APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2006, p. 1771–1776 Vol. 72, No. 3
0099-2240/06/$08.00+0 doi:10.1128/AEM.72.3.1771–1776.2006

Transcriptional Analysis of the Toxin-Coding Plasmid pBtoxis from *Bacillus thuringiensis* subsp. *israelensis*

Claudia Stein, Gareth W. Jones, Tanya Chalmers, and Colin Berry*

Cardiff School of Biosciences, Cardiff University, Museum Avenue, Cardiff CF10 3US, United Kingdom

Received 13 December 2005/Accepted 14 December 2005

In *Bacillus thuringiensis* subsp. *israelensis* all of the insecticidal toxins are encoded on a single, large plasmid, pBtoxis. Sequencing of this plasmid revealed 125 potential coding sequences, many of which have predicted functions in gene regulation and physiological processes, such as germination. As a first step in understanding the possible role of pBtoxis in its host bacterium, a survey of the transcription of genes with predicted functions was carried out. Whereas many coding sequences, including those previously identified as probable pseudogenes, were not transcribed, mRNA was detected for 29 of the 40 sequences surveyed. Several of these sequences, including eight with similarities to the sequences of known transcriptional regulators, may influence wider gene regulation and thus may alter the phenotype of the host bacterium.

Bacillus thuringiensis subsp. *israelensis* is widely used for control of dipteran pest insects and vectors of human disease, such as mosquitoes and blackflies. This bacterium has an excellent safety record, and there have been no reports of insect resistance in the field. An important factor in this lack of resistance is the complex arsenal of toxins produced by this organism (5), all of which are encoded by a single, large plasmid, pBtoxis. Sequencing of this plasmid (3) revealed 125 coding sequences (CDSs), including the sequences of the previously identified toxin genes (*cry4Aa*, *cry4Ba*, *cry11Aa*, *cry10Aa*, *cyt1Aa*, and *cyt2Ba*); a new, putative toxin gene, *cyt1Ca*, encoding a previously undescribed fusion toxin; several CDSs corresponding to fragments of toxin-encoding sequences; and numerous transposition-associated CDSs. Whereas such sequences accounted for 30% of the CDSs, it is likely that other plasmid CDSs may also have an effect on the host cell phenotype. Several of the CDSs appeared to be related to genes encoding transcriptional regulators in other organisms. Production of such regulators might alter the transcription of genes located both on the plasmid and on the chromosome and thereby have significant effects on the phenotype and, perhaps, on virulence.

As a first step in analyzing the possible roles of such plasmid genes, we performed a transcriptional survey of the pBtoxis genes using previously identified orthologs in other organisms. Clearly, effects on the phenotype can be exerted only through expression of the plasmid CDSs. Thus, we identified potentially important genes that could be investigated further to increase our understanding of the molecular biology of *B. thuringiensis* subsp. *israelensis*.

MATERIALS AND METHODS

Bacterial strains. For this survey, we utilized *B. thuringiensis* subsp. *israelensis* strain 4Q7 (also known as 4Q2-81), a plasmidless strain, in order to prescreen CDS-specific primers to ensure that there were no genomic copies of the genes that might produce false-positive results for pBtoxis-derived gene expression. *B. thuringiensis* subsp. *israelensis* strain 4Q5 (also known as 4Q2-72) has been cured of all plasmids except pBtoxis and was used to determine whether transcripts were produced from CDSs on this plasmid (since full sequences have not been determined for all *B. thuringiensis* subsp. *israelensis* plasmids, the use of strain 4Q5 eliminated false-positive results if related CDSs were present on other plasmids).

CDSs used. Table 1 shows the subset of pBtoxis CDSs surveyed in this study and their relatedness to previously reported genes, as identified during sequencing of the plasmid (3). As a positive control, the gene encoding the Cry11Aa toxin (pBt023) was chosen since production of this protein, like production of Cry4Aa, Cry4Ba, and Cyt1Aa, is known to be significant (13).

Detection strategy. For each CDS surveyed, a pair of oligonucleotide primers was designed so that they had annealing temperatures in the approximate range from 58 to 66°C and produced 128- to 703-bp amplicons from the target sequences (Table 2). The abilities of the primer pairs to produce the desired amplicons were confirmed by colony PCR using vegetative cells of strain 4Q5 as the template; the PCR conditions were 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min and then a final extension at 72°C for 10 min. Each primer pair was then used in a similar colony PCR using strain 4Q7 to test for the presence of related CDSs in the host genomic DNA. As a further control, PCRs were carried out with primer pairs in the absence of any template.

To detect transcription, RNA was isolated from *B. thuringiensis* subsp. *israelensis* 4Q5 cultures grown at 30°C with shaking in NYSM medium (11), using an RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. Samples (1 ml) were removed for RNA isolation at approximately 12, 24, and 36 h after inoculation of 30 ml of medium with one colony from a plate that was incubated overnight. These times were chosen since they corresponded to times when there were rapidly dividing vegetative cells at the early times and when there were cells in different phases of the sporulation process later. RNAs from these different times were then pooled for reverse transcription since it was concluded that the reverse transcription-PCR technique is so sensitive that information concerning the stage specificity of transcription could not be derived reliably. For reverse transcription, Superscript RNase H$^-$ reverse transcriptase (Invitrogen) was used to obtain cDNA from approximately 200 ng of isolated RNA with 12.5 μg/μl random hexamer primers in a 20-μl (final volume) mixture according to the manufacturer's instructions. In each case, a duplicate sample to which no reverse transcriptase was added was prepared as a negative control. This sample was used in subsequent PCRs in parallel with the reverse-transcribed RNA to ensure that there was no bacterial DNA contamination of the RNA samples that would lead to false-positive results. PCR was carried out under the conditions described above for the colony PCR except that the initial step consisting of 95°C for 5 min was omitted. Amplified products were visualized in 1.5% agarose gels (>200-bp amplicons) or 2% agarose gels (<200-bp amplicons) that were stained with ethidium bromide.

RESULTS AND DISCUSSION

Genomic homologs. No CDS-specific primer pairs produced amplicons in template-free controls, indicating that artifactual

* Corresponding author. Mailing address: Cardiff School of Biosciences, Cardiff University, Museum Avenue, Cardiff CF10 3US, United Kingdom. Phone: 44-29-20874508. Fax: 44-29-20874116. E-mail: Berry@cf.ac.uk.

TABLE 1. CDSs included in the transcriptional survey

CDS[a]	Predicted product	Transcriptional activity
pBt011	Putative DNA-binding protein	Yes
pBt014	Probable transcriptional regulator	Yes
pBt020	Hypothetical protein	No
pBt021	Cyt1Aa toxin	Yes
pBt023	Cry11Aa crystal toxin	Yes
pBt029	Putative DNA-binding protein	No
pBt031	Putative *N*-acetylmuramoyl-L-alanine amidase (peptidoglycan hydrolase)	Yes
pBt036	Cyt2Ba cytolytic delta-endotoxin	Yes
pBt054	Cyt1Ca possible two-domain toxin	Yes
pBt056	Hypothetical protein (potential pseudogene; matches pBt152 with two frameshifts and an in-frame stop)	No
pBt060	Putative spore germination protein (pseudogene; contains two potential frameshifts)	No
pBt063	Putative spore germination protein (pseudogene; truncated by IS*240* insertion)	No
pBt075	Hypothetical protein; weakly similar to a *Yersinia pestis* plasmid hypothetical protein	Yes
pBt084	Putative spore germination protein; similar to *B. subtilis* GerAC	Yes
pBt085	Putative spore germination protein; similar to *B. subtilis* GerBB	Yes
pBt086	Putative spore germination protein; similar to *B. subtilis* GerKA	Yes
pBt087	Putative 1-phosphatidylinositol phosphodiesterase precursor; contains an in-frame TGA stop after amino acid 86	No
pBt091	Putative transcriptional regulator, ArsR family	Yes
pBt092	Small DNA-binding protein (bacterial histone-like family)	Yes
pBt093	HfQ protein (RNA-binding protein)	No
pBt094	Putative transcriptional regulator; similar to *B. subtilis* transition state regulators	Yes
pBt97	Putative class II aminotransferase	Yes
pBt98	Pyridoxal phosphate-dependent enzyme	Yes
pBt100	tRNA synthetase-related protein	Yes
pBt101	Possible kinase	Yes
pBt102	GntR family transcriptional regulator containing aminotransferase domain	N/D[b]
pBt108	Putative sigma factor, ECF family	Yes
pBt120	Putative DNA-binding protein	Yes
pBt131	Putative ABC transporter permease protein	No
pBt132	Putative ABC transporter ATP-binding protein	N/D
pBt133	Putative ABC transporter exported solute-binding protein	N/D
pBt136	Possible peptide antibiotic precursor	Yes
pBt137	Integral membrane protein (possible peptide antibiotic maturation and biosynthesis protein)	Yes
pBt138	Integral membrane protein (possible accessory factor in peptide antibiotic secretion)	Yes
pBt139	Putative ABC transporter ATP-binding protein	No
pBt145	Putative spore coat-associated protein; similar to *B. subtilis* spore coat-associated protein N CotN	Yes
pBt147	HfQ protein (RNA-binding protein)	No
pBt148	Putative transcriptional regulator; similar to *B. subtilis* transition state regulators	Yes
pBt149	Putative transcriptional regulator, ArsR family	Yes
pBt152	Hemagglutinin-related protein	Yes
pBt156	FtsZ/tubulin-related protein	Yes
pBt157	Putative DNA-binding protein; contains predicted helix-turn-helix motif	Yes
pBt158	Putative transcriptional regulator, MerR family	No

[a] The CDS designations are those described by Berry et al. (3), who describe each CDS and its database homologs in more detail. The transcriptional activity determined in this study is indicated.

[b] N/D, not determined (activity of the gene was not assessed due to the presence of closely related genomic copies of the gene).

products from primers alone were not generated. Prescreening of primer pairs with plasmidless *B. thuringiensis* subsp. *israelensis* strain 4Q7 resulted in production of amplicons with the pBt102, pBt132, and pBt133 oligonucleotide pairs. The resulting fragments were cloned into the vector pGEM-T (Promega) and subjected to DNA sequencing. Analysis of these sequences revealed very closely related genomic homologs of the plasmid target genes (and also homologs in the complete *B. thuringiensis* subsp. *konkukian* genome [accession number AE017355]). As a result, analysis of the expression of the corresponding plasmid CDSs could not be performed reliably. No other primer pairs produced amplicons from strain 4Q7 cells, whereas all other primer pairs produced fragments of the expected sizes when strain 4Q5 cells (containing the pBtoxis plasmid) provided the template. Products of the expected sizes were also generated by reverse transcription-PCR from some but not all CDSs (data not shown), and the amplicons were taken to indicate the transcriptional activities of the genes. In the case of four genes (pBT036, pBt084, pBt108, and pBt138), the amplicon was also cloned and sequenced to verify that the products were derived from the intended genes. In each case, the sequence corresponded to the expected amplicon from pBtoxis. Table 1 shows the results of the transcriptional survey. It should be noted that in our experiments several CDSs were transcriptionally inactive. However, we cannot completely rule out the possibility that these genes are transcribed (for instance, under other growth conditions or under the influence of unknown inducers). Nonetheless, our survey did indicate that there was mRNA production from many of the plasmid genes assessed, as discussed below.

Toxin-related CDSs. As expected, transcripts for the *cry11Aa* (pBt023) and *cyt1Aa* (pBt021) genes were detected, which is consistent with the fact that the corresponding proteins are produced abundantly by *B. thuringiensis* subsp. *israelensis* during sporulation. In addition, transcripts were also detected for the *cyt1Ca* (pBt054) and *cyt2Ba* (pBt036) genes, for which spore-associated proteins have not been found in strain 4Q5 (although possible detection of Cyt2Ba was observed by Western blotting in *B. thuringiensis* subsp. *israelensis* strain 1884 [6]). The reason for the apparently low levels of these Cyt proteins may include instability of the transcript and/or the resultant protein, failure in translation of the message, and, in the case of Cyt2Ba, possible masking of the product on protein gels by Cyt1Aa, which is more abundant but a similar size.

CDS pBt020 encodes a hypothetical protein with no known homologues in the NCBI nonredundant database. We decided to analyze this CDS because of its location in the pBtoxis plasmid, in which it is immediately downstream of the *cyt1Aa* gene, and because it and *cyt1Aa* are convergently transcribed and there are only 2 nucleotides (nt) between their stop codons. As a result, transcriptional read-through from both genes to produce antisense RNA with respect to the other gene is a theoretical possibility. In our experiments, an amplicon was produced using the pBt020 primers; however, our method of cDNA production using random hexamers would have produced an amplicon from this CDS whether RNA was produced as sense RNA (from a putative pBt020 promoter) or as antisense RNA (from the *cyt1Aa* promoter). As a result, further experiments were performed with this pair of CDSs to determine the nature of the transcripts detected in the initial experiment

TABLE 2. Oligonucleotide primer sequences based on the CDS of pBtoxis

CDS	Orientation	Oligonucleotide primer (5′–3′)	T_m (°C)[a]	Amplicon size (bp)
pBt011	Forward	GACAATTATCGAAGTTGAAAAGG	62	430
	Reverse	ACTATTGAATCCTTCCGTTTCC	62	
pBt014	Forward	ACAGTAATCTATCTAAAATTGAGC	62	278
	Reverse	TCTAAAGTTTCACGTAGCTTCC	62	
pBt020	Forward	GAAGAACAAATTTTAGAGAAAGG	60	128
	Reverse	TAGTAGCTATATTTATTAAATATGG	60	
pBt021	Forward	CTGGGGCAAGGAAACTGCTA	62	270
	Reverse	TTAGAGGGTTCCATTAATAGCG	62	
pBt023	Forward	AGATAGTTCTTTAGATACTTTAAG	60	314
	Reverse	CATCACTAACCTCAATAATCC	60	
pBt029	Forward	TATTAGGTTCAGAAAAGAATCCG	62	223
	Reverse	CAACAGAACTTATATTTCTTTCC	60	
pBt031	Forward	GGTGCACATGATTCAGGTGC	62	679
	Reverse	CGATGTGCATTACCTTCAAGG	62	
pBt036	Forward	AGGCATATTGCATTAACAGTTCC	64	411
	Reverse	TGTTTGAGTAGCTGATAAATTACG	64	
pBt054	Forward	GCACTAGTACCTACTACATCC	62	703
	Reverse	GGTTTTGAATACTGTAAGCACG	62	
pBt056	Forward	GATGATTTGTTTAATCCTGAATGG	64	323
	Reverse	TTCATTGTGGGGAAACTTTGC	60	
pBt060	Forward	GAATGAAAATATTGAAACCATCC	60	391
	Reverse	AGAAATAGGTTCCTGAATCG	60	
pBt063	Forward	TTATTCATTCGCTACAACTTGC	60	192
	Reverse	CAAAATAACTCGTTAGAATCTGG	62	
pBt075	Forward	AGGAGATAGTCTAAGATATGTACG	66	324
	Reverse	GTATTACCATTTAGATCTGTGACG	66	
pBt084	Forward	TAATTGCCTTAGATAGAACACC	60	541
	Reverse	GAGATTTTGGTTAAGTACTTTCC	62	
pBt085	Forward	TATTCACTCACTGCAACTGGC	62	623
	Reverse	CTGTATTCGCTATCAGTATTCC	62	
pBt086	Forward	GAATGAGATTGTTGAAACTATCC	62	641
	Reverse	AGTTAGATAATTCTCCTATGCC	60	
pBt087	Forward	GACTTGTGTAACATTAGTAGGC	62	598
	Reverse	TATACCGTATTCTTGGGATGC	62	
pBt091	Forward	CGATCTTAATTATGAACTCGAGC	64	244
	Reverse	CACTTGTTTATCGTCCACTCG	62	
pBt092	Forward	TCAGAATTAATCAAACAAGTCGC	62	257
	Reverse	ACAGCTTCTTTTAATGCTTTTCC	62	
pBt093	Forward	TCTTTGCAGGAACAATTGTTGC	62	180
	Reverse	GAATCGAATCGTCGAGATTGC	62	
pBt094	Forward	GAAAGCAACCGGTATTGTACG	62	270
	Reverse	ACTTTGTATTGTTCTAATTCTTCC	62	
pBt097	Forward	GGCTGAAGAGAAGGCATGGC	64	343
	Reverse	CACAGTGCTCCTAGTCTCTCC	66	
pBt098	Forward	TGCCTATGAGTCATTGGCGG	62	356
	Reverse	CTGCATATGATGCCGCTGAGC	66	

Continued on following page

TABLE 2—*Continued*

CDS	Orientation	Oligonucleotide primer (5′–3′)	T_m (°C)[a]	Amplicon size (bp)
pBt100	Forward	CGAAAATTATATCTCCAAGATCC	62	582
	Reverse	GCATGGAATATGCATATCACC	60	
pBt101	Forward	CTATATGCTAGTGCTGAAGCC	62	403
	Reverse	CATTATCAACGATAACCAATCGC	64	
pBt102	Forward	AGAGGTACACGTGTTAGTACAC	64	358
	Reverse	GTTGTGCGCCTGATGTAATCATA	66	
pBt108	Forward	ACGTTGTGAAACAAATTCTATGG	62	303
	Reverse	GTTCATTCATATTACTAAGTACG	60	
pBt120	Forward	CTATTAGGTTCAGAAAATAATCC	60	246
	Reverse	TAACTCTATTTCTTTGCTCATGC	62	
pBt131	Forward	CCATGCTTGGGATTATTATCG	60	683
	Reverse	AGTTGCATCACTTGGCTTGCG	64	
pBt132	Forward	CGTATTATCAAGGAAAATTGGC	60	455
	Reverse	CGTTGGATTATTCGCAATCGC	62	
pBt133	Forward	CACTAATTGTTATTGTGGCAGC	62	686
	Reverse	TTGGATTTGTGACTTCTGAAGC	62	
pBt136	Forward	TGTTCGAGATTATGGGTTATTTTG	64	218
	Reverse	CCATGCAACAGCTTGTGCTTTTA	66	
pBt137	Forward	CCTTATTATTTGAGGGTATTACG	62	652
	Reverse	GCTGAAATTATGTATAAAATTAGG	60	
pBt138	Forward	CACATAAGTTTGAAATATATATGG	60	425
	Reverse	GTATATTGAATTTTACGAAGCCG	62	
pBt139	Forward	CTACATAATATCTCATTTTCTGC	60	377
	Reverse	GACCAATACAATTTGTGACGC	60	
pBt145	Forward	GAAATTAGGTGCTGGAGTGG	60	446
	Reverse	ATTTTAATCCACCTGTTTCTGC	60	
pBt147	Forward	CGAAATTACAATCATTTCAAAAGG	62	225
	Reverse	AGAATCGAATCGTAGAGATTGC	62	
pBt148	Forward	TGAAAGCAACAGGTATTGTACG	62	252
	Reverse	CAGCTAACAGTTGTTTTAGACC	62	
pBt149	Forward	TCTATATGGATATTGAGTATTACG	62	273
	Reverse	CCTCTATTCTCTTCATGCTCTC	64	
pBt152	Forward	GGAGATTTGACTGCCTTTGG	60	675
	Reverse	CCAATCATTACAATTAACAGCG	60	
pBt156	Forward	AAGAGGCTGATTTATTCGCAGG	64	425
	Reverse	GTTAATTTCATCAGGATCACCG	62	
pBt157	Forward	TGAATAGGGATCACTTTTATACG	62	279
	Reverse	AACACTCTACTATTTCTACACC	60	
pBt158	Forward	TCTGAACTTACTGGGCTTTCG	62	397
	Reverse	TAAGATCCTGATTTCTACGTGC	62	

[a] Approximate denaturation temperatures (T_m) (based on approximations for short sequences) were determined as follows: $T_m = 4(G + C) + 2(A + T)$.

described above. Using the same RNA sample, separate reverse transcriptase reactions in which the random hexamer primers were replaced by the following primers were carried out: pBt020 forward or pBt021 reverse (for subsequent detection of a transcript from *cyt1Aa*) or pBt020 reverse or pBt021 forward (for subsequent detection of a transcript from pBt020). Reactions primed with the latter primers produced no product during PCR with the pBt020 forward and pBt020 reverse primers, in-

dicating that there was no transcription from the pBt020 CDS. In contrast, PCR performed with the pBt021 forward and pBt021 reverse primers produced amplicons of the expected size following reverse transcription with either pBt020 forward or pBt021 reverse. This indicated not only that a transcript was produced from *cyt1Aa* (as expected) but that there was significant read-through into the pBt020 region since pBt020 forward must have primed reverse transcription in this region. Thus, it appears that termination of the *cyt1Aa* transcript does not occur at the stem-loop structure that begins 27 nt downstream of the *cyt1Aa* gene and within the pBt020 CDS (GGTAATATCACAAGT ATAAATACTTGTGGTATTACC; $\Delta G = -20.8$ kcal/mol [17]). This sequence lacks the T tract of a classical factor-independent transcriptional termination sequence, although not all such terminators require the T tract (18). In this case, however, it appears that the feature described above is not sufficient to cause transcript termination. The lack of pBt020 transcription means that this CDS is unable to interfere with production of Cyt1Aa from its gene. Analysis of the region upstream of the pBt020 CDS revealed a region with some similarity to a σ^G-like promoter sequence (−35 GTATA-14 nt-CATATTA, 200 nt upstream of the pBt020 ATG initiation codon; compared to the σ^G consensus G[A/C]AT[A/G]-18 nt-CAT[A/T][A/C]TA [8]) that might permit this gene to be transcribed during sporulation, the period during which the *cyt1Aa* gene would be convergently transcribed. The suboptimal spacing of the features identified may explain the apparent lack of pBt020 transcription in our experiments.

Like pBt020, pBt075 encodes a hypothetical protein. However, protein derived from this gene has been identified in association with spores of a transconjugant *Bacillus sphaericus* strain to which an erythromycin-resistant variant of pBtoxis was transferred (4a). Our experiments also indicated that there was production of transcript from this gene in *B. thuringiensis*, although the function of the protein product remains unknown. The CDS apparently encoding a sigma factor (pBt108, sigma E-like) is transcriptionally active, which supports the suggestion (3) that the pBtoxis plasmid may contribute a supplementary sigma factor of a type involved in toxin gene transcription (4, 19–21) that may aid in the production of its own toxins.

Sporulation-, germination-, and cell division-related CDSs. Several CDSs that might have direct effects on the host phenotype were identified during the sequencing of pBtoxis (3). The present study showed that several such CDSs are transcriptionally active. Two CDSs that may be associated with sporulation, pBt145 (related to *cotN*, which produces a secreted protein incorporated into the spore of *Bacillus subtilis* and may be involved in its production [15, 16]) and pBt031 (which produces a protein that has similarities to cell wall hydrolases), are transcribed from pBtoxis. The plasmid also appears to encode proteins with possible germination functions (pBt084, pBt085, and pBt086). Transcript was detected for each CDS in this group, implying that the CDSs are organized as an operon. To analyze this further, PCRs between CDSs were performed using primers pBt084 reverse and pBt085 forward and, separately, primers pBt085 reverse and pBt086 forward. Amplicons of the expected sizes were produced with each of these primer pairs, indicating that there was cotranscription of the genes and confirming that the CDSs are organized in a single operon. No transcript was produced from the pBt060 and pBt063 CDSs, which are related to *ger* and were postulated to be pseudogenes (3). The effect of the plasmid *ger* genes on the host bacterium will be the subject of a separate report (K. Gammon, C. Berry, and B. N. Dancer, unpublished data). Plasmid-directed production of peptide antibiotic factors may be encoded by pBt136, pBt137, and pBt138. In this study we detected mRNA for each of these CDSs, implying that they may be active in directing antibiotic peptide production. To determine the possible operon organization of these genes, PCRs were performed using primers pBt136 forward and pBt137 reverse and, separately, primers pBt137 forward and pBt138 reverse. A product of the predicted size was obtained with the pBt136 forward and pBt137 reverse primers, indicating that pBt136 and pBt137 are cotranscribed. No product was detected in the PCR performed with primers pBt137 forward and pBt138 reverse, suggesting that pBt138 does not form part of the operon with pBt136 and pBt137. It is possible that this two-gene operon may be regulated by PlcR, the pleiotropic regulator of transcription of several extracellular virulence factors in *B. thuringiensis* (1), since it is preceded by a possible −10 sequence (TATAAT; nt 111884 to 111889) and the conserved palindromic sequence TATGNAN$_4$TNCATA (pBtoxis nt 111835 to 111850) associated with PlcR regulation. PlcR-regulated genes are usually turned on at the end of the vegetative phase in cells grown in rich media, such as NYSM (10). The putative ABC transporter genes (pBt131 to pBt133) are orientated divergently with respect to the peptide antibiotic genes described above and encode proteins with low levels of similarity to Bac components involved in bacteriocin production and secretion. We surveyed the transcription of only pBt131 (since genomic homologs of pBt132 and pBt133 were identified [see above]) and found that this CDS appeared to remain untranscribed (similarly, pBt139, the gene encoding the other predicted ABC-type protein on pBtoxis, was transcriptionally silent). Another CDS, pBt152, encodes a protein related to hemagglutinin, and our results indicated that this gene is transcribed, although the significance of this in *B. thuringiensis* remains to be determined. The putative deletion pseudogene pBt056, which matches pBt152 with two frameshifts and an in-frame stop codon, is not transcribed. The plasmid also contains a gene encoding a protein with similarity to the cell division protein FtsZ (pBt156). This gene appears to be transcriptionally active, and this suggests a possible influence of the plasmid on cell division.

Enzyme-encoding CDSs. CDSs encoding putative enzymatic products were also identified in pBtoxis. Phosphatidylinositol-specific phospholipase C is known to have roles in virulence in *Bacillus cereus* (14). The pBtoxis homolog pBt087, however, contains an in-frame stop codon and appeared in this study to be transcriptionally inactive. The genome of *B. thuringiensis* subsp. *israelensis*, however, contains a distinct but related phosphatidylinositol-specific phospholipase C gene (7) that may be transcribed. CDSs encoding other putative enzymes, including pBt097, pBt098, pBt100, and pBt101, all appear to be transcribed.

Regulator CDSs. The pBtoxis plasmid contains 14 CDSs that may have functions in the regulation of other genes based on their similarity to genes encoding DNA-binding proteins, RNA-binding proteins, or known transcriptional regulators. Many of these 14 CDSs (pBt029, pBt093, pBt120, pBt147, and pBt158) were not

transcribed in our experiments (pBt102 was not examined due to the presence of a genomic homolog). However, pBt011, pBt014, pBt091, pBt092, pBt094, pBt148, pBt149, and pBt157 did produce transcripts and therefore may produce regulatory proteins. Some of these active CDSs have homologues with known roles in bacilli; for instance, pBt149 encodes a protein similar to the PagR protein of the anthrax plasmid pXO1, which is known to regulate other genes in bacilli (9), and pBt094 and pBt148 are related to the gene encoding the *Bacillus subtilis* transition state regulator AbrB, a regulator of other regulator proteins (12). Transcription of the genes encoding the putative regulators described above may have great significance for the phenotype and behavior of *B. thuringiensis* strains. Such effects could parallel the recent report (2) of a plasmid-encoded regulator that alters extracellular proteinase production in *Bacillus anthracis*, which, like *B. thuringiensis*, is a member of the *B. cereus* sensu lato group. Of course, genomic regulators would be expected to influence transcription of plasmid genes, but our results may indicate that there is production of plasmid-encoded regulators that may be able to participate in plasmid-genome "cross talk" to influence expression of both plasmid and genomic loci. *B. thuringiensis* subsp. *israelensis* is by no means unique in *B. thuringiensis*, in which the toxins are encoded on large extrachromosomal elements in most strains. We expect that these plasmids, like pBtoxis, encode not only the toxins that are directly responsible for insect pathogenicity but also a variety of other proteins that may affect the phenotype and behavior of the host organism. In this study, we established that many of the pBtoxis genes with putative functions other than toxicity are transcribed. The production of the corresponding proteins and their roles in host processes and their possible contributions to virulence remain to be established, but in this study we took the first step in the investigation of the molecular role of the toxin-coding plasmid in the host cell.

ACKNOWLEDGMENTS

This work received funding from the BBSRC (G.W.J.) and the European Community through the UK Socrates-Erasmus Council (C.S.).

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