129]). The motifs that are conserved across all GNAT enzymes are coloured as follows: motif C—green, motif D—blue, motif A—red, motif B—magenta. Non-conserved N-terminal and C-terminal regions are coloured wheat. The AcCoA cofactor is drawn in black using a stick representation

reaction. The crystal structure of a homolog from *C. jejuni* with a similar fold has also been reported [140].

Step four

The fourth step of the Pse pathway (Fig. 2) is catalysed by an inverting nucleotide sugar hydrolase (UDP-6-deoxy-AltDiNAc hydrolase, PseG, EC 3.6.1.57) that belongs to the glycosyltransferase B (GT-B) family [141]. The enzyme hydrolyses UDP-6-deoxy-AltDiNAc to generate the free sugar 2,4-diacetamido-2,4,6-trideoxy- β -L-altropyranose [142]. The enzyme also inverts the stereochemistry at the C-1 atom of the substrate [128, 143]. Insertional inactivation of the *H. pylori* *pseG* (HP0326B) gene abolished flagellin production and resulted in a non-motile phenotype [92].

Biochemical and biophysical analyses showed that *C. jejuni* PseG (CjPseG, 282 aa) exists as a monomer both in solution and in crystal [142]. Structural analysis of CjPseG revealed that it has two domains: N-terminal domain (residues 1–142) and C-terminal domain (residues 153–282), connected by a short α -helix (residues 143–152) [142]. The N-terminal domain harbors a parallel β -sheet made up of seven β -strands ($\uparrow\beta_3\text{--}\uparrow\beta_2\text{--}\uparrow\beta_1\text{--}\uparrow\beta_4\text{--}\uparrow\beta_5\text{--}\uparrow\beta_6\text{--}\uparrow\beta_7$), sandwiched between two α -helices on one side and three on the other (Fig. 6). The C-terminal domain contains a six-stranded parallel β -sheet, where strands are arranged in the order $\uparrow\beta_{10}\text{--}\uparrow\beta_9\text{--}\uparrow\beta_8\text{--}\uparrow\beta_{11}\text{--}\uparrow\beta_{12}\text{--}\uparrow\beta_{13}$, and sandwiched

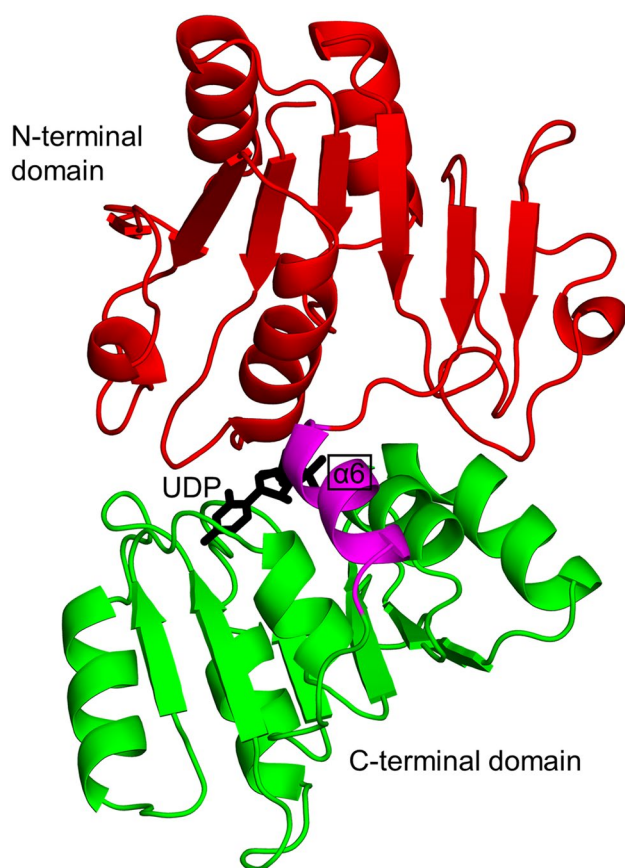


Fig. 6 Cartoon representation of the structure of *C. jejuni* PseG in complex with uridine-5'-diphosphate (UDP) (PDB ID: 3HBN) [142]. The N-terminal domain (red) is connected with the C-terminal domain (green) by an α -helix (magenta coloured). The UDP molecule is drawn as black sticks

between two α -helices on one side and three α -helices on the other. X-ray crystallographic analysis of CjPseG in complex with UDP revealed that the enzyme active site is located in the long cleft at the domain interface, with residues from both domains contributing to the ligand binding pocket [142]. Detailed analysis of the modelled complex with the nucleotide sugar substrate suggested that the UDP-sugar adopts a twist-boat conformation at the active site, thereby exposing the anomeric bond for the nucleophilic attack via an active site water molecule, which facilitates inversion at the C-1 atom [142]. The active site has three highly conserved residues (H17, Y78, and N255) that interact with the sugar moiety of the substrate. A mutagenesis study showed that H17 is crucial for substrate recognition and serves as a catalytic base in the reaction [142].

Mechanistic studies showed that the elimination of the nucleotide moiety by CjPseG occurs via a metal-independent C–O bond cleavage mechanism. In the course of the reaction, a catalytic water molecule (hydrogen bonded to H17 and main-chain carbonyl of I13) directly attacks the

anomeric carbon (C-1), followed by cleavage of the C–O anomeric bond to remove UDP from the UDP-6-deoxy-AltdiNAc sugar [143].

Step five

The fifth step of the Pse synthesis pathway, a condensation between phosphoenolpyruvate (PEP) and sugar 2,4-diacetamido-2,4,6-trideoxy- β -L-altropyranose to generate pseudaminic acid (Pse), is catalysed by Pse synthase (also known as pseudaminic acid biosynthesis protein I, PseI, EC 2.5.1.97) (Fig. 2). Inactivation of the *pseI* gene *H. pylori* (HP0178), or of the homologous gene *neuB3* in *C. jejuni*, abolished the synthesis of functional flagella and thereby impaired bacterial motility [92, 144]. Biochemical analysis of *C. jejuni* NeuB3 (CjNeuB3) showed that a divalent metal ion is required for its activity [145]. Apart from *C. jejuni*, functional homologues of HpPseI have also been found in many other organisms, where they are involved in the biosynthesis of sialic acid (NeuAc) by catalysing the reaction of a condensation between PEP and *N*-acetylmannosamine (ManNAc) (or ManNAc-6-P in mammalian cells) [146].

Biophysical and structural analyses of *N. meningitidis* NeuB (NmNeuB, 349 aa, sharing 30 and 35% sequence identity with CjNeuB3 and HpPseI, respectively) showed that NmNeuB exists as a homodimer both in solution and in crystal [147]. The crystal structure of NmNeuB in complex with PEP and substrate analog *N*-acetylmannosaminol (rManNAc, an unreactive, reduced form of ManNAc) [148] revealed that the NmNeuB monomer contains an N-terminal catalytic domain with a $(\beta/\alpha)_8$ barrel fold (also known as triosephosphate isomerase (TIM)-barrel fold), connected via a long linker with the C-terminal antifreeze protein-like (AFPL) domain (74 aa) (Fig. 7) [147]. In the homodimer, the AFPL domain from one monomer binds over the active site in the TIM barrel domain of the opposite monomer, capping the active site cavity. This structural architecture is crucial for the activity of the enzyme, as the residues from the linker region and AFPL domain form part of the active site. This feature is common among enzymes with a TIM barrel fold [149]. In the crystal structure of the NmNeuB/PEP/rManNAc complex, the substrate analog forms hydrogen bonds with highly conserved residues D247, Q55, and Y186. Another important conserved residue, R314 on the AFPL domain of the second monomer, interacts with the *N*-acetyl group of rManNAc via a water molecule. A combination of mutagenesis, biochemical and kinetic analyses have shown that R314 plays an important role in the proper positioning of the sugar substrate at the active site, and thereby contributes to the catalysis [150]. The R314A substitution resulted in the inactivation of NmNeuB [150].

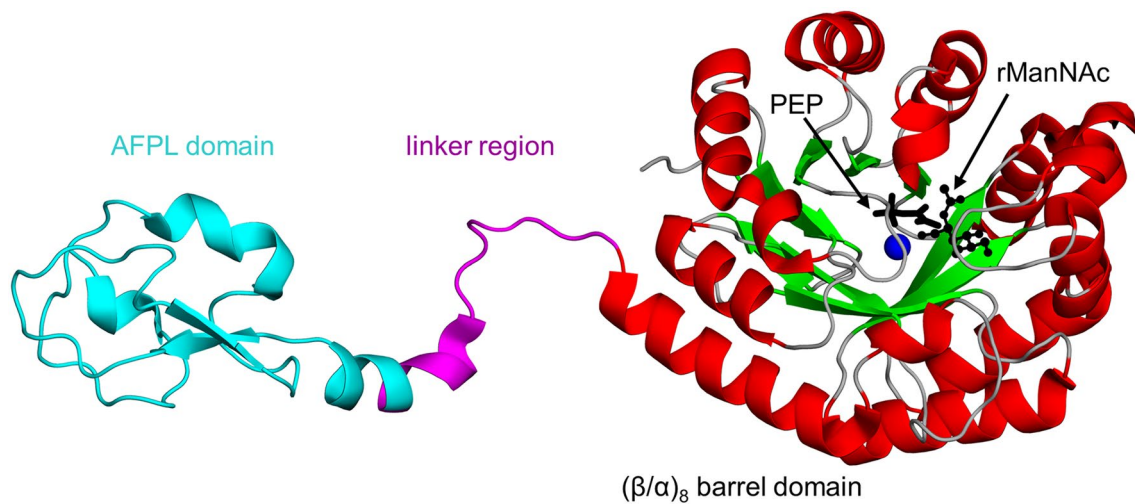


Fig. 7 Cartoon representation of the structure of *N. meningitidis* NeuB in complex with substrate analog *N*-acetylmannosaminol (rManNAc), phosphoenolpyruvate (PEP) and Mn^{2+} (PDB ID: 1XUZ [147]). The linker region and antifreeze protein-like (AFPL) domain

are coloured magenta and cyan, respectively. The PEP cofactor is drawn as black sticks, whereas the rManNAc is shown using a ball-and-stick representation; the Mn^{2+} ion is shown as a blue sphere

Kinetic analysis of *C. jejuni* NeuB3 showed that the NeuB3-catalysed reaction follows the Michaelis–Menten kinetics, and the condensation reaction occurs by a C–O bond cleavage mechanism that proceeds via an oxocarbenium ion and tetrahedral intermediates [145]. The divalent cation (Mn^{2+} or Co^{2+}), which is absolutely required for catalysis, acts as an electrophile that activates the carbonyl of the aldehyde for an attack by the C-3 carbon of PEP. Three conserved glutamate residues in the active site were identified as candidates for the role of the catalytic base in the reaction [147].

Step six

The final step of the Pse synthesis pathway (Fig. 2), activation of Pse with cytidine 5'-monophosphate (CMP), is catalysed by the metal-dependent pseudaminic acid cytidyltransferase, also known as CMP-pseudaminic acid synthetase or pseudaminic acid biosynthesis protein F (PseF, EC 2.7.7.81). This enzyme belongs to the nucleotide diphosphate sugar transferases superfamily [128]. Insertional inactivation of the *pseF* gene in *H. pylori* (HP0326A) resulted in the non-flagellated, non-motile phenotype [92]. A functional homolog of PseF found in *N. meningitidis* catalyses the biosynthesis of CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac), and is termed CMP-5-*N*-acetylneuraminic acid synthetase (also known as CMP-Neu5Ac synthetase CNS, EC 2.7.7.43) [151].

N. meningitidis CNS (NmCNS) activates 5-*N*-acetylneuraminic acid (Neu5Ac) by transferring the CMP moiety of CTP to the anomeric OH-group of Neu5Ac in a Mg^{2+} -dependent manner. The Neu5Ac is then transferred

and incorporated into the bacterial cell surface components, such as LPS and the polysialic acid capsule, which are important virulence factors. Structural analysis of NmCNS revealed that it exists as a dimer in the crystal [152]. The NmCNS subunit has an α/β -type fold with an ~ 35 -residue-long insertion—a β -hairpin (HP) domain—that plays an important role in dimerisation [152]. The core hydrolase domain has an $\alpha\beta\alpha$ three-layer sandwich architecture which consists of seven β -strands with the topological order ($\uparrow\beta 3-\uparrow\beta 2-\uparrow\beta 1-\uparrow\beta 4-\downarrow\beta 8-\uparrow\beta 5-\uparrow\beta 9$) (Fig. 8). The central β -sheet is flanked by four α -helices on one side and three on the other. The HP domain protrudes out from the central β -sheet and contains two antiparallel β -strands ($\beta 6$ and $\beta 7$) and two α -helices. The active site of NmCNS is located at the interface of the core domain of one monomer and the HP domain of the second (Fig. 8). Analysis of the structure of the NmCNS active site revealed that it contains a hydrophobic pocket formed by Y179, F192, and F193 to aid binding of the methyl group of the *N*-acetyl moiety of Neu5Ac [153]. This feature is important for substrate recognition, as alanine substitutions at these positions resulted in a significant loss of enzymatic activity [153]. Residue K142 in the HP domain serves to neutralise the negative charge of the carboxylate group of sialic acid by the proper position of R196 via a hydrogen-bonding network. Furthermore, residues D211 and D209 that coordinate the catalytic Mg^{2+} ions were shown by mutagenesis to play an important role in catalysis. Finally, mutations of Q104 at the active site suggested its role in the metal-binding site of an intermediate complex.

The CNS enzyme follows an ordered sequential kinetic mechanism where the CTP molecule binds to the enzyme first, followed by sialic acid [153]. Horsfall and colleagues

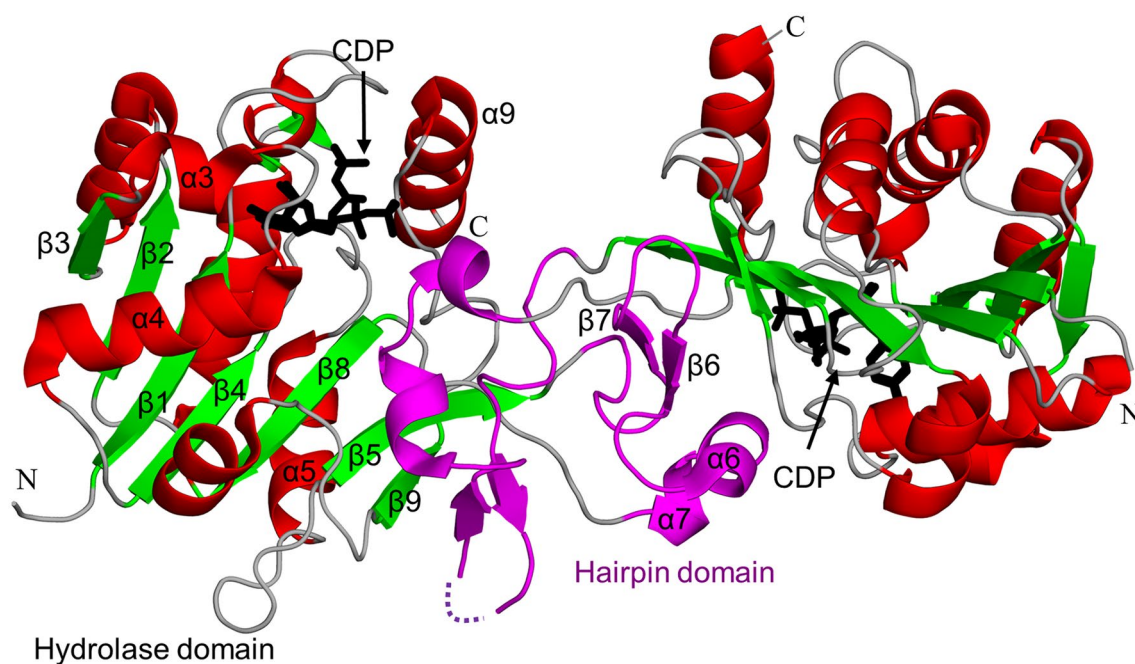


Fig. 8 Cartoon representation of the structure of the *N. meningitidis* CNS homodimer in complex with cytidine-5'-diphosphate (CDP) (PDB ID: 1EYR [151]). The dimerisation hairpin (HP) domain is coloured magenta. The substrate analog CDP is shown using a stick representation

proposed a catalytic mechanism for CNS that involves two Mg^{2+} ions [153]. The role of the Mg^{2+} ions is to facilitate the correct orientation of the substrates and to activate the α -phosphate moiety of CTP and the sugar hydroxyl group of Neu5Ac. An ordered solvent molecule has been proposed to serve as the general base for the reaction [151]. Kinetic analysis showed that other nucleotides (ATP, GTP, TTP, or UTP) cannot replace CTP as a donor of nucleotide monophosphate in the NmCNS-catalysed reaction.

Current knowledge on inhibitors targeting Pse biosynthesis pathway

The Pse synthesis pathway enzymes are considered promising targets for the development of novel therapeutics since bacterial motility is essential for colonisation and development of persistent infection. This section summarises efforts to identify inhibitors targeting this pathway and investigate their mode of action. Kinetic studies and structure–activity relationship analysis revealed that the activated final product of the Pse pathway, cytidine 5'-monophosphate (CMP)-conjugated Pse, can serve as a natural inhibitor of the first enzyme (PseB) of the Pse biosynthesis pathway [128]. The enzymatic activity of PseB is also inhibited by UDP- α -D-galactose [130]. Recently, Ménard and colleagues have identified five additional PseB inhibitors using a combination of high-throughput screening and in silico approaches [154].

Three out of the five inhibitors were able to penetrate the cell membrane and inhibit flagellin production in *C. jejuni* with an IC_{50} (50% inhibitory concentration) of 14 μ M. Interestingly, these inhibitors also showed dose-dependent activity against the fourth enzyme of the Pse pathway in *H. pylori*, PseG [154]. Analysis of the binding mode of the inhibitors, predicted using in silico docking, suggested that they bind in the active site of PseB and PseG, competing with their respective substrates [154]. Since they showed inhibitory activity at concentrations in the micromolar range, it is thought that this class of molecules can be developed into novel anti-infective agents to combat multidrug-resistant bacterial infection.

Although no inhibitors have so far been identified for the remaining four enzymes of the Pse biosynthesis pathway in *H. pylori* or *C. jejuni*, the current knowledge on inhibitors of homologous enzymes from other species may guide efforts towards this goal. In a study on the *N. meningitidis* sialic acid synthase (NmNeuB), a structural and functional homolog of the fifth enzyme (PseI) of the Pse pathway [148], a stable 2-deoxy analog of the putative tetrahedral intermediate of the reaction was identified, which inhibits the NmNeuB activity with an apparent K_i of 3.1 μ M. The structure of the NmNeuB/inhibitor/ Mn^{2+} complex revealed that the inhibitor binds at the active site in a similar manner to the cognate substrate. A study on the CMP-5-*N*-acetylneuraminic acid synthetase (NmCNS) from *N. meningitidis* (a structural and functional homolog of PseF) revealed that

sulfo-CTP and sulfo-UTP analogs inhibit the NmcNS activity [155], although no structures of the inhibitor complexes are available yet.

Conclusions and therapeutic prospects

Glycoconjugates are widespread in nature and have diverse biological functions. Importantly, most of the characterised bacterial glycoproteins are linked with virulence factors [156]. In fact, many of the glycoproteins produced by bacteria contribute directly to cell adhesion, invasion, immune activation, or evasion of host defence mechanisms [66, 157–160].

Flagellar glycosylation is essential for the biosynthesis of functional flagella and hence for motility and pathogenesis [156, 161]. Inactivation of the glycosylation genes generated mutants that were either unable to produce flagellins or possessed inactive flagella [69, 127]. The structural similarity between the flagellar glycans of GI tract pathogens and the host's sialic acids makes a significant contribution to host–pathogen interactions [157]. A recent report showed that Pse residues modulate cytokine interleukin 10 expression, and thereby facilitate bacterial colonisation of the host [162].

The glycosylation sites in flagellins showed variation in terms of their biological functions. In *C. jejuni*, some glycosylation sites appear to be indispensable for the assembly of the flagellum, while other locations were shown to be crucial for autoagglutination and microcolony formation, which is a prerequisite to the development of biofilms in the host [69, 163]. In *H. pylori*, Pse biosynthesis is essential not only for the assembly of functional flagella but also for the production of a wide range of virulence factors, including urease and LPS [133]. It has been suggested that glycosylation in *H. pylori* could also serve to disguise the antigenic epitopes of outer membrane proteins, thereby reducing host immune responses and influencing the clinical outcome of *H. pylori* infection [133].

The presence of nonulosonates, such as pseudaminic acid and related sugars, on the bacterial cell surface components including pilli, LPS O-antigen, and capsular polysaccharide [164–166] has attracted considerable attention over recent years. Prior to its incorporation in biological macromolecules, Pse must be synthesised and activated [128]. Due to the unstable nature of the activated nucleotide sugars and high cost of synthesis by chemical methods, Pse-synthesising enzymes are of great interest in the field of biotechnology, as an alternative route to cost-efficient production of Pse and its analogs. In addition, as highlighted in a recent report, bacterial O-glycosylation systems can be exploited for the production of bioconjugate vaccines targeting a variety of pathogens [167].

Pseudaminic acid is found only in bacterial pathogens. It is not produced or utilised by humans, nor is it present in commensal bacteria that dominate the human gut microbiome [168, 169]. Therefore, the Pse biosynthesis pathway represents an attractive target for selective inhibition, and further detailed studies of the enzymes from this pathway and their validation as targets for novel antimicrobials are well warranted in the view of the global spread of resistance to the existing antibiotics.

Compliance with ethical standards

Conflict of interest The authors have no conflict interest.

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