

Capsule Production in *Bacillus cereus* Strains Associated with Severe Pneumonia

David Sue,^{1†} Alex R. Hoffmaster,¹ Tanja Popovic,² and Patricia P. Wilkins^{1*}

Bacterial Zoonoses Branch, Division of Foodborne, Bacterial and Mycotic Diseases, National Center for Zoonotic, Vector-borne and Enteric Diseases,¹ and Office of the Chief Science Officer,² Centers for Disease Control and Prevention, 1600 Clifton Rd. NE, Atlanta, Georgia 30333

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We identified three encapsulated *Bacillus cereus* strains, isolated from patients with severe pneumonia, in a collection of *B. cereus* isolates associated with human illness. We found that the extent of capsule expression was influenced by culturing conditions. Our findings highlight consequent clinical and laboratory diagnostic challenges posed by such isolates.

Bacillus cereus is a gram-positive, spore-forming, opportunistic pathogen (5, 13). Although it has been implicated in severe respiratory infections (9, 12), the majority of reported illnesses involving *B. cereus* are food-borne intoxications (5, 6, 11). Phylogenetically, *B. cereus* is closely related to both *Bacillus anthracis* and *Bacillus thuringiensis*, and these species are commonly referred to as the *B. cereus* group (1, 3, 10). Classically these species are differentiated by multiple phenotypic tests (2). One recognized phenotype used to distinguish *B. anthracis* from *B. cereus* and *B. thuringiensis* is the presence of a poly- γ -D-glutamic acid capsule in *B. anthracis* (7); *B. cereus* and *B. thuringiensis* typically are not encapsulated (2).

Recently, Hoffmaster et al. reported a unique strain of encapsulated *B. cereus* (G9241) associated with severe pneumonia (9). The encapsulated cells of G9241 did not react with antibodies specific for the *B. anthracis* poly- γ -D-glutamic acid capsule and did not contain the *capBCA* operon, which encodes the enzymes necessary for the production of the *B. anthracis* capsule. However, a plasmid-encoded (pBC218), putative capsule biosynthesis operon was identified in G9241 that was hypothesized to encode genes necessary for capsule biosynthesis in this strain.

To examine the prevalence of encapsulated *B. cereus* strains associated with human disease, *B. cereus* isolates from the CDC's Special Bacteriology Reference Laboratory culture collection were screened using India ink staining to demonstrate the presence of capsule and PCR to detect putative capsule biosynthesis genes, which were identified in G9241 (9). A total of 45 *B. cereus* strains from the culture collection, which were isolated in the United States from 1954 to 2000, were included in this study. This convenient sample represents a spectrum of human illness associated with *B. cereus*: 21 isolates from 21 sporadic severe systemic human infections (atypical, unusual presentations) of the blood or lungs and 24 isolates from cases of gastrointestinal illness or from food remnants associated

with food-borne outbreaks. *B. cereus* strains G9241 and ATCC 14579 were used throughout as positive and negative controls, respectively.

Isolates were grown on Trypticase soy agar plates containing 5% (vol/vol) sheep blood (SBA) (Becton Dickinson Microbiology Systems, Cockeysville, MD) overnight at 37°C in 5% CO₂ and stained using India ink (Remel, Lenexa, KS) (9). Of 45 *B. cereus* isolates tested, only 1 *B. cereus* strain, G9898 (12), expressed capsule (Table 1).

The same 45 isolates were screened by PCR to detect the putative polysaccharide polymerase and translocase genes, hypothesized to be required for capsule biosynthesis in G9241 (9). DNA was isolated using the High Pure PCR template preparation kit (Roche Diagnostics, Indianapolis, IN), with lysozyme (100 mg/ml) to lyse the cells. Forward and reverse primers were designed to amplify 607 nucleotides of the putative polysaccharide polymerase (forward, 5'-TTAATAAAGTGGCCGTTACAT-3'; reverse, 5'-GTACAACAAATCCTGC GAATA-3') and 432 nucleotides of the putative polysaccharide translocase (forward, 5'-AATTTGGGATGTATTTCGATGT-3'; reverse, 5'-ATGAACCTACTTCCGCCAACA-3') genes. PCR amplification was performed using Platinum *Taq* polymerase (Invitrogen, Foster City, CA). Amplicons were analyzed using E-gel agarose (2%) gel electrophoresis (Invitrogen). G9898 was the only *B. cereus* strain that contained the putative capsule biosynthesis genes.

Two additional *B. cereus* strains (03BB87 and 03BB102), identified after our study began (8), were also analyzed. *B. cereus* strains 03BB87 and 03BB102 were isolated from a

TABLE 1. Laboratory and epidemiologic findings associated with reported encapsulated *B. cereus* isolates

Strain	State where isolated	Yr isolated	Occupation of patient	Capsule expression	PCR for pBC218 genes ^a	Reference
G9241	LA	1994	Welder	+	+	9
G9898	LA	1996	Welder	+	+	12
03BB87	TX	2003	Muller	+	+	8
03BB102	TX	2003	Welder	+	–	8

^a PCR was performed to detect the putative capsule biosynthesis genes, polysaccharide polymerase and polysaccharide translocase.

* Corresponding author. Mailing address: Centers for Disease Control and Prevention, 1600 Clifton Road, Mailstop D-11, Atlanta, GA 30333. Phone: (404) 639-3297. Fax: (404) 639-3023. E-mail: pwilkins@cdc.gov.

† Present address: Department of Microbiology and Immunology, Emory University, School of Medicine, Atlanta, GA 30322.

TABLE 2. Capsule production by *B. cereus* strains grown under various culturing conditions

Growth conditions ^a	Growth of <i>B. cereus</i> strain ^b				
	G9898	03BB87	03BB102	G9241 (+ ctrl)	14579 (- ctrl)
SBA plates					
30°C (5% CO ₂)	V	V	-	+	-
30°C (12% CO ₂)	-	-	-	+	-
37°C (5% CO ₂)	V	V	V	+	-
37°C (12% CO ₂)	-	-	+	+	-
Broth media					
30°C (HI broth)	V	-	V	+	-
30°C (HI + H. serum)	+	+	+	+	-
30°C (HI + NaHCO ₃)	+	+	+	+	-
30°C (HI + H. serum + NaHCO ₃)	+	+	+	+	-
37°C (HI broth)	-	-	V	+	-
37°C (HI + H. serum)	-	-	+	+	-
37°C (HI + NaHCO ₃)	-	-	+	+	-
37°C (HI + H. serum + NaHCO ₃)	-	-	+	+	-

^a H. serum, horse serum.

^b Each symbol represents three independent experiments, where each of the three test strains and two control strains was cultured under the designated growth conditions on three separate occasions and then observed by light microscopy at magnification $\times 100$ after staining. +, capsule visualized; -, capsule not visualized; V, capsule visualized on less than 5% of cells observed; + ctrl, positive control; - ctrl, negative control.

welder and a muller, respectively, from two unrelated fatal pneumonia cases in Texas in 2003. A detailed report of these cases and the epidemiologic investigation is in preparation (S. B. Avashia, submitted for publication). Capsule was observed in both strains, using India ink staining (Table 1). When these two *B. cereus* strains were tested by PCR, the putative capsule biosynthesis genes were detected in strain 03BB87 only (Table 1). The failure to detect these capsule genes in 03BB102 may be due to slightly divergent sequences in primer binding regions, or capsule genes unrelated to these may be responsible for the capsule in this isolate.

We performed direct fluorescent-antibody staining for the detection of *B. anthracis*-specific capsule antigens to determine if the capsule of these *B. cereus* isolates contained poly- γ -D-glutamic acid (4). *B. anthracis* Pasteur (ATCC 4229) and *B. cereus* (ATCC 14579) were used as positive and negative controls, respectively, for capsule direct fluorescent-antibody assays. The three encapsulated *B. cereus* strains did not react with the poly- γ -D-glutamic acid capsule antibody following incubation at either 30°C or 37°C.

To determine if culturing conditions affected capsule expression, we evaluated capsule production for the three *B. cereus* strains G9898, 03BB87, and 03BB102 and the positive control,

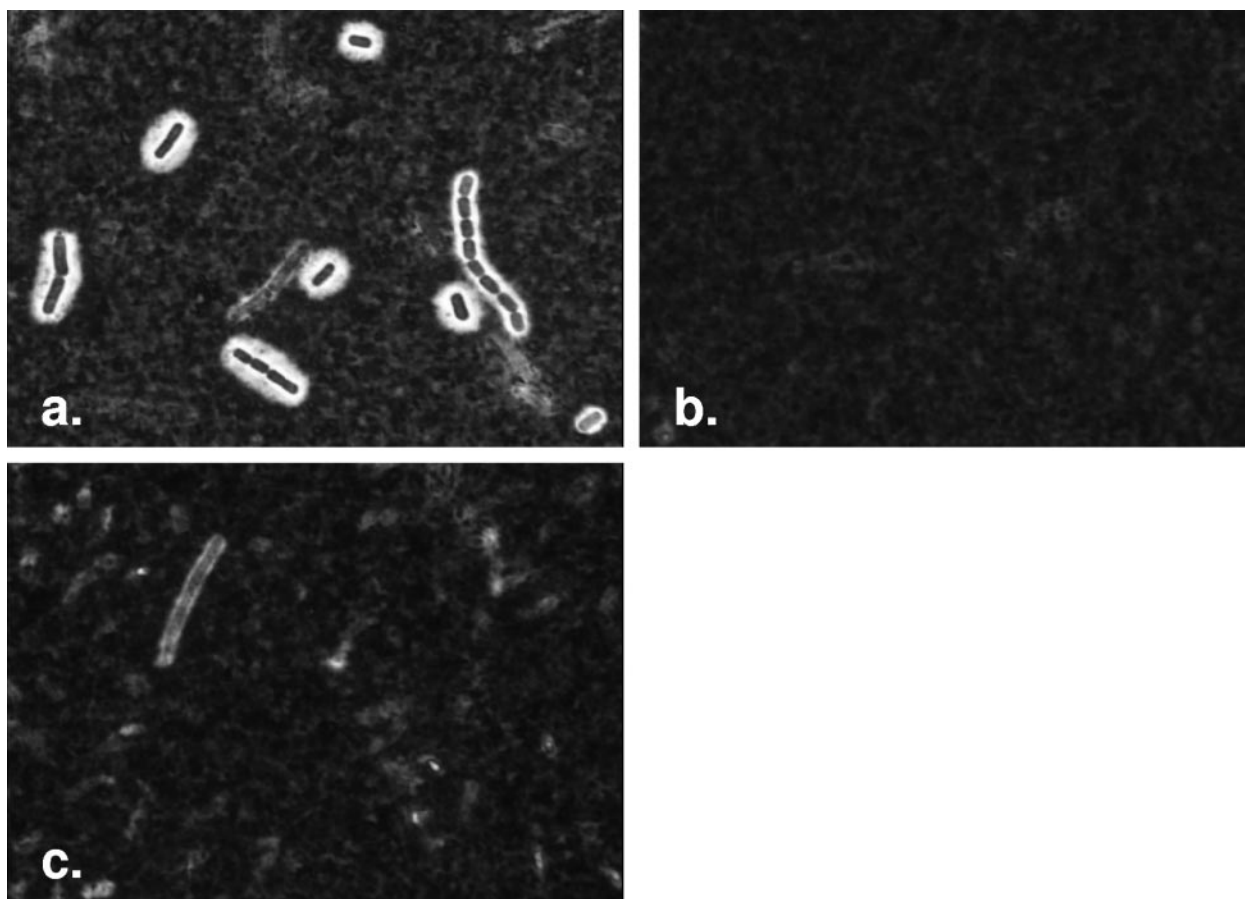


FIG. 1. Culture conditions affecting capsule production in rare, encapsulated *B. cereus* strains. Microscopic images captured (at $\times 100$) of *B. cereus* 03BB87 following incubation (a) at 30°C in HI plus horse serum plus sodium bicarbonate, (b) at 30°C in HI broth, and (c) at 30°C on SBA in 5% CO₂. These images represent the degree of capsule formation observed with India ink staining of cells, noted in Table 1 as +, -, and V, respectively. Each represents three independent experiments, where each of the three test strains and two control strains was cultured under the various growth conditions.

G9241, using India ink staining following growth on SBA plates incubated overnight at 30°C or 37°C in 5% or 12% CO₂ atmosphere (four combinations) or in broth culture. Approximately 10⁷ cells grown on SBA at 30°C in 5% CO₂ were used to inoculate broth cultures, which contained the following: (i) 450 µl of heart infusion (HI) broth alone, (ii) HI broth containing 50% (vol/vol) heat-inactivated horse serum (Sigma, St. Louis, MO), (iii) HI broth containing 0.8% (wt/vol) sodium bicarbonate (ICN Biomedicals, Aurora, MO), or (iv) HI broth containing both 50% heat-inactivated horse serum and 0.8% sodium bicarbonate. Cultures were incubated without shaking at 30°C or 37°C in 5% CO₂ for 3 h, and pelleted cells were stained by India ink (9). Tests were performed in triplicate. The level of capsule production varied for the three strains according to culturing conditions and the media used (Table 2; Fig. 1). After observing variation in capsule expression from these three strains, we retested 10 of the nonencapsulated strains from the original collection in HI plus horse serum and HI plus sodium bicarbonate broths at 30°C and 37°C. None of these strains formed a capsule under these conditions.

Our findings demonstrate that capsule expression in *B. cereus* is rare; we observed capsules in only 3 of the 47 total *B. cereus* isolates we examined. Therefore, the total number of reported encapsulated *B. cereus* strains is four, including G9241. The clinical sources of these four strains are notably similar (9, 12). Each of the encapsulated *B. cereus* strains was implicated in either fatal (three strains) or near-fatal (one strain) pneumonia in individuals characterized as immunocompetent (9, 12). Furthermore, each of these individuals was a metal worker and resided in either Texas or Louisiana (9, 12).

Discovery of these encapsulated *B. cereus* strains shows that the distinctions classically used to define *B. cereus* group organisms may not always be clear-cut, highlighting the danger of relying exclusively upon a single phenotypic trait to characterize an isolate and emphasizing the importance of using multiple tests when identifying isolates associated with human disease.

Our findings demonstrate the challenges for clinical and public health laboratories charged with the identification and differentiation of *B. cereus* and *B. anthracis*. With an increased awareness of the importance of rapid and accurate identification of *B. anthracis* in today's world, the discovery of several capsule-producing *B. cereus* strains, all isolated from patients with severe pneumonia, also emphasizes the need for a better

understanding of the prevalence and risk factors associated with the diseases caused by these organisms.

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