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The monofunctional glycosyltransferase of *Escherichia coli* is a member of a new class of peptidoglycan-synthesising enzymes

Overexpression and determination of the glycan-polymerising activity

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Abstract Using conserved fingerprints in the glycosyltransferase (GTase) domain of high-molecular-weight penicillin-binding proteins (PBP), a gene (*mgt*) encoding a putative monofunctional glycosyltransferase has been identified in *Haemophilus influenzae* and in other bacterial species. Here we report the cloning of the homologous *Escherichia coli* gene and show that the solubilised membrane fraction of *E. coli* cells overexpressing the *mgt* gene contain a significantly increased peptidoglycan synthesis activity. In contrast to the high-molecular-weight PBPs, this activity is not inhibited by Flavomycin.

Key words: Escherichia coli; Monofunctional glycosyltransferase; Penicillin-binding protein; Peptidoglycan synthesis; Cell wall

1. Introduction

The synthesis of a cell wall is one of the most intensively studied processes of bacterial metabolism. Efforts are directed mostly towards understanding of the last steps of peptidoglycan synthesis which take place in the periplasmic space and are therefore regarded as suitable physiological targets for antibiotics. In this compartment two major enzymatic activities are responsible for the formation of peptidoglycan: (1) the glycosyltransferase (GTase) which polymerises the glycan strands using lipid-linked disaccharide-pentapeptide as substrate and (2) the transpeptidase (TPase) which cross-links the formed strands via their peptide chains. Transpeptidases are also known as penicillin-binding proteins (PBP), since penicillin and other β-lactam antibiotics were found to covalently bind to their active sites, thereby inhibiting their function which eventually leads to cell lysis. In *Escherichia coli* the high-molecular-weight PBPs 1A, 1B, 2 and 3 are involved in various cellular processes such as cell shape determination, cell elongation and cell division. Among these enzymes, PBP1A and PBP1B were shown to carry both glycosyltransferase and transpeptidase activity [1]. These periplasmically located PBPs are bound to the cytoplasmic membrane via a N-terminal hydrophobic signal sequence. Their activities reside in two different domains of the polypeptide: GTase is found in the N-terminal half of the protein, while the C-terminal half carries the TPase activity and is therefore responsible for penicillin-binding [2]. *E. coli* mutants lacking either PBP1A or PBP1B, but not both, are viable which shows that each of these PBPs can compensate for the absence of the other [3–5]. Since mutants lacking PBP1B retain only 10% of the peptidoglycan synthesis activity, the major peptidoglycan-synthesising activity is assigned to PBP1B [3,6]. The bifunctional nature of the high-molecular-weight PBPs 2 and 3 is still controversial. While the TPase activity of both proteins was proven, the GTase activity could not be demonstrated unambiguously [7]. PBP2 in conjunction with the RodA protein was shown to be involved in cell elongation; PBP3 together with the FtsW protein is believed to play an important role during cell septation [8–11]. Low-molecular-weight PBPs, namely PBPs 4, 5 and 6, are monofunctional and usually have a D,D-carboxypeptidase activity that is required for the maturation of the peptidoglycan and for the regulation of the level of cross-linking [12].

In the mid-1980s, monofunctional, non-penicillin-binding enzymes from different bacterial species were described that possess GTase activity. Hara and Suzuki [13] purified and characterised an *E. coli* enzyme of M_r 34000, which synthesised peptidoglycan from lipid-linked precursors. Enzymes with similar catalytic properties were also found in the Gram-positive *Staphylococcus aureus*, *Micrococcus luteus* [14] and *Streptococcus pneumoniae* [15]. It is noteworthy that these enzymes were shown to be responsible for the major part of the total in vitro GTase activity measured. So far, little attention was paid to this new class of peptidoglycan-synthesising proteins. One reason for the fact that these enzymes were not investigated in more detail may be that such a GTase could be purified only from *E. coli* and only in very small quantities [13]. However, the present availability of complete bacterial genome sequences as well as the progress made in the heterologous overexpression and purification of bacterial proteins, may render them more accessible for functional and structural studies. Here we report the cloning of the *E. coli mgt* gene, of which the sequence shows significant homologies to well-conserved fingerprints found in the N-terminal GTase domain of the bifunctional PBPs. Furthermore, we show that overexpression of the *mgt* gene leads to a significant increase in the rate of peptidoglycan synthesis in in vitro GTase assays, demonstrating for the first time that the *mgt* gene product indeed exhibits the assumed activity.

In this paper, the term glycosyltransferase (GTase) describes the glycan-synthesising polymerase activity of the high-molecular-weight PBPs and of the MGT protein. We deliberately avoided the term transglycosylase, which is confusing because it has been used in the literature to describe both glycan synthesising as well as glycan degrading enzymes.

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2. Materials and methods

2.1. Cloning of mgt *encoding the putative monofunctional GTase from* Escherichia coli

The monofunctional GTase of *E. coli* was identified by DNA sequence data base searches using the conserved fingerprints indicated in Fig. 1 as search patterns. A homology search in the complete *Haemophilus influenzae* genome revealed the presence of an ORF (HI0831) encoding a possible monofunctional GTase. A homologous gene was then found in the known *E. coli* genome sequence in the 72.2 min region. Primer 84/2 (5′-CGCGTGCTGGTTCTGGC**catAT**GAGT AAAAGCCGCTTAAC-3′) introducing by the three base substitutions (lower case letters) a *Nde*I site (bold letters) and primer 84/10 (5′-CACTTCGCGCG**AAGCTT**AATCCAGCTG-3′) containing a natural *Hin*dIII site were used to amplify *mgt* from *E. coli* strain W3110 (ATCC 27325). The PCR reaction was performed with *Taq* DNA-Polymerase and reagents from Boehringer Mannheim (Mannheim, Germany) with the following cycle protocol: melting at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min for a total of 20 cycles. The resulting 0.8 kb PCR product was digested with *Nde*I and *Hin*dIII, isolated and integrated into plasmid pDS56/RBSII,NdeI yielding plasmid pMGT. Plasmid pDS56/RBSII,NdeI was derived from plasmid pDS56/RBSII,NcoI [16] by replacing its *Nco*I site with a *Nde*I site and by eliminating the *cat* gene.

2.2. Expression of mgt in E. coli

The expression plasmid containing the putative monofunctional GTase gene (*pMGT1*) was used to transform W3110 cells, which already contained plasmid pREP4 [16]. pREP4 harbours a neomycin/kanamycin cassette and the *lacI* repressor gene, which guarantees a very low basal expression of the cloned PCR product. For the expression, W3110 cells bearing pMGT and pREP4 were grown in Super Medium (25 g bacto-tryptone, 15 g bacto-yeast extract and 5 g NaCl per litre) containing ampicillin (100 μg/ml) and kanamycin (25 μg/ml) to an OD_{600} of 0.6. The expression was then induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 50 μM. The cells were grown for another 30 min, harvested by centrifugation, washed with 20 mM Tris-HCl, pH 8.0 and stored at −20°C. Plasmid pDS781/RBSII-DHFRS, expressing the mouse dihydrofolate reductase gene [16] was used in the same expression system as vector control.

2.3. Preparation of solubilised membranes and determination of the peptidoglycan synthesis activity

Wet packed cells (1 g) were suspended in 4 ml of disruption buffer (50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM 1,4-dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride and traces of DNAse) and disintegrated by two successive passages through a French Press chamber at 83 MPa. Whole cells and cell debris were removed by centrifugation at 15 000×*g* for 20 min, and the membrane vesicles were sedimented by centrifugation at 200 000×*g* for 1 h. The membrane vesicles were resuspended in 1 ml of disruption buffer containing 2% Elugent (Calbiochem, La Jolla, CA), the extract incubated for 10 min on ice and finally centrifuged for 40 min at 200 000×*g*. The supernatant (=Elugent extract) was either directly used for the determination of the peptidoglycan synthesis activity or stored in aliquots at −20°C, where it retains its glycan-polymerising activity without considerable loss for several freeze/thaw cycles.

The radiolabelled lipid II precursor was prepared with *E. coli* membranes from UDP-*N*-acetyl-[^{14}C]glucosamine (Amersham, Buckinghamshire, UK) essentially as described [13]. Peptidoglycan synthesis activities were determined in 50 μl reactions containing 50 mM PIPES, pH 6.1, 10 mM $MgCl_2$, 0.2 mM DTT, 1 mM ATP, 26.7% dimethylsulfoxide (DMSO), Elugent extract and ^{14}C-labelled lipid II (6000 cpm, 14.2 pmol). The reaction was started by the addition of the labelled compound and incubated for 30–60 min at room temperature. The reaction mixtures were finally filtered through hydrophilic Durapore PVDF membranes (0.65 μm; Millipore, Bedford, MA), on which synthesised peptidoglycan was retained, while the unincorporated ^{14}C-labelled lipid II precursor was washed out by three successive washing steps with 0.4 M ammonium acetate in methanol. The filter-bound radioactivity was determined by liquid scintillation counting. Flavomycin (a mixture of different moenomycins), which was used for inhibition studies, was from Hoechst (Frankfurt, Germany). In control experiments demonstrating the effect of Flavomycin on glycan polymerisation of high-molecular-weight PBPs, *E. coli* SP1026 cells (ED3184 Δ*ponB*:: Spc^r, [4]) lacking PBP1B were transformed with plasmid pMC56-PBP1b, which was shown to functionally overexpress the *E. coli* homologue PBP1B from *Citrobacter freundii* (Löchle et al., submitted).

2.4. Molecular cloning methods and analytical procedures

General DNA manipulations and cloning procedures were performed according to described protocols [17]. DNA sequence analysis was performed according to the dideoxynucleotide chain termination method [18] using a *Taq* DyeDeoxy terminator cycle sequencing kit and the model 377A DNA sequencer from Applied Biosystems. The protein content of samples was determined as described by Bradford [19] and SDS/polyacrylamide gel electrophoresis was performed according to Laemmli [20].

3. Results and discussion

Based on characteristic fingerprints found in the N-terminal GTase domains of bifunctional PBPs (Fig. 1) we discovered in *Haemophilus influenzae* an unassigned ORF (HI0831, EMBL/GenBank/DDJB, accession number L42023) encoding a putative monofunctional GTase. Sequence comparison with the known *E. coli* genomic sequences also led to the identification of an unassigned ORF (ORF242; EMBL/GenBank/DDJB, accession number U18997) of which the product may carry a putative GTase activity. Similarly, a homologous MGT was identified in the known genomic sequence of *Staphylococcus aureus* (EMBL/GenBank/DDJB, accession number L19300) and in *Alcaligenes eutrophus* (EMBL/GenBank/DDJB, accession number Z22737; Paik, J., Jendrossek, D. and Hakenbeck, R., in preparation). Recently, Spratt and coworkers found by a different approach other putative monofunctional GTases [21].

In order to elucidate the enzymatic activity of this protein, we amplified the *E. coli mgt* gene (ORF 242) by PCR and cloned it in an expression vector. Although the expression system used usually leads to a high expression of the desired gene, a fractionation of the cells into cytoplasmic and membrane fraction was necessary to clearly visualise the resulting product by SDS-PAGE (Fig. 2). The sequence of the gene showed an uncleavable N-terminal membrane-anchor suggesting that the protein would be membrane-bound. A band of the expected size (27 kDa) appeared in the membrane fraction of W3110 cells expressing the putative monofunctional GTase. Such a band could not be detected in control cells, which contained pDS781/RBSII-DHFRS. Interestingly, the host cells (W3110) expressing *mgt* stopped growing, but did not lyse, immediately after induction with IPTG, indicating that the gene product was deleterious for the cells. Therefore, the cells were grown for only half an hour after induction.

Much more relevant than the synthesis of this monofunctional enzyme was the demonstration of its peptidoglycan synthesis activity. The assays to measure the peptidoglycan synthesis activity of this monofunctional enzyme were performed using solubilised membranes and lipid II, the specific substrate of the known periplasmic *E. coli* GTases. Since we expressed the *mgt* gene in wild-type W3110 cells, it was important to keep the interference of the other peptidoglycan-synthesising enzymes, namely PBPs 1A and 1B, as low as possible. PBP1B, which is the major peptidoglycan-synthesising protein of *E. coli*, has its pH optimum between pH 7.5 and 8.0 [13]. On the other hand, the only monofunctional GTase in *E. coli* described so far [13] was shown to have a pH

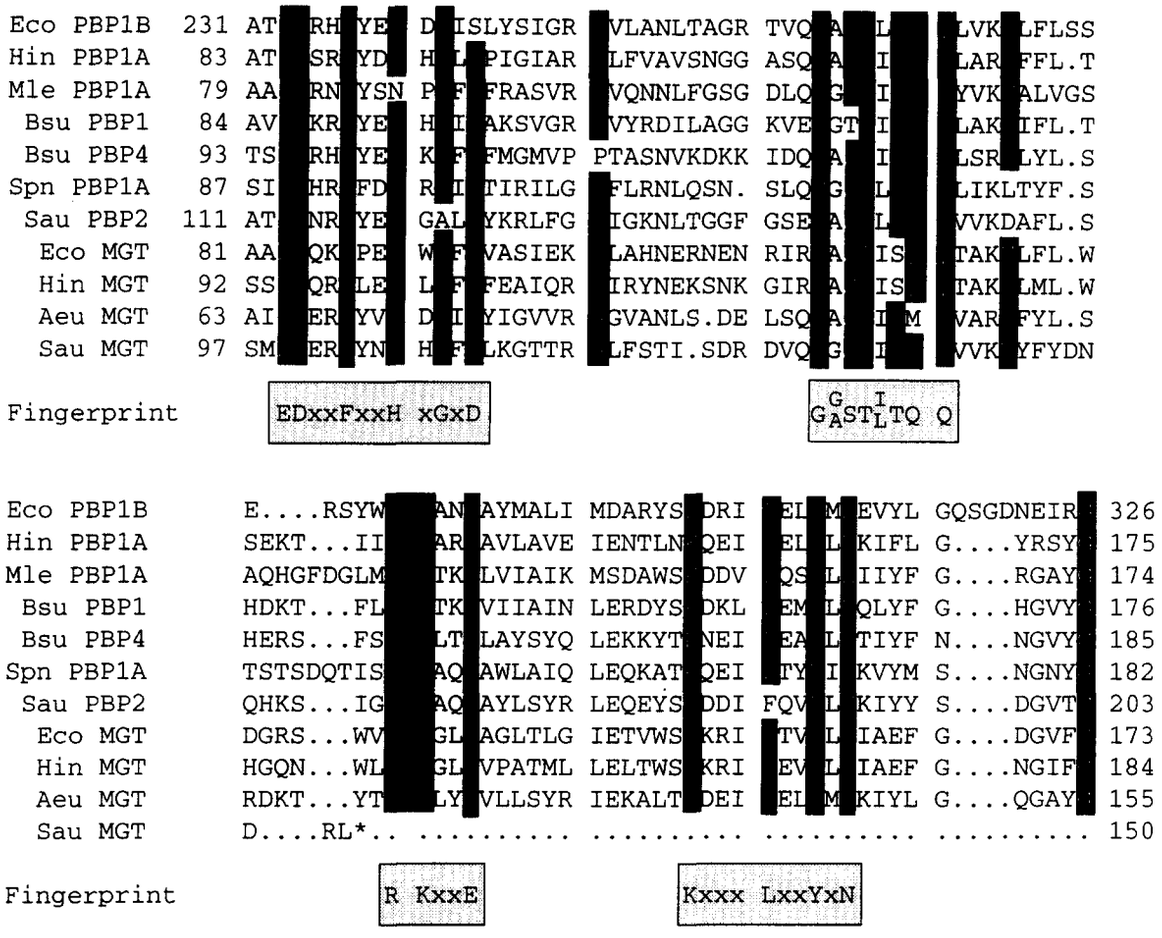

Fig. 1. Alignment of the amino acid sequences of the N-terminal domains of the indicated high-molecular-mass PBPs from *E. coli* (Eco), *H. influenzae* (Hin), *Mycobacterium leprae* (Mle), *Bacillus subtilis* (Bsu), *Streptococcus pneumoniae* (Spn), *Staphylococcus aureus* (Sau) and the new putative monofunctional glycosyltransferases (MGTs). The fingerprints used for the search of the MGTs are indicated by the shadowed boxes (x = any amino acid). Numbers indicate the N- and C-terminal residues of the listed proteins.

optimum between pH 6.0 and 6.5. We therefore decided to determine glycan polymerisation activity at a rather acidic pH. In Fig. 3 the peptidoglycan synthesis activities of induced *E. coli* W3110/pMGT1 cells are compared with those of the same cells that carry plasmid pDS781/RBSII-DHFRS. The results clearly demonstrate that the expression of the cloned gene leads to a significantly increased glycan polymerisation activity. Furthermore, subsequent incubation of the synthesised peptidoglycan with lysozyme led to an almost complete loss of filter-bound radioactivity (data not shown), indicating that the polymerised glycan chains serve as substrate for lysozyme. Glycan polymerisation by the synthesised gene product showed a pH dependence, the pH optimum being between pH 6.0 and 6.5. On the other hand, peptidoglycan-synthesising activity of W3110/pDS781/RBSII-DHFRS increased with increasing pH, which is a result of the fact that PBP1B becomes active under more alkaline conditions. The initial velocity of polymerisation was about 6.0 pmol/mg protein per min, which is three orders of magnitude lower than the specific activity of the glycan polymerase described by Hara and Suzuki [13]. One must take into account, however, that the activity determined here was performed with a detergent extract which is also presumed to contain peptidoglycan hydrolysing enzymes.

An interesting property of the synthesised monofunctional GTase results from inhibition assays performed in the presence of Flavomycin. This phosphoglycolipid antibiotic is known to prevent glycosyl transfer, acting probably as a substrate analogue of lipid II [22]. As expected, peptidoglycan synthesis of W3110/pDS781/RBSII-DHFRS cells was severely impaired in the presence of low (0.2 μg/ml) Flavomycin concentrations. In contrast, the polymerisation activity of cells expressing *mgt* was not significantly affected by Flavomycin (Fig. 3). Since the inhibitory mechanism of Flavomycin relies on the stoichiometric binding to the GTase domain, the failure of this antibiotic to inhibit glycan polymerisation by the MGT enzyme may have been due to the overexpression of *mgt*. However, as shown in Fig. 4A, even after a 100–500-fold dilution of the Elugent extract from *mgt* expressing cells, this inhibition was negligible and probably affected only the low polymerisation activity deriving from PBPs 1A and 1B under the applied conditions. In a control experiment, PBP1B from *Citrobacter freundii*, which is 94% identical to the *E. coli* homologue, was synthesised in *E. coli* SP1026 cells lacking a PBP1B background (Löchle et al., submitted) and the effect of

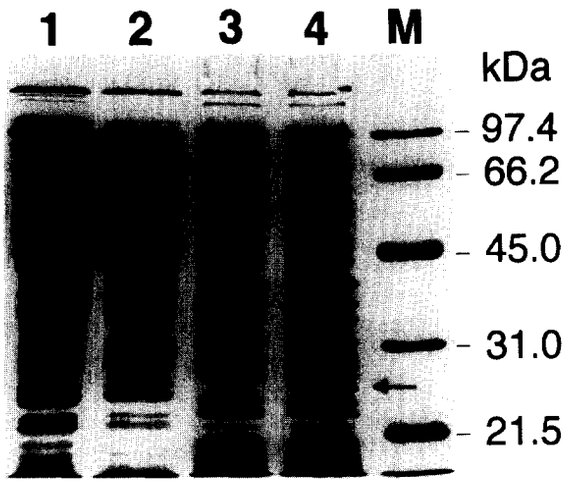

Fig. 2. Expression of *mgt* in *E. coli*. W3110/pREP4 cells carrying expression plasmids pDS781/RBSII-DHFRS (lanes 1 and 3) or pMGT (lanes 2 and 4) were grown to an $OD_{600} = 0.6$, the expression of the cloned genes was induced with 50 μM IPTG and the cells were grown for another 30 min. The cytoplasmic (lanes 1 and 2) and membrane fractions (lanes 3 and 4) were analysed by SDS-PAGE. 30 μg of protein (25 μg in lane 2) were loaded on a 10% SDS/Polyacrylamide gel which was stained with Coomassie blue. The arrow indicates the synthesised MGT product. M = molecular mass marker.

Flavomycin on the glycan polymerisation activity was analysed. Although in these cells the heterologous enzyme was overproduced and highly active, a 10-fold dilution of the corresponding Elugent extract already allowed Flavomycin (0.2 μg/ml) to inhibit the glycan-polymerising activity by 50%, and a further 5-fold dilution of the extract resulted in complete inhibition of glycan polymerisation by Flavomycin (Fig. 4B). At present, the reasons remain speculative for why Flavomycin is a potent inhibitor of the GTase activity of PBPs 1A and 1B while it does not affect the GTase activity of MGT. Acting as a substrate analogue or possibly as a transition state analogue, Flavomycin is believed to compete with lipid II for binding to the active site of GTases. Differential affinities of monofunctional and bifunctional GTases for lipid II and Flavomycin might explain the observed differences in the effect of Flavomycin on the two types of peptidoglycan polymerising GTases. The failure of Flavomycin to inhibit the *mgt* product as well as the pH optimum of the enzyme in the acidic pH range minimise the background peptidoglycan synthesis of the high-molecular-weight PBPs and represent an easy way to measure the GTase activity of MGT in wild-type cells. Evidence for the presence of peptidoglycan-degrading hydrolases in the Elugent extracts is provided by the fact that most of the radioactivity was incorporated for the 5- and 10-fold diluted samples. This can be explained by a complete polymerisation of the lipid-linked precursor, which is fastest for the undiluted sample, offering thereby more time to the endogenous hydrolases, during the 1 h incubation, for the subsequent peptidoglycan degradation.

In summary, the data presented here confirm the presence of a membrane-bound monofunctional GTase in *E. coli* that polymerises glycan chains using lipid II as substrate. This enzyme activity can now be assigned to the *mgt* gene product. However, it is not clear whether the cloned and expressed *mgt* gene encodes the monofunctional glycan polymerase described by Hara and Suzuki [13]. While the enzyme purified by Hara and Suzuki has a molecular weight of 34 kDa, the *mgt* gene encodes a product of 27.3 kDa, which corresponds well to the overexpressed protein observed by SDS-PAGE in this study. A discrepancy of a few kDa may appear in the acceptable range of inaccuracy of the gel electrophoresis method and is frequently encountered when apparent and calculated molecular weights of, mostly hydrophobic, proteins are compared. However, there is a considerable difference in M_r between the GTase presented in this study and the enzyme purified by Hara and Suzuki, taking into account that the observed M_r of both proteins derive from similar SDS-PAGE analyses. Furthermore, the purified glycan polymerase of Hara and Suzuki [13] was shown to be stimulated by Ca^{2+} ions. Preliminary experiments with MGT did not confirm those results. On the other hand, both monofunctional enzymes have pH optima in the range between pH 6.0 and 6.5 and are not inhibited by Flavomycin, indicating that these enzymes possess some similar characteristics. More information on the nature of the glycan polymerase purified by Hara and Suzuki would require the determination of the N-terminal amino acid sequence of this enzyme.

At present, the functional role of the MGT enzyme in the *E. coli* cell cycle remains unclear. Expression of *mgt* seems to be transcriptionally and translationally coupled to a gene encoding a putative 'σ cross-reacting protein' of unknown function [23]. Whether these genes are expressed under laboratory growth conditions remains to be determined. Since PBP $1A^-/1B^-$ double mutants are not viable [3–5] we must assume that either the *mgt* gene is not or only poorly expressed, or that the product polymerised by the monofunctional GTase, i.e., non-crosslinked glycan strands, is not the right substrate for transpeptidation by the high-molecular-weight PBPs 2 and 3. Further studies are envisaged (1) to analyse the polymerisation product formed by purified MGT enzyme (excluding thereby the cross-linking PBPs), and (2) to assess the possible

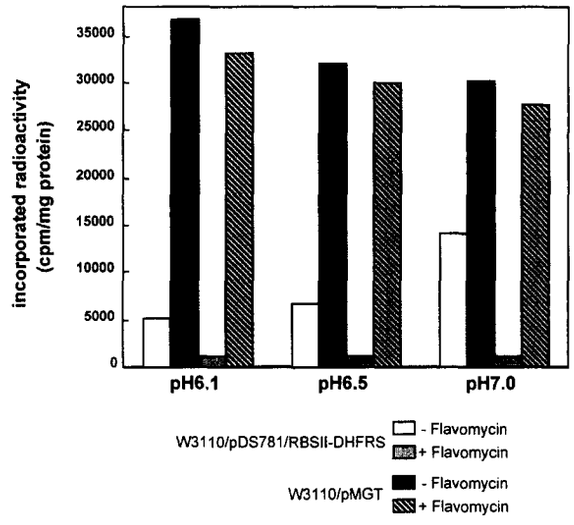

Fig. 3. Peptidoglycan synthesis by Elugent extracts using ^{14}C-labelled lipid II precursor and inhibition by Flavomycin. Elugent extracts of W3110/pDS781/RBSII-DHFRS (118 μg protein) or W3110/pMGT cells (65 μg protein) were prepared as described under Section 2 and incubated for 50 min in 50 mM PIPES buffer of the indicated pH containing ^{14}C-labelled lipid II (14.2 pmol, ~6000 cpm) in absence or presence of 0.2 μg/ml Flavomycin. Polymerised peptidoglycan was determined as filter-bound radioactivity by scintillation counting.

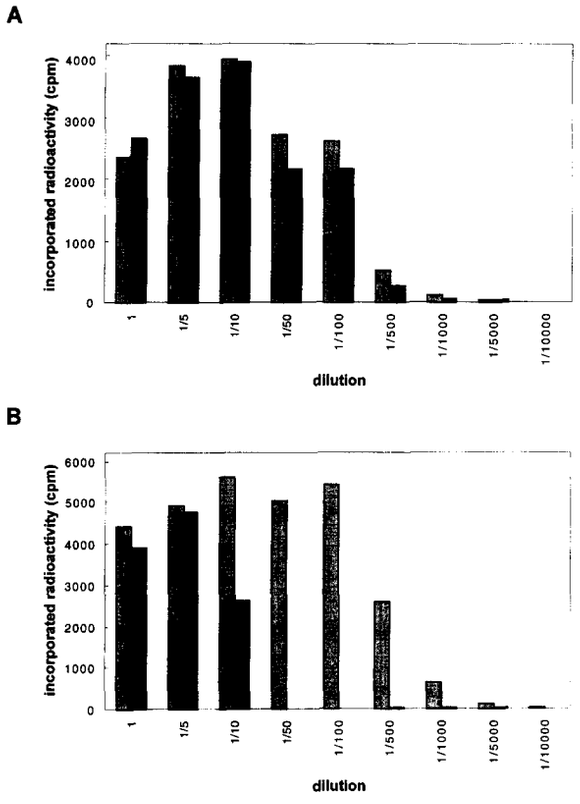

Fig. 4. Effect of Flavomycin on peptidoglycan synthesis of serially diluted Elugent extracts. Elugent extracts of W3110/pMGT (A) or SP1026/pMC56-PBP1b (B) were diluted as indicated in 50 mM PIPES, pH 6.1, 1 mM $MgCl_2$ containing 2% Elugent and peptidoglycan polymerisation was determined after 1 h incubation in absence (light bars) or presence of 0.2 μg/ml Flavomycin (dark bars) as described under Section 2.

function of this enzyme in the cell cycle by determining gene expression levels and by deletion mutagenesis.

Independent of the function of the enzyme described in this paper, monofunctional GTases may become very important for the design of new antibiotics. The increasing resistance of pathogenic bacteria against β-lactam antibiotics demands the establishment of new targets, such as the GTase activity of MGTs and PBPs. The presence of major non-PBP related glycan polymerase activities in pathogenic Gram-positive bacteria like *S. aureus* and *S. pneumoniae* [14,15] emphasizes the importance of this new class of enzymes and should incite further studies in this direction. Another reason why MGTs deserve more consideration relies on the fact that until now no structural data are available for the bifunctional high-molecular-weight PBPs, a class of enzymes which has been under investigation for more than 20 years. The smaller MGTs might prove to be more accessible for structural investigations.

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