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REVIEW

Modeling bacterial UDP-HexNAc: polyprenol-P HexNAc-1-P transferases

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Protein N-glycosylation in eukaryotes and peptidoglycan biosynthesis in bacteria are both initiated by the transfer of a D-*N*-acetylhexosamine 1-phosphate to a membrane-bound polyprenol phosphate. These reactions are catalyzed by a family of transmembrane proteins known as the UDP-D-*N*-acetylhexosamine: polyprenol phosphate D-*N*-acetylhexosamine 1-phosphate transferases. The sole eukaryotic member of this family, the D-*N*-acetylglucosamine 1-phosphate transferase (GPT), is specific for UDP-GlcNAc as the donor substrate and uses dolichol phosphate as the membrane-bound acceptor. The bacterial translocases, MraY, WecA, and WbpL, utilize undecaprenol phosphate as the acceptor substrate, but differ in their specificity for the UDP-sugar donor substrate. The structural basis of this sugar nucleotide specificity is uncertain. However, potential carbohydrate recognition (CR) domains have been identified within the C-terminal cytoplasmic loops of MraY, WecA, and WbpL that are highly conserved in family members with the same UDP-*N*-acetylhexosamine specificity. This review focuses on the catalytic mechanism and substrate specificity of these bacterial UDP-D-*N*-acetylhexosamine: polyprenol phosphate D-*N*-acetylhexosamine 1-P transferases and may provide insights for the development of selective inhibitors of cell wall biosynthesis.

Key words: MraY/tunicamycin/undecaprenol/WecA/WbpL

Introduction

The UDP-HexNAc: polyprenol-P HexNAc-1-P transferase enzyme family, which includes both eukaryotic and prokaryotic transmembrane proteins, catalyzes the biosynthesis of polyprenol-linked oligosaccharides (Lehrman, 1994). These transferase-catalyzed reactions involve a membrane-associated polyprenol phosphate acceptor and a cytoplasmic UDP-D-*N*-acetylhexosamine sugar nucleotide as the donor substrate. The eukaryotic UDP-GlcNAc: dolichol-P GlcNAc-1-P transferase (GPT) transfers *N*-acetylglucosamine-1-phosphate (GlcNAc-1-P) from UDP-GlcNAc to the polyisoprenoid acceptor dolichol phosphate to form GlcNAc-PP-dolichol (Lehrman, 1991; McLachlan and Krag, 1992). This is the first committed step in the assembly of dolichol-linked oligosaccharide intermediates and is essential for eukaryotic protein N-glycosylation (Burda and Aebi, 1999; Imperiali *et al.*, 1999). The prokaryotic enzymes catalyze reactions analogous to those of the eukaryotic GPTs, but which lead to the formation of polyprenol-linked oligosaccharides involved in bacterial cell wall and peptidoglycan assembly (Bugg and Brandish, 1994; Anderson *et al.*, 2000).

There are significant sequence similarities amongst the UDP-GlcNAc/MurNAc enzyme family members (Lehrman, 1991, 1994; Anderson *et al.*, 2000; Amer and Valvano, 2001; Bouhss *et al.*, 2004; Lloyd *et al.*, 2004) (Figure 1). A series of six conserved sequences (designated A through F) ranging from 5 to 13 amino acid residues have been identified within both the bacterial and eukaryotic members of the family (Nogare *et al.*, 1998). Systematic deletion of the internal A–F microdomains results in Chinese hamster ovary (CHO) cells that lack enzyme activity, but are still able to bind tunicamycin, a substrate analog inhibitor (Nogare *et al.*, 1998). These conserved sequences all occur at or near an interface between hydrophilic and hydrophobic domains. Their distribution suggests a possible involvement in substrate binding and/or catalysis because the sugar-nucleotide donors are hydrophilic, whereas the polyprenol acceptor is hydrophobic (Bugg and Brandish, 1994; Anderson *et al.*, 2000). The relative order and positions of these conserved sequences strongly imply that this group of enzymes constitutes a conserved family, and for that reason, they have been termed the UDP-GlcNAc/MurNAc family (Lehrman, 1994).

Four subgroups of bacterial enzymes have been identified within the family based on their specific substrate preference, MraY, WecA/TagO, WbcO/WbpL, and RgpG (Anderson *et al.*, 2000). These subgroups are highly homologous and use a common, membrane-bound acceptor substrate, undecaprenol phosphate, but differ in their selectivity for soluble sugar nucleotide donor. MraY-type transferases are highly specific for UDP-*N*-acetylmuramate-pentapeptide (UDP-MurNAc-pp) (Meadow *et al.*, 1964; Hammes and Neuhaus, 1974), whereas WecA proteins are selective for UDP-*N*-acetylglucosamine (UDP-GlcNAc) (Barr *et al.*, 1989), sugar nucleotides that differ only by the UDP-MurNAc-pp substituent, 3-*O*-lactyl-pentapeptide. The WbcO/WbpL substrate specificity has not yet been determined, but the structure of their biosynthetic endproducts implies that

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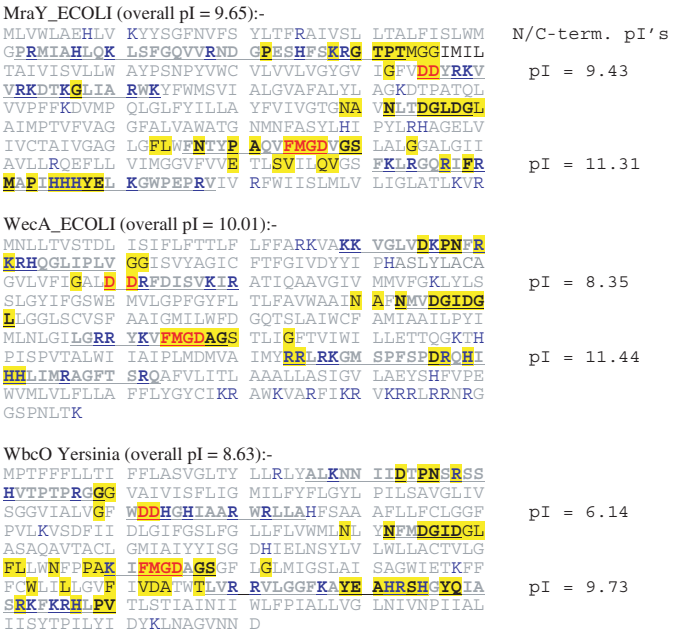

Fig. 1. Conserved functional domains for the MraY/WecA/WbcO transferase family. The postulated active site Asp nucleophile (V/IFMGD motif) and the Mg^{2+}-cofactor binding site (DD) are highlighted in red. The cytoplasmic domains (cytoloops I–V) are indicated (bold underline) with the most highly conserved motifs boxed in yellow. The postulated carbohydrate recognition (CR) domains are located on cytoloop V. Calculated isoelectric points (pI) for the entire sequences and their N- and C-terminal distribution are as shown. Basic residues are shown in blue.

UDP-*N*-acetyl-D-fucosamine (UDP-FucNAc) and/or UDP-*N*-acetyl-D-quinosamine (UDP-QuiNAc) are used (Skurnik, 1999; DiGiandomenico *et al*., 2002; Raymond *et al*., 2002; Skurnik and Bengoechea, 2003). Similar reasoning suggests that the RgpG subgroup is composed of relatively nonspecific transferases that can use either UDP-FucNAc or UDP-GlcNAc (Shibata *et al*., 2002). The transferred sugar phosphates in these transferase-catalyzed reactions are evidently all 2-deoxy-2-*N*-acetyl-D-hexosamine-1-phosphates, suggesting that it is this distinguishing structural motif that determines the sugar nucleotide substrate specificity (Anderson *et al*., 2000).

This review focuses specifically on the bacterial UDP-HexNAc: polyprenol-P HexNAc-1-P transferases and will discuss the MraY, WecA/TagO/Llm, WbpL/WbcO, and RgpG subfamily members. Details of the carbohydrate recognition (CR) domains, DDxxD Mg^{2+}-binding motif, and the active site nucleophile motif will be discussed. Mechanistic and enzyme inhibitor studies are also reviewed, with particular reference to the substrate analog inhibitor, tunicamycin.

The MraY transferase subfamily

The D-MurNAc-pentapeptide-1-P transferase (MraY, translocase I) is an essential and ubiquitous bacterial enzyme that catalyzes the first membrane-associated step in peptidoglycan biosynthesis (van Heijenoort, 2001a,b). Peptidoglycan biosynthesis is initiated in the cytoplasm where the precursor sugar nucleotide UDP-MurNAc-L-Ala-D-Glu-X-D-Ala-D-Ala (where X is either m-DAP or L-Lys) is assembled by the stepwise addition of L-alanine, D-glutamate, and *meso*-diaminopimelic acid (*meso*-DAP) to UDP-*N*-acetyl-muramic acid, followed by the addition of the D-Ala-D-Ala dipeptide (van Heijenoort, 2001a). These additions are catalyzed by the Mur group of enzymes and constitute an important target for antibacterial agents (Wong and Pompliano, 1998; Trias and Yuan, 1999; El Zoeiby *et al*., 2003; Silver, 2003).

Subsequent biosynthetic steps for peptidoglycan occur at the periplasmic membrane (van Heijenoort, 2001b). MraY catalyzes the transfer of MurNAc-pentapeptide-1-P from the sugar nucleotide precursor to the membrane-associated lipid carrier (undecaprenol phosphate) to give MurNAc-pentapeptide-PP-undecaprenol (lipid I), with release of uridine monophosphate (UMP). Translocase II (MurG), an *N*-acetylglucosamine-β-1,4-transferase then catalyzes the addition of *N*-acetylglucosamine (GlcNAc) to lipid I to yield GlcNAc-MurNAc-pentapeptide-PP-undecaprenol (lipid II) and release UDP. This second transferase is encoded by the *murG* gene and has been shown to be peripherally associated with the cytoplasmic face of the membrane (Ha *et al*., 2000, 2003; van den Brink-van der Laan *et al*., 2003). The lipid II intermediate is then "flipped" to the outer face of the membrane where it becomes the initial substrate for the peptidoglycan cross-linking reactions (van Heijenoort, 2001a,b).

The D-MurNAc-pentapeptide-1-P transferase (MraY) belongs to the UDP-GlcNAc/MurNAc enzyme family and has been studied in *Escherichia coli* (Geis and Plapp, 1978), *Micrococcus lysodiekticus* (Anderson *et al*., 1965), and *Staphylococcus aureus* (Pless and Neuhaus, 1973). In *E. coli*, the *mraY* gene encoding the transferase has been found in the *mra* (murein) gene cluster and has been cloned and sequenced (Ikeda *et al*., 1991). The *mra* region contains genes involved in either cell division or murein synthesis. The MraY protein is an essential enzyme, and cells depleted of MraY first swell and then rupture (Boyle and Donachie, 1998). It is one of the most basic proteins found in bacteria with a theoretical pI around 9.5 (Anderson *et al*., 2000). Overexpression of MraY is often detrimental, and it is only recently that *E. coli* MraY protein has been overexpressed and purified (Bouhss *et al*., 2004; Lloyd *et al*., 2004).

The membrane topologies of the MraY transferases from both the Gram-negative *E. coli* and Gram-positive *S. aureus* have been established by protein fusion to beta-lactamase and used to generate a common topological model (Bouhss *et al*., 1999). This model was in agreement with many structural features predicted from the comparative sequence analysis of 25 other MraY proteins, which confirmed its validity as a topological model for all eubacterial MraYs (Bouhss *et al*., 1999). Putative MraY proteins contain 10 transmembrane domains, with the N- and C-termini exposed on the periplasm face of the bacterial membrane. Five cytoplasmic loops (designated cytoloop I–V) and four periplasmic loops (designated exoloop I–IV) are apparent, the topology and distribution of which are highly conserved in other MraY subfamily members (Bouhss *et al*., 1999; Anderson *et al*., 2000; Amer and Valvano, 2001, 2002) (Figure 1).

The MraY proteins generally contain either 324 or 360 amino acid residues. The *Bacillus subtilis* MraY protein is typical of the 324 amino acid type, and the *E. coli* MraY of the longer, 360 amino acid type. The major differences between the two types occur at the N-terminus (Figure 2). The 324 amino acid type MraYs have only 3–5 periplasmically located residues before the first transmembrane domain. In addition, the 324 amino acid type lacks a 15-residue sequence that maps to the third periplasmic loop (Figure 2). Exceptions to these two general types are the unusually large MraYs of *Bacteroides* species (typically 419 amino acid, 47 kDa) and the smaller MraY homolog (Q6W3M8, 149 amino acid, 16.7 kDa) of *Alvinella pompejana* epibiont 6C6, a symbiont from a deep-sea polychaete that colonizes tubes on the sides of black smoker chimneys. This latter gene encodes a putative MraY protein with close homology to 360 amino acid type *mraY*'s at the C-terminus, but lacks the N-terminal domains (Figure 2). It is adjacent to a gene with homology to *murD* (UDP-MurNAc-Ala-D-Glu ligase) with just nine nucleotides separating start and finish sites (Campbell *et al*., 2003).

Hydrophobity profiling indicates that the larger *Bacteroides* MraY's are still comprised of 10 transmembrane (TM) domains (N. Price, unpublished). However, two additional sequences, LSPDVVIRENIEVQKSENEIEVIHGTH and EHAQTAG, are inserted into the second periplasmic loop (cytoloop II) and an extra 17 residues, SLVEPGCSVKFTK-PDQL, are inserted into cytoplasmic loop V immediately

```
tr|Q6W3M8                ..........  ..........  ..........  ..........  ..........
sp|Q03521|MRAY_BACSU     ..........  ..........  .MLEQVILFT  ILMGFLISVL  LSPILIPFLR
sp|P15876|MRAY_ECOLI     MLVWLAEHLV  KY.YSGFNVF  SYLTFRAIVS  LLTALFISLW  MGPRMIAHLQ
tr|Q64ZL8                MLYYLFEWLH  KLNFPGAGMF  GYTSFRALMA  IILALLISSI  WGDKFINLLK

tr|Q6W3M8                ..........  ..........  ..........  ..........  ..........
sp|Q03521|MRAY_BACSU     RLKFGQSIRE  EGPK.SHQKK  SGTPTMGGVM  IILSIIVTTI  VMTQKFSEIS
sp|P15876|MRAY_ECOLI     KLSFGQVVRN  DGPE.SHFSK  RGTPTMGGIM  ILTAIVISVL  LWAYPS..N.
tr|Q64ZL8                RKQITETQRD  AKIDPFGVNK  VGVPSMGGVI  IIVAILIPCL  LLGKLHN...

tr|Q6W3M8                ..........  ..........  ..........  ..........  ..........
sp|Q03521|MRAY_BACSU     PEMVLLLFVT  LGYGLLGFLD  DYIKVVMKRN  LGLTSKQKLI  GQIIIAV.VF
sp|P15876|MRAY_ECOLI     PYVWCVLVVL  VGYGVIGFVD  DYRKVVRKDT  KGLIARWKYF  WMSVIALGVA
tr|Q64ZL8                IYMILMLITT  VWLGSLGFAD  DYIKIFKKDK  EGLHGKFKII  GQVGLGLIVG

tr|Q6W3M8                ..........  ..........  ..........  ..........  ..........
sp|Q03521|MRAY_BACSU     YAVYHY....  ..........  ..........  .YNFATDIRI  PG........
sp|P15876|MRAY_ECOLI     FALYLAG...  ..........  ..........  KDTPATQLVV  PFF.......
tr|Q64ZL8                LTLYLSPDVV  IRENIEVQKS  ENEIEVIHGT  HDLKSTQTTI  PFFKSNNLDY

tr|Q6W3M8                ..........  ..........  ..........  ..........  ..........
sp|Q03521|MRAY_BACSU     TDLSFDLG..  .....WAYFI  LV.LFMLVGG  SNAVNLTDGL  DGLLSGTAAI
sp|P15876|MRAY_ECOLI     KDVMPQLG..  .....LFYIL  LA.YFVIVGT  GNAVNLTDGL  DGLAIMPTVF
tr|Q64ZL8                ADLVGFMGEH  AQTAGWILFV  IITIFVVTAV  SNGANLNDGM  DGMAAGNSAI

tr|Q6W3M8                ....MAIIVY  ITGNYVLSKY  LLLPNIGGVG  EITVLASSLS  GALFGFLWYN
sp|Q03521|MRAY_BACSU     AFGAFAILAW  NQSQYDVA..  ..........  ...IFSVAVV  GAVLGFLVFN
sp|P15876|MRAY_ECOLI     VAGGFALVAW  ATGNMNFASY  LHIPYLRHAG  ELVIVCTAIV  GAGLGFLWFN
tr|Q64ZL8                IGLTLGILAY  VSSHIEFAGY  LNIMYIPGSE  ELVIFICAFI  GALIGFLWYN

tr|Q6W3M8                CYPAELFMGD  TGSLSIGGVI  AYMAILAKSE  FLLLLIGFIF  VIETISVIVQ
sp|Q03521|MRAY_BACSU     RDPAKVFMGD  TGSLALGGAI  VTIAILTKLE  ILLVIIGGVF  VIETLSVILQ
sp|P15876|MRAY_ECOLI     TYPAQVFMGD  VGSLALGGAL  GIIAVLLRQE  FLLVIMGGVF  VVETLSVILQ
tr|Q64ZL8                AYPAQVFMGD  TGSLTIGGII  AVFAIIIHKE  LLIPILCGIF  LVENLSVLLQ

tr|Q6W3M8                VGSYKVRQK.  ....RIFLMA  PIHHHFEMKQ  ..........  .......WAE
sp|Q03521|MRAY_BACSU     VISFKTTGK.  ....RIFKMS  PLHHHYELVG  ..........  .......WSE
sp|P15876|MRAY_ECOLI     VGSFKLRGQ.  ....RIFRMA  PIHHHYELKG  ..........  .......WPE
tr|Q64ZL8                RFYYKAGKRK  GIKQRLFKRA  PIHDHFRTSM  SLVEPGCSVK  FTKPDQLFHE

tr|Q6W3M8                NKIIVRFWMV  AFVANILALI  TIKIR
sp|Q03521|MRAY_BACSU     WRVVVTFWAA  GLLLAVLGIY  IEVWL
sp|P15876|MRAY_ECOLI     PRVIVRFWII  SLMLVLIGLA  TLKVR
tr|Q64ZL8                SKITVRFWIV  TIVLAAITII  TLKIR
```

Fig. 2. Pileup alignment of representative MraY sequences of different lengths. *Alvinella pompejana* epibiont 6C6 (Q6W3M8), 149 amino acids; *Bacillus subtilis* (Q03521), 324 amino acids *Escherichia coli* (P15876), 360 amino acids; and *Bacteroides fragilis* (Q64ZL8), 422 amino acids.

after the CR domain (Figure 2). The *Bacteroides* MraYs are also amongst the least conserved within the CR domain itself, with the sequence RAPIHDHFRT in place of the usually highly conserved MAPIHHHFEL (note particularly the His-Asp-His instead of the usual triple-His motif). Also less conserved in this region are the glutamine-containing MAPL*Q*HHFEL motifs of *Streptomyces coelicolor* and *Streptomyces avermitilis* (P56833 and Q82AD9, respectively); the *CS*PLHHHYE*Y* motif characteristic of *Chlamydia* species; the MAP*F*HHHFE motif of mycobacterial MraYs; and, in several miscellaneous bacteria, the replacement of the Pro by a fourth His residue, that is, MA*H*LHHHFEL. Interestingly, the atypical MraY CR motif in mycobacterial species, with Phe replacing the usual Leu/Ile at position 4, correlates with their N-glycolylated peptidoglycan (Raymond *et al*., 2005), suggesting that this replacement may extend the MraY substrate specificity to include UDP-MurN-glycolyl-pentapeptide substrates.

The enzyme kinetics of *S. aureus* (Copenhagen) MraY have been studied in detail (Heydanek *et al*., 1969). The reaction pathway occurs by a double displacement mechanism according to the following sequence:

UDP-MurNAc-pentapeptide + MraY-Nuc$^+$ $\rightarrow$ MraY-MurNAc-pentapeptide + UMP (step 1)

MraY-MurNAc-pentapeptide + undecaprenol-P $\rightarrow$ MraY + MurNAc-pentapeptide-PP-undecaprenol (step 2)

The first reaction (step 1) involves the formation of an acyl-enzyme intermediate, due to a nucleophilic attack by an active site residue on the sugar nucleotide alpha phosphate. The leaving group for this reaction is UMP. Note also that step 1 is reversible (Pless and Neuhaus, 1973), and that this UMP exchange reaction has been used as the basis for several MraY activity assays (Geis and Plapp, 1978; Zawadzke *et al*., 2003; Bouhss *et al*., 2004). Step 1 is competitively inhibited in the forward direction by 5-fluoro-UDP-MurNAc-pentapeptide (Ki = 0.12 mM) and in the reverse, exchange reaction by 5-fluoro-UMP (Ki = 50 μM) (Stickgold and Neuhaus, 1967). The second reaction (step 2) involves the displacement of the acyl-enzyme intermediate with transfer of the D-MurNAc-pentapeptide-1-P group to the membrane-bound undecaprenol phosphate. The product of this reaction, D-MurNAc-pentapeptide-PP-undecaprenol (lipid I), represents the first membrane-bound intermediate in the biosynthesis of peptidoglycan (Neuhaus, 1970; van Heijenoort, 2001b).

MraY substrate specificity

The MraY sugar nucleotide substrate, UDP-MurNAc-pentapeptide, may vary in the composition of its 3-*O*-lactyl pentapeptide substituent. The pentapeptide most often has a DAP residue in position 3 as in *E. coli* or a lysine residue as in *S. aureus* (Hammes and Neuhaus, 1974; Bouhss *et al*., 1999). However, complementation analyses have shown that the *S. aureus* MraY is functional in *E. coli* (Bouhss *et al*., 1999). This tolerance of MraY toward different peptide moieties in the nucleotide substrate shows a low specificity of the enzyme for the peptide side chain. MraY has been shown to accept shorter or longer peptides as well as modified peptides as substrates: dipeptides (Ornelas-Soares *et al*., 1994), tripeptides (Hammes and Neuhaus, 1974; van Heijenoort *et al*., 1992), tetrapeptides (Hammes and Neuhaus, 1974), acetylated and dansylated pentapeptides (Ward and Perkins, 1974; Weppner and Neuhaus, 1977; Brandish *et al*., 1996a, b; Stachyra *et al*., 2004), and hexa- and heptapeptides (Billot-Klein *et al*., 1997).

The *S. aureus* MraY is selective for its native precursor, UDP-MurNAc-Ala-D-Glu-Lys-D-Ala-D-Ala, and there is high specificity for L-Ala in position 1 and the D-Ala at position 4 (Hammes and Neuhaus, 1974). Replacement of both L-Asp-1 and D-Asp-4 by glycine reduced the *S. aureus* MraY Vmax/Km by 135-fold (Hammes and Neuhaus, 1974). UDP-MurNAc-tetrapeptide, lacking the outermost D-Ala, or UDP-MurNAc-tripeptide were 4-fold and 77-fold, respectively, less active than the native substrate. The UDP-MurNAc-tripeptide rate is characterized by a high Km and at higher concentration is comparable to that of the native substrate. The rate of transfer from 5-fluoro-UDP-MurNAc-pentapeptide is <2% of that observed with the native substrate (Stickgold and Neuhaus, 1967; Neuhaus, 1970), and 5-fluoro-UDP-MurNAc-pentapeptide is a competitive inhibitor (Ki = 1.2×10^{-4} M) in the transfer reaction (Neuhaus, 1970). 4-Fluorinated UDP-MurNAc pentapeptide has also been synthesized and inhibits the growth of gram-positive bacteria in culture when added at 0.01 mg/mL (Ueda *et al*., 2004).

The specificity of the MraY-catalyzed UMP exchange reaction has also been studied. 5-Fluoro-UMP is a competitive inhibitor (Ki = 5×10^{-4} M) of the exchange reaction (Stickgold and Neuhaus, 1967). When 5-fluoro-UMP is used in the exchange reaction, the rate is <0.5% of that observed with UMP. It was also observed that UDP-MurNAc-Ala-D-Glu-Lys-D-Ala-Gly is a moderate competitive inhibitor of the transferase reaction (Ki = 0.84 mM), indicating that the analogs interact at the same binding site(s) (Stickgold and Neuhaus, 1967). UDP-MurNAc-tripeptide is also a poor substrate for the UMP exchange reaction, 150-fold less active than UDP-Mur-pentapeptide (Neuhaus, 1970). In peptidoglycan of other bacteria, the Lys residue at position 3 is often replaced by DAP, and indeed, the *S. aureus* MraY enzyme has low specificity for this substitution (Hammes and Neuhaus, 1974). This lack of specificity for the diamino acid residue has been exploited in fluorescence-based MraY assays that use UDP-MurNAc-pentapeptide dansyl substituted on the Lys/DAP amino group (Brandish *et al*., 1996a; Stachyra *et al*., 2004). Quenching experiments with this fluorescent substrate show that the dansyl group is located *in vivo* close to the membrane interface (Weppner and Neuhaus, 1978).

The specificity of *E. coli* MraY for the polyprenol phosphate substrate has also been studied. The preferred substrate is the natural one, undecaprenol-P, although the enzyme can also accept heptaprenyl phosphate (C_{35}) and dodecaprenyl phosphate (C_{60}) as substrates (Brandish *et al*., 1996a). The ability of MraY to use the eukaryotic lipid carrier, dolichol-P, has not as yet been tested, but the bacterial GlcNAc-1-P transferase WecA is unable to utilize dolichol-P as a lipid carrier (Rush *et al*., 1997).

The WecA/TagO/Llm transferase subfamily

The *wecA* gene (formerly called *rfe*) specifies the UDP-GlcNAc: undecaprenol phosphate GlcNAc-1-P transferase (WecA) that catalyzes the first step in the biosynthesis of enterobacterial common antigen (ECA) (Schmidt *et al*., 1976; Meier-Dieter *et al*., 1992) and lipopolysaccharide (LPS) O-antigen (Schmidt *et al*., 1976; Alexander and Valvano, 1994; Rick *et al*., 1994). ECA is a glycolipid produced by all members of the Enterobacteriaceae (Barr and Rick, 1987; Meier-Dieter *et al*., 1992). The early steps in the biosynthesis of ECA involve the assembly of the initial sugar repeat unit onto undecaprenol phosphate, to yield a lipid-linked intermediate (Rick *et al*., 1985; Barr and Rick, 1987). The genetic determinants of the ECA biosynthesis are located in the *rfe–rff* gene cluster on the *E. coli* chromosome and have been characterized in *E. coli* mutants defective in ECA biosynthesis (Meier-Dieter *et al*., 1992). One of these genes, the *wecA* (*rfe*) gene, is essential for the synthesis of ECA and also for the biosynthesis of LPS O-antigen (Schmidt *et al*., 1976). *Escherichia coli wecA* mutants lack the ability to synthesize GlcNAc-PP-undecaprenol (Barr and Rick, 1987; Meier-Dieter *et al*., 1992).

The general topology of WecA/TagO/Llm subfamily members is similar to MraY and typically consists of 11 transmembrane domains (Anderson *et al*., 2000). However, unlike MraY, the C-terminus of WecA is cytosolic, giving rise to the 11th TM domain and hence to five cytoplasmic loops and five external loops (Anderson *et al*., 2000). The terminal transmembrane helix (TM-11) is essential for the stability of the protein, perhaps because of its interactions with other transmembrane segments or because it targets the WecA protein to the plasma membrane (Amer and Valvano, 2000). The C-terminal region following TM-11 has a net positive charge, further evidence for its cytosolic location (Amer and Valvano, 2000). For the *E. coli* MraY and WecA proteins, these structural differences may underlie the different detergent requirements to maintain their activity in vitro. The *E. coli* WecA is active in Chaps but inactivated by Triton X-100, whereas the opposite is true for MraY activity (Umbreit and Strominger, 1972; Hyland and Anderson, 2003), a difference that has been used to distinguish WecA, MraY, and MurG activities in a high-throughput solid-phase extraction assay (Hyland and Anderson, 2003).

Removal of the first three transmembrane domains from the *E. coli* WecA protein, including the cytoplasmic and periplasmic loops between them, results in nonfunctional WecA enzymes (Amer and Valvano, 2000). However, these truncated WecA mutants are still correctly inserted in the cytoplasmic membrane, suggesting that the first N-terminal 110 amino acids of WecA are required for enzyme function but not for membrane insertion (Amer and Valvano, 2000). Hence, WecA may be inserted in the membrane by a *sec*-independent mechanism involving residues in the middle and/or the C-terminal part of the protein (Amer and Valvano, 2000). A comparison of the bacterial WecA sequences with the eukaryotic GlcNAc-1-P transferases (GPTs) found in yeast and in CHO cells revealed overall general similarities (Zhu and Lehrman, 1990; McLachlan and Krag, 1992; Lehrman, 1994). The similarity and identity of the *E. coli* WecA protein with the GPT from CHO cells were 57.5 and 23.8%, respectively (Meier-Dieter *et al*., 1992).

Proteins in the WecA/TagO/Llm subfamily typically contain 350–360 amino acids. Exceptionally large WecA homologs are those of *Pirellula baltica* (Q7UQ81, 532 amino acid, 57 kDa) and *Nitrosomonas europaea* (Q82SN0, 540 a.a) (N. Price, unpublished). The *N. europaea* gene has strong homology to the other WecA subfamily members at the N-terminus and low similarity to SpaA, a two-component sensor histidine kinase, at the C-terminus. The *Pirellula* gene has similarity to *tagO* at the N-terminus (BLAST E-value = $1e^{-32}$), but with a unique 173 amino acid C-terminal sequence. Importantly, the predicted DDxxD Mg^{2+}-binding motif and the WecA-type HIHH CR motif (discussed below) are conserved for both of these sequences (N. Price, unpublished).

Homologs of WecA are also found in *Bacillus* sp. and on the genomes of mycobacteria. *Bacillus subtilis* 168 *tagO* gene has relatively low overall homology to *E. coli wecA* (BLAST E-value = $2e^{-17}$), but has more pronounced similarity in the CR domain. TagO is required for the biosynthesis of two teichoic acids (TAs) in *B. subtilis* 168, a major TA required for growth and a nonessential minor TA (Soldo *et al*., 2002). Given the known hypersensitivity of *Bacillus* bacteria to tunicamycin (Tamura *et al*., 1976), a nonselective inhibitor, it is likely that TagO is a more important target than MraY in these strains. If so, it will be interesting to compare the tunicamycin sensitivity of *Bacillus* strains relative to their sensitivity to an MraY-specific inhibitor, such as mureidomycin or liposidomycin. A lipophilic protein (Llm) in *S. aureus* with homology to TagO affects the rate of bacterial lysis and resistance to methicillin (Maki *et al*., 1994).

A global sequence alignment of the cytoplasmic domain V within the WecA subgroup revealed a highly conserved CR motif that is specific for the WecA enzymes (Anderson *et al*., 2000). The WecA CR domain contains three conserved histidine residues in close proximity, separated by a small hydrophobic amino acid (typically, HIHH) (Anderson *et al*., 2000; Amer and Valvano, 2001, 2002). Except for their highly basic nature and the presence of a central histidine residue, no other significant homology has been found between the consensus sequences in WecA and MraY. Interestingly, no significant sequence similarity has been observed between the bacterial WecA cytoplasmic domain V and the eucaryotic GPT proteins, even though both enzymes transfer GlcNAc-1-phosphate onto the lipid carrier (Anderson *et al*., 2000).

The WbpL/WbcO and RgpG subfamilies

The WbpL/WbcO subgroup of bacterial UDP-HexNAc: polyprenol-P HexNAc-1-P transferases includes the WbcO protein from *Yersinia enterocolitica* and the WbpL protein from *Pseudomonas aeruginosa* (Dean *et al*., 1999; Skurnik, 1999; Anderson *et al*., 2000; DiGiandomenico *et al*., 2002; Raymond *et al*., 2002). These two transferases initiate LPS O-antigen biosynthesis as putative-D-FucNAc-1-P transferases (Rocchetta *et al*., 1998; Belanger *et al*., 1999; Anderson *et al*., 2000). Similar to other GlcNAc/MurNAc-1-P transferase family members, WbpL is a highly hydrophobic protein possessing 11 predicted transmembrane domains

(Belanger *et al*., 1999; Anderson *et al*., 2000). The *wbcO* gene (formerly called *trsF*) is located on the *Y. enterocolitica* serotype 3 core 3 cluster (Skurnik, 1999; Skurnik and Bengoechea, 2003). The gene *wbpL* has been found in the O-antigen biosynthetic gene cluster in several *P. aeruginosa* serogroups (Dean *et al*., 1999; Raymond *et al*., 2002). It initiates the biosynthesis of both the common and the serotype-specific LPS antigen by transferring GlcNAc-1-P and FucNAc-1-P to undecaprenol-P (Rocchetta *et al*., 1998; Belanger *et al*., 1999). Some serotypes of *P. aeruginosa* have QuiNAc in their O-antigen and may possess a WbpL-like transferase that uses UDP-QuiNAc as the sugar nucleotide substrate (DiGiandomenico *et al*., 2002; Raymond *et al*., 2002).

Sequence analysis of the deduced WbpL/WbcO protein sequences showed a topological arrangement similar to the MraY and the WecA proteins (Anderson *et al*., 2000). WbpL and WbcO, as well as the other members of their subgroup, possess a highly conserved consensus sequence unique to the whole subgroup (Anderson *et al*., 2000). This WbpL/WbcO-specific sequence motif, YEAHRSHxYQx-AxR, is situated within cytoplasmic domain V, similar to the location of the MraY- and WecA-consensus motifs (Figure 1). Complementation analyses have shown that functional mutations in the *P. aeruginosa wbpL* can be restored by the *E. coli wecA* gene, but only for the biosynthesis of the common LPS (Rocchetta *et al*., 1999). This suggests that the substrate specificity of the WecA protein does not extend to either of the sugar nucleotides, UDP-QuiNAc or UDP-FucNAc (Rocchetta *et al*., 1998; Belanger *et al*., 1999).

Genes homologous to *wbcO*/*wbcL* homologs are also found on the genomes of other *Pseudomonas* and *Yersinia* species. The *wbiH* gene of *Burkholderia* sp. (e.g., Q79TO2_BURMA and O69129_BURPS) and *Azoarcus* (Q5P6P6) are assigned to the WbcO/WbpL family by CR domain homology (Anderson *et al*., 2000). A WbcO/WbpL subfamily member is also present in the recently sequenced genome of *Methylococcus capsulatus* str. Bath, where it has a potential role in the biosynthesis of an insoluble capsule (Ward *et al*., 2004). The putative WbcO protein encoded by this gene (Q60AG0) contains a DDxxD-type motif, a conserved active site aspartyl nucleophilic (145-FMG*D*-148), and a characteristic YEAHRSHxYQxAxR CR domain typical of other WbcO/WbpL subfamily members. However, it is considerably smaller (hypothetical 274 residues, 28.7 kDa) than other subfamily members and lacks a 60–70 residue N-terminal domain (N. Price, unpublished).

The WbpL/WbcO consensus motif also shows sequence similarity to another UDP-HexNAc: polyprenol-P transferase subfamily type, typified by the RgpG protein from *Streptococcus mutans* (Anderson *et al*., 2000). RgpG is a putative HexNAc-1-phosphate transferase which is required for rhamnose–glucose polysaccharide (RGP) synthesis (Yamashita *et al*., 1999). The *rgpG* gene can complement a *wecA*-deficient *E. coli* strain, which suggests a functional similarity between the RgpG and the WecA proteins (Yamashita *et al*., 1999). Based on sequence homology in the cytoplasmic domain V, the RgpG CR domain appears to be a hybrid that aligns with both the WecA and the WbcO consensus motifs (Anderson *et al*., 2000).

CR domains

C-terminal cytoplasmically located CR domains have been identified for the UDP-HexNAc: polyprenol-P HexNAc-1-P transferase family that are unique to specific subfamily members (Anderson *et al*., 2000). These CR domains typically consist of 28 residues that form a cytoplasmically located loop (cytoloop V), which is highly basic (pI range 10–12) and is immediately adjacent to the active site of the transferase at the membrane–cytoplasm interface.

There is an absolute correlation between the presence of a particular cytoloop V and the UDP-*N*-acetyl-D-hexosamine substrate specificity (Anderson *et al*., 2000; Amer and Valvano, 2002). The MraY CR domain contains a highly conserved, short internal sequence motif of 13 amino acids: MAPIHHHFELKGW (Anderson *et al*., 2000) that is not found in any of the other subgroups within the transferase family and correlates with the subfamily usage of UDP-MurNAc-pp. Similarly, the WecA CR domain consists of 13 conserved residues RRxxxGxSPFSP-DxxHIHH and is unique to the WecA subfamily members, which selectively use UDP-GlcNAc (Anderson *et al*., 2000; Amer and Valvano, 2001, 2002). Mutational analysis of the CR domain of *E. coli* WecA supports the hypothesis that it is involved in the recognition of UDP-GlcNAc (Amer and Valvano, 2002). Replacement of the entire 279-HIHH-282 motif by GGGG resulted in a 93% reduction of WecA activity. A similar loss of activity was observed for a conservative $WecA_{H279S}$ point mutation, highlighting the importance of the first His residue in the CR domain. These mutations also reduced tunicamycin binding, by 90 and 85% (Amer and Valvano, 2002), suggesting that the initial UDP-GlcNAc–WecA recognition might occur via the UDP-HexNAc sugar motif. A less dramatic phenotype was observed for a conservative Arg→Lys substitution of the second Arg (Arg-265) at the start of the *E. coli* WecA CR domain. This reduced the enzymatic activity by about 55% and tunicamycin binding by a similar amount (Amer and Valvano, 2002). Basic amino acid residues are also present at the start of the CR domains for MraY and WbcO/WbpL family members (Figure 1). It has been suggested that the *E. coli* WecA Arg-265 could be involved in binding the acidic phosphates of UDP-GlcNAc (Amer and Valvano, 2002), although it is difficult to reconcile this with the binding of UDP-GlcNAc:Mg^{2+} as a complex to the DDxxD motif on cytoloop II (see discussion below). One plausible explanation is that these basic residues help to stabilize the charge on the HexNAc-PP-undecaprenol product or on the covalent HexNAc-1-P: enzyme intermediate that is formed as a consequence of the double displacement reaction mechanism (Heydanek *et al*., 1969).

The WbcO/WbpL family members carry a WbcO-specific cytoloop V that contains the conserved CR domain YEAHRSHxYQxAxR, which potentially determines the specificity for a third sugar nucleotide type, UDP-D-FucNAc and/or UDP-D-QuiNAc (Anderson *et al*., 2000). The CR domain of the RgpG subfamily has a hybrid WecA–WbcO sequence. Complementation data for a *rgpG*-deleted *S. mutans* (Yamashita *et al*., 1999) and the dual nature of its CR domain predicted it to be capable of both GlcNAc1-P and FucNAc-1-P transfer (Anderson *et al*., 2000).

Although the CR domains for MraY, WecA, and WbpL/WbcO have no homology at the sequence level, they are all characterized by exceptionally high isoelectric points (Anderson *et al*., 2000). They may interact selectively with the acidic HexNAc-1-P: enzyme intermediates, as suggested above. Alternatively, this interaction could be with the next glycosyltransferase in the three respective biosynthetic pathways, all of which have a presumed peripheral membrane location (Hu *et al*., 2003; van den Brink-van der Laan *et al*., 2003). The extent to which the CR domains alone dictate transfer specificity will ultimately require the construction of hybrid proteins whose defining cytoloop V domains have been interposed with those from other family members.

The catalytic mechanism and the DDxxD motif

Models of the membrane topology of MraY and WecA have been proposed based on the localization of β-lactamase and FLAG epitope inserts, respectively (Bouhss *et al*., 1999; Amer and Valvano, 2001), and have been refined by comparative sequence alignment of the transferase family (Lehrman, 1994; Nogare *et al*., 1998; Anderson *et al*., 2000). These studies implicate conserved sequence motifs on the five cytoplasmic loops as the UDP-sugar binding site and the catalytic site. Adjacent conserved aspartyl residues located on loop II, D-115/D-116 for MRAY_ECOLI and D-90/D-91 for WECA_ECOLI (Figure 3a), are postulated to be part of a DDxxD motif and are potentially involved in binding a Mg^{2+} cofactor (Amer and Valvano, 2002; Lloyd *et al*., 2004; Xu *et al*., 2004).

Ashby and Edwards first noted DDxxD motifs as a conserved feature of prenyltransferases and proposed that the aspartates in these motifs bind diphosphate-containing substrates through Mg^{2+} bridges (Ashby and Edwards, 1990). Evidence supporting this comes from the X-ray crystal structure of recombinant avian farnesyl diphosphate synthase (FPS), which contains two DDxxD Asp-rich regions (D-117 to D-121 and D-257 to D-261) located at the end of α-helices D and H, respectively (Tarshis *et al*., 1994). Further evidence arose from the binding of samarium heavy metal atoms into the Mg^{2+}-binding sites within the crystal structure of avian FPS (Tarshis *et al*., 1994). The side chains of D-117, D-118, D-121 and of D-257, D-258, D-261 are positioned so that their carboxylate groups all point into the active site cleft. The DDxxD motif generally has a basic residue (Arg or Lys), 10 residues downstream, which is also implicated in binding the metal ion. For avian FPS, these conserved basic residues (Arg-126 and Lys-271) were found on the loops between helices D/E and helices H/I, respectively.

Mutational studies have confirmed the importance of the DDxxD motif for FPS (Joly and Edwards, 1993; Song and Poulter, 1994) and for the D-GlcNAc-1-P transferases (Amer and Valvano, 2002). A substitution mutation of the first Asp in the DDxxD domain II of FPS to Glu decreased the Vmax by over 90-fold, whereas mutation of the third Asp had little effect (Joly and Edwards, 1993). The Michaelis constant remained unchanged by either mutation (Joly and Edwards, 1993). These results suggest that the first aspartate in domain II is involved in the catalysis by FPS, but not in substrate binding.

```
CLUSTAL W multiple sequence alignment
sp|P24140|GPT_CRIGR    105-ICCMIFLGFADDVLNLRWRH-124
sp|P24235|WECA_ECOLI     80-AGVLVFIGALDDRFDISVKI-99
sp|P15876|MRAY_ECOLI    105-LVGYGVIGFVDDYRKVVRKD-124
tr|Q9F8C9|WBCO_YEREN     82-GGVIALVGFWDDHGHIAARW-101
                                  .:*  **  .:  :
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CLUSTAL W multiple sequence alignment
sp|P24140|GPT_CRIGR   240-YHNWYPSQVFVGDTFCYFAGMTFAVVGILGHFSKT-274
sp|P24235|WECA_ECOLI  205-GILGRRYKVFMGDAGSTLIGFTVIWILLETTQGKT-239
sp|P15876|MRAY_ECOLI  255-WFNTYPAQVFMGDVGSLALGGALGIIAVLLRQEFL-289
tr|Q9F8C9|WBCO_YEREN  203-LWNFPPAKIFMGDAGSGFLGLMIGSLAISAGWIET-237
                               ::*:**. .   *     : :
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Fig. 3. (**a**) The putative DDxxD motif for Chinese hamster ovary (CHO) cell UDP-GlcNAc: dolichol-P GlcNAc-1-P transferase (GPT) (P24140), *Escherichia coli* WecA (P24235), *E. coli* MraY (P15876), and *Yersinia enterocolitica* WbcO (Q9F8C9). The conserved Asp-Asp (highlighted in bold) are predicted to be coordinated to the Mg^{2+} cofactor, which forms ligands to the pyrophosphate moiety of the UDP-HexNAc sugar nucleotide substrate or to the 5′-OH, 8′-OH, and 7′-ring oxygen of the inhibitor tunicamycin (Lloyd *et al*., 2004; Xu *et al*., 2004). (**b**) The putative active site nucleophilic Asp V/IFMGD motif for CHO cell GPT (P24140), *E. coli* WecA (P24235), *E. coli* MraY (P15876), and *Y. enterocolitica* WbcO (Q9F8C9). Topology analyses map the conserved Asp to be five residues (highlighted in bold) inside the bilayer on the predicted transmembrane domain following cytoplasmic loop IV.

The Mg^{2+}-binding DDxxD motif identified for *E. coli* WecA is highly conserved for all of the HexNAc-1-P transferase family members (Lehrman, 1994; Anderson *et al*., 2000) (Figure 3a). Hence, the 90-DDxxD-94 in WECA_ECOLI corresponds to 115-DDxxK-119 for MRAY_ECOLI and to 92-DDxxH-96 for the *Y. enterocolitica* WbcO sequence (Figure 3). These motifs are invariably located on cytoplasmic loop II, and for the bacterial transferases are usually 43–45 residues downstream of the conserved Gly-Gly motif at the C-terminal end of cytoloop I. Noticeably, although the DD is invariably present, the adjacent region is not especially conserved (Figures 1 and 3). Substitution mutations that affect the DD motif in *E. coli* WecA or MraY result in decreased enzymatic activities (Amer and Valvano, 2002; Lloyd *et al*., 2004), but substitution of the 3rd Asp (i.e., D-94) in WecA resulted in no observable change. These results are comparable with the mutational analysis of the DDxxD domain II of FPS (Joly and Edwards, 1993) and highlight the importance of the two adjacent DD residues.

Other conserved aspartyl residues are also implicated in the HexNAc-1-P transferase catalytic process (Amer and Valvano, 2002; Lloyd *et al*., 2004). Mutations in D-156 and D-159 of *E. coli* WecA result in <10% of wild-type activity and have significantly reduced binding to tunicamycin

(Amer and Valvano, 2002). These two resides form part of the highly conserved NxxNxxDGIDGL motif that is located on cytoloop III for all the bacterial subfamily transferases (Figure 1). This indicates their probable role in catalytic activity, with the reduced tunicamycin binding suggesting a possible interaction with the UDP-HexNAc (Amer and Valvano, 2002). However, because they are conserved in all family members, including the eukaryotic GPT proteins (Nogare *et al*., 1998), these residues are unlikely to be directly involved in the UDP-HexNAc substrate specificity of the bacterial transferases.

The active site nucleophile

Site-specific mutagenesis at residue D-267 of the *E. coli* MraY has also been shown to eliminate enzyme activity (Lloyd *et al*., 2004). This led to the hypothesis that D-267 is the active site nucleophile responsible for the cleavage of the substrate pyrophosphate bond (Lloyd *et al*., 2004) (Figure 4). The comparable aspartyl residue, D-252, of the CHO cell GPT is highly conserved with other members of the MraY/WecA/WbcO family (Figure 3b) and is also essential for activity (Scocca and Krag, 1997). The hydrophobicity profile of the CHO GPT sequence shows that Asp-252 is located on TM-8, just inside the cytoplasmic side of the membrane (Anderson *et al*., 2000) (Figures 1 and 3b). Hydrophobicity analysis places the *E. coli* MraY Asp nucleophile (D-267) identified by Lloyd *et al*. (2004) at five residues inside the cytoplasmic face of TM-8. Topological analysis of the *E. coli* MraY by *blaM* (β-lactamase) fusion insertion places this residue on cytoloop IV between TM-7 and TM-8 (Bouhss *et al*., 1999). However, it is noticeable that the transmembrane domain TM-8 is predicted to contain only 14 residues (Bouhss *et al*., 1999), typically too small a helix to traverse the membrane bilayer. Moreover, the equivalent active site Asp nucleophile for *E. coli* WecA (D-217) and for *Y. enterocolitica* WbcO (D-215) are similarly placed five residues inside of TM-8 (i.e., 213-VFMGD-217 and 211-IFMGD-215, respectively) (Figure 3b) and are therefore also on the transmembrane helix immediately after cytoloop IV.

The bacterial V/IFMGD motifs are highly conserved with the equivalent region around Asp-286 in the CHO cell GPT (248-VFVGD-252) and are believed to be the active site nucleophile regions (Figure 3b). The terminal aspartyl group in this motif undergoes nucleophilic attack on the beta-phosphate of the UDP-HexNAc substrate, leading to a displacement of UMP and to the formation of an acyl-enzyme intermediate, in which the HexNAc-1-phosphate is covalently attached to the active site Asp (Figure 4). This is in agreement with the double displacement mechanism proposed from kinetic studies on MraY (Heydanek *et al*., 1969; Brandish *et al*., 1996a). Moreover, for *E. coli* MraY, the predicted acyl-enzyme intermediate, MurNAc-pentapeptide-1-P–MraY, has been trapped following the incubation of solubilized MraY with radiolabeled UDP-MurNAc-pentapeptide. The radiolabeled enzyme–substrate complex was observed after gel filtration (Lloyd *et al*., 2004), although unfortunately, the comparable experiment with the *E. coli* MraY D-267 residue mutated was not reported.

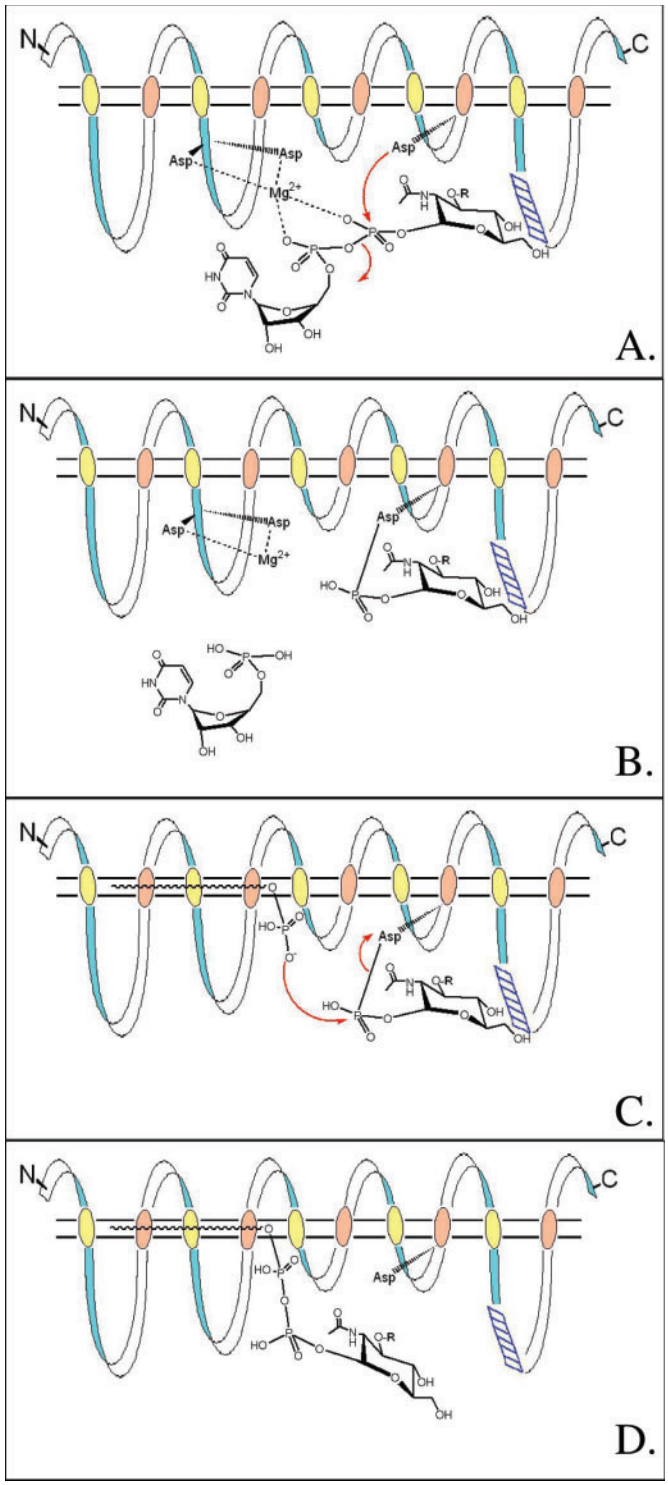

Fig. 4. Generalized mechanism for the UDP-HexNAc: polyprenol-P HexNAc-1-P transferase enzyme family. (**A.**) UDP-D-HexNAc coordinated via the Mg^{2+} cofactor to the DDxxD motif on cytoloop II and nucleophilic attack by active-site Asp located on cytoloop IV/TM-8. (**B.**) Displacement of UMP and formation of a HexNAc-phospho-acyl-enzyme covalent intermediate. (**C.**) Nucleophilic attack by membrane-associated polyprenol phosphate. (**D.**) Formation of the HexNAc-PP-polyprenol product. The putative carbohydrate recognition (CR) domain located on cytoloop V is depicted by the blue cross-hatching.

Enzyme inhibitor studies

Peptidoglycan biosynthesis is a well-documented target for antimicrobial agents, and there are several antibiotics that block the MraY-catalyzed step (Bugg and Walsh, 1992; Kimura and Bugg, 2003). MraY inhibitors can be classified based on their mode of inhibition, being either direct inhibitors of MraY, such as tunicamycin, or indirect inhibitors such as amphomycin and bacitracin (Kimura and Bugg, 2003). Direct inhibitors contain pharmacophores that mimic the nucleotide substrate and have additional hydrophobic moieties attached (Figure 5). Indirect inhibitors act to limit the availability of the undecaprenol-P substrate by interfering with its recycling.

Tunicamycin inhibits both MraY and GPT, which account for its known bacterial and mammalian toxicity (Heifetz *et al*., 1979; Brandish *et al*., 1996b). It reversibly inhibits the transfer of MurNAc-pentapeptide-1-P to undecaprenol-P and the transfer of GlcNAc-1-P to undecaprenol-P or dolichol-P in bacterial or eukaryotic cells, respectively (Tamura *et al*., 1976; Brandish *et al*., 1996b). Several new classes of antibiotics have recently been characterized as potent and specific inhibitors of the MraY-catalyzed step in peptidoglycan assembly (Kimura and Bugg, 2003). These compounds include mureidomycins (Inukai *et al*., 1989; Isono and Inukai, 1991; Inukai *et al*., 1993), liposidomycins (Isono *et al*., 1985; Muroi *et al*., 1997), pacidamycins (Karwowski *et al*., 1989), napsamycins (Chatterjee *et al*., 1994), muraymycins (Lin *et al*., 2002; McDonald *et al*., 2002), FR-900493 (Kimura and Bugg, 2003), capuramycins (Yamaguchi *et al*., 1986), A-503083 (Muramatsu *et al*., 2004), and caprazamycins (Igarashi *et al*., 2003). Like tunicamycin, these compounds share a uridine nucleoside analog moiety found in the enzyme sugar nucleotide substrate (Figure 5). In addition, the φX-174 bacteriophage lysis protein E has been shown to inhibit the MraY-type transferases by a direct interaction with a periplasmic domain (Bernhardt *et al*., 2001).

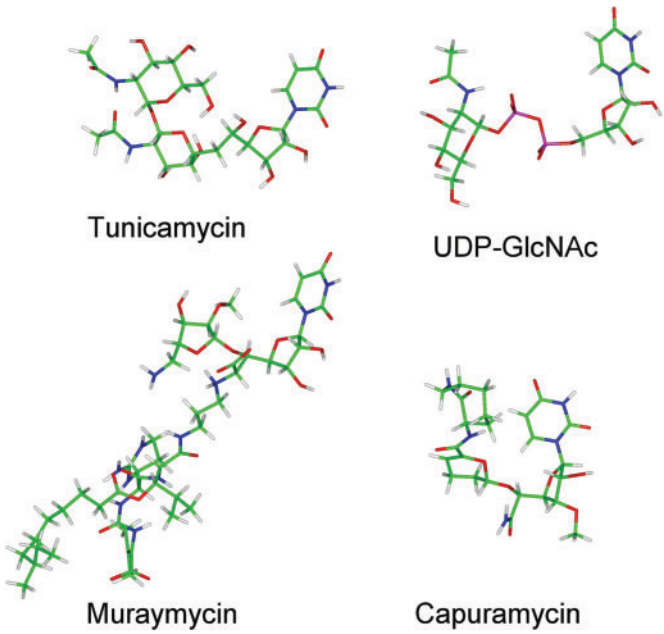

Fig. 5. Conformational alignment of a sugar nucleotide substrate (UDP-D-GlcNAc) and several substrate analog inhibitors, tunicamycin, muraymycin, and capuramycin. The inhibitors share a uridine nucleoside analog moiety found in the sugar nucleotide substrate.

Tunicamycin and mureidomycin inhibit the MraY and the mammalian GPT transferases to varying extents (Heifetz *et al*., 1979; Brandish *et al*., 1996b). Mureidomycin A is >2000-fold more selective for the bacterial MraY MurNAc-pp-1-P transferase, whereas tunicamycin is ~1500-fold more selective for the mammalian GlcNAc-1-P transferase (Brandish *et al*., 1996b). Further, mureidomycin is a poor inhibitor for the formation of lipid-linked *N*-acetylglucosamine for TA synthesis in *B. subtilis* (IC50 > 100 μg/mL) (Bouhss *et al*., 2004). Mureidomycin is competitive with respect to both the fluorescent substrate analog dansyl-UDP-MurNAc-pentapeptide and dodecaprenyl-P (Bouhss *et al*., 2004). Tunicamycin acts as a competitive inhibitor (Ki = 55 μM) with respect to the nucleotide substrate analog dansyl-UDP-MurNAc-pentapeptide, but it is noncompetitive toward the lipid acceptor substrate, undecaprenol phosphate. This suggests that the *N*-acyl chain of tunicamycin may be involved in its localization to the membrane layer rather than in binding to the active site of the enzyme (Bouhss *et al*., 2004). In contrast, the inhibition of the eukaryotic target enzyme GPT by tunicamycin has been reported to be irreversible and noncompetitive with respect to both substrates (Heifetz *et al*., 1979). Thus, the conserved eukaryotic dolichol recognition sites in GPT (Albright *et al*., 1989; Datta and Lehrman, 1993) may represent a potential binding site for the hydrophobic chain of tunicamycin. In a screen for peptidoglycan, biosynthesis inhibitors using permeabilized *E. coli* cells inhibition by tunicamycin was <25% at 50 μM, demonstrating the intrinsic resistance of *E. coli* (Barbosa *et al*., 2002). In contrast, resistance of *E. coli* to mureidomycin A and C is due to the expression of the AcrAB-TolC multidrug efflux system (Gotoh *et al*., 2003). Most *Bacillus* sp. are highly sensitive to tunicamycin, and resistance in *B. subtilis* has been linked to a specific gene, *tmrB* (Noda *et al*., 1992, 1995).

Mureidomycin A and liposidomycin B are both slow binding inhibitors of *E. coli* MraY, with inhibition resulting from either a reversible covalent interaction with the active site or from the conversion of the E.I. complex into a more tightly bound E.I.* state (Brandish *et al*., 1996a,b). Liposidomycin B is competitive with respect to the undecaprenol-P, but noncompetitive with the sugar nucleotide, the reverse of that seen for tunicamycin. Molecular modeling studies suggest that when overlaid on a model of UDP-MurNAc-pentapeptide, the lipid chains of liposidomycin and tunicamycin align in different directions when the uracil fragments are superimposed (Dini *et al*., 2000). This model also suggests that the MurNAc analog of liposidomycin B and the GlcNAc residue of tunicamycin superimpose, and that the pyrophosphate unit of UDP-MurNAc-pentapeptide is mimicked by the ribosamine ring of liposidomycin and the pseudogalactosamine ring of tunicamycin. In this model, the 5′,2″-diazopanone ring of liposidomycin is aligned in the same direction as the tunicamycin 5′-OH group (Dini *et al*., 2000).

The synthesis of a series of simplified pharmacophores based on the nucleotide moiety of liposidomycin also

highlights the importance of stereochemistry at the nucleotide 5′ position. For these synthetic 6-*O*-β-D-ribofuranosyl nucleosides only the (S)-5′-hydroxymethyl isomer showed significant activity against MraY (Dini *et al.*, 2002). The importance of the 5′ position was also probed, with primary and secondary amino groups being 10–50 times more active against MraY than amides or tertiary amino groups (Dini *et al.*, 2001a). Structure-activity relationship studies of these analogs also pointed to the uracil ring as being crucial and catalytic hydrogenation of the uracil 5,6-double bond reduced the inhibitory activity by >20-fold (Dini *et al.*, 2001a). The presence of the 3″-hydroxy group was important to retain activity, and further the 3′-deoxyribosyl uridinyl analogs were even more active, being up to 5-fold more active than the intact ribosyl analog (Dini *et al.*, 2001b).

Inhibition of MraY has also been investigated using 5′-uridinyl dipeptide analogs based on mureidomycin (Gentle *et al.*, 1999; Howard and Bugg, 2003). A uridine 5′-alanine-*N*-methyl-β-alanine ester showed greatest activity, with the *N*-methyl group, a requirement for potent inhibition (Gentle *et al.*, 1999). Hence, the positively charged amine side chain of mureidomycin A was proposed to bind directly to the MraY DDxxD moiety, in lieu of the Mg^{2+} cofactor. In addition, inhibition of MraY by nucleoside analogs of mureidomycin can be relieved by Mg^{2+} suggesting a common binding site within the enzyme (Howard and Bugg, 2003).

Further insight into the active site architecture of the UDP-HexNAc: polyprenol-P HexNAc-1-P transferases has come from consideration of the conformation of the substrate analog tunicamycin (Xu *et al.*, 2004). The conformational flexibility of tunicamycin is somewhat constrained by its four ring systems (Figure 6). Mobility is restricted to the 1″,11′-glycosidic linkage between the A and B rings, the 6′-methylene bridge between the B and C rings, and to restricted rotation about the *N*-glycosidic linkage to the uracil (ring D). Nuclear magnetic resonance (NMR)-derived through bond proton couplings from the uracil H-6 to the tunicamycin H-2′, H-3′, and H-5′ protons established that the pseudoribosyl ring assumes an *endo*-2′-*exo*-3′ puckered conformation with the uracil group *anti*-aligned (Xu *et al.*, 2004). Similar couplings from the tunicamycin H-11′ to H-7′ and H-9′ protons assigned the structure of the pseudogalactosamine pyranose ring as having a $^4C_1$ chair conformation, in which the *N*-acyl group is extended from the back face (Figures 6 and 7). The pseudogalactosamine pyranose ring is presumed to mimic the pyrophosphate linkage of the sugar nucleotide within the enzymatic active site.

The conformational state about the 6′-methylene bridge was rationalized by the preparation of chirally deuterated tunicamycin (Xu and Price, 2004; Xu *et al.*, 2004). *Streptomyces chartreusis*, a tunicamycin-producing streptomycete was cultured on medium containing chemically synthesized (S)-D-(6-^{2}H)-glucose, resulting in deuterium incorporation at the 6″-methylene of the GlcNAc (ring A) and 6′-methylene in the 6′-methylene bridge (Xu *et al.*, 2004). NMR correlation spectroscopy (COSY and HSQC) of the resultant chirally labeled (R)-(6′-^{2}H) 6′-methylene bridged tunicamycin assigned H-7′–H-6′a, H-6′b–H-5′, and H-5′–H-4′ as coupled systems. Newman projections of the C-7′–C-6′, C-6′–C-5′, and C-5′–C-4′ bonds were consistent with the NMR data and predicted a C-5′(+), C-6′(+), and C-7′(+) bridge

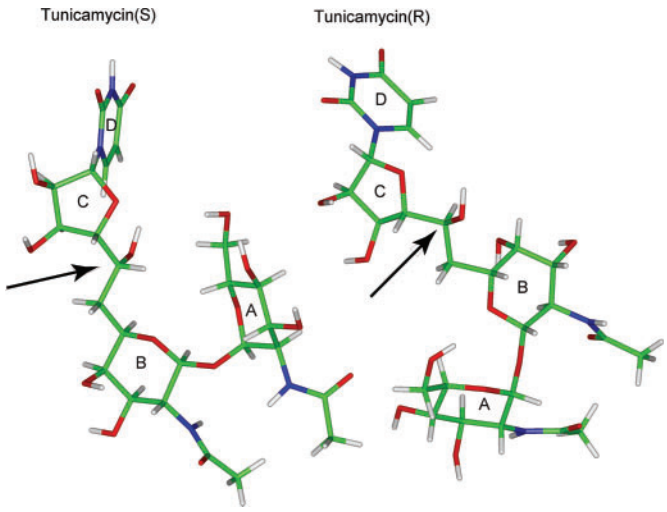

Fig. 6. Energy-minimalized conformational differences for native tunicamycin [S]-5′-hydroxy epimer and its [R]-5′-hydroxy epimer. Conformation flexibility is confined by the four ring systems, *N*-acetylglucosamine, pseudogalactosamine, pseudoribose, and uracil (rings A, B, C, and D, respectively). The epimeric 5′-carbon is indicated by an arrow.

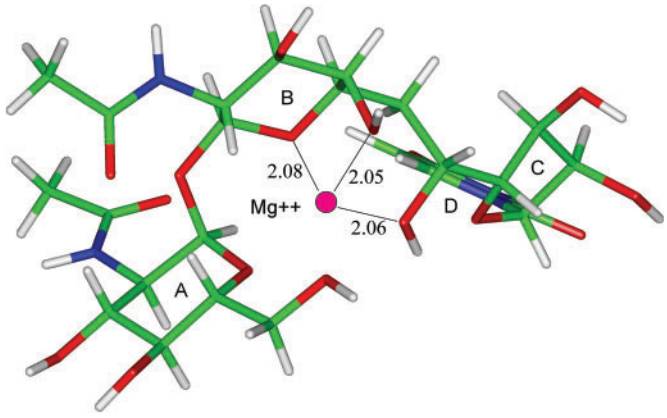

Fig. 7. Conformation of tunicamycin with Mg^{2+} coordinated to positions 5′-OH, 8′-OH, and the pseudoGalN pyranosyl ring 7′-O. The deduced O–Mg^{2+} coordination distances shown are energy minimalized and in accordance with those in the catalytic sites of F1-ATPase (Weber *et al.*, 1998).

conformation (Xu *et al.*, 2004). Molecular modeling by the authors indicates that this arrangement facilitates the chelation of divalent metal ions to the 5′-OH, 8′-OH, and the pseudogalactosamine ring ether oxygen 7′-O, and weaker interactions with the A ring of tunicamycin (Figure 7). Mg^{2+} has a strong propensity to assume octahedral coordination, with the average distance of 2.1 angstroms between the Mg^{2+} and coordinating oxygen atoms (Weber *et al.*, 1998). Molecular dynamics simulations of the tunicamycin (R)-5′-hydroxymethyl epimer in a periodic box of water molecules showed that a totally different extended conformation was taken that persisted throughout the simulation (Figure 6). This result suggests that the 5′-epimer of tunicamycin may never arrive at a conformation that is recognized

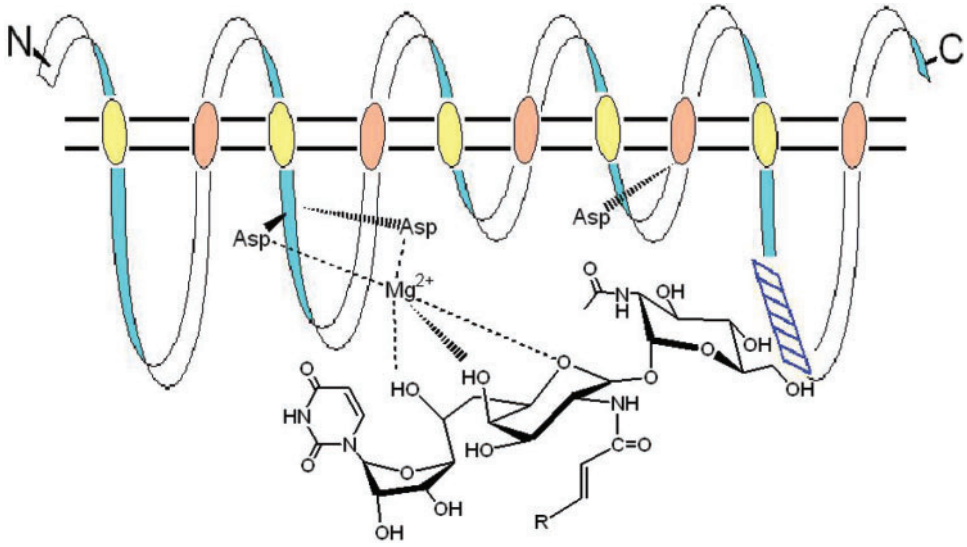

Fig. 8. Schematic model for tunicamycin inhibition of UDP-HexNAc: polyprenol-P HexNAc-1-P transferase. The enzyme-bound Mg^{2+} cofactor is coordinated to tunicamycin at positions 5′-OH, 8′-OH, and the 7′-O ring oxygen, occupying the binding site for the UDP-HexNAc substrate, and thereby inhibiting the first step in the double displacement mechanism. The sixth coordination position of the Mg^{2+} is presumed to be occupied by a molecule of water.

by the receptor protein, or is incorrectly folded when complexed with Mg^{2+} and so does not bind. Consistent with our model of tunicamycin binding to the Mg^{2+} cofactor, tunicamycin–metal complexes were identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy, including a Mg^{2+}–tunicamycin complex and a dimeric complex of Mg^{2+}–$(tunicamycin)_2$ (Xu *et al.*, 2004).

The coordination of Mg^{2+} to the tunicamycin structural motif implicated in mimicking the pyrophosphate linkage of UDP-HexNAc suggests a probable mechanism for its inhibition of the UDP-HexNAc: polyprenol-P HexNAc-1-P transferases family of enzymes. Tunicamycin may be coordinated to the Mg^{2+} cofactor which is in turn ligated to the Asp-Asp of the transferase DDxxD motif (Figure 8). This arrangement would block the binding of the UDP-HexNAc substrate to the active site, thereby causing reversible inhibition of the displacement mechanism at the first step. This proposed mechanism is consistent with the known double displacement kinetics of the transferases, the absolute requirement for Mg^{2+} cofactor, and for the well-defined UMP exchange reaction catalyzed by the transferases. Moreover, the proximity of the tunicamycin GlcNAc moiety (ring A) to the transferase CR domain in this model suggests that the sugar-nucleotide substrate recognition is mediated by this interaction. Hence, the CR domain on cytoloop V may determine the specificity for the UDP-HexNAc substrate before its coordination to the DDxxD motif. The rational design of selective inhibitors for MraY, WecA, WbcO, and RgpG remains a laudable goal of antibiotic research.

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Abbreviations

CHO, Chinese hamster ovary; ECA, enterobacterial common antigen; FPS, farnesyl diphosphate synthase; FucNAc, *N*-acetyl-D-fucosamine; GlcNAc, *N*-acetylglucosamine; GPT, UDP-GlcNAc: dolichol-P GlcNAc-1-P transferase; HexNAc-1-P, *N*-acetylhexosamine 1-phosphate; LPS, lipopolysaccharide; MurNAc-pp, *N*-acetylmuramate pentapeptide; NMR, nuclear magnetic resonance; QuiNAc, *N*-acetyl-D-quinosamine; TAs, teichoic acids; UMP, uridine monophosphate.

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