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Review

N-acetylmuramic acid 6-phosphate lyases (MurNAc etherases): role in cell wall metabolism, distribution, structure, and mechanism

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Abstract. MurNAc etherases cleave the unique D-lactyl ether bond of the bacterial cell wall sugar *N*-acetylmuramic acid (MurNAc). Members of this newly discovered family of enzymes are widely distributed among bacteria and are required to utilize peptidoglycan fragments obtained either from the environment or from the endogenous cell wall (i.e., recycling). MurNAc etherases are strictly dependent on the substrate MurNAc possessing a free reducing end and a phosphoryl group at C6. They carry a single conserved sugar phosphate isomerase/sugar phosphate-binding (SIS) domain to which MurNAc 6-phosphate is bound. Two subunits form an enzymatically active homodimer that structurally resembles the isomerase module of the double-SIS domain protein GlmS, the glucosamine 6-phosphate synthase. Structural comparison provides insights into the two-step lyase-type reaction mechanism of MurNAc etherases: β-elimination of the D-lactic acid substituent proceeds through a 2,3-unsaturated sugar intermediate to which water is subsequently added.

Keywords. Murein autolysis, cannibalism, peptidoglycan dissimilation, cell wall recycling, MurNAc-6P etherase, SIS domain, sugar phosphate binding, C-O lyase.

Introduction

N-acetylmuramic acid (MurNAc) etherases are involved in the degradation of the bacterial cell wall component murein. This huge macromolecule surrounds the cells of almost all bacteria and has the attributes of a strong firm fabric conferring a back pressure against the high intracellular turgor, thereby stabilizing the cells. Chemically, murein is a peptidoglycan that builds a network of heteroglycan chains interlinked by short peptides. The glycans of variable length are composed of alternating β-1,4-linked amino sugars, *N*-acetylglucosamine (GlcNAc) and its 3-*O* lactyl ether derivative, MurNAc. The amino sugar MurNAc is unique to bacteria and functions as the branching point in the peptidoglycan network (Fig. 1): the carboxylic acid moiety of the ether-bonded D-lactyl substituent of MurNAc is amidated by peptides that cross-link the heteroglycan chains (..-GlcNAc-MurNAc-..) [1]. Generally, murein does not accumulate in the environment, since it is cleaved by a variety of murein hydrolases or autolysins that target virtually every covalent bond linking monomer building blocks in the peptidoglycan. Very recently we identified a new family of

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enzymes that targets the unique lactylether bond of MurNAc [2].

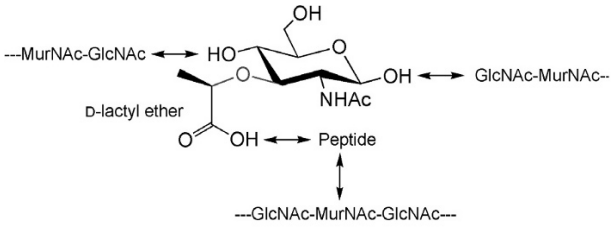

Figure 1. The structure of the amino sugar *N*-acetylmuramic acid (MurNAc) which is an essential component of the peptidoglycan (murein) network of the bacterial cell wall. Together with *N*-acetylglucosamine (GlcNAc), it builds up the heteroglycan strands of murein and also connects them via short peptides which are linked to its unique D-lactyl ether substituent at C3.

Here we present an overview of what is currently known about this new family of enzymes. We first summarize the role of MurQ, the etherase of *Escherichia coli*, in cell wall recycling and murein dissimilation. We then survey the distribution of etherase orthologs in species other than *E. coli* and speculate on their function. Insight into the evolutionary relationships and enzyme mechanism is provided by the classification of MurNAc etherases among the sugar phosphate isomerase (SIS) domain proteins [3]. Finally, we present a structural model of MurQ which is the basis for further investigations on the detailed catalytic mechanism of this unique enzyme family.

Role of MurNAc etherases in cell wall recycling

Little is known about the degradation of MurNAc, the ether component of murein, and – with the exception of the Gram-negative model organism *E. coli* – the dissimilatory pathways of this abundant natural polymer have not been enzymatically and genetically characterized. This is astonishing given that cell wall lytic enzymes, muramidases or autolysins, are present in virtually all bacteria [4] and that possibly the best studied enzyme of all, egg white lysozyme, is a muramidase that hydrolyzes the glycosidic bond between MurNAc and GlcNAc in the peptidoglycan [5]. Extensive studies have been performed on the action and mechanism of lysozymes [6–8], but these enzymes were long considered to be solely involved in defense mechanisms against bacteria. It is now clear that they also play an important role in the dissimilation and (re)utilization of the bacterial cell wall.

Related to the lysozymes, structurally and functionally, are the so-called lytic transglycosylases [9–11]. They also cleave the glycosidic bond between MurNAc and GlcNAc in the peptidoglycan network, but – in contrast to the former group of enzymes – introduce an intramolecular glycosidic bond involving the 6-hydroxyl group of MurNAc, yielding 1,6-anhydro-MurNAc. AnhydroMurNAc-containing compounds are the major cell wall turnover products of Gram-negative bacteria and are released by the cell's own lytic transglycosylases and murein endopeptidases during growth [1]. In this vein, *E. coli* removes about half of the murein of the cell wall within one generation, a dramatic loss of cell wall material. However, only a minor fraction of murein turnover products are found in the growth medium, because *E. coli* efficiently reutilizes, i.e., recycles, the released fragments [12, 13]. Cell wall recycling has been intensively studied in the model organism *E. coli* in the last two decades and is now quite well understood (Fig. 2). Goodell originally observed that *E. coli* recycles the murein-derived tripeptide L-Ala-γ-D-Glu-meso-Dap (diaminopimelate), for short Ala-Glu-Dap. The muropeptide ligase Mpl which he had predicted was later identified [14]. This enzyme links the tripeptide (it also accepts tetra- and pentapeptide as substrates [15]) to UDP-MurNAc thereby generating the murein precursor UDP-MurNAc-Ala-Glu-Dap which is otherwise synthesized *de novo* through three ligases that add each amino acid in a single step [16]. A further ligase adds a D-Ala-D-Ala dipeptide to yield UDP-MurNAc-pentapeptide (UDP-MurNAc-Ala-Glu-Dap-Ala-Ala), the last soluble precursor of murein synthesis. Murein-derived peptides are taken up by the oligopeptide transporter Opp which recruits the periplasmic binding protein MppA [17]. We know now that recycling of the peptides is only a minor pathway in *E. coli*, presumably required during cell division [18], and that murein is almost entirely recycled in the form of anhydro-disaccharide-peptides. This pathway requires the secondary transporter AmpG and the cytoplasmic anhydroMurNAc-L-Ala amidase AmpD [19]. The designation 'Amp' stands for ampicillin, because it was found that these proteins influence the induction of a chromosomal β-lactamase: AmpG acts as a positive and AmpD as a negative regulator for induction of β-lactamase AmpC [20–22]. The first protein is required for the uptake of the inducer, anhydroMurNAc-peptide, and the latter for its rapid degradation [23]. In the group of Jochen Höltje, a cytoplasmic L,D-carboxypeptidase was later identified that is crucial for correct muropeptide recycling. This enzyme removes the carboxy-terminal D-Ala from tetrapeptide-containing recycling fragments [24]. In the absence of the enzyme, tetrapeptides, instead of tripeptides, are linked to the murein precursors by Mpl, yielding UDP-MurNAc-Ala-Glu-Dap-Ala instead of the pentapeptide deriv-

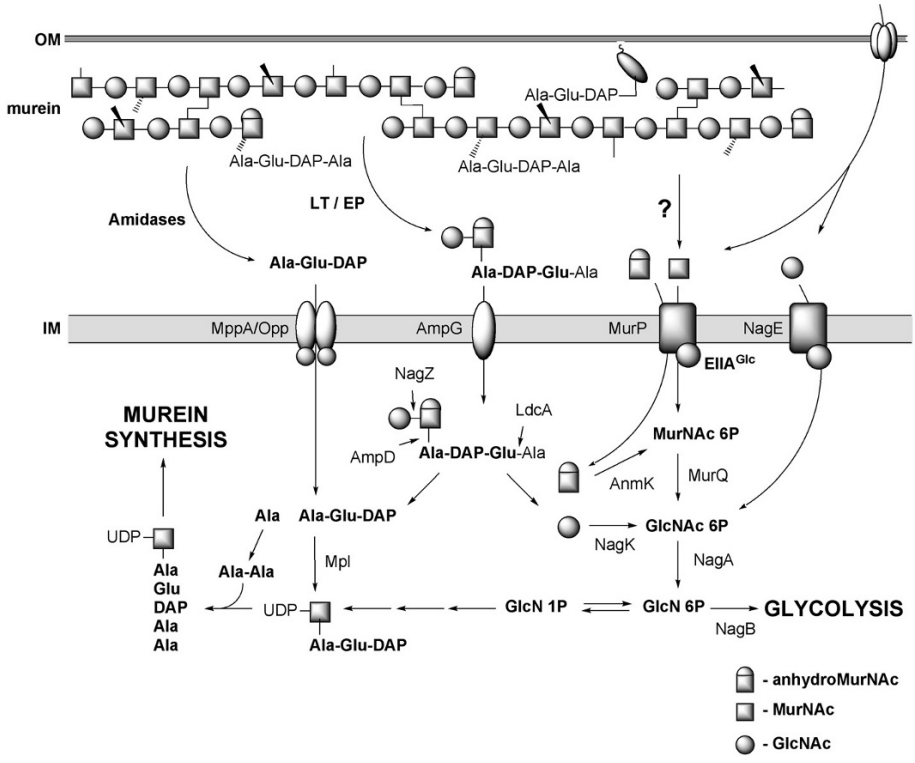

Figure 2. Peptidoglycan (murein) turnover and metabolic pathways of *E. coli*, in which about 50 % of the murein of the endogenous cell wall is recycled, and murein fragments from the environment are utilized. GlcNAc-anhydroMurNAc-tetrapeptides are released from the murein by the action of lytic transglycosylases (LT) and endopeptidases (EP) during cell elongation, and tripeptides (Ala-Glu-DAP) are released by amidases from the cell wall during division. The former are taken up by the secondary transporter AmpG and the latter by the ABC transporter MppA/Opp. Individual amino sugars, GlcNAc and MurNAc, from the environment and possibly of the endogenous cell wall (?) can also be taken up separately by the phosphotransferase systems NagE and MurP and are concomitantly phosphorylated. The etherase MurQ is required for the conversion of MurNAc 6-phosphate to GlcNAc 6-phosphate, which is required for the utilization of MurNAc and anhydroMurNAc. The latter is released from anhydroMurNAc-peptides in the cytoplasm via NagZ, an *N*-acetylglucosaminidase, AmpD, an anhydroMurNAc-peptide amidase, and LdcA, a D-Ala-carboxypeptidase. Recycling of the amino sugars of the cell wall for murein synthesis proceeds through the glucosamine (GlcN) phosphate pathway, and recycling of the peptides released from the cell wall proceeds through the muropeptide ligase, Mpl. For further information on the pathways refer to the text.

ative. This leads to incorporation of 'wrong' tetrapeptides into the peptidoglycan network instead of pentapeptides which are needed for the cell wall cross-linking. As a consequence, the cells lose the integrity of the murein sacculus and eventially lyse [15, 24].

The recycling pathways for the amino sugars of the cell wall (GlcNAc and anhydroMurNAc) were elucidated only recently. NagZ, a cytoplasmic *N*-acetylglucosaminidase (along with AmpD), releases the amino sugars from the recycled anhydro-disaccharide-peptides [25, 26]. In his 2001 paper, Ted Park [27] laid out a pathway by which *E. coli* can recycle these cell wall-derived amino sugars and suggested the existence of an etherase required for the breaking of the lactyl ether bond in the conversion of anhydroMurNAc to GlcNAc. Later, Uehara, Park and colleagues identified two kinases specific for these amino sugars, AnmK and NagK, respectively, which convert them to the 6-phosphoryl derivatives [28, 29], thereby returning them to the pathway for utilization. Together with this group, we showed that the etherase MurQ – which we had previously implicated in MurNAc utilization (see below) – is required for the recycling of the cell wall via 1,6-anhydroMurNAc and that this enzyme is the only MurNAc 6-phosphate etherase in *E.* coli [2, 30]. Hence, reutilization of the amino sugars of the cell wall requires the conversion of the recycling product anhydroMurNAc ultimately to glucosamine 6-phosphate. This molecule can then be isomerized to glucosamine 1-phosphate, entering the pathway for cell wall synthesis, or is converted to fructose 6-phosphate entering glycolysis (Fig. 2).

The complex murein recycling pathway of *E. coli* contains a large number of enzymes that are dedicated to the process of salvaging all components of the cell wall murein. This is remarkable, because in *E. coli*, murein accounts for only 1–2 % of the cell dry mass and, therefore, the benefit in terms of energy conservation is rather limited. Even so, *E. coli* has preserved the recycling pathway although it is not essential, at least under laboratory conditions. A

recycling pathway has not been recognized in Gram-positive bacteria, so far, although the amount of cell wall material in these organisms is much higher and, hence, cell wall recycling would be of even greater benefit than in Gram-negative bacteria. We show later that MurNAc etherases are found mainly in Gram-positive bacterial species, which demonstrates that Gram-positive organisms do have the potential to reutlize their murein via an etherase pathway.

Role of the etherase in MurNAc/anhydroMurNAc dissimilation

Beside recycling their own cell wall, *E. coli* and other bacteria are able to utilize cell wall components, such as the monosaccharides GlcNAc and MurNAc, as their sole source of carbon, nitrogen, and energy [31–33]. Growth of *E. coli* on GlcNAc and MurNAc requires the *nag* genes (*nagEBACD*), in particular *nagA*, encoding the GlcNAc 6-phosphate deacetylase, and *nagB*, encoding the glucosamine 6-phosphate deaminase [34]. *nagE*, which is divergently transcribed from *nagBA*, encodes a permease of the phosphotransferase system (PTS) responsible for the transport and phosphorylation of GlcNAc [35]. The uptake of MurNAc, however, does not depend on the GlcNAc transporter but on another PTS which requires the soluble glucose-specific PTS component $EIIA^{Glc}$ [33]. The MurNAc-specific PTS MurP concomitantly transports and phosphorylates MurNAc yielding MurNAc 6-phosphate. Since the further metabolism of MurNAc 6-phosphate requires the *nag* pathway, we suggested a hypothetical etherase that would cleave the lactylether substituent of MurNAc 6-phosphate yielding GlcNAc 6-phosphate [33]. We found that this enzyme, an etherase, is encoded by a gene adjacent to *murP*. This gene, which we named *murQ*, is, like *murP*, essential for growth on MurNAc as the sole source of carbon and energy [2]. In addition, recycling of the cell wall-derived anhydroMurNAc depends on MurQ along with a bifunctional glycosidase/kinase (AnmK, see above and Fig. 2) that opens the anhydro ring and phosphorylates the product at C6. MurP was found to be involved in the uptake of anhydroMurNAc coming from the environment [30], which, however, is not phosphorylated by the PTS. We showed that a *murQ* deletion mutant accumulates MurNAc 6-phosphate [2, 30] and that purified MurQ generates GlcNAc 6-phosphate and D-lactate, which were identified by electrospray ionization mass spectrometry and a coupled enzyme assay with D-lactate dehydrogenase, respectively.

The MurNAc catabolic and the anhydroMurNAc recycling pathways merge at the level of MurNAc 6-phosphate and both require the etherase MurQ. However, in the one case, only a low level of etherase is needed for the continuous recycling of anhydroMurNAc derived from the own cell wall during 'normal' growth, since only about 10^6 anhydroMurNAc molecules are released per generation throughout exponential growth. This is reflected in the low-level constitutive rate of transcription of *murQ*. In the other case, growth on external MurNAc requires high uptake and metabolic rates and, hence, high-level expression of both the etherase and the MurNAc-PTS. Expression of both genes is controlled by the repressor MurR, a member of the relatively uncharacterized RpiR/AlsR family of transcriptional regulators [36–38]. MurR binding interferes with transcription from the *murQP* promoter and is released from the operator by binding of MurNAc 6-phosphate. *murR* and *murQP* are expressed from non-overlapping face-to-face (convergent) promoters, yielding transcripts that are complementary in their 5′ ends. Interference of the transcripts might affect protein expression. Furthermore, high-level *murQ* transcription depends on activation by cyclic AMP (cAMP)-catabolite activator protein (CAP) bound upstream of the *murQ* promoter [36].

The function of *murQP* in *E. coli* is still puzzling. To our knowledge, *E. coli* generates only anhydroMurNAc from its own cell wall and, hence, the source of the reducing sugar MurNAc is enigmatic. However, a periplasmic protein with lysozyme-like activity has been identified in *E. coli* [39]. In addition to their function in recycling anhydroMurNAc derived from murein, *E. coli* etherase and the MurNAc-PTS might be involved in scavenging cell wall fragments from the environment or in cannibalism, as has been found in sporulating Gram-positive bacteria [40–42]. Surprisingly, two paralogs of the etherase are present in some *Vibrio*, *Lactococcus and Enterococcus* spp., one apparently in an operon together with the anhydroMurNAc kinase and therefore likely to have a function in cell wall recycling. The other is found in an operon together with the MurNAc-PTS and, hence, is probably required for dissimilation of murein or MurNAc from the environment. In most bacteria, however, there is only one paralogous gene.

Phylogenetic distribution and the role of etherases

Open reading frames displaying high amino acid sequence identity with MurQ, the etherase of *E. coli*, were found in the genome of several organisms. Therefore, the MurNAc/anhydroMurNAc degradation pathway seems to be widely spread among bacteria and it is also present in some other organisms,

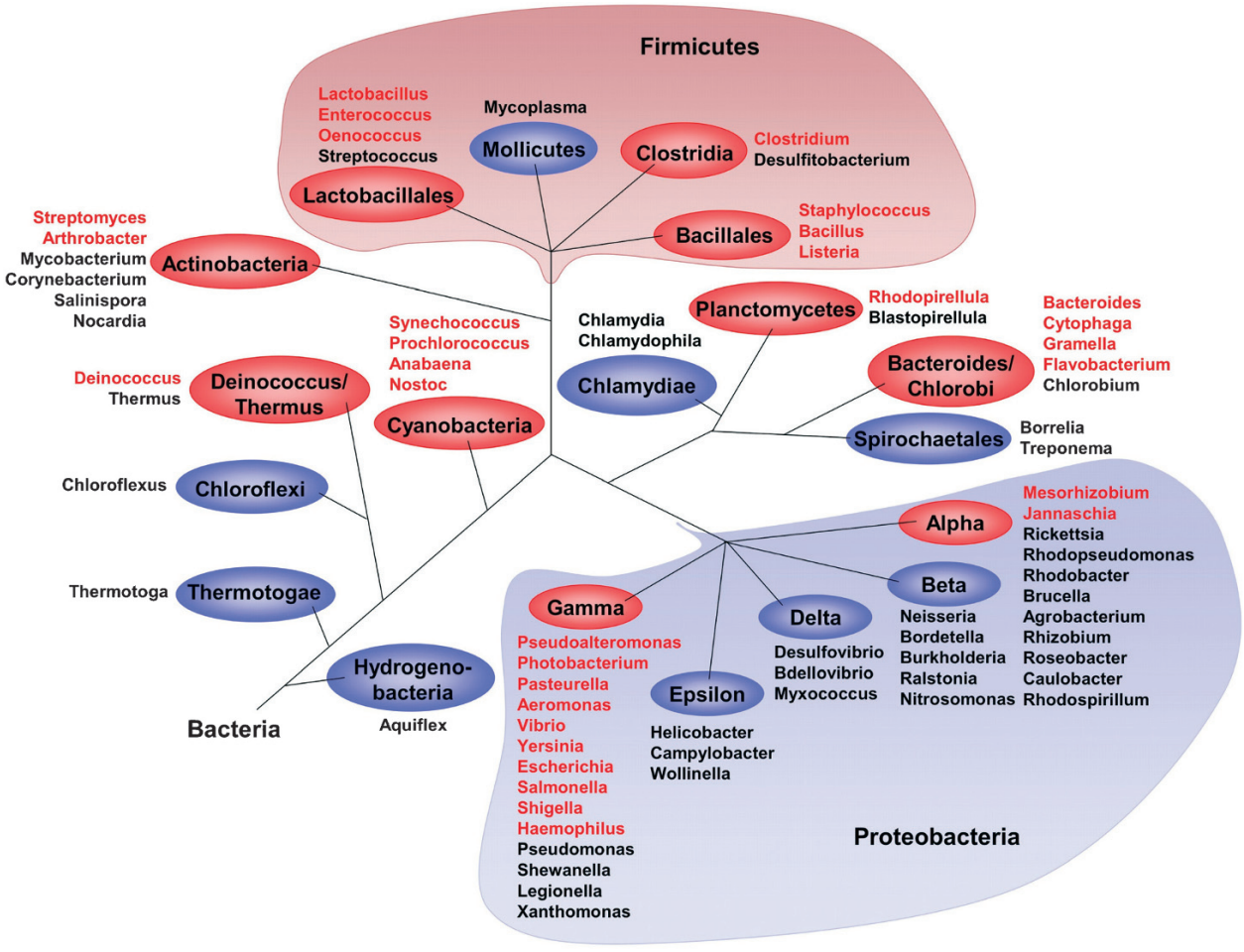

Figure 3. Distribution of MurNAc etherase within the major lineages of bacteria. The phylogenetic tree is based on 16S rRNA data of Carl R. Woese, but the lengths of the branches do not correlate with evolutionary distances. Bacterial lineages and species which carry open reading frames with significant amino acid sequence identity (>25%) to MurQ of *E. coli* are shown in red; bacterial lineages where an ortholog is apparently missing are shown in blue and the respective species in black. Note that etherases have also been recognized in fungi and the archaeon *Picrophilus torridus*.

e.g., in fungi of the *Aspergillus* genus and the archaeon *Picrophilus torridus*. The distribution of etherases within the bacterial phylum is presented in Figure 3. Putative orthologs of MurQ are found in some Gram-negative bacterial species, mostly γ-proteobacteria, and their role in cell wall recycling is proposed for these organisms since they likely contain a cell wall recycling pathway similar to that of *E. coli*. However, in most other Gram-negative species, an etherase is apparently missing. For some bacteria that do not carry an ortholog of the MurQ etherase on their chromosome, the reason is obvious – they do not have a murein cell wall – as in intracellular pathogens of the chlamydiae, mollicutes (mycoplasmae), and thus there is no need for murein recycling. In others it is not clear why an etherase should be absent, and why MurQ is apparently absent in most Gram-negative species. The reason for this needs to be further investigated. Surprisingly, an etherase is also missing in some close relatives of *E. coli*, i.e., in γ-proteobacteria of the genus *Pseudomonas*, which are known to degrade their own cell wall in a manner analogous to *E. coli* with lytic transglycosylases [11]. It is not clear if these species indeed lack a reycling pathway, which would be quite surprising, or if these organisms possess etherase(s) unrelated to MurQ that fulfill its function. Some organisms might use a different strategy to reutilize their cell wall. Possibly a direct short-cut pathway of intact MurNAc (or larger fragments) could lead to cell wall synthesis without the need for cleaving the ether bond. In *E. coli*, however, the recycling of the amino sugars proceeds via the GlcNAc phosphate pathway which is delicately balanced and regulated [43–45].

Interestingly, an ortholog of MurQ is present in most Gram-positive bacteria, e.g. in the bacillales, clostridiae, lactobacillales. Cell wall recycling has not been investigated in Gram-positive bacteria so far, and the

general view is that these organisms do not reutilize their cell wall. This, however, would be a waste of resources, since the murein of Gram-positive bacteria accounts for a much larger proportion of cellular mass compared to that of Gram-negatives. Turnover of cell wall material has been observed in some Gram-positive species [46–49] and, very recently, in some Gram-positive species, a form of cannibalism or fratricide has been documented, which is the killing of genetically identical cells (siblings) [41]. Cannibalism in *Bacillus subtilis* occurs during the early stages of sporulation [42]. Cells that have entered the sporulation pathway are able to block sister cells from sporulating and cause them to lyse. The sporulating cells feed on the nutrients thereby released, which allows them to grow and, hence, delay their commitment to spore formation. The presence of a MurQ ortholog in most Gram-positive bacteria indicates that a form of cell wall recycling (e.g., the shedding of old cell wall material) or cannibalism takes place in these organisms. We had suggested earlier that the *murQP* operon of *E. coli* might actually have been acquired by horizontal gene transfer from Gram-positive bacteria [2]. Interestingly, a MurQ-like etherase is present in an organism which certainly does not recycle murein, the archaeon *P. torridus*. Archaea do not contain a murein cell wall and there is no need for an etherase in cell wall recycling. We cloned the etherase of the thermoacidophilic archaeon *P. torridus* and heterologously expressed it in *E. coli* [U. Dahl, S. Litzinger, C. Mayer, unpublished data]; the enzyme is active at elevated temperatures and is thermostable. The role of etherases in this organism might be the scavanging of cell wall fragments from the environment in an 'active or passive' mode, which means that they are either utilizing 'dead' cell wall material or are feeding on living bacteria and killing them. Interestingly, however, some organisms which are known to feed on bacteria, like the predatory bacteria *Bdellovibrio* or *Myxoccus* spp., do not possess MurNAc phosphate etherase orthologs and hence a MurNAc dissimilation pathway is apparently missing.

MurNAc etherases, a family of mono-SIS domain proteins

MurQ and other MurNAc etherases can be assigned to the SIS domain protein family (PF01380 [3] and www.sanger.ac.uk/Users/agb/SIS/SIS.html). SIS domains are found in many sugar phosphate-binding proteins (Fig. 4A), such as isomerases, C-N lyases, and bacterial transcriptional regulators involved in the metabolism of sugar phosphates. MurR, the transcriptional repressor of MurQ of *E. coli* carries an SIS domain with some 30 % amino acid sequence identity with the SIS domain of MurQ [36]. Both proteins bind MurNAc 6-phosphate. Binding of this effector inactivates the repressor MurR and leads to dissociation of the protein from the operator DNA [36]. A stretch of about 140 amino acid residues defines the SIS domain, but the level of sequence identity between SIS domains usually is rather low. Therefore, the SIS domain is a conserved structural rather than a concerved sequence domain. It is characterized by an α/β structure consisting of a five-stranded parallel β sheet flanked on both sides by α helices, forming a three-layered α-β-α sandwich (Fig. 5). The parallel β sheet contains five strands, of the order 21345. A well-studied SIS domain protein is glucosamine 6-phosphate synthase (GlmS). GlmS converts fructose 6-phosphate to glucosamine 6-phosphate, catalyzing the first step in a pathway leading to the eventual formation of murein and other amino sugar-containing macromolecules via uridine 5′-diphospho-*N*-acetylglucosamine (UDP-GlcNAc). The isomerase domain alone as well as the entire protein in the absence of glutamine are enzymatically active but catalyze solely the isomerization of fructose 6-phosphate to glucose 6-phosphate. The sugar phosphate isomerization involves a ring opening and an enolization step. The endiole intermediate is stabilized by the ε-amino group of Lys603 which forms a Schiff base. In the entire GlmS protein in the presence of glutamine, ammonia is channeled from the aminotransferase module to the isomerase module and replaces Lys603 for binding to the sugar, hence introducing an amino group [50, 51].

The SIS domain proteins come in two flavors: double-SIS domain proteins like GlmS and mono-SIS domain proteins, shown in Figure 4A. Crystal structures of some SIS proteins have been determined and, intriguingly, it is evident from these structures that homodimers are the active biological molecules of mono-SIS proteins and that the two SIS domains of double-SIS proteins come in close contact to constitute the active sites.

Mono-SIS family members catalyze sugar isomerization reactions which are characterized by the abstraction of a proton α-positioned to the carbonyl (reducing end) of the sugar and an enolization. Phosphoheptose isomerases (sedoheptulose 7-phosphate isomerases; GmhA) catalyze the first step in the biosynthesis of the heptose portion of the lipopolysaccharide (LPS) [52, 53]. Three proteins from *Campylobacter jejuni*, *Vibrio cholerae* and *Pseudomonas aeruginosa* have been crystallized and their structures determined (structure codes: 1TK9, 1X94, 1X92, respectively; [54]). Closely related to GmhA proteins is DiaA, a DNA-binding protein, that is required for the timely

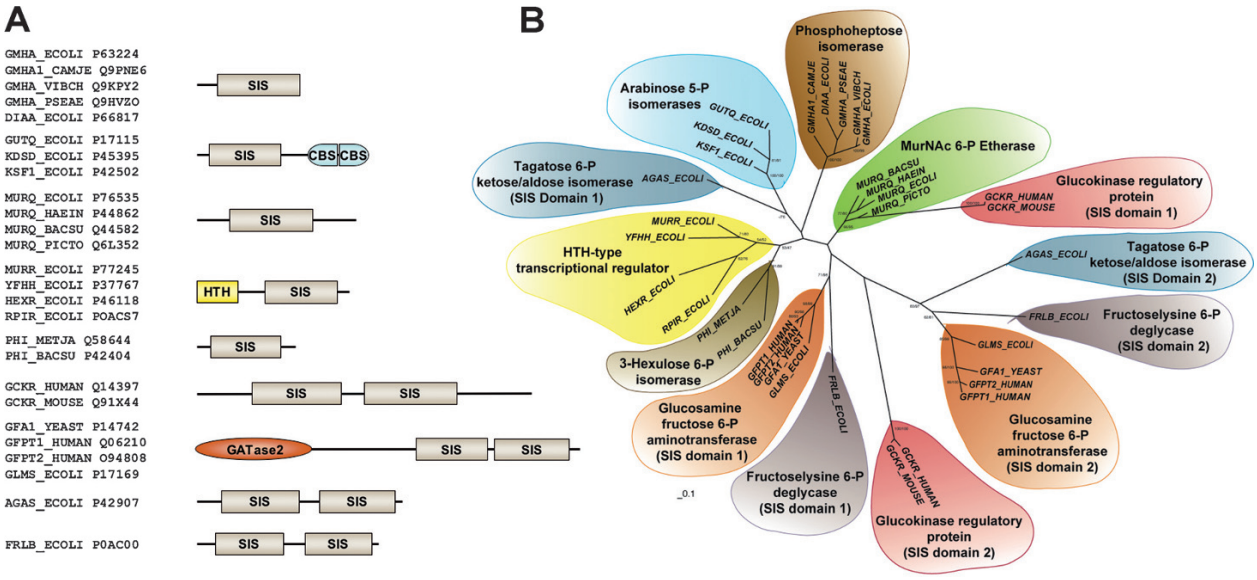

Figure 4. Domain structure (*A*) and phylogenetic/evolutionary relationship (*B*) of mono- and double-SIS domain proteins including MurNAc etherases. Selected members of the SIS family, which are indicated by the identifier of the SwissProt, database, are shown. Mono-SIS domain proteins can be divided into the following subfamilies: (1) GmhA/DiaA, the sedoheptulose 7-phosphate (phosphoheptose) isomerases; (2) GutQ/KdsD/KpsF, the arabinose 5-phosphate isomerases, which in addition to the SIS domain carry two copies of the CBS (cystathionine β-synthase) domain; (3) MurQ, the MurNAc etherases; (4) MurR/RpiR(AlsR), the helix-turn-helix (HTH)-SIS domain transcriptional regulators; (5) PHI(YckF), the 3-hexulose 6-phosphate isomerases. Double-SIS domain proteins can be assigned to the follwing subfamilies: (1) GckR, the glucokinase regulatory proteins; (2) GlmS, the glucosamine-fructose 6-phosphate aminotransferases, which have an N-terminal glutamine aminotransferase class II module and a C-terminal isomerase module which carries two SIS domains; (3) AgaS, putative tagatose 6-phosphate ketose/aldose isomerases; (4) FrlB, fructoselysine 6-phosphate deglycases. Phosphoglucose isomerases (pgi) which are related to the SIS family of proteins but are characterized by a permutation of the SIS-fold have been excluded. (B) Maximum likelihood tree of an alignment of SIS domains (based on 132 amino acid positions) of the selected proteins as obtained by PhyML [77]. The first support values shown are maximum likelihood bootstraps as obtained by 500 replicates in PhyML, the second values are posterior probabilities as obtained by MrBayes 3.1 [78] with 200 000 generation and a burnin of 5000. The tree was generated by the program TreeViewX [79]. While all SIS2 domains form a weakly supported monophyletic group (i.e., were derived from a common ancestor), the origin of SIS1 domains is unclear. However, independent tandem duplications in several proteins, leading to two consecutive domains within a protein, can be rejected by this tree. For this case, domains from one protein would form a monophyletic group. Note that the SIS domains of the MurNAc etherases are related to the first domain (SIS1) of the glucosekinase regulator proteins. It can be speculated that originally the MurNAc etherases contained two SIS domains, but secondarily lost the less conserved SIS2 domain.

initiation of chromosomal replication [55–57]. The exact mechanism of their action, however, remains to be investigated.

An SIS domain followed by two copies of the cystathionine β-synthase (CBS) domain is present in proteins of the GutQ/KdsD/KpsF family. GutQ is a protein of the glucitol operon of *E. coli* [58], and KpsF is a virulence factor involved in capsular polysialic acid biosynthesis in some pathogenic strains of *E. coli* [59]. KpsF and KdsD (or YrbH), an additional member of this family, are D-arabinose-5-phosphate isomerases, which are required for biosynthesis of 3-deoxy-D-manno-oculosonic acid, a precursor for LPS and capsular polysaccharides [60–63].

MurQ-like etherases form a subfamily of mono-SIS domain proteins which is characterized by additional helices at the N and C termini (Fig. 5). These helices might be involved in dimer stabilization and catalysis, respectively. From the structural model of the MurNAc etherase (see below), it can be seen that the latter helix covers the active site. The MurNAc etherase regulator MurR and other transcriptional regulators (e.g., the hexose regulator HexR and the allose/ribose regulator RpiR/AlsR) which affect sugar metabolism and bind phosphosugars are classified in an SIS-family that contains an additional N-terminal helix-turn-helix (HTH) DNA-binding motif [36–38]. Finally, the PHI proteins including the protein YckF of *B. subtilis* [64, 65], constitute a last subfamily of mono-SIS domain proteins that catalyzes the isomerization between 3-hexulose 6-phosphate and fructose 6-phosphate in the dissimilatory ribulose monophosphate cycle. 3-Hexulose 6-phosphate synthase (HPS) and 6-phospho 3-hexuloisomerase (PHI) are the key enzymes of the ribulose monophosphate pathway. This pathway, which was originally found in methylotrophic bacteria, is now recognized as a widespread prokaryotic pathway involved in formaldehyde fixation and detoxification [66, 67].

Double-SIS domain proteins carry two SIS domains related by pseudo dyad symmetry. The two SIS domains, however, seem not to be evolutionarily

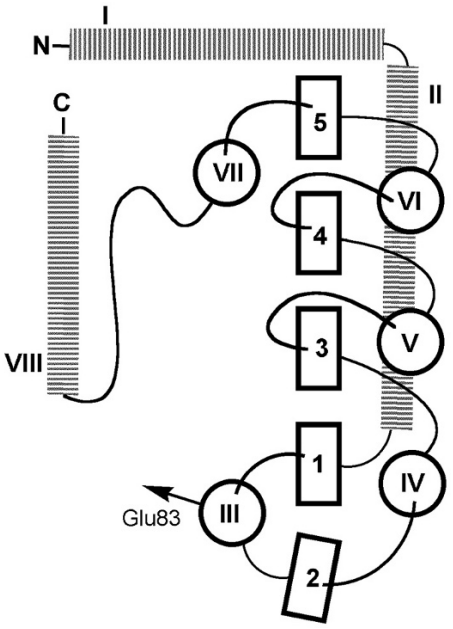

Figure 5. Schematic topology of the SIS domain fold. Shown is a general scheme as for MurNAc etherases. β strands (open boxes) and α helices (shaded boxes and open circles) are numbered according to their appearance in the amino acid sequence. The position of the catalytic residue Glu83 is indicated. Note that the Glu83 residue from the other subunit of the MurQ homodimers comes into close contact with the active site constituted by the SIS domain shown here (cf. Fig. 6A).

linked but have developed from unrelated ancestors (Fig. 5). Some double-SIS domain proteins might catalyze isomerization reactions like the mono-SIS proteins, e.g., the putative tagatose 6-phosphate keto/aldo-isomerase AgaS [68] and the isomerase part of GlmS-like proteins (see above). Others are C-N lyases, like the fructoselysine deglycase (FrlB [69]) and the full GlmS protein (MurNAc etherases are the only C-O lyases in the SIS family). Evolutionarily linked to the MurNAc etherases are the members of the glucokinase regulatory protein family which plays a role in the control of blood glucose homeostasis (GckR, [2, 70]). This vertebrate enzyme inhibits glucokinase by forming an active complex with the enzyme [71, 72].

Etherase structure

In an attempt to generate a structural model of the etherase of *E. coli*, we surprisingly obtained an unexpected hit from the protein database (Protein Database at URL: www.pdb.org). The crystal structure of a protein of unknown function from *Haemophilus influenzae* is highly similar to MurQ (56% overall amino acid sequence identity) and had been solved as part of a structural genomics project and deposited in the database (structure code: 1NRI; K. Kim, P. Quartey, R. Ng, T. I. Zarembinski, A. Joachimiak, posted to the database 24 January 2003). Here, a structural model of MurQ is presented that is based on the structure of this putative etherase (Fig. 6A). We recognized that two monomers within the MurQ structure display a high degree of surface contacts, which indicate that the biologically active unit is represented by a homodimer (Fig. 6B). The formation of stable dimers of MurQ is consistent with gel filtration and dynamic light-scattering experiments with purified protein [T. Jaeger, unpublished results]. The overall structure of the MurQ dimer resembles the structure of GlmS which forms a pseudodimer carrying two SIS domains on one polypeptide strand (Fig. 6C). It had been shown for GlmS that the active site is formed by amino acids of one SIS domain that accommodate the phosphosugar. However, the amino acid residues important for catalysis are provided by other parts of the protein: the glutamate residue (Glu488) that acts as a general base in catalyzing a hydrogen transfer from C1 to C2 of the substrate, as well as the lysine residue (Lys603) that forms a Schiff base with the substrate [50, 51]. Accordingly, the MurQ dimer carries two putative active sites that are formed by the SIS domain of one subunit and the C-terminal part of the other, and vice versa. By comparing the structure of GlmS and the structural model of the MurQ dimer, we were able to identify amino acids that might affect enzyme activity and/or sugar phosphate binding; e.g., Glu83 of *E. coli* MurQ was found to be positioned similarly to Glu488 of GlmS in the SIS domain (Fig. 6). The site-directed mutant Glu83Ala was found to be inactive [C. Mayer, unpublished results], indicating that Glu83 has a function similar to the Glu488 of GlmS. A lysine resembling the function of Lys603 of GlmS is apparently missing. Detailed kinetic studies with enzyme mutants are underway. In analogy to the mechanism of GlmS, we suggest that the etherase involves (1) a sugar ring opening step, (2) a general base, presumably Glu83, that removes the acidic H2 proton α-positioned to the carbonyl, (3) an additional acidic residue that facilitates lactic acid departure, and (4) the closure and opening of the active site presumably by the movement of the C-terminal α helix of the protein, which covers the active site (Fig. 7).

The mechanism of MurQ action

Enzymes that catalyze the scission of ether bonds are remarkable catalysts, because ethers (C-O-C), in general, are highly resistant to biodegradation due to their high bond energy and chemical inertness [73]. As

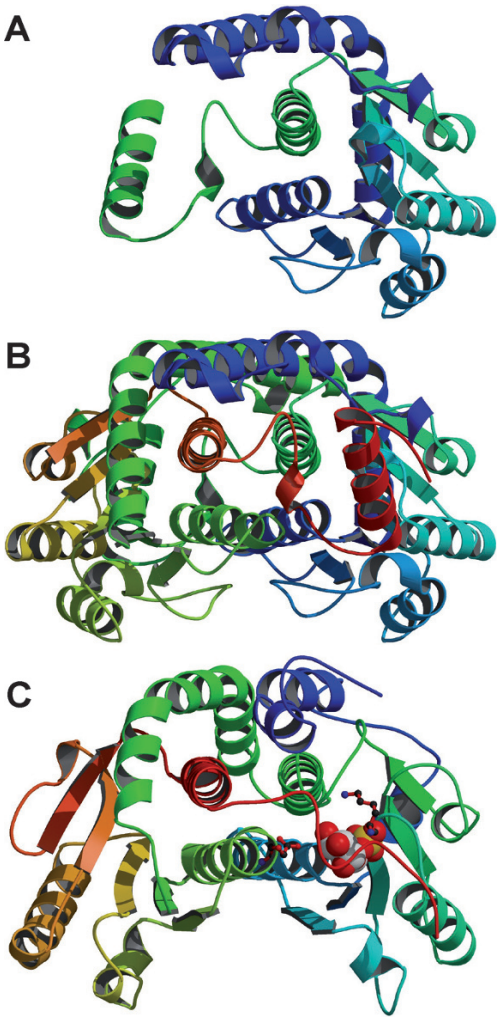

Figure 6. Structural model of the etherase MurQ of *E. coli* as monomer (*A*), as homodimer (*B*), and the structure (structure code: 1MOR) of the isomerase module of GlmS (*C*). The two catalytic residues in the active site of GlmS, the general base Glu488 (left side; similarly positioned to Glu83 of MurQ) and Lys603 (right side) which forms a Schiff base with the substrate, are shown as ball and sticks. In the presented GlmS structure, the product of the isomerization reaction, glucose 6-phosphate (the phosphate is yellow colored) is shown as a space-filled model in the active site. The modeling was performed with Swiss model [80] and the figures were generated with the program Molscript [81].

a result, ether compounds frequently accumulate in the environment. These include some of the most problematic pollutants. In fact, this relative inertness may be the reason that nature sometimes makes use of the ether bond, e.g., in recalcitrant substances like the lignin of wooden plants [74] and in cytotoxic or antibiotic substances like the polycyclic ethers synthesized by marine organisms [75]. The trivial name etherase classifies enzymes that catalyze the scission of ether bonds exhibiting a wide variety of mechanisms: (1) oxygenative cleavage via monooxygenases; (2) oxidation of the carbon atom α-linked to the ether bond, followed by hydrolysis of the resulting ester; (3) hydroxyl shift mechanisms; (4) direct hydrolysis of the C-O bond; (5) anaerobic cleavage of methyl-aryl ethers; (6) oxidative mechanisms; (7) reductive mechanisms and, finally (8) carbon-oxygen lyase-mediated cleavage [73].

MurNAc etherases catalyze a lyase-type reaction [2] and can be assigned to the carbon-oxygen lyases (EC 4.2). They are the first members within this enzyme family that catalyze the elimination of an 'alcohol' from a monosaccharide – β-elimination of the lactic acid alkoxyl group at C3 and the C2 proton – and not from a polysaccharide, as catalyzed by the hyaluronate, pectate, or chondroitin lyases. Usually, an alkoxyl group is not a leaving group, and so the lactyl ether alkoxyl substituent of MurNAc might be protonated prior to cleavage. Unlike other ether compounds, the lactyl ether substituent of MurNAc is rather unstable under alkaline conditions. At pH >12, the proton (H2) α-positioned to the carbonyl of the sugar is readily eliminated [76]. Likewise, the enzyme might provide a basic residue that catalyzes the same deprotonation reaction. Hence, at least two catalytic residues are required: a general base that removes the H2 and an acid catalyst that protonates the leaving group. The acidity of the H2 is greatly increased in the open-chain form of the sugar, hence a ring-opening step, as in other SIS proteins is suggested. In addition, some SIS domain proteins (e.g. GlmS) involve the formation of a Schiff base. However, an obvious candidate which might form a Schiff base (a conserved lysine residue) can not be found in etherases, and a Schiff base adduct could not be detected by mass spectrometry on $NaBH_4$-reduced MurQ that had been incubated with MurNAc 6-phosphate [C. Mayer, unpublished observations].

The unsaturated phosphosugar intermediate, which is released by the elimination of lactic acid, has a considerable lifetime and can be detected either by a colorimetic assay (Morgan-Elson assay) or by mass spectrometry. In the Morgan-Elson reaction, the very same unsaturated amino sugars are generated by heating (boiling for 3 min) under alkaline conditions (borate buffer pH >10). This step can be substituted by an incubation with etherase. The second part of the Morgan-Elson reaction is the reaction of Ehrlich reagent (dimethylaminobenzaldehyde, DMBA in acidic acid) which leads – in both cases – to the formation of a violet-colored adduct. The intermediate of the etherase reaction can be visualized, in particular when the reaction is run 'in reverse,' indicating that an equilibrium of intermediate and product is formed; in the forward reaction, the intermediate is readily converted into product by hydration of the double bond. The same catalytic

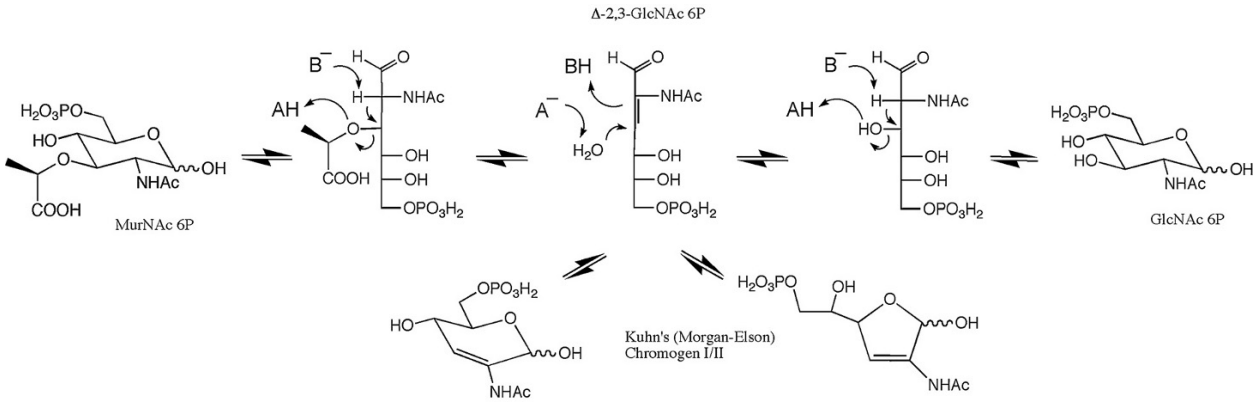

Figure 7. Proposed reaction mechanism of MurNAc etherases. The substrate MurNAc 6-phosphate is transformed to the open-chain form. The acidic H2 proton α-positioned to the carbonyl is detached by enzymic base catalysis (likely Glu83 in *E. coli* MurQ). Subsequent elimination of the alkoxyl group (lactic acid) is facilitated by protonation and leads to the formation of the 2,3-unsaturated intermediate, which eventually leaves the active site and can be detected by a Morgan-Elson-type colorimetric assay. However, after activation of a water molecule by deprotonation, the double bond of the intermediate is hydrated yielding *N*-acetylglucosamine 6-phosphate.

amino acid residues are possibly required for the hydration step as for the elimination step: the former acid catalyst would then function as a base, deprotonating the incoming water molecule, and the protonated former general base would then provide its proton (Fig. 7).

Concluding remarks

MurNAc etherases are rare examples of cloned and characterized enzymes that cleave ether bonds. They are required to entirely degrade the natural polymer murein. Since bacteria have undergone 3.5 billion years of evolution, it is not surprising that enzymes have arisen that are able to target the unique lactylether bond of the murein. The evolutionary relationship of MurNAc etherases to sugar phosphate isomerases of the SIS family sheds light on the possible mechanism of the etherase reaction. However, the precise catalytic steps and the amino acids involved remain to be discovered. The structural model of the *E. coli* etherase and the assignment of a protein of unknown function of *H. influenzae,* for which a structure is available, to the MurNAc etherases should greatly facilitate this work. The etherases might also become interesting catalysts for enzymatic synthesis; the addition of molecules other than water to the unsaturated intermediate or running the equilibrium reactions in reverse are two possible strategies. MurNAc etherases exist in a range of bacteria. However, it remains to be discovered why many bacteria do not contain them. Evolution might have found other ways to cleave lactylether bonds. However, it is not clear if cell wall recycling/reutilization is unique to some bacteria or if it is generally present. Recycling of the cell wall material may be especially critical in pathogenic bacteria which have to hide from the immune system and for environmental species which have to deal with a limited food supply. We hope that this review of MurNAc etherases will stimulate future research on this interesting new family of enzymes.

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