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Cloning and Expression of the Toxin in Sporogenic and Asporogenic Strains of *Bacillus subtilis*

E. S. Ward[1], A. R. Ridley[2], D. J. Ellar[1]† and J. A. Todd[3]

[1] *Department of Biochemistry, University of Cambridge Tennis Court Road, Cambridge CB2 1QW, England*

[2] *Imperial Cancer Research Fund Lincolns Inn Fields, London, England*

[3] *Department of Medical Microbiology, Sherman Fairchild Science Building Stanford University School of Medicine, Stanford, CA 94305, U.S.A.*

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A plasmid-borne gene from *Bacillus thuringiensis* var. *israelensis* encoding a 27,340 M_r insecticidal δ-endotoxin has been cloned on a bifunctional multicopy plasmid in a wild-type sporogenic strain and two asporogenic mutants of *Bacillus subtilis*. The δ-endotoxin gene is expressed at a low level during vegetative growth in all three strains, but the synthesis of the toxin increases markedly during the third hour of stationary phase for both the sporogenic strain and an asporogenic mutant containing the OJ lesion. However, in a stage OA mutant, this increase in δ-endotoxin synthesis is not observed. In both the wild-type sporogenic *B. subtilis* and the asporogenic OJ strain, phase-bright inclusions, resembling the *israelensis* crystal in appearance, are visible during late stationary phase. The insoluble inclusions from the *B. subtilis* transformants, consisting solely of the 27,340 M_r polypeptide, were purified by density gradient centrifugation and found to be extremely toxic to *Aedes aegypti* larvae. After solubilization in alkaline buffer, this polypeptide was also shown to be haemolytic for human erythrocytes and to lyse *Aedes albopictus* cells with the same LC_{50} value as native *israelensis* δ-endotoxin crystals. During stationary phase, novel mRNA species appear in both the wild-type strain and the OJ mutant, but not in the OA mutant, and these appear to be the major gene-specific transcripts. Transcriptional mapping of δ-endotoxin-specific mRNA has shown that the same region of initiation is used at a relatively low level in all three strains during vegetative growth.

1. Introduction

During the process of sporulation, strains within the Gram-positive bacterial species *Bacillus thuringiensis* produce intracellular protein crystals (Somerville, 1978; Bulla *et al.*, 1980) that are toxic to a wide range of lepidopteran and some dipteran larvae (Huber & Luthy, 1981). *B. thuringiensis* var. *israelensis* (Goldberg & Margalitt, 1977) produces a δ-endotoxin that is extremely toxic to the larvae of mosquitoes and blackfly (de Barjac, 1978; Thomas & Ellar, 1983). Of the several proteins in the native *israelensis* crystal the 27,000 M_r polypeptide is the most prominent and controversial. Using purified preparations obtained after solubilization of the native crystal in alkaline conditions, this polypeptide was found to be larvicidal (Sriram *et al.*, 1985) and a 25,000 M_r derivative of the polypeptide produced by partial proteolysis was found to be both larvicidal and haemolytic (Davidson & Yamamoto, 1984; Thomas, 1984; Armstrong *et al.*, 1985). In contrast to these findings, no larvicidal activity was detected by Wu & Chang (1985) in the 26,000 M_r polypeptide, and several other groups have isolated 25,000 to 26,000 M_r *israelensis* polypeptides that they report to be haemolytic, but not mosquitocidal (Lee *et al.*, 1985; Hurley *et al.*, 1985; Cheung & Hammock, 1985). The availability of the cloned 27,000 M_r crystal protein (Ward *et al.*, 1984; Ward & Ellar, 1986) provided us with an opportunity to resolve this controversy by examining the haemolytic and mosquitocidal

† Author to whom all correspondence should be addressed.

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activity of this protein in the absence of the other polypeptides that are present in the native crystal.

Recent reports have demonstrated the isolation of δ-endotoxin genes for several serotypes of *B. thuringiensis* (Schnepf & Whiteley, 1981; Klier *et al.*, 1982; Held *et al.*, 1982; Ward *et al.*, 1984; Sekar & Carlton, 1985; Adang *et al.*, 1985; Shibano *et al.*, 1985; Waalwijk *et al.*, 1985). In some cases, the nucleotide sequence of the cloned gene has also been reported (Schnepf *et al.*, 1985; Adang *et al.*, 1985; Shibano *et al.*, 1985; Waalwijk *et al.*, 1985; Ward & Ellar, 1986). In addition Wong *et al.* (1983) have identified the transcriptional start points for the var. *kurstaki* δ-endotoxin gene encoding the P1 protein (Yamamoto & McLaughlin, 1981) in both var. *kurstaki* and *Escherichia coli*, and Waalwijk *et al.* (1985) have identified a transcriptional start site for the var. *israelensis* 27,000 M_r δ-endotoxin. However, the factors that determine the temporal expression of the δ-endotoxin genes of *B. thuringiensis* have not been characterized. The molecular basis for the programmed gene expression that occurs during sporulation is much better understood for *Bacillus subtilis* than for *B. thuringiensis*. Changes in RNA polymerase specificity, brought about by the temporal binding of different sigma subunits to the core enzyme, are thought to contribute to the regulated expression of genes (Losick & Pero, 1981). Different σ factors have been found for *B. thuringiensis*, but have not been characterized (Klier *et al.*, 1973, 1983; Petit-Glatron & Rapoport, 1976).

Initiation of sporulation in *B. subtilis* depends upon the products of at least eight genes (*spoOA*, *spoOB*, *spoOC*, *spoOE*, *spoOF*, *spoOH*, *spoOJ* and *spoOK*) called the *spoO* loci (Piggot & Coote, 1976; Hoch *et al.*, 1978). Recent data suggest that the *spoO* gene products act in conjunction with the different forms of RNA polymerase to stimulate transcription of certain temporally regulated genes (Zuber & Losick, 1983; Gilman & Chamberlin, 1983; Hoch *et al.*, 1985; Ferrari *et al.*, 1985*b*; Price & Doi, 1985). In addition, several *spoO* loci have now been cloned (Hirochika *et al.*, 1981; Ikeuchi *et al.*, 1983; Shimotsu *et al.*, 1983; Weir *et al.*, 1984; Bouvier *et al.*, 1984; Ferrari *et al.*, 1985*a*,*b*; Kudoh *et al.*, 1985) and, in some cases, their gene products characterized.

This paper reports the cloning of the var. *israelensis* δ-endotoxin gene on a multicopy plasmid in a wild-type strain and two stage O mutants of *B. subtilis*. In an attempt to understand the factors determining the control of δ-endotoxin synthesis in these recombinants the expression of the δ-endotoxin gene at both the transcriptional and translational level has been studied. The haemolytic and larvicidal activity of the cloned 27,000 M_r δ-endotoxin is also reported.

2. Materials and Methods

(a) *Strains and plasmids*

B. subtilis 168 Sueoka *trpC*2 (a kind gift from Dr T. Leighton); two asporogenic mutants *B. subtilis* 0J87 *metC*2 *tal*-1 and *B. subtilis* OA34 *phe*-12 *rif*-2 *tal*-1 were kind gifts from Dr P. Piggot, Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, Penn. U.S.A. Camtaq 10 and Camtaq 11 were constructed using pUC12 (Messing, 1983), pC194 (Horinouchi & Weisblum, 1982) and a 1·2 kb† *Taq*I restriction fragment derived from pIP173 (Ward *et al.*, 1984). pC194 DNA was obtained by restricting the cloning vector pHV33 (Primrose & Ehrlich, 1981) with *Hin*dIII followed by gel purification using DEAE-cellulose (Dretzen *et al.*, 1981). The bifunctional plasmids Camtaq 10 and Camtaq 11 were constructed using *E. coli* TG1 = K12, Δ(*lac-pro*), *supE*, *thi*, *hsd*D5/F′*tra*D36, *pro*A$^+$B$^+$, *lacI*^{q}, *lacZ*ΔM15 (a kind gift from Dr T. Gibson) (Gibson, 1984) as a cloning host.

(b) *Enzymes and reagents*

Restriction enzymes were obtained from either New England Biolabs or Bethesda Research Laboratories. S_1 nuclease was from Bethesda Research Laboratories. Klenow fragment of DNA polymerase and phage T4 DNA ligase were generous gifts from Dr T. Hunt, University of Cambridge. Lysozyme and polyethylene glycol were from BDH. Deoxynucleotide and dideoxynucleotide triphosphates were from Boehringer-Mannheim. [α-^{35}S]dATP and [α-^{32}P]dATP were from Amersham. Restriction enzyme digestions and ligations were carried out as described (Ward & Ellar, 1986).

(c) *Protoplast transformation*

The method of Chang & Cohen (1979) was used.

(d) *Plasmid isolation*

For plasmid isolation from *E. coli*, the Triton lysis method as described by Ward *et al.* (1984) was used. To isolate plasmid DNA from transformants of *B. subtilis*, the alkaline extraction method described by Birnboim & Doly (1979) was used.

(e) *Purification of δ-endotoxin inclusions from* B. subtilis

A transformant of the sporogenic strain of *B. subtilis* (168-11), harbouring the δ-endotoxin gene was grown in PWYE medium (peptone/water/yeast extract) overnight, diluted into fresh PWYE and grown to mid-exponential phase before diluting into CCY medium (Stewart *et al.*, 1981). Media contained chloramphenicol at a concentration of 4 μg/ml. Spores and crystals were harvested from the culture as described (Thomas & Ellar, 1983) at a stage when 90% of the sporulated cells had lysed. Inclusions were purified using differential ultracentrifugation on a discontinuous sucrose density gradient (Thomas & Ellar, 1983). For the isolation of inclusions produced by transformants of the OJ mutant (OJ-19), cells were cultured in 2×SG medium (Leighton & Doi, 1971) containing chloramphenicol at 4 μg/ml. A partially purified preparation of the 27,000 M_r δ-endotoxin inclusions was obtained from the OJ-19 cells by lysing late stationary cultures, after harvesting and resuspension in 10 mM-NaCl, 1 mM-EDTA, by sonication. The majority of unlysed cells were removed by brief centrifugation, and the resulting supernatant, containing phase-bright inclusions, used for assays. Purified and

† Abbreviation used: kb, 10^3 bases or base-pairs.

partially purified inclusions from 168-11 and OJ-19 cells, respectively, were assayed for toxicity *in vivo* using 3rd instar *Aedes aegypti* larvae by published procedures (Ward *et al.*, 1984). Controls using non-recombinant *B. subtilis* 168 or OJ87 cells were assayed in the same way.

(f) *Toxicity assays* in vitro

Inclusions purified from 168-11 and partially purified inclusions from OJ-19, were assayed for toxicity *in vitro* using *Aedes albopictus* cells and human erythrocytes as described by Thomas & Ellar (1983).

(g) *Polyacrylamide gel electrophoresis and immunoblotting*

Polyacrylamide gel electrophoresis and immunoblotting were as described in the accompanying paper, except that for the preparation of lysates, recombinants were grown in 2×SG medium (Leighton & Doi, 1971) containing chloramphenicol at a concentration of 4 μg/ml, and for the wild-type *trpC2* strain, tryptophan was added to a final concentration of 50 μg/ml. Cells were grown to mid-exponential phase and diluted in fresh 2×SG medium and regrown. This procedure was repeated once before the use of the culture.

(h) *RNA preparation and S_1 nuclease mapping*

RNA preparation and S_1 nuclease mapping were as described in the accompanying paper, except that for RNA preparations cells were grown in 2×SG medium (Leighton & Doi, 1971) containing chloramphenicol at a concentration of 4 μg/ml, and for the wild-type *trpC2* strain tryptophan was added to a final concentration of 50 μg/ml.

3. Results

(a) *Construction of plasmids Camtaq 10 and Camtaq 11*

These plasmids were constructed using the strategy shown in Figure 1. The 1·2 kb *Taq*I fragment containing the *israelensis* δ-endotoxin gene was ligated into the *Acc*I site of the cloning vector pUC12, and used to transform *E. coli* to ampicillin resistance. Both orientations of the insert with respect to the vector were obtained. DNA extracted from recombinants with orientation A (designated pUCtaqA) was used for the subsequent step, in which gel-purified pC194 was ligated into the *Hin*dIII site of the pUC12 polylinker in the construct pUCtaqA. The resulting plasmids, called Camtaq 10 and Camtaq 11, are capable of replication in both *E. coli* and *B. subtilis*, conferring ampicillin and chloramphenicol resistance, respectively on these hosts.

(b) *Transformation of* B. subtilis *168, OJ87 and OA34 with plasmids Camtaq 10 and Camtaq 11*

Using the protoplast method of transformation (Chang & Cohen, 1979), chloramphenicol-resistant regenerants of all three strains of *B. subtilis* were obtained with Camtaq 10 and Camtaq 11 DNA. Extraction of plasmid DNA, followed by restriction enzyme analysis (not shown), indicated that all three strains maintained the intact plasmid. In subsequent experiments a Camtaq 11 transformant of *B. subtilis* 168 (168-11) and Camtaq 10 transformants of *B. subtilis* OA (OA-1) and *B. subtilis* OJ (OJ-19) were used.

(c) *Expression of the δ-endotoxin gene*

Immunoblotting using antisera raised against the 27,000 M_r δ-endotoxin (Ward *et al.*, 1984) was used to detect the presence of this protein in lysates of 168-11, OA-1 and OJ-19. Cells were harvested during the mid-exponential and early stationary phases of growth and at the times after the onset of stationary phase indicated in Figure 2. It is apparent that both 168-11 and OJ-19 show similar

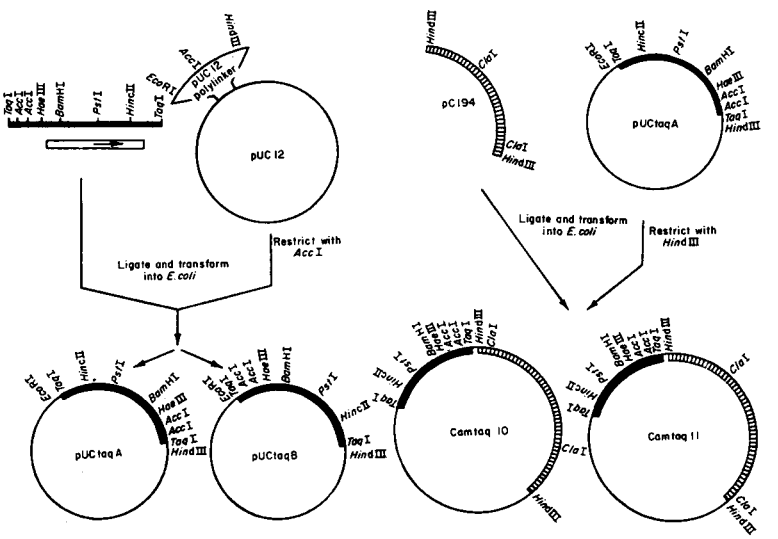

Figure 1. Construction of Camtaq 10 and Camtaq 11. A 1·2 kb *Taq*I restriction fragment encoding the δ-endotoxin gene was ligated into the *Acc*I site of pUC12, followed by ligation of pC194 into the *Hin*dIII site of the pUC12 polylinker Filled bars represent var. *israelensis* DNA; hatched bars represent pC194; and the continuous line represents pUC12.

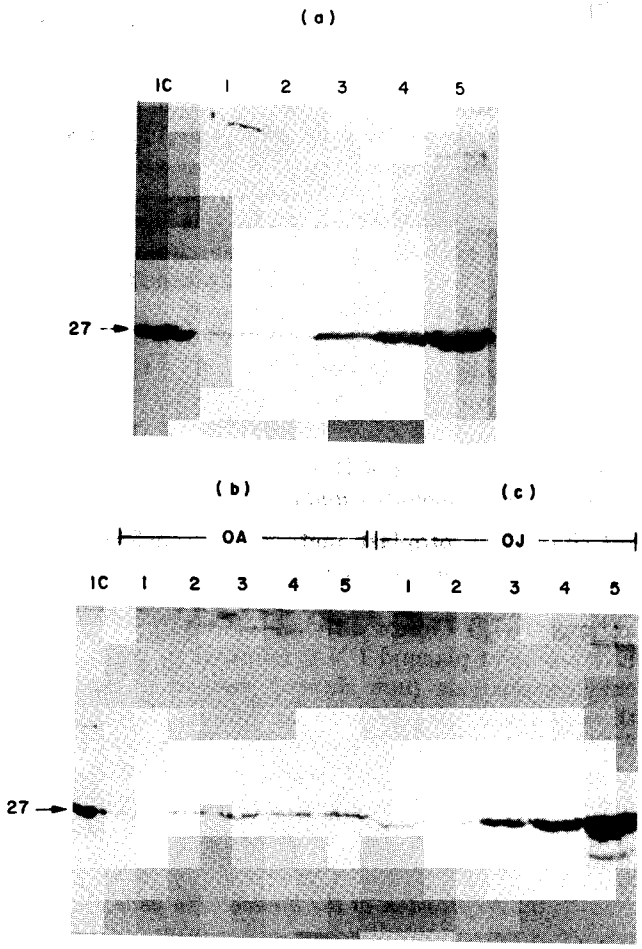

Figure 2. Expression of the 27,000 M_r δ-endotoxin in (a) *B. subtilis* 168 (Camtaq 11 transformant, 168-11); (b) OA34 (Camtaq 10 transformant, OA-1); and (c) OJ87 (Camtaq 10 transformant, OJ-19), using immunoblotting with antibodies raised against the 27,000 M_r polypeptide. Lysates of cells were made of cells harvested at the following growth points: Lane 1, mid-exponential; lane 2, early stationary; lane 3, 2 to 3 h after onset of stationary phase (stage II of sporulation for wild-type cells, as observed by phase-contrast microscopy); lane 4, 5 h after onset of stationary phase (stage III to IV for wild-type cells); lane 5, 16 to 17 h after onset of stationary phase (stage VI for wild-type cells). Approximately 150 μg of protein was loaded onto each lane for the cell lysates, and lanes 1 to 5 for each strain correspond to the same stage of growth; 15 μg of var. *israelensis* crystal protein was loaded onto lanes labelled IC. The position of the 27,000 M_r δ-endotoxin is indicated by an arrow. The polypeptide of M_r 54,000, which reacts with anti-δ-endotoxin sera, is thought to be a dimer of the 27,000 M_r δ-endotoxin.

patterns of δ-endotoxin synthesis. During mid-exponential and stationary phases of growth, Figure 2(a) and (c) shows that they both exhibit a low level of δ-endotoxin synthesis, and then at about two hours after the onset of stationary phase a substantial increase in the amount of δ-endotoxin synthesized occurs. The level of δ-endotoxin continues to increase for the next 14 to 15 hours of stationary phase. The presence of chloramphenicol at a concentration of 4 μg/ml in the growth medium caused a considerable decrease in the rate of sporulation of both 168-11 and a pHV33 transformant of *B. subtilis* 168, with the result that stage VI did not occur until about 16 hours after the onset of stationary phase.

Although the Camtaq 10 transformant, OA-1, synthesizes a low level of δ-endotoxin during the mid-exponential and stationary phases of growth similar to that of 168-11 and OJ-19, Figure 2(b) shows that in this recombinant no increase in δ-endotoxin synthesis occurs at a time two hours, or even 16 hours, after the onset of stationary phase.

(d) *Toxicity and haemolysis assays*

Phase-bright inclusions, with the same irregular shape, but smaller than the *israelensis* crystals, could be seen by phase-contrast microscopy at a stage 16 hours into sporulation for 168-11, or into stationary phase for OJ-19 (Fig. 3(a) and (b)). In contrast, inclusion bodies were not visible at any stage of growth for OA-1 (data not shown). These inclusions were purified from sporulating cultures of 168-11 using differential ultracentrifugation and found to contain only the 27,000 M_r polypeptide by sodium dodecyl sulphate/polyacrylamide gel electrophoresis. Following solubilization in alkaline buffer (Thomas & Ellar, 1983) the inclusions were assayed *in vitro* for toxicity against *Aedes albopictus* cells. After 20 hours, solubilized protein inclusions at a concentration of 0·5 μg/ml had completely lysed 5×10^4 *A. albopictus* cells, with cytopathic effects that were indistinguishable from those caused by 2 μg/ml of alkali-solubilized native *israelensis* crystals (Thomas & Ellar, 1983). In *in-vivo* assays, purified δ-endotoxin inclusions isolated from sporulated cultures of 168-11 caused death of third instar *Aedes aegypti* larvae, with an LC_{50} value of 125 ng/ml, whereas a lysate prepared from non-recombinant *B. subtilis* 168 cells had no detectable effect. Partially purified inclusions from OJ-19 showed similar potent larvicidal activity, but an LC_{50} value was not obtained in this case.

In addition, solubilized inclusions harvested from lysed cultures of OJ-19 were haemolytic to human erythrocytes: 5 μg of solubilized 27,000 M_r δ-endotoxin at a dilution of 1 : 128 causing cytolysis within three hours. Control lysates obtained from *B. subtilis* OJ87 cultures did not cause haemolysis under the conditions of assay. Cytolysis of human erythrocytes using 20 μg of solubilized *israelensis* crystal protein at a dilution of 1 : 64 has been observed (Thomas, 1984).

(e) *S_1 nuclease mapping of the transcriptional start points*

The start points for transcription of the δ-endotoxin gene were determined using high-resolution S_1 mapping. RNA was isolated from the cells at the growth stages indicated in the Figure legends (Fig. 4(a), (b) and (c)). The 393 base-pair

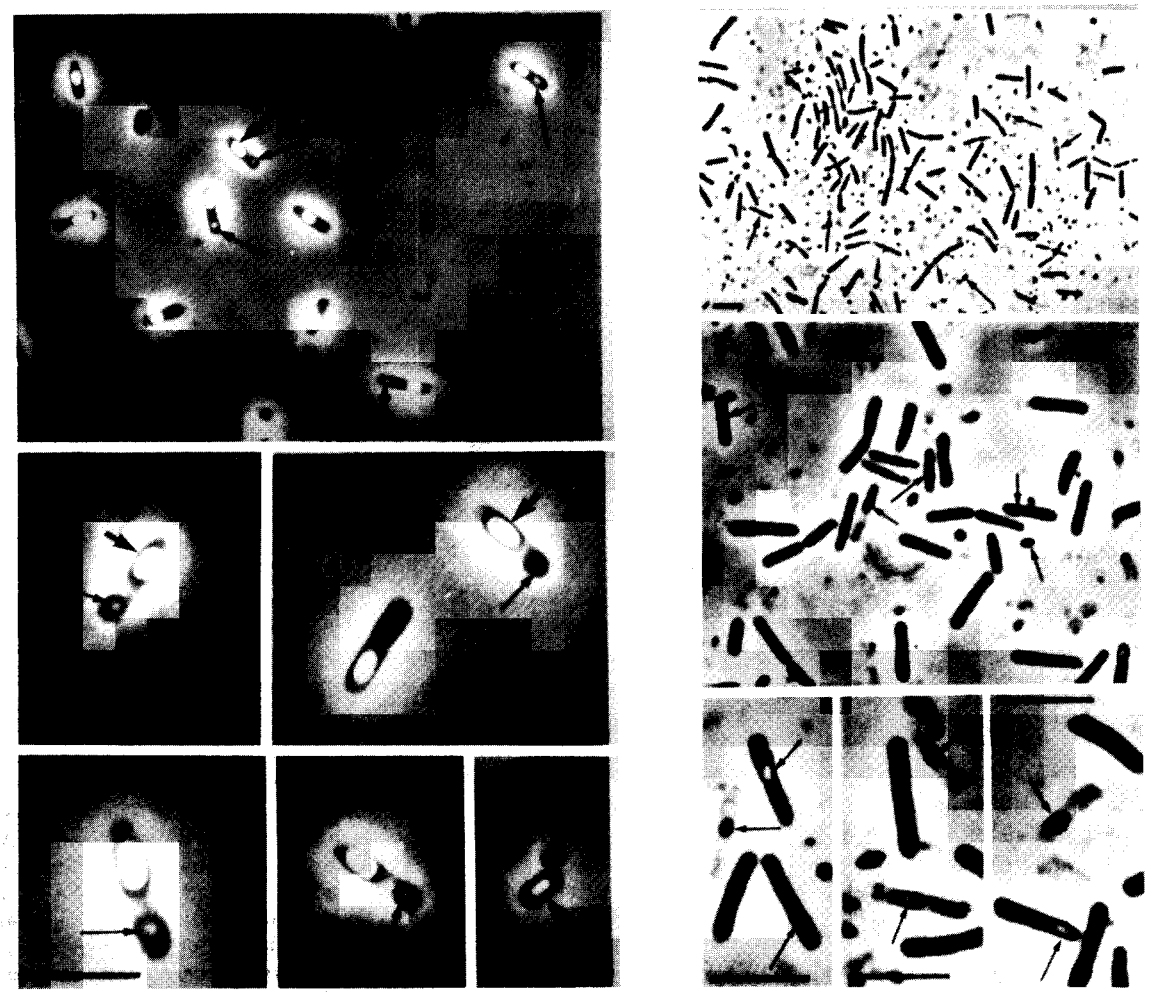

Figure 3. Phase-contrast micrographs of: (a) Camtaq 11 transformant of *B. subtilis* 168, during stage VI of sporulation; (b) Camtaq 10 transformant of *B. subtilis* OJ87, cultured for the same period as cells in (a). Large arrows indicate spores in the wild-type strain, small arrows indicate phase-bright inclusions. The bars represent 5 μm.

*Taq*I–*Bam*HI probe, described in the accompanying paper, was used for the hybridizations.

As the three recombinants enter the third hour of stationary phase, Figure 4(a), (b) and (c) shows that they differ markedly in the pattern of recognition of transcriptional start points. Firstly, in both 168-11 and OJ-19, initiation appears to occur at the start point PB1 used by *B. thuringiensis* var. *israelensis* (see Ward & Ellar, 1986), albeit at a low level. A diffuse heterogeneous start site, not clearly visible in Figure 4, but seen on prolonged exposure (data not shown), is used at a low level by all three recombinants during exponential growth. For OA-1, this appears to be the only region of transcriptional initiation used throughout exponential and stationary phases (Fig. 4(b)), and is discussed in more detail below. Interestingly, in both 168-11 and OJ-19 a different start point appears to be used during the third hour of stationary phase, centred at nucleotide 466 (PBS1), shown in Figures 4(a), 4(c) and 5. For OJ-19, the level of transcripts originating from PBS1 appears to show a further increase from the third to the sixth hour of stationary phase and then remains relatively constant, and these transcripts represent the major gene-specific RNA for the following 11 hours. In contrast, the PBS1 transcripts in 168-11 do not show this increase, and their level becomes undetectable at stages III to IV of sporulation. Concomitant with the decrease in the level of PBS1 transcripts, Figure 4(a) shows that in 168-11 an increase in δ-endotoxin transcription from other initiation points centred at nucleotide 390 (PBS2) and 238 (PB1) occurs. The location of these start points in the nucleotide sequence 5′ to the δ-endotoxin structural gene is shown in Figure 5. For 168-11, PBS2 and PB1 transcripts are the major gene-specific RNA species during stages IV to VI of sporulation, whereas during the stationary phase of growth for OJ-19 these transcripts are only detectable at a low level (Fig. 4(a) and (c)).

Although it is not clearly visible in Figure 4(a), (b) and (c), during exponential growth 168-11, OJ-19 and OA-1 all initiate transcription in the

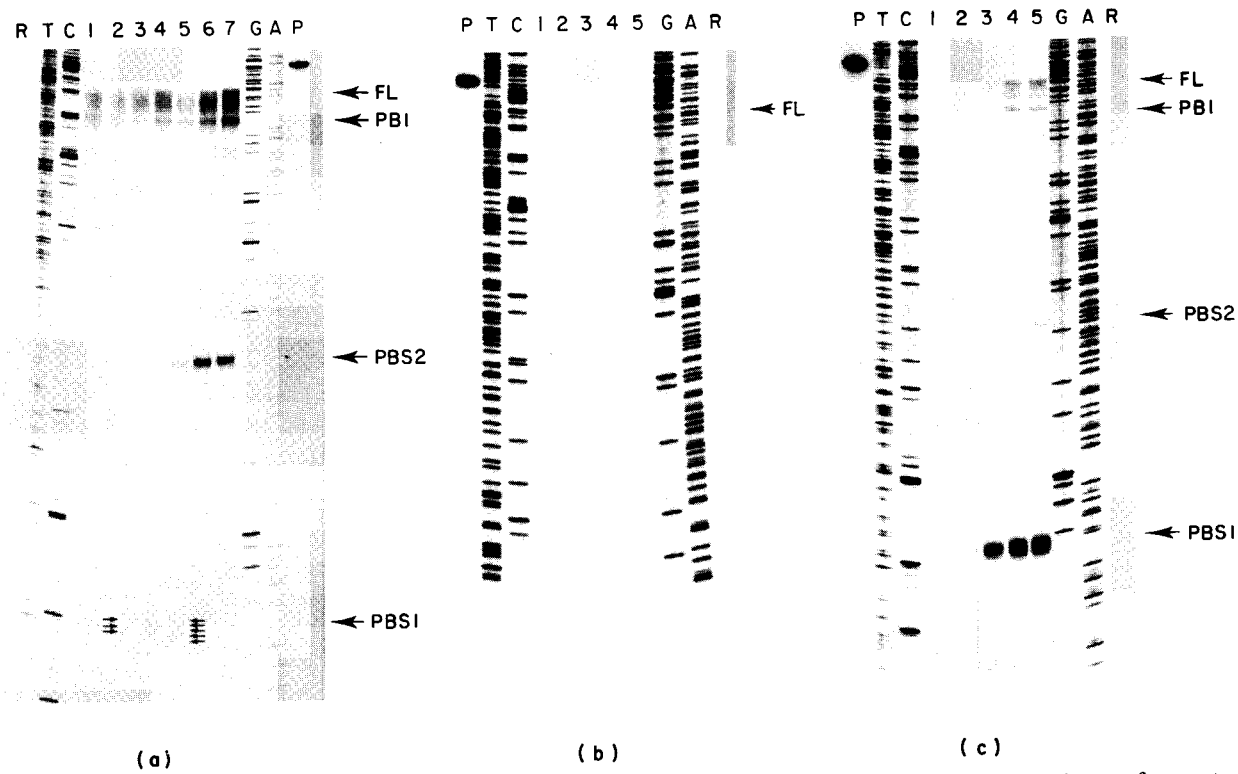

Figure 4. S_1 nuclease mapping of transcriptional start points used in: (a) *B. subtilis* 168 (Camtaq 11 transformant, 168-11); (b) OA34 (Camtaq 10 transformant, OA-1); and (c) OJ87 (Camtaq 10 transformant, OJ-19). The 393 base-pair *Taq*I–*Bam*HI probe was used, and the fragment of probe protected from S_1 nuclease digestion by *B. subtilis* mRNA was analysed on 6% acrylamide/8 M-urea sequencing gels. The dideoxy-T, -C, -G and -A sequence reactions of the appropriate M13 clone were used to determine the length of the protected probe (lanes T, C, G and A). For 168-11, RNA samples were extracted from cells at the following stages of growth and development: lane 1, mid-exponential; lane 2, early stationary; lane 3, 1 h after onset of stationary phase (stage I); lane 4, 2·5 h after onset of stationary phase (stage II as determined by phase-contrast microscopy); lane 5, 4 h after onset of stationary phase (stage III); lane 6, 5·5 h after onset of stationary phase (stages III to IV), lane 7, 16 to 17 h after stationary phase (stage VI). For OJ-19 and OA-1, RNA was extracted from cells at the same stages of growth as for 168-11, except that samples were not taken at 1 h and 4 h after the onset of stationary phase, and the stage of sporulation is not applicable for the stage O mutants. Lanes 1 to 5 for these 2 strains correspond to the same stage of growth as lanes 1, 2, 4, 6 and 7 for the 168-11 strain in (a). For (a), (b) and (c), lane R is a control using 15 μg of carrier tRNA; lane P corresponds to untreated probe (1 to 2%) of the amount used in each hybridization. The arrows in (a) indicate transcripts initiating at PBS1 in 168-11 transformants: in (a), (b) and (c), FL indicates complete protection of the probe, and in (a) and (c), PB1, PBS1 and PBS2 correspond to the transcriptional start points discussed in the text.

same region, mapping to a position that is very close to the *Taq*I site at nucleotide 214 (Fig. 5). Initiation in this region appears to be diffuse and heterogeneous, although longer exposure of gels (data not shown) reveals that the diffuse region consists of approximately ten discrete, closely spaced bands. Although this heterogeneous site appears to be utilized throughout exponential and stationary phase for all three recombinant strains, both 168-11 and OJ-19 initiate transcription at PB1, PBSI and PBS2 at about two to three hours after cessation of exponential growth (Fig. 4(a) and (c)). In contrast, as OA-1 cells enter stationary phase, no change in the pattern of transcription can be observed (Fig. 4(b)). At present we cannot decide whether the diffuse bandset observed for all three strains during exponential growth reflects heterogeneity of transcriptional initiation *in vivo* or, alternatively, an *in-vitro* artefact generated during the S_1 mapping procedure. Under the experimental conditions used, however, distinct start points (PB1, PBS1 and PBS2) have been observed for 168-11 and OJ-19, and additional experiments have indicated that the appearance of the diffuse bandset is not affected by the concentration of S_1 nuclease (data not shown). This suggests that the former explanation is the more likely.

A possible reason for the heterogeneity and low levels of initiation shown by the three recombinant strains during exponential growth may be that the appropiate transcription factors necessary to direct accurate and efficient initiation from this region are not available. The possibility that the heterogeneous start site is due to readthrough transcription from fortuitously placed promoter sequences in the pC194 DNA is made unlikely by the fact that the pC194 orientation differs in Camtaq 10 and Camtaq 11. In addition, it should be emphasized that the *Taq*I site is at a position 24 nucleotides upstream from the transcriptional start site PB1,

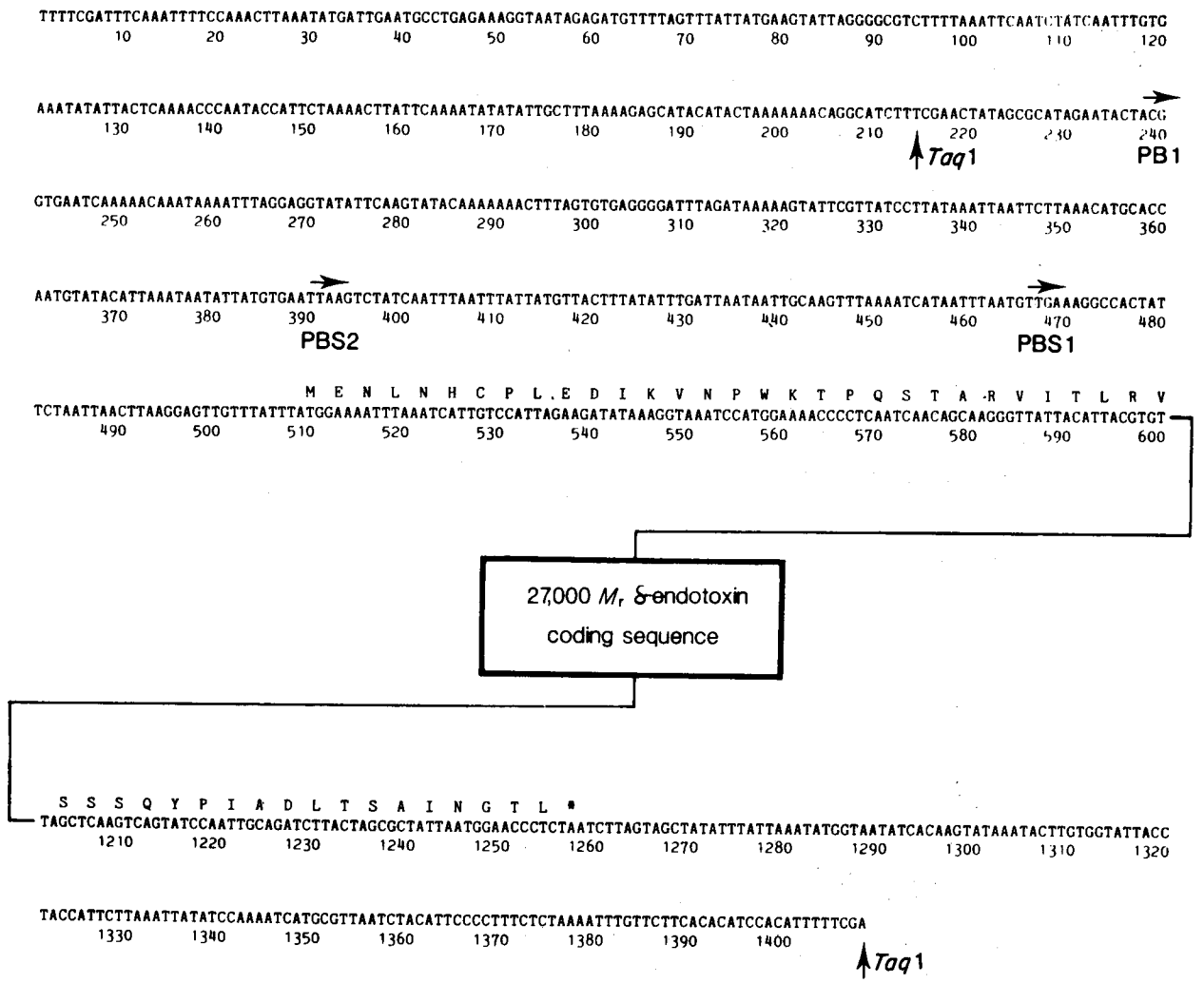

Figure 5. Transcriptional start sites used in *B. subtilis* recombinants harbouring Camtaq 10 or Camtaq 11. The 1·2 kb *Taq*I fragment used to construct Camtaq 10 and 11 extends from nucleotide position 214 to 1404, and the *Taq*I sites are shown. The δ-endotoxin structural gene extends from nucleotides 509 to 1258, and not all the structural gene sequence is shown. The initiation sites for transcription, determined by S_1 nuclease mapping, used in the *B. subtilis* recombinants 168-11 and OJ-19 are indicated by PB1, PBS1 and PBS2. Sequence hyphens are omitted for clarity.

and therefore in Camtaq 10 and 11 the −35 region is lacking from this promoter. This could account for the heterogeneity of initiation that has been observed for the three recombinant strains. Interestingly, despite the lack of some of the promoter sequences, *B. subtilis* 168 and OJ appear to utilize the start point PB1 as the cells enter the stationary phase of growth, presumably reflecting differences in transcriptional regulation between these two strains and the OA strain.

4. Discussion

When the *israelensis* 27,000 M_r δ-endotoxin gene is cloned in a multicopy plasmid in three strains of *B. subtilis*, the toxin is expressed at a low level during vegetative growth in all three strains. During stationary phase there is a marked increase in expression in both *B. subtilis* 168 and OJ, but not in OA. During sporulation of 168-11 and continued incubation of OJ-19, toxin synthesis is maintained at the high level and the protein is deposited as inclusions that are visible by phase-contrast microscopy. It is of significance that the 27,000 M_r δ-endotoxin is deposited as phase-bright inclusions when it is produced in sufficient quantities. This has been observed for other cloned genes when they are expressed at high levels in *E. coli* (Botterman & Zabeau, 1985) and *B. subtilis* (Mongkolsuk *et al.*, 1983). Clearly the simultaneous synthesis of the other polypeptides that occurs in the native *israelensis* crystals is not essential for formation of a protein inclusion body. The insoluble inclusions from the *B. subtilis* transformants consisting solely of the 27,000 M_r polypeptide were purified by density gradient centrifugation and found to be extremely toxic to *A. aegypti* larvae. After solubilization of the inclusions in alkaline buffer, this polypeptide was also shown to be haemolytic for human erythrocytes and to lyse *A. albopictus* cells with the same LC_{50} value as native *israelensis* δ-endotoxin crystals (Thomas, 1984).

These data indicate that the 27,000 M_r δ-endotoxin produced by recombinant *B. subtilis* cells is at least equally as cytolytic to both *A. albopictus* cells and human erythrocytes as the solubilized native crystal. Since our assays show that this protein is also highly active *in vivo* against mosquito larvae, the unequivocal conclusion from these results is that the 27,000 M_r polypeptide possesses both potent larvicidal and cytolytic activities. This is consistent with observations made by Davidson & Yamamoto (1984), Thomas (1984) and Armstrong *et al.* (1985). In the light of our results the significance of the observations made by Lee *et al.* (1985), Hurley *et al.* (1985) and Cheung & Hammock (1985), who could detect only haemolytic, but not mosquitocidal activity in preparations of the 27,000 M_r *israelensis* δ-endotoxin, purified after first solubilizing the native crystal in alkali, is unclear. However, it is noteworthy that, in our demonstration of the potent larvicidal activity of the 27,000 M_r protein, the larvae were fed this protein in the form of native insoluble inclusions produced in the *B. subtilis* transformants. Conceivably, after alkali solubilization the 27,000 M_r δ-endotoxin may under certain conditions be degraded to a form that retains haemolytic activity but has lost larvicidal activity. This suggestion is supported by the finding that an unrelated *B. thuringiensis* δ-endotoxin can be converted from a lepidopteran-specific toxin to a dipteran-specific form by differential proteolytic processing of a single polypeptide precursor (Haider *et al.*, 1986).

Although these experiments demonstrate the potent larvicidal activity of the 27,000 M_r polypeptide, it is noticeable that the LC_{50} value of the native *B. thuringiensis* var. *israelensis* crystal (7·5 ng/ml: Schnell *et al.*, 1984) is approximately 16-fold less than this. One reason for the difference may be that the inclusions produced in *B. subtilis* are approximately one-half of the size of those produced in *B. thuringiensis*. Because mosquito larvae are filter feeders that selectively concentrate particles 0·5 to 10 μm in diameter (Dadd, 1975), the smaller *B. subtilis* inclusions may more readily avoid entrapment. Since the native *israelensis* crystal contains several polypeptides it is possible that one of these may be a more potent larvicide than the 27,000 M_r polypeptide, or that synergism occurs between the 27,000 M_r protein and one or more of the other polypeptides to enhance toxicity. Cloning of these other *israelensis* polypeptides and expression singly and in combination will undoubtedly help to resolve this question.

S_1 nuclease mapping has been used to identify the transcriptional start points used by these three different recombinants. All three utilize a very heterogeneous start point close to the *Taq*I site (at position 214) during vegetative growth, and transcripts from this region are presumably responsible for the low level of δ-endotoxin synthesis detected by immunoblotting. Possible reasons for the heterogeneity of initiation from this region are discussed in Results.

Interestingly, the δ-endotoxin gene is being expressed during vegetative growth in a heterologous spore-forming organism (in *B. subtilis* 168-11), whereas in *israelensis* it is only expressed during sporulation. This is possibly due to the fact that the δ-endotoxin gene is present in a multicopy plasmid in 168-11, and under these conditions *trans*-acting regulatory factors are titrated. This phenomenon has been observed for the cloned *spoVG* promoter (Zuber & Losick, 1983). Alternatively, the lack of repression of the δ-endotoxin gene in 168-11 may be due to the absence of suitable regulatory proteins in this recombinant, or sequences upstream from the *Taq*I site may be required for repression. To investigate these possibilities it will be necessary to clone the δ-endotoxin gene in single and multicopy with 5′-flanking sequences extending beyond those used in this study.

The data obtained from the S_1 mapping and immunoblotting experiments, taken together, indicate that the appearance of transcripts originating from PB1, PBS1 and PBS2 coincides with a marked increase in δ-endotoxin synthesis in 168-11 and OJ-19. This strongly suggests that it is the appearance of these transcripts after the onset of stationary phase that results in the high level of δ-endotoxin expression observed for these two recombinants. Clearly, transcriptional initiation at these points is temporally regulated, and two observations made during the S_1 experiments are of particular interest.

Firstly, the OJ lesion, which prevents the first morphological manifestation of the sporulation process, does not appear to affect the temporally regulated transcription of the δ-endotoxin gene from PBS1. The OJ lesion is much less pleiotropic than OA, and recent reports (Zuber & Losick, 1983; Gilman & Chamberlin, 1983) have shown that OJ does not affect the transcription of genes that are affected by other stage O mutations. In a recent report (Ferrari *et al.*, 1985*b*) of the cloning and sequencing of the OA gene product, it has been shown to have a high degree of homology with the OmpR protein (Wurtzel *et al.*, 1982), a positive regulator of porin synthesis in *E. coli*. In addition, the *spoOF* protein has been shown to share 56% homology with the *spoOA* product in the N-terminal region (Trach *et al.*, 1985). This finding suggests that the structurally related *spoOA* and *spoOF* products may form a functional complex *in vivo* (Trach *et al.*, 1985). Alternatively, the observations that second site suppressors of other stage O mutations map to, or very close to, the *spoOA* locus (Hoch *et al.*, 1985; Kawamura & Saito, 1983; Sharrock *et al.*, 1984) suggest that the altered *spoOA* protein might be able to accomplish the function of the defective stage O polypeptide. The absence of a functional *spoOA* product in OA-1 appears to prevent utilization of PBS1 and PBS2, suggesting that this protein may interact directly or indirectly with the transcription factors involved in expression from these start sites.

Secondly, during the stationary phase of growth, 168-11 and OJ-19 use PBS1 and PBS2 differentially; 168-11 uses the start point at PBS1 transiently, and at a low level from stages I to III of sporulation, and PBS2 and PBI transcripts become the major gene-specific mRNA during stages III to VI. In contrast, for OJ-19, PBS1 transcripts increase during the third to fourth hours of stationary phase, and are then maintained at a relatively high level for the following 14 hours. These observations are presumably due to differences in the availability and/or activity of certain regulatory proteins in *B. subtilis* 168 and OJ87. The possibility that the orientation of the pC194 fragment in Camtaq 10 and 11 plays a role in affecting transcription from PBS1 and PBS2, although unlikely, cannot be excluded.

Table 1 shows a comparison of the −10 and −35 regions of PBS1 and PBS2 with the consensus sequences for the −10 and −35 regions of promoters recognized by *B. subtilis* RNA polymerase containing different σ species (Moran *et al.*, 1982; Johnson *et al.*, 1983; Gilman *et al.*, 1981; Wong *et al.*, 1984; Wang & Doi, 1984). The −10 and −35 regions of PBS1 both have significant homologies (6/7 and 5/7 bases, respectively) with the −10 and −35 consensus sequences for σ^{29} RNA polymerase, whereas for PBS2, only the −10 region (6/7 bases), but not the −35 region, has homology with the corresponding consensus sequences. Homologies between this −35 region and the consensus −35 regions corresponding to the different forms of *B. subtilis* RNA polymerase could not be found.

Since σ^{29} is only detectable in extracts of *B. subtilis* for a limited period during the stationary phase of growth (Haldenwang *et al.*, 1981; Trempy *et al.*, 1985), this sigma factor could, in part account for the temporal expression of the δ-endotoxin from the PBS1 promoter. It is possible that σ^{29} cannot be detected in extracts of *B. subtilis* after stage III because of its compartmentalization into the forespore (Nakayama *et al.*, 1981). This sequestration of σ^{29} from the mother cell could therefore account for the transient nature of transcriptional initiation from PBS1 in 168-11. Clearly this compartmentalization cannot occur in OJ, since stage O mutations prevent the formation of an asymmetric septum (Piggot & Coote, 1976; Hoch *et al.*, 1978). In addition, the OJ lesion differs from the other stage O mutations in that it does not appear to affect the synthesis of σ^{29} (Trempy *et al.*, 1985). The continued availability of σ^{29} in OJ cells could therefore account for the prolonged and high level of expression from PBS1 observed in OJ-19. In contrast, the expression of the δ-endotoxin in 168-11 during stages III to VI of sporulation appears to be due to transcription from PBS2 and PB1. It is conceivable that, in OJ-19, continued utilization of PBS1 impairs transcriptional initiation at PBS2 and PB1. Alternatively, the availability of different regulatory factors in the two recombinants may be the major determinant of promoter recognition.

The ability of *B. subtilis* to utilize multiple transcriptional initiation sites has been observed for several other *Bacillus* genes (Wong *et al.*, 1983; Johnson *et al.*, 1983; Wang & Doi, 1984; Wong *et al.*, 1984). For the wild-type 168 strain particularly, the data in this report suggest that this property contributes to the prolonged expression of the δ-endotoxin gene throughout sporulation. It will be necessary to carry out transcription assays *in vitro* to characterize the factors involved in the utilization of these promoter sites.

Table 1

Comparison of the conserved −10 and −35 regions

Holoenzyme/ promoter	−35	Spacing	−10
B. subtilis			
$E\sigma^{43}$	TTGACA	17–18	TATAAT
$E\sigma^{37}$	AGG-TT	13–16	GG-ATTG-T
$E\sigma^{32}$	AAATC	14, 15	TA-TG-TT-TA
$E\sigma^{29}$	A-TT-AAAA	14–17	CATATT-T
$E\sigma^{28}$	CTAAA	16	CCGATAT
var. *israelensis*			
PBS1	GATTAATAA	15	CATAATTT
PBS2	ACATGCACC	16	AATATTAT

Comparison of the conserved −10 and −35 regions of *B. subtilis* promoters (Johnson *et al.*, 1983; Tatti & Moran, 1985; Cowing *et al.*, 1985) recognized by different holoenzyme forms of RNA polymerase with the promoter sequences of the cloned var. *israelensis* δ-endotoxin gene utilized in *B. subtilis*. Nucleotides corresponding to PBS1 and PBS2 which show identity with the $E\sigma^{29}$ conserved sequences are shown by single and double underlining, respectively. Sequence hyphens are omitted for clarity.

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