



**OPINION OF THE SCIENTIFIC COMMITTEE ON ANIMAL NUTRITION
ON THE SAFETY OF USE OF *BACILLUS* SPECIES IN ANIMAL NUTRITION**

(EXPRESSED ON 17 FEBRUARY 2000)

1. BACKGROUND

In its report on the use of certain micro-organisms as feed additives expressed on 26 September 1997, the Scientific Committee on Animal Nutrition (hereafter SCAN) stated that the use of *Bacillus* species may be ill-advised and should be accepted only for clearly defined strains which have been tested negative for toxicity and pathogenicity *in vitro* and *in vivo*.

In June 1999, Denmark drew the attention of the Commission to a number of scientific publications describing the detection of toxigenic strains of *Bacillus cereus* and other *Bacillus* spp. Strains of these species are used in animal nutrition either as microbial feed additives or as a source of enzymes used as feed additives.

2. TERMS OF REFERENCE

In the light of its previous report and of newly available scientific data, SCAN is requested to reassess the safety of the use of bacteria of the genus *Bacillus* (*Bacillus cereus* and other species) in animal nutrition. SCAN is also requested to identify the scientific data which should be provided for the safety evaluation of products using strains of *Bacillus* species submitted for authorisation as feed additives.

3. INTRODUCTION

The genus *Bacillus* contains a number of industrially important species. The large range of physiological types found amongst the bacilli (attributed to the genetic diversity of the genus) and the fact that most species are non-pathogenic and are relatively easy to manipulate and to grow, makes *Bacillus* spp. preferred hosts in the fermentation industry (Arbige *et al.*, 1993). Approximately half of the present commercial production of bulk enzymes derives from strains of *Bacillus* spp. These include proteases (from *B. alcalophilus*, *B. amyloliquefaciens*, *B. lentus*, *B. licheniformis*), α -amylases (from *B. amyloliquefaciens*, *B. licheniformis*, *B. stearothermophilus*) glucose isomerase (from *B. coagulans*) and pullulanase (from *B. acidopullulyticus*). Strains of *B. subtilis* are used for the preparation of nucleic acid bases such as inosine which are precursors of flavour enhancing nucleotides for use in the food industry (Priest and Harwood, 1994). These bacteria also produce

lipopeptide surfactants and a diversity of polypeptide “antibiotics” with activity against bacteria and fungi. Some *Bacillus* species (*B. cereus*, *B. subtilis*, *B. licheniformis*) have also found use in the animal feed industry. Their addition to diets of pigs, poultry and calves is said to improve performance and the health of livestock. Several products of this nature have temporary approval and are now seeking permanent authorisation for use as feed additives.

Publications appearing in the scientific literature in 1998/9 have suggested that toxin production amongst *Bacillus* species may be far more widespread than previously thought (Beattie and Williams, 1999). One reason for this is that the introduction of more sensitive test methods has allowed the detection of toxigenic effects at much lower concentrations (Andersson *et al.*, 1999; Finlay *et al.*, 1999; Salkinoja-Salonen *et al.*, 1999). The detection of toxin production by current industrial strains would bring into question their continuing use despite a history of *apparent* safe use. Application of a precautionary approach would argue that if genes encoding toxins are present, the level of expression could not be predicted or guaranteed under all circumstances. Where the organism itself may enter the human food chain it would appear prudent to avoid the use of those strains which are potentially toxigenic. However, where bacilli are used as a source of fermentation products the same stringency may not be required. Fermentation conditions are standardised and it is reasonable to assume that toxins, in the unlikely event of their presence, would be produced at a constant low concentration. In these cases, the hazard arises from the possible inclusion and concentration of the toxin(s) in the final product (e.g. enzyme). Since the producer organism itself does not enter the food chain, monitoring of the final product for the absence of toxigenic material may provide sufficient safeguard.

This Opinion examines the extent to which toxin production may be an unrecognised problem amongst some species of *Bacillus* and the implications this may have for their continuing commercial use. Knowledge of the genetic and biochemical basis for toxin production and methods for the detection of *Bacillus* toxins are reviewed and recommendations made for how best to ensure the absence of toxins (or a capacity for toxin production) given the present state of knowledge.

4. TAXONOMY OF *BACILLUS CEREUS* AND RELATED SPECIES

Bacteria that differentiate into endospores under aerobic conditions have traditionally been placed in the genus *Bacillus*. Over the past three decades, this genus has expanded to accommodate more than 100 species (see www.dsmz.de/bactnom/nam0379.htm). A pioneering analysis of 16S ribosomal RNA sequences from numerous *Bacillus* species indicated that the genus *Bacillus* should be divided into at least five genera or rRNA groups (Ash *et al.*, 1991). With the subsequent isolation of many new species this number of “genera” has increased to about 16. Within this framework, *Bacillus subtilis*, the type species, is accommodated in rRNA group 1 or *Bacillus sensu stricto*. Two species groups of interest to this report are included in rRNA group 1, the *B. cereus* group and the *B. subtilis* group. These present very different taxonomic structures.

4.1. The *Bacillus cereus* group

Bacillus anthracis, *B. cereus*, *B. mycoides*, *B. thuringiensis* and more recently *B. pseudomycoides* (Nakamura 1998) and *B. weihenstephanensis* (Lechner *et al.*, 1998) comprise the *B. cereus* group. These bacteria have highly similar 16S and 23S rRNA sequences indicating that they have diverged from a common evolutionary line relatively recently. The guidelines for the delineation of a bacterial species require strains within a species to share more than 70% chromosomal DNA hybridisation and between species less than 70% hybridisation. Strains of *B. anthracis* conform to these guidelines; it is the most distinctive member of this group, both in its highly virulent pathogenicity and taxonomically. On the other hand, DNA from strains of *B. cereus* and *B. thuringiensis* hybridises beyond the 70% limit and extensive genomic studies have shown that there is no taxonomic basis for separate species status (Carlson *et al.*, 1996). Nevertheless, the name *B. thuringiensis* is retained for those strains that synthesise a crystalline inclusion (Cry protein) or delta-endotoxin that may be highly toxic to insects. The *cry* genes are usually located on plasmids and loss of the relevant plasmid(s) makes the bacterium indistinguishable from *B. cereus*. It is now clear that most strains in the *B. cereus* group, including *B. thuringiensis*, carry enterotoxin genes (see section 7).

4.2. The *Bacillus subtilis* group

The *B. subtilis* group traditionally comprises four species: *B. amyloliquefaciens*, *B. licheniformis*, *B. pumilus* and *B. subtilis* itself (Claus and Berkeley, 1986; Priest *et al.*, 1988). Recent ecological studies, however, have identified some very close relatives of *B. subtilis*: *B. atrophaeus* (Nakamura, 1989) *B. mojavensis* (Roberts *et al.*, 1994) and *B. vallismortis* (Roberts *et al.*, 1996) and have subdivided *B. subtilis* into subsp. *subtilis* and subsp. *spizizenii* (Nakamura *et al.*, 1999). These taxa all conform to the DNA hybridisation guidelines for bacterial species noted above (section 4.1). The 16S rRNA gene sequences differ between representative species of the *B. subtilis* group, but such data are not available for the recently-described “ecological” group. Species of the traditional group can be distinguished phenotypically, but *B. mojavensis*, *B. subtilis* and *B. vallismortis* are indistinguishable and can only be identified by molecular means while *B. atrophaeus* is distinguished from *B. subtilis* only by pigmentation. One of the main implications of the inability to distinguish the members of the ecological group is that strains of “*B. subtilis*” being used by industry may actually belong to *B. mojavensis*, *B. vallismortis* or to other species.

5. BACILLUS SPP. AS A HUMAN HEALTH PROBLEM

5.1. Gastrointestinal diseases caused by *Bacillus cereus* and related species

B. cereus is well recognised as a food poisoning organism. Outbreaks can be divided into two types according to their symptomatology. The diarrhoeal type is far more frequent in Europe and USA while the emetic type appears more prevalent in Japan. While the poisonings are usually mild, both types of intoxications have caused deaths. Typical foods implicated are stews,

puddings, sauces, and flour and rice dishes (Drobniewski, 1993). When expressed as proportion of all reported food poisonings, outbreaks ascribed to *Bacillus* spp. seem to concentrate in Scandinavia and Canada (10 - 47% of total) and are less frequent in Central Europe, UK, USA and the Far East (1 - 5 % of total)(Granum, 1997; Granum pers. communication). These figures may, however, be misleading since they also reflect different reporting practices. Thus in the Netherlands in 1991 *B. cereus* was responsible in 27% of outbreaks in which the causative agent was identified. However, the incidence was only 2.8 % of the total, since the majority of cases of food poisoning were of unknown aetiology (Schmidt, 1995). In addition, when the number of food poisoning cases ascribed to *B. cereus* are expressed on a per head of population basis many of the large regional differences in incidence disappear.

The diarrhoeal type of food poisonings is caused by enterotoxins (see section 7) formed by vegetative *B. cereus* in the intestine. The fact that *B. cereus* spores can survive the conditions of the gastrointestinal tract and adhere to the gut epithelium may be another contributing factor (Drobniewski, 1993, Andersson *et al.*, 1998). Symptoms start within a few hours after ingestion of contaminated food. A profuse diarrhoea with abdominal cramps develops, usually continuing for 10 - 12 hours.

The emetic toxin (cereulide) is a cyclic dodecadepsipeptide, which is resistant to heat, to proteases and to both low and high pH (Agata *et al.* 1995a). Consequently it can cause poisonings as a pre-formed toxin present in the ingested food. The poisoning, characterised by violent vomiting, has led to hepatic failure and death (Mahler *et al.*, 1997). The absence of starch hydrolysis is a special phenotypic character associated with most cereulide-producing strains (Nishikawa *et al.* 1996).

B. mycoides and *B. thuringiensis* have also been isolated in food poisoning outbreaks, and the toxins produced by these species are apparently identical or very similar to those of *B. cereus* (Jackson *et al.*, 1995; Damgaard *et al.* 1996).). Since *B. cereus* and *B. thuringiensis* differ only in the presence of *cry* plasmids in the latter, it is likely that the incidence of food poisoning from *B. thuringiensis* has been under-reported for two important reasons:

- most food laboratories identify *B. cereus* at the culture level only which is insufficient to distinguish the two taxa; thus *B. thuringiensis* will be reported as *B. cereus*.
- food processing treatments such as high temperature may lead to plasmid loss and "conversion" of *B. thuringiensis* spores or cells into *B. cereus*.

Strains of *B. alvei*, *B. circulans*, *B. licheniformis*, *B. pumilus*, *B. sphaericus* and *B. subtilis* have occasionally been reported as causative agents in food poisoning. Both diarrhoeal and emetic types of outbreaks have been recorded, but very little is known about the nature of the toxins associated with these species (Drobniewski, 1993, Salkinoja-Salonen *et al.*, 1999).

5.2. Other diseases associated with *Bacillus* spp.

Local infections caused by different bacilli are common traumatic or post-surgical complications. Eye infections are particularly serious, since in very aggressive cases the condition may lead to blindness in less than 24 h. Cases of fulminant necrotising soft tissue infections also have been reported. The species most often indicated or isolated is, again, *B. cereus*. Exotoxins, phospholipases, proteinases and haemolysins produced by the organism may all have a role in the pathogenesis (Drobniewski, 1993; Åkesson *et al.* 1991).

Opportunistic systemic infections caused by *B. cereus* are relatively frequently reported in the literature. Respiratory infections, septicaemia and endocarditis, and even central nervous system infections have been observed. Patients are often compromised and have some other underlying illness or health problem (i.e. cardiac valve disease, intravenous drug use, haemodialysis). *B. popilliae* has also been reported in connection with endocarditis and *B. pumilus* in infections mimicking listeriosis. In many clinical reports on opportunistic infections the causative *Bacillus* has not been identified to species level (Drobniewski, 1993; Steen *et al.*, 1992; Workowski and Flaherty, 1992; Meredith *et al.*, 1997; Wu *et al.*, 1999;).

The classic *Bacillus* species causing fatal systemic infections is *B. anthracis*, the causative agent of anthrax. The disease now is rare in Europe, but sporadic cases do occur. In animals *B. anthracis* causes a septic disease, while in humans infected by the diseased animals, anthrax often starts as a pustular skin infection. The most fatal form of the disease is, however, the pulmonary type, typically occurring in individuals handling wool and animal skins. The intestinal form of the disease is less frequent. The pathogenicity of *B. anthracis* is associated with the presence of two plasmids, pXO1 and pXO2, the former coding for the anthrax toxin and the latter for capsule formation (Drobniewski, 1993; Pfisterer, 1991).

6. ANIMAL DISEASES CAUSED BY *BACILLUS* SPP.

Amongst strains of bacilli, *B. anthracis* is the single most important animal pathogen. Only occasionally do other species of this genus cause disease in livestock. They are more commonly found as contaminating agents in clinical specimens (e.g. bovine milk) (Frank, 1997). However, within the *B. cereus* group, *B. cereus* itself may cause mastitis of varying severity and abortion in cattle herds. *B. licheniformis*, a member of the *B. subtilis* group, occasionally is also occasionally associated with bovine toxemia and abortions (Johnson *et al.*, 1994). The incidence of bovine abortion caused by strains of *B. licheniformis* and *B. cereus* together over a ten-year period in southern USA was estimated to be 3.5% of total abortions and stillbirths examined. Although many reports implicate *B. licheniformis*, it is evident that this species is only weakly virulent and usually will multiply freely only in animals which, for various reasons, are immune compromised (Anon, 1997).

7. TOXIN PRODUCTION BY *BACILLUS CEREUS* AND RELATED SPECIES – GENETIC BASIS AND MODE OF ACTION

7.1. Mechanism of toxicity

To date, five enterotoxins have been described for *B. cereus* and a single ionophore-like emetic toxin. The mechanisms of toxicity for the known enterotoxins listed in Table 1 are not well understood. Two studies have been completed on the action of the haemolytic (Hbl) and non-haemolytic (Nhe) toxins (Beecher and Wong, 1997; Lund and Granum, 1997). Although the receptors are not known it appears that one of the components of the tripartite toxin binds first to the receptor followed by binding of the two other (lytic?) components. The final result of the action of the enterotoxins is epithelial cell membrane damage. The depsipeptide emetic toxin (cereulide), in addition to inducing membrane damage, has been shown to cause vomiting by stimulation of the vagus afferent through binding to the 5-HT₃ receptor (Agata *et al.*, 1995a).

Table 1. Toxins known to be produced by *Bacillus spp.*

Toxin (the first 6 from <i>B. cereus</i>)	Type	Food poisoning	References*
Hemolysin BL (Hbl)	Protein, 3 components	Probably	1, 2, 3, 4
Non-haemolytic enterotoxin (Nhe)	Protein, 3 components	Yes	5, 6, 7
Enterotoxin T (BceT)	Protein, 1 component, 41 kDa	?	8
Enterotoxin FM (EntFM)	Protein, 1 component, 45 kDa	??	9,10
Enterotoxin K (EntK)	Protein, 1 component, 35 kDa	Yes, 3 deaths	10
Emetic toxin (cereulide)	Cyclic peptide, 1.2 kDa	Yes, 1 death	11, 12, 13, 14
Emetic toxin from <i>B. licheniformis</i>			15, 16

*References: 1, Beecher and Wong, 1997; 2, Heinrichs *et al.*, 1993; 3, Ryan *et al.*, 1997; 4, Beecher *et al.*, 1995; 5, Lund and Granum, 1996; 6, Lund and Granum, 1997; 7, Granum *et al.*, 1999; 8, Agata *et al.*, 1995b; 9, Asano *et al.*, 1997; 10, De Buyser ML and Granum PE Unpublished results; 11, Agata *et al.*, 1994; 12, Agata *et al.*, 1995a; 13, Yokoyama *et al.*, 1999; 14, Mahler *et al.*, 1997; 15, Salkinoja-Salonen *et al.*, 1999; 16, Mikkola *et al.*, 1999.

7.2. Genetic basis of toxin production

All the enterotoxins in Table 1 have been sequenced. The two tripartite enterotoxins Hbl and Nhe described in Table 2 are both transcribed from single operons and are positively regulated by *plcR* (Agaisse *et al.*, 1999), a gene first described to regulate the *plcA* (phospholipase C). The binding sequence of *PlcR* is TATGNANNNTNCATA. This sequence is found from about 30 to more than 200 bases upstream of transcription start for the different genes it regulates. Nothing is known about regulation of the other enterotoxins or the genetic basis for the production of the emetic toxins.

Table 2. The genetics of the haemolytic and non-haemolytic enterotoxins of *B. cereus*.

Enterotoxin	1st gene in operon	2nd gene in operon	Third gene in operon	
Non-haemolytic (Nhe)	<i>nheA</i> (45kDa)	<i>nheB</i> (39 kDa)	<i>nheC</i> (?)	105 kDa protein (collagenase) may contribute to Nhe activity
	1st gene in operon	2nd gene in operon	3rd gene in operon	4th gene in operon
Haemolytic (Hbl or haemolysin BL) (<i>hblA</i> and <i>hblB</i> were sequenced first)	<i>hblC</i> L ₂ -component	<i>hblD</i> L ₁ -Component	<i>hblA</i> B-component	<i>hblB</i> Similar to B. Function not known but not necessary for activity

Based on data from Heinrichs *et al.*, 1993; Ryan *et al.*, 1997 and Granum *et al.*, 1999;

7.3. Toxin production in species other than *B. cereus*

Production of Hbl and Nhe enterotoxins has been shown by use of the OXOID- and TECRA kit for strains of *B. licheniformis*, *B. circulans*, and *B. lentus* (Beattie and Williams, 1999). Recently Chaithong *et al.* (1999) have also shown by PCR that *Bacillus* strains other than those from the *B. cereus* taxonomic group possess *hbl* genes.

Bacillus cereus/B. thuringiensis and three other members of the genus, *B. subtilis*, *B. licheniformis* and *B. pumilus*, are known to have caused two different types of food poisoning (Kramer and Gilbert, 1989; Jackson *et al.*, 1995). It is still unclear which of the five enterotoxins (Table 1) are the major virulence factors, but it is clear that Nhe, EntK and probably Hbl have caused diarrhoeal food poisoning (Granum and Lund, 1997).

For the three other *Bacillus* species implicated in food poisoning it is not clear if the enterotoxins are the same as those of *B. cereus* or if other enterotoxins are involved. The indirect evidence of the presence for genes similar to Hbl provided by PCR, and for Hbl and Nhe by the Oxoid- and the TECRA kits is not conclusive. Without purification of toxins and sequencing of the PCR products it is impossible to be sure about the presence of similar or identical virulence factors. However, *B. licheniformis* has been shown to produce a toxin that shows similar physico-chemical properties to cereulide but has a different pattern of biological activity (Salkinoja-Salonen *et al.*, 1999).

8. METHODS OF DETECTION

8.1. Commercially available test kits

Two commercial kits for the detection of *B. cereus* enterotoxins are available; one specific for one component of the haemolytic (Hbl) toxin and the other for a single component of the non-haemolytic toxin. Detection of the haemolytic toxin is by a reversed passive latex agglutination method developed by Denka Seiken Co. in Japan but marketed in Europe by Oxoid/Unipath. The kit is specific for the L₂ component of the tripartite toxin at a sensitivity claimed by the manufacture of 1 ng/ml in the test wells (Beecher and Wong, 1994a). All three components of the Hbl toxin need to be present for full activity (Beecher and Wong, 1994b). However, the L₂ component appears present in only approximately 60% of food poisoning isolates of *B. cereus* (Granum *et al.*, 1995). Reliance on the Oxoid kit alone will fail to detect some toxigenic strains. The Tecra ELISA kit is also specific for only one element of the non-haemolytic toxin, NheA (Lund and Granum, 1996). Insufficient is known at present about the necessity for all three elements of the toxin to be present for the demonstration of cytotoxicity and, thus, whether this is a limitation of this test kit.

Both the Tecra and the Oxoid test kits have been shown to cross-react with supernatants from the culture of strains other than *B. cereus*, although the number of strains examined to date is small. Amongst the 95 isolates of the *B. cereus* group examined by Beattie and Williams (1999), 86% were positive with the Tecra and 59% with the Oxoid test kit. Of these, 13/13 isolates of *B. thuringiensis* were positive with the Tecra ELISA kit while 15/15 isolates of *B. mycooides* were positive with the Oxoid kit. Flecher and Logan (1999) similarly found 4/4 isolates of *B. mycooides* and *B. thuringiensis* positive with both test kits. Positive reactions with one or both kits have been reported for some strains of *B. lentus*, *B. licheniformis*, and *B. circulans* (Beattie and Williams, 1999), but not in others (Tan *et al.*, 1997).

8.2. Laboratory bioassays

The ileal loop test, normally performed using rabbits, has been widely used for the detection of enterotoxins, including those from *B. cereus*. However, loop test methods, even in the presence of positive and negative controls, can be confounded by a number of factors. Different strains produce different degrees of fluid accumulation in the loops, young rabbits are more sensitive

than older animals and the media used to culture the test strain can markedly affect toxin production. The vascular permeability reaction (VPR) is another standard assay for enterotoxins, in which the test material is injected intradermally into the back of an adult rabbit or guinea pig. This is followed some hours later by a blue dye and the extent of diffusion of the dye from the site of introduction measured. There is usually good correlation between the ileal loop and VPR assays for semi-purified toxin fractions but this is not always the case when crude culture filtrates are tested (Drobniewski, 1993). These methods do not detect the presence of the emetic toxin.

Cell cytotoxicity assays have proved more sensitive and often more reliable than other assay systems (Jackson, 1993). Various cell lines have been used with different responses to *Bacillus* toxins (Szabo *et al.*, 1991) and none can be considered as standard. However, Vero cells are a common choice with the advantage of ready availability and fast growth. Early experiments of this type were dependent on the visual assessment of toxin-induced cell damage. More recently attempts have been made to remove the subjective element from this assay. Two approaches have been adopted. The first makes use of a tetrazolium salt (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide [MTT]) which is cleaved in the mitochondria of metabolically-active cells with a resultant colour change (Beattie and Williams, 1999; Finlay *et al.*, 1999). The second approach is the measurement of the uptake of radio-labelled amino acids by the cell culture (Sandvig and Olsnes, 1982).

Cytotoxicity assays using concentrated but otherwise untreated culture filtrates do not generally distinguish between the effects of emetic and enterotoxins. However, *B. cereus* diarrhoeal toxins are heat-labile and are fully denatured by autoclaving unlike the emetic toxin, which resists such treatment. This is the basis of the Hep-2 cell vacuolation assay for the emetic toxin in which any toxic effect of autoclaved culture supernatant is detected by the swelling of mitochondria (Hughes *et al.*, 1988). The use of MTT as an alternative detection method has been recommended as mitochondrial swelling is transient and easily missed (Finlay *et al.*, 1999). Loss of motility of boar spermatozoa also has been suggested as a rapid bioassay for the presence of cereulide, the emetic toxin of *B. cereus* (Andersson *et al.*, 1999). Use of this assay with strains of *B. licheniformis* also resulted in the inhibition of motility, but without the mitochondrial swelling observed in spermatozoa exposed to cereulide (Salkinoja-Salonen *et al.*, 1999). Unfortunately, spermatozoa are notoriously sensitive to a large number of factors with consequent loss of mobility. Further work would be needed to confirm the specificity and robustness of this assay method before it could be considered as reliable. Direct methods for the detection of cereulide have not been described, but its low molecular weight (1.2 kDa) would make it an ideal candidate for mass spectrometric detection (e.g. by MALDI-TOF mass spectrometry).

Comparisons between the ileal loop or VPR assays and those based on cell cultures have been rarely made. However, comparisons have been made between cell cytotoxicity assays and the two commercial systems in an effort to confirm the sensitivity and validity of the latter (Lund and Granum, 1996; Beattie and Williams, 1999). In every case where this has been done the

detection rate was higher with the cell-culture based systems, implying a greater sensitivity.

8.3. PCR-based methods

Safety is ensured ultimately by the absence of genetic elements coding for toxin production. Genes encoding all five of the *B. cereus* enterotoxins (Table 1) have been sequenced but only four published. The remaining sequence for the EntK will be published in 2000. PCR primers have been developed and shown effective for the three enterotoxins Hbl, Nhe and BceT (Agata *et al.*, 1995b; Granum *et al.*, 1996; Granum *et al.*, 1999). However, experience has shown that there is some heterogeneity in the two tripartite enterotoxins, which might make it difficult to select ideal primers (Schoeni and Wong, 1999). In addition, the degree of homology that exists between the *B. cereus* toxins and those from other species remains unknown and thus the value of the existing primers for species other than *B. cereus* is uncertain. For these reasons it remains necessary to use tissue culture assays even for strains that are PCR negative, to secure the safe use of strains of *Bacillus*.

9. CONCLUSIONS

- The incidence of food poisoning involving strains of the *B. cereus* taxonomic group is common. Cases where the causative organism is a strain of *Bacillus* other than a member of the *B. cereus* group are rare.
- Probably all members of the *B. cereus* taxonomic group are toxin producers, although large differences occur in the nature and amount of toxins produced. Thus, probably all strains of this group pose a hazard in the human food chain.
- There is no taxonomic difference between *B. cereus* and *B. thuringiensis* other than the presence of plasmids encoding delta-endotoxins in the latter. From the viewpoint of safety assessment they should be considered to belong to the same species.
- Strains of the *B. cereus* group associated with outbreaks of food poisoning generally produce higher concentrations of toxins in laboratory cultures than those isolated from other sources.
- Strains from six species, three from the *B. subtilis* group (*B. subtilis*, *B. licheniformis* and *B. pumilus*) and three from other groups (*B. alvei*, *B. circulans* and *B. sphaericus*) also have been implicated in food poisoning. Although little is known about their virulence factors, both emetic and enterotoxin(s) must be involved. Within the *B. subtilis* group there are strains that pose a similar hazard to those in the *B. cereus* group. However, a majority of strains evidently lack the gene(s) encoding toxin(s) or if present, a capacity for toxin production or fail to produce detectable levels of toxin under the conditions employed and these could be considered safe for use.
- At this time insufficient is known about the genetic basis for toxin production in *Bacillus* species to rely wholly on PCR-based evidence of the absence of a toxigenic potential. Identification of hazard will continue to depend on the detection of the toxin(s) and/or their effects on biological systems.

10. RECOMMENDATIONS

- (1) It is evident that a substantial majority, if not all, strains of *Bacillus cereus* and closely related species produce toxins that may be damaging to human health.
 - (a) SCAN recommends that, for all future applications involving the addition of living organisms to animal feeds, the use of strains from the *B. cereus* taxonomic group be strongly discouraged.
 - (b) For additives based on strains belonging to the *B. cereus* taxonomic group with an existing history of use, or for new products where benefits of use can be shown to substantially outweigh any risks, the best available methods should be used to demonstrate the absence of toxin production. A scheme for testing for the presence of toxins in strains of bacilli is proposed in the Annex to this Opinion.
 - (c) Since knowledge of toxin production amongst strains of *Bacillus* is incomplete, it is further recommended that any authorisation for use granted should be subject to periodic review and that, in the light of any new and relevant information, additional testing should be required.
- (2) SCAN recognises that the risks posed by strains of *Bacillus* other than those from the *B. cereus* taxonomic group are less severe and that, with the exception of strains from the *B. subtilis* taxonomic group, detection of toxin production is the exception. Accordingly, provided toxin production cannot be detected, products based on species of *Bacillus*, other than those from the *B. cereus* taxonomic group, should be accepted. SCAN recommends the use of the scheme for testing proposed in the Annex to this Opinion.
- (3) For enzyme production or for other products of *Bacillus* species which do not constitute the whole organism, the producing strain should ideally be shown not to produce toxins under production conditions. However, where low levels of toxins are produced, detectable only after concentration of the culture medium, SCAN recognises it may be possible to introduce adequate monitoring to ensure that the product itself is free from toxins or live organisms.

Finally, SCAN draws to the Commission's attention the widespread use of certain species of *Bacillus* as plant biopesticides. Several *Bacillus* species are registered as pesticides (see <http://www.epa.gov/pesticides/biopesticides/>) elsewhere in the world, but in Europe only strains of *B. thuringiensis* are presently used for this purpose. *B. thuringiensis* is essentially indistinguishable from *B. cereus* and virtually all strains tested produced enterotoxins. If the Commission adopts SCAN's recommendations for the more stringent testing of *Bacillus* strains used as animal feed additives because of the risk associated with the indirect contamination of animal products, then authorisation for the direct application of living strains of *B. thuringiensis* to human food crops would seem to require at least the same degree of safety assurance.

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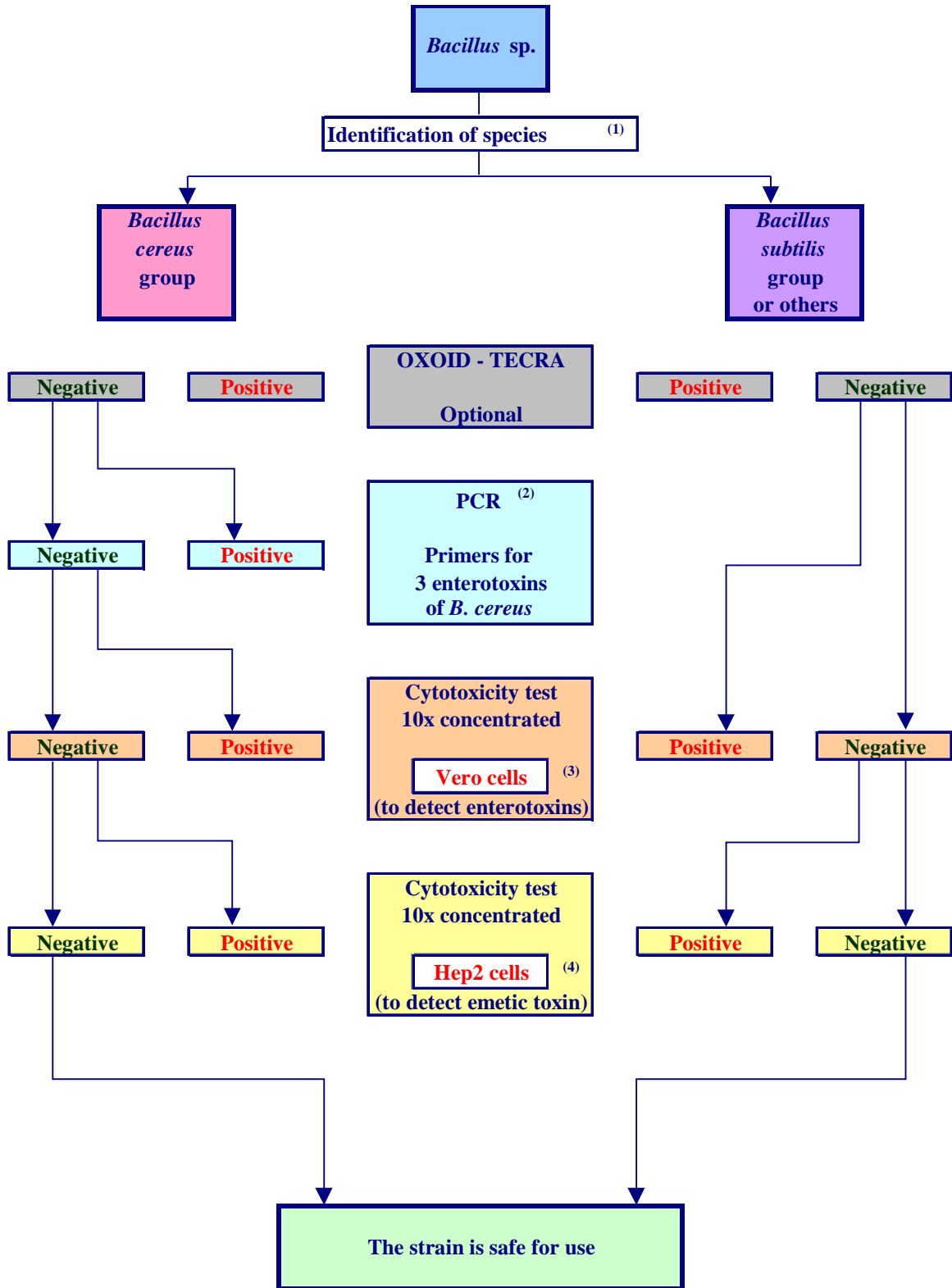
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Annex

Recommended scheme for the testing *Bacillus* species intended for use as feed additive or for other purposes for toxin production



Notes

The following notes, which expand on the recommendations embodied in the proposed testing scheme, are not intended to be exclusive or mandatory. Methods which use other cell lines or detection methods but which achieve the same purpose and with approximately the same degree of sensitivity could be substituted.

- (1) Strains of the *B. cereus* group can be readily distinguished from most other aerobic, endospore-forming bacteria by their characteristic colonial morphology on various selective media described in the literature. Confirmation can be achieved using commercially available identification kits (e.g. the Biolog or Biomerieux systems) or ideally by small subunit ribosomal RNA sequencing.
- (2) PCR detection is recommended for the following enterotoxins produced by *B. cereus*:
 - Hemolysin BL (Hbl)
 - Non-haemolytic enterotoxin (nhe)
 - Enterotoxin K (EntK)

Recommended primers:

Gene	Forward primer	Reverse primer	Product size
<i>NheB-nheC</i>	CGGTTCATCTGTTGCGA CAGC	CGACTTCTGCTTGTGCT CCTG	1437
<i>HblD-hblA</i>	CGCTCAAGAACAAAAA GTAGG	CTCCTTGTAATCTGTA ATCCCT	≈1650 ^a
<i>EntK</i> ^b			

^a The exact sequence and length of the spacer region between *hblD* and *hblA* is not known

^b Available after submission of publication in early spring 2000.

- (3) The suggested cytotoxicity test for the detection of enterotoxins using Vero cells is based on that described by Sandvig and Olsnes (1982).

Toxin production. Start with an overnight 2-10 ml culture of the test strain in BHI with 1 % extra glucose (BHIG). Transfer 1 ml of the pre-culture into 50 ml of BHIG, and incubate with shaking (100 rpm) for 6 h at 32°C. Cells then are removed by centrifugation at 5,000g for 10 min, and the supernatant used for cell toxicity tests. The supernatant is concentrated ten-fold by protein precipitation with ammonium sulphate to 80 % saturation (561 g/l). After recovery of the protein by centrifugation (10,000g for 20 min) the pellet is resuspended in 20 mM phosphate buffer at pH 6.8 in about 1/20 of the original volume. The remaining ammonium sulphate is removed by dialysis against the same buffer at 4°C for at least 6 h and the volume then adjusted to one-tenth of the original volume. Cytotoxins usually are easily detected in supernatants before concentration.

Growth medium for Vero cells

MEM	500 ml
Foetal calf serum	50 ml
Penicillin-streptomycin solution	5 ml

Use one flask with growing Vero cells, and pour out the growth medium. Remove the cells from the flask with a cell scraper. Resuspend the cells in 15 ml growth medium. Transfer 5 ml of the cell suspension to each of three new 250 ml flasks and add 15 ml of fresh growth medium. Incubate the flasks in 5 % CO₂ at 37°C.

Cytotoxicity test. Loosen the cells from one 250 ml flask with a cell scraper and add 20 ml of fresh growth medium. Mix and add 30 ml fresh growth medium. This suspension contains enough Vero cells for a single 24 well plate. Transfer 1 ml of cell suspension to each of the wells in the multi-well plate and incubate as above for two days. Before the start of the toxicity test wells should be completely covered with Vero cells. Remove the growth medium from the wells and wash each once with MEM (1 ml). Add 1 ml of preheated (37°C) MEM without leucine to each well and 50 µl of toxin immediately thereafter. Incubate the cells with toxins in the incubator for 2 h. Remove the medium and wash once with 1 ml of preheated (37°C) MEM without leucine. Add 16 µl isotope (¹⁴C-leucine) to 8 ml (enough for one 24 well plate) of MEM without leucine, and add 300 µl of this solution to each well. Incubate the plates at 37°C without CO₂ for 1 h, then remove the medium and add 1 ml 5 % TCA to each well. Incubate the plate at room temperature for 10 min, then remove the TCA and wash each well twice with 1 ml 5 % TCA. Add 300 µl 0.1 M KOH and incubate at room temperature for a further 10 min. Transfer all of the solution from each well into individual scintillation tubes, add 2 ml scintillation liquid, shake well and count for 1 min.

For positive control use cells incubated with 50 µl supernatant for a positive *B. cereus* strain (see toxin preparation). For calculation the average of two samples of the same toxin is used.

Calculation of % inhibition of ¹⁴C-leucine uptake. After subtraction of the value for background counts (which is usually 30-60 cpm):

$$(\text{cpm for Vero cells without toxin added} - \text{cpm test sample}) \times 100 / \text{cpm for Vero cells without toxin added}$$

If the inhibition of the leucine uptake is less than 20 % after ten-fold concentration, the tested strain should be considered toxin negative.

- (4) The recommended Hep-2 cell vacuolation assay for the emetic toxin in which any toxic effect of autoclaved culture supernatant is detected by the swelling of mitochondria is that described by Hughes *et al.* (1988). The use of MTT as an alternative detection method (Finlay *et al.*, 1999) could also be considered.