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ORIGINAL ARTICLE

Carotenoids found in *Bacillus*

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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.*

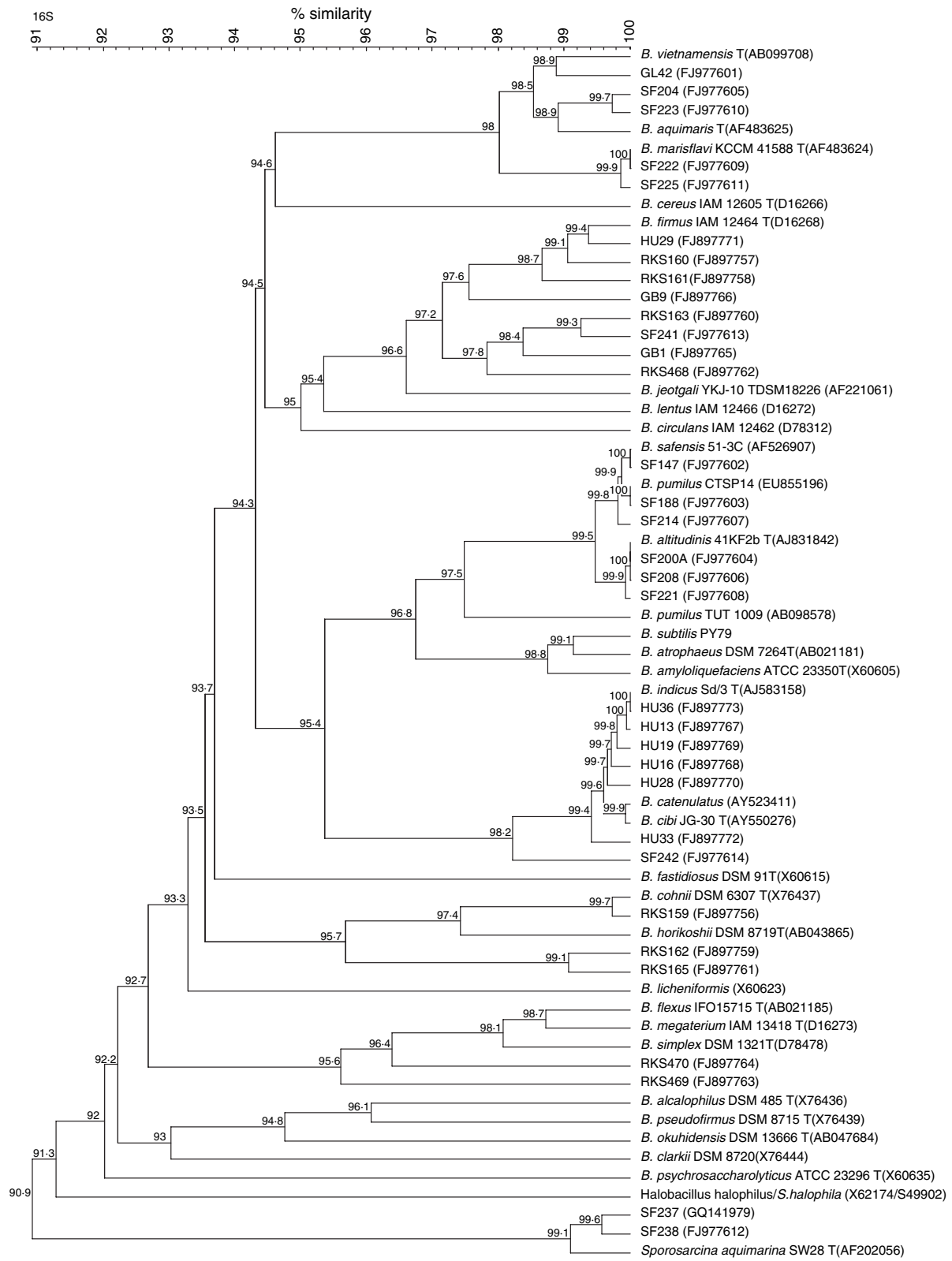

Figure 1 Phylogenetic relationship of pigmented spore formers. Dendograms of strains based on 16S rRNA (*rrnE*) sequence alignment using CLUSTALW ver. 1.83 (http://align.genome.jp/). GenBank accession numbers are shown in brackets.

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.

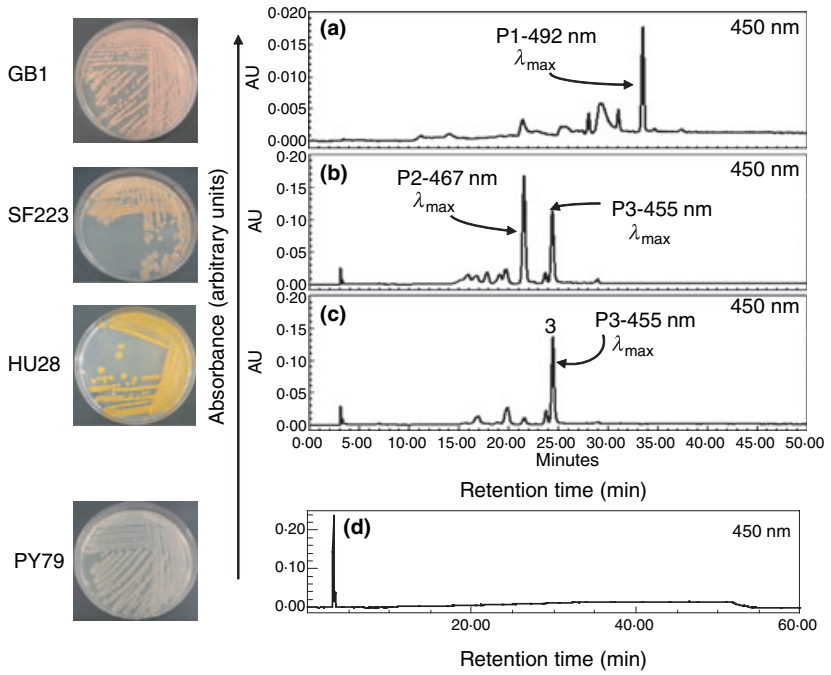

Figure 2 Characteristic high-performance liquid chromatography-photo diode array (HPLC-PDA) profiles recorded at 450 nm. Representative cultures of the red (GB1), orange (SF223), yellow (HU28) strains along with a colourless control (PY97) strain. These cultures, shown on the left of the figure, were 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

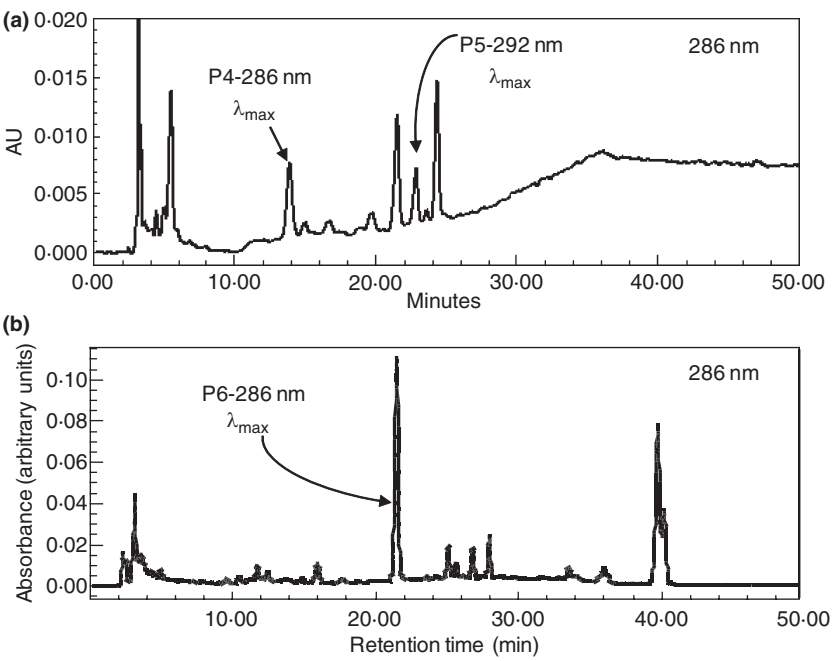

Figure 3 High-performance liquid chromatography-photo diode array profiles of organic extracts recorded at 286 nm. Panel a shows representative extracts prepared from orange/yellow pigmented strains. Peak (P) 4 has been identified as diapophytoene and P5 menaquinone. A diapophytoene reference compound 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,

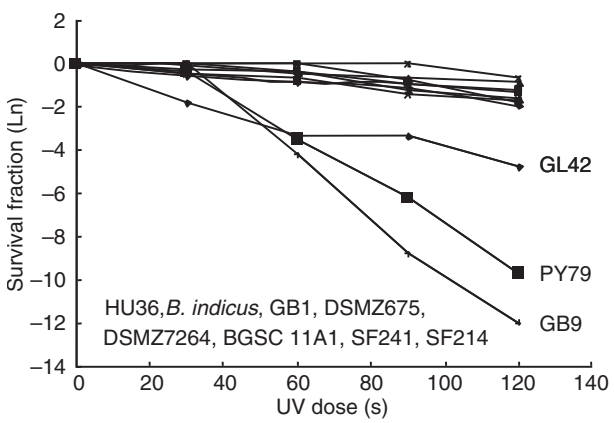

Figure 4 UV-C resistance. Suspensions of purified spores were irradiated with UV-C (254 nm) using a germicidal lamp. The surviving fraction (Ln) was determined as a function of time.

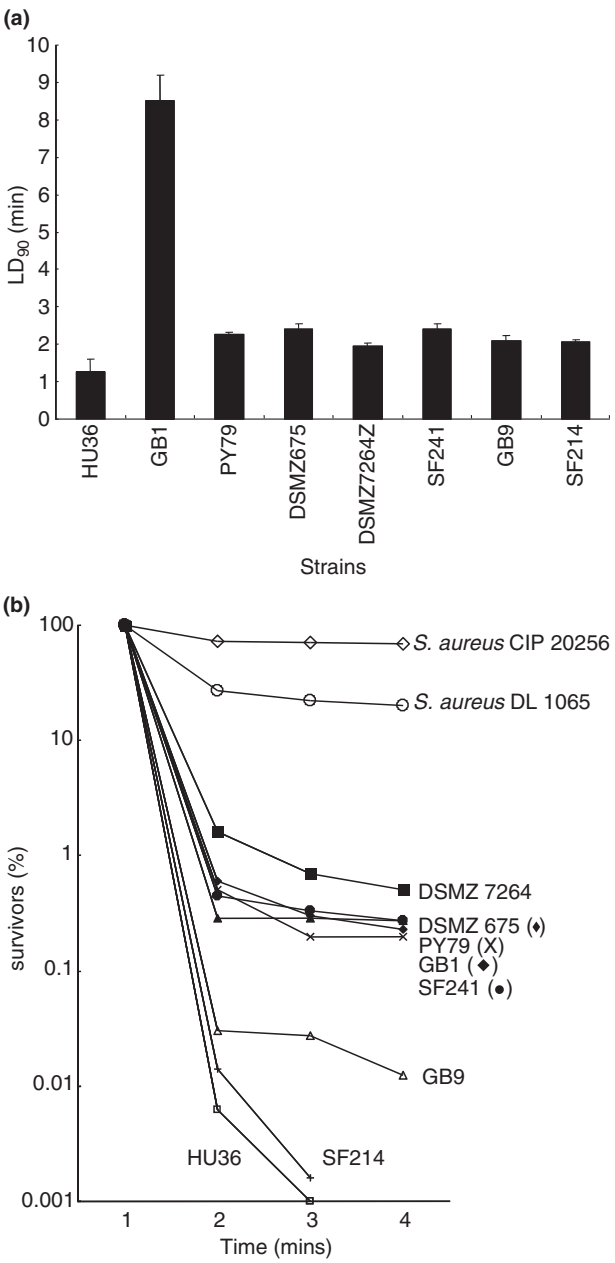

Figure 5 Resistance to hydrogen peroxide. Suspensions of purified spores (Panel a) or exponentially growing cells (Panel b) were assayed for resistance to 5% (spores) or 1·5% (vegetative cells) hydrogen peroxide. The experiment was repeated two times.

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).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Biotypes

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