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REVIEW ARTICLE

Bacillus and relatives in foodborne illness

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Summary

Species of *Bacillus* and related genera have long been troublesome to food producers on account of their resistant endospores. These organisms have undergone huge taxonomic changes in the last 30 years, with numbers of genera and species now standing at 56 and over 545, respectively. Despite this expansion, relatively few new species have been isolated from infections, few are associated with food and no important new agents of foodborne illness have been reported. What has changed is our knowledge of the established agents. *Bacillus cereus* is well known as a cause of food poisoning, and much more is now understood about its toxins and their involvement in infections and intoxications. Also, although *B. licheniformis, B. subtilis* and *B. pumilus* have occasionally been isolated from cases of food-associated illness, their roles were usually uncertain. Much more is now known about the toxins that strains of these species may produce, so that their significances in such episodes are clearer; however, it is still unclear why such cases are so rarely reported. Another important development is the use of aerobic endosporeformers as probiotics, as the potentials of such organisms to cause illness or to be sources of antibiotic resistance need to be borne in mind.

Introduction

The genus *Bacillus* was of key importance in the early history of microbiology, for with observations upon *Bacillus subtilis* and its spores Ferdinand Cohn (1876) was finally able to discredit the theory of spontaneous generation, and Robert Koch's (1876) study of the life history of *B. anthracis* marked the genesis of clinical bacteriology. However, most aerobic endosporeformers are saprophytic organisms living in the natural environment, and their main habitats are soils of all kinds and the water columns and bottom deposits of fresh and marine waters. They have also been isolated from air at high altitudes and from deep subterranean sources.

Bacillus species are known to have roles in the postharvest processing and flavour development of cocoa, coffee and vanilla and, with floras often dominated by *B. subtilis*, in the production of several traditional fermented foods based on leaves and seeds (Logan and De Vos 2009). More recently, it has become appreciated that aerobic endosporeformers growing in the rhizosphere may promote the growth of plants by nitrogen fixation, the production of phytohormones, increasing nutrient availability, interactions with symbiotic bacteria and fungi, enhancement of root nodulation, biological control of plant pathogens and remediation of metal toxicity (Logan and De Vos 2009; Pérez-García *et al.* 2011; Ruiz-Lozano and Azcón 2011). Representatives of several species, including *B. amyloliquefaciens*, *B. cereus, B. licheniformis, B. megaterium, B. pumilus* and *B. subtilis*, have been isolated from the inner tissues of healthy plants, and some strains appear to have important roles in growth promotion and plant protection (Reva *et al.* 2002). Also, it is now better appreciated that some aerobic endosporeformers may have adapted to life in the gastrointestinal tracts of animals – both small ones such as sowbugs (Swiecicka and Mahillon 2006; König 2011) and large ones such as humans (Hong *et al.* 2005, 2009; Fakhry *et al.* 2008) – and should be regarded as commensals of the gut rather than merely as soil micro-organisms in

transit. In the sow bug or wood louse (*Porcellio scaber*), a trichome-forming organism known as '*Arthromitus*', with endospore-forming filaments over 100 μm long and up to 180 cells per filament, has been identified as *B. cereus* (Jorgensen *et al.* 1997), and similar filamentous organisms have been isolated from moths, roaches and termites (Margulis *et al.* 1998). *Bacillus oleronius* was first isolated from the hindgut of the termite *Reticulitermes santonensis*, and cellulolytic strains of the *B. cereus* group and *B. megaterium* have been found in the gut of another termite *Zootermopsis angusticollis* (Wenzel *et al.* 2002).

The resistance of endospores to heat, radiation, disinfectants and desiccation and the adhesive characters of particular spores that facilitate their attachment to processing equipment and resistance to cleaning procedures (Andersson *et al.* 1995; Ryu and Beuchat 2005) result in these organisms frequently being troublesome contaminants in clinical environments, biotechnological processes and food production. Dried foods such as spices, milk powders and cereal products are often quite heavily contaminated with spores, and when water becomes available during food preparation these spores may germinate, leading to spoilage or food poisoning. Although food spoilage usually manifests as changes in texture or the production of off-flavours, food defects can also simply be caused by unwanted microbial growth in commercially sterile products. Such problems cause significant financial losses, despite the contributions of modern food technology and preservation techniques. Recent concerns are the tolerance, adaptation or resistance of spores or vegetative cells of some species to conditions of low temperature or low pH that were previously presumed to stop growth, or to treatments such as ultrahigh heat treatment (UHT) that were expected to inactivate all living material (Heyndrickx 2011).

Taxonomic change

Bacillus was defined in 1920 as a genus of Gram-positive, aerobic, sporeformers, and the production of resistant endospores in the presence of oxygen has long been the defining feature. Members of the genus may be aerobic or facultatively anaerobic, and they are also expected to possess Gram-positive cell wall structures (although their staining reactions, even in young cultures, may be Gram-variable or frankly Gram-negative). These characters formed part of the definition of the genus for some 75 years, but since 1995, three strictly anaerobic and seven asporogenous *Bacillus* species have been proposed. This undermining of the definition occurred because 16S rRNA gene sequence analysis permits the recognition of genus boundaries, whereas genera were previously defined phenotypically, as pragmatic collections of species sharing key (i.e. diagnostic) features. Also, only coccoid cells have been observed in the single available strain of *B. saliphilus,* so that *Bacillus* is no longer exclusively a genus of rods. Furthermore, the genus *Sporosarcina*, established to accommodate aerobic endospore-forming cocci, now contains 12 species, most of which are rod shaped (Logan *et al.* 2009).

With the accumulation of 16S rRNA gene sequence data, the genus *Bacillus* has been divided. Fourteen new genera containing species originally allocated to *Bacillus* have been published since 1990, and together with *Bacillus* itself the species in these genera now number 397. *Bacillus,* with 165 species, has acquired more novel species than the number it has lost to new genera. In addition, 42 genera containing aerobic endospore-forming species that were not originally allocated to *Bacillus* have also been described. *Sporosarcina* was proposed in 1936, but the other 41 genera have been proposed since 1990, and together they contain 149 species. Following a handful of mergers, there are now 56 genera of aerobic endosporeformers overall and over 545 species (Logan and Halket 2011).

This explosion in the recognition of bacterial diversity and huge expansion in the number of bacterial taxa, which is not restricted to the aerobic endosporeformers, has been fuelled by discoveries of strains from novel, and often exotic, environments. Many new species have been delineated largely on the basis of 16S rRNA gene sequence similarity and DNA–DNA relatedness; their phenotypic descriptions may be brief – often limited to observations of a single isolate – so that routine phenotypic characters for distinguishing some of them may be very few in number and of little practical value.

Many of the one hundred and thirty or more *Bacillus* species that have been proposed since 1990 were based upon study of single isolates. The descriptions of several other species were based upon only two strains, so that nearly 40% of the species in this genus (and about 50% of those published since 1990) are poorly represented, and we have little or no information about their within-species variation.

Lack of knowledge of the between-strain variation of a species will nearly always lead to difficulties in identification, and further isolations regularly result in emendations of descriptions and, not infrequently, in taxonomic and nomenclatural changes (Logan *et al.* 2009). However, because many of the novel organisms have been isolated from exotic sources or extreme environments, food and clinical bacteriologists need not be greatly concerned at this taxonomic expansion; for the species relevant to their interests, it does not represent taxonomic upheaval. *Bacillus* is still the largest genus, and it continues to accommodate most of the best-known names such as *B. subtilis*

(the type species), *B. cereus, B. licheniformis, B. pumilus* and *B. thuringiensis*; these, it so happens, are the species that will figure most prominently in the remainder of this article. Rather few other familiar names, some of which are of potential clinical interest, have been transferred to newer genera – such as *Brevibacillus brevis*, *Lysinibacillus sphaericus*, *Paenibacillus polymyxa* and *P. macerans* – and several new *Bacillus* and *Paenibacillus* species have been proposed on the basis of single isolates of unknown significance from clinical sources (Logan *et al.* 2011).

Bacillus cereus and foodborne illness

Bacillus cereus (Fig. 1a) is the aerobic endosporeformer next in importance to *B. anthracis* as a pathogen of humans and other animals. It has been reported from a wide range of opportunistic infections both in immunocompromised and in immunocompetent patients (Bottone 2010; Logan *et al.* 2011) and causes two distinct foodborne illness syndromes and a wide range of opportunistic infections. However, it is only in the last 30–40 years that the importance of this species as a pathogen has been truly appreciated, although the diarrhoeal illness was described in Norway as early as the 1950s (Hauge 1955). Clinical isolates are phylogenetically diverse (Hoffmaster *et al.* 2008), and the organism's ubiquity ensures that cases of foodborne illness are not uncommon. El Saleeby *et al.* (2004) presented an unusual example of *B. cereus* illness following ingestion: an association between the consumption of tea and invasive infection in immunocompromised children.

The true burden of illness is unknown, since episodes involving less commonly identified pathogens such as *B. cereus* are less likely to have their aetiologies confirmed – because these organisms are not always considered in clinical, epidemiological and laboratory investigations of foodborne illness (Logan *et al.* 2011) and because they commonly occur as sporadic cases, rather than in major outbreaks. In figures from the late 20th century, the diarrhoeal syndrome was reported as a common occurrence in Norway (where *B. cereus* was the most frequent isolate from foodborne illness in 1990), Finland, Hungary,

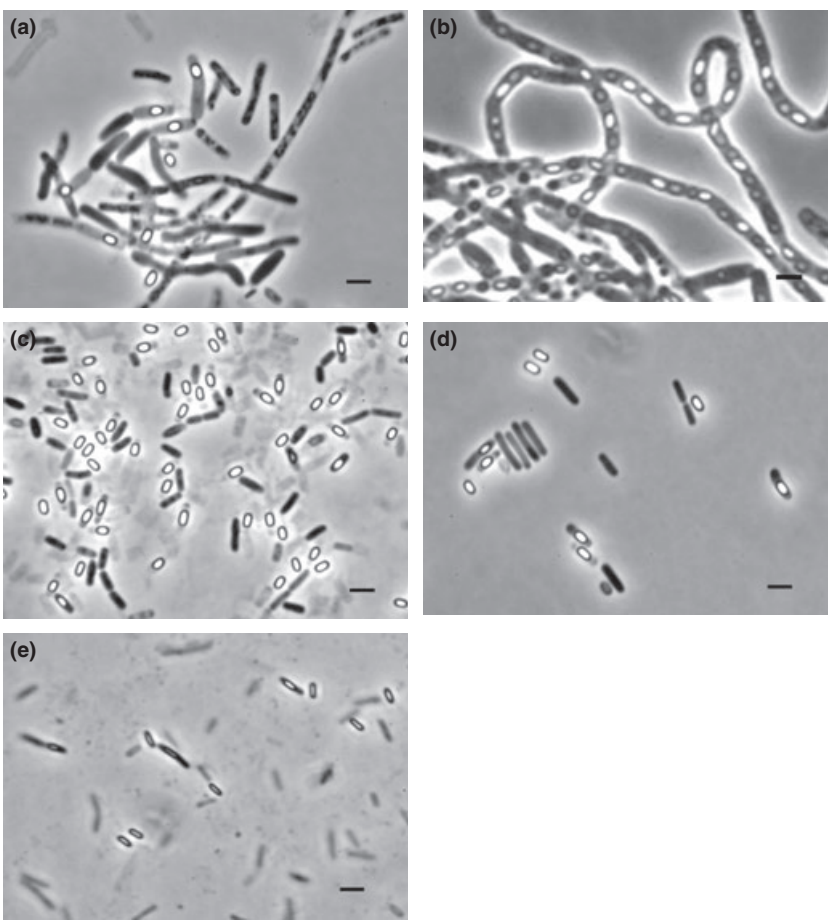

Figure 1 Photomicrographs of *Bacillus* species viewed by phase-contrast microscopy. Bar markers represent 2 μm. (a) *Bacillus cereus*: showing some poly-β-hydroxybutyrate inclusions, which are smaller and less phase-bright than the spores; (b) *Bacillus thuringiensis*: showing parasporal crystals of insecticidal toxin, which are less phase-bright than the spores; (c) *Bacillus licheniformis*; (d) *Bacillus subtilis*; (e) *Bacillus pumilus*.

Taiwan and the Netherlands, but rare in the UK and Japan – where the emetic syndrome was more prevalent (Kotiranta *et al.* 2000).

Both syndromes arise as direct results of the fact that *B. cereus* spores can survive normal cooking procedures. Under improper storage conditions after cooking, the spores germinate and the vegetative cells multiply. The capacity of the strain concerned to produce toxin(s) will, of course, influence the infective or intoxicating dose in both types of illness. Cases with both diarrhoeal and emetic symptoms may be caused by single strains producing both diarrhoeal and emetic toxins, or by the presence of both diarrhoeal strains and emetic strains in the food responsible (Pirhonen *et al.* 2005). Regulation of production of *B. cereus* toxins and its implications for food safety were reviewed by Ceuppens *et al.* (2011); they concluded that the complexity of toxin expression is still not well understood and that the influences of food components, temperature and other environmental factors need investigation within the relevant foods and intestinal environments.

Diarrhoeal illness

This syndrome is characterized by abdominal pain with watery diarrhoea 8–16 h after ingestion of the contaminated food, and it is associated with a diversity of foods from meats and vegetable dishes to pastas, desserts, cakes, sauces and milk. Symptoms usually resolve within 24–48 h. However, in a large outbreak that affected 44 elderly persons and that was associated with the consumption of vegetable purée, six patients developed bloody diarrhoea and three died of necrotic enteritis (Lund *et al.* 2000).

Following the ingestion of spores or vegetative cells, one or more enterotoxins are produced by vegetative cells in the small intestine, where they are believed to cause diarrhoea by damaging the integrity of ileal epithelial cell membranes. Infective doses range from 10^4 to 10^9 cells per gram of food; variations in infective dose will reflect the proportion of ingested cells that are sporulated, and which therefore can survive the acid barrier of the stomach.

Bacillus cereus produces a range of protein toxins, of which three have been implicated in diarrhoeal illness. These enterotoxins are chromosomally encoded:

1. Haemolysin BL (Hbl) is a proteinaceous toxin that also has dermonecrotic and vascular permeability activities and that causes fluid accumulation in ligated rabbit ileal loops. Genes encoding Hbl are carried by about 50–66% of strains tested (Granum 2002; Ngamwongsatit *et al.* 2008; Ankolekar *et al.* 2009), and it was formerly believed to be the primary virulence factor in *B. cereus* diarrhoea, but outbreaks associated with strains lacking this toxin have occurred (Granum *et al.* 1996). It is believed to cause osmotic lysis by forming a transmembrane pore, following independent binding of its three components B, L_1 and L_2 to the host cell (Stenfors Arnesen *et al.* 2008).

2. Nonhaemolytic enterotoxin (Nhe) is another three-part proteinaceous, pore-forming toxin that is structurally similar to Hbl; its components are a cytolytic protein NheA and two binding components NheB and NheC. It appears that all *B. cereus* strains carry genes encoding Nhe (Ngamwongsatit *et al.* 2008; Stenfors Arnesen *et al.* 2008; Ankolekar *et al.* 2009). It was discovered following a Norwegian outbreak that was caused by an Hbl-negative strain, and it is now believed to be the most dominant diarrhoeal toxin (Stenfors Arnesen *et al.* 2008). Production of both Hbl and Nhe is believed to be restricted to members of the *B. cereus* group (From *et al.* 2005).

3. Cytotoxin K (CytK) is a single-component, β-barrel pore-forming toxin that belongs to the same family of toxins as *Clostridium perfringens* β-toxin. It is dermonecrotic, cytotoxic and haemolytic, and nearly 90% *B. cereus* strains may carry the gene for it (Ngamwongsatit *et al.* 2008). It was originally isolated from the strain responsible for a French outbreak of necrotic enteritis in which three people died (Lund *et al.* 2000) and was considered responsible for the severity of illness and bloody diarrhoea; however, Nhe may also have been involved (Stenfors Arnesen *et al.* 2008). This toxin occurs in two forms that have 89% amino acid sequence homology, CytK-1 and CytK-2 (Fagerlund *et al.* 2004). The former was associated with the French necrotic enteritis outbreak and is the more aggressively cytotoxic. The phylogenetically distinct strain concerned has a smaller genome than other members of the *B. cereus* group (Lapidus *et al.* 2008); the names '*Bacillus cytotoxis*', '*Bacillus cytotoxicus*' and '*B. cereus* subsp. *cytotoxis*' have been used for it, but the taxon has not been validly published.

Although a psychrotolerant member of the *B. cereus* group, *B. weihenstephanenis,* has been described, it is not the only psychrotolerant species in the group (Stenfors and Granum 2001). Samapundo *et al.* (2011) examined 380 isolates of *B. cereus* from foods in Belgium, and although they found only 10 strains were psychrotolerant (growing at ≤7°C), 88% of their strains could grow at ≤10°C. Of the 80 strains that they examined for toxin genes, over 50% carried all the genes for Hbl, Nhe and CytK production, 84% carried all three *hbl,* 70% carried all three *nhe* and 72% carried *cytK*, and only three strains lacked the capacity to produce any of the enterotoxins.

Although there is evidence that Nhe is the most dominant in diarrhoeal illness (Stenfors Arnesen *et al.* 2008), it is possible that combinations of toxins may act synergistically. As most strains produce more than one enterotoxin,

it is difficult to determine the *in vivo* activity of an individual toxin. Other cytotoxins (including enterotoxin FM, or EntFM) and haemolysins (such as HlyII), collagenases and phospholipases, are produced by *B. cereus*, and they may contribute to diarrhoeal illness, but their individual roles remain uncertain (Ceuppens *et al.* 2011).

Toxicity was initially detected nonspecifically in laboratory animals, such as by observing for fluid accumulation after injection of ligated ileal loops of rabbits, and tissue culture assays followed. Hbl and Nhe may be specifically detected in foods by two commercially available, antibody-based kits, the Oxoid BCET-RPLA and the TECRA-BDE, respectively. The former uses reversed passive latex agglutination to detect the L_2 component of Hbl, while the latter is based upon an enzyme-linked immunosorbent assay for the NheA component of Nhe. Because only one component of each three-component toxin is detected by these kits, they cannot demonstrate with certainty the presence of the complete, active toxin. However, where tissue culture assay results have been compared with those from kits, they have correlated quite well (Fermanian *et al.* 1996; Fletcher and Logan 1999). Although the genes for these toxins are evidently widely carried by soil and food isolates of *B. cereus*, it seems certain that toxin production must vary widely among strains – because percentages of strains positive for toxicity by tissue culture assay (Fletcher and Logan 1999; From *et al.* 2005) are not as high as those for toxin gene carriage. Indeed, if all strains carrying enterotoxin genes did produce appreciable amounts of these toxins, we might expect incidents of *B. cereus* diarrhoeal food poisoning to be very much more frequent.

Emetic illness

The emetic syndrome emerged in the UK in the 1970s (Mortimer and McCann 1974) and is characterized by nausea and vomiting 0·5–6 h after eating the offending food – predominantly oriental rice dishes, although occasionally other foods such as pasteurized cream, milk pudding, pasta dishes and reconstituted infant formulas have been implicated. It is an intoxication that is caused by a single highly heat-, proteolysis-, acid- and alkali-resistant toxin that is preformed in the food and then ingested – hence its rapid onset. Foods responsible usually carry about 10^5–10^8 cells per gram to produce sufficient toxin, and levels of toxin production are influenced by the nature of the food (Agata *et al.* 2002; Shaheen *et al.* 2006), but any cooking or reheating that may kill the cells will leave the toxin unscathed. One incident involving seven people was attributed to indirect ingestion via contaminated hands, following the use of contaminated rice in a children's craft activity (Briley *et al.* 2001). Cases are usually sporadic, but a large emetic outbreak affecting 43 children and three adults who had consumed rice pudding has been reported from Berlin. The organism was isolated from one vomit specimen, but could not be shown to produce the toxin or to carry the *ces* gene for it, nor was cereulide detected in the vomit. However, epidemiological and clinical findings were consistent with cereulide being responsible (Kamga Wambo *et al.* 2011). Recovery usually occurs within 24 h, but some outbreaks have included fatalities, particularly in children, with cases of liver failure following consumption of pasta salad (Dierick *et al.* 2005), liver failure with brain oedema after eating pasta (Mahler *et al.* 1997), and brain oedema with liver necrosis (Shiota *et al.* 2010) associated with reheated fried rice. A 9-year-old girl with fulminant hepatitis, renal and pancreatic insufficiency, shock and seizures recovered owing to rapid diagnosis and supportive care (Posfay-Barbe *et al.* 2008), while the recovery of an 11-year-old boy who contracted acute encephalopathy and liver failure following the consumption of fried rice was marred by a mild impairment of intelligence (Ichikawa *et al.* 2010). A fatal case associated with fatty degeneration of the heart and liver, in a boy who had eaten Chinese noodles (Takabe and Oya 1976), was also probably owing to emetic toxin.

The toxin, cereulide, is a small ring-formed dodecadepsipeptide comprising a ring of four amino- and/or oxy-acids: $[\text{D-}O\text{-Leu-D-Ala-L-}O\text{-Val-L-Val}]_3$. Chemically speaking, it is closely related to the potassium ionophore valinomycin (Agata *et al.* 1994). It is produced by a non-ribosomal peptide synthetase at the end of logarithmic growth, and the cereulide synthetase gene (*ces*) cluster is borne on a megaplasmid, called pBCE, that is related to the pXO1 virulence plasmid of *B. anthracis* (Ehling-Schulz *et al.* 2006). Cereulide production is largely restricted to a single evolutionary lineage within the *B. cereus* group that also contains *B. anthracis* (Ehling-Schulz *et al.* 2005; Guinebretière *et al.* 2010), and is associated with a specific biotype and particular serovars of *B. cereus* (Logan *et al.* 1979). The toxin has fungistatic activity that may be of value to a producing organism in the natural environment (Ladeuze *et al.* 2011). Its mechanism of action in food poisoning is unclear, but it has been shown to stimulate the vagus afferent through binding to the 5-HT3 receptor (Agata *et al.* 1995) and, of relevance to liver failure, it inhibits fatty acid oxidation by toxicity to mitochondria (Mikkola *et al.* 1999). Cereulide production does not appear to be connected with sporulation (Finlay *et al.* 2000), it is produced in aerobic and microaerobic, but not in anaerobic conditions (Finlay *et al.* 2002), and transcription of *ces* is lowered by salt (Dommel *et al.* 2011). It is produced in larger amounts at lower incubation temperatures (12–22°C), yet although its production

by *B. cereus* has not been demonstrated at temperatures below 12°C, the related psychrotolerant species *B. weihenstephanensis* may produce detectable cereulide at 8°C (Thorsen *et al.* 2006). Frenzel *et al.* (2011) found that the addition of polyphosphates to foods, even at levels too low to affect vegetative cell integrity, interfered with and reduced the production of cereulide.

The toxin is not antigenic, and there is no commercial assay available for it – although attempts have been made to develop one. The earliest detection system for emetic toxin involved monkey-feeding tests (Logan *et al.* 1979); this was followed by cell culture assays (Finlay *et al.* 1999) and a boar sperm motility inhibition assay (Andersson *et al.* 1998). While these are not specific for the toxin, heat treatment of extracts prior to assay will destroy any heat-labile toxins. Specific detection can be achieved using HPLC-MS or LC-MS (Häggblom *et al.* 2002; Ladeuze *et al.* 2011), and a real-time PCR-based assay for detecting *ces* has been developed (Fricker *et al.* 2007). Ghelardi *et al.* (2002) used PCR amplification of toxin genes in conjunction with RAPD-PCR and multiplex RAPD-PCR to trace the source of two outbreaks of emetic food poisoning. Bauer *et al.* (2010) developed a stable isotope dilution analysis using $^{13}C_6$-cereulide as an internal standard for the quantification of cereulide in foods by LC-MS/MS.

Bacillus thuringiensis

Bacillus thuringiensis (Fig. 1b) is well known as an insect pathogen. Preparations of certain strains are widely used as biopesticides, and transgenic crop plants that express the insecticidal crystal toxin genes have been developed, but there is as yet no evidence of infections directly associated with the use of this organism as an insecticide. Occupational exposure to the organism has been connected with presence of the organism in faeces, but without gastrointestinal symptoms. A review of the safety of using *B. thuringiensis* as a biopesticide on crop plants found that the main pesticide strains assayed produced low titres of enterotoxin (Bishop 2002). There have been few reports of gastroenteritis outbreaks in which *B. thuringiensis* was implicated (McIntyre *et al.* 2008; Stenfors Arnesen *et al.* 2008), but cases of illness caused by this species may have been attributed to *B. cereus*, as the former may not produce its characteristic insecticidal toxin crystals when incubated at 37°C, owing to the loss of the plasmids carrying the genes encoding them. Strains of *B. thuringiensis* commonly carry genes for *B. cereus* enterotoxins chromosomally; indeed, Ngamwongsatit *et al.* (2008) found that strains of this species carried *hblCDA* more frequently (nearly 87%) than *B. cereus* strains, but that their carriage of *nheABC* and *cytK* were at similar levels (100 and 84%, respectively) to *B. cereus*. Ankolekar *et al.* (2009) found their *B. thuringiensis* isolates from rice to be positive for Hbl and Nhe by the Oxoid and TECRA assays, but they did not detect the gene for cereulide. Even isolates of *B. thuringiensis* from Antarctic soils (Forsyth and Logan 2000) have been found to carry enterotoxin genes (Prabhakar and Bishop 2011). Fletcher and Logan (1999) also found that strains of *B. thuringiensis* were positive in the commercial tests for enterotoxin and in a cytotoxicity assay.

Bacillus weihenstephanensis and *Bacillus mycoides*

Bacillus weihenstephanensis was proposed (Lechner *et al.* 1998) to accommodate a group of psychrotolerant strains of *B. cereus* that appeared to be phylogenetically distinct from mesophilic strains of *B. cereus.* These organisms belong to the same phylogenetic group as *B. mycoides*, and neither species appears to carry *cytK* (Guinebretière *et al.* 2010). They are also distinct from the *B. anthracis* and *B. cereus* emetic biotype lineage (Priest *et al.* 2004; Guinebretière *et al.* 2010). However, not all psychrotolerant members of *B. cereus* belong to *B. weihenstephanensis*, and intermediate strains carrying markers for both psychrotolerance and mesophily exist (Stenfors and Granum 2001). These authors found that of 26 food, dairy and clinical strains of *B. cereus* – four of which could be identified as *B. weihenstephanensis* – all carried genes for Nhe, while the distribution of genes for Hbl did not show any correlation between the species. Later work on 39 dairy isolates also found only four strains that were identifiable as *B. weihenstephanensis*, and none of these tested consistently as cytotoxic in cell culture assay (Stenfors Arnesen *et al.* 2007). There have been no reports of diarrhoeal outbreaks associated with *B. weihenstephanensis*, but any such outbreak would almost certainly be ascribed to *B. cereus* unless the organism concerned underwent extensive investigation.

Although cereulide production is largely restricted to a narrow range of *B. cereus* strains, the genetic determinants for cereulide are located on a plasmid and so are potentially transferrable to other species (Van der Auwera *et al.* 2007). Thorsen *et al.* (2006) screened 921 *B. cereus* group isolates from soil and found that the only two strains that produced cereulide, as determined by liquid chromatography-high-resolution mass spectrometry, were identifiable as *B. weihenstephanensis*. These two strains hydrolysed starch and produced acid from salicin, unlike the characteristic *B. cereus* emetic biotype. Study of an emetic *B. weihenstephanensis* strain and mesophilic *B. cereus* emetic strains showed that cereulide was not produced at 5°C and was only weakly produced at 8°C (Thorsen *et al.*

2009). This is consistent with the findings of Finlay *et al.* (2000) and Carlin *et al.* (2006) and with the circumstances of many emetic outbreaks where inadequately refrigerated foods have been implicated. There have been no reports of emetic outbreaks associated with *B. weihenstephanensis* but, as with diarrhoeal outbreaks, only thorough characterization of an implicated isolate could identify it to this species.

Bacillus mycoides is an occasionally encountered soil inhabitant and long-established member of the *B. cereus* group that is characterized by its rhizoid ('hairy-looking') colonial morphology (Fig. 2). Fletcher and Logan (1999) demonstrated cytotoxicity, and positive results for the Oxoid and TECRA assays in several strains of this species, but there have been no reports of illness associated with this species.

Other species

Reports of infections with aerobic endosporeformers outside the *B. cereus* group are comparatively rare, but very diverse, and usually occur in immunocompromised patients, or those with implanted devices or trauma (Logan *et al.* 2011). The species most commonly reported from these infections are the same ones that have been isolated in association with foodborne illness: *B. licheniformis, B. subtilis* and *B. pumilus.*

Bacillus licheniformis (Fig. 1c) can cause foodborne illness, with nausea, vomiting, diarrhoea and stomach cramps occurring 5–12 h after consumption of a variety of foods that have included ice cream, deserts, meat pies and sandwiches; counts, where made, ranged from 3×10^5 to 1×10^8 CFU g^{-1} of the implicated food (Salkinoja-Salonen *et al.* 1999) but ingested doses are unknown. In the case of a child fatality associated with infant feed (formula), a surfactant called lichenysin A, which is a heat-stable cyclic lipopeptide, was implicated (Mikkola *et al.* 2000). *Bacillus licheniformis* and *B. subtilis* strains may produce surfactin, a very powerful surfactant with structural similarity to lichenysin A (Peypoux *et al.* 1999) and to cereulide. Neiminen *et al.* (2007) isolated from mastitic milk two *B. licheniformis* strains that carried the lychenysin synthetase genes *lchAA, lchAB* and *lchAC* and which produced heat-stable toxins capable of immobilizing boar sperm. However, even though it is structurally similar to cereulide, the toxic action of lichenysin A appears to be different; it has the potential to form ion channels in host cell membranes and has a surfactant effect, rather than the toxicity for mitochondria that is observed with cereulide (Mikkola *et al.* 2000).

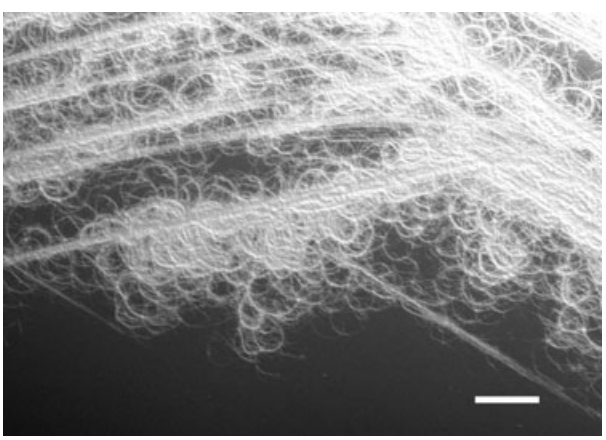

Figure 2 *Bacillus mycoides* on blood agar after 24–36 h at 37°C. Bar marker represents 2 mm.

The name *B. subtilis* (Fig. 1d) was often used prior to the 1970s as an identity for any clinical *Bacillus* isolate, but in the last 30–40 years there have been reports of infection in which this species appears to have been identified accurately. *Bacillus subtilis* has been implicated in foodborne illness with vomiting as the commonest symptom, but accompanying diarrhoea was frequently reported too. The onset periods have been short (ranging from 10 min to 14 h; median 2·5 h), the bacterial loads of the organism were high (10^5–10^9 CFU g^{-1}) and so it may be inferred that the ingested doses were also high, and the implicated foods were often prepared dishes in which meat or fish were served with cereal-based components such as bread pastry, rice or stuffing. Duc *et al.* (2005) reported repeated attacks of projectile vomiting in two children, of nine years and of 12 months age following consumption of an infant cereal product. When the mother ate the product from a new can of the same batch she experienced nausea without vomiting. Strains of *B. subtilis* and *B. cereus* were isolated, but the latter was not of the biotype associated with emetic toxin production, and heat-stable toxin production could not be demonstrated. It was therefore suspected that the *B. subtilis* may have, at least in part, been responsible for the illness, but it was not possible to confirm it. This species is the main cause of a kind of spoilage in bread called ropiness, and foodborne illness has occasionally been associated with the consumption of ropy bread; usually, however, such spoiled bread is too unattractive to be consumed, and so illness is avoided (Rosenkvist and Hansen 1995). Stickel *et al.* (2009) reported further examples of this species being implicated in food-associated illness; two patients presented with liver damage following long-term consumption of nutritional supplements. *B. subtilis* was isolated from the products and one strain was shown to be hepatotoxic by Hep2G cell culture assay.

Apertroaie-Constantin *et al.* (2009) found that a strain of *B. subtilis* from foodborne illness following consumption

of a chicken korma in the UK, and four strains of this species and one of *B. mojavensis* from illness associated with pumpkin curry in Finland, all produced the heat-stable, nonprotein toxin known as amylosin. Amylosin has been characterized as an ionophore that forms K^+ and Na^+ channels in host cell membranes (Mikkola *et al.* 2007), and it was first detected in *B. amyloliquefaciens* (see below). *Bacillus mojavensis* (Roberts *et al.* 1994) was proposed on the basis of isolates from desert soils, and it is a very close relative of *B. subtilis*; indeed, it is indistinguishable from it by routine phenotypic tests and the practical value of its distinction has been questioned (Logan and De Vos 2009). However, strains identified as belonging to this species have been isolated from food and found to be toxigenic by cytotoxicity assay (From *et al.* 2005). From *et al.* (2007b) found that a strain of *B. mojavensis* isolated from herbs produced cytotoxic, surfactin-like, heat-stable cyclic lipopeptide with putative emetic character, and Apertroaie-Constantin *et al.* (2009) found that this strain also produced amylosin. Two other strains of this species from spices and food have also been shown to produce heat-stable toxins (From *et al.* 2005).

Bacillus amyloliquefaciens, another close relative of *B. subtilis*, is widely used industrially for enzyme and amino acid production, but human consumption of L-tryptophan manufactured in an organism genetically engineered from a strain of this species was associated with a large epidemic of eosinophilia-myalgia syndrome with 37 deaths; the causative agent has not been identified with certainty (Mikkola *et al.* 2004). Environmental strains of this species producing amylosin were first isolated in association with building-related ill health problems (Mikkola *et al.* 2004).

Strains of *B. pumilus* (Fig. 1e) have been isolated in association with foodborne illness and from clinical to environmental specimens, and toxicity was first demonstrated in strains from building-associated ill health, recycled wood pulp, a spruce tree and the type strain (Suominen *et al.* 2001). Strains producing heat-stable toxin have also been isolated from mastitic cow milks (Neiminen *et al.* 2007). Another heat-stable cyclic lipopeptide that is structurally similar to surfactin, pumilacidin (Naruse *et al.* 1990), was implicated in an outbreak associated with reheated rice in a Chinese restaurant (From *et al.* 2007a); acute symptoms of dizziness, headache and back pain developed during the meal, and diarrhoeal illness and stomach pain followed and recurred for 2 weeks.

Heat-stable toxin production has also been demonstrated in strains of *Bacillus firmus*, *B. megaterium* and *B. simplex* (Taylor *et al.* 2005) and heat-labile toxin from *Bacillus* (now *Lysinibacillus*) *fusiformis* (From *et al.* 2005), but there have been no reports of these species being associated with foodborne illness. The only report of gastro-enteric illness connected with an aerobic endospore-former outside the genus *Bacillus* was of a waterborne outbreak in Sweden following the accidental overload of a purification plant with raw water. Strains subsequently identified as *Brevibacillus agri* were isolated. Four towns with a total population of 84 500 were involved, with most affected households complaining about water odour and taste and many persons reporting abdominal pain, diarrhoea, myalgia, vomiting and fever just short of 24 h after drinking the water. Most patients recovered in 1–4 days (Logan *et al.* 2002).

Given that spores of *Bacillus* species are so widely distributed and that they so commonly contaminate our food and survive processing, it is surprising that they are not isolated from cases of foodborne illness more frequently. It is also not clear why episodes involving *B. licheniformis, B. subtilis* and *B. pumilus* are so rarely reported. However, the pre-eminence of *B. cereus* as an endospore-forming, food-poisoning organism may be explained in part by the ability of strains of this species to grow faster than members of the *B. subtilis* group and to out-compete them. Crielly *et al.* (1994) found that spores of *B. cereus* were found in smaller numbers than those of *B. licheniformis* in raw and pasteurized milk and in reconstituted milk powders. However, these *B. cereus* spores germinated more rapidly and their vegetative cells grew faster in milks incubated at ambient temperature – so that *B. cereus* soon came to dominate the *Bacillus* population.

Probiotics

Bacillus species are attractive as probiotics because their spores will not only survive the acid barrier of the stomach, but also permit long shelf lives in commercial products. Such products are currently more widely marketed in SE Asia, especially Vietnam, than in Europe and the USA, but interest in the West is increasing rapidly (Cutting 2011). The probiotic use of aerobic endosporeformers goes back over 50 years, and there has been a huge increase in scientific interest over the last 15 years. *B. cereus, B. clausii, B. coagulans, B. licheniformis, B. pumilus* and *B. subtilis* have attracted most interest and appear in a range of products for human and animal use; also used are *Brevibacillus laterosporus* and *Paenibacillus polymyxa*. However, some products are mislabelled and contain different species to what is listed on the label – in several cases, for example, they contain *B. cereus* instead of *B. subtilis* (Cutting 2011). Another source of confusion is the widespread use of scientifically invalid names; '*Bacillus polyfermenticus*' is a trade name for a member of the

B. subtilis group, while '*Lactobacillus sporogenes*' is an invalid synonym for *B. coagulans*.

It is a matter of potential concern that strains representing all of the aerobic endospore-forming species used in probiotics have been isolated in association with human infections or foodborne illness. On the other hand, it is perhaps surprising that reports of illness associated with such dietary supplements are not seen more often, just as it was noted previously that cases of foodborne illness attributable to *Bacillus* strains are fewer than might be expected. The author is aware of only one report of human infection associated with probiotic *Bacillus* use. In this case, a long-established product for preventing intestinal disorders, labelled as containing *B. subtilis* (but which actually contained several strains of *B. clausii*), led to a fatal septicaemia in an immunocompromised patient (Spinosa *et al.* 2000). A further concern about the use of aerobic endosporeformers in probiotic preparations, both for humans and for other animals, arises from the potential of these organisms to disseminate any antibiotic resistance genes that they might carry. The European Commission's Scientific Committee on Animal Nutrition has accordingly discouraged the use of strains from the *B. cereus* group, because of their potential to cause illness in humans and animals, and has deemed certain other animal products as unsafe because of the dangers of disseminating resistance to clinically important antibiotics such as erythromycin and lincosamides (e.g. lincomycin and clindamycin) (Logan 2004).

As the foregoing has attempted to show, our understanding of aerobic endospore-forming bacteria as members of the gut flora, as agents of foodborne illness and infection and as nutritional supplements has greatly increased over the last decade, and it is noteworthy that a rather small number of species names appear in all three of these contexts. We might hope to develop a more integrated view of their roles in the gut over the decade to come.

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