Analysis of \textit{Bacillus subtilis} \textit{tag} gene expression using transcriptional fusions

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Five of the genes known to encode the synthesis of poly(glycerol phosphate), the major teichoic acid of \textit{Bacillus subtilis} 168, are organized in two divergently transcribed operons (a divergon), denoted \textit{tag}ABC and \textit{tag}DEF. To monitor their expression, the 399 bp intergenic region separating the first structural genes of these operons was fused, in both orientations, to a \textit{lacZ} reporter gene, allowing measurement of promoter activity under specific physiological conditions. Under all experimental conditions, \textit{tag}A and \textit{tag}D appeared coordinately expressed, the level of \textit{tag}D being always higher than that of \textit{tag}A. No influence of the chromosomal context was observed. Phosphate limitation was accompanied by reduced \textit{tag} gene expression. Following the onset of sporulation, expression of \textit{tag} genes diminished rapidly and was essentially abolished by stage II. During germination, the activity of \textit{tag} genes was detectable before the rise in culture turbidity associated with spore outgrowth. In contrast to \textit{tagC} (\textit{dinC}), the expression of which is DNA-damage-inducible, the induction of \textit{SOS} functions had no effect on \textit{tagA} and \textit{tagD} gene expression. The biological significance of these results is discussed.

\textbf{Keywords:} \textit{Bacillus subtilis}, teichoic acids, poly(ribitol phosphate), \textit{tag} gene expression, \textit{lacZ} transcriptional fusions

INTRODUCTION

Poly(glycerol phosphate) [poly(groP)], the major cell wall teichoic acid of \textit{Bacillus subtilis} 168 (Baddiley, 1970), has been shown to be essential for growth (Karamata \textit{et al}., 1987; Mauel \textit{et al}., 1989). However, presently, the precise role(s) of teichoic acids is not understood. So far, nine genes apparently concerned with glucosylated poly(groP) synthesis have been sequenced (Honeyman & Stewart, 1989; Mauel \textit{et al}., 1991; Soldo \textit{et al}., 1993; Lazarevic & Karamata, 1993). It was proposed (Mauel \textit{et al}., 1991) that six of them are organized in two divergently transcribed operons (a divergon) denoted \textit{tag}ABC and \textit{tag}DEF, separated by a 399 bp intergenic region. Subsequently, it appeared (V. Lazarevic, unpublished) that \textit{tag}C corresponds to \textit{dinC} (Cheo \textit{et al}., 1991) and thus belongs to the \textit{SOS} regulon. Its function, if any, in poly(groP) synthesis remains unknown. Expression of \textit{tag} genes is likely to be subject to an elaborate control mechanism. First, there is evidence of an interdependence between teichoic acid and peptidoglycan synthesis (Ward, 1981), as well as between poly(groP) and polyglucose galactosamine phosphate, a so-called secondary teichoic acid (Shibaev \textit{et al}., 1973; Rosenberger, 1976; Estrella \textit{et al}., 1991). Second, under phosphate-limitation, teichoic acid is replaced by teichuronic acid, a phosphate-free anionic polymer (Janczura \textit{et al}., 1961; Ellwood & Tempest, 1972). Finally, in contrast to vegetative cells, spores of \textit{B. subtilis} strain W23 were shown not to contain teichoic acid (Chin \textit{et al}., 1968).

We report here the construction of different transcriptional fusions between reporter genes and the regulatory, \textit{tagA}–\textit{tagD}, intergenic region. They were exploited to determine \textit{tag} gene expression under a variety of conditions: (i) chromosomal location, (ii) phosphate limitation, (iii) sporulation and germination, and (iv) damage to cell DNA.

\textbf{METHODS}

\textbf{Bacterial strains and plasmids.} These are listed in Table 1.

\textbf{Growth and maintenance of strains.} \textit{Escherichia coli} cells harbouring plasmids were grown in LB medium (Miller, 1972),

\textbf{Abbreviations:} MMC, mitomycin C; poly(groP), poly(glycerol phosphate).
Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source/reference</th>
</tr>
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<tbody>
<tr>
<td>Escherichia coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F- endA1 hisR17(i-cm') supE44 thi-1 recA1 gyrA96(NalR) relA1 Δ(argF-lacZYA)U169 ø80d1lacZAM15 deoR</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td>JM83</td>
<td>F' ara Δ(lac-proAB) rpsL ø80d1lacZAM15</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>168</td>
<td>trpC2</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>SG64</td>
<td>trpC2 lacA17 lacR1</td>
<td>Errington &amp; Vogt (1990)</td>
</tr>
<tr>
<td>L4532</td>
<td>trpC2 tag&quot;TpDZ5326 (tagD::lacZ)</td>
<td>Transformation of 168 with pDZ5326 → CmR</td>
</tr>
<tr>
<td>L4561</td>
<td>trpC2 ampyEΩpTAG101 (tagA::lacZ, tagD::xylE)</td>
<td>Transformation of 168 with SmaBI-linearized pTAG101 → CmR Amy-</td>
</tr>
<tr>
<td>L4562</td>
<td>trpC2 ampyEΩpTAG110 (tagD::lacZ, tagA::xylE)</td>
<td>Transformation of 168 with SmaBI-linearized pTAG110 → CmR Amy-</td>
</tr>
<tr>
<td>L4563</td>
<td>trpC2 ampyEΩpTAG102 (tagA::lacZ, tagD::xylE)</td>
<td>Transformation of 168 with SmaBI-linearized pTAG102 → CmR Amy-</td>
</tr>
<tr>
<td>L4564</td>
<td>trpC2 ampyEΩpTAG103 (tagD::lacZ, tagA::xylE)</td>
<td>Transformation of 168 with SmaBI-linearized pTAG103 → CmR Amy-</td>
</tr>
<tr>
<td>L4565</td>
<td>trpC2 tag&quot;QTAG101 (tagA::lacZ, tagD::xylE)</td>
<td>Transformation of 168 with pTAG101 → CmR</td>
</tr>
<tr>
<td>L4566</td>
<td>trpC2 tag&quot;QTAG110 (tagA::lacZ, tagD::xylE)</td>
<td>Transformation of 168 with pTAG110 → CmR</td>
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<tr>
<td>L4572</td>
<td>hisA1 argC4 xin-15</td>
<td>A. Grecescu-Monsurt, University of Lausanne</td>
</tr>
<tr>
<td>L4578</td>
<td>hisA1 argC4 xin-15 tagC::lacZs</td>
<td>Transformation of L4572 with Sacl-linearized pAG8 → CmR</td>
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<tr>
<td>L4581</td>
<td>trpC2 tag&quot;ΩpAZ5335 (tagA::lacZ)</td>
<td>Transformation of 168 with pAZ5335 → CmR</td>
</tr>
<tr>
<td>L4582</td>
<td>trpC2 tag&quot;ΩpDZ5336 (tagD::lacZ)</td>
<td>Transformation of 168 with pAZ5336 → CmR</td>
</tr>
<tr>
<td>L4583</td>
<td>trpC2 ampyEΩpAZ5335 (tagA::lacZ)</td>
<td>Transformation of 168 with SmaBI-linearized pAZ5335 → CmR Amy-</td>
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<tr>
<td>L4584</td>
<td>trpC2 ampyEΩpDZ5336 (tagD::lacZ)</td>
<td>Transformation of 168 with SmaBI-linearized pDZ5336 → CmR Amy-</td>
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<tr>
<td>L4609</td>
<td>hisA1 argC4 metC3 tag&quot;ΩpAZ5335 (tagA::lacZ)</td>
<td>Transformation of L5087 with L4581 → CmR</td>
</tr>
<tr>
<td>L4610</td>
<td>hisA1 argC4 xin-15 tag&quot;ΩpAZ5335 (tagA::lacZ)</td>
<td>Transformation of L4572 with L4581 → CmR</td>
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<tr>
<td>L4611</td>
<td>hisA1 argC4 tag&quot;ΩpDZ5336 (tagD::lacZ)</td>
<td>Transformation of L5087 with L4582 → CmR</td>
</tr>
<tr>
<td>L4612</td>
<td>hisA1 argC4 xin-15 tag&quot;ΩpDZ5336 (tagD::lacZ)</td>
<td>Transformation of L5087 with L4582 → CmR</td>
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<tr>
<td>L4642</td>
<td>trpC2 ampyEΩpAZ5365 (tagA::lacZ)</td>
<td>Transformation of 168 with SmaBI-linearized pDZ5365 → CmR Amy-</td>
</tr>
<tr>
<td>L4643</td>
<td>trpC2 ampyEΩpDZ5366 (tagD::lacZ)</td>
<td>Transformation of 168 with SmaBI-linearized pDZ5366 → CmR Amy-</td>
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<tr>
<td>L5087</td>
<td>hisA1 argC4 metC3</td>
<td>Briehl et al. (1989)</td>
</tr>
<tr>
<td>SA355</td>
<td>trpC2 lacR1 lacA17 ampyEΩpTAG102 (tagA::lacZ, tagD::xylE)</td>
<td>Transformation of SG64 with SmaBI-linearized pTAG102 → CmR Amy-</td>
</tr>
<tr>
<td>SA356</td>
<td>trpC2 lacR1 lacA17 ampyEΩpTAG103 (tagA::xylE, tagD::lacZ)</td>
<td>Transformation of SG64 with SmaBI-linearized pTAG103 → CmR Amy-</td>
</tr>
<tr>
<td>SA370</td>
<td>trpC2 ampyEΩpTAG102 (tagA::lacZ, tagD::xylE)</td>
<td>Transformation of 168 with SA355 DNA → CmR</td>
</tr>
<tr>
<td>SA371</td>
<td>trpC2 ampyEΩpTAG103 (tagA::xylE, tagD::lacZ)</td>
<td>Transformation of 168 with SA356 DNA → CmR</td>
</tr>
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<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristics*/construction</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMTL20P</td>
<td>2.85 kbp; ApR</td>
<td>Chambers et al. (1988)</td>
</tr>
<tr>
<td>pMTL20EC</td>
<td>6.66 kbp; ApR, EmR, CmR</td>
<td>Berth et al. (1988)</td>
</tr>
<tr>
<td>pMTL21</td>
<td>4.87 kbp; ApR</td>
<td>Chambers et al. (1988)</td>
</tr>
<tr>
<td>pDG268</td>
<td>1.5 kbp; ApR, lacZ</td>
<td>P. Stagier, Institut de biologie physico-chimique, Paris, France</td>
</tr>
<tr>
<td>p620</td>
<td>4.15 kbp; ApR, 1.3 kbp Sacl-Kpol (Klenow) fragment encoding XylE from Pseudomonas putida in Sacl site of pMTL20P</td>
<td>J. K. Berth, PHLS, Porton, UK</td>
</tr>
</tbody>
</table>
supplemented with ampicillin (50–100 µg ml⁻¹). *Bacillus subtilis* strains were grown either in LB or in SA medium (Karamata & Gross, 1970), supplemented with chloramphenicol (3–5 µg ml⁻¹) or erythromycin (1 µg ml⁻¹), as appropriate. Starch plates were grown either in LB or in SA medium (Karaniata et al., 1990), supplemented with chloramphenicol (3–5 µg ml⁻¹) and into pMTL21 (Stml). DNA manipulations. Plasmids were extracted from *E. coli* strains by the method of Del Sal et al. (1988). For all DNA manipulations, routine procedures (Maniatis et al., 1982) were followed.

**Table 1 (cont.)**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristics*/construction</th>
<th>Source/reference</th>
</tr>
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<tbody>
<tr>
<td>p5305</td>
<td>5.96 kbp; Ap⁹ Em⁹ Cm⁸; EcoRI– BamHI fragment encompassing the amino-terminal part of tagC</td>
<td>Mauel et al. (1991)</td>
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<tr>
<td>p5314</td>
<td>5.76 kbp; Ap⁹ Em⁹ Cm⁸; 1108 bp HindIII fragment encompassing tagA–tagD intergenic region in pMTL20EC</td>
<td>Mauel et al. (1991)</td>
</tr>
<tr>
<td>p5361</td>
<td>2.93 kbp; Ap⁶; 455 bp NdeI–HpaI (Klenow) fragment from p5314 in pMTL21 (Stml)</td>
<td>This investigation</td>
</tr>
<tr>
<td>pAG7</td>
<td>3.81 kbp; Ap⁶; EcoRI–BamHI fragment from p5305 in pMTL21</td>
<td>This investigation</td>
</tr>
<tr>
<td>pAG8</td>
<td>8.9 kbp; Ap⁶ Cm⁸; tagC::lacZ; lacZ–CAT cassette from pSGMU38 cloned into SphI site of pAG7</td>
<td>This investigation</td>
</tr>
<tr>
<td>pDGxylE</td>
<td>10.68 kbp; Ap⁶ Cm⁸; lacZ, xylE; 1.36 kbp EcoRI–HindIII fragment from p620 containing xylE in pDG268</td>
<td>This investigation</td>
</tr>
<tr>
<td>pTAG101</td>
<td>11.78 kbp; Ap⁶ Cm⁸; tagA::lacZ; 455 bp NdeI–HpaI (Klenow) fragment from p5314 in pDGxylE</td>
<td>This investigation</td>
</tr>
<tr>
<td>pTAG102</td>
<td>11.14 kbp; Ap⁶ Cm⁸; tagA::lacZ; 455 bp NdeI–HpaI (Klenow) fragment from p5314 in pDGxylE (HindIII + Klenow)</td>
<td>This investigation</td>
</tr>
<tr>
<td>pTAG103</td>
<td>11.14 kbp; Ap⁶ Cm⁸; tagA::lacZ; 455 bp NdeI–HpaI (Klenow) fragment from p5314 in pDGxylE (HindIII + Klenow)</td>
<td>This investigation</td>
</tr>
<tr>
<td>pTAG110</td>
<td>11.78 kbp; Ap⁶ Cm⁸; tagA::lacZ; 455 bp NdeI–HpaI (Klenow) fragment from p5314 in pDGxylE</td>
<td>This investigation</td>
</tr>
<tr>
<td>pAZ5335</td>
<td>10.43 kbp; Ap⁶ Cm⁸; tagA::lacZ; 1108 bp HindIII fragment from p5314 in pDG268</td>
<td>This investigation</td>
</tr>
<tr>
<td>pAZ5365</td>
<td>8.9 kbp; Ap⁸ Cm⁸; tagA::lacZ; 455 bp NdeI–HpaI fragment from p5361 in pDG268 (HindIII–BamHI)</td>
<td>This investigation</td>
</tr>
<tr>
<td>pDZ5326</td>
<td>8.9 kbp; Ap⁸ Cm⁸; tagD::lacZ; 1108 bp HindIII fragment from p5314 in pSGMU38</td>
<td>This investigation</td>
</tr>
<tr>
<td>pDZ5336</td>
<td>10.43 kbp; Ap⁶ Cm⁸; tagD::lacZ; 1108 bp HindIII fragment from p5314 in pDG268</td>
<td>This investigation</td>
</tr>
<tr>
<td>pDZ5366</td>
<td>9.85 kbp; Ap⁶ Cm⁸; tagD::lacZ; 455 bp NdeI–HpaI fragment from p5361 in pDG268 (HindIII–EcoRI)</td>
<td>This investigation</td>
</tr>
</tbody>
</table>

* Km⁸, Ap⁶, Em⁸, resistance to kanamycin, ampicillin and erythromycin, respectively.
C. MAUEI, and OTHERS

Fig. (0.1 ml (Mauck Phosphate limitation.
(0.75 mM) and MgSO₄ (1 mM) (Spizizen, 1958)
composite medium consisting of: Tris/HCl (0.1 M) pH '7.6
Catechol 2,3-dioxygenase was assayed as described previou;
37 °C.
Alkaline phosphatase was assayed by the method of Torriani
(1960), modified by Grant (1974). One unit of alkaline phos-
phatase hydrolysed 1 nmol of p-nitrophenyl phosphate min⁻¹ at
37 °C.

Construction of transcriptional fusions. DNA fragments
a promoterless copy of hisA (Antoniewski et al., 1990) or into its derivative containing, next
tagD: :lacZ

Sporulation. The replacement sporulation procedure of Sterlini
& Mandelstam (1969) was employed. At an OD₆₀₀ of 0.7–0.8,
cells growing in CH medium were resuspended in SM medium.
β-Galactosidase and alkaline phosphatase were assayed on
periodically removed samples.

Spor germination. Bacteria, allowed to sporulate for 36 h at
37 °C on nutrient agar supplemented with sporulation salts
(Sterlini & Mandelstam, 1969), were harvested. Spores were
purified by the 'lysozyme, salt and detergent' procedure
(Nicholson & Setlow, 1990), omitting the treatment with
PMSF. They were heat-shocked for 35 min at 70 °C and
germinated in CH medium (Sterlini & Mandelstam, 1969)
supplemented with 20 mM L-alanine. The OD₆₀₀ of germinating
spores was monitored and samples were removed periodically
for the β-galactosidase assay.

SOS induction. Cultures, grown to a density of 4 × 10⁹ cells ml⁻¹
in SA medium at 37 °C, were treated for 10 min with mitomycin
C (MMC; 2 μg ml⁻¹), filtered, washed and resuspended in
prewarmed medium (Mauel & Karamata, 1984). Before and
after the MMC treatment, samples were removed for the β-
galactosidase assay.

RESULTS

Characterization of the experimental system

Transcriptional fusions with different vectors. The lacZ
genesis in plasmids pSGMU38 and pDG268 have been
adapted for the construction of lacZ fusions in B. subtilis,
by fusion, in frame, to the ribosome-binding site and the
first 24 codons of the spoIIA gene of B. subtilis
(Errington, 1986), and to the ribosome-binding site and
the first nine codons of the spoVGA gene of B. subtilis
(Antoniewski et al., 1990; P. Stragier, personal com-
munication), respectively. Single-copy insertion of
pDZ5326 and pDZ5336 (tagD::lacZ fusions in pSGMU38
and pDG268, respectively) into the tag region of the
bacterial chromosome yielded strains L4532 and L4582
(Table 1). Strain L4582 produced about ten times more
β-galactosidase than strain L4532

Expression from tag promoters in different genetic
contexts. The B. subtilis genes so far identified that are
specifically devoted to the synthesis of teichoic acid are
located to a chromosomal region at about 310° which

Alkaline phosphatase was assayed by the method of Torriini
(1960), modified by Grant (1974). One unit of alkaline phos-
phatase hydrolysed 1 nmol of p-nitrophenyl phosphate min⁻¹ at
37 °C.
Catechol 2,3-dioxygenase was assayed as described previously
(Zukowski et al., 1983).

Phosphate limitation. TLP (H. M. Pooley, unpublished) is a
composite medium consisting of: Tris/HCl (0.1 M) pH 7.6
(Mauel & Glaser, 1972); (NH₄)₂SO₄ (9 mM), trisodium citrate
(0.75 mM) and MgSO₄ (1 mM) (Spizizen, 1958); special salts mix
(0.1 mol %) (Schlaeppe et al., 1982); CaCl₂ (0.1 mM); sodium L-

glutamate (50 mM); glucose 0.5 % and, as needed, amino acids
and bases at 20 μg ml⁻¹ and 100 μg ml⁻¹, respectively. Phosphate
was added as KH₂PO₄ at 1 mM (TLP1) or 220 μM (TLP2).
Strains, grown overnight at 30 °C in TLP1 medium, were
diluted in TLP2 and incubated at 37 °C. Samples for the β-
galactosidase assay were periodically removed.

Sporulation. The replacement sporulation procedure of Sterlini
& Mandelstam (1969) was employed. At an OD₆₀₀ of 0.7–0.8,
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Expression from tag promoters in different genetic
contexts. The B. subtilis genes so far identified that are
specifically devoted to the synthesis of teichoic acid are
located to a chromosomal region at about 310° which
encompasses many other genes involved in cell envelope metabolism (Mauel et al., 1991; Margot & Karamata, 1992; Lazarevic & Karamata, 1993; Margot et al., 1994). To assess whether this clustering of functionally related genes reflects some regulatory interdependence, tagA::lacZ as well as tagD::lacZ fusions were inserted at either the tag or the amyE locus located at 25° (Anagnostopoulos et al., 1993). At both locations, the levels of gene expression were identical, suggesting that the chromosomal context plays no major role in regulation (Table 2).

An attempt to monitor expression from tagA and tagD promoters simultaneously by fusing each of them to a different reporter gene -lacZ or xylE- with plasmid pDGxyIE as vector was unsuccessful. Indeed, although when on a multicopy plasmid xylE could function as a reporter gene in B. subtilis (Zukowski et al., 1983), the carehol 2,3-dioxigenase activity expressed from single-copy chromosomal insertions was barely detectable in a variety of strains with tagA::xylE and tagD::xylE fusions. Therefore, in our conditions, xylE was inappropriate for monitoring gene expression. This low xylE reporter gene sensitivity in B. subtilis, previously observed by Leonhardt & Alonso (1988), remains unexplained. Incidentally, fusion of xylE to one of the promoters did not influence the level of lacZ expression from the other (Table 2).

When fusions were made with two DNA segments of different length (see Methods), expression of tagD was somewhat reduced in those obtained with the shorter DNA fragment (Table 2).

In conclusion, the genetic contexts examined did not influence significantly the level of β-galactosidase activity. Therefore, expression of tag genes, in various physiological conditions (see below) known, or supposed, to influence the synthesis of teichoic acids in B. subtilis 168, was determined using different constructs and different genetic backgrounds.

### Phosphate limitation

Under conditions of phosphate deprivation, newly synthesized walls incorporate teichuronic acid, a phosphate-free acidic polymer, instead of poly(groP) (Janczura et al., 1961; Ellwood & Tempest, 1972). To assess whether this phenomenon is associated with an arrest of tag gene transcription, expression of tagA::lacZ and tagD::lacZ fusions was monitored during transfer of strains L4609 and L4611 to a phosphate-limited medium. About one generation time before cessation of growth, β-
galactosidase activity from both fusions began to decrease, levelling off, after 7 h, at about 60% of its initial activity (Fig. 2). That phosphate was the growth limiting factor was confirmed by culturing the strain at three different phosphate concentrations (110 μM, 220 μM and 1 mM). All three cultures grew with approximately 1 h generation time. However, the growth ceased at different ODs, probably corresponding to phosphate exhaustion.

**Sporulation**

It has been reported that spores of *B. subtilis* W23 do not contain teichoic acid (Chin et al., 1968). Assuming that the same holds for strain 168, we attempted to determine the sporulation stage at which the expression of tag genes was shut off. To avoid endogenous β-galactosidase activity, absent in exponential growth but induced during sporulation (Dubnau et al., 1987; Errington & Mandelstam, 1986), gene fusions were transferred to the lac A17 lac R1 genetic background of strain SG64, a 168 derivative (Errington & Vogt, 1990). The level of expression of tagA::lacZ and tagD::lacZ fusions during sporulation was monitored with strains SA355 and SA356, respectively. Following sporulation induction in SM medium, β-galactosidase activity from both fusions declined rapidly, reaching a near zero level 2 h later, before appearance of the alkaline phosphatase, an enzyme specific to sporulation stage II (Fig. 3).

**Spore germination and outgrowth**

Since spores produced from strains SA355 and SA356 germinated extremely poorly, relevant gene fusions were transferred from *B. subtilis* SG64 to *B. subtilis* 168, yielding strains SA370 and SA371, the spores of which readily respond to alanine as germinant. Both tagA::lacZ and tagD::lacZ fusions were expressed following completion of germination but before any rise in the culture turbidity (OD₆₀₀) could be detected (Fig. 4).

**SOS induction**

Since tagC (dinC) was shown to be inducible by DNA damaging agents (Cheo et al., 1991), strains with tagA and tagD transcriptional fusions were treated with MMC and assayed for β-galactosidase activity. To avoid cell lysis resulting from vegetative phage development (Mauel & Karamata, 1984), experiments were performed with strains carrying a xin-15 mutation (non-inducible PBSX prophage). Induction of SOS functions, in strains L4610
tagA::lacZ and LA612 tagD::lacZ, did not affect the expression of tagA and tagD genes from promotors of their intergenic region (Fig. 5a). However, the level of expression of a transcriptional fusion to tagC in strain L4578, barely detectable in the absence of SOS induction, increased rapidly upon MMC treatment (Fig. 5b), in agreement with a previous report (Cheo et al., 1991). Incidentally, in this construct, tagC was fused to lacZ from pSGMU38, the expression of which is about ten times lower than that of lacZ from pDG268 (see above). Accordingly, Miller units in Figs 5(a) and 5(b) are not readily comparable.

**DISCUSSION**

Teichoic acids, shown to be essential for vegetative growth of *B. subtilis* 168 (Mauel et al., 1991), can nevertheless be replaced by phosphorus-free teichuronic acids during phosphorus starvation (Janczura et al., 1961; Ellwood & Tempest, 1972). They are however not incorporated into the cell wall of *B. subtilis* W23 and *B. licheniformis* during spore formation (Chin et al., 1968). Five of the teichoic-acid-encoding genes are organized in two divergently transcribed operons (Mauel et al., 1991), the expression of which was assessed by the construction of transcriptional fusions to the reporter lacZ gene. One of them, tagDEF, was shown to encode glycerol-3-phosphate cytidylyltransferase (Mauel et al., 1991; Pooley et al., 1991), UDP-glucose:poly(groP)glycosyltransferase (Brooks et al., 1971) and CDP-glycerol:poly(groP)glycerophosphotransferase (Pooley et al., 1992), respectively, while the identification of the enzymes encoded by operon tagAB remained elusive (see below).

At first sight, observations on tag gene expression during phosphate starvation do not seem compatible with previous analyses suggesting an extensive replacement of teichoic by phosphate-devoid teichuronic acids (Janczura et al., 1961; Ellwood & Tempest, 1972). Reducing the phosphate concentration in the growth medium from 1 mM to 220 nM produced a 40% reduction of the β-galactosidase activity from lacZ fusions to both tagAB and tagDEF operons (Fig. 2). However, this value is an overestimate of the residual tag gene expression, the extent of which depends on intracellular β-galactosidase stability. Indeed, enzyme molecules present at the time of phosphate exhaustion are not immediately degraded. The plot of β-galactosidase activity per ml of culture versus OD₆₀₀ (not presented) revealed a biphasic kinetics: the
original slope, characteristic of the first 3 h of growth, was significantly reduced during the following 80 min. Finally, the curve reached a near-plateau at the time coinciding with the arrest of growth. Therefore, under our experimental conditions, no conclusion can be drawn as to the level of residual tag gene expression. In a chemostat culture at a growth-limiting phosphate concentration of 220 μM, corresponding to a dilution rate of 0.2 h⁻¹, cell walls contained negligible quantities of glycosylglycerol (measuring teichoic acid) as compared to glucuronic acid (measuring teichuronic acid) (Lang et al., 1982). Nevertheless, studies on B. subtilis var. niger (De Boer et al., 1981) aimed at determining wall teichoic acid content as a function of phosphorus-concentration-dependent growth rate showed that at dilution rates lower than 0.5 h⁻¹ teichoic acid continued to be synthesized but, instead of being incorporated into the wall, was excreted into the medium.

Induction of sporulation was accompanied by a rapid decrease of β-galactosidase activity which appeared essentially abolished by stage II (Fig. 3). The disappearance of enzyme activity cannot be directly related to an arrest in its synthesis but must, at least partly, result from extensive degradation of the molecule. It is therefore difficult to determine, even approximately, the time at which expression of tag genes is switched off. This result is however consistent with the finding that spores of B. subtilis W23 and B. licheniformis do not contain teichoic acids (Chin et al., 1968), as well as with the hypothesis attributing to teichoic acids a more specific role in the elongation of bacilli (Boylan et al., 1972; Pooley et al., 1993). Indeed, unlike the vegetative cell wall, neither the spore septum, nor its cortex, undergoes longitudinal expansion. Moreover, expression of tag genes prior to spore outgrowth (Fig. 4) is compatible with an essential role for teichoic acids in cell surface expansion.

Our observations (Fig. 5) confirmed that tagC (dinC) was expressed from a DNA damage-inducible promoter (Choe et al., 1991). This contradicts previous results from insertional mutagenesis experiments (Mauel et al., 1989) as well as nucleotide sequence analyses, which suggested that the transcription terminator for tagAB lay downstream of tagC (Mauel et al., 1991). The latter observations led to the proposal that genes tagA, B and C formed a single operon (Mauel et al., 1991). However, a more recent experiment (C. Mauel, unpublished) has revealed that integration of a plasmid containing a DNA segment overlapping the tagB stop codon and the tagC start codon did not affect the expression of tagC (Choe et al., 1991). In conclusion, tagC, expressed from its own DNA-damage-inducible promoter, should no longer be considered as part of the tagAB operon. Whether it plays a role in teichoic acid synthesis remains an open question.

Under all experimental conditions, expression of tagD and tagA genes went hand in hand, indicating that operons tagDEF and tagAB, forming the tag divergon, are likely to be co-ordinately regulated. However, expression of the former was always up to threefold higher than that of the latter, raising the possibility that the level of expression of the tagAB and tagDEF operons could be related to the functions they encode. While the products of genes tagDEF were shown to be involved in precursor synthesis, as well as in the polymerization of the main glucosylated poly(groP) chain (see above), the identity of the TagA and TagB proteins is poorly characterized. Recent inspection of data banks revealed a similarity between genes tagA and the rffM gene of E. coli (E. Lawlor, personal communication). Their predicted products show 31% identity and 55% similarity according to the GAP alignment algorithm. Gene rffM encodes UDP-N-acetyl-D-mannosaminuronic transferase, which adds N-acetylmannosaminuronic acid to N-acetylglucosamine-pyrophosphoryl undecaprenol (Barr et al., 1988; Meier-Dieter et al., 1990, 1992). It plays an essential role in the synthesis of the enterobacterial common antigen, an acidic cell surface glycolipid found in Enterobacteriaceae (Mäkelä & Mayer, 1976). These similarities suggest that TagA may link N-acetylmannosamine to N-acetylglucosamine-pyrophosphoryl undecaprenol, i.e. be involved in the polymerization of the linkage unit that joins the main poly(groP) chain to the peptidoglycan. Further, the substantial local similarity between tagB and tagF products (Mauel et al., 1991) suggests that TagB might add the three gro(P) residues completing the linkage unit. This tentative assignment of the two tag operons to different functions is consistent with the present finding that expression of tagA is significantly weaker than that of tagD, responsible for the synthesis of the precursor of both the main chain (about 50 residues; F. Neuhaus, personal communication) and the linkage unit (three residues; Araki & Ito, 1989).

ACKNOWLEDGEMENTS

The financial support of the British Council and the Fonds national suisse de la recherche scientifique (grants 83BC-032045 and 31.32588-91) is gratefully acknowledged. S. A. M. is the grateful recipient of an SERC CASE award studentship (in conjunction with SKB, Pharmaceuticals). We are grateful to A. Bauduret, J. Paturiaux, G. Price and D. Matthews for expert technical assistance, as well as to C. Chervet for complementary experiments.

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