Cloning, Nucleotide Sequence, and Expression of the Gene Encoding the Bacteriocin Boticin B from *Clostridium botulinum* Strain 213B

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Bacteriocins are antimicrobial peptides or proteins formed by certain bacterial species that typically have inhibitory activity against closely related organisms (5). *C. botulinum* is a gram-positive, endospore-forming anaerobic bacterium that causes the severe neuroparalytic illness known as botulism in humans and animals (10). Our laboratory demonstrated that *Clostridium botulinum* strain 213B produces a heat-stable bacteriocin, called boticin B, that is bactericidal to certain *C. botulinum* strains and related species (13; H. Sugiyama, E. A. Johnson, R. Sandler, and S. Cole, unpublished data). Purification and characterization of the boticin B peptide was performed in this laboratory (13) and will be described in a separate communication.

**Isolation of the boticin B structural gene.** In order to determine the location of the boticin B gene, plasmid DNA and total genomic DNA (containing plasmid and chromosomal DNAs) were isolated from *C. botulinum* strain 213B. *C. botulinum* 213B contains two plasmids (18.8 and 12.1 kb). Undigested plasmid DNA and HindIII- and EcoRI-digested plasmid and total DNAs were separated on an agarose gel by electrophoresis, transferred to a nylon membrane, and hybridized to a degenerate 18-mer oligonucleotide (BB1) (5'-GC[A/T][A/T]GTT[A/T]TGGTT[A/T]-3') probe. The sequence of the probe was designed based on the previously identified molecular mass of 5,138 Da. A putative ribosome binding site (RBS), AAGGAGG, was located 8 bp upstream of the start codon of the *btcB* gene. Sequences exhibiting similarity to a promoter region are located upstream of the RBS (nucleotides 503 to 550) (Fig. 2). A region of dyad symmetry that could form a stem-loop structure is located downstream of the *btcB* gene (nucleotides 730 to 745 and 753 to 768) (Fig. 2). This region has the features associated with bidirectional rho-independent transcription termination (8). Sequence analyses revealed several open reading frames (ORFs) in the vicinity of the *btcB* gene. One of these, ORF-30, is located just 63 bp downstream of the *btcB* gene (Fig. 2). Interestingly, the 30-amino-acid putative peptide encoded by this ORF contains 20% lysine, 17% phenylalanine, 10% serine, and 10% glutamic acid residues. However, it is not known if ORF-30 is transcribed as a monocistronic message or is part of an operon with *btcB* and possibly other bacteriocin-associated genes such as genes coding for regulation, immunity, extracellular translocation, and amino acid modification, as has been described for certain other bacteriocin genetic systems (5, 6). No protein in the GenBank databases revealed significant homology to boticin B, the ORF-30 product, or products of other ORFs surrounding *btcB*.

The *btcB* gene encodes a 50-amino-acid protein with a calculated molecular mass of 5,138 Da. Amino acid analysis of purified boticin B estimated that the active protein contained 39 amino acids, and mass spectroscopic analysis determined its molecular mass to be 4,003 Da (13). The smaller size of the purified protein could indicate that the *btcB* gene encodes a prepeptide that is posttranslationally modified to form the active peptide, which is relatively common for bacteriocins from other gram-positive bacteria (5). The amino acid sequence of one of the chymotrypsin fragments (11 amino acids)
analyzed previously did not contain the first 10 amino acids as deduced from the gene (Fig. 3). Interestingly, this region does not contain a chymotrypsin cleavage site, indicating that these amino acids may not be part of the active protein. The molecular mass of the peptide without these 10 amino acids would correlate with the molecular mass of 4,003 Da as determined previously by mass spectroscopy. In addition, the peptide started with a valine residue, not asparagine, which is in this position according to the nucleotide sequence of the \textit{btcB} gene (Fig. 3). The other chymotryptic peptide, a 14-amino-acid fragment, is missing a stretch of three amino acid residues (Asn-Leu-Asp) that are encoded by the \textit{btcB} gene (Fig. 3). However, it is not clear whether these amino acid residues are deleted from the mature peptide or whether the discrepancy is due to technical difficulties involved in sequencing this very hydrophobic peptide fragment. These findings further support the possibility that the boticin B encoded by the \textit{btcB} gene is synthesized as a prepeptide, followed by cleavage of leader peptide sequences, and further undergoes several modifications before assuming its active form.

Expression of the boticin B gene in \textit{C. botulinum} strain 62A. Experiments designed to express boticin B in \textit{Escherichia coli} were unsuccessful (data not shown); therefore, we attempted to express the bacteriocin in a strain of \textit{C. botulinum} that does not produce boticin B. The 3.0-kb \textit{HindIII} fragment containing the \textit{btcB} gene with its putative regulatory sequences was inserted into the mobilizable plasmid \textit{pJIR1457} (7), yielding \textit{pMVP1113}. The plasmid \textit{pMVP1113} was transferred into \textit{C. botulinum} strain 62A by conjugation from the \textit{E. coli} donor strain S17-1(\textit{pMVP1113}) as described previously (2).

The \textit{C. botulinum} 62A transconjugant carrying \textit{btcB} expression construct \textit{pMVP1113} was analyzed for boticin B production using a well diffusion assay. The transconjugant containing vector \textit{pJIR1457} alone was used as a negative control. \textit{C. botulinum} strain 213B and \textit{C. botulinum} transconjugants were grown in M1 broth (5% Trypticase peptone, 2.5% proteose peptone, 1% glucose, 2% yeast extract, and 0.1% sodium thioglycolate) without antibiotics for 3 days at 37°C. Bacterial cells...
were removed by centrifugation, and the culture supernatants were filtered through a 0.2-μm-pore-size membrane filter. Fifty-microliter culture filtrates of 62A(pMVP1113), 62A(pJIR1457), and C. botulinum strain 213B were loaded into precut wells in M1 agar plates, incubated anaerobically for 6 h at room temperature to allow inhibitory agents to diffuse through the agar medium, and then overlaid with 6 ml of M1 broth containing 0.75% agar and ca. 10⁶ cells of C. botulinum strain 62A per ml. The plates were incubated anaerobically for 24 h at 30°C and examined for the presence of inhibition zones surrounding the wells. No inhibitory activity was detected in the culture media of both transconjugant strains, while strain 213B exhibited inhibitory activity (data not shown). To determine if bacteriocin was produced but not released into the culture media by the C. botulinum strain 62A transconjugant carrying boticin B expression construct pMVP1113, bacterial cells were sonicated for 10 min and cell-free lysates were also analyzed for boticin B production. However, no inhibitory activity was observed (data not shown). To test whether boticin B was expressed at levels too low to detect inhibitory activity in culture filtrates, culture filtrate proteins were concentrated by ammonium sulfate precipitation. Ammonium sulfate was added to 63% saturation to 150 ml of culture filtrates and incubated for 18 h at 4°C to allow precipitation of proteins (1). Precipitated proteins were collected by centrifugation and dissolved in 1 ml of 25 mM sodium phosphate buffer (pH 6.8), and 50 μl was added to wells in M1 agar plates. The 150-fold-concentrated culture filtrate of 62A(pMVP1113) produced an inhibition zone in well diffusion plates overlaid with C. botulinum strain 62A (Fig. 4). The inhibition zone (2 mm) was smaller than that observed with the 213B culture filtrate (8 mm). The concentrated culture filtrate of 62A(pJIR1457) did not reveal any inhibitory activity, indicating that the activity of 62A(pMVP1113) was due to the presence of the 3.0-kb HindIII fragment from C. botulinum strain 213B containing the boticin B gene. The low levels of phenotypic expression of boticin B that were observed in the C. botulinum strain 62A transconjugant could possibly be explained by the lack of additional genes needed for expression of functional boticin B, such as regulatory genes or genes coding for immunity, peptide modification, and extracellular translocation, in this heterologous strain.

The presence of sequences homologous to btcB was examined in other clostridial strains. Total genomic DNA was isolated from the following strains: C. botulinum strains 62A, Hall A, 588 Ab, NCTC 2916 A(B), 2B, 17B, Okra B, Alaska E, Kyoto F, and Langeland F; Clostridium barattii strain ATCC 27638; Clostridium butyricum strains 1024 and ATCC 19398; and Clostridium sporogenes strain PA3679. HindIII- and EcoRI-digested total DNA from each strain was separated on an agarose gel by electrophoresis, transferred to a nylon membrane, and hybridized to the BB1 oligonucleotide probe. C. botulinum strain 213B HindIII- and EcoRI-digested total DNAs were included as positive controls. The probe hybridized to the same-size 3.0-kb HindIII and 9.0-kb EcoRI DNA fragments from strains 213B and 588 Ab. No hybridization was observed with chromosomal or plasmid DNA from the other clostridial strains (data not shown). Plasmid DNA was isolated from strain 588 Ab and compared to plasmids from strain 213B. Both strains contained two similar-sized plasmids, and their HindIII and EcoRI digestion band profiles were identical (data not shown). These data suggest that strain 588 Ab carries the same plasmids as strain 213B or closely related plasmids. Culture filtrates of strain 588 Ab, similar to those of strain

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**Val-Leu-Gly-Leu-Xaa-Ala-**

**Met-Gln-Lys-Pro-Glu-Ile-Ile-Ser-Ala-Asp-Leu-Gly-Leu-Cys-Ala-**

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**-Val-Asn-Glu-Phe-Val**

**-Val-Asn-Glu-Phe-Val-Ala-Leu-Ala-Ala-Ile-Pro-Gly-Gly-Ala-Ala-**

↓

**Ala-Val-Xaa-Gln-Met-Pro---------------------Glu-Ile-Val-Ser-**

**-Thr-Phe-Ala-Val-Cys-Gln-Met-Pro-Asn-Leu-Asp-Glu-Ile-Val-Ser-**

↓

**-Asn-Ala-Ala-Tyr**

**-Asn-Ala-Ala-Tyr-Val**

**FIG. 3.** Alignment of chymotryptic peptide fragment sequences and the putative boticin B sequence. The sequences of the 11- and 14-amino-acid chymotryptic fragments isolated by Yu (13) are underlined. The arrows indicate chymotrypsin cleavage sites.

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**FIG. 4.** Expression of the boticin B gene in C. botulinum strain 62A. Culture filtrates were tested for inhibitory activity in well diffusion plates. A, C. botulinum 213B; B, C. botulinum 62A(pMVP1113); C, C. botulinum 62A(pJIR1457). The latter two culture filtrates were concentrated 150-fold by ammonium sulfate precipitation.
213B, were inhibitive to growth of C. botulinum strain 62A (data not shown). Interestingly, culture filtrates of strain 213B were also inhibitive to growth of strain 588 Ab (data not shown), indicating that strain 213B may produce other bacteriocins. Although btcB is located on a plasmid, the absence of btcB in the variety of strains analyzed suggests that the plasmid is not frequently transferred among the clostridia. However, we feel that it would be of interest to examine other clostridial strains that cause intestinal infections, such as strains of Clostridium difficile and Clostridium perfringens, for the presence of btcB.

To our knowledge, these data provide the first genetic evidence of a bacteriocin being produced by a toxigenic strain of proteolytic, group I C. botulinum. Boticin B appears to be unique since it shows no detectable homology to other bacteriocins, including those from clostridial species (4). The production of boticin B could have important practical implications in regard to intestinal and infant botulism. Although it has been suspected that C. botulinum strains that have the capability to colonize the infant and microbially altered adult intestinal tracts produce substances with antibiosis properties against competitors (10), inhibitory compounds other than fermentation end products have not been previously reported. Also, since C. botulinum 213B is commonly used in strain mixture cocktails for challenge studies in foods (3, 11), it is possible that the production of boticin B could inhibit other C. botulinum strains in the mixture, contributing to low toxin production in a permissive food environment. Based on our results, it may be useful to evaluate C. botulinum strains in a food inoculum mixture for antagonistic activity.

**Nucleotide sequence accession number.** The nucleotide sequence presented in this paper was submitted to GenBank and was given the accession number AF278540.

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**REFERENCES**