Transcriptional analysis of the *Bacillus subtilis* teichuronic acid operon

Maryam Lahooti and Colin R. Harwood

**INTRODUCTION**

A major growth-limiting substrate in the natural habitat of *Bacillus subtilis* and other soil-living micro-organisms is phosphorus (Hulett, 1993) and consequently this bacterium has developed a complex series of responses to limit the effect of phosphate starvation. These responses include the induction of extracellular hydrolytic enzymes that allow the cell to recover inorganic phosphate from organic sources such as teichoic acids (Grant, 1979; Eymann et al., 1996), transporters that increase the affinity of the cell for the uptake of phosphate at low concentrations (Takemaru et al., 1996; Qi et al., 1997) and mechanisms that reduce the phosphorus requirement of the cell (Ellwood & Tempest, 1969; Lang et al., 1982; Müller et al., 1997).

During the transition to phosphate starvation, *B. subtilis* co-ordinately induces approximately 40 genes (Eymann et al., 1996). Prominent amongst these are genes of the Pho regulon (Seki et al., 1987; Hulett, 1993; Hulett et al., 1994a, b), whose products include alkaline phosphatases (APases), alkaline phosphodiesterase (APDase), a high affinity phosphate transporter (pst) and genes required for teichuronic acid synthesis (Müller et al., 1997). The latter represent an important mechanism for conserving phosphate in *B. subtilis* since their induction brings about a major change in the anionic polymer composition of the cell wall, from one that contains almost exclusively phosphate-containing teichoic acids to one which contains significant amounts of non-phosphate-containing teichuronic acids (Ellwood & Tempest, 1969; Lang et al., 1982). Under phosphate-replete conditions, teichoic acids represent nearly 50% of the wall by weight and comprise as much as 15% of total cell phosphorus (Archibald et al., 1993). Not only does switching from teichoic to teichuronic acids reduce the phosphorus requirement of the cell, but teichoic-acid-containing cell wall released into the growth medium as a result of wall turnover (Merad et al., 1989) represents a significant potential source of phosphorus (Grant, 1979) that can be recovered by the combined activities of APases and APDases.

We and others have shown that teichuronic acid biosynthesis in *B. subtilis* is under the control of the Pho regulon (Müller et al., 1997; Qi & Hulett, 1998; Liu & Hulett, 1998). The Pho regulon is regulated by a two-component signal transduction pathway consisting of proteins PhoP and PhoR (Seki et al., 1987, 1988), equivalent to PhoB and PhoR of *Escherichia coli*.
respectively (Tommassen et al., 1982; Makino et al., 1985, 1986). PhoR is a membrane-spanning sensor protein with histidine protein kinase (HPKase) activity that, at low phosphate concentrations, activates its cognate response regulator, PhoP. Two other signal transduction pathways interact with PhoP/PhoR: Spo0A ~ P, the activated product of the phosphorelay (Hoch, 1998) that is responsible for the induction of sporulation, terminates the phosphate response (Jensen et al., 1993), while ResD/ResE are required for the full induction of the Pho regulon (Hulett, 1995) by modulating the expression of the phoPR operon. In this paper we identify a large operon (tua) required for the synthesis of teichuronic acid and confirm that the influence of PhoR on teichuronic acid biosynthesis is due to its effect on controlling the transcriptional activity of this operon.

METHODS

Bacterial strains and plasmids. The strains of Bacillus subtilis used in this study were all derivatives of B. subtilis 168 (Anagnostopoulos & Spizizen, 1961) and are shown in Table 1. Integration vector pMutin2 (Vagner et al., 1994) was used to generate both a transcriptional reporter and translational fusion of tuaA. Escherichia coli XL-1 Blue (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F’ proAB lacI’ZM15, Tn10 (Tet’)]) was used as a host for the construction and propagation of recombinant plasmids. pUC19 (Amersham Pharmacia Biotech) was used to clone inverse PCR products and gel-extracted DNA fragments. Phagemid pBluescript SK + (Stratagene) was used to generate the RNA probe for Northern blotting.

Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype/phenotype</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td>B. subtilis 168</td>
<td>Prototrophic derivative of B. subtilis 168</td>
<td>Laboratory collection</td>
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<tr>
<td>GCH504</td>
<td>trpC pheA1 phoRBall1::Te’</td>
<td>Hulett et al. (1994a)</td>
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<td>MH5124</td>
<td>GCH504 (phoRBall1::Te’) constructed by transformation of GCH504 with chromosomal DNA from MH5124</td>
<td>This study</td>
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<td>GCH504 (tuaA::pMutinTUA) constructed by transformation of GCH504 with integrational plasmid pMutinTUA</td>
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<td>GCH931</td>
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<td>Plasmids</td>
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<td>pUC19</td>
<td>General purpose E. coli cloning vector</td>
<td>Pharmacia</td>
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<td>pUC19 with ΔtuaA tuaBA containing EcoRI–PstI fragment</td>
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<td>pUC19 with S’ end of tuaA</td>
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<td>E. coli phagemid</td>
<td>Stratagene</td>
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<td>pBluescript SK + with EcoRI–PstI fragment containing ΔtuaA and tuaBA</td>
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<td>pMutin2</td>
<td>B. subtilis integrative vector with promoterless lacZ, constitutive lacI, IPTG-inducible PSpo1 promoter; Ap’ (E. coli), Em’/Lm’ (B. subtilis)</td>
<td>Vagner et al. (1998)</td>
</tr>
<tr>
<td>pMutinTUA</td>
<td>pMutin2 with 351 bp fragment from the S’ end of tuaA</td>
<td>This study</td>
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</table>

Media and growth conditions. B. subtilis was grown at 37 °C in vigorously shaking flasks. LB broth consisted of 1% Bacto tryptone, 0.05% Bacto yeast extract and 1% NaCl. When required, LB broth was solidified with 1.5% agar (Difco). Minimal medium used for the transformation of B. subtilis consisted of Spizizen’s minimal salts (Spizizen, 1958) supplemented with 0.5% glucose and 0.02% Casamino acids. Starvation medium, used in transformation, was the same except that Casamino acids was omitted. To analyse the phosphate starvation response in batch cultures, cultures were grown in low (LPDM) or high (HPDM) phosphate defined medium, as described previously (Müller et al., 1997). The phosphate concentration was 0.065 mM in LPDM and 3.5 mM in HPDM. The composition of chemostat medium has been described previously (Müller et al., 1997). The concentration of phosphate was 0.25 mM under phosphate-limiting conditions and 5 mM under Mg2+ limitation. The concentration of Mg2+ was kept constant at 75 μM under both limitations. SOC medium, used for the electrotransformation of E. coli, contained 20% (w/v) Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4 and 20 mM glucose. When required antibiotics were added to media at the following concentrations (μg ml−1): ampicillin (Ap), 100; erythromycin (Em), 1; lincomycin (Ln), 25; tetracycline (Tc), 10. The Pspo1 promoter was induced by the inclusion of IPTG (1 mM) in the growth medium.

Continuous cultures. Batch cultures, grown in high phosphate chemostat medium (pH adjusted to 7.0) to exponential phase, were used as inocula. Addition of culture medium was
controlled using a peristaltic pump (Watson–Marlow) and delivered at a rate of about 200 ml h⁻¹, equivalent to a mean generation time of approximately 3.5 h. Continuous cultures were grown in a 1 l fermentation vessel (Braun Biotech) with an impeller speed of 500 r.p.m. and an air flow of 2 l min⁻¹. Temperature and pH were maintained at 37 °C and 7.0, respectively. Foaming was controlled by the addition of polypropylene glycol 2025 (BDH) anti-foaming agent. Samples for monitoring growth and for enzyme assays (20 ml) were collected directly from the fermentation vessel while samples for cell wall analyses (200 ml) were collected via the overflow line and cooled to 4 °C. Growth was monitored by determining the dry weight of the bacteria retained on pre-weighed cellulose acetate membrane filters (pore size 0.45 µm, Whatman).

DNA manipulations. Restriction endonuclease digestion and ligation of DNA were performed essentially as described by Sam brook et al. (1989). Inverse and long-range PCR were carried out using the GeneAmp XL PCR kit (Perkin Elmer), according to the manufacturer’s instructions. Rib DNA polymerase XL was used for long-range PCR to ensure high efficiency DNA synthesis and the correction of mis-incorporated nucleotides. The reaction conditions were as follows: an initial denaturation step at 94 °C for 1 min, followed by 16 cycles of 30 s at 94 °C and 10 min at 66 °C, and 12 cycles of 30 s at 94 °C and 10 min at 66 °C; the latter being extended for 15 s for each cycle. Finally, the samples were incubated at 72 °C for 10 min to complete the reaction. General purpose PCR reactions used Taq DNA polymerase (Promega). The reaction conditions were 35 cycles of denaturation step at 92 °C for 1 min, followed by primer annealing for 1 min and primer extension at 72 °C for 1 min. The reaction was incubated for 72 °C for 10 min to ensure the completion of any prematurely terminated DNA strand. DNA amplifications for primer extension reactions used the proof-reading Pfu DNA polymerase (Stratagene) according to the manufacturer’s instructions. Restriction endonucleases and other enzymes were obtained from Boehringer Mannheim.

Plasmids were purified using plasmid DNA isolation kits (Qiagen). DNA extracted from agarose gels was purified using the QIAquick gel extraction kit (Qiagen). DNA sequencing was carried out using either the T7 Sequenase DNA sequencing kit (version 2.0; Amersham Pharmacia Biotech) or was carried out using either the T7 Sequenase DNA polymerase (Stratagene) according to the manufacturer’s instructions. Restriction endonucleases and other enzymes were obtained from Boehringer Mannheim.

Enzyme assays. The presence of APase activity in colonies growing on the surface of agar plates was determined qualitatively by overlaying the colonies with sterile filter paper moistened with p-nitrophenylphosphate (pNPP; 1 mg ml⁻¹ in 1 M Tris/HCl, pH 8.0; Yamane & Maruo, 1978). The development of a bright yellow colour, resulting from the hydrolysis of pNPP, was indicative of a positive reaction. APase activity of liquid cultures was determined as described by Nicholson & Setlow (1990). One unit of enzyme activity was defined as the amount of enzyme hydrolysing 1 µmol pNPP in 1 min at 30 °C. Specific activity was determined in relation to culture optical density (600 nm) in batch cultures and to protein in continuous cultures. β-Galactosidase activity was assayed using the method of Miller (1972). One unit of enzyme activity was defined as the amount of enzyme hydrolysing 109 µmol ONPG in 1 min at 28 °C. The specific activity was based on the concentration of protein in the extracts. Protein concentration was determined using a Coomassie-brilliant-blue-based protein assay kit (Bio-Rad).

Cell wall analysis. Cell walls were isolated and purified as described previously (Merad et al., 1989). Following digestion of dry cell walls with ethanolic magnesium nitrate (Ames, 1966), teichoic acid content was estimated as total organic phosphorus by the method of Chen et al. (1956). Teichuronic acid was estimated as total uronic acid in aqueous suspension of cell walls by the method of Blumenkrantz & Asboe-Hansen (1973), using glucuronolactone as standard.

Transformation. B. subtilis was transformed with plasmid and chromosomal DNA using the ‘Groningen’ method as described by Bron (1990). E. coli was transformed by electrotransformation. Electrocompetent cells were transformed in electroporation cuvettes (0.2 cm electrode gap) using a Gene Pulser, according to the manufacturer’s instructions (Bio-Rad). A pulse of 25 F, capacitance, 2.5 kV and 200 µl resistance was applied to the mixture, giving a time constant of 4.5–5.0 ms. Aliquots (200 µl) were plated onto LB agar plates containing the required selective antibiotics and incubated at 37 °C overnight.

Labelling and hybridization of nucleic acid probes. A non-radioactive digoxigenin (DIG) system was used to label DNA and RNA for hybridization and subsequent luminescent detection. DNA was labelled with a DIG DNA labelling kit (Boehringer Mannheim), as recommended by the manufacturer. Labelled antisense mRNA probes were prepared in vitro. The EcoRI–PstI fragment of the B. subtilis chromosome downstream of the lytC gene, encoding most of the tuaA gene and the 5′ end of tuaB, was cloned into phagemid Bluescript SK + adjacent to the T3 promoter (pM12). The vector, linearized with EcoRI, was used to generate labelled RNA using T3 RNA polymerase and a DIG RNA labelling kit (Boehringer Mannheim), as recommended by the manufacturer.

Northern blot analysis. Total RNA was isolated from cultures of B. subtilis using the RNEasy mini kit (Qiagen), according to manufacturer’s instructions. Strains were grown in LBDM or HPDM and samples were taken approximately 30 min after transition to stationary phase. Total RNA (approx. 40 µg) was separated in 1% agarose gels containing 2 µg ml⁻¹ formaldehyde and blotted by capillary transfer to positively charged nylon membrane (Boehringer Mannheim) using 20 × SSC buffer, as described by Sam brook et al. (1989). RNA was cross-linked to the membrane by exposure to UV light. Prehybridization was carried out for 1–2 h in hybridization solution [5 × SSC, 50% (v/v) deionized formamide, 0.1% sodium laurylsarcosine, 0.02% SDS, 2% blocking reagent] at 68 °C. The DIG-labelled probe was antisense RNA synthesized in vitro from pML12. The denatured probe (100 °C, 10 min) was added to hybridization solution and hybridized overnight at 68 °C. Membranes were washed twice for 15 min with 2 × SSC, 0.1% SDS at room temperature, twice for 15 min with 0.5 × SSC at room temperature and once with 0.1 × SSC, 0.1% SDS at 68 °C. The membranes were exposed to Kodak BioMax MS Film. The sizes of transcripts were determined by comparison with an RNA size marker (0.28–6.58 kb; Promega).

RESULTS

Cloning and sequencing of the 5′ end of the tua operon

The tua operon was previously reported to be located downstream of the lytC gene on the B. subtilis chromosome (Soldo et al., 1999) and this region was therefore amplified by inverse PCR. Chromosomal DNA from B. subtilis strain GCH504 was digested with PstI, which cuts the chromosome upstream of lytC, and the
generated fragments self-ligated at very low DNA concentrations. The circularized fragments were used as templates for inverse long-range PCR, using primers annealing upstream of the EcoRI site at the end of the known tuaA sequence (Lazarevic et al., 1992): Pr1 (downstream primer), 5′-GTGCATGTATACTGAG-3′; and Pr2 (upstream primer), 5′-TCATAAC-3′. A fragment of approximately 3 kb was generated and this was purified and subsequently amplified by long-range PCR, using the same primer pair. Digestion with EcoRI and PstI resulted in two main fragments, one of which was the expected size (1.9 kb) for the region between the Pr2 primer site and the PstI site. The other fragment (1.2 kb) was presumed to correspond to sequences downstream of the original EcoRI site and terminating at the next PstI site (within tuaB); this fragment was cloned into pUC19 and sequenced using a combination of universal and specific primers. The location of this region downstream of lytC was confirmed by PCR, using primers within lytC and the newly sequenced region. The sequence was identical to that published for the entire genome sequence of B. subtilis strain 168 (Kunst et al., 1997) which also indicated that the tua operon contains eight genes (tuaA–H) and is 9.0 kb in size.

Analysis of the DNA sequence immediately downstream of lytC revealed the presence of a stem–loop structure (ΔG value of –24.2 kcal; Tinoco et al., 1973), showing a strong resemblance to an intrinsic (i.e. ρ-independent) transcription termination element, and two ORFs, one starting just before the newly sequenced EcoRI–PstI fragment, the other within this fragment and extending beyond the PstI site. The first of these ORFs, tuaA, uses the relatively unusual GUG start codon (Kunst et al., 1997) and is preceded by a putative ribosome-binding site (RBS) with a ΔG value of –13.8 kcal (Tinoco et al., 1973). TuaA has a calculated molecular mass of 20.2 kDa and shows homology to glucosyl transferases, such as WcaJ of E. coli encoding a UDP-glucose lipid carrier transferase (Stevenson et al., 1996), which are involved in the linkage of sugars to carrier lipids. TuaA is therefore likely to be involved in the linkage of N-acetylmuramaldehyde to undecaprenyl carrier lipid. The second ORF, tuaB, was located 150 nt downstream of the stop codon of tuaA. It was preceded by a putative RBS with a ΔG value of –8.6 kcal (Tinoco et al., 1973) and uses AUG as the start codon. TuaB shows homology with membrane transport proteins involved in exopoly-saccharide biosynthesis, such as E. coli WzxC, an integral membrane transport protein involved in lipopolysaccharide biosynthesis (Itoh et al., 1996). TuaB may therefore play a role in the transport of repeating teichuronic acid subunits, prior to assembly of the polymer on the trans side of the cytoplasmic membrane (Archibald et al., 1993).

Construction of strain 168tuaA::pMutin2TUA

To confirm the involvement of the putative tua operon in the synthesis of teichuronic acid, a fragment from the 5′ end of the first ORF (tuaA) was cloned into the integrational vector pMutin2 (Fig. 1), using E. coli as an intermediate host. The first 351 nt of the putative ORF, including a putative RBS and start codon, was amplified by PCR using primers Pr10 (5′-GCGCGCCGCGGATCCGAGGTGTGTGTCAGTGAGTGC-3′) and Pr11 (5′-GAGGTGTGTGTCAGTGAGTGC-3′) and uses the relatively unusual GUG start codon (Kunst et al., 1997) and is preceded by a putative ribosome-binding site (RBS) with a ΔG value of –13.8 kcal (Tinoco et al., 1973). TuaA has a calculated molecular mass of 20.2 kDa and shows homology to glucosyl transferases, such as WcaJ of E. coli encoding a UDP-glucose lipid carrier transferase (Stevenson et al., 1996), which are involved in the linkage of sugars to carrier lipids. TuaA is therefore likely to be involved in the linkage of N-acetylmuramaldehyde to undecaprenyl carrier lipid. The second ORF, tuaB, was located 150 nt downstream of the stop codon of tuaA. It was preceded by a putative RBS with a ΔG value of –8.6 kcal (Tinoco et al., 1973) and uses AUG as the start codon. TuaB shows homology with membrane transport proteins involved in exopoly-saccharide biosynthesis, such as E. coli WzxC, an integral membrane transport protein involved in lipopolysaccharide biosynthesis (Itoh et al., 1996). TuaB may therefore play a role in the transport of repeating teichuronic acid subunits, prior to assembly of the polymer on the trans side of the cytoplasmic membrane (Archibald et al., 1993).

![Fig. 1. Integration of pMutinTUA into the chromosomal copy of tuaA via a single crossover recombination. Ap, ampicillin resistance gene (E. coli); Δ, 3′ deletion; Em, erythromycin/lincomycin resistance gene (B. subtilis); lacI, gene encoding the E. coli lactose repressor; lacZ, gene encoding the E. coli β-galactosidase; Pp spac, IPTG-inducible promoter; Ptua, tua operon promoter.](image-url)
GCGCGCAGCTCCCGATCTTTGGTCAACCG-
CG-3'), both of which had GC clamps and BamHI recognition sites (indicated in bold) incorporated at their 5' ends. The fragment was cloned into BamHI-digested pMutin2 (Vagner et al., 1998) to generate pMutinTUA, which was subsequently integrated into the chromosome of *B. subtilis* 168 via a single (Campbell-type) crossover recombination (Fig. 1).

The presence and orientation of the insert was confirmed by PCR using combinations of primers flanking the pMutin2 multiple cloning site and primers internal to the cloned fragment. The presence of a single copy of pMutin2, omitting the smallest fragment that contains EcoRI or HindIII. The digested DNA was probed with EcoRI or HindIII. The digested DNA was probed with EcoRV-digested fragments of pMutin2, omitting the smallest fragment that contains DNA derived from the spoV region of the *B. subtilis* chromosome. Appropriately sized fragments hybridizing to the pMutin2 probe were detected in chromosomal DNA from strain GCH504 but not strain GCH504 (data not shown).

Strain GCH931 has a lacZ reporter gene located downstream of a 3'-truncated version of tuaA (tuaΔ) that is transcriptionally fused to the putative tua promoter (P tua). A second, intact copy of tuaA, together with the remaining genes in the tua operon, is located downstream of an IPTG-inducible Pspac promoter. This construct therefore permits not only the monitoring of P tua activity via β-galactosidase synthesis, but also controlled expression of an intact tua operon (Fig. 1).

To study the involvement of PhoP/PhoR in the regulation of P tua, a deletion mutation was introduced into the phoR gene of strain GCH931. Chromosomal DNA from MH5124 (*phoRΔBall::Tc*), containing a phoR gene insertionally inactivated by the replacement of an internal Ball fragment with a tetracycline resistance gene (Hulett et al., 1994a), was transformed into strains GCH504 and GCH931. The resulting strains, GCH930 (*phoRΔBall::Tc*) and GCH932 (tuaA::pMutinTUA phoRΔBall::Tc), had inactivated phoR genes, as confirmed by their inability to induce APase activity under phosphate starvation. The presence of phoRΔBall::Tc was also confirmed by PCR, using primers that flank the phoR gene. Derivatives with phoRΔBall::Tc produced a larger PCR product with respect to strain GCH504.

Expression of the tua operon

The transcriptional activity of the tua operon promoter was monitored using the lacZ transcriptional reporter located downstream of a 3'-truncated copy of tuaA (Fig. 1). Strains GCH504, GCH931 and GCH932 were grown in batch culture under phosphate starvation (LPDM) and phosphate-replete (HPDM) conditions. APase activity was used as a reporter for the induction of the Pho regulon.

In the case of the strain GCH504 and GCH931, APase was induced towards the end of exponential phase and during early stationary phase in LPDM but not in HPDM (Fig. 2a, b). In the case of GCH932, APase was not induced during growth on either medium (Fig. 2c).

![Fig. 2. Growth, APase and β-galactosidase activities of (a) wild-type strain GCH504, (b) pMutinTUA integrant GCH931 and (c) phoR-deficient version of the integrant (GCH932). Strains were grown in LPDM (□) or HPDM (■) and growth was monitored by measuring OD600. APase (○, ●) and β-galactosidase (△, ▲) activities were determined in LPDM (○, △) and HPDM (●, ▲).](Image)
PhoR for induction (Müller et al., 1997; Qi & Hulett, 1998).

To confirm the role of the tua operon in teichuronic acid synthesis, cell walls were extracted from strains GCH504, GCH930, GCH931 and GCH932 after growth in LPDM and HPDM (Table 2). Walls of GCH504 showed approximately half the amount of teichoic acid and five times the amount of teichuronic acid in LPDM-grown cells compared to HPDM-grown cells. The presence of IPTG in the culture medium had no influence on the anionic polymer composition of the walls. There was no change in the anionic polymer composition of GCH930 (phoR::Tc), irrespective of the growth conditions. In the case of strain GCH931 (tuaA::pMutinTUA), the concentration of teichuronic acid was similarly low on both media in the absence of IPTG. However, when the tua operon was induced with IPTG via the Pspac promoter, the amount of teichuronic acid in the wall increased about fivefold. The teichoic acid concentration reflected the phosphate status of the culture rather than the amount of teichuronic acid present, indicating that the synthesis of these anionic polymers is, to a large extent, controlled independently. This was confirmed by the data obtained with GCH932 (tuaA::pMutinTUA phoR::Tc) which showed IPTG-inducible teichuronic acid synthesis, but little or no repression of teichoic acid synthesis.

A limitation of applying phosphate starvation to batch cultures is that the resulting growth arrest is accompanied by a marked decrease in cell wall synthesis and consequently the cells have only a limited ability to replace their wall anionic polymers by turnover (Merad et al., 1989). To study the changes in cell wall composition under conditions of cell growth and wall turnover, B. subtilis was grown in continuous cultures under phosphate-replete (Mg²⁺-limited) or phosphate-limited conditions.

Growth of the wild-type strain (GCH504) under phosphate-replete conditions (Table 3) resulted in a wall consisting almost exclusively (> 95%) of teichoic acids. Under phosphate-limited growth the amount of anionic polymer in the wall fell by ca 35% and, in line with previously published data (Müller et al., 1997), the wall contained a mixture of anionic polymers: about 60% teichoic acids and 40% teichuronic acids.

When strain GCH931 (tuaA::pMutinTUA) was grown under phosphate-replete conditions, the amounts of teichoic and teichuronic acids in the wall were similar to strain GCH504 under the same conditions. APase and β-galactosidase activities were low, reflecting a lack of induction of the Pho regulon. However, when the culture was shifted to phosphate limitation there was a large (> 25-fold) increase in APase and β-galactosidase activities, accompanied by a marked (~ 3-fold) reduction in the amount of teichoic acids; there was no increase in teichuronic acids since, in this mutant, the expression of the putative tua operon is under the control of the Pspac promoter. The inclusion of IPTG in the culture medium led to the induction of teichuronic acid synthesis to a level that was about 75% that of the wild-type under phosphate limitation, while the amount of teichoic acid remained similar. At this time in the chemostat cycle, the amounts of β-galactosidase and APase were about 50–75% of those observed under phosphate limitation in the absence of IPTG. This may indicate that prolonged growth under phosphate limitation induces

### Table 2. Anionic polymer composition of B. subtilis strain 168 and constructed mutants grown to stationary phase in defined medium with or without phosphate limitation and with or without IPTG, an inducer of the Pspac promoter located immediately upstream of the tua operon

Data are the means of two separate measurements that varied by less than 20%.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth medium</th>
<th>IPTG</th>
<th>Teichuronic acid [µmol (mg wall)⁻¹]</th>
<th>Teichoic acid [µmol (mg wall)⁻¹]</th>
<th>Anionic polymers [µmol (mg wall)⁻¹]</th>
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</tbody>
</table>
secondary regulators, such as Spo0A, that depress the Pho regulon (Hulett et al., 1994b). When IPTG was added to phosphate-replete cultures, normal amounts of teichoic acids were observed, but there was a threefold increase in teichuronic acid synthesis compared with growth in the absence of IPTG and with that of the wild-type. The amounts of β-galactosidase and APase were more comparable to those observed in phosphate-replete (Mg\textsuperscript{2+}-limited) conditions in the absence of IPTG, than to those under phosphate limitation.

Transcription analysis of the tua operon

RNA was isolated from stationary-phase cultures of strains GCH504 and GCH930 (phoR::BalI::Tc\textsuperscript{+}), grown in either HPDM or LPDM. The RNA was subjected to Northern blot analysis, using as probe DIG-labelled antisense RNA derived from the EcoRI–PstI fragment of pML12. In the case of the GCH504 grown on LPDM, a number of well distinguished bands were observed on the autoradiograph, while no hybridizing bands were observed under phosphate-replete conditions or with strain GCH930 on either growth medium (Fig. 3). The largest product, which was approximately 9 kb in size, is consistent with a full-length transcript for the putative tua operon, confirming that the tua genes are organized as a single operon and transcribed as a single, polygenic mRNA. The smaller products, of approximately 4, 3·5, 2·5, 2·0 and 1·8 kb, are presumably either prematurely terminated transcripts or processed products.

Conclusions

The data in this paper confirm that an operon of eight genes, the putative tua operon located downstream of the B. subtilis lytC gene, is required for the synthesis of teichuronic acid. The operon is located at position 3658–3649 kb in relation to the B. subtilis origin of replication (oriC; 0/4215 kb) (Kunst et al., 1997).

We have used the integration vector pMutin2 to interrupt the first gene in the operon, namely tuaA, simultaneously generating a transcription fusion to a lacZ reporter gene and placing the entire tua operon under the control of the IPTG-inducible P\textsubscript{spac} promoter. When the resulting strain, GCH931, was grown under phosphate-replete conditions, either in batch or continuous culture, teichoic acids were the main cell wall polymer and negligible amounts of teichuronic acids were detected. However, when phosphate was growth-limiting, the amount of teichoic acids decreased and, in the absence of teichuronic acid synthesis, the total amount of wall-associated anionic polymer was reduced. The deficiency in teichuronic acid synthesis was to some extent compensated by the incomplete shutdown in teicholic acid synthesis that is observed in some strains of B. subtilis 168. The data were therefore consistent with those of Mauel et al. (1994), obtained using lacZ transcriptional reporters to the divergent tag operons, which show a marked reduction in tag gene expression during phosphate starvation, followed by a low level of constitutive tag gene expression.

The observation that, in the wild-type, teichuronic acid synthesis was not able to compensate completely for the

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**Table 3.** Anionic polymer composition of wild-type B. subtilis GCH504 and mutant GCH931 grown in continuous culture under phosphate or Mg\textsuperscript{2+} limitation and with or without the addition of IPTG, the inducer of the P\textsubscript{spac} promoter.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Limiting substrate</th>
<th>IPTG</th>
<th>β-Galactosidase [U (mg protein)\textsuperscript{−1}][U (mg protein)\textsuperscript{−1}]</th>
<th>APase</th>
<th>Teichuronic acid [µmol (mg wall)\textsuperscript{−1}]</th>
<th>Teichoic acid [µmol (mg wall)\textsuperscript{−1}]</th>
<th>Total anionic polymers [µmol (mg wall)\textsuperscript{−1}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCH504</td>
<td>Mg\textsuperscript{2+}</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0·03</td>
<td>0·92</td>
<td>0·95</td>
</tr>
<tr>
<td></td>
<td>Phosphate</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0·24</td>
<td>0·38</td>
<td>0·62</td>
</tr>
<tr>
<td>GCH931</td>
<td>Mg\textsuperscript{2+}</td>
<td>−</td>
<td>75</td>
<td>0·5</td>
<td>0·03</td>
<td>0·83</td>
<td>0·86</td>
</tr>
<tr>
<td>(tuaA::pMutinTUA)Phosphate</td>
<td>−</td>
<td>1614</td>
<td>23·3</td>
<td>0·02</td>
<td>0·28</td>
<td>0·30</td>
<td>0·30</td>
</tr>
<tr>
<td></td>
<td>Phosphate</td>
<td>+</td>
<td>1228</td>
<td>11·5</td>
<td>0·18</td>
<td>0·35</td>
<td>0·53</td>
</tr>
<tr>
<td></td>
<td>Mg\textsuperscript{2+}</td>
<td>+</td>
<td>136</td>
<td>2·3</td>
<td>0·10</td>
<td>1·00</td>
<td>1·10</td>
</tr>
</tbody>
</table>

Data are the means of three separate measurements that varied by less than 20%. NA, Not applicable.
observed reduction in teichoic acid synthesis under phosphate limitation was in contrast to the data obtained by Lang and colleagues (Lang et al., 1982) who reported an almost complete replacement of one polymer type with the other. The incomplete shutdown of teichoic acid synthesis was particularly clear from the chemostat studies in which continued growth and associated cell wall turnover under phosphate-limited growth means that the relative rates of anionic polymer synthesis are fully reflected in cell wall composition (Table 3). In the case of GCH4931 (tuaA::pMutinTUA), the absence of compensatory teichuronic acid synthesis under phosphate limitation resulted in a marked decrease in wall anionic polymer content to about one-third of that present under phosphate-replete conditions. The cells exhibited a coccal cell morphology (data not shown), which accords with previous observations with conditional mutants in which teichoic acid biosynthesis is inhibited (Boylan et al., 1972). The inclusion of IPTG into phosphate-limited chemostat medium induced the tua operon, resulting in a greater than sixfold increase in teichuronic acid synthesis and increasing total wall anionic polymers. Under these conditions the cells exhibited a more normal rod-shaped morphology (data not shown).

Transcriptional analysis showed that the tua operon is transcribed on a single transcript of ~9 kb, although the presence of other smaller products hybridizing to the tuaA probe suggests either extensive premature termination or mRNA processing.

REFERENCES


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