Assay for UDPglucose 6-dehydrogenase in phosphate-starved cells: gene *tuaD* of *Bacillus subtilis* 168 encodes the UDPglucose 6-dehydrogenase involved in teichuronic acid synthesis

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A novel assay permitting the detection of UDPglucose 6-dehydrogenase activity in cell-free extracts obtained from phosphate-starved cultures of *Bacillus subtilis* is described. The critical step, the separation of phosphate-starvation-induced exo-enzymes, phosphatases and phosphodiesterases from the cytoplasmic fraction containing the UDPglucose dehydrogenase, was achieved by protoplasting and removal of the periplasmic fraction by protoplast washing. Using this method, the following were unambiguously demonstrated: (i) the presence in the cytoplasm of an enzymic activity oxidizing UDPglucose to UDPglucuronic acid, and (ii) that detection of the activity in whole-cell-free extracts is prevented by the presence of 'periplasmic' enzymes catalysing the degradation of the sugar nucleotides. With this method, several *B. subtilis* 168 mutants unable to synthesize teichuronic acid were examined. Strains inactivated in gene *tuaD*, whose product shares homology with UDPglucose 6-dehydrogenase and GDPmannose 6-dehydrogenase from other organisms, were shown to lack UDPglucose 6-dehydrogenase activity. Anion exchange chromatography revealed that mutants deficient in *tuaD* lacked a cytoplasmic UDPglucuronate pool.

Keywords: UDPglucose 6-dehydrogenase, enzyme assay, *Bacillus subtilis*, teichuronic acid

INTRODUCTION

UDPglucose 6-dehydrogenase (UDPG-DH, EC 1.1.1.22) converts UDPglucose (UDPG) into UDPglucuronic acid (UDPGUA). The latter, as well as UDP-N-acetylgalactosamine, are precursors of teichuronic acid of *Bacillus licheniformis* 94 (Hughes, 1970; Janczura *et al.*, 1961) and *Bacillus subtilis* W23 (Wright & Heckel, 1975). Ellwood & Tempest (1969) and Kruyssen *et al.* (1980) have incorporated UDPG-DH into the hypothetical metabolic pathway leading to teichuronic acid. Although UDPG-DH activity was assayed in cell-free extracts from many organisms, its activity was never conclusively established in any

Bacillus species. Indeed, the only evidence in favour of UDPG-DH activity in a cell-free extract of *B. subtilis* 168 *trp* was obtained by an indirect (Rosenberger, 1976) and possibly inappropriate method (see below).

Analysis of recently obtained nucleotide sequences of *B. subtilis* 168 allowed the identification of an operon consisting of eight genes, *tuaA–H*, involved in the synthesis of teichuronic acid (Soldo *et al.*, 1999). The product of *tuaD* exhibits a high similarity with proteins belonging to the UDPglucose/GDPmannose dehydrogenase family, as well as with the products of *ywqF* and *ytca*, two *B. subtilis* 168 genes, located in the same chromosomal region as the *tua* operon.

In this paper, we report (i) a reliable method for preparing cell-free extracts allowing a spectrophotometric assay of UDPG-DH activity, (ii) a demonstration that *tuaD* of *B. subtilis* 168 is the structural gene of UDPG-DH, a conclusion that could not have been

Abbreviations: UDPG, uridine 5'-diphosphoglucose (UDPglucose); UDPGUA, uridine 5'-diphosphoglucuronic acid (UDPglucuronic acid); UDPG-DH, UDPglucose 6-dehydrogenase.

reached from sequence comparison, and (iii) that *TuaD* is responsible for the synthesis of the entire UDPGUA pool in phosphate-starved *B. subtilis*.

METHODS

Bacterial strains. Strain 168, the *B. subtilis* reference strain, whose genome nucleotide sequence was reported by Kunst *et al.* (1997), was used as the parent strain for the construction of mutants deficient in teichuronic acid synthesis. Strains L16050 *tuaC*, L16051 *tuaD-H*, L16054 *tuaD* and L16071 *tuaG* were obtained by Campbell insertion of plasmids constructed with pMTL20EC (Chambers *et al.*, 1988), a vector conferring chloramphenicol resistance and inserts corresponding to *SubtiList* (Moszer *et al.*, 1995) base numbers 3655664–3654953, 3654128–3653562, 3654439–3654016 and 3650860–3650480, respectively. Strains L16050 *tuaC*, L16054 *tuaD* and L16071 *tuaG* are able to express *tua* genes located downstream of the inactivated ones, owing to the presence of a plasmid-borne promoter. Details on the molecular sequence of the *tua* operon and on the inability of the mutants to incorporate teichuronic acids in their cell wall can be found elsewhere (Soldo *et al.*, 1999).

Growth conditions. Wild-type and mutants were grown in low-phosphate medium (Grant, 1979) supplemented with 300 μ M inorganic phosphate. For *tua*-deficient mutants, 3 μ g chloramphenicol ml^{-1} was added to prevent reversion to *tua*⁺, due to plasmid excision. Exponentially growing cells at a low density were used to inoculate 1 l cultures in 5 l flasks which were further incubated, by shaking at 37 °C, and harvested about 12 h after the onset of phosphate limitation, which was assessed as described by Grant (1979). Cells at a concentration of 0.4 g dry weight l^{-1} were pelleted, washed once with ice-cold bidistilled water and stored frozen at –20 °C.

Preparation and chromatography of nucleotide precursors. A frozen cell pellet corresponding to 330 ml culture was thawed in 3 ml boiling water, incubated for 15 min at 100 °C and centrifuged. The supernatant was mixed with 1 ml activated charcoal GR (Merck) that had been washed with acetic acid and ethanolic ammonia, dried and resuspended in water at 50 mg ml^{-1} . The mixture was incubated for 1 h at room temperature with gentle shaking. Charcoal was collected by centrifugation, washed three times with 5 ml 100 mM acetic acid and extracted twice for 60 min at 70 °C with 2 ml 25 mM NH_3 in 50% ethanol. The two extracts were pooled, frozen, lyophilized and resuspended in 1 ml water to yield a crude preparation of nucleotide precursors.

A 500 μ l portion of the nucleotide precursors preparation was diluted to 2 ml with 10 mM ammonium acetate, filtered through the 0.22 μ m membrane filter to remove charcoal particles and loaded onto a strong anion-exchanger FPLC column (MonoQ HR 5/5, Pharmacia) preconditioned in 10 mM ammonium acetate. Elution at 0.5 ml min^{-1} with a 50 min linear concentration gradient was performed with 1 M ammonium acetate as the second buffer and monitored by absorbance at 275 nm. The peak corresponding to UDPGUA was identified by (i) its retention time, (ii) its UV absorption spectrum and (iii) the Blumenkrantz & Asboe-Hansen (1973) assay for uronic acid, for which the peak material reacted positively, providing an amount of UDPGUA within 10% of that deduced from the absorbance spectrum. The UDPGUA peak was integrated and quantified by comparison to a reference curve obtained by chromatography of known amounts of commercial UDPGUA. To determine the yield of

the UDPGUA preparation, known amounts of UDPGUA were added to samples of the crude extract of nucleotide precursors before charcoal adsorption. Purification and chromatography resulted in a 25–45% loss of the internal standard. UDPGUA amounts in unspiked samples were corrected accordingly. The method was capable of detecting 5 nmol UDPGUA.

Preparation of the cell-free extract. A thawed pellet corresponding to a 330 ml sample of phosphate-limited culture was resuspended in 8 ml protoplasting buffer (50 mM Tris/HCl pH 8.6; 100 mM KCl; 25%, w/v, sucrose; 1 g lysozyme l^{-1}) and incubated for 20 min at 37 °C. The sample, cooled on ice, was subsequently processed at 4 °C. Protoplasts were harvested by centrifugation, washed once with 8 ml lysozyme-free protoplasting buffer and lysed by resuspension in 2 ml lysis buffer (50 mM potassium phosphate pH 6.8, 100 mM KCl, 1 mM EDTA, 0.1 mM PMSF). The lysate was homogenized by a brief ultrasonication and centrifuged for 30 min at 100 000 g. Finally, the supernatant was dialysed overnight against 50 mM potassium phosphate pH 6.8, 100 mM KCl, 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, in a Slide-A-Lyzer cassette (molecular mass cut-off 10 000 Da; Pierce). The presence of Triton X-100 and DTT, associated with slightly higher activity recovery, was not essential.

UDPG-DH assay. The dialysed cell-free extract (25 μ l) was diluted with 175 μ l 500 mM glycylglycine/NaOH pH 9.2, to which 200 μ l 10 mM NAD^+ and 10 mM UDPG, dissolved in the same buffer, were added. The sample was placed in a thermostated 8-cell changer of a Perkin-Elmer spectrophotometer at 37 °C and absorbance at 340 nm was recorded at 1 min intervals for 30 min. A control sample without UDPG was run in parallel.

The stoichiometry of NADH and UDPGUA production was determined in a 2.5 ml reaction mixture from strain L16071 *tuaG* characterized by a high level of UDPG-DH. The absorbance spectrum of the assay mixture was periodically recorded to quantify NADH. At regular intervals of time, 400 μ l aliquots were removed, mixed with 1.6 ml ethanol, kept on ice for 1 h and centrifuged. The supernatant was collected, lyophilized, resuspended in 10 mM ammonium acetate and chromatographed to quantify UDPGUA as described above.

Other methods. Protein content was assayed with the BCA reagent (Pierce) using bovine serum albumin as standard. UDPG was assayed enzymically with beef liver UDPG-DH (Boehringer Mannheim) according to Strominger *et al.* (1957). Phosphoglucosomutase was assayed according to Forsberg *et al.* (1973). Glucose-6-phosphate dehydrogenase was assayed according to Ujita & Kimura (1982) using NAD^+ as electron acceptor. Nucleoside diphosphate sugar hydrolase (Mauck & Glaser, 1970) was detected on isoelectric focusing gels by the direct phosphodiesterase staining method of Némoy *et al.* (1983) using UDPG as substrate.

RESULTS AND DISCUSSION

UDPG-DH is required for the synthesis of UDPGUA, one of the precursors of teichuronic acid. In *B. subtilis*, the synthesis of the latter polymer (Soldo *et al.*, 1999) is induced under conditions of phosphate limitation. However, it is well known that phosphate starvation leads to induction of the synthesis of several phosphatases, phosphodiesterases and nucleases. The parallel appearance of these hydrolytic enzymes may significantly complicate assays for enzymes involved in

Fig. 1. Assay of UDPG-DH activity in the cell-free extract of strain L16071 *tuaG*. Kinetics of appearance of NADH (○) and UDPGUA (□) in the reaction mixture.

nucleotide-diphosphate-sugar metabolism through rapid nucleotide degradation. The report of Merchante *et al.* (1995), strongly suggesting the existence of a *B. subtilis* periplasmic fraction containing these hydrolytic enzymes, offers a possibility to devise reliable assays by avoiding the mixing of the periplasmic fraction with the relevant cytoplasmic fraction. The advantages of the periplasm/cytoplasm separation are illustrated by the straightforward spectrometric assay for UDPG-DH described below.

UDPG-DH assay in *B. subtilis*

Cell-free extracts were obtained from cells grown as a batch culture in a low-phosphate medium. Twelve hours after the onset of phosphate limitation, cells were harvested, washed and protoplasted. Protoplasts were washed and disrupted. Membrane debris was removed by ultracentrifugation and the enzyme-containing supernatant was dialysed. UDPG-DH activity, monitored by NAD^+ reduction, was detected only when UDPG was added to the assay mixture. NAD^+ reduction in the presence of UDPG increased linearly until the A_{340} reached a value of 1. The rate of NADH formation decreased subsequently, possibly due to an inhibitory effect of the produced NADH (Ordman & Kirkwood, 1977). Indeed, addition of 40 μM NADH to the reaction mixture led to a loss of over 90% of the UDPG-DH activity. Quantification of NADH and UDPGUA, simultaneously accumulating in the reaction mixture, provided a ratio of 2.25 NAD^+ reduced per UDPG oxidized, as calculated from the slopes of regression lines (Fig. 1), in good agreement with the expected stoichiometrical coefficient of 2. These observations established that NAD^+ reduction in the assay is essentially due to the activity of UDPG-DH, which was stable for at least 24 h in extracts stored on ice.

Protoplasting and removal of the periplasmic fraction was essential for the detection of UDPG-DH activity. Cell-free extracts obtained from sonicated or French-

press-broken cells exhibited no UDPG-DH activity in the spectrophotometric assay. It should, however, be pointed out that complete protoplasting was unnecessary. Indeed, even a very limited lysozyme treatment – up to 5 min incubation in osmotically stabilized medium – was sufficient for the release of the periplasmic fraction (H. M. Pooley, unpublished). Observations of Merchante *et al.* (1995), Mauck & Glaser (1970) and M. Pagni (unpublished) revealed that, compared with the cytoplasmic fraction, phosphodiesterases of the periplasmic fraction are more diverse and their specific activity is at least 10 times higher. The final purification step, i.e. the dialysis of the ultracentrifugation supernatant, was also crucial for the recovery of UDPG-DH activity, which is hardly detectable in undialysed extract. The effect of dialysis might be accounted for by the removal of low-molecular-mass inhibitory compounds. For example, NADH and UDPxylose have been reported to inhibit the beef UDPG-DH (Zalitis & Feingold, 1969) and, as shown above, NADH was inhibitory to the *B. subtilis* enzyme.

The only previously reported UDPG-DH assay in *B. subtilis* (Rosenberger, 1976) employed cell-free extracts obtained by breaking cells with a French press. Without dialysis, these extracts were assayed for UDPG-DH activity with the radioassay of Davies & Dickinson (1972). The specificity of this radioassay, established on germinating lily pollen, was never confirmed for *Bacillus* extracts. The results obtained by Rosenberger (1976) with *B. subtilis* extracts are incompatible with our failure to detect UDPG-DH activity in undialysed extracts obtained with a French press. However, we believe that the activity obtained with this radioassay was an artefact most likely due to contamination of the cell-free extract by the nucleoside-diphosphate-sugar hydrolase activity originating from the periplasmic fraction. Indeed, the latter enzyme was shown to liberate glucose 1-phosphate from UDPG (Mauck & Glaser, 1970; see Methods). The highly active cytoplasmic phosphoglucumutase and the (NAD^+ -reducing) glucose-6-phosphate 1-dehydrogenase (Ujita & Kimura, 1982), whose presence in the extracts was confirmed (M. Pagni, unpublished), would, together with enzymes inherent to the Davies & Dickinson assay, eventually convert [^{14}C]glucose 1-phosphate into [^{14}C]gluconate, an acidic sugar, most probably indistinguishable from glucuronate by the paper chromatographic method of Davies & Dickinson (1972).

Sequence comparison and analysis of *tua*-deficient strains

Inspection of the *B. subtilis* 168 genome sequence (Kunst *et al.*, 1997) yields three genes – *tuaD*, *ytca* and *ywqF* – which encode enzymes belonging to the UDPglucose/GDPmannose dehydrogenase family. At the protein level, *TuaD* exhibits 45% and 51% identities with *Ytca* and *YwqF*, respectively. A search for similar proteins in the release 35 of the SWISS-PROT database yielded nine proteins with BLAST scores to either of *TuaD*, *Ytca* or

Table 1. UDPG-DH activity and UDPGUA precursor pools in *tua*-deficient strains, and in *B. subtilis* 168

Samples from batch cultures were collected 12 h after the onset of phosphate limitation.

Strain	Genotype*	UDPG-DH†	UDPGUA (µM)‡
168	<i>Tua</i> ⁺	2.8	203
L16050	<i>tuaC</i> §	1.0	60
L16051	<i>tuaD-H</i>	<0.1	<3
L16054	<i>tuaD</i> §	<0.1	<3
L16071	<i>tuaG</i> §	7.3	325

* Only markers relevant to teichuronic acid synthesis are indicated.

† µmol UDPG oxidized min⁻¹ (g protein)⁻¹.

‡ Concentration in a 1 ml solution containing nucleotide precursors extracted from a 330 ml culture.

§ Genes distal to the inactivated one are expressed from the constitutive plasmid-borne promoter.

|| Below the detection threshold.

YwqF of above 400, an arbitrarily chosen figure. Although all were members of the UDPglucose/GDPmannose dehydrogenase family, unambiguous biochemical identification of their substrates has been achieved for only two of them, i.e. KfD, a UDPG-DH of *Escherichia coli* (Petit *et al.*, 1995) and AlgD, a GDPmannose dehydrogenase, of *Azotobacter vinelandii* (Campos *et al.*, 1996). Further sequence analyses – multiple alignments of sequences and computation of their pairwise distances – revealed that the distance between KfD and AlgD is roughly comparable to that separating the three *B. subtilis* dehydrogenases, precluding the assignment of a specific substrate to any of the putative dehydrogenases *TuaD*, *YtcA* or *YwqF*. However, since *tuaD* belongs to an operon shown to be involved in teichuronic acid synthesis (Soldo *et al.*, 1999), it is likely that it encodes the UDPG-DH required for teichuronic acid synthesis. Therefore, several *tuaD*-deficient mutants were examined for UDPG-DH activity as well as for the accumulation of UDPG and UDPGUA.

Data presented in Table 1 show that specific inactivation of *tuaD*, achieved by insertional mutagenesis, is accompanied by the complete loss of UDPG-DH activity. Under the growth conditions used, this unambiguously identifies the product of gene *tuaD* as UDPG-DH and indicates that the products of neither of the other genes with sequence homology are involved in the oxidation of UDPG under phosphate-limiting conditions. To ascertain that UDPG is the *in vivo* substrate of UDPG-DH in *B. subtilis* 168 and to examine the effect of UDPG-DH deficiency on the cytoplasmic pools of UDPG and UDPGUA, we have measured the latter compounds in all *tuaD*-deficient strains, as well as in the parent strain. UDPGUA was present in *tuaD*⁺ strains and absent in *tuaD* strains L16051 *tuaD-H* and L16054 *tuaD* (Fig. 2,

Fig. 2. FPLC of nucleotide precursors extracted from *B. subtilis* 168 and mutant L16051 *tuaD-H* on a MonoQ column. Elution profiles as detected by absorbance at 275 nm (—) using a linear concentration gradient of 1 M ammonium acetate (---). The shaded peak corresponds to UDPGUA.

Table 1), confirming that *TuaD* is responsible for UDPGUA production during phosphate-limited growth. Enzymic quantification of UDPG in crude nucleotide-precursor preparations of the five examined strains did not reveal a significant accumulation of UDPG (not presented). The measured amounts were low, an order of magnitude lower than those of UDPGUA in strain 168. The apparent absence of UDPG accumulation in *tuaD* mutants may be accounted for by its channelling into other metabolic pathways, or by feedback inhibition of its synthesis.

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