Undecaprenol Kinase: Function, Mechanism and Substrate Specificity of a Potential Antibiotic Target

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Abstract

The bifunctional undecaprenol kinase/phosphatase (UdpK) is a small, prokaryotic, integral membrane kinase, homologous with *Escherichia coli* diacylglycerol kinase and expressed by the *dgkA* gene. In Gram-positive bacteria, UdpK is involved in the homeostasis of the bacterial undecaprenoid pool, where it converts undecaprenol to undecaprenyl phosphate ($C_{55}P$) and also catalyses the reverse process. $C_{55}P$ is the universal lipid carrier and critical to numerous glycopolymer and glycoprotein biosynthetic pathways in bacteria. *DgkA* gene expression has been linked to facilitating bacterial growth and survival in response to environmental stressors, as well being implicated as a resistance mechanism to the topical antibiotic bacitracin, by providing an additional route to $C_{55}P$. Therefore, identification of UdpK inhibitors could lead to novel antibiotic treatments. A combination of homology modelling and mutagenesis experiments on UdpK have been used to identify residues that may be involved in kinase/phosphatase activity. In this review, we will summarise recent work on the mechanism and substrate specificity of UdpK.

1. Introduction

Undecaprenyl phosphate ($C_{55}P$) (**1**) is a lipid phosphate composed of 11 isoprene units with the (Z_8, E_2, ω)-configuration (**Figure 1A**).[1–3] In Gram-positive bacteria, $C_{55}P$ can be synthesised from (Z_8, E_2, ω)-undecaprenol ($C_{55}OH$) (**2**) through phosphorylation by undecaprenol kinase (UdpK), an enzyme that also catalyses the reverse process.[4] $C_{55}P$ is commonly referred to as the universal lipid carrier, as it is utilised in numerous essential bacterial biosynthetic pathways. However, exceptions are known, *Mycobacterium tuberculosis* for example uses a decaprenyl phosphate carrier[5–7]. Undecaprenol-containing glycolipids (UCGs), wherein a saccharide head group is linked to the C_{55} chain through an anomeric pyrophosphate bond (**Figure 1B**), are key building blocks used in the biosynthesis of several important glycopolymers[8,9] and glycoproteins.[10]

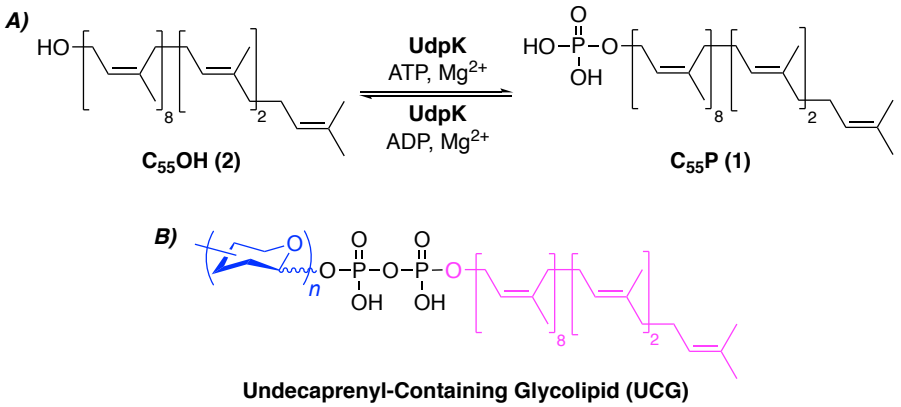


Figure 1. A) Phosphorylation of undecaprenol by UdpK. This enzyme can catalyse both phosphorylation and dephosphorylation. **B**) Generic structure of an undecaprenol-containing glycolipid (UCG) with the saccharide head group shown in blue and the undecaprenyl tail in purple. UCGs are used in bacterial glycopolymer and glycoprotein synthesis. ATP = Adenosine triphosphate and ADP = adenosine diphosphate.

$C_{55}P$ effectively functions as a transmembranal carrier of hydrophilic saccharide subunits from the location of their initial synthesis in the cytoplasm, across the cytoplasmic membrane and into the extracytoplasmic space (**Figure 2**).[11,12] This is the site of further processing and polymerisation, where complex glycopolymers and glycoproteins are formed. These form the scaffold of peptidoglycan, a key component of the bacterial cell wall (**Figure 2**), and confers structural support and environmental fortification to the cell.[8,13] Additionally, phylogenetic studies indicate that a relationship exists between peptidoglycan biosynthetic genes present in plant chloroplasts and bacteria.[14] Both Gram-positive and Gram-negative bacteria contain peptidoglycan in varied quantities due to differing functional requirements. While Gram-negative bacteria have a considerably thinner peptidoglycan layer than Gram-positive bacteria it performs essential structural, chemical and biological roles in all bacterial species where it has been found.[15] Peptidoglycan is the major constituent of the Gram-positive glycan cell wall, in which it forms a cross-linked polymeric structure. Formation of this key component is reliant on $C_{55}P$, which is converted into lipid I (by MraY) and then lipid II (by MurG), the final monomeric intermediate involved in peptidoglycan biosynthesis.[16] Lipid II is transferred across the plasma membrane by a flippase (MurJ[17] and/or

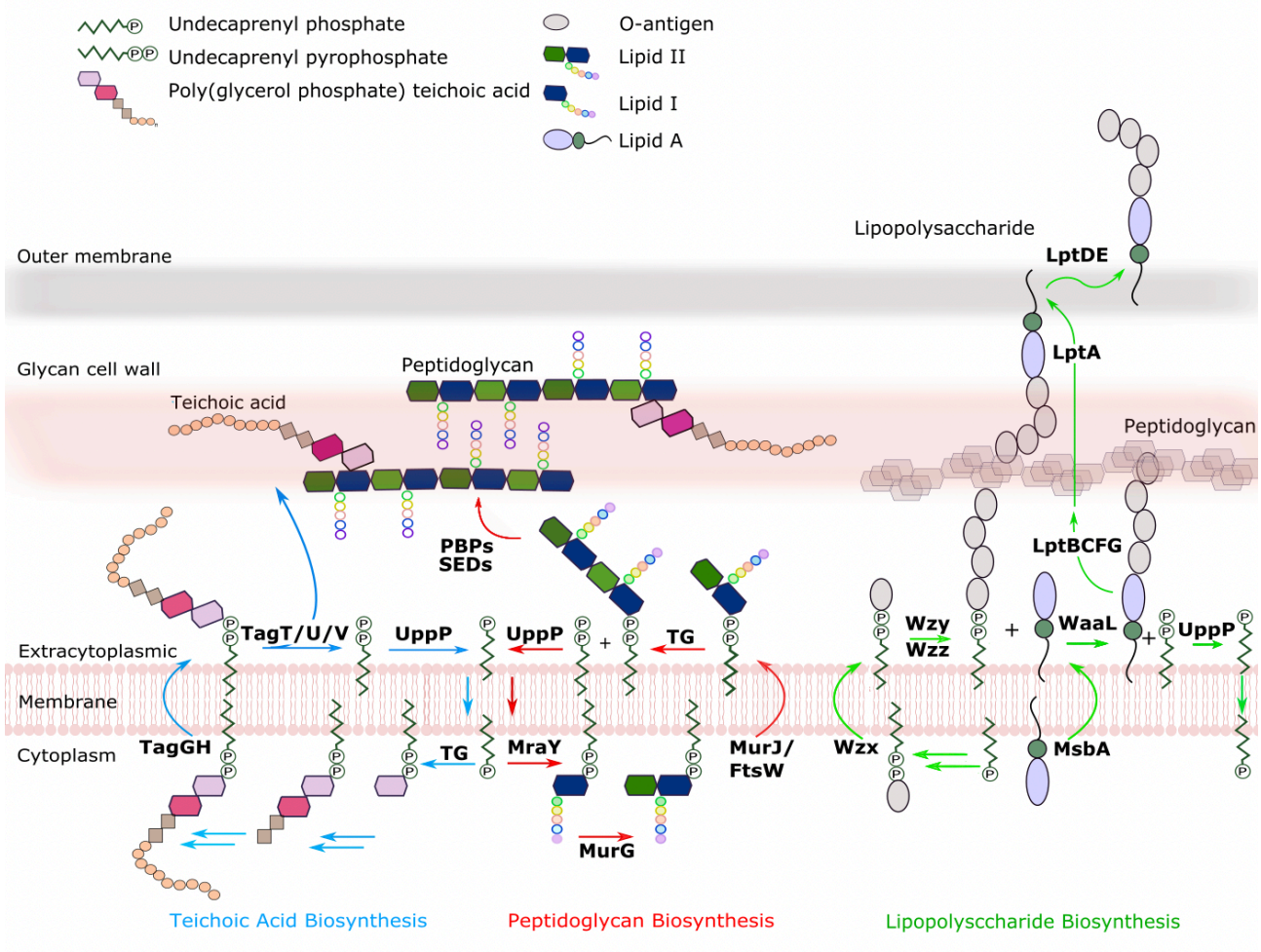


Figure 2. Schematic showing cellular processes in Gram-positive and/or Gram-negative bacteria that are reliant on $C_{55}P$ (Gram-positive bacteria do not have an outer membrane). Peptidoglycan (centre, red arrows) is an essential structural polymer in both Gram-positive and Gram-negative bacteria. $C_{55}P$ is sequentially converted to lipid I (by MraY) and then lipid II (by MurG), which is flipped across the membrane for polymerization.[12] Lipopolysaccharide (right, green arrows) is only found in Gram-negative bacteria and protects them from bile salts and lipophilic antibiotics. $C_{55}P$ is used in the synthesis of the *O*-antigen subunit.[20] This is then flipped across the membrane and polymerised by Wzz/Wzy, before the glycan head is transposed on to lipid A. Teichoic acid (left, blue arrows) is exclusive to Gram-positive bacteria and provides extra rigidity to the cell. After synthesis of the UCG and flipping across the membrane, the glycan is appended to the growing peptidoglycan chain. In all processes, undecaprenyl pyrophosphate ($C_{55}PP$) is liberated into the outer leaflet of the cytoplasmic membrane. This is converted to $C_{55}P$ by undecaprenyl pyrophosphate phosphatase (UppP, BacA) before being recycled back to the inner leaflet of the membrane. PBP = penicillin-binding protein and SEDS = shape, elongation, division and sporulation. Polymerization is performed by class A PBPs and SEDs-class B PBP combinations. TG = transglycosylase. UdpK has been omitted for clarity.

FtsW[18],[19]). Its polar head group extending into the extracytoplasmic space undergoes polymerisation and transfer reactions that result in its incorporation into the growing peptidoglycan layer. The peptidoglycan network of Gram-positive bacteria is further functionalised with teichoic acid and lipoteichoic acid, polymers which provide extra rigidity to the cell-wall by attracting divalent cations.[9] Teichoic acid biosynthesis also utilises $C_{55}P$ as a transmembrane shuttle to transport carbohydrate, carbohydrate-peptide and glycerol phosphate building blocks across the cytoplasmic membrane and is essential in both Gram-positive and Gram-negative bacteria.[21,22][23–25] As well as being involved in peptidoglycan biosynthesis, it is also required for the synthesis of lipopolysaccharide (LPS).[26,27] LPS are large molecular protrusions which are anchored in and extend from the outer leaflet of the outer membrane. They differ according to bacterial strain, relaying differing bacterial toxicity and virulence.[28,29] These essential structures protect Gram-negative bacteria from bile salts and lipophilic antibiotics. Structurally, LPS is composed of lipid A (endotoxin), which anchors it to the outer membrane, a core oligosaccharide and the *O*-antigens. It is the synthesis of the *O*-antigen that is reliant on $C_{55}P$, with *O*-antigen subunits synthesised as UCGs in the cytoplasm before being flipped across the membrane for further processing.[30–32] $C_{55}P$ also is employed in the synthesis of glycoproteins.[33] The appropriate UCGs are synthesised on the inner leaflet of the cytoplasmic membrane before being flipped to the outer leaflet for use in *O*- and *N*-glycosylation of proteins.[10,34–36] Peptidoglycan synthesis, teichoic acid synthesis and the *O*-linked mannose glycosylation of glycoproteins in Actinomycetes[37] are all essential processes in Gram-positive bacteria.

2. Biosynthesis of Undecaprenyl Phosphate

$C_{55}P$ can be biosynthesised via three different pathways (**Figure 3**).[4] In the *de novo* synthesis of $C_{55}P$ (**Figure 3A)**, a multi-step cascade of reactions occurs in the cytoplasm that involves the initial generation of $C_{55}PP$ through a series of contiguous condensations between farnesyl

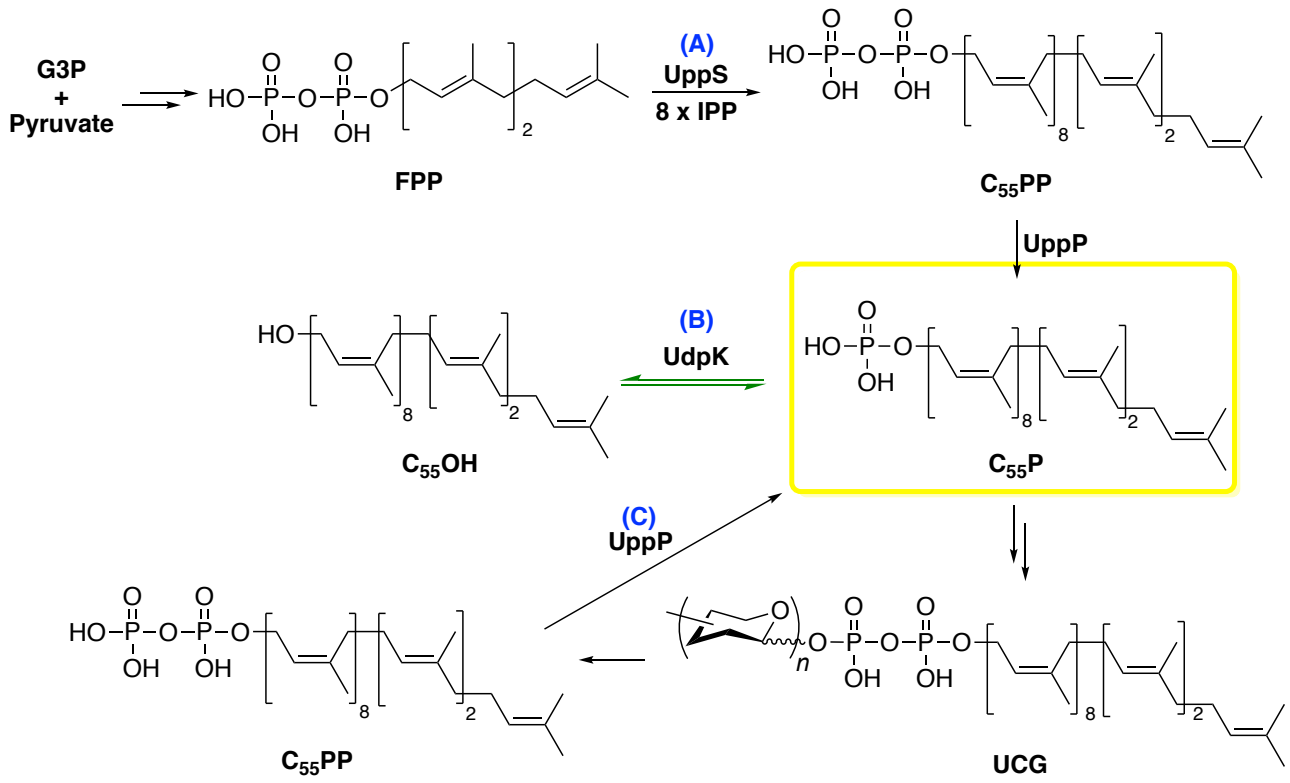


Figure 3. An overview of three different biosynthetic pathways that produce undecaprenyl phosphate ($C_{55}P$). **A**) *De novo* synthesis via the glyceraldehyde-3-phosphate (G3P) pathway; **B**) Phosphorylation of $C_{55}OH$ by UdpK; and **C**) Recycling $C_{55}P$ from $C_{55}PP$, a by-product of glycol-polymer/protein synthesis;.[4,39]

pyrophosphate and eight isopentenyl pyrophosphate units, catalysed by undecaprenyl pyrophosphate synthase (UppS).[25,38] $C_{55}P$ is then generated through undecaprenyl pyrophosphate phosphatase (UppP)-catalysed dephosphorylation of $C_{55}PP$. Alternatively, in glyco-polymer/protein syntheses, $C_{55}PP$ is a by-product left in the outer leaflet of the cytoplasmic membrane. UppP converts this $C_{55}PP$ to $C_{55}P$, before it is translocated back to the inner leaflet of the membrane for further use in UCG synthesis (**Figure 3C**).[39] Analysis of X-ray crystal structures of *Escherichia coli* UppP lead both the Caffrey and Strynadka groups to speculate that UppP may also function as a flippase for transport of $C_{55}P$ back to the inner leaflet of the cytoplasmic membrane.[40,41] This is because its structure resembles that of membrane channels (TagGH,[21] MsbA[42] and MurJ[43]), with the possibility of alternating active sites on either side of the membrane. Additionally, $C_{55}P$ can be generated through kinase mediated phosphorylation of $C_{55}OH$ (**Figure 3B**), a membrane bound lipid alcohol found

exclusively in Gram-positive bacteria. $C_{55}OH$ accounts for a high proportion of the total bacterial undecaprenoid pool found in Gram-positive bacteria but has not been found in Gram-negatives, suggesting this pathway is exclusive to Gram-positive organisms.[44] Knowledge of the active undecaprenol kinase and the gene(s) encoding it remained limited until a homologue of *E. coli* diacylglycerol kinase (DgK) was identified in *Streptococcus mutans* and the resulting *dgkA*-encoded protein was confirmed to be an active undecaprenol kinase.[45][46] More recently, Rock et al. identified the *dgkA* gene in *Bacillus subtilis* also operating as an undecaprenol kinase.[44] The *dgkA* gene codes for a membrane protein in both Gram-negative bacteria (DgkA) and in Gram-positive (UdpK).[46] Given the role that $C_{55}P$ plays in vital bacterial processes, the enzymes and numerous independent intermediates involved in its biosynthesis could make good antibiotic targets. Note, literature describing these enzymes can be confusing, as both the gene encoding undecaprenol kinase in Gram-positive bacteria and the gene encoding diacylglycerol kinase in Gram-negatives is called *dgkA*. For clarity, henceforth we will refer to the *udpk* gene when referencing the gene that encodes undecaprenol kinase and the *dgkA* gene when referring to the gene that encodes diacylglycerol kinase.

3. Role of UdpK in Gram-Positive Bacteria

In 2017, Wong and co-workers showed that UdpK has bifunctional enzymatic activities, in that it can catalyse both the phosphorylation of $C_{55}OH$ and the dephosphorylation of $C_{55}P$.[4] The latter secondary function was certified by the recombinant expression of *S. mutans* UdpK in *E. coli* (which does not contain detectable levels of $C_{55}OH$).[4] Upon expression of UdpK in *E. coli*, $C_{55}OH$ was detected, suggesting that UdpK converted native *E. coli* $C_{55}P$ to $C_{55}OH$. UdpK offers an alternative pathway for bacterial cell wall homeostasis and function.

Under static conditions, biosynthesis of $C_{55}P$ by *de novo* synthesis and recycling pathways is favoured. However, the UdpK-mediated pathway is reserved for bacterial survival under an applied stressor, growth in inhospitable environments and/or to facilitate periods of rapid growth.[4,45]

Phosphorylation and dephosphorylation by UdpK are favoured at different points in cellular development. Under accelerated growth conditions, phosphorylation of $C_{55}OH$ is favoured as it generates larger quantities of $C_{55}P$, facilitating rapid glycopolymer synthesis.[45] Phosphatase activity is favoured when growth is static to allow $C_{55}OH$ reserves to build up.[25] Additionally, *udpk* plays a role in phosphate homeostasis.[4] During periods of phosphate starvation, the gene is upregulated and promotes the generation of inorganic phosphate via the dephosphorylation of $C_{55}P$ to sustain cellular requirements.[4] UdpK is also instrumental to bacterial physiological regulation of bacterial biofilm formation and colony morphology.[47,48] Daugelat *et al.* described the influence UdpK has upon biofilm formation and bacterial infections.[47] *Mycobacterium smegmatis* UdpK deficient mutants form incomplete biofilms, characterised by isolated colonies of abnormal "caved-in" morphology.[47] Mice inoculated with *udpk*-deficient bacteria showed a reduction in penile smegma formation due to insufficient biofilm formation resulting from the lack of *udpk* gene expression.[47] This phenotypical influence was further noted in oral biofilm formation and the development of dental caries. Similar to *M. smegmatis* mutants, *S. mutans udpk*-knock-out strains exhibited reduced biofilm formation to the extent of approximately 40% when compared to wild-type bacteria. Additionally, UdpK was shown to be essential in maintaining bacterial function in cariogenic acidic environments[48] leading to the development of dental cavities and oral infections.[49,50] Yamashita *et al.* reported kinase activity to be critical for bacterial virulence at acidic pH.[49] Mutants of *S. mutans* grew at similar rates to wild-type bacteria at pH 7.4. The *udpk* gene reliance was seen when mediated at pH 5.5, as *S. mutans* mutants displayed restricted growth due to a heightened acid-sensitivity. Reintroduction of the *udpk* gene on a plasmid restored growth in acidic media by improving acid-tolerance.[45] A reduction in growth rate was further evident in systems doped with the eukaryotic DgkA inhibitor R59949 when incubated at pH 5.4 due to heightened acid-sensitivity imparted presumably by UdpK inhibition. This shows a correlation between *udpK* bacterial expression, bacterial acidic tolerance and virulence in acidic enviroments.[49]

Further studies of *B. subtilis* found that deletion of the *udpk* gene reduced their ability to correctly form an endospore.[51] The mutant endospores were shown to be unstable by observation with phase-contrast microscopy and to have a defective cortex structure as confirmed by electron microscopic examination. Additionally, the deletion was shown to have adverse effects upon bacterial adaption with a significant decrease in levels of dipicolinic acid being present in mutant spores, a compound involved in bacterial endospore heat resistance and dormancy.[52]

Due to its ability to offer an alternative mechanism for Gram-positive bacteria to access $C_{55}P$, upregulation of UdpK-mediated $C_{55}P$ synthesis has also been implicated in antibiotic resistance.[53] Bacitracin is a topical antibiotic used in the treatment of minor injuries and skin infections that kills bacteria by sequestering $C_{55}PP$, inhibiting dephosphorylation and recycling of $C_{55}P$.[54] Colicin M, a polypeptide toxin produced by *E. coli*, also sequesters $C_{55}PP$, disabling the regeneration of $C_{55}P$ and blocking $C_{55}P$-reliant pathways.[55] In the presence of these antibiotics, upregulation of UdpK provides an alternative synthesis of the lipid carrier through phosphorylation of C_{55}-OH. As the C_{55}-OH pool is maintained on the inner-leaflet of the cell membrane, and neither bacitracin nor colicin M interact with $C_{55}OH$, peptidoglycan synthesis can continue during antibiotic exposure. This resistance mechanism was observed by Kuramitsu and Lis, with *udpk*-deficient *S. mutans* strains displaying bacitracin hypersensitivity and abolished growth compared to wild type organisms.[45] Additionally, the *udpk* gene in Gram-positive organisms is part of a 3-gene operon. The operon consists of *Sgp,* a GTPase involved in ribosomal biogenesis and/or regulation and potential physiological membrane stress response pathways, and *ygfG,* a homologue of *E. coli* YbeY involved in ribosome regulation. The presence of the *udpk* gene in the operon suggests involvement in other stress-invoked response pathways potentially centred around ribosomal function.[56]

4. Structure and Mechanism of UdpK

UdpK is an ATP-dependent enzyme that consists of 130 amino acids and is a homologue of DgkA from *E. coli*.[56] Both enzymes contain sequences with homologous regions and are distinct from other classes of kinase, which lead to unique biochemical functionality within this pair of enzymes. Due to there being no published structure of UdpK, DgkA has been used as a model for mechanistic studies on UdpK.[4] Caffrey and co-workers reported the crystal structure of DgkA up to 2.05 Å resolution.[57,58] The enzyme is a homotrimer, in which each monomer has three membrane-embedded helical domains and an *N*-terminal aliphatic α-helix (**Figure 4A, B**). There are three active sites of the shared site type per kinase. The location of evolutionary conserved and essential residues when mapped to the crystal structure provided an explanation for the substrate docking mechanism which involves proximal alignment of the ATP γ-phosphate region and the primary hydroxyl group of the lipid (**Figure 4C**). Phosphorylation was deemed to proceed through direct phosphate transfer from nucleotide to lipid and not through an enzyme-phosphate intermediate. Glutamate and aspartate residues in the active site position the lipid substrate deprotonate the terminal hydroxy group to form an alkoxide. The alkoxide ion reacts with the adjacent γ-phosphate of the divalent metal-nucleotide complex, generating a pentavalent phosphate transition state, which subsequently collapses, releasing the phosphorylated lipid and ADP.

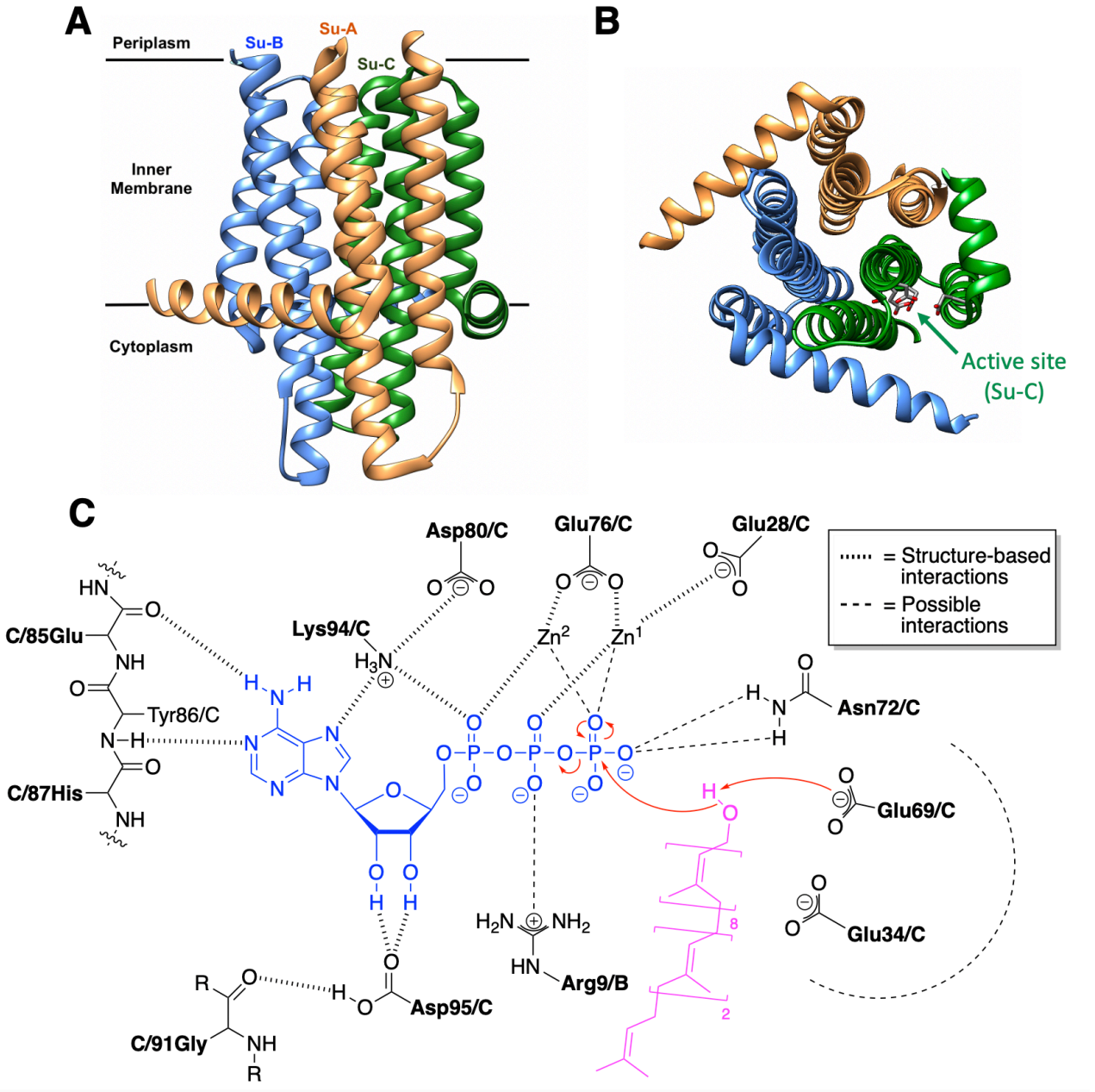


Figure 4. A) View from the membrane and **B)** from the cytoplasm of the homotrimeric membrane embedded DgkA from *E. coli* (PDB 4UXX). Subunit A (Su-A) is orange, subunit B (Su-B) is blue, and subunit C (Su-C) is green. Enzyme active sites are found toward the cytoplasmic region of the protein. **C)** Proposed mechanism for phosphorylation of undecaprenol by DgkA.

In the absence of a crystal structure of UdpK, a homology model of the enzyme has been created using DgkA as a template (**Figure 5A, B**).[4] UdpK from *S. mutans* shares ~37% sequence identity with DgkA and predicted key amino acid residues are conserved between both enzymes. *S. mutans* UdpK was shown to require a number of amino acid residues for enzymatic function (**Figure 5C**). These include Glu79, Asn82, Glu86, Asp90 and Asp106 in *S. mutans* UdpK.[4] Similar residues (e.g. Glu69, Asn72, Glu28, Asp76 and Asp80) are conserved in DgkA.[58] Phosphatase activity was also compromised in mutant strains, displaying a bi-functioning enzyme dependence on identical key residues.[4] No mutant strain was identified that could solely catalyse phosphorylation over dephosphorylation (or *vice versa*), lending credence to the conclusion that the two enzyme functions operate co-dependently and indispensably of each other, within a shared active site. Due to the structural and biochemical similarities between DgkA and UdpK, it is assumed that the phosphorylation mechanism is similar. In this proposed mechanism, Glu86 and Asn82 stabilise and position the nucleotide terminal phosphate (**Figure 5C**). Concurrently, a basic Glu79 initiates kinase activity through deprotonation of the alcohol to give an alkoxide, which then reacts with ATP to yield $C_{55}P$ and ADP. In the proposed phosphatase mechanism, Glu86 coordinates with the ADP β-phosphate, initiating deprotonation of water and subsequent attack of $C_{55}P$ by the resulting hydroxide ion. This releases the $C_{55}OH$ product, as well as ADP and inorganic phosphate. It is interesting to note that the proposed role of ADP is that of a nucleophilic catalyst.

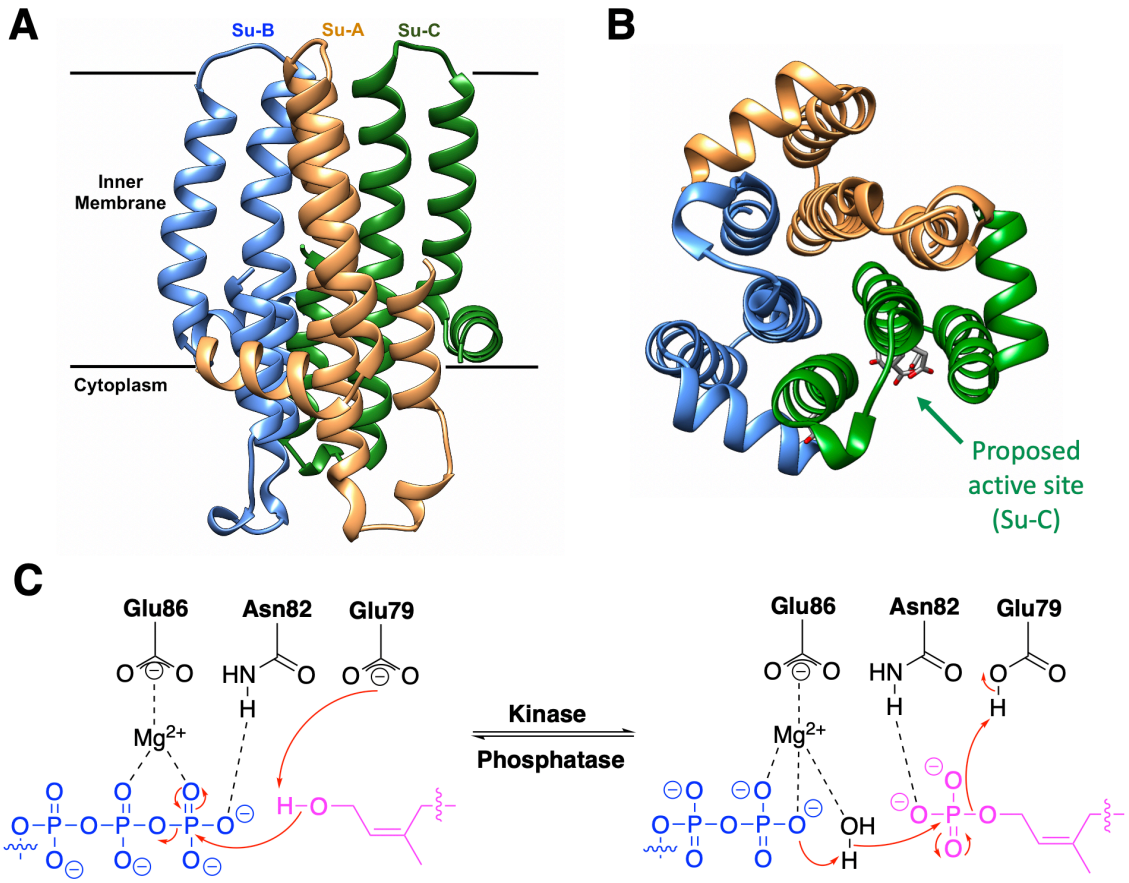
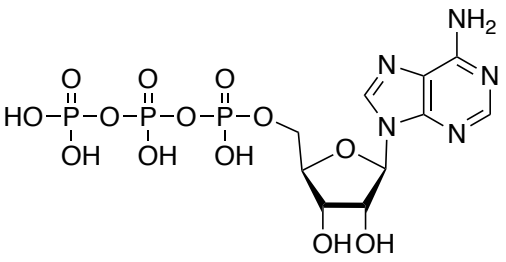


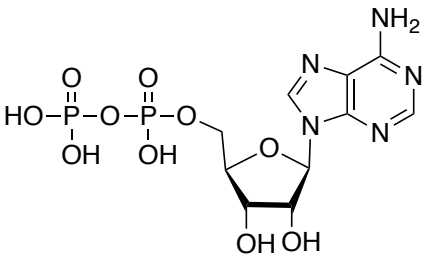
Figure 5. A) View from the membrane plane and **B)** from the cytoplasm of homology model of UdpK from *S. mutans*. Model generated using SWISS-MODEL with *E. coli* DgKA-ACP complex (PDB 4UXX) as a template. Subunit A (Su-A) is orange, subunit B (Su-B) is blue, and subunit C (Su-C) is green. Enzyme active sites are found in the cytoplasmic region of the protein. **C)** Proposed active-site residues and mechanism of UdpK for both kinase and phosphatase reactions.

5. UdpK Nucleotide Specificity

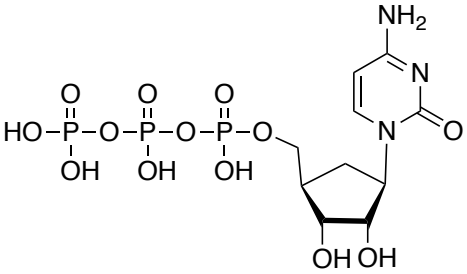
The kinase and phosphatase reactions catalysed by UdpK use different nucleotides (ATP (**3**) for kinase, ADP (**4**) for phosphatase). Whether the kinase or phosphatase reaction dominates partly depends on the ratio of ATP and ADP.[4] For the kinase reaction, only ATP analogues with the adenine base are tolerated (**Figure 6**).[59] This selectivity can be rationalised through analysis of the DgkA structure reported by Caffrey and co-workers, which can operate as a putative model for UdpK. The purine base of adenine nucleotides are optimally aligned within the active site via hydrogen bonds



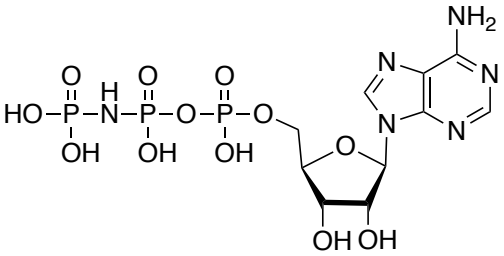
ATP (**3**)
Active



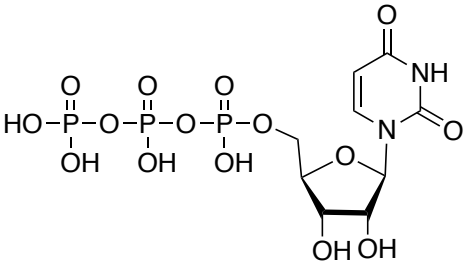
ADP (**4**)
Active



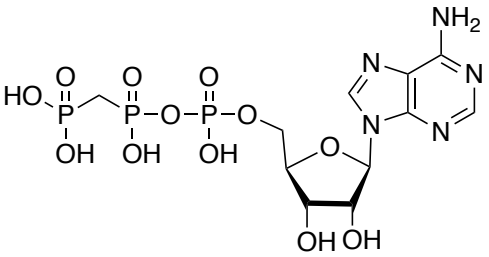
CTP (**5**)
Not active



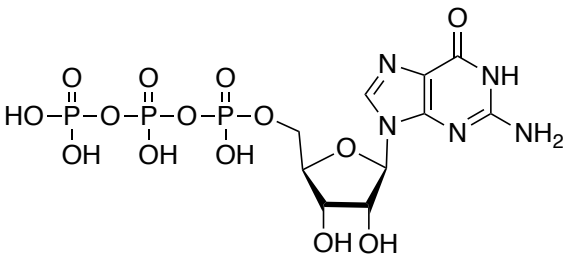
AMPPNP (**6**)
Not active



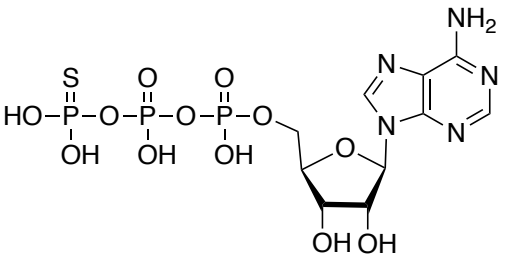
UTP (**7**)
Not active



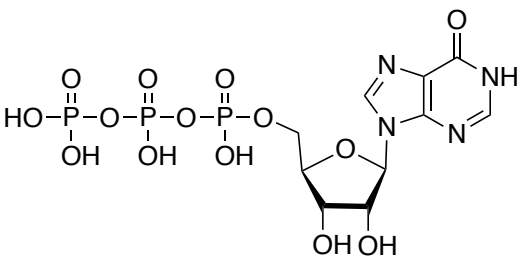
AMPPCP (**8**)
Not active



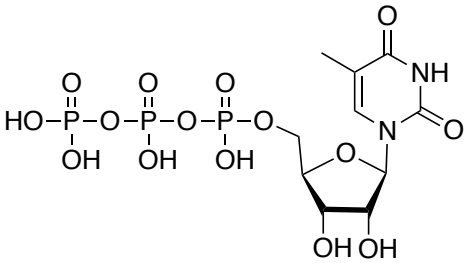
GTP (**9**)
Not active



Adinosine 5′-(γ-Thio)triphosphate (**10**)
Active



ITP (**11**)
Active (DgK)



TTP (**12**)
Not active (DgK)

Figure 6. Nucleotides tolerated by UdpK.

with backbones of amino acid residues His87C and Glu85C (**Figure 4**). Additionally, Tyr86C located between the binding residues further embeds the nucleotide via a π -stacking interaction between the adenyl core and the tyrosyl ring. Changing adenine to guanine in the nucleotide triphosphate results in replacement of the amino group at position 6 with a carbonyl. Given that the adenine amino group is involved in a hydrogen bonding interaction with the Glu85 backbone carbonyl in DgkA, this substitution with oxygen likely leads to a repulsive interaction between the guanine ring and protein, explaining the decreased binding affinity.[4] Under similar conditions, crystal soaking with non-adenine containing nucleotides (GTP, thymidine triphosphate (TTP), CTP or UTP) had no effect on the DgkA crystal.[57] In another study with DgkA, a reduction in k_{cat} was observed when GTP (**9**) or inosine triphosphate (ITP) (**11**) were used, compared to the optimal ATP system[58]. Screening ATP analogues has provided insight into the phosphoryl transfer mechanism and more information on substrate selectivity. Adenosine-5'-(*y*-thio)triphosphate (**10**) was shown to be an active substrate of UdpK but is turned over at a slower rate than ATP,[4] presumably due to the reduced electrophilicity and sterics of the thiophosphate.[60] The activity of this substrate reinforces the proposal that phosphate transfer occurs at the γ-phosphate position of ATP.[4,57] Analogues containing a different functional linkage between the β- and γ-phosphorus atoms, such as adenosine-5'-(β,γ-imido)triphosphate (AMPPNP) (**6**) and adenosine-β,γ-methyleneadenosine-5'-triphosphate (AMPPCP) (**8**), are not substrates as the terminal phosphate cannot be cleaved.[4] Both UdpK and DgkA are ATP dependent, with the most significant sequence conservation found in the ATP binding regions. The phosphatase activity of UdpK was shown to be ADP (**4**) dependent.

6. UdpK Lipid Substrate Specificity

UdpK has been shown to phosphorylate a broad range of lipid alcohol substrates (**Table 1**). UdpK and DgkA, while both structurally similar, display different substrate selectivity, with *B. subtilis* UdpK catalysing the phosphorylation of undecaprenol but not diacylglycerol.[44] In DgkA

and UdpK, the largest proportion of amino acids are conserved in the active site between the transmembrane domains 2 and 3, which is associated with nucleotide selectivity[4]. However, in the N-terminal helix and the C-terminal transmembrane domains 1 and 3, which are associated with lipid binding, there is less conservation, which is likely responsible for the differing lipid selectivity.[4]

The first significant analysis of UdpK's lipid substrate-specificity was performed by Wong and coworkers.[39] UdpK processes polyprenols of varied lengths, with shorter isoprenols such as nerols (**14**, **15**), farnesols **(16** – **18**), geranylgeraniol (**19**) and heptaprenol (**20**) performing better than the larger polyprenols.[59] (E_8, ω)-solanesol (**22**) is two isoprene units shorter than bacterial (Z_7, E_3, ω) undecaprenol and all of its alkenes are in the *trans*-configuration. It is processed by UdpK at a similar rate to (Z_7, E_3, ω)-undecaprenol, suggesting that the double bond stereochemistry does not significantly impact enzyme activity. Polyprenols containing a saturated α-isoprene (dolichol, **25**) or all saturated isoprene units (phytol, **24**) are processed at similar rates to undecaprenol but aliphatic alcohols (**26** – **28**) are poorer substrates, suggesting that hydrocarbon branching is important. Synthetic pentaprenols containing a dabsyl group at the ω-terminus (**29** – **32**) are also readily processed by UdpK. Wong and co-workers also found that common hydroxy-containing surfactants such as Tween 20 (**33**), Triton X-100 (**34**) and Tergitol NP-40 (**35**) were phosphorylated by UdpK. This was qualitatively determined by checking for the presence of phosphorylated surfactants by TLC and LCMS. In another study, Rock and co-workers found that UdpK was incapable of phosphorylating ceramide (**36**), D-erythro-sphingosine (**37**), 1-oleoyl-*rac*-glycerol (**38**), 1,2-dioleoyl-*sn*-glycerol (**39**) and phosphatidylinositol (**40**). These respective studies by Wong and Rock suggest that UdpK preferentially processes linear primary alcohols. It appears that additional hydrophilic moieties close to the alcohol group and/or increased steric hinderance, i.e., secondary alcohols, are poorer substrates.

The Cochrane lab recently reported the semi-synthesis of a library of novel labelled undecaprenol analogues.[61] d_1-(Z_7, E_3, ω)-Undecaprenol (**41**) was synthesised from (Z_7, E_3, ω)-undecaprenol (which is extracted from bay leaves) and found to be readily processed at similar rates

to (Z_7, E_3, ω)-undecaprenol. Additionally, the terminal ω-isoprene unit was modified with a variety of different chemical labels or functional groups, including azides (**42**, **48**), thioesters (**43**), alcohols (**44**), epoxides (**45**), alkynes (**46**, **47**), spin-labels (**49**), fluorophores (**50** – **52**) and photoaffinity labels (**53**). Rates were broadly comparable for all of these ω-modified substrates, showing that this position is an excellent site for chemical labelling on undecaprenol.

7. Conclusions and Future Outlook

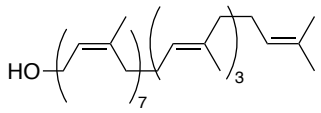
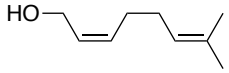
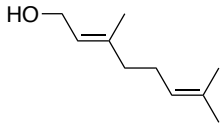
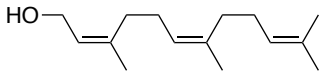
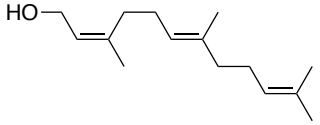
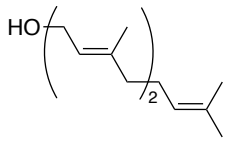
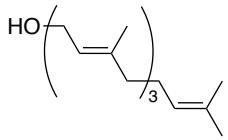
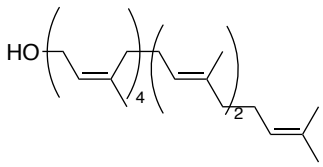
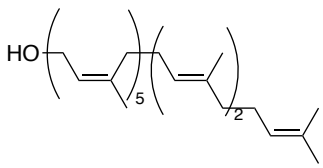
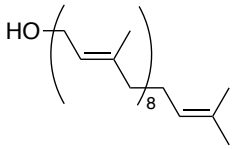
Due to the bacterial adaptations imparted by UdpK expression and the number of unique biosynthetic pathways reliant on undecaprenyl phosphate, this enzyme could make a good antibiotic target in Gram-positive bacteria. Inhibition of this kinase could impede bacterial survival by inhibiting the synthesis of essential glycopolymers and glycoproteins in already stressed bacteria (for example, by another antibiotic), as well as restore sensitivity to antibiotics such as bacitracin, whose efficacy is reduced by UdpK-mediated $C_{55}P$ synthesis. At present the only structure of UdpK available is a homology model and although mechanistic hypotheses have been derived from this, rational drug-design would be better aided by a high-resolution X-ray crystal structure. Given the promiscuity of UdpK towards lipid alcohols, substrate-mimics or novel scaffolds that bind to the kinase active site may offer new antibiotic candidates and in turn may aid in the generation of a crystal structure of undecaprenol kinase via complexation with these novel inhibitors. To date all UdpK assays have relied on either a coupled pyruvate kinase/lactate dehydrogenase coupled enzyme assay and/or thin-layer chromatography (TLC). High-throughput screening of large compound libraries using the coupled enzyme assay system is problematic as there are three enzymes that could be inhibited. TLC provides a clear result but is not high throughput. Chelation-enhanced fluorescence (CHEF) screening of kinases, which was first reported by the Imperiali group,[62,63] allows the high-throughput screening of protein kinases.[64,65] This method was adapted by the Anslyn group for the differential sensing of MAP Kinases using chemically labelled peptides.[66] If analogous lipid

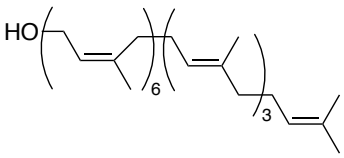
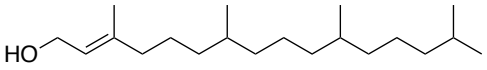
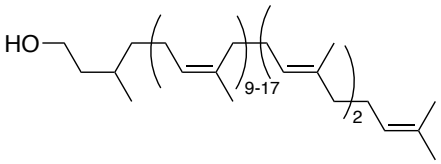
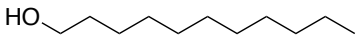
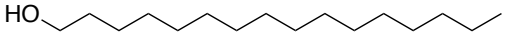
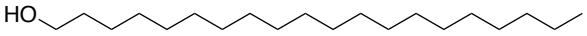
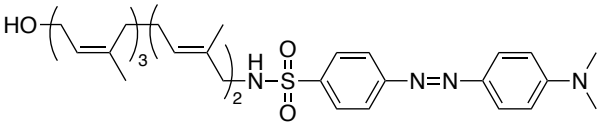
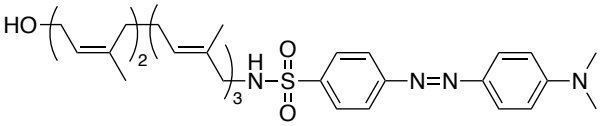
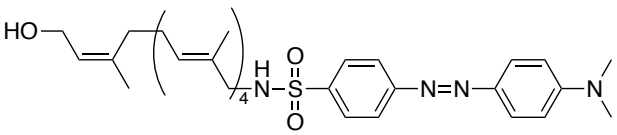
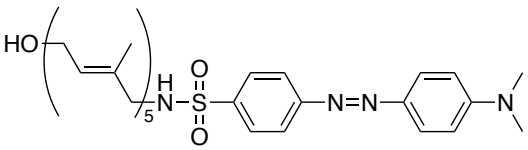
substrates could be prepared that allow CHEF-monitoring of UdpK, such a system could enable high-throughput screening of large compound libraries for identification of new antibiotic candidates. The studies reported herein highlight UdpK as a vital enzyme in Gram-positive bacteria under environmental or antibiotic stressors, and emphasise its potential as an antibiotic target.

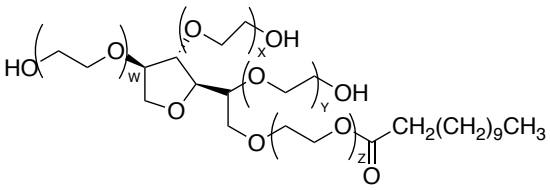
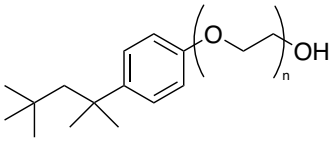
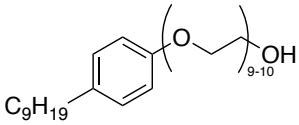
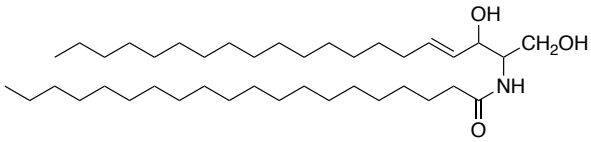
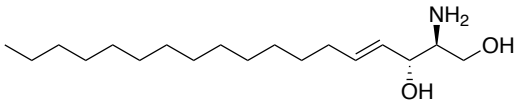
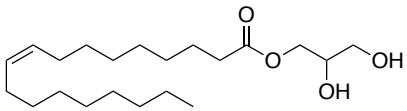
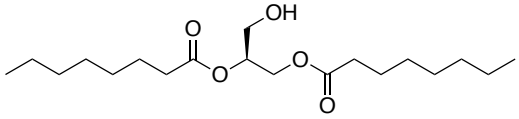
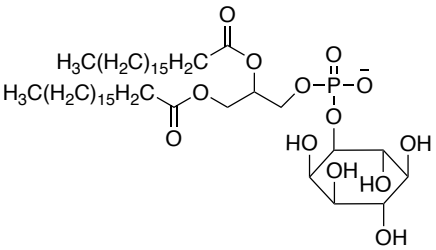
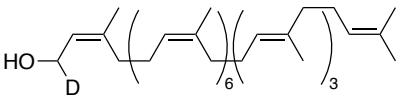
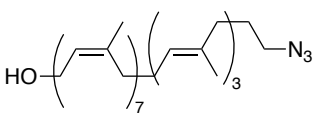
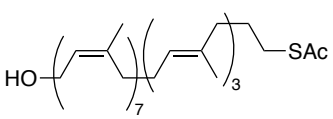
Acknowledgements

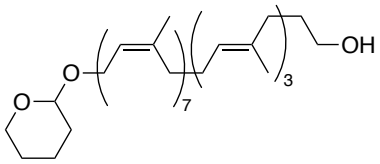
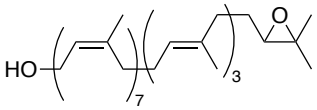
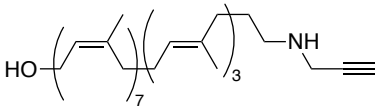
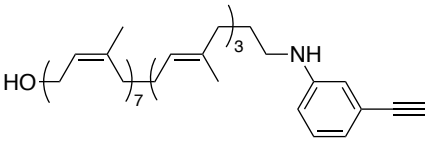
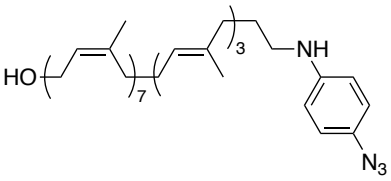
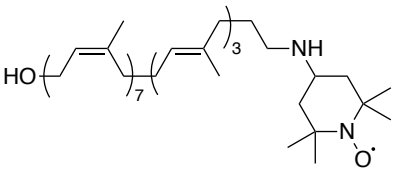
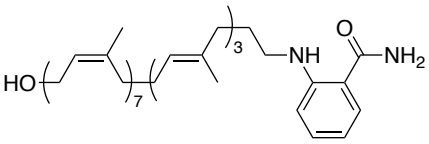
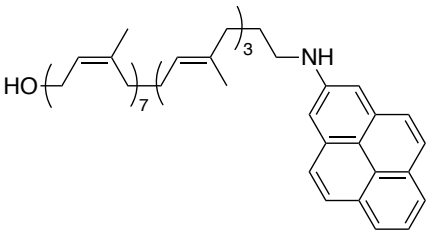
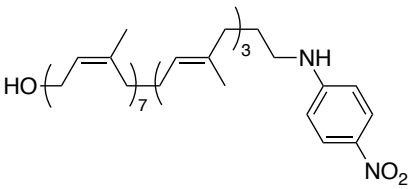
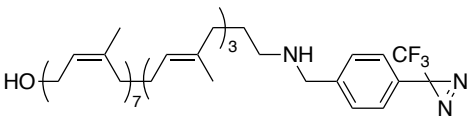
We thank Dr Rachel Cochrane (QUB) and Prof. Ulrich Zachariae (U Dundee) for helpful suggestions during the preparation of this manuscript. We acknowledge financial support from the EPSRC (EP/S015892/1, S. A. C), Science Foundation Ireland (16/IA/4435, M. C) and an MRC 4-year PhD studentship (C. M. I).

Table 1. Substrate selectivity of UdpK

	Structure	Substrate	Activity[a] [µmol mg^{-1} min^{-1}]	Ref
13		(Z_7,E_3,ω)-Undecaprenol	5.1 ± 0.2	[59]
14		(*Z*, ω)-Nerol	8.7 ± 0.3	[59]
15		(*E*, ω)-Nerol	6.2 ± 0.1	[59]
16		(Z_2,ω)-Farnesol	21.2 ± 0.4	[59]
17		(*Z*, *E*, ω)-Farnesol	5.4 ± 0.4	[59]
18		(E_2, ω)-Farnesol	11.5 ± 0.2	[59]
19		(E_3, ω)-Geranylgeraniol	9.3 ± 1.0	[59]
20		(Z_4, E_2, ω)-Heptaprenol	8.8 ± 0.2	[59]
21		(Z_5, E_2, ω)-Octaprenol	3.8 ± 0.1	[59]
22		(E_8, ω)-Solanesol	4.9 ± 0.3	[59]

23		(Z_6, E_3, ω)-Decaprenol	3.8 ± 0.1	[59]
24		Phytol	5.6 ± 0.8	[59]
25		Dolichol (α-dihydro, $Z_{9\text{-}17}$, E_2)	4.5 ± 0.2	[59]
26		Undecanol	2.1 ± 0.1	[59]
27		Hexadecanol	1.9 ± 0.1	[59]
28		Eicosanol	1.3 ± 0.1	[59]
29		(Z_3, E_2)-Pentaprenol (dabsyl)	4.1 ± 0.1	[59]
30		(Z_2, E_3)-Pentaprenol (dabsyl)	4.0 ± 0.4	[59]
31		(Z, E_4)-Pentaprenol (dabsyl)	5.9 ± 0.0	[59]
32		(E_5)-Pentaprenol (dabsyl)	3.3 ± 0.1	[59]

33		Tween 20	Active[b]	[59]
34		Triton X-100	Active[b]	[59]
35		Tergitol NP-40	Active[b]	[59]
36		Ceramide	Inactive[b]	[44]
37		D-Erythro-sphingosine	Inactive [b]	[44]
38		1-Oleoyl-*rac*-glycerol	Inactive [b]	[44]
39		1,2-dioleoyl-*sn*-glycerol	Inactive [b]	[44]
40		Phosphatidylinositol	Inactive [b]	[44]
41		(Z_7,E_3)-Undecaprenol (α-d_1)	9.4 ± 0.5	[61]
42		(Z_7,E_3)-Undecaprenol ω-azide	10.8 ± 0.6	[61]
43		(Z_7,E_3)-Undecaprenol ω-thioacetate	11.8 ± 0.1	[61]

44		(Z_7,E_3)-Undecaprenol α-tetrahydropyran, ω-OH	3.4 ± 0.3 [61]
45		(Z_7,E_3)-Undecaprenol ω-epoxide	8.1 ± 0.7 [61]
46		(Z_7,E_3)-Undecaprenol ω-propargylamine	7.1 ± 0.5 [61]
47		(Z_7,E_3)-Undecaprenol ω-(3-ethynylaniline)	9.0 ± 0.5 [61]
48		(Z_7,E_3)-Undecaprenol ω-(4-azidoaniline)	9.4 ± 0.2 [61]
49		(Z_7,E_3)-Undecaprenol ω-(4-amino-TEMPO)	5.1 ± 0.3 [61]
50		(Z_7,E_3)-Undecaprenol ω-(2-aminobenzamide)	6.9 ± 0.8 [61]
51		(Z_7,E_3)-Undecaprenol ω-(pyrenemethylamine)	6.2 ± 0.1 [61]
52		(Z_7,E_3)-Undecaprenol ω-(4-nitroaniline)	9.0 ± 0.9 [61]
53		(Z_7,E_3)-Undecaprenol ω-diazirine	5.1 ± 0.3 [61]

[a]UdpK activity determined colorimetrically using a pyruvate kinase/lactate dehydrogenase coupled assay unless otherwise stated. [b] Actual rate unavailable.

References

[1] M. Lee, D. Hesek, J. Zajíček, J. F. Fisher and S. Mobashery, *Chem. Commun.*, 2017, **53**, 12774–12777.

[2] T. TouzÉ and D. Mengin-Lecreulx, *EcoSal Plus*, 2008, **3**, doi: 10.1128/ecosalplus.4.7.1.7

[3] M. Scher, W.J. Lennarz, C.C. Sweeley, Proc. Natl. Acad. Sci. U. S. A. 59 (1968) 1313–1320.

[4] L.Y. Huang, S.C. Wang, T.J.R. Cheng, C.H. Wong, Biochemistry 56 (2017) 5417–5427.

[5] G. Manat, S. Roure, R. Auger, A. Bouhss, H. Barreteau, D. Mengin-Lecreulx, T. Touzé, Microb. Drug Resist. 20 (2014) 199–214.

[6] M.A. Valvano, Mol. Microbiol. 67 (2008) 232–235.

[7] E. Layre, L. Sweet, S. Hong, C.A. Madigan, D. Desjardins, D.C. Young, T.-Y. Cheng, J.W. Annand, K. Kim, I.C. Shamputa, M.J. McConnell, C.A. Debono, S.M. Behar, A.J. Minnaard, M. Murray, C.E. Barry 3rd, I. Matsunaga, D.B. Moody, Chem. Biol. 18 (2011) 1537–1549.

[8] W. Vollmer, D. Blanot, M.A. De Pedro, FEMS Microbiol. Rev. 32 (2008) 149–167.

[9] S. Brown, J.P. Santa Maria, S. Walker, Annu. Rev. Microbiol. 67 (2013) 313–336.

[10] Y.J. Lee, A. Ishiwata, Y. Ito, Tetrahedron 65 (2009) 6310–6319.

[11] J. van Heijenoort, Cell. Mol. Life Sci. 54 (1998) 300–304.

[12] A.C.K. Teo, D.I. Roper, Antibiotics 4 (2015) 495–520.

[13] W. Vollmer, P. Born, in: O. Holst, P.J. Brennan, M. von Itzstein, A.P.B.T.-M.G. Moran (Eds.), Microb. Glycobiol., Academic Press, San Diego, 2010, pp. 15–28.

[14] N. Sato, H. Takano, J. Plant Res. 130 (2017) 635–645.

[15] T. J. Silhavy, D. Kahne and S. Walker, *Cold Spring Harb. Perspect. Biol.*, 2010, **2**, doi:10.1101/cshperspect.a000414

[16] A. Bouhss, A.E. Trunkfield, T.D.H. Bugg, D. Mengin-Lecreulx, FEMS Microbiol. Rev. 32 (2008) 208–233.

[17] N. Ruiz, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 15553–15557.

[18] T. Mohammadi, V. van Dam, R. Sijbrandi, T. Vernet, A. Zapun, A. Bouhss, M. Diepeveen-de Bruin, M. Nguyen-Distèche, B. de Kruijff, E. Breukink, EMBO J. 30 (2011) 1425–1432.

[19] N. Ruiz, Lipid Insights 8 (2015) 21–31.

[20] S. Kalynych, R. Morona, M. Cygler, FEMS Microbiol. Rev. 38 (2014) 1048–1065.

[21] V. Lazarevic, D. Karamata, Mol. Microbiol. 16 (1995) 345–355.

[22] J.B. Ward, Microbiol. Rev. 45 (1981) 211–243.

[23] M.S. Trent, A.A. Ribeiro, W.T. Doerrler, S. Lin, R.J. Cotter, C.R. Raetz, J. Biol. Chem. 276 (2001) 43132–43144.

[24] M.A. Jorgenson, K.D. Young, J. Bacteriol. 198 (2016) 3070–3079.

[25] N. Kawakami, S. Fujisaki, Biosci. Biotechnol. Biochem. 82 (2018) 940–946.

[26] M.J. Osborn, Annu. Rev. Biochem. 38 (1969) 501–538.

[27] D. Liu, R.A. Cole, P.R. Reeves, J. Bacteriol. 178 (1996) 2102 LP – 2107.

[28] J.W. Peterson, in: S. Baron (Ed.), Med. Microbiol., 4th ed., Galveston (TX), 1996.

[29] C. Galanos, M.A. Freudenberg, Mediators Inflamm. 2 (1993) S11–S16.

[30] B.C. McGrath, M.J. Osborn, J. Bacteriol. 173 (1991) 649 LP – 654.

[31] G. Samuel, P. Reeves, Carbohydr. Res. 338 (2003) 2503–2519.

[32] I.M. Weiner, T. Higuchi, L. Rothfield, M. Saltmarsh-Andrew, M.J. Osborn, B.L. Horecker, Proc. Natl. Acad. Sci. U. S. A. 54 (1965) 228–235.

[33] I. Hug, M.F. Feldman, Glycobiology 21 (2010) 138–151.

[34] R.K. Upreti, M. Kumar, V. Shankar, Proteomics 3 (2003) 363–379.

[35] E. Weerapana, K.J. Glover, M.M. Chen, B. Imperiali, J. Am. Chem. Soc. 127 (2005) 13766–13767.

[36] M.F. Feldman, M. Wacker, M. Hernandez, P.G. Hitchen, C.L. Marolda, M. Kowarik, H.R. Morris, A. Dell, M.A. Valvano, M. Aebi, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 3016–3021.

[37] A. Dell, A. Galadari, F. Sastre, P. Hitchen, Int. J. Microbiol. 2010 (2010) X.

[38] O.C. Olabisi, P. Prasit, African J. Microbiol. Res. 5 (2011) 2555–2565.

[39] L.D. Tatar, C.L. Marolda, A.N. Polischuk, D. van Leeuwen, M.A. Valvano, Microbiology 153 (2007) 2518–2529.

[40] S. D. Workman, L. J. Worrall and N. C. J. Strynadka, *Nat. Commun.*, 2018, **9**, doi:10.1038/s41467-018-03547-8

[41] M. El Ghachi, N. Howe, C. Y. Huang, V. Olieric, R. Warshamanage, T. Touzé, D. Weichert, P. J. Stansfeld, M. Wang, F. Kerff and M. Caffrey, *Nat. Commun.*, 2018, **9**, doi:10.1038/s41467-018-03477-5

[42] W. Mi, Y. Li, S.H. Yoon, R.K. Ernst, T. Walz, M. Liao, Nature 549 (2017) 233–237.

[43] A. C. Y. Kuk, A. Hao, Z. Guan and S. Y. Lee, *Nat. Commun.*, 2019, **10**, doi:10.1038/s41467-019-09658-0

[44] A. Jerga, Y.J. Lu, G.E. Schujman, D. De Mendoza, C.O. Rock, J. Biol. Chem. 282 (2007) 21738–21745.

[45] M. Lis, H.K. Kuramitsu, Infect. Immun. 71 (2003) 1938–1943.

[46] M.D. Hartley, A. Larkin, B. Imperiali, Bioorganic Med. Chem. 16 (2008) 5149–56.

[47] L. Röse, S.H.E. Kaufmann, S. Daugelat, Microbes Infect. 6 (2004) 965–971.

[48] A. Yoshida, H.K. Kuramitsu, Appl. Environ. Microbiol. 68 (2002) 6283–6291.

[49] Y. Shibata, M. Kawada-Matsuo, Y. Shirai, N. Saito, D. Li, Y. Yamashita, J. Med. Microbiol. 60 (2011) 625–630.

[50] Y. Yamashita, T. Takehara, H.K. Kuramitsu, J. Bacteriol. 175 (1993) 6220–6228.

[51] S. Amiteye, K. Kobayashi, D. Imamura, S. Hosoya, N. Ogasawara, T. Sato, J. Bacteriol. 185 (2003) 5306 LP – 5309.

[52] M. Paidhungat, B. Setlow, A. Driks, P. Setlow, J. Bacteriol. 182 (2000) 5505–5512.

[53] J.M. Manson, S. Keis, J.M.B. Smith, G.M. Cook, Antimicrob. Agents Chemother. 48 (2004) 3743–3748.

[54] W. Lee, K. Schaefer, Y. Qiao, V. Srisuknimit, H. Steinmetz, R. Müller, D. Kahne, S. Walker, J. Am. Chem. Soc. 138 (2016) 100–103.

[55] R.E. Harkness, T. Ölschläger, FEMS Microbiol. Lett. 8 (1991) 27–41.

[56] W.D. Van Horn, C.R. Sanders, Annu. Rev. Biophys. 41 (2012) 81–101.

[57] D. Li, J.A. Lyons, V.E. Pye, L. Vogeley, D. Aragão, C.P. Kenyon, S.T.A. Shah, C. Doherty, M. Aherne, M. Caffrey, Nature 497 (2013) 521–524.

[58] D. Li, P.J. Stansfeld, M.S.P. Sansom, A. Keogh, L. Vogeley, N. Howe, J.A. Lyons, D. Aragao, P. Fromme, R. Fromme, S. Basu, I. Grotjohann, C. Kupitz, K. Rendek, U. Weierstall, N.A. Zatsepin, V. Cherezov, W. Liu, S. Bandaru, N.J. English, C. Gati, A. Barty, O. Yefanov, H.N. Chapman, K. Diederichs, M. Messerschmidt, S. Boutet, G.J. Williams, M.M. Seibert, M. Caffrey, *Nat. Commun.*, 2015, **6**, doi:10.1038/ncomms10140.

[59] L.Y. Huang, S.H. Huang, Y.C. Chang, W.C. Cheng, T.J.R. Cheng, C.H. Wong, Angew. Chemie - Int. Ed. 53 (2014) 8060–8065.

[60] M.L. Peck, D. Herschlag, RNA 9 (2003) 1180–1187.

[61] R. V. K. Cochrane, F. M. Alexander, C. Boland, S. K. Fetics, M. Caffrey and S. A. Cochrane, *Chem. Commun.*, 2020, **58**, 8603–8606.

[62] D.A. Pearce, N. Jotterand, I.S. Carrico, B. Imperiali, J. Am. Chem. Soc. 123 (2001) 5160–5161.

[63] M.D. Shults, B. Imperiali, J. Am. Chem. Soc. 125 (2003) 14248–14249.

[64] J.A. González-Vera, E. Luković, B. Imperiali, Bioorganic Med. Chem. Lett. 19 (2009) 1258–1260.

[65] L.B. Peterson, B. Imperiali, in: H. Waldmann, P. Janning (Eds.), Concepts Case Stud. Chem. Biolo, 2014, pp. 1–16.

[66] D. Zamora-Olivares, T.S. Kaoud, J. Jose, A. Ellington, K.N. Dalby, E. V Anslyn, Angew. Chem. Int. Ed. Engl. 53 (2014) 14064–14068.