Regulatory sequences of two flagellin genes in Bacillus thuringiensis subsp. alesti

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Two highly homologous flagellin genes, flaA and flaB, are expressed in Bacillus thuringiensis subsp. alesti. Both genes were found to be transcribed during vegetative growth. After the onset of sporulation, transcripts could not be detected. Both flaA and flaB were found to be transcribed from σ70-like promoters. In addition, the 3'-terminal half of flaA was cloned and sequenced, completing the sequence of flaA.

Keywords: Bacillus thuringiensis, flagellin gene, regulatory sequence

INTRODUCTION

Bacillus thuringiensis (Bt) is a well characterized entomopathogen found in diseased insects, soil and in the phylloplane. Its insecticidal activity is mainly due to the δ-endotoxin, a crystalline, proteinaceous inclusion body produced during sporulation. When toxin crystals are ingested by susceptible insects, the crystals are solubilized in the midgut, toxins are activated and the insect gut epithelium is fatally damaged, presumably by toxin-mediated formation of pores through the epithelial cell membrane (Lereclus et al., 1989). Spore-containing crystal preparations are used in protection against various insect pests. Previous reports indicate that the spores also may play a role in killing certain insect species (Li et al., 1987). The δ-endotoxin lacking strain Bt75 subsp. alesti is virulent to Trichoplusia ni both when injected and when fed orally (Lövgren et al., 1993; Lövgren, 1992). The δ-endotoxin lacking strain Bt13 subsp. gelechiae has an LD₅₀ of 0.52 c.f.u. per T. ni larva when injected (Zhang et al., 1993). Together, these observations show that the δ-endotoxin is not an absolute prerequisite for Bt virulence and that vegetative Bt can establish an infection in a live host.

Apart from the δ-endotoxin, several potential virulence factors have been identified in Bt, such as the β-exotoxin thuringiensis (an ATP analogue), phosphatidylcholine-degrading (PC-PLC, lecininase) and phosphatidylinositol-specific (PI-PLC) C-type phospholipases (Faust, 1977; Zhang et al., 1993), immune inhibitor A (InA) (Lövgren et al., 1990) and flagella. Earlier observations have shown that purified Bt flagella, as well as flagellated Bt, can bind to insect cells in culture (Zhang et al., 1995), suggesting that Bt adheres to host cells with the aid of flagella. In this paper, we have analysed the transcription of two flagellin genes in Bt5 subsp. alesti, showing transcription from vegetative promoters during early- to mid-exponential growth phase.

METHODS

Strains and media. Bt subsp. alesti strain 5 (Bt5) (Somerville & Pockett, 1975) was used for studies of flagellin transcription and as a source of Bt chromosomal DNA. Escherichia coli DH5α was used as a host strain for screening of Bt5 genomic libraries and cloning. Bacteria were grown in LB or TYB medium at 37 °C as previously described (Lövgren et al., 1993).

DNA extraction, library construction and screening. Bt5 DNA was isolated as previously described (Lövgren et al., 1993). A library made of partially Sac3A1-digested Bt5 genomic DNA previously used for cloning the 5' terminus of flaA (Lövgren et al., 1993) was screened with a 21-mer oligonucleotide derived from the 3'-terminal sequence of flaA (Lövgren et al., 1993), to obtain a flaA 3'-terminal clone. Plasmids and oligonucleotides used are listed in Table 1.

DNA methods. Oligonucleotides were end-labelled using T4 polynucleotide kinase according to a standard procedure (Maniatis et al., 1982). Double-stranded DNA sequencing was performed with the Sequenase kit (USB/Amersham). PCR was performed using a DNA Thermal Cycler (Perkin-Elmer Cetus). Taq polymerase was purchased from Perkin-Elmer. PCR
conditions were: 95 °C, 1 min; 55 °C, 2 min; 72 °C, 3 min; 40 cycles. All other DNA work was done according to standard procedures.

**RNA methods.** Primer extension was performed using a Promega kit. End-labelled primer (10 ng, 42-mer) was annealed to 20 μg Bt5 total RNA for 20 min at 70 °C, in the annealing buffer provided in the kit. Extension was carried out at 42 °C for 30 min. Extension products were separated on a 6% (w/v) polyacrylamide gel. As a size marker, a sequencing reaction with the flaA 5'-terminal clone and primer P1 was used.

For RACE-PCR (rapid amplification of cDNA-PCR), the 5'-AmpliFINDER RACE kit (Clontech) was used. For the synthesis of cDNA, flaA-specific (P8) or flaB-specific (P4) primer was annealed to 10 μg Bt5 total RNA in the presence of the T4 RNA ligase. The ligated anchor-cDNA was then used as a template for PCR, using anchor-complementary and gene-specific primers (P1, P2). To 100 μl of the PCR reaction mixture, 5 μl [α-32P]dATP was added. RACE-PCR products were analysed as described above for primer extension products.

**RNA extraction.** Bt5 RNA was isolated by sonication from Bt5 grown in LB or TYB as previously described (Zhang & Lövgren, 1995).

**Northern blots.** Total RNA was isolated at different times from Bt5 grown in TYB. Equal amounts of RNA were separated on a 1.5% (w/v) agarose gel according to Klemenz et al. (1985) and blotted onto Hybond N membranes (Amersham) by the capillary blot method. Membranes were hybridized at 42 °C for 18-20 h with end-labelled oligonucleotide probes (C7 for flaA, C4 for flaB) in a buffer of 5 x Denhardt's solution (1 x Denhardt's is 0.2 g Ficoll l−1, 0.2 g polyvinyl pyrrolidone l−1, 0.2 g BSA l−1), 5 × SSC (1 × SSC is 0.15 M sodium chloride, 0.015 M sodium citrate) and 0.5% SDS, at intermediate (50%, v/v) and low (45%, v/v) formamide stringency conditions. Following hybridization, the membranes were washed twice for 10 min at 42 °C in 2 × SSPE (1 × SSPE is 0.18 M sodium chloride, 0.001 M sodium phosphate, 0.001 M EDTA, pH 7.7) and 0.1% SDS followed by one wash for 20 min at 42 °C in 1 × SSPE and 0.1% SDS. For the quantification of fla transcripts, autoradiograms were scanned and analysed in a PhosphorImager using the ImageQuant program (Molecular Dynamics).

**RESULTS AND DISCUSSION**

We have previously cloned one partial and one complete flagellin gene from Bt5 (flaA and flaB, respectively). The flaA clone, pT33-1, ends with a Sau3AI site 365 bp into the putative flaA ORF. This site is not present in flaB (pT33-2), due to a single base substitution (Fig. 1; Lövgren et al., 1993). To clone the flaA 3' terminus, a Bt5 library made with partially digested total DNA was screened using an oligonucleotide probe derived from the 3' terminus-encoding sequence of flaB.

One clone (pT33-C) was obtained, containing a 0.6 kb insert. Sequence analysis of this insert revealed a high degree of homology with the flaB 3' terminus (Fig. 1). Homology was lowest in a region corresponding to the middle part of flaB. Together, these facts suggested that we had indeed cloned the 3' terminus of flaA. Simply combining the 5' and 3' terminals of flaA sequences to one gene showed that the homology to flaB extended past the

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**Table 1. Plasmids and oligonucleotides used**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Vector/insert</th>
<th>Reference</th>
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<tr>
<td>pT33-1</td>
<td>pUC8/1072 bp of flaA (5'-terminal)</td>
<td>Lövgren et al. (1993)</td>
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<tr>
<td>pT33-2</td>
<td>pUC8/1604 bp of flaB (complete)</td>
<td>Lövgren et al. (1993)</td>
</tr>
<tr>
<td>pT33-C</td>
<td>pUC8/600 bp of flaA (3'-terminal)</td>
<td>This work</td>
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**Oligonucleotide**

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<tr>
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<th>Position A/B†</th>
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<td>21-mer</td>
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<td>939-959 B</td>
</tr>
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<td>42-mer</td>
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<td>778-737 A</td>
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<tr>
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<td>-</td>
<td>901-869 A</td>
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<td>P2</td>
<td>ACCTGTGACAGGTTTACGGGCCTTCTTTCGC</td>
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<td>P4</td>
<td>CTTTACCTCTTGAGATTAGTACC</td>
<td>-</td>
<td>794-770 B</td>
</tr>
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<td>GTGATAATCTTTCCACACCCCTT</td>
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<td>C4</td>
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<td>-</td>
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<td>C7</td>
<td>AGGCTTACCTGCCGCTGACTGAGTACG</td>
<td>-</td>
<td>1252-1227 A</td>
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</tbody>
</table>

* +, coding strand; −, complementary to coding strand. 
† A and B denote sequence positions in flaA and flaB, respectively.

**Fig. 1.** Sequence comparison of flaA and flaB. Gaps have been introduced in the coding sequences for maximum alignment. Deduced transcriptional start sites are denoted as +1. Vegetative promoters (−35, −10) and a putative σ24 promoter (−24, −12) are indicated by solid lines. The 3'-terminal flaA clone, pT33-C, begins at the indicated Sau3AI site (Sau3A). The 355 bp repeat present in flaB lies at nucleotide positions 48-402 and 968-1323. Parts of the repeat are present in flaA, at positions 694-756 and 1313-1544.
Fig. 2. Mapping of the transcription initiation points of flaA and flaB. RACE-PCR was performed as described in Methods. RACE-PCR products were run in parallel with a DNA sequence reaction as a marker. (a) pT33–1 (flaA 5′-terminal clone), primer P1; (b), pT33–2 (flaB full-length clone), primer P2. Start sites were deduced by counting off the size of the AmpliFINDER anchor-specific primer (48 nt), as well as the terminal A residue unspecifically added by Taq polymerase, from the PCR product. The sequences of the size markers are complementary to mRNA.

San3AI site present in flaA without apparent insertions or deletions at this site (Fig. 1). However, since there was no overlap between the new sequence and the previously published flaA 5′-terminal-encoding sequence, further evidence was required to establish the identity of the new clone.

PCR was performed using Bt5 chromosomal DNA and primers corresponding to 5′- and 3′-terminal flaA sequences, respectively. Specific primer sets were chosen to confirm that the 5′- and 3′-terminal clones of flaA were derived from the same gene. As a control the flaB gene was amplified. The resulting PCR products were separated on an agarose gel and their sizes were calculated. None of the PCR reactions gave rise to more than one band on the agarose gel and the PCR product sizes were as expected (data not shown). Subsequent cloning of flaA PCR products was unsuccessful, but direct sequencing of one of these products showed that no additional San3AI fragment was present in the internal San3AI site of flaA, and that the 5′- and 3′-terminus-encoding clones comprise one gene (data not shown).

Comparison of the coding regions of flaA and flaB reveals a high degree of identity in the 5′- and 3′-terminal regions, and a region of variability in the middle region. flaA and flaB share a 90% overall identity at both the DNA and amino acid levels. Both Fla proteins are homologous to flagellins from other bacterial species in their N- and C-terminal regions. The 355 bp direct repeat present in flaB is in part, with only minor alterations, present in flaA (Lövgren et al., 1993). This suggests that flaA has arisen through duplication of flaB, though the mechanism of this duplication remains unknown. In Campylobacter coli and
C. jejuni, duplicated flagellin genes have been shown to provide a genetic reservoir, protecting against mutations in the major flagellin gene by acting as a substrate for recombination (Alm et al., 1993). In this case, the minor (duplicate) flagellin gene does not normally contribute to motility or the ability to colonize host cells. In the case of Bt5 fla genes, both are transcribed simultaneously and both gene products may be assembled into flagella.

Transcriptional analysis of flaA and flaB was performed to confirm that both genes are expressed and to determine their respective promoters. Primer extension cannot easily distinguish between flaA and flaB transcripts because of the extensive identity in the 5' region of their coding sequences. Primer extension gave one extension product and the deduced transcriptional start points of flaA and flaB were indistinguishable from those determined by RACE-PCR (data not shown). RACE-PCR allows proper identification due to the cDNA-synthesis step where primers specific to the middle regions of flaA and flaB are used. Transcription of flaA starts at an A residue 9 bp downstream of a σ70-like promoter (−35, TGGACA; −10, TACAAT). Transcription of flaB starts at a A residue 6 bp downstream of a similar σ70-like promoter (−35, TCCACA; −10, TACAAT) (Fig. 2).

Northern blot analysis of fla transcripts shows transcription of both flaA and flaB during early- to mid-exponential growth phase, coinciding with the maximal growth rate. Following the drop in growth rate in late-exponential phase, fla transcripts are no longer detectable (Fig. 3). This correlates well with transcription from vegetative promoters. A similar expression pattern, with a slight time lag, is seen when flagellin released into the growth medium is measured (Lövgren et al., 1993). E. coli DH5α harbouring the full-length flaB clone expresses Bt5 flagellin during exponential growth, presumably through transcription from the vegetative flagellin promoter (Lövgren et al., 1993).

Previously, flagellin expression in Bt has been shown to be independent of catabolite repression (Lövgren et al., 1993), which also suggests vegetative promoter regulation. Major vegetative promoters governing flagellin transcription have only rarely been reported (Dons et al., 1992; Penn & Luke, 1992). Usually, bacterial flagellin genes are transcribed from the σ28, or σ54-dependent specialized minor promoters (Helmann, 1991; Mirel & Chamberlin, 1989; Guerry et al., 1991; Suerbaum et al., 1993; Kutsukake et al., 1990; Arnosti & Chamberlin, 1989). In Campylobacter, the major flagellin gene (flaA) is under the control of σ28, whereas the minor gene (flaB) is regulated by σ54. A σ54-dependent promoter is present 322 bp upstream of the dedicated transcription start point of flaA (Fig. 1). To investigate the role of this promoter, studies can be done fusing different parts of the flaA upstream region to a promoterless reporter gene and expressing the constructs in Bt. One might also identify transcriptional control elements other than the −35, −10 vegetative promoter. It is unlikely that vegetative transcription ceases altogether as soon as the cells enter the transition state, in which growth continues, albeit at a lower rate (Fig. 1).

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**REFERENCES**


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