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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Received 26 December 2005
Revised 30 January 2006
Accepted 3 February 2006

The *ggaAB* 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 σ^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 this polymer is equivalent to 10% of that present in poly(glycerol phosphate) [poly(GroP)], the most abundant

and essential WTA of strain 168 (H. M. Pooley, personal communication).

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 ϕ 3T and ρ 11 (Estrela *et al.*, 1991). The isolation and analysis of ϕ 3T-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*-acetylglucosamine (UDP-GlcNAc) 4-epimerase (Soldo *et al.*, 2003), and *gga*, a locus to which most mutants conferring ϕ 3T-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*-acetylglucosamine 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

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Abbreviations: CDP-Gro, CDP-glycerol; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; GlcNAc 1-P, *N*-acetylglucosamine 1-phosphate; GroP, glycerol phosphate; LU, linkage unit; ManNAc, *N*-acetylmannosamine; ND, nephelometric density; PG, peptidoglycan; P-GlcNAc-ManNAc-(GroP)₁₋₂, phospho-*N*-acetylglucosaminyl-*N*-acetylmannosaminyl (glycerol phosphate)₁₋₂; poly(GlcGalNAc 1-P), poly(glucosyl *N*-acetylgalactosamine 1-phosphate); poly(GroP), poly(glycerol phosphate); poly(RboP), poly(ribitol phosphate); Tm, tunicamycin; UDP-Glc, UDP-glucose; UDP-GalNAc, UDP-*N*-acetylgalactosamine; UDP-GlcNAc, UDP-*N*-acetylglucosamine; UP, undecaprenyl phosphate; UPP-GlcNAc-ManNAc, undecaprenyl pyrophosphoryl-*N*-acetylglucosaminyl-*N*-acetylmannosamine; WTA, wall teichoic acid.

phospho-*N*-acetylglucosaminyl-*N*-acetylmannosaminyl (glycerol phosphate)₁₋₂, [P-GlcNAc-ManNAc-(GroP)₁₋₂], 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 elements of 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* DH5 α or JM83.

Chemicals and isotopes. CDP-glycerol (CDP-Gro), UDP-glucose (UDP-Glc), UDP-GlcNAc and UDP-*N*-acetylgalactosamine (UDP-GalNAc) (D-isomers) were from Sigma. UDP-[1-¹⁴C]GlcNAc (~ 60 mCi mmol⁻¹; ~ 2.22 GBq mmol⁻¹), [1-¹⁴C]GlcNAc (~ 2.22 GBq mmol⁻¹), [α -³⁵S]dATP (> 1000 Ci mmol⁻¹; > 37 TBq mmol⁻¹) and [γ -³²P]ATP (> 5000 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 μ g ml⁻¹). *B. subtilis* cells were grown in liquid LB, SA + trp [(0.2 % (NH₄)₂SO₄, 1.4 % K₂HPO₄, 0.6 % KH₂PO₄, 0.1 % trisodium citrate.2H₂O, 0.02 % MgSO₄.7H₂O, 5 μ M MnSO₄.H₂O, 0.5 % glucose, 1 % casein acid hydrolysate (Difco), 20 μ g tryptophan ml⁻¹)] or SAT2T (SA + trp, 40 μ g thymine ml⁻¹) medium, and on LB agar. When required, SA + trp and SAT2T media were supplemented with adenine (100 μ g ml⁻¹). Chloramphenicol and kanamycin were added at final concentrations of 3 and 5 μ g ml⁻¹, respectively. Amylase deficiency, due to *amyE* locus disruption, was demonstrated as described previously 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. ϕ 3T and ρ 11 phage stocks were obtained as described previously by Soldo *et al.* (2003). Phage susceptibility was tested by spotting 5 μ l of the phage stock (10⁹–10¹⁰ 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 μ g 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 sequencing was performed with a Sequenase Version 2.0 Kit (USB) and [α -³⁵S]dATP, according to the supplier's recommendations.

RNA isolation, and primer extension. *B. subtilis* strain L5047 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'-labelling with [γ -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 Mauël *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/HCl, 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 substrates 500 μ M UDP-Glc, 500 μ M UDP-GlcNAc, 400 μ M CDP-Gro, 2.5 μ M UDP-GalNAc and 1.6 μ M UDP-[1-¹⁴C]GalNAc 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 unincorporated 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 μ M 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 μ Ci 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 *gne*, all known mutations conferring deficiency in poly(GlcGalNAc 1-P), as well as resistance to bacteriophages ϕ 3T and ρ 11, have been mapped between *tagF* 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 glucosyl/glycerophosphate 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 : undecaprenyl pyrophosphoryl-*N*-acetylglucosaminyl-*N*-acetylmannosamine (UPP-GlcNAc-ManNAc) GroP transferase (Pooley *et al.*, 1992) and the CDP-Gro : poly(GroP) GroP transferase, respectively (Mauël *et al.*, 1991).

The involvement of the *ggaAB* operon in poly(GlcGalNAc 1-P) synthesis was confirmed by insertional mutagenesis with plasmids p6361 (strain L4664) and p6362 (strain L4665) containing fragments of *ggaB* (Fig. 1), as well as by a partial deletion of *ggaA* (strain L4660). All of these mutants exhibited resistance to bacteriophages ϕ 3T and ρ 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'-TCCTTTGTAGAC-AATCGTACGATT-3', spanning residues -905 to -928 upstream of the *ggaA* start codon. The transcriptional starts (Fig. 3) were located on a DNA segment almost identical to the regulatory region of the strain W23 *tarD* gene (Lazarevic *et al.*, 2002a) (Fig. 1), and corresponded to a sequence differing from that of the σ^A -dependent promoter consensus (Helmann & Moran, 2002) 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 pAZ8102, which is a plasmid with the *ggaAB* promoter region inserted upstream of the *lacZ* gene, and selecting chloramphenicol-resistant and amylase-deficient mutants. β -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 OD₆₀₀ 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 *tag* thermosensitive mutants, we placed the *ggaAB* operon of strains L6601 *tagD11*, L6476 *tagB1* and L6477 *tagF1* under an IPTG-inducible P_{spac} promoter. The constructs obtained, as well as the reference thermoresistant strain L4674, were incubated in SAT2T medium supplemented with [1- 14 C]GlcNAc. For each strain, two cultures were grown: one with 100 μ g 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 *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 *tagD*, the structural gene encoding glycerol-3-phosphate cytidyltransferase (Pooley *et al.*, 1991), or *tagB*, encoding the enzyme putatively involved in the completion of the poly(GroP) LU, i.e. attachment of GroP to UPP-GlcNAc-ManNAc (Mauël *et al.*, 1991), allowed only residual synthesis of poly(GlcGalNAc 1-P). The incorporation of 14 C in *tagB*- or *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_{spac}-*ggaA tagF1* (not presented) revealed, in addition, that synthesis of poly(GlcGalNAc 1-P), expressed from the P_{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 [14 C]GalNAc, supplied as UDP-[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-[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 [14 C]GalNAc at a nearly steady rate, an incorporation that was attributed to

Table 1. Strains, plasmids and phages

Strain, plasmid or phage	Genotype or description	Reference or origin*
<i>E. coli</i>		
DH5 α	F ⁻ ϕ 80dlacZ Δ M15 Δ (argF-lacZYA)U169 <i>endA1 recA1</i> <i>hsdR17</i> (r _k ⁻ m _k ⁺) <i>deoR thi-1 supE44 λ⁻ gyrA96 relA1</i>	Hanahan (1983)
JM83	F ⁻ ϕ 80dlacZ Δ M15 Δ (lac-proAB) <i>ara rpsL</i>	Yanisch-Perron <i>et al.</i> (1985)
<i>B. subtilis</i>		
168	<i>trpC2</i>	Laboratory stock
W23	Prototroph Sm ^r	Laboratory stock
L5047	<i>pheA3 purA16 hisA35 trpC2 metB5</i>	Estrela <i>et al.</i> (1991)
L5054	<i>pheA3 purA16 hisA1 trpC2 gtaB515</i>	Estrela <i>et al.</i> (1991)
L5703	<i>metB5</i> hybrid W23/168, W23-like	Karamata <i>et al.</i> (1987)
L5705	<i>metB5</i> hybrid W23/168, W23-like	Karamata <i>et al.</i> (1987)
L5706	<i>metB5</i> hybrid W23/168, mixed-type	Karamata <i>et al.</i> (1987)
L5773	<i>hisA1 argC4</i> hybrid W23/168, mixed-type	Karamata <i>et al.</i> (1987)
L4660	<i>pheA1 purA16 hisA35 trpC2 metB5 ΔggaA</i>	Transformation of <i>gga</i> locus with p6363 $\rightarrow$ Cm ^r . Deletion of a part of <i>ggaA</i> by plasmid excision. Selection for ϕ 3T-resistance
L4661	<i>pheA3 purA16 hisA35 trpC2 metB5 P_{Em}-ggaB</i>	Campbell integration of p6315 in <i>ggaA</i> of L5047 $\rightarrow$ Cm ^r
L4664	<i>pheA3 purA16 hisA35 trpC2 metB5 ggaB::p6361</i>	Campbell integration of p6361 in <i>ggaB</i> of L5047 $\rightarrow$ Cm ^r
L4665	<i>pheA3 purA16 hisA35 trpC2 metB5 ggaB::p6362</i>	Campbell integration of p6362 in <i>ggaB</i> of L5047 $\rightarrow$ Cm ^r
L4670	<i>hisA1 argC4</i> hybrid W23/168, mixed-type <i>ggaB::p6362</i>	Campbell integration of p6362 in <i>ggaB</i> of L5773 $\rightarrow$ Cm ^r
L4671	<i>metB5</i> hybrid W23/168, mixed-type; <i>ggaB::p6362</i>	Campbell integration of p6362 in <i>ggaB</i> of L5706 $\rightarrow$ Cm ^r
L4673	<i>pheA3 purA16 hisA35 trpC2 metB5 Ω(amyE::cat P_{ggaA}-lacZ)</i>	Integration of the pAZ8102 insert in <i>amyE</i> of L5047 $\rightarrow$ Cm ^r
L4674	<i>pheA3 purA16 hisA35 trpC2 metB5 P_{spac}-ggaA</i>	Campbell integration of p6360 into L5047 $\rightarrow$ Km ^r
L4675	<i>hisA1 argC4 metC3 tagD11 P_{spac}-ggaA</i>	Campbell integration of p6360 into L6601 $\rightarrow$ Km ^r
L4676	<i>hisA1 argC4 metC3 tagB1 P_{spac}-ggaA</i>	Campbell integration of p6360 into L6476 $\rightarrow$ Km ^r
L4677	<i>hisA1 argC4 metC3 tagF1 P_{spac}-ggaA</i>	Campbell integration of p6360 into L6477 $\rightarrow$ Km ^r
L6601	<i>hisA1 argC4 metC3 tagD11</i>	Pooley <i>et al.</i> (1991)
L6476	<i>hisA1 argC4 metC3 tagB1</i>	Pooley <i>et al.</i> (1991)
L6477	<i>hisA1 argC4 metC3 tagF1</i>	Pooley <i>et al.</i> (1991)
Phages		
ϕ 3Tc	Clear-plaque mutant	Estrela <i>et al.</i> (1991)
ρ 11c	Clear-plaque mutant	Estrela <i>et al.</i> (1991)
Plasmids		
pDG268	Vector designed for insertion of transcriptional <i>lacZ</i> fusions into <i>amyE</i> , Ap ^r Cm ^r	Antoniewski <i>et al.</i> (1990)
pLP1C	Ap ^r Cm ^r	Longchamp <i>et al.</i> (1994)
pMTL20EC	Ap ^r Em ^r Cm ^r	Oultram <i>et al.</i> (1988)
pSGMU441	Ap ^r Km ^r ; P _{spac}	J. Errington, Sir William Dunn School of Pathology, Oxford, UK
p6310	Ap ^r Em ^r Cm ^r	Cloning of the 625 bp <i>SacI</i> - <i>BglII</i> (<i>ggaB</i> 1125 ⁺ - <i>ggaB</i> 1776) fragment in pMTL20EC
p6315	Ap ^r Em ^r Cm ^r , P _{Em} - <i>ggaB</i>	Cloning of the 1176 bp <i>BglII</i> - <i>EcoRI</i> (<i>ggaA</i> 253-87 downstream of <i>ggaA</i>) fragment in pMTL20EC
p6321	Ap ^r Em ^r Cm ^r	Cloning of the 840 bp <i>BamHI</i> - <i>SacI</i> (<i>ggaB</i> 291- <i>ggaB</i> 1130) fragment into pMTL20EC
p6356	Ap ^r Cm ^r	Cloning of the 752 bp <i>BglII</i> - <i>ScaI</i> (<i>ggaA</i> 253- <i>ggaA</i> 1004) into <i>SmaI</i> - <i>ScaI</i> of pLP1C
p6357	Ap ^r Cm ^r	Cloning of the 398 bp <i>ScaI</i> fragment (<i>ggaA</i> 999-55 downstream of <i>ggaA</i>) into <i>SmaI</i> site of pLP1C
p6360	Ap ^r Km ^r P _{spac} - <i>ggaA</i>	Cloning of the 1483 bp <i>BglII</i> (1225 upstream of <i>ggaA</i> - <i>ggaA</i> 258) fragment in pSGMU441

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 Δ ggaA (▲).

temperature (Fig. 5), and with the observed stimulating effect of CDP-Gro on WTA synthesis by protoplasts of strain W23 (Bertram *et al.*, 1981). However, it should be recalled that the most abundant WTA of W23 is poly(ribitol phosphate) [poly(RboP)], which contains GroP in the LU only, whereas in strain 168, CDP-Gro is overwhelmingly drained into poly(GroP) synthesis. Therefore, since the stimulating effect of CDP-Gro was observed with poly(RboP) synthesis (Bertram *et al.*, 1981), we resorted to hybrids between strains 168 and W23, in which the poly(GroP)-synthesizing genes

Fig. 3. Localization by primer extension of start site(s) of the *ggaAB* operon. The oligonucleotide 5'-TCCTTTGTAGACAA-TCGTACGATT-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 μ g 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 thermoresistant 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.

Fig. 6. Kinetics of incorporation of [1-¹⁴C]GalNAc, supplied as UDP-[1-¹⁴C]GalNAc, into protoplasts of 168-derived strains. Protoplasts obtained by lysozyme treatment at 30 °C were transferred into the assay mixture containing UDP-[1-¹⁴C]GalNAc, and incubated at 30 °C. Samples were withdrawn at regular intervals of time. The incorporated radioactivity was separated by paper chromatography, and counted. Reference strain L5047 (●); L4660 ΔggaA (▲); L4661 PEm-ggaB (◆); L5054 gtaB (■).

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 [1-¹⁴C]GalNAc from UDP-[1-¹⁴C]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 [1-¹⁴C]GalNAc from UDP-[1-¹⁴C]-GalNAc by protoplasts of strain L5054 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 [1-¹⁴C]GalNAc supplied as UDP-[1-¹⁴C]GalNAc by protoplasts of the 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 cytidyltransferase, and TagB, which most likely couples GroP to UPP-GlcNAc-ManNAc, shows that both ManNAc

Table 2. [1-¹⁴C]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-[1-¹⁴C]GalNAc was separated by paper chromatography, and counted. ND, Not determined.

Strain	Radioactivity obtained with specified compounds added to the assay mixture (d.p.m.)				
	UDP-Glc UDP-GalNAc	UDP-Glc UDP-GalNAc UDP-GlcNAc	UDP-Glc UDP-GalNAc CDP-Gro	UDP-Glc UDP-GalNAc UDP-GlcNAc CDP-Gro	UDP-Glc UDP-GalNAc UDP-GlcNAc CDP-Gro Tm
L5773 Mixed hybrid	262	249	262	2109	265
L5706 Mixed hybrid	297	268	336	2040	296
L4670 Mixed hybrid ggaB (L5773)	ND	ND	ND	231	ND
L4671 Mixed hybrid ggaB (L5706)	ND	ND	ND	185	ND
L5703 W23-like hybrid	ND	ND	ND	232	ND
L5705 W23-like hybrid	ND	ND	ND	208	ND
L5047	1717	2845	1889	1820	1077
L5054 gtaB	5127	4402	5259	4245	4077

Fig. 7. Proposed biosynthetic pathway for poly(GlcGalNAc 1-P). Modified from Lazarevic *et al.* (2002b).

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 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 exposal 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 P_{spac} 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 (Mauël *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).

ACKNOWLEDGEMENTS

This work was supported by grants from the Swiss National Science Foundation (93.0257 to D. K., and 3100A0-102205 to V. L.). We thank Harold M. Pooley (Department of Fundamental Microbiology, University of Lausanne, Switzerland) and François-Xavier Abellan (Biokema SA, Crissier, Switzerland) for communicating unpublished results.

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