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Requirement of *flhA* for Swarming Differentiation, Flagellin Export, and Secretion of Virulence-Associated Proteins in *Bacillus thuringiensis*

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***Bacillus thuringiensis* is being used worldwide as a biopesticide, although increasing evidence suggests that it is emerging as an opportunistic human pathogen. While phospholipases, hemolysins, and enterotoxins are claimed to be responsible for *B. thuringiensis* virulence, there is no direct evidence to indicate that the flagellum-driven motility plays a role in parasite-host interactions. This report describes the characterization of a mini-Tn*10* mutant of *B. thuringiensis* that is defective in flagellum filament assembly and in swimming and swarming motility as well as in the production of hemolysin BL and phosphatidylcholine-preferring phospholipase C. The mutant strain was determined to carry the transposon insertion in *flhA*, a flagellar class II gene encoding a protein of the flagellar type III export apparatus. Interestingly, the *flhA* mutant of *B. thuringiensis* synthesized flagellin but was impaired in flagellin export. Moreover, a protein similar to the anti-sigma factor FlgM that acts in regulating flagellar class III gene transcription was not detectable in *B. thuringiensis*, thus suggesting that the flagellar gene expression hierarchy of *B. thuringiensis* differs from that described for *Bacillus subtilis*. The *flhA* mutant of *B. thuringiensis* was also defective in the secretion of hemolysin BL and phosphatidylcholine-preferring phospholipase C, although both of these virulence factors were synthesized by the mutant. Since complementation of the mutant with a plasmid harboring the *flhA* gene restored swimming and swarming motility as well as secretion of toxins, the overall results indicate that motility and virulence in *B. thuringiensis* may be coordinately regulated by *flhA*, which appears to play a crucial role in the export of flagellar as well as nonflagellar proteins.**

Bacillus thuringiensis is a gram-positive, rod-shaped, spore-forming bacterium that is motile by peritrichous flagella. During sporulation this organism produces a variety of δ-endotoxins (Cry proteins) that are primarily responsible for a highly specific insecticidal activity (53). For this reason, *B. thuringiensis* is being used presently as a biological insecticide, accounting for more than 90% of the biopesticides employed worldwide (37).

Despite a long record of being safe, there is increasing public concern about the potential pathogenicity of *B. thuringiensis* to humans. This is due to the sporadic but sometimes severe infections caused by this organism, which include diarrheal food poisoning (34), corneal ulcer (52), cellulitis (57), and burn (13) as well as war wound (28) infections. Moreover, the pathogenicity of *B. thuringiensis* has been demonstrated in an animal model of infection by administering spores to mice via nasal inoculation (27, 50). Similar to *Bacillus cereus*, *B. thuringiensis* produces a variety of virulence factors, which include phosphatidylcholine-preferring phospholipase C (PC-PLC) and phosphatidylinositol-specific phospholipase C (38, 40), hemolysins, and enterotoxins (1, 18, 21). Heierson et al. (24) isolated an avirulent pleiotropic mutant of *B. thuringiensis* that was unable to express both hemolytic activities and flagella and suggested that flagella and motility in this organism played a role in parasite-host interactions. Such a finding, moreover, was thought to indicate that the regulation of genes encoding flagellar components and virulence factors could be coordinated in *B. thuringiensis* (60).

Several lines of evidence convincingly support the idea that virulence and motility are intimately linked in many pathogenic bacteria (reviewed in reference 49); however, little information is available on their genetic or structural relationships, most likely because of the complexity of both the assembly of the flagellar/chemotactic apparatus and the molecular mechanisms involved in the secretion of virulence factors. To the best of our knowledge, nothing is known as to whether common pathways governing expression of flagella and secretion of virulence factors occur in *B. thuringiensis*.

The flagellar components are known to be sequentially assembled from the cytoplasmic membrane outward (55). The ordered assembly process arises, at least in part, from the hierarchical nature of flagellar gene expression, flagellin being the last and major flagellar protein expressed. Flagellar genes are organized into several classes in both the enteric bacteria

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and *Bacillus subtilis*, and the function of each gene product in a given class is required for the expression of genes in a lower class (44, 45). While class II genes encode structural and accessory proteins needed for the assembly of the flagellar hook-basal body (HBB) complex, class III genes, the expression of which depends on the late-flagellar sigma factor (σ^{28} in the enterics and σ^{D} in *B. subtilis*), encode proteins required for the maturation of the flagellum and the chemosensory system. Coupling of class III gene expression to the HBB complex is achieved through the action of FlgM, an anti-sigma factor, which inactivates σ^{28} (or σ^{D}) until a functional HBB is complete (44, 48). FlgM is then secreted from the cell by the flagellar-dedicated secretory apparatus, which is localized within the flagellar basal body and contains a membrane-associated and specialized pore for protein export (46). Indeed, with the P- and L-ring subunits as the only exception (30, 32), none of the external proteins of the flagellum possess a signal peptide sequence at their amino termini (29, 31) and, therefore, cannot be exported by the bacterial general secretory pathway. The flagellar export apparatus encompasses six integral proteins (FlhA, FlhB, FliO, FliP, FliQ, and FliR in *Salmonella*) (46) that share substantial homology with components of the type III virulence secretion pathway found in gram-negative bacteria (reviewed in reference 33). Interestingly, it has been demonstrated that the virulence-associated protein YplA of *Yersinia enterocolitica*, although not involved in motility, is secreted by the flagellar export apparatus (58). This finding supports the idea that this export system and the type III virulence secretion pathway may have had a common evolutionary origin and share overlapping functions.

A relationship between virulence and motility is also supported by the demonstration that, in some microorganisms, a substantial increase in the secretion of virulence factors is associated with a specialized form of flagellum-driven motility, the swarming motility that enables bacterial cells to collectively move across solid surfaces (3, 14, 43, 54). The ability to swarm depends on a complex surface-induced differentiation process which is characterized by the production of elongated and aseptate cells that exhibit a remarkable increase in the number of flagella in comparison with that of the short oligoflagellated swimmer cells (reviewed in reference 22). A link between swarming motility and the type III virulence secretion pathway has been further suggested by the finding that null mutations in genes encoding subunits of the flagellum type III export machinery abolish swarming migration in *Proteus mirabilis*, *Y. enterocolitica*, and *Salmonella* (20, 59). On the other hand, the decreased expression of the *hpm*A hemolysin gene in an *flhA* null mutant of *P. mirabilis* indicates that a motility gene could have a regulatory role in the production of virulence factors (20). The relationship between swarming and virulence has been investigated mostly in gram-negative bacteria (2, 3, 14), although several species belonging to the *Bacillus* and *Clostridium* genera have been described to be active swarmers (26, 43, 54). Swarming differentiation has never been described for *B. thuringiensis*, and nothing is known as to whether molecular components coupling motility and virulence do exist in this organism.

This report describes the swarming behavior exhibited by *B. thuringiensis* in response to surface sensing and the isolation of a nonswarming mutant with a transposon insertion in *flhA*, a flagellar class II gene involved in the type III export of flagellar components (46). The *flhA* mutant of *B. thuringiensis* was defective in both swimming and swarming motility because it lacked flagella. The impairment in the flagellar filament assembly was due to defective export of flagellin, as both flagellin gene transcription and flagellin production could be detected inside the cell. The mutant was also defective in the secretion of the virulence factors hemolysin BL (HBL) (9) and PC-PLC, the intracellular production of which was demonstrated to occur in the *flhA* mutant as well as in the parental strain. The requirement of *flhA* for flagellin export, swarming differentiation, and secretion of virulence-associated proteins was confirmed by complementing the *flhA* mutant with a plasmid harboring the *flhA* gene. These findings suggest that a putative hierarchy in the expression of *B. thuringiensis* motility genes may differ from that described for *B. subtilis* and the enteric bacteria and indicate that a coordinate secretion for flagellin and virulence-associated proteins does exist in *B. thuringiensis*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. thuringiensis* strain IP2 (407 Cry-[*plcA′Z*]) (19) was obtained from strain 407 Cry$^-$ (H1 serotype). Strain MP02 is a nonmotile *flhA* null mutant derived from strain IP2 (produced in this investigation). *B. subtilis* strain ATCC 6633 was used to prepare a FlgM-specific probe (described above). The *Escherichia coli* strain TOP 10 (Invitrogen BV, Gronigen, The Netherlands) was used as the intermediary host. *B. thuringiensis* strains were grown at 30°C either in tryptone-NaCl (1% tryptone, 0.5% NaCl), brain heart infusion broth supplemented with 0.1% glucose (BHIG), HBL sheep blood agar (8), or Luria-Bertani (LB) medium. LB was supplemented with either spectinomycin (250 μg ml^{-1}) or erythromycin (25 μg ml^{-1}) when necessary. Media were routinely solidified with 1.5% agar unless otherwise specified. *E. coli* was grown at 37°C in LB supplemented with either ampicillin (100 μg ml^{-1}) or spectinomycin (60 μg ml^{-1}) when necessary.

Motility assays. Swimming motility was evaluated on semisolid (0.25% agar) LB medium at 30°C and was confirmed by microscopic examination of bacteria under a phase-contrast microscope. Phenotypic assays for swarming were initiated by spotting 2 μl of an overnight culture at the center of tryptone-NaCl or LB plates containing 0.45 to 1.5% agar. Plates were analyzed after growth at up to 24 to 48 h of incubation at 30°C. Bacteria were Gram stained for microscopy to evaluate the presence of elongated swarm cells (54). Flagella staining was performed as described by Harshey and Matsuyama (23).

Mutant screening, sequencing, and complementation of *flhA*. The thermosensitive plasmid pIC333, carrying the mini-Tn*10* insertion that contains a spectinomycin resistance gene (56), was introduced by electroporation (39) into strain IP2 to produce a library of insertional mutants as previously described (19). Spectinomycin-resistant mutants were isolated on 1% agar LB plates containing spectinomycin and were screened a second time on semisolid LB medium to isolate nonmotile mutants. Chromosomal DNA from the nonmotile strain MP02 was cut with *Eco*RI or *Hin*dIII and was ligated. The ligation mixture was used to transform *E. coli*, and transformants were screened for resistance to spectinomycin (60 μg ml^{-1}). Plasmid DNA was prepared from *E. coli* transformants, and restriction maps of the plasmid were determined to verify the presence of the mini-Tn*10* insertion (a 2.2-kb *Bam*HI fragment is characteristic of the mini-Tn*10* insertion). The junction fragments between the ends of the mini-Tn*10* insertion and chromosomal DNA were sequenced by using the oligonucleotides 5′-CGT TGGCCGATTCATTAATGC-3′ and 5′-CGATATTCACGGTTTACCCAC-3′ that matched the ends of the mini-Tn*10* insertion. DNA sequencing was performed by using the ALFexpress AutoRead Sequencing Kit (Pharmacia Biotech, Uppsala, Sweden) and the ALFexpress DNA sequencer (Pharmacia). Sequences were analyzed by GCG software, and database searching was performed with the BLAST algorithm. Theoretical protein molecular weight and pI values were calculated by the Compute pI/M_w program. Predicted transmembrane segments were identified by the TMpred program at the EMBnet node.

flhA was amplified from strain IP2 by using primers (*flhA*U, 5′-CAGACGAA TGAACTT-3′; *flhA*L, 5′-CTTTCCATTACTTCACC-3′) designed on the basis of sequences external to the coding region and was cloned into the pGEM-T vector (Promega, Madison, Wis.). *flhA* was excised from the plasmid by using *Sph*I and *Spe*I and was cloned into pHT304 (4). pHT304*flhA* was introduced into

strain MP02 by electroporation, and recombinant clones were screened on LB agar containing erythromycin.

RNA isolation, cDNA synthesis (RT), and PCR. Total RNA was purified from *B. thuringiensis* cultures grown in LB broth for 3 h. Briefly, after being washed with diethylpyrocarbonate-treated water, 10^8 bacterial cells were resuspended in 450 μl of lysis buffer (Rneasy Mini Kit; Qiagen) containing 0.35 g of glass beads (diameter, 0.1 mm) and was vortexed for 15 min to break the cells. Samples were centrifuged for 2 min at 10,000 × *g* and the aqueous phase was removed. Two hundred-fifty microliters of absolute ethanol was added, and the mixture was applied to an RNeasy mini spin column (Qiagen). After being digested with 40 Kunitz units of RNase-free DNase (Qiagen) for 20 h, total RNA was eluted from the column by following the instructions of the manufacturer. An aliquot of the RNA was examined on agarose gel to ensure its integrity and lack of DNA contamination. For cDNA synthesis, up to 2 μg of RNA was annealed with 1 μg of random hexamer primers at 70°C for 5 min, chilled on ice for 5 min, and mixed with reverse transcription (RT) buffer (50 mM Tris [pH 8.3], 75 mM KCl, 3 mM MgCl2), 13 mM dithiothereitol, 0.5 mM desoxynucleoside triphosphate, and 200 U of Superscript II reverse transcriptase (Gibco BRL, Life Technologies). Reactions were carried out at 42°C for 50 min and were stopped by incubation at 72°C for 15 min. For detection of flagellin gene expression, cDNA was amplified with the primers *fla*BU2 (5′-TAACAGCATGCGTACTCAAGAG-3′) and *fla*BL1 (5′-CATTTGTGGAGTTTGGTTAGC-3′). For detection of *flgM* the primers *flgM*F1 (5′-AATCAATTTGGAACACA-3′) and *flgM*R3 (5′-ATTTGC GTCTACTTTGTA-3′) were used in PCR to amplify *B. thuringiensis* or *B. subtilis* chromosomal DNA. For Southern analysis, *B. thuringiensis* and *B. subtilis* genomes were digested with either *Eco*RI or *Pst*I, electrophoresed, and transferred to positively charged nylon membranes. The amplification product obtained with *flgM*F1 and *flgM*R3 on the *B. subtilis* genome was labeled with the ECL Direct Labeling and Detection kit (Pharmacia). Filters were hybridized with this probe, and chemiluminescence was detected by Hyperfilm ECL (Pharmacia).

Preparation of culture supernatants and cell lysates. Bacterial cells were grown in BHIG in a rotary shaker at 200 rpm for 5 h at 30°C and were pelleted by centrifugation at 10,000 × *g*. Culture supernatants were concentrated with Microcon YM-10 filters (Millipore, Bedford, Mass.). Cells were washed with cold Tris-buffered saline (TBS; pH 7.4) and lysed in 1 ml of TBS with zirconium beads (diameter, 0.1 mm) by shaking at 4°C for 4 min with a mini-bead beater (Biospec Products, Barltesville, Okla.). Residual cells and debris were removed from the lysate by centrifugation at 12,000 × *g*. Concentrated culture supernatants and cell lysates were stored at 20°C and were used within 1 week.

Protein gel and immunoblot analysis. Protein samples were resuspended in sample buffer containing β-mercaptoethanol (51) and were normalized with respect to the optical density at 600 nm of the culture. After being heated at 95°C for 10 min, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and gels were either silver stained (11) or were used for protein blotting. Immunoblotting was performed as described previously (10), and membranes were probed with rabbit antisera to the individual HBL components, with an anti-B monoclonal antibody, or with a mouse antiserum to *B. thuringiensis* strain IP2 flagellin (produced during this investigation). Isoelectric points were determined on a PhastSystem (Pharmacia) by using Pharmacia isoelectric focusing (IEF) standards on Phastgel IEF gels with a pH range of 9 to 3.

Preparation of a mouse antiserum to *B. thuringiensis* flagellin. Purified flagellin was prepared with cultures of strain IP2 grown in BHIG for 5 h at 30°C. Bacterial cells were vortexed, and after centrifugation to remove cells flagellar filaments were collected from supernatants by high-speed centrifugation at 100,000 × *g* for 1 h. Flagellin was analyzed by SDS-PAGE separation as described by Gygi et al. (20) and were electroeluted. BALB/c mice were immunized by intraperitoneal injection of 30 μg of the protein preparation dissolved in Tris-HCl buffer and emulsified in Freund's incomplete adjuvant (1:3). At day 21 animals received a booster injection (30 μg of the protein in adjuvant), and serum was obtained after 48 h.

Detection of hemolysin and PC-PLC activities. HBL activity was determined on sheep blood agar plates by the formation of a discontinuous zone of hemolysis around colonies (8). Thuringiolysin O and hemolysin II, III, and IV were detected by IEF sheep blood agar overlay of culture supernatants with or without cholesterol (Sigma Chemical Co., St. Louis, Mo.). PC-PLC activity was measured in culture supernatants and cell lysates by a gel diffusion assay with a gel containing 1.5 mg (wt/vol) of crude PC (Sigma)/ml in TBS. Different amounts of pure PC-PLC (Sigma) were used to generate a standard curve. Slides were kept at 37°C in a humidified chamber, and after 12 to 18 h the activity was recorded as the diameter of the opaque zone.

RESULTS

Swarming differentiation by *B. thuringiensis*. The ability to swarm is thought to be a ubiquitous behavioral trait exhibited by flagellated eubacteria in response to surface sensing. In contrast to swimming, which is brought about by individual cells growing in liquid media, swarming is a collective cell movement that can be viewed when cells are propagated on solid culture media (reviewed in reference 22). *B. thuringiensis* strain IP2 exhibited a characteristic swimming motility when grown in liquid media or on semisolid media containing 0.25 to 0.30% agar: the swimmer cells appeared as short (2 to 3 μm) and oligoflagellated rods with fewer than 10 flagella per cell (Fig. 1A). Swarming could be observed on solid media, the minimum agar concentration inducing swarm-cell differentiation being 0.45%. Optimal agar concentration for swarming ranged from 0.7 to 1.2%, although the strain was able of swarming over solid media containing an agar concentration as high as 2.5%. Swarming occurred at a wide range of temperature values, the optimum being between 25 and 38°C. Under optimal swarming conditions (tryptone-NaCl medium containing 1% agar), the cell organization within the growing colony appeared similar to that described for *B. cereus* (54). All around the colony rim cells were viewed as long, filamentous swarm cells that maintained close cell-to-cell contact along their long axis while coordinately moving outward from the colony border in groups or rafts of tightly bound cells (Fig. 1B). Swarm cells were elongated (14 to 16 μm) (Fig. 1C) and were hyperflagellated (Fig. 1D), and they generated a continuous layer of collective cell migration all around the colony border. The swarm cells eventually stopped moving, underwent reductive divisions, and dedifferentiated into short, oligoflagellated vegetative swimmer cells, thus giving rise to a phase of colony consolidation. However, alternate cycles of swarming and consolidation, such as those exhibited by *P. mirabilis* swarm colonies (26), were not observed during the development of *B. thuringiensis* colonies. Indeed, both swarm cell migration and dedifferentiation into swimmer cells appeared to occur continuously within the layer constituting the advancing front of the developing community. Consequently, as already reported for several gram-negative and -positive bacteria (15, 43, 54, 59), the macroscopic appearance of the swarm colonies produced by *B. thuringiensis* was characterized by the presence of a central and large consolidation phase surrounded by the swarming layer localized at the colony rim.

Isolation and characterization of the nonmotile and nonswarming mutant MP02. Several mutants of *B. thuringiensis* strain IP2 were obtained by transposon mutagenesis with the mini-Tn*10* insertion (56) that contains a spectinomycin resistance gene. Among the mini-Tn*10* mutants obtained, we selected a strain that was unable to move on semisolid (0.25% agar) media even after prolonged incubation (24 h). Microscopic examination of the mutant, referred to as MP02, showed that it was no longer motile, and flagella staining revealed no flagella on the cell surface (Fig. 1E). When propagated over solid media, strain MP02 was unable to swarm, giving rise to compact colonies (Fig. 1F) in which swarm cell differentiation was never observed. Only short rods (Fig. 1G) never exhibiting flagella (Fig. 1H) were observed both in the center and at the colony rim.

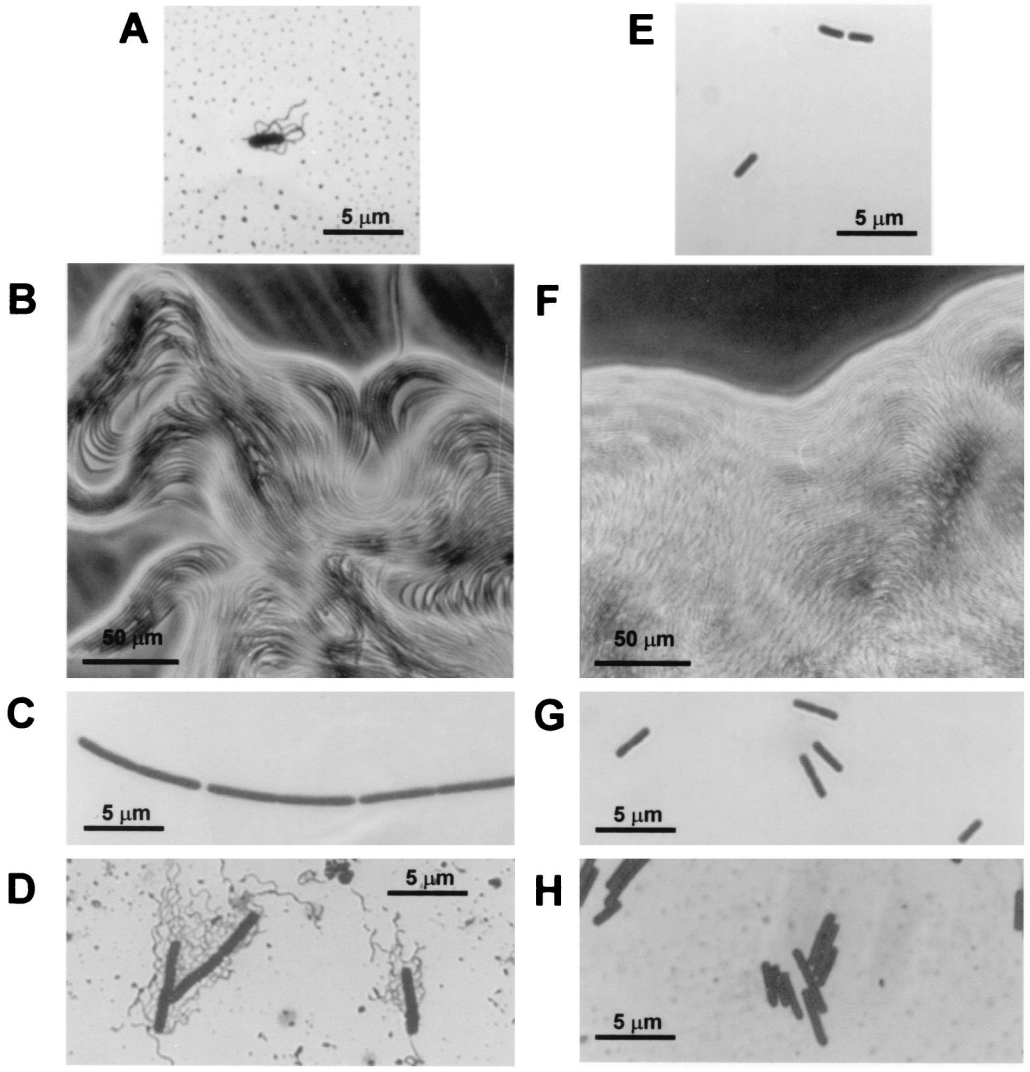

FIG. 1. Morphological traits of *B. thuringiensis* IP2 and its mutant, MP02. Short and oligoflagellated swimmer cell of strain IP2 (A); nonflagellated swimmer cell of mutant MP02 (E). (B and F) Rim of a colony produced by strains IP2 (B) and MP02 (F). Elongated (C) and hyperflagellated (D) swarm cells of strain IP2 are also shown. Short (G) and nonflagellated (H) cells of the mutant MP02 picked up from the colony rim are depicted as well.

The mini-Tn*10* element contains a pUC replicon allowing recovery of the chromosomal DNA sequences flanking the insertion site. Therefore, chromosomal DNA from the mutant MP02 was digested with *Hin*dIII or *Eco*RI (restriction sites for these two enzymes are not present in the mini-Tn*10* element) and was treated with T4 DNA ligase. The ligation mixture was used to transform *E. coli* cells, and transformants were selected for resistance to spectinomycin. The recombinant plasmids isolated from the $Spec^R$ transformants were used as templates to determine the nucleotide sequence of the ends of the mini-Tn*10* element and the chromosomal DNA adjacent to the insertion site. Sequencing showed that the transposon had inserted into a 2,082-bp coding sequence sharing similarities (58% identity in 977 overlapping nucleotides) with *B. subtilis flhA*; therefore, the *B. thuringiensis* gene was named *flhA* (GenBank accession number AJ438180). This gene has a putative ribosome-binding site 9 nucleotides upstream of the first nucleotide and encodes a 688-residue protein having a calculated size of 76.8 kDa and a pI of 5.19. The predicted amino acid sequence of *B. thuringiensis* FlhA showed significant homology to the FlhA protein of *B. halodurans* (37% identity, 57% positives), *B. subtilis* (35% identity, 56% positives), extremophilic eubacteria (*Thermotoga maritima* and *Aquifex aeolicus*), and several other gram-negative species (*Helicobacter pylori*, *E. coli*, *Campylobacter coli*, *Salmonella enterica* serovar Typhimurium, *P. mirabilis*, *Treponema pallidum*, *Y. enterocolitica*, and *Borrelia burgdorferi*). Analysis of the predicted amino acid sequence identified seven hydrophobic regions (putative transmembrane segments) in the N-terminal sequence and a long hydrophilic C-terminal domain. This finding suggests that the protein encoded by *B. thuringiensis flhA* is bound to the cytoplasmic membrane and possesses a soluble domain protruding into the cytoplasm, as already demonstrated for *Salmonella* FlhA (46). Like many other proteins constituting the flagellar export ap-

paratus in gram-negative and -positive organisms, *B. thuringiensis* FlhA displayed a high degree of relatedness to a family of membrane proteins (LcrD of *Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Y. enterocolitica*; PcrD of *Pseudomonas aeruginosa*; MxiA of *Shigella flexneri*; InvA and SsaV of *S. enterica* serovar Typhimurium; and SctV of *Chlamydia* spp.) involved in the type III secretion of virulence factors (reviewed in reference 33). The highest sequence similarity was observed in the hydrophobic N-terminal region, predicted to contain transmembrane segments, while less similarity was found in the putative cytoplasmic C terminus.

***B. thuringiensis flhA* is necessary for the export of flagellin but not for its synthesis.** Enteric bacteria and *B. subtilis* carrying mutations in *flhA* exhibit a nonflagellated phenotype, and the mutation in *flhA* has been demonstrated to be the only factor responsible for the lack of flagellar assembly in the mutant strains (12, 16, 20, 35). Indeed, *flhA* mutants are completely defective in the export of the anti-sigma factor FlgM, which downregulates the expression of flagellar class III genes, flagellin included (44). Furthermore, it is generally assumed that the protein encoded by *flhA* is an essential component for the export of structural flagellar proteins outside the cell. In *Salmonella*, FlhA is associated with the flagellar basal body, physically interacts with the MS ring, and strongly binds to flagellin, giving rise to a complex that is necessary for promoting the export of flagellin outside the cell (47).

The complete lack of flagellar-filament assembly observed in the *flhA* mutant of *B. thuringiensis* prompted us to investigate whether the *flhA* mutation in *B. thuringiensis* affected both flagellin gene transcription and flagellin export. Flagellin mRNA was searched for in both strains MP02 and IP2 by the use of RT-PCR by using primers designed on the basis of the available flagellin gene sequence of *B. thuringiensis* subsp. *alesti* (41). The flagellin transcript was detected in the wild type as well as in the *flhA* mutant (Fig. 2). The ability of the *flhA* mutant of *B. thuringiensis* to transcribe a purported class III gene product, flagellin, suggested that *B. thuringiensis flhA* cannot be considered a flagellar class II gene.

To evaluate whether a gene similar to *flgM* could be found in *B. thuringiensis*, chromosomal DNAs from strain IP2, the mutant MP02, and *B. subtilis* ATCC 6633 (as the positive control) were subjected to amplification with the primers *flgM*F1 and *flgM*R3, constructed on the basis of the *B. subtilis flgM* sequence (http://genolist.pasteur.fr). Although a variety of PCR conditions were used, no amplification product was ever obtained with both strains IP2 and MP02 of *B. thuringiensis*, while amplification was achieved with *B. subtilis* ATCC 6633. Moreover, a *flgM*-specific probe prepared from *B. subtilis* always gave a complete lack of hybridization when used with *B. thuringiensis* (Fig. 3). The lack of PCR amplification and DNA-DNA hybridization might be due to the distant relationship between *B. thuringiensis* and *B. subtilis*. However, *B. thuringiensis* is closely related to *B. cereus* and *Bacillus anthracis* (25). Thus, a search of sequences similar to that of *flgM* was performed with the available genome sequences of *B. anthracis* (http://www.tigr.org) and *B. cereus* (http://www.integratedgenomics.com). No significant similarity was found, thus supporting the hypothesis that the lack of *flgM* could be a common feature of species belonging to the *B. cereus* group.

The question of whether flagellin was synthesized and not exported by strain MP02 was addressed by performing immunoblot assays on cell lysates and culture supernatants from strains MP02 and IP2. As shown in Fig. 4, flagellin was synthesized by both strains but the protein was not exported by strain MP02. Moreover, the amount of flagellin that accumulated intracellularly in the mutant appeared to be higher than that in the parental strain. This finding clearly shows that, in contrast to *B. subtilis* (12), the *flhA* mutation in *B. thuringiensis* does not influence flagellin production. Therefore, the lack of functional flagellar export machinery due to the *flhA* mutation appears to be the only factor responsible for the lack of flagellar assembly in this species.

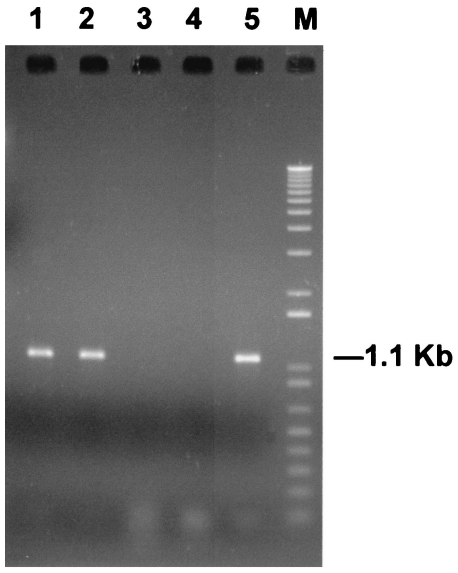

FIG. 2. Flagellin gene transcription by *B. thuringiensis* IP2 and MP02. Electrophoretic separation of the amplified products obtained by RT-PCR (lanes 1 and 2) or PCR (lanes 3 and 4) on total RNA from strain IP2 or MP02, respectively. Lane 5 shows control PCR amplification on the chromosomal DNA of strain IP2. M, molecular size marker.

The activity of *flhA* is necessary for the export of nonflagellar proteins. Mutations in *flhA* have been reported to affect the expression of the hemolysin gene *hpmA* in *P. mirabilis* (20) and

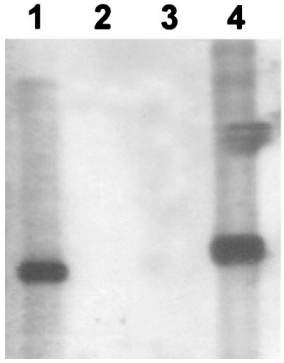

FIG. 3. Southern hybridization of an *flgM*-specific probe with *B. thuringiensis* IP2 and *B. subtilis* ATCC 6633. Hybridization with *Eco*RI (lanes 1 and 2)- or *Pst*I (lanes 3 and 4)-digested chromosomal DNAs from *B. subtilis* (lanes 1 and 4) and *B. thuringiensis* (lanes 2 and 3).

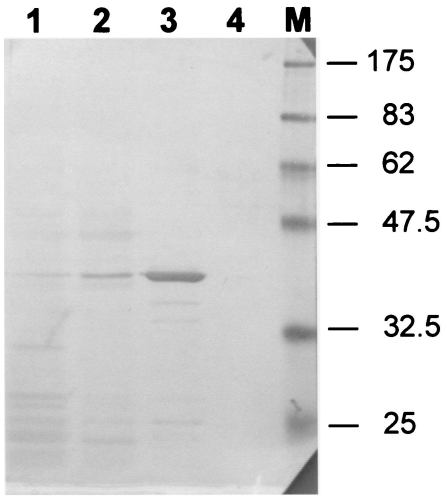

FIG. 4. Immunoblot with an antiflagellin antiserum on cell lysates (lanes 1 and 3) and culture supernatants (lanes 2 and 4) prepared from *B. thuringiensis* strain IP2 (lanes 1 and 2) and from its mutant MP02 (lanes 3 and 4). M, molecular size standard.

the secretion of the virulence-associated phospholipase YplA in *Y. enterocolitica* (58). Since the *flhA* mutant of *B. thuringiensis* was found to be completely defective in the export of flagellin, preliminary investigations were focused on establishing whether the lack of functional flagellar export machinery could also affect the secretion of nonflagellar proteins in this species. SDS-PAGE of culture supernatants from strains MPO2 and IP2 revealed that the mutant was defective in the secretion of several proteins possessed by the wild type (Fig. 5). Moreover, in contrast to strain IP2, the *flhA* mutant did not produce the

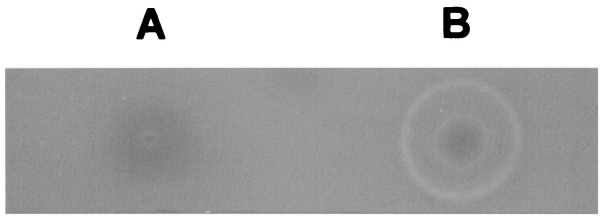

FIG. 6. Hemolytic activity of *B. thuringiensis* IP2 and MP02 on HBL agar plates. Shown are the absence and presence of the discontinuous pattern of hemolysis caused by HBL around the colonies produced by strain MP02 (A) and IP2 (B).

typical discontinuous pattern of hemolysis caused by the secretion of the enterotoxic tripartite HBL (8, 9, 10) on sheep blood agar plates (Fig. 6). None of the HBL components (B, L1, or L2) could be detected in culture supernatants (100-fold concentrated) from the mutant strain when subjected to immunoblotting with polyclonal antisera specific to the individual components (data not shown). As expected, immunoblot analysis performed with a monoclonal antibody to the B protein showed that the wild-type strain yielded a positive signal in both culture supernatant and cell lysate (Fig. 7). However, while only one protein, with a predicted molecular size of 37.5 kDa (corresponding to that estimated for the secreted B component [9]), was detectable in the supernatant, more than one reactive band was observed in the cell lysate. On the basis of these results we hypothesized that the 40-kDa band corresponded to the unprocessed B component still containing the signal peptide, the 37.5-kDa band corresponded to the mature protein, and the lower band (about 36 kDa) corresponded to a degraded form of the protein. No signal was obtained from the

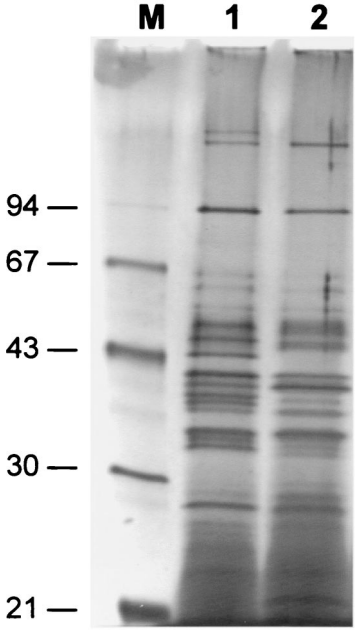

FIG. 5. Extracellular protein secretion by *B. thuringiensis* IP2 and MP02. Extracellular proteins from culture supernatants were separated by SDS-PAGE and were stained with silver. Lane 1, strain IP2; lane 2, strain MP02. M, molecular size standard.

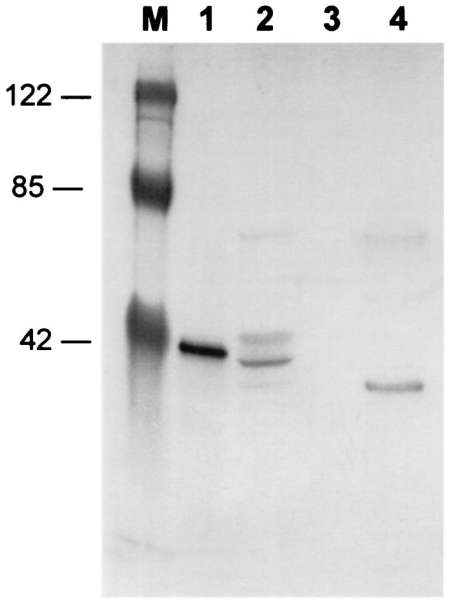

FIG. 7. Immunoblot with a monoclonal antibody to the B component of HBL on cell lysates (lanes 2 and 4) and culture supernatants (lanes 1 and 3) prepared from *B. thuringiensis* strain IP2 (lanes 1 and 2) and from its mutant MP02 (lanes 3 and 4). M, molecular size standard.

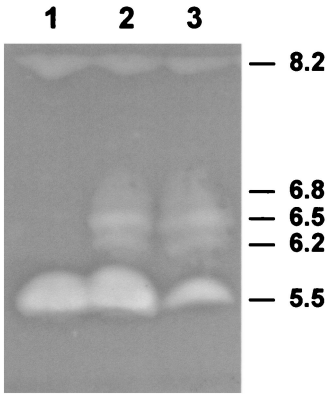

FIG. 8. IEF sheep blood agar overlay of culture supernatants from *B. thuringiensis* IP2 and MP02. Shown are culture supernatants from strain IP2 with (lane 1) or without (lane 2) cholesterol and from the mutant MP02 (lane 3). Numbers at the right side of the figure indicate pI values.

culture supernatant of the mutant, while one reactive band (about 36 kDa) was observed in the cell lysate (Fig. 7). In the mutant, the mature form of B, which was not released into the culture supernatant, could be subjected to rapid hydrolysis, thus accounting for the substantial increase in the intracellular concentration of the purported B degradation product. These observations suggested that in strain MP02 the B component of HBL was synthesized, blocked in its secretion, and intracellularly degraded.

To investigate whether the *flhA* mutation influenced the secretion of other *B. thuringiensis* hemolysins, culture supernatants from strains IP2 and MP02 were subjected to IEF sheep blood agar overlay assays (Fig. 8). Zymograms of the mutant were similar to those of the wild type, since both strains were able to secrete thuringiolysin O (pI values of about 6.2, 6.5, and 6.8), which was identified through the inhibition of its activity by cholesterol (36), hemolysin II (6), and/or hemolysin III (5), which have similar isoelectric points (pI values of about 8.2), and hemolysin IV (pI 5.5) (7, 42).

PC-PLC activity was evaluated in culture supernatants and cell lysates from strains IP2 and MP02 by a gel diffusion assay with PC as the substrate. Intracellular PC-PLC activity was 2.7 times lower in strain MP02 than in strain IP2 (0.021 U/ml versus 0.056 U/ml); however, the mutant showed a significant reduction in the secretion of PC-PLC compared to that secreted from the wild type (0.05 U/ml versus 3.46 U/ml). This result indicates that the mutation in *flhA*, although weakly reducing PC-PLC synthesis, dramatically reduces its secretion.

Complementation of the *flhA* mutant. Complementation of the *flhA* mutant with a plasmid harboring the *flhA* gene was performed to establish if the defect in *flhA* was the only one responsible for the phenotype exhibited by strain MP02. *flhA* was subcloned into the *Sph*I/*Spe*I sites of pHT304, an *E. coli-B. thuringiensis* shuttle and expression vector (4). pHT304*flhA* was introduced into strain MP02, generating strain MP03 that displayed flagella on the cell surface and, as in the parental strain, exhibited a swimming or swarming behavior depending on whether it was grown in liquid or solid medium (Fig. 9). The ability to produce HBL and PC-PLC was regained, although the level of both toxins was lower than that produced by the wild type. Weak HBL activity was observed when MP03 was propagated on sheep-blood agar plates, and all the HBL components were detected by immunoblot analysis. Moreover, extra- and intracellular PC-PLC (0.64 and 0.048 U/ml, respectively) were 12.8 and 2.3 times higher than the corresponding activities measured with the mutant.

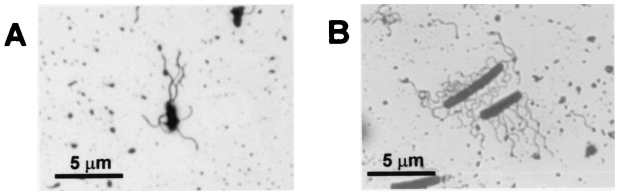

FIG. 9. Morphological traits of *B. thuringiensis* strain MP03. Shown are a short and oligoflagellated swimmer cell (A) and an elongated and hyperflagellated swarm cell (B) picked up from a swarming colony.

These findings demonstrate that in *B. thuringiensis* the flagellum-aided swimming and swarming motility, the export of flagellin, and the secretion of virulence-associated proteins, such as HBL and PC-PLC, depend on the activity of *flhA*.

DISCUSSION

Flagellum-driven motility was suggested to play a role in the virulence of *B. thuringiensis* following the demonstration that an avirulent pleiotropic mutant was unable to produce hemolytic activities and flagella (24). However, no evidence has been produced showing that genes involved in the flagellum assembly may also play a role in the production of specific virulence factors by *B. thuringiensis*. This is the first report to demonstrate that the activity of *B. thuringiensis flhA* is required for swimming motility, swarming differentiation, and secretion of virulence-associated proteins.

***B. thuringiensis flhA* and motility.** The identification of the *flhA* gene in *B. thuringiensis* was achieved by sequencing the DNA regions flanking the insertion site of the mini-Tn*10* element in a mutant (MP02) that was completely devoid of flagella (Fig. 1E). The nonflagellated phenotype observed for the mutant suggested that *B. thuringiensis flhA* could have a role similar to that played by *flhA* in enteric bacteria and *B. subtilis* (12, 16, 20, 44, 46), where: (i) *flhA* has been classified as a flagellar class II gene regulating the expression of flagellar class III genes, flagellin included, and (ii) FlhA is a membrane protein involved in the export of structural flagellar proteins outside the cell. *flhA* has not been described previously for *B. thuringiensis*, and there is no report demonstrating that, in this organism, flagellar gene expression is governed by a given hierarchical pathway. Therefore, it was interesting to address the question of whether a *B. thuringiensis* mutant having a genetic defect in *flhA* transcribed the flagellin gene, synthesized flagellin, and exported flagellin outside the cell.

The predicted amino acid sequence of *B. thuringiensis flhA* suggested that FlhA is a membrane protein also in this organism and, by analogy with FlhA of other organisms, was thought to behave as a component of the flagellar export apparatus. Evidence is provided to demonstrate that the flagellin transcript and the intracellular production of flagellin occurred in the wild type as well as in the *flhA* mutant of *B. thuringiensis*

(Fig. 2 and 4). In contrast, the export of flagellin outside the cell could be detected only in the parental strain, while an increased amount of intracellular flagellin was observed in the mutant (Fig. 4). These findings clearly show that the *flhA* mutation in *B. thuringiensis*, in contrast to the *flhA* mutants of *B. subtilis* and enteric bacteria (12, 20, 35), does not abolish flagellin production. The protein is accumulated in the mutant apparently because it is not exported outside the cell. Therefore, *flhA* cannot be regarded as a flagellar class II gene in *B. thuringiensis*, since the expression of a purported class III gene, the flagellin gene, does not depend on its activity. Since the expression of flagellar class III genes is coupled with that of class II genes through the action of FlgM, we hypothesized that a gene similar to *flgM* could not be involved in the regulation of genes encoding external flagellar proteins in *B. thuringiensis*. This was supported by three findings: (i) the absence of a PCR amplification product with primers designed on the sequence of *B. subtilis flgM*, (ii) the complete lack of hybridization of a *flgM*-specific probe with *B. thuringiensis* DNA (Fig. 3), and (iii) the absence of a gene related to *flgM* in the genomic sequences of *B. anthracis* and *B. cereus*. Therefore, the lack of functional export machinery due to the *flhA* mutation was considered to be the only factor responsible for the impairment of flagellar assembly in this species.

The *flhA* mutant of *B. thuringiensis* was unable to swarm in response to surface sensing (Fig. 1). Swarming by *B. thuringiensis*, previously unobserved in this organism, was found to be very similar to that recently described for its closest relative, *B. cereus* (54). *B. thuringiensis* swarm colonies did not produce regularly spaced rings of swarming migration and consolidation; rather, the macroscopic appearance of swarm colonies was characterized by a central consolidation phase surrounded by a continuous layer of an advancing front movement of swarm cells all around the colony rim (Fig. 1B). The ability of *B. thuringiensis* to swarm was documented by the demonstration that swarming motility is brought about by elongated and hyperflagellated swarm cells organized as multicellular masses rapidly and coordinately moving outward to the colony border (Fig. 1B to D). Swarming differentiation was completely lacking in the *flhA* mutant, which never produced hyperflagellated or elongated swarm cells (Fig. 1F to H). Characterization of nonswarming mutants has firmly established that all defects in genes encoding subunits of the flagellar export apparatus completely abolish swarming differentiation (20, 59), since swarming differentiation requires functional flagella. The observation that *flhA* is required for exhibiting a swarming behavior by *B. thuringiensis* is in agreement with the involvement of *flhA* in both the assembly of a functional flagellar filament and the control of cell differentiation, as already reported for a nonswarming *flhA* mutant of *P. mirabilis*, for which a negative feedback from the *flhA* defect to the *flhDC* master operon has been demonstrated (17).

The requirement of *flhA* activity for flagellar filament assembly, flagellum-driven motility, and swarming behavior of *B. thuringiensis* was demonstrated by complementing the *flhA* mutant with a plasmid harboring an intact *flhA* gene. The complemented strain regained the ability to export flagellin, swim in liquid media, and differentiate from swimmer cells into the elongated and hyperflagellated swarm cells when propagated over solid culture media (Fig. 9).

***B. thuringiensis flhA* and virulence.** In addition to the motility defects, the *flhA* mutant of *B. thuringiensis* was impaired in the secretion of HBL (Fig. 6 and 7), whereas the secretion of thuringiolysin O and hemolysins II to IV were similar in the wild type and the *flhA* mutant (Fig. 8). Our results showed that HBL was synthesized intracellularly, completely blocked in its secretion, and most likely degraded inside the cell in the *flhA* mutant of *B. thuringiensis* (Fig. 7).

The finding that HBL secretion is blocked in a mutant having a defective flagellar export apparatus is of great interest. Secretion of a nonflagellar protein through the flagellar export machinery has already been reported for *Y. enterocolitica* (58); however, in such a case the protein was exported in a process similar to that of the type III secretion, which implies that the protein is not modified as it crosses the plasma membrane (reviewed in reference 33). The amino acid sequences of the HBL proteins showed that they all possess a signal peptide sequence at their amino termini, thus indicating their secretion through an *S*-dependent secretion pathway. However, since the molecular mass of the secreted B component of HBL corresponded to that estimated for the protein lacking the amino-terminal signal sequence, we speculate that signal peptidases could cooperate with the flagellar export apparatus in the secretion of HBL. This hypothesis is supported by the observation that the flagellar P- and L-ring subunits, which do possess a signal peptide sequence before being secreted (30, 32), are exported and assembled in the flagellar structure only when all the components of the flagellar export apparatus are active (44).

Impairment in HBL secretion was not the only defect we observed in the secretion of virulence-associated proteins in the *flhA* mutant of *B. thuringiensis*. The secretion of PC-PLC also appeared to be significantly influenced by the mutation in *flhA*, although in this case the amount of intracellular PC-PLC activity detected in the mutant was reduced to about one third of the activity measured in the wild type. However, the amount of the enzyme activity secreted by the mutant was about 70 times lower than that by the wild type; indeed, the ratio between the amount of extra- and intracellular PC-PLC activity was higher than 60 and lower than 3 when calculated for the wild type and the mutant, respectively. Such a difference does not merely reflect the reduced amount of PC-PLC synthesized intracellularly in the mutant; rather, the remarkable lowering in the extracellular PC-PLC activity could be explained by assuming that impairment in PC-PLC secretion occurs in the *flhA* mutant. Therefore, whatever the mechanism of PC-PLC secretion is, this observation strongly suggests that *flhA* is required for or in some way coupled with the export of PC-PLC outside the cell.

The overall results produced by this investigation provide the first evidence that in *B. thuringiensis* the activity of a single gene, *flhA*, is crucial for the assembly of flagellar filament, swimming motility, swarming differentiation, and export of flagellar as well as nonflagellar proteins, such as flagellin and the virulence-associated proteins HBL and PC-PLC. Moreover, the observation that *B. thuringiensis flhA* cannot be considered a flagellar class II gene is of intrinsic interest, since it opens a new perspective in studying the flagellar gene expression hierarchy of gram-positive bacteria. Finally, the involvement of *flhA* in the virulence potential of *B. thuringiensis* could

be exploited to produce a *B. thuringiensis* product that is demonstrably nonpathogenic to humans but still active against insects; this could provide an added margin of safety and allay public concern.

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