Poly(glucosyl-N-acetylgalactosamine 1-phosphate), a wall teichoic acid of *Bacillus subtilis* 168: its biosynthetic pathway and mode of attachment to peptidoglycan

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The *gga*AB operon of *Bacillus subtilis* 168 encodes enzymes responsible for the synthesis of poly(glucosyl N-acetylgalactosamine 1-phosphate) [poly(GlcGalNAc 1-P)], a wall teichoic acid (WTA). Analysis of the nucleotide sequence revealed that both GgaA and GgaB contained the motif characteristic of sugar transferases, while GgaB was most likely to be bifunctional, being endowed with an additional motif present in glucosyl/glycerophosphate transferases. Transcription of the operon was thermosensitive, and took place from an unusually distant *A*-controlled promoter. The incorporation of the poly(GlcGalNAc 1-P) precursors by various mutants deficient in the synthesis of poly(glycerol phosphate), which is the most abundant WTA of strain 168, revealed that both WTAs were most likely to be attached to peptidoglycan (PG) through the same linkage unit (LU). The incorporation of poly(GlcGalNAc 1-P) precursors by protoplasts confirmed the existence of this LU, and provided further evidence that incorporation takes place at the outer surface of the protoplast membrane. The data presented here strengthen the view that biosynthesis of the LU, and the hooking of the LU-endowed polymer to PG, offer distinct widespread targets for antibiotics specific to Gram-positive bacteria.

INTRODUCTION

Poly(glucosyl N-acetylgalactosamine 1-phosphate) [poly(GlcGalNAc 1-P)], a wall teichoic acid (WTA) of *Bacillus subtilis* 168, accounts for up to 18 % of total cell wall hexosamines (Estrela et al., 1991; Pooley et al., 1987; Shibaev et al., 1973; Soldo et al., 2003). During exponential growth in phosphate-replete medium, the phosphorus contained in poly(glycerol phosphate) is not essential for cell growth (Estrela et al., 1991). The nucleotide sequence of the *gga*AB operon was thermosensitive, and took place from an unusually distant *A*-controlled promoter. The incorporation of the poly(GlcGalNAc 1-P) precursors by various mutants deficient in the synthesis of poly(glycerol phosphate), which is the most abundant WTA of strain 168, revealed that both WTAs were most likely to be attached to peptidoglycan (PG) through the same linkage unit (LU). The incorporation of poly(GlcGalNAc 1-P) precursors by protoplasts confirmed the existence of this LU, and provided further evidence that incorporation takes place at the outer surface of the protoplast membrane. The data presented here strengthen the view that biosynthesis of the LU, and the hooking of the LU-endowed polymer to PG, offer distinct widespread targets for antibiotics specific to Gram-positive bacteria.

Under laboratory conditions, the synthesis of poly(GlcGalNAc 1-P) is not essential for cell growth (Estrela et al., 1991), and its only known function is to serve as adsorption site for bacteriophages φ3T and ρ11 (Estrela et al., 1991). The isolation and analysis of φ3T-resistant mutants has led to the identification of two linkage groups specifically involved in poly(GlcGalNAc 1-P) synthesis: *gne* (*gneA*), the structural gene of the UDP-N-acetylgalactosamine (UDP-GlcNAc) 4-epimerase (Soldo et al., 2003), and *gga*, a locus to which most mutants conferring φ3T-resistance have been mapped, and which is assumed to be involved in the polymerization of poly(GlcGalNAc 1-P) (Estrela et al., 1991). The nucleotide sequence of the *gga* locus has been determined within the *B. subtilis* genome sequencing project (Lazarevic et al., 1995).

The synthesis of poly(GlcGalNAc 1-P) is inhibited by tunicamycin (Tm) (Pooley & Karamata, 2000), an antibiotic interfering with the coupling of N-acetylgalactosamine 1-phosphate (GlcNAc 1-P) to undecaprenyl phosphate (UP), i.e. the first step of the synthesis of the poly(GroP) linkage unit (LU) carrier (Araki & Ito, 1989). The LU, consisting of...
phospho-N-acetylglucosaminyl-N-acetylmannosaminyl (glycerol phosphate)
joins the poly(GroP) chain to peptidoglycan (PG). Furthermore, the
deficiency in TagO, the UDP-GlcNAc : UP GlcNAc 1-P transferase,
prevents the synthesis of poly(GlcGalNAc 1-P) (Soldo et al., 2002). These observations suggest that poly-
(GlcGalNAc 1-P) is attached to PG by a LU containing the poly(GroP) LU.

In this study, we analyse the ggaAB operon responsible for
poly(GlcGalNAc 1-P) polymerization and discuss the
relevance of the incorporation of poly(GlcGalNAc 1-P)
precursors by whole cells and protoplasts to the mode of
polymerization and attachment of this polymer to PG.

**METHODS**

**Bacterial strains, plasmids and phages.** These are listed in Table 1. Chromosomal fragments of *B. subtilis* subcloned in plasmid vectors were maintained in *Escherichia coli* DH5α or JM83.

**Chemicals and isotopes.** CDP-glycerol (CDP-Gro), UDP-glucose (UDP-Glc), UDP-galactose and UDP-N-acetylgalactosamine (UDP-
GalNAc) (d-isomers) were from Sigma. UDP-[1-14C]GalNAc (~60
mCi mmol⁻¹; ~2-22 GBq mmol⁻¹), [1-14C]GlcNAc (~2-22 GBq
mmol⁻¹), [1-35S]ATP (>1000 Ci mmol⁻¹; >37 TBq mmol⁻¹) and
[32P]ATP (>500 Ci mmol⁻¹; >185 TBq mmol⁻¹) were from
Amersham.

**Media and growth conditions.** *E. coli* strains were routinely
grown in Luria–Bertani (LB) medium (Difco) or on LB agar (Difco)
containing, when appropriate, ampicillin (50 µg ml⁻¹). *B. subtilis*
cells were grown in liquid LB, SA+trp [0-2-2% (NH₄)₂SO₄, 1-4%
K₂HPO₄, 0-6% KH₂PO₄, 0-1% trisodium citrate, 2H₂O, 0-02%
MgSO₄.7H₂O, 5 µM MnSO₄, 0-5% glucose, 1% casein acid
hydrolysate (Difco), 20 µg tryptophan ml⁻¹)] or SAT2T (SA+trp,
40 µg thymine ml⁻¹) medium, and on LB agar. When required, SA+trp and SAT2T media were supplemented with adenine (100 µg ml⁻¹), Chloramphenicol and kanamycin were added at final
concentrations of 3 and 5 µg ml⁻¹, respectively. Amylase deficiency,
due to *amyE* locus disruption, was demonstrated as described pre-
viously by Soldo et al. (1993). Growth was followed by measuring
nephelometric density (ND) or OD₆₀₀ for *B. subtilis*; an ND of 100
 corresponds to about 10⁸ cells ml⁻¹.

**Phage susceptibility test.** φ3T and ρ11 phage stocks were
obtained as described previously by Soldo et al. (2003). Phage
susceptibility was tested by spotting 5 µl of the phage stock (10⁻¹
phage ml⁻¹) onto fresh streaks of *B. subtilis* strains on LB agar
plates. Plates were incubated for 8 h at 30 °C.

**Transformation.** *E. coli* competent cells were prepared and transformed
by the procedure of Chung & Miller (1988). Transformation of
*B. subtilis* was performed as described previously by Karamata &
Gross (1970). For the selection of kanamycin-resistant recombinants,
the transformation mixture was incubated for an additional 90 min
with a sublethal concentration (0-1 µg ml⁻¹) of the antibiotic prior
to plating.

**DNA preparation and sequencing.** Plasmid DNA was prepared
by the boiling miniprep method (Del Sal et al., 1988). DNA sequenc-
ing was performed with a Sequenase Version 2.0 Kit (USB) and
[35S]dATP, according to the supplier’s recommendations.

**RNA isolation, and primer extension.** *B. subtilis* strain LS047
was grown in SA+trp medium at 37 °C, and harvested at an ND of
60. Total cellular RNA was isolated as previously described (Soldo
et al., 1999). Oligonucleotide 5'-labeling with [32P]ATP, and the
primer extension reaction, were carried out as described previously
by Lazarevic et al. (1992). Extension products were separated on a
6% polyacrylamide sequencing gel, alongside a sequencing ladder
obtained with the same primer on plasmid p6360.

**β-Galactosidase assay.** The assay was performed as described by
Mauel et al. (1994).

**Polymer synthesis by protoplasts.** The protocol was essentially
that described by Bertram et al. (1981). *B. subtilis* cells were grown in
SAT2T. At an ND of 60, the cells were harvested by centrifugation,
concentrated 40-fold, and incubated in protoplasting medium
(50 mM Tris/Cl⁻, PH 7.5, 0-625 M sucrose, 10 mM MgCl₂,
and 0-5 mg lysozyme ml⁻¹) for 30 min at 30 °C. Polymer synthesis was
assayed at 30 °C on a 0-2 ml sample of protoplasts, to which sub-
strates 500 µM UDP-Glc, 500 µM UDP-GlcNAc, 400 µM CDP-Gro,
2-5 µM UDP-GalNAc and 1-6 µM UDP-[1-14C]GlcNAc were added
in different combinations. The final volume of the assay mixture was
0-25 ml. The reaction was stopped by immersion in boiling water
for 2 min. Following separation of polymerized material from unin-
corporated precursors by descendent paper chromatography
(Bertram et al., 1981), radioactivity was determined by scintillation
counting in 10 ml Optifluor (Packard).

**Labelling and extraction of poly(GlcGalNAc 1-P).** Cells were
grown in SAT2T medium containing 100 µM GlcNAc at 30, 37, 42
and 45 °C. At an ND of 6, [1-14C]GlcNAc was added at a final
concentration of 0-1 µCi ml⁻¹ (3-7 kBq ml⁻¹), and the incubation
was continued until the ND reached 100. Selective acid extraction of
poly(GlcGalNAc 1-P) was as described previously by Soldo et al.
(2002).

**RESULTS**

**Sequence and function of the ggaAB operon.** To characterize the genes involved in poly(GlcGalNAc 1-P) synthesis, we resorted to sequence analysis. With the exception of *gna*, all known mutations conferring deficiency in poly(GlcGalNAc 1-P), as well as resistance to bacteriophages φ3T and ρ11, have been mapped between tagP and gtaB loCi (Estrela et al., 1991; Lazarevic et al., 1995; Soldo et al., 2003). Analysis of the nucleotide sequence of this region has revealed two ORFs, ggaA and ggaB. The 26 nt sequence located 34 nt downstream of the stop codon of ggaB may form a hairpin structure corresponding to a terminator. Absence of any obvious terminator-like structure in the ggaA–ggaB intergenic segment implies that the two ORFs form an operon. The operon is separated from the upstream tagH and the downstream gtaB gene by non-coding regions, which consist of about 2-2 and 0-7 kbp, respectively (Lazarevic et al., 1995). The 2-2 kbp non-coding region exhibits a high degree of similarity to strain W23 tar genes (Lazarevic et al., 2002a). The G+C contents of ggaA and ggaB are 28-2 and 28-8%, respectively, i.e. very low compared with 43-5%, which is the mean G+C content of the *B. subtilis* 168 chromosome (Kunst et al., 1997). The low G+C content of the ggaAB operon, as well as its location between two non-coding regions, leave little doubt that poly(GlcGalNAc 1-P)-encoding genes are acquired through
horizontal transfer.
The 320 residue N-terminal regions of GgaA and GgaB share 34% identity, and contain the pfam00535 domain (Marchler-Bauer et al., 2003), which is characteristic of enzymes transferring a sugar from its NDP-form. The C-terminal moiety of GgaB (residues 520–894) corresponds to the glucosylglycerophosphate transferase domain COG1887 (Marchler-Bauer et al., 2003). This domain is present in the WTA biosynthetic enzymes TagB and TagF, which are the putative CDP-Gro: undecaprenylpyrophosphoryl-N-acetylgulosaminyl-\( \gamma \)-acetylmannosamine (UPP-GlcNAc-ManNAc) GroP transferase (Pooley et al., 1992) and the CDP-Gro: poly(GroP) GroP transferase, respectively (Mauè et al., 1991).

The involvement of the ggaAB operon in poly(GlcGalNAc 1-P) synthesis was confirmed by insertion mutagenesis with plasmids p6361 (strain L4664) and p6362 (strain L4665) containing fragments of ggaA (Fig. 1), as well as by a partial deletion of ggaA (strain L4660). All of these mutants exhibited resistance to bacteriophages \( \Phi 3T \) and \( \rho 11 \), and apparently normal morphology and growth rate. The poly(GlcGalNAc 1-P)-deficient phenotype, i.e. considerably reduced amount of acid-extractable cell wall hexosamines, was confirmed on strain L4660 with a partly deleted ggaA (Fig. 2).

**Expression of the ggaAB operon**

Transcription start signals in the region upstream of ggaA were sought by extension of primers. Since assay for wall hexosamine revealed a thermosensitive synthesis of poly(GlcGalNAc 1-P) (Fig. 2), RNA was prepared from cultures of strain L5047 growing exponentially in SA + trp medium at 30 and 45 °C. Total RNAs were incubated with a series of \( ^{32}P \)-labelled primer oligonucleotides at a stepwise increasing distance from the ggaA start codon. A start signal was obtained with oligonucleotide 5’-CTCCTTGTAGAC-AATCGTAGGATT-3’, spanning residues 905 to 928 upstream of the ggaA start codon. The transcriptional start (Fig. 3) were located on a DNA segment almost identical to the regulatory region of the strain W23 tarD gene (Lazarevich et al., 2002a) (Fig. 1), and corresponded to a sequence differing from that of the \( \sigma \)-independent promoter consensus (Helmann & Moran, 2002b) in 3 nt only (Figs 1 and 3). The innermost start point was separated from the ggaA start codon by 1114 nt, which is a considerable distance. Visual inspection of the gel revealed that the intensity of the start signal at 45 °C was significantly weaker than that at 30 °C (Fig. 3). This thermosensitivity was examined with a lacZ reporter gene inserted into the \( amyE \) locus. The relevant construct was obtained by transforming B. subtilis competent cells with pA2Z8102, which is a plasmid with the ggaAB promoter region inserted upstream of the lacZ gene, and selecting chloramphenicol-resistant and amylase-deficient mutants. \( \beta \)-Galactosidase activity was measured on cultures growing at 30, 37, 42 and 45 °C. The reporter gene was expressed at a constant rate until the OD\( \text{max} \) reached about 0-6. It appeared that the relatively weak activity of this ggaAB promoter was comparable during growth at 30 and 37 °C, but decreased with increasing temperature (Fig. 4). In conclusion, the expression of the ggaAB operon was thermosensitive, and took place from a promoter located over 1 kbp away from the ggaA start codon.

**How is poly(GlcGalNAc 1-P) attached to PG?**

Susceptibility of poly(GlcGalNAc 1-P) synthesis to Tm (Pooley & Karamata, 2000), as well as its dependence on TagO, the UDP-GlcNAc: UP GlcNAc 1-P transferase (Soldo et al., 2002), strongly suggest the existence of a LU that shares certain elements with the poly(GroP) LU. To characterize this LU, we resorted to a genetic approach by examining whether other tag genes are involved in the synthesis of poly(GlcGalNAc 1-P). For that purpose, since thermosensitivity of poly(GlcGalNAc 1-P) synthesis (see above) prevents a straightforward utilization of \( \text{tag} \) thermosensitive mutants, we placed the ggaAB operon of strains L6601 tagD11, L6476 tagB1 and L6477 tagF1 under an IPTG-inducible P\( _{\text{spac}} \) promoter. The constructs obtained, as well as the reference thermostable strain L4674, were incubated in SAT2T medium supplemented with \( [1^{-14}C] \)GlcNAc. For each strain, two cultures were grown: one with 100 µg IPTG ml\(^{-1} \), and the other without IPTG. At an ND of 15, cultures were shifted from 30 to 45 °C, and the cells were harvested at an ND of 100. It appeared (Fig. 5) that deficiency of \( \text{tagF} \), encoding the CDP-Gro: poly(GroP) GroP transferase (Pooley et al., 1992), did not affect the poly(GlcGalNAc 1-P) synthesis, while that of either \( \text{tagD} \), the structural gene encoding glycerol-3-phosphate cytidylyltransferase (Pooley et al., 1991), or \( \text{tagB} \), encoding the enzyme putatively involved in the completion of the poly(GroP) LU, i.e. attachment of GroP to UPP-GlcNAc-ManNAc (Mauè et al., 1991), allowed only residual synthesis of poly(GlcGalNAc 1-P). The incorporation of \( ^{14}C \) in \( \text{tagB} \)- or \( \text{tagD} \)-deficient strains was comparable with that obtained with ggaA- (see above) or gne-deficient strains (Soldo et al., 2003). These observations strongly suggest that poly(GroP) and poly(GlcGalNAc 1-P) are linked to PG through the same LU. Thermosensitivity of strain L4677 \( P_{\text{spac}^{-}}\text{ggaA tagF1} \) (not presented) revealed, in addition, that synthesis of poly(GlcGalNAc 1-P), expressed from the \( P_{\text{spac}} \) promoter, cannot compensate for the missing poly(GroP).

**Incorporation of poly(GlcGalNAc 1-P) precursors by protoplasts**

The synthesis of poly(GlcGalNAc 1-P) was assessed by the experimental system devised by Bertram et al. (1981). Here, the incorporation of \( [1^{-14}C] \)GalNAc, supplied as UDP-[\( 1^{-14}C \)]GalNAc, was measured. Protoplasts of strains derived from 168 were obtained by lysozyme treatment. Assay mixtures containing 40-fold concentrated protoplast suspensions were incubated with UDP-[\( 1^{-14}C \)]GalNAc, and an excess of cold UDP-GlcNAc, to prevent UDP-GalNAc 4-epimerization, and incorporation of the label through UDP-GlcNAc (Bertram et al., 1981). Protoplasts of the parent strain incorporated significant amounts of \( [1^{-14}C] \)GalNAc at a nearly steady rate, an incorporation that was attributed to...
Table 1. Strains, plasmids and phages

<table>
<thead>
<tr>
<th>Strain, plasmid or phage</th>
<th>Genotype or description</th>
<th>Reference or origin*</th>
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<td>DH5α</td>
<td>F− φ80d lacZ ΔM15 Δ(argF–lacZYA) U169 endA1 recA1 hsdR17 (rK− mK−) deoR thi-1 supE44 Δ− gyrA96 relA1</td>
<td>Hanahan (1983)</td>
</tr>
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<td>JM83</td>
<td>F− φ80d lacZ ΔM15 Δ(lac−proAB) ara rpsL</td>
<td>Yanisch-Perron et al. (1985)</td>
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<td><strong>B. subtilis</strong></td>
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<td>168</td>
<td>trpC2</td>
<td>Laboratory stock</td>
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<tr>
<td>W23</td>
<td>Prototroph Smr</td>
<td>Laboratory stock</td>
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<td>L5047</td>
<td>pheA3 purA16 hisA35 trpC2 metB5</td>
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<td>pheA3 purA16 hisA1 trpC2 gtaB15</td>
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<td>Karamata et al. (1987)</td>
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<td>L5095</td>
<td>metB5 hybrid W23−168, W23−like</td>
<td>Karamata et al. (1987)</td>
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<td>metB5 hybrid W23−168, mixed-type</td>
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<td>L5773</td>
<td>hisA1 arg4 hybrid W23−168, mixed-type</td>
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<td>L4660</td>
<td>pheA1 purA16 hisA35 trpC2 metB5 ΔggaA</td>
<td>Transformation of gga locus with p6363 → Cm'. Deletion of a part of ggaA by plasmid excision. Selection for φ3T− resistance</td>
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<td>pheA3 purA16 hisA35 trpC2 metB5 PEm−ggaB</td>
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<td>L4664</td>
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<td>L6601</td>
<td>hisA1 arg4 metC3 tagD11</td>
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<td>Pooley et al. (1991)</td>
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<td><strong>Phages</strong></td>
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<td>ρ11c</td>
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<td>Ap′ Cm'</td>
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<td>Ap′ Em' Cm'</td>
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<td>pSGMU441</td>
<td>Ap′ Km' Pqspac</td>
<td>J. Errington, Sir William Dunn School of Pathology, Oxford, UK</td>
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<td>p6310</td>
<td>Ap′ Em' Cm'</td>
<td>Cloning of the 625 bp SacI−BglII (ggaB1125−ggaB1776) fragment in pMTL20EC</td>
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<td>Cloning of the 1176 bp BglII−EcoRI (ggaA253−87 downstream of ggaA) fragment in pMTL20EC</td>
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<td>Cloning of the 1483 bp BglII (1225 upstream of ggaA−ggaA258) fragment in pSGMU441</td>
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the extension of existing chains of poly(GlcGalNAc 1-P) (Fig. 6). The absolute requirement of UDP-Glc for $^{14}$C-GalNAc incorporation (not presented) left no doubt that the label was incorporated into poly(GlcGalNAc 1-P). This conclusion was confirmed by the absence of $^{14}$C-GalNAc incorporation into the $ggaA$-deficient strain, as well as by a higher rate of incorporation into protoplasts of strain L4661 (Fig. 6), in which expression of $ggaB$ was increased through a promoter of the erythromycin-resistance gene from the integrated plasmid. Attempts to increase the rate of incorporation by adding CDP-Gro and UDP-GlcNAc to the assay mixture, with the aim to de novo generate LUs (Bertram et al., 1981), failed (Table 2). This result is in apparent contradiction with the reduced incorporation of $^{1-14}$C-GalNAc from exogenously supplied $^{1-14}$C-GlcNAc into cells of $tagB$- and $tagD$-deficient mutants at the non-permissive

<table>
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<td>Ap’ Cm’</td>
<td>Cloning of the HindIII–EcoRI fragment from p6310 [containing the SacI–BglII (ggaB1125–ggaB1776) fragment] in pLP1C.</td>
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<td>Cloning of the HindIII–EcoRI fragment from p6321 [containing the 840 bp BamHI–SacI (ggaB291–ggaB1130) fragment in pLP1C]</td>
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<td>p6363</td>
<td>Ap’ Em’ Cm’</td>
<td>Insertion of the EcoRI–BamHI fragment [containing the 398 bp Scal fragment (ggaA999–55 downstream of ggaA)] from p6357 into EcoRI–BamHI of p8102</td>
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<td>p8102</td>
<td>Ap’ Em’ Cm’</td>
<td>Cloning of the BglII fragment from p6360 in pDG268</td>
</tr>
<tr>
<td>pAZ8102</td>
<td>Ap’ Cm’</td>
<td>Lazarevic et al. (1995)</td>
</tr>
</tbody>
</table>

$*$→Cm’ and →Km’ indicate selection for resistance to chloramphenicol or kanamycin, respectively.

†Numbers refer to nucleotide position in the indicated gene.

Fig. 1. Organization of the DNA segment containing the $ggaAB$ operon, and the similarity between promoter regions upstream of $ggaA$, and those upstream of the $B. subtilis$ W23 $tarD$ gene. Inserts of plasmid clones are represented by bold horizontal lines. Arrows correspond to putative genes. The drawing respects the relative sizes of these entities. Relevant restriction sites are indicated. The nucleotide sequences correspond to the identified promoter regions upstream of $ggaA$ and the $B. subtilis$ W23 $tarD$ genes (Lazarevic et al., 2002a), respectively. Identical residues are aligned by vertical bars. The number of nucleotides between the promoter and the corresponding translation start codon (italic) is indicated. –35 and –10 promoter regions are labelled. Transcriptional starts are denoted by triangles.
Fig. 2. Synthesis of poly(GlcGalNAc 1-P) as a function of temperature. Cultures were grown in SAT2T medium at 30, 37, 42 and 45 °C, and labelled with [1-14C]GlcNAc at ND values from 6 to 100. Measurements corresponding to acid-extractable radioactivity corresponding to poly(GlcGalNAc 1-P) are expressed as percentages of total [14C] incorporated into the cell walls. L5047 (○); L4660 ΔggaA (▲).

Fig. 3. Localization by primer extension of start site(s) of the ggaAB operon. The oligonucleotide 5’-TCTTTTGTAGACAA-TGTTAGATT-3’ was used to prime DNA sequencing reactions on plasmid p6360 with ddG, ddA, ddT and ddC (lanes 1–4), as well as the cDNA synthesis using 25 μg RNA extracted from strain L5047 grown in SA+trp medium at 30 (lane 5) and 45 °C (lane 6).

Fig. 4. Expression of the ggaAB operon determined with the lacZ reporter gene. Cultures of strain L4673 Ω(amyE::cat P_{ggaA-lacZ}) were grown in LB medium at 30 (■), 37 (●), 42 (▲) and 45 °C (▲). Samples were withdrawn at regular intervals of time, and processed and assayed for β-galactosidase activity, which is expressed in Miller units.

Fig. 5. Synthesis of poly(GlcGalNAc 1-P) in tag-deficient mutants. Mutants L4675 tagD11 P_{spac-ggaA}, L4676 tagB1 P_{spac-ggaA}, L4677 tagF1 P_{spac-ggaA}, and the reference thermostable strain L4674 P_{spac-ggaA} were grown in SAT2T medium supplemented with [1-14C]GlcNAc in the presence (black bars) and absence (white bars) of IPTG. At an ND of 15, cultures were progressively shifted to 45 °C, and incubated until the ND was 100. Cells were harvested, and the percentage of radioactivity extracted at pH 4 was determined.
had been replaced by poly(RboP)-synthesizing genes, but which retained the capacity to synthesize poly(GlcGalNAc 1-P). In protoplasts of these so-called mixed-type hybrids (Karamata et al., 1987), the incorporation of $[1-\text{14C}]\text{GalNAc}$ from UDP-$[1-\text{14C}]\text{GalNAc}$ was hardly detected. This was not surprising in view of the low quantities of GalNAc in walls of whole cells, relative to amounts present in strain 168 (H. M. Pooley, personal communication). However, with these hybrid strains, addition of CDP-Gro and UDP-GlcNAc to the assay mixture did increase the rate of incorporation by a factor of 10 (Table 2), a relative increase comparable to that obtained for poly(RboP) (Bertram et al., 1981). This increase was most likely due to newly synthesized LU, since it was completely inhibited by Tm, an antibiotic known to interfere with the synthesis of the LU by blocking the coupling of GlcNAc 1-P to the lipid carrier.

The incorporation of $[1-\text{14C}]\text{GalNAc}$ from UDP-$[1-\text{14C}]\text{GalNAc}$ by protoplasts of strain L5054 $\text{gtaB}$, a mutant deficient in the UDP-Glc pyrophosphorylase, was substantially higher than that obtained with protoplasts of the reference strain L5047 (Table 2, Fig. 6). It also appeared that the excess incorporation relative to that obtained with strain L5047 was insensitive to Tm, i.e. it was achieved on an excess of apparently idle LUs. Again, as with the parent L5047 strain, addition of CDP-Gro and UDP-GlcNAc, two out of three LU precursors, did not increase the rate of incorporation of $[1-\text{14C}]\text{GalNAc}$ supplied as UDP-$[1-\text{14C}]\text{GalNAc}$ by protoplasts of the $\text{gtaB}$-deficient strain (Table 2).

**DISCUSSION**

From our experiments, the complete pathway of poly-(GlcGalNAc 1-P) synthesis can be deduced (Fig. 7). We have obtained strong evidence that the main chain of this WTA is attached to PG through the same LU as poly(GroP), which is the most abundant WTA of strain 168. First, the synthesis of poly(GlcGalNAc 1-P) requires TagO (Soldo et al., 2002), and is sensitive to Tm (Pooley & Karamata, 2000), revealing that GlcNAc is the first element of the LU. Second, requirement of TagD, the glycerol-3-phosphate cytidylyltransferase, and TagB, which most likely couples GroP to UPP-GlcNAc-ManNAc, shows that both ManNAc

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**Table 2.** $[1-\text{14C}]\text{GalNAc}$ incorporation by protoplasts: role of strain 168 LU elements

Protoplast samples of different *B. subtilis* strains, obtained by lysozyme treatment at 30°C, were incubated for 20 min at 30°C in the assay mixture containing WTA precursors and Tm, as specified in Methods. Radioactivity incorporated from UDP-$[1-\text{14C}]\text{GalNAc}$ was separated by paper chromatography, and counted. ND, Not determined.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Radioactivity obtained with specified compounds added to the assay mixture (d.p.m.)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>UDP-Glc</td>
</tr>
<tr>
<td></td>
<td>UDP-GalNAc</td>
</tr>
<tr>
<td></td>
<td>CDP-Gro</td>
</tr>
<tr>
<td>L5773 Mixed hybrid</td>
<td>262</td>
</tr>
<tr>
<td>L5076 Mixed hybrid</td>
<td>297</td>
</tr>
<tr>
<td>L4670 Mixed hybrid $\text{ggaB (L5773)}$</td>
<td>ND</td>
</tr>
<tr>
<td>L4671 Mixed hybrid $\text{ggaB (L5706)}$</td>
<td>ND</td>
</tr>
<tr>
<td>L5703 W23-like hybrid</td>
<td>ND</td>
</tr>
<tr>
<td>L5705 W23-like hybrid</td>
<td>ND</td>
</tr>
<tr>
<td>L5047</td>
<td>1717</td>
</tr>
<tr>
<td>L5054 $\text{gtaB}$</td>
<td>5127</td>
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</table>
and GroP, the second and the third elements of the poly(GroP) LU, are part of the poly(GlcGalNAc 1-P) LU. Third, incorporation of [1-14C]GalNAc, supplied as UDP-[1-14C]GalNAc, by protoplasts of 168/W23 hybrid strains was strongly stimulated by addition of both CDP-Gro and UDP-GlcNAc to the assay mixture. The two latter precursors provide a complete set of LU constituents, since UDP-ManNAc is obtained from UDP-GlcNAc through the action of the UDP-GlcNAc 2-epimerase, a cytoplasmic enzyme that is released to the medium following lysis of a small proportion of protoplasts. This is substantiated by Harrington & Baddiley (1985) who reported that a very small contamination of B. subtilis membrane preparations by UDP-GlcNAc 2-epimerase allows in vitro synthesis of the LU. The polymerization of the poly(GlcGalNAc 1-P) chain is most likely to be achieved by alternate incorporation of GalNAc-P and glucose, mediated by GgaB and GgaA, which are two proteins whose N-terminal moieties contain similar motifs characteristic of sugar transferases (Marchler-Bauer et al., 2003). Despite an exhaustive search, no other gene specifically involved in poly(GlcGalNAc 1-P) has been found (Estrela et al., 1991). Therefore, it appears that the large GgaB protein endowed with the motif characteristic of glycerol- and ribitol-metabolizing enzymes (Marchler-Bauer et al., 2003) is bifunctional; its roles being the initiation of the main polymer chain, as well as the addition of one of its elements in alternation with GgaA. According to the structural formula of the GlcGalNAc 1-P disaccharide (Shibaev et al., 1973), the precursor phosphate remains attached to the C-1 of GalNAc, strongly suggesting that the main chain begins by the coupling of GalNAc 1-P to the C-1 of the LU glycerol. Finally, the poly(GlcGalNAc 1-P) chain polymerized on the LU is translocated to the outer membrane surface by the TagGH ABC transporter (Lazarevic & Karamata, 1995), and hooked to the nascent PG chain.

Experiments of Bertram et al. (1981) strongly suggest that the incorporation by protoplasts of WTA components, supplied as NDP precursors, takes place at the outer surface of the cytoplasmic membrane. Our experiments provide additional evidence, by revealing that incorporation of label, supplied as UDP-[1-14C]GalNAc, into poly(GlcGalNAc 1-P) has an absolute requirement for UDP-Glc. Had the label been transported into the cytoplasm, some incorporation would have taken place during residual synthesis of poly(GlcGalNAc 1-P), without any exogenously supplied UDP-Glc, since UDP-Glc is present in the cytoplasm. It should be recalled that, in cases examined to date, addition of Tm to the reaction mixture reduces the incorporation by 35 % (Bertram et al., 1981) and about 50 % (this work), revealing that a residual synthesis of new LUs and chains does take place in protoplasts. Therefore, it would seem that the export of the polymer by the TagGH transporter is coupled with a temporary exposure of the polymerizing enzymes at the outer surface (Bertram et al., 1981; Lazarevic & Karamata, 1995). The latter enzymes could incorporate...
exogenously supplied precursors, and further elongate the exported chains, which, due to lysozyme-mediated removal of PG, would remain idle. This would be the case for poly(GlcGalNAc 1-P), as well as for poly(RboP) (Bertram et al., 1981).

The relatively high [14C]GalNAc incorporation rate into protoplasts of L5054, a gtaB-deficient strain (Fig. 6), was likely to be due to accumulation of idle LU destined for the synthesis of poly(GlcGalNAc 1-P). First, in absolute terms, the inhibitory effect of Tm on the gtaB-deficient strain was comparable with that obtained with protoplasts of the reference strain L5047. This implies that the increased rate of synthesis of the gtaB-deficient mutant is due to accumulated idle elements on which synthesis can readily begin when appropriate precursors are provided. Second, since gtaB-deficient cells can supply UDP-GalNAc through UDP-GlcNAc 4-epimerization (Soldo et al., 2003), and since the synthesis of the poly(GlcGalNAc 1-P) chain probably begins with GalNAc (see above), it seems that the LUs accumulated during growth of L5054 gtaB are endowed with GalNAc, and therefore cannot serve for poly(GroP) synthesis. It is most likely that the same mechanism is responsible for a higher rate of poly(GlcGalNAc 1-P) synthesis by protoplasts of strains with increased expression of ggaB (Fig. 6).

One of the main roles of WTAs is the maintenance of the global negative charge of the cell surface. As previously discussed (Estrela et al., 1991), whenever the rate of synthesis of poly(GroP) is reduced, that of poly(GlcGalNAc 1-P) is increased (Rosenberger, 1976; F.-X. Abellan & H. M. Pooley, personal communication). However, at 45 °C, synthesis of the poly(GlcGalNAc 1-P) from a Pspac promoter can not compensate for the absence of poly(GroP) (see above), either because of an insufficient rate of expression of the ggaAB operon, or because the main polymer may have a more specific role.

It has been shown that WTAs are essential for growth of B. subtilis strains 168 and W23 (Mauel et al., 1991; unpublished), as well as for Staphylococcus epidermidis ATCC 14990 (Fitzgerald & Foster, 2000). Therefore, the presence of a LU common to many Gram-positive bacteria (Araki & Ito, 1989) suggests that it represents an interesting and a relatively widespread target for antibiotics (Pooley & Karamata, 1988). This conclusion is strengthened by the recently reported evidence that in B. subtilis, under laboratory conditions, teichuronic acid cannot substitute for a deficiency in poly(GroP) (Bhavsar et al., 2004).

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