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InhA1, NprA, and HlyII as Candidates for Markers To Differentiate Pathogenic from Nonpathogenic *Bacillus cereus* Strains[▽]

Céline Cadot,[1,2] Seav-Ly Tran,[1] Marie-Léone Vignaud,[2] Marie-Laure De Buyser,[2] Anne-Brit Kolstø,[3] Anne Brisabois,[2] Christophe Nguyen-Thé,[4,5] Didier Lereclus,[1] Marie-Hélène Guinebretière,[4,5]* and Nalini Ramarao[1]*

INRA, Unité MICALIS, GME, La Minière, Guyancourt, France[1]; AFSSA, LERQAP, Unité CEB, Maisons-Alfort, France[2]; University of Oslo, Laboratory for Microbial Dynamics, Oslo, Norway[3]; INRA, Unité SQPOV, Avignon, France[4]; and Université d'Avignon, UMR408, Avignon, France[5]

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Bacillus cereus **is found in food, soil, and plants, and the ability to cause food-borne diseases and opportunistic infection presumably varies among strains. Therefore, measuring harmful toxin production, in addition to the detection of the bacterium itself, may be key for food and hospital safety purposes. All previous studies have focused on the main known virulence factors, cereulide, Hbl, Nhe, and CytK. We examined whether other virulence factors may be specific to pathogenic strains. InhA1, NprA, and HlyII have been described as possibly contributing to *B. cereus* pathogenicity. We report the prevalence and expression profiles of these three new virulence factor genes among 57 *B. cereus* strains isolated from various sources, including isolates associated with gastrointestinal and nongastrointestinal diseases. Using PCR, quantitative reverse transcriptase PCR, and virulence *in vivo* assays, we unraveled these factors as potential markers to differentiate pathogenic from nonpathogenic strains. We show that the *hlyII* gene is carried only by strains with a pathogenic potential and that the expression levels of *inhA1* and *nprA* are higher in the pathogenic than in the nonpathogenic group of strains studied. These data deliver useful information about the pathogenicity of various *B. cereus* strains.**

Food poisoning is a common yet distressing and sometimes life-threatening problem for millions of people throughout the world. *Bacillus cereus* is reported to be the fourth major cause of verified food-borne outbreaks in the European Union (4). However, *B. cereus*-associated outbreaks are likely to be underestimated, as they do not constitute a reportable disease and usually go undiagnosed. If *B. cereus* is suspected, several identification tests can be performed: morphology tests on selective media, resistance to polymyxin B, lecithinase synthesis, hemolytic capacity, mannitol fermentation, and starch hydrolysis (52). These tests do not, however, reveal whether the isolated strains are pathogenic. The capacity of *B. cereus* to sporulate and to form biofilms (31, 39) allows the bacterium to resist the usual cleaning procedures used in the food industry, and *B. cereus* is consequently found in many raw and processed foods, such as rice, spices, milk, vegetables, meat, and various desserts (61). *B. cereus* can cause two types of food-borne illness, emetic and diarrheal syndromes. The emetic type is characterized by vomiting after a short incubation period of 1 to 6 h and is due to cereulide, a peptide preformed in food (15). The emetic symptoms are similar to those caused by *Staphylococcus aureus*. The diarrheal type is characterized by abdominal cramps and watery diarrhea occurring within 8 to 16 h after the ingestion of contaminated foods. The clinical symptoms are similar to those caused by *Clostridium perfringens* (26). In general, both food-borne illnesses are relatively mild, but more-severe cases involving hospitalization or even death have occasionally been reported (46, 47, 61). Interestingly, the spectrum of potential *B. cereus* toxicity ranges from strains used as probiotics for humans (37) to highly toxic food-poisoning strains (46).

Rare but serious opportunistic nongastrointestinal infections have also been attributed to *B. cereus*. The main affections observed are endophthalmitis, periodontitis, meningitis, and encephalitis (5, 8, 13, 14, 22, 42). Moreover, a *B. cereus* strain has caused lethal infections resembling anthrax (36), and several cases of bacteremia in preterm neonates have been described, highlighting this public health problem (35, 49). Infections are characterized by bacterial accumulation despite the induction of inflammation (33, 34). Therefore, *B. cereus* is able to persist and to counteract the host immune system.

B. cereus produces several secreted toxins, the expression of which is controlled by the pleiotropic transcriptional activator PlcR (23, 44). These include the enterotoxins Hbl and Nhe and the cytotoxin CytK. All three compounds are toxic to Vero cells (18, 32, 45, 46), and there is a strong correlation between the concentration of Nhe in the supernatant of a given strain and cytotoxicity (51). The *nhe* gene is present in all of the *B. cereus* strains tested, whereas *hbl* and *cytK* are present in less than 50% of randomly sampled strains (1, 16, 38, 53, 56). CytK was originally isolated from the food-borne-outbreak strain NVH 0391/98, which killed three people (46). Nhe and Hbl protein production is higher in clinical and food-poisoning

* Corresponding author. Mailing address for N. Ramarao: Unité GME, INRA, La Minière, 78285 Guyancourt Cedex, France. Phone: 00 33 1 30 83 36 36. Fax: 00 33 1 30 43 80 97. E-mail: nalini.ramarao@jouy.inra.fr. Mailing address for M.-H. Guinebretière: Unité SQPOV, INRA, UMR 408, Domaine St. Paul, Site Agroparc, F-84914 Avignon Cedex 9, France. Phone: 00 33 4 32 72 25 24. Fax: 00 33 4 32 72 24 92. E-mail: guinebre@avignon.inra.fr.

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strains than in environmental strains (28). However, deletion of *plcR* reduces but does not abolish the virulence of the bacterium in various infection models (cultured cells, insects, mice, and rabbits), suggesting that other factors not regulated by PlcR are involved in specific stages during the infection process (7, 54, 57).

For example, the metalloprotease InhA1 provides *B. cereus* spores with the ability to escape from macrophages and to induce cell mortality (54). InhA1, for which gene expression starts at the onset of stationary phase (25), is secreted and associated with the spore exosporium (9). It has a lethal effect following injection into the insect hemocoel (58) and hydrolyzes cecropin and attacin, two antibacterial proteins found in the hemolymph of insects (12), allowing the bacteria to counteract the host immune system. *Bacillus anthracis* InhA1 shares 96% identity with InhA1 of *B. cereus* and is one of the major proteases isolated in the culture supernatant in minimal medium (10). It digests various substrates, including extracellular matrix proteins, and cleaves tissue components, such as fibronectin, laminin, and collagen (11). This may help the bacteria to cross the host barrier and gain access to deeper tissues (50).

Another metalloprotease, NprA (bacillolysin, also designated NprB or Npr599 in *B. anthracis*), for which regulation is also independent of PlcR, represents 60 to 80% of the *B. anthracis* and *B. cereus* secretome in a sporulation-favorable medium (10; M. Gohar, personal communication). NprA is part of the thermolysin M4 peptidase family, which also contains pseudolysin, the major *Pseudomonas aeruginosa* virulence factor. Very few studies have been performed on NprA, but it has been shown to cleave tissue components, such as fibronectin, laminin, and collagen, and thus displays characteristics related to pathogenic factors (11). These data suggest that InhA1 and NprA play a significant role in the overall pathogenesis of *B. anthracis* and *B. cereus*, by enhancing tissue degradation and by counteracting host defense responses.

Hemolysin II (HlyII) may also play a role during bacterial immune escape. HlyII is, like CytK, a member of the β-barrel pore-forming toxins, such as *S. aureus* alpha-toxin (24) or *C. perfringens* β-toxin (60). HlyII hemolytic activity toward rabbit blood cells is over 15 times more potent than that of *Staphylococcus* alpha-toxin (48). It forms heptameric pores in synthetic lipid bilayers and is hemolytic and cytotoxic to various human cell lines (2, 3). HlyII induces apoptosis in human and mouse macrophages and is strongly implicated in *B. cereus* virulence toward mice and insects (S. Tran, E. Guillemet, C. Clybouw, A. Moris, M. Gohar, D. Lereclus, and N. Ramarao, submitted for publication).

The objective of this study was to identify genetic determinants specific to pathogenic strains. We report the prevalence and expression profiles of three new virulence factor genes, *inhA1*, *nprA*, and *hlyII*, among a panel of strains representative of *B. cereus* diversity and showing different pathogenic profiles. Using PCR and quantitative reverse transcriptase PCR (RT-qPCR), we highlight these factors as potential markers to differentiate pathogenic from nonpathogenic strains.

MATERIALS AND METHODS

Bacterial strains. This study includes 57 *Bacillus cereus* strains isolated from various sources (Table 1). The panel of strains was chosen to be representative of the various pathogenic profiles found among the *B. cereus* group: strains associated with food poisoning (19 isolates associated with diarrheal or emetic outbreaks), strains associated with other clinical diseases (20 isolates from human samples associated with nongastrointestinal infection), and strains not associated with diseases (18 isolates from the environment or from food samples with no collective food-borne-poisoning history). Strains not associated with disease were all low-Nhe producers, as measured by a Tecra test kit (*Bacillus* diarrheal enterotoxin visual immunoassay), and low-Hbl producers, as measured by an Oxoid test kit (BCET-RPLA toxin detection kit) as reported in reference 28. They were presumed to be nonpathogenic. In contrast, strains associated with food poisoning were all high-Nhe producers (15, 17, 28). Strains were selected from the six major *B. cereus* phylogenetic groups (groups II to VII as defined in reference 30) to obtain a strain collection representative of *B. cereus* diversity. Most strains not associated with disease were affiliated with group VI; moreover, there were no strains associated with food-borne diseases or clinical infection from group VI, as previously observed (30).

Detection of the virulence factor genes by PCR. (i) Primer design. For PCR primer design, nucleotide sequences from available databases were aligned to identify regions that were conserved across all strains and regions that differed within paralogous genes. Indeed, *in silico* analysis revealed the presence of up to three zinc metalloproteases that were highly similar to *inhA1* (around 66% identity) in the genomes of various strains (*inhA2*, *inhA3*, and *inhA4*) (41, 27). Several strains possessed the *nprA* paralogue *nprB* (41). Moreover, *inhA1* is characterized by a high degree of sequence polymorphism between the strains; thus, some bases had to be replaced with inosine (which can pair with A, C, or T) or a degenerate sequence had to be used to account for all cases of genetic variability. In some strains, if the targeted gene had not been detected with the first primer pair, a second screen was performed with another primer pair (Table 2).

(ii) DNA extraction and PCR. The genomic DNA from each strain was prepared as previously described (29), with minor modifications. Briefly, bacteria were grown on LB agar plates overnight at 32°C. Bacteria were collected from the plates, resuspended in extraction buffer (1.7% SDS, 200 mM Tris, pH 8, 20 mM EDTA, 200 mM NaCl), and incubated at 55°C for 1 h with proteinase K. DNA was extracted with phenol-chloroform, washed with ethanol, dissolved in sterile MilliQ water, and treated with RNase for 15 min. DNA was quantified by measuring absorbance at 260 nm.

The PCR mixture for gene detection contained 50 to 125 ng DNA, 1 μM primer (forward and reverse), 1.2 U RedGoldStar DNA polymerase (Eurogentec), 200 μM deoxynucleoside triphosphate (dNTP) mix, and 1× buffer supplemented with 2 mM $MgCl_2$ in a final volume of 15 μl. Thermal cycling was carried out in a GeneAmp PCR system 9700 (Applied Biosystems) with the following program: a start cycle of 3 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at the annealing temperature (Table 2), and 1 min at 72°C, and a final extension time of 5 min at 72°C. PCR fragment sizes were revealed on 2% agarose gels.

Detection of the *hlyII* gene by Southern dot blot assay. Strains that were negative for *hlyII* in PCR experiments were subjected to Southern dot blotting. Strains with sequenced genomes were included as positive and negative controls. We generated a biotinylated probe by PCR amplification of the *hlyII* gene using the primer pair F-hlyII-p and R-hlyII-p (Table 2) and the ATCC 14579 strain as template. The reaction mixture contained 0.7 μM primers, 200 μM dNTPs, and 68 μM biotin-11-dUTP (Fermentas), and the amplification was performed with high-fidelity *Taq* DNA polymerase (Roche).

Three micrograms of genomic DNA of each strain to be tested was denatured by heating at 98°C for 6 min, spotted onto a Hybond N+ membrane (Amersham), and fixed under UV light (0.150 J/cm^2). Denatured probe at a concentration of 30 ng/ml was hybridized to membranes for 4 h in Rapid-hyb buffer according to the manufacturer's instructions (Amersham). The membranes were washed, and then genomic DNA-biotinylated probe complexes were revealed with a biotin chromogenic kit (Fermentas) according to the manufacturer's protocol; a deep purple coloration indicated spots in which the *hlyII* gene was present.

Quantification of virulence factor expression by RT-qPCR. The amounts of *inhA1*, *nprA*, and *hlyII* transcripts in each strain were measured by RT-qPCR. The expression levels of the three genes were calculated based on the comparative quantification cycle (*Cq*) method and relative to the mean expression of two housekeeping genes, *rpoA* and *rpoB*, used as endogenous references. The whole experiment (from strain culture to RT-qPCRs) was performed twice for empirical assessment of accuracy and repeatability.

(i) Strain culture and preparation. Bacteria were cultivated overnight in standard LB medium. Fresh LB or sporulation-specific medium (43) was then inoculated with the overnight culture and grown for 105 min, by which time all strains were in exponential growth phase. The synchronized cultures were inoculated at an optical density at 600 nm (OD_{600}) of ~0.02 in LB or in sporulation medium in identical test tubes and incubated in a 32°C water bath with agitation.

TABLE 1. Strain table and detection of *inhA1*, *nprA*, and *hlyII* genes by PCR and Southern blotting

Pathogenic profile and genetic group	Strain	Source	Reference or source[a]	Detection of:		
				inhA1	*nprA*	*hlyII*
Nonpathogenic						
II	INRA BC′	Vegetable	28	+	+	−
III	INRA PF	Milk protein	28, 30	+	+	−
IV	INRA A3	Starch	28, 30, 16	+	+	−
VI	INRA 1	Pasteurized purée	28, 30, 16	+	+	−
	INRA 5	Pasteurized purée	28, 30	+	+	−
	INRA BK	Vegetable	28	+	+	−
	INRA BL	Vegetable	28	+	+	−
	INRA C1	Pasteurized vegetables	28, 30, 16	+	+	−
	INRA C46	Pasteurized vegetables	28	+	+	−
	INRA C64	Pasteurized vegetables	28	+	+	−
	INRA C74	Pasteurized vegetables	28, 30	+	+	−
	ADRIA I3	Cooked foods	28, 30	+	+	−
	ADRIA I20	Cooked foods	28, 30, 16	+	+	−
	ADRIA I21	Cooked foods	28, 30, 16	+	+	−
	INRA SL′	Soil	28, 30	+	+	−
	INRA SO	Soil	28, 30	+	+	−
	INRA SV	Soil	28, 30	+	+	−
	WSBC 10204	Pasteurized milk	30, 16	+	+	−
Food poisoning						
II	NVH 0861/00	Diarrheal outbreak	28, 30, 16	+	+	−
III	NVH 0500/00	Diarrheal outbreak	28, 30, 16	+	+	+
	NVH 0075/95	Diarrheal outbreak	30, 16	+	+	−
	06 ceb 04 bac	Emetic outbreak	This study	+	+	−
	NVH 1519/00	Diarrheal outbreak	30, 16	+	+	−
	198R 2003	Emetic outbreak	This study	+	+	−
	F3371/93	Diarrheal outbreak	28, 30, 16	+	+	+
	F4433/73	Diarrheal outbreak	28, 30, 16	+	+	−
	F4810/72	Emetic outbreak	30, 16	+	+	−
	LMG 17615 (F289/78)	Diarrheal outbreak	28, 16	+	+	+
	NC 7401	Emetic outbreak	30, 16	+	+	−
	NVH 200	Diarrheal outbreak	30, 16	+	+	−
IV	NVH 0230/00	Diarrheal outbreak	28, 30, 16	+	+	−
	NVH 1230/88	Diarrheal outbreak	28, 30, 16	+	+	+
	98HMPL63	Diarrheal outbreak	28, 30, 16	+	+	−
	F2081A/98	Diarrheal outbreak	28, 30, 16	+	+	+
	F352/90	Diarrheal outbreak	28, 30, 16	+	+	+
	F4430/73	Diarrheal outbreak	28, 30, 16	+	+	−
VII	NVH 0391/98	Diarrheal outbreak	28, 30, 46, 16	+	+	−
Clinical, nongastrointestinal infection						
II	AH1125	Human	§	+	+	+
III	DSM 4222 (F837/76)	Human, postoperative infection	28, 30, 16	+	+	+
	AH728	Human, urine	§	+	+	−
	AH825	Human, periodontitis	§	+	+	−
	AH891	Human, blood	§	+	+	−
	AH893	Human, wound	§	+	+	−
	AH1127	Human, eye	§	+	+	−
	AH1131	Human, eye	§	+	+	+
	B06_007	Human, cutaneous	§	+	+	−
	B06_018	Human, peritoneal	§	+	+	−
IV	AH716	Human, pus	§	+	+	−
	AH726	Human, urine	§	+	+	−
	AH815	Human, periodontitis	§	+	+	+
	AH1129	Human, eye	§	+	+	−
	AH1293	Human, blood	§	+	+	+
	B06_019	Human, wound	§	+	+	−
	B06_034	Human	§	+	+	−
	B06_036	Human, blood	§	+	+	−
V	AH1308	Human, feces	§	+	+	−
	B06_015	Human, drainage tube	§	+	+	−

[a] §, University of Oslo's *Bacillus cereus* group multilocus sequence typing Website (http://mlstoslo.uio.no).

TABLE 2. Characteristics of primers used in this study

Primer purpose and target gene (locus tag[a])	Primer[b]	Primer sequence[c] (5′–3′)	Location within gene[a]	Annealing temp (°C)	Product size (bp)	Reference or source
PCR for *inhA1*, *nprA*, and *hlyII* detection						
inhA1 (BC1284)	F-inhA1-d1	ACGCITTIAAATTTGCICG	1937–1955	55	257	This study
	R-inhA1-d1	ACGCGTTGGAGATACAACTT	2193–2174			
	F-inhA1-d2	CCAGCTTGGAAAGTTGTATC	2164–2183	55	166	This study
	R-inhA1-d2	CAGCTTGTCCTACTACTTCA	2329–2310			
nprA (BC0602)	F-nprA-d	GTATACGGAGATGGTGATGG	1111–1130	55	263	This study
	R-nprA-d	GGATCACTCATAGAGCGAAG	1373–1354			
hlyII (BC3523)	Fhly-II	GATTCTAAAGGAACTGTAG	94–112	48	868	19
	Rhly-II	GGTTATCAAGAGTAACTTG	961–943			
	F-hlyII-d	CAAGTTACTCTTGATAACC	943–961	48	194	This study
	R-hlyII-dq	TCACCATTTACAAAGATACC	1136–1117			
PCR amplification of *hlyII* probe						
hlyII (BC3523)	F-hlyII-p	GCATTTGCAGATTCTAAAGG	85–104	55	1,019	This study
	R-hlyII-p	TCGTAACTGATACCATAACC	1103–1084			
RT-qPCR						
inhA1 (BC1284)	F-inhA1-q	GATAAAAC(A/G)CCAGCTTGGAAAG	2155–2176	60	189	This study
	R-inhA1-q	TGCAGA(A/T)TTATCATCAGCTTG	2343–2323			
nprA (BC0602)	F-nprA-q	TGCAGCAGCAGTAGATGCTC	951–970	60	188	This study
	R-nprA-q	ATGTTACACCATCACCATC	1138–1120			
hlyII (BC3523)	F-hlyII-q	CTGGAAAAACCATCAAGTTACTC	930–952	60	207	This study
	R-hlyII-dq	TCACCATTTACAAAGATACC	1136–1117			
rpoA (BC0158)	F-rpoA-q	TAACTCCTTACGTCGTATTC	111–130	60	186	This study
	R-rpoA-q	ATTTCCAACGTCTTCTCTTC	296–277			
rpoB (BC0122)	F-rpoB-q	TCAGTGGTTTCTTGATGAGG	111–130	60	176	This study
	R-rpoB-q	CTTTTACACGAAGTGGTGCT	286–267			

[a] Reference strain *Bacillus cereus* ATCC 14579 (NCBI accession no. NC_004722).
[b] F, forward primer; R, reverse primer.
[c] I, inosine.

After exactly 6 h of incubation, a 1-ml sample was taken from each culture and centrifuged at 12,000 × *g* for 3 min at 4°C, and the bacterial pellet was placed immediately at −80°C until processing.

(ii) RNA extraction. *B. cereus* cells, thawed in 500 μl of TRI reagent (Ambion), were disrupted by bead beating (FastPrep; MP Biomedicals) with about 350 mg of 0.1-mm-diameter silica beads (lysing matrix B; MP Biomedicals). RNA was separated from proteins by adding chloroform and was purified with an RNeasy kit (Qiagen) according to the manufacturer's instructions. Remaining traces of DNA were eliminated by two rounds of DNase treatment (TURBO DNA-free kit; Ambion). RT-qPCR controls without reverse transcriptase showed that no genomic DNA was detectable in the RNA samples. The concentration of RNA was assessed by spectrophotometry (NanoDrop; Thermo Scientific). Between 10 and 30 μg of RNA were recovered from a 1-ml bacterial culture.

(iii) RNA purity and quality and determination of the RIN. The purity and integrity of RNA are critical elements for the overall success of RNA-based analyses. We measured the ratio of absorbance at 260 and 280 nm. All RNA samples had an OD_{260}/OD_{280} ratio of ≥1.95, which is considered a good indicator of RNA purity (40). Moreover, RNA integrity was further examined by measuring the RNA integrity number (RIN) using a user-independent strategy. The RIN algorithm allows the calculation of RNA integrity using a trained artificial neural network based on the determination of various features. These variables include the total RNA ratio (ratio of the area of ribosomal bands to the total area of the electropherogram) and the height of the 16S and 23S peaks (40). The RIN was calculated for a representative set of strains and was between 7 and 9.6. RIN values above 5 indicated RNA samples that were of very good quality (21).

(iv) Primer design. The primers used for RT-qPCR experiments were designed to amplify specific gene fragments of 170 to 210 bp (Table 2). The efficiency of the primer pairs was determined experimentally on one representative strain of each genetic group by measuring the *Cq* of each RNA sample over a 4-log RNA dilution (6). Primer pairs with an efficiency of ≤85% were eliminated. In the case of *inhA1*, several primer pairs were tested but none of the pairs could be validated for all strains. The primers chosen, F-inhA1-q and R-inhA1-q, were validated for the genetic groups II, III, IV, and VI (efficiency of >85%) but were not suitable to quantify *inhA1* expression in strains belonging to genetic groups V and VII (3 strains out of 57).

Primer specificity was validated by sequencing the amplification product obtained for the target and reference genes of one representative strain in each genetic group (data not shown).

(v) RT-qPCR experiments and analysis. We used the mean expression values of two reference genes to normalize our data, as suggested in several publications (6, 63). Thereafter, target gene amounts measured by RT-qPCR were normalized against the amounts of *rpoA* and *rpoB* genes, for which the difference in the mean *Cq* between strains from the three pathogenic profiles was <0.08.

Target and reference gene mRNA abundance was measured by one-step RT-qPCR with a QuantiFast SYBR green RT-PCR kit (Qiagen). RT-qPCRs and cycling were set up as recommended by the manufacturer. RT-qPCR samples contained 1 ng of RNA and 1 μM each primer in a final volume of 25 μl and were run in a LightCycler 480 (Roche) in 96-well plates. Expression levels of the target genes relative to the endogenous standard were calculated using the basic relative quantification method (Roche) and the LightCycler 480 software. All RT-qPCRs were performed in duplicate and repeated if the *Cq* difference between both duplicates exceeded 0.5. For each strain and each gene, the mean expression value of the two experiments carried out in duplicate was calculated and spotted as a single value. All plates also contained a positive control (control RNA sample loaded on each plate) to detect interrun variations.

The specificity of each amplification reaction was verified with the melting curve profile (55): a single peak was obtained, attesting to the amplification of a unique product. For each gene, a control with no RNA template (no-template control) was included in all plates to check for the absence of reagent contamination or small amounts of primer dimeric forms.

Insects and *in vivo* experiments. Infection by injection into the hemolymph of *Galleria mellonella* larvae was performed as described previously (20, 27, 57). Ten-microliter suspensions containing 10^4 *B. cereus* vegetative bacteria were injected onto the base of the last proleg of groups of 20 last-instar larvae. All tests were run twice. Insect mortality was recorded after a 24-h incubation time at 32°C.

Cell culture and toxicity. The cytotoxicity of *B. cereus* cell-free supernatant, collected after 6 h of culture in LB medium, to J774 murine macrophage-like cells was assessed as previously described (54).

Statistics. For statistical analysis of RT-qPCR results, we determined the Spearman's rank correlation coefficient and carried out the Mann-Whitney test using Statext software (www.statext.com).

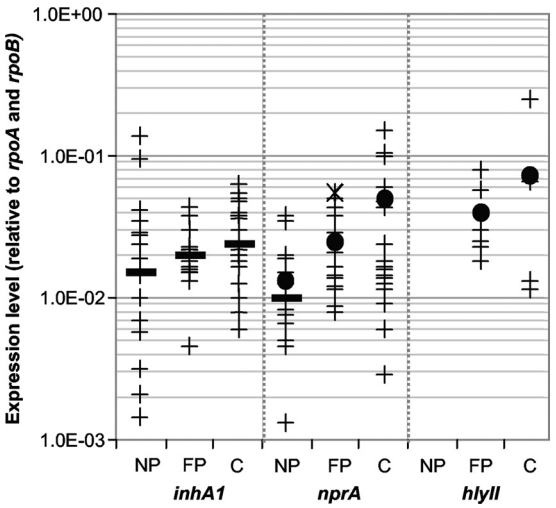

FIG. 1. Expression profiles of *inhA1*, *nprA*, and *hlyII* among *B. cereus* strains. Logarithmic representation of the expression levels of *inhA1*, *nprA*, and *hlyII* among 57 (54 for *inhA1*) *B. cereus* nonpathogenic (NP), food-poisoning (FP), and clinical (C) strains normalized to the mean expression value of the two reference genes *rpoA* and *rpoB*. For each strain, the mean value of two independent experiments done in duplicate is represented as a cross. For each pathogenic profile, the mean value is represented as a dot and the median value as a bold line. The × symbol represents the *nprA* expression level of the NVH 0391/98 strain.

RESULTS

Prevalence of *inhA1*, *nprA*, and *hlyII* genes among pathogenic and nonpathogenic *B. cereus* strains. The prevalence of the three virulence factor genes *inhA1*, *nprA*, and *hlyII* among our panel of 57 *B. cereus* isolates was investigated by PCR (Table 1). With *inhA1*- and *nprA*-targeting primers, a PCR product of the expected size was amplified from all strains, indicating that the *inhA1* and *nprA* genes are present in all strains tested. In the case of *hlyII*, amplification was observed for 11 of the 57 strains studied. The absence of *hlyII* from all other isolates was confirmed by Southern blotting (not shown).

Thus, *inhA1* and *nprA* genes were carried by all strains studied, whereas *hlyII* was found in only 19% of them (Table 1). Interestingly, the distribution of *hlyII* was unequal among the three groups: none of the strains affiliated to the nonpathogenic group carried the *hlyII* gene, whereas it was present in 6/19 food-poisoning and in 5/20 clinical strains (Table 1). Thus, *hlyII* was not found in nonpathogenic strains and its prevalence among pathogenic strains, isolated from food-poisoning outbreaks or clinical infections, was around 30%.

Quantification of *inhA1*, *nprA*, and *hlyII* expression in *B. cereus* strains. It has often been suggested that the expression level rather than the mere presence of genes encoding virulence factors accounts for most *B. cereus* pathogenic potential (28, 51). This study sought, therefore, to measure and compare the expression profiles of *inhA1*, *nprA*, and *hlyII* in pathogenic and nonpathogenic *B. cereus* isolates (Fig. 1). We calculated the mean expression value of two independent experiments, done in duplicate, for each strain and for each gene. Moreover, we also calculated the mean and median gene expression values for each pathogenic group.

(i) *nprA* expression. There is a clear difference in *nprA* expression profiles between nonpathogenic and pathogenic strains (food poisoning and clinical) ($P < 0.002$). The mean expression values were 2- and 3-fold greater for food-borne and clinical infection strains, respectively, than for nonpathogenic strains. The median *nprA* expression value (middle value of the distribution, for which 50% of the strains have superior values and 50% of the strains have inferior values) also provided an interesting indicator. It was used to evaluate the number of pathogenic strains with high *nprA* expression levels (high NprA producers were defined as strains for which the expression value is superior to the nonpathogenic strain median value). In both pathogenic groups, from food-borne and clinical infections, over 85% of the strains were high-NprA producers. Interestingly, strain NVH 0391/98, which was isolated from a fatal food-poisoning outbreak, had the highest *nprA* expression level among strains associated with diarrhea (× symbol in Fig. 1).

The results were similar for strains cultured in sporulation-specific medium: 100% of food-borne and 80% of clinical infection-associated strains were high-NprA producers (not shown). In this medium, on average, the expression values were 5- to 14-fold higher than in LB medium, suggesting that the *nprA* gene was expressed better in a relatively poor, sporulation-specific medium, in which NprA is the major secreted protein at this physiological stage (10).

(ii) *inhA1* expression. In the case of *inhA1* expression, the mean value for nonpathogenic strains was not statistically different from the mean value for pathogenic strains. However, for this gene, the expression profiles of nonpathogenic strains varied significantly from strain to strain and there were up to 100-fold differences in transcript abundance between isolates. Thereafter, the median value rather than the mean value of each group was considered. Seventy percent of both food-borne and clinical pathogenic strains were high-InhA1 producers, i.e., had expression values superior to the nonpathogenic strain median value.

(iii) *hlyII* expression. Nonpathogenic strains do not carry the *hlyII* gene; thus, we could not compare mean or median expression values between pathogenic and nonpathogenic strains. However, the mean *hlyII* expression value for clinical strains was 2-fold higher than the mean value for strains associated with food poisoning, as observed for *nprA* expression.

(iv) Relative *inhA1*, *nprA*, and *hlyII* expression. In all the strains carrying them, the *inhA1*, *nprA*, and *hlyII* genes were expressed, although not at the same level, depending on the strain. The expression levels of the two metalloproteases genes *inhA1* and *nprA* in LB medium appeared to be closely related in all strains (Spearman's rank correlation coefficient, $P < 0.001$). Indeed, if for each strain the *inhA1* expression value (x axis) was plotted against the *nprA* expression value (y axis), the average ratio of expression of *inhA1* to *nprA* was 1 (Fig. 2), and almost all the strains had values that were close to the regression line. Thereafter, for a given strain, the expression profiles for *inhA1* and *nprA* were similar and a strain was either a high or a low producer of both metalloproteases.

We observed a similar trend among strains with *hlyII* expression (Fig. 2), even though the correlation coefficient calculated with this third gene was slightly weaker ($P < 0.05$) due to the limited available data, as only 11 isolates were found to be *hlyII*

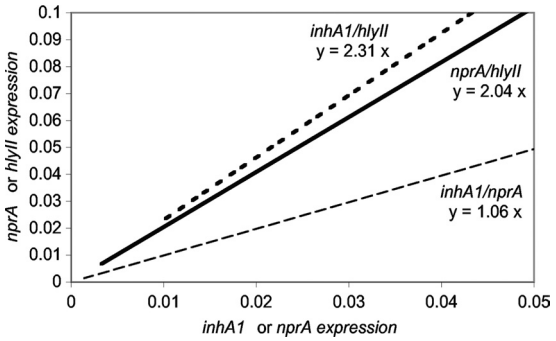

FIG. 2. Relative expression profiles. For each strain, the *inhA1* (*x* axis) expression value was plotted against the *nprA* (*y* axis, gray dashed line) or the *hlyII* (dark dashed line) expression value. Alternatively, *nprA* expression values (*x* axis) were plotted against the *hlyII* values (*y* axis, plain line). The regression line was drawn, and the average ratio between expression values was calculated.

positive. For these strains, the ratio of *hlyII* to *inhA1* was 2.3 and the ratio of *hlyII* to *nprA* was 2. This suggests that when a strain expresses one of these genes to a high level, first, it also has high expression of the other two genes, and second, it expresses twice as much *hlyII* as *inhA1* and *nprA*.

Virulence of nonpathogenic and pathogenic strains in *G. mellonella* insects. Nine strains, belonging to the three pathogenic groups and with various *inhA1*, *nprA*, and *hlyII* expression profiles, were tested for their virulence by injection into the hemocoel of *G. mellonella* larvae (Fig. 3A). Nonpathogenic strains were all avirulent (less than 25% mortality). In contrast to nonpathogenic strains, clinical pathogenic strains and those associated with food poisoning were all virulent (inducing 55% to 98% mortality).

All strains containing *hlyII* (F2081A/98, AH1293, and AH1131) were strongly virulent to insects, suggesting that HlyII may be a good indicator of pathogenicity. However, several *hlyII*-negative strains were still virulent (3/6 in the insect infection test and 70 to 75% of the food poisoning and clinical isolates), indicating that the induction of diseases is probably multifactorial.

We observed good correlation between the expression levels of *inhA1* (Fig. 3B) and *nprA* (Fig. 3C), and virulence in *G. mellonella* insects for eight of the nine strains tested. Thus, high-InhA1 and -NprA producers are likely to be highly pathogenic to *G. mellonella* insects, as also observed for the two food-poisoning and clinical pathogenic strain profiles. We consistently found the two most virulent strains, F2081A/98 and AH1293 (mortality of >77%), to be high-NprA, -InhA1, and -HlyII producers. Moreover, five of the six virulent strains carried the *hlyII* gene and/or were high-NprA producers. Thus, it appears that InhA1, NprA, and HlyII confer an advantage on the strains expressing them to establish an infection.

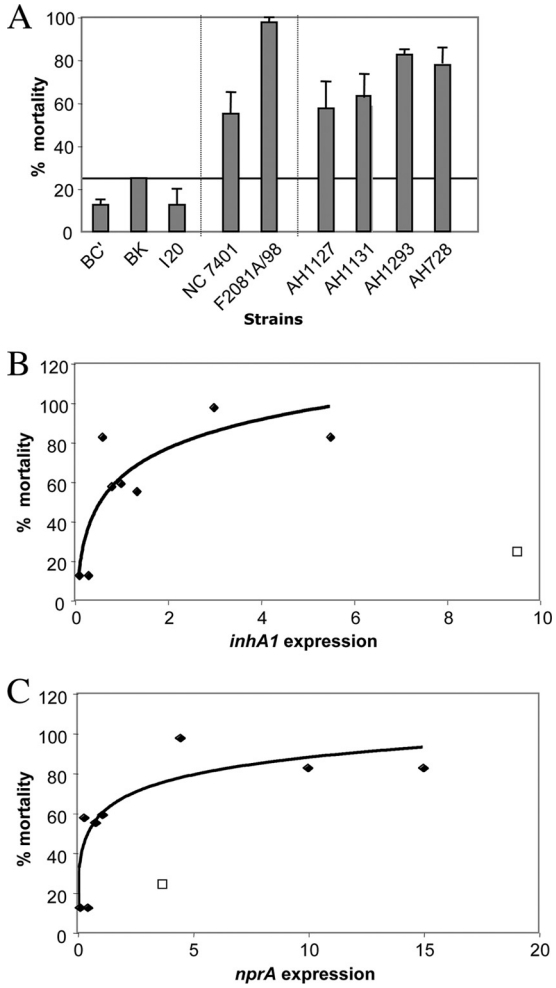

FIG. 3. Virulence of strains to *G. mellonella* insects and expression correlations. Nine strains were tested for their virulence toward *G. mellonella* insects by injection into the insect larvae hemocoel. (A) Mortality was recorded 24 h postinfection. Error bars show standard deviations of the means of the results from two independent experiments. (B and C) A logarithmic regression line represents the correlation between levels of *inhA1* (B) or *nprA* (C) expression of these strains and virulence. The square represents the BK strain, for which data were not included in the regression line calculation. Black diamonds represent all of the other strains that were tested for virulence.

DISCUSSION

Altogether, we show that the *hlyII* gene is present in only 19% of the strains. This is consistent with a study by Fagerlund et al. (19) in which *hlyII* was detected in 6 of the 29 (21%) *B. cereus* strains tested. We further show that *hlyII* is found only in pathogenic strains isolated from food-poisoning outbreaks or clinical infections. This is the first example of a *B. cereus* gene that is clearly present only in pathogenic strains. This sheds further light on the important role of hemolysin II and highlights that the presence of the *hlyII* gene may be a good indicator of pathogenicity. However, as only 30% of pathogenic strains carry *hlyII*, the absence of *hlyII* cannot be interpreted as absence of pathogenicity.

In addition, we show that the two genes *inhA1* and *nprA*, encoding two zinc metalloproteases implicated in *B. anthracis* and *B. cereus* pathogenesis, are carried by all strains studied but are expressed at a higher level in pathogenic strains, especially in strains associated with clinical nongastrointestinal disease.

It appears that *B. cereus* may use the three virulence factors InhA1, NprA, and HlyII (when present) simultaneously rather

than separately. Indeed, we observed correlations between the expression levels of the three factors, first, with one another, and second, with virulence to insects, suggesting that *B. cereus* likely uses these factors concomitantly during pathogenicity. Although it is not possible to discriminate pathogenic from nonpathogenic strains based on their *inhA1* or *nprA* expression levels alone, as the expression profiles of the groups partially overlap, on average, pathogenic strains express *inhA1*, *nprA*, and *hlyII* to higher levels than nonpathogenic strains. Thus, we speculate that the expression of these genes may confer an advantage on the strains carrying them for their ability to induce infection.

The expression levels of these three genes had never been studied before, as all the previous works have focused mainly on the main known virulence factors, Hbl, Nhe, CytK, and cereulide (1, 16, 38, 53, 56). *B. cereus* causes many different pathologies, and the importance of each virulence factor may vary accordingly. For instance, the diarrheal disease is attributed to enterotoxins because *B. cereus* culture filtrates cause fluid accumulation in rabbit ileal loop (59). The toxins may elicit diarrhea by inducing membrane permeability of epithelial cells in the small intestine. Hbl, Nhe, and CytK are all toxic to Vero cells and are considered good diarrheal toxin candidates. InhA1, NprA, and HlyII may play a role in addition to the enterotoxins. InhA1 and NprA are proteases cleaving various membrane components (11), and HlyII has cytotoxic and hemolytic activities (2). These properties may allow the bacteria to gain access to deeper tissues and to disseminate.

In the case of nongastrointestinal clinical infections, several strains express *inhA1*, *nprA*, and *hlyII* at very high levels. Among these, the strains isolated from human blood (AH1293, AH891, B06_036, and B06_019) express *inhA1* and *nprA* to a higher level than strains isolated from the eye (AH1127 and AH1131). These strains induce nongastrointestinal diseases and may therefore enter the blood system quickly after infection. To persist inside the host, these strains will have to survive the bactericidal action of phagocytes. Phagocytes, such as macrophages, are essential effectors of the immune response against infective microorganisms, as they engulf pathogens and activate the late immune response. InhA1, NprA, and HlyII are likely to be involved in the bacterial capacity to counteract the host immune system. In particular, InhA1 allows the bacteria to escape from macrophages (54), HlyII induces macrophage mortality (Tran et al., submitted), and InhA1 and NprA cleave various host cell components (11). These properties may therefore confer an advantage on strains expressing these genes, in their capacity to induce severe clinical diseases.

We hope that our findings on InhA1, NprA, and HlyII and the RT-qPCR methods allowing quantification of the expression of toxin genes will improve knowledge of *B. cereus* factors implicated in diseases for better clinical diagnosis and food safety.

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