

# Carotenoids present in halotolerant *Bacillus* spore formers

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## Keywords

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## Abstract

Six isolates of pigmented spore-forming bacteria were recovered from human faeces from subjects in Vietnam. 16S rRNA analysis demonstrated close association with known pigmented *Bacillus* species. All isolates were able to tolerate growth on 8% NaCl and were resistant to arsenate, characteristics that make them most related to *Bacillus indicus*. Two visible pigments were apparent, a yellow pigment found in vegetative cells and an orange pigment found only in spores. We used high-performance liquid chromatography to characterize and quantify these pigments and found them to be carotenoids. The biosynthetic pathway that generates them branches with one that could lead to the spore-associated orange pigmentation. Although these bacteria were found in faeces, the seafood-rich diet of Vietnam and the recovery of other pigmented *Bacillus* species from seafood and marine environments makes it highly probable that the true origin of these bacteria is from ingested seafood.

## Introduction

Carotenoids are the most widespread group of naturally occurring pigments. These yellow, orange and red coloured molecules are found in both eukaryotes and prokaryotes. At least 600 structurally different compounds are now known, with an estimated yield of 100 million tonnes per annum (Harborne, 1991; Britton *et al.*, 2003). One of the principal functions of carotenoids within the cell is to provide protection against photooxidative damage by quenching singlet oxygen as well as other harmful radicals that are formed when cells are illuminated (Demmig-Adams & 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 in mammals can prevent the onset of chronic disease states (Giovannucci, 2002; Mares-Perlman *et al.*, 2002). These health-promoting properties have led 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, and food and feed industries as precursors, colourants and supplements. The global market is expand-

ing and in 2005 has been estimated at \$935 million (Lee & Schmidt-Dannert, 2002). Total chemical synthesis is the method of choice used to produce carotenoids industrially. The disadvantages of this approach include the production of *stero* isomers not found in the natural product, 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 & 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 & 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 metabolic 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 C<sub>40</sub> carotene precursor. Following a number of desaturation reactions phytoene is converted, sequentially, to phytofluene,  $\zeta$ -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 catalysing 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 *M. 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).

Carotenoids then, are high-value fine chemicals with attractive biotechnological properties. Biological 'natural' sources of carotenoid production are becoming commercially attractive. In this paper, as part of a study of the microflora of the human gastrointestinal tract we have characterized six isolates of yellow–orange pigmented *Bacillus* recovered from freshly voided human faeces from volunteers in Vietnam. We have characterized the carotenoid content of these isolates qualitatively and quantitatively, showing that the yellow carotenoid pigment is produced during vegetative cell growth and the orange carotenoid pigment is associated with spores.

## Materials and methods

### Isolation of pigmented spore formers from faeces

Samples of freshly voided faecal material were collected from volunteers, diluted (1:10) in phosphate-buffered saline (PBS) and resuspended by vigorous vortexing until a

homogenous suspension was obtained. Next, to recover heat-resistant spores, 1 mL of the suspension was heated at 65 °C for 1 h and serial dilutions made in PBS before plating on Difco sporulation medium (DSM) agar and incubation for 2 days at 37 °C. For each sample *c.* 50 colonies were chosen at random and checked by phase-contrast microscopy for the presence of spores.

### General methods

Vegetative cell growth was made on Luria Bertani (LB) agar and sporulation on DSM agar (Nicholson & Setlow, 1990). To prepare large quantities of spores free from vegetative cells sporulation was made in DSM liquid medium using the exhaustion method as outlined elsewhere (Nicholson & Setlow, 1990). In this method sporulation was allowed to proceed for 24 h at 37 °C before removal of contaminating vegetative cells by lysozyme treatment. Vegetative cells were prepared by growth of bacteria in LB medium (37 °C) until cultures reached an OD<sub>600 nm</sub> of approximately 2.0. Resistance to arsenate and arsenite was determined as described (Suresh *et al.*, 2004). Determinations of tolerance to NaCl was made on LB agar containing NaCl at different concentrations (5%, 8%, 10% and 12%). Sporulation efficiency was determined by growth and sporulation on DSM agar (3 days at 37 °C) followed by measurement of heat-resistant (65 °C 1 h) CFU mL<sup>-1</sup> vs. unheated CFU mL<sup>-1</sup>. A nonpigmented spore former, *Bacillus subtilis* strain, PY79 (Youngman *et al.*, 1984), was used as a control. Anaerobic growth was determined using sealed containers and the Oxoid Gas-Pak system (Oxoid, UK).

### 16rRNA analysis

To assign strains to bacterial species for each isolate the almost entire 16S rRNA gene (*rrnE*) was amplified as described previously (Hoa *et al.*, 2000). The 1400 bp amplicon was then sequenced and subjected to nucleotide databases using the NCBI web-based BLAST programme (<http://www.ncbi.nlm.nih.gov/BLAST/>). Closest known species were recorded with percentages of identity. Sequences were aligned and phylogenetic trees were drawn using CLUSTALW programme (<http://align.genome.jp/>).

### Carotenoid extraction and analysis

Bacterial biomass was lyophilized to complete dryness (3 days). The lyophilized material was ground into a homogenous powder using a mortar and pestle. Typically carotenoids and other isoprenoids were extracted from 30 mg of ground material using chloroform (Fraser *et al.*, 2000). In brief, methanol (250  $\mu$ L) was added to the dried powder and mixed, then 500  $\mu$ L of chloroform (Analar) added. The suspension was incubated on ice for 20 min to minimize

degradation of carotenoids. To the suspension water (250  $\mu$ L) was added and vortexed (10 s). In order to form a partition, the suspension was centrifuged for 3 min at 12 000 g. The organic hypophase (lower phase) was removed and the aqueous hyperphase (upper phase) re-extracted twice. The organic extracts were pooled and reduced to complete dryness under a stream of nitrogen gas. The dried extracts can be stored at this stage at  $-20^{\circ}\text{C}$  under nitrogen.

The component carotenoids were subsequently separated and analysed using Waters Alliance (Milford, MA) 2600S high-performance liquid chromatography (HPLC) with on-line PDA detection following the procedure described in Fraser *et al.* (2000). The dried extracts were re-dissolved in ethyl acetate (HiperSolv, VWR, Wickford, UK) 50  $\mu$ L and then centrifuged for 3 min at 12 000 g to remove any particulate material. Separation of isoprenoids was performed using a RP C<sub>30</sub> 5  $\mu$ m column (250  $\times$  4.6 mm) coupled to a 20  $\times$  4.6 mm C<sub>30</sub> guard column (YMC Inc., Wilmington, NC) operating at a constant temperature of 25  $^{\circ}\text{C}$ . Carotenoids were eluted from the column with a gradient of 95% (A) – methanol, 5% (B) – 20% aqueous methanol containing 0.2% [weight in volume (w/v)] ammonium acetate for 12 min, a step to 80% A, 5% B and 15% (C) – *tert*-butyl methyl ether 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 1 mL min<sup>-1</sup> was employed and the eluate monitored continuously with a diode array detector between 200 and 600 nm. Identification was performed on the basis of co-chromatography and spectral comparison with authentic standards. Where authentic standards were not available correlation to reference spectral characteristics were carried out and relative polarities deduced from chromatographic behaviour. For quantification, dose–response curves for  $\beta$ -carotene (standard coloured carotenoid) were prepared. Ubiquinone was also identified by co-chromatography and spectral comparison with authentic standards and dose–response curves prepared for quantification. All solvents were purchased from VWR (Poole, UK).

## Results

### Isolation and characterization of pigmented spore formers

Heat-resistant spores present in freshly voided human faeces were isolated as described in Methods. On average, spore counts found in faeces were in the range of 10<sup>4</sup> CFU g<sup>-1</sup>. Using this approach six yellow–orange pigmented colonies were readily discernable on sporulation agar plates. These isolates were labelled, HU13, HU16, HU19, HU28, HU33 and HU36. Basic characteristics are shown in Table 1. All

produced ellipsoidal spores within swollen sporangia. These isolates were further distinguished by being nonmotile, able to hydrolyse starch (amylase positive) and failing to grow anaerobically. Strains of at least one yellow pigmented *Bacillus* species, *B. indicus*, is arsenic resistant (Suresh *et al.*, 2004) so we tested for tolerance to both arsenate and arsenite and found all six isolates were able to tolerate up to 20 mM arsenate but not to arsenite. All six isolates were able to grow in up to 8% NaCl. Three isolates, HU28, HU33 and HU36 exhibited poor sporulation efficiencies using the exhaustion method for sporulation in DSM medium.

### Pigmentation

Colonies were isolated on their ability to produce pigmented colonies. When grown on LB agar colonies initially were yellow after overnight incubation at 37  $^{\circ}\text{C}$ . As incubation was continued colonies gradually assumed an orange hue. By contrast, sporulation on DSM agar plates produced colonies that were orange. Figure 1 shows the appearance of colonies grown on LB or DSM agar. To determine whether the orange colour was specific to spore formation we made cultures of spores grown by exhaustion in DSM medium and ensured that there was no residual vegetative cells using an established protocol of treatment with lysozyme followed by extensive washing. Similarly, cultures of vegetative cells were made using incubation in LB medium until the culture reached an OD<sub>600</sub> of 2.0. Cultures prepared in this way would be free of any spores, which was checked both microscopically and by determination of heat-resistant CFU. In both cases, spores and vegetative cells were lyophilized and as shown in Fig. 1 the difference in pigment was clearly distinguished following desiccation with vegetative cells being yellow and spores orange.

### Phylogenetic analysis

To determine the relatedness of strains at the genetic level the entire 16S rRNA gene (*rrnE*) was sequenced from each isolate. Neighbour-joining trees are shown in Fig. 2. All were closely related to *Bacillus catenulatus*, *Bacillus indicus* and *Bacillus cibi* (similarity greater than 99%). *Bacillus indicus* and *B. cibi* all form yellow–orange pigmented colonies (Suresh *et al.*, 2004; Yoon *et al.*, 2005).

### Carotenoids

All six yellow–orange pigmented isolates were grown in LB medium and screened to reveal the presence of coloured carotenoid pigment. The pigmentation was released from the freeze-dried cells upon the addition of chloroform but not methanol. Therefore the pigment was hydrophobic in nature akin to the physical properties of carotenoids. Crude

**Table 1.** Phenotypic characterization of pigmented strains

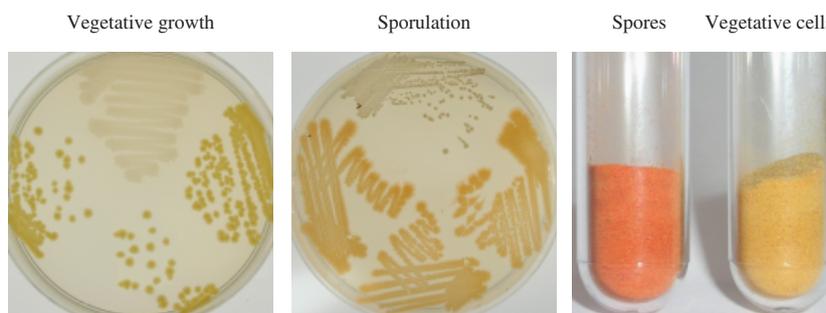
	<i>Bacillus</i> species*									
	1	2	3	4	5	6	7	8	9	10
Colour	YO	YO	YO	YO	YW	Y	YO	CrY	YW	Y
Spore position	S	S	S	S	T	T	C/S	ND	S	C/S
Swollen sporangium	+	+	+	+	+	–	+	+	+	–
Starch hydrolysis	+	+	+	+	–	+	+	+	–	+
Growth at										
10 °C	+	±	±	ND	ND	–	+	+	–	+
15 °C	+	+	+	+	ND	–	+	+	+	+
20 °C	+	+	+	+	ND	–	+	+	+	+
40 °C	+	+	+	–	+	+	+	+	+	+
45 °C	+	–	–	–	+	+	+	+	+	+
50 °C	–	–	–	–	+	+	–	±	–	–
Anaerobic	–	–	–	–	+	ND	–	+	ND	ND
Growth in presence of										
Arsenate Na <sub>2</sub> HAsO <sub>4</sub>	+	+	+	+	ND	ND	ND	ND	ND	ND
5 mM	+	+	+	+	ND	ND	ND	ND	ND	ND
9 mM	+	+	+	+	ND	ND	ND	ND	ND	ND
20 mM										
Arsenite As <sub>2</sub> O <sub>3</sub>	–	–	–	+	ND	ND	ND	ND	ND	ND
1 mM	–	–	–	+	ND	ND	ND	ND	ND	ND
3 mM										
Maximum NaCl concentration <sup>†</sup>	8%	8%	8%	2%	8%	10%	12%	13–14%	16–17%	16–17%
Sporulation efficiency <sup>‡</sup>	60.8%	1.1%	3.5%	ND	ND	ND	ND	ND	ND	ND
Motile	–	–	–	–	+	+	+	+	ND	ND

\**Bacillus* species. 1, HU13, HU16, HU19. 2, HU28. 3, HU33, HU36. 4, *Bacillus indicus* sp. nov. Sd/3<sup>T</sup> (Suresh et al., 2004). 5, *Bacillus vedderi* sp. nov. DSM 9768<sup>T</sup> (Agnew et al., 1995). 6, *Bacillus okuhidensis* sp. nov. JCM 10945<sup>T</sup> (Li et al., 2002). 7, *Bacillus cibi* sp. nov. JG-30<sup>T</sup> (Yoon et al., 2005). 8, *Bacillus jeotgali* sp. nov. YKJ-10<sup>T</sup> (Yoon et al., 2001). 9, *Bacillus clarkii* sp. nov. DSM 8720<sup>T</sup> (Nielsen et al., 1995). 10, *Bacillus pseudofirmus* sp. nov. DSM 8715<sup>T</sup> (Nielsen et al., 1995).

<sup>†</sup>Maximum concentration of salt (w/v) in which growth occurred.

<sup>‡</sup>Sporulation determined on DSM agar plates after 3 days at 37 °C.

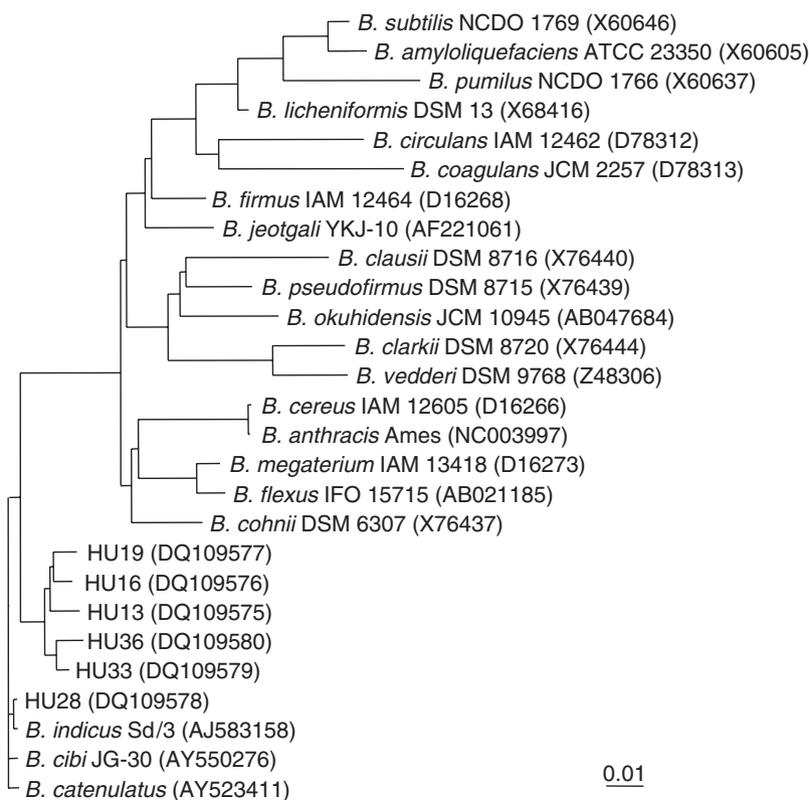
ND, no data; ±, weak; Cr, cream; O, orange; W, white; Y, yellow; C, central; S, subterminal; T, terminal; DSM, Difco sporulation medium.



**Fig. 1.** 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 cells ( $6.8 \times 10^9$  CFU g<sup>-1</sup>) or spores ( $1.7 \times 10^{10}$  cfu g<sup>-1</sup>) of HU19.

organic extracts were screened by HPLC–PDA without pre-fractionation using an unbiased HPLC separation that facilitates separation and identification of both polar and nonpolar carotenoids. The profiles recorded at 250–600 nm of all isolates were found to be similar (data not shown). The predominant peaks at 450 nm showed characteristic signa-

ture carotenoid spectra. Those isolates containing the highest level of pigment (e.g. HU19 and HU36; Table 2) were subjected to further detailed analysis. Accordingly, pure cultures of either vegetative cells or spores as described above were prepared together with *B. subtilis* strain PY79 that served as a nonpigmented control *Bacillus* species.



**Fig. 2.** Phylogenetic relationship of isolates. Dendrogram of the six pigmented isolates is based on 16S rRNA (*rrnE*) sequence alignment by CLUSTALW version 1.83 (<http://align.genome.jp/>). Bar is 0.01 nucleotide substitutions per site. Also shown are other related species with strain designations. Numbers in brackets are GenBank accession numbers. The *rrnE* sequences have been deposited to GenBank with accession numbers indicated.

**Table 2.** Preliminary screening of yellow–orange pigmented isolates

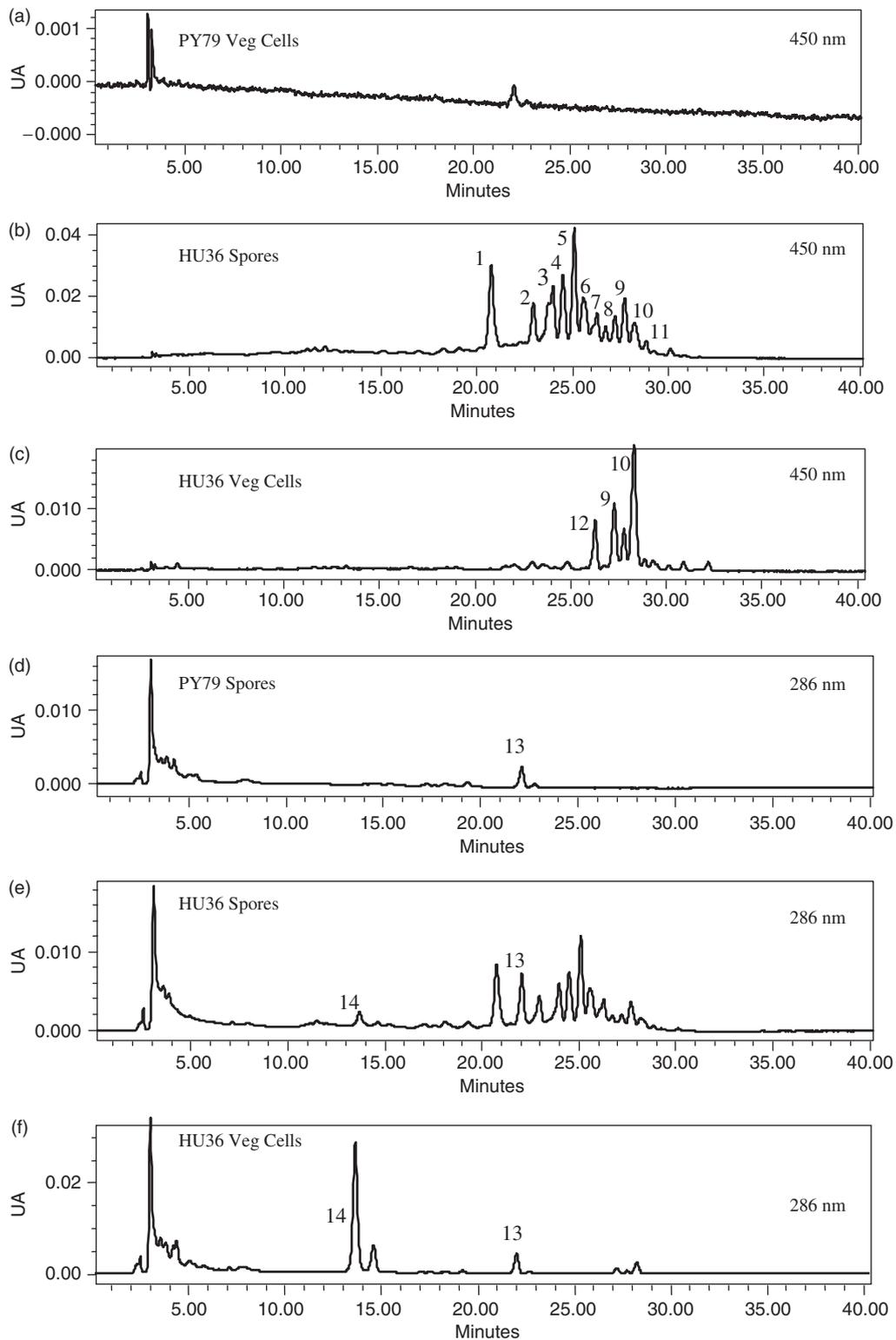
Form	Isolate	Carotenoid	Carotenoid content (area $\times 10^3$ mL <sup>-1</sup> culture)
Vegetative cell	HU13	ODMS	39
	HU16	ODMS	31
	HU19	ODMS	152
	HU28	ODMS	122
	HU33	ODMS	42
	HU36	ODMS	143

ODMS, hydroxy-demethylspheroidene.

Figure 3 illustrates the HPLC profiles of the carotenoids found in the isolate HU36 (spores and vegetative cells, panels b and c, respectively) compared to the control PY79 (panel a). The presence of coloured carotenoids is recorded at 450 nm (panels a–c), while colourless carotenoids and ubiquinone are displayed in panels d–f. These profiles are characteristic for all isolates analysed. No chromatographic components indicating the presence of coloured or colourless carotenoids were observed in vegetative cells or spores of the PY79 strain. By contrast, extracts prepared from spores exhibited the presence of at least 11 chromatographic components showing characteristic coloured carotenoids (panel b). Extracts prepared from vegetative cells contained three predominant coloured carotenoids (panel c). The

carotenoids predominant in vegetative cells possessed spectral maxima at 453.6 nm, the persistence (i.e. repeated inflexions) in the spectra suggested that the carotenoid was acyclic in nature. Using authentic standards (described in Badenhop *et al.*, 2003) HPLC peaks 8–11 were identified as 1-HO demethylspheroidene (ODMS) (Table 3). The separation of multiple chromatographic peaks with identical spectra and similar retention times is likely to be due to different geometric isomers. The separation of carotenoid isomers is a common feature of the C<sub>30</sub> separation stationary phase. Although, a suitable authentic standard was not readily available in comparison with reference spectra (Britton *et al.*, 2003), and the relative retention times suggest the identity of HPLC peak 12 to be 3,4-dihydro-spheroidene (DHS) (Table 3).

The presence of ODMS was also found in vegetative cells. However, additional coloured carotenoids were observed in spore extracts. These carotenoids were relatively more polar in their nature than ODMS. HPLC peaks 1 and 4–7 (Fig. 3; panel b) all exhibited similar chromatographic and spectral properties and thus were structurally related (Table 3). The maximum wavelength ( $\lambda$ ) of the carotenoids ranged from 465.7 to 468.4 nm. Thus a shift in their  $\lambda$  max was observed with the carotenoids isolated from spores. These shifts will theoretically, result in a colour change from those carotenoids determined in vegetative cells. Such an alteration in



**Fig. 3.** High-performance liquid chromatography profiles. UV/VIS recorded at 450 nm: (a) PY79 wild type vegetative cell material; (b) HU36 spores; (c) HU36 vegetative material. UV recorded at 286 nm; (d) PY79 wild type spores; (e) HU36 spores; (f) HU36 vegetative material. Each peak numbered in b and c indicates characteristic carotenoid signature spectrum. Peak 13 represents phytoene and peak 14 represents ubiquinone.

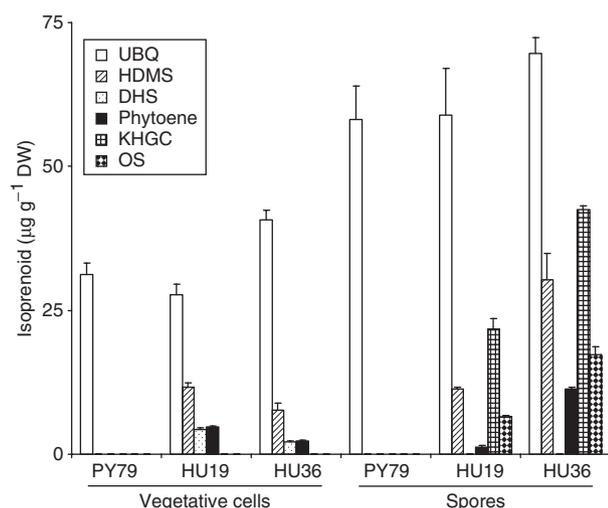
**Table 3.** Carotenoid identification based on co-chromatographic and comparative spectral properties with authentic standards and reference data

HPLC peak no.*	UV/VIS (nm) <sup>†</sup>	Retention time	Carotenoid	Reference spectra
	Authentic standards <sup>‡</sup>			
1	<u>465.7</u> , 493.5	20.756	NI	Keto/hydroxy- $\gamma$ -carotene derivatives (Britton <i>et al.</i> , 2003)
2	<u>468.1</u>	22.70	NI	Keto/hydroxy- $\gamma$ -carotene derivatives (Britton <i>et al.</i> , 2003)
3	428.2, <u>453.6</u> , 485.0	23.48	NI	Hydroxy-spheroidene (Britton <i>et al.</i> , 2003; Badenhop <i>et al.</i> , 2003)
4	<u>466.9</u> , 494.7,	24.25	NI	Keto/hydroxy- $\gamma$ -carotene derivatives (Britton <i>et al.</i> , 2003)
5	<u>468.4</u> , 494.7	24.85	NI	Keto/hydroxy- $\gamma$ -carotenederivatives (Britton <i>et al.</i> , 2003)
6	<u>466.9</u> , 494.7	25.33	NI	Keto/hydroxy- $\gamma$ -carotene derivatives (Britton <i>et al.</i> , 2003)
7	<u>468.1</u> , 494.5	26.03	NI	Keto/hydroxy- $\gamma$ -carotene derivatives (Britton <i>et al.</i> , 2003)
8	429.5, <u>453.6</u> , 486.2	26.98	1-HO-Demethylspheroidene	1-HO-demethylspheroidene (Badenhop <i>et al.</i> , 2003)
9	428.2, <u>453.6</u> , 483.8	27.28	1-HO-Demethylspheroidene	1-HO-demethylspheroidene (Badenhop <i>et al.</i> , 2003)
10	429, <u>454.8</u> , 486.2	28.31	1-HO-Demethylspheroidene	1-HO-demethylspheroidene (Badenhop <i>et al.</i> , 2003)
11	428.7, <u>454.8</u> , 485.0	28.60	1-HO-demethylspheroidene	1-HO-demethylspheroidene (Badenhop <i>et al.</i> , 2003)
12	414, <u>438</u> , 468.0	26.29	NI	3,4-dihydrospheroidene (Britton <i>et al.</i> , 2003)
13	<u>269.8</u> , 330.5	21.88	Ubiquinone	NA
14	<u>286.4</u>	13.56	Phytoene	Phytoene (Britton <i>et al.</i> , 2003)

\*Corresponding to the numbered peaks in Fig. 3.

<sup>†</sup>Highest peak underlined.

HPLC, high-performance liquid chromatography; NI, not identified; NA, not available.



**Fig. 4.** Quantification of carotenoids found in spores and vegetative cells. Carotenoids determined were UBQ – ubiquinone, ODMS – hydroxy-demethylspheroidene, KHGC – keto/hydroxy  $\gamma$ -carotene, DMS – demethylspheroidene and OS – 3,4-dihydrospheroidene. Material from either spores or vegetative cells were examined of the following strains, PY79 (wild type lab strain of *Bacillus subtilis*) and HU19 and HU36 examined in this work. DW, dry weight. Experiments were repeated three times and standard deviations indicated.

colour (e.g. yellow to orange) was clearly visible when comparing vegetative cells and spores (Fig. 1). Besides increases in the maxima other features of the spore-derived carotenoids included the disappearance of spectral persistence with a more bell shaped spectra. Inflections within the spectra were however still observable. Collectively, these features indicate structurally the likely presence of a mono-

cyclic end group as well as keto and/or perhaps hydroxy moieties. Comparison with reference spectra also matched the identity of carotenoids to keto/hydroxyl derivatives of  $\gamma$ -carotene (Table 3) (Britton *et al.*, 2003).

Recording of spectra on-line from 280 to 600 nm enabled searching for other essential pathway carotenoids such as  $\zeta$ -carotene, phytofluene and phytoene. The presence of  $\zeta$ -carotene or phytofluene was not found. At 286 nm components of the chromatogram were observed that matched typical spectra exhibited by authentic phytoene (Fig. 3; panels e and f). The earlier retention time suggested that the phytoene determined in the vegetative and spore extracts was probably not 15-*cis* or all-*trans* in its geometric configuration (Table 3). The isoprenoid ubiquinone was found in all samples.

Besides the presence of different carotenoids in vegetative cells and spores quantitative determination revealed a greater carotenoid content in spores as was the ubiquinone content (Fig. 4). The HU36 isolate also exhibited higher levels compared to HU19.

## Discussion

A number of studies have reported the existence of pigmented species of *Bacillus*. The best-known example is *B. subtilis* var *niger* (now known as *B. atrophaeus*) which produces a soluble black pigment (Nakamura, 1989). More recently reports have appeared, mostly from Central and SE Asia of yellow and yellow-orange pigmented *Bacillus* species (Table 1). These include, *B. cibi* (Yoon *et al.*, 2005), *B. jeotgali* (Yoon *et al.*, 2001), *B. indicus* (Suresh *et al.*, 2004), *B. clarkii* (Nielsen *et al.*, 1995), *B. okuhidensis* (Li *et al.*, 2002), *B.*

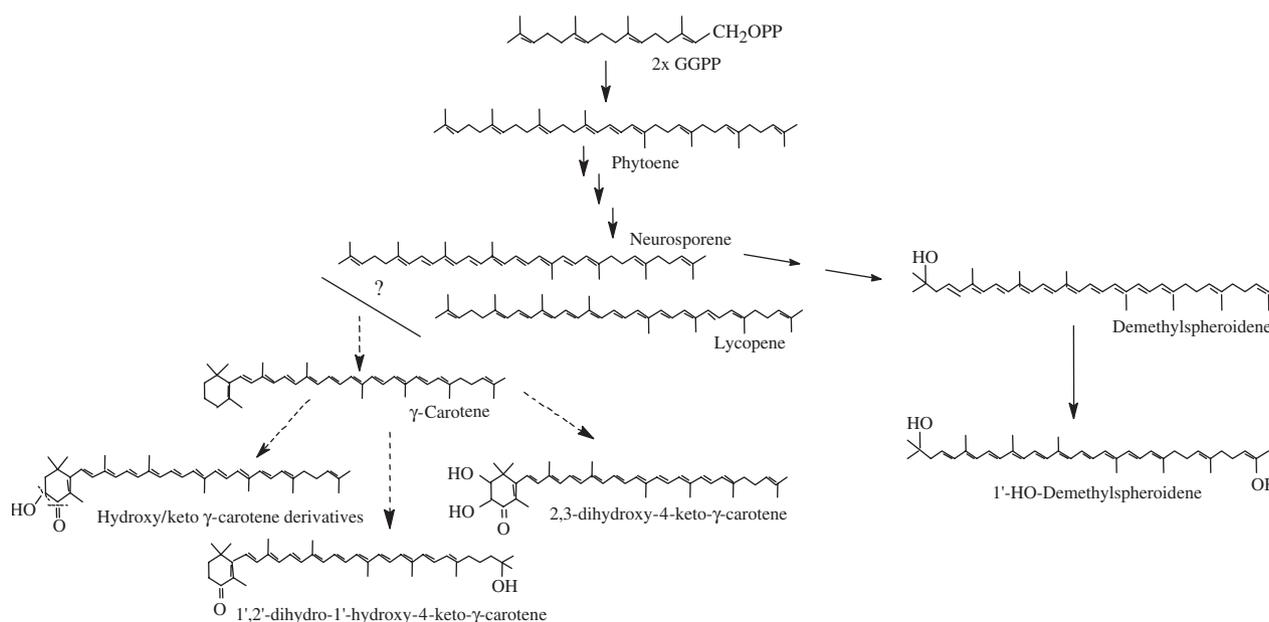
*vedderi* (Agnew *et al.*, 1995) and *B. pseudofirmus* (Nielsen *et al.*, 1995). With the exception of *B. indicus* all appear able to tolerate high levels of NaCl although not at amounts sufficient for them to be considered halophiles. Interestingly, both *B. cibi* and *B. jeotgal* were recovered from Jeotgal, a traditional Korean seafood product made from fermented fish, shellfish, shrimp, oysters, fish roe, intestines and other ingredients.

We have identified six yellow–orange pigmented colonies from heat-treated human faeces. All colonies were found to contain phase-bright endospores and 16S rRNA analysis identified these as close relatives of *B. cibi*, *B. jeotgali* and *B. indicus*. Based on their nonmotility, ability to hydrolyse starch and their arsenic resistance these new isolates could possibly be isolates of *B. indicus* although they appear to be able to tolerate higher levels of NaCl and temperature.

It is highly likely that the presence of these pigmented *Bacillus* species in faeces is linked with the Vietnamese diet, which is rich in seafood and particularly a fermented seafood condiment known as Nuoc Mam or Vietnamese fish sauce. Like, Jeotgal, Nuoc Mam is made from fermented seafood products. A recent report has identified a new *Bacillus* species, *Bacillus vietnamensis* sp. nov. in Nuoc Mam (Noguchi *et al.*, 2004) and in studies in this laboratory (data not shown) we can readily isolate *Bacillus* spore formers from Vietnamese fish sauce. The fact that the pigmented strains are halotolerant suggests that these are marine bacteria that are associated with aquatic animals or crustaceans. It is possible that these bacteria are actually resident members of the gut microflora of aquatic animals

since species of *Bacillus* have been isolated from the gastrointestinal tracts of shrimps (Sharmila *et al.*, 1996; Gate-soupe, 1999). An interesting possibility is that these pigmented *Bacillus* species could contribute to the pigmentation of aquatic organisms by acting as a dietary source of carotenoids.

Using a combination of HPLC analysis and UV/VIS spectral data we have ascertained that the pigmentation in these *Bacillus* isolates is due to the presence of carotenoids. Based on the physical characteristics of the carotenoids determined in this study and existing reference data available we have assigned the predominant carotenoid species in vegetative cells as 1-HO-demethylspheroidene and in spores to keto and/or hydroxy- $\gamma$ -carotene derivatives. Thus, there is a quantitative and qualitative difference in end-product carotenoids formed during different developmental stages. From the identity of the end product and intermediate carotenoids determined putative biosynthetic pathways present in vegetative cells and spores can be predicted (Fig. 5). Both vegetative cells and spores appear to have the ability to form neurosporene. Therefore, two GGPP molecules are condensed to form phytoene. This C<sub>40</sub> hydrocarbon skeleton with a chromophore of three conjugated double bonds is then subjected to three sequential desaturations at positions 11, 12, 12', 13' and 7, 8 yielding neurosporene which possess nine conjugated double bonds. In vegetative cells this acyclic carotene can be further methylated, hydroxylated and desaturated. During spore formation it would appear that a mono-cyclization of an acyclic precursor occurs, to which keto and hydroxy moieties can be incorporated.



**Fig. 5.** Putative pathways involved in carotenoid formation during vegetative growth and spore formation. Those reactions that appear unique to carotenogenesis in spores are shown as dashed arrows.

One important question is that of the role of these *Bacillus* carotenoids. In vegetative cells, the pigment could help protect the cells from photooxidative damage to which aquatic organisms would be more exposed. On the other hand, spores are, by their very nature, dormant and, at least, in *B. subtilis*, have an established system for protection to UV damage. This has been extensively studied and is based on the synthesis of the small acid-soluble proteins (SASP). SASPs are low molecular weight proteins that are synthesized 2–3 h after the initiation of spore formation (Setlow, 1992). Thus, their structural genes (*ssp* genes) are developmentally expressed. The SASPs are made only in the forespore chamber of the sporulating cell and bind to the forespore chromosome protecting it from UV irradiation and damage. For a marine microorganism the presence of carotenoids in spores must presumably provide an additional level of UV protection.

As a spore-forming organism an ordered programme of differential gene expression using alternative transcription factors controls differentiation and it is possible that one or more carotenoid biosynthetic enzymes are developmentally controlled. To our knowledge, this article reports the first example in bacteria of separate developmental biosynthetic pathways responsible for carotenoid formation. It is therefore of considerable interest to perform further studies on carotenoid formation in these isolates at the gene, enzyme and metabolite level. These isolates also have the potential to be exploited from a biotechnological perspective as the habitat from which they have been isolated indicates that they are compatible with the human diet providing a natural source of carotenoids. From an environmental perspective, it is also noteworthy that these bacteria are arsenic resistant indicating the presence of pollution. The presence of an active endogenous isoprenoid/carotenoid pathway suggests they are amenable to metabolic engineering and the well-characterized stability and robustness of the spore could provide a matrix or platform to stabilize carotenoids. Finally, induced germination of orange pigmented spores to yellow vegetative cells could form the basis of a biosensor (Rotman & Cote, 2003).

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