Arch Microbiol (2001) 176 : 217–223
DOI 10.1007/ s002030100315

ORIGINAL PAPER

**Philipp Krubasik · Shinichi Takaichi · Takashi Maoka
Miki Kobayashi · Kazumori Masamoto
Gerhard Sandmann**

# Detailed biosynthetic pathway to decaprenoxanthin diglucoside in *Corynebacterium glutamicum* and identification of novel intermediates

Received: 9 March 2001 / Revised: 14 May 2001 / Accepted: 17 May 2001 / Published online: 30 June 2001


**Abstract** Carotenogenic mutants of *Corynebacterium glutamicum* were analyzed for their carotenoid content. Mutant MV10 accumulated the same carotenoids as the wild-type, decaprenoxanthin, decaprenoxanthin monoglucoside, and (2*R*,6*R*,2′*R*,6′*R*)-decaprenoxanthin di-(β-D)-glucoside, but in three-fold higher amounts. In addition, decaprenoxanthin diglucoside fatty acid esters and the intermediates nonaprene, 2-(3-methyl-2-butenyl)-ε,ψ-carotene, and sarcinene, 2,2′-bis(3-methyl-2-butenyl)-ε,ε-carotene were identified as minor carotenoids. The pink mutants MV40 and MV60 synthesized only lycopene. From another pink mutant, MV70, novel $C_{50}$-carotenoids were isolated. By NMR and mass spectroscopy, nonaflavuxanthin, 2-(4-hydroxy-3-methyl-2-butenyl)-1,16-didehydro-1,2-dihydro-ψ,ψ-carotene, and flavuxanthin, 2,2′-bis(4-hydroxy-3-methyl-2-butenyl)-1,16,1′,16′-tetradehydro-1,2,1′,2′-tetrahydro-ψ,ψ-carotene, were identified. The identification of these intermediates revealed the detailed pathway for the formation of decaprenoxanthin derivatives in *Corynebacterium glutamicum*.

P. Krubasik · G. Sandmann (✉)
Botanisches Institut, J.W. Goethe Universität,
P.O. Box 111932, 60054 Frankfurt, Germany
e-mail: sandmann@em.uni-frankfurt.de,
Tel.: +49-69-798 24746, Fax: +49-69-79824822

S. Takaichi
Biological Laboratory, Nippon Medical School,
Kawasaki 211–0063, Japan

T. Maoka
Kyoto Pharmaceutical University, Kyoto 607–8414, Japan

M. Kobayashi
Mitsubishi Chemical Co., Ltd., Tsukuba Research Center,
Ibaraki 300-03, Japan

K. Masamoto
Faculty of Education, Kumamoto University,
Kumamoto 860–8555, Japan

**Keywords** $C_{50}$-carotenoid biosynthesis · *Corynebacterium glutamicum* · Carotenogenic mutant · Decaprenoxanthin diglucoside fatty acid ester · Novel $C_{50}$-carotenoid intermediate

## Introduction

Carotenoids are yellow-to-red colored pigments with antioxidative and light-protecting properties that originate from the terpenoid biosynthetic pathway. In photosynthetic organisms, they are essential components of the light-harvesting apparatus and of protective functions. Carotenoids are synthesized by plants, algae, fungi, and bacteria. Among the bacteria, a high degree of variation of carotenoid structures is observed (Goodwin 1980).

Most carotenoids consist of 40 carbon atoms, but in a few species carotenoids with a variation of the carbon atom number are formed. In *Staphylococcus aureus* (Marshall and Wilmoth 1981) and heliobacteria (Takaichi et al. 1997), carotenoids with only 30 carbons are found, whereas in other species the carbon skeleton can be extended to $C_{45}$ and to $C_{50}$. Typical $C_{50}$-carotenoids are decaprenoxanthin [(2*R*,6*S*,2′*R*,6′*S*)-(2,2′-bis(4-hydroxy-3-methyl-2-butenyl)-ε,ε-carotene)] with two substituted ε-end groups in *Agromyces mediolanus* (formerly *Flavobacterium dehydrogenans)*, C.p. 450 [(2*R*,2′*R*)-2,2′-bis(4-hydroxy-3-methyl-2-butenyl)-β,β-carotene] with two substituted β-end groups in *Curtobacterium flaccumfaciens* (formerly *Corynebacterium poinsettiae*), sarcinaxanthin (2,2′-bis(3-methyl-2-butenyl)-ε,ε-carotene) with two substituted γ-end groups in *Micrococcus luteus* (formerly *Sarcina lutea*) (Férézou 1992), and bacterioruberin [(2*S*,2′*S*)-2,2′-bis(3-hydroxy-3-methylbutyl)-3,4,3′,4′-tetradehydro-1,2,1′,2′-tetrahydro-ψ,ψ-carotene-1,1′-diol] with two substituted acyclic ψ-end groups in *Halobacterium* species. The details of the biosynthetic pathways leading to $C_{50}$-carotenoids are not yet known, the enzymes involved have not been isolated, and the genes of the pathway are unknown. Among the coryneform bacterial groups, $C_{50}$-carotenoids are abundant. Molecular biological techniques for genetic manipulation of these species have been developed and a transposable element

from the gram-positive *C. glutamicum* has been isolated (Vertès et al. 1994b). Thus, it was possible to construct an artificial transposon and demonstrate its function by transposon mutagenesis of a *C. glutamicum* strain so far mainly used for amino acid production. Several pigment mutants have also been isolated (Vertès et al. 1994a). Here, we present the carotenoid composition of *C. glutamicum* and a detailed analysis of the pigment mutants obtained by transposon mutagenesis. Based on these results, we propose a biosynthetic pathway of $C_{50}$-carotenoid biosynthesis in *C. glutamicum*.

## Materials and methods

### Bacterial strains

Strain MJ233C *Corynebacterium glutamicum*, formerly assigned as *Brevibacterium flavum* (Kurusu et al. 1990) but now renamed (Liebl et al. 1991), and its transposon mutants MV10, which has a darker yellow color than the wild-type; MV40, MV60, and MV70 which are pink; and MV240 which is white (Vertès et al. 1994a) were used in this study. The bacteria were grown in AR medium (Kurusu et al. 1990) or in LB medium (Sambrook et al. 1989) at 30 °C with aeration. Kanamycin was added to the transposon mutants to a final concentration of 25 mg/l.

### Carotenoid analysis

Cells were harvested by centrifugation and freeze-dried. Carotenoids from *C. glutamicum* were extracted with methanol at 60 °C for 20 min and partitioned into 10% ether in petrol. Lycopene (ψ,ψ-carotene) and its hydroxy derivatives were extracted from an *Escherichia coli* transformant with acetone at 50 °C for 20 min and partitioned into 10% ether in petroleum ether. The collected upper phase was evaporated to dryness and the residue dissolved in either methanol or acetone.

Carotenoids were separated by HPLC on a Nucleosil $C_{18}$ 3-µm column (4×250 mm Macherey-Nagel, Düren, Germany) with acetonitrile/methanol/2-propanol (85:10:5, by volume) (Albrecht et al. 1997) or on a non-endcapped polymeric 3-µm $C_{30}$ column (4× 250 mm YMC Wilmington N.C., USA) with methanol/*tert*-butylmethyl-ether/$H_2O$ (44:50:6, by volume) (Sander et al. 1994). The flow rate was 1 ml/min. Absorbance spectra in the eluent were recorded on-line with a photodiode array detector 440 (Kontron, Straubenhard, Germany). Carotenoids were identified by comparison of retention time on HPLC and absorbance spectra with isolated standards that were characterized by their absorbance and mass spectra. The reference compounds lycopene, 1-hydroxylycopene (1,2-dihydrol-ψ,ψ-caroten-1-ol), and 1,1′-dihydroxylycopene (1,2,1′,2′-tetrahydro-ψ,ψ-carotene-1,1′-diol) were isolated from *E. coli* transformed with the plasmids pACCRT-EBI$_{Eu}$ and pUCCRT-C (Albrecht et al. 1997). Decaprenoxanthin was isolated from *Pseudomonas* sp. KK10206C (Miki et al. 1994) as the major carotenoid of this strain and was reevaluated to be decaprenoxanthin (Dr. A. Yokoyama, Marine Biotechnology Institute, Shimizu Laboratories, personal communication). Decaprenoxanthin monoglucoside (2-[4-(β-D-glucopyranosyloxy)-3-methyl-2-butenyl]-2′-(4-hydroxy-3-methyl-2-butenyl)-ε,ε-carotene) and decaprenoxanthin diglucoside (2,2-bis[4-(β-D-glucopyranosyloxy)-3-methyl-2-butenyl]-ε,ε-carotene) were isolated from *Arthrobacter* sp. M3 (Arpin et al. 1972). For carotenoid purification, the crude extract disolved in *n*-hexane was loaded on a column of silica gel 60 (Merck, Germany), and then carotenes were eluted with *n*-hexane. Carotenes were separated by HPLC on a Novapak $C_{18}$ column (8×100 mm, RCM type, Waters, USA) with acetonitrile/tetrahydrofuran/methanol (58:7:35 by volume) and a flow rate of 2 ml/min (Takaichi 2000).

Relative molecular masses of carotenoids were determined by field-desorption mass spectrometry using a double-focusing gas chromatograph mass spectrometer equipped with a field-desorption apparatus (M-2500; Hitachi, Japan) (Takaichi 1993). Carotenoids were acetylated as described previously (Takaichi and Ishidsu 1992). $^{13}C$- (125 MHz) and $^{1}H$-NMR (500 MHz) spectra of the carotenoids in $CDCl_3$ were recorded with a system UNITY *INOVA*-500 (Varian, USA). Assignments of $^{1}H$ and $^{13}C$-NMR spectra were based on $^{1}H$-$^{1}H$ COSY and NOESY spectra, HMBC and ROESY correlations, and by comparison with the data from lycopene (Hengartner et al. 1992). The NMR spectrum of decaprenoxanthin diglycoside was determined in acetone-$d_6$.

The circular dichroism (CD) spectra of carotenoids were measured with a J-720 spectropolarimeter (JASCO, Japan) in diethyl ether/*i*-pentane/ethanol (5:5:2, by vol.) at 25 °C.

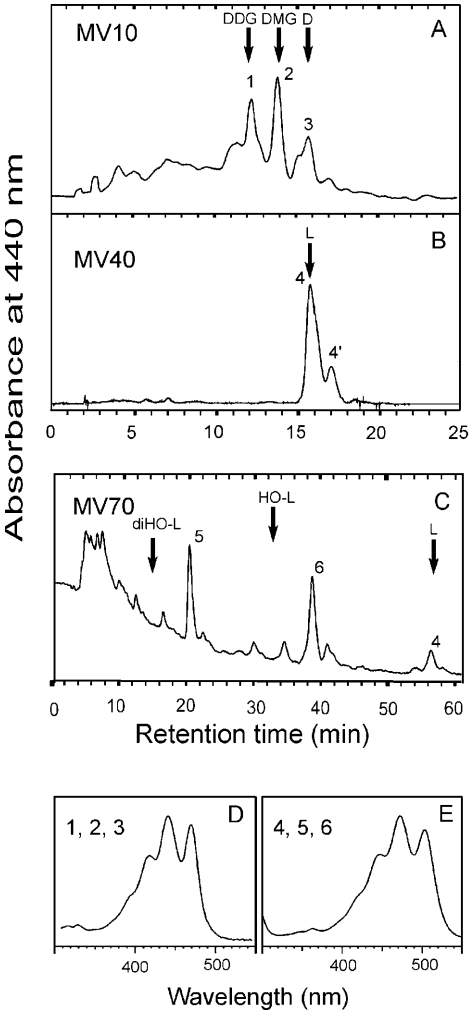


**Fig. 1** Separation of carotenoids in different strains of *Corynebacterium glutamicum* by Nucleosil $C_{18}$ (**A**, **B**) and $C_{30}$-column (**C**) HPLC. The retention times for cochromatography with isolated standards are indicated by *arrows* at top of the chromatograms. *D* Decaprenoxanthin, *DMG* decaprenoxanthin monoglucoside, *DDG* decaprenoxanthin diglucoside, *L* lycopene, *HOL* 1-hydroxylycopene, *DHOL* 1,1′-dihydroxylycopene. Absorption spectrum of peaks 1–3 with maxima at 418, 440 and 470 nm in the HPLC eluent (**D**) and those of peaks 4–6 with 446, 472, and 504 nm (**E**) are also indicated (*upper part of figure*). Peaks are referred to in the text by the numbers shown in the figure

**Table 1** Characteristics of carotenoids from *Corynebacterium glutamicum* wild-type, and trivial and IUPAC-IUB semi-systematic names of carotenoids found. For HPLC separation of peaks 1–3, the system with the Nucleosil $C_{18}$ column was used, and for peaks 4–6 the system with the $C_{30}$-column was used

| Peak no. | Carotenoid (trivial and IUPAC-IUB names) | $\lambda_{max}$ (nm) in the HPLC eluent | | | Relative molecular mass (*m/z*) | Retention time (min) |
|---|---|---|---|---|---|---|
| 4 | Lycopene; ψ,ψ-carotene | 446 | 472 | 504 | 536 | 57 |
| 6 | Nonaflavuxanthin; 2-(4-hydroxy-3-methyl-2-butenyl)-ψ, ψ-carotene | 446 | 472 | 504 | 620 | 38 |
| 5 | Flavuxanthin 2,2′-bis(4-hydroxy-3-methyl-2-butenyl)-ψ, ψ-carotene | 446 | 472 | 504 | 704 | 21 |
| 3 | Decaprenoxanthin; (2*R*,6*R*,2′*R*,6′*R*)-2,2′-bis(4-hydroxy-3-methyl-2-butenyl)-ε,ε-carotene | 418 | 440 | 470 | 704 | 15.8 |
| 2 | Decaprenoxanthin monoglucoside | 418 | 440 | 470 | 866 | 13.9 |
| 1 | Decaprenoxanthin diglucoside | 418 | 440 | 470 | 1028 | 12.1 |

[a]See Fig.1

## Results

Carotenoids of cells of the *C. glutamicum* strains were extracted and analyzed by HPLC (Fig. 1). The carotenoids of the wild-type strain showed the same elution profile as the mutant MV10 (Fig. 1A), but MV10 had a total carotenoid content of 191 µg/g dry weight, which was three times higher than in the wild-type. In the pink mutants MV40 and MV60, only one type of carotenoid was found (Fig. 1B). The retention time of the major peak (peak 4 in Fig. 1B) on HPLC and its absorption spectrum in the HPLC eluent (Fig. 1E) were identical to a standard all-*trans* lycopene, and a Z isomer eluted 1.3 min later (peak 4′). The white mutant MV240 was completely devoid of carotenoids, even the colorless phytoene could not be detected.

The mutant MV10 had three major carotenoid peaks (Fig. 1A). These components showed the same absorption spectra (Fig. 1D), and the absorption maxima were 418, 440, 470 nm in the HPLC eluent (Table 1). These spectral properties indicate that these carotenoids have the same chromophore, and the number of conjugated double bonds is 9 (Takaichi and Shimada 1992). Retention times of these peaks were identical to the standard decaprenoxanthin (peak 3), decaprenoxanthin monoglucoside (peak 2), and decaprenoxanthin diglucoside (DDG) (peak 1) isolated from the bacteria indicated in Materials and methods. Their relative molecular masses were 704, 866 and 1,028, respectively. The CD spectrum of DDG (data not shown) was almost the same as that of synthetic (2*R*,6*R*,2′*R*,6′*R*)-decaprenoxanthin (Gerspacher and Pfander 1989) indicating that DDG and its intermediates from *C. glutamicum* have the same 2*R*,6*R*,2′*R*,6′*R*-stereochemistry. The glycoside moiety of DDG was identified as β-D-glucoside based on the $^1$H-NMR spectrum in acetone-$d_6$, due to low solubility in $CDCl_3$, including $^1$H-$^1$H COSY correlation and comparison of the coupling constants with those of the β-D-glucoside moiety of dihydroxylycopene diglucoside (Takaichi et al. 2001). The same sugar moiety was identified in the decaprenoxanthin derivatives of another *Corynebacterium* and an *Arthrobacter* species (Goodwin 1980). Based on our data, the components of peaks 3, 2 and 1 have been assigned as (2*R*,6*R*,2′*R*,6′*R*)-decaprenoxanthin, (2*R*,6*R*,2′*R*,6′*R*)-decaprenoxanthin mono-β-D-glucoside and (2*R*,6*R*,2′*R*,6′*R*)-decaprenoxanthin di-β-D-glucoside, respectively (Fig. 2). IUPAC-IUB semi-systematic names and also trivial names of the identified carotenoids used here are shown in Table 1.

Although carotenes eluted close to peak 3 on the Nucleosil $C_{18}$ HPLC, they eluted separately on the Novapak $C_{18}$ HPLC (Takaichi 2000). The major component in the carotene fraction was lycopene, and two minor components were tentatively identified as nonaprene [(2*R*,6*R*)-(2-(3-methyl-2-butenyl)-ε,ψ-carotene] and sarcinene [2,2-bis(3-methyl-2-butenyl)-ε,ε-carotene] (Fig. 2) based on the absorption spectra, relative molecular masses, and retention times on HPLC (Table 2).

Other minor components of MV 10 were eluted at around 30 min on the Nucleosil $C_{18}$ HPLC (not shown in Fig. 1A). These carotenoids appeared to be very hydrophobic on $C_{18}$-HPLC and also apparently behaved as highly polar carotenoids on silica gel TLC. These chromatographic properties are characteristic of the carotenoid glycoside fatty acid esters, since the glycosyl group adsorbed strongly on silica gel and the fatty acid moiety adsorbed on the $C_{18}$-column (Takaichi and Ishidsu 1992). The relative molecular masses were 1,508, 1,534 and 1,560. The value 1,508 corresponded to two C16:1 fatty acid esters of DDG. Similarly, 1,534 and 1,560 corresponded to one C16:1 and one C18:0, and two C18:0, respectively. Thus these carotenoids were determined to be DDG diesters.

The pink mutant MV70 (Fig. 1C) had different carotenoid components than the pink mutants MV40 (Fig. 1B) and MV60. The component in peak 4 was identified as lycopene based on the absorption spectrum, relative molecular mass of 536 and retention time on HPLC (Table 1). Two major components (peaks 5 and 6) showed the same absorption spectrum as lycopene (Table 1), therefore the number of conjugated double bonds is 11 (Takaichi and Shimada 1992). 1-Hydroxylycopene and 1,1′-dihydroxylycopene standards eluted faster than the components of peaks 6 and 5, respectively, on the $C_{30}$-column (Fig. 1C). The relative molecular masses of compounds 5 and 6

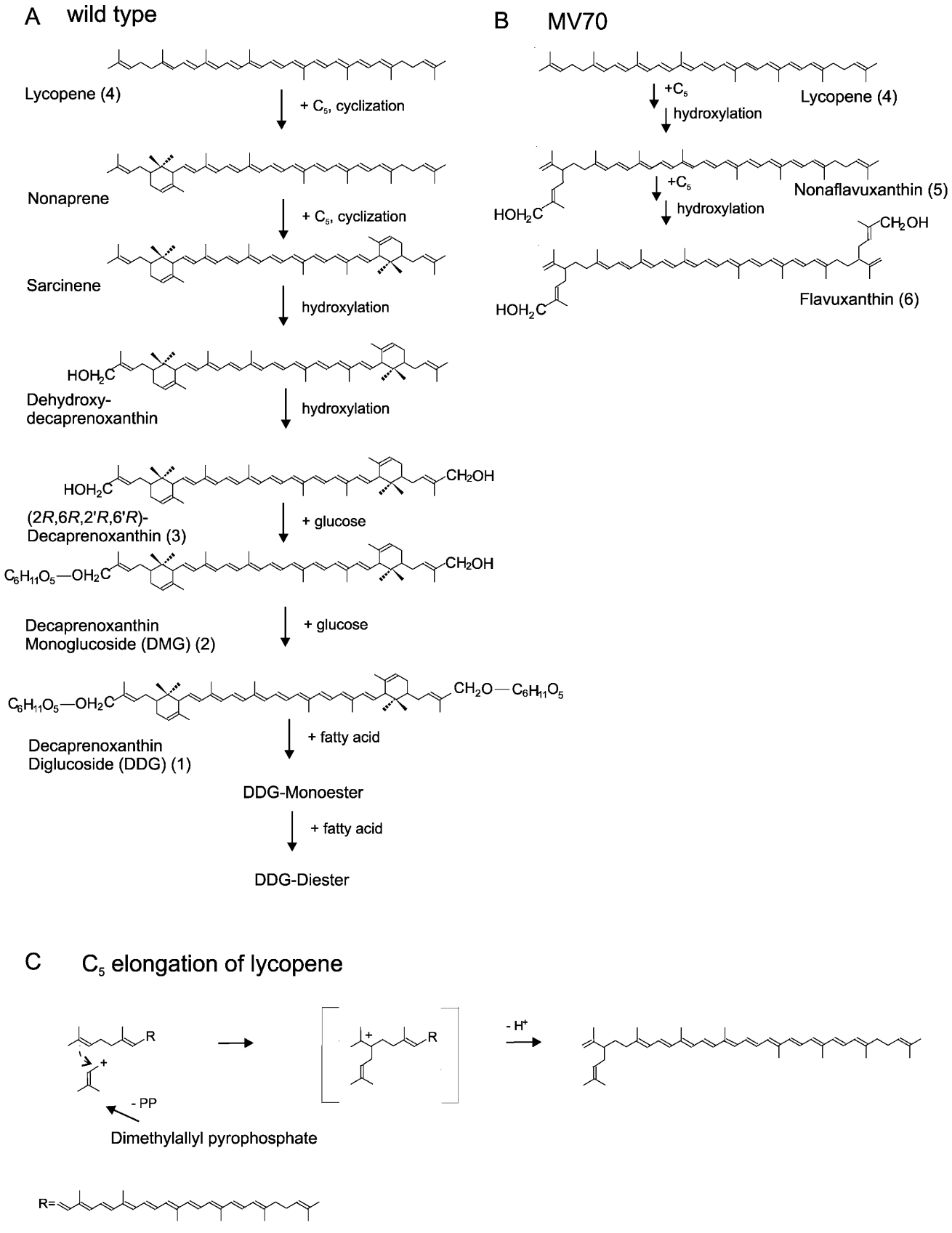


**Fig. 2** Postulated pathway for the synthesis of decaprenoxanthin diglucoside from lycopene in *Corynebacterium glutamicum* wild-type (**A**) and flavuxanthin in mutant MV70 (**B**). The proposed mechanism for $C_5$-elongation is given in **C**. Numbers in parentheses indicate the peak numbers in Fig. 1

**Table 2** Characteristics of carotenes from *Corynebacterium glutamicum* mutant MV10, and trivial and IUPAC-IUB semi systematic names of carotenes found. For HPLC separation, the system including the Novopak $C_{18}$ column was used

| Carotenoid (trivial and IUPAC-IUB names) | $\lambda_{max}$ (nm) in the HPLC eluent | | | Relative molecular mass (*m*/*z*) | $R_t$ (min) |
|---|---|---|---|---|---|
| Lycopene; ψ,ψ-carotene | 447 | 473 | 504 | 536 | 11.4 |
| Nonaprene; (2*R*,6*R*)-2-(3-methyl-2-butenyl)-ε,ψ-carotene | 430 | 456 | 486 | 604 | 20.9 |
| Sarcinene; (2*R*,6*R*,2′*R*,6′*R*)-2,2′-bis(3-methyl-2-butenyl)- ε,ε-carotene | 416 | 438 | 468 | 672 | 39.0 |

**Table 3** $^{13}C$- (125 MHz) and $^{1}H$-NMR (500 MHz) data of flavuxanthin in $CDCl_3$ (δ, ppm). Numbers in parentheses indicate the coupling constant in Hz

| Position | $^{13}C$ | Assignment | $^{1}H$ | Multiplicity | | HMBC correlations (from $^{1}H$ to $^{13}C$) |
|---|---|---|---|---|---|---|
| 1 | 147.1 | qC | – | | | |
| 2 | 47.0 | $CH_2$ | 2.08 | m | | |
| 3 | 31.1 | $CH_2$ | 1.56 | m | | C-1 |
| 4 | 37.9 | $CH_2$ | 2.00 | m | | C-2 |
| 5 | 139.6 | qC | – | | | |
| 6 | 125.7 | CH | 5.93 | d | (11) | |
| 7 | 124.8 | CH | 6.48 | dd | (15, 11) | |
| 8 | 135.4 | CH | 6.24 | d | (15) | C6, 10 |
| 9 | 136.1 | qC | – | | | |
| 10 | 131.6 | CH | 6.18 | d | (12) | C-19 |
| 11 | 125.2 | CH | 6.63 | dd | (15, 12) | |
| 12 | 137.4 | CH | 6.36 | d | (12) | C-10, 14, 20 |
| 13 | 136.5 | qC | – | | | |
| 14 | 132.6 | CH | 6.25 | m | | |
| 15 | 130.1 | CH | 6.63 | m | | |
| 16 | 111.8 | $CH_2$ | 4.78 | br.s | | C-2, 17 |
| | | | 4.70 | br.s | | |
| 17 | 18.5 | $CH_3$ | 1.634 | s | | C-2, 16 |
| 18 | 17.0 | $CH_3$ | 1.802 | s | | C-3, 4, 5, 6 |
| 19 | 12.9 | $CH_3$ | 1.97 | s | | C-9, 10 |
| 20 | 12.9 | $CH_3$ | 1.97 | s | | C13, 14 |
| 1″ | 31.7 | $CH_2$ | 2.10 | m | | |
| 2″ | 124.8 | CH | 5.36 | t | (5) | |
| 3″ | 135.3 | qC | – | | | |
| 4″ | 13.9 | $CH_3$ | 1.670 | s | | C-1″, 2″, 5″ |
| 5″ | 69.1 | $CH_2$ | 4.00 | s | | C-1″, 2″, 4″ |

were 704 and 620, and by acetylation they formed monoacetyl and diacetyl derivatives, respectively. The $^{13}C$- and $^{1}H$-NMR spectra of the latter (Table 3) indicated that the carotenoid was symmetrical, and the olefinic region was similar to that of lycopene. From HMBC and ROESY correlations, the structure of the extra isoprene is as indicated in Fig. 3. This E structure of the hydroxy group is the same as that in decaprenoxanthin. Thus the component in peak 5 was determined to be 2,2′-bis (4-hydroxy-3-methyl-2-butenyl)-1,16,1′,16′-tetradehydro-1,2,1′2′-tetrahydro-ψ,ψ-carotene, and has been tentatively named flavuxanthin. Similarly, the component in peak 6 was 2-(4-hydroxy-3-methyl-2-butenyl)-1,16-didehydro-1,2-dihydro-ψ,ψ-carotene, and has been tentatively named nonaflavuxanthin (Fig. 2).

## Discussion

In this study, the structures of the main carotenoids of *C. glutamicum* and of several minor intermediates were elucidated. Furthermore, the carotenoids of different mutants of *C. glutamicum* were analyzed. Based on these data, the biosynthetic pathway of cyclic $C_{50}$-carotenoids in *C. glutamicum* is proposed in Fig. 2A. Although none of the *C. glutamicum* mutants accumulated precursors of lycopene, i.e. phytoene, phytofluene, ζ-carotene and neurosporene, lycopene, which was found in the wild-type as well as in several mutants, is most likely synthesized according to the known carotenoid biosynthetic pathway of other bacteria. Lycopene is the $C_{40}$-carotenoid substrate for extension of the carbon chain. The intermediates nonaprene, sarcinene, and decaprenoxanthin were identified in the mutant MV10 and the wild-type of *C. glutamicum*.

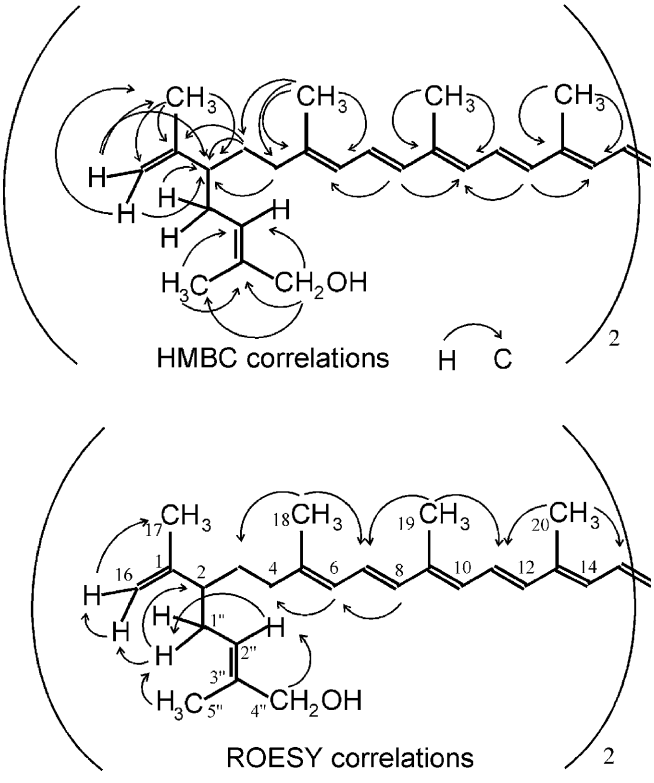


**Fig. 3** Structure of the end group of flavuxanthin. *Arrows* indicate HMBC and ROESY correlations. The *numbers* indicate the carbon atoms of flavuxanthin

Thus the following reaction sequence can be proposed (Fig. 2A). Lycopene is converted into the $C_{45}$-carotenoid nonaprene by addition of a $C_5$ unit and subsequent cyclization. At the other side of the molecule, a second addition of another $C_5$ unit and cyclization results in the formation of the $C_{50}$-carotenoid sarcinene. In the following step, hydroxylation leads via dehydroxydecaprenoxanthin to decaprenoxanthin and glucosylations of the hydroxy groups to decaprenoxanthin monoglucoside and DDG, the main carotenoids of the wild-type of *C. glutamicum*. A final esterification at the glucosides yields DDG monoester and DDG diester, which were found in small amounts in *C. glutamicum*. The esterified fatty acids are C16:0 and C18:1. Since the fatty acid composition of *C. glutamicum* is not known, their incorporation into DGG may be selective or may reflect the occurrence of the major fatty acids present in this species. Major fatty acids in the carotenoid glycoside esters from *Rhodococcus rhodochrous* (Takaichi and Ishidsu 1992), *Myxococcus* sp. MY-18 (Takaichi et al. 1995), and *Meiothermus ruber* (Burgess et al. 1999) are different from those in the cellular lipids.

In the mutant MV70, the acyclic carotenoids nonaflavuxanthin, with 45 carbon atoms, and flavuxanthin, with 50 carbon atoms, were identified by spectroscopic analysis. The exo-methylene group at C16 of a ψ-end group present in both carotenoids is only known in aleuriaxanthin, isolated from the fungus *Aleuria aurantia* (Eschenmoser et al. 1983). The occurrence of acyclic $C_{45}$ and $C_{50}$ carotenoids in mutant MV70 is a strong indication against a simultaneous elongation/cyclization reaction as previously proposed (Britton 1988). We suggest that the likely mechanism for the formation of the $C_{50}$-carotenoid decaprenoxanthin involves the $C_5$ extension of lycopene and a subsequent cyclization step (Fig. 2A). This conclusion is further supported by the $C_{45}$- and $C_{50}$-carotenoids identified in *Curtobacterium flaccumfaciens* (Norgard et al. 1970). In this bacterium 2-(dihydroxy-isopentenyl)-2′-isopentenyl-β-carotene is the cyclic end-product of $C_{50}$-carotenoid biosynthesis and the corresponding acyclic $C_{45}$ and $C_{50}$ precursors have been identified. Thus, it can be proposed that in MV70 lycopene is converted to acyclic $C_{50}$-carotenoids via $C_{45}$-carotenoids by addition of a $C_5$ group at position C-2 and C-2′ because the mutation affects the ability to form cyclic carotenoids. Based on the carotenoid products identified in MV70 and in analogy to the formation of bacterioruberin (Britton 1988), the mechanism of $C_5$-extension can be specified as outlined in Fig. 2C.

The mechanism of subsequent formation of an ε-end group may be similar to the lycopene cyclization reaction. Protonation of the C-1,16 double bond should be the initial reaction in the ring formation. In the wild-type, hydroxylations at C-4″ follow the formation of the ε-end groups (Fig. 2A), whereas the accumulating acyclic precursors are also hydroxylated in the mutant MV70 (Fig. 2B).

In conclusion, carotenoid analysis of several mutants of *C. glutamicum* revealed all possible intermediates of the biosynthetic pathway to DDG and the corresponding fatty acid esters and thus revealed the detailed biosynthetic pathway to $C_{50}$ end-products. Among the precursors of DDG, four novel carotenoids, nonaprene, nonaflavuxanthin, flavuxanthin and DDG diester, have not been reported in any other organisms so far (Straub 1987).

## References


Albrecht M, Takaichi S, Misawa N, Schnurr G, Böger P, Sandmann G (1997) Synthesis of atypical cyclic and hydroxy carotenoids in *Escherichia coli* transformants. J Biotechnol 58: 177–185

Arpin N, Liaaen-Jensen S, Trouilloud M (1972) Bacterial carotenoids. XXXVIII $C_{50}$-carotenoids. 9. Isolation of decaprenoxanthin mono- and diglucoside from an *Arthrobacter* sp. Acta Chem Scand 26:2524–2526

Britton G (1988) Biosynthesis of carotenoids. In: Goodwin TW (ed) Plant pigments. Academic, London, pp 133–182

Burgess ML, Barrow KD, Gao C, Heard GM, Glenn D (1999) Carotenoid glycoside esters from the thermophile bacterium *Meiothermus ruber*. J Nat Prod 62:859–863

Eschenmoser W, Uebelhard P, Eugster CH (1983) Synthesen der enantiomeren Aleuriaxanthine. Nachweis eines vorherrschenden (*Z*)-Aleuriaxanthins in *Aleuria*. Helv Chim Acta 66:82–91

Férézou JP (1992) $C_{50}$ bicyclic carotenoids: sarcinaxanthin synthesis. Methods Enzymol 213:75–86

Gerspacher M, Pfander H (1989) $C_{45}$ und $C_{50}$-Carotinoide. Synthese eines optisch aktiven cyclischen $C_{20}$-Bausteins und von Decaprenoxanthin (=(2*R*,6*R*,2′*R*,6′*R*)-2,2′-Bis(4-hydroxy-3-methylbut-2-enyl)-ε,ε-carotin). Helv Chim Acta 72:151–157

Goodwin TW (1980) The biochemistry of carotenoids, vol 1. Chapman and Hall, London

Hengartner U, Bernhard K, Meyer K, Englert G, Glinz E (1992) Synthesis, isolation, and NMR-spectroscopic characterization of fourteen (*Z*)-isomers of lycopene and of some acetylenic didehydro- and tetradehydrolycopenes. Helv Chim Acta 75: 1848–1865

Kurusu Y, Kainuma M, Inui M, Sato Y, Yukawa H (1990) Electroporation-transformation system for coryneform bacteria by auxotrophic complementation. Agric Biol Chem 54:443–447

Liebl W, Ehrmann M, Ludwig W, Schleifer KH (1991) Transfer of *Brevibacterium divaricatum* DSM 20297$^T$, "*Brevibacterium flavum*" DSM 20411, "*Brevibacterium lactofermentum*" DSM 20412 and DSM 1412, and *Corynebacterium glutamicum* and their distinction by rRNA gene restriction patterns. Int J Syst Bacteriol 41: 255–260

Marshall JH, Wilmoth GJ (1981) Pigments of *Staphylococcus aureus*, a series of triterpenoid carotenoids. J Bacteriol 147:900–913

Miki W, Otaki N, Yokoyama A, Izumida H, Shimidzu N (1994) Okadaxanthin, a novel $C_{50}$-carotenoid from a bacterium, *Pseudomonas* sp. KK10206C associated with marine sponge, *Halichondria okadi*. Experientia 50:684–686

Norgard S, Aasen AJ, Liaaen-Jensen S (1970) Bacterial carotenoids. XXXII. $C_{50}$-Carotenoids. 6. Carotenoids from *Corynebacterium poinsettiae* including for new $C_{50}$-diols. Acta Chem Scand 24:21832197

Sambrook J, Fritsch EF, Maniatis, T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Sander LC, Epler Sharpless K, Craft NE, Wise SA (1994) Development of engineered stationary phases for the separation of carotenoid isomers. Analyt Chem 66:1667–1674

Straub O (1987) Key to carotenoids, Birkhäuser Verlag, Basel

Takaichi S (1993) Usefulness of field desorption mass spectrometry in determining molecular masses of carotenoids, natural carotenoid derivatives and their chemical derivatives. Org Mass Spectrom 28:785–788

Takaichi S (2000) Characterization of carotenes in a combination of a $C_{18}$ HPLC column with isocratic elution and absorption spectra with a photodiode-array detector. Photosynth Res 65: 93–99

Takaichi S, Ishidsu J (1992) Carotenoid glycoside ester from *Rhodococcus rhodochrous*. Methods Enzymol 213:366–374

Takaichi S, Shimada K (1992) Characterization of carotenoids in photosynthetic bacteria. Methods Enzymol 213:347–385

Takaichi S, Yazawa H, Yamamoto Y (1995) Carotenoids of the fruiting gliding myxobacterium, *Myxococcus* sp. MY-18, isolated from lake sediment: accumulation of phytoene and ketomyxocoxanthin glucoside esters. Biosci Biotechnol Biochem 59:464–468

Takaichi S, Inoue K, Akaike M, Kobayashi M, Oh-oka H, Madigan MT (1997) The major carotenoid in all known species of heliobacteria is the $C_{30}$ carotenoid 4,4′-diaponeurosporene, not neurosporene. Arch Microbiol 168:277–281

Takaichi S, Maoka T, Hanada S, Imhoff JF (2001) Dihydroxylycopene diglucoside diesters: a novel class of carotenoids from the phototrophic purple sulfur bacteria *Halorhodospira abdelmalekii* and *Halorhodospira halochloris*. Arch Microbiol 175: 161–167

Vertès AA, Asai Y, Masayuki I, Kobayashi M, Kurusu Y, Yukawa H (1994a) Transposon mutagenesis of coryneform bacteria. Mol Gen Genet 254:397–405

Vertès AA, Inui M, Kobayashi M, Kurusu Y, Yukawa H (1994b) Isolation and characterization of IS31831, a transposable element from *Corynebacterium glutamicum*. Mol Microbiol 11: 739–746