Biochimica et Biophysica Acta 1811 (2011) 177–185

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbalip

Identification and the developmental formation of carotenoid pigments in the yellow/orange *Bacillus* spore-formers

Laura Perez-Fons [a,b], Sabine Steiger [c], Reena Khaneja [b], Peter M. Bramley [a,b], Simon M. Cutting [b], Gerhard Sandmann [c], Paul D. Fraser [a,b,*]

[a] Centre for Systems and Synthetic Biology, Royal Holloway, University London, Egham Hill, Egham, Surrey, TW20OEX, UK
[b] School Biological Sciences, Royal Holloway, University London, Egham Hill, Egham, Surrey, TW20OEX, UK
[c] Goethe University Frankfurt, Department Biological Sciences, Biosynthesis Group, Molecular Biosciences 213, POB111932, D-60054, Frankfurt, Germany

ARTICLE INFO

Article history:
Received 27 August 2010
Received in revised form 30 November 2010
Accepted 17 December 2010
Available online xxxx

Keywords:
Apocarotenoids
Bacillus
Diphenylamine (DPA)
Sporulation
Mass spectrometry

ABSTRACT

Spore-forming *Bacillus* species capable of synthesising carotenoid pigments have recently been isolated. To date the detailed characterisation of these carotenoids and their formation has not been described. In the present article biochemical analysis on the carotenoids responsible for the yellow/orange pigmentation present in *Bacilli* has been carried out and the identity of the carotenoids present was elucidated. Chromatographic, UV/Vis and Mass Spectral (MS) data have revealed the exclusive presence of a C_{30} carotenoid biosynthetic pathway in *Bacillus* species. Apophytoene was detected representing the first genuine carotenoid formed by this pathway. Cultivation in the presence of diphenylamine (DPA), a known inhibitor of pathway desaturation resulted in the accumulation of apophytoene along with other intermediates of desaturation (e.g. apophytofluene and apo-ζ-carotene). The most abundant carotenoids present in the *Bacillus* species were oxygenated derivatives of apolycopene, which have either undergone glycosylation and/or esterification. The presence of fatty acid moieties (C_9 to C_{15}) attached to the sugar residue via an ester linkage was revealed by saponification and MS/MS analysis. In source fragmentation showed the presence of a hexose sugar associated with apolycopene derivatives. The most abundant apocarotenoids determined were glycosyl-apolycopene and glycosyl-4′-methyl-apolycopenoate esters. Analysis of these carotenoids over the developmental formation of spores revealed that 5-glycosyl-4′-methyl-apolycopenoate was related to sporulation. Potential biosynthetic pathways for the formation of these apocarotenoids in vegetative cells and spores have been reconstructed from intermediates and end-products were elucidated.

1. Introduction

Carotenoid pigments represent the largest and most diverse class of natural products known to mankind. To date over 700 structures have been reported from plants, fungi and bacteria [1]. A defining feature of carotenoids is their chromophore, which consists of a series of conjugated double bonds. The length of the conjugated bonds determines the colour of the molecule. It is the ability of carotenoids to confer colour that has fuelled commercial interest in these molecules, predominantly as natural colorants. In addition to their utilisation as colorants many carotenoids when consumed in the diet can confer health promoting properties. For example, the dietary intake of lycopene-containing products is associated with the prevention and more recently the treatment of certain cancers such as prostate cancer [2–4], while β-carotene is provitamin A, a dietary component that is essential for human health [5,6]. To date those carotenoids possessing a C_{40} backbone have received the greatest attention. This is likely to reside in their greater availability in nature and existing route to commercial exploitation. For example, canthaxanthin is an oxygenated C_{40} bicyclic carotenoid, with an annual sales market of $280 million due to its use as a feed supplement in the aquaculture and poultry industry [7]. Despite their intense colour the disadvantages of using carotenoids as natural colorants include their poor solubility in aqueous solutions and instability. These properties are unavoidable with the existing carotenoids due to their hydrophobic nature.

The present production method of choice for carotenoids is chemical synthesis. However, the consumer preference for natural products, high costs, and the dependence on by-products derived from fossil fuels and the detrimental impact on the environment have together intensified efforts to identify alternative biosources. Not all organisms are capable of synthesising carotenoids *de novo*, instead their formation is restricted to the secondary metabolism of plants, algae and certain groups of fungi and bacteria [8,9]. In higher plants, algae and fungi the carotenoids produced contain a C_{40} scaffold [9,10], whilst bacteria can produce a diverse range of carotenoids with both C_{40} and C_{30} backbones [11–22]. The origin of this structural diversity

* Corresponding author. Tel.: +44(0)1784 43894; fax: +44 (0)1784 414224.
E-mail address: p.fraser@rhul.ac.uk (P.D. Fraser).

1388-1981/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbalip.2010.12.009

resides from the prenyl diphosphates precursors utilised in the first committed step of carotenoid biosynthesis [23]. This initial reaction in carotenoid formation is catalysed by variant phytoene synthases. Thus, the combination of two C_{20} precursors (GGPP) gives the C_{40} backbone (Fig. 1A) and the conjugation of two C_{15} molecules (FPP) produces the backbone of C_{30} carotenoids (Fig. 1C). The latter are generally referred to as 4,4′-diapocarotenoids and are typically found in a limited number of Gram +ve bacteria such as *Methylobacterium rhodinum* (formally *Pseudomonas rhodos*) [12,24,25], *Streptococcus faecium* [26], *Heliobacteria* [20,27], and *Staphylococcus aureus* [15,28,29]. However, triterpenoids recently identified in the Gram +ve bacteria *Planococcus* [18] and *Halobacillus* have been described as 8′-apocarotenoids [17] instead of 4,4′-diapocarotenoids. C_{40} and C_{30} 4,4′-diapocarotenoids are symmetric terpenoids in which a system of three conjugated double bounds is located in the centre of the molecule, as a result of the combination of two identical precursors. However, NMR analysis [11,17,18] revealed that C_{30} apo-8′-carotenoids are not symmetric as the 3 conjugated double bounds are not located in the core of the structure. This leads to the hypothesis that the original prenylated precursors might be C_{20} and C_{10} precursors instead of two C_{15} molecules (Fig. 1B). The diapo/apo-carotenoids from these bacterial sources have gained interest in the past years due to their ability to function as virulence factors in some pathogenic bacteria [30–32] and their potential to provide a suitable biosource of carotenoids with improved solubility and stability for the food and feed industry. A diverse range of spore-forming *Bacillus* spp. has also been isolated that contain carotenoid pigments [33]. Interestingly, these bacteria also possess the ability to act as probiotics [34]. The aim of the present studies was to identify the carotenoid pigments produced by these bacteria in relation to other Gram +ve and define their developmental formation in these spore-forming bacteria. A combination of different analytical techniques have been employed for defining the structures of these novel compounds [8,35].

2. Materials and methods

2.1. Bacterial strains and cultivation

The yellow and orange pigmented *Bacillus* spp. used in this study have been described previously [33]. To generate biomass for the structural elucidation of pigments *Bacillus indicus* HU36 was used routinely as a representative model strain. General cultivation was carried out in tryptone-yeast (TY) media on agar or in shake culture for 48 h, 30 °C at 200 rpm. Induction of sporulation and the production of spores were performed using Difco Sporulation Media (DSM) as described elsewhere [36].

2.2. Determination of sporulation efficiency and time-course experiments

From a master glycerol stock DSM agar plates were inoculated with a HU36 streak and incubated for 2 days at 30 °C. From this plate a single colony was used to inoculate DSM liquid media (10 ml). The culture was incubated at 30 °C and agitated (200 rpm), until the mid-exponential phase of growth was reached (OD_{600} ~0.6). An aliquot (1 ml) of this culture was then used to seed DSM liquid media (50 ml) present in 250 ml baffled flasks. These cultures were then incubated at 30 °C (200 rpm) for 3 days.

To determine the percentage of sporulation viable counting of vegetative cells and heat-resistant spores (HRS) was carried out. Data points were collected every 24 h from inoculation. At each time point

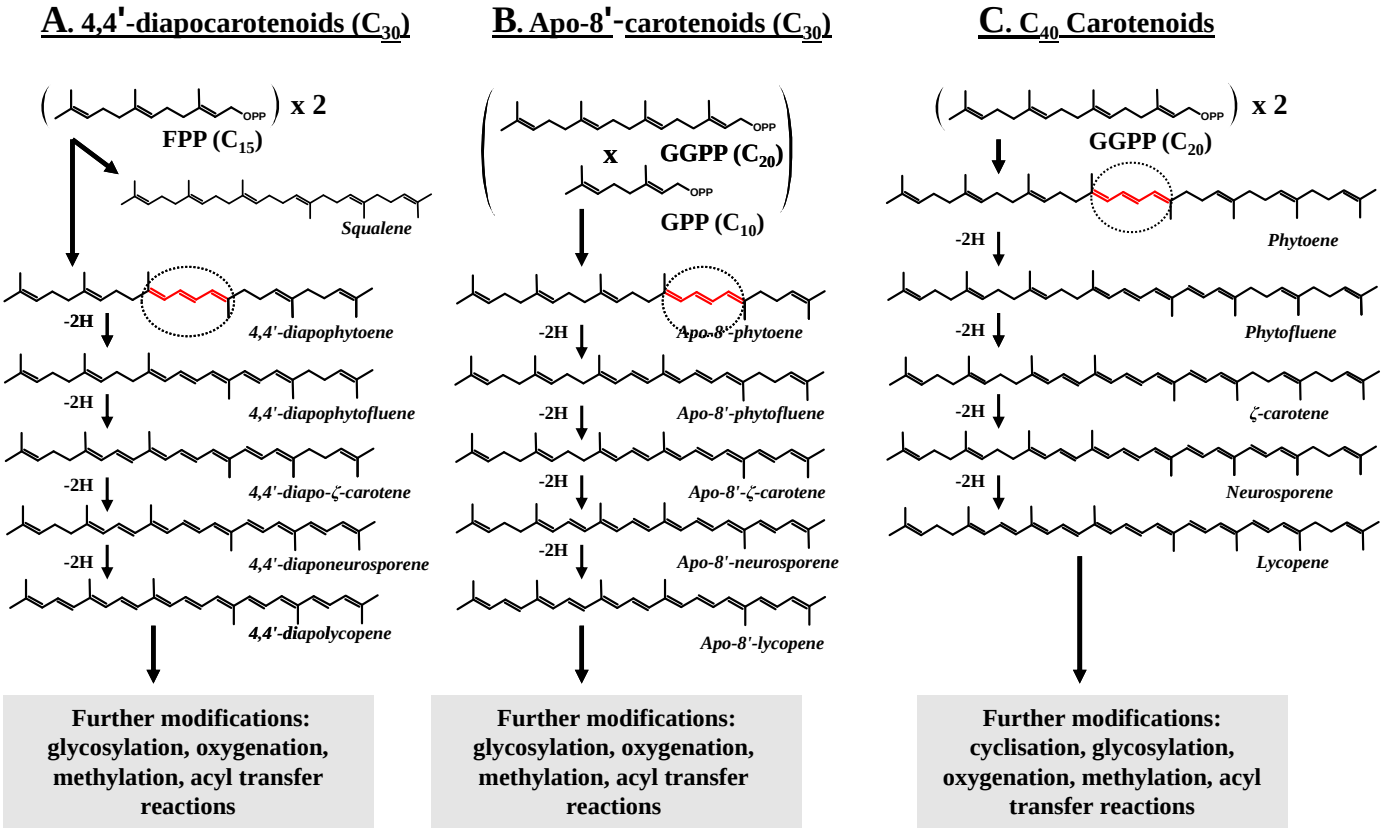

Fig. 1. The diversity of biosynthetic carotenoid pathways. Two identical prenyl diphosphate precursors, GGPP for C_{40} carotenoids (C) and FPP for C_{30} 4,4′-diapocarotenoid (A), lead to the formation of a symmetric structure whereas the combination of two different precursors C_{20} and C_{10} produces the asymmetric C_{30} apo-8′-carotenoids (B). C_{40} biosynthetic pathway is widespread in plants and fungi [9,10] and the C_{30} 4,4′-diapocarotenoid biosynthesis has been described in *S. aureus* [28,29,39], *Methylobacterium* [24,25] and *Streptococcus* [43]. C_{30} apo-8′-carotenoids have been found in *Planococcus* [18], halophilic cocci [11] and *Halobacillus* [17]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a culture aliquot (0.5 ml) was taken and 0.1 ml was used for serial dilution in phosphate buffer [0.85% (w/v) pH 7]. Each dilution was plated onto DSM agar and after 24 h at 37 °C colonies were counted. The remaining 0.4 ml of culture was heated at 65 °C for 1 h to eliminate vegetative cells and retain heat-resistant spores. Serial dilutions of the heat-treated solution were then carried out. Data were expressed as colony forming units (c.f.u.) per milliliter and the sporulation efficiency was provided as a percentage of heat-resistant spores divided by the number of vegetative cells. To routinely ascertain the sporulation state cultures were examined by phase-contrast microscopy. Material for carotenoid analysis was obtained every 24 h following inoculation by centrifugation at 10,000*g* performed at 4 °C for 10 min. The pelleted cells were freeze-dried and stored at −80 °C.

2.3. Carotenoid extraction, separation, identification and quantification

Material for carotenoid analysis was prepared by growing strain HU36 in baffled flasks (2 l) containing TY liquid media (500 ml), incubated at 30 °C (agitated at 200 rpm). Cells were harvested after 2 days by centrifugation. The cellular biomass was frozen and lyophilized to completed dryness (about 3 days). This material was either used immediately or stored at −80 °C. A homogeneous powder was prepared using a mortar and pestle or with a tissue lyser (Qiagen, Crawley, UK). An aliquot (50 mg) of the homogenised freeze-dried material was taken for extraction. Firstly, direct extraction without saponification was carried out with methanol (2 ml) added to the powdered cellular material. The suspension created then was sonicated at room temperature (RT) for 10 to 15 min. Centrifugation (12,000 rpm) for 5 min was carried out to remove the cell debris and create the carotenoid containing supernatant. The cellular debris was re-extracted until no extractable colour was recovered from the cellular material. The methanolic extracts were pooled and then dried under vacuum (centrifugal evaporator EZ-2 Plus, Genevac, Ipswich, Suffolk, UK) and the dried carotenoid extract was stored at −20 °C under nitrogen until further analysis.

Saponification prior to extraction was performed by treating the freeze-dried cells (50 mg) with a solution of NaOH (10% w/v; 1 ml) and sonicating the suspension for 15–20 min at room temperature. NaOH was removed by centrifugation and from the digested cellular material methanol (250 µl) and chloroform (500 µl) was added, mixed and then a partition created with Tris-buffered saline (50 mM Tris–HCl, NaCl 1 M, pH 7.0). After centrifugation the carotenoid yielding hypophase was removed and the aqueous hyper-phase was re-extracted with chloroform (500 µl) twice (at this point no colour was detectable in the cellular debris). The pooled organic extracts were reduced to dryness under a stream of nitrogen gas; typically these dried extracts were stored at this stage at −20 °C, until further analysis.

Due to their lack of solubility in chloroform or ethyl acetate, dried extracts were routinely dissolved in chloroform: methanol (1:1; by vol.). TLC separations of these *Bacillus* derived carotenoids were carried out using two systems. Firstly, system I comprised of an activated silica gel stationary phase and the mobile phase acetone (35%) in petroleum ether (b.p. 80–100 °C) [15] and system II an identical stationary phase with toluene/ethyl acetate/methanol (50:25:25 by vol.) [21].

For chemical modification, the non-hydrolysed carotenoid extracts were dissolved in methanol containing 10% w/v KOH and heated for 20 min at 60 ° C. They were further fractionated by direct partitioning into diethyl ether (alkaline fraction) followed by pH adjustment to 3 with HCl and partitioning into ether again (acid fraction).

High Performance Liquid Chromatography (HPLC)-Photodiode array (PDA) analysis of carotenoids was performed with a Waters Alliance (Milford, MA) 2600S system as previously described [37] Detection was carried out with an on-line PDA. Separations were performed on a reverse phase (RP) C_{30} 5 µm column (250 × 4.6 mm i.d.) with a C_{30} guard column (20 × 4.6 mm), (YMC Inc., Wilmington, NC), which were maintained at 25 °C. Prior to injection onto the column extracts were filtered through a PTFE membrane (0.2 µm: Chromacol Ltd., Herts, UK.) and then centrifuged at 12,000 rpm for 3 min. The mobile phase used for routine analysis comprised of (A) methanol, (B) methanol: water (80:20 by vol.) containing 0.2% (w/v) ammonium acetate and (C) *tert*-butyl methyl ether [37]. Elution from the column was carried out from 95% (A) and 5% (B) for 12 min, then a step to 80% (A), 5% (B) and 15% (C) followed by a linear gradient to 30% A, 5% B and 65% C at 30 min. The column was then returned to the initial conditions and equilibrated over 30 min. A flow rate of 1 ml/min was employed and elution was monitored continuously with the on-line PDA (200–600 nm). Identification was performed by the comparison of spectral and chromatographic characteristics to authentic and similar carotenoids as well as reference parameters in the literature [8,9,11,18,35]. Quantification was carried out using dose-response curves prepared from authentic standards previously purified by HPLC. For purification saponified extracts were separated by HPLC using identical conditions as described above and pure compounds were isolated using a fraction collector. Absorption coefficients (ε) calculated for both yellow and orange pigments were 149,621 and 122,300 ($M^{-1}\,cm^{-1}$), respectively [8,11,38]. Menaquinone was also indentified by spectral comparison with authentic standards. All solvents were purchased from VWR (Poole, UK).

To complement the UV/Vis and chromatographic properties used to identify the carotenoids in question Mass Spectrometry (MS) was also employed. Separations were performed by HPLC prior to on-line MS in a similar manner to that detailed above, with the exception that a RP C_{30} 3 µm column (150 × 2.1 mm i.d.) coupled to a 20 × 4.6 mm C_{30} guard column was used. The mobile phase was altered to facilitate ionisation and was comprised of (A) methanol containing 0.1% formic acid (by vol.) and (B) *tert*-butyl methyl ether containing 0.1% formic acid (by vol.). These solvents were used in a gradient mode starting at 100% (A) for 5 min, then stepped to 95% (A) for 4 min, followed by a linear gradient over 30 min to 25% (A). After this gradient (A) was a step down to 10% over 10 min. Initial conditions (100% A) were restored for 10 min after the gradient to re-equilibrate the system. The flow rate used was 0.2 ml/min. The ionisation mode employed was Atmospheric Pressure Chemical Ionisation (APCI) operating in positive mode (Thermo Scientific, San Jose, California, USA). Capillary and APCI vaporisation temperatures were set at 225 °C and 450 °C respectively and the gas flow (nitrogen) at 80 units. APCI source settings were as follows: source voltage at 4.5 kV, source current 5 µA and a capillary voltage of 3 V. A full MS scan was performed from 300 to 1500 *m/z* and MS/MS spectra were recorded at normalised collision energy of 35% and isolation width of 1 *m/z*.

3. Results and discussion

3.1. Isolation of carotenoid pigments from Bacillus material

Bacillus spp. producing yellow, orange and red carotenoids have been bio- and genotyped [33]. Of particular interest is the *B. indicus* HU36 strain and its related species due to their amenable probiotic properties [34]. The yellow/orange carotenoids formed by these species are the focus of the present study and *B. indicus* HU36 was used as a representative of this collection.

When grown on either solid or in liquid TY or DSM based media carotenoids were produced. It was evident that the type and quantity of carotenoids produced varied with the culture conditions used. However, growth in TY broth for 48 h resulted in a comparatively higher biomass (5-fold and 3-fold increase at days 2 and 3 respectively) with a carotenoid composition that was representative of all the culture conditions assessed. Thus, this procedure was standardised for the generation of material to be used in the structural elucidation of the carotenoids present. The carotenoids produced were retained in an

exclusive manner within the cellular pellet; no carotenoids were present in the spent media. Extraction from fresh, frozen and freeze-dried material was evaluated in conjunction with different solvent treatments (e.g. methanol, acetone, chloroform, diethyl ether, DMSO and ethyl acetate) and homogenisation procedures including French press, manual grinding with liquid nitrogen, tissue lysers and sonication. Methanol extraction of ground freeze-dried material was comparatively the most effective approach but large volumes and multiple re-extractions of methanol were required. Alternatively, rapid chemical treatment with 10% (w/v) NaOH at room temperature followed by sonication released the carotenoids into $CHCl_3$:MeOH (2:1). The carotenoids could then be enriched into the $CHCl_3$ phase with the addition of aqueous buffer. The profile and UV/Vis spectra of the carotenoids extracted with methanol solely and $CHCl_3$ after saponification were identical. Therefore, the chemical treatment procedure used represented an efficient extraction procedure that did not modify the core structure of the native carotenoids present.

3.2. The separation and identification of Bacillus carotenoids

Glycosylated C_{30} carotenoids have been reported previously in some Gram +ve bacteria such as *S. aureus* [15,21,29], *Planococcus maritimus* [18] and *Halobacillus* [17]. Therefore the separation of *Bacillus* pigments was initially performed on TLC, using systems devised for the triterpenoids and their glycosides [15,21]. TLC system I revealed both yellow and orange *Bacillus* pigments with R_f values of 0.95 and 0.91 respectively, indicating that the orange pigment was more polar. The yellow and orange pigments were designated C455 and C467 respectively according to their adsorption maxima in the visible range e.g. C-carotenoid with a maxima of 455 nm. Following saponification with KOH the C455 carotenoid accumulated in an alkaline ether fraction, whereas the C467 product was found in the acidified methanolic fraction. TLC analysis on a system suitable for glycosyl diapocarotenoic acid derivatives indicated that the C455 hydrolysis product had an R_f of 0.56 being less polar than the C467 product which had an R_f of 0.50. The non-hydrolyzed carotenoids remained on the origin. Table 1A and B summarises the chromatographic behaviour of the *Bacillus* pigments on both TLC systems used and prior to and after saponification. Comparison with the chromatographic and spectral properties of the diapocarotenoids found in *S. aureus* showed that the *Bacillus* carotenoids were not diaponeurosporene derivatives, but similarity to the *P. maritimus* methyl glucosyl-3, 4-dehydro-apo-8′-lycopenonate was feasible.

Collectively the data suggest that the *Bacillus* carotenoids are apolycopene derivatives. Increased polarity after saponification treatment infers the presence of ester, not ether linked fatty acids presumably on glycosyl moieties. Furthermore, the ability of the C467 hydrolysis product to reside in the acid fraction demonstrates that it is carrying an additional carboxylic acid group which is absent in C455.

Following TLC, HPLC-PDA/MS analysis was performed. Fig. 2 details the chromatographic profiles obtained with non-saponified extracts at 286 nm (A), 450 nm (B) and saponified extracts at 450 nm (C). Tables 1 and 2 document the chromatographic components, their UV/Vis and mass spectral properties. At 286 nm the predominant peak (1) at 9.8 min, had an absorption spectrum typical of phytoene (Fig. 2D-I) adjacent was an apparent geometric isomer (peak 2). These compounds did not co-chromatograph with authentic C_{40} phytoene but did possess identical properties to apophytoene and its isomers isolated from *Planococcus* [18], *Heliobacteria* [20,27] and *S. aureus* [39]. The identification of apophytoene in the bacterial extracts was confirmed by the mass spectrum of peaks 1 and 2 at 409.2 *m/z* (Fig. 3A), corresponding to a $[M+H]^+$ for the parent ion of C_{30} H_{40}. Besides apophytoene (1) and (2), menaquinone-7 (3) was identified ($[M+H]^+ = 649.2$).

The chromatographic profile at 450 nm of the non-saponified methanolic extracts was complex with over 20 components (Fig. 2B). These peaks could be categorised into several groups according to their UV/Vis spectra. Group II contained peaks 4 to 11, eluting between 23 to 27.5 min. These chromatographic components all had a visible spectral maximum of 452 nm (Fig. 2D-II), with persistence at 426 and 478 nm and pronounced *cis* peaks at 342 nm. The intensity of

Table 1
Chromatographic and spectral properties of apocarotenoids detected in non-saponified extracts prepared from the orange/yellow *B. indicus* HU36.

	TLC analysis		HPLC	Spectral properties			
Carotenoid	R_f	Colour	R_t	λ_{max} (nm)	% III/II	% A_B/A_{II}	$[M+H]^+$
Trace at 286 nm, saponified and unsaponified extract (Fig. 2A):							
Apo-8′-phytoene **(1)**	–	NC	9.49	274, 286, 298	< 1	NA	409.20
Apo-8′-phytoene isomer **(2)**	–	NC	10.35	274, 286, 298	< 1	NA	409.20
Menaquinone-7 **(3)**	–	NC	20.65	240, 262, 270, 330	NA	NA	649.29
Trace at 450 nm, unsaponified extract (Fig. 2B):							
cis-1-glycosyl-apo-8′-lycopene ester **(4)**	0.71	LY	23.22	342, 426, 448, 478	53	57.1	401.3, n.d.
cis-1-glycosyl-apo-8′-lycopene ester **(5)**	0.71	LY	24.12	342, 426, 448, 476	56.7	51	401.2, n.d.
cis-1-(6-$C_{11:0}$)-glycosyl-apo-8′-lycopene **(6)**	0.71	LY	24.75	344, 426, 450, 478	53	49.8	401.2, 749.2
cis-1-(6-$C_{11:0}$)-glycosyl-apo-8′-lycopene **(7)**	0.71	LY	25.05	344, 424, 448, 476	58.3	48	401.2, 749.2
cis-1-(6-$C_{9:0}$)-glycosyl-apo-8′-lycopene **(8)**	0.71	LY	25.5	344, 424, 448, 476	91.2	30	401.2, 721.1
cis-1-(6-$C_{9:0}$)-glycosyl-apo-8′-lycopene **(9)**	0.71	LY	26	344, 426, 448, 476	17.3	30	401.2, 721.2
cis-1-(6-$C_{11:0}$)-glycosyl-apo-8′-lycopene **(10)**	0.71	LY	27.09	344, 428, 452, 480	51.6	18.4	401.2, 581.1, 749.1
cis-1-(6-$C_{11:0}$)-glycosyl-apo-8′-lycopene **(11)**	0.71	LY	27.55	428, 448, 476	71.8	NA	401.1, 581.1, 749.1
Methyl 1-(6-$C_{9:0}$)-glycosyl-apo-8′-lycopenoate **(12)**	0.91	O	28.39	440, 466, 492	0.35	NA	445.1, 765.1
Methyl 1-(6-$C_{10:0}$)-glycosyl-apo-8′-lycopenoate **(13)**	0.91	O	29.00	438, 466, 492	< 1	NA	445.1, 779.1
Trace at 450 nm, unsaponified extract (Fig. 2B):							
Methyl 1-(6-$C_{11:0}$)-glycosyl-apo-8′-lycopenoate **(14)**	0.91	O	29.42	438, 466, 492	< 1	NA	445.1, 793.1
Methyl 1-(6-$C_{11:0}$)-glycosyl-apo-8′-lycopenoate **(15)**	0.91	O	29.64	440, 466, 492	8	NA	445.1, 793.1
1-(6-$C_{8:0}$)-glycosyl-apo-8′-lycopene **(16)**	0.94	Y	30.02	430, 454, 484	68.4	NA	401.1, 707.2
1-(6-$C_{9:0}$)-glycosyl-apo-8′-lycopene **(17)**	0.94	Y	30.65	430, 454, 484	39.4	NA	401.1, 721.2
1-(6-$C_{10:0}$)-glycosyl-apo-8′-lycopene **(18)**	0.94	Y	31.22	428, 454, 484	47.4	NA	401.1, 735.2
1-(6-$C_{11:0}$)-glycosyl-apo-8′-lycopene **(19)**	0.94	Y	31.84	430, 454, 484	48.3	NA	401.1, 749.2
1-(6-$C_{12:0}$)-glycosyl-apo-8′-lycopene **(20)**	0.94	Y	32.49	430, 454, 484	59	NA	401.1, 763.2
1-(6-$C_{13:0}$)-glycosyl-apo-8′-lycopene **(21)**	0.94	Y	32.91	430, 454, 484	64.3	NA	401.1, 777.2
1-(6-$C_{13:0}$)-glycosyl-apo-8′-lycopene **(22)**	0.94	Y	33.27	430, 454, 484	66	NA	401.1, 777.2
1-(6-$C_{14:0}$)-glycosyl-apo-8′-lycopene **(23)**	0.94	Y	34.05	430, 454, 484	64.7	NA	401.1, 791.2
1-(6-$C_{15:0}$)-glycosyl-apo-8′-lycopene **(24)**	0.94	Y	35.03	430, 454, 484	66	NA	401.1, 805.2

Analysis was performed by TLC, HPLC-PDA and LC/MS. NC: not coloured; Y: yellow (glycosyl-apolycopene); LY: light yellow and OR the orange carotenoid (methyl glycosyl-apolycopenoate); NA: not applicable; R_t: retention time; R_f: retention factor. The experimental details of the methods used are provided in the Materials and methods section. Fatty acids were present in their saturated form.

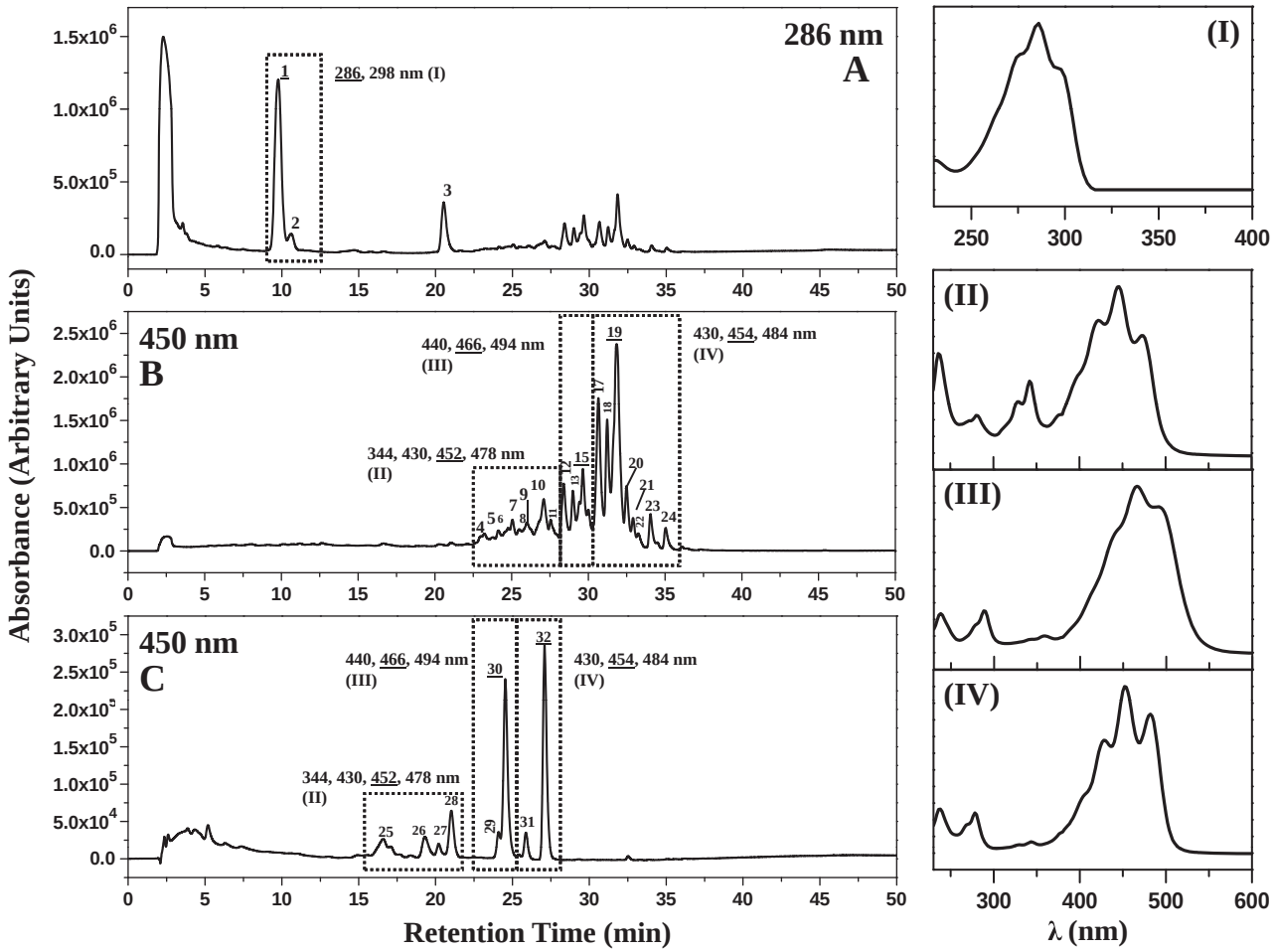

Fig. 2. HPLC analysis of extracts prepared from freeze-dried cells of HU36. Chromatograms (panel A and panel B) represent unsaponified extracts and are recorded at 286 and 450 nm respectively. A profile following saponification with NaOH (10% w/v) is shown in (panel C). Chromatographic peaks were grouped (I–IV) according to their UV/Vis spectra as shown in panel D.

the *cis* peak was indicated by the A_B/A_{II} ratio provided in Table 1. It appears that the higher the ratio and thus greater degree of *cis* geometric isomerisation [35] correlated with an earlier retention time or more polar the compound. A mass of 401.2 $[M+H]^+$ corresponding to an apolycopene skeleton was found in all these chromatographic components included in group II. Higher parent masses were also observed e.g. 749.2 $[M+H]^+$ or 721.2 $[M+H]^+$, but the presence of a hexose sugar (e.g. $\Delta m/z$ of 162) and fatty acids (e.g. $\Delta m/z$ of 186 for undecanoic acid) was revealed upon in-source fragmentation of the higher mass parent ions. The esterified fatty acids moieties found ranged from nonanoic, decanoic and undecanoic acids (Table 1). Thus the components of group II were designated as esterified glycosides of apolycopene existing in the *cis* geometric configuration predominantly.

Chromatographic peaks 12 to 15, eluting from 28 to 29.5 min represent group III. These chromatographic components all had UV/Vis maxima of 466 nm; persistence was reduced, with shoulders present at 438 and 492 nm. The mass of the parent carotenoid found in these peaks was determined to be 445.1 $[M+H]^+$ instead of 401, indicating the presence of a methylated carboxylic acid. The occurrence of the acid group or more precisely the keto group explains the reduced persistence in the spectra and increased λ maxima. The higher parent masses of these peaks could be accounted for by the presence of a hexose sugar and fatty

Table 2
Chromatographic and spectral properties of carotenoids detected in saponified extracts prepared from *B. indicus* HU36.

	TLC analysis		HPLC	Spectral properties			
Carotenoid	R_f	Colour	R_t	λ_{max} (nm)	% III/II	% A_B/A_{II}	$[M+H]^+$
Trace at 450 nm, saponified extract (Fig. 2C):							
cis-1-glycosyl-apo-8′-lycopene **(25)**	0.21	LY	16.57	342, 424, 446, 478	29.8	47.8	n.d.
cis-Methyl-1-glycosyl-apo-8′-lycopenoate **(26)**	0.21	LO	19.3	358, 460, 488	NA	38.7	n.d.
cis-1-glycosyl-apo-8′-lycopene **(27)**	0.21	LY	20.2	344, 422, 446, 476	62.5	49	581.12
cis-1-glycosyl-apo-8′-lycopene **(28)**	0.21	LY	21.02	342, 428, 448, 476	55.3	28.3	n.d.
cis-Methyl-1-glycosyl-apo-8′-lycopenoate **(29)**	0.34	O	24.10	356, 436, 464, 492	< 1	14.6	445.1, 624.9
Methyl-1-glycosyl-apo-8′-lycopenoate **(30)**	0.34	O	24.53	436, 464, 492	6.1	NA	445.1, 463.1, 625.06
cis-1-glycosyl-apo-8′-lycopene **(31)**	0.38	Y	25.87	342, 426, 450, 478	66.3	11.2	581.02
1-glycosyl-apo-8′-lycopene **(32)**	0.38	Y	27.08	428, 454, 484	64.2	NA	401.1, 419.06, 581.05

Analysis was preformed by TLC, HPLC-PDA and LC/MS. The yellow colour indicates (glycosyl-apolycopene), while light yellow and orange colouration indicates the presence of methyl glycosyl-apolycopenoate. NA represents not applicable and n.d, not detected. The analytic procedures are described in the Materials and methods section.

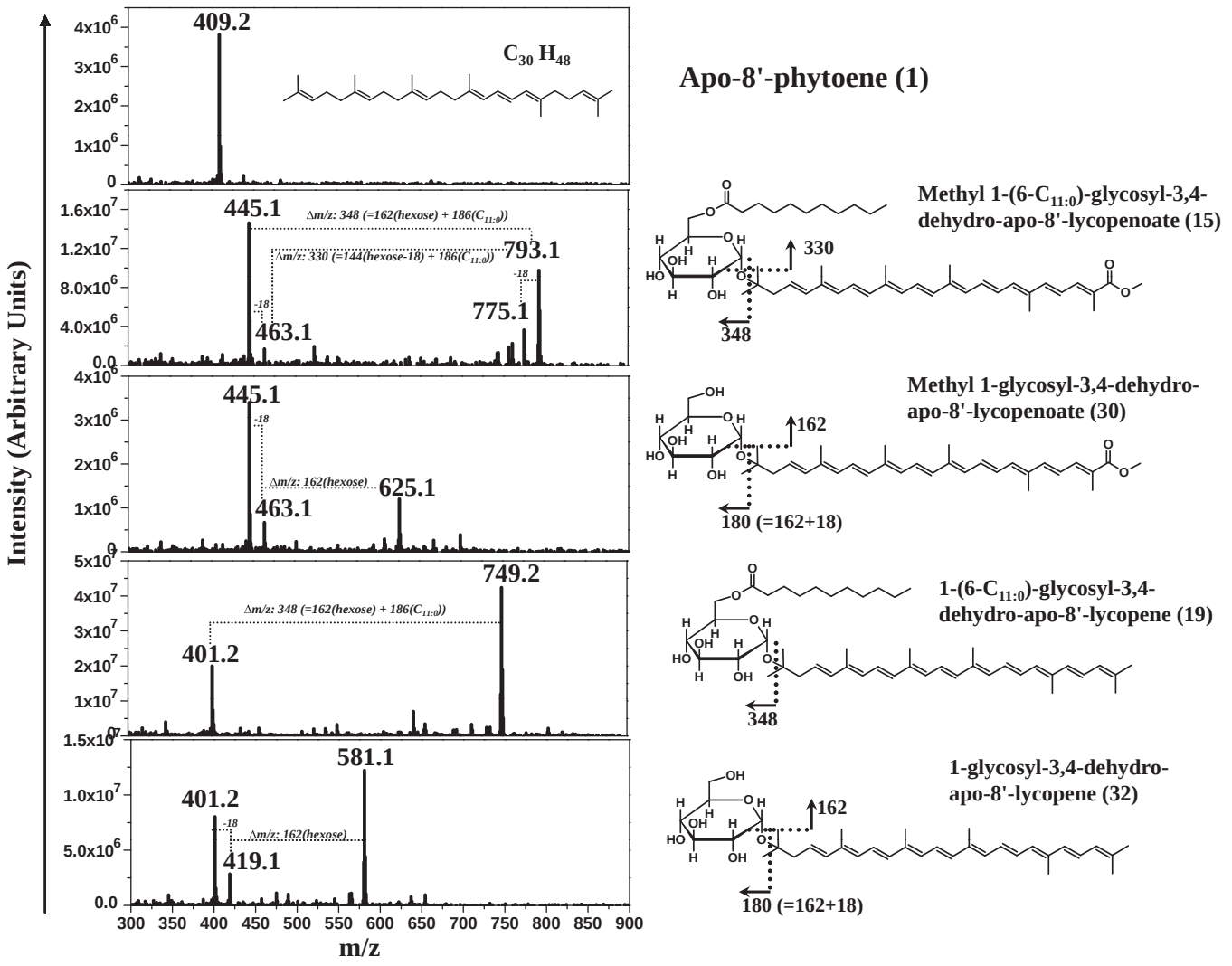

Fig. 3. Characteristic MS spectra of the major apocarotenoids found in saponified and non-saponified extracts prepared from *B. indicus* HU36 (panels A–E). Corresponding structures and fragmentation patterns are provided.

acid derivatives, as differences in masses of 162 (hexose moiety) and 158–186 ($C_{9:0}$–$C_{11:0}$ esters) were detected upon fragmentation (Table 1). For example peak 15 was designated as methyl 1-(6-$C_{11:0}$)-glycosyl-3, 4-dehydro-apo-8′-lycopenoate (Fig. 3B), derived from a parent mass of 793.1 $[M+H]^+$ carrying a hexose sugar ($\Delta m/z$ 162) esterified to undecanoic acid ($\Delta m/z$ 186) and attached to 445.1 $[M+H^+]$ dehydro-methyl-apo-8′-lycopenoate. Alternatively, this parent apocarotenoid can be obtained from 793.1 $[M+H]^+$ through the loss of a hydroxyl group as water to 775.1, followed by the removal of hexose sugar ($\Delta m/z$ 144 = hexose-18) and fatty acids [$\Delta m/z$ 186 ($C_{11:0}$)] yielding 463.1 from which the loss of another hydroxyl group as water would result in 445.7 $[M+H]^+$ (Fig. 3B). The UV/Vis and MS properties of this chromatographic group match that found previously in *Planococcus* where methyl glucosyl-apolycopenoate was elucidated [17].

The peaks 17 to 24 in the chromatogram (Fig. 2B) are designated group IV where no methylated carboxylic acid forms of apolycopene occurred, instead the carotenoid skeleton apolycopene with a mass of 401.2 $[M+H^+]$ was found. The UV/Vis spectra were also more persistent and the maximum reduced to 454 nm, which was indicative of no keto group being present. Using peak 19 as an example the parent molecule possessed an m/z of 749.2 $[M+H]^+$, which yielded the apocarotenoid skeleton of 401.2 m/z through the excision of an esterified-glycosyl component. This mass difference of 348 m/z is equivalent to the sum of a sugar (162 m/z) and fatty acid, in this case saturated undecanoic acid (186 m/z) (Fig. 3D). Similar analysis was performed on the rest of the yellow chromatographic components detected in group IV, permitting the identification of eight different esterified saturated fatty acids ranging from octanoic ($C_{8:0}$) to pentadecanoic ($C_{15:0}$) acid (Table 1). Several components (e.g. 21 and 22) showed identical spectral properties but different retention times. This was probably due to the presence of geometric isomers or the fatty acid present being branched (*iso*- or *anteiso*) as this type of fatty acids are typical of those found in bacteria [40].

Saponification reduced the complexity of the chromatogram (Fig. 2C). The chromatographic grouping of II, III, and IV was effectively identical as Fig. 2B but without the presence of fatty acid moieties on the glycosylated apolycopene and apolycopenoate. These were clear from the mass spectra (Fig. 3C and E). For example, peaks 30 and 32 possessed $[M+H]^+$ of 625.1 (Fig. 3C) and $[M+H]^+$ 581.1 (Fig. 3E) respectively, loss of hexose sugar ($\Delta m/z$ of 162) yielding 445.1 $[M+H]^+$ for methyl-dehydro-apo-lycopenoate and 401.2 $[M+H]^+$ for dehydro-apo-lycopene. Thus, it is clear that the fatty acids are connected to the sugar residue by ester not ether bonds.

3.3. Identification of pathway intermediates using diphenylamine (DPA) treatment

In order to identify intermediates in the *Bacillus* apocarotenoid biosynthetic pathway the known inhibitor of carotenoid desaturation

Table 3
Determination of apocarotenoid contents in *B. indicus* HU36 cells following treatment with the desaturase inhibitor diphenylamine (DPA).

			Carotenoid content (μg/g DW)			
	Biomass (mg DW)	Colour*	Total	Yellow	Orange	Apo-8′-phytoene
0 μM DPA	48.9 ± 6.5	Y-O	186.8 ± 27.8	78.2 ± 27.8	106.3 ± 19.5	2.3 ± 0.9
10 μM DPA	40.8 ± 3.8	Y-O	195.6 ± 2.9	83.8 ± 1.4	107.1 ± 5.2	4.6 ± 1.8
100 μM DPA	43.7 ± 5.5	NC	241.7 ± 24.1	30.7 ± 16.3	58.8 ± 16.3	152.1 ± 10.7

Quantification was performed by HPLC-PDA as described in the Materials and methods section. Y-O indicates a yellow/orange colouration of colonies (*) while NC represents the visualisation of colonies with a white-creamy appearance (not coloured). The yellow pigment is glycosyl-apolycopene, orange, methyl glycosyl-apolycopenoate and the colourless precursor, apophytoene. DW: dry weight; Y-O: yellow/orange; NC: not coloured. Data represent the mean of three independent determinations ± standard error (SEM).

diphenylamine (DPA) was used. Treatments were carried out by seeding agar plates with DPA over a 0 to 100 μM range. The plates were inoculated with an aliquot (0.1 ml) of *Bacillus* culture broth taken at the mid-exponential growth phase. After 24-h cultivation the biomass was harvested from the plates and carotenoids present in the material were analysed. The most dramatic feature of the HPLC profiles (Supplementary Fig. 1A) was the increase in apophytoene (peak 1) and its geometric isomer (peak 2). In addition, the chromatogram recorded at 350 nm (Supplementary Fig. 1B) indicated the presence of chromatographic peaks unique to DPA treatment. These peaks were identified from their characteristic UV/Vis and mass spectra as apophytofluene (peaks 3–5) and apo-ζ-carotene (peak 6). The C_{30} apophytofluene had a visible spectrum with two persistent peaks at 350 and 365 nm, and a $[M+H]^+$ of 403 *m/z*, whereas apo-ζ-carotene with two extra conjugated double bonds had a $[M+H]^+$ of 405 *m/z* and a UV/Vis spectrum with clear persistence giving rise two peaks at 400 and 425 nm. Interestingly, the amount of total carotenoid increased with the highest DPA concentration (Table 3) although it was not statistically significant. This was mainly due to the high concentration of apophytoene accumulating to 60% of the total carotenoid, compared to 1.2% in the wild type without DPA treatment ($p<0.001$) (Table 3). The amounts of the yellow (1-glycosyl-3,4-dehydro-apo-8′-lycopene) and orange (methyl 1-glycosyl-3,4-dehydro-apo-8′-lycopenoate) pigments were reduced to a level about half that found in the untreated e.g. about 24% of the orange pigment accumulated at 100 μm DPA compared to 57% in the untreated ($p<0.05$). The increase in the occurrence of total carotenoid in the presence of DPA has been reported in other organisms [41], where it has been attributed to the elimination of regulation by end-product feedback inhibition or alternatively it is feasible that the pigments undergo further catabolism in these *Bacillus* species.

In some Gram +ve bacteria squalene formed via the condensation of two farnesyl diphosphate (FPP) molecules is the precursor for 4,4′-diapophytoene [25]. In the present study squalene was not detectable in the yellow/orange *Bacillus* species tested, using either LC-MS or GC-MS. Following DPA treatment the build up of precursors indicated no detectable squalene. The greatest similarity between the *B. indicus* HU36 apocarotenoids and other bacteria is clearly *Planococcus*, which through NMR characterisation has been shown to form 8′-apocarotenoids. Therefore, it would appear that the apocarotenoids in these *Bacillus* yellow/orange strains are similar to those determined in *Planococcus* and 8′-apo in nature [17,18]. The confirmation of the 4,4′-diapo or apo-8′ nature of these *Bacillus* carotenoids will be further carry out by NMR analysis. To form 8′-apocarotenoids geranylgeranyl diphosphate (C_{20}) and geranyl diphosphate (C_{10}) must be used as precursors instead of FPP.

3.4. Apocarotenoid formation during the cellular development of *Bacillus spp.*

The ability of *B. indicus* HU36 and other orange/yellow species to form spores during development has been reported previously [42] along with a concurrent colour change from yellow to orange. A detailed analysis of development was thus carried out to define pigment formation during sporulation. Cultivation in DSM media revealed the presence of vegetative cells at day 1 exclusively, by day 2 55% sporulation had occurred, reaching a peak at day 3 (Table 4). The content of the yellow pigment 1-glycosyl-3,4-dehydro-apo-8′-lycopene was comparatively constant over development. However, the orange pigment methyl 1-glycosyl-3,4-dehydro-apo-8′-lycopenoate significantly increased ($p<0.05$) by about 43% with the onset of sporulation (Table 4; Supplementary Fig. 2). In TY media sporulation did not occur and consistent with our earlier observations in DSM medium the orange pigment methyl 1-glycosyl-3,4-dehydro-apo-8′-lycopenaoate did not accumulate (Table 4; Supplementary Fig. 2). These data suggest that apocarotenoid formation in these pigmented *Bacilli* is, in part, under developmental regulation with the formation of apocarotenoid methyl 1-glycosyl-3,4-dehydro-apo-8′-lycopenoate strictly coordinated with this developmental event.

The detailed analysis of pigments over development and flowing DPA treatment has enabled the prediction of a putative biosynthetic pathway in these *Bacilli* (Fig. 4). The formation of apolycopenoic acid which is subsequently methylated, will presumably require the formation of an aldehyde and then an acid. The sequence of these oxidation reactions will be important in ascertaining the developmental regulation of the pathway associated with sporulation.

Table 4
Changes in carotenoid content associated with different sporulation states of *B. indicus* HU36.

				Carotenoid content (μg/ g DW)				
Media	Time (day)	% Sporulation	Biomass (mg DW)	Total	UNK-8 min	Yellow	Orange	Apo-8′-phytoene
DSM	**1**	0	20.5	111.03 ± 13.8	1.23 ± 0.06	43.6 ± 13.7	66.23 ± 0.06	ND
	2	50.7 ± 8.9	19.2	175.5 ± 41.5	1.96 ± 0.42	39.6 ± 1.8	133.8 ± 39.3	ND
	3	60.2 ± 10.5	17.3	88.8 ± 2.01	1.58 ± 0.43	10.7 ± 0.4	76.5 ± 2.04	ND
TY	**1**	0	37.3	123.1 ± 34.1	3.9 ± 0.9	110.03 ± 45.9	ND	18.1 ± 0.00
	2	0.03 ± 0.01	120	130.2 ± 3.3	15.9 ± 6.3	86.5 ± 2.5	14.1 ± 0.4	13.71 ± 0.04
	3	0.02 ± 0.005	69	34.1 ± 7.8	24.3 ± 0.3	34.1 ± 7.8	11.5 ± 2.8	12.2 ± 3.3

Analysis and quantification of the carotenoids present has been described in the Materials and methods section. The yellow pigment is glycosyl-apolycopene, the orange pigment is methyl glycosyl-apolycopenoate and the colourless precursor is apophytoene. UNK-8 min indicates a non-identified carotenoid eluting at 8 min. Its presence was confirmed in extracts prepared from HU36 grown in either on DSM or TY media. Sporulation efficiency (% Sporulation) equals to the c.f.u. of HRS divided by c.f.u. of veg. cells ×100. Where c.f.u. represents colony forming unit and HRS, heat-resistant spores. ND indicates not detected and DW, dry weight. The data presented are the mean of three independent determinations ± standard error of the mean (SEM).

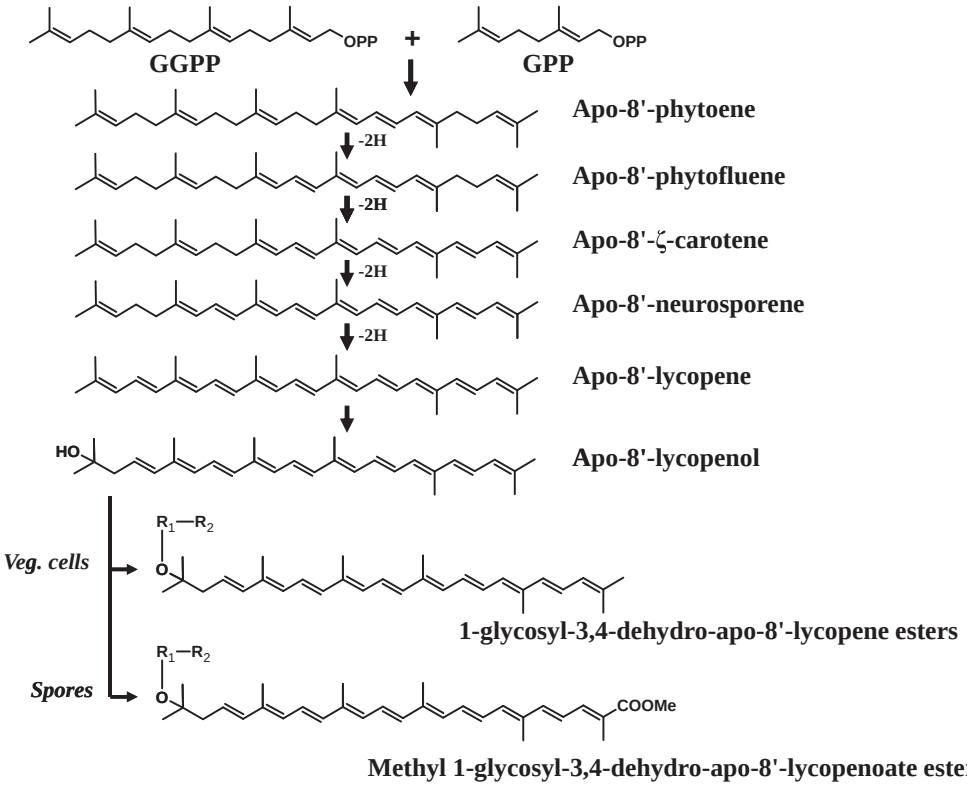

Fig. 4. Proposed biosynthetic pathway of apocarotenoids in yellow/orange pigmented *Bacillus* species. GGPP—Geranylgeranyl diphosphate, GPP—Geranyl diphospahte, R_1—hexose and R_2—fatty acid.

4. Conclusions

A combination of biochemical techniques has been used in the present study to demonstrate the presence of C_{30} apocarotenoid derivatives in certain pigmented *Bacillus* spp. The yellow pigment 1-glycosyl-3-4-dehydro-8′-apolycopene ester predominates in vegetative cells, while the formation of the orange pigment methyl 1-glycosyl-3,4-dehydro-8′-apolycopenate ester is enhanced during sporulation. Thus developmental regulation of the biosynthetic pathway appears to occur in these *Bacillus* spp. The detailed analysis of pigments through cellular development and following treatment with inhibitors has enabled a putative biosynthetic pathway to be proposed. Whether these apocarotenoids are generated by the condensation of geranylgeranyl diphosphate (C_{20}) and geranyl diphosphate (C_{10}) to generate apo-8′ carotenoids or via two farnesyl diphosphate (C_{15}) molecules to yield 4,4′-diapocarotenoids awaits further elucidated by NMR. In summary the carotenoids present in the yellow/orange pigmented *Bacillus* spp. such as *B. indicus* HU36 have been characterised. This work can now serve as a platform to elucidate the developmental regulation of the pathway as well as determining the potential biotechnological importance of these molecules in the health, food and feed sectors.

Acknowledgments

The authors are grateful for funding through the EU FP7 Colorspore project number 207948. Prof. John Halket is thanked for utilisation of MS equipment, Mr Christopher Gerrish for excellent technical assistance and Dr Enrique Sentandreu for initial LC-MS familiarisation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbalip.2010.12.009.

References

[1] G. Britton, S. Liaaen-Jensen, H. Pfander (Eds.), Carotenoids Handbook. Carotenoids, Birkhauser Verlag, Basel, 2004.
[2] N. Barber, The tomato: an important part of the urologist's diet? BJU Int. 91 (2003) 307–309.
[3] E. Giovannucci, Tomatoes, tomato-based products, lycopene, and cancer: review of the epidemiologic literature, J. Natl. Cancer Inst. 91 (1999) 317–331.
[4] E. Giovannucci, A. Ascherio, E.B. Rimm, M.J. Stampfer, G.A. Colditz, W.C. Willett, Intake of carotenoids and retinol in relation to risk of prostate cancer, J. Natl. Cancer Inst. 87 (1995) 1767–1776.
[5] J.A. Olson, O. Hayaishi, The enzymatic cleavage of beta-carotene into vitamin A by soluble enzymes of rat liver and intestine, Proc. Natl. Acad. Sci. USA 54 (1965) 1364–1370.
[6] D.M. Snodderly, Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins, Am. J. Clin. Nutr. 62 (1995) 1448S–1461S.
[7] P.M. Bramley, Phytochemical functional foods, in: I. Johnson, G. Williamson (Eds.), Phytochemical Functional Foods, CRC Press, Boca Raton, 2003, pp. 253–279.
[8] G. Britton, S. LiaaenJensen, H. Pfander (Eds.), Carotenoids, vol. 1A. Isolation and Analysis. Carotenoids. Vol. 1A, Birkhauser Verlag, Basel, 1994.
[9] G. Sandmann, Carotenoid biosynthesis in microorganisms and plants, Eur. J. Biochem. 223 (1994) 7–24.
[10] G. Sandmann, N. Misawa, Fungal carotenoids, in: H.D. Osiewacz (Ed.), The Mycota — Industrial Applications, Springer-Verlag, Heidelberg, 2002, pp. 248–262.
[11] A.J. Aasen, G.W. Francis, S. Liaaen-Jensen, Bacterial Carotenoids XXIX. The carotenoids of two yellow halophilic cocci including a new glycosidic methyl apo-lycopenoate, Acta Chem. Scand. 23 (1969) 2605–2615.
[12] H. Kleinig, R. Schmitt, W. Meister, G. Englert, H. Thommen, New C-30 carotenoic acid glucosyl esters from pseudomonas-rhodos, Z. Fur. Naturforsch. C-J. Biosci. 34 (1979) 181–185.
[13] S. Kocher, J. Breitenbach, V. Muller, G. Sandmann, Structure, function and biosynthesis of carotenoids in the moderately halophilic bacterium *Halobacillus halophilus*, Arch. Microbiol. 191 (2009) 95–104.
[14] B.F. Lutnaes, A. Oren, S. Liaaen-Jensen, New C(40)-carotenoid acyl glycoside as principal carotenoid in *Salinibacter ruber*, an extremely halophilic eubacterium, J. Nat. Prod. 65 (2002) 1340–1343.
[15] J.H. Marshall, G.J. Wilmoth, Pigments of *Staphylococcus aureus*, a series of triterpenoid carotenoids, J. Bacteriol. 147 (1981) 900–913.
[16] J.C. McDermott, A. Ben-Aziz, R.K. Singh, G. Britton, T.W. Goodwin, Recent studies of carotenoid biosynthesis in bacteria, Pure Appl. Chem. 35 (1973) 29–45.
[17] A. Osawa, Y. Ishii, N. Sasamura, M. Morita, S. Kocher, V. Muller, G. Sandmann, K. Shindo, Hydroxy-3,4-dehydro-apo-8′-lycopene and methyl hydroxy-3,4-

dehydro-apo-8′-lycopenoate, novel C(30) carotenoids produced by a mutant of marine bacterium *Halobacillus halophilus*, J. Antibiot. Tokyo 63 (2010) 291–295.
[18] K. Shindo, M. Endo, Y. Miyake, K. Wakasugi, D. Morritt, P.M. Bramley, P.D. Fraser, H. Kasai, N. Misawa, Methyl glucosyl-3, 4-dehydro-apo-8′-lycopenoate, a novel antioxidative glyco-C-30-carotenoic acid produced by a marine bacterium *Planococcus maritimus*, J. Antibiot. 61 (2008) 729–735.
[19] K. Shindo, K. Mikami, E. Tamesada, S. Takaichi, K. Adachi, N. Misawa, T. Maoka, Diapolycopenedioic acid xylosyl ester, a novel glyco-C-30-carotenoic acid produced by a new marine bacterium *Rubritalea squalenifaciens*, Tetrahedron Lett. 48 (2007) 2725–2727.
[20] S. Takaichi, K. Inoue, M. Akaike, M. Kobayashi, H. Oh-oka, M.T. Madigan, The major carotenoid in all known species of heliobacteria is the C30 carotenoid 4,4′-diaponeurosporene, not neurosporene, Arch. Microbiol. 168 (1997) 277–281.
[21] R.F. Taylor, B.H. Davies, The triterpenoid carotenoids and related terpenoids in *Staphylococcus aureus* 209P, Can. J. Biochem. Cell Biol. 61 (1983) 892–905.
[22] M. Teramoto, N. Rahlert, N. Misawa, G. Sandmann, 1-hydroxy monocyclic carotenoid 3,4-dehydrogenase from a marine bacterium that produces myxol, FEBS Lett. 570 (2004) 184–188.
[23] F. Bouvier, A. Rahier, B. Camara, Biogenesis, molecular regulation and function of plant isoprenoids, Prog. Lipid Res. 44 (2005) 357–429.
[24] H. Kleinig, R. Schmitt, On the biosynthesis of C30 carotenoic acid glucosyl esters in *Pseudomonas rhodos*—analysis of car-mutants, Z Fur Naturforsch CJ Biosci. 37 (1982) 758–760.
[25] L. Tao, A. Schenzle, J.M. Odom, Q. Cheng, Novel carotenoid oxidase involved in biosynthesis of 4,4′-diapolycopene dialdehyde, Appl. Environ. Microbiol. 71 (2005) 3294–3301.
[26] B.H. Davies, R.F. Taylor, The biosynthesis of triterpenoid carotenoids in *Streptococcus faecium* UNH 564P, Can. J. Biochem. 60 (1982) 684–692.
[27] S. Takaichi, H. Oh-Oka, T. Maoka, D.O. Jung, M.T. Madigan, Novel carotenoid glucoside esters from alkaliphilic heliobacteria, Arch. Microbiol. 179 (2003) 95–100.
[28] J.H. Marshall, G.J. Wilmoth, Proposed pathway of triterpenoid carotenoid biosynthesis in *Staphylococcus aureus*—evidence from a study of mutants, J. Bacteriol. 147 (1981) 914–919.
[29] A. Pelz, K.P. Wieland, K. Putzbach, P. Hentschel, K. Albert, F. Gotz, Structure and biosynthesis of staphyloxanthin from *Staphylococcus aureus*, J. Biol. Chem. 280 (2005) 32493–32498.
[30] C.I. Liu, G.Y. Liu, Y. Song, F. Yin, M.E. Hensler, W.Y. Jeng, V. Nizet, A.H. Wang, E. Oldfield, A cholesterol biosynthesis inhibitor blocks *Staphylococcus aureus* virulence, Science 319 (2008) 1391–1394.
[31] G.Y. Liu, A. Essex, J.T. Buchanan, V. Datta, H.M. Hoffman, J.F. Bastian, J. Fierer, V. Nizet, *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity, J. Exp. Med. 202 (2005) 209–215.
[32] Y. Song, C.I. Liu, F.Y. Lin, J.H. No, M. Hensler, Y.L. Liu, W.Y. Jeng, J. Low, G.Y. Liu, V. Nizet, A.H. Wang, E. Oldfield, Inhibition of staphyloxanthin virulence factor biosynthesis in *Staphylococcus aureus*: in vitro, in vivo, and crystallographic results, J. Med. Chem. 52 (2009) 3869–3880.
[33] R. Khaneja, L. Perez-Fons, S. Fakhry, L. Baccigalupi, S. Steiger, E. To, G. Sandmann, T. C. Dong, E. Ricca, P.D. Fraser, S.M. Cutting, Carotenoids found in *Bacillus*, J. Appl. Microbiol. 108 (2010) 1889–1902.
[34] H.A. Hong, J.M. Huang, R. Khaneja, L.V. Hiep, M.C. Urdaci, S.M. Cutting, The safety of *Bacillus subtilis* and *Bacillus indicus* as food probiotics, J. Appl. Microbiol. 105 (2008) 510–520.
[35] G. Britton, S. LiaaenJensen, H. Pfander (Eds.), Carotenoids, vol. 1B. Spectroscopy. Vol. 1B, Birkhauser Verlag, Basel, 1995.
[36] W.L. Nicholson, P. Setlow, Sporulation, germination and outgrowth, in: C.R. Harwood, S.M. Cutting (Eds.), Molecular Biological Methods for Bacillus, John Wiley & Sons, Ltd, Chichester, UK, 1990.
[37] P.D. Fraser, M.E. Pinto, D.E. Holloway, P.M. Bramley, Technical advance: application of high-performance liquid chromatography with photodiode array detection to the metabolic profiling of plant isoprenoids, Plant J. 24 (2000) 551–558.
[38] G. Britton, S. LiaaenJensen, H. Pfander, Carotenoids Handbook. Carotenoids Handbook, Birkhauser Verlag Ag, 2004.
[39] B. Wieland, C. Feil, E. Gloria-Maercker, G. Thumm, M. Lechner, J.M. Bravo, K. Poralla, F. Gotz, Genetic and biochemical analyses of the biosynthesis of the yellow carotenoid 4,4′-diaponeurosporene of *Staphylococcus aureus*, J. Bacteriol. 176 (1994) 7719–7726.
[40] T. Kaneda, Fatty acids in the genus *Bacillus*. I. Iso- and anteiso-fatty acids as characteristic constituents of lipids in 10 species, J. Bacteriol. 93 (1967) 894–903.
[41] R. Candau, E.R. Bejarano, E. Cerda-Olmedo, In vivo channeling of substrates in an enzyme aggregate for beta-carotene biosynthesis, Proc. Natl Acad. Sci. USA 88 (1991) 4936–4940.
[42] L.H. Duc, P.D. Fraser, N.K.M. Tam, S.M. Cutting, Carotenoids present in halotolerant *Bacillus* spore formers, FEMS Microbiol. Lett. 255 (2006) 215–224.
[43] R.F. Taylor, B.H. Davies, A cell-free system from *Streptococcus faecium* for studies on the biosynthesis of triterpenoid carotenoids, Can. J. Biochem. 60 (1982) 675–683.