Molecular Cloning and Characterization of Two Genes Encoding Sigma Factors That Direct Transcription from a *Bacillus thuringiensis* Crystal Protein Gene Promoter†

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Two sigma factors, σ^35 and σ^28, direct transcription from the Bt I and Bt II promoters of the cryIA(a) gene of *Bacillus thuringiensis*; this gene encodes a lepidopteran-specific crystal protoxin. These sigma factors were biochemically characterized in previous work (K. L. Brown and H. R. Whiteley, Proc. Natl. Acad. Sci. USA 85:4166–4170, 1988; K. L. Brown and H. R. Whiteley, J. Bacteriol. 172:6682–6688, 1990). In this paper, we describe the cloning of the genes encoding these two sigma factors, as well as their nucleotide and deduced amino acid sequences. The deduced amino acid sequences of the σ^35 and σ^28 genes show 88 and 85% identity, respectively, to the sporulation-specific σ^2 and σ^K polypeptides of *Bacillus subtilis*. Transformation of the σ^35 and σ^28 genes into *B. subtilis* shows that the respective *B. thuringiensis* sigma factor genes can complement *spolIGS5* (σ^K) and *spolIC94* (σ^K) defects. Further, *B. thuringiensis* core polymerase reconstituted with either the σ^35 or σ^28 polypeptide directly transcribes from *B. subtilis* promoters recognized by *B. subtilis* RNA polymerase containing σ^K and σ^K, respectively. Thus, σ^35 and σ^28 of *B. thuringiensis* appear to be functionally equivalent to σ^K and σ^K of *B. subtilis*. However, unlike the situation for σ^K in *B. subtilis*, the homologous σ^K gene in *B. thuringiensis* does not result from a late-sporulation-phase chromosomal rearrangement of two separate, partial genes.

The various subspecies of *Bacillus thuringiensis* produce parasporal crystalline inclusions which are toxic to the larvae of insects within the orders Lepidoptera, Diptera, and Coleoptera (reviewed in references 2, 18, 32, and 58). The timing of crystal protein expression suggests that this process is a sporulation-regulated event (31, 48), and researchers have speculated that a modified form of RNA polymerase is involved (23, 58). However, novel sigma factors directing crystal protein expression have only recently been isolated from *B. thuringiensis* (6, 7).

The first crystal protein gene cloned and sequenced, cryIA(a) (45, 47, 60), is expressed during sporulation of *B. thuringiensis* subsp. *kurstaki* HD-1-Dipel from two overlapping promoters, designated Bt I and Bt II (58). Comparison of mRNA levels and immunoblots of the 133,000-molecular-weight protein product indicated that regulation of the cryIA(a) gene is primarily at the level of transcription initiation. Brown and Whiteley (6) have shown that transcription from Bt I is directed in vitro by a form of RNA polymerase containing an alternate sigma factor, designated σ^K, based on its apparent molecular mass in kilodaltons. This sigma factor also recognizes promoters for three other crystal protein genes: cytA, the gene encoding a hemolytic and mosquitoicidal polypeptide from *B. thuringiensis* subsp. *israelensis* crystals (56, 57); cryB (formerly cryA4), a crystal toxin-encoding gene from *B. thuringiensis* subsp. *thuringiensis* HD-2 (4); and obtI of the cryIA (formerly cryBl) operon of *B. thuringiensis* subsp. *kurstaki* HD-1 (59). Brown and Whiteley (7) have also described the isolation and biochemical characterization of the second factor, σ^K, that directs transcription from the upstream Bt II promoter of cryIA(a) in vitro. This second sigma factor also recognizes other sporulation stage-specific gene promoters (7), including additional promoters for cytA and cryIB (5), as well as a promoter for cotT, a coat protein gene from *B. subtilis* presumably recognized by RNA polymerase containing the σ^K polypeptide (3).

To understand more completely the regulation of crystal protein gene expression in *B. thuringiensis*, we have cloned and sequenced the genes encoding two sporulation-specific sigma factors that direct transcription of the cryIA(a) gene in vitro. Notably, we find that the deduced amino acid sequences for the σ^K and σ^28 genes are highly homologous to those of two known *B. subtilis* sigma factors, σ^K and σ^K, respectively. Further, we find that σ^K and σ^28 genes can complement σ^K and σ^K defects of *B. subtilis* and that σ^K and σ^28 polypeptides can recognize in vitro many of the same *B. subtilis* promoters recognized by σ^K and σ^K-containing polymerases. However, we show that the σ^K gene does not result from a chromosomal recombination event. The gene encoding σ^K in *B. subtilis*, on the other hand, results from a very late sporulation-stage juxtaposition, within the mother cell, of the *spolVCB* and *spolIC* genes, which are normally separated by 42 kb of DNA (29). Despite the differences in physical organization of the σ^K and the σ^K genes, the high degree of sequence and functional homology between the *B. thuringiensis* and *B. subtilis* sigma factors permits us to localize crystal protein expression, both temporally and spatially, to well-characterized *Bacillus* sporulation events.

**MATERIALS AND METHODS**

Purification of sigma factors for Edman degradation. Approximately 100 g (wet weight) of *B. thuringiensis* subsp.
kurstaki HD-1-Dipel cells from either stage II or stage IV of sporulation was disrupted by passage through a French pressure cell. The \( \sigma^{35} \) and \( \sigma^{28} \) transcription factors were then isolated as previously described (6, 7), yielding approximately 50 \( \mu \)g of each purified polypeptide. Approximately 15 \( \mu \)g of each sigma factor was then electrophoresed and transferred to Immobilon P for N-terminal sequencing as described by Matsudaira (37).

Host strains and plasmid constructions. Host strains Escherichia coli JM83 (54) and GD32, a recA mutant of JM83 (13), were used for all cloning steps and preparation of template for DNA sequencing. Vectors for sequencing were either pUC118 or pUC119 (55). Complementation analyses were performed by cloning fragments containing the \( \sigma^{35} \) or the \( \sigma^{28} \) gene (see Fig. 1 and 3) into pPL703E, a variant of pPL703 (1) containing the ColE1 origin of replication (59). This vector permitted plasmid constructions in E. coli that could be subsequently transformed into B. subtilis strains and maintained extrachromosomally.

DNA sequencing and sequence analysis. Unidirectional nested deletions were made by the method of Henikoff (17). Double-stranded template was prepared by alkaline lysis of 6 ml of plasmid-containing host strain (34), followed by alkali and Sepharose CL-6B purification as described by Murphy and Kavanagh (40). Dideoxy sequencing reactions (44) were performed with Sequenase according to the manufacturer's instructions (U.S. Biochemical Corporation, Cleveland, Ohio), except that termination reactions were performed at 49°C. All presented nucleotide data were confirmed by sequencing of the opposite strand. Compilation and analysis of nucleotide sequence were performed with The DNA Inspector IIe (TextCo, Lebanon, N.H.) or the IntelliGenetics Suite (Release 5.35; IntelliGenetics, Mountain View, Calif.). Amino acid alignments were performed with the FastP algorithm (33). Identical residue pairs were marked with two dots; conservative changes were marked with one dot and represent a log-odds matrix score of 0 or better (12, 41). The sequence data presented in this paper have been assigned accession numbers X56696 and X56697 in the EMBL Data Library.

Hybridization of Southern DNA transfers, colony hybridization. DNA from 0.8% agarose slab gels was transferred overnight to nitrocellulose; colonies from agar plates were transferred to nitrocellulose, lysed with NaOH, and washed as described (34). Prehybridization buffer contained 6\( \times \) SSC (1\( \times \) SSC = 0.15 M NaCl plus 0.015 M sodium citrate), 1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 200 \( \mu \)g of calf thymus DNA ml\(^{-1}\), and 50 mM Tris, pH 7.5. Probe concentrations were kept below 5 \( \times \) 10\(^5\) counts ml\(^{-1}\). Nitrocellulose filters hybridized with imperfect probes were washed in the same buffer lacking the DNA block; filters hybridized with probes of expected perfect homology were washed in the same buffer lacking the DNA and diluted fivefold (to approximately 1.2 \( \times \) SSC-0.2% SDS, 0.2 mM EDTA-10 mM Tris, pH 7.5) before use. Probes were labeled with T4 kinase in the presence of 100 \( \mu \)Ci [\( \gamma ^32P \)]ATP or by random-primed labeling of 200 ng of DNA in the presence of 50 \( \mu \)Ci of [\( \alpha ^32P \)]dATP with a commercial kit (Boehringer-Mannheim, Indianapolis, Ind.).

Complementation of B. subtilis spoIIG55 and spoIIC94 defects with the B. thuringiensis \( \sigma^{35} \) and \( \sigma^{28} \) genes. B. subtilis PY79 or mutant strains were grown overnight on LB plates (38) and then inoculated into LB broth and grown to stationary phase. Cells were made competent (34) following by SP salts as described by Dubnau and Davidoff-Abelson (14), transformed with pPL703E and derivative constructs containing the \( \sigma^{35} \) or \( \sigma^{28} \) genes (see Fig. 1 and 3), and plated on LB medium containing 2 \( \mu \)g of neomycin ml\(^{-1}\). Alternatively, cells were placed in a 0.2-cm cuvette, electrophoresed at 25 \( \mu \)F, 1.30 kV, and 400 ohms in the presence of the aforementioned plasmids, and plated as described above. Complementation was determined by the ability of the various plasmid constructs to restore production of viable spores in B. subtilis spoIIG55 and spoIIC94 strains. Viable spore counts were determined by overnight growth in cultures in 2 \( \times \) SG medium (16 g/l of nutrient salts, 2 mM MgSO\(_4\) 30 mM KCl, 1 mM Ca(NO\(_3\))\(_2\), 100 \( \mu \)M MnCl\(_2\), and 1 \( \mu \)M FeSO\(_4\)) serial dilution of stationary-phase cultures in T-base (29), incubation at 93°C for 15 min to kill vegetative cells, and plating on LB medium. Only freshly transformed cells were used to inoculate liquid cultures in the complementation assays, and the assays were performed three times with similar results (the results of a representative complementation assay are shown in Table 1).

In vitro transcription assays. \( \sigma^{35} \) and \( \sigma^{28} \) polypeptides were purified by elution from polycrylamide gels and renaturation as described by Hager and Burgess (16). These polypeptides were reconstituted with core-delta polymerase prepared from vegetative B. thuringiensis cells (19), and their activity in their absence in transcription from B. thuringiensis and B. subtilis promoters was measured by runoff transcription as described by Brown and Whiteley (6, 7). The transcriptional activity of the \( \sigma^{35} \) polypeptide was tested on the \( \sigma^{E} \)-dependent promoter of the spoIID gene contained on plasmid pSR3 (42). The \( \sigma^{28} \) polypeptide was tested on the following \( \sigma^{D} \)-dependent promoters: spoIVCA (\( \alpha^E \)) on pBK28, cotD on pLRK100 (25), gerE on pSCI146, and cotA on pKS24. The \( \sigma^{28} \) polypeptide was also tested with spoVG on pCB1291 (39) as a negative control.

Polymerase chain reaction amplification of the \( \sigma^{28} \) gene. Polymerase chain reaction amplification of the \( \sigma^{28} \) gene was performed with Taq polymerase as described by the manufacturer (U.S. Biochemical Corp.). Temperature cycling was provided by a Perkin Elmer-Cetus programmable heat block. Template was either vegetative DNA isolated from B. thuringiensis subsp. kurstaki HD-1-Dipel at a final reaction concentration of 100 ng ml\(^{-1}\) or plasmid DNA containing the cloned gene at 50 ng ml\(^{-1}\). The following primers for PCR amplification were used: GGA ATT CAT GAA TAG TGT CCC CTT TAA TAG C, containing an EcoRI linker at its 5' end, and CCT TCT AAT ATT ACA GAT AGA ATC GC. These oligomers correspond to nucleotides (nt) 183 to 208 and 1215 to 1189, respectively, in Fig. 4A.

Reagents, enzymes, and oligonucleotides. Restriction endonucleases were purchased from Boehringer-Mannheim, New England Biolabs (Beverly, Mass.), or Bethesda Research Laboratories (Gaithersburg, Md.). Exonuclease III and nuclelease S1 were from Bethesda Research Laboratories. T4 polynucleotide kinase, ligase, and DNA polymerase were from Boehringer-Mannheim. [\( \alpha ^32P \)]dATP (3,000 Ci mmol\(^{-1}\), 10 mCi ml\(^{-1}\)), [\( \gamma ^32P \)]dATP (3,000 Ci mmol\(^{-1}\), 10 mCi ml\(^{-1}\)), and [\( \alpha ^35S \)]dATP (1,000 Ci mmol\(^{-1}\), 12.5 to 17 mCi ml\(^{-1}\)) were obtained from New England Nuclear (Boston, Mass.). Oligonucleotides were synthesized on a model 8600 multiple column DNA synthesizer (BioSearch, Inc., San Rafael, Calif.). In the case of the probe used for isolation of the \( \sigma^{35} \) gene, GA(G,A) GCI (C,T)I GCI CCI GCI (C,T)I ACI AA(G,A) GA(G,A) GA(G,A) GA(G,A) TA (see Results), mixed deoxynucleotides were used at the twofold reduced concentration by response, and 

\[ \text{transfection} \text{coefficient} \] was used at the fourfold redundant positions to decrease base discrimination at mismatches (35).
**RESULTS**

Cloning of the $\sigma^{35}$ gene. Edman degradation of the purified $\sigma^{35}$ polypeptide yielded the following amino acid sequence: YIGGSEALPPPLTKEEYYVLXNKL, where the P in parentheses is uncertain and the X is an unknown residue. We designed an oligomer corresponding to the region EALP PPLTKEEYY (see Materials and Methods). This oligomer bound specifically to a ca. 4.5-kb XbaI-EcoRI fragment from digested genomic *B. thuringiensis* subsp. *kurstaki* HD-1 Dipel DNA after being washed at 36°C. Hybridization of the probe to colonies of *GD32* cells harboring a library of 4- to 6-kb XbaI-EcoRI fragments in pUC118 yielded a colony with a plasmid containing a 4.4-kb insert with the restriction map depicted in Fig. 1. Hybridization of the oligomer to various digests of this plasmid suggested that part or all of the gene resided within the XbaI-HindIII region of the insert (Fig. 1).

Nucleotide and deduced amino acid sequence of $\sigma^{35}$ and surrounding region. Sequencing of plasmids pLA30 and pLA31 (Fig. 1) revealed three large open reading frames (ORFs); the middle ORF encodes a protein whose deduced amino acid sequence includes a region near the N terminus that is nearly identical to that determined by N-terminal sequencing (underlined in Fig. 2A). The discrepancy occurs between the tyrosine determined by N-terminal sequencing and the glutamate (number 4 in the series of glutamates) deduced from the nucleotide sequence. Despite this discrepancy, and on the strength of the results presented below, we conclude that we have cloned and sequenced the gene encoding $\sigma^{35}$. FastP analysis (33) shows that the deduced amino acid sequence of this gene has 87.9% identity (Fig. 2B) to $\sigma^{E}$ (50), a well-studied sporulation-phase sigma factor from *B. subtilis*. The presence of a reasonable ribosome binding site at an appropriate spacing from the deduced start codon for $\sigma^{35}$ (Fig. 2A) and alignment of the $\sigma^{35}$ and $\sigma^{E}$ polypeptides (Fig. 2B) suggest that the actual start codon for $\sigma^{35}$ is at the methionine at nt 506, even though the ORF extends 10 amino acids upstream. A GC-rich palindrome (underlined) lies between nt 1226 and 1263, inclusive, immediately following the stop codon TAA. The alignment of the deduced polypeptides, in conjunction with the N-terminal amino acid sequence data obtained from Edman degradation, also suggests that $\sigma^{35}$, like $\sigma^{E}$, is a processed polypeptide. The deduced amino acid sequence indicates a molecular mass of 27,604 Da for $\sigma^{35}$ (24,213 for the processed protein), which is substantially less than that predicted by SDS-polyacrylamide gel electrophoresis but consistent with the aberrant migration of sigma factors noted by many researchers.

The region surrounding the $\sigma^{35}$ gene contains two other significant ORFs. The upstream partial ORF (nt 1 through 483) has 44.4% identity to the corresponding region of SpoIAG of *B. subtilis*, the putative protease required for processing of pro-$\sigma^{35}$ to its active form (49, 53). The downstream partial ORF has very high identity (92.1%) to the corresponding region of the SpoIIIG polypeptide, $\sigma^{3}$, a forespore-specific sigma factor (22, 36, 52). This ORF also has significant identity (36.5%) to the corresponding region of $\sigma^{35}$, by comparison, the corresponding stretch of 121 amino acids of $\sigma^{35}$ has 35.5% identity to $\sigma^{3}$. Thus, over the region of DNA sequenced, *B. thuringiensis* has the same gene arrangement as found in *B. subtilis*.

Cloning of the $\sigma^{28}$ gene, nucleotide and deduced amino acid sequence. Sequential Edman degradation yielded the following encoding for the N terminus of $\sigma^{28}$: YVKNNAFQP PLSSDDERK. We were unable to synthesize a mixed oligomer based on this sequence that would hybridize to a specific restriction fragment of *B. thuringiensis* subsp. *kurstaki* DNA. However, the high similarity between the above sequence and that determined for the N-terminal region of the $\sigma^{28}$ polypeptide from *B. subtilis* (51) suggested that a gene fragment from *spolVCB* could serve as a probe for $\sigma^{28}$.

The 0.35-kb SstI fragment from pBK28 (26), which contains all but the last 27 nt of *spolVCB* encoding the amino terminus of $\sigma^{28}$ (51), hybridized specifically to a 2.2-kb XbaI fragment from *B. thuringiensis* subsp. *kurstaki* DNA extracted from vegetative cells. Cloning of this fragment into pUC118 yielded plasmid pLA40 with the restriction map shown in Fig. 3; hybridization of DNA to Southern transfers of pLA40 digested with various enzymes suggested that the $\sigma^{28}$ ORF resides partly within the 0.3-kb HindIII-XbaI fragment, which was subcloned to generate pLA41. The insert from pLA41 was then used as a probe to clone the remaining downstream piece of the $\sigma^{28}$ gene from a library of *Psil*-SstI fragments, yielding the insert contained in plasmid pLA42, which was subsequently ligated to the *BamHI*-Psil fragment from pLA40 to make pLA43 and pLA44. These latter plasmids encode the entire $\sigma^{28}$ polypeptide (see below).

Sequencing of the region encoding $\sigma^{28}$ showed an ORF (Fig. 4A) whose deduced amino acid sequence has high identity to the $\sigma^{28}$ polypeptide of *B. subtilis* (Fig. 4B). Surprisingly, the $\sigma^{28}$ polypeptide is homologous not only to the N terminus (SpoIVCB region) of $\sigma^{28}$ but to the recombination site and the C terminus (SpoIIC region) of $\sigma^{35}$ as well. In *B. subtilis*, the (partial) genes *spolVCB* and *spoIIC* recombine in the mother cell to form a complete ORF encoding $\sigma^{28}$ only after stage IV of sporulation (15, 28, 29, 51). However, we were able to clone a complete $\sigma^{28}$ ORF from vegetative *B. thuringiensis* DNA.

The deduced amino acid sequence of the $\sigma^{28}$ gene, when compared with the sequence obtained by Edman degradation (Fig. 4B), suggests that $\sigma^{28}$, like $\sigma^{28}$ from *B. subtilis*, is a processed polypeptide. The deduced amino acid sequence indicates a protein of 27,087 Da (24,959 Da after processing) with 85.0% identity to the $\sigma^{28}$ polypeptide. Surprisingly, the deduced molecular mass of the processed $\sigma^{28}$ polypeptide slightly exceeds that deduced for the processed $\sigma^{35}$ polypeptide (24,213 Da), even though the apparent molecular masses of these two polypeptides are quite distinct as measured by
FIG. 2. (A) Nucleotide and deduced amino acid sequence of the region encoding the *B. thuringiensis* subsp. *kurstaki* σ35 gene. The TAA stop codons are indicated with asterisks. A potential ribosome binding site, a sequence corresponding to that determined by sequential Edman degradation of the σ35 polypeptide, and a potential palindrome immediately following the TAA stop codon at nt 1223 are underlined. Partial sequences for the ORFs upstream and downstream of σ35 are also presented. (B) Alignment of σ35 of *B. thuringiensis* with σ6 of *B. subtilis*. Identical residue pairs are indicated with two dots; conservative changes (score of 0 or better in the PAM log-odds matrix; see Materials and Methods section) are flagged with a single dot. Sequences determined by sequential Edman degradation for σ35 (this study) and σ6 (30) are underlined to emphasize that both polypeptides are apparently processed.
The \(\sigma^{28}\) gene does not result from late-sporulation-stage chromosomal rearrangement. Our ability to clone the \(\sigma^{28}\) gene from \(B.\) thuringiensis DNA isolated from vegetative cells suggested that it does not result from a late-sporulation-stage chromosomal recombination event, as occurs for the \(\sigma^{K}\) gene in \(B.\) subtilis, which is the recombination product of the \(spol\) and \(spo\) partial genes. Two tests were performed to confirm this result. First, DNA was purified from \(B.\) thuringiensis vegetative and late-sporulation-stage cells (obtained ca. 1.5 h before release of mature endospores) and digested with \(XbaI\) and \(PstI\). Both of these enzymes cut within the \(\sigma^{28}\) gene (Fig. 3 and 4). After electrophoresis and transfer to nitrocellulose, the digested DNA was probed with radiolabeled \(HindIII-XbaI\) fragment from pLA41 (Fig. 3). No change could be detected in the sizes of the hybridizing bands of DNA from late-sporulation-stage cells when compared with DNA taken from vegetative cells (Fig. 5A). As a positive control, DNA was purified from vegetative and late-sporulation-stage \(B.\) subtilis 168 S cells and digested with EcoRI. Labeled 0.35-kb \(SstI\) fragment from pBK28 hybridized to a 4.2-kb EcoRI fragment from both vegetative (Fig. 5B, lane a) and late-sporulation-stage (lane b) DNA. Importantly, the same probe also hybridized to a new 2.8-kb EcoRI fragment from late-sporulation-stage DNA, the latter fragment resulting from chromosomal recombination of the \(spol\) and \(spo\) genes to generate the \(\sigma^{K}\) gene (51). As a second test for a \(B.\) thuringiensis chromosomal rearrangement generating the \(\sigma^{28}\) gene, DNA was amplified by the polymerase chain reaction from the \(\sigma^{28}\) gene-containing plasmid pLA43 described above or genomic DNA from early vegetative \(B.\) thuringiensis subsp. \(kurstaki\) cells. Both templates yielded the same, expected 1.0-kb DNA fragment (Fig. 5C) containing an internal \(HindIII\) site, suggesting that an entire, intact \(\sigma^{28}\) gene is present during vegetative growth and that the gene does not result from a chromosomal recombination event.

The \(\sigma^{28}\) and \(\sigma^{K}\) polypeptides restore sporulation in \(\sigma^{K}\)- and \(\sigma^{K}\)-defective \(B.\) subtilis strains. To test the functional equivalence between \(\sigma^{28}\) and \(\sigma^{K}\), the \(XbaI-HindIII\) fragment from pLA30, containing the \(\sigma^{28}\) gene and, presumably, most if not all of the upstream ORF (which shows homology in the region sequenced to SpoIIA) was inserted into pPL703E. The resulting construct, pLA32, was transformed into a \(B.\) subtilis \(\sigma^{28}\) mutant, spoIIG55. Similarly, pLA44 encoding \(\sigma^{28}\) was transformed into the \(\sigma^{K}\)-defective mutant \(spo\)IIC94.

Both of these plasmids caused a substantial reduction in the production of viable, heat-resistant spores in wild-type \(B.\) subtilis PY79 (Table 1), a result similar to that observed by previous researchers (24). However, plasmid pLA32, encoding the \(\sigma^{28}\) polypeptide, restored sporulation of the \(spo\)IG55 strain to nearly wild-type levels. Similarly, \(\sigma^{28}\) encoded on pLA44 complemented the \(spo\)IIC94 defect in \(B.\) subtilis; the number of viable spores was three orders of magnitude less than in the wild-type strain but at least three orders of magnitude greater than in \(spo\)IIC94 transformed with vector alone. Phase-bright, free spores were visible in both of the complemented strains. These results show that the \(\sigma^{35}\) and \(\sigma^{28}\) polypeptides can at least partially restore viable spore production in \(\sigma^{E}\)- and \(\sigma^{K}\)-defective \(B.\) subtilis strains.

**b.** thuringiensis core-delta polymerase reconstituted with \(\sigma^{35}\) and \(\sigma^{28}\) can recognize \(\sigma^{E}\) and \(\sigma^{K}\) promoters. We tested the ability of the \(\sigma^{35}\) and \(\sigma^{28}\) polypeptides, reconstituted with core-delta polymerase, to direct transcription in vitro from a number of promoters. The polypeptides directed transcription from the Bt I and Bt II promoters, respectively, of the cryI(a) gene (Fig. 6A and B). Not surprisingly, addition of \(\sigma^{35}\) permitted the recognition of the \(\sigma^{E}\)-dependent promoter of the \(spo\)IIID gene (Fig. 6A, transcript size of 600 nt when cut with \(HindIII\)). However, \(\sigma^{28}\) did not permit recognition of the "S promoter" residing on the same plasmid (42). The \(\sigma^{28}\) polypeptide directed core polymerase to the known \(\sigma^{K}\)-dependent promoters of conD and gerE; conA was not well recognized (Fig. 6B). The \(\sigma^{28}\) polypeptide also enabled core polymerase to recognize a promoter upstream of its own gene and, weakly, the \(\sigma^{K}\)-dependent promoter of the \(\sigma^{K}\) (\(spol\)VCB) gene. An additional, weak promoter upstream of the known \(\sigma^{K}\)-dependent promoter of \(\sigma^{28}\) was also recognized by \(\sigma^{28}\) (Fig. 6B). That \(\sigma^{28}\) recognizes a promoter preceding its own gene is consistent with the finding that its homolog, \(\sigma^{K}\), is autogenuously regulated (25).

As a measure of its specificity, the \(\sigma^{28}\) polypeptide did not enable core polymerase to recognize the promoter of the \(spo\)VG gene, which is transcribed in vitro by RNA polymerase in association with any one of the \(\sigma^{35}\), \(\sigma^{35}\), or \(\sigma^{35}\) polypeptides (8, 20).

**DISCUSSION**

The genes encoding the \(\sigma^{35}\) and \(\sigma^{28}\) polypeptides that direct RNA polymerase to the Bt I and Bt II promoters of the cryI(a) gene of \(B.\) thuringiensis subsp. \(kurstaki\) HD-1-Dipel have been isolated and sequenced. The deduced amino acid sequences of these genes have substantial identity, respectively, to the \(\sigma^{E}\) (SpoIIGB) and \(\sigma^{K}\) (SpoVCB/SpoIIC) polypeptides of \(B.\) subtilis. The high degree of identity for the \(\sigma^{28}/\sigma^{E}\) and \(\sigma^{35}/\sigma^{K}\) sets of polypeptides is surprising in light of the difference in %G+C between the two organisms (34 and 43% for the type species, respectively; 9). Indeed, FastN analysis shows that the nucleotide conservation between \(B.\) thuringiensis and \(B.\) subtilis is substantially lower: 75% identity for the \(\sigma^{28}/\sigma^{35}\) pair and 65% for the \(\sigma^{K}/\sigma^{28}\) pair on the nucleotide level, versus 88 and 86% at the amino acid level (see results above). As expected, most nucleotide differences between each gene pair occur at the third position of the codon (data not shown).

Interestingly, the N-terminal amino acid for the processed \(\sigma^{35}\), \(\sigma^{35}\), and \(\sigma^{K}\) polypeptides is a tyrosine. The reported N-terminal amino acid for the processed \(\sigma^{28}\) protein, glycine (reference 30 and Fig. 2B), differs from the tyrosines of the mature \(\sigma^{28}\), \(\sigma^{K}\), and \(\sigma^{35}\) polypeptides. However, two amino acids upstream of the glycine of the mature \(\sigma^{28}\) polypeptide

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**FIG. 3.** Restriction map of the region encoding the \(B.\) thuringiensis subsp. \(kurstaki\) \(\sigma^{28}\) gene. Nucleotide distances are indicated in kilobases; subclones are indicated below with parent vectors listed in parentheses. The abbreviations for restriction sites are given in Fig. 1, with the following additions: B, BamHI; A, AccI; S, SstI; P, PstI.
are two tyrosine residues, in perfect alignment with those adjacent to the processing site in $\sigma^{35}$ (Fig. 2B). These observations suggest that the processing enzymes for each of the four sigma polypeptides might have similar amino acid specificities or recognition sites. Isolation of the processing enzymes will permit comparison of amino acid recognition sequences and proteolytic function.

Despite the high conservation of amino acid sequence between $\sigma^{28}$ and $\sigma^{K}$, the $\sigma^{28}$ gene does not result from a chromosomal recombination event. This result validates and extends the conclusions of Kunkel et al. (28), who provided molecular and genetic evidence that the rearrangement per se is not necessary for regulation of the timing of $\sigma^{K}$ expression, maturation, or function. Southern hybridization

**FIG. 4.** (A) Nucleotide and deduced amino acid sequence of the region encoding the B. thuringiensis subsp. kurstaki $\sigma^{28}$ gene. The presumed initiation codon (TTG) at nt 369, determined by alignment with the highly homologous $\sigma^{K}$ polypeptide, is preceded by a strong potential ribosome binding site (underlined). A sequence determined by Edman degradation and an imperfect palindrome following the TAA stop codon at nt 1080 (signaled with asterisks) are also underlined. The region in the $\sigma^{28}$ gene corresponding to the recombination site in the $\sigma^{K}$ gene (51) is marked with asterisks. (B) Alignment of $\sigma^{28}$ with $\sigma^{K}$ of B. subtilis. Amino acid identities and conservative changes are indicated with two dots and one dot, respectively. Sequence determined by Edman degradation for $\sigma^{28}$ (this study) and $\sigma^{K}$ (51) is underlined.
of\(\sigma^{28}\) or \(\sigma^K\) probes to genomic digests of vegetative and sporulation-stage DNA from other Bacillus strains would indicate how widespread the recombination phenomenon is.

The high amino acid sequence identity between \(\sigma^{35}\) and \(\sigma^E\) and between \(\sigma^{28}\) and \(\sigma^E\) suggests functional homology as well. This idea is supported by the finding that \(\sigma^{35}\) and \(\sigma^{28}\) can restore sporulation in \(\sigma^E\) and \(\sigma^K\)-defective strains. Notably, \(\sigma^{28}\) complementation of the spoIIC94 defect was substantially less than \(\sigma^{35}\) complementation of the spoIG55 defect. This result may reflect the absence of an ORF encoding a proper processing enzyme in the \(\sigma^{28}\)-containing plasmid pLA44; judging by total insert size, such an ORF should exist upstream of \(\sigma^{35}\) in construct pLA32 that was transformed into the spoIG55 strain. The amino acid sequence encoded by the latter ORF has significant identity over the region sequenced to the spoIG4 gene product, which is believed to be a protease responsible for processing and activation of \(\sigma^E\) in B. subtilis (21, 49). Thus, complementation of the \(\sigma^E\) defect with \(\sigma^{35}\) may be effective because of sufficient protease for \(\sigma^{35}\) activation. Poor complementation of the spoIIC94 defect with the \(\sigma^{28}\) gene may also reflect excessive copy number of the latter gene, whose promoter region may titrate a transcription factor essential for the continuation of sporulation (46).

Functional homology between \(\sigma^{35}\) and \(\sigma^E\) and between \(\sigma^{28}\) and \(\sigma^E\) is also suggested by the results of the in vitro transcription assays. The \(\sigma^{35}\) polypeptide directs polymerase to a \(\sigma^E\) promoter preceding the spoIIG gene; this promoter matches the \(-10\) region consensus developed for \(\sigma^{35}\)-dependent promoters (6) and differs by one nucleotide, an A residue instead of a G, from the consensus \(-35\) region 5'-GCAATNT-3'. The \(\sigma^{28}\) polypeptide enables specific polymerase recognition of \(\sigma^E\) promoters preceding cotD and gerE. However, \(\sigma^{28}\) polymerase recognition of cotA was very weak. The cotA promoter (43) has a change from adenine to thymine at position +5 relative to the 5' T of the proposed \(-10\) consensus sequence for \(\sigma^{28}\) promoters, 5'-TNATANAGag-3' (7). This adenine is conserved among the promoters for gerE and cotD (11, 61) as well as for cotT (3), all of which are recognized strongly by \(\sigma^{28}\) (this work; 7). The change from adenine to thymine may therefore explain weak recognition of cotA by \(\sigma^{28}\)-containing polymerase. Additional evidence for functional homology between \(\sigma^{35}\) and \(\sigma^E\) and between \(\sigma^{28}\) and \(\sigma^E\) has been provided by studies of cryI(a) promoter function in various B. subtilis spo backgrounds. The blocks in promoter expression obtained also suggest that the cryI(a) promoter is under \(\sigma^E\) and \(\sigma^K\) control (5).

![FIG. 5.](image)

![FIG. 6.](image)

### TABLE 1. Complementation of \(\sigma^E\) (spoIG55) and \(\sigma^K\) (spoIIC94) mutants of B. subtilis with plasmids encoding \(\sigma^{35}\) and \(\sigma^{28}\) from B. thuringiensis

<table>
<thead>
<tr>
<th>B. subtilis strain</th>
<th>No. of viable spores per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>PY79</td>
<td>(2 \times 10^6)</td>
</tr>
<tr>
<td>PY79(pLA 32) (encodes (\sigma^{35}))</td>
<td>(3 \times 10^6)</td>
</tr>
<tr>
<td>PY79(pLA 44) (encodes (\sigma^{28}))</td>
<td>(7 \times 10^6)</td>
</tr>
<tr>
<td>spoIG55(pPL703E)</td>
<td>(&lt;10^2)</td>
</tr>
<tr>
<td>spoIIC94(pPL703E)</td>
<td>(4 \times 10^7)</td>
</tr>
<tr>
<td>spoIIC94(pLA 44)</td>
<td>(&lt;10^6)</td>
</tr>
<tr>
<td>spoIIC94(pLA 32)</td>
<td>(1 \times 10^6)</td>
</tr>
</tbody>
</table>
The data presented in this paper agree with the conclusion of previous researchers that crystal protein expression is a sporulation-mediated event. The data also permit localization of crystal protein expression both temporally and spatially within the sporulation regime of Bacillus spp. Thus, we predict crystal protein to first appear in B. thuringiensis at the time of septum formation (stage II) separating the mother cell from the nascent endospore. This time period would correspond to processing of the σ^{35} (σ^{3}) polypeptide to the mature, active form, by analogy to σ^{3} processing in B. subtilis (possibly by the spoIVF gene product; 10). Because σ^{X} expression is confined largely, if not exclusively, to the mother cell (51), we presume that σ^{35} expression is similarly restricted, thus explaining the large, continuous accumulation of cryIA(a) gene product in the form of a bipyrimal crystalline inclusion within the mother cell and not within the forespore compartment. Available evidence does not permit us to speculate on the presence or the effect of a “switch factor” on transcription of the Bt II promoter of the cryIA(a) gene. Such a factor (the product of the spoIID gene in B. subtilis) activates transcription from the σ^{35} gene itself but represses transcription of a spore coat gene, cotD (25; 27). Such a factor may greatly influence late-sporulation expression of cryIA(a) from the Bt II promoter.

The fact that the σ^{35} and σ^{28} genes recognize additional crystal protein gene promoters in vitro (6, 7) permits us to predict that the same sporulation sigma factors or factors similar to σ^{35}/σ^{2} and σ^{28}/σ^{X} may drive transcription of other crystal protein genes as well. However, crystal protein expression in organisms such as B. thuringiensis subsp. finitimis, which occurs within the forespore compartment, may be directed by different sporulation-phase sigma factors such as one homologous to the forespore-specific σ^{2} polypeptide. These suppositions await cloning or purification of sigma factors from additional B. thuringiensis strains and testing of their ability to direct transcription from crystal protein gene promoters.

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