UNIVERSITÀ DEGLI STUDI DI NAPOLI

"FEDERICO II"

DOTTORATO IN BIOLOGIA APPLICATA INDIRIZZO MICROBIOLOGIA

Characterization of *Lactobacilli* and *Bacilli* of intestinal origin

Tutor :

Ch.mo Prof.

Ezio Ricca

Candidato:

Fakhry S. Saad

Coordinatore:

Ch.mo Prof.

Ezio Ricca

INDICE

Chapter one

Introduction

INTRODUCTION

1. Intestinal microflora and probiotics

The concept that the endogenous microflora has a crucial role in host health has been indipendently suggested by Pasteur, Koch, Metchnikoff and Escherich during the very early years of modern microbiology (Hopper et al. 1998). Since then, it has been claimed that the composition of the microflora is essential for the well-being of the host and the importance of interactions between host and bacteria has been emphasized (Hopper et al. 1998). Despite these early insights, the study of bacteria-host interactions has been for many years focused exclusively on those involving pathogenic organisms. Only recently, scientists have started to study the molecular mechanisms mediating the interaction of non-pathogenic bacteria with the mucosal surfaces of their host (Hopper et al. 1998).

Very recently, metagenomic data have shown that the adult human gut houses a bacterial community (*microbiota*) containing trillions of members comprising thousands of species-level phylogenetic types (phylotypes). Two bacterial phyla, the Firmicutes and the Bacteroidetes, commonly dominate this ecosystem (Mahowald et al. 2009), as they do in the guts of at least 60 mammalian species (Ley et al. 2008). Progress made with 16S rDNA-based enumerations has disclosed significant differences in community membership between healthy adults (Gill et al. 2006), differences that may contribute to variations in normal physiology between individuals or that may predispose to disease. For example, studies of gnotobiotic mouse models indicate that mutualistic relations with the gut microbiota influence maturation of the immune system, modulate responses to epithelial cell injury, affect energy balance, and support biotransformations that we are not equipped to perform on our own, including processing of xenobiotics.

Several bacterial species are used in commercial preparations (*probiotics*) that contain live bacteria and that, when orally administered, are thought to have a positive effect on human health by restoring an active and balanced microflora after antibiotic treatments or other stressful conditions. This practice has started in a rather empirical way much before that the interactions between the microbiota and the host were studied in details. There are now many proposed mechanisms by which probiotics may positively affect the animal health. Listed below is a brief

description of mechanisms by which probiotics may protect the host cells against intestinal diseases.

Production of inhibitory substances. Probiotic bacteria produce a variety of substances that are inhibitory to both gram-positive and gram-negative bacteria. These inhibitory substances include organic acids, hydrogen peroxide and bacteriocins. These compounds may reduce not only the number of viable cells but may also affect bacterial metabolism or toxin production.

Blocking of adhesion sites. Competitive inhibition for bacterial adhesion sites on intestinal epithelial surfaces is another mechanism of action for probiotics (Conway et al. 1987; Goldin et al. 1992; Kleeman and Klaenhammer. 1982). Consequently, some probiotic strains have been chosen for their ability to adhere to epithelial cells.

Competition for nutrients. Probiotics may utilize nutrients otherwise consumed by pathogenic microorganisms. However, the evidence that this occurs *in vivo* is lacking.

Degradation of toxin receptor. The postulated mechanism by which *S. boulardii* protects animals against *C. difficile* intestinal disease is through degradation of the toxin receptor on the intestinal mucosa (Castagliuolo et al. 1996 and 1999; Pothoulakis et al. 1993).

Stimulation of immunity. Recent evidence suggests that stimulation of specific and nonspecific immunity may be another mechanism by which probiotics can protect against intestinal diseases (Fukushima et al. 1998; Kaila et al. 1992; Link-Amster et al. 1994; Malin et al. 1996; Perdigon et al. 1986). For example, oral administration of *Lactobacillus* GG during acute rotavirus diarrhea is associated with an enhanced immune response to rotavirus (Kaila et al. 1992). This may account for the shortened course of diarrhea seen in treated patients. The underlying mechanisms of immune stimulation are not well understood, but specific cell wall components may act as adjuvants and increase humoral immune responses.

Most probiotics marketed today contain live cells of various bacterial species belonging to the *Lactobacillus* and *Bifidobacterium* genera, commensal bacteria normally found in the GIT, although not as numerically predominant genera. More

surprising is the use in several commercial probiotic preparations of bacteria not considered as normal inhabitant of the intestine, i.e. bacteria of the *Bacillus* genus, aerobic spore formers normally considered as soil organisms (Serror et al. 2002).

2. The *Lactobacillus, Bifidobacterium* and *Bacillus* genera

2.1 *Lactobacillus*

Lactobacillus is a genus belonging to the group of Lactic Acid Bacteria (LAB), a widespread group of gram-positives that are catalase-negative, anaerobic (microaerophilic) and non motile organisms. To this heterogeneous group of bacteria, that includes mesophilc as well as thermophilic species, belogs several genera of cocci (*Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus*) and rods (*Carnobacterium, Lactobacillus*). Most species of these genera are ubiquitous in the environment, occupying niches ranging from plant surfaces to the gastrointestinal tracts of many animals. They are, in general, nutritonally fastidious organisms requiring several amino acids, vitamins, cofactors and metabolic intermediates.

With a few exceptions, LAB derive all of their energy from conversion of glucose to lactate or to lactate and a mixture of other products via the homofermentative or heterofermentative pathway, respectively. During homolactic fermentation, 85 to 95 % of the sugar utilized is converted to lactic acid; in contrast during heterolactic fermentation only 50 % of the sugar utilized is converted to lactic acid. The remaining products of heterolactic fermentation are carbon-dioxide and ethanol, which is sometimes further converted to acetate. These pathways generate ATP by nonoxidative substrate-level phosphorylation; in the homofermentative pathway, the net energy yield is of 2 molecules of ATP per molecule of glucose. In the heterofermentative pathway the net energy yield is 1 molecule of ATP per molecule of glucose under strictly anaerobic conditions and 2 molecules of ATP per molecule of glucose in aerobic or microaerophilic conditions, because of the presence of an alternative electron sink for cofactor regeneration. Some strains have also been shown to derive energy from a pH gradient established by carrier-mediated lactate efflux (Chassy et al. 1993). Phylogenetically all LAB constitute with Bacilli and Streptococci a cluster within the clostridial subbranch of the gram-positive eubacteria that has low G+C contents in their DNAs.

Several species of the genus *Lactobacillus* are used for many food fermentations, and although their use in the dairy industry is of particular economic importance, they are also essential for non-dairy fermentations (production of sausages, olives, pickled vegetables and silage). Lactobacilli are also the source of various bacteriocins (Adlerberth et al. 1996) and are found as commensals in the gut of human and animals. Although several species of this genus contain plasmids, of particular interest are three plasmids of *L. gasseri*, of 150, 50 (Roussel et al. 1993) and 48 (El-Osta et el.1996) kbp, that share the uncommon feature of being linear. A number of strains have insertion sequences (IS), like the ISL*1* and IS*1201* of *L. casei* (Shimizu et al.1985) and *L. helveticus* (Serror et al. 2002), respectively, and genetic tools have been developed for various species (Serror et al. 2002; Hols et al 1994).

The positive effects exerted by several strains of *Lactobacillus* on animal health have been attributed to a reduced or excluded pathogen adhesion by steric hindrance, to the production of biosurfactants or antimicrobial substances and/or to the stimulation of host immune system. All these mechanisms represent examples of antagonistic activities exerted commensal bacteria against pathogens.

In LAB the ability to adhere to epithelial cells does not depend on a unique and ubiquitous mechanism and a great variability, depending on the LAB strain, is generally observed. For example, for some strains of *L. fermentum* isolated from intestinal and urogenital epithelia, protein-mediated mechanisms have been described. In *L. fermentum* 104R a surface protein of 29 kDa has been purified and its ability to bind intestinal mucus has been tested by *in vitro* experiments (Rojas et al. 2002). This protein does not contain carbohydrate moieties, it is anchored to the cell surface by non-covalent interactions and its sequence has only poor homologies with previously published sequences. Dot blot assays showed that this protein is responsible of bacterial adhesion to both partially purified gastric mucin and small intestinal mucus layer from pig (Rojas et al. 2002). Independently, a different *L. fermentum* strain (RC-14) isolated from the urogenital tract of an healty woman, was reported to produce a 29 kDa protein responsible of *L. fermentum* adhesion and able to inhibit adhesion of the uropathogenic strain of *Enterococcus faecalis* 1131 to intestinal mucus (Heinemann et al. 200)). Although more information is needed to compare the two proteins of similar size, it is tempting to hypothesize a common protein-mediated mechanism to promote the adhesion of *Lactobacillus* strains to mucus. For some lactobacilli adhesion has been attributed

to the presence of the S-layer, a proteinaceus structure assembled around the cell to form a two-dimensional layer, generally constituted by a single polipeptide (Sara and Slevtr. 2000). Schneitz et al. (1993) described the potential involvment of S-layer in promoting adhesion of *L. acidophilus* to chicken intestinal epithelial cells (Sara and Slevtr. 2000). More recently, the analysis of S-layer genetics revealed the presence of two interchangeable genes coding for S-layer proteins. It has been proposed that a phase-variation mechanism in certain intestinal conditions may induce a phase-switch, thus conferring a competitive advantage during host colonization (Schneitz et al. 1993). For the industrial strain *L. johnsonii* LA1, lipoteichoic acids (LTA) have a role in bacterial adherence. As shown by Granato et al. (1999), a preincubation of Caco-2 cells with increasing amount of LTA resulted in a strong inhibition of adhesion in a dose-dependent manner, thus demonstrating their involvement in the adhesion process (Granato et al. 1999).

Many molecules with antimicrobial activity are produced by *Lactobacillus* species. A, so far, uncharacterized factor with an *in vitro* and *in vivo* antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria is produced by a human isolate of *L. acidophilus*. Such molecule is peculiar in that it is able to cross the epithelium and penetrate the host cells playing an antagonistic activity against *Salmonella enterica* serovar *Typhimurium* SL1344 one hour after infection, when the intracellular pathogen is localized inside the epithelial cell (Coconnier et al. 2000).

L. casei strain Shirota has been reported to have an antagonistic activity against Shiga toxin-producing *E. coli* (STEC) (Ogawa et al. 2001). Newborne rabbits, used as experimental animal model, daily administrated with *L. casei* cells showed a significant decrease in the severity of diarrhea and histological damages to the intestinal mucus induced by STEC infection with respect to control animals (Ogawa et al. 2001). In this case, the antagonistic effect was attributed to the enhancement of the local immune response to STEC cells and Shiga toxins leading to the elimination of the pathoghen and the decrease of toxin concentration in the intestine.

Several *Lactobacillus* strains have been reported to display stimulatory properties on cells of the innate immune system *in vitro* (Haller et al. 2000; Kato et al. 1983; Kato et al. 1984) and *in vivo* (Perdigon et al.1991; Perdigon et al. 1995; Perdigon et

al. 1986; Perdigon et al. 1988) and immunomodulatory effects have been observed also in humans administered with fermented milk products containing LAB strains

2.2 *Bifidobacterium*

Bifidobacterium is a group of Gram-positive, anaerobic, branched rod-shaped bacteria. In the intestines, they ferment sugars to produce lactic acid.

These bacteria are able to metabolize so-called "nondigestible" plant polymers or host-derived glycoproteins and glycoconjugates. It is thought that Bifidobacteria's ability to colonize the gastrointestinal tract is partly partly due to the large variety of molecules that they are able to use as energy source (Schell *et al*. 2002).

Bifidobacteria produce a range of antimicrobial agents that are active against both Gram-positive and -negative organisms (Gibson et al. 1994).

Bifidobacterium infantis, B. brevi, and *B. longum* are the largest group of bacteria in the intestine of infants, where they are particularly abundant to decline in number with age. In breast-fed infants, Bifidobacteria have been reported to constitute about 90% of the intestinal bacteria; however, this number is lower in bottle-fed infants. When breast-fed infants' diets are changed to cows milk and solid food, *Bifidobacteria* are joined by rising numbers of other bacteria found in the human body such as *Bacteroides*, *Streptococci* and *Lactobacilli*. The lower number of *Bifidobacteria* in formula-fed babies might account for a higher risk of diarrhea and allergies that is usually associated with babies who aren't breast-fed. In addition, Bifidobacteria only produce lactic acid as fermentation end-products while other bacteria, for example Enterobacteria, produce several other end-products. This is thought to be the reason that explain why infants carrying more Bifidobactria than Enterobacteria have less digestive problems.
B. longum is often the dominant *Bifidobacterium* species detected in humans and is the only species to regularly harbor plasmids. It is a leading member of the probiotic bacteria due to numerous studies that have provided a growing body of evidence for its role in a myriad of potential health benefits. These include diarrhea prevention in antibiotic treated patients (Black et al. 1991); cholesterol reduction (Dambekodi and Gilliland, 1998); alleviation of lactose intolerance symptoms (Jiang et al. 1996); immune stimulation (Takahashi et al. 1998); and cancer prevention (Reddy and Rivenson, 1993).

2.3 *Bacillus*

In 1872, Ferdinand Cohn, a contemporary of Robert Koch, recognized and named the bacterium *Bacillus subtilis.* The organism is Gram-positive, capable of growth in the presence of oxygen, and forms a unique type of resting cell called an endospore. Koch relied on Cohn's observations in his classic work (1876), *The etiology of anthrax based on the life history of Bacillus anthracis*, which provided the first proof that a specific microorganism could cause a specific disease.

The ubiquity and diversity of these bacteria in nature, the unusual resistance of their endospores to chemical and physical agents, the developmental cycle of endospore formation, the production of antibiotics, the toxicity of their spores and protein crystals for many insects, and the pathogen *Bacillus anthracis,* have attracted ongoing interest in these bacteria since and Cohn and Koch's discoveries in the 1870s.

The genus *Bacillus* remained intact until 2004, when it was split into several families and genera of endospore-forming bacteria, justifiable on the basis of ssRNA analysis.

There is great diversity of physiology among the aerobic spore formers; their common features include degradation of a great variety of substrates derived from plant and animal sources, including cellulose, starch, pectin, proteins, agar and hydrocarbons; antibiotic production; and endospore formation.

2.3.1 Spores

Bacterial spores (Fig. 1) are highly specialized, differentiated cell types, designed for the survival to adverse conditions. They are formed inside the bacterial cell and hence called endospores and characterized by a structure is very different from that of vegetative cells.

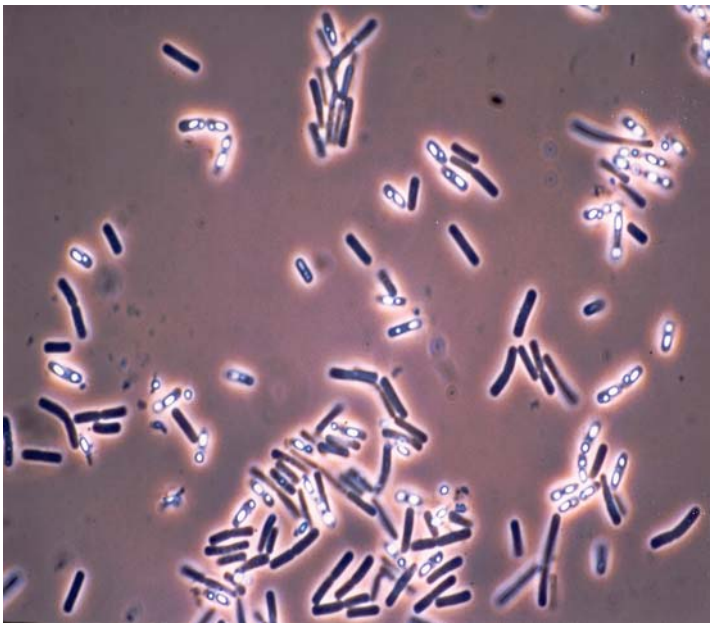


Figure 1: *Bacillus* with fluorescence microscope

Spores formation involves an unique process of asymmetric cell division, followed by engulfment of the smaller cell and eventually leading to the sacrifice of the original bacterial cell for the production of a single spore (Fig. 2). Spores are metabolically dorment (Lewis 1969) and highly resistant to adverse conditions, such as starvation, high temperatures, ionizingradiation, mechanical abrasion, chemical solvents, detergents, hydrolytic enzymes, desiccation, pH extremes and antibiotics (reviewed in Setlow, 2000).

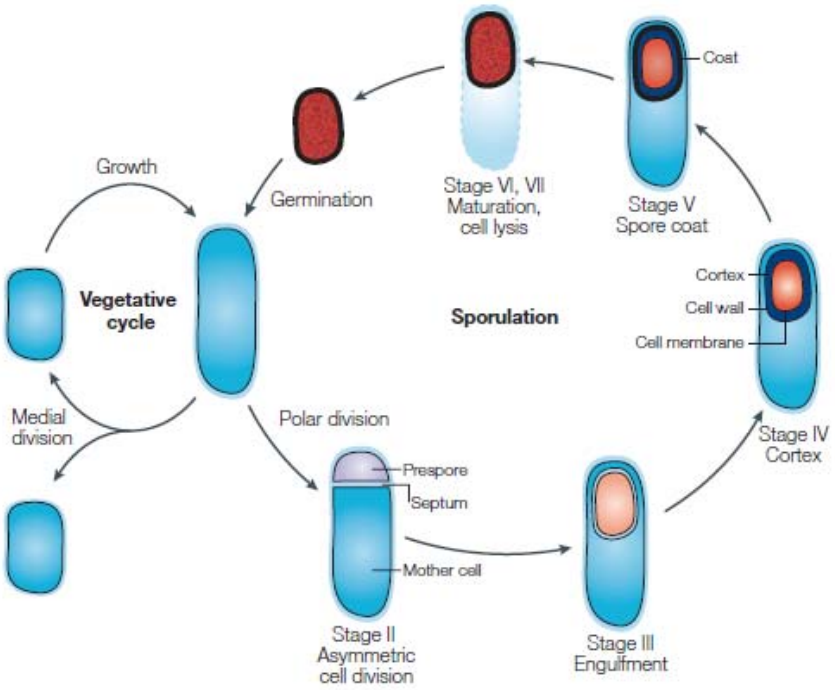


Figure. 2 : The life cycle of spore-forming bacteria (Errington 2003).

2.3.2 Spore structure

The remarkable properties of bacterial spores are due to their unique structure. Spores consist of a core, surrounded by the inner membrane, the cortex, the outer membrane, the coat and in some species the exosporium.

The innermost part of the spore, the core, contains the spore cytoplasm with the regular cellular components, such as cytoplasmic proteins, ribosomes and DNA. The physical state of the core cytoplsm, however, is far from regular in comparsion to vegetative cell cytoplasm, having a water content of only 30-50%, instead of the 70-88% in vegetative cytoplasm (Potts. 1994; Setlow. 1994; 2000). This dehydrated state plays an importantrole in spore longevity, dormancy and resistance (Beaman et al. 1982; 1984; Gould. 1986; Nakashio and Gerhardt. 1985). The core cytoplasm lacks most of the common high-energy compounds found in the cytoplasm of vegetative cells (reviewed in

Setlow, 1994; 2000). The core cytoplasmic contains very large amounts of divalent cations, mainlt calcium, which are complexed with the spore specific-compound Pyridine-2,6-dicarboxylic acid (dipicolinic acid or DPA; Powell. 1953). DPA is associated with core dehydration (Paidhungat et al., 2000), and plays a role in wet heat resistance (Halvorson and Howitt. 1961; Paidhungat et al., 2000). DPA and calcium are excreted from the core during germination, and play important roles in the germination process (Paidhungat et al. 2001).

The spore core is surrounded by the inner membrane. The inner membrane becomes the cytoplasmic membrane of the nascent vegetative cell upon germination, and swells 2-fold without *de novo* lipid synthesis. Therefore, the inner membrane is in a special, compressed state. The inner membrane was proposed to be the main permeability barrier of spores (Setlow. 1994; 2000), and is the site where the spore germination receptores are located (Hudseon et al. 2001; Paidhungat et al. 2001). Furthermore, the inner membrane is a key target for several sporocidal chemicals (Cortezzo et al. 2004; Genest et al. 2002; Setlow et al . 2003). Thus, the inner membrane is a structure of prime importance for spore resistance and germination.

The cortex is a thick wall composed of specifically modified peptidoglycan, built aroud the inner membrane (Warth and Strominger. 1972). The cortex is of crucial importance for the maintenance of spore core dehydration and thus resistance and dormancy (reviewed in Pophan. 2002). The specific structure of the cortex is conserved among species, and may play a role in spore heat resistance.

Around the cortex lies the relateviely poorly studied outer membrane. The outer membrane has opposite polarity to the inner membrane. Around the outer membrane is built the coat, a dynamic, intricate protein structure generally consisting of three distinct layers (Henriques et al. 2004). The coat shields the cortex peptidoglycan from enzymatic attack (Dricks. 1999; 2002; Seltow. 2000). Furthermore, the coat is involved in resistance to environmental UV radiation (Riesenman and Nicholson. 2000) and to a variety of chemicals includings oxidative agants (Genest et al. 2002; Kim et al. 2004). The inner layer of the coat harbors a lytic enzymes, which helps degrading the cortex during germination (Paidhungat et al. 2001; Bagyan and Setlow. 2002). Several other coat proteins are also involved in spore germination, by

facilitating the passage of specific germinant molecules through the coat (Behravan et al. 2000).

In many species the spore coat is surrounded by a lose, membrane-like structure called the exosporium, important for spore hydrophobicity and adherence properties (Koshikawa et al. 1989).

2.3.3 Spore germination

Spore germination involves a series of rapid degradative reactions, leading to dismantlement of the unique spore structure and loss of spore dormancy and resistance (Fig. 3). The subsequent steps that lead to cell-enlargement and cell-division are termed outgrowth, which is considered a separate process, distinct from germination (Campbell and Leon. 1985).

Spores of *Bacillus* species are found in soil, dust, and water as well as in the air (Nicholson 2002), however, their primary reservoir has long been considered the soil. Spores are often found in the gastrointestinal tract of insects and animals, including humans. Their presence in the gut has always been considered as a consequence of the ingestion of contaminated food.

The acid-resistance of the spore is thought to allow a safe transit of the stomach to ingested spores that in the nutrient-rich intestine could germinate. Spore germination in the murine intestine has been clearly established and it has been suggested when a dose of $1x10^9$ spores is ingested by a mouse, about 10% of the spores germinate in the jejunum and ileum (Casula and Cutting. 2002). It has also been demonstrated that vegetative cells (derived from spore germination) are able to perform few rounds of growth and, then, to enter into the sporulation cycle in the lower part of the intestine where nutrients are limited and environmental condition do not favor cell growth.

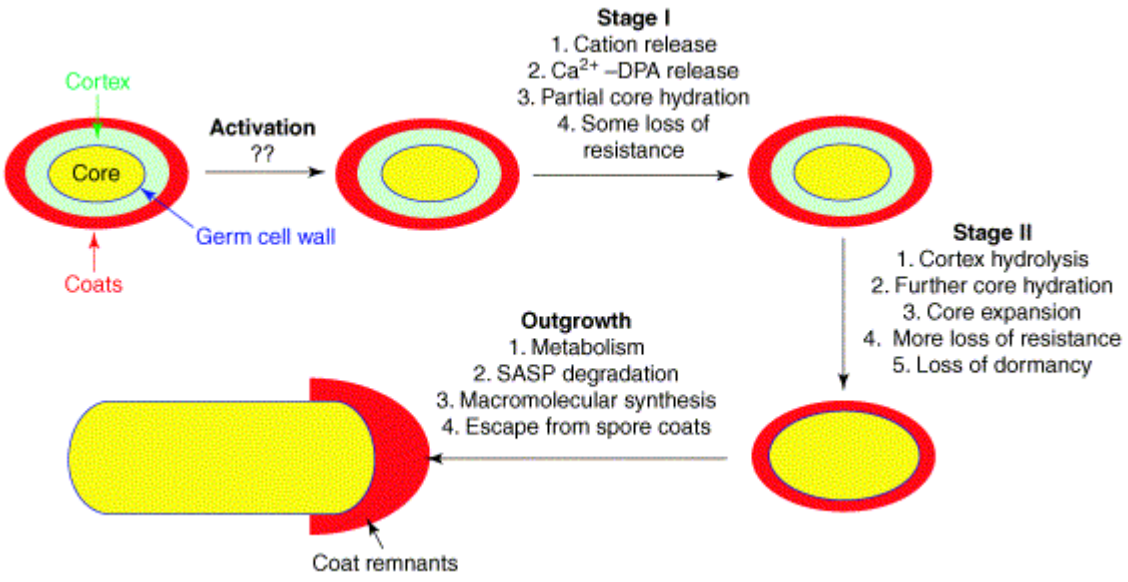


Fig. 3: Schematic representation of spore germination. (Setlow 2003).

A recent *in vitro* study has shown that *B. subtilis* spores can be phagocitated by human THP-1 macrophage cells (Ceragioli et al., 2009). Phagocytated spores are able to germinate in the macrophages, were they are, then, killed before cell growth commenced (Ceragioli et al., 2009).

3. Pigmented Bacilli

Most Bacillus species are unpigmented. Some strains of *Bacillus megatrium* are known to form a yellow pigment especially on casein agar. A yellowish pigment is also found in colonies of *B. fastidiosus* upon prolonged incubation on special agar (Fahmy, personal communication). Some pigmented isolates have been indicated as belonging to the *B. firmus* (pink), *B. licheniformis* (brwnish red), *B. pumilus* (slightly yellowish), *B. pulvifaciens* (red, orange), *B. sphaericus* (pink) and *B. subtilis* (pink, yellow, orange, brown) species.

Bacillus subtilis: some strains produce a brown or red pigment, in fewer cases orange or black pigments. Occurrence of each pigment is dependent on

composition of medium. Strains forming a black pigment exclusively on carbohydrate-containing media have been named *B. subtilis var. aterrimus*. Other strains forming a black pigment only in the presence of tyrosine have been described as *B. subtilis var. niger* .

Bacillus cereus: the red pigment pulcherrimin is produced by some strains in starch media containing sufficient iron. Some strains produced a yellowish-green fluorescent pigment in various media. On nutrient agar some strains darken the medium slightly, and some produced a pinkish brown diffusible pigment.

Bacillus firmus: pigmented strains mostly from salt marshes.

Bacillus licheniformis: red pigment (presumably pulcherrin) formed by some strains on carbohydrates media containing sufficient iron. Aged culture may become brown.

Bacillus indicus: different pigments are produced by cells and spores. A chemical characterization has revealed that a mixute of several different carotenoids contribute to the observed colours (Fig. 4).

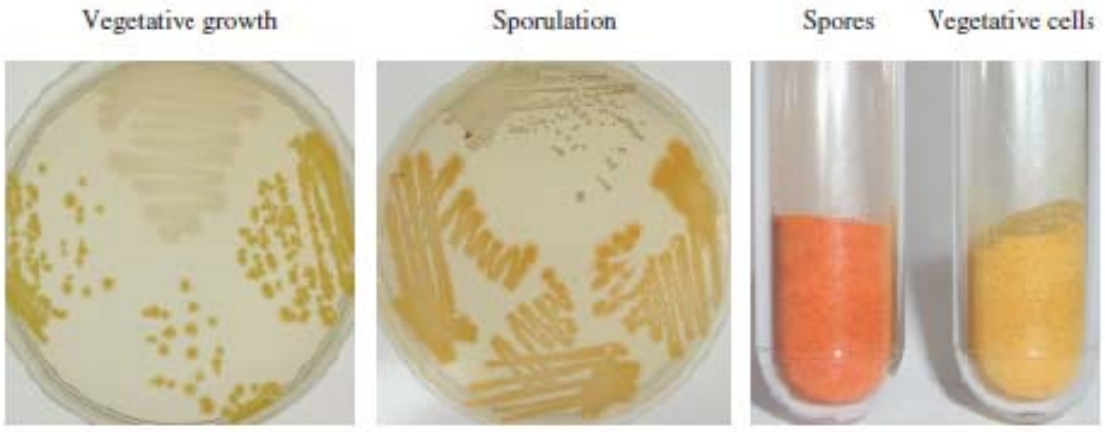


Fig. 4: Pigmented isolates. The figure shows the growth of Bacillus isolates on agar allowing vegetative cell growth (LB agar, 1 day at 37 °C) and sporulation (Difco sporulation medium (DSM) agar, 2 days, 37 °C). Strains shown are clockwise from top, PY79, HU13, HU28 and HU33. Bacillus subtilis strain PY79 was used to show the normal cream-grey, appearance of Bacillus colonies on solid agar. The third panel shows lyophilized vegetative cellsor spores (Duc et al. 2006)

4. Carotenoids

Carotenoids are the most widespread group of naturally occurring pigments. These yellow, orange and red coloured molecules are found in both eukaryotes and prokaryotes and at least 600 structurally different compounds are now known (Harborne. 1991; Britton et al. 2003). One of the principal functions of carotenoids within the cell is to provide protection against photoxidative damage by quenching singlet oxygen as well as other harmful radicals that are formed when cells are illuminated (Demmig-Adams and Adams. 2002). In photosynthetic organisms, they play a vital role as light-harvesting pigments, while in mammals the cleavage of some carotenoids (e.g. beta-carotene) plays an important role in nutrition (Vitamin A), vision (retinal) and its development (retinoic acid). In addition, it is the inherent potent antioxidant properties of carotenoids that protect cells from environmental extremes and that in mammals can prevent the onset of chronic disease states (Giovannucci. 2002; Mares-Perlman et al. 2002). These health-promoting properties have lead to substantial interest in carotenoids as nutritional supplements, particularly as mammals (most notably humans) cannot synthesis carotenoids de novo and they must be acquired from the diet.

Commercially, carotenoids are used in the pharmaceutical, cosmetic, food and feed industries as precursors, colourants and supplements. Total chemical synthesis is the method of choice used to produce carotenoids industrially. The disadvantages of this approach include the production of steroisomers not found in the natural products, contamination with reaction intermediates/products and lack of potential synergistic nutrients present in biological mixtures. Thus, a commercial opportunity exists for carotenoid production from natural sources (Ausich. 1997; Borowitzka. 1999). Microbial sources of carotenoids, currently used commercially, include the unicellular algae *Dunaliella salina*, *Spirulina* (Borowitzka. 1999) and *Haematococcus* (Lorenz and Cysewski. 2000; Guerin et al. 2003) as well as the filamentous fungus *Blakeslea trispora* (Quiles-Rosillo et al. 2005). At present there is only

one higher plant source (Tagetes flowers) from which carotenoids are produced commercially (Piccaglia et al. 1998).

The availability of genes encoding biosynthetic enzymes from microbial and plant sources has also facilitated the opportunity to engineer the pathway into more suitable hosts (Fraser and Bramley. 2004). This approach has been used successfully in *Escherichia coli* (Misawa et al., 1995) and with the food yeast *Candida utilis* (Miura et al. 1998). The enhancement of nutritionally valuable carotenoids in crop plants has also been achieved (Ye et al. 2000; Fraser et al. 2002). However, consumer concern over genetically modified foods has prevented exploitation of carotenoid production by genetic engineering.

The amenability of carotenoid formation to genetic manipulation has in part been due to similarities in the biosynthetic pathways found in carotenogenic eukaryotes and prokaryotes. Carotenoids are isoprenoid compounds and thus biosynthetically related to other isoprenoids (such as ubiquinone) via the five-carbon precursor isopentenyl diphosphate (IPP). From the common isoprenoid-forming pathway, geranylgeranyl diphosphate (GGPP) is the precursor utilized in the formation of carotenoids. Condensation of two GGPP molecules results in the formation of phytoene, the first C40 carotene precursor. Following a number of desaturation reactions phytoene is converted, sequentially, to phytofluene, z-carotene, neurosporene and lycopene. In some organisms, neurosporene is formed as the end product of desaturation instead of lycopene. Either neurosporene or lycopene can be subject to additional hydroxylation, cyclization or other modifications dependant on species. In bacteria, carotenoids have been comprehensively studied in purple nonsulfur anoxygenic photosynthetic bacteria (e.g., Rhodobacter capsulatus), nonphotosynthetic bacteria (e.g. Erwinia herbicola and Myxococcus xanthus) and cyanobacteria (e.g. Synechococcus sp.) (Armstrong 1994). In all cases the basic biosynthetic pathways, carotenoids and carotenoid genes have been identified and shown to overlap with those in fungi and plants. Enzymes catalyzing specific carotenoids have been shown to be homologous between bacteria, fungi and plants. In all carotenogenic bacteria studied to date the biosynthetic genes have been clustered in specific operons. For example, in *Myxococcus xanthus* a 12 kb DNA cluster carries 11

different carotenoid genes with evidence that carotenoid biosynthesis is under the control of an alternative transcription factor (Botella et al. 1995).

5. Aim of this work

My main interest is the study of commensal bacteria, their physiological characterization and the understanding of their functional role. To this aim I started my Thesis project characterizing bacteria of intestinal origin, using as starting samples ileal biopsies of human healthy volunteers. Those samples were collected at the "Rummo" hospital in Benevento, Italy, by Dr. Sorrentini, a gastroenterologist that collaborates with the laboratory where I performed my Thesis work.

Initially, I focused my attention on Lactobacilli and Bifidobacteria, organisms normally found in the animal and human gut. I noted that most manuscripts describing the isolation of microrganisms from intestinal samples either use fecal samples or colon biopsies. When biopsies are used, samples are treated without considering that epithelial cell are covered by a mucus layer and that is subject *in vivo* to a turn over that leads to the replacement of the layer itself and of the bacteria associated with it. I decided to use a previously published method to separate the mucus layer from the epithelial cells and to characterize the microrganisms associated with each of the two fractions. As described in Chapter 2 of this Thesis, I found that only a very limited number of bacteria were associated to the epithelial cells. I, then, decided to characterize the Lactobacilli and Bifidobacteria associated to the epithelial cells to understand whether they belonged to known species and to analyze their physiological properties. Results of this study highlighted that *L. mucosae* and *L. gasseri* are the predominant *Lactobacillus* species associated to the epithelial cells and that they share very similar physiological properties, suggesting that they form a sub-population of bacteria tightly associated to epithelial cells of the intestine.

As reported before in this Chapter, members of the *Bacillus* genus are spore formers thought to have as their main natural habitat the soil. I was intrigued by the fact that a number of commercial probiotic products contain spores of the *Bacillus* genus and that such products are used to balance the intestinal microflora and to solve intestinal disorders. I used the ileal biopsies described before to see if people, that were not under probiotic treatment, contained bacteria of the *Bacillus* genus in their intestine. As reported in Chapter 3, I was able to isolate several Bacilli belonging to various species and found that they differed from the reference species strains for various properties. For example, isolates of the *B. subtilis* species were

found to efficiently form biofilm and grow in anaerobic conditions, in which the domesticated strains of that species were not able to form biofilm or grow.

The presence of Bacilli in ileal samples prompted me to study in more details the natural habitat of those organisms. In collaboration with the laboratory of Prof. S. M. Cutting in UK, over 100 fecal samples were collected, analyzed and an average of $1x10^4$ CFU of aerobic Bacilli found. As discussed in Chapter 4, such an high number of spores in the gut is difficul to be due to the ingestion of spore-contaminated foods and suggests that the animal gut is a natural habitat of spore former bacteria, that in the gut have been shown to germinate, grow and sporulate.

The analysis of a large number of aerobic spore formers isolated from fecal samples (Chapter 4) showed that many of those organisms are pigmented with colors ranging from yellow, to pink and red. Samples from soil or sea water also contained a large number of pigmented Bacilli and the appearance of the colors seemed to be regulated by a nutrient and/or cell density signal, with no coloured colonies on high-density plates and many coloured colonies on low density plates. This was a surprising finding since only few species of the *Bacillus* genus have been reported as pigmented. In addition, since Bacilli are not photosynthetic species the presence of pigments raised questions about their functinal role. In the frame of a larger project performed in collaboration with other european laboratories, I isolates and characterized several pigmented Bacilli. As described in Chapter 5, the pigment produced by several of those Bacilli was identified as a carotenoid and attempts were made to understand their physiological role. One of the isolates, belonging to the *B. pumilus* species, was particularly interesting because the pigment was water-soluble and was produced in a temperature-regulated fashion.

In conclusion, with my Thesis work I have found that: i) *L. mucosae* and *L. gasseri* are the predominant *Lactobacillus* species attached to the ileal epithelial cells and that they form a sub-population of bacteria with very similar physiological properties; ii) aerobic spore formers are normally found in ileal samples; iii) the animal gut is a natural habitat of aerobic spore formers; and iv) pigmented Bacilli are very common in nature, their pigment is a carotenoid involved in cell protection mechanisms against oxydative stress.

References

Adlerberth, I., Ahrnè, S., Johansson, M., Molin, G., Hanson. L., Wold, A.E. (1996) *Appl Environ Microbiol* 62,22-44.

Armstrong G.A. (1994) Eubacteria show their true colors: genetics of carotenoid pigment biosynthesis from microbes to plants. *J Bacteriol* 176, 4795–4802.

Ausich R.L. (1997) Commercial opportunities for carotenoid production by biotechnology. *Pure Appl Chem* 69, 2169–2173.

Beaman, T. C., Greenmyre, J.T., Corner, T.R., Pankratz, H.S., Gerhardt, P. (1982) Bacterial spore heat resistance correlated with water content, wet density, and protoplast/ sporoplast volume ratio. *J Bacteriol* 150, 870-877.

Beaman, T. C., Koshikawa, T., Pankratz, H.S., Gerhardt, P. (1984) Dehypartitional within core protoplast.

Black. F., Einarsson. K., Lidbeck. A., Orrhage. K., Nord, C. E. (1991). Effect of lactic acid producing bacteria on the human intestinal microflora during ampicillin treatment. Scand. *J. Infect. Dis.* 23,247-254

Berhaven, J., Chirakkal, H., Masson, A., Moir, A. (2000) Mutations in the *gerP* locus of *Bacillus subtilis* and *Bacillus cereus* affect access of germinants to their targets in spores. *J Bacteriol* 182,1987-1994.

Britton, G., Liaaen-Jensen, S., Pfander, H. (eds) (2003) Carotenoids, Handbook, Birkhauser Verlag, Basel, Switzerland.

Borowitzka, M.A. (1999) Commercial production of microalgae: ponds, tanks, tubes and fermenters. *J Biotechnol* 70, 313–321.

Dambekodi, P. C., Gilliland, S. E.(1998) Incorporation of cholesterol into the cellular membrane of *Bifidobacterium longum. J. Dairy Sci*. 81,1818-1824.

Casula, G., Cutting, S.M. (2002) *Bacillus* probiotics: spore germination in the gastrointestinal tract*. Appl. Environ. Microbiol*. 68,2344–2352.

Campbell, L. L. J. (1958). Bacterial spore germination. Definitions and methods os study, p. 33-38 *In* H. O. Halverson (ed.), Spores, vol. I. Americans Institute of Biological Sciences, Washington.

Castagliuolo, I., LaMont, J. T., Nikulasson, S. T., Pothoulakis, C. (1996) *Saccharomyces boulardii* protease inhibits *Clostridium difficile* toxin A effects in the rat ileum. *Infect. Immun*. 64, 5225–5232.

Chassy, B.M., and Murphy, C.M. (1993) *Bacillus subtilis* and other gram-positive bacteria, A.L. Sonenshine, J.A. Hoch and R. Losick (Eds.), American Society for Microbiology Press, Washington, DC, US, 65

Ceragioli, M., Cangiano, G., Esin, S., Ghelardi, E., Ricca, E., Senesi, S. (2009) Phagocytosis, germination and killing of *Bacillus subtilis* spores presenting heterologous antigens in human macrophages. *Microbiolog* 155(Pt 2),338-46.

Coconnier, M.H., Lievin, V., Lorrot, M., Servin, A.L. (2000) *Appl. Environ. Microbiol*. 66, 1152.

Conway, P. L., Gorbach, S. L.,Goldin, B. R. (1987) Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. *J. Dairy Sci*. 70, 1–12.

Cortezzo, D. E., Koziol-Dube, K., Setlow, B., Setlow, P. (2004). Treatment with oxidizing agents damages the inner membrane of spores of *Bacillus subtilis* and sensitizes spores to subsequent stress. *J Appl Microbiol* 97,838-852.

Demmig-Adams, B., Adams, I.W.W . (2002) Antioxidants in photosynthesis and human nutrition. *Science* 298, 2149–2153.

El-Osta, Y., Dobos, M., Hillier, A.J., Davidson, B. E. (1996) Proc. Fifth Symp. Lactic Acid Bact.

Fraser, P.D., Bramley, P.M., (2004) The biosynthesis and nutritional uses of carotenoids. *Prog Lipid Res* 43, 228–265.

Fukushima, Y., Kawata, Y., Hara, H., Terada, A., Mitsuoka, T. (1998) Effect of a probiotic formula on intestinal immunoglobulin A production in healthychildren. *Int. J. Food Microbiol.* 42, 39–44.

Gerhardt, P. C., Beaman. C., Corner. T. R., Greenamyre, J. T., Tisa, L. S. (1982) Photometric immersion refractometry of bacterial spores. *J. Bacteriol.* 150, 643-648.

Genest, P. C., Setlow, B., Melly., E., Setlow, P. (2002) Killing of spores of *Bacillis subtilis* by peroxynitrite appears to be caused by membrane damage. *Microbiology* 148, 307-314.

Goldin, B. R., Gorbach, S. L., Saxelin, M., Barakat, S., Gualtieri, L., Salminen, S. (1992) Survival of *Lactobacillus* species (strain GG) in human gastrointestinal tract. *Dig. Dis. Sci.* 37, 121–128.

Gould, G. W. (1986) Water and the survival of bacterial spores, p. 143-156. *In* A. C. Leopold (ed.), Membranes, Metabolism, and Dry Organisms. Cornell University Press, Ithaca, USA.

Granato, D., Perotti, F., Masserey, I., Rouvet, M., Golliard, M., Servin, A., Brassart, D.(1999) *Appl. Environ. Microbiol.* 65, 1071.

Guerin, M., Huntley, M.E., Olaizola, M. (2003) Haematococcus astaxanthin: applications for human health and nutrition. *Trends Biotechnol* 21, 210–216.

Gill et al. (2006) *Science* 312,1355 - 1359

Halverson, H., C. Howitt. (1961) The role of DPA in bacterial spores, p.149-165. *In* H. O. Halverson (ed.), Spores, vol. II. Burgess Publishing Company, Minneapolis.

Harborne, J.B. (1991) Recent advances in the ecological chemistry of plant terpenoids. In Ecological Chemistry and Biochemistry of Plant terpenoids (Harborne JB & Tomas-Barberan RA, eds), pp. 399–426. Clarendon, Oxford.

Henriques, A. O., Costa, T.V., Martins, L.O., Zilhão, R. (2004) the functional architecture and assembly of the spore coat, p. 65-86. *In* Ricca, E., A. O. **Henriques, A.O., Cutting, S.M**.(ed.), Bacterial spore former: probiotics and emerging applications. Horizon Bioscience, Wymondham, Norfolk UK.

Jiang, T. A., Mustapha. A., Savaiano, D. A. (1996) Improvement of lactose digestion in humans by ingestion of unfermented milk containing *Bifidobacterium longum*. *J. Dairy Sci*. 79,750-757.

Kaila, M., Isolauri, E., Soppi, E., Virtanen, E., Laine, S., Arvilommi, H. (1992) Enhancement of the circulating antibody secreting cell response in human diarrhea by a human *Lactobacillus* strain. *Pediatr. Res.* 32, 141–144.

Kato, I., Yokokura, T., Mutai, M.(1983) *Microbiol. Immunol*. 27, 611.

Kato, I., Yokokura, T., Mutai, M.(1984) *Microbiol. Immunol*. 28, 209.

Kim, H. S., Sherman, D., Johnson, F., Arnoson, A.I. (2004) Characterization of a major *Bacillus anthracis* spore coat protein and its role in spore inactivation. *J Bacteriol* 186, 2413-2417.

Kleeman, E. G., Klaenhammer, T. R. (1982) Adherence of *Lactobacillus* species to human fetal intestinal cells. *J. Dairy Sci*. 65, 2063–2069.

Koshikawa, T., Yamazaki, M., Yoshimi, M., Ogawa, S., Yamada, A., Watabe, K., Torii, M. (1989) Surface hydrophobicity of spores of *Bacillus* spp. *J Gen Microbiol* 135 (Pt 10),2717-2722.

Lewis, J. C. (1969) Dormancy, p. 301-358.*In* G. W. Gould and A. Hurst (ed.), the Bacterial Spore, vol. 1. Academic Press, London and New York.

Link-Amster, H., Rochat, F., Saudan, K. Y., Mignot, O., Aeschlimann, J. M. (1994) Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. *FEMS Immunol. Med. Microbiol*. 10, 55–63.

Haller, D., Blum, S., Bode, C., Hammes, W.P., Schiffrin, E.J. (2000) *Infect. Immun*. 68, 752.

Haller, D., Bode, C., Hammes, W.P., Pfeifer, A.M., Schiffrin, E.J., Blum, S. (2000) Gut, 47, 79.

Heinemann, C., Johan, E.T., Vlieg, v.H., Janssen, D.B., Busscher, H.J., Mei, H.C., Reid, G. (2000) *FEMS Microbiol. Lett*. 190, 177.

Henriques, A. O., Costa, T.V., Martins, L.O., Zilhão, R. (2004) the functional architecture and assembly of the spore coat, p. 65-86. *In* Ricca, E., A. O. Henriques, A.O., Cutting, S.M.(ed.), Bacterial spore former: probiotics and emerging applications. Horizon Bioscience, Wymondham, Norfolk UK.

Hols, P., Ferain, T., Garmyn, D., Bernard, N., Delcour, J.(1994) *Appl. Environ. Microbiol.* 60, 1401.

Hooper, L.V., Bry, L., Falk, P.G., Gordon, J.I. (1998) BioEssays 20,336

Jiang, T. A., Mustapha. A., Savaiano. D. A. (1996) Improvement of lactose digestion in humans by ingestion of unfermented milk containing *Bifidobacterium longum*. *J. Dairy Sci*. 79,750-757.

Kaila, M., Isolauri, E., Soppi, E., Virtanen, E., Laine, S., Arvilommi, H. (1992) Enhancement of the circulating antibody secreting cell response in human diarrhea by a human *Lactobacillus* strain. *Pediatr. Res.* 32, 141–144.

Kim, H. S., Sherman, D., Johnson, F., Arnoson, A.I. (2004) Characterization of a major *Bacillus anthracis* spore coat protein and its role in spore inactivation. *J Bacteriol* 186, 2413-2417.

Kleeman, E. G., Klaenhammer, T. R. (1982) Adherence of *Lactobacillus* species to human fetal intestinal cells. *J. Dairy Sci*. 65, 2063–2069.

Koshikawa, T., Yamazaki, M., Yoshimi, M., Ogawa, S., Yamada, A., Watabe, K., Torii, M. (1989) Surface hydrophobicity of spores of *Bacillus* spp. *J Gen Microbiol* 135 (Pt 10),2717-2722.

Lewis, J. C. (1969) Dormancy, p. 301-358.*In* G. W. Gould and A. Hurst (ed.), the Bacterial Spore, vol. 1. Academic Press, London and New York.

Ley et al. (2008) *Science* 320, 1647–1651.

Link-Amster, H., Rochat, F., Saudan, K. Y., Mignot, O., Aeschlimann, J. M. (1994) Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. *FEMS Immunol. Med. Microbiol*. 10, 55–63.

Lorenz, R.T., Cysewski, G.R. (2000) Commercial potential for Haematococcus microalgae as a natural source of astaxanthin. *Trends Biotechnol* 18, 160–167.

Mahowald et al. PNAS (2009) 106:5859-5864.

Malin, M., Suomalainen, H., Saxelin, M., Isolauri, E. (1996) Promotion of IgA immune response in patients with Crohn's disease by oral bacteriotherapy with *Lactobacillus* GG. *Ann. Nutr. Metab*. 40, 137–145.

Mares-Perlman, J.A., Millen, A.E., Ficek, T.L., Hankinson, S.E. (2002) The body of evidence to support a protective role for lutein and zeaxanthin in delaying chronic disease. *Overview. J Nutr* 132, 518S–524S.

Miura, Y., Kondo, K., Saito, T., Shimada, H., Fraser, P.D., Misawa, N. (1998) Production of the carotenoids lycopene, beta-carotene, and astaxanthin in the food yeast Candida utilis. *Appl Environ Microbiol* 64, 1226–1229.

Nakashio, S., Grehardt, P. (1985) Protoplast deyadration correlated with heat resistance of bacterial spores. *J Bacteriol* 162,571-578.

Nicholson, W.L., Fajardo-Cavazos, P., Rebeil, R., Slieman, T.A., Riesenman, P.J., Law, J.F., Xue, Y. (2002) Bacterial endospores and their significance in stress resistance. *Antonie Van Leeuwenhoek* 81,27-32.

Ogawa, M., Shimizu, K., Nomoto, K., Takahashi, M., Watanuki, M., Tanaka, R., Tanaka, T., Hamabata, T., Yamasaki, S., Takeda, Y. (2001*) Infect. Immun*. 69, 1101.

Paidhungat, M., Ragkousi, K., Setlow, P. (2001) Genetic requirements for induction of germination of spores of *Bacillus subtilis* by Ca(2+)- dipicolinate. *J Bacteriol* 183,4886-4893.

Paidhungat, M., Stelow, M., Driks, A., Stelow, P. (2000) Characterization of spores of *Bacillus subtilis* which lack dipicolinic acid. *J. Bacteriol.* 182, 5505-5512.

Popham, D. L. (2002) Specialized peptidoglycan of the bacterial endospores: the inner wall of the lockbox. Cell Mol Life Sci 59, 426-433.

Perdigon, G., Alvarez, S., and Pesce de Ruiz, H.A. (1991) *J. Dairy Res.* 58, 485.

Perdigon, G., Alvarez, S., Rachid, M., Aguero, G., Gobbato, N. (1995*) J. Dairy Sci,* 78, 1597.

Perdigon, G., de Macias, M.E., Alvarez, S., Oliver, G., de Ruiz, H. (1986) *Infect. Immun*. 53, 404.

Perdigon, G., de Macias, M.E., Alvarez, S., Oliver, G., de Ruiz, H. (1988) *Immunology*, 63, 17.

Piccaglia, R., Marotti, M., Grandi, S.(1998) Lutein and lutein ester content in different types of Tagetes patula and T. erecta. *Indust. Crops Products* 8, 45–51.

Pothoulakis, C., Kelly, C. P., Joshi, M. A., Gao, N., O'Keane, C. J., Castagliuolo, **I.,Lamont, J. T.** (1993) *Saccharomyces boulardii* inhibits *Clostridium difficile* toxin A binding and enterotoxicity in rat ileum. *Gastroenterology* 104, 1108–1115.

Potts, M. (1994) Desication tolerance of prokaryotes. *Microbiol Rev* 58,755-805.

Quiles-Rosillo MD, Ruiz-Vazquez RM, Torres-Martinez S & Garre V (2005) Light induction of the carotenoid biosynthesis pathway in Blakeslea trispora. *Fungal Genet Biol* 42,141–153.

Reddy, B. S., and Rivenson. A. (1993) Inhibitory effect of Bifidobacterium longum on colon, mammary, and liver carcinogenesis induced by 2-amino-3-methylimidazo[4,5-f]quinoline, a food mutagen. *Cancer Res*. 53,3914-3918.

Rojas, M., Ascencio, F., and Conway, P.L. (2002)*Appl. Environ. Microbio*l. 68, 2330.

Riesenman, P. J., Nicolson, W.L. (2000) Role of the spore coat layers in *Bacillus subtilis* spore resistance to hydrogen peroxide, artificial UV-C, UV-B, and solar UV radiation. *Appl Env Microbiol* 66, 620-626.

Roussel, Y., Colmin, C., Simonet, J.M., Decaris, B. (1993) *J. Appl. Bacterio*l. 74, 549.

Sara, M., Sleytr, U.V. (2000) *J. Bacteriol*. 182, 859.

Schneitz, C., Nuotio, L., Lounatma, K. (1993) *J. Appl. Bacteriol*. 74, 290.

Serror, P., Sasaki, T., Ehrlich, S.D., Maguin, E. (2002) *Appl Environ Microbiol*. 68, 46.

Setlow, P. (1994) Mechanisms which contribute to the long-term survival of spores of Bacillus species. *Soc Appl Bacteriol Symp Ser* 23,49S-60S.

Setlow, P. (2000) Resistance of bacterial spores, p. 217-230. *In* G. Storz and R. Hengge-Aronis (ed.), Bacterial stress responses. ASM press, Washington, D.C.

Schell, Mark A., et al. (2002) The genome sequence of Bifidobacterium longum reflects its adaptation to the human gastrointestinal tract. *Proc Natl Acad Sci USA* 22, 14422-14427.

Shimizu-Kadota, M., Kiwaki, M., Hirokawa, H., Tsuchida, N. (1985) *Mol. Gen. Genet*. 200, 193.

Tailliez, P., Ehrlich, S.D., Chopin, M.-C. (1994)*Gene* 145, 75.

Takahashi, T., Nakagawa, E., Nara, T., Yajima, T., Kuwata,T. (1998) Effects of orally ingested *Bifidobacterium longum* on the mucosal IgA response of mice to dietary antigens. *Biosci. Biotechnol. Biochem*. 62,10-15.

Warth, A. D., Strominger, J.L. (1972) Structure of the peptidoglycan from spores of *Bacillus subtilis. Biochemistry* 11,1389-1396.

Ye, X., Al-Babili, S., Kloti, A., Zhang, J., Lucca, P., Beyer, P., Potrykus, I. (2000) Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science* 287, 303–305.

Chapter two

Characterization of intestinal bacteria tightly bound to the human ileal epithelium

Published on: Research in Microbiology

Fakhry, S., Manzo, N., D'Apuzzo, E., Pietrini, L., Sorrentini, I., Ricca, E., De Felice, M. and Baccigalupi, L. "Characterization of intestinal bacteria tightly bound to the human ileal epithelium" Res. Microbiol. (*in press*) (2009).

Abstract

In order to perform a selective isolation of bacteria tightly bound to the human gut, ileal biopsies of healthy volunteers were treated to wash out the mucus layer and loosely bound bacterial cells. Rod-shaped, anaerobic bacteria that had remained attached to the epithelial cells were isolated and identified at the species level by analysis of the 16S rDNA sequence and biochemical tests. One isolate was identified as belonging to the *Bifidobacterium breve* species while all the others were Lactobacilli of only two species, *L. mucosae* and *L. gasseri*. Members of these species were found previously along with many others in intestinal samples but their predominance among bacteria strictly associated to the epithelium, as shown here, was not suspected before and suggests that these species may represent a specific sub-population of tissue-bound bacteria. A series of physiological tests was performed and indicated that all isolates were able to produce antimicrobials active against selected pathogens and survive simulated gastric conditions. All isolates were able to grow and produce biofilm in simulated intestinal fluid after exposure to gastric conditions. Some isolates were able to degrade mucin while none of them showed cytotoxicity *in vitro* on HT29 cells. The tight association of the strains isolated with ileal epithelial cells is presumably indicative of a direct interaction with the host cells. For this reason and for the absence of cytotoxicity *in vitro,* those isolates can be proposed as potential probiotic strains for human use.

1. Introduction

The human intestinal microflora establishes a complex symbiotic interaction with epithelial and immune cells of the gastrointestinal tract (GIT). In this interaction the microbial role is essential in providing nourishment, forming a first line of defense against invasion by pathogenic organisms, regulating epithelial development and inducing innate immunity (Eckburg et al. 2005). These contributions are reciprocated by stable conditions of temperature, pH, osmolarity and food supply for the micro-organisms.

Recent metagenomic experiments have indicated that the vast majority of the intestinal

bacteria belong to two phyla, the Firmicutes, including the large class of Clostridia and the lactic acid bacteria (LAB), and the Bacteroidetes (Eckburg et al. 2005; Mahowald et al. 2009). Most of these organisms are anaerobes and not cultivable in laboratory conditions and only 0.1% of the total gut bacteria are facultative anaerobes (Eckburg et al. 2005). However, the composition of the gut microbiota is known to vary transiently as a consequence of diet changes, enteral infections, antibiotic or anti-acid treatments and immune suppression (Schiffrin et al. 2002). Recently, it has been shown that phylum-level changes in the microflora are associated with diseases, such as obesity (Turnbaugh et al. 2009). In particular, the coexistence of H_2-producing bacteria and H_2-utilizing methanogenic Archaea in the GIT of obese individuals induced to hypothesize that H_2 transfer between eubacteria and archaeal species increases the energy uptake by the large intestine of obese individuals (Zhou et al. 2001).

At the species level a large diversity exists in the human gut. It is known that the type of neonatal feeding influences the species composition of the microflora (Benno et al. 1984) and that in the elderly a general reduction in species diversity occurs, with an increase of facultative anaerobes and a decrease of Lactobacilli and Bifidobacteria (Hopkins et al. 2001; Woodmansey 2007).

Lactobacilli and Bifidobacteria, the most frequently used bacteria in probiotic products for human consumption, belong to different phyla. Lactobacilli, with all other LAB, are low G+C Gram-positives and belong to the phylum of the Firmicutes, while Bifidobacteria are high G+C Gram-positives and belong to the phylum of the Actinobacteria (Vaughan et al. 2005).

Bifidobacteria, like all Actinobacteria, are not numerically important in the intestine, their number is higher in breast-fed than in formula-fed infants and decreases in adults (Benno et al. 1984; Schiffrin et al. 2002). In spite of their number, Bifidobacteria are thought to play a relevant role in keeping the gut health (Kullen et al. 2000) and cells of *Bifidobacterium longum* have been shown to contribute to the reduction of intestinal inflammation by efficiently inhibiting pancreatic and neutrophilic elastases (Ivanov et al.

2006).

Culturing and PCR-based studies on faecal samples from healthy humans reported the isolation of various LAB species with *Lactobacillus ruminis*, *L. crispatus*, *L. gasseri, L. plantarum, L. acidophilus, L. delbrueckii, L. casei, L. paracasei* and *Leuconostoc argentinum* as the most abundant species (Vaughan et al. 2005).

Similar analysis performed on infant faecal samples confirmed *L. acidophilus, L. casei/paracasei* and *L. salivarius* as predominant (Vaughan et al. 2005).

The microbiological analysis of intestinal biopsies from healthy adults identified *L. ruminis, L. gasseri, L. vaginalis* and *Leuconostoc mesenteroides* as the most abundant species (Vaughan et al. 2005), while a plasmon resonance approach showed that *L. salivarius* (26%), *L. fermentum* (13%), *L. gasseri* (10%), *L. paracasei* (7%), *L. casei* (3%), *L. mucosae* (3%) and *L. plantarum* (3%) are the predominant species associated to human colonic mucin (Kinoshita et al. 2006).

Not much attention has been given so far to whether bacteria isolated from the animal gut bind tightly or loosely to the epithelium and whether physical interactions between bacterial cells and animal tissue is relevant for probiotic activity of intestinal microflora. To gain insight into this problem we followed a previously reported procedure (Martin et al. 2004) to separate the mucus layer from the epithelial tissue of ileal biopsies of healthy human volunteers and found that only a small minority of ileal bacteria remains attached to the human cells after this treatment. Among these tightly bound bacteria we focused our attention on eleven anaerobic rod-shaped strains that would more likely display a probiotic function in the gut and report here on some of their relevant properties.

2. Materials and Methods

2.1 Collection of ileal samples

Mucosal samples were collected by forceps biopsy in the distal ileum from 7 adult human volunteers (M/F 4/3, mean age ± SD 45.0 ± 13) undergoing routine diagnostic endosocopy for colorectal cancer (CRC) screening. All patients recruited gave their informed consent to the study. The study was approved by the appropriate ethics committee and have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. The patients did not follow any special dietary regimen, and had not recently received any antibiotic or probiotic treatment. Samples were stored at -80°C in phosphate-buffered saline (PBS) containing 15% glycerol before subsequent analysis. Endoscopic appearance as well as histology of the ileum was normal in all patients.

2.2 Bacterial isolation and culture conditions

Ileal samples (10-20 mg/each) were treated with DTT as previously reported (Martin *et al*. 2004), extensively washed with PBS to eliminate loosely attached bacteria. Tissue samples after the washes, as well as buffer recovered from each wash, were plated on LB (8g/l NaCl, 10 g/l tryptone, 5 g/l yeast extract) and MacConkey (DIFCO) media in aerobic conditions to determine the total aerobic count and on deMan, Rogosa and Sharpe (MRS) (Difco) medium in anaerobic condition to isolate lactic acid bacteria. Anaerobic conditions were obtained by incubating liquid and solid cultures in an anaerobic chamber (Oxoid).

2.3 Physiological and biochemical analysis

Exponentially growing cells of the various isolates were used for biochemical analysis by the use of API 50CHL kit (Biomerieux) following the manufacturer's instructions. For biofilm formation bacteria were grown in modified TSB (mTSB) medium as described by Lebeer et al., (2007): 15 g/liter TSB (BD Biosciences) enriched with 20 g/liter Bacto peptone no. 3 (BD Biosciences).

Mucin degradation assays were performed as previously reported (Woodmansey 2007). In brief, cells were grown overnight in MRS broth at 37°C in anaerobic conditions and spotted on Medium B plates: trypton (Oxoid) 7.5 g/l ; casitone (Difco) 7.5 g/l; yeast extract (Oxoid) 3.0 g/l; meat extract (Merck) 5.0 g/l; NaCl (BDH) 5.0 g/l; $K_2HPO\text{-}3H_2O$ (BDH) 3.0 g/l; KH_2PO (BDH) 0.5 g/l; $MgSO\text{-}7H_2O$ (BDH) 0.5 g/l; cysteine HCl (Sigma) 0.5 g/l; resazurin (BDH) 0.002. g/l; D-(1)-glucose (BDH) 10 or 30, purified HGM 5 g/l and agarose (Sigma) 1.5 g/100 ml. The pH of medium was adjusted to 7.0 with 2 N NaOH. Mucin degradation activity was evaluated by the diameter of the halo observed after plate staining with amido black 0.1% in glacial acetic acid 3.5 M and washing with glacial acetic acid 1.2 M. Mucin used in this study was from porcine stomach type III (Sigma).

Antimicrobial activity was determined as previously described (Baccigalupi et al. 2005) with the following modifications: 10 µl of each culture in stationary growth phase were spotted on the surface of an MRS agar plate and the spots air dried. A 100 µl of an exponential culture of the indicator bacterial strain were mixed with 5 ml of soft agar (0.7%) and poured over the plate. The plates were incubated aerobically overnight at 37°C and the inhibition halos were measured and expressed in mm.

2.4 Simulated gastric and intestinal fluids

Gastric and intestinal fluids (SGF and SIF) were simulated as previously reported (Fakhry *et al*. 2008). Exponentially growing cells were washed, resuspended in SGF (PBS 0.5% w/v, pepsin 3 g/l, pH 2.0) or SIF (PBS 0.5% w/v, pancreatin 1 g/l, pH 8.0) and incubated 1 hour at 37°C. Cells were then diluted, plated on MRS plates and incubated at 37°C in anaerobic conditions. To monitor growth in simulated intestinal condition after gastric treatment, exponentially growing cells were washed, resuspended in SGF (PBS 0.5% w/v, pepsin 3 g/l, pH 2.0) and incubated 1 hour at 37°C. Cells were then diluted at 0.05 OD_{600nm} in MRS supplemented with pancreatin (1 g/l, pH 8.0), incubated at 37°C in anaerobic conditions and growth monitored 18 hours.

2.5 PCR conditions and primers

Oligonucleotides Ribo-for (5'-AGTTTGATCCTGGCTCAG-3') and Ribo-rev (5'-ACCTACGTATTACCGCGGC-3') were used to prime an amplification reaction (PCR) with chromosomal DNA of the various isolates as a template. The amplification product of 500 bp was gel purified and used to obtain the nucleotide sequence. Species were assigned on the base of homologies found in the databank of the Ribosomal Database Project (Michigan State University, USA).

2.6 *In vitro* biofilm assay

Biofilm formation was assayed as in reference (Kullen et al. 2000). Briefly, a platform carrying 96 polystyrene wells was filled with 200 µl medium. Approx. $3x10^7$ CFU were added and incubated without shaking for 24 h at 37°C. To quantify biofilm formation, the wells were briefly washed in phosphate-buffered saline (PBS). The remaining attached bacteria were stained for 30 min with 200 µl 0.1% (wt/vol) crystal violet in an isopropanol-methanol-PBS solution (1:1:18 [vol/vol]). Excess stain was washed with water. Wells were air dried (30 min), the dye bound to the wells was extracted with 200 µl ethanol-acetone (80:20) and the optical density (OD) of 135 µl of each well was measured at 570 nm. Each strain was tested in at least three independent experiments, each with three biological replicates. Data were normalized to the indicated positive control (LGG), which was taken as 100% to compare different experiments. Additionally, sterile medium was always included (negative control).

2.7 *In vitro* cytotoxicity assays

Cytotoxic activity of the 11 intestinal isolates was investigated in human cancer cell line HT29 (colon adenocarcinoma) using the MTS assay (Promega kit) following the manufacter's instructions. In brief, cells were grown in high-glucose Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% vol/vol fetal bovine serum (FBS), 50 ug/ml streptomycin, 50 ug/ml penicillin and incubated at 37°C under a 5% CO_2 humidified

atmosphere. Cells ($3x10^3$/well) were seeded in 96 well plates and allowed to attach and reach 80% confluence; then the media was removed and replaced by fresh medium containing 30% vol/vol bacteria conditioned media (CM). Cells were incubated for 48 h. MTS assay was done, by using MTS: DMEM in a ratio 1:5 with 2 hours incubation at 37°C, 5% CO_2. The optical density of the formazan was measured at 490 nm by using a Multiskan Spectrum (Thermo Electron Corporation). Optical density was a direct measure of the cell viability. The experiments were done in three independent experiment and samples run in quintuplicate.

2.8 Preparation of bacterial supernatant

Bacterial strains were grown anaerobically in overnight MRS broth at 37°C till stationary phase. Cells were then pelletted and the supernatant was filter-sterilized through a 0.22 um filter and stored at -20°C.

3. Results

3.1 Isolation of Lactobacilli tightly associated to ileal epithelial cells

In order to restrict our isolation to bacteria tightly associated to the human tissue, samples of ileal biopsies of healthy human volunteers, collected as described in Materials and Methods. Most bacteria were found in the buffer recovered after the washes with an average of $3.54x10^3$/mg and $2.21x10^3$/mg of aerobic bacteria and LAB, respectively. A lower number of bacteria with an average of 16/mg and 10/mg of aerobic bacteria and LAB, respectively, were found tightly attached to ileal epithelial cells. Bacteria able to grow anaerobically on MRS medium were analyzed for their colony morphology on MRS plate, Gram-staining and catalase phenotype. Only one colony of those apparently identical from each ileal sample was selected, analysed for their cell shape under the light microscope and divided into two groups: round- and rod-shaped bacteria. By this procedure we ended up with 24 isolates: 13 round-shaped and 11 rod-shaped. With the aim of characterising potential probiotic Lactobacilli, we focused our attention on the rod-shaped bacteria that

were characterised at the species level by analysis of the 16S rDNA sequence and by biochemical tests (API gallery). As a result, of the eleven rod-shaped isolates one was identified as belonging to the *Bifidobacterium breve* species while the remaining ten were all Lactobacilli, either of the *L. mucosae* (eight isolates) or *L. gasseri* (two isolates) species (Table 1). Since most isolates either come from different samples or belong to different bacterial species (Table 1), only strains SF1087, SF1091 and SF1108, belonging to the same species and coming from the same ileal biopsy, could be potential siblings.

Table 1. Rod-shaped bacteria tightly associated to ileal epithelial cells

Biopsy n°	Strain name	Species [a]	Gene bank accession number
1	SF1031	*Lactobacillus mucosae*	FN400925
1	SF1036	*Bifidobacterium breve*	FN400926
2	SF1087	*Lactobacillus mucosae*	FN400927
2	SF1091	*Lactobacillus mucosae*	FN400928
2	SF1108	*Lactobacillus mucosae*	FN400929
2	SF1109	*Lactobacillus gasseri*	FN400930
3	SF1111	*Lactobacillus mucosae*	FN400931
4	SF1146	*Lactobacillus mucosae*	FN400932
5	SF1183	*Lactobacillus gasseri*	FN400933
6	SF1232	*Lactobacillus mucosae*	FN400934
7	SF1233	*Lactobacillus mucosae*	FN400935

[a] Assessed on the base of the nucleotide sequence of the gene coding for the 16S RNA and of biochemical (API) tests.

3.2 Production of antimicrobial activity

All Lactobacilli were analysed for the production of antimicrobial molecules active against selected pathogens (Table 2). As summarized in Table 2, all strains produced antimicrobial molecules, active against the Gram-positive and the Gram-negative pathogens used in our study. As a control we used strains LGG, a commercially available probiotic strain of *L. rhamnosus*, L38 and VL36, two food-isolated strains identified as *L. rhamnosus* and *L. fermentum*, respectively (Baccigalupi et al. 2005).

Table 2. Antimicrobial activity[a,]

Strani	*Bacillus cereus* 6A2[b]	*Staphylococcus aures* ATCC6538	*Listeria monocitogenes* ATCC7644	*Salmonella typhimurium* ATCC14028	*Shigella sonneii* ATCC25931	*Escherichia coli* ATCC13762
SF1031	++	++	++	++	++	+
SF1036	++	++	++	++	++	++
SF1087	++	++	++	++	++	+
SF1091	++	++	++	++	++	++
SF1108	+	++	++	++	+	++
SF1109	++	++	++	++	++	++
SF1111	++	++	++	++	++	++
SF1146	+	++	++	++	++	++
SF1183	++	++	++	++	++	++
SF1232	++	++	++	++	++	++
SF1233	+++	+++	++	++	++	++
LGG	++	+	++	+/-	+/-	++
VL36[c]	+/-	+/-	-	+	-	-
L38[c]	+	-	-	+++	-	++

[a] Symbols refer to the size of the inhibition halo observed with growing cells: - no halo; +/- < 1 cm; + between 1 and 2 cm; ++ between 2 and 4 cm; +++ > 4 cm; [b] Naclerio *et al.*, 1993; [c] Baccigalupi *et al.* 2005.

It is interesting to note that, while all our ileal isolates produced similar antimicrobial activities, the three *Lactobacillus* strains from other sources showed a more heterogeneous profile of production of antimicrobial molecules.

3.3 Resistance to simulated GIT conditions

We measured the survival of all isolated Lactobacilli in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) as previously reported (Fakhry et al. 2008). Almost identical numbers of cells were recovered on MRS plates from treated and untreated cells of all 11 isolates, indicating an almost total resistance to both treatments (data not shown). We then analysed whether cells exposed to simulated gastric fluid were still able to grow in simulated intestinal conditions. All strains were able to grow and very similar data were collected for all strains. Fig. 1 reports the growth curves of treated and untreated cells for four representative strains. In all cases a long lag phase was observed, but once that growth was started the growth rates appeared similar to that of untreated cells (Fig. 1). We cannot exclude the possibility that pancreatin inhibited growth and that after 8-14 hours the enzyme was no longer active, thus allowing the cells to enter the exponential growth phase. However, in such case we would expect cell growth to start at similar time points in all samples. As can be observed in Fig. 1, for the various isolates cell growth started at different time points (i.e. about after 8 hours for *B. breve*, about 11 hours for *L. mucosae* SF1233 and about 13 hours for *L. mucosae* SF1091 and *L. gasseri*).

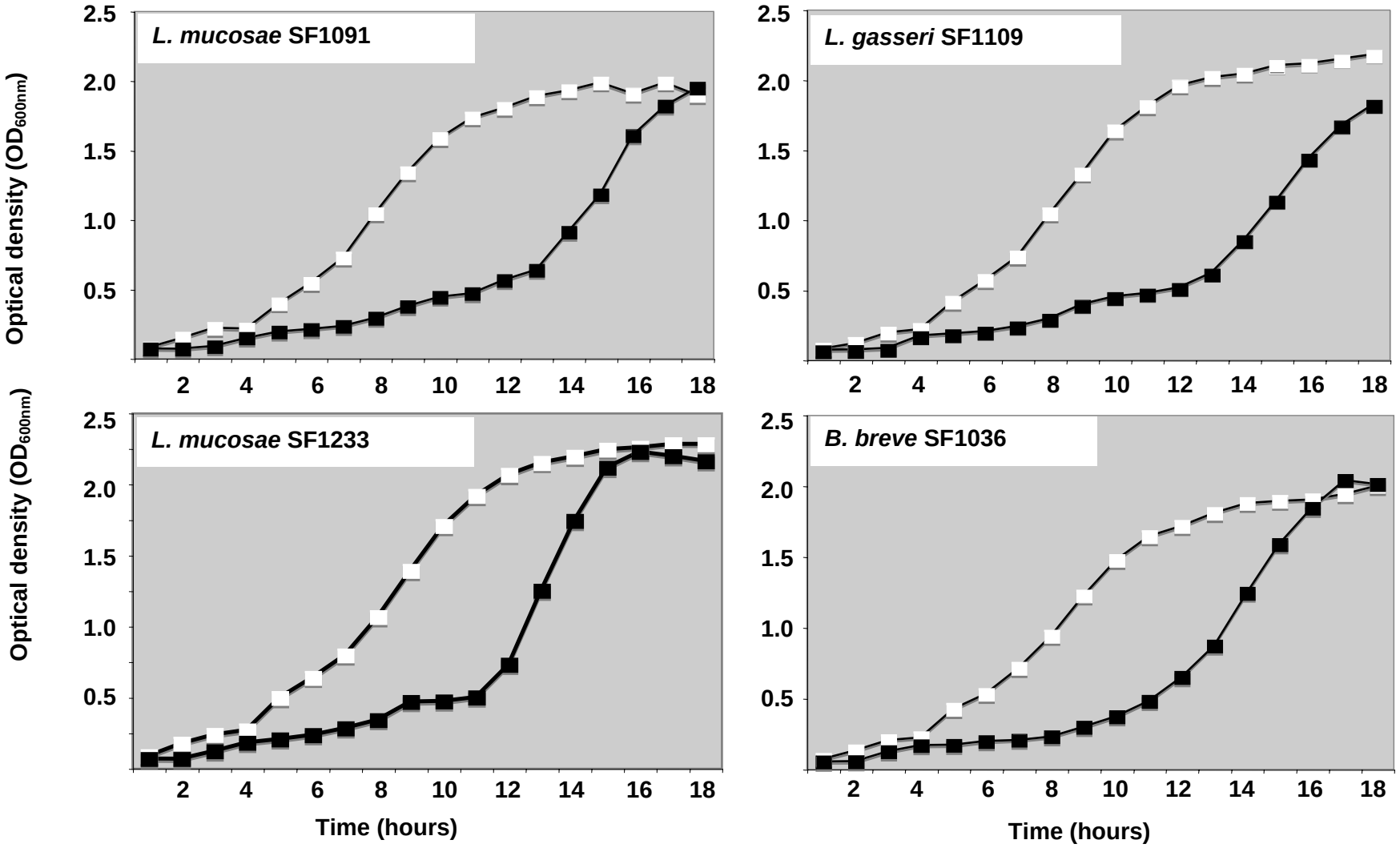


Fig.1:Growth curves of treated (closed symbols) and untreated (open symbols) cells. Overnight cultures of the various strains were either treated with pepsin at pH 2 for 1 hour at 37°C or left in MRS broth at 37°C, then diluted at OD 0.05 (600 nm) and used to inoculate MRS(open symbols) or MRS pH 8-supplemented with pancreatin (closed symbols) and OD measured every hour for 18 hours.

Since we did not observe any mortality on plates due to the treatment in SGF conditions, we believe it is unlikely that the long delay is due to reduced number of live cells after the SGF treatment. For these reasons we favor the idea that the long lag phase experienced by cells subjected to SGF and then grown in SIF was due to the need of treated cells to adapt their metabolism to the new growth conditions.

3.4 Biofilm formation

All 11 Lactobacilli were tested for their ability to form biofilm in microtiter plate assays (Lebeer et al. 2007). *L. rhamnosus* GG (LGG), one of the clinically best-studied probiotic organisms (Banasaz *et al.* 2002) and a known biofilm producer (Lebeer et al. 2007), was used as a reference strain. Since biofilm formation is known to depend on environmental conditions (Lebeer et al. 2007), we measured the production of biofilm after exposure of our isolates to simulated gastric fluid and in simulated intestinal conditions, as described above. As reported in (Fig. 2), all isolates produced a biofilm and the SGF-SIF treatments did not significantly affect the amount of biofilm produced.

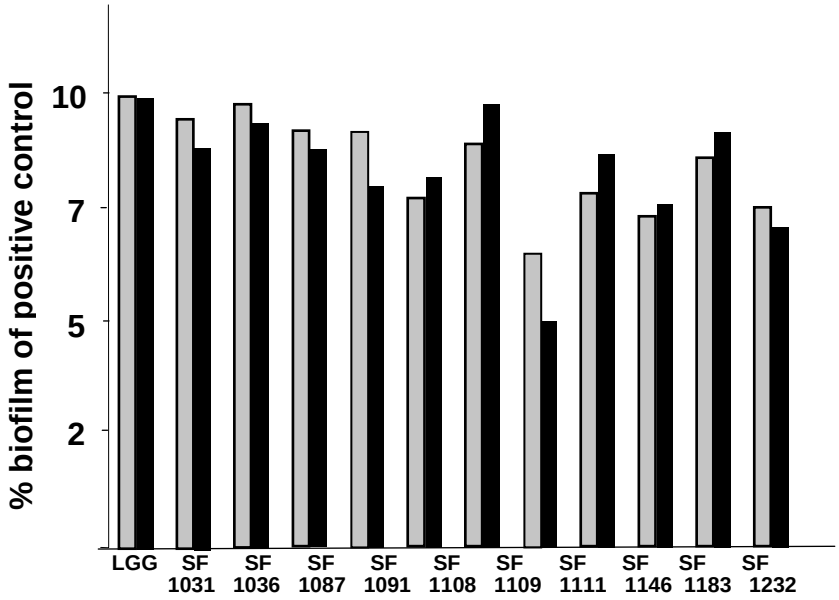


Fig. 2: Biofilm formation. The abilities of biofilm formation by the various isolates are expressed in comparison with that of strain LGG. Values obtained for strain LGG were taken as 100%. Biofilm formation was monitored in MRS broth (grey bars) and in MRS pH 8-supplemented with pancreatin (black bars). The data shown are representative of at least three independent experiments, each with three biological replicates.

3.5 Mucin degradation

The ability of our isolates to degrade mucin has been evaluated by a previously described plate assay (Zhou et al. 2001). As summarized in Table 3, we found that 8 of our 11 isolates were able to degrade mucin. While none of the two *L. gasseri* strains had mucin degradation activity, the *B. breve* strain and 7 out of 8 *L. mucosae* strains showed a clear ring of mucin degradation on plate (Table 3). As a control strain we also tested *L. rhamnosus* GG (LGG) that also showed a clear mucin degradation activity (Fig. 3).

Table 3. Mucin degradation assay.

Strain name	Species [a]	Mucin degradation[a]
SF1031	*Lactobacillus mucosae*	++
SF1036	*Bifidobacterium breve*	++
SF1087	*Lactobacillus mucosae*	+
SF1091	*Lactobacillus mucosae*	-
SF1108	*Lactobacillus mucosae*	++
SF1109	*Lactobacillus gasseri*	-
SF1111	*Lactobacillus mucosae*	+
SF1146	*Lactobacillus mucosae*	+
SF1183	*Lactobacillus gasseri*	-
SF1232	*Lactobacillus mucosae*	+
SF1233	*Lactobacillus mucosae*	++
LGG	*Lactobacillus rhamnosusi*	++

[a] Symbols refer to the size of the halo observed : - no halo; +less than 1 cm; ++ between 1 and 2 cm.

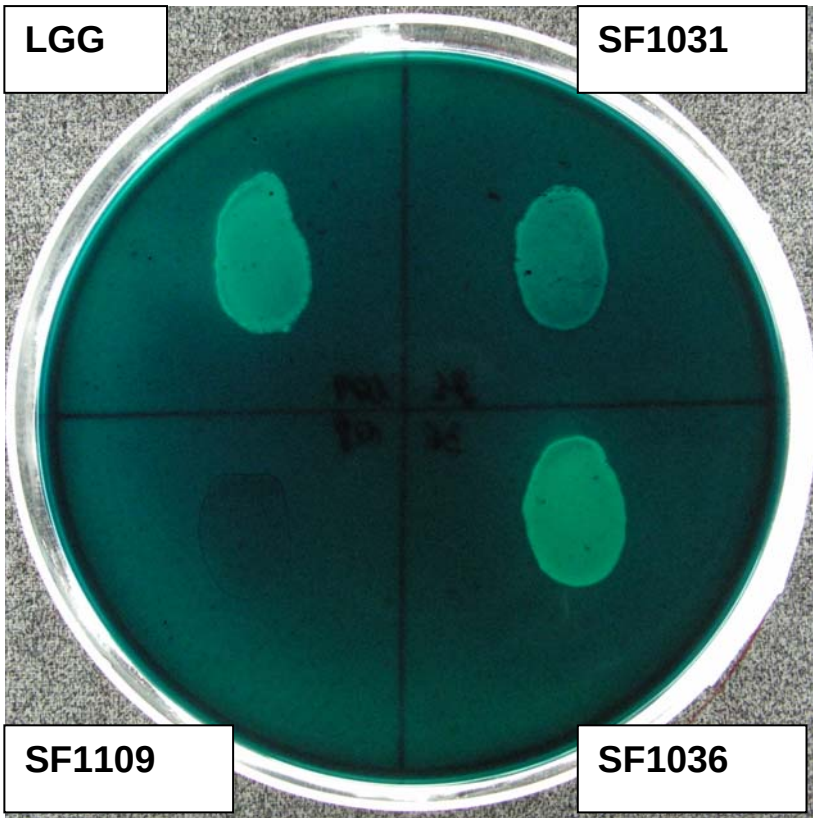


Fig. 3: Mucin degradation assay. An exponential culture of each strain was used to 'spot' sterile Medium B plates. After 48-72 hour of incubation at 37°C in anaerobic conditions, plates were stained and washed as described in Materials&Methods. The appearance of a halo around the bacterial spot was taken as an indication of the presence of mucin degradation activity.

3.6 *In vitro* cytotoxicity assay

The cytotoxic activity of culture supernatants was assayed in vitro using cultured human colon cancer cells HT29 as previously described (Arafa et al. 2009; Kullen et al. 2000).

The optical density, indicative of the cell viability, was measured at 490 nm and resulted similar for all strain tested (data not shown). The similar behaviour of all eleven isolates and of the commercial probiotic strain LGG can be taken as an indication of the absence of cytotoxic activity.

4. Discussion

In this work, we have isolated and characterised anaerobic rod-shaped bacteria attached tightly attached to human ileum. Our analysis at the species level revealed the presence of *Bifidobacterium breve* (1 isolate), *Lactobacillus gasseri* (2 isolates) or *Lactobacillus mucosae* (8 isolates), all species that are normally found in the gastrointestinal tract of humans (Honda et al. 2007; Turroni et al. 2009) and various mammals (Busconi et al. 2008; Korhonrn et al. 2008). Bifidobacteria constitute the prevalent bacterial population in newborn infants, but are also present in adult individuals (Bennon et al. 1984; Ross et al. 2000; Turroni et al. 2009). Some species such as *B. breve, B. longum and B. bifidum* are mostly associated with the human intestine and only occasionally found in animals (Turroni et al. 2009). These species belong to mucosa-adherent members of the genus and are always found in association with intestinal mucosa and not in fecal samples (Turroni et al. 2009). Many beneficial effects have been attributed to various *Bifidobacterium* strains, such as improvment in digestion and regulation of the host immune response (Mrtin et al. 2004; Ouwehand et al. 2008). *L. gasseri* is a well characterized species, known to represent the major homofermentative Lactobacillus species of the human intestine (Kullen et al. 2000; Mitsuoka et al. 1992).

L. mucosae is, instead, a poorly characterised species, first isolated in a study aimed at the isolation of *L. reuteri* (Ross et al. 2000). The similarities between *L. reuteri* and *L. mucosae* also depend on the presence in both species of the *mub* gene, encoding a cell-

surface protein with mucin-binding activity. It is interesting that the G+C content of *mub* of *L. gasseri* is similar to the overall G+C content of *L*. *mucosae* and different from that of other *L*. *reuteri* genes. Based on this and on the observation that the *mub* gene has been found in all *L*. *mucosae* but only in some *L. reuteri* strains, Ross et al. (Ross et al. 2000) suggested that *L*. *mucosae* may be the source of *mub* and *L*. *reuteri* is a recipient of the gene at some point during the course of evolution.

In all studies in which *L. mucosae* and *L. gasseri* have been found associated to intestinal samples, they had never been indicated as predominant in number with respect to other *Lactobacillus* species. In a study by Kinoshita et al. (Kinoshita et al. 2006), for example, *L. gasseri* and *L. mucosae* were identified as 10 and 3% of the total LAB population, respectively, in human colonic mucin. Although the low number of isolates in our study does not allow us to draw statistically relevant conclusions, we propose that the extensive washing and DTT treatment we performed on the ileal samples removed most of the loosely attached bacteria, allowing us to isolate a subpopulation of bacteria tightly associated to the epithelial cells characterized by very similar properities. *L. mucosae* and *L. gasseri* would then be predominant species among those bacteria tightly attached to the epithelial cells of the ileal tract of the human intestine. Although we have not performed typing experiments, we speculate that our isolates could belong to the same or very closely related strains of the *L. gasseri* and *L. mucosae* species, since they were selected from different ileal samplesby the same treatment. Our hypothesis is supported by the extremely similar physiological properties that characterise this sub-population of intestinal bacteria: i) all produce antimicrobial molecules similarly active against all tested pathogens. This is a peculiar feature since LAB from different sources have a more heterogeneous profile of antimicrobial activity (see strain LGG, VL36 and L38 in Table 2 or reference (2)); ii) all are resistant to simulated gastric fluid and able to grow efficiently in simulated intestinal fluid after exposure to gastric conditions; iii) all produce biofilm also in conditions that mimick gastric and intestinal environments; iv) 8 out of 11 isolates degrade mucin in an on plate assay; v) none of the isolates showed any cytotoxic activity.

The ability to survive, grow and produce biofilm in conditions that mimic what happens when bacteria transit the stomach tract are important featuresof potential probiotic strains, sincde they allow them to reach the intestinal environment without injury. Moreover, biofilms have protective and adhesive properities and have been associated with longer persistence of bacteria in the GIT of animals (Fakhry et al. 2008; Lebeer et al. 2007). The degrade mucin is a controversial property. The mucus layers are essential for preventing adhesion of pathogens, toxins, and other antigens present in the intestinal lumen to epithelial cells (Moncada *et al.* 2003). For this reason mucin degradation has been associated to pathogens by some authors (Moncada et al. 2003; Prizont et al. 1982; Pultz et al. *2006*). However, this activity has been also observed in several non-pathogenic and probiotic bacteria (Ruas- Madiedo et al. 2008., Yang et al. 2007). In those cases the ability to degrade mucin was seen as a beneficial feature of intestinal bacterial that with their surface-exposed glycoside hydrolases participate in the regulation of mucin turnover and, as a consequence, contribute to the integrity of the intestinal epithelium (Yang et al. 2007). As also suggested in a previous study (Ruas-Madiedo et al. 2008), we hypothesized that the ability to degrade mucin may be an adaptative advantage for intestinal bacteria, that using mucin as a source of nutrients, can colonize the epithelial cell-surface, underneath the mucus layers, more efficiently. *In vivo* studies will be needed to verify whether this degradative activity increases the intestinal persistence of bacteria in the GIT.

The tight association with the epithelial cells, presumably indicative of a direct interaction with the host cell, together with the physiological properties discussed above and with absence of cytotoxic activity makes the sub-population of intestinal bacteria that we isolated very promising as probiotic strains for human use.

References

Arafa, **H.M.M. (2009)** Possible contribution of B-glycosidases and caspases in the cytotoxicity of novel glycoconjugates in colon cancer cells. *Invest. New Drugs* (*published on line on May 5th 2009).*

Baccigalupi, L., Di Donato, A., Parlato, M., Luongo, D., Carbone, V., Rossi, M., et al. (2005) Two small, surface-associated factors mediate adhesion of a food-isolated strain of *Lactobacillus fermentum* to Caco-2 cells. *Res. Microbiol*. 156, 830-836.

Banasaz, M., Norin, E., Holma, R., Midtvedt, **T.** (2002) Increased enterocyte production in gnotobiotic rats mono-associated with *Lactobacillus rhamnosus* GG. *Appl. Environ*. *Microbio*l. 68, 3031-3034.

Benno, Y., Sawada, K., Mitsuoka, **T.** (1984) The intestinal microflora of infants: composition of fecal flora in brest-fed and bottle-fed infants. Microbiol. Immunol. 28, 975-986.

Bun, S.-S., Laget, M., Chea, A., Bun, H., Ollivier, E., Elias, **R.** (2009) Cytotoxic activity of alkaloids isolated from *Stephania rotunda in vitro* cytotoxic activity of cephranthine. *Phytotherapy Res*. 23, 587-590.

Busconi, M., Reggi, S., Fogher, C. (2008) Evaluation of biodiversity of lactic acid bacteria microbiota in the calf intestinal tracts. *Antoine van Leeuwenhoek* 94, 145-155.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., et al. (2005) Diversity of the human intestinal microbial flora. *Science* 308, 1635-1638.

Fakhry, S., Sorrentini, I., Ricca, E., De Felice, M., Baccigalupi, **L.** (2008) Characterization of spore forming Bacilli isolated from the hhuman gastrointestinal tract. *J Appl. Microbiol*. 105, 2178-2186.

Heuvelin, E., Leberton, C., Grangette, C., Pot, P., Cerf-Bensussan, N., Heyman, M. (2009) Mechanisms involved in alleviation of intestinal inflammation by Bifidobacterium breve soluble factors. *PLoS ONE* 4 e5184.

Honda, H., Kataoka, F., Nagaoka, S., Kawai, Y., Kitazawa, H., Itoh, H., et al. (2007) β-galactosidase, phospho-b-galactosidase and phospho-b-glucosidase activities in lactobacilli strains isolated from human faeces. *Lett. Appl. Microbiol*. 45, 461-466.

Hopkins, M.J., Sharp, R., Macfarlane, **G.T.** (2001) Age and disease related changes in intestinal bacteria populations assessed by cell culture, 16S rRNA abundance and community fatty acid profile. *Gut* 48, 198-205.

Ivanov, D., Emonet, C., Foata, F., Affolter, M., Delley, M., Fisseha, M., et al. (2006) A serpin from the gut bacterium *Bifidobacterium longum* inhibits eukaryotic elastase-like serine proteases. *J. Biol. Chem*. 281, 17246-17252.

Kinoshita, H., Uchida, H., Kawai, Y., Kitazawa, H., Miura, H., Shiiba, K., et al. (2006) Quantitative evaluation of adhesion of Lactobacilli isolated from human intestinal tissues to human colonic mucin using surface plasmon resonance (BIACORE assay). *J. Appl. Microbiol*. 102, 116-123.

Korhonrn, J.M., Sclivagnotis, Y., von Wright, **A.** (2008) Characterization of dominant cultivable *lactobacilli* and their antibiotic resistance profile from faecal samples of weaning

piglets. *J. Appl. Microbiol*. 103, 2496-2503.
Kullen, M.J., Klaenhammer, **T.R.** (2000) Genetic modification of intestinal *Lactobacilli* and *Bifidobacteria. Curr Issues Mol Biol*. 2, 41-50.

Lebeer, S., Verhoeven, T.L.A., Perea Velez, M., Vanderleyden, J., De Keersmaecker, **S.C.J.** (2007) Impact of Environmental and genetic factors on biofilm formation by the probiotic strain *Lactobacillus rhamnosus* GG. *Appl. Environ. Microbiol*. 73, 6768-6775.

Mahowald, M.A., Rey, F.E., Seedorf, H., Turnbaugh, P.J., Fulton, R.S., Wollam, A., et al. (2009) Characterizing a model human gut microbiota composed of members of ita two dominant bacterial phyla. *Proc. Natl. Acad. Sci*. USA 106, 5859-5864.

Manninen, T.J.K., Rinkinen, M.L., Beasley, S.S., Saris, **P.E.** (2006) Alteration of the canine small-intestine lactic acid bacterium microbiota by feeding of potential probiotics. *Appl. Environ. Microbiol*. 72, 6539-6543.

Martin, H.M., Campbell, B.J., Hart, C.A., Mpofu, C., Nayar, M., Singh, R., et al. (2004) Enhanced *Escherichia coli* adherence and invasion in Crohn's disease and colon cancer. Gastroenterology 127, 80-93.

Mitsuoka, **T.** (1992) Intestinal flora and aging. *Nutr Rev*. 50, 438-446.

Moncada, D.M., Kammanadiminti, S.J., Chadee, **K.** (2003) Mucin and Toll-like receptors in host defense against intestinal parasites. Trends in Parassitol. 19, 305-311.

Cuwehand, A.C., Bergsam, N., Parhial, R., Lahtinen, S., Gueimonde, M., Finne-Soveri, H., et al (2008) Bifidobacterium microbiota and parameters of immune function in elderly subjects. FEMS Immunol. Med. Microbiol. 53, 18-25.

Prizont, R. (1982) Degradation of intestinal glycoproteins by pathogenic *Shigella flexneri*. *Infect Immun*. 36, 615-620.

Pultz, N.J., Hoskins, L.C., Donskey, C.J. (2006) Vancomycin-resistant Enterococci may obtain nutritional support by scavenging carbohydrate fragments generated during mucin degradation by the anaerobic microbiota of the colon. *Microb Drug Resist*. 12, 63-67.

Ross, S., Karner, F., Axelsson, L., Jonsson, **H.** (2000) *Lactobacillus mucosae* sp. nov., a new species with *in vitro* mucus-binding activity isolated from pig intestine. *Int. J. System Evol. Microbiol*. 50, 251-258.

Ruas-Madiedo, P., Gueimonde, M., Fernandez-Garcia, M. de los Reyes-Gavilan, C., Margolles, A. (2008) Mucin degradation by *Bifidobactrium* strain isolated from human intestinal microbiota. *Appl. Environ. Microbiol*. 74, 1936-1940.

Schiffrin, E.J., Blum, S. (2002) Interactions between the microbiota and the intestinal mucosa. European J. Clin. Nutrution 56, 560-564.

Turnbaugh, P.J., Harnady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., et al. (2009) A core gut microbiome in obese and lean twins. *Nature* 457, 480-485.

Turroni, F., Foroni, E., Pizzetti, P., Giubellini, V., Ribbera, A., Merusi. P., et al. (2009) Exporing the diversity of the Bifidobacterial population in the human intestinal tract. Appl. Environ. Microbiol. 75, 1534-1545.

Vaughan, E.E., Heilig, H.G., Ben-Amor, K., de Vos,**W.M.** (2005) Diversity, vitality and

activities of intestinal lactic acid bacteria and bifidobacteria assessed by molecular approaches. *FEMS Microb. Rev*. 29, 477-490.

Woodmansey, **E.J.** (2007) Intestinal bacteria and ageing. *J. Appl. Microbiol*. 102, 1178-1186.

Yang, F., Wang, J., Li, X., Ying, T., Qiao, S., Li, D. (2007) 2-DE and MS analysis of interactions between *Lactobacillus fermentus* I5007 and intestinal epithelial cells. *Electrophoresis* 28, 4330-4339.

Zhang, H., DiBiase, J.K., Zuccolo, A., Kudrna, D., Braidotti, M., Yu, Y., et al. (2009) Human gut microbiota in obesity and after gastric bypass. *Proc. Natl. Acad. Sci.* USA 106, 2365-2370.

Zhou, J.S., Gopal, P.K., Gill, **H.S.** (2001) Potential probiotic lactic acid bacteria *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019) do not degrade gastric mucin in vitro. Int. *J. Food Microbiol*. 63, 81–90.

Chapter three

Characterization of spore forming Bacilli isolated from the human gastrointestinal tract

Published on: Journal of Applied Microbiology

Fakhry, S., Sorrentini, I., Ricca, E., De Felice, M. and Baccigalupi, L. "Characterisation of spore forming Bacilli isolated from the human gastrointestinal tract" J. Applied Microbiology 105:2178-2186 (2008).

Abstract

A total of 25 spore-formers was isolated from faeces and ileal biopsies of healthy human volunteers and identified at the species level. Physiological analysis was performed to evaluate the ability of the various isolates to form biofilms, to swarm, to produce surfactants and molecules that have antimicrobial activity against selected pathogens. To assess the potential probiotic activity of the isolates, we tested the resistance of cells and spores to simulated gastric conditions, the ability to grow and sporulate in anaerobic conditions and the presence of toxin-encoding genes in their genome. Conclusions: Spore-formers belonging to various bacterial species have been isolated from the gut of healthy human volunteers. These strains appear to be well adapted to the intestinal environment and we propose them as potential probiotic strains for human use and as oral vaccine vehicles. Significance and Impact of the Study: To our knowledge this is the first detailed characterization of spore-forming Bacilli from the human GIT. Our data suggest that the isolated species do not transit, but rather colonize this specific habitat and propose them as probiotic strains for human use.

1. Introduction

Endospore-forming bacteria are Gram positive organisms belonging to various genera that, all together, include more than 200 species (Fritze 2004). These organisms are generally divided into two main groups of aerobic and anaerobic bacteria, with each group further subdivided into three genera: *Bacillus, Sporosarcina, Sporolactobacillus and Clostridium, Desulfotomaculum*, *Sporomusa*, respectively (Fritze 2004). However, few exceptions have been found and members of the aerobic *Bacillus* genus have been described as Gram-negatives (*B. azotoformans, B. oleronius* and *B. horti*) (Fritze 2004) or as capable of anaerobic metabolism *(B. subtilis*) (Nakano and Zuber 1998; Tam et al. 2006).

The common feature of spore-forming Bacilli is the ability to differentiate a peculiar cell form, the endospore (spore). Formation of the spore initiates when vegetative growth can no longer occur because of food shortage or other nonphysiological conditions in the environment. The spore is a quiescent cell form, characterized by several protective layers surrounding the dehydrated cytoplasm that contains the nucleoid (Henriques and Moran 2007). This structural organization makes the spores extremely resistant to external physical and chemical insults and able to survive almost indefinitely in the absence of water and nutrients. The exceptional longevity of the spore in the environment is the main reason for the ubiquitous distribution of these organisms, in particular, of the aerobic ones (Fritze 2004).

It is generally accepted that the primary reservoir of spore-forming Bacilli is the soil and the ability of spores to be dispersed in dust and water has been identified as the cause of their presence in almost every conceivable habitat. Several species of spore-formers are commonly found also in the gastrointestinal tract (GIT) of a variety of animals (Barbosa et al. 2005; Tam et al. 2006). Only few *Bacillus* species are pathogens of animals (*B. cereus* and *B. anthracis*) or insects (*B. thuringiensis*), while the majority of them are nonpathogenic. Their presence in the GIT has been considered as due to the ingestion of bacteria associated with soil, water, air or foods.

However, a new theory is now emerging in which spore-former species are thought to establish an endosymbiotic relationship with their host, being able to survive and proliferate within the GIT and specifically interact with immuno and intestinal cells (Hong et al. 2005). Recent work has shown that in a murine model ingested spores can safely cross the stomach barrier and germinate in the intestine (Casula and Cutting 2002). In the same experimental model it has been also shown that spores can perform a complete life cycle, with germination in the upper part of the intestine, vegetative growth and sporulation before being expelled in the faeces (Tam et al. 2006). Other studies have

established that *B. subtilis*, in combination with *Bacteroides fragilis*, is able to induce the development of gut-associated lymphoid tissue (GALT) and preimmune antibody repertoire in rabbits (Rhee et al. 2004). This study also showed that sporulation, as opposed to vegetative cell growth, is essential for GALT development. An in vitro analysis has also shown that the Competence and Sporulation Factor (CSF) of *B. subtilis*, a five amino acid peptide secreted during exponential growth and acting as a quorum-sensing molecule for the induction of DNA uptake and sporulation, is able to induce heat-shock response in human enterocytelike (Caco-2) cells (Fujiya et al. 2007).

In a rather empirical way, spores of several Bacillus species have been widely used as human and animal probiotics for decades. Some commercial products have proven to contain *Bacillus* species different from those declared on their label (Green et al. 1999; Hoa et al. 2000), some strains are of unknown origin, some are multidrug resistant and some even harbor toxin genes (Green et al. 1999). Moreover, little is known about how spores exert their beneficial action on humans and animals. An in vivo study with a murine infection model has shown that the oral administration of 1×10^9 spores of *B. subtilis* one day before infection with 1.5×10^3 CFU of the murine enteropathogen *Citrobacter rodentium* was able to drastically reduce the mortality rate and some signs of enteropathy but without affecting the animal immune-response to the pathogen (D'Arienzo et al. 2006). All of the studies mentioned above have been performed with domesticated strains of B. subtilis. There is evidence that laboratory strains of *B. subtilis* differ from undomesticated strains, in several aspects including factors that are likely to affect their efficacy as probiotics (Branda et al. 2001; Earl et al. 2007, 2008). For these reasons, in this study, we aimed to isolate and identify aerobic spore-formers from the human GIT. Strains were characterized and tested for properties that would be beneficial to their survival in the gut and that could be desirable for probiosis. The collection of wild Bacilli of human origin described here will most likely provide a useful source of potential probiotics for human

use, since it has been suggested that probiotic strains originate from the target animal microflora (Barbosa et al. 2005).

2. Materials and methods

2.1 Collection of ileal and faecal samples

Ileal biopsy samples were collected from eight adult human volunteers (M／F 5／3, mean age ± SD 45.0 ± 13) undergoing routine diagnostic colonoscopies. All patients recruited gave their informed consent to the study. The study was approved by the appropriate ethics committee and has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. The patients did not follow any special dietary regimen, and had not recently received any antibiotic or probiotic treatment. Samples were stored at -80 °C in phosphatebuffered saline (PBS) containing 15% glycerol before subsequent analysis. Endoscopic appearance as well as histology of the ileum was normal in all patients. Faecal samples were collected from five healthy adult human volunteers that did not follow any special dietary regimen and who had not received any antibiotic treatment for at least 3 months.

2.2 Bacterial isolation and characterization

Ileal and faecal samples (20–40 and 50 mg／each, respectively) were heat-treated (80 °C for 10 min) to kill all vegetative cells and individually placed on LB plates. After 36 h of incubation at 37 °C, colonies were recovered and purified by streaking on fresh LB plates. Pure cultures were streaked on Difco sporulation medium (DSM), incubated at 37 °C for 24–37 h and checked by light microscopy for the presence of spores.

Exponentially growing cells were used to extract chromosomal DNA as previously reported (Green et al. 1999). DNA coding for 16S RNA was PCR amplified by using chromosomal DNA as a template and oligonucleotides Ribo-For (5’ AGTTTGATCCTGGCTCAG-3’ ; annealing at position +9 ／ +28) and Ribo-Rev (5’-CCTACGTATTACCGCGGC-3’ annealing at position +549 ／ +531). Those two

oligonucleotides were designed to amplify a 540 bp DNA fragment (MicroSeq 500 16S ribosomal DNA) previously indicated as sufficient for species identification (Woo et al. 2003). Amplified DNA was used to determine the nucleotide sequence (BMR Genomics, Padova, Italy) which was used for an online computerassisted analysis of homology.

Exponentially growing cells of the various isolates were used for biochemical analysis by the use of API 50 CHL kit (Biomerieux) following the manufacturer's instructions.

Unless otherwise specified, bacteria were grown in LB medium (for 1 l: 10 g Bacto-Tryptone, 5 g Bacto-yeast extract, 10 g NaCl, pH 7.0). Anaerobic conditions were obtained by incubating liquid and solid cultures in an anaerobic chamber (Oxoid).

2.3 Physiological analysis

Swarming motility was tested as previously reported (Connelly et al. 2004). Overnight cultures of all strains were spotted on LB or B (Julkowska et al. 2004) medium plates. LB and B plates were incubated at 37 °C and 30 °C, respectively, for 24–36 h. Surfactin production was assessed as described by Youssef et al. (2004) and by growing cells on B medium (Julkowska et al. 2004). To test biofilm production overnight cultures were used to inoculate liquid MSgg medium (100 mmol l^{1} MOPS pH 7.0, 0.5% glycerol, 0.5% glutamate, 5 mm potassium phosphate pH 7.0, 50 μg ml^{-1} tryptophan, 50 μg ml^{-1} phenylalanine, 2 mmol l^{-1} $MgCl_2$, 0.7 mmol l^{-1} $CaCl_2$, 50 μmol l^{-1} $FeCl_3$, 50 μmol l^{-1} $MnCl_2$, 2 μmol l^{-1} thiamine, 1 μmol l^{-1} $ZnCl_2$) (Branda et al. 2001) and cells grown at 37 °C in static conditions for up to 48 h. Cells forming a solid layer at the liquid–air interface were considered as biofilm producers.

Resistance to GIT conditions was assessed as previously reported (Duc et al. 2004). Cells or spores were suspended in simulated gastric fluid [SGF: 1 mg of pepsin (porcine stomach mucosa; Sigma) per ml; pH 2.0] or small intestine fluid [SIF: 1 mg of pancreatin (porcine pancreas; Sigma) per ml and 0.2% bile salts (50% sodium cholate–50% sodium deoxycholate; Sigma); pH 7.4] and incubated at 37°C for 1 h. Samples were serially

diluted and plated to determine the number of CFU per ml on LB agar plates. Resistance to antibiotics was assessed on plates by adding to LB plates the following antibiotics: neomycin (20 lg ml^{-1}), erythromycin (3 lg ml^{-1}), spectinomycin (200 lg ml^{-1}) or rifampicin (50 lg ml^{-1}). Production of antimicrobials was tested as previously reported (Baccigalupi et al. 2005).

2.4 Analysis of enterotoxins and virulence traits

Methods to detect putative B. cereus enterotoxin genes from Bacillus species by PCR amplification from chromosomal DNA have been reported previously (Duc et al. 2004). Primer sets were those described by Guinebretiere et al. (2002). Haemolysis was detected by streaking cells on horse blood (Oxoid) agar plates and 48-h incubation at 37°C.

3. Results

3.1 Isolation of spore-formers from human gut

Samples of faeces and ileal biopsies of healthy human volunteers, collected as described in 'Materials and methods', were heat-treated to kill all cells and incubated on a solid medium to allow germination and growth of heat-resistant spores. All recovered bacteria were purified and analysed for colony morphology and the presence of spores by light microscopy. From a total of eight ileal biopsies (20–40 mg⁄ each from different individuals) and of five samples of faeces (50 mg/each from different individuals) 13 and 12 spore-formers were isolated, respectively.

Together with the spore-formers, other bacteria were also isolated, but only partially characterized and not used in the present study. Those organisms were either members of thermophilic species or mesophilic but probably part of abundant population not totally killed by the heat-treatment.

The 25 spore-formers isolated were characterized at the species level by analysis of the 16S rDNA sequence and biochemically by the use of API 50 CHL kit (Table 1). As shown

in Table 1, mostly similar species of aerobic sporeformers were isolated from the two sources, whereas, due to the isolation procedure, we did not recover clones of anaerobic spore-formers. In addition, a *B. thuringiensis* clone was isolated from a faecal sample and *B. megaterium* and *Paenibacillus chibensis* isolates derived from ileal biopsies. For strain SF170, also of ileal origin, a species was not assigned since its 16S DNA sequence showed homology with an uncultured *Bacillus* (GenBank entry: AY493970). Because of the low number of samples and isolates, our results can not be taken as an indication that some species proliferate preferentially in one source or the other, but they do suggest that a very similar population of Bacilli can be found in both.

Table 1 List of intestinal strains isolated

Strain	Species[a]	Source	Accession number[b]
SF119	*Bacillus pumilus*	feces	FM178952
SF120	*Bacillus licheniformis*	feces	FM178953
SF147	*Bacillus pumilus*	feces	FM178954
SF148	*Bacillus subtilis*	feces	FM178955
SF149	*Bacillus subtilis*	feces	FM178956
SF150	*Bacillus clausii*	feces	FM178957
SF151	*Bacillus subtilis*	feces	FM178958
SF152	*Bacillus subtilis*	feces	FM178959
SF153	*Bacillus subtilis*	feces	FM178960
SF154	*Bacillus subtilis*	feces	FM178961
SF155	*Bacillus subtilis*	feces	FM178962
SF168	*Bacillus thuringiensis*	feces	FM178963
SF85	*Bacillus pumilus*	ileum	FM178964
SF106	*Bacillus subtilis*	ileum	FM178965
SFB2	*Bacillus subtilis*	ileum	FM178966
SFB3	*Bacillus subtilis*	ileum	FM178967
SF128	*Bacillus subtilis*	ileum	FM178968
SF169	*Bacillus licheniformis*	ileum	FM178969
SF170	*Bacillus* sp.	ileum	FM178970
SF173	*Bacillus megaterium*	ileum	FM178971
SF174	*Bacillus clausii*	ileum	FM178972
SF185	*Bacillus subtilis*	ileum	FM178973
SF186	*Paenibacillus chibensis*	ileum	FM178974
SF188	*Bacillus pumilus*	ileum	FM178975
SF195	*Bacillus subtilis*	ileum	FM178976

*Species assignment was based on 16S rDNA sequence analysis and on the results of the API50 CHL kit. Accession numbers of 16S rDNA sequences deposited to the EMBL nucleotide sequence database.

3.2 Swarming motility and biofilm formation

As an initial characterization, all 25 isolates were tested for their ability to swarm and produce surfactin and biofilm (Table 2). Swarming is a typical movement of bacterial cells on a solid surface (Fig. 1) and, together with biofilm formation, is a property of Bacilli that is often lost (or much reduced) in laboratory strains. In confirmation of this, none of the strains in our lab collection was able to swarm or produce biofilm in control experiments (data not shown).

Table 2. Physiological properties of the intestinal isolates.

Strain	Swarming		Surfactin ring	Biofilm formation
	LB	B		
SF119	X	X		X
SF120	X	X	X	X
SF147	X	X	X	X
SF148	X	X	X	X
SF149	X	X	X	X
SF150				X
SF151				
SF152				X
SF153				
SF154				
SF155	X			X
SF168				
SF85	X	X	X	X
SF106				
SFB2				
SFB3	X	X	X	X
SF128	X	X	X	X
SF169	X	X	X	X
SF170				
SF173				
SF174				
SF185				X
SF186				X
SF188	X	X	X	X
SF195				X

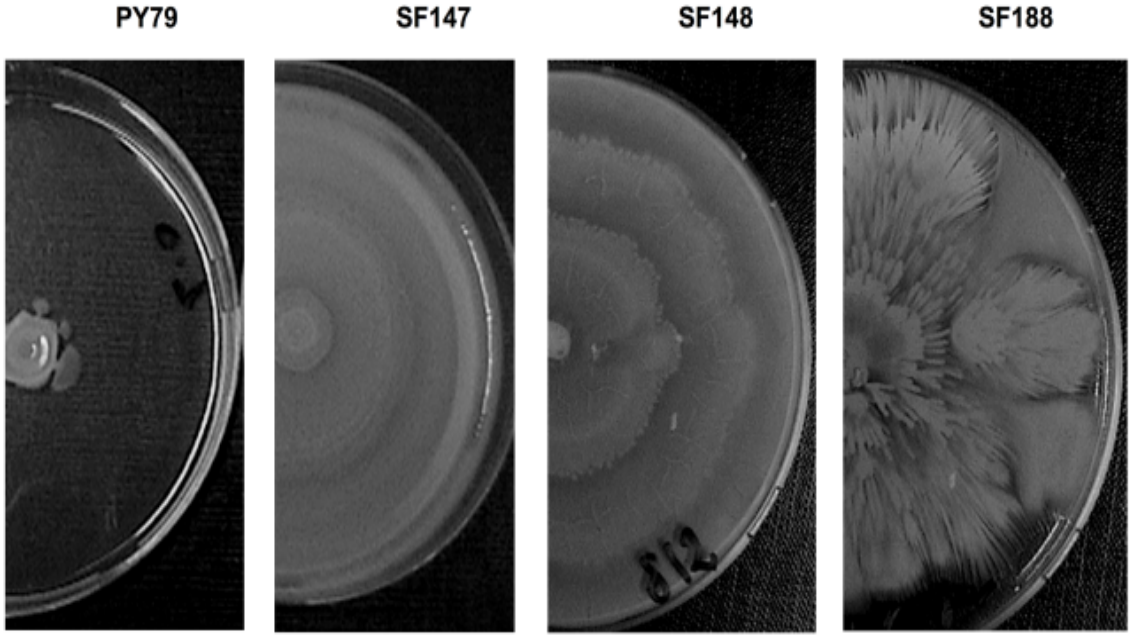


Fig. 1: Examples of swarming motility on LB medium additioned of 0.7% agar. PY79 is a laboratory collection strain of *B. subtilis* (Youngman *et al.* 1984) and does not show swarming motility. The other strains are three of the GIT isolates described in Table 1 and all show various types of swarming motility.

Studies in *B. subtilis* have shown that swarming is dependent on the presence of a flagellum in various physiological conditions and, only in a minimal medium, also on the ability of the strain to produce surfactin (Julkowska et al. 2005). For this reason we tested our strains for the ability to swarm in rich (LB) and synthetic (B; Julkowska et al. 2004) medium and also assayed their ability to produce surfactin.

The analysis of Table 2 indicates that all isolates belonging to the *B. subtilis* species behaved as previously reported for that species: those that were able to swarm in minimal medium also produced surfactin. This behaviour was not observed with SF119 (*B. pumilus*) and SF155 (*B. subtilis*) as they were able to swarm in minimal medium but did not produce surfactin. A possible explanation is that those two isolates produce a different surfactant that does not respond to the assay we used to detect surfactin (Materials and methods).

The majority of the strains (16 out of 25) formed biofilms. This is an interesting observation since biofilms have protective and adhesive properties and have been associated to a longer persistence of Bacilli in the GIT of animals (Huang et al. 2008).

The analysis of Table 2 indicates that all strains able of swarming mobility are also able to produce biofilms, whereas some of the biofilm-producers do not swarm, either in rich or minimal media. These results induced us to speculate that biofilm formation is essential but not sufficient for swarming motility. Additional studies will be needed to properly address this point.

3.3 Production of antimicrobial activity

All isolated Bacilli were then analysed for the production of antimicrobial molecules active against selected pathogens (Table 3). An exponential culture of each of the 25 isolates was used to 'spot' sterile LB plates. As previously reported (Baccigalupi et al. 2005), the spots were air-dried and used to overlay soft agar (0.7%) containing exponential cells of one of the indicator strains. Solidified plates were then incubated at the appropriate temperature for 18–24 h and the appearance of a growth–inhibition halo taken as an indication of the presence of an antimicrobial activity. As summarized in Table 3, most of the strains produced antimicrobial molecules, mainly active against the Gram-positive pathogens used in our study. In particular, most of the strains were active against *Listeria monocytogenes*. Only four strains, all isolated from fecal samples, were active against *Salmonella enterica* (SF148, SF149 and SF168) or *Shigella sonnei* (SF147).

Table 3. Antimicrobial activities produced by the intestinal isolates.

Strain	*Bacillus cereus*	*Staphylococcus aureus*	*Listeria monocytogenes*	*Salmonella enterica*	*Shigella sonneii*
SF 119			X		
SF 120	X		X		
SF 147		X	X		X
SF 148	X		X	X	
SF 149	X		X	X	
SF 150					
SF 151		X	X		
SF 152		X	X		
SF 153		X	X		
SF 154	X	X	X		
SF 155			X		
SF 168			X	X	
SF85		X	X		
SF 106		X			
SF B2		X	X		
SF B3	X		X		
SF 128	X		X		
SF 169					
SF 170					
SF 173					
SF 174					
SF 185					
SF 186			X		
SF 188					
SF 195					

Although the low number of isolates do not allow us to draw statistically significant conclusions, it is interesting to note that, while almost all fecal isolates (11 out of 12) produced antimicrobial molecules, less than 50% (6 out of 13) of the strains isolated from the ileal biopsies showed that property.

3.4 Resistance of spores and cells to simulated GIT conditions

We measured the survival of spore suspensions in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) as previously reported (Duc et al. 2004). Spores were prepared by the exhaustion method originally developed for *B. subtilis* (Nicholson and Setlow 1990). As previously reported for fecal-isolates of *B. subtilis* (Tam et al. 2006), we also noticed that some strains were faster to sporulate than other isolates, including a laboratory strain [PY79, a derivative of the 168 type strain (Youngman et al. 1984)].

Spores were then purified as previously described (Nicholson and Setlow 1990) and aliquots of 3–5 x 10^8 spores suspended for 1 h in SGF and SIF. Almost identical numbers of cells were recovered on LB plates from treated and untreated spores of all 25 isolates and of the strain PY79, indicating an almost total resistance of spores to the condition used (data not shown).

We also measured the survival of vegetative cells to SGF and SIF. Exponentially growing cells (always between 1.0 x 10^7 and 1.0 x 10^8 CFU) were exposed for 1 h to either PBS or SGF or SIF, washed and plated on LB plates. While cells of most isolates and of the control strain PY79 were totally killed by both treatments (data not shown), cells of strains SF119 (*B. pumilus*) and SF128 (*B. subtilis*), showed only a minor reduction in CFUs (Table 4).

Table 4. Survival to intestinal conditions.

Strain	Initial CFU	CFU after 1 h in PBS	CFU after 1 h in SGF[a]	CFU after 1 h in SIF[a]
PY79	9.5 x 10^7	8.8 x 10^7	n. d.[b]	n.d.[b]
SF119	2.0 x 10^7	1.8 x 10^7	1.7 x 10^6	2.8 x 10^6
SF128	5.3 x 10^7	5.4 x 10^7	8.0 x 10^6	6.0 x 10^6

[a] SIF: Simulated Gastric Fluid; SIF: Simulated Intestinal Fluid. [b] n.d.: not detectable

it is well known that several *Bacillus* isolates produce such protective structure that surrounds the cell and reduces cell surface accessibility (Candela et al. 2005; Huber et al. 2005). We tested those two strains, as well as all other isolates, for sensitivity to some antibiotics. SF119 and SF128 were both sensitive to all antibiotics tested (not shown) suggesting that their resistance to SGF and SIF is not due to an impermeable surface. We do not have an explanation for the high level of resistance of cells of those two strains to simulated GIT conditions and can only speculate that it may reflect an adaptation of the cells to the environment they lived in.

The antibiotic inhibition test indicated that strains SFB3 and SF168 were resistant to erythromycin (3 lg ml^{-1}), strain SF195 was resistant to spectinomycin (200 lg ml^{-1}) and strain SFB2 was resistant to neomycin (20 lg m^{-1} l) and spectinomycin (200 lg ml^{-1}). All other strains were sensitive to the antibiotic tested (Material and methods).

3.5 Growth and sporulation in anaerobic conditions

Members of the *Bacillus* genus are aerobic bacteria, unable to grow anaerobically. However, there are exceptions and lab collections strains (derivatives of the 168 type strain of *B. subtilis*) are able to grow anaerobically when nitrate is provided as electron acceptor (Nakano and Zuber 1998). Tam et al. (2006) have reported that two wild isolates *of B. subtilis* can grow and sporulate on a solid sporulation-inducing medium (DSM) in anaerobic conditions, whereas the domesticated strain PY79 grew but sporulated at very low efficiency. In their experimental conditions, addition of nitrate to the medium did not improve significantly either growth or sporulation of the tested strains (Tam et al. 2006).

We analysed all 25 human isolates for their ability to grow in anaerobic conditions in a rich (LB) and a sporulation- inducing medium (DSM). As reported in Table 5, most of the isolates were able to grow anaerobically in DSM whereas only three of them grew anaerobically in rich LB medium. Of the 19 isolates that grew anaerobically on DSM plates, 13 were also able to sporulate in the absence of oxygen. This observation confirms the previous observation reported on *B. subtilis* isolates (Tam et al. 2006) and expands it to the other species present in our collection.

Table 5. Growth and sporulation of the intestinal isolates in anaerobic conditions.

Strain	Vegetative growth on LB	Vegetative growth on DSM	Sporulation
SF119			
SF120	X	X	X
SF147			
SF148		X	X
SF149		X	X
SF150		X	X
SF151		X	
SF152		X	
SF153			
SF154		X	
SF155			
SF168		X	X
SF85			
SF106		X	X
SFB2		X	X
SFB3		X	X
SF128	X	X	X
SF169	X	X	X
SF170			
SF173		X	X
SF174		X	
SF185		X	X
SF186		X	
SF188		X	X
SF195		X	

The analysis of Table 5 also reveals that three out of four *B. pumilus* isolated from the human GIT were strictly aerobic (SF119, SF147 and SF85) and that the *B. subtilis* isolates were mostly anaerobic since of the 13 isolated strains two were strictly aerobic (SF153 and SF155), four were able to grow but not to sporulate (SF151, SF152, SF154 and SF195) and seven were able togrow and sporulate (SF148, SF149,SF106, SFB2, SFB3,SF128 and SF185)

3.6 Presence of potential virulence factors

We used a PCR approach to evaluate the presence of known B. cereus enterotoxin genes in the chromosome of all isolates using, as a control, the B. cereus strain GN105 (Naclerio et al. 1993). This method has been applied previously to profile putative food-poisoning

Bacillus strains (Duc et al. 2004; Guinebretiere et al. 2002; Phelps and McKillip 2002). Figure 2 reports the results obtained for the PCR amplification of two isolates (SF150 and SF188) and the control strain GN105. Those reported in Fig. 2 were the only two strains positive for the presence of known enterotoxins. All other isolates were negative in our PCR-based analysis allowing us to conclude that 23 out of 25 tested strains did not contain genes encoding known Bacillus toxins in their genome.

In vivo analysis showed that only strains SF128 produced a-haemolysis while four strains produced b-haemolysis (SF119, SF147, SF168 and SF188). All other strains did not produce haemolysis and, therefore, can be consideredn as c-haemolytic.

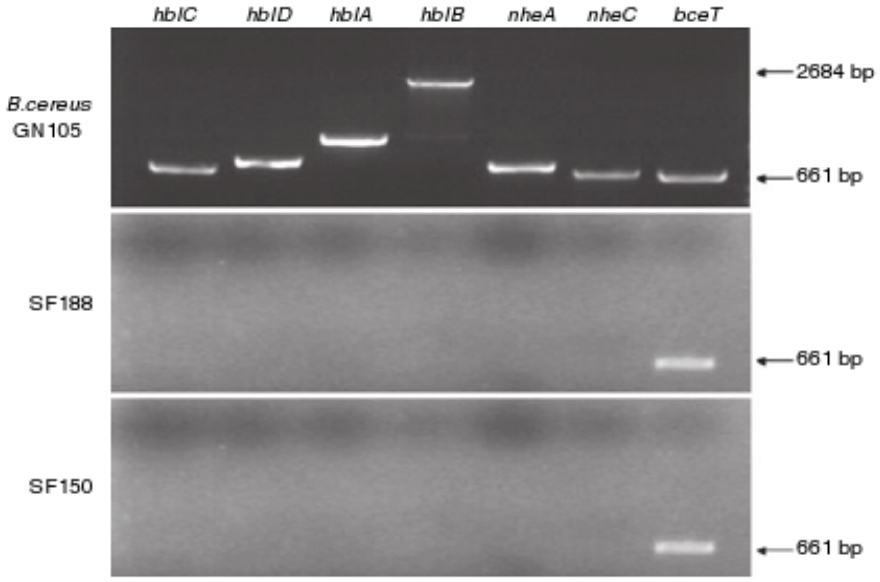


Figure 2 Agarose gel electrophoresis of PCR amplification products. All selected oligonucleotide pairs amplified specific fragments of the expected size using chromosomal DNA of the Bacillus cereus strain GN105 (Naclerio et al. 1993) as a template. Only the oligonucleotide pair used to amplify part of the bceT gene amplified a fragment of the expected size using chromosomal DNA of strains SF150 or Sf188 as a template.

4. Discussion

We used fecal samples and ileal biopsies of healthy human volunteers to retrieve 13 and 12 spore-forming isolates, respectively. Those bacteria were first characterized at the species level and then analysed for various physiological properties, some of which may be relevant for future use in probiotic preparations containing defined strains.

Some interesting conclusions can be drawn from this work. In agreement with Bacilli isolated from the soil (Branda et al. 2001; Earl et al. 2007), those described here, isolated from a seemingly peculiar environment such as the human gut, displayed swarming motility and biofilm formation. It is not yet known whether those Bacilli are able to swarm or form biofilm within the gut, but the observation that these properties have not been lost (as it has, instead, occurred in laboratory strains) allows us to hypothesize that they are important in the environment where these bacteria inhabit.

Biofilms have been proposed to have protective and adhesive roles for the bacteria producing them. These functions are potentially relevant for bacteria in the gut, and because of the protective environment of the biofilm, could enable survival in the intestinal conditions and adhere to mucus and epithelial cells more effectively than planktonic cells. In addition, within biofilms bacteria can respond to quorum-sensing molecules more easily than planktonic cells. Sporulation is known to be induced by quorum-sensing signals (e.g. the CSF of *B. subtilis)* and this has been shown to be essential for GALT development (Rhee et al. 2004).

Also the ability to grow and sporulate in an anaerobic environment appears as a common property of Bacilli isolated from the anaerobic gut. Rather than aerobes, Bacilli should be considered as facultative anaerobes, able to use oxygen or a different electron acceptor depending on the environmental conditions. The observation that some isolates were able to grow but not to sporulate in the anaerobic conditions obtained in the laboratory may suggest that different electron acceptors and／or different metabolic pathways are used during growth and sporulation.

While it is not surprising that all isolates survived the simulated GIT conditions in the spore form, it is striking that two isolates survived also in the vegetative cell form. The observation that the same two isolates were sensitive to common antibiotics allowed us to exclude that resistance to pepsin and pancreatin, present in SGF and SIF, respectively, was due to an unusual cell surface, impermeable to many external molecules. The

mechanism of that resistance remains not known and further experiments will be needed to address this point.

Some features of the 25 human isolates, such as growth and sporulation in anaerobic conditions, cell survival to simulated GIT condition and biofilm formation, allow us to hypothesize that those strains are well adapted to the gut environment and potentially able to colonize that habitat.

Most of the isolates were sensitive to common antibiotics and did not contain genes encoding for known *Bacillus* toxins. Only two isolates, SF150 and SF188, contained a gene homologous to the bceT gene of *B. cereus*. However, bceT codes for enterotoxin T, a factor that has been shown not to contribute to food poisoning (Choma and Granum 2002). Although a proper safety assessment, with cytotoxicity and in vivo tests, is needed before these strains can be considered as probiotics, the preliminary data presented here are an encouraging starting point to identify Bacilli of human origin to be used as probiotics for human use.

An additional potential application of those strains is as oral vaccine vehicles. Spores of B. subtilis have been used to display heterologous antigens (Isticato et al. 2001; Mauriello et al. 2004). Recombinant spores, orally administered to mice, were able to induce a specific humoral (Duc et al. 2003) and cellular (Mauriello et al. 2007) response. The immune response induced by spores exposing a fragment of the tetanus toxin resulted protective in a challenge experiment, with immunized mice able to survive the injection of a lethal dose of the toxin (Duc et al. 2003). It has been proposed that part of the observed immune response is not due to antigens present on the spores orally administered to the animals but rather to the antigens produced inside the animal body when recombinant spores germinate and sporulate (Uyen et al. 2007). All those studies have been performed with a laboratory strain of B. subtilis, not producing biofilms and incapable of efficient sporulation in anaerobic conditions. It is then reasonable to hypothesize that natural Bacilli expressing heterologous antigens may perform better than lab strains. It is, then, clear that a more efficient biofilmassisted adhesion of cells to the intestinal epithelium and a more efficient

sporulation in anaerobic conditions of the gut would result in a more efficient expression of the antigens and, presumably, in a stronger immune response.

References

Baccigalupi, L., Di Donato, A., Parlato, M., Luongo, D., Carbone, V., Rossi, M., Ricca, E. and De Felice, M. (2005) Small, surface-associated factors mediate adhesion of a food-isolated strain of *Lactobacillus fermentum* to Caco-2 cells*. Res Microbiol* 156, 830–836.

Barbosa, T.M., Serra, C.R., La Ragione, R.M., Woodward, M.J. and Henriques, A.O. (2005) Screening for Bacillus isolates in the broiler gastrointestinal tract. *Appl Environ Microbiol* 71, 968–978.

Branda, S.S., Gonza´lez-Pastor, J.E., Ben-Yehuda, S., Losick, R. and Kolter, R. (2001) Fruiting body formation by *Bacillus subtilis*. *Proc Natl Acad Sci USA* 98, 11621–1166.

Candela, T., Mignot, T., Hagnerelle, X., Haustant, M. and Fouet, A. (2005) Genetic analysis of *Bacillus anthracis* Sap S-layer protein crystallization domain. *Microbiology* 151, 1485–1490.

Casula, G. and Cutting, S.M. (2002*) Bacillus* probiotics: spore germination in the gastrointestinal tract. *Appl Environ Microbiol* 68, 2344–2352.

Choma, C. and Granum, P.E. (2002) The enterotoxin T (BcET) from *Bacillus cereus* can probably not contribute to food poisoning. *FEMS Microbiol Lett* 217, 115–119.

Connelly, M.B., Young, G.M. and Sloma, A. (2004) Extracellular proteolytic activity plays a central role in swarming motility in *Bacillus subtilis. J Bacteriol* 186, 4159–4167.

D'Arienzo, R., Maurano, F., Mazzarella, G., Luongo, D., Stefanile, R., Ricca, E. and Rossi, M. (2006*) Bacillus subtilis* spores reduce susceptibility to *Citrobacter rodentium* mediated enteropathy in a mouse model. *Res Microbiol* 157, 891–897.

Duc, L.H., Huynh, H.A., Fairweather, N., Ricca, E. and Cutting, S.M. (2003) Bacterial spores as vaccine vehicles*. Infect Immun* 71, 2810–2818.

Duc, le H., Hong, H.A., Barbosa, T.M., Henriques, A.O. and Cutting, S.M. (2004) Characterization of *Bacillus* probiotics available for human use. *Appl Environ Microbiol* 70, 2161–2171.

Earl, A.M., Losick, R. and Kolter, R. (2007) *Bacillus subtilis* genome diversity. *J Bacteriol* 189, 1163–1170.

Earl, A.M., Losick, R. and Kolter, R. (2008) Ecology and genomics of *Bacillus subtilis*. *Trends Microbiol* 16, 269–275.

Fritze, D. (2004) Taxonomy of the genus *Bacillus* and related genera: the aerobic endospore-forming bacteria. *Phytopathology* 94, 1245e1248.

Fujiya, M., Musch, M.W., Nakagawa, Y., Hu, S., Alverdy, J., Kohgo, Y., Schneewind, O., Jabri, B. et al. (2007) The *Bacillus subtilis* quorum-sensing molecule CSF contributes to intestinal homeostasis via OCTN2, a host cell membrane transporter. *Cell Host Microbe* 1, 299–308.

Green, D., Wakeley, P., Page, A., Barnes, A., Baccigalupi, L., Ricca, E. and Cutting, S. (1999) Characterization of two *Bacillus* probiotic species. *Appl Environ Microbiol* 65, 4288–4291.

Guinebretiere, M.-H., Broussolle, V. and Nguyen-The, C. (2002) Enterotoxigenic profiles of food-poisoning and food-borne *Bacillus cereus* strains. *J Clin Microbiol* 40, 3053–3056.

Henriques, A.O. and Moran, C.P., Jr (2007) Structure, assembly and function of the spore surface layers. *Ann Rev Microbiol* 61, 555–588.

Hoa, N.-T., Baccigalupi, L., Huxham, A., Smertenko, A., Van, P.-H., Ammendola, S., Ricca, E. and Cutting, S. (2000) Characterization of *Bacillus* species used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders. *Appl Environ Microbiol* 66, 5241–5247.

Hong, H.A., Duc, le H. and Cutting, S.M. (2005) The use of bacterial spore formers as probiotics. *FEMS Microbiol Rev* 29, 813–835.

Huang, J.M., La Ragione, R.M., Nunez, A. and Cutting, S.M. (2008) Immunostimulatory activity of *Bacillus* spores*. FEMS Immunol Med Microbio*l 53, 195–203.

Huber, C., Ilk, N., Ru¨nzler, D., Egelseer, E.M., Weigert, S., Sleytr, U.B. and Sa´ra, M. (2005) The three S-layer-like homology motifs of the S-layer protein SbpA of *Bacillus sphaericus* CCM 2177 are not sufficient for binding to the pyruvylated secondary cell wall polymer. *Mol Microbio*l 55, 197–205.

Isticato, R., Cangiano, G., Tran, T.-H., Ciabattini, A., Medaglini, D., Oggioni, M.R., De Felice, M., Pozzi, G. et al. (2001) Surface display of recombinant proteins on *Bacillus subtilis* spores. *J Bacteriol* 183, 6294–6301.

Julkowska, D., Obuchowski, M., Holland, I.B. and Se´ror, S.J. (2004) Branched swarming patterns on a synthetic medium formed by wild-type *Bacillus subtilis* strain 3610: detection of different cellular morphologies and constellations of cells as the complex architecture develops. *Microbiology* 150, 1839–1849.

Julkowska, D., Obuchowski, M., Holland, B. and Seror, S. (2005) Comparative analysis of the development of swarming communities of *Bacillus subtilis* 168 and

a natural wild type: critical effects of surfactin and the composition of the medium. *J Bacteriol* 187, 65–76.

Mauriello, E.M.F., Duc, L.H., Isticato, R., Cangiano, G., Hong, H.A., De Felice, M., Ricca, E. and Cutting, S.M. (2004) Display of heterologous antigens on the *Bacillus subtilis* spore coat using cotc as a fusion partner. *Vaccine* 22, 1177–1187.

Mauriello, E.M.F., Cangiano, G., Maurano, F., Saggese, V., De Felice, M., Rossi, M. and Ricca, E. (2007) Germination independent induction of cellular immune response by *Bacillus subtilis* spores displaying the C fragment of the tetanus toxin. *Vaccine* 25, 788–793.

Naclerio, G., Ricca, E., Sacco, M. and De Felice, M. (1993) Antimicrobial activity of a newly identified bacteriocin of *Bacillus cereus*. Appl *Environ Microbiol* 59, 4313–4316.

Nakano, M.M. and Zuber, P. (1998) Anaerobic growth of a ''strict aerobe'' (*Bacillus subtilis*). *Annu Rev Microbiol* 52, 165–190.

Nicholson, W. L. and Setlow, P. (1990) Sporulation, germination and outgrowth. In Molecular Biological Methods for *Bacillus* eds. **Harwood**, **C and Cutting, S**. pp. 391–450. Chichester, UK: John Wiley and Sons.

Phelps, R.J. and McKillip, J.L. (2002) Enterotoxin production in natural isolates of Bacillaceae outside the *Bacillus cereus* group. *Appl Environ Microbiol* 68, 3147–3151.

Rhee, K-J., Sethupathi, P., Driks, A., Lanning, K.D. and Knight, K.L. (2004) Role of commensal bacteria in the development of gut-associated lymphoid tissue and preimmune antibody repertoire. *J Immunol* 172, 1118–1124.

Tam, N.K., Uyen, N.Q., Hong, H.A., Duc, le H., Hoa, T.T., Serra, C.R., Henriques, A.O. and Cutting, S.M. (2006) The intestinal life cycle of *Bacillus subtilis* and close relatives*. J Bacteriol* 188, 2692–2700.

Uyen, N.Q., Hong, H.A. and Cutting, S.M. (2007) Enhanced immunisation and expression strategies using bacterial spores as heat-stable vaccine delivery vehicles. *Vaccine* 25, 356–365.

Woo, P.C.Y., Ng, K.H.L., Lau, S.K.P., Yip, K.T., Fung, A.M.Y., Leung, K.W., Tam, D.M.W., Que, T.I. et al. (2003) Usefulness of the MicroSeq 500 16S ribosomal DNA-based bacterial identification system for identification of clinically significant bacterial isolates with ambiguous biochemical profiles*. J Clin Microbiol* 41, 1996– 2001.

Youngman, P., Perkins, J.B. and Losick, R. (1984) A novel method for the rapid cloning in *Escherichia coli* of *Bacillus subtilis* chromosomal DNA adjacent to Tn917 insertion. *Mol Gen Genet* 195, 424–433.

Youssef, N.H., Duncan, E.K., Nagle, D.P., Savage, K.N., Knapp, R.M. and McInerney, M.J. (2004) Comparison of methods to detect biosurfactant production by diverse microorganisms. *J Microbiol Methods* 56, 339–347.

Chapter four

Defining the natural habitat of Bacillus spore - formers

Published on: Research in Microbiology

Hong, H.A., To, E., **Fakhry, S.**, Baccigalupi, L., Ricca, E. and Cutting, S.M. "Defining the natural habitat of *Bacillus* spore-formers". Res. Microbiol. 160:375-379 (2009)

Abstract

Our understanding of the genetics and physiology of the spore-forming genus Bacillus is remarkable. On the other hand, though, where these Gram-positive bacteria live and grow is far from clear. The soil, once considered their habitat, may simply serve as a reservoir. A growing number of studies show that Bacillus spores can be found in the intestinal tracts of animals, raising the question of whether this could be where they live and grow. In this study, we have conducted the first evaluation of Bacillus spore formers in soil and in human faeces. Our aim is simply to determine the abundance of aerobic spore-formers. Our results show that soil carries approximately w10^6 spores/g while human faeces an average of up to 10^4 spores/g. The numbers of spores found in faeces, we reason, is too high to be accounted for principally by ingestion of food contaminated with spores from soil. This provides further evidence that Bacillus spore formers may have adapted to survival within the intestinal tract of insects and other animals that ingest them; if so they may well be hitherto undiscovered gut commensals.

1. Introduction

Bacterial endospore-formers typically fall under two major groupings of Gram-positives, the *Bacilli* and *Clostridia*. Many other spore-forming genera exist, including Gram-negatives, but for the most part, these remain poorly understood (Fritze 2004). In the case of *Bacillus*, most members have long been considered soil organisms (Nicholson et al. 2002*).* This assumption is based upon culturedependant methods of isolation that enrich for the presence of endospores, implying abundance (Felske et al*.* 2004). In recent years, it has become apparent that this may be an oversimplification and *Bacillus* endospores have been found in diverse environments including rocks, dust, aquatic environments and the gut of various insects and animals (Felske et al*.* 2004). Endospores are uniquely robust life forms enabling them to be dispersed easily and, as a result, found everywhere (Nicholson et al. 2000; Nicholson et al. 2002*)*. From a purely academic viewpoint, it is ironic that, for an organism that is genetically so well defined, on a par with *Escherichia*

coli, its true habitat and life cycle is so poorly understood. One question that has been raised is whether there is a symbiotic relationship between Bacillus spp. and insects and animals (Nicholson et al*. 2002)*. For insects, in particular, there is stronger evidence for commensalism where the host benefits. For example, cockroaches fed with *Bacillus cereus* exhibited direct and positive weight gains (Feinberg et al. 1999). An obligate, endosymbiotic, relationship with the Grampositive endospore former, Metabacterium polyspora has been shown in the gut of the guinea pig (Angert et al*.* 1998) demonstrating that for some endospore formers, at least, there must also be direct benefits to the bacterium. *Bacillus spp*. are being used as probiotics for livestock, aquaculture as well as functional foods for human consumption (Hong et al. 2005). For animal use, there are bdirect and substantiated benefits to animal health including weight gains and prevention of disease. In rabbits for example, *Bacillus subtilis* has been shown to have a direct effect on the development of the gut-associated lymphoid tissue (GALT). Interestingly, these studies showed that sporulation, per se, was required for this phenomenon. This has been supported by murine studies that have shown, in vivo, that ingested endospores are able to germinate, proliferate and then resporulate in the small intestine (Yoon et al. 2005). *Bacillus* endospores then must have a much more intimate relationship with insects and animals than might be expected if their primary habitat were only the soil. We have raised the possibility that *Bacillus* species are able to exploit the intestinal tract as a habitat (Yoon et al*.* 2005). This has been supported by a number of studies that have recovered *Bacillus spp*. from the small intestine and from faecal samples (Fakhry et al*.* 2008; Hong et al. 2009). In this work, we have examined the abundance of aerobic endospore formers in samples of human faeces as well as soil samples. Our results suggest that the human gastrointestinal (GI) tract is populated with *Bacillus* spore-forming species but these counts are at least 100-fold lower than the counts found in soil.

2. Materials and methods

2.1 Collection of faecal samples

Freshly voided faeces was collected from healthy volunteers and weighed by difference. Volunteers had not taken antibiotics or probiotics within the preceding 12 months and were recruited from the vicinity of either London or Naples. Faeces was stored at 4 °C and processed within 2-3 h of collection.

2.2 Soil samples

Approximately 10-20 g of soil (weighed by difference) was collected from selected locations in the London region. In each case, soil at a depth of 5-10 cm below the surface was collected. Samples were stored at 4 °C prior to analysis.

2.3 Determination of counts of spore-forming bacteria

Bacterial endospore formers were counted using two methods, heat or ethanol treatment. Faecal or soil samples (approx. 1-10 g) were suspended in the minimal volume of sterile phosphate-buffered saline (PBS) that allowed adequate suspension of solid matter by vigorous vortexing. In some cases, sterile glass beads (2 mm) were used to aid homogenisation of solid matter. For heat treatment, homogenised samples were heated in an oven at 65 °C for 45 min. For ethanol treatment 1 volume of homogenised sample was mixed with an equal volume of absolute alcohol and incubated for 1 h at room temperature (RT). After both heat or ethanol treatment, samples were immediately serially diluted and plated out on three solid media, Difco-Sporulation medium (DSM; [Nicholson et al. 1990]), MRS agar (Oxoid) and MacConkey's agar

(Oxoid). Plates were incubated for 2-3 days at 30 °C and in the case of MRS agar plates were incubated anaerobically using a Don Whitley anaerobic cabinet.

2.4 Other methods

Catalase activity was confirmed by emulsification of single colonies in a 3% solution of hydrogen peroxide (Sigma). The one-way ANOVA test was used to compare the significance between groups.

3. Results and Discussion

Faecal and soil samples were examined for quantification of bacterial endospores using a culture-dependent approach. Spore counts from faecal samples were taken from volunteers in an Italian study (Fig. 1A and Supplementary Table 1) and 20 from the UK (Fig. 1B and Supp. Table 2). As controls, the number of enterobacteria and lactobacilli were enumerated using growth on MacConkey's and MRS agar (H respectively. Faecal counts for the enterobacteria and lactobacilli ranged between 10^6-10^7 for the former and 10^7-10^9 for the latter. Counts of lactobacilli using MRS medium were in agreement with other work (Hartemink et al. 1997) as were counts for the enterobacteria, demonstrating that our methodology was sound. In addition, 50 soil samples were examined (Fig. 2 and Supp. Table 3). Our analysis wasdesigned to identify aerobic endospore formers and therefore excluded species of the strict anaerobes, i.e., *Clostridia*. Colonies were randomly tested for their catalase reaction and always tested positive, further excluding the possibility of *Clostridia*. The Gram stain was also performed on some isolates confirming in all cases the presence of Gram-positive rods. Thus, the vast majority of recovered strains were most probably species of *Bacillus*.

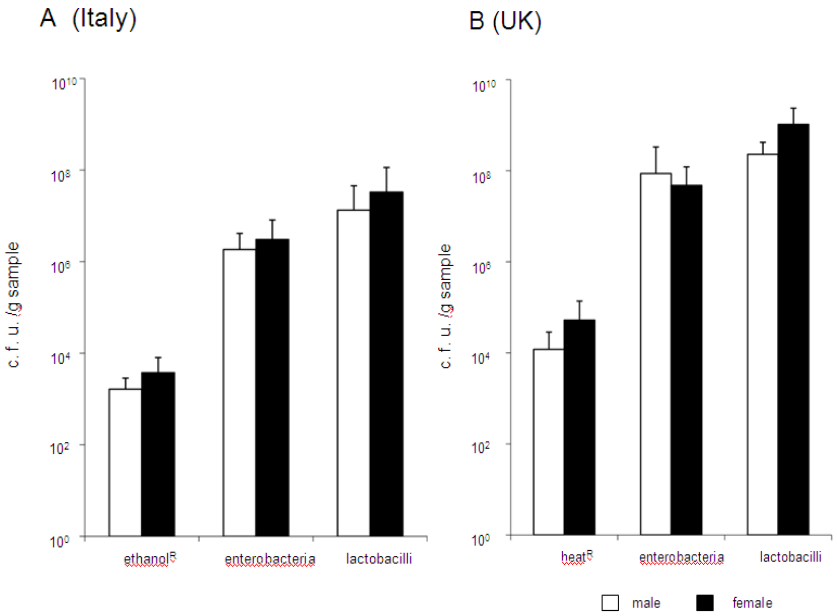


Figure 1. Aerobic spore-formers isolated from human feces. Counts of spores (cfu/g) obtained by ethanol treatment of freshly voided samples in Italy (A) and in UK (B). Plates were grown aerobically. Levels of enterobacteria and lactobacilli were determined by plating untreated samples on MacConkey's and MRS agar respectively. Raw data is given in Supp. Tables 1 and 2.

Supp. Table 1: Counts of spore-formers[1], enterobacteria and lactobacilli in human feces obtained from Italy[2]							
		CFU/g feces for:					
		Aerobically growth				Anaerobically growth	
		Ethanol Treatment		No Treatment			
Sample No.		DSM agar		MacConkeys agar		MRS agar	
		Male	Female	Male	Female	Male	Female
1		1.00×10^3	6.00×10^3	5.00×10^5	1.50×10^7	5.00×10^6	2.00×10^6
2		2.64×10^3	2.90×10^3	2.00×10^6	1.41×10^6	3.00×10^6	2.83×10^8
3		2.28×10^3	8.54×10^2	8.65×10^6	2.92×10^4	3.74×10^7	6.84×10^7
4		4.40×10^2	1.23×10^3	5.73×10^6	8.77×10^5	8.40×10^6	1.75×10^7
5		1.87×10^3	5.70×10^2	5.00×10^6	8.55×10^5	7.50×10^5	1.14×10^6
6		1.74×10^3	1.62×10^3	8.71×10^4	1.25×10^7	4.35×10^5	3.75×10^6
7		5.49×10^2	5.00×10^3	1.03×10^6	1.18×10^6	1.37×10^8	1.48×10^7
8		4.40×10^2	1.50×10^4	5.73×10^5	9.00×10^4	9.55×10^6	2.50×10^6
9		7.00×10^2	2.21×10^2	9.80×10^5	7.70×10^5	4.90×10^4	7.00×10^5
10		2.60×10^3	1.42×10^2	8.00×10^5	4.26×10^4	3.00×10^6	2.25×10^5
11		1.74×10^3	4.00×10^3	1.74×10^6	2.00×10^6	1.74×10^7	1.60×10^6
12		4.40×10^3	7.50×10^3	1.00×10^5	1.90×10^6	1.30×10^5	1.80×10^6
13		8.49×10^2		1.00×10^6		2.80×10^5	
14		3.28×10^2		7.20×10^5		1.00×10^6	
15		8.80×10^2		1.37×10^5		8.00×10^4	
16		1.40×10^3		0.90×10^6		1.20×10^6	
17		4.00×10^3		1.40×10^6		1.65×10^6	
AVG		$\mathbf{1.81 \times 10^3}$	$\mathbf{3.75 \times 10^3}$	$\mathbf{1.84 \times 10^6}$	$\mathbf{3.05 \times 10^6}$	$\mathbf{1.33 \times 10^7}$	$\mathbf{3.38 \times 10^7}$

[1]selected using ethanol treatment; [2]Volunteers aged between 2 and 46.

Supp. Table 2: Counts of spore-formers[1], enterobacteria and lactobacilli in human feces obtained from the UK[2]							
		CFU/g feces for:					
		Aerobically growth				Anaerobically growth	
		65°C Treatment		No Treatment			
Sample No.		DSM agar		MacConkeys agar		MRS agar	
		Male	Female	Male	Female	Male	Female
1		4.78×10^{4}	3.93×10^{2}	2.21×10^{6}	9.59×10^{7}	4.31×10^{7}	2.85×10^{8}
2		3.50×10^{1}	1.63×10^{3}	8.21×10^{6}	3.60×10^{7}	4.46×10^{8}	4.29×10^{9}
3		2.78×10^{4}	1.86×10^{4}	4.12×10^{7}	4.13×10^{6}	1.25×10^{8}	1.92×10^{8}
4		3.05×10^{2}	1.70×10^{4}	6.27×10^{6}	3.77×10^{6}	5.14×10^{8}	1.23×10^{9}
5		3.83×10^{2}	7.18×10^{4}	2.74×10^{5}	6.95×10^{6}	1.62×10^{8}	5.14×10^{8}
6		2.72×10^{4}	1.51×10^{4}	4.69×10^{5}	1.75×10^{5}	3.26×10^{8}	1.00×10^{8}
7		9.21×10^{3}	6.20×10^{3}	2.87×10^{6}	9.13×10^{4}	2.30×10^{7}	1.17×10^{8}
8		4.93×10^{2}	6.91×10^{4}	2.19×10^{7}	6.74×10^{6}	3.18×10^{7}	1.67×10^{9}
9		2.85×10^{2}	3.93×10^{4}	5.71×10^{4}	9.56×10^{7}	4.66×10^{8}	1.91×10^{8}
10		5.89×10^{3}	2.83×10^{5}	7.83×10^{8}	2.28×10^{8}	1.52×10^{8}	1.79×10^{9}
AVG		**1.19×10^{4}**	**5.22×10^{4}**	**8.66×10^{7}**	**4.78×10^{7}**	**2.29×10^{8}**	**1.04×10^{9}**

[1]selected using ethanol treatment; [2]Volunteers aged between 2 and 46.

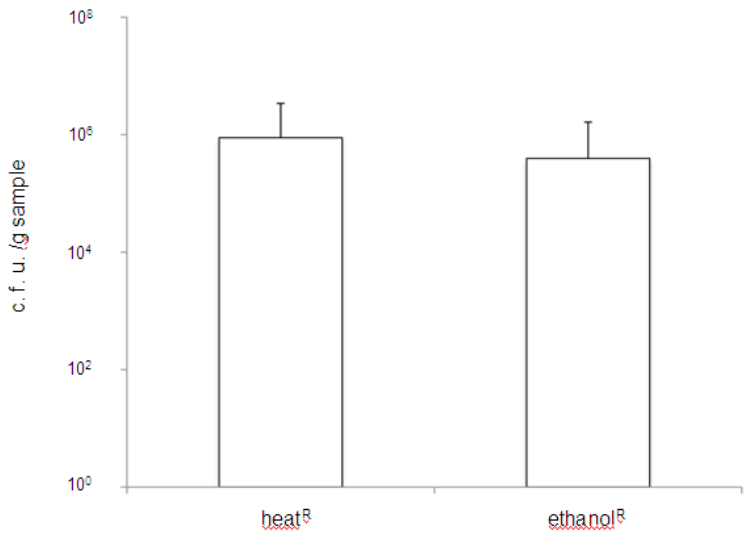


Figure 2: Aerobic spore-formers isolated from soil samples. Soil samples (50) were examined for the presence of aerobic spore formers (cfu/g) using ethanol or heat-treatment. Raw data is given in Supp. Table 3.

3.1 . Spore formers are recovered from humanfaecal samples

Heat treatment (65 °C, 45 min.) should kill all vegetative cells, but it is worth mentioning that endospores of some species are resistant to only 60 °C. For example, endospores of some strains of B. cereus showed a reduction in viability after heat treatment at 65 °C (Hoa et al. 2000). We used 65 °C since, at 60 °C, residual fungal contamination was sometimes observed, and therefore our counts are probably a slight underestimate of the true numbers of aerobic endospore formers. While a surprisingly high number of bacterial species produce endospores (Fritze 2004), the most ubiquitous is *Bacillus* and it is likely that these represent the majority of the recovered counts. Our counts averaged at about 10^4 CFU/g and were therefore similar to those from a study of heat-treated human samples in Vietnam (Tam et al. 2006), although counts for adults were somewhat higher in the Vietnamese study. Using ethanol produced somewhat different results with lower endospore counts (~10^3) compared to heat treatment. There are a number of explanations: first, the study was performed in two different laboratories and two

Supp. Table 3: Counts of spore former in soil samples					
Sample No.[1]	Heat-resistant cfu/g	Ethanol-resistant cfu/g	Sample No.[1]	Heat-resistant cfu/g	Ethanol-resistant cfu/g
1	9.24×10^4	1.83×10^5	26	1.54×10^5	2.10×10^5
2	7.38×10^4	1.58×10^5	27	5.10×10^4	4.44×10^4
3	1.80×10^5	1.33×10^5	28	5.22×10^5	6.12×10^5
4	8.04×10^4	6.90×10^4	29	7.26×10^4	4.74×10^4
5	8.04×10^5	3.84×10^4	30	2.46×10^5	4.68×10^4
6	2.52×10^5	7.14×10^4	31	7.74×10^5	7.02×10^5
7	8.40×10^4	8.40×10^4	32	8.10×10^5	4.26×10^5
8	7.04×10^4	5.28×10^4	33	2.06×10^6	8.76×10^5
9	4.44×10^5	4.86×10^5	34	3.18×10^5	9.90×10^4
10	3.00×10^5	3.06×10^5	35	1.80×10^4	3.42×10^4
11	1.82×10^6	1.21×10^6	36	4.20×10^3	7.80×10^3
12	6.48×10^5	3.72×10^5	37	6.54×10^5	6.00×10^2
13	7.80×10^5	5.28×10^5	38	3.44×10^4	7.20×10^4
14	2.52×10^5	6.48×10^4	39	5.58×10^4	6.48×10^4
15	3.24×10^5	6.66×10^4	40	7.80×10^6	8.82×10^6
16	2.70×10^5	2.58×10^4	41	4.74×10^5	1.30×10^5
17	5.34×10^5	8.52×10^4	42	2.28×10^5	6.72×10^4
18	2.88×10^5	2.58×10^5	43	6.36×10^5	2.22×10^5
19	5.34×10^4	1.04×10^5	44	1.80×10^3	1.20×10^3
20	8.40×10^4	5.10×10^4	45	6.96×10^5	3.00×10^5
21	2.92×10^6	5.84×10^5	46	1.10×10^5	3.18×10^5
22	2.70×10^5	3.00×10^5	47	2.23×10^5	1.45×10^5
23	1.87×10^5	3.66×10^4	48	1.63×10^7	2.28×10^5
24	4.50×10^5	9.24×10^4	49	5.10×10^4	7.50×10^4
25	6.72×10^5	6.84×10^5	50	1.30×10^5	1.43×10^5
AVG				**8.86×10^5**	**3.95×10^5**

different countries and we cannot rule out the possibility of a slight variation in the methodology or reagents. A second possibility is that this reveals a real difference perhaps caused, for example, by diet. We believe, though, that the lower counts reflect the choice of ethanol for detection of endospore counts. The premise for using ethanol as a selective treatment was a previous study suggesting better enumeration of endospore counts compared to heat treatment (Koransky et al. 1978). In this work though, endospores evaluated were, at most, 5 days old. It is well understood that 'aged' spores are less capable of germinating synchronously and they must be heat-activated prior to culture on rich media (Moir 1997). If endospores found in the faeces (and also the soil samples; see below) are in this state of heightened dormancy, then this might explain the slight difference in endospore counts between the two studies. Although some differences existed between sexes, endospore counts were always higher in females, although the differences were not significant ($P > 0.05$).

3.2 Aerobic spores present in soil

Counts of aerobic endospore formers were, on average, 10- to 100-fold higher than faecal counts with an average of 10^5 CFU/g. As with faeces, heat-treatment generated somewhat higher numbers. Colonies identified were, in general, more pleiomorphic in appearance with rhizoid-type colonies, pinpoint as well as crenated forms. We also found that up to 15% of colonies identified from soil were pigmented. Pigmentation in endospores provides them with an extra level of protection against UV irradiation and an attribute of highvalue if they are to remain dormant in the soil for long periods of time (Moeller et al. 2005). A variety of pigments have been found in *Bacillus* species including reds (*Bacillus atrophaeus* (Moeller et al. 2005) and *Bacillus megaterium* [Mitchell et al. 1986]), dark-grey (*B. atrophaeus* [Moeller et al. 2005]), yellow and orange *(Bacillus indicus* [Suresh et al. 2004] and *Bacillus cibi* [Yoon et al. 2005]). In many cases, the pigments are carotenoids that provide natural antioxidative properties (Duc et al. 2006., Moeller et al. 2005). Intriguingly, we found that at low dilutions,

pigmentation was difficult to detect even when colonies were well isolated. On the other hand, at higher dilutions, for example, with 20e100 colonies on a plate, as many as 15% of colonies derived from heat-or ethanol-treated soil were pigmented (data not shown). It is possible that in a nutrient-rich environment (i.e., at high dilution), pigmentation is enhanced implying some form of catabolite repression. In any event, the main discovery, we surmise, is that a large number of endospore-formers are able to form pigments and the use of classical culture-dependant methods of identifying soil endospore formers may have failed to identify these. We believe then that the soil potentially offers a large reservoir of yet undiscovered pigmented endospore formers.

3.3 Endospores found in food products

A potential source of the spores found in the human GI tract is through food. Endospores are commonly found in food products where their presence can be linked to the soil. For example, populations of endospores (typically, *B. cereus, B. licheniformis* and *B. subtilis*) in pasteurised milk can reach 10^3 CFU/ml and they have been shown to contaminate milk from silage, bedding as well as faeces (Yoon et al. 2005). During prolonged storage, germination, outgrowth and proliferation of endospores can substantially increase counts of live bacteria to as high as 10^6 CFU/ml (Notermans et al. 1997). Other food sources that carry *Bacillus* endospores at levels reaching 10^2 CFU/g are rice, grain and vegetables. In all cases, the origin of these endospores can be attributed to soil but as with milk storage, if endospores can germinate and proliferate the numbers of bacteria can increase substantially (Ankolekar et al. 2009; Rosenkvist et al. 1995).

3.4 The true habitat of Bacillus species

If we assume that soil is the true habitat of *Bacillus*, then their presence in faeces is a direct consequence of the host having consumed food contaminated with soil. Our data reveals a basal level of endospores in the human GI tract of about 10^4 spores/g of

faeces. For a healthy adult living on a Western diet they would be expected to have a mean daily stool weight of about 200 g (Cummings et al. 1992) which, using our findings here, would contain in total approximately 2 x 10^6 endospores. To produce this a person would need, for example, to consume 2 litres of milk a day, or 20 kg of rice and cereals. While these

are generalizations we doubt that the counts found in human faeces can be accounted for based solely on intake with food. A more reasonable explanation is that intake with food introduces endospores into the GI tract which then germinate and proliferate as part of their life cycle. Germination is a process designed to occur in the presence of nutrients and nowhere else is this more apparent than in the small intestine. If endospores are designed to survive within the GI tract we might ask what attributes they possess that facilitates this. One important finding is that *Bacillus* can grow and sporulate under anaerobic conditions (Nakano et al. 1997., Tam et al. 2006), as well as molecular studies showing endospore germination, proliferation and re-sporulation (Casula et al. 2002; Tam et al. 2006) . The endospore itself, is encased in a protective coat of protein, the spore coat, whose natural protective role has surprisingly, until recently, been poorly understood. Work has now shown that the spore coat enables protection from immersion in gastric juices (Duc et al. 2006., Spinosa et al. 200). Interestingly, a role for the spore coat in avoiding phagocytic predation by the protozoan *Tetrahymena theromophila* has also been demonstrated (Klobutcher et al. 2006). Perhaps then, the endospore is designed to survive predation whether by simple microbes or large animals. Intriguingly, few studies have been made on the analysis of live bacillus in the soil environment and, as with faecal analysis, there are a number of logistic and technical reasons why this approach is problematic. Still, those studies that examined soil using fluorescent antibodies failed to convincingly prove the existence of vegetative *B. subtilis* in the soil other than an association with decaying plant matter (Siala et al. 1974). Other studies have demonstrated that sporulation of *B. subtilis* cannot occur at temperatures below 15 °C (Budde et al. 2006). For an organism

purported to live in the soil, this result is difficult to explain, but rather supports the hypothesis that endospores, while found in the soil, have adapted to survive within the GI-tract of animals that ingest them. How these Gram-positive endospore formers have adapted to life within a host remains to be seen, yet *B. subtilis* is now being subject to microarray-based comparative genomics, revealing a remarkable diversity within this single species (Earl et al. 2008).

References

Angert, E.R., Losick, R.M. (1998) Propagation by sporulation in the guinea pig symbiont *Metabacterium polyspora. Proc. Natl. Acad. Sci*. U.S.A. 95, 10218-10223.

Ankolekar, C., Rahmati, T., Labbe, R.G. (2009) Detection of toxigenic *Bacillus cereus* and *Bacillus thuringiensis* spores in U.S. rice. *Int. J. Food Microbiol*. 128, 460-466.

Budde, I., Steil, L., Scharf, C., Volker, U., Bremer, E. (2006) Adaptation of *Bacillus subtilis* to growth at low temperature: a combined transcriptomic and proteomic appraisal. *Microbiology* 152, 831-853.

Casula, G., Cutting, S.M. (2002) *Bacillus* probiotics: spore germination in the gastrointestinal tract. *App. Env. Microbiol*. 68, 2344-2352.

Cummings, J.H., Bingham, S.A., Heaton, K.W., Eastwood, M.A. (1992) Fecal weight, colon cancer risk, and dietary intake of nonstarch polysaccharides (dietary fiber). *Gastroenterology* 103, 1783-91789.

Duc, L.H., Fraser, P., Cutting, S.M. (2006) Carotenoids present in halotolerant *Bacillus* spore formers. *FEMS Microbiol. Lett*. 255, 215-224.

Duc, L.H., Hong, H.A., Cutting, S.M. (2003) Germination of the spore in the gastrointestinal tract provides a novel route for heterologous antigen presentation. *Vaccine* 21, 4215-4224.

Earl, A.M., Losick, R., Kolter, R. (2008) Ecology and genomics of *Bacillus subtilis*. *Trends Microbiol.* 16, 269-275.

Fakhry, S., Sorrentini, I., Ricca, E., De Felice, M., Baccigalupi, L. (2008) Characterization of spore forming *Bacilli* isolated from the human gastrointestinal tract. *J. Appl. Microbiol*. 105, 2178-2186.

Feinberg, L., Jorgensen, J., Haselton, A., Pitt, A., Rudner, R., Margulis, L. (1999) Arthromitus (Bacillus cereus) symbionts in the cockroach *Blaberus giganteus*: dietary influences on bacterial development and population density. *Symbiosis* 27, 109-123.

Felske, A.D.M. (2004) Ecology of *Bacillus* species in soil. In E. Ricca, A.O. Henriques, & S.M. Cutting (Eds.), *Horizon Bioscience* (pp. 35-44). Waondham.

Fritze, D. (2004) Taxonomy of the genus *Bacillus* and related genera: the aerobic endospore-forming bacteria. *Phytopathology* 94, 1245-1248.

Hartemink, R., Domenech, V.R., Rombouts, F.M. (1997) LAMVAB-A new selective medium for the isolation of lactobacilli from faeces. *J. Microbiol.* Methods 29, 77-84.

Hartemink, R., Rombouts, F.M. (1999) Comparison of media for the detection of *bifidobacteria, lactobacilli* and total anaerobes from faecal samples. *J. Microbiol*. Methods 36, 181-192.

Hoa, N.T., Baccigalupi, L., Huxham, A., Smertenko, A., Van, P.H., Ammendola, S., Ricca, E., Cutting, S.M. (2000) Characterization of *Bacillus* species used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders. *Appl. Environ. Microbiol*. 66, 5241-5247.

Hong, H.A., Duc, L.H., Cutting, S.M. (2005) The use of bacterial spore formers as probiotics. *FEMS Microbiol*. Rev. 29, 813-835.

Hong, H.A., Khaneja, R., Tam, N.M., Cazzato, A., Tan, S., Urdaci, M., Brisson, A., Gasbarrini, A., Barnes, I., Cutting, S.M. (2009) *Bacillus subtilis* isolated from the human *gastrointestinal tract. Res. Microbiol.* 160, 134-143.

Klobutcher, L.A., Ragkousi, K., Setlow, P. (2006) The *Bacillus subtilis* spore coat provides ‘‘eat resistance’’ during phagocytic predation by the protozoan Tetrahymena thermophila*. Proc. Natl. Acad. Sci*. U.S.A. 103, 165-170.

Koransky, J.R., Allen, S.D., Dowell Jr., V.R. (1978) Use of ethanol for selective isolation of sporeforming microorganisms. *Appl. Environ*. *Microbiol*. 35, 762-765.

Mitchell, C., Iyer, S., Skomurski, J.F., Vary, J.C. (1986) Red pigment in *Bacillus megaterium* spores. *Appl. Environ. Microbiol*. 52, 64-67.

Moeller, R., Horneck, G., Facius, R., Stackebrandt, E. (2005) Role of pigmentation in protecting *Bacillus* sp. endospores against environmental UV radiation. *FEMS Microbiol. Ecol*. 51, 231-236.

Moir, A. (2006) How do spores germinate? *J. Appl. Microbiol*. 101, 526-530.

Nakano, M.M., Dailly, Y.P., Zuber, P., Clark, D.P. (1997) Characterization of anaerobic fermentative growth of *Bacillus subtilis*: identification of fermentation end products and genes required for growth. *J. Bacteriol*.179, 6749-6755.

Nicholson, W.J., Munakata, N., Horneck, G., Melosh, H.J., Setlow, P. (2000) Resistance of *Bacillus* endospores to extreme terrestial and extraterrestrial environments. *Microbiol. Mol. Biol*. Rev. 64, 548-572.

Nicholson, W.L. (2002) Roles of *Bacillus* endospores in the environment. *Cell. Mol. Life Sci*. 59, 410-416.

Nicholson, W.L., Setlow, P. (1990) Sporulation, germination and outgrowth. In C.R. Harwood, & S.M. Cutting (Eds.), *Molecular Biological Methods for Bacillus* (pp. 391-450). Chichester, UK: John Wiley & Sons Ltd.

Notermans, S., Dufrenne, J., Teunis, P., Beumer, R., Te Giffel, M.C., **Peeters Weem, P**. (1997) A risk assessment study of *Bacillus cereus* present in pasteurized milk. *Food Microbiol.* 14, 143-151.

Rhee, K.J., Sethupathi, P., Driks, A., Lanning, D.K., Knight, K.L. (2004) Role of commensal bacteria in development of gut-associated lymphoid tissues and preimmune antibody repertoire. *J. Immunol*. 172, 1118-1124.

Rosenkvist, H., Hansen, A. (1995) Contamination profiles and characterisation of *Bacillus* species in wheat bread: raw materials for bread production. *Int. J. Food Microbiol*. 26, 353-363.

Siala, A., Hill, I.R., Gray, T.R.G. (1974) Populations of spore-forming bacteria in an acid forest soil, with special reference to *Bacillus subtilis. J. Gen. Microbiol*. 81, 183-190.

Spinosa, M.R., Braccini, T., Ricca, E., De Felice, M., Morelli, L., Pozzi, G., Oggioni, M.R. (2000) On the fate of ingested *Bacillus* spores.**n** *Res. Microbiol*. 151, 361-368.

Suresh, K., Prabagaran, S.R., Sengupta, S., Shivaji, S. (2004) *Bacillus indicus* sp. nov., an arsenic-resistant bacterium isolated from an aquifer in West Bengal, India. *Int. J. Syst. Evol. Microbiol.* 54, 1369-1375.

Tam, N.M.K., Uyen, N.Q., Hong, H.A., Duc, L.H., Hoa, T.T., Serra, C.H., Henriques, A.O., Cutting, S.M. (2006) The intestinal life cycle of *Bacillus subtilis* close relatives. *J. Bacteriol*. 188, 2692-2700.

Vissers, M.M., Te Giffel, M.C., Driehuis, F., De Jong, P., Lankveld, J.M. (2007) Minimizing the level of *Bacillus cereus* spores in farm tank milk. *J. Dairy Sci.* 90, 3286-3293.

Yoon, J.H., Lee, C.H., Oh, T.K. (2005*) Bacillus cibi* sp. nov., isolated from jeotgal, a traditional Korean fermented seafood. Int. *J. Syst. Evol*. *Microbiol.* 55, 733-736.

Chapter five

Carotenoids Found in Spore - Forming Bacteria

Published on: Journal of Applied Microbiology

Khaneja, R., Perez-Fons, L., **Fakhry, S.**, Baccigalupi, L., Steiger, S., To, E., Sandmann, G., Dong, T.C., Ricca, E., Fraser, P.D., and Cutting, S.M. "Carotenoids Found in Bacillus" J. Appl. Microbiol. *in press* (2009).

Abstract

Pigmented spore-formers were isolated from a number of sources including seawater, soil, fermented foods and from the human gastrointestinal tract. The most commonly found pigments were yellow, orange and pink. Isolates were nearly always members of the *Bacillus* genus and in most cases were related with known species such as *Bacillus marisflavi*, *Bacillus indicus*, *Bacillus firmus*, *Bacillus altitudinsis* and *Bacillus safensis*. Our screen revealed that pigmented spore formers are quite common and we show that this ubiquity has probably gone unnoticed. Pigments were profiled using HPLC-PDA analysis and three types of carotenoid were found with absorption peaks at 455nm, 467nm and 492nm corresponding to the visible colours of yellow, orange and pink respectively. Although we cannot rule out the presence of other carotenoids these three predominant carotenoids appear to account for the pigments obtained in most pigmented *Bacilli* and our analysis reveals a C30 biosynthetic pathway. Interestingly, we report the presence of a water-soluble pigment that may also be a carotenoid. The function of carotenoids is that of photoprotection and carotenoid containing spores exhibited significantly higher levels of resistance to UV radiation than non-carotenoid containing *Bacillus* species. How these carotenoids are produced is not yet known but are likely to reveal new isoprenoid biosynthetic pathways which have industrial importance.

1. Introduction

Pigments are widespread in nature and are found in both eukaryotes and prokaryotes. In photosynthetic organisms pigments mainly function in light harvesting but they can also serve as photoprotective agents (Chew et al. 2007; Coesel et al. 2008). For others, pigmentation can help protect the cell from predation, for example, prodigiosin, the red pyrrole-containing pigment commonly found in *Serratia*, *Streptomyces* and

Vibrio species has well-defined antibacterial properties (Harris et al. 2004; Perez-Tomas et al. 2003). Similarly, the purple violacein of *Chromobacterium* species is able to provide resistance to being consumed by predatory protozoa together with inherent antibiotic properties ((Lopes et al. 2009). Photoprotective pigments include the melanins and carotenoids (Hullo et al. 2001, Moeller etal. 2005). Interestingly, the latter have also been shown to act as virulence factors in a number of pathogens, including *Staphylococcus aureus* (Liu et al. 2005) and *Mycobacterium* spp. (Gao et al. 2003; Provvedi et al. 2008). Here the carotenoid helps protect bacteria attempting to survive within an intracellular environment, that is, following phagocytosis where they provide resistance to oxidation and neutrophil attack (Liu et al. 2005). Presumably, carotenoids, that originally evolved to protect the cell from UV damage, have assumed an additional and more sophisticated role as part of a pathogenic life cycle.

Bacterial spores are well known for their robust resistance properties being able to withstand extremes of heat, desiccation, exposure to noxious chemicals and UV irradiation (Nicholson et al. 2000; Riesenman et al. 2000). As ubiquitous, yet dormant, entities they are found in soils, rocks, water as well as the intestinal tracts of numerous insects and mammals (Fakhry et al. 2008; Hong et al. 2009; Jensen et al. 2003; Nicholson et al. 2002). *Bacillus subtilis* spores carry a melanin-like compound in their coats that helps protects spores against solar radiation (i.e., UV-A and UV-B) (Hullon et al. 2001; Riesenman et al. 2000). The natural pigmentation of *B. subtilis* sporulating colonies is therefore brown but other colours have been documented in spores. Examples include a red pigmented *Bacillus megaterium* (Mitchell et al. 1986), a pink pigment in some isolates of *Bacillus firmus* (Pane et al. 1996) and red and grey pigments found in *Bacillus atrophaeus* (Fritze et al. 2001; Nakamura et al. 1989). A variable yellow-orange pigmentation has been found in a number of species including, *B. indicus* (Suresh et al. 2004), *B. cibi* (Yoon et al. 2005) , *B. vedderi* (Agnew et al. 1995), *B. jeogali* (Yoon et al. 2001), *B. okuhidensis* (Li et al. 2002), *B. clarkii* ((Nielsen et al. 1995), *B. pseudofirmus* (Nielsen et al 1995) and *B. firmus* (Ruger et al. 1980).

With the exception of melanin, some of these pigments are probably carotenoids and a number of spore-forming species have been shown to have carotenoids associated with either the vegetative cell or the spore (Duc et al. 2006; Mitchell et al. 1986; Moeller et al. 2005) . Carotenoids are isoprenoids and thus originate from the five-carbon building block, isopentenyl pyrophosphate (IPP), which is the universal precursor of all isoprenoids. Supporting this squalene has been identified in numerous Gram-positive bacteria including *Bacillus* (Amdur et al. 1978). Besides a chromophore (which is responsible for the colour) another structural feature is their long hydrocarbon chain that conveys a pronounced hydrophobic chemical nature. These lipophilic properties ensure that virtually all carotenoids are found in membranous structures.

Providing resistance to UV irradiation is a necessity of bacterial endospores and generally, levels of resistance, 10-50 times higher than growing vegetative cells are common ((Moeller et al. 2005). Resistance has generally been attributed to two unique features of the spore that are centred on protecting the germ-line DNA (Nichelson et al. 2000., Setlow et al. 2001). First, a difference in the photochemistry of spore DNA caused by a conformational change in the DNA (referred to as the A-form) induced by the binding of small, acid-soluble proteins (SASP) to spore DNA. Second, a relatively error-free mechanism for repairing UV damaged DNA. Membranes are also susceptible to UV damage where reactive oxygen species (generated by UV irradiation) may attack and interfere with the integrity of lipids leading to lipid peroxidation and/or inactivation of membrane-associated proteins (Moeller et al. 2005). Membrane damage would have catastrophic consequences to survival of the newly germinated spore and so it is likely that spore formers have evolved mechanisms to protect themselves from long-term exposure to UV. As antioxidants carotenoids are capable of scavenging reactive oxygen species generated by UV irradiation. Located in the membranes they would protect against membrane damage rather than from protecting spore DNA. Supporting this, a red-orange pigment, thought to be a carotenoid, has been implicated in providing resistance to UV-A in a strain of *B. atrophaeus* (Moeller et al. 2005).

In this study we have screened a number of different sample sites for the presence of pigmented, spore-forming bacteria. These were found in abundance and found to be either species of *Bacillus* or *Sporosarcina aquimarina*. Although the visible colour of colonies varied pigmentation was found to be due to the presence of one or more of three distinct carotenoids.

2. Materials & Methods

2.1 General methods.

Vegetative cell growth was made on LB solid or liquid medium unless indicated otherwise in the text. Sporulation was made in DSM (Difco sporulation medium) agar or liquid medium (Nicholson et al. 1990). For analysis of sporulation efficiencies spores recovered from plate cultures (30^{o}C, 3 days old) were examined microscopically using a haemocytometer counting chamber or by determination of heat-resistance (65^{o}C, 45 min.). Starch hydrolysis was determined as described elsewhere using agar plates containing 1% soluble starch ((Cutting et al. 1990). Haemolysis was evaluated by streaking onto tryptose blood agar containing sheep's blood at 5% and incubation for 24h at 37^{o}C. Motility was tested by the method of Hendrickson (Hendrickson et al. 1985) using growth in 0.4% agar and incubation for 2 days at 30^{o}C. Resistance to arsenate and arsenite was determined as described (Suresh et al. 2004). Surfactin was measured by an oil-displacement method as described elsewhere (Hong et al. 2009). Tolerance to NaCl was determined by streaking strains on LB agar supplemented with NaCl at different concentrations (5%, 8%, 10% and 12%). For determination of anaerobic growth strains were streaked on solid DSM agar plates containing potassium nitrate (at a concentration of 5 mM) or potassium nitrite (at 2.5 mM) as electron acceptors (Nakano et al. 1997; Ye et al. 2000). Streaked plates with test and control strains were incubated in a Don Whitley anaerobic chamber and growth at 30ºC was monitored after three days. *Clostridium perfringens* isolate *fD00385* was included as a positive 'anaerobic' control and *Bacillus pumilus* SC2200 as a negative control.

2.2 Isolation of pigmented spore-formers

Different sources were used for isolation of strains. For solid samples the basic procedure consisted of homogenizing the sample in PBS (phosphate-buffered saline), heating for 1h at 65^{o}C and then plating serial dilutions on DSM agar. The isolation of human samples has been described previously (Fakhry et al. 2008, Tam et al. 2006). Seawater was filtered (250 ml water / filter) using a 0.22 micron (Millipore) filter. Each filter was heat treated (80^{o}C for 10 min), placed on top of a DSM plate and incubated at 25^{o}C or 30^{o}C for 2-3 days. Colonies were purified, grown on DSM and checked for the presence of spores under the light microscope.

2.3 Reference strains

Reference strains used in this study were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany) or the BGSC (*Bacillus* Genetic Stock Center, USA) culture collections.

2.4 16S rRNA analysis

To assign strains to bacterial species for each isolate the entire 16S rRNA gene (*rrnE*) was amplified as described previously (Hoa et al. 2000). The 1,400 bp amplicon was then sequenced and subjected to nucleotide database analysis using the Ribosomal Database Project II sequence database; http://rdp.cme.msu.edu/). Closest known species were recorded as percentages of identity. Sequences were aligned and phylogenetic trees assembled using the CLUSTALW programme (http://align.genome.jp/).

2.5 Determination of UV resistance

Methods used were as described by Moeller et al (Moeller et al. 2005), with some modifications. Briefly, spores in aqueous suspension (10^7 spores/ml) were exposed to UV-C radiation from a germicidal lamp (VL 50C; Vilber Lourmat, France) with a major emission line at 254nm. During irradiation the spore suspensions were stirred continuously to ensure homogenous exposure. Immediately following UV radiation, at defined time points, 0.1ml of the aqueous suspension was taken for evaluation of the viable count by serial dilution in PBS and plating on DSM agar. The surviving fraction (Ln) was determined from the quotient N/N_0 where N = the number of c.f.u. of the irradiated sample and the N_0 that of the non-irradiated controls. Survival curves were obtained by plotting the Ln of N/N_0 as a function of fluence (exposure time).

2.6 Hydrogen peroxide assays

Spore suspensions (1×10^8 spores/ml) were treated with H_20_2 as described elsewhere (Riesenman et al. 2000).

2.7 Pigment extraction.

For pigment analysis isolates were grown as patches on tryptone-yeast extract (TYE) agar at 30°C. After 3-4 days of growth colonies were scraped from the agar surface and centrifuged to remove residual media. The cell pellet was frozen and then lyophilised for 3 days. The resulting lyophilised material was stored at -20°C until extraction. In order to obtain a homogeneous powder for extraction the freeze-dried cells were milled using a tissue lyser (Qiagen, Crawley, UK). Aliquots of this material were then taken for further analysis. An aliquot of the powdered freeze-dried material (typically 20mg) was suspended in 1M NaOH (500 µl) and sonicated at room temperature for 5 min. After removing the NaOH phase by centrifugation carotenoids were extracted as previously described (Duc et al. 2006). To the digested cells methanol (250 µl) and chloroform (500 µl) was added and mixed. Water (250 µl) was

then added and the suspension vortexed in order to create a phase separation. After centrifugation, the organic layer (lower phase) was collected and the aqueous (upper) layer re-extracted with chloroform twice (or until no colour was observed in the debris). The organic extracts were pooled and reduced to complete dryness under a stream of nitrogen gas. The dried extracts were stored at this stage at -20°C under nitrogen.

2.8 Pigment analysis.

Dried extracts were routinely dissolved in chloroform with the exception of the samples originating from pink coloured isolates that were dissolved in methanol. In this instance methanol was necessary because of their lack of solubility in chloroform (or ethyl acetate). Prior to injection onto the HPLC column extracts were filtered through a PTFE membrane (0.2µm; Chromacol Ltd, Herts, UK) and centrifuged at 12,000 rpm for 3 min. Carotenoids were separated and then detected on-line using a Waters Alliance (Milford, MA) 2600S high-performance liquid chromatography (HPLC) system with an online Photo Diode Array (PDA) detector. Injections (20µl) were made and separations performed on a RP C_{30} 5 µm column (250 x 4.6 mm i.d.) coupled to a 20 x 4.6mm C_{30} guard column (YMC Inc., Wilmington, NC) maintained at a constant temperature of 25 °C as described previously (Fraser et al. 2000). The mobile phases used were (A) methanol, (B) MeOH:H_2O (80:20) (v/v) containing 0.2% (w/v) ammonium acetate and (C) *tert*-butyl methyl ether. Carotenoids were eluted from the column with a gradient of 95% (A) methanol, 5% (B) for 12 min, a step to 80% A, 5% B and 15% (C) at 12 min, followed by a linear gradient to 30% A, 5% B and 65% C by 30 min. The column was returned to the initial conditions and equilibrated over 30 min. A flow rate of 1ml min^{-1} was employed and the eluate monitored continuously with an on-line PDA set to monitor between 200 and 600 nm. Identification was performed by the comparison of spectral and chromatographic characteristics associated with similar or authentic carotenoids. In cases where no authentic standards existed comparison with reference compounds in the literature (Britton et al. 2003) were made. For quantification, dose–

response curves for β-carotene (standard coloured carotenoid) were prepared. Menaquinone was also identified by spectral comparison with authentic standards. All solvents were purchased from VWR (Poole, UK).

2.9 Detection of water-soluble pigments

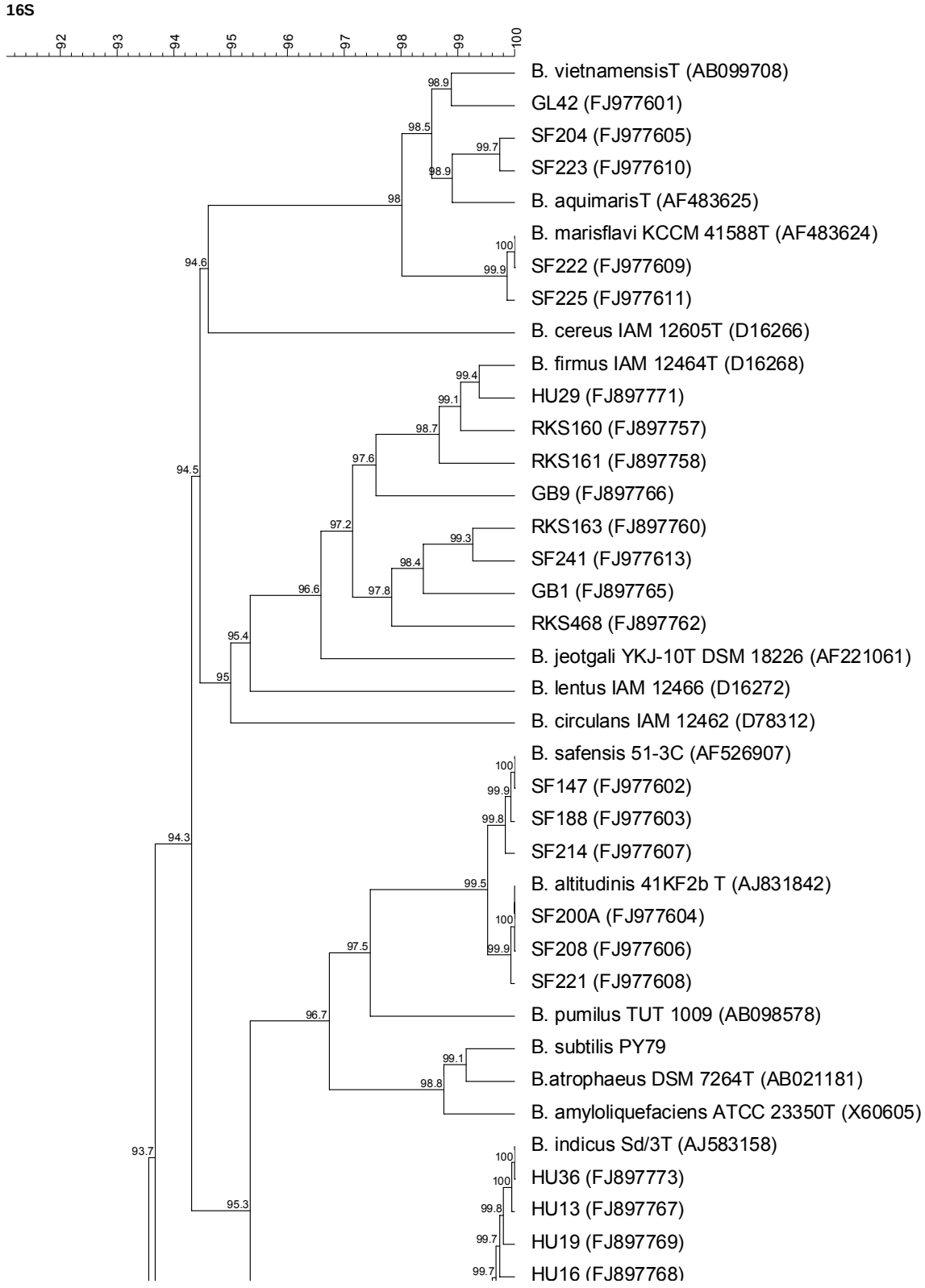
Bacillus cells were grown on solid or in liquid media for 2 to 3 days and then harvested by centrifugation at 9,000*g* for 5min. In the case of solid agar, material was first scraped from the surface with an inoculation loop, placed into a micro-centrifuge tube and washed with dH_2O (1ml). Pelleted cells were resuspended in dH_2O (1ml) and then passed through a French pressure cell at 20MPa. The homogenate was then centrifuged at 40,000*g* for 20min at 4^{o}C. The resulting coloured supernatant was removed and a UV/VIS spectral trace recorded from 250 to 600nm (Beckman Coulter DU800 spectrophotometer, High Wycombe, UK).

3. Results

3.1 Biotypes of pigmented spore formers

Environmental samples were evaluated for the presence of heat-resistant bacteria by plating on a rich medium (DSM) from which colonies would only arise if the sample carried endospores. Plates were incubated for a sufficient period of time to allow the visual identifcation of pigmented colonies. Samples examined were a fermented rice condiment (‘Tuong Nep Dac Biet’) obtained from Vietnam, soil, sea-water as well as samples from human feces and human gut biopsies that had been previously described (Fakhry et al. 2008; Hong et al. 2009). Pigmented heat-resistant isolates could be identified with ease and colours obtained were mostly yellow, orange, pink and red and subtle variations between these colours, e.g., yellow-orange, orange-red (Figure 1 shows some examples). We found the colony could vary, somewhat, depending upon the medium used, for example, colours were often more vibrant on LB agar compared to DSM agar. Similarly, it was also observed that pigment formation in

some strains would be very much dependant upon the growth temperature, for example, isolate SF214 would develop an orange pigment at 25°C but at 42°C white colonies were formed.



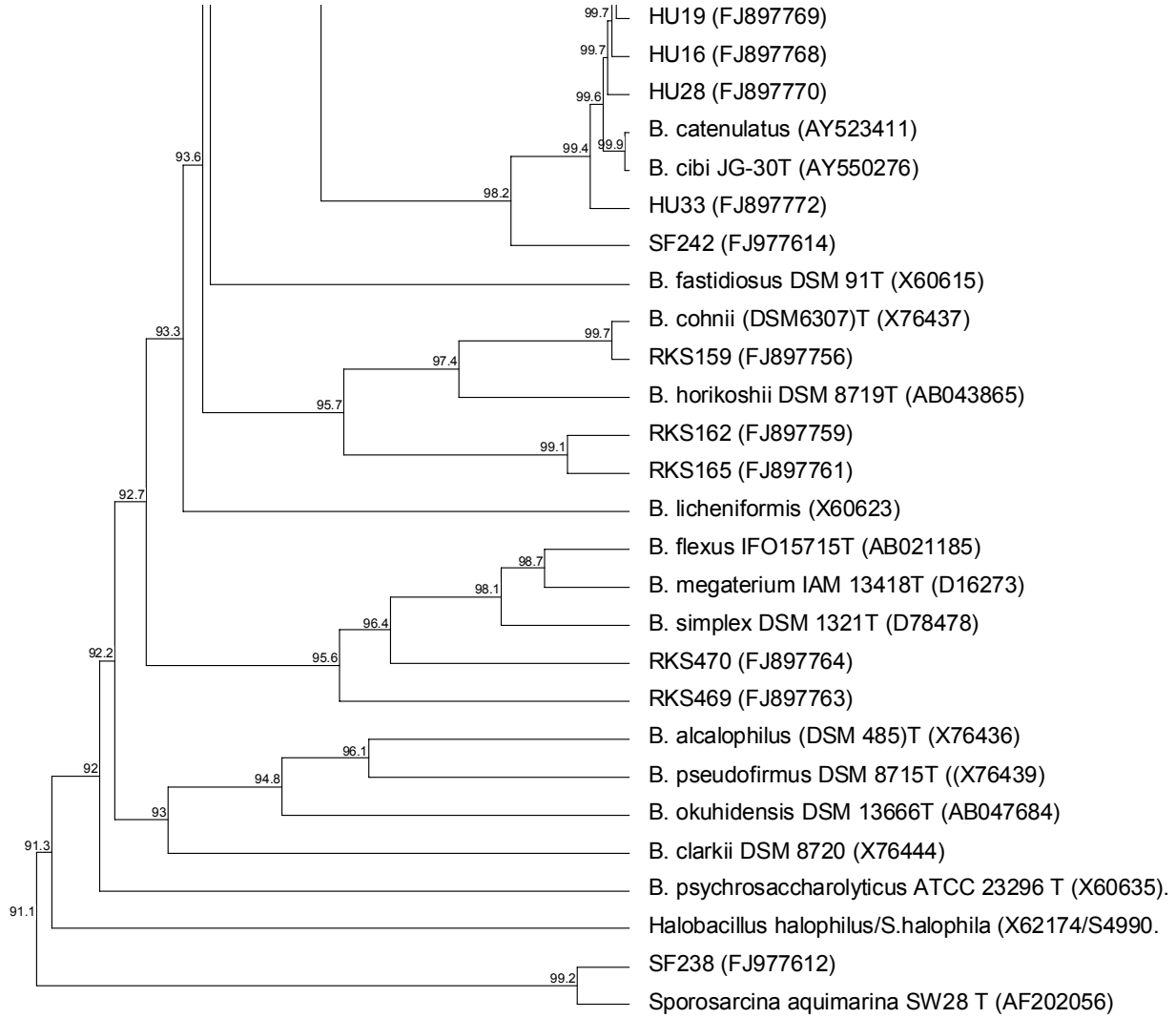


Figure 1: Phylogenetic relationship of pigmented spore formers. Dendograms of strains based on 16S rRNA (*rrnE*) sequence alignment using CLUSTALW version 1.83 (http://align.genome.jp/). Bar is 0.01 nucleotide substitutions per site. Numbers in brackets are Genbank accession numbers.

In an analysis of soil samples taken from fifty locations in the London area the most abundant pigmented spore-formers isolated were yellow colonies (26 out of the 50

sites tested) and in some samples up to 13% of the heat-resistant colonies were yellow. Dark grey colonies were found at 8 sites, pink colonies from 6 sites and orange at 3 sites. These figures, while undoubtedly generalizations, demonstrate that pigmented spore-formers can readily be found in soil. Another finding was that when soil samples were processed, following heat-treatment, serial dilution and plating out, the pigmentation was most obvious only at the highest serial dilutions. That is, on lower dilutions, where colonies were crowded, pigmentation was barely detectable, if at all, suggesting that pigmentation was subject to some form of nutritional or extracellular input.

The *rrnE* (16S rRNA) gene was sequenced in its entirety for the isolates selected for further analysis. Initial examination against type strains (Table 1) together with phylogenetic analysis (Figure 1) revealed that many isolates were identical to known pigmented species including *B. marisflavi* (Yoon et al. 2003)), *B. aquimarina* (Yoon et al. 2003), *B. firmus* ((Pane et al. 1996), *B. vietnamensis* (Noguchi et al. 2004), *B. cibi* (Yoon et al. 2005) and *B. indicus* (Suresh et al. 2004) as well as one isolate of *Sporsarcina aquamarina* (Yoon et al. 2001). Isolates related to *B. altitudinis*, *B. safensis* and *B. cohnii* were also found for which no pigmentation has previously been described. Other isolates appeared unrelated to known species (e.g., RKS162, RKS165, RKS469, RKS470, GB9 and possibly RKS468) and probably will define new ones.

Selected isolates were chosen for further analysis (Table 1). This included the characterization of sporulation efficiencies (Table 2) and basic biotypes (Supp. Table 1). The *Sporosarcina* isolate (SF238) and most SF colonies tested were unable to grow anaerobically in marked contrast to *Bacillus* spp. that had been isolated from the human gut (Fakhry et al. 2008; Hong et al. 2009). Sporulation was generally straightforward to induce using growth on a rich, meat-derived, medium with the exception of two isolates SF238 and RKS469 and probably reflects a unique nutritional requirement for optimal sporulation.

Table 1: Pigmented *Bacillus* spore formers

Strain ID	Colony colour	Accn. No.[a]	Source/Reference	Closest match
RKS159	Yellow	FJ897756	Fermented rice condiment, this work	*B. cohnii*
RKS162	Yellow	FJ897759	Fermented rice condiment, this work	*B. horikoshii*
RKS165	Yellow	FJ897761	Fermented rice condiment, this work	*B. horikoshii*
RKS469	Yellow	FJ897763	Fermented rice condiment, this work	*B. simplex*
RKS470	Yellow	FJ897764	Fermented rice condiment, this work	*B. flexus*
SF147	Yellow	??	Human feces (8)	*B. pumilus*
SF188	Yellow	??	Human feces (8)	*B. pumilus*
SF222	Yellow	??	Sea water, this work	*B. aquimaris*
SF242	Yellow	??	Sea water, this work	*Sporosarcina spp.*
SF225	Yellow	??	Soil, this work	*B. pumilus*
HU36	Yellow-orange	FJ897773	Human feces (7)	*B. indicus*
HU13	Yellow-orange	FJ897767	Human feces (7)	*B. indicus*
HU16	Yellow-orange	FJ897768	Human feces (7)	*B. indicus*
HU19	Yellow-orange	FJ897769	Human feces (7)	*B. indicus*
HU28	Yellow-orange	FJ897770	Human feces (7)	*B. indicus*
HU33	Yellow	FJ897772	Human feces (7)	*B. cibi*
SF200A	Yellow-orange	??	Sea water, this work	*B. pumilus*
SF208	Yellow-orange	??	Sea water, this work	*B. pumilus*
SF221	Yellow-orange	??	Sea water, this work	*B. pumilus*
SF204	Orange	??	Sea water, this work	*B. aquimaris*
SF223	Orange	??	Sea water, this work	*B. aquimaris*
SF239	Orange	??	Soil, this work	*Sp. aquimarina*
GL42	Orange	??	Shrimp pond (Vietnam)	*B. alcalophilus*
SF214	Orange-red	??	Sea water, this work	*B. pumilus*
SF237	Red	??	Soil, this work	
RKS160	Pink	FJ897757	Fermented rice condiment, this work	*B. firmus*
HU29	Pink	FJ897771	Human feces, (16)	*B. firmus*
GB9	Pink	FJ897766	Human ileum, (16)	*B. firmus*
RKS163	Pink	FJ897760	Fermented rice condiment, this work	*B. firmus*
SF238	Pink	??	Soil, this work	*Sp. aquimarina*
SF241	Pink	??	Sea water, this work	*B. firmus*
GB1	Deep pink	FJ897765	Human ileum, (16)	*B. firmus*
RKS161	Deep pink	FJ897758	Fermented rice condiment, this work	*B. firmus*
RKS468	Deep pink	FJ897762	Fermented rice condiment, this work	*B. firmus*
Type strains[b]				
Strain ID	**Colony colour**	**Accn. No.[a]**	**Source/Reference**	**Closest match**
DSMZ 7264	Grey	AB021181	Soil (25)	*B. atrophaeus*
BGSC 11A1	Yellow-orange	Not known	Soil	*B. atrophaeus*
DSMZ 675	Yellow orange[c]	NBRC 1372	Soil (10)	*B. atrophaeus*
DSMZ 8715	Yellow	X76439	Soil and animal manure (30)	*B. pseudofirmus*
DSMZ 9768	Yellow	Z48306	Bauxite waste (1)	*B. vedderi*
DSMZ 18226	Yellow	AF221061	Fermented seafood condiment, (40)	*B. jeotgali* YKJ-10
DSMZ 8720	Yellow	X76444	Soil (30)	*B. clarkii*
DSMZ 13666	Yellow-brown	AB047684	Hot spa (20)	*B. okuhidensis*
DSMZ 15820	Yellow-orange	AJ583158	aquifier, (37)	*B. indicus* Sd/3
DSMZ 16189	Yellow-orange	AY550276	Fermented seafood condiment, (41)	*B. cibi* JG-30

[a]nucleotide sequence of the 16S rRNA gene (*rrnE*)
[b]obtained from the BGSC or DSMZ collections.
[d]described as the 'red strain' in (10, 24).

Table 2: Sporulation efficiencies

Strain	Sporulation efficiency[a]	Spore charact.[b]	Strain	Sporulation efficiency[a]	Spore charact.[b]
RKS159	79	E, T	SF200A	68	E, C
RKS162	100	E, T	SF208	92	E, C
RKS165	116	E, C	SF221	100	E, C
RKS469	<1	E, T	SF204	60	E, ST
RKS470	92	E, T, Sw	SF223	58	E, T
SF147	97	E, C	SF239	<1	S, C
SF188	96	E, C	GL42	25	E, T
SF222	10	S, ST	SF214	77	E, St
SF242	ND	S, C	SF237	<1	S, C
SF225	62	E, C	RKS160	100	E, St
HU36	67	E, St	RKS161	100	E, T
HU13	12	E, T	HU29	100	E, C
HU16	29	E, T	GB9	128[c]	E, St
HU19	37	E, T	RKS163	166[c]	E, T
HU28	34	E, T	RKS468	87	E, T
HU33	42	E, T	SF238	<1	S, C
GB1	153[c]	E, C	SF241	47	E, C
PY79	86	E, T/St	DSMZ 18226	11	E, T, Sw
DSMZ 7264	91	E, C	DSMZ 8720	31	E, St
BGSC 11A1	83	E, St	DSMZ 13666	10	E, T
DSMZ 675	106	E, C	DSMZ 15820	32	E, St, Sw
DSMZ 8715	46	E, C/St	DSMZ 16189	16	E, C/St

[a]Percentage of heat-resiatnt (65^{o}C, 45 min.) spores after three-four days culture at 37^{o}C on DSM agar.

[b]E, ellipsoidal spore shape; S, spherical spore shape; T, terminal spore position; C, central spore position; St, sub central terminal position; Sw, swollen sporangium.

[c]values greater than 100% reflect heat-induced germination of spores, i.e., the unheated sample does not reflect the true count of spores in the crop due to the failure of spores to germinate unless heat-activated, brought about the heat-treatment itself.

Supplementare Table 1: Biotypes

Strains	Haem[a]	Surfactin[b]	Motility	Starch hydrolysis	Max. Arsenic tolerance [c](mM)	Max. Arsenate tolerance[d] (mM)	NaCl tolerance (%)			
							5	8	10	12
B. subtilis PY79	□	-	+	+	0.5	20	+	+	+	±
SF147	□	+	+	-	0	0	+	+	-	-
SF188	□	+	+	-	0	0	+	+	-	-
SF200A	□	+	-	-	0	0	+	+	+	±
SF204	□	+	-	+	2	60	+	+	+	+
SF208	□	+	+	-	0	0	+	+	±	-
SF214	□	-	+	-	0	5	+	+	±	-
SF221	□	+	+	-	0	5	+	+	-	-
SF222	□	-	-	-	1	0	+	+	+	+
SF223	□	-	-	+	1	60	+	+	±	±
SF225	□	+	-	-	1	5	+	+	+	+
SF239	□	+	-	-	0.5	0	+	-	-	-
SF241	□	-	+	+	2	40	+	-	-	-
SF242	□	-	+	+	2	60	+	-	-	-
HU36	γ	-	+	+	0.5	20	+	+	±	-
HU13	γ	-	±	+	1	30	+	+	+	-
HU16	γ	+	±	+	1	30	+	+	-	-
HU19	γ	+	+	+	1	30	+	+	-	-
HU28	γ	+	+	+	1	30	+	+	-	-
HU33	γ	-	+	+	1	30	+	+	+	±
RKS159	α	-	+	+	1	60	+	-	-	-
RKS162	γ	-	+	+	2	50	+	-	-	-
RKS165	γ	+ +	+	+	2	60	+	-	-	-
RKS469	α	-	+	+	0.5	30	+	+	+	-
RKS470	γ	+ +	+	+	1	30	+	+	+	-
RKS160	α	+	+	+	1	20	+	+	-	-
HU29	γ	-	+	+	0.5	20	+	+	-	-
GB9	γ	-	+	+	3	60	+	+	-	-
RKS163	γ	+	+	+	1	40	+	-	-	-

Strains	Haem[a]	Surfactin[b]	Motility	Starch hydrolysis	Max. Arsenic tolerance[c](mM)	Max. Arsenate tolerance[d] (mM)	NaCl tolerance (%)			
							5	8	10	12
GB1	γ	-	+	+	2	30	+	+	-	-
RKS161	γ	-	+	+	2	50	+	-	-	-
RKS468	γ	+	+	+	2	30	+	-	-	-
GL42	γ	-	-	+	2	20	+	+	±	-
B. pseudofirmus (DSMZ 8715)	γ	-	+	+	1	60	+	+	+	±
B. atrophaeus (DSMZ 675)	γ	-	+	+	2	20	+	±	-	-
B. atrophaeus (BGSC11A1)	γ	-	+	+	2	5	+	±	-	-
B. atrophaeus (DSMZ 7264)	γ	-	+	+	2	5	+	±	-	-
B. jeotgali (DSMZ 18226)	β	-	+	+	2	60	+	±	-	-
B. clarkii (8720)	γ	-	±	-	0.5	60	+	+	+	±
B. okuhidensis (DSMZ 13666)	α	-	+	+	0.5	50	+	+	±	-
B. indicus (DSMZ 15820)	γ	-	±	+	2	50	+	+	±	-
B. cibi (DSMZ 16189)	γ	-	+	+	1	40	+	+	±	-

[a]Haem = haemolysis, hemolysis on sheep blood agar; α, complete haemolysis with a clear zone around colonies; β, partial haemolysis; γ, no changes.

[b]Surfactin was measured using an oil displacement method; - is no measurable activity, +, represents a diameters of oil displacement of <10 mm, ++, 10-15mm.

[c]Max. Arsenic tolerance[e] (mM)= Arsenite (As (III), As_2O_3), Arsenic tolerance on LB agar, growth monitored after 24 hours.

[d]Max. Arsenate tolerance[e] (mM)= Arsenite (As (V), Na_2HAsO_4), Arsenic tolerance on LB agar, growth monitored after 24 hours

3.2 Carotenoid profiling

The present study has focused on the screening of pigmented *Bacillus* strains isolated from diverse environments. These strains can be visually classified on the basis of colour, with three categories yellow, orange and pink predominating. The intensity of the colour varied depending on the strain (Table 3).

In order to ascertain the nature of the pigments present carotenoid analysis was performed. Direct extraction of the cellular pellet with organic solvent proved to be ineffective. However freeze-drying the cells, grinding the material into a fine homogeneous powder and then directly saponifying the material, assisted the release of pigments into organic solvents such as chloroform. Extracts were then analysed by HPLC-PDA using a separation system capable of resolving both polar and non-polar like carotenoid molecules. Typical HPLC profiles recorded at 450nm are illustrated in Figure 2. Figure 2A is representative of all pink coloured strains analysed (e.g., GB1), the predominant peak having a maxima of 492nm (Table 3). Figure 2B shows a profile associated with the orange/yellow pigmented strains (e.g., *B. indicus*), in this instance the major component peaks 2 and 3 had a maxima of 455 and 467 nm respectively. Those strains with a yellow pigmentation (e.g., HU19) had the characteristic profile similar to that shown in Figure 2C with peak 3 having a maxima of 455nm (Table 3).

The UV/VIS spectra of these compounds were characteristic of carotenoids, typically those of an acyclic nature. As found previously the lack of available authentic standards precludes definitive identification. On the basis of reference spectra in the literature the predominant *Bacillus* carotenoids were putatively identified to be acyclic carotenoids and potentially monocyclic, while other reports have stated the presence of astaxanthin (Pane et al. 1996). However, further investigation of the chromatographic components recorded at 286 nm in the *Bacillus* strains suggest the presence of C30 apophytoene (Figure 3). For example in Figure 3A the component with the UV/VIS spectra characteristic of phytoene has a retention time of 13 minutes (Peak-4). For comparison, an enriched carotenoid extract from ripe tomato fruit has been chromatographed concurrently (Figure 3B).

Table 3: Carotenoid content determined in different spore-forming isolates[a]

Strain identifier	Carotenoid production (low to high producers)	UV/VIS spectral characteristics (nm)[b]
Yellow		
B. jeotgali	Low	Below LOI
B. okuhidensis	Low	Below LOI
B. clarkii	Low	Below LOI
RKS159	Low	Below LOI
SF242	Low	Below LOI
SF200A	Low	P3-(400)[d], 429, **455**, 485
SF221	Low	P3-(400)[d], 429, **455**, 485
SF225	Medium	P3-(400)[d], 429, **455**, 485
B. pseudofirmus	Medium	P3-(400)[d], 429, **455**, 485
HU28	Medium	P3-(400)[d], 429, **455**, 485
SF208	Medium	P3-(400)[d], 429, **455**, 485
HU16	High	P3-(400)[d], 429, **455**, 485
HU19	High	P3-(400)[d], 429, **455**, 485
SF147	High	P3-(400)[d], 429, **455**, 485
SF188	High	P3-(400)[d], 429, **455**, 485
Orange		
SF208	Low	Below LOI
SF223	Low	Below LOI
SF214[c]	Low	Below LOI (soluble fraction 410nm)
SF237[c]	Low	Below LOI (soluble fraction 410nm)
B. atrophaeus DSMZ 675	Low	Below LOI
B. atrophaeus 11A1	Low	Below LOI
RKS470	Medium	P3-(400)[d], 429, **455**, 485 P2-440, **467**, 495
RKS162	Medium	P3-(400)[d], 429, **455**, 485 P2- 440, **467**, 495
RKS165	Medium	P3-(400)[d], 429, **455**, 485 P2-440, **467**, 495
SF222	Medium	P3-(400)[d], 429, **455**, 485 P2-440, **467**, 495
SF204	Medium	P2-440, **467**, 495
SF223	Medium	P2-440, **467**, 495
SF239	Medium	P2-440, **467**, 495
B. indicus Sd/3	High	P3-(400)[d], 429, **455**, 485 P2-440, **467**, 495
B. cibi JG-30	High	P3-(400)[d], 429, **455**, 485 P2-440, **467**, 495
HU33	High	P3-(400)[d], 429, **455**, 485 P2-440, **467**, 495
HU36	High	P3-(400)[d], 429, **455**, 485 P2-440, **467**, 495
HU13	High	P3-(400)[d], 429, **455**, 485 P2-440, **467**, 495
Pink		
SF238	Low	Below LOI
RKS160	Low	Below LOI
HU29	Low	Below LOI
GB9	Low	Below LOI

RKS163	Low	P1-(434)[d], 463, **492**, 524
GL42	Low	P1-(434)[d], 463, **492**, 524
SF238	Medium	P1-(434)[d], 463, **492**, 524
SF241	Medium	P1-(434)[d], 463, **492**, 524
GB1	Medium	P1-(434)[d], 463, **492**, 524
RKS161	Medium	P1-(434)[d], 463, **492**, 524
RKS468	Medium	P1-(434)[d], 463, **492**, 524
Dark Grey		
B. atrophaeus DSMZ 7264	ND	ND
SF116	ND	ND
SF120	ND	ND
SF120A	ND	ND

[a]UV/Vis spectral characteristics are provided for the predominant carotenoid observed from the characteristic HPLC-PDA profiles. P1 to P3 represent chromatographic peaks and can be cross referenced with Figure 2. LOI = level of identification. [b]main peaks in the spectra with the □maxima of the predominant peak is underlined. [c]Poor extraction with organic solvent, but water-soluble pigment observed when extracted with a French press. ND-carotenoids not detected, or below limits of detection using the experimental procedure described. Below LOI-indicates that the carotenoid is present but below the level of identification typically 0.01□g per g dry weight (DW). Low carotenoid producers represents levels in the range of 0.05 to 0.2 □g per g DW, medium carotenoid producers means 0.2 to 2.0 □g per g dry DW and high producers 2.0 up to 20 μg per g dry DW. [d] Brackets indicate the presence of shoulders in the characteristic UV/VIS spectra.

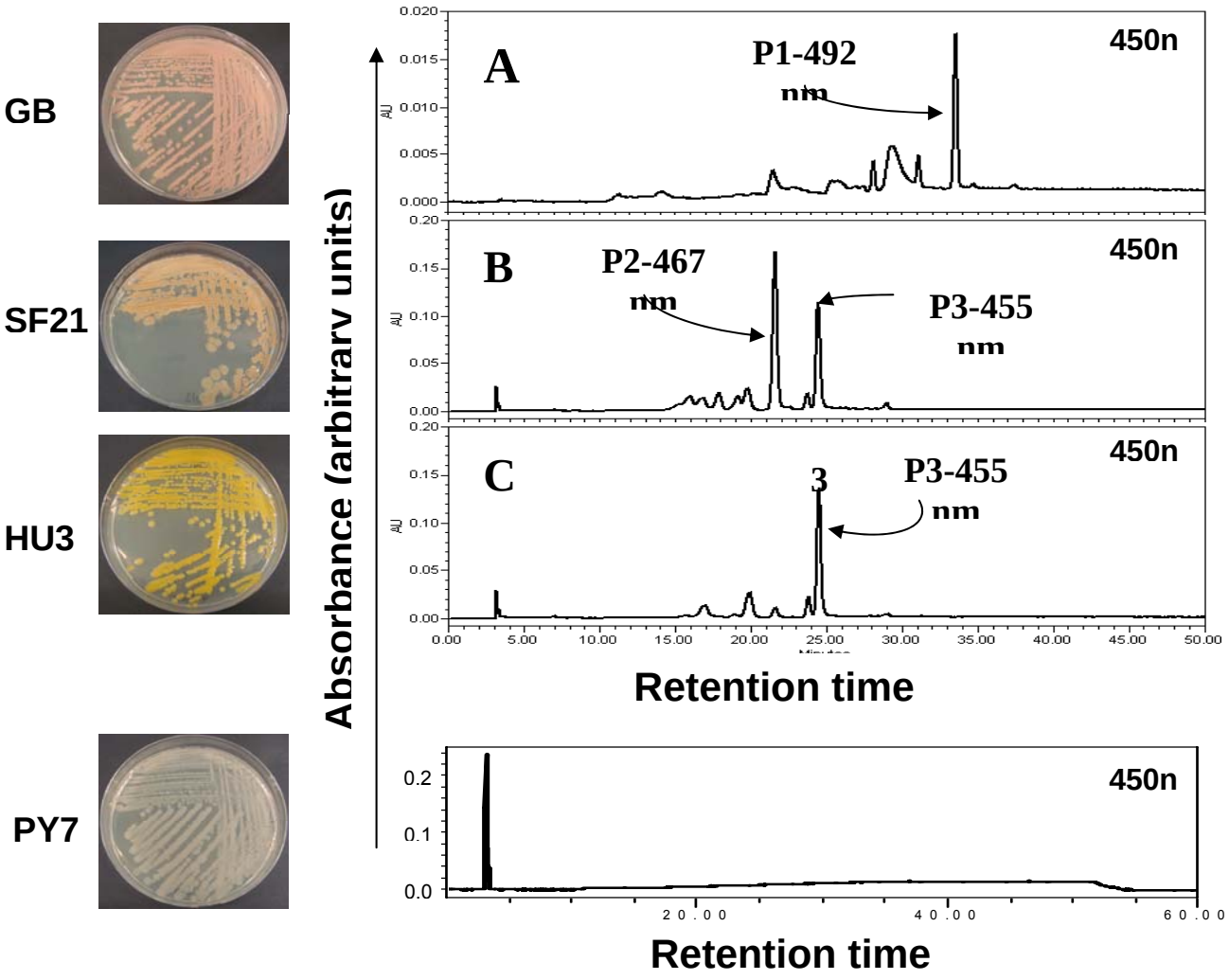


Figure 2: Characteristic HPLC-PDA profiles recorded at 450nm. Representative cultures of the red (GB1), orange (SF214), yellow (HU36) strains along with a colourless control (PY97) strain. These cultures, shown on the left of the figure, were grown at 37^{o}C (2 days) on LB agar. Typical HPLC-PDA profiles are shown in the panels on the right. Panel **A**, pink coloured *Bacillus* strains, Panel **B**, orange pigmented strains and Panel **C**, yellow coloured strains. P1 (P = peak) represents the carotenoid unique to pink strains with an absorption lmax of 492 nm. Peak-2 has a spectral lmax at 467nm, while peak-3 has a maxima at 455nm. Panel D illustrates the absence of carotenoids in the control strain

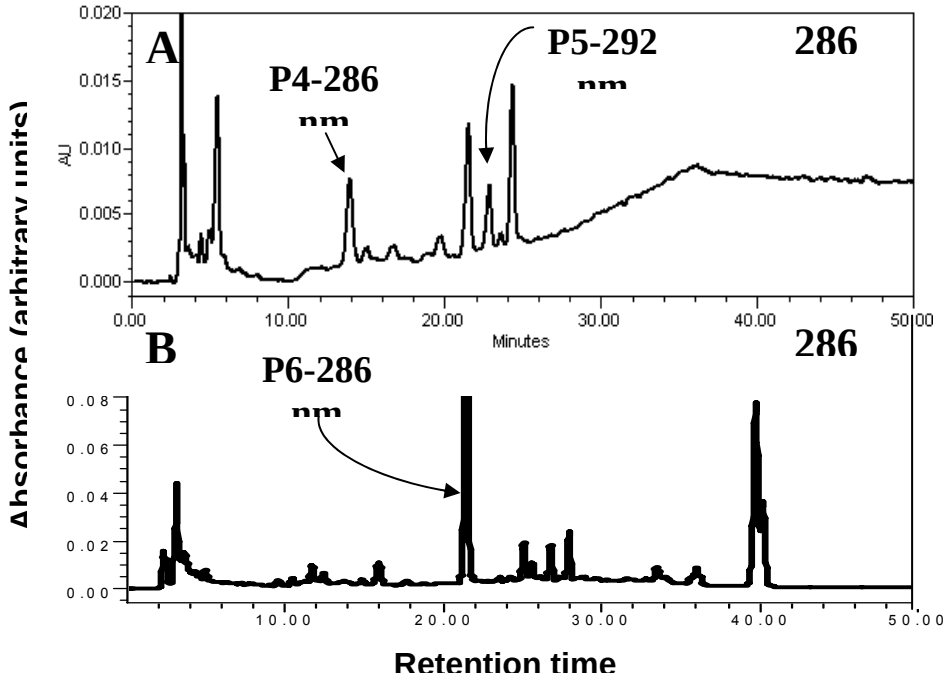


Figure 3: HPLC-PDA profiles of organic extracts recorded at 286nm.
Panel **A** shows representative extracts prepared from orange/yellow pigmented strains. Peak (P)-4 has been identified as diapophytoene and P-5 menaquinone. Panel **B** shows an organic extract from ripe tomato fruit, the retention time of C40 phytoene (P-6) is shown for comparison.

The phytoene (C40) present in this extract elutes with a retention time of 21 min. (indicated by P-6). Thus, the identical spectra but earlier retention time suggests that di-apophytoene exists in these *Bacillus* strains and that a C30 pathway exists. This evidence is supported by the presence of C30 apocarotenoids in other closely related bacteria such as *Staphylococcus aureus*. Several of the strains isolated displayed visually coloured colonies (Table 1) but attributing this colour to the presence of carotenoid was not possible under the experimental conditions applied. This is because the detection limits prevented definitive carotenoid-like UV/VIS spectra being acquired.

3.3 Detection of water-soluble pigments

Intense visual pigmentation of cells was observed in several *Bacillus* isolates (such as SF241). However, it was not possible to release the pigments responsible into organic solvents from both cellular suspensions and homogenates treated with organic solvent. The solvents tested included methanol, ethyl acetate, hexane, chloroform and hot (40^{o}C) acetone individually and in combination. Following cell breakage of aqueous cellular suspensions using a French pressure cell, the resulting supernatant extracts were intensely coloured. A UV/VIS spectral scan (250 to 600 nm) of these aqueous extracts revealed the presence of a pronounced peak occurring at 410nm. This peak was not present in control non-pigmented *Bacillus* strains or those strains where the pigments could be released following treatment with organic solvents

3.4 Pigment-conferred resistance properties

We determined the resistance of purified suspensions of selected pigmented spores to monochromatic UV-C (254 nm) (Figure 4). UV-C radiation is not entirely representative of the natural environmental (solar) radiation, which is primarily UV-A and UV-B, but it is the most energetic form of UV radiation (Riesenman et al. 2000)). Eight pigmented isolates, HU36, *B. indicus* Sd/3, GB1, DSMZ 675, DSMZ 7264, BGSC 11A, SF241 and SF214 all showed no significant reduction in viability after 120 seconds of UV-C exposure. This was in striking contrast to *B. subtilis* PY79 spores that showed a clear sensitivity to UV-C. Not all pigmented spores proved resistant to UV-C though, GL42 and GB9 both showed sensitivity. In data not shown we found that in vegetative cells of the same strains no resistance to UV-C was observed demonstrating that the protective role of the carotenoid was probably of importance only in the dormant state.

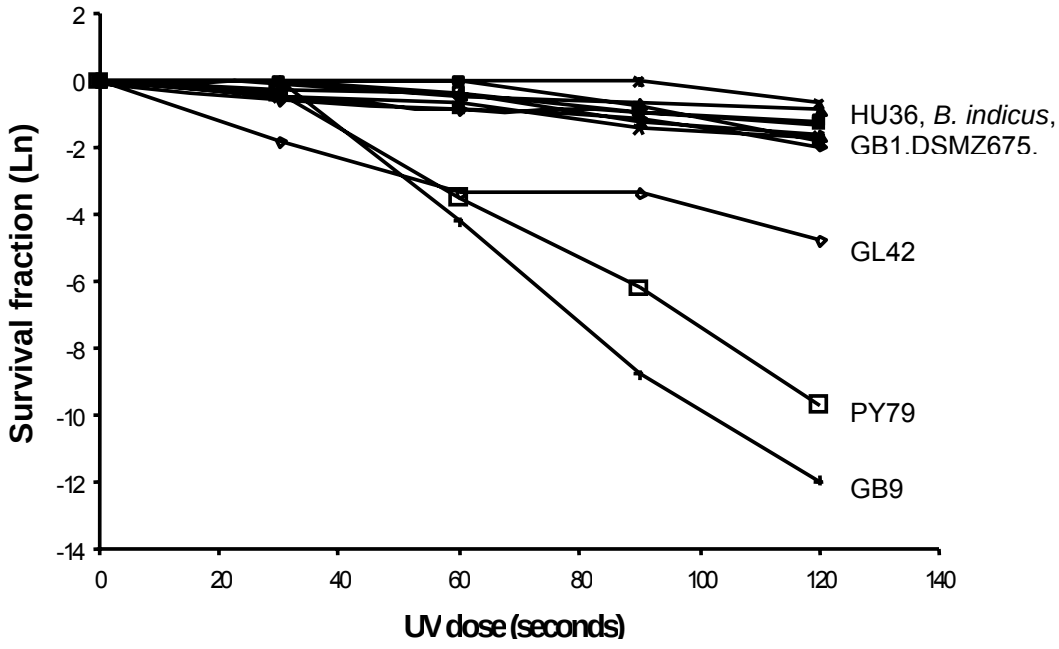


Figure 4: UV-C resistance. Suspensions of purified spores were irradiated with UV-C (254nm) using a germicidal lamp. The surviving fraction (ln) was determined as a function of time.

Bacterial spores have resistance to hydrogen peroxide and in *B. subtilis* this has been attributed to the CotA laccase present in the spore coat ((Hullo et al. 2001., Riesenman et al. 2000). Carotenoids, as antioxidants, could also provide a protective role in inactivating H_2O_2 as has been found for the pathogen *S. aureus* which is exposed to reactive oxygen species within a phagocyte (Liu et al. 2005). We examined resistance of spores of selected isolates to 5% H_2O_2 (Figure 5A).

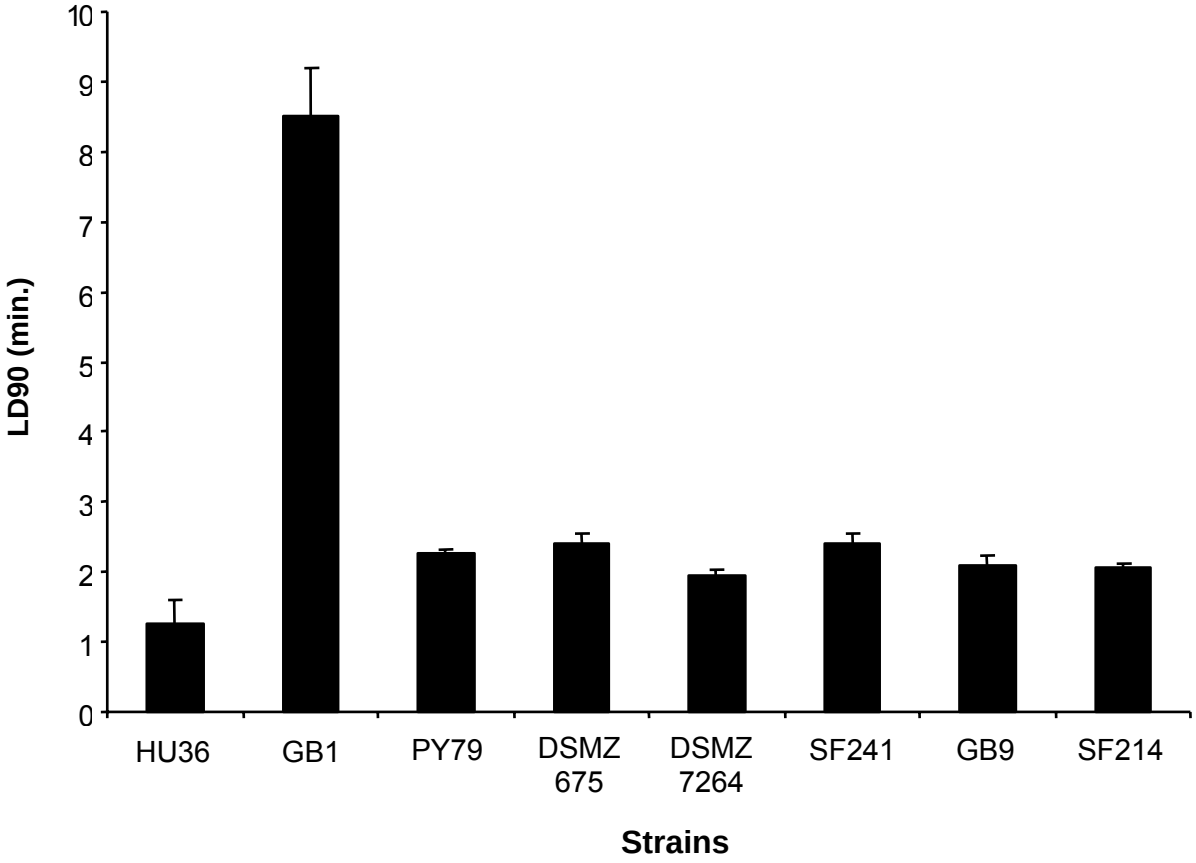


Figure 5A: Resistance to hydrogen peroxide. Suspensions of purified spores were assayed for resistance to 5% hydrogen peroxide.The experiment was repeated two times.

We found that with the exception of GB1 spores the LD_{90} was equivalent in all strains and were not significantly different ($P < 0.05$). The LD_{90} values we obtained were substantially (25-times) lower than those reported in previous work (Riesenman et al. 2000) even though we used the same methodology. Our data though, showed that resistance was the same between the pigmented spores and *B. subtilis* PY79 implying that the carotenoid content cannot contribute to hydrogen peroxide resistance. Vegetative cells were however extremely sensitive to hydrogen peroxide (Figure 5B).

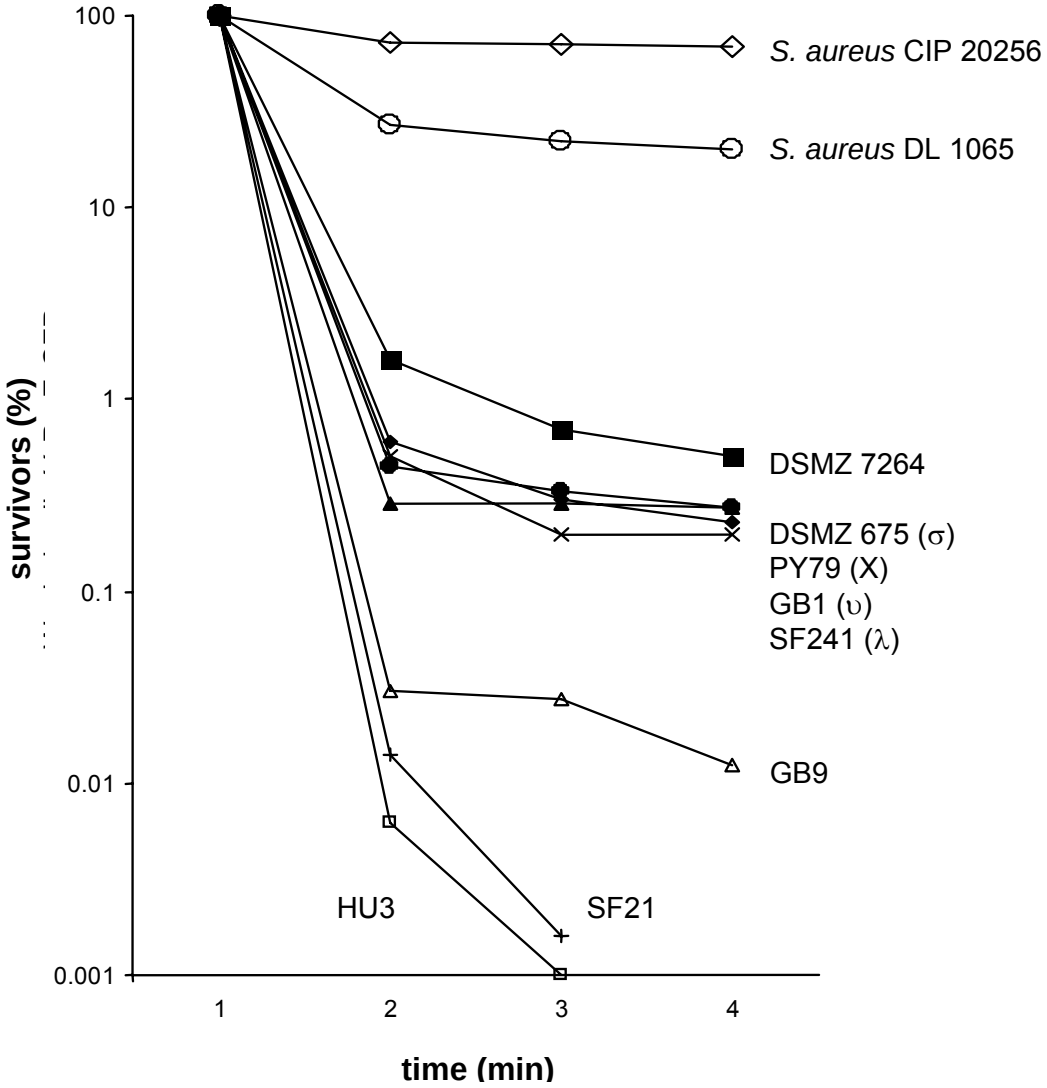


Figure 5B: Resistance to hydrogen peroxide.Suspensions of exponentially growing cells were assayed for resistance to 1.5% hydrogen peroxide. The experiment was repeated two times.

To demonstrate data shown in Fig. 5B shows the kinetics of cell killing. Pigmented strains were killed rapidly and showed no greater resistance than PY79 vegetative cells. As a comparison we also, in parallel, examined two yellow-pigmented strains of *S. aureus* that has been shown elsewhere, and confirmed here, to have resistance to hydrogen peroxide enabling this organism to survive within a phagocyte.

4. Discussion

As mentioned in the introduction to this paper, a relatively small number of publications have reported the identification and species determination of pigmented *Bacilli*. Of these Duc *et al* (Du et al. 2006) provide one of the first detailed reports of pigmented marine *Bacillus* species. We have extended this work to examine different habitats for pigmented spore formers and then determine the nature of the pigments. At the outset we emphasise that this study is not exhaustive, nor was this the intention. Rather, to show the diversity and the chemical basis for spore pigmentation. Our study has revealed that for the most part, the abundance of coloured *Bacilli*, has probably gone unnoticed. We attribute this to the technicalities of identifying coloured colonies where plating out bacteria at low dilution masks the true abundance of pigmented species. Only at high dilutions do the pigments become apparent and in some cases one in ten colonies were found to be pigmented. Another contributing factor is the medium and temperature used to culture *Bacilli* where we have found that significant variation in the colony colouration can result. The variation in colour suggests that environmental or nutritional factors could be important. In a previous study of a strain of *B. indicus* (HU36) we demonstrated that the pigment was due to one or more carotenoids (Duc et al. 2006). For this reason we made the assumption that the pigments found in the *Bacilli* isolated in this study would also be carotenoids. In order to demonstrate the presence of carotenoids the following criteria have been used; (i) extraction into organic solvents, (ii) separation of carotenoid components on chromatographic systems used routinely for carotenoids, (iii) the presence of the di-apophytoene, which is the first unique intermediate of the carotenoid pathway and (iv) characteristic UV/VIS spectral properties of the enriched carotenoid preparations. Further systematic analysis is now required to unequivocally identify these carotenoids present in *Bacillus* using combinations of hyphenated MS and possibly NMR approaches. One of the limitations of analysis presently encountered is the poor extraction recoveries for the carotenoids, which is likely to be associated with the chemical nature of the pigments.

Therefore, to date, we cannot rule out the presence of further carotenoids in these *Bacillus* isolates or that carotenoids are not the sole pigments responsible for the colour present in these *Bacillus* strains isolated. The physical characteristics of the carotenoids detected in this study are however in good agreement with the visualised colour of the colonies. For example the carotenoid exhibiting maxima at 455 nm predominates in isolates classified as being yellow, while orange strains contain the carotenoid with the maxima at 467 nm and the red strains posses a carotenoid with a 492 nm maxima. Thus, the light energy absorbed by these carotenoids matches the visual colour of the strains. This finding suggests that carotenoids are the sole pigments responsible for the colour and in cases where coloured colonies could be visualised but no carotenoids detected, the poor extraction properties are responsible for the lack of detection.

In the case of the water-soluble pigments, our inability to partition pigments into organic solvents, to resolve them by HPLC separations and obtain on-line characteristic carotenoid UV/VIS spectra, precludes assignment of carotenoids as the pigments responsible for colouration of these *Bacillus* isolates (e.g., SF214). It is possible that the spectral peak at 410nm determined in aqueous extracts is representative of a water-soluble carotenoid, carotenoprotein (Cremades et al. 2001) or another type of pigment absorbing light in the visible region. Interestingly, in study of a red pigment found in *B. atrophaeus* DSMZ 675 (Moeller et al. 2005) two absorption peaks of 377 and 398nm were observed yet in our analysis we could not repeat this finding. Further experimentation is required to ascertain the true nature of these pigments though. It is of note that such water-soluble pigments have potential utility in the industrial sector as colorants both for foods and other valuable commodities.

Why then are *Bacillus* spores pigmented; most likely is protection against UV radiation? This is consistent with the likely fate of a dormant spore where it is exposed to excessive levels of solar radiation in the environment. This is particularly true of marine bacteria and may explain why pigmented isolates can so readily be recovered from aquatic environments and fermented seafood products (Noguchi et al. 2004; Yoon et al. 2001;

Yoon et al. 2005). We have shown here that pigmented isolates can also be recovered from soil as well as the GI-tract. In the case of the GI-tract pigmented isolates may occur simply due to diet.

In other work we have developed the hypothesis that spore formers may actually carry out their life cycle of vegetative growth within the intestinal tracts of animals that ingest them (Fakhry et al. 2008; Hong et al. 2009). Excreted in the feces spores would remain in a state of dormancy for indefinite periods of time in the environment and so pigmentation would help shield spores from the harmful effects of radiation. Interestingly, there was no obvious correlation between UV-C resistance and carotenoid content. For example, while HU36 carried high levels of carotenoids and was fully resistance to UV-C, this was not the case for SF214, DSMZ 675 or DSMZ 7264, the latter containing no extractable carotenoids. Similarly conflicting reports have been reported in other studies, for example, the red carotenoid pigment of DSMZ 675 spores appears important in protecting against UV-A but not against UV-B or UV-C (Moeller et al. 2005). Other studies with the coat-associated melanin of *B. subtilis* spores have shown that in the absence of the spore outer coat, resistance to UV-C was actually increased (Riesenman et al. 2000). It is clear then that individual carotenoids may have evolved differently with regard to their protective role and that this may also differ significantly between species. We also found that the carotenoid content of spores appeared not to serve any role in protection against hydrogen peroxide, or at least there were no differences with melanin-containing *B. subtilis* PY79 spores. This property has been assigned to the CotA laccase found in the spore coat of *B. subtilis* and it is possible that the pigmented spores also contained a melanin-like compound in their coats which is visibly suppressed by the carotenoid pigmentation (Hullon et al. 2001). However, GB1 spores did show a noticeable four-fold higher level of resistance than spores of the other strains so we cannot rule out the possibility that this is due to the spore carotenoid content. Until non-pigmented mutations can be genetically engineered attempting to establish a link between resistance and carotenoid content will remain a subjective issue. As has been summarised elsewhere a

number of other enzymes present in the spore coat could also provide resistance to reactive oxygen species including melanins, oxalate decarboxylase (OxdD) and a managanese-dependant superoxide dismutase (SodA) ((Henriques et al. 2007).

Our studies are now focused on identifying the compounds and their biosynthetic pathways and with this aim the genomes of GB1 and HU36 are currently being sequenced. The carotenoids are formed from the isoprenoid biosynthetic pathways and as such are normally found in membranes (Amdur et al. 1978., Daum et al. 2009)). Our work has shown pigmentation can vary dependant upon growth conditions (nutrition, temperature) as well as cell density and in other work we have shown that for *B. indicus* HU36, the yellow colouration of vegetative cells changes to an orange pigmentation as cells sporulate (Duc et al. 2006). This suggests that developmental signals may affect the biosynthetic pathways. Deciphering the carotenoid biosynthetic pathways will therefore prove a complex task yet there are a number of incentives for attempting this. First and foremost is the ability to metabolically engineer bacteria to synthesise high levels of endogenous isoprenoids. If this can be achieved these bacteria could be included amongst the cohort of metabolically engineered bacteria now under development as second and third generation biofuels (Klein-Marcuschamer et al. 2007).

References

Agnew, M. D., S. F. Koval, and K. F. Jarrell. 1995. Isolation and characterisation of novel alkaliphiles from bauxite-processing waste and description of *Bacillus vedderi* sp. nov., a new obligate alkaliphile. Systematic and Applied Microbiology **18:**221-230.

Amdur, B. H., E. I. Szabo, and S. S. Socransky. 1978. Presence of squalene in Gram-positive bacteria. J Bacteriol **135:**161-3.

Britton, G., S. Liaaen-Jensen, and H. Pfander (ed.). 2003. Carotenoids, Handbook. Birkhauser Verlag, Basel, Switzerland.

Chew, A. G., and D. A. Bryant. 2007. Chlorophyll biosynthesis in bacteria: the origins of structural and functional diversity. Annu Rev Microbiol **61:**113-29.

Coesel, S., M. Obornik, J. Varela, A. Falciatore, and C. Bowler. 2008. Evolutionary origins and functions of the carotenoid biosynthetic pathway in marine diatoms. PLoS ONE **3:**e2896.

Cremades, O., E. Ponce, R. Corpas, J. F. Gutierrez, M. Jover, M. C. Alvarez-Ossorio, J. Parrado, and J. Bautista. 2001. Processing of crawfish (*Procambarus clarkii*) for the preparation of carotenoproteins and chitin. J Agric Food Chem **49:**5468-72.

Cutting, S. M., and P. B. Vander-Horn. 1990. Genetic Analysis, p. 27-74. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular Biological Methods for *Bacillus*. John Wiley & Sons Ltd., Chichester, England.

Daum, M., S. Herrmann, B. Wilkinson, and A. Bechthold. 2009. Genes and enzymes involved in bacterial isoprenoid biosynthesis. Current Opinion in Chemical Biology **13:**1-9.

Du, H., N. Jiao, Y. Hu, and Y. Zeng. 2006. Diversity and distribution of pigmented heterotrophic bacteria in marine environments. FEMS Microbiol Ecol **57:**92-105.

Duc, L. H., P. Fraser, and S. M. Cutting. 2006. Carotenoids present in halotolerant *Bacillus* spore formers. FEMS Microbiology Letters **255:**215-224.

Fakhry, S., I. Sorrentini, E. Ricca, M. De Felice, and L. Baccigalupi. 2008. Characterisation of spore forming *Bacilli* isolated from the human gastrointestinal tract. Journal of Applied Microbiology **In press**.

Fakhry, S., I. Sorrentini, E. Ricca, M. De Felice, and L. Baccigalupi. 2008. Characterization of spore forming *Bacilli* isolated from the human gastrointestinal tract. J Appl Microbiol **105:**2178-86.

Fraser, P. D., M. E. Pinto, D. E. Holloway, and P. M. Bramley. 2000. Technical advance: application of high-performance liquid chromatography with photodiode array detection to the metabolic profiling of plant isoprenoids. Plant J **24:**551-8.

Fritze, D., and R. Pukall. 2001. Reclassification of bioindicator strains *Bacillus subtilis* DSM 675 and *Bacillus subtilis* DSM 2277 as *Bacillus atrophaeus*. Int J Syst Evol Microbiol **51:**35-7.

Gao, L. Y., R. Groger, J. S. Cox, S. M. Beverley, E. H. Lawson, and E. J. Brown. 2003. Transposon mutagenesis of *Mycobacterium marinum* identifies a locus linking pigmentation and intracellular survival. Infect Immun **71:**922-9.

Harris, A. K., N. R. Williamson, H. Slater, A. Cox, S. Abbasi, I. Foulds, H. T. Simonsen, F. J. Leeper, and G. P. Salmond. 2004. The *Serratia* gene cluster encoding biosynthesis of the red antibiotic, prodigiosin, shows species- and strain-dependent genome context variation. Microbiology **150:**3547-60.

Hendrickson, D. A. 1985. Reagents and stains, p. 1093-1107. *In* E. H. Lennette, A. Balows, W. J. Hausler, and H. J. Shadomy (ed.), Manual of Clinical Microbiology. ASM Press, Washington, D.C.

Henriques, A. O., and C. P. Moran, Jr. 2007. Structure, assembly, and function of the spore surface layers. Annu Rev Microbiol **61:**555-88.

Hoa, N. T., L. Baccigalupi, A. Huxham, A. Smertenko, P. H. Van, S. Ammendola, E. Ricca, and S. M. Cutting. 2000. Characterization of *Bacillus* species used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders. Appl. Env. Microbiol. **66:**5241-7.

Hong, H. A., R. Khaneja, N. M. Tam, A. Cazzato, S. Tan, M. Urdaci, A. Brisson, A. Gasbarrini, I. Barnes, and S. M. Cutting. 2009. *Bacillus subtilis* isolated from the human gastrointestinal tract. Res Microbiol **160:**134-43.

Hullo, M. F., I. Moszer, A. Danchin, and I. Martin-Verstraete. 2001. CotA of *Bacillus subtilis* is a copper-dependent laccase. J Bacteriol **183:**5426-30.

ensen, G. B., B. M. Hansen, J. Eilenberg, and J. Mahillon. 2003. The hidden lifestyles of *Bacillus cereus* and relatives. Environmental Microbiology **5:**631-640.

Klein-Marcuschamer, D., P. K. Ajikumar, and G. Stephanopoulos. 2007. Engineering microbial cell factories for biosynthesis of isoprenoid molecules: beyond lycopene. Trends Biotechnol **25:**417-24.

Li, Z., Y. Kawamura, O. Shida, S. Yamagata, T. Deguchi, and T. Ezaki. 2002. *Bacillus okuhidensis* sp. nov., isolated from the Okuhida spa area of Japan. Int J Syst Evol Microbiol **52:**1205-9.

Liu, G. Y., A. Essex, J. T. Buchanan, V. Datta, H. M. Hoffman, J. F. Bastian, J. Fierer, and V. Nizet. 2005. *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. J Exp Med **202:**209-15.

Lopes, S. C., Y. C. Blanco, G. Z. Justo, P. A. Nogueira, F. L. Rodrigues, U. Goelnitz, G. Wunderlich, G. Facchini, M. Brocchi, N. Duran, and F. T. Costa. 2009. Violacein extracted from *Chromobacterium violaceum* inhibits *Plasmodium* growth *in vitro* and *in vivo*. Antimicrob Agents Chemother.

Mitchell, C., S. Iyer, J. F. Skomurski, and J. C. Vary. 1986. Red pigment in *Bacillus megaterium* spores. Applied and Environmental Microbiology **52:**64-67.

Moeller, R., G. Horneck, R. Facius, and E. Stackebrandt. 2005. Role of pigmentation in protecting *Bacillus* sp. endospores against environmental UV radiation. FEMS Microbiol Ecol **51:**231-6.

Nakamura, L. K. 1989. Taxonomic relationship of black-pigmented *Bacillus subtilis* strains and a proposal for *Bacillus atrophaeus* sp. nov. International Journal of Systematic Bacteriology **39:**295-300.

Nakano, M. M., Y. P. Dailly, P. Zuber, and D. P. Clark. 1997. Characterization of anaerobic fermentative growth of *Bacillus subtilis*: identification of fermentation end products and genes required for growth. J Bacteriol **179:**6749-55.

Nicholson, W. J., N. Munakata, G. Horneck, H. J. Melosh, and P. Setlow. 2000. Resistance of *Bacillus* endospores to extreme terrestial and extraterrestrial environments. Microbiology and Molecular Biology Reviews **64:**548-572.

Nicholson, W. L. 2002. Roles of *Bacillus* endospores in the environment. Cellular and Molecular Life Sciences **59:**410-416.

Nicholson, W. L., and P. Setlow. 1990. Sporulation, germination and outgrowth., p. 391-450. *In* C. R. Harwood. and S. M. Cutting. (ed.), Molecular Biological Methods for *Bacillus*. John Wiley & Sons Ltd., Chichester, UK.

Nielsen, P., D. Fritze, and F. G. Priest. 1995. Phenetic diversity of alkaliphilic *Bacillus* strains: proposal for nine new species. Microbiology **141:**1745-1761.

Noguchi, H., M. Uchino, O. Shida, K. Takano, L. K. Nakamura, and K. Komagata. 2004. *Bacillus vietnamensis* sp. nov., a moderately halotolerant, aerobic, endospore-forming bacterium isolated from Vietnamese fish sauce. Int J Syst Evol Microbiol **54:**2117-20.

Pane, L., L. Radin, G. Franconi, and A. Carli. 1996. The carotenoid pigments of a marine *Bacillus firmus* strain. Boll Soc Ital Biol Sper **72:**303-8.

Perez-Tomas, R., B. Montaner, E. Llagostera, and V. Soto-Cerrato. 2003. The prodigiosins, proapoptotic drugs with anticancer properties. Biochem Pharmacol **66:**1447-52.

Provvedi, R., D. Kocincova, V. Dona, D. Euphrasie, M. Daffe, G. Etienne, R. Manganelli, and J. M. Reyrat. 2008. SigF controls carotenoid pigment production and affects transformation efficiency and hydrogen peroxide sensitivity in *Mycobacterium smegmatis*. J Bacteriol **190:**7859-63.

Riesenman, P. J., and W. L. Nicholson. 2000. Role of the spore coat layers in *Bacillus subtilis* spore resistance to hydrogen peroxide, artificial UV-C, UV-B, and solar UV radiation. Appl Environ Microbiol **66:**620-6.

Ruger, H.-J., and J. A. C. Koploy. 1980. DNA base composition of halophilic and nonhalophilkic *Bacillus firmus* strains of marine origin. Microb. Ecol. **6:**141-6.

Setlow, P. 2001. Resistance of spores of *Bacillus* species to ultraviolet light. Environ Mol Mutagen **38:**97-104.

Suresh, K., S. R. Prabagaran, S. Sengupta, and S. Shivaji. 2004. *Bacillus indicus* sp. nov., an arsenic-resistant bacterium isolated from an aquifer in West Bengal, India. Int J Syst Evol Microbiol **54:**1369-75.

Tam, N. M. K., N. Q. Uyen, H. A. Hong, L. H. Duc, T. T. Hoa, C. H. Serra, A. O. Henriques, and S. M. Cutting. 2006. The intestinal life cycle of *Bacillus subtilis* and close relatives. Journal of Bacteriology **188:**2692-2700.

Ye, R. W., W. Tao, L. Bedzyk, T. Young, M. Chen, and L. Li. 2000. Global gene expression profiles of *Bacillus subtilis* grown under anaerobic conditions. J Bacteriol **182:**4458-65.

Yoon, J. H., S. S. Kang, K. C. Lee, Y. H. Kho, S. H. Choi, K. H. Kang, and Y. H. Park. 2001. *Bacillus jeotgali* sp. nov., isolated from jeotgal, Korean traditional fermented seafood. Int J Syst Evol Microbiol **51:**1087-92.

Yoon, J. H., I. G. Kim, K. H. Kang, T. K. Oh, and Y. H. Park. 2003. *Bacillus marisflavi* sp. nov. and *Bacillus aquimaris* sp. nov., isolated from sea water of a tidal flat of the Yellow Sea in Korea. Int J Syst Evol Microbiol **53:**1297-303.

Yoon, J. H., C. H. Lee, and T. K. Oh. 2005. *Bacillus cibi* sp. nov., isolated from jeotgal, a traditional Korean fermented seafood. Int J Syst Evol Microb **55:**733-6.

Yoon, J. H., K. C. Lee, N. Weiss, Y. H. Kho, K. H. Kang, and Y. H. Park. 2001. *Sporosarcina aquimarina* sp. nov., a bacterium isolated from seawater in Korea, and transfer of *Bacillus globisporus* (Larkin and Stokes 1967), *Bacillus psychrophilus* (Nakamura 1984) and *Bacillus pasteurii* (Chester 1898) to the genus *Sporosarcina* as *Sporosarcina globispora* comb. nov., *Sporosarcina psychrophila* comb. nov. and *Sporosarcina pasteurii* comb. nov., and emended description of the species. Int J Syst Evol Microbiol **51:**1079-86.

Appendix

ELSEVIER

INSTITUT PASTEUR

Research in Microbiology xx (2009) 1–7

www.elsevier.com/locate/resmic

Characterization of intestinal bacteria tightly bound to the human ileal epithelium

Saad Fakhry [a], Nicola Manzo [a], Enrica D'Apuzzo [a], Lorena Pietrini [b], Italo Sorrentini [b], Ezio Ricca [a], Maurilio De Felice [a], Loredana Baccigalupi [a,*]

[a] *Dipartimento di Biologia Strutturale e Funzionale, Università Federico II, via Cinthia 4, complesso MSA, 80126 Napoli, Italy*
[b] *UOC di Gastroenterologia ed Endoscopia Digestiva, Azienda Ospedaliera di Benevento, ''G. Rummo'', Benevento, Italy*

Received 29 July 2009; accepted 9 September 2009

Abstract

In order to perform selective isolation of bacteria tightly bound to the human gut, ileal biopsies of healthy volunteers were treated to wash out the mucus layer and loosely bound bacterial cells. Rod-shaped anaerobic bacteria that had remained attached to the epithelial cells were isolated and identified at the species level. One isolate was identified as belonging to the *Bifidobacterium breve* species, while all the others were lactobacilli of only two species, *Lactobacillus mucosae* and *Lactobacillus gasseri*. Members of these species were found previously in intestinal samples, but their predominance among bacteria strictly associated with the epithelium was not suspected before and suggests that these species may represent a specific subpopulation of tissue-bound bacteria. Physiological analysis indicated that all isolates were able to produce antimicrobials, grow and form biofilm in simulated intestinal fluid after exposure to gastric conditions. Some isolates were able to degrade mucin while none showed cytotoxicity in vitro on HT29 cells. The tight association of the strains isolated with ileal epithelial cells is presumably indicative of a direct interaction with the host cells. For this reason and for the absence of cytotoxicity in vitro, those isolates can be proposed as potential probiotic strains for human use.

Keywords: *Lactobacillus*; Gut; Commensals; Probiotics; Mucus

1. Introduction

The human intestinal microflora establishes a complex symbiotic interaction with epithelial and immune cells of the gastrointestinal tract (GIT). In this interaction, the microbial role is essential in providing nourishment, forming a first line of defense against invasion by pathogenic organisms, regulating epithelial development and inducing innate immunity [7]. These contributions are reciprocated by stable conditions of temperature, pH, osmolarity and food supply for the microorganisms.

Recent metagenomic experiments have indicated that the vast majority of the intestinal bacteria belong to two phyla, the *Firmicutes*, including the large class of *Clostridia* and the lactic acid bacteria (LAB), and the *Bacteroidetes* [7,17]. Most of these organisms are anaerobes and not cultivable in laboratory conditions, and only 0.1% of the total gut bacteria are facultative anaerobes [7]. However, the composition of the gut microbiota is known to vary transiently as a consequence of diet changes, enteral infections, antibiotic or anti-acid treatments and immune suppression [27]. Recently, it has been shown that phylum-level changes in the microflora are associated with diseases such as obesity [28]. In particular, the coexistence of H_2-producing bacteria and H_2-utilizing methanogenic *Archaea* in the GIT of obese individuals triggered the hypothesis that H_2 transfer between eubacterial and archaeal

* Corresponding author. Tel.: +39 081 679037; fax: +39 081 679233.
E-mail address: lorbacci@unina.it (L. Baccigalupi).

0923-2508/$ - see front matter © 2009 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.resmic.2009.09.009

species increases the energy uptake by the large intestine of obese individuals [33].

At the species level, wide diversity exists in the human gut. It is known that the type of neonatal feeding influences the species composition of the microflora [4] and that, in the elderly, a general reduction in species diversity occurs, with an increase in facultative anaerobes and a decrease in lactobacilli and bifidobacteria [11], [31].

Lactobacilli and bifidobacteria, the most frequently used bacteria in probiotic products for human consumption, belong to different phyla. Lactobacilli, with all other LAB, are low G + C Gram-positives and belong to the phylum of the *Firmicutes*, while bifidobacteria are high G + C Gram-positives and belong to the phylum of the *Actinobacteria* [30].

Bifidobacteria, like all *Actinobacteria*, are not numerically important in the intestine; their number is higher in breast-fed than in formula-fed infants and decreases in adults [4], [27]. In spite of their number, bifidobacteria are thought to play a relevant role in keeping the gut healthy [15] and cells of *Bifidobacterium longum* have been shown to contribute to the reduction of intestinal inflammation by efficiently inhibiting pancreatic and neutrophilic elastases [12].

Culturing and PCR-based studies on fecal samples from healthy humans reported the isolation of various LAB species with *Lactobacillus ruminis*, *L. crispatus*, *L. gasseri, L. plantarum, L. acidophilus, L. delbrueckii, L. casei, L. paracasei* and *Leuconostoc argentinum* as the most abundant species [30]. Similar analysis performed on infant fecal samples confirmed *L. acidophilus, L. casei/paracasei* and *L. salivarius* as predominant [30]. The microbiological analysis of intestinal biopsies from healthy adults identified *L. ruminis, L. gasseri, L. vaginalis* and *Leuconostoc mesenteroides* as the most abundant species [30], while a plasmon resonance approach showed that *L. salivarius* (26%), *L. fermentum* (13%), *L. gasseri* (10%), *L. paracasei* (7%), *L. casei* (3%), *L. mucosae* (3%) and *L. plantarum* (3%) are the predominant species associated with human colonic mucin [13].

Not much attention has been paid thus far to whether bacteria isolated from the animal gut bind tightly or loosely to the epithelium or whether physical interactions between bacterial cells and animal tissue are relevant for probiotic activity of intestinal microflora. To gain insight into this problem, we followed a previously reported procedure [19] to separate the mucus layer from the epithelial tissue of ileal biopsies of healthy human volunteers, and found that only a small minority of ileal bacteria remains attached to the human cells after this treatment. Among these tightly bound bacteria, we focused our attention on eleven anaerobic rod-shaped strains that would more likely display a probiotic function in the gut, and we report here on some of their relevant properties.

2. Materials and methods

2.1. Collection of ileal samples

Mucosal samples were collected by forceps biopsy in the distal ileum from 7 adult human volunteers (M/F 4/3, mean age ± SD 45.0 ± 13) undergoing routine diagnostic endoscopy for colorectal cancer (CRC) screening. All patients recruited gave their informed consent to the study. The study was approved by the appropriate ethics committee and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. The patients did not follow any special dietary regimen, and had not recently received any antibiotic or probiotic treatment. Samples were stored at −80 °C in phosphate-buffered saline (PBS) containing 15% glycerol before subsequent analysis. Endoscopic and histological analysis of ileum samples did not reveal any sign of inflammation.

2.2. Bacterial isolation and culture conditions

Ileal samples (10–20 mg/each) were treated with DTT as previously reported [19], and extensively washed with PBS to eliminate loosely attached bacteria. Tissue samples after the washes, as well as buffer recovered from each wash, were plated on LB (8 g/l NaCl, 10 g/l tryptone, 5 g/l yeast extract) and MacConkey (DIFCO) media in aerobic conditions to determine the total aerobic count and on deMan, Rogosa and Sharpe (MRS) (Difco) medium in anaerobic condition to isolate lactic acid bacteria. Anaerobic conditions were obtained by incubating liquid and solid cultures in an anaerobic chamber (Oxoid).

2.3. Physiological and biochemical analysis

Exponentially growing cells of the various isolates were analyzed by the API 50 CHL kit (Biomerieux) following the manufacturer's instructions.

For biofilm formation, bacteria were grown in modified TSB (mTSB) medium as described by Lebeer et al., (2007): 15 g/liter TSB (BD Biosciences) enriched with 20 g/liter Bacto peptone no. 3 (BD Biosciences).

Mucin degradation assays were performed as previously reported [34]. In brief, cells were grown overnight in MRS broth at 37 °C in anaerobic conditions and spotted on Medium B plates: tryptone (Oxoid) 7.5 g/l; casitone (Difco) 7.5 g/l; yeast extract (Oxoid) 3.0 g/l; meat extract (Merck) 5.0 g/l; NaCl (BDH) 5.0 g/l; K_2HPO-$3H_2O$ (BDH) 3.0 g/l; KH_2PO (BDH) 0.5 g/l; $MgSO$-$7H_2O$ (BDH) 0.5 g/l; cysteine HCl (Sigma) 0.5 g/l; resazurin (BDH) 0.002. g/l; D-(1)-glucose (BDH) 10 or 30 g/l, purified hog gastric mucin (HGM) 3 g/l and agarose (Sigma) 1.5 g/100 ml. The pH of medium was adjusted to 7.0 with 2 N NaOH. Mucin degradation activity was evaluated by the diameter of the halo observed after plate staining with amido black 0.1% in glacial acetic acid 3.5 M and washing with glacial acetic acid 1.2 M. Mucin used in this study was from porcine stomach type III (Sigma).

Antimicrobial activity was determined as previously described [2] with the following modifications: 10 µl of each culture in stationary growth phase were spotted on the surface of an MRS agar plate and the spots air-dried. 100 µl of an exponential culture of the indicator bacterial strain were mixed with 5 ml of soft agar (0.7%) and poured over the plate.

Please cite this article in press as: Fakhry, S., et al., Characterization of intestinal bacteria tightly bound to the human ileal epithelium, Research in Microbiology (2009), doi:10.1016/j.resmic.2009.09.009

The plates were incubated aerobically overnight at 37 °C and the inhibition halos were measured and expressed in mm.

2.4. Simulated gastric and intestinal fluids

Gastric and intestinal fluids (SGF and SIF) were simulated as previously reported [8]. Exponentially growing cells were washed, resuspended in SGF (PBS 0.5% w/v, pepsin 3 g/l, pH 2.0) or SIF (PBS 0.5% w/v, pancreatin 1 g/l, pH 8.0) and incubated 1 h at 37 °C. Cells were then diluted, plated on MRS plates and incubated at 37 °C in anaerobic conditions. To monitor growth in simulated intestinal conditions after gastric treatment, exponentially growing cells were washed, resuspended in SGF (PBS 0.5% w/v, pepsin 3 g/l, pH 2.0) and incubated 1 h at 37 °C. Cells were then diluted at 0.05 OD_{600nm} in MRS supplemented with pancreatin (1 g/l, pH 8.0), incubated at 37 °C in anaerobic conditions and growth monitored 18 h. As control; each strain was incubated under the same conditions in MRS medium without supplements.

2.5. PCR conditions and primers

Oligonucleotides Ribo-for (5′-AGTTTGATCCTGGCTCAG-3′) and Ribo-rev (5′-ACCTACGTATTACCGCGGC-3′) were used to prime an amplification reaction (PCR) with chromosomal DNA of the various isolates as a template. The amplification product of 500 bp was gel-purified and used to obtain the nucleotide sequence. Species were assigned on the basis of homologies found in the databank of the Ribosomal Database Project (Michigan State University, USA).

2.6. In vitro biofilm assay

Biofilm formation was assayed as in reference [16]. Briefly, a platform carrying 96 polystyrene wells was filled with 200 µl of modified TSB medium (mTSB). Approximately $3x10^7$ CFU were added and incubated without shaking for 24 h at 37 °C. To quantify biofilm formation, the wells were briefly washed in phosphate-buffered saline (PBS). The remaining attached bacteria were stained for 30 min with 200 µl 0.1% (wt/vol) crystal violet in an isopropanol-methanol-PBS solution (1:1:18 [vol/vol]). Excess stain was washed with water. Wells were air-dried (30 min), the dye bound to the wells was extracted with 200 µl ethanol-acetone (80:20) and the optical density (OD) of 135 µl of each well was measured at 570 nm. Each strain was tested in at least three independent experiments, each with three biological replicates. Data were normalized to the indicated positive control (LGG), which was taken as 100% to compare different experiments. Additionally, sterile medium was always included (negative control).

2.7. In vitro cytotoxicity assays

Cytotoxic activity of the isolates was investigated in human cancer cell line HT29 (colon adenocarcinoma) using the MTS assay (Promega kit) following the manufacter's instructions. In brief, cells were grown in high-glucose Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% vol/vol fetal bovine serum (FBS), 50 ug/ml streptomycin, 50 ug/ml penicillin and incubated at 37 °C under a 5% CO_2 humidified atmosphere. Cells (3×10^3/well) were seeded in 96-well plates and allowed to attach and reach 80% confluence; then the media was removed and replaced by fresh medium containing 30% vol/vol bacteria-conditioned media (CM). Cells were incubated for 48 h. MTS assay was carried out using MTS:DMEM at a ratio of 1:5 with 2 h incubation at 37 °C, 5% CO_2. The optical density of formazan was measured at 490 nm using a Multiskan Spectrum (Thermo Electron Corporation). Optical density was a direct measure of cell viability. The experiments were done in three independent experiment and samples run in quintuplicate.

2.8. Preparation of bacterial supernatant

Bacterial strains were grown anaerobically in overnight MRS broth at 37 °C till stationary phase. Cells were then pelletted and the supernatant was filter-sterilized through a 0.22 um filter and stored at −20 °C.

3. Results

3.1. Isolation of lactobacilli tightly associated with ileal epithelial cells

In order to restrict our isolation to bacteria tightly associated with the human tissue, samples of ileal biopsies were treated as described in Material and methods. Most bacteria were found in the buffer recovered after the washes with an average of 3.54×10^3/mg and 2.21×10^3/mg of aerobic bacteria and LAB, respectively. A lower number of bacteria with an average of 16/mg and 10/mg of aerobic bacteria and LAB, respectively, were found tightly attached to ileal epithelial cells. Bacteria able to grow anaerobically on MRS medium were analyzed for their colony morphology on MRS plates, Gram-staining and catalase phenotype. Only one colony among those which were apparently identical from each ileal sample was selected and analyzed for cell shape under the light microscope. Selected bacteria were divided into two groups: round- and rod-shaped bacteria. By this procedure we ended up with 24 isolates: 13 round-shaped and 11 rod-shaped. With the aim of characterizing potential probiotic lactobacilli, we focused our attention on the rod-shaped bacteria that were characterized at the species level by analysis of the 16S rDNA sequence and by biochemical tests (API gallery). As a result, among the eleven rod-shaped isolates, one was identified as belonging to the *Bifidobacterium breve* species while the remaining ten were all lactobacilli, either of the *L. mucosae* (eight isolates) or *L. gasseri* (two isolates) species (Table 1). Since most isolates either came from different samples or belonged to different bacterial species (Table 1), only strains SF1087, SF1091 and SF1108, belonging to the same species and from the same ileal biopsy, could be potential siblings.

Please cite this article in press as: Fakhry, S., et al., Characterization of intestinal bacteria tightly bound to the human ileal epithelium, Research in Microbiology (2009), doi:10.1016/j.resmic.2009.09.009

Table 1
Rod-shaped bacteria tightly associated with ileal epithelial cells.

Biopsy n°	Strain name	Species[a]	Gene bank accession number
1	SF1031	*Lactobacillus mucosae*	FN400925
1	SF1036	*Bifidobacterium breve*	FN400926
2	SF1087	*Lactobacillus mucosae*	FN400927
2	SF1091	*Lactobacillus mucosae*	FN400928
2	SF1108	*Lactobacillus mucosae*	FN400929
2	SF1109	*Lactobacillus gasseri*	FN400930
3	SF1111	*Lactobacillus mucosae*	FN400931
4	SF1146	*Lactobacillus mucosae*	FN400932
5	SF1183	*Lactobacillus gasseri*	FN400933
6	SF1232	*Lactobacillus mucosae*	FN400934
7	SF1233	*Lactobacillus mucosae*	FN400935

[a] Assessed on the basis of the nucleotide sequence of the gene coding for 16S RNA and of biochemical (API) tests.

3.2. Production of antimicrobial activity

All lactobacilli were analyzed for the production of antimicrobial molecules active against selected pathogens. As summarized in Table 2, all strains produced antimicrobial molecules active against the Gram-positive and the Gram-negative pathogens used in our study. As a control, we used strain LGG, a commercially available probiotic strain of *L. rhamnosus*, and strains L38 and VL36, two food-isolated strains identified as *L. rhamnosus* and *L. fermentum*, respectively [2]. It is interesting to note that, while all our ileal isolates produced similar antimicrobial activities, the three *Lactobacillus* strains from other sources showed a more heterogeneous profile of production of antimicrobial molecules.

3.3. Resistance to simulated GIT conditions

We measured the survival of all isolated lactobacilli in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) as previously reported [8]. Almost identical numbers of cells were recovered on MRS plates from treated and untreated cells of all 11 isolates, indicating almost total resistance to both treatments (data not shown). We then analyzed whether cells exposed to simulated gastric fluid were still able to grow in simulated intestinal conditions. All strains were able to grow and very similar data were collected for all strains. Fig. 1 reports the growth curves of treated and untreated cells for four representative strains. In all cases, a long lag phase was observed, but once that growth was started the growth rates appeared similar to that of untreated cells (Fig. 1).

We cannot exclude the possibility that pancreatin inhibited growth and that after 8–14 h the enzyme was no longer active, thus allowing the cells to enter the exponential growth phase. However, in such a case we would expect cell growth to start at similar timepoints in all samples. As can be observed in Fig. 1, for the various isolates cell growth started at different time points (i.e. about after 8 h for *B. breve*, about 11 h for *L. mucosae* SF1233 and about 13 h for *L. mucosae* SF1091 and *L. gasseri*). Since we did not observe any mortality on plates due to treatment under SGF conditions, we believe it unlikely that the long delay was due to a reduced number of live cells after the SGF treatment. For these reasons, we favor the idea that the long lag phase experienced by cells subjected to SGF and then grown in SIF was due to either the stress encountered or the need for treated cells to adapt their metabolism to the new growth conditions.

3.4. Biofilm formation

All 11 lactobacilli were tested for their ability to form biofilm in microtiter plate assays [16]. *L. rhamnosus* GG (LGG), one of the clinically best-studied probiotic organisms [3] and a known biofilm producer [16], was used as a reference strain. Since biofilm formation is known to depend on environmental conditions [16], we measured production of biofilm after exposure of our isolates to simulated gastric fluid and under simulated intestinal

Table 2
Antimicrobial activity.[a]

Strain	*Bacillus cereus* 6A2[b]	*Staphylococcus aures* ATCC6538	*Listeria monocytogenes* ATCC7644	*Salmonella typhimurium* ATCC14028	*Shigella sonnei* ATCC25931	*Escherichia coli* ATCC13762
SF1031	++	++	++	++	++	+
SF1036	++	++	++	++	++	++
SF1087	++	++	++	++	++	+
SF1091	++	++	++	++	++	++
SF1108	+	++	++	++	+	++
SF1109	++	++	++	++	++	++
SF1111	++	++	++	++	++	++
SF1146	+	++	++	++	++	++
SF1183	++	++	++	++	++	++
SF1232	++	++	++	++	++	++
SF1233	+++	+++	++	++	++	++
LGG	++	+	++	±	±	++
VL36[b]	±	±	−	+	−	−
L38[b]	+	−	−	+++	−	++

[a] Symbols refer to the size of the inhibition halo observed with growing cells: - no halo; ± < 1 cm; + between 1 and 2 cm; ++ between 2 and 4 cm; +++ > 4 cm.
[b] [2].

Please cite this article in press as: Fakhry, S., et al., Characterization of intestinal bacteria tightly bound to the human ileal epithelium, Research in Microbiology (2009), doi:10.1016/j.resmic.2009.09.009

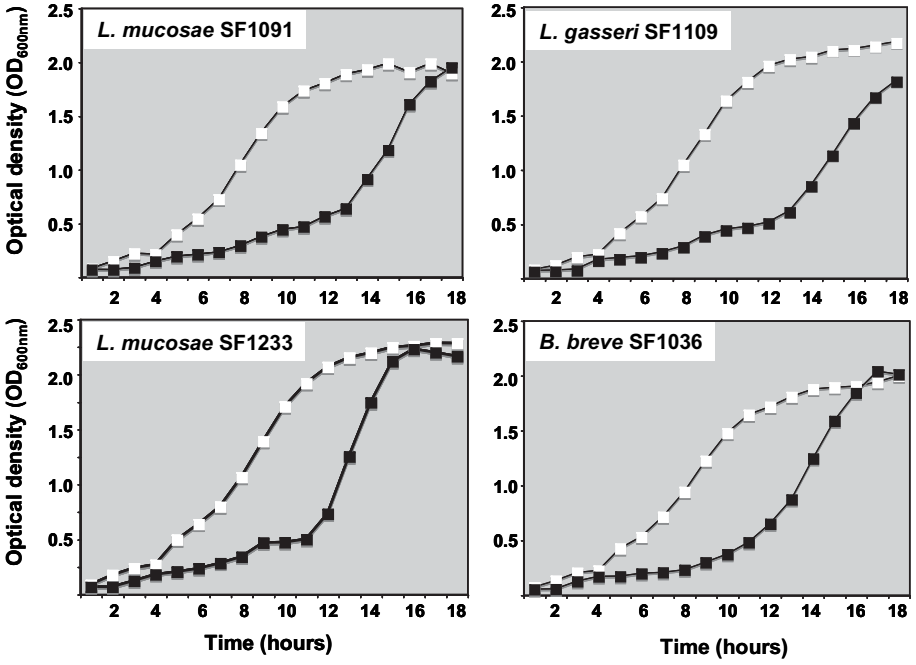


Fig. 1. Growth curves of treated (closed symbols) and untreated (open symbols) cells. Overnight cultures of the various strains were either treated with pepsin at pH 2 for 1 h at 37 °C or left in MRS broth at 37 °C, then diluted at OD 0.05 (600 nm) and used to inoculate MRS (open symbols) or MRS pH 8 supplemented with pancreatin (closed symbols) and OD measured every hour for 18 h.

conditions, as described above. As reported in Fig. 2, all isolates produced a biofilm and SGF-SIF treatments did not significantly affect the amount of biofilm produced.

3.5. Mucin degradation

The ability of our isolates to degrade mucin has been evaluated by a previously described plate assay [34]. As summarized in Table 3, we found that 8 of our 11 isolates were able to degrade mucin. While none of the two *L. gasseri* strains had mucin degradation activity, the *B. breve* strain and 7 out of 8 *L. mucosae* strains showed a clear ring of mucin degradation on plate (Table 3). As a control strain, we also tested *L. rhamnosus* GG (LGG) and it also showed clear mucin degradation activity (Fig. 3).

3.6. In vitro cytotoxicity assay

The cytotoxic activity of culture supernatants was assayed in vitro using cultured human colon cancer cells HT29 as previously described [1], [5].

The optical density, indicative of the eucaryotic cell viability, was measured at 490 nm and was shown to be similar for all strains tested (data not shown). The similar behavior of all eleven isolates and of the commercial probiotic strain LGG can be taken as an indication of the absence of cytotoxic activity.

4. Discussion

In this work, we have isolated and characterized anaerobic rod-shaped bacteria tightly attached to human ileum. Our analysis at the species level revealed the presence of *Bifidobacterium breve* (1 isolate), *Lactobacillus gasseri* (2 isolates) and *Lactobacillus mucosae* (8 isolates), all species that are normally found in the gastrointestinal tract of humans [10], [29] and various other mammals [6], [14], [18]. Bifidobacteria constitute the prevalent bacterial population in newborn infants, but are also present in adult individuals [4], [25], [29]. Some species such as *B. breve*, *B. longum* and *B. bifidum* are mostly associated with the human intestine and only occasionally found in animals [29]. These species belong to

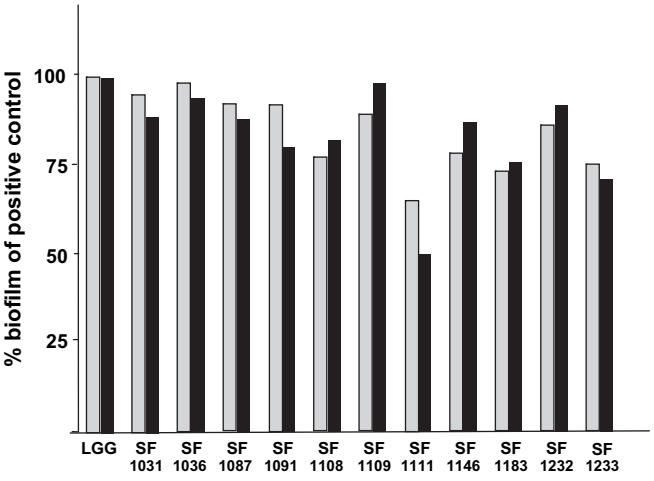


Fig. 2. Biofilm formation. The capacity for biofilm formation by the various isolates is expressed in comparison with that of strain LGG. Values obtained for strain LGG were taken as 100%. Biofilm formation was monitored in MRS broth (gray bars) and in MRS pH 8 supplemented with pancreatin after exposure to simulated gastric juice (black bars). The data shown are representative of at least three independent experiments, each with three biological replicates.

Please cite this article in press as: Fakhry, S., et al., Characterization of intestinal bacteria tightly bound to the human ileal epithelium, Research in Microbiology (2009), doi:10.1016/j.resmic.2009.09.009

Table 3
Mucin degradation assay.

Strain name	Species[a]	Mucin degradation[a]
SF1031	*Lactobacillus mucosae*	++
SF1036	*Bifidobacterium breve*	++
SF1087	*Lactobacillus mucosae*	+
SF1091	*Lactobacillus mucosae*	−
SF1108	*Lactobacillus mucosae*	++
SF1109	*Lactobacillus gasseri*	−
SF1111	*Lactobacillus mucosae*	+
SF1146	*Lactobacillus mucosae*	+
SF1183	*Lactobacillus gasseri*	−
SF1232	*Lactobacillus mucosae*	+
SF1233	*Lactobacillus mucosae*	++
LGG	*Lactobacillus rhamnosus*	++

[a] Symbols refer to the size of the halo observed: − no halo; + less than 1 cm; ++ between 1 and 2 cm.

mucosa-adherent members of the genus and are always found in association with intestinal mucosa and not only in fecal samples [29]. Many beneficial effects have been attributed to various *Bifidobacterium* strains, such as improvement in digestion and regulation of the host immune response [9], [22]. *L. gasseri* is a well characterized species, known to represent the major homofermentative *Lactobacillus* species of the human intestine [15], [20].

L. mucosae is, instead, a poorly characterized species, first isolated in a study aimed at isolation of *L. reuteri* [25]. The similarities between *L. reuteri* and *L. mucosae* also depend on the presence in both species of the *mub* gene, encoding a cell-surface protein with mucin binding activity. It is interesting that the G + C content of *mub* of *L. gasseri* is similar to the overall G + C content of *L. mucosae* and different from that of other *L. reuteri* genes. Based on this and on the observation that the *mub* gene has been found in all *L. mucosae,* but only in some *L. reuteri* strains, Ross et al. [25] suggested that *L. mucosae* may be the source of *mub* and *L. reuteri* is a recipient of the gene at some point during the course of evolution.

In all studies in which *L. mucosae* and *L. gasseri* were found to be associated with intestinal samples, they had never been indicated as predominant in number with respect to other *Lactobacillus* species. In a study by Kinoshita et al. [13], for example, *L. gasseri* and *L. mucosae* were identified as 10 and 3% of the total LAB population, respectively, in human colonic mucin. Although the low number of isolates in our study does not allow us to draw statistically relevant conclusions, we propose that the extensive washing and DTT treatment we performed on the ileal samples removed most of the loosely attached bacteria, allowing us to isolate a subpopulation of bacteria tightly associated with the epithelial cells characterized by very similar properties. *L. mucosae* and *L. gasseri* would then be predominant species among those bacteria tightly attached to the epithelial cells of the ileal tract of the human intestine. Although we have not performed typing experiments, we speculate that our isolates could belong to the same or very closely related strains of the *L. gasseri* and *L. mucosae* species, since they were selected from different ileal samples by the same treatment. Our hypothesis is supported by the extremely similar physiological properties that characterize this subpopulation of intestinal bacteria: i) all produce antimicrobial molecules similarly active against all tested pathogens. This is a peculiar feature, since LAB from different sources have a more heterogeneous profile of antimicrobial activity (see strain LGG, VL36 and L38 in Table 2 or reference [2]); ii) all are resistant to simulated gastric fluid and able to grow efficiently in simulated intestinal fluid after exposure to gastric conditions; iii) all produce biofilm in conditions that mimic gastric and intestinal environments; iv) 8 out of 11 isolates degrade mucin in an on-plate assay; v) none of the isolates showed any cytotoxic activity.

The abilities to survive, grow and produce biofilm in conditions that mimic what happens when bacteria transit the stomach tract are important features of potential probiotic strains, since they allow them to reach the intestinal environment without injury. Moreover, biofilms have protective and adhesive properties and have been associated with longer persistence of bacteria in the GIT of animals [8], [16].

The ability to degrade mucin is a controversial property. The mucus layers are essential for preventing adhesion of pathogens, toxins and other antigens present in the intestinal lumen to epithelial cells [21]. For this reason, mucin degradation has been associated with pathogens by some authors [21], [23], [24]. However, this activity has also been observed in several non-pathogenic and probiotic bacteria [26], [32]. In those cases, the ability to degrade mucin was seen as a beneficial feature of intestinal bacterial that, along with their surface-exposed glycoside hydrolases, participates in regulation of mucin turnover and, as a consequence, contribute to the integrity of the intestinal epithelium [32]. As also suggested in a previous study [26], we hypothesized that the ability to

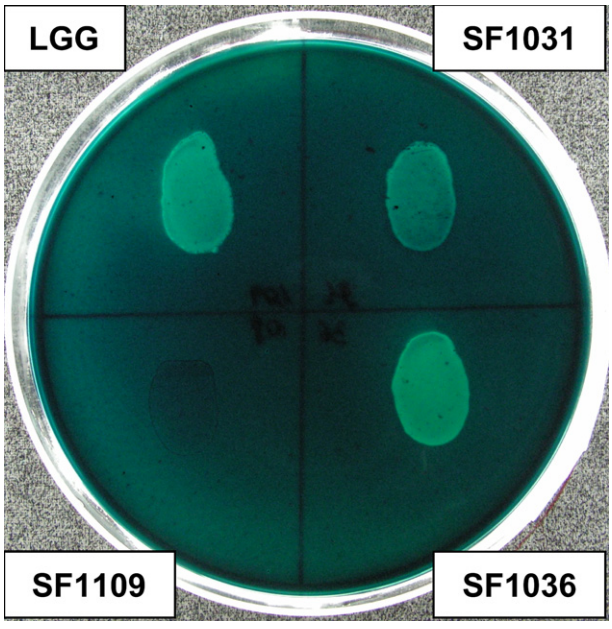


Fig. 3. Mucin degradation assay. An exponential culture of each strain was used to 'spot' sterile Medium B plates. After 48–72 h of incubation at 37 °C in anaerobic conditions, plates were stained and washed as described in Materials and Methods. The appearance of a halo around the bacterial spot was taken as an indication of the presence of mucin degradation activity.

Please cite this article in press as: Fakhry, S., et al., Characterization of intestinal bacteria tightly bound to the human ileal epithelium, Research in Microbiology (2009), doi:10.1016/j.resmic.2009.09.009

degrade mucin may be an adaptive advantage for intestinal bacteria, that using mucin as a source of nutrients, can more efficiently colonize the epithelial cell surface underneath the mucus layers. In vivo studies will be needed to verify whether this degradative activity increases the intestinal persistence of bacteria in the GIT.

The tight association with epithelial cells, presumably indicative of a direct interaction with the host cell, together with the physiological properties discussed above and with the absence of cytotoxic activity, make the subpopulation of intestinal bacteria that we isolated very promising as probiotic strains for human use.

Acknowledgments

We thank Luciano Di Iorio for technical assistance. This work was supported by a grant from the European Union (7th Framework n° 207948: COLORSPORE) to ER.

References

[1] Arafa, H.M.M. (2009) Possible contribution of B-glycosidases and caspases in the cytotoxicity of novel glycoconjugates in colon cancer cells. Invest. New Drugs. (published on line on May 5th 2009).

[2] Baccigalupi, L., Di Donato, A., Parlato, M., Luongo, D., Carbone, V., Rossi, M., et al. (2005) Two small, surface-associated factors mediate adhesion of a food-isolated strain of *Lactobacillus fermentum* to Caco-2 cells. Res. Microbiol. 156, 830–836.

[3] Banasaz, M., Norin, E., Holma, R., Midtvedt, T. (2002) Increased enterocyte production in gnotobiotic rats mono-associated with *Lactobacillus rhamnosus* GG. Appl. Environ. Microbiol. 68, 3031–3034.

[4] Benno, Y., Sawada, K., Mitsuoka, T. (1984) The intestinal microflora of infants: composition of fecal flora in brest-fed and bottle-fed infants. Microbiol. Immunol. 28, 975–986.

[5] Bun, S.-S., Laget, M., Chea, A., Bun, H., Ollivier, E., Elias, R. (2009) Cytotoxic activity of alkaloids isolated from *Stephania rotunda in vitro* cytotoxic activity of cephranthine. Phytother. Res. 23, 587–590.

[6] Busconi, M., Reggi, S., Fogher, C. (2008) Evaluation of biodiversity of lactic acid bacteria microbiota in the calf intestinal tracts. Antoine Van Leeuwenhoek 94, 145–155.

[7] Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., et al. (2005) Diversity of the human intestinal microbial flora. Science 308, 1635–1638.

[8] Fakhry, S., Sorrentini, I., Ricca, E., De Felice, M., Baccigalupi, L. (2008) Characterization of spore forming Bacilli isolated from the human gastrointestinal tract. J. Appl. Microbiol. 105, 2178–2186.

[9] Heuvelin, E., Lebreton, C., Grangette, C., Pot, P., Cerf-Bensussan, N., Heyman, M. (2009) Mechanisms involved in alleviation of intestinal inflammation by *Bifidobacterium Breve* soluble factors. PLoS ONE 4, e5184.

[10] Honda, H., Kataoka, F., Nagaoka, S., Kawai, Y., Kitazawa, H., Itoh, H., et al. (2007) β-galactosidase, phospho-b-galactosidase and phospho-b-glucosidase activities in lactobacilli strains isolated from human feces. Lett. Appl. Microbiol. 45, 461–466.

[11] Hopkins, M.J., Sharp, R., Macfarlane, G.T. (2001) Age and disease related changes in intestinal bacteria populations assessed by cell culture, 16S rRNA abundance and community fatty acid profile. Gut 48, 198–205.

[12] Ivanov, D., Emonet, C., Foata, F., Affolter, M., Delley, M., Fisseha, M., et al. (2006) A serpin from the gut bacterium *Bifidobacterium longum* inhibits eukaryotic elastase-like serine proteases. J. Biol. Chem. 281, 17246–17252.

[13] Kinoshita, H., Uchida, H., Kawai, Y., Kitazawa, H., Miura, H., Shiiba, K., et al. (2006) Quantitative evaluation of adhesion of Lactobacilli isolated from human intestinal tissues to human colonic mucin using surface plasmon resonance (BIACORE assay). J. Appl. Microbiol. 102, 116–123.

[14] Korhonrn, J.M., Sclivagnotis, Y., von Wright, A. (2008) Characterization of dominant cultivable lactobacilli and their antibiotic resistance profile from fecal samples of weaning piglets. J. Appl. Microbiol. 103, 2496–2503.

[15] Kullen, M.J., Klaenhammer, T.R. (2000) Genetic modification of intestinal Lactobacilli and Bifidobacteria. Curr. Issues Mol. Biol. 2, 41–50.

[16] Lebeer, S., Verhoeven, T.L.A., Perea Velez, M., Vanderleyden, J., De Keersmaecker, S.C.J. (2007) Impact of Environmental and genetic factors on biofilm formation by the probiotic strain *Lactobacillus rhamnosus* GG. Appl. Environ. Microbiol. 73, 6768–6775.

[17] Mahowald, M.A., Rey, F.E., Seedorf, H., Turnbaugh, P.J., Fulton, R.S., Wollam, A., et al. (2009) Characterizing a model human gut microbiota composed of members of its two dominant bacterial phyla. Proc. Natl. Acad. Sci. U.S.A. 106, 5859–5864.

[18] Manninen, T.J.K., Rinkinen, M.L., Beasley, S.S., Saris, P.E. (2006) Alteration of the canine small-intestine lactic acid bacterium microbiota by feeding of potential probiotics. Appl. Environ. Microbiol. 72, 6539–6543.

[19] Martin, H.M., Campbell, B.J., Hart, C.A., Mpofu, C., Nayar, M., Singh, R., et al. (2004) Enhanced *Escherichia coli* adherence and invasion in Crohn's disease and colon cancer. Gastroenterology 127, 80–93.

[20] Mitsuoka, T. (1992) Intestinal flora and aging. Nutr. Rev. 50, 438–446.

[21] Moncada, D.M., Kammanadiminti, S.J., Chadee, K. (2003) Mucin and Toll-like receptors in host defense against intestinal parasites. Trends Parasitol. 19, 305–311.

[22] Ouwehand, A.C., Bergsma, N., Parhiala, R., Lahtinen, S., Gueimonde, M., Finne-Soveri, H., et al. (2008) Bifidobacterium microbiota and parameters of immune function in elderly subjects. FEMS Immunol. Med. Microbiol. 53, 18–25.

[23] Prizont, R. (1982) Degradation of intestinal glycoproteins by pathogenic *Shigella flexneri*. Infect. Immun. 36, 615–620.

[24] Pultz, N.J., Hoskins, L.C., Donskey, C.J. (2006) Vancomycin-resistant Enterococci may obtain nutritional support by scavenging carbohydrate fragments generated during mucin degradation by the anaerobic microbiota of the colon. Microb. Drug Resist. 12, 63–67.

[25] Ross, S., Karner, F., Axelsson, L., Jonsson, H. (2000) *Lactobacillus mucosae* sp. nov., a new species with *in vitro* mucus-binding activity isolated from pig intestine. Int. J. Syst. Evol. Microbiol. 50, 251–258.

[26] Ruas-Madiedo, P., Gueimonde, M., Fernandez-Garcia, M., de los Reyes-Gavilan, C., Margolles, A. (2008) Mucin degradation by *Bifidobactrium* strain isolated from human intestinal microbiota. Appl. Environ. Microbiol. 74, 1936–1940.

[27] Schiffrin, E.J., Blum, S. (2002) Interactions between the microbiota and the intestinal mucosa. Eur. J. Clin. Nutr. 56, 560–564.

[28] Turnbaugh, P.J., Harnady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., et al. (2009) A core gut microbiome in obese and lean twins. Nature 457, 480–485.

[29] Turroni, F., Foroni, E., Pizzetti, P., Giubellini, V., Ribbera, A., Merusi, P., et al. (2009) Exploring the diversity of the Bifidobacterial population in the human intestinal tract. Appl. Environ. Microbiol. 75, 1534–1545.

[30] Vaughan, E.E., Heilig, H.G., Ben-Amor, K., de Vos, W.M. (2005) Diversity, vitality and activities of intestinal lactic acid bacteria and bifidobacteria assessed by molecular approaches. FEMS Microbiol. Rev. 29, 477–490.

[31] Woodmansey, E.J. (2007) Intestinal bacteria and ageing. J. Appl. Microbiol. 102, 1178–1186.

[32] Yang, F., Wang, J., Li, X., Ying, T., Qiao, S., Li, D., et al. (2007) 2-DE and MS analysis of interactions between *Lactobacillus fermentus* I5007 and intestinal epithelial cells. Electrophoresis 28, 4330–4339.

[33] Zhang, H., Di Biase, J.K., Zuccolo, A., Kudrna, D., Braidotti, M., Yu, Y., et al. (2009) Human gut microbiota in obesity and after gastric bypass. Proc. Natl. Acad. Sci. U.S.A. 106, 2365–2370.

[34] Zhou, J.S., Gopal, P.K., Gill, H.S. (2001) Potential probiotic lactic acid bacteria *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019) do not degrade gastric mucin in vitro. Int. J. Food Microbiol. 63, 81–90.

Please cite this article in press as: Fakhry, S., et al., Characterization of intestinal bacteria tightly bound to the human ileal epithelium, Research in Microbiology (2009), doi:10.1016/j.resmic.2009.09.009

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Characterization of spore forming Bacilli isolated from the human gastrointestinal tract

S. Fakhry[1], I. Sorrentini[2], E. Ricca[1], M. De Felice[1] and L. Baccigalupi[1]

1 Dipartimento di Biologia Strutturale e Funzionale, Università Federico II, Napoli, Italy
2 UOC di Gastroenterologia ed Endoscopia Digestiva, Azienda Ospedaliera di Benevento, ''G. Rummo'', Benevento, Italy

Keywords
Bacillus, commensals, gut, probiotics, spores, vaccine vehicles.

Correspondence
Loredana Baccigalupi, Dipartimento di Biologia Strutturale e Funzionale, Università Federico II,via Cinthia 4, complesso MSA 80126, Napoli, Italy. E-mail: lorbacci@unina.it

2008/1127: received 2 July 2008, revised and accepted 18 July 2008

doi:10.1111/j.1365-2672.2008.03934.x

Abstract

Aims: To isolate and characterize spore-former bacteria able to colonize the human gastrointestinal tract (GIT).
Methods and Results: A total of 25 spore-formers was isolated from faeces and ileal biopsies of healthy human volunteers and identified at the species level. Physiological analysis was performed to evaluate the ability of the various isolates to form biofilms, to swarm, to produce surfactants and molecules that have antimicrobial activity against selected pathogens. To assess the potential probiotic activity of the isolates, we tested the resistance of cells and spores to simulated gastric conditions, the ability to grow and sporulate in anaerobic conditions and the presence of toxin-encoding genes in their genome.
Conclusions: Spore-formers belonging to various bacterial species have been isolated from the gut of healthy human volunteers. These strains appear to be well adapted to the intestinal environment and we propose them as potential probiotic strains for human use and as oral vaccine vehicles.
Significance and Impact of the Study: To our knowledge this is the first detailed characterization of spore-forming Bacilli from the human GIT. Our data suggest that the isolated species do not transit, but rather colonize this specific habitat and propose them as probiotic strains for human use.

Introduction

Endospore-forming bacteria are Gram positive organisms belonging to various genera that, all together, include more than 200 species (Fritze 2004). These organisms are generally divided into two main groups of aerobic and anaerobic bacteria, with each group further subdivided into three genera: *Bacillus*, *Sporosarcina*, *Sporolactobacillus* and *Clostridium*, *Desulfotomaculum*, *Sporomusa*, respectively (Fritze 2004). However, few exceptions have been found and members of the aerobic *Bacillus* genus have been described as Gram-negatives (*B. azotoformans*, *B. oleronius* and *B. horti*) (Fritze 2004) or as capable of anaerobic metabolism (*B. subtilis*) (Nakano and Zuber 1998; Tam *et al.* 2006).

The common feature of spore-forming Bacilli is the ability to differentiate a peculiar cell form, the endospore (spore). Formation of the spore initiates when vegetative growth can no longer occur because of food shortage or other nonphysiological conditions in the environment. The spore is a quiescent cell form, characterized by several protective layers surrounding the dehydrated cytoplasm that contains the nucleoid (Henriques and Moran 2007). This structural organization makes the spores extremely resistant to external physical and chemical insults and able to survive almost indefinitely in the absence of water and nutrients. The exceptional longevity of the spore in the environment is the main reason for the ubiquitous distribution of these organisms, in particular, of the aerobic ones (Fritze 2004).

It is generally accepted that the primary reservoir of spore-forming Bacilli is the soil and the ability of spores to be dispersed in dust and water has been identified as the cause of their presence in almost every conceivable habitat. Several species of spore-formers are commonly found also in the gastrointestinal tract (GIT) of a variety

of animals (Barbosa *et al.* 2005; Tam *et al.* 2006). Only few *Bacillus* species are pathogens of animals (*B. cereus* and *B. anthracis*) or insects (*B. thuringiensis*), while the majority of them are nonpathogenic. Their presence in the GIT has been considered as due to the ingestion of bacteria associated with soil, water, air or foods.

However, a new theory is now emerging in which spore-former species are thought to establish an endosymbiotic relationship with their host, being able to survive and proliferate within the GIT and specifically interact with immuno and intestinal cells (Hong *et al.* 2005). Recent work has shown that in a murine model ingested spores can safely cross the stomach barrier and germinate in the intestine (Casula and Cutting 2002). In the same experimental model it has been also shown that spores can perform a complete life cycle, with germination in the upper part of the intestine, vegetative growth and sporulation before being expelled in the faeces (Tam *et al.* 2006). Other studies have established that *B. subtilis*, in combination with *Bacteroides fragilis*, is able to induce the development of gut-associated lymphoid tissue (GALT) and preimmune antibody repertoire in rabbits (Rhee *et al.* 2004). This study also showed that sporulation, as opposed to vegetative cell growth, is essential for GALT development. An *in vitro* analysis has also shown that the Competence and Sporulation Factor (CSF) of *B. subtilis*, a five amino acid peptide secreted during exponential growth and acting as a quorum-sensing molecule for the induction of DNA uptake and sporulation, is able to induce heat-shock response in human enterocyte-like (Caco-2) cells (Fujiya *et al.* 2007).

In a rather empirical way, spores of several *Bacillus* species have been widely used as human and animal probiotics for decades. Some commercial products have proven to contain *Bacillus* species different from those declared on their label (Green *et al.* 1999; Hoa *et al.* 2000), some strains are of unknown origin, some are multidrug resistant and some even harbor toxin genes (Green *et al.* 1999). Moreover, little is known about how spores exert their beneficial action on humans and animals. An *in vivo* study with a murine infection model has shown that the oral administration of 1×10^9 spores of *B. subtilis* one day before infection with $1{\cdot}5 \times 10^3$ CFU of the murine enteropathogen *Citrobacter rodentium* was able to drastically reduce the mortality rate and some signs of enteropathy but without affecting the animal immune-response to the pathogen (D'Arienzo *et al.* 2006).

All of the studies mentioned above have been performed with domesticated strains of *B. subtilis*. There is evidence that laboratory strains of *B. subtilis* differ from undomesticated strains, in several aspects including factors that are likely to affect their efficacy as probiotics (Branda *et al.* 2001; Earl *et al.* 2007, 2008). For these reasons, in this study, we aimed to isolate and identify aerobic spore-formers from the human GIT. Strains were characterized and tested for properties that would be beneficial to their survival in the gut and that could be desirable for probiosis. The collection of wild Bacilli of human origin described here will most likely provide a useful source of potential probiotics for human use, since it has been suggested that probiotic strains originate from the target animal microflora (Barbosa *et al.* 2005).

Materials and methods

Collection of ileal and faecal samples

Ileal biopsy samples were collected from eight adult human volunteers (M/F 5/3, mean age ± SD 45·0 ± 13) undergoing routine diagnostic colonoscopies. All patients recruited gave their informed consent to the study. The study was approved by the appropriate ethics committee and has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. The patients did not follow any special dietary regimen, and had not recently received any antibiotic or probiotic treatment. Samples were stored at −80°C in phosphate-buffered saline (PBS) containing 15% glycerol before subsequent analysis. Endoscopic appearance as well as histology of the ileum was normal in all patients. Faecal samples were collected from five healthy adult human volunteers that did not follow any special dietary regimen and who had not received any antibiotic treatment for at least 3 months.

Bacterial isolation and characterization

Ileal and faecal samples (20–40 and 50 mg/each, respectively) were heat-treated (80°C for 10 min) to kill all vegetative cells and individually placed on LB plates. After 36 h of incubation at 37°C, colonies were recovered and purified by streaking on fresh LB plates. Pure cultures were streaked on Difco sporulation medium (DSM), incubated at 37°C for 24–37 h and checked by light microscopy for the presence of spores.

Exponentially growing cells were used to extract chromosomal DNA as previously reported (Green *et al.* 1999). DNA coding for 16S RNA was PCR amplified by using chromosomal DNA as a template and oligonucleotides Ribo-For (5′-AGTTTGATCCTGGCTCAG-3′; annealing at position +9/+28) and Ribo-Rev (5′-CCTACGTAT-TACCGCGGC-3′ annealing at position +549/+531). Those two oligonucleotides were designed to amplify a 540 bp DNA fragment (MicroSeq 500 16S ribosomal DNA) previously indicated as sufficient for species identification (Woo *et al.* 2003). Amplified DNA was used to

determine the nucleotide sequence (BMR Genomics, Padova, Italy) which was used for an online computer-assisted analysis of homology.

Exponentially growing cells of the various isolates were used for biochemical analysis by the use of API 50 CHL kit (Biomerieux) following the manufacturer's instructions.

Unless otherwise specified, bacteria were grown in LB medium (for 1 l: 10 g Bacto-Tryptone, 5 g Bacto-yeast extract, 10 g NaCl, pH 7·0). Anaerobic conditions were obtained by incubating liquid and solid cultures in an anaerobic chamber (Oxoid).

Physiological analysis

Swarming motility was tested as previously reported (Connelly *et al.* 2004). Overnight cultures of all strains were spotted on LB or B (Julkowska *et al.* 2004) medium plates. LB and B plates were incubated at 37°C and 30°C, respectively, for 24–36 h. Surfactin production was assessed as described by Youssef *et al.* (2004) and by growing cells on B medium (Julkowska *et al.* 2004). To test biofilm production overnight cultures were used to inoculate liquid MSgg medium (100 mmol l^{-1} MOPS pH 7·0, 0·5% glycerol, 0·5% glutamate, 5 mm potassium phosphate pH 7·0, 50 μg ml^{-1} tryptophan, 50 mg ml^{-1} phenylalanine, 2 mmol l^{-1} $MgCl_2$, 0·7 mmol l^{-1} $CaCl_2$, 50 μmol l^{-1} $FeCl_3$, 50 μmol l^{-1} $MnCl_2$, 2 μmol l^{-1} thiamine, 1 μmol l^{-1} $ZnCl_2$) (Branda *et al.* 2001) and cells grown at 37°C in static conditions for up to 48 h. Cells forming a solid layer at the liquid–air interface were considered as biofilm producers.

Resistance to GIT conditions was assessed as previously reported (Duc *et al.* 2004). Cells or spores were suspended in simulated gastric fluid [SGF: 1 mg of pepsin (porcine stomach mucosa; Sigma) per ml; pH 2·0] or small intestine fluid [SIF: 1 mg of pancreatin (porcine pancreas; Sigma) per ml and 0·2% bile salts (50% sodium cholate–50% sodium deoxycholate; Sigma); pH 7·4] and incubated at 37°C for 1 h. Samples were serially diluted and plated to determine the number of CFU per ml on LB agar plates. Resistance to antibiotics was assessed on plates by adding to LB plates the following antibiotics: neomycin (20 μg ml^{-1}), erythromycin (3 μg ml^{-1}), spectinomycin (200 μg ml^{-1}) or rifampicin (50 μg ml^{-1}). Production of antimicrobials was tested as previously reported (Baccigalupi *et al.* 2005).

Analysis of enterotoxins and virulence traits

Methods to detect putative *B. cereus* enterotoxin genes from *Bacillus* species by PCR amplification from chromosomal DNA have been reported previously (Duc *et al.* 2004). Primer sets were those described by Guinebretiere *et al.* (2002). Haemolysis was detected by streaking cells on horse blood (Oxoid) agar plates and 48-h incubation at 37°C.

Results

Isolation of spore-formers from human gut

Samples of faeces and ileal biopsies of healthy human volunteers, collected as described in 'Materials and methods', were heat-treated to kill all cells and incubated on a solid medium to allow germination and growth of heat-resistant spores. All recovered bacteria were purified and analysed for colony morphology and the presence of spores by light microscopy. From a total of eight ileal biopsies (20–40 mg/each from different individuals) and of five samples of faeces (50 mg/each from different individuals) 13 and 12 spore-formers were isolated, respectively.

Together with the spore-formers, other bacteria were also isolated, but only partially characterized and not used in the present study. Those organisms were either members of thermophilic species or mesophilic but probably part of abundant population not totally killed by the heat-treatment.

The 25 spore-formers isolated were characterized at the species level by analysis of the 16S rDNA sequence and biochemically by the use of API 50 CHL kit (Table 1). As shown in Table 1, mostly similar species of aerobic spore-formers were isolated from the two sources, whereas, due to the isolation procedure, we did not recover clones of anaerobic spore-formers. In addition, a *B. thuringiensis* clone was isolated from a faecal sample and *B. megaterium* and *Paenibacillus chibensis* isolates derived from ileal biopsies. For strain SF170, also of ileal origin, a species was not assigned since its 16S DNA sequence showed homology with an uncultured *Bacillus* (GenBank entry: AY493970). Because of the low number of samples and isolates, our results can not be taken as an indication that some species proliferate preferentially in one source or the other, but they do suggest that a very similar population of Bacilli can be found in both.

Swarming motility and biofilm formation

As an initial characterization, all 25 isolates were tested for their ability to swarm and produce surfactin and biofilm (Table 2). Swarming is a typical movement of bacterial cells on a solid surface (Fig. 1) and, together with biofilm formation, is a property of Bacilli that is often lost (or much reduced) in laboratory strains. In confirmation of this, none of the strains in our lab collection was

Table 1 List of intestinal strains isolated

Strain	Species*	Source	Accession number†
SF119	*Bacillus pumilus*	Feces	FM178952
SF120	*Bacillus licheniformis*	Feces	FM178953
SF147	*Bacillus pumilus*	Feces	FM178954
SF148	*Bacillus subtilis*	Feces	FM178955
SF149	*Bacillus subtilis*	Feces	FM178956
SF150	*Bacillus clausii*	Feces	FM178957
SF151	*Bacillus subtilis*	Feces	FM178958
SF152	*Bacillus subtilis*	Feces	FM178959
SF153	*Bacillus subtilis*	Feces	FM178960
SF154	*Bacillus subtilis*	Feces	FM178961
SF155	*Bacillus subtilis*	Feces	FM178962
SF168	*Bacillus thuringiensis*	Feces	FM178963
SF85	*Bacillus pumilus*	Ileum	FM178964
SF106	*Bacillus subtilis*	Ileum	FM178965
SFB2	*Bacillus subtilis*	Ileum	FM178966
SFB3	*Bacillus subtilis*	Ileum	FM178967
SF128	*Bacillus subtilis*	Ileum	FM178968
SF169	*Bacillus licheniformis*	Ileum	FM178969
SF170	*Bacillus* sp.	Ileum	FM178970
SF173	*Bacillus megaterium*	Ileum	FM178971
SF174	*Bacillus clausii*	Ileum	FM178972
SF185	*Bacillus subtilis*	Ileum	FM178973
SF186	*Paenibacillus chibensis*	Ileum	FM178974
SF188	*Bacillus pumilus*	Ileum	FM178975
SF195	*Bacillus subtilis*	Ileum	FM178976

*Species assignment was based on 16S rDNA sequence analysis and on the results of the API50 CHL kit.

†Accession numbers of 16S rDNA sequences deposited to the EMBL nucleotide sequence database.

Table 2 Physiological properties of the intestinal isolates

Strain	Swarming		Surfactin ring	Biofilm formation
	LB	B		
SF119	X	X		X
SF120	X	X	X	X
SF147	X	X	X	X
SF148	X	X	X	X
SF149	X	X	X	X
SF150				X
SF151				
SF152				X
SF153				
SF154				
SF155	X			X
SF168				
SF85	X	X	X	X
SF106				
SFB2				
SFB3	X	X	X	X
SF128	X	X	X	X
SF169	X	X	X	X
SF170				
SF173				
SF174				
SF185				X
SF186				X
SF188	X	X	X	X
SF195				X

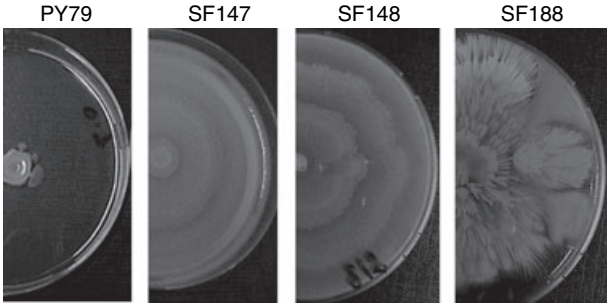


Figure 1 Examples of swarming motility on LB medium additioned of 0·7% agar. PY79 is a laboratory collection strain of *Bacillus subtilis* (Youngman *et al.* 1984) and does not show swarming motility. The other strains are three of the GIT isolates described in Table 1 and all show various types of swarming motility.

able to swarm or produce biofilm in control experiments (data not shown).

Studies in *B. subtilis* have shown that swarming is dependent on the presence of a flagellum in various physiological conditions and, only in a minimal medium, also on the ability of the strain to produce surfactin (Julkowska *et al.* 2005). For this reason we tested our strains for the ability to swarm in rich (LB) and synthetic (B; Julkowska *et al.* 2004) medium and also assayed their ability to produce surfactin.

The analysis of Table 2 indicates that all isolates belonging to the *B. subtilis* species behaved as previously reported for that species: those that were able to swarm in minimal medium also produced surfactin. This behaviour was not observed with SF119 (*B. pumilus*) and SF155 (*B. subtilis*) as they were able to swarm in minimal medium but did not produce surfactin. A possible explanation is that those two isolates produce a different surfactant that does not respond to the assay we used to detect surfactin (Materials and methods).

The majority of the strains (16 out of 25) formed biofilms. This is an interesting observation since biofilms have protective and adhesive properties and have been associated to a longer persistence of Bacilli in the GIT of animals (Huang *et al.* 2008).

The analysis of Table 2 indicates that all strains able of swarming mobility are also able to produce biofilms, whereas some of the biofilm-producers do not swarm, either in rich or minimal media. These results induced us

to speculate that biofilm formation is essential but not sufficient for swarming motility. Additional studies will be needed to properly address this point.

Production of antimicrobial activity

All isolated Bacilli were then analysed for the production of antimicrobial molecules active against selected pathogens (Table 3). An exponential culture of each of the 25 isolates was used to 'spot' sterile LB plates. As previously reported (Baccigalupi *et al.* 2005), the spots were air-dried and used to overlay soft agar (0·7%) containing exponential cells of one of the indicator strains. Solidified plates were then incubated at the appropriate temperature for 18–24 h and the appearance of a growth–inhibition halo taken as an indication of the presence of an antimicrobial activity. As summarized in Table 3, most of the strains produced antimicrobial molecules, mainly active against the Gram-positive pathogens used in our study. In particular, most of the strains were active against *Listeria monocytogenes*. Only four strains, all isolated from fecal samples, were active against *Salmonella enterica* (SF148, SF149 and SF168) or *Shigella sonnei* (SF147).

Table 3 Antimicrobial activities produced by the intestinal isolates

Strain	*Bacillus cereus*	*Staphylococcus aureus*	*Listeria monocytogenes*	*Salmonella enterica*	*Shigella sonneii*
SF119			X		
SF120	X		X		
SF147		X	X		X
SF148	X		X	X	
SF149	X		X	X	
SF150					
SF151		X	X		
SF152		X	X		
SF153		X	X		
SF154	X	X	X		
SF155			X		
SF168			X	X	
SF85	X	X	X		
SF106		X			
SFB2		X	X		
SFB3	X		X		
SF128	X		X		
SF169					
SF170					
SF173					
SF174					
SF185					
SF186					
SF188			X		
SF195					

Although the low number of isolates do not allow us to draw statistically significant conclusions, it is interesting to note that, while almost all fecal isolates (11 out of 12) produced antimicrobial molecules, less than 50% (6 out of 13) of the strains isolated from the ileal biopsies showed that property.

Resistance of spores and cells to simulated GIT conditions

We measured the survival of spore suspensions in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) as previously reported (Duc *et al.* 2004). Spores were prepared by the exhaustion method originally developed for *B. subtilis* (Nicholson and Setlow 1990). As previously reported for fecal-isolates of *B. subtilis* (Tam *et al.* 2006), we also noticed that some strains were faster to sporulate than other isolates, including a laboratory strain [PY79, a derivative of the 168 type strain (Youngman *et al.* 1984)].

Spores were then purified as previously described (Nicholson and Setlow 1990) and aliquots of $3–5 \times 10^8$ spores suspended for 1 h in SGF and SIF. Almost identical numbers of cells were recovered on LB plates from treated and untreated spores of all 25 isolates and of the strain PY79, indicating an almost total resistance of spores to the condition used (data not shown).

We also measured the survival of vegetative cells to SGF and SIF. Exponentially growing cells (always between $1·0 \times 10^7$ and $1·0 \times 10^8$ CFU) were exposed for 1 h to either PBS or SGF or SIF, washed and plated on LB plates. While cells of most isolates and of the control strain PY79 were totally killed by both treatments (data not shown), cells of strains SF119 (*B. pumilus*) and SF128 (*B. subtilis*), showed only a minor reduction in CFUs (Table 4).

We reasoned that such resistance could be due to a peculiar cell surface of the two strains, both biofilm-producers (Table 2), and that secreted molecules could make the surface proteins unaccessible for the lytic enzymes present in SGF and SIF. Although we did not analyse the two strains for the presence of an S-layer,

Table 4 Survival to intestinal conditions

Strain	Initial CFU	CFU after 1 h in PBS	CFU after 1 h in SGF	CFU after 1 h in SIF
PY79	$9·5 \times 10^7$	$8·8 \times 10^7$	n.d.	n.d.
SF119	$2·0 \times 10^7$	$1·8 \times 10^7$	$1·7 \times 10^6$	$2·8 \times 10^6$
SF128	$5·3 \times 10^7$	$5·4 \times 10^7$	$8·0 \times 10^6$	$6·0 \times 10^6$

SIF, simulated gastric fluid; SIF, simulated intestinal fluid; n.d., not detectable.

it is well known that several *Bacillus* isolates produce such protective structure that surrounds the cell and reduces cell surface accessibility (Candela *et al.* 2005; Huber *et al.* 2005). We tested those two strains, as well as all other isolates, for sensitivity to some antibiotics. SF119 and SF128 were both sensitive to all antibiotics tested (not shown) suggesting that their resistance to SGF and SIF is not due to an impermeable surface. We do not have an explanation for the high level of resistance of cells of those two strains to simulated GIT conditions and can only speculate that it may reflect an adaptation of the cells to the environment they lived in.

The antibiotic inhibition test indicated that strains SFB3 and SF168 were resistant to erythromycin (3 μg ml^{-1}), strain SF195 was resistant to spectinomycin (200 μg ml^{-1}) and strain SFB2 was resistant to neomycin (20 μg ml^{-1}) and spectinomycin (200 μg ml^{-1}). All other strains were sensitive to the antibiotic tested (Material and methods).

Growth and sporulation in anaerobic conditions

Members of the *Bacillus* genus are aerobic bacteria, unable to grow anaerobically. However, there are exceptions and lab collections strains (derivatives of the 168 type strain of *B. subtilis*) are able to grow anaerobically when nitrate is provided as electron acceptor (Nakano and Zuber 1998). Tam *et al.* (2006) have reported that two wild isolates of *B. subtilis* can grow and sporulate on a solid sporulation-inducing medium (DSM) in anaerobic conditions, whereas the domesticated strain PY79 grew but sporulated at very low efficiency. In their experimental conditions, addition of nitrate to the medium did not improve significantly either growth or sporulation of the tested strains (Tam *et al.* 2006).

We analysed all 25 human isolates for their ability to grow in anaerobic conditions in a rich (LB) and a sporulation-inducing medium (DSM). As reported in Table 5, most of the isolates were able to grow anaerobically in DSM whereas only three of them grew anaerobically in rich LB medium. Of the 19 isolates that grew anaerobically on DSM plates, 13 were also able to sporulate in the absence of oxygen. This observation confirms the previous observation reported on *B. subtilis* isolates (Tam *et al.* 2006) and expands it to the other species present in our collection.

The analysis of Table 5 also reveals that three out of four *B. pumilus* isolated from the human GIT were strictly aerobic (SF119, SF147 and SF85) and that the *B. subtilis* isolates were mostly anaerobic since of the 13 isolated strains two were strictly aerobic (SF153 and SF155), four were able to grow but not to sporulate (SF151, SF152, SF154 and SF195) and seven were able to grow and sporulate (SF148, SF149, SF106, SFB2, SFB3, SF128 and SF185).

Table 5 Growth and sporulation of the intestinal isolates in anaerobic conditions

Strain	Vegetative growth on LB	Vegetative growth on DSM	Sporulation
SF119			
SF120	X	X	X
SF147			
SF148		X	X
SF149		X	X
SF150		X	X
SF151		X	
SF152		X	
SF153			
SF154		X	
SF155			
SF168		X	X
SF85			
SF106		X	X
SFB2		X	X
SFB3		X	X
SF128	X	X	X
SF169	X	X	X
SF170			
SF173		X	X
SF174		X	
SF185		X	X
SF186		X	
SF188		X	X
SF195		X	

Presence of potential virulence factors

We used a PCR approach to evaluate the presence of known *B. cereus* enterotoxin genes in the chromosome of all isolates using, as a control, the *B. cereus* strain GN105 (Naclerio *et al.* 1993). This method has been applied previously to profile putative food-poisoning *Bacillus* strains (Duc *et al.* 2004; Guinebretiere *et al.* 2002; Phelps and McKillip 2002). Figure 2 reports the results obtained for the PCR amplification of two isolates (SF150 and SF188) and the control strain GN105. Those reported in Fig. 2 were the only two strains positive for the presence of known enterotoxins. All other isolates were negative in our PCR-based analysis allowing us to conclude that 23 out of 25 tested strains did not contain genes encoding known *Bacillus* toxins in their genome.

In vivo analysis showed that only strains SF128 produced α-haemolysis while four strains produced β-haemolysis (SF119, SF147, SF168 and SF188). All other strains did not produce haemolysis and, therefore, can be considered as γ-haemolytic.

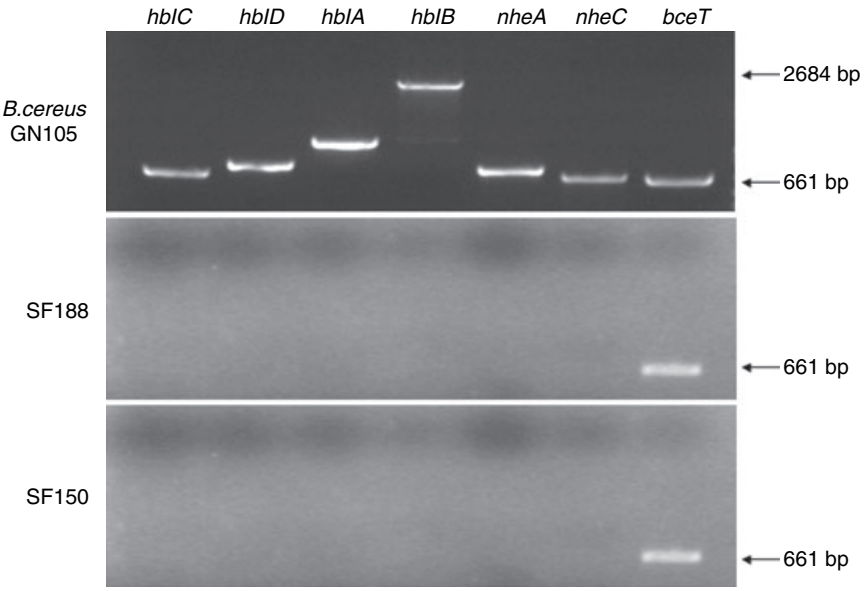


Figure 2 Agarose gel electrophoresis of PCR amplification products. All selected oligonucleotide pairs amplified specific fragments of the expected size using chromosomal DNA of the *Bacillus cereus* strain GN105 (Naclerio *et al.* 1993) as a template. Only the oligonucleotide pair used to amplify part of the *bceT* gene amplified a fragment of the expected size using chromosomal DNA of strains SF150 or Sf188 as a template.

Discussion

We used fecal samples and ileal biopsies of healthy human volunteers to retrieve 13 and 12 spore-forming isolates, respectively. Those bacteria were first characterized at the species level and then analysed for various physiological properties, some of which may be relevant for future use in probiotic preparations containing defined strains.

Some interesting conclusions can be drawn from this work. In agreement with Bacilli isolated from the soil (Branda *et al.* 2001; Earl *et al.* 2007), those described here, isolated from a seemingly peculiar environment such as the human gut, displayed swarming motility and biofilm formation. It is not yet known whether those Bacilli are able to swarm or form biofilm within the gut, but the observation that these properties have not been lost (as it has, instead, occurred in laboratory strains) allows us to hypothesize that they are important in the environment where these bacteria inhabit.

Biofilms have been proposed to have protective and adhesive roles for the bacteria producing them. These functions are potentially relevant for bacteria in the gut, and because of the protective environment of the biofilm, could enable survival in the intestinal conditions and adhere to mucus and epithelial cells more effectively than planktonic cells. In addition, within biofilms bacteria can respond to quorum-sensing molecules more easily than planktonic cells. Sporulation is known to be induced by quorum-sensing signals (e.g. the CSF of *B. subtilis*) and this has been shown to be essential for GALT development (Rhee *et al.* 2004).

Also the ability to grow and sporulate in an anaerobic environment appears as a common property of Bacilli isolated from the anaerobic gut. Rather than aerobes, Bacilli should be considered as facultative anaerobes, able to use oxygen or a different electron acceptor depending on the environmental conditions. The observation that some isolates were able to grow but not to sporulate in the anaerobic conditions obtained in the laboratory may suggest that different electron acceptors and/or different metabolic pathways are used during growth and sporulation.

While it is not surprising that all isolates survived the simulated GIT conditions in the spore form, it is striking that two isolates survived also in the vegetative cell form. The observation that the same two isolates were sensitive to common antibiotics allowed us to exclude that resistance to pepsin and pancreatin, present in SGF and SIF, respectively, was due to an unusual cell surface, impermeable to many external molecules. The mechanism of that resistance remains not known and further experiments will be needed to address this point.

Some features of the 25 human isolates, such as growth and sporulation in anaerobic conditions, cell survival to simulated GIT condition and biofilm formation, allow us to hypothesize that those strains are well adapted to the gut environment and potentially able to colonize that habitat.

Most of the isolates were sensitive to common antibiotics and did not contain genes encoding for known *Bacillus* toxins. Only two isolates, SF150 and SF188, contained a gene homologous to the *bceT* gene of *B. cereus*. However, *bceT* codes for enterotoxin T, a factor that has been shown not to contribute to food poisoning (Choma and Granum 2002). Although a proper safety assessment, with cytotoxicity and *in vivo* tests, is needed before these strains can be considered as probiotics, the preliminary

data presented here are an encouraging starting point to identify Bacilli of human origin to be used as probiotics for human use.

An additional potential application of those strains is as oral vaccine vehicles. Spores of *B. subtilis* have been used to display heterologous antigens (Isticato *et al.* 2001; Mauriello *et al.* 2004). Recombinant spores, orally administered to mice, were able to induce a specific humoral (Duc *et al.* 2003) and cellular (Mauriello *et al.* 2007) response. The immune response induced by spores exposing a fragment of the tetanus toxin resulted protective in a challenge experiment, with immunized mice able to survive the injection of a lethal dose of the toxin (Duc *et al.* 2003). It has been proposed that part of the observed immune response is not due to antigens present on the spores orally administered to the animals but rather to the antigens produced inside the animal body when recombinant spores germinate and sporulate (Uyen *et al.* 2007). All those studies have been performed with a laboratory strain of *B. subtilis*, not producing biofilms and incapable of efficient sporulation in anaerobic conditions. It is then reasonable to hypothesize that natural Bacilli expressing heterologous antigens may perform better than lab strains. It is, then, clear that a more efficient biofilm-assisted adhesion of cells to the intestinal epithelium and a more efficient sporulation in anaerobic conditions of the gut would result in a more efficient expression of the antigens and, presumably, in a stronger immune response.

Acknowledgements

We thank Krzysztof Hinc for advise on swarming motility and surfactin production and Luciano Di Iorio for technical assistance. This work was supported by grants of the Italian Ministry of the University (MIUR-COFIN 2006) and of the European Union (seventh Framework no. 207948: Colorspore) to E.R.

References

Baccigalupi, L., Di Donato, A., Parlato, M., Luongo, D., Carbone, V., Rossi, M., Ricca, E. and De Felice, M. (2005) Small, surface-associated factors mediate adhesion of a food-isolated strain of *Lactobacillus fermentum* to Caco-2 cells. *Res Microbiol* **156**, 830–836.

Barbosa, T.M., Serra, C.R., La Ragione, R.M., Woodward, M.J. and Henriques, A.O. (2005) Screening for *Bacillus* isolates in the broiler gastrointestinal tract. *Appl Environ Microbiol* **71**, 968–978.

Branda, S.S., González-Pastor, J.E., Ben-Yehuda, S., Losick, R. and Kolter, R. (2001) Fruiting body formation by Bacillus subtilis. *Proc Natl Acad Sci USA* **98**, 11621–1166.

Candela, T., Mignot, T., Hagnerelle, X., Haustant, M. and Fouet, A. (2005) Genetic analysis of *Bacillus anthracis* Sap S-layer protein crystallization domain. *Microbiology* **151**, 1485–1490.

Casula, G. and Cutting, S.M. (2002) *Bacillus* probiotics: spore germination in the gastrointestinal tract. *Appl Environ Microbiol* **68**, 2344–2352.

Choma, C. and Granum, P.E. (2002) The enterotoxin T (BcET) from *Bacillus cereus* can probably not contribute to food poisoning. *FEMS Microbiol Lett* **217**, 115–119.

Connelly, M.B., Young, G.M. and Sloma, A. (2004) Extracellular proteolytic activity plays a central role in swarming motility in *Bacillus subtilis*. *J Bacteriol* **186**, 4159–4167.

D'Arienzo, R., Maurano, F., Mazzarella, G., Luongo, D., Stefanile, R., Ricca, E. and Rossi, M. (2006) *Bacillus subtilis* spores reduce susceptibility to *Citrobacter rodentium*-mediated enteropathy in a mouse model. *Res Microbiol* **157**, 891–897.

Duc, L.H., Huynh, H.A., Fairweather, N., Ricca, E. and Cutting, S.M. (2003) Bacterial spores as vaccine vehicles. *Infect Immun* **71**, 2810–2818.

Duc, le H., Hong, H.A., Barbosa, T.M., Henriques, A.O. and Cutting, S.M. (2004) Characterization of *Bacillus* probiotics available for human use. *Appl Environ Microbiol* **70**, 2161–2171.

Earl, A.M., Losick, R. and Kolter, R. (2007) *Bacillus subtilis* genome diversity. *J Bacteriol* **189**, 1163–1170.

Earl, A.M., Losick, R. and Kolter, R. (2008) Ecology and genomics of *Bacillus subtilis*. *Trends Microbiol* **16**, 269–275.

Fritze, D. (2004) Taxonomy and systematics of the aerobic endospore forming bacteria: *Bacillus* and related genera. In *Bacterial Spore Formers* ed. Ricca, E., Henriques, A.O. and Cutting, S.M. pp. 17–34. Norfolk, UK: Horizon Bioscience.

Fujiya, M., Musch, M.W., Nakagawa, Y., Hu, S., Alverdy, J., Kohgo, Y., Schneewind, O., Jabri, B. *et al.* (2007) The *Bacillus subtilis* quorum-sensing molecule CSF contributes to intestinal homeostasis via OCTN2, a host cell membrane transporter. *Cell Host Microbe* **1**, 299–308.

Green, D., Wakeley, P., Page, A., Barnes, A., Baccigalupi, L., Ricca, E. and Cutting, S. (1999) Characterization of two *Bacillus* probiotic species. *Appl Environ Microbiol* **65**, 4288–4291.

Guinebretiere, M.-H., Broussolle, V. and Nguyen-The, C. (2002) Enterotoxigenic profiles of food-poisoning and food-borne *Bacillus cereus* strains. *J Clin Microbiol* **40**, 3053–3056.

Henriques, A.O. and Moran, C.P., Jr (2007) Structure, assembly and function of the spore surface layers. *Ann Rev Microbiol* **61**, 555–588.

Hoa, N.-T., Baccigalupi, L., Huxham, A., Smertenko, A., Van, P.-H., Ammendola, S., Ricca, E. and Cutting, S. (2000) Characterization of *Bacillus* species used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders. *Appl Environ Microbiol* **66**, 5241–5247.

Hong, H.A., Duc, le H. and Cutting, S.M. (2005) The use of bacterial spore formers as probiotics. *FEMS Microbiol Rev* **29**, 813–835.

Huang, J.M., La Ragione, R.M., Nunez, A. and Cutting, S.M. (2008) Immunostimulatory activity of Bacillus spores. *FEMS Immunol Med Microbiol* **53**, 195–203.

Huber, C., Ilk, N., Rünzler, D., Egelseer, E.M., Weigert, S., Sleytr, U.B. and Sára, M. (2005) The three S-layer-like homology motifs of the S-layer protein SbpA of *Bacillus sphaericus* CCM 2177 are not sufficient for binding to the pyruvylated secondary cell wall polymer. *Mol Microbiol* **55**, 197–205.

Isticato, R., Cangiano, G., Tran, T.-H., Ciabattini, A., Medaglini, D., Oggioni, M.R., De Felice, M., Pozzi, G. *et al.* (2001) Surface display of recombinant proteins on *Bacillus subtilis* spores. *J Bacteriol* **183**, 6294–6301.

Julkowska, D., Obuchowski, M., Holland, I.B. and Séror, S.J. (2004) Branched swarming patterns on a synthetic medium formed by wild-type *Bacillus subtilis* strain 3610: detection of different cellular morphologies and constellations of cells as the complex architecture develops. *Microbiology* **150**, 1839–1849.

Julkowska, D., Obuchowski, M., Holland, B. and Séror, S. (2005) Comparative analysis of the development of swarming communities of *Bacillus subtilis* 168 and a natural wild type: critical effects of surfactin and the composition of the medium. *J Bacteriol* **187**, 65–76.

Mauriello, E.M.F., Duc, L.H., Isticato, R., Cangiano, G., Hong, H.A., De Felice, M., Ricca, E. and Cutting, S.M. (2004) Display of heterologous antigens on the *Bacillus subtilis* spore coat using cotc as a fusion partner. *Vaccine* **22**, 1177–1187.

Mauriello, E.M.F., Cangiano, G., Maurano, F., Saggese, V., De Felice, M., Rossi, M. and Ricca, E. (2007) Germination-independent induction of cellular immune response by *Bacillus subtilis* spores displaying the C fragment of the tetanus toxin. *Vaccine* **25**, 788–793.

Naclerio, G., Ricca, E., Sacco, M. and De Felice, M. (1993) Antimicrobial activity of a newly identified bacteriocin of *Bacillus cereus*. *Appl Environ Microbiol* **59**, 4313–4316.

Nakano, M.M. and Zuber, P. (1998) Anaerobic growth of a "strict aerobe" (*Bacillus subtilis*). *Annu Rev Microbiol* **52**, 165–190.

Nicholson, W. L. and Setlow, P. (1990) Sporulation, germination and outgrowth. In *Molecular Biological Methods for Bacillus* eds. Harwood, C and Cutting, S. pp. 391–450. Chichester, UK: John Wiley and Sons.

Phelps, R.J. and McKillip, J.L. (2002) Enterotoxin production in natural isolates of *Bacillaceae* outside the *Bacillus cereus* group. *Appl Environ Microbiol* **68**, 3147–3151.

Rhee, K-J., Sethupathi, P., Driks, A., Lanning, K.D. and Knight, K.L. (2004) Role of commensal bacteria in the development of gut-associated lymphoid tissue and preimmune antibody repertoire. *J Immunol* **172**, 1118–1124.

Tam, N.K., Uyen, N.Q., Hong, H.A., Duc, le H., Hoa, T.T., Serra, C.R., Henriques, A.O. and Cutting, S.M. (2006) The intestinal life cycle of *Bacillus subtilis* and close relatives. *J Bacteriol* **188**, 2692–2700.

Uyen, N.Q., Hong, H.A. and Cutting, S.M. (2007) Enhanced immunisation and expression strategies using bacterial spores as heat-stable vaccine delivery vehicles. *Vaccine* **25**, 356–365.

Woo, P.C.Y., Ng, K.H.L., Lau, S.K.P., Yip, K.T., Fung, A.M.Y., Leung, K.W., Tam, D.M.W., Que, T.I. *et al.* (2003) Usefulness of the MicroSeq 500 16S ribosomal DNA-based bacterial identification system for identification of clinically significant bacterial isolates with ambiguous biochemical profiles. *J Clin Microbiol* **41**, 1996–2001.

Youngman, P., Perkins, J.B. and Losick, R. (1984) A novel method for the rapid cloning in *Escherichia coli* of *Bacillus subtilis* chromosomal DNA adjacent to Tn917 insertion. *Mol Gen Genet* **195**, 424–433.

Youssef, N.H., Duncan, E.K., Nagle, D.P., Savage, K.N., Knapp, R.M. and McInerney, M.J. (2004) Comparison of methods to detect biosurfactant production by diverse microorganisms. *J Microbiol Methods* **56**, 339–347.

INSTITUT PASTEUR

Research in Microbiology 160 (2009) 375–379

www.elsevier.com/locate/resmic

Defining the natural habitat of *Bacillus* spore-formers

Huynh A. Hong [a], Ellen To [a], Saad Fakhry [b], Loredana Baccigalupi [b], Ezio Ricca [b], Simon M. Cutting [a,*]

[a] *School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey TW20 0EX, UK*
[b] *Department of Structural and Functional Biology, Federico II University, Naples, Italy*

Received 11 May 2009; accepted 24 June 2009
Available online 7 July 2009

Abstract

Our understanding of the genetics and physiology of the spore-forming genus *Bacillus* is remarkable. On the other hand, though, where these Gram-positive bacteria live and grow is far from clear. The soil, once considered their habitat, may simply serve as a reservoir. A growing number of studies show that *Bacillus* spores can be found in the intestinal tracts of animals, raising the question of whether this could be where they live and grow. In this study, we have conducted the first evaluation of *Bacillus* spore formers in soil and in human faeces. Our aim is simply to determine the abundance of aerobic spore-formers. Our results show that soil carries approximately $\sim 10^6$ spores/g while human faeces an average of up to 10^4 spores/g. The numbers of spores found in faeces, we reason, is too high to be accounted for principally by ingestion of food contaminated with spores from soil. This provides further evidence that *Bacillus* spore formers may have adapted to survival within the intestinal tract of insects and other animals that ingest them; if so they may well be hitherto undiscovered gut commensals.

Keywords: *Bacillus* species; Endospores; Spore-formers; Soil microorganisms; Gut commensals

1. Introduction

Bacterial endospore-formers typically fall under two major groupings of Gram-positives, the *Bacilli* and *Clostridia*. Many other spore-forming genera exist, including Gram-negatives, but for the most part, these remain poorly understood [12]. In the case of *Bacillus,* most members have long been considered soil organisms [25]. This assumption is based upon culture-dependant methods of isolation that enrich for the presence of endospores, implying abundance [11]. In recent years, it has become apparent that this may be an oversimplification and *Bacillus* endospores have been found in diverse environments including rocks, dust, aquatic environments and the gut of various insects and animals [25]. Endospores are uniquely robust life forms enabling them to be dispersed easily and, as a result, found everywhere [24,25]. From a purely academic viewpoint, it is ironic that, for an organism that is genetically so well defined, on a par with *Escherichia coli*, its true habitat and life cycle is so poorly understood. One question that has been raised is whether there is a symbiotic relationship between *Bacillus* spp. and insects and animals [25]. For insects, in particular, there is stronger evidence for commensalism where the host benefits. For example, cockroaches fed with *Bacillus cereus* exhibited direct and positive weight gains [10]. An obligate, endosymbiotic, relationship with the Gram-positive endospore former, *Metabacterium polyspora* has been shown in the gut of the guinea pig [1] demonstrating that for some endospore formers, at least, there must also be direct benefits to the bacterium. *Bacillus* spp. are being used as probiotics for livestock, aquaculture as well as functional foods for human consumption [16]. For animal use, there are direct and substantiated benefits to animal health including weight gains and prevention of disease. In rabbits for example, *Bacillus subtilis* has been shown to have a direct effect on the development of the gut-associated lymphoid tissue (GALT)

* Corresponding author. Tel.: +44 (0)1784 443760; fax: +44 (0)1784 414224.
E-mail address: s.cutting@rhul.ac.uk (S.M. Cutting).

0923-2508/$ - see front matter © 2009 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.resmic.2009.06.006

[28]. Interestingly, these studies showed that sporulation, per se, was required for this phenomenon. This has been supported by murine studies that have shown, in vivo, that ingested endospores are able to germinate, proliferate and then re-sporulate in the small intestine [33]. *Bacillus* endospores then must have a much more intimate relationship with insects and animals than might be expected if their primary habitat were only the soil. We have raised the possibility that *Bacillus* species are able to exploit the intestinal tract as a habitat [33]. This has been supported by a number of studies that have recovered *Bacillus* spp. from the small intestine and from faecal samples [9,17]. In this work, we have examined the abundance of aerobic endospore formers in samples of human faeces as well as soil samples. Our results suggest that the human gastrointestinal (GI) tract is populated with *Bacillus* spore-forming species but these counts are at least 100-fold lower than the counts found in soil.

2. Materials and methods

2.1. Collection of faecal samples

Freshly voided faeces was collected from healthy volunteers and weighed by difference. Volunteers had not taken antibiotics or probiotics within the preceding 12 months and were recruited from the vicinity of either London or Naples. Faeces was stored at 4 °C and processed within 2–3 h of collection.

2.2. Soil samples

Approximately 10–20 g of soil (weighed by difference) was collected from selected locations in the London region. In each case, soil at a depth of 5–10 cm below the surface was collected. Samples were stored at 4 °C prior to analysis.

2.3. Determination of counts of spore-forming bacteria

Bacterial endospore formers were counted using two methods, heat or ethanol treatment. Faecal or soil samples (approx. 1–10 g) were suspended in the minimal volume of sterile phosphate-buffered saline (PBS) that allowed adequate suspension of solid matter by vigorous vortexing. In some cases, sterile glass beads (2 mm) were used to aid homogenisation of solid matter. For heat treatment, homogenised samples were heated in an oven at 65 °C for 45 min. For ethanol treatment 1 volume of homogenised sample was mixed with an equal volume of absolute alcohol and incubated for 1 h at room temperature (RT). After both heat or ethanol treatment, samples were immediately serially diluted and plated out on three solid media, Difco-Sporulation medium (DSM; [26]), MRS agar (Oxoid) and MacConkey's agar (Oxoid). Plates were incubated for 2–3 days at 30 °C and in the case of MRS agar plates were incubated anaerobically using a Don Whitley anaerobic cabinet.

2.4. Other methods

Catalase activity was confirmed by emulsification of single colonies in a 3% solution of hydrogen peroxide (Sigma). The one-way ANOVA test was used to compare the significance between groups.

3. Results and discussion

Faecal and soil samples were examined for quantification of bacterial endospores using a culture-dependent approach. Spore counts from faecal samples were taken from volunteers in an Italian study (Fig. 1A and Supplementary Table 1) and 20 from the UK (Fig. 1B and Supp. Table 2). As controls, the number of enterobacteria and lactobacilli were enumerated using growth on MacConkey's and MRS agar [14] respectively. Faecal counts for the enterobacteria and lactobacilli ranged between 10^6–10^7 for the former and 10^7–10^9 for the latter. Counts of lactobacilli using MRS medium were in agreement with other work [13] as were counts for the enterobacteria, demonstrating that our methodology was sound. In addition, 50 soil samples were examined (Fig. 2 and Supp. Table 3). Our analysis was designed to identify aerobic endospore formers and therefore excluded species of the strict anaerobes, i.e., *Clostridia*. Colonies were randomly tested for their catalase reaction and always tested positive, further excluding the possibility of *Clostridia*. The Gram stain was also performed on some isolates confirming in all cases the presence of Gram-positive rods. Thus, the vast majority of recovered strains were most probably species of *Bacillus*.

3.1. Spore formers are recovered from human faecal samples

Heat treatment (65 °C, 45 min.) should kill all vegetative cells, but it is worth mentioning that endospores of some species are resistant to only 60 °C. For example, endospores of some strains of *B. cereus* showed a reduction in viability after heat treatment at 65 °C [15]. We used 65 °C since, at 60 °C, residual fungal contamination was sometimes observed, and therefore our counts are probably a slight underestimate of the true numbers of aerobic endospore formers. While a surprisingly high number of bacterial species produce endospores [12], the most ubiquitous is *Bacillus* and it is likely that these represent the majority of the recovered counts. Our counts averaged at about 10^4 CFU/g and were therefore similar to those from a study of heat-treated human samples in Vietnam [33], although counts for adults were somewhat higher in the Vietnamese study. Using ethanol produced somewhat different results with lower endospore counts ($\sim 10^3$) compared to heat treatment. There are a number of explanations: first, the study was performed in two different laboratories and two different countries and we cannot rule out the possibility of a slight variation in the methodology or reagents. A second possibility is that this reveals a real difference perhaps caused, for example, by diet. We believe, though, that the lower counts

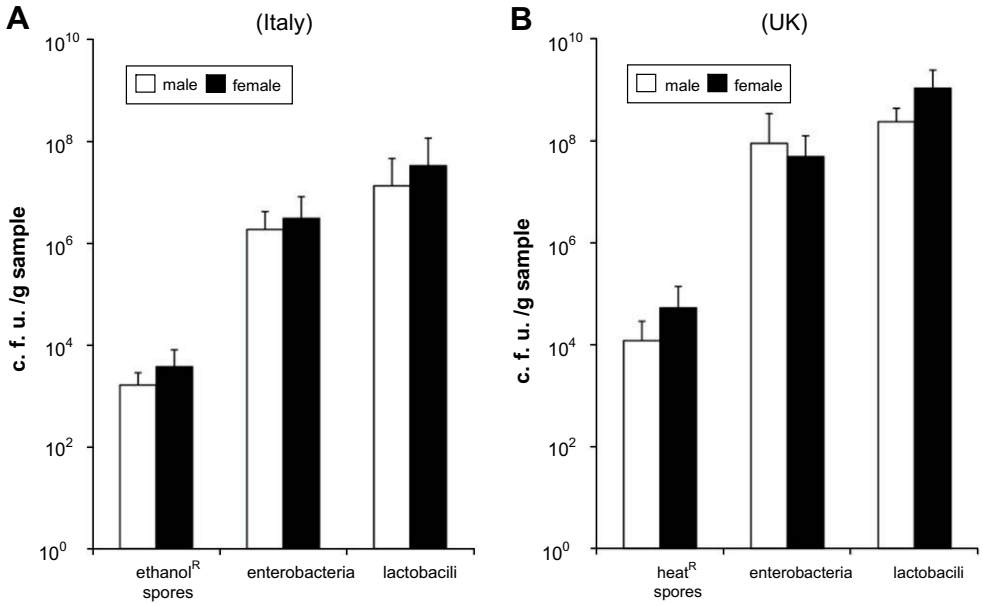


Fig. 1. Aerobic spore formers isolated from human faeces. Panel A shows counts of spores (CFU/g) obtained by ethanol treatment of freshly voided samples. Panel B shows counts obtained by heat-treatment. Plates were grown aerobically. Levels of enterobacteria and lactobacilli were determined by plating untreated samples on MacConkey's and MRS agar respectively. Raw data is given in Supp. Tables 1 and 2.

reflect the choice of ethanol for detection of endospore counts. The premise for using ethanol as a selective treatment was a previous study suggesting better enumeration of endospore counts compared to heat treatment [19]. In this work though, endospores evaluated were, at most, 5 days old. It is well understood that 'aged' spores are less capable of germinating synchronously and they must be heat-activated prior to culture on rich media [22]. If endospores found in the faeces (and also the soil samples; see below) are in this state of heightened dormancy, then this might explain the slight difference in endospore counts between the two studies. Although some differences existed between sexes, endospore counts were always higher in females, although the differences were not significant ($P > 0.05$).

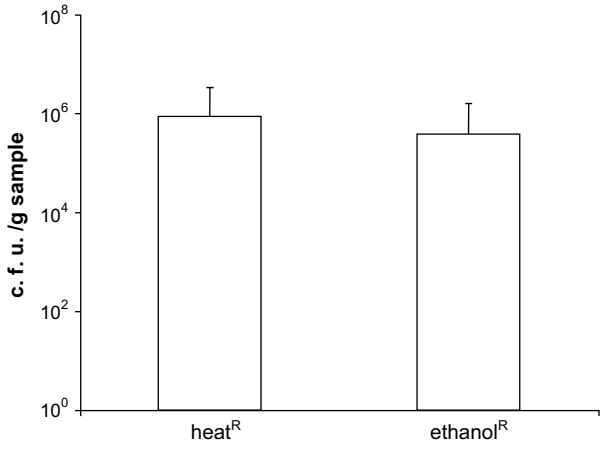


Fig. 2. Aerobic spore formers isolated from soil samples. Fifty soil samples were examined for the presence of aerobic spore formers (CFU/g) using ethanol or heat treatment. Raw data is given in Supp. Table 3.

3.2. Aerobic spores present in soil

Counts of aerobic endospore formers were, on average, 10- to 100-fold higher than faecal counts with an average of 10^5 CFU/g. As with faeces, heat-treatment generated somewhat higher numbers. Colonies identified were, in general, more pleiomorphic in appearance with rhizoid-type colonies, pinpoint as well as crenated forms. We also found that up to 15% of colonies identified from soil were pigmented. Pigmentation in endospores provides them with an extra level of protection against UV irradiation and an attribute of high-value if they are to remain dormant in the soil for long periods of time [21]. A variety of pigments have been found in *Bacillus* species including reds (*Bacillus atrophaeus* [21] and *Bacillus megaterium* [20]), dark-grey (*B. atrophaeus* [21]), yellow and orange (*Bacillus indicus* [32] and *Bacillus cibi* [35]). In many cases, the pigments are carotenoids that provide natural antioxidative properties [6,21]. Intriguingly, we found that at low dilutions, pigmentation was difficult to detect even when colonies were well isolated. On the other hand, at higher dilutions, for example, with 20–100 colonies on a plate, as many as 15% of colonies derived from heat-or ethanol-treated soil were pigmented (data not shown). It is possible that in a nutrient-rich environment (i.e., at high dilution), pigmentation is enhanced implying some form of catabolite repression. In any event, the main discovery, we surmise, is that a large number of endospore-formers are able to form pigments and the use of classical culture-dependant methods of identifying soil endospore formers may have

failed to identify these. We believe then that the soil potentially offers a large reservoir of yet undiscovered pigmented endospore formers.

3.3. Endospores found in food products

A potential source of the spores found in the human GI tract is through food. Endospores are commonly found in food products where their presence can be linked to the soil. For example, populations of endospores (typically, *B. cereus*, *B. licheniformis* and *B. subtilis*) in pasteurised milk can reach 10^3 CFU/ml and they have been shown to contaminate milk from silage, bedding as well as faeces [34]. During prolonged storage, germination, outgrowth and proliferation of endospores can substantially increase counts of live bacteria to as high as 10^6 CFU/ml [27]. Other food sources that carry *Bacillus* endospores at levels reaching 10^2 CFU/g are rice, grain and vegetables. In all cases, the origin of these endospores can be attributed to soil but as with milk storage, if endospores can germinate and proliferate the numbers of bacteria can increase substantially [2,29].

3.4. The true habitat of Bacillus species

If we assume that soil is the true habitat of *Bacillus,* then their presence in faeces is a direct consequence of the host having consumed food contaminated with soil. Our data reveals a basal level of endospores in the human GI tract of about 10^4 spores/g of faeces. For a healthy adult living on a Western diet they would be expected to have a mean daily stool weight of about 200 g [5] which, using our findings here, would contain in total approximately 2×10^6 endospores. To produce this a person would need, for example, to consume 2 litres of milk a day, or 20 kg of rice and cereals. While these are generalizations we doubt that the counts found in human faeces can be accounted for based solely on intake with food. A more reasonable explanation is that intake with food introduces endospores into the GI tract which then germinate and proliferate as part of their life cycle. Germination is a process designed to occur in the presence of nutrients and nowhere else is this more apparent than in the small intestine. If endospores are designed to survive within the GI tract we might ask what attributes they possess that facilitates this. One important finding is that *Bacillus* can grow and sporulate under anaerobic conditions [23,33], as well as molecular studies showing endospore germination, proliferation and re-sporulation [4,33]. The endospore itself, is encased in a protective coat of protein, the spore coat, whose natural protective role has surprisingly, until recently, been poorly understood. Work has now shown that the spore coat enables protection from immersion in gastric juices [7,31]. Interestingly, a role for the spore coat in avoiding phagocytic predation by the protozoan *Tetrahymena theromophila* has also been demonstrated [18]. Perhaps then, the endospore is designed to survive predation whether by simple microbes or large animals. Intriguingly, few studies have been made on the analysis of live bacillus in the soil environment and, as with faecal analysis, there are a number of logistic and technical reasons why this approach is problematic. Still, those studies that examined soil using fluorescent antibodies failed to convincingly prove the existence of vegetative *B. subtilis* in the soil other than an association with decaying plant matter [30]. Other studies have demonstrated that sporulation of *B. subtilis* cannot occur at temperatures below 15 °C [3]. For an organism purported to live in the soil, this result is difficult to explain, but rather supports the hypothesis that endospores, while found in the soil, have adapted to survive within the GI-tract of animals that ingest them. How these Gram-positive endospore formers have adapted to life within a host remains to be seen, yet *B. subtilis* is now being subject to microarray-based comparative genomics, revealing a remarkable diversity within this single species [8].

Acknowledgements

This work was supported by a grant (KBBE-2007-207948) from the EU 7th Framework to SMC and ER.

Appendix. Supplementary information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.resmic.2009.06.006.

References

[1] Angert, E.R., Losick, R.M. (1998) Propagation by sporulation in the guinea pig symbiont *Metabacterium polyspora*. Proc. Natl. Acad. Sci. U.S.A. 95, 10218–10223.
[2] Ankolekar, C., Rahmati, T., Labbe, R.G. (2009) Detection of toxigenic *Bacillus cereus* and *Bacillus thuringiensis* spores in U.S. rice. Int. J. Food Microbiol. 128, 460–466.
[3] Budde, I., Steil, L., Scharf, C., Volker, U., Bremer, E. (2006) Adaptation of *Bacillus subtilis* to growth at low temperature: a combined transcriptomic and proteomic appraisal. Microbiology 152, 831–853.
[4] Casula, G., Cutting, S.M. (2002) *Bacillus* probiotics: spore germination in the gastrointestinal tract. App. Env. Microbiol. 68, 2344–2352.
[5] Cummings, J.H., Bingham, S.A., Heaton, K.W., Eastwood, M.A. (1992) Fecal weight, colon cancer risk, and dietary intake of nonstarch polysaccharides (dietary fiber). Gastroenterology 103, 1783–91789.
[6] Duc, L.H., Fraser, P., Cutting, S.M. (2006) Carotenoids present in halotolerant *Bacillus* spore formers. FEMS Microbiol. Lett. 255, 215–224.
[7] Duc, L.H., Hong, H.A., Cutting, S.M. (2003) Germination of the spore in the gastrointestinal tract provides a novel route for heterologous antigen presentation. Vaccine 21, 4215–4224.
[8] Earl, A.M., Losick, R., Kolter, R. (2008) Ecology and genomics of *Bacillus subtilis*. Trends Microbiol. 16, 269–275.
[9] Fakhry, S., Sorrentini, I., Ricca, E., De Felice, M., Baccigalupi, L. (2008) Characterization of spore forming *Bacilli* isolated from the human gastrointestinal tract. J. Appl. Microbiol. 105, 2178–2186.
[10] Feinberg, L., Jorgensen, J., Haselton, A., Pitt, A., Rudner, R., Margulis, L. (1999) Arthromitus (*Bacillus cereus*) symbionts in the cockroach *Blaberus giganteus*: dietary influences on bacterial development and population density. Symbiosis 27, 109–123.
[11] Felske, A.D.M. (2004) Ecology of *Bacillus* species in soil. In E. Ricca, A.O. Henriques, & S.M. Cutting (Eds.), Horizon Bioscience (pp. 35–44). Waondham.

[12] Fritze, D. (2004) Taxonomy of the genus *Bacillus* and related genera: the aerobic endospore-forming bacteria. Phytopathology 94, 1245–1248.
[13] Hartemink, R., Domenech, V.R., Rombouts, F.M. (1997) LAMVAB-A new selective medium for the isolation of lactobacilli from faeces. J. Microbiol. Methods 29, 77–84.
[14] Hartemink, R., Rombouts, F.M. (1999) Comparison of media for the detection of bifidobacteria, lactobacilli and total anaerobes from faecal samples. J. Microbiol. Methods 36, 181–192.
[15] Hoa, N.T., Baccigalupi, L., Huxham, A., Smertenko, A., Van, P.H., Ammendola, S., Ricca, E., Cutting, S.M. (2000) Characterization of *Bacillus* species used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders. Appl. Environ. Microbiol. 66, 5241–5247.
[16] Hong, H.A., Duc, L.H., Cutting, S.M. (2005) The use of bacterial spore formers as probiotics. FEMS Microbiol. Rev. 29, 813–835.
[17] Hong, H.A., Khaneja, R., Tam, N.M., Cazzato, A., Tan, S., Urdaci, M., Brisson, A., Gasbarrini, A., Barnes, I., Cutting, S.M. (2009) *Bacillus subtilis* isolated from the human gastrointestinal tract. Res. Microbiol. 160, 134–143.
[18] Klobutcher, L.A., Ragkousi, K., Setlow, P. (2006) The *Bacillus subtilis* spore coat provides "eat resistance" during phagocytic predation by the protozoan *Tetrahymena thermophila*. Proc. Natl. Acad. Sci. U.S.A. 103, 165–170.
[19] Koransky, J.R., Allen, S.D., Dowell Jr., V.R. (1978) Use of ethanol for selective isolation of sporeforming microorganisms. Appl. Environ. Microbiol. 35, 762–765.
[20] Mitchell, C., Iyer, S., Skomurski, J.F., Vary, J.C. (1986) Red pigment in *Bacillus megaterium* spores. Appl. Environ. Microbiol. 52, 64–67.
[21] Moeller, R., Horneck, G., Facius, R., Stackebrandt, E. (2005) Role of pigmentation in protecting *Bacillus* sp. endospores against environmental UV radiation. FEMS Microbiol. Ecol. 51, 231–236.
[22] Moir, A. (2006) How do spores germinate? J. Appl. Microbiol. 101, 526–530.
[23] Nakano, M.M., Dailly, Y.P., Zuber, P., Clark, D.P. (1997) Characterization of anaerobic fermentative growth of *Bacillus subtilis*: identification of fermentation end products and genes required for growth. J. Bacteriol. 179, 6749–6755.
[24] Nicholson, W.J., Munakata, N., Horneck, G., Melosh, H.J., Setlow, P. (2000) Resistance of *Bacillus* endospores to extreme terrestial and extraterrestrial environments. Microbiol. Mol. Biol. Rev. 64, 548–572.
[25] Nicholson, W.L. (2002) Roles of *Bacillus* endospores in the environment. Cell. Mol. Life Sci. 59, 410–416.
[26] Nicholson, W.L., Setlow, P. (1990) Sporulation, germination and outgrowth. In C.R. Harwood, & S.M. Cutting (Eds.), Molecular Biological Methods for *Bacillus* (pp. 391–450). Chichester, UK: John Wiley & Sons Ltd.
[27] Notermans, S., Dufrenne, J., Teunis, P., Beumer, R., Te Giffel, M.C., Peeters Weem, P. (1997) A risk assessment study of *Bacillus cereus* present in pasteurized milk. Food Microbiol. 14, 143–151.
[28] Rhee, K.J., Sethupathi, P., Driks, A., Lanning, D.K., Knight, K.L. (2004) Role of commensal bacteria in development of gut-associated lymphoid tissues and preimmune antibody repertoire. J. Immunol. 172, 1118–1124.
[29] Rosenkvist, H., Hansen, A. (1995) Contamination profiles and characterisation of *Bacillus* species in wheat bread: raw materials for bread production. Int. J. Food Microbiol. 26, 353–363.
[30] Siala, A., Hill, I.R., Gray, T.R.G. (1974) Populations of spore-forming bacteria in an acid forest soil, with special reference to *Bacillus subtilis*. J. Gen. Microbiol. 81, 183–190.
[31] Spinosa, M.R., Braccini, T., Ricca, E., De Felice, M., Morelli, L., Pozzi, G., Oggioni, M.R. (2000) On the fate of ingested *Bacillus* spores. Res. Microbiol. 151, 361–368.
[32] Suresh, K., Prabagaran, S.R., Sengupta, S., Shivaji, S. (2004) *Bacillus indicus* sp. nov., an arsenic-resistant bacterium isolated from an aquifer in West Bengal, India. Int. J. Syst. Evol. Microbiol. 54, 1369–1375.
[33] Tam, N.M.K., Uyen, N.Q., Hong, H.A., Duc, L.H., Hoa, T.T., Serra, C.H., Henriques, A.O., Cutting, S.M. (2006) The intestinal life cycle of *Bacillus subtilis* close relatives. J. Bacteriol. 188, 2692–2700.
[34] Vissers, M.M., Te Giffel, M.C., Driehuis, F., De Jong, P., Lankveld, J.M. (2007) Minimizing the level of *Bacillus cereus* spores in farm tank milk. J. Dairy Sci. 90, 3286–3293.
[35] Yoon, J.H., Lee, C.H., Oh, T.K. (2005) *Bacillus cibi* sp. nov., isolated from jeotgal, a traditional Korean fermented seafood. Int. J. Syst. Evol. Microbiol. 55, 733–736.

Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Carotenoids found in *Bacillus*

R. Khaneja[1], L. Perez-Fons[2], S. Fakhry[3], L. Baccigalupi[3], S. Steiger[4], E. To[1], G. Sandmann[4], T.C. Dong[5], E. Ricca[3], P.D. Fraser[2] and S.M. Cutting[1]

1 School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey, UK
2 Centre for Systems and Synthetic Biology, Royal Holloway, University of London, Egham, Surrey, UK
3 Department of Structural and Functional Biology, Federico II University, Naples, Italy
4 J.W. Goethe Universität Frankfurt, Frankfurt, Germany
5 University of Medicine & Pharmacy, Ho Chi Minh City, Vietnam

Keywords
Bacillus, carotenoids, isoprenoids, spores.

Correspondence
Simon M. Cutting, School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey TW20 0EX, UK.
E-mail: s.cutting@rhul.ac.uk

2009/1179: received 30 June 2009, revised 10 September 2009 and accepted 2 October 2009

doi:10.1111/j.1365-2672.2009.04590.x

Abstract

Aims: To identify the diversity of pigmented aerobic spore formers found in the environment and to characterize the chemical nature of this pigmentation.
Materials and Results: Sampling of heat-resistant bacterial counts from soil, sea water and the human gastrointestinal tract. Phylogenetic profiling using analysis of 16S rRNA sequences to define species. Pigment profiling using high-performance liquid chromatography-photo diode array analysis.
Conclusions: The most commonly found pigments were yellow, orange and pink. Isolates were nearly always members of the *Bacillus* genus and in most cases were related with known species such as *Bacillus marisflavi*, *Bacillus indicus*, *Bacillus firmus*, *Bacillus altitudinis* and *Bacillus safensis*. Three types of carotenoids were found with absorption maxima at 455, 467 and 492 nm, corresponding to the visible colours yellow, orange and pink, respectively. Although the presence of other carotenoids cannot be ruled out, these three predominant carotenoids appear to account for the pigments obtained in most pigmented bacilli, and our analysis reveals the existence of a C30 biosynthetic pathway. Interestingly, we report the presence of a water-soluble pigment that may also be a carotenoid. The function of carotenoids is photoprotection, and carotenoid-containing spores exhibited significantly higher levels of resistance to UV radiation than non–carotenoid-containing *Bacillus* species.
Significance and Impact of the Study: This study demonstrates that pigmented bacilli are ubiquitous and contain new carotenoid biosynthetic pathways that may have industrial importance.

Introduction

Pigments are widespread in nature and are found in both eukaryotes and prokaryotes. In photosynthetic organisms, pigments mainly function in light harvesting, but they can also serve as photoprotective agents (Chew and Bryant 2007; Coesel *et al.* 2008). For others, pigmentation can help protect the cell from predation, for example, prodigiosin, the red pyrrole-containing pigment commonly found in *Serratia*, *Streptomyces* and *Vibrio* species has well-defined antibacterial properties (Perez-Tomas *et al.* 2003; Harris *et al.* 2004). Similarly, the purple violacein of *Chromobacterium* species is able to provide resistance to being consumed by predatory protozoa together with inherent antibiotic properties (Lopes *et al.* 2009). Photoprotective pigments include the melanins and carotenoids (Hullo *et al.* 2001; Moeller *et al.* 2005). Interestingly, the latter have also been shown to act as virulence factors in a number of pathogens, including *Staphylococcus aureus* (Liu *et al.* 2005) and *Mycobacterium* spp. (Gao *et al.* 2003; Provvedi *et al.* 2008). Here, the carotenoid helps protect bacteria attempting to survive within an intracellular environment, that is, following phagocytosis where they provide resistance to oxidation and neutrophil

attack (Liu *et al.* 2005). Presumably, carotenoids, that originally evolved to protect the cell from UV damage, have assumed an additional and more sophisticated role as part of a pathogenic life cycle.

Bacterial spores are well known for their robust resistance properties being able to withstand extremes of heat, desiccation, exposure to noxious chemicals and UV irradiation (Nicholson *et al.* 2000; Riesenman and Nicholson 2000). As ubiquitous, yet dormant, entities they are found in soils, water as well as the intestinal tracts of numerous insects and mammals (Nicholson 2002; Jensen *et al.* 2003; Fakhry *et al.* 2008; Hong *et al.* 2009). *Bacillus subtilis* spores carry a melanin-like compound in their coats that helps protects spores against solar radiation (i.e. UV-A and UV-B) (Riesenman and Nicholson 2000; Hullo *et al.* 2001). The natural pigmentation of *B. subtilis*-sporulating colonies is therefore brown, but other colours have been documented in spores. Examples include a red-pigmented *Bacillus megaterium* (Mitchell *et al.* 1986), a pink pigment in some isolates of *Bacillus firmus* (Pane *et al.* 1996) and red- and grey-pigmented *Bacillus atrophaeus* (Nakamura 1989; Fritze and Pukall 2001). A variable yellow-orange pigmentation has been found in a number of species including, *Bacillus indicus* (Suresh *et al.* 2004), *Bacillus cibi* (Yoon *et al.* 2005), *Bacillus vedderi* (Agnew *et al.* 1995), *Bacillus jeotgali* (Yoon *et al.* 2001a), *Bacillus okuhidensis* (Li *et al.* 2002), *Bacillus clarkii* (Nielsen *et al.* 1995), *Bacillus pseudofirmus* (Nielsen *et al.* 1995) and *B. firmus* (Ruger and Koploy 1980). With the exception of melanin, some of these pigments are probably carotenoids, and a number of spore-forming species have been shown to have carotenoids associated with either the vegetative cell or the spore (Mitchell *et al.* 1986; Moeller *et al.* 2005; Duc *et al.* 2006). Carotenoids are isoprenoids and thus originate from the five-carbon building block, isopentenyl pyrophosphate, which is the universal precursor of all isoprenoids. Supporting this, squalene has been identified in numerous Gram-positive bacteria including *Bacillus* (Amdur *et al.* 1978). Besides a chromophore (which is responsible for the colour), another structural feature is their long hydrocarbon chain that conveys a pronounced hydrophobic chemical nature. These lipophilic properties ensure that virtually all carotenoids are found in membranous structures.

Providing resistance to UV irradiation is a necessity of bacterial endospores, and generally, levels of resistance of 10–50 times higher than growing vegetative cells are common (Moeller *et al.* 2005). Resistance has generally been attributed to two unique features of the spore that are centred on protecting the germ-line DNA (Nicholson *et al.* 2000; Setlow 2001). First, a difference in the photochemistry of spore DNA caused by a conformational change in the DNA (referred to as the A-form) induced by the binding of small, acid-soluble proteins to spore DNA. Second, a relatively error-free mechanism for repairing UV damaged DNA. Membranes are also susceptible to UV damage where reactive oxygen species (generated by UV irradiation) may attack and interfere with the integrity of lipids leading to lipid peroxidation and/or inactivation of membrane-associated proteins (Moeller *et al.* 2005). Membrane damage would have catastrophic consequences to survival of the newly germinated spore, and so it is likely that spore formers have evolved mechanisms to protect themselves from long-term exposure to UV. As antioxidants carotenoids are capable of scavenging reactive oxygen species generated by UV irradiation. Located in the membranes, they protect against membrane damage rather than from protecting spore DNA. Supporting this, a red-orange pigment, thought to be a carotenoid, has been implicated in providing resistance to UV-A in a strain of *B. atrophaeus* (Moeller *et al.* 2005).

In this study, we have screened a number of different sample sites for the presence of pigmented, spore-forming bacteria. These were found in abundance and found to be species of either *Bacillus* or *Sporosarcina*. Although the visible colour of colonies varied, pigmentation was found to be because of the presence of one or more of three distinct carotenoids.

Materials and methods

General methods

Vegetative cell growth was made on Luria–Bertani (LB) solid or liquid medium unless otherwise indicated in the text. Sporulation was made in Difco sporulation medium (DSM) agar or liquid medium (Nicholson and Setlow 1990). For analysis of sporulation efficiencies, spores recovered from plate cultures (30°C, 3-day-old) were examined microscopically using a haemocytometer counting chamber or by determination of heat resistance (65°C, 45 min). Starch hydrolysis was determined as described elsewhere using agar plates containing 1% soluble starch (Cutting and Vander-Horn 1990). Haemolysis was evaluated by streaking onto tryptose blood agar containing sheep's blood at 5% and incubation for 24 h at 37°C. Motility was tested by the method of Hendrickson (1985) using growth in 0·4% agar and incubation for 2 days at 30°C. Resistance to arsenate and arsenite was determined as described by Suresh *et al.* (2004). Surfactin was measured by an oil-displacement method as described elsewhere (Hong *et al.* 2009). Tolerance to NaCl was determined by streaking strains on LB agar supplemented with NaCl at different concentrations (5, 8, 10 and 12%). For determination of anaerobic growth, strains were streaked on solid DSM agar plates containing potassium nitrate (at a concentration of 5 mmol l^{-1}) or potassium nitrite (at 2·5 mmol l^{-1}) as

electron acceptors (Nakano *et al.* 1997; Ye *et al.* 2000). Streaked plates with test and control strains were incubated in a Don Whitley anaerobic chamber, and growth at 30°C was monitored after 3 days. *Clostridium perfringens* isolate *fD00385* was included as a positive 'anaerobic' control and *Bacillus pumilus* SC2200 as a negative control.

Isolation of pigmented spore formers

Different sources were used for isolation of strains. For solid samples, the basic procedure consisted of homogenizing the sample in phosphate-buffered saline (PBS; pH 7·4), heating for 1 h at 65°C and then plating serial dilutions on DSM agar. The isolation of human samples has been described previously (Tam *et al.* 2006; Fakhry *et al.* 2008). Sea water was filtered (250 ml water/filter) using a 0·22-micron (Millipore, Milan, Italy) filter. Each filtrate was plated on top of a DSM plate and incubated at 25 or 30°C for 2–3 days. All colonies were collected in liquid DSM, heat treated (80°C for 10 min), plated on DSM and incubated at 25 or 30°C for 2–3 days. Colonies were purified, grown on DSM and checked for the presence of spores under the light microscope. Strains were prefixed HU to denote human origin, RK, from rice condiments and SF as from soil or sea water.

Reference strains

Reference strains used in this study were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) or the BGSC (*Bacillus* Genetic Stock Center, Columbus, OH, USA) culture collections.

16S rRNA analysis

To assign strains to bacterial species for each isolate, the entire 16S rRNA gene (*rrnE*) was amplified as described previously (Hoa *et al.* 2000). The 1400-bp amplification product was then sequenced and subjected to nucleotide database analysis using the Ribosomal Database Project II sequence database (http://rdp.cme.msu.edu/). Closest known species were recorded as percentages of identity. Sequences were aligned, and phylogenetic trees assembled using the ClustalW programme (http://align.genome.jp/).

Determination of UV resistance

Methods used were as described by Moeller *et al.* (2005), with some modifications. Briefly, spores in aqueous suspension (10^7 spores per ml) were exposed to UV-C radiation from a germicidal lamp (VL 50C; Vilber Lourmat, Marne-la-Vallee, France) with a major emission line at 254 nm. During irradiation, the spore suspensions were stirred continuously to ensure homogenous exposure. Immediately following UV radiation, at defined time points, 0·1 ml of the aqueous suspension was taken for evaluation of the viable count by serial dilution in PBS and plating on nutrient agar. The surviving fraction (Ln) was determined from the quotient N/N_0, where N is the number of CFU of the irradiated sample and N_0 is that of the nonirradiated controls. Survival curves were obtained by plotting the Ln of N/N_0 as a function of fluence (exposure time).

Hydrogen peroxide assays

Spore suspensions (1×10^8 spores per ml) were treated with H_2O_2 as described elsewhere (Riesenman and Nicholson 2000), and statistical analysis was carried out using the Student's *t*-test with a *P*-value of >0·05 that was considered nonsignificant.

Pigment extraction

For pigment analysis, isolates were grown on tryptone-yeast extract agar at 30°C. After 3–4 days of growth, colonies were scraped from the agar surface and centrifuged to remove residual media. The cell pellet was frozen and then lyophilized for 3 days. The resulting lyophilized material was stored at −20°C until extraction. To obtain a homogeneous powder for extraction, the freeze-dried cells were milled using a tissue lyser (Qiagen, Crawley, UK). Aliquots of this material were then taken for further analysis.

An aliquot of the powdered freeze-dried material (typically 20 mg) was suspended in 1 mol l^{-1} NaOH (500 μl) and sonicated at room temperature for 5 min. This treatment rendered the cellular material amenable to solvent extraction. The NaOH solution was completely removed by centrifugation to avoid possible saponification. The carotenoids were then extracted as previously described (Duc *et al.* 2006). To the digested cells, methanol (250 μl) and chloroform (500 μl) were added and mixed. Water (250 μl) was then added, and the suspension vortexed to create a phase separation. After centrifugation, the organic layer (lower phase) was collected, and the aqueous (upper) layer re-extracted twice with chloroform (or until no colour was observed in the debris). The organic extracts were pooled and reduced to complete dryness under a stream of nitrogen gas. The dried extracts were stored at this stage at −20°C under nitrogen.

Pigment analysis

Dried extracts were routinely dissolved in chloroform with the exception of the samples originating from

pink-coloured isolates that were dissolved in methanol. In this instance, methanol was necessary because of their lack of solubility in chloroform (or ethyl acetate). Prior to injection onto the high-performance liquid chromatography (HPLC) column, extracts were filtered through a polytetrafluoroethylene (PTFE) membrane (0·2 μm; Chromacol Ltd, Herts, UK) and centrifuged at 12 000 rev min^{-1} for 3 min. Carotenoids were separated and then detected online using a Waters Alliance (Milford, MA, USA) 2600S HPLC system with an online photo diode array (PDA) detector. Injections (20 μl) were made, and separations performed on a reverse phase (RP) C_{30} 5-μm column (250 × 4·6 mm i.d.) coupled to a 20 × 4·6 mm C_{30} guard column (YMC Inc., Wilmington, NC, USA) maintained at a constant temperature of 25°C as described previously (Fraser *et al.* 2000). The mobile phases used were methanol (solvent A), MeOH : H_2O (80 : 20) (v/v) containing 0·2% (w/v) ammonium acetate (solvent B) and *tert*-butyl methyl ether (solvent C). Carotenoids were eluted from the column with a gradient of 95% solvent A and 5% solvent B for 12 min, followed by a step to 80% solvent A, 5% solvent B and 15% solvent C at 12 min. Then, at 12 min, a linear gradient was initiated to reach 30% A, 5% B and 65% at 30 min. The column was returned to the initial conditions and equilibrated over 30 min. A flow rate of 1 ml min^{-1} was employed, and the eluate monitored continuously with an online PDA set to monitor between 200 and 600 nm. Identification was performed by the comparison of spectral and chromatographic characteristics associated with similar or authentic carotenoids. In cases where no authentic standards existed, comparison with reference compounds in the literature (Britton *et al.* 2003) were made. For quantification, dose–response curves for β-carotene (standard-coloured carotenoid) were prepared. Menaquinone was also identified by spectral comparison with authentic standards. All solvents were purchased from VWR (Poole, UK).

Detection of water-soluble pigments

Bacillus cells were grown on solid or in liquid media for 2–3 days and then harvested by centrifugation at 9000 ***g*** for 5 min. In the case of solid agar, material was first scraped from the surface with an inoculation loop, placed into a microcentrifuge tube and washed with dH_2O (1 ml). Pelleted cells were resuspended in dH_2O (1 ml) and then passed through a French pressure cell at 20 MPa. The homogenate was then centrifuged at 40 000 ***g*** for 20 min at 4°C. The resulting coloured supernatant was removed, and a UV/VIS spectral trace recorded from 250 to 600 nm (Beckman Coulter DU800 spectrophotometer, High Wycombe, UK).

Results

Biotypes of pigmented spore formers

Environmental samples were evaluated for the presence of heat-resistant bacteria by plating on a rich medium. Plates were incubated for a sufficient period of time to allow the visual identification of pigmented colonies. Samples examined were a fermented rice condiment ('Tuong Nep Dac Biet'), obtained from Vietnam, soil, sea water as well as samples from human faeces and human gut biopsies that had been previously described (Fakhry *et al.* 2008; Hong *et al.* 2009). Pigmented heat-resistant isolates could be identified with ease, and colours obtained were mostly yellow, orange, pink and red and subtle variations between these colours, e.g. yellow-orange, orange-red (Fig. 1 shows some examples). We found the colony could vary, somewhat, depending upon the medium used, for example, colours were often more vibrant on LB agar compared to DSM agar. Similarly, it was also observed that pigment formation in some strains would be very much dependant upon the growth temperature, for example, isolate SF214 would develop an orange pigment at 25°C but at 42°C white colonies were formed.

In an analysis of soil samples taken from 50 locations in the London area, the most abundant pigmented spore formers isolated were yellow colonies (26 out of the 50 sites tested), and in some samples up to 13% of the heat-resistant colonies were yellow. Dark grey colonies were found at eight sites, pink colonies at six sites and orange at three sites. These figures, while undoubtedly generalizations, demonstrate that pigmented spore formers can readily be found in soil. Another finding was that when soil samples were processed, following heat treatment, serial dilution and plating, the pigmentation was most obvious only at the highest serial dilutions. That is, on lower dilutions, where colonies were crowded, pigmentation was barely detectable, if at all, suggesting that pigmentation was subject to some form of nutritional or extracellular input.

The *rrnE* (16S rRNA) gene was sequenced in its entirety for the isolates selected for further analysis. Initial examination against type strains (Table 1) together with phylogenetic analysis (Fig. 1) revealed that many isolates were identical to known pigmented species including *Bacillus marisflavi* (Yoon *et al.* 2003), *Bacillus aquimaris* (Yoon *et al.* 2003), *B. firmus* (Pane *et al.* 1996), *Bacillus vietnamensis* (Noguchi *et al.* 2004), *B. cibi* (Yoon *et al.* 2005) and *B. indicus* (Suresh *et al.* 2004) as well as two isolates of *Sporsarcina* (Yoon *et al.* 2001b). Isolates of *Bacillus altitudinis* and *Bacillus safensis* were also found for which no pigmentation has previously been described. Other isolates appeared unrelated to known species (e.g.

Table 1 Pigmented *Bacillus* spore formers

Strain ID	Colony colour*	Source/reference	Closest match†
RKS159	Yellow	Fermented rice condiment, this work	*Bacillus cohnii* (0·998)
RKS162	Yellow	Fermented rice condiment, this work	*Bacillus horikoshii* (0·991)
RKS165	Yellow	Fermented rice condiment, this work	*B. horikoshii* (0·987)
RKS469	Yellow	Fermented rice condiment, this work	*Bacillus simplex* (0·963)
RKS470	Yellow	Fermented rice condiment, this work	*B. simplex* (0·976)
SF147	Yellow	Human faeces (Fakhry *et al.* 2008b)	*Bacillus safensis* (1·00)
SF188	Yellow	Human faeces (Fakhry *et al.* 2008b)	*B. safensis* (0·997)
SF222	Yellow	Sea water, this work	*Bacillus marisflavi* (1·00)
SF242	Yellow	Sea water, this work	*Bacillus cibi* (0·996)
SF225	Yellow	Soil, this work	*B. marisflavi* (0·998)
HU36	Yellow-orange	Human faeces (Duc *et al.* 2006)	*Bacillus indicus* (0·998)
HU13	Yellow-orange	Human faeces (Duc *et al.* 2006)	*B. cibi* (0·998)
HU16	Yellow-orange	Human faeces (Duc *et al.* 2006)	*B. indicus* (0·999)
HU19	Yellow-orange	Human faeces (Duc *et al.* 2006)	*B. cibi* (0·999)
HU28	Yellow-orange	Human faeces (Duc *et al.* 2006)	*B. indicus* (0·998)
HU33	Yellow	Human faeces (Duc *et al.* 2006)	*B. indicus* (0·998)
SF200A	Yellow-orange	Sea water, this work	*Bacillus altitudinis* (1·00)
SF208	Yellow-orange	Sea water, this work	*B. altitudinis* (1·00)
SF221	Yellow-orange	Sea water, this work	*B. altitudinis* (0·996)
SF204	Orange	Sea water, this work	*Bacillus aquimaris* (0·994)
SF223	Orange	Sea water, this work	*B. aquimaris* (0·993)
GL42	Orange	Shrimp pond (Vietnam)	*Bacillus vietnamensis* (0·992)
SF214	Orange-red	Sea water, this work	*Bacillus pumilus* (1·00)
SF237	Red	Soil, this work	*Sporosarcina aquimarina* (0·996)
RKS160	Pink	Fermented rice condiment, this work	*Bacillus firmus* (0·992)
HU29	Pink	Human faeces (Hong *et al.* 2009)	*B. firmus* (0·995)
GB9	Pink	Human ileum (Hong *et al.* 2009)	*B. firmus* (0·995)
RKS163	Pink	Fermented rice condiment, this work	*B. firmus* (0·983)
SF238	Pink	Soil, this work	*Sporo. aquimarina* (0·992)
SF241	Pink	Sea water, this work	*B. firmus* (0·988)
GB1	Deep pink	Human ileum (Hong *et al.* 2009)	*B. firmus* (0·987)
RKS161	Deep pink	Fermented rice condiment, this work	*B. firmus* (0·990)
RKS468	Deep pink	Fermented rice condiment, this work	*B. firmus* (0·981)
Type strains‡			
DSMZ 7264	Grey	Soil (Nakamura 1989)	*Bacillus atrophaeus*
BGSC 11A1	Yellow-orange	Soil	*B. atrophaeus*
DSMZ 675	Yellow orange§	Soil (Fritze and Pukall 2001)	*B. atrophaeus*
DSMZ 8715	Yellow	Soil and animal manure (Nielsen *et al.* 1995)	*Bacillus pseudofirmus*
DSMZ 9768	Yellow	Bauxite waste (Agnew *et al.* 1995)	*Bacillus vedderi*
DSMZ 18226	Yellow	Fermented seafood condiment (Yoon *et al.* 2001a)	*Bacillus jeotgali* YKJ-10
DSMZ 8720	Yellow	Soil (Nielsen *et al.* 1995)	*Bacillus clarkii*
DSMZ 13666	Yellow-brown	Hot spa (Li *et al.* 2002)	*Bacillus okuhidensis*
DSMZ 15820	Yellow-orange	Aquifier (Suresh *et al.* 2004)	*B. indicus* Sd/3
DSMZ 16189	Yellow-orange	Fermented seafood condiment (Yoon *et al.* 2005)	*B. cibi* JG-30

*As observed on Luria–Bertani or Difco sporulation medium agar.
†Using 16S rDNA sequence analysis in this work. The similarity score is shown in brackets. SeqMatch reports the per cent sequence identity over all pairwise comparable positions when run with aligned sequences. (Ribosomal Database Project II sequence database).
‡Obtained from the *Bacillus* Genetic Stock Center (BGSC) or Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) collections.
§Described as the 'red strain' in (Fritze and Pukall 2001; Moeller *et al.* 2005).

RKS162, RKS165, RKS469, RKS470, GB9 and possibly RKS468) and probably will define new ones (Satomi *et al.* 2006; Shivaji *et al.* 2006).

Selected isolates were chosen for further analysis (Table 1). This included the characterization of sporulation efficiencies (Table 2) and basic biotypes (Supporting information Table S1). The *Sporosarcina* isolates (SF237 and SF238) and most SF colonies tested were unable to grow anaerobically in marked contrast to *Bacillus* spp. that had been isolated from the human gut (Fakhry *et al.*

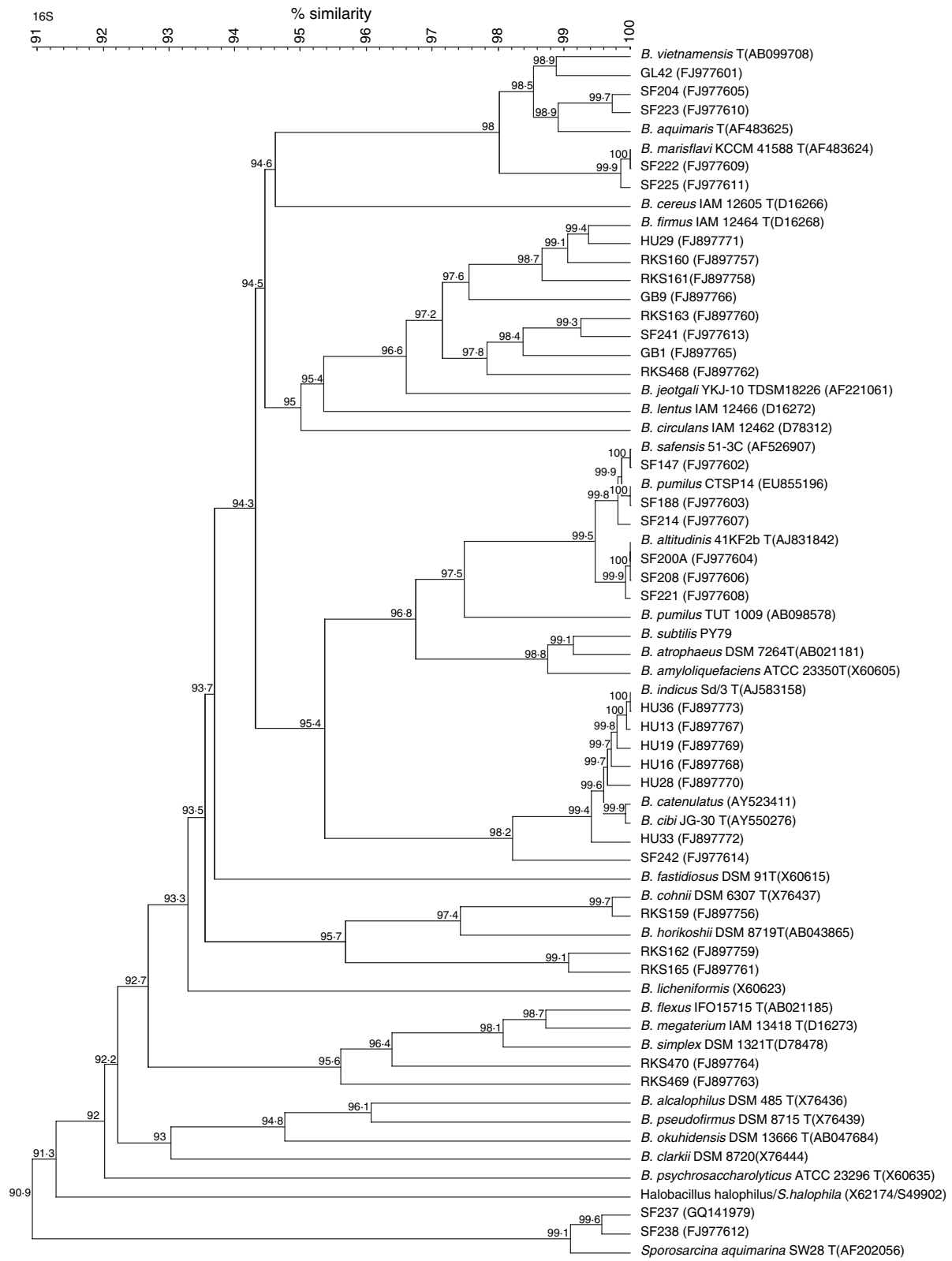


Figure 1 Phylogenetic relationship of pigmented spore formers. Dendograms of strains based on 16S rRNA (*rrnE*) sequence alignment using CLUSTALW ver. 1.83 (http://align.genome.jp/). GenBank accession numbers are shown in brackets.

Table 2 Sporulation efficiencies

Strain	Sporulation			Strain	Sporulation		
	%*	Anaerobic†	Shape‡		%*	Anaerobic†	Shape‡
RKS159	79	–	E, T	SF200A	68	–	E, C
RKS162	100	–	E, T	SF208	92	–	E, C
RKS165	116	–	E, C	SF221	100	–	E, C
RKS469	<1	++	E, T	SF204	60	–	E, ST
RKS470	92	++	E, T, Sw	SF223	58	–	E, T
SF147	97	–	E, C	SF241	47	–	E, C
SF188	96	–	E, C	GL42	25	–	E, T
SF222	10	–	E, ST	SF214	77	–	E, St
SF242	ND	–	E, St, Sw	SF237	<1	–	S, T, Sw
SF225	62	–	E, C	GB1	153§	–	E, C
HU36	67	–	E, St	RKS160	100	–	E, St
HU13	12	+	E, T	RKS161	100	–	E, T
HU16	29	–	E, T	HU29	100	+	E, C
HU19	37	–	E, T	GB9	128§	+	E, St
HU28	34	–	E, T	RKS163	166§	–	E, T
HU33	42	+	E, T	RKS468	87	–	E, T
Bacillus subtilis PY79	86	+	E, T/St	SF238	<1	–	S, T
Bacillus pseudofirmus DSMZ 8715	46	–	E, C/St	*Bacillus cibi* DSMZ 16189	16	–	E, C/St
Bacillus atrophaeus DSMZ 7264	91	+	E, C	*Bacillus jeotgali* DSMZ 18226	11	–	E, T, Sw
B. atrophaeus BGSC 11A1	83	+	E, St	*Bacillus clarkii* DSMZ 8720	31	–	E, St
B. atrophaeus DSMZ 675	106	+	E, C	*Bacillus okuhidensis* DSMZ 13666	10	–	E, T
				Bacillus indicus DSMZ 15820	32	–	E, St, Sw

*Percentage of heat-resistant (65°C, 45 min) spores after 3–4-day culture at 30°C on Difco sporulation medium agar.
†Anaerobic growth in presence of nitrate. ++ indicates strong growth.
‡E, ellipsoidal spore shape; S, spherical spore shape; T, terminal spore position; C, central spore position; St, sub central terminal position; Sw, swollen sporangium.
§Values >100% reflect heat-induced germination of spores, i.e. the unheated sample does not reflect the true count of spores in the crop because of the failure of spores to germinate unless heat-activated, brought about the heat-treatment itself.

2008; Hong *et al.* 2009). Sporulation was generally straightforward to induce growth on a rich, meat-derived medium with the exception of three isolates (SF238, SF237 and RKS469), and which may reflect a unique nutritional requirement for optimal sporulation.

Carotenoid profiling

The present study has focused on the screening of pigmented *Bacillus* strains isolated from diverse environments. These strains can be visually classified on the basis of colour, with three categories yellow, orange and pink predominating. The intensity of the colour varied depending on the strain (Table 3). To ascertain the nature of the pigments present, carotenoid analysis was performed. Direct extraction of the cellular pellet with organic solvent proved to be ineffective, however, freeze-drying the cells, grinding the material into a fine homogeneous powder followed by rapid treatment with NaOH (1 N) to assist the release of pigments into organic solvents such as chloroform. Extracts were then analysed by HPLC-PDA using a separation system capable of resolving both polar and nonpolar-like carotenoid molecules. Typical HPLC profiles recorded at 450 nm are illustrated in Fig. 2. Figure 2(a) is representative of all pink-coloured strains analysed (e.g. GB1), the predominant peak having a maxima of 492 nm (Table 3). Figure 2(b) shows a profile associated with the orange/yellow-pigmented strains (e.g. SF223) – in this instance, the major component peaks 2 and 3 had a maxima of 467 and 455 nm, respectively. Those strains with a yellow pigmentation (e.g. HU28) had the characteristic profile similar to that shown in Fig. 2(c) with peak 3 having a maximum of 455 nm (Table 3). The UV/VIS spectra of these compounds were characteristic of carotenoids, typically those of an acyclic nature. As found previously, the lack of available authentic standards precludes definitive identification. On the basis of reference spectra in the literature, the predominant *Bacillus* carotenoids were putatively identified to be acyclic carotenoids and potentially monocyclic, while other reports have stated the presence of astaxanthin (Pane *et al.* 1996); however, further investigations into the chromatographic components recorded at 286 nm in the *Bacillus* strains suggest the presence of C30

Table 3 Carotenoid content determined in different spore-forming isolates*

Strain identifier	Carotenoid production (low to high producers)	UV/VIS spectral characteristics (nm)†
Yellow		
Bacillus jeotgali	Low	Below LOI
Bacillus okuhidensis	Low	Below LOI
Bacillus clarkii	Low	Below LOI
RKS159	Low	Below LOI
SF200A	Low	P3-(400)‡, 429, **455**, 485
SF221	Low	P3-(400)‡, 429, **455**, 485
SF242	Medium	P3-(400)‡, 429, **455**, 485
SF225	Medium	P3-(400)‡, 429, **455**, 485
Bacillus pseudofirmus	Medium	P3-(400)‡, 429, **455**, 485
HU28	Medium	P3-(400)‡, 429, **455**, 485
SF208	Medium	P3-(400)‡, 429, **455**, 485
HU16	High	P3-(400)‡, 429, **455**, 485
HU19	High	P3-(400)‡, 429, **455**, 485
SF147	High	P3-(400)‡, 429, **455**, 485
SF188	High	P3-(400)‡, 429, **455**, 485
RKS469	High	P3-(400)‡, 429, **455**, 485
Orange		
SF208	Low	Below LOI
Bacillus atrophaeus DSMZ 675	Low	Below LOI
B. atrophaeus 11A1	Low	Below LOI
RKS470	Medium	P3-(400)‡, 429, **455**, 485 P2-440, **467**, 495
RKS162	Medium	P3-(400)‡, 429, **455**, 485 P2- 440, **467**, 495
RKS165	Medium	P3-(400)‡, 429, **455**, 485 P2-440, **467**, 495
SF222	Medium	P3-(400)‡, 429, **455**, 485 P2-440, **467**, 495
SF204	Medium	P3-(400)‡, 429, **455**, 485 P2-440, **467**, 495
SF223	Medium	P3-(400)‡, 429, **455**, 485 P2-440, **467**, 495
SF239	Medium	P3-(400)‡, 429, **455**, 485 P2-440, **467**, 495
Bacillus indicus Sd/3	High	P3-(400)‡, 429, **455**, 485 P2-440, **467**, 495
Bacillus cibi JG-30	High	P3-(400)‡, 429, **455**, 485 P2-440, **467**, 495
HU33	High	P3-(400)‡, 429, **455**, 485 P2-440, **467**, 495
HU36	High	P3-(400)‡, 429, **455**, 485 P2-440, **467**, 495
HU13	High	P3-(400)‡, 429, **455**, 485 P2-440, **467**, 495
Pink		
RKS160	Low	Below LOI
HU29	Low	Below LOI
GB9	Low	Below LOI
RKS163	Low	P1-(434)‡, 463, **492**, 524
GL42	Low	P1-(434)‡, 463, **492**, 524
SF237§	Low	Water soluble P2 related
GB1	Medium	P1-(434)‡, 463, **492**, 524
RKS161	Medium	P1-(434)‡, 463, **492**, 524
RKS468	Medium	P1-(434)‡, 463, **492**, 524
SF241	Medium–high	P1-(434)‡, 463, **492**, 524
SF214§	High	Water soluble P2 related
Dark grey		
B. atrophaeus DSMZ 7264	ND	ND
SF116	ND	ND
SF120	ND	ND
SF120A	ND	ND

*UV/Vis spectral characteristics are provided for the predominant carotenoid observed from the characteristic high-performance liquid chromatography-photo diode array profiles. Peak 1–Peak 3 represent chromatographic peaks and can be cross-referenced with Fig. 2. LOI, level of identification.

†Main peaks in the spectra with the λ_{max} of the predominant peak is underlined.

‡Brackets indicate shoulder on the UV/VIS spectra.

§Poor extraction with organic solvent, but water-soluble pigment observed when extracted with a French press. ND-carotenoids not detected, or below the limits of detection using the experimental procedure described. Below LOI indicates that the carotenoid is present but below the level of identification typically 0·01 μg (g DW)$^{-1}$. Low carotenoid producers represents levels in the range of 50–150 μg (g DW)$^{-1}$, medium carotenoid producers means 150–200 μg (g DW)$^{-1}$ and high producers 200–300 μg (g DW)$^{-1}$. DW, dry weight.

apophytoene (Fig. 3). For example in Fig. 3(a), the component with the UV/VIS spectra characteristic of phytoene has a retention time of 13 min (Peak-4). This component shows co-chromatography with diapophytoene generated by transgenic *Escherichia coli*. For further comparison and for further clarification, the phytoene (C40) present in a carotenoid extract from ripe tomato elutes with a retention time of 21 min (indicated by Peak-6 in Fig. 3b). Thus, the identical spectra but earlier retention time suggests that diapophytoene exists in these *Bacillus* strains and that a C30 pathway exists. This evidence is supported by the presence of C30 apocarotenoids in other closely related bacteria such as *Staph. aureus*. Several of the strains isolated displayed visually coloured colonies (Table 1), but attributing this colour to the presence of carotenoid was not possible under the experimental conditions applied. This is because the detection limits prevented definitive carotenoid-like UV/VIS spectra being acquired.

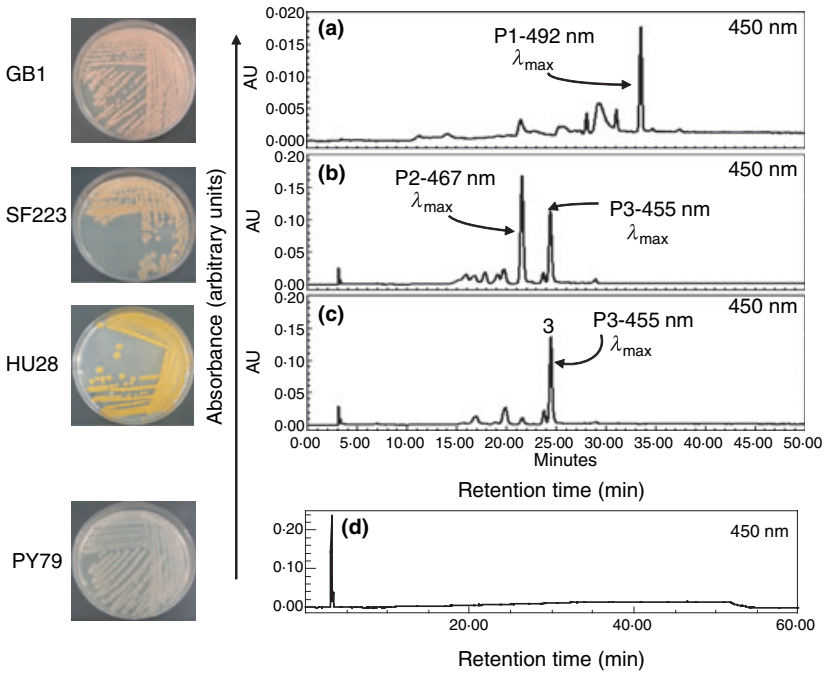


Figure 2 Characteristic high-performance liquid chromatography-photo diode array (HPLC-PDA) profiles recorded at 450 nm. Representative cultures of the red (GB1), orange (SF223), yellow (HU28) strains along with a colourless control (PY97) strain. These cultures, shown on the left of the figure, were grown at 37°C (2 days) on Luria–Bertani agar. Typical HPLC-PDA profiles are shown in the panels on the right. Panel a, pink-coloured *Bacillus* strains, Panel b, orange-pigmented strains and Panel c, yellow-coloured strains. P1 (P = peak) represents the carotenoid unique to pink strains with an absorption λ_{max} at 492 nm. P2 has a spectral λ_{max} at 467 nm, while P3 has a λ_{max} at 455 nm. Panel d illustrates the absence of carotenoids in the control strain.

Detection of water-soluble pigments

Intense visual pigmentation of cells was observed in several *Bacillus* isolates (SF214 and SF237); however, it was not possible to release the pigments responsible into organic solvents from both cellular suspensions and homogenates treated with organic solvent (Table 3). The solvents tested included methanol, ethyl acetate, hexane, chloroform and hot (40°C) acetone individually and in combination. Following cell breakage of aqueous cellular suspensions using a French pressure cell, the resulting supernatant extracts were intensely coloured. A UV/VIS spectral scan (250–600 nm) of these aqueous extracts revealed the presence of a pronounced peak occurring at 410 nm. This peak was specific for SF214 and SF237. Nevertheless, it may be from a compound that masks the

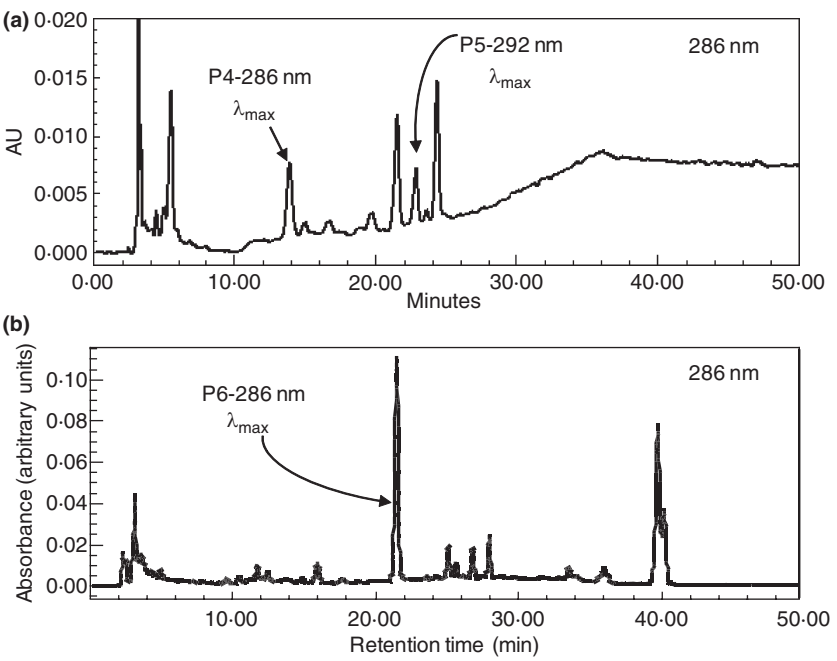


Figure 3 High-performance liquid chromatography-photo diode array profiles of organic extracts recorded at 286 nm. Panel a shows representative extracts prepared from orange/yellow pigmented strains. Peak (P) 4 has been identified as diapophytoene and P5 menaquinone. A diapophytoene reference compound co-chromatographs at 13 min. Panel b shows the retention time of C40 phytoene (P-6) from ripe tomato for comparison.

carotenoid spectrum. According to the colour of the extract, maxima in the range between 450 and 500 nm should be expected.

Pigment-conferred resistance properties

We determined the resistance of purified suspensions of selected pigmented spores to monochromatic UV-C (254 nm) (Fig. 4). UV-C radiation is not entirely representative of the natural environmental (solar) radiation, which is primarily UV-A and UV-B, but it is the most energetic form of UV radiation (Riesenman and Nicholson 2000). All eight pigmented isolates, HU36, *B. indicus* Sd/3, GB1, DSMZ 675, DSMZ 7264, BGSC 11A, SF241 and SF214 showed no significant reduction in viability after 120 s of UV-C exposure. This was in striking contrast to *B. subtilis* PY79 spores that showed a clear sensitivity to UV-C. Not all pigmented spores proved resistant to UV-C; however, both GL42 and GB9 showed sensitivity. In data not shown, we found that in vegetative cells of the same strains no resistance to UV-C was observed demonstrating that the protective role of the carotenoid was probably of importance only in the dormant state.

Bacterial spores have resistance to hydrogen peroxide, and in *B. subtilis* this has been attributed to the CotA laccase that has been shown to be present in the spore coat (Riesenman and Nicholson 2000; Hullo *et al.* 2001). Carotenoids, as antioxidants, could also provide a protective role in inactivating H_2O_2 as has been found for the pathogen *Staph. aureus* that is exposed to reactive oxygen species within a phagocyte (Liu *et al.* 2005). We examined resistance of spores of selected isolates to 5% H_2O_2 (Fig. 5a). We found that with the exception of GB1 spores, the LD_{90} (lethal dose required to kill 90% of bacteria) was equivalent in all strains and was not significantly different ($P < 0{\cdot}05$). The LD_{90} values we obtained were substantially (25 times) lower than those reported in previous work (Riesenman and Nicholson 2000), even though we used the same methodology. Our data, though, showed that resistance was the same between the pigmented spores and *B. subtilis* PY79,

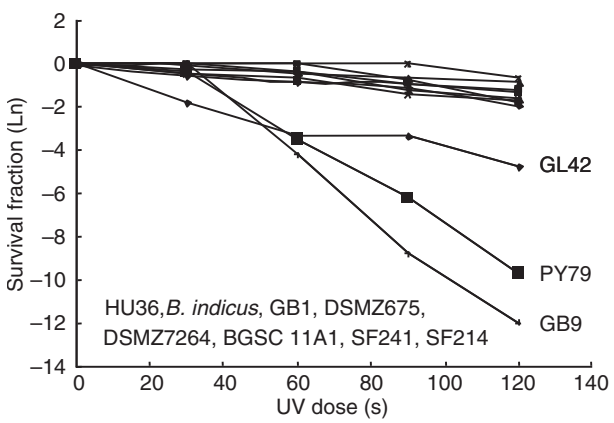


Figure 4 UV-C resistance. Suspensions of purified spores were irradiated with UV-C (254 nm) using a germicidal lamp. The surviving fraction (Ln) was determined as a function of time.

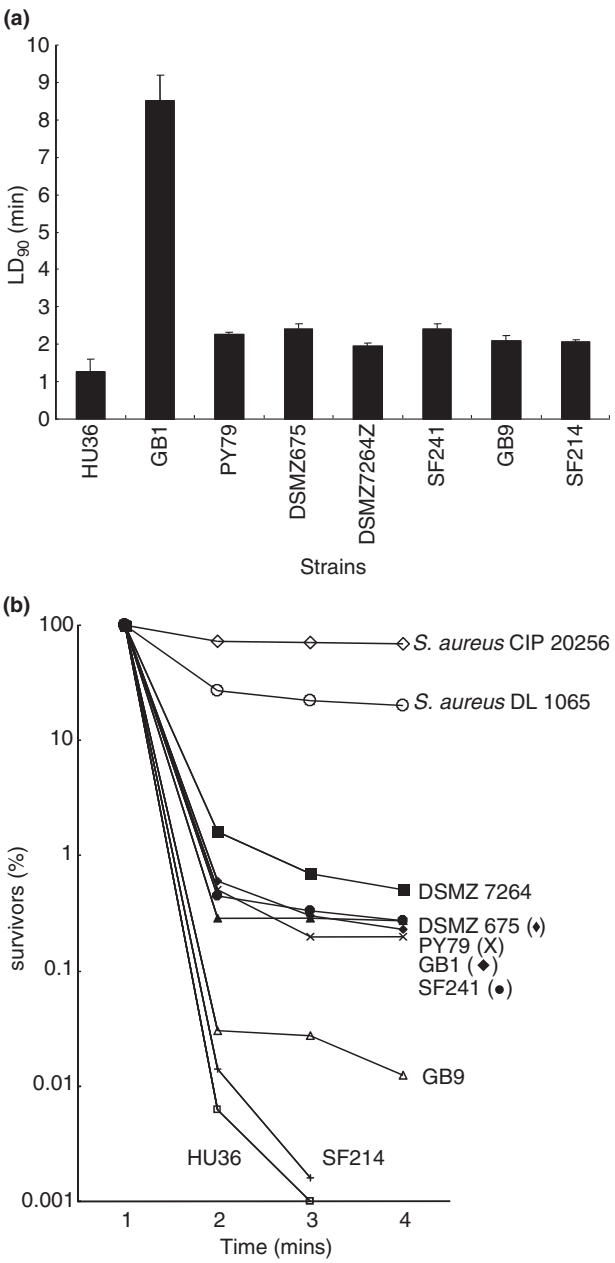


Figure 5 Resistance to hydrogen peroxide. Suspensions of purified spores (Panel a) or exponentially growing cells (Panel b) were assayed for resistance to 5% (spores) or 1·5% (vegetative cells) hydrogen peroxide. The experiment was repeated two times.

implying that the carotenoid content cannot contribute to hydrogen peroxide resistance. Vegetative cells were, however, extremely sensitive to hydrogen peroxide (Fig. 5b). To demonstrate data shown in Fig. 5(b) shows the kinetics of cell killing. Pigmented strains were killed rapidly and showed no greater resistance than PY79 vegetative cells. As a comparison, we also, in parallel, examined two yellow-pigmented strains of *Staph. aureus* that has been shown elsewhere and confirmed here to have resistance to hydrogen peroxide enabling this organism to survive within a phagocyte.

Discussion

As mentioned in the introduction to this article, a relatively small number of publications have reported the identification and characterization of pigmented bacilli. Of these, Duc *et al.* (2006) provide one of the first detailed reports of pigmented marine *Bacillus* species. We have extended this work to examine different habitats for pigmented spore formers and then determine the nature of the pigments. At the outset, we emphasize that this study is not exhaustive, nor was this the intention; rather, to show the diversity and the chemical basis for spore pigmentation. Our study has revealed that for the most part, the abundance of coloured bacilli has probably gone unnoticed. We attribute this to the technicalities of identifying coloured colonies, where plating out bacteria at low dilution masks the true abundance of pigmented species. Only at high dilutions do the pigments become apparent, and in some cases one in ten colonies were found to be pigmented. Another contributing factor is the medium and temperature used to culture bacilli where we have found that significant variation in the colony colouration can result. The variation in colour suggests that environmental or nutritional factors could be important. In a previous study of a strain of *B. indicus* (HU36), we demonstrated that the pigment was because of one or more carotenoids (Duc *et al.* 2006). For this reason, we made the assumption that the pigments found in the bacilli isolated in this study would also be carotenoids. To demonstrate the presence of carotenoids, the following criteria have been used: (i) extraction into organic solvents, (ii) separation of carotenoid components on chromatographic systems used routinely for carotenoids, (iii) the presence of the diapophytoene, which is the first unique intermediate of the carotenoid pathway and (iv) characteristic UV/VIS spectral properties of the enriched carotenoid preparations. Further systematic analysis is now required to unequivocally identify these carotenoids present in *Bacillus* using combinations of hyphenated mass spectroscopy and possibly nuclear magnetic resonance approaches. One of the limitations of analysis presently encountered is the poor extraction recoveries for the carotenoids, which is likely to be associated with the chemical nature of the pigments. Therefore, to date, we cannot rule out the presence of further carotenoids in these *Bacillus* isolates or that carotenoids are not the sole pigments responsible for the colour present in these *Bacillus* strains isolated. The physical characteristics of the carotenoids detected in this study are however in good agreement with the visualized colour of the colonies. For example, the carotenoid exhibiting maximum at 455 nm predominates in isolates classified as being yellow, while orange strains contain the carotenoid with the maximum at 467 nm and the pink strains possess a carotenoid with a 492-nm maximum. Thus, the light energy absorbed by these carotenoids matches the visual colour of the strains. This finding suggests that carotenoids are the sole pigments responsible for the colour and in cases where coloured colonies could be visualized but no carotenoids detected, the poor extraction properties are responsible for the lack of detection. Carotenoids exist in most *Bacillus* strains. However, only the presence of the pink carotenoids with the main absorbance maximum at 492 nm seems to be species specific, to the *B. firmus*-related isolates.

In the case of the water-soluble pigments, i.e. pigments that could not be partitioned into organic solvents but were retained in the aqueous phase, our inability to partition pigments into organic solvents, to resolve them by HPLC separations and obtain online characteristic carotenoid UV/VIS spectra, precludes assignment of carotenoids as the pigments responsible for colouration of these *Bacillus* isolates (e.g. SF214 and SF237). It is possible that the spectral peak at 410 nm determined in aqueous extracts is representative of a water-soluble carotenoid, carotenoprotein (Cremades *et al.* 2001) or another type of pigment absorbing light in the visible region. Interestingly, in a study of red pigment found in *B. atrophaeus* DSMZ 675 (Moeller *et al.* 2005), two absorption peaks of 377 and 398 nm were observed, yet in our analysis, we could not repeat this finding. Further experimentation is required to ascertain the true nature of these pigments, though. It is of note that such water-soluble pigments have potential utility in the industrial sector as colourants for both foods and other valuable commodities.

Why then are *Bacillus* spores pigmented – most likely, is protection against UV radiation? This is consistent with the likely fate of a dormant spore where it is exposed to excessive levels of solar radiation in the environment. This is particularly true of marine bacteria and may explain why pigmented isolates can so readily be recovered from aquatic environments and fermented seafood products (Yoon *et al.* 2001a, 2005; Noguchi *et al.* 2004). We have shown here that pigmented isolates can also be

recovered from soil as well as the gastrointestinal tract (GI)-tract. In the case of the GI-tract, pigmented isolates may occur simply because of diet.

In other work, we have developed the hypothesis that spore formers may actually carry out their life cycle of vegetative growth within the intestinal tracts of animals that ingest them (Fakhry *et al.* 2008b; Hong *et al.* 2009). Excreted in the faeces, spores would remain in a state of dormancy for indefinite periods of time in the environment, and so pigmentation would help shield spores from the harmful effects of radiation. Interestingly, there was no obvious correlation between UV-C resistance and carotenoid content. For example, HU36 carried high levels of carotenoids and was fully resistant to UV-C, but this was also the case for SF214, DSMZ 675 or DSMZ 7264, the latter containing no extractable carotenoids. In contrast, GB9 was highly susceptible to UV-C radiation. Similarly, conflicting reports have been reported in other studies, for example, the red carotenoid pigment of DSMZ 675 spores appears important in protecting against UV-A but not against UV-B or UV-C (Moeller *et al.* 2005). Other studies with the coat-associated melanin of *B. subtilis* spores have shown that in the absence of the spore outer coat, resistance to UV-C was actually increased (Riesenman and Nicholson 2000). It is clear then that individual carotenoids may have evolved differently with regard to their protective role and that this may also differ significantly between species. We also found that the carotenoid content of spores appeared not to serve any role in protection against hydrogen peroxide, or at least there were no differences with melanin-containing *B. subtilis* PY79 spores. This property has been assigned to the CotA laccase found in the spore coat of *B. subtilis*, and it is possible that the pigmented spores also contained a melanin-like compound in their coats, which is visibly suppressed by the carotenoid pigmentation (Hullo *et al.* 2001). However, GB1 spores did show a noticeable 4-fold higher level of resistance than spores of the other strains, so we cannot rule out the possibility that this is because of the spore carotenoid content. Until nonpigmented mutations can be genetically engineered, attempting to establish a link between resistance and carotenoid content will remain a subjective issue. As has been summarized elsewhere, a number of other enzymes present in the spore coat could also provide resistance to reactive oxygen species including melanins, oxalate decarboxylase and a managanese-dependent superoxide dismutase (Henriques and Moran 2007).

Our studies are now focused on identifying the compounds and their biosynthetic pathways, and with this aim the genomes of GB1 and HU36 are currently being sequenced. The carotenoids are formed from the isoprenoid biosynthetic pathways and as such are normally found in membranes (Amdur *et al.* 1978; Daum *et al.* 2009). Our work has shown that pigmentation can vary dependent upon growth conditions (nutrition, temperature) as well as cell density, and in other work we have shown that for *B. indicus* HU36, the yellow colouration of vegetative cells changes to an orange pigmentation as cells sporulate (Duc *et al.* 2006). This suggests that developmental signals may affect the biosynthetic pathways. Deciphering the carotenoid biosynthetic pathways will therefore prove a complex task, yet there are a number of incentives for attempting this. First and foremost is the ability to metabolically engineer bacteria to synthesize high levels of endogenous isoprenoids. If this can be achieved, these bacteria could be included amongst the cohort of metabolically engineered bacteria now under development as second- and third-generation biofuels (Klein-Marcuschamer *et al.* 2007).

Acknowledgements

This work was supported by a grant (KBBE-2007-207948) from the EU 7th Framework to S.M. Cutting, P.D. Fraser, G. Sandmann, T.C. Dong and E. Ricca.

References

Agnew, M.D., Koval, S.F. and Jarrell, K.F. (1995) Isolation and characterisation of novel alkaliphiles from bauxite-processing waste and description of *Bacillus vedderi* sp. nov., a new obligate alkaliphile. *Syst Appl Microbiol* **18**, 221–230.

Amdur, B.H., Szabo, E.I. and Socransky, S.S. (1978) Presence of squalene in Gram-positive bacteria. *J Bacteriol* **135**, 161–163.

Britton, G., Liaaen-Jensen, S. and Pfander, H. ed. (2003) *Carotenoids, Handbook*. Basel, Switzerland: Birkhauser Verlag.

Chew, A.G. and Bryant, D.A. (2007) Chlorophyll biosynthesis in bacteria: the origins of structural and functional diversity. *Annu Rev Microbiol* **61**, 113–129.

Coesel, S., Obornik, M., Varela, J., Falciatore, A. and Bowler, C. (2008) Evolutionary origins and functions of the carotenoid biosynthetic pathway in marine diatoms. *PLoS ONE* **3**, e2896.

Cremades, O., Ponce, E., Corpas, R., Gutierrez, J.F., Jover, M., Alvarez-Ossorio, M.C., Parrado, J. and Bautista, J. (2001) Processing of crawfish (*Procambarus clarkii*) for the preparation of carotenoproteins and chitin. *J Agric Food Chem* **49**, 5468–5472.

Cutting, S.M. and Vander-Horn, P.B. (1990) Genetic analysis. In *Molecular Biological Methods for Bacillus* ed. Harwood, C.R. and Cutting, S.M. pp. 27–74. Chichester, England: John Wiley & Sons Ltd.

Daum, M., Herrmann, S., Wilkinson, B. and Bechthold, A. (2009) Genes and enzymes involved in bacterial isoprenoid biosynthesis. *Curr Opin Chem Biol* **13**, 1–9.

Duc, L.H., Fraser, P. and Cutting, S.M. (2006) Carotenoids present in halotolerant *Bacillus* spore formers. *FEMS Microbiol Lett* **255**, 215–224.

Fakhry, S., Sorrentini, I., Ricca, E., De Felice, M. and Baccigalupi, L. (2008) Characterization of spore forming *Bacilli* isolated from the human gastrointestinal tract. *J Appl Microbiol* **105**, 2178–2186.

Fraser, P.D., Pinto, M.E., Holloway, D.E. and Bramley, P.M. (2000) Technical advance: application of high-performance liquid chromatography with photodiode array detection to the metabolic profiling of plant isoprenoids. *Plant J* **24**, 551–558.

Fritze, D. and Pukall, R. (2001) Reclassification of bioindicator strains *Bacillus subtilis* DSM 675 and *Bacillus subtilis* DSM 2277 as *Bacillus atrophaeus*. *Int J Syst Evol Microbiol* **51**, 35–37.

Gao, L.Y., Groger, R., Cox, J.S., Beverley, S.M., Lawson, E.H. and Brown, E.J. (2003) Transposon mutagenesis of *Mycobacterium marinum* identifies a locus linking pigmentation and intracellular survival. *Infect Immun* **71**, 922–929.

Harris, A.K., Williamson, N.R., Slater, H., Cox, A., Abbasi, S., Foulds, I., Simonsen, H.T., Leeper, F.J. *et al.* (2004) The *Serratia* gene cluster encoding biosynthesis of the red antibiotic, prodigiosin, shows species- and strain-dependent genome context variation. *Microbiology* **150**, 3547–3560.

Hendrickson, D.A. (1985) Reagents and stains. In *Manual of Clinical Microbiology* ed. Lennette, E.H., Balows, A., Hausler, W.J. and Shadomy, H.J. pp. 1093–1107. Washington, D.C: ASM Press.

Henriques, A.O. and Moran, C.P. Jr (2007) Structure, assembly, and function of the spore surface layers. *Annu Rev Microbiol* **61**, 555–588.

Hoa, N.T., Baccigalupi, L., Huxham, A., Smertenko, A., Van, P.H., Ammendola, S., Ricca, E. and Cutting, S.M. (2000) Characterization of *Bacillus* species used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders. *Appl Environ Microbiol* **66**, 5241–5247.

Hong, H.A., Khaneja, R., Tam, N.M., Cazzato, A., Tan, S., Urdaci, M., Brisson, A., Gasbarrini, A. *et al.* (2009) *Bacillus subtilis* isolated from the human gastrointestinal tract. *Res Microbiol* **160**, 134–143.

Hullo, M.F., Moszer, I., Danchin, A. and Martin-Verstraete, I. (2001) CotA of *Bacillus subtilis* is a copper-dependent laccase. *J Bacteriol* **183**, 5426–5430.

Jensen, G.B., Hansen, B.M., Eilenberg, J. and Mahillon, J. (2003) The hidden lifestyles of *Bacillus cereus* and relatives. *Environ Microbiol* **5**, 631–640.

Klein-Marcuschamer, D., Ajikumar, P.K. and Stephanopoulos, G. (2007) Engineering microbial cell factories for biosynthesis of isoprenoid molecules: beyond lycopene. *Trends Biotechnol* **25**, 417–424.

Li, Z., Kawamura, Y., Shida, O., Yamagata, S., Deguchi, T. and Ezaki, T. (2002) *Bacillus okuhidensis* sp. nov., isolated from the Okuhida spa area of Japan. *Int J Syst Evol Microbiol* **52**, 1205–1209.

Liu, G.Y., Essex, A., Buchanan, J.T., Datta, V., Hoffman, H.M., Bastian, J.F., Fierer, J. and Nizet, V. (2005) *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. *J Exp Med* **202**, 209–215.

Lopes, S.C., Blanco, Y.C., Justo, G.Z., Nogueira, P.A., Rodrigues, F.L., Goelnitz, U., Wunderlich, G., Facchini, G. *et al.* (2009) Violacein extracted from *Chromobacterium violaceum* inhibits *Plasmodium* growth in *vitro* and in *vivo*. *Antimicrob Agents Chemother* **53**, 2149–2152.

Mitchell, C., Iyer, S., Skomurski, J.F. and Vary, J.C. (1986) Red pigment in *Bacillus megaterium* spores. *Appl Environ Microbiol* **52**, 64–67.

Moeller, R., Horneck, G., Facius, R. and Stackebrandt, E. (2005) Role of pigmentation in protecting *Bacillus* sp. endospores against environmental UV radiation. *FEMS Microbiol Ecol* **51**, 231–236.

Nakamura, L.K. (1989) Taxonomic relationship of black-pigmented *Bacillus subtilis* strains and a proposal for *Bacillus atrophaeus* sp. nov. *Int J Syst Bacteriol* **39**, 295–300.

Nakano, M.M., Dailly, Y.P., Zuber, P. and Clark, D.P. (1997) Characterization of anaerobic fermentative growth of *Bacillus subtilis*: identification of fermentation end products and genes required for growth. *J Bacteriol* **179**, 6749–6755.

Nicholson, W.L. (2002) Roles of *Bacillus* endospores in the environment. *CMLS, Cell Mol Life Sci* **59**, 410–416.

Nicholson, W.L. and Setlow, P. (1990) Sporulation, germination and outgrowth. In *Molecular Biological Methods for Bacillus* ed. Harwood., C.R. and Cutting., S.M. pp. 391–450. Chichester, UK: John Wiley & Sons Ltd.

Nicholson, W.J., Munakata, N., Horneck, G., Melosh, H.J. and Setlow, P. (2000) Resistance of *Bacillus* endospores to extreme terrestial and extraterrestrial environments. *Microbiol Mol Biol Rev* **64**, 548–572.

Nielsen, P., Fritze, D. and Priest, F.G. (1995) Phenetic diversity of alkaliphilic *Bacillus* strains: proposal for nine new species. *Microbiology* **141**, 1745–1761.

Noguchi, H., Uchino, M., Shida, O., Takano, K., Nakamura, L.K. and Komagata, K. (2004) *Bacillus vietnamensis* sp. nov., a moderately halotolerant, aerobic, endospore-forming bacterium isolated from Vietnamese fish sauce. *Int J Syst Evol Microbiol* **54**, 2117–2120.

Pane, L., Radin, L., Franconi, G. and Carli, A. (1996) The carotenoid pigments of a marine *Bacillus firmus* strain. *Boll Soc Ital Biol Sper* **72**, 303–308.

Perez-Tomas, R., Montaner, B., Llagostera, E. and Soto-Cerrato, V. (2003) The prodigiosins, proapoptotic drugs with anticancer properties. *Biochem Pharmacol* **66**, 1447–1452.

Provvedi, R., Kocincova, D., Dona, V., Euphrasie, D., Daffe, M., Etienne, G., Manganelli, R. and Reyrat, J.M. (2008) SigF controls carotenoid pigment production and affects

transformation efficiency and hydrogen peroxide sensitivity in *Mycobacterium smegmatis*. *J Bacteriol* **190**, 7859–7863.

Riesenman, P.J. and Nicholson, W.L. (2000) Role of the spore coat layers in *Bacillus subtilis* spore resistance to hydrogen peroxide, artificial UV-C, UV-B, and solar UV radiation. *Appl Environ Microbiol* **66**, 620–626.

Ruger, H.-J. and Koploy, J.A.C. (1980) DNA base composition of halophilic and nonhalophilkic *Bacillus firmus* strains of marine origin. *Microb Ecol* **6**, 141–146.

Satomi, M., La Duc, M.T. and Venkateswaran, K. (2006) *Bacillus safensis* sp. nov., isolated from spacecraft and assembly-facility surfaces. *Int J Syst Evol Microbiol* **56**, 1735–1740.

Setlow, P. (2001) Resistance of spores of *Bacillus* species to ultraviolet light. *Environ Mol Mutagen* **38**, 97–104.

Shivaji, S., Chaturvedi, P., Suresh, K., Reddy, G.S., Dutt, C.B., Wainwright, M., Narlikar, J.V. and Bhargava, P.M. (2006) *Bacillus aerius* sp. nov., *Bacillus aerophilus* sp. nov., *Bacillus stratosphericus* sp. nov. and *Bacillus altitudinis* sp. nov., isolated from cryogenic tubes used for collecting air samples from high altitudes. *Int J Syst Evol Microbiol* **56**, 1465–1473.

Suresh, K., Prabagaran, S.R., Sengupta, S. and Shivaji, S. (2004) *Bacillus indicus* sp. nov., an arsenic-resistant bacterium isolated from an aquifer in West Bengal, India. *Int J Syst Evol Microbiol* **54**, 1369–1375.

Tam, N.M.K., Uyen, N.Q., Hong, H.A., Duc, L.H., Hoa, T.T., Serra, C.H., Henriques, A.O. and Cutting, S.M. (2006) The intestinal life cycle of *Bacillus subtilis* and close relatives. *J Bacteriol* **188**, 2692–2700.

Ye, R.W., Tao, W., Bedzyk, L., Young, T., Chen, M. and Li, L. (2000) Global gene expression profiles of *Bacillus subtilis* grown under anaerobic conditions. *J Bacteriol* **182**, 4458–4465.

Yoon, J.H., Kang, S.S., Lee, K.C., Kho, Y.H., Choi, S.H., Kang, K.H. and Park, Y.H. (2001a) *Bacillus jeotgali* sp. nov., isolated from jeotgal, Korean traditional fermented seafood. *Int J Syst Evol Microbiol* **51**, 1087–1092.

Yoon, J.H., Lee, K.C., Weiss, N., Kho, Y.H., Kang, K.H. and Park, Y.H. (2001b) *Sporosarcina aquimarina* sp. nov., a bacterium isolated from seawater in Korea, and transfer of *Bacillus globisporus* (Larkin and Stokes 1967), *Bacillus psychrophilus* (Nakamura 1984) and *Bacillus pasteurii* (Chester 1898) to the genus *Sporosarcina* as *Sporosarcina globispora* comb. nov., *Sporosarcina psychrophila* comb. nov. and *Sporosarcina pasteurii* comb. nov., and emended description of the species. *Int J Syst Evol Microbiol* **51**, 1079–1086.

Yoon, J.H., Kim, I.G., Kang, K.H., Oh, T.K. and Park, Y.H. (2003) *Bacillus marisflavi* sp. nov. and *Bacillus aquimaris* sp. nov., isolated from sea water of a tidal flat of the Yellow Sea in Korea. *Int J Syst Evol Microbiol* **53**, 1297–1303.

Yoon, J.H., Lee, C.H. and Oh, T.K. (2005) *Bacillus cibi* sp. nov., isolated from jeotgal, a traditional Korean fermented seafood. *Int J Syst Evol Microbiol* **55**, 733–736.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Biotypes

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.