**Bacillus cytotoxicus** sp. nov. is a novel thermotolerant species of the *Bacillus cereus* Group occasionally associated with food poisoning

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An aerobic endospore-forming bacillus (NVH 391-98T) was isolated during a severe food poisoning outbreak in France in 1998, and four other similar strains have since been isolated, also mostly from food poisoning cases. Based on 16S rRNA gene sequence similarity, these strains were shown to belong to the *Bacillus cereus* Group (over 97% similarity with the current Group species) and phylogenetic distance from other validly described species of the genus *Bacillus* was less than 95%. Based on 16S rRNA gene sequence similarity and MLST data, these novel strains were shown to form a robust and well-separated cluster in the *B. cereus* Group, and constituted the most distant cluster from species of this Group. Major fatty acids (iso-C₁₅:₀, C₁₆:₀, iso-C₁₇:₀, anteiso-C₁₅:₀, iso-C₁₆:₁, iso-C₁₃:₀) supported the affiliation of these strains to the genus *Bacillus*, and more specifically to the *B. cereus* Group. NVH 391-98T taxon was more specifically characterized by an abundance of iso-C₁₅:₀ and low amounts of iso-C₁₃:₀ compared with other members of the *B. cereus* Group. Genome similarity together with DNA–DNA hybridization values and physiological and biochemical tests made it possible to genotypically and phenotypically differentiate NVH 391-98T taxon from the six current *B. cereus* Group species. NVH 391-98T therefore represents a novel species, for which the name *Bacillus cytotoxicus* sp. nov. is proposed, with the type strain NVH 391-98T (=DSM 22905T=CIP 110041T).

Abbreviations: DDH, DNA–DNA hybridization; MLST, multilocus sequence typing.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene of strains are described in Tables 1 and S1. The sequences of the MLST genes can be found at http://mlstoslo.uio.no/index.html.

A supplementary figure and three supplementary tables are available with the online version of this paper.
INTRODUCTION

Bacillus species comprise psychrophilic to thermophilic organisms, which allows them to colonize a wide range of environments. Like other species of Bacillus, the Bacillus cereus Group species are metabolically diverse which enables them to develop in various environmental conditions. This phylogenetically distinct group at present comprises six closely related species B. pseudomycoides (Nakamura, 1998), B. mycoides (Lechner et al., 1998; Nakamura, 1998; Nakamura & Jackson, 1995), B. weihenstephanensis (Lechner et al., 1998), B. cereus (Smith et al., 1952; Somerville & Jones, 1972), B. thuringiensis (Nakamura, 1994; Smith et al., 1952; Somerville & Jones, 1972) and B. anthracis (Logan et al., 1985; Smith et al., 1952; Somerville & Jones, 1972). Adaptation to the environment has played a major role in shaping the evolution of these organisms, triggering a shift in growth temperature range for some species and emergence of specific ecotypes (Guinebretière et al., 2008). For example, B. mycoides and B. weihenstephanensis mainly derived from the natural environment (soils, rivers and plants) are preferentially associated with cold thermal niches compared with other species (Guinebretière et al., 2008; Lechner et al., 1998). The six species are generally recognized as free-living soil organisms but some of them are also potential pathogens of mammals and insects. Due to their broad ability to adapt, B. cereus Group strains are readily recovered in foods, leading to food poisoning when conditions are favourable. It was during a food poisoning outbreak in France in 1998 that the singular strain NVH 391-98T was first isolated and initially allocated to B. cereus. This event, resulting in three fatalities, represented the most severe known case of diarrhoeal food poisoning in France caused by a presumed B. cereus Group strain. The strain was intensively studied, and an important diarrhoeic enterotoxin, called cytotoxin K, was identified (Lund et al., 2000) and further characterized (Brillard & Lereclus, 2004; Fagerlund et al., 2004). Since then, four similar isolates have been reported, three of which were also linked to food poisoning outbreaks. Two such cases were detected in France and one in Germany. The strains were isolated from vegetable purees and semolina incriminated in the food poisoning cases. Together, they displayed phenotypic and genetic differences to other B. cereus Group strains (Afchain et al., 2008; Auger et al., 2008; Fagerlund et al., 2007; Rau et al., 2009) and a particular thermotolerant ecotype known as phylogenetic group VII (Guinebretière et al., 2008). Here, it is shown that these strains are closely related and represent a novel species of the B. cereus Group, for which the name Bacillus cytotoxicus sp. nov. is proposed.

METHODS

Strains and DNA sequences. NVH 391-98T (=DSM 22905T =CIP 110041T) and close relative strains are listed in Table 1. Type strains used in this work (B. pseudomycoides DSM 12442T, B. mycoides CIP 103472T, B. weihenstephanensis WSBC 10204T, B. cereus ATCC 14579T, B. thuringiensis CIP 53.137T and B. subtilis CIP 52.65T) were all obtained from international collections, except strain WSBC 10204T which was provided by the University of Munich. All strains were maintained at −80 °C in micro-tubes containing 40 % glycerol and all cultivated well on trypticase soy agar (30 g trypticase soy broth and 15 g bacto agar; pH 8.0) and Luria agar (25 g Luria–Bertani broth and 15 g Bacto agar; pH 7.0) at 30–37 °C.

Genomic sequences were obtained from Integrated Microbial Genomes (IMG) (Markowitz et al., 2010) for all type strains (finished or draft sequences), except for the B. weihenstephanensis type strain whose sequence was substituted for a genomic sequence of closely related strain B. weihenstephanensis KBAB4. They included complete genomic sequence of NVH 391-98T, B. cereus ATCC 14579T, B. thuringiensis ATCC 10792T, B. anthracis A0488T, B. anthracis Ames, B. mycoides DSM 2048T, B. pseudomycoides DSM 12442T, B. weihenstephanensis KBAB4, B. subtilis NCIB 3610T.

The entire 16S rRNA gene sequences from type strains of the genus Bacillus were obtained from NCBI databases (refseq_rna and refseq_genomic) when available, otherwise from the IMG genome database from which a consensus sequence from all 16 rRNA gene copies was generated and used for analysis. For the recent isolates 08CEB44BAC and CVUAS2833, 16S rRNA genes were amplified by PCR and sequenced as previously described (Guinebretière et al., 2008).

The concatenated sequences integrated into MLST analysis were those related to food poisoning Bacillus strains (Afchain et al., 2006) and were obtained from http://mlstoslo.uio.no/index.html.

Table 1. Bacillus cytotoxicus strains included in this work

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin and year of isolation</th>
<th>Other code/name</th>
<th>Initial references</th>
<th>16S rRNA GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>INRA AF2</td>
<td>Potato purée, France, 2003, related to food poisoning</td>
<td>B. cereus INRA AF2</td>
<td>Guinebretière et al. (2006), Fagerlund et al. (2007)</td>
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<tr>
<td>NVH 883/00</td>
<td>Spices, Norway, 2000</td>
<td>B. cereus NVH883/00</td>
<td>Guinebretière et al. (2006), Fagerlund et al. (2007)</td>
<td>AM747233</td>
</tr>
<tr>
<td>AFSSA 08CEB44 BAC</td>
<td>Cooked Semolina, France, 2008, related to food poisoning</td>
<td></td>
<td>This paper</td>
<td>JN790693</td>
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<tr>
<td>CVUAS2833</td>
<td>Potato purée, Germany, 2007, related to food poisoning</td>
<td></td>
<td>Rau et al. (2009)</td>
<td>JN790694</td>
</tr>
</tbody>
</table>
For the recent isolates 08CEB44BAC and CVUAS2833, sequences were obtained as previously described (Tourasse et al., 2006).

**Phylogenetic analysis.** BLASTN software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to find the most closely related sequences from the NCBI's RNA database (Refseq_rna). A preliminary broad 16S rRNA gene phylogeny including 103 species was generated (as described in the following paragraph but running approximate likelihood ratio tests instead of bootstraps for faster processing) and used to select 29 representative strains from the major robust clusters (accession numbers can be found in Table S1, available in IJSEM Online). The five *B. cytotoxicus* sequences (accession number found in Table 1), four *B. cereus* Group sequences (*B. weihenstephanensis* AM747230, *B. mycoides* AM747229, *B. cereus* AF176322, *B. pseudomykoides* AM747226), and consensus sequences from *B. weihenstephanensis* KBAB4, *B. anthuras* A0488 and *B. anthuras* Ames genotypes were added to the 29 representatives to the final phylogeny.

The 16S rRNA gene sequences were aligned using MUSCLE (Edgar, 2004). Phylogeny was reconstructed using PhyML 3.0 (Guindon et al., 2010). Maximum-likelihood searches were run under the GTR (generalized time reversible) evolutionary model estimating all the GTR model parameters and starting from a tree obtained using the modified neighbour-joining algorithm ikon (Gascuel, 1997). Node support was measured with 1000 bootstrap replicates. This same procedure was applied to analyse the concatenated genes of the MLST scheme.

**G+C content and DNA–DNA hybridizations.** The G+C content of DNA was determined by using HPLC (Mesbah et al., 1989) using the further specifications given by Logan et al. (2000). DNA–DNA hybridization (DDH) was performed using a modification of the microplate method of Ezaki et al. (1989), as described by Willems et al. (2001). A hybridization temperature of 37 °C (calculated with correction for the presence of 50 % formamide) was used.

Predicted DDH value was calculated for all possible pairs of type strains in the *B. cereus* Group (21 pairs) using a regression analysis including experimental DDH values and genome similarity values: predicted DDH = 1.066 (Index SIM) – 1.9689 (Fig. S1). The similarity index between two genomes (Index SIM) was calculated by multiplying the ANI value (Goris et al., 2007) against the conserved DNA % of the genome (Goris et al., 2007): (ANI% × conserved DNA%) threshold 85 % / 100 (Fig. S1 and Table S2). This index has the advantage that it takes into account both the mean similarity of conserved regions (represented by ANI) and the size of the conserved genome DNA, which is suitable to compare strains that significantly differ in terms of size of conserved DNA, such as species of the *B. cereus* Group. Both parameters were calculated using the ANI script (kindly provided by K. T. Konstantinidis, Michigan State University). The final data are means of reciprocal values for each pair of strains.

**Detection of the cytK-1 form of cytoxin K.** A pronounced polymorphism for cytK gene has been previously observed, resulting in a specific form of the gene for NVH 391-98T (Fagerlund et al., 2004). As this form has been shown to be present in NVH 391-98T, INRA AF2 and NVH883-00 (Fagerlund et al., 2007) but absent from all other species of the *B. cereus* Group among 420 tested strains (Guinebretière et al., 2010), we used PCR to screen for the presence of this cytK-1 form in the two other closely related strains, as described previously (Guinebretière et al., 2006).

**Chemotaxonomic characterization.** Extraction of Fatty Acid Methyl Esters (FAMEs) was performed according to the standardized MIDI protocol (http://www.microbialid.com/PDF/Technote_101.pdf). In our experiment, bacterial cells were obtained from culturing at 30 °C on trypticase soy broth agar (TSBA, 30 g trypticase soy broth and 15 g Bacto agar; 1 °C) for 24 h. The growth temperature used was 2 °C higher than in the MIDI standard because it was found to be close to the optimal temperature for all seven *B. cereus* Group species (see Table 3 for temperature growth ranges). The effect of physiological age was minimized by harvesting cells from a streak plate on the overlap between the second and third quadrant on the plate. After preparation, FAMEs were analysed by using gas chromatography-mass spectrometry (GC-MS) (Shimadzu QP2010 system), as previously described (Brillard et al., 2010).

The cell-wall diamino acid was determined from whole-cell hydrolysate (4 M HCl, 100 °C, 16 h) subjected to thin-layer chromatography on cellulose plates using the solvent system of Rhuland et al. (1955).

**Phenotypic characterization.** The strains were characterized phenotypically using API strips according to the method of Logan & Berkeley (1984). Vegetative cells and sporangea were observed by phase-contrast microscopy for rod shape, spore shape, spore position, swollen sporangea and parasporal crystal. Electronic microscopy analysis was done by using the Technical platform MINMAZ (INRA, Jouy-en-Josas, France). Catalase production, starch hydrolysis, Voges–Proskauer reaction, egg yolk reaction, anaerobic growth, reduction of nitrates, minimum and maximum growth temperatures all followed the standard tests given in Bergey’s Manual (Claus & Berkeley, 1986; Logan & De Vos, 2009). For anaerobic growth, aerobiologically incubated tubes were used as controls. Motility was tested on Luria agar with 0.25 % agar, as described previously (Houry et al., 2010). The tryptophan auxotrophy of NVH 391-98T and close relatives was checked by observing the presence or absence of growth on Minimum Medium MOD (Duport et al., 2004; Rosenfeld et al., 2005) supplemented with 5 g glucose l−1 and with or without tryptophan (1 g l−1), compared to growth on a rich medium (TSA) and to growth of *B. cereus* ATCC 14579 as controls.

**RESULTS AND DISCUSSION**

**Genomic delimitation.** The 16S rRNA gene sequence of strain NVH 391-98T and of close relatives was a continuous stretch of 1532 bp. The closest relatives of NVH 391-98T were the six species of the *B. cereus* group (97–98 % identity, Table S1 and Fig. 1). Lower sequence similarities (≤ 95 % identity) were found with all other validly described *Bacillus* species (Table S1). The phylogenetic analysis indicated that strain NVH 391-98T and close relative strains (INRA AF2, CVUAS2833, 08CEB44BAC and NVH 883-00) formed a homogeneous and robust cluster (Fig. 1) and that this cluster was easily differentiated from all *B. cereus* Group species, as supported by high bootstrap values. In contrast, 16S rRNA gene sequence analysis was not able to clearly differentiate species of the *B. cereus* Group, except *B. pseudomykoides*. It is well known that these six species are genetically very close (Turnbull et al., 2002). What is important here is that all these six species of the *B. cereus* Group are genetically distinct from the NVH 391-98T cluster based on 16S rRNA gene sequence analysis.

MLST data allowed a more discriminatory analysis in the *B. cereus* Group (Fig. 2), supporting the previous data and also highlighting NVH 391-98T cluster as a robust genetic entity and as the most distant cluster from other species in the *B. cereus* Group, followed by *B. pseudomykoides*. Sequences of
Fig. 1. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences of all strains under study. The phylogenetic position of *B. cytotoxicus* (indicated in bold type) is shown among type strains of a selection of representatives in the genus *Bacillus*. Bootstrap values are given at each branch point. *Geobacillus stearothermophilus* was used as an outgroup to root the tree. The accession numbers of the strains used to generate the tree are given in Table S1. Bar, 0.05 substitutions per site.
the seven genes selected for one of the proposed MLST schemes (Tourasse et al., 2006) appeared to be identical for the strains NVH 391-98T, INRA AF2 and CVUAS2833. Sequencing types for the two other strains (08CEB44BAC and NVH883-00) each contained only two alleles that are different (not shown). Therefore, all five strains can be considered as members of a single clonal complex. Since the strains were isolated from very different locations, this indicates that the ‘B. cytotoxicus’ species represents a fairly narrow lineage of closely related strains.

DNA–DNA hybridization (DDH) values were experimentally measured for 11 pairs of type strains in the B. cereus Group (mean of reciprocal values) and genome similarities of type strains (Table 2). Strain NVH 391-98T showed very low DDH similarity to values and genome similarities of type strains (Table 2). Using a regression analysis including experimental DDH predicted for all possible pairs of type strains (21 pairs) around 13–20 % with the six other species of the B. cereus Group, there were specific between-species differences in the amounts of certain major fatty acids, particularly for B. pseudomycoides DSM 12442T and NVH 391-98T cluster; but this is congruent with previous DDH data. Except for B. pseudomycoides, the distinctiveness of these species seems to not always be strictly based on formal genomic delimitation referring to DNA–DNA reassociation values previously obtained (Lechner et al., 1998; Nakamura, 1994; Somerville & Jones, 1972). This creates difficulties in genetically distinguishing B. thuringiensis strains from B. cereus strains (Guinebretière et al., 2008; Helgason et al., 2000; Hill et al., 2004) and B. weihenstephanensis strains from B. mycoides strains (Guinebretière et al., 2008). In any case, NVH 391-98T cluster is clearly distinct based on DDH values, as well as B. pseudomycoides.

The DNA G+C content of NVH 391-98T is 35.8 mol%, similar to the other B. cereus Group species.

**Phenotypic features**

The fatty acid profiles of strains belonging to the NVH 391-98T cluster (available as Table S3) were highly similar, composed of iso-C15:0 (36 %), C16:0 (11 %), anteiso-C15:0 (11 %), iso-C17:0 (8 %), iso-C16:0 (7 %), iso-C13:0 (7 %), iso-C14:0 (5 %), C16:0-6 (3 %), anteiso-C17:0 (3 %), C14:0 (2 %), anteiso-C13:0 (2 %), iso-C16:0-5 (1 %), and others that were found at <1 %. Most of the major fatty acids (iso-C15:0, anteiso-C15:0, iso-C16:0, C16:0, iso-C17:0, anteiso-C17:0) were those typically found in the Bacillus genus (Kämpfer, 1994; Song et al., 2000). Lower amounts of anteiso-C15:0 and the presence of some particular fatty acids (iso-C12:0, iso-C13:0, anteiso-C13:0) are specific to the B. cereus Group (Song et al., 2000), supporting the affiliation of NVH 391-98T cluster to the B. cereus Group. In the B. cereus Group, there were specific between-species differences in the amounts of certain major fatty acids, particularly for B. pseudomycoides DSM 12442T and NVH 391-98T cluster;

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**Fig. 2.** Maximum-likelihood phylogenetic tree based on concatenated sequences from genes included in MLST scheme of Tourasse et al. (2006). The phylogenetic position of B. cytotoxicus strains (indicated in bold type) is shown among type strains of B. cereus Group species. Bootstrap values above 75 % are given at each branch point. Bar, 0.02 substitutions per site.

http://ijs.sgmjournals.org
NVH 391-98T cluster presented significantly higher amounts of iso-C₁₅:₀ and anteiso-C₁₅:₀ (see Table S3) and significantly lower amounts of iso-C₁₃:₀ compared to other B. cereus Group species. These characteristics are thus specific to the NVH 391-98T cluster in our experimental conditions.

As for members of B. cereus Group and for most species in the genus Bacillus, the strain NVH391-98T possessed a cell-wall type based on meso-diaminopimelic acid as the diagnostic diamino acid, which is consistent with assignment of the strain to the genus Bacillus. Considering the phylogenetic position of strain NVH 391-98T, it shows the peptidoglycan type A₁γ.

Strains of the NVH 391-98T cluster were all endospore-forming, rod-shaped, motile and catalase-positive bacteria, which confirm their affiliation to the genus Bacillus. On API20E and API50CH, they demonstrated the major specificities of the B. cereus Group (Table 3). In the B. cereus Group, strains of the NVH 391-98T cluster could be differentiated from most of the other species by assimilation of D-mannose, absence of starch hydrolysis, absence of assimilation of sucrose, starch and glycogen, and a weak VP reaction. Two highly specific features of the NVH 391-98T taxon were the absence of trehalose assimilation and absence of growth without tryptophan. The absence of trehalose assimilation may be explained by the absence of genes coding for trehalose-6-phosphate hydrolase and the trehalose-specific PTS system, as observed in the genome of NVH 391-98T. Concerning the absence of growth without tryptophan, we observed that the genome of NVH 391-98T does not contain the entire tryptophan biosynthesis operon (trp), which differentiates it from all other available genome sequences of the B. cereus Group (85 in total). Therefore, this strain and its close relatives were dependent on tryptophan for growth on minimal media.

Minimum and maximum growth temperatures tested according to the standard test in Bergey’s Manual were unable to distinguish most of the known species in the B. cereus Group (Table 3). In particular, the large growth temperature ranges observed for B. cereus and B. thuringiensis are indicative of broad-ranging diversity and may be explained by the fact that they each belong to several ecotypes (Guinebretière et al., 2008). However, strains of the NVH 391-98T cluster showed specificity in that they grow between 20 and 50 °C whereas strains of the other species grew between 5–15 °C and 35–45 °C. A shift in growth temperature range makes the NVH 391-98T cluster the only thermotolerant taxon in the B. cereus Group.

### Other typical characteristics

As for many B. cereus Group strains, spores of NVH 391-98T are characteristically surrounded by the exosporium structure (Fig. 3). The exosporium, being relatively hydrophobic, is believed to be responsible for the high ability of B. cereus Group bacteria to attach to inert surfaces (Faille et al., 2007; Tauveron et al., 2006). This is one of the properties of these
Table 3. Characteristics of B. cereus Group type strains, and of other strains of the same species in bracket (data from this study and from Logan & Berkeley, 1984; Logan et al., 1985)

Strains: 1, B. cytotoxicus NVH 391-98T (n=5 strains); 2, B. pseudonycoids DSM 12442T (n=7 strains); 3, B. cereus ATCC 14579T (n=11 strains); 4, B. thuringiensis CIP 53137T (n=8 strains); 5, B. weihenstephanensis WSBC 10204T (n=5 strains); 6, B. mycoides CIP 102472T (n=3 strains); 7, B. anthracis species (data from Logan et al., 1985). Symbols: +, positive for the type strain and 90 % or more of strains; —, negative for the type strain and for 90 % or more of strains; w, weakly positive; d, 11–89 % of positive B. anthracis strains. Numbers in round or square brackets indicate the percentage of positive strains recorded in the species when this character appears variable with strains of this study (square brackets) or with strains from Logan & Berkeley (1984) (round brackets and italics). All strains gave positive results in API50CH for D-glucose, D-fructose, aesculin, maltose, and negative results for erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, D-adonitol, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, 2-methyl-D-mannoside, melibiose, inulin, melezitose, raffinose, xylitol, D-lyxose, D-tagatose, D-fucos, L-fucos, D-arabitol, L-arabitol, 2-ketogluconate, 5-ketogluconate. All strains gave negative results in API20E tests for lysine decarboxylase, ornithine decarboxylase, H₂S production, urease, tryptophan desaminase and indole production.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<td><strong>Common general characteristics</strong></td>
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<tr>
<td>Cell diameter &gt;1.0 µm</td>
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<td>+ [34]</td>
<td>+</td>
<td>+</td>
<td>[33]</td>
<td>-</td>
</tr>
<tr>
<td>Gelatin</td>
<td>+</td>
<td>+ [86]</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>API 50CH tests</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>-</td>
<td>+w [17w]</td>
<td>-</td>
<td>(90)</td>
<td>-</td>
<td>(92)</td>
<td>+w</td>
</tr>
<tr>
<td>Ribose</td>
<td>+ [67]</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>[81]</td>
<td>+</td>
</tr>
<tr>
<td>Galactose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>[38]</td>
<td>-</td>
<td>(32)</td>
<td>-</td>
</tr>
<tr>
<td>2-Methyl-D-glucoside</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>[33]</td>
<td>-</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>[69]</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Amygdalin</td>
<td>+w [67]</td>
<td>-</td>
<td>+w [23w]</td>
<td>-</td>
<td>+w [83]</td>
<td>-</td>
<td>(50)</td>
</tr>
<tr>
<td>Arbutin</td>
<td>+</td>
<td>-</td>
<td>[33]</td>
<td>+</td>
<td>[82]</td>
<td>+</td>
<td>[63]</td>
</tr>
<tr>
<td>Salicin</td>
<td>+</td>
<td>+ [67]</td>
<td>+</td>
<td>[64]</td>
<td>+</td>
<td>[75]</td>
<td>+</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>+</td>
<td>-</td>
<td>[33]</td>
<td>+</td>
<td>[55]</td>
<td>+</td>
<td>[63]</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
<td>-</td>
<td>[33]</td>
<td>+</td>
<td>[64]</td>
<td>+</td>
<td>[38]</td>
</tr>
<tr>
<td>Trehalose</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Starch</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>[73]</td>
<td>+</td>
<td>[80]</td>
<td>+</td>
</tr>
<tr>
<td>Glycogen</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>[73]</td>
<td>+</td>
<td>[88]</td>
<td>+</td>
</tr>
<tr>
<td>P-Gentiobiose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>[9]</td>
<td>-</td>
<td>-</td>
<td>[33]</td>
</tr>
<tr>
<td>Turanose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(16)</td>
<td>-</td>
<td>(11)</td>
<td>-</td>
</tr>
<tr>
<td>Gluconate</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(36)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Minimum growth temperature</td>
<td>20 °C</td>
<td>10 °C</td>
<td>10 °C</td>
<td>10 °C</td>
<td>5 °C</td>
<td>5 °C</td>
<td>&gt;10 °C</td>
</tr>
<tr>
<td>Maximum growth temperature</td>
<td>50 °C</td>
<td>45 °C</td>
<td>40 °C</td>
<td>45 °C</td>
<td>37 °C</td>
<td>37 °C</td>
<td>&lt;50 °C</td>
</tr>
</tbody>
</table>
bacteria that allow them to persist on washable industrial surfaces and thus spark food contamination issues and, in the worst-case scenario, food poisoning.

All strains of the NVH 391-98T cluster carried the cytK gene under the cytK-1 form that corresponds to a pronounced polymorphism (Fagerlund et al., 2004) and is the higher cytotoxic form (Fagerlund et al., 2007). The cytK-1 form is distinctive of the NVH 391-98T taxon, since it has never been shown by tracking on 425 B. cereus Group strains for any other species of the B. cereus Group (Guinebretière et al., 2010). The resulting enterotoxin (cytotoxin K-1) was first isolated for NVH 391-98T (Lund et al., 2000), which is able to synthesize this toxin in high amounts (Brillard & Lereclus, 2004; Fagerlund et al., 2007) and is considered particularly virulent since it has been connected to three deaths. The CytK-1 form has been exploited for a PCR diagnostic method that is able to detect B. cytotoxicus strains and has been validated on numerous B. cereus Group strains (Guinebretière et al., 2006, 2010).

Among MLST genes, panC originated from a pilot experiment on multiple locus sequencing of B. cereus strains (Candelon et al., 2004). As the phylogeny of panC closely follows the global phylogeny of the B. cereus Group (Guinebretière et al., 2008), it can be used as a tool for tracking strains of NVH 391-98 taxon (phylogenetic group VII) at https://www.tools.symprevius.org/Bcereus/english.php (Guinebretière et al., 2010). This is thus another rapid method for identifying strains of B. cytotoxicus, through their highly specific panC polymorphism.

The complete genomic sequence of the strain NVH 391-98T confirmed genetic divergence with other representatives of the B. cereus Group, especially highlighted by large difference in the chromosome sizes (4.1 compared to 5.2–5.4 Mb) (Lapidus et al., 2008).

**Conclusion**

The very high genetic proximity of NVH 391-98T taxon to the six B. cereus Group species, and its similarity with their phenotypic features, prompts us to consider this taxon as belonging to the B. cereus Group. In addition, MLST data and DDH values provide ample evidence that this taxon should be considered as a novel genomic species. Chemotaxonomic, biochemical and physiological data correlate the genetic results, providing phenotypic differentiation of this novel species from the six current B. cereus Group species.

**Description of Bacillus cytotoxicus**

*Bacillus cytotoxicus* (cy.to.to’xic.us. Gr. n. kutos, hollow, hold of a ship; N.L. pref. cyt-, prefix denoting pertaining to a cell; N.L. adj. toxicus -a -um (from L. n. toxicum, poison), toxic; N.L. masc. adj. cytotoxicus, cytotoxic, referring to cytotoxin K, an enterotoxin isolated and described from the type strain).

Isolated from rehydrated foods connected to food poisoning outbreaks, unknown natural niche. Gram-positive, facultative anaerobic (although aerobic growth is faster), motile rods (≥1.0 µm). Cell morphology similar to B. cereus: cells occur singly, in pairs, occasionally in short chains or filaments. Endospores are mainly ellipsoidal and lie central to subterminal position in non-swollen sporangia. When grown for 24 h on TSA at 37 °C, colonies are 1 mm diameter, cream-coloured, round, with a flat and shiny surface; may become matt and with slightly irregular margins with age. Like other species of the B. cereus group, is egg-yolk lecithinase-positive and mannitol-negative, resulting in typical pink colony with white precipitate around the colony.
on Mossel medium, generally less pronounced than for B. cereus, or resulting in atypical precipitate under the colony. Optimal growth occurs at 30–37 °C, the maximum growth temperature is 50 °C. Can be differentiated from other species of the B. cereus Group by maximum growth at 50 °C and minimum growth at 20 °C, by absence of starch hydrolysis, by absence of growth on synthetic media without tryptophan, and weak VP reaction. API20E and API50CH are summarized in Table 3 highlighting the following specific characteristics for B. cytotoxicus: VP weak, d-mannose-positive, sucrose-negative, trehalose-negative, starch and glycogen-negative. The major fatty acids are similar to those of other B. cereus Group species, with the particularity that iso-C15:0 is in lower amounts and iso-C15:0 and anteiso-C15:0 are in higher amounts. The cell-wall peptidoglycan contains meso-diaminopimelic acid.

The type strain is NVH 391-98T (=DSM 22905T = CIP110041T) isolated during a severe food poisoning outbreak in France in 1998. The genomic DNA G+C content of the type strain is 35.8 mol%.

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