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

Keywords: *Bacillus subtilis*, teichoic acids, poly(ribitol phosphate), *tag* gene expression, *lacZ* transcriptional fusions

INTRODUCTION

Poly(glycerol phosphate) [poly(groP)], the major cell wall teichoic acid of *Bacillus subtilis* 168 (Baddiley, 1970), has been shown to be essential for growth (Karamata *et al.*, 1987; Mauël *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; Mauël *et al.*, 1991; Soldo *et al.*, 1993; Lazarevic & Karamata, 1993). It was proposed (Mauël *et al.*, 1991) that six of them are organized in two divergently transcribed operons (a divergon) denoted *tagABC* and *tagDEF*, separated by a 399 bp intergenic region. Subsequently, it appeared (V. Lazarevic, unpublished) that *tagC* corresponds to *dinC* (Cheo *et al.*, 1991) and thus belongs to the SOS regulon. Its function, if any, in poly(groP) synthesis remains unknown. Expression of *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 *et al.*, 1973; Rosenberger, 1976; Estrela *et al.*, 1991). Second, under phosphate-limitation, teichoic acid is replaced by teichuronic acid, a phosphate-free anionic polymer (Janczura *et al.*, 1961; Ellwood & Tempest, 1972). Finally, in contrast to vegetative cells, spores of *B. subtilis* strain W23 were shown not to contain teichoic acid (Chin *et al.*, 1968).

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

METHODS

Bacterial strains and plasmids. These are listed in Table 1.

Growth and maintenance of strains. *Escherichia coli* cells harbouring plasmids were grown in LB medium (Miller, 1972),

Abbreviations: MMC, mitomycin C; poly(groP), poly(glycerol phosphate).

Table 1. Bacterial strains and plasmids

Strain	Genotype	Source/reference
<i>Escherichia coli</i>		
DH5 α	F ⁻ <i>endA1 hsdR17(r_m⁺) supE44 thi-1 recA1 gyrA96(Nal^R) relA1</i> Δ (<i>argF-lacZYA</i>)U169 ϕ 80d/ <i>lacZ</i> Δ M15 <i>deoR</i>	Hanahan (1983)
JM83	F ⁻ <i>ara</i> Δ (<i>lac-proAB</i>) <i>rpsL</i> ϕ 80d/ <i>lacZ</i> Δ M15	Yanisch-Perron <i>et al.</i> (1985)
<i>Bacillus subtilis</i>		
168	<i>trpC2</i>	Laboratory stock
SG64	<i>trpC2 lacA17 lacR1</i>	Errington & Vogt (1990)
L4532	<i>trpC2 tag⁺ΩpDZ5326 (tagD::lacZ)</i>	Transformation of 168 with pDZ5326 $\rightarrow$ Cm ^R
L4561	<i>trpC2 amyEΩpTAG101 (tagA::lacZ tagD::xylE)</i>	Transformation of 168 with <i>Sna</i> BI-linearized pTAG101 $\rightarrow$ Cm ^R Amy ⁻
L4562	<i>trpC2 amyEΩpTAG110 (tagD::lacZ tagA::xylE)</i>	Transformation of 168 with <i>Sna</i> BI-linearized pTAG110 $\rightarrow$ Cm ^R Amy ⁻
L4563	<i>trpC2 amyEΩpTAG102 (tagA::lacZ tagD::xylE)</i>	Transformation of 168 with <i>Sna</i> BI-linearized pTAG102 $\rightarrow$ Cm ^R Amy ⁻
L4564	<i>trpC2 amyEΩpTAG103 (tagD::lacZ tagA::xylE)</i>	Transformation of 168 with <i>Sna</i> BI-linearized pTAG103 $\rightarrow$ Cm ^R Amy ⁻
L4565	<i>trpC2 tag⁺ΩpTAG101 (tagA::lacZ tagD::xylE)</i>	Transformation of 168 with pTAG101 $\rightarrow$ Cm ^R
L4566	<i>trpC2 tag⁺ΩpTAG110 (tagD::lacZ tagA::xylE)</i>	Transformation of 168 with pTAG110 $\rightarrow$ Cm ^R
L4572	<i>bisA1 argC4 xin-15</i>	A. Grecescu-Monsutti, University of Lausanne
L4578	<i>bisA1 argC4 xin-15 tagC::lacZ</i>	Transformation of L4572 with <i>Sca</i> I-linearized pAG8 $\rightarrow$ Cm ^R
L4581	<i>trpC2 tag⁺ΩpAZ5335 (tagA::lacZ)</i>	Transformation of 168 with pAZ5335 $\rightarrow$ Cm ^R
L4582	<i>trpC2 tag⁺ΩpDZ5336 (tagD::lacZ)</i>	Transformation of 168 with pAZ5336 $\rightarrow$ Cm ^R
L4583	<i>trpC2 amyEΩpAZ5335 (tagA::lacZ)</i>	Transformation of 168 with <i>Sna</i> BI-linearized pAZ5335 $\rightarrow$ Cm ^R Amy ⁻
L4584	<i>trpC2 amyEΩpDZ5336 (tagD::lacZ)</i>	Transformation of 168 with <i>Sna</i> BI-linearized pAZ5336 $\rightarrow$ Cm ^R Amy ⁻
L4609	<i>bisA1 argC4 metC3 tag⁺ΩpAZ5335 (tagA::lacZ)</i>	Transformation of L5087 with L4581 $\rightarrow$ Cm ^R
L4610	<i>bisA1 argC4 xin-15 tag⁺ΩpAZ5335 (tagA::lacZ)</i>	Transformation of L4572 with L4581 $\rightarrow$ Cm ^R
L4611	<i>bisA1 argC4 tag⁺ΩpDZ5336 (tagD::lacZ)</i>	Transformation of L5087 with L4582 $\rightarrow$ Cm ^R
L4612	<i>bisA1 argC4 xin-15 tag⁺ΩpDZ5336 (tagD::lacZ)</i>	Transformation of L5087 with L4582 $\rightarrow$ Cm ^R
L4642	<i>trpC2 amyEΩpAZ5365 (tagA::lacZ)</i>	Transformation of 168 with <i>Sna</i> BI-linearized pDZ5365 $\rightarrow$ Cm ^R Amy ⁻
L4643	<i>trpC2 amyEΩpDZ5366 (tagD::lacZ)</i>	Transformation of 168 with <i>Sna</i> BI-linearized pDZ5366 $\rightarrow$ Cm ^R Amy ⁻
L5087	<i>bisA1 argC4 metC3</i>	Briehl <i>et al.</i> (1989)
SA355	<i>trpC2 lacR1 lacA17 amyEΩpTAG102 (tagA::lacZ tagD::xylE)</i>	Transformation of SG64 with <i>Sna</i> BI-linearized pTAG102 $\rightarrow$ Cm ^R Amy ⁻
SA356	<i>trpC2 lacR1 lacA17 amyEΩpTAG103 (tagA::xylE tagD::lacZ)</i>	Transformation of SG64 with <i>Sna</i> BI-linearized pTAG103 $\rightarrow$ Cm ^R Amy ⁻
SA370	<i>trpC2 amyEΩpTAG102 (tagA::lacZ tagD::xylE)</i>	Transformation of 168 with SA355 DNA $\rightarrow$ Cm ^R
SA371	<i>trpC2 amyEΩpTAG103 (tagA::xylE tagD::lacZ)</i>	Transformation of 168 with SA356 DNA $\rightarrow$ Cm ^R
Plasmid		
Relevant characteristics*/construction		Source/reference
pMTL20P	2.85 kbp; Ap ^R	Chambers <i>et al.</i> (1988)
pMTL20EC	4.66 kbp; Ap ^R Em ^R Cm ^R	Brehm <i>et al.</i> (1988)
pMTL21	2.47 kbp; Ap ^R	Chambers <i>et al.</i> (1988)
pSGMU38	7.8 kbp; Ap ^R Cm ^R ; 'lacZ	Errington (1986)
pDG268	9.32 kbp; Ap ^R Cm ^R ; 'lacZ	P. Stragier, Institut de biologie physico-chimique, Paris, France
p620	4.15 kbp; Ap ^R ; 1.3 kbp <i>Sca</i> I- <i>Kpn</i> I (Klenow) fragment encoding XylE from <i>Pseudomonas putida</i> in <i>Sma</i> I site of pMTL20P	J. K. Brehm, PHLS, Porton, UK

Table 1 (cont.)

Plasmid	Relevant characteristics*/construction	Source/reference
p5305	5.96 kbp; Ap ^R Em ^R Cm ^R ; <i>EcoRI</i> – <i>Bam</i> HI fragment encompassing the amino-terminal part of <i>tagC</i>	Mauël <i>et al.</i> (1991)
p5314	5.76 kbp; Ap ^R Em ^R Cm ^R ; 1108 bp <i>Hind</i> III fragment encompassing <i>tagA</i> – <i>tagD</i> intergenic region in pMTL20EC	Mauël <i>et al.</i> (1991)
p5361	2.93 kbp; Ap ^R ; 455 bp <i>Nde</i> I– <i>Hpa</i> I (Klenow) fragment from p5314 in pMTL21 (<i>Stu</i> I)	This investigation
pAG7	3.81 kbp; Ap ^R ; <i>EcoRI</i> – <i>Bam</i> HI fragment from p5305 in pMTL21	This investigation
pAG8	8.9 kbp; Ap ^R Cm ^R ; <i>tagC</i> :: <i>lacZ</i> ; <i>lacZ</i> –CAT cassette from pSGMU38 cloned into <i>Sph</i> I site of pAG7	This investigation
pDG _{xylE}	10.68 kbp; Ap ^R Cm ^R ; ' <i>lacZ</i> ', ' <i>xylE</i> '; 1.36 kbp <i>EcoRI</i> – <i>Hind</i> III fragment from p620 containing <i>xylE</i> in pDG268	This investigation
pTAG101	11.78 kbp; Ap ^R Cm ^R ; <i>tagA</i> :: <i>lacZ</i> <i>tagD</i> :: <i>xylE</i> ; 1108 bp <i>Hind</i> III fragment from p5314 in pDG _{xylE}	This investigation
pTAG102	11.14 kbp; Ap ^R Cm ^R ; <i>tagA</i> :: <i>lacZ</i> <i>tagD</i> :: <i>xylE</i> ; 455 bp <i>Nde</i> I– <i>Hpa</i> I (Klenow) fragment from p5314 in pDG _{xylE} (<i>Hind</i> III + Klenow)	This investigation
pTAG103	11.14 kbp; Ap ^R Cm ^R ; <i>tagA</i> :: <i>xylE</i> <i>tagD</i> :: <i>lacZ</i> ; 455 bp <i>Nde</i> I– <i>Hpa</i> I (Klenow) fragment from p5314 in pDG _{xylE} (<i>Hind</i> III + Klenow)	This investigation
pTAG110	11.78 kbp; Ap ^R Cm ^R ; <i>tagA</i> :: <i>xylE</i> <i>tagD</i> :: <i>lacZ</i> ; 1108 bp <i>Hind</i> III fragment from p5314 in pDG _{xylE}	This investigation
pAZ5335	10.43 kbp; Ap ^R Cm ^R ; <i>tagA</i> :: <i>lacZ</i> ; 1108 bp <i>Hind</i> III fragment from p5314 in pDG268	This investigation
pAZ5365	9.83 kbp; Ap ^R Cm ^R ; <i>tagA</i> :: <i>lacZ</i> ; 455 bp <i>Nde</i> I– <i>Hpa</i> I fragment from p5361 in pDG268 (<i>Hind</i> III– <i>Bam</i> HI)	This investigation
pDZ5326	8.9 kbp; Ap ^R Cm ^R ; <i>tagD</i> :: <i>lacZ</i> ; 1108 bp <i>Hind</i> III fragment from p5314 in pSGMU38	This investigation
pDZ5336	10.43 kbp; Ap ^R Cm ^R ; <i>tagD</i> :: <i>lacZ</i> ; 1108 bp <i>Hind</i> III fragment from p5314 in pDG268	This investigation
pDZ5366	9.85 kbp; Ap ^R Cm ^R ; <i>tagD</i> :: <i>lacZ</i> ; 455 bp <i>Nde</i> I– <i>Hpa</i> I fragment from p5361 in pDG268 (<i>Hind</i> III– <i>Eco</i> RI)	This investigation

* Km^R, Ap^R, Em^R, resistance to kanamycin, ampicillin and erythromycin, respectively.

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 contained 0.2% starch. Amylase activity was detected by the presence of an unstained halo after the plates were flooded with 0.5% I₂/1% KI (O'Kane *et al.*, 1986).

Transformation. Transformation of *E. coli* was described by Mandel & Higa (1970) or Chung & Miller (1988), and that of *B. subtilis* as described by Karamata & Gross (1970). *B. subtilis* recombinants were selected on LB plates containing 3–5 µg chloramphenicol ml⁻¹.

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.

tagA::*lacZ* and *tagD*::*lacZ* transcriptional fusions were obtained by inserting DNA fragments bearing the 399 bp intergenic region between *tagA* and *tagD* into pSGMU38 (Errington, 1986), specifically to target fusions into the *tag* locus, and into pDG268 (Antoniewski *et al.*, 1990), or its derivative pDG_{xylE} (Fig. 1), to target fusions into the *amyE* locus. The latter vector was constructed by inserting a promoterless copy of the *Pseudomonas putida xylE* gene, carried on p620, into pDG268. Thus, it contained two promoterless

reporter genes, *lacZ* and *xylE*, oriented back to back, and separated by unique *Bam*HI and *Hind*III sites. However, under our conditions, *xylE* turned out to be inappropriate for monitoring gene expression. Two DNA segments of different lengths were inserted into these vectors in both orientations: first, a 455 bp *Nde*I–*Hpa*I segment containing the first 36 and 20 bp of *tagA* and *tagD*, respectively; second, a 1108 bp *Hind*III segment containing the first 565 and 144 bp of *tagA* and *tagD*, respectively. The plasmids were established at the *amyE* locus by replacement recombination, thereby inactivating the *amyE* gene, or at the *tag* locus by a Campbell-type mechanism. The site of plasmid integration was determined by Southern hybridization, transformation linkage to *tagD*12(Ts) or the Amy⁻ phenotype on starch plates.

Enzyme assays. The β-galactosidase assay developed for *E. coli* (Miller, 1972) was adapted to our conditions. Culture samples (0.5 ml) were frozen in liquid nitrogen and stored at –70 °C. Upon addition of Z buffer (0.5 ml; Miller, 1972) and lysozyme (50 µl; 20 mg ml⁻¹), samples were preincubated for 30 min at 28 °C (or 20 min at 37 °C). At time zero, 200 µl *o*-nitrophenyl β-D-galactopyranoside (4 mg ml⁻¹) was added. The reaction was measured at 28 °C. Upon development of a yellow colour, it was stopped by the addition of 0.5 ml Na₂CO₃ (1 M). The absorbance was recorded at 420 and 550 nm and Miller units calculated (Miller, 1972). Under these cell lysis conditions, the factor correcting for light scattering by the cell debris was 2.3.

Fig. 1. Construction of transcriptional fusions. DNA fragments encompassing the intergenic regulatory region of the *tag* divergon were inserted in both orientations into pDG268 (Antoniewski *et al.*, 1990) or into its derivative containing, next to *lacZ*, a promoterless copy of *xylE* of opposite transcriptional orientation. Fusions were made with DNA segments of 1108 bp (*HindIII*–*HindIII*), or 455 bp (*NdeI*–*HpaI*), corresponding to the intergenic region plus the first 565 and 144 bp, or 36 and 20 bp, of *tagA* and *tagD*, respectively. The orientation of fusions as well as the names of the corresponding plasmids are indicated.

Alkaline phosphatase was assayed by the method of Torriani (1960), modified by Grant (1974). One unit of alkaline phosphatase 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 (Mauck & Glaser, 1972); (NH₄)₂SO₄ (9 mM), trisodium citrate (0.75 mM) and MgSO₄ (1 mM) (Spizizen, 1958); special salts mix (0.1 ml %) (Schlaeppli *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, cells growing in CH medium were resuspended in SM medium. β-Galactosidase and alkaline phosphatase were assayed on periodically removed samples.

Spore 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 30 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 × 10⁷ 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 (Mauël & 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* genes 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 *spoVG* gene of *B. subtilis* (Antoniewski *et al.*, 1990; P. Stragier, personal communication), 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; replicate experiments provided 58 and 51 vs 6 and 3 Miller units. Such discrepancies between pSGMU38 and pDG268 constructs, exhibited by other fusions too (not presented), cannot be explained by a significant difference between the ΔGs of the Shine-Dalgarno sequences [–18 kcal (75 kJ) mol⁻¹ and –19 kcal (79 kJ) mol⁻¹ for pSGMU38 and pDG268, respectively; Tinoco *et al.*, 1973] nor by the spacing between the latter and the translational start points (8 and 10 bp for pSGMU38 and pDG268, respectively). With the exception of *tagC*::*lacZ*, only fusions in plasmid pDG268 were used in experiments reported below.

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

Table 2. Expression of *lacZ* in different genetic contexts

	Strain	Fusion(s)*	Location†	Size of fragment‡ (bp)	β -Galactosidase (Miller units)§
1	L4561	<i>tagA::lacZ tagD::xylE</i>	<i>amyE</i>	1008	22, 26
2	L4563	<i>tagA::lacZ tagD::xylE</i>	<i>amyE</i>	455	22, 32
3	L4583	<i>tagA::lacZ</i>	<i>amyE</i>	1008	22, 28
4	L4642	<i>tagA::lacZ</i>	<i>amyE</i>	455	27, 28
5	L4565	<i>tagA::lacZ tagD::xylE</i>	<i>tag</i>	1008	24, 25
6	L4581	<i>tagA::lacZ</i>	<i>tag</i>	1008	24, 26
7	L4562	<i>tagD::lacZ tagA::xylE</i>	<i>amyE</i>	1008	58, 60
8	L4564	<i>tagD::lacZ tagA::xylE</i>	<i>amyE</i>	455	32, 33
9	L4584	<i>tagD::lacZ</i>	<i>amyE</i>	1008	47, 52
10	L4643	<i>tagD::lacZ</i>	<i>amyE</i>	455	40, 38
11	L4566	<i>tagD::lacZ tagA::xylE</i>	<i>tag</i>	1008	58, 54
12	L4582	<i>tagD::lacZ</i>	<i>tag</i>	1008	51, 58

* The presence of *xylE* fused to one of the promoters had no influence on the level of *lacZ* expression from the other: *tagA::lacZ* compare lines 1, 5 (+*xylE*) and 3, 6 (−*xylE*); *tagD::lacZ* compare lines 7, 11 (+*xylE*) and 9, 12 (−*xylE*).

† Integration of plasmids at the *tag* locus and at the *amyE* locus occurred by Campbell and by reciprocal (double crossover) recombination, respectively. The level of expression was not affected by the chromosomal context: *tagA::lacZ*, compare lines 1, 3 (*amyE* locus) and 5, 6 (*tag* locus); *tagD::lacZ*, compare lines 7, 9 (*amyE* locus) and 11, 12 (*tag* locus).

‡ The distance separating *lacZ* from the intergenic regulatory region did not influence the level of expression of *tagA::lacZ*: compare lines 1, 3 (long fragment) and 2, 4 (short fragment). The level of *tagD::lacZ* expression was somewhat reduced in the fusions made with the shorter DNA fragment: compare lines 7, 9 (long fragment) and 8, 10 (short fragment).

§ Results of two independent experiments are given. Samples were removed for β -galactosidase assay at an OD₆₀₀ of about 0.2.

encompasses many other genes involved in cell envelope metabolism (Mauël *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 pDG*xylE* 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 catechol 2,3-dioxygenase 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 -

Fig. 2. Effect of phosphate limitation on the expression of *tagA* and *tagD*. Cultures, grown overnight in TLP1 medium (1 mM phosphate), were diluted at time 0 in TLP2 (220 μ M phosphate). Growth was monitored by OD₆₀₀ and the β -galactosidase activity assayed on periodically removed samples. (a) Strain L4611, *tagD::lacZ*. (b) Strain L4609, *tagA::lacZ*. $\square$, OD₆₀₀; $\circ$, Miller units.

Fig. 3. Expression of *tagA* and *tagD* genes during sporulation. Sporulation was achieved by the replacement procedure of Sterlini & Mandelstam (1969). Strains, grown overnight in CH medium, were resuspended in SM medium (time 0). Samples were periodically removed for β -galactosidase and alkaline phosphatase assays. (a) Strain SA356, *tagD::lacZ*. (b) Strain SA355, *tagA::lacZ*. $\circ$, Miller units; $\triangle$, alkaline phosphatase units.

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 μ M, 220 μ M 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 *lacA17 lacR1* 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 (Mauël & Karamata, 1984), experiments were performed with strains carrying a *xin-15* mutation (non-inducible PBSX prophage). Induction of SOS functions, in strains L4610

Fig. 4. Expression of *tagA* and *tagD* genes during spore germination. Germination was induced by heat shock and resuspension at time 0 in CH medium (Sterlini & Mandelstam, 1969) supplemented with 20 mM L-alanine. The OD₆₀₀ was monitored and samples periodically removed for the β-galactosidase assay. (a) Strain SA371, *tagD::lacZ*. (b) Strain SA370, *tagA::lacZ*. □, OD₆₀₀; ○, Miller units.

Fig. 5. Effect of SOS induction on the expression of *tagA*, *tagD* and *tagC*. Cultures at a density of 4×10^7 cells ml⁻¹ were treated with MMC. The time of MMC addition is indicated by an arrow. Before and after treatment, samples were removed for the β-galactosidase assay. (a) Strain L4612, *tagD::lacZ* (○, ●) and strain L4610, *tagA::lacZ* (□, ■). (b) Strain L4578, *tagC::lacZ* (□, ■). Open symbols, without MMC treatment; solid symbols, MMC treated.

tagA::lacZ and L4612 *tagD::lacZ*, did not affect the expression of *tagA* and *tagD* genes from promoters 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 (Mauël *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 (Mauël *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 cytidyltransferase (Mauël *et al.*, 1991; Pooley *et al.*, 1991), UDP-glucose:poly(groP)glucosyl transferase (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 μM 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 (Cheo *et al.*, 1991). This contradicts previous results from insertional mutagenesis experiments (Mauël *et al.*, 1989) as well as nucleotide sequence analyses, which suggested that the transcription terminator for *tagAB* lay downstream of *tagC* (*dinC*) (Mauël *et al.*, 1991). The latter observations led to the proposal that genes *tagA*, *B* and *C* formed a single operon (Mauël *et al.*, 1991). However, a more recent experiment (C. Mauël, 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* (Cheo *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-pyrophosphorylundecaprenol (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-pyrophosphorylundecaprenol, 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 (Mauël *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).

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