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ORIGINAL PAPER

The biosynthetic pathway to a novel derivative of 4,4′-diapolycopene-4,4′-oate in a red strain of *Sporosarcina aquimarina*

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Abstract In a red bacterial strain SF238 belonging to *Sporosarcina aquimarina,* a C_{30} carotenoid biosynthetic pathway was identified. It has been reconstructed by analysis of intermediates that accumulate in two different pigment mutants. It starts with the synthesis of 4,4′-diapophytoene and proceeds with its desaturation to 4,4′-diapolycopene, which is then oxidized to 4,4′-diapolycopene-4,4′-dioate. Using a combination of HPLC–PDA and LC–MS/MS analyses, the final product of this pathway was identified as acetyl-4,4′-diapolycopene-4,4′-dioate. This is a novel carotenoid not reported in any organisms to date. It could be demonstrated that this carotenoid has excellent antioxidative properties to protect from photosensitized peroxidation reactions like other related 4,4′-diapolycopene-4,4′-dioate derivatives.

Keywords Acetyl-4 · 4′-Diapolycopene-4 · 4′-Dioate · Antioxidative property · C_{30} biosynthesis pathway · Chemical modification

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Introduction

Among the spore-forming bacteria, non-pigmented strains dominate. However, several pigmented isolates have been reported over the years (Rüger and Koploy 1980; Duc et al. 2006). They show either an orange/yellow or a reddish/pink phenotype. The latter pigmentation was found for *Bacillus megaterium* (Mitchell et al. 1986) and some isolates of *Bacillus firmus* (Pane et al. 1996). A recent paper (Khaneja et al. 2010) describes a selection of pigmented spore-forming bacteria isolated from soil and sea water. Spectral (UV/Vis) differences between extracts suggested the presence of different acyclic carotenoids including those of a C_{30} pathway.

In most bacteria, the carotenoids are of the C_{40}-type with an acyclic carbon chain or ß-ionone groups at both ends. These C_{40}-carotenoids originate from the condensation of two molecules of C_{20} geranylgeranyl pyrophosphate (Sandmann 2001). Bacteria are the only organisms which in addition can synthesize C_{50}, C_{45} and C_{30} carotenoids. While C_{45} and C_{50} carotenoids are derived from C_{40} structures by extensions with C_5 prenyl groups (Krubasik et al. 2001), formation of C_{30} carotenoids proceeds via the condensation of two molecules of C_{15} farnesyl pyrophosphate (Taylor 1984).

Recently, the carotenoids from yellow/orange spore-forming *Bacillus indicus* and *Halobacillus halophilus* have been structurally elucidated (Osawa et al. 2010; Perez-Fons et al. 2011). Both synthesize the C_{30} glycosyl-4′-methyl-diapolycopenoate fatty acid esters. In addition, several carotenoid intermediates of the C_{30} pathway to the end product have been found in both species. Staphyloxanthin, a diaponeursoporene derivative, is the major carotenoid in *Staphylococcus aureus* (Pelz et al. 2005). In contrast to the yellow/orange carotenoids, very little is known about the

nature of the reddish pigments in spore-forming strains nor are any data available about their biosynthesis pathway to date. In a recently isolated *Sporosarcina aquimarina*-related strain SF238 (Khaneja et al. 2010), a unique absorbance spectrum pointed at the formation of an atypical carotenoid. In this investigation, we isolated this carotenoid from *S. aquimarina* strain SF238 and elucidated its structure by HPLC and MS analyses. Two mutant strains helped to establish the individual steps of this carotenogenic pathway towards the C_{30} end product.

Materials and methods

Growth and carotenoid isolation

By phylogenetic analysis, *S. aquimarina* strain SF238 (Khaneja et al. 2010) belongs to *S. aquimarina* (Yoon et al. 2001) in the order Bacillales. It was cultivated as shake culture for three days in LB medium. Cells were freeze-dried after harvest. Carotenoids were extracted with methanol, partitioned into 50 % ether in petrol and evaporated to dryness. After resuspension in acetone, TLC on silica plates developed with CH_2Cl_2/methanol/acetone (8:1:1, v/v/v) was used for enrichment and pre-purification. The eluted carotenoid was further used for HPLC and MS analyses. In case of saponification, which was used as an analytical to cleave the carotenoid, 10 % KOH for hydrolysis in methanol (final concentration) was added and then heated for 15 min at 60 °C.

Mutagenesis of *S. aquimarina* strain SF238 was carried out with ethyl methanesulfonate (EMS) as described (Dubnau et al. 1973). A white mutant 238M1 and a pale yellow-pigmented 238M2 were selected from the plate. Singlet oxygen suppression was determined by photooxidation of linoleic acid with methylene blue as photosensitizer under illumination (7000 lux at 22 °C for 3 h). Formation of conjugated dienes was determined photometrically by measuring the absorbance at 235 nm (see Osawa et al. (2010) for experimental details). Activity is given as the IC_{50} value that indicates the carotenoid concentration responsible for 50 % quenching compared to a control without added carotenoid.

Mass analysis

Identification of carotenoids was carried out using the high-resolution Q-TOF mass spectrometer UHR-MAXIS (Bruker Daltonics, Bremen, Germany) on-line with a UHPLC UltiMate 3000 equipped with a PDA detector (Dionex Softron, Germering, Germany). Chromatographic separation was performed in system I in a similar manner to previously detailed for HPLC–PDA (Fraser et al. 2000) 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 (YMC Inc., Wilmington, NC, USA). The mobile phase was altered to facilitate ionization 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 1 min, held it for 5 min and followed by a linear gradient over 30 min to 10 % (A). This last condition was kept for 10 min in isocratic mode, and after that, initial conditions (100 % A) were restored for 2 min. The column was then re-equilibrated for 15 min. The flow rate used was 0.2 ml/min, and the injection volume was 10 µl. The ionization mode used was Atmospheric Pressure Chemical Ionisation (APCI) operating in positive mode. Capillary and APCI vapourization temperatures were set at 200 and 350 °C, respectively, and the dry gas (nitrogen) and nebulizer were set at 3 L min-1 and 3 bar, respectively. APCI source settings were as follows: corona discharge voltage at 4000 nA and a capillary voltage of 4 kV. A full MS scan was performed from 50 to 1000 m/z, and target mass window was set at 500-1,000 m/z, giving a transfer time of 65 µs and pre-pulse storage of 16.1 µs. MS/MS spectra were recorded at an isolation width of 0.5 m/z. Collision energy ramp from 40 to 80 eV was applied for target masses between 650 and 900 m/z. Instrument calibration was performed externally prior to each sequence with APPI/APCI calibrant solution (Fluka). Automated post-run internal calibration was performed by injecting identical APPI/APCI calibrant solution at the end of each sample run via a six-port divert valve equipped with a 20-µl loop.

HPLC analysis

HPLC analysis of the intermediates of the pathway accumulating in mutants 238M1 and 238M2 involved system II with a Nucleosil C18 5 μ column and acetonitrile/methanol/2-propanol (85:10:5, by volume) as the mobile phase. Separation was performed isocratically at a column temperature of 20 °C. Reference compounds diapophytoene, diaponeurosporene and diapolycopene (Raisig and Sandmann 2001) or 4,4′-diapolycopene-4,4′-dioate (Tao et al. 2005) were synthesized in *Escherichia coli* transformed with the genes of the C_{30} pathway.

Results and discussion

Carotenoid analysis and structure elucidation

The untreated carotenoid extract from *S. aquimarina* strain SF238 was separated in HPLC system I on a C_{30} column

(Fig. 1a). The three peaks 1, 1′ and 1″ appearing at retention times between 22 and 25 min represent the all-*trans* and two *cis* isomers of the major carotenoid from *S. aquimarina* strain SF238 due to their slightly shorter retention times compared to all-*trans* peak 1 (501 nm, Fig. 2), a downstream shift of the main absorbance maximum by 3 and 6 nm, respectively, and the appearance of a *cis* peak at 390 nm for 1″ indicating the presence of a *cis* double bond in the central region of the molecule. The absorbance maxima of the *trans* isomer was at 472, 501 and 535 nm (Fig. 2). In addition, a small peak 2 at 20.8 min was detected. After pre-purification and enrichment by TLC, its spectrum exhibited the absorbance maxima at 463 and 485 nm and a shoulder at 430 nm (Fig. 2). Saponification of the carotenoid extract from *S. aquimarina* strain SF238 used in Fig. 1a, modified carotenoid 1. This is indicated by the longer retention times of the isomers of the hydrolysis product of carotenoid 1, peaks 3, 3′ and 3″, which run between 23 and 27 min (Fig. 1b). In addition, the absorbance maxima were shifted to a shorter wavelength of 460, 491 and 422 nm (Fig. 2). The *cis* isomers 3′ and 3″ show the same spectral differences to all-*trans* (peak 3) as the isomers of compound 1. Upon chromatography of 4,4′-diapolycopene-4,4′-dioate as a standard (Fig. 1c), we found exactly the same retention times as for compound 3 generated by saponification of compound 1 and its *cis* isomers, the same spectrum (Fig. 2) and the same isomer composition.

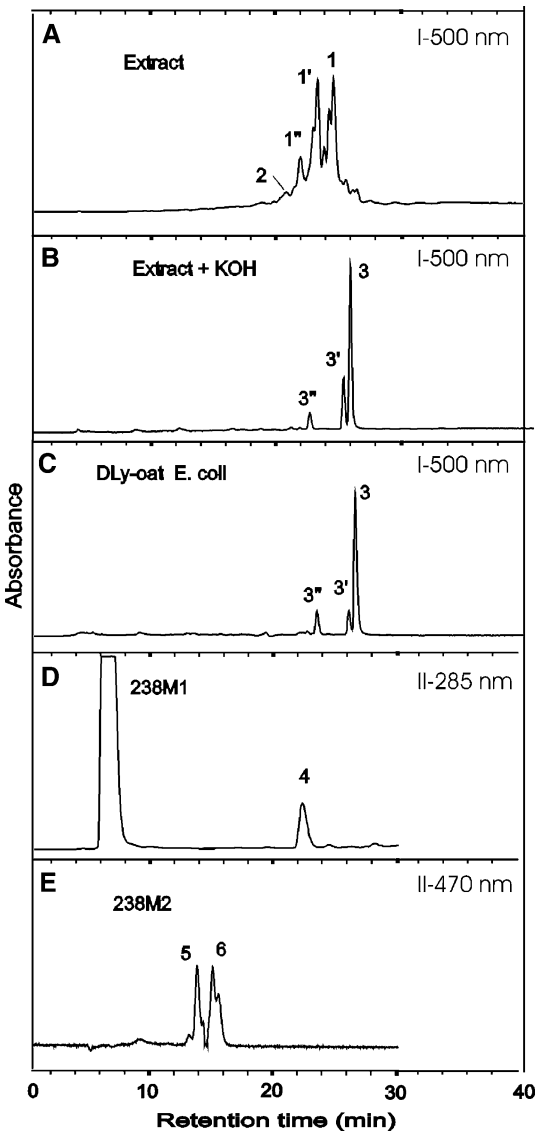

Fig. 1 HPLC separation of carotenoids from *Sporosarcina aquimarina* strain SF238. **a** Untreated extract, **b** Saponified extract, **c** 4,4′-Diapolycopene-4,4′-dioate as reference compound, **d** Extract from mutant 238M1 and **e** From mutant 238-M2. Separation of **a**–**c** was on a C_{30} column (system I), **d** and **e** on a C_{18} column (system II)

The mass and the fragmentation pattern of the major carotenoid 1 (without saponification) from *S. aquimarina* strain SF238 were determined by HPLC–MS. Fig. 3a shows the full scan of carotenoid 1. [M + H]+ of the highest mass corresponded to m/z 503.278. The calculated formula for this compound based on the accurate mass and the isotopic pattern was $C_{32}H_{38}O_5$. Formulas calculated for the most abundant m/z values are listed in Table 2. The second highest intensity peak was at [M + H]+ with m/z of 461.266. This corresponds to the mass of 4,4′-diapolycopene-4,4′-dioate probably generated by in-source fragmentation of the m/z 503.278 carotenoid during the ionization/nebulization step. Fragmentation pattern of the peak m/z 503.278 is shown in the MS/MS spectrum in Fig. 3. In addition to some residual m/z 503, prominent fragments that can be identified were at m/z 485.262, 443.257 and 425.247. This fits well into the fragmentation pattern for acetyl-4,4′-diapolycopene-4,4′-dioate together with its mass [M + H]+ of 503 as outlined in Fig. 3. The fragments were obtained by loss of water -18 Da or loss of acetoxyl -59 Da, and simultaneous loss of both residues by -77 Da. This corresponds to the change in HPLC retention time after hydrolysis (Fig. 1) and the short-range shift of the main absorbance maximum by 12 nm (Fig. 2). This indicates a loss of one double bond from the conjugated system (Takaichi 1992).

Acetyl-4,4′-diapolycopene-4,4′-dioate from SF238 is a unique carotenoid. It shares the basic C_{30} diapolycopene dicarboxylate structure, which is uncommon and restricted to only a few red-pigmented bacteria. These bacteria like *Methylobacterium rhodium* (formerly *Pseudomonas rhodos*) (Kleinig et al. 1979) and *Rubritalea squalenifaciens* (Shindo et al. 2007) accumulate 4,4′-diapolycopene-4,4′-dioate acylglyco ester. This carotenoid from *Rubritalea squalenifaciens* and also ethyl glucosyl-3,4-dehydro-apo8′-lycopenoate from *Planococcus maritimus* (Shindo et al. 2008b) also shows the highest $^1O_2$ quenching activity exhibiting IC_{50} values for 50 % quenching of 5.1 μM

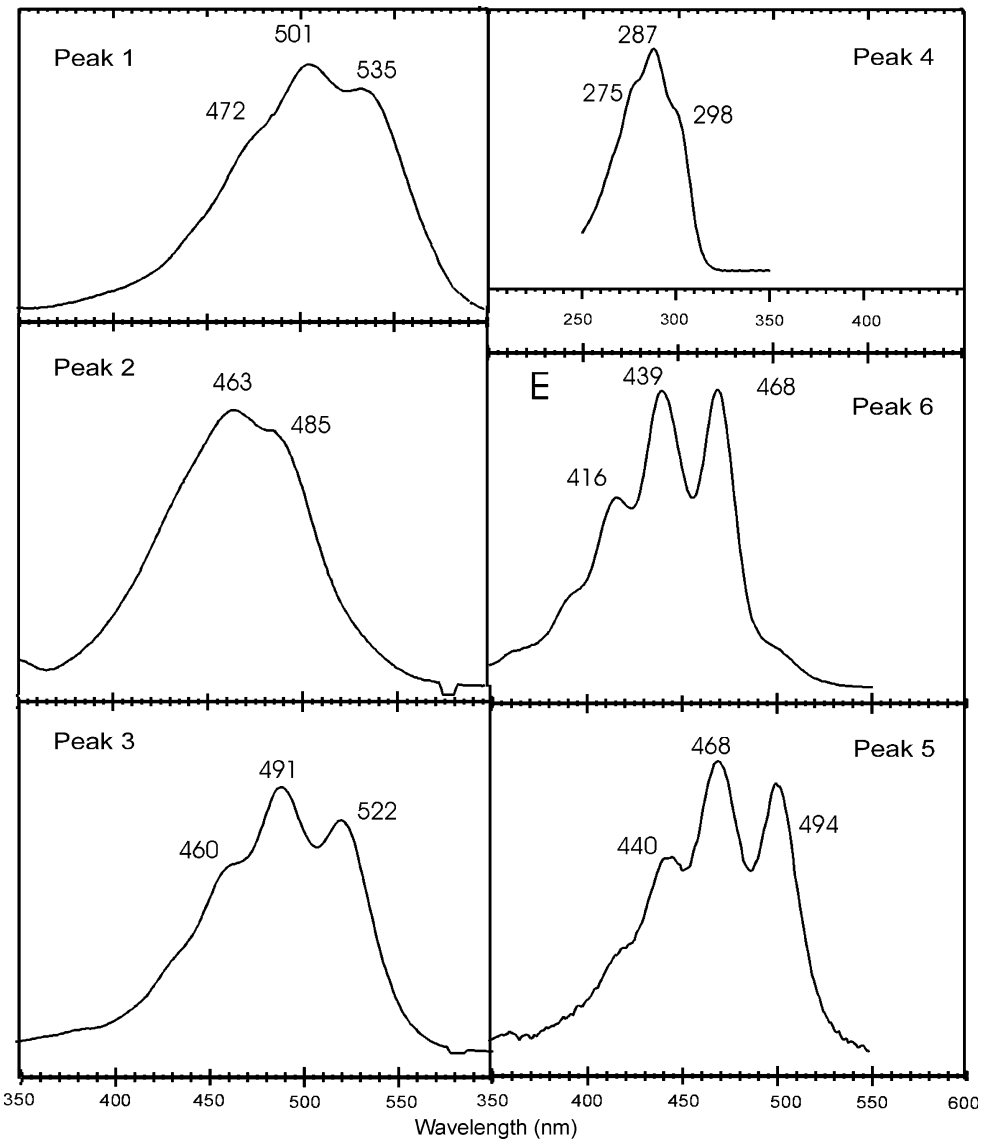

Fig. 2 Absorbance spectra of carotenoids from *Sporosarcina aquimarina* strain SF238. Peak number refers to HPLC separation in Fig. 1. The spectrum from compound/peak 3 is identical to that of 4,4′-diapolycopene-4,4′-dioate, compound 4 to 4,4′-diapophytoene, compound 5 to 4,4′-diapolycopene and compound 6 to 4,4′-diaponeurosporene

(Shindo et al. 2008a). Acetyl-4,4′-diapolycopene-4,4′-dioate was less effective with a slightly higher value of 6.8 µM (Table 1) but was superior to astaxanthin, which is regarded as a powerful antioxidant with an IC_{50} value of 8.9 µM (Tables 1, 2). Other C_{30} carotenoids are an order of magnitude less effective.

Establishment of the pathway

The C_{30} biosynthesis pathway leading to acetyl-4,4′-diapolycopene-4,4′-dioate via 4,4′-diapolycopene-4,4′-dioate was elucidated by analysis of carotenoid intermediates in pigment mutants 238M1 and 238M2. Fig. 1 trace D from 238M1 shows the carotenoid (peak 4) at 22.6 min at the same retention time as reference 4,4′-diapophytoene and the same spectrum with maxima at 275, 287 and 298 nm (Fig. 2). In 238M2, two different carotenoids could be identified (Fig. 1e) with the help of reference compounds (same retention times, same spectra). These were 4,4′-diapolycopene at a retention time of 13.4 min (peak 5) with absorbance maxima at 440, 468 and 494 nm and 4,4′-diaponeurosporene (peak 6) at 15.5 min with absorbance maxima at 416, 439 and 468 nm (Fig. 2). Due to the identified metabolites and the end product, a C_{30} carotenoid biosynthesis pathway can be established for *S. aquimarina* strain SF238 (Fig. 4). After synthesis of 4,4′-diapophytoene (compound 4), the desaturation steps proceed via 4,4′-diaponeurosporene (compound 6) to 4,4′-diapolycopene (compound 5), which is then terminally oxidized at both ends to carboxylic groups. The final step in the pathway is a condensation of one of the carboxylic groups with acetic acid. From *S. aquimarina* strain SF238 cultures, a minor carotenoid 2 could be enriched with a main maximum of 463 nm (Fig. 2, peak 2). The difference of 40 nm to acetyl-4,4′-diapolycopene-4,4′-dioate with a main maximum at 503 nm accounts for a lack of three double bonds in the

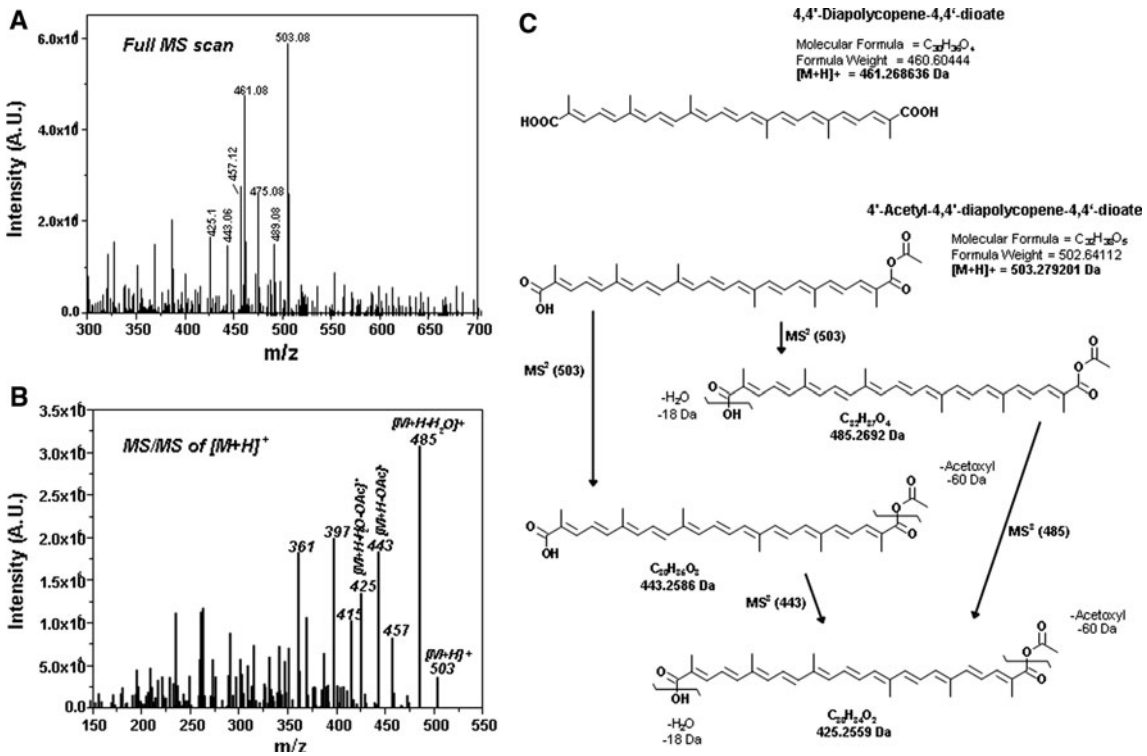

Fig. 3 Full scan (**a**) and MS/MS (**b**) analysis and fragmentation pattern (**c**) of the main carotenoid compound 1 from *Sporosarcina aquimarina* strain SF238

Table 1 Carotenoid $^1O_2$ quenching activity of acetyl-4,4′-diapolycopene-4,4′-dioate compared to other bacterial C_{30} carotenoids

IC_{50} value	
Acetyl-4,4′-diapolycopene-4,4′-dioate (compound 1)	6.8 μM (this work)
Acyl-4,4′-diapolycopene-4,4′-dioate xylosyl ester	5.1 μM (Shindo et al. 2008a)
Hydroxy-3,4-dehydro-apo-8′-lycopene	79 μM (Osawa et al. 2010)
Methyl hydroxyl-3,4-dehydro-apo-8′-lycopenoate	44 μM (Osawa et al. 2010)
Methyl glucosyl-3,4-dehydro-apo8′-lycopenoate	5.1 μM (Shindo et al. (2008b)
Astaxanthin	8.9 μM (Shindo et al. 2008a)
β-Carotene	>100 μM (Shindo et al. 2008a)

Table 2 Formulas calculated for the most abundant m/z values found in the mass spectrum of 4,4′-diapolycopene-4,4′-dioate derivatives and their corresponding fragments

Measured m/z	Formula calcd.	Calculated m/z	Error (mDa)
461.2663	$C_{30}H_{37}O_4$	461.2686	2.3
503.2781	$C_{32}H_{39}O_5$	503.2792	1.1
485.2620	$C_{32}H_{37}O_4$	485.2686	6.6
443.2572	$C_{30}H_{35}O_3$	443.2581	0.9
425.2474	$C_{30}H_{33}O_2$	425.2475	0.1

conjugated system. Therefore, we assume that this minor carotenoid 2 in *S. aquimarina* strain SF238 is acetyl-4,4′-diaponeurosporene-4-oate. It fits well into the biosynthetic pathway as a parallel reaction to acetyl-4,4′-diapolycopene-4,4′-dioate formation by the oxidation of 4,4′-diaponeurosporene instead of 4,4′-diapolycopene (Fig. 4). This C_{30} pathway in *S. aquimarina* resembles partially the one in *Staphylococcus aureus* along with 4,4′-diaponeurosporene from which both divert (Raisig and Sandmann 2001; Pelz et al. 2005). Carotenogenesis in *S. aquimarina* is much closer to the pathway in *Methylobacterium* or *Methylomonas* (Tao et al. 2005) sharing the reactions from 4,4′-diapophytoene to 4,4′-diapolycopene-4,4′-dioate (Fig. 4). Although 4,4′-diapolycopene-dial could not be detected in *S. aquimarina*, we assume a two oxidation reaction from 4,4′-diapolycopene via 4,4′-diapolycopene-dial to 4,4′-diapolycopene-4,4′-dioate in analogy to bacteria.

Conclusion

In *S. aquimarina* strain SF238 (Khaneja et al. 2010), which belongs to *Sporosarcina aquimarina* (Yoon et al. 2001), the C_{30} acetyl-4,4′-diapolycopene-4,4′-dioate was identified as a novel carotenoid and its pathway from

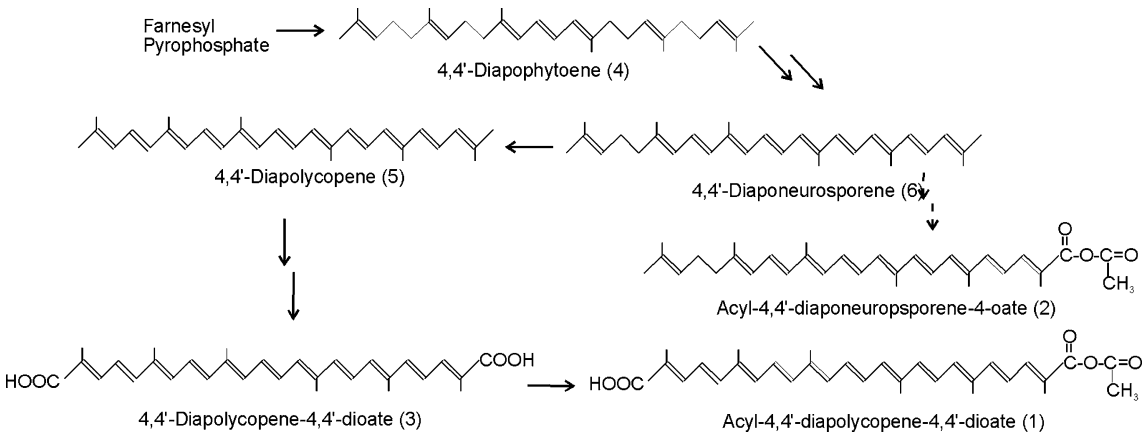

Fig. 4 The biosynthesis pathway in Sporosarcina *aquimarina* strain SF238 to acyl-4,4′-diapolycopene-4,4′-dioate

4,4′-diapophytoene established. The end product belongs to a series of diapolycopenoate derivatives with well-known antioxidant behaviour suppressing the generation of $^1O_2$ (Shindo et al. 2008a). This property was also demonstrated for acetyl-4,4′-diapolycopene-4,4′-dioate, indicating a function in protecting SF238 from photosensitized peroxidation reactions.

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