TagO is involved in the synthesis of all anionic cell-wall polymers in Bacillus subtilis 168

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Sequence homologies suggest that the Bacillus subtilis 168 tagO gene encodes UDP-N-acetylglucosamine:undecaprenyl-P N-acetylglucosaminyl 1-P transferase, the enzyme responsible for catalysing the first step in the synthesis of the teichoic acid linkage unit, i.e. the formation of undecaprenyl-PP-N-acetylglucosamine. Inhibition of tagO expression mediated by an IPTG-inducible Pspac promoter led to the development of a coccoid cell morphology, a feature characteristic of mutants blocked in teichoic acid synthesis. Indeed, analyses of the cell-wall phosphate content, as well as the incorporation of radioactively labelled precursors, revealed that the synthesis of poly(glycerol phosphate) and poly(glucosyl N-acetylgalactosamine 1-phosphate), the two strain 168 teichoic acids known to share the same linkage unit, was affected. Surprisingly, under phosphate limitation, deficiency of TagO precludes the synthesis of teichuronic acid, which is normally induced under these conditions. The regulatory region of tagO, containing two partly overlapping σA-controlled promoters, is similar to that of sigA, the gene encoding the major σ factor responsible for growth. Here, the authors discuss the possibility that TagO may represent a pivotal element in the multi-enzyme complexes responsible for the synthesis of anionic cell-wall polymers, and that it may play one of the key roles in balanced cell growth.

Keywords: teichoic acid, teichuronic acid

INTRODUCTION

In addition to peptidoglycan, the cell wall of Bacillus subtilis 168 contains two teichoic acids (TAs), poly(glycerol phosphate) [poly(groP)], the major TA essential for cell growth (Mauel et al., 1989), and poly(glucosyl N-acetylglactosamine 1-phosphate) [poly(GlcGalNAcP)], the non-essential minor TA (Shibaev et al., 1973). These polymers, corresponding to up to 60% of the wall dry weight (Boylen & Ensign, 1968), are attached to the peptidoglycan through a linkage unit that is formed by successive transfer to the phosphorylated isoprenoid membrane lipid carrier of N-acetylglucosamine (GlcNAc) 1-phosphate, N-acetylmannosamine and most likely one glycerol phosphate residue (Yokoyama et al., 1989). The linkage unit is the acceptor for the polymerization of the main TA chain, which proceeds at its distal end (Archibald et al., 1993). Concomitantly with this process or, alternatively, following completion of polymerization, TAs are translocated through the membrane by an ABC transporter (Lazarevic & Karamata, 1995), and are finally attached to the peptidoglycan.

The genes identified so far that are known to be specifically involved in TA metabolism are clustered between 308° and 311° on the genetic map of B. subtilis 168 (Lazarevic et al., 1995; Kunst et al., 1997). They form the divergents tagAB–tagDEF (Mauel et al., 1991; Honeyman & Stewart, 1989) and mnaA(formerly orfX or yvyH)–gtaB (Soldo et al., 1993; Soldo et al., 2002), as well as the operons ggaAB (Freymond, 1995) and tagGH (Lazarevic & Karamata, 1995). Thermosensitive strains bearing mutations in the tagB, tagF or tagD genes, involved in the synthesis of poly(groP), are impaired in growth and exhibit a coccoid morphology at the non-permissive temperature (Rogers et al., 1989).
µ strains were grown in SPIZ I, SPIZ II, SA, LB and PL media, grown in LB medium containing, when appropriate, 50 µg chloramphenicol ml⁻¹, 3 µg kanamycin ml⁻¹, 5 µg kanamycin ml⁻¹ or 200 µg IPTG ml⁻¹.

**Transformation.** E. coli DH5α competent cells were prepared and transformed according to the procedure of Chung & Miller (1988). The transformation of B. subtilis competent cells was carried out as described previously (Karamata & Gross, 1970). Prior to plating onto LA plates containing 5 µg kanamycin ml⁻¹, the transformation mixture was incubated for an additional 90 min with a sub-lethal concentration (0.1 µg ml⁻¹) of the antibiotic.

**DNA preparation.** Plasmid DNA was prepared by the boiling method of Del Sal et al. (1988).

**Sequencing.** Both DNA strands of the EcoRI insert of plasmid p5504 were sequenced by primer walking and the dye-terminator chain termination method, using the Sequenase Version 2.0 Kit (USB) and [α-³²P]dATP (Amersham). Assembly and analysis of the sequence data were done by using the University of Wisconsin Computer Group software (Devereux et al., 1984).

**PCR conditions.** PCRs were set up in 100 µl of reaction buffer (Pharmacia Biotech) containing 2.5 U Tag DNA polymerase (Pharmacia Biotech), 1 ng of B. subtilis genomic DNA, 100 pmol of each primer and 20 nmol of each of the four dNTPs (Pharmacia Biotech). Reactions were run with denaturation at 95 °C for 2 min, followed by 30 cycles of amplification (95 °C for 30 s, 45 °C for 1 min, 72 °C for 1 min), with a final extension at 72 °C for 10 min.

**RNA isolation and primer-extension mapping.** RNA was isolated from B. subtilis 168 cells by using the RNeasy Total RNA Kit (Qiagen). The poly(A)⁺ RNA was isolated by using the Oligotex mRNA Kit (Qiagen). Reverse transcription (RT) reactions were carried out in 20 µl of reaction buffer containing 400 µM of each dNTP, 100 pmol of each primer and 2.U of reverse transcriptase (SuperScript II, Invitrogen). The RNA was reverse transcribed for 1 h at 37 °C and the cDNA was amplified by PCR using the same primers as for the expression experiments. The PCR products were resolved on 0.8% agarose gel in 0.5x TBE buffer and visualized by staining with ethidium bromide. The RNA bands were excised from the gel and digested with DNase I (Invitrogen) before being ligated into the pBluescript II KS⁺ plasmid vector (Stratagene) using the T4 DNA ligase (Roche). The plasmids were transformed into E. coli DH5α and the resulting colonies were screened by colony hybridization using a radiolabelled insert from the plasmid p5504.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** Escherichia coli strain DH5α (Hanahan, 1983) was used as the host for plasmid clones. The plasmids and B. subtilis strains used in this study are listed in Table 1. E. coli strains were routinely grown in LB medium containing, when appropriate, 50 µg ampicillin ml⁻¹ and 10 µg chloramphenicol ml⁻¹. B. subtilis strains were grown in SPIZ I, SPIZ II, SA, LB and PL media, as described previously (Karamata & Gross, 1970; Grant, 1979). When required, media were supplemented with 20 µg tryptophan ml⁻¹, 3 µg chloramphenicol ml⁻¹, 5 µg kanamycin ml⁻¹.

**Table 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant markers</th>
<th>Origin/construction</th>
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<tr>
<td><strong>B. subtilis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>168</td>
<td>trpC2</td>
<td>Anagnostopoulos &amp; Spiizizen (1961)</td>
</tr>
<tr>
<td>L16055</td>
<td>trpC2 tagOAp5525 (P₃p₃−tagO)</td>
<td>Integration of p5525 containing P₃p₃ into tagO of strain 168</td>
</tr>
<tr>
<td>L16058</td>
<td>trpC2 tagOApPS008 (tagO–lacZ)</td>
<td>Integration of pPS008 into tagO of strain 168</td>
</tr>
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<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCR941</td>
<td>Ap⁺ Cm⁻; lacZ</td>
<td>C. Rivolta, University of Pavia, Italy</td>
</tr>
<tr>
<td>pMTL20EC</td>
<td>Ap⁺ Em⁺ Cm⁻</td>
<td>Chambers et al. (1988)</td>
</tr>
<tr>
<td>pSGMU441</td>
<td>Km⁻ Ap⁺; P₃p₃</td>
<td>J. Errington, Sir William Dunn School of Pathology, Oxford, UK</td>
</tr>
<tr>
<td>pBluescript II KS⁺</td>
<td>Ap⁺</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pWD1</td>
<td>Cm⁻</td>
<td>H. Wood, Trinity College, Dublin, Ireland</td>
</tr>
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<td>pVL23</td>
<td>Ap⁺ Cm⁻</td>
<td>Cloning of the ~1-kb EcoRI (cat) fragment of pWD1 into pBluescript II KS⁺</td>
</tr>
<tr>
<td>pPS008</td>
<td>Ap⁺ Cm⁻; tagO–lacZ</td>
<td>Cloning of the B. subtilis 1592 bp EcoRI–Stul segment into EcoRI–PmeI of pCR941</td>
</tr>
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<td>p5504</td>
<td>Ap⁺ Em⁺ Cm⁻</td>
<td>Cloning of the B. subtilis 2068 bp EcoRI segment into pMTL20EC</td>
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<td>p5514</td>
<td>Ap⁺</td>
<td>Cloning of the EcoRI insert of p5504 into pBluescript II KS⁺</td>
</tr>
<tr>
<td>p5515</td>
<td>Ap⁺</td>
<td>Cloning of the ~1.1 kb EcoRV–Smal (cat) fragment of pVL23 into Stul site of the EcoRI insert of plasmid p5514</td>
</tr>
<tr>
<td>p5525</td>
<td>Km⁻ Ap⁺</td>
<td>Segment (577 bp; positions 160–736) amplified from B. subtilis 168 chromosomal DNA using oligonucleotides BS096 (5’TTCGCCGCGAGACTCATCTTTAT TCCCCGAGACC-3’) and BS097 (5’-GGCGATGCATAGCGCCATGACAG CAATCGTGACA-3’). The PCR product was digested with Sphi and Smal (sites shown in bold), and cloned into the same sites of pSGMU441</td>
</tr>
<tr>
<td>p5530</td>
<td>Ap⁺ Em⁺ Cm⁻</td>
<td>Cloning of the Sphi–Smal insert of p5525 into pMTL20EC</td>
</tr>
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</table>
RNA Kit (QiaGen). Labelling of the 5'-end of the oligonucleotide with [γ-32P]ATP and cDNA synthesis were performed with the Primer Extension System (Promega). One picomole of the 5'-end labelled oligonucleotide VL307 (5'-GTTATGATTAAAACCTGTCCAGCCGCA3') was incubated with 20 μg of total RNA at 95 °C for 1 min, at 55 °C for 2 min and then on ice for 15 min. cDNA products were analysed on a 6% (w/v) denaturing polyacrylamide gel, alongside the sequencing ladder.

Enzyme assay. β-Galactosidase activity assays were performed according to Nicholson & Setlow (1990). Cells were grown in LB medium and then pelleted for 2 min in a microcentrifuge. The pellets (from 0.3 to 1 ml samples) were resuspended in 100 μl of Z buffer (Miller, 1972). Toluene (10 μl) was added to the suspension; the suspension was vortexed for 15 s and then transferred to 900 μl of Z buffer containing 0.89 μg ONPG ml⁻¹, which had been pre-equilibrated for 5 min at 28 °C. For cultures grown in PL medium (Grant, 1979), 100 μl aliquots were toluene-treated and transferred to 900 μl of ONPG-supplemented Z buffer. Reactions were stopped with 400 μl of 1 M Na₂CO₃; the samples were then centrifuged. The A₄₂₀ of the supernatant was read against the reaction that had been stopped at time zero. The activity of the supernatants is expressed in units calculated according to the formula A₄₂₀ × 1000/[reaction time (min) × OD₅₉₅ of culture].

Cell-wall preparation. Cell walls were prepared as previously described (Soldo et al., 1999).

Estimation of cell-wall phosphate. An aliquot of the culture that had been grown at 37 °C in SA medium supplemented with 20 μg tryptophan ml⁻¹, 5 μg kanamycin ml⁻¹ and 200 μg IPTG ml⁻¹ was centrifuged, washed with the IPTG-free medium and resuspended in either fresh IPTG-free medium or in IPTG-containing medium to an OD₅₉₅ of 0.020. Cell walls were isolated from the cultures when OD₅₉₅ 0.75 was reached. Lyophilized cell walls were mineralized (Ames, 1966), and the phosphate concentration was determined according to Chen et al. (1956).

Estimation of cell-wall uronate. Exponentially growing cells in PL medium supplemented with IPTG were washed and transferred to fresh medium with or without IPTG to an OD₅₉₅ of 0.1. Walls were isolated from phosphate-starved cells collected 5 h after growth had slowed down. Uronic acid was determined as described by Blumenkrantz & Asboe-Hansen (1973).

Labelling of cell-wall polymers with [2-3H]glycerol and [1-14C]GlcNAc. Cells were grown at 37 °C in SA medium supplemented with 20 μg tryptophan ml⁻¹, 5 μg kanamycin ml⁻¹, 100 μM GlcNAc, 5 mM glycerol, 1.5 mM MgCl₂ and 200 μg IPTG ml⁻¹. At an OD₅₉₅ of 0.3, a sample from the exponentially grown culture was washed with the IPTG-free medium and diluted in fresh medium with or without IPTG to an OD₅₉₅ of 0.060. [1-14C]GlcNAc or [2-3H]glycerol were added at a final concentration of 0.2 mCi ml⁻¹ (7.4 MBq ml⁻¹) and 1 mCi ml⁻¹ (37 MBq ml⁻¹), respectively, and incubation was continued until OD₅₉₅ 0.300 was reached. To determine the incorporation of radioactive glycerol into the cell-wall fraction, 2 ml samples were washed and resuspended in TMS/lysozyme buffer [1 M sucrose, 50 mM Tris/HCl (pH 8), 8 mM MgCl₂, 200 μg lysozyme ml⁻¹]. After the conversion of over 95% of the cells into protoplasts (which generally required up to 30 min incubation at 37 °C), the protoplasts were sedimented and the radioactivity of the supernatant, corresponding to that released by wall digestion (Pooley & Karamata, 2000), was counted. The incorporation of [1-14C]GlcNAc was measured in 1 ml samples, which were washed, boiled for 5 min in 0.1 M phosphate buffer (pH 7) and then centrifuged. To selectively extract the N-acetylgalactosamine-containing polymer (Estrela et al., 1991), pellets were resuspended in 1 ml of 0.1 M sodium citrate buffer (pH 4), incubated for 30 min at 100 °C and then centrifuged. The radioactivity present in the supernatants, corresponding to poly(GlcGalNAcP), was counted. The pellets, containing essentially radioactive hexosamine incorporated into the peptidoglycan, were resuspended in 1 ml of water and their radioactivity was counted. Radioactivity was always determined by scintillation counting in 10 ml of Optifluor (Packard).

RESULTS

Nucleotide sequence

Sequence analysis of the 1.3 kb segment located immediately downstream of tuaABCDEFGH, the teichuronic acid operon (Soldo et al., 1999), reveals an ORF designated tagO (Fig. 1), followed by a transcriptional terminator adjacent to the terminator of ywhKJ, a convergently transcribed operon of unknown function (Soldo et al., 1996). The potential initiation codon of tagO, ATG, is preceded at an appropriate distance by a sequence complementary to the 5' end of 16S rRNA (Fig. 1). The deduced protein, TagO, corresponds to a 358 residue hydrophobic protein with 11 putative transmembrane domains (Sonnhammer et al., 1998). Screening of the sequence database revealed 26% identity between TagO and Rfe, the E. coli protein endowed with the UDP-N-acetylgalactosamine:undecaprenyl-P > N-acetylgalcosaminyl 1-P transferase (Meier-Dieter et al., 1992). When compared to a series of proteins from different bacterial species thought to have the same enzymic activity (not shown), TagO exhibited the highest similarity (44% identity) with the product of the Staphylococcus aureus llm gene, which is known to affect the level of methodillin resistance as well as the autolysis rate in S. aureus (Maki et al., 1994). To a somewhat lesser extent, TagO is similar to UDP-N-acetylmuramoyl-pentapeptide:undecaprenyl-P phospho-N-acetyl-D-glucosaminyl 1-P transferase (not shown), the enzymes which catalyse the initial step of peptidoglycan synthesis. However, mraY, the unique tagO parologue in the B. subtilis 168 chromosome (Kunst et al., 1997), was shown to encode the latter enzymic activity (Daniel & Errington, 1993). Therefore, it is most likely that TagO does indeed catalyse the formation of undecaprenyl-PP-GlcNAc and UMP from undecaprenyl-P and UDP-GlcNAc, a reaction known to be inhibited by tunicamycin (Hancock et al., 1976).

Expression of the tagO operon

Expression of tagO in phosphate-replete as well as in low-phosphate media (i.e. LB and PL, respectively) was examined by following the β-galactosidase activity in L16058, a strain carrying a transcriptional tagO-lacZ fusion. The transcription profile in LB medium (Fig. 2) reveals a constant level of expression until the mid-
exponential phase of growth, followed, after a short transition, by a slightly but significantly higher level of expression throughout the late-exponential and stationary phases of growth. During the transition period, a peak corresponding to a twofold increase in tagO expression was observed in four independent experiments. In PL medium, the rate of tagO transcription is nearly constant during the several hours following phosphate exhaustion (Fig. 2), a behaviour intermediary between Pho-P regulated tag and tua operons (Liu & Hulett, 1998; Liu et al., 1998). Indeed, whereas the expression of the TA operons tagAB and tagDE is turned off during phosphate starvation (Mauël et al., 1994), the expression of the tuaABCDEFGH operon is hyperinduced (Soldo et al., 1999).

The transcriptional initiation sites of the tagO gene were located by primer-extension experiments. A 26-mer oligonucleotide complementary to the potential transcript between nucleotides +46 and +71, numbered from the start codon of the tagO gene, was annealed to total cellular RNA and extended with the AMV reverse transcriptase. Results obtained with RNA extracted from cells at OD\text{595} 0·3 (not shown) or OD\text{595} 0·7 were

Fig. 1. Genetic organization of the DNA segment containing tagO and the nucleotide sequence of the tagO regulatory region. Inserts of plasmids used for sequencing and integration into the B. subtilis chromosome are represented by bold lines. Arrows correspond to putative genes. Stem–loop structures indicate putative transcriptional terminators. EcoRI and StuI restriction sites are indicated. Repeated pentamer and octamer motifs are underlined and dot-underlined, respectively. Putative RBSs are double-underlined. Asterisks denote stop codons. Regions of dyad symmetry are shown by converging arrowheads. Promoter regions (−10 and −35) are boxed. The transcriptional starts of the upstream (P1) and downstream (P2) promoters are denoted by solid triangles.

Fig. 2. Growth curves for strain L16058, carrying the tagO–lacZ fusion, when grown in phosphate-rich (LB medium; left graph) and phosphate-limited (PL medium; right graph) media. ■, OD\text{595}; ●, β-galactosidase activity. The means for two samples are given.
distance, both of the transcriptional start sites are preceded by a perfectly conserved −10 region (TATAAT) of promoters that are recognized by the RNA polymerase containing σ^A, the major σ factor of *B. subtilis*. The downstream promoter P2, corresponding to the stronger signal, has a reasonable −35-like sequence (TTGgtA) that is separated from the −10 region by 18 nt. In the upstream P1 promoter, a questionable −35 region (gTGrrt) is also separated from the −10 region by an 18 nt spacer which contains a TG dinucleotide at −15/−14, a characteristic common to many *B. subtilis* σ^A-dependent promoters lacking a conserved −35 region (Helmann, 1995). Interestingly, the −35-like region of both promoters is preceded at a distance of 5 nt by the octamer motif TTTTTGAT (Fig. 1).

The biological role of tagO

Sequence analysis strongly suggests that TagO, the putative N-acetylglucosaminyl 1-P transferase, mediates the first step in the synthesis of the TA linkage unit. To investigate if tagO, like other tag genes involved in the synthesis of the major TA [i.e. *tagA*, *tagB*, *tagD*, *tagF*, *tagG*, *tagH* and *mnaA(yvyH)*] (Mauël et al., 1989; Honeyman & Stewart, 1989; Soldo et al., 1993; Lazarevic & Karamata, 1995; Soldo et al., 2002]), is essential for cell growth, we have attempted to inactivate it by gene disruption.

Insertion of the chloramphenicol-resistance cassette (cat) gene from linearized p5515 into the *tagO StuI* site or a Campbell-type integration of p5530 (Fig. 1) did not yield viable recombinants. Thus, recombination events generating truncated TagO proteins containing 233 and 182 N-terminal residues, respectively, were apparently lethal. This observation is compatible with the proposed function of TagO in the synthesis of the TA linkage unit.

To control the level of *tagO* expression and to study the phenotype of conditional *tagO* deficient strains, the operon was placed under the control of a P_spac promoter (Yansura & Henner, 1984). A DNA fragment containing the proximal part of *tagO* and its RBS was cloned into vector pSGMU441; the resulting plasmid, p5525, was integrated into the chromosome. Proper plasmid integration into the selected kanamycin-resistant recombinant (L16055) was confirmed by PCR (not shown).

Strain L16055 harbours a truncated copy of *tagO* which is under the control of its native promoter, as well as a complete *tagO* gene whose expression depends on the vector-borne IPTG-inducible P_spac promoter. In the absence of IPTG, the LacI protein represses transcription from the P_spac promoter. Following cell transfer from IPTG-containing to IPTG-free medium, typical rods are progressively converted into aggregates of coccoid cells (Fig. 4), a morphology characteristic of mutants deficient in TA synthesis. The implication of this result – that TA metabolism is impaired by reduced *tagO* expression – was confirmed by determining the amounts of phosphate, glycerol and N-acetylglucosamine [the cell-wall compounds which correspond to poly(groP) and

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**Fig. 3.** Determination of the sequence of the 5‘ end of *tagO* mRNA. The 5‘-end-labelled oligonucleotide was used to prime DNA sequencing reactions on p5514 (lanes G, A, T and C) and extension reactions with reverse transcriptase and 20 µg of RNA isolated from the *B. subtilis* 168 cells grown to an OD_595 of 0.7 (lane E). Products were resolved by electrophoresis on a 6% polyacrylamide gel. The upstream (P1) and downstream (P2) transcriptional starts are indicated.

**Fig. 4.** Cell morphology of strain L16055 after 24 h incubation on LA plates containing 200 µg IPTG ml⁻¹ (A) and no IPTG (B).
Table 2. Effect of reduced tagO expression in strain L16055 (Pspac–tagO) on the amount of anionic cell-wall polymer compounds

<table>
<thead>
<tr>
<th>IPTG (µg ml⁻¹)</th>
<th>Expt</th>
<th>Phosphate* [mmol (g cell wall)⁻¹]</th>
<th>Incorporation of:</th>
<th>Uronate‡ [mmol (g cell wall)⁻¹]</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>[²⁻³H]glycerol into poly[gluP]†</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>[¹⁴C]GlcNAc into poly[GlcGalNAcP]†</td>
<td>[¹⁴C]GlcNAc into peptidoglycan†</td>
</tr>
<tr>
<td>200</td>
<td>I</td>
<td>1·02 ± 0·01‡</td>
<td>25·1 ± 0·5</td>
<td>2·63 ± 0·09</td>
</tr>
<tr>
<td></td>
<td>II</td>
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<td>1·26 ± 0·01</td>
<td>26·8 ± 0·5</td>
</tr>
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<td>0</td>
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<td>0·11 ± 0·01</td>
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<td></td>
<td>II</td>
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<td>1·11 ± 0·02</td>
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</tr>
<tr>
<td>R</td>
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<td>0·61</td>
<td>0·48</td>
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</tr>
<tr>
<td></td>
<td>II</td>
<td>0·60</td>
<td>0·51</td>
<td>0·96</td>
</tr>
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</table>

* Growth in supplemented SA medium from OD₅₅₀ of 0·02 to 0·75. The phosphate content of the purified and mineralized cell walls was determined.
† Two independent experiments, designated I and II, were performed. Cells were labelled with [¹⁴C]GlcNAc or [²⁻³H]glycerol during growth in supplemented SA medium from OD₅₅₀ of 0·06 to 3·3 (2·25 generations). During this period, culture growth, measured as OD₅₅₀, was identical in IPTG-supplemented and in IPTG-free medium.
‡ Growth in PL medium starting from OD₅₅₀ 0·1. Samples were collected 5 h after the onset of teichuronic acid synthesis, which coincided with growth slowing down. Final OD₅₅₀ of the IPTG-supplemented and the IPTG-free culture was 1·05 and 1·11, respectively.
§ Means ± SD for two samples are given.
∥ Value obtained for IPTG-uninduced cells (0 µg IPTG ml⁻¹) divided by the value obtained for the IPTG-induced cells (200 µg IPTG ml⁻¹).

poly[GlcpNACP] present in the cell walls of the mutant and wild-type strains (Table 2). In the presence of IPTG, the cell-wall phosphate of the mutant strain L16055 (Pspac–tagO) was close to 0·95 mmol (g cell wall)⁻¹, the value obtained with strain 168. However, in the IPTG-free medium, the amount of cell-wall phosphate in strain L16055 cells was drastically reduced when compared to that of cells grown in the IPTG-supplemented culture and collected at the same optical density (Table 2). During a relatively limited labelling period, i.e. 2·25 generations after IPTG depletion, the incorporation of [¹⁴C]GlcNAc and of [²⁻³H]glycerol into the cell-wall fraction was significantly reduced. The reduced level of cell-wall glycerol is in agreement with an impaired incorporation of the major TA, whereas low levels of acid-extractable hexosamine reflect a deficiency in the synthesis of the secondary polymer. These observations are compatible with the involvement of TagO in the synthesis of the linkage unit that appears to be common to poly[gluP] and poly[GlcpNACP] (Pooley & Karamata, 1994; Frymond, 1995; Lazarevic & Karamata, 1995). Incidentally, the reduced level of incorporation of TA radioactive precursors due to tagO repression parallels the reduction in incorporation obtained upon the addition of tunicamycin to the growth medium (Pooley & Karamata, 2000). Tunicamycin is an antibiotic whose precise target is the reaction involved in the formation of undecaprenyl-PP-GlcNAc (Hancock et al., 1976), the reaction most likely mediated by TagO. Impaired tagO expression during labelling with [¹⁴C]GlcNAc was accompanied by a slight reduction in the radioactivity count in the cell-wall fraction that was non-extractable at pH 4, i.e. the fraction which essentially corresponds to peptidoglycan. However, this reduction is probably due to the absence of the TA linkage unit. Indeed, under our experimental conditions (mild acid treatment of the cell wall) the linkage unit, when synthesized, remains attached to the cell wall and, due to labelled GlcNAc and N-acetylmannosamine, contributes to the overall radioactivity count.

To investigate if TagO, which is expressed under phosphate limitation (see above), participates in the synthesis of teichuronic acid, strain L16055 was cultured in PL, the low-phosphate medium, with or without the inducer. The exhaustion of phosphate from this medium slows down growth and, in wild-type cells, induces replacement of the cell-wall TA by the phosphate-free teichuronic acid (Grant, 1979). It appeared that the amount of uronate in the cell walls of strain L16055 was considerably lower in the absence of IPTG, strongly suggesting the involvement of TagO in teichuronic acid synthesis. This is a rather unexpected observation, since the putative enzymic reaction catalysed by TagO is not known to be involved in the synthetic pathway of poly(gluconoyl N-acetylgalactosamine), the supposed teichuronic acid of B. subtilis 168 (see below). However, in the presence of the inducer, the amount of cell-wall uronate (g cell wall)⁻¹ was significantly below 0·502, the value obtained with the parent strain 168 (Soldo et al., 1999). Therefore, in contrast to the situation with the cell-wall phosphate in cells grown in phosphate-rich medium, IPTG did not restore the wild-type cell-wall uronate levels in strain L16055 during phosphate starvation. This may be due to some adverse effect of the truncated copy of tagO generated by p5525 integration, or it may be due to deregulated expression of tagO by Pspac. 
**DISCUSSION**

The involvement of *tagO* in the synthesis of all *B. subtilis* 168 anionic polymers, teichoic as well as teichuronic acid, on one side, and the complex regulation of its expression, on the other side, suggest that *tagO* plays one of the central roles in balanced cell growth.

Although the precise enzymic activity of TagO has not been confirmed by an assay, nucleotide sequence homologies strongly suggest that *tagO* encodes a UDP-N-acetylglucosamine:undecaprenyl-P N-acetylglucosaminyl 1-P transferase, the enzyme that mediates the first step in the synthesis of the TA linkage unit. This is in full agreement with the observation that interference in the expression of *tagO* can impair the synthesis of poly(groP) as well as poly(GlcGalNAcP), since both polymers have a common linkage unit (Freymond, 1995; Pooley & Karamata, 1994). The absence of *tagO* expression in phosphate-depleted conditions, in which teichuronic acid normally replaces TA, is accompanied by inhibition of uronate incorporation, which is less severe but nevertheless comparable to the reduction of phosphate incorporation into TA in the absence of *tagO* expression (Table 1). This unexpected result, revealing an involvement of TagO in teichuronic acid synthesis, implies (Soldo *et al.*, 1999) that, in *B. subtilis* 168, teichuronic acid either has a more complex chemical composition than previously reported (Wright & Heckels, 1975) or that it is hooked to peptidoglycan through a linkage unit, a situation observed in *Micrococcus luteus* (Araki & Ito, 1989). Unlike the TA genes *tagA*, *tagB*, *tagD*, *tagE* and *tagF*, whose expression is turned off by phosphate starvation (Mauël *et al.*, 1994), *tagO* is efficiently transcribed for several hours following phosphate exhaustion (Fig. 2), formally in agreement with the possibility that *tagO* is involved in teichuronic acid synthesis. Incidentally, in phosphate-depleted conditions, the replacement of TAs by teichuronic acids is at least partly mediated by PhoP/R, a two-component regulatory system (Seki *et al.*, 1988). The binding of PhoP to the Pho boxes in the *tagA–tagD* and *tuaA* regulatory regions results in repression of the relevant TA genes (Liu *et al.*, 1998) and induction of the teichuronic acid operon genes (Liu & Hulett, 1998). Inspection of the *tagO* regulatory region did not reveal repeats of TTAACA-like motifs corresponding to the *B. subtilis* Pho box (Liu & Hulett, 1998). Therefore, the balance between TA and teichuronic acid does not seem to include PhoP binding to the *tagO* regulatory region. This is in agreement with the expression pattern of *tagO*, which is not significantly altered by phosphate exhaustion, as well as with the involvement of TagO in the synthesis of both phosphate-containing and phosphate-free anionic cell-wall polymers.

Sequence analysis and primer-extension experiments revealed that the expression of the *tagO* operon takes place from a regulatory region organized in a way similar to the regulatory region that controls the expression of the structural gene of the σ^A^ factor.

Indeed, several features of the *tagO* regulatory region resemble those of the three-gene *sigA* (*rpoD*) operon (Wang & Doi, 1987): (i) the −10 region of P1 overlaps the −35 region of P2 by 2 nt, (ii) P2 is a much stronger promoter than P1, and (iii) the relevant transcriptional starts lie within short direct repeats, which are, in the case of *tagO*, represented by the AAGGA pentamer. Overlapping promoters and direct repeats could also contribute to a complex regulation of *tagO* expression, probably in response to the cell growth rate. To our knowledge, TagO is the first example of a gene apparently co-regulated with *sigA*, a gene whose product is intimately associated with cell growth.

Should chemical analyses confirm the presence of GlcNAc in a putative teichuronic acid linkage unit, the pivotal structural role, as well as the regulatory role, of TagO in the balanced growth of *B. subtilis* would appear very likely. For instance, TagO, a membrane-anchored protein, may have the potential to associate itself with either TagG and TagH, the ABC transporter for TA (Lazarevic & Karamata, 1995), or with TuaB, the transmembrane protein possibly responsible for the transloca(s) related to teichuronic acid synthesis (Soldo *et al.*, 1999). Assembling the other, relevant, *tag* or *tua* gene products around their respective pivotal blocks would generate multi-enzyme complexes responsible for anionic polymer synthesis. Analogies at the level of their regulatory regions would suggest that, like σ^A^, TagO too is not dissociable from balanced cell growth which, as clearly shown, cannot take place without the concomitant synthesis of anionic polymers (Mauël *et al.*, 1989). By controlling the very first step of anionic polymer synthesis, *tagO* would be the rate-limiting factor in this synthetic process in *B. subtilis* 168. The significant homology between the sequences of TagO and MraY, a protein capable of recognizing the peptidoglycan subunit, leaves open the possibility that TagO may also take part in the hooking of complete TA chains to peptidoglycan, a reaction for which no candidate enzyme has so far been identified.

**ACKNOWLEDGEMENTS**

This work was supported by Grant 96.0245 from the Office Fédéral de l’Education et de la Science (OFES), Switzerland.

**REFERENCES**


Bacillus subtilis tagO gene


Received 12 December 2001; revised 6 February 2002; accepted 20 March 2002.