Analysis of teichoic acid biosynthesis regulation reveals that the extracytoplasmic function sigma factor $\sigma^M$ is induced by phosphate depletion in *Bacillus subtilis* W23

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The expression of the *Bacillus subtilis* W23 *tar* genes specifying the biosynthesis of the major wall teichoic acid, the poly(ribitol phosphate), was studied under phosphate limitation using lacZ reporter fusions. Three different regulation patterns can be deduced from these $\beta$-galactosidase activity data: (i) *tarD* and *tar*, gene expression is downregulated under phosphate starvation; (ii) *tarA* and, to a minor extent, *tarB* expression after an initial decrease unexpectedly increases; and (iii) *tarO* is not influenced by phosphate concentration. To dissect the *tarA* regulatory pattern, its two promoters were analysed under phosphate limitation: The $P_{tarA}$-ext promoter is repressed under phosphate starvation by the PhoPR two-component system, whereas, under the same conditions, the $P_{tarA}$-int promoter is upregulated by the action of an extracytoplasmic function (ECF) $\sigma$ factor, $\sigma^M$. In contrast to strain 168, $\sigma^M$ is activated in strain W23 in phosphate-depleted conditions, a phenomenon indirectly dependent on PhoPR, the two-component regulatory system responsible for the adaptation to phosphate starvation. These results provide further evidence for the role of $\sigma^M$ in cell-wall stress response, and suggest that impairment of cell-wall structure is the signal activating this ECF $\sigma$ factor.

INTRODUCTION

In addition to peptidoglycan (PG), cell walls of *Bacillus subtilis* contain teichoic acids (WTA), polymers essential for growth and survival (Mauël *et al*., 1989). The *B. subtilis* strains 168 and W23 are endowed with chemically different major WTA, i.e. poly(glycerol phosphate) [poly(Gro-P)] and poly(ribitol phosphate) [poly(Rbo-P)], respectively. The linkage unit by which WTA is fixed to PG seems to be similar in the two strains (Lazarevic *et al*., 2002a). It apparently consists of one or two Gro-P residues in 168 and W23, respectively, followed by N-acetylmannosamine-N-acetylglycaminosamine-phosphate (ManNAc-GlcNAc-P), which is linked to the C-6 of the PG N-acetylmuramylate (MurNAc).

In *B. subtilis* W23, the chromosome region encompassing most of the genes that specify the synthesis of Rbo-P-containing WTA has been cloned, sequenced and analysed (Lazarevic *et al*., 2002a; Young *et al*., 1989). The *tar* (teichoic acid ribitol) genes are organized in two divergently transcribed operons, *tarDF* and *tarABIJKL*. This organization is analogous to that of strain 168 *tag* (teichoic acid glycerol) genes.

Studies of *tarA* gene regulation in *B. subtilis* W23 revealed two growth-phase-dependent increases, which are driven by $\sigma^X$ and $\sigma^M$, two extracytoplasmic function (ECF) $\sigma$ factors (Minnig *et al*., 2003). In strain 168, $\sigma^M$ is involved in the response to diverse stresses, all of which disturb the cell envelope: $\sigma^M$ is essential for growth and survival at high salt concentration (Horsburgh & Moir, 1999), and is activated in response to ethanol, heat, acid and superoxide stress (Thackray & Moir, 2003). Moreover, $\sigma^M$ is induced, together with $\sigma^W$, by antibiotics that inhibit cell wall synthesis, e.g. vancomycin (Cao *et al*., 2002b), and the bacitracin-resistance gene *bcrC* was shown to be under control of $\sigma^M$ and $\sigma^X$ (Cao & Helmann, 2002). Cao *et al.* (2002a) suggested that the signal activating $\sigma^M$ is not the antibiotic molecule itself, but structural changes induced by the attack of the cell wall. Thus, $\sigma^M$ is considered to be part of a network coordinating antibiosis stress response and cell envelope homeostasis (Mascher *et al*., 2003). In strain W23, $\sigma^M$ plays an important role not only in wall synthesis and homeostasis, but also in cell division (Minnig *et al*., 2003). In both 168...
and W23, sigM gene expression is driven by two promoters, recognized by $\sigma^A$ and $\sigma^M$, respectively (Horsburgh & Moir, 1999).

Under phosphate-limiting conditions, *B. subtilis* WTAs are substituted by phosphate-free teichuronic acids (TUAs) (Ellwood & Tempest, 1972). In *B. subtilis* 168, this process, referred to as WTA–TUA switch, is regulated by the PhoPR two-component system, which is activated, together with the general stress response, when the extracellular P$_i$ concentration drops below 100 $\mu$M (Hulett, 2002). During phosphate deprivation, PhoPR activates, among others, the tuaABCDEFGH operon specifying TUA synthesis, while repressing the expression of WTA operons tagAB and tagDEF.

In strain W23, only the biochemical aspects of the replacement of WTA by TUA have been studied so far (Cheah et al., 1982; Wright & Heckels, 1975). However, the presence of a putative PhoP-binding region, referred to as the Pho-box, in the tarA–tarD intergenic region (Fig. 2a and Lazarevic et al., 2002a), suggests that the WTA–TUA switch should also be mediated by the PhoPR system.

Here, we report the different expression patterns of several genes encoding ribitol WTA biosynthesis, as measured by *lacZ* reporter fusions in *B. subtilis* W23 under phosphate limitation. The role of the ECF sigma factor $\sigma^M$ and the two-component system PhoPR was studied under the same conditions.

### METHODS

#### Bacterial strains and growth conditions

Bacterial strains and plasmids are listed and described in Table 1. *B. subtilis* was cultured in PL medium (Grant, 1979), a defined medium containing 250 $\mu$M P$_i$, which is the limiting nutrient. Cultures were aerated either by bubbling or by vigorous shaking. *Escherichia coli* was cultured in LB supplemented with 200 $\mu$g ml$^{-1}$ ampicillin (Ap) for plasmid replication.

#### DNA manipulations

DNA manipulations were performed using standard methods (Sambrook et al., 1989). Plasmids were prepared with the NucleoSpin Plasmid Kit (Macherey-Nagel). PCRs were set up with 10 ng DNA, and 20 pmol of each primer in 40 ml Hot Start MasterMix (Qiagen) containing 4 units HotStarTaq DNA polymerase. Reactions were run according to the manufacturer’s instructions, with 1 min polymerization time at 72 °C per kb.

#### Transformations and construction of strains

Competent *E. coli* DH5$\alpha$ and JM83 cells were prepared by treatment with calcium and manganese (Hanahan et al., 1991). Transformation of *B. subtilis* was performed as described by Karamata & Gross (1970). Recombinants were selected on solid media supplemented with appropriate antibiotics [for *E. coli*, ampicillin (Ap) 100 $\mu$g ml$^{-1}$; for *B. subtilis* kanamycin (Km) 5 $\mu$g ml$^{-1}$, erythromycin (Em) 0·3 and 100 $\mu$g ml$^{-1}$, chloramphenicol (Cm) 5 $\mu$g ml$^{-1}$].

Since strain W23 can be transformed with chromosomal DNA, but not with integrative plasmids, the latter were first inserted into 168, and the DNA of the resulting strains was used to transform W23. Construction of the relevant strains is outlined in Table 1. The pDG268 plasmid (Antoniewski et al., 1990) was used for the construction of strains bearing transcriptional *lacZ* fusions. To insert these reporter fusions into the *amyE* locus, strain 168 was transformed by linearized pDG268-derived plasmids. To obtain strain W14900, featuring a *lacZ* fusion at the end of the *tar* gene, circular pWW11146 plasmid was used to transform strain L5607, a 168-derived interstrain hybrid containing the *tar* gene region of W23 (Karamata et al., 1987). Genomic DNA of the resulting strain was used to transform W23.

#### Sequencing of the *B. subtilis* W23 *tarO* gene

Plasmid pV019 carrying a segment of strain W23 *tarO* gene was integrated into the *B. subtilis* W23 chromosome by homologous recombination. Salt chromosomal fragments of a chosen Cm-resistant transformant were treated with ligase, and used to transform *E. coli* TP610 (Hedegaard & Danchin, 1985). The resulting plasmid pV021, recovered by Ap selection, was used as template to determine the nucleotide sequence of the *tarO* gene (GenBank accession no. AB889011). Sequencing was performed at Microsynth (Balgach, Switzerland).

#### $\beta$-Galactosidase assays

*Overnight* *B. subtilis* cultures with different inoculum sizes were grown in PL medium (Grant, 1979), with aeration by bubbling at 35 °C. The overnight culture with an OD$_{600}$ of $<0·4$ was diluted to an OD$_{600}$ of 0·03, and divided in two tubes containing 40 ml fresh prewarmed PL medium. During 9 h growth at 37 °C with aeration, the OD$_{600}$ was measured periodically, and 0·5 ml samples were withdrawn, frozen in liquid nitrogen, and stored at −80 °C. After 7 h growth, i.e. 3 or 4 h after phosphate depletion for *phoPR* or *phoPR* strains, respectively, sodium phosphate buffer (pH 7·0) was added to one of the parallel cultures to obtain a final P$_i$ concentration of 5 mM.

The $\beta$-galactosidase assay developed for *E. coli* (Miller, 1972) was adapted to our conditions (Mauel et al., 1994). $\beta$-Galactosidase activity is expressed in Miller units, i.e. the absorbance $\times 1000$ min$^{-1}$ ml$^{-1}$ divided by the OD$_{600}$ of the culture at the sampling time.

### RESULTS

#### *tar* expression is affected by the extracellular P$_i$ concentration

To investigate the regulation of different *tar* genes in response to phosphate concentration, strains carrying *lacZ* fusions were constructed and cultured in PL medium (Grant, 1979). This defined synthetic medium contains an excess of carbon and nitrogen sources. The limiting nutrient is phosphate, present at an initial concentration of 250 $\mu$M (Grant, 1979). Exponential growth of *B. subtilis* in PL up to an OD$_{600}$ of about 0·4 is followed by a slow-down of growth rate resulting from phosphate limitation. When an excess of phosphate is added to post-exponential-phase cultures, growth resumes immediately (Fig. 1a).

To investigate the regulation of *tar* genes in PL medium, two parallel cultures of the relevant strains were performed, and phosphate was added to one of them after 7 h growth, to a final concentration of 5 mM. Analysis of strain W4839 (*tarA*::*lacZ*) revealed that $\beta$-galactosidase activity of the *tarA*::*lacZ* fusion reaches a maximum just after the end of exponential growth. In contrast to a continuous decrease after phosphate exhaustion, as observed for *tagA* and *tagD* in *B. subtilis* 168 (Mauel et al., 1994), $\beta$-galactosidase activity of the *tarA*::*lacZ* fusion declined for about 2 h only. Subsequently, it rose again, even when no phosphate was added.
Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or description</th>
<th>Reference or construction</th>
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<tr>
<td><strong>E. coli</strong></td>
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</tr>
<tr>
<td>DH5x</td>
<td>F(^{-}) endA1 hisD17((r_{w}) m(_{w})) supE44 (\lambda^{+}) thi-1 recA1 gyrA96(NalR) relA1 ((\lambda argF)--lacZYA)U169 (&amp;)80dlacZ(\Delta)M15 deoR</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td>JKM38</td>
<td>F(^{-}) ara (\Delta)(lac--proAB) rpsL (&amp;)80dlacZ(\Delta)M15</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>TP610</td>
<td>F(^{-}) thi-1 thr-1 leuB6 lacY1 tonA21 supE44 (\lambda^{+}) hisD1 rdsM recBC lop11 ligB cya-610</td>
<td>Hedegaard &amp; Danchin (1985)</td>
</tr>
<tr>
<td><strong>B. subtilis</strong></td>
<td></td>
<td></td>
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<tr>
<td>168</td>
<td>trpC2</td>
<td></td>
</tr>
<tr>
<td>L5706</td>
<td>metBS, Tar(^{+})+</td>
<td>Karamata et al. (1987)</td>
</tr>
<tr>
<td>L24071</td>
<td>trpC2, P(_{M})::lacZ, Cm(^{r})</td>
<td>Integration of pPM into 168→Cm(^{r})</td>
</tr>
<tr>
<td>W23</td>
<td>Prototroph, Sm(^{r})</td>
<td>Laboratory stock</td>
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<td>W4824</td>
<td>P(_{tarA\text{-int}})::lacZ, Cm(^{r})</td>
<td>Minnig et al. (2003)</td>
</tr>
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<td>W4838</td>
<td>P(_{tarA\text{-ext}})::lacZ, Cm(^{r})</td>
<td>Minnig et al. (2003)</td>
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<td>W4839</td>
<td>tarA::lacZ, Cm(^{r})</td>
<td>Minnig et al. (2003)</td>
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<td>W4840</td>
<td>tarD::lacZ, Cm(^{r})</td>
<td>Integration of pW1152DZ into amyE of 168→W23→Cm(^{r})+</td>
</tr>
<tr>
<td>W4844</td>
<td>tarB::lacZ, Cm(^{r})</td>
<td>Integration of pW11129 into amyE of 168→W23→Cm(^{r})</td>
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<td>W14900</td>
<td>tarL::lacZ, Cm(^{r})</td>
<td>Integration of pW11126 into 168→W23→Cm(^{r})</td>
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<td>W14943</td>
<td>P(_{tarA\text{-int}})::lacZ, sigM(\Delta)pSigM(\Delta)lacI, Cm(^{r}) Km(^{r})</td>
<td>Minnig et al. (2003)</td>
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<td>W14947</td>
<td>sigMyhdLyhdK::lacZ, Cm(^{r})</td>
<td>Campbell integration of pSigM(\Delta)lacI into 168→W4839→Km(^{r})</td>
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<td>Minnig et al. (2003)</td>
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<td>W24069</td>
<td>P(_{tarA\text{-ext}})::lacZ, phoPR(\Delta)Em(^{r}), Cm(^{r}) Em(^{r})</td>
<td>Integration of pPhoPR(\Delta)Em(^{r}) into 168→W4824→Em(^{r})</td>
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<td>Integration of pPhoPR(\Delta)Em(^{r}) into 168→W14963→Em(^{r})</td>
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<td>Integration of pPM into 168→W23→Cm(^{r})</td>
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<td>W24078</td>
<td>tarO::lacZ, Cm(^{r})</td>
<td>Integration of pTarO into amyE of 168→W23→Cm(^{r})</td>
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<td><strong>Plasmids</strong></td>
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<td>pDIA5304</td>
<td>Cm(^{r}) Ap(^{r})</td>
<td>Glaser et al. (1993)</td>
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<td>pDG268</td>
<td>Vector designed for insertion of transcriptional lacZ fusions into amyE, Cm(^{r}) Ap(^{r})</td>
<td>Antoniewski et al. (1990)</td>
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<td>pDG641</td>
<td>Em(^{r}) Ap(^{r})</td>
<td>Guérot-Fleury et al. (1995)</td>
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<td>pUC18</td>
<td>Ap(^{r})</td>
<td>Stratagene</td>
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<td>pPhoPR</td>
<td>Cm(^{r}) Ap(^{r})</td>
<td>Cloning of a 2907 bp PCR product (mdh572(\Delta)--phoRI1616) in pUC18</td>
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<td>pPhoPR-Em(^{r})</td>
<td>phoRI1616(\Delta)Em(^{r}), Cm(^{r}) Em(^{r}) Ap(^{r})</td>
<td>Cloning of an Em(^{r}) cassette (Smal--AfII from pDG641) in Eco47III--AfII (153 bp upstream of phoRI1822) in pPhoPR</td>
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<td>pPM</td>
<td>P(_{M})::lacZ, Cm(^{r}) Ap(^{r})</td>
<td>Cloning of a 184 bp PCR product (69 upstream of sigM--sigM161) in pDG268</td>
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<td>pSigM(\Delta)lacI</td>
<td>Km(^{r}), Ap(^{r})</td>
<td>Minnig et al. (2003)</td>
</tr>
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<td>pTarO</td>
<td>tarO::lacZ, Cm(^{r}) Ap(^{r})</td>
<td>Cloning of a 244 bp PCR product (117 upstream of tarO--tarO127) in pDG268</td>
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<td>pV015</td>
<td>Ap(^{r})</td>
<td>Cloning of a 572 bp PCR product (tuaE58--tuaE629) into Smal of pUC18</td>
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<td>pV019</td>
<td>Cm(^{r}) Ap(^{r})</td>
<td>Cloning of the EcoRI--HindIII pV015 fragment in pDIA5304</td>
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<td>pV021</td>
<td>Cm(^{r}) Ap(^{r})</td>
<td>Ligation of the SaII fragment of the B. subtilis W23 chromosome carrying integrated pV019</td>
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<td>pW1152DZ</td>
<td>tarD::lacZ, Cm(^{r}) Ap(^{r})</td>
<td>Cloning of the 860 bp HindIII--BstI (tarA294--tarD59) fragment in pDG268</td>
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<td>pW11129</td>
<td>tarB::lacZ, Cm(^{r}) Ap(^{r})</td>
<td>Cloning of the 2396 bp BanHI--SphI (tarF339--tarB187) W23 chromosomal fragment in pDG268</td>
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<td>pW11146</td>
<td>tarL::lacZ, Cm(^{r}) Ap(^{r})</td>
<td>Cloning of 517 bp PCR product (tarL1344, 3 bp downstream of tarL) in pDG268</td>
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</table>

*Produces ribitol WTA instead of glycerol WTA.
†168→W23→Cm\(^{r}\): transformation of W23 with 168 DNA, and selection for Cm\(^{r}\).
‡Numbers refer to nucleotide position in the indicated gene.

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β-Galactosidase activity resulting from a tarB::lacZ fusion in strain W4844 followed a similar pattern, but with smaller amplitudes (Fig. 1c). β-Galactosidase activity profiles during phosphate limitation were measured for tarD and tarL::lacZ fusions (Fig. 1d, e) using strains W4840 (tarD::lacZ) and W14900 (tarL::lacZ). The β-galactosidase activity of these fusions decreased continuously as soon as phosphate was exhausted. After phosphate addition, β-galactosidase activity rose again for both strains. Furthermore, the tarO gene, located in a monocistronic operon downstream of the TUA operon, was sequenced. TarO and its 168 homologue TagO, sharing 97% identity, most likely catalyse the first reaction in the WTA-linkage-unit biosynthesis (Soldo et al., 2002). TarO expression was measured by quantification of the β-galactosidase activity of strain W24078 (tarO::lacZ) during growth in PL medium (Fig. 1f). β-Galactosidase activity of the tarO::lacZ fusion was about 30% higher after phosphate depletion than in exponential phase. However, tarO does not seem to be regulated by phosphate concentration, as addition of an excess of Pi in stationary phase did not influence its expression.

Thus, three different regulation patterns of tar genes can be deduced from these β-galactosidase activity data: (i) tarD and tarL expression are repressed under phosphate starvation; (ii) tarA and tarB expression first decreases, then increases under phosphate starvation; and (iii) tarO does not seem to be regulated by the extracellular phosphate concentration.

**P** _tarA*-ext is controlled by the PhoPR two-component system

To dissect the regulatory events that determine the biphasic pattern of tarA expression under phosphate starvation, the activity profiles of its promoters _P_ _tarA*-ext and _P_ _tarA*-int were analysed (Fig. 2a). _P_ _tarA*-ext overlaps the putative PhoP-binding site (Lazarevic et al., 2002a), and may therefore be repressed under phosphate starvation. The expression data obtained by monitoring β-galactosidase activity of a _P_ _tarA*-ext::lacZ fusion (strain W4824) confirmed this hypothesis: _P_ _tarA*-ext-driven β-galactosidase activity presented a steady level during exponential phase (Fig. 3a); upon phosphate depletion, its activity decreased continuously. A sharp upregulation of _P_ _tarA*-ext was observed after addition of an excess of Pi in stationary phase. β-Galactosidase assays of _P_ _tarA*-ext activity in PL showed that the response to

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**Fig. 1.** Regulation of _tar_ genes under phosphate limitation. (a) Growth curve of _B. subtilis_ W4839 in PL medium. Growth curves of other W23-derived strains carrying lacZ fusions are similar. (b–f) β-Galactosidase activity of strains in PL: (b) W4839 (tarA::lacZ), (c) W4844 (tarB::lacZ), (d) W4840 (tarD::lacZ), (e) W14900 (tarL::lacZ), and (f) W24078 (tarO::lacZ). Two parallel cultures of the indicated strains were grown in PL. The 0 h time point corresponds to the end of exponential growth. After 7 h growth (arrow), phosphate was added to one of the cultures to a final concentration of 5 mM (open symbols, dashed lines). Results of one representative experiment are plotted.
Phosphate was abolished in a *phoPR* mutant, W24069 (Fig. 3a). The β-galactosidase level of W24069 was higher than in the wild-type, and no repression upon phosphate depletion, or induction after phosphate addition, was detected. Thus, it appears that *P*tarA-ex is controlled by the PhoPR two-component system, which may indeed act on the putative Pho-box present on the fragment cloned upstream of *lacZ*.

**P*tarA-int is induced by phosphate starvation**

Analysis of *P*tarA-int (Fig. 2a) fused to *lacZ* in strain W4838 cultivated in PL medium revealed an opposite response to phosphate starvation (Fig. 3b). The resulting β-galactosidase activity was very low in the exponential phase, and sharply increased upon phosphate depletion. When phosphate was added to a final concentration of 5 mM, this increase stopped. As shown previously, *P*tarA-int is controlled by the two ECF σ factors σX and σM (Minnig *et al*., 2003). To assess the possibility that one of these ECF σ factors may be induced by phosphate starvation, and mediate the *tarA* expression increase in stationary phase, we resorted to *sigX*- and *sigM*-knockout mutants (Minnig *et al*., 2003). Inactivation of *sigX* did not affect the profile of *P*tarA-int::*lacZ* expression in PL medium (not presented). Inactivation of *sigM*, however, almost completely abolished *P*tarA-int-driven *lacZ* expression (Fig. 3b).

Thus, *tarA* expression under phosphate limitation seems to be controlled by two counteracting phenomena: *P*tarA-ext is repressed under phosphate starvation by means of the PhoPR two-component system, while *P*tarA-int is induced under phosphate starvation by means of σM.

**Regulation of *sigM* expression and σM activity under phosphate limitation**

The importance of σM for *tarA* expression under phosphate limitation was confirmed by monitoring β-galactosidase activity of the *tarA*: *lacZ* fusion in a *sigM* mutant strain (Fig. 4a). Indeed, after phosphate depletion, β-galactosidase activity of the *tarA*: *lacZ* fusion constantly decreased, the induction present in *sigM*+ strains being abolished. The impact of σM on *tarA* expression in PL (Figs 4a and 3b)
suggests that $\sigma^M$ itself is activated by phosphate starvation. To test this hypothesis, W14963, a strain carrying a sigMyhdLK::lacZ fusion, was cultured in PL medium. Indeed, sigM expression, as measured by $\beta$-galactosidase activity assays, increased upon phosphate depletion (Fig. 4b). This increase was immediately stopped by the addition of excess phosphate (5 mM). To get an idea of the activation of the $\sigma^M$ factor by an extracellular stimulus, we decided to monitor the activity of a promoter that is dependent solely on $\sigma^M$. Hence, the P$_M$ promoter upstream of the sigMyhdLK operon, which was shown to be regulated exclusively by $\sigma^M$ (Horsburgh & Moir, 1999; Thackray & Moir, 2003), was fused to lacZ in the amyE locus, yielding strain W24076. P$_M$-driven $\beta$-galactosidase activity was monitored by cultivation of strain W24076 (P$_M$::lacZ) in PL medium, and subsequent enzyme assays (Fig. 4c). The resulting activity profile confirms the sigM expression data, i.e. the P$_M$ promoter is induced by phosphate starvation in B. subtilis W23. Thus, $\sigma^M$ seems to be activated by a stimulus depending on phosphate depletion in the medium. $\sigma^M$ has been studied in more detail in strain 168, but, so far, phosphate starvation has not been identified as its activator. Indeed, the P$_M$-driven $\beta$-galactosidase activity, assayed in strain L24071, a 168-derived strain carrying the P$_M$::lacZ fusion, showed no induction of the P$_M$ promoter after phosphate depletion (Fig. 4c). Therefore, the response of $\sigma^M$ to phosphate concentration seems to be specific to strain W23.

**PhoPR is required for the induction of $\sigma^M$ under phosphate starvation**

In B. subtilis W23, sigM expression and $\sigma^M$ activity depend on extracellular phosphate concentration. To test whether the sigM expression profile under phosphate limitation is dependent on the PhoPR two-component system, which is the major system regulating adaptation to phosphate limitation, a phoPR deletion mutant was constructed. Both genes were deleted, and replaced by an Em$^\text{r}$ cassette in W14963 carrying the sigMyhdLK::lacZ fusion. Growth in PL of the resulting mutant W24070 was different from the W23 wild-type in that there was no post-exponential growth after phosphate depletion (Fig. 5a). Upon phosphate addition in stationary phase, growth resumed immediately.

Strain W24070 (phoPR::Em$^\text{r}$, sigMyhdLK::lacZ) was cultured in PL, and sigM expression was monitored by $\beta$-galactosidase assays (Fig. 5b). No response to changing phosphate concentration was detected, and deletion of phoPR resulted in a steady and low level of $\beta$-galactosidase activity, suggesting that the response of $\sigma^M$ to phosphate concentration depends on the PhoPR two-component system. Inspection of the sequence of sigM and its upstream region did not reveal any putative Pho-box motif (Fig. 2b). Therefore, the dependency of $\sigma^M$ on the PhoPR two-component system is thought to be indirect.

**DISCUSSION**

In B. subtilis 168, the phosphate-dependent regulation of tag genes, which encode WTA biosynthesis as well as the WTA–TUA switch, has been studied previously (Liu et al., 1998; Mauël et al., 1994; Müller et al., 1997; Soldo et al., 1999). In strain W23, only biochemical studies were performed, and these showed that cell wall composition depends on phosphate concentration (Cheah et al., 1982; Wright & Heckels, 1975). Investigation of the regulation of tar genes during growth in rich medium has shown that tarA regulation involves two ECF $\sigma$ factors (Minnig et al., 2003) and possibly other regulators (unpublished data). Moreover, in the intergenic region of the tarAB1JKL–tarDF divergon, a putative Pho-box was determined by sequence similarity with the equivalent region of strain 168 (Lazarevic et al., 2002a). In order to study tar gene regulation under phosphate limitation, we monitored expression of tarA, tarB, tarD, tarL and...
Phosphate starvation activates *B. subtilis* W23 θ^M

Dissection of the regulatory elements governing *tarA* expression by *lacZ* reporter fusions revealed opposite responses to phosphate concentration of its two promoters: *P*~*tarA*-ext is inhibited under phosphate starvation (Fig. 3a), whereas *P*~*tarA*-int is induced under the same conditions (Fig. 3b). The sum of activities of those two promoters results in the biphasic response of *tarA* expression to phosphate exhaustion (Fig. 1b).

The decrease in *tarA* expression following phosphate depletion was shown to be dependent on the PhoPR two-component system, which is at least partially responsible for the WTA–TUA switch in *B. subtilis* 168 (Hulett, 2002; Liu *et al.*, 1998). Repeats of TTHACA-like motifs, with an opposite orientation to the direction of transcription, corresponding to the *B. subtilis* 168 Pho-box, were identified in the W23 *tar* regulatory region, overlapping *P*~*tarA*-ext (Lazarevic *et al.*, 2002a). Indeed, in a *phoPR* mutant, this promoter no longer responds to phosphate concentration (Fig. 3a), indicating that PhoP binding could be responsible for *P*~*tarA*-ext repression upon phosphate exhaustion.

In rich medium, *P*~*tarA*-int is recognized by two ECF σ factors, σ^X^ and σ^M^ (Minnig *et al.*, 2003). Therefore, we investigated the influence of those two ECF σ factors on *P*~*tarA*-int-driven β-galactosidase activity in PL medium. Indeed, inactivation of σ^M^ abolished the activity increase that took place after phosphate depletion (Fig. 3b), whereas inactivation of σ^X^ did not have any effect (data not shown). Different activation factors have been identified for σ^M^, all of them disturbing the cell envelope (Cao *et al.*, 2002a; Horsburgh & Moir, 1999; Thackray & Moir, 2003). By monitoring *sigM* expression and *P*~*M*~ promoter activity, we were able to show that in *B. subtilis* W23, but not in strain 168, σ^M^ is also induced under phosphate starvation (Fig. 4c). The underlying mechanism could consist of either (i) the direct perception of low phosphate concentration by its regulators YhdL and YhdK, or (ii) the regulation of *θ*^M^ by another, higher-level regulatory system activated by low phosphate concentration. At least two important regulatory systems have been shown to be directly induced by phosphate starvation in *B. subtilis* 168: the PhoPR two-component system and the general stress response θ factor θ^B^.

Consensus sequences of the Phop-binding site (Hulett, 2002) and the θ^B^-dependent promoter (Helmann & Moran, 2002) have been defined. Inspection of the sequence upstream of the *B. subtilis* W23 *sigMyhdLK* operon (Minnig *et al.*, 2003) revealed neither a putative Pho-box nor a θ^B^-controlled promoter. Moreover, no evidence of a θ^B^-mediated control of *sigM* expression was reported (Horsburgh & Moir, 1999; Price *et al.*, 2001; Thackray &

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tarO using *lacZ* fusion constructs. Three different response patterns to phosphate concentration were observed. According to the proposed biosynthesis pathway (Lazarevic *et al.*, 2002a), downregulation of *tarD* and *tarL* during phosphate depletion most likely prevents WTA synthesis. On the other hand, a steady-state level of tarO, and an increase in *tarA* and *tarB* expression in a later stage of phosphate starvation, suggest that TarO, TarA and TarB may play a role under these conditions. Based on sequence comparisons, putative functions for TarO and TarA were proposed (Lazarevic *et al.*, 2002a; Soldo *et al.*, 2002). TarO most probably adds the first molecule of the WTA linkage unit, i.e. GlcNAc-P, to the carrier lipid. TarA seems to catalyse the second step of linkage-unit synthesis, i.e. the addition of ManNAc on the undecaprenyl-P-P-GlcNAc. TarB subsequently adds the first Gro-P residue. Our results suggest that a truncated WTA linkage unit, either GlcNAc-ManNAc or GlcNAc-ManNAc-GroP, is synthesized under phosphate starvation. Two possible destinations can be imagined for this compound: (i) it may be incorporated in this form into the cell wall, or (ii) it may be used for TUA biosynthesis. Biochemical analysis of the cell-wall polysaccharide content will be needed to shed light on this question.

Fig. 5. Role of the PhoPR two-component system in the regulation of *sigM* expression under phosphate limitation. (a) Growth of W14963 (sigMyhdLK::lacZ, circles) and W24070 (phoPR::Em^r, sigMyhdLK::lacZ, triangles). (b) β-Galactosidase activity of strains W14963 (circles) and W24070 (triangles). Two parallel cultures were grown in PL. The 0 h time point corresponds to the end of exponential growth. After 7 h growth (arrow), phosphate was added to one of the cultures to a final concentration of 5 mM (open symbols, dashed lines). Results of one representative experiment are plotted.

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Moir, 2003). Therefore, dependency of sigM expression on σ^B has not been investigated here.

Even though inspection of the sequence upstream of sigM did not reveal a putative Pho-box, induction of sigM expression under phosphate starvation depends on the PhoPR two-component system. The influence of PhoPR on σ^M thus seems to be indirect. Globally, a phoPR mutant lost its capacity to adapt to phosphate starvation, e.g. the WTA are not replaced by TUA and no growth in phosphate depleted medium can be observed. The WTA–TUA switch, which brings about a transient impairment of the wall, is probably sensed by the bacterium as stress. In the phoPR mutant, this cell-wall stress does not happen, and σ^M remains inactive. However, in the wild-type cell, σ^M would be induced by the cell-wall impairment caused by the WTA–TUA switch. This hypothesis is consistent with previous studies showing that σ^M is induced by different kinds of stresses, all of them disturbing the cell envelope, i.e. high salt concentration, heat, ethanol, superoxide stress and cell-wall-targeted antibiotics (Horsburgh & Moir, 1999; Cao & Helmann, 2002; Cao et al., 2002a; Thackray & Moir, 2003).

As shown in this contribution, σ^M seems to play a role in the adaptation to phosphate-depleted conditions in B. subtilis W23. However, the exact role of σ^M, and the advantage brought about by the induction of σ^M and its regulon under phosphate starvation, remain unknown. For example, cultures of sigM mutant strains in PL medium did not reveal any growth defect, which was reported to be characteristic for several 168-derived mutants affected in WTA biosynthesis (Lazarevic et al., 2002b). Moreover, in B. subtilis 168, σ^M was not induced under phosphate starvation (Fig. 4c), and this strain is able to adapt well to phosphate starvation.

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REFERENCES


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Minnig, K., Barblan, J.-L., Kehl, S., Beggah Möller, S. & Mauël, C. (2003). In \textit{Bacillus subtilis} W23, the duet \(\sigma^X \sigma^M\), two sigma factors of the extracytoplasmic function subfamily, are required for septum and cell wall synthesis under batch culture conditions. \textit{Mol Microbiol} 49, 1435–1447.


