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ORIGINAL PAPER

Structure, function and biosynthesis of carotenoids in the moderately halophilic bacterium *Halobacillus halophilus*

Saskia Köcher · Jürgen Breitenbach · Volker Müller · Gerhard Sandmann

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Abstract Inhibitor studies and mutant analysis revealed a C_{30} pathway via 4,4′-diapophytoene and 4,4′-diaponeurosporene to 4,4′-diaponeursoporene-4-oic acid esters related to staphyloxanthin in *Halobacillus halophilus*. Six genes may be involved in this biosynthetic pathway and could be found in two adjacent gene clusters. Two genes of this pathway could be functionally assigned by functional pathway complementation as a 4,4′-diapophytoene synthase and a 4,4′-diapophytoene desaturase gene. These genes were organized in two operons together with two putative oxidase genes, a glycosylase and an acyl transferase ortholog. Pigment mutants were obtained by chemical mutagenesis. Carotenoid analysis showed that a white mutant accumulated 4,4′-diapophytoene due to a block in desaturation. In a yellow mutant carotenogenesis was blocked at the stage of 4,4′-diaponeurosporene and in an orange mutant at the stage of 4,4′-diaponeurosporene-4-oic acid. The protective function of these pigments could be demonstrated for *H. halophilus* after inhibition of carotenoid synthesis by initiation of oxidative stress. A degree of oxidative stress which still allowed 50% growth of carotenogenic cells resulted in the death of the cells devoid of colored carotenoids.

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S. Köcher · V. Müller
Molecular Microbiology and Bioenergetics,
Institute of Molecular Biosciences, Goethe University,
60054 Frankfurt/Main, Germany

J. Breitenbach · G. Sandmann (✉)
Biosynthesis Group, Institute of Molecular Biosciences,
Goethe University, Biocampus 213, P.O. Box 111932,
60054 Frankfurt/Main, Germany
e-mail: sandmann@bio.uni-frankfurt.de;
sandmann@em.uni-frankfurt.de

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Introduction

In contrast to photoautotrophic organism for which the presence of carotenoids as photoprotectants is essential, formation of carotenoids is found only in some heterotrophic microorganisms (Goodwin 1980). However, it is striking that carotenoids are widely distributed in extremophiles. Examples are the thermophilic bacterium *Thermus thermophilus* which synthesizes zeaxanthin and ß-cryptoxanthin glucoside fatty acid esters which help for membrane stabilization (Yokoyama et al. 1995, 1996), high salt requiring *Salinibacter ruber* with the glycosyl fatty acid ester salinixanthin (Lutnaes et al. 2002) and the psychrotrophic bacterium *Arthrobacter agilis* which increases its content in C_{50} bacterioruberin glycosides in response to low temperature (Fong et al. 2001). Bacterioruberin is also the major carotenoid found in the radioresistant bacterium *Rubrobacter radiotolerans* (Saito et al. 1994) and typical for halophilic archaea like *Halobacterium salinarum* (Kelly et al. 1970). Quite often, extremophiles accumulate glycosides of carotenoids of C_{30}, C_{40} and C_{50} carbon chain length as integral constituents of their membranes. By spanning through the membrane (Yokoyama et al. 1995), these carotenoids with the polar end groups influencing membrane fluidity and stability (Britton 1995). Furthermore, they prevent oxidation of the membranes due to their antioxidative properties (Woodall et al. 1997).

The basic carotenoid biosynthetic pathway in these bacteria starts with the synthesis of C_{40} phytoene from two molecules of geranylgeranyl pyrophosphate catalyzed by

the *crtB* gene product (Sandmann and Misawa 1992). Alternatively, the C_{30} equivalent diapophytoene is formed from farnesyl pyrophosphate by the ortholog CrtM (Wieland et al. 1994). In each case, a variable number of double bonds is introduced either by CrtI into phytoene or by CrtN into diapophytoene (Raisig and Sandmann 2001). Depending on the species, subsequent steps may include an extension of the C_{40} molecule to a C_{50} carbon skeleton, cyclization and formation of oxo groups (Britton 1988). Especially hydroxy or carboxy groups are important for glycosilation or esterification.

Halobacillus halophilus (formerly *Sporosarcina halophila*) is a moderatly halophilic bacterium (Claus et al. 1983; Spring et al. 1996). It can tolerate up to 3 M sodium chloride and compensates this osmotic burden by the accumulation of compatible solutes (Roeßler and Müller 1998, 2001; Müller and Saum 2005; Saum et al. 2006). Like several other halophilic and halotolerant bacteria (Aasen et al. 1969; Duc et al. 2006), *H. halophilus* is pigmented. First spectral analysis indicated that the orange pigmentation of *H. halophilus* is due to the presence of carotenoids (H. Flach, Thesis Maximilians-Universität, München, 2003). Therefore, the present study is focused on the analysis of the carotenoids in *H. halophilus* including the function and the organization of the genes involved in the biosynthesis of these pigments. Furthermore, first indications on the role of carotenoids to protect from oxidative stress under saline growth were obtained.

Materials and methods

Organism and cultivation

Halobacillus halophilus (DSM 2266^T) was grown in complex medium (Nutrient Broth, Merck, Darmstadt, Germany, supplemented with 50 mM $MgCl_2$). The final concentration of NaCl varied depending on the assay conditions (values are given in the text). The pH was adjusted to 7.8 with NaOH. For inhibition experiments, diphenylamine (DPA) was applied in a concentration of 40 µM and duroquinone (DQ) at 100 µM. Growth experiments were done in 15 ml tubes filled with 5 ml of complex medium. After inoculation (5%) the cultures were incubated on a rotary shaker at 30°C. The optical density was determined in a Hitachi photometer at 578 nm. All data points given reflect the means of duplicate tubes from one experiment, and experiments were performed at least three times. For the preparation of spores, *H. halophilus* was grown on agar-solidified marine broth (Difco, Augsburg, Germany) supplemented with 0.08 mM $MnCl_2$ until sufficient refractile endospores were observed by phase-contrast light microscopy. Preparation of spores was done as described previously (Dohrmann and Müller 1999). *Escherichia coli* DH5α and JM101 were used for plasmid amplification, and genetic pathway complementation, respectively. They were grown in LB medium (Sambrook et al. 1989) at 37°C with added antibiotics according to the plasmids carried by *E. coli*, ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml). *Staphylococcus carnosus* transformants carrying pTXcrtPQMN (Pelz et al. 2005) were grown as described.

Random mutagenesis

Mutagenesis of *H. halophilus* was carried out with cells grown to the mid exponential growth phase in NB-medium containing 1.0 M NaCl. After harvesting, the cells were washed once with NB-medium and re-suspended in the original volume of minimal G10 medium 1% glucose, NH_4Cl (2 g/l), $FeSO_4 \cdot 7\ H_2O$ (10 mg/l), Tris base (12 g/l), K_2HPO_4 (0.49 g/l), yeast extract (0.1 g/l), vitamin solution DSM 141 (1 ml/l), and artificial sea water DSM 79 (250 ml/l) in the presence of 1.0 M NaCl without any carbon source. To 2 ml of the cell suspension, 20 µl of EMS (ethyl methane sulfonate, Sigma-Aldrich, Steinheim, Germany) were added and mixed vigorously. After 20 min of incubation at 30°C, cells were washed twice in G10 medium, diluted 50-fold and grown in broth overnight. Different dilutions were plated on non-selective agar plates. Colonies were visually screened for white and yellow/orange pigmentation differing from the deep orange color of the wild type.

Gene sequences and plasmid constructions

DNA sequences were retrieved from the genome sequence of *H. halophilus* that will be described elsewhere. In the genome sequences of *H. halophilus* a gene orthologous to phytoene synthase genes and three genes orthologous to phytoene desaturase genes were identified. Their DNA sequences were deposited in the GenBank database with accession numbers FJ040212. All four genes were amplified by PCR and cloned in frame into the vector pUC19 (Yanisch-Perron et al. 1985). The primers used were listed in Table 1. All genetic manipulations were carried out according to Sambrook et al. (1989). Plasmids pACCRT-M and pACCRT-MN were used for pathway complementation and the generation of 4,4′-diapophytoene, 4,4′-diaponeurosporene and 4,4′-diapolycopene in *E. coli* (Raisig and Sandmann 1999). Plasmid pDCQ150ΔcrtNa was constructed from pDCQ150 (Tao et al. 2005) by deletion of the *crtNa* by digestion with *Bsm*I and re-ligation.

Database searches were carried out with the similarity search tool BLAST P 2.2.10 (Altschul et al. 1997).The phylogenetic tree was generated with programme Clustal X,

Table 1 Primers used in this study

Primer	Sequence	Use
crtNa(op)_for	5′ TACATTAGCGCCAGAGGGTC 3′	Bridge between *crtNa* and *crtNc* (Fig. 4a)
crtNc(op)_rev	5′ CTTCTCATCATCTTCTTAAC 3′	Bridge between *crtNa* and *crtNc* (Fig. 4a)
crtNc(op)_for	5′ CATTTCATCCCTCGATCGTTG 3′	Bridge between *crtNc* and *crtM* (Fig. 4a)
crtM(op)_rev	5′ ATAAGCCCAACCGTACTTGC 3′	Bridge between *crtNc* and *crtM* (Fig. 4a)
crtNb(op)_for	5′ CAGACGGTAGAGCTGCTGC 3′	Bridge between *crtNb* and *orf_GT* (Fig. 4a)
orf-GT(op)_rev	5′ AACGCCTATGAAGCGATCGG 3′	Bridge between *crtNb* and *orf_GT* (Fig. 4a)
orf-GT(op)_for	5′ CGATGATTGAAGGTGCAACG 3′	Bridge between *orf-GT* and *orf-AT* (Fig. 4a)
orf-AT(op)_rev	5′ GAGCAAGGCAGCACGGTATG 3′	Bridge between *orf-GT* and *orf-AT* (Fig. 4a)
crtNa-for-*Bam*HI	5′ CGGGATCCGATGAAGAAAGTGGCAG 3′	Amplification of *crtNa*
crtNa-rev-*Eco*RI	5′ CGGAATTCTATCATGAGGTTTCCGCC 3′	Amplification of *crtNa*
crtM-for-*Pst*I	5′ TTCTGCAGCATGATGGACTTGAATAATGCTTATGAC 3′	Amplification of *crtM*
crtM-rev-*Eco*RI	5′ CCGGAATTCTTATTGCATTTCCGCAATAATCTGC 3′	Amplification of *crtM*

Version 1.83 (Thompson et al. 1997) with correction settings for multiple substitution and the alignments.

visualized with TreeView. The number of bootstrap trials was 1000.

Transcriptional analysis

RNA isolation was carried out as described before (Saum et al. 2006). *H. halophilus* cells were harvested in the mid exponential growth phase (OD_{578} 0.7–1.0) and broken after lysozyme treatment (40 mg/ml, 5 min, room temperature) in a MM 301 mixer mill (Retsch, Germany) at 30 Hz for 5 min with zircon oxide balls (2 mm in diameter). Total RNA was prepared by using a NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. cDNA synthesis was accomplished using Moloney murine leukemia virus reverse transcriptase RNase H Minus, point mutant (Promega, Mannheim, Germany). To prevent RNA degradation during this step, 48 U of recombinant RNasin RNase inhibitor (Promega, Mannheim, Germany) was added. The cDNA was then used as a template in a PCR.

Carotenoid analysis

For carotenoid extraction, 20 mg of *H. halophilus* freeze dried cells or spores were suspended in 10-ml 10 mM Tris–HCl and broken in a French pressure cell (95 MPa). The homogenate was mixed with 20 ml acetone and heated for 15 min at 60°C. Carotenoids were extracted from freeze-dried cells of *E. coli* or *Staphylococcus carnosus* directly with methanol and heating. Partitioning against 50% ether in petrol concentrated the carotenoids in the upper phase. The extracts were used for TLC on silica gel developed with varying acetone concentrations in petrol and for HPLC analysis. The HPLC system involved a HyPurity C18 5-µm column with acetonitrile/methanol/2-propanol (85:10:5, by volume) as the mobile phase for isocratic elution at a column temperature of 20°C (Steiger and Sandmann 2004). Detection was with a Kontron DAD 440 photodiode array detector with on-line registration of the spectra. Reference compounds were extracted from different sources. Staphyloxanthin was obtained from *S. carnosus* transformants carrying pTXcrtOPQMN (Pelz et al. 2005) and enriched by TLC on silica plates using 35% acetone in petrol (Marshall and Wilmoth 1981). The major carotenoids from *H. halophilus* were purified by two TLC steps on silica gel involving first 35% acetone in petrol to remove the carotenes and then methanol/ethylacetate/petrol (25:25:50 by volume; Taylor and Davies 1983). 4,4′-Diaponeurosporene-4-oic acid was obtained from *E. coli* carrying plasmids pACCRT-MN together with pDCQ150ΔcrtNa. 4,4′-Diapolycopene-4-al together with 4,4′-diaponeurosporene-4-al were generated by expression of plasmids pDCQ166 and pDCQ177 in combination in *E. coli* (Tao et al. 2005). The mono aldehydes were enriched and isolated on silica TLC plates with 10% ethylacetate in toluene. Carotenoid samples were hydrolyzed in 10% KOH in methanol for 12 h at room temperature (Marshall and Wilmoth 1981).

Results

Carotenoid analysis

Extraction of carotenoids from *H. halophilus* cells was not effective by direct solvent extraction of fresh or freeze-dried cell which is routine for most other bacteria. Instead, cells had to be broken in a French pressure cell followed by acetone extraction. Upon separation by HPLC (Fig. 1a), two prominent peaks (nos. 3 and 5) appeared. The spectra are shown in Fig. 2. The absorbance maxima were 430, 453 and 485 for peaks 1, 2 and 6 (spectrum A) and 445, 466,

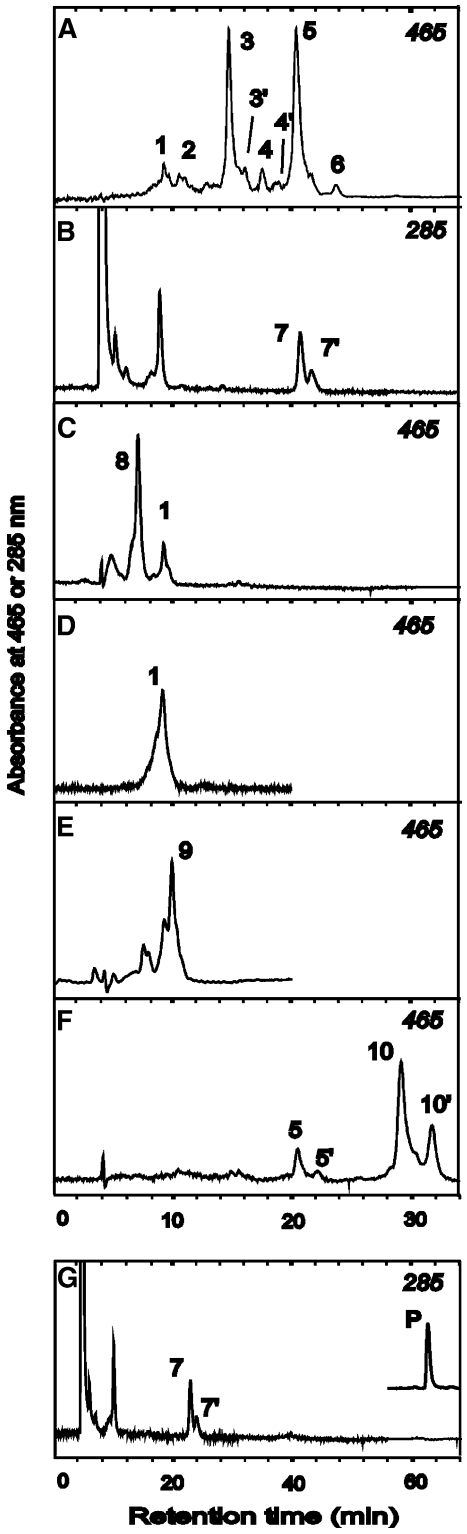

Fig. 1 HPLC separation of carotenoids from *Halobacillus halophilus* DSM 2266. **a** Cell extract, **b** cells treated with diphenylamine, **c** cell extract hydrolyzed with 10% KOH, **d** 4,4′-diapolycopene-4-oic acid (peak 1), **e** 4,4′-diaponeurosporene-4-al (peak 9), and F. staphyloxanthins with different fatty acids (peaks 5, 5′, 10 and 10′)

and 495 nm from peaks 3 to 5 (Fig. 2b). The spectrum of compounds 3–5 exhibits a low fine structure. This is typical for conjugated terminal aldehyde groups which shifts the absorbance maxima by 25–30 nm (Britton 1995). Peaks 3′ and 4′ can be regarded as *cis*-isomers due to a *cis*-peak at 358 nm. Co-chromatography with 4-keto-γ-carotene and 3-hydroxy-4-keto-γ-carotene with similar absorbance maxima and spectrum shape as in Fig. 2b (Sandmann et al. 2006) demonstrated the absence of both carotenoids.

First indication on the nature of the *H. halophilus* pigments was obtained by inhibition of carotene desaturation with diphenylamine (DPA). This inhibitor prevents the insertion of double bonds into phytoene and diapophytoene (Sandmann and Fraser 1993). In our case, the latter C_{30} carotenoid was accumulated (Fig. 1b) and identified as 15-*cis* (peak 7) and all-*trans* 4,4′-diapophytoene (peak 7′) by co-chromatography with an authentic standard generated by expression of a *crtM* gene in *E. coli*. The spectra (Fig. 2c) showed the typical maxima at 275, 285 and 295 nm. The C_{40} phytoene with an identical spectrum runs at a retention time of about 58 min in our HPLC system (Fig. 1g, peak P) and was absent in DPA-treated *H. halophilus* as indicated by the HPLC run of Fig. 1g which extends the separation from Fig. 1b to 60 min.

Due to their spectra and retention times, potential C_{30} candidates for compounds 3–5 are lipophilic glycosyl-4,4′-diaponeurosporene-4-oate esters (Marshall and Wilmoth 1981) including staphyloxanthin (Pelz et al. 2005). Related carotenoids with the spectrum of compound 1, 2 and 6 (Fig. 2b) could be other substituted 4,4′-diaponeurosporene-4-oic acids (Marshall and Wilmoth 1981; Pelz et al. 2005). They all can be hydrolyzed under alkaline conditions to 4,4′-diaponeurosporene-4-oic acid (Marshall and Wilmoth 1981). Hydrolysis of a *H. halophilus* carotenoid extract resulted in two different carotenoid products with retention times of 7.2 and 9.8 min (Fig. 1c, peaks 8 and 1). They both had a spectrum corresponding to the one in Fig. 2a, which is typical for the free acid (Marshall and Wilmoth 1981; Pelz et al. 2005). 4,4′-Diaponeurosporene-4-oic acid was extracted from *E. coli* carrying a plasmid for the synthesis of diapophytoene and a plasmid responsible for its subsequent conversion into the acid. The latter was used as a reference compound in the HPLC run of Fig. 1d. 4,4′-Diaponeurosporene-4-oic acid co-chromatographed with peak 1 and had the same spectrum. Therefore, compound 1 was identified as 4,4′-diaponeurosporene-4-oic acid. Another reference carotenoid was 4,4′-diaponeurosporene-4-al (Fig. 1e, peak 9) which was synthesiszed by *E. coli* expressing all genes for the generation of this aldehyde (Tao et al. 2005). It ran close behind compound 1 but had a different spectrum as shown in Fig. 2b. Its presence

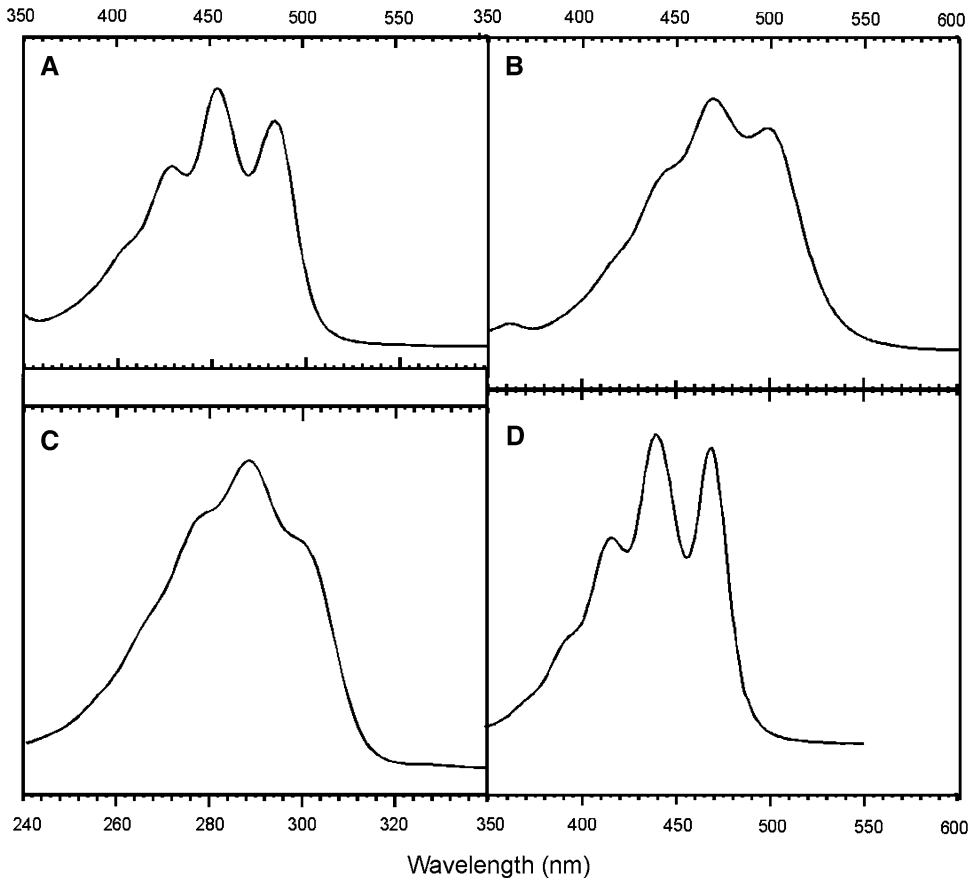

Fig. 2 Representative spectra of carotenoids from Figs. 1 and 3. **a** Compounds 1, 2 and 6; **b** compounds 3, 4 and 5; **c** compound 7 and **d** compound 11

as a *H. halophilus* carotenoid could be excluded. As a final standard we included a staphyloxanthin preparation from *S. carnosus* transformed with all genes for the synthesis of staphyloxanthin (Pelz et al. 2005). HPLC separation revealed two minor peaks around 20 min (Fig. 1f) and two larger ones around 30 min (peaks 5, 5′, 10 and 10′). Their spectra were all the same corresponding to that in Fig. 2b. They may resemble two different glycosyl fatty acid esters of staphyloxanthin with C_{15} and C_{17} fatty acids which were previously found in *S. aureus* (Marshall and Wilmoth 1981) together with the corresponding *cis*-isomers. The early-running staphyloxanthin was labeled as peak 5 since it had the same retention time (20.2 min) and the same spectroscopic property as compound 5 from *H. halophilus* (Fig. 1a). The carotenoid pattern of spores from *H. halophilus* was the same as in cells (Fig. 3a) with the exception of the minor compound 6 (Fig. 1a) which was completely missing.

Carotenoid mutants

In addition to inhibitors, pigment mutants are a useful tool to elucidate a carotenogenic pathway. Several mutants from *H. halophilus* were generated by chemical mutagenesis. They fell into three categories according to their pigmentation: light orange (mutant OC), bright yellow (mutant YC) and white (mutant WH). The carotenoids which accumulated were separated and compared to the carotenoids from wild type *H. halophilus* (Fig. 3). In OC the prominent carotenoid (trace B, peak 1) had the same retention time and spectrum as diaponeurosporenoic acid. In WH the only detectable carotenoid was 15-*cis* diapophytoene and the all-*trans* isomer (trace C, peaks 7 and 7′). In YC only diaponeurosporene (trace D, peak 11) was detectable. Its spectrum is that of Fig. 2d.

Identification of carotenogenic genes

By searching the genome of *H. halophilus*, two clusters with three putative carotenoid biosynthesis genes each were identified by BLAST analysis. The organization of these biosynthesis genes is shown in Fig. 4a. Genes c*rtNa*, *crtNc* and *crtM* are arranged in the same orientation. *CrtNa* and *crtNc* show an overlap of 3 bp, and *crtNc* and *crtM* are separated by only 3 bp; 429 bp downstream of *crtM crtNb, orf-GT* and *orf-AT* build a second cluster in the opposite orientation. They are separated by 21 bp between *orf-GT* and *orf-AT* and overlap 117 bp in the case of *crtNb* and *orf-GT*.

Transcriptional organization of carotenogenic genes

The arrangement of the putative carotenoid biosynthesis genes is indicative of their organization in transcriptional

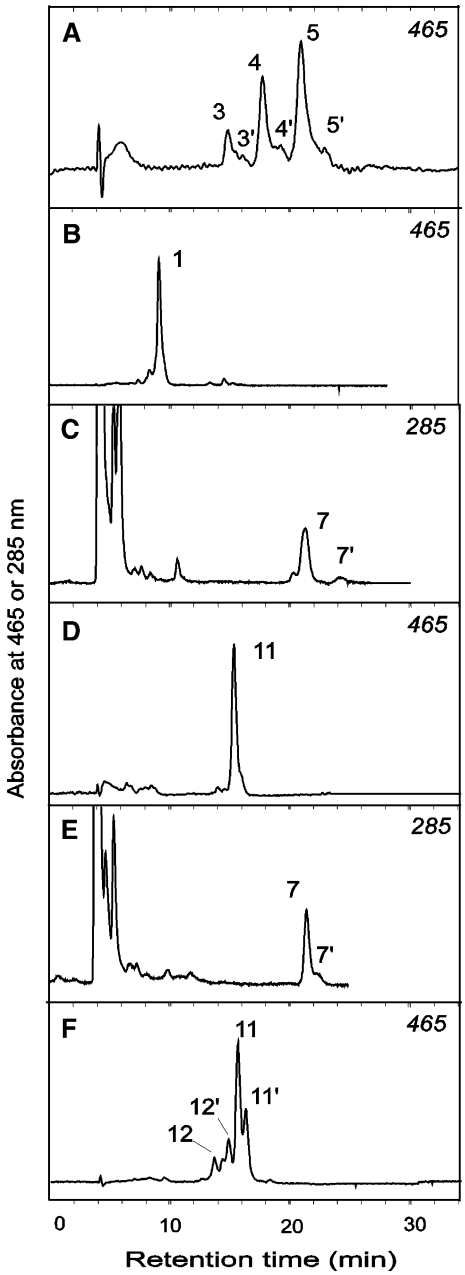

Fig. 3 HPLC separation of carotenoids from *Halobacillus halophilus* DSM 2266 and from complementation of *crt* genes. **a** spore extract from wild type, **b** mutant OC1, **c** mutant WH, **d** mutant YC, **e** complementation with $crtM_{Hh}$, **f** complementation with $crtNa_{Hh}$. Peaks of compounds already shown in Fig. 1 have the same number as there

units. To prove the existence of such a polycistronic messenger, mRNA was isolated from *H. halophilus* cells and transcribed into cDNA. This cDNA was then used as a template in a PCR using primers that bridge the intergenic regions between *crtNa* and *crtNc,* between *crtNc* and *crtM*, between *crtNb* and *orf-GT* and between *orf-GT* and *orf-AT* as indicated in Fig. 4a. Products with the expected fragment sizes of 928, 848, 811 and 765 bp were obtained indicating that the cDNA originated from two different polycistronic mRNA. This result demonstrates that *crtNa*, *crtNc* and *crtM* as well as *crtNb*, *orf-GT* and *orf-AT* are part of an operon, respectively.

Properties of deduced gene products

Three of the gene products, designated CrtNa, CrtNb and CrtNc, show sequence homology to bacterial carotene desaturases. CrtNa has 48% amino acid identity (similarity 71%) with that of a CrtN homologue in *Methylomonas* sp. 16a. CrtNb had 31% amino acid identity (similarity 54%) with that of a 4, 4′-diapolycopene oxidase in *Methylomonas* sp. 16a and 31% amino acid identity (similarity 54%) with the diapophytoene desaturase in *Psychroflexus torques* ATCC 700755. We could also identify a third homologue of a diapophytoene desaturase. CrtNc is highly similar to the diapophytoene dehydrogenase of *Heliobacillus mobilis* (AAC84034) (55%). A phylogenetic tree was constructed involving the genes $crtNa_{Hh}$, $crtNb_{Hh}$ and $crtNc_{Hh}$ from *H. halophilus* (Fig. 4b). All *crtN* genes were related to *crtI*. Nevertheless, they were separated from this gene encoding phytoene desaturase from the C40 pathway. The gene $crtNa_{Hh}$ clustered with functionally identified diapophytoene desaturases, $crtNb_{Hh}$ with ORFcrtP from *S. aureus* (Pelz et al. 2005) and $crtN_b$ *Methylomonas*, and $crtNc_{Hh}$ with the gene for an aldehyde oxidase from the latter bacterium (Tao et al. 2005). Gene *crtM* compares best to a putative phytoene/diapophytoene synthase that catalyses the condensation of two molecules of farnesyl-pyrophosphate. The protein of the latter has a length of 301 amino acids and is 55 and 44% identical (similarities, 71 and 61%) to CrtM from *Bacillus* sp. NRRL B-14911 and *Oceanobacillus iheyensis*, respectively.

Besides these typical proteins for carotenoid biosynthesis, we could identify two additional proteins that appear to be involved in pigment biosynthesis in *H. halophilus*. The product of orf-GT with a deduced length of 369 amino acids is similar to a glycosyl transferase from *Chlorobium tepidum* TLS (57% similarity, 35% identity) involved in carotenoid synthesis. Orf-AT is a putative acyl-transferase with a deduced length of 236 amino acids. It is 57% similar (35% identity) to the protein found in *Exiguobacterium sibiricum* 255-15.

Functional analyses of carotenogenic gene products from *H. halophilus*

The functions of $crtM_{Hh}$ and $crtNa_{Hh}$ were analyzed by pathway complementation in *E. coli* (Fig. 3e, f). With plasmid pUC19CRT-M_{Hh} containing the *crtM* gene from *H. halophilus*, diapophytoene was synthesized in *E. coli* (trace E, peaks 7 and 7′) from pre-existing farnesyl pyrophosphate. Upon expression of pACCRT-M, diapophyto-

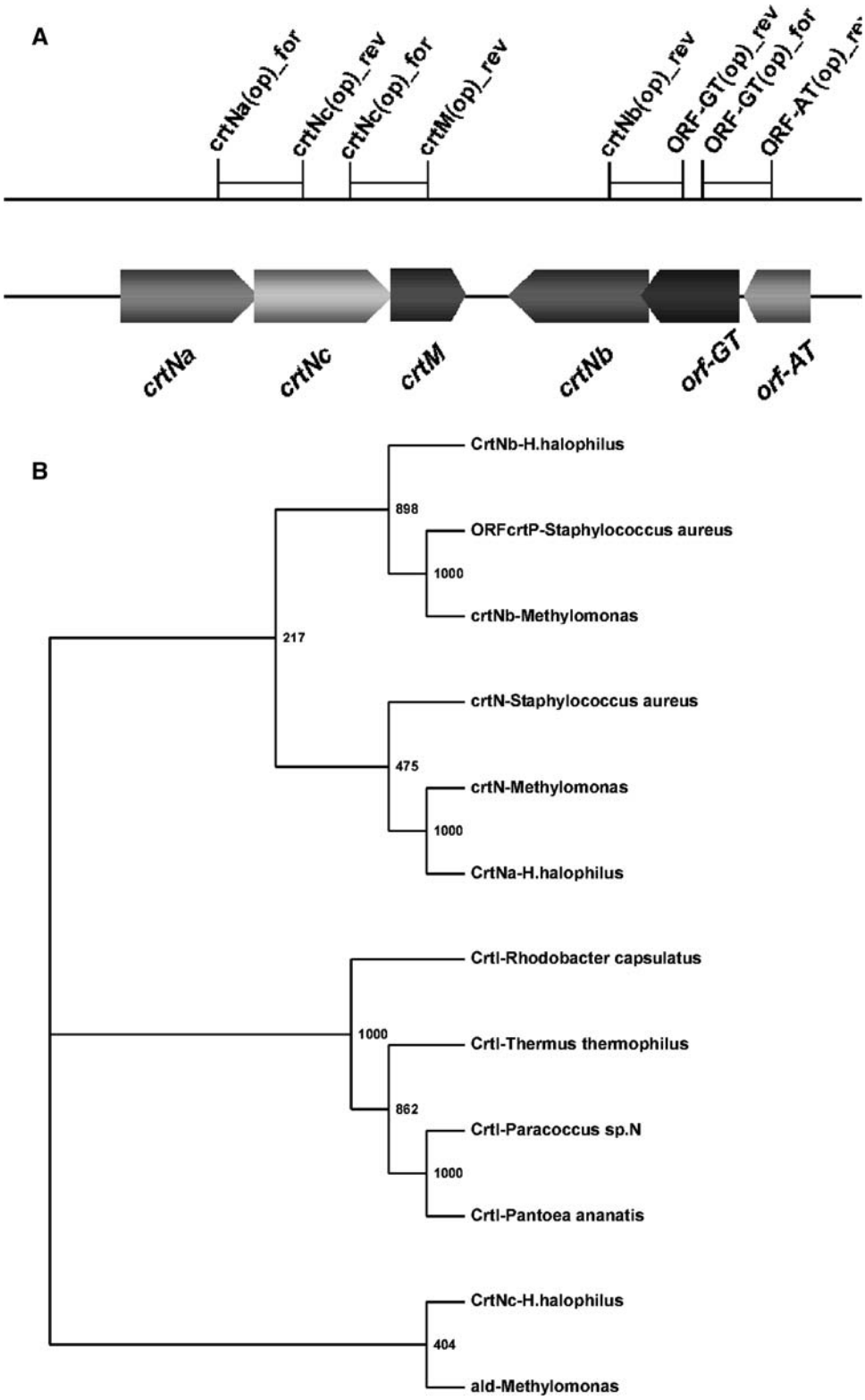

Fig. 4 Organization of carotenogenic gens in *Halobacillus halophilus* DSM 2266 (**a**) and phylogenetic relationship of three types of *crtI*-related genes (**b**). The top of part A indicates the regions of the two operons amplified by PCR from the cDNA

ene is synthesized in *E. coli*. Simultaneously, co-existing pUC19CRTNa$_{Hh}$, with the *crtNa* gene from *H. halophilus* mediates the formation of diaponeurosporene (trace F, peaks 11 and 11′) as the major carotenoid in *E. coli* accompanied with smaller amounts of diapolycopene (peaks 12 and 12′). For the genes *crtNb*$_{Hh}$ and *crtNc*$_{Hh}$, we were unable to obtain a co-expression with plasmids producing either diaponeurosporene or diaponeurosporene-4-al.

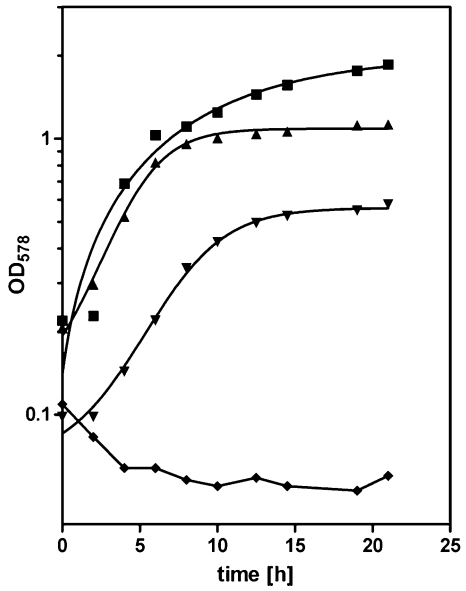

Fig. 5 Effect of inhibition of carotenoid biosynthesis on growth of *H. halophilus* DSM 2266 under oxidative stress conditions. Cells were grown on NB with 1.0 M NaCl in the absence (*square*) or presence of 40 µM diphenylamine (*triangle*), 100 µM duroquinone (*inverted triangle*) or a combination of 40 µM diphenylamine and 100 µM duroquinone (*diamond*)

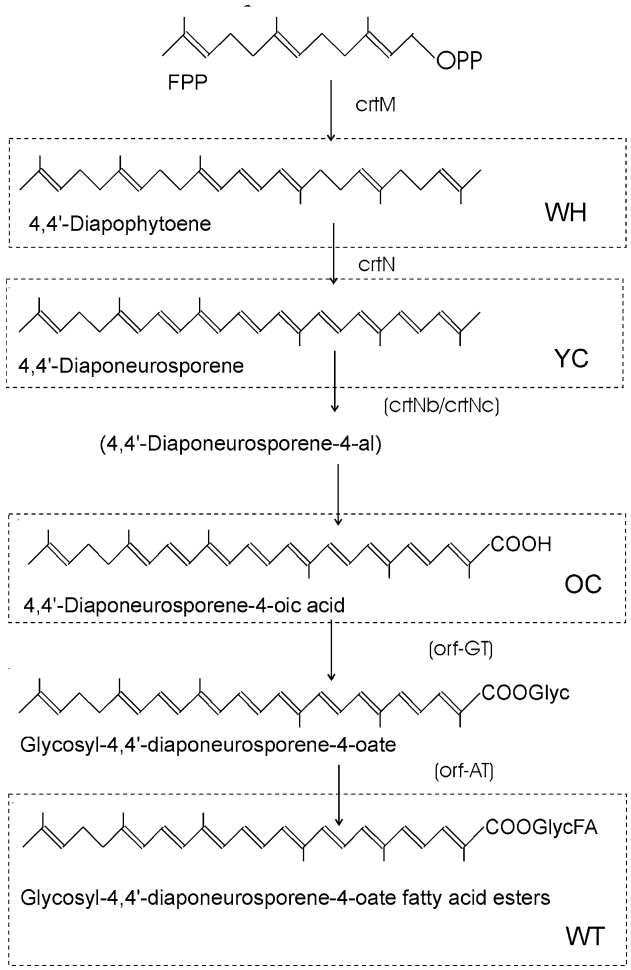

Fig. 6 Proposed carotenoid biosynthetic pathway in *Halobacillus halophilus* DSM 2266. Gene products catalyzing the individual reactions are indicated next to the arrow. Putative gene products and intermediates are in brackets. Carotinoid end products in wild type (WT) and mutants WH, OC and YC are *boxed*

Carotenoids and oxidative stress

The relevance of the C_{30} carotenoids for growth of *H. halophilus* under oxidative stress was tested (Fig. 5). By application of diphenylamine, the formation of colored carotenoids was completely inhibited. Duroquinone was used to establish peroxidative conditions by generating O_2^- (Moore et al. 1989). Cell density of control cultures at 1 mM NaCl was comparable over the first 10 h to diphenylamine-treated cells. At the stationary phase, the density of the diphenylamine culture was slightly lower. With duroquinone, initial growth was slower than in the control and final optical density was only about half which is indicative of the initiation of peroxidation by this quinone. When both compounds were added, growth was completely inhibited. Obviously, carotenoids are essential for *H. halophilus* to survive oxidative stress conditions.

Discussion

Accumulation of 4,4′-diapophytoene by inhibition of desaturation and formation 4,4′-diaponeuroporenoic acid as carotenoid hydrolysis product (Fig. 1) indicated that the *H. halophilus* carotenoids are synthesized in a C_{30} pathway via 4,4′-diaponeurosporene-4-oic acid (Fig. 6). All intermediates from 4,4′-diapophytoene to 4,4′-diaponeurosporene to 4,4′-diaponeurosporenoic acid accumulated in individual mutants with different color phenotype (Fig. 3). Compound 5 is the one that is also formed not only in *H. halophilus* but also in *S. carnosus* transformed with the complete carotenogenic gene cluster from *S. aureus* (Pelz et al. 2005) corresponding to glycosyl-4,4′-diaponeurosporene-4-oic acid fatty acid esters. In this respect, they resemble staphyloxanthins. These are typical pigments of *S. aureus* (Marshall and Wilmoth 1981; Pelz et al. 2005). Their common feature is a core of 4,4′-diaponeurosporenoic acid with an ester linkage to a sugar which is also esterified with a fatty acid. The different staphyloxanthins identified to date can vary by the sugar moiety which is glucose but also mannose or galactose, and can contain C_{17} or C_{15} fatty acids (Marshall and Wilmoth 1981). Furthermore, isomers exist differing in the position of the ester linkage between the sugar and the diaponeurosporenoic acid and fatty acid in addition to *cis*/*trans*-isomers. Based on spectral properties, HPLC retention time and especially the

formation of 4,4′-diaponeurosporenoic acid upon alkaline hydrolysis, compounds 3, 4 and 5 most likely resemble some of these staphyloxanthins. We attempted to obtain mass spectra from compound 3, 4 and 5 by APCI and by MALDI-TOF but were not successful. It was difficult to ionize the carotenoids and fragmentation was very strong. Other groups working with staphyloxanthin derivatives also encountered instability upon ionization (Pelz et al. 2005). The spectrum of the minor compound 6 (Fig. 2a) indicates that this carotenoid is unrelated to staphyloxanthin. But it could be a 4′-glycosyloxy-4,4′-diaponeurosporene-4-oic acid derivative. A similar carotenoid with an additional double bond was identified in an unspecified halophilic coccus (Aasen et al. 1969) and as a minor carotenoid in *S. aureus* (Taylor and Davies 1983). This would involve an additional oxidase in *H. halophilus* to generate a 4′-hydroxy group.

The proposed carotenoid biosynthesis pathway of *H. halophilus* is outlined in Fig. 6. The carotenoids from mutants and wild type are marked. Although not found in *H. halophilus*, 4,4′-diaponeurosporene-4-al and glycosyl-4,4′-diaponeurosporene-4-oate are included as intermediates of staphyloxanthins. The genes with assigned functions are *crtM* and *crtNa* catalyzing the first two steps of the pathway are indicated. The desaturase gene *crtNa* and the two following genes in the biosynthesis pathway, *crtNb* and *crtNc* although encoding proteins with oxidase function, belong to the same *crtI*-related gene family (Fig. 4b). Another closely related gene is *crtO* of a ketolase (Fernández-Gonzalez et al. 1997). The reason why the function of *crtNb* and *crtNc* could not be revealed by genetic pathway complementation in *E. coli* may be the low expression level in this bacterium. These and the other genes assigned only by similarity are shown in brackets and are positioned according to the function of their homologues. All six genes are organized in two adjacent operons transcribed in opposite directions (Fig. 4a). The only other known C_{30} carotenoid synthesis gene clusters from *S. aureus* and from *Methylomonas* consist of a single operon. Apart from a tandem arrangement of *crtNa* and *crtNc* in *Methylomonas*, the carotenoid gene organization of all three bacteria is completely unrelated.

Although carotenoids of various structures are synthesized in bacteria, their common feature is their function as lipophilic antioxidations. For several bacteria, the presence of the antioxidative enzyme catalase was shown to be important for osmoprotection (Cho et al. 2000; Lee et al. 2005). Also *H. halophilus* is a halophilic carotenogenic bacterium. Therefore, we analyzed the growth of *H. halophilus* after formation of colored carotenoids was inhibited (Fig. 5). The effect was minor under standard conditions. However, when oxidative conditions were applied that allowed about 50% growth of the non-inhibited culture, the culture devoid of colored carotenoids died. This result indicates that the glycosy-4,4′-diaponeurosporenoic fatty acid esters are very important protectants for *H. halophilus* to cope with oxidative stress. A similar antioxidative action of staphyloxanthins was also demonstrated in *S. aureus*. When wild type and cells with inactivated carotenoid biosynthesis were treated with hydrogen peroxide or radical generating compounds, the pigmented cells showed a much higher survival rate (Clauditz et al. 2006). This supports the postulated membrane integration and stabilizing function of C30 carotenoid glycolipids (Taylor 1984).

In conclusion, the carotenoids accumulating in *H. halophilus* are C_{30} compounds structurally related to staphyloxanthins. As lipophilic antioxidants, they promote survival of the cells under oxidative stress. A carotenogenic gene cluster has been identified in which all genes necessary for the synthesis of staphyloxanthins are organized in two operons. The knowledge on the biosynthesis pathway in *H. halophilus* and the identification of the initial genes for C_{30} carotenoid synthesis allows future studies on carotenoid protective function, analysis of salt-dependent carotenoid synthesis and carotenoid pathway regulation in this halophilic bacterium.

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