Sequencing and analysis of the divergon comprising *gtaB*, the structural gene of UDP-glucose pyrophosphorylase of *Bacillus subtilis* 168

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(Received 6 April 1993; revised 25 June 1993; accepted 5 July 1993)

Nucleotide sequencing revealed that *gtaB*, the structural gene of UDP-glucose pyrophosphorylase (EC 2.7.7.9), is part of a divergon-like genetic entity. The latter consists of two monocistronic operons *gtaB* and *orfX*, transcribed from a 245 bp regulatory region, each encoding an acidic protein with a molecular mass of 33.0 and 42.6 kDa, respectively. *gtaB* is transcribed from a distal P^A promoter, and a proximal P^B promoter which is negatively controlled by the Sin protein. Sin-mediated transcriptional attenuation and enhancement of P^B and P^D, respectively, suggest that these promoters control functions which antagonize each other. Transcription of *orfX* is mediated by a P^A promoter. The regulatory region comprises four ATGAAA hexamers, present as two inverse repeats. Protein GtaB exhibits high homology to analogous prokaryotic enzymes, while OrfX shows 55.4% homology with the product of *Escherichia coli* o389, which is part of a regulatory unit involved in sugar processing. Mutations *gtaB*⁵¹⁵ and *gtaB*¹⁰⁰, which define different bacteriophage adsorption patterns, were sequenced. They are transitions leading to substitution of amino acids which occupy conserved positions, and are thus likely to be part of an enzyme active site. The nature of the possible receptors for defective bacteriophages PBSY and PBSZ is discussed.

Introduction

UDP-glucose pyrophosphorylase (UDPGPPase) catalyses the synthesis of UDP-glucose (UDPG) from glucose 1-phosphate and UTP. UDPG participates in a variety of enzymic reactions which include: (i) metabolism of polysaccharides (glycogen, starch, laminarin and cellulose),

disaccharides (sucrose, trehalose), β -glucosides, UDP-glucuronate and glucosyl-ceramide (Hassid, 1969; White *et al.*, 1973); (ii) galactose entry into glycolysis; (iii) synthesis of periplasmic membrane-derived oligosaccharides (Miller *et al.*, 1986) which could play a role in osmotic adaptation of Gram-negative bacteria (Schulman & Kennedy, 1977); (iv) glucosylation of hydroxymethylcytosine residues in cells infected by T-even bacteriophages (Shedlovsky & Brenner, 1963); and (v) synthesis of the core region of lipopolysaccharides in Gram-negative bacteria (Sundararajan *et al.*, 1962). In the Gram-positive bacterium *Bacillus subtilis*, strain 168, UDPG is required for the glucosylation of the poly(glycerol phosphate) [poly(groP)] (Young, 1967), the major cell wall teichoic acid (Baddiley, 1970), and for the polymerization of the secondary anionic polymer, the poly(3-*O*- β -D-glucopyranosyl-*N*-acetylgalactosamine 1-phosphate [poly(Glc-GalNAc 1-P)]) (Shibaev *et al.*, 1973).

The *B. subtilis* 168 gene *gtaB*, which encodes UDPGPPase, has been mapped in the *hisA* region (Young *et al.*, 1969; Pooley *et al.*, 1987), and subsequently physically localized close to the *tag* genes (Mauël *et al.*, 1989). The UDPGPPase activity in extracts of nine strains, each bearing a different *gtaB* mutation, was found to be below 2% of that of the wild-type. All

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Abbreviations: GalNAc, *N*-acetylgalactosamine; Cm^r, chloramphenicol-resistant; Em^r, erythromycin resistant; phage^{r/s}, phage resistance/sensitivity; poly(Glc-GalNAc 1-P), poly(3-*O*- β -D-glucopyranosyl-*N*-acetylgalactosamine 1-phosphate); poly(groP), poly(glycerol phosphate); RBS, ribosome-binding site; UDPG, UDP-glucose; UDPGPPase, UDP-glucose pyrophosphorylase.

The GenBank and EMBL accession number for the sequence reported in this paper is Z22516.

mutations were associated with resistance to bacteriophage ϕ 29 (Pooley, 1987). Study of phage sensitivity spectra has, however, revealed the existence of two classes of mutants. Those carrying a *gtaB* mutation were resistant to defective bacteriophages PBSY and PBSZ, while those endowed with the so-called *gtaB^s* mutation were, like the wild-type strain, sensitive to these phages. Although in both classes the content of cell-wall-associated galactosamine was significantly reduced, it was five times higher in strains with *gtaB^s* mutations than in those bearing *gtaB* ones.

To understand the regulation of UDPGPPase synthesis, as well as the difference between the above-mentioned phenotypes, we have sequenced the *gtaB* regulatory unit, and the mutant alleles *gtaB515* and *gtaB^s100*. We report the complete nucleotide sequence of the *gtaB*-containing divergon, which consists of two divergently transcribed monocistronic operons.

Methods

Bacterial strains, plasmids and phages. These are listed in Table 1. Fragments from the *B. subtilis* chromosomal DNA insert in phage λ 63 (Mauël *et al.*, 1989) were subcloned in pMTL20EC (Chambers *et al.*, 1988), except for the insert in plasmid 6301, which was cloned in pJH101 (Ferrari *et al.*, 1983) and maintained in *Escherichia coli* strain DH5.

Media and growth. *E. coli* strains were grown in LB medium containing the following selective agents: ampicillin (50 μ g ml⁻¹), erythromycin (10 μ g ml⁻¹), and chloramphenicol (10 μ g ml⁻¹). *B. subtilis* strains were grown in SPIZ I, SPIZ II, SA and LB media as previously described (Karamata & Gross, 1970). When required, media were supplemented with amino acids (20 μ g ml⁻¹), adenine (100 μ g ml⁻¹), chloramphenicol (3 μ g ml⁻¹) or both erythromycin (1 μ g ml⁻¹) and lincomycin (25 μ g ml⁻¹).

DNA preparation. *B. subtilis* chromosomal DNA was isolated by the method of Marmur (1961). Plasmid DNA was prepared by the boiling method miniprep (Del Sal *et al.*, 1988). DNA fragments, separated by electrophoresis on 0.8% agarose gels, were extracted from gel slices and purified by centrifugation through siliconized sterile glass wool (Heery *et al.*, 1990).

Transformation. Competent cells of *E. coli* DH5 were prepared and transformed by the procedure of Chung & Miller (1988). Transformation of *B. subtilis* was performed as described by Karamata & Gross (1970). Chloramphenicol-resistant (Cm^r) transformants were selected on LB plates supplemented with chloramphenicol (3 μ g ml⁻¹). Before plating on LA plates containing erythromycin and lincomycin, the transformation mixture was incubated for an additional 90 min period with a sublethal concentration of erythromycin (0.1 μ g ml⁻¹).

Determination of phage susceptibility. Phage ϕ 29 was propagated on strain BD70. Stocks of the defective bacteriophages PBSY and PBSZ were obtained by mitomycin C induction of cultures of *B. subtilis* strains S31 and W23, respectively, and prepared as previously described (Mauël & Karamata, 1984; Karamata *et al.*, 1987). Phage susceptibility was assessed by spotting the stocks onto fresh streaks of purified Cm^r transformants, and incubating at 37 °C for 6 h.

Polymerase chain reaction (PCR). PCRs were performed on DNA substrates, purified by CsCl/ethidium bromide gradient centrifugation

(Sambrook *et al.*, 1989) with the GeneAmp PCR reagent kit (Perkin Elmer Cetus). After an initial 1 min denaturation step at 95 °C, the amplification was carried out for 30 rounds: 1 min denaturation at 95 °C alternating with 1 min annealing at 37 °C and 1 min extension at 72 °C. The polymerization was completed by a 3 min incubation at 72 °C. Samples were extracted with chloroform, digested with the appropriate restriction enzymes and cloned in pMTL20EC.

DNA sequencing and analysis. DNA fragments cloned into pMTL20EC were deleted using the double-stranded nested deletion kit (Pharmacia), and sequenced with the Sequenase version 2.0 kit (US Biochemical). The [³⁵S]dATP α S was from Amersham. Sequence data were compiled and analysed with the University of Wisconsin Computer Group software (Devereux *et al.*, 1984).

Isolation of total RNA and primer extension. Cultures grown in SA medium at 37 °C were harvested at a concentration of 2×10^8 cells ml⁻¹, and total RNA was isolated according to the method of Gilman & Chamberlin (1983). Relevant oligodeoxynucleotides, identified from the sequence analysis, i.e. 5'-AAGAAAACGTGTTCTAAGCCTG3' for *gtaB* and 5'-TCTTGATCGCTTCAGGCCTGGTC3' for *orfX*, both situated between nucleotides 17 and 40 downstream of their respective start codons, were synthesized with a Beckman SM oligonucleotide synthesizer and purified on a NAP-25 (Pharmacia) column. Twenty picomoles was radiolabelled in a 50 μ l mixture containing 20 units of T4 polynucleotide kinase (Biofinex) and 100 μ Ci (3.7 MBq) of [γ -³²P]ATP (> 5000 Ci mmol⁻¹, Amersham), and purified by ethanol precipitation (Sambrook *et al.*, 1989). Primer extensions were performed with 1.5 pmol of these probes and 50 μ g of total cellular RNA (Jones *et al.*, 1985). The extension products were resuspended in 6 μ l 50% (v/v) formamide, and loaded onto polyacrylamide gels, together with sequencing reaction products.

Amylase detection. Cm^r transformants of strain L5047 were tested for the inactivation of the amylase gene as follows. After 18 h incubation on chloramphenicol and 1% (w/v) starch-supplemented LA plates, colonies were flooded with 0.5% (w/v) iodine in 1% (w/v) potassium iodide. Amylase deficiency, due to *amyE* locus disruption, was revealed by absence of clearing around colonies.

Results

The nucleotide sequence of the *gtaBorfX* divergon

Transformation of a *gtaB*-bearing strain with p6312 (Fig. 1) yielded recombinants with the wild-type phage susceptibility spectrum (C. Mauël, unpublished results). Thus, the relevant 2.5 kb region, comprising the insert of p6312 (Fig. 1), was sequenced on both strands by the dideoxy chain-termination method. Computer analysis of the sequence revealed two open reading frames (Figs 1 and 2): *orfX*, transcribed in the direction of DNA replication, and *gtaB*, the structural gene of the UDPGPPase, transcribed in the opposite direction. Potential AUG translation initiation codons for *gtaB* and *orfX* are separated by an intergenic regulatory region of 245 bp, and preceded by Shine-Dalgarno sequences with ΔG s of -11.9 and -12.4 kcal mol⁻¹ (-49.8 and -51.9 kJ mol⁻¹) respectively. The UAA stop codons of both ORFs are followed by inverted repeats, which could generate stem-loop structures with a ΔG of

Table 1. Bacterial strains, plasmids and bacteriophage

Name	Genotype or description	Origin
<i>Bacillus subtilis</i>		
L5047	<i>pheA1 purA16 hisA35 trpC2 metB5</i>	Mauël <i>et al.</i> (1989)
L6200	<i>hisA1 argC4 ilvA1 gtaB⁸¹⁰⁰</i>	Pooley <i>et al.</i> (1987)
L5054	<i>pheA1 purA16 hisA35 trpC2 gtaB515</i>	Pooley <i>et al.</i> (1987)
L6331	<i>purA16 leuA8 ilvA1 gtaB515</i>	Pooley & Karamata (1984)
L4598	<i>pheA1 purA16 hisA35 trpC2 metB5 gtaB::p6344</i>	Integration of p6344 into the <i>gtaB</i> locus of L5047
L4599	<i>pheA1 purA16 hisA35 trpC2 metB5 amyE::cat</i>	Insertion of fragment containing <i>cat</i> , 0.6 kb fragment of <i>orfX</i> and <i>lacZ</i> , into the <i>amyE</i> locus of L5047
L4600	<i>pheA1 purA16 hisA35 trpC2 metB5 amyE::cat orfXΔ(Δ::p6350) lacZ (orfX⁺)</i>	Integration of p6350 into the partial copy of <i>orfX</i> of L4599
S31	Prototroph	Ionesco <i>et al.</i> (1964)
W23	Prototroph Sm ^r	Dubnau <i>et al.</i> (1967)
BD70	<i>metC3 trpC2</i>	Dubnau <i>et al.</i> (1967)
IS432	<i>leuA8 metB5 hisA1 sinΔ</i> Cm ^r	I. Smith, Public Health Research Institute, New York, USA
<i>Escherichia coli</i>		
DH5	F ⁻ <i>endA1 hsdR17 (r⁻m⁺) supE44 thi-1 λ⁻ recA1 gyrA96 relA1Δ(argF-lacZYA)U169 φ80ΔlacZΔM15</i>	P. Shaw, Université de Lausanne
Phage		
λ63	Contains a 17.3 kb fragment of <i>B. subtilis</i> 168 chromosomal DNA	Mauël <i>et al.</i> (1989)
Plasmids		
pMTL20EC	Ap ^r Em ^r Cm ^r	Chambers <i>et al.</i> (1988)
pMTL500C	Ap ^r Cm ^r <i>ori</i> pAMβ1	N. P. Minton, Porton, UK
pJH101	Ap ^r Tc ^r Cm ^r	Ferrari <i>et al.</i> (1983)
pDG268	Ap ^r Cm ^r <i>amyE::cat lacZ</i>	P. Stragier, Université Paris-Sud, France
pVL22	Ap ^r Em ^r Cm ^r	<i>Hind</i> III- <i>Bgl</i> II deletion in the polylinker of pMTL20EC
p6344	Ap ^r Em ^r Cm ^r	Cloning of the 0.6 kb <i>Dra</i> I- <i>Bgl</i> II fragment from p6312 into the <i>Sma</i> I and <i>Bgl</i> II sites of pMTL20EC
p6344A	Ap ^r Cm ^r <i>ori</i> pAMβ1	Cloning of the 0.6 kb <i>Kpn</i> I- <i>Bgl</i> II fragment from p6344 into the same sites of pMTL500C
p6346	Ap ^r Em ^r Cm ^r	The 0.6 kb <i>Hpa</i> I- <i>Sac</i> I insert of p6320 cloned into <i>Sma</i> I- <i>Sac</i> I sites of pMTL20EC.
p6347	Ap ^r Em ^r Cm ^r	The 0.75 kb <i>Hind</i> III- <i>Bgl</i> II chromosomal fragment of L5054 cloned into the same sites of pMTL20EC
p6348	Ap ^r Em ^r Cm ^r	The 0.75 kb <i>Hind</i> III- <i>Bgl</i> II chromosomal fragment of L6200 cloned into the same sites of pMTL20EC
p6349	Ap ^r Cm ^r <i>amyE::cat lacZ</i>	Cloning of the 0.6 kb <i>Bam</i> HI- <i>Eco</i> RI fragment from p6346 into the same sites of pMTL20EC
p6350	Ap ^r Em ^r	Cloning of the 0.6 kb <i>Eco</i> RI- <i>Hpa</i> I insert from p6320 into the <i>Eco</i> RI- <i>Sma</i> I of pVL22

Fig. 1. Sequencing strategy. The sequence was determined on both strands using sets of nested deleted inserts derived from plasmid subclones (—). Thin arrows indicate the direction and the length of each independently determined sequence using M13 (—) or synthesized primers (—). ORFs are indicated under the restriction map (—). DNA replication proceeds from right to left.

Fig. 2. Nucleotide sequence of the 2.5 kb region containing *gtaB* and *orfX*, and their deduced amino acid sequences. Major restriction sites are indicated. -35 and -10 promoter regions are underlined. Putative ribosome-binding sites (RBS) are doubly underlined. Uppercase letters in the nucleotide sequence indicate transcription start points. Asterisks denote stop codons. Regions of dyad symmetry are shown by converging arrowheads. Hexamer ATGAAA repeats are in *italic*.

–22.8 kcal mol⁻¹ (–95.4 kJ mol⁻¹) for *gtaB* and –24.9 kcal mol⁻¹ (–104.2 kJ mol⁻¹) for *orfX*, and function as ρ -independent transcriptional terminators. The inverted repeat downstream of *orfX* acts as the terminator for the convergently transcribed *lytR* gene (Lazarevic *et al.*, 1992).

Deduced products of the *gtaB* and *orfX* genes

gtaB encodes an apparently cytoplasmic 33 kDa protein, consisting of 292 amino acid residues, and having a pI of 4.94 (Table 2). Screening of the GenBank database revealed 42.1%, 45.4% and 42.4% homologies between the GtaB protein and UDPGPPases of *Acetobacter xylinum* (Brede *et al.*, 1991), *E. coli* (A. C. Weissborn *et al.*, unpublished results) and *Salmonella typhimurium* (Jiang *et al.*, 1991), respectively (Fig. 3). The putative product of gene *orfX*, containing 380 residues, has a molecular mass of 42.6 kDa and a pI of 5.76. It exhibits 55.4% amino acid identity with an *E. coli* protein (Fig. 4) that is possibly involved in sugar processing (Daniels *et al.*, 1992).

Table 2. Properties of the nucleotide sequence of the *gtaB* and *orfX* genes and their deduced products

Property	<i>gtaB</i>	<i>orfX</i>
Start codon	AUG	AUG
Stop codon	UAA	UAA
Size of ORF (bp)	876	1140
TA in wobble position (%)	63	53.6
TA (%)	56.8	53.5
ΔG for RBS (kcal mol ⁻¹)*	–11.9	–12.4
Length (amino acids)	292	380
Molecular mass (kDa)	33.0	42.6
Isoelectric point	4.9	5.7
Net charge	–11	–14

* Calculated according to the rules of Tinoco *et al.* (1973) (1 kcal = 4.184 kJ).

Transcription analysis of the *gtaB* and *orfX* genes

UDPG, whose synthesis is mediated by GtaB, is involved in the glucosylation of poly(groP) and in the synthesis of the main chain of poly(Glc-GalNAc 1-P) (Shibaev *et al.*, 1973). Although neither of these roles is essential for the growth of *B. subtilis* 168, the analysis of the *gtaB* and,

<i>B. subtilis</i>	MKKVRKAIIPAAGLGTRFLPATKAMPKEMLPVVDKPTIQYIIIEAVEAGIEDIIIVTGKSKRAIEDHFDYSPLEERNLEEKKTTELLEKVKKASNL	96
<i>A. xylinum</i>	MIKPLKKAVLPVAGLGTRFLPATKCVPKMLTVVDRPLIQYAIIDEAREAGIEEFCLVSSRGKDSLIDYFDISYELEDTLKARKKTSALKALEATRVI	97
<i>E. coli</i>	MAAINTKVKKAVIPVAGLGTRMLPATKAIPEKMLPLVDKPLIQYVNVNCAAGITEIVLVTHSSKNSIENHFDTSFELEAMLEKRVKRLQLLDEVQSICPP	100
<i>S. typhimurium</i>	MMNLKAVIPVAGLGTHMLPATKAIPEKMLPLVDKPMIPIQYIVDEIVAAGIKIEIVLVTHASKNAVENHFDTSYELESLLQVRKRLQLLAEVQSICPP	95

<i>B. subtilis</i>	-ADIHYIRQKEPKGLGHAVWCARNFVIGDEPFAVLGGDDIVQAEPTGLRQ-----LMDEYEKTLSSIIIGVQVPEEETHRYGIID---PLTSEGRYVQVKNFV	189
<i>A. xylinum</i>	PGTMSVPPAGTAGPWHAIWCAREFIGNDFAILLPDDVVQSKKSCIGQ-----LVEVYNKTTGGNVLAIVTEVPREQTGSGYILD---VGKDDGKTVEVKGVLV	191
<i>E. coli</i>	HVTIMQVRQGLAKGLGHAVLCAHPVVGDEPFAVILPDVILDEYESDLSQDNLAEMIRRFDETHGSQIMVEPV-ADVTA-YGVVDCKGVELAPGESVPMVGVV	200
<i>S. typhimurium</i>	GVTIMNVRAQPLGLGHSIILCARPVVGDNPFIIVLPDIIIDDATADPLRYNLAAMVARFNETGRSQVLAKRMKGDLS-YSVIQTKPELDNEGKVSRIVEFI	196

<i>B. subtilis</i>	EKP-PKGTAPSNLAILGRYVFTPEIFMYLEEQQVQAGGEIQLTDAIQKLEIQRVFAYDFEGKRYDVGEKLGFIITTTLEFAMQDKELRDQLVFPMEGLNKEEI	292
<i>A. xylinum</i>	EKP-DPKDAPSTLSVIGRYVLTADVLKHLAKLEKAGGEVQLTDAMAKTIGHVFPFHGYRYEGKRFDCGSKIASWKPRSPPLWSVRNWLPAVNS	284
<i>E. coli</i>	EKP-KADVAPSNLAIVGRYVLSADIWPLAKTPPGAGDEIQLTDAIDMLIEKETVEAYHMKGKSHDCGNKLGVMQAFVEYGIHNTLTGTEFKAWLEEEMGIKK	302
<i>S. typhimurium</i>	EKPDQPQTLSDMLMAVGRYVLSADIWAELETERTEPGAWGRIQLTDAIAELAKKQSVDAAMLMTGDSYDCGKKMGYMAFVKYGLRNLKEGAKFRKSIEQLLHE	297

Fig. 3. Alignment of the UDPGPPase amino acid sequences of *B. subtilis*, *A. xylinum*, *E. coli* and *S. typhimurium*. Gaps (-) were introduced to maximize homologies. Conserved residues are given in bold characters.

OrfX	MKKLVMTVFGRPEAIKMAPLVLELKKYPEIDSYVTVTAQHQRMLDQVLDAFHIPKDFDLNIMKERQTLAEITSNALVRLDELFDKDIK	89
O389	MSAKALAAYSKRIDVKVLTVFGTRPEXIKMAPLVHALAKDPFFEAKVCVTAQHREMLDQVLKLSIVPDYDLNIMQPGGLTEITCRILEGLKPILAEFK	100

OrfX	PDIVLVHGDTTTTFAGSLAAFYHQIAVGHVEAGLRTGNKYSPFPEELNRQMTGAIADLHFAPTQGAQDNLLKENKKADSIFVTGNTAIDALNTTVRDGYS	189
O389	PDVVLVHGDTTTTLATSLAAFYQRIPVGHVEAGLRTGDLVSPWPEEANTRTLTHGLAMVHFSPTETSRQNLLENVADSRIFTGNTVIDALLVWRDQVMS	200

OrfX	HPVLDQVG-----EDKMILLTAHRRENLEGPENMFKAIRRVIGFEDVQVVPVHLNFPVREAAHKHFGSDRVHLIEPLEVIDFHNFAAKSHFIL	281
O389	SDKLSELAANYPFIXPDKKMILVTGHRRESFGRGFEEICHALADIATTHQDIQIYYPVHLNPNVREPVRNRLGHVKNVILIDPQEYLPFVWLMNHAWLIL	300

OrfX	TDSGGVQEEAPSLGKPVVLVRDTERPEAGVEAGTLKLAGTDEENIYQLAKQLLTDPDDEYKMSQASNPYGDGEASRRIVEELLFHYGKYRKEQPDSTGK	380
O389	TDSGGIQQEAPSLGKPVLMVRDTERPEAVTAGTVRLVGTQKQRIVEEVTRLLKDENEYQAMSRANPNYGDGQACSRILEALKNNRISL	389

Fig. 4. Comparison of the amino acid sequences of the products of *orfX* and the *E. coli* o389 gene. Identical residues are indicated (|). Numbers refer to the C-terminal residue of each fragment.

Fig. 5. Identification of transcriptional start points of *gtaB* and *orfX* genes by primer extension. The sequencing reactions (lanes G, A, T, C) and the corresponding extension reactions (lanes 1 to 3) were performed using the same primer. Lanes: 1, RNA from strain IS432 (*sin*Δ); 2 and 3, RNA from the reference strain 5047.

possibly, *orfX* expression may provide useful information on the regulation of teichoic acid synthesis. In addition to sequence analysis (see above), we have examined the transcription of these genes by the primer extension method. The ^{32}P 5' end-labelled 24-base oligonucleotide primers, complementary to potential *gtaB* and *orfX* transcripts between nucleotides +40 and +17, were annealed to total RNA and extended with the AMV reverse transcriptase. The 5' end of *orfX* mRNA was thus located 39 bases upstream of the putative translation initiation codon (Figs 2 and 5). A reasonable -10 region of an σ^A -controlled promoter (5 out of 6 matches) lies at an appropriate distance from the transcriptional start (Fig. 2). The -35 σ^A promoter region (4 out of 6 matches) is separated from the -10 one by a 16-base spacer. The start point of *gtaB* transcription was localized 80 bases upstream of the potential start codon. It corresponds to a P^A promoter whose -35 and -10 regions, separated by a perfect 17-base spacer, exhibit 3/6 and 5/6 matches to P^A consensus sequences (Fig. 2). Gels of the *gtaB* transcript reveal a few additional,

shorter, primer extension products, characterized by very weak signals (Fig. 5). Absence of any established promoter site, corresponding to all but one of these signals (see below), suggests that they are likely to be artefacts.

Search for a possible role of *gtaB* and *orfX* products in the regulation of the divergon expression was restricted to *GtaB* since *OrfX* is essential for cell growth (see below) and, so far, no conditional lethal mutants affected in *orfX* have been isolated. Primer extension analysis of mutants L5054 *gtaB*⁵¹⁵, L6200 *gtaB*¹⁰⁰ and L4598, in which *gtaB* was inactivated by plasmid insertion, all exhibited start signals identical to that of the wild-type (not presented). Thus, at least in the exponential growth phase, *GtaB* does not seem to play a role in the regulation of the *gtaBorfX* divergon. Finally, we examined the role of *Sin*, a key regulatory protein in *B. subtilis* differentiation (Gaur *et al.*, 1986), which was shown to be involved in the regulation of the expression of the *N*-acetylmuramoyl-L-alanine amidase (Fein & Rogers, 1976; Pooley & Karamata, 1984; Kuroda & Sekiguchi, 1993; V. Lazarevic, unpublished results). Primer extension of mutant IS432, *sin*Δ with an inactivated *sin* gene, reveals that one of the very weak start points for *gtaB* transcription (see above), hardly detectable in *sin*⁺ strains, becomes as intense as the one associated with the P^A promoter (Fig. 5). This start point corresponds to a P^B promoter previously identified and localized in this region of the *B. subtilis* chromosome (Boylan *et al.*, 1990). Thus, in addition to its numerous functions (Gaur *et al.*, 1986), *Sin* appears to negatively regulate the transcription from the P^B promoter of *gtaB*. Primer extension analysis has not revealed any effect of *Sin* on *orfX* transcription (not presented).

Nucleotide sequences of *gtaB*⁵¹⁵ and *gtaB*¹⁰⁰ mutant alleles

Phenotypic differences between strains bearing *gtaB* and *gtaB*^s type mutations, revealed by bacteriophage adsorption spectra (Pooley *et al.*, 1987), prompted us to determine the nature of mutations *gtaB*⁵¹⁵ and *gtaB*¹⁰⁰. Firstly, we identified the DNA fragment which can correct both these mutations. p6344A was constructed by insertion of the wild-type (*gtaB*⁺) sequence, encoding residues 1–213 of the UDPGPPase, into the polylinker region of pMTL500C, a replicative plasmid in *B. subtilis*. Transformation of the *B. subtilis* ϕ 29-resistant strains, L5054 *gtaB*⁵¹⁵ (ϕ 29^r PBSZ^r PSBY^r) and L6200 *gtaB*¹⁰⁰ (ϕ 29^r PBSZ^s PBSY^s) (Table 3) by p6344A (Cm^r) yielded Cm^r strains, a small proportion of which exhibited the *GtaB*⁺ phenotype. The latter strains were obtained by the restoration of the *gta*⁺ allele through recombination. Thus, the two investigated mutations were localized in

Table 3. Phage resistance pattern of strains examined

Strain	Bacteriophage		
	ϕ 29	PBSY	PBSZ
L5047 <i>gtaB</i> ⁺	S	S	S
L5054 <i>gtaB</i> 515	R	R	R
L6200 <i>gtaB</i> ⁸ 100	R	S	S
L4598 <i>gtaB</i> ::p6344	R	R	R

S and R denote sensitivity and resistance, respectively.

Table 4. Base changes and resulting amino acid replacement in mutants *gtaB*515 and *gtaB*⁸100

Strain (mutation)	Codon and amino acid change		Position
	From	To	
L5054 (<i>gtaB</i> 515)	GAC (Asp)	GGC (Gly)	132
L6200 (<i>gtaB</i> ⁸ 100)	GCA (Ala)	GTA (Val)	117

Fig. 6. Integration of p6347 or p6348 into the *gtaB* locus by recombination upstream (a) and downstream (b) of the *gtaB*515 and *gtaB*⁸100 mutation, respectively. The white circle represents the position of the point mutation in the *gtaB* gene.

the cloned fragment. Chromosomal regions of relevant *GtaB*-deficient mutants, expected to carry *gtaB*515 and *gtaB*⁸100, were amplified by PCR. Direct sequencing of thus obtained fragments revealed, in each of the mutants, one single base-pair transition (Table 4). Mutation *gtaB*515 corresponds to an AT to GC transition at position 1975, resulting in a replacement of an aspartate by a glycine residue at position 132 of the UDPGPPase.

Mutation, *gtaB*⁸100 is due to a CG to TA transition at position 1930, changing alanine into valine at position 117 of the enzyme (Table 4). To confirm that the identified mutations are indeed responsible for the mutant phenotypes, the relevant mutated chromosomal fragments amplified by PCR were introduced into the parent strain. PCR products, digested with *Hind*III/*Bgl*II, and cloned into pMTL20EC, gave rise to p6347 (*gtaB*515) and p6348 (*gtaB*⁸100). Their integration into the *gtaB* locus of strain L5047 (*gtaB*⁺) led to a partial duplication of *gtaB* (Fig. 6). All ϕ 29-resistant transformants obtained with p6347 (*gtaB*515) were also PBSY^r and PBSZ^r, while those obtained with p6348 (*gtaB*⁸100) were PBSY^s and PBSZ^s. Phage-resistance patterns were therefore identical to those of the original *gtaB* mutants, confirming that the two identified point mutations in the *gtaB* gene were responsible for the specific modifications of phage susceptibility. However, in both crosses, a proportion of Cm^r recombinants exhibited the wild-type phage sensitivity pattern, i.e. ϕ 29^s PBSY^s PBSZ^s. Appearance of the latter is readily accounted for by recombinational events upstream of the mutation (Fig. 6a), which leave the wild-type allele in the complete gene copy and place the mutated base pairs in the truncated *gtaB* copy, conferring wild-type phage sensitivity upon such recombinants.

Insertional mutagenesis of *gtaB* and *orfX* genes

Inactivation of the *gtaB* gene by insertion of p6344 yielded viable Cm^r recombinants, which were all resistant to bacteriophages ϕ 29, PBSY and PBSZ. Thus, the resulting, truncated, *GtaB* protein, which lacks the 79 C-terminal amino acid residues, exhibited the phenotype corresponding to mutation *gtaB*515 (Table 3).

Inactivation of *orfX* was attempted by transformation of competent cells of strain L5047 with p6346 or p6350, and selection of Cm^r and erythromycin-resistant (Em^r) recombinants. Theoretically, a Campbell-type integration of either of these plasmids should have taken place and led to interruption of *orfX* at codon position 334, and thus to a protein devoid of its 46 C-terminal amino acids. However, absence of such transformants suggests that the product of *orfX* is essential for cell growth. To eliminate the possibility that p6350 is intrinsically impaired in the integration into the chromosome, the 0.6 kb *Hpa*I-*Sac*I fragment of *orfX* was translocated into gene *amyE*, generating a partial diploidy of *orfX*. To that effect, it was cloned in pDG268 (Fig. 7). The resulting p6349, linearized with *Sca*I, was introduced by transformation into cells of strain L5047. Cm^r and amylase-deficient transformants, generated by insertion of the *orfX* fragment into the *amyE* gene following a double-crossover were obtained (Fig. 7). When one chosen *amyE*

Fig. 8. Schematic drawing of the regulatory region of the *gtaBorfX* divergon. Black segments refer to the ATGAAA hexamers and the corresponding arrows to their orientation.

Discussion

Sequence analysis of *gtaB*, the structural gene of the UDPGPPase in *B. subtilis* 168, reveals an acidic, apparently soluble, protein with a molecular mass of 33 kDa. The enzyme displays a relatively high amino acid sequence homology with analogous enzymes of bacteria *A. xylinum*, *E. coli* and *S. typhimurium* (Fig. 3), but apparently none with the analogous UDPGPPases in the eukaryotes *Dictyostelium discoideum* (Ragheb & Dottin, 1987) and potato tuber (Katsube *et al.*, 1990). Inactivation by insertional mutagenesis of the *B. subtilis* gene led to a phenotype characteristic of *gtaB*-bearing mutants, i.e. resistance to bacteriophages $\phi 29$, PBSY and PBSZ. Strains with mutated *gtaB* were previously shown to be deficient in poly(Glc-GalNAc 1-P), as well as in glucosylation of the glycerol residue of poly(groP), the main anionic polymer. The divergently transcribed gene *orfX*, which shares the regulatory region with *gtaB*, was shown to be essential for cell growth. Absence of conditional mutants did not allow the identification of its biological role. However, a relatively high amino acid sequence homology to the product of the *E. coli* genetic entity *o389*, belonging to a cluster possibly involved in sugar processing, might suggest a role for OrfX in the metabolism of cell surface polymers, such as teichoic or lipoteichoic acids, for instance.

From the structural point of view, the divergently transcribed monocistronic operons *gtaB* and *orfX*, flanked by two strong ρ -independent terminators and sharing a 245 bp regulatory region, form a divergon. However, so far, no evidence of a functional or regulatory interdependence of GtaB and OrfX proteins has been obtained. Sequence and primer extension analyses of the exponentially growing wild-type and *sin* Δ -bearing cells show that *orfX* is transcribed from a

P^A promoter, and *gtaB* from a P^A and from a Sin-repressed P^B promoter. The transcription start site corresponding to P^B , hardly detectable in exponentially growing *sin*⁺ cells, reveals transcription in strains with an inactivated *sin* gene.

Interestingly, the Sin protein, while apparently attenuating the activity of the P^B promoter of *gtaB*, was shown to enhance that of the P^D promoter of the *lytABC* operon (Kuroda & Sekiguchi, 1993). Should such an effect of Sin be valid for transcription from other P^B and P^D promoters, it would appear that σ^B - and σ^D -controlled regulons encode antagonizing functions. The regulatory regions of the Sin-repressed *gtaB* and the Sin-depressed *lytABC* operons each contain two pairs of similar inverted repeats: in *gtaB*, the inverted repeat is an ATGAAA hexamer (Fig. 8), and in *lytABC*, it is the ANAATGAAA nonamer (Lazarevic *et al.*, 1992), which includes the former hexamer. A more general confirmation of our observation should help to correlate the effect of Sin with these regulatory elements.

Mutations *gtaB515* and *gtaB⁸100*, which confer different phage adsorption spectra, correspond, respectively, to the following amino acid substitutions: glycine for aspartate, which appreciably alters the UDPGPPase hydropathy profile; and valine for alanine. Strains bearing either of these mutations are resistant to phage $\phi 29$ due to absence of its receptor, the glucosylated poly(groP) (Young, 1967). Loss of receptor(s) for defective bacteriophages PBSY and PBSZ, associated with *gtaB* mutations seems to be correlated with a very severe reduction of the cell wall GalNAc content (Pooley *et al.*, 1987). The presence of small amounts of this compound in *gtaB⁸*-bearing mutants, possibly due to leakiness of the UDPGPPase, only slightly altered by the replacement of a valine by an alanine residue, might be correlated with their sensitivity to PBSY and PBSZ.

However, poly(Glc-GalNAc 1-P) is not the specific receptor for these phages: firstly, *gga*-bearing mutants, characterized by complete absence of this polymer (Estrela *et al.*, 1991), are sensitive to PBSZ (Estrela, 1991), and secondly, a double *gtaB^g gga* mutant remains sensitive to both PBSY and PBSZ (B. Soldo, unpublished results). Therefore, other receptors for PBSY and PBSZ adsorption should be envisaged. For instance, adsorption may require another GalNAc-containing polymer (Duckworth *et al.*, 1972), whose residual synthesis might take place in *gtaB^g*-bearing strains, or possibly either the glucosylated poly(groP) or the poly(Glc-GalNAc 1-P) may allow adsorption. Incidentally, the presence of a UDP sugar hydrolase, external to the cytoplasmic membrane in phosphate-starved cultures of *B. subtilis* strain W23 (Mauck & Glaser, 1970), leaves open the question of a more direct role of this enzyme in the phage-bacterium interaction.

The observation that each of the two characterized mutations of *gtaB* corresponds to a substitution of an amino acid conserved in the four UDPGPPase prokaryotic genes that have been sequenced so far suggests that the mutated domain encodes the active site of the enzyme. A confirmation of such a correlation, i.e. that missense mutations with a detectable phenotype are due to substitution of conserved amino acids, may not only account for the uneven distribution of thermosensitive mutations (Karamata & Gross, 1970; D. Karamata, unpublished results) but may, conversely, allow the identification of an active site by sequencing of these mutations.

We are grateful to Dr Catherine Mauël for the generous gift of material and for making available unpublished data. B. Soldo was partly supported by a grant from the Centre for genetic engineering and biotechnology, Sarajevo.

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